Exploring host-immune-microbial interactions during intestinal schistosomiasis

Costain, A.H.

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Alice H. Costain

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Alice Harriet Costain

Colophon

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Prof. dr. H.H. Smits
Prof. dr. A. MacDonald (University of Manchester)

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Prof. dr. M. Roestenberg
Prof. dr. J. Samsom (Erasmus University, Rotterdam)
Prof. dr. E.J Kuijper
Prof. dr. D. Thornton (University of Manchester)

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List of Abbreviations

AA; Alternatively activated

AAI; Allergic airway inflammation

Ag; Antigen

APC; Antigen presenting cell

AWA: Adult worm antigen

BCR; B cell receptor

Breg; Regulatory B cell

BAL; Bronchoalveolar lavage

CAA: Circulating anodic antigen

CBA; Cytometric bead array

CCA: Circulating cathodic antigen

CD; Cluster of differentiation

cDC; Conventional dendritic cell

COPD; Chronic obstructive pulmonary disease (COPD)

CTLA-4; Cytotoxic T lymphocyte antigen-4

DC; Dendritic cell

DAMP; Damage associated molecular pattern

DAPI; 4’,6-diamidino-2-phenylindole

DNA; Deoxyribonucleic acid

DTr; Diphtheria toxin receptor

DTx; Diphtheria toxin

EDTA; Ethylenediaminetetraacetic acid

ELISA; Enzyme linked immunosorbent assay

ES products; Extracellular secretory products

FACS; Fluorescence assisted cell sorting

FBS; Fetal bovine serum

FSC; Forward scatter

FO; Follicular B cell

GITR; Glucocorticoid-Induced TNFR-Related (GITR) protein

GFP; Green fluorescent protein

GM-CSF; Granulocyte macrophage colony stimulating factor

HDM; House dust mite

HK; House keeper

HMGB1; High box mobility protein 1

HRP; Horseradish peroxidase

IDO; Indoleamine-pyrrole 2,3-dioxygenase

IFN-γ; interferon gamma

Ig; immunoglobulin

IL; Interleukin

i.p; intraperitoneal

I,v; intravenous

i.n; intranasal

LAP; Latency associated peptide

LPS; Lipopolysaccharide

LUMC; Leiden University Medical Centre

MHC: Multiple histocompatibility complex

MLN; Mesenteric lymph node

MZ; Marginal zone B cells

MZ_MZP; Marginal zone B cells and their precursors

OVA; Ovalbumin

NGS; Next generation sequencing

PAMPs; Pathogen associated molecular patterns

PBMCs; Peripheral blood mononuclear cells

PBS; Phosphate buffered saline

pDCs; Plasmocytoid dendritic cell

RBC; Red blood cell

RELMα; Resistin-like molecule alpha

RNA; Rubonucleic acid

RT; Room temperature

SCFAs; short chain fatty acids

S. haematobium; Schistosoma haematobium

S. mansoni; *Schistosoma* mansoni

S. Japonicum; Schistosoma japonicum

SEA: Schistosome egg antigen

SSC; Side scatter

T1; Transitional type 1 B cells

T2; Transitional type 2 B cells

T2_MZPs; Transitional type 2 Marginal zone precursor B cells

TCR; T cell receptor

TGFβ; Transforming growth factor beta

Th: T helper

TLR; Toller like receptor

TMA; Trimethylamine

TMAO; Trimethylamine oxide

Treg; Regulatory T cell

TSLP; Thymic stromal lymphopoiten

UOM; university of Manchester

Abstract

Schistosomes are extraordinarily successful parasites and experts of immune calibration. Like other helminths, schistosomes have evolved sophisticated mechanisms to circumvent and modulate immune responses, ultimately permitting their long-term persistence within the host. This recalibration reflects the spectrum of immunomodulatory molecules produced by schistosomes, alongside the host's reparative response to parasite-inflicted damage. Importantly, as schistosome eggs cause considerable destruction to the host as they rupture the intestinal wall, it is possible that intestinally-derived signals, including those from the microbiota, may contribute to the immune responses found during schistosomiasis. In the work detailed in thesis, we attempt to dissect the involvement of parasite, host, and microbial factors in the instruction of schistosome associated Type 2 and Regulatory cell networks. First, we systematically characterise immune profiles across the course of conventional egg producing infections, providing a concise narrative of how schistosomiasis associated immune profiles evolve over time in effector and priming sites. Here, though the depletion of $CD11c^+$ cells at peak stages of disease, we reveal a role for $CD11c^+$ DCs in the maintenance of *S. mansoni* elicited Type 2 immunity. Next, by using a combination of high vs low dose, and egg producing vs non egg producing infections, we show elevated intestinal permeability during chronic and high dose egg producing infections, with hints towards enhanced bacterial translocation. Infection patency evoked a Type 2 dominated immune response in the mesenteric lymph nodes and colon that coincided with significant intestinal microbiome alterations. Significantly, through the use of germ free mice and faecal transplants, we provide evidence that the schistosome infection associated microbiota can influence the character of host Immunity. Moving on from the intestine and Type 2 immunity, we next sought to better define the signals endorsing schistosome elicited Regulatory B cell (Breg) and T cell (Treg) expansion. Through the interrogation of Type-I signalling, we show IFN-I to assist in murine Breg generation an *in vitro* but not *in vivo setting.* Finally, we show chronic egg-producing and non-egg producing *S. mansoni* infections to expand phenotypically distinct Treg and Breg and populations, with microbiotas from these mice capable of modulating the severity of experimental allergy. Together, our data elevates the mechanistic understanding of parasite-host-microbial relations and provides a strong platform for the future study of schistosome or microbial factors in the modulation of inflammatory disease.

Lay Abstract

Schistosomes are parasitic worms that cause the disease schistosomiasis. To survive and thrive within the mammalian host, schistosomes manipulate host immunity in a variety of ways, including the production of certain factors that interact with immune and non-immune cells. In addition, as part of the schistosome lifecycle, schistosome eggs move across the gut wall causing intense damage. This damage might make the gut 'leakier' and allow substances within the gut (including bacteria) to enter other parts of the body and influence the immune system. In this thesis we investigate how factors produced by parasites, the host and our gut bacteria, influence the immune system during schistosomiasis. Firstly, we look broadly at immune responses generated during conventional egg producing *Schistosoma mansoni* infections, and characterise how immune cells respond in the spleen, liver, and mesenteric lymph nodes at different time points of infection. We show that a specific type of innate immune cell called dendritic cells are important for the upkeep certain immune responses during infection, referred to as Type 2 immunity. Next, by infecting mice with egg producing and non-egg producing schistosomes, we show that intestinal leakiness is provoked by the movement of eggs across the gut wall, and that increasing the infection dose makes leakiness occur at earlier stages of infection. We show the movement of eggs to evoke a Type 2 immune response within the intestine and to also disrupt the diversity and abundance of bacteria within the gut. Next by transferring faeces from schistosome infected mice into naïve mice without intestinal bacteria, we show that microbes from schistosome infected mice can promote aspects of the gut immune response found during schistosome infections, even in the absence of parasites. Next, we investigate the role of certain secreted immune proteins, called cytokines, during schistosomiasis. We show that Type-I IFNs (cytokines) can help support a specific adaptive immune cell, the Regulatory B cell (Breg), to produce another cytokine called IL-10. Finally, previous work has shown *S. mansoni* infections to protect against allergy, with evidence that certain immune cells called Regulatory T cells (Treg) and Bregs mediate this effect. We show that both egg producing and non-egg producing infections are capable of triggering expansion of these immune cells and that the gut bacteria from these mice can modulate the severity of allergic disease in mice. Together this work provides new and exciting insights into the factors governing immune responses during schistosomiasis, with strong hints towards bacterial involvement.

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.

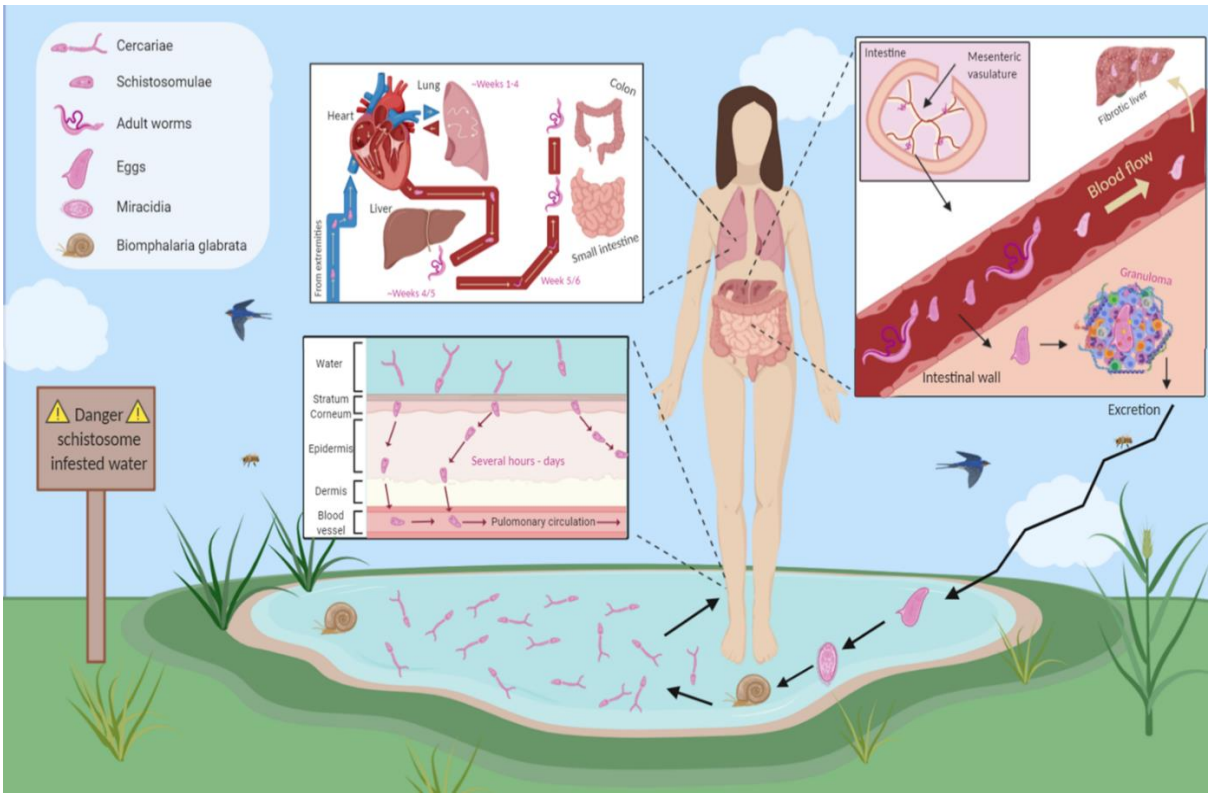

Diagram 1 – The lifecycle of *Schistosoma mansoni*

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.

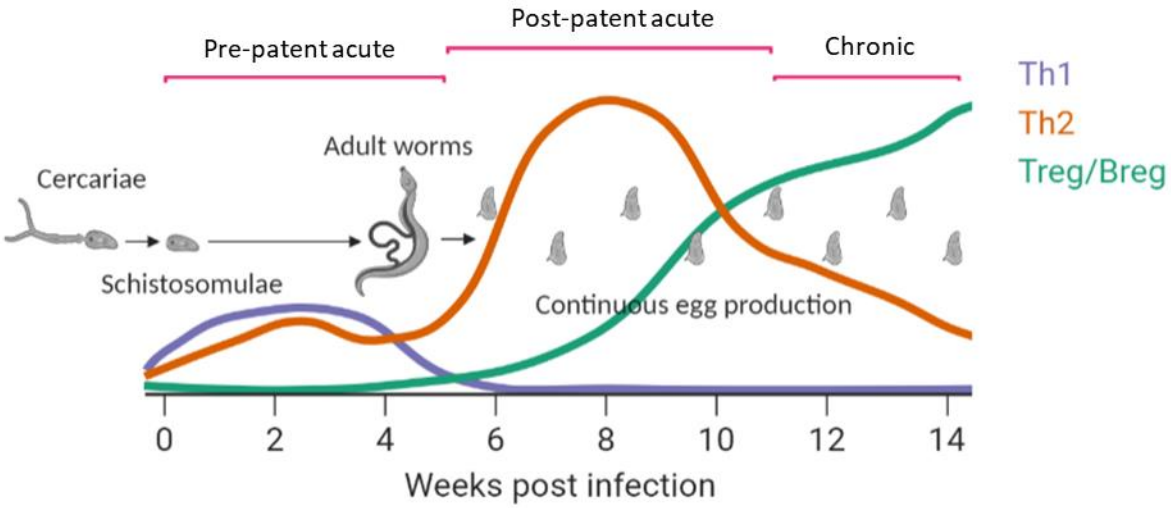

Diagram 2 – The immunological profile of schistosomiasis.
Over the course of S. *mansoni* infections, the host's immune response progresses through at-least three immunologically distinct phases: pre-patent acute, post-patent acute and chronic disease, each of which reflect antigenically distinct lifecycle 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.

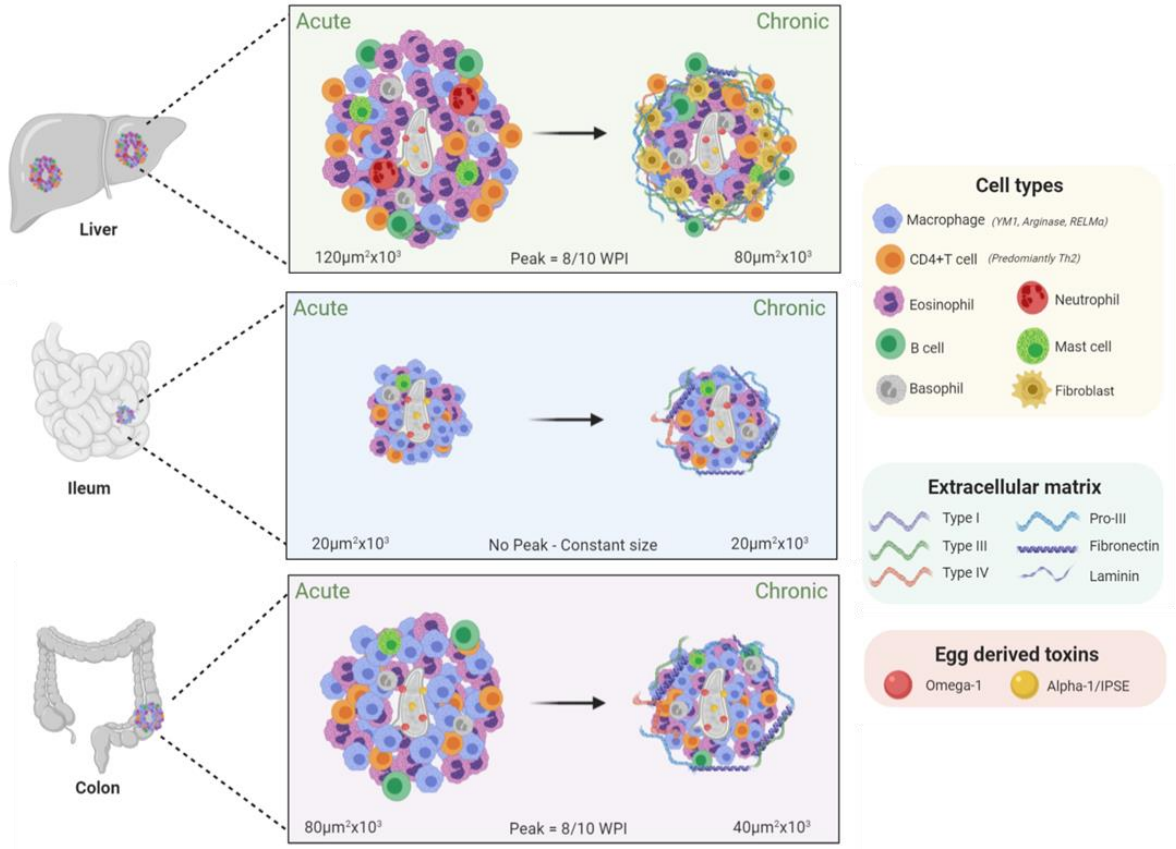

Diagram 3 -The Granuloma. Granulomas serve two functions: the encapsulation or sequestration of egg-derived molecules from host tissue and enabling egg transit across the intestine[63]. Over the years, granuloma structure and cellular composition has been characterised via variety of approaches, including 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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CHAPTER 2

Schistosome egg migration: mechanisms, pathogenesis, and host immune responses

Alice H. Costain, Andrew S. MacDonald and Hermelijn H. Smits

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Schistosome egg migration: mechanisms, pathogenesis and host immune responses

AUTHOR INFORMATION

Alice H. Costain[1,2], Andrew S. MacDonald[2] and Hermelijn H. Smits[1*]

1. Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands
2. Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, UK

*Co-corresponding authors: Andrew S MacDonald (andrew.macdonald@manchester.ac.uk) and Hermelijn Smits (h.h.smits@lumc.nl)

ABSTRACT

Many parasitic worms possess complex and intriguing life cycles, and schistosomes are no exception. To exit the human body and progress to their successive snail host, *Schistosoma mansoni* eggs must migrate from the mesenteric vessels, across the intestinal wall and into the faeces. This process is complex and not always successful. A vast proportion of eggs fail to leave their definite host, instead becoming lodged within intestinal or hepatic tissue, where they can evoke potentially life-threatening pathology. Thus, to maximise the likelihood of successful egg passage whilst minimising host pathology, intriguing egg exit strategies have evolved. Notably, schistosomes actively exert counter-inflammatory influences on the host immune system, discreetly compromise endothelial and epithelial barriers, and modulate granuloma formation around transiting eggs, which is instrumental to their migration. In this review, we discuss new developments in our understanding of schistosome egg migration, with an emphasis on *S. mansoni* and the intestine, and outline the host-parasite interactions that are thought to make this process possible. In addition, we explore the potential immune implications of egg penetration and discuss the long-term consequences for the host of unsuccessful egg transit, such as fibrosis, coinfection, and cancer development.

Keywords

Schistosoma mansoni, intestine, endothelium, type 2 immunity, immune modulation

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The image in Figure 2 was captured by Angela Marley during her PhD studies in the MacDonald laboratory. The authors declare no conflict of interest.

INTRODUCTION

Schistosomiasis is a chronic and potentially lethal tropical disease, mainly caused by the parasitic blood flukes *Schistosoma mansoni, S. haematobium, and S. japonicum.* Schistosomes have evolved to develop and thrive in their infected hosts, with untreated infections generally persisting for 3-10 years and a minority of infected individuals developing severe, life-threatening pathology[1]. Common among parasites, schistosomes possess rather peculiar life cycles. This includes stages within definitive human hosts and secondary snail vectors, transformation through various larval forms, and - importantly - a unique process of egg migration to leave their human host. In this essential life cycle event, schistosome eggs pass from the host vasculature, across intervening tissue and into the environment via host excretions. This enigmatic process, and its pathological/immunological consequences, is the focus of this review, with particular emphasis placed on the intestinal response to *S. mansoni.*

Adult *S. mansoni* worms reside deep within the mesenteric veins of the intestine, where they feed on blood and acquire nutrients necessary for growth, development, and egg production[2]. Each worm pair produces ~300 eggs daily, which exit the host by moving from the depths of the mesenteric vessels, across the intestinal wall and into the intestine lumen[3,4]. Importantly, as schistosome eggs are not in possession of any obvious motility mechanisms themselves, their expulsion is likely to be heavily reliant on host-driven processes. However, successful egg passage is not guaranteed. Approximately half of all deposited eggs never reach the intestine, but instead are swept to the liver, where they evoke strong granulomatous inflammation, as characterised by the infiltration of alternatively activated (AA) macrophages, eosinophils and T-helper 2 (Th2) cells, with additional fibroblast proliferation and generation of extracellular matrix[3–5] For the remainder of intestinally-bound eggs, success is still not certain. Firstly, eggs remain viable for a mere 2-3 weeks following oviposition, providing them with a relatively short timeframe to make this journey[6,7]. Secondly, due their high antigenicity and continual release of antigens and other metabolites, transiting eggs are easily detected by the host immune system, becoming the focal point of inflammatory granulomatous reactions. If these responses are too extreme, a variety of immune-pathologic sequelae will follow[8].

As our understanding of schistosome immunobiology has increased, it has become increasingly obvious that schistosomes implement a variety of strategies to ensure efficient egg transit. Within the vasculature, egg extravasation is promoted by angiogenesis, endothelial activation, and interactions with blood clotting components[9,10]. In the intestinal tissues, schistosomes exert a

variety of immunomodulatory influences to support granuloma formation around transiting eggs, which is an essential process in egg excretion[11–14]. Directly related to this, and to prevent overwhelming immunopathology, schistosomes guide the immune response towards a more regulatory phenotype during chronic disease. In this review we discuss the strategies employed by schistosomes to favour egg passage, and outline the potential immune implications and pathological consequences that may follow (Diagram 1).

ENDOTHELIAL EXTRAVASATION

Maturation and mesenteric migration

Before egg production can commence, schistosomes need to mature to adulthood while navigating from the skin, via the lungs to the mesenteric veins of the intestine (*S. mansoni* and *S. japonicum*, causing hepatosplenic disease) or bladder (*S. haematobium*, causing urinary schistosomiasis[15–17]). Worm maturation occurs in the blood vessels and requires the transduction of host-derived signals from male worms to their female partner[18]. Signals from the adaptive and innate immune system are thought to be intimately linked with this process[18]. Notably, worm growth and reproductive activity is severely stunted in the absence of $CD4^+$ T cells, but can be sufficiently restored through repeated stimulation of innate immunity via toll-like receptor signaling or inflammasome activation by endogenous danger signals[18–21]. The specific immunological factors that guide parasite development remain poorly defined and controversial. For instance, while a functional role for interleukin (IL)-7 in parasite development is generally agreed upon, there is ongoing controversy surrounding the ole of TNF, with studies showing that both TNF neutralisation and administration can promote egg production[20,22,23].

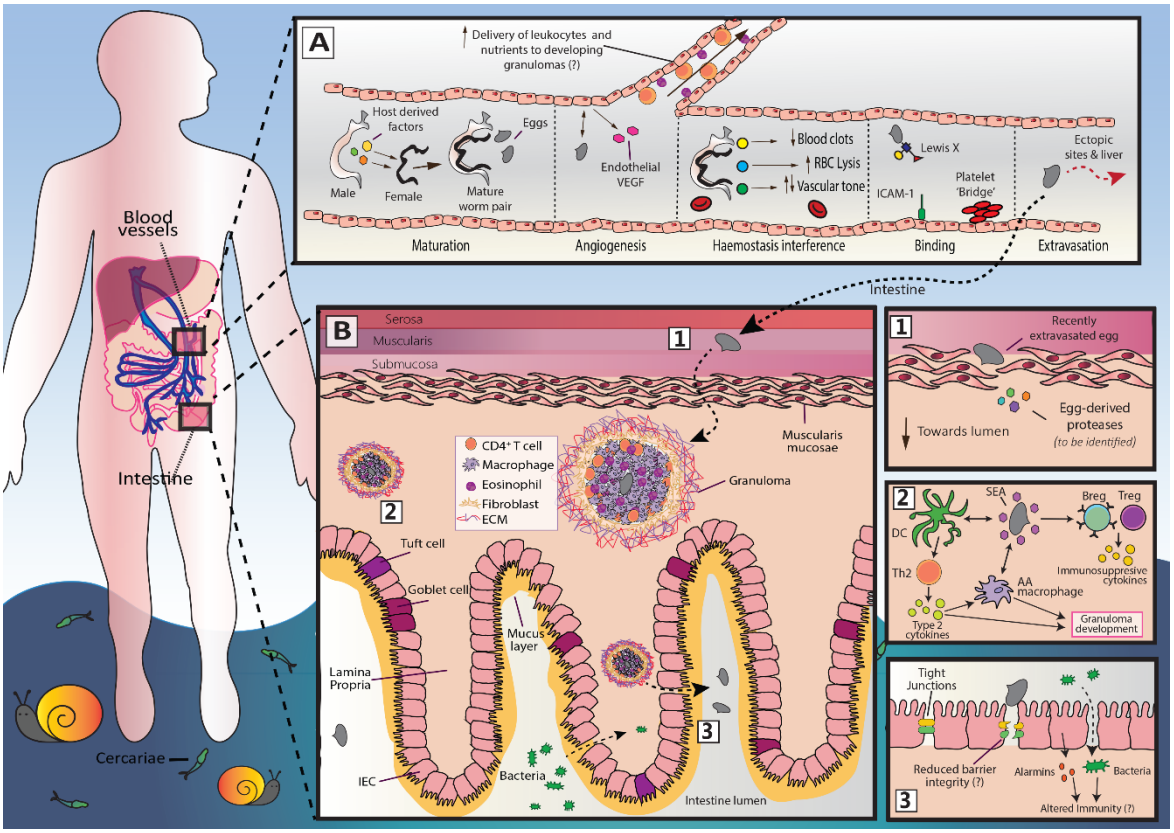

Diagram 1. An overview of *S. mansoni* egg migration. Schistosome egg transit is facilitated by a series of host interactions at the intestinal and vascular interface. **(A)** The development of schistosomes into sexually mature, egg-producing adults occurs within the portal vein (~3-5 weeks post infection) and requires the transduction of host-derived signals (including those from the innate and adaptive immune system) to the developing worm pair. Once sexual maturity is reached, worm pairs migrate towards the mesenteric vessels, where the females lay approximately 300 eggs per day and actively modulate the intravascular environment to support their long-term survival. The production of eggs at ~5-6 weeks post infection is a milestone event in the schistosome life cycle, that is characterised by induction of a marked Th2 response, and angiogenesis. Notably, the generation of a Th2 response by the host is critical for egg passage, and new vessel formation may favour egg transit, promoting the recruitment of immune cells and nutrients to developing granulomas. Freshly deposited eggs cannot move by themselves and must somehow attach and extravasate the endothelium. Although yet to be fully defined, this process may involve E-selectin:-Lewis-x interactions, and participation from platelets, ICAM-1 and VCAM-1. While a large proportion of eggs successfully penetrate the endothelium and reach intestinal tissue, many are swept to the liver or other distal locations (e.g. brain or spinal cord). Since schistosome eggs are unable to transit through these organs, overwhelming tissue pathology and inflammation may ensue.

(B) Once schistosome eggs have passed across the host endothelium and out of the vasculature, they must cross the multi-layered intestinal wall. The host immune system responds to transiting eggs via an inflammatory granuloma response, in which individual eggs are encapsulated by immune cells (including alternatively activated (AA) macrophages, Th2 cells and eosinophils) and extracellular matrix (ECM), which protects host tissues from egg-derived toxins, but ultimately leads to formation of fibrotic lesions. For unknown reasons, granulomatous responses need to successfully develop for effective egg excretion from the host. Accordingly, schistosomes and their host have co-evolved a wide range of mechanisms to skew the host immune response towards granuloma-inducing Th2 profile. These include the ability of soluble egg antigens (SEA) to promote alternative activation in macrophages and to condition dendritic cells (DCs) for Th2 polarisation. However, to prevent unwanted bystander tissue damage and potentially fatal immunopathology, schistosomes also implement various strategies to dampen host immunity and expanded regulatory networks (Bregs and Tregs).

There remain many unknowns surrounding egg migration. This includes the molecules secreted by eggs to disrupt host barriers and modulate immune responses and, importantly, how egg penetration and intestinal 'leakiness' may influence local and systemic immune reactions.

At approximately 4-6 weeks post infection, sexually mature worm pairs move to the mesenteric vessels, which is the site of oviposition[16]. Although eggs can be found throughout the vasculature, certain sites may be favoured[15]. In mice, oviposition appears to be concentrated in the Peyer's patches of the small intestine, while in primates and man, eggs are more readily detected in the colon and rectum[15,17,24,25]. Why oviposition shows such patterns is subject to speculation. It is possible that worm migration is dictated by host-derived signals (e.g hormones or digestion products absorbed across the intestine wall) or that worms preferentially exploit regions of low sheer stress and high vascularisation to avoid eggs being swept away with the blood stream[15]. Alternatively, blood vessel diameter could be the major determinant, with adult worm pairs being relatively large (~0.5 mm in diameter and up to 10 mm long) in comparison to the mesenteric vessels that they reside in [26]. Furthermore, while *S. mansoni* eggs are laid diffusely across the intestine and seldom produce bulky, concentrated lesions, *S. haematobium* and *S. japonicum* worms tend to deposit eggs in a few areas where a large number of them are concentrated[15,17]. These deposition patterns may reflect the unavoidable clumping of worms within host vessels or, alternatively, worms may be attracted to factors at the site of eggs-induced lesions, including substances released from breached blood vessels[15]. Finally, it is possible that egg aggregation supports extravasation, with the build-up of egg-derived proteases creating channels from the intravascular to intraluminal space[27].

Even though migrating worm pairs are clear potential obstructions to blood flow, schistosome infections are not associated with enhanced blood clotting. In fact, individuals with advanced hepatosplenic schistosomiasis have a reduced level of blood coagulation factors, and blood clots are not observed around worms in host vessels[28–31]. Experimental studies have also shown schistosome infections to impact blood-coagulation, where blood from 7-week infected mice coagulates more rapidly than control, with faster lysis of the clot formed[26]. However, ex vivo studies involving the exposure of adult worms to blood from infected or non-infected mice, demonstrate an anticoagulant effect of the adult parasite[26]. Mechanistically, there is strong evidence indicating that that schistosomes directly modulate the host haemostatic system via a variety of bioactive secretory products and molecules on the schistosome's outer-surface (tegument)[9]**.** For instance, schistosomes inhibit blood clot formation and/or promote blood clot lysis through the activities of several tegumental enzymes, including enolase, SmSP2, SmAP and SmCalp1&2, and vascular tone is modified through the activities of SmSP2 and SmPOP[32–35]. Altogether, such processes can be viewed as a schistosome survival mechanism in the blood stream, likely promoting residence and movement while preventing unwanted vessel occlusion.

Endothelial adherence

Schistosome eggs are striking structures, encased by a rigid network of cross-linked proteins and, in the case of *S. mansoni*, characterised by a large protruding lateral spine. Due to the high rigidity and inflexibility of their outer shell, schistosome eggs must rely on external forces to bring them towards to the endothelial lining[36].It has long been suggested that the active migration of endothelial cells over schistosome eggs, brings the eggs into close contact with the vessel lining[37]. More recently, video imaging has suggested that female worms prompt egg-endothelial associations via strong muscular contractions at their genital pore (dorsiflexion) that thrusts their eggs into the endothelium[7]. Once brought into close contact with the endothelium, eggs likely tether themselves to endothelial surface adhesion molecules, including ICAM-1, VCAM-1 and E-selectin[38,39]. However, while binding to E-selectin is likely mediated by egg-shell components glycosylated with Lewis-x motifs[39], there are no obvious integrin-like structures within the egg-shell that bind ICAM-1 or VCAM-1. Interestingly, ICAM-1 shows strong upregulation in response to eggs and SEA[38], and soluble ICAM-1 levels are constitutively higher in schistosome-infected individuals and positively correlate with egg excretion rates[40]. Additionally, there is evidence to show that ICAM-1 not only mediates egg binding, but also participates in the generation of granulomatous inflammation around parasite eggs, by regulating leukocyte trafficking, vascular permeability and modulating T cell responsiveness to soluble egg antigens (SEA)[38,40,41]. As later discussed, intact granuloma formation is essential for successful egg expulsion.

Freshly deposited eggs are immediately surrounded by cells and proteins of the haemostatic system, including the plasma proteins von Willebrand factor (VWF), fibrin and fibrinogen [7,42,43]. In addition to ICAM-1, E-selectin and VCAM-1, schistosome eggs may bind to these haemostatic components to promote their anchorage to the endothelium and to prevent them from being swept away with circulation. Indeed, the administration of platelet inactivating drugs to *S.mansoni*-infected mice results in significantly diminished egg excretion rates[44]. By closely analysing the interactions between eggs and such components, deWalick and colleagues demonstrated that the schistosome egg-shell directly binds to VWF: an adhesive glycoprotein that tethers clotting material (such as platelets) to the activated endothelium[42,45]. This binding is suggested to benefit egg extravasation in two ways. VWF could form a direct bridge between eggs and the extracellular matrix, and/or the binding of VWF to clotting material may induce stable clot formation, making it easier for eggs to adhere to the endothelium.

The role of the schistosome spine in egg migration is not known. Given that *S. japiconium* eggs are virtually spineless, it is unlikely that the schistosome spine plays a major function. In fact, a recent report comparing egg morphology between Praziquantel resistant and susceptible *S. mansoni* infection suggests that the spine actually hinders egg transit[46]. More specifically, resistant strain eggs were shown to have smaller lateral spines than susceptible strain eggs, and were also more frequently shed into host faeces[46]. Thus, from an evolutionary standpoint, perhaps *S. japiconium* has taken advantage of spine absence. However, based on the repeated observation that *S.mansoni* eggs clump to one another at their spine tip, it is also possible that spine-to-spine clumping enhances the cytotoxicity of freshly deposited eggs, promoting channel formation through host tissues and/or accelerating granuloma development[27,47].

Eggs and angiogenesis

Schistosomes not only reside and produce eggs within blood vessels, but also appear to promote their formation[48]. Angiogenesis is a complex process in which new vessels develop from pre-existing ones, creating an environment that favours tissue growth and repair [49]. This sequential process is guided by pro-angiogenic factors (such as VEGF, angiopoietin and inflammatory cell-derived chemokines) which instruct endothelial cell activation, proliferation and reorganisation[49]. Evidence for schistosome-induced angiogenesis can be found in both human studies and experimental models. During murine infection, vascularity is significantly enhanced in areas of high egg concentration (including the Peyer's patches) and, when angiogenesis is inhibited, there is a reduction in worm load and hepatic egg deposition[10,24,50]. In human studies, mucosal biopsies containing *S. haematobium* eggs are more vascularised than healthy, egg-free control tissue[51]. In addition, schistosomiasis patients have significantly higher serum VEGF levels than healthy individuals, or those with active hookworm infections[10]. Schistosomes likely promote neovascularisation to sustain their life cycle, for several reasons. First, the remodelling of intestinal vasculature may increase the number of worms the blood vessels can accommodate and reduce egg 'spill over' into hepatic tissue[24]. Second, angiogenic responses could enable the recruitment of leukocytes to developing granulomas, and ensure an adequate supply of oxygen and nutrients at these sites[52]. Third, increased vessel density may impair intestinal tissue, making it easier for eggs to disrupt and move through[51]. Finally, similar to what has been observed in cancer, growth of new vessels would maintain blood flow in scenarios of vessel occlusion (e.g. by worm pairs and their eggs). Furthermore, conditions created by vessel occlusion such as hypoxia, acidic PH and low glucose concentration, may also contribute to the neovascularisation observed[53].

While adult worms have poorly defined roles in the induction of angiogenesis, secretory products of schistosome eggs (soluble egg Ags, or SEA) have been shown to instruct angiogenesis via direct and indirect mechanisms. Investigations using human umbilical vein endothelial cells have shown that SEA can directly encourage endothelial cell proliferation, migration, sprouting and production of VEGF[53,54]. The extent of angiogenic activity can be influenced by host genetics, and lies within the glycoprotein fraction of SEA[55,56]. Indirectly, SEA induces angiogenesis through the actions of alternatively activated (AA) macrophages and hedgehog signalling[57]. In this case, SEA stimulates macrophage secretion of biologically active hedgehog ligands, which subsequently activate hedgehog-responsive endothelial cells to proliferate and secrete angiogenic factors. The fact that sprouting blood vessels are more frequently observed around viable ova as opposed to dead or dying calcified ova, strongly suggests that substances actively secreted from eggs are responsible for new blood vessel formation[51].

In addition to angiogenesis, endothelial activation by schistosomes may also support granuloma formation. Schistosome triggered VEGF significantly increases proliferation of and extracellular matrix deposition by hepatic stellate cells, which are the main source of extracellular matrix around schistosome eggs in the liver[58]. Whether similar mechanisms are involved in intestinal granuloma formation is unknown, though it is tempting to speculate that endothelial cells lining mesenteric vessels could be activated upon worm encounter to secrete pro-fibrotic factors, such as VEGF, IL-13, TGF-β or IL-33, to intestinal-resident cells involved in fibrosis. IL-33, for instance, is constitutively expressed by endothelial cells, and has recently been shown to support liver granuloma pathology through the induction of pro-fibrotic AA macrophages[59,60]. In addition, VEGF partially regulates Th2 inflammatory responses in the lung and liver in response to schistosome eggs[61]. Given that type 2 immune responses are essential for adequate granuloma formation, it would be interesting to define a role for VEGF in intestinal granulomatous reactions.

Overall, schistosomes employ a variety of strategies within host blood vessels that favour their survival and life cycle propagation. Increased understanding of these interactions could identify new targets for the prevention of severe disease during schistosomiasis. For instance, direct targeting of adult worms could prevent or reduce the production of eggs and their accumulation within host tissues, which is the primary cause of pathogenesis during schistosome infection.

INTESTINAL PASSAGE

After successfully extravasating the mesenteric vessels schistosome eggs are confronted with a much larger anatomical hurdle: the intestinal wall. This extensive barricade is the host's largest interface with the external environment, it's luminal side in constant exposure to the contents of the intestines, including innocuous (food) Ags, commensals and pathogens[62]. To efficiently segregate the host from this hazardous environment, the intestinal wall incorporates a dense network of immune cells and an innermost monolayer of tightly aligned intestinal epithelial cells (IECs). IECs and resident immune cells communicate with one another to reinforce barrier integrity, maintain homeostasis and mount robust responses against invading threats - schistosome eggs included. However, despite the host response against them, a large proportion of eggs successfully transit across the intestinal tissues and exit the host body. Crucially, to enable this process while limiting enteric inflammation, schistosomes have evolved several strategies to modulate host immune responses and manipulate host barriers. For example, as illustrated in Figure 1, schistosome eggs are capable of digesting through the intestinal muscular layer (muscularis mucosae) without triggering significant inflammation or immune infiltration. This process likely involves a collaboration between egg-derived proteases (yet to be defined) and immune cells that have been recruited to the serosal tissue and/or mesenteric vessels[24]. In particular, the strategies employed by schistosomes to favour migration of eggs through the intestine likely heavily involve Th2 polarisation and granuloma maintenance.

Moreover, Due to infection longevity and the continuous passing of eggs, it is currently unclear and difficult to assess which intestinal layer schistosome eggs predominantly enter (i.e serosa, submucosa or mucosa). However, H&E images from infected mouse intestine suggest that eggs tend to penetrate through the muscularis-serosa layer[63].

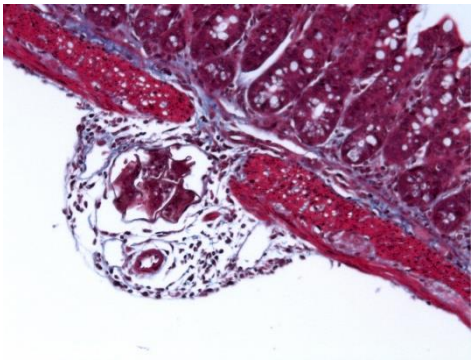

Figure 1. *S. mansoni* egg penetrating through the intestinal wall. Light microscope image of Masson's Trichrome stained ileal section (5 µm) from a C57BL/6 mouse 6 weeks after percutaneous infection with approximately 40 *S. mansoni* cercariae.

Promoting Granuloma formation

S. mansoni eggs are highly antigenic structures that continuously secrete a variety of innocuous, toxic or antigenic substances into host tissues[6]. Accordingly, infiltrating eggs are focal points for the host immune system, which mounts a distinct attack and sequestration strategy in response: the granuloma. Granulomas are highly organised, multi-cellular structures that are enriched with a range of immune cells including Th2 cells, macrophages and eosinophils, with mast cell infiltration, accumulation of type 2 cytokines (such as IL-4, IL-13 and IL-5) and additional involvement from stromal cells/fibroblasts. Type-2 cytokines instruct the polarisation of macrophages towards an alternatively activated (AA) phenotype, whom are essential to the resolution of egg-induced inflammation and tissue damage[64–66]. After reaching maximal responses, granulomatous lesions decline in size and become fibrotic, with IL-13 being the main cytokine responsible for fibrosis, mediating its effects through AA macrophages and fibroblasts[5,67,68].

It is important to highlight that although these egg-triggered inflammatory reactions can also be found throughout infected liver, there are distinct differences between intestinal and hepatic granulomas in terms of size, cellular composition and extracellular matrix deposition[7,69]. These finding arguably reflect the higher proportion of dead eggs within infected liver, and thus, differences in eggs secretions between the two tissues. Alternatively, these findings suggest that the local microenvironment has a large bearing on granuloma function and development. In the case of intestinal granulomas, it would be very interesting to disentangle intestinal bacterial involvement, as studies on antibiotic-treated mice show only intestinal granulomas, but not hepatic granulomas, to be influenced by antibiotic administration[70]. Moreover, it is unknown whether intestinal granulomas are better 'designed' for egg excretion than their hepatic counterparts. Furthermore, while the immunology of hepatic granulomas has been studied extensively over past decades, our understanding of intestinal granulomas is considerably less. Accordingly, most of the data presented in the following section has been obtained from studies on hepatic granulomas, and we cannot conclusively rule out the possibility that intestinal granuloma development differs to that described here.

The schistosome granuloma is both beneficial and detrimental for the host[5]. On the one hand, granulomas protect host tissues from egg-derived toxins such as Omega-1 and IPSE/alpha-1, which can cause severe damage[71]. On the other hand, the fibrotic sequelae that follow granuloma resolution is the main cause of pathology and lethality in schistosomiasis [5]. At the same time, in a remarkable example of co-evolution, granulomas are thought to be essential for schistosomes and

continuation of their life cycle. Early studies in immunodeficient mice demonstrated the crucial role of granulomas in egg passage, with egg excretion rates plummeting in mice failing to generate intact granulomas, leading to lethal microvascular damage[12–14,72]. These data also underscored the importance of host T cells in granuloma development, results that were later corroborated by studies in HIV^+ infected people, in whom egg excretion rates were found to positively correlate with circulating $CD4^+$ T cell levels[73]. Furthermore, Th2 immunity is critical for intact granuloma development and host survival, since mice deficient in IL-4 and IL-13 signaling die during acute disease due to elevated intestinal and hepatic pathology, oxidative damage, endotoxemia and cachexia[64,67,74–76]. Therefore, it comes as no surprise that schistosomes actively modulate host immune responses to ensure a Th2 bias is achieved.

Infection with *S.mansoni* follows a predictable immunological pattern, with a mixed, low level Th1/Th2 profile prevailing in the initial 4-5 weeks, then Th2 responses dominating from the point of egg production, with parallel expansion of regulatory cell networks that exert their influence most dramatically during chronic infection[77]. Although many innate cell types help establish the Th2 milieu, dendritic cells (DCs) are essential[78,79]. Using their vast array of pattern-recognition receptors (PRRs), DCs sense, process and present schistosome egg-derived Ags to naïve CD4+ T cells, instructing Th2 development by yet to be fully defined mechanism[80].

SEA is a complex mixture of immune-stimulatory Ags, that is well known for its capacity to condition DCs for Th2 priming[81–83]. Omega-1, a glycosylated T2 RNase, is a single component of SEA and potentially, the most powerful inducer of Th2 [84,85]**.** Omega-1 is taken up through mannose receptors on DCs and enhances Th2 polarisation by degrading mRNA and ribosomal RNA, leading to reduced co-stimulatory molecules expression (CD83 and CD86) and reduced production of the Th1 promoting cytokine IL-12[84,85] Somewhat unexpectedly, SEA and Omega-1 can also stimulate DC production of Type-I IFN (normally associated with anti-viral responses), which enables their initiation of Th2 responses[86,87]. However, it is important to note that Omega-1 is not the only SEA component involved in DC conditioning, as SEA depleted of Omega-1 and *S.mansoni* eggs with specific Omega-1 knock-down still retain some of their Th2 polarising potential[88]. In addition, Kaisar and colleagues very recently identified a novel pathway in Th2 polarisation that entails Dectin1/2 signaling on DCs and works independently of the actions of Omega-1[89]. Overall, the impact of egg Ags on host innate immune cells is complex and multifactorial, often flouting convention, but with the cumulative effect being initiation of a strong Th2 response.

It is important to realise that although Th2 skewing is primarily ascribed to eggs, adult worms are also capable of instructing a Th2 environment before the onset of egg production[90]. In fact, by comparing granuloma formation between models of natural infection and portal vein egg injection, Leptak and colleagues demonstrate that granuloma development requires both worm- and egg-derived signals[91]. Notably, while the inflammatory reaction towards portal vein injected eggs is minimal in terms of reaction volume, cellular infiltration and collagen deposition, these reactions can be sufficiently restored if eggs are injected into mice previously infected with single-sex male cercariae or exposed to adult worm homogenates[91]. Mechanistically, this 'priming' effect could be explained by the sharing of Ags between adult worms and eggs[91]. Further, cytokines produced in response to adult worms may be key to granuloma induction[23,41,91]. In particular, the cytokine TNF is thought to be exclusively produced in response to adult worms and may be able to restore granuloma formation in naïve mice injected with eggs alone[91]. it has been suggested that the production of TNF may be linked to phagocytosis of worm-regurgitation products by local macrophage populations[91,92].

The schistosome granuloma is hallmarked by the accumulation of AA macrophages, which predominantly arise from the recruitment of Ly6C^{Hi} monocytes, as opposed to the proliferation of local macrophage populations[93,94]. AA macrophages are activated by type-2 cytokines (IL-4/IL-13), characterised by the expression of various signature genes (such as arginase 1, Ym1, Relmα) and are thought to prevent potentially lethal host pathology via a variety of mechanisms[66,95].This includes coordinating the recruitment of collagen and cells to developing granulomas, regulating T-cell proliferation, facilitating tissue repair and regeneration, and inhibiting the differentiation of classically activated macrophages (CAMs), which generate pro-inflammatory cytokines and cause oxidative tissue damage[66,96,97.] Crucially, thanks to the wound healing capacities of AA macrophages, intestinal barrier integrity is sufficiently maintained during egg migration and the host is shielded from enteric bacteria[66]. Indeed, in mice deficient in IL-4/IL-13 signaling specifically on macrophages and neutrophils ((LysM(Cre)IL-4Ralpha(-/flox) mice), AA macrophages do not develop and mice are extremely susceptible to *S. mansoni* infection (100% mortality during acute disease)[64]. Importantly, acute mortality was associated with exaggerated Th1 pathology, severe hepatic and intestinal damage, impaired egg excretion and sepsis[64]. In more recent studies, AA macrophages have also shown to maintain tissue integrity in models of urogenital schistosomiasis[65]and their protective function has been partially attributed to the enzyme arginase-1 and resistin-like molecule (RELM)-α[97–100] . Notably, arginase-1 contributes to the long-term survival of schistosomiasis by restricting

Th1 and Th17 associated immunopathology, and RELMα acts as a negative regulator of Th2 responses [97–100]. Moreover, while Ym1 is an additional phenotypic marker of AA macrophages, its contribution to Th2 responses during schistosomiasis remains undefined. However, various reports suggest a role for Ym1 in Th2 differentiation[101,102]. Of note, our understanding of the complexity of 'M2' macrophages, and the range of cytokines that can promote aspects of M2 polarisation, is continually growing, with IL-10, TGF-β and IL-33 also shown capable of instructing alternative activation[60,103–105].

Schistosomes not only rely on cytokines to instruct AA macrophages induction, but also play a direct role in their polarisation. Schistosome-derived molecules that may contribute to alternative activation include SEA, peroxiredoxin, lysophosphatidylcholine and hemozoin [106–110]. SEA, for instance, appears able to directly interact with macrophages to instruct an AA phenotype, or indirectly elicits an AA profile via IL-33 and its receptor ST2[60,108]. Interestingly, in a model of intravenous *S.mansoni* egg injection, mice deficient in ST2 demonstrated impaired production of Th2 cytokines and primary granuloma[111]. However, this study did not assess the influence of ST2 deficiency on macrophage function or polarisation. Another molecule that influences macrophage polarisation is hemozoin. Hemozoin is a neutralised version of heme that schistosomes regurgitate into the bloodstream following the digestion of host erythrocytes[109]. Hemozoin crystals spontaneously aggregate in the liver, where their consumption by patrolling macrophages promotes an alternatively activated phenotype, including the expression of the Th2 negative regulator RELM-α [98,110,112]. Moreover, the uptake of these worm-regurgitation products by antigen presenting cells has been suggested to induce the production TNF, which has previously shown a key role in granuloma formation[23,77,91].

In terms of other myeloid cells, eosinophils and mast cells are cardinal features of the schistosome granuloma, but their function there remains elusive. While older studies suggest that eosinophils favour egg passage by digesting the epithelial basal membrane**,** more recent models of eosinophil ablation found no obvious role for them in this process or for granuloma formation, fibrosis or worm burden[68, 113–115]In this context, mast cell function also remains a mystery. During *Trichinella spiralis* infections, mast cells mediate parasite expulsion and disrupt epithelial barrier function through the release of mast cell protease 1 (mMCP-1), a serine protease that degrades TJ proteins[116]. These observations prompted investigations into the role of mMCP-1 in barrier integrity during schistosome infections, but no functional role was found in this setting[117]. However, mast cell secretion of pro-angiogenic factors could support egg extravasation[118]. The strong Th2 milieu may

simply promote the recruitment of eosinophils and mast cells to the area, where it is possible that both cells types represent (minor) innate sources of Th2 associated cytokines, and so help sustain granulomas.

The alarmins IL-33, IL-25 and TSLP (thymic stromal lymphopoietin) are important initiators of type 2 immune reactions that are released from the epithelium, endothelium and other stromal compartments upon damage and stress[119]. Although yet to be formally demonstrated, their release could be triggered by egg migration. While individual ablation of TSLP, IL-25 or IL-33 has no discernible impact on hepatic granuloma formation and fibrosis during chronic *S. mansoni* infection, simultaneous blockade of all three signaling pathways results in a modest decrease in Th2 associated pathology by 9 weeks of infection, including a reduction in granuloma-associated eosinophilia, hepatic fibrosis and IL-13 producing type 2 innate lymphoid cells (ILC2s)[120,121]. However, such effects were transient in nature and no longer significant by 12 weeks post infection, suggesting that these alarmins are dispensable for the development and maintenance of egg-induced pathology [121]. Similarly, the observed reduction in ILC2 activity appeared to be compensated for by enhanced CD4+ T cell responses, indicating that ILC2s also have no major function in the maintenance of type 2 inflammation in this particular setting. Considering the essential role of Th2 responses in granuloma formation and egg expulsion, it makes sense that compensatory mechanisms are at play, and that schistosomes are not overly reliant on the unregulated release of stromal mediators for development or type 2 immunity. However, given that ILC2s have been shown to be important initiators of Th2 responses during gastrointestinal helminth infections, and to significantly contribute to wound repair responses at mucosal surfaces, further studies are required to reach a formal conclusion on the involvement of ILC2s in schistosome-associated Th2 inflammation[122–124]. In addition, although the aforementioned study signposts the redundancy of alarmins in type 2 immunity during schistosome infection, there remains the possibility that they contribute to regulatory cell expansion during chronic stages of disease[125].

Exiting the epithelium

Tight junctions (TJs) are the final hurdle that schistosome eggs must overcome to transit through the intestinal wall and exit the host. TJs are multi-protein complexes that tightly link adjacent IECs at their apical membranes, creating virtually impermeable seals. In this manner, TJs regulate para-cellular permeability (the movement of substances between adjacent cells) and are essential for maintenance of intestinal barrier function[126]. In scenarios of TJ disruption, intestinal barrier function is impaired, allowing for enhanced permeation of luminal substances (such as bacteria, Ags, toxins

and metabolites) into mucosal tissues and the systemic circulation[126]. Both experimental models and human infection studies hint towards increased intestinal permeability during schistosome infections**.** Mice experimentally infected with *S. mansoni* demonstrate reduced ileal integrity from 8 weeks post infection, and *S. mansoni* infections in humans are associated with extremely high levels of endotoxin in the bloodstream[117,127]. In addition, the lethality of experimental schistosomiasis in scenarios of AA macrophage impairment is partially attributed to mass disruption of the intestinal wall and sepsis, with a common gastrointestinal symptom of intestinal schistosomiasis being blood in stool[64,128].

One future goal is to better define schistosome-epithelial interactions. Namely, is intestinal epithelial disruption solely driven by damage caused by egg migration and/or, do schistosomes actively modulate components of the epithelial barrier to favour egg migration? In a unique model of urogenital schistosomiasis, in which *S. haematobium* eggs are directly introduced to the bladder wall, eggs were shown to suppress the transcription of multiple genes implicated in urothelial barrier maintenance, including junctional adhesion molecules claudin and uroplakin[129]. These data indicate that schistosome eggs not only physically disrupt TJs as they pierce through the intestinal lining, but also suppress the transcription of various TJ related genes, which could facilitate egg transit. However, in a separate study, *S. japonicum* eggs were instead found to reinforce intestinal epithelial barrier function and relieve inflammation in the TNBS colitis model[130]**.** Crucially, prevention of experimental colitis was accompanied by reduced bacterial translocation and enhanced levels of tight junction molecules (Zo-1 and occludin). This study suggests that eggs can fortify the intestinal barricade to prevent potentially lethal bacterial translocation, and thereby enhance host survival. Perhaps the discrepancies between these two studies reflect the manner of egg introduction: direct egg injection into the bladder wall vs. injection into the peritoneal cavity. More specifically, could the introduction of eggs into the peritoneal cavity influence the function of intestinal cell types? Direct injection of eggs into intestinal tissue, as very recently described[131], may provide a novel approach to study the impact of *S. mansoni* eggs on intestinal barrier integrity.

In addition to direct effects of schistosome eggs, intestinal permeability can be influenced by cytokines such as IFN-γ, TNF and IL-13, as well as epithelial cell apoptosis and exogenous factors such as diet and non-steroidal anti-inflammatory drugs[126,132]. With regards to cytokine-mediated barrier dysfunction, it was recently demonstrated that schistosome eggs are incapable of penetrating through epithelial cells themselves, but require pro-inflammatory mediators TNF and/or IFN-γ to disrupt tight junctions and reduce intestinal epithelial resistance**.** Schistosome eggs

appear not to stimulate production of pro-inflammatory cytokines by DCs, which suggests that other local mediators or cell types may be involved[70,80]. Alternatively, the tissue damage caused by egg transit, compounded by involvement of intestinal bacteria, could conceivably stimulate production of such cytokines[70]. Furthermore, IL-13 has shown to induce epithelial apoptosis and increase the expression of the pore forming TJ molecule claudin-2, indicating that Th2 responses themselves can be instrumental to decreased barrier integrity[132].

Once eggs rupture past IECs they are met by a stratified layer of mucus, whose nature and function in schistosomiasis remains undefined. Mucus secretion is likely enhanced during schistosome infections, given that the IL-4/IL-13 signaling axis is critical to goblet-cell hyperplasia and mucus secretion[133,134]. Whether such alterations influence egg expulsion is unclear. In gastrointestinal nematode infections, mucus mediates the rapid expulsion of these parasites by limiting their motility and preventing their establishment within the gastrointestinal tract[133]. Applying these observations to schistosome infections, it is possible that increased mucus generation leads to accelerated expulsion of eggs into the environment. In addition, since intestinal mucins are capable of instructing important pro-inflammatory functions in DCs, it is tempting to suggest that mucins may contribute to modulation of the inflammatory environment during schistosomiasis[135]. Directly related to this, the exposure of DCs to mucins may be increased by egg-induced disruption of the intestinal barrier.

While larval and adult gastrointestinal nematodes likely interact with IECs for prolonged periods of time, schistosome eggs transiently move past or through them during transit. It is unknown whether these different exposure times influence how IECs sense and respond to the parasite infections. Very recently, intestinal tuft cells (a rare IEC population) were shown to instigate type 2 immunity in response to gastrontestinal nematodes infections[136–139]. As tuft cell research is still in its infancy, defining the interactions between tuft cells and schistosome eggs remains an open and interesting point of further study.

MICROBIAL MEDIATED MIGRATION

In the face of the complexity of the immunopathology they generate, schistosome eggs ultimately transit from the mesenteric vessels via the serosa, the muscularis, the epithelium and the mucosa into the intestinal lumen, where a dense and vibrant microbiota surrounds them. As discussed above, successful egg penetration requires carefully coordinated interactions between host and

parasite. These interactions can be extended to a third partner: the intestinal microbiome (herein, defined as commensal bacteria, viruses and fungi).

Helminths and the microbiome have co-evolved with their mammalian hosts over millennia, so extensive interactions exist between the three parties[140]. Many helminths favour the establishment of defined microbial communities to support their own infectious life cycle and improve the overall wellbeing of the host**.** For instance, *Trichuris muris* uses intestinal bacteria as environmental hatching cues and *Heligmosomoides polygyrus* infections alter cecal microbiome composition, leading to a greater availability of short chain fatty acids (SCFAs) that dampen allergic responses[141,142]. While the interface between host, microbiota and intestinal-dwelling nematodes is beginning to be understood, there is currently a paucity of information on this topic about schistosomes, and whether they also remodel gut microbiome composition to support their own life cycle and lessen host pathology.

In mice, experimental *S. mansoni* infections are accompanied by profound changes in microbiota composition from at the point of egg production, including the expansion of *Akkermansia muciniphila* and bacterial populations from the Family Lactobacillaceae [143] . The expansion of these bacterial communities may favour chronic schistosome infection by elevating the frequency of regulatory cell populations, or repairing egg-induced damage to the intestinal wall[144,145]. Further, a recent study using a broad spectrum of antibiotics showed that bacterial depletion reduces faecal egg excretion and intestinal granuloma development during murine infection with *S. mansoni*[70]. In contrast, worm fecundity and liver pathology was not influenced by antibiotic administration, indicating that intestinal granuloma development and egg transit is particularly dependent on local, bacterially-derived factors. These factors could include bacterially-induced cytokines, such as IFN-γ and TNF, that are needed for tight junction severance[70,126]. Complementing these studies, metabolic analysis of faeces from mice experimentally infected with *S. mansoni* reveals several alterations in gut-bacteria related metabolites from day 41 of infection, including a greater availability of the SCFA, propionate[146]. Additionally, differences in intestinal microbiome structure have been reported between children with or without *S. haematobium* infection[147]. As *S. haematobium* is a urogenital parasite, this observation indicates that the schistosomes have a systemic influence on microbiome composition. Similar to that observed in *Trichuris* infection, it is possible that the schistosome-induced Th2 responses promote the establishment of a particular microbiome[148]. Moreover, as signals from the gut microbiome are known to influence immune responses at both local and distal locations (e.g the lung), it is possible that schistosome-induced alterations to the

intestinal microbiome influence the development and/or protection against a range of diseases[149,150].

THE IMMUNE IMPLICATIONS OF EGG PENETRATION

In mice, granulomatous responses are maximal by approximately 8-10 weeks post infection, before gradually declining in magnitude[151,152]. This decline corresponds with a reduction in lymphocyte proliferation and responsiveness, and represents the transition from acute to chronic disease[77,153,154].Chronic schistosomiasis is characterised by high circulating levels of anti-inflammatory IL-10 and/or TGF-β and the profound expansion of regulatory T cells (Tregs) and B cells (Bregs), which function to suppress potentially deleterious activities of T-helper cells and limit granulomatous pathology[77]. Schistosome eggs are continually produced during this chronic period, but thanks to the immune-modulatory capacities of these regulatory cell networks, the majority of chronically infected individuals do not develop lethal pathology.

Schistosomes drive regulatory cell induction via a variety of immunomodulatory influences[155]. The egg-derived glycoprotein IPSE-1/ alpha-1,was recently shown to promote B cell IL-10 and equip B cells with Treg inducing capacities[156]**.** In the case of worm-derived products, both Cyclophilin A and schistosomal lysophosphatidylserine (lyso-PS) can modulate DC function, leading to preferential expansion of IL-10 producing Tregs[157,158]. Small exosome-like extracellular vehicles (EVs) from schistosomes could also represent untapped and important sources of immunomodulation[159,160]. In addition to such direct effects of worm and egg-derived products on inflammation and the immunological environment, it is tempting to speculate that egg penetration itself will shape the immunoregulatory landscape, perhaps in part via facilitating the systemic spread of immune-modulating luminal molecules.

Such molecules could include LPS and bacterially-derived metabolites. SCFAs, for instance, are known to increase Treg responses, mediate the dampening of allergic airway inflammation in the context of *H. polygyrus bakeri* infection, and down-regulate the pro-inflammatory effector functions of intestinal macrophages[141,150,161,162]. A role for SCFAs in schistosome-driven immune regulation has not yet been demonstrated, but this would make for an interesting point of further study. Intriguingly, although reported endotoxemia levels are 10 times higher in sera from individuals with human schistosomiasis than that observed in lethal toxic shock, study participants have neither poor health nor systemic inflammation[127]. This observation has been suggested to reflect the type of LPS in the blood stream, where the structure of LPS (specifically Lipid A acylation) dictates whether it

has an antagonistic or agonistic effect[127,163]. As the structure of LPS varies between different bacterial communities, potential differences in LPS immunogenicity could reflect schistosome-induced alterations to the gut microbiome[143,163]. Alternatively, it is possible that schistosomes modulate innate cell responsiveness to TLR ligands and/or reprogram innate cell function to acquire a more regulatory phenotype[70,157,164,165]. In support of these hypotheses, schistosome-derived lysophosphatidylserine has been shown to be capable of modifying TLR2 signalling pathways in DCs, leading to altered maturation and enhanced induction of Tregs[157]. In addition, *S. japonicum* infections can increase the survival rate of mice with LPS-induced sepsis, in a mechanism that likely entails the polarisation of macrophages to an M2 phenotype[166,167]. Molecules that may mediate this effect include schistosome-derived cysteine proteases, which were recently shown to protect against sepsis challenge and lower the production of pro-inflammatory cytokines and NO from LPS-stimulated macrophages[168]. Interestingly, similar protective mechanisms appear to be induced during infections with the filarial worm *Litomosoides sigmodontis*[164] and the trematode *Fasciola hepatica*[169]. Notably, *F. hepatica* reduces the release of inflammatory mediators from macrophages via mechanisms of molecular mimicry, and *L. sigmodontis* protects from bacterial sepsis by down-regulating the expression of macrophages genes involved in TLR signaling162,167.

The translocation of bacterial ligands into host tissues likely has immunomodulatory consequences. In the absence of Myd88 signaling[170], which is central to bacterial-innate cell interactions, schistosome infected mice demonstrate impaired Th1 responses and reduced granulomatous responses[170.] The regulatory environment may also be influenced by microbial translocation, where translocated LPS could combine with SEA to trigger the release of inflammasome-derived IL-1β**,** which partakes in Breg induction[171,172].Finally, there remains the possibility that bacterial leakage influences the dynamics of macrophage proliferation and alternative activation at the site of inflammation. For example, during experimental schistosomiasis, it is possible that intestinal bacteria act as a trigger for monocyte recruitment from the bone marrow and resultant macrophage accumulation, as opposed to the in situ proliferation of resident macrophage populations[94,173].

By strategically expanding regulatory cell populations, schistosomes not only limit egg-induced pathology, but can also suppress the development of various inflammatory diseases including allergy[174]. Indeed, many epidemiological studies have found inverse correlations between schistosomiasis and allergies, and in experimental models schistosome infection provides relief against allergic airway inflammation and OVA-induced airway hyper-responsiveness, with optimal protection achieved during chronic but not acute disease stages[175–179]. There is also evidence to

show that schistosome eggs can protect from allergic asthma in the absence of adult worms, despite the strong Th2 responses they evoke[180,181]. A current research goal is to better define the immunomodulatory mechanisms employed by schistosomes, where this new-found knowledge could potentially be used to reengineer and recover regulatory cell function in allergic individuals.

EGGS THAT FAIL

Despite the multiple strategies employed, many eggs fail to exit their mammalian host. Experimental infections indicate that only 20-55% of eggs are successful excreted, while the remainder inevitably become trapped within host tissues[3,4]. Although egg deposition is targeted for the urinary bladder (*S. haematobium*) and the intestine (S*. mansoni* and *S. japonicum*), eggs swept systemically through the blood stream can readily be detected at various other locations including the eyes, skin, kidney, spleen and central nervous system (CNS)[3,17]. Egg deposition at intended and unintended sites can have serious pathological consequences for the host. The more severe disease complications generally manifest many years after infection, reflecting gradual egg accumulation in host tissues and the resolution of granulomas by fibrosis and calcification[128].

This aspect of schistosome infection is perhaps most evident in liver pathology during *S. mansoni* and *S. japonicum* infection. Granuloma formation around hepatic egg deposits leads to severe fibrosis, blood flow obstruction and, subsequently, the formation of ascites and blood vessels that bypass the liver (portosystemic shunting)[128]. If these vessels rupture, life-threatening bleeding may follow. Pathology in the intestine is generally less severe than in the liver, and can be characterized by pseudopolyposis, ulceration and stricture formation. In *S. haematobium* infections, egg entrapment within pelvic organs (urinary bladder, ureters, cervix, vagina, prostate gland and seminal vesicles) can also result in severe pathology, including obstructive bladder and ureteral fibrosis, bladder cancer, kidney failure and the formation of gross genital sores[128,182]. In females, these lesions may give rise to infertility, ectopic pregnancies and menstrual irregularities, while in men, higher rates of sperm apoptosis have been reported[182,183].

Importantly, reduced integrity of the genital epithelium constitutes a significant risk factor for the acquisition of HIV and other pathogens, including oncogenic viruses and bacteria[184,185]. Indeed, a recent longitudinal study in Tanzania demonstrated a clear gender bias in schistosomiasis and HIV acquisition, with schistosome-infected women demonstrating a 3 fold higher chance of acquiring HIV than uninfected women, whereas odds remained the same for men with or without infection[186]. The increased risk of HIV acquisition in females very likely reflects the local physical changes caused

by schistosome eggs at the female genitalia[184].T he sequestration of eggs within the mucosal tissue of the vagina and cervix leads to ulceration, erosion and the formation of tiny cervical abnormalities (yellow and/or grainy sandy patches) that are surrounded by an irregular network of blood vessels. These blood vessels are believed to represent egg-induced angiogenesis and could accelerate the transmission of HIV during intercourse[51,184]. In contrast, *S. haematobium* infected men are at lesser risk of acquiring HIV because eggs do not infiltrate male genital organs that are exposed to the virus. However, other studies have shown mild associations between male urogenital schistosomiasis and HIV-1 acquisition, and even though *S. mansoni* eggs do not localise to the genitalia, *S. mansoni*-infected individuals also appear more susceptible to HIV[184,186,187]. These associations are believed to be a consequence of schistosome-associated immunomodulation, as opposed to local tissue damage caused by eggs[184]. In particular, the co-receptors for HIV are more highly expressed on the surface of CD4+ T cells from schistosome-infected individuals, allowing for greater viral uptake[187]

Similar to HIV, there is a clear link between urogenital schistosomiasis and bladder cancer, with promotion of carcinogenesis likely due to *S. haematobium* enhancing entry sites for oncolytic viruses and bacteria[185,188]. Alternatively, egg-driven inflammation may have a bystander effect on host cells, or immunosuppressive cytokines (produced during chronic stages) may reduce host ability to clear oncolytic viral and bacterial infections. In addition, *S. haematobium* eggs have shown to directly influence the transcription of carcinogenesis-associated genes within the bladder wall[129.] This effect could potentially be mediated by schistosome-derived estrogenic molecules, albeit their carcinogenic activity has yet to be defined[189].

Curiously, while a clear association between *S. haematobium* and bladder cancer has been established, there is limited and controversial evidence to implicate S. *japonicum* and S*. mansoni* in intestinal and liver cancer[185,188,190]. The reason for these strain-specific differences is unclear, but it has been suggested this reflects the site of egg deposition and/or the contribution of environmental carcinogens, including tobacco smoke or industrial and agricultural dyes[185,188].

The spreading of eggs to the CNS (neuroschistosomaisis) is one of the more devastating outcomes of schistosome infection that can result in seizure or paralysis, depending on brain or spinal cord involvement. While *S. japonicum* eggs are more frequently found in the brain, eggs from *S. mansoni* and *S. haematobium* appear to have a predilection towards the spinal cord[128]. These differences could reflect the smaller size of *S. japonicum* eggs and/or their absence of a protruding spine[128]. Moreover, given that schistosome eggs disperse to many ectopic locations, it is possible that they affect co-infections encountered at these sites too. At a local and mechanistic level, the

inflammatory environment established by eggs may exacerbate already established pathology or create an environment that favours other pathogen survival. At an immunological level, schistosome co-infections may suppress immune responses towards viral and bacterial Ags, leading to ineffective pathogen clearance and chronicity[191,192]

CONCLUSION AND OUTLOOK

How schistosome eggs successfully exit the host body has long been a focus of many researchers. Very early studies revealed the severity of egg-driven pathogenesis and the curious migration patterns taken by adult worms to reach favored sites of oviposition[16,17,50,182]. In more recent years, research has shed light on the molecular and immunological mechanisms that govern this process. Within host blood vessels, schistosomes establish a suitable environment for maturation, movement and egg extravasation by interfering with host haemostasis and angiogenesis[9,10]. For intestinal egg passage and granuloma formation, schistosomes actively tamper with host immune responses, to achieve a delicate balance between immune effector and immune regulatory activity, and to limit bystander tissue damage. However, despite the exit strategies employed, egg transit is not a certainty and lethal pathology may follow egg entrapment.

Our understanding of the interactions between schistosomes and the mammalian host is continually growing, with many interesting avenues still open for exploration (*See 'Outstanding questions' box).* For example, while schistosome infection influences gut microbiome composition, we are yet to define how these fluctuations impact egg migration, disease pathogenesis, immunomodulation and long-term host/parasite survival[143]. With powerful sequencing techniques emerging and our knowledge of the intestinal microbiome constantly expanding, we anticipate that the relationship between the mammalian host, schistosomes and the microbiome will soon be much better understood. Moreover, we are optimistic that the study of schistosomes will continue to increase fundamental understanding of the mechanisms governing immune-regulation and type 2 immunity. In turn, this could lead to the identification and generation of new vaccine candidates and targets for the treatment of egg-induced pathogenesis in schistosomiasis, as well as other type 2 inflammatory diseases.

Outstanding questions

1. Recent studies show schistosome infections to influence the composition of the gut microbiota. Do these changes reflect bacterial adaptations to the inflammatory environment established by schistosomes infections, or do schistosomes actively promote the colonization of select bacterial communities? Furthermore, do these microbiome fluctuations influence i) egg excretion, ii) pathogenesis, iii) the immune landscape and/or iv) schistosome long-term survival?

2. Which exact molecules mediate egg binding to the vascular endothelium, and which process(s) ultimately allow for egg extravasation?

3. The effects of schistosome infections on cancer require further attention. Notably, why is the association between carcinogenesis and schistosome infection stronger for S. haematobium than for S. mansoni and S. japonicum?

4. Many cells of the intestinal immune system have undefined roles in the instigation and maintenance of type 2 immunity during schistosome infection. Notably, what is the actual function of eosinophils and mast cells in the granuloma? Are chemosensory tuft cells involved in egg detection and the initiation of type 2 responses? Which intestinal macrophage subsets are involved? Do ILC2s participate in these immune reactions?

5. Do worms and/or eggs secrete molecules that influence host intestinal barrier integrity? Does the secretion of such molecules promote egg penetration through host tissues?

6. Which co-infections/pathologies in the gut are worsened by the inflammatory environment established by eggs?

7. Does egg migration trigger the release of alarmins (e.g IL-25, IL-33 and TSLP) in the intestine, and do these molecules influence the development of Th2 or regulatory responses in the gut?

8. Serosal immune responses are poorly characterised. Do serosa-resident immune populations play a role in egg migration and how do eggs transit through these tissues?

9. Mucins - Do they play a functional role in intestinal egg-migration or local immune modulation?

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CHAPTER 3

Dynamics of host immune response development during *Schistosoma mansoni* infection

Alice H. Costain, Alexander T. Phythian-Adams, Stefano A.P. Colombo, Angela K. Marley, Christian Owusu, Peter C. Cook, Sheila L. Brown, Lauren M. Webb, Rachel J. Lundie, Hermelijn H. Smits, Matthew Berriman and Andrew S. MacDonald

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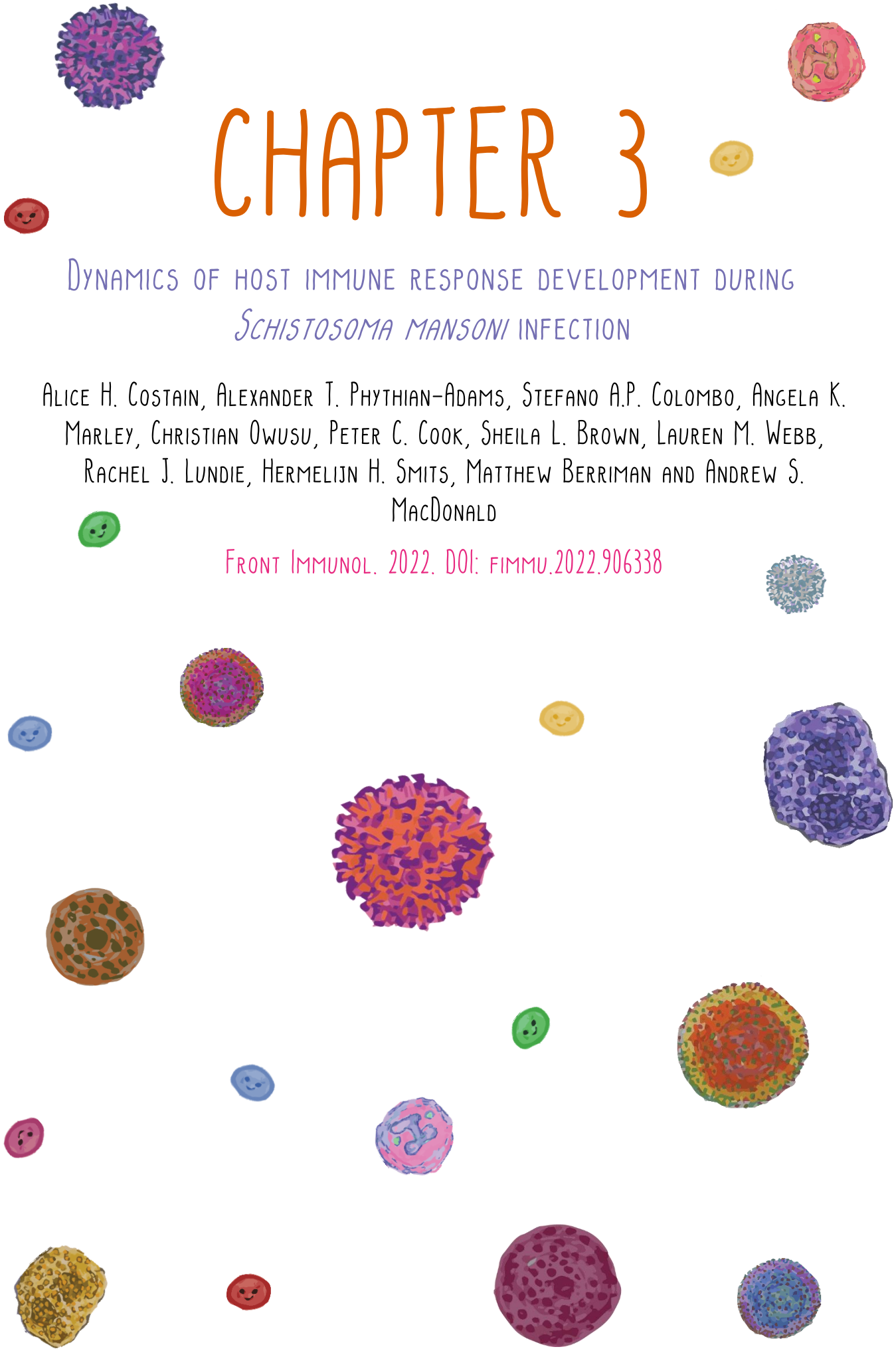


Dynamics of host immune response development during *Schistosoma mansoni* infection

AUTHOR INFORMATION

Alice H. Costain[1,2†], Alexander T. Phythian-Adams[1†], Stefano A.P. Colombo[1†], Angela K. Marley[1], Christian Owusu[3], Peter C. Cook[1,4], Sheila L. Brown[1], Lauren M. Webb[1,5], Rachel J. Lundie[6], Hermelijn H. Smits[2], Matthew Berriman[3,7] and Andrew S. MacDonald[1*]

[1]Lydia Becker Institute of Immunology and Inflammation, University of Manchester, UK

[2]Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands

[3]Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, UK

[4]MRC Centre for Medical Mycology, University of Exeter, UK

[5]Department of Immunology, University of Washington, USA

[6]360biolabs, Melbourne, Australia

[7]Wellcome Centre for Integrative Parasitology, University of Glasgow, UK

[†]These authors contributed equally

Corresponding author: Andrew S MacDonald (andrew.macdonald@manchester.ac.uk)

ABSTRACT

Schistosomiasis is a disease of global significance, with severity and pathology directly related to how the host responds to infection. The immunological narrative of schistosomiasis has been constructed through decades of study, with researchers often focussing on isolated time points, cell types and tissue sites of interest. However, the field currently lacks a comprehensive and up-to-date understanding of the immune trajectory of schistosomiasis over infection and across multiple tissue sites. We have defined schistosome-elicited immune responses at several distinct stages of the parasite lifecycle, in three tissue sites affected by infection: the liver, spleen, and mesenteric lymph nodes. Additionally, by performing RNA-seq on the livers of schistosome infected mice, we have generated novel transcriptomic insight into the development of schistosome-associated liver pathology and fibrosis across the breadth of infection. Through depletion of $CD11c^+$ cells during peak stages of schistosome-driven inflammation, we have revealed a critical role for $CD11c^+$ cells in the co-ordination and regulation of Th2 inflammation during infection. Our data provide an updated and high-resolution account of how host immune responses evolve over the course of murine schistosomiasis, underscoring the significance of $CD11c^+$ cells in dictating host immunopathology against this important helminth infection.

Keywords

Schistosomiasis, Dendritic cells, Pathology, Chronic infection, CD11c, transcriptomics

Abbreviations

Antigen (Ag); Antigen presenting cells (APCs); Dendritic cells (DCs); Interleukin (IL); Masson's Trichrome (MT); Mesenteric lymph nodes (MLN); Regulatory B cells (Bregs); Regulatory T cells (Tregs); Schistosome soluble egg Ag (SEA); T helper (Th); Weeks post infection (WPI).

Schistosomiasis is a complex and potentially life-threatening parasitic disease, caused by infection with blood flukes of the genus *Schistosoma. S. mansoni,* S. *haematobium* and S. *japonicum* are responsible for most human cases, with approximately 240 million individuals infected globally, predominantly clustering in specific regions of Africa, Asia, the Middle East, and South America[1].

Schistosomiasis has a distinctive triphasic immune profile that reflects transformative events within the parasite lifecycle[2]. In brief, the host first progresses through a (pre-patent) acute phase (0-5 WPI; wks post infection), where it responds to immature, lung migrating schistosomula and developing worm pairs[3]. In the second (post-patent) acute phase (5-12 WPI), the host is confronted daily with hundreds of highly immunogenic eggs and must contend with accompanying inflammation and associated tissue damage[4]. In the final chronic phase (12 WPI+), the host enters a more regulated or 'exhausted' state that follows prolonged exposure to parasite antigens (Ags)[5,6]. At each lifecycle stage schistosomes employ a wide range of immune evasion and manipulation strategies that enable mitigation against collateral tissue damage and promotion of long-term survival within the host[7]. For example, as an appropriately regulated T Helper (Th) 2 response is essential for host and parasite survival[8–10], schistosomes produce an array of molecules that guide host immunity towards a Type 2 phenotype[11–17], whilst concurrently encouraging the expansion of regulatory networks that downregulate and resolve chronic inflammation[14,15,18–20].

The immune response triggered at each lifecycle stage has been thoroughly dissected in experimental murine infections and, to a lesser extent, human and primate work. Together, the following immune narrative has been established: during the initial 4-5 wks of pre-patency, migrating schistosomula and juvenile worms evoke a low-level mixed T helper 1 (Th1)/Th2 immune response[21–23]. This profile takes a dramatic shift at around 5-6 WPI, with egg production skewing host immunity towards a Th2-dominated profile, including dramatic upregulation of interleukin (IL-) 4, IL-5, IL-13, IgE and eosinophilia. This Type 2 environment dwarfs and counteracts the early Type 1 component[21], peaks approximately 8-10 WPI, and is critical for coordinated granulomatous inflammation[8,10,24]. Moreover, although Th17 responses are acknowledged during schistosomiasis, they are generally considered low level in relation to Th2 or Th1 responses, and typically only emerge in specific mouse strains (i.e. CBA mice but not C57BL/6 or BALB/C)[25,26]. The majority of data suggests Th17 responses to promote immune pathology rather than benefit the host[27,28]. As infections progress into chronicity, Type 2 responses decline and regulatory responses prevail[5,29–31]. Down-modulation of Type 2 responses and suppression of severe disease is thought to be primarily

mediated by IL-10[32], with its secretion attributed to Regulatory B cells (Bregs)[33,34] and T cells (Tregs)[30,35–37]. Tissue fibrosis (scarring) is central to schistosomiasis associated pathology, particularly in the liver, with IL-13 proposed as the main pro-fibrotic mediator in mice[38]. This immunological picture has been established over decades of research, often focusing on a single tissue site, timepoint or event within the schistosome lifecycle. While time-course studies exist, very few have simultaneously evaluated the Th1/Th2/regulatory balance over the course of infection, in a range of tissue sites. Pioneering work in the early 1990s provided two splenic time-course studies, which form the basis of our understanding of the Th1-Th2 switch from the point of egg production[21,22]. However, these studies reflect the technical limitations of the time, and have received minimal follow-up with the resolution that is possible today.

Dendritic cells (DCs) are central players in the host immune system, poised to recognize and respond to invading threats and inflammation, and translate these danger signals into the activation and differentiation of T cells[39]. DCs have proven key to orchestration of Th1 and Th2 inflammation, as well as the promotion of regulatory responses. In the context of schistosomiasis, we have previously shown that they play a fundamental role in the priming of $CD4^+$ T cell responses, with depletion of $CD11c^+$ DCs early in murine *S. mansoni* infection (wks 4 – 6) leading to a stark reduction in splenic and hepatic Th2 cells (IL-4, IL-5, and IL-13) and Tregs[40]. However, the involvement of DCs in maintenance and/or regulation of schistosome infection beyond wk 6 remains unclear. Similarly, it is currently unknown whether DCs in later stages of infection remain vital for coordinating $CD4^+$ T cell function and cytokine secretion, or if other antigen presenting cells (APCs) adopt more dominant roles in this context.

We now provide a comprehensive account of schistosome-elicited immune responses over the course of infection and across multiple tissue sites (liver, spleen, and mesenteric lymph nodes [MLNs]). In addition to refined characterization of cellular networks and cytokine dynamics across these compartments, we have interrogated how the host responds to infection and attempts to resolve egg-driven damage by RNA-seq analysis of liver samples isolated over the course of infection. Finally, we have revealed a critical role for $CD11c^+$ cells in the maintenance of hepatic Type 2 inflammation, with CD11c depletion from 6 WPI leading to compromised cytokine production and altered granulomatous pathology. These novel data elevate and update our fundamental understanding of immune response development over the course of murine schistosomiasis, and the significance of $CD11c^+$ cells in coordinating immunopathology against this important and relevant helminth infection.

Mice

Experiments were performed using female C57BL/6 mice (Envigo), CD11c.DOG[41] x C57BL/6 or CD11c.DOG x 4get IL-4-eGFP F1 mice[42], housed under specific pathogen free conditions at the University of Manchester or the University of Edinburgh, and used at 8-12 wks of age. Experiments were performed under a project license granted by the Home Office UK following ethical review by the University of Manchester or University of Edinburgh, and performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

Schistosoma mansoni infections and cell depletions

Biomphalaria glabrata snails infected with *S. mansoni* parasites were obtained from Biomedical Research Institute, Rockville, MD. Mice were percutaneously infected[43] with 40-80 *S. mansoni* cercariae, with infections lasting 4, 6, 8, 12 or 15 wks in duration. To quantify parasite burden, *S. mansoni* eggs were isolated and counted from pieces of liver (approx. 1g in weight) or intestine, and digested overnight in 5% KOH[43]. For CD11c depletion experiments, mice received daily intraperitoneal injections with 8 ng/g diphtheria toxin (Sigma-Aldrich) in PBS or with PBS alone[24,41], from wk 6 of infection (days 42-51), and culled on day 52.

Cell isolation

Single-cell suspensions of liver, MLN and spleen were prepared using the following methods, and as described previously[24]. Spleens and MLNs were diced and digested at 37°C with 0.8 U/ml Liberase TL and 80 U/ml DNase I type IV in HBSS (all Sigma), supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen). After 30 min the digestion reaction was halted by the addition of 100 µl/ml 0.1 M, pH 7.3, EDTA (Ambion) stop solution, followed by addition of DMEM containing 50 U/ml penicillin and 50 µg/ml streptomycin. This was then passed through a 70 µm cell strainer to generate a cellular suspension, with splenocytes undergoing an additional RBC lysis incubation to remove contaminating erythrocytes. Livers were perfused, minced with sterile scalpel blades, and incubated at 37°C for 45 min using the digestion media detailed above. After addition of EDTA stop solution and DMEM, digested livers were passed through a 100 µm cell strainer. To separate leukocytes, liver cells were resuspended in 33% isotonic Percoll (GE healthcare) and centrifuged at 700 g. Pelleted cells were passed through a 40 µm cell strainer (for removal of *S. mansoni* eggs), followed by RBC lysis, counting and resuspension in PBS supplemented with 2% FBS, 2 mM EDTA, for flow cytometry or culture/stimulation.

Quantification of cytokines

Cytokine assays were performed as previously described[44,45]. Single-cell suspensions of livers, MLNs and spleens were stimulated *ex vivo* with plate bound anti-CD3 (0.5 µg/well) or SEA (0.25 µg/well) in X-vivo 15 media (Lonza) supplemented with 2 mM L-Glutamine and 50 µM 2-ME (Sigma). Stimulation was carried out in 96 well U bottom plates, with 1 x 10^6 cells seeded per well in 200µl volumes. Cell supernatants were harvested 72 h later and stored at -20°C until further analysis. Paired capture and detection Abs were used for analysis of murine IL-4, IL-10, IL-17A (Biolegend) IL-5, IFNγ (produced from hybridomas in-house) and IL-13 (eBioscience).

Serum antibody and IgE levels

SEA-directed antibodies and IgE levels were measured as described previously[24]. In brief, to quantify serum levels of SEA-specific IgG, IgG1, IgG2a, IgG2c and IgG3, 96 well plates (NUNC Maxisorp) were coated with 5 µg/mL SEA overnight, then blocked with 1% bovine serum albumin (BSA, Sigma) in PBS for 1 h at room temperature. Plates were then incubated with sera at doubling dilutions (1:50-1:6400). Alkaline phosphatase conjugated goat anti mouse IgG, IgG1, IgG2a, IgG2c and IgG3 (SouthernBiotech) were added to plates, followed by liquid PNPP substrate, with absorbances read at 405nm. Serum levels of IgE (BD) were measured using paired capture and biotinylated detection antibodies, with quantity assessed via standard curve and absorbances read at 450nm. For all ELISAs, between individual incubation steps, plates were washed 3 times with PBS containing 0.05% Tween (Sigma). Data presented as endpoint dilutions.

Flow cytometry

Single cell suspensions were washed with PBS and stained for viability with ZombieUV (1:2000; Biolegend) or LIVE/DEAD™ Fixable Aqua (1:500; Invitrogen). Cells were then incubated with 5 µg/ml FC block (αCD16/CD32; 2.4G2; Biolegend) in FACS buffer (PBS containing 2% FBS and 2 mM EDTA), before staining for surface markers for 30 min. After surface staining, cells were washed twice with FACS buffer before fixation in 1% PFA for 10 min. For intracellular staining, cells were permeabilised with eBio Fixation/Permeabilisation buffer for 1 h, before staining with relevant intracellular antibodies for 30 min. Samples were analysed by flow cytometry (LSR Fortessa, BD) and data analysed using FlowJo v10 software. A list of antibodies used is provided **Table 1**.

Imaging

For histological analysis by light microscopy, median liver lobes were fixed in 10% NBF for 24 h, dehydrated through a series of graded alcohols and embedded in paraffin blocks. Tissues were cut into 5 µm thick sections using a microtome and stained with Masson's Trichrome (MT;

granulomatous inflammation). For quantification of granulomatous pathology and fibrosis, MT stained liver sections were scanned with a dotslide digital slide scanner (Olympus BX51, VS-ASW FL Software), creating bright field (BF) overview images of the whole slide (see Fig. 2.1). Using FIJI imaging analysis software (ImageJ 1.48r)[46] individual liver sections were cropped to obtain solely liver sections. Colour thresholds were set using hue, saturation and brightness (HSB) settings to select either granulomas alone (blue staining) or liver parenchyma (purple staining). HSB thresholds were set using an algorithm developed by Dr. Tim Kendall (Western General Hospital, Edinburgh), enabling the quantification of the number of pixels for the region of interest.

For immunohistochemistry (IHC) analysis by confocal miscopy, pieces of the left liver lobe were placed in optimum cutting temperature formulation (OCT) and stored at -80°C until further processing. Tissues were cut into 20 µm cyrosections, dried overnight at RT, then fixed in ice-cold acetone for 5 min before storage at -20°C. For confocal Immunohistochemistry, cryosections were thawed and rehydrated with 1X PBS before incubation for 1 h at RT with 10% blocking solution: 1% BSA, 10% FCS and 0.005 µg/µl FcR-Block (2.4G2) in PBS-T (Tween-20; 1:1000 in PBS). Samples were incubated overnight at 4°C with fluorescently labelled monoclonal antibodies (all eBioscience) for CD11c (N418; 1:250), Siglec-F (E50-2440; 1:100) TCRβ (H57-597; 1:50), in 2% blocking solution (0.2% BSA, 2% FCS and 0.005 µg/ul FcR-Block in PBS-T). Stained cryosections were washed once with PBS-T followed by 2 washes with PBS, then stained with 1 µg/ml DAPI dissolved in PBS for 15 min at RT. Cryosections were subsequently mounted with coverslips using ProLong Gold antifade reagent (LifeTechnologies) and blinded before analysis. Analysis of confocal photographs was carried out using Volocity imaging software (PerkinElmer). For each photograph, the contrast was enhanced to remove background staining and schistosome eggs were cropped out of individual images to exclude possible autofluorescence during subsequent quantification steps. Snapshots of cropped images were captured separately for each fluorochrome used, and for each snapshot the surface area of positive staining above a fixed intensity was measured. Intensity was set using isotype controls for each fluorochrome labelled.

RNA extraction and sequencing

RNA extraction was performed in line with Peña-Llopis & Brugarolas[47]. In brief, liver tissue samples weighing between 15 mg and 40 mg were homogenized in lysis buffer, followed by an acid-phenol chloroform extraction of total RNA. Polyadenylated mRNA was purified from liver total RNA using oligo(dT) Dynabead selection, followed by metal ion hydrolysis fragmentation with the Ambion RNA fragmentation kit. First strand cDNA was synthesized using randomly primed oligos followed by

second strand synthesis using DNA polymerase I to produce double-stranded cDNA. This was followed by end repair with T4 and Klenow DNA polymerases and T4 polynucleotide kinase to blunt-end the DNA fragments. A single 3' adenosine nucleotide was added to the repaired ends using a Klenow fragment (3'→5' exo-) and dATP to deter concatemerization of templates, limit adapter dimers and increase the efficiency of adapter ligation. Adapters were then ligated (containing primer sites for sequencing). Ligated fragments were run on an agarose gel, size selected for 100-200 bp inserts and the DNA extracted according to the Qiagen gel extraction kit protocol, except for the dissolution of gel slices, which was done at room temperature rather than 50°C. Libraries were then amplified by PCR to add primers for flow cell surface annealing and to increase yield; sample cleanup was performed with AMPure SPRI beads. The libraries were quantified on an Agilent Bioanalyzer and Kapa Illumina SYBR Fast qPCR kit, and sequenced on the Illumina HiSeq 2500 platform as 75 bp paired end reads.

RNAseq analysis

mRNA reads were aligned to the mouse reference transcriptome (GRCm38) using Bowtie2 v2.2.1[48] and quantified using eXpress v1.3.0[49]. Briefly, the mouse cDNA reference was downloaded from the Ensembl Genomes FTP server and indexed prior to mapping using the Bowtie2-build command. Reads were aligned with Bowtie2 using default parameters apart from -X 800, which sets the maximum fragment length for concordant pairs at 800 bp, thus ensuring that reads from all valid fragments are considered; and -a, which reports all alignments. The Bowtie2 alignments were passed directly to eXpress for transcript quantification. As eXpress reports transcript-level counts, a custom perl script was used to aggregate and collapse the reported counts into their corresponding gene-level counts. The results were organized into a tab-delimited matrix with each row representing a gene and each column representing a sample.

Read counts were normalised and differential expression analysis performed using the DESeq2 package[50]. Genes with low expression were removed prior to downstream analysis by removing those where the sum of the total read counts in all samples was fewer than 50. Gene names were assigned to ensembl IDs using the biomaRt package[51]. Principle component analysis was performed using the DESeq2 package. For the production of volcano plots and heatmaps genes were considered significantly differentially expressed between conditions if they had an adjusted p value (padj) < 0.01 and a log2FoldChange in expression >1. Gene set enrichment analysis for GO terms associated with biological process was performed using the clusterProfiler package[52]. Genes with

padj < 0.01 (both up- and down-regulated) were selected for gene set enrichment analysis. The statistical significance of enriched terms was adjusted using the Holm method. GO terms with a padj > 0.01 were considered statistically significant. Redundant GO terms were removed using the 'simplify' function in clusterProfiler using a cutoff value of 0.5 when selecting by padj.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 or JMP software. As described in corresponding figure legends, experimental groups were compared using an unpaired t-test, one-way or two-way ANOVA, followed by appropriate post-hoc tests. Significance for all statistical tests was shown in figures as P < 0.05 (*), P < 0.01 (**), P < 0.001 (***)

Company	Target	Clone		Company	Target	Clone
BD	Siglec-F	E50-2440		Biolegend	Ly6G	1A8
Biolegend	CD11b	M1/70		Biolegend	PDCA-1	927
Biolegend	CD11c	N418		Biolegend	XCR1	ZET
Biolegend	CD169	3D6.112		Ebioscience	B220	RA-6B2
Biolegend	CD172a	P84		Ebioscience	IL-13	ebio13A
Biolegend	CD26	DPP-4		Ebioscience	IL-5	TRFK.5
Biolegend	CD4	RM4-5		Ebioscience	MHCII	MS/114.15.2
Biolegend	CD45	30-f11		Ebioscience	Ter119	TER-119
Biolegend	CD64	x54-5/7.1		Invitrogen	CD19	Ebio(ID3)
Biolegend	CD8	53-6.7		Invitrogen	CD3	17A2
Biolegend	F4/80	BM8		Invitrogen	FoxP3 (IN)	FJK-16s
Biolegend	IFNy	XMG1.2		Invitrogen	NK1.1	PK136
Biolegend	IL-10	JES5-16E3		Invitrogen	TCRβ	H57-597
Biolegend	IL-17	TC11-1810.1		Biolegend	CD25	PC61
Biolegend	IL-4	11B11		Biolegend	CD49b	HMα2
Biolegend	Ly6C	HK1.4				

Table 1. A list of Flow cytometry antibodies and their clones

Dynamics and development of hepatic pathology

Experimental schistosome infections of mice are commonly employed to model the pathological and immunological features of human infections[53,54]. To assess host responses at several distinct stages of schistosomiasis, mice were percutaneously infected with 40 cercariae, and animals taken for analysis after 4, 6, 8, 12 or 15 wks (Figure 1A). In brief, these wks correspond to pre-egg production/adult pairing and maturation (wk 4), the initial phase of egg production (wk 6), the peak of post-patent active disease (wk 8), progression into chronic disease (wk 12) and established chronicity (wk 15)[2,3 4]. Egg-driven granulomatous responses generally peak at 8 wks post infection before declining in magnitude, with calcified and fibrotic granulomas accumulating during chronic infection stages, coincident with the immune response against freshly laid eggs being down-regulated[37,55,56]. Importantly, as egg production is an ongoing, asynchronous process, infected tissues simultaneously present with newly formed, mature and resolving granulomas. To visualise the extent of granulomatous inflammation in the liver, sections from the median lobes of infected mice were stained with Masson's Trichrome (MT) (Figure 1B). Before the start of egg-laying (wk 4), there were no visible signs of damage or eggs in the livers of infected mice, whilst at later infection stages, eggs continued to accumulate within hepatic tissue (Figure 1B & Supplementary Figure 1A). Granulomatous inflammation became more pronounced at each ensuing stage, with infected livers showing extensive MT staining from wk 8, which accounted for approximately 50% of the total tissue section by wks 12 and 15 (Figure 1C). Closer assessment of wks 6-8 revealed that granulomatous inflammation in this transition phase started at approximately 45 days post infection, with significantly greater inflammation visualised by day 51 (Supplementary Figure 1B&C) and with evidence of enhanced DC, T cell and eosinophil infiltration to hepatic tissue, as assessed by confocal microscopy paired with quantification of $CD11c^+$, $TCR\beta^+$ and $Siglec\text{-}F^+$ staining (Supplementary Figure 1D). Furthermore, mirroring the exaggeration of hepatic pathology during later stages of infection (Figure 1B&C), infected mice displayed significantly higher serum levels of SEA-specific antibodies and IgE by wks 12 and 15 (Supplementary Figure 1E&F), as well as hepatosplenomegaly from wk 8, as defined by increased spleen and liver weight as a proportion of total body weight (Supplementary Figure 1G).

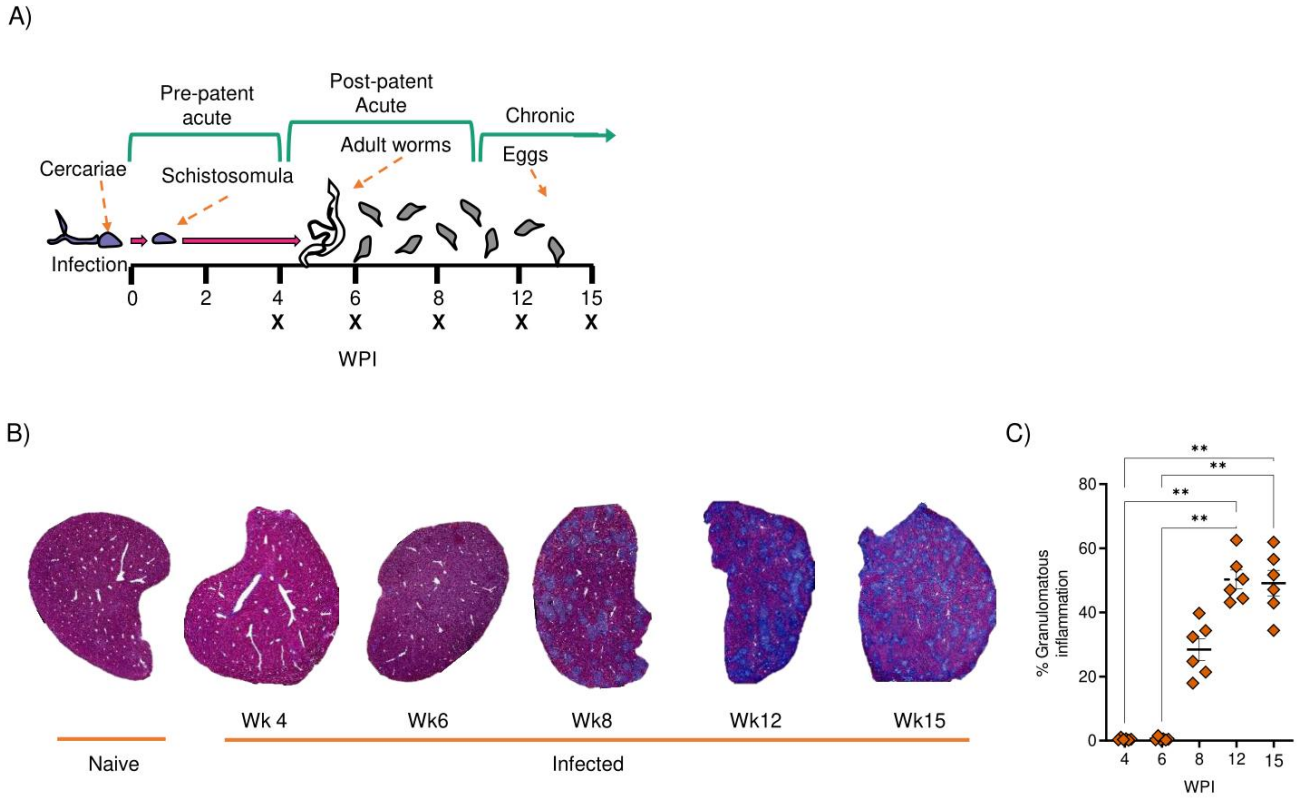
Tissue-specific cytokine responses during infection

Next, to define how parasite-specific cytokine responses develop across infection, we focussed on three tissue sites that are differentially affected by schistosomiasis: the liver, spleen, and MLNs.

More specifically, while the liver represents a principal effector site in which eggs deposit and granulomas form, the spleen and MLNs represent important immune priming sites against parasite Ags from systemic circulation and intestinal sites, respectively.

To evaluate Ag-specific cytokine secretion, isolated liver, spleen and MLN cells were cultured in the presence of SEA (Figure 1D). Irrespective of the tissue site in question, immune cells from infection produced very low levels of SEA-specific Th2 associated cytokines (IL-4, IL-5 and IL-13) at wk 4 post infection, with no statistically significant differences relative to naïve mice. In contrast, the Th1 associated cytokine IFNγ peaked at this time-point in infected animals. As infections progressed into wk 6 and beyond, splenic, MLN and hepatic IFNγ dramatically reduced. All tissues showed increased production of IL-4, IL-5 and IL-13 from wk 6, which either peaked at 8-12 wks post infection or remained elevated into chronic stages. IL-10 responses tended towards an increase during later stages of infection, which was particularly evident in the MLNs and liver. Similar cytokine profiles were seen upon stimulation of isolated cells with anti-CD3 (Supplementary Figure 2). This included peak Th2 cytokine potential at approximately 8 wks post infection, and increased IL-10 during chronicity, particularly in the liver and MLNs (Supplementary Figure 2). Moreover, while SEA-specific IL-17A production was minimal in all three sites, anti-CD3 stimulated liver cells from infected mice produced significantly increased levels of IL-17A than their naïve counterparts.

Figure 1. Development of granulomatous pathology and a dominant Type 2 response during *S. mansoni* infection (A) Schematic of infection setup. C57BL/6 mice were infected with 40 *S. mansoni* cercariae with infections lasting, 4, 6, 8, 12 or 15 wks in duration (indicated by X). (B) Representative images of liver sections stained with Masson's Trichrome (MT) at indicated wks, allowing for visualisation of inflammatory cell infiltration and type I collagen deposition. (C) The proportion of granulomatous inflammation per tissue section, using an objective algorithm to quantify the number of pixels of granulomatous inflammation in a defined region of interest. (D) At specified wks, liver, spleen and MLN cells from naïve and schistosome infected mice were cultured for 72 h with 0.25 µg of schistosome egg antigen (SEA; antigen-specific stimulation). Supernatants were collected and cytokine production (medium alone values subtracted) was assessed by ELISA. Data are from a single experiment (B&C) or pooled from 2 (D) separate experiments (n=36-10 animals per time point). Significance calculated by one-way (C) or two-way ANOVA (D). Data presented as mean +/- SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$


A)
Pre-patent acute
Post-patent Acute
Chronic
Cercariae
Schistosomula
Adult worms
Eggs
Infection
0 2 4 6 8 12 15
X X X X X
WPI
B)
Wk 4
Wk6
Wk8
Wk12
Wk15
Naive
Infected
C)
% Granulomatous inflammation
WPI
D)
Naive
Infected
Liver
Spleen
MLN
IL-4
IL-5
IL-13
IL-10
IFNγ
IL-17A
ng/ml
WPI

Cellular responses to schistosome infection vary across tissues

We next looked to identify the cell types responding at different sites over the course of infection. From as early as 4 wks post infection, despite the lack of egg production, cellular infiltration to the liver was significantly increased in infected animals compared with naïve (Figure 2A). Closer inspection of immune cell numbers (Figure 2A&C) and frequencies (Figure 2B&D) revealed a dramatic increase in hepatic eosinophils, macrophages, DCs, plasmacytoid DCs (pDCs), B cells, $CD4^+$ T cells and NK cells in comparison to naïve mice, but without any significant increase in their frequency. Similarly, while wk 6 saw an increase in overall cell numbers in the liver (aside from pDCs), cellular frequencies remained comparable to naïve controls. However, cell proportions altered significantly by wk 8 of infection, with an increased frequency of hepatic eosinophils, neutrophils, and monocytes, coinciding with a decline in B cell, T cell, NK cell, DC and macrophage proportions. These differences in percentages persisted into later wks of infection. Akin to the earlier observed peak in Th2 cytokine production (Figure 1D), liver cell counts peaked at 8 wks post infection before a gradual decline (Figure 2A). By 15 wks post infection, eosinophils and monocytes were the only cell types found to be significantly more numerous in infected livers than naïve.

Although there were clear differences in basal immune cell composition between priming (MLN and spleen) and effector (liver) sites (Figure 2B), changes in cellularity evoked by schistosome infection were largely comparable in all three tissues. MLN and spleen cell counts remained unchanged at wks 4 and 6 when compared to naïve mice but, similarly to the liver, a significant infiltration in immune cells was observed from wk 8 onwards in these sites that persisted into chronic stages of disease. Eosinophilia was a prominent feature of both MLNs and spleens from the 8 wk time-point onwards, but less dramatically than in the liver. Neutrophilia was also evident within MLN and splenic responses by wks 8 and 12 that, akin to the liver, was no longer significant by 15 wks post infection. Notably, the liver showed a significant decrease in B cell frequency from wk 8-15 of infection, while increased B cell proportions were observed in the spleen and MLNs.

Tregs are key immunosuppressive cells that aid in control of immunopathology during *S. mansoni* infection[30,37,57,58], commonly defined by their expression of the transcription factor FoxP3[59] and/or the activation marker CD25 (IL-2 receptor α)[60] (Figure 3A). When looking at relative Treg proportions, we observed significant expansion of $CD25^+Foxp3^+$ Tregs within the liver of infected mice from 8 wk onwards (Figure 3B), whilst in the spleen and MLNs, an increase in $CD25^+Foxp3^+$ Treg frequency was observed at wk 12 in the MLNs only (Figure 3B). However, reflecting the infection-induced increase in liver, spleen, and MLN cellularity (Figure 2A), we observed significant

numerical expansion of $CD25^{+}Foxp3^{+}$ Tregs by wks 8, 12 and 15 in the liver, spleen and MLNs (Figure 3B). Assessment of $CD25^{+}Foxp3^{-}$ $CD4^{+}$ T cells, representing activated effector $CD4^{+}$ T cells populations[61], revealed their numerical and proportional expansion in the liver from wk 8 of infection (Figure 3C). These $Foxp3^{-}$ populations showed numerical expansion in the MLNs at wks 8, 12 and 15, with significant proportional increases met at wk 15, while the least striking expansion of these effector cells was evident in the spleen (Figure 3C).

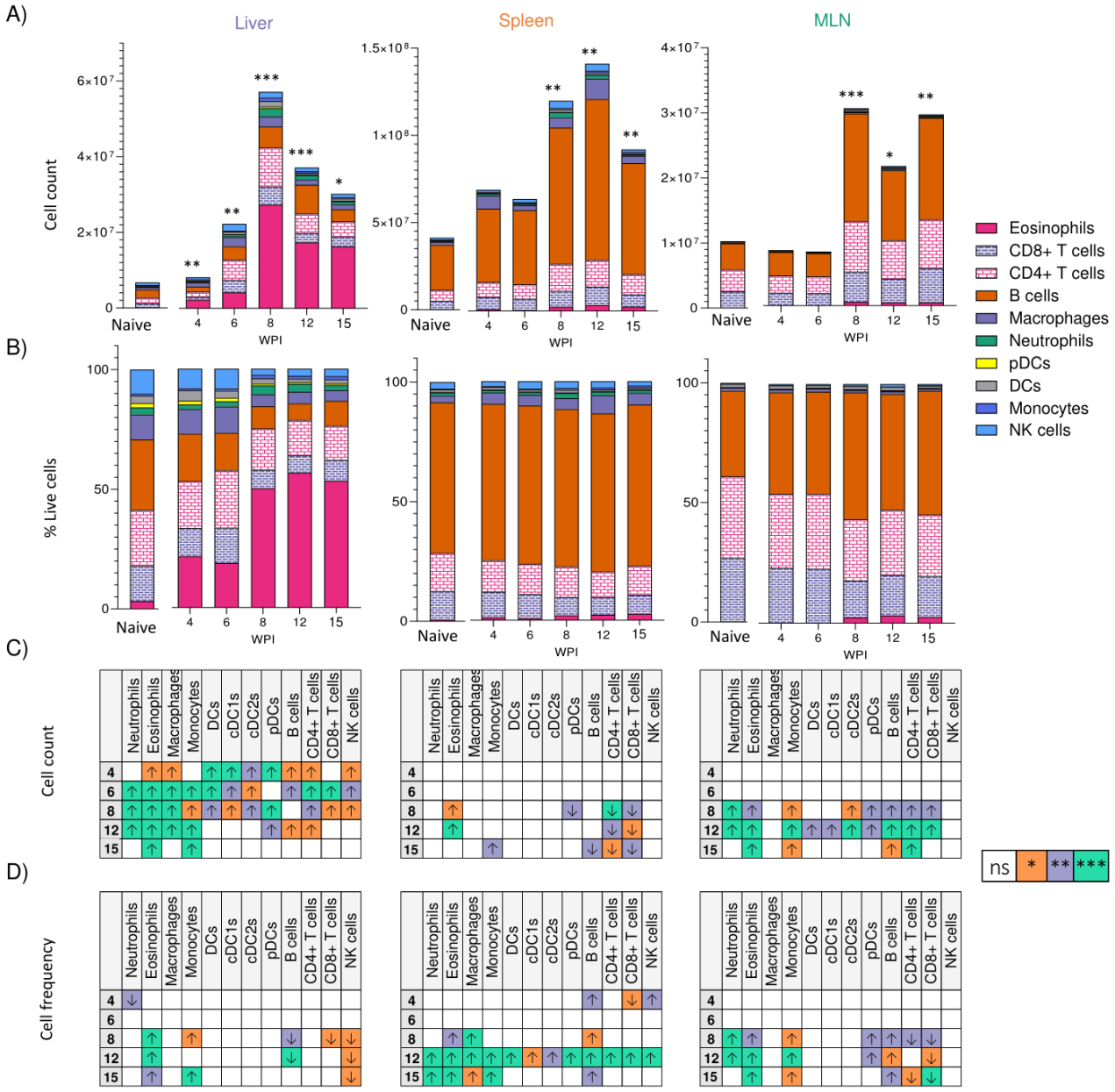


Figure 2. Tissue-specific cellular responses to schistosomiasis. Stacked bar charts showing hepatic splenic and mesenteric (A) cell counts and (B) cell frequencies (as a proportion of total live cells) at indicated wks of infection. For infected mice, data is presented as mean values for each given time point, with averages calculated from two pooled experiments per time point. n=6-8 per timepoint from two pooled experiments. Naïve data is presented as mean values for the entire infection, with averages calculated from two pooled time course experiments. n=30. Significance in (A) reflects comparison of total cell counts between naïve and infected mice. Statistics tables showing differences in (C) cell counts and (D) cell frequencies between naïve and infected mice, for the liver spleen and MLN. Arrows in table (D) represent the direction of cell frequency change in infected animals in comparison to naïve. Significance calculated by Kruskal-Wallis followed by Dunn's multiple comparisons test, with comparison between naïve and infected groups. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

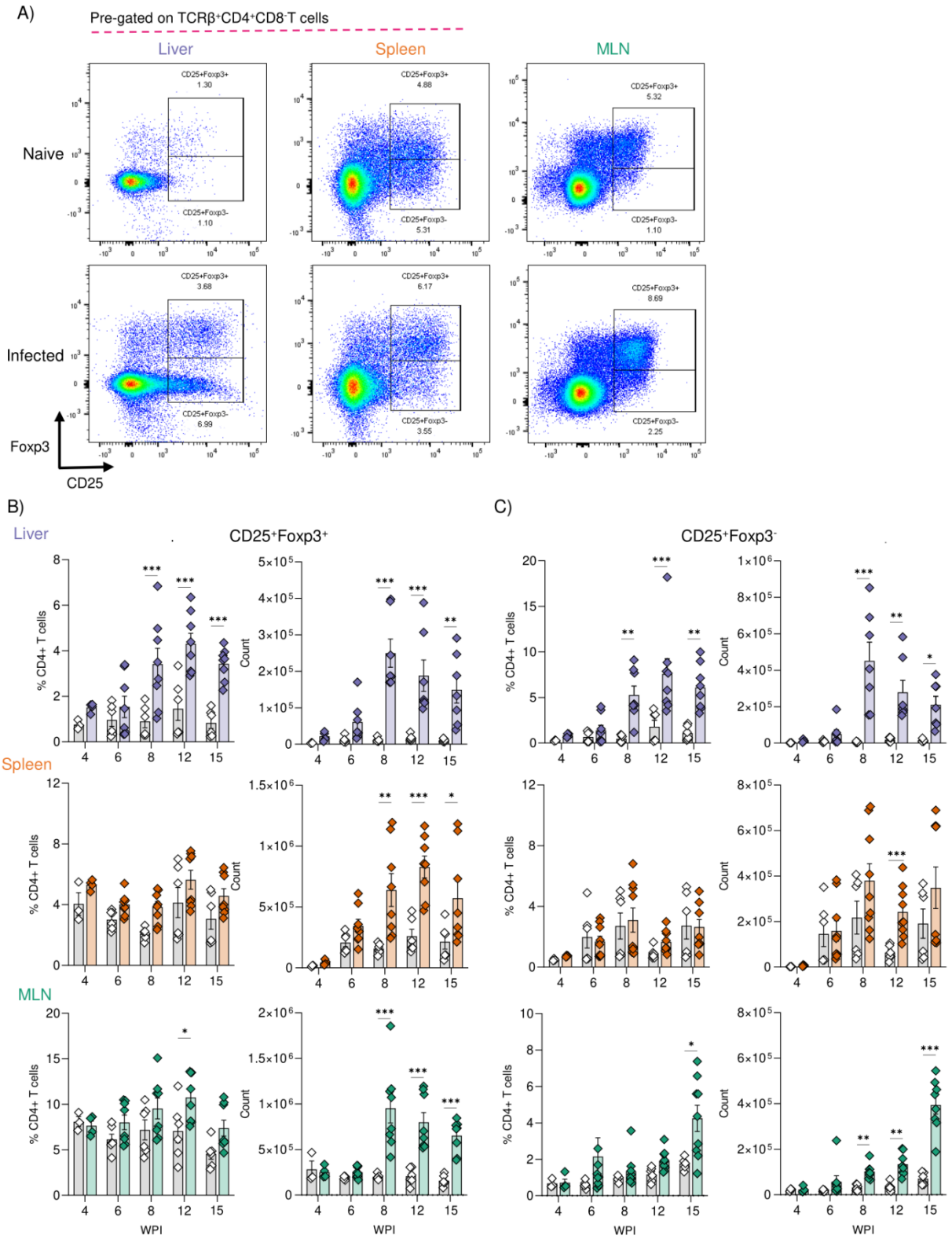


Figure 3. Regulatory T cell dynamics across *S. mansoni* infected tissues. (A) Representative flow plots for CD25+Foxp3+ and CD25+Foxp3- gating, pre-gating on live CD45+TCRβ+CD4+CD8- cells. The frequency and total numbers of (B) CD25+Foxp3+ T cells and (C) CD25+Foxp3- cells in the liver, spleen and MLN of naïve and infected mice, with frequency presented as % of total CD4+ T cells. (B&C). Results are mean +/- SEM from two experiments pooled (wks 6-15) or a single experiment (wk4) (n=3-8 mice per group per time-point). Significance calculated by two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

Transcriptional signature of schistosomiasis associated liver pathology

Given the dramatic changes in liver pathology and granulomatous inflammation observed over the course of *S. mansoni* infection, we next sought to obtain a broader, deeper and less biased understanding of the changes occurring in the liver tissue over the course of *S. mansoni* infection, RNA was isolated from the livers of infected mice and matched naïve controls at wks 3, 4, 6, 8, 12, and 15, with transcriptional profiling performed by RNAseq (Supplementary Excel file 1). As early as wk 3 post infection we were able to observe distinct transcriptional profiles in infected mice compared to naïve controls (Figure 4A&B) including increased expression of chemokines (Cxcl9 and Cxcl10) and genes associated with MHCII expression (*H2-Aa*, *H2-Ab1* and *H2-Eb1*) (Figure 4B). From wk 4-6 onward, significant upregulation of transcripts associated with maturation of myeloid cells and B cells (*Irf8*, *Cd74*, *Ly6d* and *Ear2*) was observed. Starting at wk 8 and continuing through wks 12 and 15 we observed collagens (*Col6a1* and *Col6a2*) and other genes associated with the extracellular matrix (ECM) (*Lpl*, *Lum*, *Anxa2*) among the most significantly upregulated differentially expressed genes (DEGs; Figure 4B).

To obtain an overview of the processes influenced by infection we performed gene set enrichment analysis (GSE) of gene ontology (GO) terms associated with biological processes using genes differentially expressed at each timepoint (Figure 4C, Supplementary Excel file 2). Whilst there were too few differentially expressed genes at wk 3 and wk 4 post infection to identify statistically meaningful enrichment of GO terms, we identified a range of terms to be enriched (both positively and negatively) from wk 6 onwards. As expected, we observed strong positive enrichment of terms associated with the immune response, including those related to antigen presentation, immune cell migration and cytokine production. Notably, our GSE analysis identified an number of terms associated with the synthesis of collagen and restructuring of the extracellular matrix at wk 12 and wk 15. Interestingly, we identified strong negative enrichment of a number of GO terms associated with metabolic processes, particularly those associated with the metabolism of nucleotides.

As we had observed substantial hepatic changes by MT staining (Figure 1B) we examined genes associated with extracellular matrix remodelling. Selecting the 15 most significant DEGs associated with GO terms relating to tissue remodelling revealed strong upregulation of matrix metalloproteases (MMPs) (*Mmp2* and *Mmp14*); a collagen (*Col1a2*) and ER-resident chaperon associated with collagen biosynthesis (*Serpinh1*); other secreted components of the extracellular matrix (*Dpt*, *Postn*, *Efemp2*, *Smoc2* and *Elna*); a catalytic enzyme required for matrix component crosslinking (*Loxl1*), a matrix binding protein (*Ccdc80*); and receptors associated with the regulation

of matrix deposition (*Anxa2* and *Pdgfra*) (Figure 4D). In total we identified 76 DEGs associated with the extracellular matrix, including 13 encoding collagens, 5 Adamts (A Disintegrin and Metalloproteinase with Thrombospondin motifs) family members, 8 MMPs, and the transcription factor *Sox9* (Supplementary Excel file).

Interrogation of the most significantly differentially expressed genes associated with the immune response revealed a diverse array of genes linked to different aspects of immune function. We identified genes associated with immune cell chemotaxis (*Cd34*, *Cxcl14, Ear2* and *Pf4* (encoding CXCL4)); TLR4/RAGE binding S100 family members associated with triggering migration via NF-κB signalling (*S100a8* and *S100a9*); an actin regulator associated with macrophage function (*Gsn*); genes associated with interaction with and remodelling of the extracellular matrix (*Col3a1, Mmp2, Spp1*); genes related to B cell function including an alarmin receptor involved in B cell development (*Crlf2*) and components of immunoglobulins (*Iglc2* and *Jchain*); a signal transduction enzyme (*Smpd3*); and an antimicrobial peptide (*Slpi*) (Figure 4E).

These data demonstrated a dramatic change in the transcriptional profile of the liver during *S. mansoni* infection with strong enrichment of genes linked to the attraction and activation of leukocytes, and the restructuring of ECM. Additionally, we found significant downregulation of GO terms associated with various metabolic and catabolic processes. Although some of these changes were evident as early as 3 wks post infection, the major changes in gene expression occurred between wk 6 and wk 8, indicating that transcriptional reprogramming of the liver environment is associated with the start of egg production.

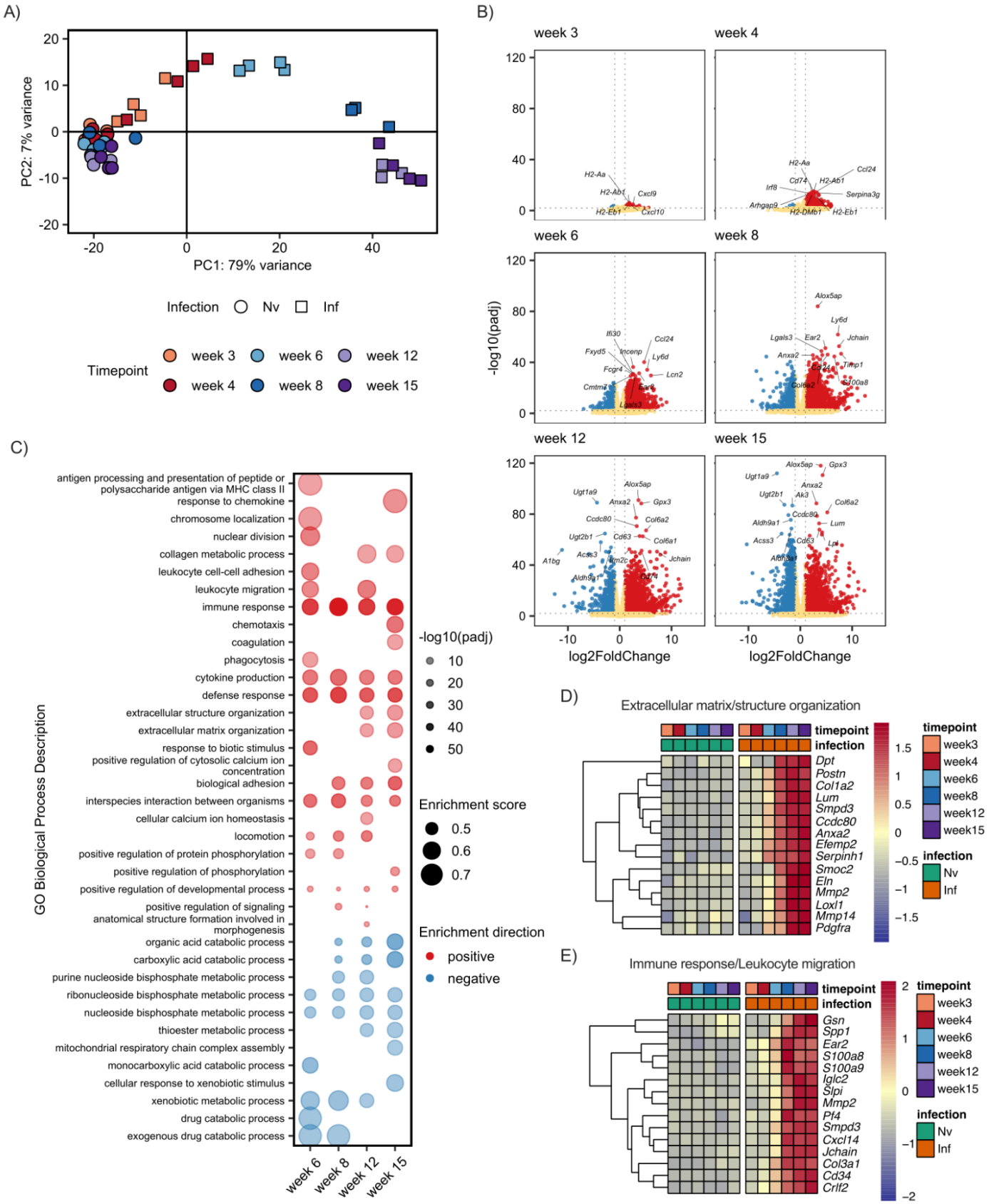

A)
PC2: 7% variance
PC1: 79% variance
Infection Nv Inf
Timepoint week 3 week 4 week 6 week 8 week 12 week 15
B)
week 3
week 4
week 6
week 8
week 12
week 15
-log10(padj)
log2FoldChange
C)
GO Biological Process Description
antigen processing and presentation of peptide or polysaccharide antigen via MHC class II
response to chemokine
chromosome localization
nuclear division
collagen metabolic process
leukocyte cell-cell adhesion
leukocyte migration
immune response
chemotaxis
coagulation
phagocytosis
cytokine production
defense response
extracellular structure organization
extracellular matrix organization
response to biotic stimulus
positive regulation of cytosolic calcium ion concentration
biological adhesion
interspecies interaction between organisms
cellular calcium ion homeostasis
locomotion
positive regulation of protein phosphorylation
positive regulation of phosphorylation
positive regulation of developmental process
positive regulation of signaling
anatomical structure formation involved in morphogenesis
organic acid catabolic process
carboxylic acid catabolic process
purine nucleoside bisphosphate metabolic process
ribonucleoside bisphosphate metabolic process
nucleoside bisphosphate metabolic process
thioester metabolic process
mitochondrial respiratory chain complex assembly
monocarboxylic acid catabolic process
cellular response to xenobiotic stimulus
xenobiotic metabolic process
drug catabolic process
exogenous drug catabolic process
week 6
week 8
week 12
week 15
-log10(padj)
Enrichment score
Enrichment direction
positive
negative
D)
Extracellular matrix/structure organization
timepoint
infection
Dpt
Postn
Col1a2
Lum
Smpd3
Ccdc80
Anxa2
Efemp2
Serpinh1
Smoc2
Eln
Mmp2
Loxl1
Mmp14
Pdgfra
E)
Immune response/Leukocyte migration
Gsn
Spp1
Ear2
S100a8
S100a9
Iglc2
Slpi
Mmp2
Pf4
Smpd3
Cxcl14
Jchain
Col3a1
Cd34
Crlf2

Figure 4. Schistosome egg deposition dramatically alters the liver transcriptome. Livers from schistosome infected mice (Inf) or matched naïve controls (Nv) were harvested at 3, 4, 6, 8, 12 and 15 wks post infection (n=3-4 per timepoint). RNA was isolated from the liver tissue and transcriptionally profiled by RNAseq. (A) Principal components analysis of total read counts. Points represent individual replicates. Point shape indicates group, Nv (○) Inf (□), and colour indicates timepoint. (B) Volcano plots of differentially expressed protein-coding genes in Inf vs Nv mice at each time point. Genes were considered significantly differentially expressed in Inf mice when the adjusted p value (padj) was < 0.01 and the log2FoldChange was > 1. Red dots indicate genes significantly up-regulated in Inf mice, blue dots indicate genes significantly down-regulated in Inf mice, yellow dots represent genes not significantly differentially expressed between Inf and Nv mice. (C) Gene set enrichment (GSE) analysis for Inf mice from wk 6 to wk 12 using GO Biological Process terms based on genes with a padj < 0.01 vs Nv mice. Points represent the enrichment score for a given GO term at each time point. Size of point indicates the enrichment score. Opacity of point indicates the significance value for that enrichment score expressed as -log10(padj). Point colour indicates whether a given GO term was positively (red) or negatively (blue) enriched. Blank spaces indicate that GO term was not significantly enriched at that timepoint. GO terms were considered significantly enrinched if padj < 0.01. Heatmaps representing the mean expression of top 15 most significantly differentially expressed genes identified in specific GO terms: (D) "extracellular matrix structure" and "extracellular matrix organisation" and (E) "Immune response", and "Leukocyte migration". All genes presented by heatmap had a p value < 0.01. Differential expression analysis was performed using DESeq2, GSE analysis was performed using clusterProfiler.

Hepatic granuloma composition over the course of Infection

We next sought to investigate some of the transcriptional changes we had observed at a location level, in hepatic granulomas (Figure 5). Schistosome granulomas consist of ECM, fibroblasts and a range of immune cells (predominantly Th2 cells), whose frequency and spatial distribution varies across the course of infection and the site in question[56,62]. To examine the spatial dynamics of granuloma formation and resolution and assess the localisation of key cell types, confocal microscopy was performed on liver sections at each stage of infection (Figure 5A).

While no eggs were found in livers from infected mice at wk 4, we observed disorganised clustering of immune cells with sporadic Siglec-F staining throughout that was not present in naïve mice (Figure 5A). By wk 6, immune cells began to assemble into more organised, compact, areas that enveloped tissue-trapped eggs and harboured $CD11c^+$ and $Siglec\text{-}F^+$ cells, and a small number of $TCR\beta^+$ cells, at their core. By wks 8 and 12, $CD11c^+$ and $Siglec\text{-}F^+$ cells now appeared distributed across granulomas, with new infiltration and co-localisation of $TCR\beta^+$ cells with $CD11c^+$ cells. By wk 15, $CD11c^+$ and $TCR\beta^+$ cells reduced in frequency but remained dispersed and colocalised across granulomas, while $Siglec\text{-}F^+$ cells were again evident within the innermost layer, along with some staining across granulomas. By quantifying the surface area for positive staining around individual eggs, we were able to assess the kinetics of these cell types within granulomas (Figure 5B). From wks 6-8, the staining for each marker increased markedly, but was only significant for CD11c and Siglec-F. After wk 8, Siglec-F decreased while CD11c remained consistently elevated until wk 15.

TCRβ patterns were somewhat comparable to CD11c, but with a more dramatic reduction by wk 15. These histological data were generally consistent with the hepatic flow cytometry readouts for DCs, eosinophils and T cells (Figure 2).

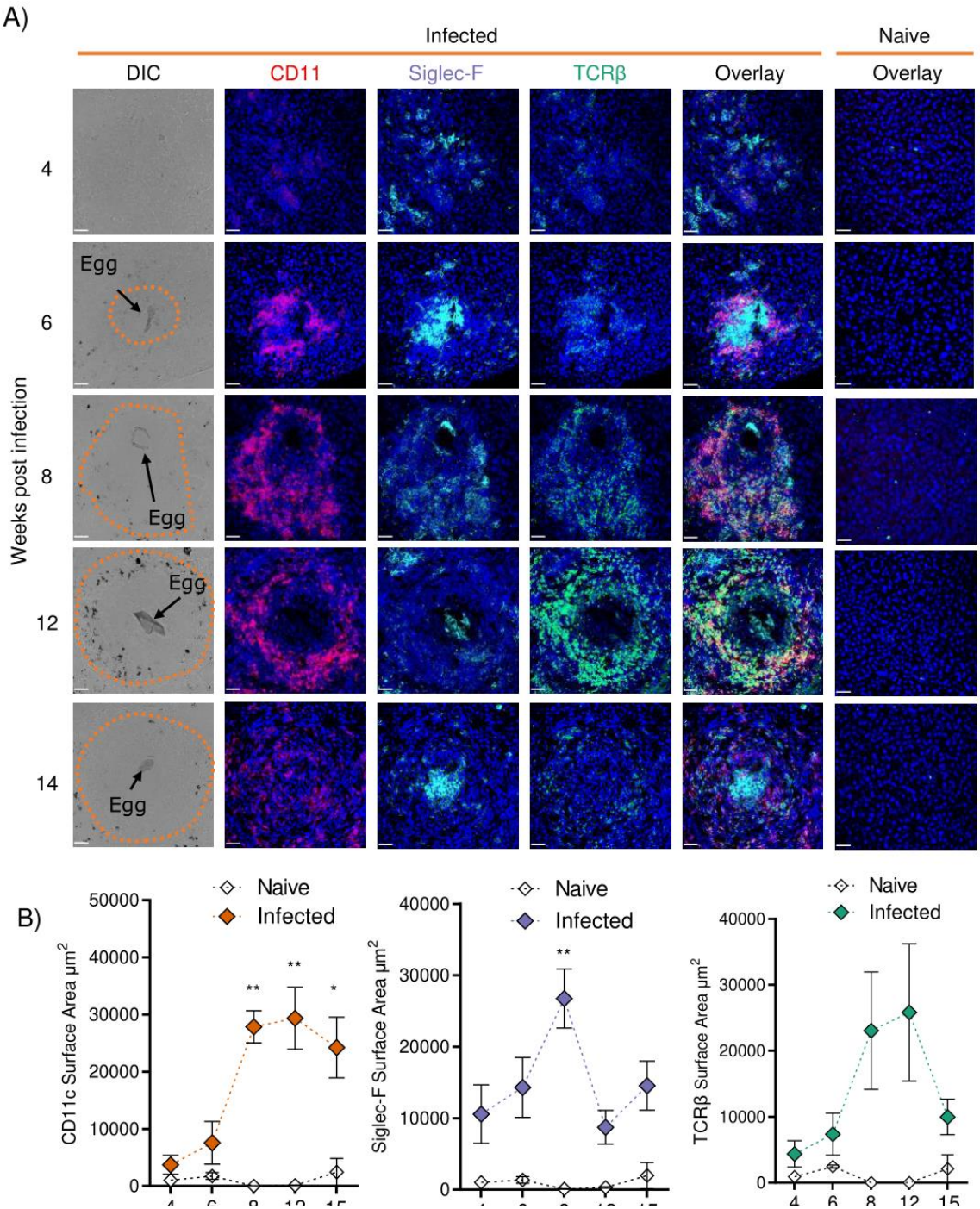


Figure 5. Confocal microscopy analysis reveals distinct alterations in hepatic granuloma composition across infection timeline. At indicated stages of infection, the infiltration and localisation of CD11c$^+$, Siglec-F$^+$ and TCRβ$^+$ cells in hepatic granulomas was assessed by IHC. (A) Representative confocal microscopy images taken from livers of 5 *S. mansoni* infected mice at each timepoint. Top row showing differential Interference Contrast (DIC) images, with eggs indicated by arrows and dotted lines outlining granuloma periphery. (B) Quantification by Image J of positive Siglec-F, CD11C and TCRβ staining. 1 experiment. Significance calculated by Two-way ANOVA. Data presented as mean +/- SEM. * = p<0.05, ** = p<0.01, *** = p<0.001. Bars indicate SEM of from 3 sections of naïve hepatic tissue vs 10 granulomas from infected mice.

CD11c depletion dramatically impairs hepatic immune cell polarisation, provoking neutrophilia

In previous work, we used targeted CD11c$^+$ cell depletion to reveal a crucial role for CD11c$^+$ cells in Th2 induction during initial egg producing stages of *S. mansoni* infection (wk 4-6)[24], prior to the dramatic pathological and immunological changes that occur from 6–8-wks post infection (Figures 1-5). We next sought to explore how CD11c depletion would affect immunopathology and the maintenance of immune responses at later stages of infection using CD11c.DOG mice, in which the CD11c promotor controls expression of the human diphtheria toxin (DTx) receptor (DTR)[41]. CD11c$^+$ cells were depleted from day 42 to 51 of infection, and host responses evaluated at day 52 (Figure 6A). CD11c depletion had no discernible impact on hepatosplenomegaly (Figure 6B), egg burden (Figure 6C) or the extent of hepatic granulomatous inflammation, as measured by quantification of MT staining (Figure 6D). However, closer inspection of hepatic granuloma composition revealed more inflammatory pathology following CD11c depletion (Figure 6E). As expected, CD11c$^+$ staining was significantly reduced in granulomas from DTx treated mice, indicating effective depletion (Figure 6F&G), coincident with a significant decrease in the proportion of TCRβ$^+$ and Siglec-F$^+$ cells (Figure 6F&G).

In support of our histological data (Figure 6F&G), assessment of liver cell populations by flow cytometry showed that DTx administration significantly depleted CD11c$^+$MHC-II$^+$ DCs (approximately 50% depletion across experiments),, while hepatic macrophage proportions remained intact (Figure 7A). These changes were accompanied by a significant reduction in hepatic CD4$^+$ T cells, CD8$^+$ T cells and eosinophils, alongside a stark increase in hepatic neutrophilia (Figure 7A). Next, to explore the impact of CD11c depletion on cytokine dynamics, we evaluated hepatic responses following culture of isolated liver cells with SEA (Figure 7B) or anti-CD3 (Figure 7C). While CD11c depletion did not significantly alter Ag-specific cytokine production by isolated liver cells in culture, their potential to produce IL-4 and IL-10 in response to anti-CD3 was significantly diminished (Figure 7C). Together, these data showed that CD11c depletion significantly influenced the hepatic granulomatous response during schistosome infection, reducing eosinophilia and T cells, while increasing neutrophilia and impairing IL-4 and IL-10 potential, without dramatically affecting Ag-specific cytokine production.

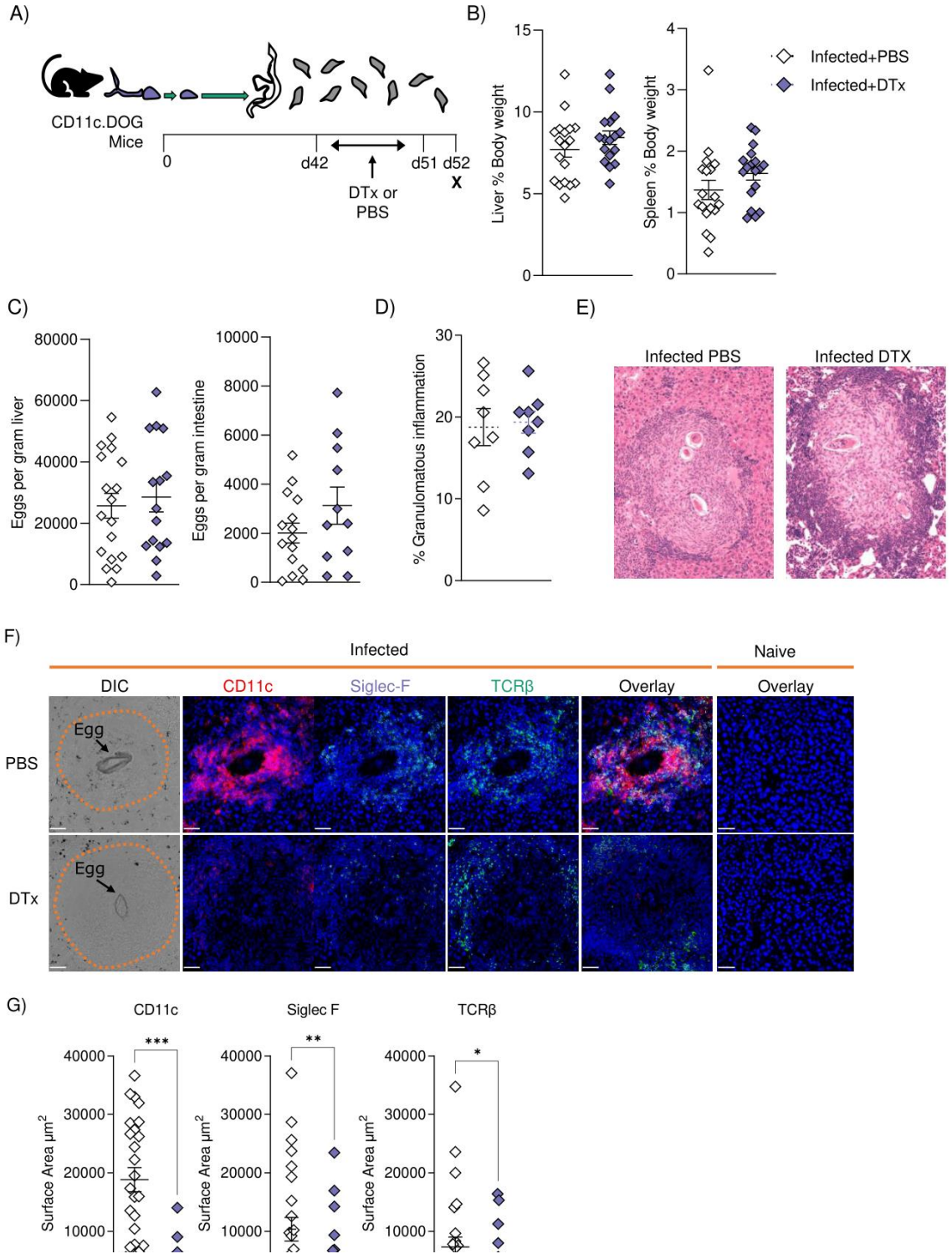


Figure 6. CD11c depletion disrupts granulomatous pathology (S. mansoni infection (A) Schematic of infection setup. CD11c.DOG mice were infected with 40 S. mansoni cercariae with CD11c+ cells depleted via Dtx administration on days 42-51, and mice culled at d52. (B) Liver and spleen weights for infected mice with data represented as a proportion of total body weight. (C) The total number of schistosome eggs per gram of liver or intestinal tissue (D) Quantification of granulomatous inflammation (E) Representative images of hepatic granulomas stained with H&E (F) Representative confocal microscopy granuloma images, with staining for CD11c, Siglec-F and TCRβ. First column showing differential Interference Contrast (DIC) images, with eggs indicated by arrows and dotted lines outlining granuloma periphery. (G) Quantification of positive Siglec-F, CD11C and TCRβ staining. Data are from a single experiment (D, E, F&G) or pooled from 3 (A-C) 3 separate experiments. Significance calculated by unpaired T-test. Data presented as mean +/- SEM. * = p<0.05, ** = p<0.01, *** = p<0.001.

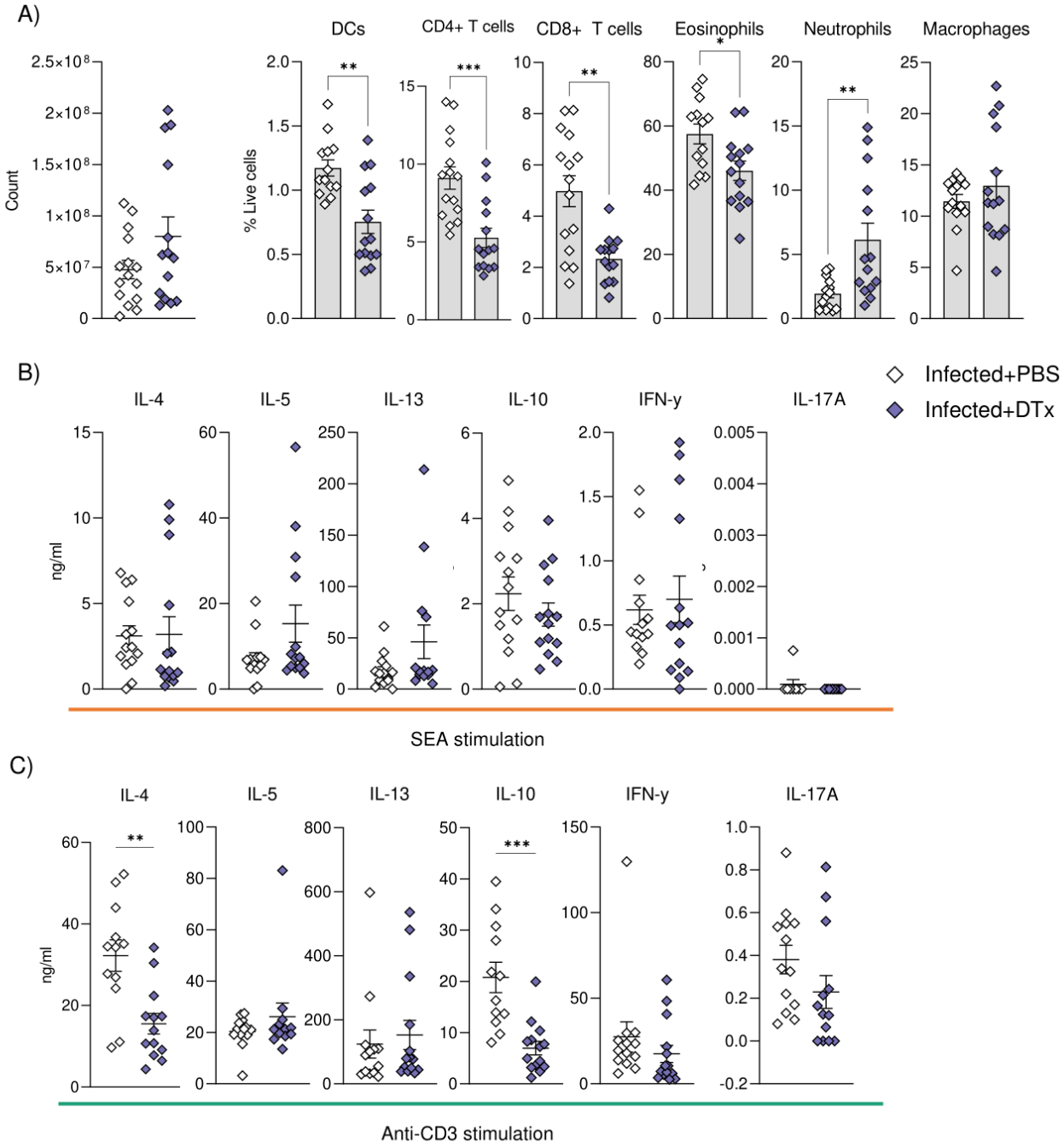


Figure 7. CD11c depletion compromises hepatic cellular dynamics during *S. mansoni* infection. The total number of liver cells (A) and the frequency of various immune cells in the liver of Dtx of PBS treated infected mice. Liver cells were cultured for 72 h with (B) 0.25 µg of SEA or (C) 0.5µg of anti-CD3. Supernatants were collected and cytokine production (medium alone values subtracted) was assessed by ELISA. Data are pooled from 3 separate experiments (n=12-18 animals per time point). Significance calculated by unpaired T-test. Data presented as mean +/- SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

In addition to elevating our basic understanding of immune response development over the course of murine *S. mansoni* infection, in both effector and priming sites, we have revealed a crucial role for $CD11c^+$ cells in hepatic granuloma coordination and maintenance in the post-patent phase of schistosome infection. The onset of egg production evoked pronounced pathological (Figure 1 & Supplementary Figure 1), immunological (Figures 1-3 & Supplementary Figure 2) and transcriptional host responses (Figure 4), with clear immunopathology by the eighth wk of infection (Figures 1,4,5 & Supplementary Figures 1&2). As demonstrated through cytokine analysis of cultured liver, spleen or MLN cells *ex vivo* (Figure 1 & Supplementary Figure 2), infection elicited a distinctive triphasic kinetic, including a Th1-skewed phase prior to egg production, a Th2-dominated response from egg deposition, and a more regulated profile in the chronic stage (Figure 1 & Supplementary Figure 2). Egg deposition drastically remodelled the liver transcriptome, including dramatic enrichment of genes associated with granulocyte recruitment, tissue remodelling and leukocyte activation, and with the most pronounced transcriptional changes starting between wk 6 and 8 of infection (Figure 4). Histologically, distinctive hepatic granulomatous inflammation was evident by wk 6 of infection, which subsided by more chronic stages (Figures 1 & 5). Importantly, depletion of $CD11c^+$ cells between wks 6-8 of infection had a dramatic impact on the hepatic response, including disrupted granuloma formation and cellularity, increased neutrophilia, and impaired cytokine production (Figures 6&7)

Time course kinetics

We found that the dramatic granulomatous inflammation that develops in the liver from wk 6 of infection (Figure 1) became evident over the course of a matter of days, with day 45 representing a distinct tipping point between 'homeostasis' and stark pathology (Supplementary Figure 1). However, it is important to note that, although we selected representative images for each timepoint, granuloma development is asynchronous, due to differences in timing and location of egg deposition, egg maturity and secretions, and previous exposure to cross-reactive worm Ags [53,63]. To investigate granuloma development in a more synchronous manner, investigations could be made using intravenous injections into the portal vein or lung[53].

Schistosome infection actively modifies T cell responses in order to promote the Th1-Th2 switch, trigger regulatory cell networks, and induce a state of T cell hyperresponsiveness[8,64,65]. We observed a reduced propensity for isolated cells from livers, spleens or MLNs of more chronically infected

mice to produce cytokines in response to Ag-specific (SEA) stimulation (Figure 1), in support of previous studies suggesting $CD4^+$ T cell exhaustion during later stages of active disease[66,676]. In contrast, upon polyclonal (anti-CD3) stimulation, many cytokines continued to increase in the liver and MLNs beyond wk 12 of infection (Supplementary Figure 2). This might suggest that down-modulation of T cell activation during chronic infection is restricted to Ag-specific responses. Alternatively, it is possible that while Ag-specific T cells enter an exhausted state following repeat Ag stimulation[5], a pool of T cells undergo Ag-independent bystander activation towards circulating cytokines and TLR ligands, thus sustaining cytokine secretion across infection[68]. Finally, the reduction in T cell activity may reflect the direct activity of schistosome products on T cells or modulation of APC activity[19,67,69,70], which could include promoting the emergence of more tolerogenic APCs[67,70] or those with a reduced capacity to elicit effector Th2 cell proliferation[71].

In keeping with regulatory responses being more apparent during chronicity[5,29], we observed evidence for enhanced immunoregulation in later stages of infection (Figure 1, 2 & Supplementary Figure 2). $CD25^+Foxp3^+$ Tregs were expanded across all tissues at later infection phases, but with the most statistically robust expansion (both proportional and numerical) within the liver (Figure 3). These observations support previous studies showing enhanced Treg frequencies in the liver, MLN, spleen and colonic granulomas during infection[30,37,58] and suggest the hepatic inflammatory environment to most effectively support Treg activation and recruitment. This may result from the costimulatory environment[72], TGFβ[73], RELMα[74,75] egg antigens[35,58], or the higher levels of IL-4 and IL-13 (Figure 1) which have shown capable of inducing $CD25^+$ Tregs from peripheral naïve $CD25^-CD4^+$ T cells[76,77]. Although previous work has assessed Treg development in the spleen, MLN and liver [30,37,58], we are the first to show side by side comparison of tissue-specific Treg kinetics over the course of infection. It remains to be addressed whether Breg and $CD8^+$ Treg[78] generation mirrors these $CD4^+$ Treg dynamics, and whether *S. mansoni* elicited Tregs harbour tissue-specific functions.

We also observed increased IL-10 levels as infection progressed, from isolated and cultured spleen, MLN and liver cells, with this profile being most evident with anti-CD3 stimulation (Figure 1 & Supplementary Figure 2). This may reflect our choice of SEA for Ag-specific *ex vivo* restimulation, given that adult worm-derived molecules may preferentially expand regulatory responses[19,70]. Splenic IL-10 levels were low in comparison to the liver and MLN, which again could reflect the choice of stimulation or the relative abundance of cultured cell types. Furthermore, B cells can be meaningful sources of IL-10 during schistosomiasis[29,34], quantification of which would require

alternative approaches, such as stimulation via TLRs and CD40[79], or the use of IL-10 reporter mice, which constitute an improved tool for visualisation of which cell types contribute to the IL-10 pool[80].

In terms of other cellular responses, $CD8^+$ T cells comprised a third of all T cells in the liver (Figure 2), with previous literature accrediting them with 'suppressor' functions in the downmodulation of egg-driven pathology [81,82]. Eosinophils showed increases across tissues during infection, a likely reflection of elevated levels of IL-5 (Figure 1 & Supplementary Figure 2) or chemokines such as CCL24[83] (Figure 4) or CXCL12[84]. Despite their dominating presence within schistosome infected tissues, the true function of eosinophils in schistosomiasis is not yet known, with eosinophil ablation[85] or IL-5 removal[86] failing to impede granuloma formation or fibrosis, influence hepatocellular damage, or impact Th2 development[87]. Analysis of Siglec-F staining of liver sections showed eosinophilia during early stages of granuloma formation within the inner perimeter of circumoval inflammation (Figure 5). In previous reports, this localisation has only been shown through morphometric inspection of Giemsa or H&E stained granulomas[56,62, 85], and this pattern suggests that eosinophils may play an important role in granuloma formation and protection of parenchymal tissue, and/or in the destruction of the entrapped eggs[88]. Indeed, an interesting feature of the hepatic response was that liver cells from infected mice had a greater potential to produce IL-5 as early as wk 4 of infection (Figure 1 & Supplementary Figure 2), which could support the rapid recruitment of eosinophils during granuloma initiation and development from around wk 6. Moreover, we suggest that the hepatic immune infiltration observed at wk 4 (Figure 2) is encouraged by the deposition of worm-derived antigens or regurgitation products within the liver. Indeed, schistosome-derived hemozoin has shown to deposit in large quantities the liver and influence components of host immunity, including alternative activation of macrophages89. Further interrogation of this pre-patent inflammation would ideally entail comparison between naïve, mixed-sex and single-sex infections.

Transcriptomics is a powerful investigative tool, that offers a high-resolution and unbiased description of host processes at an RNA level. The transcriptional profile of the mammalian host during schistosomiasis has been explored in a range of primate and rodent models[89–91], with more recent work exploring the human transcriptome during active infection[92,93]. However, only a few of these studies have interrogated the dynamics of host gene expression at multiple time points of schistosome infection[91], and no comprehensive overview of how the hepatic transcriptional environment changes across the course of infection yet exists. By defining hepatic transcriptional signatures during pre-patency, our data offers insight into first cellular responders and the

mechanisms underlying their recruitment (Figure 4). Notably, the IFNγ-inducible chemokines *Cxcl9* and *Cxcl10*[94] were among the most upregulated genes at wks 3 and 4 post infection, which is consistent with reports from murine *S. japonicum* infection[89] and suggests Th1-associated chemokines[94] may be associated with initial inflammatory cell recruitment and instigation of granulomatous inflammation. Similarly, expression of IFNγ-driven *Tgtp1* (T cell-specific GTPAse-1), which is associated with M1 effector functions[95] links with literature showing enhanced frequency of M1-like macrophages during acute schistosomiasis[96,97] and provides further clarity into the timing of their arrival and/or proliferation.

The transcriptome of the post-patent liver (Figure 4) clearly reflected the diverse populations of immune cells populating the tissue (Figure 2&3), as well as the structural and fibrotic changes the liver undergoes to counteract and/or compensate for egg-induced inflammation (Figures 1&5). From wk 6, infected livers transitioned into an inflammatory state, as evidenced by enhanced transcripts of acute-phase serum amyloid proteins, SAA1 and SAA2, and moderate upregulation (relative to later wks of infection) of chemokines *Ccl8* and *Ccl24*. Interestingly, and in line with our histological analysis for Siglec-F^{+} cells (Figure 5), the chemotactic ligand for eosinophils, *Ccl24* (encoding eotaxin-2[98]), reached peak expression at wk 8 of infection before declining at more chronic phases. The list of upregulated genes from wk 8 onwards was dominated by genes associated with tissue restructuring and fibrosis, as well as the recruitment, activation and function of key immune cells, including mast cell proteases, eosinophilic elastases and a marker of macrophage alternative activation, chitinase-like 3 (*Chil3,* encoding Ym1), whose function during schistosomiasis has yet to be resolved. Our transcriptomic analysis may assist in the identification of novel cellular and molecular targets for the therapeutic control of schistosomiasis, or treatment of schistosomiasis associated hepatic fibrosis. Notably, the ratio of MMPs to tissue inhibitors of matrix metalloproteinases (TIMPs), is a suggested determining factor in the severity of schistosomiasis and may provide clarity on the differences in wound healing response and outcome between *S. japonicum* and *S. mansoni* infections[89,99.]. Moreover, in terms of individual genes of interest, *Timp-1, Ccl24,* and *Sox9* all demonstrate sustained upregulation from the point of egg production and are all implicated in the exaggeration of fibrosis[100–102]. Finally, the transcriptome of the chronically infected liver showed very interesting metabolic changes, including the downregulation of the cytochrome P450 family (CYPs), amongst other drug metabolising enzymes. Importantly. the downregulation of Cyp2b and Cyp3a may have significant implications for the metabolism and clearance of the anti-schistosome drug praziquantel[103] and thus this could represent a therapeutically fruitful area of follow-up.

CD11c depletion

Having generated a high-resolution picture of hepatic schistosomiasis at a transcriptional (Figure 4) and cellular (Figures 2, 3&5) level, we next sought to identify the importance of $CD11c^+$ cells in coordinating the liver response to infection. The development of Th2 immunity is crucial for the control of excessive schistosome associated pathology[8,10,32,38]. Notably, mice in Th1 polarised settings (including IL-4 deficient or IL-10/IL-4 double-deficient mice) exhibit rapid weight loss at the onset of egg production, elevated expression of proinflammatory Th1 cytokines and mediators, and high levels of mortality between wks 8-10 of infection[10,32,104]. The mechanisms underlying the dramatic transition from a mixed, low level Th1/Th2 to a Th2 dominated immune response have been thoroughly dissected, with central involvement of STAT-6[105], IL-4[10,32] and IL-4Rα[9,106] signalling, and DCs[40]. However, there is less understanding of how granulomas and hepatic Th2 responses are maintained and regulated over the course of infection. Herein, we show CD11c depletion during peak development of schistosome-elicited Type 2 inflammation (wks 6-8) compromised hepatic granuloma composition (Figure 6). Although CD11c depletion at this stage of infection resulted in impaired liver cell potential to produce IL-4 and IL-10 (Figure 7B), it did not significantly impact Ag-specific cytokine responses (Figure 7A). This contrasts CD11c depletion at wks 4-6, which we have previously shown significantly reduces hepatic cell Ag-specific IL-4, IL-5 and IL-13 production[40]. These data indicate that, while $CD11c^+$ DCs may be crucial for the induction and expansion of $CD4^+$ T cells in priming stages of infection[40], at later stages effector T cells can competently produce cytokines without their assistance, with other APCs such as B cells or macrophages potentially playing larger roles in effector T cell activation at later phases of infection[107]. Alternatively, as hepatic CD11c depletion was incomplete in our experiments (Figures 6&7), it is possible that residual $CD11c^+$ DCs were sufficient to maintain hepatic Th2 effector responses. While Ag-specific liver cell cytokine responses were not significantly affected following CD11c depletion at this timepoint, production of both IL-4 and IL-10 was impaired following stimulation with anti-CD3, implicating $CD11c^+$ cells in dictating hepatic $CD4^+$ T cell potential to produce these cytokines during schistosomiasis. Taken together, these results suggest that $CD11c^+$ APCs play a supportive role in stimulation of T cell cytokine production in the liver effector site. In contrast, CD11c depletion dramatically altered granuloma and hepatic cellularity (Figures 6 & 7), in particular significantly reducing eosinophilia and T cells, without reducing overall granulomatous inflammation (Figure 6D). Deficits in hepatic recruitment and/or retention of eosinophils and T cells following CD11c depletion may fit with the known ability of DCs to produce a range of chemokines[108–110]. In line with this, in a

model of schistosome egg induced pulmonary granulomatous inflammation, depletion of known eosinophil[108] and Th2 cell[111] chemokines CCL17 and CCL22, which can be produced by DCs[108–110], led to altered granuloma structure, including a dramatic reduction in eosinophilia[112].

In our experiments, neutrophils, which are normally minor constituents of *S. mansoni* granulomas due to the release of egg-derived chemokine-binding proteins and proteases that inhibit neutrophil function or recruitment[16,113], significantly increased in the liver following CD11c depletion (Figure 7). The fact that schistosome eggs actively secrete neutrophil inhibiting molecules suggests that their presence is undesirable during infection, and their expansion may give rise to more damaging hepatic inflammation, as seen in murine *S. japonicum* infection[114]. The cause of neutrophilic expansion is unclear, but this has been reported in other studies using CD11c DTR transgenic mice[115]. We predict that the dysregulated granulomas observed in CD11c-depleted mice could have severe pathological consequences in later disease stages, with the absence of intact granuloma barrier leading to increased perfusion of hepatotoxic egg molecules into the tissues.

Together, these data indicate that $CD11c^+$ cells play a critical role in recruitment or retention of eosinophils and T cells in the liver from wk 6 of schistosome infection, while other $CD11c^-$ cells are generally sufficient for reactivation of cytokine production by hepatic effector T cells. Our results provide a platform for future interrogation of the role of $CD11c^+$ cells in more chronic stages of infection, alongside employment of more targeted CD11c depletion approaches[116], to enable a more holistic understanding of the role and importance of $CD11c^+$ APC subsets in governing immunopathology in effector sites such as the liver over the course of schistosome infection

In conclusion, this study provides a detailed and comprehensive analysis of immune response development over the course of schistosome infection at a resolution not previously achieved, providing a valuable resource to inform future work aiming to better understand the mechanisms that govern immunity, inflammation and pathology in this important neglected tropical disease.

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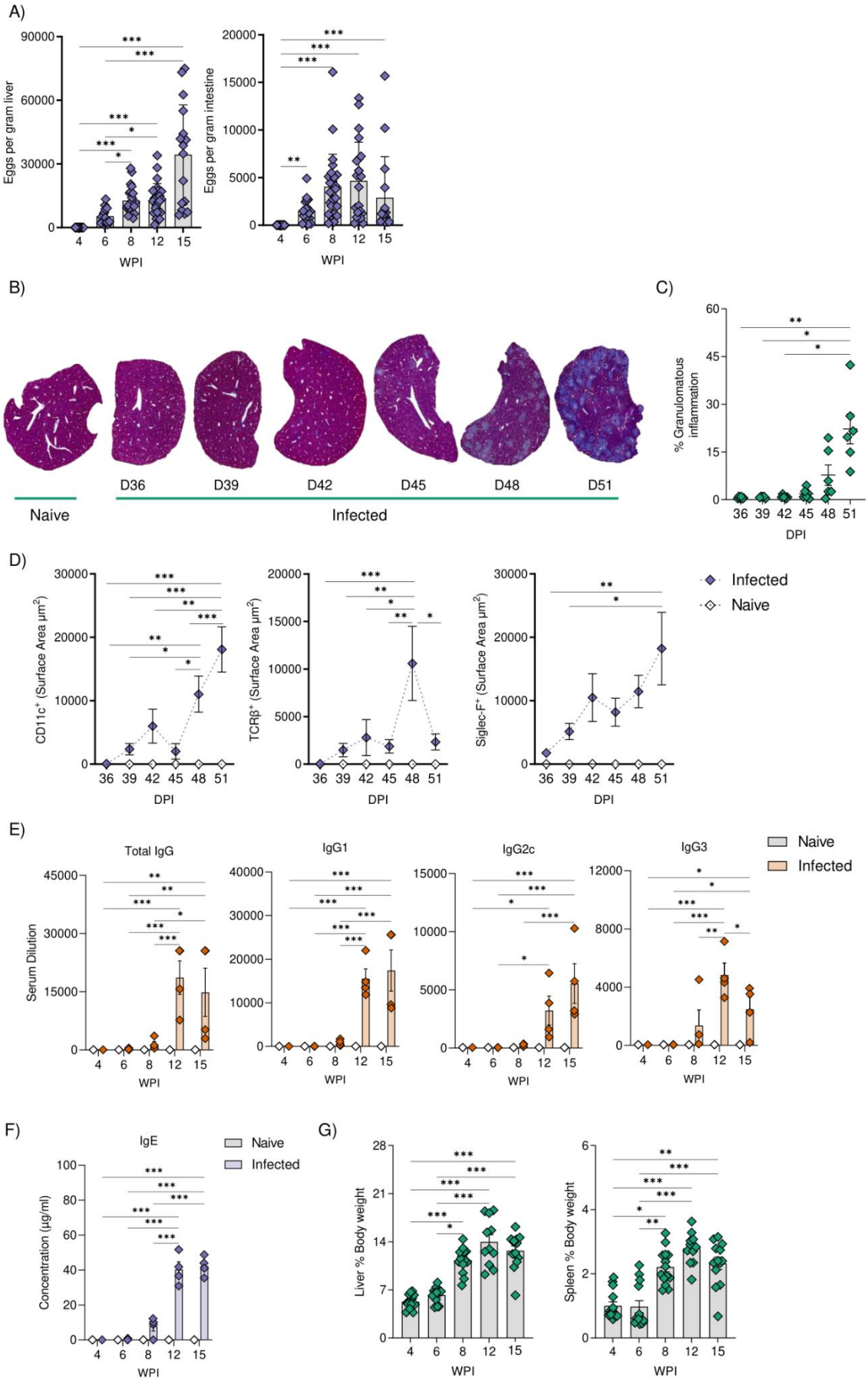
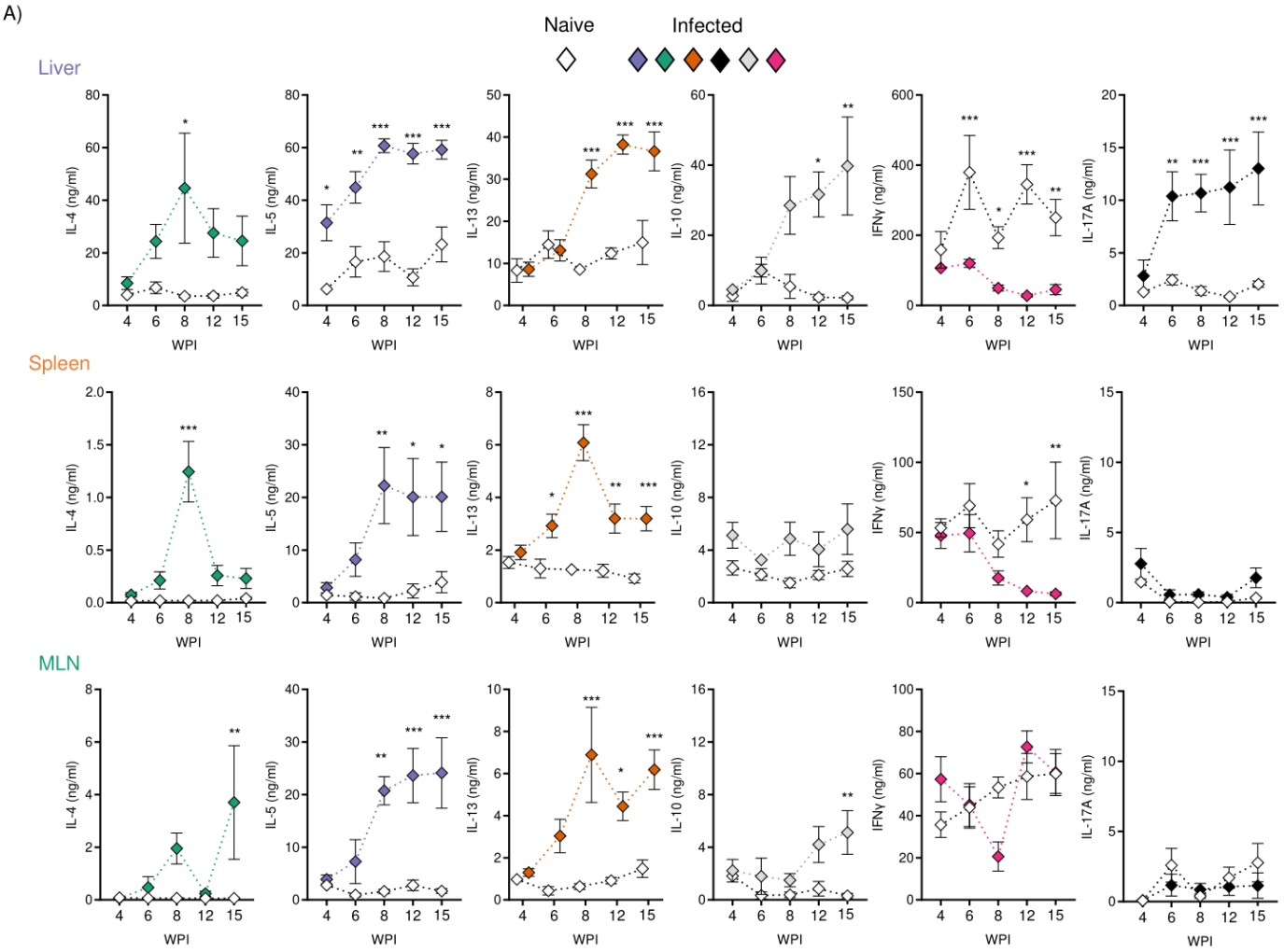

A)
Eggs per gram liver
Eggs per gram intestine
WPI
B)
D36
D39
D42
D45
D48
D51
Naive
Infected
C)
% Granulomatous inflammation
DPI
D)
CD11c+ (Surface Area µm2)
TCRβ+ (Surface Area µm2)
SiglecF+ (Surface Area µm2)
Infected
Naive
DPI
E)
Total IgG
IgG1
IgG2c
IgG3
Serum Dilution
Naive
Infected
WPI
F)
IgE
Concentration (µg/ml)
Naive
Infected
WPI
G)
Liver % Body weight
Spleen % Body weight
WPI

Supplementary Figure 1. Development of schistosome associated pathology. (A) The total number of schistosome eggs per gram of liver or intestinal tissue. (B) Visualisation of hepatic granulomas at 36, 39, 42, 45, 48 and 51 days post infection (DPI). Representative images of liver sections stained with Masson's Trichrome (MT) (C) Quantification of granulomatous inflammation. (D) Quantification of positive CD11C, TCRβ and Siglec-F staining in liver sections. (E) SEA-specific IgG, IgG1, IgG2c and IgG3 titres in the serum of naïve and infected mice, presented as endpoint serum dilutions. (F) Serum IgE titres, presented as concentration. (G) Liver and spleen weights for infected mice with data represented as a proportion of total body weight. Data are from a single experiment (B-F) or pooled from 2 (G) or 3 separate experiments (A), with 3-18 mice per timepoint, per infection group. Significance calculated by one-way or two-way ANOVA. Data presented as mean +/- SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.



Supplementary Figure 2. Cytokine profiles in response to anti-CD3 stimulation. Liver, spleen and MLN cells from naïve or infected mice were cultured for 72 h in the presence of 0.5ug anti-CD3. Supernatants were collected and cytokine production (medium alone values subtracted) was assessed by ELISA. Data are pooled from two separate experiments. Significance calculated by two-way ANOVA. Data presented as mean+/- SEM, * = p<0.05, ** = p<0.01, ***= p<0.001.

Supplementary Excel Sheet 1: RNAseq_timecourse_supplement_1:

https://docs.google.com/spreadsheets/d/1NGJ4Mrx2A8jUeHuUhvWZ0Vedi_BYIgKO/edit?usp=sharing&ouid=100566158862055693850&rtpof=true&sd=true

Supplementary Excel Sheet 2: RNAseq_timecourse_supplement_2:

https://docs.google.com/spreadsheets/d/1MSa8F3Ytuv3iJfEdX0le_OfxxlYepwmw/edit?usp=sharing&ouid=100566158862055693850&rtpof=true&sd=true

CHAPTER 4

Tissue damage and microbiota modifications provoke intestinal type 2 immunity during *Schistosoma mansoni* infection

Alice H. Costain, Alba Cortés, Stefano A. P. Colombo, Arifa Ozir-Fazalalikhan, Emma L. Houlder, Melissa Lawson, Frank Otto, Gordana Panic, Amanda Ridley, Irene Nambuya, Jaqueline J. Janse, Claudia J. de Dood, Meta Roestenberg, Angela van Diepen, Cornelis H Hokke, Jonathan R. Swann, Gabriel Rinaldi, Cinzia Cantacessi, Andrew S. MacDonald, Hermelijn H. Smits

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AUTHOR INFORMATION

Alice H. Costain[1,2], Alba Cortés[3,4], Stefano A. P. Colombo[2], Arifa Ozir-Fazalalikhan[1], Emma L. Houlder[2], Melissa A.E. Lawson[2], Frank Otto[1], Gordana Panic[5], Amanda Ridley[2], Irene Nambuya[2], Jaqueline Janse[1], Claudia J. de Dood[1], Meta Roestenberg[1], Angela van Diepen[1], Cornelis H Hokke[1], Jonathan R. Swann[5], Gabriel Rinaldi[6], Cinzia Cantacessi[3], Andrew S. MacDonald[2]*, Hermelijn H. Smits[1]*

1. Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands
2. Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, UK
3. Department of Veterinary Medicine, University of Cambridge, UK
4. Departament de Farmàcia i Tecnologia Farmacèutica i Parasitologia, Facultat de Farmàcia, Universitat de València, Spain
5. School of Human Development and Health, University of Southampton
6. Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, CB101SA, UK

*Co-corresponding authors: Andrew S. MacDonald (andrew.macdonald@manchester.ac.uk) and Hermelijn Smits (h.h.smits@lumc.nl)

ABSTRACT

During mammalian infection with *Schistosoma mansoni*, parasite eggs pierce the intestinal wall as they cross into the lumen. Despite this destructive process being central to schistosome-associated pathology, there are limited studies addressing the impact of egg transit on intestinal barrier integrity, microbiota composition, and host immune responses. Here, we present a detailed characterisation of the intestinal environment during murine schistosomiasis, using a combination of high vs low dose, and mixed sex (egg production) vs male worm only (no egg production) infections. We show that intestinal barrier function was compromised during patent (egg producing) infections, with evidence for increased intestinal permeability and systemic responses to gut bacteria. Infection intensity altered the kinetics of these barrier changes. Patent infections induced Type 2 dominated immune responses in the mesenteric lymph nodes and colonic lamina propria, as characterised by eosinophilia, elevated Th2 cell cytokine and transcription factor expression, and increased Type 2 associated factors (Relmα and Ym1) in host faeces. Importantly, this dramatic Type 2 shift coincided with significant alterations to the intestinal microbiota that became more marked as infection progressed, and with increased infection intensity. Finally, using germ free mice and faecal transplants, we found that the schistosome-associated microbiota induce a Type 2 profile in recipients. Our data elevates mechanistic understanding of schistosome-mediated immune modulation and provides evidence for a novel tripartite interaction between host, schistosome and commensal bacteria in development of the intestinal immune response during infection.

Keywords

Schistosoma, microbiota, Th2, intestinal permeability

Abbreviations

Ag, Antigen; CAA, circulating anodic antigen; DC, Dendritic cell; FITC-dextran, Fluorescein isothiocyanate–Carboxymethyl–Dextran; GF, Germ-free; IL-, Interleukin; LP; Lamina Propria; MLN; mesenteric lymph node; pDC, Plasmacytoid dendritic cell; SEA, Soluble egg antigen; SIC, small intestinal content; SPF, specific pathogen free; Th, T Helper; TMA, Trimethylamine; TMAO, Trimethylamine oxide; Treg, Regulatory T cell; WPI, weeks post infection.

INTRODUCTION

Schistosomiasis is a neglected yet significant tropical disease, spread by exposure to infectious *Schistosoma* larvae (cercariae) found in infested water in endemic regions. An estimated 250 million individuals have overt disease[1], with *S. haematobium*, *S. mansoni* and *S. japonicum* being the three main causative species. The disease that develops is indicative of where mature adult worms live and deposit their eggs within the host. During *S. mansoni* and *S. japonicum* infections, adult worms reside and lay eggs within the mesenteric vessels, with these eggs either piercing through intestinal tissue and escaping the host or being swept by portal blood flow to trap them within the liver. Tissue transiting or entrapped eggs become the focal points of T helper 2 (Th2) dominated, granulomatous inflammation. These inflammatory reactions serve as protective shields that destroy eggs and, enigmatically, facilitate their movement into the intestinal lumen[2]. If the host immune reaction ineffectively or overzealously deals with persistent egg-driven Th2 inflammation, severe obstructive and potentially life-threatening disease may result[3–5].

Schistosoma parasites are adept regulators of the host immune system, crafting an immunological environment that promotes their long-term survival whilst minimising bystander tissue damage within their host[6]. Schistosome modification of host immunity is in part achieved through the active secretion of immunomodulatory molecules[7–9], each mediating their effects through distinct pathways, cell types and at different stages of infection[10]. In addition to parasite products, host immune responses are instructed by stress signals, endogenous molecules released upon tissue damage[11], and environmental stimuli such as the microbiota[12,13].

The mammalian microbiota has a profound impact on the calibration of host immunity, with almost every disease and tissue system reporting sensitivity to microbiota composition[14]. In schistosomiasis, qualitative and quantitative changes in intestinal microbiota composition have been reported across experimental and natural infections[15–18], with accompanying pathology and immune reactions showing sensitivity to shifts in bacterial community structure[16,17,19,20]. It remains unclear whether schistosomes actively modify the composition of the microbiota in support of their infectious lifecycle, or whether the perturbed microbiota is a consequence of egg-driven tissue destruction and/or pronounced Type 2 immunity.

In this study, we investigated the extent of intestinal inflammation caused by schistosome egg transit during infection and explored the impact this process has on the intestinal microbiota, as well as how that microbiota influences host immunity. Our results revealed the dramatic impact

schistosome eggs have on the intestinal environment, including a reduction in intestinal barrier function, skewed immune profiles, and strikingly altered microbiota composition. Then, through faecal transplant studies we have identified a critical role for the intestinal microbiota in dictating host intestinal Type 2/17 immune responses. Combined, our data suggests a novel, microbial-based mechanism by which parasitic worms skew host immunity.

MATERIALS AND METHODS

Animals and Ethics statement

Age matched, female C57BL/6 mice (Envigo) were housed under specific pathogen free (SPF) conditions at Leiden University Medical Centre (The Netherlands) or the University of Manchester (UK). For germ-free (GF) experiments, GF mice were bred in isolators at the University of Manchester Gnotobiotic Facility and maintained in GF isolators or individually ventilated cages for experiment duration. For recolonisation experiments, GF mice were colonised by a single oral gavage of faeces isolated from either uninfected or schistosome infected mice. Mice were culled 2 or 3 weeks post gavage and maintained under GF conditions for this duration. Experiments based in the Netherlands were performed in accordance with the guidelines and protocols approved by the Ethics Committee for Animal Experimentation of the University of Leiden. UK experiments were performed under a project license granted by the Home Office UK and performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

Schistosome infection

Biomphalaria glabrata snails infected with a Puerto Rican strain of *S. mansoni* were maintained at Leiden University Medical Centre or obtained from K. Hoffman (Aberystwyth University, UK). Mice were percutaneously infected with male and female *S. mansoni* cercariae (mixed-sex infection) or male cercariae only (single-sex infection). Infections were performed at doses of 40 cercariae (low dose infection) or 180 cercariae (high dose infection). Cercariae sex was determined by purpose-made multiplex PCR, targeting schistosome sex-specific sequences, as described previously[21]. At the time of euthanasia, infection was confirmed in single-sex and mixed-sex infected mice by the detection of the regurgitated worm product, Circulating Anodic Antigen (CAA) in mouse serum[22]. At time points following egg deposition (week 7 onwards), mixed-sex infections were further confirmed by macroscopic evaluation of the liver and intestine, followed by whole intestine digestion in 4% KOH (24hr, 37°c) for egg counts. Mixed-sex infected mice with no visible sign of infection were excluded from analysis. A separate cohort of mice were evaluated at each specified timepoint.

FITC dextran intestinal permeability assay

Intestinal permeability was assessed *in vivo* by administration of the fluorescent tracer, fluorescein isothiocyanate-conjugated (FITC) dextran (4kDa) at dose of 0.6mg/g BW (Sigma Aldrich). Mice were fasted for 4h prior to the oral administration of FITC-dextran. 2h later, ~100µl of blood was collected by tail vein bleed, placed into heparinised tubes, and centrifuged (500xg, 10 minutes, RT) to collect plasma. FITC concentration was analysed using fluorimetry at excitation and emission wavelengths of 485nm and 525nm respectively. For standard curve creation, FITC-dextran was serially diluted in plasma obtained from non-infected control mice. Permeability testing was conducted one week prior to mice being culled for further sampling.

Cell isolation

Single cell suspensions were prepared from the mesenteric lymph nodes (MLNs), colonic lamina propria (LP) and spleen. Leukocytes were isolated from colonic LP as previously described[23,24], with some modifications to account for the high toxicity associated with schistosome infections. Colons were removed by cutting below the caecum, with care taken to remove as much fat as possible from the colon exterior. Using blunt tip scissors, colons were opened longitudinally and washed vigorously in ice-cold PBS (Sigma). In studies involving microbiome analysis, intestines were removed using sterile scissors, and a sterile razor blade was used to squeeze out any luminal content, which was transferred directly into sterile tubes, snap frozen and stored at -80 °C. After removal of luminal content, intestines were chopped into smaller segments (1-2cm) and placed in ice-cold RPMI 1640 (Sigma) containing 3% fetal bovine serum (FBS), 20 mM HEPES (Sigma) and 2mM EDTA (Sigma). After vigorous vortexing for 10 seconds, colon segments were washed with pre-warmed PBS, and the epithelial fraction removed by incubation in prewarmed RPMI 1640 containing 3% FBS, 20 mM HEPES, 5mM EDTA, and 0.5 mM freshly thawed dithiothreitol (DTT; Life Technologies) for 10 minutes at 37°C with shaking at 200rpm. Samples were vortexed, media was drained and replaced for an additional 10 minute incubation. The remaining tissue (LP and muscularis) was digested at 37°C for 30 minutes with continuous stirring (450rpm) in pre-heated RPMI 140 containing 10% FBS, 20 mM HEPES, 2 mM L-glutamine (Sigma), 1x non-essential amino acids (Sigma)**,** 1mM sodium pyruvate (Sigma), 0.5mg/ml Liberase TL (Roche), and 0.25 mg/ml DNase I type VI (Sigma). Digestion was quenched by placing on ice and topping up with ice cold media containing 10% FBS. Samples was sequentially passed through a 70µm and 40-µm cell strainer, and after pelleting, resuspended in 10% FBS containing media. MLNs were carefully removed from the exterior of the small intestine and mesenteries respectively, before 30 minute incubation with

0.15mg/ml Liberase TL and 0.05µg/ml DNase I type VI in HBSS at 37°C in a shaking incubator. Digested tissue was subsequentially passed through a 40-µm cell strainer, and after pelleting, were resuspended in 10% FBS supplemented media. Spleens were processed in a similar manner to the MLNs, with the inclusion of an additional RBC lysis step after cell straining. Single-cell suspensions were counted using haemocytometers and re-suspended in X-VIVO™ Serum-free media (Lonza), before taken further for flow cytometry or PMA/ionomycin stimulation.

Flow cytometry

Equal numbers of cells were stained per sample, washed with PBS and stained with live/dead fixable aqua dead cell stain kit (1:400; Thermo Scientific) or Zombie UV dye (1:2000, BioLegend) for 10 minutes at room temperature. Samples were subsequently blocked with 5 µg ml^{-1} FcγR-binding inhibitor (2.4G2; Biolegend) in FACS buffer (PBS containing 2% FBS and 2mM EDTA), before staining with relevant antibodies at 4 °C for 30 minutes (Table 1). For detection of intracellular markers, cells were further fixed and permeabilized with BD Cytofix/Cytoperm™ for 1h at 4°C, then stained for specified intracellular markers. Cell surface markers and intracellular cytokines were stained using combinations of fluorescently labelled primary or secondary antibodies. For intracellular cytokine staining, cells were plated at 0.4-1 × 10^6/well in volumes of 100µl and stimulated for 3h in the presence of 30ng/ml PMA, 1 µg/ml Ionomycin and 1 µg/ml Golg Stop (BD). Cells were further processed for flow cytometry as described above.

Company	Target	Clone		Company	Target	Clone
Invitrogen	CD3	17A2		Invitrogen	CD11b	M1/70
Biolegend	CD4	RM4-5		Invitrogen	CD11c	N418
Biolegend	CD8	53-6.7		BD	CD19	Ebio(ID3)
Biolegend	CD25	PC61		Biolegend	CD64	x54-5/7.1
Ebioscience	CD44	Im7		Biolegend	F4/80	BM8
Biolegend	CD62L	MeL-14		Biolegend	Ly6C	HK1.4
Biolegend	IFN-y	XMG1.2		Biolegend	MHC-II	M5/114.15.2
Biolegend	IL-10	JES5-16E3		Biolegend	PDCA-1	927
Ebioscience	IL-13	ebio13A		BD	Siglec-F	E50-2440
Biolegend	IL-17	TC11-1810.1		Ebioscience	Ter-119	TER-119
Biolegend	IL-4	11B11		Biolegend	XCR1	ZET
Ebioscience	IL-5	TRFK.5		Ebioscience	Gata-3	TWAJ
Invitrogen	TCRb	H57-597		Biolegend	GITR	DTA-1
Invitrogen	Foxp3	FJK-16s		Ebioscience	RORyt	B2D
Biolegend	CD45	30-f11		Biolegend	T-bet	4Bio

Table 1. List of Flow cytometry antibodies and their clones

DNA Isolation and Microbial 16S rRNA Gene Sequencing

Genomic DNA was isolated from faecal samples collected directly form the colon of mice infected with either mixed- or single-sex *S. mansoni* at several weeks post-infection (i.e., 4, 7, 12 and 14 weeks) and naïve counterparts using a PowerSoil DNA Isolation Kit (Qiagen) according to manufacturers' instructions. Parallel to the samples, two non-template negative controls were subjected to the DNA isolation protocol and downstream analysis to ensure no undesired contamination occurred. High-throughput sequencing of the prokaryotic 16S rRNA gene was performed on an Illumina MiSeq platform[25]. Briefly, the V3-V4 region was PCR-amplified using universal primers[26] that contained the Illumina adapter overhang nucleotide sequences, 5 ng/µl of template DNA, and the Q5® NEBNext hot start high-fidelity DNA polymerase (New England Biolabs) with thermocycling as follows: 2 minutesat 98 °C, 20 cycles of 15 s at 98 °C – 30 s at 63 °C – 30 s at 72 °C, and a final elongation step of 5 minutes at 72 °C. Resulting amplicons were purified using AMPure XP beads (Beckman Coulter) and set up for the index PCR using Nextera XT index primers (Illumina), Q5® NEBNext hot start high-fidelity DNA polymerase, and the following thermocycling protocol: 3 minutes at 95 °C, 8 cycles of 30 s at 95 °C – 30 s at 55 °C – 30 s at 72 °C, and 5 minutes at 72 °C. Indexed samples were purified using AMPure XP beads, quantified using the Qubit dsDNA high sensitivity kit (Life Technologies), and equal amounts from each sample were pooled. The resulting pooled library was quantified by real-time PCR using the NEBNext library quantification kit (New England Biolabs) and sequenced using the v3 chemistry (2x300 bp paired-end reads, Illumina).

Paired-end demultiplexed Illumina reads were processed using the Quantitative Insights Into Microbial Ecology (QIIME2; 2019.1 release) software suite[27] .Sequences were then quality filtered, dereplicated, chimeras identified, and paired-end reads merged in QIIME2 using DADA2[28]with default settings. A phylogenetic tree was generated using the align-to-tree-mafft-fasttree pipeline in the q2-phylogeny plugin. Bray-Curtis dissimilarity between samples was calculated using core-metrics-phylogenetic method from the q2-diversity plugin. Classification of Operational Taxonomic Units (OTUs) was performed using a Naïve Bayes algorithm trained using sequences representing the bacterial V3-V4 rRNA region available from the SILVA database (https://www.arb-silva.de/download/archive/qiime; Silva_132)[29] (Quast et al., 2013), and the corresponding taxonomic classifications were obtained using the q2-feature-classifier plugin in QIIME2. The classifier was then used to assign taxonomic information to representative sequences of each OTU.

Statistical analyses were performed using the online software Calypso (cgenome.net/calypso/)[30]. For data normalisation, cumulative-sum scaling (CSS) was applied to the OTU table, followed by log2 transformation (CSS+log) to account for the non-normal distribution of taxonomic counts data. Samples were ordinated using unsupervised Principle Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities, or Non-metric multidimensional scaling (NMDS). Supervised Canonical Correspondence Analysis (CCA) was performed on samples collected at individual time points and setting the type of infection as explanatory variable. Following rarefaction of raw data, differences in microbial alpha diversity (Shannon diversity), richness and evenness were evaluated using ANOVA. Where genera were ranked by abundance this was achieved by calculating the mean of the total normalized readcounts per genera across all samples. Heatmaps were generated using pheatmap (version 1.0.12). All other graphs were produced using ggplot2 (version 3.3.3).

Metabolomics

Small intestinal content (SIC) samples were randomised and 20-50 mg of sample weighed out into a 2 ml sterile screw-top tube. 10-20 zirconium beads were proportionally added, as well as $_{d}H2O$ at a 2:7 weight to water ratio. The contents were homogenized (6,500 rpm, 2 cycles of 45 seconds), then centrifuged at 13,000 x g for 20 minutes. Subsequently, 54 µl of the supernatant was transferred to an Eppendorf tube and 6 µl of phosphate buffer solution (1.5 M KH2PO4, 2 mM NaN3, 1 % TSP, pH 7.4) was added and the contents vortexed. 50 µl of the mixture was added to 1.7 mM tubes. A quality control sample was generated by pooling from each sample and combining with buffer as described above.

One dimensional 1H NMR spectra were acquired on a Bruker Advance III HD 600 MHz NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), equipped with a SampleJet system and a cooling rack of refrigerated tubes at 6° C. For serum samples, a relaxation edited spin-echo using the 1D-Carr-Purcell-Meiboom-Gill (CPMG) presat pulse sequence was applied to all samples, analysed at a temperature of 300 K. For SIC samples, a standard one-dimensional solvent suppression pulse sequence was used (relaxation delay, 90° pulse, 4 µs delay, 90° pulse, mixing time, 90° pulse, acquire FID), analysed at a temperature of 310 K. For each sample, 128 transients were collected in 64K frequency domain points with a spectral window set to 20 ppm.

Automated processing of the spectra was performed using TopSpin 3.6 (Bruker Corporation, Germany) including spectral calibration, phase and baseline correction. The resulting raw NMR spectra were imported into MATLAB (Version 2018a, Mathworks Inc). After digitization of the

spectra, redundant peaks (TSP, H2O) were removed. The spectra were manually aligned to quality control samples and reference peaks using Recursive Segment-Wise Peak Alignment 1 (University of Southampton). Metabolite identification was performed using an in-house metabolite library, cross-referencing with the Human Metabolome Database (https://hmdb.ca/) and Statistical Total Correlation Spectroscopy (STOCSY) 3. The relative concentrations of all age associated metabolites were calculated from the spectral data using trapezoidal numerical integration.

Faecal ELISAs and occult blood detection

Faecal pellets were reconstituted in PBS containing 0.1% Tween 20 (100mg/ml) and vortexed thoroughly to attain a homogenous faecal suspension. Samples were centrifuged (10 minutes, 4°C, 12,000 rpm) to collect a supernatant that was stored at -20°C until later analysis. Faecal levels of lipocalin (Lcn-2; R&D Systems), Ym1 and RELMα (both PeproTech) were analysed by ELISA as per manufacturer's instructions. Faecal blood was detected using Hemdetect occult blood detection kits (Dipro).

Cytokine and serum Ab analysis

Serum levels of Ym1, RELMα (both Peprotech) and IgE (BD**)** were measured using paired capture and detection antibodies, with quantity assessed via standard curve. Levels of IFNγ, IL-5, IL-12 and TNFα were measured by Cytometric Bead Array (CBA) according to manufacturer's instructions (BD). Commensal bacteria-specific ELISAs were conducted as previously described[31]. Briefly, caecal content from a naïve C57BL/6 SPF mouse was homogenised and centrifuged at 1,000 rpm to remove large aggregates. The resulting supernatant was washed twice with sterile PBS by centrifugation for 1 minute at 8,000 rpm. For the final wash, the bacterial pellets were re-suspended in 2 ml ice-cold PBS and sonicated on ice. Samples were subsequently centrifuged at 20,000xg for 10 minutes, and supernatants recovered for a crude commensal bacterial Ag preparation. To measure levels of commensal bacteria specific Total IgG, 96 well plates (NUNC Maxisorp) were coated with 5µg/mL of commensal bacterial Ag and sera were incubated at dilutions of 1:50. Alkaline phosphatase–conjugated goat anti–mouse IgG (SouthernBiotech) was added to plates (1:1000), followed by liquid PNPP substrate, with absorbances read at 405nm. For the presence of IgG1 and IgG2c reactive against SEA, 96 well plates were coated with 5 µg/mL and serum was added at dilutions of 1:1000. SEA-specific isotypes were detected using alkaline phosphatase–conjugated goat anti–mouse IgG1 or IgG2c antibodies (Southern Biotech). Absorbance at 405 was determined as above. For all ELISAs, blocking steps were conducted with 1% bovine serum albumin (BSA, Sigma) for 1h at room

temperature. After blocking and between individual incubation steps, plates were washed 3 times with PBS containing 0.05% Tween (Sigma).

RNA extraction and quantitative PCR analysis

RNA from snap-frozen MLNs or ileum was extracted using TriPure isolation reagent (Roche) and translated to cDNA using SuperScript™ III Reverse Transcriptase and Oligo (dT; Life Technologies). Quantitative PCR was performed with SYBR Green Master Mix (Applied Biosystems) using a Biorad CFX96 Real-time system C1000 thermal cycle. Expression levels were normalized to housekeeping gene Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0). The primers used are given in the table below.

Target gene	Forward primer	Reverse primer
Cdh1	CCAAGCACGTATCAGGGTCA	ACTGCTGGTCAGGATCGTTG
Chi3l3	ACAATTAGTACTGGCCCACCAGGAA	TCCTTGAGCCACTGAGCCTTCA
Cldn2	ATCACCACAGCTTGTGACCC	TCTAGAAAACGGAGCCGTCC
Cldn3	CCTAGGAACTGTCCAAGCCG	CCCGTTTCATGGTTTGCCTG
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
Ifng	CGGCACAGTCATTGAAAGCC	TGTCACCATCCTTTTGCCAGT
Il10	GACAACATACTGCTAACCGACTC	ATCACTCTTCACCTGCTCCACT
Il12a	GGTGAAGACGGCCAGAGAAA	GTAGCCAGGCAACTCTCGTT
Il13	CCCTGGATTCCCTGACCAAC	GGAGGCTGGAGACCGTAGT
Il17a	TCATCCCTCAAAGCTCAGCG	TTCATTGCGGTGGAGAGTCC
Il33	CTGCAAGTCAATCAGGCGAC	ACGTCACCCCTTTGAAGCTC
Il4	CCTCACAGCAACGAAGAACA	ATCGAAAAGCCCGAAAGAGT
Il5	TGGGGGTACTGTGGAAATGC	CCACACTTCTCTTTTTGGCGG
Lcn2	GCCACTCCATCTTTCCTGTTG	AAGAGGCTCCAGATGCTCCTT
Nod2	CTGTCCAACAATGGCATCACC	GTTCCCTCGAAGCCAAACCT
Nos2	TCCTGGACATTACGACCCCT	CTCTGAGGGCTGACACAAGG
Ocln	GTCCTCCTGGCTCAGTTGAA	AGATAAGCGAACCTTGGCGG
Rn18s	GACTCAACACGGGAAACCTC	AGACAAATCGCTCCACCAAC
Rplp0	TCTGGAGGGTGTCCGCAACG	GCCAGGACGCGCTTGTACCC
Tnf	GTCCCCAAAGGGATGAGAAG	CACTTGGTGGTTTGCTACGA
Tjp	GGAGATGTTTATGCGGACGG	CCATTGCTGTGCTCTTAGCG

Table 2. Primers for qPCR

Statistics

Statistical analysis of 16S rRNA Gene Sequencing was performed as described. All other statistics were performed using GraphPad Prism 9 software. Data are shown as mean values ± S.E.M. Where applicable, experimental groups were analysed by unpaired t-test, one-way or two-way analysis of variance (ANOVA) followed by Tukey's post-test as appropriate. Significant differences were defined at $P < 0.05$.

RESULTS

Increased intestinal barrier permeability during chronic egg producing *S. mansoni* infections

During active *S. mansoni* infections, eggs continuously transit from the mesenteric vasculature across the intestinal wall for eventual release into the environment via the faeces[32]. With several hundred eggs produced per day per worm pair[33], this process causes significant damage to the intestinal wall[6]. To investigate whether egg transit reduces mucosal barrier function, we assessed intestinal leakage and inflammation at several key stages of the *S. mansoni* lifecycle: pre-patent acute (week 3), the start of post-patent acute (week 6), the midst of post-patent acute disease (week 8) and chronic stages of disease (weeks 11 and 13) (Figure 1A). In addition, to distinguish the influence of worm or egg-derived signals on intestinal function, comparison was made between mice infected with patent, dual-sex schistosome infections (mixed-sex infection), or mice infected with male schistosomes only (single-sex infection), in which no eggs are produced[21]. Measurement of serum circulating anodic antigen (CAA)[34] levels confirmed similar infection burdens between the single- and mixed-sex infection groups (Supplementary Figure 1A).

Intestinal permeability, as evaluated via oral administration of FITC labelled dextran (Figure 1C), showed that until 8 weeks post infection, no measurable differences in FITC-dextran levels were detected in the plasma of mixed-sex, single-sex or uninfected control mice. However, from week 11 of infection onwards, intestinal permeability was significantly higher in mixed-sex infected mice in comparison to single-sex infected or uninfected controls. These permeability changes did not correlate with individual mouse infection intensity, as measured by intestinal egg burden (Figure 1B) or levels of the regurgitated worm Ag, CAA (Supplementary Figure 1A).

To test whether, as a consequence of increased gut barrier permeability, luminal content (including intestinal bacteria and their products) may have permeated local tissues or systemic circulation, serum levels of commensal bacteria-specific IgG were measured across the course of infection (Figure 1D). In keeping with observed permeability kinetics (Figure 1C), significantly higher levels of

commensal bacteria-specific IgG were detected in the serum of mixed-sex infected mice at weeks 12 and 14 post infection, but not in naïve or single-sex infected mice (Figure 1D). Similarly, faecal occult blood was detected in faeces from mixed-sex infection at weeks 8, 12 and 14, at respective percentages of 12.5%, 87.7% and 75% (Figure 1F&G). In addition to occult blood, faecal pellets were analysed for levels of lipocalin (LCN-2), an anti-microbial peptide that can be found within neutrophilic granules or readily released from the intestinal epithelium upon damage[35]. Faecal lipocalin levels were significantly higher in mixed-sex infected mice from week 8 onwards (Figure 1E). Despite identifying multiple signs of disrupted intestinal barrier integrity during chronic disease, we observed no differences in mRNA expression of intestinal tight junction molecules such as claudin-2, claudin-3, occludin, E-cadherin and tight junction protein (TJP) (Supplementary Figure 1B).

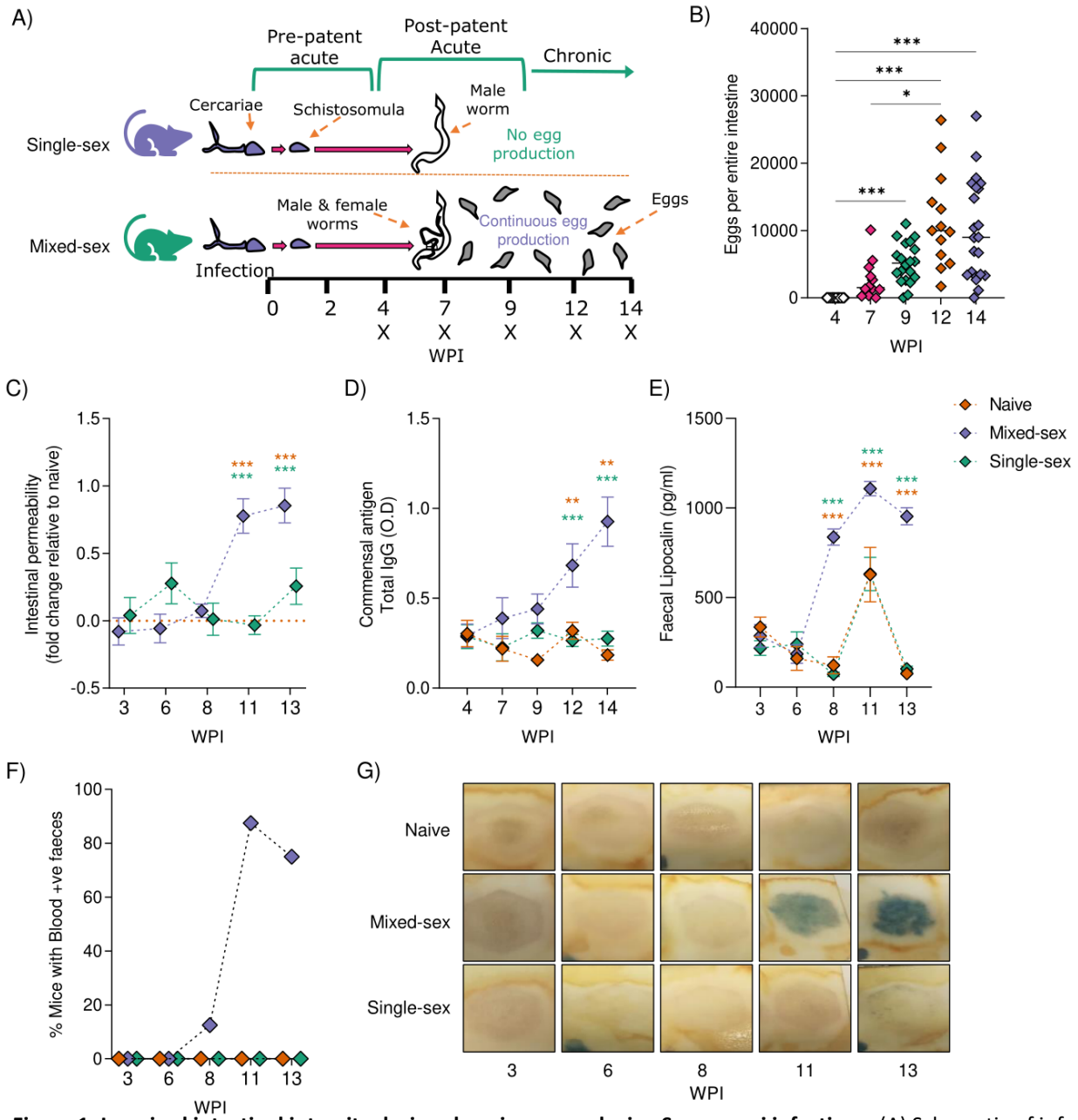


Figure 1. Impaired intestinal integrity during chronic egg producing S. mansoni infections. (A) Schematic of infection setup (WPI, weeks post infection). C57BL/5 mice were infected with ~40 S. mansoni cercariae, of either male and female gender (mixed-sex infection), or male only (single sex infection). Infections lasted 4, 6, 9, 12 or 14 weeks (indicated by X) and permeability was measured 1 week prior by FITC-dextran oral gavages. (B) Intestinal egg counts in mixed-sex infected mice at indicated time points. (C) Changes in intestinal permeability over the course of infections as measured by the detection of FITC-dextran (ELISA) in serum of mice 2 hours after oral gavage. Data presented as fold change relative to naïve controls. (D) Relative serum IgG specific to commensal bacteria throughout time-course. (E) Faecal Lipocalin, a marker of intestinal epithelial damage, was measured in the faeces of mice on the same weeks of FITC dextran permeability measurement. (F) Percentage of mice positive for blood in faeces. (G) Images depicting occult blood detection (blue) in faecal samples of indicated mice groups. Data presented as mean +/- SEM. n=12-24 mice per group pooled across two (D-G) or three separate experiments (B&C). Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and calculated by one-way ANOVA (B) or two-way ANOVA (C-E). Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively. Significant differences between naïve and single-sex infected mice are indicated by #. n= 18-24 mice per group from 3 pooled experiments.

Pronounced intestinal Th2 inflammation in patent mixed-sex infections only

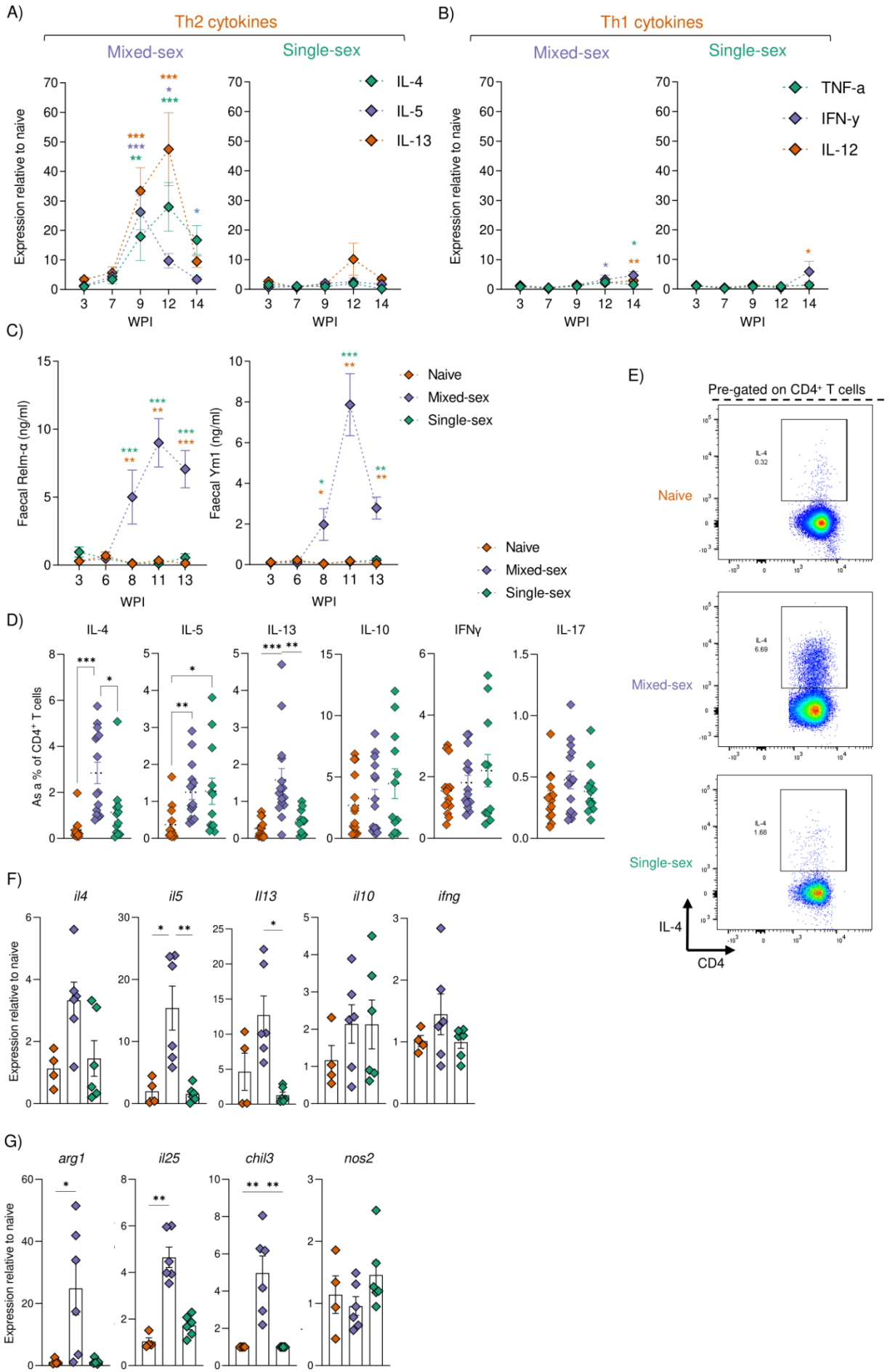
After identifying distinct differences in intestinal integrity between patent and non-patent infections, we next addressed the influence of egg migration on local immune responses. We first evaluated the kinetics of mRNA expression of cytokines within the MLNs of mixed-sex and single-sex infected mice across the course of infection, and naïve controls (Figure 2 A&B). In support of existing literature on systemic responses[36,37], mixed-sex infections were accompanied by pronounced Th2 polarisation in terms of *il4*, *il5* and *il13* expression, from week 9 onwards (Figure 2A). After peaking at 9 or 12 weeks post infection, Th2 cytokine expression declined, but remained elevated in comparison to pre-patency. In contrast to mixed-sex infected mice, the expression of Th2-associated cytokines in single-sex infections remained comparable to naïve irrespective of time-point. Regarding Th1 associated cytokines, *ifng*, *il12* and *tnfa* mRNA expression remained similar between all groups at weeks 4-9 (Figure 2B). However, by week 14 we observed a modest but significantly increased Th1 cytokine expression profile in both mixed- and single-sex infection groups (Figure 2B). These MLN gene expression patterns were reflected at a systemic level (Supplementary Figure 2), with only mixed-sex infections showing elevated serum levels of Th2 associated mediators IL-5, RELMα, Ym1 (Supplementary Figure 2A), total IgE and SEA-specific IgG1 (Supplementary Figure 2C). From a Th1 perspective, we observed significantly higher serum levels of IFNγ, IL-12, TNF-α (Supplementary Figure 2B), and SEA-specific IgG2c (Supplementary Figure 2C), in single-sex infected mice at chronic disease stages.

Next, reasoning that mediators produced locally due to egg induced tissue damage may leak into the intestinal lumen, faecal pellets were analysed for various inflammatory mediators and cytokines (Figure 2C). Interestingly, while many cytokines showed no difference between the experimental groups (IL-6, IL-1β, and IFN-β; data not shown) or could not be detected (IL-13, IL-4, IL-10; data not shown), faeces from mixed-sex infected mice showed significantly higher levels of Type 2 mediators, Ym1 and RELMα from week 8 of infection onwards.

Focussing on week 14, a time point at which egg migration has long been established and permeability is pronounced (Figure 1), we observed similar trends for MLN $CD4^+$ T cell cytokine production (Figure 2D&E) and ileal mRNA expression (Figure 2F). Notably, the production and expression of Th2 associated cytokines was elevated in mixed-sex infected mice only, with the exception of IL-5, whose secretion from $CD4^+$ T cells was comparable in single-sex infected mice. Moreover, despite the damage caused by egg transit, patent infection did not significantly influence levels of MLN cell expression or production of inflammatory IFNγ or counter regulatory IL-10.

Additionally, ileal tissue from mixed-sex infected mice expressed similar mRNA expression profiles of Th2 and Th1 cytokines to MLN (Figure 2F), as well as elevated expression of genes associated with alternative activation of macrophages (*arg1* and *chil3*), and the damage associated alarmin *il25* (Figure 2G). No differences were observed in the expression of *nos2*, which is linked to classical macrophage activation[38].

Figure 2. Pronounced Th2 orientated immune profiles in mixed-sex infections. (A&B) The mRNA expression of Th2 (IL-4,5 &13) and Th1 (IL-12,TNF & IFN-y) cytokines in the mesenteric lymph nodes (MLNs) of naïve, mixed-sex and single-sex infected mice (both infected with 40 cercariae) at indicated time-points. Data normalized against HK gene RPLP0 and represented as fold change relative to naïve. (C)) Faecal levels of RELMα and Ym1 (D) Cytokine secretion from PMA ionomycin stimulated MLN cells at week 14 of infection.(E) Representative flow plots for IL-4 secretion, pre-gating on live CD45+ TCRβ+ CD4+ cells. (F&G) The colonic mRNA expression of indicated genes at 14 weeks post infection. Data normalised against HK pool (RPLP0, β2m, β-actin and s18) and represented as fold change relative to nave. Data presented as mean +/- SEM. n=4-7 from one single experiment (F&G) or pooled across two separate experiments (A,B,C,D&E). Significant differences were determined by one-way (D,F&G) or two-way ANOVA (A-C) followed by Tukey post hoc tests. *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively.


A)
Th2 cytokines
Mixed-sex
Single-sex
IL-4
IL-5
IL-13
Expression relative to naive
WPI
B)
Th1 cytokines
TNF-a
IFN-y
IL-12
C)
Faecal Relm-α (ng/ml)
Faecal Ym1 (ng/ml)
Naive
Mixed-sex
Single-sex
D)
IL-4
IL-5
IL-13
IL-10
IFNγ
IL-17
As a % of CD4+ T cells
E)
Pre-gated on CD4+ T cells
Naive
Mixed-sex
Single-sex
IL-4
CD4
F)
il4
il5
Il13
il10
ifng
Expression relative to naive
G)
arg1
il25
chil3
nos2
Expression relative to naive

Schistosome egg driven microbial disruption increases as infection progresses

Alterations in intestinal microbiota composition, as investigated through 16s rRNA sequencing, have been reported during human[39–42] and murine infections[16,17,43] with *Schistosoma spp.* including male-sex infections lacking egg production[17]. These studies either focus on one [16,17,40,42] or two isolated time points[41,43] with or without comparison into anthelminthic administration[41]. However, to our knowledge, there are no studies detailing how the microbiota changes over the course of schistosome infection and whether any changes are driven by worms, eggs or the immune response accompanying infection. To assess the influence of egg transit on microbial communities, large intestinal content was recovered from naïve, single-sex and mixed-sex infected mice at 4, 7, 12 and 14 weeks post infection, and bacterial community composition was assessed by 16s sequencing. In line with previous reports[15], intestinal microbiota profiles were comparable between experimental groups prior to the onset of egg production (4 weeks post infection), but by week 7 of infection dimensionality reduction by non-metric multidimensional scaling (NMDS) revealed a distinct shift in the bacterial composition of mixed-sex infected microbiota relative to naïve and single-sex infected groups (Figure 3A). This shift in composition became more pronounced at weeks 12 and 14 post infection suggesting that sustained egg accumulation/transit has a large influence on intestinal microbial communities. Supporting this, we observed no clear separation of naïve or single-sex samples at any time-point of infection. However, when analysed using supervised Canonical Correspondence analyses (CCA) to test whether observed trends could be explained by infection group, significant differences in colonic microbial communities became apparent between single-sex, naïve and mixed-sex infected groups (Figure 3B).

To gain insight into these microbial changes, differences in alpha diversity (Shannon diversity), richness and abundance of individual taxa was evaluated at each timepoint (Supplementary Figure 3A). While no significant differences were detected across groups at weeks 4 and 7, during chronic weeks 12 and 14, mixed-sex infections showed a significant decrease in microbial evenness without affecting either species richness or overall alpha diversity. With the above data hinting towards overgrowth or overrepresentation of particular bacterial species, we inspected bacterial communities at the genus level (Figure 3C), which revealed greater representation of bacteria of the genera *Alistipes*, *Alloprevotella*, *Bacteroides* and *Rikenellaceae* in mixed-sex infected mice at weeks 12 and 14, in comparison to earlier stages.

The intestinal microbiota plays a crucial role in host physiological processes and homeostasis, mediated in part through the vast range of metabolites they produce[44]. NMR spectroscopy was used

to capture the metabolic profile of the small intestine in naïve, single-sex and mixed-sex infected mice (Supplementary Figure 3B). Of the metabolites investigated, we observed increased levels of dihydroxyacetone in mixed-sex infected mice at week 14. Conserved across single-sex and mixed-sex infections were decreased intestinal levels of cholates and bile acids, and elevations in tartrate and lysine. We found no significant changes in intestinal levels of the short chain fatty acid butyrate.

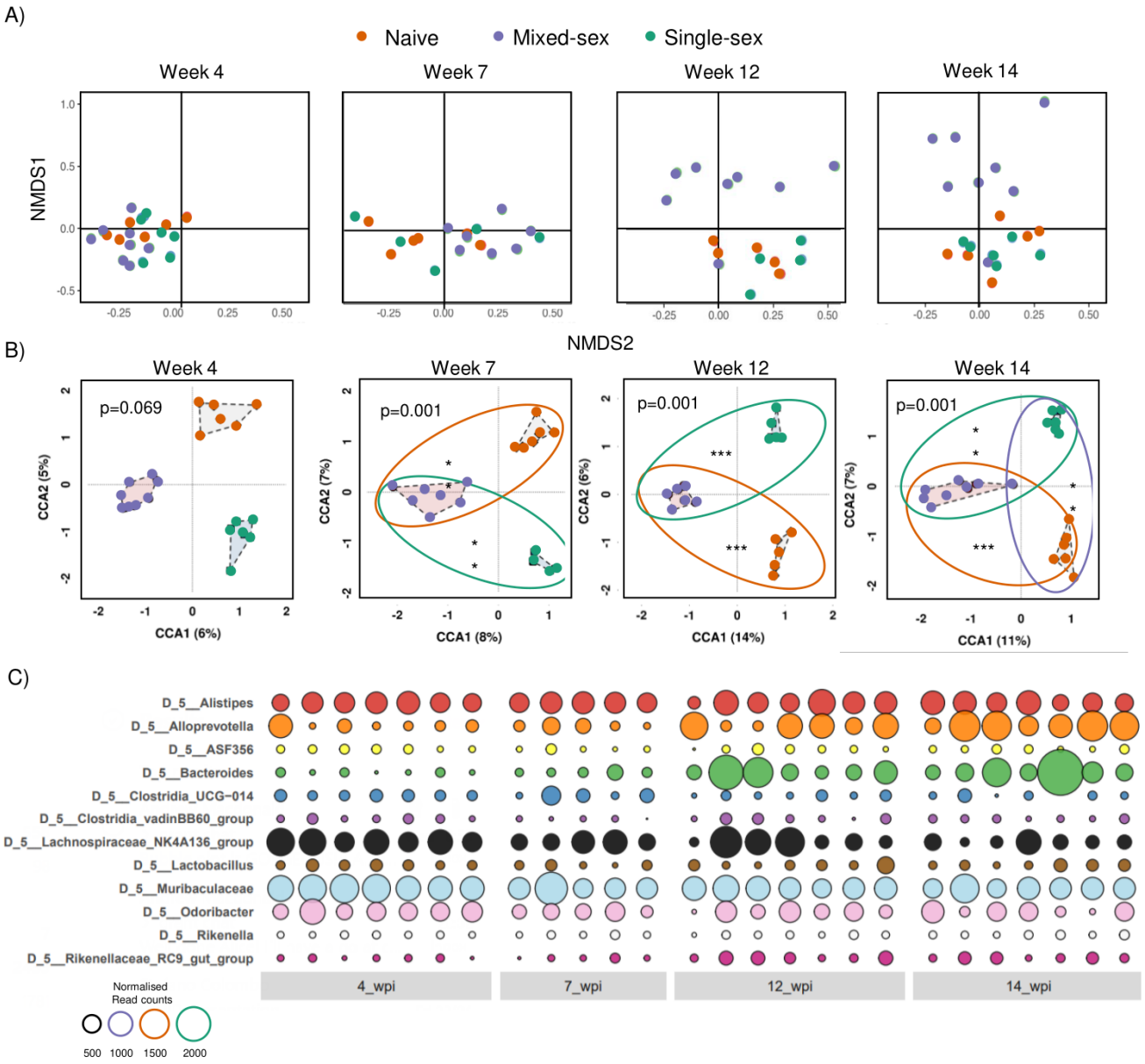


Figure 3. Egg producing schistosome infections progressively alter the of colonic microbial communities. Colonic microbial profiles of mice infected with mixed-sex or single-sex S. mansoni parasites, at weeks 4, 7, 12 and 14. Comparison made to uninfected controls. Ordinated by (A) Non-metric multidimensional scaling (NMDS) and (B) Canonical Correspondence Analysis (CCA). (C) Microbial composition at genus level in mixed sex infections only. Bubble plot representing the relative abundance of the 12 most prevalent genera. Circle diameter reflects the proportion of microbiota comprised of that genus. Empty spaces reflect no detection of a given genus by 16S sequencing. Data from one single experiment with n=5-7. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Schistosome egg transit impairs intestinal barrier function in a dose dependent manner

Having established that egg producing infections diminish intestinal barrier function during chronic infection stages (Figure 1), we next asked how these permeability alterations could be influenced by increasing infection intensity. To test this, direct comparison was made between mice conventionally infected with 40 (low) or 180 (high) mixed sex cercariae, with infections lasting 7 weeks in duration and permeability measured 3 days prior to mouse culling (Figure 4A). As expected, high dose infected mice showed a higher burden of eggs per gram of liver tissue (Figure 4B). In line with our time-course experiment using low dose infection (Figure 1), we detected no difference in plasma FITC levels between naïve mice and those infected with 40 mixed-sex cercariae at this 6 week time-point (Figure 4C). However, significantly increased permeability was evident in mice infected with 180 cercariae at this early stage of infection, similar to low dose infected mice at later time points (i.e. week 11 onwards (Figure 1C)). Importantly, these high dose infected mice also demonstrated higher titres of commensal-bacteria specific IgG (Figure 4D) and faecal RELMα (Figure 4E) and Ym1 (Figure 4F) and increased faecal blood (Figure 4G&H). Together, these data demonstrated that intestinal barrier function was compromised to a similar degree in high dose infected mice (week 7) and mice harbouring chronic low dose infections (from week 11), and suggested that infection intensity and egg number was a strong determinant of changes in gut barrier function, rather than time after infection per se.

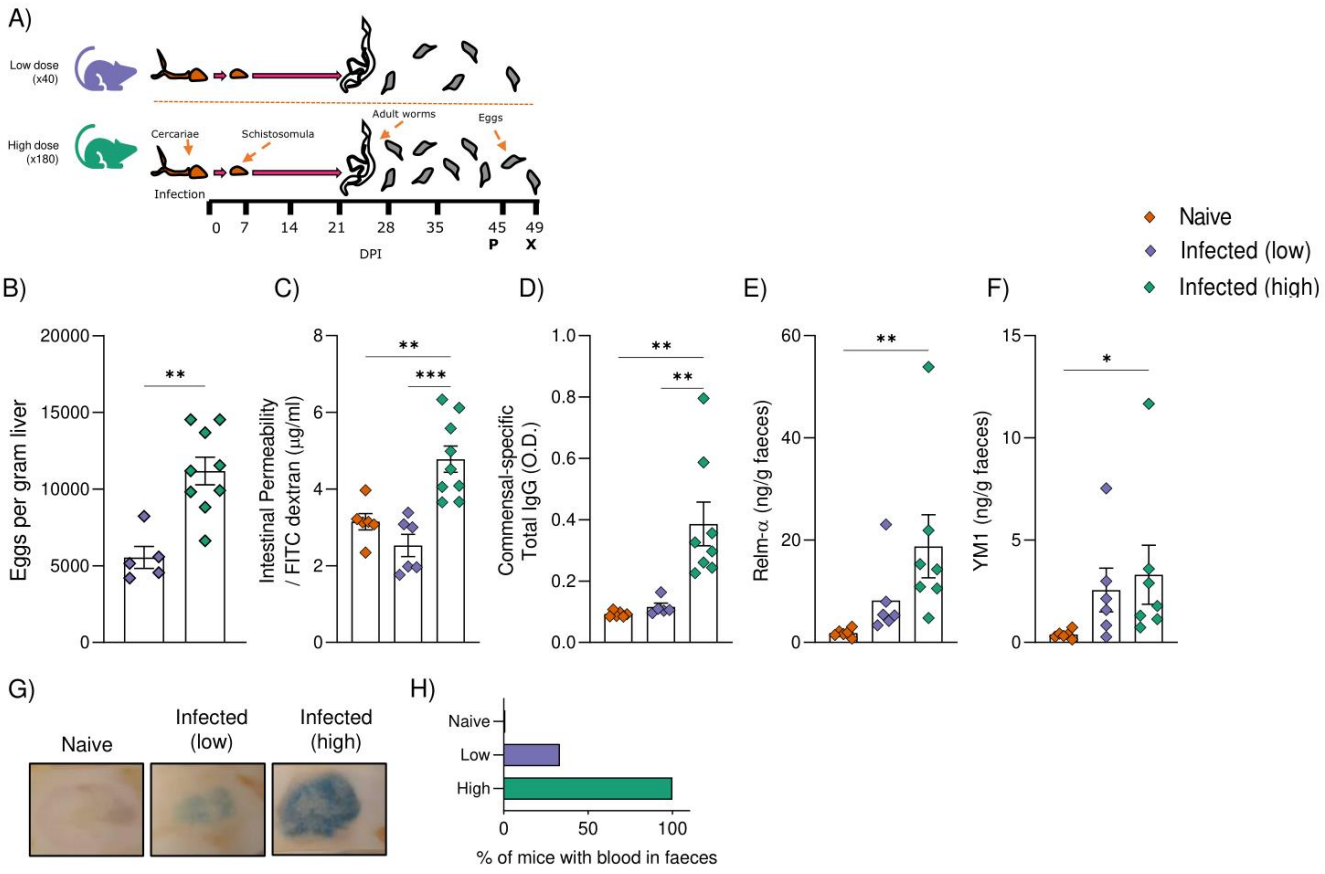


Figure 4. High dose infections evoke earlier impairments in barrier function. (A) Schematic of infection setup (weeks post infection; WPI). C57BL/5 mice were infected with 40 (low dose) or 180 (high dose) of egg producing, mixed gender S. mansoni cercariae. Infections lasted 7 weeks (indicated by X) and permeability was measured 4 days prior by FITC-dextran permeability assay (day 45). (B) Eggs per gram liver tissue (C) Changes in intestinal permeability as measured by serum FITC-dextran. (D) Serum IgG specific to commensal bacteria at week 7. (E) Faecal levels of RELMα and (F) Ym1. (G) Images depicting occult blood detection (blue) in faecal samples of indicated mice groups. (H) Percentage of mice positive for blood in faeces. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and determined by one-way ANOVA and Tukey's post hoc test. Data presented as mean +/- SEM, * = $p<0.05$. n = 5–9 from two pooled experiments.

Immune profiles in high vs low dose infection differ more in colons than MLNs

Akin to other helminth infections[24,45] it is extremely challenging to isolate live intestinal immune cells from schistosome infected mice, with no published reports of this, and so no data available yet to assess whether MLN cellular profiles are an accurate reflection of the intestinal tissue response to schistosome infection. However, using an adapted version of recently published protocols for naïve[23] and *Heligmosomoides polygyrus* infected intestinal tissues[24,45], we were able to successfully isolate live LP leukocytes from the colons of *S. mansoni* infected mice and characterise them by multiparameter flow cytometry (Figure 5).

Irrespective of dose, we observed dramatic eosinophilia and neutrophilia in the MLNs of infected mice, alongside decreased proportions of $CD4^+$ and $CD8^+$ T cells (Figure 5A). While macrophages are typically infrequent in LNs[46], they significantly increased in both high and low dose infection groups. A clear infiltration of monocytes was observed in high dose infected mice only, while B cell frequency was moderately increased in low dose infection alone. Within the colon, we also observed significantly increased eosinophilia (low dose and high dose) and neutrophilia (high dose only) in infected mice (Figure 5B). However, in contrast to the MLNs, we observed no significant change in colonic $CD4^+$ T cell, macrophage, monocyte, or B cell frequency during infection. Taken together, similar cellular profiles were generally observed in low and high dose infection, cellular changes were generally more dramatic and evident in high dose infections, particularly in the MLN.

Having compared cellular changes in MLNs and colons from high vs low dose infection, we next investigated T cell functionality in these compartments. Focusing first on mesenteric responses, we assessed cytokine production in *ex vivo* stimulated MLN $CD4^+$ T cells (Figure 6A). Irrespective of dose, $CD4^+$ T cells from infected mice showed a significantly greater capacity to express IL-4, IL-5, IL-13, IL-10 and IFNγ than uninfected mice, and a trend towards increased IL-17. While $CD4^+$ T cells from high dose infected mice generally demonstrated a greater potential to produce these cytokines than low dose infected mice, no statistically significant difference was found. To complement these data, we evaluated MLN $CD4^+$ T cell expression of transcription factors Foxp3, Gata-3, T-bet and Rorγt, which demarcate regulatory T cells (Tregs), Th2, Th1 and Th17 cells, respectively (Figure 6B). Similar to their cytokine profiles, MLN $CD4^+$ T cells from high or low dose infection expressed higher levels of Gata-3, Foxp3 and Rorγt than uninfected controls, which was also evident for T-bet in high dose infection alone, with no significant difference between high and low dose infected mice for any of these transcription factors.

While the colonic LP contained a relatively low percentage of CD45$^+$ cells (~10-20% of total), sufficient numbers were recovered to characterise the CD4$^+$ T cell compartment (Figure 6C&D). In clear contrast to the comparable phenotype of MLN CD4$^+$ T cells isolated from high vs low dose infection, only colonic CD4$^+$ T cells from high dose infection had a significantly increased capacity to produce IL-5, IL-13, IL-10 and IFNγ in comparison to naïve mice, and a trend towards higher IL-4 (Figure 6C)**.** Similarly, colonic CD4$^+$ T cell expression of Gata-3 and T-bet was increased in high dose infections only (Figure 6D), and no significant differences in Foxp3 or Rorγt were detected.

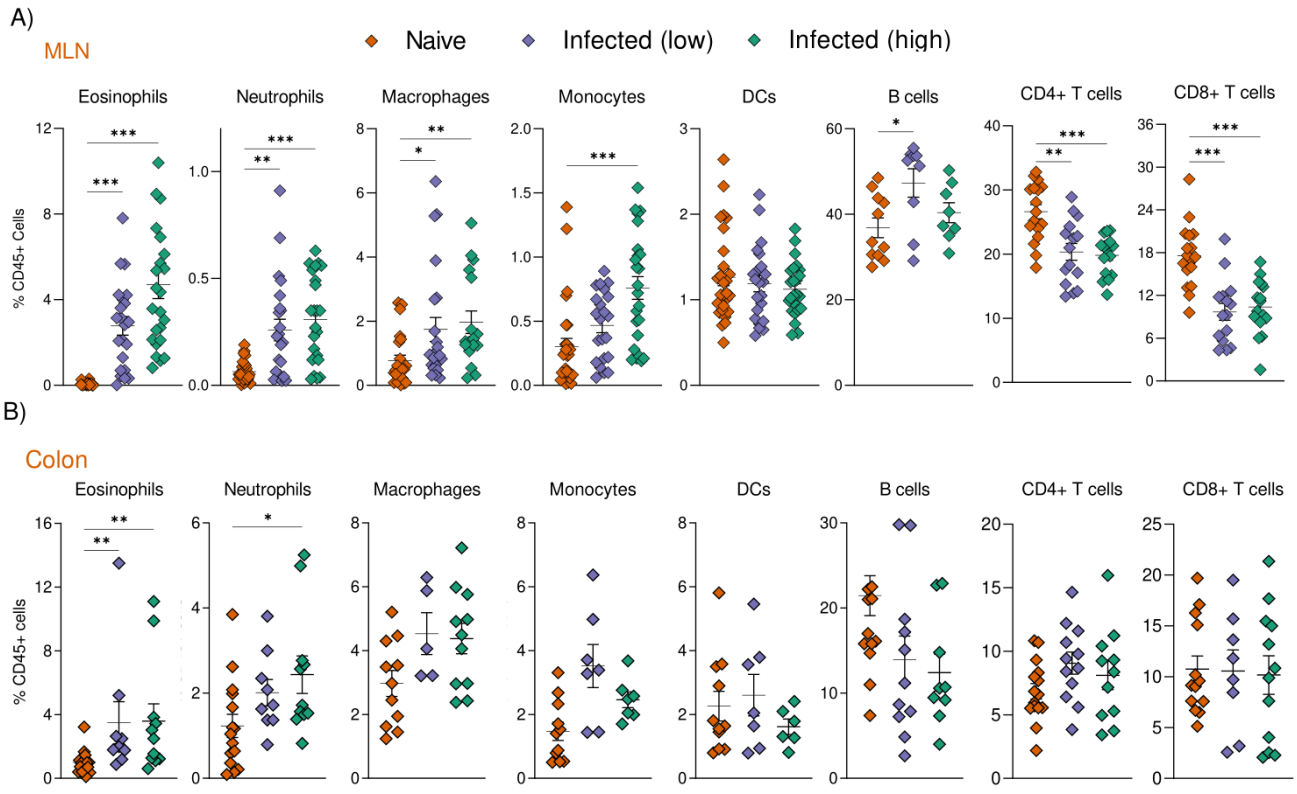


Figure 5. Increased appearance of granulocytes in colon and MLN of schistosome infected mice. The frequency of various immune cells in the (A) MLN and (B) colon of schistosome infected mice. Data shown are from 7 (A) or 5 (B) pooled experiments, with 2-4 mice per experimental group per experiment. Data presented as mean +/- SEM. Significant differences were determined by one-way ANOVA with suitable post-hoc testing. *p < 0.05, **p < 0.01, ***p < 0.001

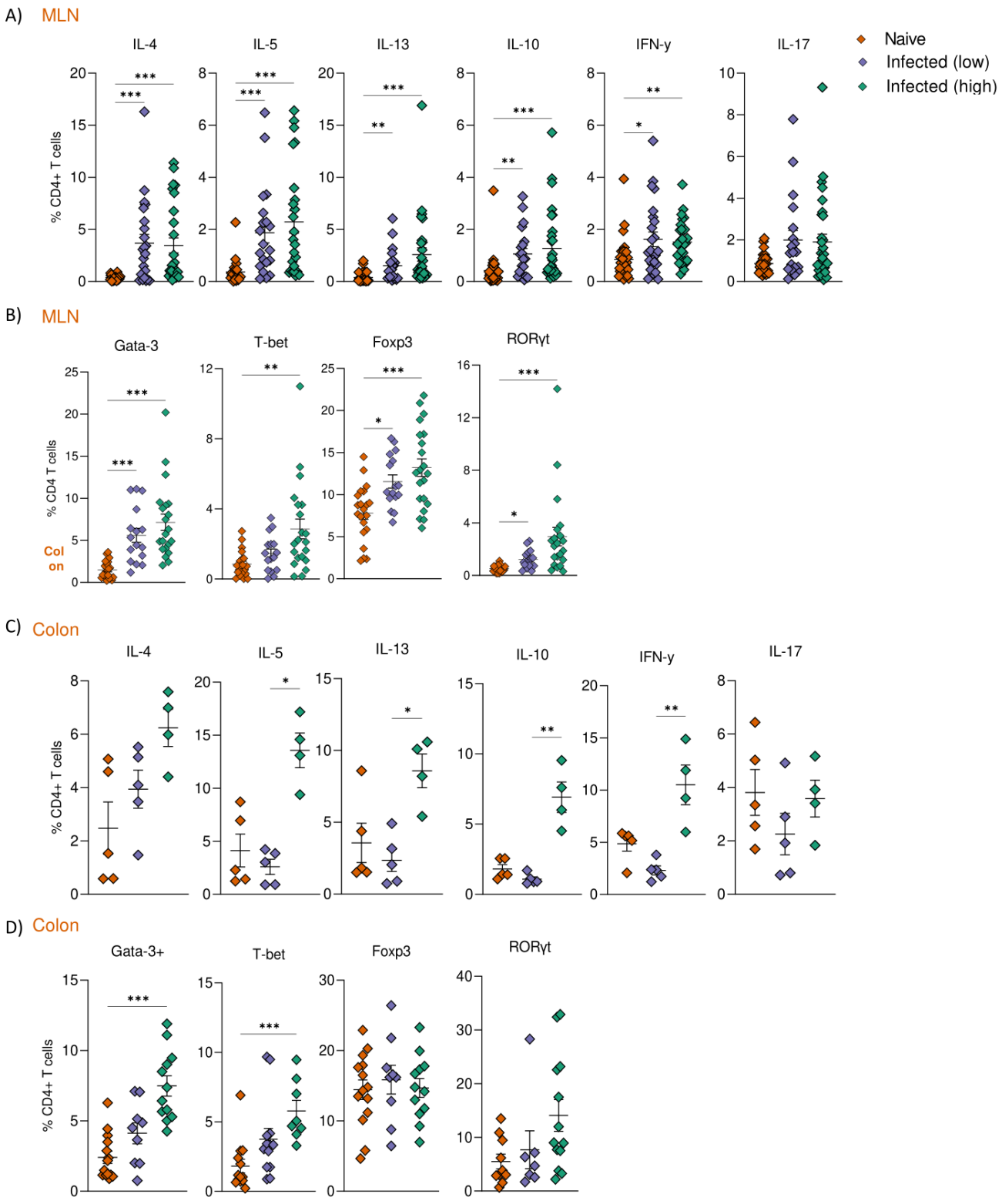


Figure 6. T cell profiles differ between infection doses in the colon. Cytokine secretion from PMA ionomycin stimulated (A) MLN and (C) colonic CD4+ T cells. The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors in (B) MLN and (D) colonic CD4+ T cells. Data presented as mean +/- SEM. Data shown are from 2 (C), 3 (4) or 7 (A&B) pooled experiments n= 2-4 per experimental group per experiment. Significant differences were determined by one-way ANOVA with Tukey post hoc testing. *p < 0.05, **p < 0.01, ***p < 0.001

Egg driven microbial alterations are dose dependent

Having demonstrated accelerated intestinal permeability and damage (Figure 4), along with more marked intestinal immune responses (Figures 4-6), in high vs low dose infection, we next addressed the impact of infection dose on bacterial community structure. 16s sequencing was performed on the large intestinal content of naïve mice and those infected for 7 weeks with 40 or 180 schistosome parasites (Figure 7). As expected, schistosome infection, irrespective of dose, evoked shifts in colonic bacterial communities with visualisation of clustering by PCoA showing clear separation between naïve, low dose and high dose infected mice (Figure 7A). When analysed by CCA, a significant difference in microbial profiles was detected between naïve and high dose infected mice but, due to a shortage of numbers, statistical testing was not possible for low dose groups (Figure 7B). A significant decrease in alpha diversity (quantified by Shannon index) was observed in high dose infection, with a trend towards lower species richness and abundance (Figure 7C).

With the data indicating a more marked impact of high dose infection on the host microbiota, we next assessed how infection altered bacterial communities at the genus level. Looking first at the 12 most abundant bacterial genera (Figure 7D), high dose infection led to an outgrowth of *Staphylococcus* and *Lachnospiraceae NK4A136* in comparison to low dose infected and uninfected mice. *Bacteroides*, *Alistipes* and *Biophilla* were also expanded upon infection but irrespective of infection dose. Mixed-sex infected mice at week 12 and 14 also showed greater representation of *Bacteroides* and *Alistipes* (Figure 3C), suggesting a conserved effect of heavy egg burden.Focusing on the more significant alterations in bacterial genera between groups (Figure 7E), high dose infection led to a decline in the abundance of *Muribacterium, RF39*, *Parasutterlla*, while bacteria of the genera *Ruminatium*, *Siraeum Dubosiella* and *Runmioccocus* were now undetectable in the colonic content of high dose infected mice.

We next sought to characterise the small intestinal metabolomic profile associated with high and low dose infection (Supplementary Figure 4) and saw a general trend towards decreased levels of certain amino acids (isoleucine, leucine, methionine and sarcosine) and metabolites associated with liver function (bile acids and cholates) in infected mice. This trend towards reduced bile acids and cholate mirrors that observed in our low dose time course experiment (Figure 3B).

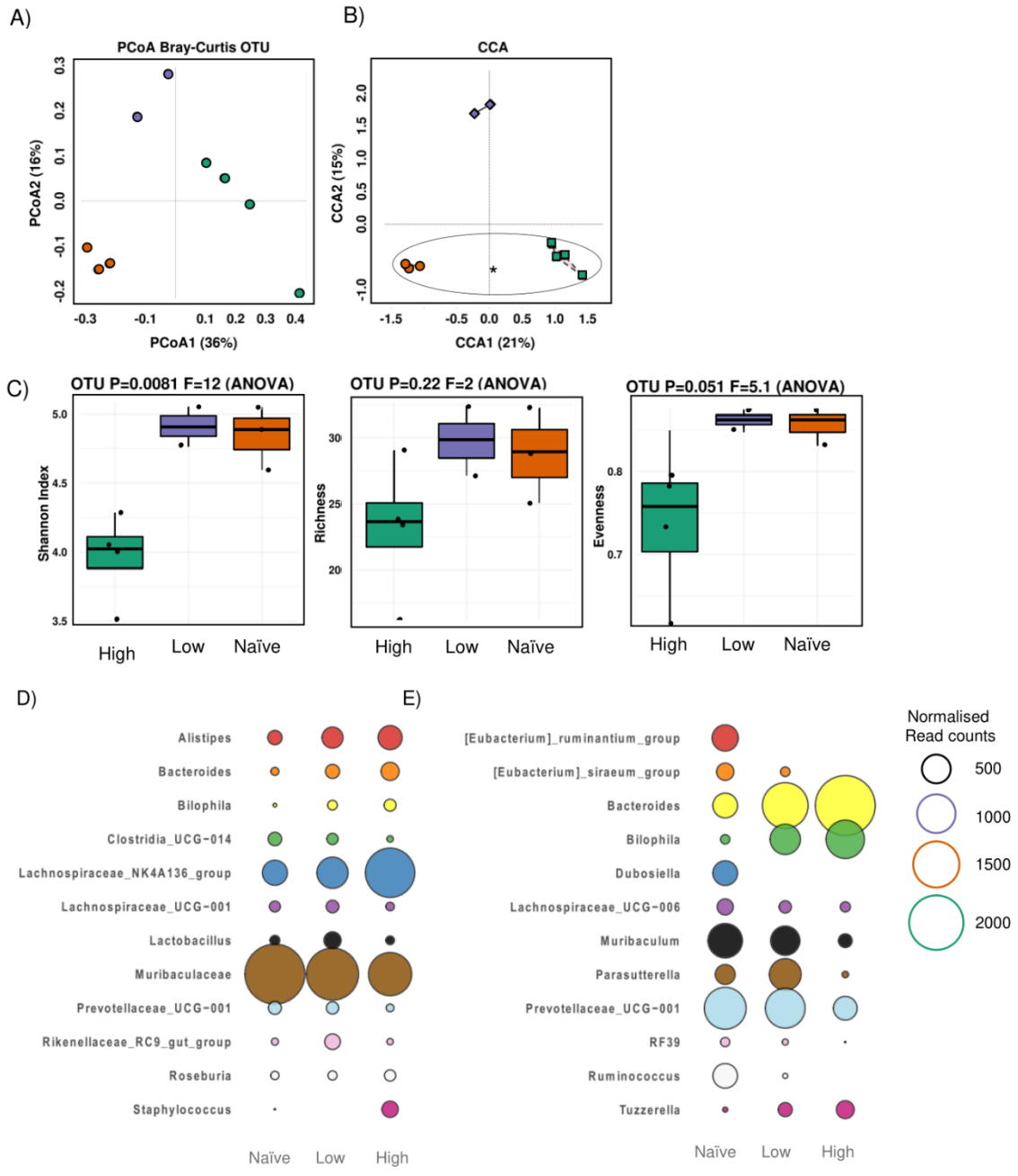


Figure 7. Schistosome infections provoke dose dependent dysbiosis. Colonic microbial profiles in low and high dose infected mice at week 7 of infection, as displayed as (A) Principal Coordinate Analysis (PCoA) and (B) Canonical Correspondence Analysis (CCA). (C) Alteration in microbial alpha as measured by species evenness, abundance and Shannon diversity. (D) Bubble plots representing the relative abundance of the (D) 12 most prevalent genera or (E) 12 genera showing the largest fluctuations between groups. Circle size reflects the proportion of microbiota comprised of that genus. Empty spaces reflect no detection of a given genus by 16S sequencing. Data from one single experiment with n=2-4. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Transfer of schistosome infection-associated microbiota into GF recipients promotes MLN CD4$^+$ T cell IL-4 production

After identifying pronounced differences in immune profiles and intestinal microbiota compositions between naïve mice and those infected with egg producing schistosomes, we next sought to directly address the relationship between the two. More specifically, we questioned whether the schistosome infection-associated microbiota contributes to intestinal immune polarisation. GF C57BL/6 mice were colonised for 2 or 3 weeks with defined microbiotas by transfer of faeces from SPF mice that had been infected with *S. mansoni* for 7 weeks, or from naïve controls (Figure 8A) and characterised the microbiota of recipient mice by 16s rRNA sequencing (Supplementary figure 5A). Given the relatively comparable immune profiles observed between low and high dose infected mice, faeces were pooled across infection groups to generate the faecal transplant 'slurry'. Strikingly, mesenteric CD4$^+$ T cells from mice recolonised with a schistosome infection-associated microbiota expressed significantly more of the Th2 associated cytokine IL-4 than mice receiving naïve faeces or PBS alone (Figure 8 B&C)**.** This trend could not be recapitulated with gavage of schistosome eggs alone (Supplementary Figure 5B) CD4$^+$ T cells from the same experimental group also trended towards greater IL-17 production, while secretion of IL-5, IL-13, IL-10 and IFNγ was comparable between groups. As faeces was pooled from high and low dose infected mice, we cannot distinguish which microbiota is the MLN IL-4 inducing trigger. However, given the preceding permeability (Figure 4) and immune (Figure 5&6) we anticipate these effects to be associated with high dose microbiotas.

Although IL-4 was increased in MLN CD4$^+$ T cells from GF mice that received faeces from schistosome infected mice, the expression of the Th2 defining transcription factor GATA-3 was comparable in those cells across experimental groups (Figure 8D). However, we noted a significantly increased frequency of Rorγt$^+$ CD4$^+$ T cells, and decreased Foxp3$^+$ CD4$^+$ T cells, in MLNs of mice colonised with schistosome infection-associated microbiotas (Figure 8D). Finally, irrespective of donor, faecal transplant failed to have a significant impact on the proportion of myeloid or granulocytic cells in the MLN of recipient GF mice, with the exception of monocytes, whose frequency was significantly enhanced upon receipt of a schistosome infection associated microbiota (Supplementary Figure 5C). Numerically, faecal transfer recipients tended towards greater MLN counts than PBS controls, which was reflected by significantly higher absolute counts of eosinophils, monocytes and plasmacytoid dendritic cells (pDCs) (Supplementary Figure 5D).

Together, these data reveal that recolonisation of GF mice with the microbiota associated with schistosome infection is sufficient to induce IL-4+ CD4+ T cells in the MLNs of recipient mice.

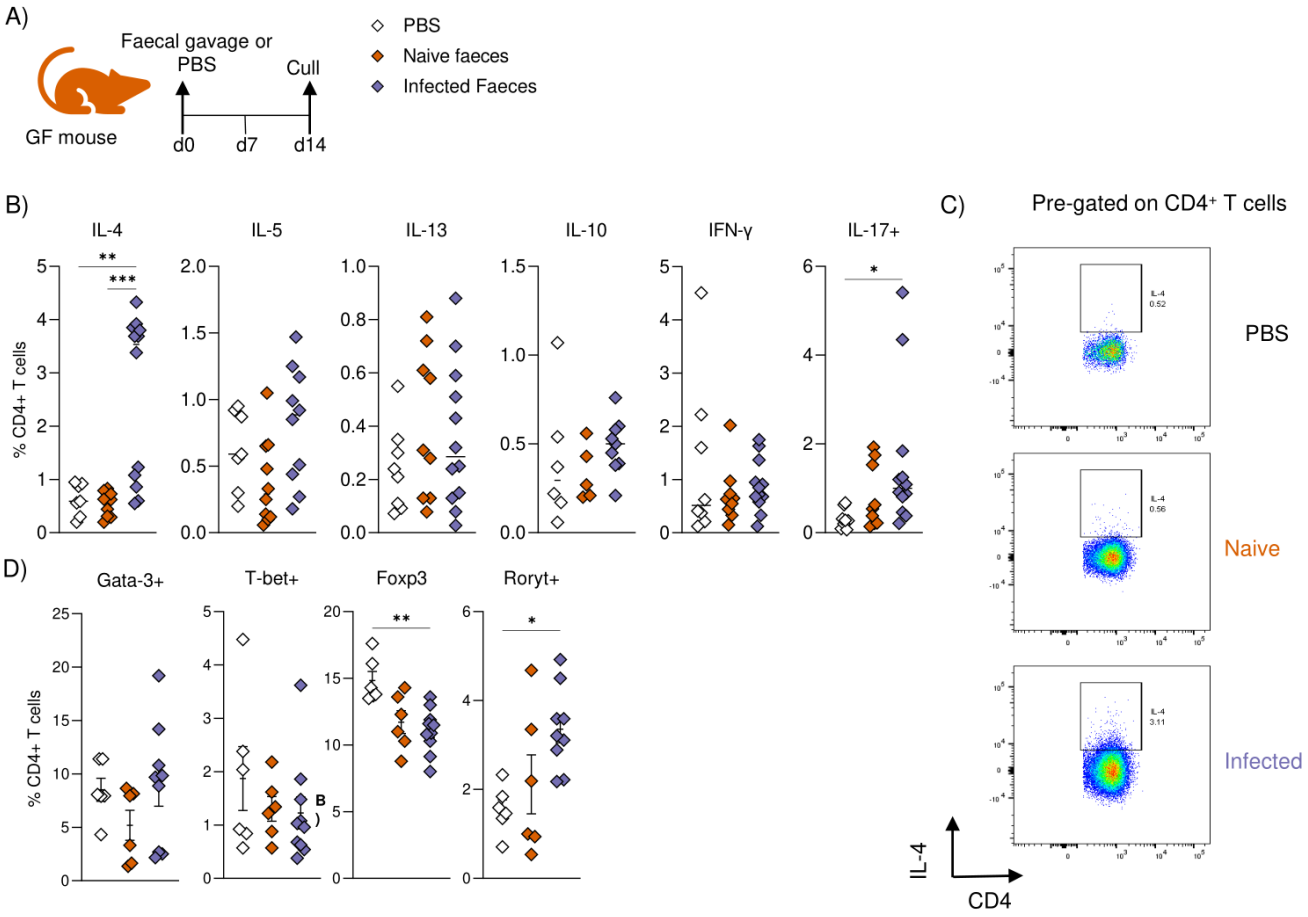


Figure 8. Transfer of faeces from schistosome infected mice into germ free mice evokes local Th2 and Th17 response. (A) Experiment schematic. Germ free mice were colonised for 2 or 3 weeks with faeces from naïve or mice infected with schistosomes for 7 weeks. Faeces was pooled from high and low dose infected mice for the generation of the 'infected' faecal slurry. (B) Cytokine secretion from PMA ionomycin stimulated CD4+ T cells. (C) Representative flow plots for IL-4 secretion, pre-gating on live CD45+ TCRβ+ CD4+ cells. (D) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors in MLN CD4+ T cells. Data presented as mean +/- SEM. Data pooled from two (D) or three pooled experiments. n=6-13 mice per group. Significant differences were determined by one-way ANOVA followed by Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.

Schistosomes, like many helminths, are renowned for their capacity to modulate the host immune response[10,47]. This includes their ability to limit collateral tissue damage to the host, regulate immunity towards bystander Ags and promote immune environments that enable their longevity[1410]. Despite the continuous transit of schistosome eggs across the intestinal tissues, and the damage this process causes, we currently lack understanding as to how egg-driven intestinal responses are regulated, and how local immune responses and commensal microbes influence these processes. Here we have directly investigated the impact of schistosome egg production on intestinal barrier function, local immune polarisation, and colonic bacterial communities by comparing mixed-sex vs single-sex, and high vs low dose, *S. mansoni* infections. We show that egg production is central to intestinal damage and permeability, cytokine production, and altered microbiome, with levels of each of these outcomes dictated by chronicity and/or intensity of infection. By transferring faeces from mixed-sex *S. mansoni* infected SPF donor mice into GF recipients, we have discovered the ability of the schistosome infection-associated microbiota to direct host MLN $CD4^+$ T cells towards a Type 2 phenotype. Together, these data suggest an active role for the intestinal microbiota, shaped by the egg stage of the parasite, in priming host intestinal immunity during schistosomiasis.

Previous researchers have suggested that a 'leaky gut' and subsequent luminal content exposure may[48–51], but this has never been directly tested. Here, we provide the first evidence that altered barrier integrity during schistosomiasis influences the immune trajectory of the host. Intestinal permeability was increased during chronic and high-intensity *S. mansoni* infections, with evidence of barrier damage from the onset of egg production (Figures 1&4). While in murine low dose infections permeability was evident by week 11 of infection, in high dose infections alterations could be visualised as early as week 6. These data highlight the resilience of the intestinal barrier and indicate that although egg migration provokes intestinal damage from the onset, sustained egg accumulation or high intensity production is required for intestinal leakiness to become evident. In addition, it is possible that in high dose settings, the intestines recuperative capacities are overwhelmed, leading to earlier permeability changes. Extrapolating this data to a human setting, we anticipate that these permeability alterations may take longer to manifest, reflecting sheer size differences in mouse-human anatomy. Individuals hyper-exposed to *S. mansoni* (i.e fisherman in endemic regions) display significantly higher endotoxemia in comparison to uninfected persons[52]. While these data imply enhanced intestinal permeability in infected persons, this has not been

addressed through more direct methods of assessment (i.e oral gavages with saccharides[53,54]) and the kinetics of these changes are unknown.

It is important to note that, although we focussed on the influence of egg migration on paracellular permeability (i.e movement between adjacent epithelial cells), luminal content can cross the epithelium via other means. This includes specialised microfold (M) cells that overlay specialised lymphoid follicles, and the extension of dendritic cell (DC) dendrites through the epithelial layer[55]. Although not directly assessed here, it is plausible to suggest that egg migration disrupts the homeostatic function of these cell types/processes, resulting in altered Ag uptake via these routes. Similarly, Ag uptake could be facilitated by increased cellular apoptosis and epithelial cell turnover[17]. Finally, with high-level mucus production hallmarking helminth infections[56,57] it is possible that mucin-producing goblet cells act as conduits for DC Ag delivery, in a process known of retrograde endocytosis[55,58]

Schistosomes are not the only parasites known to damage the intestinal barrier during their infectious life-cycles, including *Toxoplasma gondii*, *H. polygyrus* and *Fasciola hepatica*[59]. Despite the physical damage they impose on the gastrointestinal tract, none of these infections are typically associated with sepsis or secondary bacterial infection[59], even though schistosome-infected individuals are reported to have systemic endotoxin levels at 10 times greater than that observed in lethal toxic shock[52]. This enigma may reflect a greater capacity of the host immune system to deal with these high LPS burdens, as observed in F. hepatic infections[60,61], or immune hyporesponsiveness in chronic disease[62]. Additionally, this could be due to the dynamic regenerative capacity of the intestinal tissues, counteracting parasite-evoked damage and generating a robust mucosal immune response that limits bacterial spread[59.] Given that host survival is vital for the parasite, they likely manipulate host immune responses in a manner that improves bacterial control, and thus their own longevity. Indeed, chronic filarial infections have shown to promote bacterial clearance and sepsis survival via the functional reprogramming of macrophages[63.] How schistosomes prevent sepsis is relatively uncharted research. One molecule that warrants attention includes cDC2 -derived hepcidin, which was recently shown to promote intestinal wound healing and limit microbiota tissue infiltration following DSS challenge[64]. In addition, resident macrophage populations in the liver (Kupffer cells) have recently been shown to be crucial for the capture of intestinal bacteria and prevention of their dissemination[65]. Given the large impact of schistosomiasis on host liver function, further studies to assess this mechanism are warranted.

The capacity to isolate live immune cells from the intestine of schistosome infected mice (Figure 5&6) brings forth many exciting opportunities. Here, we provide a broad snapshot into schistosome-evoked immunity within the intestine, and thus, there remain many fascinating avenues of adaptive and innate immunity still open for exploration. For example, macrophages play important roles in the promotion or resolution of tissue damage, where surrounding cytokine environments dictate their 'M1' proinflammatory or 'M2' wound healing capacities[38]. Considering the intestinal tissue damage evoked by *S. mansoni* infection (Figures 1&4), schistosome infections represent a compelling system to unearth the core cell types and effector molecules that regulate mucosal inflammation, and the signals underlying their generation. Colonic macrophage frequencies were comparable between high and low dose infected mice (Figure 5), but their relative activation and M1/M2 status was not evaluated. Refined assessment of M2/pro-repair factors (e.g Ym1/2, Relmα Arginase) vs M1/pro-inflammatory (iNOS, TNF) factors would be of particular interest here. Such high dimensional flow analysis, paired with techniques of RNA seq and imaging mass cytometry, and analysis across the whole course of infection, would provide an unprecedented image into the schistosome intestine. Inclusion of in situ hybridization, could also provide insight into the localisation of infiltrating bacteria relative to immune cells and sites of damage[100].

While MLN immune profiles were similar between low dose and high dose infected mice (Figure 5&6), we observed dose dependant differences in PMA/ionomycin stimulated cytokine secretion (Figure 6), with high dose infected mice producing greater levels of IL-5, IL-10, IL-13 and IFNγ than low dose infected mice. These results suggest that while LN priming of $CD4^+$ T cells is comparable between the two infection intensities, their colonic environments vary and differ in their capacity to support or maintain T cell polarisation. This may reflect differences in colonic egg burden or exposure to damage or luminally-derived products. Instalment of cytokine reporter mice, or conduction of e*x vivo* SEA and commensal bacteria specific stimulation will provide further clarity on this matter.

The composition of the intestinal microbiota is intricately linked to the fine tuning of host immunity, with GF mice showing stunted immune development[66] and skewed Th2 responses[67], and with certain microbial species shown to endorse the conversion of naïve $CD4^+$ T cells into immunosuppressive Tregs[68,69] or more proinflammatory Th17 cells[70]. Here we reveal the capacity of the schistosome infection-associated microbiota to boost mesenteric $CD4^+$ T cell production of IL-4 and induce the appearance of $CD4^+$ T cells with a greater expression of $Ror\gamma t^+$ (Figure 8). While previous research has shown bacterially-derived molecules to reverse the Th2 'bias' observed in GF

animals[67], to our knowledge this is the first report demonstrating the ability of infection to promote resident microbes that enhance intestinal T cell IL-4 production. As our analysis showed no significant increase in $CD4^+$ T cell IL-5, IL-13, IL-10 or GATA-3 expression (Figure 8), our data may indicate the generation of T follicular helper (Tfh) cells that are pre-primed for release of IL-4[71] . Alternatively, our data could suggest for a transient wave of Type 2 immunity, which has been described in the developing lung at maximum periods of lung remodelling[72,73]. In these pulmonary studies, Type 2 innate immune cells flux into the lungs in response to rising IL-33 and display a lower threshold for responses towards allergens[73]. Thus, it would be of particular interest to inspect IL-33 and Tfh levels in our experiments, with use of IL-4 reporter mice providing greater insight into the contribution of individual $CD4^+$ subsets to the IL-4 pool. Finally, comparison of high dose and low dose microbiotas, or mixed-sex vs single-sex microbiotas is also warranted, with this analysis highlighting whether the MLN IL-4 phenotype is exclusive to particular doses or time points of *S. mansoni* infection.

In the context of schistosomiasis, where the generation of an appropriately regulated Th2 responses underpins host survival[48,49,51], the ability of the host immune system to rapidly respond to recurring infection would be of immediate benefit. In support of this line of reasoning, certain *Lactobacillaceae* species have shown to prime host immunity in a manner that alters susceptibility to enteric helminth *H. polygyrus*[74]. Moreover, susceptibility to schistosomiasis has shown partial dependence on baseline microbiota composition[16,19], and the degree of schistosome evoked granulomatous inflammation can be altered through antibiotic administration[20]. Future studies should address the relevance of commensal driven MLN IL-4 populations to barrier repair or promotion of Type 2 immunity, for instance by challenging faecal transplant recipients with *S. mansoni* or models of colitis.

The induction of Th17 cells by the intestinal microbiota has primarily been accredited to segmented filamentous bacteria (SFB)[70], but may also involve other commensals[75]. Th17 responses are not typically associated with schistosomiasis infections in C57BL/6 mice[76], but in CBA/J mice, they are associated with severe egg-driven pathology[77]. Although we did not see significantly increased Th17 development in our infection experiments, our observation that MLN $ROR\gamma t^+$ $CD4^+$ T cell responses were somewhat elevated in high dose infection (Figure 5) or following faecal transplant from high dose infection into naïve GF mice (Figure 8), suggests that this immune phenotype may be conferred by a component of the schistosome infection-associated microbiota, caused by egg transit and tissue damage. In support of this idea, schistosome infection-associated microbiotas have shown to

be pro-inflammatory/ 'colitogenic' in co-housing studies[78], and inflammatory IBD associated microbiotas are accompanied by an abundance in mucosal Th17 cells and a disproportionate ratio of Tregs[79]. Furthermore, given the crucial role of Th17 cells in maintenance of intestinal barrier function and compartmentalisation of the intestinal microbiota[80], perhaps the promotion of mucosal RORγt$^+$ CD4$^+$ T cell responses by the schistosome microbiota represents a conserved evolutionary mechanism that could help fortify the intestinal wall against egg transit. Finally, it is plausible to suggest that the microbiota-instructed RORγt$^+$ T cells may play a role in the regulation of Type 2 immunity[81]

It remains unclear which particular microbial species, metabolites or global changes within the intestinal system may be responsible for the development of MLN IL-4$^+$ and RORγt$^+$ CD4$^+$ T cells in faecal transplant mice (Figure 8). Similarly, it is unknown whether certain microbial components contribute to barrier integrity and repair during egg transit. While 16s sequencing revealed structural differences between faecal transplant recipients (Supplementary Figure 5), and naïve or infected mice (Figures 3&7), this analysis was not capable of fully capturing the metabolic activity or functionally of the identified groups. For instance, we observed conserved expansion of *Bacteroides* and *Alistipes* genera across chronic (12-14 week) low dose mixed-sex and 7 week high dose mixed-sex infections (Figures 3&7). However, with some species within these genera showing pro-inflammatory[82,83] potential, and others showing anti-inflammatory[83,84] effects, it remains unclear how these bacterial modifications impact the outcome of *S. mansoni* infection. Shotgun metagenomic sequencing provides taxonomic resolution at a species level, offers information on their functional potential, and allows characterisation of fungal and viral communities. Integrating this approach with metabolomic analysis, alongside inspection of microbiotas from different regions of the intestine, would provide further clarity on microbial factors responsible for the observed immune phenotype. Furthermore, with advances in culturomics and metagenomics, it may soon be possible to identify and capture candidate bacteria and move away from cruder faecal transplant work.

Enteric helminths disrupt microbiota composition through a variety of approaches[14], including the anti-microbial activity of their extracellular secretary (ES) products, competing with microbial species for nutrients and the intestinal niche, and altering mucin dynamics and so settlement of certain mucin-colonising bacteria[85–87]. Our data reveals a clear-cut role for egg transit in schistosome infection-associated modification of host microbiota structure, with egg-driven microbial alterations shown to increase over the course of infection (Figure 3), and differences in bacterial communities

also more dramatic with increasing infection dose (Figure 7). However, it remains unclear whether these bacterial changes reflect the secretion of antimicrobial compounds from schistosome eggs, the Th2 dominated immune response associated with infection, or the destructive inflammatory response evoked by egg transit. Our data generally supports egg-driven tissue damage as the dominant candidate for altering the host microbiota. More specifically, by comparing chronic low dose mixed-sex and post-patent acute high dose mixed-sex infections, we revealed significant impairment in barrier function associating only with later timepoints in low dose infections (Figure 1), and only with high dose post-patent acute infections (Figure 4), even though clear Type 2 immune responses were present earlier or in low intensity infections (Figures 2&5). The association of infection chronicity and/or intensity with intestinal damage was also evident for changes in microbial profiles (Figures 3&7). Mechanistically, a higher level of egg transit likely exposes cells within damaged tissues to several forms of stress, leading to enhanced necrosis and apoptosis[17], and potential release of endogenous danger molecules, which may then generate an intestinal niche that favours certain microbes. It is important to note that, although intestinal leakiness increased with chronicity or intensity of mixed-sex infections, the intestinal barrier appeared intact through histological inspection and analysis of tight junction associated genes (Supplementary Figure 1B). Thus, the intestine during schistosomiasis is able to rapidly heal several aspects of egg-associated damage, through mechanisms that remain to be determined. It is possible that these processes may involve the host microbiota, given that some intestinal bacteria can promote wound healing[88]. In terms of immune-mediated modifications to the microbiota, Type 2 immune responses may promote the expulsion or expansion of defined intestinal bacteria, as already show in in the context of murine infection with *Trichuris muris* infecton[89] or *H. polygyrus*[90]. In the context of our work, this could in part be facilitated by schistosome infection increasing Th2 associated mediators such as Ym1 and RELMα in the luminal content (Figures 2&4), anti-commensal antibodies (Figures 1&4), or IL-25 production – all of which could influence intestinal epithelial cells and IL-13 responses[91]**.** Additionally, RELMα has shown bactericidal effects within the skin[92], but its intestinal antibacterial properties are uncharacterised. Similarly, antimicrobial peptides and proteins (AMPs) induced by helminth infection could selectively tailor the host microbiota and modify their tissue location[90]. Moreover, it is possible that an impaired barrier function alters sampling of gut bacteria by innate immune cells, leading to modified inflammation, IgA induction and capacity to compartmentalise the microbiota[93].

In addition to schistosome eggs, the fact that single-sex infections are associated with changes in the intestinal microbiota by week 14 (Figure 3) indicated that the worms alone, even though they

live in the mesenteric vasculature, can impact the composition of the intestinal microbiota. Indeed, intestinal microbiota alterations has been reported in individuals infected with the urogenital parasite, S. haematobium, which are distally located within the bladder plexus[18,39]. Additionally, helminth-derived secretions have shown to elicit direct antimicrobial activities[94], though this has not yet been investigated for schistosomes. However, given that single-sex infections were also accompanied by small but significant elevations in systemic and local Th1 mediators at the 14 week timepoint (Figure 2 & Supplementary Figure 2) where we observed concurrent changes in the intestinal microbiome (Figure 3), our data likely indicate immune involvement as opposed to the direct actions of the worm secretions, which will have been produced throughout infection.

We have found that schistosome infection not only impacts intestinal microbial composition, but also has consequences in terms of metabolites (Supplementary Figures 3&4). Alterations in the intestinal metabolome may reflect differences in host, parasite and/or bacterial production or utilisation of metabolites, as well as their absorption across the intestinal wall[95]. Of particular interest, in both single-sex and mixed-sex infections we detected significant reductions in levels of bile acids and cholates within the small intestine (Supplementary Figure 3). Recent studies have pinpointed a role for bile acids in shaping the composition of intestinal immune populations[96,97],including the promotion of haemopoiesis and subsequent conferred protection against infection[98]. While the alterations in bile acids may reflect schistosome worms influencing liver function, it is possible this may also have a role in shaping schistosome associated immune responses, and the ability of the host to fight off concurrent infections or leakage of intestinal derived Ags or pathogens across the intestinal wall[99].

In conclusion, we have identified profound alterations in barrier integrity, immune polarisation, and colonic microbial structure over the course of murine schistosomiasis, with changes coinciding with chronicity and/or intensity of intestinal tissue-damaging parasite eggs. Additionally, we have shown that the schistosome infection-associated microbiota promotes development of MLN IL-4$^+$ and RORγt$^+$ CD4+ T cells, which may be of further consequence or benefit to parasite and host survival. This work provides a robust experimental platform for further interrogation, and discovery of novel mechanisms that link parasite, microbial and immune factors to 'regulated' intestinal inflammation.

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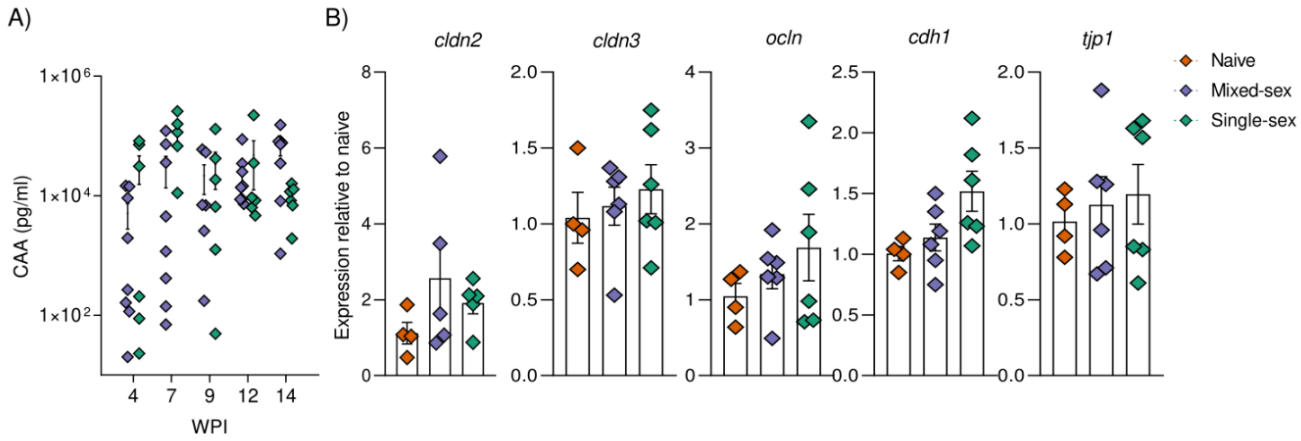
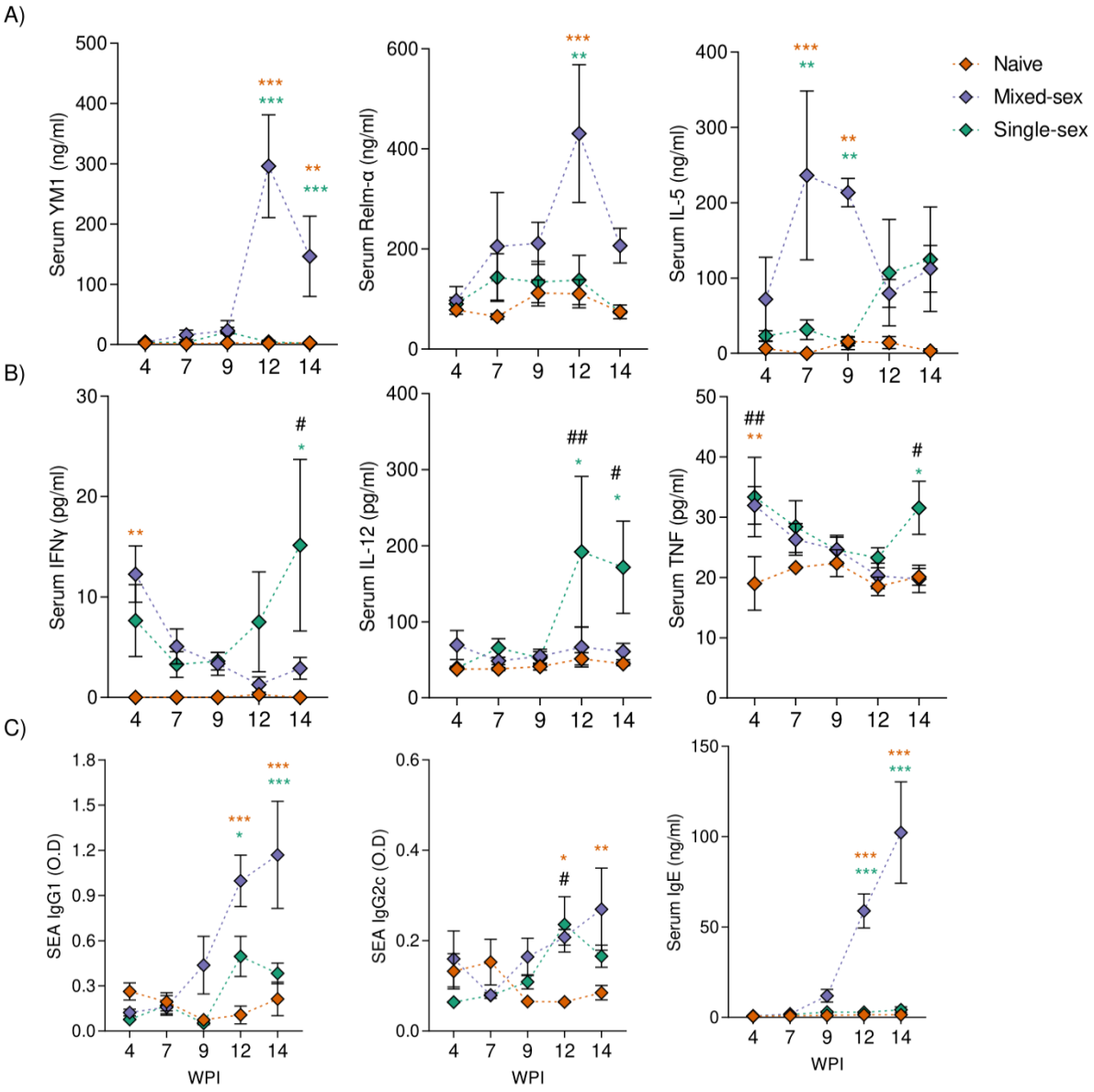
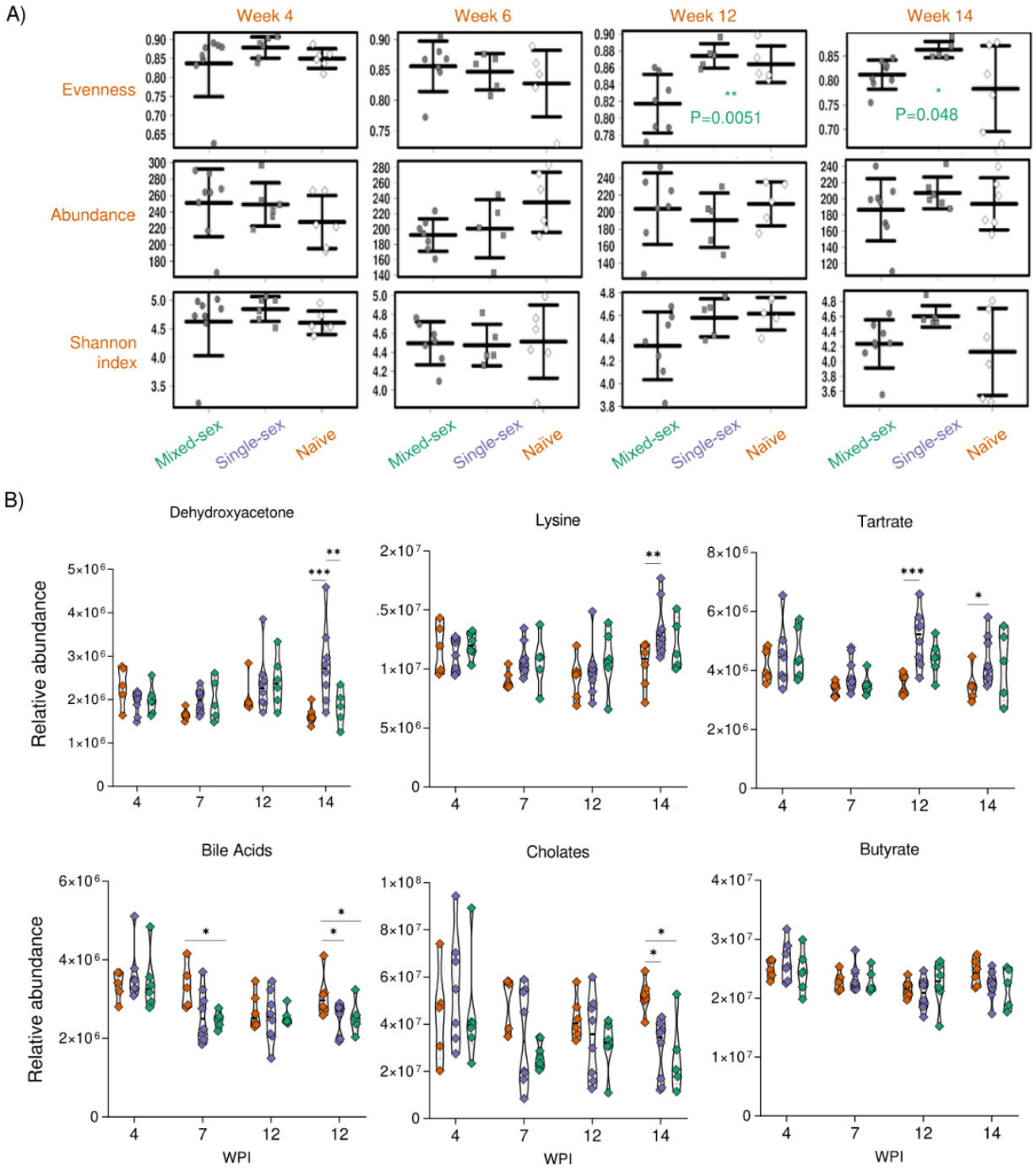


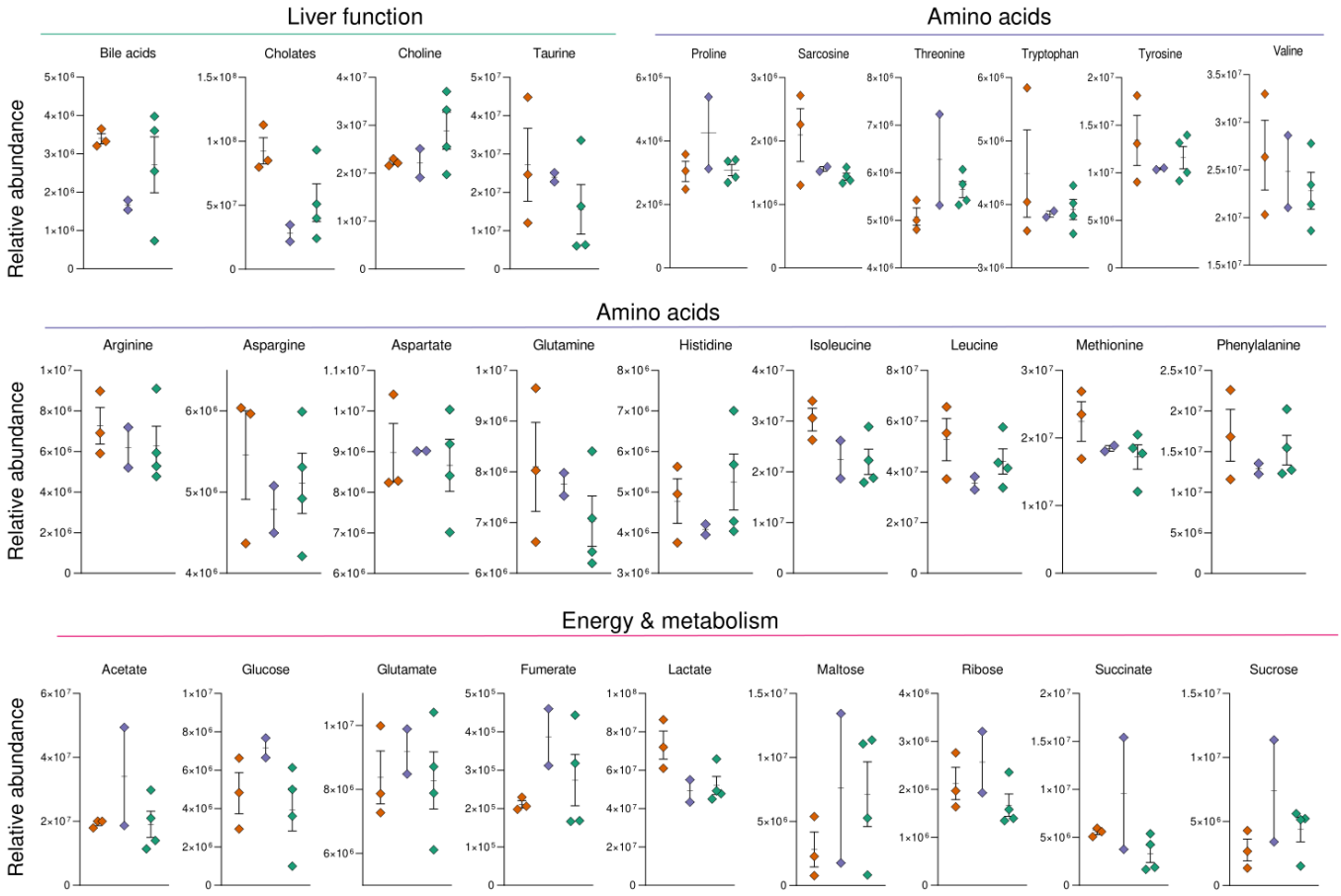
Supplementary Figure 1. Comparable infection loads and expression of tight junction genes in egg producing and non-egg producing infections. (A) Serum levels of the regurgitated worm product Circulating anodic antigen (CAA) in mixed-sex and single-sex infected mice (B) The expression of tight junction associated genes in the colonic tissue of naïve, mixed-sex and single-sex infected mice at week 14 of infection. Data normalized against HK pool (RPLP0, β2m, β-actin and s18) and represented as fold change relative to naïve. Data presented as mean +/- SEM. n=4-8 from one single experiment. Statistical analysis conducted by one-way ANOVA.



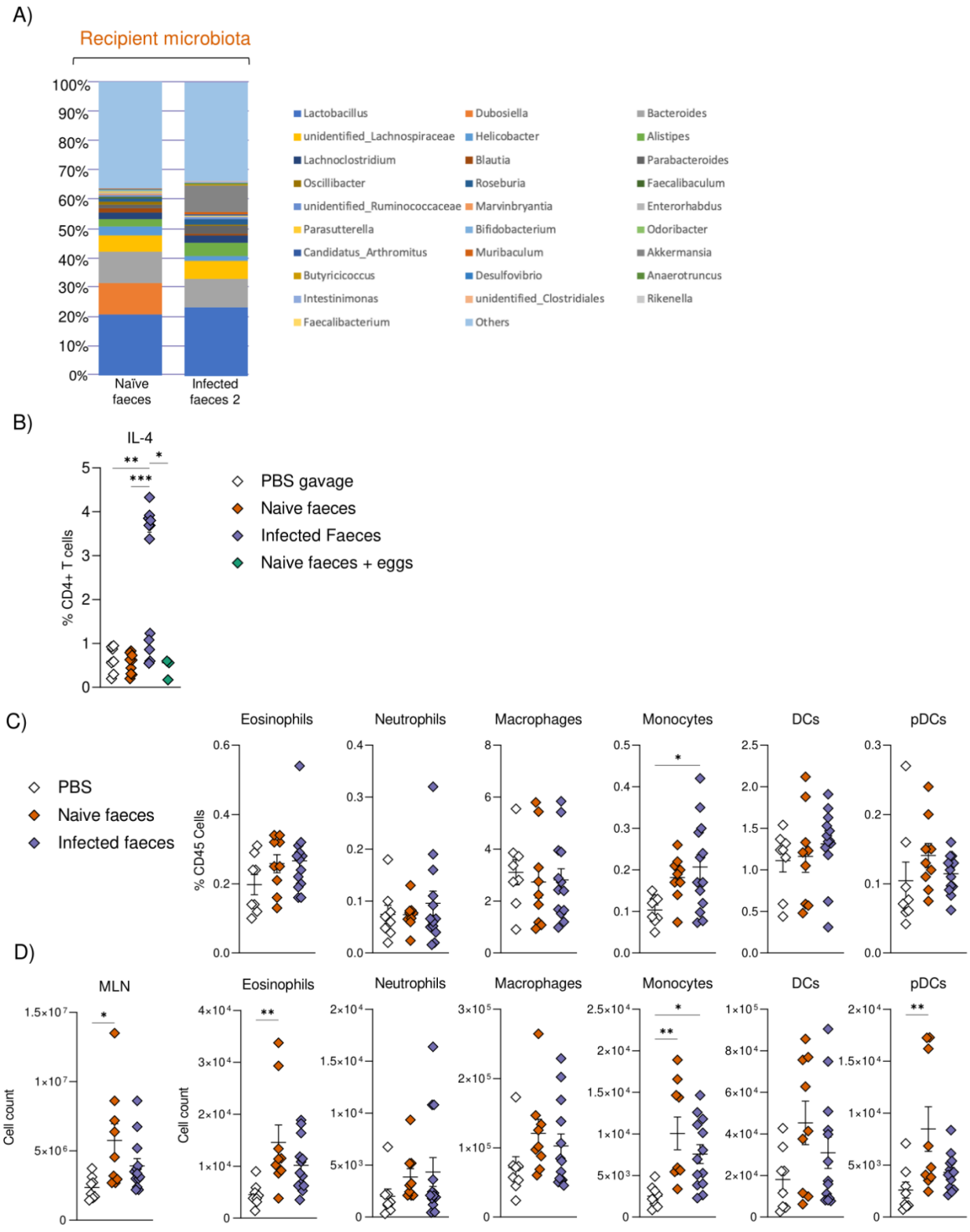
Supplementary Figure 2. Systemic Th2 polarisation in mixed-sex infections only. (A) Serum levels of Th2 associated mediators (Ym1, RELMα) and cytokines (IL-5), (B) Th1 associated cytokines (IFNγ, TNFα and IL-12) and (C) SEA-specific antibodies (IgG1 and IgG2c) and total IgE across the course of infection. Data presented as mean +/- SEM. n=6-8 from a single experiment. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and determined by two-way ANOVA. Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively. Significant differences between naïve and single-sex infected mice are indicated by #.



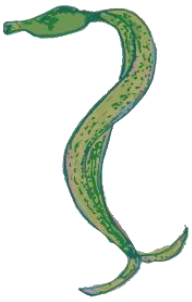
Supplementary figure 3. Schistosome egg transit reduces microbial evenness and the impacts metabolic profile of the small intestine. (A) Differences in microbial alpha diversity in infected and non-infected mice as measured by species evenness, abundance, and Shannon diversity. (B) The relative abundance of small intestinal metabolites was determined by NMR spectroscopy. Data from one single experiment with n=5-7. Significant differences between each group are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and calculated by one-way ANOVA (A) or two-way ANOVA (B) followed by Tukey's post doc.



Supplementary Figure 4. Intestinal metabolomics. The abundance of small intestinal metabolites was determined by NMR spectroscopy. Data from one single experiment with n=5-7. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$



Supplementary Figure 5. Characterisation of faecal transplant microbiotas and immune responses. (A) Bar charts representing the 20 most abundant genera detected in the colonic content of faecal transplant recipients. 16s sequencing was conducted on pooled faecal donor or recipient samples, with 3-4 samples pooled per group. (B) IL-4 secretion in PMA stimulated MLN CD4+ T cells after 2 weeks of faecal recolonisation. To control for the effect of egg-derived antigens, a separate cohort of mice were gavaged with naïve faeces spiked with approx. 1000 S. mansoni eggs. The (C) frequency and (D) absolute numbers of indicated cell types in the MLNs and of GF mice receiving faecal transplant. (B=D) Data presented as mean +/- SEM. Data pooled from two (B) or three pooled experiments (C&D). n=3-13 mice per group. Significant differences were determined by one-way ANOVA followed by Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.



CHAPTER 5

Type I interferons provide additive signals for murine regulatory B cell induction by *Schistosoma mansoni* eggs

Katja Obieglo, Alice Costain, Lauren M. Webb, Arifa Ozir-Fazalalikhan, Shelia L. Brown, Andrew S. MacDonald, Hermelijn H. Smits

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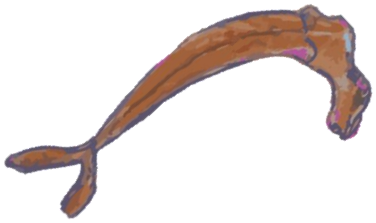

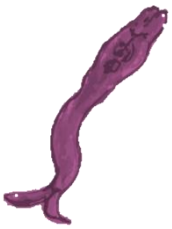

Type I interferons provide additive signals for murine regulatory B cell induction by Schistosoma mansoni eggs

AUTHOR INFORMATION

Katja Obieglo[1], Alice Costain[1,2], Lauren M. Webb[2], Arifa Ozir-Fazalalikhan[1], Shelia L. Brown[2], Andrew S. MacDonald[2], Hermelijn H. Smits[1]

1. Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands
2. Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, UK

Corresponding authors: Hermelijn Smits (h.h.smits@lumc.nl)

ABSTRACT

The helminth *Schistosoma mansoni (S. mansoni)* induces a network of regulatory immune cells, including interleukin (IL)-10-producing regulatory B (Breg) cells. However, the signals required for the development and activation of Breg cells are not well characterized. Recent reports suggest that helminths induce type I interferons (IFN-I), and that IFN-I drives the development of Breg cells in humans. We therefore assessed the role of IFN-I in the induction of Breg cells by *S. mansoni*. Mice chronically infected with *S.mansoni* or intravenously injected with *S.mansoni* soluble egg antigen (SEA) developed a systemic IFN-I signature. Recombinant IFNα enhanced IL-10 production by Breg cells stimulated with *S. mansoni* soluble egg antigen (SEA) *in vitro*, while not activating Breg cells by itself. IFN-I signalling also supported *ex vivo* IL-10 production by SEA-primed Breg cells but was dispensable for activation of *S. mansoni* egg-induced Breg cells *in vivo*. These data indicate that while IFN-I can serve as a co-activator for Breg cell IL-10 production, they are unlikely to participate in the development of Breg cells in response to *S. mansoni* eggs.

Keywords

Type I interferons; regulatory B cells; *Schistosoma mansoni*; chronic infection; interleukin (IL)-10

INTRODUCTION

The helminth *Schistosoma mansoni* induces a network of regulatory immune cells during the chronic phase of infection[1]. The induction of B cells with regulatory properties, so called regulatory B (Breg) cells, by *S. mansoni* has been studied extensively[2-5]. Breg cells as part of the regulatory network play an important role in limiting immunopathology and attenuate responses to bystander Ags such as allergens[6]. Breg cell-induction, as observed during chronic infection, can be replicated by *in vitro* stimulations with soluble egg antigens (SEA)[7,8] and the even single, egg-derived molecule IPSE/alpha-1[8] in the absence of infection. While it is currently unclear which receptors and pathways *S. mansoni*-derived molecules engage, factors consistently reported to be important for Breg cell development and activation are stimulation through the BCR[9-12], CD40[9,13-16] and the toll-like receptors (TLR) TLR2/4[17-19], TLR7[20] and TLR9[17]. Moreover, different cytokines including IL-21[21], IL-35[22,23], BAFF[24,25], APRIL[26] and type I interferons (IFN-I)[27] have been described to support Breg cell development.

IFN-I are a large family of cytokines, containing 14 IFNα subtypes and a single IFNβ, central in the immune response to viral infections[28]. Induced, amongst others, by ligation of pattern recognition receptors (PRRs) of immune and non-immune cells, IFN-I act in an auto- and paracrine manner to induce an antiviral state, but can also interfere with innate and adaptive immune responses[29,30]. IFN-I can enhance Ag presentation and chemokine production in innate cells, promote effector T cell responses and induce B cell antibody production in viral infection (reviewed in [30]). The role of IFN-I in bacterial, fungal and intracellular parasitic (mainly *Leishmania*, *Plasmodium* and *Trypanosoma* spp.) infections is complex, with possible beneficial and detrimental outcomes for the host (reviewed in [28]). Only recently, reports have highlighted the potential of helminths or their products to induce IFN-I in mouse models. Infection with the gastrointestinal helminth *Heligmosomoides* (*H.) polygyrus* has been shown to induce IFN-I signalling in gut and lung in a microbiota-dependent manner, protecting mice from RSV infection [31]. *S. mansoni* eggs and soluble egg antigens (SEA) have been shown to induce an IFN-I signature both in splenic DCs and in *in vitro* differentiated bone marrow DCs (BMDCs)[32,33], and *Nippostrongylus (N.) brasiliensis* induces IFN-I in skin DCs[34]. A more generalized expression of IFN-stimulated genes (ISGs) in response to *S. mansoni* products has so far only been shown by Webb et al. for whole lung tissue following i.p. sensitization and i.v. challenge with *S. mansoni* eggs[33].

B cells express the IFNα/β receptor (IFNAR) and respond to IFN-I[35-37]. B cell responses to IFN-I are most extensively studied in autoimmunity. In systemic lupus erythematosus (SLE), IFN-I are considered to promote the activation of autoreactive B cells, maturation into plasmablasts and autoantibody production, contributing to disease pathology[38]. Menon et al. add important knowledge to the picture by showing that plasmacytoid DCs (pDCs) drive the formation of IL-10-producing Breg cells by IFNα production and CD40 ligation in healthy individuals, but fail to do so in SLE patients. While Breg cell-derived IL-10 normally provides an important feedback loop that limits IFNα production, SLE patients have hyperactivated pDCs that fail to induce Breg cells, possibly due to Breg cells being less responsive to supra-optimal concentrations of IFNα[39]. In patients with certain types of multiple sclerosis (MS) IFNβ therapy is a treatment option commonly applied. It has been reported that IFNβ therapy not only increased IL-10 production by monocytes and T cells[40,41], but also B cells and plasmablasts[42].

Whereas Breg cells can be induced by *S.mansoni*-derived Ags *in vitro*, this is less potent than the induction of Breg cells during chronic infection, and the induction of Breg cells by IPSE/alpha-1 has only been demonstrated *in vitro*[8]. Helminth infections trigger a multitude of different immune responses in the host *in vivo*, and it is likely that additional signals, in addition to helminth molecules, are required for optimal Breg cell induction. Here, we sought to address whether IFN-I are central to the induction of Breg cells by *S. mansoni*. We show that both *S. mansoni* infections and intravenous injections with SEA induce a systemic IFN-I signature *in vivo*. Recombinant IFNα enhanced B cell IL-10 production in response to SEA and SEA+aCD40 *in vitro*, while blocking antibodies against IFNAR alpha chain (IFNAR1) reduced the *ex vivo* IL-10 production by *in vivo*-primed B cells. However, B cell induction in response to egg administration *in vivo* was not affected in IFNAR$^{-/-}$ mice. Collectively, these data show that IFN-I provide additive signals for Breg cell induction by *S. mansoni in vitro*, but are not crucial for *S. mansoni*-induced Breg cells *in vivo*.

MATERIAL AND METHODS

Animals

Female C57BL/6 mice (Harlan) were housed under SPF conditions in the animal facility of the Leiden University Medical Center (Leiden, The Netherlands). *Ifnar1*$^{-/-}$ mice on an C56BL/6 background were housed at the University of Manchester. All animals were used for experiments at 6-12 weeks of age. All animal studies were performed in accordance with either the Animal Experiments Ethical Committee of the Leiden University Medical Centre or under a license granted by the home office (UK) in accordance with local guidelines.

Preparation of SEA and Eggs

S. mansoni eggs were isolated from trypsinized livers or guts of hamsters after 50 days of infection, washed in RPMI medium supplemented with penicillin (300U/mL), streptomycin (300µg/mL) and amphotericin B (300µg/mL) and stored at -80°C until use. SEA was prepared as previously described[47]. Protein concentration was determined by BCA. SEA preparations were routinely tested for endotoxin contamination by Limulus Amoebocyte Lysate (LAL) assay or TLR4-transfected HEK reporter cell lines.

S.mansoni infections and in vivo injections

For the high dose infection model, mice were percutaneously infected with approximately 180 cercariae and serum collected on day 49 after infection. For the evaluation of splenic ISG expression in SEA/IFNα treated mice, mice were intravenously injected with 50ug SEA in PBS or intraperitoneally injected with PBS or IFNα ($20x10^3$ units). Splenocytes were harvested 12 hours after injection, snap-frozen and stored -80°C for later analysis. For the egg challenge model in IFNAR$^{-/-}$ mice, mice received two intraperitoneal injections (day 0 and day 7) of 5000 *S.mansoni* eggs diluted in sterile PBS. Mice were sacrificed 7 days after the last injection.

Splenocyte and B cell isolation

Spleens were homogenized by passage through a 70µM cell strainer (BD Biosciences) and erythrocytes depleted from the single cell suspension by lysis. B cells were purified from splenocytes by anti-CD19 MicroBeads (Miltenyi Biotech) following the manufacturer's instructions. For cell sorting experiments, MACs-isolated $CD19_+$ B cells were sorted by flow cytometry into FO B cells ($CD23^+CD21^{low}$) and MZ B cells ($CD23^-CD21^{hi}$).

In vitro stimulation

Splenic CD19^{+} B cells, MZ B cells and FO B cells (1.5x10^{6}/mL) were cultured in medium (RPMI 1640 GlutaMAX; Thermo Fisher Scientific) supplemented with 5% heat-inactivated fetal calf serum (FCS; Greiner Bio-One) 2-mercaptoethanol (5x10^{-5} M), penicillin (100U/mL) and streptomycin (100µg/mL; all Sigma-Aldrich). Cells were stimulated with the following stimuli as indicated in the figures: SEA (20µg/mL), aCD40 (clone 1C10; 0.5µg/mL; Biolegend), recombinant IFNα (Biolegend), CpG ODN 1826 (class B; 0.2-1µM; Invivogen), aCD40L blocking antibody (clone MR1; 10µg/mL; kind gift from L. Boon, Bioceros), aIFNAR1 blocking antibody (clone MAR1-5A3; 10µg/mL; eBioscience). After 2 days (FO and MZ B cells) or 3 days (total B cells) culture at 37°C, supernatants were harvested for cytokine analysis by ELISA or CBA. For flow cytometric analysis of IL-10, cells were re-stimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) for 4 hours in the presence of Brefeldin A (10 µg/ml; all Sigma-Aldrich).

Flow cytometry

Cells were stained with antibodies against B220 (clone RA3-6B2), CD21 (clone 7G6), CD23 (clone B3B4) and IL-10 (clone JESS-16E3). Dead cells were stained with live/dead fixable aqua dead cell stain kit (ThermoScientific). FcγR-binding inhibitor (2.4G2, kind gift of L. Boon, Bioceros) was added to all stainings. Flow cytometry was performed on a FACS Canto II using FACSDiva software (BD Biosciences) followed by data analysis using FlowJo.

ELISA and CBA

The concentration of IL-6 and IL-10 in cell-free culture supernatants was assessed by OptEIA ELISA kits (BD Biosciences) (Total B cells) or BD cytometric bead array (CBA) Flex-set kits (BD Biosciences) (MZ and FO B cells). The concentration of cytokines in serum of chronically infected mice was also assessed by CBA Flex-set kits, except for IFNα3 and IFNβ which were measured by ELISA (PBL).

RNA extraction and qPCR analysis

RNA from frozen splenocytes was extracted using TriPure isolation reagent (Roche) and translated to cDNA using SuperScript™ III Reverse Transcriptase and Oligo (dT; Life Technologies). Quantitative PCR was performed using SYBR Green Master Mix (Applied Biosystems) using a Biorad CFX96 Real-time system C1000 thermal cycle. Expression levels were normalized to *Gadph* The following primers were used:

RPLP0: 5'- TCTGGAGGGTGTCCGCAACG–3' 5'- GCCAGGACGCGCTTGTACCC-3'; *MX1*: 5'-TTCAAGGATCACTCATACTTCAGC–3' 5'-GGGAGGTGAGCTCCTCAGT-3'; *Oas1a*: 5'-GCTGCCAGCCTTTGATGT–3' 5'-TGGCATAGATTGTGGGATCA-3';

Ifit3 5′-TGAACTGCTCAGCCCACA–3′ 5′-TCCCGGTTGACCTCACTC-3′;

Stat11 5′-GTGCCTCTGGAATGATGGGT–3′ 5′-GAAGTCAGGTTCACCTCCGT-3′;

Ifi30 5′-GAACATGGTGGAGGCCTGTC–3′ 5′-TGGCGCACTCCATGATACTC-3′;

Ifit1 5′-TCTAAACAGGGCCTTGCAG–3′ 5′-GCAGAGCCCTTTTTGATAATGT-3′

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.02). All data are presented as mean ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

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Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

RESULTS

S.mansoni infections and SEA injections induce a systemic IFN signature *in vivo*

We first sought to assess whether chronic *S. mansoni* infection induces a systemic IFN-I signature. High-dose infection with 180 *S. mansoni* cercariae significantly increased the serum concentration of IFNα3 in the majority of animals (Figure 1A), while lower doses of 20-80 cercariae did not (Suppl. Figure 1). Systemic levels of IL-5 and IL-12/23p40 were similarly increased, while IFNβ, IL-10, and IL-17 were only elevated in a minority of animals (Figure 1A). The production of IFN-I subtypes is often difficult to assess, as they are frequently produced at low levels and transiently, or consumed by neighbouring cells following production, which might explain the high dose of infection necessary to reliably detect IFN-I in the serum. Irrespective, the significant increase in serum IFN-I following high-dose infection supports the notion that *S. mansoni* induces a systemic IFN-I signature.

Next, to investigate the contribution of egg-products to IFN-I induction, we examined the expression of interferon stimulated genes (ISGs) in the splenocytes of mice intravenously injected with SEA after 12 hr (Figure 1B). SEA-treated mice demonstrated a clear IFN-I signature, with significant upregulation shown for the *Mx1* and *Oas1a*, and a trend towards enhanced expression for *Ifit3* and *Ifit1*. Additionally, SEA-treatment induced *Oas1a* expression to a similar level to that observed for IFNα-treated control mice (Supplementary Figure 2). Collectively, these data suggest that egg-exposure alone, in the absence of worms, is sufficient to drive IFN-I responses.

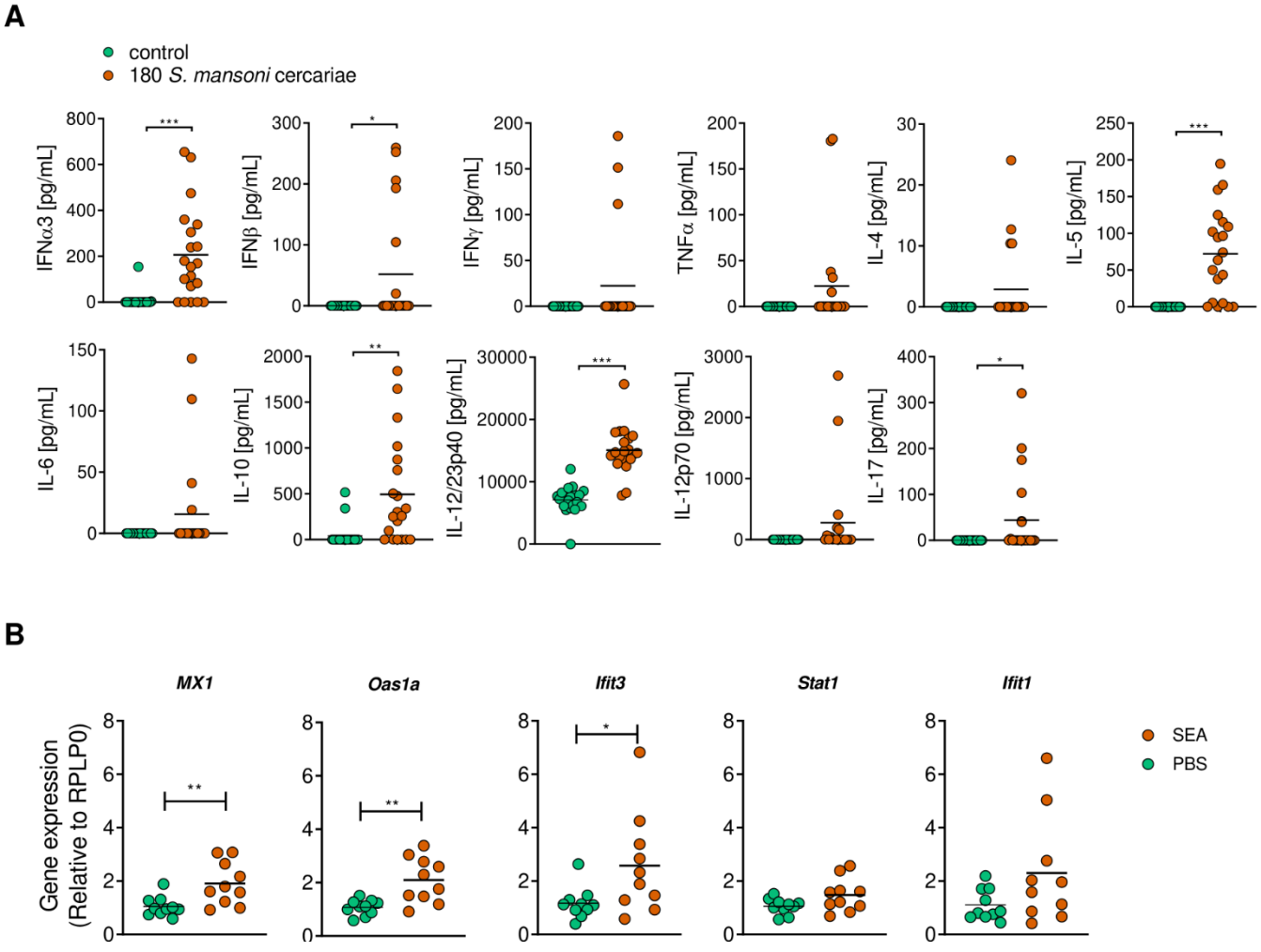

Figure 1: S. mansoni infections and SEA injections induce a systemic type I IFN signature. (A) Mice were infected with 180 S. mansoni cercariae and serum samples taken at d49 of infection for assessment of cytokine levels by ELISA/CBA. Pooled data from 2 experiments, n=20/group. (B) Splenocytes from SEA injected mice were harvested 12 hours post injection. The mRNA expression of interferon responsive genes (ISGs) was evaluated by qPCR (normalised against RPLP0). Data from 2 experiments n=2-8 per group. Significant differences were determined by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Recombinant IFNα enhances SEA/aCD40-induced B cell IL-10 production *in vitro*

We have previously demonstrated that SEA induces B cell IL-10 production and that CD40 ligation enhances SEA-induced Breg cell development[8], while others have reported a synergistic effect of IFNα and CD40 ligation on the development of IL-10-producing human B cells[39]. We therefore tested

the effect of simultaneous stimulation of splenic B cells with SEA, anti-CD40 and recombinant IFNα *in vitro*. After 3 days of culture, the concentration of IL-10 in culture supernatants of SEA-stimulated B cells increased with increasing doses of IFNα, whereas IFNα alone had no effect (Figure 2A; Supplementary Figure 3B for gating scheme). The strongest induction of B cell IL-10 production could be observed when cells were co-stimulated with SEA and anti-CD40 compared to SEA alone (Figure 2A). IFNα at concentrations of 10^3-10^4 U/mL (equivalent to circa 15-150ng/mL) significantly enhanced IL-10 production in response to SEA and SEA+anti-CD40, whereas IL-10 production seemed to plateau at 10^5 U/mL IFNα (Figure 2A). IL-10 production after co-stimulation with IFNα increased up to 4-fold compared to the control condition without addition of IFNα. IFNα also enhanced IL-6 production, a pro-inflammatory cytokine known to be produced by B cells, in response to SEA and anti-CD40, albeit to a lesser extent (Figure 2A). This indicated a pattern of cytokine expression characteristic for Breg cells. Conversely, the percentage of IL-10-producing B cells after 3 days of stimulation with SEA or SEA+anti-CD40 in the presence of IFNα did not increase (Figure 2B), which suggests that the peak of the stimulatory activity of IFNα occurs early and has passed, possibly due to a decline in the IFNα concentration in culture supernatant due to consumption, when the intracellular staining was performed after 3 days of culture. As a control, we also stimulated B cells with CpG ODN1826 (class B) and IFNα. Already a low concentration of 10^3 U/mL IFNα strongly amplified the CpG ODN1826-induced cytokine production (Supplementary Figure 4A) and percentage of 10-producing B cells (Supplementary Figure 4B). These data show that IFNα provides additional signals for the induction of B cell IL-10 production in cells activated with known Breg cell-inducing stimuli SEA or CpG ODN1826.

To identify which B-cell subset produces IL-10 in response to IFNα stimulation, splenic B cells were sorted into the two main subsets, $CD23^{low}CD21^{+}$ marginal zone (MZ) B cells and $CD23^{hi}CD21^{-}$ follicular (FO) B cells, for subsequent 2-day in vitro stimulation with SEA, anti-CD40 and recombinant IFNα (10^4 U/mL) (Supplementary Figure 3B for gating scheme). Unlike their FO counterparts, MZ B cells reacted potently to the addition of IFNα to the culture media (Figure 2C), with IFNα-treated MZ B cells demonstrating a 4-fold increase in IL-10 secretion compared to MZ B cells cultured in media alone. Importantly, the effect of IFNα-stimulation was further potentiated by the addition of SEA or SEA+antiCD40 to the culture media, with the highest production of IL-10 shown for MZ B cells cultured in the presence of IFNα, SEA and antiCD40. In contrast, FO B-cells produced relatively little IL-10 irrespective of IFNα-, SEA or SEA+antiCD40 stimulation. In comparison to unstimulated FO B cells, FO B-cells treated with IFNα-, SEA +/- antiCD40 produced significantly higher amounts of IL-

10. However, these IL-10 levels were considerably lower to that produced by MZ B cells under the same stimulation conditions. Like that observed for total B cells (Figure 2B), the percentage of IL-10 producing MZ B cells remained unaltered by IFNα-stimulation (Figure 2D), supporting the notion that peak time of IFNα stimulatory activity has already been reached. On the other hand, the percentage of IL-10 producing FO B cells even appeared to decrease following IFNα-stimulation (Figure 2D). As for IL-6, only MZ B-cells but not FO B cells showed IL-6 production following IFNα stimulation (Figure 2C). However, similar to that described for total B cells (Figure 2A), these IL-6 levels were considerably lower than that detected for IL-10. Altogether, these data demonstrate a heightened responsiveness of MZ B cells to IFNα and schistosome Ag stimulation, and support the notion that IFN-Is and schistosome Ags synchronously drive regulatory B cell activity *in vitro.*

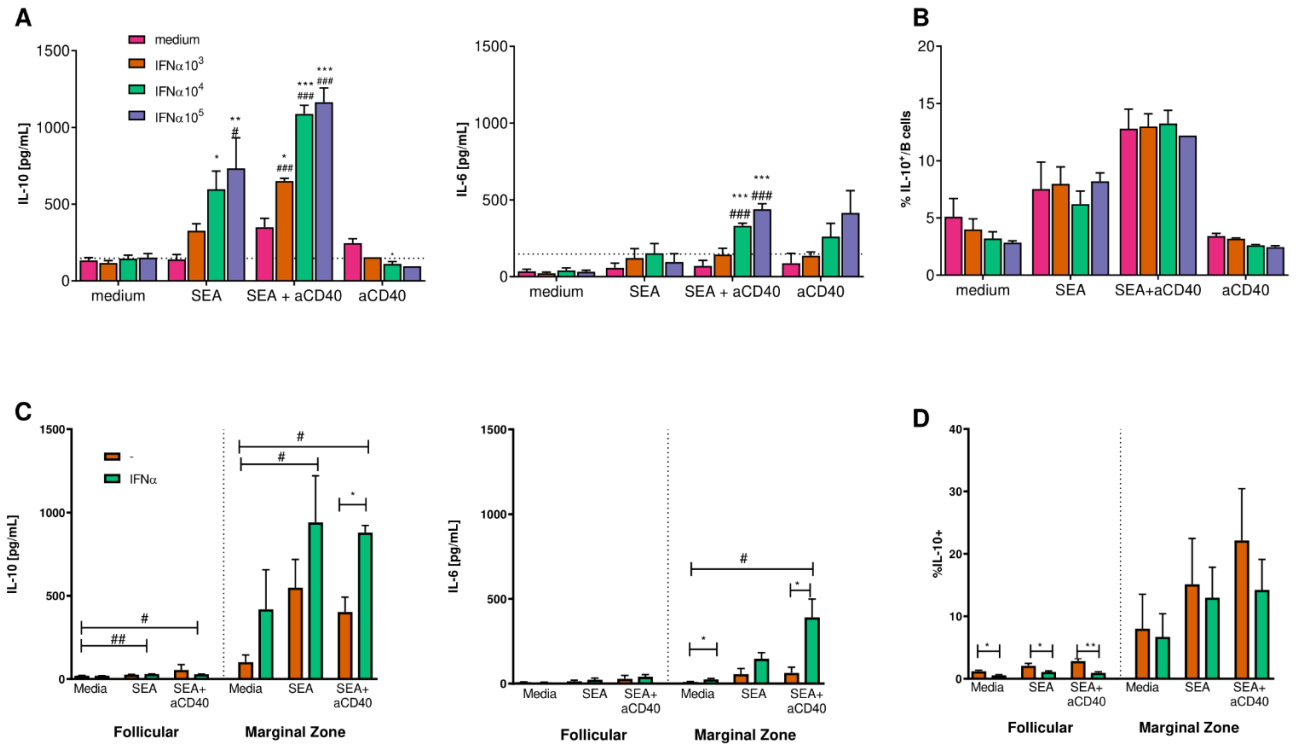

Figure 2: Recombinant IFNα enhances SEA/aCD40-induced B cell IL-10 production. (A-B) B cells were isolated from the spleen of naïve mice and stimulated in vitro with SEA (20µg/mL), anti-CD40 (0.5 µg/mL) and IFNα (103-105U/mL) as indicated. After 3 days of culture, supernatants were analysed for IL-10 and IL-6 concentration by ELISA (A), and the percentage of IL-10+ B cells assessed by flow cytometry (B). (C- D) Splenic marginal zone and follicular B cells from naïve mice were sorted using flow cytometry and cultured for 2 days in the presence of SEA (20µg/mL), anti-CD40 (0.5 µg/mL) and IFNα (104/mL). IL-6 and IL-10 production as measured by CBA (C) and frequency of IL-10+ cells in each respective subset as determined by flow cytometry (D). Summary of 4 (A) or 3 (B-D) experiments, each data point is the mean of two-four technical replicates. Data are presented as mean ± SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and determined by one-way ANOVA followed by Dunnett's multiple comparisons test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ indicates significant difference relative to medium only control.

IFNAR1 signalling provides co-signals for IL-10 production by *in vivo* primed B cells

To assess whether IFN-I signalling provides important signals for IL-10 production by *in vivo* primed Breg cells, we treated mice with SEA i.p. and subsequently restimulated total splenocyte cultures *ex vivo* with SEA, in the presence or absence of blocking antibodies against IFNAR1. We also used blocking antibodies against CD40 ligand (CD40L) upon *ex vivo* restimulation to assess the importance of CD40 co-ligation on B cells for IL-10 induction. While blocking CD40L alone, or in combination with blocking IFNAR1, had either no or no additional effect, blocking IFNAR1 signalling significantly reduced the concentration of IL-10 in 2-day culture supernatants (Figure 3A). The production of IL-6 was not affected by either of the blocking agents (Figure 3A), while the percentage of IL-10 producing B cells in culture was mildly but significantly reduced by both blocking agents (Figure 3B). We concluded that signalling via IFNAR1, but not the ligation of CD40, is essential for SEA-induced B cell IL-10 production in this setting.

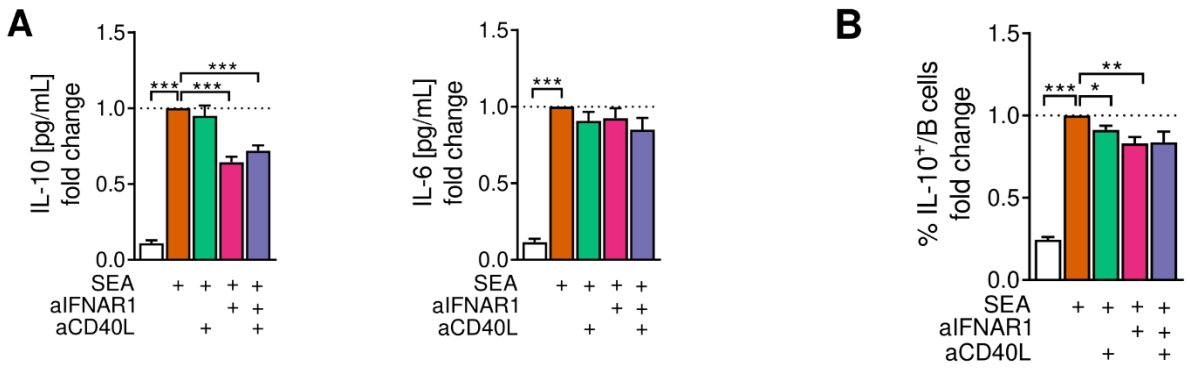

Figure 3. Ex vivo block of IFNAR1 reduces B cell IL-10 production. Splenocytes from SEA-injected mice (100µg SEA i.p. on d0 & d7; section d14) were re-stimulated ex vivo with SEA (20ug/mL) for 2 days in the presence or absence of anti-CD40L (10µg/mL) and anti-IFNAR1 (10µg/mL) blocking antibodies as indicated. After 2 days of culture, supernatants were analysed for IL-10 and IL-6 concentration by ELISA (A), and the percentage of IL-10+ B cells assessed by flow cytometry (B). Summary of 2 experiments, n=10/group. Data are presented as mean ± SEM. Significant differences were determined by RM-One Way ANOVA & Dunnett's post test comparing all groups to the SEA-stimulated positive control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IFNAR1 signalling is dispensable for Breg cell induction *in vivo*

To assess whether IFN-I signalling provides important signals for Breg cell development and IL-10 production in response to *S. mansoni* egg products not only *in vitro* but also *in vivo*, we induced Breg cell development by two doses of i.p. administered *S. mansoni* eggs (5000) in WT control or IFNAR1$^{-/-}$ mice, a model we previously showed to be very suitable to demonstrate schistosome-induced splenic Breg cell development[8]. The absence of IFNAR1 did not affect the concentration of IL-10 in B cells and total splenocyte culture supernatants in response to restimulation with SEA and anti-CD40 (Figure 4A). In addition, the percentage of IL-10^{+} B cells seemed increased rather than

decreased in IFNAR1$^{-/-}$ mice (Figure 4B). Additionally, no changes in IL-10 production could be observed when blocking IFNAR1 signalling by means of *in vivo* administration of anti-mouse IFNAR1 blocking antibody (suppl. Figure 5). Thus, IFNAR1 signalling seems to be dispensable for the induction of Breg cells to *S. mansoni* egg challenge *in vivo*.

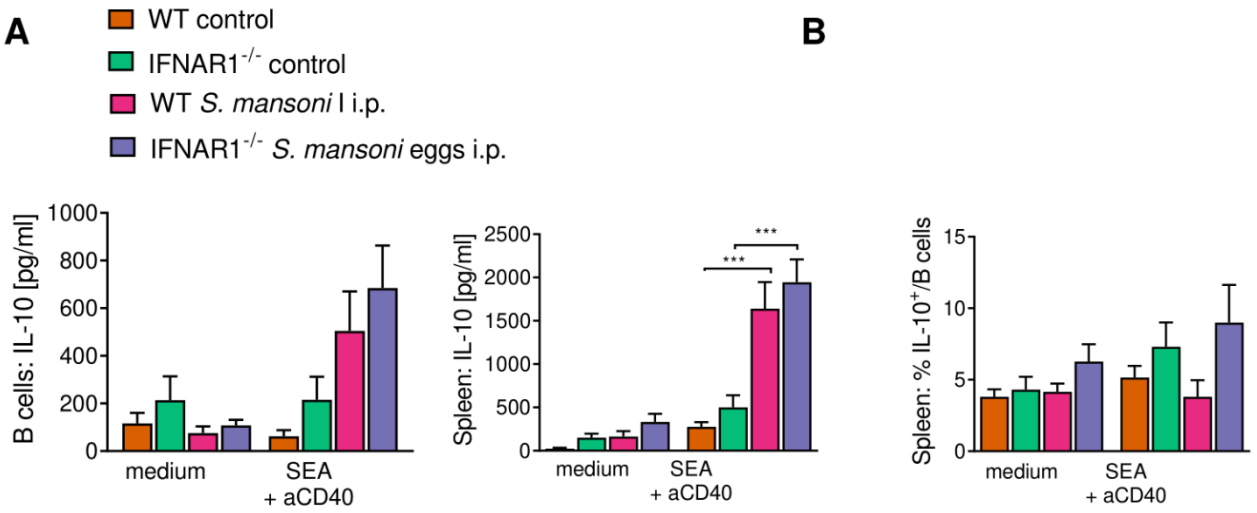

Figure 4. IFNAR1 signalling is dispensable for Breg cell induction in vivo. Splenocytes and MACS-isolated CD19+ B cells from S. mansoni egg-injected mice (5000 S. mansoni eggs i.p. on d0 & d7; section d14) were re-stimulated ex vivo with SEA (20µg/mL) and anti-CD40 (2µg/mL) for 2 days. After 2 days of culture, supernatants of isolated B cell and total spenocyte cultures were analysed for IL-10 concentration by ELISA (A), and the percentage of IL-10+ B cells within splenocyte cultures assessed by flow cytometry (B). Summary of 2 experiments, n=8-10/group. Data are presented as mean ± SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$

In this study we sought to address whether IFN-I might provide the 'missing link', synergizing with *S. mansoni*-derived signals for the induction of Breg cell IL-10 production. We show that, although *S. mansoni* infections and injections with egg Ags induce systemic IFN-I signature, and IFN-I signalling enhances *in vitro* IL-10 production by Breg cells exposed to *S. mansoni* Ags, IFN-I responsiveness is ultimately dispensable for Breg cell induction by *S. mansoni* eggs *in vivo*.

We and others have previously shown that chronic *S. mansoni* infection induces Breg[3, 4, 43, 44] and that this Breg cell-inducing effect can be replicated by isolated eggs, SEA and even the single, egg-derived molecule IPSE/alpha-1 in the absence of adult worms and a natural infection [7, 8.] Components of SEA directly bind to splenic B cells[8], but the receptors ligated and signalling pathways activated by these Ags remain to be identified. Moreover, SEA immunization is less potent than chronic infection at Breg cell induction *in vivo*, and the induction of Breg cells by IPSE/alpha-1 has only been demonstrated *in vitro*[8]. Helminth infections trigger a multitude of different immune responses in the host *in vivo*, and it is likely that additional signals, in addition to helminth molecules, are required for optimal Breg cell induction.

We found an increased concentration of IFN-I in serum of mice actively infected with *S. mansoni,* and enhanced expression of ISGs in the spleen of SEA-injected mice. These data are in line with previous reports on the capacity of *S. mansoni* eggs or egg Ags, *H polygrus* infection and *N. brasiliensis* Ags to induce IFN-I[31-43]. pDCs are considered an important source of IFN-I[45]. IFN-I were however produced by conventional DCs (cDCs) rather than pDCs after SEA-stimulation of BMDCs *in vitro*[33]. We have not addressed the cellular source of IFN-I in our study, therefore both pDCs and cDCs remain possible sources. Notably, little is reported to date regarding IFN-I production by human DCs in response to helminths but work of our own group suggests that *S. mansoni* egg Ags do not induce IFN-I in human monocyte-derived DCs (Everts, personal communication).

We show here that recombinant IFNα, while having no measurable effect on its own, significantly, and dose-dependently increased IL-10 production by B cells in response to *in vitro* stimulation with SEA alone or SEA+aCD40. Additionally, in harmony with previous mechanistic studies[8] and models of chronic *S.mansoni* infection[46], we provide evidence that MZ B cells, as opposed to FO B cells, are responsible for this increase in IL-10. IFNα was also shown to have a synergistic effect on SEA+aCD40-induced IL-6 production, albeit to lesser extent. Conversely, the percentage of IL-10^{+} B cells was unchanged or slightly reduced after 3 days of culture in the presence of increasing amounts of IFNα, suggesting that IFN-I may change the dynamics and timing of IL-10 production. Menon et

al. observe an optimal IL-10 induction in naïve TLR9-stimulated B cells at $50x10^5$ U/mL IFNα and a less effective stimulation at higher concentrations[39], whereas we find an additive effect even at $1x10^6$ U/mL on both SEA- and TLR9-stimulated B cells on IL-10 concentration in culture supernatants. The fact that IFNα has no effect at all on IL-10 or IL-6 expression by itself underpins that IFN-I signalling modulates responses in pre-activated B cells rather than providing an activation signal to B cells by itself, which has been similarly reported by others[27,42]. In this context, it is plausible that stimulation with *S. mansoni*-derived Ags *in vitro* provides this pre-activation signal, rather than SEA- and IFN-I-specific signalling pathways synergizing to promote B cell IL-10 production. This is in line with previous reports describing IFN-I signalling to regulate B cell responses to other pre-activating stimuli such as BCR or TLR7 ligation[35,36]. In this context, Braun et al. show that murine, mature splenic B cells get partially activated by treatment with IFNα/β, characterized by the upregulation of activation markers and increased survival in the absence of proliferation or terminal differentiation, and display enhanced response to BCR ligation[35]. Poovassery and colleagues report that both BCR and IFNAR signalling restore TLR7-induced B cell hyporesposiveness[36]. That the percentage IL-10^+ B cells tends to decrease at the end of culture might suggest that the peak of IFNα stimulatory activity has occurred earlier and that after 3 days of culture the IFN-I concentration in culture supernatant has already declined, making an earlier time point for the assessment of IL-10^+ B cells preferable.

Arguably, *in vitro* stimulation of isolated B cells with recombinant IFNα does not mimic the natural situation very well. We therefore also assessed the role of IFN-I signalling on Breg cell recall responses *ex vivo*. Blocking IFNAR1 upon *ex vivo* restimulation of *in vivo* SEA-induced Breg cells significantly reduced IL-10, but not IL-6 production. Adding blocking antibody against CD40L to the cultures, and thereby preventing the ligation of CD40 expressed on B cells by accessory cells present in whole splenocyte cultures, had only negligible effects. This might indicate that, while CD40 ligation has previously been shown to enhance B cell IL-10 expression[8,9,15], it does not provide additional signals for B cell IL-10 production in this restimulation setting. This might point at a difference in the contribution of CD40 signalling to Breg cell induction upon concurrent priming of B cells with an Ag and agonistic anti-CD40[8] and upon *ex vivo* restimulation as performed in this study. Alternatively, it is also possible that insufficient CD40 ligation was occurring in our chosen culture conditions, and so explains why blocking CD40 had a negligible effect. Finally, although injections with *S.mansoni* egg-products can effectively drive both systemic IFN-I responses and Breg development[8], we found B cell IL-10 production to be unaltered in IFNAR1$^{-/-}$ mice upon egg i.p. administration, suggesting that IFN-I signalling is dispensable in this setting. However, it is unknown

whether other signals have compensated for the lack of IFN-I, and so, a full blown Breg response is still driven in IFNAR$^{-/-}$ mice. In addition, we have not tested the role of IFN-I during schistosome infections in IFNAR$^{-/-}$ mice and therefore cannot exclude that IFN-I produced in response to repetitive stimulations and chronic inflammation may still contribute to Breg cell development during schistosomiasis. Combined, our data suggests that *in vivo*, where multiple pathways are activated simultaneously and potentially act synergistically, IFN-I signalling is not essential for the development and activation of Breg cells against *S. mansoni* eggs.

The physiological role of IFN-I in helminth infections has not been extensively studied to date. Enteric *H. polygyrus*-induced IFN-I protects from RSV co-infection[31]. SEA-stimulated BMDCs induce IFN-I[32], and SEA-stimulated cDCs as well as skin DCs exposed to *N. brasiliensis* were shown to be dependent on IFN-I signalling for their effective induction of Th2 response[33,34]. Therefore, more research is needed to fully understand the role of IFN-I in helminth, and more specifically in *S. mansoni* infections.

Collectively, the data presented here show that, while IFN-I can enhance IL-10 production by *S. mansoni*-activated Breg cells both *in vitro* and *ex vivo*, IFN-I signalling is dispensable for the formation and activation of *S. mansoni*-induced Breg cells *in vivo*. A better understanding of the signals for optimal Breg cell development and activation is required to develop novel therapies around Breg cells.

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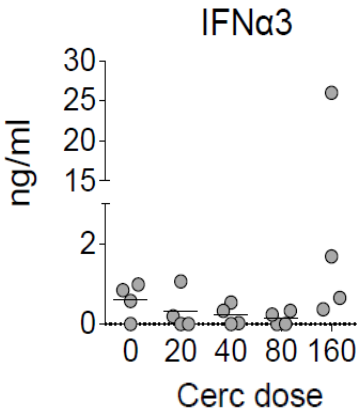
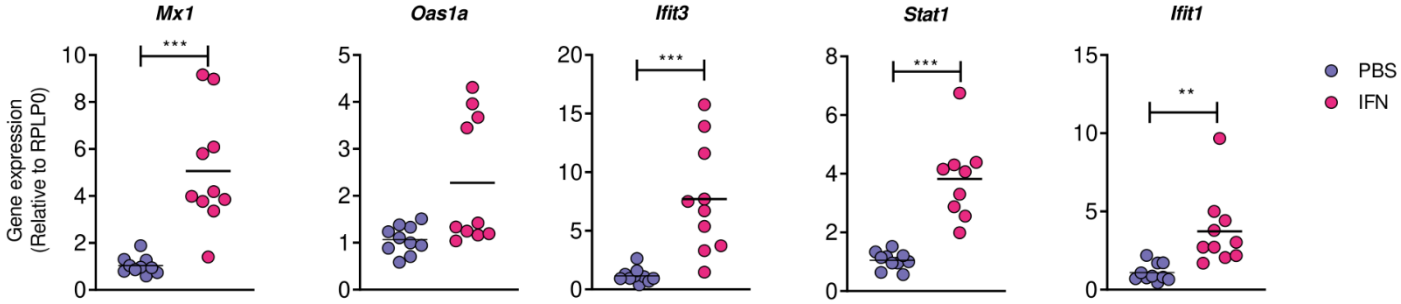
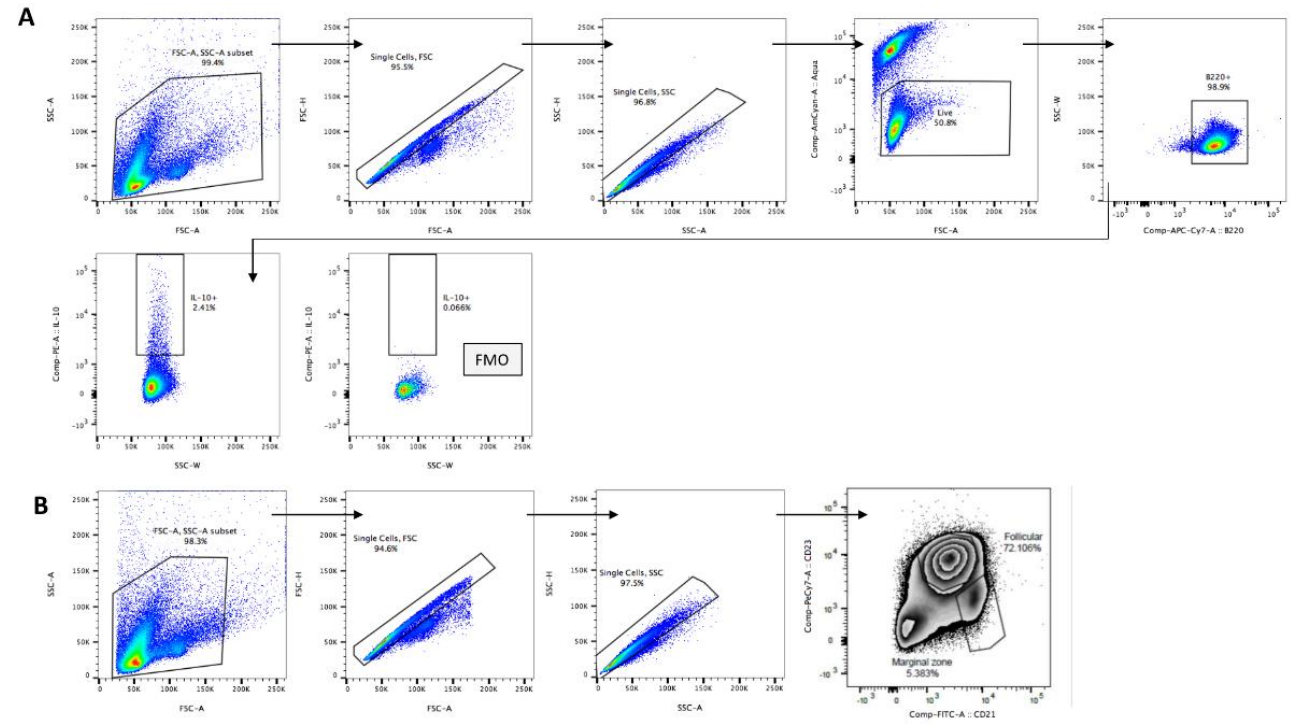


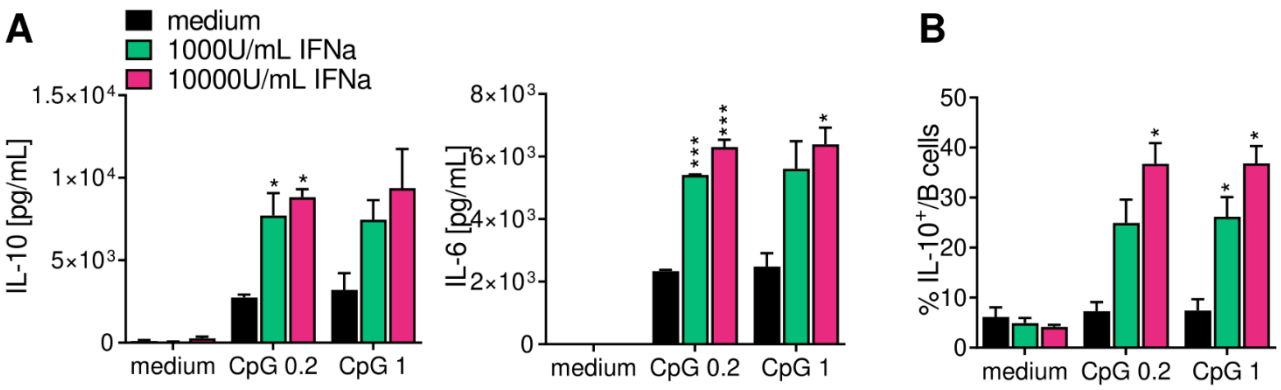
Supplementary Figure 1. A trend towards enhanced IFNα3 levels in the serum of mice infected with 20-160 cercariae. Mice were infected with 20-160 cercariae and serum samples were taken at d56 of infection for assessment of cytokines by ELISA. Data from 1 experiment, with 4 mice per group.



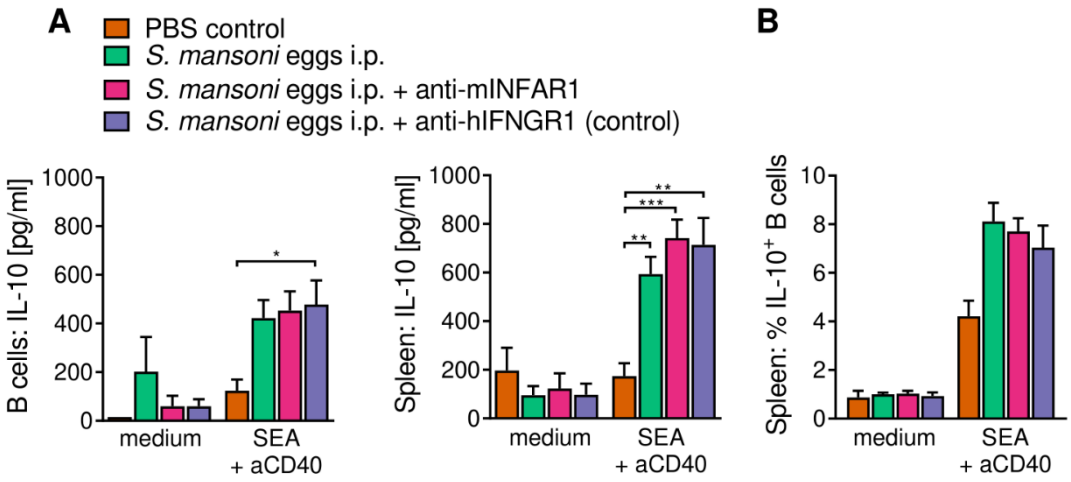
Supplementary Figure 2: Expression of interferon stimulated genes in IFNα injected mice. 12 hours post-injection, splenocytes were isolated from PBS- and IFNα-treated mice. The mRNA expression of interferon-stimulated genes (ISGs) was assessed by qPCR (relative to RPLP0). Data from 2 experiments, n=2-8 per group. Significant differences were determined by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplementary Figure 3. Flow cytometry gating schemes. (A) Identification of IL-10 production from 3-day cultured B cells. (B) Cell sorting scheme for follicular and marginal zone B cell subsets from MACs isolated B cell.



Supplementary Figure 4. Recombinant IFNα enhances CpG-induced B cell IL-10 and IL-6 production. B cells were isolated from the spleen of naïve mice and stimulated in vitro with CpG ODN1826 (class B; 0.2-1µM) and IFNα (103-104 U/mL) as indicated. After 3 days of culture, supernatants were analyzed for IL-10 and IL-6 concentration by ELISA (A), and % IL-10 B cells assessed by flow cytometry (B). Summary of 2-3 experiments, each data point is the mean of two technical replicates. Data are presented as mean ± SEM. Significant differences were determined by one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$.



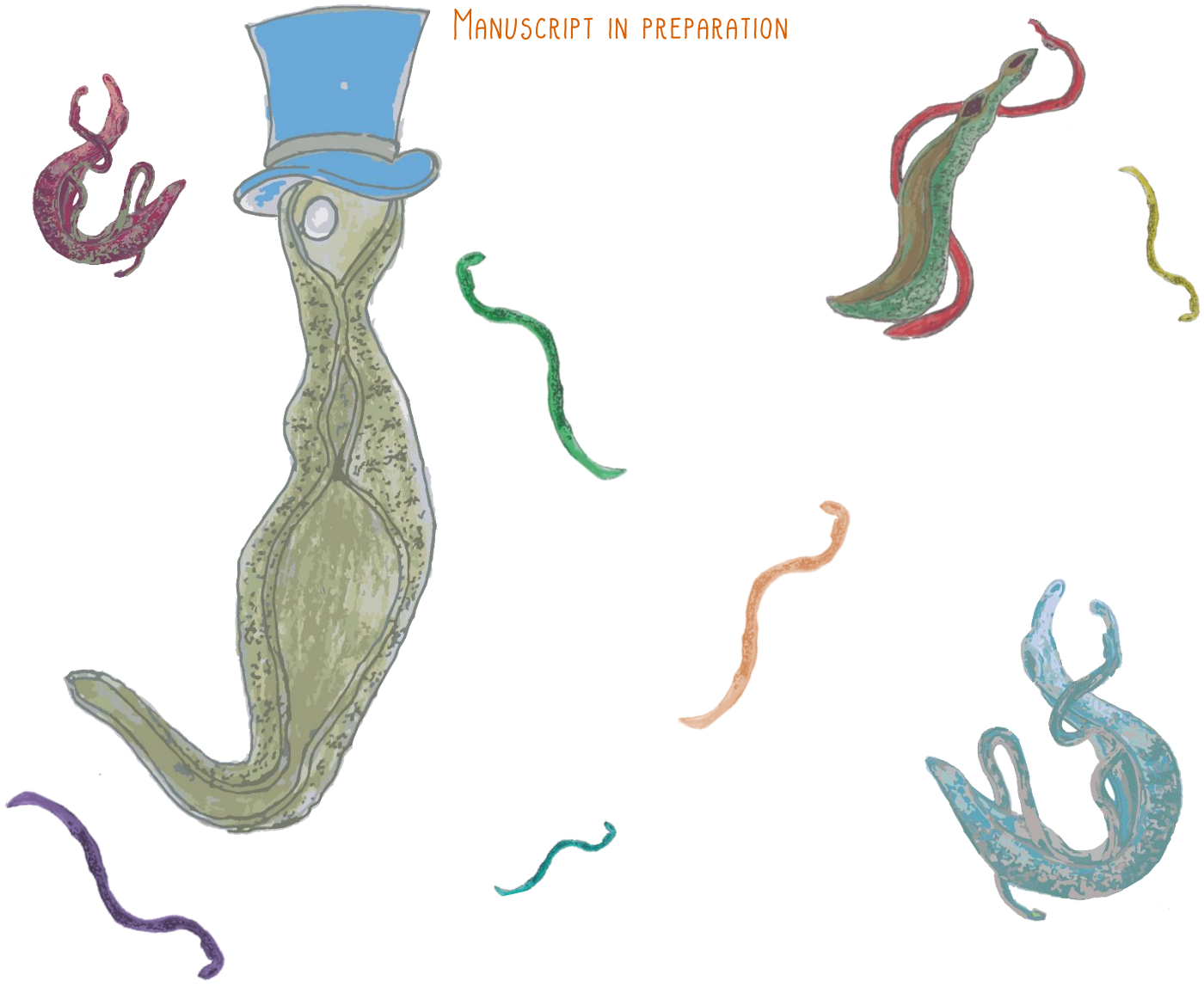
Supplementary Figure 5. IFNAR1 signalling is dispensable for Breg cell induction in vivo. Mice were treated as depicted in A. On day 14, spleens were harvested and total splenocyte cell suspensions and isolated CD19 + B cells restimulated with SEA (20ug/ml) and anti-CD40 (2ug/ml) for 2 days. Supernatants were analyzed for IL-10 and IL-6 concentration by ELISA (B), and the percentage of IL-10+ B cells assessed by flow cytometry (C). Data from one experiment, n=5/group. Data are presented as mean ± SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER 6

PATENT *SCHISTOSOMA MANSONI* INFECTIONS ENDORSE DISTINCT REGULATORY CELL NETWORKS, WITH EVIDENCE FOR MICROBIAL ASSISTED ALLEVIATION OF ALLERGIC AIRWAY INFLAMMATION

ALICE H. COSTAIN, ARIFA OZIR-FAZALALIKHAN, KATJA OBIEGLO, FRANK OTTO, AMANDA R. RIDLEY, HELEN E. PARKER, NICHOLAS A. SCOTT, GORDANA PANIC, JAQUELINE J. JANSE, ANGELA VAN DIEPEN, META ROESTENBERG, CORNELIS H. HOKKE, JONATHON R. SWANN, ANDREW S. MACDONALD, HERMELIJN H. SMITS

MANUSCRIPT IN PREPARATION



Patent Schistosoma mansoni infections endorse distinct regulatory cell networks, with evidence for microbial assisted alleviation of allergic airway inflammation

AUTHOR INFORMATION

Alice H. Costain[1,2], Arifa Ozir-Fazalalikhan[1], Katja Obieglo[1], Frank Otto[1], Amanda R. Ridley[2], Helen E. Parker[2], Nicholas A. Scott[2], Gordana Panic[3], Jaqueline J. Janse[1], Angela van Diepen[1], Meta Roestenberg[1], Cornelis H. Hokke[1], Jonathon R. Swann[3], Andrew S. MacDonald[2], Hermelijn H. Smits[1*]

1. Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands
2. Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, UK
3. School of Human Development and Health, University of Southampton

*Co-corresponding authors: Andrew S MacDonald (andrew.macdonald@manchester.ac.uk) and Hermelijn Smits (h.h.smits@lumc.nl)

ABSTRACT

Chronic schistosome infections drive systemic activation of the regulatory arm of the immune system, including Regulatory B cell (Breg) and T cell (Treg) populations that curb and restrain excessive inflammatory responses, partially through the provision of anti-inflammatory IL-10. Murine schistosome-induced Breg and Treg cells have repeatedly proven proficient down-regulators of allergic airway inflammation (AAI) and suppressors of T effector responses. However, the signals and life stages dictating their expansion are far from understood. Comparing conventional mixed-sex (egg production) and male worm only (no egg production) *Schistosoma mansoni* infections, we show both patent and non-patent infections to elicit the generation of phenotypically distinct splenic regulatory cell networks. However, at chronic stages of infection, B cells and $CD4^+$T cells from egg producing infections displayed a heightened propensity to produce IL-10 compared to non-patent infections. As we recently observed substantial differences in intestinal microbiota composition between chronic single-sex and mixed-sex infections, we next questioned the involvement of their associated microbiotas in regulatory cell induction and modulation of AAI. In comparison to allergic mice recolonised with naïve microbiotas, the transfer of single-sex and mixed-sex microbiotas lead to a significant reducing in cellular infiltration to the airways, with mixed-sex microbiotas imprinting a distinctive M1 phenotype on pulmonary macrophages and skewing towards mild Th1 $CD4^+$ responses. Although no large alterations were observed in pulmonary regulatory populations, schistosome microbiota recipients displayed an enhanced frequency of splenic $Foxp3^+$ and CTLA-4^+CD4^+ T cells. Importantly, when transferred into non allergic IL-10 reporter mice, schistosome microbiotas evoked subtle elevations in splenic and mesenteric IL-10. These data shed further light on the contribution of worm and egg derived signals in the promotion of schistosome-mediated regulation and suggest that the regulatory capacity of *Schistosoma* parasites is partially mediated through changes in the microbiota.

Keywords

Schistosoma mansoni, microbiota, Breg, Treg, chronic infection, allergic airway inflammation.

Abbreviations

Adult worm Antigen (AWA) Allergic airway inflammation (AAI); Alternative activation (AA); B Cell receptor (BCR); Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4); Extracellular secretory products (ES products); Follicular (FO); Glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR); Interleukin (IL); Latency associated peptide (LAP); Marginal Zone (MZ); Mesenteric lymph nodes (MLNs), Regulatory B cell (Breg); Regulatory T cell (Treg); Schistosome egg antigen

(SEA); Short chain fatty acids (SCFAs); Transitional Type 1 (T1); Transitional Type 2 (T2); transitional type 2 marginal zone precursors (T2-MZPs); T Helper (Th); Toll like receptor (TLR); Trimethylamine (TMA); Trimethylamine oxide (TMAO);

INTRODUCTION

The mammalian immune system has evolved in tandem with environmental microbes and parasites and is thus reliant on their encounter for adequate immune education and development[1]. Indeed, allergy and autoimmunity are rising within westernised populations, and have shown inversely correlated with helminth infections, including schistosomiasis[1,2]. In human infections, *Schistosoma* parasites appear to lessen the severity of asthma[3] and allergic skin reactivity[4,5], with evidence for the induction of strong regulatory networks[6]. Experimentally, schistosome-mediated protection against models of allergic airway inflammation (AAI) is facilitated by the actions of discrete regulatory cell populations, including Regulatory T cells (Tregs)[7–9] and B cells (Bregs)[9–12], that typically emerge during infection chronicity[11–13]. A current research ambition is to learn from these parasites, identify the mechanisms underlying Breg and Treg induction, and strategically implement this understanding for the treatment of hyper-inflammatory conditions such as allergy.

Tregs are essential mediators in the control of helminth mediated pathology and are thus commonly targeted by helminths to enhance their longevity and limit attack from the host immune system[14]. Treg populations are typically hallmarked by the master transcription factor Foxp3^{+}[15], as well as putative markers such as the IL-2α receptor (CD25)[16], glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR)[17], and the inhibitor receptor Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)[18,19]. In schistosomiasis, both CD25^{+} and CTLA-4^{+}CD4^{+} T cells are important providers of the immunosuppressive cytokine IL-10 and are capable of modulating Th2-dominated granulomatous inflammation[20–24] and subverting potentially lethal Interleukin-4 (IL-4) /IL-13 driven pathology[25]. In allergic disease, Tregs have pivotal roles in maintaining tolerance to innocuous Ags[26,27]. The transfer of helminth-induced Tregs into uninfected sensitised hosts downregulates AAI[28], and Treg abolishment, by means of anti-CD25 antibodies[8] or genetic ablation[7], reverts schistosome mediated AAI suppression and aggravates allergic responses. Moreover, regulatory cell populations are known to work in synergy, with one allergic system showing *S. mansoni* induced Bregs to confer protection against allergy through activation of their Treg counterparts[9]. In fact, while IL-10 is critical to the downmodulation of schistosome evoked inflammation[25,29,30], schistosome-induced Tregs can work independently of IL-10 production[22], with the majority of IL-10 coming from a non CD4+ T cell source[23], including B cells[31,32].

Bregs expand during experimental and natural infections with *Schistosoma* species[12,32–35]; which may represent an immunoevasive strategy contributing to long-term parasite survival[36]. In murine infections, Bregs are enriched splenic marginal zone (MZ)[12,32] and pulmonary compartment[32], and protect AAI through means of IL-10 production and/or Treg expansion[9–12,35]. Similarly, *S. haematobium* infected individuals have an elevated percentage of IL-10 producing peripheral B cells in comparison to uninfected persons, with IL-10 levels returning to baseline following anti-helminthic administration[11]. Importantly, these schistosome-driven Bregs demonstrate an enhanced capacity to restrain T effector responses, induce Tregs, and are characterised by regulatory associated markers such as CD1d or the surface latency associated peptide (LAP)/ TGF-β1[31,32]. Unlike Tregs, there is no lineage-specific marker to define Breg functionality[37]**.** Instead, Bregs adopt a wide variety of phenotypes depending on the inflammatory threat encountered and are standardly identified via their provision IL-10, albeit other IL-10 independent effector mechanisms do exist[10,38–42]. In terms of instructive signalling, engagement with inflammatory cytokines [37,43,44], CD40[45] and the B cell receptor (BCR) are considered crucial for their appearance and IL-10 production. Toll-like receptor (TLRs) ligands [46,47] support this instruction, with described roles bacteria and/or their metabolites[48–51], and direct interaction with *Schistosoma* products[52]. On the basis that Breg cells are not lineage specific but emerge in response to discrete inflammatory cues[37], closer interrogation of chronic inflammatory scenarios may provide novel insight into the molecular determinants controlling the Breg differential program and IL-10 transcription.

The identification of Breg and Treg generating factors is made more complex by the intricacy of the schistosome life-cycle. Notably, with schistosomes undergoing multiple transformative and tissue-migratory stages within their host, it is difficult to ascertain whether regulatory cell generation is purely parasite driven, and/or a result of host-derived reparative or inflammatory mediators[14]. It is also possible that microbial derived signals play a participatory role, with schistosome infections shown to drive intestinal dysbiosis[53–56] (*Costain et al., manuscript in preparation*) and antibiotic administration influencing the instigation of schistosome-specific immune responses[57]. Importantly, multiple reports have shown microbial members (of their derived products) to promote Breg[48,50,51,58] and Treg[59,60] generation, and helminth-derived microbiotas to modulate the severity of airway[61] and intestinal inflammation[54,62,63]. Taken together, these observations warrant closer inspection of the role of schistosome associated microbiotas in regulation of host immunity.

In the current study we provide further clarity on the signals driving splenic regulatory cell expansion during murine schistosomiasis. By virtue of infections with egg producing or non-egg producing *S.*

mansoni parasites, we demonstrate that patent schistosomiasis endorses the expansion of phenotypically distinct splenic B cell and T cell networks, coinciding with heightened production of IL-10. While non-patent infections failed to enhance the propensity for $CD4^+$ T cells to produce IL-10, B cell IL-10 levels were elevated in these worms only infections as well suggesting that worm derived molecules, in the absence of eggs or their associated inflammation, can support Breg programming. Interestingly, the transplant of schistosome-associated microbiota into antibiotic treated recipients led to subtle increase in splenic and mesenteric IL-10, as well as a shift to Th1-like allergen-specific responses in the lung, and downmodulation of HDM-evoked allergic airway inflammation. Together, these data strongly suggest schistosomes to not only modulate host immunity directly via their own secretions or molecules, but also through changes in the microbiota composition.

MATERIALS AND METHODS

Animals

Age-matched C57BL/6 mice (Envigo) and IL-10GFP Vertx reporter mice (raised in house) on a C57BL/6 background were housed under SPF conditions at the Leiden University Medical Centre (LUMC, Leiden, The Netherlands) or the University of Manchester (Manchester UK). For germ-free (GF) experiments, GF mice were bred in isolators at the University of Manchester Gnotobiotic Facility. All animal experiments were conducted in accordance with either the Animal Experiments Ethical Committee of the Leiden University Medical Centre or under a project license granted by the Home Office UK and performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

Schistosome infection

Biomphalaria glabrata snails infected with a Puerto Rican strain of *S. mansoni* were maintained at LUMC or obtained from K. Hoffman (Aberystwyth University, UK). Mice were percutaneously infected with 40 male and female parasites (mixed-sex infection), or 40 male parasites (single-sex infection), with parasite sex determined by purpose-made multiplex PCR, targeting schistosome-sex specific sequences[64]. Mice were culled at 4, 7, 9, 12 or 14 weeks post infection. Infections in mixed-sex infected mice were confirmed by macroscopic evaluation of livers, followed by digestion of the whole intestine in 4% KOH (24hr, 37°c) for egg counts. Evaluation of circulating cathodic antigen (CCA) levels in mice urine allowed for confirmation of single-sex infections[65]. For GF schistosome infections, GF mice were infected with 180 male and female *S. mansoni* cercariae with infections

lasting 7 weeks. To minimise exposure to the environment, infections were conducted under strict sterile condition, and mice were fed an autoclaved diet and water.

Antibiotic treatment and faecal transplant

For depletion of intestinal bacteria communities, mice were treated with metronidazole, ampicillin, gentamycin, neomycin (all 1mg/ml; Sigma) and vancomycin (0.5mg/ml; Alfa Aesar) in drinking water for 2 weeks, with replenishment every 3-4 days[66]. 2 days after the removal of Abx from the drinking water, mice were recolonised by oral gavage with faeces from naïve or schistosome infected mice, with faeces taken at week 14 of infection. 2 days post primary colonisation, mice received an additional 'booster' gavage to promote bacterial recolonisation[67]. Mice were euthanised 3 weeks post the first faecal gavage or taken further for induction of HDM evoked allergic airway inflammation.

House dust mite evoked allergic airway inflammation

Mice were anesthetised with isoflurane and sensitised intranasally with 100µg of HDM (Greer Laboratories) dissolved in 30µl of sterile PBS. 1 week following HDM sensitisation, mice were challenged for 4 consecutive days with 10µg of HDM in 30µl volumes under isoflurane anaesthetic. Mice were culled 2 days after the final allergen challenge, with collection of BAL, lung and mediastinal LNs.

Isolation of immune cells

Immune cells were isolated from the spleen, lung, bronchiolar lavage fluid (BAL) and mesenteric lymph nodes (MLNs). Spleens and MLNs were homogenized by passage through a 70µM cell strainer, with spleens undergoing an additional incubation with RBC lysis for erythrocytes depletion. Cells were manually counted by haemocytometer and resuspended in FACs buffer (PBS supplemented with 2mM EDTA (Sigma) and 2% FBS) or in complete RPMI 1640 GlutaMAX media (Invitrogen) containing 5% FBS, 5×10^{-5} M 2-Mercaptoethanol (Sigma) and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin; Invitrogen). Cells were then taken further for cell culture, flow cytometry or B cell isolation. For HDM based experiments, BAL cells were obtained by flushing the lungs 2 times with FACs buffer. Lungs were sliced and digested at 37 °C for 40 minutes with 0.15mg/ml Liberase TL (Roche) and 0.05µg/ml DNase I type VI (Sigma) in HBSS (Sigma). Digestion was stopped by the addition of FACs buffer followed by resuspension and passage of cells though a 70µm cell strainer. Lung samples underwent an additional RBC lysis step before counting by haemocytometer and resuspension in X-VIVO™ (Lonza) or FACs buffer.

B cell isolation and in vitro stimulation

Total B cells were purified from splenocytes by anti-CD19 Microbeads (Miltenyi Biotech) following manufacturer's instructions. Total B cells (6×10^6/mL) were cultured for 72h at 37 °C in complete RPMI 1640 GlutaMAX with or without SEA (20µg/mL), anti-CD40 (0.5µg/mL), adult worm antigen (AWA) (20 µg/mL) and worm Extracellular secretory (ES) products (20 µg/mL). Following culture, supernatants were harvested for cytokine analysis by ELISA. For flow cytometry based detection of IL-10, cells stimulated with PMA (100 ng/mL) and ionomycin (1µg/mL) in the presence of Brefeldin A (10µg/mL; all Sigma–Aldrich) for the final 4 hours of incubation.

Flow cytometry

Cells of equivalent numbers were washed twice in PBS, before staining with live/dead fixable aqua dead cell stain kit (1:400; Thermo Scientific) or Zombie UV dye (1:2000, BioLegend) for 10 minutes at room temperature. Cells were subsequently stained with B cell or T cell specific antibodies (Table 1) with FcγR-binding inhibitor added to all samples. For staining panels involving the analysis of intracellular makers, samples were further fixed and permeabilized with BD Cytofix/Cytoperm™ (BD) or eBioscience™ Foxp3 / Transcription Factor Fixation/ Permeabilization for 1h at 4 °C, before staining with specified intracellular markers.

ELISA

Concentrations of IL-10 and IL-6 in cell-free culture supernatants was assessed by OptEIA ELISA kits (BD) according to manufacturer's instructions.

Company	Target	Clone		Company	Target	Clone
Ebioscience	B220	RA-6B2+A2:D39		Invitrogen	CD11b	M1/70
BD	CD19	Ebio(ID3)		Invitrogen	CD11c	N418
Biolegend	CD1d	1B1		Biolegend	CD62L	MeL-14
BD	CD21	7G6		Biolegend	CD64	x54-5/7.1
BD	CD23	B3B4		Biolegend	F4/80	BM8
Biolegend	CD24	M1/69		Biolegend	Ly6C	HK1.4
BD	CD5	53-7.3		Biolegend	MHC-II	M5/114.15.2
BD	CD86	B7-2		Biolegend	PDCA-1	927
BD	CD9	MZ2		BD	Siglec-F	E50-2440
BD	Galectin-1	RG9-35		Ebioscience	Ter-119	TER-119
Biolegend	CD4	RM4-5		Biolegend	XCR1	ZET
Biolegend	CD8	53-6.7		Biolegend	CD25	PC61
Biolegend	IFNγ	XMG1.2		Invitrogen	CD3	17A2
Biolegend	IL-10	JES5-16E3		Biolegend	CD4	RM4-5
Ebioscience	IL-13	ebio13A		Ebioscience	CD44	Im7
Biolegend	IL-17	TC11-1810.1		BD	CTLA-4	UC10-4B9
Biolegend	IL-4	11B11		Ebioscience	Gata-3	TWAJ
Ebioscience	IL-5	TRFK.5		Biolegend	GITR	DTA-1
Invitrogen	TCRb	H57-597		Ebioscience	RORyt	B2D
Invitrogen	Foxp3	FJK-16s		Biolegend	T-bet	4Bio

Table 1. Flow cytometry antibody list

Serum metabolomics

Serum samples were randomized, thawed at room temperature, briefly vortexed then centrifuged for 20 minutes at 13,000 x g in a centrifuge cooled to 4° C. In an Eppendorf tube, 30 µl of the serum supernatant was combined with 30 µl of a buffer solution (pH 7.4, 100% D2O) containing 0.075 M Na2HPO4, 2 mM NaN3 (an antimicrobial) and 0.08% of the internal standard 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate (TSP). The mixture was transferred to 1.7 mm glass NMR tubes. A quality control sample was generated by pooling from each sample and combining with buffer as described above.

One dimensional 1H NMR spectra were acquired on a Bruker Advance III HD 600 MHz NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), equipped with a SampleJet system and a cooling rack of refrigerated tubes at 6° C. A relaxation edited spin-echo using the 1D-Carr-Purcell-Meiboom-Gill (CPMG) presat pulse sequence was applied to all samples, analysed at a temperature of 300 K. For each sample, 128 transients were collected in 64K frequency domain points with a spectral window set to 20 ppm.

Automated processing of the spectra was performed using TopSpin 3.6 (Bruker Corporation, Germany) including spectral calibration, phase and baseline correction. The resulting raw NMR spectra were imported into MATLAB (Version 2018a, Mathworks Inc). After digitization of the spectra, redundant peaks (TSP, H2O) were removed. The spectra were manually aligned to quality control samples and reference peaks using Recursive Segment-Wise Peak Alignment 1. For the SIC profiles, the data was normalized using a probabilistic quotient, to account for variability in sample dilutions 2. Metabolite identification was performed using an in-house metabolite library, cross-referencing with the Human Metabolome Database (https://hmdb.ca/) and Statistical Total Correlation Spectroscopy (STOCSY) 3. The relative concentrations of all age associated metabolites were calculated from the spectral data using trapezoidal numerical integration.

Statistics

Statistical analyses were performed using GraphPad Prism 9 software. Data are shown as mean values ± S.E.M. Where applicable, experimental groups were analysed by unpaired t-test, one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test as appropriate. Significant differences were defined at $P < 0.05$.

Distinct Treg networks emerge during patent and non-patent schistosomiasis

Phenotypically distinct Treg populations emerge within the liver[22–24], colon[21] and spleen[20] of chronically infected animals, with both worm[68,69] and egg-derived products[70,71] demonstrating Treg inducing capacity in *in vitro* settings or through challenge models. However, to our knowledge, it has yet to be investigated whether worm-alone signals, in the absence of egg driven inflammation, may support Treg expansion during active infection.

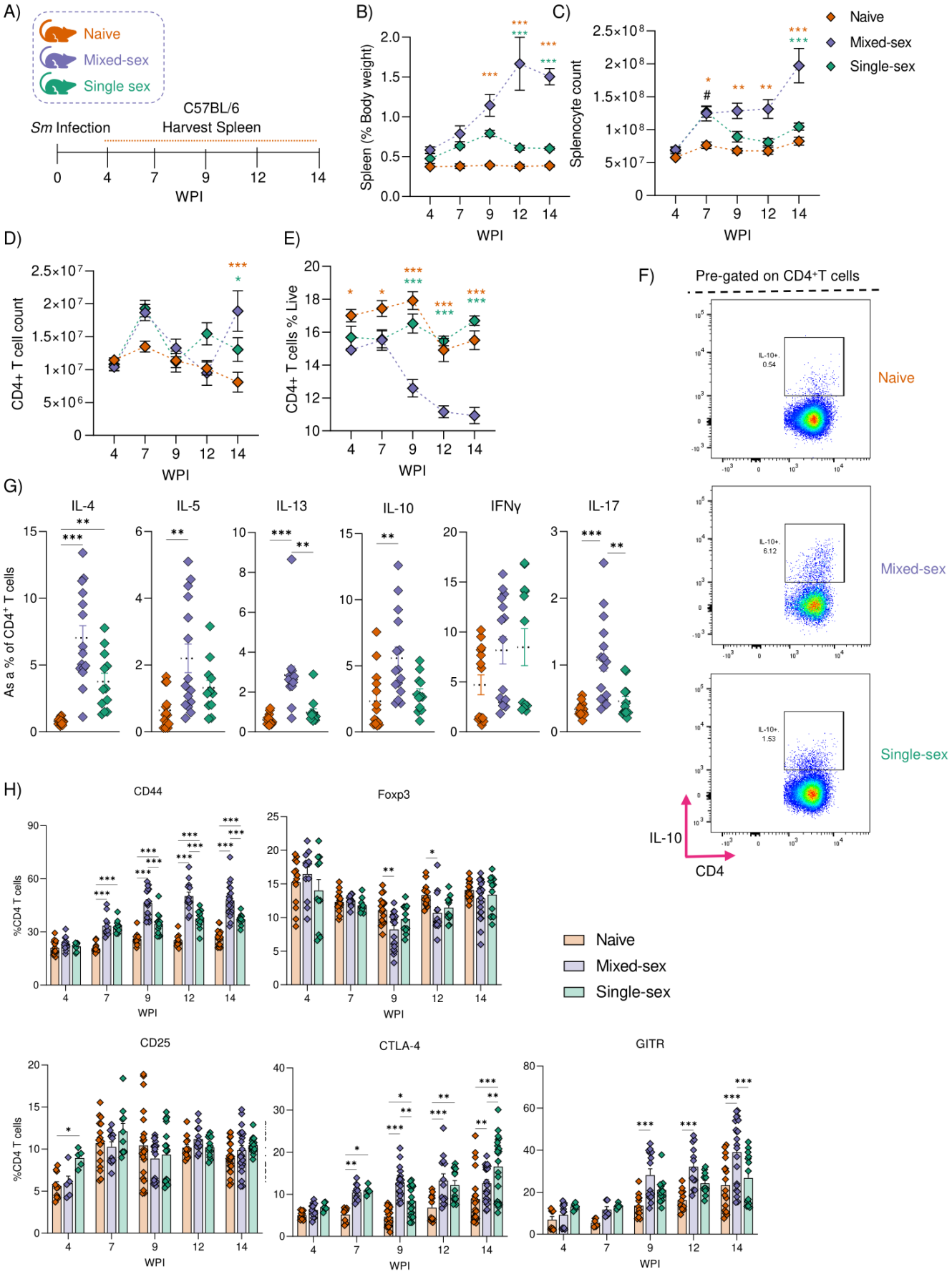
In attempt to define the schistosome-derived signals that support Treg induction, we monitored splenic Treg frequency in mice conventionally infected with 40 male and female schistosome cercariae (mixed-sex model), or male cercariae alone (single-sex model) in which no eggs are produced, with infections lasting 4, 7, 9, 12 or 14 weeks in duration (Figure 1A). Firstly, looking at broad splenic responses, only conventional mixed-sex infections were associated with splenomegaly, as defined as the increase in spleen weight relative to overall body weight (Figure 1B). These increases began at 9 weeks post infection, persisted into chronicity, and enlarged overtime. Expectedly, these increases in spleen size translated into enhanced splenocyte numbers (Figure 1C) and equivocal CD4+ T cell counts between groups (Figure 1D), despite a decrease in total $CD4^+$ T cell proportions in mixed-sex infected mice (Figure 1E). Focusing on week 14, a time-point at which Treg numbers are known to expand during schistosomiasis[20,22,23], only mixed-sex infected mice were typified by greater expression of T cell IL-10 following *ex vivo* PMA/iono stimulation (Figure 1F&G). Mixed-sex infected $CD4^+$ T cells also expressed greater levels of IL-17 and Th2 associated cytokines, IL-5 and IL-13 than naïve or single-sex infected mice in response to PMA/iono (Figure 1G). Whilst IL-4 expression was upregulated in both single-sex and mixed-sex CD4+ T cells (Figure 1G).

We next tested the frequencies of different Treg populations over the course of single-sex and mixed-sex schistosomiasis (Figure 1H). The activation and Treg associated marker CD44[72] was significantly enhanced in infected mice groups from week 7 onwards, with the more prominent upregulation in egg producing infections from week 9. Corroborating previous reports showing little evidence for Foxp3 induction during acute or chronic schistosomiasis[20], Foxp3 expression remained consistent across all groups, with the exception of weeks 9 and 12 in which a decrease in $Foxp3^+CD4^+$T cells was observed in single-sex and mixed-sex mice groups. Very interestingly, despite multiple studies showing $CD25^+CD4^+$ T cells to play important roles in the regulation of schistosomiasis immunopathology[23,25], this does not necessarily seem to coincide with an increase

in their frequency here. In line with a previously reported role for CTLA-4$^+$ T cell populations in the regulation of schistosomiasis pathology[20] we observed a marked increase in CTLA-4 expression in both single and mixed-sex infections. Interestingly, at week 14 CTLA-4 was more prominently expressed on single-sex CD4$^+$ T cells

than those from mixed-sex mice. Finally, upregulation of GITR was limited to mixed-sex infected mice, with significant up-regulation observed in mixed-sex infected mice from week 9 when compared to naïve controls or mice infected with male cercariae only.

Figure 1. Definition of splenic Treg networks during schistosomiasis. (A) C57BL/6 WT mice were infected with ~40 male and female S. mansoni cercariae (mixed-sex infection) or ~40 male S. mansoni cercariae (single-sex infection). Spleens were harvested from naïve or infected mice at weeks 4, 7, 9 12 or 14 of infection. (B) Spleen weights as a proportion of total body weight. (B) The total number of leukocytes recovered from the spleens of naïve or infected mice. (C) CD4+ T cells as a % of total liver cells. (D) Total numbers of CD4 T cells. (F) Representative flow plots for IL-10 secretion, pre-gating on live CD45+TCRβ+CD4+ cells. (G) Cytokine secretion from PMA ionomycin stimulated splenocytes cells at week 14 of infection. (H) The proportion of CD44+, Foxp3+,CD25+,CTLA-4+ and GITR+ CD4+ T cells across the course of infection, presented as a % of total CD4+ T cells. Results are mean +/- SEM from two (F&G) or three (B,C,D,E&H) experiments pooled (n=12-24 mice per group per time-point). Significant differences were determined by one-way (G) or two-way ANOVA (B,C,D,E&H) followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B-E) Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively. Significant differences between single-sex and naïve mice are indicated by #


A)
Naive
Mixed-sex
Single sex
C57BL/6
Sm Infection
Harvest Spleen
0
4
7
9
12
14
WPI
B)
Spleen (% Body weight)
C)
Splenocyte count
Naive
Mixed-sex
Single-sex
D)
CD4+ T cell count
E)
CD4+ T cells % Live
F)
Pre-gated on CD4+T cells
IL-10+
0.54
6.12
1.53
Naive
Mixed-sex
Single-sex
IL-10
CD4
G)
IL-4
IL-5
IL-13
IL-10
IFNγ
IL-17
As a % of CD4+ T cells
H)
CD44
Foxp3
%CD4 T cells
Naive
Mixed-sex
Single-sex
CD25
CTLA-4
GITR

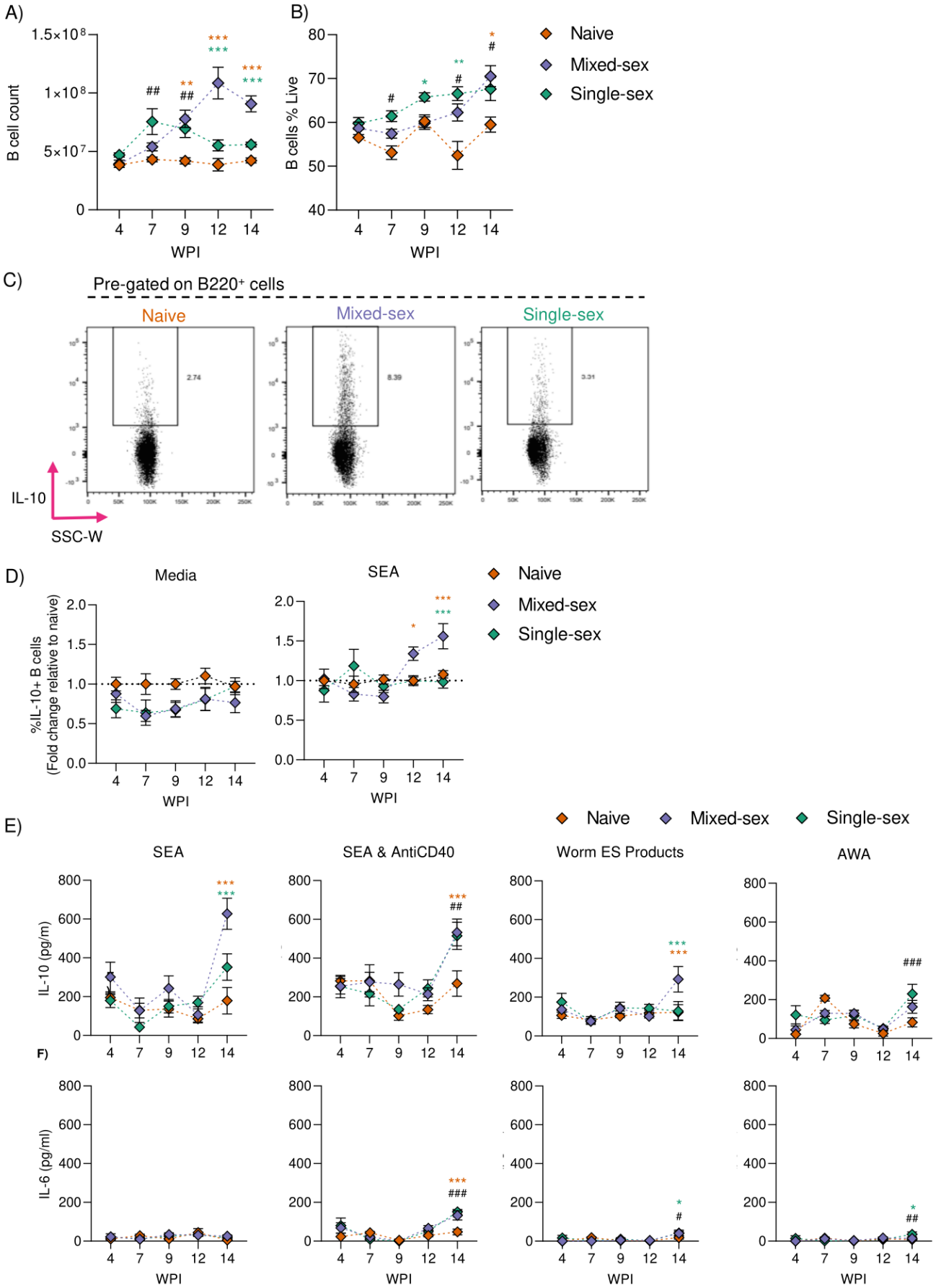
Regulatory B cell expansion during patent and non-patent infection

Several studies demonstrate an expansion of splenic IL-10 producing Bregs during chronic stages of murine schistosomiasis[12,23,31,32,35] with evidence as well for Breg induction in infections with male worms alone[35], but this was in different mouse strains (BALB/C vs C57BL/6). Here, we monitored Breg frequency across the course of conventional mixed-sex or single-sex infections in C57BL/6 mice, allowing better comparisons. Coinciding with enhanced splenomegaly (Figure 1B) and splenocyte numbers (Figure 1C), splenic B cell counts were increased in mixed-sex infected mice during chronic weeks of infection in comparison to single-sex infected mice and uninfected controls (Figure 2A). However, when looking at relative B cell proportions, their frequency remained comparable to naïve and mixed-sex infected mice until week 12 of infection, whereby enhanced B cell frequency was observed in mixed-sex mice (Figure 2C). On the contrary, the proportion of B cells in single-sex infected mice remained significantly elevated in single-sex infected mice from week 9 onwards in comparison to both naïve and mixed-sex infected groups.

To assess the propensity of B cells to produce IL-10, splenic B cells were isolated across the course of infection and stimulated for 72 hrs in the presence of schistosome egg antigen (SEA) and analysed for intracellular IL-10 by flow cytometry (Figure 2C&D). Unlike their single-sex or naïve counterparts, B cells from mixed-sex infected mice demonstrated a startling increase in IL-10 production at week 14 post infection, with evidence for enhanced IL-10 production in comparison to naïve B cells at week 12. To corroborate these findings and further define which parasite derived molecules or stimulation conditions may promote B cell IL-10, or oppositely the more pro-inflammatory IL-6, we analysed the supernatants of 3 day cultured B cells, whom had been exposed to SEA with or without anti-CD40, worm extracellular secretions (ES) or adult worm antigens (AWA) (Figure 2E). In response to SEA and worm ES products, the pattern for IL-10 by ELISA was near identical to that observed by flow cytometry, with mixed-sex B cells significantly increasing IL-10 production at week 14. However, upon stimulation with SEA and antiCD40, B cells from single-sex infected mice demonstrated the same propensity to produce IL-10 as mixed-sex B cells, though with the important exception that anti-CD40 + SEA also induce IL-6 production in B cells as compared SEA alone, which did not induce IL-6 production in B cells. For AWA only single-sex B cells showed heightened IL-10 and IL-6 secretion at week 14, but only half of amounts detected with SEA or SEA + anti-CD40. Together, these data highlight the presence of Breg cell differentiation in both worm only and egg-producing infections though the potency of the Breg cells – at least in terms of IL-10 production – seems less in worm only-infected mice. Furthermore, given the large increase in spleen size (Figure

1B) and B cell numbers (Figure 2A) in mixed-sex infections at week 14, one can infer that the total levels of IL-10 during conventional egg producing infections to be considerably greater than that of naïve or single-sex infected mice. In this stage, it is unclear why B cells from mixed-sex infected mice respond differently to worm-derived products (worm ES and/or AWA) as compared to single-sex infected mice, as they both have been exposed to adult worms throughout the infection. It cannot be excluded that separate Breg populations have developed in mixed-sex infected mice that primarily respond to eggs, showing the much stronger response there. Together, these data highlight the induction of Breg cell populations in both single-sex and mixed-sex infections to evoke a Breg phenotype and elucidate differences in IL-10 secretion in response to either worm or egg-derived signals in the presence or absence of costimulatory pathways.

Figure 2. Signal specific expansion of IL-10 producing B cells during S. mansoni infections. The total number (A) and proportion (B) of CD19+ B cells in the spleens of naïve, mixed-sex and single-sex infected mice. (C-E) CD19+ B cells were isolated from the spleens of naïve and infected mice and stimulated in vitro for 3 days with SEA (20 µg/mL), anti-CD40(0.5µg/mL), adult worm antigen (AWA) (20 µg/mL) and worm extracellular secretory (ES) products (20 µg/mL). (C) Representative flow plots for IL-10 secretion in isolated B cells at week 14 of infection. (D) Percentage of IL-10+ B cells as assessed by flow cytometry and presented as fold change relative to naïve. SEA and media conditions only. (E) B cell supernatants were analysed for IL-10 and IL-6 concentrations by ELISA. Data are from one single experiment (E&F; AWA and worm ES products), or from two (E&F) or three (A-D) experiments pooled (n= 6-24 per time point). Data are presented as mean+/-SEM. Significant differences are indicated by *p<0.05, **p<0.01, ***p<0.001 and determined by two-way ANOVA followed by Tukey's post hoc test. Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively. Significant differences between single-sex and naïve mice are indicated by #.


A)
B)
B cell count
B cells % Live
WPI
Naive
Mixed-sex
Single-sex
C)
Pre-gated on B220⁺ cells
Naive
Mixed-sex
Single-sex
IL-10
SSC-W
D)
Media
SEA
%IL-10+ B cells
(Fold change relative to naive)
E)
SEA
SEA & AntiCD40
Worm ES Products
AWA
IL-10 (pg/m)
IL-6 (pg/ml)
F)

Signature B cell phenotypes in single-sex and mixed-sex infections

After having established that splenic B cells from schistosome infected mice have a greater propensity to produce IL-10, our attention turned to phenotyping the B cell subsets responsible. A variety of Breg subsets have been described in mice and men, with their exact phenotypes and immunosuppressive activities differing depending on the inflammatory threat encountered[37,73]. In previous studies, we demonstrated that MZ B cells**,** as opposed to FO B cells, are responsible for the majority of B cell IL-10 during chronic infection stages[11]. To accommodate for the wider range of Breg populations described[37,43,73], we broadened our gating scheme allowing for the demarcation of Transitional type 1 (T1), Type 2 (T2) and transitional type 2 marginal zone precursors (T2-MZPs) (Supplementary Figure 1 A&B).

Schistosome infections dramatically altered the splenic B cell profiles, as visualised by and representative flow plots (Supplementary Figure 2A) and summarised in statistical tables (Supplementary Figure 2B). Whilst FO and T1 proportions were fairly comparable between infection groups and time-points, we observed dramatic enrichment for T2 and T2_MZP B cell subsets within mixed-sex infected mice, with single-sex infections also accompanied by increased T2 frequency at weeks 7, 9 and 14 (Supplementary Figure 1C). These increases coincided with an overall reduction in MZ_MZP and MZ frequency within infected mice groups. CD1d and CD5 are commonly used to capture the murine B Cell IL-10 compartment[6,37,73,74]. Proportionally, CD5 expression was shown significantly higher on mixed-sex and single-sex T2 B cells than naïve T2 B cells, with no discernible differences in expression amongst other B cell subsets (Supplementary Figure 1D). Mixed-sex T2_MZP B cells expressed higher levels of $CD1d^{+}$ than naïve or single-sex T2_MZPs.

Although attempts were made to evaluate the IL-10 production and Treg induction capacity of individual B cell subsets, technical issues precluded firm conclusions to be drawn (Data not shown).

Evidence for microbial instructed IL-10 production and regulation of splenic Treg populations

The obvious explanation behind the differential Breg and Treg signatures in single-sex and mixed-sex infections is the presence or absence of eggs, their complex mixture of Ags and the vigorous inflammation and damage that accompanies their tissue penetration and entrapment. In addition, our group previously reported reduced barrier integrity during patent schistosomiasis with evidence for enhanced bacterial translocation (*Costain et al., manuscript in preparation*). As such we reasoned that regulatory cell expansion may (additionally) be supported by intestinally-derived signals, that leak into systemic circulation as a result of egg transit. ^{1}H nuclear magnetic resonance (NMR) spectroscopy–based metabolic profiling was performed on the sera of infected mice groups

to evaluate the relative metabolic profiles (Figure 3). Several metabolites were shown to differ amongst groups, including higher amounts of the bacteria related product Trimethylamine (TMA) in chronically infected mixed-sex mice. Although other experimental helminth infections were accompanied by greater levels of short-chain fatty acids (SCFA; e.g acetate, butyrate and propionate)[62,75], we observed no differences in butyrate levels between infection groups throughout the infection period. Several amino acids were decreased in the circulation of infected mice, with both mixed-sex and single-sex infected mice showing decreased levels of isoleucine, leucine and valine levels between weeks 9 and 12.

Next, given the considerable differences between mixed-sex and single-sex intestinal microbiotas and metabolomes (*Costain et al., manuscript in preparation*), we next aimed to directly assess the

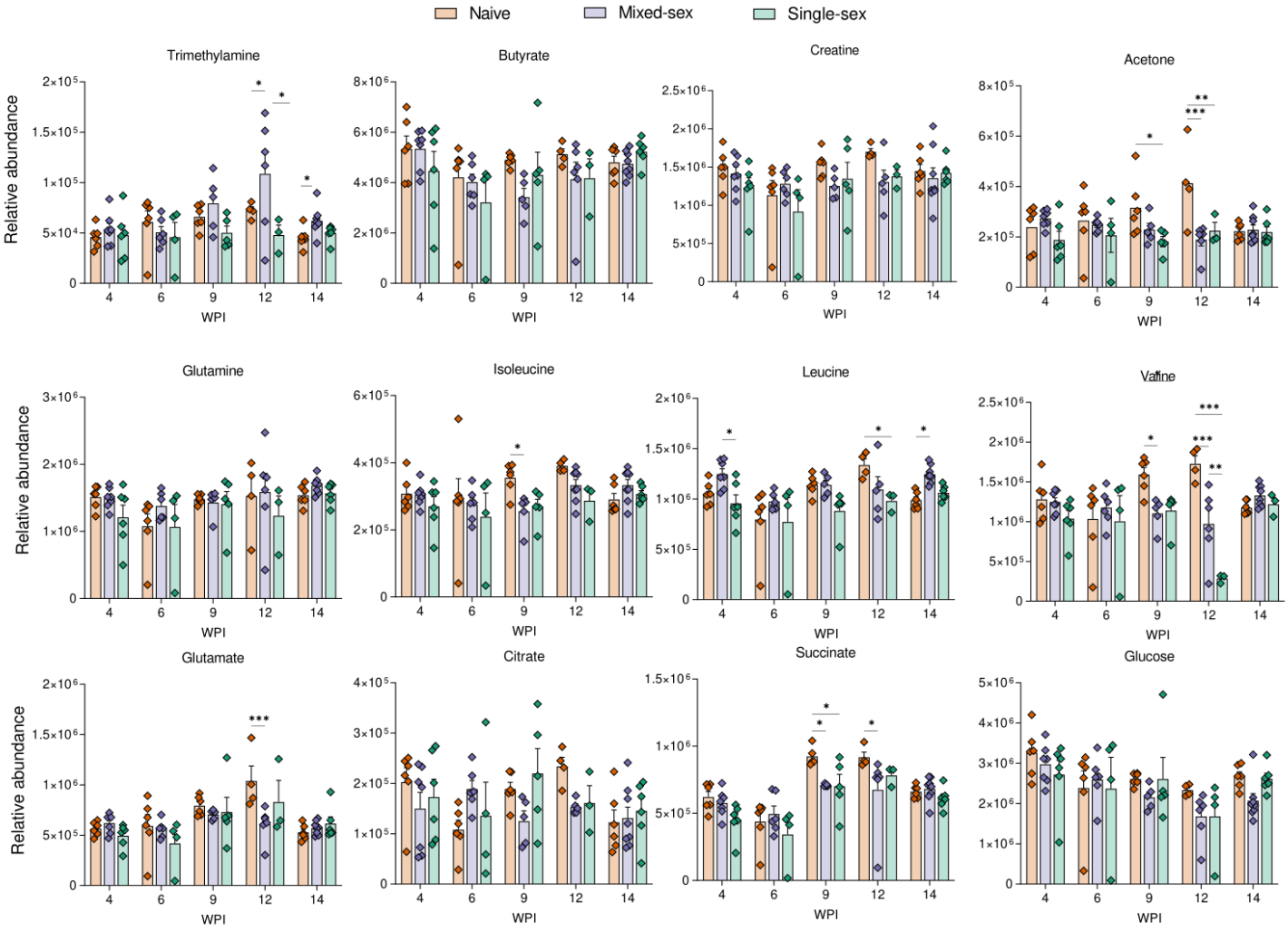


Figure 3. Serum metabolite profiles across infection. Relative abundance of metabolites in the serum of naïve and schistosome infected mice. Results are mean +/- SEM from one single experiment (n= 5-8). Significant differences were determined individual two-way ANOVAs followed by Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.

capacity of their associated microbiotas to support the production of T cell and B cell IL-10. Antibiotic-treated Vert-X mice (express GFP under control of the IL-10 promotor) were recolonised for 3 weeks with naïve or chronically infected schistosome-associated microbiotas (Figure 4). Although deemed statistically insignificant, splenic B cells from mixed-sex and single-sex microbiota recipients had an increased tendency to produce IL-10 compared to those from naïve microbiota recipients (Figure 4A). Within the mesenteric lymph nodes (MLNs), B cells and T cells from single-sex microbiota recipients also displayed an enhanced tendency to produce IL-10 compared to other microbiota transfer groups (Figure 4B). No Considerable differences were shown for B cell or T Cell phenotypes otherwise (Data not shown).

To further assess whether microbially derived signals may contribute the generation of schistosome induced regulatory networks GF mice were infected with egg producing schistosomes, and splenic

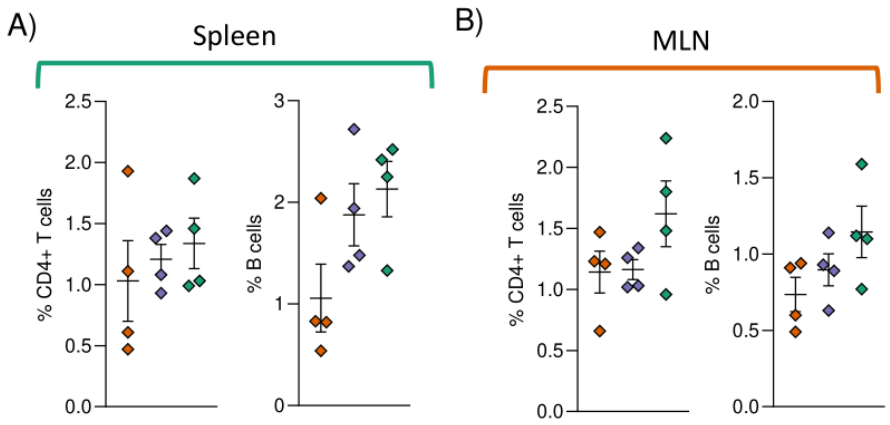


Figure 4. Receipt of schistosome microbiota promotes splenic and mesenteric IL-10. Abx-treated Vertx mice (IL-10 reporters) received two faecal transplants from naïve mice, or 14-week infected mixed-sex or single-sex mice. After 3 weeks recolonisation (A) splenic and (B) mesenteric CD4+T Cell and B cells were analysed for IL-10 expression. Data are from one single experiment (n=4). Data are presented as mean+/-SEM. Significant differences are indicated by *p<0.05, **p<0.01, ***p<0.001 and determined by one-way ANOVA.

CD4+ T cell phenotypes and cytokine secretion was analysed after 7 weeks. $CD4^+$ T cell proportions (Supplementary Figure 3A) and their expression of Th2 (Gata-3), Th17 (Roryt) and Th1 (T-bet) associated transcription factors remained unaltered between SPF and GF mice (Supplementary Figure 3B). However, we observed a significant increase in the expression of CD25 and CTLA-4 in GF infected mice in comparison to their SPF counterparts, with a slight tendency towards heightened Foxp3. Likewise, the potential for IL-10 production was heightened in GF schistosome infected mice, as was IFNγ (Supplementary Figure 3C). Analysis of B cell profiles was not conducted here due the 7 week time point of infection, thus closer inspection at chronic disease is needed. Together, these

data provide a convincing argument for microbial involvement in schistosome-elicited immune responses. Knowing the capacity of schistosomes to alleviate allergic inflammation[9–12,35], these data also provide strong rationale to investigate microbial participation here.

Faeces transfer from schistosome-infected mice modulates the severity off allergic airway inflammation

Several studies have shown helminth-associated microbiotas and experimental schistosome infections to attenuate allergic airway disease[9,11,12,35,76]. To determine whether intestinal bacteria from schistosome infected mice could also influence AAI, we transferred faeces from naïve, single-sex or mixed-sex infected mice to antibiotic-treated SPF mice, and after a 3 week colonisation period, challenged them to house dust mite (HDM) intranasally (Figure 5A). Transfer of both mixed-sex and single-sex schistosome infected microbiotas lessened allergic airway inflammation as evidenced by a reduction in cellular infiltration and a trend towards less eosinophils in the bronchial alveolar lavage (BAL) fluid in comparison to HDM allergic control mice (treated with PBS) (Figure 5B). On closer interrogation of differential cell counts, mice recolonised with single sex faeces showed a stronger reduction in the total number of neutrophils, $CD4^+$ T cells, $CD8^+$ T cells and monocytes in the BAL fluid as compared to mice recolonised with mixed-sex faeces. Interestingly, evaluating inflammatory cells in the lung tissue, revealed that recipients of mixed-sex of single-sex microbiotas were typified by a general increase in total lung $CD45^+$ cell counts, with a significant increase in total CD4+ T cells and a trend towards heightened B cell numbers while other cell types remained unchanged or similar across all HDM-sensitized and challenged mice (Figure 5C). Lung CD4+ T cells from HDM challenged mice did not produce elevated Th2 associated cytokines in response to PMA/iono restimulation in comparison to non HDM controls, despite elevated BAL-eosinophilia. However, as we did not evaluate HDM-specific T cell responses, it might be speculated that putative differences related to Th2 cytokines will be mostly found in the Ag-specific T cell responses (Figure 6A). Interestingly, $CD4^+$ T cells from single-sex and mixed-sex faeces recipient mice showed a trend towards greater IL-10 production in response to PMA/iono stimulation, and $CD4^+$ and $CD8^+$ T cells from mixed-sex faeces recipients showed significantly greater expression of Th1 associated IFNγ, but not IL-17.

HDM-treatment altered the ratio of alveolar to interstitial macrophages, with the proportion of interstitial macrophages significantly increasing in the lungs of HDM-treated mice (Figure 6B), while feacal transplant was partially reversing this phenomenon. Coinciding with the greater IFNγ propensity of CD4+ T cells from mixed-sex microbiota recipients (Figure 6A), alveolar and interstitial

macrophages from mice receiving mixed-sex schistosome faeces were typified by a shift towards classical 'M1'-like macrophage signature (Figure 6C), such as less of the Th2-associated mediator RELMα, and more of MHC-II (alveolar MF) and less of the M2-marker mannose receptor CD206 (interstitial MF). HDM treatment evoked a large alteration in pulmonary CD4+ T cell activation ($CD44^{+}CD62L^{-}$) status and transcription factor expression (Figure 6D). While no differences in lung CD4+ T cell activation or expression of Foxp3, Gata-3 or T-bet were found between HDM treated groups, we observed a trend towards heightened Roryt and CTLA-4 expression in mice receiving schistosome infected faeces (Figure 6D). Finally, HDM-treatment did not evoke significant systemic inflammation, as evidenced by equivocal splenic cell counts across all treatment groups (Supplementary Figure 4A). However, the infiltration of splenic CD4+ T cells (Supplementary Figure 4B), and their expression of Foxp3 (Supplementary Figure 4C) was significantly increased upon single-sex and mixed-sex microbiota transfer. Curiously, upregulation of CTLA-4 was exclusive to splenic CD4+ T cells from single-sex microbiota recipients.

Collectively, these data show the potential of a schistosome associated microbiota, from both egg-producing and non-egg producing chronic infections, to modulate the severity of allergic airway disease. These data hint that schistosomes not only modulate host immunity directly but may also act through changes in gut microbial composition.

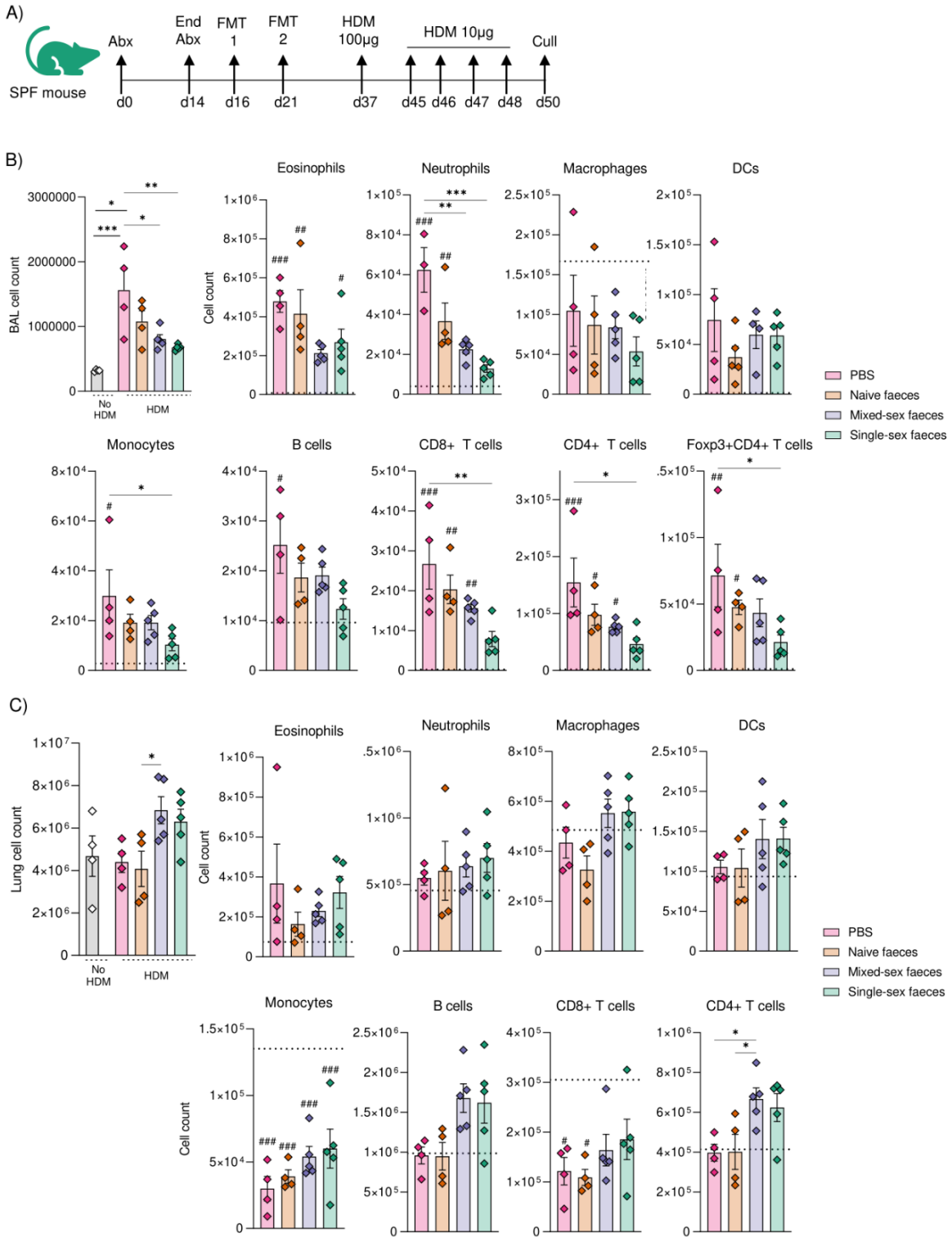


Figure 5. Schistosome-associated microbiota modulates the severity of HDM-evoked allergic airway inflammation. (A) C57BL/6 SPF mice were treated with broad-stream antibiotics (Abx) in drinking water for 2 weeks, followed by two oral gavages with sterile PBS or faeces. Gavages were performed two days apart, with faeces collected from naïve mice or 14 week infected mixed-sex or single-sex infected mice. After a 3 week recolonisation period, mice were subjected to HDM challenge and sensitisation. Mice were sacrificed 2 days after the final HDM challenge, and the severity of airway inflammation was evaluated. Mice not subjected to HDM treatment were gavaged with sterile PBS. Abx; Vancomycin (0.5mg/ml), Ampicillin (1mg/ml), Neomycin (1mg/ml), Metronidazole (1mg/ml) and Gentamycin (1mg/ml). Differential cell counts in the (B) Bronchiolar lavage fluid (BAL) or (C) lung. Dotted line represents mean cell count in no HDM control group. Results are mean +/- SEM from one single experiment (n=4-5 mice per group). Significant differences were determined by one-way ANOVA followed by suitable Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Significant differences between HDM treated and non HDM treated mice are indicated by #.

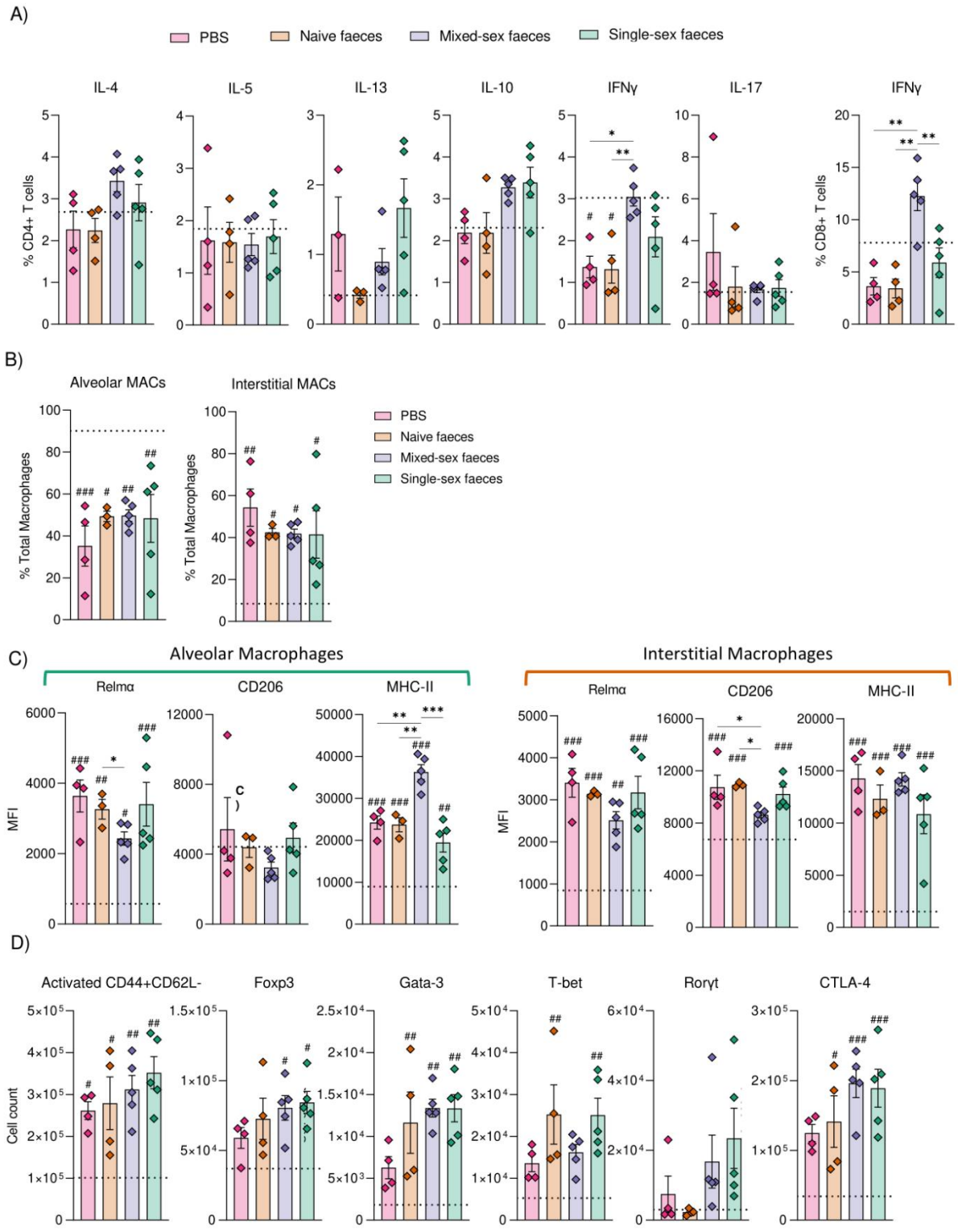


Figure 6. Faeces from schistosome infected mice imprints distinct T cell and macrophage phenotypes in allergic mice. (A) Cytokine secretion from PMA ionomycin stimulated lung cells. (B) The proportion of alveolar and interstitial macrophages as a % of total lung macrophages. (C) The geometric mean fluorescence intensity of RELMα, CD206 and MHC-II on the surface of alveolar and interstitial macrophages. (D) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors and IL-2R (CTLA-4) of pulmonary CD4+ T cells. Dotted line represents mean values in the no HDM control group. Data presented as mean +/- SEM. Data shown are from 1 single experiment (n=3-4 mice per group) Significant differences were determined by one-way ANOVA with Tukey's post hoc testing. *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences between HDM treated and non HDM treated mice are indicated by #.

Helminths tactfully tamper with host immunological processes through the direct actions of their immunogenic secretions or by driving dysbiosis of the host microbiota[1,62,76]. The potential for schistosome infections to inhibit inflammatory disease is well documented[1,12,31–33,54,77–79], with both experimental[6,9,10,12,35] and epidemiological studies[6] advocating a role for regulatory cells in the control of airway inflammation. Here we reveal the capacity of a schistosome associated microbiota to modulate the severity of AAI (Figures 5&6), with evidence for microbial support in schistosome-associated splenic Breg expansion (Figure 4) and Treg signatures (Supplementary Figure 3).

Infections with male and female egg producing schistosomes or male schistosomes alone evoked the expansion of distinct splenic regulatory networks (Figure 1 & Supplementary 1 &2). Breg generation is nourished by inflammatory environments including the presence apoptotic cells[80], inflammatory cytokines such as Type I IFNs[44], IL-1β[58]and IL-6[58], and receptor ligation with pathogen specific ligands[52,81]. Herein and supporting previous studies[35], we show worm only infections in the absence of egg-evoked inflammation to endorse B cell IL-10 production (Figure 2), further highlighting the known regulatory potential of the worm secretome[68]. However, the potential seems less as compared to patent infection, with less IL-10 secretion, at least with the ex vivo stimulations used here. In addition, as we did not identify the exact B cell subset responsible for B cell IL-10 in mixed-sex and single-sex infections, it is possible that differing B cell subsets harbour Breg capacities in each infection, and that different compounds/ products trigger their functionality and IL-10 production[82]

B cells of mixed-sex and single-sex mice were shown phenotypically unique, with notable expansion of T2_MZP B cells in mixed-sex infected mice only (Supplementary Figures 1 &2). The expansion of T2_MZPs is of considerable interest given their anti-inflammatory and IL-10 production capacities and knowing that microbially-derived components[50,83] and -stimulated cytokines endorse their functionality[58]. For schistosomiasis, a helminth infection accompanied with compromised barrier integrity and evidence of dissemination of luminal products (*Costain et al., Manuscript in preparation*)[84] it is exciting to consider a schistosome-specific microbial element supports T2_MZP proliferation and IL-10 production. Although, we should also consider the involvement of female worm-derived products on Breg generation, knowing that female only infection promote a more immune dampening environment than males[85] . Although we did not address the B cell subset responsible for the majority of IL-10 here, these data highlight the capacity of schistosome-elicited

environments to endorse Breg phenotypes and warrant further investigation into the microbial assistance in their generation.

We demonstrate that *S. mansoni* infections are accompanied by the expansion of splenic $CD4^+$ cells expressing CD44, GITR and CTLA-4, but not CD25 or Foxp3 (Figure 1). The lack of Foxp3 expression corroborates previous work[20] and fits with the narrative that most $CD4^+$ T cell derived IL-10 comes from a non Foxp3+ source[70]. The unaltered expression of CD25 is somewhat surprising given previous reports showing enhanced CD25 expression during both experimental and natural infections[20,21,23,86]. However, given that CD25 has proven unessential in the regulation of murine schistosomiasis [20], it makes sense that we do not see enhanced enumeration of these cell types. CTLA-4 is a crucial negative regulator of T cell activation[18] that is upregulated during *S.mansoni* infection, regulates Type 2 cytokine production and immune cell recruitment[20], and whose gene polymorphisms are suspected to influence schistosomiasis susceptibility[87]. Indeed, patients with severe schistosomiasis fibrosis have lower frequencies of CTLA-4^+ T cells than fibrosis free patients[88]. Our data suggests a role for worm-derived signals and microbial elements in the regulation of CTLA-4, with enhanced expression on splenic $CD4^+$ T cells of single-sex infected mice (Figure 1), albeit we did not observe an increase in CTLA-4 expression on antibiotic-treated Vertx mice recolonised with single-sex faeces (Data not shown). These observations support previous work showing the adult worm Ag PIII to increase the percentage of human $CD4^+$ T-cells expressing CTLA-4[89] and suggests that adult worms instruct CTLA-4 expression as to suppress anticipated egg driven inflammation. Curiously, with the exception of IFNγ, only regulatory associated markers CTLA-4, CD25 and IL-10 were affected by the absence of an intestinal microbiota in GF infections (Supplementary Figure 3), hinting for a key role for the bacteria in modulating regulatory cell networks during schistosomiasis.

The capacity of a schistosome associated microbiota to modulate disease severity has been reported in experimental colitis[54]. Floudas *et al* report an alleviating effect of microbiotas from male only infected mice on colitis progression, whereas conventional dual-sex microbiotas evoked a 'colitogenic' inflammatory profile. The presence of a more 'colitogenic' microbiota in conventional egg-producing infections supports our observation of an M1 phenotype in mixed-sex faecal transfer recipients (Figure 6). However, as these researchers achieved faecal transplant by means of co-housing with schistosome-infected mice for 3 weeks, and microbiota structure is known to alter considerably in this length of time (*Costain et al., Manuscript in Preparation*) we cannot assert whether faecal delivery via oral gavage (as conducted in this study), or faeces uniquely from infection chronicity, would have the same colitogenic effect. Moreover, although out lab recently

reported on the microbial structure of male only and mixed-gender schistosome infections (*Costain et al., Manuscript in preparation*), we did not inspect the microbial composition of faecal transplant recipients here. As many microbial species are lost and are unable to re-establish during the faecal transplant process[90] we are not able to comment on their inflammatory status. Thus, future studies teasing apart the bacterial elements involved in the regulation of inflammation would be of great interest.

The airway macrophages of HDM-treated mixed-sex microbiota recipients, were skewed towards a slight M1 profile (Figure 6). Although too early to draw firm conclusions, this data suggests the presence of an M1 aggravating component within the faeces of mixed-sex infected mice, that promotes an inappropriate inflammatory profile response following allergen exposure. Given the strong Th2 polarising nature of schistosome eggs and their Ags[91,92], we speculate that the polarising agent responsible is likely of microbial origin. However, it is worth noting that schistosome eggs contain a homologue of high-mobility group box 1 (HMGB1) protein, which is a potent stimulator of M1 macrophages[93]. As dietary supplementation with SCFAs has shown to restore macrophage homeostasis following antibiotic treatment and microbiota rexposure[66], it is also possible that the aggravation of an M1 phenotype upon mixed-sex microbiota transfer reflects an absence of certain metabolites or lack of bacterial species that generate them. Moreover, it is conceivable that the consortium of microbes found within a mixed-sex schistosome-associated microbiota are less capable of supporting the regrowth of SCFA-generating bacterial species, that are depleted using this same antibiotic regime[66]. Additionally, while mice receiving mixed-microbiotas showed a general reduction in airway inflammation in response to HDM in comparison to uncolonized mice or those recolonised with naïve faeces, it remains unclear whether the instruction of an M1 phenotype contributes to this phenotype.

Microbial derived components have a well-established role in the calibration of systemic and local immunity[94], with SCFAs (e.g acetate, butyrate and propionate) commonly commended for their immunomodulatory and anti-inflammatory capacities[50,62,76,95]. While our analysis did not detect differences in serum butyrate levels between infected and naïve mice, the pro-inflammatory bacterial-derived metabolite TMA was shown significantly higher in the serum of mice chronically infected with egg producing schistosomes (Figure 3), in support of previous metabonomic studies[96,97]. TMA is microbial derived metabolite, produced from the breakdown of dietary choline and carnitine, and delivered to the liver for conversion into trimethylamine N-oxide (TMAO)[98]. While TMAO is known to promote atherosclerosis, cardiovascular disease, and inflammation[98], the

influence of TMAO metabolism on helminth infection and disease regulation has not been established. Given that TMAO levels are increased in inflammatory bowel disease patients[99], it is possible that enhanced circulating TMAO levels simply reflects the pronounced egg-driven mucosal inflammation. In addition, given that TMA is known to influence macrophage activation[100], and promote platelet activation and thrombosis, it is conceivable that TMA might influence the severity of granulomatous inflammation or promote the attachment and diapedesis of freshly deposited schistosome eggs across the endothelim[101]. With a ubiquitous range of bacterial taxa responsible for TMA production[102], it is currently unknown whether schistosomes actively support the generation of TMA-producing bacteria.

Corroborating our metabolomic analysis (Figure 3), previous metabolic phenotyping during schistosomiasis has revealed disturbances in amino acids, metabolites related to energy (glycolysis, TCA cycle), inflammation and microbial function, but it is unclear how these metabolic profiles arise. Both mixed-sex and single-sex schistosome infections are known to alter microbiota structure[54] (*Costain et al., manuscript in preparation*), indicating that schistosome metabolic profiles are driven by shifts in bacterial colonisation or function. In addition, schistosomes use host-derived metabolites for their own growth and development[103], and metabolic alterations could be indicative of compromised liver function[96]. Finally, similar to that described for *H. polygyrus*[75], it is exciting to consider that schistosomes actively modulate host metabolite profiles as to support specific immune profiles, sustain their own longevity or downmodulate bystander inflammation. Indeed, transfer of schistosome-associated microbiota into Abx recipients suggests for greater IL-10 capacity (Figure 4) and modulation of airway disease (Figures 5&6), but the microbial or metabolic mediators involved required further inspection.

In summary we demonstrate egg producing and non-egg producing schistosome infections to prompt the emergence of distinct splenic regulatory populations, with evidence that schistosomes associated microbiotas promote their instruction and assist in the modulation of unrelated inflammation. Closer interrogation of schistosome microbial crosstalk will likely unravel new and exciting molecules and pathways for the targeting of allergic disease and expansion of regulatory cell types.

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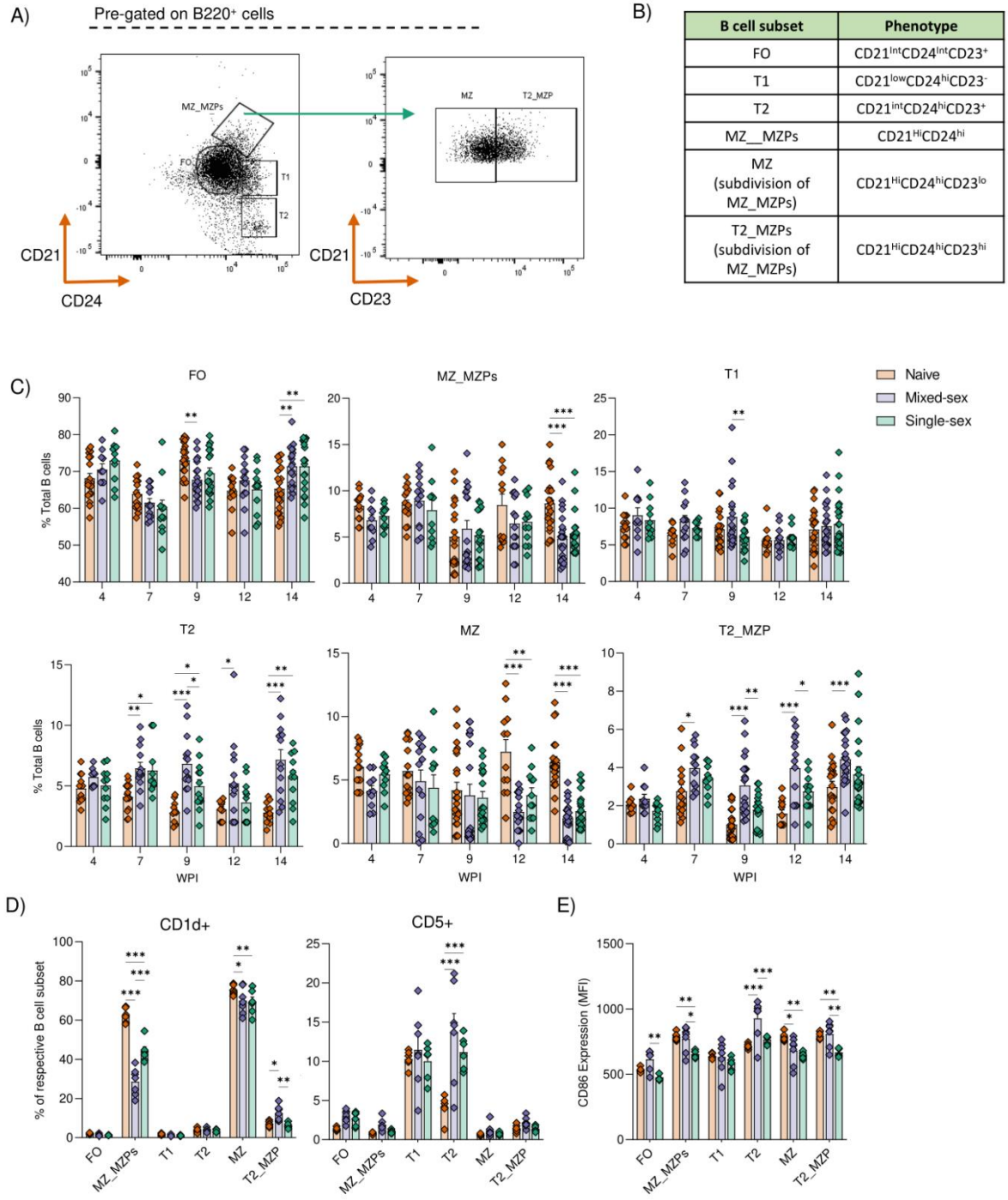
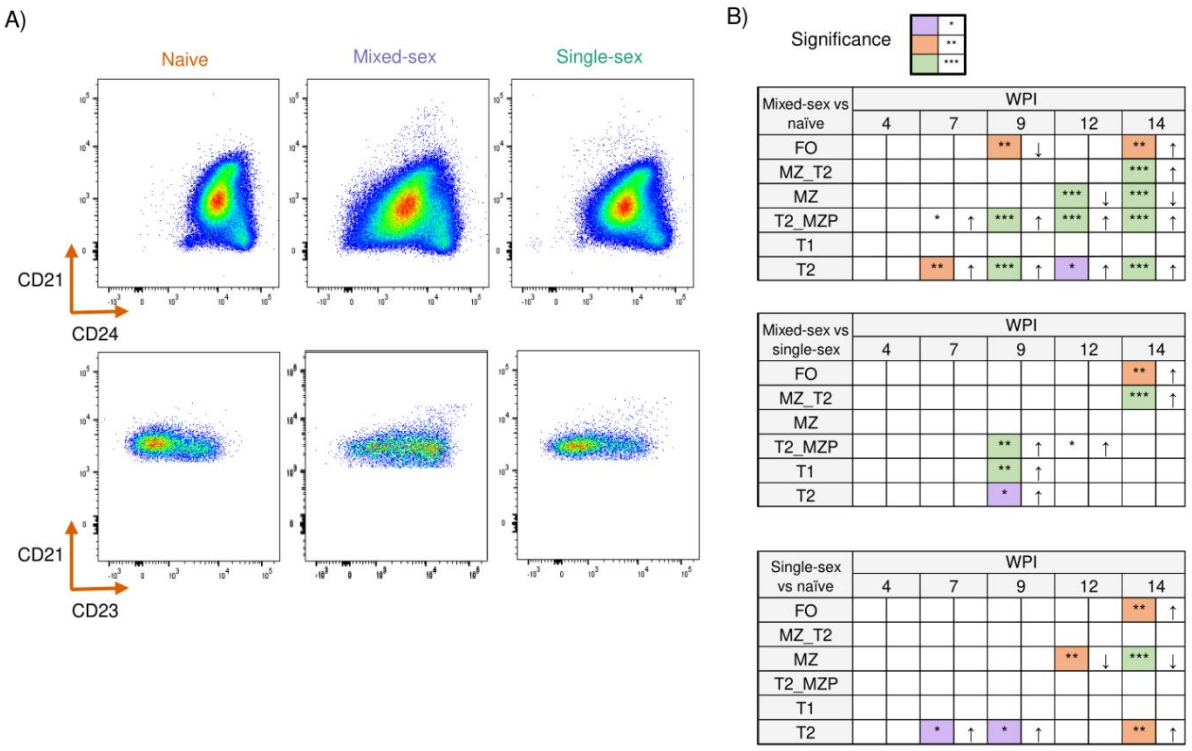


B cell subset	Phenotype
FO	$CD21^{Int}CD24^{Int}CD23^{+}$
T1	$CD21^{low}CD24^{hi}CD23^{-}$
T2	$CD21^{Int}CD24^{hi}CD23^{+}$
MZ__MZPs	$CD21^{Hi}CD24^{hi}$
MZ (subdivision of MZ_MZPs)	$CD21^{Hi}CD24^{hi}CD23^{lo}$
T2_MZPs (subdivision of MZ_MZPs)	$CD21^{Hi}CD24^{hi}CD23^{hi}$

Supplementary Figure 1. S. mansoni infections elicit distinct splenic B cell profiles. (A) Representative FACs plot of B cell gating. CD19+ B cells were subsetted into Follicular (FO; CD21IntCD24IntCD23), Marginal zone and marginal zone precursors (MZ_MZPs; CD21HiCD24hi) Transitional type 1 (T1;CD21lowCD24hiCD23-) and Transitional type 2 (T2;CD21intCD24hiCD23+). Within the MZ_T2 subset, B cells were further divided into marginal zone (MZ; CD21HiCD24hiCD23lo) and Transitional two-marginal-zone precursor B cells (T2-MZPs; CD21HiCD24hiCD23hi) based on CD expression. (B) Reference table for B cell phenotypes. (C) Percentage of total B cells of FO, MZ_T2, T1, T2, MZ and T2_MZP B cells in naïve or infected mice. (D) The percentage of respective B cell subsets positive for CD1d and CD5. (E) the geometric mean fluorescence intensity of CD86 on the surface of B cell subsets. Results are mean+/- SEM. Data from (D-E) one representative experiment or from (C) three experiments pooled (n=6-24 mice per group). Significant differences were determined by individual one-way ANOVAs (D&E) or two-way ANOVA (C). *p < 0.05, **p < 0.01, ***p < 0.001.

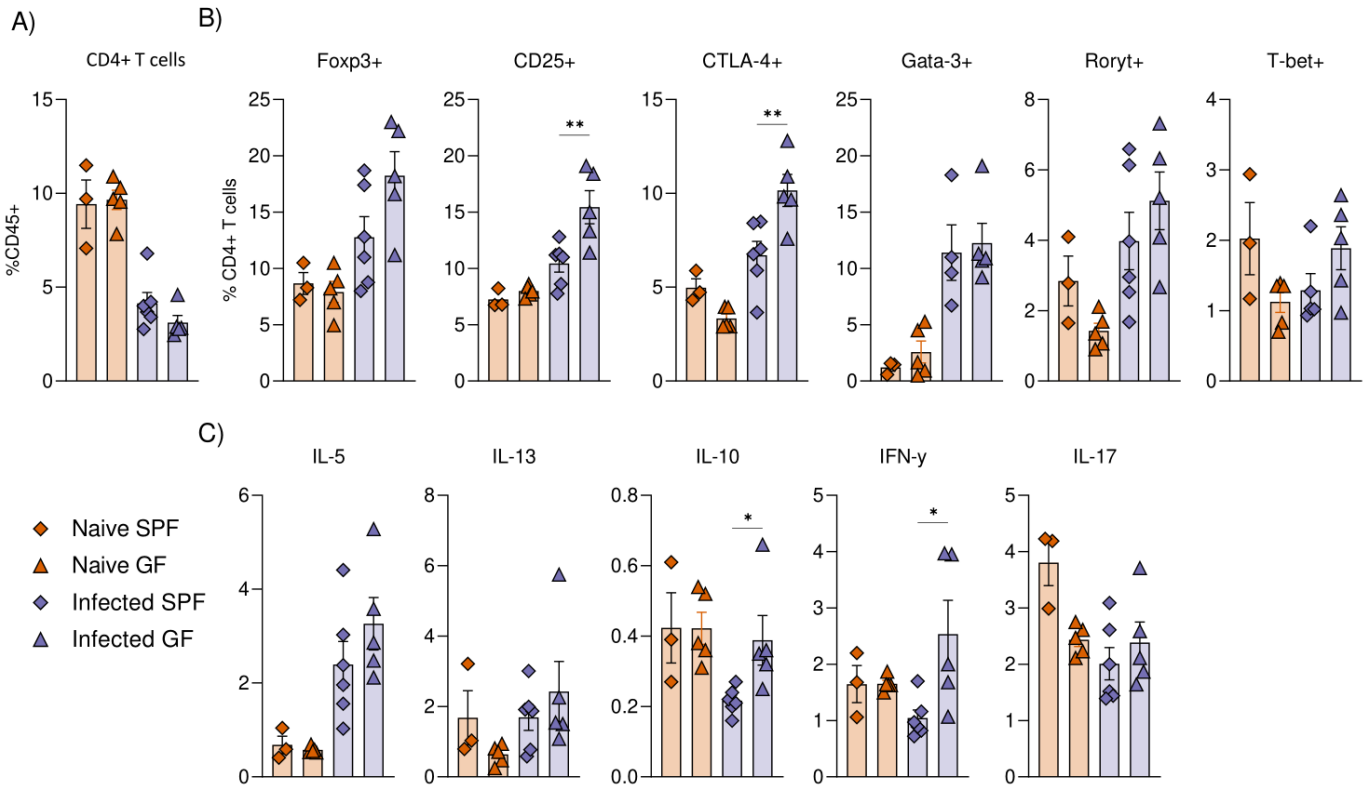


Mixed-sex vs naïve	WPI									
	4		7		9		12		14	
FO					**	↓			**	↑
MZ_T2									***	↑
MZ							***	↓	***	↓
T2_MZP			*	↑	***	↑	***	↑	***	↑
T1										
T2			**	↑	***	↑	*	↑	***	↑

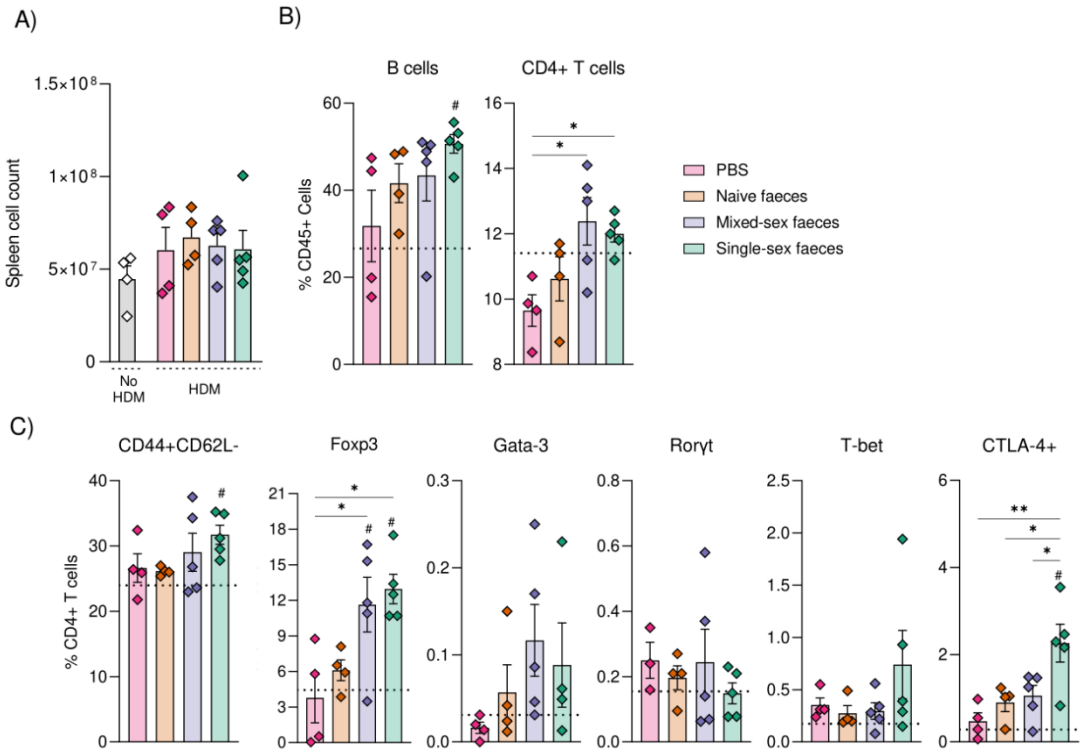
Mixed-sex vs single-sex	WPI									
	4		7		9		12		14	
FO									**	↑
MZ_T2									***	↑
MZ										
T2_MZP					**	↑	*	↑		
T1					**	↑				
T2					*	↑				

Single-sex vs naïve	WPI									
	4		7		9		12		14	
FO									**	↑
MZ_T2										
MZ							**	↓	***	↓
T2_MZP										
T1										
T2			*	↑	*	↑			**	↑

Supplementary Figure 2. B cell phenotypes during schistosomiasis. (A) Representative FACs plots of naïve, mixed-sex and single-sex infected B cells at week 14 of infections. (B) Summary statistics tables showing differences in FO, MZ_T2, MZ, T2_MZP, T1 and T1 proportions (as a % of total B cells) between naïve, mixed-sex and single-sex infected mice. Arrows reflect significant increases or decreases in B cell frequencies, and colours indicate statistical significance.



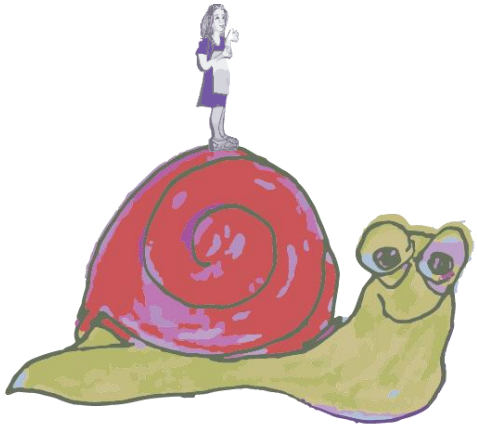
Supplementary Figure 3. GF or SPF mice were infected with mixed-sex cercariae with infection lasting 7 weeks. (A) Frequency CD4+T cells as a proportion of total CD45+ Cells. (B) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors, as well as CD25 and CTLA-4 in splenic CD4+ T cells. (C) Cytokine secretion from PMA ionomycin stimulated splenocytes cells at week 7 of infection. Results are mean +/- SEM from one single experiment (n=3-6 mice per group). Significant differences were determined by unpaired t test between infected groups. *p < 0.05, **p < 0.01, ***p < 0.001.



Supplementary Figure 4. Splenic alterations following Abx, FMT and HDM challenge. Abx-treated SPF C57BL/6 mice received 2 gavages of PBS (control) or faeces from naïve mice, or those chronically infected (week 14) with single-sex or mixed-sex S.mansoni parasites. After 3 weeks recolonisation, mice were challenged and sensitised to HDM, and culled 2 days after the last HDM challenge for analysis of airway and systemic inflammation. (A) Total spleen counts and (B) the proportion of splenic CD4+ T cells and B cells. (C) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors and IL-2R (CTLA-4) in splenic CD4+ T cells. Dotted line represents mean values in no HDM control group. Results are mean +/- SEM from one single experiment (n=4-5 mice per group). Significant differences were determined by one-way ANOVA followed Tukey Post hoc testing. *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences between HDM treated and non HDM treated mice are indicated by #.

CHAPTER 7

Summarising discussion & choice of methodology



As this thesis is presented in journal format, each individual paper has their own study-specific methods section. However, as a caveat of the journal style, these sections fail to disclose the benefits and limitations of each technique and provide a justification as to why each method was chosen. In this section I discuss the pros and cons associated with the various methods used in this thesis. As the studies presented in this thesis are linked, much of the methodology will be shared between them. For each methodological consideration, a statement will detail which study(s) it relates to.

- **Chapter 2** – Literature Review. *Schistosome Egg Migration: Mechanisms, Pathogenesis and Host Immune Responses*
- **Chapter 3** – Data Chapter. Dynamics of host immune response development during *Schistosoma mansoni* infection
- **Chapter 4** – Data Chapter. Tissue damage and microbiota modifications provoke intestinal Type 2 immunity during *Schistosoma mansoni* infection
- **Chapter 5** – Data Chapter. *Type I interferons provide additive signals for murine regulatory B cell induction by Schistosoma mansoni eggs.*
- **Chapter 6** – Data Chapter. Patent *Schistosoma mansoni* infections endorse distinct regulatory cell networks, with evidence for microbial assisted alleviation of allergic airway inflammation

Measuring intestinal barrier function

Intestinal barrier function involves the regulation of luminal content (including bacteria and foodstuff) between epithelial cells (paracellular route) or through them (transcellular route) and into the underlying mucosa or more systemic sites. Although 'leaky' guts are associated with gastrointestinal disorders[1], the dissemination of luminal products can stimulate beneficial immune responses[2]. Intestinal permeability and injury can be measured through a variety of approaches, including histology, advanced imagine techniques, cell cultures and the movement of tracers from the intestinal lumen into circulation (vice versa) (Reviewed in [3]). In Chapter 4, the influence of schistosome egg transit on intestinal integrity was primarily investigated through gavages with the oral tracer FITC-dextran. Whilst performing this technique there were lots of discussions into whether complementing approaches were needed, and if so, which should be selected.

Ingestion of oral probes

Intestinal permeability is commonly evaluated the by the ingestion of non-digestible probes, which pass from the intestinal lumen into extraintestinal sites (such as urine, blood, and organs) before their subsequent detection by techniques including organ cultures, fluorimetry, chromatography and mass spectrometry[3]. Several types of probes are used for these assays including saccharides, radioisotopes, and polyethylene glycols (PEG). These tests benefit from their real-time tracking, non-invasiveness, and the ability to conduct them under *in vivo* conditions. However, there exist many confounding variables that may cloud results, including person to person differences in gastrointestinal motility, intestinal surface area, mucosal blood flow, kidney function and drug abuse. The test conditions themselves are also unethical; often consisting of a large fasting period and the collection of bodily excretions (i.e blood or urine) over a 24-hour timeframe.

In humans, the most common method of intestinal permeability assessment is the dual-saccharide lactulose-mannitol test, with both sugars passing from the gut into urinary stream with minimal metabolism. The test works on the basis that while mannitol (monosaccharide) has a high transcellular permeation rate at baseline, the passing of lactulose (disaccharide) is much slower due to its crossing via the controlled paracellular pathway. As such, mannitol recovery is considered reflective of surface area, while a higher excretion ratio of lactulose/mannitol is suggestive of epithelial damage and altered integrity. In addition, by evaluating the time of urinary excretion, researchers gain information on regional permeability changes. For example, secretion within the first 2 hours reflects small intestinal permeability, whilst excretion within the 8-24 hour window is indicative of colonic leakiness. However, the validity of this assay has recently been brought into question, with studies showing both saccharides to cross the intestinal barrier via the same pathway[1], and the test itself lacks difficulties in test standardisation and uncertainty on normal value ranges.

In murine experiments, urine-based approaches are often bypassed due to the large volume of urine required for analysis and the unideal methods used for urine acquisition (i.e metabolic chambers). Instead, permeability is commonly evaluated in rodents through oral gavage with fluorescently labelled tracers, whose passage and subsequent recovery in the blood permits assessment of permeability via fluorimetry. These assays are commonly employed using dextran polysaccharides conjugated to various fluorophores (e.g FITC-dextran), where alteration of dextran size allows inference into the permeation of large or small macromolecules across the intestinal barrier. Similar to that observed with the dual saccharide test, regional changes in permeability can be assessed by

measuring FITC concentration in plasma at various times after oral application. Moreover, while many researchers choose to cull experimental mice at the time of plasma collection, it is possible to collect blood over time (e.g via tail vein) with plasmatic fluorescence still readily detectable.

Intestinal loops

The permeability of isolated intestinal segments can be assessed through the creation of intestinal loops. Working on the same basis as that described above, these assays rely on the flow of markers from the intestinal loop segments into extraintestinal space / media. These assays can be done ex vivo, by the creation of intestinal sacs submerged in media, or in vivo / in anaesthetised animals by carefully tying off individual loops of the intestine. After carefully tying off the chosen region, the probe of choice is injected into the loop, and the probe is recovered from the extraintestinal site after a set time-point. An obvious disadvantage of this assay is the skill and dexterity required to perform *in vivo*, and the artificiality of creating loops ex vivo, with the potential of cell death and time delays clouding the results.

Imaging approaches

Thanks to advances in imaging technology intestinal injury can be visualised by non-invasive means. For example, by coupling the oral ingestion of fluorescently labelled probes with far infrared technology and spectral imaging instruments (e.g IVIS machines), researchers can successfully monitor intestinal injury in real time and without sacrifice of experimental animals[4]. For these whole-body imaging approaches animals are imaged under light anaesthesia and assessment can be made easily quickly and non-invasively. The drawbacks come from the expense and expertise of the equipment, and the capacity to detect and visualise certain probes.

Detection of luminal products in extraintestinal sites

The *in vitro* measurement of luminal products (i.e bacteria, PAMPs, metabolites) into extra-intestinal sites is a popular method used to measure barrier integrity. Common sites of assessment include systemic circulation, mesenteric lymph nodes and the spleen. In addition, as the majority of intestinal venous blood feeds into portal circulation, the liver is highly vulnerable to exposure to bacterial products that have translocated from the intestinal lumen[3]. Within these tissue sites or circulation, various ELISAs can be used to measure luminal products or PAMPs. LPS/endotoxin can be measured using traditional Limulus assays, and bacteria ribosomal DNA can be amplified by 16s ribosomal primers or deep 16s sequencing. Another exciting approach is the use of Fluorescence in situ hybridization (FISH), used for the identification and localisation of bacteria based on 16S RNA

sequences. Furthermore, viable bacteria can be detected via aerobic and anaerobic culture techniques (although not all bacteria are culturable) and chromatography approaches can be used to detect metabolites.

In addition to luminally-derived products, substances normally found within the intact epithelium can be found within circulation or urinary stream. For example, intestinal fatty acid binding protein (iFABP) is located with small intestinal enterocytes, but readily shed into circulation upon damage[5]. Additionally, the tight junction protein zonulin (ZO-1) can be measured via urinary excretion-based assays, where greater urinary recovery supposedly equates to greater barrier damage. However, a recent report has cast doubts on the validity of currently available commercial detection kits, and thus, the reliability of results published to date[6].

Excretion of Inflammatory markers and blood products

Intestinal permeability is not only influenced by the integrity of the epithelium, but also the endothelium. In scenarios of barrier dysfunction, markers normally restricted to the blood during healthy conditions may pass across the mucosal barrier into the intestine lumen, where they are subsequently non-invasively measured in the faeces[3]. The measurement of occult blood or albumin (highly abundant carrier protein within the blood) in faecal pellets is an example of such an approach. In addition, in a more visual albeit invasive approach, endothelial permeability can be measured in experimental animals through intravenous injection of Evans blue (binds to albumin), whose leakage into organs (approximately 1 hour post administration) provides a measure of plasma extravasation. Faeces can also be analysed for levels of inflammation-associated molecules such as calprotectin, Alpha-1-Antitrypsin (A1AT) and lipocalin. However, elevations in their levels may be reminiscent of inflammation as opposed to reduced barrier integrity.

Evaluation of tight junctions, intestinal integrity, and inflammation

The integrity of the intestinal barrier is standardly evaluated by light microscopy and H&E staining, with stereological sectioning to gain a composite picture. H&E staining allows visualisation of intestinal pathology (e.g ulcerations and inflammation), which may cause or contribute to permeability changes. To gain greater resolution, H&E staining is often complemented with more specific stains (e.g for collagen deposition and mucin expression), or immunohistochemistry/ immunofluorescence to assess for markers of interest. The visualisation of tight junction complexes provides information on the integrity of the boarder, with fragmented or jarred staining patterns reflecting potential alterations in permeability. This approach can be further supplemented by protein and mRNA transcript levels of tight junctions.

Cell lines

Human intestinal epithelial cell lines (e.g Caco-2, T84, and HCT-8 cells) are commonly employed to model the gastrointestinal environment (Reviewed in[5]). When seeded on trans-wells, and under the correct culture conditions, these immortalised cell lines differentiate into confluent epithelial monolayers that adopt many enterocyte-like features, including apical microvilli, enzymes, membrane transporter molecules and functional tight junction proteins. Thanks to its ease of manipulation, this *in vitro* system can be used to study innumerable topics, including drug-absorption, molecule toxicity and intestinal permeability, with the latter parameter often measured via transepithelial electric resistance (TEER) or assessing tight junction dynamics. However, this system does have its drawbacks. Namely, as these cell lines generally consist of a single-cell type, they fail to recapitulate the full diversity of cell types found within the native intestinal epithelium, let alone their complex interactions and structural organisation. However, more recent years have seen the advent of 'gut-on-a-chip' and intestinal organoid technology. Intestinal organoids are established from adult intestinal stem cells (ISCs), or more specifically, from intestinal crypts isolated from surgical resections or endoscopic biopsies. Unlike immortalised epithelial cell lines, ISCs/organoids can give rise to the entire breadth of cell types that line the intestinal epithelium: goblets cells, Paneth cells, tuft cells, microfold cells, enteroendocrine cells, enterocytes and ISCs. In addition, they also faithfully retain the physiological and pathological attributes of the tissue from which they derive[7]. For instance, adopting different gene expression profiles depending on the intestinal region of origin (jejunum/ileum/ duodenum) or whether the tissue was healthy or diseased. Intestinal organoids are also grown as 3 dimensional scaffolds, bringing forth challenges with imaging and co-culturing with other cell-types. These challenges have been addressed through the creation of gut-on-a-chip technology, in which organoid-derived monolayers and microvascular endothelial cells are incorporated and contained within engineered microenvironments. These intestinal chips contain many critical elements needed to fully recreate the intestinal environment, including blood vessels, vascular flow, mechanical forces, and oxygen gradient. Researchers have even used these technologies to assess microbial interactions[8].

Schistosome infections

Schistosome infections were performed as part of all data chapters. Schistosome cercariae can be administered via a variety of routes, including subcutaneous, intramuscular, and intraperitoneal and intradermal[9]. However, percutaneous administration is most common approach albeit not necessarily the most effective[9]. In this thesis, cercariae were topically applied to the shaved

abdomen of anesthetised mice, with controlled exposure to the parasite for 30 minutes. This approach has various advantages. Firstly, this infection route is the most reminiscent of what occurs in nature, where other infection routes require injection and mechanical transformation of cercariae to schistosomula *in vitro,* with the artificial separation of the tail from the head of cercariae via vortexing and syringe suction [10]. Injection methods are also less reproducible in terms of the level of infection achieved[11]. Secondly, the other injection-based approaches on unanaesthetised mice are associated with a greater risk of injecting yourself with schistosome cercariae. Finally, although intramuscular administration is the most effective[9], it is considerably more painful to the mouse. Moreover, while cercariae can be topically applied to the tail, this approach often involves the use of un-anesthetised mice in debatably unethical restraining devices for 20-30 minutes, and the tail skin is potentially more difficult for cercariae to penetrate than the abdomen[11].

Mice are permissive hosts to the three main culpable species of schistosomiasis (*S. mansoni, S. haematobium* and *S. japonicum*) with the timing of worm maturation and egg deposition similar between mice and man. The relative 'ease' of performing experimental schistosome infections means that a wide range of transgenic mice can be infected with these parasites, and in the future, CRISPR-CAS9 technologies may permit the generation of transgenic schistosome lines[12]. However, there several flaws in murine models of schistosomiasis that need consideration[13].

Firstly, whilst humans are naturally exposed to schistosomes over a series of years, mice are typically exposed to one bolus dose of cercariae. This infection method fails to consider repeat exposure, gradual tolerance to schistosomes, and that many individuals in endemic regions first encounter parasite Ags whilst *in utero*[14]. Although investigations of maternal influences are possible[15], maternal schistosome infections of mice are painstakingly long and not feasible for every experimental set up. In addition, mice appear more resistant to secondary infection than humans[16]. As part of the 'Leaky Liver' hypothesis, murine concomitant immunity (resistance to secondary infection) to schistosomes is common due to the generation of portal anastomose and shunts, that redirect migrating schistosomula away from the liver and prevent their maturation[17].

Secondly, replicating a natural infection dose is difficult to achieve in mice, with estimations that a single worm pair in mice is equivalent to more than 1000 pairs in a human[13]. As there are inevitably inconsistencies in cercariae viability and the infection efficacy, murine infections are typically conducted using 20-200 parasites[18]. These large infection doses are often required to elicit a consistent phenotype, perform intervention studies, and recover back parasite material. However, with high cercarial doses come lethal infections, and the cause of death between humans and mice

[13]. Notably, whilst most deaths in chronically infected persons come from severe Symmers' fibrosis and bleeding of oesophageal varices, this advanced disease cannot be replicated in mouse infections. Instead, mouse death is thought a consequence of gastrointestinal haemorrhaging with evidence of cytokine shock[13,19].

Finally, there are discrepancies in the final site worm residency and egg entrapment between mice and human studies. One prominent example is that of *S. haematobium*, whose worms reside in the bladder plexus during human infections, but in mice, dwell within the intestinal vasculature[20].For *S. mansoni* and *S. Japonicum* the location of adult worms is more consistent between mice and humans, but minor locational disparities could still influence the location of ectopic egg spread the organs struck by egg evoked damage.

Single sex infections

Schistosomes are unique amongst blood-flukes in that they are dioecious (as opposed to hermaphroditic) which means they can form separate sexes. This unique property allows researchers to perform unisexual infections with male or female worms alone, and thus, dissect the contribution of each gender or worm-derived signals to the immune landscape described above. In Chapters 4 & 6 we make comparison between egg producing infections with male and female parasites, and non-egg producing infections with male worms alone. In Chapter 4 this approach allowed us to infer how egg penetration impacts the intestinal environment (microbially, immunologically and integrity-wise), whilst in Chapter 6**,** such comparison allowed us to dissect the signals contributing to splenic regulatory cell generation. While female only infections are possible, the reliance of female worms on their male partner for complete sexual maturation[21–24], means that female only infections often do not persist long into chronic phases, and the worms themselves are developmentally and reproductively stunted[24]. There are also concerns that female worms may sporadically produce eggs in the absence of a partner[25], resulting inadvertent exposure to egg Ags.

Transgenic mice

CD11c depletion

In Chapter 3 we investigate the role of DCs in the maintenance and regulation of host responses through the use of an established CD11c.DTR transgenic mouse line (CD11c.DOG mice)[26,27-]. In this mouse strain, conditional ablation of CD11c+ cells is achieved by inserting the human diphtheria toxin (DTx) receptor (DTR) under the control of the CD11c gene. While these transgenic mice are highly susceptible to DTx induced depletion, WT mice are resistant due to a mutation in the murine

DTR gene making them low affinity to the toxin. As shown from published work from our lab, this mouse strain allows for non-toxic and reliable depletion of CD11c+ DCs through Dtx administration[27] This contrasts with other strains of CD11c.DTR mice, whereby DTx injection proves lethal after several days of consecutive administration[28].

A large flaw of using CD11c depletion to investigate DC involvement is that many other cell types may express CD11c on their surface, including macrophages, monocytes, B cell subsets and some granulocytes. As such, we cannot state that the phenotype observed upon CD11c depletion is purely results from DC loss. In future studies, it would be interesting to assess DC depletion by focusing into individual DC subsets. For instance, the use of $BAT^{F-/-}$ mice or $IRF4^{-/-}$ mice who lack cDC1s and cDC2s respectively. However, for the studies presented in this thesis, we chose to look broadly at global depletion before homing down into individual subsets.

IL-10 reporters

In Chapter 6 we briefly report on the capacity of schistosome-associated microbiotas to promote B cell and T cell IL-10. The identification of IL-10 producing cell types (especially B cells) is tediously challenging, and often requires several days of artificial *ex vivo* stimulation followed by flow cytometry staining of IL-10. A variety or transgenic IL-10 reporter lines avoid the need for ex vivo stimulation and intracellular staining, including the IL-10GFP (VeRT-X) strain[29]. In our hands and others[30], we have been able to reliably identify IL-10+ lymphoid cells. However, when using these cell types of care must be taken not to use buffers that will deplete the GFP signal. In addition, these strains are not suitable for the detection of myeloid IL-10 due to autofluorescence coinciding with the GFP emission[31].

Faecal transplant studies and gnotobiotic approaches

The intestinal microbiota is a major calibrator of host physiology and immune development[32,33]. Both germ free (GF) and antibiotic (Abx)- treated mice allow for the study of set microbiotas or defined bacterial species (and their metabolites) on host responses. In Chapter 4 we evaluate the role of the schistosome-elicited microbiota in skewing of intestinal and mesenteric immune responses through means of faecal transplant into GF animals. Whilst in Chapter 6 we investigate the capacity of defined microbiotas to alleviate allergic airway disease, through antibiotic) mediated depletion of intestinal bacteria, followed by faecal transplant and a model of house dust mite (HDM) evoked allergic inflammation.

Experimental design and setup are incredibly important in microbiota studies, as the composition, diversity and metabolic activity of bacterial populations are heavily influenced by external

factors[34,35]. This includes factors such as diet, water, handling of animals, bedding, lighting, and temperature. Although every attention was made to control environmental factors during our investigations, we cannot exclude the possibility that our results our confounded by external influences.

The majority of mouse experiments are conducted with specific pathogen free (SPF) mice, whose microbiota is frequently screened for certain pathogens. However, as there is no surveillance or monitoring of commensal organisms, murine microbiotas will vary greatly across institution and different mouse houses will have different definitions of 'SPF' mice. When performing experiments with SPF mice, great care must be taken to ensure all experimental groups are housed under the same conditions, with use of litter mate controls where possible. In Chapter 4 microbiota composition was scrutinised in naïve and schistosome infected mice via 16s sequencing, with experiments taking place across two institutions. By the inclusion of naïve mice groups at both institutions, and with schistosome infections having such a dramatic impact on microbiota structure, it is possible to tease apart schistosome evoked alterations, and those that are mouse-house associated. However, for our more mechanistic microbiota studies, in which we investigated the immune contributions of defined microbiotas, we turned to more controlled and rigorous systems of Abx-treatment and GF mice.

GF mouse models are the most nuclear and 'gold standard' approach to investigate microbial-host relations[34,36]. GF mice are bred and maintained within internally sterilised isolators, with the introduction of sterilised cages, bedding, food, and water via peracetic acid sprayed and autoclaved drums[34].Through this approach, GF mice colonies are completely shielded from the external environment, and as such, allow for investigations into the complete absence of microbes, or introduction of defined consortiums. However, the unsurprising expense associated with these experiments, alongside the required technical support, skill and labour needed to maintain the facilities means these experiments are inaccessible to many researchers. Logistic and economic caveats aside, GF mice are immunologically and physiologically stunted in comparison to SPF mice[36]. This brings forth questions of reliability and translatability, when experimenting an already handicapped system. For example, does the immune system react in the same manner as it would do in an already microbially rich and pre-primed system?

Abx-treatment models are commonly employed to avoid the complications associated with GF studies. As each Abx varies in their mechanism of action and consequentially, the groups of bacteria

they target, Abx can be selectively administered to deplete certain microbes or evoke certain compositional shifts. Alternatively, global depletion can be achieved by administrating cocktails of varied Abx classes. The inclusion of anti-fungals within the Abx regimen is recommended to prevent fungal overgrowth that may confound experimental results. In comparison to GF mice models, antibiotic approaches are relatively inexpensive, accessible and do not need the same degree of technical knowhow or expertise. Immunologically, as these mice have been colonised from birth, they will generally not show impairments in the development or early immune training. However, there are several pitfalls associated with an antibiotic treatment approach[33]: i) Abx cannot completely clear out the mouse microbiota. ii) Depending on starting bacterial composition, the type of Abx administered, the presence of Abx resistance and the duration of the Abx regime, it may not be possible to achieve the desired depletion and you may select for overgrowth of certain residential organisms, further confounding experimental results. iii) Mice often refrain from drinking Abx water, which may lead to dehydration, mice nearing the severity limit of the experiment and/or uneven depletion of the microbiota across experimental groups. iv) Due to ethical concerns surrounding antibiotic treatment, the duration of administration is often limit to a 2-4 week period (institution / animal facility guidance dependent).

In our studies, we chose to assess the influence of a schistosome microbiota on host immunity through faecal transplant into GF mice. We selected GF mice because we wanted to assess how the schistosome microbiota would influence the host in a completely clean system, without prior exposure to other organisms. We chose to perform faecal transplants rather than co-housing studies because the schistosome-associated microbiota varies over the course of infection (Chapter 4) [37,38], and thus, if co-housing was performed, we would not know the exact nature of the microbiota we were transferring. 16s sequencing was performed on the large intestinal content of recipient and donor mice in order to ascertain which microbial species were able to colonise GF mice and potentially evoke immunological changes. However, as frozen material was used for these transplant studies (due to logistic reasons), bacterial viability would have been stunted significantly in the freeze thaw process. In addition, many anaerobic bacteria are lost during the recolonisation period in spite of strict anaerobic conditions[39]. As such, in our studies the bacterial profile of recipient mice was not a full emulation of the donor faeces we placed in. Future studies should be performed using fresh faecal material as to avoid the loss of potentially immunogenic bacteria, emphasis on rapid processing in an anaerobic environment.

In Chapter 6 we evaluated the capacity of a schistosome associated microbiota to relief experimental AAI in Abx-treated mice. We chose Abx-treated mice rather than GF mice for feasibility reasons. Namely, due to the strict and sterile conditions of GF animal units, it is very arduous to anesthetise mice and perform intranasal allergen administration within a GF isolator. Accordingly, the microbiota was ablated through 2 week administration of a broad-stream Abx cocktail: ampicillin, gentamycin metronidazole, neomycin and vancomycin.

HDM-evoked allergic airway inflammation

In Chapter 6 the influence of a schistosome-associated microbiota on the exacerbation of inflammatory disease, was evaluated in a model of HDM-evoked airway inflammation.

Mice do not spontaneously develop asthma, and so it must be artificially induced[40].The induction of allergic disease requires two distinct phases: sensitisation and challenge. Briefly, the first phase is characterised by IgE production by B cells, which binds to high affinity receptors (FcεR1) on surface of basophils and mast cells. In the challenge phase, allergen exposure and cross-linking reactivate airway basophils and mast cells, evoking an immediate hypersensitivity reaction. The selection of an appropriate allergic protocol and allergen allows for successful recapitulation of many aspects of human disease, including flux of cells to the airways, epithelial hypertrophy, goblet cell hyperplasia and airway hyper-responsiveness.

While there are a wide range of allergens that are considered relevant to human asthma (including *Aspergillus fumigatus* and cockroach allergens) HDM is one of the most commonly employed. There are a variety of reasons why we chose HDM. Firstly, HDM encompasses a large breadth of Ags that are believed involved in human allergic responses such as LPS, faecal matter and the glucose derivative chitin. This is in contrast to the frequently used OVA-alum model, where OVA is not an allergen in humans. Secondly, standardised allergens inevitably have batch to batch variations (due to the large mixture of extracts their composed of) leading to a range in effectiveness of allergic induction. Our lab amongst others have shown HDM administration, and our selected allergic protocol, to reliably stimulate Th2 immunity [40,41] and batch testing prior to this study confirmed its suitability. Finally, the intranasal route of administration is less artificial than other allergic models that using intraperitoneal sensitisations. HDM can also be administered intranasally over months, evoking more clinically relevant chronic disease[42]

Confocal microscopy

In Chapter 3 we provide images of hepatic granulomas over the course of infection that were generated by Immunohistochemistry (IHC) combined with confocal microscopy. While this imaging approach provided valuable insight into the localisation and infiltration of select immune cells to the schistosome granuloma, there are various limitations. Firstly, since conducting these investigations (Angela Marley; 2015) imaging technologies have advanced tremendously. For example, the introduction of imaging mass cytometry (Hyperion; Fluidgm) has allowed for the unprecedent insight into tissue microenvironments, with resolution at a single-cell level and simultaneous analysis of 4 to 37 targets a single scan. Similarly, multiphoton microscopy would have allowed for three-dimensional imaging or even the live of imaging of granulomas within infected mice, facilitating the observation of cellular presence and movement within the granuloma. Secondly, by exclusively imaging granulomas within the median and left liver lobe, we assumed that egg / granuloma distribution was equivocal across the liver (i.e left right, median and caudate lobes). Further evaluation of granuloma formation within right and caudate lobes is required in order to ascertain whether the left and median lobes fully represent granulomatous inflammation and the timing of egg delivery.

Microbiome analysis

As part of Chapter 4, 16s rRNA gene sequencing was used to evaluate the structure of the colonic microbiota in schistosome infected mice or mice receiving faecal transplant (16s sequencing reviewed in[43]). Before the introduction of next-generation sequencing (NGS), bacterial identification typically revolved around the enrichment of bacteria in broth or agar-based media, followed by biochemical analysis for identification. Although these techniques will likely remain a mainstay within the microbiology field, they do have multiple drawbacks including bias towards culturable bacterial strains, the readouts are purely qualitative and they unlikely to reveal the entire diversity of the sample under scrutiny. NGS on the other hand, is a molecular diagnostic technique that has revolutionised our capacity to profile microorganisms. In this technique, hypervariable regions of the bacterial 16s rRNA gene (typically V3–V4 and V4–V5 regions) are amplified and sequenced, and then generated sequences are compared to publicly available reference libraries, ultimately allowing for the identification of OTUs in question. This technique has many advantages over traditional lab based approaches. Namely, NGS can show the relative abundance of an organism, it does not rely on the given sample being culturable, and hundreds of samples can be ran in parallel. However, 16s rRNA sequencing has now had its heyday, with more innovative techniques such as shotgun metagenomics stealing the limelight. The largest pitfall of 16s rRNA gene sequencing is its

incapacity to differentiate between bacteria of the same strain; i.e due to similarities in their 16S rRNA gene profiles. Accordingly, in this thesis bacterial communities were characterised no further than the genus level and as a result, we can only speculate on the functional and immunomodulatory consequences of the dysbiosis observed. Future studies interrogating the schistosome associated microbiota by shotgun metagenomics are warranted[44]. In this technique, the sequencing of all genomic DNA within a given sample (rather than just 16s rRNA genes) allows not only for the generation of a taxonomic profile, but also provides further information on antibiotic resistance metabolic functionally. This would be especially exciting in schistosomiasis with preliminary data in this thesis hinting towards certain microbial communities playing an active role in host immune modulation. However, due the greater depth in taxonomy resolution, shotgun metagenomics comes at a much higher and often inaccessible price.

Flow cytometry

Flow cytometry has been integral to all data chapters presented in this thesis. In this technology, cells are stained with fluorescently conjugated antibodies, each with their own unique spectral profile. Stained cells are subsequently passed through a flow cytometer, whose collection of lasers and detectors allows the for the definition of cell types within a heterogenous population based on their size and marker expression/ antibody binding. As this thesis evolved, and more expertise was attained, the flow cytometry panels used became more elaborate, albeit still largely constrained to the capacity of a 16 colour LSR-Fortessa. Each fluorochrome added to a conventional flow cytometry panel adds a greater risk spectral spillover, leading to clouding and difficulties in data interpretation. While compensation matrixes can correct for overlapping fluorescence, sometime the overlap is too extensive and manual compensation can be painstakingly long. In recent years, spectral flow cytometry has been introduced. This technology gets around the problems of conventional flow cytometry by summing the florescence together and using unmixing technology to mathematically sperate out individual fluorophores.

Colonic Lamina propria preps

The vigorous Type 2 immune response elicited by intestinal worms (e.g mucus production, cell infiltration & tissue remodelling) makes it near impossible to isolate live cells from heavily infected helminth infected intestine. As such, to our knowledge there is next to no publishable works on immune responses in the schistosome infected intestine as analysed by flow cytometry. In Chapter 4 we used recently published cell isolation protocols[45,46] (in the context of H. *polygyrus* infected

small intestine) and in-house expertise[47] to successfully isolate live immune cells from schistosome infected colon segments.

Intestinal isolation protocols can be broken down broadly into 3 stages: I) Collection and washing of the intestine ii) removal of surface layers and iii) enzymatic digestion of tissues, allowing for generation of a single-cell suspensions. Due the strength of the anti-worm immune response evoked speed, is essential across in all three stages.

For the first stage, collection of all intestinal samples should take no longer than 1 hour to avoid mass scale cell death. After having characterised the colonic microbiota of schistosome infected mice (Chapter 4) and knowing that schistosome eggs penetrate the length of the intestine, with a predilection towards the ileum and proximal colon[48], we chose to focus on characterising intestinal immune responses within the colon of infected animals. With the colon being approximately 1/3 the length of the small intestine, and generally harbouring less fat and no Peyer's Patches, our focus on the colon sped up our collection time within the animal unit, allowed us to collect and analyse other tissues in concert, and undoubtedly, helped us attain live colonic cells.

In the second stage, outer surface and epithelial layers were removed through two 10 minute incubations (37°C, orbital shaker) in RPMI-1640 media containing 5mM EDTA, 0.25mM DTT, 10mM HEPES and 3% FBS. The chelating agent EDTA was added for the dissociation and removal of intestinal epithelial cells, and DTT was included for its mucolytic properties. Between each 10 minute incubation samples were vigorously vortexed, and liquid poured off via a sieve. In initial experiments, samples were incubated for 20 minutes without a break This led to poorer cell yields; likely due to a build-up of toxic material within the incubation media.

Next samples were digested sliced with scissors and digested with 1.25mg/ml DNAse-1 and 0.1mg/ml Liberase TL in volumes of 5ml. We attained approximately 5 million live cells from each colon, within the downfall that only 5-15% of live cells obtained were CD45+. In future work, I would strongly advocate for further enzyme optimisation, generating a cocktail that can more effectively digest through the thicker layers of the colon and deal with the intestinal remodelling evoked by infection. This could include a concoction of collagenase D & V and dispase[49].

Intravenous and intraperitoneal egg injections

In Chapter 5, we use intraperitoneal (i.p) and intravenous (i.v) injections with *Schistosoma* eggs or SEA to investigate whether Type-I IFNs provide additive signals for schistosome egg-driven Breg expansion. In these models, eggs are considered Ag reservoirs, that allow synchronous delivery of

Ags to the host without additional complexity of an ongoing infection. In part of the paper, mice (WT or IFNAR$^{-/-}$) were challenged with two doses of i.p administered *S. mansoni* eggs (5000), with injections administered on day 0 and day 7, and mice culled 7 days later. We chose this model to assess whether Type-I IFNs are instrumental in Breg generation in an *in vivo* system. Further, our lab has previous shown the egg challenge model to trigger Breg development[50]. Regarding i.v injections, we chose to deliver SEA via the intravenous route to maximise Ag delivery to the spleen. Splenocytes were harvested 12 hours post injection based on previous literature showing rapid upregulation of Type-I IFN associated genes following SEA exposure[51], as well as a preliminary time course experiment from our lab investigating the kinetics of gene upregulation (data not shown). Egg and SEA dosages were also based on previous studies[50,51].

Assessment of Breg generation

In Chapter 6 we evaluate the splenic regulatory environment during egg producing and non-egg producing infections and attempt to ascertain which signals promote Breg generation. One way we chose to evaluate Breg development was through flow cytometry based phenotyping. A variety of Breg subsets have been described in mouse and man[52], with our lab previously demonstrating high provision of IL-10 from CD19^{+}CD21^{hi}CD23-CD24^{hi} MZ B cells[50,53,54]. To build upon our previous work and knowing that certain Breg subsets are induced by microbially derived signals [55], we expanded on our flow cytometry panel through the inclusion of CD24. This further allowed demarcation and identification of B cell precursor subsets: Transitional type 1 (T1;CD19^{+}CD21^{low}CD24^{hi}CD23^{--}) and Type 2 (T2; CD19^{+}CD21^{int}CD24^{hi}CD23^{+}), which have recently emigrated from the bone marrow. After identifying large phenotypic discrepancies between infected and non-infected mice, we next to identify which B cell subset was responsible for the majority of B cell IL-10 during schistosomiasis. By culturing bulk splenic B cells (isolated by CD19 MACs isolation) with a mixture of parasite derived Ags (SEA; AWA; worm ES products), we showed a greater IL-10 production propensity from infected mice B cells, as determined by flow cytometry and ELISA. However, were unable to identify which B cell subset was responsible as the expression of CD21, CD23 and CD24 changed upon activation (data not shown). Accordingly, we next attempted flow based approaches to sort subsets of interest (based on CD21, CD23 and CD24 expression) and assess their individual potential to produce IL-10 after several days culture. However, multiple difficulties were encountered when trying these approaches, which ultimately prevented us from attaining meaningful results. Namely, for each aforementioned culture technique, we would minimally isolate ~1,000,000 B cells of each subset of interest allowing for two technical replicates per splenic sample, and ideally 3+ biological replicates

per experimental group per experiment. While B cells are plentiful in the spleen (~60% of total CD45 cells) some candidate B cell subsets encompass as little as 1% of the total B cell pool, resulting in insufficient numbers for some of our downstream readouts. In the B cells that were successfully sorted, the high pressure, decompression and sheer force of the flow cytometer had a detrimental impact on B cell vitality, with the bulk majority dying after several days in culture. Moreover, as signals such as CD40 engagement is key for Breg survival and function[52], culturing B cells in isolation likely escalated our hardships.

As to avoid cell sorting associated cell death, for our final attempt of B cell subset isolation we used a newly developed FO and MZ B cell MACs isolation kit (Miltenyi Biotech). This approach led to us successfully attaining viable MZ and FO B cells, that we subsequentially cultured for 72hrs alone or in the presence of CD25 depleted CD4+ T cells to assess their IL-10 production and Treg induction potential. We were unable to look at more specific B cell subsets via this approach, and further investigation is warranted. Moreover, it is important to mention that while we had difficulty isolating viable splenic B cell subsets from schistosome infected mice via cell assisted sorting (Chapter 6), we were able to successfully sort viable splenic FO and MZ B cells from experimental mice (WT or IFNR-/-) challenged with i.p *S. mansoni* eggs (Chapter 5). The reason for this discrepancy ultimately comes down to the scale of the experiments and the frequency of desired B cell subsets. Future work is required assess the immunological importance of individual splenic B cell subsets during schistosomiasis.

SUMMARISING DISCUSSION

Parasites employ a range of peculiar tactics to fine tune host immune responses, ultimately permitting them to persist and prosper within the host[56,57]. For schistosomes, their potent immunosuppressive capacities not only allow them to downregulate the destructive Th2 orientated inflammation they inflict on host tissues, but also enable them to assist in the alleviation of unrelated immunological and inflammatory diseases[48,58–60]. A major research aim is to identify, understand and harness the therapeutic potential of helminth-derived molecules, and better ascertain the pathways and cell types that mediate their effects.

By systematically characterising host immunity in egg producing and non-egg producing *S. mansoni* infections, egg challenge models, and recipients of schistosome infection derived faecal transplants, we have increased our understanding of how schistosome-elicited immune responses are orchestrated. Moreover, we provide a platform for future study, focusing on delineating the microbial and/or metabolomic components that endorse schistosome associated immune responses, suppress bystander inflammation, and reinforce mucosal barrier integrity. Diagram 1 provides a broad schematic overview of the key results of this thesis and avenues for exploration, with more detailed schematics (Diagrams 2-4) later in this chapter.

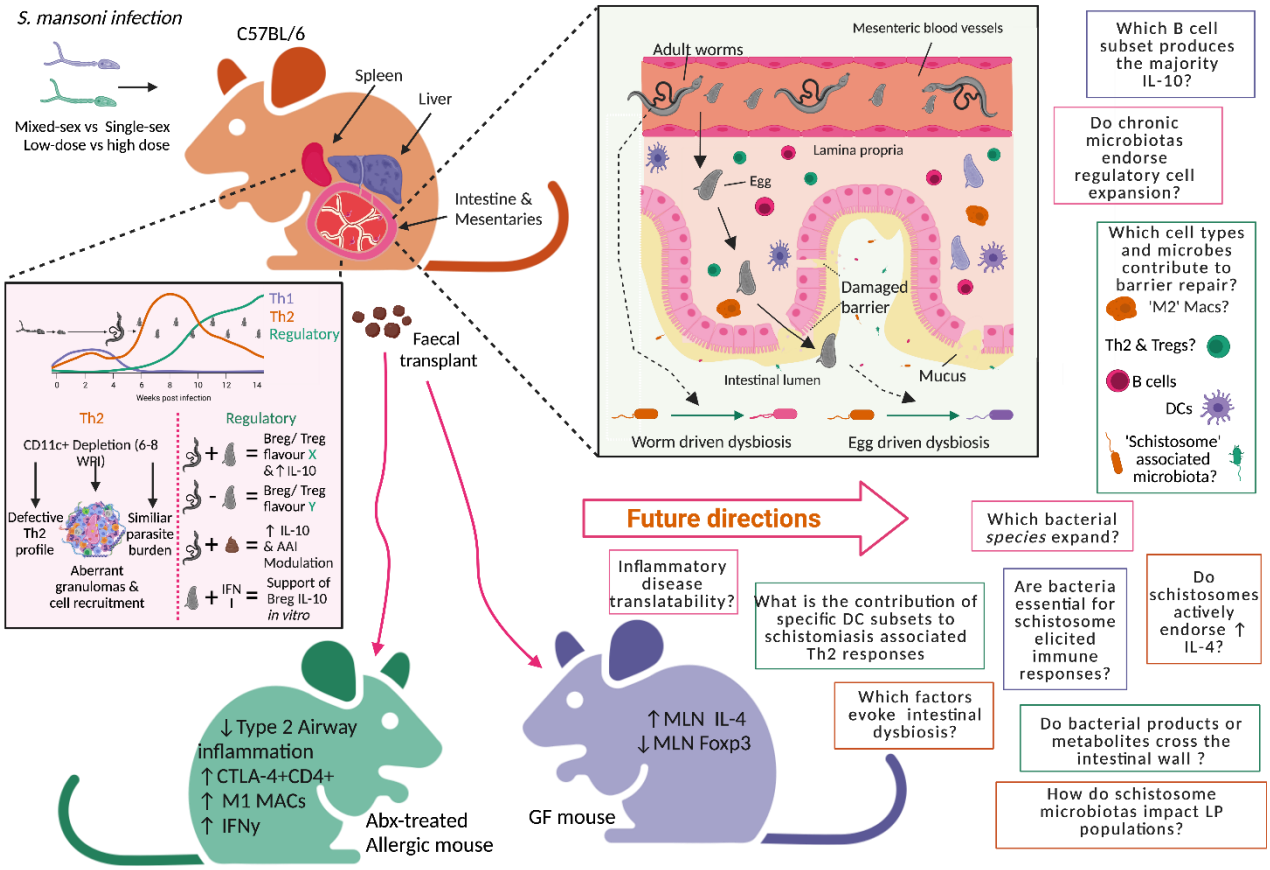


Diagram 1. Summary of key results and future directions. Experiments in this thesis were primarily conducted with female C57BL/6 mice, infected with either single-sex or mixed-sex *S. mansoni* parasites, at infection doses of 40 cercariae (low dose) or 180 cercariae (high dose). Infections lasted 3-14 weeks and immune response responses were characterised in the spleen, MLN, liver, and colon. Corroborating existing literature, conventional egg producing infections were typified by a triphasic immune profile, with distinctive Th1, Th2 and Regulatory components (Chapters 3&4). Th2 polarisation was not apparent in worm only infections (Chapter 4) and was abrogated upon depletion of $CD11c^+$ cells during peak stages of post-patent acute disease (weeks 6-8) (Chapter 3). Further studies are required to address the functional importance of individual $CD11c^+$ cells, including DC subsets, here. Regulatory cell expansion was visible in both chronic mixed-sex and single-sex infections, but with Bregs and Tregs adopting different phenotypes in each type of infection, and hints towards greater IL-10 production propensity in mixed-sex infections (Chapter 6). It has yet to be determined whether Bregs and Tregs from different infection types harbour different suppressive functions, and which subsets possess the greatest immunoregulatory capacities (e.g. T2_MZP B cells or MZ). While schistosome Ags are capable of directly endorsing Breg generation, it is likely that they work alongside other additive signals within the environment. Although Type-I IFNs were elevated in experimental infections and promoted SEA-induced Breg expansion *in vitro*, they proved redundant in Breg induction *in vivo* (Chapter 5). This thesis provides an extensive overview of the influence of egg transit on intestinal barrier function, immune responses, and microbial and metabolic landscapes (Chapter 4). Significantly, chronic and high dose infections increased intestinal leakiness, with evidence for enhanced systemic exposure to gut luminal substances (Chapter 4). It is not known which cell types help maintain intestinal integrity during patent schistosomiasis, and whether intestinal leakiness influences schistosome-associated immune responses (e.g. splenic Breg expansion during infection chronicity (Chapter 6)). Both worm-only and egg-producing infections altered intestinal microbial and metabolic profiles, with more profound alterations observed in the presence of eggs (Chapter 4). Higher resolution next generation sequencing (NGS) is required to define the inflammatory or pro-inflammatory nature of these bacterial shifts. Moreover, it is currently unclear what factors triggers these microbial modifications (e.g. parasite derived products, damage, or Type 2 inflammation) and whether bacteria are essential to schistosomiasis survival or schistosome-elicited immune responses. We have also optimised an intestinal isolation protocol that reliably enables live cell extraction and flow cytometry on the schistosome-infected intestine, and resultingly, a broad overview of how egg migration impacts the intestinal environment (Chapter 4). Future analyses should interrogate the involvement of local cell types in barrier repair (e.g. M2 macrophages), alongside techniques such as mass cytometry imaging and FISH to visualise cellular and bacterial localisation relative to sites of damage. Finally, through the transplant of schistosome infection derived faeces into GF or Abx-treated mice, we have demonstrated the capacity of schistosome infection associated microbiotas to alleviate bystander allergy (Chapter 6) and induce the emergence of a unique population of IL-4 committed MLN CD4+ T cells (Chapter 4). The results presented in this thesis provide a strong platform for the study of schistosome-induced cell types and microbiotas in the regulation of mucosal inflammation.

The immune trajectory of schistosomiasis and insight into the infected LP

The immune profile of schistosomiasis includes subtle Th1 orientation during the first 4-5 weeks of infection and potent Th2 skewing from the onset of egg production[61,62], which is scaled back during chronicity coinciding with regulatory cell expansion[63–65] (Diagram 2) In Chapters 3, 4 and 6**,** we closely inspected this trajectory in egg producing or non-egg producing infections, and across multiple tissue sites. Together, we have provided an up to date and detailed picture of how schistosome elicited immune responses evolve and highlighted which signals may dictate this triphasic immune profile (Diagram 2). In terms of some of the more novel findings, we have defined the 'tipping' point for development of overt granulomatous pathology (Chapter 3), demonstrated a key role for $CD11c^+$ cells in the maintenance of Type 2 responses at peak disease (Chapter 3; Diagram 1), showed unique differences in splenic regulatory cell induction in conventional egg producing infections vs male only infections (Chapter 6; Diagram 4), and provided a first look into anti-parasite immune responses within the colonic LP of schistosome infected mice (Chapter 4; Diagram 3).

Adult worms have been suggested to establish a Th2 environment prior to egg deposition, in a supposed attempt to ensure adequate adoption of Type 2 immune responses from the onset of egg production[66]. We observed no indication of Th2 priming by male worms alone, as evidenced by similar Th2 gene expression patterns (MLN and colon) and levels of systemic Type mediators (Ym1, RELM-α and IgE) to that of naïve mice (Chapter 4). However, upon inspection of cytokine secretion potential (PMA/iono stimulation) of MLN (Chapter 4) and splenic (Chapter 6) $CD4^+$ T cells, we observed an enhanced potential for $CD4^+$ T cells from male worm infected mice to produce IL-5 (MLN) and IL-4 (spleen) at week 14 of infection (Diagram 2). Together, these data suggest that worm Ags alone are not sufficient to induce systemic or local Type 2 responses, but they are capable of priming $CD4^+$ T cells for the release of Type 2 cytokines upon stimulation. The factors governing this are unclear but may stem from cross-reactivity between worm and egg derived Ags[67]. Moreover, it is worth mentioning that while egg antigens are most accredited for Type 2 induction, AWA have also shown capable of eliciting Type 2 responses (IL-5 and IL-13) in $CD4^+$ T cells of previously infected traveller populations and inducing cytokine secretion at a similar level to SEA[68]. Carrying on with the theme of this thesis, an obvious next step would be to assess the capacity of the microbiota induced by single-sex infection to endorse Type 2 cytokine secretion through the use of faecal transplant studies. In addition, it would also be of interest to evaluate $CD4^+$ cytokine production at periods other than infection chronicity.

Due to the exuberant Type 2 immune response elicited by schistosome infection, it has proven to be extremely challenging to extract live immune cells from heavily infected helminth intestine[46,48,69]. Accordingly, most of our understanding of immune responses in the intestinal tissues during schistosome infection has been derived from mRNA or histological/image based approaches or use of the MLNs as a proxy readout. Chapter 4 provides a broad insight into the cell types and mediators that respond to infection within the colonic LP and highlights discrepancies between colonic and mesenteric responses (Diagram 3). For example, whilst the MLN T cell expression of transcription factors and cytokines is largely comparable between low and high dose infections, within the colon only high-dose infections induced significant upregulation of Th2 (IL-5, IL-13 and Gata-3) and Th1 (IFNγ and T-bet) associated products. The MLNs are an important site of intestinal T cell activation and differentiation[70]**.** After this, effector subsets will migrate to the intestinal tissues, where re-encounter with specific Ag triggers them to execute effector functions[70]. Importantly, $CD4^+$ T cells are a plastic and versatile population, that can acquire a mixed phenotype or switch to a different lineage altogether[70], with secondary immunological challenge playing an integral role in this transition[70]**.** We put forth the following hypotheses to explain the differences between mesenteric and colonic responses against *S. mansoni* infection. Firstly, higher dose infection will provide a higher Ag burden while also causing greater tissue damage than low dose infections, which together could lead to greater T cell activation and recruitment to the tissues. Secondly, within the MLNs, $CD4^+$ T cells from low and high dose infections will be exposed to a similar range of Ags, perhaps leading to similar activation and differentiation profiles. However, upon their migration to the intestinal tissues, $CD4^+$ T cells from heavily infected mice would be confronted with large scale tissue damage, and potential exposure to damage associated molecules or luminal content that is not present during low dose infections. Accordingly, colonic $CD4^+$ T cells from high dose infection may adopt a more heterogenous / mixed T cell profile in attempt to deal with the encountered threat, downmodulate the high scale inflammation and maintain intestinal homeostasis. In addition, previous studies have shown the dose of Ag exposure to regulate the development of effector T cells into Th1 or Th2 cells[71]. Taken together, it would be fascinating to assess the Ag specificity of responding cell types within the colon. This could be achieved through the use of commensal bacterial and SEA specific tetramers or TCR transgenic mice with TCRs specific to bacterial Ags or SEA. Although to our knowledge no SEA specific MHC-II tetramers exist, a TCR Tg line has previously been generated against the major egg Ag Sm-p40[72]. Moreover, similar to that observed during *H. polygyrus* infections, it would be fascinating to assess whether S*chistosoma* spp. evoke the generation of Th1/Th2 hybrid cells that simultaneously express T-bet and Gata-3, and whose dual

expression allows endorsement of both Th1 and Th2 profiles but triggering less pathology than their single identity counterparts[70,73]. Finally, it is important to stress that the cytokine profiles shown here are in response to PMA/ionomycin stimulation, so represent cellular secretion *potential* but not necessarily what occurs *in vivo*. For example, upon PMA/ionomycin stimulation MLN $CD4^+$ T cells from infected mice were capable of producing IL-5 and IL-13. However, lymph nodes are known to lack adequate signalling to support full production of IL-5 and IL-13, with Th2 cells instead acquiring full effector functions upon migration from the MLNs to the tissues, potentially in response to local damage cues[74,75]. Use of cytokine reporter mice, as informatively employed in other helminth infections[76], would provide better clarity on the actual contribution of cellular subsets to the cytokine production during active schistosome infection.

After developing a reliable protocol for intestinal immune cell isolation from schistosome infected mice, deeper interrogation of immune cell phenotypes, activation statuses and effector functions are warranted. Multi-parameter flow cytometry and imaging would allow for probing into cellular dynamics, while FISH could identify the localisation of bacteria to infiltrating cell types and RNA-seq (single cell and bulk tissue) would highlight cells, molecules and mediators of interest. Tying together the work of Chapters 4 & 6, establishing the dynamics of colonic Breg and Treg expansion would be of particular interest. For instance, B cells are known to contribute to downmodulation of hepatic[77] and intestinal[78] granulomas, but a convincing role for IL-10 producing B cells specifically has not been established. B cell IgA production could also be addressed, with IgA shown to prevent bacterial invasion into underlying tissue and to select for colonisation of beneficial commensals[79].

A novel result that would be interesting to pursue further is our detection of Th2 mediators RELMα and Ym1 within the faeces of infected mice. These data not only highlight the exuberance of Type 2 immunity during schistosomiasis, but also provide an innovative and non-invasive approach to characterise intestinal immunopathology, which could be of great value to human studies in particular. Indeed, faecal profiling has been used in cirrhosis patients to investigate local inflammation and barrier permeability[80]. Future studies could address which mediators are detectable in human faecal samples, as well as whether molecules such as AMPs can be quantified within murine or human faeces.

The small intestine and colon are drained by spatially segregated MLNs which, during *S. mansoni* infections, have shown to differ in their Ag specific cytokine responses[81]. This includes similar levels of Th2 responses between LNs draining the small intestine and colon, but restriction of Th1

responses to the colonic LNs[81], in line with several studies indicating that Ag specific immune responses can be influenced by the microbiota[82]. For example, MyD88$^{-/-}$ mice, which have disrupted responsiveness to a wide range of TLRs, display intact Th2 responsiveness but impaired IFNγ production during *S. mansoni* infection[82,83]. As the colon is exposed to a higher density of microbes than the small intestine[84], it is possible that the microbiota has a greater 'bystander' influence on schistosome-specific immune responses int this location. The experiments in this thesis were conducted using combined MLNs, as opposed to separating those that specifically drain the small intestine and colon. Experiments investigating cellular composition of individual LNs and use of microbial-specific stimulations, would help clarify the extent to which the microbiota governs individual MLN responses during infection. This approach could also be of value when attempting to ascertain the effect of schistosome faecal transplant on local MLN responses.

Finally, some evidence suggests a 1-2 week delay between mesenteric egg deposition and the appearance of eggs in the faeces[85]. Within this time frame deposited eggs have been suggested to release cytotoxic secretions that remodel proximal tissue, enhance vascularity and modulate inflammatory cell function in a manner that renders them dysfunctional in terms of anti-egg defences [85]. Ultimately, this may facilitate greater egg accumulation in gut associated lymphoid tissue, reduced egg 'spill-over' into hepatic tissue and reduced intestinal tissue mass for eggs to transit[85]. The studies in this thesis focused on cellular alterations within the MLNs and colon at week 7 of infection, but it would of value to investigate and compare colonic and lymph node cellular and immune changes in the first few days following egg production.

Microbial contribution to schistosome elicited immune responses

The intestinal microbiota can have a dramatic influence on the establishment and resolution of intestinal and extra-intestinal diseases, with faecal microbiota transplants showing great potential to rectify 'diseased' / dysbiotic microbiotas and confer a health benefit to the transplant recipient[86]. From the work in this thesis, we are not suggesting schistosome faecal transplants to be used clinically. Rather, we advocate for more refined identification of the microbial and metabolic components within the schistosome-infection associated microbiota, with the aim of identifying immunogenic and therapeutically rewarding candidate molecules. As later discussed, we believe the findings of this thesis will have particular implications for study and treatment of inflammatory mucosal disorders, as well as enhancing our broad understanding of parasite adaptation to the host.

Type 2 immunity is commonly mounted at mucosal surfaces to ward off helminth infections and promote wounder repair[87]. As made clear by GF and Abx experiments, the microbiota is strongly

involved in the regulation of Type 2 immunity[88–90]. However, the precise factors involved in this coordination are poorly defined. Thus far, microbiota elicited Treg populations have shown to regulate Type 2 immunity[90,91]. However, unlike Th17 and Treg responses[32,92–94], no studies have directly implicated specific bacteria in the generation of Type 2 immune responses. In Chapter 4, we show that receipt of a schistosome-infection associated microbiota provokes development of intestinal Type 2 immunity, as evidenced by increased MLN $CD4^+$ T cell IL-4 production, following a 2 or 3 week recolonisation period (Diagram 2). These data strongly suggest that the schistosome microbiota harbours Type 2 polarising agents. However, to solidify these conclusions, more in-depth analysis of faecal donor recipient microbiotas should be performed. For instance, shotgun metagenomics to define bacterial species and their functional potential, alongside advanced culturomics[95] and more refined gavage with candidate molecules. It would also be of interest to evaluate the kinetics of IL-4 expression in these faecal transfer experiments, to identify the timepoint of peak of IL-4 production, and thus the ideal timepoint post gavage to evaluate host immune responses.

In addition to MLN IL-4, $CD4^+$ T cells from these same mice had a slightly an enhanced propensity to produce IL-17 and had lower expression of Foxp3, which is interesting given the that microbiotas derived from IBD patents elicit similar responses[96]. Experiment repetition to boost n numbers and confirm these trends is required. With no coinciding increase in the Th2-associated transcription factor Gata-3, these data suggest a unique population of pre-primed T cells, that is able to rapidly act and produce IL-4. Alternatively, this may represent a transient Type 2 wave, similar to that observed in the newly developed lung[97]. From an evolutionary stance, the microbial instruction of IL-4 could ensure rapid Th2 polarisation following egg exposure or repeat infection, which would be of benefit to the parasite and host considering the importance of intact Th2 responses in schistosomiasis survival[19,98,99]. On this basis, it would be interesting to assess whether schistosome microbiota recipients produce greater levels of IL-4 than naïve microbiota recipients upon local egg challenge or schistosome infection, or whether these faecal transfers impact the development of other mucosal inflammatory conditions, including colitis. Further clarification on the identity of this T cell subset is required, placing particular emphasis on Gata-$3^{lo/-}$ T follicular helper (Tfh) cells[100], which have previously shown commitment to IL-4 production in a HDM system and able to produce greater IL-4 than neighbouring Th2 cells[75]. In addition, upon HDM challenge, IL-4 committed Tfhs appears capable of differentiation into Th2 cells[100]. Moreover, previous studies have described Tfh cells as IL-4+ in Type 2 settings, whilst negative for IL-13 and IL-5[101,102] and with our data fitting this

trend. Future studies ascertaining the presence of these IL-4 committed TFhs in schistosomiasis are warranted (e.g. by IL-4 cytokine protein reporter mice alongside inclusion of XCR5, PD1 and BCL6 in future flow cytometry panels), with such analysis potentially revealing a new mechanism by which schistosomes ensure a Th2 polarised environment is rapidly developed by the host.

The faecal transplants described in Chapter 4 were performed using faeces from naïve mice, or faeces pooled from high dose and low dose infected mice (week 7 of infection). Although it would have been ideal to compare faecal transplant from the two infection groups, this was not possible for technical reasons, including limited numbers of GF mice. Thus, it currently remains unclear whether the MLN IL-4$^+$ phenotype is driven by components of the high and/or low dose microbiota, which should be investigated in the future. Given the immune and permeability data presented in this chapter (and depicted in Diagram 3), we anticipate that factors in the high dose microbiota will be more apparent and so more effective at evoking this outcome. However, it's also completely possible that both low and high dose infections select for common microbiota that induce this MLN IL-4$^+$ CD4$^+$ T cell phenotype. From an evolutionary perspective, it makes sense that, irrespective of infection dose, schistosomes (and the microbiotas they induce in their host) select for this unique T cell subset, so that IL-4 can be produced in response to imminent damage. Moreover, if we do visualise an enhanced IL-4 propensity upon receipt of both high and low dose microbiotas, this would help us narrow down which microbiota element is responsible. i.e. by identifying which metabolites or bacteria show conserved expansion in both low dose and high dose infections. Finally, through the employment of control gavages (naïve faeces spiked with parasite eggs), we showed that this immune modulation is not driven by egg Ags. On the contrary, the administration of eggs could evoke tolerance to SEA, as opposed to immune activation[103].

Our faecal transplant studies principally focused on MLN responses. Future scrutiny of how the schistosome infection associated microbiota impacts local LP responses and myeloid activation profiles is warranted. For example, it is possible that we are missing a key immune phenotype within the LP. Moreover, our data from high dose and low dose infected mice demonstrates that MLN responses do not always mirror what occurs in the intestine (Chapter 4;). Macrophages are of specific interest in this context, due to their known role in wound repair[104], and also given our observation that microbiotas of chronically infected mice were capable of skewing pulmonary macrophages of Abx-treated allergic mice towards an M1-like profile while enhancing the potential of pulmonary CD4$^+$ and CD8$^+$ T cells to produce IFN-γ (Chapter 6; Diagram 4). This curious observation somewhat contradicts our earlier observation of enhanced IL-4 production from MLN

$CD4^+$ cells upon a receipt of a schistosome microbiota (Chapter 4; Diagram 2). However, it is possible that in response to antibiotic treatment these airway macrophages now respond inappropriately to HDM treatment and develop a more pro-inflammatory profile[49], which can be restored through recolonisation with naïve or single-sex microbiotas, but not mixed-sex. In addition, as Abx-treatment does not completely eliminate all intestinal bacteria[33], it could be that remaining bacteria (i.e. those not cleared by Abx) have impacted the recolonisation potential of transferred microbiotas, and led to the growth of different bacterial species. Thus, future HDM experiments should be repeated in a GF system, with additional inspection of MLN responses to evaluate whether the IL-4 phenotype persists here. Moreover, it is fascinating to consider that M1 polarisation in the lung may be 'intentionally' induced by the schistosome infection associated intestinal microbiota. If so, this instruction may help counteract pulmonary Type 2 inflammation during post-patent acute infection which could influence the survival of lung stage schistosome larvae and/or promote immune regulation across egg breached tissues (i.e. by counteracting zealous egg evoked Type 2 reactions).

As mentioned above, it remains unclear which microbial factors transferred via faecal transplant may support the induction of MLN $IL\text{-}4^+$ $CD4^+$ T cells. We performed 16s rRNA sequencing on the large intestinal content of faecal transplant donor and recipient samples in an attempt to ascertain the bacterial communities responsible for this immunogenic effect. The output of this analysis was inconclusive, with the microbiotas of faecal transplant recipients being fairly comparable in terms of the top 20 most abundant bacterial genera. However, we did observe considerable levels of *Alistipes* and *Akkermancia* in schistosome microbiota recipients. *Alistipes* members are potential sources of SCFAs and coordinators of liver fibrosis[105], while the *Akkermancia* genus has been associated with preservation of gut barrier integrity[106]. A critical next step will be to perform shotgun metagenomics and metabolomics analysis on recipient faecal samples. Such analyses will provide species level information on bacterial, fungal and viral communities, allowing greater inference into the pro- or anti-inflammatory nature of these microbial shifts. In addition, it is important to realise that the immunogenic effect we observed is unlikely caused by one single microbial species but instructed by alterations in the entire ecosystem.

Given that we have shown that the microbiota composition continuously changes across the course of infection (Chapter 4; Diagram 2) it would be interesting to perform the same faecal transfer experiments into GF mice using donor material from chronically infected mice. With regulatory cell networks prevailing during chronicity (Chapter 6; Diagram 4), we predict receipt of microbiotas from chronically infected mice will elicit a more immunosuppressive phenotype. Indeed, when faeces

were transferred from chronic schistosome infection into Abx-treated IL-10 reporter mice, we saw subtle increases in MLN and splenic IL-10 ((Diagram 4), but more experimental repeats are required to confirm whether these observations are meaningful (Chapter 6). It is possible that these increases could be clearer in a GF system, without residual recipient bacteria (i.e. those not completely cleared by Abx treatment) interfering with colonisation by transferred samples. However, for the interpretation of these results and studies going forward, it is important to realise that recipient microbiotas are often poor emulations of donor microbiotas. This is due to high bacterial loss during the transfer process (emphasis on anaerobes) and the inability of some bacterial species to be primary colonisers[39]. Indeed, even when processed under strict anaerobic conditions, approximately 50% of processed material is unviable, and is reduced further upon in ambient air or upon freeze-thawing[39]. To promote bacterial engraftment, future studies should include multiple faecal inoculations, as opposed to one single gavage. This modification would theoretically allow for initial colonisation with oxygen-tolerant microbes, which through their consumption of oxygen (and alterations in gut redox state) would create an environment suitable for oxygen-sensitive strains to colonise[107,108]. Further, when fresh material is unavailable, gavages should ideally be performed with 5 minutes of sample thawing to limit oxygen exposure[107]. Culturomics (the definition of microbial composition, and the conditions needed for their growth, through high throughput bacterial culturing[109]) will also advance future transplant or oral gavage based studies[95].

The studies in this thesis open the gateway to many new and exciting experiments, aiming to elucidate the immunogenic properties of schistosome infection associated microbiotas and metabolomes. For example, to assess whether faecal metabolites specifically contribute to host immune skewing, we could perform similar faecal transplant studies but making comparison between schistosome microbiotas and their egg free or faecal filtrates. Using a similar approach to that of *Shute* et al, these faecal filtrates could be applied rectally as to limit their absorption of destruction within the upper GI tract[110]. Although, with this technique, there can be unpredictable absorption and damage to the rectum, and ensuing infection[86]. To determine the pathways and cell types by which these metabolites act, our investigations could be conducted in a range of transgenic mice. Strains of particular interest include free fatty-acid receptor-2 (ffar2) $^{-/-}$ mice, which lack the SCFA-sensing G-protein-coupled receptor (GPCR; GPCR43) on colonic epithelium and immune cells[110,111], 4get/KN2 mice that allow simultaneous analysis of IL-4 transcripts and protein secretion[76] and, given our data highlighting a central for CD11c^{+} cells in the regulation of schistosomiasis pathology (Chapter 3), use of transgenic mice permitting global deficiency or inducible depletion of cDC2s (*Cd11c*^{cre}x*Irf4*^{flox} [112] and *Mgl2*^{DTR} [113] mice) and cDC1s (*Batf3*$^{-/-}$ [114]and

Xcr1^{DTR} [115]mice). Moreover, given the enhanced levels of TMA in the serum of mixed-sex infected mice (Chapter 6), it would be interesting to inhibit TMA in future transplant and infection studies through administration of its dietary inhibitor iodomethylcholine[116], or as a reciprocal experiment, placing mice on TMAO rich diets[117].

While we and others have previously shown that schistosomes can alter composition of the host intestinal microbiota[37,118,119], the exact factors mediating these changes are unknown. In Chapter 4, we showed that high and low dose infections differ in their influence over microbiota structure but display similar immune profiles in terms of $CD4^+$ T cell cytokines, transcription factors and myeloid cell frequencies within the MLNs. Although these microbiota sequencing experiments were performed with low numbers (n= 2-4), and thus require repeating, these data may imply that that schistosome-driven dysbiosis is primarily evoked by egg migration and its associated tissue damage, as opposed to host immune responses. However, it is important to highlight that in contrast to the MLNs, colonic T cell profiles differed drastically between low dose and high dose infections (*discussed in greater detail in previous sections*). To better ascertain the factors underlying schistosome-evoked microbial modifications further comparison of low vs high dose infections should be conducted, with emphasis on IgA secretion and binding to bacteria, mucins, and antimicrobial peptides or proteins (AMPs). IgA dictates microbial composition and excludes bacterial dissemination to underlying tissue through toxin neutralisation and select bacterial agglutination[120]. Mucus structure determines bacterial colonisation by provision of nutrients and attachment sites[121], while Intestinal AMPs can rapidly destroy and inactivate microbes, sculpting microbiotal diversity and abundance, while restricting bacterial contact with the epithelium to limit their invasion of host tissues[122]. In our high vs low dose experiments (Chapter 4) we showed levels of host derived RELMα to be greatly increased in the faeces of high dose infected mice in comparison to low dose counterparts. Although RELMα is commonly associated with the negative regulation of type 2 immunity[123], it has recently been ascribed antibacterial properties within the skin[122]. Whether RELMα hails similar properties within the intestine is unknown, but it's relative AMP RELMβ, has been shown to eliminate Gram negative bacteria within the intestine and limit their contact with underlying host tissue[124]. Another AMP that demands further attention is small proline-rich protein 2A (SPRR2A), which is elevated in in the intestine of *H. polygyrus* infected mice, which shapes bacterial communities via its bactericidal activity against Gram positive bacteria, and limits their invasion into the intestinal barrier[125]. Importantly, SPRR2A production is triggered by Type 2 cytokines (IL-4 and IL-13), suggesting that helminths use their strongly Th2 polarised environment

to promote selective AMP secretion, and thus microbial modifications. Going forward, techniques such as IHC, qPCR and ELISA should be employed to assess intestinal levels of candidate AMPs during schistosomiasis. If promising, we could address their role in modifying the schistosome infection associated microbiota through their neutralisation or use of global deficient mice (e.g., Retnla$^{-/-}$ mice) combined with bacterial profiling (i.e. 16s rRNA sequencing or shotgun metagenomics).

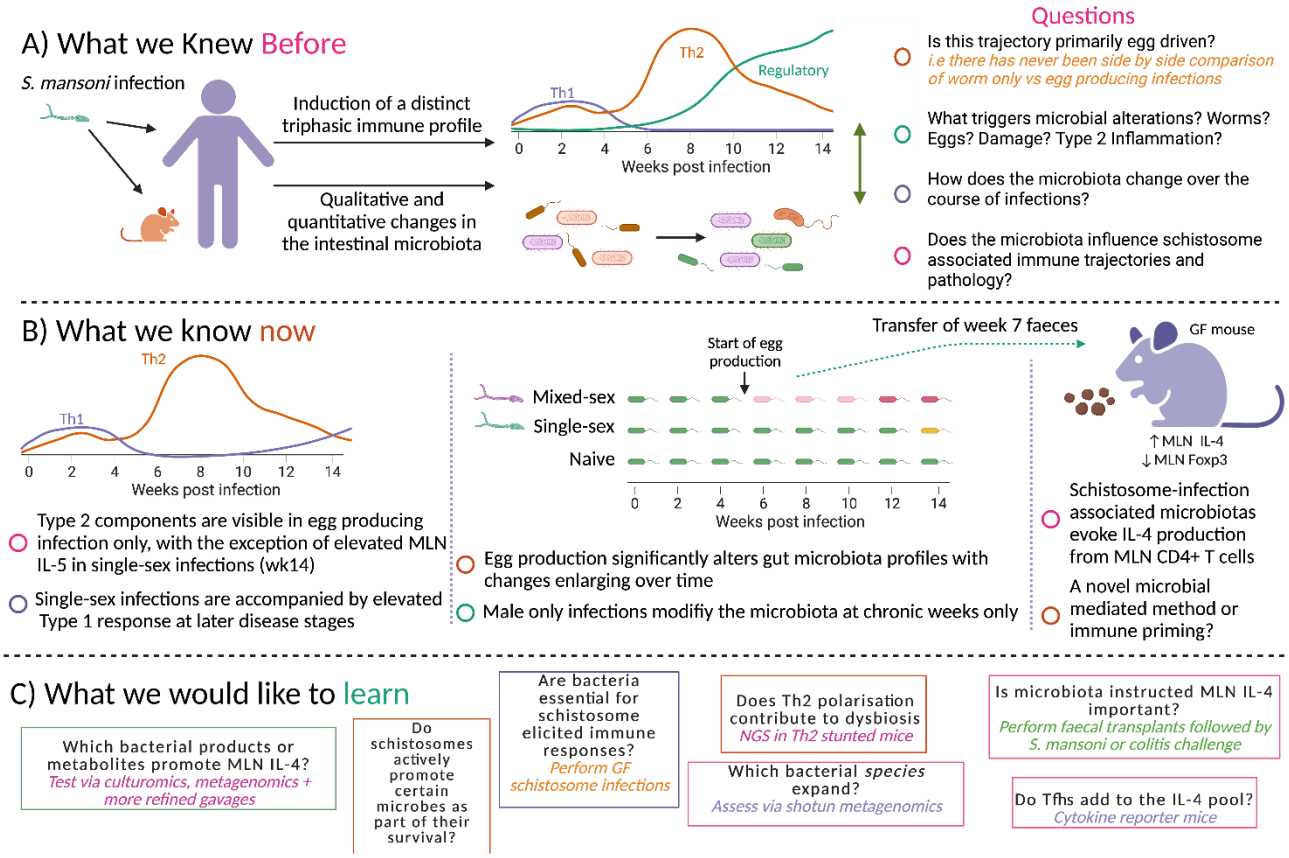


Diagram 2. Investigating the consequences of *Schistosoma mansoni* infection and associated faecal transplants on host immunity, and areas of follow-up study. (A) Before the work detailed in this thesis, infections with *S. mansoni* parasites were known to evoke triphasic immune profiles (i.e. Th1, Th2 and regulatory elements) and alter the composition of the intestinal microbiota at the onset of egg production (mice) or at undefined points of patent infection (human). However, it was unknown whether microbiota profiles changed over the course of infection, whether these microbial modifications were evoked by worms, eggs or egg driven damage, and whether schistosome-infection associated microbiotas contribute to the immune trajectory of schistosomiasis. (B) We have found that conventional egg producing infections provoke pronounced Type 2 immune responses, with evidence for minor Th2 polarisation by male worms alone at late stages of chronic infection (14 wk). Although Th1 profiles are often only acknowledged during pre-patent acute stages of schistosomiasis, we found evidence for more pro-inflammatory Th1 orientated responses during chronic disease in worm only infections, at a time point when microbiota composition was also shown to differ between single-sex infected, mixed-sex infected and naïve mice. Microbiota structure changed dramatically from the onset of egg production, with considerable differences between low dose and high dose microbiotas, and acute vs chronic microbiotas. The transfer of faeces from mice infected with schistosomes for 7 weeks into GF animals induced local MLN IL-4$^+$ CD4$^+$ T cells. To our knowledge this is the first report showing helminths ability to promote resident microbes that support intestinal IL-4. (C) The work in this thesis raises many new questions and experiments, focussing on deciphering which components of the schistosome-infection associated microbiota possess immunomodulatory potential.

Chronic or high dose infections compromise intestinal barrier integrity

Schistosome eggs rupture across the intestinal wall, and in this process evoke chronic tissue damage, GI bleeding and enhance epithelial leakiness (Chapter 4; Diagram 3). Remarkably, despite the intense damage infection causes, we found very little evidence for septicaemia or bacterial translocation, with the exception of increased serum levels of TMA (luminal product produced by bacterial mediated fermentation; Chapter 6) and commensal specific antibodies (Chapter 4). Although not presented in this thesis, we failed to detect elevations in systemic IFN-γ or TNF-α, two proinflammatory mediators commonly associated with septic cytokine storms[126]. Hence schistosomes, like other helminths[127,128], likely induce potent mechanisms to facilitate rapid and effective repair of the intestinal wall, whilst preventing the passage of harmful luminal content into underlying tissues and circulation.

The work presented in this thesis sets the groundwork for future studies uncovering the mechanisms involved in the regulation and repair of the intestinal wall during schistosomiasis. This may have broader implications and translatability to other inflammatory GI conditions, including inflammatory bowel disease. Thus far *Schistosoma* parasites have been shown to be capable of nullifying high endotoxin exposure through the actions of their secreted proteases, which can induce Tregs and inhibit the release of pro-inflammatory factors (such as TNF-α, IL-6, NO and IL-12) from macrophages[129–132]. Based on the work shown in this thesis, it would be very interesting to evaluate whether factors within the schistosome infection associated microbiota contribute to barrier repair or promote the anti-bacterial activities of local immune cells or epithelial cells. [53]Moreover, as LPS from different bacteria have different immunostimulatory profiles (based on side chain acylation)[133] it is tempting to suggest that schistosomes may actively promote the growth of select, anti-inflammatory microbes.

The role of a schistosome-associated microbiota in barrier defence could be investigated through a variety of means. For instance, after identifying candidate microbes or their derived products by a combination of shotgun metagenomics and metabolomic platforms, these bacterial cocktails or isolated products could be applied to experimental models of GI inflammation, with readouts of intestinal leakiness and inflammation. Moreover, with these candidate microbial factors likely mediating their effects through local immune populations, this work should be conducted in select transgenic mouse lines lacking candidate immune cells or effector molecules. If time permitted, our work would ideally be supplemented with Fluorescence in situ hybridization (FISH) analysis of intestine from infected mice, with a bacteria-specific 16S rDNA probe to look for bacterial

localisation relative to sites of damage and granuloma development[134]. This, in combination with Hyperion imaging mass cytometry (enabling visualisation of up to 44 targets simultaneously) would allow us to spatially define the cell types responding to bacterial invasion, and or contributing to intestinal restoration and repair.

With the liver being central to schistosomiasis immunobiology and the clearance of intestinally derived bacteria and toxins[135,136], future studies should address the hepatic contribution to sepsis control during schistosomiasis. For example, resident Kupffer cells are uniquely poised for the rapid elimination of intestinal pathogens, with their antimicrobial functions programmed by commensal derived D-Lactate[136]. It is possible that factors specific to the schistosome microbiota also program bactericidal properties of Kupffer cells or other hepatic populations. Alternatively, this could be influenced by the deposition of worm regurgitation products (such as hemozoin[137]) in the liver.

Finally, in terms of human work and translatability, an important next step would be to obtain stool, serum, liver, and intestinal biopsy samples from schistosome infected individuals, pre and post praziquantel administration (depending on the invasiveness of the sample collected). This could allow for high-resolution histological assessment of egg-driven damage, paired with information on infection intensity, intestinal microbiota structure, and the reactivity of circulating T cells to schistosome specific Ags or intestinally-derived commensals. It would also be fascinating to assess intestinal permeability in infected persons, for instance by performing dual saccharide mannitol lactulose permeability tests[3].

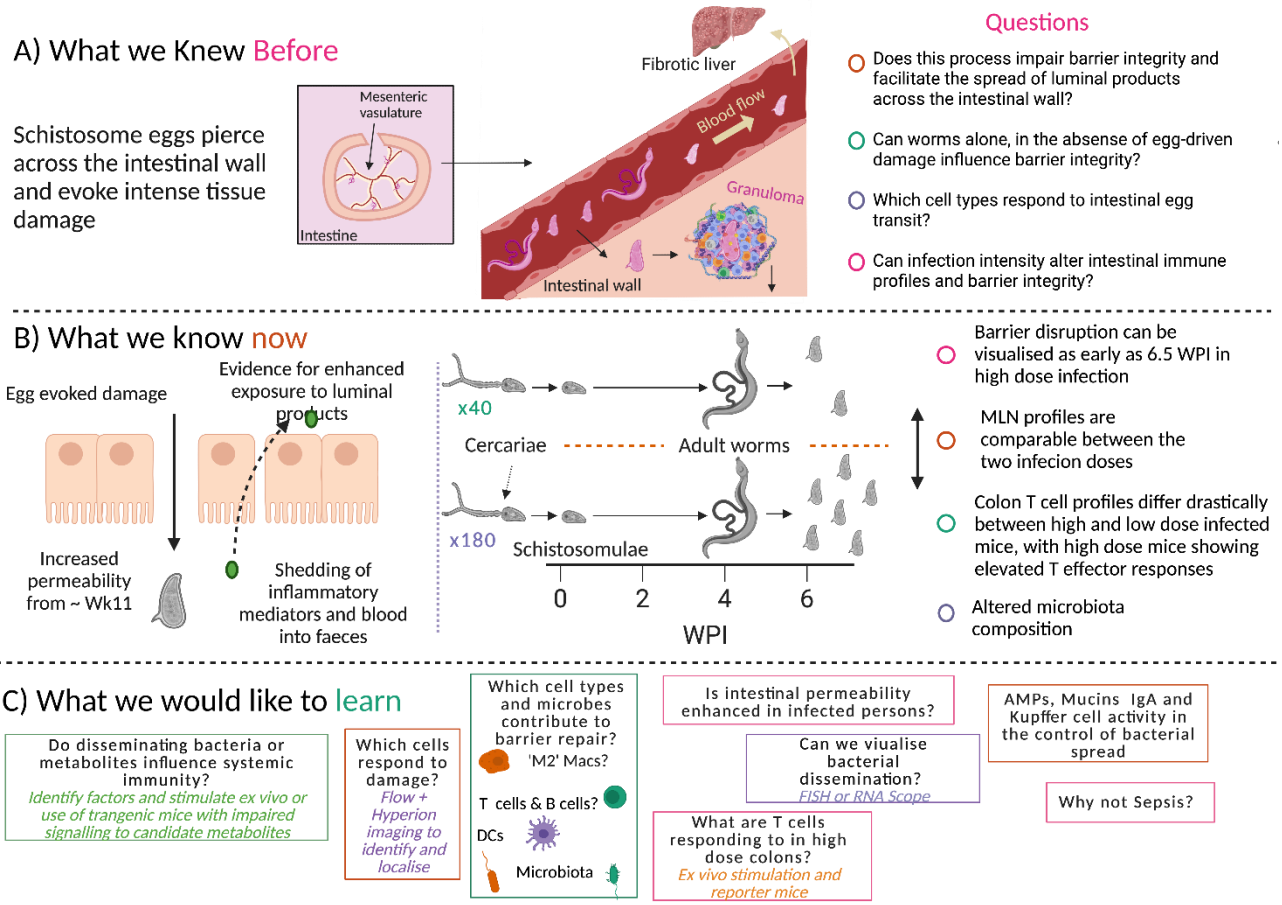


Diagram 3. How schistosome egg migration impacts the intestine and avenues for future study (A) Schistosome eggs pierce across intestinal tissues and cause extensive damage in the process. It was previously unknown how this process influences barrier integrity, and whether infection dose impacts the kinetics of these changes. Furthermore, due to the exuberant nature of schistosome evoked mucosal immune responses, isolation of cells from the intestines of schistosome infected mice is challenging, so our understanding of cellular responses to intestinal egg migration was previously lacking. (B) Chronic and high dose infection impaired intestinal integrity, with release of inflammatory mediators into host faeces and evidence for enhanced systemic exposure to gut-derived products. Generation of a reliable and reproducible intestinal cell isolation protocol demonstrated significant differences in immune profiles between patent high dose and low dose infections, most evident in terms of T cell polarisation and cytokine production potential. (C) It is currently unclear whether disseminating luminal products have the capacity to influences systemic responses to schistosomiasis, and what cell types and effector molecules underly repair of the intestinal barrier and sepsis control. Furthermore, greater resolution is required regarding the cell types responding to egg damage, and the contribution of humoral immunity, mucins, AMPs and liver macrophages in bacterial localisation.

Evidence for microbial assistance in schistosome-mediated protection against AAI

Hyper-inflammatory disorders such as allergy and autoimmune disease are less prevalent in helminth-endemic regions[59,138–140], and children infected with *Schistosoma* parasites show less skin prick test reactivity to HDM[141]. Animal models have yielded similar results, with many studies showing *Schistosoma mansoni* infections or egg exposure to alleviate inflammatory disease[142–147]. Given these findings and knowing that the capacity of some experimental helminth infections to downmodulate mucosal inflammation is partly microbially mediated[110,148,149], in Chapter 6 we questioned the potential of a schistosome infection-associated microbiota to influence AAI driven by HDM. Our results indicate that the microbiota associated with schistosome infection, both egg-producing and non-egg producing, has the potential to downmodulate airway disease (Diagram 4), although further experiments are required to warrant firm conclusions. This includes repeat experiments in different allergic models as well as in GF mice to control for the immune perturbating effects of Abx[49]. Histological analysis is also necessary to confirm AAI reduction by faecal transplant from schistosome infection, which time limitations prevented.

The microbiota is intimately involved in the training, maturation, and function of host immunity at both local and distal locations[150,151]. Recent experimental and epidemiological studies highlight the extensive cross-talk between the intestinal microbiota and the airways, in what is referred to as the 'gut-lung axis'[151], with both microbiota composition and early life exposure having a huge impact on allergic airway disease development[150,152,153]. We currently have only a very basic mechanistic understanding of which specific microbial factors or metabolites may confer protection against allergic diseases, with microbial-derived SCFAs having been acknowledged for their anti-allergic properties[150,151]. For example, high childhood levels of butyrate and propionate are associated with reduced atopic sensitisation and asthma development[154], with protection potentially conferred by the seeding of lungs with highly phagocytic DCs with impaired Th2 inducing capacities[2]. Aside from SCFAs, certain gut commensals associated with allergic relief show probiotic potential[150], but no other candidate molecules have received such thorough attention. We did not observe significantly increased SCFA levels in the serum (Chapter 6) or faeces (Chapter 4) of schistosome infected mice, suggesting the presence novel immunomodulatory molecules conferring disease protection in our system, but due to low n numbers, experiment repetition is required to confirm this. One obvious candidate may be TMA, although this luminal product was only shown significantly elevated in the serum of mixed-sex infected mice, and in other disease systems this metabolite is associated with disease exacerbation, especially cardiovascular disease [155].

One possible next step would be to perform next generation sequencing (NGS) and metabolomic analysis on various compartments of microbiota recipients (e.g. serum and intestinal content), allowing for candidate molecule identification and subsequent testing in allergic systems. Importantly, the therapeutic application of single, well-defined microbial/metabolic products circumvents the uncertainties and risks associated with faecal microbiota transplant or live helminth infection and would therefore be of great advantage. For example, it is unclear how long the microbiota is reprogrammed following bacterial therapy, and probiotics on the whole have yielded little clinical evidence to support their use[150]. Regarding helminth therapy, the promise and potential of initial trials cannot consistently be replicated[156], and mixed-sex egg producing S*chistosoma* infections are highly unethical due to the pathological damage elicited by eggs. Indeed, prophylactic application of live helminths is generally not feasible, risks side effects and would require identification of those with a genetic pre-disposition to allergy. Moreover, as the mammalian immune system is at its most malleable during early life (i.e. infancy and gestation)[59], exposure to potential immunomodulators should ideally be carried out during early years. The use of individual, well characterised helminth products would offer a clear advantage here.

We have shown that the microbiota associated with schistosome infection reduced the infiltration of cells to the airways but increased total lung cell counts (Chapter 6), suggesting factors able to affect chemotaxis. Inspection of BAL chemokine levels could confirm this speculation, but time restraints prevented this. With regards to cellular analysis, closer inspection should be paid to pulmonary macrophage and DC subsets, which are capable of producing distinct soluble mediators (e.g. CCL11, CCL17, CCL22, CCL24 and TGF-β1) that can inhibit or promote cellular recruitment during experimental allergy[157,158]. To confirm the M1 pulmonary macrophage skewing that was evident following microbiota transfer from egg-producing schistosome infection (Chapter 6; (Diagram 4), assessment of cytokine secretion (including proinflammatory TNFα or anti-inflammatory IL-10) could be conducted, with these macrophages derived from GF or Abx-treated recipients, in case Abx treatment contributes to dysregulated cytokine secretion. Moreover, the lung is not a sterile organ, but contains a diverse consortium of microbes suggested to influence allergic development[159]. Upon faecal transplant, intestinal bacteria will inevitably be seeded within the airways[160], and so potentially influence the function of local airway immune cells. Indeed, colonisation of the airways with certain bacterial species enhances the risk of developing airway diseases in children[153], and asthmatic patients showing resistance or sensitivity to corticosteroid treatment demonstrate[161]. NGS with metabolomic analysis should be conducted on the respiratory and intestinal microbiota of faecal transplant recipients.

Our lab previously showed schistosome infection-induced pulmonary[142]and splenic Bregs[143] to reduce AAI, with splenic B cells gaining additional support via the induction/recruitment of active FoxP3$^+$ Treg cells[142]. Future studies should address whether the microbiota associated with schistosome infection can instruct Breg and Treg generation in the absence of infection, and whether these microbial-driven regulatory cells contribute to AAI alleviation. For example, this could be achieved through the transfer of naïve or schistosome infection microbiota instructed B cells into HDM sensitised mice and assessment of allergic inflammation following challenge. Taking a similar approach to Van der Vlugt *et al*[143], these studies could include blocking IL-10 signalling, or use of Foxp3$^+$ T cell depleted DEREG mice, in order to ascertain whether these B cells provide protection in an IL-10 and Treg independent manner. Moreover, and as mentioned previously, although our preliminary IL-10 reporter experiments (Chapter 6) suggested components of the schistosome infection associated microbiota promote B cell and T cell IL-10 (Diagram 4), repeat experiments are required to draw concrete conclusions. It is also possible that subtle increases in IL-10 could become more convincing upon additional *ex vivo* stimulation, or *in vivo* challenge (e.g. HDM exposure or schistosome infection). Finally, it is worth mentioning here that in our 'simple' GF faecal transplant experiments (Chapter 4), transfer of a schistosome-infection associated microbiota reduced MLN CD4$^+$ T cell Foxp3 expression, and in our HDM experiments (Chapter 6) we did not observe differences in pulmonary CD4$^+$Foxp3$^+$ expression between faecal transfer groups. Thus, although these experiments suggests that a schistosome infections associated microbiota is not capable of supporting Foxp3$^+$Treg activation or recruitment, these microbiotas could be capable of supporting the expression of other putative Treg associated markers, including CTLA-4, GITR or Helios.

Another interesting observation from our experiments was the capacity of the schistosome infection-associated microbiota, particularly from single-sex infections, to enhance pulmonary and splenic CD4$^+$ T cell expression of CTLA-4, where CTLA-4 an indispensable regulator of T cell activation, that is expressed on Tregs and whose absence leads to lethal lymphoproliferative disease[162]. Our data suggest microbial factors may promote the expression of these putative regulatory cell markers, potentially revealing a new mechanism by which schistosomes modulate host immunity. Although the intestinal microbiota has shown to influence of the efficacy of CTLA-4 blockade in cancer treatment[163] the contribution of intestinal communities or products in CTLA-4 induction is unknown.

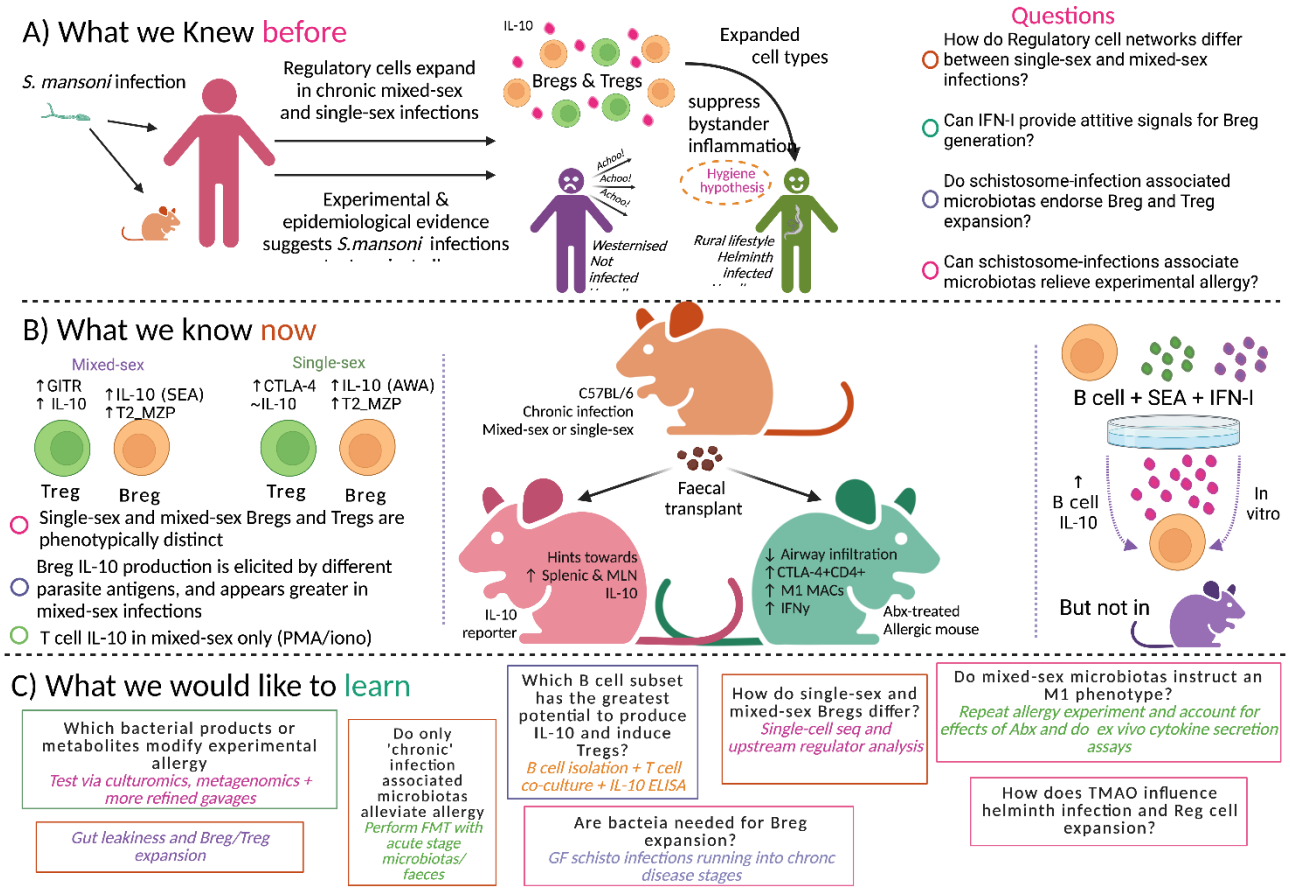


Diagram 4. Defining the contribution of schistosome and microbial signals in regulatory cell expansion and allergy prevention, and areas for future study. (A) Chronic schistosome infections are accompanied by the profound expansion of Treg and Breg networks, whose immunosuppressive activities are thought to partially underly schistosome-mediated protection against hyper inflammatory conditions, including allergy. However, the signals dictating Treg and Breg expansion during schistosomiasis are not fully known, and it is possible that the microbiota may play a participatory role. (B) In this thesis we show both single-sex and mixed-sex infections are capable of generating splenic IL-10 producing B cells, with phenotypically distinct B cells emerging in each type of infection. IL-10 production by CD4+ T cells was only visible in mixed-sex infections, but further parasite specific stimulation is needed to confirm this. Transfer of mixed-sex and single-sex infection associated microbiotas was shown to modify the severity of allergic disease, strongly hinting for a role of the microbiota in schistosome mediated suppression of bystander inflammation. Finally, although IFN-I were shown to provide additive signals for Breg generation *in vitro*, they appeared *in vivo.* (C) In future work we would like to unravel which specific bacteria, bacterial products or metabolites alleviate allergic disease or promote IL-10 production. However, before these advanced experiments are performed, further repetition of our experimental faecal transplant – allergy experiments are required. In addition, we would like to identify which specific B cell subset is responsible for the majority of B cell IL-10 in each type of infection and further characterise their other immunosuppressive functions. Finally, greater attention on the contribution of TMAO and mixed-sex microbiotas in M1 macrophage polarisation is warranted.

Signals driving schistosome-induced Treg and Breg expansion

Chronic *S. mansoni* infections support the generation of $CD4^+$ T cell and B cell populations with regulatory properties and that can protect against AAI[64,142,143]**.** In Chapter 6, we showed that phenotypically distinct splenic T cell and B cell populations emerge during egg-producing and non-producing infections, with highest B cell IL-10 production propensity during infection chronicity (Diagram 4). These observations contribute to the body of evidence that Breg and Treg populations can be generated by patent and non-patent *S. mansoni* infections[37,63,64,142,143,164], and demonstrate that while single egg-derived molecules (IPSE/alpha-1) are capable of potentiating B cell IL-10[50], worm derived Ags also possess Breg inducing potential. In terms of T cell IL-10, PMA/ionomycin stimulation evoked greater IL-10 expression in splenic $CD4^+$ T cells of mixed-sex infected mice only (Diagram 4). However, as these stimulation conditions bypass TCR interactions, they reflect cytokine *potential* and do not necessarily reveal what occurs during active infection. Thus, further interrogation of T cell cytokine secretion via IL-10 reporter mice or through *ex vivo* stimulation with relevant Ags (e.g. SEA, AWA or worm/egg ES products) are warranted for firm conclusions.

Guided by preliminary transcriptome analysis (*Katja Obliego, unpublished observations*) and previous studies showing that Type I-IFNs can support human[165] and murine Breg generation, we investigated a supportive role for Type-I IFNs in S. *mansoni* driven Breg generation (Chapter 5; Diagram 4). Type-I IFNs were shown to enhance SEA-induced Breg generation in an *in vitro* system, with the majority of B cell IL-10 produced by stimulated MZ B cells, in support of previous work from our lab[50,143]. However, impairment of Type-I IFN signalling, as assessed via egg challenge in $IFNR^{-/-}$ mice, had no measurable impact on splenic Breg IL-10 secretion. These data suggest that while Type-I IFNs provide additive signals for Breg cell expansion, their contribution in a more complex, *in vivo* system is redundant.

Bregs are a heterogeneous population of cells, that can arise from different stages of B cell development and adopt a range of phenotypes and effector functions depending on the stimulus encountered[166]. In the absence of a clear identifying marker, the study of Bregs by flow cytometry is difficult, with a single flow cytometry panel unable to incorporate all potential Breg-associated markers. The Breg field and the work following on from this thesis will undoubtedly benefit from advancement and application of spectral flow cytometry and mass cytometry, allowing incorporation of many more markers into a single panel and thus greater analysis of Breg heterogeneity.

The signals required for Breg induction *in vivo* are complex and highly context specific**,** with phenotypically and functionally divergent Breg populations arising in different disease scenarios and at different stages of B cell development[166,167]. Breg activation signals include a combination of CD40-ligand, BCR Ags, TLR ligands and epigenetic regulation, but building evidence suggests inflammation to be key[166,168]. The generation of B cells in responses to inflammatory signals may represent an evolved mechanism to circumvent overwhelming inflammation and tissue damage. In the absence of cytokine reporter mice, and in order to gage which parasite signals may endorse Breg IL-10, we stimulated isolated splenic B cells from naïve and infected mice with a range of parasite-specific stimuli (i.e. SEA, AWA and worm ES products). B cells from infected mice produced greater IL-10 levels than their naïve counterparts, with mixed-sex and single-sex B cells differing in their IL-10 responsiveness to each stimulation (Chapter 6; Diagram 4). These data hint that separate Breg populations arise in each type of infection. Although we were able to identify the emergence of phenotypically distinct B cell subsets across single sex and mixed-sex infections, technical issues precluded their isolation and subsequent testing of regulatory function (i.e. subset specific IL-10 production and Treg inducing potential). These experiments should be revisited and, cost permitting, complemented with mRNA sequencing and pathway analysis to assess transcriptional changes and to predict pathways leading to their generation.

The expansion of IL-10 producing B cells during male worm only infections has been shown previously[164] but is nonetheless puzzling given the lack of egg driven damage, and thus lack of Breg inducive inflammatory signalling. It is possible that adult worms actively endorse Breg expansion to pre-empt egg mediated tissue destruction. However, if true, why do Breg populations emerge in response to worms alone only during chronicity? It is possible that the worm secretome contains more Breg inducing factors or molecules cross-reactive with SEA during late stage infection, but this has not yet been evaluated.

Many studies have shown the microbiota, microbiota induced products and food derived substance to support Breg induction[55,169–171]. At the same time as Breg expansion, we observed significant alterations in microbiota composition in mixed-sex and single-sex infected mice (Chapter 4), with faecal transplant studies suggesting that these microbiotas may support splenic and MLN B cell and T cell IL-10 (Chapter 6). These studies would benefit from the inclusion of IL-10 reporter mice, interrogation of kinetics of IL-10 responses and inspection of local LP B cell populations. Indeed, although most Breg populations are described in the spleen and lymph nodes, studies from our lab[142] and others[169,172] show non lymphoid structures to also harbour suppressive B cell populations.

Importantly, in non-helminth settings, faecal transplant into GF mice has been shown to significantly increase colonic B cell IL-10 levels, with a clear kinetic timeline of when IL-10 reaches its peak[169]. Furthermore, it would be of special interest to further define microbial-schistosome-Breg relations, with emphasis on T2_MZP B cells, which are exclusively expanded during mixed-sex infections and whose regulatory potential in models of rheumatoid arthritis is dependent on microbially-induced cytokines[55]. This may be particularly relevant to mixed-sex infections, given egg-driven damage and reduction of intestinal barrier integrity (Chapter 4), with potential for luminal products to disseminate systemically.

It remains unclear which signals might mediate *S. mansoni* induction of Treg expansion. Thus far, the schistosome worm derived molecules Cyclophilin A[37] and phosphatidylserine (PS)[173] have shown to endorse Treg expansion through modulation of DC functionality. Schistosome eggs also induce marked Treg response *in vitro* and *in vivo*[174–176]. However, like adult worm products, single-egg derived molecule that can directly induce Tregs have yet to be identified, with studies to date suggesting eggs may indirectly promote Treg functionality through Bregs[50] and DCs[174]. While *H. polygyrus* parasites can directly induce Tregs through the actions of their secreted TGF-β mimic[177,178], no such mechanisms have yet been found during schistosomiasis.

Our work in Chapter 6 suggests that expression of the Treg-associated co-inhibitory marker CTLA-4 may be partially driven by male-worm Ags, while GITR expression is promoted by eggs or their associated inflammation. As certain Breg subsets have been shown to be capable of inducing CTLA-4 expression on Tregs[179], it would be interesting to evaluate whether single-sex evoked Bregs can exert this function.

In our experiments, the expression of Foxp3 or CD25 by splenic $CD4^+$ T cells was unchanged by infection (Chapter 6). Whilst the lack of altered CD25 was unexpected, and contradicts previous reports[180], other research groups have shown unaltered numbers and proportions of splenic $FoxP3^+$ Tregs in infected mice[181], but expansion in the liver and MLNs [63,181]. The work in this thesis indicates that $CD25^+FoxP3^+$ T cell induction is tissue specific and influenced by infection intensity. Indeed, when GF or SPF mice were infected with 180 parasites, we observed upregulation of both CD25 and Foxp3 in splenic $CD4^+$ T cells by week 7 of infection (Chapter 6). Similarly, within the MLNs at this same time point, Foxp3 expression was more markedly increased in high dose infected mice than their low dose counterparts (Chapter 4). In terms of tissue specify, we detected significant numerical and proportional expansion of $CD25^+Foxp3^+CD4^+$ T cells in the liver of infected mice (weeks 8-14),

but only numerical expansion in the spleen and MLNs (with the exception of proportional expansion in the MLN at week 12) (Chapter 3). These data likely reflect the enhanced recruitment of Tregs to the liver to deal with egg driven granulomatous inflammation and suggest that the hepatic environment may be particularly supportive for Treg activity. In the MLNs, it is possible that $CD25^{+}Foxp3^{+}$Treg expansion is more evident than in the spleen[181] due to greater exposure to Ags draining from colonic and small intestinal granulomas or substances within the intestinal lumen.

In Chapter 6 we revealed a potential role for the microbiota in the regulation of splenic Treg populations. In particular, $CD4^{+}$ T cells from schistosome-infected GF mice expressed significantly higher levels of CTLA-4, CD25 and IL-10 than their schistosome infected SPF counterparts, with additional strong trends towards heightened Foxp3 expression. With these GF T cells also demonstrating an enhanced potential to produce IFNγ, our data suggests that in normal SPF conditions, the microbiota may suppress the activity of Treg and Th1 populations, which may result in stronger type 2 activity. In support of this speculation, GF or Abx-treated *S. mansoni* infected mice may develop milder granulomatous pathology than their conventionally SPF raised controls[83,182]. Importantly, aside from this historic GF study[182], the work in thesis provides the first snap-shot of how the intestinal microbiota contributes to T cell polarisation through GF approaches. However, more repeats are required to draw firm conclusions for many of these preliminary experiments. For instance, Breg expansion was not investigated in these GF schistosome infection experiments due to experiments stopping at week 7 of infection (a time-point prior to Breg induction).

Breg and Treg deficiencies have been reported in a variety of autoimmune, metabolic, carcinogenic, and allergic conditions[183,184]. The identification of Breg and Treg inducing agents, or those that bolster their activity, is therefore of huge therapeutic interest - especially if this bypasses the deleterious effects of parasite infections or uncertainties of faecal transplants. In allergic inflammation, promising therapeutic strategies include restoration of airway barrier function and the suppression of Th2 responses through the promotion of Th1 and regulatory responses[185]. The work in thesis highlights schistosomiasis as a compelling model to uncover the mechanisms underpinning regulatory cell expansion and restoration of mucosal tissue injury.

CONCLUSION

Together, the work in this thesis expands on our understanding of schistosome elicited immunity: defining immune disparities between low dose, high dose, patent, and non-patent infections, characterising intestinal barrier dysfunction, and providing detailed analyses of immune cell populations across priming and effector tissues. We have revealed the contribution of $CD11c^{+}$ cells

and Type-I interferons to schistosome induced Type 2 inflammation and Breg expansion, respectively, and highlighted the capacity of factors within the schistosome infection associated microbiota to promote intestinal Type 2 immunity and alleviate AAI. Combined, this thesis provides a platform for future work aiming to identify the cell types, parasite and microbial molecules involved in the regulation and repair of mucosal inflammation and tissue damage, with the potential to harness this information for development of future treatments for diverse mucosal inflammatory diseases.

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ADDENUM

English summary, Nederlandse Samenvatting, Curriculum vitae, List of publications, Contributions, Acknowledgements
















English summary

Parasites are incredibly fascinating organisms, that are hailed for their undesirable attributes (e.g., bizarre appearances and unique behavioural practices) and defined by their need to live off another organism (a host) to grow, feed and thrive. To maintain their long-term survival, complete their infectious lifecycle and lessen infection associated pathology, many parasites have evolved sophisticated techniques and factors to configure, suppress and evade the host immune system. Importantly, although parasites are primarily portrayed negatively, their capacity to modulate immune cells can prove beneficial to host health. More specifically, mounting evidence suggests exposure to parasites and symbiotic microbes (e.g., bacteria and fungi) during childhood is crucial for adequate immune education and protection against inflammatory diseases. This thesis strives to better delineate the mechanisms by which the parasitic Schistosoma flatworm calibrates and modulates host immunity, with emphasis on their impact on the intestinal environment and interactions with tolerogenic B cells.

Schistosomiasis, also known as Bilharzia, is a neglected yet significant tropical disease, with over 240 million cases annually, an estimated 200,000 deaths per year, and second only to Malaria in terms of debilitation. This parasitic disease is widespread across sub-Saharan Africa, regions of South America and Asia, and heavily impacts those in impoverished communities with poor access to safe drinking water. The three most common species of *Schistosoma* blood flukes to infect humans are *S. mansoni*, S. *japonicum* and S. *haematobium*. The two former species are responsible for intestinal disease, whilst *S. haematobium* has consequences for the urinary tract. The focus of this thesis is on *S. mansoni.*

Schistosoma infection starts upon exposure to freshwater harbouring the free-swimming larval form of the parasite, cercariae. These fork-tailed parasites penetrate the skin of their prospective mammalian host, and over the course of several weeks, navigate from the skin, across the pulmonary system, to the blood vessels of the liver and intestine. Here, immature *Schistosoma* parasites (schistosomula) mature into male and female adult worms, which pair and produce egg (up to 300 per day per worm pair) for the remainder of their lifespan, which can be years without pharmaceutical intervention. In an impressive case of immune modulation and protective mechanisms by the host, schistosome eggs are enclosed by host immune cells and fibrotic material, in an immune lesion known as a granuloma. Granuloma formation is required for the translocation of eggs across the intestinal wall and elimination into faeces, where the eggs hatch and complete their lifecycle outside of the mammalian host, in a suitable freshwater snail. Alternatively, many

eggs fail to transit across the intestine and are instead swept with the blood flow to the liver where they lodge and evoke inflammatory granulomas. These egg-evoked inflammatory lesions are central to schistosome-associated pathology, and in severe advanced disease, can lead to fatality.

To enhance the fitness of their host, and thus promote their own survival, schistosomes employ a variety of techniques to calibrate and influence host immunity. This includes the endorsement of Type 2 immunity to support granuloma formation and egg egress, as well as the bolstering of tolerogenic immune cell networks, which serve to down-modulate the potentially exuberant responses directed against them and lessen egg associated pathology. Moreover, as an important bystander effect, the induction of tolerogenic cell types, including regulatory B cells (Bregs) and T cells (Tregs), provides the host relief against non-related immune diseases typified by an overactive immune system, including allergy and autoimmunity.

Experimental (murine) schistosomiasis forms an ideal system to study the pathways and molecules exploited by the parasite to drive host immune modulation. For example, the potent capacity of schistosomes to support Breg and Tregs generation can be studied over time. Similarly, as both schistosomiasis and allergy are hallmarked by Type 2 immune responses, though with completely different clinical symptoms, detailed inspection during experimental schistosomiasis may provide insight into novel cell types and pathways involved in type-2 immune driven host defence and repair, which may have repercussions for novel therapeutic allergy treatments. Finally, by bolstering our understanding of the impact of schistosome egg transit on the intestinal interface, we may identify core mechanisms involved in the regulation and repair of mucosal inflammation, including the involvement of intestinal bacteria.

The first part of this thesis provides a high-resolution narrative of the immune response evoked by schistosomiasis over the course of infection (Chapter 3**)** and offers novel insight into the consequences of schistosome egg transit on intestinal barrier integrity, microbiota structure and mucosal immunity (Chapters 2&4).

The movement of schistosome eggs from the intestinal vasculature across the intestinal wall into host faeces is an enigmatic and intriguing process, involving the passing of multiple barriers (epithelial and endothelial), the recruitment and modulation of immune cells for granuloma formation, and the instruction of repair networks to heal egg driven damage. In Chapter 2, we discuss our current understating of schistosome egg migration, outline the host-parasite-microbial interactions that may enable this process and speculate on the consequences of successful (or unsuccessful) egg transit on the fitness of the host and unrelated diseases.

Schistosomiasis is hallmarked by a distinctive triphasic immune response. This includes a mixed, low-level Type 1 and Type 2 immune response during the initial weeks of infection, an exuberant Type 2 response from the point of egg production (week 5-6), and the emergence of potent regulatory cell networks during infection chronicity (from week 12 onwards). This narrative has slowly been pieced together from hundred of independent research articles, all focusing on different time points of infection and different organs of interest. In Chapter 3 we provide a concise and up-to-date narrative of the immune trajectory of schistosomiasis across the course of experimental infection, focusing on three tissue sites affected by infection: the liver, mesenteric lymph nodes and spleen, using histological, cellular (flow cytometry) and transcriptomic (RNA-seq) based techniques. Further, through the depletion of $CD11c^+$ antigen-presenting cells (including dendritic cells and macrophages) during the initial stages of egg production, we reveal a critical role for $CD11c^+$ cells in the coordination of granuloma responses and Th2 inflammation.

Despite the large impact of schistosomiasis on the intestine, the bulk of immunological studies on *S. mansoni* to date have focussed on the liver (principal site of egg deposition) or more distal sites, including the spleen. This, in part, stems from the notorious technical difficulty to obtain live cells from the schistosome-infected murine intestine, due to the hostile anti-parasite Type 2 immune response evoked here, including excessive mucus production, tissue remodelling, pH alterations and cellular infiltration. We have overcome these technical difficulties and were able to islate live immune cells from the guts of infected mice. In Chapter 4, we provide a detailed characterisation of the intestinal environment during experimental schistosomiasis, with specific emphasis on intestinal microbiota structure, integrity of the intestinal barrier and contrasts between mesenteric and intestinal immune responses. Through the employment of egg producing and non-egg producing infections – by making use of infections with male and female larvae (egg production) or with male larvae only (no egg production) - we show egg deposition to dramatically reduce the integrity of the intestine, modify the composition of the intestinal microbiota and reconfigure the frequency and profile of colonic and mesenteric immune cells. Further, using germ-free mice (devoid of microbiota) and faecal microbiota transfers, we demonstrate the capacity of a schistosome-associated microbiota to instruct components of Type 2 immunity.

The second part of this thesis focus on the induction of splenic regulatory cell networks (Chapter 5&6) and the potential for the microbiota of schistosome infected animals to alleviate experimental allergy (Chapter 6). Previous work from our lab shows chronic schistosome infections to support the expansion of tolerogenic Bregs, which provide relief against experimental allergy, and that both

IL-10 producing Bregs from the spleen and the lung are involved. The cellular mechanisms driving Breg induction remain relatively undefined, but previous studies and unpublished gene expression analysis from our lab points towards a role of cytokines, like Type I interferons, in Breg activation during chronic schistosomiasis. We found Type I interferons to support the expansion of Breg cells in vitro culture systems, but not within the body.

In **Chapter 6**, we continued our search to define regulatory cell inducing molecules during schistosomiasis, using egg producing and non-egg producing infections to tease signals apart. We show both type of chronic infections to expand distinct splenic regulatory cell networks, but with T cells and B cells from egg producing infections to have a superior capacity to produce IL-10. With work in Chapter 4 demonstrating that the intestinal microbiota differs between egg producing and non-egg producing infections, we next questioned whether the microbiota was involved in regulatory cell induction and alleviation of allergic disease. In comparison to allergic mice recolonised with naïve uninfected faeces, the transfer of faeces from egg producing and nonegg producing infections reduced recruitment of cells characteristic to an allergic response into the airways and imprinted a distinctive Type 1 phenotype on innate and adaptive immune cell types instead of Type 2.

Finally, in Chapter 7, we discuss our main findings, and its implications for our current understanding of host-parasite-microbial relations.

Nederlandse Samenvatting

Parasieten zijn ongelooflijk fascinerende organismen, die bekend zijn vanwege hun afschuwwekkende eigenschappen (bijv. hun bizarre uiterlijk en unieke gedragingen). Deze hebben vorm hebben gekregen door hun afhankelijkheid om te leven van/in een ander organisme (een gastheer) en zo te groeien, zich te voeden en verder te gedijen. Om hun overleving op de lange termijn te garanderen, hun levenscyclus te voltooien en infectie-gerelateerde pathologie te verminderen, hebben veel parasieten allerlei ingenieuze strategieën ontplooid om het immuunsysteem van de gastheer te moduleren, te onderdrukken en te omzeilen. Belangrijk is dat, hoewel parasieten voornamelijk als negatief worden neergezet, hun vermogen om immuun cellen te moduleren ook gunstig kan zijn voor de gezondheid van de gastheer. Toenemend bewijs suggereert dat blootstelling aan parasieten en symbiotische microorganismen (bijv. bacteriën en schimmels) tijdens de kindertijd cruciaal is voor een adequate uitrijping van het immuunsysteem en dat dit kan beschermen tegen immuungerelateerde ziekten, zoals auto-immuniteit en allergieën. In dit proefschrift worden mechanismen in kaart gebracht waarmee de parasitaire Schistosoma platworm de immuniteit van de gastheer kalibreert en beïnvloedt, met nadruk op hun impact op de darmomgeving en interacties met tolerogene B-cellen.

Schistosomiasis, ook bekend als Bilharzia, is een genegeerde maar belangrijke tropische ziekte, met meer dan 240 miljoen gevallen en naar schatting 200.000 sterfgevallen per jaar, en is als de tweede geplaatst, alleen voor Malaria, in termen van verzwakking en morbiditeit. Deze parasitaire ziekte is wijdverbreid in Afrika ten zuiden van de Sahara, regio's van Zuid-Amerika en Azië, en heeft grote gevolgen voor mensen uit gemeenschappen met een lage sociaaleconomische status en beperkt toegang tot veilig drinkwater. De drie meest voorkomende soorten Schistosoma wormen die mensen infecteren zijn *S. mansoni*, *S. japonicum* en *S. haematobium*. De twee eerstgenoemde soorten zijn verantwoordelijk voor darmziekten, terwijl *S. haematobium* gevolgen heeft voor de urinewegen. De focus van dit proefschrift ligt op *S. mansoni*.

De Schistosoma infectie begint bij contact met zoet water waarin vrijzwemmende parasitaire larven voorkomen, zogenaamde cercariae. Deze vorkstaartvormige parasieten dringen de huid binnen van hun toekomstige gastheer en migreren in de loop van enkele weken vanuit de huid, via de longen naar de bloedvaten van de lever en de darmen. Hier rijpen de onvolwassen Schistosoma wormen (schistosomula) uit tot volwassen mannelijke en vrouwelijke wormen, die vervolgens gaan paren en eieren produceren (tot 300 per dag per wormpaar) voor de rest van hun levensduur; dit kan jaren zijn zonder farmacologische interventies. In een ultiem geval van immuunontwijking en

bescherming van de gastheer, worden Schistosoma-eieren omsloten door immuuncellen van de gastheer en fibrotisch weefsel en vormen ze een immuunlaesie die bekend staat als een granuloom. De vorming van deze granuloma's is noodzakelijk voor de verplaatsing van eieren door de darmwand en de uitscheiding via de feces, zodat de eieren uiteindelijk buiten het lichaam terecht komen en hun levenscyclus kunnen voltooien in een geschikte zoetwaterslak. In praktijk slagen veel eieren er niet in om de darmwand te passeren en worden ze in plaats daarvan met de bloedstroom meegenomen naar de lever, waar ze vervolgens vastlopen in het lokale vaatbed en ook weer granuloma structuren vormen. Deze ontstekingshaarden nemen een centrale plek in de pathologie die geassocieerd is met Bilharzia en kunnen bij ernstige gevorderde ziekte tot de dood leiden.

Om de gezondheid van hun gastheer optimaal te houden en zo hun eigen overleving te veilig te stellen, gebruiken Schistosoma parasieten verschillende methodes om ontstekingsreacties te vervormen. Dit omvat als eerste het bevorderen van type 2-immuniteit om de granuloomvorming en het passeren van darmwand door de eieren te ondersteunen. Als tweede betreft het de ontwikkeling en activatie van tolerogene of onderdrukkende immuuncellen, die ontstekingsreacties gericht tegen de wormen maar ook de pathologie als gevolg van vastgelopen eieren (bv in de lever) zullen verminderen. De aanmaak van tolerogene immuuncellen, zoals regulerende B-cellen (Bregs) en T-cellen (Tregs), heeft ook een belangrijk ‘bijwerking’, nl dat dit de gastheer beschermd tegen immuunziektes, die worden gekenmerkt door een overactief immuunsysteem, zoals allergie, astma en auto-immuniteit.

Een experimenteel infectie model van schistosomiasis in muizen vormt een ideaal systeem om de mechanismen en moleculen te bestuderen die de parasieten gebruiken om de immuunreacties van de gastheer te moduleren. Allereerst het feit dat Schistosoma wormen zo meesterlijk zijn in het aansturen van Breg en Treg cellen kan op deze manier onderzocht worden en beter in kaart gebracht. Verder is het een heel interessant gegeven dat zowel schistosomiasis als allergie gekenmerkt door type 2-immuunreacties, maar beiden zich manifesteren met hele andere klinische symptomen. Het experimentele schistosomiasis model kan ook hier uitkomst bieden en meer inzicht verschaffen in de celtypes en de overdracht van signalen die betrokken zijn bij type-2 afweer en herstel van de gastheer. Deze verworven kennis kan gebruikt worden voor de ontwikkeling van nieuwe behandelingen van allergie. Ten slotte kunnen we dit experimentele model gebruiken om impact van schistosomiasis op de integriteit van de darmwand en het omliggende weefsel te onderzoeken tijdens het uittreden van parasitaire eieren, zoals bijvoorbeeld de regulatie en herstel van slijmvliesontstekingen en interacties met lokale darmbacteriën.

Het eerste deel van dit proefschrift omvat een hoofdstuk met een gedetailleerd verslag van immuunreacties die optreden tijdens de loop van een Schistosoma infectie (Hoofdstuk 3) en biedt nieuwe inzichten in de effecten van Schistosoma eieren op de integriteit van de darmbarrière, de samenstelling van de microbiota en de mucosale immuniteit (Hoofdstuk 2&4).

De passage van parasitaire eieren vanuit de bloedvaten rondom de darmen, door de darmwand heen naar de feces van de gastheer is een raadselachtig en intrigerend proces. Om dit succesvol te laten verlopen, moeten de eieren meerdere barrières (zowel bloedvat- en slijmvlieswanden) passeren, moeten immuuncellen gerekruteerd en gemoduleerd worden voor de vorming van granuloma's, en moeten er herstelwerkzaamheden in gang gezet worden om weefselschade te genezen. In hoofdstuk 2 bespreken we onze huidige kennis van het migratieproces van Schistosoma eieren. We schetsen de interacties die optreden tussen de gastheer, de parasiet en de darmflora bacteriën om dit proces mogelijk te maken en speculeren we over de gevolgen van succesvolle (of niet-succesvolle) migratie van eieren op de gezondheid van de gastheer en de bescherming tegen immuunziektes.

Het verloop van een Schistosoma infectie kan in drie fasen onderverdeeld worden met bijbehorende kenmerkende immuunreacties. Gedurende de eerste paar weken worden zowel wat lichte type 1- als type 2-immuunreacties gevonden. Vervolgens vindt er een omslag plaats vanaf het punt dat de eiproductie op gang komt (week 5-6), wat tevens gepaard gaat met een sterk ontwikkelde type 2-immuunreactie. Tijdens de chronische fase van de infectie (vanaf week 12) zwakken de type 2-immuunreacties wat af en worden vooral sterk regulerende immuunreacties gevonden. Ook dit proces is gedetailleerd beschreven **in** Hoofdstuk 2, alsmede de laatste inzichten hierin gebaseerd op honderden onafhankelijke onderzoeksartikelen, allemaal gericht op verschillende tijdstippen van de infectie en bij verschillende aangedane organen.

In Hoofdstuk 3 beschrijven onderzoek naar de verschillende immuunreacties tijdens een experimentele infectie met schistosomiasis, waarbij we inzoomen op drie verschillende weefsels die tijdens de infectie worden aangetast: de lever, mesenteriale lymfeklieren (drainerende de darmen) en de milt. De immuuncellen in deze weefsel worden bestudeerd met behulp van histologische, cellulaire (flow cytometrie) en op gen-expressie (RNA-seq) gebaseerde technieken. Verder laten we zien door specifieke cellen tijdelijk uit het lichaam te verwijderen tijdens de beginfase van de eiproductie, dat bepaalde CD11c+ antigeen-presenterende cellen (inclusief dendritische cellen en macrofagen), een cruciale rol spelen bij het ontstaan van granuloomreacties en type 2-ontstekingen.

Ondanks de grote impact van schistosomiasis op de darm, heeft het grootste deel van de immunologische onderzoeken naar *S. mansoni* zich tot nu toe vooral gericht op de lever (waar ook ei-afzetting optreedt en dit leidt tot lokale fibrose vorming) of verder weg gelegen plekken, zoals de milt. Deze lacune komt gedeeltelijk voort uit technische beperkingen om immuuncellen levend te kunnen isoleren uit de darmen van schistosoom-geïnfecteerde muizen. De verwachting is dat dit wort bemoeilijkt door de sterke anti-parasiet type 2-immuunreacties die hier ontwikkeld is, wat o.a. leidt tot een overmatige slijmproductie, weefselschade en herstelwerkzaamheden, pH-veranderingen en infiltratie van immuuncellen. Het is ons gelukt deze isolatie techniek werkend te krijgen. Daarom kunnen wij in Hoofdstuk 4 een gedetailleerde karakterisering van de darmomgeving geven tijdens experimentele schistosomiasis. We geven hier een specifieke nadruk op de samenstelling van de darmflora, integriteit van de darmbarrière en de vergelijking van immuunreacties in de mesenteriale lymfeklieren en de darm zelf. Door gebruik te maken van ei-producerende en niet-ei-producerende schistosome infecties – dit door infecties met vrouwelijke en mannelijke larven (wel eieren) of alleen mannelijke larven (geen eieren) toe te passen - laten we zien dat de passage van eieren de integriteit van de darm drastisch vermindert, de samenstelling van de darmflora wijzigt en de frequentie/profiel van immuuncellen in de darm en mesenteriale lymfeklieren aanpast. Verder bewijzen we met behulp van muizen zonder darmflora (bacterie-vrije muizen) en met fecestransplantaties dat de darmflora van schistosoom-geinfecteerde dieren elementen van Type 2-immuniteit kan instrueren.

Het tweede deel van dit proefschrift richt zich op de instructie van regulerende cellen in de milt (Hoofdstuk 5&6) en het potentieel van darmflora bacteriën afkomstig uit schistosoom-geïnfecteerde dieren om allergische reacties in de luchtwegen te voorkomen (Hoofdstuk 6). Eerdere studies uit ons laboratorium toonde aan dat chronische infecties met Schistosoma allergische reacties in de luchtwegen kunnen voorkomen en dat zowel IL-10-producerende Breg cellen uit de milt en uit de longen hierbij betrokken zijn. De mechanismen die tot Breg cel vorming leiden tijdens een Schistosoma infectie zijn nog relatief ongedefinieerd. Eerdere studies en ongepubliceerde genexpressie-analyse uit ons laboratorium wijzen, onder anderen, in de richting van cytokines, zoals type I interferonen, voor de activering van Breg cellen tijdens chronische schistosomiasis. Alhoewel we vonden dat type I interferonen de expansie van Breg-cellen in laboratoriumkweken kunnen ondersteunen, de rol voor deze cytokines voor Breg cell ontwikkeling in het lichaam bleek zeer beperkt (Hoofdstuk 5).

In Hoofdstuk 6 gingen we verder met onze zoektocht naar het definiëren van immuunregulerende cellen, de vorming hiervan en de factoren die deze processen ondersteunen tijdens schistosomiasis, door wederom gebruik te maken van ei-producerende en niet-ei-producerende infecties. We vonden dat beide typen chronische infecties verschillende immuunregulerende cellen kunnen aansturen en bevorderen in de milt. Echter de T- en B-cellen van ei-producerende infecties bleken een superieur vermogen te hebben om het immuunregulerende cytokine IL-10 te produceren in vergelijking met T- en B-cellen van niet-ei-producerende infecties. Omdat het werk in Hoofdstuk 4 heeft aangetoond dat de darmflora samenstelling verschilt tussen ei-producerende en niet-ei-producerende infecties, vroegen we ons vervolgens af of de darmflora-bacteriën ook betrokken zijn bij de vorming van immuunregulerende cellen tijdens schistosomiasis en mogelijk het voorkomen van allergische luchtwegaandoeningen. Hiertoe hebben we fecestransplantaties vergeleken van ei-producerende en niet-ei-producerende geïnfecteerde muizen in bacterie-vrije allergische muizen. We vonden minder recruitering van typische 'allergische' immuuncellen in de luchtwegen van dieren die feces van ei-producerende infecties hadden ontvangen in vergelijking met dieren die feces kregen van niet-ei-producerende infecties. Tevens vonden we verschillen in type 1-reacties in zowel aangeboren en adaptieve immuuncellen.

Ten slotte bespreken we in Hoofdstuk 7 onze belangrijkste bevindingen en de implicaties ervan voor ons huidige begrip van gastheer-parasiet-microbiële relaties.

Curriculum vitae

Alice Harriet Costain was born on the 2nd of March 1994 on the Isle of Man (British Isle). She completed her primary and secondary education on the island, before moving to the UK, where she undertook a Bachelor of Science (Bsc Hons) in Physiology at the University of Manchester (2015). Whilst studying for her Bsc, Alice developed a strong enthusiasm towards the immune system and parasitic worms, with a particular admiration towards *Schistosoma* blood flukes. Driven by her fascination in immunology, Alice enrolled for an MSc in Integrated Immunology at the University of Oxford (2016). Here, Alice completed an intestinally focused research project, was introduced to the fascinating concept of Regulatory B cells (Bregs) and her excitement towards parasites grew stronger. Combing her love for worms and mucosal immunology, Alice applied for an internship with Prof Hermelijn Smits (2016; LUMC; Boehringer Ingelheim funded), where she began investigating the link between schistosome infections, leaky guts and Breg generation. This 6 month internship soon transpired into a collaborative PhD project between Leiden University and the University of Manchester, under the supervision of Prof Hermelijn Smits and Prof Andrew MacDonald (starting April 2017). During the writing of this thesis, Alice was offered a post-doctoral position with Prof Andrew MacDonald, where she continues to explore the enigmatic process of schistosome egg migration, and its impact on barrier integrity, host immunology and the local microbiota.

List of publications

1. **Alice H. Costain**, Alexander T. Phythian-Adams, Stefano A.P. Colombo, Angela K. Marley, Christian Owusu, Peter C. Cook, Sheila L. Brown, Lauren M. Webb, Rachel J. Lundie, Hermelijn H. Smits, Matthew Berriman and Andrew S. MacDonald (2022). *Dynamics of host immune response development during Schistosoma mansoni infection.* ***Frontiers in Immunology***, *13:906338*

2. Lauren M. Webb, Alexander T. Phythian-Adams, **Alice H. Costain**, Sheila L. Brown, Rachel J. Lundie, Peter C. Cook, Lucy H. Jackson-Jones, Angela K. Marley, Hermelijn H. Smits, Elia D. Tait Wojno and Andrew S. MacDonald. (2021). *Plasmacytoid dendritic cells facilitate T helper cell cytokine responses throughout Schistosoma mansoni infection.* ***Immunohorizons****,* 5, 721-732.

3. Emma L. Houlder, **Alice H. Costain**, Peter C. Cook and Andrew S. MacDonald (2021). *Schistosomes in the Lung: Immunobiology and Opportunity.* ***Frontiers in Immunology***, 12:635513

4. Alba Cortés, Simon Clare, **Alice H. Costain**, Alexandre Almeida, Catherine McCarthy, Katherine Harcourt, Cordelia Brandt, Charlotte Tolley, James Rooney, Matthew Berriman, Trevor Lawley, Andrew S. MacDonald, Gabriel Rinaldi and Cinzia Cantacessi (2020). *Baseline Gut Microbiota Composition Is Associated With Schistosoma mansoni Infection Burden in Rodent Models.* ***F*rontiers in Immunology**, 11:593838

5. Saeed Katiraei, Margreet R. de Vries, **Alice H. Costain,** Kathrin Thiem , Lisa R. Hoving Janna A. van Diepen, Hermelijn H. Smits, Kristien E. Bouter, Patrick C. N. Rensen, Paul H. A. Quax, Max Nieuwdorp, Mihai G. Netea , Willem M. de Vos , Patrice D. Cani, Clara Belzer, Ko Willems van Dijk, Jimmy F. P. Berbée, Vanessa van Harmelen (2019) *A. muciniphila exerts lipid-lowering and immunomodulatory effects without affecting neointima formation in hyperlipidemic APOE*3-Leiden.CETP mice.* ***Molecular Nutrition & Food Research,*** *64(15):1900732*

6. Katja Obieglo, **Alice H. Costain**, Lauren M. Webb, Arifa Ozir-Fazalalikhan, Shelia L. Brown, Andrew S. MacDonald, Hermelijn H. Smits (2019). *Type I interferons provide additive signals for murine regulatory B cell induction by Schistosoma mansoni eggs.* ***European Journal of Immunology****, 49(8)*

7. **Alice H Costain**, Andrew S. MacDonald and Hermelijn H Smits (2018), Schistosome Egg Migration: Mechanisms, Pathogenesis and Host Immune Responses. ***Frontiers in Immunology****, 10*

8. Grigory Ryzhakov, Nathaniel R. West, Fanny Franchini, Simon Clare, Nicholas E. Ilott, Stephen N. Sansom, Samuel J. Bullers, Claire Pearson, **Alice H. Costain**, Alun Vaughan-Jackson, Jeremy A. Goettel, Joerg Ermann, Bruce H. Horwitz, Ludovico Buti, Xin Lu, Subhankar

Mukhopadhyay, Scott B. Snapper and Fiona Powrie (2018). *Alpha kinase 1 controls intestinal inflammation by suppressing the IL-12/Th1 axis.* ***Nature Communications***, 9 (3797)

Contributions

Prof Hermelijn Smits and Prof Andrew MacDonald were involved in the planning and critical analysis of all thesis chapters and pushed for the publications of Chapter 2, 3 and 5.

Many members of the Smits Lab, MacDonald Lab, and Leiden Immunoparasitology group contributed to the experimental work in this thesis (i.e mouse monitoring and tissue processing) and provided invaluable discussion. In particular, Arifa Ozir-Fazalalikhan, Frank Otto, Dr Emma Houlder and Dr Stefano Colombo helped perform many schistosome infections, schistosome egg injections and faecal transplants.

Dr Alba Cortes, Dr Stefano Colombo and Dr Cinzia Cantacessi helped acquire and analyse the 16s sequencing of intestinal content in Chapter 4 and contributed to the planning of this chapter alongside Dr Gabriel Rinaldi.

Dr Melissa Lawson, Vicky Taylor, Hayley Jenkins and Paul Smith helped coordinate all GF experiments and provided great technical support and advice.

Dr Gordano Panic and Prof Jonathon Swann performed metabolomics analysis on the serum and small intestinal content samples shown in Papers 2 and 3

Dr Tovah Shaw helped with optimising schistosome intestinal preps.

Prof Meta Roestenberg, Dr Jacqueline Janse and Arifa Ozir-Fazalalikhan organised single-sex schistosome infections. Claudia J. de Dood performed the CAA analysis.

Dr Alexander Phythian-Adams, Sheila Brown, Dr Peter Cook, Dr Angela Marley, Dr Lauren Webb, Christian Owusu, Dr Rachel Lundie performed the experiments outlined in Chapter 3. Dr Stefano Colombo aided with data collation, data analysis, and planning of this chapter.

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