Schistosome Vaccine Development Using the Local Immune Response

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Amendments

ADDENDA

Chapter 2:

P.32, Fig. 2A: *Reviewer comment:* Negative control samples would be helpful. *Rebuttal:* Negative control ASC-probes were not performed in this assay since they did not contain sufficient antibody, I agree it would have been useful for completeness, however it would not change the conclusion drawn.
Reviewer comment: How were the optimal ASC-probe concentrations determined? *Rebuttal:* These were determined by titrating ELISA. The concentration which provided the largest dynamic range in a range of samples was used as outlined in section 2.2.6.

P.35, Fig.4: *Reviewer comment:* Why was western data from only 1 rat shown? *Rebuttal:* Reactivity from only one rat was used to highlight the profound differences within one individual in each tissue region.

P.38, 2nd last paragraph: *Reviewer comment:* How can an unchallenged rat have high egg reactivity? *Rebuttal:* the unchallenged rat did have a prior primary schistosome infection as outlined in section 2.2.3.

General reviewer comment: a valuable experiment would have been to perform proteomics techniques to identify novel antigens, such as immunoprecipitations with ASC-probes followed by LC-MS/MS. *Rebuttal:* This was attempted but due to the scarcity of larval schistosome material and very low immunoglobulin concentration in ASC-probes, this was unsuccessful.

Chapter 3:

P.56, Fig. 6: *Reviewer comment:* Why was IL-4 not measured in the skin? *Rebuttal:* Additional data has been generated and has been published (see McWilliam *et al.* (2013) PLoS Negl Trop Dis 7(9): e2460.doi:10.1371/journal.pntd.0002460). Additional data includes the transcript levels of IL-4 and IL-10 in buffalo skin samples.

P.59, Fig. 8: *Reviewer comment:* What happens to the cytokine ratio when the cells are stimulated with schistosome antigen? *Rebuttal:* While it would have been interesting to stimulate the buffalo lymph node cells with schistosome antigen, a non-specific stimulation was chosen because of the scarcity in larval material.

P.60, Section 3.3.4: *Reviewer comment:* Could you not have cultured cells in media depleted of Ig? *Rebuttal:* the media supplement was described as 'serum-free', however unfortunately the media still contained a residual amount of Ig which interfered with the sensitive ELISA used.

P.64, Fig. 11: *Reviewer comment:* Why were lung LN responses not analysed? *Rebuttal:* These were not shown because they recognised relatively few antigens, unlike the skin LN samples shown here.
Reviewer comment: Why were the 2 low MW antigens not isolated and sequenced? *Rebuttal:* Unfortunately these antigens were not identified. This was due to the scarcity of larval schistosome material and very low immunoglobulin concentration in the ASC-probes.

P.67, last paragraph: *Reviewer comment:* Protection in humans is complicated...there are however naturally resistant people who are protected by a Th1 driven process. *Rebuttal:* While the majority of studies have correlated Th2 responses with protection against schistosomiasis (as described here) there is a report of a group of individuals in an endemic area of Brazil in which protection is linked with a Th1 response (see ref. 117). This is addressed in Section 6.2.4.

Chapter 4:

Reviewer comment: It would be helpful to elaborate on the antigens on the array. How were they selected? *Rebuttal:* in this chapter a protein microarray was employed to identify novel antigens. Antigens were selected for inclusion on the array by the following criteria: high sequence homology among schistosome species; expression in the important larval stage; predicted or known to be localised on the parasite surface; and limited sequence similarity with mammalian orthologues (see ref. 231).

Reviewer comment: The use of the array provides a fundamental limiting flaw as you can only identify what has been selected on the array. *Rebuttal:* While this limitation is true of the approach taken here, there are advantages over conventional proteomics techniques, which are biased to the most abundant proteins and possibly least immunologically meaningful cytosolic proteins (231).

Reviewer comment: It is not explained why the buffalo ASC probes were not also screened against the array. *Rebuttal:* Unfortunately due to the unavailability of arrays at the time, this could not be performed, however it is currently underway.

Chapter 5:

Reviewer comment: It appears that the vaccine trial was only conducted once. Typically one conducts at least two independent mouse trials before reaching a conclusion on protective efficacy (or not) given the difficulty in reproducing these outcomes. *Rebuttal:* Unfortunately additional trials were not possible due to the fact that we did not have the capability to perform vaccine trials in our laboratory, and relied on collaborators' resources. A new vaccine trial is currently underway however.

Reviewer comment: Why choose a formulation that is likely to drive IgE responses? I understand that IgE is associated with PZQ-induced resistance in human endemic areas, but a vaccine that induces an IgE response is fraught with danger. *Rebuttal:* DEAE-dextran was selected as an adjuvant in the vaccine trial because it induced a type-2 response including specific IgE. For a human vaccine this is not ideal, and has led to vaccines being abandoned (see ref. 150), however for veterinary vaccines, such as the buffalo, this is less of a concern.

P.97, Section 5.2.2: *Reviewer comment:* Why use just two vaccinations? Many other schistosomiasis mouse trials use three vaccinations. *Rebuttal:* In this experiment, a third vaccination was not possible due to time constraints. The study highlights the significant difference in immune response induced by each adjuvant, and this result is highly unlikely to be different between two or three vaccinations.

P.97, Section 5.2.2: *Reviewer comment:* Was the recombinant protein cleared of LPS prior to use in T cell stimulation assays? *Rebuttal:* The recombinant Sj-L6L-1 was not cleared of bacterial contaminants which may be present (such as LPS). While such contaminants may influence the immune response to the antigen, each adjuvant group had the same antigen (and potential contaminants). Since the responses were significantly different, the adjuvants clearly had a greater effect at skewing the immune responses than contaminants.

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Summary

Schistosomiasis is one of the most prevalent infectious diseases, and contributes to the persistence of poverty in endemic regions due to its high level of morbidity. It is caused by several related species of the parasitic flatworms, known as schistosomes or blood flukes. Despite control efforts, schistosomiasis is still widespread in many regions. It has proved difficult to control, particularly for the Asian zoonotic *Schistosoma japonicum* which has the additional problem of animal reservoirs such as the water buffalo. A vaccine for humans and/or buffaloes could significantly help in the control of schistosomiasis and has been a goal for schistosome research for decades.

The aim of this thesis has been to contribute towards developing a vaccine against *S. japonicum*. The intramammalian larval stages are considered the targets of natural and experimental immunity and therefore were the focus of this research. Due to difficulties in working with this microscopic and transient developmental stage no larval specific vaccine candidates have been found. The antibody secreting cell (ASC)-probe technique has been used to identify larval vaccine targets for other helminths, therefore this promising method was applied here to study the immune response against the schistosome larvae as they penetrate, migrate and develop in the host. In this method, cells from lymph nodes draining the sites of larval migration (the skin and lungs) are cultured to allow the *in vivo*-induced ASC to release antibody into the culture media. This technique allows the capture of the local antibody response induced by the migrating larvae, effectively providing an immunological 'spotlight' on this transient developmental stage.

Initially the ASC-probe technique was successfully implemented in a rat model of schistosomiasis, indicating the applicability of the method to this disease and providing valuable samples for antigen identification. Then the technique applied to the natural host, the water buffalo. The type of immune response induced against the migrating schistosome larvae was also investigated, in the first study of its kind in this important reservoir host. The captured local antibody response from the rat experiment was subsequently used to identify potentially novel vaccine targets using a newly-generated protein microarray. Several vaccine targets were identified, and one antigen in particular, *S. japonicum* Ly-6-like protein-1 (Sj-L6L-1), showed promise and was further characterised. It was found to be primarily transcribed in the developing larval stages, uniquely antigenic in the lung site in rats, present in the tegument and antigenic during early water buffalo infection. Finally, recombinant Sj-L6L-1 was tested with various adjuvant formulations, and a vaccine trial using a type-2 response-inducing adjuvant was performed. Although the antigen failed to protect mice against schistosomiasis, there is scope to try to improve the vaccine by optimising the formulation or recombinant production, and this is discussed.

The research presented in this thesis provides a comprehensive investigation into the complex immunobiology of the migrating schistosome larvae, by investigating this developmental stage in both an experimental model and a natural host, and then identifying and characterising a novel larval vaccine target. It is hoped that this thesis will contribute towards developing an effective anti-schistosome vaccine.

General declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper accepted for publication in a peer reviewed journal and 1 unpublished publication. The core theme of the thesis is vaccine development against Asian schistosomiasis. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Physiology under the supervision of Professor Els Meeusen and Associate Professor David Piedrafita.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2 and 3 my contribution to the work involved the following:

Thesis chapter:	**Publication title:**	**Publication status:**	**Nature and extent of candidate's contribution:**
Chapter 2	The developing schistosome worms elicit distinct immune responses in different tissue regions	Accepted	I was responsible for the design of the majority of the experiments, conducting part of the rat schistosomiasis experiments and all of experiments testing the ASC-probe samples (apart from the glycan microarray screening). I analysed the data and wrote the manuscript with assistance. **Contribution: 70%**.
Chapter 3	The Local Immune Response of the Chinese Water Buffalo, *Bubalus bubalis*, Against Migrating *Schistosoma japonicum* Larvae	Submitted	I was responsible for the design of the majority of the experiments, conducting part of the buffalo experiments and the majority of experiments generating immunological samples. I analysed the data and wrote the manuscript with assistance. **Contribution: 80%**.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: .....

Date: .....

Contributions by others to the thesis

Professor Els Meeusen and Associate Professor David Piedrafita supervised the candidate, and contributed to the design of the experiments, data analysis and interpretation of the results, and provided academic and editorial advice, and critically revised the manuscripts and thesis.

Professor Don McManus and Dr. Patrick Driguez contributed to the design of particular experiments, data analysis and interpretation of the results, and critically revised the manuscripts. Dr. Patrick Driguez also performed the screening of the protein microarray and assisted with the data analysis featuring in Chapter 4, and critically revised the manuscripts. Mary Duke assisted in the parasite infection experiments and parasite material collection. The screening of the glycan microarray was performed by the Consortium for Functional Glycomics (www.functionalglycomics.org). The eosinophil and mast cell counts were performed by Fiona Tegart and Dr. Melissa Burke prepared the cDNA samples featuring in Chapter 3.

Papers published or submitted during candidature

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McWilliam, H.E.G., Driguez, P., Piedrafita, D., McManus, D.P., Meeusen, E.N.T. Novel immunomic technologies for schistosome vaccine development. *Parasite Immunology*, **2012**, 34: 276-284. (Review)

Young, A.R., Barcham, G.J., **McWilliam, H.E.G.**, Piedrafita, D.M., Meeusen, E.N. Galectin secretion and binding to adult *Fasciola hepatica* during chronic liver fluke infection of sheep. *Veterinary Immunology and Immunopathology*, **2012**, 145: 362-367.

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Thank you Els, for your constant guidance and encouragement – I hope I have acquired some of your enthusiasm and talent for research during the past few years. And thank you for always saying 'yes' to my plans for overseas trips, and even suggesting some I hadn't thought about. I am grateful to take away a strong understanding of what research is about from you.

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Thank you Professor Don McManus for welcoming me into your laboratory in Brisbane, for your willingness to collaborate, to always quickly read over manuscripts, and offer guidance during my many sojourns up north.

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During my time in Yueyang, China, I am grateful for the significant help I received from the staff at the Hunan Institute for Parasitic Diseases. Thank you to Dr. Yuesheng Li for making the water buffalo experiments possible and for welcoming me in China, and for the other staff at the Institute. In particular, thanks to Zheng Mao for the experimental assistance and also for welcoming me into Yueyang culture.

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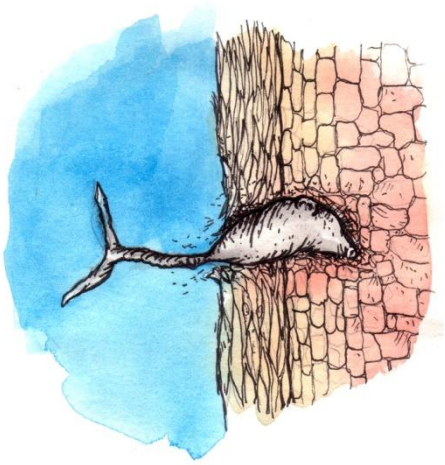
Abbreviations

A+D	Aluminium hydroxide and diethylaminoethyl dextran
ADCC	Antibody-dependant cellular cytotoxicity
Alum	Aluminium hydroxide
ASC	Antibody secreting cell
cDNA	Complementary deoxyribonucleic acid
DD	Diethylaminoethyl dextran
DPI	Days post-infection
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbant assay
EST	Expressed sequence tags
g	Gravity
GPI	Glycophosphatidylinositol
h	Hour
H&E	Haematoxylin and eosin
HRP	Horseradish peroxidise
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton
LacNAc	poly-*N*-acetyllactosamine
LDS	Lithium dodecyl sulphate
Le^X	Lewis X
LN	Lymph node
Ly-6	Lymphocyte Antigen 6
min	Minute
NI	Non-infected
NDUFV2	NADH dehydrogenase ubiquinone flavoprotein 2
OD	Optical density

p-value	Probability value
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20 (0.05% v/v)
PMA	Phorbol myristate acetate
PZQ	Praziquantel
qPCR	Quantitative real-time polymerase chain reaction
r	Recombinant
RB	Relative binding
RNA	Ribonucleic acid
r_S	Spearman's rank coefficient
RT	Room temperature
Sj-L6L-1	*Schistosoma japonicum* Ly-6-like protein-1
SM-PBST	Skim milk in phosphate buffered saline with 5% Tween-20
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI	Signal intensity
TBS	Tris-buffered saline
Th1/Th2	T helper cell type ½
TMB	3,3',5,5;-tetramethylbenzidine
Trx	Thioredoxin
TSP-2	Tetraspanin-2
UV	Ultraviolet
WHO	World Health Organisation

Chapter 1: Introduction



1.1 Introduction

Schistosomiasis is one of the world's most prevalent infectious diseases, with over 200 million people infected and nearly 800 million at risk of contracting the disease (1). Known also as bilharzia (named after Theodor Bilharz who first described the African schistosomes) or snail fever (2), it is caused by several related species of the parasitic flatworms, known as schistosomes or blood flukes, and is currently listed as one of the neglected diseases which debilitate communities in tropical developing countries (3). There is evidence of schistosomiasis in ancient Chinese (4) and Egyptians (5) indicating a long history with humans, however our respective ancestors are likely to have been co-evolving for tens of millions of years (6). Despite this strong history together, schistosomiasis remains a devastating infection and contributes to poverty in endemic regions (3). Therefore the elimination of the disease is an important goal (7). Control programs have had success in some regions, although these measures are not always effective due to limitations such as fast reinfection rates after treatment and persistence of animal reservoirs (8). In some areas the prevalence has actually increased due to changing environmental conditions and human migration (9). A vaccine would significantly aid in the eradication of schistosomiasis (10-12), however, despite decades of research and significant advances, an effective vaccine remains unavailable.

This chapter discusses the basic biology of schistosomes including their complex life cycle, their distribution, the pathology they cause and their mechanisms of immune evasion. The focus of this thesis is on Asian schistosomiasis, caused by *Schistosoma japonicum*, therefore the zoonotic aspect of this parasite is considered. Subsequently, the current control methods are considered with their successes and limitations. Finally the possibility of vaccination against this parasite is reviewed, by summarising the evidence for immunity, previous attempts at vaccine development, and outlining the case for larvae being the most important vaccine target. Finally a novel technique is introduced which may provide a new approach towards achieving this important goal of an effective vaccine against schistosomiasis.

1.2 Biology of schistosomes

Schistosomes belong to the class of parasitic flatworms (platyhelminthes) known as trematodes. While most trematodes are hermaphroditic, schistosomes are the only members which are dioecious and have separate sexes. There are at least 80 species of schistosomes, each adapted to specific animal hosts (6), however this chapter will focus only on schistosome species relevant to humans. Schistosomes are complex parasites which require two diverse hosts, an intermediate freshwater snail and a definitive host, usually a mammal. This close adaptation has resulted from a long history of co-evolution, which is likely to trace as far back as when our early mammalian forerunners diverged from cold-blooded reptiles (6).

1.2.1 Distribution of the major schistosome species

There are five main species which infect humans. Their distribution (Fig. 1) is largely restricted by the ecology of their specific intermediate amphibious snail hosts (13) and therefore near fresh water sources: *S. mansoni* and *S. haematobium* are endemic in Sub-Saharan Africa and the Arabian peninsula, with the former also present in South America; *S. japonicum* is endemic in the marsh and lake regions of China, particularly along the Yangtze River basin (9) and mountainous regions (14), and parts of the Philippines and Indonesia (13); *S. mekongi* is found in a restricted pockets along the Mekong river in Southeast Asia (15); and *S. intercalatam* is present in a small region of West Africa (16).

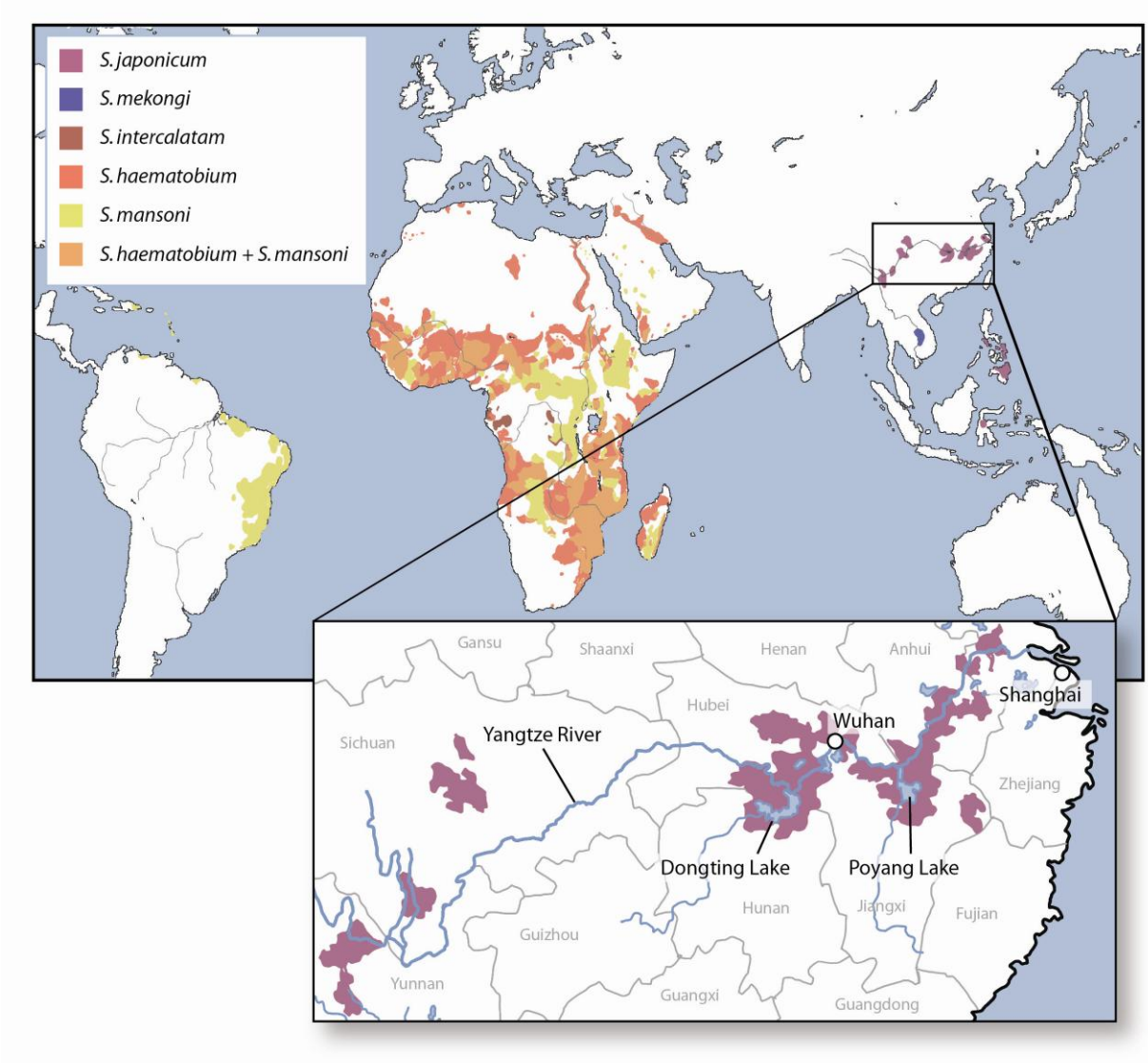


Figure 1: Distribution of the major human schistosome species

Global illustration adapted from Gryseels *et al.* (13), and endemic regions for schistosomiasis in China (2000) adapted from Spear *et al.* (17).

Conservative estimates place the number of infected people with these species at around 207 million (1), although it is possible that in fact up to three fold this number are living with the direct effects of schistosomiasis (18). Reasons for this possible underestimate are that many chronic infections are subclinical and escape detection (19) and current diagnostic techniques are not sensitive to detect low infections (18). By far the majority of schistosomiasis-infected individuals arex in Africa, where around 90% of the cases are found (20). There are around 1 million cases of Asian schistosomiasis in China, with 50 million at risk of infection (9, 21). Although *S. japonicum* infects comparatively fewer people than the African species, its control is still considered one of China's main public health priorities along with AIDS, tuberculosis and hepatitis B (22). Schistosomiasis has its greatest impact on morbidity rather than mortality, leading to disability, delayed development in children, and ultimately contributing to poverty (3).

1.2.2 Life cycle

The life cycle of schistosomes is as complex as it is fascinating. It requires navigating through the tissues of two extremely divergent hosts, as well as traversing the aquatic gap between them (Fig. 2). This section describes the life cycle with particular focus on the entry and intra-mammalian development of schistosome larvae. The development of *S. mansoni* has been more thoroughly characterised in the literature, although studies on *S. japonicum* are referenced where available. All human *Schistosoma* spp. have similarities in penetration and migration. Also both *S. japonicum* and *S. mansoni* finally dwell in the mesenteric veins, unlike *S. haematobium* pairs which end their migration in the urinary blood vessels (13).

The free-swimming larval form, the cercariae, emerges from the infected snails in large numbers, searching for the skin of their mammalian definitive host. The cercariae has a thick carbohydrate rich outer layer, known as the glycocalyx, which protects it from osmotic shock as it enters freshwater (23). Using their bifurcated tail, they swim towards the surface of the water to increase the chances of contacting an individual (24). To locate their host the cercariae use a variety of signals, such as chemical and thermal signatures of skin and also water disturbance (24-25). The detected skin components induce the cercariae to secrete the contents of several glands (26), which initially allow skin adhesion via a mucus-like secretion (24). Then the cercariae gain entry into the skin by the proteolytic activity of secreted enzymes, such as cercarial elastase (27-28). During this entry phase the tail is lost, most likely from mechanical dislocation as the tail continues to beat while the body is attached to the skin (29). The cercarial body penetrates each layer of the skin aided by lytic secretions and also repeated elongation and contraction movements (30) (Fig. 3). During this infection phase, there is a gradual loss of the outer glycocalyx, revealing a trilaminate plasma membrane (which corresponds to a single lipid bilayer) which is replaced by a heptalaminate layer (a more unusual double lipid bilayer) over several hours (31). This new outer tegument remains as a highly dynamic and protective layer through larval development and during the adult stage (32). The process when a cercaria loses its tail and glycocalyx and generates the mature tegument is collectively known as cercarial transformation, and the larvae are now known as schistosomula.

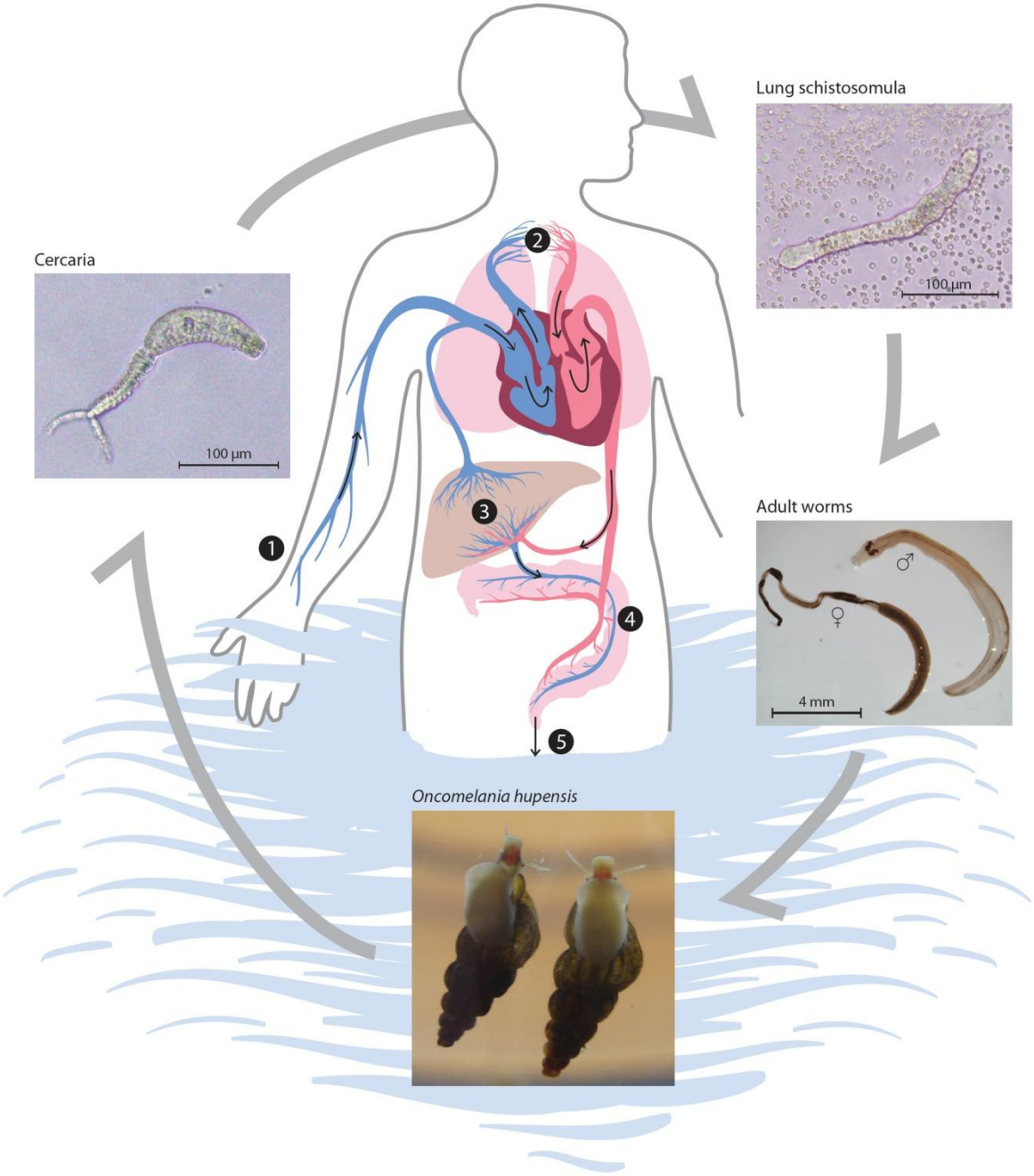


Figure 2: The Schistosoma japonicum *life cycle*

Cercariae penetrate mammalian skin (1) and transform into schistosomula as they enter the peripheral circulation. They travel with the venous blood flow to the heart and then enter the lung vasculature (2) where the larvae elongate into the lung schistosomula. After returning to the heart they continue down with the arterial blood flow and enter the liver through the hepatic artery where the larvae mature (3). Now adults, the male and female worms migrate in the hepatic portal vein and finish in the mesenteric veins (4). The attached adult pairs excrete eggs which enter the intestinal lumen and are shed during defecation (5). These eggs then hatch in the freshwater environment releasing miracidia to infect the intermediate snail host, *Oncomelania hupensis*. Larvae develop inside the snails and then infective cercariae are released.

Schistosomula can take up several days to penetrate through the skin, with *S. japonicum* larvae migrating faster through the skin than *S. mansoni* and *S. haematobium* (33-34). The majority take around two days to penetrate through mouse skin completely and exit via a dermal blood vessel (34), however He *et al.* (33) observed larvae inside these blood vessels as early as two hours post-infection, therefore there is some heterogeneity in the timing of migration after a single infection with several cercariae. The timing through skin of natural hosts such as buffalo has not been determined but it may possibly take longer due to the thicker skin barrier.

After successfully penetrating into a dermal blood vessel, the schistosomula now begin their intravascular transit (33). The venous blood flow takes them through the right side of the heart and subsequently into the fine capillaries of the lungs where they become significantly elongated (30). Movement through blood vessels is aided by the elongation/contraction movement and blood flow, and the narrow lung vasculature is thought to be the most difficult obstacle since the larvae spend the most time in this region (35). Rather than travelling *en masse*, larvae gradually trickle into the lungs over several days; the peak of *S. japonicum* schistosomula are found in the lungs around three to four days post-infection in mice (34, 36) and four days in rats (37). Again this has not been investigated in large animal models, but may be delayed by several days. Researchers at the Hunan Institute for Parasitic Diseases, with significant experience with water buffalo infection, indicate that in this host, the larvae arrive in the lungs at around day 7 post-infection (Prof. Yuesheng Li, personal communication).

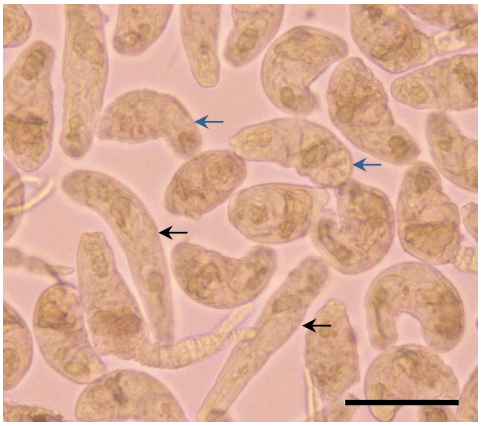


Figure 3: Schistosome larval motility

Schistosomula were obtained by manual transformation of cercariae and cultured for 24 hours at 37°C in RPMI media. Schistosomula can be seen in various stages of elongation (black arrows) and contraction (blue arrows). The scale bar represents 100 μm.

The lung-stage schistosomula then travel with the blood flow out of the lungs, returning through the heart (on the left side) and potentially make several circuits of the vascular system before appearing in the portal system (35). The larvae are then taken to the liver blood vessels via the hepatic artery (35). They can be found in the murine hepatic portal system as early as three days after infection (34) and develop and grow in the liver for a further 8-10 days (38). At around 10-15 days old, parasites begin to resemble more developed adult worms and the male and female worms can be distinguished (39). Feeding on host blood also begins around this time (40). The separate male and female worms become attached, where the male worm embraces the female in his gynecophoral canal and they then begin migrating against the blood flow through the portal vein to the mesentery. They have both an oral and a ventral sucker at their anterior end, which are used for feeding and attachment and movement along the vessel wall (41).

The mature schistosome adult worm pairs arrive and attach inside the mesenteric vessels around the intestine, where the females continuously produce eggs into the blood stream (11, 13). Secreting thousands of eggs per day (11), the females must consume significantly more erythrocytes than the larger males (40). Adult worms escape the hostile immune system through various evasive adaptations, and live typically for 3-5 years (42), but have been observed to last more than 30 years (43). In various hosts such as rodents, rabbits and Rhesus monkeys, development of *S. japonicum* worms from skin penetration to mating and egg laying takes approximately five weeks, although eggs can be detected as early as 24 days post-infection in mice (38).

While the migration of worms has finished, the journey of their deposited eggs begins. They must traverse the walls of the mesenteric vein and the intestine, to enter the gut lumen to ensure their exit from the mammalian host. To facilitate their passage across these tissues, the eggs release proteases and other products (44). Localised inflammation and a cellular immune response are induced around the eggs which further enable tissue migration (45-46). After leaving the host with the faeces, the eggs hatch and a miracidium is released into the freshwater. This free-swimming stage uses its ciliated surface to search for an *Oncomelania hupensis* intermediate host to infect, which it identifies by snail-emitted chemical cues (47). The miracidia penetrate into the snail host and multiply as sporocysts (11), and the cercarial stage is asexually produced, which leaves the snail to find a mammalian host again.

1.2.3 Schistosomiasis pathology

The disease resulting from schistosome infection is generally classed as either acute schistosomiasis, which occurs in the weeks following infection, or chronic schistosomiasis, which takes years of heavy infection to develop. The initial penetration of skin can induce a temporary rash, which is often noted in naive travellers returning from endemic regions; however it may occur in previously-exposed individuals and go unnoticed (13).

Acute schistosomiasis, known as Katayama fever, occurs in the initial phase at around 2-10 weeks following infection (48). Acute disease resulting from *S. mansoni* and *S. haematobium* infections is primarily found in previously unexposed individuals such as travellers and not subjects from endemic regions (13), whereas acute *S. japonicum* disease is more common (49) and can occur in endemic communities with a history of infection (21). It is suggested that among the human schistosomes *S. japonicum* has the highest risk of infection-related inflammation (8) which may contribute to this difference. The symptoms of acute schistosomiasis include fever, fatigue, malaise, bloody diarrhoea and a dry cough (13, 50), although it may be overlooked or misdiagnosed due to these non-specific symptoms (48). Acute schistosomiasis can also present with eosinophilia and infiltrates can be visualised by chest radiography (13). It is thought to largely begin at the onset of egg laying due to the egg antigens (21, 50-51), but may also be due to reactions against the antigens from migrating larvae and adult worms which result in circulating immune complexes (48, 50). Patients with acute disease have higher cellular responses to parasite antigens than chronically-infected individuals whose responses appear to be down-regulated (52).

Chronic schistosomiasis is caused by the immune response that ensues against schistosome eggs lodged in host tissues (50). Adult worm pairs produce thousands of eggs per day (11), and while many migrate across the intestinal wall and are shed by the host, a large number are swept with the flow of blood to become lodged into the fine liver sinusoid capillaries or remain in the intestinal wall (9). These trapped eggs cause a granulomatous response from the host, involving the recruitment of monocytes, neutrophils and eosinophils, which are replaced by fibrocytes and the deposition of collagen fibres (53). In the short term, the granulomas appear to serve a host-protective role, particularly in the liver. The egg secretions are toxic to hepatocytes but remain contained within the granuloma (54), and the eggs are eventually destroyed (53). However with prolonged years of heavy infection these granulomas result in extensive hepatic and intestinal fibrosis and collagen deposition which is the major pathology of chronic schistosomiasis (13, 50).

Liver damage by the egg granulomas is primarily mediated by hepatic inflammation and periportal fibrosis, causing a restriction of blood flow (50). Presinusoidal inflammation can cause hepatomegaly, and the reduced blood flow through the fibrotic hepatic portal vein causes portal hypertension and eventually splenomegaly and gastrointestinal varices which can rupture in severe cases (13, 50). Intestinal damage by eggs trapped in the gut wall results in inflammation, ulceration, micro-abscess formation and diarrhoea (50). Blood is often found in the stool from intestinal lesions, and this may contribute to the anaemia which is common with schistosomiasis, although other factors such as splenomegaly and inflammation may also promote anaemia (55-56). Schistosomiasis has its greatest impact on morbidity rather than mortality, leading to disability and delayed development in children (3). However death may result from ruptured gastrointestinal varices induced by portal hypertension (13). *S. japonicum* causes more severe hepatic disease in comparison to the other major schistosome species (49).

1.2.4 Masters in the art of evasion

One of the most intriguing aspects of the schistosome life cycle is how the adult worms manage to survive inside mammalian host for such an extended period. From skin penetration and transformation into schistosomula, to the final attachment of the adults in the mesenteric veins, the schistosomes are surrounded by a hostile immune system. Under normal circumstances, the host would recognise such a foreign body and mount an immune response to reject it, however schistosome worms have several adaptations to ensure their survival for decades (43). Much work has been done to understand these mechanisms of immune evasion although there are still unanswered questions on the precise details (32).

The main adaptation attributed to the worm's ability to avoid immune-mediated clearance lies at the schistosome tegument, which is a highly dynamic and protective layer (57), represented in Figure 4. In ultrastructural studies, the *S. japonicum* tegument is visible as an undulating surface of pits and invaginations and around 5 μm thick (58). It is composed of a continuous cytoplasmic layer around the worm called a syncytium, and this is covered by an unusual double lipid bilayer (32). The outermost of these membranes is called the membranocalyx, which is likely to be a protective layer to shield parasite antigens from the host

(32). Below this is the apical membrane which more closely resembles the plasma membrane of animal cells (59). While there are few mitochondria and cellular machinery in the syncytium (58), protein synthesis and vesicle packaging occurs in the nucleated cell bodies located under peripheral muscle layers and these are connected to the syncytium by cytoplasmic channels (57).

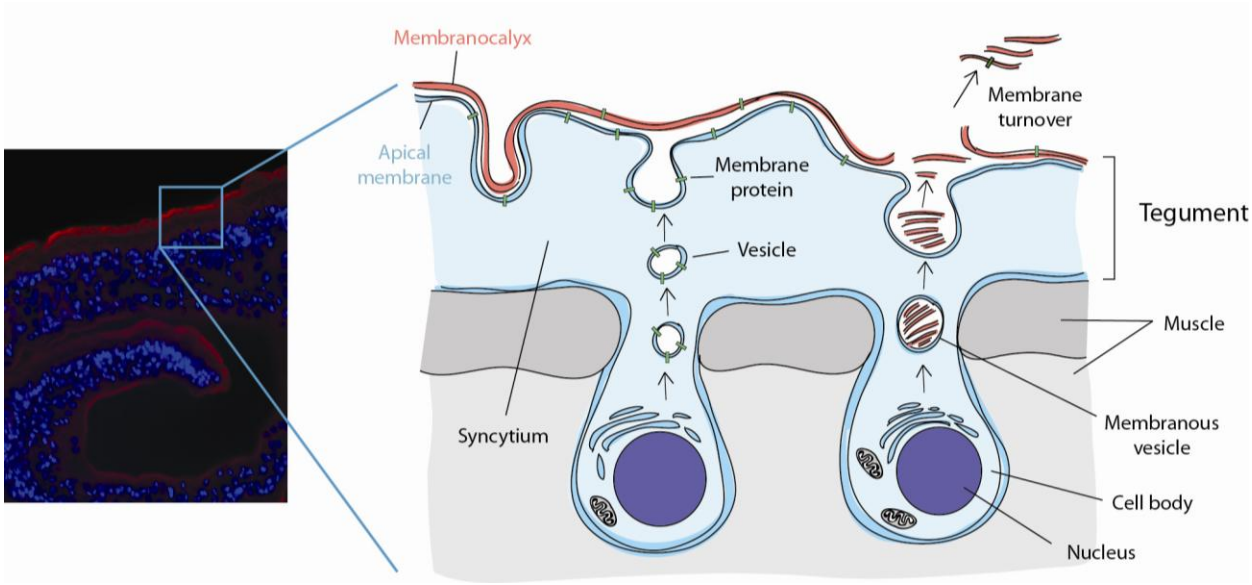


Figure 4: The schistosome tegument

Male schistosome worm section (transverse) immunofluorescently labelled with immune rat serum, detected with Alexa Fluor 647 conjugated to anti-rat IgG (red). The tegument surface is strongly recognised by rat serum. Nuclei stained with DAPI (blue) showing the tegument layer separated from underlying nuclei by the syncitial layer. On the right hand side, a diagram of the dynamic schistosome tegument is depicted with the vesicles allowing turnover of the membranocalyx and apical plasma membrane, showing the potential location of membrane proteins (green rectangles).

Numerous secretory vesicles are found in the syncytial layer, which originate in the cell bodies below and carry either structural components (proteins and carbohydrates) or packages of membranocalyx to the apical surface (32, 59). When these latter vesicles fuse with the apical plasma membrane they release fragments of the membranocalyx, which self-assemble at the surface (59). This permits the continual recycling of the outer layer, which is regularly sloughed off (58). Since the vesicle-bound membranocalyx does not derive from the endoplasmic reticulum membranes, it is postulated that it may not contain transmembrane or membrane-anchored proteins (such as glycophosphatidylinositol (GPI) anchors) (59). Therefore the current view is that the membranocalyx is a lipid-rich barrier containing few proteins, and functions to hide parasite antigens

present in or on the apical membrane (Fig. 4) (59-60). However since these two closely-apposed membranes cannot be reliably separated there is still some uncertainty which proteins are on the outermost tegument membrane (60-61).

The membranocalyx is a prominent evasive adaptation for these parasites, and is a unique feature of blood-dwelling trematodes (62). In addition to acting as a physical barrier, this membrane continuously sheds (58), with labelling studies indicates that it is replaced after around four to eight hours (59, 63), and evades the immune response by simultaneously shedding any bound antibody and reducing leukocyte adhesion (32). There is also evidence of endocytosis from the surface, which may be a way for the parasite to internalise tegument-bound antibodies and avoid immune recognition (58).

Another technique the worms use to avoid immune recognition is by acquiring host molecules on their surface, which are likely to mask any schistosome antigens (32). This is thought to occur by the possession of receptors for specific host molecules, or simply by the passive accumulation of host molecules into the tegument membranes (32). Immunoglobulins of various classes have been identified decorating the worm surface (57, 64) although these are bound by their Fc region rendering them ineffective for complement fixation or leukocyte effector function (65). Also blood-group antigens from erythrocytes have been found on the tegument (66). Host proteins with functional significance have also been found, such as the mouse complement inhibitor Crry (59) which could interrupt the complement cascade.

These adaptations which enable schistosomes to avoid immune-mediated clearance make the prospect of generating a vaccine extremely daunting, and help to explain why, after decades of research, a vaccine is still not available. Nevertheless, there are several lines of evidence to suggest that vaccination is an achievable goal and that tegument proteins are still promising vaccine candidates (61). By identifying and then vaccinating with the right surface molecules, we may overcome the schistosomes' evasive defences.

1.3 Transmission of *S. japonicum*

Schistosome transmission occurs where the freshwater habitat of the intermediate snail host overlaps with that of humans. Each schistosome species develops inside a unique genus of snails, with *S. japonicum* infecting only *Oncomelania* spp.; *O. hupensis hupensis* in China (9), and *O. quadrasi* in the Philippines (67). For the other schistosomes, *S. haematobium* and *S. intercalatum* share snails of the genus *Bulinus*; *S. mansoni* infects *Biomphalaria* sp.; and *S. mekongi* infects *Neotricula* sp. Due to fluctuating flood levels and temperatures seasonal variation significantly influences *S. japonicum* transmission in the lake and marsh regions along the Yangze River, resulting in changes to the freshwater snail populations and human activities (4, 9). The two high transmission areas, the Dongting and Poyang Lake regions in the Yangze River basin, have similar transmission dynamics with two peak periods of human transmission (9, 68). The first peak is when the rainy season begins around April, and the water levels and temperatures rise creating favourable conditions for *O.*

h. hupensis snails. Then, the second peak is after the height of flooding and the water levels subside in autumn, ending in November (4), when the snails hibernate in the mud for the dry winter season (9) (Fig. 5). Between these two peaks little transmission occurs in the Poyang Lake regions, whereas it continues at a low level in the Dongting Lake region (9). Human infections occur with occupational and social activities such as fishing, farming, clothes washing and swimming (9). In the Dongting Lake region, most of the water contact occurs in adult males (4) whereas for African schistosomiasis adolescents appear to have more exposure (69).

Figure 5: The Dongting Lake region marshlands

A. The Dongting Lake bed, near Matang Town, November 2011. The water levels have subsided to leave an expanse of grass-covered marshlands with sporadic ponds. B. A 'cow pat' suggests a recent bovine visitor, as water buffaloes frequently graze these fields, with infected animals contaminating the marshlands. C. A resident *Oncomelania hupensis* snail hidden beneath the grass in the muddy marsh, with its characteristic ribbed shell.

Unlike the other human schistosome species, *S. japonicum* is a zoonotic parasite and can invade a wide variety of mammalian hosts which complicates its transmission and control. Over 40 species can be infected; however it is the domestic animals which are important for human transmission (70-71). Around 100,000 bovines are likely to be infected (21) and of these the domestic water buffalo, *Bubalus bubalis*, is considered to

be the most important animal reservoir of schistosomiasis in China (9, 72-73) where in the lake and marsh areas it has been shown to account for up to 75% of human transmission (74). Buffaloes are used extensively for agriculture in Asia and live in close proximity with humans (21). Infected buffaloes contaminate this shared environment with copious amounts of schistosome eggs (72). This is particularly the case for the Dongting and Poyang Lake regions, where water buffalo and cattle are grazed on the marshlands, contaminating the lake basin where all transmission takes place (21) (Fig. 5). Furthermore, water buffaloes are relied upon for work power and food and chronic schistosomiasis directly impacts buffalo health and productivity (75). High infection rates in buffalo have been recently reported in the Philippines (75-76), where they are also likely to contribute to human transmission. This buffalo disease reservoir significantly complicates the control efforts of Asian schistosomiasis and must be addressed as a part of an integrated control strategy before eradication can be considered (9).

1.4 Control of schistosomiasis

1.4.1 Chemotherapeutic treatment

The most widely used treatment for schistosomiasis is with the anthelmintic drug praziquantel (PZQ) which is effective against all human schistosome species (13) and has become relatively cheap in the last few decades (77). It was discovered in the mid-1970s and was a major breakthrough for the control of schistosomes and other parasitic flatworms (78). It is recommended for both acute (79) and chronic human schistosomiasis (13), and for the treatment of livestock (80). After treatment of acute disease, fever is cured within 10 days, and for chronic cases body weight as well as work capacity increases (81).

The exact mechanism of worm killing by PZQ is not entirely understood (77), although it is known to destabilise the adult tegument and cause paralysis and worm death within hours. Coincident with the PZQ-induced tegument damage is an increased exposure of parasite antigens, and early experiments suggested the host immune response aids in killing the damaged parasites (82). Also, PZQ induces the rapid uptake of calcium ions by the worms and some evidence suggests Ca^{2+} channels may be targets of the drug (77). Recently, You *et al.* (83) showed that a protein in the calcium signalling pathway was up regulated following PZQ treatment and knocking down its expression exacerbated the drug's effects.

PZQ is not effective against the immature schistosomula of around 3-4 weeks old, but can kill the earliest migrating stages and mature adults older than four weeks (84); it is most potent against mature worms older than 6-7 weeks (77). Therefore, a re-administration of PZQ is recommended after 2-4 weeks (85), although it is still highly effective after a single dose (81). While PZQ has little toxicity (81), there can be mild side effects from treatment such as nausea, vomiting and bloody diarrhoea, particularly when treating heavy infections (13); this is thought to be due to antigen released from dying worms (86).

1.4.2 Current control strategies: successes and limitations

The cornerstone of schistosomiasis control programs in China and other endemic regions of the world is PZQ (11, 81). Before the 1980s when PZQ treatment became ubiquitous, chemotherapeutic options had significant toxicity and therefore played a minor role in control programs, which were based largely on transmission control by targeting the snail vector (81). After the mid-1980s there was a shift towards morbidity control through the use of PZQ as recommended by the WHO (87). In addition to PZQ, the current national schistosomiasis control program involves health education, improving water quality and sanitation, and snail control (88)

These programs have had great success in certain regions of Asia, reducing the prevalence of *S. japonicum* by 90% since the 1950s (89-90) and in some regions elimination has been accomplished (81). Yet despite this achievement, schistosomiasis remains a serious problem in China with approximately 1 million people infected (9, 89), and certain previously-controlled regions have shown an increase in prevalence (91-92). In endemic areas the prevalence of schistosomiasis in the human population remained constant from 1995 to 2004 at around 5% (93). The highly-endemic lake regions are difficult to control because of the large population of the *O. hupensis* snail vector (81), and also the water buffalo reservoirs which contaminate the area (4). Furthermore, the spread of schistosomiasis may actually increase in the near future due to factors such as environmental changes caused by new dam construction (9), irrigation (13), climate change (94), and also large population movements (95).

The persistence of schistosomiasis in these areas can be attributed to several limitations in the PZQ-based strategy. PZQ has a short half-life, and reinfection inevitably occurs after treatment (11, 74, 95). In endemic regions the infection levels can return to baseline within 18-24 months of mass PZQ administration (50). This necessitates regular administration which adds to the financial burden of PZQ-based programs (9). Since the majority of disease transmission is due to bovines in China (74), control programs have to routinely treat both humans and livestock (13, 91), further increasing the cost and logistics of these programs.

In response to these limitations, it is suggested that effective control will require a vaccine to complement the existing schistosomiasis containment strategies (9-12, 96-99). An anti-schistosome vaccine could give long-lasting protection for humans, reducing reinfection rates, blocking transmission, and ultimately eliminating this parasite from communities. Even an effective vaccine against *S. japonicum* for buffalo alone would significantly reduce human infection and morbidity (98). Encouragingly, there is evidence that a vaccine is a valid prospect, and this is reviewed in the following section.

1.5 Vaccinating against schistosomes

An effective vaccine has been a priority in schistosome research for many years, but despite the discovery and testing of many vaccine candidates and advances in understanding protective immunity, no vaccine is currently available (9, 100). Schistosomes do not replicate inside the mammalian host, therefore a vaccine that

is at least moderately protective could reduce pathology and transmission (9). It is suggested that a vaccine which reduced the worm burden by around 40-50% could complement the existing PZQ-based control programs and aid toward elimination of schistosomiasis (12). In the context of Asian schistosomiasis, an effective vaccine for buffalo alone would significantly reduce human morbidity as well as improve buffalo health (101), and for this reason an effort has been made to develop a transmission-blocking veterinary vaccine (98).

Evidence suggests that developing a vaccine against schistosomes is possible. Studies in endemic areas show that natural immunity develops against schistosomes after years of exposure, whereas experimental models of immunity can induce exceptional levels of protection (11). Both of these phenomena implicate the incoming larvae as the most important target of immunity (11) suggesting this stage may hold the key to a highly effective vaccine.

1.5.1 Natural immunity

There is evidence from a number of epidemiological studies that anti-schistosome immunity can develop after years of exposure in humans. This phenomenon has been more thoroughly described for *S. mansoni* and *S. haematobium* (102-103) however evidence also exists for age-related resistance against *S. japonicum* (69). A study by Li *et al.* (104) involved treating over two hundred individuals from an endemic area of Hunan province in China and then monitoring them for the following two years. The reinfection intensity was highest among the adolescent groups (<19 years old) despite adults having the greatest level of exposure (Fig. 6). An earlier study in the Poyang Lake region found a similar age-related resistance with individuals younger than 21 years old having a greater rate of reinfection compared with adults who recorded a similar level of water exposure (105).

Naturally-acquired protection against all the major schistosome species in humans is linked with type-2 responses and is positively correlated with IgE levels (106-109) and Th2 cytokines (110-111). Zhang *et al.* (106) found that individuals from Poyang Lake who had higher antigen-specific IgE levels were half as likely to re-acquire schistosomiasis following PZQ treatment. The mechanism of action is thought to be effected against the incoming larvae by eosinophils using antibody-dependant cellular cytotoxicity (9, 13, 112). Both IgG and IgE can promote eosinophil-mediated killing of schistosomula *in vitro* (113-114). Furthermore, a group of individuals naturally resistant to *S. mansoni* from an endemic area of Brazil had significantly higher levels of IgE specific for the schistosomula tegument, but not for the adult antigen (115-116).

There is also evidence that individuals can show protection after repeated PZQ treatment and reinfection, in a process of 'drug-induced resistance' (117). This may result from antigen exposure following adult worm death - resistance in this case is associated with higher worm-specific IgE levels following PZQ treatment (118-119). This process may hasten the generation of 'natural' immunity by increasing the exposure of worm antigens which otherwise takes years to develop (42).

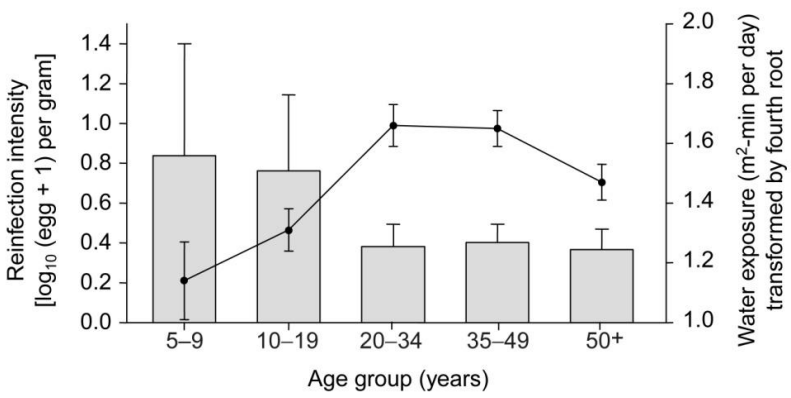


Figure 6: Age-related S. japonicum *infection intensity compared to water exposure*

Data was compiled from 213 subjects from the Dongting Lake region (1996-1998), adapted from (104). The reinfection intensity (bars) is reduced in adults older than 20 years, while the water exposure (line) is increased.

There are several reports of age-related acquired resistance against *S. japonicum* in water buffaloes, where studies show that buffaloes older than around 2 years have reduced intensity and prevalence of infection (14, 21, 72, 120). Buffaloes are also known to have a natural resistance to *S. japonicum* infection compared to other hosts, such as cattle (*Bos taurus*) (71, 121). There is a lack of studies exploring the mechanism of action in this natural host (98), and understanding this may assist in developing a transmission-blocking vaccine for buffalo.

While natural immunity does appear to occur in human endemic populations, it can take years to develop and may be only partially protective. Resistance has been consistently correlated with specific antibody levels, providing evidence that a vaccine based on the relevant antigens recognised by these antibodies could provide protection.

1.5.2 Experimental immunity

The most promising evidence that a vaccine is possible comes from experiments where animals are infected with radiation-attenuated cercariae. In this model, cercariae are attenuated with radiation (UV, gamma or X-ray) and are then used to infect various animal species, typically conferring up to 90% protection from a challenge infection with all major schistosome species (122-124). Attenuated larvae have been shown to provide protection in mice (125), rats (126-127), baboons (128) and chimpanzees (129). A high level of protection was shown in water buffaloes, where animals immunised with UV-attenuated *S. japonicum* cercariae had 89% reduction in worm burden after a challenge infection (130).

The mechanism of protection has been investigated extensively using *S. mansoni* in mice (124). After attenuated larvae infect the host, they exhibit a delayed and slightly altered migration pattern in the skin (131). Larvae may pass through the skin-draining lymph nodes during their migration to the lungs, and they do not progress beyond the lung stage (124). These attenuated larvae induce a stronger immune response than normal larvae; they release more antigen (131) and induce a more pronounced cellular immune response

(132) during their protracted migration. A mixed Th1/Th2 response is induced in the skin draining lymph nodes, with a persistence of the Th1 phenotype (132). It is thought that these skin-primed Th1 cells are recruited to the lungs following the migrating attenuated larvae, and are the effectors of immunity against challenge larvae (133).

The principal target of immunity in vaccinated mice was shown to be the lung-stage schistosomula (36, 124). Immunity in this host is thought to be largely Th1 cell-mediated although serum can transfer some protection (123). Attenuated larvae are surrounded by a dense accumulation of inflammatory mononuclear cells, including $CD4^+$ T cells and macrophages, which appear to divert the larvae from their migration without necessarily damaging them (124). The larvae appear to be often blocked or redirected out of the lung vasculature and into the airways where they eventually die (122), although macrophage-released mediators may also contribute to larval killing (123). Overall, the delayed and truncated migration including the exposure of more larval antigens is thought to result in a strong cellular and humoral response to larval antigens which is responsible for immunity (122). This vaccination model provides insight into the source and target of protective immunity, however is impractical for wide-scale use (98).

The protective Th1 response in vaccinated mice differs from the type-2 response correlated with protection in endemic human communities. However, some other experimental hosts more closely resemble the human response. For example in rhesus monkeys given repeated immunisations with attenuated *S. japonicum* larvae, the skin is the major site of larval attrition most likely mediated by eosinophils (134-135). Also schistosome resistance in the rat is suggested to be more closely related to human immunity, where anaphylactic antibodies (IgE and IgG2a) promote killing of schistosomula by antibody-dependent cellular cytotoxicity as in humans (113-114). Laboratory rats show strong resistance to reinfection after a primary infection, and this is largely antibody-mediated (113).

1.5.3 Larvae are ideal targets for an anti-schistosome vaccine

The evidence from experimental and natural immunity implicates the larvae as both a source of protective antigens, and a susceptible target of immunity. This is in line with many other parasitic helminths where the larvae are also thought to be susceptible immune targets (136-145).

As described in section 1.2.2, when the cercariae penetrate into mammalian skin they shed their outer glycocalyx revealing a single lipid bilayer, which is then protected by the membranocalyx after several hours (31). Before the defensive tegument is fully formed, the larvae are susceptible to antibody-dependant cellular cytotoxicity *in vitro* up to when they are roughly 24 hours old (146). After this time, they presumably become armed with the evasive strategies that enable them to survive as adults for decades and they no longer can be killed by the same mechanism. However the lung-stage schistosomula are still a target for immunity via potentially different mechanism; the lung vasculature provides a significant obstacle for the larvae (35) and their migration can be blocked as described for the attenuated vaccine model.

The transient vulnerability of the migrating larvae provides a brief window of opportunity when the larvae are susceptible to immune killing. The worm surface changes antigenically through development (147), and there are likely to be antigens briefly exposed during this period which may become hidden or absent in the mature tegument. These developmentally-regulated antigenic changes are potential evasive mechanisms by the parasite; by only transiently expressing surface or secreted molecules, they may escape adaptive immune responses of the host. This could explain why natural immunity takes years to develop; it takes repeated larval exposure to develop a protective response to these larval antigens.

Considering the adult worms live for decades amongst a specific antibody response apparently without harm, antigens exposed on adults may not be protective. It is possible that only antigens specific to the larvae, or uniquely exposed during this stage, will confer the high levels of protection seen with the radiation-attenuated vaccine.

1.5.4 Previous and current vaccine candidates

The past several decades of schistosome vaccine development have seen the identification and synthesis of many individual antigens. However, an effective anti-schistosome vaccine remains elusive. Table 1 lists many prominent vaccine candidates, including their expression during schistosome development and the techniques used for their discovery. While a level of protection has been seen in various animal models with these antigens (see McManus and Loukas (11)), they have failed to replicate the high level achieved with the radiation-attenuated vaccine model.

In general, parasite molecules that are exposed to the host have been considered the most promising candidates, and these include surface molecules exposed on the tegument, or secreted molecules, particularly for the larvae (148). To identify such molecules, traditional methods have used the host immune response to indicate which antigens are exposed. Immune serum and monoclonal antibodies have generally been employed, and this has resulted in numerous antigens discovered during the 1980s and 1990s. One drawback with this method is that only the most immunodominant antigens are selected, and in the case of schistosomiasis the eggs and adults are the greatest antigenic challenge (42). Hence these antigens are all present in the adult stage (Table 1).

In 1996, the WHO organised a major trial to test the six leading vaccine candidates available at the time against *S. mansoni* in independent laboratories (antigens are indicated in Table 1). Unfortunately none of the antigens reached the goal of 40% protection (149) and this was a significant setback for vaccine development. Nevertheless, one of these antigens, the 28 kDa glutathione S-transferase of *S. haematobium* (Sh28GST) has been taken through phase I and II clinical trials (96, 150). Data from these trials have not been released however (150).

More recent techniques of discovery have made use of the wealth of sequence information available since the release of genomic data for the three major human schistosomes (151-153). Several targets have been

discovered using post-genomic techniques since the early 2000s, using algorithms to predict surface-bound transmembrane antigens (11) (Table 1). Advanced proteomics methods have also been employed to explore the schistosome tegument surface (60) which have the potential to reveal exposed targets on the parasite.

Several antigens are still in development and hold promise as vaccine candidates. *S. mansoni* Tetraspanin-2 (SmTSP-2) and the 29 kDa tegumental antigen (Sm29) were identified as putative membrane proteins based on their sequences, and have shown protection in mice (150). However the *S. japonicum* TSP-2 has shown significant antigenic diversity so it is unlikely to be useful against Asian schistosomiasis (154).

Paramyosin, identified as a 97 kDa antigen originally in 1986 (155), has shown protection against *S. japonicum* in sheep, pigs and water buffalo in the range of 30-40% worm reduction (156), although higher efficacy was originally seen in mice (156). While generally considered a muscle protein, whether or not it is tegument-located has been debated (32). Gobert *et al.* (157) immunolocalised it on the surface of lung-stage schistosomula suggesting it may be briefly exposed during development. Its use as a vaccine candidate has been hampered by a difficulty in expressing it in large quantities, however this has recently been achieved and *S. japonicum* paramyosin may be tested as a transmission-blocking vaccine for water buffaloes in the near future (158).

The developing schistosomula have long been targets for vaccine development (11, 100, 148), however the majority of candidates investigated to date are not specific to these important developing stages (see Table 1). This is primarily due to the difficulties in working with schistosomula; firstly obtaining enough material for traditional antigen identification, and secondly the low antigenic challenge larvae elicit in comparison to the adult worms and deposited eggs which give an overwhelming antibody response (42). Attempts at characterising the schistosomula surface by proteomics techniques have also proven difficult because the tegument does not detach as easily as for the adults (60).

Table 1: Vaccine candidates against schistosomiasis, their stage of expression and method of discovery.

Antigen	Abbreviation	Stage expressed	Method of discovery	Year of discovery
Paramyosin	Sm97*/Sj97	Cercariae, schistosomula, adults (157)	Serum from mice vaccinated with non-living schistosomula (159) followed by cDNA library screening (155)	1986
Glutathione *S*-transferase, 26kDa	Sj26GST	All stages (11)	Serum from *S. japonicum* infected mice, used in cDNA library screening (160)	1986
Glutathione *S*-transferase, 28kDa	Sm28GST*/ Sh28GST#/ Sj28GST	All stages (10-11)	Serum raised against adult antigen extract (161), used in cDNA library screening (162)	1987
Tetraspanin, integral membrane antigen	Sm23*/Sj23	All stages (163)	A monoclonal antibody raised against schistosomula extracts (163) followed by cDNA library screening (164)	1990
Calpain (large subunit)	Sm-p80/ calpain (Sj)	All stages (11)	Serum raised against adult membrane extract, used in cDNA library screening (165-166)	1991
Fatty acid binding protein	Sm14*†/Sj14/ FABP	All stages (167)	Serum raised against soluble adult antigen extract, used in cDNA library screening (168)	1991
Triosephosphate isomerise	Sm28-TPI*/ SjTPI	All stages (169)	A monoclonal antibody raised against schistosomula extracts (169), then affinity purification and peptide sequencing (170)	1992
Irradiation vaccine antigen-5	IrV-5*	All stages (10)	Serum raised against antigens recognised by radiation-attenuated vaccine, used in cDNA library screening (171)	1992
Cytosolic superoxide dismutase	CT-SOD (Sm)	Adults primarily, schistosomula (172)	Serum raised against an adult antigen fraction, used in cDNA library screening (173)	1992
Tetraspanin-2	SmTSP-2†	Schistosomula, adults (174)	Signal sequence trapping to identify membrane proteins (175)	2003
Very low-density lipoprotein-binding protein	SjSVLBP	Adult males (176)	Sequence obtained initially from EST dataset, then a cDNA library (176)	2003
Serine protease inhibitor	Serpin (Sj)	Adults (11)	Immune serum from *Microtus fortis*, used in cDNA library screening (177)	2005
Tegumental antigen (29kDa)	Sm29	Schistosomula, adults (178)	Reverse vaccinology: mining the genome to identify membrane proteins (178-179)	2006

** Antigens from the 1996 World Health Organisation independent trial (149).*
† Antigens for which clinical trials are planned (150).
 # Sh28GST has undergone Phase I and II clinical trials (96, 150)

1.5.5 The antibody secreting cell-probe method: a spotlight on the migrating larvae

Bearing in mind the considerable potential of schistosomula as a source of effective vaccine antigens, techniques which overcome the difficulties of working with this developmental stage are required. One such method, termed the 'ASC-probe technique' developed by Meeusen and Brandon (180-181), is particularly amenable to studying migrating helminths, and has been used in a number of infections to effectively put an 'immunological spotlight' on developing larvae (181-187) (Fig. 7). In this technique, cells obtained from lymph nodes draining a particular infection site of interest are cultured, which allows the *in vivo*-primed antibody-secreting plasma cells (ASC) to secrete antibodies into the culture media. These antibodies, present in the culture supernatants (ASC-probes), are specific for the pathogen infecting that tissue region and are only present in an active infection.

ASC-probes obtained from lymph nodes draining different tissues from the same animal were shown to produce antibodies which can recognise distinct stage-specific antigens (181). Hence these ASC-probes can be considered to be a snapshot of the local antibody response, which is specific for (a) the tissue region and (b) the developmental stage of the pathogen within that tissue region. These tissue-specific ASC-probes can be used for the discovery of their target antigens, and can therefore be considered to be more relevant and directed tools for immunomic analysis compared to the complexity and non-specificity of serum antibody probes.

The ASC-probe technique was used to identify a surface antigen specific to the infective larval stage of *H. contortus* (termed *Hc*-sL3) (182), which was later found to be protective in several vaccine trials (144, 188). In these studies, ASC-probes were produced from the lymph nodes draining the abomasum, the site of parasite infection, during the larval rejection response and identified an antigenic region at 70-83 kDa, which localises to the larval surface (182). Since the ASC-probe response profile was much simpler than that obtained with immune serum, it enabled a more manageable and targeted approach for larval-specific antigen identification. Similarly, the ASC-probe technique (181, 184) was used to show that particular larval-specific antigens are recognised in distinct tissue compartments during *Ascaris suum* and *Fasciola hepatica* migration. Once again, antibodies against these antigens were not detected with serum antibodies.

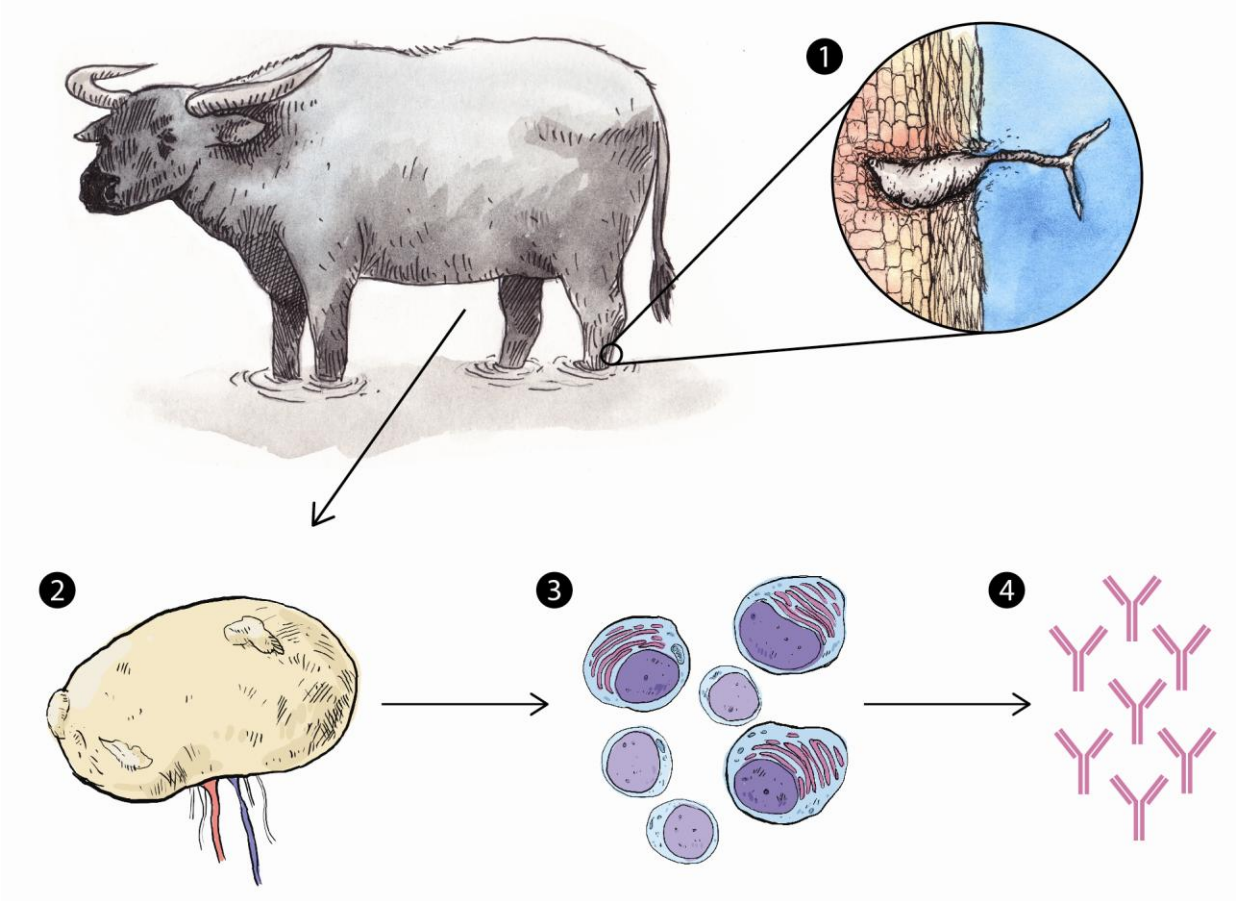


Figure 7: The ASC-probe method

The ASC-probe method allows the collection of the local antibody response induced against migrating helminth larvae, in this case *S. japonicum* cercariae (1) penetrating water buffalo skin. The draining lymph node (2) is removed and disrupted to collect the lymphocytes (including ASC) (3) which are then cultured to allow secretion of antibodies by the ASC. Antibody specific for the larval stage migrating through the site drained by the lymph node is then collected in the culture supernatant (4).

For schistosomes, as for other pathogens, antigen identification has long been performed using serum antibodies obtained from infected individuals, and has enabled the discovery of various candidates (see Table 1) which are often the most immunogenic (122). Importantly however, highly immunogenic antigens may not make effective vaccines, since the long-lived adult parasites do not seem to be affected by the high antibody titres against such molecules (189), and hence selective pressure may have forced protective epitopes to evolve towards low immunogenicity (122) or be restricted to transient parasite stages. In this respect, the ASC-probe technique offers two main advantages to the use of serum antibodies. The first is its ability to recognise antigens which have low immunogenicity, or are only transiently exposed to the immune system, which is likely to be the case for surface or secreted antigens from migrating schistosomula. By analysing the

local antibody response, it may be possible to identify antigens which are not seen when using serum as a probe, as in the previously referred studies with *H. contortus*, *A. suum*, and *F. hepatica*.

The second main advantage of lymph node- derived ASC-probes is the capacity to focus on particular tissue compartments in isolation from more immunologically dominant infection sites. Eberl *et al.* (190) showed that even a fairly significant number (2000) of schistosome cercariae used to infect chimpanzees provides a low antigenic stimulus in serum, and that the major cause of antibody production in schistosomiasis was egg deposition in the liver and intestine. Therefore to be able to focus on the antibody response caused by schistosomula alone, in isolation from that caused by the egg deposition (and even the potentially irrelevant adult response), would be a significant advantage.

This method is likely to be a promising tool for the identification of larval specific antigens for schistosome vaccine development (100). Also, by analysing the local immune responses (antibody and cellular) induced by the migrating larvae, the complex immunobiology of schistosome migration can be investigated. This can be done in experimental hosts, but in the case of *S. japonicum* a natural host such as the water buffalo can also be studied.

Once the desired tissue-specific ASC-probes are obtained, the challenge is then to use these as a tool to identify the antigens they target in the complex and scarce larval homogenates. A promising new approach is to make use of both protein or carbohydrate arrays which have become available, and provide a way to study the immunome (100).

1.6 The aims of this study

The overall aim of this study is to use the local immune response induced by migrating schistosome larvae to advance vaccine development by investigating larval immunobiology and identifying larval-specific vaccine targets.

The specific aims are:

- Establish the ASC-probe method in an experimental model of schistosomiasis and determine if migrating schistosome larvae induce distinct antibody responses at each site of migration.
    - > Chapter 2

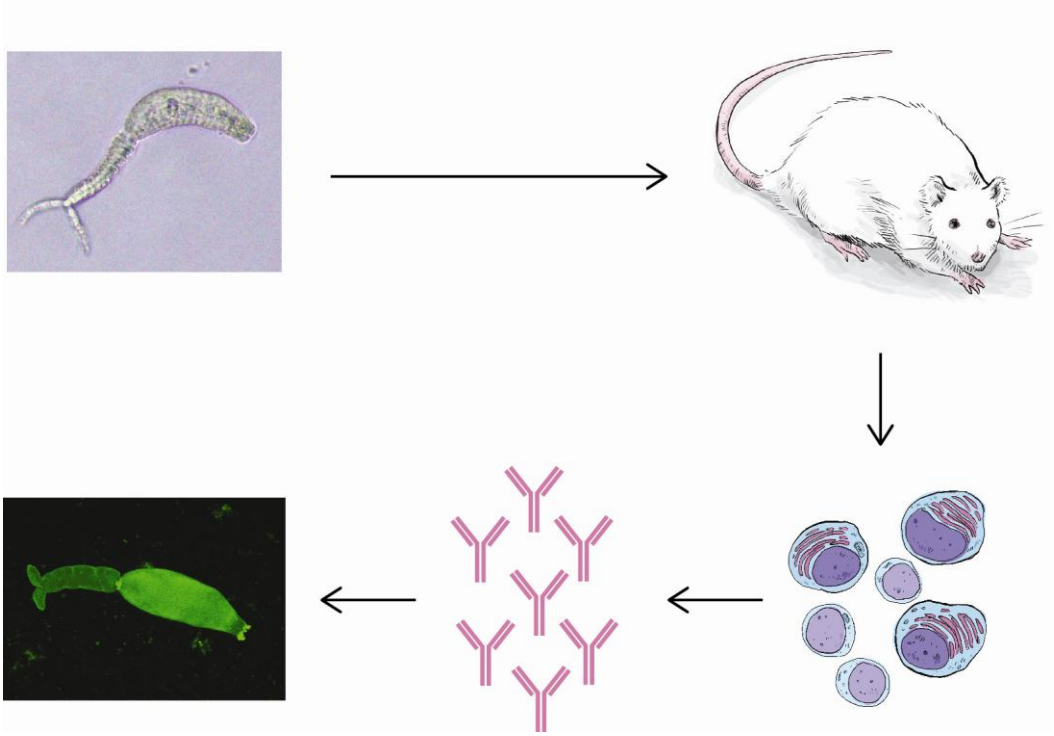
- Use the established ASC-probe method to investigate migrating larvae in the water buffalo. Also analyse the immune responses induced by the larvae in this natural host.
    - > Chapter 3

- Use these ASC-probes to identify novel and potentially larval-specific vaccine targets using novel immunomic techniques, namely protein and carbohydrate arrays.
    - > Chapter 4

- Characterise and then test any novel vaccine targets against *S. japonicum* infection.
    - > Chapter 4
    - > Chapter 5

Chapter 2:

The developing schistosome worms elicit distinct immune responses in different tissue regions



Declaration for Thesis Chapter 2

Chapter 2 has been accepted for publication in Immunology and Cell Biology:

> **McWilliam, H.E.G.**, Driguez, P., Piedrafita, D., Maupin, K.A., Haab, B.B., McManus, D.P., Meeusen, E.N.T. The developing schistosome worms elicit distinct immune responses in different tissue regions. *Immunology and Cell Biology*, **2013**, Accepted.

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution:	**Extent of contribution:**
I was responsible for the design of the majority of the experiments, conducting part of the rat schistosomiasis experiments and the majority of experiments testing the ASC-probe samples. I wrote the manuscript with assistance, as listed below.	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name:	**Nature of contribution:**	**Extent of contribution:**
Patrick Driguez	Assisted with: experimental design, execution of rat experiments at QIMR, the analysis and interpretation of results, and manuscript editing.	N/A
David Piedrafita	Assisted with: experimental design, execution of rat experiments at QIMR, the interpretation of results.	N/A
Kevin Maupin	Glycan microarray data analysis and interpretation	N/A
Brian Haab	Glycan microarray data analysis and interpretation	N/A
Donald McManus	Assisted with: experimental design, manuscript editing.	N/A
Els Meeusen	Assisted with: experimental design, execution of rat experiments at QIMR, the analysis and interpretation of results, and manuscript editing.	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate	Date	Main supervisor	Date

2.1 Introduction

Schistosomiasis remains one of the most common helminth infections, causing a chronic debilitating condition that contributes to the persistence of poverty in endemic regions (3). It has been conservatively estimated that around 200 million people are infected with schistosome blood flukes (1, 18). Limitations in the current measures to control these parasites, such as rapid re-infection rates after treatment, suggest that the elimination of schistosomiasis will require complementary strategies such as improved sanitation, elimination of the intermediate snail host and, once available, vaccination (11, 150, 191).

Schistosomes have a complex migratory path upon infecting their mammalian host, as they develop from larvae (cercariae, schistosomula) to paired adults in approximately 4-5 weeks (9), and each stage is known to vary its antigenic profile as it traverses through skin, then the vasculature of lungs, liver and intestines or bladder (depending on the species) (192). These developmentally-regulated antigenic changes are potential evasive mechanisms by the parasite; by only transiently expressing surface or secreted molecules, they may escape adaptive immune responses of the host.

Mechanisms of immunity have been proposed against specific developmental stages of schistosomes. The larval stages, principally the lung-stage schistosomula, are widely accepted as an important target of immunity in animal models and humans (193-194). Immunity against the schistosomulum larval stage has been shown to operate at least in part through antibody-mediated mechanisms which are dependent on specific isotypes (194). The radiation-attenuated larval model can result in 70-90% reduction in worm burden (124), and provides evidence that highly effective antigens are present in schistosomula (195). While experimental vaccines have mostly tested antigens present in adults, with varying levels of efficacy (11, 100), there is a lack of vaccine candidates specific to larvae (100). Therefore, a more detailed description of the complex immune response to the migrating schistosomes would be valuable, and may aid in designing a multi-stage vaccine to target antigens throughout the life cycle.

The rat is a semi-permissive host for schistosomes, and antibody-mediated immunity induced following a primary infection is directed against migrating larvae (113, 196), a mechanism similar to what is thought to operate in humans (112). In the present study, we have employed the antibody secreting cell (ASC)-probe method (180-181), a powerful technique which captures the local antibody response induced in lymph nodes draining distinct tissue regions, to focus on immune responses against distinct stages of the schistosomes, with an emphasis on the larval stages. By culturing cells of the lymph nodes draining the different sites of parasite migration after challenge with *Schistosoma japonicum*, marked differences in antibody specificity were revealed at different tissue regions. In addition isotype responses, which are indicative of immune bias (197), were distinct at each tissue site suggesting different regional immune mechanisms may be operating against the migrating parasites.

2.2 Materials and methods

2.2.1 Ethics statement

The conducts and procedures involving animal experiments were approved by the Animals Ethics Committee of the Queensland Institute of Medical Research (project no. P288). This study was performed in accordance with the recommendations of the Australian code of practice for the care and use of animals for scientific purposes, 2004.

2.2.2 Parasites and crude protein extracts

S. japonicum cercariae were shed from infected *Oncomelania hupensis* snails (from Anhui Province, P. R. China) by exposure to light for 3-4 hours in distilled water. Cercariae were collected from the surface of the water and either counted under a light microscope and used immediately for rat infections or surface staining, or collected in bulk and frozen on dry ice for preparation of antigen. Cercariae were also mechanically transformed and cultured into schistosomula for surface-staining using a similar method described by Wang *et al.* (198). Briefly, cercariae were transferred directly into warm media (RPMI +L-glutamine containing 10% (v/v) fetal bovine serum) and incubated for 2 h at 37 °C with 5% CO_2. They were then transformed by passing 25 times through a 22 gauge syringe, and after two washes were cultured for 24 h. For antigen preparations, lung-stage schistosomula and adult worms were collected as described by Gobert *et al.* (199) and stored at -80°C. Crude antigen was prepared from cercariae and schistosomula by resuspending the frozen parasites in lithium dodecyl sulphate (LDS) sample buffer (with reducing agent) (Invitrogen, Carlsbad), heating to 95°C for 10 min with vigorous vortexing, and centrifuging at 12,000 x *g* for 10 min. Adult antigen homogenate was prepared as described (200), and the protein concentration determined by BCA assay (Pierce, Rockford).

2.2.3 Experimental design and infections

Four groups of female Wistar rats (n=5) were used in this study; one non-infected control group (C) and three infected groups. The first infected group (group I) was given only a primary infection with *S. japonicum* and animals sacrificed 6 weeks post-infection. The other two infected groups were given a primary followed by a secondary challenge infection 6 weeks later, one sacrificed at day 5 (group I_5) and one at day 9 (group I_9) post-secondary infection. These two time points were chosen based on observations that *S. japonicum* schistosomula pass through the skin in the first day with the majority reaching the lungs at 4 days post-infection (37), and allowing 5 days for optimal lymph node responses to develop (185). Rats were infected percutaneously by applying the cercariae on a cover slip to the shaved abdomen for 30-40 min. The primary and secondary infections consisted of 125 and 350 cercariae, respectively.

2.2.4 Generating antibody-secreting cell (ASC)-probes

To investigate the humoral response induced by the schistosomula larvae, the axillary and inguinal lymph nodes (LN), which drain the abdominal skin, and the mediastinal LN draining the lungs, were removed. The liver-draining LN (portal node) was taken for comparison, because this tissue is stimulated by the deposited schistosome eggs and mature worm stage from the primary patent infection. The spleen was also taken since it is likely to be stimulated by secreted antigens released in the blood stream. These four lymphoid organs were carefully dissected and then teased gently in media (RPMI +L-glutamine containing 5% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml)). After two washes in cold media, viable cells were counted using a haemocytometer and trypan blue. Cells were resuspended at 3 x 10^6 cells/ml in media supplemented with 0.1 ng/ml recombinant rat IL-6 (R&D Systems, Minneapolis), and cultured at 37°C with 5% CO_2 for 5 days to allow the *in vivo* primed ASCs to release their antibodies into the culture medium. The addition of IL-6 to cultures was found to approximately double the amount of immunoglobulin (Ig) secreted into culture but did not affect isotype secretion (data not shown). Cultures were then centrifuged at 500 x *g* for 8 min at 4°C, and the supernatants (ASC-probes) collected and stored at -20°C until required.

2.2.5 Quantitation of total antibody levels in ASC-probe samples

A capture enzyme-linked immunosorbant assay (ELISA) was employed to measure total Ig levels in ASC-probe samples. Plates were coated with goat anti-rat IgG (whole molecule) (Sigma-Aldrich, Saint Louis), which binds all rat isotypes. Blocking was performed with 5% (w/v) skim milk (SM) powder in phosphate buffered saline with 0.05% (v/v) tween-20 (PBST). ASC-probes (undiluted) were added to duplicate wells then serially diluted. A standard curve was used for quantitation using purified rat IgG (Invitrogen) on each plate, and captured Ig was detected with goat anti-rat Ig (H+L):HRP (Invitrogen). TMB solution (Invitrogen) was added followed by 2M H_2SO_4, and the optical density (OD) was read at 450nm and compared to a standard curve to calculate the amount of Ig in each sample.

2.2.6 Relative isotype levels in ASC-probe samples

The relative level of each antibody isotype in ASC-probe samples was measured by capture ELISA, using reagents from the BD Pharmingen Rat Immunoglobulin Isotyping ELISA Kit. Plates were coated overnight with the capture monoclonal antibodies against rat isotypes: IgG1, IgG2a, IgG2b, IgE, IgM at 1:200; IgA and Ig2c at 1:100. Each ASC-probe sample was diluted to the same total Ig level to allow comparison of isotype level. After blocking and washing, ASC-probes were added to wells at the optimum concentration: 0.1 μg/ml for IgG1, IgG2c, IgA and IgE; 0.02 μg/ml for IgG2a and IgM; 0.04 μg/ml for IgG2b. Plates were again washed three times and anti-rat Ig:HRP was added to each well at 1:100, and the OD measured as described above. To measure antigen-specific isotype levels in serum, plates were coated with adult antigen (10 μg/ml), and serum was diluted in 1% SM in PBST (1:10 for IgE and IgM and 1:600 for total Ig). Anti-IgE and IgM

monoclonal antibodies were added (as above) and detected with goat anti-mouse IgG:HRP (Sigma-Aldrich). Total Ig was measured with goat anti-rat Ig (H+L):HRP.

2.2.7 Surface staining of live schistosome larvae

ASC-probe samples were incubated undiluted overnight at 4°C with live cercariae or schistosomula. Skin-, lung- and liver-LN ASC-probe samples from infected rats, and a control antibody sample from uninfected rats, were used. The larvae were then washed three times with media and incubated with goat anti-rat IgG(H+L):FITC (Millipore, Temecula) (1:100) for one hour at RT, followed by three washes. Fixation was then performed with 4% paraformaldehyde in PBS for 10 min at RT. Cercariae and schistosomula were then visualised using an Olympus IX81 fluorescence microscope.

2.2.8 Western blotting of schistosome extracts

Schistosome protein extracts (5 μg) were separated on 10-well 10% NuPAGE Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes, which were then sliced into individual lanes. Lanes were blocked for 2 h at RT in 5% SM-PBST. ASC-probe samples (undiluted) or serum (1:200) from one infected animal (rat 2-group I_5) were incubated with individual lanes overnight at 4°C. After three washes with PBST, 800 μl of anti-rat Ig (H+L):HRP (1:1000) was applied for 1 h at RT then washed again. Finally ECL substrate (GE Healthcare) was applied and chemiluminescence detected on Super RX film (Fujifilm).

2.2.9 Glycan array probing

Glycan array binding was performed by Core H of the Consortium for Functional Glycomics (http://www.functionalglycomics.org). The mammalian glycan array version 5.0 was used, which contains 610 glycans covalently linked to a glass slide. Undiluted ASC-probe samples from lung lymph nodes (n=3) and spleen (n=4), and serum (n=6) diluted 1:100 in binding buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 0.05% Tween-20, 1% BSA), were incubated on the array for 1 h. Arrays were washed and then incubated with goat anti-rat IgG(H+L):FITC, and the fluorescence measured.

2.2.10 Glycan array data analysis

The glycans on the array contain various combinations of common carbohydrate motifs, which are arrangements of residues commonly seen in glycobiology. To determine which motifs were recognised by the antibody samples, a program based on the 'motif segregation' method developed by Porter *et al.* (201) was used. This significantly simplifies the complex array data, and is a valuable tool to quickly identify the glycan motifs highly bound by the antibody sample screened on the array. The program ranks the motif structures by the probability that the particular structure contributes to the antibody recognition of a glycan, and this is represented by a *p*-value. Thus the lower the *p*-value, the more likely the motif is recognised by the antibody

sample. Because of the number of statistical tests performed during this analysis a stringent *p*-value ($p<0.001$) was used as the cut-off for significance.

2.2.11 Statistical analysis

To assess any differences in total Ig levels and relative isotype levels, groups were compared using one-way analysis of variance, followed by Tukey's multiple comparison post-hoc test. The significance level was set at $p<0.05$.

2.3 Results

2.3.1 The magnitude of the local antibody response follows the path of larval migration

Antibody secreting cell (ASC)-probes were generated from lymph nodes (LN) and spleens during the time of larval migration. The antibody levels gradually accumulated over the 5 day incubation period (data not shown), indicating that the antibody was secreted into culture and not from another source such as serum. The levels of total Ig at the end of the incubation period were measured in each ASC-probe sample to indicate the magnitude of the antibody response in each tissue region (Fig. 1).

For each sample type, the control group C and unchallenged infected group I had the lowest mean antibody levels, reflecting the quiescent state of the uninfected or unchallenged lymph nodes and spleens. Five and nine days after challenge, groups I_5 and I_9 generally had the highest antibody levels, reflecting active re-stimulation with migrating larvae. ASC-probes derived from skin-LN (Fig. 1A) and spleen (Fig. 1C) showed a similar profile, with the highest antibody levels at 5 days post secondary infection (group I_5), significantly higher than the primary infection rats (group I) and group C ($p=0.004$ and 0.001, respectively), reflecting initial antigenic challenge at these sites. Lung-LN ASC-probes (Fig. 1B) generally had much higher basal antibody levels than the other tissue ASC-probe samples, and showed the most variability within groups. In both challenged groups I_5 and I_9, one rat (indicated by hollow points in Fig. 1B) had dramatically higher antibody levels in lung-LN than the others with Ig levels of 19 and 36 μg/ml respectively, compared to a mean of 1.5 μg/ml in the other animals. During the dissection, the lungs of these particular rats had obvious inflammation, and were found to contain trapped schistosomula by histology (data not shown). Apart from these individuals, there was little difference in total Ig level between groups in lung-LN samples. Ig levels of liver-LN ASC-probes from groups C, I and I_5 were generally low, apart from one individual from group I (rat I-3) which had a much higher Ig level (0.88 μg/ml) than the other liver-LN samples (mean 0.12 μg/ml). This particular rat also had a significantly higher liver egg count than other infected rats (1151 eggs per gram of liver tissue compared to a mean of 109 ± 67 for the other rats), which most likely contributed to the high

antibody response in this sample. There was a consistent increase in Ig level in rats 9 day after challenge (group I_9), although this was not significant due to the outlier.

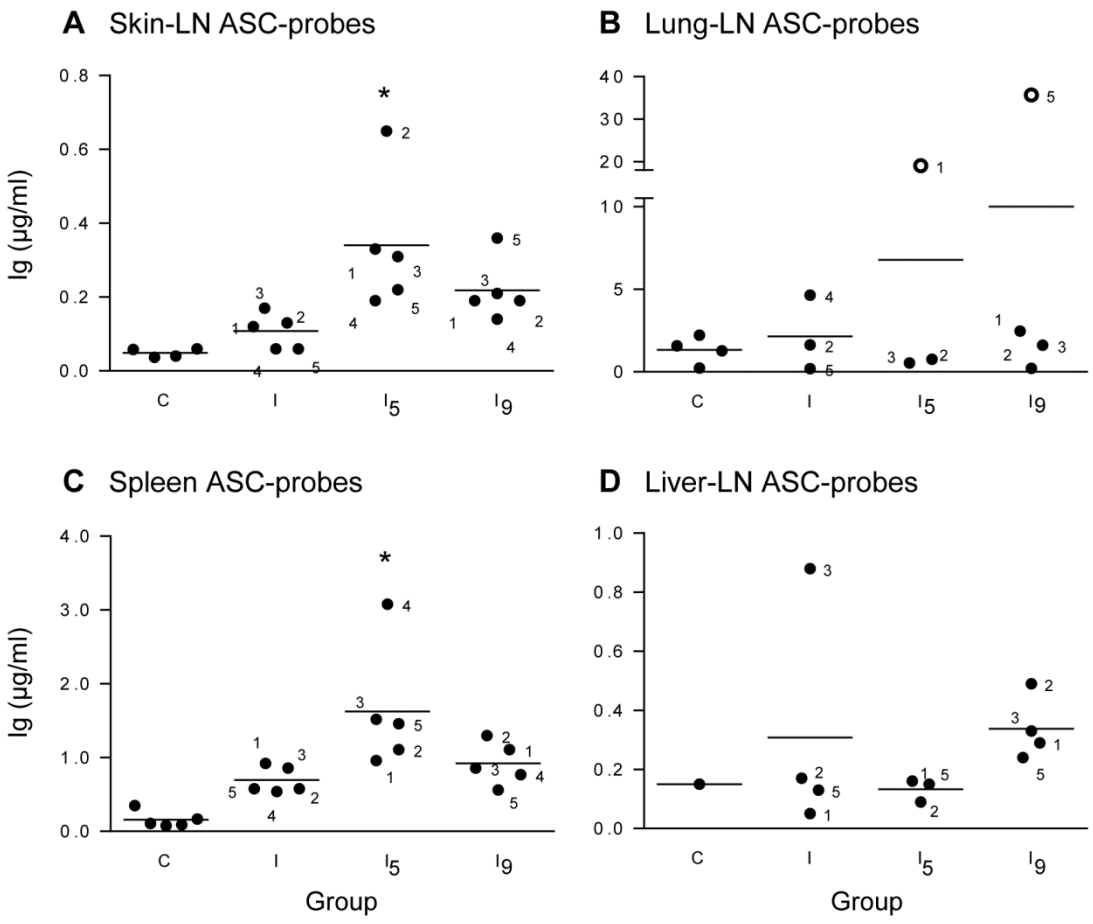


Figure 1: Total immunoglobulin (Ig) levels in each antibody secreting cell (ASC)-probe sample determined by ELISA

ASC-probe samples from skin-draining lymph node (LN) (A), lung-LN (B), spleen (C) and liver-LN (D) contain different amounts of secreted Ig, reflecting the infection challenge of the migrating parasite in each tissue region. Samples were from uninfected control (C) and from rats given a primary infection only (I) or a primary then a challenge infection and sacrificed at 5 or 9 days post-infection (I_5 and I_9, respectively). Each data point represents an individual rat sample, and infected rats are numbered for reference. Two rats which exhibited trapped schistosomula in lung tissue are indicated by hollow data points (B). Statistical significance ($p<0.05$) above C and I groups are represented by asterisks.

2.3.2 The local antibody response differs in relative isotype levels

The relative level of each antibody isotype in the ASC-probes was determined in order to characterise differences in the immune response profile stimulated in each tissue region. Six ASC-probe samples from each tissue region, adjusted for similar total Ig, were used from rats given a secondary infection (groups I_5

and I_9). The relative level of each isotype is represented by the optical density (Fig. 2). Samples from liver-LN ASC-probes had the highest IgG1 and IgE levels ($p = 0.006$ and $p < 0.0001$, respectively), and the highest levels of IgG2c (although not significant). IgG2a levels were highest in lung-LN ASC-probes (significantly above liver-LN and spleen samples; $p = 0.002$) while IgM was highest in spleen ASC-probes ($p < 0.0001$).

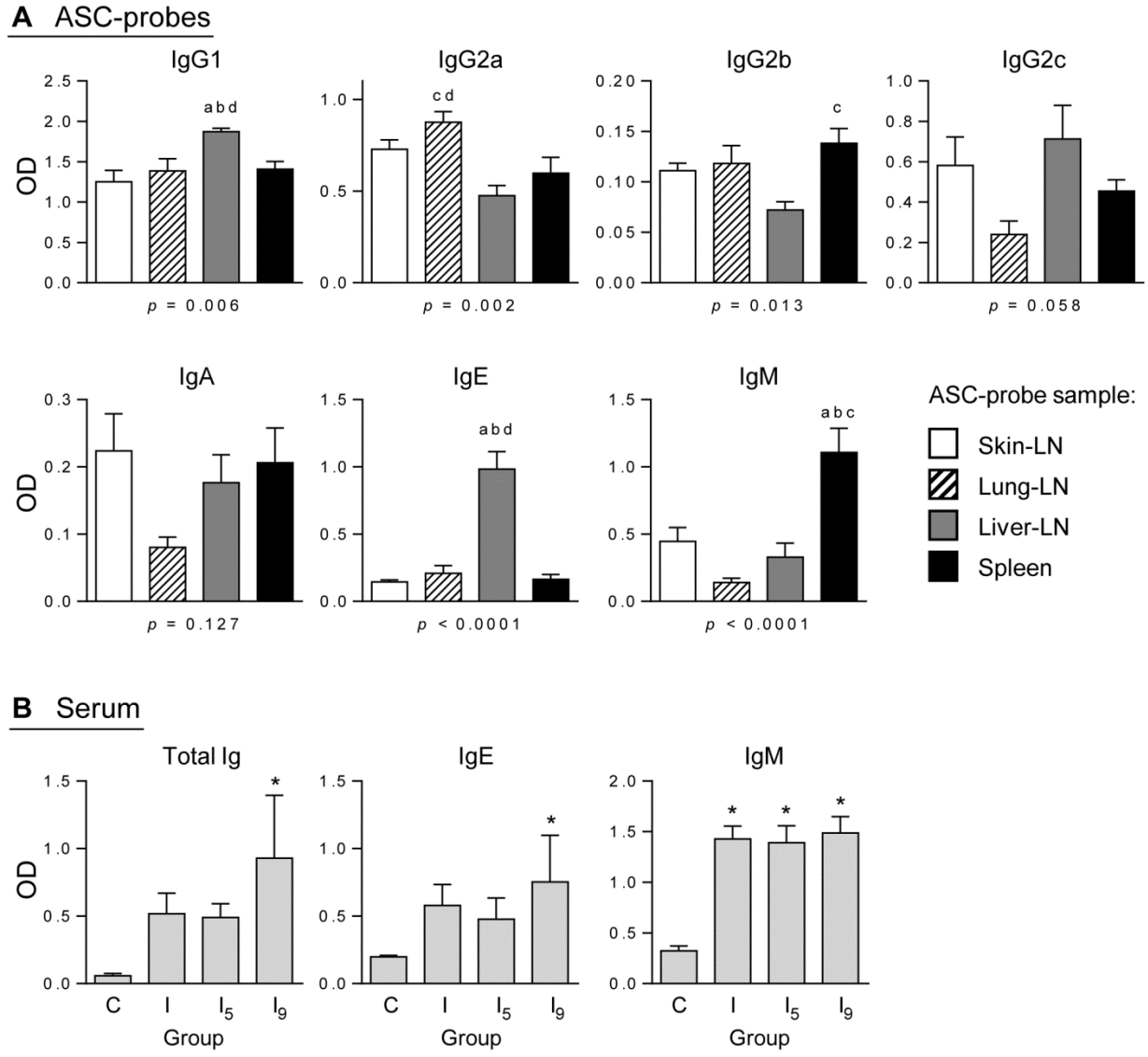


Figure 2: Each tissue region produces a qualitatively different antibody response

The level of each antibody isotype in the ASC-probe samples was measured by ELISA and shown by optical density. ASC-probe samples from each tissue region are represented: skin-LN (white); lung-LN (diagonal lines); liver-LN (grey); spleen (black). The means of six ASC-probe samples were analysed from rats with a secondary schistosome infection, standardised for total Ig levels. The error bars represent standard error of the mean, and p-values from one-way analysis of variance tests are shown underneath each graph. Statistical significance ($p<0.05$) between groups, as indicated by Tukey's post-hoc test, is represented by the letters: significantly greater than skin (a); lung (b); liver (c); or spleen (d). Statistical significance above group C is indicated by the asterisks (*).

The serum levels of schistosome-specific antibody (total Ig, IgE and IgM) were also measured for each group (Fig. 2B). All infected animals had specific antibody levels above uninfected rats, although for total Ig and IgE only group I_9 was significantly above group C. No significant differences were seen for total Ig, IgE and IgM levels between the chronically infected and challenged groups.

2.3.3 ASC-probes differentially recognise the surface of schistosome larvae

Immunofluorescent staining of live cercariae and schistosomula was performed to determine the specificity of different ASC-probes to the surface of larval stages of the parasite. The control antibody samples did not recognise the surface of either cercariae or schistosomula, as seen by the lack of a distinct outline, although some auto-fluorescence of internal structures is seen (Fig. 3D and H). Skin- and lung-LN ASC-probes from secondary infected rats strongly bound to the surface of cercariae (Fig. 3A and B). Interestingly, the liver-LN ASC-probe did not recognise the cercarial surface (Fig. 3C), although all three samples from infected rats did bind to the cercarial secretions which had adhered to the plate (data not shown). On the other hand, the surface of the schistosomula were only strongly recognised by the lung-LN ASC-probes (Fig. 3F), while the liver-LN ASC-probe showed some binding to the schistosomula ventral sucker (Fig. 3G, white arrows) which was similarly recognised by the lung-LN ASC-probes; this opening is a potential source of secretions.

2.3.4 Antigen recognition by ASC-probes

To further assess the antigen specificity of ASC-probes and serum, western blotting was performed using crude schistosome antigen extracts prepared from three developmental stages (cercariae, lung-stage schistosomula and adults). The local antibody response from different tissue regions of the same rat recognised different sets of antigens in each life cycle stage (Fig.4), and this was a consistent finding for two additional rat samples (not shown). Some bands were recognised in multiple ASC-probes, but some were only recognised by particular tissue samples and were unique to each parasite stage. In particular three low-molecular weight bands (10-16 kDa), and one high molecular weight (>200 kDa) band, were recognised only by skin-LN ASC-probes in cercarial extracts (Fig. 4, lane 2 arrows). These antigens were not recognised by other ASC-probes or serum, and importantly the same skin antibody sample did not recognise these bands in the other life stages, indicating that they are exposed only in the skin region and are cercariae-specific. One band of approximately 56 kDa was recognised by skin-LN ASC-probes only in lung-stage schistosomula, but not the other stages by the same antibody sample (Fig. 4, lane 8 arrow) and is also likely to be specific to the early larval stage. The lung ASC-probes recognised high molecular weight antigens with strong binding, particularly to many cercarial bands. For the spleen ASC-probes, a high-molecular weight smear was characteristic rather than multiple specific bands, and this smearing was also seen in the serum-probed strips (particularly lanes 6 and 18). In general, the antigens recognised by serum comprise those bound strongly by the ASC-probes but not the previously mentioned stage-specific bands, and serum showed the highest level of background binding.

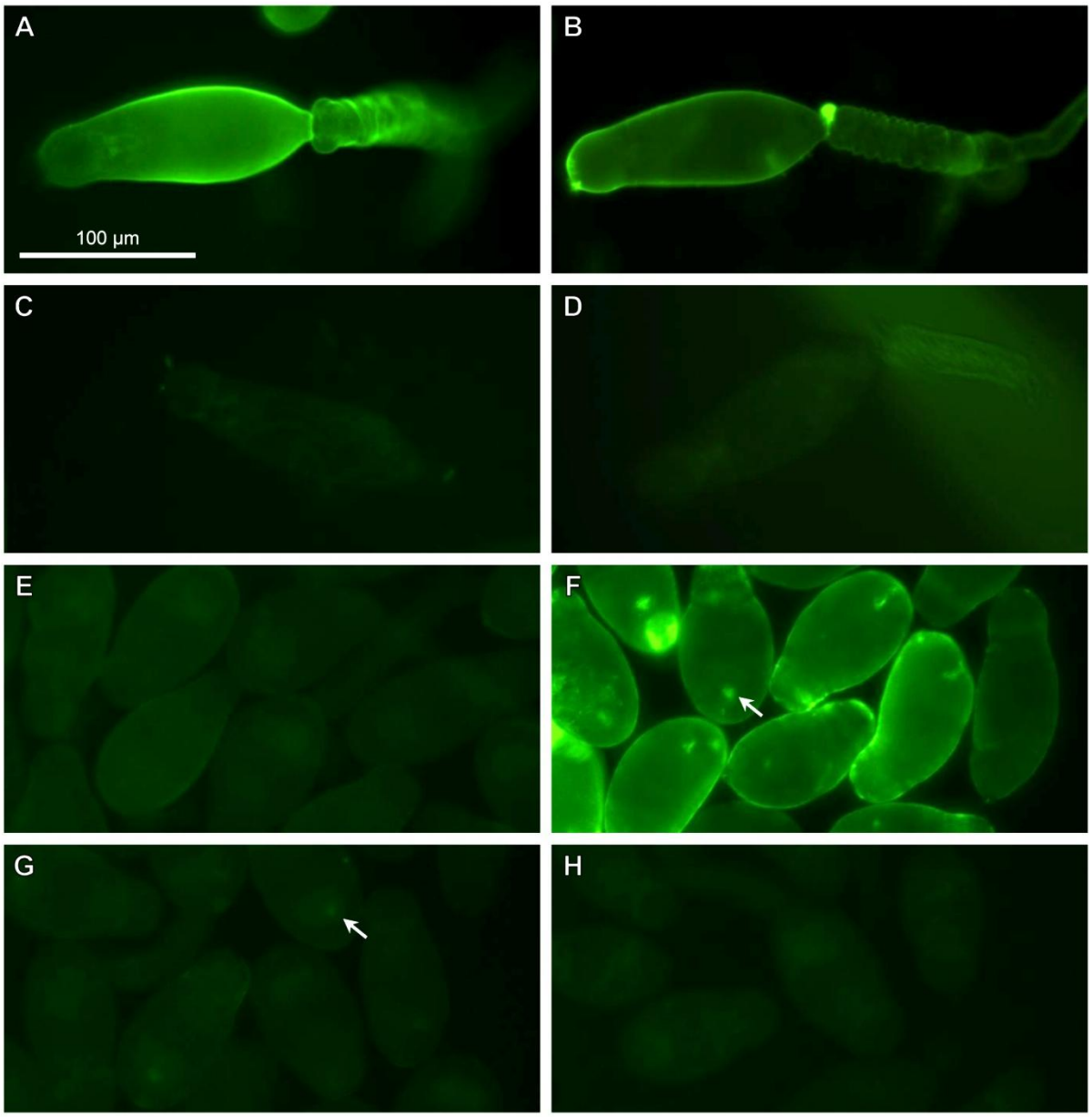


Figure 3: Immunofluorescence reveals differential surface binding of larval schistosomes by ASC-probes

Live cercariae (A-D) and 24 h schistosomula (E-H) were incubated with ASC-probes from infected skin-LN (A,E), lung-LN (B,F), liver-LN (C,G) and a control sample from uninfected rats (D,H). Surface binding of antibody was detected using FITC-conjugated anti-rat Ig, and visualised by an inverted fluorescence microscope. Binding to the schistosomula ventral sucker is highlighted by white arrows.

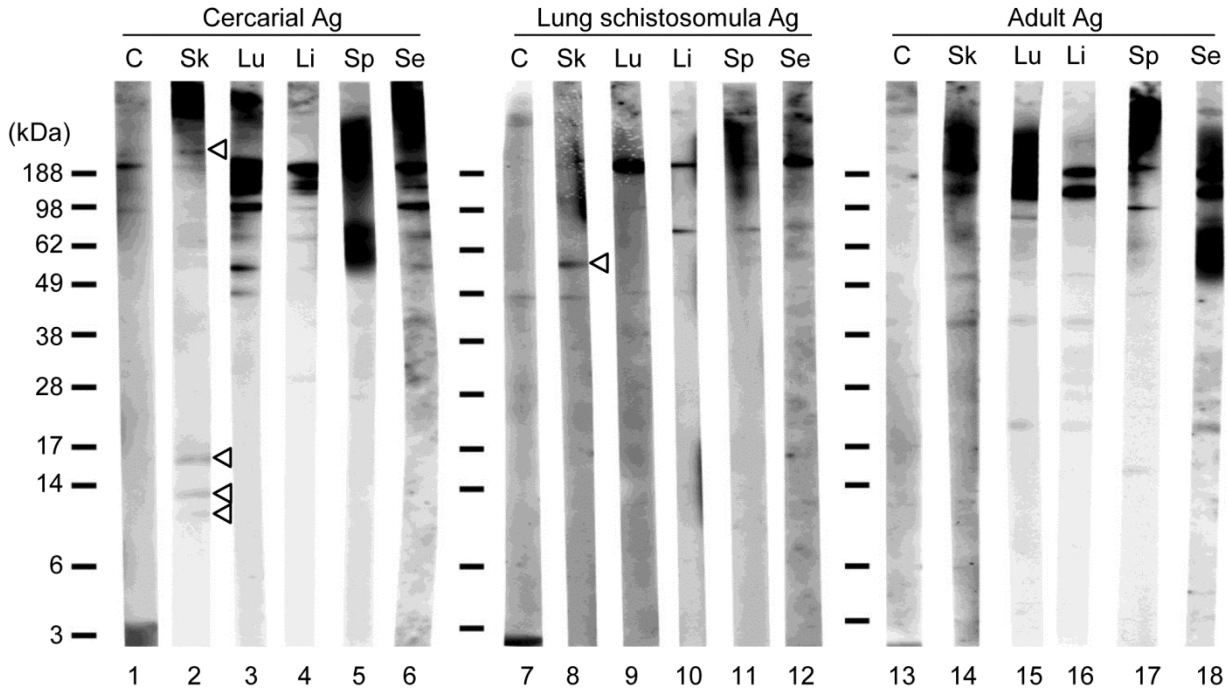


Figure 4: Each tissue region produces antibodies against a distinct set of antigens

Serum and ASC-probes from each of the tissue regions, from one individual (rat 2-group I_5), were used to probe western blots containing antigen (Ag) from three schistosome developmental stages: cercariae (lanes 1-6); lung-stage schistosomula (lanes 7-12); and adult (lanes 13-18). The ASC-probe samples were cultured from skin (Sk), lung (Lu) and liver (Li) lymph nodes, and spleen (Sp). Serum (Se) and a non-infected control lung ASC-probe (C) was used for comparison. White arrows highlight apparent stage-specific antigens.

2.3.5 Carbohydrate binding by ASC-probes

Since a large proportion of the antibody response during schistosomiasis is directed at carbohydrate epitopes,(202) the recognition of glycan structures was investigated by screening a glycan microarray. Serum, lung- and spleen-LN ASC-probes showed high levels of binding to the array and were used in the analysis. The skin- and liver-LN samples did not show adequate binding, due to their lower than recommended antibody levels (generally <0.5 μg/ml) and could therefore not be analysed on the arrays.

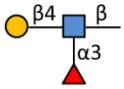
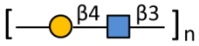
A high level of binding to a wide range of glycans was seen in samples from infected rats and, to simplify the complex carbohydrate recognition observed, the motif segregation method was applied (201). Glycan motifs significantly recognised ($p<0.001$) for each sample type are shown in Figure 5. Several motifs were commonly bound by all the samples (Fig. 5A). The motif with the highest significance was GlcNacβ1-3 at the terminal position (non-reducing end), while the blood group antigen Lewis X (Le^X), poly-*N*-acetyllactosamine (LacNAc) and terminal glucose (both structurally related to Le^X) were also commonly bound by all samples.

Differences in motif recognition between lung, spleen and serum samples were also observed (Fig. 5B). Compared with the serum, the ASC-probe samples recognised fewer motifs, with the spleen samples binding to the least number of motifs, only recognising terminal Glcα in addition to the common motifs listed above. The lung LN ASC-probes bound to a broader range of poly-LacNAc motifs and the common 'fucosylated N-glycan core' structure. The 'GlcNAcβ 6' substituted' and 'Fucα1-6' motifs, recognised by lung LN ASC-probes and serum, are present on the 'fucosylated N Glycan Core' motif. The serum recognised these same motifs, and also a broad range of mannose residues in various linkages, and fucosylation by different linkages.

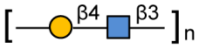
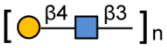
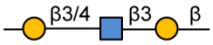
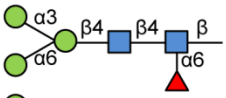
Figure 5: The glycan motifs recognised by antibody samples (next page)

Lung and spleen ASC-probes (n = 3 and 4, respectively), and serum antibody (n = 6), were screened on a glycan array and the specific motifs significantly recognised are illustrated here. The significance level for each glycan motif is indicated by the symbols; $p > 1x10^{-3}$ (-), $p = 1x10^{-3}$ to $1x10^{-4}$ (+), $p = 1x10^{-4}$ to $1x10^{-5}$ (++) and $p < 1x10^{-5}$ (+++). Some structures were commonly recognised by all three sample types (A) and some were only bound by particular antibody sources (B). To simplify the data, motifs are grouped together by structural similarity.

A Glycans recognised by all sample types

Motif Name	Structure	Lung	Spleen	Serum
GlcNAcb1-3 (terminal)		+	+ + +	+ + +
Lewis x		+	+ +	+ + +
Poly LacNAc		+	+	+ + +
Glucose (terminal any linkage)		+	+	+

B Glycans recognised by one or two sample types

Motif Name	Structure	Motif similarity	Lung	Spleen	Serum
Poly LacNAc (internal)			+	-	+ +
Poly LacNAc (terminal)		LacNAc	+	-	+ + +
Blood group i (not I)			+	-	+ + +
GlcNAcβ 6' substituted			+	-	+ + +
Fucα1-6			+	-	+ + +
N-Glycan core fucosylated		N-glycan core	+	-	+ + +
N-Glycan complex			-	-	+ + +
N-Glycan			-	-	+ + +
Mannose (any linkage)			-	-	+ + +
Manβ (anywhere)			-	-	+ + +
Manα 2' Substituted			-	-	+ + +
Manα (anywhere)		Mannose (various linkages)	-	-	+ + +
Man (sub-terminal)			-	-	+ + +
Manα1-3 (anywhere)			-	-	+ +
Manα1-6 (anywhere)			-	-	+ +
Fucα (anywhere)			-	-	+ +
Fucose (anywhere)		Fucose (various linkages)	-	-	+ +
Fucα1-3			-	-	+ +
O Glycan linkage			-	-	+
Glcα (terminal)			-	+	-

Key: = Gal = Glc = GalNAc = GlcNAc = Man = Fuc

2.4 Discussion

Schistosomes have a complex migratory path through mammalian tissues, and a deeper understanding of the local antibody response they elicit in each anatomical region would be valuable in decoding the complex immunology and designing vaccines against the crucial developmental stages. While serum is a common source of infection-induced antibodies, it does not provide information on the subtle short-lived antigenic changes in various tissues elicited by developing helminths. Therefore in this study, we characterised the local antibody response developing schistosomes elicit in each tissue region, using a technique novel to this infection, and showed the quantity and quality of the humoral response differs significantly in each tissue site.

The antibody response captured in the skin- and lung-LN ASC-probes was induced by the larvae migrating through these two tissue regions, as evidenced from the timing of the highest level of antibody secretion into culture supernatants in the different groups. Firstly looking at the skin, the initial site of infection, the largest antibody response was found in the rats killed at 5 days post-secondary infection (group I_5).. Since the peak in the number of mature plasma cells in draining LN occurs around 4-5 days following antigenic challenge (185, 203), the increase at day 5 reflects the peak antibody response against the invading cercariae around the day of infection. By 9 days post-infection, however, antibody secretion had receded in the skin-LN, demonstrating the transient nature of the LN stimulation, and that the timing is crucial to capture the local antibody response against this parasitic stage. Interestingly, the spleen antibody secretion profile was similar to the skin samples, indicating that the earliest larval migration causes some antigens to reach the spleen, presumably via the vasculature.

There was little change in total Ig levels in lung-LN ASC-probes following challenge, apart from two high-responding rats. These two rats showed lesions and trapped schistosomula in the lungs, which likely provided increased antigenic stimulation. This significant variation between individuals is most likely due to the out-bred nature of the rats, and also the fact that the timing of schistosome migration is relatively heterogeneous from the skin to the lungs, and occurs over several days (34, 36-37).

A uniform increase in antibody secretion was seen in the liver-LN ASC-probes of rats 9 days after challenge infection, indicating the possible re-stimulation of the hepatic LN by the early arrival of immature worms into this tissue at the later time point. Interestingly, one unchallenged rat with an unusual high antibody response in the liver LN was found to have a 10 fold higher than normal egg count in the liver, illustrating the exquisite sensitivity of the LN response to individual variations, as was also seen in the lung lymph nodes.

The type of antibody produced is thought to be important in protection against schistosomiasis in rats and other hosts, since particular isotypes have been shown to either participate in, or block, larval killing mechanisms (194). Furthermore, the predominance of particular antibody isotypes can indicate the generation of different types of immune responses (e.g. type 1/type 2) (197). In the present study, significant differences in antibody isotypes were detected in the larvae-induced antibody samples of the different tissue

regions. In particular, we found a high level of IgE contained in liver LN samples indicating a strong type-2 immune response. Since the liver antibody response is induced by adult worms and eggs, this result agrees with the finding in mice that egg deposition induces a type-2 response (204-205). The predominant isotype in the spleen ASC-probe samples was IgM, which peaked at 5 days post challenge. IgM antibodies are often directed against carbohydrate epitopes (178) and it is likely that the large amount of anti-carbohydrate IgM seen in schistosomiasis may originate predominantly in the spleen before making its way to the serum. In contrast to these significant local differences in IgE and IgM levels, the serum showed no significant difference for these two isotypes among infected groups. In rats, IgG2a has been shown to be a protective isotype against *S. mansoni* for immunity via ADCC (206-207) and vaccination with irradiated cercariae (208), while IgG2c has been shown to block this protective ADCC (209). It is interesting therefore that the local lung-draining LN samples in this study simultaneously had the highest IgG2a and lowest IgG2c levels, implicating an effective antibody response is induced in the lung region capable of effecting larval killing. This is consistent with the importance of the lung as the principle site of larval susceptibility (193).

The striking difference in isotype responses in each tissue region raises the interesting question of whether each parasite stage is inducing a particular immune response, as is traditionally thought, or if the tissue being infected is influencing the outcome of the immune response, as has recently been suggested (210-211). More work would be required to further elucidate this however. In addition, it would be interesting to relate the different antibody isotypes to different parasite stages or antigens. However, this would require large amounts of larval material which is difficult to obtain for this parasite, but may form part of future studies aimed at identifying larval antigens for vaccination.

The specificities of the local antibody responses were examined by immunofluorescence, western blotting and glycan array screening, and highlighted differences in antibody specificity from various lymphoid compartments. The cercariae are covered by an antigenic carbohydrate-rich layer (the cercarial glycocalyx) which is shed during skin penetration (32) as they transform into schistosomula and migrate to the lung. Consequently, the skin-LN antibody sample predominantly recognises the cercarial surface and not the schistosomula, indicating that the skin response captured here is largely induced by the glycocalyx, which the cercariae shed and leave in the skin (212). The only sample to strongly bind to the schistosomula, which is the prime target for immunity, is the lung-LN sample. Interestingly the liver-LN sample does not significantly bind to either of the larval surfaces, instead binding to the cercarial secretions and schistosomula ventral sucker. This may indicate that the antigenic challenge of the adults and eggs in the rat liver shares epitopes with the larval secretions, but not the larval surface. Together, these results show the unique local responses in each tissue region against the migrating worms, as they change their surface antigens.

Differences in antigen specificity were confirmed by western blotting and several stage-specific antigens were detected using the larvae-induced antibodies. Antigens specific to the cercarial stage were recognised by the skin-LN ASC-probe samples, and not the other ASC-probes or even serum. If serum was used alone as the source of antibody, these potentially important stage-specific antigens would therefore not be recognised. In

preliminary experiments with water buffaloes, natural hosts of *S. japonicum*, similar cercarial antigens were recognised by buffalo skin-LN ASC-probes after challenge (Chapter 3). Together, these results highlight the value in using the ASC-probe technique to discover larval-specific antigens.

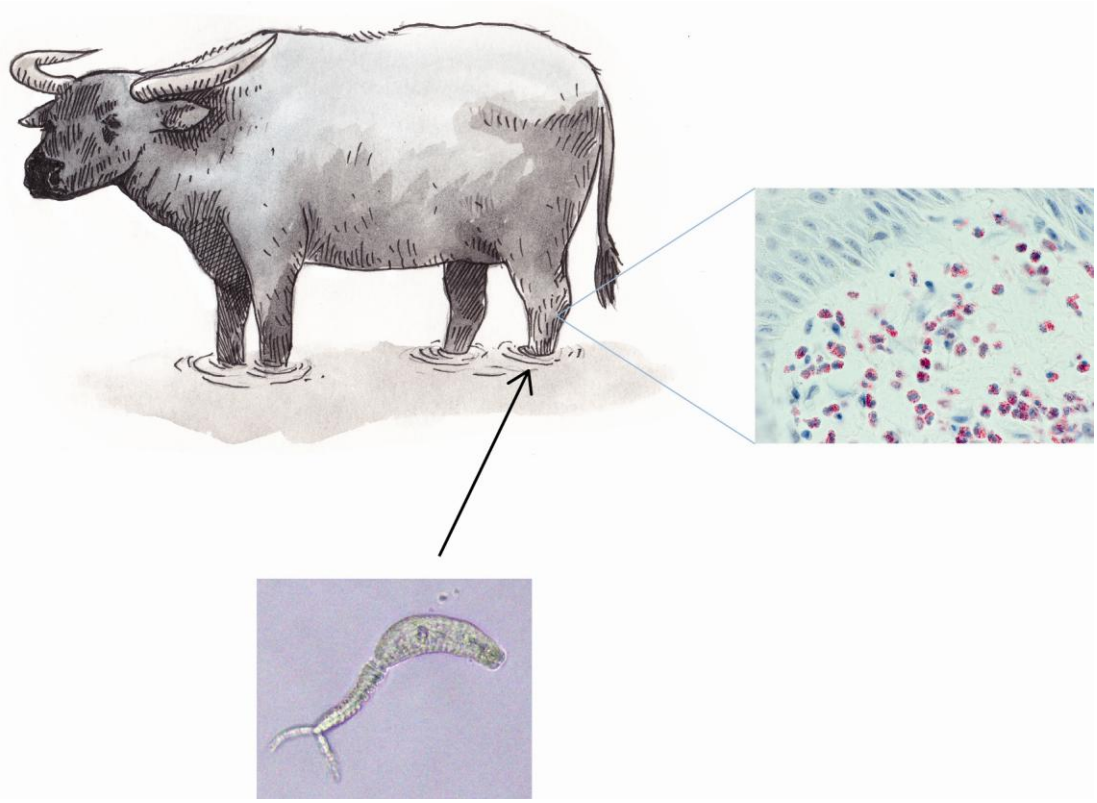
During schistosomiasis, high titres of anti-carbohydrate antibodies are found in serum (190, 202), and carbohydrates may be important vaccine candidates (213-214). Some glycans are known to be stage-specifically regulated and this was explored in the present study by screening a glycan array using serum and the tissue-specific antibody probes. Similar to the finding against protein antigens in the western blots, serum recognised the most motifs, while the ASC-probes recognised a more discrete range. The glycan array results indicated that the spleen recognised the fewest glycan motifs, suggesting that the large anti-carbohydrate response in this tissue is specific to a small set of glycans. Four motifs were common to all samples, and three of these are related to the Le^X antigen, which is present on many schistosome glycans throughout the lifecycle (215). The top motif, terminal GlcNAcβ1-3, is noteworthy in that it has not been reported as being a common terminating residue in schistosome glycans, despite being the most consistently recognised carbohydrate motif in this study.

Unlike the spleen, the lung ASC-probes recognised the fucosylated N-glycan core and related motifs, which are commonly at the core of schistosome glycans (216). The reason these are not antigenic in the spleen is not known, but it is possible that they are not found on circulating antigens which are likely to be responsible for inducing an immune response in the spleen. While this array was valuable to show differences in glycan specificities between ASC-probe samples, it is predominantly comprised of mammalian glycans and a more targeted parasite glycan array may be needed to discover unique schistosome glycans. Schistosome-specific glycans have recently been developed (217), and will be used in further studies to identify stage-specific schistosome glycan antigens.

In this study, the capture and analysis of the local antibody response induced by migrating schistosome larvae revealed significant differences in the quantity, quality and specificity of the ASC-probe samples. Antibody probes reflecting the early larval stages in the skin and lung indicated the presence of as yet uncharacterised larval-specific protein and carbohydrate antigens, highlighting the potential of this method for vaccine development. There was a strong binding of antibodies produced by lung-LN to the schistosomula surface and several high molecular weight larval antigens. Considering the importance of the lung in schistosome immunity (11), further analysis of the lung ASC-probes using the latest proteomic and glycomic technologies (100, 218) is expected to result in the identification of novel stage-specific antigens with high relevance to vaccine design.

Chapter 3:

The local immune response of the Chinese water buffalo, *Bubalus bubalis*, against migrating *Schistosoma japonicum* larvae



Declaration for Thesis Chapter 3

Chapter 3 is based on a manuscript submitted for publication in PLOS Neglected Tropical Diseases:

> **McWilliam, H.E.G.**, Piedrafita, D., Li, Y., Zheng, M., He, Y., Yu, X., McManus, D.P., Meeusen, E.N.T. The Local Immune Response of the Chinese Water Buffalo, Bubalus bubalis, Against Migrating Schistosoma japonicum Larvae. *PLOS Neglected Tropical Diseases*, **2013**, Submitted.

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution:	**Extent of contribution:**
I was responsible for the design of the majority of the experiments, conducting part of the buffalo experiments and the majority of experiments generating immunological samples. I analysed the data and wrote the manuscript with assistance, as listed below.	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name:	**Nature of contribution:**	**Extent of contribution:**
David Piedrafita	Assisted with: experimental design, execution of buffalo experiments, the analysis and interpretation of results, manuscript editing.	N/A
Yuesheng Li	Assisted with: experimental design, execution of buffalo experiments.	N/A
Mao Zheng	Assisted with: execution of buffalo experiments.	N/A
Yongkang He	Assisted with: execution of buffalo experiments.	N/A
Xinling Yu	Assisted with: execution of buffalo experiments.	N/A
Donald McManus	Assisted with: experimental design, manuscript editing.	N/A
Els Meeusen	Assisted with: experimental design, execution of buffalo experiments, the analysis and interpretation of results, manuscript editing.	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

.....	.....	.....	.....
Candidate	Date	Main supervisor	Date

3.1 Introduction

Asian schistosomiasis, caused by *Schistosoma japonicum*, is a parasitic disease endemic in the marsh and lake regions of China, particularly along the Yangtze River basin and mountainous regions (9, 14), and parts of the Philippines and Indonesia (13). Up to 1 million people are infected with 50 million at risk of infection (9, 89). Schistosomiasis causes significant morbidity in chronically infected individuals, perpetuating poverty in endemic communities (3), and is one of China's major public health priorities (22).

Control of Asian schistosomiasis relies largely on chemotherapeutic treatment with praziquantel (PZQ) however this is complicated by the fact that it is a zoonotic disease and infects a wide variety of mammalian hosts (70-71). In particular, the domestic water buffalo, *Bubalus bubalis*, is considered the most significant animal reservoir of schistosomiasis in China (9, 72-73) where it has been shown to account for up to 75% of human transmission (74). This results in the need for routine PZQ treatment of livestock in addition to humans for parasite control (9). However as a complementary strategy, a vaccine could contribute significantly to the elimination of schistosomiasis (9-12, 96-99). An effective vaccine for buffalo alone would significantly reduce human morbidity as well as improve buffalo health (101), and for this reason an effort has been made to develop a transmission-blocking veterinary vaccine (98).

Much of our understanding of the immunobiology of schistosomiasis comes from murine models (112) but there is a distinct lack of understanding of the immunological mechanisms during schistosomiasis in natural large animal hosts, including water buffaloes. Moreover, the vast majority of previous studies were performed in naive mice, and there is limited information on the response from endemically-exposed hosts, which are the intended recipients of a vaccine. There are several reports of age-related acquired resistance in buffaloes that show that older buffaloes have reduced intensity and prevalence of infection (14, 21, 72, 120). Also, buffaloes are known to have a natural resistance to *S. japonicum* infection compared with other hosts, such as cattle, *Bos taurus* (71, 121). While this acquired and natural immunity has been documented, it has not been studied in depth and the mechanism is unknown. Since the migrating schistosomula are recognised as a susceptible target in humans and other animals (11, 124, 148, 150), it is likely that this stage may be also targeted by immune effector mechanisms in buffaloes.

In order to elucidate the immunobiology of schistosomiasis in an endemic host, we investigated the local immune response directed at the migrating schistosome larvae in water buffaloes from an endemic area in China. We characterised the type of immune response at each local site of larval migration, the skin and the lungs, in comparison with the liver where the majority of eggs are trapped. The results of this study may shed light on a mechanism of immunity in a natural host of *S. japonicum* and inform the design of more effective vaccines.

3.2 Materials and methods

3.2.1 Ethics statement

All experimental animal procedures were performed with written approval from the Ethical Review Board of the Hunan Institute of Parasitic Diseases (approval no. 110818), and from the Monash University Animal Ethics Committee (no. 2011-124-FW). The handling and care of the animals were performed by trained staff adhering to good animal practice guidelines according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

3.2.2 Animals and experimental infections

Eleven mixed-sex (8 male and 3 female) water buffaloes (*B. bubalis*) were selected from a schistosomiasis-endemic area in Hunan Province, aged between 1 – 1.5 years. Animals previously exposed to *S. japonicum* were selected based on positive faecal egg counts, which required testing over 50 buffaloes from the region. Animals were then moved to *S. japonicum*-free pasture in a non-endemic region of Hunan Province to rest for 7 weeks, during which time they were treated with praziquantel (PZQ) twice to cure the existing schistosomiasis. Because PZQ is only effective on adult worms older than 4 weeks, the second dose was to cure any parasites acquired immediately prior to being moved to the parasite-free pasture (see Fig.1).

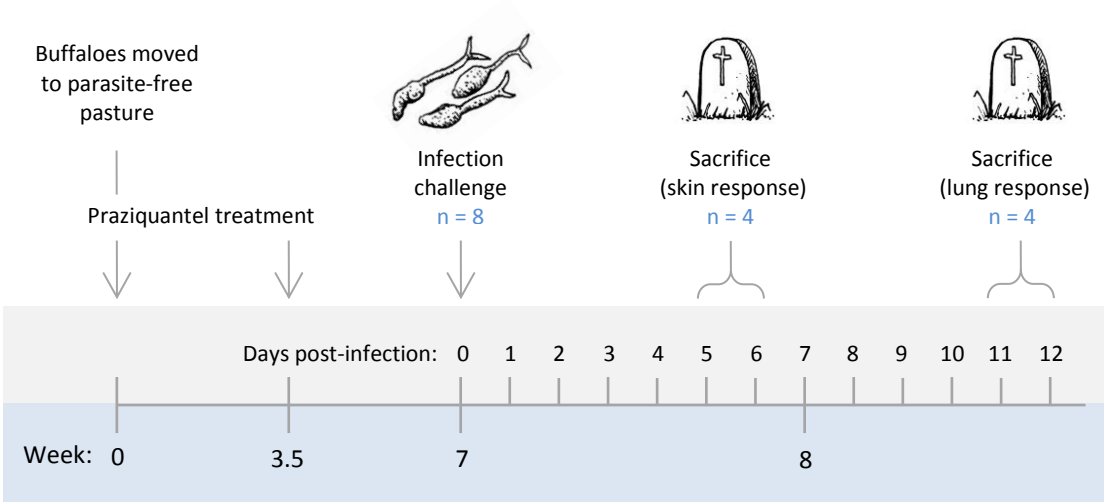


Figure 1: Water buffalo experiment timeline

Timeline depicting the treatment of water buffaloes over the experiment. Animals were moved to parasite-free pasture at the beginning (week 0) and treated twice with praziquantel. At week 7,while 3 animals were kept parasite free, 8 buffaloes were infected with *S. japonicum* cercariae and subsequently killed at 5-6 days post-infection (DPI) or 11-12 DPI.

Eight buffaloes were chosen randomly for experimental infections, and these were divided into two groups for sacrifice at two time points; either at 5 and 6 days post-infection (5-6 DPI) to study the skin-penetrating cercariae, or 11-12 DPI to focus on the lung-migrating larvae. These particular time points were chosen to study the matured adaptive response in the local draining lymph node (LN), since the peak of an adaptive immune response occurs around 4-5 days in draining LN following antigenic challenge (185, 203). The response in the skin-LN was likely to be maximal at 5-6 DPI while 11-12 DPI was chosen to study the response against the lung-stage of infection, as the peak of larvae migration through the buffalo lung is around 7 DPI (unpublished observations). Three buffaloes were left un-infected (group NI) and sacrificed at different days throughout the trial (-1, 5 and 11 DPI).

Infection with *S. japonicum* cercariae was administered percutaneously on the shaved inner thigh, by transferring cercariae with a sterile culture loop onto a glass cover slip, and then resting this on the moistened skin for at least 30 minutes. The *S. japonicum* cercariae were obtained from *Oncomelania hupensis* snails collected from the marshlands of the Dongting Lake Region, Hunan Province.

Three buffaloes in each challenge group were given a moderate infection of 200-600 cercariae, depending on availability (Table 1). Matched pairs were given the same cercarial number, and numbered according to this dose. One buffalo at each time point (#4) received a high dose of 2400 cercariae to be used as a source of highly-stimulated samples for a separate study.

Table 1: Individual water buffalo data

Group	Buffalo number	Infection dose	Cercarial number	Sex	Age (years)	Weight (kg)	DPI sacrificed
NI	1	-	-	M	1.3	165.8	-
NI	2	-	-	F	1.5	193.5	-
NI	3	-	-	M	1.2	137.1	-
5-6 DPI	1	Moderate	200	M	1	136.3	6
5-6 DPI	2		400	M	1.5	223.7	6
5-6 DPI	3		600	F	1.4	181	5
11-12 DPI	1	Moderate	200	M	1.5	202.5	11
11-12 DPI	2		400	F	1.4	170.1	11
11-12 DPI	3		600	M	1.5	229.8	12
5-6 DPI*	4	High	2400	M	1.1	134.5	5
11-12 DPI*	4		2400	M	1.5	200.9	12

NI = not infected; DPI = days post infection. *High-dose infection animals were considered separately in the analysis

3.2.3 Sample collection

At post-mortem, lymph nodes (LN) draining the skin of the inner thigh (inguinal LN), lungs (mediastinal LN) and liver (portal LN) were collected and sliced into pieces in a sterile petri dish containing approximately 5 ml of media (RPMI 1640 Glutamax, supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies)). These were stored on ice until processed. A small sample of skin (~1 cm^2) was removed from the site of parasite infection, as was a similar piece of lung tissue. Half of each was placed into RNAlater (Ambion), and the other half fixed in 10 % formal saline for up to 2 weeks, followed by storage in 70 % ethanol until they were processed into paraffin for histological analysis.

3.2.4 Skin histology

Fixed skin samples were paraffin-embedded and 4 μm sections were cut and stained with haematoxylin and eosin (H&E) for leukocyte counting, toluidine blue for mast cell differentiation, or stained overnight with Llewellyn's Sirius red for eosinophil identification (219). Cells were counted in 5-7 different fields-of-view at 400 x magnification to determine the numbers of these cell types in the skin. To count the number of leukocytes in the epidermis, sections 0.26 mm in length were enumerated. To determine the thickness of the epidermis and stratum corneum layers, ImageJ (220) was used to calculate the volume of each layer relative to the length of the skin section (determined by measuring the stratum granulosum). For each sample, three sections of skin (approximately 0.8 mm) were measured.

3.2.5 Measuring cytokine transcript levels in skin by quantitative real-time polymerase chain reaction (qPCR)

Buffalo skin samples were disrupted using a T10 Basic Ultra-Turrax homogeniser (IKA) in the presence of Qiazol (Qiagen), and the RNA was subsequently extracted according to Qiagen's protocol. After resuspending the RNA pellet in water, it was purified using the Total RNA Extraction Miniprep System (Viogene) and the concentration determined on a NanoDrop spectrophotometer (Thermo Scientific). Total RNA (300 ng) was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) which includes a genomic DNA removal step. To perform the qPCR, primers were used based on *B. bubalis* sequences, except for galectin-14 which was based on the ovine sequence since the *B. bubalis* sequence was not available. The sequence of the primers was as follows: β-actin, used as a reference gene (forward: CGCACCACCGGCATCGTGAT, reverse: TCCAGGGCCACGTAGCAGAG); galectin-14 (forward: 5'-CACCCAGCCTCCCTACATAA-3', reverse: 5'-ATGCCCATCTGAAAGTCCAC-3'); interleukin-5 (IL-5) (forward: 5'-TGGCAGAGACCTTGACACTGCT-3', reverse: 5'-CACAGCATCCCCTTGTGCAGTT-3') and interferon-γ (IFN-γ) (forward: 5'-GTCTCCTTCTACTTCAAACT-3', reverse: 5'-ATTCTGACTTCTCTTCCGCT-3'). The qPCR reactions included SYBR Master Mix (Applied Biosystems) and the above primers at 0.5 μM and 5 μl of 1:20 diluted cDNA, and were run in triplicate on an Eppendorf Realplex4 Mastercycler for 40 cycles, using an annealing temperature of 58 °C for β-actin and galectin-14 and 56 °C for IFN-γ and IL-5. Melt curve analysis was done to

ensure a single product was amplified. The relative copy number of SYBR green for each gene was calculated from a standard curve of serial dilutions of cDNA, and then the relative expression of the target gene was determined relative to the reference gene copy number.

3.2.6 Culturing lymphocytes: generating antibody secreting cell (ASC)-probes and cell stimulations

Lymph node slices were gently teased apart in cold media under sterile conditions. Cells were washed twice in cold media, each time collected by centrifugation at 400 x *g* for 10 min at 4 °C. Lymphocytes were counted with a haemocytometer, and trypan blue was used to exclude dead cells. Cells were resuspended to the appropriate cell concentration in media supplemented with AlbuMAX II (2 mg/ml; Life Technologies). To generate ASC-probe samples from each LN, lymphocytes were made up to 5 x 10^6 cells/ml and cultured for 4 days in a humidified incubator at 37 ºC under 5% CO_2. For cell stimulations, lymphocytes were cultured for 48 hours at 1 x 10^7 cells/ml and stimulated by adding phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (1 μg/ml). After culturing, the cell supernatants containing antibodies secreted by *in vivo* generated ASCs (ASC-probes) or secreted cytokines were collected and stored at -20 ºC until required.

3.2.7 Measuring cytokine protein levels in cell culture supernatants

The levels of IFN-γ, IL-4 and IL-10 were quantified in cell culture supernatants by sandwich ELISA. To determine IFN-γ levels, the Bovine IFN-γ ELISA kit (AbD Serotec) was used according to the manufacturer's instructions. For IL-4 and IL-10, monoclonal antibody pairs were used (AbD Serotec). Plates were coated with mouse anti-bovine IL-4 or IL-10 monoclonal antibody (2.5 μg/ml) overnight at 4 °C. All subsequent steps were done at room temperature, and washing consisted of 5 changes of PBS with 0.05% Tween-20 (PBST). After blocking with 0.1% bovine serum albumin in PBS for 1 hour, samples were incubated for 1.5 hours (the supernatants or recombinant bovine IL-4/IL-10 for standard curves). The supernatant samples were diluted 1:20 for IL-4 and IFN-γ and 1:50 for IL-10 measurement. After washing, biotinylated anti-bovine IL-4 or IL-10 was added at 1:1600 or 1:1000, respectively, and incubated for 1 hour and subsequently washed. Finally, horseradish peroxidise-conjugated streptavidin (Dako) was added at 1:1000 for 1 hour, and plates were developed using TMB Substrate (Life Technologies) and optical density measured using a spectrophotometer. Supernatant OD readings were compared to the linear part of the standard curve to determine the cytokine amount.

3.2.8 Measuring total antibody levels in ASC-probe samples

The level of total buffalo antibody was measured using a capture ELISA, and the results compared to a standard curve made with purified buffalo immunoglobulin from serum. This was achieved by using Protein G Agarose Resin (Thermo Scientific) according to the manufacturer's directions. Briefly, 1.5 ml of the agarose resin was washed twice in PBS then combined with 2 ml of buffalo serum and 10 ml PBS, and mixed at RT for 7 h. This was then added to a disposable column and then washed with 30 ml of PBS. Finally 5 ml of 0.1 M glycine (pH 2)

was added to elute the bound antibody and then immediately combined with 0.5 ml 1M tris to neutralise the pH, and the eluted antibody was frozen until required. Subsequently the glycine was removed by dialysis into PBS and the protein concentration determined by BCA Protein Assay (Thermo Scientific), reflects the antibody amount.

For the capture ELISA, plates were coated with rabbit anti-bovine IgG (whole molecule) (Sigma-Aldrich), diluted to 1:2000 in carbonate coating buffer (pH 9.6) overnight at 4 °C. Wells were blocked by incubating 0.5 % (w/v) Tween-20 in PBS for 2 h at 37°C, and washed between each of the following steps 3 times with PBST. ASC-probes were added to the wells and serially diluted in PBST and incubated for 1.5 h at 37 °C. A standard curve using the purified buffalo antibody was included on each plate and captured antibody was detected with rabbit anti-bovine IgG (Whole molecule):HRP at 1:4000. TMB (3,3',5,5;-tetramethylbenzidine) solution (Life Technologies) was added to each well, followed by 2M H_2SO_4, and the optical density (OD) was read at 450nm and compared to the standard curve to calculate the amount of antibody in each sample.

3.2.9 Measuring specific antibody levels in ASC-probe samples

The level of specific antibody was measured in each ASC-probe sample by ELISA against soluble adult worm antigen, which was prepared as previously described (200). Plates were coated by adding adult antigen at 20 µg/ml in carbonate coating buffer (pH 9.6) overnight at 4°C. Wells were blocked by incubating 0.5% (v/v) Tween-20 in PBS for 1.5 h at 37°C, and washed between each of the following steps 3 times with PBST. ASC-probes (undiluted) were added to duplicate wells and incubated for 1.5 h at 37°C. Finally captured antibody was detected with rabbit anti-bovine IgG (Whole molecule):HRP (Sigma-Aldrich). TMB solution (Life Technologies) was added to each well, followed by 2M H_2SO_4, and the optical density (OD) was read at 450 nm.

3.2.10 Antigen recognition of schistosome antigens by western blotting

Crude antigen was prepared from cercariae and adult worms by resuspending the frozen parasites in LDS sample buffer with reducing agent (Life Technologies), homogenising with a pellet pestle and heating to 95°C for 10 min with vigorous vortexing. Finally extracts were centrifuged at 12,000 x *g* for 10 min. Approximately 5 µg of each extract was separated on a 10-well 10 % NuPAGE Bis-Tris gel (Life Technologies) and transferred onto nitrocellulose membranes, which were then sliced into strips corresponding to individual lanes. Strips to be probed by buffalo ASC-probes were blocked overnight at 4 °C in 5 % normal rabbit serum (NRS) in PBST.

Due to high non-specific binding to western blots, the antibody was purified from ASC-probes using HiTrap Protein G Column HP (GE Healthcare Life Sciences), according to the manufacturer's instructions. Briefly, 25 ml of ASC-probe sample was cycled through the column several times, and after washing with 20 ml of PBS the antibody was eluted using 4 ml of 0.1 M glycine (pH 2), which was immediately neutralised with 500 µl 1M tris.

Purified ASC-probe antibody samples (supplemented with 0.2% NRS) from one infected buffalo #4 (from 5-6 DPI group) were incubated with individual western blot strips for 3 h at RT. After three washes with PBST, each

strip was incubated with anti-bovine Ig (H+L):HRP (1:1000 in 1% NRS-PBST) for 1 h at RT, then washed again. Finally strips were developed with Sigma*FAST*™ DAB with Metal Enhancer (Sigma). For the strip probed with rat ASC-probe sample, a similar protocol was used except blocking was done with 5% skim milk in PBST (SM-PBST). A pool of skin ASC-probe samples from infected rats was applied undiluted, and anti-rat Ig (H+L):HRP (Life Technologies) incubated at 1:1000 in 1% SM-PBST. This blot was developed with ECL substrate (GE Healthcare) was applied and chemiluminescence detected on Super RX film (Fujifilm).

3.2.11 Data interpretation and statistical analysis

This study was limited in the sample sizes that could be included for each group, due to the high cost, the large scale and the logistics involved in using water buffaloes. Therefore many of the differences are described as trends, and statistical results are only reported when significance was achieved at $p < 0.05$. When comparing three groups, a one-way analysis of variance test followed by Tukey's post-hoc test was performed, and when comparing two groups the student's t-test was employed. To measure correlations between two parameters, the Spearman's rank correlation was performed and the rank coefficient (r_S) was determined. Considering that the two high-dose buffaloes received up to 12 fold more cercariae than the moderate infections, their data are not included in the statistical analysis but are represented separately.

3.3 Results

3.3.1 Gross observations at necropsy

Buffalo were infected via the cover-slip method (Fig. 2A), and at 5 DPI the skin site where cercariae were applied had an obvious raised inflammatory reaction (Fig. 2B). By 11-12 DPI the skin reaction had reduced and was no longer raised (data not shown). No difference was seen in the lungs between infected and uninfected buffalo. The livers had evidence of pathology from trapped schistosome eggs; white nodules covered the liver, typical of a patent schistosomiasis infection (221)(Fig. 2C and D).

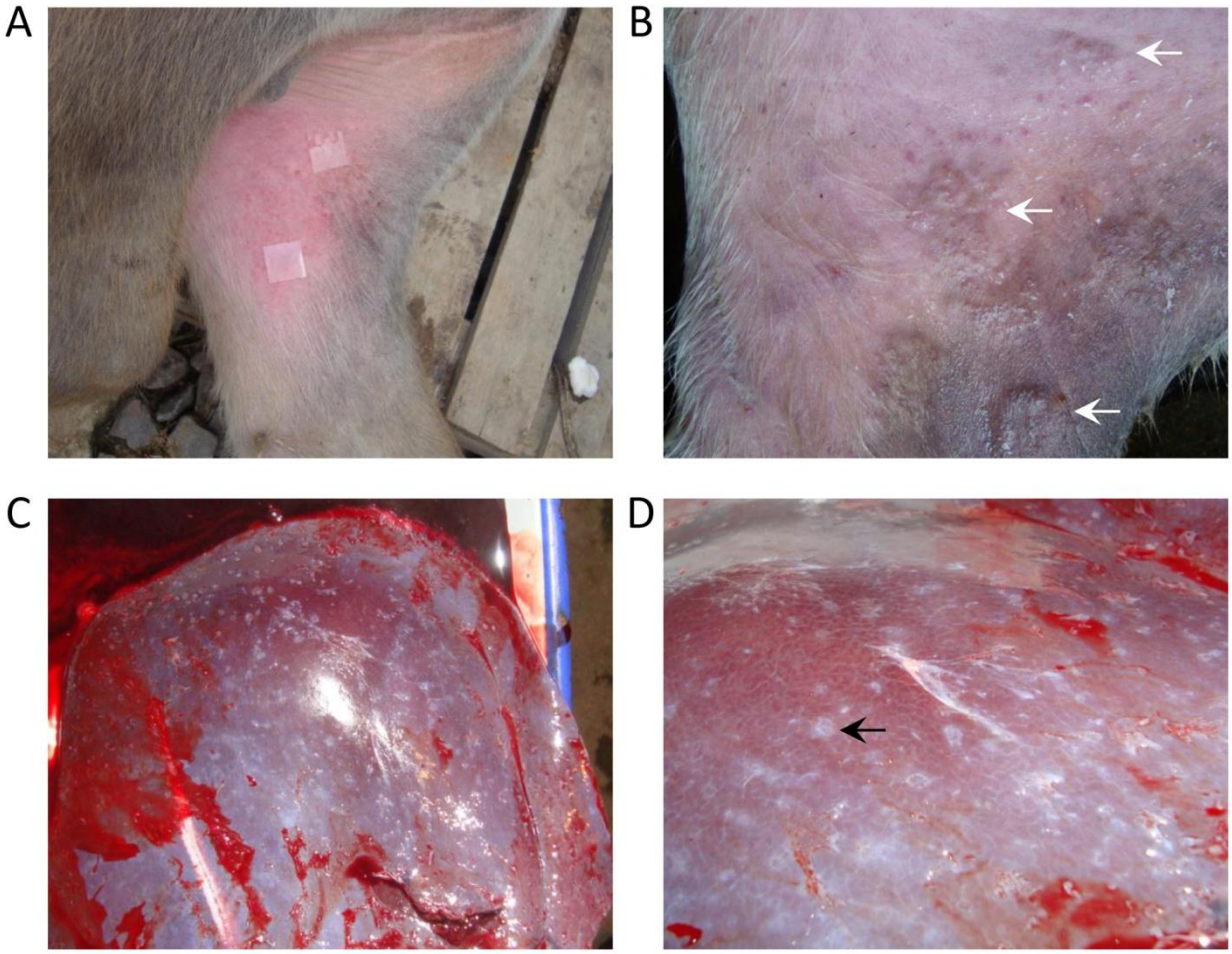


Figure 2: Skin and liver observations from infected buffaloes

Buffaloes were infected using cover slips bearing cercariae in water on the inner thigh (A) and 5 days post-infection it resulted in raised inflammatory reaction (particularly for buffalo #4, shown here) (B). At necropsy livers showed evidence of egg-induced pathology (C, and close-up in D). White arrows indicate the raised skin reaction, while black arrows highlight the white nodules.

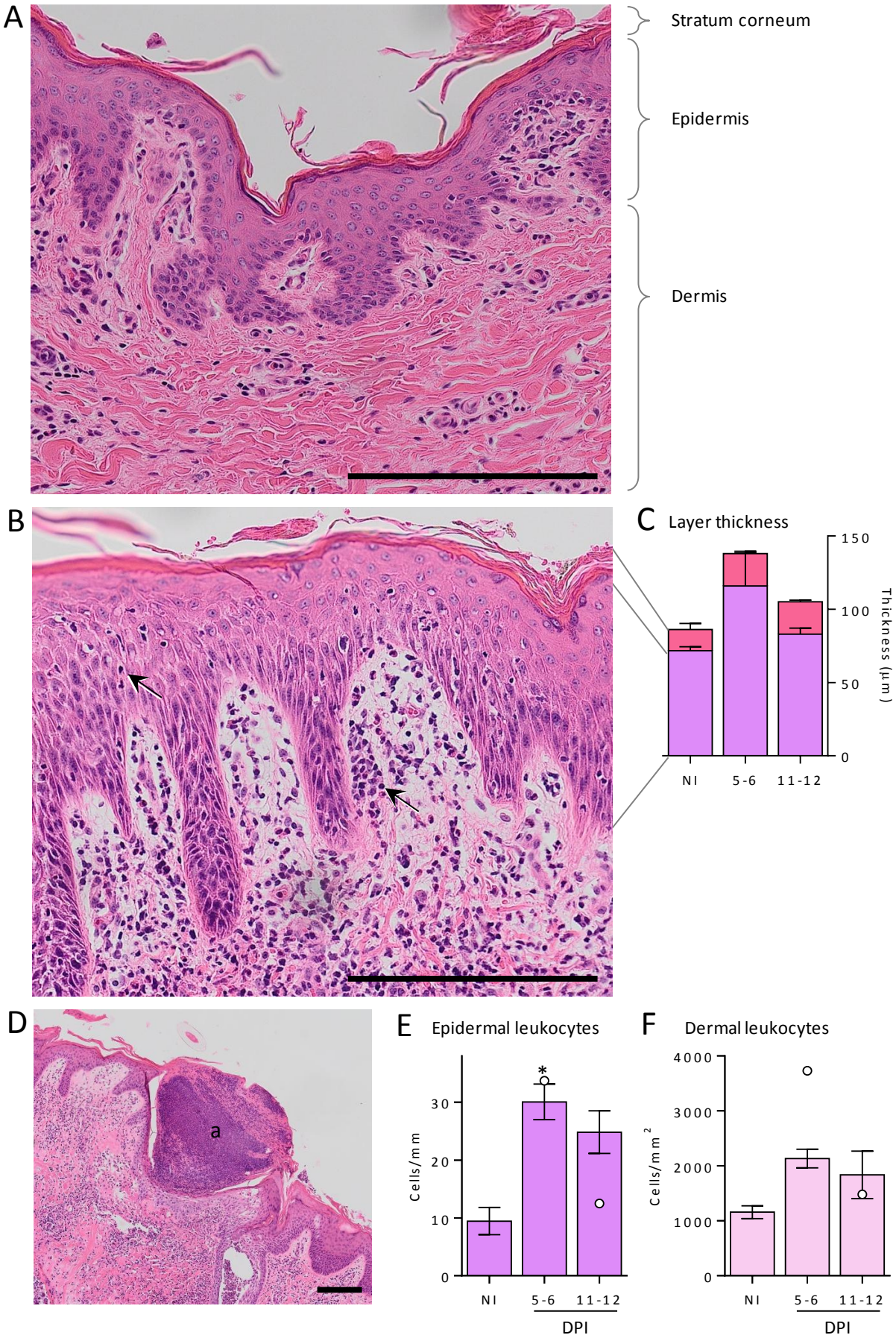
3.3.2 Cellular and cytokine responses at the skin site of larval penetration

Compared to uninfected controls, the skin of infected buffaloes at both time points showed signs of strong inflammation (Fig. 3), characterised by large numbers of infiltrating leukocytes in infected (Fig. 3B) compared to control skin (Fig. 3A). Thickening of the two outer layers, the epidermis and stratum corneum, was observed at 5-6 DPI (Fig 3C); although not statistically significant, all infected skin values were above NI values at 5-6 DPI. The epidermis was thickest at 5-6 DPI and then reduced to near NI level at 11-12 DPI, whereas the thickened stratum corneum increased slightly further at 11-12 DPI. Large, eosinophilic abscesses were seen in all skin sections at 5-6 DPI (Fig. 3D), in most cases severely disrupting the architecture as they spanned multiple skin layers; these had mostly cleared up at the later time point with only one of the four buffaloes from 11-12 DPI group (#1) showing abscesses. These granulocytic abscesses consisted of dense nuclei, likely to be infiltrating leukocytes, with eosinophilic granules staining throughout (not shown). Leukocytes were enumerated in the epidermal (Fig. 3E) and dermal layers (Fig. 3F) and showed a similar pattern: a large increase in leukocytes initially at 5-6 DPI and a slight reduction at 11-12 DPI. This was significantly higher only in the epidermal layer.

A large number of infiltrating eosinophils were seen in infected buffalo skin compared with controls (Fig. 4A-C), and this was predominantly in the dermal layer. Some eosinophils were seen invading discrete sections of infected epidermis (Fig. 4B); however this was not consistent and seen in very few sections, hence numbers were not determined. Eosinophils were enumerated in the dermal layer (Fig. 4E), showing a dramatic increase from a negligible amount (19 cells/mm^2) in control skin to 710 cells/mm^2 of dermis at 5-6 DPI and then dropping to 410 cells/mm^2 at 11-12 DPI. When individual data points of eosinophil numbers in infected dermis from buffaloes given the same dose of infecting cercariae were linked, it was observed that for all buffaloes receiving a moderate challenge dose the number of eosinophils was highest at 5-6 DPI, reducing at 11-12 DPI (Fig. 4F). However, for the pair of buffaloes given the high dose (#4) the number of eosinophils was highest at the later time point.

Figure 3: Leukocyte infiltration and thickness of buffalo skin following infection (next page)

Buffalo skin section from non-infected buffaloes (A) are compared to those following moderate infection with *S. japonicum* cercariae (B and D), which resulted in a large influx of leukocytes in dermal and epidermal layers (B, black arrows) and granulocytic abscess (a) formation (D). Stratum corneum, epidermis and dermis are indicated for reference. Staining performed with haematoxylin and eosin, and all scale bars represent 200 μm. Leukocytes were enumerated in the dermis (E) and epidermis (F) in non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes. The thickness of the epidermis (purple) and stratum corneum (pink) are also shown (C). Bars represent the mean of the moderate infections with standard error, and the high infections are also shown in E and F (white dots). Significance above NI levels is shown by the asterisk ($p = 0.015$).


A
Stratum corneum
Epidermis
Dermis
B
C
Layer thickness
150
100
50
0
Thickness (µm)
NI
5-6
11-12
D
a
E
Epidermal leukocytes
Cells/mm
30
20
10
0
*
NI
5-6
11-12
DPI
F
Dermal leukocytes
Cells/mm²
4000
3000
2000
1000
0
NI
5-6
11-12
DPI

Mast cells were observed in the dermis of all skin samples (Fig. 5), including NI skin. However there was a consistent increase in mast cell numbers only at 11-12 DPI compared to uninfected skin; despite no statistical significance, all samples at this latest time point had a higher number of mast cells than NI sections, increasing from a mean of 64 to 86 cells/mm^2 (Fig. 5C).

The immune response occurring in the skin infection site was investigated by measuring the relative transcript level of IFN-γ (a typical type-1 cytokine), IL-5 (a type-2 cytokine which promotes eosinophil proliferation and survival (222)) and galectin-14 (a molecule specific to eosinophils (223)) (Fig. 6A-C). The levels of these transcripts were normalised to a reference gene (β-actin) and then calculated relative to the mean of control samples to observe changes resulting from infection. There was a slight non-significant drop in skin IFN-γ following infection (Fig. 6B), while IL-5 was found to be expressed at approximately 7.5 times the level of normal skin at 5-6 DPI, whereas it had returned to the NI level at 11-12 DPI (Fig. 6A). The level of galectin-14 increased to approximately 14 times the level of expression in NI skin at 5-6 DPI, reducing to 5 times the level at 11-12 DPI (Fig. 6C). Furthermore, there was a positive and significant correlation with the level of galectin-14 and the number of eosinophils in the skin ($p = 0.011$, $r_S = 0.817$; Fig 6D). As for the number of eosinophils in tissues, only the #4 buffaloes given the high dose had an increase in galectin-14 expression at 11-12 DPI compared to 5-6 DPI (Fig 6C).

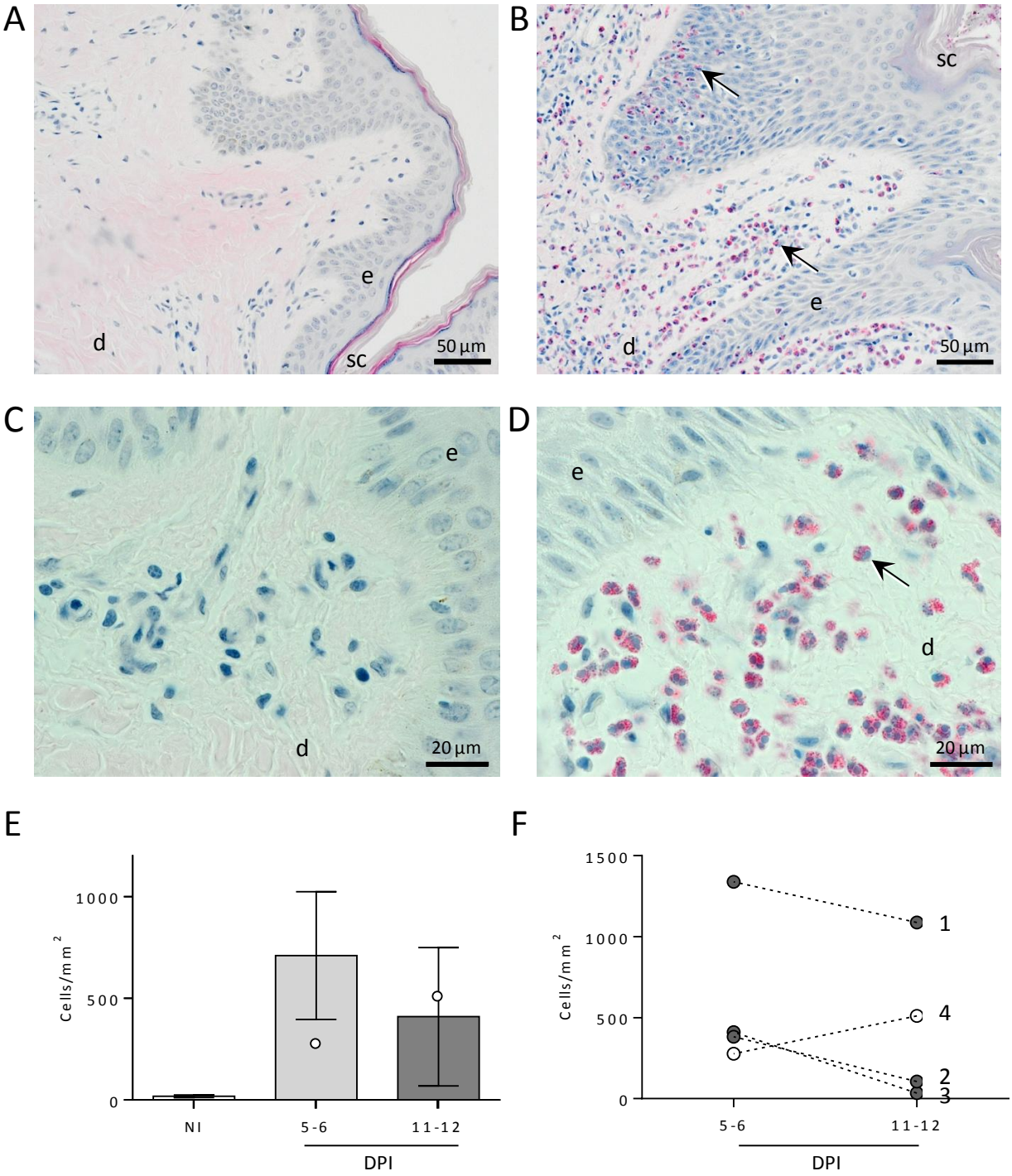


Figure 4: Eosinophil infiltration of buffalo skin following cercarial penetration

Buffalo skin sections from a non-infected buffaloes (A, C) and skin following moderate *S. japonicum* infection (B, D) stained with Llewellyn's sirius red stain highlights numerous eosinophils (black arrows) infiltrating the dermis and epidermis post-infection. Stratum corneum (sc), epidermis (e) and dermis (d) are indicated for reference. Eosinophils were enumerated in non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes (E). Bars represent the mean of the moderate infections with standard error, and the high infections are also shown (white dots). The change in eosinophil number over time for each cercarial dose is shown in (F), where buffaloes #4 (white dots) had a high dose (2400) and #1-3 (grey dots) had a moderate dose (200-600), respectively.

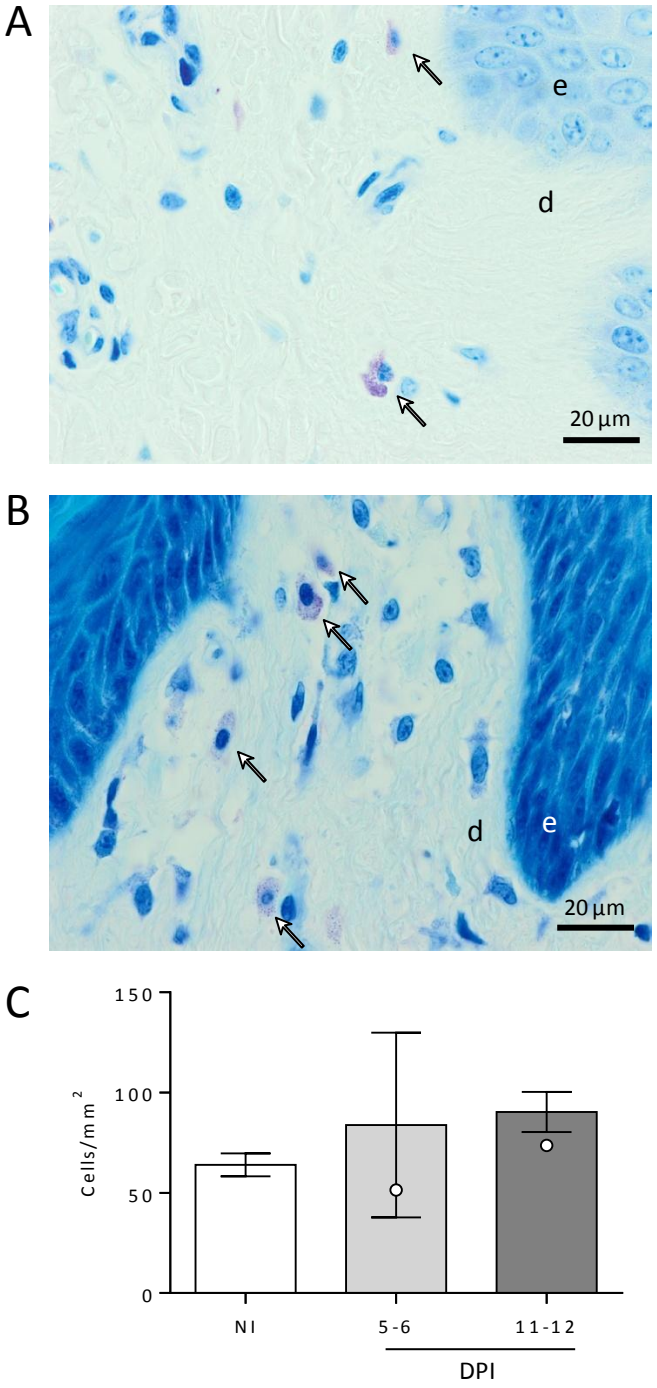


Figure 5: Mast cells in buffalo skin following cercarial penetration

Mast cells highlighted in buffalo skin sections by toluidine blue stain, seen in both uninfected (A) and 5 days post-infected skin (B). Mast cell numbers in the dermis are shown in (C) for non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes. Means of the moderate infections are shown with standard error, and the high infections are also shown (white dots). Epidermis (e) and dermis (d) are indicated for reference.

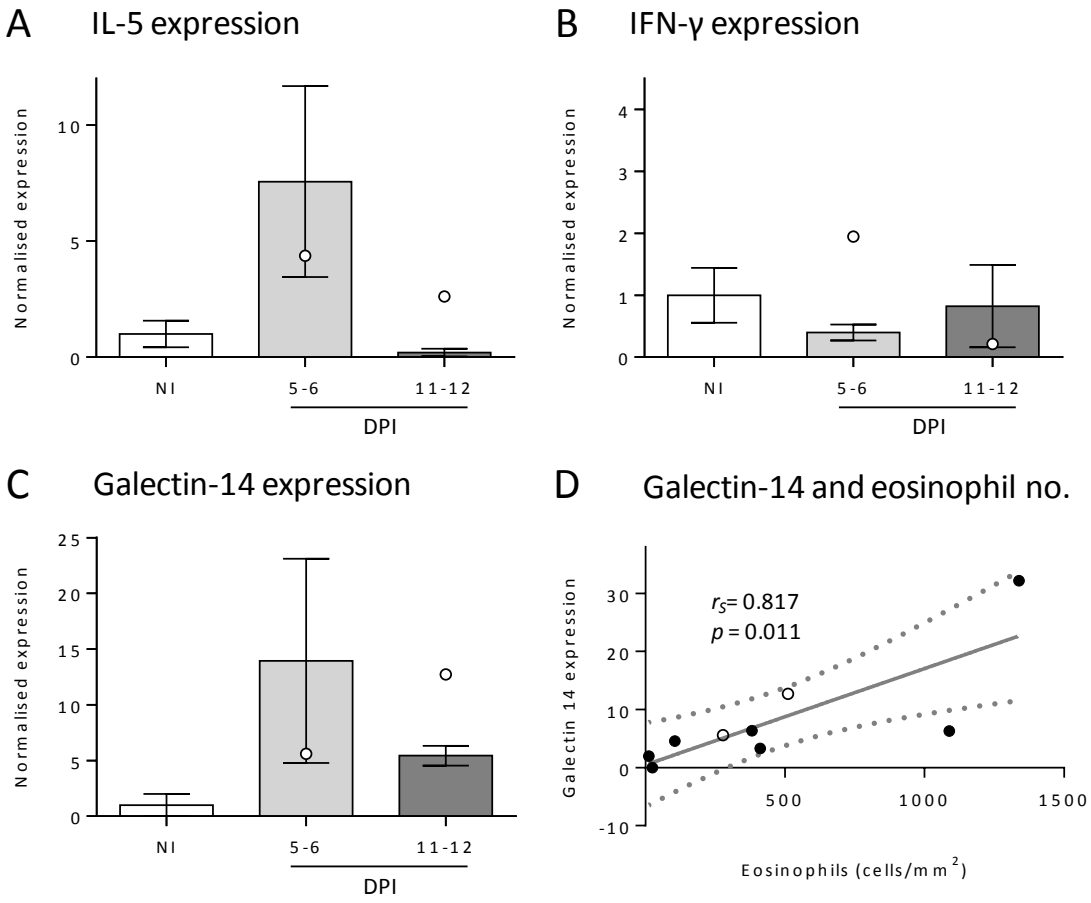


Figure 6: Transcript levels of IL-5, IFN-γ and galectin-14 in buffalo skin

The level of IL-5 (A), IFN-γ (B) and galectin-14 (C) transcripts were quantified in skin samples by quantitative real-time polymerase chain reaction (qPCR) from non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes. Each sample was normalised to the expression of β-actin, and then shown relative to the mean of the NI samples. Bars represent the mean of the moderate infections with standard error, and the high infections are also shown (white dots). The level of galectin-14 transcript and eosinophil numbers in tissue sections positively and significantly correlated (D) as determined by a Spearman's rank coefficient calculation (r_S = 0.817, p = 0.011). White points again indicate the high-dose infection buffaloes.

3.3.3 Cytokine responses in draining LN

To determine the type of immune response induced locally in the skin and lungs by migrating schistosome larvae, and compare this to the liver response induced by the resident eggs, lymphocytes from the LN draining these tissues were cultured and stimulated non-specifically and the level of secreted cytokines were determined by ELISA. Levels of IFN-γ and IL-4, the prototypical type-1 and -2 cytokines respectively, and IL-10, were measured and the means for the six moderate infection dose animals are shown in Fig. 7. Surprisingly, the two buffaloes given the high infection dose had generally impaired draining LN cytokine responses at the time points analysed; this was particularly the case for the skin-LN, where levels of each cytokine from these high-infection buffaloes was in the range of the NI buffaloes, while the moderate-infection animals were generally higher.

For the skin-LN, there was an increase in all three cytokine levels at 5-6 DPI, which was reduced at 11-12 DPI. While only IL-4 showed a statistically significant increase in 5-6 DPI above NI levels ($p = 0.047$), all buffaloes at 5-6 DPI had higher IFN-γ levels than NI animals and 2 of 3 remained higher at 11-12 DPI, indicating a prolonged IFN-γ response. Interestingly, the high-dose buffaloes had higher levels of each of these cytokines at 11-12 DPI than 5-6 DPI in the skin-LN cultures (Fig. 7), similar to the eosinophil and galectin-14 levels in the skin.

For the lung-LN response, only the IFN-γ levels showed a significant increase at the latest time point of infection compared to the NI level ($p = 0.036$); there was no change in IL-4 or IL-10 over the infection period. It was also noted that the lung-LN had a higher level of IL-4 and IL-10 than skin-LN among the NI animals, indicating a difference in basal stimulation of the lungs compared to the skin. The IFN-γ levels were consistent among each LN in NI individuals. The liver-LN response showed a trend towards an increase for each cytokine, although this was only significantly above NI for IFN-γ ($p = 0.011$).

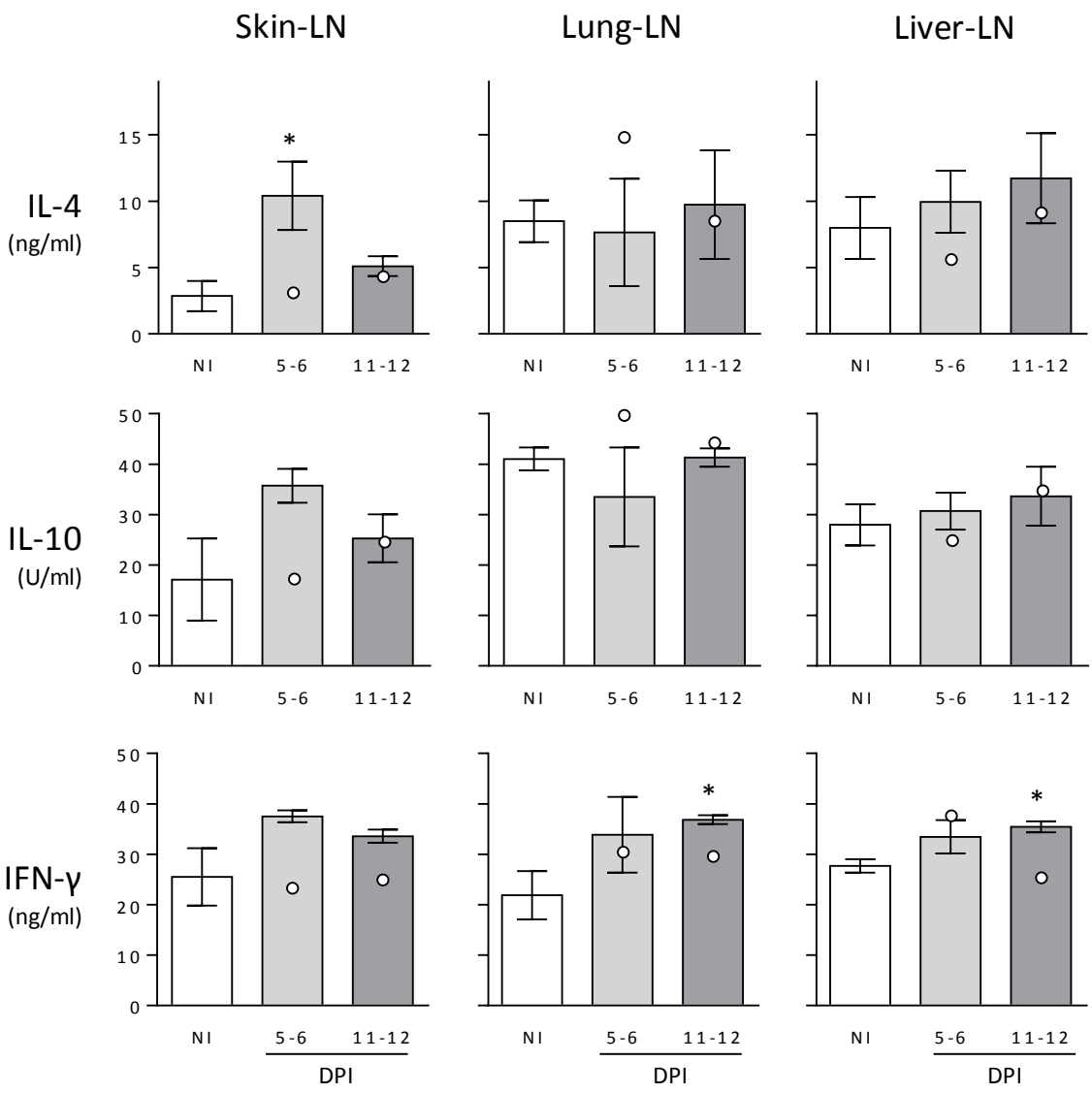


Figure 7: Cytokine levels in stimulated lymph node cell culture supernatants

Levels of IFN-γ, IL-4 and IL-10 were measured by ELISA from PMA/ionomycin stimulated lymphocytes taken from skin-, lung- and liver-lymph nodes (LN) from non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes. Bars represent the mean of the moderate infections with standard error, and the high infections are also shown (white dots). Asterisks indicate significance above NI levels ($p < 0.05$).

The ratio of IL-4 to IFN-γ levels for each stimulated LN culture is shown in Fig. 8 to indicate the balance of type-1 and -2 immune responses in each draining LN. The ratio of these two cytokines in uninfected LN cultures differs in each tissue-draining LN, indicating a difference in these tissues prior to challenge. While the skin has a relatively low IL-4/IFN-γ ratio (0.1), the lung and liver begin at a higher level (0.5 and 0.3, respectively). Although not significant, all lung-LN and 2/3 liver-LN ratios were higher than skin-LN ratios. Following infection with cercariae, the skin-LN and the lung-LN then deviate from these NI levels. In the skin-LN, the ratios for each animal are higher at 5-6 DPI (0.3) than in NI animals, as IL-4 increases relative to IFN-γ indicating a type-2 response. This then returns to the NI level in 11-12 DPI buffalo skin-LN. However in the lung the opposite occurs, where the ratio deviates lower at 5-6 DPI (0.2) and stays a similar level at 11-12 DPI (0.3), suggesting a predominant and prolonged type-1 response. In contrast, in the liver-LN no change from the NI ratio is seen at these two time points indicating a stable balance of these two cytokines.

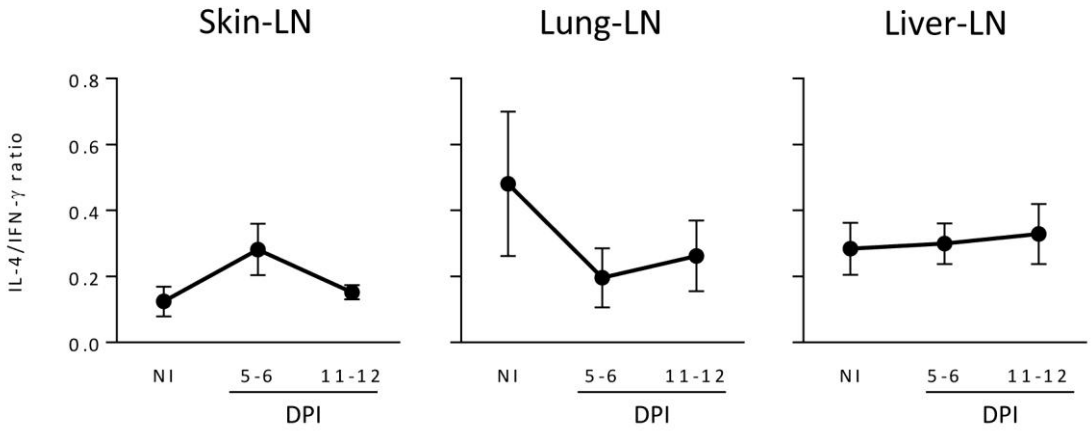


Figure 8: IL-4/IFN-γ ratios from each stimulated LN cell culture during larval schistosomiasis

The ratio of IL-4/IFN-γ levels is represented from PMA/ionomycin stimulated lymphocytes taken from skin-, lung- and liver-lymph nodes (LN). Results from non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes (moderate infection dose) are shown. Means and standard error of the mean are represented.

3.3.4 The local antibody response during larval schistosome migration

In order to measure the local antibody response against migrating schistosome larvae in the water buffalo, the level of antibodies were determined in the ASC-probe samples. Initially, the total antibody level was measured by a capture ELISA as performed in Chapter 2 for the rat ASC-probes. However high background levels were observed with this ELISA, most likely due to the finding that the media supplemented with AlbuMAX II, without any buffalo cells cultured, contained a considerable level of contaminating bovine immunoglobulin (13.4 μg/ml, data not shown). Therefore the level of specific antibodies was also measured against soluble adult worm antigen, which showed a greater and more informative difference between NI and infected groups. There was a significant positive correlation between the results of the total and specific antibody ELISAs ($p = 0.014$, $r_S = 0.422$), indicating that these two parameters are related. Also, as for LN cytokine levels, buffaloes which received the high dose of cercariae (buffaloes numbered 1) generally had impaired antibody responses (particularly for specific antibody level) and were excluded from the analysis; the results from these two buffaloes are indicated on the graphs by the hollow points.

The total antibody was quantified by ELISA, and the level of contaminating bovine antibody level in media (13.4 ug/ml) was then subtracted to give the secreted antibody level in each ASC-probe sample (Fig. 9). The only sample to show a consistent increase in total antibody following infection was the skin-LN ASC-probes (Fig. 9A). At 5-6 DPI the level of secreted antibody was almost double the NI level at 14.7 ug/ml compared to 7.5 ug/ml; and although this was not significant, each buffalo at this time point was higher than each NI animal. By 11-12 DPI the level had returned to the uninfected level. The lung- and liver-LN ASC-probes showed no consistent change after infection.

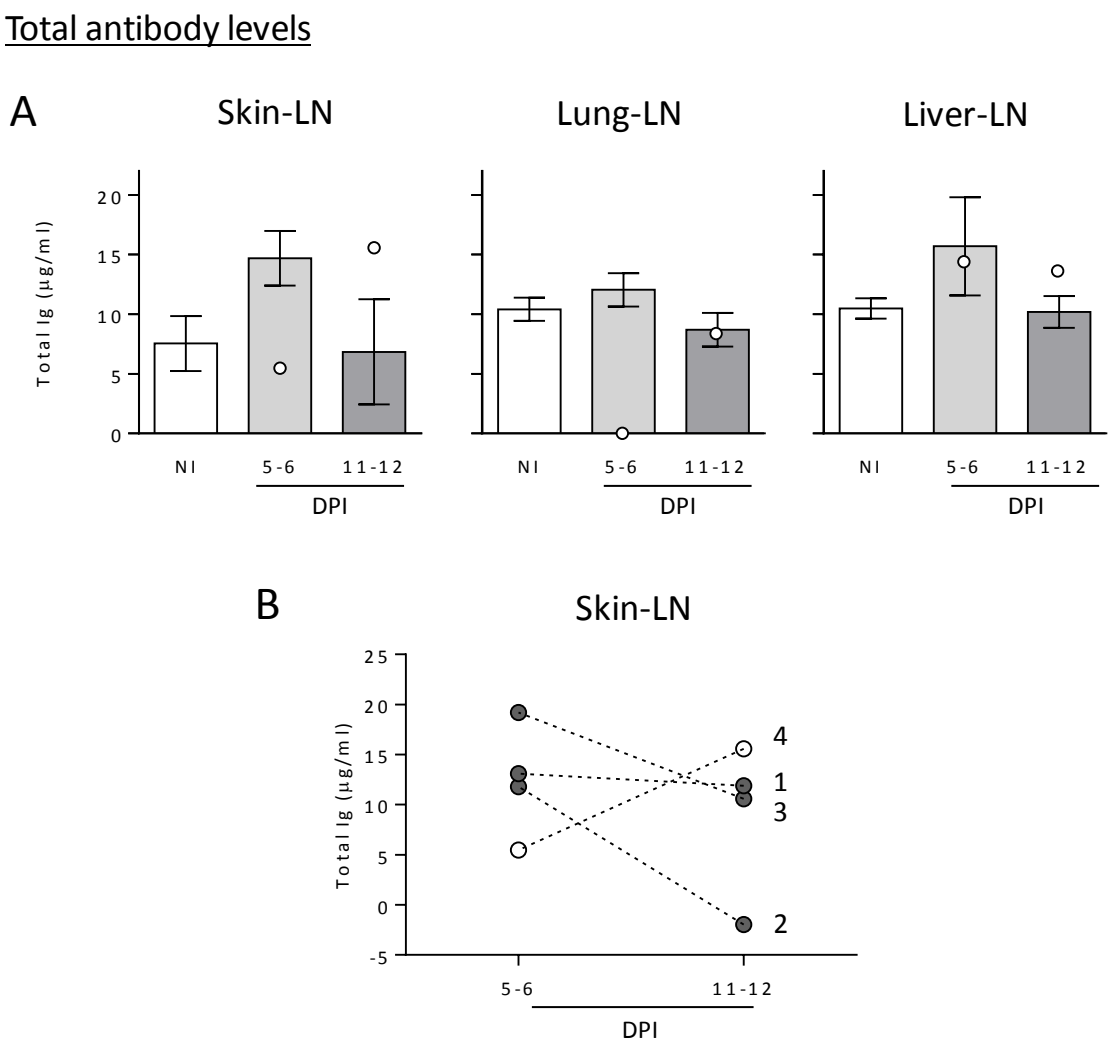


Figure 9: Total antibody levels in antibody secreting cell (ASC)-probes

Total antibody levels in each ASC-probe was measured by capture ELISA and quantified by comparison to a known quantity of purified buffalo antibody. Means and standard error of the mean from skin-, lung- and liver-draining lymph nodes (LN) from non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes are shown (A). Bars represent the mean of the moderate infections with standard error, and the high infections are also shown (white dots). The change in skin-LN ASC-probe total antibody level for each cercarial dose is shown (B), where buffaloes #4 (white dots) had a high dose (2400) and #1-3 (grey dots) had a moderate dose (200-600, respectively).

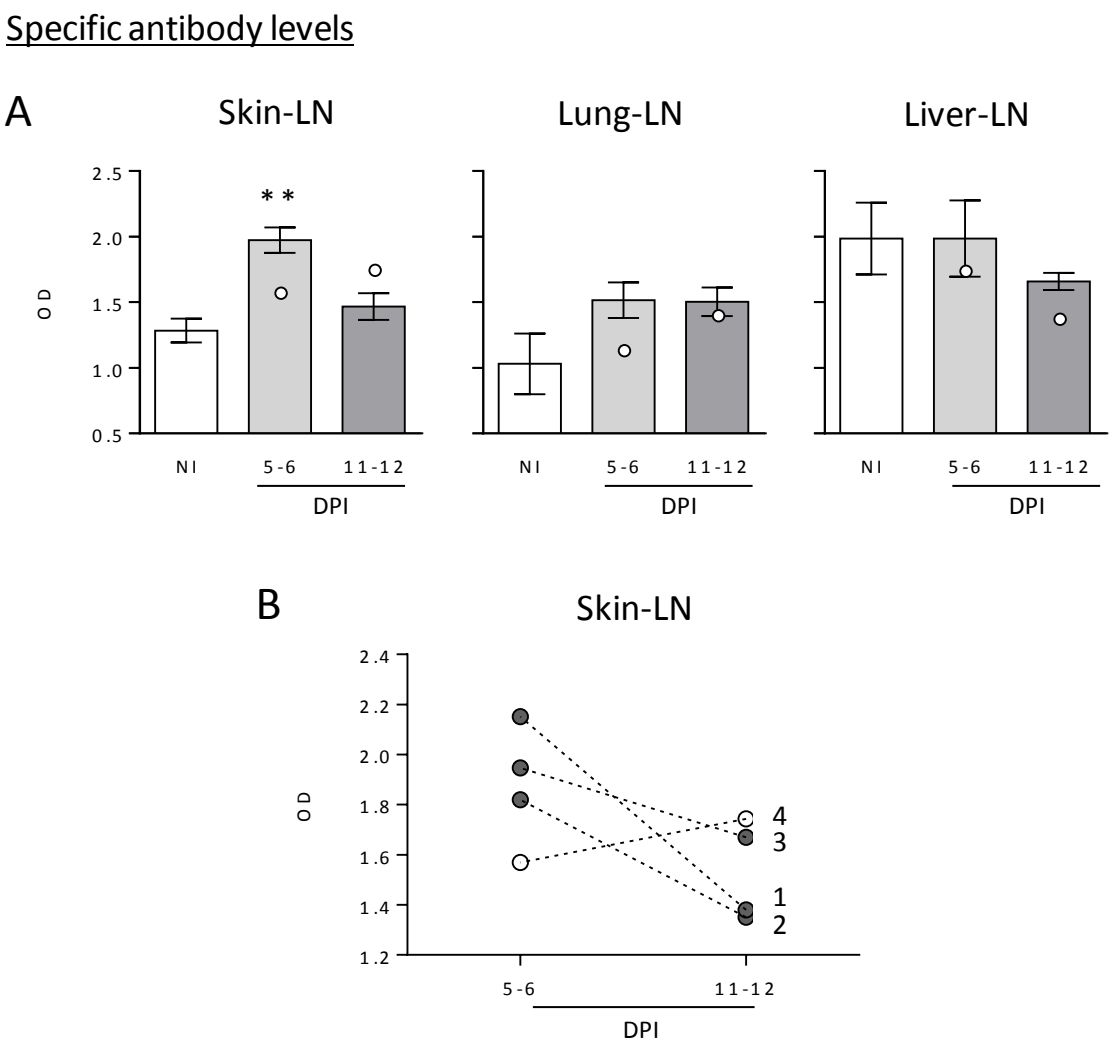


Figure 10: Specific antibody levels in antibody secreting cell (ASC)-probes

The level of antibody specific to adult worm antigen in each ASC-probe was measured by ELISA. Means and standard error of the mean from skin-, lung- and liver-draining lymph nodes (LN) from non-infected (NI), 5-6 or 11-12 days post-infection (DPI) buffaloes are shown (A). Bars represent the mean of the moderate infections with standard error, and the high infections are also shown (white dots). The skin-LN ASC-probe samples at 5-6 DPI were highly significantly different than NI or 11-12 DPI samples, shown by the asterisks ($p = 0.006$). The change in skin-LN ASC-probe specific antibody for each cercarial dose is shown (B), where buffaloes #4 (white dots) had a high dose (2400) and those #1-3 (grey dots) had a moderate dose (200-600, respectively).

Figure 9B shows the total antibody levels of individual skin-LN ASC-probe samples, and the buffaloes given the same dose of infecting cercariae are linked and numbered (see Table 1 for cercarial numbers). Similar to the dermal eosinophil numbers result (Fig. 4F), the total antibody level was increased at 11-12 DPI only in high-dose buffaloes (those numbered 1) while it was lower in moderate-dose buffaloes (numbered 2-4). The same result was found in the specific antibody ELISA as shown in Fig. 10B.

The specific antibody level results are shown in Fig. 10, and despite a high level of basal reactivity in NI groups, where optical densities (OD) were around 1.0-1.3 for NI skin- and lung-LN samples, a greater difference was seen between NI and infected buffalo ASC-probes than with the total antibody ELISA (Fig. 9). The specific antibody level in skin-LN ASC-probes was highest at 5-6 DPI, as found in total antibody ELISA, however it was highly significant in this case ($p = 0.006$). While infection did cause a slight increase in the specific antibody level of lung-LN ASC-probes, it was statistically insignificant and was similar at both time points after infection. The liver-LN samples had consistently high levels of specific antibody which did not change with infection at these time points. Among the NI samples, the specific antibody level from liver-LN samples was significantly higher than the skin- or lung-LN ASC-probes ($p = 0.046$), indicating chronic antigenic stimulation in the liver, most likely from resident eggs, and a lack of specific antigen stimulation in the skin and lung in NI animals.

3.3.5 Buffalo ASC-probes recognise stage-specific antigens

Considering the ASC-probes from skin- and liver-LN contained high levels of specific antibody, the recognition of these probes to antigens from two life stages of schistosomes was investigated by western blotting (Fig. 11). Cercariae (C; lanes 1-3) and adult worm (A; lanes 4-5) SDS-soluble fractions were probed with skin and liver ASC-probes from buffalo #1 which contained a high level of specific antibody. Also, a pooled ASC-probe sample generated from rat skin-LN was included for comparison.

While there were a number of bands consistently recognised in each blot and by each ASC-probe sample, there were several uniquely-recognised antigens which suggest the ASC-probes recognise stage-specific antigens. Highlighted in lane 1 and 2 are two low molecular weight antigens unique to the cercarial stage and only recognised by skin ASC-probe samples from both buffalo and rat. An antigen of approximately 16 kDa, present in both cercarial and adult extracts, was strongly recognised by liver-LN ASC-probes but only weakly by skin-LN samples. Finally an antigen of approximately 55 kDa, only present in the adult worm antigen (lanes 4 and 5) was predominantly recognised by the liver-LN sample and only weakly by the skin-LN sample.

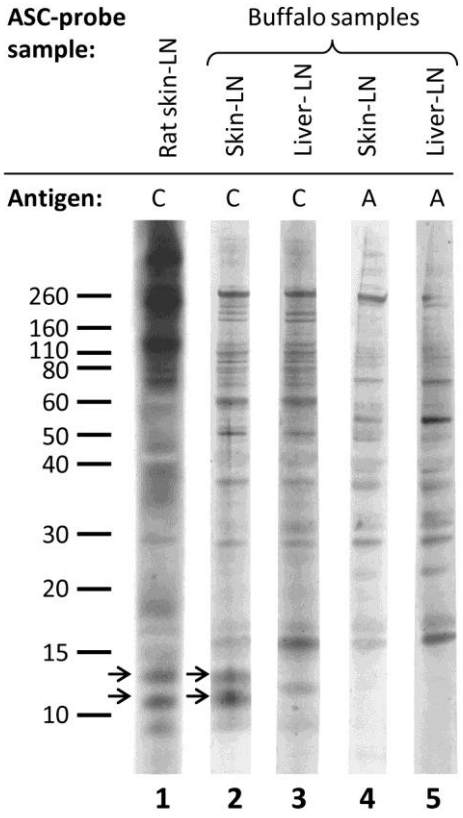


Figure 11: Binding of skin- and liver-LN ASC-probes to schistosome antigens

Total antigen preparations from cercariae (C) or adult worms (A) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Individual blot lanes were excised and then probed with either pooled rat skin-LN ASC-probes (lane 1), buffalo ASC-probes from skin-LN (lanes 2 and 4) or liver-LN (lanes 3 and 5). Molecular weight markers are shown on the left-hand side. Two larval-specific bands only recognised by skin-LN ASC-probes are indicated by the arrows.

3.4 Discussion

In this chapter, the local immune response against migrating schistosome larvae was investigated for the first time in the water buffalo, at two sites of larval migration. Despite the evidence of acquired immunity in this natural host, there have been few studies on its immune response during schistosomiasis, and none exploring the anti-larval response (11).

To study the anti-larval response, the skin and lungs were investigated at two time points after cercarial infection, estimated to reflect a mature immune response in the LN draining those tissues. A similar approach was detailed in Chapter 2, where the local antibody response against larvae was successfully captured in the rat model, and similar time points were used in the present study. A major difference in this study from Chapter 2 was that each time point spanned two days due to the inability of completing the experiments in a single day. However since there is heterogeneity in the timing of schistosomula migration, for example in rodents the bulk of larvae gradually leave the skin over a 2 day period, and then arrive into the lungs over several days (34, 36-37), the difference in response over 2 days is likely to be negligible. Also in this study the second time point after challenge was later than in Chapter 2, at 11-12 DPI instead of 9 DPI in the rat study, as it was estimated that larvae arrive later in the buffalo lung compared to rat lungs due to the significant size difference (Prof. Li Yuesheng, personal communication), although this has not been formally investigated in large animal models. Overall the findings indicated that these time points did correspond with the timing of larval migration, since we observed consecutive cytokine responses; the response for all cytokines in the skin-LN was highest at 5-6 DPI, with the lung-LN IFN-γ highest at 11-12 DPI.

3.4.1 Local immune response in the skin

The local immune response against invading cercariae in the skin was the most obvious pathological change evident during the study and was characterised by a strong type-2 inflammatory response. Examination of the skin site showed raised allergic 'wheal' type reactions at 5-6 DPI, which had reduced by 11-12 DPI. Histological and qPCR analysis, as well as skin-LN cell stimulations, confirmed a predominant type-2 response against the penetrating larvae at this tissue site. Large numbers of eosinophils were observed infiltrating the dermis even as late as 12 DPI, which was confirmed by transcript levels of the eosinophil-specific galectin-14. The significant correlation of galectin-14 transcript level to eosinophil number indicates possibility of using qPCR to quantify cell number.

Large eosinophilic abscesses were present indicating a profound inflammatory response disrupting the skin layers in these regions, and the dramatic increase in dermal and epidermal leukocytes further supports this. Similar granulocytic abscesses were described in the infection of mouse skin by Incani and McLaren (224), which contained dead larvae and discarded tails observed 6 hours post-infection, associated with eosinophils only in mice given a secondary infection. Although we did not observe trapped larvae, they may have been present at earlier time points than investigated in our study or revealed by more thorough investigation of skin samples.

The outer most skin layers experienced thickening, which in the epidermis could reflect the significant increase in leukocytes or enlarged keratinocytes. The stratum corneum, which is the principle defensive layer of the skin (225), remained thickened even at 11-12 DPI which may play a protective role from further cercarial penetration. Further examination of the skin at 11-12 DPI indicated that the larvae-associated damage had begun to heal, with several parameters returning to NI levels at this time point. The increase in mast cells at this late time point could be evidence of recovery from tissue damage, since their numbers are known to increase in skin during wound healing (226), or they may serve a defensive function to quickly respond to future cercarial challenge by enhancing the inflammatory response.

The transcript levels of type-1 and -2 cytokines, IFN-γ and IL-5 respectively, indicated a type-2 response in the skin at 5-6 DPI. IL-5 is predominantly secreted by Th2 cells and promotes eosinophil proliferation and survival (222), consistent with the large number of eosinophils observed at this time point. By 11-12 DPI, IL-5 levels had returned to baseline indicating the type-2 response was waning, consistent with a reduction in skin-resident eosinophils.

In the draining skin-LN there was an increase in both IFN-γ and IL-4 following infection, suggesting a mixed type-1 and -2 response. A similar mixed response has been reported following primary infection of mouse skin (227). However in the present study, the ratio of these two cytokines suggests it was predominantly oriented towards the type-2 response, and this agrees with the allergic-type reactions visible in the skin at the same time. Concurrent with the intense inflammatory response in the skin was a rise in IL-10 in the skin-LN, which agrees with other studies showing the induction of IL-10 following cercarial penetration (228). As a regulatory cytokine, IL-10 limits excessive inflammation by targeting leukocytes, down regulating pro-inflammatory cytokine secretion (229). In schistosomiasis it has been considered to be an immunomodulatory strategy of the schistosome parasite to limit the immune response during skin penetration to promote survival (228).

3.4.2 Local immune response in the lungs and liver

In contrast to the dramatic immune events in the skin, there was no detectable gross or histological change in lung tissue post-infection (data not shown). This could be due to the fact that the relatively small schistosomula disperse throughout the lungs, which are of considerable size in water buffalo, and the samples selected here did not happen to contain inflammatory infiltrates which are likely to be localised to small migration paths. Considering the small size of schistosomula (100-200 μm in length) compared to the buffalo lungs, it would be difficult to identify these paths compared to rodent models, where it has been studied extensively and is considered to be the major site of larval attrition (35-36).

However, there was a measurable and significant type-1 response in the lung-draining LN at 11-12 DPI, where IFN-γ increased compared to NI levels. At this time point, the lung-LN was most likely responding to the local larval migration through the lungs, predicted to peak in number through this tissue a few days previously at 7 DPI. Interestingly, in the uninfected lung-LN the levels of IL-4 and particularly IL-10 were high compared to the

skin-LN levels, and infection did not change this. This resulted in a high ratio of IL-4 to IFN-γ in uninfected buffalo lung-LN, indicating a type-2 bias in this draining LN. This could reflect a difference in LN types, between skin-LN (a peripheral node) compared to lung-LN (a mucosal node) or simply different levels of stimulation from the environment prior to infection. However in relation to the incoming schistosomula, considering the initial type-2 bias, the swing towards a type-1 response in the lung would require a strong stimulus. The significant IL-10 level in the lungs indicates a regulatory phenotype that may limit the inflammatory type-1 response and reduce larval killing in the lungs, because a strong lung Th1 response is considered the mechanism of immunity in the murine radiation-attenuated vaccine model (195).

The liver-LN also had more of a type-2 response than uninfected skin-LN, likely due to trapped liver eggs from previous infections which are a strong stimulus for Th2 differentiation (112). Similar to the lung-LN response at 11-12 DPI, the liver-LN had a late IFN-γ response, although this was less pronounced. This slight type-1 response was possibly due to the arrival of immature worms to the liver, which also were found to induce an antibody response in the rat liver at the latest time point in Chapter 2. The timing of larval entering the liver is likely to be shortly after leaving the lungs, as seen in the mouse model (36), since travel time between organ vasculature is minimal (35).

3.4.3 Immune responses to larvae: evidence of immunity?

Overall, the data show that a significant type-2 response was generated following cercarial invasion of water buffalo skin, with a mixed but predominantly type-2 response in the skin draining LN. Then as the larvae migrated to the lungs, a moderate type-1 response was seen in the lung-LN. To our knowledge, this is the first time the immune response to migrating schistosome larvae has been investigated in an endemically-exposed host, shedding light on the immune mechanisms induced during a natural infection. The current paradigm of schistosomiasis holds that the migrating larvae induce a primarily Th1 response, which then becomes a strong Th2 response several weeks later after the worms mature and eggs are deposited in tissues (112). This has largely been elucidated from primary infections in naive rodent models, where the eggs provide a strong Th2-inducing stimulus (205). The radiation-attenuated vaccine has also been studied extensively in mice, where immunity is mostly attributed to a protective Th1 response primed in the skin, which is then effected in the lungs (123-124, 195).

In contrast to the Th1-protective mechanism in the mouse, protection in humans from schistosomiasis-endemic communities is linked with type-2 responses and is positively correlated with specific IgE levels (107-109) and Th2 cytokines (110-111); protection is thought to be effected by eosinophils against the incoming larvae using antibody-dependant cellular cytotoxicity (9, 13, 112). A similar allergic inflammatory skin response described here for the water buffalo was seen in rhesus monkeys given repeated immunisations with radiation-attenuated *S. japonicum* larvae (134). In this model the skin is the major site of larval attrition during the first 24 h, and larvae were cleared by 3 days (135). Since buffaloes have a natural and acquired immunity, it is conceivable that the strong inflammatory type-2 response in the skin region observed here is a mechanism for immunity against

penetrating larvae in a similar fashion to that seen in immunised rhesus monkeys. It is possible that the increased skin thickness of large animal hosts compared to murine skin may delay larval migration and allow more effective immunity to develop at this tissue site. While trapped or killed larvae were not observed in buffalo skin here, dead larvae would most likely be cleared before 5-6 DPI as in immunised rhesus monkeys, and further studies at earlier time points would be required to confirm this.

Despite such a strong type-2 response in the skin and a mixed response in the skin-LN, it was interesting that there was a shift towards a type-1 response in the lung-LN during larval lung migration. As mentioned, much work has been done on the lung immune response following the radiation-attenuated vaccine model in mice, because this model results in high levels of protection that is effected predominantly in the lung by a strong Th1 cell response (123-124, 195). In an investigation into the protective radiation-attenuated vaccine model, Mountford et al. (195) found that while skin-stage larval antigens induced a mixed-type response, with some IFN-γ and higher levels of IL-4, lung-stage larval antigens induced a uniquely Th1 type response. This finding closely reflects the buffalo response observed in this study, where a mixed but largely type-2 response occurred in the skin followed by a type-1 response in the lung region. Therefore the lung response in buffalo may also protect in a similar mechanism as the radiation-attenuated vaccine studied in rodent models.

3.4.4 Local antibody response to larvae

The last parameter of the immune response investigated against the larvae was the local antibody response, by measuring the total and specific antibody levels secreted by draining lymph nodes (ASC-probes). It was previously found that migrating schistosome larvae induce a detectable antibody response that could be captured by the ASC-probe technique in the rat model (see Chapter 2), and this allowed insight into the complex immunology of larval migration. Therefore this technique was applied to the natural host, the water buffalo, in the hope that it could add to the understanding of the local anti-larval immune response. Furthermore since immunity in humans and animal models is thought to be in part antibody-mediated against the migrating larvae (11, 113), the local antibody response could be a tool in novel antigen selection.

The data presented here indicated there was a significant antibody response in the skin region induced against the penetrating larvae, consistent with the robust type-2 response observed in this tissue site, since Th2 cells drive strong antibody responses (230). However there was little change in the lung or liver antibody responses. There was a slight increase in the lung specific antibody level for both time points post-infection, although this was not as dramatic as in the skin-LN. In the rat experiment from Chapter 2, a very similar profile was seen in the skin-LN following infection, however the largest increase in antibody response was in some lung samples, in contrast to the buffalo experiment shown here. As suggested previously, the low immune activation seen in the lungs by these experiments may be due to the large size of the buffalo lungs and the comparatively minute larval challenge. Despite the vast difference in lung size, rats are commonly challenged with several hundred cercariae (37), while the moderate infections in buffaloes were 200-600 cercariae. Even the high-dose buffaloes (2400 cercariae) had little response in the lungs (these animals are discussed further below). This highlights the vast

difference in models of infection from the large natural hosts and may suggest different immunity effector mechanisms occurring in the water buffalo from rodent models.

While infection did not change the local antibody response in the liver-LN, it was noted for its high resting specific antibody production in uninfected animal LN, in comparison to skin- and lung-LN. This may indicate the presence of specific ASC in the liver-LN before infection as a result of liver-deposited eggs, since these are known to be the most significant antigenic challenge in schistosomiasis (190). All buffaloes were sourced from an endemic area and evaluated for positive faecal egg counts before commencement of the trial and would therefore have high probability of egg deposition in the liver from previous infections.

The antigen recognition profile of the anti-larval skin antibody response was compared to the liver LN response by western blotting of schistosome antigens, since these were the most pronounced antibody responses and were induced by different parasite stages. While many similar antigens were recognised probably due to cross-reactive epitopes in cercariae and adult worms, there was an indication that larval-specific antigens were recognised only by the skin antibody response, particularly two antigens consistently recognised by both buffaloes and rat skin ASC-probes. One-dimensional SDS-PAGE western blotting can indicate larval-specific antigen binding, but is not suitable for antigen identification. Further experiments using 2D gels or protein microarrays (231), and carbohydrate arrays (217), should be conducted in the future in order to identify these novel vaccine targets.

3.4.5 Observations on cercarial infection doses on the local immune response

In this study the number of cercariae required to induce a measurable immune response in water buffalo against the larvae was not known. Due to a limited number of cercariae, a moderate dose of 200-600 cercariae was used to infect most of the buffaloes. One buffalo at each time point was however infected with a much higher dose (2400 cercariae) in an attempt to generate high local antibody levels for future antigen identification.

It was initially anticipated that the high-dose buffaloes would have increased immune responses against the large antigenic and immune stimulus considering they received 4-12 fold more cercariae than the moderate doses. Surprisingly, in almost every parameter this was not the case and the high-dose buffaloes consistently had the lowest response. While the immune responses in the local skin site were similar to the moderate infection animals at the early time point of 5-6 DPI, the immune response in the draining lymph node was consistently low. This indicates that while the cercariae did infect the skin with these high numbers, the draining skin-LN either did not receive the immune stimulus to the same degree as the moderate infections, or there was a higher down-regulation of the immune response in the skin-LN.

Another interesting finding was that in several parameters of the skin region, the immune response was greater in the high-dose animal at 11-12 DPI than at 5-6 DPI, whereas the inverse was true for each of the moderate infection buffaloes. The number of eosinophils and IL-5 transcript in the skin, as well as the cytokine and antibody responses in the skin-LN, were higher at 11-12 DPI than 5-6 DPI only in high-dose buffaloes. This

indicates a delayed or persistent immune response with the higher challenge whereas the moderate-infection doses had begun to resolve the damage by 11-12 DPI.

A possible explanation for this reduced and delayed immune response in the skin and draining LN following a high dose is a greater down-regulation of the immune response. It is well documented that cercarial secretions have an immunomodulatory function which reduces inflammation and facilitates immune evasion (228), therefore a greater cercarial number could explain the down-regulated LN response. If there are more cercariae present in the skin they would leave more products such as the secretions, the cercarial surface (the glycocalyx), and their tails, which could result in immunomodulatory or immunosuppressive effects. In particular, while cercariae are generally thought to discard their tails before skin penetration, Wang *et al.* (232) recently showed that with high-density cercariae infections more tails enter the skin, and products derived from these tails may suppress or delay immune responses in the high-dose buffaloes.

In future experiments the moderate dose should be adopted if analysing the immune response to migrating larvae, since 200-600 cercariae was sufficient to study the immune response at the local site and the draining LN. Indeed the lowest dose buffaloes often had the highest response, and it is worth noting that there was no consistent dose response within the moderate dose animals.

3.4.6 Study limitations

The numbers of buffalo in this study was a significant limitation, since most comparable studies on schistosome larvae have been performed in mice which have much less logistical restrictions and group sizes can be considerably larger. Also, since few immunological studies had been performed on the water buffalo, the number of infecting cercariae required was unknown. From this study several hundred parasites was sufficient to study the anti-larval immune response, and in future studies a consistent number of cercariae would be better in each group to maximise the small numbers available. Also, to better analyse the skin response, earlier time points should be studied to determine if larval attrition is a feature of the buffalo skin immune response.

Regarding the ASC-probe samples generated in this study, as mentioned previously there was a significant amount bovine antibody contained in the media supplemented with Albumax II, and this resulted in a high level of background in the ELISA and western blotting experiments. While the specific antibody ELISA did show a difference in the skin region post-infection, perhaps if there was less background other subtle differences would have been observed, e.g. in the lung samples. An attempt was made to measure the antibody isotype levels in the ASC-probes, as done in Chapter 2, however this was not successful (data not shown) since it was difficult to separate the difference between contaminating bovine and the secreted buffalo immunoglobulins. Also, in order to reduce the high non-specific binding in the western blotting, the antibody needed to be purified from the ASC-probes, and a lengthy process to reduce the background for these experiments was also required. Therefore this was a significant limitation in these experiments, and in the future different culture supplements without any bovine Ig contamination need to be investigated. Further experiments should be performed in an attempt to

identify the antigens recognised by the skin ASC-probes. This could include immunoprecipitation or two-dimensional Western blotting.

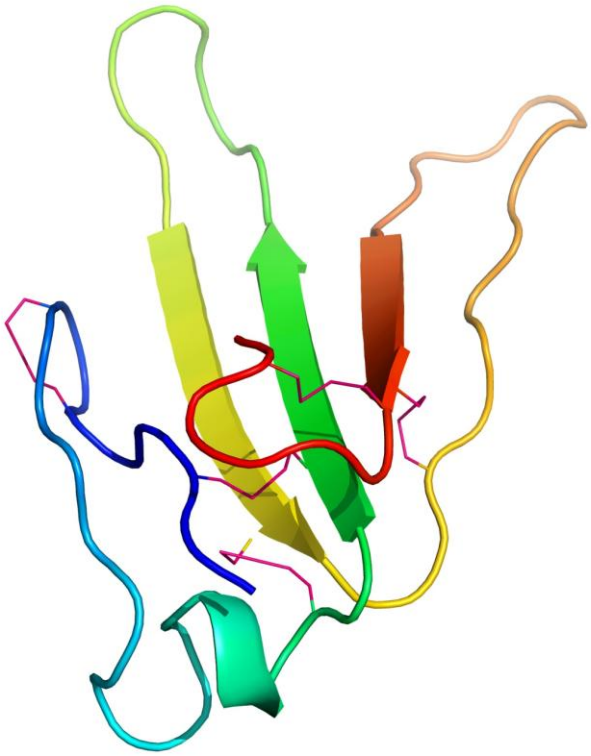
3.4.7 Conclusions

This chapter outlines the immune response of the endemically-exposed natural host, the water buffalo, to migrating schistosome larvae. After infection, a strong inflammatory type-2 response was observed in the skin, which is similar to responses observed in animals given repeated infections to induce immunity; it is therefore possible that the skin is the site of larval killing in this partially-immune host. The type-2 response resulted in the induction of a strong local antibody response in the skin region, which was captured by generating lymph node-derived ASC-probes and may provide a tool for the discovery of novel larval antigens recognised by the buffalo humoral response. The local lung immune response was comparatively weaker, and shifted to a type-1 response, although this could be influenced by the high IL-10 levels and type-2 bias in the resting lung-LN.

This study provides a novel insight into the immune response of water buffaloes, important transmission reservoir hosts for schistosomiasis japonica, and indicates that distinct type-1 and -2 responses occur in distinct tissue regions against migrating larvae. Finally, since this host may be able to generate partial immunity to this parasite, this study suggests that vaccination formulations which replicate this response could be more effective.

Chapter 4:

Identification and characterisation of a novel vaccine target against schistosomiasis



4.1 Introduction

An effective vaccine against schistosomiasis would be a major step towards eliminating this devastating and widespread tropical parasitic disease, and has been the focus of intense research for decades (11). Numerous schistosome vaccine candidates have been discovered with varying levels of protection, and several molecules are in development (150); however a commercial vaccine still remains elusive. It has been suggested that more effective vaccine candidates remain undiscovered; hence there is a need to find novel targets (11-12).

The radiation-attenuated larval vaccine model provides the best evidence that high levels of protection can be generated against schistosome infection. When a range of animal models are infected with radiation-attenuated cercariae, the resulting immunity rejects up to 90% of the challenge infection (122, 124). In this model the larvae do not progress to adult worms, and the consensus is that the early developing schistosomula represent a potent source of protective antigens and are the target of this protective response (122, 124). Furthermore, these larval stages are also suspected of being the target of naturally acquired immunity in humans (9, 13, 112). In contrast, the adult worms can live in the host bloodstream for decades despite being surrounded by a specific immune response (32, 43). Therefore larval-specific antigens, or simply molecules exposed uniquely in larvae, could serve as effective novel vaccine candidates. Despite this the current schistosome vaccine targets are largely adult antigens and there is a lack of larval antigens (100).

Recently the genomes of all three major schistosome species have been sequenced (151-153), and several post-genomic approaches and high throughput methods have been developed to cope with this wealth of information. One such approach is a schistosome-specific protein microarray, which contains 232 unique antigens displayed on nitrocellulose slides (231). These proteins were selected from bioinformatic data based on criteria biased towards promising vaccine candidates i.e. up-regulated expression in larvae; predicted/known parasite surface expression; and limited similarity with mammalian sequences (231). Many of these are novel molecules and the majority are from *Schistosoma japonicum*, with the remainder from *S. mansoni*. The arrayed proteins can be probed with infection-induced antibodies as a powerful new technology for vaccine antigen discovery (100).

This chapter describes the screening of this protein microarray with the anti-larval antibodies generated in Chapter 2, in order to identify antigens exposed in the vulnerable skin and lung larval stages. These samples were generated from rats are known to have antibody-mediated immunity against the migrating larvae during a challenge infection (113, 196); therefore these samples potentially recognise protective antigens. Several novel antigens were discovered, one of which was produced in recombinant form and further characterised.

4.2 Materials and methods

4.2.1 Animal ethics statement

The conducts and procedures involving animal experiments were approved by the Monash University animal ethics review board, and the Animals Ethics Committee of the Queensland Institute of Medical Research (project no. P288). This study was performed in accordance with the recommendations of the Australian code of practice for the care and use of animals for scientific purposes, 2004.

4.2.2 Protein microarray screening

A protein microarray consisting of 232 unique schistosome proteins, as described by Driguez et al. (231), was screened using ASC-probes from the rat study from Chapter 2. The microarray slides were hydrated using Blocking Buffer (BB; Whatman) for 30-60 min at RT, using 16-pad incubation chambers and frames (Whatman) to separate arrays. The ASC-probes (undiluted) or serum (1:100 in BB) were then incubated on the arrays, and put in a sealed box overnight at 4 °C. The ASC-probe samples used were four skin- and four lung-LN ASC-probes from infected rats (groups I_5 and I_9). Considering non-infected (NI) ASC-probes contain little antibody and are not ideal controls for non-specific antibody binding, NI rat sera (n = 15) was used instead. Arrays were washed 3 times with tris-buffered saline (TBS) with 0.05% Tween-20 (TBST), and biotin-conjugated anti-rat IgG was then incubated on the array (1:1000 in BB) for 1 h at RT. After 3 washes in TBST, streptavidin-conjugated Cy5 fluorophore (Surelight P3, Columbia Biosciences; 1:200 in BB) was incubated for 1 h RT. After a final 3 washes in TBST the array slides were separated from the chambers and were washed in distilled water and dried by centrifuging for 5 minutes at 500 x *g*. The slides were stored in the dark until scanned.

4.2.3 Microarray scanning and antigen identification

Scanning was performed on a confocal laser microarray scanner (Genepix 4300A, Molecular Devices), and the signal intensity (SI) was quantified using image analysis software (Genepix Pro 7, Molecular Devices) and transformed and normalised using the vsn statistical package (http://www.r-project.org). Finally the data was re-transformed (inverse $\log_2$) to a normalised SI (233-235).

Antigens were considered positively recognised by an ASC-probe sample when the mean SI for skin- or lung-LN ASC-probes was greater than two standard deviations above the mean of NI rat sera. Amino acid sequences of the identified antigens were then analysed for features of potentially important vaccine candidates: developmental expression was based on EST Profile Viewer (http://www.ncbi.nlm.nih.gov/unigene); presence of a signal peptide using SignalP -4.1 (www.cbs.dtu.dk/services/SignalP); and a predicted transmembrane domain with TMpred (www.ch.embnet.org/software/TMPRED_form.html). Also, literature

searches were performed to determine if each antigen is novel. The antigens were considered novel if it has not been tested for vaccine efficacy against *S. japonicum* and has not had significant characterisation.

4.2.4 RNA isolation from schistosome life-stages

Developmental life-stages of schistosomes were processed for transcript expression analysis. Cercariae were obtained from freshly-shed infected *Oncomelania hupensis* snails and transferred using a sterile bacterial loop directly to Qiazol Lysis Reagent (Qiagen). Two-day (2d) schistosomula were manually transformed from cercariae using the syringe method as described in Chapter 2 (Section 2.2), and cultured *in vitro* for 2 days at 37 °C with 5% CO_2. Three-day (3d) schistosomula were obtained from infected mice lungs 3 days post-infection (lung-stage schistosomula) as described by Gobert et al. (199), and were lysed directly in Qiazol. Adult worm pairs and separate males and females were obtained from freshly-perfused mice and washed in PBS before homogenising in Qiazol. The RNA was then extracted from each stage following the manufacturer's protocol and stored in water at -80 °C until required

4.2.5 Developmental expression of novel antigens by quantitative real-time PCR (qPCR)

After determining the RNA concentration using on a NanoDrop spectrophotometer (Thermo Scientific), total RNA (300 ng) was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) which includes a genomic DNA removal step. To perform the qPCR, primers were designed based on the novel antigen sequences identified in the protein microarray screening and using NADH dehydrogenase ubiquinone flavoprotein 2 (NDUFV2) as the reference gene (236). The primer sequences were: AY815838 (forward: 5'- CGTCGACATTCAAGTTGGTC -3', reverse: 5'- GGGGCATAATCTTCACTTTGA -3'); Ly-6-like-1 (forward: 5'- TGAAAGTTTTGGGACTTTGTATG -3', reverse: 5'- CGAATGGATTCGGACAGTCT -3'); calponin-like (forward: 5'- CATGTCATTCGGTGCTCAAC -3', reverse: 5'- TTCAGCAATATGACGTTGATTACTA -3') and NDUFV2 (forward: 5'- CGAGGACCTAACAGCAGAGG -3', reverse: 5'- TCCGAACGAACTTTGAATCC -3'). Because calponin-like protein has homology to another calponin homologue in *S. japonicum* (accession #AAD11976), the primers were designed in the most dissimilar region. The qPCR reactions included SYBR Master Mix (Applied Biosystems) and the above primers at 0.5 μM and 5 μl of 1:20 diluted cDNA, and were run in triplicate on an Eppendorf Realplex4 Mastercycler for 35 cycles, using an annealing temperature of 55 °C. Melt curve analysis was done to ensure a single product was amplified. The relative copy number of SYBR green for each gene was calculated from a standard curve of serial dilutions of cDNA, and the relative expression of the target gene was determined relative to the reference gene copy number. Finally, the expression of each gene was calculated relative to the cercarial level to observe the change through development.

4.2.6 Sequence analysis of S. japonicum *L6L-1 (Sj-L6L-1)*

Orthologous sequences to Sj-L6L-1 were identified by BLAST searching the amino acid sequence against various databases. The NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to identify related *S. japonicum*, *S. mansoni*, and mammalian species (*Mus musculus* and *Homo sapiens*). For other parasitic trematodes (*S. haematobium*, *Fasciola hepatica*, *F. gigantica*, *Opisthorchis viverreni* and *Clonorchis sinensis*), searches were performed using the databases at www.gasserlab.org. Sequence alignments were performed by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The presence of a signal peptide and omega site (which denotes the position where the C-terminal propeptide is cleaved off in the mature protein and is replaced with a glycosylphosphatidylinositol (GPI)-anchor) was predicted by SignalP -4.1 (www.cbs.dtu.dk/services/SignalP/) and big-PI predictor (http://mendel.imp.ac.at/gpi/gpi_server.html), respectively. Structural homology searches were performed using the Phyre2 server (www.sbg.bio.ic.ac.uk/phyre2) (237), and the protein structure was displayed using PyMOL (v1.3; www.pymol.org).

To assess the evolutionary relationships of Sj-L6L-1 with related orthologues, a phylogenetic tree was constructed using Mega5.1 (238). Amino acid sequences were aligned using ClustalW, and the tree was constructed using the neighbour-joining method with bootstrapping (1000 replicates) to test branch reliability. The p-distance method was use to estimate evolutionary distances.

4.2.7 Production and purification of recombinant Sj-L6L-1 (rSj-L6L-1)

The Sj-L6L-1 sequence was amplified from the pXi T7 vector used in the construction of the protein arrays (231). Primers were designed to produce the mature Sj-L6L-1 protein without the predicted N-terminal signal peptide and C-terminal propeptide; beginning at M-20 and truncated at the predicted GPI-anchor attachment site at N-95 (see Fig. 3) (forward primer: 5' – ATGAAAAATAAAAAGGTCAAATG – 3', reverse: 5' – ATTACAATAATCTTCATCACAAC – 3'). Amplication of the 231 bp Sj-L6L-1 fragment was performed using Phusion High Fidelity DNA Polymerase (New England Biolabs). Next 3' adenine overhangs were added by incubating the PCR product with 1 unit of Platinum Taq DNA Polymerase (Life Technologies) and 0.2 mM dNTP mix for 10 min at 72 °C. This was purified and then cloned in-frame into the pBAD/TOPO ThioFusion plasmid (Life Technologies) according to the manufacturer's directions. This resulted in a fusion protein with thioredoxin (*E. coli*) as a solubility tag and V5/6His purification tag at the amino- and carboxy-terminus, respectively.

The plasmid was sequenced to confirm the correct sequence, and then transformed into TOP10 *Escherichia coli* cells (Life Technologies). Expression was induced by adding arabinose (0.005% w/v), and soluble rSj-L6L-1 present in the bacterial lysate supernatant was purified on a HisTrap HP column (GE Healthcare) according to their instructions. Briefly, the concentration of imidazole was increased from 20 mM to 500 mM. Fractions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-

PAGE) and the purest fraction was dialysed into tris-buffered saline (TBS; 20 mM tris, 150 mM NaCl, pH 8.0) and stored at -20 °C. The thioredoxin fusion partner alone (with the V5/6His tag; referred to as rTrx) was also produced using the 'empty' pBAD vector, and purified in the same way to be used as a control.

Antiserum against rSj-L6L-1 and rTrx was generated by injecting a rat with 50 μg of rSj-L6L-1 or rTrx with 200 μg QuilA in 0.1 ml PBS. A secondary immunisation was administered 2 weeks later, after which a test bleed indicated a specific antibody response to the administered antigen.

4.2.8 Schistosome protein extracts

Crude adult worm extract was prepared by homogenising 10 adult worm pairs with 100 μl of sample buffer (with or without reducing agent), heating to 70 °C for 10 min, followed by centrifugation at 12 000 x *g* for 10 min at RT. To determine if Sj-L6L-1 is soluble and present in the tegument, adult worms were separated into different fractions. Tegument was gently removed by a modified freeze/thaw/vortex technique (57, 239). Briefly, washed and snap-frozen worm pairs were thawed in TBS (pH 7.5) on ice for 5 min, followed by vortexing for 5 x 1 sec to allow gentle removal of tegument. The supernatant was removed and centrifuged at 13 000 x *g* for 10 min at 4 °C. The tegument pellet was resuspended in sample buffer, and heated as above. The remaining worms were washed in 20 mM tris (pH 7.4) and then homogenised in the same buffer. After centrifugation as before, the supernatant was kept as the soluble fraction. The pellet was washed twice with buffer followed by another centrifugation step, and then solubilised in 1% SDS. After spinning again, the supernatant was kept as the aqueous-insoluble fraction.

4.2.9 Western blotting for detection of native and recombinant L6L

Purified rSj-L6L-1 and rTrx was prepared by adding sample buffer with or without reducing agent (50 mM dithiothreitol; DTT). Then 1 μg of each was run on 10% NuPAGE Bis-Tris gels, along with Novex Sharp Pre-stained Protein Standards (Life Technologies) and stained with Coomassie.

For western blotting, rSj-L6L-1 (0.5 μg) or worm extracts (approximately 10 μg) were separated by SDS-PAGE (with and without reducing agent) and transferred onto nitrocellulose membranes, which were then blocked overnight at RT in 5% w/v skim milk powder in PBST (SM-PBST). After washing three times in PBST, the primary antibodies were added: rSj-L6L-1 was probed with neat pooled rat lung-LN ASC-probes from infected rats (obtained in Chapter 2); worm extracts were probed with either anti-rSj-L6L-1 or anti-rTrx antiserum (1:500 in 1% SM-PBST). After washing, anti-rat Ig (H+L):HRP (1:1000) (Life Technologies) was incubated with the membranes for 1 h at RT, and washed again. Finally ECL substrate (GE Healthcare) was applied and chemiluminescence detected on Super RX film (Fujifilm).

4.2.10 Recognition of rSj-L6L-1 by enzyme-linked immunosorbant assay (ELISA)

An ELISA was used to quantify the antigenicity of rSj-L6L-1 during schistosomiasis, using samples from infected rats (Chapter 2) and water buffaloes (Chapter 3). Some samples showed binding to the Trx fusion partner alone (data not shown), therefore binding was measured to both rSj-L6L-1 and rTrx. Wells were coated with either recombinant protein (3 μg/ml) in carbonate coating buffer (pH 9.6) overnight at 4 °C. Wells were blocked with either 5% SM-PBST (rat samples) or 0.5% Tween-20 in PBS (buffalo samples). The rat samples used for probing consisted of non-infected rat serum at 1:200 (n = 3) or neat ASC-probes from infected rats: the skin (n = 5); lung (n = 5); liver (n = 5); and spleen (n = 4). The buffalo samples consisted of purified IgG from buffalo serum (purified as described in Section 3.2.9). Duplicate samples were incubated in the wells for 2 h at 37 °C. After washing, either anti-rat Ig (H+L):HRP (1:5000) or anti-bovine Ig (H+L):HRP (1:1000) was incubated for 1 h at 37 °C. TMB solution (Life Technologies) was added to each well, followed by 2M H_2SO_4, and the optical density (OD) was read at 450 nm.

4.2.11 Statistical analysis

When comparing two groups the student's t-test was performed, while the one-way analysis of variance was used for multiple groups. The respective non-parametric analyses were used when data did not have a normal distribution or the variance was not consistent between groups. Differences were considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1 Screening a protein microarray with ASC-probes for antigen identification

A schistosome protein microarray largely containing uncharacterised proteins was employed in order to discover novel vaccine candidates. The array was screened using rat antibody samples generated at the local site of larval infection, the skin and lungs (ASC-probes; see Chapter 2). Of the 232 unique proteins on the microarray, 11 antigens were recognised by the lung and/or skin ASC-probes above the background level (Table 1). Nine of these were recognised by the lung antibody, three by the skin antibody and one (tetraspanin-2; TSP-2) was recognised by both ASC-probe types. The lung samples bound with a higher signal intensity than the skin, likely due to having greater total antibody levels than skin samples. Only 3 antigens were consistently recognised by all 4 of the lung ASC-probes; these were the novel antigen hypothetical protein AY815838, and the known vaccine candidates TSP-2 and 21.7 kDa antigen (Sj21.7). Other known molecules that were recognised by the ASC-probes include 22.6 kDa antigen (Sj22.6), *S. mansoni* filamin, dynein light chain 1 (DLC1), *S. mansoni* tetraspanin-3, and 29 kDa antigen (Sj29).

To select antigens for further characterisation, each of the 11 sequences was investigated as to its novelty, its potential for up-regulated larval expression, and predicted to be exposed to the host (indicated by the presence of a signal peptide and/or transmembrane domain). Four of the eleven identified were novel and recognised by lung ASC probes, and three of these had indications that they were highly expressed in the schistosomula stage. Two of the novel antigens, a hypothetical protein (AY815838) and Ly-6-like protein-1 (Sj-L6L-1) were predicted to have signal peptides and transmembrane domains. The third novel antigen identified, calponin-like protein, had no predicted host-exposed features.

The three novel and potentially larval antigens were further investigated. The AY815838 protein is unknown, but has limited amino acid sequence identity to two surface antigens of *S. mansoni*: Sm25 (accession #AAA29943; 34% identity) and Sm13 (accession #AAC25419.1; 30% identity). Sj-L6L-1 has significant homology to a *S. mansoni* antigen that was briefly investigated in a DNA vaccine study (240). Finally calponin-like protein has a predicted size of 27 kDa and has some homology with a 38 kDa *S. japonicum* calponin homologue (accession #AAD11976; 63% identity) previously investigated (241).

Table 1: S. japonicum *protein microarray antigen recognition by rat ASC-probes*

		Recognition* by ASC-probes				Expression			
Name	**Accession**	**Skin (No.)**		**Lung (No.)**		**Novel**[Δ]	**profile#**	**SignalP†**	**TM∞**
Tetraspanin-2 (TSP-2)	EF553319.1	++	(3)	+++	(4)	No	S	No	Yes
Hypothetical protein	AY815838	-	(1)	+++	(4)	Yes	S	Yes	Yes
22.6 kDa antigen (Sj22.6)	L08198	-	(0)	++	(3)	No	S, A	No	Yes
Calponin-like	AY813467	-	(0)	++	(3)	Yes	S	No	No
Filamin (*S. mansoni*)	XM_002571418	-	(0)	++	(2)	No	C, S, A, Sp	No	Yes
21.7 kDa antigen (Sj21.7)	AF048759	-	(1)	++	(4)	No	S,A	No	No
Dynein light chain 1 (DLC1)	AF072327.1	-	(0)	+	(2)	No	E, S	No	No
Zinc finger protein	AY223099	-	(1)	+	(2)	Yes	E, A	No	No
Ly-6-like	AY816003	-	(1)	+	(1)	Yes	S	Yes	Yes
Tetraspanin-3 (TSP-3; *S. mansoni*)	XM_002579498	+	(3)	-	(0)	No	U^	No	Yes
29 kDa antigen (Sj29)	DQ873812.1	+	(3)	-	(1)	No	S, A	Yes	Yes

Recognition level*

+++	= High (>10000 SI)
++	= Moderate (500-9999 SI)
+	= Low (0-499 SI)
-	= Negative (0 SI)

*Recognition level represented as positive when signal intensity greater than the mean+2SD of control serum. Number of ASC-probe samples which recognise each antigen is indicated in parentheses. [Δ] Novelty: defined as not having been tested as a vaccine candidate against *S. japonicum* and limited characterisation. # Expression profile: The stage(s) with >2-fold expression is indicated, based on EST Profile Viewer, UniGene, NCBI. †Signal presence as determined by SignalP -4.1 (www.cbs.dtu.dk/services/SignalP/). ∞Transmembrane prediction by TMpred (www.ch.embnet.org/software/TMPRED_form.html) ^Data on sequence not present in UniGene however up-regulated in schistosomula (Gobert et al. 2010). Ideal novel antigen characteristics are highlighted in blue.

4.3.2 Developmental expression of novel antigens

The developmental expression of the three novel antigen transcripts was investigated by qPCR (Fig. 1). The AY815838 transcript was most highly expressed in the 2 day schistosomula, elevated to 65 times the cercarial expression which then reduced to 5 and 10 times cercarial expression levels in 3-day schistosomula and adult pairs, respectively. Calponin-like protein showed a steady increase in expression throughout the development of the intravascular stages, peaking in the adult worms at 41 times the cercarial expression. Finally Sj-L6L-1 was very highly expressed in the developing schistosomula; the 2-day *in vitro* cultured and the 3-day *in vivo* lung-stage larvae had 23 and 27 times the cercarial expression (respectively), whereas adult males had just 3 times the transcript level. For all three genes, expression in adult worms was predominant or restricted to the males.

From this experiment AY815838 and Sj-L6L-1 were promising novel targets with high larval expression. However since AY815838 had been identified in a previous screening of the protein microarray, and was being pursued as a vaccine candidate in our collaborator's laboratory (P. Driguez and D.P. McManus, personal communication), only Sj-L6L-1 was characterised further.

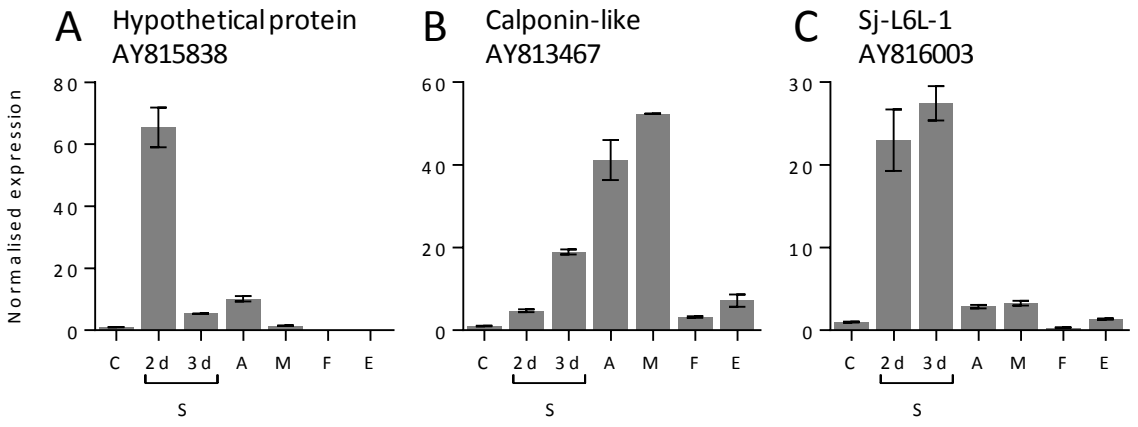


Figure 1: Developmental expression of novel S. japonicum *antigens recognised by lung ASC-probes.*

Expression levels of AY815838 (A), calponin-like (B) and Ly-6-like protein-1 (Sj-L6L-1; C) genes in developmental stages of *S. japonicum* were determined by quantitative real-time PCR. Stages examined were cercariae (C), 2 day- (2d) and 3 day-old (3d) schistosomula (S), adult pairs (A), adult males (M), adult females (F) and eggs (E). Normalized fold expression of the genes relative to the expression in cercariae is presented, and bars represent standard error of the mean.

4.3.3 Sequence analysis of Sj-L6L-1

Since Sj-L6L-1 had several promising characteristics for a novel vaccine candidate it was further characterised. Database searching revealed it had similarity with the Lymphocyte Antigen 6 (Ly-6) family of proteins, present in many eukaryotic species. The membrane-anchored members of this family share several features: a predicted N-terminal signal peptide; a glycosylphosphatidylinositol (GPI)-anchor omega site where the C-terminal propeptide is cleaved off in the mature protein; and the Ly-6/uPAR (LU) protein domain characterised by 8-10 conserved cysteine residues (242).

Highly related orthologues to Sj-L6L-1 were found in *S. mansoni* (Sm-L6L-1; accession # XP_002570561) and *S. haematobium* (Sh-L6L-1; B_00489) which have 78 and 79% amino acid identity, respectively. All three have most of the classic Ly-6 family features; predicted signal peptides, GPI-anchor omega site, and ten cysteines in the mature region (eight of these align with conserved mammalian residues but two are in different positions) (Fig. 2A). However domain searching using the amino acid sequence does not predict the presence of an LU domain in these schistosome orthologues, most likely due to the altered positions of the 3rd and 4th cysteines (see Fig 2A); hence these are referred to as 'Ly-6-like' proteins. These altered cysteines were common in all trematode orthologues examined (Fig 2A and data not shown).

The full pre-protein sequence of Sj-L6L-1 is predicted to be 14.1 kDa, while the mature protein after the signal peptide and propeptide are cleaved is estimated to be 8.9 kDa. However the addition of the GPI anchor is estimated to add a similar amount to the cleaved propeptide (243), so the native form would be approximately 12 kDa.

S. japonicum also has several orthologous sequences to Sj-L6L-1, the closest of which is referred to as Sj-L6L-2 (accession # AAW26563.1) but has only 38% identity. Apparent homologues of Sj-L6L-2 were found in *S. mansoni* (Sm-L6L-2; accession # XP_002573383) and *S. haematobium* (Sh-L6L-2; A_06850) with 70 and 78% identity to Sj-L6L-2, respectively. Other parasitic trematode sequences with low identity (31-34%) to Sj-L6L-1were identified in *F. hepatica* (Fh_Contig6273), *F. gigantica* (Contig25430), *C.sinensis* (CS1_c757) and *O. viverrini* (OV1_c8524). The closest mammalian orthologues are the mouse Ly-6D (also known as ThB; 28% identity; accession # EDL29445), the human E48 protein (32% identity; accession # CAA73189), and human CD59 (25% identity; accession # CAG46523) which is the most well-characterised because of its role in complement inhibition. Phylogenetic analysis, predicting evolutionary relationships between orthologues, indicates that the trematode sequences are all more closely related than these mammalian Ly-6 family proteins (Fig. 2B). The schistosome L6L-1 and L6L-2 sequences branch separately in relatively tight groups, whereas the other trematode sequences appeared more diverse. The mammalian CD59 sequences were the most evolutionarily distant, and branched together.

Despite not having a traditional LU domain by sequence searching, structural homology searching of the mature Sj-L6L-1 sequence predicts structural similarity with Ly-6 proteins. The highest scoring template following the Phyre2 server search was the Ly-6 protein Lynx1, with 98.3% confidence. As shown in Fig.3,

modelling predicted a three-fingered structure stabilised by the 5 disulfide bonds (Fig. 3A), which is common to Ly-6-like proteins (244). The disulfide binding pattern is in the same order as other Ly-6 proteins with elucidated structures, such as human Lynx1 (245) and CD59 (246-247) (Fig. 3B).

Figure 2: Sequence analysis of Sj-L6L-1 and orthologues (next page)

A. The Sj-L6L-1 sequence aligned with the highly similar orthologous sequences from *S. mansoni* (Sm-Ly-6-1) and *S. haematobium* (Sh-Ly-6-1) and compared to similar Ly-6 family proteins from mice (Mm-Ly-6D) and humans (Hs-E48 and Hs-CD59). Sequences were aligned using ClustalW followed by manual alignment of the structurally-important cysteine residues in the mature protein region. The Ly-6/uPAR protein domain is shown between the blue brackets in the mammalian sequences. The N-terminal signal peptide and the C-terminal propeptide are highlighted in grey, while structurally important conserved cysteine residues are highlighted in red. The predicted omega site (GPI-anchor site) is highlighted in blue. The predicted mature Sj-L6L-1 sequence is indicated within the black triangles. B. Phylogenetic tree showing similarity between Sj-L6L-1, trematode and mammalian orthologues was constructed using the neighbour-joining method. Two orthologous ly-6-like proteins in *Schistosoma* spp. (*S. japonicum* (Sj), *S. mansoni* (Sm), *S. haematobium* (Sh)) and the closest sequence to Sj-L6L-1 from *Fasciola gigantica* (Fg), *F. hepatica* (Fh), *Opisthorchis viverrini* (Ov) and *Clonorchis sinensis* (Cs) are shown. The bootstrap method (1000 replicates) was used to test branch reliability, with values shown at branch points. The scale bar represents the evolutionary distances (the number of amino acid substitutions per site) as estimated by the p-distance method. Sequences branching together (evolutionary distances < 0.3) are grouped in colours, and the amino acid sequence identity to Sj-L6L-1 is shown in blue text.

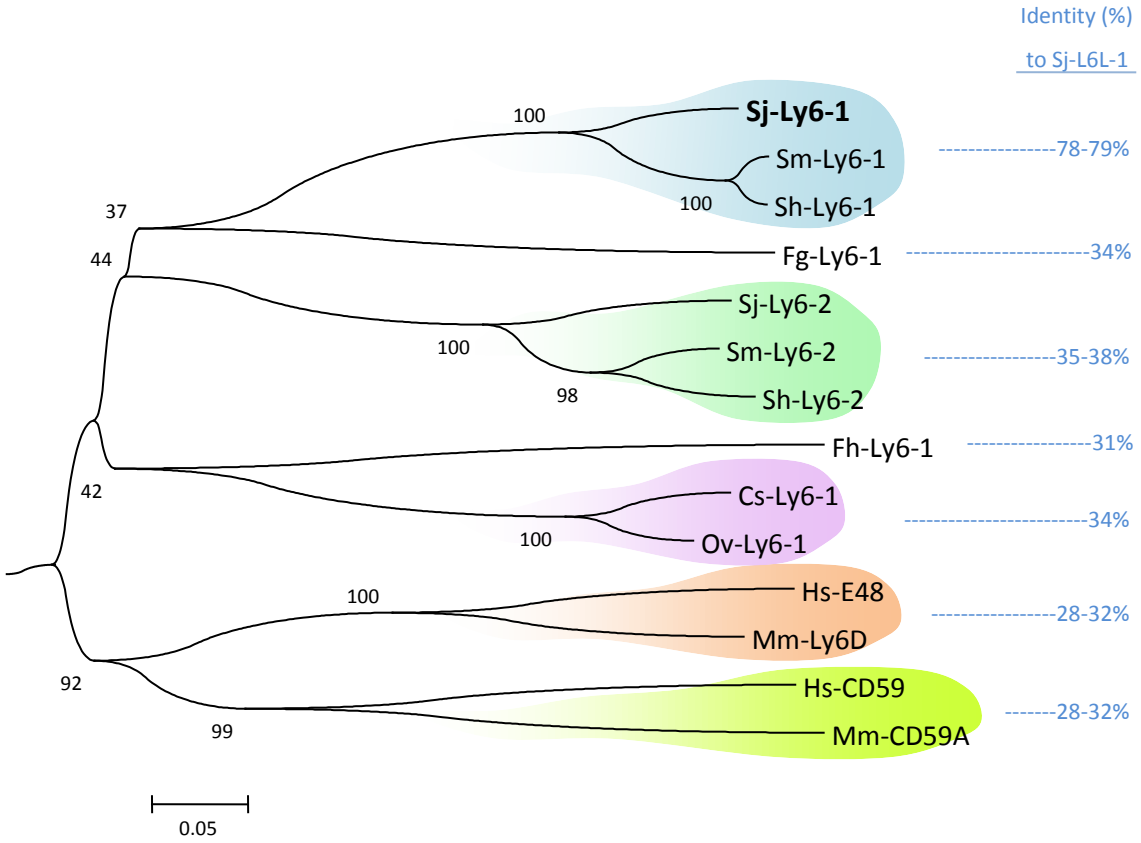
A

```
Sj-L6L-1 MKVLGLCMLI-TLLLNGINCMKNKKVKCYRCSDCPNPFDKTQVTELANCNFCR 52
Sm-L6L-1 MKVLGICVIL-TLIFNGINCIKNKKVKCYRCSDCPNPFDKTQITELGNCNFCR 52
Sh-L6L-1 MKVLGICIVV-TLIFSGINCVKNKKVKCYRCTDCPNPFDKTQITELANCNFCR 52
Mm-Ly6D  MMFRMKTALL-VLLVLAVATSPAWALRCHVCTNSAN--CKNPQVCPSNFYFCK 50
Hs-E48   ----MRTALL-LLATLAVATGPALTLRCHVCTSSSN--CKHSVVCPASSRFCK 46
Hs-CD59  MGIQGGSVLFGLLLVLAVFCHSGHSLQCYNCPNPTAD-CKTAVNCSSDFDACL 52
                :.  *   .:       ::*: *.. .    *      ..   *

Sj-L6L-1 TVYT-YRDEDNYRIAKDCVTSCVPQDRRGGKTG---LVTECCDEDYCNTASKQ 101
Sm-L6L-1 TVYT-YRDEDNYRIAKDCVASCVPQDRRGGKAG---LVTECCDEDYCNASPKH 101
Sh-L6L-1 TVYT-YRDEDNYRIAKDCVASCVPQDRRGGKAG---LVTECCDEDYCNASPKH 101
Mm-Ly6D  TVTS-VEPLNGNLVRKECANSCTSDYSQQGHVSSGSEVTQCCQTDLCNERLVS 102
Hs-E48   TTNT-VEPLRGNLVKKDCAESCTPSYTLQGQVSSGTSSTQCCQEDLCNEKLHN  98
Hs-CD59  ITKAGLQVYNKCWKFEHCNFNDVTTRLRENELT-----YYCCKKDLCNFNEQL 100
          . :   .         :.*   . ..      ..          **. * **

Sj-L6L-1 SSISFLLISSITMLLIYTNHLMH------- 124
Sm-L6L-1 YSISFLLITSFTIFITYTNKFIY------- 124
Sh-L6L-1 YSISFLLITSFTIFIIYANKFIY------- 124
Mm-Ly6D  AAPGHALLSSVTLGLATSLSLLT-VMALCL 131
Hs-E48   AAPTRTALAHSALSLGLALSLLAVILAPSL 128
Hs-CD59  ENGGTSLSEKTVLLLVTPFLAAAWSLHP-- 128
                          .: :  .
```

B



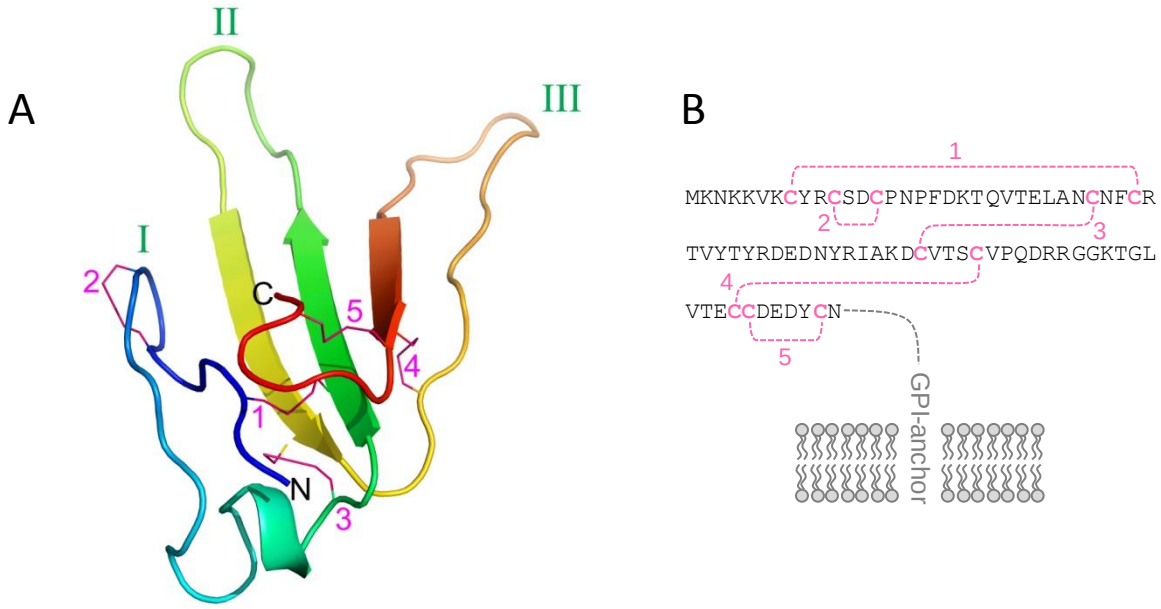


Figure 3: Predicted structure of mature Sj-L6L-1

A. The predicted structure of Sj-L6L-1 was modelled based on a Phyre2 structure homology search, revealing a structure with the typical three-finger fold common to Ly-6 family proteins. B. The mature Sj-L6L-1 amino acid sequence with the predicted disulfide bonding pattern, also characteristic of this family, including the GPI-anchor at the C-terminus. Each finger is labelled with green roman numerals, and the disulfide bonds are shown and numbered in magenta.

4.3.4 Recombinant Sj-L6L-1 is in the native antigenic conformation

Recombinant Sj-L6L-1 was produced fused to thioredoxin (Trx) as solubility tag. Additionally, the Trx tag was expected to increase the recombinant's immunogenicity since small proteins generally elicit weak antibody responses. Induction with arabinose caused a significant expression of a soluble protein band at approximately 27 kDa (slightly larger than the predicted 25 kDa) as seen by reducing SDS-PAGE (Fig. 4). This was purified by the 6His tag using a nickel column (Fig. 4A). The protein band was excised and the sequence confirmed by LC-MS/MS using a HCT ULTRA ion trap mass spectrometer (Bruker Daltonics; Monash Biomedical Proteomics Facility).

In contrast, under non-reducing conditions the protein ran at 25 kDa (Fig. 4B). This reduction in size was likely due to a change in the Sj-L6L-1 portion of the fusion protein, since reducing or non-reducing conditions had no effect on the size of rTrx which remained at 16 kDa. Finally, rSj-L6L-1 was recognised by the rat lung ASC-probes from infected rats, but only in the non-reduced form (Fig. 4B). This indicates that

the recombinant protein shares conformational epitopes with the native protein, and these are abolished by treating with reducing agent, presumably disrupting the disulfide bonds between the structurally-important cysteines.

It is also important to note that there was a ladder effect of rSj-L6L-1 in the non-reduced sample (lane 4, Fig. 4B). These corresponded to multimers of the protein, visible at 50 kDa (dimer) and 75 kDa (trimer) and then increasing masses that are not distinguishable. In the reduced sample (lane 5) only an additional 55 kDa band is present, likely to be a dimer of the reduced form. These bands also reacted with an anti-His tag antibody (data not shown), indicating that they are aggregates if rSj-L6L-1. Interestingly none of the multimers were recognised by the lung ASC-probes compared to the 25 kDa monomer.

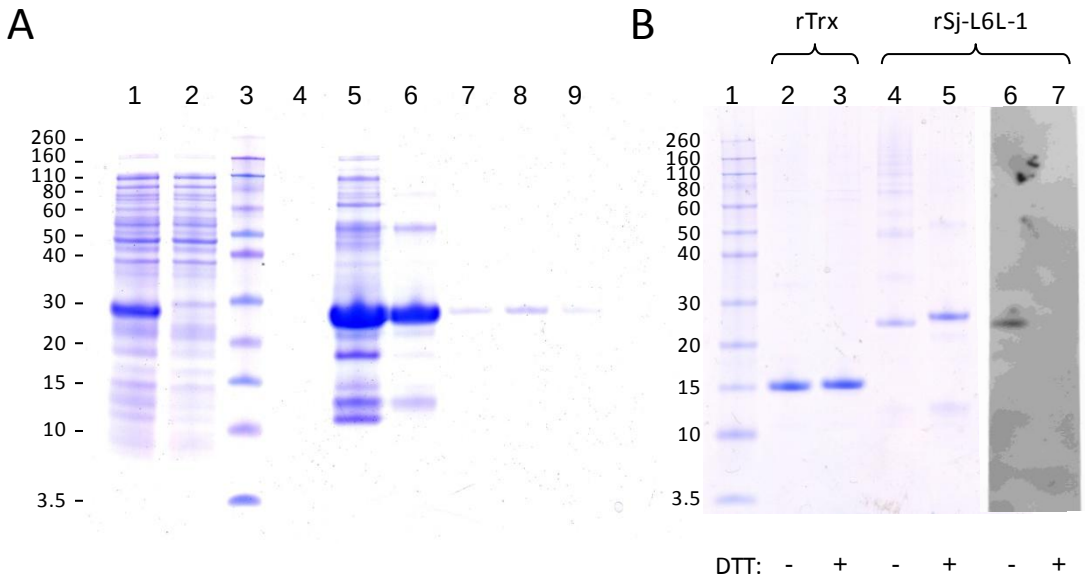


Figure 4: Recombinant Sj-L6L-1 (rSj-L6L-1) was produced with conformational epitopes

A fusion protein of rSj-L6L-1 with thioredoxin (Trx) was expressed in *Escherichia coli* and purified by affinity chromatography. Panel A shows the SDS-PAGE steps of rSj-L6L-1 purification: soluble bacterial lysate with arabinose-induced expression of rSj-L6L-1 before chromatography (lane 1) and after (lane 2); molecular weight markers (lane 3); and column eluates with increasing concentrations of imidazole: 50 mM (lane 4); 200 mM (lane 5); 350 mM (lane 6); 500 mM (lanes 7-9). Panel B shows the effect of reducing agent (DTT) on rSj-L6L-1 (lanes 4-7) or the fusion partner alone (rTrx; lanes 2-3). Including reducing agent (lanes 3, 5, 7) had no effect on rTrx but slightly increased the size of rSj-L6L-1. On a western blot (lanes 6-7), Sj-L6L-1 was recognised by lung-LN ASC-probes only in non-reduced form (lane 6).

4.3.5 Native Sj-L6L-1 is present in a tegument extract

Due to the scarcity of larval material, adult worm extracts were used to detect native Sj-L6L-1 from the parasite. Antiserum to rSj-L6L-1 recognises a band at approximately 11 kDa under non-reducing SDS-PAGE, and a 12.5 kDa band under reducing SDS-PAGE (Fig. 5A), while anti-rTrx antiserum does not recognise either band. The same 12.5 kDa band was seen in the worm tegument and insoluble fractions by reducing SDS-PAGE, but not in the aqueous-soluble fraction. This indicates rSj-L6L is located in the tegument and is membrane-associated. The corresponding band is not clearly visible in the tegument or aqueous-insoluble fractions by Coomassie staining (Fig. 5B), indicating that it may not be a major protein constituent of adult worms. However this should be confirmed with quantitative proteomics techniques.

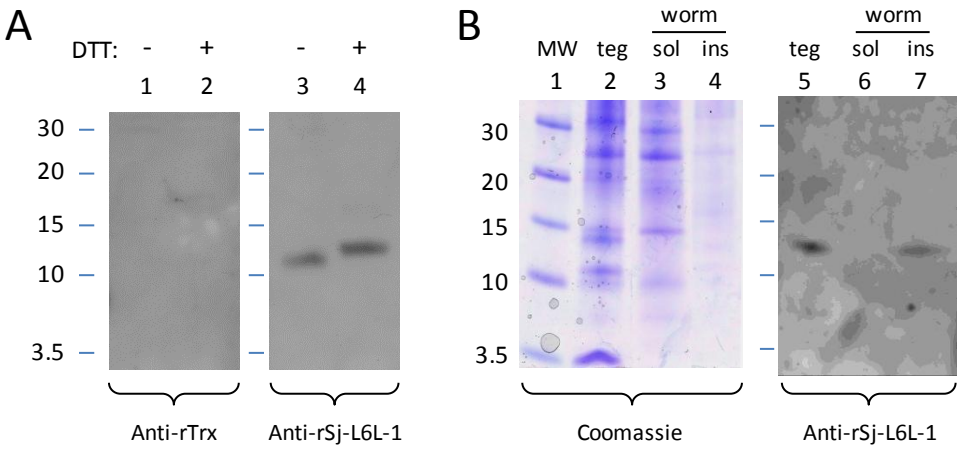


Figure 5: Detection of native Sj-L6L-1 in S. japonicum *extracts.*

A. Crude adult worm extract was treated without (-) or with (+) reducing agent (DTT), and then separated by SDS-PAGE and transferred by western blotting. These were then probed with antisera against rTrx (lanes 1-2) or rSj-L6L-1 (lanes 3-4). Molecular weights are shown on the left (kDa). B. Adult worms were fractionated into tegument (teg), aqueous-soluble (sol) and -insoluble (ins) worm fractions, and separated by reducing SDS-PAGE. The Coomassie stain of the gel is shown in lanes 1-4 and western blot of the same fractions in lanes 5-7, probed with antiserum to rSj-L6L-1.

4.3.6 Schistosome larvae induce anti-rSj-L6L-1 antibodies

Since lung ASC-probes were already found to recognise non-reduced rSj-L6L-1 (Fig. 4), an ELISA was used to investigate the binding of all rat ASC-probe sample types and purified buffalo sera to rSj-L6L-1 (Fig. 6). To account for some background binding to the fusion tag alone seen with the sera, the data is presented as relative binding (RB) to rTrx. Figure 5A illustrates that the non-infected (NI) rat sera has no recognition of rSj-L6L-1 with a mean of 0.8 RB, indicating preferential recognition of rTrx. The only sample type to have statistically significant recognition of rSj-L6L-1 is the lung ASC-probes, with a mean of 4.8 RB ($p = 0.039$). The skin had some slight recognition of rSj-L6L-1 at 1.6 RB, although this was not significantly higher than other groups.

Serum samples taken from water buffalo shortly after *S. japonicum* infection (described in Chapter 3) were also tested for reactivity against rSj-L6L-1 (Fig. 6B). During this infection, a slight increase in anti-rSj-L6L-1 antibodies occurs in the serum at 11-12 days post-infection (DPI) (1.11 RB) compared to 5-6 DPI (0.95 RB), although this was not significant. There was only one sample from NI buffalo, and this was similar to the mean recognition by NI rats at 0.84 RB. The buffalo ASC-probe samples were also tested but no reactivity was observed (data not shown).

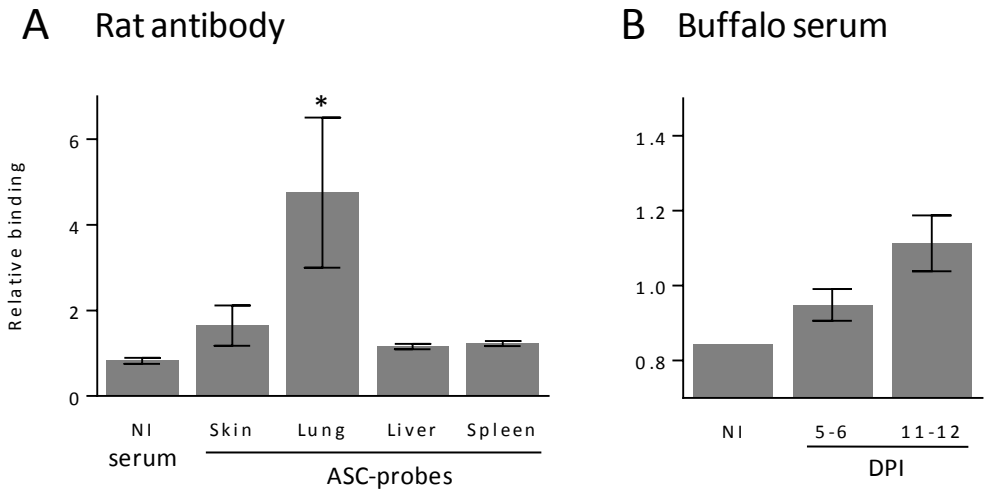


Figure 6: Recognition of rSj-L6L-1 during schistosomiasis

A. Rat ASC-probes from skin-, lung- or liver-LN or spleen are compared to non-infected (NI) rat serum. B. Purified antibody from buffalo serum during an active larval infection; NI animal (n = 1) was compared to buffaloes 5-6 (n = 4) or 11-12 (n = 4) days post infection (DPI). Relative binding to rSj-L6L-1 is shown, which is the ratio of rSj-L6L-1 optical density to that of the fusion partner alone, rTrx. Bars represent the mean ± standard error.

4.4 Discussion

In this chapter a novel post-genomic technology, a protein microarray, was employed to identify novel schistosome vaccine candidates. The local larvae-induced rat ASC-probes (generated in Chapter 2) were used to screen this microarray and several novel and also known vaccine candidates were recognised. Two promising candidates with high larval expression were identified. One of these, AY815838, had been identified in a previous screening of the protein microarray, and was being pursued as a vaccine candidate in our collaborator's laboratory (P. Driguez and D.P. McManus, personal communication). The remaining candidate, named Sj-L6L-1, was further characterised and has several features suggesting it may be an efficacious vaccine candidate.

4.4.1 Antigen recognition by protein microarray screening

The skin and lung ASC-probes recognised relatively few proteins on the array, binding to approximately 5% of the 232 different proteins. In comparison, sera from chronically infected humans recognised an average of 18% of the proteins (Patrick Driguez, personal communication). The low reactivity of ASC-probes on the array could be a product of the low antibody level, especially the skin ASC-probes which contain less than 0.3 μ/ml of total antibody (see Chapter 2). Alternatively, it could reflect a high specificity of the ASC-probes for a limited number of antigens which are exposed in the respective tissue regions from which they are derived, as observed on western blots (Chapter 2).

Nevertheless, four novel antigens were recognised by the ASC-probes, predominantly by lung samples. AY815838 was recognised the most strongly, calponin-like protein moderately and the zinc finger protein and Sj-L6L-1 weakly. However, it should be noted that the degree of antibody binding to proteins on the array does not necessarily indicate antigenicity, because the amount of protein in each spot is not standardised due to the cell-free expression system used (248-249). These four novel antigens were then ranked for their vaccine potential, as described below in Section 4.4.2.

Known vaccine candidates were also recognised by screening the microarray. One antigen is TSP-2, a tegumental antigen which is currently in development against *S. mansoni* and is a promising candidate (150). Data here suggests that TSP-2 is exposed to the host in both the skin and lung sites during rat schistosomiasis, since it was strongly recognised by skin and lung ASC-probes. TSP-2 is known to have tegument expression in larvae and adults (154) and the transcript is up-regulated in the lung stage for both *S. japonicum* and *S. mansoni* (154, 174). Since the skin and lung are important sites of immunity against larvae, and TSP-2 is antigenic in these sites, it is possible that larval killing occurs here and contributes to the protective effect of this antigen.

Other known vaccine candidates recognised by the lung ASC-probes were: Sj22.6 and Sj21.7, both tegument associated proteins from the TAL protein family (250); *S. mansoni* filamin, a large structural protein shown to

confer some protection in a mouse model (251); and DLC1 which has been found associated with the worm surface (252-253). Finally two candidates, *S. mansoni* TSP-3 and Sj29, were recognised by the skin ASC-probes, indicating that they are antigenic predominantly during skin invasion. These are both purported to be surface molecules and Sj29 has been investigated as a vaccine candidate. Gobert et al. (193) found that in *S. mansoni* both of these genes had significant up-regulated transcript expression after transformation from cercariae to larvae, and interestingly Sm29 (the homologue of Sj29) had the highest expression in the 3 hour schistosomula, which is the stage shortly after skin penetration.

4.4.2 Ranking the novel antigens for characterisation

A major criterion used in the present study to select antigens for characterisation was restricted or predominant larval expression. Initially, the NCBI EST database was used as a preliminary measure of developmental expression. It indicated that only one of these was not up-regulated in schistosomula, the zinc finger protein (AY223099). This antigen also lacked a signal peptide and transmembrane domain, and is likely to be an intracellular protein and not host-exposed; therefore this protein was not pursued further.

The developmental expression of the remaining three antigens was then confirmed by qPCR to more reliably assess if they were indeed up-regulated in larvae, and both AY815838 and Sj-L6L-1 showed larval gene expression. AY815838 was dramatically up-regulated in the 2 day *in vitro*-cultured, but not the 3 day *in vivo* lung-isolated, schistosomula. This high expression in the *in vitro* larvae could indicate an early high expression which is then reduced after 3 days, or could be an artefact of *in vitro* culturing. Sj-L6L-1 however showed a pronounced and consistent larval expression, with similar up-regulated levels in the 2 day and 3 day larvae compared to other stages. There was a low level of expression in the adult worms, 8-fold less than larvae, which was attributed solely to males. In contrast, calponin-like protein showed a progressive increase in expression from cercariae to adults, suggesting that it is not likely to be a larval-specific antigen.

Based on these analyses, Sj-L6L-1 and AY815838 ranked the highest as novel larval vaccine targets. Since AY815838 was being pursued as a vaccine candidate in our collaborator's laboratory, Sj-L6L-1 was further characterised and produced in recombinant form.

4.4.3 Previous reports on schistosome L6L proteins

While Sj-L6L-1 is completely novel for *S. japonicum*, the closest homologue to Sj-L6L-1 in *S. mansoni* (Sm-L6L-1) has been mentioned previously in the literature. Following the release of the genome sequence, Wilson and Coulson (148) discussed the presence of "six orthologues of human CD59" in *S. mansoni*, which are the Ly-6-like family of proteins. They then reveal from a personal communication that four of these were found at the worm surface by proteomics, however in a subsequent publication it was reported that just two of these orthologues were identified by proteomics experiment. They term these CD59a and CD59b (corresponding to Sm-L6L-2 and Sm-L6L-1, respectively), and identified them by enzymatic shaving of the

adult worm tegument by PiPLC treatment, which specifically cleaves GPI-anchored proteins (254). Finally, Farias et al. (240) analysed the *S. mansoni* transcriptome for genes up-regulated from the cercariae to the schistosomula, identifying several including Sm-L6L-1 (which they refer to as 'dif 5'). They performed a brief DNA vaccine trial with several novel antigens and found that 'dif 5' resulted in a slight (but non-significant) reduction in worm burden (22%). In this study the developmental expression was also investigated, and Farias et al. (240) report that Sm-L6L-1 is expressed 4-fold above cercariae in one week-old schistosomula and around 7-fold greater in adults. While this suggests a different expression profile in the *S. mansoni* compared to the *S. japonicum* homologue, it is possible that even higher expression would be found in early-stage (2-3 day old) schistosomula, as described in this chapter. Together these reports suggest that Sm-L6L-1 is a promising vaccine target, since it is surface-exposed and conferred slight protection in a DNA vaccine model; although further reports on its characterisation have not been published.

4.4.4 What are Ly-6 proteins?

The Ly-6 family of proteins was originally described in mice, but Ly-6-like proteins have since been found in many animal species from *C. elegans* to humans and comprise the Ly-6 super gene family (255). These are broadly grouped together based on the presence of 8-10 conserved cysteines which comprise the LU domain (242). These conserved cysteines create 4-5 disulfide bonds resulting in a three-finger structure, a motif also common to the related snake venom toxins (244). Generally those containing 10 cysteines are GPI-anchored to the plasma membrane, while those with 8 are secreted (such as snake venom toxins) and lack a GPI-anchor signal sequence (242). Since Sj-L6L-1 is related to this family by containing most of these features, it is referred to here as Ly-6-like and part of the Ly-6 super family.

The Ly-6 family members and Ly-6-like proteins appear to have extremely diverse roles, although their precise functions are as yet unclear (256). These proteins also exhibit limited sequence identity (255), and makes assigning a putative function to Sj-L6L-1 difficult. They are generally thought to participate in development, cell adhesion, and cell signalling, although how the latter occurs is still unknown (255). The best known member is CD59, which inhibits the complement cascade and protects self-cells (e.g. erythrocytes) from the membrane attack complex (257). Murine Ly-6 members are expressed in lymphocytes and are used as differentiation markers through hematopoietic cell development (255), while the human protein E48 (the closest orthologue to Sj-L6L-1) is found on keratinocytes and mediates cell adhesion (258).

The schistosome surface tegument is known to have various adaptations to evade the mammalian immune response, including factors which inhibit complement-mediated attack (32). Considering that the tegument-located Sj-L6L-1 has some identity with human CD59 which inhibits the membrane attack complex, it is attractive to hypothesise that it exert a complement-inhibitory effect in schistosomes, as proposed by Wilson and Coulson (148). However based on sequence phylogenetics, the diverse trematode Ly-6-like proteins including Sj-L6L-1 all appeared more related than any of the mammalian sequences. For example, the human E48 and CD59 proteins, while having vastly different functions, are more related than any of the trematode

sequences. Further, there is marginally more similarity to E48, the adhesion protein, than CD59. Therefore while this analysis does not suggest a function for Sj-L6L-1, it is unlikely that it serves a similar function to CD59, and may play an endogenous role in the parasite such as cell adhesion.

4.4.5 Characterisation of Sj-L6L-1

The *E. coli*-expressed rSj-L6L-1 fusion protein was produced in a soluble form and was recognised by the schistosomiasis-induced antibody response, indicating that it was at least partly in the correct antigenic conformation. By treating with reducing agent, the protein structure was altered sufficiently to ablate recognition by these antibodies. The disulfide bonds in Ly-6 proteins are known to be important for the structural conformation, stabilising the typical three-finger motif; for example, when CD59 is treated with reducing agent it loses its ability to inhibit the complement system (247). This indicates that the schistosomiasis-induced antibodies recognise only conformational epitopes on the recombinant, and potentially only conformational native epitopes (although this was not examined here). In contrast, the antiserum raised against rSj-L6L-1 bound to both the non-reduced and reduced forms of native Sj-L6L-1 in the schistosome extracts, indicating that the antiserum recognised both conformational and linear epitopes.

It was apparent that there were several aggregates of the non-reduced recombinant protein creating a ladder-like pattern seen by SDS-PAGE. After reduction most of these were removed, suggesting they are multimers linked by aberrant disulfide bonding. However these were not recognised by the schistosomiasis-induced lung ASC-probes, and therefore likely mis-folded. These aggregates were also highly immunogenic when injected, since the anti-rSj-L6L-1 antiserum reacted strongly against these species, with greater intensity than the 25 kDa monomer (data not shown). These aggregates may be more antigenic due to the fact that they are multimers and contain more epitopes for antibody binding compared to the 25 kDa monomer.

The molecular weight of native Sj-L6L-1 was found to be 11 kDa, slightly larger than the predicted mature protein of 8.9 kDa, which can be attributed to the presence of a GPI anchor. Reduction resulted in a slight increase in molecular weight to around 12 kDa, presumably because the non-reduced protein is tightly folded into the three-finger structure and opened when reduced. In the same way, rSj-L6L-1 increased in size after reduction which is further confirmation on the structural similarity between the native and recombinant form.

The native protein was identified in a tegument extract from adult *S. japonicum* worms, and the same band was found in the insoluble fraction from the 'denuded' adult worms. This indicates that Sj-L6L-1 is at least associated with the outer tegument, and is highly likely to be on the external surface as for its homologue in *S. mansoni* (254). It also indicates that the protein is attached to the plasma membrane, since it was only detected in the insoluble fraction; hence it is unlikely to be secreted like some Ly-6 proteins. Since tegumental proteins are synthesised by the cell bodies under the tegument (32), it is unsurprising that it was found in both the tegument and 'denuded' fractions.

Unfortunately enough larval crude antigen could not be obtained to compare the protein level in this developmental stage. However it is anticipated that early-stage schistosomula, around 2-3 days, produce comparatively greater amounts of the antigen considering the high transcript expression, and this needs to be investigated in future studies. Also, attempts were made to locate the antigen on the adult and larval surface by immunofluorescence using anti-Sj-L6L-1 antiserum, however this was unsuccessful. This could be due to the low antibody titres against the conformational native protein, and should be repeated with antiserum raised solely against the 25 kDa monomer.

4.4.6 Sj-L6L-1 is recognised specifically during larval infection

Finally, rSj-L6L-1 was recognised by the antibody response during rat and water buffalo schistosomiasis, an experimental model and the natural host for the parasite, respectively. In rat schistosomiasis, specifically the local lung antibody response bound to the antigen, while there was minimal binding with the skin antibodies, and none in the liver lymph nodes. This indicates that in this model, Sj-L6L-1 is antigenic in the lung region probably derived from the migrating schistosomula, whereas it is not antigenic in the liver where the adults reside. This agrees with the transcript analysis suggesting the antigen is highly expressed in the lung-stage larvae (3 days). While it is clearly expressed by the adult males also, the fact that it does not generate an antibody response in the liver could be because it is expressed at a significantly reduced level than larvae. Alternatively, since schistosomes acquire host proteins resulting in the masking of their own antigens (32), Sj-L6L-1 may be more protected from the immune system in the adult worms.

The buffalo serum response also indicates that Sj-L6L-1 is antigenic approximately at the lung-stage of schistosomula migration. There was a slight increase in reactivity to the antigen at 11-12 days post-infection (DPI), compared to 5-6 DPI and the uninfected buffalo. The larvae are predicted to enter the lungs at around 7 DPI in the buffalo (Prof. Yuesheng Li, personal communication), which would cause an antibody response to this stage several days later. This promising result indicates that antibodies to Sj-L6L-1 are part of the natural response against the larvae, and this stage could be targeted as an anti-infection vaccine. Also, it is likely that natural infection in the field could further boost and maintain the immunity against Sj-L6L-1 since it is recognised in a natural infection.

4.4.7 Conclusions

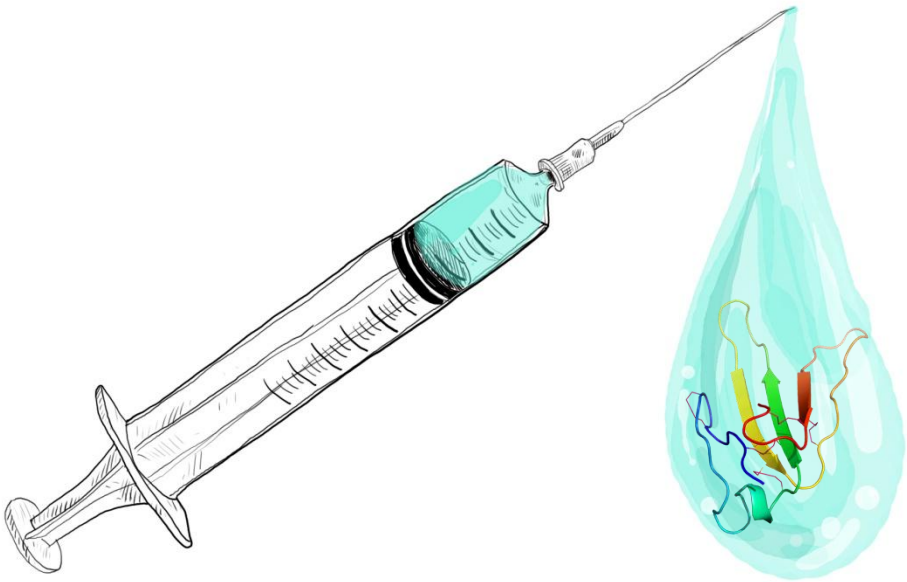
In summary, two novel vaccine targets against *S. japonicum* were identified by probing a protein microarray with the local larval-specific antibodies generated previously in Chapter 2. Additionally, by combining two immunomic technologies, the ASC-probe samples which provide tissue-specific reactivity with the protein microarray providing multiple antigen binding data, new insights on existing vaccine antigens was gained.

One of the novel antigens discovered, Sj-L6L-1, was identified as being a Ly-6-like protein by sequence and predicted structural homology, and is present on the worm tegument. It is likely to be virtually larval-specific,

or at the least only antigenic in the larval stage, fulfilling the aim of this study to find antigens specific to schistosomula. Finally, it is recognised by the water buffalo host during larval infection and may be useful as a transmission blocking vaccine against this neglected tropical disease. In addition to providing novel targets for vaccination, the discovery of these two novel schistosome molecules may allow an insight into the development and invasion process of schistosome larvae.

Chapter 5:

Testing of a novel vaccine target against *Schistosoma japonicum*



5.1 Introduction

An anti-schistosome vaccine remains an unrealised goal, and is widely considered a requirement for the elimination of schistosomiasis (10-12, 97-98). Since current vaccine candidates have not reached the level of protection seen with the attenuated larval vaccine, there may be more effective antigens which are as yet undiscovered (11). Furthermore antigens specific to larvae may be more protective (100). In Chapter 4, the novel antigen *Schistosoma japonicum* Ly-6-like-1 (Sj-L6L-1) was identified and characterised, and was shown to have several promising features such as tegument location, up-regulated expression and antigenicity in lung-stage schistosomula, and recognition by the parasite's natural host. In this chapter, the efficacy of rSj-L6L-1 is tested against *S. japonicum* infection in a mouse vaccine trial.

An effective vaccine not only requires a protective antigen but also the induction of the appropriate immune response. Subunit vaccines are routinely administered with an adjuvant to augment the immunogenicity of the antigen. It has recently been discovered that adjuvants are also capable of skewing the immune system towards different responses (259-260). The most common adjuvants used in human vaccines are aluminium salts, generally referred to as alum, which drive a Th2 response (259, 261). In contrast, Quil A which is a purified saponin derived from the bark of *Quillaja saponaria* Molina, induces strong Th1 responses (262-263). There are a range of other adjuvants which can skew the immune response against the co-administered antigen, and the mechanisms by which this is achieved is still being investigated (259).

In the rational design of vaccines, antigens can be formulated with the adjuvant that will induce the type of response required for protective immunity (if known). This is an important consideration for novel schistosome vaccines (11), and several studies have examined the effect of different adjuvants on the efficacy of existing schistosome vaccine candidates. For example, El Ridi et al. (264) compared the abilities of different adjuvants combined with *S. mansoni* vaccine targets to skew towards the protective Th2 response, whereas Xu et al. (265) achieved a reduced liver egg burden after vaccination with *S. japonicum* paramyosin adjuvanted with the Th1-inducing adjuvant, Montanide ISA 70M.

Natural protection against schistosomes is consistently correlated with type-2 cytokines and antibody isotypes (107-111). Furthermore, in Chapter 3 the potentially protective response of the water buffalo against *S. japonicum* larvae was found to be mixed but predominantly biased towards type-2, consistent with the view that the mechanism of immunity against the migrating larvae is effected by antibody-dependant cellular cytotoxicity (9, 13, 112). In contrast, in mice type-2 responses are thought to be less important in the radiation-attenuated model of immunity, with type-1 responses mediating higher levels of protection (123). However Horowitz et al. (266) found higher protection when immunising mice using crude larval antigen adjuvanted with alum compared with Freund's complete adjuvant, which they attributed to high specific IgE level and larval killing. With this in mind, the larval antigen rSj-L6L-1 was first tested with different adjuvant formulations to compare which can induce a type-2 response with high antibody titres.

Alum was selected since it is known to drive type-2 immunity (259, 261), and Quil A was used as a comparison since it typically induces a type-1 response (263). Diethylaminoethyl (DEAE)-dextran is a relatively uncharacterised adjuvant, although it is used in some veterinary vaccines (267). Recently DD was effective as an adjuvant in a vaccine trial against the sheep parasitic nematode, *Haemonchus contortus*, and induced a specific IgE response (D. Piedrafita and E.N.T. Meeusen, personal communication). Therefore DD was tested in this study to examine its ability to skew towards a type-2 response in a mouse model. Finally, rSj-L6L-1 was tested in a murine vaccine trial formulated with the selected adjuvant.

5.2 Materials and methods

5.2.1 Animal ethics statement

The conducts and procedures involving animal experiments were approved by the Animal Ethics Committees of Monash University (ethics no. MARP/2012/009) and the Queensland Institute of Medical Research (project no. P288). This study was performed in accordance with the recommendations of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2004.

5.2.2 Optimising rSj-L6L-1 vaccine formulation with different adjuvants

In order to prepare for a vaccine trial with rSj-L6L-1, several adjuvants were combined with rSj-L6L-1 in order to determine which generated the desired immune response in CBA mice (6-8 weeks old). Mice were divided into 4 groups (n = 5), and immunised with 25 μg rSj-L6L-1 with either 20 μg Quil A (Brenntag Nordic, Frederikssund, Denmark), 500 μg DD (Sigma-Aldrich), 50 μg aluminium hydroxide (alum; Rehydragel LV, Reheis Inc.) or a combination of DD and alum. Two injections (50 μl) were administered subcutaneously in the inner thigh region, two weeks apart.

One week after the final vaccination, mice were sacrificed by CO_2 inhalation. Blood was taken by cardiac puncture and serum collected. Spleens and inguinal lymph nodes (LN) (draining the injection site) were removed and placed into sterile media (RPMI 1640 Glutamax with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies)). Tissues were macerated by forcing through a 100 μm cell strainer (BD Biosciences), and cells washed twice in media supplemented with 10% fetal bovine serum. Live cells were counted using a haemocytometer with trypan blue, and resuspended at 1 x 10^6 cells/ml (1 ml per well in 24-well plates). Cells were stimulated with 20 μg/ml rSj-L6L-1 and cultured for 48 h at 37°C with 5% CO_2, after which time the supernatants containing secreted cytokines were collected by centrifugation and kept at -80°C until required.

5.2.3 Measuring anti-rSj-L6L-1 antibody levels following vaccinations

Anti-rSj-L6L-1 antibody titres in serum were measured by ELISA. Wells were coated with rSj-L6L-1 (3 μg/ml) in bicarbonate coating buffer (0.1 M, pH 9.6) and then blocked with 5% SM-PBST. To determine IgG1, IgG2a and IgG2b titres, each serum sample was serially diluted across the plate in 1% SM-PBST. For IgE and IgA, the sera were diluted at 1:320 and 1:20, respectively. The secondary antibodies were biotin-conjugated rat anti-mouse isotype-specific antibodies (BD Pharmingen), and were used at the optimal dilution (in 1% SM-PBST): anti-IgG1, -IgG2a and -IgG2b at 1:1000; anti-IgE and -IgA at 1:500. Finally streptavidin-conjugated HRP (Dako) was incubated at 1:1000 for 45 min at 37°C, and then TMB was added to each well, followed by 2M H_2SO_4, and the optical density (OD) was read at 450 nm.

To determine the total antibody titres to rSj-L6L-1 during the vaccine trial, serum samples were taken from mice at two points during the trial: immediately prior to challenge (which is 2 weeks after the final vaccination); and at necropsy (6 weeks after challenge). Then a similar ELISA as above was performed, with the following differences: plates were coated with rSj-L6L-1 at 2 μg/ml, and after samples were serially diluted (1:2 dilutions) across the plate the bound total immunoglobulin was detected using goat anti-mouse Ig (H+L):HRP (1:1000) (Koma).

The titre was defined as the final dilution of the serum sample where the optical density (OD) was above the cut-off value, which was calculated as twice the mean of naive mouse sera OD. For IgE and IgA, absolute OD levels were used due to the low titres of these isotypes in most individuals.

5.2.4 Measuring cytokine profile of stimulated lymphocytes following vaccination

The level of cytokines associated with type 1 responses (IFN-γ, IL-2, TNF-α and IL-17A) and type-2 responses (IL-4, IL-5, IL-6 and IL-10) were measured in the cell stimulation supernatants by cytometric bead array (CBA; BD Biosciences) according to the manufacturer's protocol. The supernatants were used undiluted. The beads were analysed and the fluorescence measured using a LSR II Flow Cytometer (BD Biosciences), and the level of cytokines in the supernatants was calculated using FCAP Array Software v1.0.1 (BD Biosciences).

5.2.5 Testing rSj-L6L-1 against S. japonicum *infection*

To test the efficacy of rSj-L6L-1 as a vaccine candidate against schistosomiasis, a vaccine trial was performed using two groups of 10 CBA mice (7-8 weeks old). Each group was administered 25 μg of either rSj-L6L-1 or rTrx formulated with 500 μg DD. Since rSj-L6L-1 contains a thioredoxin solubility tag, the thioredoxin tag alone (rTrx) was considered the most appropriate control. Mice were given 3 subcutaneous injections at 2 week intervals (see Fig. 1). Two weeks after the final injection, a blood sample was taken, and mice were then challenged with 34 *S. japonicum* cercariae (as described in Section 2.2.3). Mice were weighed before immunisation, before challenge and then immediately prior to sacrifice. The day prior to necropsy, mice were

separated into sub-groups of 2-3 for 24 h to capture faecal samples, which were collected, weighed and fixed in 10% formalin (v/v) in PBS.

Six weeks after challenge, a final blood sample was taken, and then mice were given an intra-peritoneal injection of heparin sodium (Pfizer; 100 µl). Mice were killed by CO_2 inhalation 10 min later and the abdominal cavity opened. The abdominal blood vessels were then perfused to collect adult worms; after the portal vein was punctured 10 ml of PBS was slowly injected into the thoracic aorta. The perfusate was collected into a petri dish and carefully inspected for adult worms. The numbers of mature pairs and single worms were determined, and the worms were then placed into 10% formalin/PBS for 48 h. The livers were removed, weighed and kept at -20°C until required. Images of each worm were captured using a Leica MZ FL III stereo microscope, carefully separating the pairs. The length of each worm was determined using ImageJ software (220); briefly, the length of the worm was measured in pixels and converted to millimetres based on the scale bar incorporated into the microscope.

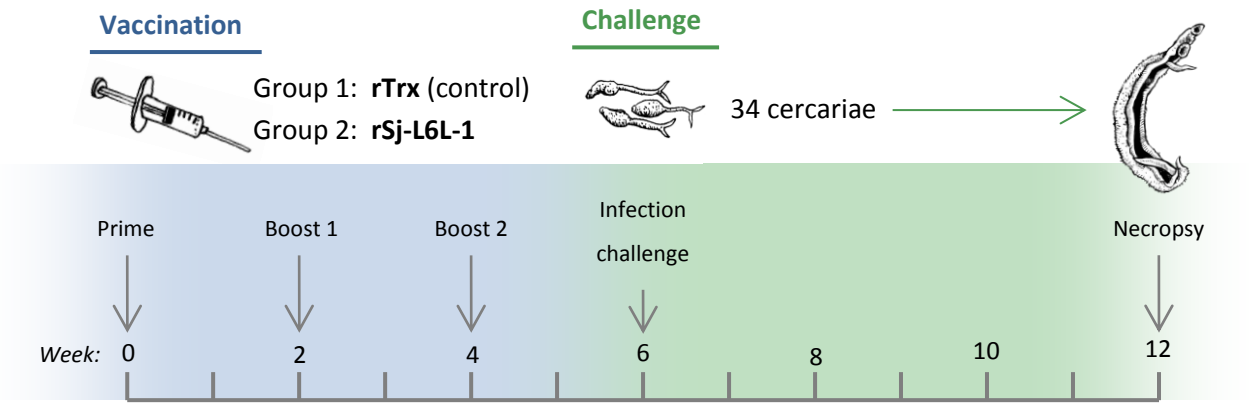


Figure 1: rSj-L6L-1 vaccine trial protocol

To test rSj-L6L-1 as a vaccine candidate against schistosomiasis, mice were given 3 vaccinations with either recombinant thioredoxin (rTrx) or recombinant *S. japonicum* Ly-6-like protein-1 (rSj-6L-1). Two weeks later mice were challenged with 34 *S. japonicum* cercariae and, after 6 weeks the numbers of adult worms were determined at necropsy.

To determine the faecal egg count, faecal samples were gently mixed by rotation for 6 h at 4°C. Samples were centrifuged for 15 min at 2000 x *g* and the supernatant was aspirated. The faecal pellet was then resuspended in 3% (v/v) formalin in PBS and forced through a 110 µm nylon membrane. After centrifuging again, the faecal matter was digested with 4% (w/v) potassium hydroxide (KOH) overnight at 37°C with vigorous shaking. The remaining matter was washed once in PBS and finally resuspended in 5% (v/v) formalin in PBS. The eggs in this digested matter were counted using a dissecting microscope, and the value represented

as eggs per gram (epg) of faeces. The liver egg burden was determined by digesting the thawed livers in 5% KOH for 20 h at 37°C. After centrifuging at 2000 x *g* for 10 min, the supernatant was removed and the pellet was washed in 5% KOH. Finally the pellet was resuspended in 5% formalin/PBS and counted as for the faecal egg count.

5.2.6 Statistical analysis

When comparing two groups, the student's t-test was performed, while the one-way analysis of variance was used for multiple groups. The respective non-parametric analyses were used when data did not have a normal distribution or the variance was not consistent between groups. When analysing the serum antibody levels, the data was log-transformed before statistical analysis. To measure correlations between two parameters, the Pearson correlation was performed and the rank coefficient (r) was determined. Differences were considered statistically significant when $p < 0.05$.

5.3 Results

5.3.1 Immune responses to rSj-L6L-1 induced by formulation with different adjuvants

Four different adjuvant formulations with Sj-L6L-1 were examined to determine which induces the most appropriate immune response in preparation for testing its efficacy against schistosomiasis. Mice were immunized twice with rSj-L6L-1 in Quil A, alum, DD or a combination of alum and DD (A+D). The specific antibody levels and cytokine responses were then measured for each antigen formulation.

In general all formulations induced high titres of anti-rSj-L6L-1 antibodies; however the type and magnitude of antibody response differed between groups (Fig. 2). Similar IgG1 titres were induced by Quil A, DD and A+D while alum induced a significantly lower IgG1 titre by around 1 log ($p < 0.0001$). For IgG2a and IgG2b, Quil A induced significantly higher titres than the others ($p < 0.0001$), but DD and A+D induced an intermediate titre that was significantly higher than alum. Only alum and DD stimulated a measurable IgE response above background and DD elicited specific IgE levels that were significantly higher than alum ($p < 0.0001$). Interestingly, combining alum and DD reduced the titres of IgG2a and IgG2b slightly and completely ablated the IgE response seen with these adjuvants individually. Finally IgA induction was only seen when rSj-L6L-1 was adjuvanted with Quil A, albeit at a very low level.

Next, the cytokine profile from antigen-stimulated lymphocytes was measured for each adjuvant formulation, and each induced a unique response (Fig. 3). Overall, Quil A and A+D formulations elicited the highest levels of type-1 (IFN-γ, IL-2 and TNF-α) and inflammatory cytokines (IL-17A and IL-6); DD induced mostly type-2 (IL-4 and IL-10) and a low level of type-1 cytokines; while alum induced the lowest levels of all cytokines. Quil A and A+D induced the highest levels of IFN-γ, which was significantly above alum and DD

in the LN ($p = 0.019$). Similarly they induced the highest IL-2 levels; in the LN A+D produced the highest IL-2 ($p < 0.0001$) whereas in the spleen Quil A produced the highest level ($p < 0.0001$). Quil A induced the most pronounced level of inflammatory cytokines, with significant amounts of TNF-α, IL-17A and IL-6. A+D also induced higher levels of these cytokines than alum and DD alone, although generally this was not significant.

While DD alone elicited a low level of IFN-γ and modest amounts of TNF-α and IL-17A and IL-6, it elicited significantly higher IL-4 levels than Quil A or alum in the draining LN ($p = 0.001$). A+D also produced a similarly high IL-4 level. DD and A+D elicited the highest IL-10 levels in the LN, significantly higher than alum ($p = 0.004$) but not Quil A. Finally, Quil A induced the most significant IL-5 level in the LN ($p = 0.027$) and spleen ($p = 0.003$) than all others.

To examine differences in local and systemic lymphocyte populations, the response from both LN and splenocyte cell stimulations were examined. Generally each formulation induced similar responses at both sites, although some differences were evident. The amount of IL-6 produced by splenocytes was significantly higher than LN cells, for all adjuvants. While both Quil A and A+D had the highest IL-6 levels at the local LN, only Quil A had the highest level in the spleen cells. The IL-10 and IL-4 levels were only higher in DD and A+D formulations in the draining LN cells, while the splenocytes showed no differences between groups. Finally, Quil A induced a much higher IL-5 response in the LN than in the splenocyte population.

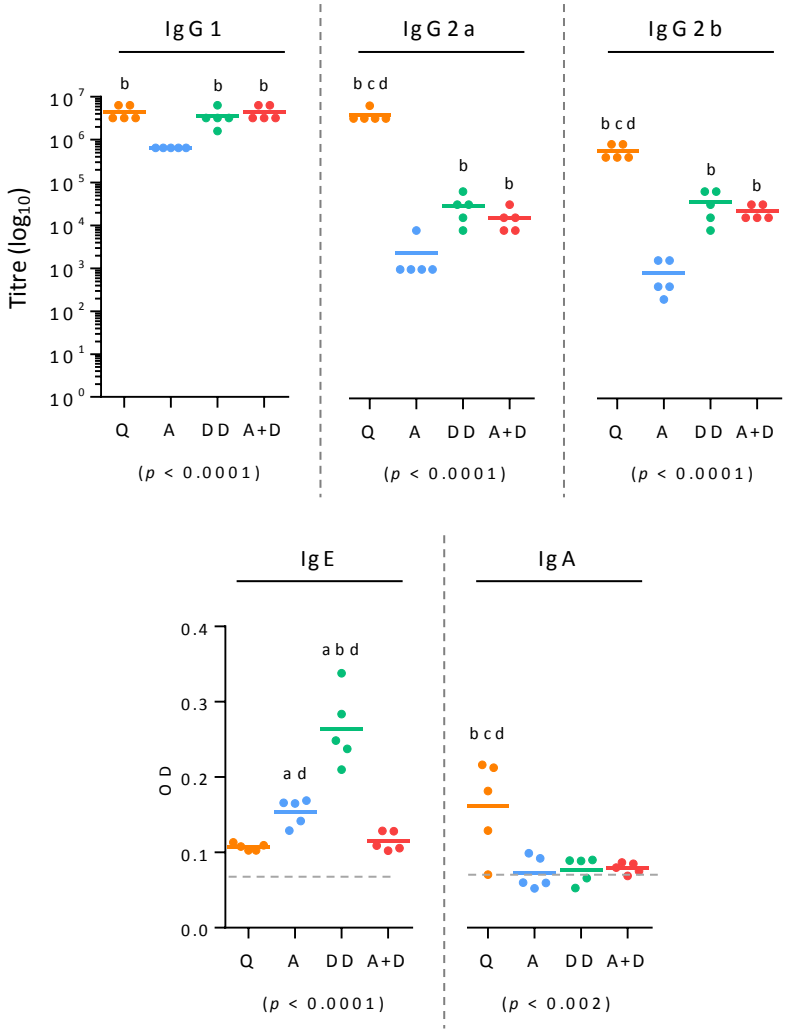
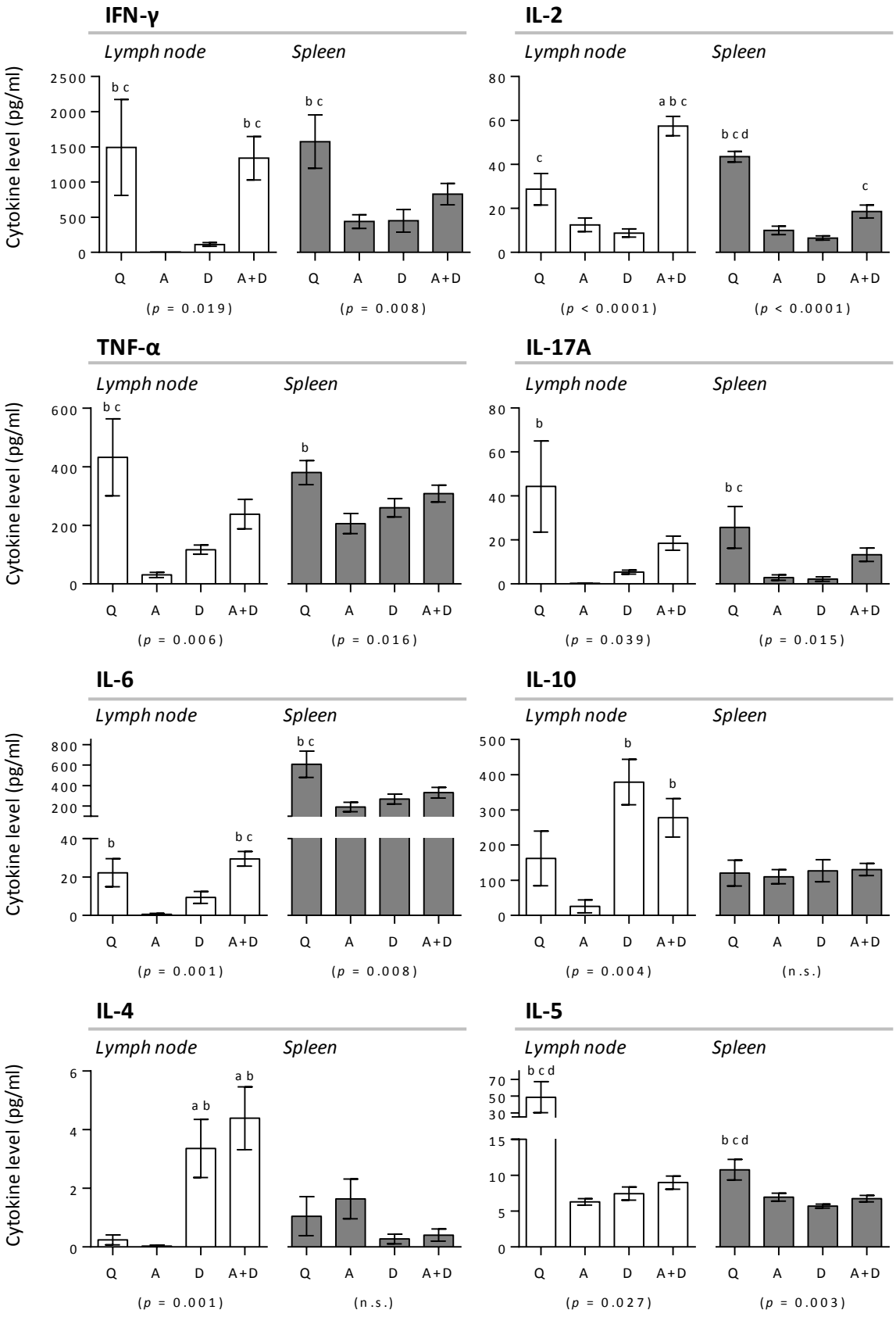


Figure 2: Antibody isotype titres to rSj-L6L-1 following different adjuvant formulations

Anti-rSj-L6L-1 serum titres (IgG1, IgG2a, IgG2b) were measured by ELISA after mice were vaccinated with rSj-L6L-1 formulated with different adjuvants; Quil A (Q), alum (A), DEAE-dextran (DD) or combined A and DD (A+D). Titres are the serum dilution the optical density (OD) was above twice the mean of naive mouse sera OD. Specific IgE and IgA levels are shown by OD, with naive sera mean is shown by the dotted horizontal line. One-way analysis of variance significance levels are indicated below each graph, and significant differences between adjuvant groups are indicated by the following letters: a = significantly above Quil A; b = sig. above alum; c = sig. above DD; d = sig. above A+D. For each adjuvant group, n = 5.

Figure 3: Cytokine profiles following different adjuvant formulations (next page)

Lymph node cells and splenocytes were stimulated with rSj-L6L-1 and cultured from mice were vaccinated with rSj-L6L-1 formulated with different adjuvants, and the secreted cytokines were measured in the culture supernatants by cytometric bead array. Adjuvants were Quil A (Q), alum (A), DEAE-dextran (DD) or combined A and DD (A+D). One-way analysis of variance significance levels are indicated below each graph, and significant differences between adjuvant groups are indicated by the following letters: a = significantly above Quil A; b = sig. above alum; c = sig. above DD; d = sig. above A+D. For each adjuvant group n = 5.


IFN-γ
Lymph node
Spleen
Cytokine level (pg/ml)
2500
2000
1500
1000
500
0
b c
b c
b c
Q A D A+D
(p = 0.019)
Q A D A+D
(p = 0.008)
IL-2
Lymph node
Spleen
80
60
40
20
0
c
a b c
b c d
c
Q A D A+D
(p < 0.0001)
Q A D A+D
(p < 0.0001)
TNF-α
Lymph node
Spleen
Cytokine level (pg/ml)
600
400
200
0
b c
b
Q A D A+D
(p = 0.006)
Q A D A+D
(p = 0.016)
IL-17A
Lymph node
Spleen
80
60
40
20
0
b
b c
Q A D A+D
(p = 0.039)
Q A D A+D
(p = 0.015)
IL-6
Lymph node
Spleen
Cytokine level (pg/ml)
800
600
400
200
40
20
0
b
b c
b c
Q A D A+D
(p = 0.001)
Q A D A+D
(p = 0.008)
IL-10
Lymph node
Spleen
500
400
300
200
100
0
b
b
Q A D A+D
(p = 0.004)
Q A D A+D
(n.s.)
IL-4
Lymph node
Spleen
Cytokine level (pg/ml)
6
4
2
0
a b
a b
Q A D A+D
(p = 0.001)
Q A D A+D
(n.s.)
IL-5
Lymph node
Spleen
70
50
30
15
10
5
0
b c d
b c d
Q A D A+D
(p = 0.027)
Q A D A+D
(p = 0.003)

5.3.2 Testing of rSj-L6L-1 vaccination against S. japonicum *infection*

Finally, a vaccine trial was conducted to test the ability of rSj-L6L-1 to induce protection against *S. japonicum* infection. Ideally, each adjuvant would have been tested to determine the efficacy of each formulation with rSj-L6L-1, however due to limitations in resources this was not possible. Therefore DD was chosen as the adjuvant since it was shown to induce a desirable mixed but predominantly type-2 response with specific IgE levels. After immunisation and challenge, the worm and egg burdens were enumerated and compared between the rTrx (control) and rSj-L6L-1 (test) vaccinated groups.

The rSj-L6L-1 vaccination did not protect against schistosomiasis in this experiment (Table 1). The total worm burden and number of worm pairs in this group was in fact slightly higher in the rSj-L6L-1 group, although not statistically different. However the number of males was significantly higher in the rSj-L6L-1 group ($p = 0.002$), but not the number of females. Worm lengths (male or female) were similar for both groups. The faecal egg counts were similar between both groups, whereas the liver egg burdens were significantly higher in the rSj-L6L-1 group ($p = 0.043$). As a measure of health, the mice were weighed before the trial, before challenge and just before necropsy (which was 6 weeks after the schistosome infection). While the rTrx group gained weight at each measurement, the rSj-L6L-1 group lost weight between challenge and necropsy, and had significantly less body weight than the rTrx control group at this point ($p = 0.004$). The weight change from pre-challenge to necropsy negatively correlated with very high significance to the liver egg burden ($p = 0.0009$, $r = -0.682$), the total worm burden ($p < 0.0001$, $r = -0.857$), number of worm pairs ($p = 0.0009$, $r = -0.683$) and male burden ($p < 0.0001$, $r = -0.808$), but not the female burden.

The specific antibody titres to rSj-L6L-1 were measured before and after challenge for each animal (Figure 4). Both vaccine groups had similar specific antibody titres to the antigen at each point in the trial; the rTrx-vaccinated group recognised rSj-L6L-1 because it contained the Trx solubility tag and hence had shared epitopes. There was a significant reduction in specific antibody levels from immediately prior to the parasite challenge to 6 weeks after this, for both vaccine groups ($p < 0.0001$).

Table 1: rSj-L6L-1 vaccination trial results

		Vaccination group		
		rTrx	**rSj-L6L-1**	**Significance**
Body weight (g)	*Pre-vaccination*	22.4 ± 0.9	21.4 ± 0.5	n.s.
	Pre-challenge	24.4 ± 0.4	23.9 ± 0.5	n.s.
	Necropsy	25.0 ± 0.5	22.4 ± 0.6	$p = 0.004$
Worm burden (#)	*Total*	15.0 ± 1.6	18.9 ± 0.7	n.s.
	Pairs	6.3 ± 0.8	7.9 ± 0.5	n.s.
	Males	6.7 ± 0.8	10.8 ± 0.7	$p = 0.002$
	Females	8.3 ± 1.1	8.1 ± 0.5	n.s.
Worm length (mm)	*Male*	6.8 ± 0.4	6.5 ± 0.2	n.s.
	Female	9.8 ± 0.3	10.0 ± 0.2	n.s.
Faecal egg count (eggs/gram faeces)		15.1 ± 8.4	14.2 ± 4.6	n.s.
Liver egg count (eggs/gram liver)		52 090 ± 6 459	65 029 ± 3 578	$p = 0.043$

Data shown as mean ± standard error, with the percentage change from the rTrx group in parentheses. Not significant = n.s.

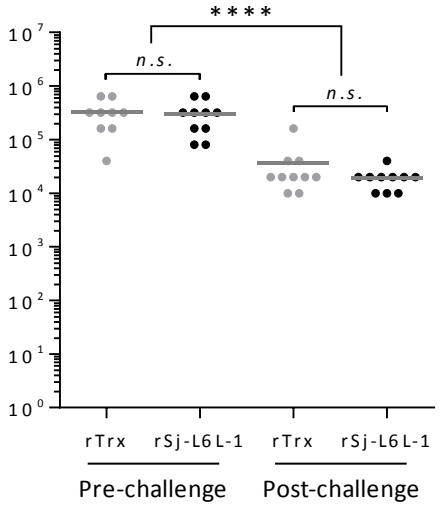


Figure 4: Total antibody titres to rSj-L6L-1 before and after challenge

Anti-rSj-L6L-1 serum titres (total Ig) were measured by ELISA during the vaccine trial after three vaccinations with either rTrx or rSj-L6L-1. Blood was taken before, and 6 weeks after mice were challenged with *S. japonicum* cercariae. The post-challenge samples were significantly lower than pre-challenge ($p < 0.0001$); however there was no significance (*n.s.*) between either vaccine groups.

5.4 Discussion

In this chapter the optimisation and testing of a novel vaccine candidate against schistosomiasis is described. The antigen Sj-L6L-1 was identified, characterised and produced in a recombinant form in Chapter 4, and was shown to have several promising features as a vaccine target. In this chapter, different adjuvant formulations were compared to determine which induced the most desirable immune response. Each adjuvant tested induced a different type of response against rSj-L6L-1, providing a novel insight into their adjuvant effects and a rationale for selecting the most appropriate formulation. Finally a preliminary vaccine trial was conducted, and under these conditions rSj-L6L-1 did not confer any protection from schistosomiasis in mice. Despite this lack of protection in this setting, several conclusions can be drawn to inform further testing of this Sj-L6L-1.

5.4.1 A comparison of adjuvant formulations with rSj-L6L-1

It is suggested that anti-schistosome immunity occurs by a type-2 response against the migrating larvae in humans (107-111), therefore the aim of the first part of this chapter was to test the ability of several adjuvants to direct the immune response against rSj-L6L-1 toward this type of immunity. The majority of current vaccines operate by inducing high specific antibody titres (268), and the antibody isotypes induced can indicate the type of immune responses generated (197). Therefore the type of response each adjuvant induced was determined by measuring the specific antibody isotypes in serum, and the cytokine profile of antigen-stimulated cells from the spleen and draining LN

While Quil A and alum induced the expected type-1 and -2 responses, respectively, DD induced the strongest type-2 response most closely related to the anti-schistosome protective correlates. The Quil A response against rSj-L6L-1 was characterised by the highest levels of type-1 and inflammatory cytokines, IFN-γ, IL-2, TNF-α, and IL-17A. It also induced high levels of specific IgG2a and IgG2b, isotypes which are induced by type-1 cytokines (269-270). Quil A was the only adjuvant to induce a significant amount of IL-5, which is typically a Th2-associated cytokine. However apart from its effect on eosinophils, in mice IL-5 also induces B cell differentiation to become antibody secreting cells (222). It also can promote IgA production (269) which was induced by Quil A. The adjuvant effect of Quil A is thought to be due to local tissue damage at the injection site which results in a damage signal (271). It is likely that the inflammatory cytokine response seen here is a result of tissue damage, which contributes to the high antibody levels, particularly increased IgG2a and IgG2b levels. In contrast, alum induced the lowest IgG subtype levels and very little cytokine response, evident that it is a relatively poor inducer of cellular responses (261-262). However specific IgE was induced indicative of a type-2 response.

DD induced a more pronounced type-2 response than alum, with significantly higher IgE in serum and IL-4 from stimulated LN cells. It also generated a weak type-1 response, with levels of type-1 isotypes (IgG2a and IgG2b) and cytokines (IFN-γ, TNF-α and IL-17A) between those of Quil A and alum levels. High levels of

IL-10 were also released from LN cells, although not significantly higher than Quil A. IL-10 inhibits inflammatory Th1 cytokines and is therefore often classed as a feature of the type-2 response (272) or a marker of regulatory T cells.

The mixed but predominantly type-2 response induced by DD was similar to that observed in the buffalo against migrating larvae (Chapter 3), which is potentially protective. More importantly, schistosome-specific IgE and IL-4 levels have both been correlated to resistance to re-infection with schistosomes (107-111). Therefore considering these similarities, and the high specific antibody titres, DD was selected for testing rSj-L6L-1 against *S. japonicum* infection.

DD is a polycationic derivative of dextran, a branched polysaccharide common to microorganisms (267). It has been shown to increase the specific antibody response when used as an adjuvant in mice and Rhesus monkeys (273-274), although its adjuvant mechanism is not fully understood. Carbohydrate adjuvants such as DD are promising adjuvants because they are generally biodegradable and have low toxicity (267), and are thought to promote immunogenicity through interaction with carbohydrate receptors on immune cells such as antigen presenting cells (267). Another mode of action is suggested by the ability of DD to facilitate the entry of protein inside cells (275), and the observation that DD-coating microparticles increases entry into dendritic cells (276). This suggests that DD may assist the uptake of the co-administered antigen by antigen presenting cells resulting in an increased adaptive response. In contrast with the damage-associated mechanism of Quil A adjuvant, DD may operate by interacting directly with antigen presenting cells and facilitating antigen uptake. Further work should be done to confirm this mode of action however.

Interestingly, when DD was combined with alum, the type-2 effect seen with each adjuvant alone was significantly reduced, instead causing a stronger type-1 response resembling that of Quil A, resulting in a mixed type-1/-2 response. There was no specific IgE detected, while the level of IgG isotypes was similar to DD alone. The levels of type-1 cytokines were generally similar to Quil A, particularly the high levels of IFN-γ and IL-2, although a similar IL-4 and IL-10 level to DD alone was secreted by LN cell stimulations. Since IFN-γ negatively regulates IgE secretion (269), the high level of this cytokine with the DD and alum combination possibly served to inhibit IgE. The observation that combining these two adjuvants changes the immune response dramatically from their use alone is interesting and warrants further study, although this is beyond the scope of this chapter.

5.4.2 Testing rSj-L6L-1 against S. japonicum *infection*

In this study, rSj-L6L-1 was tested as a vaccine adjuvanted with DD, however it conferred no protection against *S. japonicum* challenge. On the contrary, vaccination with the novel antigen significantly increased the male worm burden compared to the control group. An increased liver egg burden was also observed in this vaccination group, presumably due to the higher number of males and resulting slight increase in egg-excreting worm pairs, ultimately resulting in a loss of body mass as a reflection of poor health. While this

effect was negative, it was nevertheless significant, and warrants further investigation to understand and potentially improve this effect.

This lack of protection was somewhat unanticipated considering the promising features of Sj-L6L-1 as a vaccine candidate, as discussed in Chapter 4. Vaccination resulted in specific antibody titre against rSj-L6L-1, which still remained high when mice were killed 6 weeks after challenge, although one caveat is that much of this antibody may have been directed at the large Trx fusion partner. It would be more informative to express the antigen without this tag to monitor the antigen-specific antibody level after the vaccine trial. However serum from rSj-L6L-1 vaccinated rats (Chapter 4, Fig. 5) and mice (data not shown) was found to bind to native Sj-L6L-1 from a crude parasite extract by Western blot. So while vaccine-induced antibodies should recognise the antigen on the tegument surface, there are multiple possible reasons why rSj-L6L-1 was not protective.

The simplest explanation is that Sj-L6L-1 is not a protective antigen, and there could be several rationalisations for this. One is that although Sj-L6L-1 is on the worm tegument, as was found in Chapter 4 (Fig. 5) and on the *S. mansoni* tegument (254), it is possible that it is not available for antibody binding. Schistosomes use several mechanisms to hide their endogenous surface antigens, such as coating themselves with host molecules (32). Sj-L6L-1 is a tightly folded antigen of relatively small size (~11 kDa), so it is conceivable that it could be hidden under larger structural or host molecules. This is also reinforced by the inability of rSj-L6L-1 antiserum to surface label larvae or adults by immunofluorescence assays. An additional explanation is that the antigen is not expressed in abundance on the worm tegument, so even though it is recognised there is not enough Sj-L6L-1 present to induce worm killing by the immune system. It should be investigated whether Sj-L6L-1 is a major constituent of the tegument preparagraphtion by quantitative proteomic methods. An argument against this proposition is that vaccination with rSj-L6L-1 did have an effect, albeit a negative one, and some specific recognition must therefore have occurred.

An alternative reason for the lack of protection is that the recombinant antigen preparation and/or the adjuvant used in this trial were not optimal to test Sj-L6L-1 as a vaccine candidate. While the rSj-L6L-1 monomer (~25 kDa) did contain epitopes similar to the native schistosome antigen, and it could induce antibodies which recognised the schistosome-derived antigen, it also contained a significant amount of other mis-folded and aggregated forms of the recombinant (Chapter 4, Fig. 4). These were not recognised by schistosome-induced antibodies, indicating that they did not contain native epitopes. Therefore the rSj-L6L-1 vaccine given to the mice contained a large amount of irrelevant epitopes, and this most likely reduced the titre of antibodies specific for native Sj-L6L-1. In future testing of Sj-L6L-1, an attempt should first be made to improve the yield of rSj-L6L-1 in the native conformation, by investigating protein folding techniques or separating the monomer from the aggregates by chromatography. Alternatively, mammalian expression could be used to produce a form closer to the native antigen, or the native antigen itself could be isolated and tested.

The other explanation is that DD was not the ideal adjuvant for protection using this antigen against schistosomiasis in the mouse model. In this study it was rationally selected based on the strong type-2 immune response and specific IgE it induced against Sj-L6L-1, but this may not be effective against larvae in the mouse model. While the radiation-attenuated vaccine can operate by Th2-mediated mechanisms (for example in IFN-γ knockout mice (277)), type-1 mediated-immunity is more protective in mice (123). Also, the role of IgE in murine schistosomiasis has been controversial; one study showed that mice deficient in IgE had significantly enhanced worm burdens (278) although others have failed to showed effector function for IgE against schistosomes in mice (279-281).

In light of this, mice may not the ideal model to use a type-2-skewing adjuvant against a larval antigen. Ideally during the vaccine trial several adjuvants inducing different immune profiles should be tested simultaneously; however in the present study, this was not possible due to time and resource constraints as this would have doubled the number of mice used. It is possible Sj-L6L-1 is protective if tested with a stronger type-1 adjuvant such as Quil A, and this should be pursued.

5.4.3 A consideration on the vaccine-induced male worm survival

The finding that there was a significant effect with rSj-L6L-1 vaccination on the schistosome infection, albeit negative, is interesting and worth consideration. It was apparent that inducing a specific immune response against Sj-L6L-1 promoted the survival of male schistosome worms, increasing their numbers by just over 60% compared to the control group. It is tempting to speculate that there is a relationship between the fact that there was a higher number of male worms, and that Sj-L6L-1 is expressed uniquely by male worms (see Chapter 4, Fig. 1).

It has been established that early schistosome worm development is enhanced by host immune signals. Although the precise mechanism is not understood, several studies have indicated that $CD4^+$ T cells are required for worm development and pairing (282-284). Recently it was shown that naïve $CD4^+$ T cells allow normal worm development and this is most likely indirectly by T cell-induced mononuclear phagocyte maturation (285). How these mature mononuclear cells promote worm survival and development was not revealed, however Lamb et al. (285) speculate that it could be via release of local inflammatory mediators which increase blood flow, thereby facilitating larval transit as they navigate the narrow capillaries. Alternatively, the released mediators themselves may be cues for worm development.

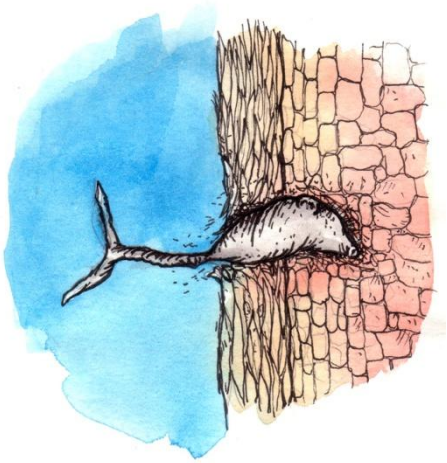
These studies suggest that an intact immune system is required for normal schistosome development. However in light of the result from this trial, the intriguing possibility exists that a specific immune response to a male larval antigen could actually promote larval survival. As discussed, it is likely that the vaccine used was not optimal; meaning the specific anti-Sj-L6L-1 immune response induced by this vaccine may have been just weak enough to promote larval survival without inducing killing. While this is highly speculative, it does explain the significant increase in male schistosome number in this trial. Further experiments could

investigate this observation which could reveal an important insight into the exploitation of the host specific immune response for larval survival.

5.4.4 Conclusions

In this chapter, the novel larval vaccine target Sj-L6L-1 was initially tested with four adjuvant formulations, which gave an insight into their ability to skew the immune response against rSj-L6L-1. DD induced a mixed but predominantly type-2 response, and was selected for use in a vaccine trail. Despite the promising characteristics of Sj-L6L-1 and the rational selection of DD as a potentially effective adjuvant, the vaccine failed to protect mice from *S. japonicum* challenge. On the contrary, vaccination with the antigen actually increased the number of surviving male worms, suggesting the intriguing possibility that the vaccine-induced specific immune response served to promote male worm survival. Further research should be pursued to produce a better recombinant form of Sj-L6L-1 which may result in protection from the disease. Also, testing with other adjuvants would more thoroughly assess its protective capacity. Finally, the increase in worm number suggests a capacity for schistosomes to exploit an antigen-specific immune response for their survival and should be investigated further to understand the extent of host manipulation by this highly adapted parasite.

Chapter 6: General Discussion



6.1 Summary

Schistosomiasis is one of the world's most prevalent infectious diseases, and contributes to the persistence of poverty in endemic regions due to its high level of morbidity (3). Despite concerted efforts to limit its prevalence, schistosomiasis is still widespread in many regions. It has proved difficult to control, particularly for the zoonotic *Schistosoma japonicum* which has the additional problem of animal reservoirs. A vaccine could significantly help in the control of schistosomiasis and may aid in its elimination, and has been a goal for schistosome research for decades (9).

This thesis aimed to contribute towards developing a vaccine against *Schistosoma japonicum*. The intramammalian larval stages are widely considered the targets of natural and experimental immunity (11) and therefore were the focus of this research. Attempts have been made to discover larval vaccine targets in the past (60), although due to difficulties in working with this transient developmental stage no larval specific vaccine candidates have been found (100). The antibody secreting cell (ASC)-probe technique has been used to identify larval vaccine targets for other helminths (181-187), therefore this promising method was applied here to study the immune response against the schistosome larvae as they penetrate, migrate and develop in the host.

Firstly, the implementation of the ASC-probe technique in a rat model of schistosomiasis is outlined in Chapter 2. This chapter shows that this method can be successfully applied to schistosomiasis, and the local antibody response was captured against the skin and lung migrating stages of *S. japonicum*. Then Chapter 3 shows the technique applied to the natural host, the water buffalo. The type of immune response induced against the migrating schistosome larvae was also concurrently investigated, in the first study of its kind in this important reservoir host. There were similarities in the local antibody responses to migrating larvae between the rat and buffaloes, but also differences; for example the lungs had high reactivity in rats but not buffaloes. There were also difficulties encountered using the buffalo ASC-probes compared to the rat samples. This highlights important discrepancies between small animal models and larger hosts, and the technical difficulties in using unusual animals such as water buffaloes, and these issues will be discussed later.

In Chapter 4 the captured local antibody response from the rat experiment was used to identify potentially novel vaccine targets using a newly generated protein microarray. One antigen in particular, *S. japonicum* Ly-6-like protein-1 (Sj-L6L-1), showed promise and was further characterised, revealing important features for a larval vaccine candidate: primarily transcribed in the developing larval stages; uniquely antigenic in the lung site in rats; tegument location; and antigenic during early water buffalo infection.

Finally, Chapter 5 outlined the optimising of recombinant (r) Sj-L6L-1 with various adjuvant formulations, and a vaccine trial using a type-2-inducing adjuvant was performed. Unfortunately no protection was induced against schistosomiasis; in fact vaccination increased male schistosome worm numbers significantly. This indicates that rSj-L6L-1 did have a significant effect, although it was a negative outcome for the mice.

Perhaps using a different adjuvant formulation, or optimising the recombinant construct, may change the effect of this antigen from being negative to positive against schistosomiasis. This chapter highlighted potential problems with the current models for vaccine testing and adjuvant selection, and this will also be discussed.

In summary, the research outlined in this thesis has used a novel approach to identify new targets for schistosome vaccines, investigated the local immune response against the migrating larvae in an important reservoir host and characterised and tested a novel vaccine candidate. However a number of important issues have been raised which may need to be addressed to progress vaccine development against schistosomes. These are discussed in the remainder of this chapter along with suggestions for the way forward.

6.2 Considerations for the future of schistosome vaccine development

The development of anti-parasite vaccines has been difficult over the years with very few successful attempts (286). The area relies largely on insights gained from successful vaccines against viral and bacterial pathogens, such as the use of single subunit vaccines and adjuvants. However there are vast differences with helminths compared to viruses and bacteria: for example the size, and hence their inability to be phagocytosed; and the different arm of the immune response adapted to eliminate them (287). The future of schistosome vaccines therefore may require a shift in thinking toward novel ways to deal with these complex multicellular pathogens. This may be accomplished by advances in several areas outlined below.

6.2.1 Finding the 'right' antigen(s)

There has been a drive to identify novel schistosome antigens because the existing antigens may not be the most effective (i.e. the 'right') candidates (11). Recent attempts at antigen identification have predominantly targeted tegumental antigens, for example through proteomics (60). This thesis describes a similar, rational approach to antigen identification, by combining the ASC-probe method with recent advances in genomics and immunomics (100), and resulted in the discovery of several antigens, one of which is tegument-associated and upregulated in larvae.

The question remains however: are single tegumental molecules the most effective targets? While it is rational to assign these as host exposed, in reality there is much we do not know about the worm tegument. For example, which antigens are actually exposed in the tegument? It is likely that some are present on the underlying apical membrane but are still covered by the outer membranocalyx (60), although the picture is not clear since current proteomic methods can not accurately separate the two outer membranes to investigate this (61). Furthermore there has been great difficulty in studying the proteome of the schistosomula tegument (60). Recently, Wilson (60) suggested a model of the tegument where some antigens were placed in the membranocalyx (e.g. tetraspanin-2 (TSP-2)) because they have been shown on the surface

of live worms by immunofluorescence, while others were hidden in the underlying apical membrane. Since this publication however Schulte *et al.* (288) used electron microscopy on *S. mansoni* adults to find TSP-2 only inside the tegument layer associated with the tegumental vesicles, and not on the outer tegument surface. TSP-2 is one of the most promising vaccine candidates against *S. mansoni* (150) (but not *S. japonicum* due to antigenic variation (289)), and must be host-exposed to have a protective effect when vaccinated. It is therefore surprising that it was not found on the outer tegument surface in this recent study, and suggests that antigens under the outer membranocalyx can still be protective. It is also possible however that the lack of TSP-2 localisation on outer the surface by Schulte *et al.* (288) could be due to the fixation technique used, or due to masking of the protein on the outer surface as discussed by the authors. The tegument is clearly a complex host-parasite interface and studies that can explain how and which antigens are truly exposed to the host, particularly the developing larvae, will be valuable for progressing vaccine development.

Schistosome secretions are known to be a source of immunomodulators and suppressors, particularly for the migrating larvae which use secretions to down regulate various aspects of the immune response (228). Vaccinating against these active immune suppressors could block their function and therefore increase the immune effector response against the penetrating larvae; hence these are valuable vaccine targets and may constitute a successful vaccine. Attempts have been made to identify the soluble proteins in the cercarial secretions (23, 290), although no studies have investigated the mucus-like part of the cercarial secretions which are likely to contain significant proteoglycans which are difficult to characterise (60).

Another consideration is that vaccinating with a single antigen may not induce a sufficient immune onslaught to overcome the rapid turnover of the dynamic, multi-layered tegument. Natural and experimental immunity is suggested to result after exposure to whole parasites (either the attenuated larvae or adult worms) which presents many antigens. Therefore multivalent vaccines could be more effective, although these are rarely explored due to the increase in cost with additional antigens. Single subunit vaccines have been effective against some viruses and bacteria, but complex multicellular parasites may require multivalent vaccines (286).

Further antigen identification should be explored, and novel targets could be used in combination with existing candidates in a multivalent approach (286). We intend to use the water buffalo ASC-probes to identify antigens in future studies, for example using them to probe the protein microarray, and this may result in discovery of antigens relevant for vaccinating this host. There is also another schistosome microarray containing significantly more proteins in development (291), and this would be extremely valuable to explore.

6.2.2 Producing the novel antigens

The way schistosome antigens are synthesised must also be considered, and this has been a problem for parasite vaccines for many years (286). Chapter 4 describes the production of Sj-L6L-1 in *E. coli*, fused to thioredoxin and histidine tags, and the antigen appeared to possess at least some of the conformational

epitopes present on the native counterpart. While bacteria are commonly used for protein expression, there are several drawbacks; some native epitopes could be excluded due to the addition of the tags which may interfere with the normal protein folding; and since bacteria do not glycosylate proteins, potentially relevant carbohydrate epitopes will not be present (286).

As an alternative, the native antigens could be isolated and tested. Historically this was more common for testing vaccines before the advent of molecular biology, or in cases when the identity of an antigen was unknown. This could give a more definitive answer to whether or not the antigens are protective without the confounding aspect of recombinant expression. A larval antigen from the sheep nematode, *Haemonchus contortus*, was discovered using the ASC-probe method (182) and has been shown to be protective in its native form (144). Despite several attempts its peptide sequence remains unknown, although it is likely to be predominantly carbohydrate in nature therefore current recombinant techniques won't be able to reproduce this. Recently, Martins *et al.* (292) cleaved GPI-anchored proteins from live adult *S. mansoni* worms and found they gave protection in a vaccine trial. Previously the same enzymatic cleaving technique was shown to contain various GPI-anchored proteins including the *S. mansoni* orthologue of Sj-L6L-1 (Sm-L6L-1) (254), although this is likely only a minor constituent of this preparation (292). While these small-scale trials are possible for adult antigens, purifying native larval-specific antigens requires a prohibitively large number of schistosomula.

Another possibility is to produce 'native' antigens using schistosome cell lines. Although not available yet, attempts have been made to culture schistosome cells, and oncogenes can be inserted for immortalised cell lines (293). If successful, these could be transfected to produce the antigens which are likely to be in the relevant conformation and with native glycan moieties (293), and this would be a significant advantage for schistosome vaccine development.

6.2.3 Carbohydrate antigens – promising vaccine targets?

Carbohydrate structures, or glycans, coat the surface of many organisms via attachment to protein or lipid components and are therefore exposed to the host's immune system. Schistosomes in particular have abundant glycoconjugates present on their surface and secreted products (213, 215), and a strong humoral response is mounted against them. The protective antibody response induced after vaccination with radiation-attenuated larvae is predominantly against carbohydrates (202), and *in vitro* experiments show that an antibody against one of the most abundant surface glycans, lacdiNAc, can induce complement-mediated killing of newly-transformed schistosomula (213). Furthermore, glycan expression appears to be developmentally regulated (294), and there is evidence of stage-specific glycans, such as the cercarial glycolipid structures (295). There is a contrasting opinion on the importance of carbohydrate epitopes as vaccine targets however; some researchers have proposed that the anti-glycan response is not in fact protective, and that these abundant carbohydrates may function as evasive tools to divert and modulate the

immune response (202). While this may be true of the abundant glycans, it is conceivable that the less abundant surface glycans of the larvae are protective and these should be explored as novel antigens.

Glycans are an abundant and relatively novel source of molecular vaccine targets for schistosomes (213-214). Glycomics is an emerging area of schistosome research, and has the potential to transform the status quo for schistosome research, which is currently focussed on protein antigens. However this would require the development of new methodologies to identify novel schistosome glycans, and to improve the synthetic production of glycan structures, although this is rapidly being overcome with advances in this area (296).

Glycan microarrays could provide a way to identify novel antigenic glycans. In Chapter 2 a glycan microarray was probed with ASC-probes which indicated that these two techniques could be used to study the tissue-restricted anti-carbohydrate response against migrating larvae. A limitation with the microarray used was that it was composed largely of mammalian glycan structures, which are not ideal targets since their vaccination may induce auto-reactivity. To look for glycans unique to schistosomes, Cornelis Hokke and colleagues at Leiden University Medical School have generated a schistosome-specific glycan microarray (297). To achieve this, carbohydrates from various schistosome developmental stages were isolated, fractionated and then arrayed on a glass surface, and this microarray can be probed with antibodies or immune serum (297). If a particular fraction is recognised, it can be interrogated to identify its constituent structures. We have initiated a collaboration with this group and hope to use the ASC-probes from buffaloes and rats on these arrays, which may identify novel vaccine targets for schistosomiasis.

6.2.4 Models for understanding immunity and developing vaccines

There has been considerable research into understanding anti-schistosome immunity. Large correlative surveys have been performed within endemic human populations, and a substantial number of studies have investigated the mechanism in animal models. The understanding of immunity gained from these studies then informs the design of vaccines in an attempt to replicate this protection. However there are significant discrepancies in our understanding of immunity which complicates the design of schistosome vaccines.

The natural expression of immunity in humans is consistently correlated with type-2 factors such as the levels of specific IgE, IL-4 and IL-5 (106-111), and this arm of the immune response is generally thought to target the migrating larvae with antibody dependant cellular cytotoxicity (298). This is also reflected in some animal models, most notably rats (113). However in the mouse model, which is the most prominent model of schistosomiasis immunity, a type-1 mediated protection against the larvae is thought to act by blocking their path through the lung vasculature (123). The picture is even further complicated by the fact that a few studies correlate type-1 factors with human immunity (117), and mice can be protected by a polarised type-2 response (123).

With this in mind, what sort of response should a vaccine induce? This is one of the prominent issues today in schistosome vaccine research. Clearly both type-1 and -2 mechanisms are capable of eliciting immunity,

although the extent of protection may depend on the particular animal. Since *S. japonicum* vaccines are aimed at humans and water buffaloes, a deeper understanding of immunity in these hosts is needed. Conclusive evidence is lacking on the exact protective mechanisms in humans, which have been extrapolated by *in vitro* studies and type-2 correlates (298) and this is due to experimental constraints of working in humans. The mechanism may never be fully demonstrated in humans, forcing the continued reliance on animal models and *in vitro* studies.

Water buffaloes offer a unique advantage in that immune mechanisms can be studied in greater detail in a host which is the intended recipient of a vaccine, and this should be pursued. The data from Chapter 3 suggests the possibility that buffaloes target larvae in the skin, but this needs to be studied in greater detail for confirmation. However in the murine model, the skin is not considered to be a significant obstacle for the larvae. It is likely that in larger hosts, where the skin is thicker, larvae take longer to penetrate and can be targeted by antibody dependant cellular cytotoxicity more readily than in mice. The normal buffalo epidermis from the inner thigh was found to be approximately 70 μm thick in Chapter 3, while the mouse abdominal epidermis (where infections are commonly administered) is around 12-16 μm thick (299). There are limitations to working with water buffaloes however: there are few reagents compared to mice (11); and their large size and cost necessitating small sample sizes. Both of these issues were encountered in Chapter 3, although this chapter demonstrates that valuable investigations are still possible.

Finally, another way to approach the question of which immune response is most efficacious is to simply test promising targets using various adjuvants and models. Since both type-1 and -2 responses can provide protection, it has been suggested that the type of immune response may not be vital (11). To investigate this antigens could be compared with different adjuvants in tandem, although these studies are scarce (267). In Chapter 5 a comparison of the immune response was measured with several adjuvants, and while this is informative from an immunological perspective, the logical next step would be to compare the level of protection induced by each formulation. Mouse models are extremely useful to assess vaccines relatively quickly; however considering the probable differences in the modes of protection, such mouse trials may not be relevant to buffaloes and humans. Testing antigens in water buffaloes is more relevant to vaccine development against Asian schistosomiasis, and ideally adjuvant optimisation would be performed in this important host.

6.3 Final conclusion

The development of an anti-schistosome vaccine continues along a slow and difficult path which has been littered with setbacks, but also fragments of hope. Significant advances in the areas discussed in this chapter may achieve progress in the eventual development of a highly effective anti-schistosome vaccine. It is hoped that the research presented in thesis represents at least a small step towards this goal by providing an investigation into the immunobiology of the migrating larvae.

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