# The Role of Key *Schistosoma mansoni* Invadolysins in Infection Establishment and Persistence in *Biomphalaria glabrata* and Mammalian Hosts: Functional Characterization of SmLeish and SmCI-1

by

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**Abstract**

Despite significant advancements in treatment coverage, schistosomiasis remains the second most impactful parasitic disease to human health after malaria. This deadly and debilitating parasite infects more than 230 million people worldwide and causes an estimated 200,000 deaths every year. The parasite's complex life cycle sees it cycle between a vertebrate definitive host in which sexual replication takes place, and an intermediate gastropod mollusc host in which the worm asexually replicates. Despite the significant differences between the immune system of a mammal and a snail, all life cycle stages of schistosomes have developed complex immunomodulatory mechanisms to fight the cellular and humoral immune responses put forward by both their human and snail hosts. During the initial infectious stages of the *Schistosoma mansoni* life cycle, a particular class of metalloproteases known as invadolysins have been shown to be released into the host. To date, these invadolysins have yet to be functionally characterized, despite their prominent featuring in larval excretory/secretory (E/S) products.

My doctoral research focused on the immune manipulation employed by *S. mansoni* via its release of invadolysins during penetration and development in a host. One invadolysin, which we termed *S. mansoni* Leishmanolysin (SmLeish), was found to be released by developing sporocysts inside of the snail host *Biomphalaria glabrata* during the first 48 hours of infection. I discovered that SmLeish features canonical MMP activity, is present in host E/S products, and has the capacity to alter susceptible snail (M-line) haemocyte chemokinesis, but not resistant snail (BS-90) haemocyte chemokinesis. In addition to this, I showed that specific knock down of SmLeish is sufficient to alter infection kinetics and success of *S. mansoni* infecting M-line *B. glabrata.* While SmLeish was then found to be present during the cercarial stages of the *S. mansoni* life cycle, it did not successfully target relevant mammalian substrates.

I then moved to characterize an invadolysin comprising roughly 12.8% of cercarial acetabular glands, which I termed *S. mansoni* cercarial invadolysin (SmCI-1). This protein was found to be localized to the acetabular glands and released upon transformation into schistosomula. It featured canonical MMP activity and was able to cleave key structural and immunological human proteins such as collagen type IV, fibrinogen, and complement component C3. It was also demonstrated to be capable of altering the production of key inflammatory cytokines in human leukocytes exposed to various stimulants such as whole cercarial lysate and lipopolysaccharides. This effect was confirmed to require MMP activity, as an inactive SmCI-1 mutant did not downregulate inflammatory cytokines. Despite this observation, both active and inactive SmCI-1 were able to elicit the production of IL-10. While SmLeish also featured an ability to alter cytokine production in a similar manner, its low abundance during mammalian infection suggests less of a role during mammalian infection than SmCI-1.

All together, this research helped further our understanding of host immune system modulation by schistosomes, while also serving as the first known characterization of invadolysins in the context of a parasitic helminth.

## Preface

This thesis is a compilation of the work done during my doctoral research. The research projects, of which this thesis is a part, received animal research ethics approval from the University of Alberta Research Ethics Office, Project Name "Immunobiology of Snail-Schistosome Interactions", No. AU00000057_REN1, AU00000057_REN2 and AU00000057_REN3.

The general introduction and discussion in chapters 1,6 are my original work. The literature review in chapter 2 is partially my original work (section 2.3 on proteases), and partially part of a review I authored (section 2.2 on schistosome immune evasion), published as:

- Hambrook, J. R., & Hanington, P. C. (2021). Immune evasion strategies of schistosomes. Frontiers in Immunology, 11, 624178.

I was responsible for research design and analysis, along with manuscript preparation. Patrick C. Hanington was the supervisory author and was responsible for the same roles outlined above.

Chapter 4 of this thesis has also been published as:

- Hambrook, J. R., Kabore, A. L., Pila, E. A., & Hanington, P. C. (2018). A metalloprotease produced by larval *Schistosoma mansoni* facilitates infection establishment and maintenance in the snail host by interfering with immune cell function. PLoS Pathogens, 14(10), e1007393.

I was responsible for the design of the research, performing the research and data analysis, as well as the manuscript composition. Kabore, A.L assisted with research design, performing the research, and data analysis. Hanington, P.C. was the supervisory author and was involved in research design, data analysis and composition of the manuscript.

Chapters 5 and 6 are also my original work, and much of the data contained therein is currently under review for publication, and will be accepted pending revisions.

I was responsible for the design of the research, performing the research and data analysis, as well as the manuscript composition. Hanington, P.C. was the supervisory author and was involved in research design, data analysis and composition of the manuscript.

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Finally, I give thanks to God for his providence. While science concerns itself with that which St. Thomas Aquinas would refer to as the "nature and activity of secondary causes", it is ultimately subservient to the Primary Cause.

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## List of abbreviations

**α -** Alpha

**~ -** Approximately

**β -** Beta

**β-PFT** - β-pore forming toxin

**% -** Percent

**A360/A980** - Ratio of Absorbance at 360 nM to Absorbance at 390 nM

**A2M -** Alpha2 Macroglobulin

**ANOVA** - Analysis of Variance

**ASW -** Artificial Spring Water

***Bg*GRN** - *Biomphalaria glabrata* progranulin

***Bg*TLR** - *B. glabrata* Toll-like receptor

***Bg*MIF** - *B. glabrata* Migration Inhibitory Factor

***Bg*TEP** - *B. glabrata* Thioester-containing Protein

**BLAST** - Basic Local Alignment Search Tool

**bp -** Base Pairs

**BSA** - Bovine Serum Albumin

**°C** - Degrees Celsius

**CBSS** – Chernin's Balanced Salt Solution

**cDNA** - Complementary Deoxyribonucleic Acid

**$CO_2$ -** Carbon dioxide

**CREP** - C-type Lectin-related Protein

**Ct -** Cycle threshold

**Cu/Zn** - Copper/Zinc

**3D** - Three-dimensional

**ΔΔCt** - Delta-Delta Cycle threshold

**DAMP** - Damage-associated Molecular Pattern

**DAPI -** 4,6-Diamidino-2-phenylindole

**DMEM** - Dulbecco's Modified Eagle's Medium

**dNTP** - Deoxynucleotide xxiii

**dpc** - Day(s) Post- challenge

**dsRNA** - Double-stranded Ribonucleic Acid

**EDTA** - Ethylenediaminetetraacetic acid

**ELISA** - Enzyme-linked Immunosorbent Assay

**ERK1/2** - Extracelluar Signal-Regulated Kinase 1 and 2

**E/S** – Excretory/secretory

**FBS** - Fetal Bovine Serum

**FREP** - Fibrinogen-related Protein

**g -** Gram or Gravity

**GAPDH** - Glyceraldehyde 3-Phosphate Dehydrogenase

**GRC -** Guadeloupe Resistance Complex

**GREP** - Galectin-related Protein

**GRN -** Granulin

**Hr(s) -** Hour(s)

**HCl -** Hydrochloric acid

**hpc** - Hour(s) Post challenge

**HRP -** Horseradish peroxidase

**ICR** - Interceding Region

**IgG** - Immunoglobulin isotype G

**iNOS** – inducible Nitric Oxide Synthase

**IgSF** - Immunoglobulin Superfamily

**IL -** Interleukin

**kDa** - Kilodalton

**LC-MS/MS** - Liquid Chromatography, tandem Mass Spectrometry

**LPS** - Lipopolysaccharide

**M -** Molar

**mA -** Milliampere

**mg -** Milligram

**MIF** - Macrophage Migration Inhibitory Factor

**MIP –** Macrophage Inflammatory Protein

**mL -** Milliliter

**mm -** Millimeter

**mM -** Millimolar

**MMP** – Matrix-Metalloprotease

**mRNA** - Messenger RNA

**n -** Sample size

**NF-kB** - Nuclear factor kappa-light-chain-enhancer of activated B cells

**ng -** Nanogram

**nM -** Nanomol**ar**

**Oligo** - Oligonucleotide

**PAMP** - Pathogen-associated Molecular Pattern

**PBL** - Polymorphic blood Leukocytes

**PBMC** - Polymorphic Blood Mononuclear Cells

**PBS** - Phosphate-buffered Saline

**PGRN** - Progranulin

**PKC** - Protein Kinase C

**PMA** - Phorbol 12-Myristate 13-Acetate

**PRR** - Pattern Recognition Receptor

**qRT-PCR** - Quantitative Real-Time Polymerase Chain Reaction

**ROS -** Reactive Oxygen Species

**s -** Second(s)

**SD -** Standard Deviation

**SDS-PAGE** - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

**SEM** - Standard Error of the Mean

**siRNA** - Short Interfering Ribonucleic Acid

**SmCI-1** – *S. mansoni* Cercarial Invadolysin - 1

**SmLeish** – *S. mansoni* Leishmanolysin

***Sm*PoMuc** - *Schistosoma mansoni* Polymorphic Mucins

**SP -** Signal Peptide

**SSS -** Sterile Snail Saline

**TEP** - Thioester-containing Protein

**TLR** - Toll-like receptor

**TBS** - Tris-buffered Saline

**TBS-T** - Tris-buffered Saline containing 0.1% Tween-20

**TNF –** Tumour necrosis factor

**U - Unit(s)**

**µg -** Microgram

**µL -** Microliter

**µm -** Micrometer

**µM -** Micromolar

**V -** Voltage

**WHO -** World Health Organization

**WSH** - Whole Snail Homogenate

**X -** Times

# Chapter 1 – General Introduction

## 1.1 Overview

### 1.1.1 Overview of schistosomiasis

Among the parasitic diseases humanity currently faces, schistosomiasis ranks second to only malaria in terms of both death and disease burden. This deadly and devitalizing disease is caused by parasitic flatworms of the genus *Schistosoma*. Three species: *Schistosoma mansoni*, *japonicum*, and *haematobium* result in most human cases worldwide, while *Schistosoma mekongi*, *guineesis* and *intercalatum* are responsible for smaller localized outbreaks (1). These parasites employ a complex life cycle featuring an obligatory molluscan intermediate host in which they undergo asexual replication, and a human or other mammalian definitive host in which mating and egg production occurs. Initially, eggs hatch in a freshwater environment, and infective miracidia are released into the environment, where they actively seek out a suitable snail intermediate host, a process which is mediated by their capacity to recognize mucous-originating glycoconjugates emanating from the mollusc (2,3). The presence of a suitable snail host restricts the geographical distribution of these parasites, with *S. mansoni* requiring the presence of snails of the genus *Biomphalaria*, *S. haematobium* requiring snails of the genus *Bulinus*, and *S. japonicum* requiring snails of the genus *Oncomelania*. Once having arrived inside of a suitable snail, the miracidia shed epidermal plates and begin transformation into the larval stage termed sporocysts. These sporocysts undergo asexual replication and will go on to release the human infectious life cycle stage known as cercaria into the surrounding environment. Cercaria will then begin to seek out a human host, which they penetrate via the skin (4–7). These cercaria then undergo significant morphological and biochemical changes before using either the lymphatic or circulatory system to

migrate to the host's lungs, and then continuing to the venules of either the hepatic portal system (in the case of *S. mansoni* and *S. japonicum*) or the venus plexus surrounding the bladder in the case of *S. haematobium* (8). Finally, mated adults will go on to produce and fertilize hundreds of eggs per day, which are subsequently released in either the feces or urine, thereby beginning the life cycle anew.

### 1.1.2 Pathologies associated with human schistosomiasis

Unfortunately, schistosomiasis causes a significant amount of pathology throughout the duration of infection in human hosts. Current estimates suggest between 200,000 to 300,000 deaths caused annually, while conservative estimates regarding disability adjusted life years (DALYs) lost to schistosomiasis range from 1.9 million to 3.3 million (9–11). Some have even argued that such DALY estimates fail to consider the full effect of subclinical infections and suggest that DALYs lost to schistosomiasis may in fact range from 3 to 70 million (12,13). While a precise quantification of the worldwide effects of this disease may remain in question, meta analyses reveals a clear association between infections and lowered school attendance, scholastic achievement, learning, and memory outcomes among school aged children (9).

While most of this pathology may be due to the chronic stages of the disease, acute pathology is also seen with schistosomiasis, especially in travellers visiting endemic areas. Such individuals have been shown to develop what is known as Katayama syndrome, a disease named after the region in Japan in which it was first recorded (14). Due to the observation that this syndrome appears 18-84 days after contact with infected water sources, it is expected that it is caused by both the antigens associated with migrating larvae, as well as the abundance of antigens associated with the beginning of egg deposition. Pathologies associated with this acute form of the disease include fever, headache, bloody diarrhea, myalgia, and respiratory problems likely to be

caused by the passage of larvae through the lungs (14,15). Fortunately, treatment resolves these issues in most cases.

Most of the pathology and deleterious effects of schistosome infections are caused by the chronic form of the disease. In the cases of both *S. mansoni* and S. *japonicum*, much of this is caused by the immunological response to schistosome eggs seen in both the GI tract and liver. In order to isolate eggs from the rest of the body, there is a large granulomatous response to eggs in the liver, which features the encapsulation of the eggs with monocytes, neutrophils, mast cells, eosinophils, alternatively activated macrophages, fibroblasts, and lymphocytes (16). This inflammatory response has the unfortunate side effect of calcifying parts of the liver, while also blocking capillaries in the hepatic portal system. This results in a considerable building up of pressure, which eventually leads to hepatomegaly, splenomegaly, fibrosis of the liver, and, in advanced cases, pipestem periportal cirrhosis (17,18).

In the case of *S. haematobium,* most pathology is the result of immune responses to eggs in individual's bladders, as well as their genital tracts. Women bear the brunt of these negative effects, with eggs causing secondary infections, bleeding, decreased fertility, and increased chances of miscarriage (19). An ill-fated side effect of S. *haematobium* is the increased susceptibility to HIV that accompanies infections, with odds ratios of infection ranging from 2.9 to 4.0 (20–22). Many different reasons have been suggested for this observance, including an increased permeability of the genital tract due to egg migration, an increased presence of T helper cells in the genital tissue, which in turn are stimulated by the trematode to display more HIV co-receptors, as well as an increased presence of leukocytes and pro-inflammatory cytokines in male semen (23,24). In addition to increased chances of infection, it appears that coinfection with urogenital schistosomiasis aids in the growth of HIV, an observation which can be partially

canceled by the administration of the schistosomicidal compound praziquantel (25). Finally, some evidence has emerged that *S. haematobium* infections are associated with bladder cancer (26).

Many have attributed much of the child malnutrition and cognitive delays associated with *Schistosoma* infections to the anemia present therein. Decreases in iron and hemoglobin are seen in these infections, correlating negatively with egg outputs, and this decrease can be countered with praziquantel treatments (27–29). Many different reasons for this anemia have been suggested, with a loss of iron via bleeding, sequestration of RBCs in the spleen, autoimmune hemolysis of RBCs, (interleukin) IL-6 mediated increase in hepcidin mediated iron sequestration, and TNF-α mediated decrease in erythropoietin production all having been suggested (28,30). Additionally, in heavy infections, it may be that the consumption of RBCs by adult worms may also contribute to anemia, with adult males and females consuming an estimated 39,000 and 330,000 cells respectively per hour (31,32).

The deposition of eggs in the blood stream often leads to their aberrant migration throughout the rest of the body. This results in the presence of eggs throughout humans, which are seen most often in abdominal organs, as well as the lungs, where calcification can lead to pain, a persistent cough, and bronchitis (18). Most interesting is the observation that the haphazard dispersal of eggs can occasionally result in their presence in human brains. All three of the main schistosome species have been found to occasionally feature egg placement in the human brain and spinal cord, an unfortunate event which results in severe headaches, epileptic seizures, loss of balance and motor function, as well as occasional instances of paraphasia (15,33). Some have even suggested the possibility that such occurrences may be due to the presence of mated adult pairs in the vasculature surrounding brain who continuously deposit eggs in the immediate vicinity of neural tissue (34).

### 1.1.3 Drugs available for combatting schistosomiasis

The predominant drug used for the treatment of schistosomiasis in the modern era remains praziquantel (PZQ). Broad screening of a variety of compounds led to the identification of PZQ in the mid to late 1970s by Bayer AG and Merck KGaA in Germany (Elberfeld and Darmstadt), followed by the capacity to synthesize the drug in large quantities (35,36). Another drug, oxamniquine, has been used but has fallen out of favor due to its inability to effectively kill *S. japonicum* and *S. haematobium* (37). Praziquantel remains capable of killing all three of the major human schistosomiasis causing *Schistosoma* species, and retains a great deal of popularity due to its high cure rates (regularly between 70 and 90%), its relative absence of notable side effects, its stability during transport, its ease of administration (via tablet, taken orally), as well as its cost, which remains under 50 US cents for administration and under 1.00 USD for administration and delivery (13,38). Efforts have been made to make the drug widely available, with Merck KGaA pledging in 2018 to donate 200 million tablets over 10 years (38). Fortunately, Merck expanded significantly upon their promise, and have donated over 1 billion tablets since 2007, allowing for the treatment of over 400 million people (39). While this has allowed for an increase in the percentage of people requiring treatment to go from 13% in 2010 to 42.4% in 2018, there has not been a corresponding decrease of proportional magnitude in the amount of infected individuals, as an estimated 237 million people had schistosomiasis in 2010, while 229 million people were still estimated to have the disease in 2018 (40). This is due in large part to the fact that re-exposure to environments harbouring infected snails is commonplace, and infection prevalence in school aged children can return to baseline 1-2 years after treatment with PZQ (41). Fortunately, mass drug administration efforts have not been in vain, seeing as this lack of reduction in overall infections has been accompanied by an apparent reduction in cognitive delays associated with childhood

malnutrition, a decrease in the spread of HIV, and an estimated 1/4$^{th}$ to 1/3$^{rd}$ total reduction in the amount of DALYs lost to the disease annually (9,42–44).

Despite its widespread use, a comprehensive understanding as to the precise mechanisms by which praziquantel enacts its schistosomicidal activity remain somewhat elusive. To date, several different hypotheses have emerged as to how praziquantel can kill adult schistosomes. The first suggested is that PZQ can increase the Ca2+ influx into worms, resulting in muscle fibers being forced into a permanent state of contraction, thereby dislodging, and killing adults. This is supported by the finding that calcium channel beta subunits from *S. mansoni* and *S. japonicum* (*Sm*Ca$_v$βA and *Sj*Ca$_v$β) are significantly different from mammalian beta subunits, and that their pairing with mammalian alpha subunits results in a lowered current which can be significantly heightened by the addition of praziquantel (45,46). Another suggestion has been that praziquantel may target ATP-binding cassette transport proteins, seeing as PZQ has been shown to bind and inhibit SMDR2, a homologue of the efflux pump P-glycoprotein (47). More recently, a transient ion receptor channel (*Sm*TRPM$_{PZQ}$) has been identified as a binding partner of PZQ, thereby emerging as a target for further drug development (48).

Seeing as praziquantel remains the only drug utilized in widespread mass drug administration efforts, considerable time has been spent examining the possibility of praziquantel resistance in schistosomes. To date, significant evidence for the emergence of such resistant populations fails to reach levels of considerable concern, but a quick examination of the evidence is merited. In a study performed in Egypt some individuals (2-4%) treated with the standard 40mg/kg PZQ continued to shed eggs, while a study in Senegal revealed suboptimal cure rates (18-36%) that remained consistent despite varying treatment concentrations (49,50). In such cases, parasites isolated from individuals failing to clear infection after treatment yielded parasite

subpopulations slightly more resistant to praziquantel treatment than their counterparts isolated from individuals who cleared infection after treatment (51). Such heightened resistance levels could be preserved for 5-6 years via passage through mice (51,52). In addition to this, selection for PZQ resistance in the lab has been successfully achieved, although the fact that sequence and expression levels of *Sm*$Ca_V\beta A$ remain the same between strains of varying susceptibility have left researchers looking for possible explanations as to how such resistance emerges (53,54). Fortunately, observed increases in resistance have only been slight, and their 3X increases in ED50s pale in comparison to those that can result from selection for oxamniquine resistance, which can approach a 1000-fold increase in ED50s (37,55). To date, praziquantel resistance has not been shown for *S. haematobium*, although sometimes individuals may fail to clear infections after standard doses of the drug (56). Interestingly, PZQ's inability to effectively kill larval schistosomes may explain the phenomena of failed treatments, as reinfection resulted from re-exposure or the ongoing presence of larval worms in the body are a more likely explanation of failed treatments than widespread resistance (57). Regrettably, reinfection can return infection rates to pre-treatment levels in as little as two years, and recent investigations in Zanzibar demonstrate that high reinfection rates can occur even after 7 years of regular MDA in hotspot communities (41,58). Fortunately, new drugs are beginning to emerge as candidates for schistosome treatment programs such as the anti-malarial drug artemisinin, which has been shown to kill larval schistosomes, and may therefore help shed more light on any potential development of PZQ resistance (59). This all serves to highlight that although PZQ resistance is not a huge problem as of now, the possibility of increased resistance exists, and is something to monitor for and prepare alternative treatment methods for should such an unfortunate development present itself. Fortunately, numerous research groups continue to look at alternative treatment options for

schistosomiasis, with high throughput screening leading to the discovery of several candidate treatments showing high schistosomicidal activity in mouse model systems (60). In addition to high throughput screening of new candidates, modification of well known schistosomicidal drugs has also been undergone, with modification of oxamniquine leading to an increased worm-killing capacity (61).

### 1.1.4 The use of molluscicides in snail control efforts

Historically, numerous control mechanisms have been employed for the elimination of schistosomiasis including water quality improvement, drug administration, and snail control. Recently, a comprehensive review and analysis determined that the control of the snail intermediate host is the most effective manner by which to control the spread of schistosomiasis (62). Some of the earlier schistosomiasis control initiatives began in Egypt, where copper sulphate was used for the targeted killing of snail populations. Despite its success in achieving snail management and its minimal effects on both human and crop health, copper sulphate's popularity declined due to issues regarding difficulty of procurement (63). Other compounds would emerge as high quality molluscicides such as sodium pentachlorophenate and niclosamide (commonly referred to as Bayluscide), which are prized for their capacity to kill not only adult molluscs but also their eggs (64). During the 1950s to the 1970s, the absence of a suitable drug for mass drug administration tactics would lead to molluscicides, led by Bayluscide during the 1960s, to be the predominant method of schistosomiasis control (64,65). After the advent of praziquantel, schistosome treatment programs would go on to be largely composed of mass drug administration efforts, with places such as China shifting towards a more comprehensive strategy composed of a mixture of MDA, molluscicides, and snail habitat control (62,66). Despite the appreciable effect of mass drug administration (MDA) campaigns in improving the health of people receiving

treatment, the frustratingly low success in reducing infected population sizes led both the 2012 London Declaration on Neglected Tropical Diseases, as well as the 2012 World Health Assembly to both call for a renewed look at non-pharmaceutical interventions for schistosomiasis (65,67). Given that successful elimination of the disease is linked most strongly with successful snail control, future control efforts might consider the regular use of niclosamide, coupled with either MDA or targeted treatment of heavily infected/ high risk individuals.

### 1.1.5 Possible alternatives to molluscicides and mass drug administration efforts

Alternatives to mass drug administration efforts and molluscicide use have also been brought forward in the fight against schistosomiasis. In some cases, groups have argued for the implementation of water recreation areas for children to swim in a snail free environment. Such areas have been used before and shown to decrease infection rates, although their feasibility remains in question due to high costs associated with installation (68). A move away from using rivers to wash and swim in is often seen as undesirable, as numerous social interactions occur during these events, while swimming pools are often viewed as "dirty" due to the amount of mud and dirt brought into them on the feet of children (69). Others have suggested a more biological approach, featuring the reintroduction of natural snail predators such as freshwater prawns into habitats in order to effectively target and eliminate disease transmitting snails (70). Such a tactic has already been shown to be promising in the field (71). Despite initial success, questions remain as to the safety of introducing such predators to environments in which they would be novel, seeing as unperceived effects on an environment are notoriously difficult to predict. Finally, some groups have simply sough to reduce snail habitat, a mechanism most famously employed in China, where both flooding and draining of natural *Oncomelania* habitat has been employed with success in reducing *S. japonicum* transmission (66).

### 1.1.6 Progress towards a schistosomiasis vaccine

To date, there is no regulatory-body-approved and phase-3-proven schistosomiasis vaccine available for use in humans. This is unfortunate, as a safe, reliable, and effective vaccine could aid a great deal in accompanying mass drug administration efforts to control schistosome infections in developing countries severely in need of sustainable control efforts. The biological basis for an effective vaccine has been demonstrated, given the observation that infection prevalence and intensity peak around 10-14 years of age before declining in both regards as individuals age (1). To add to this both IgG and IgE responses to adult worm antigens continue to increase with age (72). These observations suggest the development of an anti-schistosome immune response accompanied by the appearance of noticeable levels of concomitant immunity.

Many different vaccine candidates have been suggested for each of the three predominant human-schistosomiasis causing schistosomes. These potential targets vary in their function and cellular location, as well as the life cycle stage at which they are expressed. Some are excreted, others are surface bound, and recently even more targets have been located within extracellular vesicles secreted by adult worms (73). A list of examined antigens is laid out in Table 1 of the work of Wilson et al (74). Ultimately, it has been suggested that the protective results seen in many vaccine trials in mice may not in fact be due to the generation of effective immune responses, but rather may be a symptom of cytokine induced inflammation in the lungs leading to an increased number of migrating schistosomula ending up trapped within the pulmonary alveoli (74). This is supported heavily by the observations that a low (~32%) proportion of cercaria will go on to develop into adult worms in mice, an observation which is not altered by rendering mice severely immunocompromised by irradiation, thereby suggesting a non-immune based mechanism of parasite death (75). Additionally, antigens often lack an additive effect when co-administered

which would be considered highly unusual given an immune based parasite clearance (76). It has been suggested that delayed hypersensitivity reactions in mice lead to vascular leak syndrome in lung capillaries, thereby expelling developing larvae from the circulatory system (74). This seems likely, given that extending infections from 15 to 30 days post last immunization leads to a drop off in protection induced by both the Sm22.6 and Sm29 vaccines (77–79). Fortunately, some schistosome vaccines have been shown to be functional in non human primates such as baboons in which a radiated larvae vaccine led to 80% protection, thereby suggesting the existence of an appropriate model system in which to study such phenomena (80).

The World Health Organization has been a key proponent of several candidate schistosomiasis vaccines and has continued to help fund research into promising targets. To date, three schistosomiasis vaccines are currently undergoing various stages of human clinical trials, while another may soon begin trials after showing high levels of protection in baboons (10,11,81). The latter is Sm-p80, a cysteine protease identified and examined by Dr. Afzal Siddiqui at Texas Tech University Health Sciences which has been shown to reduce worm burden, the amount of eggs in tissue, and the amount of eggs in the feces of baboons (82). Varying formulations of this vaccine, from recombinant protein to DNA based vaccines have all shown promise in reducing infection parameters in the baboon model system (83). Phase 1 clinical trials are set to begin, with Sm-p80 set to be combined with the adjuvant glucopyranosyl lipid A under the names "SchistoShield" (84). Other groups have employed a Sm-p80-based DNA vaccine, which has also been shown to reduce worm burden and egg output in baboons by eliciting a strong immunoglobulin and Th1 type response, but has as of yet not been employed in human clinical trials (85).

Of the three vaccines to undergo clinical trials in humans, the Sm-TSP-2 vaccine is in the earliest of stages of testing, with only phase 1 trials having been performed. Sm-TSP-2 is composed of the extracellular loop portion of a key *S. mansoni* tetraspanin involved in tegument integrity (10,11). After showing high levels of protection in murine models, phase 1 clinical trials in Texas showed acceptable safety and immunogenicity levels, and another phase 1 trial is underway in an endemic area in Brazil (86). Phase 2 trials in Uganda are currently being planned (11).

Another of the three candidates undergoing trials is Sm14, otherwise known as the *S. mansoni* cytoplasmic fatty acid-binding protein. Given the incapacity of helminths to properly synthesize the fatty acids they require, a dependence on host synthesis of these essential molecules renders such a protein to be an interesting target for vaccine development. After having been developed by the team of Dr. Mariam Tender in Rio de Janeiro, Brazil, this molecule has been combined with a GLA-SE adjuvant and has completed a phase 1a clinical trial in 20 male volunteers from a non-endemic area in Brazil which proved its safety, with no significant side effects seen (86). Phase 2a trials in 30 men in an endemic area in Senegal resulted in increased antibody titers to the protein, while also proving to be safe. Phase 2b has been planned to take place with 95 school children aged 7-11 years old in the same endemic area of Senegal (87). Interestingly, this vaccine does not result in a significant increase in IgE production, throwing doubt into the future effectiveness of such a treatment (10).

Lastly, there is the rSh28GST vaccine, which has progressed the farthest in terms of human clinical trials among schistosome vaccine targets. This recombinant vaccine was designed by a group of researchers at the Institute Pasteur de Lille in France and is based on the 28kDa glutathione S-transferase of *Schistosoma haematobium*, although it has been shown to bear

significant similarity to the homologous protein in *S. mansoni.* This molecule has been shown to be present in many of the mammalian life cycle stages of schistosomes, and aids in the production of IL-10 from host cells, making it a potent immunomodulator (88). Unfortunately, after proving to be safe and immunogenic in phase 1 and phase 2 trials, phase 3 trials in Senegalese children given 4 doses of vaccine, treated with praziquantel, and assessed for eggs in urine 3 years latter, proved that the vaccine is incapable of generating protective immunity. The entire reason for this failure is not known but it has been hypothesized that the vaccine's tendency to induce the production of IgG1,2, and 4 instead of the protective IgG3 may be partially to blame (89).

### 1.1.7 Rationale for thesis work

The proceeding discussion of the history of the fight against schistosomiasis highlights several key needs. The first of which is that alternative strategies for controlling and eliminating schistosomiasis are necessary given the shortcomings of current control programs, as well as the severity of disease burden that persists in endemic nations.

Given the key observation that schistosome control efforts are most successful when snail control efforts are employed, a better understanding of the determinants of infection at the host/parasite molecular interface are required to better comprehend transmission dynamics, population infection rates, and resistance profiles. This could theoretically allow for the selection of naturally resistant snail populations, or the design of resistant snails via genetic modification techniques. Important in such an endeavor is not only an understanding of the snail immune system, but also a deeper knowledge of the factors used by *S. mansoni* in order to overcome the immune response of a snail.

While a better understanding of the determinants of infection in the intermediate host are important, a more comprehensive knowledge of the factors involved in survival in the human host are essential in the development of novel therapeutic and vaccine targets. To date, despite a wealth of knowledge about the immunomodulatory factors employed by schistosomes, PZQ treatment remains unaccompanied by a successful vaccine, leaving millions at risk of reinfection. To design better treatments for humans, a better understanding of the immune modulating factors produced by schistosomes at a larval stage is necessary, given that larval schistosomes are capable of being killed by components of the immune response in humans that they likely encounter early during infection, but can circumvent.

## 1.1 Outline of the Thesis

This thesis focuses on understanding the functions of key *S. mansoni* invadolysins in combatting the immune system of *Biomphalaria glabrata,* as well as their function in combating mammalian immune responses in the skin. It is divided into 7 chapters and an appendix. Chapter 1 serves as an introductory chapter that summarizes the state of schistosomiasis worldwide, outlines the thesis, and discusses the specific aims and objectives associated with the work performed therein. Chapter 2 is a literature review summarizing what is known about immune evasion techniques of schistosomes, as well as the form and function of invadolysins in facilitating pathogenesis in parasitic organisms, and what is known about schistosome invadolysins to date. Chapter 3 lays out the materials and methodologies I employed while conducting the research laid out in this thesis. In chapter 4, the function of SmLeish in facilitating infection establishment in *B. glabrata* is presented. The work in chapter 5 shows my investigations into the roles of SmLeish and SmCI-1 in cleaving mammalian host factors and targeting immune processes. Chapter 6 is an in-depth investigation of the roles of SmLeish and, more importantly, SmCI-1, in altering cytokine

production in mammalian immune cells. Chapter 7 is a general discussion of the results of this thesis work, their significance, what remains to be investigated, and the translational value of the work that was conducted.

### 1.2 Aim and specific objectives of the thesis

The overarching aim of my thesis was to further our understanding of the immune evasion mechanisms of schistosomes. I was able to do this by furthering our understanding of how key schistosome invadolysins are used to downregulate immune responses in both the snail and mammalian hosts of *S. mansoni* with specific focus on the parasite's use of invadolysins. The specific objectives of my thesis were to:

1. Molecularly and functionally characterize the role of an *S. mansoni* Leishmanolysin like protein (SmLeish) during infection of *B. glabrata* and examine possible involvement during mammalian infections.

To achieve the characterization of SmLeish, the following four sub-objectives were pursued:

i. Determine SmLeish transcript expression and develop an antibody to examine protein expression during infection.
ii. Develop a recombinant SmLeish and assess its MMP activity and effect on *S. mansoni*-resistant (BS-90) and susceptible (M-line) *B. glabrata* haemocyte chemokinesis.
iii. To knock down SmLeish expression in *S. mansoni* miracidia and assess its role in infection establishment in M-line *B. glabrata.*
iv. Assess SmLeish's ability to cleave key mammalian immune and structural proteins and examine its ability to alter cytokine production in mammalian leukocytes.

2. Molecularly and functionally characterize the role of an *S. mansoni* invadolysin (SmCI-1) featured prominently during the initial stages of mammalian infection.

To achieve the characterization of SmCI-1 the following five sub-objectives were pursued:

i. Develop an anti-SmCI-1 antibody and determine the location of SmCI-1 in *S. mansoni* cercaria.
ii. Make a recombinant SmCI-1, as well as a SmCI-1 mutant with an inactive catalytic site and assess their MMP activity.
iii. Examine the ability of SmCI-1 to cleave key host structural and immunological proteins.
iv. Test the ability of SmCI-1 an MutSmCI-1 to alter cytokine production in mammalian immune cells activated with various stimulants.
v. Determine the feasibility of dsRNA-mediated knock down of SmCI-1 in cercaria for subsequent *in vivo* infections.

# Chapter 2 - Literature Review[1]

## 2.1 Introduction

### 2.1.1 Immune evasion strategies of pathogens

It is, perhaps, one of the most obvious statements to make in the field of infectious disease to say that pathogens have amassed a complex variety of mechanisms to survive and thrive within their hosts. The evolution of these survival mechanism has been a key factor in the development of life on Earth and has led to the development of complex immune systems designed to fight off invading pathogens. The constant immunological battle between pathogen and host, and the ensuing immunological arms race between them, has commonly been referred to as the Red Queen hypothesis, a play on the idea that one must run as fast as they can merely to stay in one place (90). This race between pathogens and their hosts in a bid to survive and thrive has led to a high degree of variability in the manner in which different types of pathogens combat immune responses.

Each of these survival methods is tailored to the necessities of a particular pathogen type. Viruses, for example, must overcome T cells, NK cells, and host cell apoptosis. To do so, viruses have evolved different immune evasion techniques, including blockage of antigen presentation on major histocompatibility complex (MHC) class I, the synthesis of cytokine mimics (virokines) and their receptors, the evasion of NK cell-mediated killing via production of (MCH) mimics, and

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[1]Sections of this chapter (2.2 Immune evasion strategies of Schistosomes) have been published in the following manuscript

Hambrook, J. R., & Hanington, P. C. (2021). Immune evasion strategies of schistosomes. *Frontiers in Immunology*, *11*, 624178.

a hypervariability of surface binding proteins (91–93). Bacteria, requiring ways of surviving attack via macrophages, employ different survival mechanisms, including the avoidance of macrophages, as well as different methods of either escaping from the phagosome, or preventing its fusing with a lysosome (94). Multicellular helminths, once having grown to a size where lone immune cells are incapable of killing them on their own, often develop immune-resistant exteriors capable of self-renewal, and able to withstand attacks by granulocytes (95). The exterior surface of such worms often also features proteins capable of host-immune-factor uptake and molecular mimicry. Additionally, helminths release factors capable of reducing the development and recruitment of eosinophils, given the importance of such cells in anti-helminth immune responses, as well as molecules capable of modulating the functions of other key cell types during infection (96).

What is true of different types of pathogens in general is also true of different types of parasites. The designation of an organism as a "parasite" is not made by a location in the evolutionary tree of life, but rather by participation in a particular lifestyle, meaning parasites exist as a wide variety of organisms, both single celled and multicellular in nature. This large diversity of parasite organisms has resulted in numerous immune evasion techniques, which vary from species to species. Some parasites, such as the blood borne *Trypanosoma brucei* rely on variable surface glycoproteins, others, such as schistosomes, have been hypothesized to employ molecular mimicry of host factors, while yet others, such as *Leishmania spp.* prefer to avoid death by inhabiting host phagocytic cells (97). These different immune evasion strategies depend not only on the type of pathogen, but also on the type of host. Parasites with complex life cycles featuring more than one host are ideal candidates for studying different methods of immune evasion, given the large differences in immune responses seen between different hosts. This is especially true of

digenean trematodes, who must contend not only with the immune system of a vertebrate host, but also the immune system of gastropod snails (95).

### 2.2 Immune evasion strategies of Schistosomes

Human schistosomes combat the unique immune systems of two vastly different hosts during their indirect life cycles. In gastropod molluscs, they face a potent innate immune response composed of variable immune recognition molecules and highly phagocytic haemocytes. In humans, a wide variety of innate and adaptive immune processes exist in proximity to these parasites throughout their lifespan. To survive and thrive as the second most common parasitic disease in humans, schistosomes have evolved many techniques to avoid and combat these targeted host responses. Among these techniques are molecular mimicry of host antigens, the utilization of an immune resistant outer tegument, the secretion of several potent proteases, and targeted release of specific immunomodulatory factors affecting immune cell functions. This review seeks to describe these key immune evasion mechanisms, among others, which schistosomes use to survive in both of their hosts. After diving into foundational observational studies of the processes mediating the establishment of schistosome infections, more recent transcriptomic and proteomic studies revealing crucial components of the host/parasite molecular interface are discussed. In order to combat this debilitating and lethal disease, a comprehensive understanding of schistosome immune evasion strategies is necessary for the development of novel therapeutics and treatment plans, necessitating the discussion of the numerous ways in which these parasitic flatworms overcome the immune responses of both hosts.

### 2.2.1 Introduction to schistosomes/immunosuppression

All organisms must deal with threats in their environments to survive and thrive. The same is true of parasites, whose environments put them constantly at odds with host immune systems. Whereas some parasites have only one host and must therefore develop immune evasion/suppression tactics against only one type of immune system, others employ indirect life cycles, which means they encounter diverse immune systems that utilize unique strategies geared towards their death and destruction. Schistosomes, which are digenean trematodes of the genus *Schistosoma*, are one such parasite. These flatworms employ an indirect life cycle, alternating between a gastropod mollusc as an obligate intermediate host and a definitive vertebrate host such as a human (1). Schistosomiasis, the disease caused by schistosome infection of humans, is widely considered the second most important parasitic disease from a public health perspective, trailing only malaria. It afflicts an estimated 206 million people, killing up to 200,000 annually, and resulting in an estimated loss of between 1.9 and 3.3 million disability adjusted life years (9–11). Thus, a comprehensive examination and understanding of how these parasites evade the immune systems of such drastically different hosts has been and continues to be the focus of extensive investigation (13,38,98). This review seeks to highlight the foundational work which demonstrated schistosome evasion/suppression of the immune response in both host systems. Those specific components that have been implicated as being immunomodulatory, and other uncharacterized factors that bear similarities to known parasite immunomodulatory factors will be discussed.

### 2.2.2 The gastropod immune system

After emerging from its egg into freshwater, the miracidial stage of the schistosome life cycle seeks out a suitable snail host. Although the immune systems of snails lack the overall complexity and antibody mediated adaptive response seen in mammals, these invertebrates combat

schistosome infections using a multitude of immune strategies resulting in larval damage and ultimately the killing of invading schistosomes (**Figure 2.1A**) (99,100). Research into the immunological underpinnings of snail/schistosome interactions has been undertaken largely using the *Schistosoma mansoni/Biomphalaria glabrata* model system that exploits numerous *B. glabrata* strains that display different compatibility profiles with specific strains of *S. mansoni*. This allows for detailed examination of both the host immune response as well as parasite survival strategies (101,102).

Killing and elimination of invading miracidia and the sporocysts that they transform into relies primarily on coordination between the cellular and humoral immune responses of the snail. The immune cells, termed haemocytes, locate, surround, and encapsulate invading parasites. *B. glabrata* also features numerous immune molecules hypothesized to function in recognition of invading schistosomes such as the wide variety of leucine rich repeat-containing receptors including some canonical toll-like receptors (TLRs) (**Figure 2.1A**) and other pattern recognition receptors (PRRs) such as peptidoglycan recognition receptors (PGRPs), variable immunoglobulin and lectin domain-containing molecules, and the proteins encoded for in the Guadeloupe resistance complex (GRC) (99,103–106).

As mentioned above, the humoral branch of the *B. glabrata* immune system is characterized as being important for recognition and clearance of *S. mansoni* and is largely composed of pattern recognition receptors (PRRs). Many of these PRRs have been found to contain at least one immunoglobulin superfamily (IgSF) domain next to an interceding region (ICR) (**Figure 2.1A**). Some of these ICRs are linked to galectin like domains, while others are linked to C-type lectin domains. These PRRs are referred to as Galectin-related proteins (GREPs) and C-type lectin related proteins (CREPS) (103).The most well characterized PRR in *B. glabrata*

are the Fibrinogen related proteins (FREPs), which feature one or two IgSF domains linked to a fibrinogen like domain by an ICR (105). At least one FREP, FREP3, has the capacity to bind the surface of sporocysts and act as a opsonin, while siRNA mediated knockdown of FREP3 reduces resistance to *S. mansoni* challenge (107). *B. glabrata* FREPs (*Bg*FREPs) possess the capacity to somatically diversify, which is unique among invertebrates (108). Given the observation that schistosomes are in possession of polymorphic surface mucins that interact with FREPs, this somatic diversification suggests a possible form of adaptive immunity. This potential adaptive immunity is the subject of numerous investigations (109–111). In addition to these immunoglobulin-like domain containing PRRs, *B. glabrata* also possesses several thioester containing proteins (TEPs), which resemble complement component C3 in both form and function (112). The humoral immune response is not merely relegated to pathogen recognition; a β-pore forming toxin (β-PFT) known as biomphalysin is also able to directly kill *S. mansoni* sporocysts (113). Rather than functioning independently, at least three humoral immune components (*Bg*FREPS, *Bg*TEP, and biomphalysin) function as a unit to target and facilitate elimination of developing sporocysts (**Figure 2.1 D**) (109).

### 2.2.3 Molecular mimicry in the gastropod host

Avoiding immune recognition and destruction is mediated by a wide array of immune evasion techniques employed by invading miracidia and developing sporocysts (**Figure 2.1B**). First referred to as molecular mimicry in 1964, the process of parasites displaying antigens similar to those of their hosts has been a research topic of interest in numerous pathogen model systems (114,115). The phenomenon has been shown to span numerous parasitological phyla, with some helminths utilizing glycans to mask their presence to their hosts (116). Some parasitic nematodes

even employ glycans typically only found in vertebrates, suggesting that their use in parasites acts to hide the pathogen from the immune response (117).

The study of molecular mimicry in intermediate hosts was first reported in 1965 when in was observed that *B. glabrata* possessed similar antigens to those found in developing *Schistosoma mansoni* larvae (**Figure 2.1B**) (118). This was later confirmed by the development of polyclonal antibodies to haemolymph from *S. mansoni* resistant (10-R2) and susceptible (M-line) *B. glabrata* strains, with both reacting strongly with the surface of *S. mansoni* miracidia and sporocysts (119,120). This association persisted for at least 48 hours post transformation from miracidia to sporocyst, suggesting the developing larvae consistently share at least certain surface proteins during the first 48 hours of infection, during which, they are most likely to be targeted by the snail immune response (119). Similarly, antibodies raised against whole sporocysts interact with the surface of *B. glabrata* haemocytes (121). Although these cross-reactive immunoglobulin tests suggest some form of molecular mimicry, such studies lack the necessary specificity for examination of shared antigens. Observed cross reactivity may simply be due to shared glycosylation patterns between the two invertebrates, with a variety of carbohydrate epitopes being found in both animals (122,123). Often, such shared glycosylation patterns are featured at the surface of miracidia and sporocysts, although they may also be found in E/S products (3,124). Although such similarities have suggested the employment of molecular mimicry for several decades, potential targets for such a phenomenon have emerged more recently. Differences in N-glycosylation patterns of haemolymph in the resistant BS-90 strain *B. glabrata* featured less glycan epitope similarities to *S. mansoni* than a susceptible *B. glabrata* strain (*Bg*PR). These data provide support for the hypothesis that molecular mimicry might aid *S. mansoni* to avoid recognition by the varying lectins employed by *B. glabrata* as a means of immune recognition (125). This is

further supported by the observation that haemolymph from susceptible *B. glabrata* strains, such as the NMRI snail strain, features a greater abundance of schistosome-like glycan epitopes in their haemolymph than resistant BS-90 strain snails (**Figure 2.1B**). Larval transformation products (LTPs) released by the parasite during miracidium-to-sporocyst transformation also participate in glycan mimicry (**Figure 2.1B**). Host haemolymph proteins and circulating haemocytes are capable of reacting with LTPs (126,127). A differential binding pattern between LTPs and haemolymph proteins isolated from *B. glabrata* strains of varying compatibility with *S. mansoni* is observed using far-western blotting. This suggests the exploitation of differing glycan epitopes by *S. mansoni* during larval transformation (127). The attempts at mimicry made by *S. mansoni* are not limited to surface level epitopes, as sporocysts possess the capacity to produce host-like adrenocorticotropic hormone, which is processed by host haemocytes to become melanocyte-stimulating hormone, resulting in the rounding of haemocytes near the sporocyst (**Figure 2.1C**) (128).

### 2.2.4 Polymorphic mucins

While molecular mimicry may very well function in hiding the parasite from the immune response of the snail, a new set of molecules that are distinct to the parasite have emerged as possible determinants of survival: *S. mansoni* polymorphic mucins or *Sm*PoMucs (**Figure 2.1D**). These diverse proteins were first identified as part of a proteomics screen seeking to identify differentially abundant proteins produced by *B. glabrata*-compatible and incompatible strains of *S. mansoni,* and have gone on to be one of the most intensely studied components of resistance polymorphism in the *B. glabrata/ S. mansoni* system (111,129,130). *Sm*PoMucs consist of three distinct groups, each containing a characteristic C-terminal domain attached to a variable number of tandem repeats ranging from n=1 to n~55 (131). These repeats, heavily composed of serine,

threonine, and proline residues, allow for heavy glycosylation of these proteins, aiding in their classification as mucin-like molecules (131). Through a series of events including recombination, expression polymorphism, inheritable acetylation-based epigenetics, various mechanisms of post translational splicing, and differential glycosylation patterns, *S. mansoni* is capable of generating numerous different polymorphisms while only being in possession of an estimated 10 *Sm*PoMuc genes (**Figure 2.1E**) (132–134). While different *S. mansoni* strains vary in their *Sm*PoMucs transcript sequences and expression patterns, such differences are also seen on an individual level between sporocysts (132,133). In 2010, Moné and associates made the key observation that these diverse molecules could be found in association with somatically diversifying host immune molecules; FREPs and *Bg*TEP (110). This is significant seeing as research demonstrating that a *Bg*FREP/*Bg*TEP complex can render susceptible snail haemocytes and plasma nearly as deadly to *S. mansoni* sporocysts as plasma from *S. mansoni*-resistant *B. glabrata* strains. Given the fact that *Sm*PoMucs are found associated with the apical glands and E/S products of miracidia and sporocysts, *Sm*PoMucs variability should be considered a key mechanism by which the parasite discourages recognition by key humoral immune complexes both at the surface of the parasite and in the surrounding tissues (109,110,130,131). Given the somatic variation in *Bg*FREPs, and the highly polymorphic nature of *Sm*PoMucs, these interactions may prove to be supportive of the Red Queen Hypothesis whereby host and pathogen vary their respective molecular determinants of infection in order to survive (**Figure 2.1D**) (130).

### 2.2.5 Miracidia

When penetrating a suitable snail host, schistosomes are not restricted to molecular mimicry to evade detection and attack from molluscan immune cells. The first barrier miracidia face during intermediate host infection is passage through host tissues, a process thought to be

facilitated by a thiol proteinase located in the lateral penetration glands of miracidia developing inside eggs (135). During initial penetration, larval transformation products (LTPs) released from the schistosome are also expected to aid in penetration while subsequently facilitating immune evasion (136). LTP contents have been examined using mass spectrometry, which revealed the presence of numerous factors involved in immune modulation, including but not limited to: proteases (a calpain, serine peptidase, leishmanolysin like protein), protease inhibitors (alpha-macroglobulin, cystatin B), ion binding proteins, antioxidant enzymes, and venom allergen like proteins, which are the most abundantly featured proteins present (137). One such venom allergen like protein (*Sm*VAL9) has been shown to be key in facilitating the upregulation of extracellular matrix remodeling genes during penetration, as evidenced by *in vitro* observations using the *B. glabrata* embryonic cell line, suggesting that it may also be involved in movement throughout host tissues (**Figure 2.1F**) (138).

### 2.2.6 Developing sporocysts

After miracidial penetration of the snail host, the developing sporocyst goes on to synthesize excreted/secreted (E/S) factors designed to facilitate survival and immune evasion (**Figure 2.1G**). Although nomenclature differs throughout the literature, for the purposes of this discussion, E/S products differ from LTPs in that they are produced during primary sporocyst culture, while LTPs are released during the miracidium-to-sporocyst transformation. These combinations of carbohydrates and proteins are predominantly produced 1 day post entry into the snail, implicating them as determinants of infection success in avoiding encapsulation by host haemocytes, a process which leads to damage of the parasite via ROS production, followed by death and elimination by phagocytosis of dead parasite material (139). Both microarray and serial analysis of gene expression techniques have been used to examine expression profiles in

sporocysts, with a variety of proteases (cathepsin C, preprocathepsin L, hemoglobinase and elastase) and antioxidants (Cu/Zn superoxide dismutase, peroxiredoxins 1 and 2, and glutathione peroxidase) having been identified as upregulated factors potentially responsible for some of the inhibitory effects seen in the host (140,141).

The effects of excreted/secreted schistosome products have largely been investigated by examining the effect of whole E/S products on various biochemical and immune related functions in host haemocytes. They alter haemocyte metabolism, as evidenced by their capacity to modulate protein secretion from haemocytes in various *B. glabrata* strains (**Figure 2.1G**) (142). They also modulate cell signalling, as evidenced by the fact that direct exposure of susceptible *B. glabrata* to E/S products reduces both MEK and ERK phosphorylation, two hallmarks of phosphokinase based signalling (143). Since MEK and ERK phosphorylation are cell signalling pathways used to facilitate granulin mediated haemocyte proliferation, this reduction in phosphorylation may also serve to decrease the availability of appropriate levels of cytotoxic haemocyte populations (104). Another downstream result of ERK signalling is the presence of heat shock proteins, such as HSP70. HSP70 abundance decreases in susceptible snail haemocytes exposed to *S. mansoni* E/S products (143). Given the role of HSPs in the stress response and as damage-associated molecular patterns (DAMPs), this may also be a mechanism by which schistosomes persist in their intermediate hosts (144). Exposure of haemocytes to E/S products can impact parasite targeting/recognition and killing (**Figure 2.1G**). These products are capable of suppressing haemocyte chemotaxis while also reducing their capacity to phagocytize foreign particles (145–147). They also prevent the production of superoxide anions and nitric oxide, a key method by which haemocytes kill invading sporocysts (148,149).

To date, four specific factors present in the LTPs produced by *S. mansoni* have been functionally characterized. Upon entry into the snail, *S. mansoni* releases Venom allergen like protein 9 (*Sm*VAL-9), which results in the upregulation of a *B. glabrata* matrix metalloprotease (138). Given the role of such metalloproteases in remodelling tissue, it is hypothesized that this facilitates entry and penetration of the parasite into host tissue (**Figure 2.1F**). Two more immune modulators were identified as part of an examination into E/S product synthesis by sporocysts *in vitro* (139). The first is a polypeptide of roughly 24 kDa found to be capable of inhibiting protein synthesis by snail haemocytes, an effect that was seen in susceptible M-line snails, but not observed in the more resistant 10-R2 strain (148). The second is a molecule of 108 kDa in size, predicted to be composed of more than one 50 kDa domains. This 108 kDa protein was shown to be able to scavenge superoxide anions produced by phagocytosis-stimulated M-line *B. glabrata* haemocytes, thereby protecting the parasite from this toxic oxygen species (148). A fourth and more recently characterized protein was an invadolysin upregulated by *S. mansoni* 33.2-fold at 12 hours post infection in *B. glabrata.* This matrix metalloprotease is similar to Leishmanolysin (GP63), the predominant immunomodulatory protein found on the surface of *Leishmania sp.* (150). This invadolysin, termed *Sm*Leish, was found to be capable of reducing the motility of susceptible M-line *B. glabrata* haemocytes (**Figure 2.1H**). This function is essential in reducing the frequency at which sporocysts are encapsulated by haemocytes *in vitro*, and was also found to be crucial for survival within *B. glabrata* (145).

### 2.2.7 Introduction to the human host

After leaving their snail intermediate host, cercaria move to the surface of the water column in the hopes of encountering a compatible mammalian host. For *S. mansoni* and *S. haematobium*, this means encountering a human or small rodent such as a mouse or rat (151). *S. japonicum*, on

the other hand , is capable of infecting over 40 different mammalian species, although humans are one of the primary hosts (152). The environment inside of a human host differs drastically from that of a gastropod mollusc. While snails mount a capable and robust innate response, in humans, the innate and adaptive arms of a schistosomicidal immune response are brought to bear (153). Each life cycle stage that takes place within the mammalian host is presented with a unique set of immune challenges based upon the location in the host. The exact efficacy of these host immune processes remains an investigated matter. Early work demonstrated that mammalian effector cells such as neutrophils and eosinophils are capable of killing schistosomulae *in vitro*, especially in the presence of complement proteins and anti-schistosome immunoglobulins (153–155). Whether or not this occurs *in vivo* remains unascertained. Resistance to schistosome infection does vary between age groups, with children and teenagers featuring higher reinfection rates than adults, although it is not known for certain if this increase in resistance is age mediated or antibody based (156–159). It is clear that sterile immunity is not induced in humans after infection, but models suggest that the levels of protective immunity that are developed are likely caused by exposure to dead worm antigens (160). To counter the numerous challenges schistosomes face in their definitive hosts, these worms have developed several immune evasion mechanisms allowing for high infection loads and lifespans up to 37 years, indicating their capacity to survive and thrive despite the host immune response (161).

### 2.2.8 Initial penetration

The first immune barrier that schistosomes encounter is the skin. The skin functions as a barrier to all pathogens, and helps prevent the entry of parasites, fungi, bacteria, and viruses. The components of the skin relevant to schistosome infections are the epidermis, the dermis, and the basement membrane separating the two. The epidermis is largely composed of keratinocytes,

which secrete lipids to aid in the formation of a barrier, but specialized dendritic cells known as Langerhans cells are also present and are capable of taking up antigen and migrating into the lymph system. The basement membrane is a network of connective molecules composed largely of collagen type IV and collagen type VII. (7,162,163). On the inner side of the basement membrane, the dermis exists as a layer of skin featuring nerve endings, hair follicles, muscles, and blood and lymph vessels (162,163).

In order to successfully develop as a schistosomula, cercariae must penetrate through the epidermis, basement membrane, and dermis in order to locate a venule or lymphatic vessel that will subsequently lead them to the lungs (164). A healthy debate exists as to the exact mechanism and kinetics by which this skin penetration occurs, but a variety of model systems have allowed for a better understanding of how cercariae complete this task. In mice, hamsters and rats, the time needed for half of *S. mansoni* to leave the skin is 88 hours, 65 hours, and 70 hours, respectively (7). Studies using human skin explants have demonstrated that *S. mansoni* and *S. haematobium* seemingly have similar invasion kinetics to what is seen in rodent model systems, with penetration into the dermis and location of a venule taking between 48 and 72 hours (5,165,166). Penetration by *S. japonicum* has been shown to proceed at a faster rate, with up to 90% of the parasites reaching the dermis/host venules within 24 hours post infection, some even doing so in only 12 hours (166). Such an rapid migration is also supported by the observation that *S. japonicum* reach the lungs of experimentally infected mice after 3 days, while *S. mansoni* and *S. haematobium* take 6 days (167). The time it takes for penetration to occur suggests that movement in the skin is not easy for the invading larvae and suggests the need for developing mechanisms of avoid immune cells within both the epidermis and dermis, where numerous innate immune cells are present (**Figure 2.2A**).

Penetration through the uppermost layers of the epidermis is aided by the aqueous environment in which they encounter their host, seeing as the lipid interactions holding together the stratum corneum are lessened/removed in such an environment (4). Once the schistosomes have passed through the upper layers of keratinocytes, they encounter resistance at the stratum spinosus, and proceed to facilitate the killing of nearby cells while also degrading the connections (largely composed of cadherins) between such cells. This process, as well as the subsequent degradation of host structural components, may be facilitated by both mechanical movement as well as factors that degrade cell-cell adhesions. Possible sources for such factors include the head gland, sub-tegumental cell bodies, and acetabular glands (7). While each of these sources may have roles in facilitating penetration, the most likely suspect remains the acetabular glands, cells featuring long duct-like cytoplasmic extensions leading to the anterior of the parasite that are packed with proteases (168). Acetabular gland secretions are produced during migration through the collagen rich basement membrane of the skin and have been confirmed to be secreting up to three days post infection. The cells of the acetabular gland atrophy between 48-72 hours post infection, and are not thought to be a key factor in penetrating into host venules, a process which is thought to be mediated by the parasite's head gland (164).The acetabular glands are, however, necessary for percutaneous infection using mechanically transformed cercariae. Thus, it is likely that these glands remain the predominant method by which human schistosomes facilitate entry through the skin (4,6,7,169,170).

Investigations into the proteolytic factors responsible for skin penetration have revealed of a wide variety of serine proteases as a means by which to degrade host structural components. *S. mansoni*, which has been studied extensively in this area, produces numerous serine proteases, which are expelled from its acetabular glands during penetration (**Figure 2.2A**). Among these proteases are

a 28/30 kDa protease capable of cleaving casein, gelatin, C3, C3b, laminin, fibronectin, keratin, and collagens type IV and VIII; a 47 kDa protease capable of cleaving gelatin, casein, collagen type VI, and elastin; and a 60 kDa protease capable of cleaving casein and gelatin (171–173). Inhibition of these serine proteases using serine protease inhibitors reduces the likelihood of successful penetration into human skin (174). Among these serine proteases, the 28/30 kDa variant that is referred to as *Sm*CE (*Schistosoma mansoni* Cercarial Elastase) is the most crucial, as it composes roughly 36% of the total volume of acetabular gland contents (175). Inhibition of *Sm*CE using the protease inhibitor succinyl-alanyl-alanyl-prolyl-phenylalanine chloromethyl ketone (AAPF-CMK) is capable of reducing cercarial penetration by up to 80% (176). Highlighting the importance of *Sm*CE is its persistent presence throughout the intra-mammalian portion of the *S. mansoni* life cycle. A membrane bound version of the protein is found in cercariae, lung stage schistosomulae, and adult worms (177). While *S. haematobium* possess a protease like *Sm*CE, it was long held that *S. japonicum* lacked any serine protease production whatsoever during initial penetration events because *Sm*CE antibodies failed to react with *S. japonicum* cercarial extracts (178). More recent proteomic analysis of the host/parasite molecular interface during *S. japonicum* penetration into mouse skin revealed a single *S. japonicum* cercarial elastase (*Sj*CE2b) made in cercaria and localized to the acetabular glands, although levels of this protein pale in comparison to what is found in *S. mansoni* and *S. haematobium* (179).

Given the relative difference in serine protease abundance between varying species of human schistosomes, other factors must participate in the cercarial penetration event. Cathepsins have emerged as an alternative facilitator of penetration (**Figure 2.2A**). *S. japonicum* cercaria, despite lower protein output during transformation to schistosomulae than *S. mansoni*, exhibit up to 40-fold more cathepsin-B-like activity than their *S. mansoni* counterparts (178). Additionally,

proteomic analysis of the proteases found in *S. japonicum* cercaria and schistosomulae have identified five different cathepsins and only one serine protease (179). Given that *S. japonicum* is considered to be a more ancestral species of schistosome than *S. mansoni* or *S. haematobium*, it is possible that these cathepsins are an evolutionarily older mechanism of facilitating penetration into a wider variety of hosts. Seeing as *S. japonicum* has been observed to elicit more of a swollen red bump at the site of penetration, it is possible that the expanded use of serine proteases in *S. mansoni* in particular could have resulted from an evolutionary attempt to move away from cathepsins as a penetration facilitating molecule given their immunostimulatory properties in humans (178). *S. mansoni* still produces cathepsins, two of which (Cathepsin L1 and Cathepsin B) are present in the post acetabular glands of the parasite. Given the function of post acetabular glands in producing mucous like substances to facilitate attachment to host skin, it is possible that these cysteine proteases may have a role in overcoming the skin as an immune barrier. Alternatively, the involvement of cathepsin activity in the adult schistosome gut suggests that the presence of cathepsins in cercaria also has the potential to serve as nothing more than evidence of the development of factors necessary for digestion in subsequent life cycle stages (180).

Cercaria, as well as the skin stage schistosomula into which they develop, must also contend with a variety of different immune cells in the skin. The immune response commences early during penetration in the epidermis, with HLA-DR+ cells, likely Langerhans cells, having been shown to aggregate at the sight of infection within 48 hours, while keratinocytes respond to penetration by the release of the proinflammatory cytokines IL-1α and IL-1β (166,181). Additionally, fluorescent imaging has revealed that neutrophils, macrophages and dendritic cells are all capable of internalizing the products released by cercariae upon penetration (182). Eosinophils and neutrophils can kill developing schistosomula with the aid of complement

components and immunoglobulins *in vitro*. Thus, one can assert that humans possess a more than adequate array of schistosomicidal immune cells in the skin that could kill the developing larvae in the absence of immunomodulatory factors (155).

Several candidate immune modulators produced by skin staged schistosomula have been identified (**Figure 2.2A**). However, only a few of which have been functionally characterized to date. Chief among these modulators is an anti-inflammatory protein termed *Sm*16/*Sm*SLP/*Sm*SPO-1. This 16.8 kDa protein composes roughly 3-4% of the protein secreted from cercariae during 0-3 hours post infection, suggesting a role in parasite survival (175). It has been demonstrated that *Sm*16 can alter cytokine profiles. It downregulates IL-1α production in keratinocytes, lowers ICAM-1 expression in endothelial cells, prevents LPS induced neutrophil movement into the dermis, and reduces LPS mediated IL-6, TNF-α, and IL-1β production. In mice, it lessens the capacity of mouse bone marrow derived macrophages from producing IL-12p40, IL-10, and IFN-γ-induced $NO_2^-$ production, while also slowing antigen processing by phagocytic cells (183–186). The *Sm*16 counterpart in *S. japonicum*, termed *Sj*16, has also been shown to possess immunomodulatory properties, including a reduction in macrophage maturation, while also modulating cytokine production in thioglycolate-induced peritoneal mouse cells by upregulating IL-10 and IL-1RA (receptor antagonist), while downregulating (macrophage inflammatory protein) MIP-2, IL-1β and IL-12p35 (187). Interestingly, *Sj*16 is also capable of increasing the abundance of $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells, thereby suggesting that it not only has the capacity to downregulate inflammatory responses, but may also contribute to the development of a regulatory response (188). Originally, the roughly 30% similarity that *Sm*16 features with human stathmin led researchers to hypothesize that *Sm*16 functioned as a microtubule destabilizing protein. This suggestion has been disproven, and it has been shown that *Sm*16 actually assembles

as a 9 unit lipid bilayer-associated oligomer that is capable of altering both TLR4 and TLR3 signalling (185,189). Despite this plethora of functions, immunization of mice against *Sm*16, as well as infections with *Sm*16 RNAi knockdown parasites reveals that elevated humoral and cellular immunity to *Sm*16 do not confer protective immunity in mice, with worm burden and egg laying remaining unchanged (190). Given that natural infections with *S. mansoni* (in humans) and *S. japonicum* (in rabbits) fail to elicit strong antibody mediated responses, *Sm*16, long considered a possible vaccine candidate, does not merit investigation as such (187,191).

Other, perhaps less well known/examined immunomodulators are also features of schistosome infections in the human skin (**Figure 2.2A**). Some, such as the 23 kDa *S. mansoni*-derived apoptosis inducing factor, seemingly exert their immunomodulatory properties by direct targeting of T cells. Schistosome E/S fractions containing a 23 kDa protein have been shown to specifically target T lymphocytes for apoptosis, a process thought to be mediated by causing an upregulation of both the Fas Ligand and Fas receptor on CD3+ cells (192). This destruction of T lymphocytes during initial penetration may be partially to blame for the inability of immunized mouse lymphocytes to recognize the E/S products of invading parasites, as a proper T cell mediated response would be severely hampered (193). Other molecules may act by instead modulating the cytokine environment in which the parasite finds itself. *S. mansoni* can produce prostaglandin E2 ($PGE_2$), while also producing an E/S product of less than 30 kDa in size that can upregulate the production $PGE_2$ and IL-10 from human keratinocytes. This seems to be of significant importance to the kinetics of infection, as IL-10 deficient mice are able to slow schistosomula travel through the skin and into the lungs (88). Both the 23 kDa and 30 kDa immunomodulatory factors were identified via fractionation of schistosome E/S products, with the exact molecular identity having not yet been ascertained. The use of prostaglandins is not limited to $PGE_2$, however, as $PGD_2$

produced by the parasite inhibits the migration of epidermal Langerhans cells to nearby lymph nodes (194). Given that production of $PGD_2$ by *S. mansoni* has been demonstrated to require a 28kD glutathione S-transferase, an interest in exploiting such a factor as a possible vaccine candidate was explored during the early 1990s. Unfortunately, recent phase 3 clinical trials of the *S. haematobium* derived r*Sh*28GST (Bilhvax) vaccine proved ineffective in granting significant immunity (89,195–197). Finally, a family of venom allergen like proteins was identified as possible immune modulators. The *S. mansoni* genome was demonstrated to feature 29 of these proteins, which are defined by the presence of a Sperm-coating protein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) domain (198). *Sm*VAL 4, *Sm*VAL 10 and *Sm*VAL 18 were identified as being present in the E/S components from cercaria and compose roughly 3% of the normalized proteins found therein using mass spectrometry. The presence of *Sm*VAL24 in the acetabular glands has been observed via whole-mount *in situ* hybridization (175,199). To date, only two of these *Sm*VAL proteins have been functionally characterized. SmVAL4 possesses lipid and cholesterol binding capacity, although how this might result in the modulation of the host immune response has not yet been examined (200). *Sm*VAL18, on the other hand, has been shown to bind plasminogen, and help facilitate its cleavage into plasmin, which plays a role in the degradation of complement components, extracellular matrix proteins, and fibrinolysis. Thus, *Sm*VAL18 could conceivably help the parasite migrate through the skin and avoid blood clotting during penetration into a venule (201). *In S. japonicum*, only one VAL, *Sj*-VAL-1 has been examined, and has been shown to localize to the penetration and head glands of cercariae, suggesting a possible role in migration into host venules (202).

In addition to those immune mediators that have been characterized, numerous other parasite-derived factors hypothesized to be involved in overcoming the immune response in the

skin have been identified by genomic, transcriptomics and proteomic approaches (**Figure 2.2A**). One interesting possible series of targets are metalloproteases. In *S. mansoni*, genomic data suggests the presence of 114 metalloproteases, 35 of which were found to have differential transcription through different live cycle stages (198,203). Of note was the presence of seven invadolysins, five of which were upregulated preferentially in the germ ball stage of development, and two of which were most upregulated in the cercaria. Given that one invadolysin (Smp_090100.1) constitutes 12.8% of the protein secreted during the first 3 hours of infection, while another (Smp_135530) was shown to be a key determinant of infection status in the intermediate host, investigations into the role of such proteases is merited in the context of mammalian infection (145,175). This is supported by recent findings examining proteins expressed during *S. japonicum* penetration, which suggest the expression of six different invadolysins, five of which continued to be expressed by schistosomula after successful migration through the skin (179). Another potential immunomodulator yet to be functionally characterized is *Sm*KK7, a protein bearing significant homology to K+ channel blockers in scorpion venom, which could foreseeably function in inhibiting the activation of surrounding lymphocytes (175).

Despite the diverse and plentiful existence of these factors secreted by invading cercaria/skin stage schistosomula, schistosomes do not merely rely on the release of immunomodulatory molecules into the skin meant to ward off effector immune cells. The parasite must also deal with the potential of being opsonized by complement components and immunoglobulins and has therefore evolved both surface-associated factors and the use of secreted products to combat host innate and adaptive responses. This process begins during the first three hours of invasion, wherein the schistosomula rapidly seeks to shed the proteins and carbohydrates composing its glycocalyx (**Figure 2.2A**). While the glycocalyx serves an important role in

mediating survival under the high osmotic pressures seen during the free living aquatic cercarial stage of life, once in the skin, it is a potent target for both the classical and alternative pathways for the complement system (204). While the process of losing the glycocalyx is facilitated in part by mechanical movement throughout the epidermis, the close association of *Sm*CE has been suggested as a possible aid during this process.

While the glycocalyx is shed, schistosomulae also undergo a complex reorganization of their outer membrane in which it goes from a trilaminate state into a heptalaminate state that persists into adulthood (205). This newly formed heptalaminate membrane then begins to display several surface-bound factors geared towards disabling complement and immunoglobulin-based attacks. One such molecule is paramyosin, which has been shown in association with both schistosomula and adult worms. Paramyosin has been shown to bind complement components C1,C8, and C9, thereby inhibiting polymerization and deposition of the membrane attack complex on schistosomulae exposed to human serum (206). The membrane has also been shown to feature a receptor capable of binding to the Fc fragment of human IgG (but not IgE, IgA and IgM), while also binding to the $\beta_2$-microglublin found in the human major histocompatibility complex (207). Such binding could conceivably function in masking the schistosomula with host proteins, but the orientation of binding also suggests it is a mechanism of rendering IgG unable to signal to surrounding effector cells. With the Fc portion of IgG bound to the schistosome, the antibody-dependent cytotoxicity employed by cells such as macrophages, neutrophils and eosinophils can not occur due to the inability of IgG to bind the Fc receptor on the surface of such cells. Immunoglobulins in the immediate area of the infection appear to be targeted by serine proteases secreted from the schistosomula, as surface bound IgG is cleaved into Fc and soluble Fab fragments in a manner inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride

(PMSF) (208). Additionally, soluble IgE is cleaved by a schistosomula derived serine protease likely to be *Sm*CE, as a specific inhibitor of this factor inhibits IgE cleavage (209).

### 2.2.9 Immunosuppression by schistosomula in the lungs

After successfully reaching either a venule or lymphatic vessel, schistosomes begin their migration through the bloodstream towards the lungs, where they will mature for a few days prior to continuing to the liver. While some immune mediators used in the skin continue to be used in the lungs (*Sm*16, *Sj*16, Venom Allergen Like proteins, surface paramyosin), exposure of the larvae to lung epithelial cells elicits new immunomodulatory mechanisms (210). This is particularly crucial given the observation that vaccination with radiated parasites results in protective immunity that largely results in parasite killing in the lungs, suggesting that immunosuppression in the lungs is key to survival and establishment in the host (211). Although the entirety of how this resistance is mediated is poorly understood, it appears that several E/S products of lung-stage schistosomula elicit a strong Th1 type immune response, that requires cooperation with Th17 and Th2 factors in order to facilitate larval death (212,213).

To date, the factors produced by lung stage schistosomes remain poorly examined due to difficulty of isolation, prior focus on the skin stage larvae as potential vaccine targets, and a lack of standardization of the transformation and culture conditions during *in vitro* culturing of lung-stage larvae. Despite this, some studies have gone on to perform both transcriptomic analysis of lung stage larvae, as well as proteomic screens, and have identified factors thought to target host leukocytes (214,215). One protein found to be transcriptionally upregulated was a venom allergen like protein, suggesting the presence of this protein family in yet another life cycle stage of *S. mansoni* (214). Another potential target identified by both a microarray analysis and transcriptomics screen in *S. mansoni* was a protein simply termed Antigen 5 which bears homology

to an antigen from *Echinococcus granulosis* hydatid cysts. This antigen is thought to be immunomodulatory, although a particular mechanism has yet to be described (214–216).

When mechanisms designed to hamper the activation and engagement of effector cells fail, schistosomes must be able to withstand the cytotoxic effects of reactive oxygen species and reactive nitrogen species in order to survive. In fact, immune responses that effectively generate large amounts of these reactive species have been shown to correlate with larval death (212). Schistosomes switch from relying on aerobic metabolism to anaerobic metabolism during their first two weeks in their definitive host, which is hypothesized to be at least partially responsible for their gain in resistance to nitric oxide mediated killing (217,218). This is significant given the observation that nitric oxide is a significant determinant of infection success *in vivo*, with nitric oxide synthase knockout *Rattus norvegicus* showing a significant decrease in resistance to *S. japonicum* infection (219). In order to combat $H_2O_2$, however, schistosomes appear to utilize surface-associated peroxiredoxin-1as a scavenger molecule, as evidenced by the fact that RNAi mediated knockdown of this molecule renders *S. japonicum* susceptible to $H_2O_2$ (220).

Finally, schistosomula must also overcome a significant barrier during their trip throughout the vasculature, to the lungs, as well as their adults lives thereafter: human blood. Although leukocytes are correctly pointed to as the main sources of immunity in whole human blood, it is important to recognise that blood coagulation and platelet deposition surrounding pathogens is also a key mechanism by which humans fight off infection. Schistosomes have evolved mechanisms to avoid clot formation in their immediate vicinity. In both *S. japonicum* and *S. mansoni* surface bound enolases are employed as plasminogen binding proteins that increase the amount of active plasmin in close proximity to the parasite. Active plasmin surrounding the parasite results in an increase in fibrinolytic activity (221,222). Although these enolases have been

shown to facilitate this process both *in vivo* and *in vitro*, it would appear as though the plasmin activity facilitated by all three of the major human schistosomes does not rely solely on enolases, as up to 10 plasminogen binding proteins have been identified via western blotting techniques. RNAi-mediated KD of enolases does not significantly inhibit schistosomula associated plasmin activity, suggesting other as of yet unidentified factors are also involved in the inhibition of clot formation (221).

### 2.2.10 Adults

Adult schistosomes incorporate many different strategies to avoid and overcome the host immune response (**Figure 2.2C**). The first of these strategies is the capacity of adult schistosomes to incorporate host antigens on their surface in an attempt to hide themselves from host immune factors. This was first suggested by the work of Smithers et al. in which adult worms were transplanted into a host of a different species. After these transplants, the adult worms would usually survive, unless the recipient host had been vaccinated with antigens from the donor host species prior to the infection, in which case most worms would die. This strongly suggested incorporation of host molecules onto the surface of the worm (223). This work would become a staple of parasitology classes and helped lay the foundation for future work into schistosome host antigen absorption. Subsequent work has demonstrated that this incorporation of host products is not a random process, nor a result of the generalized "stickiness" of the glycocalyx. It is, rather, due to the presence of receptors for various host antigens (**Figure 2C**). Receptors for the Fc portion of immunoglobulins, one of which has been shown to be a paramyosin as well as a $\beta_2$-microglobulin in a MCH class I receptor, and a C3 receptor explains the presence of these molecules on the surface of the worm (207,224,225). Although the binding of IgG via its Fc region renders it unable to bind the Fc receptor on circulating effector cells, the exact orientation/function

of complement component C3 binding is not known (**Figure 2C**). Adult schistosomes are also capable of incorporating host CD44 into the outer portion of their tegument, especially on the tips of the adult spines (226). To date, an immunological function of CD44 being used by the worm remains to be identified.

Another immune evasion method is molecular mimicry, which is employed by adult schistosomes via the production of molecules bearing significant homology to host signalling molecules. Schistosomes have been shown to produce levels of adrenocorticotropic hormones, which, when processed by human polymorphic blood leukocytes into alpha- melanotropin, results in the subsequent inactivation of these cells, and could potentially decrease Th1 cell activation (128,227). Additionally, schistosomes have the capacity to generate substances bearing similarities to morphine and codeine, which had the effect of decreasing leukocyte activation (227,228).

While the exploitation and mimicry of host molecules greatly aids the worm during infection, they are also capable of employing novel self-made mechanisms of interfering with the human immune response (**Figure 2.2D**). The production of immunomodulatory elements in adults occurs early in their arrival to the hepatic portal system or venous plexus. In *S. japonicum*, unique factors are shown to be produced as early as day 14 post infection. Identified among the E/S proteins from 14-day old *S. japonicum* are a cystatin and an integrin. The cystatin is hypothesized to induce the polarization of alternatively activated macrophages, which are characteristic of a Th2 type responses that are less toxic to larvae than Th1 type inflammatory response. The integrin is hypothesized to mediate T cell migration, which might alter cytokine production in the milieu surrounding the larval parasite, lessening the immune challenges it may face in the lungs (229). While these cell targeting factors have yet to be functionally characterized, at least one *S. japonicum* Thioredoxin peroxidase (*Sj*TPx) has. It is a protein released from 14 day old

schistosomula that has been shown to reduce the presence of MHCII and CD86 on LPS stimulated RAW macrophages, thereby implicating it in downregulating of antigen presentation and subsequent T cell activation (229).

Many immunomodulatory factors are found in the tegument, the multi-layered outer surface of the parasite which is capable of quick self-renewal after damage, a process mediated by a population of somatic stem cells with a propensity to differentiate into tegument associated cells (230). The factors within the tegument have been extensively examined (231,232). In *S. japonicum,* one such factor, *S. japonicum* tetraspanin orphan receptor (*Sj*TOR), proves to be yet another component in fighting complement mediated death, and has been shown to bind complement component C2, while preventing complement mediated cell lysis (233). The tegument is also home to the *S. mansoni* Kunitz type protease inhibitor (*Sm*KI-1), a potent serine protease inhibitor with similarities to the Kunitz type protease inhibitors found in the gut of *Fasciola hepatica* (234). In *S. mansoni*, it is present both in the tegument of adult worms, as well as their E/S products (**Figure 2.2D**) (235). This particular inhibitor was shown to be crucial for parasite survival within mice, as siRNA KD of *Sm*KI-1 resulted in increased killing via neutrophil elastase, a molecule the inhibitor was shown to target *in vitro* (236). *Sm*KI-1 is accompanied in the tegument by *Sm*200, a 200 kDa protein used for diagnostic purposes, but whose only characterized function to date is the causation of an increased abundance in IL-10 secretion from surrounding cells (237). In *S. japonicum*, another protease inhibitor, this one of the cysteine variety, has been shown to inhibit the lysosomal cysteine protease of dendritic cells, while also reducing their antigen processing/presentation on MHC class II (238). Clearly, these two protease inhibitors are evolved mechanisms meant to prevent cell mediated killing should the parasites attempts at avoiding cell recognition/contact fail (238). Lastly, thought-provoking work into the mechanisms

by which adult schistosomes release immunomodulatory factors into the host revealed that they are not restricted to proteins shed as part of the membrane, but that they also employ exosome-like vesicles that drive classical activation of macrophages (239–241). Earlier examinations of the surface adult surface demonstrated the release of portions of the tegument, but did not make note of observing such small, exosome like vesicles (205,242,243).

Another significant barrier to overcome that adult worms must face is survival in the host bloodstream. Given the long lifespans of adult schistosomes, in addition to their relatively small size (approximately 1cm in length), constant exposure to blood clotting factors put them at risk of immobility and subsequent death. While the presence of the enolases seen in schistosomulae have been confirmed in adults, a bevy of other possible anti-coagulation mechanisms have been identified in adult worms. This is perhaps most clearly demonstrated by the fact that addition of mated pairs of adult worms to murine blood significantly reduces coagulation, while at 7 weeks post infection with *S. mansoni*, mouse blood features clots that break down more easily (244). While this general observation of anticoagulation is interesting, the past few decades have allowed for a more refined understanding of the exact molecules present at the surface of the parasite that allow for such activity (**Figure 2.2B**). Among the specific anticoagulants discovered are the kininogen cleaving serine protease Sk1, the α- and γ-thrombin binding/inhibiting membrane bound antigen *Sm*22.6, the ATP-diphosphohydrolase enzyme *Sm*ATPDase1 that cleaves the platelet activator ADP, the more recently characterized tegumental calpains, *Sm*Calp1 and *Sm*Calp2 that cleave host fibrinogen, and finally, the secreted serine protease *Sm*SP2 that activates parts of the fibrinolytic system and promotes vasodilation (245–248). The discovery of these specific factors clearly demonstrate that schistosomes have evolved a large variety of methods by which to ensure their free movement and survival within host vasculature (**Figure 2.2C**) (249). While attacks on

the parasite outer surface by blood borne factors are well characterized, less is known about how the parasite survives the host immune mediators that it internalizes during feeding on host erythrocytes. Of note is the observation that one of the several proteases the schistosome gut produces for digestion of RBCs, cathepsin D, is also capable of cleaving IgG and complement component C3, although it is not known if this functions as an immune evasion technique for combatting gut permeabilization or is rather just part of digesting these factors as possible nutrient sources (250). The contents of the adult gut are not limited to proteases, seeing as a recent study demonstrated that a micro-exon gene (MEG) protein MEG-14 interacts with and sequesters the pro-inflammatory human calgranulin S100A9, suggesting a possible role in immune downregulation (251,252). Perhaps one of the more remarkable things about schistosomes in the blood is their capacity to utilize ingested blood components to modulate the surrounding immune response. This is made evident by the observation that hemozoin, the biproduct of erythrocyte digestion composed of heme molecules, is regurgitated by the worm, taken up by macrophages, and downregulates the Th2 types response seen during egg laying (253).

### 2.2.11 Eggs

The eggs laid by schistosomes are the primary cause of pathology associated with human schistosomiasis. This has resulted in significant amounts of research going into understanding the immunological milieu surrounding these eggs. While many aberrantly migrating eggs become lodged in the liver, they typically seek to exit the host via the intestines/bladder. The process by which the eggs exit the host have been reviewed extensively, therefore here we seek to highlight the specific factors excreted by the eggs to modulate/downplay the host response during the migration through the host intestines and bladder (16,254). Schistosome eggs are composed of a thick protein shell, a cell derived envelope responsible for the secretion of various factors, and the

developing miracidium (255,256). Adult female schistosomes are estimated to lay roughly 300 eggs per day during their peak reproductive ages, and while many of these eggs will aberrantly migrate to the liver where they reside until death, successful eggs will begin their migration through the intestine towards the intestinal lumen (257). This process is accompanied by the formation of a granuloma surrounding the eggs. While schistosome egg antigens have been shown to be capable of inducing apoptosis of host cells, the parasite appears to harness the host immune response in order to facilitate egg movement towards the lumen. This is made evident by the fact that egg laying coincides with the appearance of a Th2 type response in the host, and a similar response can by induced via the direct injection of eggs into the anterior mesenteric veins of mice (258). In mice, a balance between host Th1 and Th2 responses to schistosome eggs is necessary, seeing as deficiencies in either result in accelerated pathology and subsequent death. That having been said, the observation that IL-4 and Il-13 knock out (KO) mice rarely pass eggs in their feces suggests that a Th2 type granuloma response is imperative for the egg to reliably exit the intestinal wall successfully (259,260). To date, two specific molecules have been identified that have been shown to drastically skew the T cell response in granulomas towards a Th2 type response. The first of these is a major glycoprotein known as alpha-1 (α-1), also referred to as the IL-4 inducing principle of *S. mansoni* eggs (IPSE). IPSE is recognized as an antigen by basophils, subsequently resulting in an increased amount of IL-4 production and an alteration of the T helper cell response (**Figure 2.2E**) (261). Of note is the observation that this molecule is also capable of upregulating IL-10 production in B cells, thereby increasing the proliferation of regulatory T cells, a process that may be more important in dealing with granulomas in the liver (262). Intriguingly, α-1 is not the only Th2 inducing glycoprotein secreted by schistosome eggs, as another glycoprotein, Omega1 (ω-1), a T2 ribonuclease, has shown to be the primary method by which the eggs induce

a Th2 response (263). ω-1 does not affect IL-4 release from basophils like its counterpart, α-1, but rather it targets dendritic cell (DC) functions (**Figure 2.2E**) (264). In 2012, it was shown that ω-1's glycosylation patterns allow it to be internalized by DCs via binding to the mannose receptor, while ribonuclease activity results in lowered protein synthesis via the cleavage of host rRNA and mRNA (265). The significance of this molecule in driving the immune response surrounding schistosome eggs was recently demonstrated in the first ever published occurrence of CRISPR-mediated knockout of a *S. mansoni* gene, which resulted in a decrease in Th2 cytokine production from macrophage/T cell co cultures, while also decreasing the volume of murine pulmonary granulomas (266). This skewing of the immune response allows for granulomas composed of alternatively activated macrophages, dendritic cells, lymphocytes, basophils, and eosinophils. Each of these cell types play different roles in helping to facilitate movement through the intestinal wall via the degradation of host tissue. This degradation is thought to be aided at least in part by *Sm*Enolase and *Sm*Calp1, whose presence in the eggs is thought to aid in fibrinolysis (221,246). Additionally the egg is thought to facilitate its own survival by producing *Sm*KI-1 as a method of surviving neutrophil elastase mediate death, while also producing a chemokine binding protein (*Sm*CKBP) that reduces inflammation and inflammatory cell recruitment via the binding of CXCL8 and CCL3 (**Figure 2.2E**) (267). These immune modulating and immune evading tactics allow for the egg to migrate through the host intestine/bladder, so that they may eventually be excreted in order to commence their life cycle anew.

### 2.2.12 Concluding remarks on the use of immunomodulatory factors by schistosomes

Schistosomes have evolved an indirect life cycle featuring an intermediate gastropod host in which they undergo asexual replication, and a mammalian definitive host, including humans, in which adult worms inhabiting the bloodstream undergo sexual reproduction. This complex life

cycle has led schistosomes to develop a bevy of mechanisms to avoid being killed by the immune system of the snail or human hosts. In snails, a mixture of molecular mimicry and E/S products from the developing larvae are utilized to target host haemocytes and prevent their movement, engagement, and killing of the parasite. In humans, various proteases are used to enter the host, after which each life cycle stage produces numerous factors meant for the specific targeting of particular cell types relevant to survival at each stage of development within the host. Given recent advancements in praziquantel administration efforts correlating with a decrease in estimated schistosome infections worldwide, hope exists for the eventual elimination of this deadly and debilitating disease (40). That having been said, an estimated 200 million people are still infected with schistosomiasis, highlighting the need for alternative therapeutics, as well as the possible development of a vaccine. Research into understanding the mechanisms employed by the parasite to survive in both hosts remains crucial to better understanding infection outcomes. The progression in this field from basic observational research all the way to targeted gene deletions suggests a bright future for research into schistosome immune evasion strategies.

## 2.3 Invadolysins: Their form, function, and involvement in parasitic infections

### 2.3.1 Introduction: Proteases as frequent parasitic immunomodulators

Among immune modulatory factors employed by parasitic organisms, proteases are widely distributed and utilized in numerous different processes to avoid a host immune response (268). Various parasitic organisms employ these proteases in unique ways that are often tailored to the requirements of their specific life cycle. An excellent example of this is the employment of both cysteine proteases and metalloproteases (discussed at length in following sections) in the life cycle of *Leishmania spp*. In these parasites, various cathepsin-like proteases help facilitate the infection

of macrophages, with some even going so far as to target internal signalling molecules in order to suppress Th1 functions, thereby facilitating survival inside of leukocytes (269–273). Other protozoan parasites, such as *Entomoeba histolytica*, have different requirements for pathogenesis, namely a need to disturb the structural integrity of the gut. To this end, *E. histolytica* has been found to be able to cleave human IgG and IgA, both key humoral factors for pathogen recognition at a mucosal surface (274,275). It also employs various cysteine proteases responsible for cytopathic damage, and capable of facilitating an inflamed environment via the increased recruitment of granulocytes via the cleavage of CXCL8 (276). At least one of these cysteine proteases is also responsible for an upregulation of IL-8 and IL-1β (277). This increased inflammation and damage of host cells is hypothesized to aid in disturbing the integrity of the host gut, thereby facilitating infection (268).

In helminths, proteases are often employed in the cleavage of antibodies such as IgG (268,278). The abundance of serine and metalloproteases produced and/or featured in the genome of numerous nematodes such as hookworms and *Strongoloides* parasites may also suggest a role in skin penetration akin to that of the aforementioned *S. mansoni* cercaria elastase (279). This goes to show that the function of parasitic proteases depends on the life cycle requirements of the parasite.

Although serine and cysteine proteases remain some of the most well-studied parasite proteases, an interest in looking into the role of metalloproteases in infection has emerged due to work done on such proteases in leishmaniasis and trypanosomiasis (150,280). These parasite species utilize a particular type of metalloprotease, known broadly as invadolysins, to enact immunomodulatory functions. These invadolysins, which are members of the M8 metzincin family of metalloproteases, are also commonly referred to as “leishmanolysins” due to their discovery

and characterization in *Leishmania spp.* (150). The discovery of similar proteases in the genomes of human schistosomes, as well as their identification in developing schistosomes in both the snail and human hosts has led to an interest in examining their utilization during infection (175,198,281).

### 2.3.2 Metalloprotease structure and generic functions

Prior to an in-depth discussion of leishmanolysins, a general understanding of the structure, evolution, and typical use of metalloproteases in general is merited. Metalloproteases are one of 7 classified families of proteases, and are defined by their use of a metal ion in coordinating proteolysis at the active site of the enzyme (282). While iron, cobalt, and manganese are occasionally used as the metal of choice in such proteins, the most commonly employed metal ion seen is zinc (283). In fact, matrix metalloproteases (MMPs), the subset of metalloproteases with which leishmanolysins bear the most resemblance, exist exclusively as zinc dependent endopeptidases (284). Such MMPs are likely to have played a key role in the development of life on Earth, given their ancient origins. The presence of MMPs in plants suggests their existence prior to the divergence of vertebrates from invertebrates, and the high similarity between the active sites of human MMP-1 and *Bacteroides fragilis* metalloproteinase toxin-2 suggests the origin of MMPs may date all the way back to a time predating eukaryotic life (284). Regardless of their origins, their expansion in many different species has led to a variety of functions, despite a relatively well conserved active site structure (284–286).

Zinc metalloproteases are highly conserved in the manner by which they retain a zinc ion in their active site. Such metalloproteases employ negatively charged histidine residues to bind and retain the zinc ion. That having been said, many different classes of zinc metalloproteases feature slight variations as to the exact amino acids involved in the active site of the protease (285).

In most cases, two histidine residues are often relatively close together, with only 1-3 amino acids separating them (285,287). A subset of zinc metalloproteases, known as metzincins, often have a third histidine residue in close proximity to the first two, and display an expanded protease motif HEXXHXXGXXH, in which the third histidine occurs at the end of the active site and coordinates with the zinc ion (288). Interestingly, some metzincins have an extended space between the second and third histidine residues, ranging between 20-120 amino acids in length (285,287). A glutamic acid is present directly on the C-terminal side of the first histidine in order to act as a nucleophile (285). In metzincin proteases, a methionine residue is also present at the end of the active site alpha helices, which results in a characteristic "met-turn" from which the metzincins derive their name (288). Mutational experiments reveal the necessity of this met-turn in stabilizing the protease (289).

All MMPs are generated as zymogens in a latent form (290). In humans, all MMPs are in possession of a pro-peptide domain seen only in the inactivated peptide. This N-terminal pro-peptide is in possession of a conserved cysteine residue, which acts by binding to the zinc molecule in the protease's active site via its thiol side chain, thereby inactivating the protease (290). Oftentimes, this cysteine residue can be found as part of a semi-conserved motif containing the following sequence PRCG[V/N]PD[V/L][A/G] (284,290–292). The process of eliminating this cysteine-zinc bond, and the subsequent activation of the MMP, are commonly referred to as the cysteine switch method (290). The method by which this activation occurs varies from MMP to MMP. Some of the methods include autoproteolysis, cleavage by another protease, and conformational shifts (290). The cysteine switch may also be activated via the use of organomercurial compounds, which can bind the inhibitor cysteine, thereby activating the metalloprotease without reducing its overall molecular weight (293).

To date, the mechanism by which parasite metalloproteases such as leishmanolysins facilitate the cleavage of proteins remains to be examined. That said, inferences may be gathered from the work done on other zinc metallopeptidases, where a better picture of the cleavage mechanism has emerged (286,287,290,291,294). Once activated by the removal of the inhibitory cysteine, the positively charged zinc molecule in the active site is rendered capable of attracting a water molecule. This water molecule then helps facilitate the hydrolysis of the bound peptide substrate after being activated by the site's glutamic acid. Although some older lines of thought suggested the possibility that the glutamic acid itself may have acted as the nucleophile in the reaction, directly attacking the peptide bond, more recent work on matrix metalloproteinase 3 (MMP3) using quantum modeling methodologies suggested the activation of a water solvent molecule to be the more likely of the two suggested mechanisms (295).

Matrix metalloproteases, as the name would suggest, are primarily involved in the degradation of extracellular matrix (ECM) proteins, including but not limited to collagens, fibronectin, gelatin, laminin, proteoglycans, and elastin. Each individual MMP is not capable of cleaving all these factors, and the expansion of MMPs in humans has led to individual MMPs having particular substrate profiles. This has led to the 23 MMPs found in humans to be grouped into different classes: the collagenases, gelatinases, stromelysins, matrilysins, membrane anchored MMPs, and soluble MMPs (286). Each of these classes typically targets different substrates for proteolysis. Notably, substrate specificity seems to be partially dependent on the possession of non-metalloprotease domains within the protein, a fact highlighted by the requirement of a hemopexin domain in order to cleave non-linear collagen, and the use of three repeats homologous to the type-II module of fibronectin by gelatinases in order to bind gelatin and denatured collagen (284,286). The targeting of this multitude of ECM proteins has led to MMPs being essential factors

in embryogenesis, inflammation, cell proliferation, and even cell differentiation. In a non-free-living organism, they have been shown to be highly involved in pathogenesis.

### 2.3.3 Leishmanolysins

The first significant amount of work into the structure, evolution, and function of Leishmanolysins was done while examining their namesake, protozoan parasites of the genus *Leishmania*. These unicellular pathogens effect an estimated 1.5-2 million people each year, with 12-15 million individuals living with the disease, which unfortunately results in an estimated 70,000 deaths per year (296). Several different species with particular geographical ranges cause this disease. *Leishmania tropica* and *Leishmania major*, the causative agents of cutaneous leishmaniasis, and *Leishmania donovani*, which causes the visceral form of the disease commonly referred to as DumDum fever, are predominantly seen in the middle east and northern Africa. *Leishmania braziliensis* and *Leishmania mexicana*, which causes the mucocutaneous form of the disease, are typically seen in the Southern Americas (297).

Although diverse in the pathologies they cause, these different species employ similar indirect life cycles, with asexual replication being seen in both the sand fly invertebrate host, as well as the mammalian hosts, which often ends up being a human (297). Despite differences in the immune modulating mechanisms each species endures, a common theme emerges in mammalian hosts, with promastigotes being phagocytized by macrophages, and dendritic cells, before escaping the phagolysosome, and going on to replicate asexually within the cell (298). To perform the task of avoiding immune mediated killing, as well as to penetrate host cells, *Leishmania spp.* employ proteases in an effort to successfully establish an infection (299).

#### 2.3.3.1 Overall structure of Leishmanolysins

The most common protein found on the surface of Leishmania promastigotes happens to be a protease commonly referred to as leishmanolysin (300). This protease, which can compose up to 1% of the total cellular protein seen in *L. mexicana,* is also known by the title GP63, given its nature as a 63 kDa-sized glycoprotein (301,302). Although studies of this protein typically refer to GP63 as a singular protease, it has been determined that multiple GP63 gene copies exist, in tandem, on the same chromosome, and are constitutively expressed (303). GP63 is in possession of a canonical zinc binding active site domain, manifesting as the HEXXH (X denotes a variation in observed amino acids) amino acid sequence typically seen in metalloproteases (301). Crystal structure analysis of this molecule revealed its similarities to known metzincin proteases such as human neutrophil elastase. Metzincin proteases typically contain a HEXXHXXGXXH active site, as well as a methionine residue positioned underneath the active site hypothesized to create increased hydrophobicity conducive to substrate binding. While such a methionine is conserved in GP63, this protease is unique among metzincin proteases in that a 62 amino acid insertion is found between the catalytic site glycine and the third canonical histidine (288). A significant similarity to matrix metalloproteinases is also seen in that GP63 exists as a zymogen prior to activation (301). This zymogen is likely kept inactive by the same method employed by human MMPs, the cysteine switch, as organomercurial based activation and the presence of a conserved cysteine residue attest to this (304).

#### 2.3.3.2 Cysteine Switch/Activation of Leishmanolysins

The full-length form of GP63 is bound to the surface of *Leishmania* promastigotes via the use of a glycosylphosphatidylinositol (GPI)-linkage (9). Of note is the observation that GP63 is

not only found in association with the parasite's surface but can also be found released into the surrounding environment. This process has been shown not to be mediated by phospholipolysis, as the released protein contains no GPI (305). It has been suggested that GP63 released into the surrounding tissues may in fact have two different origins. The first is proteolytic cleavage from the surface of the parasite. Peptide substrate specificity analysis has shown that GP63 is capable of cleaving peptides composed of the last 4 amino acids of the protein pro sequence and the first 5 amino acids of the mature GP63, suggesting autoproteolysis as a possible source of activation (306). The finding that zinc chelation and mutation of the HEXXH binding site of GP63 reduces release of the active enzyme into the environment helps in substantiating this claim (305). Proteolysis is not highly likely to occur via neighboring GP63 molecules, as the addition of previously activated GP63 to GP63 zymogen does not facilitate the activation of the proprotein (304). Release of surface bound molecules is not likely the only GP63 release mechanism. The work of Silverman and associates in 2010 demonstrated that GP63 is present in the vesicles released from *L. donovani*, suggesting an excretion mechanism unbeholden to the release of the activated protein from the parasite's surface (307).

#### 2.3.3.3 Specific cleavage targets of Leishmanolysin and its functional effects

GP63 was first identified as a protease in 1986 (308). Although the exact cleavage sites of the many targets of this protease are not all known, Bouvier and associates examined the peptide substrate specificity in 1990 (306). Using synthetic peptides, it was determined that GP63 preferentially cleaves peptides with a hydrophobic amino acid in the P1' position, basic amino acids in the P2' and P3' positions, and often a tyrosine at the P1 position. The substrate specificity is not seemingly identical for all forms of GP63. In fact, it has been observed that the membrane bound form of GP63 and its soluble form appear to cleave fibronectin at differing places (309).

In many respects, GP63 acts similarly to other known matrix metalloproteinases, as it has the capacity to target several ECM components. Potential cleavage targets were initially assessed using a variety of host components, and it was revealed that GP63 could effectively cleave casein, azocasein, gelatin, albumin, and fibrinogen (306). Further examination showed that collagen type IV and fibronectin, but not laminin, are also cleavage targets (309). This activity allows GP63 to be a key component utilized for the destruction of host ECM proteins, thereby facilitating the movement of the parasite throughout their environment in the host. Utilizing a model ECM known as Matrigel, researchers were able to show that zinc chelation and GP63 mutagenesis slow *Leishmania* movement across a Matrigel coated membrane, while the addition of soluble GP63 to the system largely restores higher levels of migration by promastigotes (309). Such activity is likely to aid in movement throughout the host and could potentially serve to help promastigotes penetrate mononuclear phagocytic cells.

Leishmanolysin is not restricted solely to ECM components when it comes to cleavage targets, as many key host immunological factors have been shown to be degraded in the presence of this virulence factor. Key among humoral factors that respond to parasitic infetions are complement component C3 and immunoglobulin G (310). While cleavage of IgG is hypothesized to reduce opsonization of *Leishmania* promastigotes for subsequent phagocytosis or antibody dependent cell cytotoxicity, although this remains to be experimentally shown. The role of C3 cleavage, however, has been examined in some detail. *Leishmania* promastigotes with functional GP63 feature less terminal complement component deposition on their surface, and are more resistant to serum mediated lysis (311). To add to this, targeted gene deletion of all 7 copies of GP63 in *L. major* results in parasite populations that are completely killed by 2% human serum. *L. major* with functional GP63 copies only have complete population die off at 11% human serum

concentrations (312). When it comes to cell associated immune mediators, CD4 finds itself as a target for both membrane bound and soluble GP63 (313). This may result in a disruption of T cell signalling necessary for quick elimination of novel *Leishmania* infections.

Cleavage of complement component C3 may help *Leishmania* avoid complement mediated lysis, but GP3 also effects component C3 in another way. It helps facilitate a rapid conversion of C3b into iC3b, which is then capable of binding to CR3, which facilitates higher levels of parasite phagocytosis (301,311,312,314). Given the necessity of being phagocytized for the continuation of its life cycle, this typically deadly process is encouraged by the parasite. Early research even suggested that GP63 itself could act as a ligand for CR3. It was shown that a peptide derived from GP63 could successfully inhibit promastigote binding to macrophages, suggesting that this may occur with the membrane bound full length version of the molecule (315). Others have even suggested that the fibronectin like nature of GP63 suggests that it could bind the fibronectin receptor, a possibility that has also been examined using synthetic peptides (316,317). Whether or not the protein in its native form binds to the fibronectin receptor remains to be seen.

Once successfully phagocytized, *Leishmania* proceed to perform the next task in their survival, namely surviving within the phagolysosome. GP63 persists in providing multiple functions by being capable of protecting proteins coated with GP63 from being degraded within the phagolysosome (318). This function may be predominantly used during initial infection, given that amastigotes produce less of this protease than promastigotes (150). Regardless of the kinetics of GP63 use during infection, its usefulness during infection is well characterized, given that *L. mexicana amazonensis* promastigotes with GP63 selectively inhibited in phagolysosomes demonstrate a significant handicap in their ability to avoid cytolysis within mouse macrophage phagolysosomes (319). To add to these findings, Chen and associates showed that antisense

mRNA based knock down of GP63 in *L. amazonensis* was capable of greatly lowering infection kinetics and promastigote survival/replication in the phagolysosomes of the mouse macrophage J774 cell line (320).

Due to their location within host mononuclear phagocytic cells, *Leishmania* parasites can facilitate the degradation of intracellular signalling molecules. One such molecule is the myristoylated alanine-rich kinase substrate (MARKS)-related protein termed MRP, which is cleaved by GP63 between its Ser92 and Phe93 amino acids (321). Given that MRP is found in macrophages and is hypothesized to be involved in phagocytosis, cell motility and membrane trafficking, the GP63 caused reduction of this molecule in infected macrophages suggests the exploitation of this degradation event by *L. major* (321–324). GP63 also cleaves numerous other intracellular signalling molecules, resulting in a lack of phosphorylated p38, thereby helping to inhibit activation of leishmania-infected fibroblasts (325). Finally, GP63 also cleaves the NF-κB p65 $^{RelA}$ subunit, although the cleaved molecule forms a novel complex with NF-κB p50 and is still capable of inducing MIP-2, MCP-1, MIP-1α, and MIP-1β, thereby suggesting an alteration of the cytokine profiles within macrophages during initial infection (326).

Alterations to cell behaviour by Leishmanolysin are not strictly reserved for changes in cell signalling molecules, as functional changes may also be observed. For example, live promastigotes tend to attract macrophages. Once incubated in the presence of promastigotes, these macrophages have a tendency not to move towards other promastigotes in their environment. The reason for this ablation in movement is not perfectly understood, but evidence suggests that GP63 may be at least partially responsible for this reduction in movement, as *L. major* GP63 reduces the movement of RAW 264.7 macrophages *in vitro* (327). The reason for this is not entirely known, but it may serve as a mechanism by which *Leishmania* ensures the survival of its host's cells by reducing infections

with such high inoculum that the macrophage dies too quickly. Ablation of chemotaxis is not solely seen in macrophages, as GP63 derived from *L. major* inhibits neutrophil, but not monocyte, movement towards the chemotactic peptide f-met-leu-phe (328). Both cell types are, however, inhibited in their chemotactic response towards zymosan-activated serum (328). In both neutrophils and monocytes, GP63 also possesses the capacity to inhibit respiratory burst responses stimulated by opsonize zymosan, but not Phorbol 12-myristate 13-acetate (328). These differential chemotaxis and respiratory burst responses suggest that GP63 may target extracellular ROS inducing receptors, as well chemotactic receptors that differ between leukocyte type. This remarkable molecule does not only target phagocytic cells, but also aaffects NK cells, preventing their proliferation and thereby reducing the prevalence of Th1 response inducing molecules such as IFN- γ (329).

This multitude of activities demonstrated by GP63 leads to alterations when it comes to *in vivo* infections in the mammalian hosts. Joshi and associates were able to successfully generate *L. major* mutants without any of the 7 leishmanolysin genes typically found in *L. major* via targeted gene replacement. These mutants were able to establish infections in mice, but a 3.5-4-week delay took place before *Leishmania*-caused lesions reached 3mm in diameter. While an increase in initial lesion size could be partially restored by inserting a functional copy of GP63 gene 1, wild type (WT) lesion size was not fully restored, suggesting that each GP63 gene may be important in infection kinetics (312). The use of GP63 as an important virulence factor seems to be reserved to functions within the mammalian host, given that GP63 deficient mutants develop at the same pace as WT parasites within the sand fly vector (312).

All these findings led to an interest in examining GP63 as a possible vaccine target. Preliminary results were mixed. Vaccination of CBA mice with *L. mexicana* GP63 led to

significant levels of protection, while vaccination of BALB/c mice with *L. major* GP63 did not (330,331). The generation of synthetic peptides of T cell epitopes from *L. major* GP63 did confer significant protection to both of the above mentioned mouse strains from their associated *Leishmania* species (332). That having been said, in humans, the capacity of T cells to recognize *L. mexicana* GP63 unfortunately fails to correlate with infection outcome, suggesting the need for other parasite components or vaccine adjuvants (333). More recent attempts at GP63 utilization as a vaccine candidate have tried new lipid based delivery systems, as well as DNA based vaccines, which have shown mixed results in mice (334–336). Although there is no vaccine currently in use for Leishmaniasis in human populations, the multi-edged sword that is GP63, and its variety of immune modulating functions make it a key candidate factor in the development of novel therapeutics in fighting this disease.

### 2.3.4 Invadolysins in Trypanosomes

*Leishmania* species are by no means unique in their utilization of invadolysins as virulence factors amongst kinetoplastid parasites. These potent metalloproteases are also employed by a variety of trypanosome species. Among these varying species, several similarities can be observed regarding the use of GP63 homologues. The proteins are often encoded by numerous gene families, with numerous gene copies existing in each family, a point highlighted by the 174 true gene copies encoding 29 different GP63-like proteins in *Trypanosoma cruzi*. They also all possess the classic HEXXH active site used to facilitate proteolysis and several candidate cysteine residues used in their activation (337–340).

Despite these similarities in gene organization and protein structure, Trypanosome invadolysins have been shown to exhibit differing functions dependent upon the Trypanosome species in question. In *Trypanosoma brucei*, the causative agent of East African sleeping sickness,

three families of *T. brucei* Major Surface Protein genes exist, TbMSP-A, -B, and -C. While TbMSP -A and -C are upregulated in the human blood borne metacyclic life cycle stage of the parasite, TbMSP–B is more highly upregulated in the procyclic stage often found in the intermediate host, commonly referred to as a tsetse fly. RNAi knockdown of these genes revealed that TbMSP-B cleaves variable surface glycoproteins (VSGs) from the surface of the procyclic form of the parasite, as they are necessary for immune evasion in the mammalian host, but not the tsetse fly (337).

In *T. cruzi,* GP63 homologues were originally separated into two groups, with the Tcgp63-I group being more highly expressed than the Tcgp63-II group genes. Since then, a more rigorous classification of *T. cruzi* GP63 genes has further divided them into four groups based on differing sequence features (280). Tcgp63-1 proteins are roughly 78 kDa in size and are bound to the parasite via a GPI anchor. When purified antibodies against this group of proteins are applied to the parasite, infection of Vero cells is reduced by 50% (338). Other work examined GP63 like proteins in *T. cruzi* and found differential expression of a 61 kDa and 55 kDa protein, the first of which was found likely to be a transmembrane protein in epimastigotes, amastigotes, and trypomastigotes, while the latter was found likely to be a GPI anchored protein only seen in metacyclic trypomastigotes. Notably, preincubation of trypomastigotes with immune sera to these proteins significantly reduced their capacity to infect myoblast cultures (339). Clearly, the difference in function between GP63 homologues in *T. brucei* and *T. cruzi* is a function of their differing life cycles, with the cell-inhabiting *T. cruzi* employing these proteins for infection of host cells, and the blood borne *T. brucei* not requiring such activity.

Invadolysins are employed in Trypanosome parasites of fish. In the case of *Trypanosoma carassii*, Tcagp63 was shown to reduce the production of reactive oxygen and nitrogen

intermediates by goldfish macrophages in response to both *Aeromonas salmonicida* and recombinant goldfish TNFα-2. Application of recombinant Tcagp63 was also shown to reduce goldfish macrophage iNOS-A, TNFα-1 and TNFα-2 levels (340). Perhaps the most interesting note regarding this work is that the recombinant used lacked the traditional HEXXH active site, as recombinants containing the site rapidly degraded. This demonstrates that invadolysins may have alternate methods of enacting immunosuppressive effects that are not purely reliant upon proteolytic activity.

### 2.3.5 Invadolysins in non-parasitic systems

Other than the functional characterization of invadolysins in leishmania and trypanosomes, work on the function of these proteins is lacking. However, the work of McHugh and associates suggests that such proteins may have use even in non-pathogenic species. They found that in *Drosophila melanogaster*, the invadolysin IX-14 was essential for gamete movement and neural development, thereby demonstrating a non-virulence factor-like role for invadolysins in free living organisms (341).

### 2.3.6 Previous examination of work featuring Invadolysins in Schistosomes

Studies on the function of invadolysins in the context of parasitic helminth infections are almost entirely lacking. Recently, the discovery of metzincin family metallopeptidases in both the nematodes *Haemonchus contortus* and *Necator americanus* led to the speculation that such proteins may be more widespread in parasitic lifestyles than once imagined (342). Invadolysin expression data in helminths has only been meaningfully explored in the context of several *Schistosoma* species to date, rendering the elucidation of their function in such infections a matter of key interest.

A possible role for metalloproteases in schistosome infections and life cycle perseverance predates most proteomic, genomic, and transcriptomic work that has been completed to date. This is clearly highlighted by the findings of Amiri and associates in 1988 who demonstrated that *Schistosoma douthitti* cercaria produced an elastase of significantly higher molecular weight than the cercarial elastase commonly seen in *S. mansoni* cercaria (~30000 da vs ~50000 da) (343). This alternate elastase was able to degrade denatured collagen, elastin, gelatin and an ECM model, and was inhibited by 1,10-phenanthroline, thereby implicating a metalloprotease in the cercarial penetration process (343). Further evidence of the employment of metalloproteases in schistosome survival was demonstrated via the addition of 1,10-phenanthroline to adult worms. These worms had muscle contraction impairment, a 98% reduction in egg output, and reduced male/female pairing, all of which lead to reduced survival of parasites living in mice fed small amounts of the inhibitor (344). The impairment of these negative effects via the addition of zinc further implicates a zinc dependent mechanism of survival, likely involving some proteases (344).

A 2011 study seeking to examine peptidase families in *S. mansoni* was successful in identifying 12 leishmanolysin like proteins with a conserved metallopeptidase domain (345). Phylogenetic analysis of these peptidases suggested that the expansion of elastases, cathepsin D proteins, and the leishmanolysins via gene duplication occurred latter on in schistosome evolution after their divergence from other metazoans (345). Microarray analysis, supported by qPCR evaluation, has also been used to see which of these invadolysins may be upregulated in the life cycle stages most relevant to the initial infection of humans. Seven leishmanolysin-like proteins were found to have differential mRNA expression patterns, with 6 of those having the highest upregulation in the developing germ balls, and 1 (Smp_135530) being the only invadolysin more highly upregulated in cercaria (203). Seeing as germball stage schistosomes are known to

synthesize virulence factors in preparation for human skin penetration and immune evasion, the upregulation of invadolysins in this stage hints at such possible functions. To further evidence this point, Smp_090100 was found to be 12.8% of the normalized volume of proteins that are secreted from the acetabular glands of *S. mansoni* upon stimulation of their release via vortexing (175). In addition to its location in the acetabular glands, Smp_09010, along with the closely related Smp_090110, were found via mass spectrometry analysis to be incorporated into the apical membrane of freshly transformed schistosomulae (346).

The expansion of leishmanolysin-like genes is not an event that is exclusively seen in *S. mansoni*, with a similar expansion also being seen in *S. japonicum.* Among the 108 metalloproteases found in the *S. japonicum* genome, 12 leishmanolysin like proteins were found, which places such proteins as one of the most expanded gene families in schistosomes (281). The production of these metalloproteases is not strictly relegated to the cercarial stage of the life cycle, with microarray analysis demonstrating the upregulation of M8 type metalloproteases in both *S. japonicum* cercaria and eggs (347). Given that this microarray showed the highest expression of Sjp_0048370, Sjp_0067490, and Sjp_0067500 in cercaria, and the fact that these proteins most closely resemble the three most highly expressed invadolysins in *S. mansoni* (Smp_153930, Smp_090100, and Smp_090110, respectively), it is likely that these proteins have similar functions between the two species of schistosome (347). The observation that six of these leishmanolysins have been identified via mass spectrometry analysis of *S. japonicum* cercaria, and that 5 of these 6 can also be identified in schistosomula that have been transformed via passage through mouse skin, further implicates them in penetration events, and even suggests other immunomodulatory functions (179). Interestingly, both species have in their possession Leishmanolysin like proteins with M8-like domains, but who lack the HEXXH active site, suggesting an evolutionary loss of

function during gene duplication events, or that the active siteis not required for certain biological functions.

Prior to the investigations performed for the purposes of this thesis, the function of these invadolysins was unknown. Previous findings point towards the possible uses of invadolysins in the schistosome life cycle, and genomic analysis of *S. bovis* and *S. haematobium* points towards at least one invadolysin (the *S. bovis* Smp_127030 paralogue) being highly conserved during hybridization (348). Although strong antibody responses have not been seen to the two most highly upregulated invadolysins (Smp_090100 and Smp_090110) in infected individuals, the study of these likely virulence factors is of interest in order to elucidate the immunosuppressive properties of these proteins, which could in theory lead to the discovery of anti-schistosome therapeutics (191).

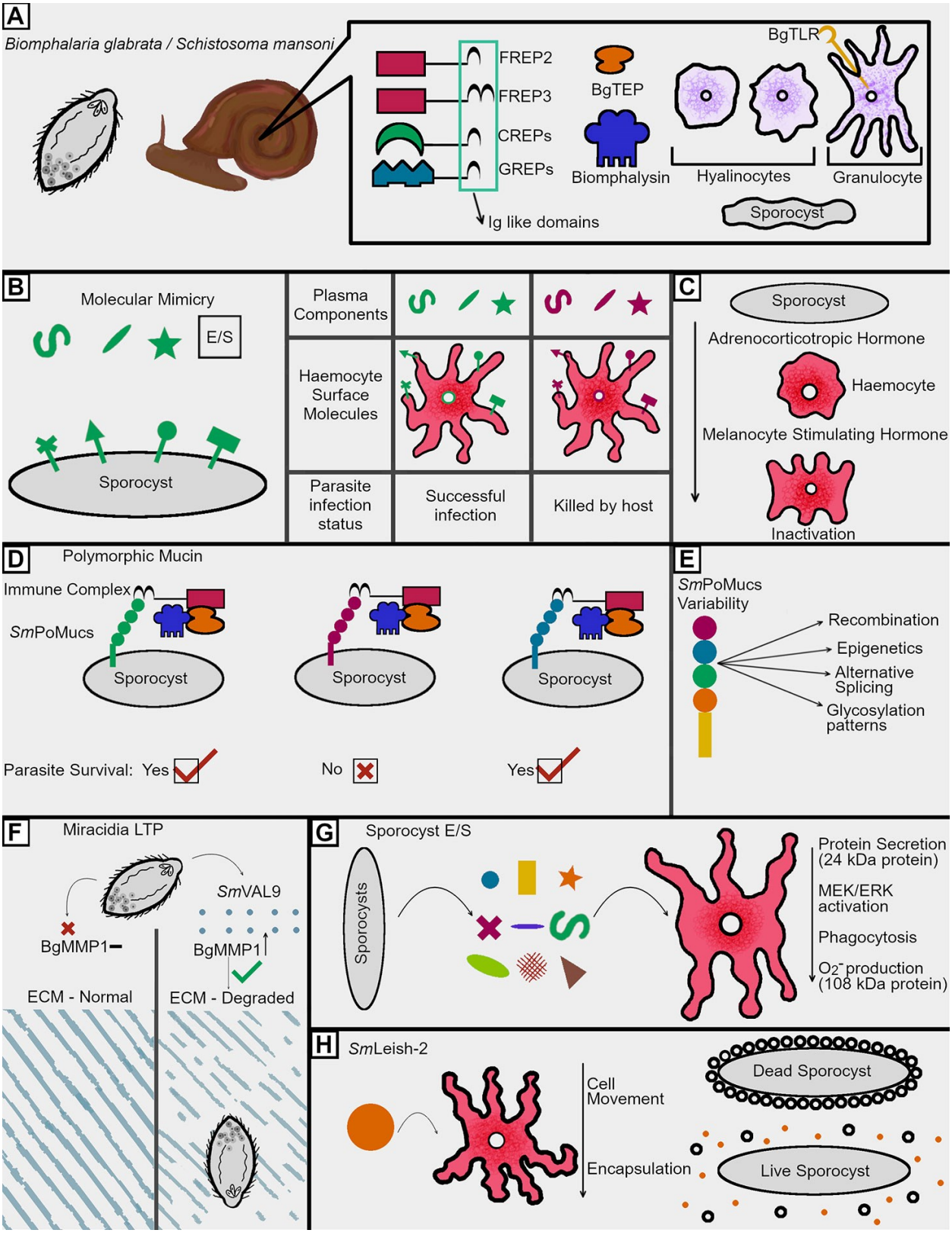
A
Biomphalaria glabrata / Schistosoma mansoni
FREP2
FREP3
CREPs
GREPs
Ig like domains
BgTEP
Biomphalysin
BgTLR
Hyalinocytes
Granulocyte
Sporocyst
B
Molecular Mimicry
E/S
Sporocyst
Plasma Components
Haemocyte Surface Molecules
Parasite infection status
Successful infection
Killed by host
C
Sporocyst
Adrenocorticotropic Hormone
Haemocyte
Melanocyte Stimulating Hormone
Inactivation
D
Polymorphic Mucin
Immune Complex
SmPoMucs
Sporocyst
Sporocyst
Sporocyst
Parasite Survival: Yes
No
Yes
E
SmPoMucs Variability
Recombination
Epigenetics
Alternative Splicing
Glycosylation patterns
F
Miracidia LTP
SmVAL9
BgMMP1
BgMMP1
ECM - Normal
ECM - Degraded
G
Sporocyst E/S
Sporocysts
Protein Secretion (24 kDa protein)
MEK/ERK activation
Phagocytosis
O2- production (108 kDa protein)
H
SmLeish-2
Cell Movement
Encapsulation
Dead Sporocyst
Live Sporocyst

**Figure 2.1. Immunosuppression tactics in the intermediate host**

**A)** Snails employ a wide variety of both humoral and cellular factors in combatting schistosome infections, many of which cooperate with each other to facilitate parasite killing. The humoral factors are largely composed of PRRs, but also feature cytotoxic components such as biomphalysin. The cellular arm of the immune response features *Bg*TLR displaying granulocytes, which envelope invading schistosomes, and hyalinocytes, which seemingly focus on the production of humoral factors and cell signaling molecules. **B)** Parasites employ molecular mimicry by utilizing surface molecules and E/S products which share glycosylation patterns seen in snail plasma and on the surface of circulating haemocytes. Sharing such glycosylation patterns has been shown to correlate with survival during infection, suggesting these shared epitopes help schistosomes avoid recognition within the snail. **C)** Molecular mimicry is also employed by the production of immune cell inactivating hormones like those produced by the snail which renders normally lethal haemocytes inactive and unable to kill invading sporocysts. **D)** In order to avoid recognition by host pattern recognition receptors, schistosomes employ a highly variable series of polymorphic mucins. These mucins are recognized by host *Bg*FREPs, and the variable nature of both the mucins and FREPs has led to the understanding that successful recognition of *Sm*PoMucs by FREPs is a key determinant of infection success. If the host FREP can recognize the *Sm*PoMucs and the surface of a sporocyst, that sporocyst will likely be killed, while having an unrecognizable *Sm*PoMuc leads to immune evasion by avoiding *Bg*FREP recognition. Such killing is thought to be at least partially dependent on a humoral *Bg*FREP/*Bg*TEP/Biomphalysin complex. **E)** In order to generate a highly variable surface mucin, numerous processes occur to give rise to the considerable amount of variability seen between different sporocyst *Sm*PoMucs. **F)** Miracidia employ a venom allergen like protein which has been shown to upregulate the production of a *B. glabrata* matrix metalloproteinase (*Bg*MMP1). This metalloproteinase is hypothesized to facilitate degradation of host connective tissues. Such degradation would allow for easier movement further into the host during initial infection. **G)** E/S products from developing sporocysts facilitate the downregulation of key anti-parasitic functions in haemocytes. While some of these proteins have been identified, others remain of an unknown composition, and are merely referred to by their size. **F)** SmLeish-2 released by the parasite reduces haemocyte motility and therefore downregulates parasite encapsulation. This allows for continued movement and development of the sporocyst within the host. (Derived from Hambrook et al. 2021)

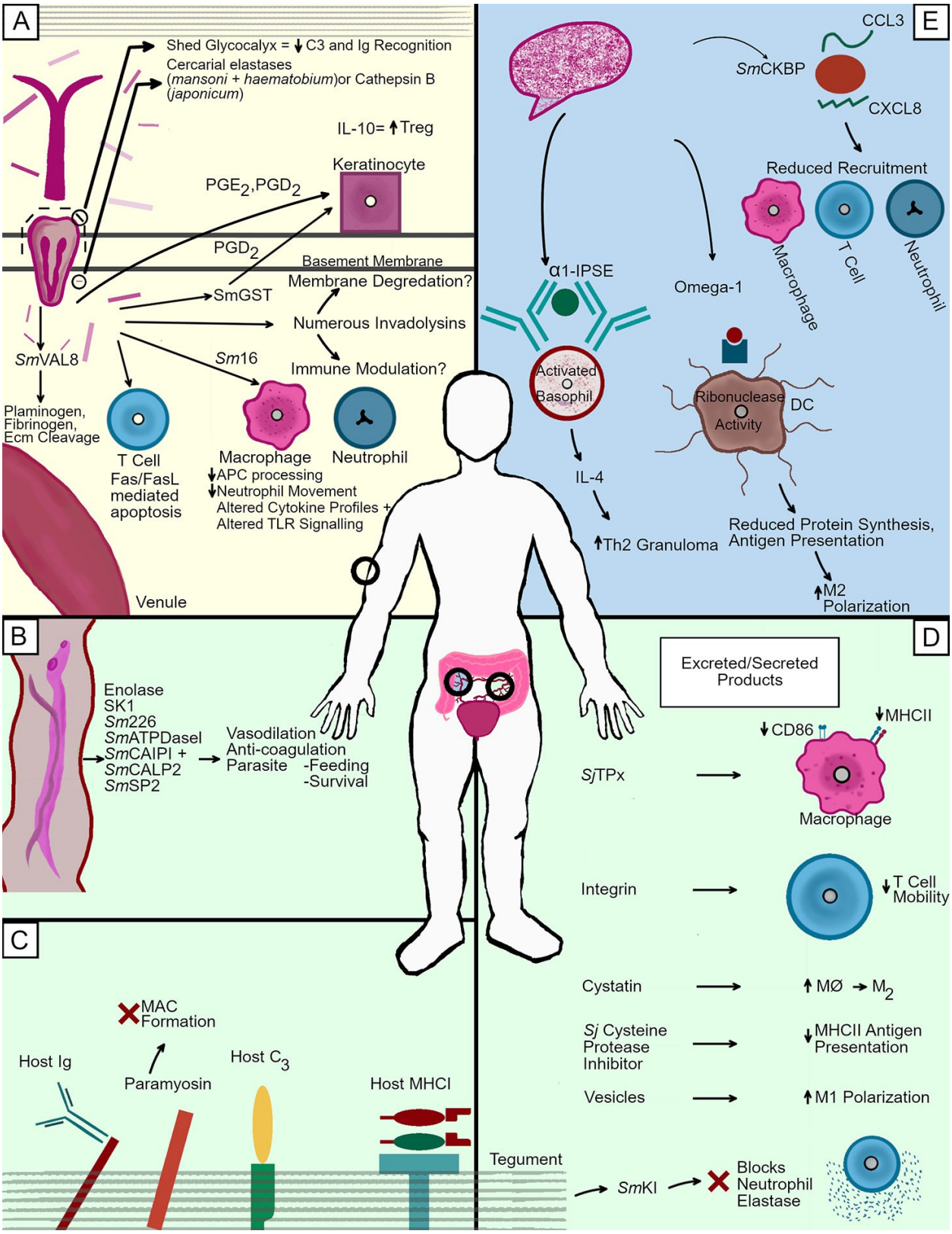
A
Shed Glycocalyx = ↓C3 and Ig Recognition
Cercarial elastases
(mansoni + haematobium)or Cathepsin B
(japonicum)
IL-10= ↑Treg
Keratinocyte
PGE2,PGD2
PGD2
Basement Membrane
Membrane Degredation?
SmGST
Numerous Invadolysins
SmVAL8
Sm16
Immune Modulation?
Plaminogen,
Fibrinogen,
Ecm Cleavage
T Cell
Fas/FasL
mediated
apoptosis
Macrophage
↓APC processing
↓Neutrophil Movement
Altered Cytokine Profiles +
Altered TLR Signalling
Neutrophil
Venule
E
CCL3
SmCKBP
CXCL8
Reduced Recruitment
Macrophage
T Cell
Neutrophil
α1-IPSE
Omega-1
Activated
Basophil
Ribonuclease
Activity
DC
IL-4
↑Th2 Granuloma
Reduced Protein Synthesis,
Antigen Presentation
↑M2
Polarization
B
Enolase
SK1
Sm226
SmATPDasel
SmCAIPI +
SmCALP2
SmSP2
Vasodilation
Anti-coagulation
Parasite -Feeding
-Survival
D
Excreted/Secreted
Products
↓MHCII
↓CD86
SjTPx
Macrophage
Integrin
↓T Cell
Mobility
Cystatin
↑MØ → M2
Sj Cysteine
Protease
Inhibitor
↓MHCII Antigen
Presentation
Vesicles
↑M1 Polarization
C
MAC
Formation
Host Ig
Paramyosin
Host C3
Host MHCI
Tegument
SmKI
Blocks
Neutrophil
Elastase

**Figure 2.2. Immunosuppression tactics in the human host**

Schistosomes travelling through humans employ different immune evasion methodologies depending upon their location and life cycle stage. **A)** As cercariae penetrate the skin, numerous proteases of different families help facilitate the cleavage of host molecules. The degradation of key structural components such as numerous collagens and elastin by these proteases helps the parasite descend through the epidermis, penetrate through the basement membrane, and eventually navigate the dermis in the search for a nearby venule. The invading larvae also release numerous immunosuppressants such as Sm16 and prostaglandins which alter leukocyte function in an attempt to avoid cell-mediated death by creating a favorable immune environment **B)** Adults can also release molecules to reduce blood clotting in the area immediately surrounding a mated pair. This wide variety of molecules may be involved in the degradation of host plasma components to facilitate feeding, but their capacity to act as anti-coagulants also implies the creating of a milieu in which the worms are able to move freely without fear of being killed by the coagulation of the blood in which they are immersed. **C)** Adult worms in the mesenteric blood vessels incorporate host molecules into their tegument. Many of these molecules are important immune factors, which are bound in such a way as to prevent proper opsonization. IgG is found via its Fc portion, making it unrecognizable to cell bound Fc receptors. Complement component C3 is also bound, although the effects of this association are not completely understood. Finally host MHCI can also be found bound to adult worms via its β2-microglobulin domain. The worm also targets mammalian factors not only for capture, but also for inactivation, as evidenced by schistosome paramyosin inhibiting membrane attack complex formation. **D)** Schistosomes release a wide variety of parasite-derived factors into their environment to create tolerable conditions for their survival. These molecules perform many different tasks, including the inhibition of cysteine proteases (*Sj* cysteine protease inhibitor), the blocking of neutrophil elastase (*Sm*KI) and the alteration of antigen processing in macrophages (*Sj*Tpx). **E)** Schistosome eggs release several factors key in skewing the T cell response towards the Th2 phenotype necessary for migration through the intestinal wall. These include α-1, which causes TH2 type granuloma formation through basophil mediated IL-4 production, and ω-1, which brings about M2 polarization by altering dendritic cell antigen presentation. The chemokine binding protein *Sm*CKBP is also present and reduces recruitment of varying leukocytes. (Derived from Hambrook et al. 2021)

# Chapter 3 - Materials and Methods

## 3.1 Snails

Two strains of *B. glabrata* snails were used in this study. The BS-90 strain is resistant to PR-1 strain *S. mansoni* infection (102,349), while the M-line strain is susceptible (101). Snails were maintained in aerated artificial spring water at 23–25°C, 12-hour day/night cycle and fed red-leaf lettuce as needed. All snail exposures were performed with the PR-1 strain of *S. mansoni* (101), which was obtained from infected Swiss-Webster mice provided by the NIH/NIAID Schistosomiasis Resource Center at the Biomedical Research Institute (350).

## 3.2 Experimental snail infections

Age-matched *B. glabrata* snails (~10mm shell diameter) were put into a 12 well plate and submersed in artificial spring water (ASW). To each well containing a snail, 10 miracidia were added, and given 24 hours to infect each snail. After infection, snails were transferred to a separate tank, and maintained as described in section 3.1 for a period of 6-12 weeks.

## 3.3 Generation of *Schistosoma mansoni* parasite materials

### 3.3.1 Collection and hatching of *S. mansoni* eggs

Mice infected with *S. mansoni* were euthanized 7 to 8 weeks post exposure and their liver extracted and homogenized using an Omni Mixer Homogenizer model $N^0$17105 for 60 seconds. The homogenized product was added to a 2L flask filled with artificial spring water. The flask was covered in aluminum foil except the top 5 cm of the flask. Light was shone at the top uncovered part of the flask to encourage the migration of newly hatched miracidia to the top of the flask thus facilitating their subsequent isolation.

### 3.3.2 Miracidia collection, transformation, and larval transformation product isolation

Miracidia were pipetted into the wells of a 12 well clear-bottom plate. After 24 hours culture in Chernin's Balanced Salt Solution (CBSS) (351) containing glucose and trehalose (1g/L each), penicillin (100 U/mL) and streptomycin (100 μg/mL), most miracidia transformed to primary sporocysts. Parasite culture supernatants containing E/S products (137) were collected and sterilized with a 0.2 μm syringe filter, concentrated and stored at −80°C.

### 3.3.3 Collection and shedding of *S. mansoni* cercaria

Snails were allocated into 6 well plates and exposed to light for 5 hours during which cercaria emerged. Snails were removed from the wells, and cercaria were transferred to a petri dish. Visual inspection using a dissecting microscope (Zeiss) was performed in order to remove contaminating vegetation, or snail faecal material.

### 3.3.4 Generation of whole cercarial lysate, drained cercarial lysate, and cercarial excretory/secretory products

Cercaria were mechanically transformed into schistosomula via vortexing in 1.5 ml tubes. Parasites were kept on ice for 15 minutes, before being centrifuged for 5 minutes at 1000g. Artificial spring water was removed, and 200 μl of RPMI 1640 was added. Cercaria were again vortexed for one minute, and visually inspected using microscopy to confirm detachment of their tails. Sub samples were taken and schistosomula were counted under a dissection scope in order to determine parasite concentration in each preparation. Parasites were centrifuged again, supernatants were collected and frozen for western blotting, and 200 μl of RPMI 1640 (1% Penicillin/Streptomycin) was again added to the transformed schistosomula. Schistosomula were cultured at 37°C for 24 hours. Untransformed cercaria, transformed schistosomula, and

schistosomula excreted/secreted (E/S) products were all gathered for western blot analysis. Whole cercarial lysate (WCL) was generated via 3x 15s sonification of freshly emerged cercaria in PBS, on ice. Drained cercarial lysate (DCL) was generated via 3x 15s sonification of cercaria that had expulsed E/S products in RPMI over 24 hours at 37°C.

### 3.4 Cell Lines

#### 3.4.1 *Biomphalaria glabrata* embryonic (Bge) cell line

*B. glabrata* embryonic (Bge) cells (352) (American Type Culture Collection NR-21959) were cultured in order to obtain supernatants in which to culture *S. mansoni* sporocysts over the span of 5 days. This was done given their similarity to *B. glabrata* haemocytes, and the requirement of *B. glabrata* materials to facilitate sporocyst growth (353). Corning$_{TM}$ 25 cm$^2$ flasks with plug seal caps were used to culture the cells at 26 °C. Cells were grown in complete Bge medium (22% Scheider's Drosophila medium, 0.45% Lactalbumin hydrolysate, 0.13% Galactose, 14.1μM phenol red, 20μg/ml Gentamycin, 10% heat-inactivated FBS), and passaged via use of a cell scraper every week by seeding at a 1:4 dilution of the cells (354).

#### 3.4.2 RAW 264.7 cell line

RAW 264.7 cells (ATCC) were employed in our examinations of invadolysin effects on phagocytosis, given their frequent use as phagocytic cells (355). Cells were cultured in complete DMEM (10% heat inactivated FBS, 1% penicillin/streptomycin (P/S)) in a Corning$_{TM}$ 25 cm2 flasks with vented seal caps at 37°C, 5% $CO_2$. Cells were passaged weekly using PBS+0.1%EDTA as a detachment buffer, followed by a 1:4 dilution during reseeding.

### 3.4.3 THP-1 cell line

THP-1 cells (ATCC) were employed in our examinations of invadolysin effects on phagocytosis, as well as analysis of their effects on cytokine production. This was done given previous use of THP-1s as cytokine producing cells when studying exposure to *S. mansoni* materials (266). Cells were cultured in complete RPMI (10% FBS, 1% (P/S)) in a Corning$_{TM}$ 25 cm2 flasks with vented seal caps at 37°C, 5% $CO_2$. Cells were passaged weekly via a 1:4 dilution into fresh media. Differentiation of THP-1 cells into macrophage-like cells was performed by treating cells with 40nM PMA for 24 hours, followed by a change to new media lacking PMA for another 24 hours.

### 3.4.4 Jurkat cell line

Jurkat cells (ATCC) were employed in our examinations of invadolysin effects on cytokine production given their high similarities to T cells. This was done given their previous use as cytokine producing cells when studying exposure to *S. mansoni* materials in co-exposure trials featuring THP-1 cells (266). Cells were cultured in complete RPMI (10% FBS, 1% (P/S)) in a Corning$_{TM}$ 25 cm2 flasks with vented seal caps at 37°C, 5% $CO_2$. Cells were passaged weekly via a 1:4 dilution into fresh media.

### 3.4.5 HL-60 cell line

HL-60 cells have long been utilized as a model for studying neutrophil function (356–358). We used HL-60 cells as such for both an examination of the effect of invadolysins on cytokine production, as well as a granulocyte-mediated killing assay for newly transformed schistosomula. Cells were cultured in complete RPMI (10% FBS, 1% (P/S)) in a Corning$_{TM}$ 25 cm2 flasks with vented seal caps at 37°C, 5% $CO_2$. Cells were passaged weekly via a 1:4 dilution into fresh media.

Differentiation of HL-60 cells was facilitated via the addition of 1.5% DMSO for 4 days prior to use in any assay, as per previously established methods (357).

Initial testing of differentiation protocols for HL-60 cells were performed for 4 days at 37 °C with 5% $CO_2$ using 1.5% DMSO, or 1μmol retinoic acid. Capacity to produce reactive oxygen species in response to PMA was assessed using a 2,7-Dichlorofluoresceine diacetate assay. 100,000 cells per well were seeded into the wells of a black walled 96 well plate in PBS. PMA was added to a final concentration of 25μM, and 490/520 readings were obtained over the course of three hours using a Spectramax fluorescence spectrophotometer, while cells were kept at 37°C.

### 3.4.6 EoL-1 cell line

EoL-1 cells have been established as a model system for studying some functions of eosinophils (359–361), and were used to determine invadolysin effects on cytokine production, as well as a granulocyte-mediated killing assay for newly transformed schistosomula. Cells were cultured in complete RPMI (10% FBS, 1% (P/S)) in a Corning$_{TM}$ 25 cm2 flasks with vented seal caps at 37°C, 5% $CO_2$. Cells were passaged weekly via a 1:4 dilution. Differentiation of EoL-1 cells was performed via treatment with 1.5% DMSO for 3 days, as per previously established methods (362).

## 3.5 Identification of *S. mansoni* Leishmanolysin (SmLeish)

Transcripts and proteins resembling leishmanolysin of *Leishmania major* (150,363) have been identified in numerous screens of schistosomes at varying life history stages including during the human infection (175,203,281,345,347,364) and snail (137,346,365) hosts. Improvement of the *S. mansoni* genome (366), identified two complete leishmanolysin-like transcripts, one of which (XP_018651919), possessed two predicted M8 protease domains (http://pfam.xfam.org).

This transcript was used for all *in silico* analyses and served as a basis for all functional assessments.

### 3.6 Sequence annotations, alignments, tertiary structure prediction and visualization

#### 3.6.1 Annotation of SmLeish and SmCI-1 MMP domains and active sites

MMP domain prediction was done by submitting the amino acid sequences for SmLeish (XP_018651919.1) and SmCI-1 (Smp_090100) as acquired from GenBank [National Center for Biotechnology Information (NCBI) to the Pfam sequence search tool (367). While Pfam denotes active sites by finding HEXXH sequences, the third histidine, methionine involved in the met-turn, and potential cysteines involved in the cysteine switch mechanism were all manually located with the aid of subsequent 3D modeling.

The predicted amino acid sequence for SmLeish (XP_018651919.1) was assessed using the program Phobius (368) to determine that SmLeish is likely a non-cytoplasmic protein with a weak signal peptide (residues 1–20) with a cut site between positions 20 and 21 as assessed using SignalP 4.1(369).

#### 3.6.2 Tertiary structure predictions and visualization

Homology modeling was performed by submitting the amino acid sequence for SmCI-1 (Smp_090100) and each of SmLeish's (Smp_135530) two MMP domains to the Robetta Server (robetta.bakerlab.org). RoseTTAFold, a deep learning-based modeling method, was used for secondary/tertiary structure prediction. The predictions with the highest confidence for SmCI-1, SmLeish D1, and SmLeish D2 (0.71,0.76,0.79) were uploaded to PyMol (PyMOL™ Version 2.4.1 Schrodinger ©) for visualization and labeling of key active site amino acids. For GP63 comparison, the GP63 protein databank file was obtained from the RCSB protein databank (288).

### 3.7 qRT-PCR assessment of transcript expression

#### 3.7.1 Total RNA extraction from infected snails

Using TRIzol Reagent with the PureLink RNA Mini Kit (Life Technologies) and following the instructions of the manufacturer, RNA from each snail was isolated and then stored at -80°C. Briefly, snails were homogenized via pestle in 1ml TRIzol for 5 minutes at room temperature. A total of 0.2 ml of chloroform was added and tubes were vigorously shaken by hand for 15s before incubation at room temperature for 3 minutes. Samples were then centrifuged for 15 minutes at 12,000g. The upper aqueous layer was then transferred to a new tube, and an equal volume of 70% EtOH was added. Samples were then purified and washed using a Qiagen RNeasy kit, as per the manufacturer's instructions.

#### 3.7.2 First strand cDNA synthesis

Complementary DNA (cDNA) was generated using this total RNA with the Quanta Biosciences qScript cDNA SuperMix Kit according to the manufacturer's instructions.

#### 3.7.3 Assessment of transcript expression

Quantitative RT-PCR primers and probe for SmLeish were designed using the qPCR assay design tools from Integrated DNA Technologies (IDT) (**Table 3.1**). Three unique qPCR assays for SmLeish were designed and utilized to assess whether alternative splicing was taking place and to ensure that assessment of transcript abundance was yielding consistent results across the entire transcript. The SmLeish qPCR reaction consisted of 10µL of TaqMan mix (Thermo Fisher Scientific), 0.4µL of the forward and reverse primers, 0.2µL of the probe, 4µL of nuclease free water, and 5µL of SmLeish template. The qRT-PCR reactions were run in triplicates in 96-well qRT-PCR plates for 40 cycles on an ABI 7500 Fast Real Time PCR machine (Thermo Fisher

Scientific) using the following thermocycling conditions: initial hold at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, with data collection every cycle. Specificity for the qRT-PCR amplicons was confirmed by continuous melt curve analysis. Significant differences between treatments were assessed by one-way analysis of variance (ANOVA) with Tukey's post-hoc test.

### 3.8 Generation and validation of polyclonal antibodies

#### 3.8.1 Antibody generation

For the work performed in chapter 4, rabbit anti-SmLeish polyclonal antibodies were generated using engineered peptides with the SmLeish peptide sequence EEDGTPRTPRDPQT (GenScript) predicted to have a high level of antigenicity based on the OptimumAntigen design tool (GenScript). Serum IgG was purified using a Protein A/G column (GE Healthcare) by FPLC. Further purification was undertaken by then running the purified IgG through an immunoaffinity column bound with the specific peptides to which the polyclonal antibody was designed.

The work done in chapter 5 and chapter 6 of this thesis required the generation of new polyclonal antibodies to SmLeish and SmCI-1. The synthesized genetic sequences for SmLeish and SmCI-1 were inserted into the pET-47b(+) bacterial expression vector (Novagen) following manufacturers specifications. BL21(DE3) (ThermoFisher Scientific) cells were transfected with SmCI-1orSmLeish-containing pET-47b(+) vector, grown at 37°C in LB media (ThermoFisher Scientific) containing 100µg/ml kanamycin (ThermoFisher Scientific) and then induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) once the cells had grown to OD 600. Following ~4 hours of growth, the cells were concentrated by centrifugation at 10 000 g for 20 minutes at 4°C. The final weight of the bacteria pellet was measured and then the lysing reagent B-PER

(Thermo Fisher Scientific) was added at a concentration of 4 ml/g of bacteria in combination with phenylmethylsufonyl fluoride (PMSF, final concentration of 1 mM), mixed gently and left to incubate at room temperature for 15 minutes. Before application to the 5ml 6xHIS column (GE Healthcare) for purification, the supernatant was diluted to a total protein concentration of 100 µg/ml in binding buffer (GE Healthcare). Following purification, the 6x HIS tag region of the recombinant protein was removed following the vector manufacturers specifications (Novagen). These recombinants were used to generate a rabbit anti-Sm-CI-1 and anti-SmLeish polyclonal antibody using the Custom pAb service offered by Genscript.

### 3.8.2 Antibody validation

The polyclonal antibodies used in chapters 5 and 6 were validated by loading samples consisting of material from approximately 15 cercaria into each lane. Samples were either treated with reducing Laemmli buffer or native Laemmli buffer, and either boiled at 95°C for 10 minutes, or left at room temperature. Western blot analysis was then performed, using either a 1:1000 dilution of the appropriate primary polyclonal antibody, or an equivalent dilution of pre-immune serum from the relevant rabbit.

## 3.9 Generation of recombinant Invadolysins

### 3.9.1 Production and purification of rSmLeish in bacterial expression system

Recombinant SmLeish was generated by using the Gateway cloning system according to the manufacturer's instructions (Life Technologies). The coding region was amplified with Phusion high-fidelity DNA polymerase from a targeted DNA template in a pUC57 plasmid (synthesized by GenScript) and cloned into the pENTR/D-TOPO vector. Plasmid DNA from this entry clone was isolated and cloned into the pET-DEST42 Gateway vector (Life Technologies) in

a Clonase recombination reaction. This DNA was then transformed into BL21-AI One Shot Chemically Competent *E. coli* (Life Technologies).

*E. coli* stably expressing SmLeish were then selected and grown up at 37°C in 100µg/mL ampicillin LB medium. Optimal expression of protein after the incorporation of L-arabinose and IPTG was then quantified by SDS-PAGE and Western blot using antibodies against the 6x His tag and V5 epitope (Life Technologies) on the recombinant protein.

Prior to purification of the recombinant protein, *E. coli* were concentrated by centrifugation at 10 000 g for 20 minutes at 4°C. The final weight of the bacteria pellet was measured and then the lysing reagent B-PER (Thermo Fisher Scientific) was added at a concentration of 4mL/g of bacteria in combination with phenylmethylsufonyl fluoride (PMSF, final concentration of 1mM), mixed gently and left to incubate at room temperature for 15 minutes. After the incubation, the samples were centrifuged at 10 000 g for 10 minutes and the supernatant kept for the purification steps. Before application to the 6x His column for purification, the supernatant was diluted to a total protein concentration of 100µg/mL in binding buffer (GE Healthcare). Fast protein liquid chromatography (FPLC) (AKTA Pure–GE Healthcare) was used to purify rSmLeish protein using 1mL nickel-agarose columns that bind to the 6x His region of the recombinant protein (GE Healthcare). Prior to quantification via BCA protein quantification assay (Thermo Fisher Scientific), the purified rSmLeish was dialyzed using a Slide-A-Lyzer Dialysis Cassette kit (Thermo Fisher Scientific) as per the manufacturer's instructions (Fig 4.9).

### 3.9.2 Production and purification of rSmLeish and rSmCI-1 in HEK expression system

The genetic sequence of SmCI-1 and SmLeish were synthesized and inserted into a pHEK293 Ultra Expression Vector I. Transient transfection of HEK293 cells was performed, with

the recombinant protein being purified via the anti-SmCI-1 and anti-SmLeish polyclonal antibody generated against a recombinant SmCI-1 or SmLeish protein generated in a bacterial expression system (see below). A mutant form of SmCI-1 (SmCI-1Mut) was synthesized in an identical manner, with the Glu232 codon (GAA) replaced with a Glycine codon (GGG).

### 3.10 Western blot and silver stain analysis

#### 3.10.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Samples were prepared in reducing Laemmli protein loading buffer (370). Samples were heated at 95 °C for 10 m, then loaded on 10, 12, or 15% (vol/vol) SDS-PAGE gels and run on the Mini Protean Tetra system (Bio-Rad) at 150 V and 300 mA. The amount of protein used varied depending on the experiment being performed.

#### 3.10.2 Silver staining

Silver staining was performed using a Silver stain kit (Pierce) as per the manufacturer's instructions. Images were acquired on the ImageQuant LAS 4000 machine (GE Healthcare).

#### 3.10.3 Blocking and immunostaining

Samples were then blotted for 2 hrs onto 0.45 μm supported nitrocellulose membranes (Bio-Rad). Blocking was performed for 1 h at room temperature in 5% (wt/vol) BSA in Tris-buffered saline (TBS) solution plus 0.1% Tween–20 (TBS–T buffer) on a rocking platform. Staining for 1 h in primary antibody (anti-SmLeishPeptide at a 1:5000 dilution, αSmLeish and αSmCI-1 at 1:1000 dilution). Membranes were washed 1X for 10 min in TBS–T, then 2X for 5 min in TBS-t, and 1X for 5 min in TBS. Following the TBS wash, membranes were probed for 1 h in a horseradish peroxidase (HRP)-conjugated secondary antibody (Goat α-rabbit IgG) in

blocking buffer, followed by a wash step as previously described. Detection was accomplished by incubating the membranes in SuperSignal West Dura Extended Duration substrate (ThermoFisher Scientific). Chemiluminescent signals were acquired on the ImageQuant LAS 4000 machine (GE Healthcare).

### 3.11 RNA-mediated gene knockdown

#### 3.11.1 siRNA-mediated SmLeish knockdown

*In vitro* transformed *S. mansoni* sporocysts were submerged in a 6nM cocktail of 27-nucleotide siRNA oligonucleotides (Integrated DNA Technologies) designed to specifically target 4 different regions of the SmLeish transcript (**Table 3.2**). The oligonucleotide sequences were confirmed to be unique to SmLeish by comparison to the *S. mansoni* genome. As has been previously reported (371,372), we found that soaking the sporocysts in a cocktail of siRNA oligos resulted in knockdown of SmLeish, this was made more consistent by using the Xfect transfection reagent (Clone Tech). Control sporocysts received siRNA oligo targeting green fluorescent protein (GFP).

#### 3.11.2 RNA oligo-mediated SmCI-1 knockdown

Recent advances in RNA mediated interference have demonstrated that long dsRNA is taken up into cells via class a scavenger receptors (373). Once internalized, these long dsRNA strand are processed into siRNA by Dicer and loaded into the RNA-induced silencing complex (RISC) which can then be used to silence gene expression (374). In order to utilize long dsRNA to silence expression of SmCI-1 in *S. mansoni* cercaria, we injected snails with 6nM of dsRNA with repeated sequences complementary to portions of the SmCI-1 transcript (**Table 3.3**). From 7 to 12 days post infection, cercaria were shed from snails as previously described, and 20 cercaria

were collected for western blot analysis. Membranes were probed with the α-SmCI-1 antibody or using a commercially available α-actin antibody (Thermo Fisher Scientific, 0.5µg/ml). A GFP KD dsRNA construct was used at an identical concentration as a control for infection assays. (Table 3.3).

### 3.12 Enzyme-linked immunosorbent assay

#### 3.12.1 ELISA of SmLeish in *B. glabrata* plasma

E/S products, M-line plasma, and infected M-line plasma were diluted 1/10 in 0.2M $NaHCO_3$ (pH 9.4) prior to addition of 100µL into a 96-well polystyrene ELISA plate. Plates were incubated at 4°C for 24 hours and then washed 3x 5 minutes with wash buffer (25mM Tris, 0.15M NaCl, 0.05% Tween 20, pH 7.2). Plates were then blocked with 2% (w/v) bovine serum albumin in wash buffer for 3 hours at room temperature. Following blocking, the buffer was replaced with 100µL of blocking buffer containing anti-SmLeish antibody (1:500) and incubated at room temperature overnight. Plates were then washed 3x 5 minutes with wash buffer and then incubated with blocking buffer containing a biotinylated anti-rabbit secondary antibody (1:250). Plates were then covered with tin foil and incubated at room temperature for 1 hour, washed 6x 5 minutes, and then incubated in the substrate solution containing streptavidin conjugated to DyLight 649 (Thermo Scientific). The reaction proceeded for 15 minutes and was then read using a 96-well plate reader (Molecular Devices).

#### 3.12.2 ELISA of SmLeish and SmCI-1 in cercarial products

To determine relative amounts of SmLeish and SmCI-1 in cercarial lysates and E/S products, 0.5 cercaria/µl of drained cercaria were sonicated 3x 15s, and their E/S products were collected. An enzyme-linked immunosorbent assay was performed on these samples using

polyclonal rabbit α-SmCI-1 or α-SmLeish antibodies. Additionally 100 ng, 10 ng, and 1 ng dilutions of the corresponding recombinants were also run for the development of standard curves. Briefly, 100 µl samples were pipetted into a black-walled 96 well plate and left overnight at 4°C. Samples were washed 3X, with 100µl, for 5 minutes on a rocking platform with washing buffer (25mM Tris, 0.15M NaCl, 0.05% Tween20, pH 7.2). Blocking buffer (Wash buffer + 2% BSA) was added for 3 hours at room temperature. A 1:500 dilution of the appropriate polyclonal antibody in blocking buffer was added and incubated at 4°C, overnight. Samples were again washed 3X 5mins. A 1:1000 dilution of goat α-rabbit IgG alkaline phosphatase-conjugated antibody was added in blocking buffer and incubated at room temperature for 1 hour. Wells were washed 6X 5 minutes, and 100µl of p-nitrophenyl phosphate substrate (ThermoFischer) was added for 1 hour. Absorbance readings were taken at 405nm.

### 3.13 MMP activity assays

#### 3.13.1 Colorimetric based generic MMP assay

Functional characterization of rSmLeish was performed using the Sensolyte Generic MMP Colorimetric Assay (Anaspec). Following manufacturer's directions, rSmLeish was incubated with the provided chromogenic substrate that is cleaved by MMPs. The sulfhydryl group reaction with Ellman's reagent yields 2-nitro-5-thiobenzoic acid (TNB) as the final product, which is detected using a microplate reader at 412 nm. Human MMP-8 obtained from Anaspec was used as a positive control. Three initial concentrations (0.25 µg/mL 0.5 µg/mL and 1µg/mL) of the enzymes were used. Human MMP-8 and rSmLeish were both exposed to 10 µg/mL trypsin for one hour at 37°C as an activation process as per the MMP-8 assay instructions that was stopped using a trypsin inhibitor (Anaspec). Recombinant SmLeish was also exposed to 5 µg/mL of trypsin for 0, 6 and 12 hours to determine whether longer exposure influenced trypsin activation or rSmLeish

cleavage. The inhibition of the human MMP-8 and rSmLeish activity was assessed by adding the MMP inhibitor Ilomastat (MMP8 and rSmLeish) or 10nM 1,10-phenanthroline (rSmLeish) to the initial enzyme before adding the substrate. These reactions were performed in 96-well plates, and the $OD_{412}$ values were obtained using a SpectroMax M2 fluorescent plate reader. These values were compared to a standard curve generated using a reference provided by Anaspec. Significant differences between treatments were assessed by one-way analysis of variance (ANOVA) with Tukey's post-hoc test.

### 3.13.2 Fluorometric based generic MMP activity assay

Generic MMP function was determined using a Fluorometric Generic MMP Activity Kit (AnaSpec, AS-72202) as per the kit's instructions. Recombinant SmCI-1, SmCI-1Mut, and SmLeish at concentrations of 0.25, 0.5, and 1.0 µg/ml were added to a kit specific reaction buffer (KSRB) and were either activated via incubation with 1mM 4-Aminophenylmercuric acetate (APMA) for one hour at 37°C or left at 37°C without activation. Inhibition was assessed via the addition of 250 µM of 1,10-phenanthroline (Sigma Aldrich) prior to addition of the substrate. As a positive control, recombinant human MMP-8 (R&D Systems) was subjected to the same conditions. Substrate only, buffer only, and APMA controls were also added. 45 µl samples were then mixed with 45 µl of substrate (Mca/Dnp fluorescence resonance energy transfer peptide) in a clear-bottom black-welled 96 well plate and 330nm/390nm fluorescence readings were obtained after 1 hour using a SpectraMax M2 microplate reader (Molecular Devices). Difference between treatments were assessed via One-Way Anova.

### 3.13.3 Specific kit-based cleavage assays

All MMPs were activated via incubation with 1mM APMA as previously described. Gelatin and collagen type 4 cleavage were examined using an EnzChek™ Gelatinase/Collagenase Assay Kit (ThermoFisher Scientific), with MMP-2 (AnaSpec) and Clostridium histolyticum collagenase (ThermoFisher Scientific) used as positive controls. Proteases were incubated with 100 µg/ml fluorescein labeled substrate in a clear-bottom black-welled 96 well plate and 495nm/515nm fluorescence readings were obtained after 20 hours incubations at 37°C using a SpectraMax M2 microplate reader (Molecular Devices). Difference between treatments were assessed via One-Way Anova.

Elastin cleavage was examined using a SensoLyte® Green Elastase Assay Kit (AnaSpec). Porcine elastase (AnaSpec) and MMP-12 (R&D Systems) were used as positive controls. Cleavage of the 5-FAM/QXL™ 520 labeled elastin was measured in a clear-bottom black-welled 96 well plate and 490nm/520nm fluorescence readings were obtained after 1 hour at 37°C using a SpectraMax M2 microplate reader (Molecular Devices). Difference between treatments were assessed via One-Way Anova.

## 3.14 Immunofluorescence

### 3.14.1 Visualization of SmLeish in *S. mansoni* infection of *B. glabrata*

*S. mansoni*-infected *B. glabrata* were frozen in Tissue-Tek OCT (VWR) and cryosectioned in 7µm sections. Sections were transferred to poly-L-lysine microscope slides (Abcam). Slides were then washed twice in TBS and 0.025% Tween-20 solution and blocked using 10% FBS with 1% BSA in TBS for two hours at room temperature. The primary antibody (anti SmLeishPeptide or Keyhole limpet hemocyanin (KLH)) diluted in TBS with 1% BSA at a

concentration of 1:250 was added to the slides and incubated overnight at 4°C in a humidified chamber. Slides were then rinsed twice for five minutes in TBS and 0.025% Tween-20 with gentle agitation. The secondary fluorophore-conjugated antibody (Alexa Fluor 488) diluted in TBS with 1% BSA following the manufacturer's recommendations was added to the slides and incubated at room temperature for one hour. In the dark, slides were rinsed three times in TBS for five minutes each and one drop of DAPI mounting medium (VWR) was added to the specimen. After five minutes, a coverslip was placed over the mounted tissue. Slides were imaged using an Axio imager A2 microscope (Zeiss),and analyzed using Zen 2011 software (Zeiss) and Photoshop CS5 (Adobe Systems Inc., USA).

### 3.14.2 Visualization of SmCI-1 in *S. mansoni* cercaria

Freshly shed cercariae were fixed using 4% paraformaldehyde for 1 hour at room temperature in 1.5 ml tubes. Fixed cercariae were washed 2X using 1000 µl Phosphate buffered saline + 0.1% Tween-20 (PBS-T). Permeabilization was performed using 500 µl of PBS + 5%BSA, 0.02% $NaN_3$, 1% TritonX-100 for 1 hour at room temperature. Cercariae were then washed 2X as described above. Parasites were blocked for 1 hour at room temperature in PBS +5% BSA after which a 1:400 dilution of rabbit anti-SmCI-1 or rabbit anti-SmLeish were added for 1 hour. Samples were washed 2X 20 minutes in PBS-T on a rocking platform, and then stained with a 1:400 dilution of donkey anti-rabbit Alexa 568 antibody and 10 µl of Alexa Fluor™ 488 Phalloidin (ThermoFisher Scientific) at 4°C overnight. Samples were again washed 2X 20 minutes with 1000 µl PBS-T and 2x 20 minutes with 1000 µl PBS. Finally, samples were resuspended in 100 µl of PBS, to which one drop of Fluoroshield™ with DAPI (Millipore Sigma) was added. Fluoresence was visualized using a Leica TCS SP5 laser scanning confocal microscope before analysis via ImageJ (NIH).

### 3.14.3 Attempted visualization of E/S product internalization

Either 2ml of RAW 264.7 macrophages or THP-1s in 50mM PMA at a concentration of 1,000,000 cells/ml in respective culture media were plated onto MatTEK Glass Bottom Dishes (LifeSciences) and cultured overnight at 37°C. Cells were washed 3X 5 mins with PBS, and 350µl of *S. mansoni* cercarial E/S products (1.5 cercaria/µl) were added to the cells for 1 hour at 37°C. Cells were again washed 3X and immediately fixed using 4% paraformaldehyde for 20 minutes at room temperature, followed by another 3X rinse in PBS. THP-1 cells were left unpermeabilized while RAW 264.7 macrophages were either left unpermeabilized or subsequently permeabilized with 0.25% Triton X-100 in PBS for 20 minutes on ice. After another 3X wash, blocking buffer (0.25% Triton X-100 in PBS + 5% heat inactivated goat serum) was added for 1 hour at room temperature. A 1:500 dilution of the polyclonal αSmCI-1 antibody was added overnight at 4°C. Samples were washed 5X, and a 1:500 dilution of F(ab')2-goat anti-rabbit IgG (H+L) cross-adsorbed antibody (Alexa Fluoro 555, ThermoFischer) in blocking buffer was added for one hour at room temperature. Samples were then washed 5X for 5mins in PBS. A 1:20 dilution of Alexa phalloidin 388 was added to for 25 minutes at room temperature, followed by a 3X 2min wash, the addition of DAPI for 15 minutes, and another 2X 2min wash. Samples were then mounted and visualized using a Leica TCS SP5 laser scanning confocal microscope before analysis via ImageJ (NIH).

## 3.15 Haemocyte-based functional assays

### 3.15.1 Assessing the impact of rSmLeish on haemocyte movement

The impact of rSmLeish and *S. mansoni* ES products on *B. glabrata* haemocyte migration was assessed using a chemokinesis assay that has been previously published (146). This approach

does not directly measure chemotaxis towards a target gradient, but instead measures the impact of a molecule of interest on cell migration behaviour compared to controls. This assay was chosen because we believed SmLeish to be inhibitory to chemokinesis but did not have any available *S. mansoni*-specific targets known to be chemo attractive to *B. glabrata* haemocytes. Also, because the specific target of SmLeish activity is not known, we were unsure of how it would affect chemotaxis induced by a known factor.

The assay was designed to test the impact of both rSmLeish and *S. mansoni* ES products on haemocyte migration of both M-line and BS-90 strains of *B. glabrata*. BS-90 snails are resistant to *S. mansoni* infection, and thus served as a control for the infection phenotype and the hypothesized role of SmLeish in facilitating infection establishment in M-line snails. In all tests, haemolymph was isolated from an individual snail, centrifuged at 500 X g for 10 minutes, and the haemocyte pellet was isolated by aspirating off the cell-free plasma. The haemocytes were resuspended in 200µL of 1x PBS (2.58 mM $NaH_2PO_4$, 7.68 $Na_2HPO_4$, 150 mM NaCl, pH7.4) and then divided into two equal volumes. Each subset of haemocytes was counted using a haemocytometer (10µL) to ensure equal cell concentrations in each subset. This was done in replicates of five snails per treatment, per strain.

Haemocyte chemokinesis was measured using a custom-built chemotaxis chamber. Haemocytes were always placed into the upper chamber of the apparatus, which is separated from the lower chamber by a 5µM pore-containing membrane. All analyses consisted of staining the membrane with hematoxylin and eosin (H and E) and then counting the number of haemocytes that migrate to the underside of the membrane. In each case, the experimental test was compared to a snail-matched control which consisted of the second haemocyte subset of each snail isolation.

Controls were exposed to CBSS in both the upper and lower chambers of the apparatus and thus represented the baseline haemocyte migration (chemokinesis) for each snail.

The experimental groups used to test SmLeish included: 0.25µg/mL rSmLeish (which was demonstrated to have enzymatic activity as a MMP and represents approximately 2.5x more SmLeish than is found in our ES products) and 0.5µg/mL *S. mansoni* ES products. 1 µM fMLP was used as a positive control. Recombinant SmLeish, *S. mansoni* ES, rSmLeish and ES products with SmLeish immunoprecipitated prior to treatment, rSmLeish incubated with 10µg/mL trypsin for 1 hour prior to treatment, rSmLeish incubated with 10nM 1,10-phenanthroline prior to treatment, fMLP contrasted to ES products or rSmLeish, SmLeish and GFP knockdown ES products and SmLeish knockdown ES products rescued with 0.25µg/mL rSmLeish were assessed for their impact on haemocyte migration in three ways: first by placing the agent in the bottom well to assess attractiveness to haemocytes, second by placing the agent in the top well with the haemocytes to assess inhibition of chemokinesis, and finally, the agent was placed in both wells, which is a traditional control for chemotaxis to assess baseline chemokinesis. Each experimental set-up was replicated five times with independent snails supplying haemocytes for each experiment. The primary experimental groups were all run with each possible combination of upper, lower and both chambers including the treatment. In the case of any treatment that includes trypsin, the treatment was inactivated using Trypsin Neutralizing Solution (ATCC) prior to incubation with haemocytes.

### 3.15.2 Quantifying the impact of SmLeish knockdown on sporocyst encapsulation

Quantifying *S. mansoni* sporocyst encapsulation by *B. glabrata* haemocytes was accomplished by fluorescently labeling primary haemocytes and incubating them for up to 30 hours with transformed sporocysts *in vitro*. Sporocyst transformation was performed as has been

previously described (137), and primary haemocytes were isolated using the head-foot retraction method (104). Prior to sporocyst transformation, the miracidia were exposed to siRNA targeting either SmLeish or GFP as described above. Haemocytes from five snails were pooled and counted using a haemocytometer. Prior to incubation with sporocysts, haemocytes were labeled using CellTracker Red CMTPX (Thermo Fisher Scientific) following the manufacturer's instructions. Following incubation, haemocytes were washed using CBSS 5x for 10 minutes each, and then resuspended in CBSS at a final concentration of 100 cells/μL. Ten microliters of the pooled haemocytes were incubated with five transformed sporocysts (either SmLeish or GFP knockdown) in a depression well microscope slide. Every hour starting from the moment haemocytes and sporocysts were co-incubated, images of each sporocyst were captured using bright field and fluorescent microscopy. In total, 35 individual sporocysts in each group were tracked and imaged at each time point. Detection of the fluorescently labeled haemocytes allowed for visualization of the encapsulation response and later quantification of the number of sporocysts encapsulated at four selected time points, 6, 12, 24 and 30 hours post incubation. The resulting encapsulation time course was visualized by treating the data as an infection study and the two encapsulation curves were analyzed using Graphpad Prism version 7a for Mac (GraphPad Software) using both a Log-rank (Mantel-Cox) test, and a Gehan-Breslow-Wilcoxian test.

### 3.16 SmCI-1 effects on human plasma

#### 3.16.1 Testing hemolytic activity of human sera pre-treated with SmCI-1

Human sera (Sigma Millipore; S7023) was preincubated with rSmCI-1 or rSmCI-1Mut at a concentration of 2μg/ml for 18 hours. Using pre-sensitized sheep erythrocytes (ssRBC) (CompTech, USA; B202), classical pathway mediated hemolysis was assessed following previously published protocols (375). Briefly, ssRBCs were washed 1x in veronal buffered saline

(VBS) (5 mM Veronal, 145 mM NaCl, 0.15 mM $CaCl_2$ 0.5 mM $MgCl_2$. 0.025% $NaN_3$, pH 7.3) by centrifugation at 2000 g for 10 minutes at 4°C and then resuspended to $10^9$ cells/ml in 5ml of gelatin veronal buffer (GVB) (VBS + 0.1% gelatin). The ssRBCs were then warmed to 37°C in a water bath. The experimental human sera control and sera with rSmCI-1 or rSmCI-1Mut added was diluted 1:50 in and then further diluted 1:3 prior to addition of 50 µl to a round bottom 96-well plate. Then 50 µl of the ssRBC suspension was added to the wells. Each treatment was replicated 8x on the plate, including a 100% lysis control which contained 50 µl ssRBCs and a cell blank which contained 50µl ssRBCs with 50 µl GVB. Plates were incubated at 37°C for 30 minutes with gentle agitation. Then 150 µl of ice-cold GVB was added to all experimental and cell-blank control wells and 200 µl of distilled water was added to the 100% lysis control wells. The plate was centrifuged at 1000 g for 5 minutes prior to the transfer of 200 µl of supernatant from each well to a new flat-bottomed 96-well plate. Plates were read using spectrophotometer at 412 nm, the cell blank absorbance was subtracted from each measurement to obtain correct absorbances. Fractional hemolysis in each well was calculated relative to the 100% lysis wells.

Alternative pathway-mediated hemolysis was assessed using rabbit erythrocytes (Innovative Research, USA; IRBRBC10ML) following a previously published protocol (375). Briefly, a 1:25 dilution of the SmCI-1 and SmCI-1Mut preincubated sera prepared as described above was prepared and then diluted 1:3 to create a working solution that was added to the wells of a round-bottom 96-well plate. Fifty µl of the working sera solution was added along with 50 µl of rabbit erythrocytes at a concentration of $10^8$ cells per ml suspended in AP buffer (GVB containing 5 m*M* $Mg^{2+}$ and 5 m*M* ethylene glycol-bis[β-aminoethyl ether]N,N'-tetraacetic acid (EGTA)). Each treatment was replicated 8x on the plate, including a 100% lysis control which contained 50 µl rRBCs in AP buffer and a cell blank which contained 50 µl rRBCs with 50 µl AP

buffer. Plates were incubated at 37°C for 30 minutes with gentle agitation. Then 150 µl of ice-cold N-saline (9 g NaCl dissolved in 1 L water) was added to all experimental and cell-blank control wells and 200 µl of distilled water was added to the 100% lysis control wells. The plate was centrifuged at 1000 g for 5 minutes prior to the transfer of 200 µl of supernatant from each well to a new flat-bottomed 96-well plate. Plates were read using spectrophotometer at 412 nm. Fractional hemolysis in each well was calculated relative to the 100% lysis wells.

### 3.16.2 Plasma mediated killing of newly transformed schistosomula

Cercaria were isolated from snails injected with our SmCI-1 dsRNA both 12 days post injection (SmCI-1 KD cercaria) as well as after only 8 days post injection as a SmCI-1 positive control. Cercaria were mechanically transformed as previously described and added to the wells of a 24 well plate in 100µl of DMEM at a concentration of 5 cercaria per well. Newly transformed schistosomula (NTS) were exposed to 10% human serum, with or without either the wild type or mutant form of rSmCI-1 at 2µg/ml, or 10% heat inactivated human serum. Larval parasite viability was measured using the previously established method published by Frahm et al., taking into account parasite motility, morphology and granularity (376). Briefly, immobile, and highly granular parasites with non-intact teguments received a score of 0, while contracting parasites with no granulation and a smooth tegument were given a score of 3. Scoring was performed blinded.

## 3.17 Human immune cell-based functional assays

### 3.17.1 Invadolysin effects on phagocytosis

The capacity of rSmCI-1 to disturb phagocytosis in RAW 264.7 macrophages and differentiated THP-1 macrophage-like cells was elucidated by examining the presence/absence of

internalized pHrodo Green *E. coli* BioParticles (ThermoFisher). For RAW cells, 300,000 cells/well were plated in DMEM+10% FBS in 12 well plates 24 hours prior to treatment. For THP-1 cells, an identical number of cells per well was differentiated with PMA as previously described. Culture supernatants were removed and replaced with DMEM or RPMI containing either 1μg/ml rSmCI-1, 10μM Cytochalasin D, or a PBS vehicle control. After 1.5hrs at 37°C, 30μg/ml pHrodo Green *E. coli* BioParticles were added to a final volume of 300μl. Cells were spun at 200g for 2 minutes, then left to incubate for one hour at 37°C. Supernatant was removed, and cells were rinsed once with PBS. Cells were detached via 10-minute incubation with 300μl of enzyme free cell disassociation buffer (Gibco). After a 30-minute fixation with 4% paraformaldehyde (pfa), cells were spun at 400g for 5 minutes and resuspended in 30μl PBS. Samples were investigate using an ImageStream X Mark II Imaging Flow Cytometer, capturing a minimum of 8000 events. Data was subsequently analyzed using IDEAS software.

Examining the effect of SmCI-1 on opsonin mediated phagocytosis was also performed. A total of 120μl human serum was mixed for 18 hrs at 37°C with 10 μl of KSRB, and either 1μg/ml rSmCI-1, 1μg/ml rSmCI-1Mut, or a PBS control. pHrodo Green *E. coli* BioParticles were opsonized via exposure to these serum samples for 1.5hrs at 37°C on a rocking platform (200rpm), then rinsed 2x with PBS (400g, 5 mins), following an altered version of the protocol established by Mankovich et al. (377). Phagocytosis by THP-1 cells was then performed as described above.

### 3.17.2 Granulocyte mediated killing of newly transformed schistosomula

Granulocyte killing assays were performed using HL-60 neutrophil-like or EoL-1 eosinophil-like cells. HL-60 and EoL-1 cells were differentiated as previously described. Then 100,000 cells (~2000 cells/parasite) were added to 384 well plates containing approximately 50

cercaria/well. Samples were treated with recombinant human C3 (45µg/ml), and 1µg/ml rSmCI-1or a 1:10 dilution of rabbit anti-SmCI-1 antibody. Cell free and cell only controls were also employed. Live/Dead counts were conducted as previously described.

### 3.18 Invadolysin effects on cytokine production

Initial examination of the effect of SmLeish and SmCI-1 on mammalian cytokine profiles was done using on mouse blood polymorphic blood leukocytes (PBLs) (Cedarlane/ATCC) using a Proteome Profiler Mouse Cytokine Array Kit (bio-techne/R&D systems). This was followed by inspecting the effect of SmLeish and SmCI-1 on cytokine production in various human cell lines. Jurkats (T-cell like), differentiated THP-1s (macrophage like), Jurkat and THP-1 combined cultures, differentiated HL-60s (neutrophil like), and differentiated EoL-1 (eosinophil like) cells using Proteome Profiler Human Cytokine Array Kit (bio-techne/R&D). systems Investigations into the effect of SmCI-1 and SmCI-1Mut on human cytokine production were examined with human blood mononuclear cells (PBMCs) (Cedarlane/ATCC) using a Human XL Cytokine Luminex Performance Panel Premixed Kit (bio-techne/R&D systems). Assays were performed and analyzed as per the manufacturer's instructions. Values are presented as X increase as compared to media-only treated cells.

In the case of mouse PBLs and human cell lines, cells were cultured in RPMI at 10,000 cells per treatment. Cells were either left unstimulated, stimulated with 5 cercaria worth of whole cercarial lysate (WCL), stimulated with 5 cercaria worth of drained cercarial lysate (DCL), or stimulated with 5 cercaria worth of E/S products. Human PBMCs were either left unstimulated, stimulated with 5 cercaria worth of whole cercarial lysate (WCL), or 1µg/ml of lipopolysaccharide (LPS). The effects of the recombinants on cytokine production were assessed by the addition of rSmCI-1 or rSmCI-1Mut at a concentration of 2µg/ml 30 minutes prior to stimulation. For mouse

PBLs and human cell lines, cytokine levels were measured 24 hours post stimulation. For human PBMCs, cytokine levels were measured 12 and 24 hours post stimulation.

### 3.19 Infection and perfusion of mice

Swiss Webster mice were used in infection assays to determine the role of SmCI-1 in *in vivo* infections. Infections were performed using a previously established method during which mice were anesthetized with sodium pentobarbite, after which a suspension of 100 cercaria in 750µl of water was placed in a plastic ring (1cm in diameter) on the surface of the mouse (378).

Five weeks post infection, mice were perfused via portal veins and adult worms were quantified using previously established methods (379,380). Briefly, mice were euthanized using a euthanasia solution containing heparin sodium salt. They were then perfused with a perfusion solution (0.85% NaCl, 0.75% $Na_3C_6H_5O_7$) using a 20-gauge needle inserted into the descending aorta. Adult worms were collected from a small slit in the hepatic portal vein, and counted via stereomicroscope.

### 3.20 Statistical analyses

Analysis of difference in variance between groups in MMP assays, substrate assays, afunctional assays, and mouse infection assays were performed using one way ANOVA with Tukey Post-Hoc tests unless otherwise indicated. Statistically significant differences in the proportion of snails shedding cercariae and the number of cercariae shed was determined using a z-test with a significance threshold of $p<0.05$. Sporocyst encapsulation assays were analysed using a Log-rank (Mantel-Cox) test, and a Gehan-Breslow-Wilcoxian test. Newly transformed schistosomula viability comparisons were performed using a Kruskal-Wallis test with Dunn's post Hoc test for multiple comparisons.

**Table 3.1 Primers and probe used in chapter 4**

| Primer (Position) | Sequence 5'→3' |
|---|---|
| SmLeish FWD Set 1 (2875–2895) | CCTCATCGCTTACCAGAATGT |
| SmLeish Probe Set 1 (2897–2920) | TTACAAATCCACCCACCCACTCTGG |
| SmLeish REV Set 1 (2948–2969) | TGGTTAGTATGCGCTCGAATTA |
| SmLeish FWD Set 2 (749–770) | GTGATCTGAGGCACTATCTTCG |
| SmLeish Probe Set 2 (790–817) | CAGTACTTTGAAGACCCGAGACTTGGTT |
| SmLeish REV Set 2 (845–865) | CGGTAAATGGCACATAAGCTG |
| SmLeish FWD Set 3 (1503–1525) | ATCCTATGCTTTCCTACGTGAAG |
| SmLeish Probe Set 3 (1536–1561) | ACCACGTACACCTAGAGACCCTCAAA |
| SmLeish REV Set 3 (1633–1652) | GCAGAAACCCAGATTCGTTG |
| SmLeish expression *Fwd | *CACC***AGGAGG**AGGAGAGCCATGGTACCCTGTTCAAG |
| SmLeish expression Rev | TTGTTTAATTGATCTACGCCT G |

*Forward primer modified by adding CACC nucleotides (italicized) and ribosome binding sequence (bold font) for efficient cloning and expression.

**Table 3.2 siRNA oligonucleotide sequences used in chapter 4**

| **Target** | **Oligonucleotide sequence 5'→3'** |
|---|---|
| siRNA-SmLeish-1 | 5′- GAAGCUUUCAGAUAUAUGCGAGACCAG -3′ |
| siRNA-SmLeish-2 | 5′- CGAACUGUAGAUGCUUUUGUGCUUAUA -3′ |
| siRNA-SmLeish-3 | 5′- UUACCUACCGCCUUCCGAAUUCGCCAG -3′ 5′- |
| siRNA-SmLeish-4 | 5′- UCGAAUCUUGUUAAACUCUACCUUCUA -3′ |
| siRNA-GFP-1 | 5′-CCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAG-3′ |
| siRNA-GFP-2 | 5′-AGGUAAUAAUACAGGACCCGGUGAUGGUCCUGUAUU-3′ |
| siRNA-GFP-3 | 5′-AUGUUGUUACUAAUGUAGCCUUGACCUACAUUAGUA-3′ |

**Table 3.3 dsRNA sequence used for SmCI-1 and GFP KD in cercaria**

| **Individual SmCI-1 KD Oligo Sequences 5'→3'** |
|---|
| 5' AAGACAAUAUUCUGCAAGUGA 3' |
| 5' AACAUGUCAUCAGUAUUCAUG 3' |
| 5' AAGCUCUAUGUUAUGAUCAUC 3' |
| 5' AAGGAGGAACUAGCGUAUUAA 3' |
| 5' AAGAGAGUAGUUGGUGAUGAA 3' |
| **Full dsRNA SmCI-1 KD sequence 5'→3'** |
| 5'AAGACAAUAUUCUGCAAGUGAAACAUGUCAUCAGUAUUCAUGAAGCUCUAUG<br>UUAUGAUCAUCAAGGAGGAACUAGCGUAUUAAAAGAGAGUAGUUGGUGAUGAA<br>AAGACAAUAUUCUGCAAGUGAAACAUGUCAUCAGUAUUCAUGAAGCUCUAUGU<br>UAUGAUCAUCAAGGAGGAACUAGCGUAUUAAAAGAGAGUAGUUGGUGAUGAAA<br>AGACAAUAUUCUGCAAGUGAAACAUGUCAUCAGUAUUCAUGAAGCUCUAUGUU<br>AUGAUCAUCAAGGAGGAACUAGCGUAUUAAAAGAGAGUAGUUGGUGAUGAAAA<br>GACAAUAUUCUGCAAGUGAAACAUGUCAUCAGUAUUCAUGAAGCUCUAUGUUA<br>UGAUCAUCAAGGAGGAACUAGCGUAUUAAAAGAGAGUAGUUGGUGAUGAAAAG<br>ACAAUAUUCUGCAAGUGAAACAUGUCAUCAGUAUUCAUGAAGCUCUAUGUUAU<br>GAUCAUCAAGGAGGAACUAGCGUAUUAAAAGAGAGUAGUUGGUGAUGAA 3' |
| **Individual GFP KD Oligo Sequences 5'→3'** |
| 5′ CCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAG 3′ |
| 5′ AGGUAAUAAUACAGGACCCGGUGAUGGUCCUGUAUU 3′ |
| 5′ AUGUUGUUACUAAUGUAGCCUUGACCUACAUUAGUA 3′ |
| **Full dsRNA GFP KD sequence 5'→3'** |
| 5′CCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAGAGGUAAUAAUACAGGA<br>CCCGGUGAUGGUCCUGUAUUAUGUUGUUACUAAUGUAGCCUUGACCUACAUUA<br>GUACCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAGAGGUAAUAAUACAG<br>GACCCGGUGAUGGUCCUGUAUUAUGUUGUUACUAAUGUAGCCUUGACCUACAU<br>UAGUACCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAGAGGUAAUAAUAC<br>AGGACCCGGUGAUGGUCCUGUAUUAUGUUGUUACUAAUGUAGCCUUGACCUAC<br>AUUAGUACCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAGAGGUAAUAAU<br>ACAGGACCCGGUGAUGGUCCUGUAUUAUGUUGUUACUAAUGUAGCCUUGACCU<br>ACAUUAGUA |

*Red lettering indicates nucleic acid at beginning of each oligo sequence.

# Chapter 4 - A metalloprotease produced by larval *Schistosoma mansoni* facilitates infection establishment and maintenance in the snail host by interfering with immune cell function[1]

## 4.1 Abstract

Metalloproteases (MPs) have demonstrated roles in immune modulation. In some cases, these enzymes are produced by parasites to influence host immune responses such that parasite infection is facilitated. One of the best examples of parasite-mediated immune modulation is the matrix metalloprotease (MMP) leishmanolysin (Gp63), which is produced by species of the genus *Leishmania* to evade killing by host macrophages. Leishmanolysin-like proteins appear to be quite common in many invertebrates, however our understanding of these proteins outside of *Leishmania spp*. is limited. Numerous proteomic and transcriptomic screens of schistosomes, at all life cycle stages of the parasite, have identified leishmanolysin-like MMPs as being present in abundance; with the highest levels being found during the intramolluscan larval stages and being produced by cercaria. This study aims to functionally characterize a *Schistosoma mansoni* variant of leishmanolysin that most resembles the enzyme produced by *Leishmania*, termed SmLeish. We demonstrate that SmLeish is an important component of *S. mansoni* excretory/secretory (ES) products and is produced by the sporocyst during infection. The presence of SmLeish interferes with the migration of *Biomphalaria glabrata* haemocytes, and causes them to present a phenotype that is less capable of sporocyst encapsulation. Knockdown of SmLeish in *S. mansoni* miracidia prior to exposure to susceptible *B. glabrata* reduces miracidia penetration success, causes a delay in reaching patent infection, and lowers cercaria output from infected snails.

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## 4.2 Author summary

Parasitic flatworms, or digenetic trematodes, cause a wide range of diseases of both medical and agricultural importance. Nearly all species of digenea require specific species of snail for their larval development and transmission. The factors underpinning snail host specificity and how they dictate infection establishment and maintenance are interesting areas of research, both from the perspective of evolutionary immunology and potential application in the design of tools that aim to prevent trematode transmission. Currently, our understanding of snail-trematode associations is one-sided, being predominantly derived from studies that have focused on the snail immune response, with almost nothing known about how the parasite facilitates infection. Metalloproteases, such as leishmanolysin, are proteolytic enzymes; some of which are produced by parasites to influence host immune responses and facilitate parasite success upon encountering the host defense response. Here, we have functionally characterized a leishmanolysin-like metalloprotease (SmLeish) from *Schistosoma mansoni*, a causative agent of human schistosomiasis, which afflicts over 260 million people globally. We demonstrate that SmLeish is associated with developing sporocysts and is also located in *S. mansoni* excretory/secretory products and interferes with snail haemocyte morphology and migration. Knockdown of SmLeish in *S. mansoni* miracidia prior to exposure to *Biomphalaria glabrata* snails reduces miracidia penetration success, delays attainment of patent infections, and lowers cercaria output from infected snails.

## 4.3 Introduction

Compatibility between parasites and their hosts is influenced by a wide variety of immune and immunosuppressive factors that arise over the co-evolutionary history of a host/parasite relationship. This is evident when examining the parasitism of gastropod molluscs by digenean

trematodes. Snails rely heavily upon an immune response comprised of soluble immune effector molecules and immune cells, termed haemocytes, that serve to encapsulate and kill sporocysts (99). Successful trematodes often dampen and/or completely negate specific functions associated with haemocytes, such as the capacity to encapsulate and kill the invading parasite, while also conferring a level of protection to other invading trematodes that would typically be targeted and killed (381–383). This process is mediated by the release of products that impact haemocyte mobility, phagocytic activity, attachment, spreading, and production of reactive oxygen species (ROS) (146,384,385).

The specific factors excreted/secreted by a trematode (termed ES products) that modulate the snail immune response to facilitate parasite infection establishment and persistence are not well known. Based on phenotypic observations of haemocyte rounding and an inability to migrate towards a trematode sporocyst when impacted by ES products of specific trematodes (386), factors that impact the extracellular matrix, such as matrix metalloproteases, might be responsible. Proteomic and transcriptomic screens of schistosomes at all life cycle stages have identified predicted proteins with the hallmark identifiers of metalloproteases, and many are produced in abundance throughout the *S. mansoni* life cycle (137,203,345–347,364,365); with the highest levels being found during the intramolluscan larval stages and in cercaria (137,346,365).

Metalloproteases are part of a larger group of proteolytic enzymes that also encompasses aspartic, glutamic, serine, cysteine, and threonine proteases. They can either be endopeptidases or exopeptidases (387). The use of a zinc metal ion to perform hydrolysis reactions is the defining characteristic of metalloproteases. In most cases, these enzymes possess a conserved zinc-binding motif (HExxH) in which the two histidines coordinate the zinc ion and the glutamate acts as a general base in the catalytic reaction. Metalloproteases can be categorized according to their

catalytic mechanism, their substrates and products, or their structural homology (387). A wide variety of physiological processes such as morphogenesis, peptide and hormone processing, cell adhesion and fusion, proliferation, migration, apoptosis, angiogenesis, and inflammation are mediated by metalloproteases (387,388).

One of the most studied metalloproteases in the context of parasitic infection is leishmanolysin, which is also referred to as Gp63. Olivier et al. (2012) (150) reported Gp63, a major leishmania surface antigen, as a zinc-dependent metalloprotease. Leishmanolysin is capable of cleaving casein, complement component C3, gelatin, albumin, haemoglobin, immunoglobulin G, C3, and fibrinogen (301,306,310). It is localized at the surface of the plasma membranes of leishmania, but also can be excreted and released into the host (150). Gp63 participates in immunomodulatory activities that facilitate infection of host macrophages and protection of leishmania within the macrophage from degradation in the phagolysosome. It is known to assist in the avoidance of complement-mediated lysis of leishmania through the cleavage of C3b (311). The product of this cleavage, iC3b triggers macrophages via the Mac-1 complement receptor, which results in increased parasite internalization, thereby facilitating infection (314). Additionally, Gp63 is thought to interact with both complement and fibronectin receptors to facilitate leishmania entrance into macrophages (311,316,317). It is also capable of degrading the extracellular matrix of host macrophages, which accelerates mobility of leishmania into these cells (309). More specifically, the hydrolysis of Protein Kinase C (PKC) substrates such as myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related proteins (MRP), found in macrophages, alters the PKC signaling pathway in the infected host macrophages leading to inhibition of the production of anti-microbial agents like reactive oxygen species (ROS) (150). Hence, via cleavage and/or degradation, leishmanolysin creates a favorable milieu in the host that facilitates parasite

survival by altering the host immune response through signaling pathway inhibition at various stages of infection.

Leishmanolysin can also be termed invadolysin in species other than leishmania, and has been identified in bacteria, plants, and invertebrate and vertebrate animals (341). These sets of homologous proteins are all generally classified in the M8 family of metzincin metalloproteases (341). They are usually endopeptidases without exopeptidase activity (306). These proteins appear to be commonly associated with host immunosuppression by trypanosomatid parasites. For example, Gp63 homologues have also been implicated in the immune evasion strategies of species of the genus *Trypanosoma*. One of the known evasive and protective functions of *T. brucei* Gp63 includes the removal of variable surface glycoproteins on the surface of the bloodstream stages of the parasite to evade host recognition and killing (337). *T. cruzi* trypomastigotes entry into red blood cells is inhibited in neutralization assays using antibodies raised against Gp63 (338,389). *T. carassii* Gp63 interacts with macrophages of the goldfish host by inhibiting ROS and nitric oxide production, alteration of the phospho-tyrosine protein patterns resulting in downregulation of pathogen and cytokine-induced inflammatory responses of monocytes and macrophages (340). This family of proteins has yet to be functionally characterized in the context of helminth infection.

Numerous analyses of the transcriptome and proteome of *S. mansoni* during various life cycle stages has implicated leishmanolysin-like factors in the establishment and maintenance of *S. mansoni* infection of the snail host. In this study, we have functionally characterized an *S. mansoni* leishmanolysin, showing that it suppresses the snail host immune response during the early stages of the intramolluscan infection. We demonstrate that this *S. mansoni* leishmanolysin (ID: CCD79314.1), termed SmLeish for the purposes of this

manuscript, is an important component of *S. mansoni* ES products and is also present on the sporocyst surface. Additionally, we demonstrate that SmLeish facilitates the infection of *Biomphalaria glabrata* snails through interference with *B. glabrata* haemocyte migration, causing them to be less capable of sporocyst encapsulation. Knockdown of SmLeish in *S. mansoni* miracidia prior to exposure to *B. glabrata* significantly influences the kinetics of the infection, reducing miracidia penetration success, the proportion of snails that shed cercaria and the number of shed cercaria per infected snail.

## 4.4 Materials and methods

### 4.4.1 Ethics statement

All animal work observed ethical requirements and was approved by the Canadian Council of Animal Care (AUP00000057).

### 4.4.2 Sporocyst transformations and isolation of *S. mansoni* excretory/secretory products

Sporocysts were collected in two separate conditions: first from regular *S. mansoni* to obtain ES products, and second from SmLeish knockdown *S. mansoni* miracidia which were later used to evaluate the success of the knockdown. Excretory/secretory products were collected in the same way as described in section 3.3.2 for SmLeish knockdown and GFP knockdown *S. mansoni*. In these cases, knockdown was performed as described in section 3.11.1 during sporocyst transformation. ES products were collected following knockdown in fresh medium.

Primary sporocysts from SmLeish knockdown miracidia were kept for 5 days in conditioned complete *B. glabrata* embryonic (Bge) cell medium prepared from culture supernatants of 4-day maintained Bge cells as previously described (390,391). Sporocysts were collected every day until day 5 post challenge. RNA was purified and underwent reverse

transcription immediately after collection of the sporocysts so that later analysis of SmLeish transcription could be undertaken to confirm knockdown efficiency.

### 4.4.3 Production of recombinant SmLeish

Recombinant SmLeish used for the studies found in this chapter was generated using a bacterial expression system as outlined in section 3.9.1 of this thesis.

### 4.4.4 Generation of an anti-SmLeish polyclonal antibody

The specificity of the polyclonal antibody was tested during a dot blot test against its cognate peptide antigen and was also tested against recombinant SmLeish as well as *S. mansoni* ES products (**SFigure 4.9**). The antibody was also used to measure the approximate concentrations of SmLeish in *S. mansoni* ES products and 2-day post challenge M-line *B. glabrata* (n = 5).

### 4.4.5 Estimation of SmLeish in *S. mansoni* ES products and snail plasma

All plasma samples were compared to plasma samples collected from non-challenged M-line *B. glabrata* (n = 3) and a standard curve generated using a serial dilution series of rSmLeish ranging from 0.0625µg/mL to 2µg/mL. This ELISA of SmLeish in *B. glabrata* plasma was performed as detailed in section 3.12.1.

### 4.4.6 Immunofluorescent detection of SmLeish during intramolluscan *S. mansoni* development

*B. glabrata* were infected with PR-1 *S. mansoni* miracidia that were then given 16 days to develop, after which cryosectioning and subsequent immunofluorescence analysis was performed as per section 3.13.1.

### 4.4.7 Evaluation of SmLeish metalloprotease activity

SmLeish metalloprotease activity was assessed using the Sensolyte Generic MMP Colorimetric Assay (Anaspec) as described in chapter 3, section 3.13.1. MMP-8 was employed as a positive control, trypsin was utilized as an activation method, and both 1,10-phenanthroline and Ilomastat were employed as inhibitors of MMP activity.

### 4.4.8 Quantifying SmLeish transcript and protein abundance during the intramolluscan development of *S. mansoni*

#### 4.4.8.1 Transcript abundance.

RNA was isolated from 5 M-line snails/timepoint, each of these snails having been infected with 5 PR-1 *S. mansoni* miracidia. Timepoints included 1hr, 3hrs, and 12hrs post infection, as well as 1, 2, 3, 4, 8, 16, and 35-days post infection. Samples of cercaria were also taken.

To confirm that challenged snails used in the SmLeish transcript expression analysis were infected with *S. mansoni*, *S. mansoni*-specific GAPDH was assessed using an established assay and protocol (392,393). This assay was also used to estimate the number of *S. mansoni* miracidia (n = 30 SmLeish-KD and 30 GFP-KD) that had successfully penetrated *B. glabrata* snails by comparing Ct values to a standard generated using known numbers of *S. mansoni in vitro*-transformed sporocysts (**SFigure 4.10**).

#### 4.4.8.2 Protein assessment.

Recombinant SmLeish, *S. mansoni* E/S products, snail plasma, and whole infected snail homogenate were separated by SDS-PAGE and then were analysed via western blot using a 1:5000 dilution of the αSmLeishPeptide polyclonal antibody, as per the methods found in chapter 3.

Timepoints post infection included 1hr, 3hrs, and 12hrs, as well as 1, 2, 4, 8, and 16 days infection. For both *B. glabrata* whole snail lysate and plasma, ~30µg of total protein was loaded into each well of the SDS-PAGE. Because no appropriate loading control exists for snail plasma, total plasma amount loaded normalizes the western blot. For total snail lysates, ß-actin was used as a loading control.

### 4.4.9 Assessing the impact of rSmLeish on haemocyte movement

In order to determine whether or not SmLeish was able to alter haemocyte chemokinesis, we employed a custom-built chemotaxis chamber. Haemocytes were isolated from either M-line or BS-90 snails and subjected to various treatments to determine whether or not SmLeish altered movement of either the susceptible or resistant snail haemocytes. Detailed methodologies are presented in section 3.14.1.

### 4.4.10 Knockdown of SmLeish using siRNA

siRNA mediated knockdown of SmLeish was performed as detailed in section 3.11.1. Confirmation of SmLeish knockdown was accomplished using the pre-existing qRT-PCR assay described above. RNA was isolated from *S. mansoni* sporocysts at 1, 2, 3, 4, and 5-days post exposure to either the SmLeish or GFP-specific siRNA oligos for 24 hours. Transcription of SmLeish *in vitro* during this period was assessed and compared to sporocysts exposed to GFP-specific siRNA. In each case, 10 sporocysts were used to generate pooled total RNA from which cDNA for qRT-PCR was synthesized as described above (**SFigure 4.11**).

Fifty M-line *B. glabrata* were challenged with *S. mansoni* miracidia exposed to either SmLeish or GFP-specific siRNA oligos 24 hours prior. Snails were exposed for 24 hours before being placed into tanks containing artificial spring water (25 snails per tank) and were fed biweekly

a diet of red leaf lettuce. Beginning at 4-weeks post challenge and continuing subsequently every week, snails were placed in 24-well plates individually and incubated for 24 hours to assess cercaria shedding. Snail mortality was also noted. The experiment culminated at week 10 post challenge. Data was assessed as a percentage of snails shedding cercaria. Statistically significant differences in the proportion of snails shedding cercariae and the number of cercariae shed was determined using a z-test with a significance threshold of $p<0.05$.

#### 4.4.11 Quantifying the impact of SmLeish knockdown on sporocyst encapsulation

Encapsulation of both SmLeish KD sporocysts and control GFP KD sporocysts was performed as detailed in section 3.14.2 by using fluorescently labeled M-line haemocytes to assess whether or not SmLeish production was involved in the prevention of encapsulation by host immune cells.

### 4.5 Results

#### 4.5.1 Abundance of the SmLeish transcript and soluble protein is highest during the early stages of the intramolluscan infection and is associated with the developing sporocyst

SmLeish is transcribed at all stages of the intramolluscan development of *S. mansoni*. Relative transcript abundance in comparison to the endogenous control, *S. mansoni* GAPDH, which allows for confirmation of *S. mansoni* presence and semi-quantification of *S. mansoni* within *B. glabrata* tissues, peaks at 12-hours post challenge (hpc) with a magnitude 33.2-fold higher than pre-challenge miracidium. SmLeish transcript abundance declines from 12-hpc until cercaria emergence begins around 35 days post challenge (dpc), at which time it rises again to 9.4-fold higher than pre-challenge miracidium. A 18.6-fold higher abundance of SmLeish transcript in isolated shed cercariae suggests that this later increase in SmLeish transcription at 35

dpc is associated with cercariae generation (**Figure 4.1A**). Quantitative RT-PCR assessment of SmLeish transcript abundance was confirmed by Western blot analysis of challenged whole-snail lysates. Probing with the anti-SmLeish antibody reveals a ~130kDa protein that is very close in size to the estimated 125.9kDa of the complete SmLeish protein. This protein displays relatively stable abundance during infection up to 16-dpc. The constitutive expression of this larger protein is contrasted by dynamic expression of a smaller ~48kDa protein that appears at 12 hpc and persists up to 16 dpc (**Figure 4.1B**). The appearance of this smaller protein in challenged whole-snail lysates correlates with a ~48kDa protein that is detected by the anti-SmLeish antibody in *S. mansoni*-infected M-line *B. glabrata* plasma, suggesting that this smaller protein is a soluble version of SmLeish (**Figure 4.1C**). Investigation using two alternative RT-qPCR assays that targeted the 5' and 3' ends of the SmLeish transcript showed little variance in SmLeish transcript abundance when compared to the primary primer and probe set used, suggesting that alternative splicing of the SmLeish transcript is not responsible for the appearance of the ~48kDa protein (**SFigure 4.7**). Immunofluorescent detection of *S. mansoni* sporocysts in histological sections of infected *B. glabrata* using the anti-SmLeish polyclonal antibody suggests that SmLeish is produced in association with the larval parasite and would likely be in close proximity to surrounding haemocytes (**Figure 4.2**).

### 4.5.2 Recombinant SmLeish possesses MMP functionality

SmLeish is predicted to possess two peptidase M8 superfamily domains, the first between amino acids 20–405, and the second between 429 and 737, that are characteristic of leishmanolysin proteins (**SFigure 4.8**). A clear signal peptide is present in the first 20 amino acids, however no traditional transmembrane region is predicted. SmLeish shares the highest amino acid identity with other leishmanolysin-like metalloproteases, however, with respect to the well-characterized

human matrix metalloproteases, SmLeish is most similar to MMP8 (14.1% amino acid identity). To test if SmLeish functions as a metalloprotease, it was compared to human MMP8 in an MMP8 activity ELISA. The human MMP8 displayed dose-dependent and trypsin-activation dependent activity. Its activity was inhibited using the human MMP8 inhibitor (Ilomastat) control provided with the ELISA kit (**Figure 4.3A and 4.3B**). Recombinant SmLeish (rSmLeish) also displayed a dose-dependent activity that was partially inhibited using the Ilomastat inhibitor, and almost completely abrogated using a different MMP inhibitor, 1,10-phanthroline (**Figure 4.3A**). Trypsin activation of SmLeish was not required for MMP activity, however, activity was diminished by ~4x if trypsin was not used (**Figure 4.3B**). Prolonged incubation of rSmLeish with trypsin at a concentration of 5µg/mL for 6 or 12 hours resulted in more efficient cleavage of the pro-rSmLeish (**SFigure 4.8B**), however, rSmLeish treated with trypsin for 12 hours did not significantly enhance the MMP activity compared to the 1-hour treatment (**Figure 4.3**).

To test if trypsin treatment cleaves rSmLeish, and to determine whether the ~48kDa protein that appears during *S. mansoni* infection of *B. glabrata* is generated by trypsin cleavage, rSmLeish was exposed to 1, 5 and 10µg/mL of trypsin for 1 hour. Five distinct bands were visualized by Western blot using the anti-SmLeish antibody, and these cleavage products became more resolved when 5 or 10µg/mL trypsin was used. The five bands were sent for tandem MS analysis and three returned conclusive results. The largest protein (~130kDa), along with two smaller proteins, one at ~50 kDa and the other at ~35kDa, all matched peptides to SmLeish. The MS analysis did not provide sufficient data to map the exact locations within SmLeish from which the smaller proteins originated, however both analyses returned only peptides from the C-terminal region of the rSmLeish protein (**SFigure 4.9C**).

### 4.5.3 Soluble SmLeish is a component of *S. mansoni* ES products and rSmLeish inhibits M-line *B. glabrata* haemocyte migration

*S. mansoni* ES products are known to negatively impact haemocyte recruitment to the sporocyst and often lead to a phenotypic rounding and loss of haemocyte adherence *in vitro*. SmLeish was confirmed to be an important component of ES products (**Figure 4.4** inset), and to test whether SmLeish contributes to alterations of haemocyte function, its impact on chemokinetic activity of haemocytes from M-line and BS-90 *B. glabrata* was assessed. Haemocytes isolated from individual snails were equally separated into two pools and placed in the top chamber of a cell migration apparatus and then one pool was treated and the other served as a control. Treatments were placed in the bottom well to stimulate migration across the membrane and assess chemoattraction, the upper chamber to determine whether contact exposure influenced cell movement to the membrane underside, or both chambers to assess chemokinesis. The ratio of migrated (underside of membrane) haemocytes in the control haemocyte pools, which was exposed to medium in both the upper and lower chambers, was compared to the experimental group of haemocytes from each snail and a ratio of experimental migration to control migration was established, with values >1 reflecting more migration in the experimental group and values <1 reflecting less.

The positive control, fMLP, induced haemocyte migration at a ratio of 2.25±0.49:1 in M-line snails and 2.53±0.43:1 in BS-90 snails. Adding fMLP to the top chamber abrogated directional migration of the haemocytes to the membrane underside (**Figure 4.4A**), as did having fMLP in both chambers (**SFigure 4.10**).

Placing either ES products or rSmLeish in the upper chamber with M-line haemocytes significantly reduced the number of haemocytes that crossed the membrane compared to controls

(0.44±0.07:1 and 0.24±0.05:1 respectively) and treating rSmLeish with trypsin prior (which had no effect on its own following deactivation (**SFigure 4.8**) to haemocyte exposure in the upper chamber slightly enhanced the observed reduction in migration (0.19±0.12:1). Having ES products or rSmLeish in the bottom chamber did not significantly impact M-line haemocyte migration compared to controls (0.61±0.12:1 and 0.66±0.12:1 respectively), although there was a trend towards reducing haemocyte migration across the membrane (**Figure 4.4A**). Removal of SmLeish from ES products or rSmLeish by immunoprecipitation prior to incubation significantly abrogated the negative effect on M-line haemocyte migration (0.96±0.13:1) (**SFigure 4.10**), as did co-treating the haemocytes in the top well with rSmLeish and 1,10-phenanthroline (1.01±0.13:1) (**Figure 4.4A**).

The impact of ES products and rSmLeish on reducing haemocyte migration were not observed when BS-90 *B. glabrata* haemocytes were assessed. In fact, ES products, but not rSmLeish, were significantly attractive to BS-90 haemocytes when placed in the bottom well (1.51±0.15:1 and 0.82±0.05:1 respectively), suggesting that the impact of SmLeish (or lack of impact) on haemocytes of BS-90 *B. glabrata* may represent one of the reasons that this strain of *B. glabrata* is refractory to infection by many strains of *S. mansoni*. Removal of SmLeish from ES products by immunoprecipitation did not significantly eliminate the attractive properties (1.46±0.33:1) (**SFigure 4.10**), nor did pre-treatment of rSmLeish with trypsin (0.98±0.15:1) or co-treatment with 1,10-phenanthroline (0.89±0.10:1) (**Figure 4.4A**).

To confirm that the loss of chemokinetic activity in M-line haemocytes incubated with *S. mansoni* ES products was in fact due to SmLeish, ES products from SmLeish knockdown parasites were tested. Knockdown of SmLeish significantly abrogated the inhibition of chemokinesis observed in M-line haemocytes (0.96±0.21:1) compared to both the normal ES

products (0.46±0.12:1) and GFP knockdown ES product (0.44±0.07:1) controls. Moreover, the loss of inhibition could be rescued if rSmLeish was added back to the SmLeish knockdown ES products prior to incubation with M-line haemocytes in the upper chamber (0.43±0.08:1) (**Figure 4.4A**). No significant impacts of SmLeish knockdown were observed when ES products from knockdown parasites were applied to BS-90 haemocytes (**Figure 4.4A**).

Additional differences between the responses of M-line and BS-90 haemocytes emerged when fMLP was included in the bottom chamber and ES products or rSmLeish in the top chamber. While fMLP was able to induce significant migration of BS-90 haemocytes across the membrane when either ES products or rSmLeish were applied in the top chamber (1.34±0.11:1 and 1.89±0.19:1 respectively), the suppressive effects of ES products (0.46±0.06:1) and rSmLeish (0.44±0.10:1) prevented migration when M-line haemocytes were used (**Figure 4.4A and 4.4B**).

Further comparison between the effects of ES products and rSmLeish on M-line and BS-90 haemocytes yielded 10 treatments in which haemocyte migration significantly differed between the two *B. glabrata* strains. Eight of the 10 treatments (depicted bottom|top; 1. fMLP|rSmLeish, 2. ES|Medium, 3. fMLP|ES 4. SmLeish-KD ES|Medium, 5. GFP-KD ES|Medium, 6. Medium|rSmLeish, 7. Medium|rSmLeish+Try and 8. rSmLeish|rSmLeish) all resulted in fewer M-line haemocytes migrating compared to controls (a migration ratio <1) while BS-90 haemocytes in these same treatments resulted in migration ratios near to, or above 1. The two remaining of the 10 significantly different treatments, Medium|SmLeish-immunoprecipitated ES and Medium|SmLeish KD ES resulted in the opposite trend, where M-line haemocytes in these treatment groups migrated similar to controls and BS-90 haemocytes significantly less than controls (**Figure 4.4B**).

### 4.5.4 SmLeish levels in *B. glabrata* plasma

Control snail plasma yielded a slight background signal that translated into an estimated 541±159pg/mL SmLeish. The average estimated SmLeish in the plasma of the five *S. mansoni*-challenged *B. glabrata* was 27,778±22,342pg/mL. This group displayed a vast range of values with the highest being from a snail in which the estimated SmLeish was 68,804pg/mL. SmLeish was estimated to be present at 106,829±4944pg/mL in *S. mansoni* ES products. For reference, the 0.25µg/mL rSmLeish yielded estimated SmLeish concentrations of 230,275±5223pg/mL (**Figure 4.14**). Thus, the lowest rSmLeish concentration used in our experiments accurately reflects the concentration present in host plasma during infection.

### 4.5.5 Knockdown of SmLeish delays *S. mansoni* infection establishment and cercarial output, but does not reduce long-term infection success in M-line *B. glabrata*

Knockdown of SmLeish using siRNA successfully reduced transcript abundance as early as 2-day post transfection *in vitro* (**SFigure 4.11**). Knockdown was statistically significant from day 3 post transfection onward compared to time-matched controls where the relative fold change in transcript abundance compared to miracidium reached 11.69±3.15 at day 4 post transfection in controls, compared to 0.94±0.33 in the knockdown group (**SFigure 4.11**). Western blot analysis indicates that the abundance of the larger ~130kDa protein declined following knockdown, however, most noticeable was the complete absence of the ~48kDa soluble SmLeish (**SFigure 4.11**).

Knockdown of SmLeish in *S. mansoni* miracidia prior to exposure to M-line *B. glabrata* significantly influenced the kinetics of the infection, reducing the proportion of snails that shed cercaria until 8-weeks post challenge. At 4-weeks post challenge, 5±4.1% of

control snails (exposed to a GFP-specific siRNA oligo cocktail) shed cercaria, compared to 0% of the snails exposed to SmLeish knockdown parasites (**Figure 4.5A**). Statistically significant differences were observed between 5 and 7-weeks post challenge when 38±6%, 72.5±3% and 93±1.1% of control snails and 6.7±9.7%, 23±16.6% and 53.9±3.7% of SmLeish knockdown snails shed cercaria at 5, 6 and 7 weeks post challenge respectively. At 8 weeks post challenge and onwards the mean proportion of snails shedding cercaria in the SmLeish knockdown group caught up to the controls and both groups had similar percentages of snails shedding cercaria until 10-weeks post challenge; 94.9±3.2, 92±7.6 and 90±9 control snails shed cercaria and 75.3±11.5, 80.8±12.3 and 76.7±18.3 SmLeish knockdown snails shed cercaria (**Figure 4.5A**).

In two of the three SmLeish knockdown trials, the average number of cercaria that was shed by each shedding snail over a 24-hour time was also enumerated. Similar to the proportion of snails that shed cercaria, the number of cercaria shed was also impacted by knockdown of SmLeish. Control parasite exposure resulted in 14±5.7, 15.8±8.7, 27±10.7, 38.2± 15.4, 52.5±21.3, 70.3±28.3 and 76.9±29.5 cercaria released per shedding snail at 4, 5, 6, 7, 8, 9, and 10 weeks post challenge (**Figure 4.5B**). Whereas in snails exposed to SmLeish knockdown parasites 0, 9±0, 8.7±3.1, 12.8±7.9, 31.6±16.6, 46.7±28.3 and 65.6±28.6 cercaria were released per shedding snail at the same time points. Between 6 and 9 weeks post challenge, snails challenged by SmLeish knockdown parasites shed statistically fewer cercaria on average than the snails exposed to GFP knockdown *S. mansoni* ($p<0.05$) (**Figure 4.5B**).

### 4.5.6 SmLeish KD *S. mansoni* are encapsulated *in vitro* by M-line *B. glabrata* haemocytes more quickly and frequently than GFP KD controls

The impact of SmLeish on *S. mansoni* infection kinetics may be mediated through an influence on miracidia penetration/establishment success and by delaying/preventing haemocyte

encapsulation. Both visual and qPCR-based assessment of intramolluscan *S. mansoni* at day-2 post challenge suggest that fewer miracidia successfully penetrated and/or established within a M-line *B. glabrata* following knockdown of SmLeish ($p<0.05$) (**SFigure 4.12**). Visualization of fluorescently labelled *S. mansoni* miracidia/sporocysts within the snail head-foot of 30 M-line *B. glabrata* following exposure to 15 miracidia identified an average of 4.9±3.6 of SmLeish knockdown and 7.4±4.3 of GFP knockdown parasites (**SFigure 4.12A**). Quantitative PCR analysis of separate 30 M-line snails targeting the GAPDH gene of *S. mansoni* estimated that 5.3±0.8 and 6.9±0.7 successfully established in the GFP knockdown and SmLeish knockdown groups respectively (**SFigure 4.12B**).

The mechanism underpinning infection success in this case was hypothesized to be associated with the ability of haemocytes to be recruited and then ultimately encapsulate the sporocyst. Encapsulation kinetics were assessed *in vitro* using transformed *S. mansoni* sporocysts and isolated primary haemocytes from M-line *B. glabrata* labelled using a cell tracking fluorescent dye. Encapsulation of SmLeish-knockdown and control GFP-knockdown sporocysts was assessed at 6, 12, 24 and 30 hours post incubation by visualizing fluorescence around the sporocyst (**Figure 4.6A and 4.6**). Out of the 35 sporocysts evaluated, 5.7%, 14.3%, 20% and 22.9% were encapsulated in the GFP knockdown control group compared to 22.9%, 40%, 54.3% and 65.7% encapsulated in the SmLeish knockdown group at 6, 12, 24 and 30 hours respectively (**Figure 4.6C**). The two infection curves were found to be significantly different using both a Log-rank (Mantel-Cox) test ($p<0.0003$), and a Gehan-Breslow-Wilcoxian test ($p<0.0005$). We ensured that we were observing differences in encapsulation rates rather than autofluorescence caused by dying parasites by examining miracidia and sporocysts unexposed to labeled haemocytes, as well as sporocysts exposed to unlabeled haemocytes, all of which failed to fluoresce (**SFigure 4.13**).

## 4.6 Discussion

It is well documented that both host and parasite produce factors that can function as determinants of host-parasite compatibility. In the snail-digenean trematode model, numerous studies have demonstrated that molecules, present on the surface of the parasite or in their ES products, can influence or suppress various aspects of the snail immune response, ranging from pathogen recognition to effector responses (129,146,385,394,395). However, very little is known about the identity and mechanism of involvement of the specific parasite factors that underlie these effects. While molecules such as the *S. mansoni* polymorphic mucins provide insight into how the parasite might evade snail immune recognition and response (396), almost nothing is known about parasite factors that dampen the snail immune response to facilitate infection establishment. In an effort to identify specific factors underpinning these effects, we have functionally characterized an *S. mansoni* metalloprotease that shares amino acid and predicted structural similarities to leishmanolysin. Functional assessment of this MMP, SmLeish, suggests that it is an important parasite-produced suppressor of the snail cellular immune response, impacting haemocyte migration and ultimately influencing parasite encapsulation and *S. mansoni* infection kinetics.

Recombinant SmLeish clearly exhibited dose-dependent MMP activity. The enzymatic activity demonstrated by the non-trypsinized recombinant suggests rSmLeish was produced in a form that was partially active despite a lack of proteolytic cleavage by any host or parasite factor. Treatment of rSmLeish with mammalian trypsin did, however, result in higher levels of MMP activity, suggesting that rSmLeish may have been activated due to non-specific removal of its N-terminal region. This finding was expected given that many metalloproteases require cleavage of their N-terminal ends in order to become functional (301). This does not necessarily lead us to believe that trypsin is the most biologically relevant activating enzyme for SmLeish, but rather

that trypsin is one of many possible enzymes that can generate a more active form of SmLeish. Even MMP-8, our positive control for this experiment, can be activated by an array of different enzymes (397–399). When treated with the human MMP inhibitor Ilomastat, both trypsinized and non-trypsinized forms of rSmLeish had reduced MMP enzymatic activity. Inhibition of rSmLeish was more evident when a different MMP inhibitor, 1,10-phenanthroline, was applied. This inhibitor has been shown to inhibit the function of leishmanolysin (321), and perhaps more relevantly, has also been shown to negatively affect the viability, motor activity and fecundity of adult *S. mansoni in vitro* (344), as well as inhibit *S. mansoni* leucine aminopeptidase activity (400). These data support the hypothesis that SmLeish is functioning as a MMP, thereby providing a foundation for understanding the phenotypic changes regarding parasite encapsulation observed in both this work and previous studies (385,386).

A role for SmLeish in host immune evasion during initial establishment within the snail is additionally supported by both the increase in transcript levels and protein expression observed throughout infection. The relative increase of SmLeish transcripts peaking at 12 hours post infection, coupled with the appearance of the ~48kDa SmLeish form in infected snail haemolymph at the same time confirms the presence of SmLeish during initial establishment and infection stages. This quick upregulation, as well as the observation that SmLeish is also present in the snail plasma early in the infection suggests a role in evading haemocyte encapsulation, which typically occurs within the first 48 hours post infection (401). Additionally, since SmLeish-like genes are present in non-parasitic flatworms that would not encounter a host immune response, it is also likely that the observed upregulation of SmLeish is also linked to the large amount of tissue remodeling occurring during the transition from miracidium to sporocyst (402). This is consistent

with the idea that parasitism is an evolved trait amongst flatworms (403) and suggests that SmLeish may have functions outside of those described here in relation to facilitating infection.

Of interest was the observation that a relative increase in transcript levels was not restricted to the early stages of infection, but was also evident at 35 days post infection, which correlates with cercarial shedding under our exposure conditions (104,392), and in isolated cercariae. Coupled with the observation that leishmanolysin-like proteins have been previously identified in schistosome cercarial secretions, this supports the hypothesis that SmLeish may also function in immunosuppressive activities during the initial stages of tissue penetration and schistosomula migration in the human hosts (175,179,347). To add further to this, Verjovski-Almeida et al. (2003) demonstrate that the transcript for SmLeish is expressed by adult *S. mansoni*, suggesting a possible role in facilitating survival within the human host as well (404).

We observed a ~130kDa form of SmLeish at 1-day post infection, association of this protein with *S. mansoni* sporocysts was confirmed via immunofluorescent detection. Additionally, a soluble ~48kDa variant which is expected to be the result of cleavage, was located in snail haemolymph. To determine whether this protein was the result of alternative splicing, three RT-qPCR assays designed to span the SmLeish transcript were performed and returned statistically similar results. Thus, it is unlikely that alternative splicing is responsible for the appearance of the 48kDa protein as one of these assays would have been expected to coincide with the appearance of the ~48kDa band. Both observations agree with the existing leishmania literature suggesting leishmanolysin exists in a full-length form, as well as a cleaved form secreted into the surrounding environment. The origins of this soluble form of SmLeish remain unknown, but research into leishmanolysin suggests it may result from both direct secretion and cleavage of the membrane bound pro-leishmanolysin (150,305). Our MMP activity assay results suggest that both forms of

SmLeish are functionally active, although the full-length recombinant we generated lacked part of the N-terminal end of the protein, thereby disallowing us to conclusively state that the membrane bound form of SmLeish demonstrates MMP activity. Indeed, the majority of MMPs, including leishmanolysin, require proteolytic cleavage of their N-terminal end to become active (150,301). Trypsin treatment of rSmLeish did produce two smaller proteins that tandem MS analysis mapped to the C-terminal end of the full-length SmLeish protein. This suggests that the higher MMP activity and slightly more substantial decrease in M-line haemocyte motility displayed by the trypsin treated rSmLeish compared to the non-trypsinized rSmLeish may be due to the presence of the smaller (soluble) SmLeish.

The haemocyte migration assay allowed us to draw two important conclusions regarding haemocyte motility and the function of SmLeish. The first is that SmLeish negatively impacts the ability of haemocytes to migrate, with this effect proving increasingly potent with M-line haemocytes relative to those from BS-90 snails. The second being that BS-90 haemocytes are attracted to sporocyst ES products, while M-line haemocytes are not. The observation that treatment of BS-90 haemocytes with rSmLeish does not result in the same chemoattractant response as treatment with sporocyst ES products suggests that SmLeish is not the chemo attractive component that BS-90 haemocytes respond to. This observation is confirmed by the fact that ES products from SmLeish knockdown *S. mansoni* are still attractive to BS-90 haemocytes. Seeing as treatment with ES products lacking SmLeish (via immunoprecipitation, 1,10-phenanthroline pre-treatment or knockdown of SmLeish) resulted in a return of M-line haemocyte mobility to baseline levels, we predict that SmLeish is playing a significant role by dampening haemocyte recruitment to the sporocyst within the snail host during *S. mansoni* infection.

It is not yet possible to make any conclusions regarding the mechanism of haemocyte migration inhibition. Cell movement towards a target is dependent upon sensing chemical gradients, adhesion to a substrate, and remodeling of the cytoskeleton, rendering these pathways possible targets. Targeting of SmLeish to these substrates is supported by the knowledge of leishmanolysin targeting myristoylated alanine-rich C kinase substrate-related proteins in humans, which are involved in cell signaling (321), and the fact that many MMPs target proteins comprising important parts of the cytoskeleton, such as collagen (284,309). Another potential target, due to the ability of leishmanolysin to cleave fibrinogen (301), would be the fibrinogen domain of the *B. glabrata* Fibrinogen-Related Proteins (FREPs), which are a unique group of molecules that play a role in parasite recognition and opsonization of sporocyst tegument-associated carbohydrates (105,107,405). Additionally, the observation that leishmanolysin assists in the avoidance of complement mediated lysis of Leishmania through the cleavage of C3b (311) suggests that the snail thioester-containing protein may also be one of the targets of SmLeish. What we can conclude is that the negative impacts of SmLeish on M-line haemocyte migration are likely due to the MMP enzymatic activity (supported by the fact that rSmLeish is inhibited by 1,10-phenanthroline), and that the negative effect of ES products is likely because of SmLeish being present (supported by the ES+1,10-phenanthroline and the fact that SmLeish knockdown abrogates the effect and can be rescued by addition of rSmLeish).

Regardless of what the target of SmLeish may be, questions remain as to why this MMP fails to impede the mobility of BS-90 haemocytes, while reducing the mobility of M-line haemocytes. It is possible that allelic differences in the SmLeish target between these strains result in an inability of SmLeish to effectively hydrolyze the target in susceptible snails but not in resistant ones. Alternatively, such allelic differences may not be present in potential SmLeish

targets, but rather in snail-associated pattern recognition receptors responsible for the targeting of and subsequent neutralization of SmLeish.

The utilization of siRNA mediated knock-down of SmLeish proved successful in reducing levels of the protein during infection. While snails infected with GFP KD miracidia still demonstrated a rise in SmLeish transcription levels peaking at four days post infection, those infected with SmLeish KD miracidia featured no such increase, confirming specific targeting of the SmLeish mRNA. While this does not control for the possibility of off-target impacts of the administration of the siRNA oligos, the inclusion of the GFP-specific siRNA control accounts for the possibility that any off-target effects might impact the outcome of the encapsulation or chemokinesis bioassays (406). Protein expression was also affected by the siRNA treatment, with the soluble ~48 kDa variant becoming undetectable in whole snail lysates, while the ~130 kDa variant exhibited a decrease in expression at 4 days post infection. The complete absence of the soluble ~48 kDa variant is possibly the result of a reduction in the amount of the full length ~130 kDa protein, resulting in less activating cleavage events.

GFP KD sporocysts were able to avoid encapsulation by M-line snail haemocytes more effectively than SmLeish KD sporocysts. Although both treatments resulted in sporocysts being encapsulated as early as 6 hours post infection, the percentage of encapsulated sporocysts was consistently higher in the SmLeish KD group until the experiment was terminated. After 30 hours, 65.7% of SmLeish KD and only 22.9% of GFP KD sporocysts had been encapsulated. These results are also consistent with the work of Joshi and associates (2002), which demonstrate that targeted gene deletion of Gp63 in *Leishmania major* results in a delay in lesion formation in its mouse host, a process reversed by the introduction of a functional copy of Gp63 (312). Although our model system does not possess the advantage of full deletion of SmLeish, the increased

frequency with which our SmLeish KD sporocysts are encapsulated by the snail immune response, and the abrogation of the negative migration effect of SmLeish in knockdown ES products draws parallels to *L. major* Gp63 gene deletion mutants, which were more effectively eliminated by the mouse immune response. This is suggestive that in both parasites, leishmanolysin functions as a key component during the interactions between the parasite and the host immune response and appears to function in favour of the parasite (312).

That SmLeish plays a role in facilitating *S. mansoni* establishment within the snail host was further supported by the changes in the time it took for infections to reach patency, and the lowered cercaria output when SmLeish KD miracidia were used to challenge snails. Both phenotypic changes were likely caused by a reduction in the number of parasites that could successfully evade the haemocyte-mediated immune response, seeing as lower numbers of parasites per infection were confirmed using both visual inspection and qPCR. The lack of a complete abrogation of patent infections suggests that although SmLeish plays a significant role in determining infection outcome, *S. mansoni* likely utilizes other means by which it dampens the ability of the snail immune response to recognize and eliminate the invading larval parasites. Alternatively, the presence of the pro-protein that remained associated with the SmLeish KD sporocysts may have retained enough function to prevent encapsulation in some cases.

Parasites are master immune modulators that utilize multiple methods of ensuring their survival inside of their hosts. *S. mansoni* proves an excellent example of this, as different life stages employ different immunomodulatory mechanisms which allow it to infect, survive, and reproduce in two vastly different hosts (261,407,408). Our work successfully demonstrates that SmLeish functions as an MMP and supports a role in facilitating sporocyst survival within the snail by impeding the encapsulation response, resulting in increased infection success, kinetics and

output. Future work should strive to discover the specific targets of SmLeish on *B. glabrata* haemocytes, while also seeking to discover the method by which it is activated during the intramolluscan stages of *S. mansoni* infection. Identifying the specific active site in SmLeish would allow for the synthesis of inactive mutants that could concretely demonstrate that the immunomodulatory roles of this MMP are due to enzymatic activity. The functional characterization of SmLeish described here provides an insightful and necessary step in understanding the schistosome/snail relationship, which serves to both deepen our knowledge of the intricacies of invertebrate immunology and will ultimately allow us to examine possible methods of reducing the worldwide prevalence and impact of schistosomiasis.

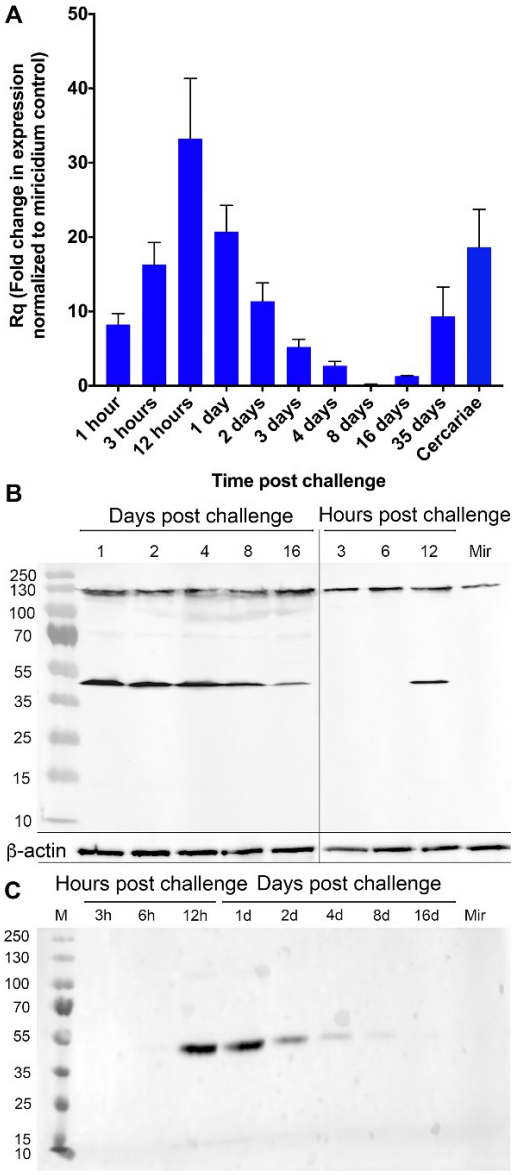



**Figure 4.1. Assessment of SmLeish expression during *S. mansoni* intramolluscan infection.**

(A) Quantitative RT-PCR analysis of SmLeish transcript abundance during critical points of *S. mansoni* intramolluscan development. M-line snails (n = 5 for each time point) were challenged with 5 miracidia of PR-1 *S. mansoni.* Fold change of SmLeish transcription normalized to a miracidium control show an increase in transcription over the initial 12 hours post infection. As infection progresses, transcript abundance relative to control declines until day 35 post challenge, a point at which cercaria begin to emerge from the infected snails. Cercaria emergence correlates with a renewed spike in SmLeish transcription. (B) Western blot analysis of whole-snail lysates confirms the qRT-PCR data, demonstrating that the increased expression is likely due to the emergence and relative increase in the abundance of a ~48kDa protein and not the larger full-length SmLeish. ß-actin is used as a loading control. (C) Western blot analysis of *B. glabrata* plasma demonstrates that the ~48kDa protein likely represents a soluble SmLeish that appears around 12 hours post challenge.

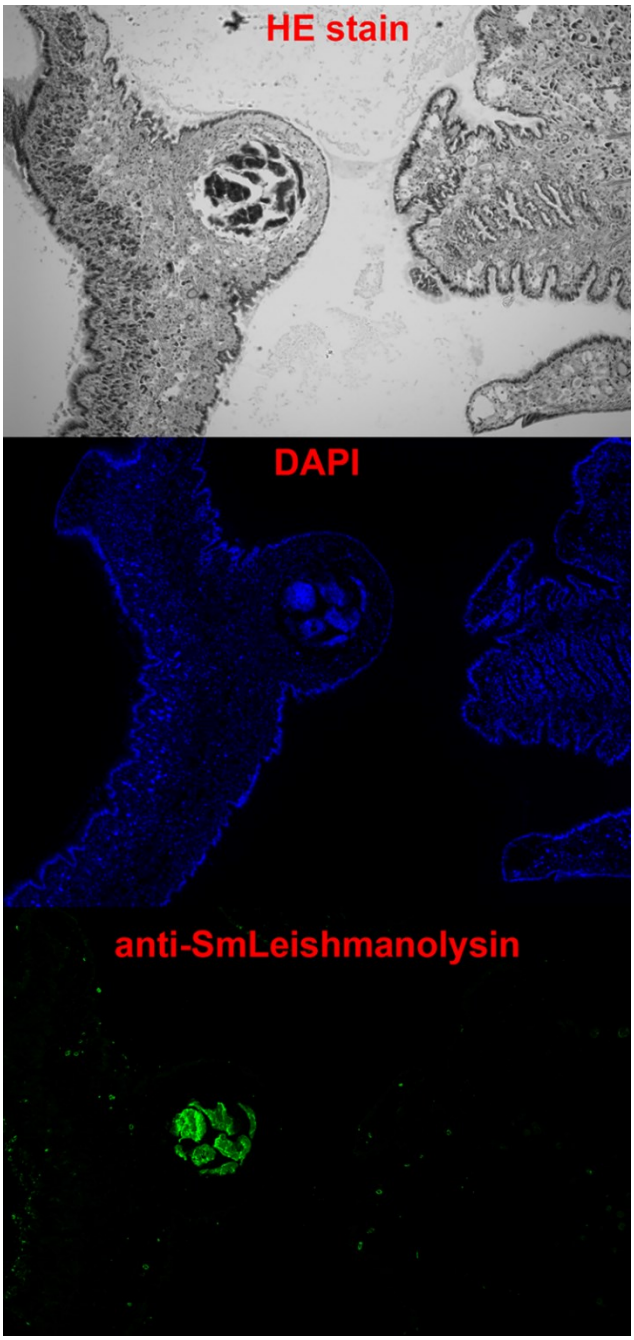



**Figure 4.2. Confirmation that SmLeish is associated with the larval *S. mansoni* sporocyst within the intramolluscan environment.**

The anti-SmLeish antibody was used to probe histological sections of *S. mansoni*-challenged *B. glabrata* to visualize the context of SmLeish expression within the snail. At all time points observed, SmLeish expression was confined to the sporocyst. Depicted is a day 16 sporocyst within the head-foot of an M-line *B. glabrata*.

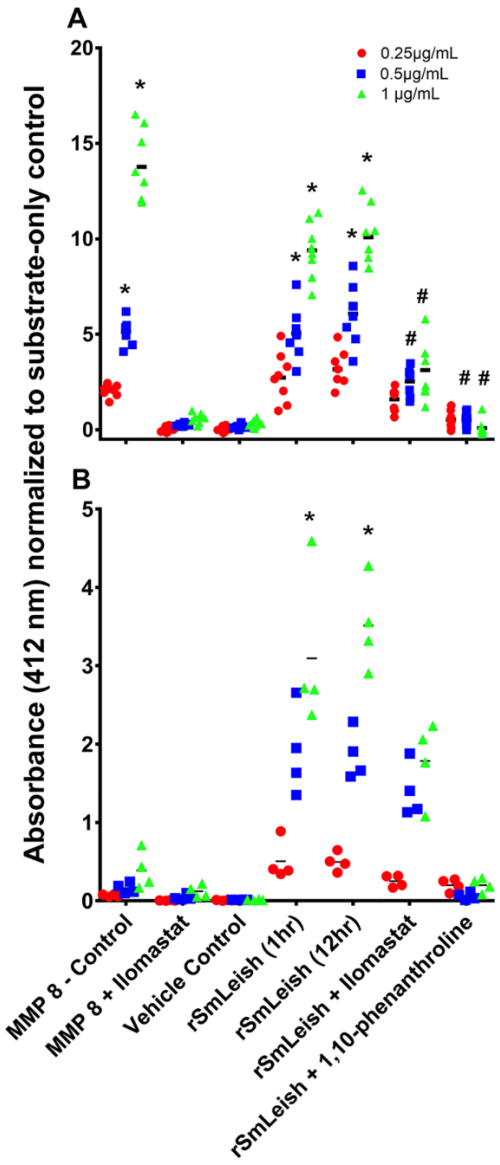



**Figure 4.3. Recombinant SmLeish is a functional matrixmetalloprotease.**
(A) Metalloprotease activity of activated (trypsinized) rSmLeish prior to incubation with the MMP8 substrate. Recombinant SmLeish is able to cleave human MMP8 substrate in a dose-dependent fashion, but is only partially inhibited by Ilomastat, and almost completely inhibited by 1,10-phenanthroline. (n=6) (B) Unlike human MMP8, which requires trypsin activation in order to display functional activity, rSmLeish does not require prior trypsinization in order to display MMP activity. However, rSmLeish enzymatic activity is significantly reduced if trypsin activation does not occur prior to assessment. Treatment of rSmLeish with 5µg/mL trypsin for 12 hours did not significantly alter the MMP activity compared to the 1 hour at 10µg/mL. (n=4) In both experiments, three initial concentrations (0.25 µg/mL 0.5 µg/mL and 1µg/mL) of the enzymes were used. Enzymatic activity indicates the concentration of the product (sulfhydryl group) formed, measured via $OD_{412}$ absorbance values after 2-hours incubation with the substrate. Significant differences between treatments and the vehicle control are signified by *, significant differences between inhibitor treatments and rSmLeish are signified by #.

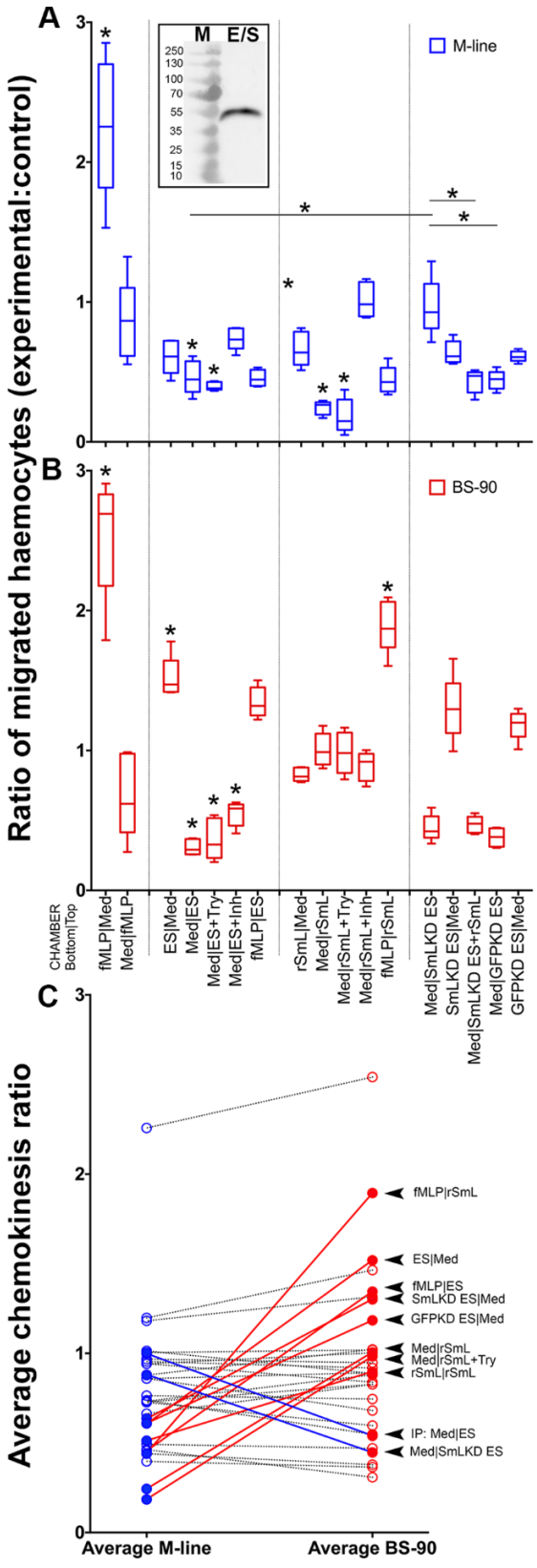

A
B
C
Ratio of migrated haemocytes (experimental:control)
M-line
BS-90
M
E/S
250
130
100
70
55
35
25
15
10
CHAMBER
Bottom|Top
fMLP|Med
Med|fMLP
ES|Med
Med|ES
Med|ES+Try
Med|ES+Inh
fMLP|ES
rSmL|Med
Med|rSmL
Med|rSmL+Try
Med|rSmL+Inh
fMLP|rSmL
Med|SmLKD ES
SmLKD ES|Med
Med|SmLKD ES+rSmL
Med|GFPKD ES
GFPKD ES|Med
Average chemokinesis ratio
Average M-line
Average BS-90
fMLP|rSmL
ES|Med
fMLP|ES
SmLKD ES|Med
GFPKD ES|Med
Med|rSmL
Med|rSmL+Try
rSmL|rSmL
IP: Med|ES
Med|SmLKD ES

**Figure 4.4. Recombinant SmLeish interferes with M-line *B. glabrata* haemocyte motility.** Directional migration of M-line and BS-90 haemocytes across a 5μM porous membrane was assessed to determine the impact of rSmLeish on haemocyte motility. Migration is presented as a ratio comparing the number of haemocytes that have migrated to the underside of the membrane in an experimental group to a control group (medium only in both upper and lower chambers) where both haemocyte groups originate from the same snail. fMLP was used as a positive control. (A) *S. mansoni* excretory/secretory (ES) products (which are shown to contain SmLeish in the inset Western blot) inhibit migration of M-line haemocytes across the membrane when incubated with the haemocytes in the upper well. Recombinant SmLeish (rSmL) inhibits migration of M-line haemocytes across the membrane when incubated with the haemocytes in the upper chamber and this effect was slightly enhanced by pre-treatment of rSmL with trypsin (Try). Knockdown of SmLeish prior to generation of ES products abrogated the negative impact of ES products on M-line haemocyte migration and the effect was rescued by addition of rSmLeish to the SmLeish KD ES products. (B) E/S products are attractive to BS-90 haemocytes when in the lower well. rSmLeish had no impact on BS-90 haemocytes and was not attractive to BS-90 haemocytes. Knock down of SmLeish in larval E/S products did not abrogate the attractive nature of E/S products to BS-90 haemocytes. * represents significant difference in haemocyte migration between a treatment and its corresponding control group, or to a specific control as indicated by a solid line. (C) Comparison of the mean values in each treatment between M-line and BS-90 *B. glabrata* haemocytes identified 10 treatments that yielded significantly different results. A value above 1 indicates migration across the membrane greater than control, a value less than 1 indicates less migration than control. Each treatment is connected between the M-line and BS-90 datasets to aid in visualizing the differences between the different snail haemocytes. A red line indicates that the treatment identified on the left induced more migration in BS-90 haemocytes than M-line, a blue line indicates more migration in M-line haemocytes than BS-90.

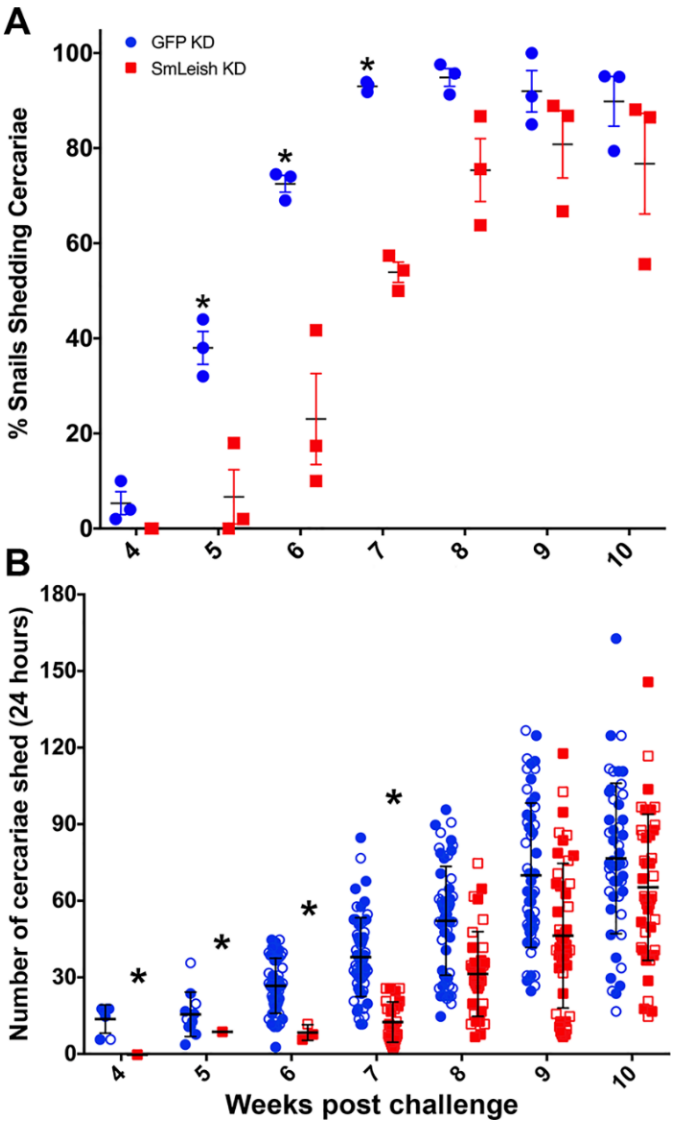



**Figure 4.5. Knockdown of SmLeish influences *S. mansoni* infection kinetics and cercarial output.**

(A)Knockdown of SmLeish prior to challenge of M-line *B. glabrata* reduced the early infection success of the parasite as measured by the number of *S. mansoni*-challenged *B. glabrata* that shed cercaria at 4, 5, 6, 7, 8, 9 and 10 weeks post challenge compared to GFP knockdown control parasites. Long-term infection success was not statistically changed after 8-weeks post challenge, suggesting that knockdown of SmLeish may reduce the number of miracidia (out of the 5 each snail was exposed to) that successfully infected the snail. However, significant differences were observed (*) at 5, 6 and 7 weeks post challenge. Shown are the mean % snails shedding cercariae resulting from three independent trials, with mean and standard error. (B) Cercaria output was also significantly influenced by SmLeish knockdown early in the shedding phase of the infection, at weeks 4, 5, 6, and 7 post challenge (*). However, parallel to the number of snails shedding, cercaria outputs were not statistically different by week 8 post challenge. Shown are the individual cercaria output numbers per infected snail at each time point. Closed and open markers distinguish between the two trials, with mean and standard deviation shown.

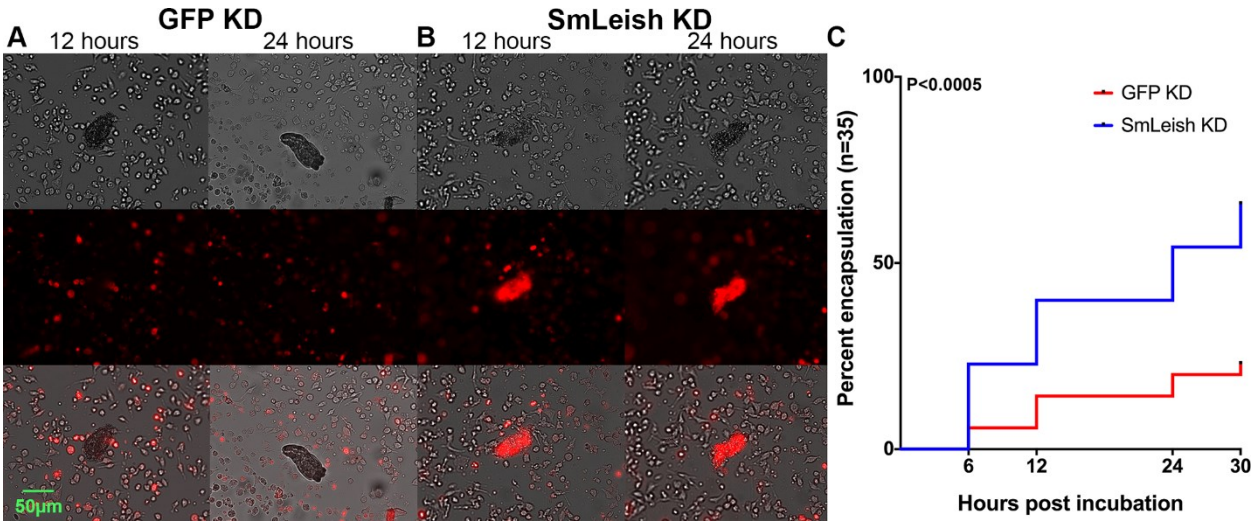
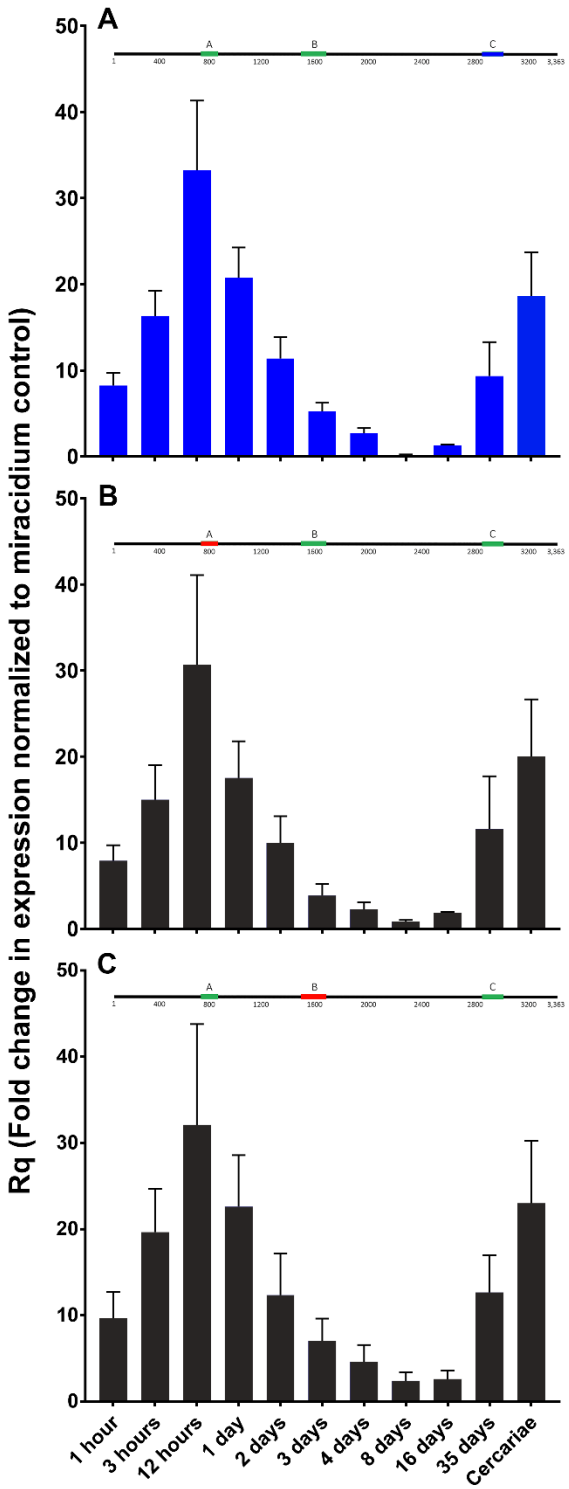



**Figure 4.6. SmLeish impacts infection success by affecting the ability of haemocytes to encapsulate *S. mansoni* sporocysts.**

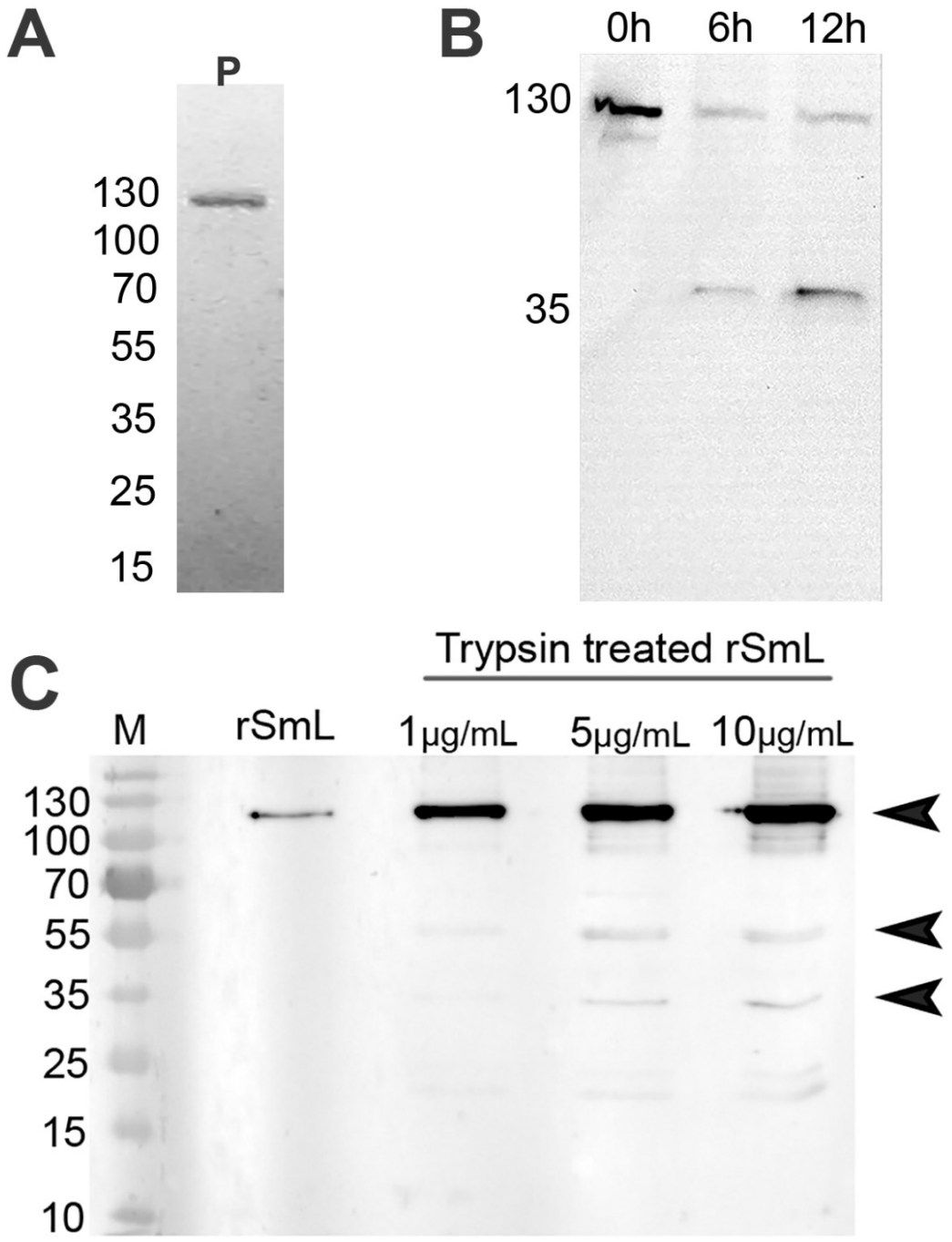
Encapsulation of *S. mansoni* sporocysts was visualized using fluorescently labelled M-line haemocytes and incubating them with GFP (A) or SmLeish (B) knockdown sporocysts that had been transformed *in vitro*. GFP knockdown control sporocysts in which SmLeish expression remains intact were infrequently encapsulated (A and C) compared to SmLeish-knockdown sporocysts, which were more frequently encapsulated by 24 hours post incubation ($p<0.0005$) (B and C). Representative images of an encapsulated and non-encapsulated sporocyst at 12 and 24 hours post incubation are shown (A and B).



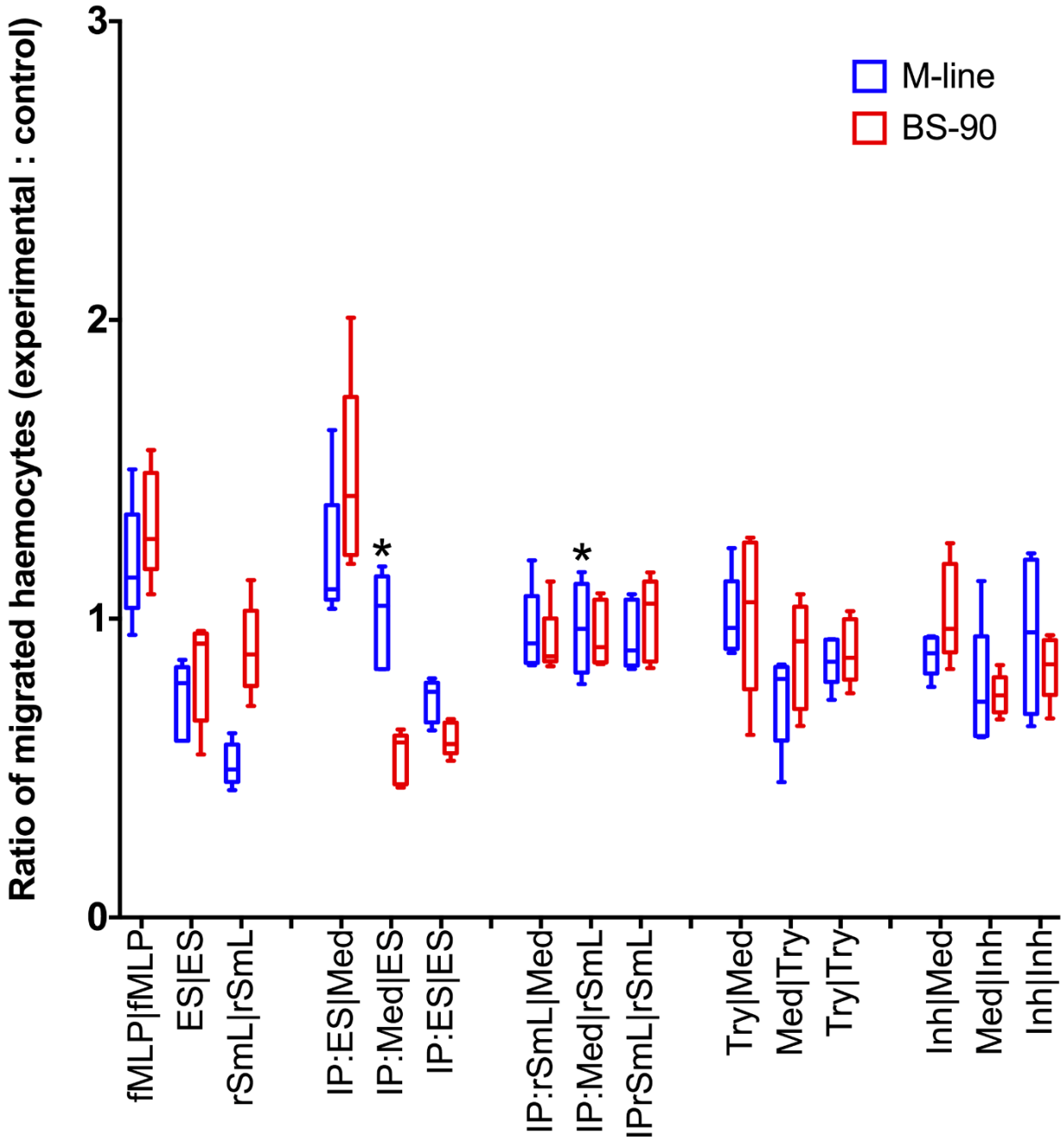

**SFigure 4.7. SmLeish transcript abundance measured using three amplified regions.**
RT-qPCR results do not vary in a statistically significant manner at any time point among three separate sets of primers and probes used to assess the transcript abundance of SmLeish. The primer/probe combination in panel A was used for further analysis such as knock down efficiency, while the primer/probe combinations in panel B and C serve to rule out alternative splicing.


**SFigure 4.8. SmLeish possesses hallmark MMP domains.**

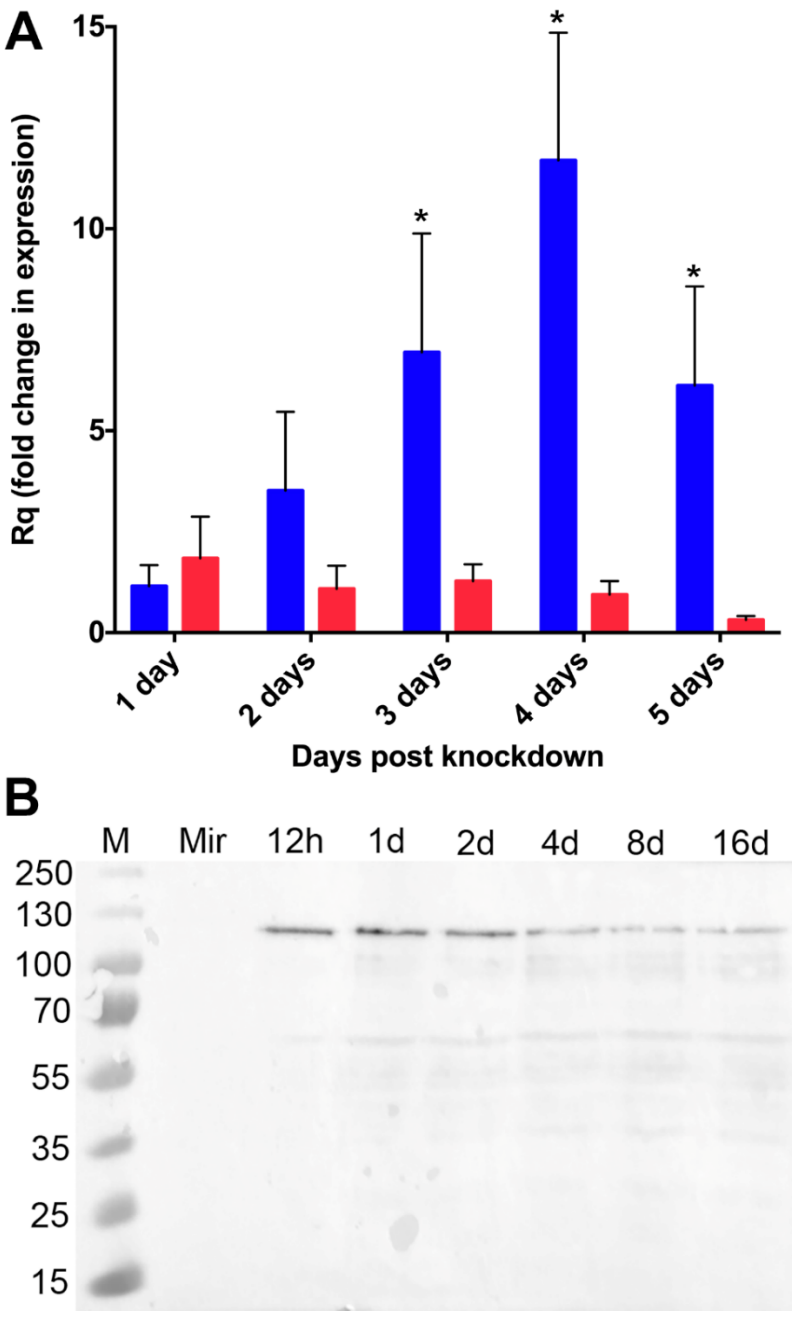
A) SmLeish features a predicted Leishmanolysin-like Peptidase/Metalloprotease (zincins) catalytic domain, which is in turn composed of two predicted Peptidase M8 domains. Within each of these domains resides the canonical HEXXH motif (blue) found in all zinc metalloproteases, which are components of the HEXXHXXGXXS motifs (green) seen across Metzincin Metalloproteases. B) The amino acid sequence of SmLeish highlights the presence of two Peptidase M8 domains and their respective active site motifs. The peptide sequence against which the anti-SmLeish antibody was derived is indicated by the black band.



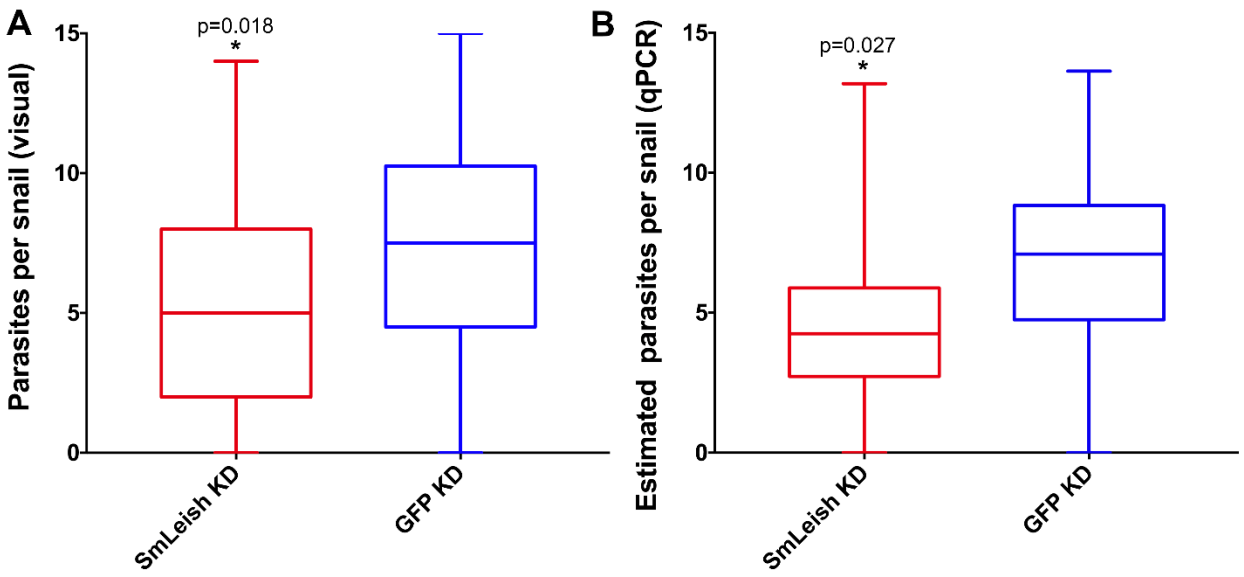

**SFigure 4.9. Recombinant SmLeish is cleaved by trypsin.**
A) SDS-PAGE of the purified rSmLeish demonstrating a pure sample. B) Recombinant SmLeish cleaved using 5ug/ml trypsin for 0, 6, and 12 hours demonstrate the occurrence of a ~48kDa cleavage product, which increases in abundance following longer incubation times. C) Western blot of purified rSmLeish (rSmL) and rSmLeish following a one-hour incubation with 1, 5 and 10µg/mL trypsin at 37C. Cleavage products were detected at ~50kDa and ~35 kDa, as well as at ~25 and 20kDa. MS/MS analysis of the 50 and 35kDa cleavage products indicates that they possess peptide fragments of SmLeish (SmLeish peptide containing products indicated by arrows).



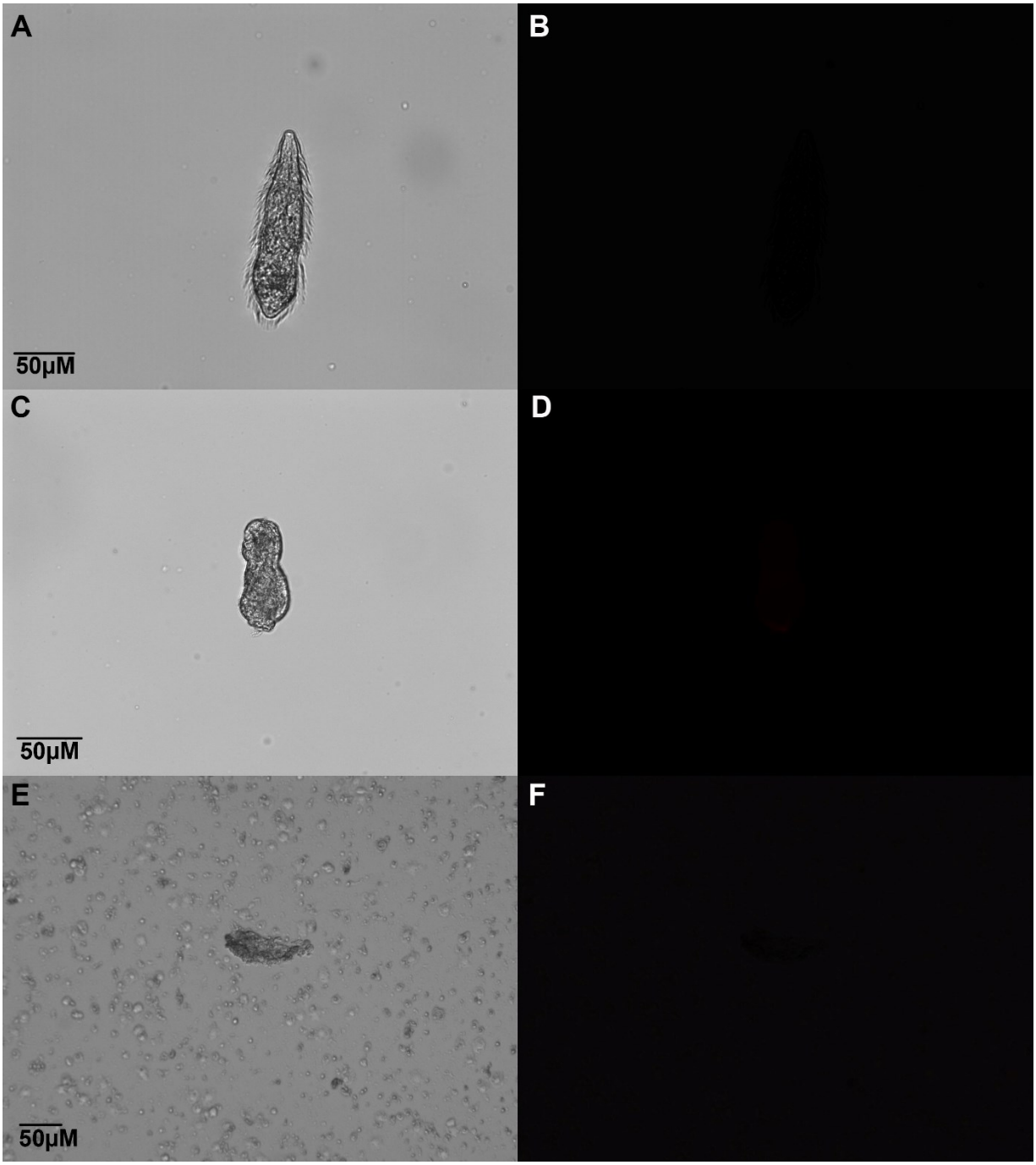

**SFigure 4.10. Controls for haemocyte migration.**
Additional dual well and trypsin/1,10-phenanthroline treatment controls for the haemocyte migration assay. Additionally, immunoprecipitation (IP) of SmLeish from ES products or out of the rSmL treatments provides further evidence that SmLeish is negatively impacting M-line haemocyte motility across the 5µm pore membrane. * indicate significant difference between indicated treatment and the non-SmLeish immunoprecipitated treatment shown in Fig 4A.



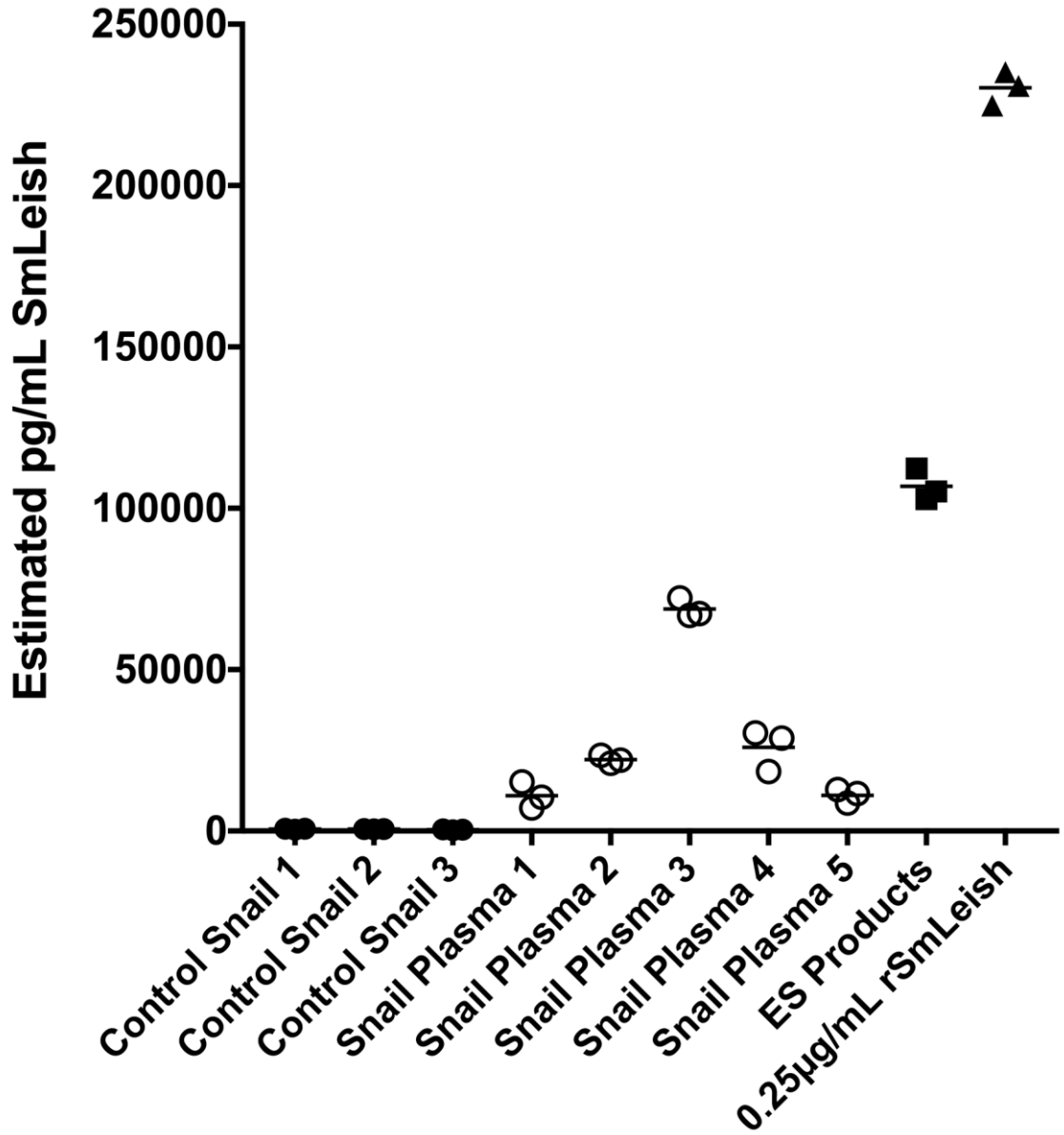

**SFigure 4.11. siRNA-mediated knockdown of SmLeish.**
Assessment of *in vitro* transformed *S. mansoni* sporocyst expression of SmLeish following SmLeish knockdown (red bars) was compared to GFP knockdown controls (blue bars). Knockdown was significant at the transcriptional level as early as 2 days post incubation and prevented increases in transcript abundance that are traditionally observed for SmLeish by 3 days post transformation (A). Western blot analysis confirmed protein-level knockdown occurs as well, but is less evident for the larger SmLeish protein, while the ~48kDa soluble form of SmLeish is not detectable following knockdown (B).




**SFigure 4.12. Infection success of SmLeish knockdown *S. mansoni* is reduced compared to knockdown controls.**
Labeling of the *S. mansoni* miracidia using a membrane fluorescent dye allowed us to visualize the larval parasites within the head-foot of challenged M-line *B. glabrata*. This approach enabled us to count the number of sporocysts within the head-foot at 4 days post challenge using 15 labelled miracidia. A) Fewer SmLeish knockdown parasites were observed via visual examination within the head-foot on average compared to GFP knockdown controls. B) qPCR results also suggested a decrease in the among of SmLeish knockdown parasites present within the snail when compared to GFP knockdown controls.




**SFigure 4.13. Haemocyte encapsulation and fluorescence controls.**
*S. mansoni* larvae fail to autofluorescence on their own and in the presence of unlabeled haemocytes. Both miracidia (A and B) and sporocysts (C and D) transformed *in vitro* for 96 hours fail to demonstrate autofluorescence. Sporocysts transformed for 30 hours and exposed to M-line haemocytes that were not fluorescently labelled (E and F) also fail to produce a fluorescent signal.




**SFigure 4.14. Analysis of SmLeish in infected M-line *B. glabrata* and *S. mansoni* ES products.**

Using the anti-SmLeish antibody in an ELISA, SmLeish was measured in infected M-line *B. glabrata*. Cell-free plasma from five snails was assessed in triplicate and compared with three non-challenged control snails as well as ES products. The ELISA was calibrated using a serial dilution of rSmLeish, and the 0.25µg/mL values are shown.

# Chapter 5 – The molecular and functional characterization of SmLeish and SmCI-1 during the initial stages of parasite infection

## 5.1 Preface

After having determined SmLeish to be a key determinant in infection outcome in *Biomphalaria glabrata*, we set about to determine whether it had a role during mammalian infection. This was spurred on by transcriptomics data showing an upregulation in SmLeish transcription that coincided with the emergence of cercaria. While transitioning into work on mammalian infections, we also became aware of several publications showing the substantial release of an invadolysin known as Smp_090100, which we would go on to name SmCI-1. from cercaria. While examining the release and ability to cleave human proteins of both SmLeish and SmCI-1's, we determined that SmCI-1 was likely to be involved more heavily than SmLeish during penetration of human skin. This explains why examination of SmLeish cleaving host factors is presented in this chapter, while other functional assays were only performed using SmCI-1. Our functional characterization of SmCI-1, including the results presented in this chapter are currently under review for publication and will be accepted pending revisions. This does not include the phagocytosis, plasma-mediated-killing, and granulocyte-mediated killing assays, as their negative results were determined to be unnecessary for publication, but important in the context of this thesis.

## 5.2 Abstract

*Schistosoma mansoni* employs immune evasion and immunosuppression to overcome immune responses mounted by its snail and human hosts. Myriad immunomodulating factors underlie this process, some of which are proteases. Here, we demonstrate that one protease, an

invadolysin we have termed SmCI-1, is released from the acetabular glands of *S. mansoni* cercaria and is involved in creating an immunological milieu favorable for survival of the parasite. We confirm the presence of SmCI-1 in the cercarial stage of *S. mansoni* and demonstrate that it is released during transformation into the schistosomula. We confirm SmCI-1 functions as a metalloprotease with the capacity to cleave collagen type IV, gelatin and fibrinogen. We also show that complement component C3b is cleaved by this protease, resulting in inhibition of the classical and alternative complement pathways. We also test the release and substrate specificity of SmLeish, an invadolysin involved in immune evasion during infection of *Biomphalaria glabrata*, and find that it differs from that of SmCI-1.

## 5.3 Introduction

Despite significant investment into treatment and prevention programs, schistosomiasis continues to afflict an estimated 230 million people worldwide (40). Key among the numerous ways in which schistosomes persist in human populations, in spite of efforts to eliminate the parasites, are immunomodulatory mechanisms that interfere with and suppress the immune response of their gastropod and human hosts (95,99,409). While the strategies used to overcome immune responses are highly effective, they are not perfect. Humans can develop low levels of non-sterilizing immunity to infection, with exposure to adult worm antigens being a likely mechanism (160,410). Lung stage schistosomula are also a possible target for immune mediated destruction given their ability to elicit a strong immune response in murine models, as well as the discovery that plasma from self-curing Rhesus macaques is capable of enhanced killing of 3-5 day old schistosomula (411,412). Such findings demonstrate that an in-depth examination of the survival mechanisms at various stages of the intra-human schistosome life cycle merits investigation as possible targets for novel therapeutics and/or vaccine development efforts.

Proteases are prominently featured amongst the immunomodulatory mechanisms employed by *Schistosoma mansoni* during the snail and human infection process (140,141,278). In snails, *S. mansoni* miracidia have been shown to release calpain, serine peptidases, and at least one metallopeptidase (137,145). Gene array data from developing sporocysts also feature proteases such as cathepsins, elastase, and hemoglobinase, each of which are upregulated during development within the snail (140,141). In humans, the employment of proteases by cercaria is widespread and well-studied (6,165). In contrast, the use of proteases and immune modulators remains less extensively examined in lung-stage schistosomula due to the relative difficulty of culturing and isolating this stage of the parasite (95). Adult schistosome-produced proteases that have been examined to date are largely involved in anti-coagulation mechanisms (246–248). Other proteases, such as metalloproteases and aminopeptidases, have been identified in adult worm extracts, but remain to be functionally characterized (278).

Proteases are especially important during the two tissue-penetrative stages of the life cycle of *S. mansoni.* These enzymes have been most extensively studied within the context of cercarial penetration of, and residence within human skin. During this time, the developing parasites strive to locate a venule through which they enter the circulation (4,6,7). Upon attachment to the surface of the skin, cercaria begin migrating through the numerous layers of the epidermis, a process involving mechanical disruption of host cell-to-cell interactions as well as the release of proteases from the acetabular glands (5,6). In *S. mansoni*, proteases released by the acetabular glands are predominantly serine proteases, which have been demonstrated to cleave key structural components in skin such as elastin, laminin, fibronectin, collagen type IV, and keratin (171–173,176). So crucial are these serine proteases that one, a 28/30kda protease termed *S. mansoni* cercaria elastase (SmCE) makes up roughly 36% of the contents of the acetabular

glands (175). Broad inhibition of serine proteases, as well as SmCE specifically, substantially reduces cercarial penetration success, highlighting the essential function of SmCE during the initial stages of *S. mansoni* infection of their human host (174,176). Cleavage targets of this serine protease are not limited to structural host components, with SmCE having been shown to cleave complement component C3 (171). This highlights the reality that cercaria must not only combat the structural components of human skin, but also encounter immunological factors. The capacity of SmCE to cleave complement component C3 suggests that cercaria have developed ways to circumnavigate the immune factors present in the skin that may target them for destruction. Accompanying these serine proteases are cysteine proteases, which are less well understood in the context of *S. mansoni,* but which are 40X more abundant in *S. japonicum* cercaria (178,180). Also present are several metalloproteases that have yet to be functionally characterized (175,404).

The *S. mansoni* genome features 114 annotated metalloproteases, 35 of which have been found to be differentially transcribed during distinct parasite life cycle stages (198,203). Key among the metalloproteases found in *S. mansoni* cercaria are a subset of matrix metalloproteases (MMPs) known as invadolysins. Invadolysins, like all MMPs, employ a zinc ion, coordinated by histidine residues, in order to attract and retain water molecules to facilitate proteolysis (287,388). They are typically synthesized as zymogens, awaiting the removal of an inhibitory-cysteine-containing pre-protein fragment in order to be rendered active (290). As their name suggests, the proteolysis performed by MMPs frequently target the extracellular matrix (ECM) components of cells such as collagen, laminin, and elastin (286,388). Invadolysins are considered M8 metzincin metalloproteases, which employ a methionine in a characteristic “met-turn” involved in the stability of the protease, and from which the name metzincin is derived

(289,291). Seven invadolysins have been shown to be differentially regulated in *S. mansoni* to date, with five being most highly transcribed in the germball stage of development, during which the necessary products for skin penetration by cercaria are generated (203). The other two are most highly upregulated in cercaria themselves, one of which is SmLeish (Smp_135530) (203). Invadolysins are also found in *S. japonicum* cercaria, with the three upregulated invadolysin genes observed in an *S. japonicum* micro-array bearing high levels of amino acid similarity to three invadolysins upregulated invadolysins in *S. mansoni* (347). The identification of these invadolysins via transcriptomics analysis, as well as their presence in both *S. mansoni* and *S. japonicum*, render them interesting targets for further study. The case for characterization of invadolysins during initial infection of a human host is bolstered by the observation that the second most highly upregulated invadolysin in *S. mansoni* germballs (Smp_090100.1) has also been identified as the second most abundant protein released from *S. mansoni* acetabular glands during transformation into schistosomula, comprising roughly 12.8% of the normalized volume of the acetabular glands (175). For the purposes of this work, we refer to Smp_090100.1 as *S. mansoni* cercarial invadolysin 1 (SmCI-1) in keeping with the nomenclature surrounding cercarial elastase (SmCE). We also continued investigating SmLeish, given it's previously demonstrated upregulation in cercaria (203).

The utilization of invadolysins as tools for invasion of a host by a parasite has been extensively studied, especially in the case of kinetoplastid parasites such as *Leishmania spp.* and *Trypanosoma spp.* Invadolysins were initially characterized in *Leishmania spp*. and are often termed leishmanolysins, or glycoprotein 63kda (GP63) (300,308). GP63 can effectively cleave gelatin, albumin, fibrinogen and collagen type IV (306,309). McGwire et al. demonstrate that the capacity for GP63 to degrade key ECM proteins is involved in facilitating leishmania movement

through a Matrigel coated membrane, implying that GP63 can aid leishmania in movement throughout its host and could help promastigotes penetrate mononuclear phagocytic cells (309). In addition to structural molecules, *Leishmania major* GP63 can cleave IgG and CD4, and facilitate the conversion of C3b into iC3b, thereby increasing uptake of the parasite into host immune cells (301,310–314). GP63 also prevents complement mediated lysis of promastigotes in human serum (312). In addition to these functions, GP63 is capable of reducing chemotactic responses in cells such as macrophages, neutrophils and monocytes (327,328). The ability for neutrophils and monocytes to initiate a respiratory burst response in response to opsonized zymosan is also hampered by the presence of *L. major* GP63 (328). To add to all of these functions, GP63 is also capable of reducing NK cell proliferation (329).

The use of invadolysins in trypanosomes highlights the fact that these remarkable immunomodulatory proteins have evolved specific functions dependent upon the requirements of the parasite. In *Trypanosoma brucei*, the blood-borne species of trypanosome responsible for sleeping sickness, some invadolysins are employed in the removal of variable surface glycoproteins (VSGs) from the surface of the procyclic form of the parasite, as they are necessary for immune evasion in the mammalian host, but not the invertebrate tsetse fly (337). In the human cell-inhabiting species *Trypanosoma cruzi*, GP63 proteins are involved in facilitating the penetration of the parasite into host cells (338,339). Finally, the fish trypanosome *T. carassi* has been shown to reduce reactive oxygen and nitrogen intermediate production by goldfish macrophages, while also decreasing iNOS-α, TNFα-1, and TNFα-2 (340).

To our knowledge, the only helminth metalloprotease to have been functionally characterized as a known invadolysin was our previous work on a protein termed SmLeish (Smp_135530) that was shown to inhibit the movement of *Biomphalaria glabrata* haemocytes,

and to negatively impact the kinetics of *S. mansoni* establishment within *B. glabrata* (145). Other helminth metalloproteases have been implicated in immunomodulation as well, with *Necator americanus* metalloproteases demonstrating a specific anti-eosinophil mechanism of action via the cleaving of eotaxin-1, but not IL-8 (413).

Given the consistent immunomodulatory role played by invadolysins during parasite infection/establishment, and the presence of SmCI-1 as the second most abundant protein in *S. mansoni* acetabular glands, we set out to characterize SmCI-1 in the context of early *S. mansoni* infection of the human host. We also compared this activity to that of SmLeish. In this study, we demonstrate that SmCI-1 acts as a MMP and can cleave specific human proteins relevant to structural and immunological functions, while SmLeish is often incapable of doing so. We also demonstrate that while several host proteins are cleaved, SmCI-1 does not have an effect on phagocytosis rates, or the ability of plasma/granulocytes to kill schistosomula. All of this was done with the intent of better understanding how *S. mansoni* is capable of surviving within human skin, as well as gaining a deeper knowledge of how parasitic organisms employ metalloproteases during infection and establishment within a host.

## 5.4 Methods

### 5.4.1 Production and Purification of Recombinant SmCI-1 and SmLeish

New recombinants for both SmCI-1 and SmLeish were generated using an HEK based expression system, and purified against polyclonal antibodies to each individual invadolysin, as laid out in section chapter. 3.9.2.

### 5.4.2 Production of Rabbit anti-SmCI-1 and SmLeish Antibodies

As is indicated in chapter 3, section 3.8.1, new polyclonal antibodies against SmLeish and SmCI-1 were generated by immunizing rabbits against full length recombinant invadolysins generated using a bacterial expression vector. Both polyclonal antibodies were validated as set out in section 3.8.2.

### 5.4.3 Western blotting

Parasite samples were loaded with a final concentration of ~20 parasites worth of material per lane, including freshly emerged cercaria, 24 post transformation cercaria, and cercarial E/S products. Primary antibodies used were αSmLeish and αSmCI-1 polyclonals.

### 5.4.4 Immunofluorescence staining

Cercaria were shed and probed using the α-SmCI-1 antibody in order to assess whether or not the native protein was localized to the acetabular glands of the larval parasite. Section 3.13.2 details the precise methodology employed.

### 5.4.5 Generic MMP assay

A generic MMP assay to determine MMP activity of the HEK generate SmLeish, SmCI-1, and SmCI-1Mut was performed as indicated in section 3.12.2.

An additional experiment was run to determine the activity levels of these invadolysins in various buffers of relevance to other assays. Generic MMP activity in KSRB, phosphate buffered saline (PBS, PH 7.4), RPMI 1640 (Gibco), DMEM (Gibco), Krebs-Ringer Phosphate Buffer (KRPG, PH7.4), and MMP buffer (50mM Tris, 10mM $CaCl_2$, 150mM NaCl, PH 7.5) was examined after 3 hours of incubation at 37°C.

### 5.4.6 Gelatin, collagen and elastin cleavage assay

Where possible, the capacity of the invadolysins to cleave structural molecules was assessed using commercially available kits. Due to the variability of activity between buffers, we elected to use the KSRB from the Generic MMP Activity Kit for these assays, after having initially tested activity in each kit's specific buffer. Individual kits were used to assess each invadolysin's capacity to cleave gelatin, collagen type IV, and elastin, as detailed in section 3.13.

### 5.4.7 Silver stain cleavage assays

For relevant structural and immunological proteins for whom no relevant cleavage assay was found, silver staining was used to determine the capacity of the invadolysins to cleave such molecules. Two micrograms of Human Fibrinogen (Sigma-Aldrich), Immunoglobulin G (Sigma-Aldrich), recombinant complement component C3 (Millipore Sigma), recombinant CD4 (Sigma Aldrich) were added to 20 µl of KSRB with 1mM APMA. Samples were then either left without protease or given 2 µg/ml SmCI-1, 2 µg/ml SmLeish or 2 µg/ml trypsin as a positive control for cleavage. Samples were incubated at 37°C for 18 hours, and then silver staining was performed as indicated in chapter 3, section 3.10.2.

### 5.4.8 Testing hemolytic activity of human sera pre-treated with rSmCI-1 and rSmCI-1(Mut)

To investigate whether the complement system was affected by SmCI-1 we pre-incubated human serum with 2µg/ml rSmCI-1 or rSmCI-1Mut and measured the hemolytic activity of both the classical and alternative complement pathways using previously establish methods. A detailed account of the process is found in section 3.16.1.

### 5.4.9 Phagocytosis

We set out to determine whether or not treatment with rSmCI-1 would impact the ability of macrophage-like cells such as RAW 264.7 mouse macrophages or differentiated THP-1 cells to internalize a highly phagocytosed target such as fluorescent *E.coli.* We also examined whether incubation with rSmCI-1 or rSmCI-1Mut would affect the opsonization and subsequent phagocytosis of such particles when they were exposed to human serum. Detailed procedures may be found in section 3.17.1.

### 5.4.10 Schistosomula plasma- and granulocyte-mediated killing assays

The effects of rSmCI-1 on the abilities of human plasma or human granulocyte-like like cells was performed as detailed in chapter 3 sections 3.16.2 and 3.17.2. The effects of human plasma and the SmCI-1 recombinants on humoral and cell mediated killing were assessed.

### 5.4.11 dsRNA mediated knockdown of SmCI-1

Ten snails with patent *S. mansoni* infections were injected with an estimated final concentration of 6 nM of the dsRNA targeting SmCI-1 for RNA interference. To assess knockdown efficacy, samples of 20 cercaria were used for western blotting from 7 to 12 days post injection using the α-SmCI-1 antibody and an anti-β-actin antibody was used as a loading control.

### 5.4.12 Mouse infections and perfusion

Infections and perfusion of Swiss Webster mice (6 per group) was performed as detailed in chapter 3 section 3.19. Cercaria treatments included parasites 12 dpi from PBS injected snails, 12 dpi GFP KD controls, 8 dpi SmCI-1 KD larvae, and 12 dpi SmCI-1 KD larvae.

### 5.4.13 Statistical analysis

Differences in activity in all cleavage, phagocytosis, and killing assays, were each assessed via One-Way Anova followed by Tukey post hoc test.

## 5.5 Results

### 5.5.1 Predicted Structure of SmCI-1 and SmLeish

Modelling the 3D structure of SmCI-1 demonstrated consistency with the core features found in invadolysins (**Figure 5.1 C,D**). SmCI-1 was predicted to feature an active site similar to GP63, with three histidines (His231, His235, and His336) present in order to coordinate the immobilization of a zinc molecule, and a glutamic acid (Glu232) utilized as a nucleophile during proteolysis. SmCI-1 also featured amino acids in locations likely to correspond to canonical features of invadolysins, such as the methionine (Met347) needed for a met-turn, and three cysteines (Cys182, Cys194, and Cys211) on the N-terminal side of the active site, each of which is a candidate for a cysteine-switch activation mechanism.

3D modeling of each of the two predicted MMP domains of SmLeish featured highly similar results (**Figure 5.1 E-H**). Both domains featured the three histidines and glutamic acid present in invadolysin active sites, while also possessing met-turn methionines and three cysteine-switch candidate cysteines apiece. Such modeling helped confirm the existence of two MMP domains in SmLeish, each possessing all the necessary features for classification as an invadolysin, as well as MMP activity.

### 5.5.2 Location and expulsion of SmCI-1

Immunofluorescence staining utilizing our polyclonal anti-SmCI-1 antibody revealed a concentrated fluorescence signal emanating from the acetabular glands of *S. mansoni* cercaria (**Figure 5.2 A**). Staining with either primary or secondary antibodies only failed to elicit such a signal. Western blot analysis of SmCI-1 release during transformation into schistosomula further confirmed the acetabular gland localization (**Figure 5.2 B**). Our antibody recognizes a protein appearing at the expected size of 65 kDa. The 65 kDa band was seen in untransformed cercaria, as well as 24 hr post transformation cercaria and their E/S products. When western blot analysis was performed using our rabbit polyclonal anti-SmLeish antibody, numerous bands were observed at ~ 40, 50, 70, and 110 kDa, with several bands appearing between 130 and 180 kDa. No protein bands were observed in the E/S products of anti-SmLeish-probed blots. Antibodies were shown to recognize parasite proteins, while pre-immune serum did not (**SFigure 5.11**).

### 5.5.3 rSmCI-1 and rSmLeish display MMP activity

Both the positive control (human MMP8), and rSmLeish also demonstrated MMP activity capable of being inhibited by the addition of 1,10-phenanthroline (**Figure 5.3 A,B**). Unlike rSmCI-1, both MMP8 and rSmLeish samples activated with APMA had significantly higher MMP activity than those that were not activated. Among MMP8 samples that were not activated, only those at 1μg/ml displayed activity levels that differed significantly from the vehicle control. Recombinant SmCI-1 displays dose-dependent MMP activity and is inhibited by the addition of [250μM] of 1,10-phenanthroline (**Figure 5.3 C**). Samples left inactivated, and those of the same concentration that were activated with 1mM APMA did not differ significantly in their activity. The mutation of Glu232→Gly232 rendered the rSmCI-1 mutant completely

inactive, with no concentration up to and including 1μg/ml differing from the vehicle control with respect to general MMP function (**Figure 5.3 D**). For rSmLeish samples that were not activated, both 1μg/ml and 0.5μg/ml samples were significantly different than the vehicle control. Activity was highest for all proteases in KSRB (**SFigure 5.12**).

### 5.5.4 Cleavage of Structural Substrates

To better understand the role of SmCI-1 and SmLeish in degradation of prominent components of the extracellular matrix of host skin cells and the basement membrane of human skin, we examine the capacity of these to cleave gelatin, collagen type IV, and elastin. In the case of each of these possible substrates, rSmCI-1 was able to weakly cleave each of them. In the cases of gelatin (**Figure 5.4 A**) and collagen type IV (**Figure 5.4 B**), rSmCI-1 cleavage activity significantly differed from the APMA negative control, but only at 2μg/ml, and not 1μg/ml. This activity was significantly lower than that seen in the positive control, *Clostridium histolytica* collagenase. In the case of elastin, rSmCI-1 displayed cleavage activity above that of the APMA control for both 2μg/ml and 1μg/ml treatments. This activity was again lower than established positive controls for the cleavage of elastin, such as porcine elastase and human MMP12 (**Figure 5.4C**). SmLeish only featured significant differences in activity from controls for gelatin (**Figure 5.4A**), but this was likely due to the presence of outlying datapoints rendering the difference significant. Alternatively, gelatin is more easily cleaved by various proteases than native collagens, given its denatured structure, which could have resulted in SmLeish being able to cleave it at low levels. Both invadolysins failed to demonstrate activity in kit-specific buffers for all three of these assays (**SFigure 5.13**).

Under reducing SDS-PAGE conditions, fibrinogen separates into the three distinct subunits of which it is composed (α, β, and γ). Our positive control, trypsin cleaves all three subunits, as evidenced by the disappearance of the bands of all three subunits and the appearance of two bands roughly 40kDa in size. Recombinant SmCI-1, alternatively, cleaves only the alpha subunit of fibrinogen, as shown by the disappearance of the α-subunit band at ~70kDa (**Figure 5.4D**), while SmLeish fails to cut any of the three subunits.

### 5.5.5 Cleavage of complement component C3 leads to increased parasite viability

Four immunologically relevant proteins were examined as possible substrates for SmCI-1: complement component C3, CD4, CR1, and Immunoglobulin G (IgG). The latter three were all cleaved by trypsin but were unaltered by rSmCI-1 treatment (**Figure 5.5, SFigure 5.14**). The only immunologically relevant protein examined that was affected by rSmCI-1 treatment was complement component C3b (**Figure 5.5 A**). Trypsin was capable of cleaving both C3b and the C3a anaphylatoxin. SmLeish failed to cleave any of the four immunological targets we examined.

The cleavage of complement component C3b led us to examine the effects of SmCI-1 on both the classical and alternative complement pathways (**Figure 5.5 C,D**). Pre-treatment of human serum with rSmCI-1 caused a significant decrease in alternative pathway-mediated lysis from 26.1±1.3% to 5.3±1.0%. A similar observation was made of the classical pathway, which displayed a significant decrease in lysis from 74.8±1.4% to 46.0±1.4% when serum was treated with rSmCI-1. Treatment with rSmCI-1Mut did not result in a significant decrease in lysis, with 26.2±1.6% and 73.2±0.7% lysis seen in the alternative and classical pathway assays, respectively (**Figure 5.5 C,D**).

Injection of our dsRNA construct was successful at reducing the amount of SmCI-1 protein in *S. mansoni* cercaria. On days 7-10 post injection, isolated cercaria were producing SmCI-1, while at 11- and 12-days post injection SmCI-1 production was drastically reduced (**SFigure 5.10 A**) despite comparable levels of loading control (**SFigure 5. B**).

This tool allowed us to assess the ability of SmCI-1 knockdown (KD) parasites to survive plasma-mediated destruction. Newly transformed schistosomula (NTS) with SmCI-1 (8 dpi) were as viable as SmCI-1 KD parasites (12 dpi) at 48,72, and 96 hours post transformation (**Figure 5.6**). At all three of the timepoints measured, NTS treated with 10% human serum or 10% human serum + rSmCI-1Mut were significantly lower than those of NTS treated with 10% human serum + rSmCI-1. Additionally, SmCI-1 KD parasites treated with 10% plasma and rSmCI-1 failed to differ in their viability scores from those SmCI-1 KD parasites treated with heat inactivated human serum.

### 5.5.6 SmCI-1 Fails to Alter Macrophage Phagocytosis

Analysis using the IDEAS software was able to accurately distinguish between RAWs and THP-1 with or without phagocytized pHrodo particles (**SFigure 5.15 A,B**). RAW 264.7 cells and stimulated THP-1 cells phagocytized the pHrodo Green *E. coli* BioParticles 48.9±1.5% and 43.1±4.2% of the time, respectively (**Figure 5.7 A,B**). Addition of 10μM cytochalasin D significantly reduced the percentage of cells undergoing phagocytosis to 17.8±2.9% and 26.6±1.9%, respectively ($p<0.01$). In the case of RAW 264.7 cells treated with rSmCI-1, phagocytosis in 45.2±0.5% of cells failed to differ significantly from negative controls. THP-1 cells treated with rSmCI-1 featured phagocytosis in 41.8±2.3% also failed to differ significantly from the negative control.

Opsonization of the BioParticles with human serum significantly increased uptake in THP-1 cells from 39.8±7.4% to 67.9±2.0%. The decrease seen in rSmCI-1 human serum samples was not significant (61.4±0.8%), whereas the decrease in rSmCI-1Mut treated human serum samples was (50.6±1.0%, p=0.037).

### 5.5.7 SmCI-1 does not alter granulocyte-mediated killing of *S. mansoni* cercaria

In the granulocyte-mediated killing assay (**Figure 5.8**), RPMI-only mediated killing was higher at both timepoints than in the plasma-mediated killing assay, with 12±2.8% dead at 24 hrs and 16±3.7% dead at 48 hours. For the neutrophil-like HL-60 cells, all treatments resulted in significantly lower death percentages than the RPMI only controls, but all failed to differ significantly from any other treatment (**Figure 5.8 A,C**). Regarding the eosinophil-like EoL-1 cells, at 24hrs, only EoL-1 cells alone, and EoL-1 cells + C3 were significantly lower than RPMI-only controls, while all treatments were significantly lower than RPMI-only controls at 48hrs. All treatments failed to differ significantly from all other treatments at both timepoints (**Figure 5.8 B,D**).

### 5.5.8 SmCI-1 KD parasites produced lowered adult worm burdens in mice

SmCI-1 knockdown was able to significantly reduce adult worm burden 5 weeks post infection in Swiss Webster mice (Figure 5.9). Of the 100 cercaria applied to each mouse, an average of 31.8 (SEM=2.4) was recovered from the PBS 12 dpi group, 32.2 (SEM=1.9) from the GFP KD 12 dpi group, 29.0 (SEM=2.0) from the SmCI-1 KD 8dpi group, and 22 (SEM=1.2) from the SmCI-1 KD 12 dpi group. The SmCI-1 KD 12 dpi group featured worm burdens significantly lower than those seen in both the PBS and GFP KD control groups, while the SmCI-1 KD 8 dpi group failed to differ significantly from controls ($p<0.01$).

## 5.6 Discussion

Surviving the early stages of infection in human skin, prior to the development of a multi-layered tegument that renders larval schistosomes highly resistant to immune mediated attack, is critical for *S. mansoni* survival (95,414). We sought to characterize the function of the most abundantly produced invadolysin in *S. mansoni* cercaria (145,175). We find compelling evidence that SmCI-1 is a potent immunomodulator that can create an environment conducive to survival of the cercaria during its transformation into a schistosomula.

### 5.6.1 The predicted structure of SmCI-1 and SmLeish are similar that of GP63

SmCI-1 and SmLeish display the highest amino acid similarity to GP63 from *Leishmania major* (288). The predicted three-dimensional structures of these proteins bear a remarkable level of similarity, especially when considering the canonical features of metzincin metalloproteases. They all possess histidine residues that all zinc metalloproteases employ in the coordination of a zinc ion in the active site of the protease (285). Typically, 1-3 amino acids separate the first and second histidine, with a glutamic acid immediately C-terminal to the first histidine (HEXXH) (285,287). The metzincins, a subset of zinc metalloproteases, frequently display an expanded protease motif (HEXXHXXGXXH) with a third histidine employed in coordinating the zinc ion, with some employing as many as 20-120 amino acids between the second and third histidine (285,287,288). While GP63 has 65 amino acids separating its second and third histidines, SmCI-1 has 100, while the first and second MMP domains of SmLeish have 80 and 103, respectively. All possess a methionine predicted to exist underneath the active site, and in the case of SmCI-1, this would be Met347. For SmLeish, MMP D1 has a methionine of interest is Met159 while MMP D2 has one at Met191. This methionine is next to third histidine in all metzincin proteases, and is the amino acid from which this subset of proteases derives its name (288). The existence

of this methionine has been found to be important in stabilizing such proteases, as mutation of this residue can lead to lowered proteolytic activity and increased autolysis (289,415,416). Finally, SmCI-1 and each SmLeish MMP domain have three cysteine residues N-terminal to the active site, as does GP63. While we are not aware which of these residues function in a potential cysteine switch mechanism in either protein, the presence of these amino acids further confirms structural similarities between SmCI-1 and other metzincin invadolysins.

### 5.6.2 SmCI-1 is found in *S. mansoni* acetabular glands and expulsed during transformation

The original identification of SmCI-1 suggests it is a prominent protein of the acetabular gland (175), an observation supported by our immunofluorescence assessment. The placement of SmCI-1 staining inside of the cercaria in a location consistent with the bulk of acetabular contents, as observed by z-stack images, was an expected finding. Our western blotting of cercarial bodies and E/S products also helped to confirm this, as the release of SmCI-1 into RPMI culture media is consistent with the fact that cercaria expulse acetabular gland contents under such conditions (175). We detect the expected ~65kda protein in Western blot along with a faint second band roughly 49kda in size in cercaria samples. Cleavage of the zymogen into an active form is a near universal requirement for MMPs, thus the substantially weaker ~49kda band could reflect the presence of an activated form of SmCI-1 retained within the cercaria (284,291,292). The exact kinetics of acetabular gland release and the location surrounding the parasite in the skin has long been contested (4–7,165). Previous research has demonstrated that acetabular gland contents can be present at the apical end of cercaria as they arrive in the dermis, wherein they are likely to encounter numerous immune cell types (168,182). Additionally, these glands have been observed to atrophy between 48-72 hours post penetration, meaning they are still present as the parasite combats the immune response in the dermis (164). The vortexing

transformation method used in this study is not ideal for studying acetabular gland release kinetics given that the cercaria are not squeezed as they would be moving between skin cells. This more natural path likely facilitates a more rapid expulsion of E/S products. However, the more controlled context of culturing suggests that as the parasite releases SmCI-1 over time, it does not seem to continue to produce SmCI-1 to replace that which it has already expulsed. This is consistent with the finding that SmCI-1 is most highly upregulated in the germ ball stage of development, as the cercaria prepares to exit the snail, and not in the emerged cercaria themselves (203).

Our anti-SmLeish Westerns gave far more inconclusive results. Unlike the antibody used in Chapter 4 of this thesis, the rabbit polyclonal we developed for this study recognized a wide variety of protein bands, leading us to doubt the specificity of the antibody. While the roughly 110 kDa band or band above 130 kDa are near to the 125 kDa expected size of SmLeish, the presence of many bands above 130 kDa, as well as the bands at ~ 40, 50, and 70 kDa render interpretations of what SmLeish is doing in the context of cercaria difficult. While the ~ 40, 50, and 70 kDa bands could be cleaved and activated forms of SmLeish, such a hypothesis remains to be tested. Given these unclear results in our Western, immunofluorescence data for anti-SmLeish probed cercaria remains uninterpretable and therefore unpresented. Given the fact that SmLeish was shown to be far less upregulated than SmCI-1 in the germball stage of development, as well as the fact that SmLeish has never been identified in the E/S products of *S. mansoni* cercaria, a difficulty in getting a strong western blot signal for the full length protein, as well as an absence of recognition of any protein in E/S products are both understandable (175,203).

### 5.6.3 MMP activity of SmCI-1 leads to the cleavage of key host proteins

SmCI-1 and SmLeish clearly display MMP activity. Whereas activation of rSmLeish and MMP-8 (positive control) using APMA significantly increased their activity, the MMP activity of SmCI-1 did not significantly differ between activated and non-activated treatment groups. Possible explanations for this are SmCI-1 being self-activating or activating neighboring SmCI-1 molecules. There is precedent for this line of thought, as *Leishmania sp.* GP63 is capable of cleaving peptides composed of the last 4 amino acids of the protein pro sequence and the first 5 amino acids of the mature GP63, suggesting autoproteolysis as a possible mechanism of activation, a finding supported by the fact that zinc chelation and mutation of the HEXXH active site of GP63 reduce its maturation (306). The concept of neighboring invadolysin molecules being responsible for such activation is less likely, given that addition of activated GP63 to GP63 zymogen does not facilitate increased activation of the zymogen (304). The release of SmCI-1 as *S. mansoni* migrates through human skin may benefit from having SmCI-1 not requiring activation, as rapid activity upon expulsion from the acetabular glands would aid in degradation of host components. Alternatively, another possibility could be the activation of SmCI-1 by another acetabular enzyme, although the lack of such enzymes in our studies relying on recombinant proteins suggests that if another enzyme facilitates activation of SmCI-1 during penetration, it is not the only way in which SmCI-1 can be activated. The requirement of APMA activation for rSmLeish prior to increased activity is consistent with our previous work demonstrating that activation of the zymogen is necessary prior to increased MMP functionality. The inhibitory effect of 1,10-phenanthroline further confirmed that SmCI-1 and SmLeish function as a zinc-based metalloprotease. Finally, mutation of the active site glutamic acid into a non-polar glycine completely abrogated the MMP activity of SmCI-1. The glutamic acid at this

site acts as a nucleophile in activating water molecules, thereby facilitating hydrolysis of substrate proteins (287,295). The mutant SmCI-1 allowed us to further assess the ability of this invadolysin to affect human cells in both an MMP activity dependent and independent manner.

Examination of the proteolysis of several host skin structural molecules by SmCI-1 was predicated upon the knowledge that *S. mansoni* products have been known to degrade host ECM at a distance from the invading larval schistosome (417,418). Of these host molecules, collagen type IV and elastin are of particular relevance, given the abundance of elastin fibres in the human skin dermis that exist as a barrier to invading pathogens, and the large quantity of collagen type IV found in the basement membrane separating the epidermis and dermis, which schistosomes must pass through on their way to finding a venule (163,419). SmCI-1 could cleave gelatin and collagen type IV. It was, however, less capable of doing so than our positive control, *C. hystolytica* collagenase. The same can be said of its ability to cleave elastin in comparison to porcine elastase and human MMP-12. Given the fact that the serine elastase has been shown to be responsible for the majority of the activity required to enter through human skin, it is possible that the low elastase/collagenase activity demonstrated by SmCI-1 is a function of it not being required for the purpose of degrading these molecules (176). Regarding fibrinogen, while not as abundant in the skin as either collagen or elastin, it may be present at the site of penetration to facilitate wound healing and blood clotting, a function that would likely be negatively impacted by the cleavage of the fibrinogen α-subunit by SmCI-1. SmLeish failed to cleave collagen type IV, elastin, and fibrinogen, and only weakly cleaved gelatin, all of which suggests it is not specialized for a role in aiding skin penetration by invasive cercaria, nor functioning in reducing blood clotting at the site of infection. The necessity of using KSRB as opposed to the cleavage-assay-provided buffers highlights the need for using a buffer in which MMPs are capable of

functioning. Although the composition of these buffers is exclusively known to the companies generating them, it is likely that KSRB contains calcium, whereas the other kit-provided buffers do not, given that it has previously been shown that MMPs often require calcium for activity (283,285,287).

### 5.6.4 SmCI-1 activity protects larval schistosomula from plasma-mediated lysis

When examining several different immunologically relevant factors in our cleavage assays, we found complement component C3b to be cleaved by SmCI-1. This aligns with studies on GP63 that demonstrate that it can cleave complement component C3. Cleavage of C3 results in an increased capacity for *Leishmania* to survive complement mediated lysis in human serum, which is suggestive of a reason for targeting C3b for cleavage (310,312). *S. mansoni* must also contend with complement-mediated killing by human serum, but only during the period in which they are undergoing shedding of the immunostimulatory glycocalyx (204). The degradation of C3b by SmCI-1 resulted in decreased complement mediated hemolysis via classical and alternative complement pathways. The abundance of SmCI-1 in the acetabular glands, as well as its release during the initial stages of infection, suggests that this invadolysin is likely a key method by which cercaria avoid complement mediated lysis during the early stages of infection. We established that this is the case by conducting NTS viability assays, where SmCI-1 KD parasites were found to be damaged by human serum. Given the inability of these parasites to generate typical levels of SmCI-1, and the ability of rSmCI-1 addition to rescue parasite viability scores, we argue that there is compelling evidence that SmCI-1 functions in protecting larval schistosomes from the membrane attack complex. This argument is further supported by the inability of heat inactivated serum to reduce viability scores in the same significant manner as regular serum with or without the catalytically inactive rSmCI-1Mut. The inability of SmCI-1 to

cleave IgG suggests that both the alternative and classical complement pathways were both affected by targeting of C3b, as opposed to targeting multiple humoral opsonins.

SmCI-1 failed to cleave IgG, CD4, and CR1. We examined IgG, given that schistosomula E/S products, containing metalloproteases, have previously been shown to cleave IgG bound to Fc receptors of *S. mansoni* (208). Serine proteases are a likely alternative IgG-targeting protease to metalloproteases, given that they have already been shown to cleave IgE (209). CD4 wasinvestigated because *L. major* GP63 degrades it, and potential CR1 cleavage was assessed because of its known association with C3b (313).

Despite possessing generic MMP activity, rSmLeish failed to cleave any of the structural or immunological molecules we examined, with the slight exception of some gelatinase activity. A likely explanation for the difference in substrate specificity between SmCI-1 and SmLeish is that the parasite has evolved to utilize different invadolysins during invasion of the invertebrate and definitive hosts. This explanation is supported by the fact that SmLeish has yet to be identified via proteomic approaches in cercaria E/S products, as well as the observation that its transcription is significantly less upregulated than that of SmCI-1 during germball development (203). There is also precedent for the use of invadolysins substrates being host-specific in other parasites, as targeted deletion of 7 GP63 genes in *L. major*, which are involved in survival and pathogenesis in mammals, had no effect on development in the invertebrate sandfly host (312). There is also precedent for the use of different invadolysins for different purposes, with *T. brucei* major surface protein – B (TbMSP-B) but not TbMSP-A being involved in the removal of variant surface glycoproteins from procyclic trypanosomes (337). While SmLeish may very well serve an as of yet undetermined role in mammalian infection, it does not appear to do so in the same capacity as SmCI-1, despite its significant role during infection of *B. glabrata*.

### 5.6.5 SmCI-1 fails to alter phagocytosis activity in phagocytic cells

To our understanding, no invadolysin has yet been implicated in reducing the generic phagocytic activity of immune cells. Despite this fact, we elected to examine the effectsof SmCI-1 on mouse macrophages and human macrophage-like cells due to the involvement of invadolysins in cell movement, as well as SmCI-1's capacity to slightly cleave human extracellular matrix components (145,341). In both RAW 264.7 macrophages and THP-1 macrophage like cells, SmCI-1's inability to significantly reduce phagocytosis suggests the parasite is not employing SmCI-1 to slow down the uptake of antigens by nearby leukocytes.

There is, however, more precedent for invadolysins altering the opsonization of targets. *L. major* promastigotes utilize GP63 to facilitate the rapid conversion of host C3b into C3bi, which helps facilitate the entrance of the parasite into host phagocytic cells via engagement with complement receptor 3 (CR3) (301,311,314,420). When we treated pHrodo *E.coli* with human serum, THP-1 cells more readily phagocytized the particles. Pre-incubation of this human serum with 2μg/ml rSmCI-1 or rSmCI-1Mut resulted in slight decreases in phagocytic rates, with only the rSmCI-1Mut treated plasma being statistically significant from human serum only treated samples. Given the inactivity of rSmCI-1Mut, it is likely that this decrease may disappear with a higher number of samples run. Alternatively, SmCI-1 may affect another human plasma component in a non-MMP activity dependent manner, resulting in a decrease in opsonization of the *E. coli* bioparticles. The fact that rSmCI-1 did not reduce phagocytosis of these particles despite cleavage of human C3 suggest that there are an adequate number of alternative opsonins such as IgG and IgM present in human serum. Given rSmCI-1's inability to cleave IgG, cleavage of C3 alone may not prove sufficient in order to result in a significant decrease in phagocytosis.

### 5.6.6 Granulocyte-like cell lines failed to kill freshly transformed schistosomula

*In vitro* killing assays using primary human neutrophils and eosinophils have demonstrated that such cells are capable of killing developing schistosomula, and that this process can be aided by the presence of human complement components and antibodies (154,155,421–423). Such killing is mediated by the presence of several reactive oxygen species, and the release of eosinophil cationic protein (ECP) (422,423). In our granulocyte-mediated killing assay, both our neutrophil-like HL-60 cells and eosinophil-like EoL-1 cells failed to kill schistosomula at a higher level than RPMI-only controls. This is in spite of the fact that HL-60 cells are often used as a model for neutrophil functions, and are capable of initiating a respiratory burst, while EoL-1 cells have been shown to be capable of synthesizing and releasing ECP (356,357,359,424,425). Our HL-60 cells initiated a respiratory burst using established measurement techniques, especially after DMSO treatment (**SFigure 5.16**), and while our EoL-1 cells were not tested for ECP production, their differentiation was confirmed phenotypically, with a change from cell clumps to individual cells post DMSO treatment. While this assay failed to further our understanding of SmCI-1's role in schistosomula survival, it is (to our knowledge), the first demonstration that HL-60 and EoL-1 cells are not a suitable system for studying granulocyte killing of developing schistosomula. While the reason for this is unknown, these cells could lack the appropriate receptors for recognition of the schistosomula, or release an insufficient amount of cytotoxic factors such as ECP or ROS to kill the developing parasites.

### 5.6.7 Effects on in vivo infection success levels

Our dsRNA construct injection marks the first successful knockdown of a *S. mansoni* cercarial factor prior to its emergence from the snail, and allowed for the generation of parasites lacking normal levels of SmCI-1. Given the roughly 30.8% reduction in adult worm burden with SmCI-1 KD parasites compared to PBS-injecedt controls, SmCI-1 has demonstrated a role for

parasite survival. Given the results of our other assays, the protective effect of SmCI-1 is likely due to a combination of degrading structural components of mammalian skin, overcoming complement mediated lysis, and downregulating inflammatory responses at the site of infection. All such functions are uniquely tailored to survival by the larval parasite during migration and maturation within the skin. Given that SmCI-1 transcript levels are significantly lower during other life cycle stages other than germball *S. mansoni*, it is likely that skin-stage parasites are the primary beneficiaries of the invadolysin's protective effects (203).

Iinfected human populations have been shown to possess antibodies to SmCI-1, although at lower levels than those seen to other numerous key schistosome antigens such as cathepsin B (191). It is possible that the inability of humans to mount a sufficient antibody response to this antigen allows the larval parasite to continue to employ the invadolysin in overcoming immune defense mechanisms. Immunization against SmCI-1 to increase circulating antibody levels may prove beneficial, not in targeting the parasite itself, but rather in altering the immunological milieu in which the parasite resides, and increasing the likelihood of parasite death in the skin.

### 5.6.8 Future work

Further examination of SmCI-1's functions in facilitating human infection by *S. mansoni* are merited. To begin with, the effect of SmCI-1 on leukocyte chemokinesis is merited, given both our observations that SmLeish affects *B. glabrata* haemocyte movement, as well as previous work demonstrating GP63's capacity to differentially alter monocyte and neutrophil chemotaxis in response to varying stimuli (145,328). Other valuable assays to consider for examination of SmCI-1's functions based upon previous work done on GP63 would include cell proliferation assays, as well as an examination of the invadolysin's affect on the initiation of respiratory burst responses to various antigens (328,329). The development of either an

inhibitory monoclonal antibody against SmCI-1, or the creation of a specific inhibitor for the same are worthy of attention in order to better characterize the role of this invadolysin in surviving granulocyte-mediated killing, using primary granulocytes isolated from donors, as opposed to cell lines. Alternatively, such experimentation could be performed using our SmCI-1 KD parasites. Finally, use of SmCI-1 KD cercaria should prove useful in further determining the role of this invadolysin *in vivo* via the use of skin punches in mice or baboons to determine the exact anatomical location of cercaria death as well as potential alterations to penetration kinetics.

## 5.7 Conclusion

SmCI-1 is the second most abundant acetabular gland protein released by *S. mansoni* cercaria during penetration into human skin. This fact alone merits an examination of its roles during infection. Here, we show that SmCI-1 possesses the hallmark structure and sequences of an invadolysin, a family of proteases shown to be important for infection success in numerous parasites. We demonstrate that SmCI-1 has numerous functions relevant to survival in human skin, including cleavage of structural components, targeting of complement component C3b for cleavage, and subsequent reduction of complement pathway activity leading to increased parasite viability. Finally, knockdown of SmCI-1 in cercaria results in reduced adult worm burdens in mice, confirming the importance of SmCI-1 in infection establishment. SmLeish fails to demonstrate comparable substrate specificity. These functions suggest SmCI-1 as an important factor for parasite survival early on during infection. While SmCI-1 fails to reduce phagocytosis levels, further research remains to be conducted on its effect on immune processes to determine its ability to influence infection establishment in mammalian hosts.

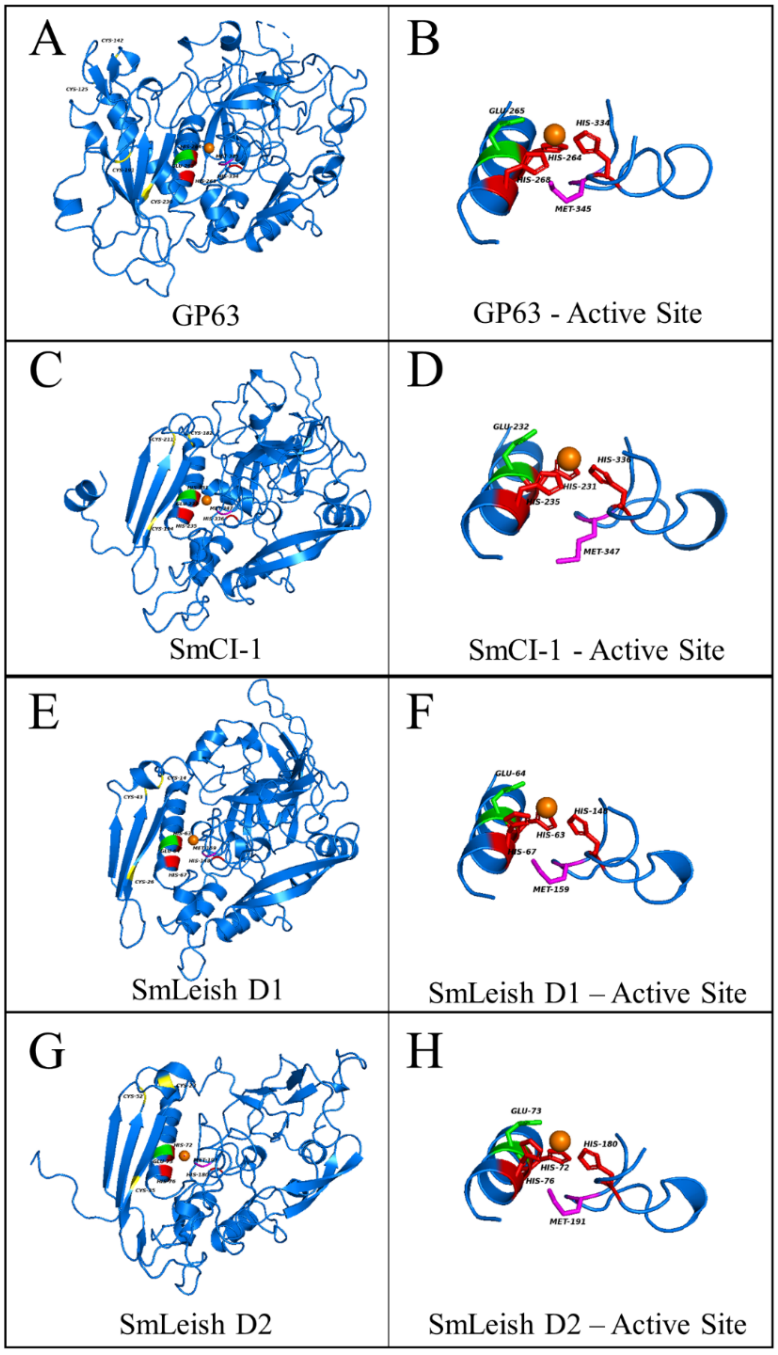



**Figure 5.1. Predicted structure of invadolysin MMP domains**

A 3D visualization of the crystal structure of GP63 (A), and a magnified view of its active site (B). The predicted structure of the full amino acid sequence of SmCI-1 is also present (C) alongside a magnified view of its active site (D). Predicted MMP domains 1 (E) and 2 (G) are also presented alongside their active sites (F,H) Active site histidines are presented in red, holding a zinc ion (orange ball) in position. Nucleophilic glutamic acids are marked as green, with likely met-turn methionines marked as purple. All cysteines N-terminal to the active site have been coloured yellow.

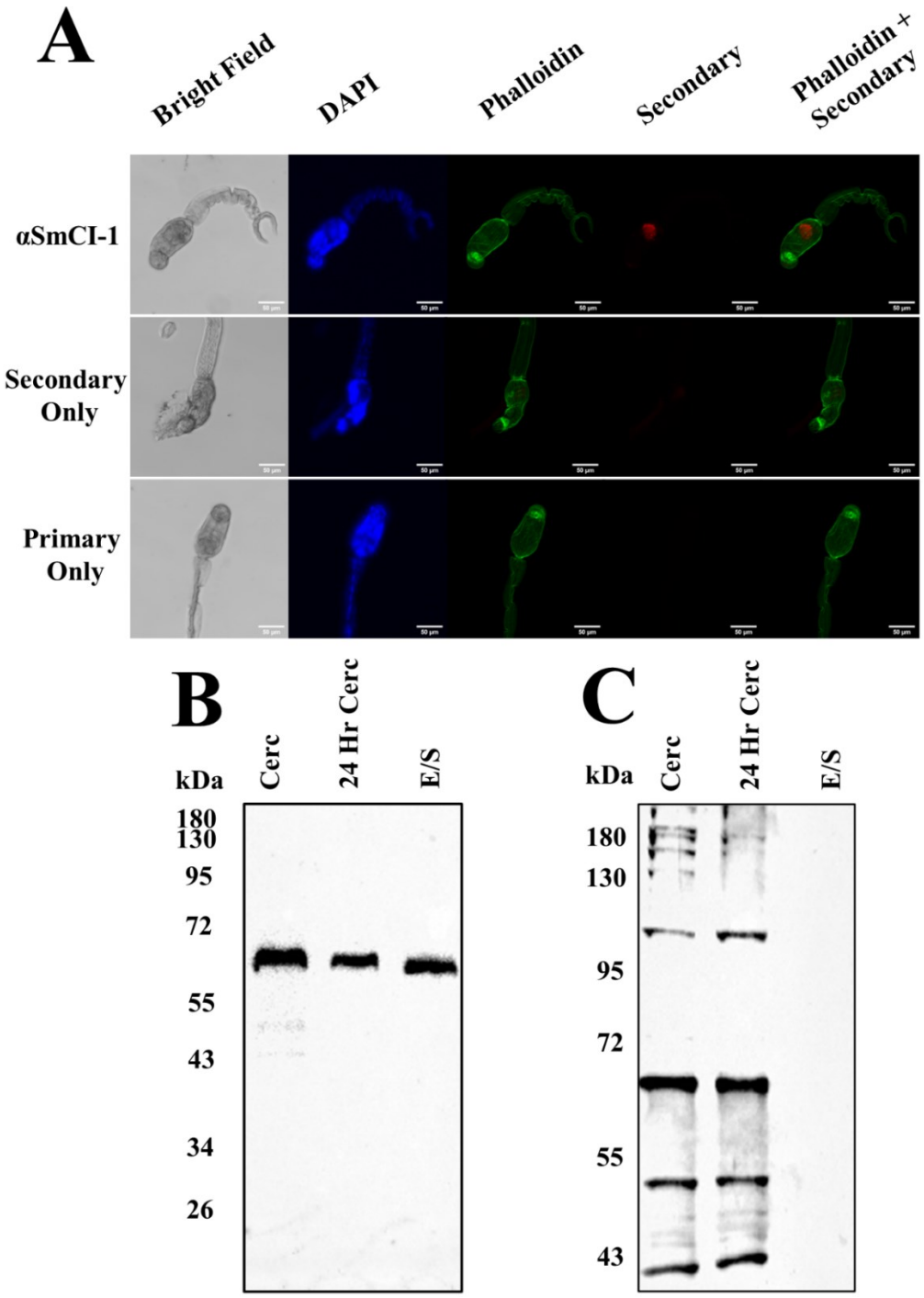



**Figure 5.2. Localization and release of SmCI-1 and SmLeish**

(A) SmCI-1 is localized to the acetabular glands of *S. mansoni* cercaria, as revealed by immunofluorescence imaging, with SmCI-1 signal appearing in red. (B) SmCI-1 is expelled from the cercaria during the initial stages of maturation into schistosomula that occur in human skin. SmCI-1 is shown via western blotting as a ~65kda band in cercaria as well as cercarial E/S products. (C) The anti-SmLeish antibody recognizes bands of various sizes in cercaria and 24 newly transformed schistsomula, but does not recognize any protein in parasite E/S products.

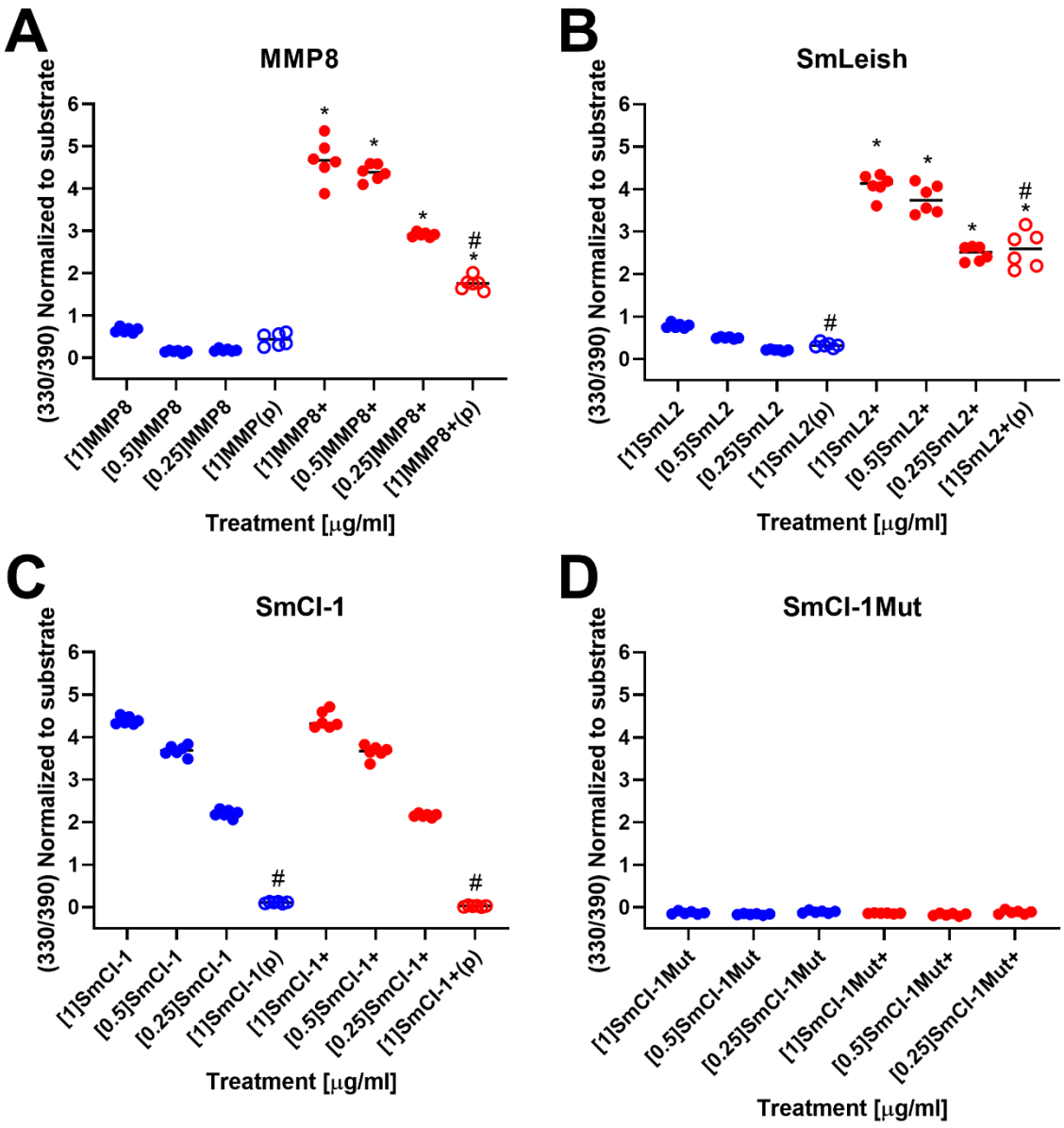



**Figure 5.3. Recombinant SmCI-1 is a functional matrix metalloprotease.**

MMP activity of (A) human MMP8, (B) rSmLeish, (C) rSmCI-1, and (D) rSmCI-1Mut. All proteases were examined at three initial concentrations of 1µg/ml, 0.5µg/ml, and 0.25µg/ml. Whereas MMP8, SmLeish, and rSmCI-1 demonstrated activity, rSmCI-1Mut did not. Human MMP8 and rSmLeish required activation with 1mM APMA(+), rSmCI-1 had similar activity levels with and without APMA-mediated activation. Significant inhibition via the addition of [250µM] (p) of 1,10-phenanthroine is denoted by (#). Activated samples (+) that differ significantly from their non-activated counterparts of an identical concentration are denoted using (*). (n=6)

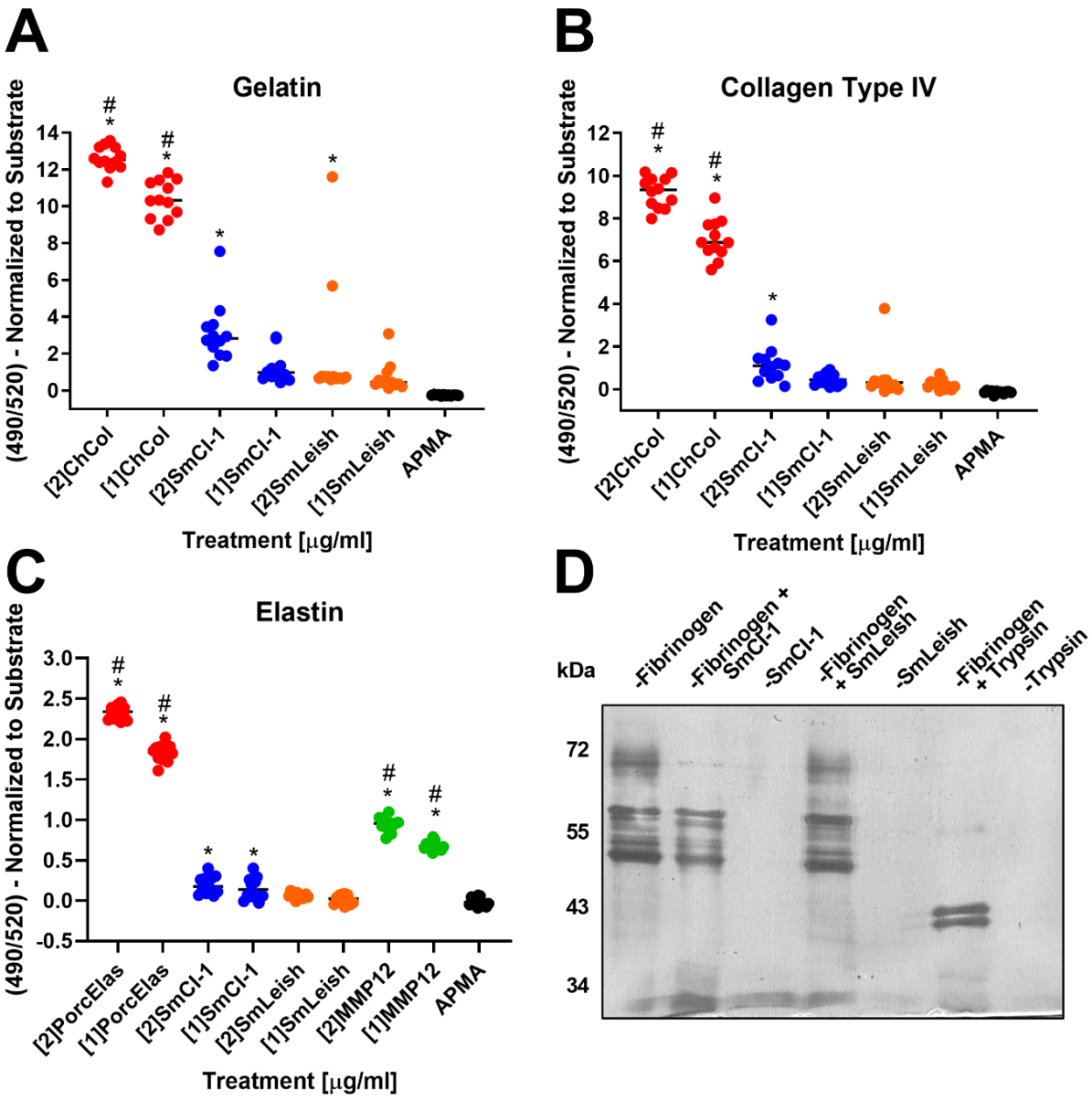



**Figure 5.4. SmCI-1 weakly cleaves key host ECM components**

Recombinant SmCI-1 and SmLeish cleave gelatin (A). Only rSmCI-1 cleaves collagen type IV (B), and elastin (C). In all cases, known positive controls for collagen type 4 and gelatin cleavage (*C. histolytica* collagenase) and elastin (porcine elastase and human MMP12) displayed higher levels of cleavage. Differences from the APMA vehicle control are denoted using (*) and samples with significantly higher cleavage activity than rSmCI-1 are denoted using (#). (D) Silver stain demonstrating the capacity of trypsin to cleave all three subunits of fibrinogen, SmCI-1's ability to cleave the alpha subunit of fibrinogen only, and inability of SmLeish to cleave any fibrinogen subunit (n=12).

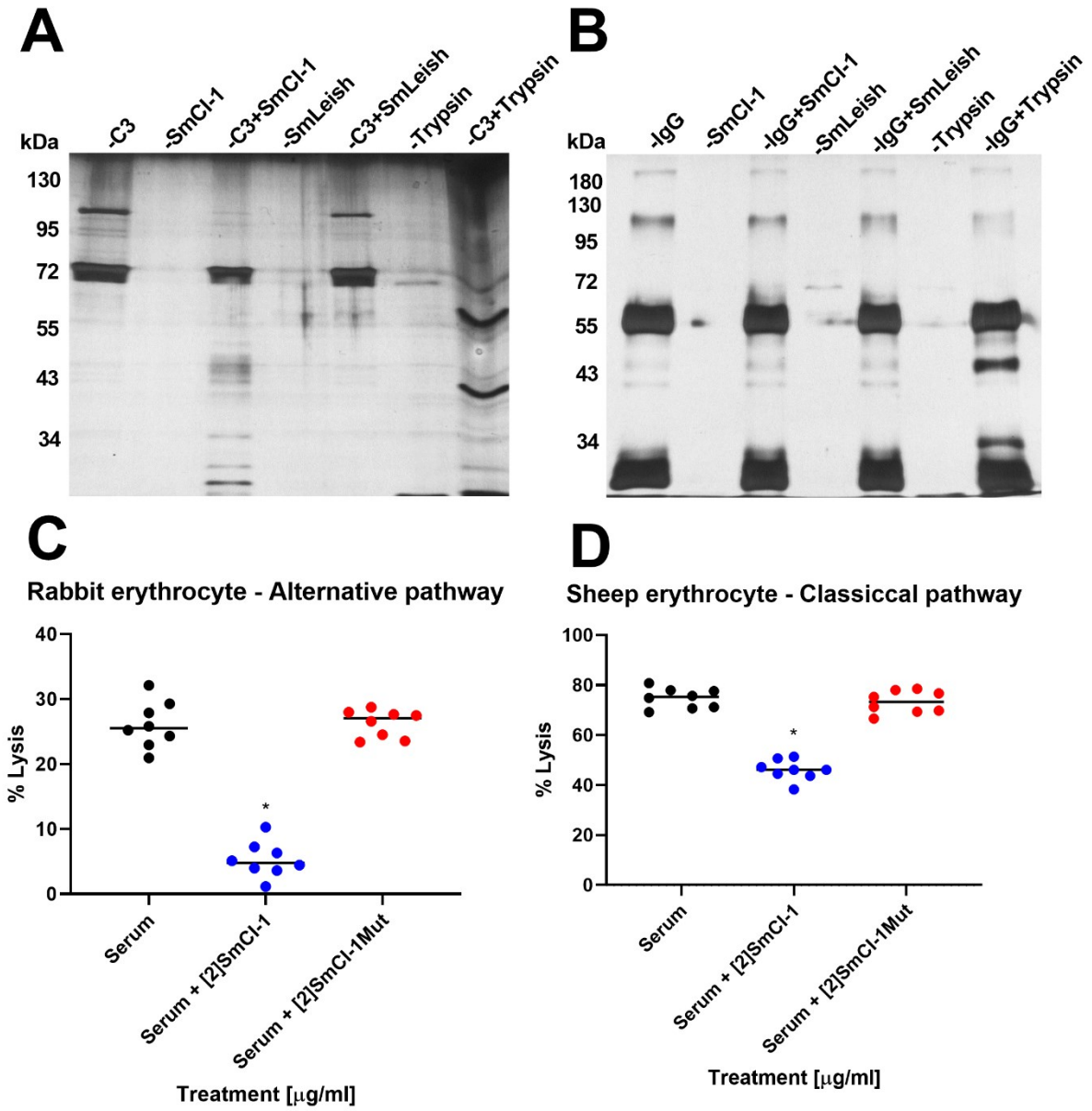



**Figure 5.5. SmCI-1 cleaves complement component C3**

Silver stain of 2µg of (A) human complement component C3 loaded into the lanes of a reducing SDS-Page Gel. Under such conditions, C3 is separated into a C3b band at roughly 110kda, and a C3a band of ~72 kDa. Trypsin treatment resulted in the degradation of both C3b and C3a, while SmCI-1 degrades only C3b into fragments visible between 26 kDa and 55 kDa. SmLeish failed to cleave C3. (B) Silver stain of human IgG separated into heavy (~50 kDa), and light (~25 kDa) chains, with residual complete antibody at higher molecular weights. (C) Rabbit erythrocyte lysis via the alternative complement pathway and (D) Sheep erythrocyte lysis via the classical complement pathway using human serum is decreased by addition of rSmCI-1, but not rSmCI-1Mut. Significant differences from untreated cells indicated by (*). (n=8)

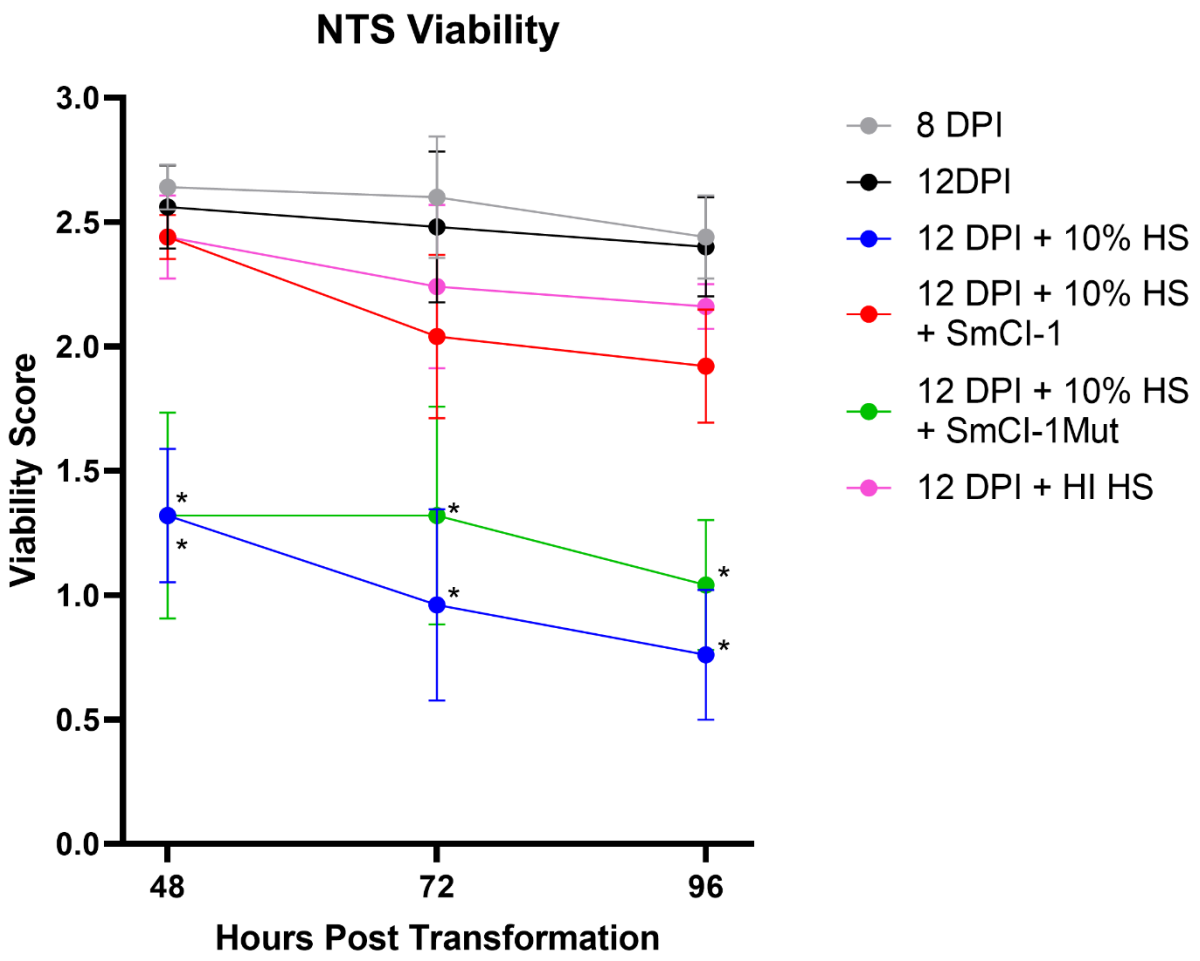



**Figure 5.6. SmCI-1 KD schistosomula are more susceptible to plasma mediated killing**
Viability scores of control (8dpi) and SmCI-1 KD (12dpi) schistosomula at 2,3 and 4 days post transformation. Newly transformed schistosomula were treated with human serum (HS), and either the wild type rSmCI-1, or the catalytically inactive mutant rSmCI-1Mut. Parasites were alternatively treated with heat inactivated human serum (HI HS). (*) indicates where treatments differed significantly from SmCI-1 KD schistosomula exposed to 10% human serum and wild type rSmCI-1. (n=5)

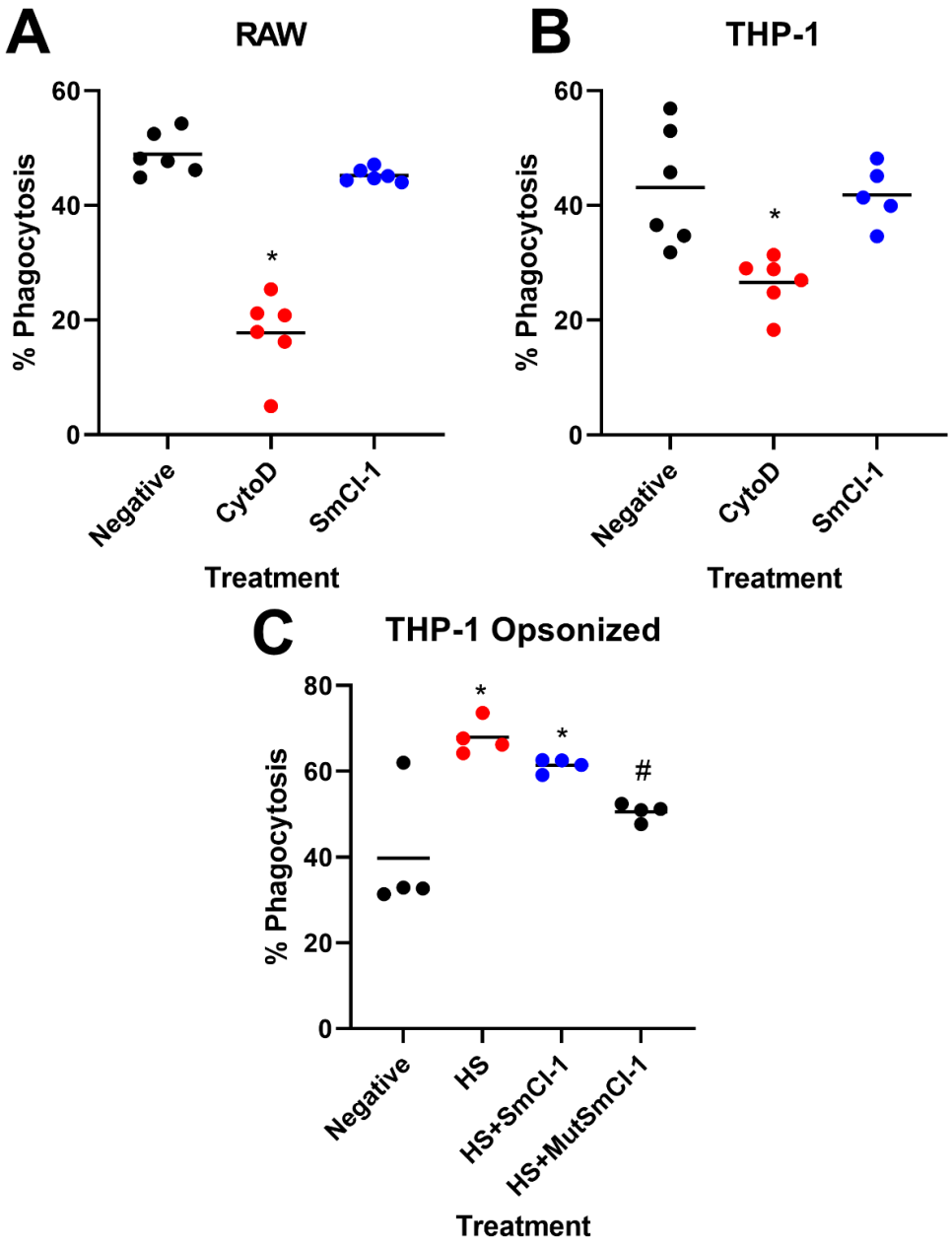



**Figure 5.7. SmCI-1 fails to reduce phagocytosis rates in macrophages**

Treatment of RAW 264.7 cells (A), or stimulated THP-1 cells (B) with recombinant SmCI-1 (1µg/ml) fails to result in a significant reduction in phagocytosis rates, unlike treatment with 10µM cytochalasin D, which significantly reduces phagocytosis (n=6). Opsonin mediated phagocytosis in THP-1 cells (C) shows that pre-incubation of human serum with rSmCI-1 does not significantly reduce phagocytosis, while rSmCI-1Mut does. (n=4) (*) indicates significant difference from negative control, while (#) indicates significant reduction from human serum treated samples.

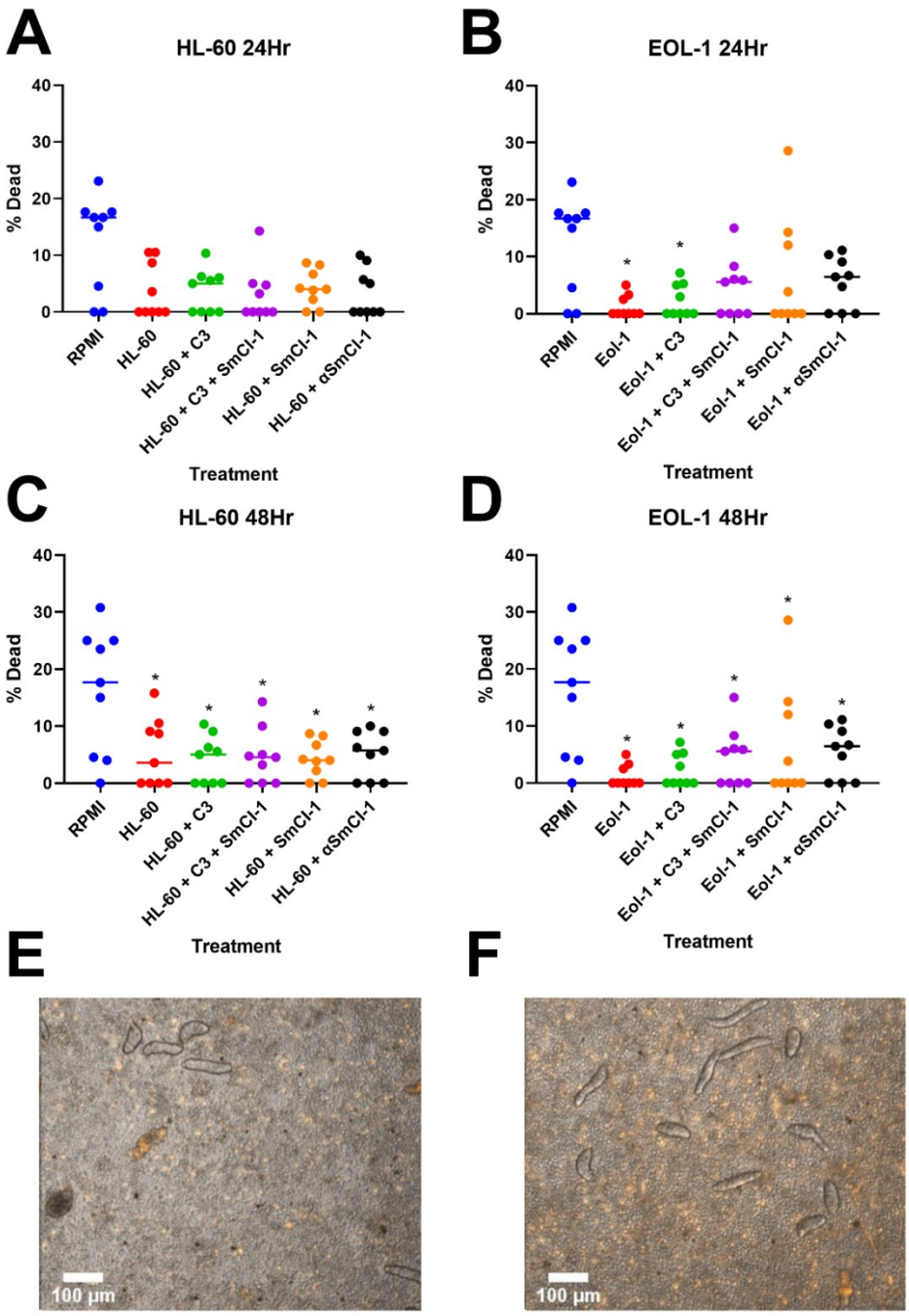



**Figure 5.8. HL-60 and EoL-1 cells fail to significantly kill schistosomula**

Death percentages for HL-60 (A,C) and EoL-1 (B,D) treated schistosomula at 24 and 48 hours post exposure. Cells were untreated, or treated with combinations of recombinant human C3 (45µg/ml) , rSmCI-1 (1µg/ml), or a 1:10 dilution of rabbit anti-SmCI-1antibody. Propidium iodide correctly distinguishes between live and dead schistosomula exposed to (E) HL-60 and (F) EoL-1 cells after 48 hours. Significant decrease from RPMI-only treated controls is indicated as follows (*).

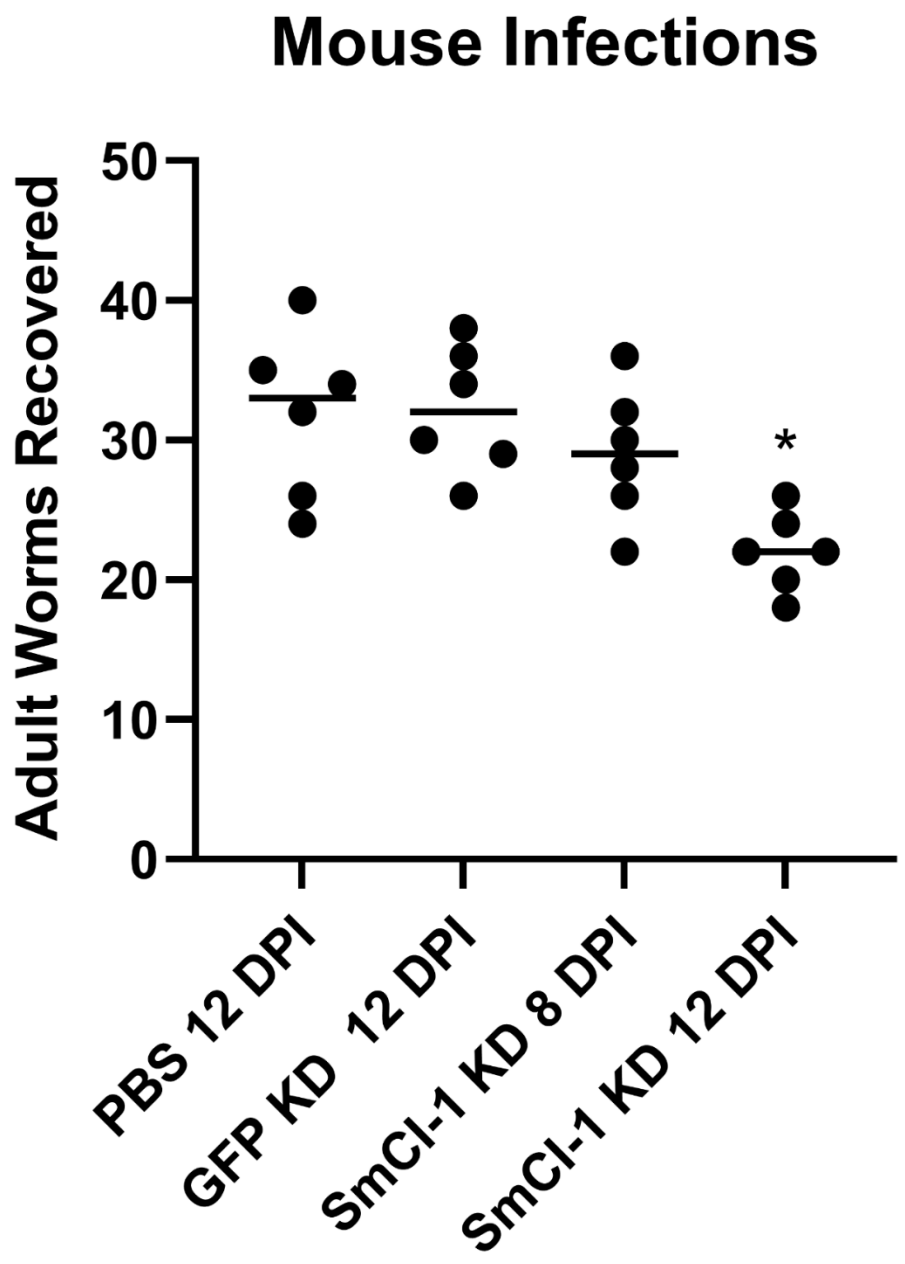
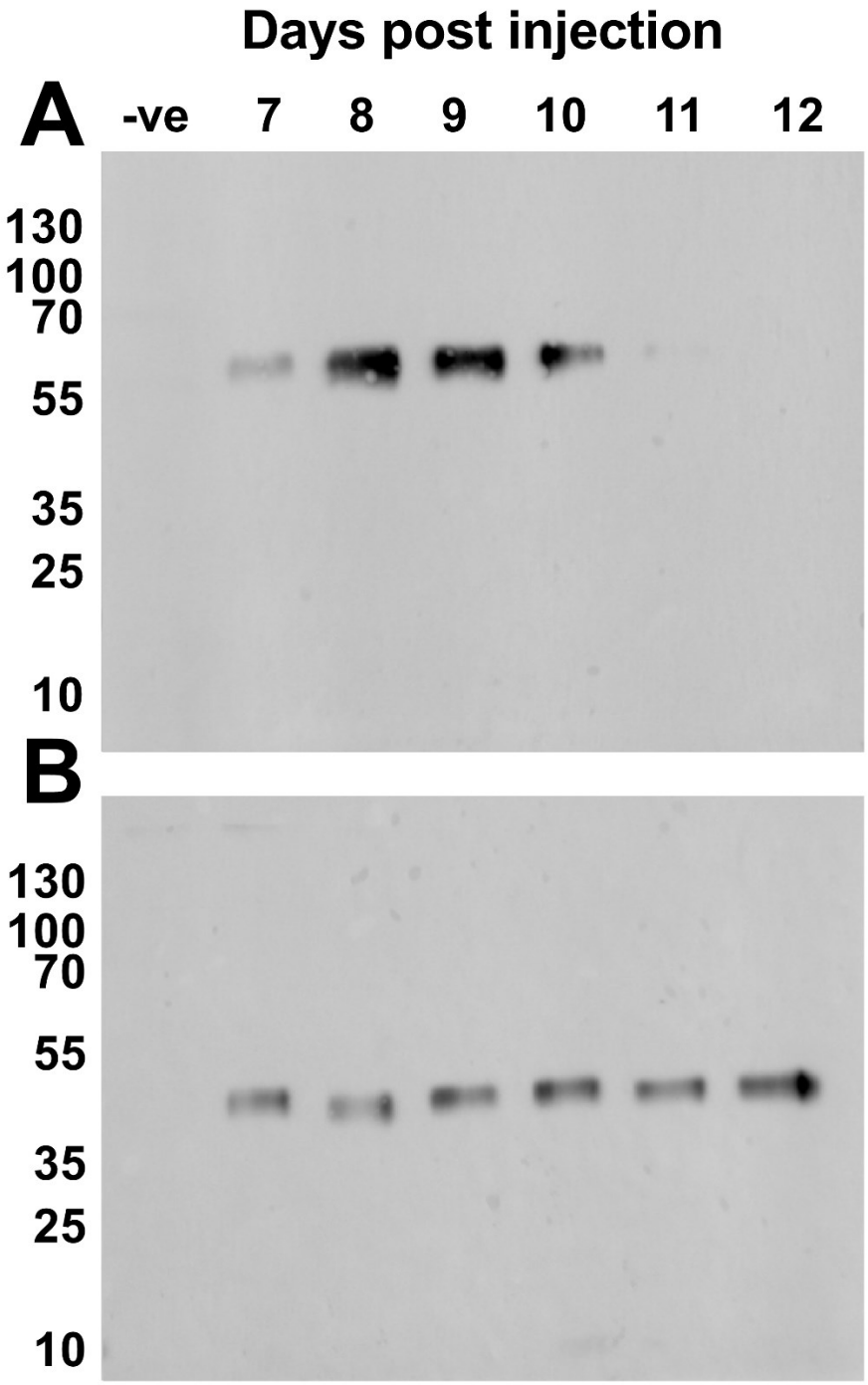


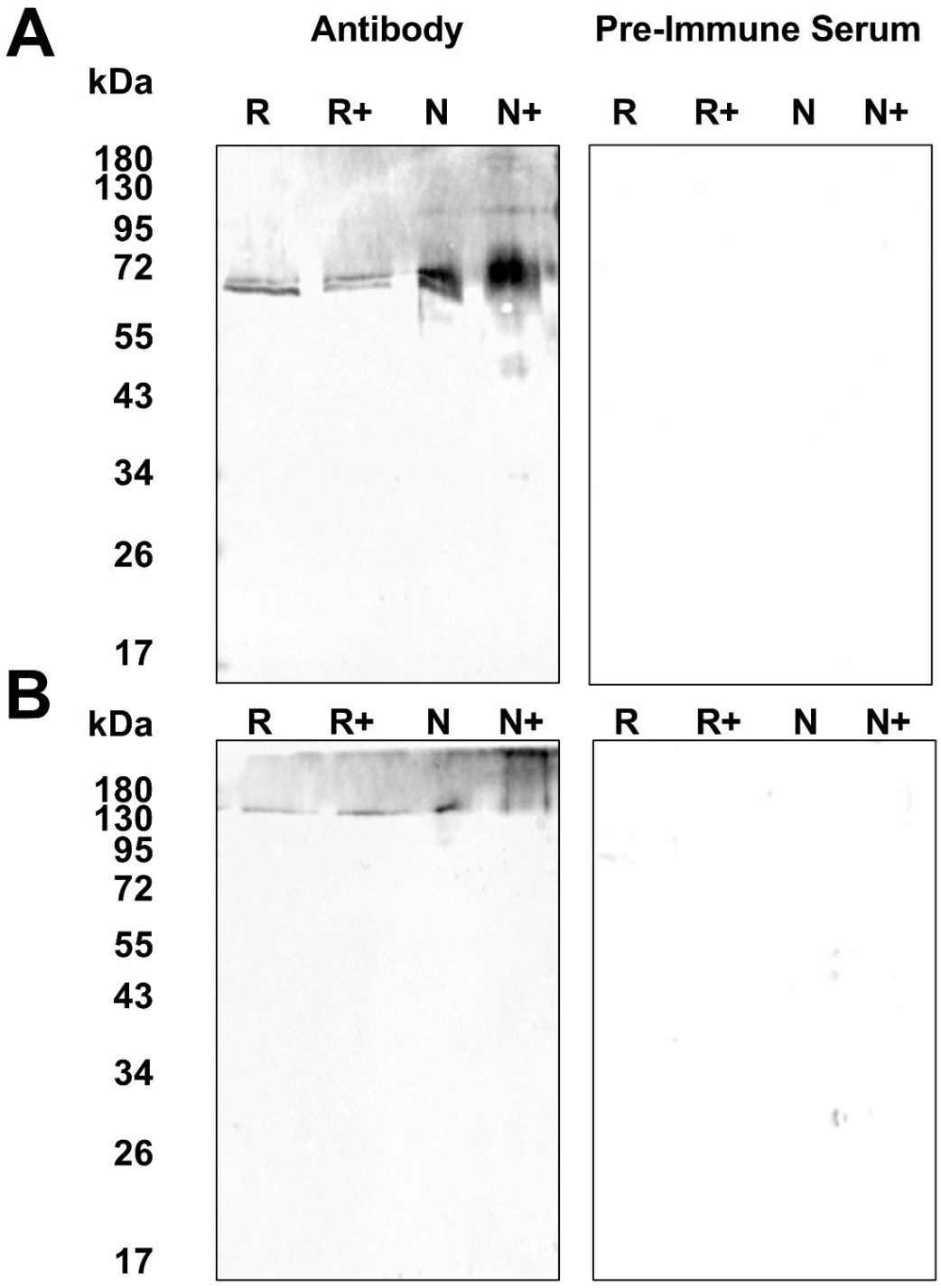

**Figure 5.9. SmCI-1 KD reduces adult worm burden in mouse model**

Infection of Swiss Webster mice using 100 *S. mansoni* cercaria results in different adult worm burdens 5 weeks post infection depending upon cercaria treatment. Cercaria emerged from *B. glabrata* 12 days post injection (DPI) with a PBS control, a GFP KD dsRNA construct, a SmCI-1 KD dsRNA construct, or 8 days post injection with a SmCI-1 KD dsRNA construct. (*) Indicates a significantly lowered adult worm burden as compared to PBS 12 dpi and GFR KD 12 dpi groups. (n=6)



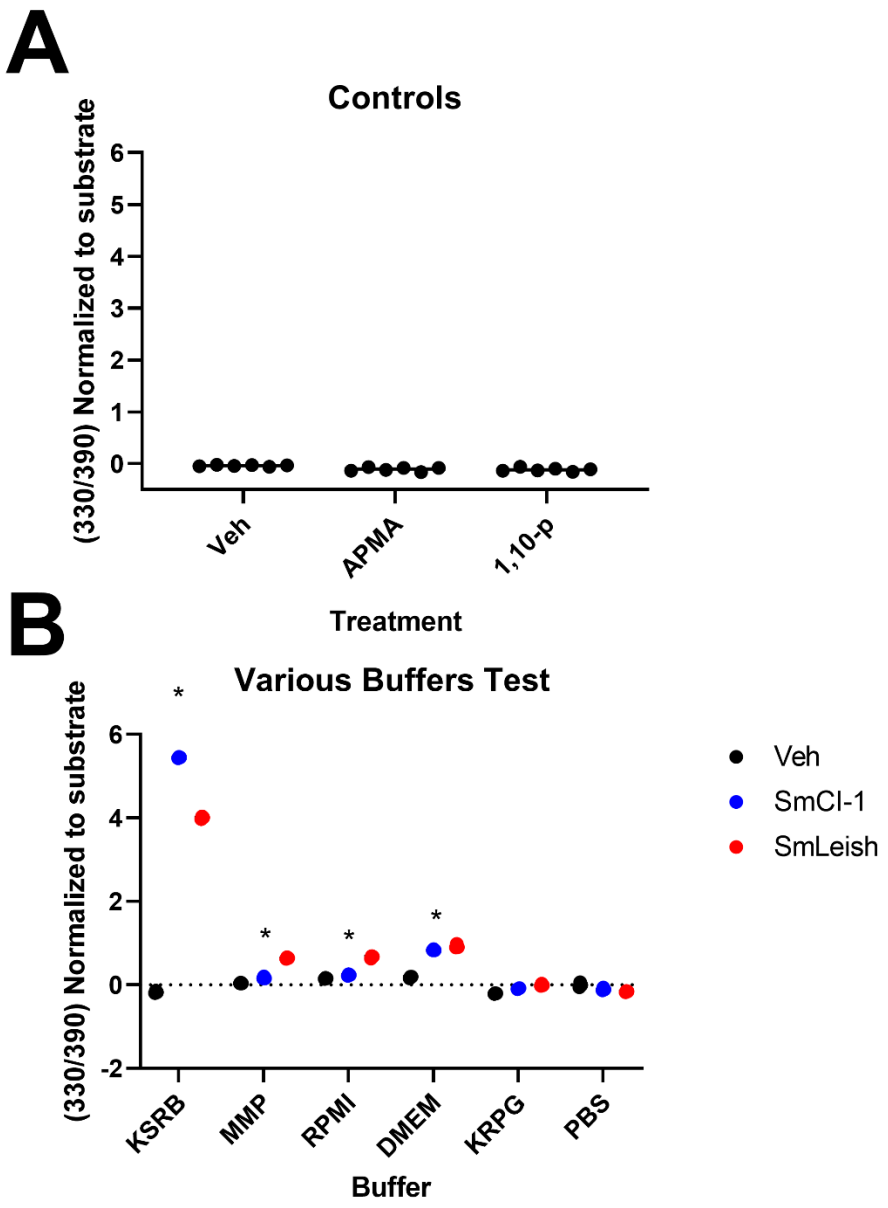

**SFigure 5.10. dsRNA successfully knocks down production of SmCI-1**

Western blot using (A) α-SmCI-1 antibody or (B) anti-β-actin antibody on samples of 20 cercaria at varying timepoints post dsRNA construct injection. At 11 and 12 days post injection, SmCI-1 levels are significantly reduced as compared to 7-10 days post injection.



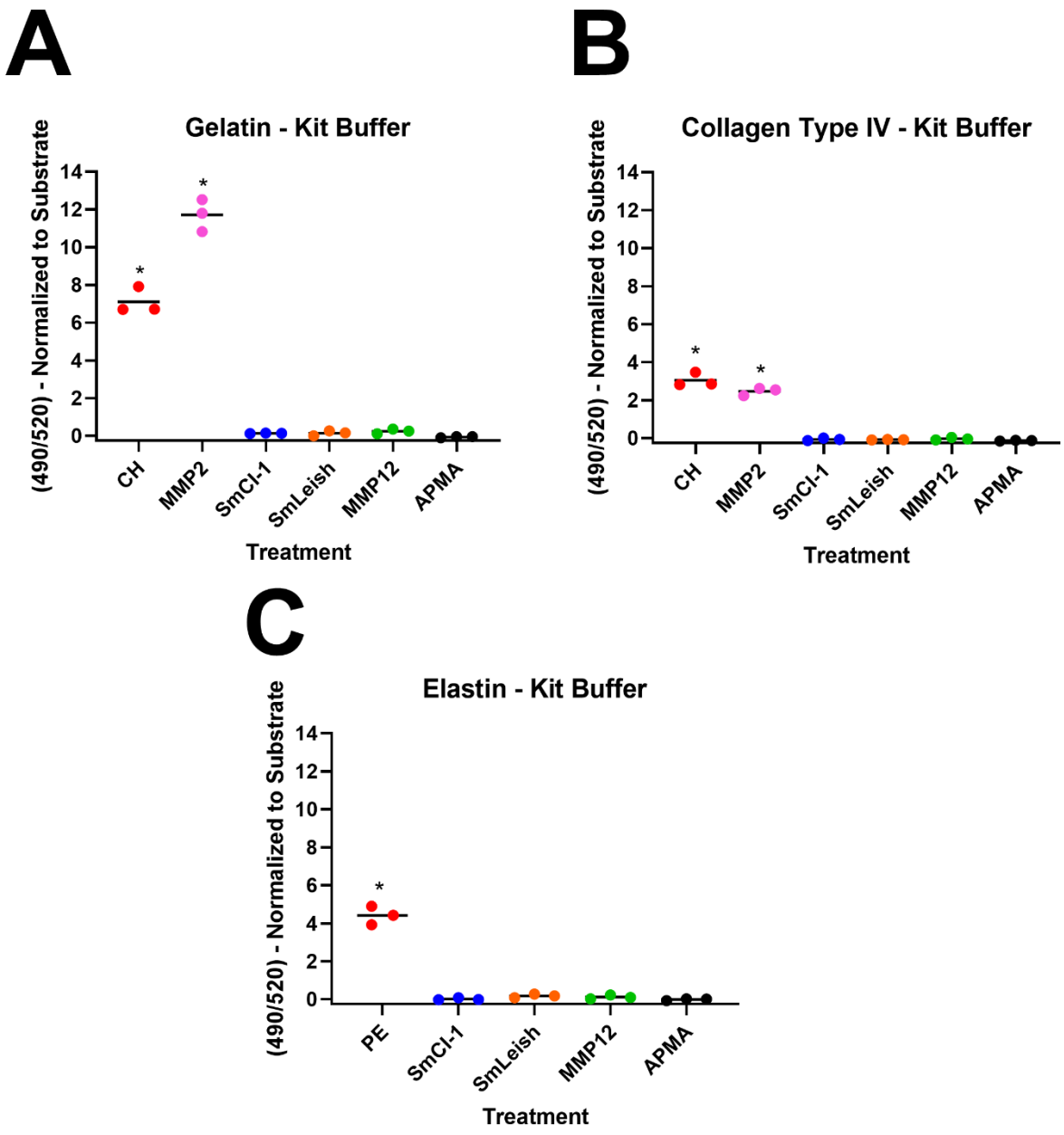

**SFigure 5.11. Validation of polyclonal anti-invadolysin antibodies**

In the case of both (A) SmCI-1, and (B) SmLeish, our polyclonal antibodies recognized proteins of the expected molecular weight in samples containing approximately 15 cercaria under both reducing (R) and native (N) conditions, both with (+) and without boiling at 95°C. Pre-immune serum from inoculated rabbits failed to recognize the invadolysins.

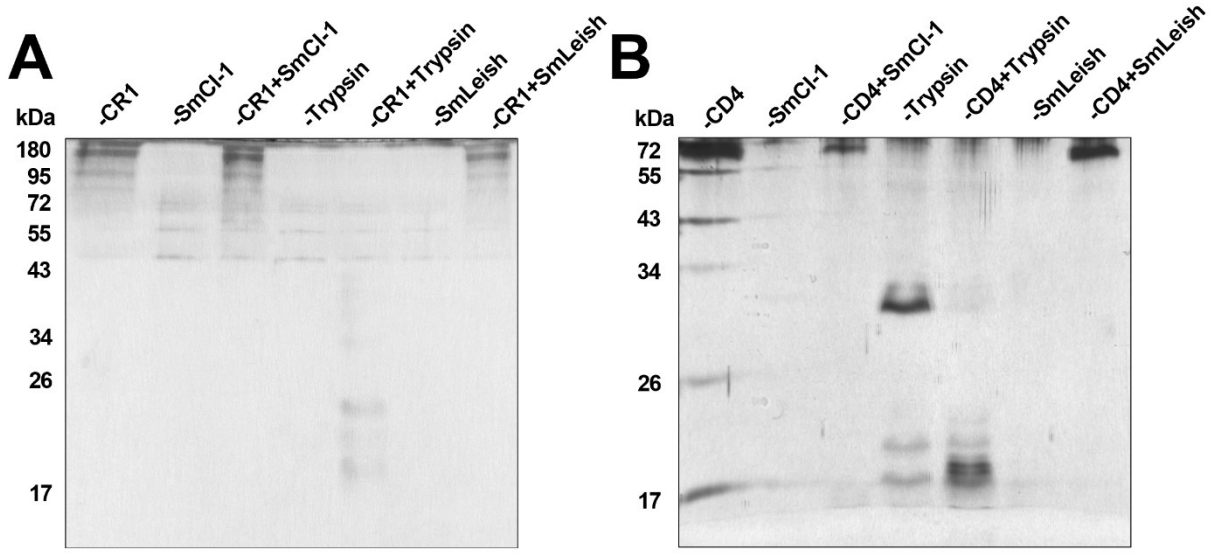



**SFigure 5.12. SmCI-1 and SmLeish are active in various buffers**

Vehicle, APMA, and 1,10-phenanthroline controls for generic MMP assay fail to vary significantly from substrate controls (A). SmCI-1 and SmLeish activity as measured using a generic fluorometric MMP assay in a variety of buffers (B). The highest activity is seen in the Sensolyte MMP assay provided buffer. Activity is also seen in our lab made generic MMP buffer (50mM Tris, 10mM $CaCl_2$, 150mM NaCl, pH 7.5), as well as RPMI and DMEM. Activity is not seen in Krebs-Ringer Phosphate Buffer (KRPG) (145mM NaCl, 6mM $Na_3PO_4$, 5mM KCl, 0.5mM $CaCl_2$, 1mM $MgSO_4$, pH 7.4) or PBS**.** Statistically significant differences from vehicle only controls indicated using (*).

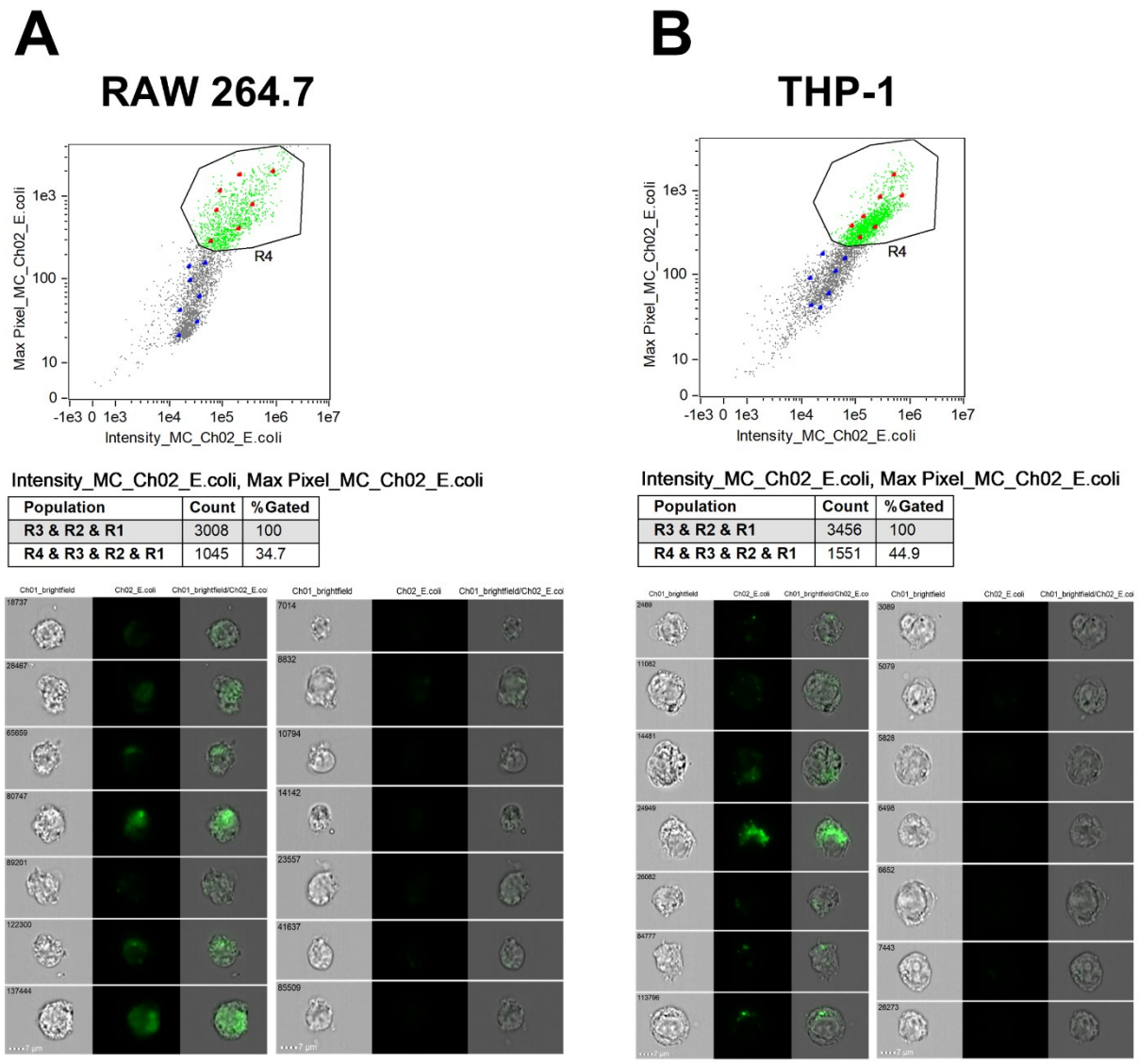



**SFigure 5.13. – Structural kit buffers lack necessary factors for invadolysin activity**
Assessment of the activity of 2µg/ml recombinant SmCI-1 and SmLeish, as well as relevant positive controls for Gelatin (A), Collagen Type IV (B), and Elastin (C) kits using the buffer provided for that specific cleavage assay. Positive controls exhibit activity whereas invadolysins do not. Differences from the APMA vehicle control are denoted using (*). (n=3)




**SFigure 5.14. – SmCI-1 and SmLeish fail to cleave key cell-surface molecules**

SmCI-1 and SmLeish fail to cleave CR1 (A), or CD4 (B). Trypsin does cleave these molecules, as evidenced by the appearance of novel bands such as the numerous bands located between 26 and 24kda when it is added to CR1, and the ~20kda bands seen when trypsin is added to CD4.




Intensity_MC_Ch02_E.coli, Max Pixel_MC_Ch02_E.coli

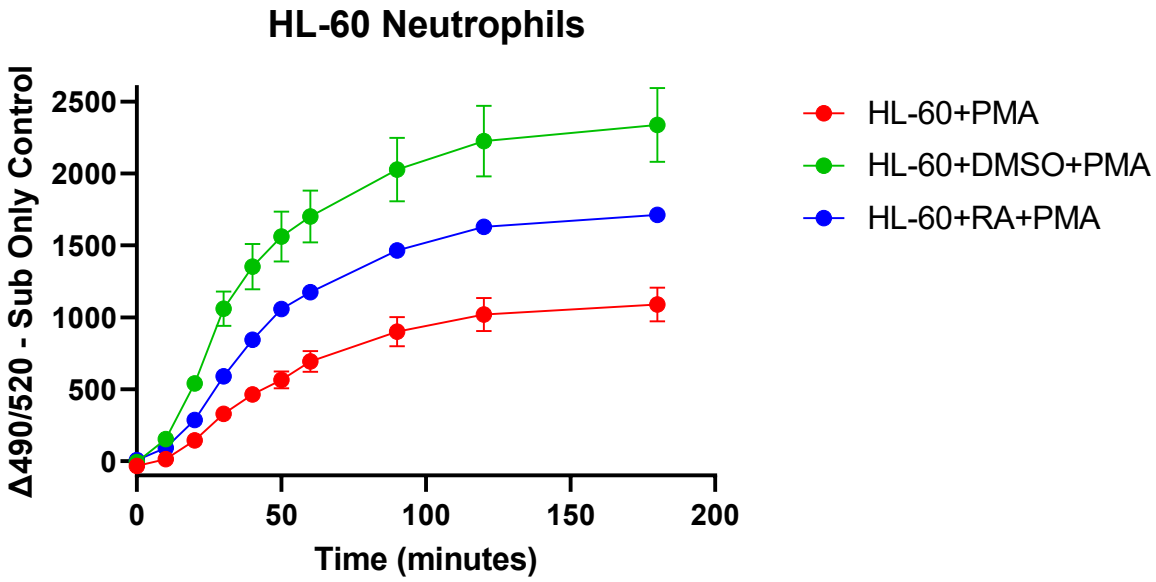
| Population | Count | %Gated |
|---|---|---|
| R3 & R2 & R1 | 3008 | 100 |
| R4 & R3 & R2 & R1 | 1045 | 34.7 |

Intensity_MC_Ch02_E.coli, Max Pixel_MC_Ch02_E.coli

| Population | Count | %Gated |
|---|---|---|
| R3 & R2 & R1 | 3456 | 100 |
| R4 & R3 & R2 & R1 | 1551 | 44.9 |

**SFigure 5.15. IDEAS software correctly distinguishes between phagocytic events and non-events**

Sample populations of both RAW 264.7 macrophages (A) and stimulated THP-1 macrophage-like cells (B) phagocytize pHrodo Green *E. coli* Bioparticles. IDEAS software correctly differentiates between those cells having taken up the fluorescent particles, and those that have not when a representative sample of cells are randomly chosen**.**




**SFigure 5.16. Differentiated HL-60 cells produce more reactive oxygen species**

Reactive oxygen species production is measured using a 2,7-Dichlorofluoresceine diacetate assay to assess the success of different differentiation protocols in rendering HL-60 cells capable of producing an ROS burst over 3 hours. Cells were differentiated with either 1μM retinoid acid (RA) or 1.5% DMSO for 4 days .HL-60 cells stimulated with 25 μM PMA at t=0.

# Chapter 6 – The role of key schistosome invadolysins in altering the cytokine output in mammalian immune cells

## 6.1 Preface

Previous research has demonstrated that both SmLeish and SmCI-1 are transcriptionally upregulated in cercaria, which motivated our efforts to examine any possible roles early on during *S. mansoni* infections in mammals. During the transitionary period between studying SmLeish in the context of *B. glabrata* infections and focusing in on SmCI-1 in the context of mammalian skin penetration, we sought to examine the function of both SmLeish and SmCI-1 on mammalian cell cytokine production. To this end, we used mouse polymorphic blood leucocytes exposed to parasite materials and our invadolysins as a hypothesis generating experiment. This was followed by examination of the effects on cytokine production in various human cell lines. Once we had determined that SmLeish was far less likely to be heavily involved in mammalian infections, we focused the remainder of our research on cytokine production in human polymorphic blood mononuclear cells. We also attempted to determine how these effects were being elicited by treating cells with an both active rSmCI-1 and inactive rSmCI-1Mut, as well as examining their effect on different stimulants. The effects we observed on human PBMCs are currently under review for publication, and pending revisions will be accepted. Assays examining cytokine output in mouse PBLs and human cell lines featuring both rSmLeish and rSmCI-1 treatments are presented in this thesis for comparison only.

## 6.2 Abstract

To survive in human skin, *Schistosoma mansoni* must evade an inflammatory response capable of killing other schistosome species. We hypothesized that schistosome invadolysins may be involved in altering cytokine production in mammalian immune cells. We examine the

anti-inflammatory capabilities of both SmLeish and SmCI-1 on mouse polymorphic blood leukocytes and numerous human cell lines and show a broad capacity to downregulate the production of various inflammatory cytokines by both MMPs. Finally, we assess the effect of SmCI-1 on cytokine release from human peripheral blood mononuclear cells, providing compelling evidence that SmCI-1 promotes an anti-inflammatory microenvironment by enhancing production of IL-10 and suppressing the production of inflammatory cytokines like IL-1B and IL-12p70 and those involved in eosinophil recruitment and activation, like eotaxin-1 and IL-5.

### 6.3 Introduction

Schistosomes produce immune evasion factors in order to facilitate infection and survival within a host (95). The specificity of these immune evasion factors, as well as that of the host immune system is one of the reasons that most schistosome species display a high degree of host specificity for their invertebrate and human hosts (99,426,427). The larval parasite's capacity to survive in the skin involves these immunosuppression mechanisms. While human schistosomes can cause inflammation and cercarial dermatitis, they fail to do so as frequently as other species. The predominant cause of cercarial dermatitis (also known as swimmer's itch) are avian schistosomes, which are typically attacked by an inflammatory response and killed in human skin (428,429). This suggests the production of factors by human schistosomes such as *S. mansoni* that function in reducing inflammation while the larval parasite migrates through the skin in search of a dermal venule.

Upon penetration into the skin, schistosome migration through both the dermis and epidermis elicits the production of numerous pro-inflammatory cytokines. Whole skin cytokine analysis has previously revealed the upregulation of inflammatory factors such as IL-6, MIP-1α,

IL-1β, and TNF-α in human skin samples and IL-6, MIP-1α, MIP-1β, and IL-1β in mouse skin samples (166,430,431). Also accompanying the release of such factors is the upregulation in anti-inflammatory factors such as IL-10 and IL-1RA, the production of which can be upregulated in keratinocytes by cercarial E/S products (166,184,430,432). A few parasite-derived factors have been implicated in facilitating the up and down regulation of select cytokines. The TLR3- and TLR4-signal altering protein Sm16 is released from the cercarial acetabular glands, and has been shown to reduce IL-1α production by keratinocytes, as well as IL-12 and IFN-γ from macrophages (183–186). Additionally, *S. mansoni* produces endogenous prostaglandin E2 ($PGE_2$), as well as releasing a factor less than 30kda in size that causes both $PGE_2$ and $PGE_2$-induced IL-10 production in keratinocytes (88). The inflammatory milieu is also altered by the release of a protein of approximately 23kda in size that induces T cell apoptosis (192). Clearly, the invading cercaria take great strides to produce factors designed to alter cell signalling/function during the initial stages of infection.

Given the high relative abundance of SmCI-1 released from cercaria during penetration into human skin, as well as the upregulation of SmLeish we have previously observed coinciding with the development and emergence of cercaria from *B. glabrata,* we hypothesized that these invadolysins may function to alter the production of cytokines by mammalian immune cells (145,175). Precedent exists for this when examining the function of invadolysins in other parasites. One such example is the function of the most highly abundant invadolysin in *Leishmania major,* GP63 which is capable of altering intracellular signalling pathways by cleaving the myristoylated alanine-rich kinase substrate (MARKS)-related protein (MRP), as well as NF-κB, key intracellular signalling molecules involved in the production of inflammatory responses (318,321,326).

To this end, we examined the effect of rSmLeish and rSmCI-1 on cytokine production in mouse polymorphic blood leukocytes and a variety of human cells lines that had been stimulated with parasite-derived products. We found broad anti-inflammatory activity and the induction of anti-inflammatory cytokines such as IL-10 and IL-1RA. After having determined SmCI-1 to be more likely to be involved in cytokine profile alterations as a result of significantly higher levels than SmLeish in cercaria, we focused on the capacity of SmCI-1 to alter cytokine output of human polymorphic blood mononuclear cells. This assessment identified the ability of SmCI-1 to downregulate key inflammatory cytokines. To follow up on this observation we used a mutant form of SmCI-1 lacking MMP activity to demonstrate that the reduction in inflammatory cytokines required MMP activity, while eliciting IL-10 production did not.

## 6.4 Methods

### 6.4.1 Recombinant invadolysin production and purification

The recombinants used in the following experiments were the same used in chapter 5, and both their method of production in human embryonic kidney (HEK) cells, and purification using polyclonal antibodies can be found in section 3.9.2.

### 6.4.2 Cytokine levels

The effects of rSmLeish and rSmCI-1 on cytokine production for mouse polymorphic blood leukocytes, THP-1s, Jurkats, THP-1s + Jurkats, EoL-1s, and HL-60s was performed using a Proteome Profiler Mouse Cytokine Array Kit (bio-techne/R&D systems) as detailed in section 3.17. The effects of rSmCI-1 and rSmCI-1Mut on human polymorphic blood mononuclear cells was performed using a Proteome Profiler Human Cytokine Array Kit (bio-techne/R&D) as detailed in section 3.18.

### 6.4.3 Invadolysin ELISAs

The relative concentration of SmCI-1 and SmLeish in cercarial E/S products and whole cercarial lysate was determined by ELISA as described in section 3.12.2.

### 6.4.4 Western blot cytokine cleavage assay

Recombinant human eotaxin-1 (R&D systems) and recombinant IL-5 (R&D systems) at an amount of 50 ng/sample were incubated overnight in kit specific reaction buffer with 1µg/ml rSmCI-1, 1 µg/ml rSmCI-1Mut or 1 µg/ml trypsin. Western blotting was performed as detailed above, using a 1:1000 dilution of monoclonal mouse anti-eotaxin-1 or IL-5 (R&D).

### 6.4.5 SmCI-1 internalization immunofluorescence

To help determine whether or not SmCI-1 could be internalized by phagocytic cells, we exposed both THP-1 and RAW 264.7 macrophages to *S. mansoni* E/S products and probed these cells using our α-SmCI-1 antibody, as outlined in section 3.14.3.

## 6.5 Results

### 6.5.1 Initial Mouse PBL and Human Cell Line Cytokine Profiles

Mouse PBLs were highly stimulated to produce a bevy of cytokines in response to parasite derived factors (**Figure 6.1**). Both whole and drained cercarial lysate (WCL and DCL) were significantly more stimulatory than cercarial E/S products for every cytokine measured other than IL-10, which was significantly higher in samples treated with excretory/secretory products. Of the 18 cytokines upregulated by WCL and DCL treatment, addition of either recombinant at both 1 or 2 µg/ml significantly reduced the abundance of 10 of them. Two cytokines, IL-10 and IL-RA, were increased in fluorescence caused by the addition of SmLeish

or SmCI-1. Notable decreases included reductions in both IL-5 and eotaxin-1, which were both more significantly reduced by SmCI-1 treatment than SmLeish treatment. rSmLeish, did, however, reduce CCL3 abundance in WCL and DCL treated PBLs more significantly than rSmCI-1.

T-cell-like Jurkat cells were stimulated by both WCL and DCL to produce a variety of Th1, Th2, and activating cytokines (**Figure 6.2**). While both invadolysins failed to significantly alter the production of IL-2 and IL-4, both significantly reduced IL-12, INF-γ, IL-6, TNF-α, and CCL3 levels. Noteworthy was the fact that both invadolysins increased IL-10 output in both WCL and DCL stimulated cells. In addition, an 11.4±0.2X increase in IL-10-caused fluorescence was seen in Jurkats stimulated with E/S products, which was significantly higher than the 1.0±0.1X increase caused by DCL stimulation.

Differentiated THP-1 cells had four of the inflammatory/activating cytokines on our panel upregulated by WCL and DCL treatment (**Figure 6.3**). All were significantly reduced by either invadolysin. Il-12 was also produced, and fluorescence levels significantly reduced by both SmLeish and SmCI-1 treatment as well. Like Jurkat cells, THP-1 cells were also seen to increase IL-10 production in response to invadolysin treatment. Additionally, this served as another case where E/S products featured significantly higher stimulation of IL-10 production than DCL.

The combination of Jurkat and THP-1 cells produced similar cytokines to the profiles seen for each cell line individually (**Figure 6.3**). New to the combination of these two cell lines,however, was the production of eotaxin-1 and IL-5, both of which were reduced by invadolysin treatment.

Both HL-60 neutrophil-like cells and EoL-1 were not as easily stimulated by either WCL or DCL as other cells lines. For HL-60 cells, both WCL and DCL treatment were able to weakly elicit the production of inflammatory cytokines and several chemokines **(Figure 6.4, Figure 6.5**). Treatment with either rSmLeish or rSmCI-1 was able to significantly reduce the output of all of these cytokines, with the exception of SmLeish failing to significantly downregulate GM-CSF production. Of note was the observation that both IL-23 and IL-27 production were stimulated by the addition of either recombinant. For EoL-1 cells significant reductions in inflammatory cytokines such as IL-1β, ICAM-1, and IL-6 were all observed in invadolysin treated samples, as were chemokines such as IP-10 and CCL3 (**Figure 6.6**). Il-5 and Il-13 production, staples of a Th2 response, were also significantly reduced by both invadolysins.

### 6.5.2 Human PBMCs Cytokine Profiles

SmCI-1 was found to have the capacity to alter the cytokine profiles of human PBMCs, as revealed by cytokine array profiling (**STable 6.1**). Amongst these data lie key observations on the effect of rSmCI-1 on the production of key cytokines, as well as whether MMP activity is required for these effects (**Figure 6.7**). WCL stimulated cells released 1060±220 pg/ml of eotaxin-1 after 24 hours. Treatment with rSmCI-1 significantly reduced the amount of eotaxin-1 to 365±36 pg/ml ($p<0.05$). In the case of IL-5, WCL stimulated cells produced 330±79 pg/ml, while treatment with rSmCI-1 significantly reduced these levels to 83±16 pg/ml ($p<0.01$). IL-1β levels were significantly lowered from 355±42 pg/ml to 104± pg/ml at 24 hours by SmCI-1 addition ($p<0.01$). Another inflammatory cytokine, IL-12 (quantified in this assay via detection of the IL-12p70 subunit), featured reduced levels from 180±34 pg/ml to 77±13 pg/ml at 24 hours when rSmCI-1 was added. Eotaxin-1 (1138±301 pg/ml), IL-5 (332±42 pg/ml), IL-1β (394±102 pg/ml), and IL-12 (189±21pg/ml) levels all failed to differ from the WCL only treated cells in a

significant manner. Despite lower concentrations overall, the same difference for these four cytokines was also observed at the 12-hour timepoints, with wild-type rSmCI-1 reducing their concentrations, and the mutant failing to do so.

We also examined anti-inflammatory molecules such as IL-10. In the case of IL-10 generated by WCL stimulated PMBCs, our treatments failed to differ significantly from each other, although a general trend towards higher IL-10 production was observed when rSmCI-1 was added (780±5 pg/ml vs 1253±348 pg/ml at 12 hours, and 478±17 pg/ml vs 767±329 pg/ml at 24 hours). Regarding PBMCs that were not stimulated with WCLs, but only treated with recombinants, rSmCI-Mut treatment resulted in the upregulation of numerous cytokines, while rSmCI-1 did not (**STable 6.1**). The only cytokine altered by both rSmCI-1 and rSmCI-1Mut was IL-10, the levels of which were raised at 12 hours from 35±9.0 pg/ml to 266±37 pg/ml and 185±37 pg/ml, respectively ($P<0.05$). The same effects were also seen at 24 hours, with unstimulated levels of IL-10 seen at 54±8.8 pg/ml, increasing significantly to 303±68 pg/ml and 697±82 pg/ml via SmCI-1 and SmCI-1Mut treatment, respectively ($p<0.01$).

A similar pattern for cytokine alteration was also seen for pro-inflammatory molecules and IL-10 in the context of LPS stimulated PBMCs (**Figure 6.8**). IL-1β production was significantly reduced 24hrs post stimulation, with LPS treated cells producing 1107±144 pg/ml, while cells treated with LPS and rSmCI-1 produced only 516±42 pg/ml. No significant reduction in IL-1β was seen for LPS+rSmCI-Mut at 24hrs, or via the addition of either recombinant for the 12hr timepoint. LPS treatment resulted in higher IL-1α and TNF-α levels than those produced by cells exposed to WCL, with observed levels 24hr post LPS stimulation of 259 ±35 pg/ml and 14648±1879 pg/ml. Both IL-1α and TNF-α levels were significantly reduced via the addition of functional rSmCI-1 ($p<0.05$), but not rSmCI-Mut. This observation was also seen at 12hr post

stimulation. IL-10 increases were also seen when adding either recombinant to LPS stimulated cells at both 12 and 24 hour timepoints. These increases were all significant, with the exception of LPS + rSmCI-1Mut after 24 hours (p=0.057).

### 6.5.3 SmLeish and SmCI-1 fail to cleave key human cytokines

Given the capacity of rSmCI-1, but not rSmCI-1Mut, to lower eotaxin-1 and IL-5 levels, as well as findings in other systems that metalloproteases can cleave eotaxin-1, we examined whether SmCI-1 is able to cleave eotaxin-1 and IL-5. Neither recombinant cytokine was cleaved by rSmCI-1, rSmCI-1Mut, or rSmLeish (**Figure 6.9**). Trypsin completely degraded eotaxin-1, and partially degraded IL-5.

### 6.5.4 Relative amounts of SmLeish and SmCI-1 in cercaria products

The ELISA based determination of invadolysin abundance in drained cercaria and E/S products revealed that SmCI-1 was present at a concentration of 659.8±0.6 ng/ml in the former, and 203.1±0.6 ng/ml in the latter, for a total of approximately 862.9 ±1.2 ng/ml (**Figure 6.10 A**). For SmLeish, 0.6±0.1 ng/ml was found in both drained cercaria and E/S products, for a total of 1.2±0.2 ng/ml (**Figure 6.10 B**). Given that cercaria were present in samples at a concentration 0.5 cercaria/µl, this translated to roughly 1.73 ng/cercaria for SmCI-1 and 0.001 ng/cercaria for SmLeish.

### 6.5.5 α-SmCI-1 antibody fails to differentiate between E/S exposed and unexposed cells

Both differentiated THP-1 (**Fig 6.11. G**) and RAW 264.7 (**Fig 6.11. H**) macrophages exposed to parasite E/S products, as well as those unexposed to these products **(Fig 6.11. D, E**) featured increased fluorescence signals as compared to secondary antibody only treated cells (**Fig 11. A, B**). For RAW 264.7 cells, permeabilization failed to alter this observation, with both

E/S exposed and unexposed cells featuring increased immunofluorescence as compared to secondary antibody only controls (**Fig 6.11 C,F,I**).

## 6.6 Discussion

In response to *S. mansoni* cercarial penetration of the skin, humans produce a variety of immunostimulatory cytokines with the aim of creating an inflammatory milieu in which invading parasites are destroyed. Even keratinocytes participate in this response by releasing IL-1α and IL-1β in response to schistosome penetration (181). Schistosomes respond to this inflammation by releasing numerous anti-immune factors (95). These factors range in form and function, from increasing anti-inflammatory molecules such as IL-10 and reducing production of inflammatory cytokines such as IL-1α and IL-1β, to directly affecting cell function by reducing neutrophil migration, facilitating T cell apoptosis and reducing migration of Langerhans cells to nearby lymph nodes (183,184,186,189,192,194,433).

Our initial hypothesis generating experiment sought to examine the effect of both rSmLeish and rSmCI-1 on mouse PBL cytokine expression in response to parasite-derived factors. This gave us several observations to consider, the first of which is that WCL and DCL are far more immunostimulatory than *S. mansoni* E/S products, which seemingly function predominantly in the elicitation of IL-10 production. This was not unexpected, given that cercarial E/S products, which heavily feature acetabular gland contents, are known to contain numerous anti-inflammatory properties (6,95,175,184). Interestingly, both invadolysins contributed to the significant reduction in several key cytokines such as IL-5, IL-12, IL-1β, and TNF-α, while also reducing levels of important chemokines such as eotaxin-1, CCL3, and CCL4. Reductions in such signalling molecules suggest an involvement by these invadolysins in

creating an environment with reduced inflammation and lowered recruitment of effector cells. Also noteworthy was that both invadolysins also elicited an increased production of anti-inflammatory factors such as IL-10 and the IL-1 receptor antagonist.

Following the initial work on mouse PBLs, we conducted similar studies in human cell lines. This helped clarify several things, primarily that the reduction in cytokine production was not seemingly caused by direct targeting of one cell type. In each of the cell lines examined, both rSmLeish and rSmCI-1 significantly downregulated the production of inflammatory factors and chemokines. The examination of THP-1 cells and Jurkat cells separately suggest edmonocytes/macrophages and T cells as likely sources of increased IL-10 output in response to the invadolysins, which could be elicited without the presence of other cell types. We also combined Jurkat and THP-1 cells, given that this combination has been previously studied in the context of schistosomiasis to help determine the effect of omega-1 on cytokine production in response to schistosome egg antigens (266). While the combination of THP-1 and Jurkat cells elicited similar cytokines in similar amounts to each cell line on its own, they also produced IL-5 and eotaxin-1, unlike their unpaired counterparts. In this case, both invadolysins were still found to reduce the output of these two cytokines. Finally, we examined granulocyte-like cell production of cytokines by using HL-60 cells as a neutrophil-like model, and EoL-1 cells as an eosinophil-like model. Both of these cells lines have been previously employed as model cell lines for cytokine release in response to varying stimuli (434–436).

Cytokine profiling following treatment of human PBMCs with recombinant SmCI-1 revealed the alteration of numerous cytokines important for an immune response in the skin. Firstly, a reduction in IL-1β and IL-12 was observed following addition of SmCI-1, suggesting a capacity to reduce inflammation at the site of infection. Additionally, we also saw changes in

cytokines that have not been previously examined in the skin, with SmCI-1 significantly reducing eotaxin-1 and IL-5 abundance compared to controls. Given the importance of eotaxin-1 in recruiting eosinophils to the site of infection, the function of IL-5 to activate eosinophils, and the capacity of eosinophils to kill schistosomulae *in vitro*, the downregulation of these cytokines by SmCI-1 likely assists in avoidance of eosinophil mediated destruction (155,437–439). Both HL-60 and EoL-1 cells were less responsive to WCL and DCL stimulation than other cells, while the cytokines they did produce were still reduced significantly by invadolysin addition. The lower cytokine stimulation was not unexpected, given the role of neutrophils and eosinophils as effector cells.

We then examined the effect of rSmCI-1 and rSmCI-1Mut on human polymorphic blood mononuclear cells. The decision to further pursue SmCI-1's affect on human PBMCs, but not SmLeish's, was predicated upon the results of our ELISA showing that SmLeish is present at concentrations several orders of magnitude lower than SmCI-1. This is consistent with previous work that failed to identify SmLeish as part of acetabular gland contents, as well as transcriptomics data suggesting that SmCI-1 transcripts in developing cercaria far exceed those of SmLeish (175,203). While SmLeish seemingly has a very similar capacity to that of SmCI-1 in reducing inflammatory cytokine production and increase IL-10 abundance, it is unlikely to be present in such an amount as to be a key driver of this process during natural infections.

Of key interest is the fact that IL-1β, IL-12, eotaxin-1 and IL-5 were all significantly reduced by the MMP-active form of SmCI-1, but not SmCI-1Mut. In addition to the reduction of proinflammatory cytokines, we also saw an increase in anti-inflammatory cytokines such as IL-10. Significant increases for both wild type and mutant SmCI-1 treated cells were seen at both 12 and 24 hours in unstimulated PBMCs. This suggests that SmCI-1 has the capacity to elicit IL-10

production in a non-MMP activity dependent manner. We did not observe a significant increase in IL-10 post SmCI-1 treatment in WCL stimulated cells, which could reflect the presence of other IL-10-inducing elements overriding the effects of SmCI-1 with respect to IL-10 production, given that levels are significantly higher than what was seen in unstimulated cells. Because our recombinant proteins were produced in HEK cells, it is unlikely that IL-10 production was caused by endotoxin contamination. Despite this fact, we treated PMBCs with pure LPS and did not see Il-10 production as high as that elicited by SmCI-1 treatment (**STable 6.1**).

On top of treating PBMCs with recombinants or whole cercarial lysate, we also examined rSmCI-1 ability to alter LPS mediated cytokine profiles. In addition to reducing IL-1β, as seen with WCL-mediateds stimulation, we also observed significant reductions in IL-1α and TNF-α. As with WCL treated cells, this reduction in pro-inflammatory cytokines was seen for active rSmCI-1, but not rSmCI-1Mut. The reduction in these three cytokines suggest that SmCI-1 may be enacting its immunomodulatory role via the targeting of inflammatory pathways common to multiple pathogen types. The reduction in IL-1α and TNF-α speak strongly to this possibility, given that they were not as upregulated in WCL-stimulated cells as they were in LPS-stimulated cells, suggesting SmCI-1 can downregulate pro-inflammatory responses that are not strongly upregulated by the schistosome itself.

Comparison of the cytokine data generated in mice PBLs, human cell lines, and human PBMCs is rendered difficult given the quantitative nature of the ELISA-based cytokine array used on human PBMCs as compared to the semi-quantitative spot-blots employed for both the mouse PBLs and human cells lines. Despite these differences in the type of data, it is noteworthy that both measurement systems featured a reduction in IL-12, IL-1β, IL-5, and eotaxin-1 in both

human and mouse PBMCs, suggesting a common function in both mouse and human. This is coupled with the observation that invadolysin treatment increased IL-10 production in both systems as well. There were several cytokines significantly reduced in mouse PBLs but not human PBMCs, such as IFN-γ and IL-13. This could be the result of the mouse fluorescence data lacking a 1:1 equivalence between intensity and cytokine concentration. It could alternatively be differences in the immune response to schistosomes between mice and human, given that the difference in immune responses remains understudied (74,440).

These findings led us to attempt to narrow down possible mechanisms by which SmCI-1 enacts its immunomodulatory properties. The ability of the active recombinant to reduce inflammatory cytokines, while the mutant failed to do so led us to attempt a cleavage assay on two of the cytokines (eotaxin-1 and IL-5), given the precedent that some hookworm metalloproteases cleave eotaxin-1 (413). Unlike in hookworms, SmCI-1 failed to cleave eotaxin-1. IL-5 was similarly unaffected. This finding suggests to us that another mechanism may be responsible for the downregulation of these cytokines other than their direct cleavage.

Alternative possible mechanisms include the cleavage of surface receptors, the targeting of intracellular signalling molecules, or affecting upstream immune stimulating pathways that led to the production of these cytokines. It is possible that SmCI-1 could cleave cell signalling molecules, given the ability of *L. major* GP63 to cleave MRP and NF-κB. To help determine whether or not SmCI-1 could be internalized by a cell, thereby giving it access to such cell signalling molecules, we attempted to do immunofluorescence staining on RAW 264.7 and THP-1 cells exposed to *S. mansoni* E/S products using the α-SmCI-1 antibody. Unfortunately, the antibody seemingly featured cross reactivity with a mammalian cell epitope, which failed to provide us with clarity regarding SmCI-1 internalization. Recently, use of an amine reactive

tracer (CFDA-SE) demonstrated that mouse macrophages, DCs, and neutrophils are all capable of internalizing schistosome acetabular gland products *in vivo* (182). While this tracer is not specific and does not definitively show the entry of SmCI-1 into host cells, it does suggest that E/S products are internalized by leukocytes in the skin, while suggesting that molecules such as SmCI-1 may have access to cell signalling molecules as cleavage targets.

The field of studying the anti-inflammatory properties of helminth antigens is continuously expanding, and seeks to exploit the hygiene/old friends hypothesis in an attempt to re-introduce worm antigens as anti-inflammatory factors for the treatment of numerous diseases (441–444). SmCI-1's ability to reduce LPS mediated inflammation positions it within this growing group of anti-inflammatory worm antigens. While different receptors and signalling pathways could be suggested as possible targets for SmCI-1 interference, the identification of such remains to be performed, and will be a significant focus of our research moving forwards.

Interpretation of the cytokine array results require several caveats. First, while PBMCs do have numerous cell types capable of giving us valuable information, they lack keratinocytes, macrophages, and granulocytes, so the possibility exists that we are not capturing the entirety of the effects of SmCI-1 observed *in vivo*. Second, these assays are performed *in vitro,* with more cells than are expected to encounter an invading schistosome during a natural infection, resulting in higher pg/ml concentrations than might be expected in the context of migration through human skin. Given that other research into the effects of *S. mansoni* immunomodulators found in the skin have employed mouse cells, as well as keratinocyte cell lines, we felt that the use of human PBMCs would allow for a more host-relevant understanding and wider capture of the effects of SmCI-1 on a variety of combined cell types (181,183,184,186). This reduction of inflammatory factors is an intriguing observation given the observation that *S. mansoni* cercaria

often do not elicit substantial cercarial dermatitis, unlike exposures to other mammalian and avian schistosomes (445). I propose that some host specificity of penetration gland proteases such as invadolysins may play a role in this difference in inflammation.

Future work should seek to determine a definitive mechanism by which the MMP activity results in reduction of IL-5, eotaxin-1, IL-1β, IL-12, and TNF-α. Given that the cleavage of pattern recognition receptors (PRRS) or intracellular signalling molecules by the active invadolysin is a likely possibility, the use of N-terminal proteomics may provide insights into which signalling pathways are being altered (446). Additionally, the examination of how the inactive MutSmCI-1 induces IL-10 production should also be elucidated. The mechanism by which this occurs is less clear. Consideration should be given to the possibilities that this occurs via binding to specific PRRs, or that SmCI-1 facilitates the differentiation of macrophages into IL-10 producing subsets (447).

### 6.7 Conclusion

I have demonstrated that both SmLeish and SmCI-1 have the capacity to reduce inflammatory cytokine output and induce the production of IL-10 in mammalian cells. While similar anti-inflammatory properties are observed, SmCI-1 is present in cercaria at a far higher concentration than SmLeish, rendering it a more likely candidate for use by the larval parasite in human skin. I demonstrate that SmCI-1 can alter the immunological environment in human cells by reducing IL-1β, IL-12, IlL5, and eotaxin-1 levels, but only while in possession of MMP activity. It can increase IL-10 levels, and MMP activity is not necessary for this function.

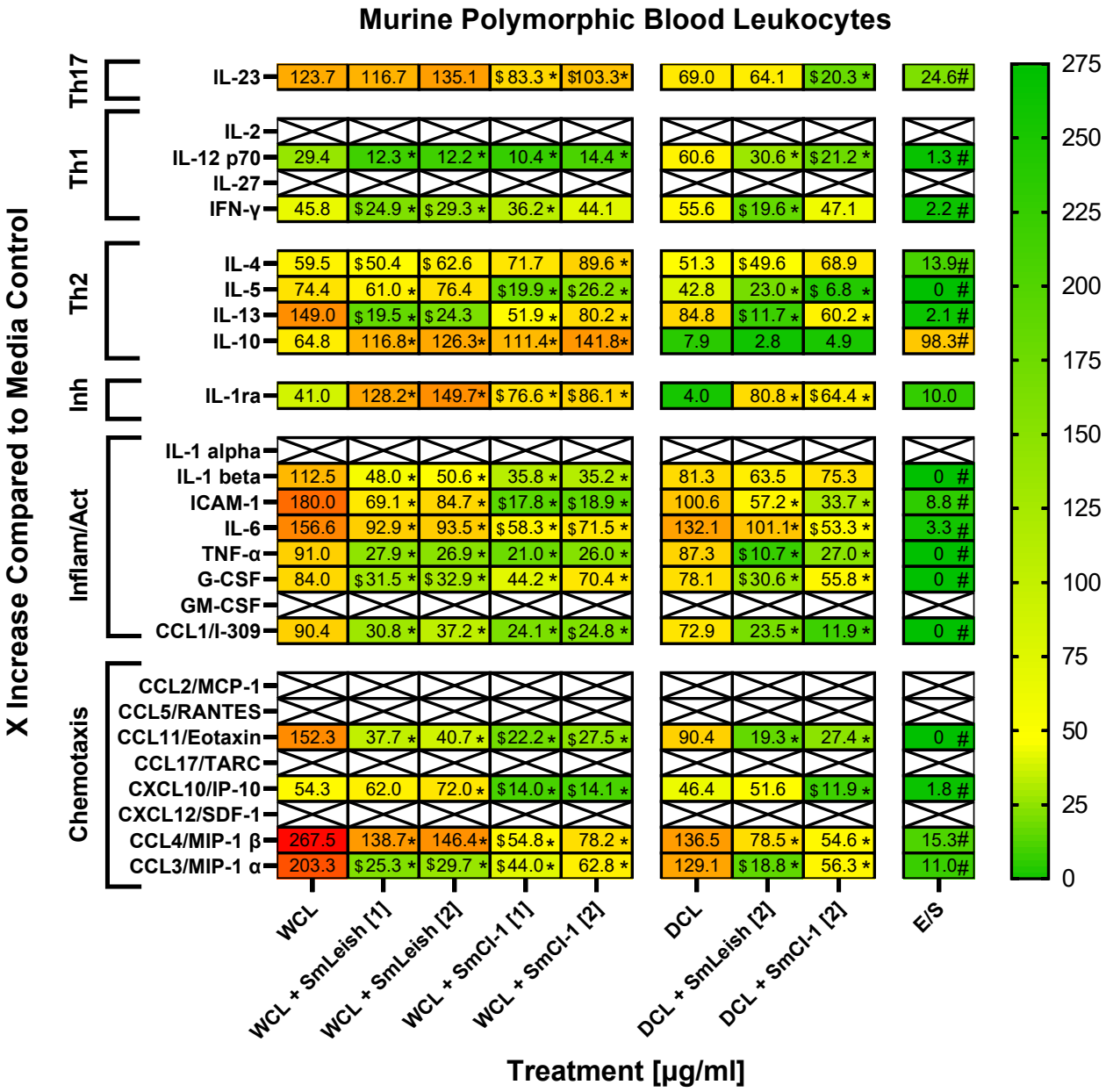



**Figure 6.1. Effects of invadolysins on cytokine production in mouse PBLs**

Cytokines stimulated via the addition of whole cercarial lysate (WCL), drained cercarial lysate (DCL), or excretory/secretory (E/S) products in mouse polymorphic blood leukocytes treated with rSmLeish and rSmCI-1. Values presented as X increase as compared to media-only stimulated controls. (*) Indicates significant difference from associated WCL or DCL only treated samples. (#) Indicates a significant difference between E/S stimulated samples and DCL treated samples. Significant difference between rSmLeish and rSmCI-1 treated samples of the same concentration are denoted by ($). Cytokines stimulated by cell lines, but not mouse PBLs are presented as blank cells.

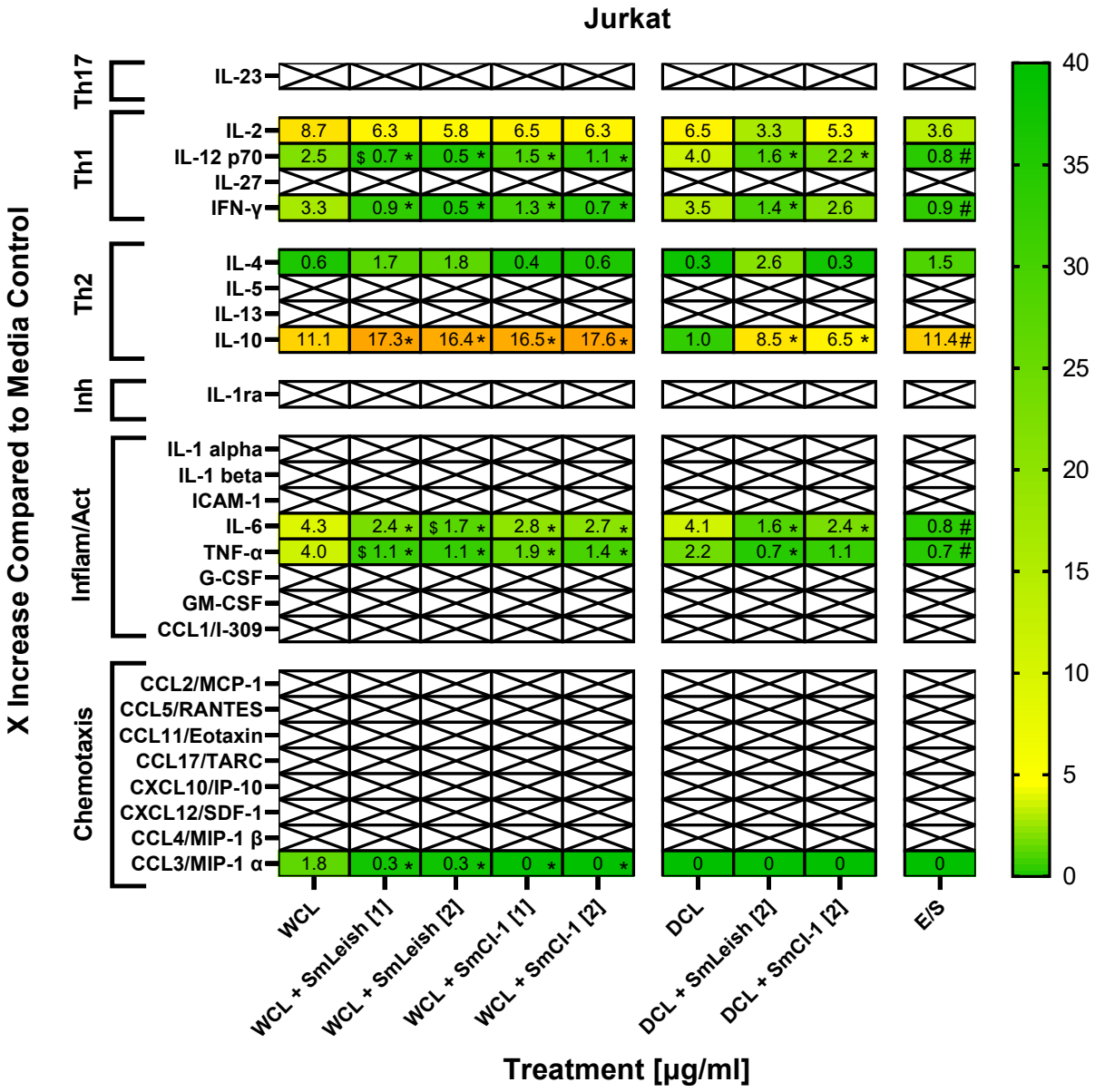



**Figure 6.2. Effects of invadolysins on cytokine production in Jurkat cells**

Cytokines stimulated via the addition of whole cercarial lysate (WCL), drained cercarial lysate (DCL), or excretory/secretory (E/S) products in Jurkat cells treated with rSmLeish and rSmCI-1. Values presented as X increase as compared to media-only stimulated controls. (*) Indicates significant difference from associated WCL or DCL only treated samples. (#) Indicates a significant difference between E/S stimulated samples and DCL treated samples. Significant difference between rSmLeish and rSmCI-1 treated samples of the same concentration are denoted by ($). Cytokines stimulated by murine PBLs or other cell lines, but not Jurkat cells are presented as blank cells.

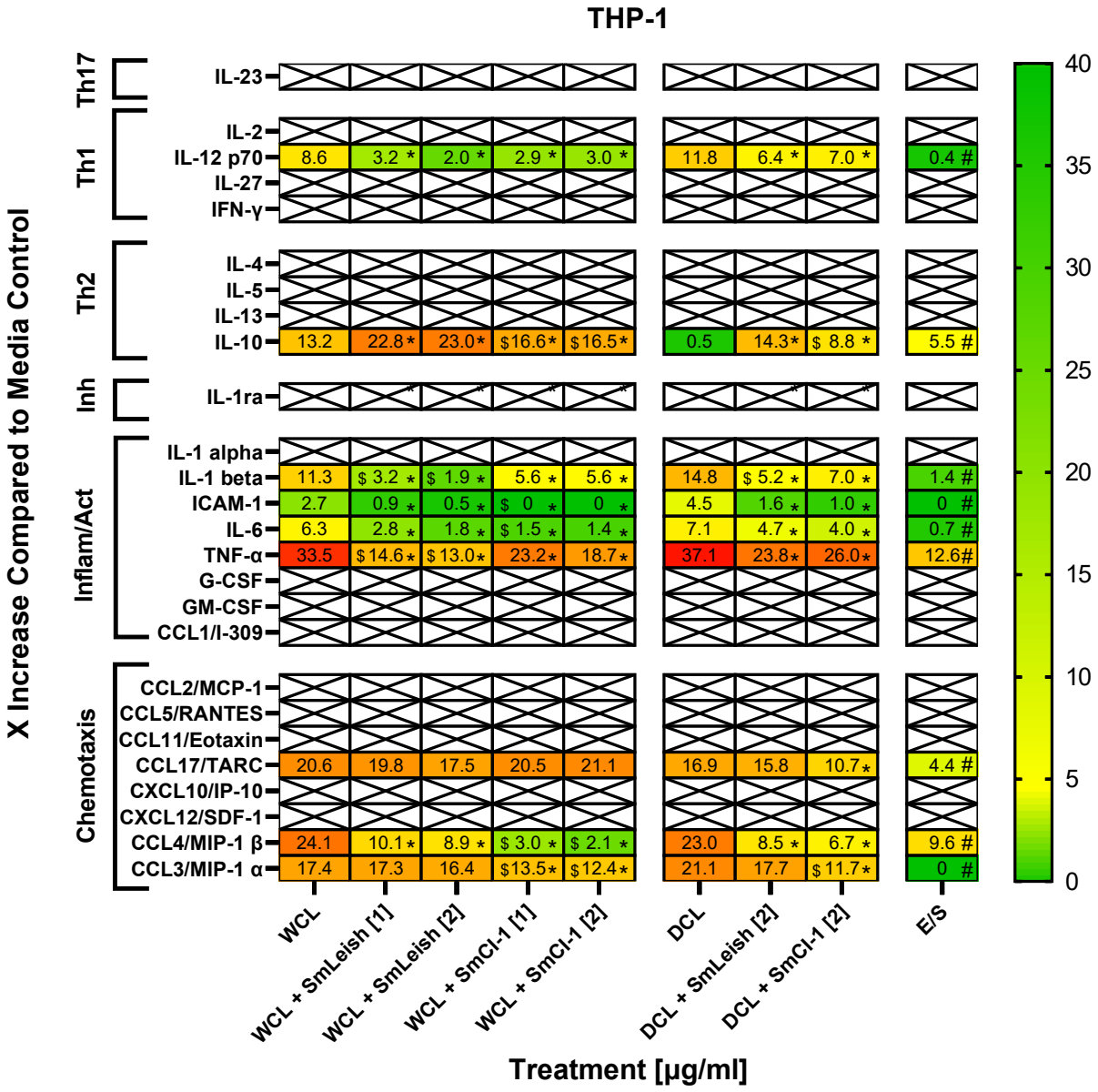



**Figure 6.3. Effects of invadolysins on cytokine production in differentiated THP-1 cells**

Cytokines stimulated via the addition of whole cercarial lysate (WCL), drained cercarial lysate (DCL), or excretory/secretory (E/S) products in differentiated THP-1 cells treated with rSmLeish and rSmCI-1. Values presented as X increased as compared to media-only stimulated controls. (*) Indicates significant difference from associated WCL or DCL only treated samples. (#) Indicates a significant difference between E/S stimulated samples and DCL treated samples. Significant difference between rSmLeish and rSmCI-1 treated samples of the same concentration are denoted by ($). Cytokines stimulated by murine PBLs or other cell lines, but not THP-1 are presented as blank cells.

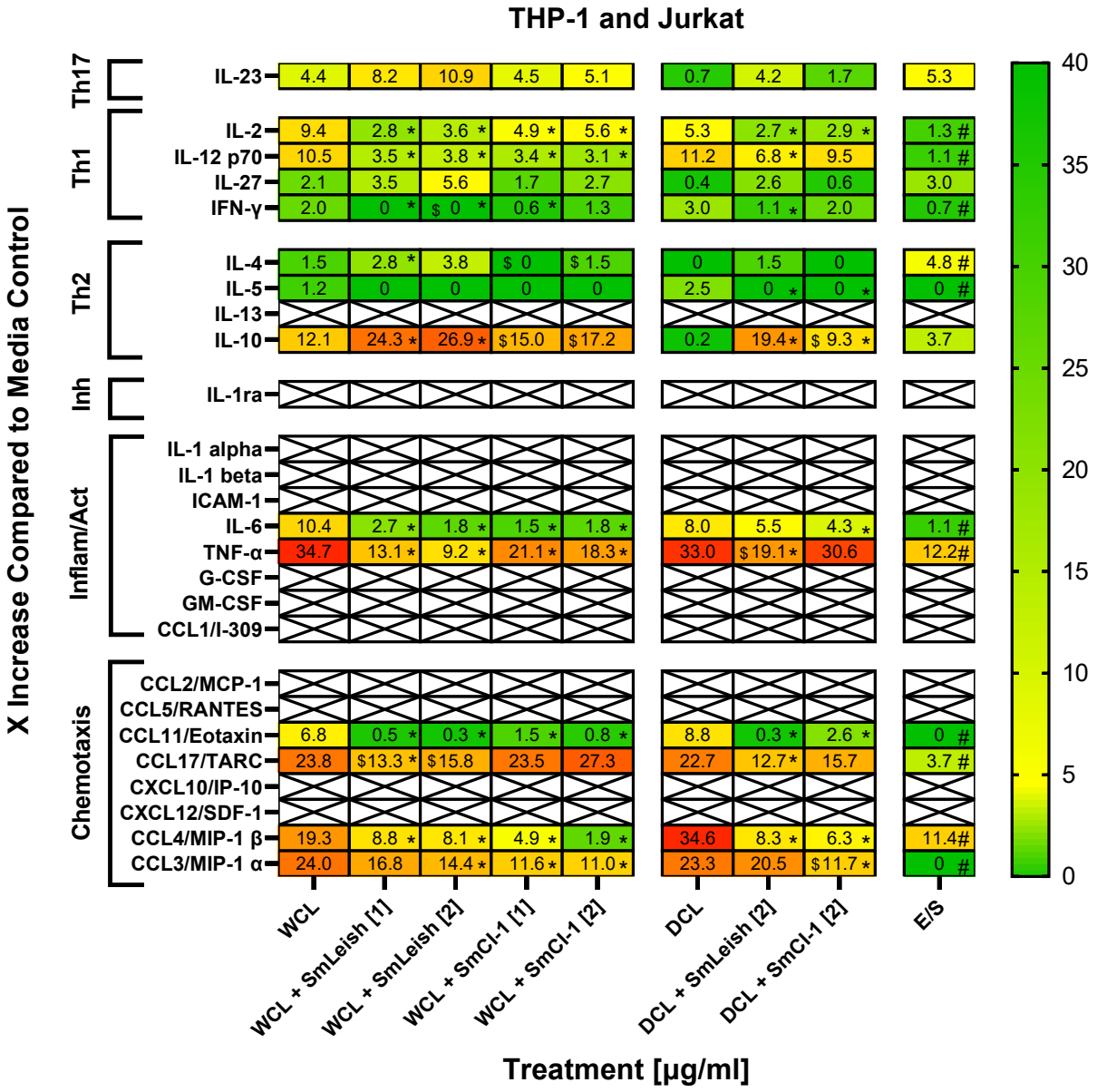



**Figure 6.4. Effects of invadolysins on cytokine production Jurkat + THP-1 cells**

Cytokines stimulated via the addition of whole cercarial lysate (WCL), drained cercarial lysate (DCL), or excretory/secretory (E/S) products in a combination of Jurkat and differentiate THP-1 cells treated with rSmLeish and rSmCI-1. Values presented as X increase as compared to media-only stimulated controls. (*) Indicates significant difference from associated WCL or DCL only treated samples. (#) Indicates a significant difference between E/S stimulated samples and DCL treated samples. Significant difference between rSmLeish and rSmCI-1 treated samples of the same concentration are denoted by ($). Cytokines stimulated by murine PBLs or other cell lines, but not combined THP-1s and Jurkats are presented as blank cells.

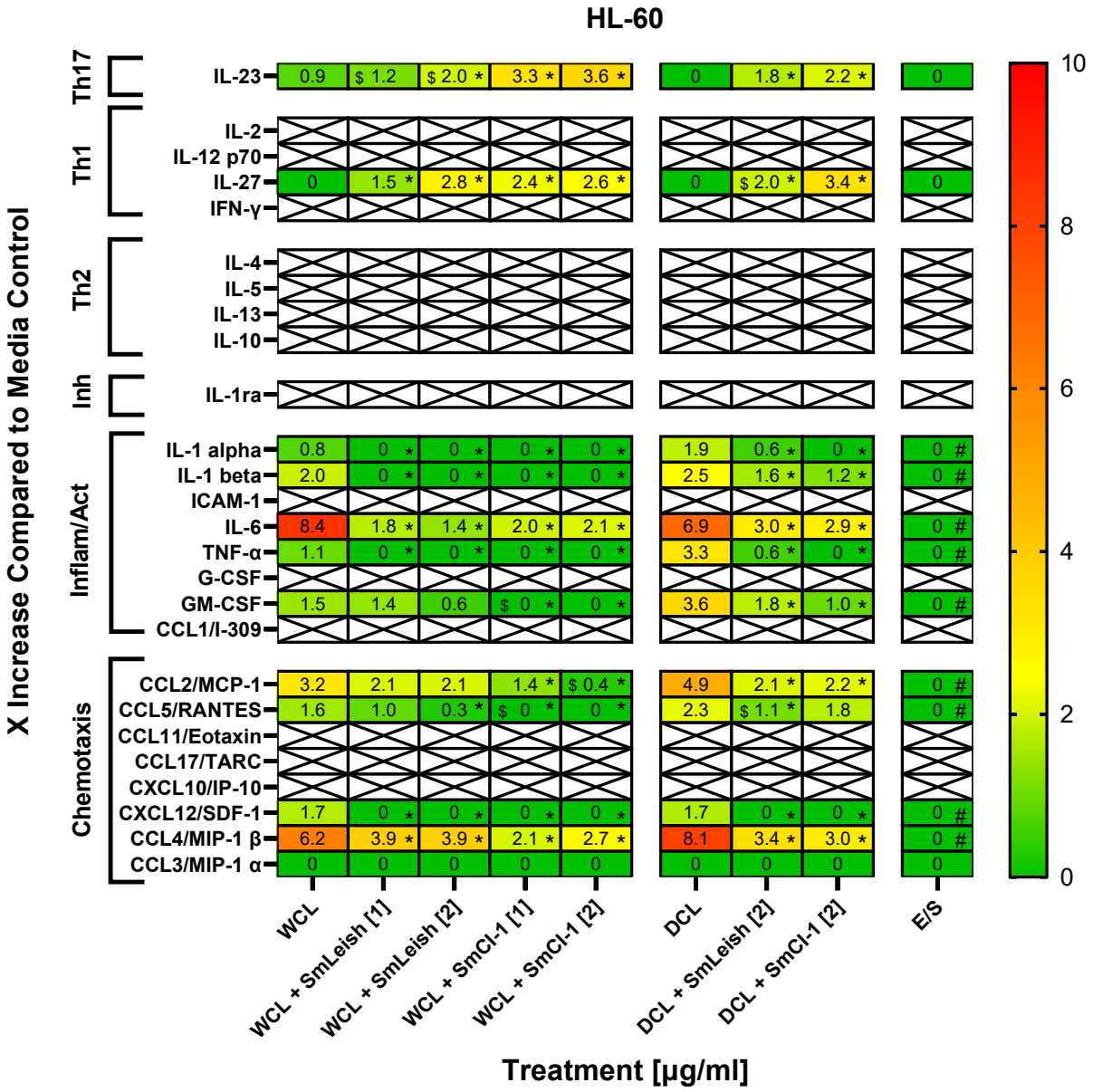



**Figure 6.5. Effects of invadolysins on cytokine production in differentiated HL-60 cells**

Cytokines stimulated via the addition of whole cercarial lysate (WCL), drained cercarial lysate (DCL), or excretory/secretory (E/S) products in HL-60 cells treated with rSmLeish and rSmCI-1. Values presented as X increase as compared to media-only stimulated controls. (*) Indicates significant difference from associated WCL or DCL only treated samples. (#) Indicates a significant difference between E/S stimulated samples and DCL treated samples. Significant difference between rSmLeish and rSmCI-1 treated samples of the same concentration are denoted by ($). Cytokines stimulated by murine PBLs or other cell lines, but not HL-60s are presented as blank cells.

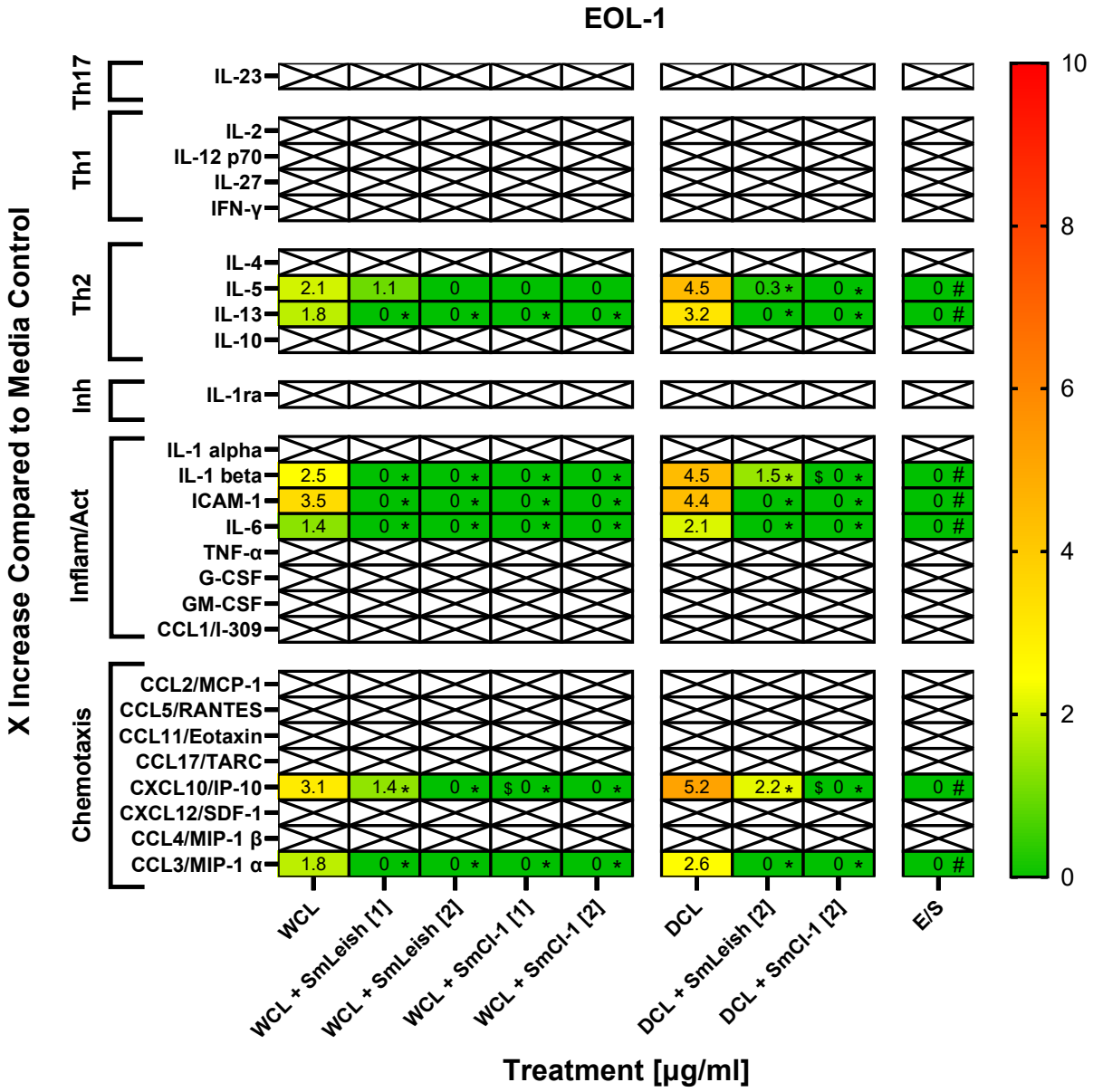



**Figure 6.6. Effects of invadolysins on cytokine production in differentiated EoL-1 cells**

Cytokines stimulated via the addition of whole cercarial lysate (WCL), drained cercarial lysate (DCL), or excretory/secretory (E/S) products in EoL-1cells treated with rSmLeish and rSmCI-1. Values presented as X increased as compared to media-only stimulated controls. (*) Indicates significant difference from associated WCL or DCL only treated samples. (#) Indicates a significant difference between E/S stimulated samples and DCL treated samples. Significant difference between rSmLeish and rSmCI-1 treated samples of the same concentration are denoted by ($). Cytokines stimulated by murine PBLs or other cell lines, but not EoL-1s are presented as blank cells.

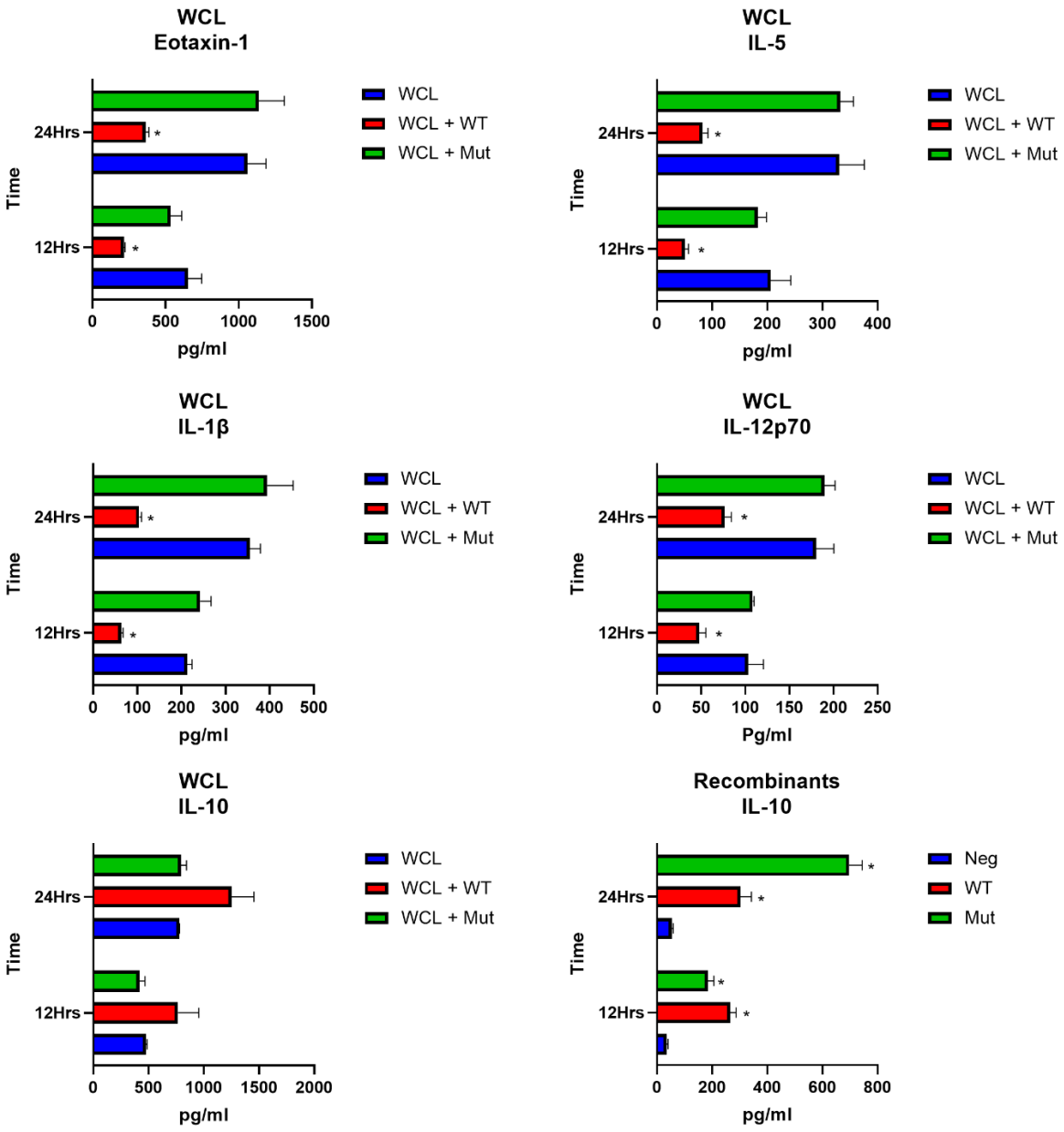



**Figure 6.7. SmCI-1 alters cytokine profiles of human PBMCs in an activity dependent manner when stimulated with whole cercarial lysate**

Cytokine levels in human PBMCx as measured by Luminex panel presented in pg/ml. Differences in levels of key cytokines unstimulated (Neg), or stimulated with whole cercarial lysate (WCL) totalling the contents of 5 *S. mansoni* cercaria. Treatments with 2µg/ml of either wild type rSmCI-1 (WT) or mutant SmCI-1Mut effects levels of these cytokines. The effect of rSmCI-1 on IL-10 levels features an increase is shown for both cells exposed to WCL, as well as cells exposed only to the recombinants themselves. (*) Indicates significant differences between the treatment and either the WCL control or untreated negative control. (n=3)

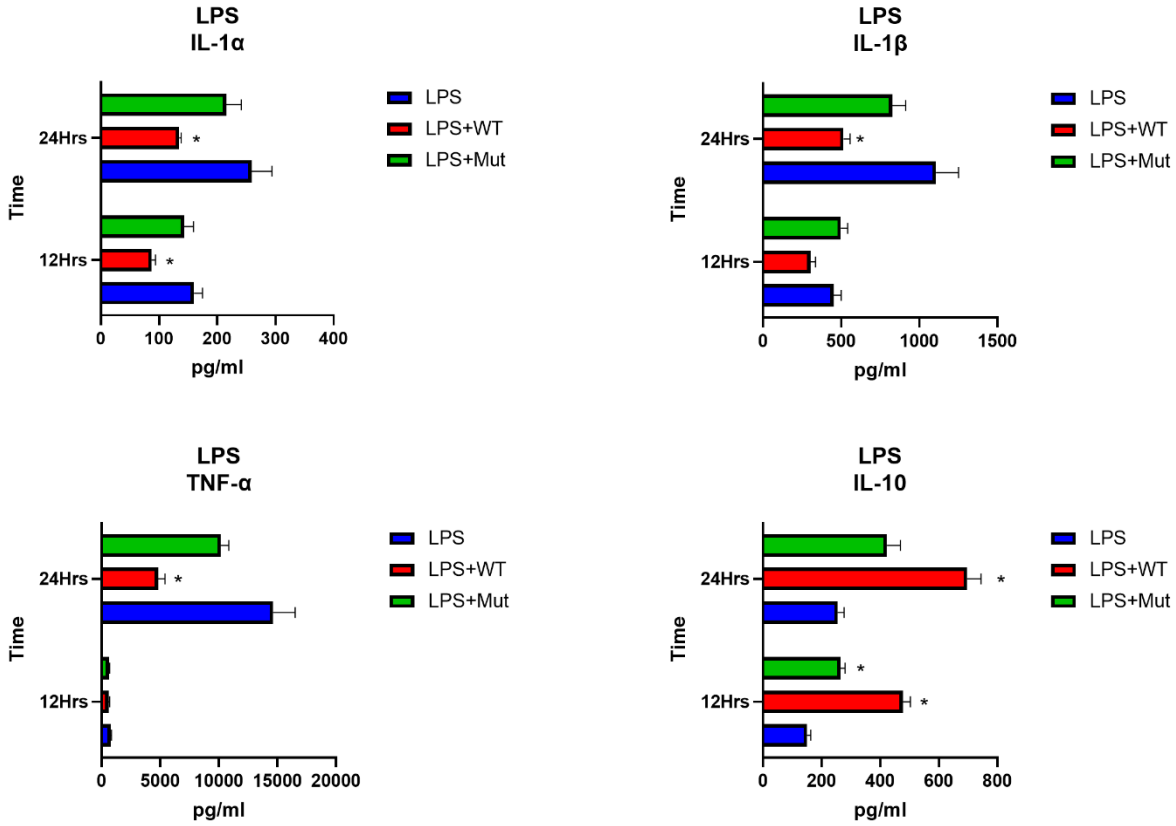



**Figure 6.8. SmCI-1 alters cytokine profiles of human PBMCs in an activity dependent manner when stimulated with lipopolysaccharides**

Cytokine levels in human PBMCs as measured by Luminex panel presented in pg/ml. Differences in levels of key cytokines stimulated with 1µg/ml lipopolysaccharides (LPS). Treatments with 2µg/ml of either wild type rSmCI-1 (WT) or mutant SmCI-1Mut effects levels of these cytokines. (*) Indicates significant differences between the treatment and either the WCL control or untreated negative control. (n=3)

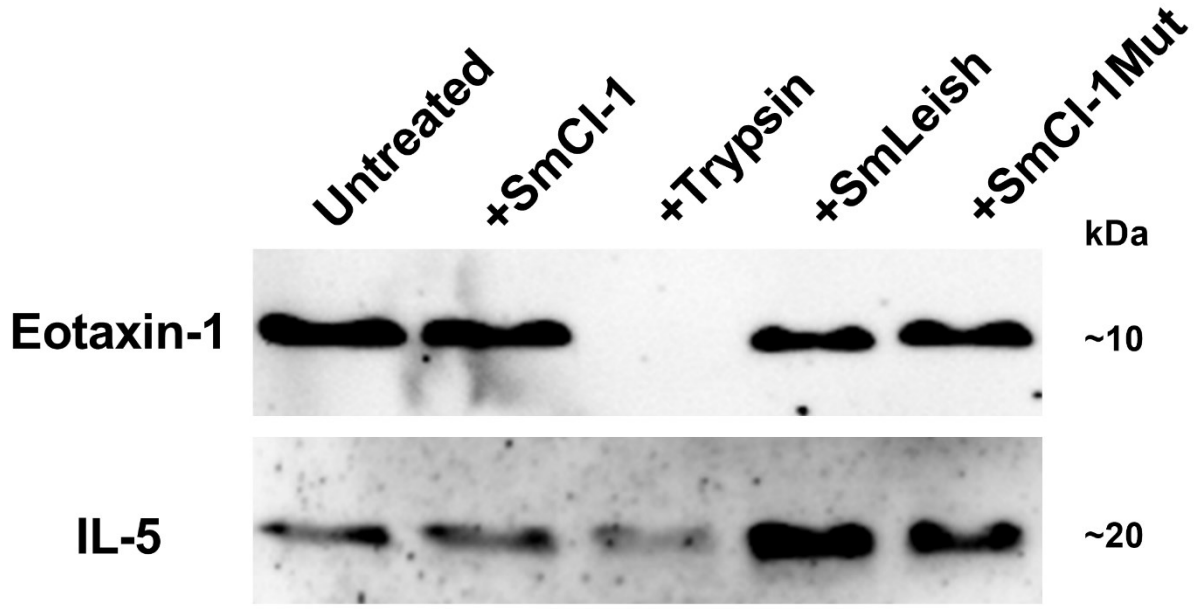



**Figure 6.9. *S. mansoni* invadolysins do not cleave key cytokines**

Western blots containing 50ng/lane recombinant human cytokines treated with 1µg/ml of the indicated proteases and probed with relevant monoclonal antibodies. Trypsin degrades eotaxin-1 while the invadolysins do not. IL-5 was weakly affected by trypsin, and not degraded by either the SmLeish, SmCI-1, or SmCI-1Mut

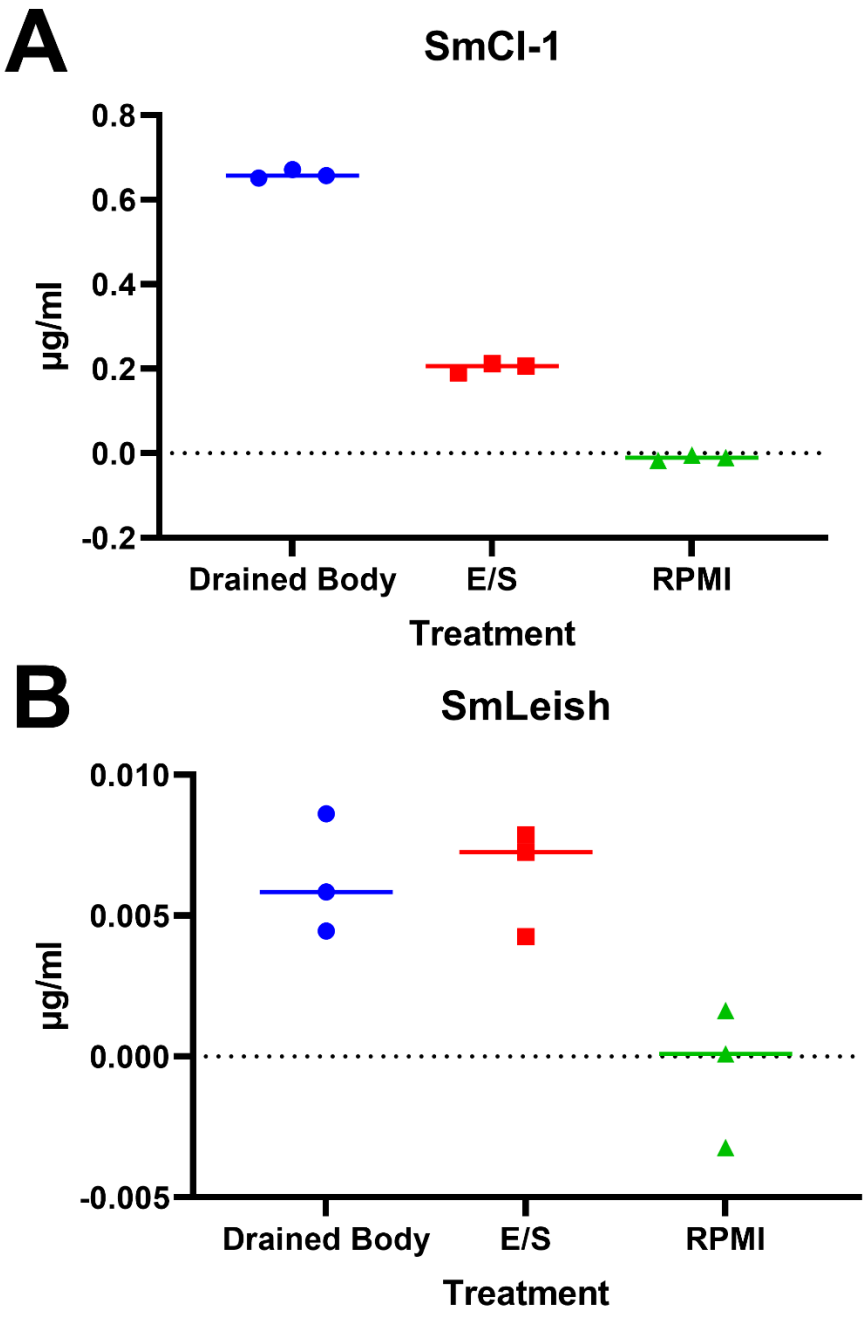



**Figure 6.10. ELISA-based determination of invadolysin concentrations**

SmCI-1 (A) and SmLeish (B) concentrations present in both drained cercarial lysate (DCL) or excretory/secretory (E/S) products, as determined using rabbit polyclonal antibodies to each of the respective invadolysins.

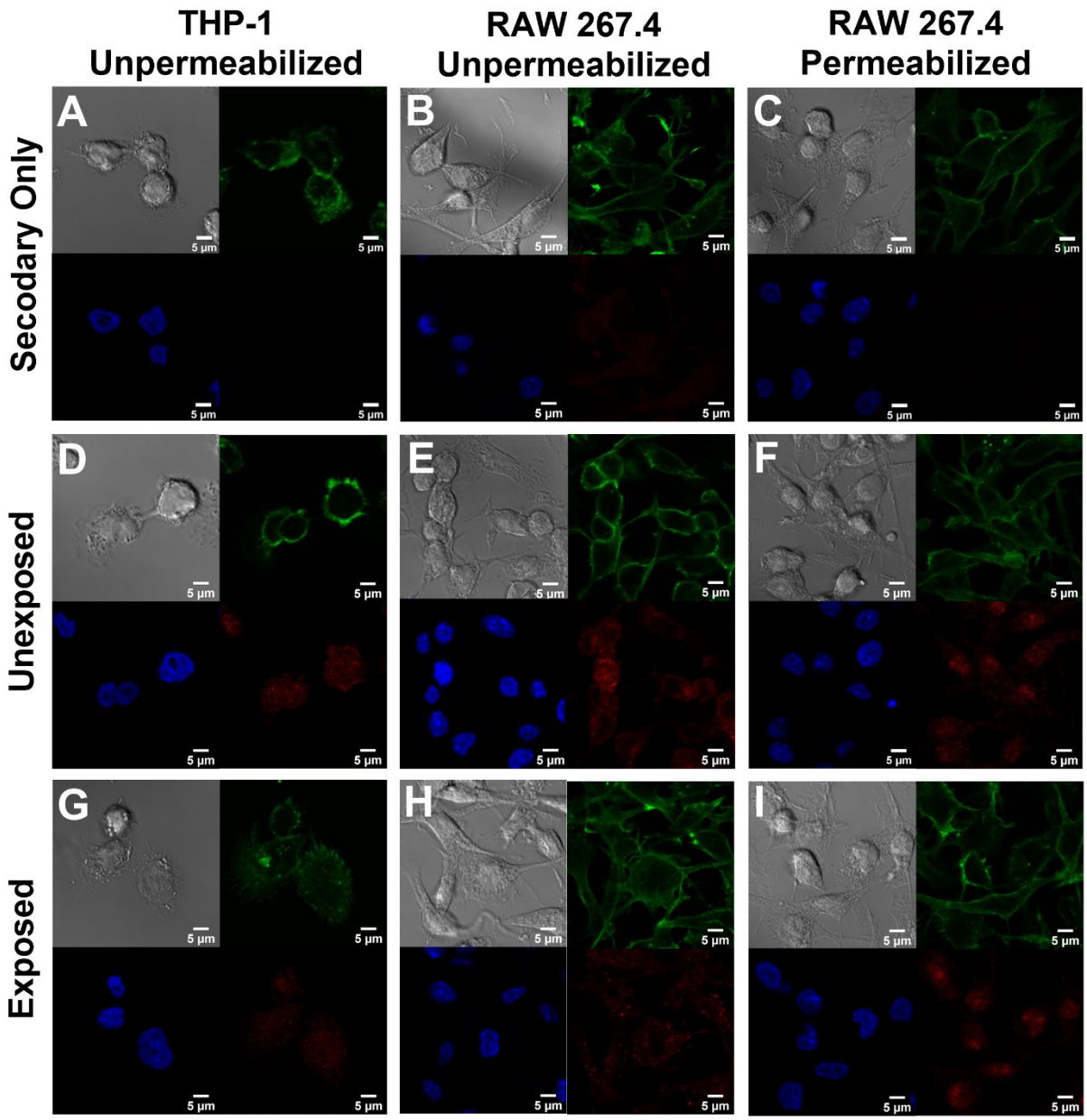



**Figure 6.11. α-SmCI-1 antibody fails to differentially recognize E/S exposed and unexposed phagocytic cells**

Immunofluorescence images of differentiated THP-1 cells, and RAW 264.7 macrophages probed with secondary antibodies only (A,B,C), cells unexposed to E/S products probed with primary and secondary antibodies (D,E,F), and cells exposed to E/S products probed with both antibodies (G,H,I). Each representative image features bright field, DAPI (blue), phalloidin Alexa 488 (green), and an Alexa Fluoro 555 secondary antibody (red).

| 24 Hrs Post Stimulation | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cytokines | Negative - Medium Control | | Whole Cercarial Lysate (~5) | | WCL + 2µg/mL SmCI-1 | | WCL + 2µg/mL SmCI-1Mut | | LPS | | LPS + 2µg/mL SmCI-1 | | LPS + 2µg/mL SmCI-1Mut | | 2µg/mL SmCI-1 | | 2µg/mL SmCI-1Mut | | Acetabular Contents | |
| (pg/ml) | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM |
| CCL2/JE/MCP-1 | 33.5 | 3.1 | 478.0 | 63.5 | 515.5 | 15.9 | 615.3 | 56.9 | 457.3 | 58.5 | 330.0 | 6.0 | 418.0 | 40.3 | 31.1 | 0.8 | 330.0 | 4.9 | 45.4 | 6.3 |
| CCL11/Eotaxin | 119.1 | 14.1 | 1059.6 | 103.8 | 364.6 | 17.0 | 1137.9 | 173.8 | 158.6 | 19.0 | 131.4 | 6.0 | 161.9 | 24.4 | 126.9 | 8.5 | 131.4 | 4.9 | 142.3 | 20.6 |
| CCL19/MIP-3 beta | 7.4 | 0.7 | 11.9 | 1.0 | 17.6 | 1.4 | 14.7 | 0.8 | 21.4 | 1.4 | 13.3 | 0.3 | 12.8 | 0.1 | 7.0 | 0.8 | 13.3 | 0.3 | 9.6 | 1.6 |
| CCL20/MIP-3 alpha | 11.9 | 1.0 | 14.0 | 1.5 | 17.9 | 1.9 | 15.5 | 2.2 | 43.4 | 4.8 | 35.1 | 5.8 | 41.7 | 5.0 | 12.5 | 0.9 | 35.1 | 4.8 | 9.6 | 0.3 |
| CCL3/MIP-1 alpha | 21.6 | 1.8 | 585.3 | 41.5 | 848.9 | 55.4 | 641.7 | 68.8 | 626.8 | 58.6 | 625.7 | 56.6 | 409.5 | 63.4 | 26.6 | 2.4 | 625.7 | 46.2 | 36.2 | 3.7 |
| CCL4/MIP-1 beta | 116.3 | 11.5 | 184.8 | 9.7 | 239.5 | 20.2 | 195.4 | 16.8 | 234.0 | 29.1 | 234.0 | 30.3 | 190.6 | 20.5 | 145.4 | 14.3 | 234.0 | 24.7 | 242.8 | 29.9 |
| CCL5/RANTES | 2257.0 | 116.8 | 14783.0 | 461.5 | 15284.6 | 1470.3 | 16110.5 | 899.9 | 11932.6 | 513.8 | 11811.3 | 912.1 | 7938.9 | 546.4 | 2893.5 | 230.4 | 11811.3 | 744.7 | 3387.4 | 276.7 |
| CXCL1/GRO alpha/KC/CINC-1 | 187.4 | 13.7 | 380.1 | 17.8 | 614.9 | 57.5 | 441.4 | 38.5 | 514.8 | 63.1 | 449.1 | 28.6 | 481.6 | 58.3 | 205.3 | 24.2 | 449.1 | 23.3 | 274.5 | 41.3 |
| CXCL10/IP-10/CRG-2 | 269.1 | 6.3 | 1296.9 | 106.5 | 1724.1 | 159.0 | 1378.4 | 101.5 | 1076.3 | 132.5 | 836.6 | 65.8 | 876.1 | 97.3 | 272.5 | 5.2 | 836.6 | 53.7 | 478.7 | 67.9 |
| CXCL2/GRO beta/MIP-2/CINC-3 | 566.4 | 23.5 | 1001.8 | 67.2 | 948.5 | 33.1 | 1257.4 | 74.6 | 1765.4 | 192.6 | 1715.9 | 207.2 | 1221.9 | 125.7 | 699.8 | 66.7 | 1715.9 | 169.2 | 1140.2 | 134.9 |
| G-CSF | 87.5 | 2.9 | 72.2 | 3.4 | 89.3 | 10.9 | 78.8 | 3.9 | 353.7 | 13.3 | 364.5 | 56.3 | 291.9 | 47.9 | 109.1 | 14.0 | 364.5 | 46.0 | 58.1 | 4.9 |
| GM-CSF | 173.4 | 12.9 | 114.7 | 10.6 | 114.1 | 4.4 | 130.2 | 12.7 | 1315.6 | 147.3 | 189.5 | 29.4 | 346.3 | 38.1 | 150.3 | 7.9 | 252.8 | 30.9 | 229.0 | 19.3 |
| IFN-alpha | 7.1 | 0.7 | 14.7 | 1.3 | 20.1 | 0.4 | 17.5 | 0.7 | 15.9 | 1.6 | 11.9 | 1.3 | 10.4 | 0.6 | 8.3 | 0.8 | 11.9 | 1.1 | 13.9 | 1.9 |
| IFN-beta | 3.1 | 0.2 | 5.8 | 0.7 | 8.5 | 1.1 | 5.9 | 0.5 | 5.1 | 0.4 | 4.0 | 0.4 | 3.1 | 0.2 | 3.5 | 0.3 | 4.0 | 0.3 | 3.7 | 0.1 |
| IFN-gamma | 148.2 | 18.3 | 213.0 | 21.1 | 238.1 | 33.6 | 247.0 | 12.0 | 205.3 | 2.1 | 168.1 | 26.2 | 193.3 | 9.3 | 119.1 | 5.5 | 168.1 | 21.4 | 150.4 | 25.5 |
| IL-1 alpha/IL-1F1 | 12.2 | 0.1 | 54.5 | 5.2 | 77.0 | 8.5 | 54.1 | 3.8 | 259.1 | 34.7 | 134.5 | 3.3 | 215.5 | 25.7 | 15.1 | 1.2 | 134.5 | 2.7 | 24.4 | 2.5 |
| IL-1 beta/IL-1F2 | 119.3 | 11.0 | 354.8 | 19.8 | 104.5 | 4.1 | 394.4 | 58.7 | 1107.3 | 144.5 | 515.9 | 41.5 | 828.7 | 85.0 | 116.1 | 2.6 | 515.9 | 33.9 | 367.0 | 46.4 |
| IL-10 | 54.1 | 4.1 | 780.1 | 2.3 | 1252.8 | 163.8 | 794.2 | 50.6 | 255.2 | 21.7 | 697.1 | 47.4 | 423.1 | 45.8 | 302.9 | 32.2 | 697.1 | 38.7 | 876.9 | 31.6 |
| IL-12 p70 | 18.0 | 1.5 | 180.4 | 16.2 | 76.5 | 6.4 | 189.4 | 12.4 | 23.5 | 2.5 | 25.3 | 2.2 | 23.8 | 2.4 | 21.8 | 1.7 | 25.3 | 1.8 | 16.8 | 0.3 |
| IL-13 | 18.9 | 2.3 | 62.0 | 4.0 | 86.9 | 7.6 | 71.0 | 7.6 | 32.8 | 4.1 | 30.5 | 2.8 | 24.6 | 2.0 | 23.1 | 1.7 | 30.5 | 2.3 | 25.8 | 3.0 |
| IL-15 | 31.9 | 2.4 | 204.0 | 12.0 | 274.1 | 7.1 | 259.0 | 14.6 | 253.0 | 34.6 | 233.7 | 35.5 | 193.7 | 17.8 | 35.2 | 3.3 | 233.7 | 28.9 | 48.4 | 6.1 |
| IL-17/IL-17A | 136.9 | 9.8 | 252.9 | 9.3 | 296.9 | 21.6 | 245.7 | 7.6 | 221.0 | 23.8 | 258.0 | 34.1 | 181.1 | 20.0 | 212.8 | 12.3 | 258.0 | 27.8 | 180.7 | 10.1 |
| IL-17E/IL-25 | 85.1 | 8.3 | 83.3 | 5.2 | 92.8 | 3.8 | 92.6 | 9.4 | 445.5 | 45.8 | 342.4 | 38.6 | 340.5 | 42.2 | 97.5 | 7.8 | 342.4 | 31.6 | 106.3 | 9.7 |
| IL-1ra/IL-1F3 | 237.8 | 21.0 | 4007.6 | 78.2 | 6520.3 | 512.6 | 4945.2 | 193.0 | 3938.7 | 72.5 | 6100.5 | 727.9 | 4833.9 | 623.0 | 414.6 | 9.7 | 6100.5 | 594.4 | 268.2 | 4.7 |
| IL-2 | 32.3 | 4.0 | 49.1 | 4.6 | 44.7 | 0.7 | 54.3 | 3.2 | 330.6 | 24.9 | 295.7 | 34.4 | 366.2 | 50.3 | 35.3 | 2.9 | 295.7 | 28.1 | 44.5 | 4.6 |
| IL-3 | 30.1 | 3.9 | 36.3 | 3.8 | 44.7 | 1.9 | 34.5 | 1.8 | 83.7 | 2.4 | 113.3 | 11.6 | 87.3 | 10.4 | 34.8 | 3.0 | 113.3 | 9.5 | 49.3 | 5.5 |
| IL-33 | 19.8 | 2.4 | 20.9 | 2.3 | 19.7 | 0.3 | 22.0 | 2.1 | 27.8 | 2.0 | 21.5 | 1.2 | 22.6 | 1.1 | 25.7 | 2.2 | 21.5 | 0.9 | 37.8 | 3.6 |
| IL-4 | 15.8 | 1.5 | 277.4 | 20.8 | 407.5 | 19.4 | 296.1 | 19.4 | 40.9 | 1.9 | 48.3 | 5.7 | 43.0 | 4.1 | 17.9 | 1.9 | 48.3 | 4.7 | 33.3 | 4.3 |
| IL-5 | 17.8 | 1.3 | 330.3 | 37.3 | 83.0 | 7.8 | 331.7 | 24.4 | 138.5 | 11.8 | 125.9 | 6.2 | 134.5 | 14.6 | 24.7 | 2.7 | 125.9 | 5.1 | 37.1 | 2.8 |
| IL-6 | 47.4 | 5.0 | 1845.2 | 122.5 | 2186.4 | 77.0 | 1619.4 | 49.8 | 3523.9 | 431.1 | 2242.0 | 100.2 | 3116.6 | 381.5 | 50.7 | 4.6 | 2242.0 | 81.8 | 183.2 | 10.6 |
| IL-7 | 21.2 | 1.9 | 24.9 | 2.6 | 32.1 | 1.6 | 29.4 | 0.9 | 89.1 | 10.9 | 91.9 | 12.3 | 64.8 | 6.6 | 22.6 | 1.4 | 91.9 | 10.1 | 30.2 | 0.7 |
| IL-8/CXCL8 | 68.2 | 6.5 | 82.5 | 2.7 | 104.3 | 7.2 | 93.8 | 11.3 | 1663.1 | 201.2 | 1616.8 | 289.1 | 1282.3 | 53.8 | 76.3 | 8.3 | 1616.8 | 236.1 | 95.5 | 5.3 |
| TGF-alpha | 244.0 | 24.2 | 219.0 | 22.4 | 272.5 | 46.8 | 278.4 | 13.8 | 254.1 | 24.7 | 208.8 | 17.8 | 150.9 | 8.8 | 276.0 | 21.9 | 208.8 | 14.6 | 289.6 | 34.0 |
| TNF-alpha | 175.1 | 10.3 | 1002.3 | 95.6 | 741.8 | 49.0 | 1126.1 | 109.7 | 14648.4 | 1878.8 | 4835.6 | 576.9 | 10175.9 | 687.7 | 178.9 | 5.7 | 4835.6 | 471.0 | 500.9 | 59.0 |
| TRAIL/TNFSF10 | 76.7 | 9.4 | 127.6 | 12.4 | 104.5 | 2.0 | 154.4 | 6.8 | 114.9 | 11.4 | 82.4 | 5.7 | 83.6 | 2.1 | 91.1 | 5.0 | 82.4 | 4.6 | 83.2 | 9.8 |
| VEGF | 269.8 | 16.3 | 2448.7 | 177.8 | 3696.3 | 374.9 | 2744.1 | 357.7 | 2113.4 | 192.4 | 1784.5 | 184.3 | 2057.3 | 185.2 | 341.8 | 26.8 | 1784.5 | 150.5 | 496.1 | 27.6 |
| TGF-beta 1 | 1289.5 | 54.9 | 1506.3 | 148.0 | 1831.8 | 65.1 | 1640.0 | 66.4 | 3799.1 | 405.5 | 3216.8 | 352.9 | 2645.5 | 252.3 | 1325.6 | 123.9 | 3216.8 | 288.1 | 1797.8 | 192.0 |
| TGF-beta 2 | 554.4 | 37.3 | 681.7 | 11.2 | 640.9 | 30.7 | 704.5 | 39.1 | 934.9 | 127.9 | 707.0 | 18.9 | 988.3 | 175.0 | 688.7 | 60.7 | 707.0 | 15.5 | 923.4 | 26.0 |

| 12 Hrs Post Stimulation | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cytokines | Negative - Medium Control | | Whole Cercarial Lysate (~5) | | WCL + 2µg/mL SmCI-1 | | WCL + 2µg/mL SmCI-1Mut | | LPS | | LPS + 2µg/mL SmCI-1 | | LPS + 2µg/mL SmCI-1Mut | | 2µg/mL SmCI-1 | | 2µg/mL SmCI-1Mut | | Acetabular Contents | |
| (pg/ml) | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM | Average | ±SEM |
| CCL2/JE/MCP-1 | 19.6 | 2.2 | 286.5 | 50.6 | 339.0 | 27.9 | 376.3 | 25.1 | 263.6 | 30.8 | 207.3 | 11.9 | 251.7 | 14.3 | 18.4 | 1.8 | 203.7 | 14.1 | 28.1 | 2.4 |
| CCL11/Eotaxin | 65.5 | 11.2 | 652.5 | 94.1 | 215.1 | 7.7 | 533.8 | 76.9 | 86.7 | 8.9 | 76.5 | 8.4 | 103.0 | 21.9 | 82.6 | 6.8 | 74.8 | 1.5 | 87.7 | 17.5 |
| CCL19/MIP-3 beta | 4.5 | 0.7 | 6.8 | 0.5 | 9.8 | 0.6 | 8.3 | 0.5 | 14.0 | 1.1 | 7.5 | 0.6 | 7.7 | 0.6 | 4.2 | 0.6 | 7.7 | 0.3 | 5.7 | 1.0 |
| CCL20/MIP-3 alpha | 7.8 | 0.7 | 8.2 | 0.9 | 9.9 | 1.7 | 10.4 | 2.0 | 27.1 | 4.1 | 19.9 | 4.6 | 26.9 | 4.0 | 7.5 | 0.3 | 19.8 | 3.7 | 6.4 | 0.1 |
| CCL3/MIP-1 alpha | 13.6 | 1.9 | 309.0 | 25.0 | 525.3 | 24.7 | 393.4 | 61.0 | 378.7 | 25.1 | 392.3 | 37.8 | 238.9 | 37.7 | 16.4 | 2.2 | 392.5 | 19.1 | 21.5 | 1.9 |
| CCL4/MIP-1 beta | 70.1 | 4.6 | 103.3 | 7.7 | 146.2 | 16.4 | 119.1 | 11.7 | 134.5 | 10.9 | 146.3 | 26.7 | 118.9 | 18.0 | 89.8 | 16.9 | 142.4 | 13.9 | 155.2 | 21.0 |
| CCL5/RANTES | 1182.9 | 77.6 | 2442.7 | 219.4 | 2492.3 | 225.4 | 2354.1 | 105.7 | 3009.9 | 493.2 | 2672.7 | 281.2 | 2077.3 | 123.8 | 1874.7 | 143.5 | 1674.7 | 109.7 | 2145.5 | 160.4 |
| CXCL1/GRO alpha/KC/CINC-1 | 113.0 | 13.4 | 235.0 | 6.7 | 363.0 | 32.7 | 294.4 | 49.2 | 336.8 | 61.4 | 277.9 | 26.6 | 260.5 | 21.8 | 133.6 | 23.1 | 265.9 | 16.5 | 159.5 | 17.0 |
| CXCL10/IP-10/CRG-2 | 172.3 | 9.4 | 790.6 | 117.5 | 1070.4 | 156.9 | 759.3 | 57.9 | 695.5 | 66.3 | 529.1 | 42.1 | 498.4 | 64.2 | 168.6 | 14.1 | 524.1 | 52.8 | 331.2 | 47.0 |
| CXCL2/GRO beta/MIP-2/CINC-3 | 364.5 | 37.9 | 595.6 | 64.5 | 567.6 | 24.1 | 790.9 | 100.0 | 861.0 | 111.7 | 933.2 | 111.7 | 782.4 | 129.0 | 399.2 | 39.0 | 1007.0 | 110.9 | 699.8 | 57.2 |
| G-CSF | 56.3 | 1.7 | 40.9 | 3.6 | 51.1 | 6.2 | 50.2 | 1.4 | 223.5 | 3.2 | 219.1 | 30.8 | 175.2 | 18.9 | 66.1 | 12.9 | 224.1 | 35.5 | 33.6 | 4.7 |
| GM-CSF | 106.7 | 15.3 | 68.1 | 6.6 | 300.2 | 29.7 | 176.4 | 9.9 | 614.9 | 94.0 | 520.4 | 42.1 | 550.2 | 27.4 | 166.8 | 17.6 | 158.2 | 22.1 | 238.2 | 10.0 |
| IFN-alpha | 4.4 | 0.2 | 8.3 | 0.5 | 12.0 | 0.4 | 11.2 | 0.8 | 9.6 | 0.9 | 7.4 | 1.0 | 6.1 | 0.4 | 4.8 | 0.8 | 8.2 | 1.0 | 7.8 | 0.8 |
| IFN-beta | 2.0 | 0.2 | 3.3 | 0.5 | 5.6 | 0.8 | 3.9 | 0.5 | 3.1 | 0.4 | 2.5 | 0.2 | 1.9 | 0.2 | 2.1 | 0.3 | 2.5 | 0.3 | 2.3 | 0.2 |
| IFN-gamma | 87.0 | 11.5 | 128.0 | 10.2 | 142.5 | 30.6 | 158.8 | 21.4 | 124.1 | 11.0 | 102.4 | 18.6 | 121.5 | 11.5 | 71.4 | 3.6 | 99.9 | 13.3 | 99.6 | 22.7 |
| IL-1 alpha/IL-1F1 | 7.1 | 0.5 | 31.3 | 3.0 | 42.6 | 3.9 | 37.8 | 3.3 | 159.7 | 14.8 | 87.3 | 6.6 | 143.2 | 16.1 | 8.9 | 0.7 | 79.0 | 2.1 | 14.2 | 1.2 |
| IL-1 beta/IL-1F2 | 70.6 | 9.6 | 213.7 | 10.4 | 63.4 | 4.3 | 242.2 | 24.8 | 452.1 | 49.0 | 303.7 | 31.3 | 498.5 | 43.8 | 69.4 | 5.2 | 92.9 | 3.9 | 136.8 | 8.5 |
| IL-10 | 35.0 | 5.2 | 477.9 | 10.0 | 766.9 | 189.7 | 417.7 | 49.7 | 150.1 | 12.7 | 478.6 | 24.3 | 265.2 | 14.9 | 265.6 | 21.3 | 184.6 | 21.6 | 532.9 | 20.3 |
| IL-12 p70 | 10.9 | 0.8 | 103.4 | 17.2 | 47.9 | 7.4 | 108.4 | 1.7 | 12.7 | 1.3 | 14.7 | 1.4 | 15.7 | 2.4 | 12.5 | 1.6 | 16.2 | 2.4 | 11.9 | 0.2 |
| IL-13 | 10.8 | 2.2 | 36.2 | 1.9 | 57.3 | 7.6 | 44.3 | 3.0 | 20.7 | 3.1 | 18.1 | 2.0 | 14.7 | 1.4 | 14.0 | 2.2 | 18.2 | 2.8 | 14.7 | 2.0 |
| IL-15 | 20.0 | 2.2 | 117.6 | 12.5 | 156.6 | 12.4 | 167.4 | 8.3 | 168.7 | 25.3 | 146.4 | 25.6 | 125.7 | 11.4 | 22.8 | 2.4 | 148.7 | 25.9 | 31.6 | 5.0 |
| IL-17/IL-17A | 82.3 | 11.8 | 158.0 | 11.8 | 170.5 | 13.5 | 145.5 | 4.5 | 127.1 | 7.8 | 155.1 | 25.2 | 106.7 | 10.1 | 142.0 | 13.2 | 161.5 | 27.9 | 116.8 | 5.3 |
| IL-17E/IL-25 | 50.7 | 8.5 | 51.7 | 2.1 | 57.3 | 5.6 | 62.6 | 8.1 | 277.3 | 37.7 | 213.3 | 17.5 | 210.4 | 18.0 | 60.1 | 5.3 | 207.8 | 28.5 | 64.1 | 8.7 |
| IL-1ra/IL-1F3 | 151.6 | 19.8 | 524.4 | 45.1 | 365.1 | 27.8 | 320.5 | 15.3 | 690.4 | 47.2 | 458.3 | 54.6 | 446.4 | 50.8 | 256.5 | 17.2 | 231.4 | 33.9 | 246.9 | 4.1 |
| IL-2 | 20.4 | 4.0 | 34.0 | 3.7 | 23.9 | 0.4 | 35.8 | 2.6 | 229.4 | 15.1 | 171.0 | 15.8 | 231.4 | 46.8 | 21.3 | 3.4 | 57.9 | 6.7 | 27.6 | 4.3 |
| IL-3 | 19.7 | 3.3 | 22.6 | 3.7 | 25.3 | 1.4 | 24.4 | 0.6 | 51.6 | 2.5 | 67.9 | 8.4 | 49.6 | 9.3 | 20.5 | 1.2 | 47.2 | 15.4 | 28.4 | 3.6 |
| IL-33 | 12.6 | 2.0 | 11.9 | 1.3 | 11.7 | 0.9 | 14.3 | 1.6 | 18.2 | 2.1 | 12.3 | 0.9 | 14.2 | 1.2 | 16.7 | 1.8 | 13.3 | 1.7 | 23.6 | 3.1 |
| IL-4 | 10.4 | 1.0 | 163.4 | 18.9 | 255.8 | 18.8 | 198.5 | 27.0 | 26.6 | 2.0 | 26.9 | 3.8 | 26.4 | 3.2 | 11.2 | 1.4 | 31.0 | 2.7 | 84.0 | 5.1 |
| IL-5 | 10.6 | 0.8 | 206.1 | 36.4 | 50.6 | 6.7 | 182.4 | 16.2 | 82.6 | 7.8 | 80.0 | 3.8 | 74.9 | 6.2 | 16.5 | 1.8 | 73.9 | 6.6 | 23.4 | 2.6 |
| IL-6 | 28.7 | 1.6 | 372.6 | 63.1 | 248.7 | 44.0 | 186.6 | 39.6 | 650.4 | 20.2 | 492.8 | 32.2 | 691.3 | 49.1 | 32.7 | 5.3 | 124.1 | 11.0 | 109.0 | 11.2 |
| IL-7 | 12.4 | 1.2 | 14.7 | 1.5 | 19.5 | 0.8 | 17.5 | 1.9 | 59.4 | 9.0 | 58.6 | 10.1 | 42.4 | 4.1 | 14.1 | 1.8 | 44.1 | 13.3 | 18.6 | 1.5 |
| IL-8/CXCL8 | 40.4 | 5.3 | 48.2 | 1.2 | 67.3 | 3.4 | 55.7 | 3.5 | 256.1 | 44.0 | 532.0 | 151.5 | 788.1 | 24.8 | 46.8 | 7.9 | 46.1 | 9.6 | 57.1 | 2.2 |
| TGF-alpha | 150.6 | 17.1 | 146.5 | 22.5 | 172.9 | 45.7 | 170.3 | 22.7 | 158.1 | 22.8 | 134.6 | 7.7 | 84.7 | 6.7 | 160.2 | 15.3 | 128.2 | 12.4 | 187.3 | 26.3 |
| TNF-alpha | 117.3 | 9.4 | 465.6 | 46.0 | 459.9 | 62.2 | 591.4 | 65.1 | 776.0 | 60.7 | 597.6 | 98.2 | 643.3 | 45.9 | 114.1 | 1.6 | 231.0 | 13.9 | 166.5 | 12.2 |
| TRAIL/TNFSF10 | 45.9 | 9.1 | 77.9 | 14.3 | 65.1 | 1.7 | 96.2 | 6.4 | 71.9 | 5.8 | 53.3 | 4.5 | 47.1 | 0.9 | 57.4 | 5.1 | 47.1 | 5.5 | 56.0 | 7.4 |
| VEGF | 161.5 | 19.3 | 1519.9 | 154.1 | 2144.8 | 211.7 | 1663.9 | 198.0 | 1245.9 | 91.7 | 1001.0 | 85.4 | 1192.4 | 119.6 | 212.0 | 13.4 | 219.1 | 70.5 | 274.9 | 14.2 |
| TGF-beta 1 | 843.8 | 23.5 | 831.6 | 80.1 | 1260.5 | 63.7 | 870.8 | 26.4 | 2185.9 | 292.0 | 2050.5 | 180.7 | 1551.3 | 133.8 | 716.7 | 76.3 | 547.1 | 189.4 | 1126.7 | 142.0 |
| TGF-beta 2 | 327.7 | 19.0 | 403.0 | 37.9 | 369.4 | 28.7 | 446.6 | 63.0 | 575.3 | 132.4 | 472.7 | 12.8 | 524.4 | 93.2 | 384.8 | 33.2 | 441.0 | 13.4 | 593.9 | 19.7 |

**STable 6.1. Cytokine array data for human PBMCs**

Average pg/ml concentrations of individually indicated cytokines treated with *S. mansoni* whole cercarial lysate, acetabular gland contents), LPS, recombinant SmCI-1, or recombinant SmCI-1Mut. Values are indicated for both 24 and 12 hour timepoints. Accompanied by Standard error of the mean values (SEM). n=3

# Chapter 7 - General Discussion

## 7.1 Overview of findings

The capacity of parasitic organisms to survive and thrive as the most common mode of eukaryotic life on planet Earth is largely dependent upon their capacity to evade and overcome the immune response mounted by their host of choice (448). To this end, parasites have been involved in a molecular arms race with their hosts, in order to develop mechanisms by which to survive (90). Schistosomes are no exception to this idea, and given their complex life cycles feature both a vertebrate (human) host as well as an invertebrate (snail) host, thereby drastically increasing the diversity of immune responses that they must overcome. Schistosomes have not taken this challenge lying down, and have amassed a significant variety of immunomodulatory mechanisms by which to survive in both mollusc and man (95).

The work presented in this thesis significantly broadens our understanding of factors termed invadolysins that schistosomes employ in order to survive in both of their hosts. Invadolysins are well known immunomodulatory matrix metalloproteases, and their abundance in the *S. mansoni* genome, as well as their upregulation during key points in the parasite's life cycle have put them forth as interesting candidates to be examined in the context of survival and immune evasion. To this end, we characterized two key invadolysins, one during the intramolluscan infection, which we termed SmLeish, and another during the initial stages of mammalian infection, which I termed SmCI-1.

Our initial assessment of SmLeish was predicated upon its identification as a factor upregulated in sporocysts during the first days after penetration into *Biomphalaria glabrata* snails. We determined that SmLeish was transcriptionally upregulated predominantly during the first 48

hours post infection, and that a full length variety could be detected via western blot in both miracidia as well as infected snail tissue, while a smaller form of the protein could be found in infected snail tissue and plasma as early as 12 hours post infection. The generation of a recombinant form of SmLeish allowed us to demonstrate that it functions as a canonical MMP and requires activation of the pre-protein prior to increased MMP activity. We then determined that siRNA-mediated knockdown of SmLeish resulted in fewer parasites surviving in the snail, resulting in decreased infection kinetics. When examining how this reduction in survival occurred, we determined that SmLeish reduced M-line, but not BS-90 haemocyte chemokinesis, resulting in a decreased rate of sporocyst encapsulation *in vitro*. Overall, this led us to determine that SmLeish is a significant factor employed by the parasite to facilitate infection and establishment within its intermediate host.

An increase in transcript abundance for SmLeish coinciding with the emergence of cercaria from the snail led us to examine a potential role of SmLeish during penetration of human skin. Shortly thereafter, I became aware of another invadolysin featured prominently in cercaria, which we termed SmCI-1. First, I used western blotting and immunofluorescence to determine that SmCI-1 was localized to the acetabular glands of cercaria. We then generated new recombinant forms of each invadolysin, and again confirmed that they possessed the canonical features of MMPs, including generic MMP activity, and inhibition by zinc-chelating molecules such as 1,10-phenanthroline. I then assessed the ability of both invadolysins to cleave key substrates targeted by invadolysins from other parasitic organisms and found that while SmCI-1 was capable of cleaving complement component C3, fibrinogen, gelatin, collagen type IV, and elastin, SmLeish was not. Given the ability of SmCI-1 to degrade C3, we then examined its ability to affect the complement system and found that it reduced complement mediated lysis by both the classical and

alternative pathways. The generation of SmCI-1 KD parasites demonstrated that this inhibition of the complement system resulted in increased parasite viability. In addition to this, I generated data that suggests SmCI-1 is not involved in a reduction of phagocytic activity in THP-1 and RAW 264.7 cell lines.

I then measured the effect of SmLeish and SmCI-1 to alter cytokine production in mouse polymorphic blood leukocytes, and a variety of human cell lines, and found that both invadolysins substantially reduced inflammatory cytokine production that was elicited by treatment with schistosome antigens. I then focused on SmCI-1, and after having generated a catalytically null mutant, demonstrated that reduction of key cytokines produced by human polymorphic blood mononuclear cells such as eotaxin-1, IL-5, IL-12 and IL-1β was dependent upon MMP activity, whereas the upregulation of IL-10 was not. Intriguingly, this reduction of inflammatory factors was also observed when cells were treated with SmCI-1, but stimulated with LPS, suggesting the targeting of immune signalling pathways shared between LPS and schistosome derived antigens.

Finally, SmCI-1 KD parasites were used in the infection of mice, and KD of this invadolysin resulted in lowered adult worm burden 5 weeks post infection, implicating the use of SmCI-1 by the invading larva in facilitating infection establishment.

**7.2 Significance of findings**

The studies performed significantly increase our knowledge both about how schistosomes facilitate infection in their intermediate and definitive hosts, but also further our understanding of the use of invadolysins as factors employed by parasites to circumnavigate a host's immune response. The publication of the studies contained in chapter 4 represent, to our knowledge, the first time a helminth invadolysin has ever been functionally characterized in the context of a

parasitic infection (145). It also represents the first time that a *S. mansoni* derived factor has been identified and demonstrated to influence *B. glabrata* haemocyte movement. The work done in chapters 5 and 6 of this thesis also serves as the first examination of a schistosome metalloprotease during mammalian infection.

The molecular basis of *S. mansoni's* ability to avoid being recognized and encapsulated by *B. glabrata* haemocytes has been a key topic of investigation for some time (95,99). Previous investigations into *S. mansoni* targeting of haemocyte function have often been performed using whole larval transformation products or fractions of the same, and have shown that only certain fractions inhibit haemocyte movement, and that this effect is dependent upon the resistance profile of the snail strain being used (146). The characterization of individual *S. mansoni* proteins has been limited to that of a 28 kDa protein that reduces protein synthesis in M-line, but not 10-R2 haemocytes, and a 108 kDa protein that functions as a superoxide anion scavenger, both of which were only known by their molecular weights (147,148). Chapter 4 of this thesis identifies a specific factor responsible for the previously observed phenotype wherein susceptible snail haemocyte movement is reduced by sporocyst LTPs, whereas resistant snail strain haemocytes are not. Additionally, it serves as the first time a protein with a known identity has been shown to influence haemocytes.

In the case of SmCI-1, the work presented in chapters 5 and 6 of this thesis represent a long overdue examination of the function of the second most abundant protein released from *S. mansoni* when penetrating into mammalian skin, given it's initial discovery in 2006 (175). The discovery that SmCI-1 protects schistosomula from the activity of the complement system, while also altering cytokine output in various cells, highlights a role in immune evasion (**Figure 7.1**). Given that schistosomes are vulnerable to killing by humoral and cellular factors early during their

infection of mammals, the knowledge that SmCI-1 participates in downregulating key processes that can kill the invading larvae is important when thinking about how to kill schistosomes. Our SmCI-1 KD schistosome infections of mice further confirm the importance of this molecule to invading larva.

Finally, the work presented in this thesis is significant in that it expands our knowledge of the function and use of invadolysins. Typically, invadolysins employed by parasites have been studied exclusively in kinetoplastid parasites, and this work is the first to examine them in the context of parasitic helminths. My thesis research demonstrates that while parasite derived invadolysins can have similar functions between different types of parasites (e.g. degradation of complement component C3, cleavage of host ECM), the use of invadolysins have likely evolved to suit the needs of the parasite in question (e.g. downregulation of eotaxin-1 has not been observed when examining kinetoplastid parasite invadolysins). Additionally, our work demonstrates that parasites can use different invadolysins to survive in their intermediate and definitive hosts, while leishmania invadolysins have been shown to be important for survival only in their mammalian host, and not in the sand fly (301,312).

### 7.3 Future research

The research undertaken for my doctoral thesis focuses on two invadolysins made by *S. mansoni,* and their involvement during different stages of development in the schistosome life cycle. While this thesis clearly demonstrates the role of SmLeish in facilitating infection in *B. glabrata*, as well as a clear immunomodulatory role of SmCI-1 in mammalian systems, work remains to be done to further our understanding of invadolysins and schistosome immunomodulation more broadly. Given that 7 invadolysins are highly upregulated during the larval stages of *S. mansoni* prior to penetration in the skin (203), as well as the observation that 6

leishmanolysin like proteins have been identified in protein extracts from *S. japonium* cercaria and schistosomula (179), characterization of these other invadolysins during infection or other life cycle stages may be merited, but I will focus on suggestions to further our understanding of SmLeish and SmCI-1 specifically.

Future studies I envisage for SmLeish predominantly focus on (i) determining how SmLeish alters M-line, but not BS-90 haemocyte movement, as well as (ii) examining the possible role of SmLeish in targeting key *B. glabrata* humoral factors responsible for haemocyte engagement of developing sporocysts. Identifying the factor(s) that SmLeish cleaves in order to reduce immune cell chemotaxis as well as the differences in these factors between strains of *B. glabrata* would allow for a more comprehensive understanding of host/parasite interactions at the molecular level, and potentially aid in the selection of naturally resistant snail populations. Examining the possibility that SmLeish could also target humoral factors for destruction would also aid in generating a more complete understanding of the molecular interface between sporocysts and the *B. glabrata* immune system.

Future studies I envision for SmCI-1 include (i) development of specific inhibitors for SmCI-1 for functional studies, (ii) continued determination of SmCI-1 cleavage targets and binding partners, (iii) an investigation into SmCI-1's effects on cell function, (iv) further examination of SmCI-1's involvement in survival *in vivo*, and finally (v) studying the effect of immunization against SmCI-1. While the work presented in this thesis greatly expands our knowledge of the use of this invadolysin by invading cercaria, the experiments above would allow for a more complete understanding of its role in parasite survival in the skin.

### 7.3.1 Determine how SmLeish alters M-line, but not BS-90 haemocyte movement

SmLeish might alter immune cell movement in a few different ways. The first is a direct cleavage of the components of the extracellular matrix. A *Drosophila melanogaster* invadolysin has been implicated in cell migration, while GP63 has been shown to help leishmania migrate through ECM (Matrigel)-impregnated 8-µm-diameter pores (Becton Dickinson, Franklin Lakes, N.J.), both of which support a role for invadolysins in ECM cleavage.

To assess this, one might begin by evaluating the ability of SmLeish treatment to cause adherent granulocytes isolated from both M-line and BS-90 snails to detach from a surface. This could be performed using commercially available 96 well plates, and cell adherence could be quantified over time by visual examination of adherent cell numbers. Given the role of the ECM in attaching to substrate surrounding a cell, detachment of M-line granulocytes, but not BS-90 granulocytes, could help point to a degradation of the ECM. Should this prove fruitful, the synthesis of M-line and BS-90 ECM components for *in vitro* cleavage assays may help to determine whether difference in such alleles corresponds to an ability of SmLeish to affect the ECM of haemocytes from different strains of *B. glabrata*.

The next possibility is that SmLeish is cleaving a cell surface receptor responsible for recognizing parasite derived molecules. To test this, the ability of SmLeish to alter chemokinesis in response to a variety of stimuli should be assessed. Possible stimulants include *E.coli*, zymosan, *S. mansoni* lysate, and *Echinostoma paraensei* lysate. While our study shows that SmLeish reduces chemokinesis in response to fMLP and sporocyst LTP, an increased number of stimulants would help determine whether targeting of surface receptors for destruction is a function of SmLeish. There is some precedent for such a study, given that *L. major* GP63 differentially effects monocyte and neutrophil movement towards many stimulants (328).

### 7.3.2 Examining the possible role of SmLeish in targeting key *B. glabrata* humoral factors

In other work I helped perform during my doctoral degree that is not contained in this thesis, we demonstrated the capacity of *B. glabrata* TEP, FREP2, and FREP3 to form a complex that was capable of facilitating sporocyst death by both humoral factors as well as in a haemocyte-dependent manner (109). This complex was so important to the *B. glabrata* immune response that addition of these three factors was sufficient to render M-line plasma and haemocytes as deadly as their BS-90 counterparts. Given our observation that SmCI-1 is capable of cleaving human complement component C3, the snails very own equivalent, BgTEP, is a prime candidate for cleavage by SmLeish. This could be done by performing the cleavage assays I have established for this thesis, using the recombinant *B. glabrata* factors produced for our other studies.

### 7.3.3 Development of specific inhibitor for SmCI-1 for functional studies

A specific inhibitor of SmCI-1 would allow for an easier way to study the effects of endogenous SmCI-1 in cercaria, without the need to overly rely on knockdown methods. An inhibitor of SmCE has previously been designed, and has been employed to show that the cercarial elastase is highly involved in penetrating mammalian skin, with an 80% reduction in penetration occurring after SmCE inhibition (174). A combination of mass spectrometry and N-terminal proteomics might allow for a specific inhibitor of SmCI-1 to be designed, which would be crucial given the non-specific nature of 1,10-phenanthroline, as well as the capacity of this inhibitor to kill schistosomes (344,446).

An alternative to the development of a specific inhibitor would be the generation of an inhibitory monoclonal antibody. Unfortunately, the polyclonal antibody we generated failed to inhibit SmCI-1 activity. Alternatively, generating a polyclonal antibody to the peptide-sequence

corresponding to the HEXXH active site of SmCI-1 might serve as an alternative to generating a monoclonal.

Such a tool would prove useful in conducting *in vitro* examinations of cercaria and newly transformed schistosomula. To begin, an inhibitory monoclonal would allow for an examination of SmCI-1 effects on plasma-mediated killing using an assay such as that presented in this thesis, especially given that SmCE inhibition has already been shown to render cercaria more prone to destruction by human plasma (449). Additionally, given that primary granulocytes have been shown to kill developing schistosomula at a higher rate when plasma is added, inhibition of SmCI-1 could highlight a role in avoiding opsonization of the parasite to avoid recognition by eosinophils and neutrophils (154,155).

### 7.3.4 Determination of SmCI-1 cleavage targets and binding partners

While the work done in this thesis does examine several known cleavage targets of invadolysins and demonstrates that SmCI-1 can in fact cleave host ECM components, complement component C3, and fibrinogen, a more comprehensive analysis of all of its substrates would go a great ways in gaining a complete understanding of its abilities. To this end, a subtiligase-mediated N-terminomics approach would be the most preferred method of finding substrates, as whole cell lysate from numerous cell types could be treated with our recombinant, and then analyzed for newly generated N-termini indicating likely substrates of our protease (446,450). Such work would be important as it would give insights as to any potential surface receptor cleavage, or degradation of an intra-cellular signalling molecule, either of which may help explain the results of our cytokine arrays. Alternatively, likely signalling pathways such as the TLR-MyD88 pathway and their activation could be assessed via in vitro cleavage assays of key components such as NF-κB, which have been previously shown to be a substrate for at least one invadolysin in leishmania (326,451).

This could also be assessed using TLR4 KO mice, or potent inhibitors of TLR4 signalling such as TAK-242.

In addition to cleavage substrates, determining binding partners of SmCI-1 would also be important, given that our inactive mutant form of the enzyme was still capable of eliciting the production of IL-10 despite no MMP activity. Co-immunoprecipitation could be performed on both cercarial E/S products and leukocyte lysate using our α-SmCI-1 polyclonal antibody to search for potential PRRs capable of recognizing the invadolysin. Alternatively, extracellular signal-regulated kinases (ERK) phosphorylation in cells exposed to our recombinants could be assessed, given their involvement in IL-10 signalling (447).

### 7.3.5 Investigation into SmCI-1's effects on cell function

While we have assessed some key functional roles of SmCI-1 in the work presented in this thesis, many other cell based functional assays should be conducted to further understand the role of this invadolysin in affecting the immune response. Primarily, an examination of the potential ability of this MMP to reduce the respiratory burst response in primary neutrophils, as well as the possibility that it could affect eosinophil degranulation should be examined, given their ability to kill schistosomula *in vitro* (421)**.** Support for such a possibility exists, given that GP63 reduces respiratory burst responses to opsonized zymosan, but not PMA, in both monocytes and neutrophils (328). Such investigations could be conducted using established methods for reading $H_2O_2$ production, such as the 2,7-Dichlorofluoreceine assay used in this thesis to assess HL-60 differentiation, as well as established methods for measuring eosinophil peroxidase activity using the peroxidase substrate, O- phenylenediamine, as performed by Adamko and associates (424,452,453).

Another possible cell assay to conduct would be to assess the effect of SmCI-1 on macrophage polarization. Given the high levels of IL-10 produced by our recombinants, it is possible that the protease is somehow driving macrophages towards an M2 phenotype. Some other schistosome-derived factors have been either shown or hypothesized to do this (229,263,265). Addition of recombinant SmCI-1 to human polymorphic blood leukocytes, followed by flow cytometry using M1 and M2 markers, could help bring to light any involvement in macrophage polarization.

Finally, chemokinesis assays should also be performed on a variety of cell types to see if SmCI-1 reduces immune cell migration, given that SmLeish was shown to do so to snail haemocytes (145).

### 7.3.6 Further examination of SmCI-1's involvement in survival *in vivo*

The generation of SmCI-1 KD parasites renders studies the role of invadolysins *in vivo* a reasonable task. While we have already determined SmCI-1 to be involved in infection, other techniques such as skin punches for subsequent immunohistochemistry could be performed in order to see where parasite death in the skin is occurring. It could also be used to see if penetration kinetics are altered in SmCI-1 KD parasites. Finally, it would allow us to examine whether a lack of SmCI-1 and its downregulatory effects on host cytokine production result in a greater influx of leukocytes to the surface of the larvae.

### 7.3.7 The effect of immunization against SmCI-1

Human populations feature a natural antibody response to SmCI-1, albeit one that is significantly lower than those seen to other key parasite antigens (191). Given the results of our mouse infection assays demonstrating a role for SmCI-1 in infection establishment, it would be

interesting to determine if immunization against this invadolysin could generate a suitable amount of antibodies capable of blocking the function of the protease, thereby protecting a host from infection. Previously established vaccination protocols to test this have already been established for both mouse and baboon model systems, with appropriate safeguards against spurious results (74,82,190).

### 7.4 Potential application of findings

The findings of my thesis work have potential applications in three different regards. The first two focus around schistosomiasis itself, and how to better conduct control and elimination efforts, while the third is the possibility of exploring invadolysins as therapeutic targets.

The first way in which these findings may apply to real world control efforts in schistosomiasis endemic areas focuses on the effects of SmLeish in *B. glabrata*. While mass drug administration strategies have been successful in reducing the morbidity associated with schistosome infections, they have not been overly effective at reducing the number of individuals worldwide requiring treatment, given the ineffectiveness of praziquantel in preventing infection (42,58,454). In order to develop more successful control programs, the elimination of the snail intermediate host has been shown to be the most effective way to reduce human infection (62). Instead of mass use of molluscicides such as niclosamide, the selection of resistant snail populations has been put forwards as a possible alternative with less potential impacts on the environment, and has already been proven in principle by demonstrating that the introduction of a resistant strain of *Biomphalaria tenagophila* to pre-existing populations reduced population-wide susceptibility to *S. mansoni* infection (455). To move this idea even further, the genetic alteration of resistant snail populations via gene-drives has been put forward (456). Knowing that sporocysts use SmLeish to survive the immune response in *Biomphalaria*, while also understanding that this

effect differs between resistant and susceptible snail strains, could eventually allow for the snail factors targeted by SmLeish (once they have been identified) to be genetically altered in order to increase resistance profiles amongst snail populations.

The second method by which the work presented in this thesis may apply to the control of schistosomiasis is by suggesting SmCI-1 as a potential vaccine candidate. Although the affects of SmCI-1 on infection *in vivo* remain to be fully elucidated, the protein's role in attacking the complement system, as well as its ability to reduce key inflammatory factors (IL-1β and IL-12) as well as key cytokines for the recruitment and development of eosinophils (IL-5, eotaxin-1), all suggest an important role in survival in the skin. Although humans have been shown to mount an antibody response to SmCI-1, it has not been shown to be particularly strong (191). Besides this, the precise type of antibody response to schistosome antigens has been highlighted as important (89). The examination of SmCI-1 as a vaccine employing a variety of different adjuvants may be able to elicit the production of a higher number of neutralizing antibodies that could impact infection in the skin. Such a vaccine would be somewhat atypical, as instead of targeting the parasite for destruction, it's predominant focus would be one of neutralizing the immunomodulatory effects of the invadolysin.

Finally, SmCI-1's ability to reduce inflammatory cytokines and elicit the production of IL-10, even as a catalytically inactive form, suggest that it may be an interesting possible tool for combatting chronic inflammatory diseases. Many groups have attempted to utilize parasite-derived factors in order to reduce excessive inflammation in mammalian models (407,441,442,444), and the identification of SmCI-1's anti-inflammatory abilities suggest that schistosome invadolysins are a group of molecules worth examining in this context. Given that SmLeish was found to have some similar anti-inflammatory properties, while failing to cleave structural molecules and

complement component C3, examination of the other 5 invadolysins generated by *S. mansoni* during cercarial development would be worthwhile in order to see if any of them are capable of altering different cytokine output, while lacking the potentially harmful side effects that could be caused by the degradation of other host factors.

### 7.5 Summary

The studies presented in my thesis demonstrate that invadolysins are key factors employed by schistosomes to alter immune responses in both *Biomphalaria glabrata*, as well as mammalian hosts. SmLeish was shown to be a determinant of host/parasite compatibility in *S. mansoni* infection of *B. glabrata*, as it reduced haemocyte movement in M-line, but not BS-90 snails, resulting in reduce parasite encapsulation, and reduced infection kinetics. In the context of mammalian infections, SmCI-1 was found to be more relevant, as it cleaved key human immune-related proteins, while SmLeish did not. SmCI-1 reduced the activity of the complement system, reduced the production of key immunostimulatory cytokines, and induced the production of IL-10, all of which suggest a role in immune evasion in the skin. Collectively, these studies show that *S. mansoni* employs unique invadolysins during the initial infectious stages of its life cycle in both its intermediate and definitive hosts in order to create an immunological milieu in which it may thrive. These studies exist as the first known investigations into invadolysin use as immunomodulatory factors by parasitic helminths.

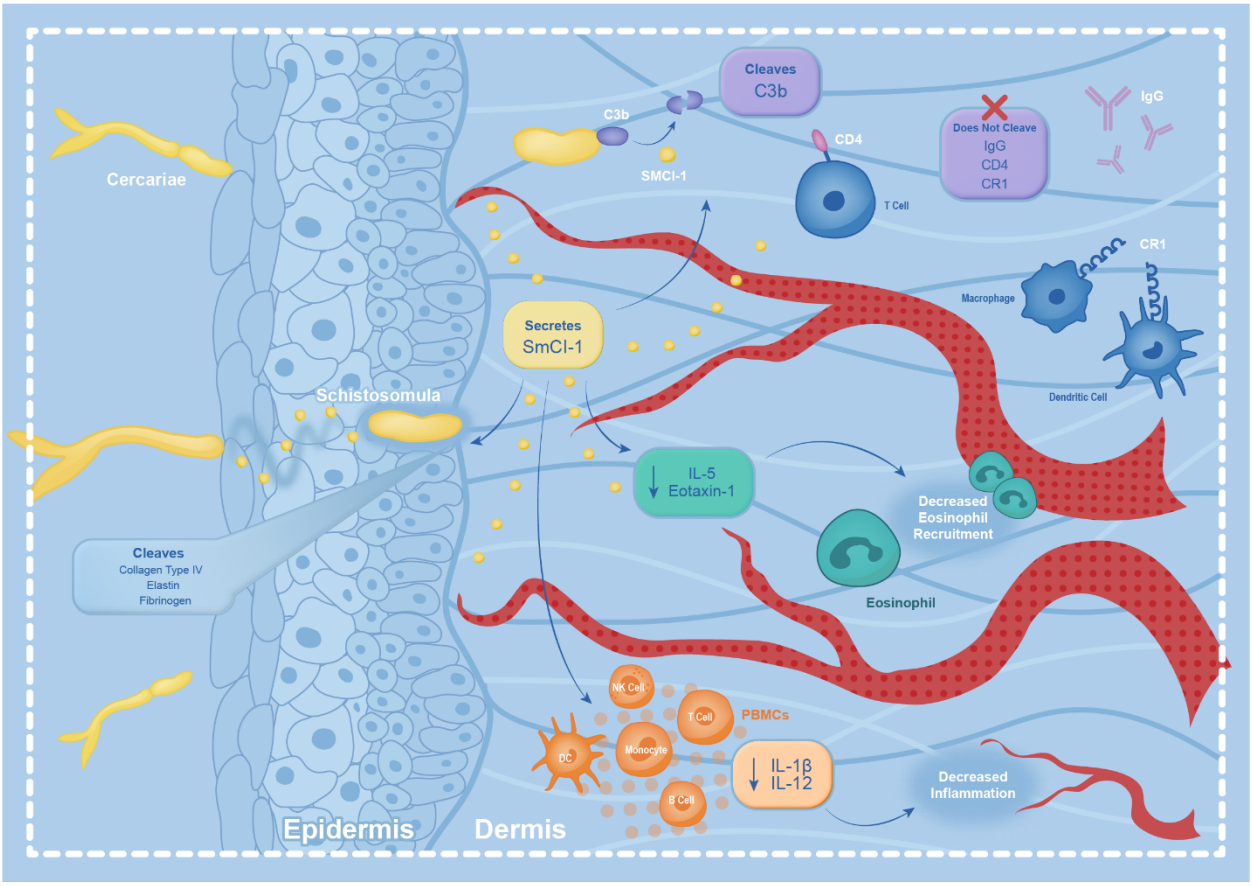



**Figure 7.1. SmCI-1 possesses numerous mechanisms of action**

SmCI-1 possess numerous mechanisms of action that suggest a key role in host penetration and survival during the initial stages of intra-human infection. These include the degradation of host ECM factors involved in the integrity of the skin. It also includes the targeted cleaving of complement component C3b, which might lead to the reduction of complement mediated lysis of the parasite, as well as reduced opsonization of the larvae, resulting in less immune cell engagement. It also includes the reduction of IL-5 and Eotaxin-1 production, an observation suggesting a role in reducing eosinophil recruitment and development. Finally, the reduction of IL-1β and IL-12 caused by SmCI-1 is indicative of a reduction in inflammation in the skin, which might explain the lack of swelling and site-specific inflammation seen during most *S. mansoni* infections.

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