Exploring host-immune-microbial interactions during intestinal schistosomiasis

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CHAPTER 1

Introduction to Schistosomiasis

The mammalian immune system contains a complex consortium of cell types that are anatomically poised and meticulously designed to defend against imminent infections, whilst remaining tolerant to innocuous antigens (Ags)[1]. This sophisticated network has been finely tuned and calibrated by years of exposure to various pathogens, including helminths[2,3]. Our immune system has evolved to not only accommodate these parasites but also prosper in their presence[2,3]. Delineating the perplexities of parasite infections, including their proficient capacity to stimulate immunoregulatory and effector cell circuits, is an exciting and therapeutically rewarding area of research[4].

THE HYGIENE / OLD FRIENDS HYPOTHESIS

A healthy immune system can vigilantly discriminate between harmless and harmful Ags: actively tolerating self-Ags, commensal bacteria, and foodstuff, whilst skilfully eradicating invading threats and malignancies. The mechanisms underlying self vs non/altered-self-tolerance, and the resolution of associated collateral tissue damage, are central themes in immunology and the subject of many reviews[1,5]. To put it very simply, this act of discrimination is achieved through collaborate efforts between cells of the innate and adaptive immune system, and the integration of complex genetic, environmental, and tissue-derived signals, including those from our nervous system, diet, and commensals/pathogens themselves[1,6]. In recent centuries, this delicate equilibrium has gone awry in industrialised-populations, who have fallen victim to an alarming surge in immune-mediated disorders, such as allergy, asthma, and autoimmune disease[6–8]. Now we face a challenge; scrambling to identify the key factors contributing to these inflammatory epidemics.

According to the 'Hygiene hypothesis', the increased prevalence of chronic-inflammatory disorders can partially be attributed to westernised lifestyle practices (i.e enhanced urbanisation and sanitisation) reducing exposure to historically ubiquitous pathogens and commensals[9]. Since being termed in 1989, the Hygiene hypothesis has been supported by countless studies, including those demonstrating a protective and/or prophylactic effect of bacteria, viruses, and parasites on inflammatory disease progression[10]. However, as not all pathogens confer disease protection[10–13], the Hygiene hypothesis has since been reformed to the 'Old-friends'[14] and 'Biodiversity'[15] hypotheses, which place greater emphasis on the immunomodulatory contribution of *select* symbionts with which we have co-evolved. These organisms are required for adequate immune education, and so in their absence, the inadequately trained immune system is liable to mount disproportional immune reactions towards harmless environmental allergens and self-Ags[3,10,14]. Moreover, in more recent years two conceptionally distinct 'hygiene hypothesis' have been established, which detail the stage in life when the immune system is most signficiantly influenced

by the envrionment[2]. Firstly, the idea that the immune system is imprinted early in life, with exposure to certain organisms having long lasting effects on how the immune system later responds and regulates inflammatory conditions. Secondly, the immune system is considered to be 'plastic' and can be recalibrated through microbes exposure in later stages of life. This latter concept underpins the idea that microbially-derived molecules can be used for the reversal and treatment of hyperinflammatory conditions.

Helminths are parasitic worms, whom by definition, derive benefit at their host organism's expense[16]. However, contrary to the debilitating and potentially life-threatening consequences of helminth infection[17], helminths are amongst the beneficial organisms thought to shape and craft the human immune system[6]. Over millennia, we have evolved to not only tolerate helminth infections, but also thrive in their presence. Indeed, helminth enriched regions have a far lower prevalence of immune pathologies than helminth-free lands[7,8,18], anthelminthic treatment during pregnancy has shown to increase the risk of allergic symptoms during infancy[8,19,20], and the offspring of migrants transitioning from rural-urban areas quickly converge with the indigenous population in terms of the risk of acquiring autoimmune disease[21,22]. Arguably, these observations could be explained by other life-style factors, including reduced physical activity, heightened pollutant exposure and greater consumption of highly processed convenience food. Indeed, anthelminthic administration does not reliably exacerbate allergy-or autoimmune related outcomes[7,8] and a fair degree of inconsistency exists within the realm of epidemiological studies[8,13]. Nevertheless, despite the complexity and confusion, the consensus still favours a role for helminths in the suppression of immune mediated pathologies, but a contributory role for other environmental and genetic signals in their development is emphasised[7].

Over the years, a variety of epidemiological, clinical, and animal studies have persuasively argued for helminth involvement in inflammatory disease (i.e. diseases hallmarked by inflammation) protection/ prevention [8,23,24], with the most consistent findings obtained from animal models[25–32]. Interestingly, mouse experiments have even underscored the importance of maternal immune responses and the presence of a critical window during early development, in which helminth-derived signals have their largest bearing on disease predisposition[33,34]. The overarching aim of most, if not all animal experiments, is to eventually translate their findings into a clinical / human setting. When the idea of helminth-mediated disease modulation first started to grow in popularity, several research groups pioneered the experimental practice of helminth therapy[35–37]. Here, live helminth larvae, worms, or eggs, are administered to various patient cohorts, who suffer from

diseases including allergy, multiple sclerosis (MS) and inflammatory bowel disease (IBD)[35–38]. Unfortunately, while initial trials showed promise, a shortfall in overwhelming clinical benefit, alongside the irrefutable ethical implications and potential allergic cross reactivity with parasite Ags[39], has since seen helminth therapy dwindle in research appeal. Instead, researchers are now taking a more refined and clinically acceptable approach, with the ultimate goal being the identification and administration of select, helminth-derived immunomodulatory molecules[4].

There exists an ever-growing list of helminth-derived immunomodulators, each directing immune function in their own distinct way[4]. Reflecting the capacity of helminths to elicit Type 2 immunity**,** a large fraction of these molecules amplify Type 2 immune responses: potentiating alternative activation of macrophages, inducing T Helper (Th)-2 cells and Type 2 innate lymphoid cells (ILC2s), boosting antibody (IgE) levels and favouring the production of Type 2-associated secreted mediators, such as RELMα, Ym1, interleukin (IL)–4, IL-5 and IL-13[4,23]. In addition, many helminth products promote layers of immune regulation. This includes the induction of Regulatory T cells (Tregs)[40] and B cells (Bregs)[29,41,42] a reduction in pro-inflammatory myeloid cell recruitment[43], and increased anti-inflammatory cytokine secretion (IL-10 and TGF-β)**.** The modulation of the immune response towards a particular phenotype not only influences helminth survival and bystander tissue damage, but also impacts host susceptibility to chronic inflammatory disease[6,7,10,44]. For example, helminth-induced polyclonal IgE or IgG4 is suggested to outcompete allergen-specific IgE, and therefore block basophil and mast cell degranulation [44,45]. In addition, helminth-driven Bregs have proven to be proficient in alleviating of allergic airway inflammation (AAI) via IL-10 production and their capacity to induce Tregs[25].

Like no man is an island, no single molecule or cell type acts in isolation (in immunity). There exists a large degree of redundancy, overlap and synchrony within the immune system, with many networks of cells and molecules acting in combination to achieve a given effector response. Similarly, it is important to stress that the beneficial aspects of helminth infection are unlikely to be governed by one single molecule or cell type. These supporting signals include host-derived inflammatory mediators (e.g cytokines and alarmins), neutrotropic factors and environmental cues from our diet and local floras[1,15]. For example, while multiple products secreted by *Heligmosomoides Polygyrus* can adeptly suppress Type 2 immunity and AAI[46–50], external signals from the *H. polygyrus*-altered microbiome have also shown instrumental[51]. Given this complexity, a current research ambition is to not only identify immunomodulators that are expressed by helminths, but also the vast range of host tissue, cellular, immune, and environmentally derived

signals that complement and enable their effects. Many laboratories have turned to *Schistosoma* parasites to realise this research goal.

SCHISTOSOMIASIS - BACKGROUND

Schistosomiasis is a neglected yet significant tropical disease caused infection with dioecious blood flukes of the genus *Schistosoma*. Spread by exposure to free-swimming larval stages (cercariae), this water-borne infection prevails in developing countries with low sanitation and poor access to safe water. With approximately 200 million individuals currently infected, schistosomiasis is a disease of epic proportions, rivalling malaria in terms of prevalence and morbidity and being directly responsible for approximately 200,000 deaths per year in sub-Saharan Africa alone[52]**.** 25 species exist within the *Schistosoma* genus, all displaying large diversity in terms of molluscan (intermediate) and mammalian (definitive) host preference, tissue migratory patterns[53,54], and egg production rates[55,56]. While their snail host preference dictates geographical distribution, the latter two parameters impact the type and severity of ensuing pathology. The three major species of schistosome to infect man are S*. haematobium,* S. *mansoni* and S*. japonicum*, which inflict either urogenital (*S.haematobium*) or intestinal (S. *mansoni* and S*. japonicum)* disease. As discussed later, schistosomiasis develops through three distinct stages of disease: pre-patent acute, post-patent acute and chronic. Each stage is typified by a distinct immune profile, that mirrors antigenically distinct stages of the parasite lifecycle.

Lifecycle

A clear comprehension of the schistosome lifecycle (Diagram 1) is essential to understanding the complex immunobiology of disease. For all species, infection begins with skin exposure to cercarial infested water. Triggered by sunlight, and so coinciding with periods of human water contact (e.g. early morning hours when daily water is collected), free-swimming cercariae emerge from their intermediate snail host in their hundreds-thousands, perishing if they fail to infect a suitable mammal within several hours[53]. Using a variety of navigational cues (e.g turbulence, sweat gradients and shadows on the water) cercariae locate and latch onto the skin of their prospective mammalian host, and in a matter of minutes breach the epidermis. Infected individuals may experience some urticarial reactions within a few hours of exposure, although this is more severe and common upon contact with schistosomes that have not evolved to infect humans (e.g. those that infect birds)[57]. Upon penetration, cercariae transform into juvenile schistosomula, shedding their defining bifurcated tail in the process. Schistosomula reside within the skin for several hours (*S. japonicum)* to days (S. *haematobium* and S. *mansoni*), where they interact with the dermal immune system

before entering blood vessels and circulating to the pulmonary system[54]. While the exact duration of pulmonary residency is difficult to define, a series of radiotracking studies suggest schistosomula remain in the lungs anywhere between day 2 and 25 of infection, with clear species-specific differences[54,58,59]. From the lungs, juveniles quickly pass through the heart[60] and disseminate to the portal vasculature, where they temporarily reside and develop into immature male and female worms. Depending on the species, development occurs within the intestinally draining hepatic portal vein and mesenteric blood vessels (*S. mansoni* and S. *japonicum*) or veins proximal to the bladder (S. *haematobium).* As parasites grow and mature, they migrate to larger vessels, seek out members of the opposite sex and form a monogamous pair, although divorces have been reported[61]. In this relationship, the males enfold the smaller female, who are reliant on male-transmitted signals (e.g. immunological, tactile, nutritional, or neurological cues) for development[62]**.** Males also require some partner-derived signalling to complete their development, but unlike females, their morphology and survival within the host is not completely dependent on pairing[62]. This pairing takes place at approximately 4-5 weeks post infection.

One paired, the worms move against blood flow to the site of oviposition, the mesenteric vasculature (S. *japonicum* and S. *mansoni*) or bladder plexus (*S. haematobium*). These long-lived flatworms bathe within the host bloodstream for years, ingesting red blood cells, regurgitating insoluble-break down products and depositing hundreds (S. *mansoni* and S. *haematobium*) or thousands (S. *japonicum*) of eggs per worm pair per day. For lifecycle completion, eggs must enigmatically migrate across the intestinal or bladder wall for subsequent release via the faecal or urinary stream[63]. This process is not a certainty, with a large fraction of eggs being inadvertently flushed to the liver or more remote organs, where they evoke intense immunological reactions that ultimately manifest as clinical disease. For the *mature* eggs[64] that successfully exit the host, in contact with freshwater triggers them to hatch and release a single ciliated larva (miracidia). Within 24 hours, miracidia must penetrate the soft tissues of a suitable freshwater snail, where they undergo rounds of asexual reproduction over the period of approximately 4 weeks, giving rise to thousands of fork-tailed cercariae.

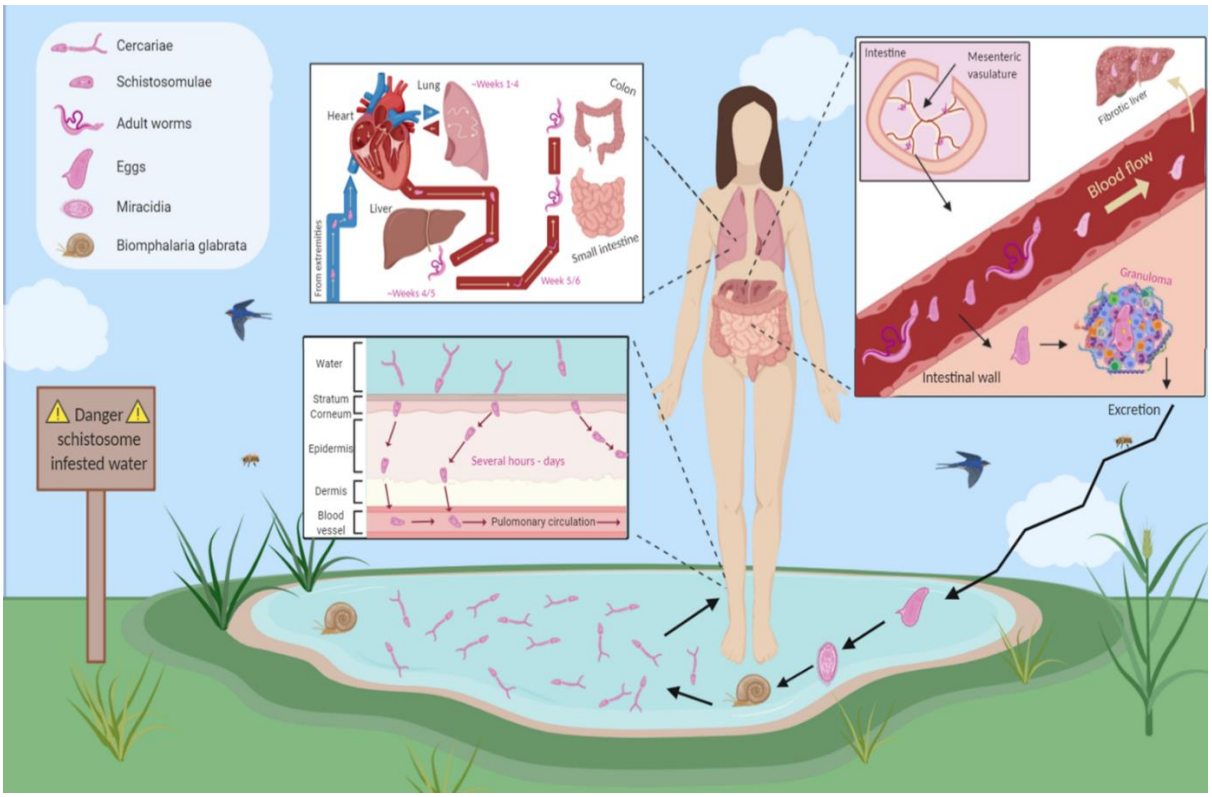

Diagram 1 – The lifecycle of *Schistosoma mansoni*

Schistosoma mansoni has a complex lifecycle that involves phases with a definitive human host and an intermediate fresh-water snail. Free-swimming miracidia infect *Biomphalaria glabrata* snails and over a 4-6-week period, parasite numbers are amplified via asexual reproduction. In response to sunlight, infected snails release thousands of cercariae, which swim towards and penetrate their definitive human host. As cercariae pierce through the skin, they transform into schistosomula and lose their defining bifurcated tail in the process. After a few hours-days of dermal migration, schistosomula enter local blood vessels and are swept to the pulmonary system via the heart, where they reside for several weeks. Cercariae make a subsequent loop of the heart, enter systemic circulation and travel to the hepatic portal system. At this temporary site of residency, schistosomula develop into immature male and female worms that pair up, transmit development cues to one another and reach full sexual maturity. The worm pairs subsequently move against the direction of blood flow to the mesenteric vasculature, where they dwell for the remainder of their lifespan and produce hundreds of eggs per day per worm pair. These eggs either inadvertently lodge within host tissues such as the liver (a dead end), or successfully transit across the intestinal tissues into the faeces. When viable eggs reach freshwater, they hatch into free-swimming miracidium, which continue the lifecycle. Adapted from [54,65,66].

Pathology

Schistosomiasis associated pathology is directly related to the location of the blood vessels in which the adult blood-flukes reside, and where their highly immunogenic eggs deposit within the body[63]. While larvae and adult worms appear somewhat able to avoid or evade immune destruction, schistosome eggs and their complex mixture of soluble egg antigens (SEA) they secrete are highly visible to the host immune system[67]. Upon deposition, schistosome eggs form the focal point of inflammatory granulomatous reactions, in which individual eggs are surrounded by extracellular matrix and a collection of cell types including, (mainly) fibroblasts, macrophages, eosinophils and Th cells[68]. This inflammatory reaction serves a dual purpose. Firstly, as a host-protective response that shields parenchymal tissue from egg-derived toxins and mitigates bystander tissue damage[67]. Secondly, via mechanisms yet to be defined, granulomas facilitate egg migration across intestinal tissues and out of the host, enabling continuation of the parasite lifecycle. In the absence of intact Type 2 responses, granulomas are disrupted, faecal egg output is diminished, pathology is exacerbated, and the host may succumb to immunopathology[69–71]. As such, schistosomes have evolved a wide variety of tactics to ensure granulomas are correctly constructed and times[64], and to skew Type 2 polarisation in their favour[68]. If these responses are not sufficiently calibrated or reined in by cells of the regulatory arm of the immune system, the ensuing pathology can be lethal[68].

For mesenteric dwelling S. *mansoni* and S. *japonicum*, severe disease is characterised clinically by pot belly (due to enlargement of the liver and spleen), emaciation, and loss of vigor[72]. The deposition of eggs within hepatic sinusoids leads to destructive collagen accumulation, impaired blood flow to the liver, portal hypertension, and the formation of rupture-prone anastomoses (collateral vessels) that extend toward the oesophagus and stomach wall[72]. Bleeding from oesophageal varices can prove deadly. In addition, intestinal schistosomiasis is clearly linked with cognitive impairment, worsening already existing poverty within schistosome endemic populations[73]. Whereas for S. *haematobium*, urogenital egg entrapment frequently results in squamous cell carcinoma of the bladder, ureteral fibrosis, and the formation of genital sores[72]. Importantly, these are not only painful, stigmatising and associated with infertility, but also enhance the risk of contracting venereal diseases such as HIV[68,74–76]. Interestingly, while only S. *haematobium* has been formally classified as a group 1 carcinogen, S. *mansoni* eggs have also shown to dysregulate apoptosis and oncogenesis-associated gene pathways[77], and infections are associated with an increased risk of developing hepatic and colorectal cancer[78]. As such, it is highly likely that the site of egg location and the surrounding tissue-microenvironment has a large bearing on tumorigenesis.

Schistosomiasis-associated pathology is not exclusive to the sites of worm residency. Ectopic lesions in schistosomiasis are defined as the spread of eggs and/or worms outside the portocaval blood channels[79]. This can have devastating consequences[67]. For instance, in the central nervous system this may give rise to seizures and paralysis, while in the lung it can lead to irreversible pulmonary hypertension[80]**.** Worms have frequently been found beyond their 'normal' locations including in the liver, lung heart, cervix, and conjunctiva[80]. However, the clinical consequence of these ectopic localisations is neither well-understood nor well-studied. Interestingly, adult worms in the lungs has been suggested to impact resistance to repeat infection[81] and, arguably, the nesting of eggs within the brain may not be possible without the presence of nearby copulating worm pairs[80,82].

Pulmonary symptoms are evident during pre- and post- patent acute and chronic schistosomiasis, but the mechanisms underlying this pathology are poorly defined[83]. In acute disease stages and in response to larval migration, worm maturation and the start of egg production, individuals from non-endemic regions are prone to develop Katayama Fever, with symptoms of general malaise, fever, coughing and breathlessness[83]. During chronic disease, the main pulmonary pathology is hypertension, which typically develops alongside sever hepatosplenic disease. In these cases, the blockage of portal vessels by schistosome eggs leads to portal hypertension and the formation of portal systemic shunts[83,84]. These shunts carry parasite eggs in the bloodstream to the lungs, where they lodge within pulmonary tissue. Interestingly, this spread of eggs may not only give rise to pulmonary pathology, but also support immunity to newly penetrated schistosomulum[84].

At this point it is important to emphasise that advanced, life-threatening disease occurs in an estimated 5-10% of infected individuals, who upon closer inspection, tend to harbour high intensity infections and generate immune responses that are unable to regulate the persistent egg-driven inflammation[85–87]. For instance, hepatosplenic individuals show defective eosinophil cytotoxicity[88] and a high level of lymphocyte proliferation[89]. There is also evidence for genetic predisposition[90] and a link with aberrant TNF and IFN-γ signalling[91,92]. In mice, severe pathology has a clear genetic association[93], which emerges in the form of persistently raised proinflammatory Th1 cytokines[94] and the emergence of pathogenic Th17 responses[95,96]. Moreover, advanced disease is more frequent in S. *japonicum* infections[66]. This could be due to substantially higher egg output the release of eggs in packages (as opposed to individually) or species specific secretion of toxic egg molecules[97,66]. Unfortunately, due differences in mouse and human anatomy, and thus disparities in disease progression[98], as well as a paucity of human studies on severe schistosomiasis, our understanding of the factors governing advanced disease is lacking. However, powerful tools such as single-cell

RNA sequencing of liver samples have recently provided great insight into the immune cell types and transcriptional landscapes involved in schistosome-associated liver fibrosis in humans[99].

THE IMMUNOLOGICAL PROFILE OF SCHISTOSOMIASIS

Schistosomiasis has a distinct immunological profile that is commonly discussed in terms of acute and chronic disease phases, some newer reviews also paying mention to an intervening 'active' phase[66]. These phases reflect antigenically distinct developmental stages within the schistosome lifecycle that differentially induce three distinct immune responses: Type 1, Type 2 and regulatory responses[100].

- **Type 1** inflammation is typically concerned with the elimination of intracellular pathogens and cancer. These responses are defined by the activity of Th1 cells, Type 1 ILCs with cytokine environments rich in IFNγ, IL-2, IL-12 and TNF. Classically activated macrophages also hallmark these responses, where they play important defensive roles against microbial pathogens[101]. The transcription factor T-bet is essential for Th1 cell commitment and Th2 program repression[102]. In schistosome infections in CBA mice, show greater genetic susceptibility to schistosomiasis[93], severe pathology and premature death is associated with prolonged persistence of Type 1 responses[94] and emergence of pathogenic Th17[93,103].
- **Type 2** immunity is involved in infection with helminths and promotion of tissue repair[100]. Th2 and ILC2 cells can produce IL-4, IL-5, IL-9 and/or IL-13[104], but other Type 2 associated cytokines include IL-25, IL-33 and TSLP[105]. Acting in synchrony, these cytokines support alternative macrophage activation (typically concerned with wound repair), priming of dendritic cells (DCs) to drive Th2 cell differentiation, B-cell IgE production and the recruitment and/or activation of eosinophils, mast cells, basophils and Type 2 ILCs. However, it is important to be aware that the cellular source of these cytokines guides host immunity trajectory, with epithelial or myeloid derived IL-33 instructing Th2 or regulatory responses respectively (at least In the context of *Nippostrongylus brasiliensis*)[106]. Moreover, Type 2 cytokines also provoke mucus production, smooth muscle contraction and fibrosis. While these elements are instrumental in helminth expulsion and tissue repair, they can also promote fibrotic disease and responses to bystander Ags leading to allergic disease[107].
- The above responses are reined in by cells of the **regulatory** network, including immunosuppressive Regulatory T cells (Tregs) and B cells (Bregs), and supposedly, more tolerogenic DC and macrophage populations[108,109]. These cells act through direct cellular interactions or secretion of anti-inflammatory mediators and cytokines such as IL-10, IL-35 or

TGF-β [107]. Moreover, the cytokines IL-22 and amphiregulin have shown important roles in wound repair and tissue resolution[110,111], although they are not typically denoted as regulatory cytokines.

- **Th17** responses are reliant on the transcription factor RORγ[112], and are commonly associated with infection with extracellular bacteria and fungi. While a Th17 response is sometimes seen during murine shistosomiasis[96], they are generally considered marginal in relation to the other archetypical responses and typically only emerge in specific mice strains[95]. The majority of data suggests Th17 responses to promote schistosome immune-pathology rather than be beneficial for the host[93,113]. Given that patients with advanced disease show signs of Th17 associated pathology[114], we should consider modelling severe disease in mice prone to Th17 pathology (i.e. the CBA mouse as opposed to the more commonly used mice strains of C57BL/6 and BALB/C)[93.]

The immune landscape of schistosomiasis is depicted in Diagram 2 and discussed much more thoroughly in the sections below. Moreover, from hereafter, I refer to infections with S. *mansoni* unless stated otherwise.

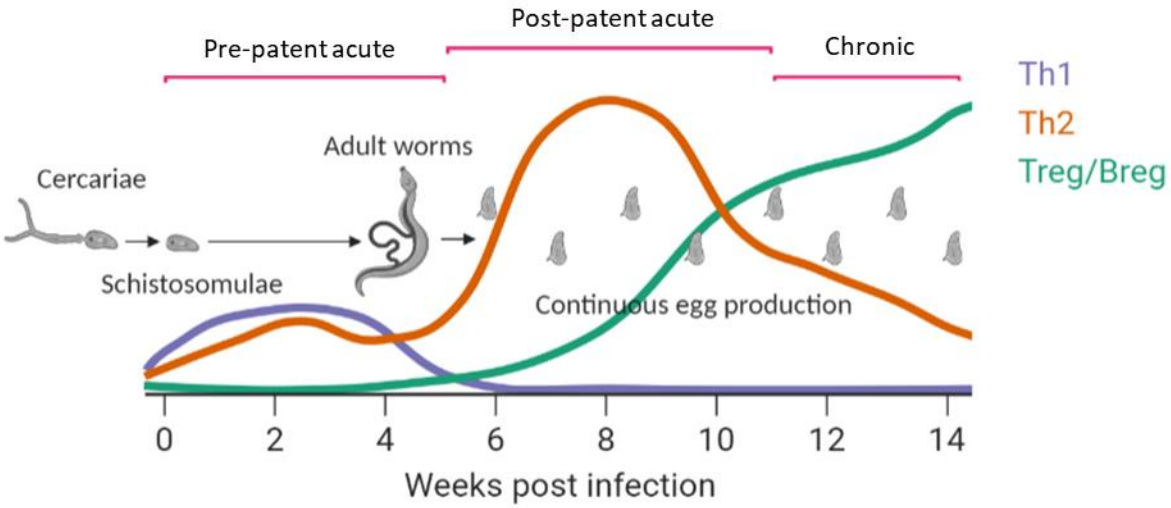

Diagram 2 – The immunological profile of schistosomiasis.
Over the course of S. *mansoni* infections, the host's immune response progresses through at-least three immunologically distinct phases: pre-patent acute, post-patent acute and chronic disease, each of which reflect antigenically distinct lifecycle events. Pre-patent acute disease refers to the first 4-5 weeks of infection, during which the host responds to lung-migrating schistosomula and developing worms in the form of a mixed, low-level T Helper 1 (Th1) /Th2 response, with neither response dominating. The immune environment takes a dramatic turn at week 5/6 (post-patent acute disease): the emergence of a vigorous Th2 response with concomitant down regulation of the earlier Th1 component. This shift is triggered by the release of hundreds of eggs by worm pairs, with eggs becoming the focal point of intense Th2-biased granulomatous inflammation as they traverse host tissue. As infections continue into chronicity the host enters a more regulated state as typified by reduced Ag responsiveness and the expansion of regulatory cell circuits, including Tregs and Bregs. Egg production persists during this period, but with diminished Th2 attributes, reduced lymphocyte activation and shrunken granulomas. A subset of infected mice or people that, failing to achieve the optimal Th1/Th2/Regulatory balance, succumb to advanced hepatosplenic disease, linked to Th1/Th17 responses. The timeframe depicted is based on murine infection and difficult to assess in humans, in which kinetics will likely differ according to prior exposure, anthelminthic administration, and whether the individual was exposed to parasite Ags *in utero.* Adapted from[115].

Rethinking disease terminology

The transition from acute to chronic disease is generally described as an immunological switch: from Ag hyper-responsiveness to Ag hypo-responsiveness[116–118]. While this terminology has been in play for decades, a recent review[80] has brought its simplicity and overgeneralisation into question. Notably, there exists much overlap between acute and chronic phase schistosomiasis in terms of pathology, and by demarcating the entire first three months of infection as 'acute', this terminology fails to distinguish between the immunologically different periods of pre- and post-patency.

The transition between acute-chronic disease has been allocated a cut-off point of 10-12 weeks post infection[80]. While this classification can be easily applied to experimental infections, it is not so straight forward in naturally infected populations. Population-based studies, although irrefutably

valuable to the study of disease transition[116–119], lack resolution due to their inability to pinpoint infection timing, dose and number of previous exposures[116]. As such, the window of acute disease is hazy and some research groups describe an additional 'subacute' timepoint, referring to the time between acute and chronic disease[116,120]. Adding another layer of complexity, it is likely that prior exposure, whether direct or *in utero*[121,122], alters these kinetics.

Although there exist many immunological discrepancies between mice and humans[98,123], murine infections provide the resolution that human studies lack. Importantly, murine models highlight that within the first 10-12 window of 'acute' disease, there are two immunologically distinct phases that can be separated by egg deposition. Specifically, while the first 4-5 weeks of prepatent infection is hallmarked by a mixed low-level, Type 1/ Type 2 response[124], the period after egg production is characterised by a vigorous Type 2 reaction that peaks around week 8-10 before subsiding in scale[125–127]. Importantly, this patent 'acute' phase harbours many hallmarks standardly associated with chronicity. For instance, Treg populations are evident as early as 8 weeks post infection[128], and although reduced Th2 cell populations only becomes 'persuasive' during week 12-15 of infection, some of these features can be visualised shortly after egg production[129]. Taken together, perhaps the schistosomiasis glossary should be altered to include 'pre-patent acute' and 'post-potent' acute phases of disease, where the latter specifically refers to the period immediately following egg release and before schistosomiasis associated immunodepression becomes convincing.

Pre-patent acute disease

The initial acute phase of disease arguably refers to the first 4-5 weeks of infection, in which egg production has yet to commence and the host responds to lung migrating schistosomula and developing worm pairs. Primary exposure often results in 'Katayama fever', a febrile syndrome whose name originates from the first reported S. *japonicum* case in 1904, Katayama District, Japan[66]. Typical disease symptoms include fever, night-sweats, headache, general malaise, and gastrointestinal symptoms, which generally develop and subside within 2-10 weeks of infection[130,131]. Katayama fever is commonly considered an immune reaction to migrating and maturing schistosomulae[80]. Indeed, individuals voluntarily infected with male schistosomes still develop Katayama fever despite the lack of eggs[132]. However, it is likely that Katayama fever also has an egg-driven component given that gastrointestinal symptoms are common, and symptoms may develop after egg production begins.

Travellers are far more susceptible to Katayama fever than individuals living in endemic areas[133]. This observation is popularly explained by the concept of prenatal and sensitisation, in which the *in*

utero transfer of schistosome Ag from mother to child may affect immunity to subsequent infections[33,34,134]. However, this hypothesis does not explain why Katayama fever is more common in S. *japonicum* than infections with S. *mansoni* and S. *haematobium*[66,135] irrespective of traveller status. Perhaps these species-specific differences reflect a higher cercarial exposure density for S. *japonicum*[135]. However, since snails shed S. *japonicum* cercariae at a much lower rate than the other two species[53], these differences in Katayama susceptibility can more likely be explained by the rapid tissue migration kinetics of *S. japonicum* schistosomulae[54], earlier onset of patency[97] or the capacity of adult worms to produce approximately 10 times more eggs per worm pair. Moreover, as S. *japonicum* has the largest host reservoir spectrum of any *Schistosoma spp.,* it may not be as well adapted to human hosts as *S. mansoni* and *S. haematobium*[58].

The low level of Katayama fever in S. *mansoni* endemic regions means that pre-patent acute disease is relatively understudied in comparison to later infection stages. The bulk of older literature in mice and humans suggests the dominance of Type 1 responses during pre-patent acute disease[125,136–140]. For instance, circulating TNF levels are higher in acutely infected persons[139], and during prepatent infection stages, Ag-stimulated mice splenocytes produce considerably lower levels of Th2-associated cytokines (IL-4 and IL-5) in comparison to Th1 (IL-2 and IFN-y)[125,126]. Ag-stimulated PBMCs from individuals with acute disease (i.e newly exposed and egg negative have a greater propensity to secrete pro-inflammatory cytokines such as TNF and IL-6[139]. However, it is unclear whether the Ags chosen in this study (vaccine candidates) are representatives of how the host would respond in a natural infection[83]. More recent studies in human and murine infections have suggested that a mixed Th1/Th2 response is mounted towards *S. mansoni* larvae[124,132,141,142]. Specifically, concurrent measurement of the production of $CD4^{+}$derived IFNγ and IL-4 in the lung draining lymph nodes of infected animals, Redpath and colleagues show IL-4-producing Th2 cells to predominate during early periods of infection (d7, 14 and 21)[124]. In addition, in voluntarily infected individuals, the production of Ag-specific Th1 and Th2-associated cytokines at 4 weeks post infection was shown relatively equivocal[132]. Finally, substantiating the presence of a Type 2 response during pre-patent acute disease, a large fraction of those infected develop blood eosinophilia between the first 4-6 of infection[143].

Systemic regulatory cell induction is not a trademark of pre-patency[116,124,128]. This suggests that i) schistosomula Ags are poor evokers of regulatory cell circuits and/or ii) regulatory cell recruitment cannot be justified by the *minimal* pathology evoked by larval migration. However, in terms of

dermal immunity, cercarial-invasion / schistosomula interaction has shown to evoke a *local* anti-inflammatory environment, rich in IL-10 and regulatory Ag presenting cells (APCs) [144,145].

Post-patent acute disease and granulomatous inflammation

In endemic regions, many infected individuals 'skip' symptomatic acute disease, and instead progress directly into the post-patent acute stage of disease, that starts approximately 5-6 weeks post infection / the start of egg deposition[125,126]. Through the implementation of various evasive techniques (e.g. tegument regeneration, molecular mimicry, manipulation of host immune responses) and due to their considerable size, adult worms are somewhat impervious to immune attack[146]. Schistosome eggs, on the contrary, are highly visible and vulnerable to the host immune system. During their obligatory transit across the intestinal wall or inadvertent entrapment within host tissues, schistosome eggs evoke a stark Type 2 response that dwarfs the earlier acute Type1/Type 2 reactions in comparison[125,126]. This immunological shift is hallmarked by eosinophilic and basophilic expansion, proliferation of Th2 cells, increased production of Th2-associated cytokine (IL-4, 5 and 13), isotype class switch towards IgE, IgG1 and IgG4 (not present in mice), and the polarisation of macrophages towards and alternatively activated phenotype[71,125,126,140,147–149]. The earlier Type 1 components are counteracted, and regulatory responses are still relatively negligible[129]. Clinically, infected individuals do not typically present with overt disease until later stages of infection. Mice on the other hand, develop a syndrome that resembles many aspects of human hepatosplenic disease, including liver and spleen enlargement, oesophageal varices, and portal fibrosis, albeit timing is cercarial dose and mouse strain dependent. To reiterate, this 'immunological disease' is provoked by the misplacement and entrapment of schistosome eggs within host tissues, and the overarching behaviour of host immunity directed against them.

Individual eggs become surrounded by a range of conglomerate of immune cells (eosinophils, B cells, T cells, basophils, macrophages and neutrophils), fibroblasts and extracellular matrix, creating a circumoval granuloma (Diagram 3), whose frequency and spatial organisation varies over the course of infection (i.e early or late stage granulomas), according to the tissue in question and the age and maturity of the egg[150,151]. These inflammatory bodies typically peak in magnitude at 8-10 weeks post infection before gradually declining in size[150]. Entrapped eggs, failing to exit the host, are eventually engulfed by the granulomas and leave behind a congested and fibrotic, calcified lesion. As previously mentioned, granulomas serve a protective function for the host while also benefitting the parasite, shielding proximal tissue from egg-derived molecules (such as Omega-1[152,153] and IPSE/alpha-1[153,154]), whilst enigmatically facilitating the movement of eggs across host tissue. However, if these

reactions are disproportional in terms of Th1/Th2/Th17 regulatory balance, life threatening hepatosplenic disease may develop.

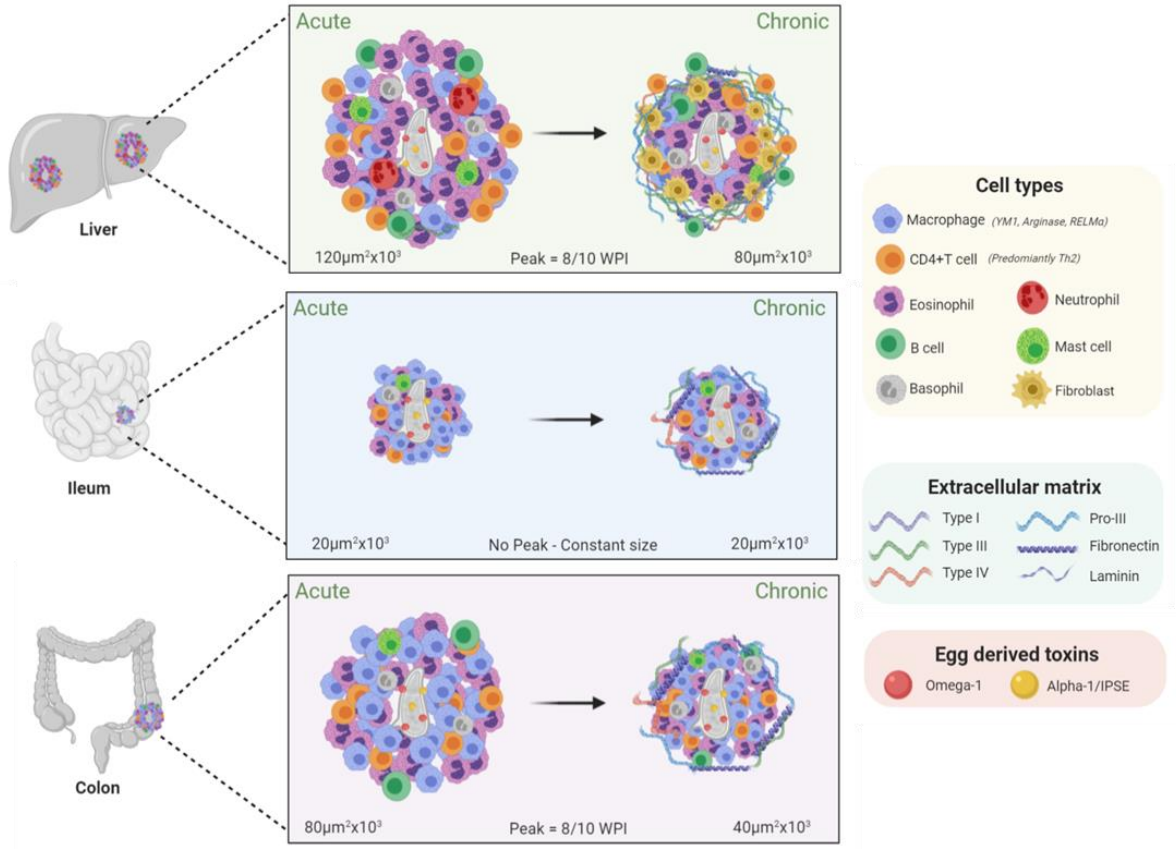

Diagram 3 -The Granuloma. Granulomas serve two functions: the encapsulation or sequestration of egg-derived molecules from host tissue and enabling egg transit across the intestine[63]. Over the years, granuloma structure and cellular composition has been characterised via variety of approaches, including standard histology and immunohistochemistry on whole tissues sections, as well staining of enzymatically dispersed granulomas[126,150,151,155]. These studies have revealed large differences in granuloma composition depending on the tissue in question and disease time-point[150,151]. Granuloma composition also alters drastically between schistosome species[97], including dense neutrophilia and marginal eosinophilia in hepatic S. *japonicum* granulomas, with the opposite trend observed for S. *mansoni*[156]. These disparities could be explained by differences in egg production (released individually or in packets) and/or the range of molecules they secrete[66,97]. The granulomas depicted in this figure represent those of *S. mansoni.*

In comparison to the liver, there is a paucity of information on intestinal granulomas beyond limited histological studies, reflecting the technical challenge of isolating live cells from intestinal tissue. At the same time, the complex architecture of the intestine, alongside the variable pattern of egg deposition, makes it more arduous to locate and characterise these inflammatory reactions. One curious observation from intestinal studies is the striking difference in granuloma composition between the large and small intestine. For example, while colonic granulomas reach a peak size of ~80µm^{2}x10^3 between weeks 8-10, ileal granulomas do not modulate over the course of infection and remain ~20µm^2 x10^3 throughout infection (based on murine work)[151]. This disparity may reflect basal immune cell populations and/or varying levels in antigenic/bacterial load, mucus, changes in pH or other aspects of small vs large intestinal environments.

Liver granulomas are marginally larger than colonic granulomas, and considerably greater than ileal[151]. Population wise, acute-stage hepatic granulomas contain dense populations of eosinophils, with an outer rim of B cells and T cells, and

diffusely spread macrophage populations[150]. Neutrophils, mast cells and basophils are present, but in marginal numbers. In comparison, both acute ileal and colonic granulomas are densely populated with macrophages, with a concurrent decrease in eosinophil numbers. No outer lymphocyte lining is present, and ileal granulomas are virtually devoid of B cells, which could reflect a lack of B cell basally.

Both colonic and liver granulomas peak in magnitude between 8-10 weeks post infection, followed by a gradual decline in size and increase in collagen deposition[151]. Ileal granulomas also enhance collagen deposition during later stages but remain a constant size throughout infection[151].

Late-stage granulomas vary highly in terms of extracellular matrix (ECM) density and constitution[157,158]. Hepatic granulomas contain an inner core of immune cells, that is encapsulated by a rim of ECM and fibroblasts, and a further exterior lining of newly infiltrated immune cells. The ECM is rich with Type I and Type II collagen, pro-collagen and patches of fibronectin. Ileal and colonic granulomas have substantially less ECM in comparison to their hepatic counterparts, but relatively similar levels in comparison to each other. The greater levels of ECM surrounding hepatic granulomas likely reflects the greater number of liver-resident cells who are capable of fibrinogenesis. By contrast, it is unsure which cell types contribute to ECM laying within the intestine, but it is likely newly recruited macrophages.

When placed in the context of successful egg migration or entrapment, these ECM deposition patterns are fascinating. It is interesting to consider that transiting eggs may harbour greater collagen degrading mechanisms, that contribute to egg movement or may signify successful transit across the wall[159].

Of note, from **6** weeks post infection, eggs can also be found within lung tissue. These granulomas are most akin to ileal granulomas, with respect to their lack of ECM[160,161].

Our first immunological insight into the factors governing active disease was provided by a series of seminal papers by Warren and Colleagues in the 1960s and 1970s[162]. Here, through the employment of unisexual and bisexual infections, with or without anthelminthic administration, the group showed that egg-driven inflammatory and fibrotic reactions (granulomas) are the driving force behind hepatosplenic disease. Soon after, certain egg-derived molecules (omega-1 and IPSE/alpha-1) were shown partially culpable for this damage[152,153], and worm-derived regurgitation products (CAA and CCA) were shown to accelerate the reactions[163,164]. A list of known schistosome-derived immunomodulators is provided in Table 2.

Since these early studies, researchers have used a variety of infection models (e.g bisexual or unisex infections and pulmonary granuloma models) and/or transgenic mouse strains to dissect the immunological contribution of various cell types, chemokines and cytokines to granuloma development and host disease. A series of independent studies in the 1990s and early 2000s, highlighted that egg excretion is highly reliant on intact adaptive immunity, where in $CD4^+$ T cell deficient or nude mice, egg excretion is impaired and granulomatous inflammation is stunted[69,165,166]**.** Cytokine-wise, IL-13 appears the main culprit of fibrogenesis[69,167], mediating its effects through hepatic stellate cells[168], with limited support from TGF-β[169] and IL-33[170,171]. IL-5 adds to the Th2 orientated environment by driving eosinophils recruitment, but neither these cytokines

nor cell type are crucial to egg excretion or host survival[147]. Interestingly, although Il-4 and IL-13 are closely related cytokines that share some overlap in their biological activity, these two cytokines have distinct and contrasting roles in schistosomiasis pathology[69]. Notably, while abolishment of IL-13 signalling improves disease prognosis and reduces overwhelming fibrogenesis, elimination of IL-4 results in a severe wasting disease in which mice succumb to infection during around the 7 week timepoint[69]. Death is associated severe Th1-biased intestinal and hepatic pathology, enhanced levels of reactive oxygen species, cachexia and septicaemia. This phenotype is further exacerbated in double deficient IL-$4^{-/-}$ IL-$10^{-/-}$ mice[172,173], which demonstrates an essential role for IL-10 in counteracting the Type-1 dominated inflammation. Moreover, an important point to make is that while both IL-13 and IL-4 both contribute to the inflammatory and fibrotic environment, neither cytokine are necessary for granuloma formation[69].

Type 1 / pro-inflammatory			
Worm derived		Egg derived	
SjP40	Enhances Th1 cytokine production and limits induction of Th2 cytokines. Simultaneously alleviating AAI model[174]	smHMGB1	Induces macrophage production of pro-inflammatory cytokines[175]
		Smp40	Promotes cytokine secretion, entirely of the Th1 type[176]

Type 2			
Worm derived		Egg derived	
CAA and CCA	Regurgitated by adult worms into the blood stream. Primes and accelerates egg induced granuloma formation[164]	Omega-1	T2 ribonuclease that conditions DCs for Th2 priming. Enhances macrophage IL-1β secretion and promotes insulin sensitivity[177–179]
Hemozoin	Regurgitated digestion product shown to modulate patrolling macrophage function[180]	IPSE/alpha-1	Promotes IL-4 production from basophils via IgE binding, reduces production of pro-inflammatory cytokine production from macrophages [181]
		PGE2	Binding of SEA to Dectin 1&2 on DCs promotes PGE2 expression. Enhances Th2 polarisation[182]

Regulatory / anti-inflammatory			
Worm derived		Egg derived	
Cyclophilin A	Modulates DC function leading to preferential Treg induction[183]	SmCKBP	Chemokine binding protein that reduces neutrophils migration via inhibition of CXCL8 [184]
Lyso-PS	Activates DCs via TLR2 binding, encouraging DC-mediated Treg induction[109]	Sj16	Anti-inflammatory and antifibrotic effects [185]
Haemazoin	Regurgitated digestion product shown to modulate patrolling macrophage function[180]	LNnt	Enhances anti-inflammatory cytokine secretion and inhibition of CD4+ T cell proliferation[186]
PIII, SM22.6 and Sm 29	Inhibits AAI. Pathway unknown[187]	ISPSE/alpha 1	Enhances B cell IL-10 production and their capacity to induce Tregs[188]

Table 1. List of worm or egg derived products with known immunomodulatory functions

The biological overlap between IL-4 and IL-13 is in part due to their receptors (type I or Type II IL-4 receptor respectively) sharing a common IL-4 receptor alpha chain (IL-4Rα). Macrophages are amongst the many cell types who express these receptors and can be alternatively activated (AA) upon ligation with these cytokines[189]. In the early 2000s, macrophage-specific expression of IL-4Rα was shown essential for host survival[71]. Specifically, Il4Rα$^{-/lox}$lyz^{2Cre} mice, who lack IL-4Rα on macrophages and neutrophils, develop a severe disease phenotype that closely resembles that of IL-4$^{-/-}$ mice, including 100% mortality early after egg onset and type-1 biased intestinal inflammation. This severe phenotype was attributed to deficits in macrophage function but not neutrophil function, as antibody mediated depletion of granulocytes failed to recapitulate the lethality[71]. However, since this seminal study, conflicting results have brought the essentiality of IL-4Rα into question[190,191]. Notably, using the same Il4Rα $^{-/lox}$lyz^{2Cre} mice strain, two separate research groups failed to reproduce the severe lethality described above, and have shown IL-4Rα signalling as dispensable in monocyte recruitment and subsequent conversion to macrophages in infected hepatic tissue[190]. It has been suggested that the lack of coherency between early and late studies may be explained by different levels of Lyz2 expression within immature (lyz^{lo})and mature (lyz2^{hi}) macrophages populations, and thus subsequent resistance to LysMCre-mediated deletion and incomplete IL-4*Rα* signalling ablation[191]**.** In addition, it is tempting to suggest that factors within the mouse-house (i.e intestinal microbiota structure) may have a large bearing on disease severity. Indeed, in the earlier seminal study, administration of antibiotics was shown to extend mouse survival time[71]. Irrespective of the incoherency described above, a persuasive line of studies indicate that several AA macrophage-associated signature genes/mediators are essential in the regulation of granulomatous inflammation[192–194]. Importantly, while Arginase-1 (Arg-1) and Resistin-like molecule (Relm)-α are commonly regarded as facilitators of Type 2 inflammation, their depletion during schistosomiasis or models of pulmonary granulomas leads to accelerated fibrosis, exacerbated inflammation and enhanced Th2 responses[192–194]*,* providing them with roles in the negative regulation of Type 2 immunity and tissue repair. Indeed, recent studies have shown RELMα to directly impair Th2 responses through the endorsement of Treg proliferation[195]. Finally, schistosomes have shown to support alternative activation of macrophages, including RELMα expression, through the regurgitation of the haem detoxification product, hemozoin[196].

While there has been a focus on macrophages and CD4+ T cells, other cell types are critically involved in the governance of granulomas, egg excretion and active disease, including DCs. DCs are versatile population of APCs, whom thanks to their vast array of pattern recognition receptors

(PRRs), inner processing machinery and unique migratory patterns, are adept orchestrators of naïve T cell activation and differentiation. In response to SEA and certain endogenous host molecules (such as Type-I IFNs[197]), DCs predominantly guide T cells towards a Th2 polarised state[198]**.** The SEA component omega-1 appears as the most potent instructor[179,199], but with other omega-1-independent Th2 priming mechanisms aknowleged[182,200]. DCs are broadly defined by their high expression of CD11c and MHC-II, but also express a wide range of co-stimulatory and co-inhibitory molecules that impact the outcome of schistosomiasis[201] and Th2 priming[202]. Global depletion of DCs is difficult to achieve due the wide-spread expression of $CD11c^+$ and MHC-II on other cell types. However, thus far, $CD11c^+$ depletion at the onset of egg production suggests DCs to be critical initiators of hepatic Th2 responses during schistosomiasis[203]**.** DCs can be further divided into plasmacytoid DCs (pDCs) and Type 1 and Type 2 conventional DCs (cDC1s and cDC2s), based on their function, morphology, and surface marker expression. In addition, in scenarios of inflammation and immunosuppression, populations of inflammatory or regulatory-like DCs may also emerge[204,205]. Data from our lab has shown that different DC subtypes respond to schistosome infection depending on the tissue bed and timepoint in question[197,206]. For example, during acute disease (week 6) both cDC and pDCs numbers increase within the liver, but only cDCs are uniquely licenced to support effector $CD4^+$ T cell responses and maintain Th2 immunity[206]. Whilst in the intestine draining MLNs, depletion of pDCs at weeks 4-6 or 6-8 impairs Th2 cytokine production[207]. Moreover, in addition to supporting effector T cell responses in the MLN during early disease, pDCs reinforce Type 2 cytokine production in the liver at later disease stages[207]. In terms of cDC subsets, IL-12 production by cDC1s has shown to restrain Th2 cytokine production and granulomatous inflammation[208], while IRF4-expressing cDC2s appear to do the opposite[209]**.** Very intriguingly, patent S. *japonicum* infections have shown to induce a novel Mar-1 expressing DC subset (potentially an inflammatory cDC2 subset[204]) whom are adept at guiding Th2 differentiation in an IL-4 dependent manner[210]. However, as DCs are infamous for their inability to produce IL-4, and thus instruct Th2 polarisation via IL-4-independent means, this particular subset requires closer interrogation / further validation[198,211]. The application of more refined targeting approaches, that allow for the depletion, tracking or enrichment of specific DC subsets will allow us to extensively characterise the contribution of DCs to active schistosomiasis.

Like DCs, B cells are capable of presenting Ags, producing cytokines, and influencing T cell function, but their main speciality is antibody production. Early after egg onset (week 7), granuloma formation appears somewhat equivocal between WT and B cell depleted mice, indicating that neither antibodies nor any other B cell effector function is required for granuloma formation or protection

against hepatoxic egg-derived molecule[212]. This finding makes sense given that early-stage granulomas are infrequently dispersed with B cells[151]. However, at chronic stage of infection, B cells appear necessary for the downregulation of granulomatous inflammation and prevention of bystander tissue pathology[212–215]. This sudden demand for B cell presence corresponds to the time point at which B cells have shown to infiltrate granulomas and create an outer lining that surround ECM-producing hepatocytes[150]. The signals that regulate hepatic humoral immunity include IL-4Rα[214] and IL-10 signalling[215], with additional contribution from parasite derived molecules themselves[188,216]. Moreover, optimal Th2 cell proliferation and cytokine production has shown dependent on signals emanating from B cells[217], immunoglobulins appear essential in the prevention of severe pulmonary disease[215], and as discussed later, IL-10 producing Breg populations emerge during chronic infection stages[29]. While schistosome induced Bregs have shown to provide relief against experimental airway inflammation[41], and IL-10 signalling has shown to orchestrate the hepatic B cell compartment[215], the specific role of IL-10 producing B cells in the downmodulation of granulomatous pathology has yet to be revealed.

In contrast to $CD4^+$ T cells, $CD8^+$ T cells do not appear to have a critical role in regulating exuberant host responses during schistosomiasis. While early depletion studies implicate $CD8^+$ T cells in the suppression of $CD4^+$ T cell responses in chronic infection stages[218], subsequent studies show $CD8^+$ T cells, and their derived IFNγ production, as dispensable in the regulation of tissue pathology[219] .

Basophils and neutrophils receive relatively little attention during active schistosome infections. Basophils contribute to the IL-4 and IL-13 pool in response to egg-derived IPSE[181,220]. However, through use of basophil deficient mice, protection against fatal schistosome infection was shown independent of basophil derived Il-4/13[148]. Regarding eosinophils, while these cell types dominate hepatic granulomas (and to a lesser extent intestinal granulomas), their depletion neither influences host survival or egg excretion[221]. It has been speculated that eosinophils may serve to destroy miracidium developing within the egg, which may account for the greater proportion of eosinophils in hepatic granulomas; i.e. eggs are stuck within the liver, and thus, a greater proportion of eggs may develop to maturity. Interestingly, neutrophils are infrequent members of S. *manson*i granulomas, but dominate those generated during S. *japonicum* infections[66]. This disparity likely reflects differences in egg-produced molecules, including SmCSKP which can inhibit neutrophil function and recruitment[222]. Mirroring their infrequency, neutrophils don't appear to participate in granuloma development. Finally, there are currently undefined roles for ILCs, but given that

depletion of ILC activating alarmins has a negligible effect on disease prognosis[171], ILCs are unlikely to have a significant role in granuloma development or associated pathology or resolution.

Although Type 1 responses decline in scale during active disease, these responses are critical, nonetheless. Notably, IFNγ is essential to the counteraction of Type 2 responses and prevention of Type 2 biased immunopathology[172,173,223–225] , and likely mediates its effects through macrophage produced Indoleamine-pyrrole 2,3-dioxygenase (IDO) and reactive oxygen species[226,227], paying specific attention to iNOS[155]. TNF on the other hand has shown to participate in the late recruitment phase of granulomas and provides vital signals for worm reproductivity and egg excretion[223,228]. Moreover, it important to note that severe disease exacerbation is associated with defective IFN-γ signalling[90,229], and dual blockade of both IL-13 and IFNγ in concert appears a therapeutically viable strategy for the alleviation of fibrosis[230].

Chronic infection

In the absence of anthelmintic administration, individuals may develop longstanding chronic infections, with fecund worm pairs living an average of 3-10 years within their host[231]. In individuals that are continuously exposed to snail infested water, worm numbers gradually decline over time due to partial immunity to new infections alongside concomitant natural death of parasites[66]. Egg production persists over this period, but due to the decrease in worm numbers, egg deposition, entrapment and excretion rates also decline. As infections progress into chronicity, lymphocytes enter a state of hyporesponsiveness[116,119,129], the vigorous egg specific Th2 responses diminishes in scale[129], new granulomas decline in magnitude and previously formed granulomas are resolved and replaced by fibrous tissue (scaring)[127,151]. This immunological downregulation reflects the recruitment of various regulatory cell networks (such as Bregs and Tregs) and lymphocyte dysfunction, including exhaustion or anergy. To reiterate, these chronic infections are not completely devoid of pathology, where persistent Ag stimulation and inflammation may lead to enhanced risk of co-infections, carcinogenesis, or various morbidities[68]. However, due to immunological downregulation, the majority of infected persons remain relatively asymptomatic and only a subset of infected individuals progress into severe, life threating disease[85–87].

Diminished lymphocyte proliferation during chronic schistosomiasis was first reported in the late 1970s[116,118], where in comparison to individuals with acute disease, chronically infected patients were essentially non-responsive to the same parasite Ag stimulation[116]. Shortly thereafter, a partial explanation for these observations was provided in the form of ‘Adherent suppressor/phagocytic’ cells, which were shown to diminish lymphocytes proliferative responses to mitogens and

schistosome-specific Ags[119,232]. Around the same era, adoptive transfer studies revealed that splenocytes from chronically infected mice are far superior at supressing granuloma reactions than splenocytes from acutely infected animals[233], and clinical and experimental studies revealed the presence of suppressive T cells[234] , parasite-derived factors[235–237] serum factors[236] and phagocytes[238]. Since these historic studies, our understanding of schistosome-mediated immunodepression has progressed tremendously, with a large emphasis on the recruitment of Tregs and Bregs, and their production of anti-inflammatory IL-10.

A variety of studies have demonstrated the importance of IL-10 in the regulation of schistosome-driven pathology and suppression of exaggerated Th1/Th2 responses[172,173,239]. Namely, mice deficient in IL-10 generate a mixed Th1/ Th2 response, that persists throughout chronic disease and leads to severe liver damage and rapid mortality kinetics[239]. Very interestingly, adoptive transfer studies reveal that while a large proportion of IL-10 is generated by $CD25^+$ Tregs (as opposed to Foxp3+ Tregs), a substantial proportion of IL-10 comes from a non T cell source[240].

Bregs are an immunosuppressive cell types that play critical roles in the governance of immunological tolerance and prevention of chronic inflammation[241]. Contrary to the well-established role of B cells in antibody production, Bregs predominantly mediate their immunosuppressive effects through the provision of anti-inflammatory cytokines such as IL-10, TGF-β and IL-35 (Reviewed in [241]). In addition, Bregs are draped in a vast array of immunosuppressive surface markers (e.g Tim-1, CD95, CD5 and CD1d) and are capable of promoting regulatory T cell (Treg) differentiation and suppressing the proliferation and/or the effector functions of pathogenic lymphocyte populations. Breg suppressive function appears highly context specific[241], with Bregs acquiring a different arsenal of effector functions depending on the insult encountered. Similarly, there is no unified consensus regarding Breg phenotype, with various Breg appearances emerging depending on the tissue-bed and inflammatory insult in question[241]. In addition, unlike Tregs who are typically identified via Foxp3 expression, there is no Breg-defining lineage transcription factor. Instead, Bregs are standardly defined by their capacity to produce IL-10, albeit, with the large caveat that many Bregs work independently of IL-10[241]. B cell IL-10 production is encouraged by an array of signals, including TLR, BCR and CD40 engagement, inflammatory cytokines, microbial signals, and parasite derived molecules[188]

IL-10 producing Bregs are visualised in live infections with egg-producing worms, or eggless, male worms alone[25,29,30,32], and can be encouraged by stimulation with schistosome-derived molecules

such as LNFPIII[242] and IPSE/alpha-1[188]. Importantly, Bregs are not restricted to murine models, with schistosome infected persons also demonstrating heightened numbers of IL-10 competent B cell popuations[42]. Moreover, the immunoregulatory capacity of both Tregs and Bregs is not exclusively reliant on IL-10 production. For example, in human infections, Bregs-like populations express IgG4 and LAP, suggesting a role for neutralising antibodies and TGF-beta activation in their effector arsenal[243,244].

The specific signals that encourage regulatory cell induction during schistosomiasis are relatively ill-defined. In terms of Bregs, these populations are likely to arise in response to the inflammatory environment established by infection, given that many inflammatory cytokines and apoptosis related signals have shown to promote Breg numbers and functionality [245,246]. In addition, the egg-derived molecule IPSE/ alpha-1 has shown to competently drive Breg expansion in an *in vitro* and *in vivo* setting[188], and factors within the adult worm secretome likely contribute as Bregs are visualised in eggless infections with male worms alone. Regarding Tregs, SEAs have shown to enhance $CD4^+$ Foxp3 expression[247] and the male-worm-derived molecule cyclophilin also supports their expansion[183]. As discussed later on, various studies hint that intestinally-derived cues, including those from commensal bacteria, may participate in the expansion of regulatory cell circuits.

In addition to the expansion of Tregs and Bregs, the immunosuppression observed during chronic schistosomiasis may partially result from lymphocyte dysfunction, including exhaustion. Persistent Ag stimulation of T cells leads to progressive loss of effector function, and their entrance into a functionally hyporesponsive state[248]. These exhausted T cells are not completely unresponsive, but rather their functionality is subpar. This could very well lead to an inability to completely clear the pathogen, but incidentally, prevent detrimental immunopathology. In murine schistosomiasis, hyporesponsive T cells have an intrinsic incapacity to produce Th2 cytokines (IL-4, 5, and 13) and are hyperproliferative towards Ag stimulation[129]. Interestingly, in these mice infections diminished responsiveness is not associated with inhibitory markers commonly associated with exhaustion (such as PD-1, CTLA-4 and LAG-3), but rather enhanced expression of the anergy-associated gene GRAIL[129]. In addition, studies indicate that growth factor deprivation (e.g IL-2) and subsequent apoptosis by neglect, is an alternative means by which $CD4^+$ T cell responses are controlled[249]. T cell dysfunction during schistosomiasis is likely driven by persistent Ag exposure, combined with the direct activity of schistosome derived products on T cells, or modulation of Ag presenting cell activity. For example, worms have shown to select for PD-L1 upregulation on macrophages[108], which

induces $CD4^+$ and $CD8^+$ T cell anergy, and schistosome-specific phosphatidylserine modulates DC function in such a manner that get enhanced Treg inducing potential [109].

SCHISTOSOMES AND THE INTESTINE

The intestinal interface

With a surface area approximately the size of a badminton court[250], the gastrointestinal tract represents the largest interface between us and the external environment. This includes exposure to trillions of luminal dwelling microbes (the microbiota), food derived Ags and toxins. The intestine has been carefully engineered to deal with this enormous Ag burden in the form of many chemical, mechanical and immunological defence mechanisms (reviewed here[251,252]). Structurally, the intestinal wall encompasses several superimposed layers (mucosa, submucosa, muscularis propria and serosa), with a specialised monolayer of intestinal epithelial cells (IECs) lining the forefront, which are specially arranged into multiple crypts and villi. The majority of IECs are absorptive cells (enterocytes), that promote food digestion and water and nutrient uptake. In addition, enterocytes are able to sense the environment, communicate with underlying immune cells, and importantly, their tight alignment creates a semi-permeable barrier that stringently regulates the movement of substances across the intestinal wall. While small molecules (<300 Da), electrolytes and nutrients may passively cross the barrier through a series of selectively permeable membrane pores (transcellular movement; within a cell) or by a series of protein complexes (tight junctions, adherins, desmosomes and intracellular junctions) that interlock adjacent IECs (paracellular movement; between cells). The movement of larger macromolecules is restricted. During intestinal homeostasis, this barrier effectively permits nutrient absorption whilst preventing the translocation of intraluminal material. The rapid regenerative rate of enterocytes further permits healing upon intestinal injury.

This physical barrier is chemically fortified by an overlaying mucus layer, that helps trap perturbing microbes, provides a nutrient rich environment for commensals, and aids in the delivery of soluble Ags from the lumen to underlying immune cells[253]. This complex web of mucin is provided by specialised IECs (goblet cells) who are found interspersed amongst enterocytes. At the base of small intestinal crypts columnar Paneth cells are found, that secrete a variety of microbial peptides into the lumen, which may become entangled in the mucin overlay. A somewhat neglected IECs is the tuft cell, whose unique chemosensory properties make it adept at detecting and instigating immune responses against helminths[254]. And further, microfold cells (M) are specifically structured and positioned for the uptake and transfer of luminal Ags to proximal immune cells. A third layer of

defence is provided by a vast population of immune cells that predominantly reside within the underlying mucosal lamina propria, but also within organised lymphoid structures (such as the mesenteric lymph nodes, Peyer's Patches, isolated lymphoid follicles or clusters) or randomly dispersed throughout the intestinal epithelium. These immune cells skilfully discriminate between harmless Ags and potential threats and coordinate with IECs to generate a given effector response. Finally, epochs of coevolution have made the microbiota an essential participant in intestinal homeostasis, where luminal bacteria, IECs and immune cells must carefully communicate and coordinate with each other to maintain a harmonious intestinal ecosystem.

Schistosome infection associated microbiota and host immunity

The gastrointestinal tract contains trillions of microbial organisms (including bacteria, fungi and viruses), who vary in abundance, pathogenicity, and function. Microbiota presence is vital for mammalian health, with the vast array of microbes collectively supporting normal gut architecture, training and stimulation of host immunity, and overall homeostasis[255]. The structure of the intestinal microbiota is intricately linked to host health, normal immune function, and parasite development, vice versa[256,257]. For example, childhood perturbations in microbiota structure (i.e via changes in hygiene practices or antibiotic consumption) lead to elevated susceptibility to immune mediated disorders in later life[258], and mice devoid of a microbiota (GF mice) are typified by exaggerated atopy[259]. Importantly, compositional changes within the microbiota can finely calibrate mucosal immunity, with certain bacterial species specifically supporting Th17 and Treg differentiation[255]. Although alterations in bacterial structure have shown to alter the balance of Th2 to Th17, to our knowledge, there are no studies that show select communities of intestinal bacteria to support a Th2 signature.

Infections with both enteric and non-enteric parasitic worms influence the composition of faecal and intestinal bacterial populations, which in turn, has far reaching effects on the pathophysiology of helminthiasis[255]. For schistosomes, qualitative and quantitative changes in gut microbiota composition have been reported in natural[260–262]and experimental infection settings[263,264], in eggless unisexual infections[263], conventional bisexual infections[260,261,264], and with both urogenital (S. *haematobium*)[260,261] or intestinal parasites (S. *mansoni* or S. *japonicum*)[262–264]. In experimental infections with S. *mansoni*, the most prominent changes are observed during periods of patency[264], which indicates that eggs themselves, or the inflammatory havoc evoked by egg migration, is responsible for these alterations. However, as gut dysbiosis is a feature of egg-free infections[263] and

remote infections with S. *haematobium* worms[260,261], it is clear that the systemic worm-induced response can also influence the gut microbiota.

Only a handful of studies have provided a snapshot into the impact of microbiota composition on host and schistosome health. For example, administration of antibiotics during active disease (weeks 6-8), causes a significant reduction in egg excretion and dampens egg-driven inflammation[265]. Interestingly, the impact of bacterial depletion was more or less exclusive to intestinal granulomas, indicating that commensals play an important role in the perpetuation of local inflammatory responses, and thus, in the mediation of egg excretion. Moreover, antibiotic depletion has shown to extend the survival rate of IL-4ra$^{-/-}$ mice[71], and susceptibility to *S.* mansoni infection (in terms of worm and egg burden) is influenced by the baseline microbiota of the host[266]. Finally, a few historic 'GF studies reveal GF mice respond to schistosomiasis in a milder fashion than conventionally reared mice, with fewer granulomas and a decline in egg output, despite similar worm burdens[267,268].

Akin to other helminth infections, the functional implications of schistosome-driven changes in microbiota structure (composition and metabolites) on host immunity are poorly understood. For example, do schistosome-driven microbial communities prime or reinforce schistosome-elicited immune responses, impact parasite development, or the extent of egg-induced pathology? Does the process of egg migration facilitate bacterial dissemination to local and distal sites? And finally, could a schistosome associated microbiota influence the severity of bystander inflammatory disease?

Thus far, several helminth infections have shown to skew microbiota composition in a manner that modulates the extent of unrelated inflammation[51,269,270]. Through means of faecal transfer, the microbiota of tapeworm (*Hymenolepis diminuta*) infected mice was shown to alleviate DNBS-induced colitis, with alleviative effects attributed to enhanced levels of SCFA (namely butyrate) and resultingly, enhanced IL-10 receptor expression[269]. Complementing this study, *H.polygyrus*-mediated suppression of airway inflammation correlates with enhanced levels of SCFAs, with the protective effect of infection abrogated upon Abx treatment[51]. Aside from assisting in protection against bystander inflammation, previous studies indicate that helminths may guide microbiota structure in favour of their survival and longevity [270]. For example, H. *polygyrus* infections are accompanied by an enhanced abundance *Lactobacillus* species, whose adept capacity to expand Treg networks may permit greater helminth establishment[270].

Intestinal integrity

The integrity of the intestinal interface has a large bearing on health and disease. If the above many defences and cell populations of the intestine are no longer synchronous, the intestinal barricade may become breached and inflamed, with enhanced intestinal permeability potentially ensuing. A 'leaky gut' refers to the escape of luminal Ags, microbes, and their products (pathogen associated molecular patterns; PAMPs) into host tissue, due to defects in barrier defences. This translocation can result in chronic intestinal disease, the modifications of immunological programming, inflammation of local tissues, and also has far reaching distal effects on distant organs that drain and come into contact with translocated luminal material. As such, the reversal of intestinal permeability appears an attractive therapeutic strategy for disease such as IBD, autoimmune hepatitis and Type 1 diabetes[271,272]**.** However, there exists a fine line between detrimental and beneficial gut leakiness, where interactions between luminal Ags and the mucosal immune system are necessary for the perseveration of homeostasis[273]. For example, transient breaches in the epithelial barrier (i.e. via administration of pharmacological agents) render mice resistant to chemically induced colitis[274], with similar results observed in mice with compromised tight junction expression[275] Under conditions of compromised barrier integrity, it is likely that specific elements of the mucosal immune system are activated and evoke compensatory mechanisms as to withstand colitis or other inflammatory conditions [273]

A leaky gut has many possible causes, including diet, alcohol consumption, stress, and infection. These factors may directly modulate barrier integrity (e.g by abrasion of the epithelium) or act indirectly through microbiota alterations[271]. To give a few examples, diets high in saturated fat have shown to alter populations of *Lactobacillus* and *Oscillibacter*, with these microbial changes correlating with enhanced colonic permeability[276]. And burn-induced gut leakiness can be reversed by the receipt of faecal microbiota transplant[277].

How do helminths fit into the permeability equation?

Intestinally dwelling helminths are in direct contact with the host epithelium and gut microbiota, providing them with ample opportunity to breach the intestinal wall and manipulate the intestinal interface. For blood-feeding hookworms, the destruction of the host epithelium is essential for their feeding and thus, survival. Whilst for whipworms, their lifecycle depends on the intestinal microbiota[278] a unique moulting stage with intestinal epithelial cells and their partial embedment within the large intestine wall. There are also many helminth species whose lifecycle continuation is reliant on the movement of their various life-stages across intestinal tissue. To name a few:

S.mansoni, H. polygyrus, *Fasciloa hepatica* and *Ascaris lumbricoides*. Taken together, it makes sense that some helminth infections would be accompanied by a leaky gut. However, proving this hypothesis is not as straight forward as one would hope[256279]. As discussed in later sections, this may reflect the complex tissue restorative mechanisms promoted by worms themselves, as to prevent worsened damage to the host, and in turn promote their own survival.

Enhanced intestinal permeability may benefit both host and helminth. From the host's perspective, increased leakiness may allow for enhanced nutrient uptake, providing the energy demand to deal with infection, or perhaps, allowing the passage of effector molecules (complement, antibodies) or cells, into the lumen to attack the luminal worms. On the contrary, for the parasite, the passage of luminal-derived immunogenic molecules may modulate inflammation and immunopathogenesis, downplaying bystander tissue damage evoked by infection and promoting survival of the host and helminth.

Mechanistically, helminths may physically disrupt the intestinal barrier by latching or burrowing into the epithelium (via hooks, abrasive surfaces, and teeth) or through the harsh breakthrough of their lifecycle stages (i.e eggs) into underlying tissue. In addition, some helminths secrete excretory/secretory (E/S) that are capable of enhancing intestinal permeability[279]. Moreover, it is unknown whether the gut microbiota alterations evoked by helminth infections, may contribute to gut leakiness. This is tempting to speculate given that certain groups of bacteria with known roles in the maintenance of intestinal barrier function (i.e. *Akkermansia muciniphila*) are altered during worm infection[280]

No sepsis during schistosomiasis

One of the more curious aspects of the schistosome lifecycle is the transit of schistosome eggs across the intestinal wall, and its utter reliance on intact granulomatous inflammation[67]. While a large fraction of studies focus on the cellular networks and effector mechanisms controlling granuloma generation, there is a shortfall of studies investigating the reparative mechanisms that counteract egg-driven damage, and potentially, reduce the likelihood of egg-facilitated sepsis.

Intestinal barrier breach has been reported in experimental and natural infections with patent *S. mansoni*[263,281,282]. However, in both settings, septicaemia is not a hallmark of infection. These observations suggest that while schistosome egg migration has a significant impact on barrier integrity, the host and/or parasite applies potent tissue restorative tactics as to mend the intestinal wall and prevent the spread of gastrointestinal content into circulation. Thus far, a viable candidate

has come in the form of the worm-derived cysteine protease inhibitor Sj-Cys (only identified in S. *japonicum* to date)[283–287]. More specifically, this cystatin has shown to limit DC Ag-presentation[283], reduce pro-inflammatory cytokine production from LPS-stimulated macrophages[286], and as a likely result of such immunomodulation, can reduce the severity of sepsis-induced cardiomyopathy[287]. Future studies are required to assess whether other immunomodulatory molecules are secreted amongst Schistosoma *spp*. and determine which cell types mediate their effects. In addition, intestinal macrophages play integral roles in the limitation of bacterial spread and wound repair[288,289], with recent studies demonstrating a role for GM-CSF and cross linking of anti-microbial IgG in this 'macrophage defence program[288,289]. Little is known about the expression of both factors during schistosomiasis, and it would be interesting to interrogate this aspect further. Unfortunately, like the wider helminth field, our knowledge of schistosome-driven intestinal immune responses is stunted by the inability to reliably obtain viable intestinal tissue preps from infected murine tissue[290,291]. This phenomenon likely reflects enhanced apoptosis, alterations in pH and exuberant mucus production in response to helminth infection. Ultimately, to this day, there has been no published cellular data on the cell types that inhabit the intestine during active schistosomiasis, and the bulk of our understanding on schistosome-driven immune regulation has been obtained from imaging based approaches. However, recent studies isolating and characterising immune cells from nematode infected intestine[290,291] provide promise for future working schistosome intestine preps.

The intestinal dysbiosis provoked by schistosome infections[260–264] may also provide inflammatory relief and protection against secondary bacterial spread. For example, patent schistosome infections are accompanied by the enrichment of *Akkermansia muciniphila* populations[264], which have shown to strengthen enterocyte monolayer integrity in an *in vitro* setting[292]. It is also possible that the schistosome expanded populations may outcompete opportunistic pathogens, or perhaps modulate host tolerance to bacterial translocation[293]. Finally, liver macrophages have recently proven critical in the capture and clearance of intestinally-derived pathogens, with their antimicrobial properties imprinted by commensal products (D-Lactate)[294]. Given the huge hepatic element of schistosomiasis, alongside known alterations in intestinal microbiota structure[280], future studies could be really fascinating.

Once the intestinal barrier has been breached, a range of cell types are recruited to the area to survey the epithelium, repair the damage, and clear penetrant microbes. During intestinal schistosomiasis, cell types already recruited to the intestine (responding to eggs) may be better anatomically placed to deal with impending barrier breakdown and penetrant microbes. For

instance, the chemokines and cytokines secreted may promote an inflammatory milieu conductive to dealing with damage or promoting barrier repair.

LEARNING FROM SCHISTOSOMES

Schistosomiasis is a systemic disease with the potential to manipulate *virtually* every crevice of immunity, and as side effect, alleviate bystander inflammation and disease[25,29,115,295]. With hyperinflammatory conditions rising across the globe, we are currently in search of novel therapeutics for their treatment and prevention[6–8]. One promising therapeutic strategy includes exploiting the immunomodulatory potential of our 'Old friends': helminths. To give an example, Bregs are functionally and/or numerically impaired in a variety of immune pathologies (including systemic lupus erythematosus, rheumatoid arthritis, and allergy[296–298]) suggesting that recuperation of Breg activity holds promise in the treatment of these conditions. With *S. mansoni* possessing potent Breg-inducing potential[29,41,188], experimental schistosomiasis represents an ideal system to identify Breg inducing molecules or the pathways leading to their expansion. Similarly, with schistosome infections sharing many Type 2 trademarks with allergy, their study will likely illuminate novel cell types, host defence and repair mechanisms that thus, will have implications for the pathogenesis and treatment of allergic disease[2,299]. To give a few examples, the use of helminth models has already led to the identification of intestinal tuft cell[300] and ILC2 populations[301] and reinterpreted our understanding of M2 macrophage function[302], the actions of their effector molecules[193,303] and the feedback systems that limit Type 2 inflammation[304]. Finally, given the large intestinal component of *S. mansoni* infections[67], these infections could provide insight into the core mechanisms involved in the regulation and repair of mucosal inflammation. However, our current understanding of the impact of schistosomiasis on the intestinal environment is lacking and requires further attention.

SCOPE OF THIS THESIS

In this bulk of work, we aimed to intricately define the immune and microbial landscape of schistosomiasis and unravel some of the mechanisms contributing to the generation schistosome-associated cell types and responses.

The first section of this thesis provides a high-resolution image of schistosome elicited immune responses over the course of infection and aims to better define the impact of *S. mansoni* egg transit on host immunity and the intestinal environment. Chapter 2 outlines our current understanding of schistosome egg transit across the intestinal wall, with emphasis on the host-parasite interactions that facilitate this process and with speculation on how egg migration impacts unrelated disease. In Chapter 3 we aimed to build upon the literature's current perspective of schistosome evoked immune responses: using cellular and histological approaches to define host immunity over the course of infection, and across effector and priming sites. We also provide a novel and crucial role for $CD11c^{+}$ cells in the upkeep of Type 2 responses during peak disease. Chapter 4 addresses the impact of egg transit on intestinal barrier function, colonic immune responses, and microbiota composition. Importantly, we provide evidence that schistosome infection associated microbiotas, are capable of guiding host immunity towards a Type 2 profile.

The second part of thesis places greater emphasis on regulatory cell induction, aiming to identify the parasite, microbial and inflammatory signals that support Breg and Treg expansion and may provide protection from AAI. In Chapter 5 we build upon previous work and show that type I interferons support *S. mansoni* Ag-driven Breg induction *in vitro* but play redundant roles *in vivo*. In Chapter 6 we disentangle the contribution of worm and egg-derived signalling in splenic Treg and Breg expansion, and by means of faecal transplant, show the microbiotas of schistosome-infected animals to modulate the severity of experimental allergy.

Finally, Chapter 7 summarises and discusses our choice of methodology and the main findings of thesis. We explore how our work translates to the wider field and consider future lines of research, with a particular focus on how the immunomodulatory potential of schistosomes or their associated microbiotas could be harnessed for the treatment of other inflammatory conditions.

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