Exploring host-immune-microbial interactions during intestinal schistosomiasis

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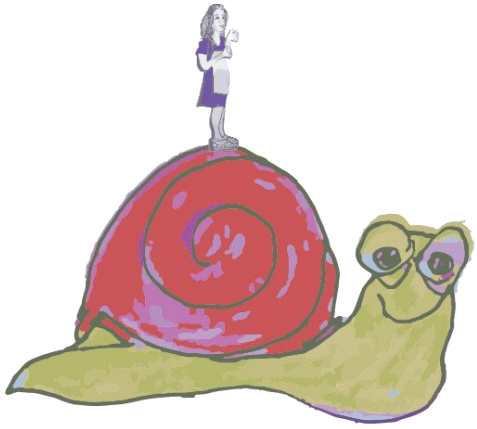
CHAPTER 7

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.

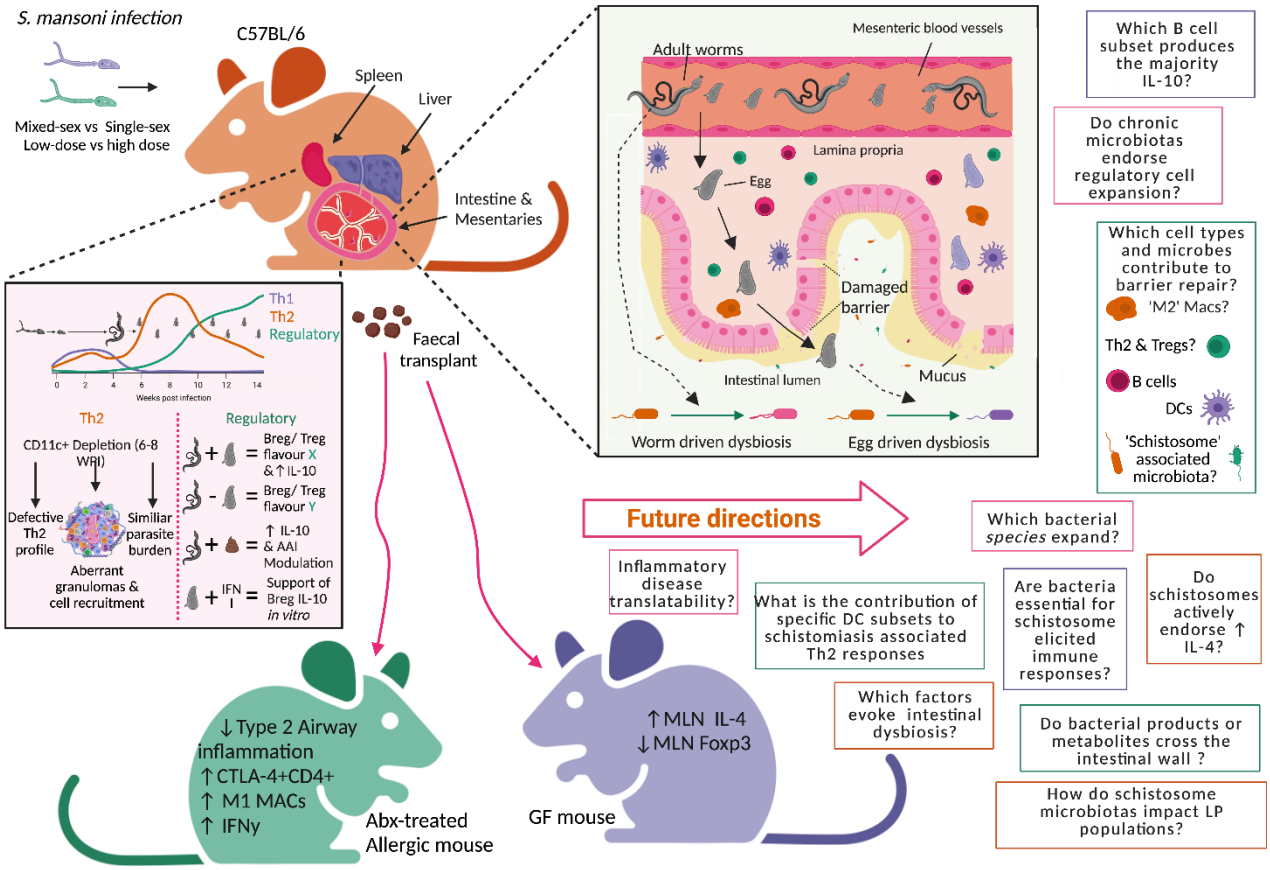


Diagram 1. Summary of key results and future directions. Experiments in this thesis were primarily conducted with female C57BL/6 mice, infected with either single-sex or mixed-sex *S. mansoni* parasites, at infection doses of 40 cercariae (low dose) or 180 cercariae (high dose). Infections lasted 3-14 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).

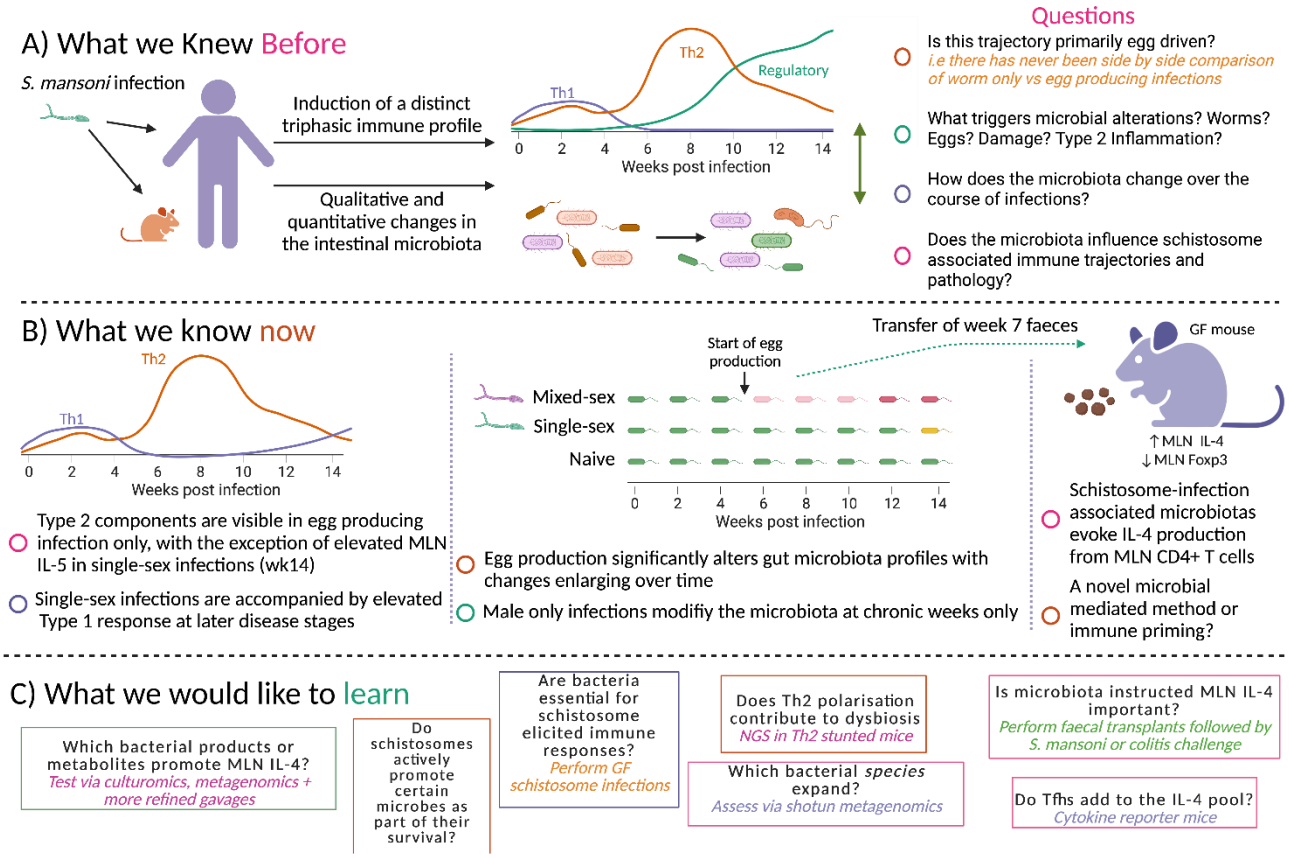


Diagram 2. Investigating the consequences of *Schistosoma mansoni* infection and associated faecal transplants on host immunity, and areas of follow-up study. (A) Before the work detailed in this thesis, infections with *S. mansoni* parasites were known to evoke triphasic immune profiles (i.e. Th1, Th2 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].

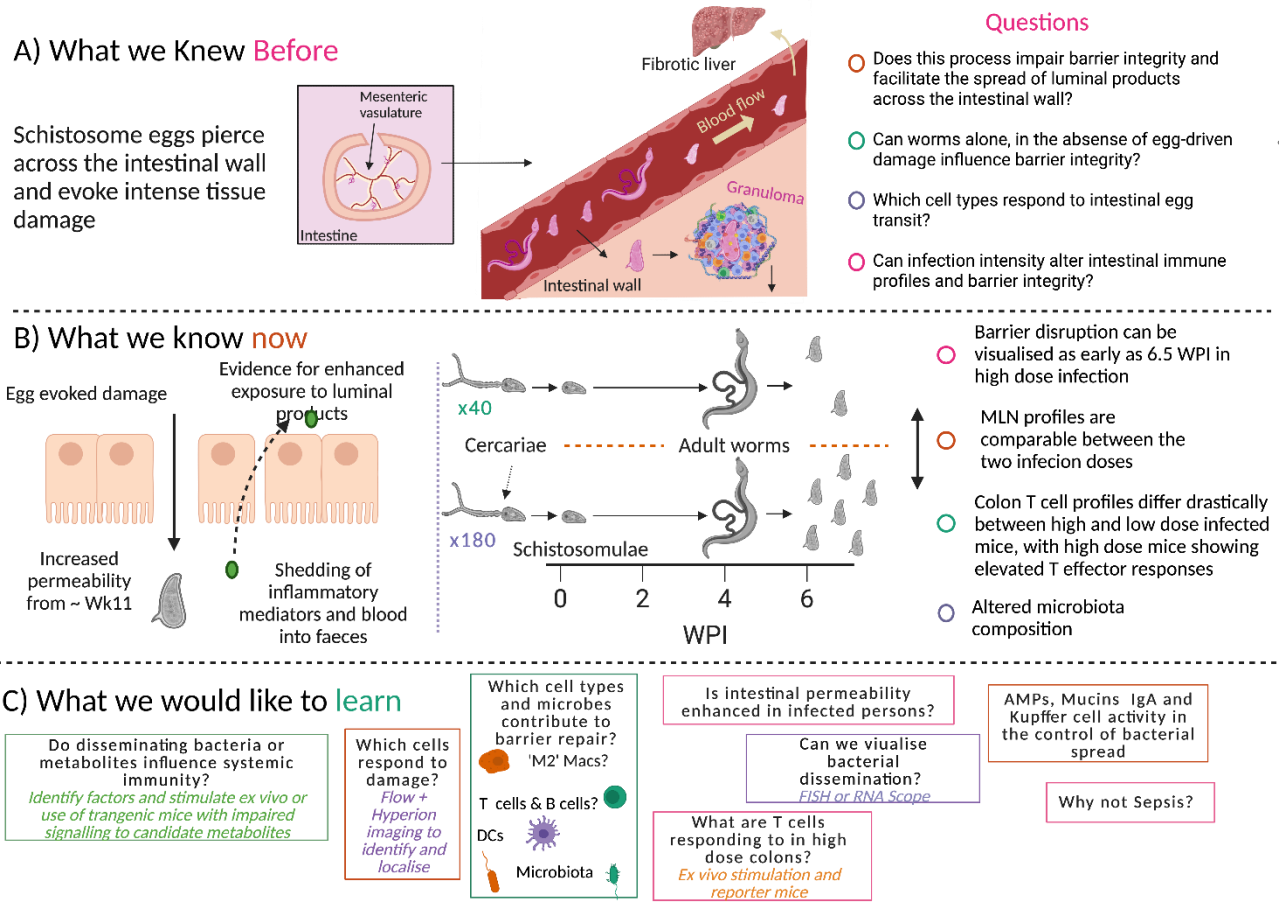


Diagram 3. How schistosome egg migration impacts the intestine and avenues for future study (A) Schistosome eggs pierce across intestinal tissues and cause extensive damage in the process. It was previously unknown how this process influences barrier integrity, and whether infection dose impacts the 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.

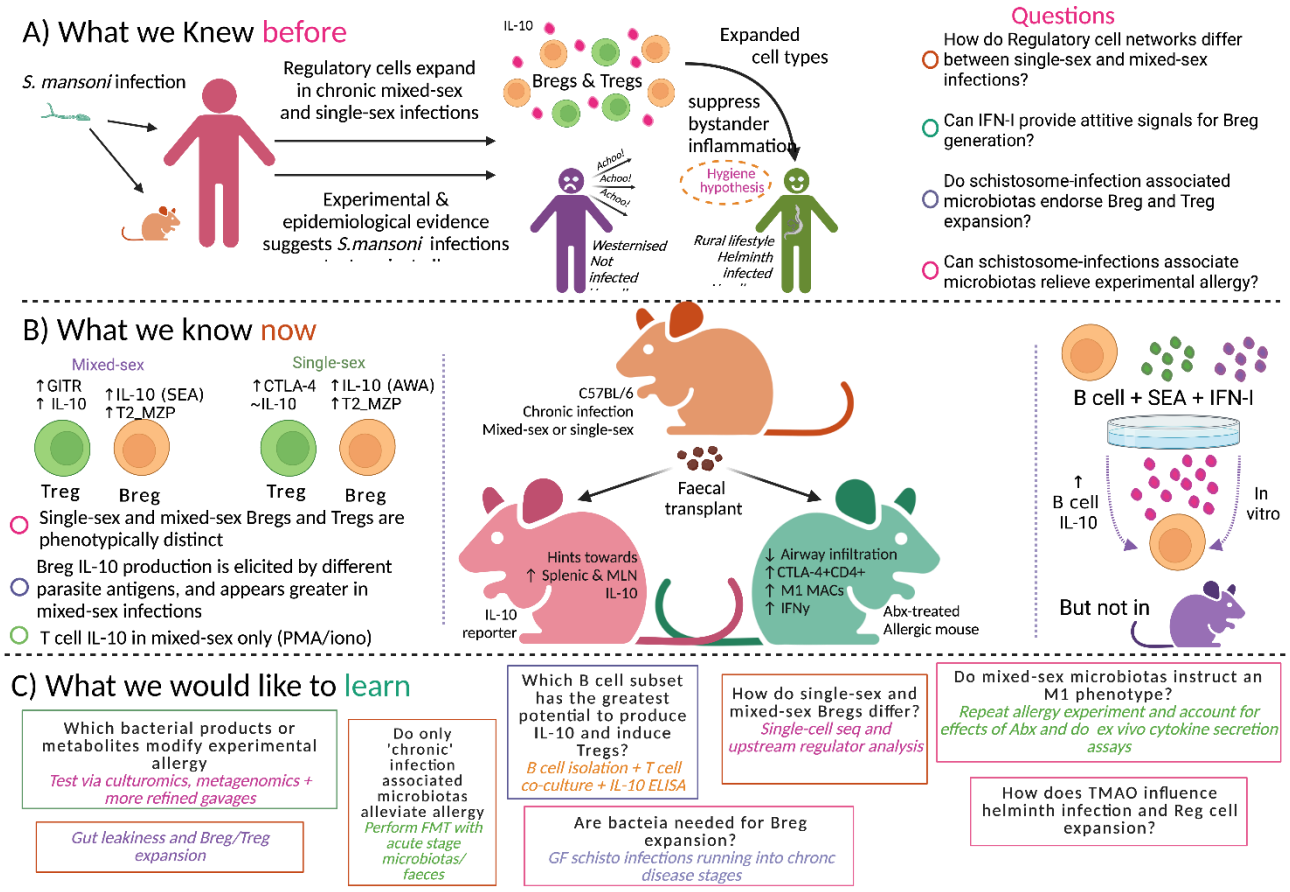


Diagram 4. Defining the contribution of schistosome and microbial signals in regulatory cell expansion and allergy prevention, and areas for future study. (A) Chronic schistosome infections are accompanied by the profound expansion of Treg and Breg networks, whose immunosuppressive activities are thought 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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