Cytokine signals and immune competition in the Th2 response to helminth infection

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Abstract

Type 2 immunity is activated in response to both allergens and helminths, but it can be detrimental or beneficial depending on the stimuli. These conflicting outcomes highlight the importance of understanding the regulation of this response. Th2 cells are key for the clearance of helminth infections in the SILP. IL-10 is a regulatory cytokine that is highly expressed during human and murine helminth infection, but its role in helminth infection remains unclear. The aim of this thesis is to assess if IL-10 acts directly or indirectly to promote the type 2 immune response while also suppressing counter-active Th1 cells which provide immune competition.

We show that helminth infection results in increased IL-10 expression in both the SILP and the draining MLN in mice, with highest expression in the infected tissue. *In vivo* blockade of IL-10 led to a significant decrease in IL-13, IL-5 and GATA3 expression by Th2 cells in the intestine and not in the MLN. *In vitro*, IL-10 induced expression of GATA3 and production of IL-5 and IL-13 in purified $CD4^+$ T cells. The impact of IL-10 was partially dependent on IFNγ signalling, and *in vivo*, surface IL-10 receptor expression was higher on Th1 cells than on Th2 cells and highest on Th1 cells in the SILP. Due to the nature of the *H. polygyrus* lifecycle, where at two-time points worms move through the wall of the intestine. We hypothesised that a possible indirect mechanism for IL-10 promotion of Th2 cells was via suppression of IFNγ responses as a result of bacteria stimuli. However, our data show no major disruption to the intestinal barrier during infection. Although, we do report IFNγ responses in the SILP, MLN and omentum over the course of *H. polygyrus* infection. Suggesting IFNγ may play multiple roles during infection. Furthermore, we found that MLN cells from *H. polygyrus* infected mice produced IFNγ when stimulated with HES, suggesting that IFNγ responses towards *H. polygyrus* itself may occur, although the function of this in the immune response to *H. polygyrus* remains unclear.

Understanding immune competition during *H. polygyrus* infection contributes towards the understanding of the immune response to helminths and with further work may contribute towards treatment strategies. In addition, IL-10 optimisation of Th2 responses during helminth infection may be applied to the treatment of helminth infections and other type 2 mediated diseases.

Table of Contents

List of Tables

List of Figures

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Impact of the COVID-19 pandemic

The sudden lab closures and switch to working from home in March 2020, in light of the COVID-19 pandemic, impacted my thesis and PhD. I unfortunately lost a significant amount of data as animals that were already on procedure and infected had to be culled. This experiment was to test the responsiveness of CD4 T cells from the MLN and SILP to bacterial antigen extract. These data would have helped to answer one of the key aims in Chapter-5. In addition, my *in vivo* experiments are complex and time dependent, requiring a team of people to ensure sample viability which unfortunately has not been possible in the past 9 months. As a result, to overcome this I carried out numerous smaller experiments which was more time consuming and delayed collection of data significantly. Furthermore, it has taken a lot of time to re-establish in house colonies after the mass cull at the start of lockdown and minimal breeding since. Some experiments for Chapter-5 involved new techniques, which I had planned to learn by collaborating with another lab group which specialises in bacterial techniques, but due to 2 metre social distancing requirements this was longer an option. In addition, I was unable to carry out optimisation of techniques to make this chapter a more complete story as this required training in the animal unit as well as in the lab and due to restrictions, this was not possible. Unfortunately, for 3 months, I was without a fully functioning laptop as its delivery was delayed due to COVID-19 and I had no access to on-site PCs, which meant important FlowJo analysis for my thesis was also significantly delayed. I have found working from home during COVID-19 tough as I thrive when in an office setting for support, help, and day to day interactions. Despite this, I feel like I have done my very best to make the most of the time I had at home to further my PhD as much as I could given the circumstances.

Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: Name: Holly C. Webster

Abbreviations

AAM	Alternatively activated macrophages
AF	Alex Fluor
AJ	Adherens junction
ANOVA	Analysis of variance
APC	Antigen presenting cell
Breg	Regulatory B cell
BSA	Bovine serum albumin
BUV	Brilliant ultra-violet
BV	Brilliant violet
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CTV	Cell trace violet
CXCR	C-X-C chemokine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
^DL	Above detection limit
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FMO	Fluorescence minus one
FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
GF	Germ-free
GFP	Green fluorescent protein
GI	Gastrointestinal
HBSS	Hanks' Balanced Salt Solution
HDM	Housedust mite
HES	*H. polygyrus* excretory-secretory
HEV	High endothelial venule
hi	high
i.p.	Intraperitoneal
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL	Interleukin
ILC	Innate lymphoid cell
iTreg	Induced regulatory T cell
JAM-A	Junctional adhesion molecule A
LN	lymph node
LP	Lamina propria
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex

MLN	Mesenteric lymph nodes
ND	Not detected
NK	Natural killer
NS	Not significant
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
pTreg	Peripheral regulatory T cell
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RORγT	Retinoic acid receptor-related orphan nuclear receptor gamma
RT	Room temperature
SCFA	Short chain fatty acid
SD	Standard deviation
SI	Small intestine
sIgA	Secretory immunoglobulin A
SILP	Small intestine lamina propria
SLO	Secondary lymphoid organ
SPF	Specific-pathogen-free
STAT	Signal transducer and activator of transcription
TBET	T-box transcription factor TBX21
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
TGFβ	Transforming growth factor β
Th	T helper
TJ	Tight junction
TLR	Toll-like receptor
Tr1	Type 1 regulatory cell
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
VAL	Venom allergen-like
WT	Wild type

Chapter-1 Introduction

1.1 CD4 T helper cells

1.1.1 Development and activation of CD4 T cells

Naïve $CD4^+$ T cells are derived from bone marrow haemopoietic progenitors which migrate to and mature in the thymus (Germain, 2002). The thymus provides an essential niche for T cell development, comprising essential stromal cells, cytokines, and chemokines (Koch and Radtke, 2011). During thymic maturation, the T cell will begin to express a T cell receptor (TCR) which undergoes a series of checks to ensure its functionality (Robey and Fowlkes, 1994, Roehm et al., 1984, Haskins et al., 1984). Positive selection ensures the TCR recognises peptide presented on major histocompatibility complex (p:MHC) by thymic antigen presenting cells (APCs) called cortical thymic epithelial cells (cTEC) at the appropriate affinity. Cells which do not meet this threshold die by apoptosis (Von Boehmer et al., 1989). It is at this stage that a T cell will either become a $CD4^+$ helper T cell, if it recognises peptide presented on major histocompatibility complex II (p:MHCII) or a $CD8^+$ cytotoxic T cell if it recognises peptide presented on major histocompatibility complex I (p:MHCI) (Teh et al., 1988, Kruisbeek et al., 1985, Kaye et al., 1989, Robey and Fowlkes, 1994). CD4 and CD8 are co-receptors that stabilise the interaction between the TCR and MHC molecules and are key for T cell activation (Guidos et al., 1990). The next TCR checkpoint is negative selection; if a T cell binds MHC presenting self-peptide on medullary epithelial cells (mTEC) too strongly, it will die by apoptosis or become anergic (unresponsive) (Robey and Fowlkes, 1994, Von Boehmer et al., 1989). This process removes T cells that respond too strongly to self-antigen. $CD4^+$ T cells that survive through these checkpoints will leave the thymus, enter the circulation, and migrate to lymph nodes.

Naïve $CD4^+$ T cells enter the lymph node (LN) via high endothelial venules (HEVs) and migrate to T cell zones, where they encounter migratory DCs presenting peripheral antigen on MHCII (Figure 1-4) (Girard and Springer, 1995, Bousso, 2008). Multiple activation signals are required for $CD4^+$ T cell activation. When a naïve $CD4^+$ T cell encounters its cognate antigen, the TCR binds to MHCII-peptide on the DC. This initial interaction is the first signal required for $CD4^+$ T cell

activation (Itano and Jenkins, 2003). Subsequent co-stimulatory signals are provided by CD28 on the $CD4^+$ T cell binding to CD80/86 present on the surface of the DC (Allison, 1994, Harding et al., 1992)(Figure 2-1). Both of these interactions are required for downstream signalling and subsequent activation, differentiation, and proliferation of $CD4^+$ T cells; the absence of co-stimulatory molecules results in $CD4^+$ T cell anergy (Smith-Garvin et al., 2009). An activated $CD4^+$ T cell during an immune response can be identified by the expression of CD44. The expression of this marker is maintained on the surface of activated $CD4^+$ T cells and remains high on memory $CD4^+$ T cells (Budd et al., 1987, Lindell et al., 2006). The expression of CD69 is also rapidly upregulated on activated $CD4^+$ T cells but is not maintained (Lindell et al., 2006, Avgustin et al., 2005). The differentiation and subsequent function of the $CD4^+$ T cell into different T helper (Th) cell subsets will depend on the cytokine microenvironment during activation.

1.1.2 CD4 T cell subset differentiation

The T helper (Th) subsets, Th1 and Th2 were first described in the late 1980s (Mosmann et al., 1986, Killar et al., 1987). The extrinsic cytokine

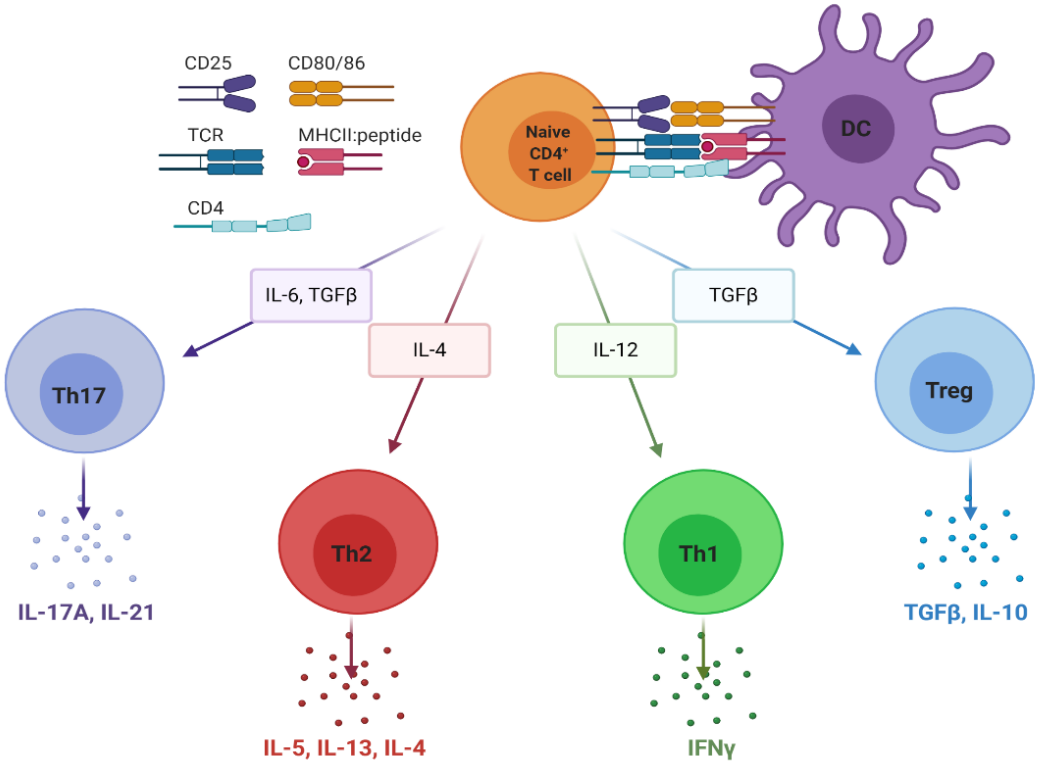

Figure 1-1 Th subset differentiation
Adapted from (Bailey et al., 2014) and created with BioRender.com.

microenvironment provided primarily by APCs in the lymph node determines the Th subset an activated $CD4^+$ T cell will differentiate in to (Bailey et al., 2014). Cytokines will bind to their cognate receptors on the surface of $CD4^+$ T cells and induce downstream signalling and activate or inhibit transcription factors (TF), this results in subsequent cytokine secretion and polarisation (Raphael et al., 2015). Presently, there are numerous described Th subsets, some of the most well characterised subsets being Th1, Th2, Th17 and regulatory T cells (Tregs)(Raphael et al., 2015). Each subset requires specific cytokines or cytokine combinations to induce $CD4^+$ T cell differentiation (Figure 1-1) (Raphael et al., 2015).

1.1.2.1 T helper 2 cells

Th2 cells are a key part of the immune response to helminths and also contribute to airway inflammation in atopic diseases such as asthma. Interleukin 4 (IL-4) is required for polarisation of Th2 cells both *in vitro* and *in vivo*. IL-4 signalling is dependent on STAT6 phosphorylation downstream of the IL-4 receptor (IL-4R) (Redpath et al., 2015). STAT6 in turn, activates the TF GATA binding protein 3 (GATA3). GATA3 is required for Th2 development and induces chromatin remodelling at the Il4, Il5 and Il13 locus and subsequent release of these cytokines (Le Gros et al., 1990, Swain et al., 1990, Zheng and Flavell, 1997). The initial source of IL-4 in the lymph node remains unclear. In IL-4 knock-out mice, the Th2 response to the murine helminth *Nippostrongylus brasiliensis* is reduced but not absent with decreased IgE switching and eosinophilia (Kopf et al., 1993). Although these data highlight the important role of IL-4 in amplifying an effective Th2 response, it is not completely essential for Th2 cell development. This suggests that there may be another stimulus for Th2 differentiation, perhaps from DCs as these cells do not produce IL-4 but are key for Th2 differentiation (Phythian-Adams et al., 2010, Smith et al., 2011). Both basophils and type 2 innate lymphoid cells (ILC2s) have been described as possible sources of IL-4 (Doherty et al., 2013, Sokol et al., 2008). Interestingly, IL-18 signalling via its receptor on natural killer T (NKT) cells has been shown to induce the release of IL-4 from these cells and may also be possible source of Th2 priming IL-4 (Yoshimoto et al., 2000).

1.1.2.2 T helper 1 and 17 cells

Both Th1 and Th17 cells are involved in autoimmune responses and chronic inflammation but have distinct cytokine signatures and effector mechanisms. IL-12 and IFNγ from DCs and surrounding innate immune cells bind to their respective receptors on $CD4^+$ T cells and are key for Th1 differentiation. IFNγ induced phosphorylation of STAT1 activates T-box transcription factor TBX21 (TBET) which is required for Th1 cell differentiation (Szabo et al., 2000). In addition, IL-12 signalling through the IL-12 receptor activates STAT4 and results in further upregulation of IFNγ (Szabo et al., 2000). Th1 cells are key for effective immunity to intracellular pathogens such as bacteria, viruses, protozoa and are also important in anti-tumour immunity (Szabo et al., 2000, Hsieh et al., 1993). IFNγ from Th1 cells activates macrophages and enhances their antimicrobial activity to facilitate clearance of intracellular pathogens (Szabo et al., 2000). The generation of T cells lacking STAT4 resulted in a Th2 bias, even in the presence of IL-12, emphasising the importance of STAT4 for Th1 cell differentiation (Kaplan et al., 1996). In addition, humans lacking the IL-12 receptor are highly susceptible to mycobacterial infections (Ramirez-Alejo and Santos-Argumedo, 2014, Kaplan et al., 1996).

The immune response to fungal and extracellular pathogens requires Th17 cells, however, Th17 cells are also drivers of numerous autoimmune conditions such as psoriasis and multiple sclerosis (Korn et al., 2009). The differentiation of these cells is dependent on IL-6 and TGFß (Korn et al., 2009). These cytokines induce subsequent activation of STAT3 and in turn the TF Retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγt) (Korn et al., 2009). $ROR\gamma t^+$ Th17 cells will release the cytokines IL-17A and IL-21 (Ivanov et al., 2006, Zhou et al., 2007, Veldhoen et al., 2006). Although IL-23 is not involved in the primary induction of Th17 cells, it is required to maintain the effector functions of pathogenic Th17 cells (Stritesky et al., 2008). It is also important to note that IL-2, which was initially described as a T cell growth factor, is a key cytokine for the activation and function of Th cell subsets (Létourneau et al., 2009). It can act in both an autocrine and paracrine fashion and is also a proposed mechanism for the regulation of effector $CD4^+$ T cell responses (Létourneau et al., 2009).

1.1.2.3 Other T helper subsets

Suppressor T cells were first discovered in 1969 (Nishizuka and Sakakura, 1969). These cells remained very controversial until 1995, when a breakthrough in the field came with the discovery of $CD4^+$ $CD25^+$ cells (Tregs) that were protective against autoimmunity in mice that had undergone a thymectomy (Sakaguchi et al., 1995). As with conventional $CD4^+$ T cells, Tregs develop in the thymus (Sakaguchi et al., 2008). The TCR on Tregs interacts with p:MHCII with an intermediate strength (Sakaguchi et al., 2008). This TCR signal strength is weak enough to allow them to avoid negative selection but not continue with conventional $CD4^+$ T cell development (Sakaguchi et al., 2008). The induction of Tregs requires the regulatory cytokine TGFβ, which switches on the TF Forkhead box protein P3 (FOXP3) via STAT5 and typically results in the secretion of the regulatory cytokines IL-10 and TGFβ (Sakaguchi et al., 2008). FOXP3 knockout mice die within the first month of life due to lymphoproliferative autoimmune syndrome (Brunkow et al., 2001). In addition, humans with a loss of function mutation in the FOXP3 gene have multi-organ autoimmunity and the inflammatory disorder IPEX (Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) (Bennett et al., 2001). Another population of Tregs that develop in the periphery from antigen specific $CD4^+$ T cells are termed peripheral Tregs (pTregs) and upregulate FOXP3 upon exposure to antigen and in the presence of TGFβ (Bluestone and Abbas, 2003). pTregs are essential for regulating local inflammation in tissues (Bluestone and Abbas, 2003, Yadav et al., 2013). In addition, type 1 regulatory T cells (Tr1) are $FOXP3^-$ and exert their inhibitory functions primarily via IL-10 (Zeng et al., 2015). Both IL-10 and IL-27 are needed for Tr1 differentiation and have been shown to prevent colitis (Groux et al., 1997, Zeng et al., 2015). Tfh cells are specialised Th cells that provide B cell help and are key for germinal centre formation (Vinuesa et al., 2005, Nurieva and Chung, 2010). These cells can be defined by their high expression of CXCR5, which promotes migration to B cell follicles and the secretion of IL-21 (Vinuesa et al., 2005, Nurieva and Chung, 2010). In addition, Tfh cells express the costimulatory molecule ICOS, the inhibitory molecule PD-1 and the TF Bcl6 (Vinuesa et al., 2005, Nurieva and Chung, 2010, King and Mohrs, 2009). Th3 cells are another regulatory subset and FOXP3 expression by these cells is still debated. These cells both require TGFβ for their differentiation but also secrete TGFβ (Gol-Ara et al., 2012). Th3 cells have been shown to inhibit

experimental autoimmune encephalomyelitis (EAE)(Gol-Ara et al., 2012, Fukaura et al., 1996). There are other well characterised Th subsets such as Th9 cells. IL-4 and TGFß in conjunction result in the differentiation of Th9 cells which secrete IL-9 and are known to promote anti-tumour immunity, contribute to Th17 recruitment in autoimmune inflammation and support the Th2 response to helminths and in allergic inflammation (Kaplan, 2013, Lu et al., 2012).

1.1.3 CD4 T cell regulation

Although effector $CD4^+$ T cells are an essential component of adaptive immunity, these responses need to be tightly regulated and this requires a fine balance; a strong enough response is needed to remove the pathogen, but that response needs to be controlled so that harmful immunopathology does not occur as a consequence. Tregs play an essential role in establishing this balance by regulating $CD4^+$ T cell responses. In the LN, Tregs can prevent naïve $CD4^+$ T cell proliferation and differentiation, in addition, in the tissues Tregs have the capacity to supress the effector functions of differentiated $CD4^+$ T cells, along with $CD8^+$ T cells, B cells, macrophages and DCs (Sojka et al., 2008, Sakaguchi et al., 2008). Numerous mechanisms of Treg-mediated suppression have been described. The secretion of anti-inflammatory cytokines is key for the suppression of both APCs and $CD4^+$ T cells. Tregs can also suppress $CD4^+$ effector cells via cell-to-cell contact via binding of inhibitory molecules on the surface of these cells (Sakaguchi et al., 2008, Sojka et al., 2008). Tregs, which express high levels of CD25 (the IL-2 receptor) have been shown to compete with effector $CD4^+$ T cells by “mopping” up IL-2 in the surrounding environment (Létourneau et al., 2009, Sakaguchi et al., 2008). In addition to the above mechanisms, Th subsets can also produce self-limiting IL-10 via a negative feedback loop which is also an essential component of $CD4^+$ T cell regulation (Ng et al., 2013). The role of IL-10 in Th responses will be discussed in detail in section 1.5.

1.2 The type 2 immune response and disease

1.2.1 Epidemiology of helminth infection

Helminths infect more than 1.5 billion people per year worldwide (Yazdanbakhsh et al., 2002, Hotez et al., 2008, W.H.O, 2020), and in areas such as sub-Saharan

Africa where these parasitic worms are endemic, morbidity levels are high (W.H.O, 2020). The four most abundant helminth species which infect humans are the roundworm *Ascaris lumbricoides,* which infects more than 1.2 billion people, the whipworm *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale* (De Silva et al., 2003). Chronic helminth infection affects an estimated 800 million children and can result in physical impairment, such as stunted growth, cognitive and developmental delays, negatively impacting their education (W.H.O, 2020, Hotez et al., 2008). Malnourishment is also associated with helminth infection, but whether this is a cause or consequence of infection remains unclear. In addition, helminth infections pose a significant burden on livestock and agriculture, adding a major challenge to global food security (Charlier et al., 2014). Helminth infections have also been shown to reduce vaccine efficacy, as demonstrated in mice vaccinated against malaria (Su et al., 2006); nematode co-infection at the time of vaccination resulted in less effective antimalarial immunity (Su et al., 2006). These factors demonstrate that, despite extensive progress in de-worming programmes, effective strategies to reduce worm burden in the human population are urgently needed. Eliminating helminth infection remains a challenge for numerous reasons. Helminth infections are largely a disease of poverty; sanitation and health care are lacking in most endemic areas (Hotez et al., 2008, W.H.O, 2020). Furthermore, different helminth species localise in specific areas of their host, and have distinct life cycles and routes of infection (Mcsorley and Maizels, 2012). However, the commonality between species is the induction of a host protective type 2 immune response which aims to eliminate the parasite. In areas where parasite infections are better controlled through de-worming, high sanitation and reduced poverty a correlation with an increase in inflammatory disorders has emerged as an adverse effect (Weinstock et al., 2002).

1.2.2 The hygiene hypothesis and old friends

Since the 1980s, there has been a steady increase in immune-mediated disorders and atopic diseases such as allergy and atopic dermatitis in the Western world (Weinstock et al., 2002). The same increase was not observed in countries such as sub-Saharan Africa, China, and East Asia where infectious diseases were still prevalent. In 1989, D. P. Strachan proposed the idea of the “hygiene

hypothesis", which suggests that reduced incidence of childhood infection resulted in increased incidence of autoimmune and atopic diseases (Strachan, 1989). In 2003, the "old friends" hypothesis was proposed by G. Rook, which explains that through evolution, humans have co-evolved with parasites such as helminths, and the absence of these organisms in western countries coincides with autoimmune and atopic disease (Rook et al., 2003). This implied a potentially beneficial role of helminths and other ancient organisms in preventing inflammatory disease. In 2000, a study showed clear evidence for a link between parasites and decreased allergy; children infected with the trematode *Schistosoma haematobium* were less prone to allergy when measuring reactivity to house dust mite (HDM) allergen (Maizels, 2019, Van Den Biggelaar et al., 2000). We now know that parasites such as helminths, which dwell in healthy hosts for long periods of time, have the ability to modulate the immune system. Immune modulatory and mimicry molecules (described in section 1.3.3) have been identified in the secretory products of helminths, which can potentially be used as treatment strategies for type 2 immune-mediated diseases (Maizels et al., 2018, Osbourn et al., 2017, Johnston et al., 2017).

1.2.3 Allergic responses

Type 2 immunity occurs in response to extracellular parasites such as helminths, however, this type of immune response is also implicated in allergic and atopic diseases such as asthma, atopic dermatitis, food-allergy, and rhinitis (Thomsen, 2015, Dharmage et al., 2019). The incidence of allergic disease is increasing globally, for example, approximately 300 million people suffer from asthma worldwide and this is predicted to increase by at least 100 million by 2025 (Dharmage et al., 2019, Thomsen, 2015). Atopy is the term that refers to a genetic tendency to mount inappropriate immunoglobulin E (IgE) responses to common allergens such as pollen and house dust mite, resulting in atopic diseases (Thomsen, 2015). IgE responses occur when the immune system becomes sensitised to an allergen; IgE released by plasma cells will bind to innate immune cells such as mast cells and once an individual is exposed to the allergen again, the allergen will bind to IgE on the surface of mast cells. This results in the release of molecules such as histamine that drive allergic reactions and the associated symptoms such as in hay fever, where histamine drives changes in blood vessel permeability resulting in sneezing, coughing and weepy

eyes (Galli and Tsai, 2012, Galli et al., 2008). Th2 and Tfh cells also play an important role in this response which are discussed fully in sections 1.1.2 and 1.3.2. The antigen HDM is used to induce specific IgE responses that drive inflammation in mouse models of allergic asthma, where the antigen sensitisation phase is sufficient for the generation of Tfh cells but not Th2 mediated inflammation which only occurs with HDM challenge after sensitisation (Galli et al., 2008, Galli and Tsai, 2012). Tfh cells facilitate the development of IgE producing plasma cells from B cells via the release of IL-21 and IL-4 (Galli and Tsai, 2012). In this model, there is evidence that Tfh cells generated in the initial sensitisation phase can differentiate in to Th2 cells upon HDM challenge (Galli et al., 2008, Ballesteros-Tato et al., 2016). In both HDM and asthma, Th2 mediated airway inflammation is implicated in disease pathogenesis. Helminths provide a niche model to not only study type 2 immune response dynamics but also to investigate natural suppression of Th2 responses due to the evolutionary mechanisms they have developed to remain undetected in the host.

1.2.4 Heligmosmoides polygyrus as an experimental model

Murine models of human helminth infection have become invaluable in unravelling the complex immune response to these parasites. A well-established mouse model of nematode roundworm infection is *Heligmosomoides polygyrus,* a natural chronic helminth infection of mice (Maizels et al., 2012). This model of infection is especially useful as the majority of mouse strains do not expel primary *H. polygyrus* infection, and infection persists for a period of weeks or months (Maizels et al., 2012). This is beneficial as human helminth infections such as *N. americanus* are also typically chronic (Reynolds et al., 2012, Nutman, 2015). In addition, *H. polygyrus* has evolved alongside its host and as a result can mimic aspects of host immune responses which are described in section 1.2.3, and as mentioned previously, these molecules have therapeutic potential for treating type 2 mediated diseases. *H. polygyrus* enters the host orally, migrates to the gut and burrows through the wall of the duodenum, encysts and moults twice over a period of 7 days (Figure 1-2)(Maizels et al., 2012). This is referred to as the tissue-dwelling stage. Around day 8, mature male and female adult worms re-enter the lumen at the proximal third of the small intestine (Monroy et al., 1992). During the luminal phase, adult parasites wrap around villi to maintain their position in the gut, mate with one another to produce eggs from

approximately day 10 onwards (Figure 1-2)(Maizels et al., 2012). Each stage of the life cycle initiates an immune response.

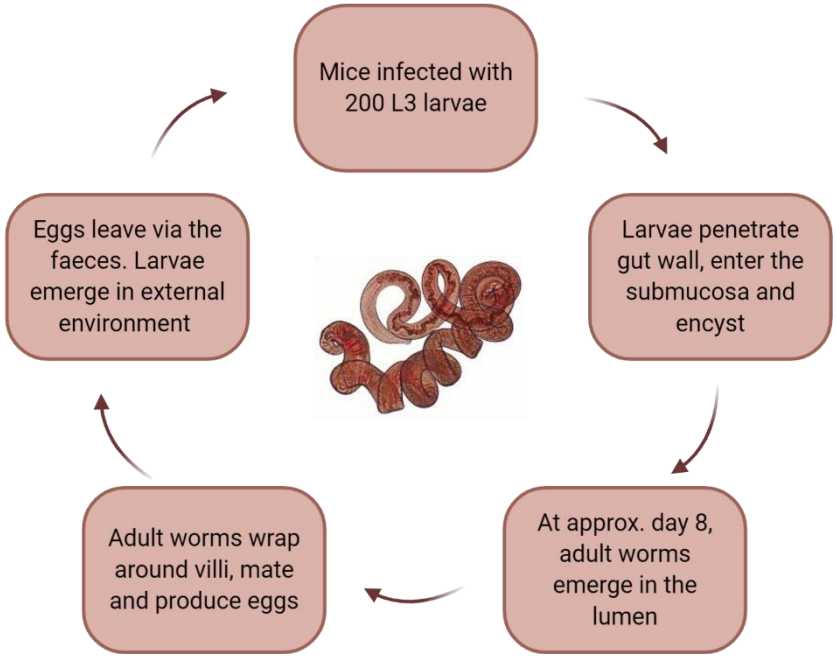

Figure 1-2 *Heligmosomoides polygyrus* life cycle
An overview of the key events in the life cycle of *Heligmosomoides polygyrus.* Created with BioRender.com and adapted from (Reynolds et al., 2012).

1.3 Anti-helminth immune response

1.3.1 Initial innate responses

H. polygyrus larvae migrate through the wall of the small intestine, however the exact mechanism of larval penetration of the submucosa remains unclear (Patel et al., 2009). As a result, epithelial cells release the alarmin cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) (Humphreys et al., 2008, Taylor et al., 2009, Owyang et al., 2006, Gause et al., 2013). There alarmins are stored in and secreted by epithelial cells and are released upon damage to the cell membranes (Bamias and Cominelli, 2015). IL-25 is essential for the expulsion of *H. polygyrus* (Zaiss et al., 2013, Hewitson et al., 2015) and for the development of type 2 immune responses against *N. brasiliensis* and *Trichuris muris* (Owyang et al., 2006, Price et al., 2010). IL-33 is also a critical cytokine involved in the initiation of adaptive T helper 2 (Th2) responses to helminth infection via stimulation of innate immune cells (Humphreys et al., 2008). TSLP also promotes the adaptive Th2 response indirectly by acting on numerous immune cells such

as DCs (Ziegler and Artis, 2010). IL-33, IL-25 and TSLP activate ILC2s, which in turn produce the type 2 cytokines IL-4, IL-5 and IL-13 (Figure 1-3) (Pelly et al., 2016, Maizels et al., 2012, Gause et al., 2013). The release of alarmins is essential for the induction of type 2 innate cell responses.

The activation of ILC2s is essential for priming an effective Th2 response to *H. polygyrus* (Pelly et al., 2016). Other innate cells including mast cells, eosinophils and basophils are also rapidly recruited to the site of infection and secrete high levels of IL-4, IL-5 and IL-13 (Gause et al., 2013, Maizels et al., 2012). Mast cells, eosinophils and basophils are activated by IgE released by plasma cells in the SILP, as discussed in section 1.3.1. IL-9 is also described as a type 2 cytokine, it is secreted by Th9 cells, ILC2s, mast cells and eosinophils (Licona-Limón et al., 2013, Gounni et al., 2000, Hültner et al., 2000, Wilhelm et al., 2011). The primary function of IL-9 is mast cell maturation, it has also been reported to promote epithelial cell release of cytokine alarmins (Faulkner et al., 1997, Matsuzawa et al., 2003, Hepworth et al., 2012). In both *T. muris* and *H. polygyrus* infection, loss of either mast cells or administration of IL-9 blocking antibody impairs parasite expulsion (Hepworth et al., 2012, Kooyman et al., 2000, Khan et al., 2003). NK cells have also recently been reported to play an important role in would healing at the early stages of *H. polygyrus* infection (Gentile et al., 2020). Depletion of NK cells resulted in intestinal bleeding but had no effect on parasite expulsion (Gentile et al., 2020). Throughout *H. polygyrus* infection IL-13 stimulates goblet cell hyperplasia and increases goblet cell production of mucins and the anti-helminthic molecule RELM-β (Artis et al., 2004). The importance of goblet cells and other specialised epithelial cells is discussed in section 1.3.2. The activation and functions of numerous innate immune cells allow a rapid response to *H. polygyrus* and also contribute towards granulomas formation. During the tissue-dwelling stage of infection, granulomas form around encysted larvae in the serosa (Anthony et al., 2007). These granulomas consist of a neutrophil core surrounding the encysted larvae (Anthony et al., 2007, Annis et al., 2009). The core is also rich in Alternatively activated macrophages (AAMs) (Annis et al., 2009, Kreider et al., 2007). Polarisation of AAMs is primarily dependent on IL-4 and IL-13, which both signal via the IL-4Rα subunit, and mice lacking IL-4Rα specifically on

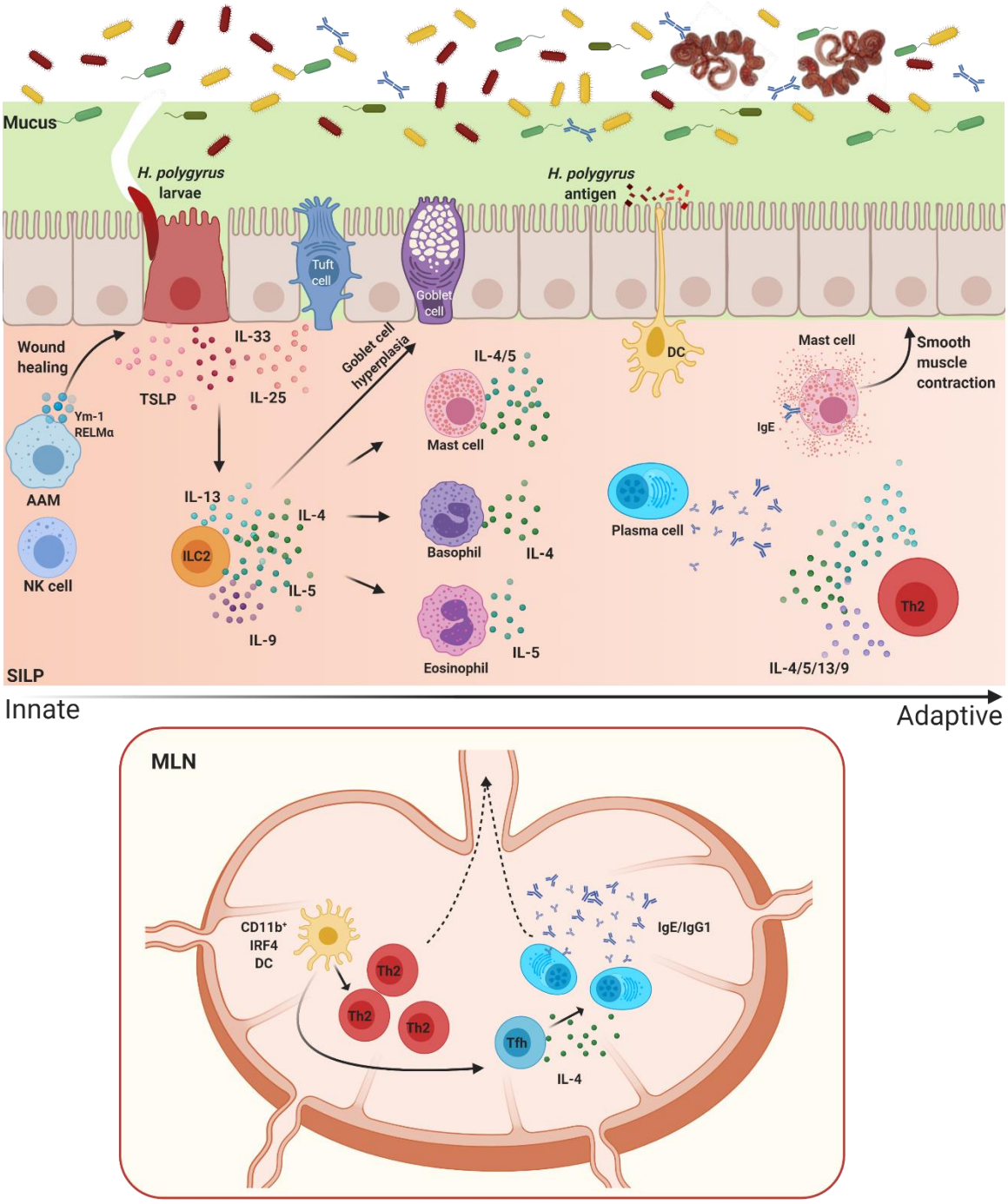

Figure 1-3 Immune response to *H. polygyrus*
Overview of the type 2 immune response to *H. polygyrus*. Created with BioRender.com and adapted from (Sorobetea et al., 2018) and (Salazar-Castañon et al., 2014).

macrophages lose the potent AAM response typically seen in helminth infection (Herbert et al., 2004). AAMs secrete the regulatory cytokines IL-10 and TGF-ß, along with secretory proteins such as RELMα, Arginase-1 and Ym1, all of which are associated with various stages of tissue repair (Annis et al., 2009, Taylor et al., 2012, Maizels et al., 2012, Gause et al., 2013). Elevated levels of these repair proteins are observed during helminth infection (Annis et al., 2009). The

importance of AAMs has been specifically demonstrated in *H. polygyrus* infection, where depleting macrophages via chlodronate liposomes resulted in increased larval mobility and reduced adult worm expulsion (Anthony et al., 2006). The outer ring of the granulomas is made up of DCs, eosinophils and Th2 cells (Anthony et al., 2007). Granulomas encase the larvae and prevent their development to adult worms, and mice with increased resistance to helminth infection have higher numbers of granulomas (Menge et al., 2003, Moreau and Chauvin, 2010). The combination of alarmin release, activation and recruitment of innate immune cells, wound healing and granulomas formation are essential for the coordination of the adaptive immune response to and expulsion of *H. polygyrus (*Figure 1-3).

1.3.2 Adaptive immune response

DCs acquire *H. polygyrus* antigens in the lumen of the small intestine and migrate to the draining MLN. The importance of DC priming of Th2 cells has been well established and is discussed in section 1.4.2 and 1.1.2.1. Mice lacking $CD11b^+$ $CD103^+$ DCs have impaired Th2 responses to the helminth *N. brasiliensis* (Gao et al., 2013). $CD11b^+$ DCs from *H. polygyrus* infected mice have been reported to promote Th2 cells differentiation (Redpath et al., 2018, Mayer et al., 2020). In *Schistosoma mansoni* infection, $CD11b^+$ $CD103^+$ DCs specifically transport parasite antigen from the SILP to the MLN (Mayer et al., 2017). In addition, the expression of the transcription factor (TF) KLF4 by DCs has been reported to be essential for priming of Th2 responses to *S. mansoni* (Tussiwand et al., 2015). The expression of OX40-liagnd (OX40L) by DCs has also been shown to be a critical costimulatory molecule for the polarisation of Th2 cells (Ito et al., 2005, Jenkins et al., 2007). Although recent studies show an important role for OX40L in Th1 responses also (Gajdasik et al., 2020). Inducible T cell co-stimulator (ICOS) is another costimulatory molecule that has been reported to contribute towards Th2 differentiation. $ICOS^{-/-}$ mice infected with *N. brasiliensis* have reduced Th2 differentiation (Kopf et al., 2000). Although similarly to OX40L, ICOS is also required for Th1 responses to infection (Wilson et al., 2006, Dong et al., 2001).

Primed Th2 cells migrate to infected tissues from the MLN and are an essential source of IL-4, IL-13 and IL-5 which further activate AAMs, ILC2s and other

components of the type 2 response (Maizels et al., 2012, Anthony et al., 2007) In addition, IL-4 and IL-13 enhance smooth muscle contractility and, coupled with increased mucus production make up the “weep and sweep” response that promotes helminth expulsion (Zhao et al., 2003, Anthony et al., 2007). Although the type 2 cytokines IL-13, IL-5 and IL-4 all have distinct roles, redundancy between these cytokines has been reported. IL-4 is reported to be essential for *T. muris* expulsion in C57BL/6 mice but not BALB/C mice, parasites persist in infected IL-4$^{-/-}$ C57BL/6 but IL-4$^{-/-}$ BALB/C mice infected with *T. muris* mice are unaffected by this deficiency (Scales et al., 2007). In both strains, lack of IL-13 impairs expulsion equally (Scales et al., 2007), perhaps due to the pivotal role of this cytokine in increasing mucus production during helminth infection. In addition, IL-4 has been reported to be non-essential for the expulsion of *N. brasiliensis* (Mckenzie et al., 1998, Liang et al., 2011, Lawrence et al., 1996). Although much of the literature depicts IL-4 as a critical cytokine for Th2 priming, there is evidence that this cytokine is dispensable. It is argued that the presence of Th2 inducing co-stimulatory molecules and low antigen dose may be sufficient for initial priming of Th2 cells which will subsequently produce IL-4 (Hosken et al., 1995, King and Mohrs, 2009, Noben-Trauth et al., 2000). Once Th2 activation, differentiation and proliferation occurs, these cells will traffic to the SILP, using homing markers including the integrin α4β7 and chemokine receptor CCR9 (Bono et al., 2016, Hosoe et al., 2004, Denucci et al., 2010). However, not all activated Th2 cells will migrate to the SILP.

A small portion of CD4^{+} T cells remain in the MLN after activation, located in the B cell follicles, referred to as Tfh cells and are characterised by the expression of the chemokine receptor CXCR5 and inhibitory molecule PD-1 (King and Mohrs, 2009, Haynes et al., 2007). IL-4 production by Tfh cells induce B cell activation and class switching to parasite-specific IgE and IgG1 (Figure 1-3) (Gause et al., 2013, Maizels et al., 2012) . IL-21 secretion by Tfh cells is key for differentiation of long-lived class switched plasma cells, although IL-21 secretion by Tfh cells is not restricted to Th2 responses (Wang et al., 2018). These plasma cells will secrete IgE and IgG1 (King and Mohrs, 2009, Maizels et al., 2012). Together these effector immune cells and molecules cumulate in a strong effective anti-helminthic Th2 immune response (Figure 1-3)(Redpath et al., 2014). However, helminths have evolved with their hosts for many years and as a result have developed multiple mechanisms to evade or counteract the host Th2 response.

1.3.3 Regulatory response and immune mimicry

The regulatory response induced by helminths involves both innate and adaptive regulatory cells, and regulatory cytokines such as IL-10 and TGF-ß (Maizels and McSorley, 2016). The mechanisms by which helminths suppress the host immune response vary between helminth species (White et al., 2020). *H. polygyrus* is an example of a helminth that actively interferes with host cytokine signalling via secreted mimicry molecules (Smyth et al., 2018, Osbourn et al., 2017). Two immune system mimic molecules have been identified in *H. polygyrus* secretory/excretory (HES) products. Firstly, *H. polygyrus* TGF-ß mimic (TGM) is a TGF-ß mimic molecule that binds to the TGF-ß receptor and, despite sharing no structural homology with the TGF-ß family, induces FOXP3 expression upon binding, similar to TGF-ß itself (Smyth et al., 2018, Johnston et al., 2017). *H. polygyrus* Alarmin Release Inhibitor (HpARI) binds to the IL-33 receptor ST2 and prevents IL-33 interacting with its receptor, therefore suppressing subsequent downstream type 2 immune responses and, preventing allergic inflammation in the airway (Osbourn et al., 2017). In addition to these cytokine mimic molecules found in HES, there are likely other active components of HES that regulate host immunity.

The excretory secretory products from helminths induce a tolerogenic phenotype in DCs, characterised by secretion of IL-10, TGFß and downregulation of MHC and co-stimulatory molecules (Segura et al., 2007, Domogalla et al., 2017). Infection with *H. polygyrus* increases the abundance of the bacteria *Lactobacillus* (Walk et al., 2010, Rapin et al., 2020). These bacteria produce short chain fatty acids (SCFA) which promote Tregs and have been reported to be protective in a mouse model of colitis (Morris et al., 2017). As well as Tregs, other regulatory populations expanded in helminth infection include regulatory B cells (Bregs) and Tr1 cells (Allen and Maizels, 2011). $CD19^{+}$ $CD23^{hi}$ Bregs adoptively transferred from helminth infected mice to recipient mice can suppress airway allergy, which is independent of IL-10. These data demonstrate an IL-10 independent role of Bregs in suppressing inflammation (Wilson et al., 2010, Allen and Maizels, 2011). Treatment with anti-CD25 mAb to deplete Tregs during infection with the helminth *T. muris* results in increased pathology (D'elia et al., 2009, White et al., 2020). However, only treatment with an anti-glucocorticoid-induced tumour necrosis factor receptor (GITR), which is

upregulated on Tregs, results in increased helminth expulsion (D'elia et al., 2009, White et al., 2020), suggesting an important role for this receptor in suppressing Th2 cells. In addition, anti-CD25 mAb treatment during *S. mansoni* infection enhances egg destruction and pathology in the gut (Layland et al., 2007). These data combined demonstrate that helminths exploit host immune responses and the suppressive role of regulatory cells which promotes parasite persistence, this mechanism can also be protective and therefore beneficial to the host.

An area of increasing interest is the consequences of highly regulatory responses to helminth infections in the context of co-infections such as HIV, *Mycobacterium tuberculosis* and malaria causing *Plasmodium* species (Salgame et al., 2013). There is evidence that having a helminth co-infection during these infections results in decreased resistance to the these infections (Salgame et al., 2013). One study in Ethiopia found an association between *M. tuberculosis* and intestinal helminth infection, with this association increasing when individuals were infected with multiple helminth species (Tristão-Sá et al., 2002, Salgame et al., 2013). Both protective and detrimental roles for helminth co-infection with malaria have been reported (Nacher et al., 2000, Lyke et al., 2005, Le Hesran et al., 2004). The conflicting outcomes of these studies may be dependent on life cycle stage of both parasites and time and order of co-infection. Similarly, the role of helminth co-infection in HIV has showed conflicting results. The suppressive nature of Tregs induced in helminth infection may dampen important immune responses required to control HIV infection, such as suppression of DCs (Salgame et al., 2013). Conversely, the type 2 cytokine IL-13 has been reported to negatively regulate HIV replication (Mikovits et al., 1994, Montaner et al., 1993). The induction of regulatory responses as an evolutionary mechanism for helminth persistence proves an interesting therapeutic target for the treatment of inflammatory conditions. Therefore, the mixed Treg/Th2 immune response described during *H. polygyrus* infection is a result of both parasite manipulation of the host via mimicry molecules to induce a regulatory response and host effector Th2 responses to the parasite itself to promote clearance.

1.3.4 Helminth expulsion and secondary challenge

The expulsion of helminths from the intestinal lumen requires the induction of Th2 mediated 'weep and sweep' response as described previously. This response comprises an increase in mucus production by goblet cells, electrolyte secretion by epithelial cells and smooth muscle contractility via IL-4 and IL-13 mediated stimulation of enteric nerves (Harris and Loke, 2017, Finkelman et al., 2004). High epithelial cell turnover during *H. polygyrus* infection has also been reported to disrupt parasite persistence (Cliffe et al., 2005). IL-5 dependent recruitment of eosinophils may promote direct killing of worms by these cells; however, this killing mechanism has only been successfully demonstrated *in vitro* (Sorobetea et al., 2018). Granulomas are key component of the immune response to *H. polygyrus* by walling off parasites which limits damage as a result of larval migration. In addition, higher numbers of granulomas correlate with resistance to infection (Menge et al., 2003, Anthony et al., 2006). Granulomas persist in the submucosa of the gut once adult worms have re-emerged in the lumen (Filbey et al., 2014, Anthony et al., 2006, Menge et al., 2003).

During *H. polygyrus* infection, there is a requirement for both killing of the parasite and subsequent wound healing. Much of these responses occur within granulomas, AAMs play a role in both parasite killing and wound repair(Ariyaratne and Finney, 2019). Binding of parasite bound IgG by CD64 on AAMs results in immobilization of parasites (Esser-Von Bieren et al., 2015, Esser-Von Bieren et al., 2013). In this study, CD11b on AAMs was required for the initial adherence to larvae but not for subsequent larval immobilization. As previously mentioned in section 1.3.1, the release of RELMα, Arginase-1 and Ym1 from AAMs in response to both IL-4 and IL-13 promotes wound healing and collagen deposition(Annis et al., 2009, Kreider et al., 2007, Herbert et al., 2004). Although, continued collagen deposition during helminth infection can result in fibrosis and subsequent loss of normal tissue structure and function (Ariyaratne and Finney, 2019, Esser-Von Bieren et al., 2015). Similarly to AAMs, eosinophils have also been reported to bind to larvae via complement receptors and IgG-Fc receptor interactions. IL-5 stimulated eosinophils bind to larvae, degranulate and release eosinophil stimulation promoter (ESP) and major basic protein (MBP), both of which can result in larval killing (Ariyaratne and Finney, 2019). In keeping with this, mice lacking eosinophils have been reported to have

increased live larvae in the submucosa (Hewitson et al., 2015). In addition, eosinophils can also promote collagen deposition and wound healing during helminth infection via mediators such as RELMα and the release of IL-4 and IL-13 which further promote AAM mediated repair (Allen and Sutherland, 2014, Chen et al., 2016). Therefore, the presence of both AAMs and eosinophils are key for both effective parasite killing and wound repair, although distinct mechanisms and immune mediators are required for these responses.

Despite these mechanisms, primary infection with *H. polygyrus* in C57BL/6 mice is typically chronic. However, parasite persistence varies between mouse strains, for example, BALB/C mice, are more resistant to infection compared to C57BL/6 mice (Scott, 1991, Reynolds et al., 2012). Upon infection with *H. polygyrus* the expression of the IL-4R on MLN cells from BALB/C mice was higher compared to C57BL/6 mice (Perona-Wright et al., 2010). This coincided with higher IL-4 expression BALB/C mice compared to C57BL/6 mice (Perona-Wright et al., 2010). Initial primary infection with *H. polygyrus* can be cleared with anti-helminthics and in most mouse strains a strong memory response is established and protects against secondary challenge (Finkelman et al., 1997, Crump and Ōmura, 2011, Reynolds et al., 2012).

The activation of the Th2 memory response upon secondary challenge with *H. polygyrus* occurs rapidly at 4 days post-infection, with the accumulation of neutrophils, Th2 cells, DCs and eosinophils around encysted larvae in the submucosa – these cell infiltrates are much greater than those found in primary infection (Anthony et al., 2006, Morimoto et al., 2004, Liu et al., 2004). In both humans and mice, memory Th2 cells can be defined by their high expression of the IL-33 receptor (ST2), allowing for TCR independent activation upon binding of IL-33, the alarmin cytokine, to its receptor (Minutti et al., 2017, Obata-Ninomiya et al., 2018, Smithgall et al., 2008). This early activation of Th2 cells drives both innate and adaptive responses, as described in sections 1.3, resulting in rapid expulsion of parasites. Successful immunity to secondary challenge with *H. polygyrus* is lost when both IL-4 and the IL-4R are blocked simultaneously but less so when blocking IL-4 alone (Urban et al., 1991). This shows that in a re-challenge setting, both IL-4 and IL-13 key for worm expulsion as both signal via the IL-4R (Urban et al., 1991).

The expulsion of helminths from the lumen is a co-ordinated process reliant on the type 2 immune response, comprising mechanisms also found in allergic disease. The ‘weep and sweep’ response induced by helminths has therefore become an important model for the study of immune mechanisms during allergic responses.

1.4 The intestinal barrier

1.4.1 Anatomy of the intestine

As the gastrointestinal (GI) tract of humans and mice have both anatomical and physiological similarities, mouse models have been used extensively in the study of gastrointestinal homeostasis and disease (Hugenholtz and De Vos, 2018, Bowcutt et al., 2014). The small intestine is the longest part of the GI tract and is made up of the duodenum, jejunum, and ileum (Figure 1-5) (Bowcutt et al., 2014). The duodenum is the proximal end of the small intestine, where bile and digestive enzymes from the pancreas enter the intestine (Bowcutt et al., 2014). The duodenum is followed by the jejunum and then the ileum. The surface of the small intestine has small finger-like projections into the lumen called villi; villi maximise the surface area of the small intestine allowing for optimal nutrient absorption (Bowcutt et al., 2014). Peyer’s patches are also an important feature of the small intestine and contain Microfold ‘M’ cells which are important for the movement of luminal antigen such as food antigen, across the wall of the intestine (Okumura and Takeda, 2017). The large intestine can be split in to two sections, the colon, and the cecum. The cecum is important for fermentation and the production of SCFA’s (Parada Venegas et al., 2019). The colon has the highest abundance of bacterial species compared to the small intestine (Berg et al., 1996, Bowcutt et al., 2014). It also contains crypts, which are small invaginations found along the intestine, where progenitor cells typically reside (Bowcutt et al., 2014). The colon lacks villi, this is because the primary function of the colon is water reabsorption (Bowcutt et al., 2014, Hugenholtz and De Vos, 2018).

Both the small intestine and colon have a mucus layer that protects the epithelial barrier, preventing microorganisms from accessing the host intestinal

epithelial cells (IECs). The mucus layer and the mucin glycans that make up the layer provide a surface for the colonisation of bacteria and are an important energy source for the microbiota (Johansson and Hansson, 2016). In response to appropriate stimuli such as pathogens, mucus production is enhanced and pathogenic bacteria, for example, become trapped in the mucus, contributing towards the clearance of pathogens (Johansson and Hansson, 2016). Damage to the intestinal epithelial layer and its associated mucosal layer can result in poor uptake of nutrients, movement of pathogens and microorganisms into the blood stream and changes to the gut microbiota (Johansson and Hansson, 2016). In the small intestine, there is one continuous diffuse mucous layer, which is penetrable to bacteria (Johansson and Hansson, 2016). However, these bacteria do not closely associate with the epithelium due to antimicrobial proteins, which are produced by most IECs including Paneth and goblet cells (Hooper and Macpherson, 2010). In addition, secreted immunoglobulin A (sIgA) from plasma cells limits bacterial interactions with the epithelium (Johansson and Hansson, 2016, Hooper and Macpherson, 2010). The colon has an inner and outer mucus layer; the inner layer is attached to the epithelium, cannot be penetrated by bacteria, and is densely made up of layers of MUC2 multimers (Ambort et al., 2012, Johansson and Hansson, 2016). Whereas, the outer mucus layer is highly colonised by bacteria (Johansson and Hansson, 2016). In both humans and mice with colitis, bacteria penetrate the inner layer of the colonic mucus (Johansson et al., 2014). Goblet cells are specialised IECs and are the predominant mucus secreting cells.

Muc2 is the dominant mucin that makes up the mucus layer (Johansson and Hansson, 2016). In $Muc2^{-/-}$ mice there is no protective mucus layer throughout the intestinal tract, resulting in inflammation along the intestine and the development of spontaneous colitis and colorectal cancer (Velcich et al., 2002, Johansson and Hansson, 2016, Van Der Sluis et al., 2006). Interestingly, goblet cells have been shown to take up luminal antigen when secreting mucus which they subsequently transfer to DCs (Mcdole et al., 2012).

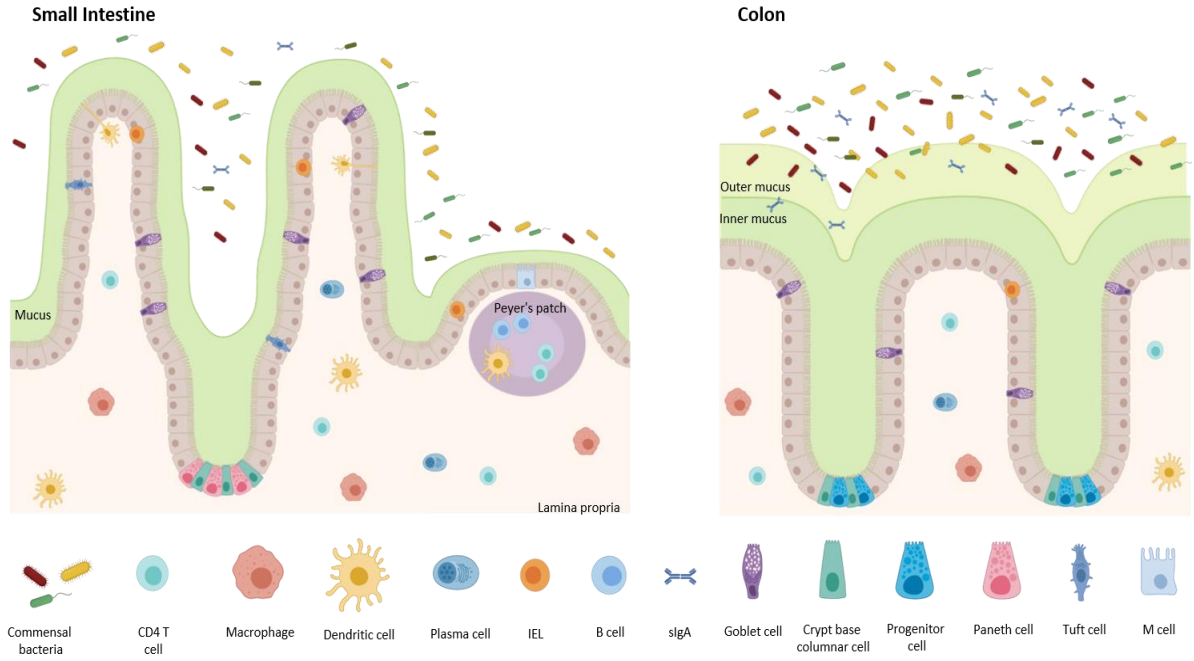

Figure 1-4 Small intestine and colon architecture and immune composition
The anatomy and immune cells of the small intestine and colon. Created with BioRender.com and adapted from (Lutter et al., 2018).

Due to the anatomical differences between the small and large intestine, these tissues are more susceptible to certain parasites (Bowcutt et al., 2014). Human parasite infections such as *Ancylostoma duodenale* and *Ascasris lumbricoides* localise to the small intestine, similar to *H. polygyrus* in mice. Whereas the human whipworm *T. trichuria* and the mouse parasite *T. muris* are found in the large intestine (Bowcutt et al., 2014). In addition, the bacterial content differs between the small and large intestine, where in humans the large intestine containing approximately 10^{10} bacteria/g of intestinal content and the small intestine containing fewer, around 10^{3} bacteria/g of intestinal content (Berg, 1996, Bowcutt et al., 2014). Indeed, due to difference in bacterial species and abundance in the small and large intestine these sites have varying susceptibility to pathogens and diseases. The pathogenic bacteria *Clostridium difficile* specifically colonises the colon, this infection can be serious if left untreated, resulting in conditions such as toxic megacolon which can result in sepsis (Johanesen et al., 2015). On the other hand, *Norovirus* is primarily found in the small intestine and is concentrated at the tips of the villi (Green et al., 2020, Bowcutt et al., 2014). In addition, cancers of the small intestine are much rarer than those in the colon (Bowcutt et al., 2014). In addition, distinct regions of the intestine correspond to draining lymph nodes that are critical for the priming of immune responses.

1.4.2 Mesenteric lymph nodes

The mesenteric lymph nodes are the draining lymph nodes for the intestine, comprising a chain of multiple lymph nodes which each drain distinct parts of the intestine (Mayer et al., 2020, Houston et al., 2016). They are often divided into the small intestine and colon draining lymph nodes, sMLN and cMLN respectively (Houston et al., 2016). These are the priming sites for adaptive immune responses in the intestine. Lymph nodes are encapsulated secondary lymphoid organs (SLOs) which have a specialised architecture that allows for

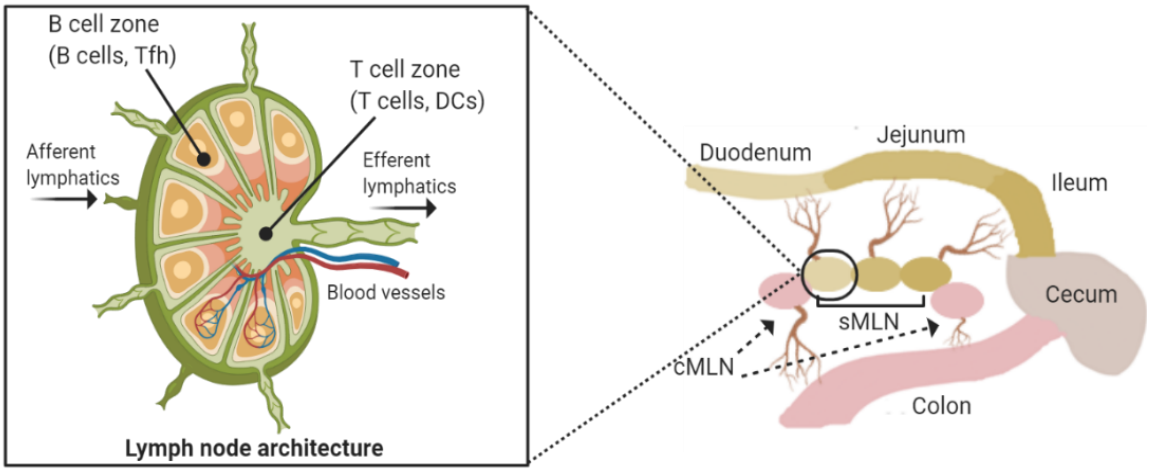

Figure 1-5 The MLN and lymph node architecture
The MLN chain drains distinct regions of the intestine referred to as the sMLN and cMLN (Houston et al., 2016, Mayer et al., 2020). The architecture of LNs (left) shows the distinct T and B cell zones and cells typically found in these areas. Created with BioRender.com and adapted from (Houston et al., 2016) and (Drayton et al., 2006).

naïve $CD4^+$ T cells to encounter their cognate antigen presented on MHCII (Jenkins et al., 2001). Lymphocytes enter LNs from the blood via HEVs (Girard and Springer, 1995). T cells will then move to T cell areas and B cells will migrate to follicles (Figure 1-5) (Girard and Springer, 1995). DCs are potent APCs with the unique capacity to capture, process, and present antigen to prime naïve T cells (García Nores et al., 2018). There are resident DCs that remain in LNs and also DCs which reside in peripheral tissues. These peripheral residing DCs are known as migratory DCs and are continually capturing both self and foreign antigen in the periphery (Segura et al., 2012, Jenkins et al., 2001). Captured antigen is internalised, processed, and presented on the surface of DCs. To maximise the chance of these DCs encountering naïve T cells with the correct TCR, DCs migrate to draining LNs (Jenkins et al., 2001, Randolph, 2001).

The subsequent activation and differentiation of T cells is described in section 1.1.1 and 1.1.2. This process requires the migration of cells throughout the MLN.

Migration into and within LNs is dependent on chemokines and their receptors (Randolph, 2001). Chemokines are chemotactic cytokines that control the migration and location of immune cells. CCR7 is a G-protein-coupled chemokine receptor which DCs upregulate upon maturation (Yanagihara et al., 1998). CCR7 binds to the homeostatic chemokines CCL21 and CCL19 which are expressed by fibroblast reticular cells (FRCs), stromal cells in T cell area (Martín- Fontecha et al., 2003). CCL21 is also expressed by endothelial cells that line the HEVs (Gunn et al., 1998). These chemokines direct DC migration to the LNs, where they arrive at the subcapsular sinus of the LN and subsequently into the LN parenchyma where they interact with naïve T cells (Jenkins et al., 2001). Chemokines and homing molecules are also required for activated T cell egress from the MLN to the intestine. During DC-T cell interactions, DCs will imprint homing receptors on the surface, for example, upregulation of the integrin α4ß7 and the chemokine receptor CCR9 is required for homing to the small intestine lamina propria, a key immunological site (Williams and Butcher, 1997, Johansson-Lindbom and Agace, 2007).

1.4.3 Epithelial barrier

The lamina propria (LP) is separated from the lumen of the gut by both the epithelium and mucus barrier. It is an important immunological site made of connective tissue, containing numerous immune cells (Figure 1-4). The LP along with the epithelium and muscular mucosae make up the mucosa of the intestine. The other three layers that make up the intestinal wall are the submucosa, the muscularis propria and the serosa. The intestinal epithelial barrier acts as a physical barrier to prevent harmful toxins and microorganisms such as bacteria, viruses, and fungi crossing from the lumen of the gut into the blood stream. The barrier is also essential for absorption and filtration of nutrients into the body. This barrier is made up of a single layer of IECs, held together by numerous tight junction (TJ) proteins. Much of the epithelial barrier is comprised of enterocytes, which play a critical role in absorption and digestion. The rest of the barrier includes specialised IECs such as goblet cells, Paneth cells and tuft cells (Figure 1-4).

1.4.3.1 Epithelial cell-cell adhesion

The cell-cell adhesion provided by TJ proteins is essential for the structural integrity of the intestinal barrier. Three of the key TJ proteins are the integral transmembrane proteins and protein families including occludin, claudins and junctional adhesion molecules (JAM). In addition, zonula occludens (ZO) are bridges that connect these transmembrane proteins to intracellular signalling cascades (Bazzoni and Dejana, 2004). Occludin and ZO-1 interactions are important for maintenance of barrier function (Bazzoni and Dejana, 2004). Occludin$^{-/-}$ mice have chronic inflammation in several tissues and intestinal epithelial hyperplasia (Saitou et al., 2000). In addition, increased expression of occludin improves and protects the integrity of the TJ (Mccarthy et al., 1996). There are 23 members of the claudin family, multiple claudin family members are expressed in the small intestine, claudin-2 specifically is expressed in the small intestine of mice and humans (Lu et al., 2013). Increased expression of claudin-2 is associated with a leaky epithelial cell barrier, and it also plays a determining role in permeability of the epithelium to ions and solutes (Rahner et al., 2001, Van Itallie et al., 2003). Absence of ZO-1 results in delayed assembly of other TJ proteins such as occludin (Tsukita et al., 2009). Adherens junctions (AJs) are also another important component of maintaining barrier integrity. AJs are protein complexes that maintain cell to cell contact and localise below the TJs (Figure 1-6) (Takeichi, 1990). Epithelial-cadherins (E-cadherins) are glycoproteins that bind to similar molecules on neighbouring epithelial cells. E-cadherins are the major cadherin found in epithelial cells (Figure 1-6) (Takeichi, 1990, Hanby et al., 1996, Doğan et al., 1995, Guo et al., 2003). AJs are key for barrier function and signalling via these junctions also regulates TJ proteins (Guo et al., 2003). Mice lacking E-cadherin in the small intestine die quickly after birth due to loss of barrier function (Bondow et al., 2012). The ability of epithelial cells to form a tightly regulated barrier is key for intestinal

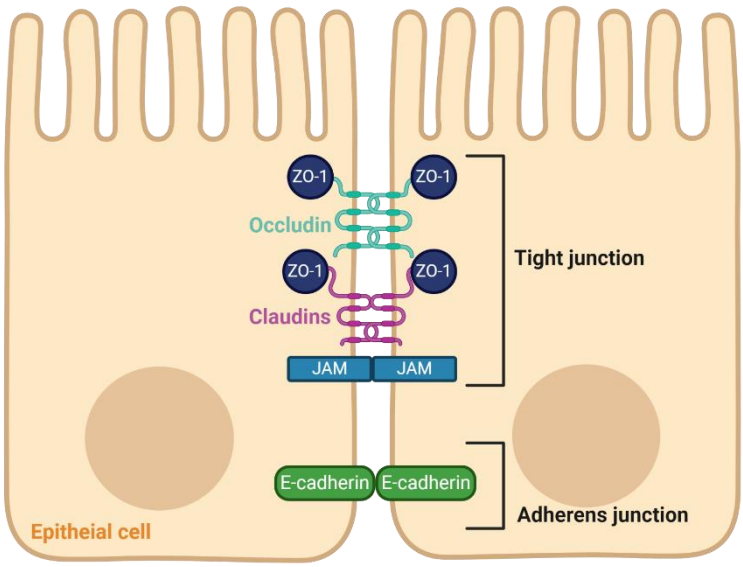

Figure 1-6 Epithelial tight junction proteins
Tight junction proteins and adherens junction proteins in epithelial cells that are essential for the formation of the intestinal epithelial barrier. Created with BioRender.com and adapted from (Hammer et al., 2015).

homeostasis, function and nutrients absorption, the rapid turnover of epithelial cells is also a key component of maintaining epithelial barrier integrity.

1.4.3.2 Epithelial cell turnover

The intestinal epithelium renews constantly (Barker et al., 2007). Crypt base columnar cells are stem cells found in the crypts that drive this rapid intestinal epithelial cell renewal (Barker et al., 2007). Typically, IECs renew every 3-5 days with older cells being shed into the lumen and new IECs differentiating in the crypts (Park et al., 2016). This process is regulated by a magnitude of factors including growth factors, Wnt ligands and commensal bacteria (Hooper and Gordon, 2001, Clevers, 2013). Interestingly, it has been reported that IL-13 is a key driver in the movement of new IECs from the crypts to the tip of the villi, this mechanism also facilitates displacement of pathogens such as *H. polygyrus* which persists in the gut by wrapping itself around villi (Cliffe et al., 2005). This migration of cells to the tip of the villi is largely reduced in germ-free (GF) mice and mice treated with antibiotics, emphasising the importance of commensal bacteria in epithelial turnover (Park et al., 2016). In IBD, epithelial cell turnover is accelerated due to increased apoptosis, resulting in loss of barrier function and inflammation (Di Sabatino et al., 2003). The balance between epithelial cell

death and proliferation is essential for the maintenance of the intestinal barrier and for the function of specialised epithelial cells.

1.4.3.3 Specialised epithelial cells

IECs are an important line of defence and some of the earliest cells to respond to pathogens. IECs express pattern recognition receptors (PRRs) that recognise and discriminate microbes. This process is important for intestinal homeostasis. Mice lacking toll-like receptors (TLRs), a type of PRR, have increased susceptibility to dextran sulphate sodium (DSS) induced colitis due to lack of microbe sensing (Rakoff-Nahoum et al., 2004). In addition, IECs can alert immune cells to pathogens and interactions between IECs and APCs can coordinate local immune responses via the release of alarmin cytokines such as TSLP, IL-25 and IL-33 as described in Section 1.3.1(Rimoldi et al., 2005, Zaph et al., 2007). Specialised IECs play specific important roles in intestinal homeostasis and in immune responses to pathogens in the gut. Paneth cells are specialised IECs found in the crypts of the small intestine. These cells secrete numerous antimicrobial molecules which are stored in granules (Bevins and Salzman, 2011). The release of antimicrobial mediators into the lumen may prevent bacteria colonising in SI crypts. These mediators can act on both commensal and pathogenic bacteria (Bevins and Salzman, 2011). Paneth cells have also been shown to sense commensal bacteria in a MyD88 dependent manner and subsequently maintain homeostasis at the barrier site (Vaishnava et al., 2008). In patients with Crohns disease, Paneth cell secretion of α-defensins, a type of antimicrobial peptide, is decreased, resulting in gradual changes and subsequent dysbiosis of the microbiota, promoting bacteria invasion across the epithelial barrier (Wehkamp et al., 2005). Along with Paneth cells, goblet cells are another subset of critical IECs. As discussed in Section 1.3.1, goblet cells are critical for formation and maintenance of the mucus layer, which protects the epithelial barrier from luminal contents. An important driver of goblet cell hyperplasia is IL-13, which is released by IL-25 stimulated ILC2s, Tuft cells are key producers of IL-25. Tuft cells are chemosensory cells found at mucosal sites, including the intestine (Ting and Von Moltke, 2019). Tuft cells are the dominant source of IL-25 which is required to drive ILC2 responses (Von Moltke et al., 2016, Ting and Von Moltke, 2019). Mice lacking tuft cells fail to induce goblet cell hyperplasia and expel the helminth *N. brasiliensis* (Gerbe et al., 2016).

Therefore, the epithelial cell barrier is an essential physical barrier that separates luminal contents from the lamina propria. In addition, specialised immune cells play essential roles in maintaining homeostasis and coordinating immune responses to pathogens.

1.4.4 Bacterial translocation

The term bacterial translocation was first proposed in 1979 (Berg and Garlington, 1979). It is defined as the movement of microbes and/or their products through the intestinal epithelial barrier, to organs such as the mesenteric lymph nodes (MLN) and spleen. The immune system has co-evolved with commensal bacteria (Berg and Garlington, 1979). This is shown in GF mice, which have no commensal bacteria. These mice have an underdeveloped mucosal immune system and SLOs that lack structure (Bauer et al., 1963, Macpherson and Harris, 2004). Bacterial translocation can occur as a direct consequence of damage to the epithelial barrier, impaired commensal immune homeostasis, or overgrowth of bacterial species (Ding et al., 2004). Sampling of the intestinal lumen, including commensal bacteria, by DCs is an important component of immune homeostasis. These DCs will prime B cells in the MLN to produce IgA, which is key for intestinal homeostasis as described in section 1.4.1 (Macpherson and Uhr, 2004). However, DCs sampling the lumen will traffic to the MLN only, not to distal sites such as the spleen or liver and so are not responsible for systemic bacterial translocation (Macpherson and Harris, 2004). In mice lacking a thymus and therefore T cells, spontaneous bacterial translocation to distal organs, including the spleen and liver, occurs (Owens and Berg, 1980). Th cells that are specific for commensal bacteria, in homeostasis, are either unresponsive to their cognate antigen or have a regulatory phenotype, preventing inappropriate inflammatory responses to commensal bacteria (Lathrop et al., 2011). However, in acute inflammatory infection mouse models such as *Toxoplasma gondii*, this commensal tolerance by T cells is lost. Commensal specific $CD4^+$ T cells become activated and induce an inflammatory response by acting as an adjuvant that exaggerates the immune response to the *T. gondii* infection (Hand et al., 2012). Therefore, the commensal bacteria that colonise the intestine are key for functional mucosal immunity. Translocation of these bacteria to distal organs in immunocompromised individuals can result in lethal sepsis (Berg and Garlington, 1979). Overall, bacterial translocation can

have levels of severity, depending on a multitude of factors. These factors include changes to the microbiota, breach of the epithelial barrier or loss of tolerance to commensal bacteria by the immune system.

1.5 The role of IL-10 in the intestine

1.5.1 IL-10 and IL-10R signalling

The regulatory cytokine IL-10 is a homodimer which binds to and signals via a tetrameric receptor made up of IL-10R1 and IL-10R2. IL-10 binds directly to IL-10R1, resulting in recruitment of IL-10R2 to form the signalling receptor (Figure 1-7) (Wei et al., 2020, Couper et al., 2008, Moore et al., 2001, Kotenko et al., 1997). Most cells express the IL-10R2 receptor constitutively as this receptor is also required for signalling of other type II cytokines, including IL-22 and IL-26 (Couper et al., 2008). IL-10R1 is expressed at basal levels on most hematopoietic cells, with receptor expression increasing once a cell becomes activated (Couper et al., 2008). In addition, IL-10R1 has been reported to be expressed on non-hematopoietic cells such as fibroblasts, and colonic epithelial cells (Denning et al., 2000, Mosser and Zhang, 2008). IL-10R expression has also been reported on intestinal stem cells (Biton et al., 2018). *In vivo* anti-CD3 treatment in mice not only results in an increase of IL-10^{+} T cells in the small intestine (Kamanaka et al., 2006b) but also an accumulation of Th17 cells which highly express IL-10R1 (Huber et al., 2011). IL-10R1 is the ligand binding component of the receptor, and thus IL-10 signalling cannot occur in its absence. Therefore IL-10R1 monoclonal antibodies have become a well-established method of blocking IL-10R signalling (Liu et al., 1994, Liu et al., 1997, Burrack et al., 2018, Ring et al., 2019). IL-10R signalling occurs via the Jak/STAT pathway. IL-10R1 and IL-10R2 are associated with the tyrosine kinases Jak1 and Tyk2 respectively. These signalling molecules become activated upon IL-10R ligation, resulting in receptor phosphorylation. Phosphorylation of the IL-10R results in the recruitment of STAT3 (Williams et al., 2004). Jak1 and Tyk2 subsequently phosphorylate STAT3. Phosphorylated STAT3 molecules form homodimers and translocate to the nucleus (Williams et al., 2004, Moore et al., 2001). Here STAT3 homodimers bind to IL-10 responsive genes (Figure 1-7). Binding of IL-10 responsive genes, including IL-10 itself, by STAT3 results in a decrease in NF-Kβ dependent inflammatory cytokine expression (Shouval et al., 2014b, Moore et al., 2001). In

addition, STAT3 also activates suppressor of cytokine signalling 3 (SOCS3) which inhibits IL-6 signalling but does not degrade the IL-10R (Murray, 2007, Mosser and Zhang, 2008). IL-10 does not exclusively activate STAT3 and has been reported to also activate both STAT1 and STAT5 but the biology and signalling cascades surrounding this remains unclear (Finbloom and Winestock, 1995, Moore et al., 2001, Weber-Nordt et al., 1996, Wehinger et al., 1996).

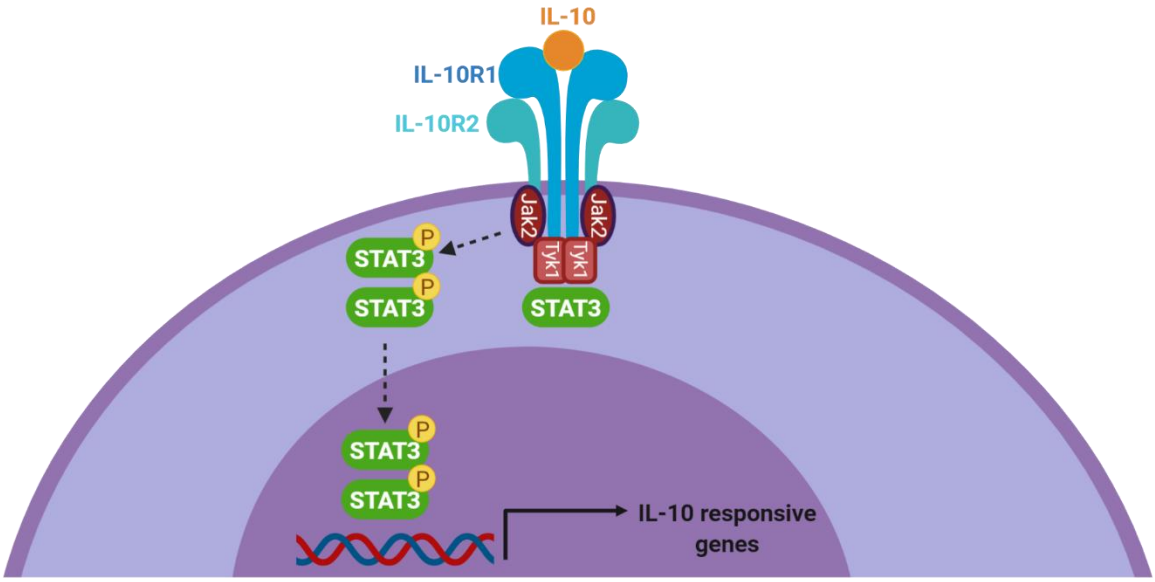

Figure 1-7 IL-10R signalling via STAT3
The main signalling cascade downstream of the IL-10R via STAT3. Created with BioRender.com.

The majority of innate and adaptive immune cells have the capacity to produce IL-10 (Saraiva and O'garra, 2010). In addition, non-immune cells such as epithelial cells have been reported to produce IL-10 (Hyun et al., 2015, Couper et al., 2008). The secretion of IL-10 by Th subsets can act as a self-limiting negative feedback loop (Couper et al., 2008, Meyaard et al., 1996). Antiviral $CD4^+$ and $CD8^+$ T cells become the prominent source of IL-10 once activated (Rojas et al., 2017). In addition, in different cell types, the secretion of IL-10 is dependent on different signalling proteins (Saraiva and O'garra, 2010). Macrophages, Th17 and Tr1 cells require the TF c-Maf for the induction of IL-10 secretion (Apetoh et al., 2010, Xu et al., 2009, Cao et al., 2005). In Th2 cells, IL-10 secretion is independent of c-Maf and instead requires GATA3 and STAT6, both of which are activated in response to IL-4R signalling (Chang et al., 2007, Shoemaker et al., 2006). The anti-inflammatory cytokine IL-27 is a potent inducer of IL-10 via STAT3 and STAT1 recruitment in T cells and bone-marrow

derived macrophages along with IL-12 and IL-21 (Batten and Ghilardi, 2007, Iyer and Cheng, 2012) and IL-27$^{-/-}$ mice, which partially resemble the phenotype of IL-10$^{-/-}$ mice, have exacerbated inflammation in numerous infection models (Batten et al., 2006, Batten et al., 2008). IL-4 has also been reported to upregulate IL-10 in macrophages stimulated with LPS (Cao et al., 2005, Mitchell et al., 2017). APCs such as DCs express PRRs, and TLR2 specifically has been reported to induce IL-10 secretion in DCs (Netea et al., 2004, Dillon et al., 2004). Also, B cells express TLR4 and TLR9, both of which have been shown to be key for IL-10 induction in B cells (Sanchez et al., 2019, Lenert et al., 2005) There are layers of complexity surrounding both the induction of IL-10 secretion and IL-10R signalling that can differ depending on the environment and cell type. The importance of this regulatory cytokine has been particularly demonstrated in the intestine.

1.5.2 IL-10 and gut homeostasis

Studies in both humans and mice have shown a crucial role for IL-10 in maintaining homeostasis in the gut. In humans, Ulcerative Colitis (UC) and Crohns Disease (CD) fall under the umbrella of Inflammatory Bowel Disease (IBD). These conditions are severely debilitating and are a result of a loss of immune homeostasis resulting in detrimental immune responses against harmless antigen such as food antigen and microbiota. Genome wide association studies (GWAS) have shown a link between single nucleotide polymorphisms in IL-10 to IBD (Franke et al., 2008, Franke et al., 2010). In addition, predisposing mutations in both IL-10 and the IL-10R have been described in association with serve early onset IBD (Glocker et al., 2009, Kotlarz et al., 2012, Moran et al., 2013). In mice, both IL-10 and IL-10R deficient mice develop spontaneous colitis (Kühn et al., 1993, Spencer et al., 1998). RAG$^{-/-}$ mice reconstituted with IL-10$^{-/-}$ T cells and treated with piroxicam (a non-steroidal anti-inflammatory drug that accelerates colitis) develop spontaneous colitis. In this model, infection with *H. polygyrus* elevated colitis by acting on distal DCs, most likely via the action of HES (Hang et al., 2010, Reynolds et al., 2012). Similarly, RAG$^{-/-}$ mice infected with the pathogenic bacteria *Helicobacter hepaticus* reconstituted with IL-10$^{-/-}$ T cells results in an innate model of colitis (Maloy et al., 2003). In addition, in a T cell transfer mouse model of colitis, administration of rIL-10 abrogated disease

(Powrie et al., 1994). Finally, spontaneous DSS-colitis in mice is prevented by the administration of IL-10 producing *Lactococcus lactis* (Steidler et al., 2000).

There are numerous mechanisms reported for IL-10 directly promoting gut homeostasis through acting on immune cells. IL-10 signalling in macrophages drives a regulatory phenotype and these macrophages can be identified by the high expression of CX3CR1 (Zigmond et al., 2014, Mantovani and Marchesi, 2014, Shouval et al., 2014a). IL-10 inhibits the expression of MHCII and costimulatory molecules such as CD80/86 on the surface of macrophages and DCs, which inhibits antigen presentation and suppresses effector responses (Fiorentino et al., 1989, Couper et al., 2008, Mosser and Zhang, 2008) . IL-10 has also been reported to antagonise inflammatory genes downstream of TLRs (Lang et al., 2002). IL-10 also prevents immune cell migration to sites of inflammation by the suppression of the chemokines such as CCL3, CXCL8 and CCL4 on activated monocytes (Moore et al., 2001, Berkman et al., 1995). The suppression of Th1 cells by IL-10 is well described, however, self-limiting IL-10 is produced by most effector Th subsets (Ng et al., 2013, Fiorentino et al., 1989). A detailed description of Treg derived IL-10 mediated inhibition of immune responses is described in section 1.1.3. It is important to note that some pro-inflammatory mechanisms of IL-10 have been described. IL-10 has been reported to contribute to B cell activation, survival, and class switching (Mosser and Zhang, 2008). In addition, IL-10 in conjunction with IL-18 stimulate natural killer (NK) cell proliferation and function (Cai et al., 1999). IL-10 may also act as a growth factor for CD8 T cells, but this is dose dependent (Rowbottom et al., 1999). However, IL-10 can also act on non-immune cells, such as epithelial cells.

IL-10 has also been reported as a key cytokine for epithelial cell barrier function. Mice with IL-10R1 depletion on epithelial cells developed more severe inflammation in a model of colitis and showed increased epithelial barrier permeability (Kominsky et al., 2014). IL-10 promotes the production of mucus from goblet cells via the suppression of endoplasmic reticulum stress, a common marker of colitis and by preventing misfolding of the MUC2 protein (Hasnain et al., 2013). Wound healing is an essential component of immune responses and in restoring homeostasis, IL-10 promotes intestinal epithelial cell proliferation and repair via Wnt1-inducible signalling protein 1 (WISP-1) (Quiros et al., 2017). In mice lacking both B and T cells, epithelial wound healing is not impaired, and

macrophages are reported to be the primary source of IL-10 for driving epithelial repair (Quiros et al., 2017). Butyrate is a SCFA produced by the microbiota, which has been shown to promote the expression of IL-10R1 on an intestinal epithelial cell line (Zheng et al., 2017). Furthermore, treatment of intestinal epithelial cell lines with both IL-10 and butyrate increased epithelial barrier integrity, more so than butyrate alone (Zheng et al., 2017). This report showed that IL-10 suppression of Claudin-2, an important epithelial TJ protein that determines intestinal permeability resulted in increased barrier integrity. Therefore, the activity of IL-10 and IL-10R signalling are key for the maintenance of gut homeostasis and also in infection settings.

1.5.3 IL-10 and helminth infection

IL-10 has been reported to be a key component in responses to helminth infection, but studies show both host protective and pro-parasitic roles for this cytokine (Schopf et al., 2002, Wynn et al., 1997, Sanchez et al., 2015, Couper et al., 2008). The expression of IL-10 increases in mice infected with *H. polygyrus* and, in a model of IBD, *H. polygyrus* limits gut injury in a manner associated with increased IL-10 (Setiawan et al., 2007, Leung et al., 2012, Redpath et al., 2013, Finney et al., 2007, Filbey et al., 2014). In addition, ICOS has been shown to be key for IL-10 expression by Tregs in *H. polygyrus* infection (Redpath et al., 2013) and co-operation between IL-10 and IL-4 in this model maximises the suppression of IL-17 in the MLN (Elliott et al., 2008). During infection with *Trichinella spiralis*, IL-10 from eosinophils indirectly suppresses nitric oxide which favours parasite persistence (Huang et al., 2014). In addition, DCs pre-treated with antigen from the tapeworm *Hymenolepis diminuta* can suppress colitis in an IL-10 dependent manner (Matisz et al., 2015). IL-10 expression by T cells but not B cells in the immune response to *Litomosoides sigmodontis* suppresses antigen specific T cell responses (Haben et al., 2013) and this infection model results in a decreased responsiveness of basophils, which is IL-10 dependent (Larson et al., 2012). These studies present a possible mechanism of parasites promoting persistence using IL-10 derived from host innate immune cells. In the whipworm *T. muris*, IL-10 is not only required for the development of resistance to this parasite but also in host survival and the absence of IL-10 results in increased susceptibility to infection and host mortality (Schopf et al., 2002). Co-operation and association between IL-10 and the Th2 response to

helminth infection have been reported. The strong Th2 response to *N. brasiliensis* requires IL-4 dependent IL-10 signalling (Balic et al., 2006). In humans, infection with *S. mansoni* results in increased expression of IL-10, along with the Th2 cytokines IL-4 and IL-13 in the blood (Marinho et al., 2016, Dias et al., 2018). Therefore, IL-10 has an expansive and important role in mediating immune responses to helminth infections. These data also demonstrate that although IL-10 is most frequently described as a suppressive cytokine, in many infection settings parasites use host IL-10 to promote persistence or to evade the host immune system. In addition, the immune environment, concentration, and tissue location of IL-10 is critical for determining its role in both homeostasis and infection settings.

1.6 Hypothesis and aims

The objectives of this thesis focus on cytokine signals and immune competition during helminth infection.

Although *H. polygyrus* is a well-established model of helminth infection and tool used to study type 2 immune response dynamics, analysing leukocytes from the SILP has remained a challenge in the field. This technical challenge places a limitation on immune cell analysis at the site of infection, which is critical for understanding the role of cytokines in orchestrating, maintaining, and controlling type 2 immune responses. IL-10 is a critical cytokine for the maintenance of intestinal homeostasis and is also important in numerous infection settings and diseases. There are numerous reports of increased IL-10 production during helminth infection but whether it is important for the type 2 immune response to helminths remains unclear. The immune response to *H. polygyrus* is described solely as a type 2 immune response, however underlying immune responses to bacteria due to barrier breach during the lifecycle of this parasite have been hypothesised in the literature. It was therefore hypothesised that **IL-10 promotes Th2 responses to helminth infection, by suppressing bacteria-specific IFNγ producing Th1 cells.** To test this hypothesis 3 objectives were addressed:

1. Develop a method for the successful isolation of leukocytes from the SILP. Allowing key questions surrounding tissue cytokine responses at the site of infection to be explored.

2. Determine the role of IL-10 in the Th2 response to helminth infection. The role of IL-10 in the context of type 2 immunity is debated and there are reports of both suppressive and promoting effects of this cytokine. Importantly, to accurately answer these questions analysis of both the priming MLN and the site of infection (SILP) is required, emphasising the importance of objective 1.

3. Investigate possible barrier breach during *H. polygyrus* infection. There are few reports surrounding barrier breach during *H. polygyrus* infection despite the fact this parasite migrates through the wall of the intestine at two points in its life cycle. To address this, IFNγ secretion by T cells in the MLN and SILP will be measured, barrier integrity will be analysed and immune responses at distal sites investigated.

By addressing these objectives, we aimed to contribute to the understanding of cytokine regulation during the type 2 immune to helminth infection and explore immune competition in *H. polygyrus* infection.

Chapter-2 Methods

2.1 Mice

C57BL/6 mice were purchased from Envigo (Huntingdon, UK), B6.4get mice were kindly provided by Professor Judi Allen (University of Manchester) and bred in-house (University of Glasgow). These mice were first developed by (Mohrs et al., 2005b) and express GFP under the control of the Il4 promoter. Il10gfp-foxp3RFP B6 mice were kindly provided by Rick Maizels (University of Glasgow) and bred in-house (University of Glasgow). These mice express two separate transgenes: IRES-eGFP inserted at the end of the last exon and before the polyadenylation site of the Il10 gene (Tiger mice) (Kamanaka et al., 2006a) and similarly have IRES-RFP inserted at this site of the Foxp3 gene (Wan and Flavell, 2005) (See figure 2-1). For each experiment mice were sex-matched and used at age 6-12 weeks. Both male and female mice were used for experiments. Animals were maintained in individually ventilated cages under standard animal house conditions at the University of Glasgow and procedures were performed under a UK Home Office license held by Rick Maizels (Project PPL number 70/8483 / PP4096415) in accordance with UK Home Office regulations and following review by the University of Glasgow Ethics Committee.

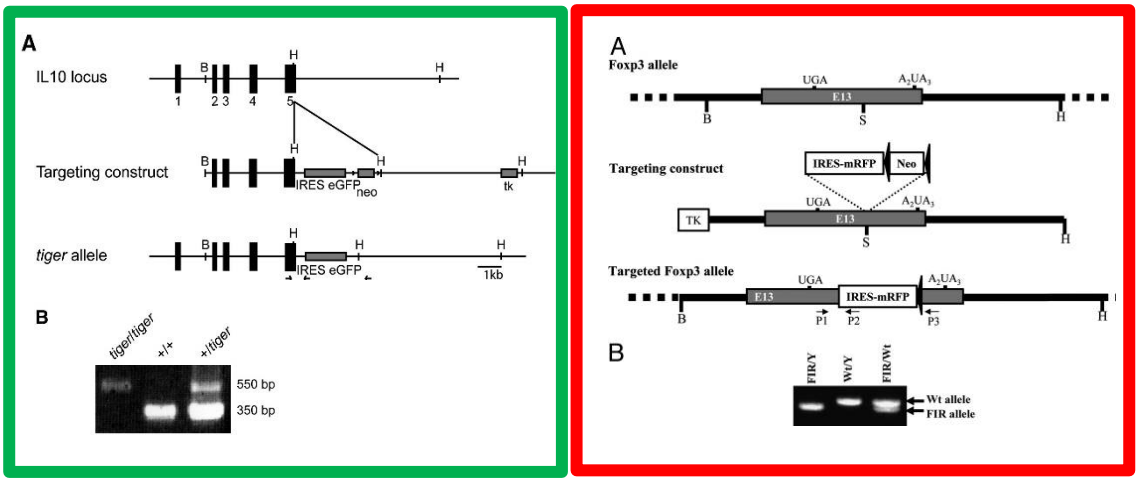

Figure 2-1 Generation of Il10gfp Foxp3rfp dual reporter mice
Adapted from (Kamanaka et al., 2006a) and (Wan and Flavell, 2005). Generation of Il10gfp (Tiger) mice (left) and Foxp3rfp mice (right).

2.2 *Heligmosomoides polygyrus* infections

The life cycle of *Heligmosomoides polygyrus* (which is also known as *Heligmosomoides polygyrus bakeri*) was maintained, prepared and counted by Nicola Britton and subsequently by Claire Ciancia (Maizels laboratory, University of Glasgow). This process is described in detail by (Johnston et al., 2015). The lifecycle of *H. polygyrus* is shown in Figure 1-2. Mice were acclimatised for 1 week after arrival in the animal unit. L3 larvae were prepared at 1 larvae/µl. Mice were infected with 200µl of L3 larvae (total 200 larvae) by oral gavage (size: 24G, round ball stainless steel tip).

2.3 IL-10R monoclonal antibody blockade

For IL-10R1 blockade, purified rat anti-mouse IL-10 receptor (IL-10R) mAb (Clone 1B1.3a BioXcell) was used and where stated a Rat IgG1 isotype (Merck) control was given. Both IL-10R1 mAb and isotype control stocks were prepared at 2.5mg/ml in sterile PBS. Each mouse was injected intraperitoneally (i.p.) (26.5G needle) with 200µl (500µg*)* of appropriate treatment. Each treatment was given at D-1, D2, and D5 of *H. polygyrus* infection, and mice were culled at D7 of infection. *H. polygyrus*-infected mice were maintained in mixed treatment group cages.

2.4 Cell Isolation

Cells were counted and dead cells excluded using trypan blue and a haemocytometer.

2.4.1 Isolation of lamina propria leukocytes

Naïve and infected animals were euthanised using carbon dioxide, and the small intestine removed (below the stomach and above the caecum). All fat was removed. Intestines were immediately transferred onto laboratory tissue paper soaked in phosphate-buffered saline (PBS) (no calcium, no magnesium, kept at room temperature). Peyer's patches were removed, and the intestines cut opened longitudinally and washed vigorously in a PBS-filled petri dish to remove intestinal content. Fine forceps were used to gently squeeze out any remaining

mucus. Intestines were then transferred onto a fresh PBS-soaked tissue and cut in to 1cm pieces and transferred to a 50ml centrifuge tube containing 30ml of HBSS (Gibco™ 14170088 no calcium, no magnesium) supplemented with 10% FCS (Gibco™ Fetal Bovine Serum, qualified, heat inactivated, E.U.-approved, South America Origin) and kept on ice.

Each sample was washed by pouring the sample into a large piece of 50-micron Nitex mesh, folded into a funnel placed in a 400ml beaker and pouring 30mls pre-warmed HBSS (Gibco™ 14170088 no calcium, no magnesium) over the Nitex mesh in the funnel. Using forceps, the samples were then transferred back into tubes containing 15ml 2mM EDTA (UltraPure™ 0.5M EDTA, pH 8.0 Cat. 15575020) in HBSS (37ºC). The samples were shaken vigorously by hand and placed into an orbital shaker (Stuart, Orbital Incubator SI500) set to 220rpm and 37ºC for 15 mins. After shaking for 15min, the samples were washed as previously and transferred to new tubes containing 15ml 2mM EDTA in HBSS and returned to the shaker for a further 15 minutes. This process of EDTA washes was repeated once more, for a total of three EDTA washes. After the 3rd wash, the samples were transferred to 15ml of RPMI 1640 (Gibco™ no glutamine, 21870076) supplemented with 10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin, 2mM L-glutamine (Life Technologies 15140122) and 62.5 CDU/ml Collagenase VIII (CDU, collagenase digestion units) (typically 0.5mg/ml, Sigma-Aldrich C2139-500MG). The samples were shaken vigorously by hand and placed back into the shaker set to 220rpm and 37ºC for 15min. Other enzymes used in optimisation steps in Chapter 2 (Table 3-1): Liberase (Roche, 05401020001), DNaseI (Sigma-Aldrich, DN25-100MG), Collagenase D (Roche) and Dispase II (Gibco).

After the 15-minute digest period digestion was stopped by adding 35ml of ice-cold RPMI 1640 (10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine) to each sample and placing on ice. Each sample was then filtered through a 100µm nylon mesh filter, followed by a 40µm nylon mesh filter – remaining tissue was not crushed through as this reduced cell viability. Samples were washed by spinning down 400g for 10 minutes at 4ºC, resuspending in 35ml of ice-cold RPMI 1640 (10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine) and this was repeated twice. The cell suspensions were resuspended in 10mls of cold RPMI 1640 (10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine) and kept on ice for further analysis.

2.4.2 Percoll gradients

Prior to optimising the SILP isolating protocol detailed in section 2.4.1, percoll gradients were used to eliminate dead cells and debris from SILP samples. An 80:40:30% percoll gradient was used as had been previously optimised in the lab. Percoll 100 (Merck) was diluted in RPMI 1640 supplemented with 10% FCS to obtain the correct percoll %. 15ml falcon tubes were pre-rinsed with RPMI 1640 supplemented with 10% FCS to ensure the gradient would settle. 2mls of 80% percoll was added first, followed by 5mls of 40% percoll, pipetting slowly to ensure not to disrupt the 80% layer. Once a single cell suspension had been obtained, cells were resuspended in 30% percoll and slowly pipetted on to the 40% percoll layer. Gradients were centrifuged at 1800rpm for 15min at 20^{o}C with no acceleration or brake. To recover cells from the gradient, the 30% layer was removed using a Pasteur pipette and the mononuclear cells collected using a fresh Pasteur pipette from the 80:40% interface. Cells were washed in 10ml RPMI 1640 supplemented with 10% FCS and kept on ice for further analysis.

2.4.3 Isolation of cells from lymphoid organs

Lymphoid organs (spleen and MLN) were harvested and collected in RPMI 1640 (10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine). Samples were crushed through a 70µm filter to obtain a single cell suspension. Splenocytes were red blood cell lysed using 1ml of ACK Lysing Buffer (Thermofisher) for 1 minute at room temperature, and lysis was stopped by adding 30ml PBS. Samples were then spun down at 400g for 5min, supernatants discarded, and samples resuspended in 10ml of PBS. Samples were kept on ice for further analysis.

2.5 Collection of blood

Animals were euthanised using carbon dioxide and the femoral artery cut using a blade, immediately afterwards the chest cavity was opened and a 25G needle with a 1ml syringe attached was used to collect blood from the left ventricle of the heart. Average volume of blood collected ranged from 200-300µl. Blood samples were left at 4^{o}C overnight in Eppendorfs to allow blood to clot. Samples

were then spun down at 12,000g for 15 min and the top layer containing serum removed and put into a new Eppendorf and stored at -20 until further analysis.

2.6 RNA extraction

Tissue samples (duodenum (1cm)/spleen/MLN/omentum) (no more than 30mg) were collected placed in RNA later (Qiagen 76104) and kept in the fridge for up to 1 month. For optimal RNA purity, samples were homogenised in a TissueLyser (Qiagen) (1min 25Hz x2, 1x 5mm steel ball (Qiagen) per sample) in Trizol (ThermoFisher), and centrifuged for 5min at 12,000g for 10 minutes at 4^{o}C. Supernatants were collected, chloroform added, and samples incubated at room temperature for 3min before being centrifuged for 15min at 12,000g at 4^{o}C. The resulting upper aqueous layer was collected and 1.5x 100% ethanol added to each sample. RNA was then purified using the RNEASY Mini Kit (Qiagen 74104), including an on-column DNase digestion (RNase-Free DNase Set Qiagen) and according to manufacturer's guidelines. RNA was eluted in 30-40µl nuclease-free water and RNA concentration determined using a nanodrop 1000. RNA concentration values ranged from 250µg/µl – 3000 µg/µl). Samples which had 260/280 of less than 2.0 had poor purity and were discarded. Where possible, cDNA was generated on the same day as RNA extraction and RNA was stored at -80^{o}C.

2.7 cDNA synthesis

cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Invitrogen 4368814). A range of 500-2000ng of RNA was transcribed (this was kept consistent for all samples in each individual experiment). Cycling parameters were as follows: 25^{o}C for 10 min, 37^{o}C for 120 min, 85^{o}C for 5 min and 4^{o}C ∞. cDNA was diluted 1:20 with nuclease-free water and kept at -20 prior to use.

2.8 RT PCR

For real time PCR, PowerUp™ SYBR™ Green Master Mix (Applied Biosystems A25742) and QuantStudio 6 Flex Real-time PCR system (Applied Biosystems) were used. For each gene master mix, 5µl of 2X SYBR master mix was added and 0.5µl

of both forward and reverse gene primers (10uM working stocks). Samples were plated in triplicate, and nuclease-free water controls added to ensure no contamination had occurred. Cycling parameters and dissociation curve conditions are stated in Table 2-1. Ct values were normalised to those for the

Cycling parameters			
UDG Activation	50°C	2 min	Hold
Dual-lock DNA polymerase	95°C	2 min	Hold
Denature	95°C	15 sec	40 cycles
Anneal/extend	60°C	1 min	
Dissociation curve conditions (melt curve stage)			
Step	**Ramp rate**	**Temp.**	**Time**
1	1.6°C/second	95°C	15 sec
2	1.6°C/second	60°C	1 min
3	0.15°C/second	95°C	15 sec

Table 2-1 Cycling parameters and dissociation conditions used in RT PCR

Gene	Forward primer	Reverse primer
IL-13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
IL-5	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCCTG
IL-10	CTGAAGACCCTCAGGATGCG	TGGCCTTGTAGACACCTTGGTC
IFNγ	TGAGTATTGCCAAGTTTGAG	CTTATTGGGACAATCTCTTCC
IL-17A	ATCCCTCAAAGCTCAGCGTGTC	GGGTCTTCATTGCGGTGGAGAG
Lcn2	AAGGCAGCTTTACGATGTACAGC	CTTGCACATTGTAGCTGTGTACC
IL-22	TTTCCTGACCAAACTCAGCA	CTGGATGTTCTCGTCGTCAC
RPS29	ACGGTCTGATCCGCAAATAC	CATGATCGGTTCCACTTGGT
Occludin	CTCCCATCCGAGTTTCAGGT	GCTGTCGCCTAAGGAAAGAG
E-cadherin	GGATCAGGACCAGGACTACG	AGGGAAGGAGCTGAAAGACC
Claudin-2	GTGGCTGTAGTGGGTGGAGT	CCAAAGAAAACAGGGCTGAG
N-cadherin	AGGTAGCTGTAAACCTGAGC	CTTGGCAAGTTGTCTAGGGA
JAM-1	CACCTTCTCATCCAGTGGCATC	CTCCACAGCATCCATGTGTGC
ZO-1	ACTCCCACTTCCCCAAAAAC	CCACAGCTGAAGGACTCACA

Table 2-2 Primers used for RT PCR

Gene	Source
IL-13	(Khaled et al., 2007)
IL-5	(Khaled et al., 2007)
IL-10	Nicolette Fonseca (University of British Columbia)
IFNγ	The Maizels Laboratory (University of Glasgow)
IL-17A	The Maizels Laboratory (University of Glasgow)
Lcn2	(Chassaing et al., 2012)
IL-22	The Maizels Laboratory (University of Glasgow)
RPS29	Graham Heieis (University of Glasgow)
Occludin	Julie Worrell (University of Glasgow)
E-cadherin	Julie Worrell (University of Glasgow)
Claudin-2	Julie Worrell (University of Glasgow)
N-cadherin	Julie Worrell (University of Glasgow)
JAM-1	(Volynets et al., 2016)
ZO-1	(Nevado et al., 2015)

Table 2-3 Source of primers

gene encoding ribosomal protein S29 (RSP29), and expression of genes of interest was determined using the 2−ΔΔC(t) method. See Table 2-2 for primer sequences. All primers were purchased from Life Technologies Ltd.

2.9 *In vitro* CD4$^+$ T cell culture

CD4$^+$ T cells were obtained from naïve splenocytes using the MojoSort™ magnetic cell separation system, which is a negative selection kit (Biolegend). Typical CD4$^+$ T cell yield from a naïve spleen was around 5 million cells. $1x10^8$ splenocytes were used per each isolation, for a full 96-well plate an average of 3 isolations were required. Isolated cells were seeded at $1x10^5$ cells per well in round bottom 96 well plates. Isolated CD4$^+$ T cells were resuspended in RPMI 1640 supplemented with 10% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 1mM Glutamax, 1mM non-essential amino acids, 1mM sodium pyruvate and 50µM 2-mercaptoethanol (ThermoFisher) for plating. Plates were pre-coated for 1hr at 37 ºC with 1µg/ml of αCD3 (17A2, BioLegend), and soluble αCD28 was added to stimulation cocktails at 1µg/ml. For polarisation of CD4$^+$ T cells recombinant mouse cytokines were added to cultures; Th0: 20ng/ml recombinant IL-2 (rIL-2) (ThermoFisher), Th2: rIL-2 (20ng/ml), rIL-4 (40ng/ml) (ThermoFisher), αIFNγ (1µg/ml) (Biolegend). Th1: rIL-2 (20ng/ml), rIL-12 (10ng/ml) (ThermoFisher). For

IL-10 stimulation, IL-10 (ThermoFisher) was added at 10ng/ml. Cultures were incubated (37ºC, 5% Co2) for 4 days and cells or supernatants harvested for further analysis. $CD4^+$ T cell purity after 4 days of culture was typically between 93-98%.

2.10 T cell proliferation

To assess $CD4^+$ T cell proliferation, the CellTrace™ Violet Cell Proliferation Kit (ThermoFisher) was used according to manufacturer's guidelines. $1x10^6$ cells were resuspended in PBS (no protein) and 1ul/ml of a 5mM stock of component A (dye) was added. Cells were then incubated in a shaking incubator (gentle shake - 90rpm) set to 37 ºC for 20 min (protected from light). To wash cells, 5 times the volume of ice-cold of RPMI 1640 supplemented with 10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine was added and cells allowed to incubate for 5 min on ice. Cells were then spun down at 400g for 5 min at 4 ºC. Cells were then resuspended at the desired concentration in RPMI 1640 supplemented with 10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine and plated as described previously. For acquisition of these cultures, samples were acquired on a low flow rate for peak separation.

2.11 T cell stimulation, intracellular staining, and flow cytometry

$3x10^6$ cells were resuspended in in 500µl of RPMI 1640 supplemented with 10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine and 2µl/ml solution of both stimulation cocktail and protein transport inhibitors (Invitrogen eBioscience™ Cell Stimulation Cocktail plus protein transport inhibitors (500X)). Cells were incubated for 4 hours (37°C, 5% CO2). After stimulation, cells were washed twice in PBS and then stained for flow cytometry. Fixable Viability Dye eFluor 780/506 (Ebioscience) was used to exclude dead cells and Fc block anti-mouse CD16/32 Antibody (Clone 93, BioLegend) was used to prevent non-specific binding. Cells were stained for surface markers in FACS buffer (PBS containing 2% FBS and 2mM EDTA (UltraPure™ 0.5M EDTA, pH 8.0 Cat. 15575020)) for 20 min at 4ºC. All surface antibodies are listed in Table 2-3. For intracellular cytokine staining, 150µl of BD Cytofix/Cytoperm™ (554714) was added to samples to permeabilise and fix cells

for 20 min at 4°C. Samples were then washed in 1ml of BD Perm/Wash™ Buffer (554714) and 50µl of intracellular anti-cytokine antibody stain: PE-Cy7-conjugated anti-IL-13 (eBio13A, Invitrogen), PE-conjugated anti-IL-5 (TRFK5, BioLegend), e450-conjugated anti-IFNγ (XMG1.2, Invitrogen)) or appropriate isotype control antibody was added to each sample. Samples were incubated at room temperature for 1 hour (protected from light). Samples were washed as previously and acquired immediately on the BD LSRII flow cytometer running FACS-Diva software (BD Biosciences). For intracellular transcription factor staining, $3x10^6$ cells were surface stained as previously and fixed and permeabilised using the eBioscience™ Foxp3 / Transcription Factor Staining Kit (ThermoFisher 00-5523-00). Cells were fixed for 1 hour at room temperature (protected from light) and then resuspended in 100µl of intracellular transcription factor stain: eFluor 450-conjugated anti-FOXP3 (FJK-16s, ThermoFisher), PE-Cy7-conjugated anti-T-bet (eBio4B10, ThermoFisher) and

Marker	Flurochrome	Clone	Dilution	Manufacturer
CD11b	BV421	M1/70	1/200	BD Horizon
CD19	APC-Cy7	1D3	1/200	eBioscience
CD4	APC	RM4-5	1/200	BioLegend
CD4	BV421	RM4-5	1/200	BioLegend
CD4	BV605	RM4-5	1/200	BioLegend
CD4	BV711	RM4-5	1/200	BioLegend
CD4	FITC	RM4-5	1/200	BioLegend
CD4	PE-Cy7	RM4-5	1/200	BioLegend
CD44	APC-Cy7	IM7	1/200	BioLegend
CD44	FITC	IM7	1/200	BioLegend
CD44	PE-Cy7	IM7	1/200	eBioscience
CD45	BUV395	30-F11	1/200	BD Biosciences
CD45	BV421	30-F11	1/200	eBioscience
CD69	FITC	H1.2F3	1/200	BioLegend
CD8a	BV605	53-6.7	1/200	BioLegend
CD8a	PE-Cy7	53-6.7	1/200	BioLegend
CXCR3	PE-Cy7	CXCR3-173	1/100	BioLegend
IL-10R	PE	1B1.3a	1/100	BioLegend
IL-4R	APC	I015F8	1/100	BioLegend
IL-7R	APC	A7R34	1/100	eBioscience
TCRß	BV421	H57-597	1/200	BioLegend
TCRß	PerCP Cy5.5	H57-597	1/200	BioLegend

Table 2-4 Surface antibodies used for flow cytometry

PE-conjugated anti-GATA3 (TWAJ, ThermoFisher), BUV395-conjugated anti-Ki67 (Clone B56, BD biosciences). Samples were washed as previously and acquired immediately on the BD LSRII or BD LSR Fortessa flow cytometer running FACS-Diva software (BD Biosciences). Analysis was performed using FlowJo (Treestar).

2.12 BAE preparation

Bacterial antigen extract (BAE) was obtained by collecting faeces (20 fresh pellets) from several cages of naïve C57BL/6 mice in 5ml of PBS containing 10µg/ml of DNase I (Sigma-Aldrich, DN25-100MG). 2x 5mm steel balls (Qiagen) were added and the sample vortexed to break up faeces. The sample was then sonicated 3x for 30 seconds and centrifuged for 10 min at 10,000g and supernatants collected and transferred to a fresh 15ml falcon. The supernatant was then filtered through a 0.2µm filter and 1ml aliquots made and frozen at -80. Prior to freezing, total protein of the BAE was measured using the Qubit™ Protein Assay Kit. Standards were prepared according to manufacturer's guidelines and all samples and standards kept in the dark once detection dye had been applied. Qubit was calibrated using standards and sample protein concentration readings given in µg/ml. A serial dilution was carried out to ensure protein concentration was not above the detection limit of the Qubit.

2.13 *Ex-vivo* re-stimulation

The MLN chain was harvested and collected in RPMI 1640 supplemented with 10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine. A single cell suspension was then achieved as described previously. Cells were washed twice using RPMI 1640 supplemented with 10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine. Cells were then counted (as described previously) and resuspended at $5x10^6$ cells/ml,100µl of cell suspension (500,000 cells per well) was added to a αCD3 pre-coated 96-well round bottom plate (coated for 1hr at 37°C with 1µg/ml of αCD3. Where stated MLN cells were stimulated with either HES (provided by the Maizels laboratory, University of Glasgow) or BAE instead of αCD3. For HES stimulation, HES was added at 1µg/ml. For BAE stimulation, BAE was added at 100µg/ml (based on total protein concentration). Cultures were incubated (37°C, 5% Co2) for 3 days and then supernatants collected for further analysis.

2.14 Cytokine measurement

Supernatants (90µl) were collected from in vitro T cell cultures or ex-vivo stimulated cultures and stored at -20ºC for further analysis. For cytokine measurements, supernatants were diluted 1/200 in sterile filtered FACS buffer. Serum was also analysed for cytokines and diluted 1/2. Cytokines (IL-10, IFNγ, IL-13, IL-5 and IL-4) were measured using BD™ CBA Flex Sets (BD Biosciences) according to the manufacturer guidelines. For a standard curve, a serial dilution of pooled recombinant cytokines was carried out using filtered FACS buffer and blank wells plated containing only filtered FACS buffer to account for background. The standards, blanks and samples were added to a 96-well round bottom plate and 50µl of capture bead mix (diluted in filtered FACS buffer) added to each well and incubated at room temperature for 1 hour (protected from light). Subsequently, 50µl of detection antibody was then added and incubated as previously. The plate was then washed twice by adding 200µl of filtered FACS buffer to each well. For acquisition, samples were resuspended in 100µl of filtered FACS buffer. A minimum of 300 beads per cytokine was acquired for analysis. The cytometric bead array was analysed using the MACSQuant® Analyser (Miltenyi Biotec). Analysis was performed using FlowJo (Treestar).

2.15 Lcn2 ELISA

Faecal samples were collected from mice infected with *H. polygyrus* and appropriate naïve controls. Three fresh faecal pellets were collected per mouse in PBS. Samples were immediately homogenised using a TissueLyser (Qiagen) (1min 25Hz) and 1x 5mm steel ball (Qiagen) per sample. Samples were then spun down at 12,000g for 15 min at 4ºC. Supernatants were collected and placed in to a new 1.5ml Eppendorf and placed at -80. Naïve samples were diluted 1/10 and infected samples 1/25 in PBS. To determine the concentration of Lipocallin-2 (Lcn2), an enzyme-linked immunosorbent assay (ELISA) was carried out using the Mouse Lipocalin-2/NGAL DuoSet ELISA (R&D systems) and DuoSet ELISA Ancillary Reagent Kit 2 (R&D systems) according to the manufacturers guidelines. ELISA wash buffer was made up of 500ml 10X PBS, 2.5ml TWEEN and made up to 5L using distilled water. Plates were read at 450nm using a VersaMax plate reader. Background was removed by subtracting the blank from all readings. The

concentration of Lcn2 was determined by plotting a standard curve and extrapolating values using GraphPad Prism. Any samples that fell above or below the standard curve limits were removed from analysis. To standardise our results, Lcn2 (pg/ml) per mg of protein was calculated to normalise Lcn2 data to total protein.

2.16 BCA Assay

Bicinchoninic acid (BCA) assay was used to measure total protein concentration in each faecal supernatant samples. Lcn2 values were then normalised to total protein of faecal supernatants to account for variability between each sample. Each sample was diluted as they were in the previous Lcn2 ELISA. Pierce™ BCA Protein Assay Kit was used according to the manufacture's guidelines. Standards were made using a serial dilution with a top standard of 2mg/ml Bovine Serum Albumin (BSA). 10µl of standard and samples were added to each plate, along with blank controls. BCA reagent (50:1 dilution solution A:B) was made up, dispensed at 200µl per well, and incubated for 30 min at 37^{o}C. Plates were read at 580nm using a TEACAN Sunrise plate reader and protein concentration readings given in µg/ml. Background was removed by subtracting the blank from all readings.

2.17 Albumin ELISA

Stored faecal samples of stated timepoints, collected for the Lcn2 ELISA (section 2.15), were thawed. To determine the concentration of faecal albumin in samples, an ELISA was carried out using Mouse Albumin ELISA Quantitation Set (Bethyl Laboratories) according to manufacturers guidelines. ELISA wash buffer was made up of 500ml 10X PBS, 2.5ml TWEEN and made up to 5L using distilled water. Plates were read at 450nm using a VersaMax plate reader. Background was removed by subtracting the blank from all readings. The concentration of Albumin was determined by plotting a standard curve and extrapolating values using GraphPad Prism. Any samples that fell above or below the standard curve limits were removed from analysis.

2.18 Histology

2.18.1 Processing, embedding, and sectioning

For duodenum cross-sections, the top 6cm of the small intestine was collected and using a blade, cut in to 1cm pieces and placed in to 10% neutral buffer formalin (NBF). Samples were left to fix overnight in NBF, washed twice in PBS and placed in 70% EtOH for 24hrs or for up to two weeks at 4^{o}C. When ready to process, samples were fixed in paraffin wax and stored at room temperature. A rotary microtome was used for sectioning paraffin embedded samples. Samples were cut at a 5-micron thickness, placed in a 40^{o}C water bath and collected on frosted microscope slides. Slides were allowed to dry for at least one hour. For small intestine swiss rolls, the small intestine was removed, and all visible fat removed. A 10ml syringe filled with PBS was used to flush out intestinal contents and repeated until all contents had been removed. A pre-soaked PBS skewer was then used to invert the intestine, which was then washed with PBS and placed into to 10% neutral buffer formalin. After 4 hours of fixation, samples on skewers were removed from 10% NBF in a fume hood, cut open longitudinally using a blade and then gently rolled on to a wooden toothpick. Toothpick was gently removed from the roll and the now complete roll placed into a tissue cassette. Tissue cassettes were labelled and placed back in to 10% NBF overnight. Samples were then placed in 70% EtOH and processed as above.

2.18.2 Staining

Prior to staining, slides were placed into a 60^{o}C oven for 30-60 min. Slides were deparaffinised by immersing in Xylene for 3 minutes and this was carried out twice. To rehydrate, the slides were immersed in 100% EtOH for 2 x 3 minutes followed by 90% EtOH for 2 x 3 minutes and finally 70% EtOH for 2 x 3 minutes. Slides were then placed in running water for 3 minutes. Slides were then stained to investigate general histology using Haematoxylin (stains nuclei blue) and Eosin (stains cytoplasm and extracellular matrix pink). For the stain, slides were immersed in Harris Haematoxylin for 3 minutes followed by running water to remove excess staining. To reduce the background of this stain, slides were briefly dipped (2 dips) in to 1% Acid/Alcohol, rinsed in running water, immersed in Scott's Tap Water Substitute for 30 seconds and rinsed in running water. To

counter stain with Eosin, slides were dipped in 70% EtOH (9-10 dips) and immersed in Eosin stain for 2-3 minutes. To dehydrate, slides were placed in 90% EtOH for 2 x 30 seconds, followed by 90% EtOH for 2 x 3 minutes and finally immersed in Xylene for 2 x 3 minutes. For quantification of goblet cells using an Alcian Blue PAS stain kit (Atom Scientific); slides were stained with Alcian blue (pH 2.5) for 10 min, washed with distilled water, treated with periodic acid 1% solution for 10 min, washed with distilled water, treated with Schiff reagent for 10 min, washed in hot tap water for 2 min and then in running water for 10 min, stained with Haemalum Mayer for 30 seconds and washed in running water. For both stains' slides were then subsequently dehydrated (gradually moving from 70%-100% EtOH) and cleared (Xylene). Slides were mounted using DPX mounting medium (CellPath) and glass cover slips (ThermoFisher). Mounted slides were left overnight to allow to dry.

2.18.3 Scoring

The severity and depth of inflammation were scored by a certified pathologist (Virginia Gamino, VetPatólogos, Madrid, Spain) in 5 high-power fields of two intestinal sections per animal, by adapting a protocol established previously (Erben et al., 2014). The severity was scored from 1-4: 1 – Minimal inflammation (<10% area evaluated), 2 – Mild inflammation (10-25% area evaluated), 3 – Moderate inflammation (26-50% area evaluated), 4 – Marked inflammation (>51% area evaluated and dense infiltrate). The depth of inflammation was scored in each field of view from 1-3: 1 – Mucosa, 2 – Mucosa and submucosa, 3 – Transmural. The combined score of inflammation and depth was scored from 0-4; 0 – minimal and mucosal, 1 – Mild/minimal and mucosa and submucosa, 2 – Moderate/mild/marked and mucosa/transmural 3 – Marked/moderate and submucosa/transmural, 4- Marked and transmural. Both Paneth and goblet cells were enumerated by counting the number of cells in 10 crypts per mouse (40X). For parasite enumeration, all visible parasites per samples were counted. All slide scoring and analysis was carried out blindly by Virginia Gamino.

2.19 Statistical analysis

All statistical analysis was carried out using GraphPad Prism (version 8/9) and data represents mean + standard deviation. A Student *t* test was used for

comparison between 2 groups and a one-way ANOVA with Tukey's multiple comparison correction was carried out for comparisons between 3 or more groups. All data sets were tested for normality using the Shapiro-Wilk normality test, this test was used as all experiments had n=<50 and this test can be applied to smaller data sets with a minimum sample number of 2. Where data were not normally distributed, a Mann Whitney U test for comparisons between 2 groups and a Kruskal-Wallis test with Dunn's multiple comparison correction was carried out for comparisons between 3 or more groups. Data represents mean with standard deviation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant

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Chapter-3 Isolation of leukocytes from helminth infected small intestine lamina propria

3.1 Introduction

Helminth models are a well-established tool for understanding type 2 immunity, and the use of these models is critical for many areas of immunological research. One of these models is *H. polygyrus* and, as previously mentioned, this nematode naturally infects the small intestine of rodents and induces a potent type 2 immune response (Monroy and Enriquez, 1992). This is described in detail in Section 1.3.

Many studies using *H. polygyrus* as an experimental model focus on fourteen days post-infection, as this is when Th2 cell expansion peaks (Perona-Wright et al., 2010). At this timepoint, adult worms emerge into the lumen and both innate and adaptive immune cells infiltrate the tissue resulting in thickening of the submucosa (Hewitson et al., 2011a, Rolot and Dewals, 2018). Furthermore, goblet cell hyperplasia occurs resulting in increased mucus production. The combination of increased mucus and fragile tissue due to oedema especially result in high cell death when digesting leukocytes from the small intestine lamina propria (SILP) for further analysis. In addition, investigating myeloid populations introduces a further level of complexity as these cells are large and become more readily trapped in dying or dead cells and tissue. Much of the literature, therefore, focuses on the immune response in the MLN or at earlier timepoints in the SILP (Mosconi et al., 2015, Pelly et al., 2016, Perona-Wright et al., 2010).

To obtain a comprehensive understanding of the immune response to infection, immune cells at both the site of infection and priming lymph node require examination. Therefore, the first step in my research was to establish a method that allowed for the examination of cell populations in the SILP during *H. polygyrus* infection. To this end, we optimised a protocol for isolating leukocytes from the SILP of key timepoints during *H. polygyrus* infection. We used both intracellular staining and flow cytometry to demonstrate that our protocol enabled us to identify leukocyte subsets from the SILP. In addition, using this

protocol, we were able to investigate the secretion of the regulatory cytokine IL-10 in both the MLN and SILP during *H. polygyrus* infection from different leukocyte subsets.

3.2 Aims

- To optimise small intestine digests from *H. polygyrus* infected mice
- To isolate different leukocyte populations from optimised digest protocols
- To phenotype Th subsets from the SILP of naïve and infected mice
- To examine IL-10 secretion by leukocyte populations in the SILP and MLN during *H. polygyrus* infection

3.3 Results

3.3.1 Optimisation of small intestine digests

Mice infected with *H. polygyrus* for 7 days have visible encysted parasites in the small intestine (Figure 3-1A, red arrows). The resulting granulomas can also be visualised at 14 days post-infection (Figure 3-1A, red arrow). In addition, at both time points there is expansion of the MLN chain and an enlarged spleen (Figure 3-1B). As previously described, there is increased mucus production in the small intestine during *H. polygyrus* infection, and at day 14, mucus production peaks (Anthony et al., 2007, Sharpe et al., 2018). Therefore, isolating leukocytes from the SILP at this timepoint is technically challenging, as mucus can result in cell death (Webster et al., 2020). We therefore aimed to isolate cells from the SILP using a protocol developed by collaborators at the University of Manchester (Liberase and DNase based enzyme cocktail: Table 3-1, Enzyme cocktail 1)(Shaw et al., 2018). Using this protocol, we found that there was large cell death when analysing single cell suspensions, which was shown in terms of scatter of SILP cells from mice 14 days post-infection and low cell yield from both naïve and D14 infected mice (Figure 3-1C & 1D). Cell scatter is a tool used to identify cells based on their size and granularity and dead cells will break up and present with a low side scatter. In addition, viability staining of these samples revealed <10%

live cells compared to the MLN, a tissue which is not technically challenging to isolate cells from, where viability of cells was >55% (Figure 3-1E and 1F).

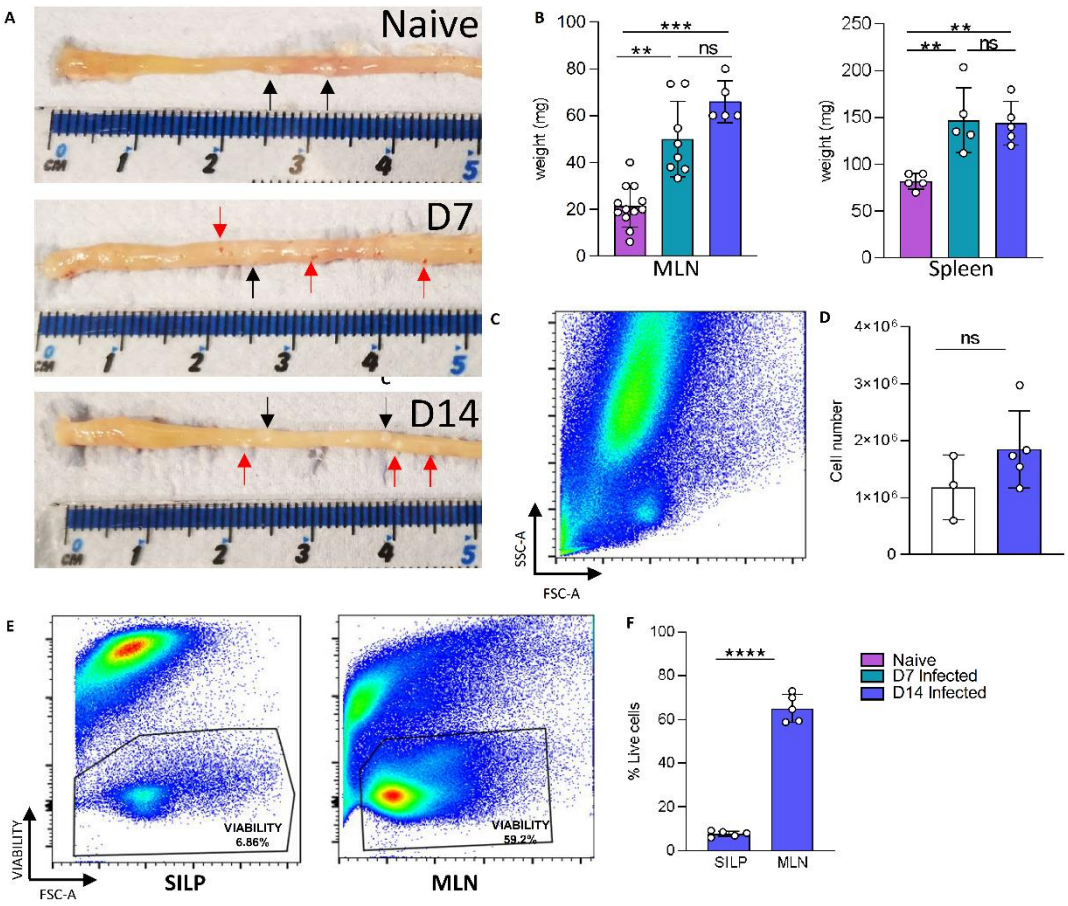


Figure 3-1 Cell death and low cell yield from helminth infected SILP digests pre-optimisation C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- or 14-days post-infection the small intestine and MLN removed. (A) Representative images of the duodenum from naïve (top) and D7 (middle) and D14 (top) infected mice. Black arrows indicate Payers patches and red arrows indicate granulomas. (B) Weight (mg) of the MLN (top) and spleen (bottom) from naïve, D7 and D14 infected mice. (C) Representative flow scatter plot of the SILP from D14 infected mice. (D) Total cell number from the SILP of naïve and D14 infected mice. (E) Representative flow plot of cell viability from cells isolated from the small intestine (left) and MLN (right) from D14 infected mice. (F) Percentage of live cells from the small intestine and MLN from D14 infected mice. Graphed data are shown with mean ± SD and are representative of 2-3 independent experiments with *n*=3-5 per experiment. Statistical significance was calculated by Student *t* test (D&F) and one-way ANOVA with Tukey's post-test for multiple comparisons between groups where data were normally distributed (B (Spleen)) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups where data were not normally distributed (B (MLN)) (Significance ***p*< 0.01, ****p*< 0.001, *****p*< .0001).

We next aimed to improve the viability of single cell suspensions digested from the small intestine of naïve and D14 infected mice. Percoll density gradients are a commonly used tool for the isolation of cells from dirty or sticky cell suspensions (Horner et al., 2019). We therefore used this technique to improve the viability and cell yield from our cell suspensions from naïve and D14 infected

mice, by removing mucus, dead cells and tissue using a density gradient. Using a percoll density gradient resulted in improved cell viability (>30%) from D14 infected mice, however, the number of cells isolated from naïve mice remained low (<10%) (Figure 3-2A & 2B). I hypothesised that enzyme cocktail 1 (Liberase and DNase based enzyme cocktail: Table 3-1) was too harsh for naïve small intestine samples and this resulted in high cell death prior to the density gradient. In addition, despite improved viability of infected samples, cell yield remained low, with <1x10^6 from naïve mice and <3x10^6 cells from D14 infected mice (Figure 3-2C & 2D). Our collaborators in the Milling & Mowat laboratories at the University of Glasgow, who routinely digest naïve SILP, estimated a yield of 1x10^7 cells from naïve SILP. Therefore, percoll gradients were no longer used for small intestine digests due to low cell yield and I next aimed to compare different digestion enzymes used to isolate leukocytes from naïve and D14 infected small intestines.

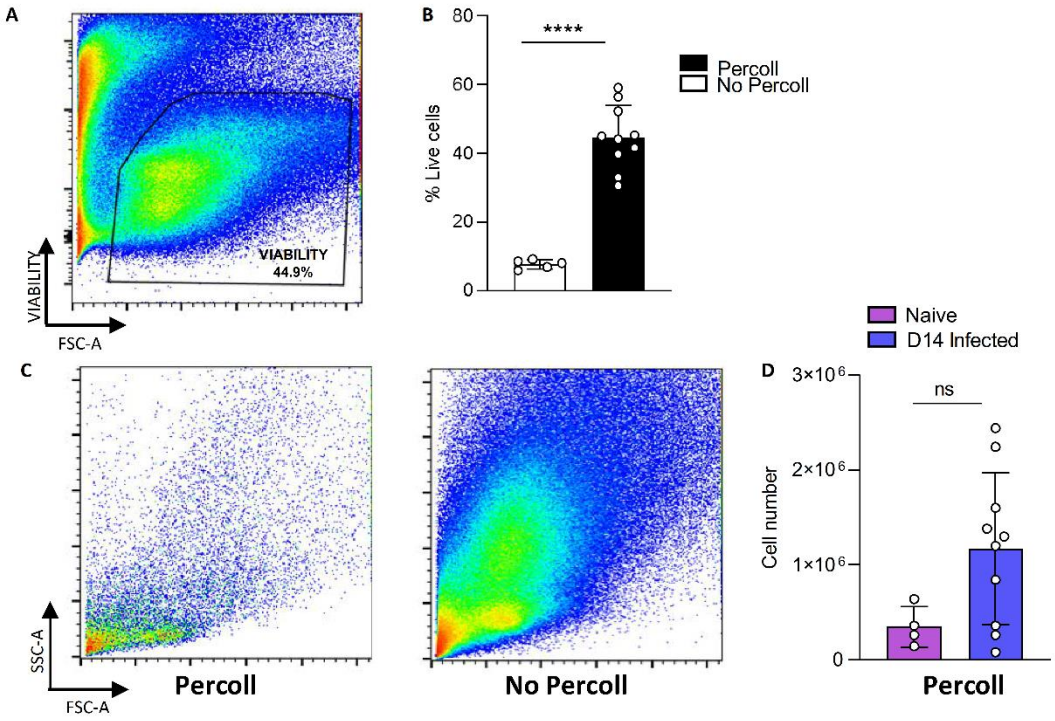


Figure 3-2 The use of percoll gradients improved cell viability but reduced cell yield
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 14 days post-infection the small intestine removed. (A) Representative flow cytometry plot of cell viability from cells isolated using percoll from the small intestine from D14 infected mice (B) Percentage of live cells from the small intestine from naive D14 infected mice isolated with and without percoll gradient. (C) Representative flow scatter plots from cells isolated with (left) and without (right) percoll from D14 infected small intestines. (D) Total cell number from small intestine cells isolated with percoll gradients from naïve and D14 infected mice. Graphed data are shown with mean ± SD and are pooled from 2-3 independent experiments with *n*=2-5 per experiment Statistical significance was calculated by Student *t* test (Significance ****p< 0.0001).

Due to poor viability and cell yield using enzyme cocktail 1, we tested new enzyme cocktails to improve viability and cell yield of SILP samples from both naïve and D14 infected mice (Figure 3-3). We tested two protocols; firstly, we tested the enzyme digest previously used to digest small intestine samples in the Perona-Wright laboratory (Table 3-1, Enzyme cocktail 2), which uses the enzymes Collagenase D, Dispase and DNase. Secondly, we adapted a protocol developed by the Mowat and Milling laboratories (Table 3-1, Enzyme cocktail 3a) which uses the enzyme Collagenase VIII. This protocol had previously been used to isolate cells from the SILP of mice infected with *Salmonella enterica* serovar Typhimurium, especially in successfully isolating myeloid cells from these infected tissues (Chirdo et al., 2005, Bravo-Blas et al., 2019, Cerovic et al., 2013). I combined this protocol with the use of DNase (Table 3-1, Enzyme cocktail 3b).

Name	Enyzme	Concentration	Protocol source
Enzyme cocktail 1	Liberase	0.1mg/ml	Dr John Grainger The University of Manchester
	DnaseI	0.02mg/ml	
Enzyme cocktail 2	Collagenase D	0.5mg/ml	Dr Stephen Redpath The Unviersity of British Columbia
	Dispase	0.5mg/ml	
	DnaseI	0.02mg/ml	
Enzyme cockatil 3a	Collagenase VIII	0.5mg/ml	Milling & Mowat Labratories The Unviersity of Glasgow
Enzyme cockatil 3b	Collagenase VIII	0.5mg/ml	Milling & Mowat Labratories The Unviersity of Glasgow
	DnaseI	0.02mg/ml	

Table 3-1 Enzyme cocktails used for SILP digest optimisation

Analysis of these digests demonstrated that the use of Collagenase VIII and DNase gave higher cell yield, improved cell viability and percentage of $CD4^+$ T cells (Figure 3-3A – 3D). In addition, during future digests, DNase was no longer used as viability improved further when this was not included in the digest (data not shown). Enzyme cocktail 3a was therefore used for all future experiments. Collagenase VIII is a mixture of enzymes, containing additional proteases including clostripain, potentially improving the efficacy of digestion. These data demonstrate that we have developed a protocol that enabled successful isolation of live cells from the SILP with high cell yield for further use in downstream analysis.

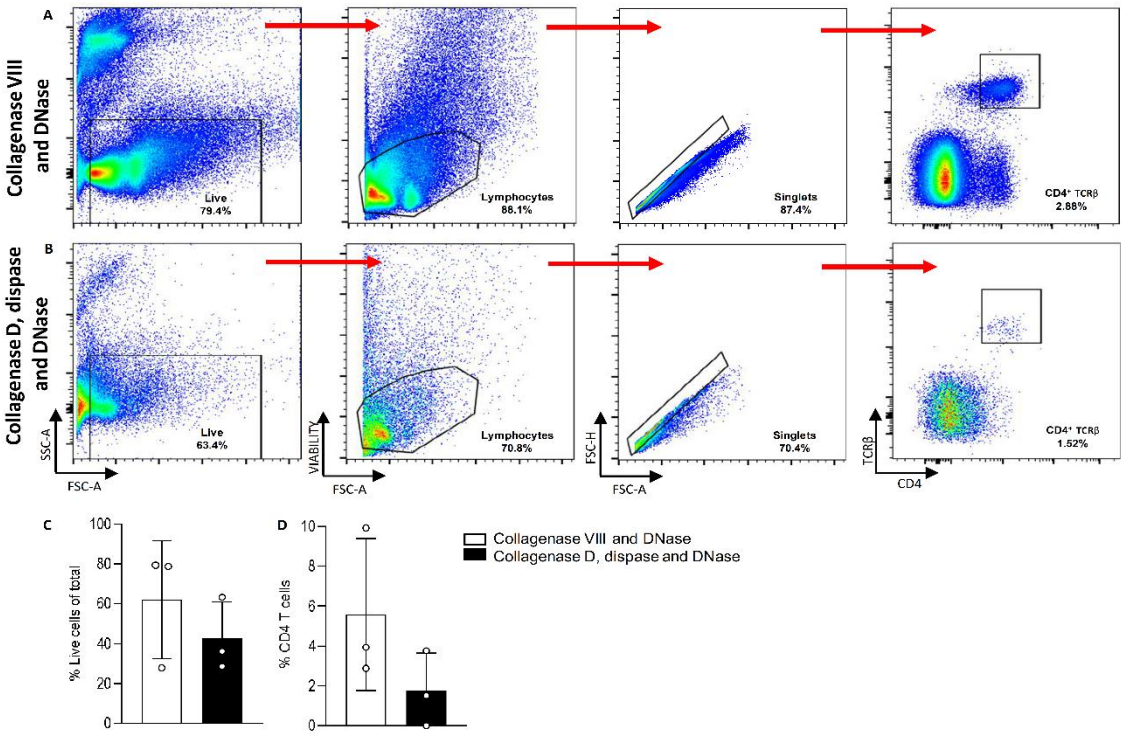


Figure 3-3 Comparison of different enzymes used for digestion optimisation
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 14 days post-infection the small intestine removed. (A-B) Representative flow gating of $CD4^+$ T cells from D14 infected small intestines digested with different enzyme cocktails. (C) Percentage of live cells and (D) Percentage of $CD4^+$ T cells of D14 infected small intestines digested with different enzyme cocktails. Graphed data are shown with mean ± SD with *n*=3 per experiment.

3.3.2 Isolation of leukocyte subsets from optimised small intestine digests

To demonstrate that $CD4^+$ T cells could be successfully isolated at our timepoints of interest (day 7 and day 14), we used enzyme cocktail 3b to digest small intestines from naïve, D7 and D14 *H. polygyrus* infected mice (Figure 3-4A-4D). $CD4^+$ T cells were successfully isolated from each of the 3 timepoints, with improved cell yield and consistent viability compared to earlier experiments (Figure 3-4E & 4F). As expected $CD4^+$ T cells increased at D14 compared to naïve mice, which is the peak T cell response to infection (Figure 3-4C & 4D)(Perona-Wright et al., 2010). Although $CD4^+$ T cells were the primary cell of interest for our experiments, we also wanted the ability to analyse other cell types for future experiments. To confirm our optimised digests would allow for these types of experiments, we carried out broad phenotyping of different cell subsets from naïve and D7 infected mice (Figure 3-5A & 5B). We were able to isolate $CD4^+$ T cells ($CD45^+$ $TCR\beta^+$ $CD8^-$ $CD4^+$, as shown in Figure 3-4B), CD8 T cells ($CD45^+$ $TCR\beta^+$ $CD4^-$ $CD8^+$), $CD45^+$ $TCR\beta^-$ $CD19^-$ $CD11b^+$ cells as broad phenotyping of

myeloid cells, B cells ($CD45^+$ $TCR\beta^-$ $CD11b^-$ $CD19^+$) and ILCs ($CD45^+$ $TCR\beta^-$ $CD11b^-$ $CD19^-$ $IL\text{-}7R^+$) (Figure 3-5A).

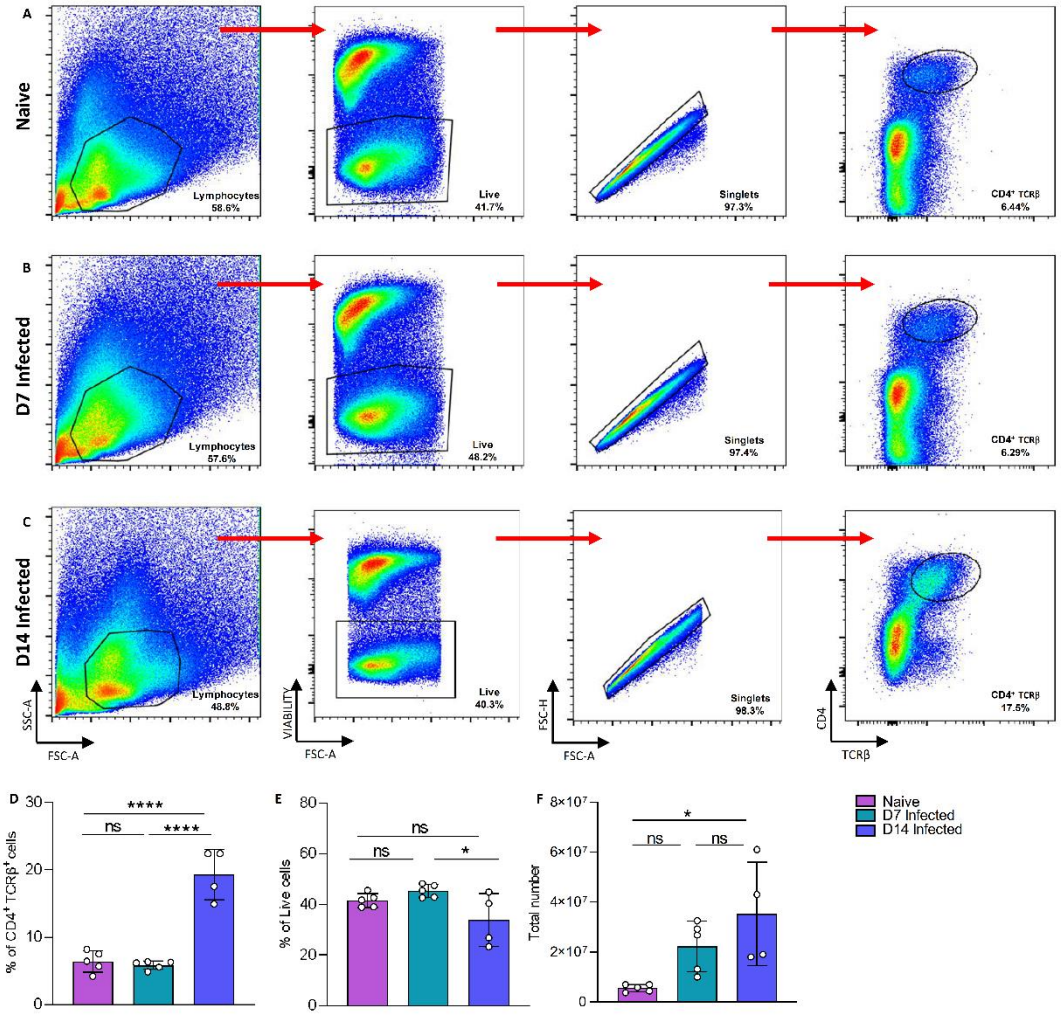


Figure 3-4 Successful isolation of CD4+ T cells from SILP using optimised digest
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14-days post-infection the small intestine removed. (A-C) Representative flow gating of $CD4^+$ $TCR\beta^+$ from naïve (top), D7 (middle) and D14 (bottom) infected small intestine samples. (D) Percentage $CD4^+$ $TCR\beta^+$ T cells (E) Percentage live cells and (F) total cell number from naïve, D7 infected small intestines digested using the optimised protocol. Graphed data are shown with mean ± SD and are representative of 2-3 independent experiments with n=4-5 per experiment. Statistical significance was calculated by one-way ANOVA with Tukey's post-test for multiple comparisons between groups (Significance $*p< 0.05$, $****p< 0.0001$)

When comparing the percentage of each cell subset of total $CD45^+$ cells from naïve and D7 infected mice (Figure 3-5B), we observed an increase in percentage of $CD4^+$, $CD8^+$ and myeloid cells in infected small intestines and a striking decrease in the percentage of B cells upon infection. Importantly, these data show that our optimised protocol enables the isolation of different leukocyte

subsets from the SILP, including myeloid cells which are more challenging to isolate from tissue with high mucus content, due to their size.

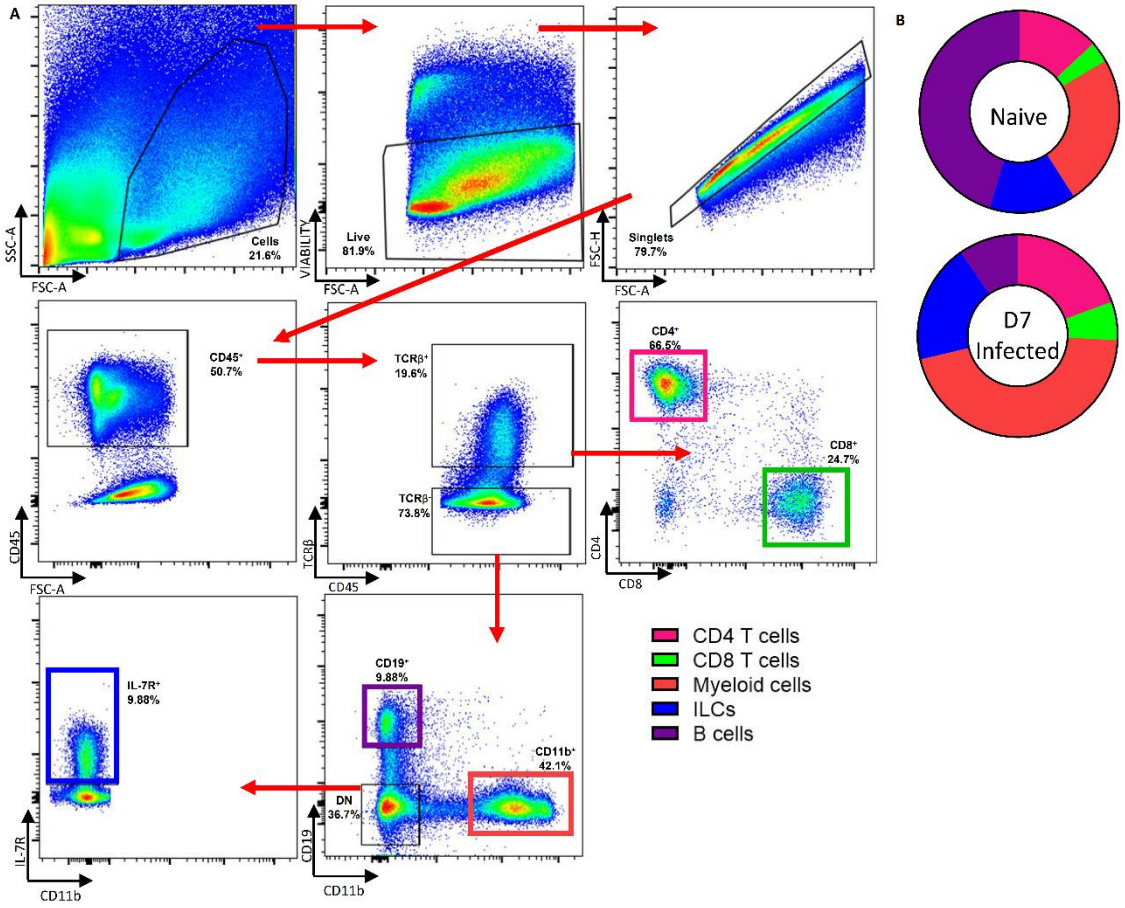


Figure 3-5 Successful isolation of leukocyte subsets from the SILP using the optimised digest
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7 days post-infection the small intestine removed. (A) Representative gating of cell subsets isolated from D7 infected small intestine samples. (B) Percentage of each cell subset of total CD45$^+$ cells from naïve and D7 infected small intestines. Gating for IL-7R$^+$, CD11b$^+$ and CD19$^+$ cells was carried out using appropriate FMO controls. Data are representative of 3 independent experiments with *n*=3-5 per experiment.

3.3.3 Phenotyping T helper cell subsets in the SILP

Having established our technique, we now wanted to examine differences in Th subsets in the SILP and MLN. In both the MLN and SILP there is an increase in activated CD4$^+$ T cells 7- and 14-days post *H. polygyrus* infection, based on high expression of CD44 (CD44^{hi}) (Figure 3-6). In the MLN, a small proportion of cells were CD44^{hi} whereas in the SILP, almost all CD4$^+$ T cells were CD44^{hi} (Figure 3-6A – 6C), reflecting that activated and primed CD4$^+$ T cells leave the lymph node and migrate to the tissue. This emphasises the importance of investigating both

the priming site and tissue as CD4⁺ T cell response dynamics are very different at both sites.

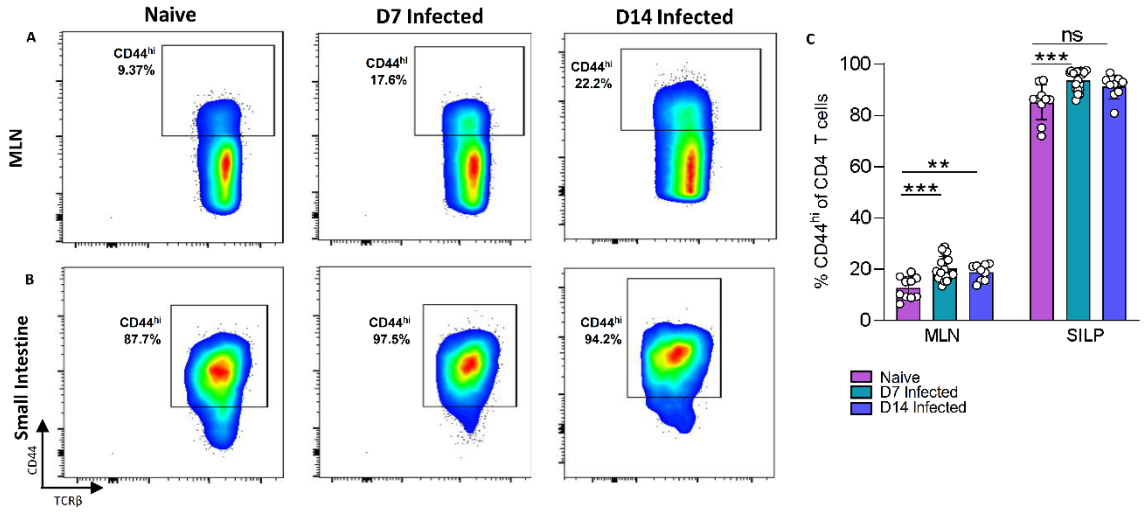


Figure 3-6 In both the MLN and SI, CD44^{hi} CD4^{+} T cells increase 7 and 14 days post-infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14-days post-infection the MLN and small intestine collected for analysis. Representative flow plots of CD44^{hi} staining of CD4^{+} TCRβ^{+} T cells in (A) the MLN and (B) the small intestine. (C) Percentage of CD44^{hi} of CD4^{+} TCRβ^{+} T cells in naïve, D7 and D14 infected mice. Graphed data are shown with mean ± SD and are pooled from 2-3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by one-way ANOVA with Tukey's post-test for multiple comparisons between groups where data were normally distributed (C (MLN)) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups where data were not normally distributed (C (SILP)) (Significance $^{**}p < 0.01$, $^{***}p < 0.001$).

We next tested if, using the optimised protocol, we could measure type 2 cytokine secretion by flow cytometry from activated CD44^{hi} T cells in the SILP and MLN from naïve, D7 and D14 infected mice. This process requires stimulation with PMA and ionomycin for 4 hours, which can increase cell death. My aim here was to ensure that the optimised cell isolation protocol was suitable for this type of analysis. Both IL-5 and IL-13 are key cytokines in the Th2 response. The percentage of IL-5^{+} CD44^{hi} CD4^{+} T cells and IL-13^{+} CD44^{hi} CD4^{+} T cells in the MLN increased 7- and 14-days post-infection compared to naïve mice (Figure 3-7A - 7D). In the SILP, the percentage of IL-5^{+} CD44^{hi} CD4^{+} T cells and IL-13^{+} CD44^{hi} CD4^{+} T cells in the SILP increased 7 days post-infection, although this increase was not significant, and increased significantly at 14-days post-infection compared to uninfected controls (Figure 3-7E – 7H). This is in keeping with peak CD4^{+} T cell responses and occurring at day 14 post-infection (Perona-Wright et al., 2010). These data confirm that our optimised small intestine digest method allows for accurate cytokine staining from naïve, D7 and D14 infected mice.

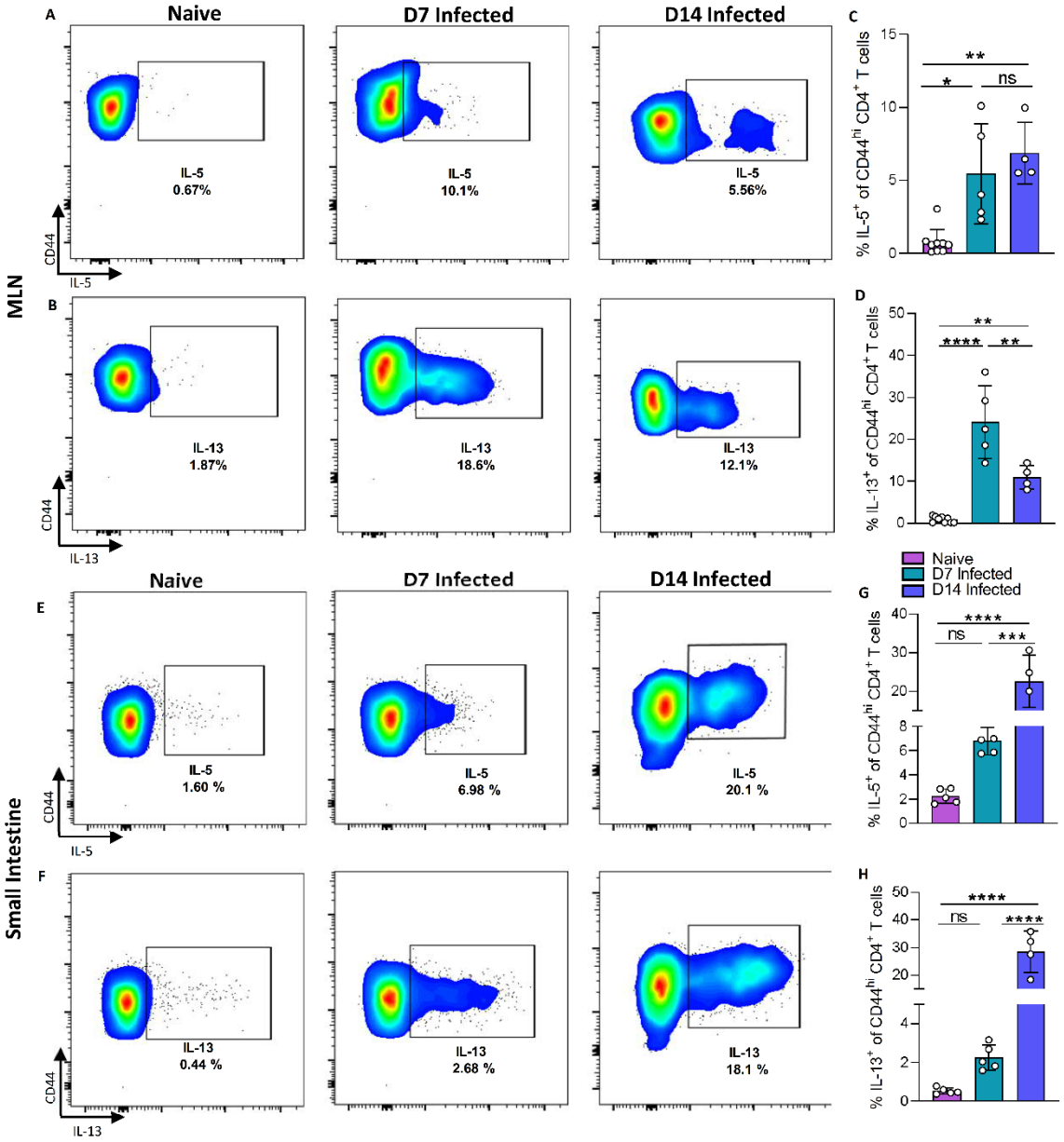


Figure 3-7 There is increased type 2 cytokine secretion in both the MLN and SILP during *H. polygyrus* infection

C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14-days post-infection the MLN and small intestine collected for analysis. Representative flow plots of (A) IL-5+ and (B) IL-13+ staining of $CD4^+$ $TCR\beta^+$ $CD44^{hi}$ T cells in the MLN from naïve, D7 and D14 infected mice. Percentage of (C) $IL\text{-}5^+$ and (D) $IL\text{-}13^+$ $CD4^+$ $TCR\beta^+$ $CD44^{hi}$ T cells in the MLN from naïve, D7 and D14 infected mice. Representative flow plots of (E) $IL\text{-}5^+$ and (F) $IL\text{-}13^+$ staining of $CD4^+$ $TCR\beta^+$ CD44hi T cells in the small intestine from naïve, D7 and D14 infected mice. Percentage of (G) IL-5+ and (H) $IL\text{-}13^+$ $CD4^+$ $TCR\beta^+$ $CD44^{hi}$ T cells in the small intestine of naïve, D7 and D14 infected mice. Gating for $IL\text{-}13^+$ and $IL\text{-}5^+$ cells was carried out using appropriate isotype controls. Graphed data are shown with mean ± SD and are representative of 2-3 independent experiments with n=4-5 per experiment. Statistical significance was calculated one-way ANOVA with Tukey's post-test for multiple comparisons between groups where data were normally distributed (D, G, H) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups where data were not normally distributed (C) (Significance *p< 0.05, **p< 0.01, ***p< 0.001, ****p< .0001).

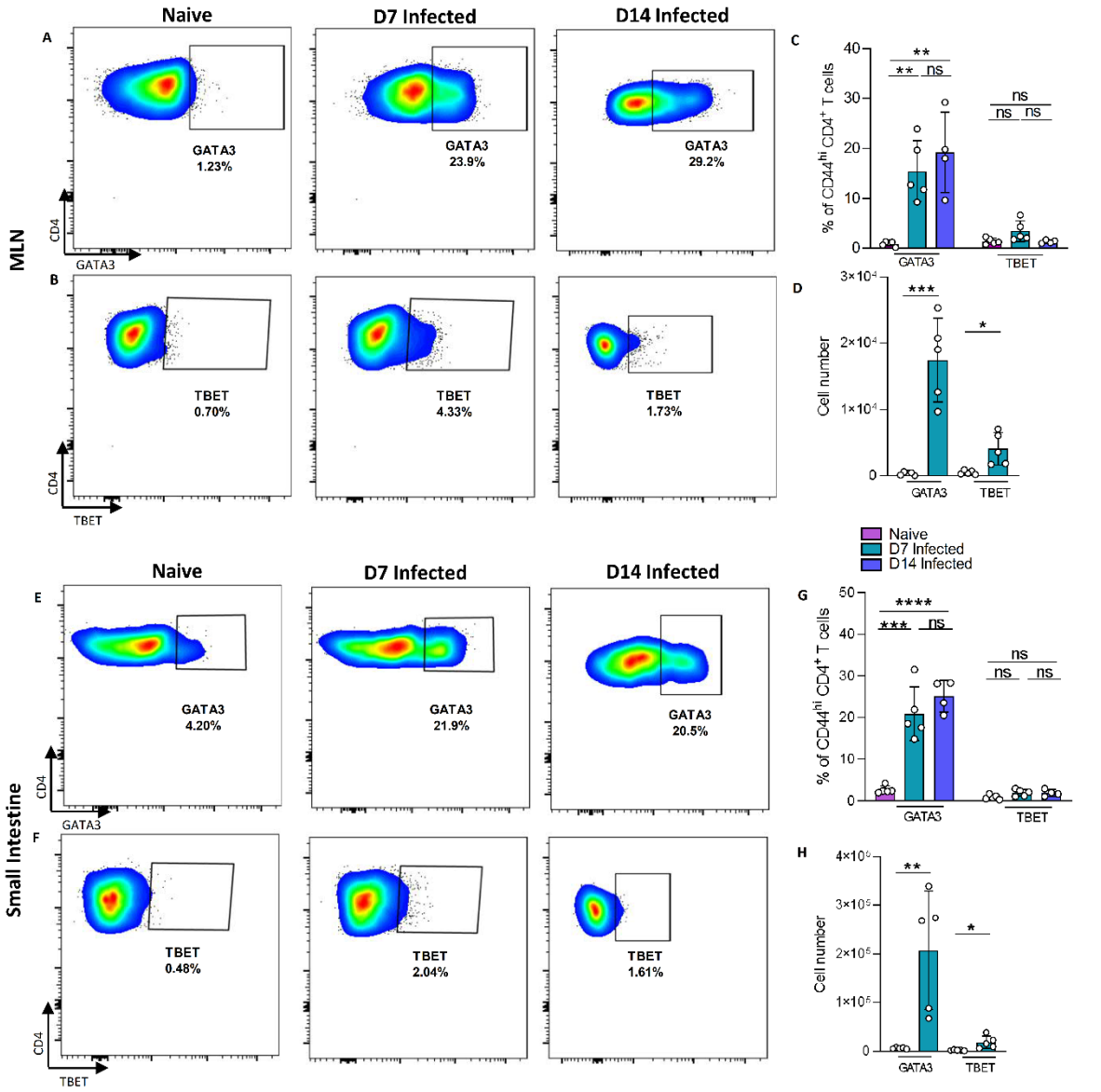


Figure 3-8 Changes in the frequency and number of Th1 and Th2 cells in the MLN and SI, 7- and 14- days post infection with *H. polygyrus* infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14-days post-infection the MLN and small intestine collect for analysis. Representative flow plots of (A) GATA3^{+} and (B) TBET^{+} staining of CD4^{+} TCRβ^{+} CD44^{hi} T cells from the MLN of naïve, D7 and D14 infected mice. (C) Percentage of GATA3^{+} and TBET^{+} of CD4^{+} TCRβ^{+} CD44^{hi} T cells from naïve, D7 and D14 infected mice. (D) Total number GATA3^{+} and TBET^{+} CD4^{+} CD44^{hi} T cells in the MLN from naïve and D7 infected mice. Representative flow plots of (E) GATA3^{+} and (F) TBET^{+} staining of CD4^{+} TCRβ^{+} CD44^{hi} T cells from the small intestine of naïve, D7 and D14 infected mice. (G) Percentage of GATA3^{+} and TBET^{+} of CD4^{+} TCRβ^{+} CD44^{hi} T cells from the small intestine of naïve, D7 and D14 infected mice. (D) Total number GATA3^{+} and TBET^{+} CD4^{+} CD44^{hi} T cells in the small intestine from naïve and D7 infected mice. Gating for TBET^{+} and GATA3^{+} cells was carried out using appropriate isotype controls. Graphed data are shown with mean ± SD and are representative of 1-3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by Student *t* test (D&H) and one-way ANOVA with Tukey's post-test for multiple comparisons between groups (C&G) (Significance *p< 0.05, **p< 0.01, ***p< 0.001, ****p< .0001).

We also aimed to confirm our definition of Th2 cells in the MLN and SILP using the TF GATA3; we also considered TBET as a marker of Th1 cells. We therefore measured the expression of GATA3 and TBET in CD44^{hi} CD4^{+} T cells from naïve,

D7 and D14 *H. polygyrus* infected mice in the SILP and MLN using our optimised protocol. Staining for transcription factors to identify Th subsets involves numerous steps of fixation, permeabilization and staining which can reduce sample viability. In the MLN, the percentage of GATA3^{+} but not TBET^{+} CD44^{hi} CD4^{+} T cells increased at D7 of infection compared to naïve controls. In addition, at D14 the increase in GATA3^{+} CD44^{hi} CD4^{+} T cells was maintained compared to uninfected controls (Figure 3-8A-D). The percentage of GATA3^{+} but not TBET^{+} CD44^{hi} CD4^{+} T cells increased in the SILP at both D7 and D14 of infection compared to naïve controls (Figure 3-8E-H). As expected, in both tissues from D7 infected mice, GATA3^{+} CD44^{hi} CD4^{+} T cells were the dominant Th subset compared to TBET^{+} CD44^{hi} CD4^{+} T cells, based on the percentage of these cells in the MLN and SILP compared to naïve mice. This was reflected in the total number of GATA3^{+} CD44^{hi} CD4^{+} T cells and TBET^{+} CD44^{hi} CD4^{+} T cells from D7 infected mice in both tissues (Figure 3-8D & 8H). Although we did see an increase in the total number of TBET^{+} CD44^{hi} CD4^{+} T cells in the MLN and SILP. Unfortunately, due to technical error, the total number of GATA3^{+} CD44^{hi} CD4^{+} T cells and TBET^{+} CD44^{hi} CD4^{+} T cells at D14 of infection were not calculated. I predict that the number of GATA3^{+} CD44^{hi} CD4^{+} T cells would have increased further at day 14 post infection as this is known to be the peak of the Th2 response (Perona-Wright et al., 2010).

Regulatory T cells are critical in preventing immune dysregulation (White et al., 2020). Through the secretion of regulatory cytokines such as IL-10 and TGF-β, Tregs have the capacity to suppress effector T cell responses (Smith et al., 2016, White et al., 2020). FOXP3^{+} CD4^{+} regulatory T cells have previously been shown to increase during *H. polygyrus* infection in the MLN and at early timepoints (D7) of infection in the SILP (Finney et al., 2007, Smith et al., 2016, Rausch et al., 2008, Redpath et al., 2013). Although suppression of Th2 cells in helminth infection has been demonstrated to be independent of IL-10 secretion by Tregs (Smith et al., 2016). Expansion of these cells in the MLN typically peaks at D21 of infection, although expansion can be seen from D7 of infection (Rausch et al., 2008, Finney et al., 2007, Smith et al., 2016). Therefore, we examined changes in Tregs 7- and 14-days post-infection in the SILP. The percentage of FOXP3^{+} CD4^{+} T cells increased at D7 in both the MLN and the SILP compared to naïve controls. At D14, the percentage of FOXP3^{+} CD4^{+} T cells increased in the MLN only (Figure 3-9A-9D). The absolute number of Tregs also increased in D7

infected mice in both tissues, compared to naïve, but sadly, as explained above, cell numbers for D14 infected mice could not be calculated. I hypothesise that similar to GATA3$^+$ CD4$^+$ T cells these cells would have continued to increase, particularly in the MLN as this has been published previously (Finney et al., 2007). Although the percentage of FOXP3$^+$ CD4$^+$ T cells did not increase in the SILP at D14 of infection compared to D7, peak Treg responses are reported at later timepoints such as D21. I predict that at timepoints such as D21 of infection, the number and percentage of Tregs would also increase in the SILP based on published reports in the MLN (Smith et al., 2016, Bowron et al., 2020). My data add to already published data by showing that Treg frequency increases not only in the MLN during *H. polygyrus* infection, but also in the infected tissue site.

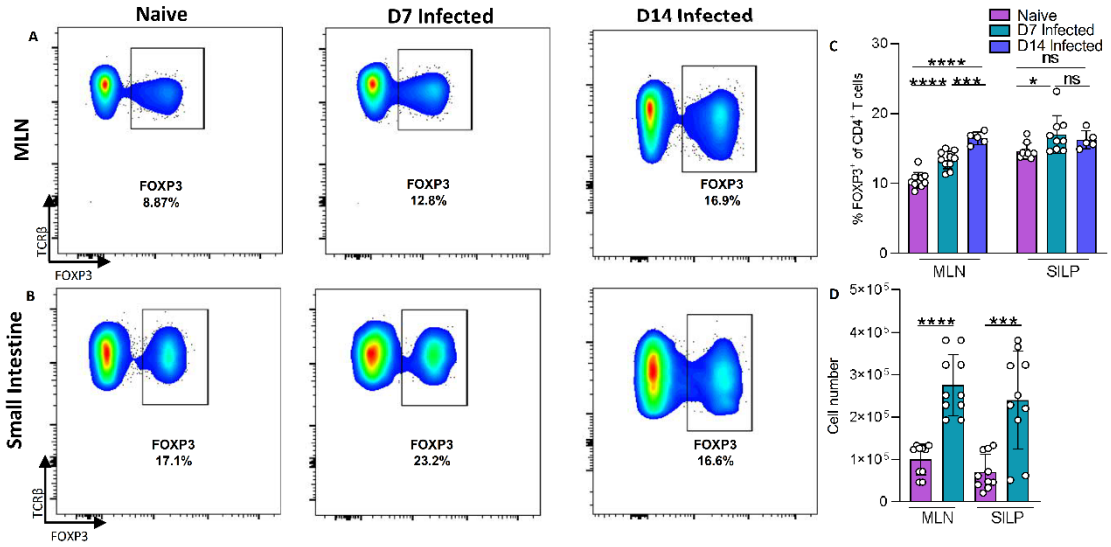


Figure 3-9 There is an increase in Tregs in the MLN and SILP, 7- and 14- days post-infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14-days post-infection the MLN and small intestine collect for analysis. Representative flow plots of FOXP3$^+$ staining of CD4$^+$ TCRβ$^+$ T cells from the (A) MLN and (B) small intestine of naïve, D7 and D14 infected mice. (C) % of FOXP3$^+$ CD4$^+$ TCRβ$^+$ T cells in the MLN and small intestine from naïve, D7 and D14 infected mice. (D) Total number of FOXP3$^+$ CD4$^+$ TCRβ$^+$ T cells in the MLN and small intestine from naïve, D7 infected mice. Gating for FOXP3$^+$ cells was carried out using an appropriate isotype control. Graphed data are shown with mean ± SD and are representative of 1-3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by Mann Whitney U test where data were not normally distributed (D (MLN)) and Student *t* test where data were normally distributed (D, SILP) and one-way ANOVA with Tukey's post-test for multiple comparisons between groups where data were normally distributed (C, MLN) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups where data were not normally distributed (C (SILP)) and (Significance *p< 0.05, ***p< 0.001, ****p< .0001).

To summarise our data so far, we have shown that despite both cytokine and transcription factor stains requiring harsh treatment of cells, viability of SILP samples was maintained from naïve, D7 and D14 infected mice (Figure 3-10A –

10F). As expected, viability was reduced, particularly at D14, when carrying out cytokine staining, as this requires 4 hours of intense stimulation and at least 10% cell death is expected (Figure 3-10D-F). These data demonstrate that the optimised digest protocol allows us to measure the Th2 immune response to *H. polygyrus* infection described in the literature.

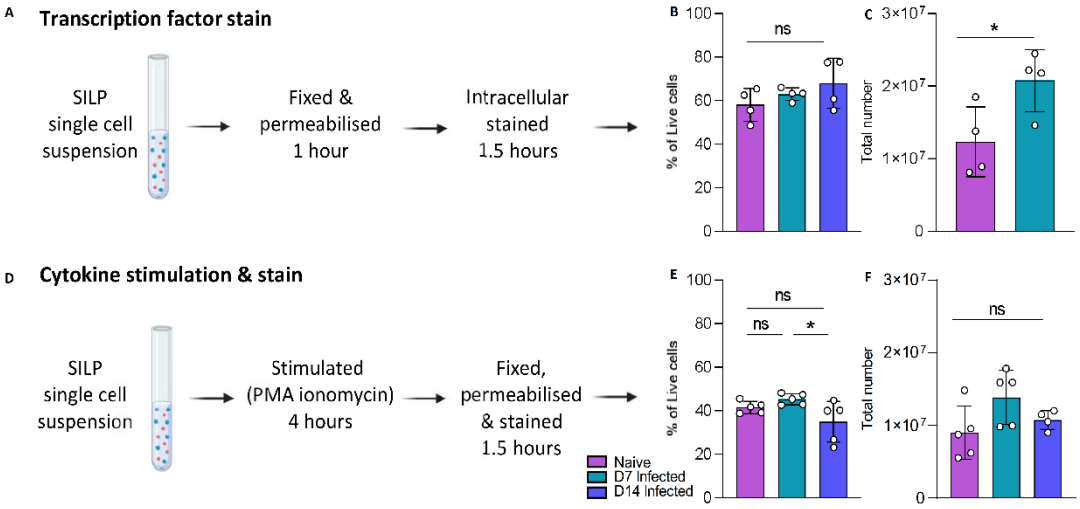


Figure 3-10 Restimulation, fixation and permeabilization does not reduce cell viability
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14-days post-infection the small intestine collected for analysis. (A) Flow chart of transcription factor stain method. (B) Percentage of live cells from naïve, D7 and D14 infected mice and (C) total number live cells from naïve and D7 infected mice. (D) Flow chart of cytokine stain method. (E) Percentage of live cells from naïve, D7 and D14 infected mice and (F) total number live cells from naïve, D7 and D14 infected mice. Graphed data are shown with mean ± SD and are representative of 1-3 independent experiments with *n*=4-5 per experiment Statistical significance was calculated by Student *t* test (C) and one-way ANOVA with Tukey's post-test for multiple comparisons between groups (B, E, F) (Significance *$p < 0.05$).

3.3.4 IL-10 secretion increases in the MLN and small intestine during infection

One of the main aims of this thesis was to understand the role of IL-10 in the immune response to *H. polygyrus* infection. Establishing a method for isolating cells from the SILP allowed us to address this question. However, we first wanted to examine IL-10 expression in the SILP during *H. polygyrus* infection using our optimised protocol. As discussed previously, IL-10 is a regulatory cytokine that is reported to have an important role in gut homeostasis in both mice and humans (Glocker et al., 2009, Kotlarz et al., 2012, Moran et al., 2013, Kühn et al., 1993, Spencer et al., 1998, Franke et al., 2008). The role of IL-10 has been debated in the context of a Th2 response, and there is evidence both that it can promote the Th2 response and that it has the capacity to suppress

Th2 cells (Laouini et al., 2003, Golebski et al., 2021). In the context *of H. polygyrus*, Treg derived IL-10 does not appear to suppress Th2 responses (Smith et al., 2016). Previously, *ex-vivo* restimulated SILP cells from *H. polygyrus* infected mice had increased IL-10 secretion compared to naïve controls (Setiawan et al., 2007). Increased IL-10 secretion by Tregs in the in the MLN

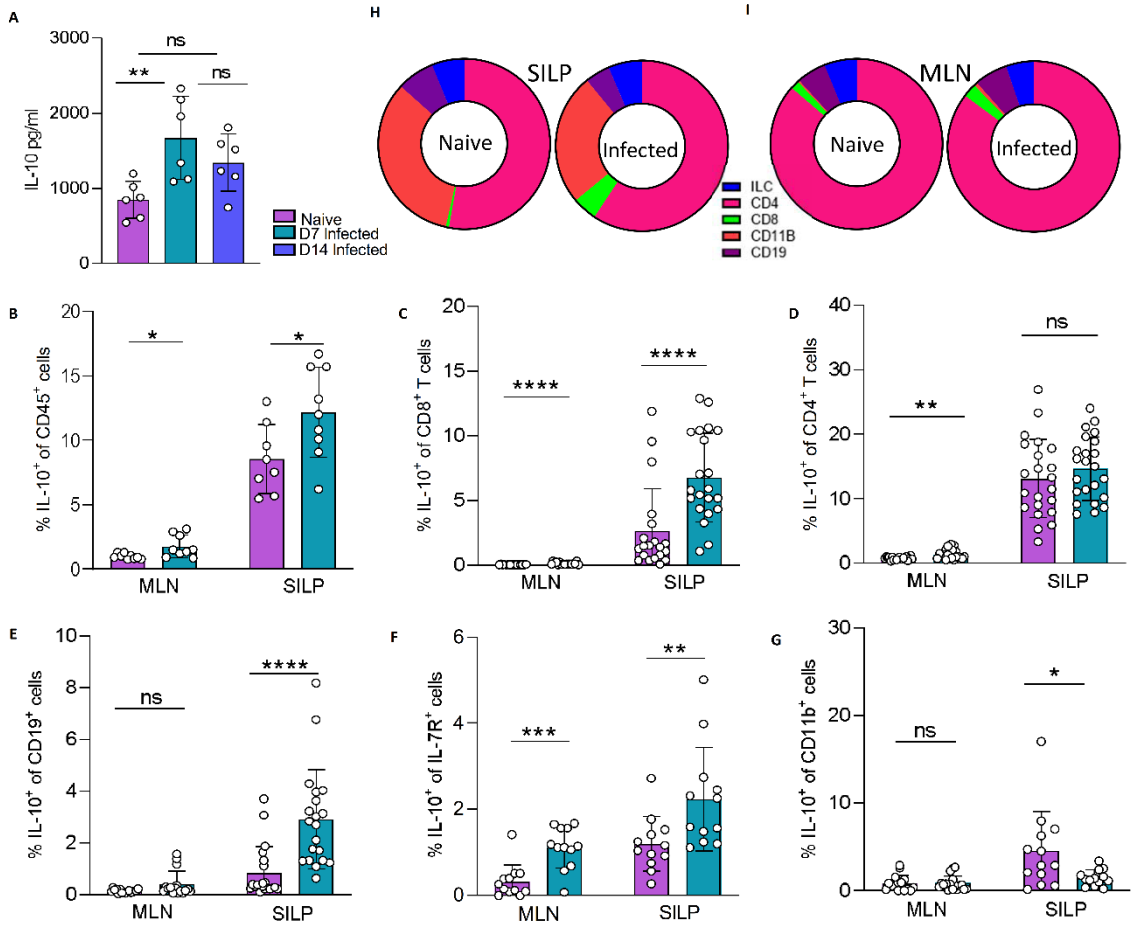


Figure 3-11 An increase in IL-10 expression in multiple cell types in both the MLN and small intestine during infection
Il10gfp-foxp3rfp B6 or C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7- and 14- post-infection the MLN and small intestine collected for analysis. (A) MLN cells from naïve, D7, D14 and D21 infected mice were stimulated *ex vivo* with anti-CD3 and 3 days later supernatants collected, and concentration of IL-10 measured. Percentage of IL-10$^+$ of (B) CD45$^+$, (C) CD8$^+$, (D) CD4$^+$, (E) CD19$^+$, (F) IL-7R$^+$ and (G) CD11b$^+$ cells from the MLN and small intestine of naïve and D7 infected report mice. Percentage of each cell subset of total IL-10$^+$ CD45$^+$ cells from (H) the small intestine and (I) the MLN of naïve and D7 infected reporter mice. Graphed data are shown with mean ± SD and are representative of 1-5 independent experiments with *n*=2-5 per experiment. Gating for IL-10$^+$ cells was carried out using samples from a WT C57BL/6 mouse, to account for any background fluorescence. Statistical significance was calculated by Student *t* test where data were normally distributed (B, D (SILP)) and Mann Whitney U test where data were not normally distributed (C, D (MLN), E, F, G) and a one-way ANOVA with Tukey's post-test for multiple comparisons between groups (A) (Significance *p< 0.05, **p< 0.01, ***p< 0.001, ****p< .0001).

and SILP during *H. polygyrus* infection has been reported (Setiawan et al., 2007, Redpath et al., 2013, Finney et al., 2007). However, its expression by different

cell types in the SILP is poorly described in the literature due to the difficulty of isolating cells from the SILP during *H. polygyrus* infection. We therefore aimed to measure IL-10 expression in the MLN and SILP during *H. polygyrus* infection. We carried out *ex-vivo* re-stimulations of whole MLN cells at D7 and D14 post infection with *H. polygyrus* and measured IL-10 in the supernatant (Figure 3-11A). We observed increased IL-10 secretion from cells isolated during infection, and this IL-10 release peaked at D7 post infection in the MLN (Figure 3-11A). We therefore focused on this timepoint for further analysis of the SILP and MLN. To measure IL-10 expression by different cell subsets in the MLN and SILP, we used Il10gfp-foxp3rfp B6 mice reporter mice. We found that, overall, the percentage of IL-10$^+$ CD45$^+$ cells increased in both the MLN and the SILP compared to naïve controls. In addition, baseline IL-10$^+$ CD45$^+$ was overall higher in the SILP compared to the MLN (Figure 3-11B). To gain a deeper understanding of the cell subsets producing IL-10 in the SILP and MLN during infection, IL-10 secretion from different cell populations was analysed using the gating strategy described in Figure 3-5A (Figure 3-11C-G). In the SILP, the percentage of IL-10$^+$ CD8 T cells, B cells and ILCs increased D7 post-infection compared to uninfected mice. Whereas the percentage of IL-10$^+$ CD4$^+$ T cells in the SILP did not increase with infection and there was decrease in the percentage of IL-10$^+$ myeloid cells compared to uninfected mice in the SILP (Figure 3-11C-G). In the MLN, the percentage of IL-10$^+$ B cells and myeloid cells remained unchanged between naïve and infected samples, whereas the percentage of IL-10$^+$ CD8$^+$ T cells, CD4$^+$ T cells, ILCs increased upon infection. IL-10 expression in both naïve and infected mice was higher in the SILP compared to the MLN (Figure 3-11B-G), which again highlights the importance of investigating both the priming site and tissue when assessing immune responses to infection.

Further analysis of these data, assessing the proportions of different cell types represented within the IL-10$^+$ CD45$^+$ population revealed that, in the SILP, CD4$^+$ T cells and myeloid cells made up the majority of the IL-10$^+$ CD45$^+$ cells (Figure 3-11H). In addition, 7 days post *H. polygyrus* infection, there was an increase in the proportion of IL-10$^+$ CD8$^+$ T cells, CD4$^+$ T cells and B cells and a decrease in IL-10$^+$ myeloid cells (Figure 3-11H). In the MLN CD4$^+$ T cells made up more than half of IL-10$^+$ CD45$^+$ cells and there were no significant changes upon infection with *H. polygyrus* (Figure 3-11I). These data show that although we did not see an increase in the percentage of CD4$^+$ T cells that expressed IL-10 upon

infection, this cell population is nonetheless one of the main producers of IL-10 in both the SILP and the MLN, in naïve as well as infected animals.

Together, the data in this chapter demonstrate that we have optimised a SILP digest protocol that allows for successful isolation of multiple immune cells from *H. polygyrus* infected SILP, with high cell yields and viability. In addition, this protocol provides a method for accurate analysis and characterisation of immune cell subsets. In keeping with the literature, we show that Th2 cells are the main effector population in the SILP. In addition, we report that the total number of Th1 cells in the SILP and MLN also increases with *H. polygyrus* infection, although the total numbers of these cells were low overall. We also report increased Tregs in the SILP and examine IL-10 producing cells in both the SILP and MLN during *H. polygyrus* infection which provides important information about the source of this cytokine during infection. By optimising this technique, we were able to address some of the main aims of this thesis and we have also provided a new insight into the immunology of *H. polygyrus* infection.

3.4 Discussion

3.4.1 Validation of optimised helminth infected SILP digestion protocol

The SILP has been investigated extensively in the literature in both naïve mice and those infected with organisms such as bacteria and viruses (Bravo-Blas et al., 2019, Perona-Wright et al., 2012, Cerovic et al., 2013, Isakov et al., 2011). The same however cannot be said for immune cell analysis in the SILP during helminth infection. The induction of a 'weep and sweep' response is a key part of the type 2 immune response but results in tissue samples that are very fragile and that quickly die *ex vivo*. This is due to elevated mucus production, infiltration of leukocytes and granulocytes, fibrosis, and the release of immune mediators such as cytokines and histamines (Allen and Maizels, 2011, Allen and Wynn, 2011, Webb and Tait Wojno, 2017). In this chapter, I aimed to study the immune response to *H. polygyrus* at the site of infection as well as in the MLN. This required the optimisation of isolating cells from infected tissues first. Our optimised protocol allowed us to identify Tregs, Th2 and Th1 cells in the SILP during *H. polygyrus* infection. In addition, we show that the regulatory cytokine

IL-10 is predominately a tissue-based cytokine and its expression is increased in numerous cell types during *H. polygyrus* infection.

The importance of studying tissue immunity is becoming increasingly important, and this is reflected in back-to-back publications of methods for isolating cells from the SILP of *H. polygyrus* infected small intestine samples (Ferrer-Font et al., 2020, Webster et al., 2020, Jarjour et al., 2020). Numerous studies have reported MLN cytokine dynamics during *H. polygyrus* infection, but studies of small intestinal cytokines during infection have been restricted to gene expression, *ex-vivo* restimulation or earlier timepoints of infection (<D7) (Redpath et al., 2013, Filbey et al., 2014, Setiawan et al., 2007, Pelly et al., 2016, Blum et al., 2012). For successful downstream analysis using techniques such as flow cytometry and intracellular staining, high cells yield, and viability are required. Our use of percoll gradients aimed to remove debris and dead cells, although this technique improved cell viability, it extended the length of our protocol and gave poor cell yields (around 1 million cells/sample). On average, our collaborators in the Milling laboratory at the University of Glasgow, digest 10 million cells from a naïve SILP. The low cell number from naïve small intestines indicated that there was high cell death prior to the use of the percoll gradient. This suggested that the digestion protocol itself (Table 1-1, Enzyme cocktail 1) was too harsh for isolating SILP cells from naïve mice, where the mucus layer is thinner compared to infected mice (Johansson and Hansson, 2016, Zhao et al., 2003, Anthony et al., 2007). Through testing of different enzyme cocktails (Table 3-1), we improved the cell yield and viability of our samples with Collagenase VIII giving the highest viability and percentage of $CD4^+$ T cells. This digest also gave the highest cell yield (data not shown). We could not show these data due to software issues resulting in the loss of data but all future experiments with this digest reflect this. Through optimisation of enzyme cocktails and timing of both processing and digestion of samples, our data demonstrate that we developed a SILP digest protocol that allows for the isolation of viable leukocytes with high cell yield from the SILP at both D7 and D14 post *H. polygyrus* infection, which are two important immunological time points.

As discussed previously, despite the high mucus content of these samples we were able to isolate various leukocyte subsets, including myeloid cells. Myeloid

cells are notoriously difficult to isolate from these kinds of tissues and prone to cell death *ex-vivo*. We used CD11b as a broad marker for myeloid cells for these experiments. Deeper analysis of these subsets to look at distinct populations of monocytes, macrophages and DCs has been carried out by our collaborators in the Milling laboratory at the University of Glasgow, published alongside our own data in our recent methods paper (Webster et al., 2020). The data from this study showed that macrophages increase with *H. polygyrus* infection and this is maintained from D7 to D14 (Webster et al., 2020). However, although DCs and neutrophils increase at D7 of infection compared to naïve mice, the total number of these cells returns to baseline at D14 of infection (Webster et al., 2020). These observations are in keeping with the overall increase in the proportion of $CD11b^+$ cells we report at D7 of infection compared to naive controls. In addition to identifying both innate and adaptive cell subsets we were also able to show changes in the proportions of these cells in the SILP 7 days post-infection. In particular, $CD4^+$ T cells were shown to significantly increase at D14 post-infection compared to naïve controls. This confirms that our protocol allows for accurate representation of the immunobiology during infection as it is well established in the literature that D14 is the peak of the $CD4^+$ T cell expansion during *H. polygyrus* infection (Perona-Wright et al., 2010).

3.4.2 Investigating Th cell subsets in both the MLN and SILP during H. polygyrus infection

The analysis of both cytokine secretion and transcription factor expression by $CD4^+$ T cells is key for the investigation of CD4-mediated immune responses. Therefore, in addition to isolating $CD4^+$ T cells, we demonstrated that we could carry out these deeper analyses without losing consistent cell viability and yield. The increase in Th2 cells observed at D7 and D14, both in terms of IL-5 and IL-13 secretion and GATA3 expression, show that the isolated cells from the SILP reflect the described immune response to *H. polygyrus* in the literature (Maizels et al., 2012). Although IL-4 is a key Th2 cytokine along with IL-5 and IL-13, particularly in the priming of Th2 cells in the MLN (Perona-Wright et al., 2010, Redpath et al., 2015), we were unable to assess IL-4 expression due to poor staining of this cytokine. The Th2 cytokines IL-13, IL-4 and IL-5 all play important roles in the type 2 immune response, however, the localisation and function of these cytokines at different sites varies. IL-4 is found in high

concentrations in the MLN during infection, and in comparison, the levels of IL-13 and IL-5 are lower (Redpath et al., 2015). Furthermore, the target cells of these cytokines differ. IL-13 primarily targets epithelial cells, ILC2s and AAMs, all of which typically localise within the tissue (Mckenzie et al., 1998, Kuhn et al., 1991, Reynolds et al., 2012, Pelly et al., 2016). Similarly, the targets of IL-5 signalling are primarily tissue-based cells, IL-5 is key for the recruitment of granulocytes and for the degranulation of basophils (Kouro and Takatsu, 2009, Obata-Ninomiya et al., 2020). Despite the main functions of IL-4 being B cell class switching and Th2 polarisation in the MLN (King and Mohrs, 2009, Kuhn et al., 1991, Redpath et al., 2015). IL-4 is also present in the infected tissue and contributes towards the activation of AAMs and further drives DC mediated Th2 cell polarisation (Cook et al., 2012, Jang and Nair, 2013). I hypothesise that IL-4 may have significantly decreased in the MLN with IL-10R blockade, similar to the decrease in IL-13 and IL-5 in the tissue, which would reflect the site-specific primary functions of these cytokines.

It is well established that Th2 cells are essential for type 2 immune responses. However, we also assessed the Th1 master transcription factor TBET and found that in the SILP and MLN, Th1 cells increased but were not nearly as prevalent as Th2 cells. This is interesting as immune competition between Th subsets is important for the outcome of an immune response to infection (Magombedze et al., 2014). There is some evidence of the presence of Th1 cells during *H. polygyrus* in the literature (Reynolds et al., 2014a, Filbey et al., 2014) and we have investigated this underlying Th1 response further in Chapter-5 as a possible source of immune competition during *H. polygyrus* infection. The presence of a Th1 response during *H. polygyrus* infection would suggest counter-regulation of the Th2 response. To further examine the idea of regulation, we considered both Tregs and the expression of the immunosuppressive cytokine IL-10.

3.4.3 IL-10 in the immune response to helminth infection

Tregs, IL-10 and TGFβ are an important component of the immune response to *H. polygyrus* infection (Finney et al., 2007, White et al., 2020, Redpath et al., 2013, Smith et al., 2016, Rausch et al., 2008) and in keeping with the literature we saw an increase in $FOXP3^{+}$ $CD4^{+}$ T cells in both the MLN and SILP at D7 of *H. polygyrus* infection (Finney et al., 2007, Redpath et al., 2013). Our data shows

that in the SILP, this increased percentage of Tregs is not observed at D14, unlike in the MLN where Tregs continue to increase at D14 of infection. Although IL-10 is secreted by Tregs, suppression of Th2 responses by Tregs is independent of IL-10 in *H. polygyrus* infection (Smith et al., 2016).

The expression of IL-10 increases during *H. polygyrus* infection (Finney et al., 2007, Setiawan et al., 2007, Redpath et al., 2013, Filbey et al., 2014, Leung et al., 2012). Its important role in helminth infection has been shown in other parasite models. IL-10 is key for host survival in *T. muris* infection, IL-$10^{-/-}$ mice infected with this parasite had a significantly lower survival rate compared top WT controls (Schopf et al., 2002). In addition, IL-4R dependent IL-10 production maintains Th2 dominance during infection with *N. brasiliensis* (Balic et al., 2006). *S. mansoni* infection reduced tissue damage in a model of airway inflammation and this was IL-10 dependent (Marinho et al., 2016). These studies demonstrate the protective effects of helminth induced IL-10 secretion. The optimised protocol allowed us to investigate IL-10 in the SILP in different cell subsets and our data demonstrate that IL-10 is highly expressed in the tissue compared to the priming lymph node. This is most likely due to its essential role in gut homeostasis as IL-$10^{-/-}$ mice develop severe colitis (Kühn et al., 1993, Spencer et al., 1998, Kole and Maloy, 2014) . Interestingly, the development of spontaneous colitis in IL-$10^{-/-}$ mice is dependent on the microbiota, as specific-pathogen-free (SPF) mice have a delayed intestinal inflammation (Kullberg et al., 1998). Furthermore, infecting IL-$10^{-/-}$ SPF mice with the pathogenic bacteria *Helicobacter hepaticus* induces chronic colitis (Kullberg et al., 1998). The presence of the microbiota in the intestine adds a layer of complexity when studying this tissue compared to the LN and highlights the requirement for IL-10 in the maintenance of homeostasis. The dominant IL-10 producing cells in the SILP were $CD4^{+}$ T cells and myeloid cells, both cell types are known to express this cytokine in naïve mice to maintain immune homeostasis (Kole and Maloy, 2014, Mantovani and Marchesi, 2014). Increased IL-10 expression during *H. polygyrus* infection by both innate and adaptive cells in both the SILP and MLN further suggests that this cytokine is important in the type 2 immune response to *H. polygyrus* infection. However, the dynamics of this remain unclear. In Chapter 3 I investigate the role of IL-10 on Th2 immunity during *H. polygyrus* infection.

3.4.4 Concluding remarks

There is an expanding need to investigate immune responses not only at lymphoid priming sites but at the sites of infection too. The idea of resident T cell memory in the tissues reshaped how we see immunity in many ways (Carbone and Gebhardt, 2019) and this may also be the case for tissue-specific immune responses to helminth infection. Type 2 cytokines can be broadly but not exclusively described as tissue and lymphoid cytokines, with IL-13 and IL-5 being recognised as tissue cytokines whereas IL-4 is concentrated in the MLN (Liang et al., 2011, Redpath et al., 2015). Using our optimised and validated protocol, we can now examine immune cells in both the MLN and SILP to obtain a deeper understanding in to key immunobiology dynamics in *H. polygyrus* infection. Our data report the presence of Tregs, Th1 and Th2 cells at both D7 and D14 of *H. polygyrus* infection in the SILP. Furthermore, we show that IL-10 expression is upregulated on numerous cell types during *H. polygyrus* infection and that the role of IL-10 may be largely tissue-based based on the expression of this cytokine.

Chapter-4 IL-10 drives tissue-based Th2 responses to helminth infection

4.1 Introduction

The type 2 effector mechanisms in the immune response to helminth infection such as mucus production, oedema and smooth muscle contractility, that are critical for helminth expulsion, are pathogenic in atopic disease such as asthma (Galli et al., 2008). Type 2 effector functions and the cytokines involved are required for helminth expulsion and subsequent remodelling and repair of tissue to restore homeostasis in the small intestine (Reynolds et al., 2012). On the other hand, dysregulation or an inappropriate Th2 response to common allergens results in tissue fibrosis and scarring, airway constriction and chronic inflammation (Galli et al., 2008). Understanding the regulation of type 2 mediated responses, in particular Th2 responses, is important in advancing the treatment of atopic disease and improving anti—helminth strategies.

IL-10 is a potent regulatory cytokine, which was initially described as a Th2 cytokine due to its ability to suppress Th1 cells (Moore et al., 2001, Fiorentino et al., 1989, Vieira et al., 1991). An increase in IL-10 is reported during *H. polygyrus* infection (Leung et al., 2012, Setiawan et al., 2007, Redpath et al., 2013, Filbey et al., 2014, Finney et al., 2007). In addition, in Figure 3-11 we demonstrate an increase in IL-10 during *H. polygyrus* infection in both the MLN and SILP. It remains unclear if increased IL-10 directly supports the Th2 response to helminth infection. However, previous studies have shown that IL-10 can enhance the activity of mast cells (Helmby and Grencis, 2003, Thompson-Snipes et al., 1991, Ghildyal et al., 1992) and promote antibody class switching (Malisan et al., 1996), both of which are key components of the type 2 immune response. There is some evidence of an underlying Th1 response during *H. polygyrus* infection (Rapin and Harris, 2018) and this will be discussed in depth in Chapter-5. Indeed, IL-10 mediated Th1 suppression may be an indirect mechanism of supporting the Th2 response, however it is currently unclear if there are direct effects of IL-10 in promoting the Th2 response. It is well established that there is an inverse relationship between IL-10 and IFNγ; IL-10 limits Th1 responses via suppression of IFNγ (Couper et al., 2008, Wilson et al., 2005, Fiorentino et al.,

1989). It also inhibits IFNγ dependent macrophage activation and disrupts the type 1 immune response to pathogens (Leng and Denkers, 2009, Gazzinelli et al., 1996, Hunter et al., 1997). On the other hand, IFNγ has been reported to limit IL-10 expression. IFNγ inhibits TLR2 dependent IL-10 expression (Hu et al., 2006). In addition, IL-10 selectively promotes the Th1 cytokine IL-12 and inhibits IL-10 in CpG stimulated bone marrow DCs (Flores et al., 2007). Intrinsic IL-10 in Th1 cells is also a critical self-limiting mechanism to prevent collateral tissue damage in response to pathogens (Hunter et al., 1997, Anderson et al., 2007). As described in section 1.5.3, the role of IL-10 in helminth infections has been reported to both inhibit and promote immune responses. These conflicting reports are most likely due to differences in parasite location, immune environment, and IL-10 concentration.

The role of IL-10 in the SILP during *H. polygyrus* is unclear and we therefore aimed to study the impact of IL-10 signalling on the Th2 response to infection. To address this question, we firstly blocked IL-10 receptor signalling during *H. polygyrus* infection and investigated the type 2 immune response. We analysed cells from the SILP, MLN and the spleen, a distal lymphoid site from the infection. We found that in the absence of IL-10R signalling, the Th2 response was significantly reduced. To determine if this was a direct effect on T cells, we measured the response of unpolarised $CD4^+$ T cells (Th0), Th1 and Th2 cells to IL-10 *in vitro*. Both Th0 and Th2 cells had increased type 2 cytokine expression upon stimulation with IL-10. To understand if Th subsets respond differently to IL-10, we measured IL-10R expression *in vitro* and how IL-10 changes the expression of its receptor. Finally, we determined if *H. polygyrus* infection resulted in changes in IL-10R expression *in vivo* and therefore IL-10 responsiveness on $IL\text{-}4^+$ "Th2" and $CXCR3^+$ "Th1" cells. In both our *in vitro* and *in vivo* systems, Th1 and Th2 subsets had differing expression of IL-10R.

4.2 Aims

- To understand the role of IL-10 signalling in the Th2 response to helminth infection

- To investigate the direct effects of IL-10 in promoting type 2 cytokines in different Th subsets *in vitro*

- To examine if Th subsets respond differently to IL-10 *in vivo* during helminth infection based on IL-10R expression

4.3 Results

4.3.1 In vivo blockade of IL-10 signalling

Our results in Figure 3-11 demonstrated an increase in IL-10 expression in both the MLN and SILP from CD45$^+$ cells at D7 of *H. polygyrus* infection. At this timepoint, CD4$^+$ T cells are migrating to the SILP and priming in the MLN is ongoing. To determine the functional importance of IL-10 in both sites, we blocked IL-10 receptor signalling, using a blocking antibody against the IL-10 receptor alpha chain, referred to as IL-10R1 (Clone 1B1.3A) (Liu et al., 1994, Liu et al., 1997, Burrack et al., 2018, Ring et al., 2019). IL-10R1 signalling was blocked at D-1, D2 and D5 of *H. polygyrus* infection to ensure that prior to and during infection IL-10R signalling was blocked. We analysed cells from the SILP,

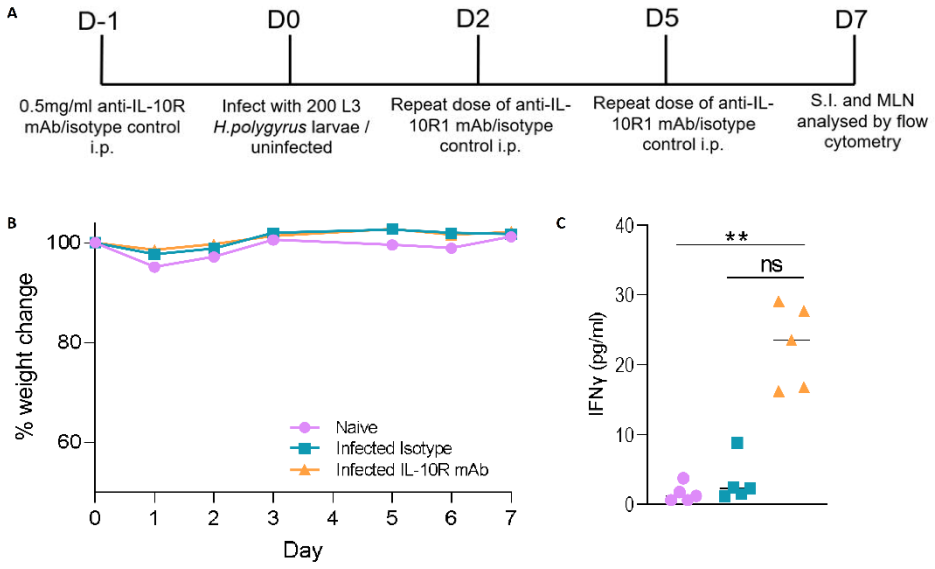


Figure 4-1 Experimental outline of IL-10R1 blockade during *H. polygyrus* infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and at D-1, D2 and D5 of infection treated with anti-IL-10R mAb or isotype control, mice were weighed daily, and 7 days post-infection serum collected for analysis. (A) Experimental outline (B) Percentage weight change of each experimental group (C) Serum concentrations (pg/ml) of IFNγ from each experimental group. Graphed data are shown with mean ± SD and are representative of 3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups (Significance $^{**}p < 0.01$).

MLN and spleen at D7 post-infection, allowing us to address the role of IL-10 during priming and on effector cells in the tissue (Figure 4-1A). To ensure there

were no non-specific effects of the IL-10R1 blockade, we used a rat IgG1 isotype control. In addition, each experimental group was weighed daily throughout the experiment to determine if blocking the IL-10R1 had any adverse effects which would result in weight loss. Percentage weight change indicated that there were no adverse effects in any experimental groups throughout the experiment (Figure 4-1B). The IL-10R1 mAb was injected intraperitoneally, resulting in a systemic signalling blockade (Ring et al., 2019). As it is well established that IL-10 inhibits IFNγ during homeostasis and during infection (Couper et al., 2008, Gazzinelli et al., 1996, Rojas et al., 2017), we therefore, used this cytokine to check our IL-10R1 blockade had worked. Serum was collected 7 days post-infection and the concentration of IFNγ measured (Figure 4-1C). In infected mice treated with the IL-10R1 blocking antibody, IFNγ increased compared to both the naïve and *H. polygyrus* isotype control groups, indicating that IL-10R signalling was successfully blocked in our system.

To determine if blocking IL-10 signalling resulted in changes in pathology of the small intestine, we assessed pathology in all experimental groups. Cross-sections of the duodenum were collected and stained with H&E (Figure 4-2A) and Alcian PAS (data not shown) for histology and goblet cells, respectively. In the small intestine of naïve mice, there was a mild multifocal to diffuse lymphocyte and plasma cell infiltrate (Figure 4-2A, top left). However, in both *H. polygyrus* infected groups there was a more mixed inflammation, consisting of lymphocytes, plasma cells, granulocytes, and macrophages (Figure 4-2A, top and bottom right). Both infected groups had variable inflammation severity depending on the area of the sample that was scored, ranging from mild and mostly mucosal at areas distal to encysted parasites (Figure 4-2A, bottom left) to severe and submucosal at parasite foci (Figure 4-2A, top and bottom right). In addition, inflammation in infected animals was transmural at specific foci and the local inflammatory influx at those foci infiltrated both the submucosa and muscular layer. Such transmural inflammation was not observed in naïve control mice. Based on these data, at 7 days post-infection, infection is associated with increased inflammation, but only at distinct foci associated with encysted larvae.

When comparing *H. polygyrus* infected isotype and IL-10R blockade groups, there were no significant changes in immunopathology and this is reflected in the

inflammation severity, depth, and combined scores (Figure 4-2B). From Alcian PAS staining for goblet cells there were no significant changes to goblet cell

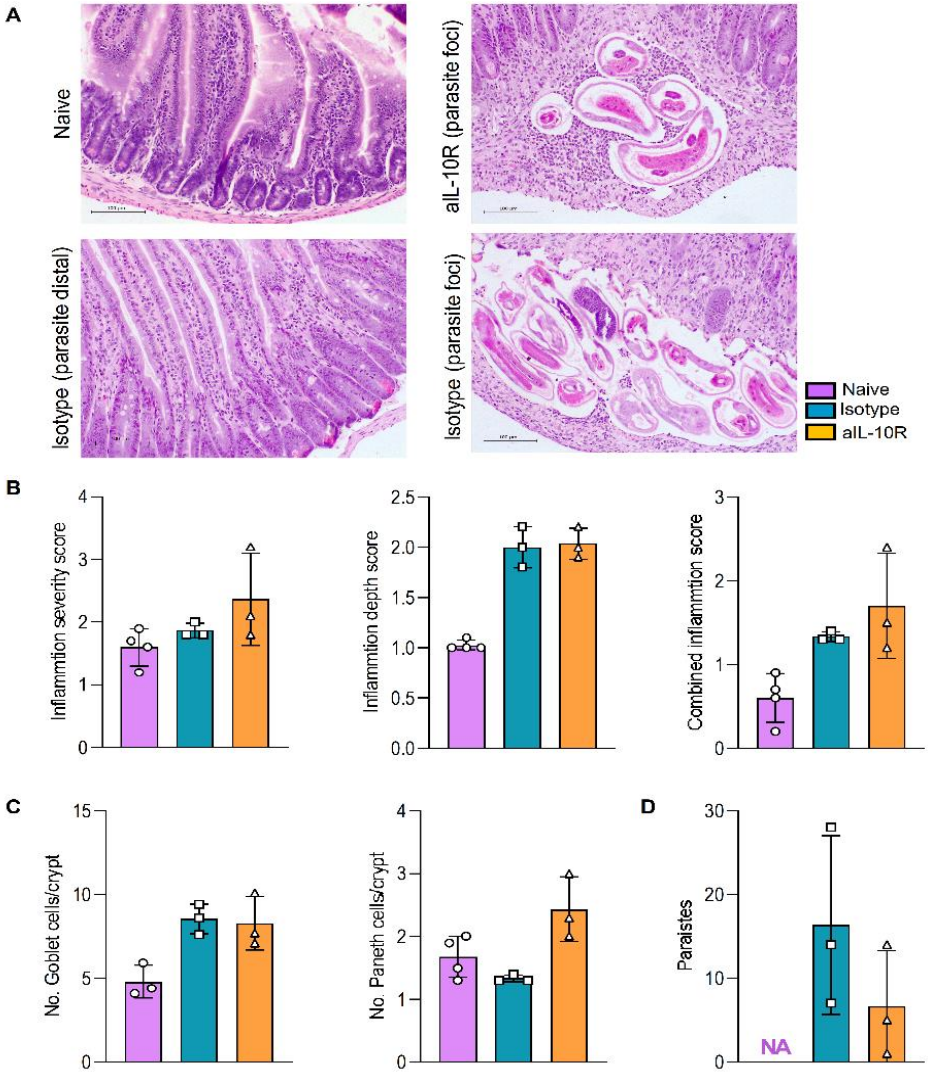
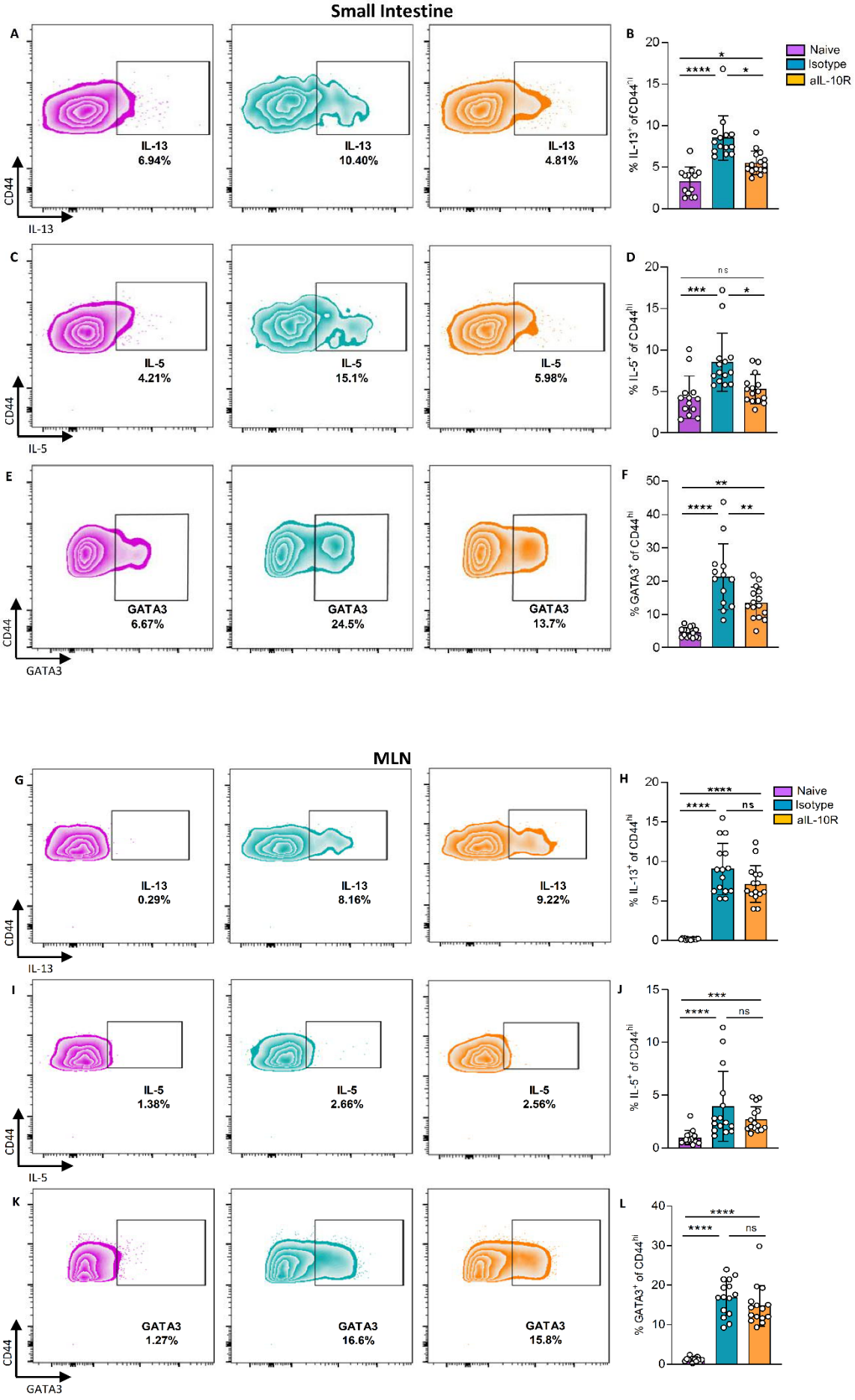


Figure 4-2 IL-10R1 blockade during *H. polygyrus* infection does not change histopathology of the duodenum
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and at D-1, D2 and D5 of infection treated with anti-IL-10R mAb or isotype control, and 7 days post-infection the small intestine collected for analysis. (A) Representative H&E staining of the duodenum from naïve (top left), anti-IL-10R mAb (top right), isotype (foci) (bottom right) and isotype (distal) (bottom left) treated mice. (B) Histology scoring of inflammation severity (left), depth (middle), combined (right). Histology scoring of number of goblet cells/per crypt (left), of Paneth cells/per crypt (middle) and number of parasites (right). Slides were scored blind by a certified pathologist from the VetPatólogos SL in Madrid, Spain. Graphed data are shown with mean ± SD and are from 1 experiment with *n*=3 per experiment.

number when comparing naïve and infected animals. In addition, there was no difference between the *H. polygyrus* infected isotype and IL-10R1 blockade groups, indicating that blocking IL-10R signalling did not alter the number of mucus producing goblet cells at this timepoint (Figure 4-2C, left). We also enumerated Paneth cells which are known to have important antimicrobial

properties and are located deep in the intestinal crypts (Bevins and Salzman, 2011). Paneth cells did not increase in small intestinal sections from the *H. polygyrus* isotype or IL-10R blockade group, compared to naïve mice (Figure 4-2C, middle). Finally, to begin to answer if blocking IL-10 signalling resulted in changes in parasite persistence, parasites encysted in the submucosa were counted in *H. polygyrus* infected isotype and IL-10R1 treated groups (Figure 4-2D). Parasites were identified in both infected groups but not in naïve mice. There was high data spread in both infected groups and this type of analysis does not account for the variability of where the worms encyst in the duodenum. Overall, these histological analyses revealed increases in immune cell infiltration and inflammation depth in infection compared to naïve mice. However, we did not observe any significant changes between our *H. polygyrus* infected isotype and IL-10R blockade groups when assessing pathology and changes in Paneth and goblet cells.

To assess the effect of IL-10 on the Th2 cytokine response, we measured the cytokines IL-5 and IL-13 and the Th2 master TF GATA3 from $CD4^+$ $TCRB^+$ $CD44^{hi}$ cells in the SILP, MLN and spleen. As expected, there was an increase in IL-13, IL-5 and GATA3 when comparing naïve and *H. polygyrus* infected isotype control groups in the SILP (Figure 4-3A-F). However, in the infected, IL-10R blockade group, there was a significant decrease in IL-5, IL-13 and GATA3 compared to infected animals treated with the isotype control (Figure 4-3B, 3D & 3F). In the MLN, a clear Th2 response was induced upon *H. polygyrus* infection compared to naïve mice, based on GATA3, IL-13 and IL-5 expression. However, unlike in the SILP a decrease in Th2 cytokines and GATA3 was not observed in the *H. polygyrus* infected IL-10R blockade group compared to infected isotype controls (Figure 4-3G-4L). Furthermore, in the spleen, a SLO not usually associated with *H. polygyrus* infection, we observed an increase in the Th2 cytokines IL-13 and IL-5 but no significant changes in $GATA3^+$ cells or between isotype and IL-10R blockade groups (Figure 4-3M-5R). *H. polygyrus* infects the small intestine and the priming response to this infection occurs in the MLN. However, in keeping


Small Intestine
A
IL-13
6.94%
IL-13
10.40%
IL-13
4.81%
CD44
IL-13
B
% IL-13+ of CD44hi
Naive
Isotype
aIL-10R
C
IL-5
4.21%
IL-5
15.1%
IL-5
5.98%
IL-5
D
% IL-5+ of CD44hi
ns
E
GATA3
6.67%
GATA3
24.5%
GATA3
13.7%
GATA3
F
% GATA3+ of CD44hi
MLN
G
IL-13
0.29%
IL-13
8.16%
IL-13
9.22%
H
% IL-13+ of CD44hi
I
IL-5
1.38%
IL-5
2.66%
IL-5
2.56%
J
% IL-5+ of CD44hi
K
GATA3
1.27%
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15.8%
L
% GATA3+ of CD44hi

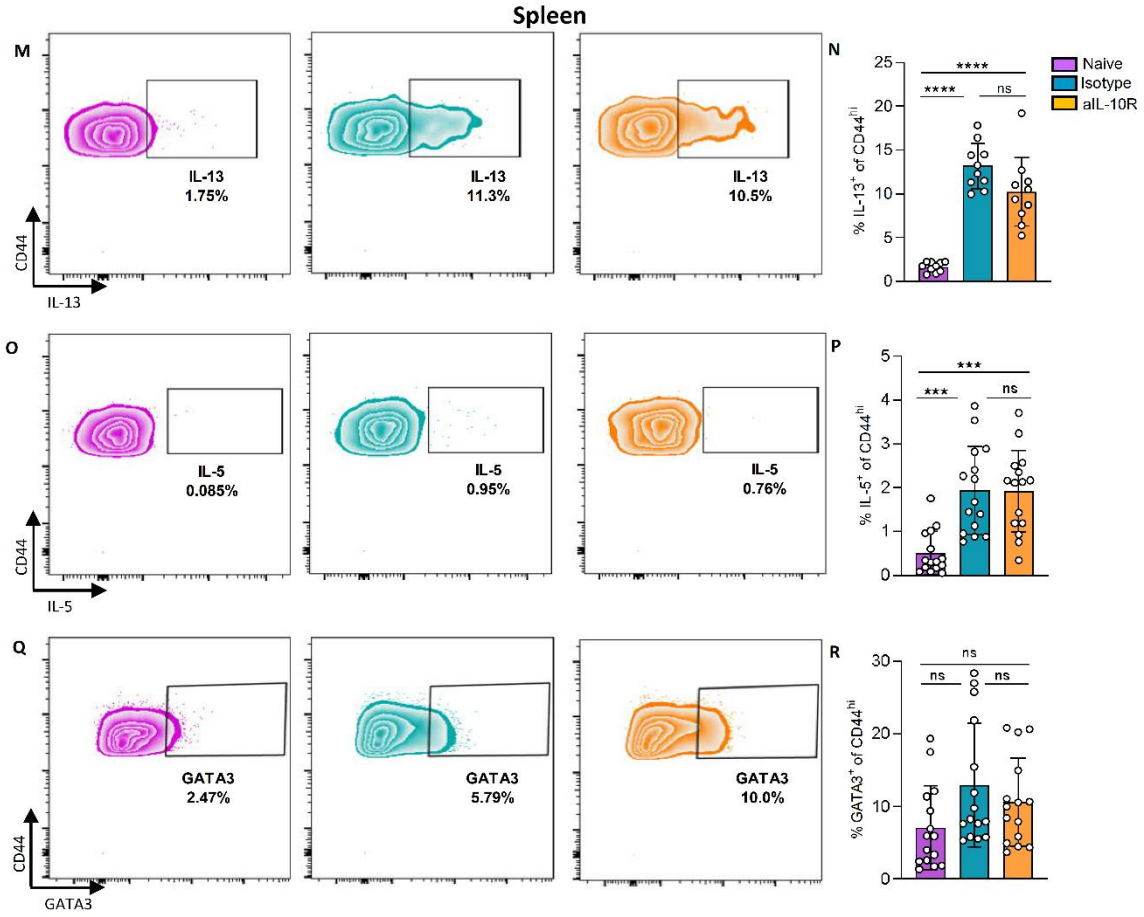

Figure 4-3 Blocking IL-10R1 signalling results in a decreased Th2 response in the small intestine during *H. polygyrus* infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and at D-1, D2 and D5 of infection treated with anti-IL-10R mAb or isotype control, *and* 7 days post-infection the small intestine, MLN and spleen collect for analysis. Top panel (small intestine), representative flow plots of (A) IL-13$^+$ (C) IL-5$^+$ (E) GATA3$^+$ staining from CD4$^+$ CD44^{hi} T cells in each experimental group in the SILP. Percentage of (B) IL-13$^+$ (D) IL-5$^+$ (F) GATA3$^+$ of CD4$^+$ CD44^{hi} in the SILP. Middle panel (MLN), Representative flow plots of (G) IL-13$^+$ (I) IL-5$^+$ (K) GATA3$^+$ staining from CD4$^+$ CD44^{hi} T cells in each experimental group in the MLN. Percentage of (H) IL-13$^+$ (J) IL-5$^+$ (L) GATA3$^+$ of CD4$^+$ CD44^{hi} in the MLN Bottom panel (spleen), Representative flow plots of (M) IL-13$^+$ (O) IL-5$^+$ (Q) GATA3$^+$ staining from CD4$^+$ CD44^{hi} T cells in each experimental group in the spleen. Percentage of (N) IL-13$^+$ (P) IL-5$^+$ (R) GATA3$^+$ of CD4$^+$ CD44^{hi} in the spleen. Gating for IL-13$^+$, IL-5$^+$ GATA3$^+$ cells was carried out using appropriate isotype controls. Graphed data are shown with mean ± SD and are pooled from 2-3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by ANOVA followed by a Tukey's post-test for multiple comparisons between groups where data were normally distributed (F, H, L, N) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups where data were not normally distributed (B, D, J, P, R) (Significance *p<0.05, **p< 0.01, ***p< 0.001, ****p< .0001).

with our data, Th2 cells have previously been reported in the spleen during *H. polygyrus* infection (Mohrs et al., 2005a). In addition, as has previously been reported and as we show in Figure 3-1B splenomegaly occurs during *H. polygyrus* infection (Brailsford and Mapes, 1987). Overall, these data suggest that IL-10 is important for maintaining an optimal Th2 response specifically in the SILP during *H. polygyrus* infection.

To validate our results, we analysed cytokine gene expression in the duodenum in our three experimental groups (Figure 4-4). Strikingly, both IL-13 and IL-5 gene expression in IL-10R blockade infected animals significantly decreased

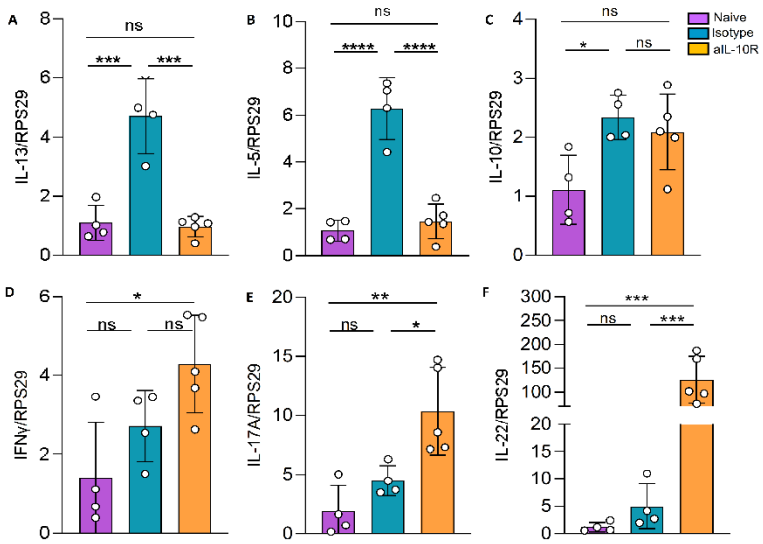

Figure 4-4 Blocking IL-10R signalling results in decreased type 2 gene expression in the duodenum during *H. polygyrus* infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and at D-1, D2 and D5 of infection treated with anti-IL-10R mAb or isotype control, and 7 days post-infection the small intestine collect for analysis. Fold change of (A) IL-13 (B), IL-5 (C), IL-10 (D) IFNγ (E), IL-17A and (F) IL-22 in the duodenum compared to housekeeping gene (RPS29). Graphed data are shown with mean ± SD and are representative of 2-3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by ANOVA followed by a Tukey's post-test for multiple comparisons between groups (Significance *p<0.05, **p< 0.01, ***p< 0.001, ****p< .0001).

compared to infected isotype controls (Figure 4-4A & B), supporting our previous observations that IL-10 is key for driving Th2 cytokine responses during *H. polygyrus* infection. IL-10 gene expression was not altered with IL-10R blockade but increased upon infection as previously described (Figure 4-4C, Figure 3-11)

We also measured genes associated with other Th subsets, IL-17A and IFNγ for Th17 and Th1 respectively (Figure 4-4D & 4E). In our *H. polygyrus* IL-10R blockade group, IL-17A increased significantly and IFNγ trended towards an increased compared to infected isotype controls, but this was not significant. These data suggest that the reduction in GATA3 and Th2 cytokines observed in the absence of IL-10 signalling is associated with an increase in the expression of canonical Th1 and Th17 cytokines, but whether this is a cause or consequence remains unclear and is discussed further in Chapter-5. IL-22 is a cytokine that is negatively regulated by and shares the IL-10R2 subunit with IL-10 (Zenewicz, 2018). We therefore included this cytokine in our analysis to determine if lack of

IL-10 signalling changes the expression of this cytokine. Indeed, IL-22 gene expression in the duodenum of infected animals was increased 100-fold during IL-10R1 blockade (Figure 4-4F). Taken together, our qPCR data further supported the conclusions from our T cell analysis, suggesting that IL-10 is required for an optimal Th2 response in the SILP during *H. polygyrus* infection.

4.3.2 In vitro, IL-10 promotes Th2 differentiation and cytokine release

Our IL-10R1 blockade data suggested a role for IL-10 in promoting the Th2 response to helminth infection. We therefore aimed to test direct effects of IL-10 on $CD4^+$ T cells in an *in vitro* setting. Our *in vitro* system involved isolating $CD4^+$ T cells from a naïve spleen and subsequently stimulating these cells with αCD3, αCD28 and IL-2 only (Th0 cells) and where stated, polarising cytokines, IL-12 for Th1 cells and IL-4 and anti-IFNγ for Th2 cells, were also added. After 4 days of *in vitro* activation, flow cytometry analysis of these cultures showed high $CD4^+$ purity and activation based on $CD44^{hi}$ expression (Figure 4-5).

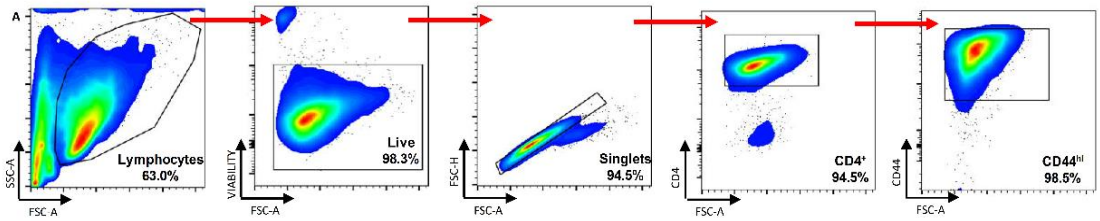

Figure 4-5 Gating strategy for *in vitro* $CD4^+$ T cell cultures
Naïve $CD4^+$ T cells were stimulated with αCD3, αCD28 and IL-2 (Th0), for 4 days and harvested for further analysis. (A) Representative gating strategy of *in vitro* activated Th0 cells.

To determine the minimum concentration of recombinant IL-10 (rIL-10) required to induce biological changes, IFNγ suppression was measured. We used IFNγ suppression as a measure of IL-10 activity as IL-10 is known to suppress IFNγ significant difference when comparing the concentration of IFNγ in 10ng/ml and 50ng/ml treated samples. We therefore used 10ng/ml of rIL-10 for all future *in vitro* experiments. Interestingly, when we measured the Th2 cytokines IL-13 and IL-5, we observed an increase in these cytokines with the addition of 10ng/ml of rIL-10 which did not increase further with 50ng/ml of rIL-10 (Figure 4-6B & 6C).

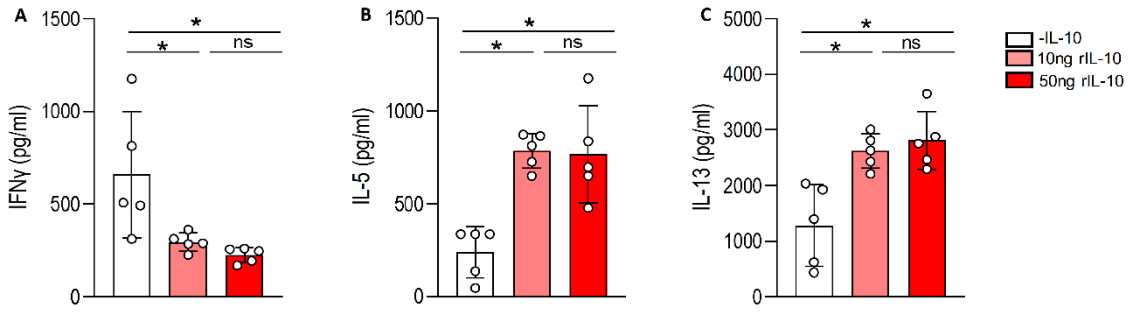

Figure 4-6 Titration of rIL-10 in CD4⁺ T cell cultures
Naïve CD4⁺ T cells were stimulated with αCD3, αCD28 and IL-2 (Th0), for 4 days with or without rIL-10 at 10ng/ml and 50ng/ml. Supernatants were collected and concentration of (A) IFNγ, (B) IL-5 and (C) IL-13 measured. Graphed data are shown with mean ± SD and are representative of 2 independent experiments with *n*=5 technical replicates per experiment. Statistical significance was calculated by ANOVA followed by a Tukey's post-test for multiple comparisons between groups where data were normally distributed (A) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups where data were not normally distributed (B&C) (Significance **p*<0.05).

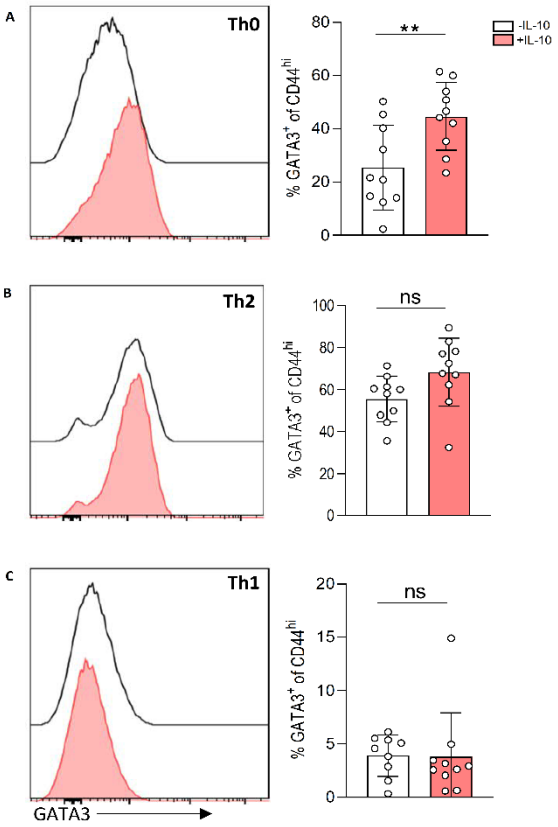

Figure 4-7 *In vitro*, IL-10 skews CD4⁺ T cells to express GATA3
Naïve CD4⁺ T cells were stimulated with αCD3, αCD28 and IL-2 (Th0) with the addition of IL-4 and anti- IFNγ for Th2 cells or IL-12 for Th1 cells, for 4 days with or without rIL-10. Representative histogram of GATA3 staining from IL-10 stimulated and unstimulated (A) Th0 cells, (B) Th2 cells and (C) Th1 cells. Percentage of CD4⁺ GATA3⁺ from (A) Th0, (B) Th2 and (C) Th1 cell cultures. Gating for GATA3⁺ cells was carried out using an appropriate isotype control. Graphed data are shown with mean ± SD and are pooled from 2 independent experiments and representative of 3 independent experiments with *n*=4-5 technical replicates per experiment. Statistical significance was calculated by Student *t* test where data were normally distributed (A&B) and Mann Whitney U test where data were not normally distributed (C) (Significance ***p*< 0.01).

Our *in vivo* data suggested that IL-10 may promote Th2 differentiation and our rIL-10 titration experiment suggested that IL-10 may achieve this by directly acting on $CD4^+$ T cells. To test this directly we investigated changes in the expression of the Th2 master TF GATA3 in our Th0 cells and also in *in vitro* polarised Th2 and Th1 cells treated with and without rIL-10 (Figure 4-7). There was a significant increase in $GATA3^+$ $CD44^{hi}$ $CD4^+$ T cells in Th0 cultures compared to untreated controls, but we did not observe the same increase in Th2 cultures (Figure 4-7A & 7B). In Th1 cultures, as expected, the percentage of $GATA3^+$ $CD44^{hi}$ $CD4^+$ T cells was low, and rIL-10 did not increase this (Figure 4-7C). These data demonstrate that rIL-10 can result in increased $GATA3^+$ expression in unpolarised cells, but not in Th1 and Th2 cells. When measuring Th2 cytokines from culture supernatants of Th0 and Th2 cultures, we found, similarly to our rIL-10 titration experiment, that Th0 cells increase their expression of Th2 cytokines with rIL-10 compared to

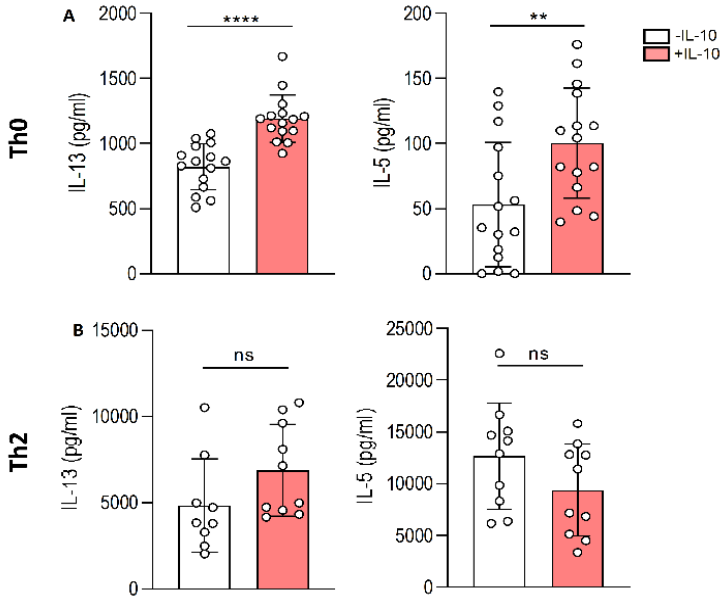

Figure 4-8 IL-10 induces type 2 cytokine expression in unpolarised $CD4^+$ T cells
Naïve $CD4^+$ T cells were stimulated with αCD3, αCD28 and IL-2 (Th0) with the addition of IL-4 and anti- IFNγ for Th2 cells, for 4 days with or without rIL-10. Supernatants were collected and concentration of IL-13 and IL-5 from (A) Th0 cultures and (B) Th2 cultures measured. Graphed data are shown with mean ± SD and are pooled from 2 independent experiments and representative of 3 independent experiments with *n*=4-5 technical replicates per experiment. Statistical significance was calculated by Student *t* test (Significance $^{**}p < 0.01$, $^{****}p < .0001$).

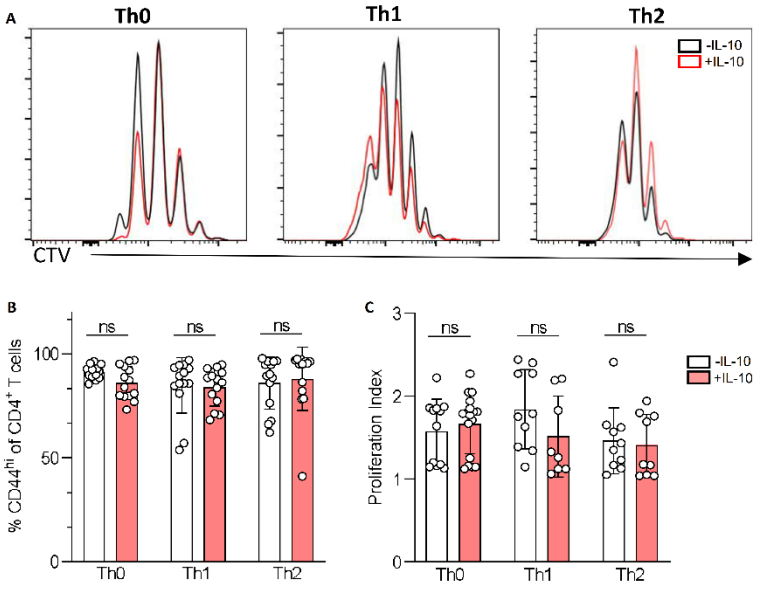

Figure 4-9 *In vitro,* IL-10 skews CD4⁺ T cells to a Th2 phenotype independently of activation and proliferation
Naïve CD4⁺ T cells were stimulated with αCD3, αCD28 and IL-2 (Th0) with the addition of IL-4 and anti- IFNγ for Th2 cells or IL-12 for Th1 cells, for 4 days with or without rIL-10. (A) Representative histograms of cell trace violet (CTV) staining from IL-10 stimulated (red) and unstimulated (black) Th0 (left), Th1 (middle) and Th2 (right) cultures. (B) Percentage of $CD44^{hi}$ CD4⁺ T cells from IL-10 stimulated and unstimulated Th0/1/2 cultures. (C) Proliferation index of CD4⁺ T cells from IL-10 stimulated and unstimulated Th0/1/2 cultures. Graphed data are shown with mean ± SD and are pooled from 2 independent experiments and representative of 3 independent experiments with *n*=4-5 technical replicates per experiment. Statistical significance was calculated by Student *t* test where data were normally distributed (B (Th0, Th2)) and Mann Whitney U test where data were not normally distributed (B (Th1), C).

untreated controls (Figure 4-8A). Th2 cytokines in Th2 cultures however did not increase (Figure 4-8B). I hypothesise that this is due to these cells already reaching their maximum cytokine output due to the extreme nature of *in vitro* polarisation.

To assess if the increase in the Th2 phenotype we observed in Th0 cultures with the addition of exogenous IL-10 was dependent on changes in activation and or proliferation of CD4⁺ T cells, we assessed CD44 expression and proliferation in Th0, Th2 and Th1 cultures with and without rIL-10 (Figure 4-9). Although proliferation varied slightly between CD4⁺ helper subsets (Figure 4-9A & 9C), we saw no significant changes in the proliferation index and in $CD44^{hi}$ CD4⁺ T cells with the addition of rIL-10. These data suggested that rIL-10 induces CD4⁺ T cell Th2 skewing independently of activation and proliferation.

I next hypothesised that due to the inverse relationship described between IL-10 and IFNγ (Fiorentino et al., 1989, Couper et al., 2008, Wilson et al., 2005), the

enhanced Th2 response we see in the presence of IL-10 may be a consequence of IFNγ suppression. To address this, we blocked IFNγ signalling in Th0 cells with or without rIL-10, and measured GATA3^{+} CD44^{hi} CD4^{+} T cells and Th2 cytokines in the supernatants (Figure 4-10). We hypothesised that the absence of IFNγ signalling would result in Th2 skewing independently of IL-10. Indeed, Th0 cells treated with anti-IFNγ alone had similar levels of both GATA3^{+} CD44^{hi} CD4^{+} T cells and Th2 cytokines to Th0 cells treated with rIL-10 alone (Figure 4-10A & 10B). Interestingly, simultaneous treatment with both rIL-10 and anti-IFNγ resulted in higher secretion of IL-13 and IL-5 than each treatment alone (Figure 4-10). These data indicate that blocking IFNγ can mimic the Th2 skewing effect of IL-10, but IL-10 still appears to have an additional role out with IFNγ suppression.

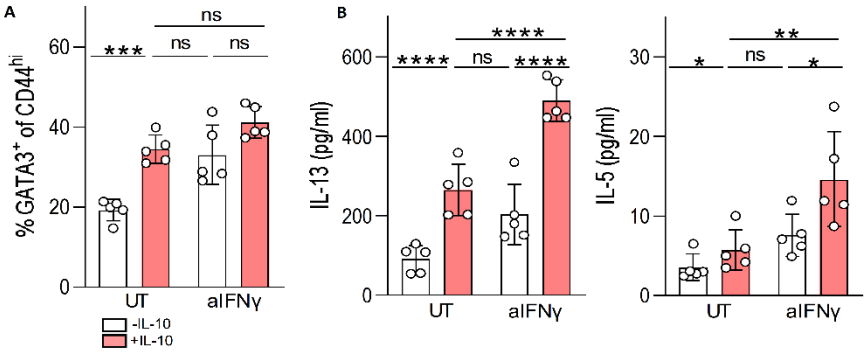

Figure 4-10 *In vitro*, Th2 induction by IL-10 is partially dependent on IFNγ suppression
Naïve CD4^{+} T cells were stimulated with αCD3, αCD28 and IL-2 (Th0), for 4 days with or without anti-IFNγ and with or without rIL-10. (A) Percentage of GATA3^{+} CD44^{hi} CD4^{+} T cells from Th0 cultures (B) Concentration (pg/ml) of IL-13 (left) and IL-5 (right) measured. UT = untreated. Gating for GATA3^{+} cells was carried out using an appropriate isotype control. Graphed data are shown with mean ± SD and representative of 1 independent experiment with n=4-5 technical replicates per experiment. Statistical significance was calculated by Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups (Significance *p<0.05, **p< 0.01, ***p< 0.001, ****p< .0001).

Taken together, our data suggest a role for IL-10 in driving Th2 polarisation that is partially dependent on the suppression of the Th1 cytokine IFNγ. We therefore hypothesised that Th1 cells may express higher levels of the IL-10R to facilitate IL-10 mediated suppression of IFNγ by these cells. To test this, we measured the expression of the IL-10R on different Th subsets and also with the addition of rIL-10 to determine if IL-10 signalling changes IL-10R expression in our *in vitro* system (Figure 4-11). Th0, Th1, Th2 cells all expressed the IL-10R, with Th2 cells having highest expression overall (Figure 4-11A-C). Upon treatment with rIL-10, IL-10R expression on both Th0 and Th1 cells decreased, whereas Th2 cells

maintained their IL-10R expression irrespective of the addition of rIL-10 (Figure 4-11B-C). These data demonstrate that in an *in vitro* setting, Th2 and Th1 cells respond differently to stimulation with rIL-10 in terms of IL-10R expression maintenance, suggesting that distinct downstream signalling pathways may occur in response to IL-10R binding in Th1 and Th2 cells.

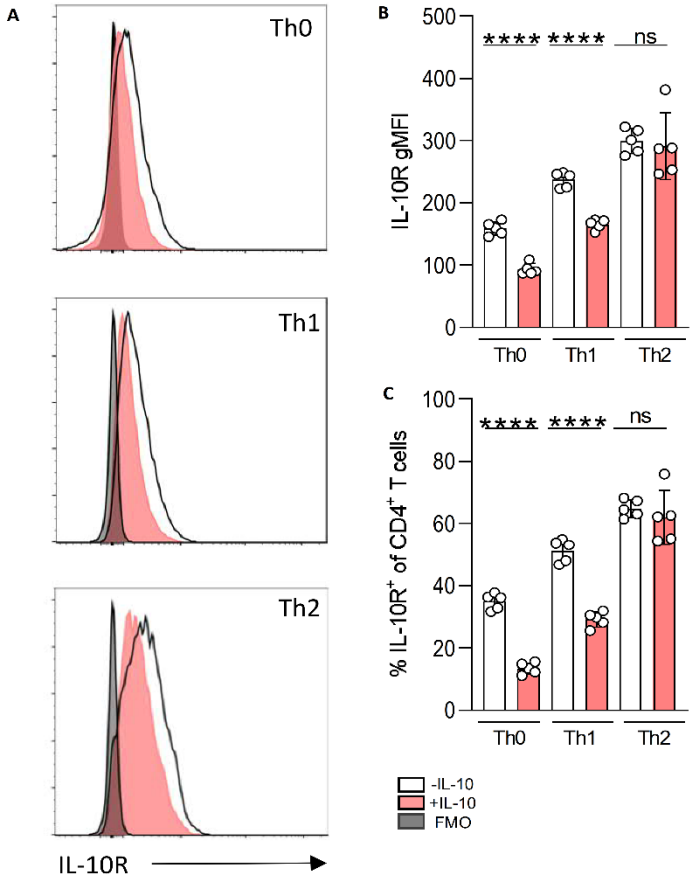

Figure 4-11 IL-10R expression on Th subsets *in vitro*
Naïve $CD4^+$ T cells were stimulated with αCD3, αCD28 and IL-2 (Th0) with the addition of IL-4 and anti- IFNγ for Th2 cells or IL-12 for Th1 cells, for 4 days with or without rIL-10. (A) Representative histograms of IL-10R staining from IL-10 stimulated and unstimulated cells from Th0/1/2 cultures compared to IL-10R FMO. (B) Geometric mean of IL-10R on Th0/1/2 cells and (C) percentage of $CD4^+$ $IL\text{-}10R^+$ of Th0/1/2 cells. Gating for $IL\text{-}10R^+$ cells was carried out using an appropriate FMO control. Graphed data are shown with mean ± SD and are pooled from 1 (panel A) or 3 (panels B & C) independent experiments with *n*=4-5 technical replicates per experiment. Statistical significance was calculated by Student *t* test (Significance ****p< .0001).

4.3.3 Th1 cells have an increased capacity of IL-10 responsiveness in vivo

Our previous *in vitro* data suggested that IL-10 can promote the Th2 response, at least partially due to suppression of IFNγ. In addition, Th1 cells which produce IFNγ at high levels may respond differently to IL-10 compared to Th2 cells based on IL-10R expression by these cells *in vitro*. We therefore aimed to assess the

responsiveness of Th1 and Th2 cells to IL-10 in vivo, based on IL-10R expression 7 days post *H. polygyrus* infection. To identify Th1 and Th2 cells *in vivo*, we considered TF or cytokine staining, but both methods involve harsh stimulation and/or fixation, which can downregulate some cell surface receptors such as cytokine receptors. To accurately measure IL-10R expression on Th1 and Th2

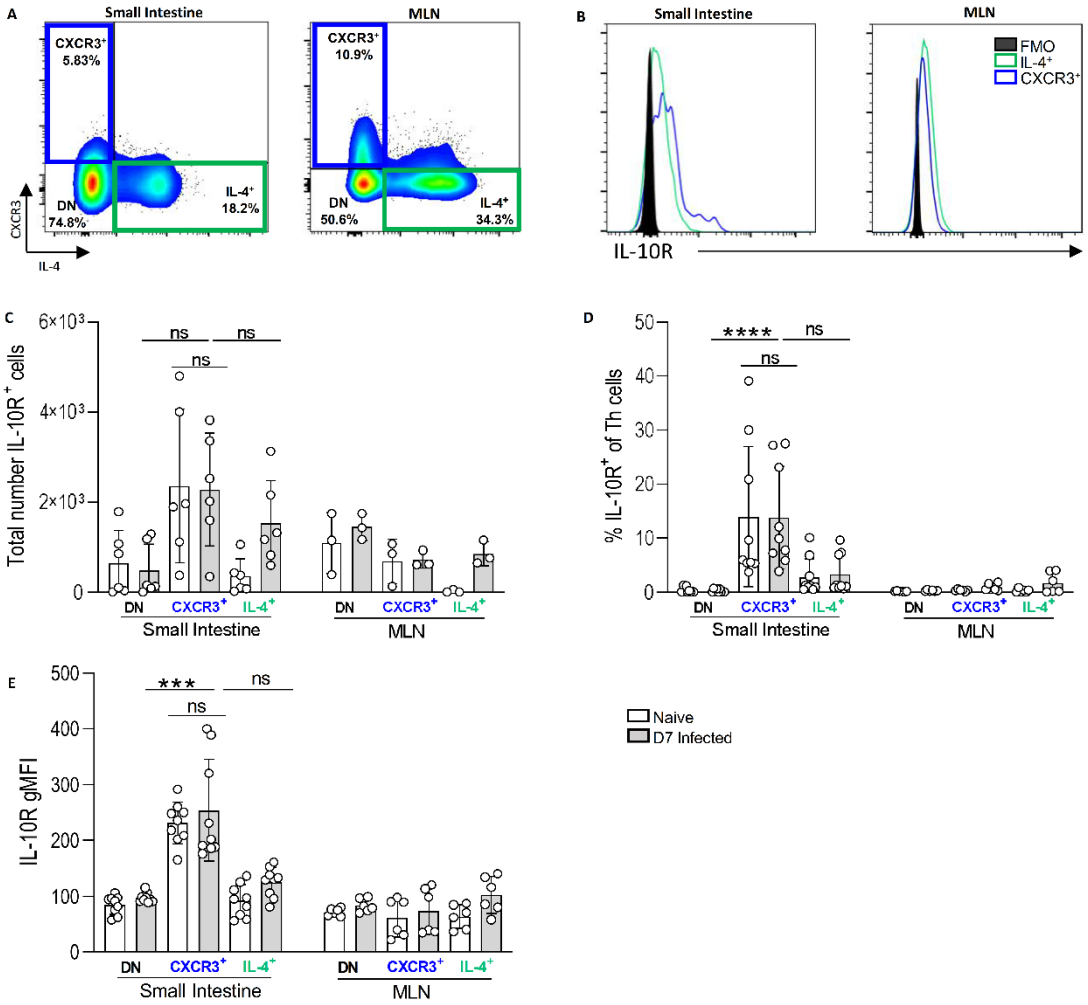

Figure 4-12 IL-10R expression is higher n CXCR3⁺ CD4⁺ T cells compared to IL-4⁺ CD4⁺ T in the SILP

B6 4get mice were infected with 200 L3 *H. polygyrus* and 7 days post-infection the small intestine and MLN removed. (A) Representative flow plots of CXCR3 and IL-4 staining of $CD44^{hi}$ $CD4^+$ T cells in the small intestine (left) and MLN (right). (B) Representative overlaid histograms of IL-10R expression of $IL\text{-}4^+$ and $CXCR3^+$ Th cells compared to IL-10R FMO. (C) Total number of IL-10R expressing CXCR3, IL-4 and DN (double negative) $CD4^+$ T cells in the small intestine and MLN. (D) Percentage of $IL\text{-}10^+$ cells of DN, $IL\text{-}4^+$ and $CXCR3^+$ Th cells in the MLN and small intestine. (E) Geometric mean of IL-10R expression of DN, $IL\text{-}4^+$ and $CXCR3^+$ Th cells in the MLN and small intestine. Gating for $IL\text{-}10R^+$ cells was carried out using an appropriate FMO control. Graphed data are shown with mean ± SD and are pooled from 3 independent experiments with n=3 per experiment. Statistical significance was calculated by Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups (Significance $^{**}p< 0.01$, $^{***}p< 0.001$, $^{****}p< .0001$).

cells in the SILP and MLN, we used the surface marker CXCR3 and IL-4 GFP reporter mice (Mohrs et al., 2005b) to identify Th1 and Th2 cells respectively (Figure 4-12A & 12B). Interestingly, in the SILP, $CXCR3^+$ $CD4^+$ T cells had significantly higher expression of the IL-10R compared to DN cells, both in frequency and intensity, a similar trend was also observed when comparing $CXCR3^+$ and $IL\text{-}4^+$ of $CD4^+$ T cells (Figure 4-12A-12E). Higher expression of the IL-10R on $CXCR3^+$ $CD4^+$ T cells was observed in cells from both naïve and infected mice (Figure 4-12A-12E). In addition, in both naïve and infected mice IL-10R expression on both $CXCR3^+$ and $IL\text{-}4^+$ $CD4^+$ T cells was higher in the SILP compared to the MLN (Figure 4-12A-12E). This is in keeping with our previous data that show that IL-10 secretion is higher in the SILP compared to the MLN (Figure 3-11). These data suggest that high IL-10R expression is a feature of $CXCR3^+$ $CD4^+$ T cells in the SILP, suggesting that the primary target of IL-10 signalling during homeostasis and helminth infection may be Th1 cells in the SILP.

4.4 Discussion

The regulation of type 2 immune responses is key for understanding numerous diseases and infections. The most prominent being helminth infection, atopy, and allergy. The role of the regulatory cytokine IL-10 in the orchestration of the Th2 response remains unclear. IL-10 is reported to suppress the Th2 response to schistosomiasis but is not implicated in Th2 suppression during *H. polygyrus* infection (Smith et al., 2016, Hoffmann et al., 2000). In addition, IL-10 has been reported to both suppress and promote Th2 responses in models of allergy and asthma (Hoffmann et al., 2000, Hawrylowicz and O'garra, 2005). However, our data demonstrate an important role for IL-10 in promoting the Th2 response in the SILP to *H. polygyrus* and that this tissue-based role of IL-10 may be, in part, due to IL-10 mediated suppression of Th1 cells and IFNγ signalling.

4.4.1 The role of IL-10R signalling in the Th2 response to H. polygyrus infection

By blocking IL-10R signalling prior to and throughout the first 7 days of *H. polygyrus* infection we could assess the role of IL-10 in both Th cell priming in the MLN and subsequent effector functions in the SILP. This time-specific and

short blockade of IL-10R signalling was a preferable system to using IL-10R$^{-/-}$ or IL-10$^{-/-}$ mice, both of which develop spontaneous colitis. Using either of these strains of mice would have skewed our results due to the severe inflammation and damage that occurs in the intestine (Kühn et al., 1993, Spencer et al., 1998). Interestingly, infecting IL-10$^{-/-}$ mice, which develop spontaneous colitis 1-3 months after birth, with *H. polygyrus* promotes the resolution of inflammation (Elliott et al., 2004). Our experimental setup did not include naïve mice treated with the IL-10R blockade. However, unlike IL-10$^{-/-}$ mice, (Kühn et al., 1993), our IL-10R1 is blockade is not prolonged and we hypothesise, would have no adverse effects on naïve mice. However, as demonstrated in IL-10$^{-/-}$ mice, IL-10 is also critical for immune homeostasis out with infection (Kühn et al., 1993), and perhaps removing IL-10 even for a short period of time may have resulted in a change in the balance of homeostatic Th1/Th2 cells. An important component of our system was to ensure the IL-10R blockade had worked systemically. The inverse relationship between IL-10 and IFNγ, described in section 4.1 of this chapter, made IFNγ a good candidate to assess the efficacy of our blockade. In the absence of systemic IL-10R signalling, we hypothesised that IFNγ would increase. Indeed, we found that in our IL-10R blockade group IFNγ increased significantly in the serum at 7 days post-infection. We had also hypothesised that due to the IL-10R mAb binding to the IL-10R, we would see an accumulation of IL-10 in the serum. However, cytokine concentrations in the serum were low and IL-10 could not be detected in the serum of any experimental groups. The increase in IL-17A gene expression observed in the infected IL-10R blockade group gave us further confidence in the effectiveness of our blockade, as previous studies using the IL-10R1 mAb also observed an increase in IL-17A (Kullberg et al., 2006). Furthermore, the dose of IL-10R1 mAb used per injection in the literature ranges from 0.2-1mg/ml, we used 0.5mg/ml which fits well in that range (Ring et al., 2019, Burrack et al., 2018, Kullberg et al., 2006, Bai et al., 2009, Brooks et al., 2008). These factors and our data combined indicate that IL-10R1 signalling was successfully blocked in our system.

IL-10$^{-/-}$ mice have abnormal intestinal histopathology (Kühn et al., 1993), we saw no changes to histopathology in the duodenum in infected mice in the absence of IL-10R signalling compared to the isotype control. This may be in part due to the protective effects of *H. polygyrus* described in IL-10$^{-/-}$ mice (Elliott et al., 2004). We hypothesise that this may also be due to the mild and short-term

nature of our blockade, however, to fully answer this question we would require the additional experimental group, where uninfected mice are treated with the IL-10R blockade. In addition, we examined specialised IECs by histology and found that despite their known contribution to mucus production for the expulsion of helminths, there were no changes in the number of goblet cells in our analysis. The Th2 cytokine IL-13 promotes goblet cell hyperplasia and due to the decrease in this cytokine observed in infected mice treated with the aIL-10R blockade, we hypothesised this would have resulted in a decrease in goblet cells (Artis et al., 2004). One explanation for the lack of change in goblet cells is the increase in IL-22 observed in our infected aIL-10R treated group, as this cytokine is also key for goblet cell hyperplasia and there may be redundancy between IL-22 and IL-13 (Turner et al., 2013). In addition, goblet cell hyperplasia typically peaks at around D14 of *H. polygyrus* infection (Sánchez-Quintero et al., 2019), so the timepoint of our analysis may also account for the lack of changes in these cells. Furthermore, Alcian PAS-stained sections were not of the best quality. The sections for this analysis were not cut at the perfect longitudinal angle, resulting in the obscuring of villi length. Goblet cells per 100μm of villi is a common method for quantifying goblet cells and we felt that our slides might not give the best representation of data as we could not carry out this type of analysis (Sánchez-Quintero et al., 2019, Wellington et al., 2020). IL-10 signalling is important for the integrity of Paneth cells in the intestine (Berkowitz et al., 2019). However, our data show no significant changes to Paneth cell number when comparing both naïve and infected mice and infected isotype and IL-10R blockade treated mice.

The decrease in Th2 cytokines observed when IL-10R signalling is blocked suggests that parasite persistence would increase due to the decrease in the Th2 response. Encysted parasites could be visualised in our H&E-stained slides and were enumerated. There were no significant changes between our infected isotype and aIL-10R treated mice in the number of encysted parasites, although it is important to note that these sections are of the top 1cm of the duodenum and encysted larvae occur along the entirety of the duodenum and top of the jejunum (Figure 3-1A). To address this question fully, visualising the entire small intestine is key. This could be achieved using the swiss roll technique which would allow for all encysted parasites to be counted (Pereira E Silva et al., 2019). A further technique to address this question would be to enumerate

visible parasites encysted in the wall of the small intestine. In addition, it would have been interesting to extend our system to 14 days post-infection. This would allow us to carry out adult worm counts in the intestine and give a better understanding if IL-10R signalling impacts parasite expulsion. The literature surrounding the role of IL-10 in parasite expulsion is conflicting. In *T. spiralis* infection, IL-10 derived from eosinophils indirectly suppresses nitric oxide and this promotes parasite persistence (Huang et al., 2014). Whereas in *T. muris*, IL-10 is required for parasite resistance and the survival of the host (Schopf et al., 2002). Overall, our histology data does not show any changes in histopathology as a result of blocking IL-10 signalling at D7 of *H. polygyrus* infection.

Although we did not observe any changes to intestinal pathology in helminth infected mice treated with aIL-10R blockade, we do report a decrease in the Th2 response to infection. The role for IL-10 in promoting the Th2 response is unclear. There is evidence that IL-10 has the capacity to suppress Th2 cells in human allergen immunotherapy (Golebski et al., 2021). However, there is also increasing data to support the concept of IL-10 promoting Th2 cells. A role for TLR-2 induced IL-10 in promoting Th2 responses in asthma has been described (Hu et al., 2006). Recently, a direct IL-10 dependent induction of the TF STAT3 and subsequently Blimp-1 was shown to be critical for Th2 development in the type 2 immune response to asthma in the lung (He et al., 2020). By measuring both GATA3 expression and the expression of the type 2 cytokines IL-13 and IL-5 from the SILP, MLN and spleen we were able to accurately assess the role of IL-10R signalling in the Th2 response to *H. polygyrus*. The effector cytokines IL-13 and IL-5 are critical for the weep and sweep response, described in section 1.3, in the SILP during *H. polygyrus* infection. This response is required for helminth expulsion and these cytokines are highly expressed in the infected tissue (Anthony et al., 2007, Maizels et al., 2009, Liang et al., 2011). IL-4 is also a critical cytokine for both priming of Th2 cells in the MLN and as an effector cytokine in the SILP (Le Gros et al., 1990, Swain et al., 1990, Redpath et al., 2015, Liang et al., 2011). However, IL-4 was not measured in these experiments as staining for this cytokine was poor despite optimisation. From these data we demonstrate a SILP specific effect of IL-10 in promoting the Th2 response. We saw a significant decrease in all Th2 markers in the SILP with IL-10R blockade during infection, and this was not observed in the MLN or the spleen. These observations were validated further when measuring gene expression of both IL-

13 and IL-5 in the duodenum, where levels of these genes were comparable to naïve mice in our IL-10R blockade group. Together, these data show a Th2 promoting effect of IL-10 in the SILP during *H. polygyrus* infection. To further elucidate the mechanism behind this, investigating Blimp-1 expression in Th2 cells would be interesting. In asthma, activation of Blimp-1 by IL-10 promoted Th2 cell development (He et al., 2020), and we hypothesise a similar mechanism may apply in the SILP. Although our data highlight a role for IL-10 in promoting the Th2 response to *H. polygyrus* infection, I hypothesise there will never be a definitive suppressing or promoting role of IL-10 that applies to all Th2 responses. This is due to the context of IL-10 signalling being important to its subsequent immunological role.

We have demonstrated a role for IL-10 in promoting Th2 cells in the SILP, we next considered if blocking IL-10 signalling would impact other important tissue cytokine responses such as Th1, Th17 and pro-repair responses. Interestingly, gene expression of both IL-17 and IFNγ increased slightly, indicating that a loss of Th2 cells allows for the expansion of other Th subsets. However, whether this is an existing underlying response that is suppressed by IL-10 or a consequence of IL-10 blockade is unclear and will be discussed further in Chapter-5. As IL-10 and IL-22 share the IL-10R2 and both play important but separate roles in maintaining epithelial barrier integrity (Morhardt et al., 2019, Gao and Xiang, 2019) we also measured gene expression of IL-22. IL-22 promotes barrier integrity by acting directly on epithelial cells as opposed to immune cells (Zenewicz, 2018). IL-22 can be pro and anti-inflammatory in different settings; co-expression with IL-17 can be pathogenic (Wei et al., 2020, Rutz et al., 2013). The striking increase in IL-22 in the IL-10R blockade group suggests there may be some redundancy between these cytokines in maintaining epithelial barrier integrity, in addition it may also indicate a pathogenic Th17 response in the absence of IL-10R1 signalling. Furthermore, IL-22 has also been shown to be key for goblet cell hyperplasia in *N. brasiliensis* infection and IL-13 is also a critical promoter of goblet cell hyperplasia (Turner et al., 2013, Artis et al., 2004). Perhaps the increase in IL-22 we observe may be to compensate for the decrease of IL-13 in infected mice treated with the IL-10R blockade. Therefore, blocking IL-10R signalling during *H. polygyrus* infection may allow other tissue cytokine responses perhaps by Th1 and Th17 cells to increase. Although further studies are required to investigate if these cytokine signatures are $CD4^+$ T cell derived

and if they are reflected at the protein level. In addition, our data suggest that the lack of IL-10 signalling may promote other cytokines such as IL-22, although the role of this cytokine in this context remains unclear.

Overall, these data demonstrate an important role for IL-10 in not only promoting Th2 cells but supporting the entire type 2 immune response in the duodenum. In addition, we have shown that the role of IL-10 may be tissue or context specific and investigating both infected tissue and priming lymph nodes is key for a complete understanding of an immune response to infection. In addition, our data provide some evidence of increases in cytokines which are associated with other Th subsets, often associated with bacterial responses, which may be suppressed by IL-10 during *H. polygyrus* infection. However, these data demonstrate that the precise direct and/or indirect mechanisms of IL-10 promoting Th2 cells are complex and understanding this IL-10-Th2 dynamic is key.

4.4.2 Using an in vitro system to understand IL-10 skewing of Th cells

Our previous data demonstrated a role for IL-10 in the Th2 response, however whether this effect was direct or indirect remains unclear. To examine direct effects of IL-10 on $CD4^+$ T cells independently of APCs, we set up an *in vitro* system to mimic $CD4^+$ T cell activation. $CD4^+$ T cells were activated by providing TCR stimulation with anti-CD3 antibodies, co-stimulation with anti-CD28 antibodies and administration of recombinant IL-2 (rIL-2) which supports T cell proliferation (Morgan et al., 1976, Klatzmann and Abbas, 2015). We hypothesise that the Th2 promoting function of IL-10 we observed in our *in vivo* IL-10R blockade was independent of APCs such as DCs, as IL-10 treated DCs have been reported to suppress Th2 cells rather than promote them in the context of airway inflammation (Koya et al., 2006). Interestingly, our *in vitro* data do demonstrate APC independent mechanisms of IL-10 in promoting Th2 polarisation. In much of our *in vitro* work, Th0 cells are used as "unpolarised $CD4^+$ T cells", as no Th subset polarising cytokines were added to these cultures. This system does come with caveats, strong TCR signals which are provided in this system have been reported to favour the polarisation of Th1 cells over Th2 cells (Bhattacharyya and Feng, 2020). As a result, cytokine independent

polarisation may occur in these cultures, however the IFNγ concentration in these cultures were low, with ~1ng/ml of IFNγ in Th0 cultures compared to ~300ng/ml in Th1 cultures. It is also important to discuss the extreme nature of *in vitro* polarisation of Th subsets. Polarising cytokine concentrations administered *in vitro* are higher than would be observed in an *in vivo* setting. For example, is has been estimated that IL-4 acts in vivo at around 5-500pg/ml, this is approximately 100-fold lower that the concentration of IL-4 added to a Th2 culture. Based on these data, Th0 cells may be a better representation of an *in vivo* $CD4^+$ T cell. Despite this, the addition of rIL-10 to Th2 cultures increased the expression of GATA3 further but did not increase the Th2 cytokines IL-13 and IL-5 concentrations in supernatants. This is most likely due to *in vitro* polarised Th2 cells reaching maximum cytokine output in our system.

The increase in GATA3, IL-5 and IL-13 in Th0 cells with the addition of rIL-10 demonstrated a potential direct effect of IL-10 signalling on T cells in promoting Th2 responses. IL-10 has previously been described to inhibit T cell proliferation and cytokine output in mice and humans (Del Prete et al., 1993, Couper et al., 2008, Ye et al., 2007). Considering this we measured proliferation and activation of Th0, Th2 and Th1 cells with and without the addition of rIL-10. Interestingly, there was no significant differences in either activation or proliferation between Th subsets and rIL-10 treated and untreated groups. I hypothesise that the strength of activation signals provided in our *in vitro* system are higher than those *in vivo* and so could not be altered by rL-10. Despite this, these data demonstrate that the direct Th2 promoting effects we observe with the addition of rIL-10 are mechanistically independent of both activation and proliferation.

We hypothesised that the mechanism behind the direct effect of rIL-10 on $CD4^+$ T cells, resulting in enhanced Th2 skewing, may be IFNγ dependent due to the inverse relationship between these cytokines (Couper et al., 2008, Wilson et al., 2005, Fiorentino et al., 1989). In keeping with this, blocking IFNγ in our $CD4^+$ T cell culture system showed similar Th2 skewing as adding rIL-10. Furthermore, the combined treatment of rIL-10 and blocking IFNγ resulted in higher concentrations of both IL-13 and IL-5 in Th0 cells than either treatment alone. Potential reasons for this are that in our *in vitro* system, anti- IFNγ treatment did not fully block all cytokine. However, these data could indicate that IL-10 is activating an additional, non-IFNγ dependent pathway, such as the activation of

Blimp-1 which is known to promote Th2 effector responses (He et al., 2020). Overall, these data suggest that Th2 skewing by rIL-10 is only partially dependent on the suppression of IFNγ. To assess and confirm this, simultaneous of blockade of IFNγ and IL-10r signalling during *H. polygyrus* infection would directly test the role of IL-10 mediated IFNγ suppression in supporting the Th2 response. T cell intrinsic MyD88 signalling induced by IL-18 is key for Th1 polarisation (Orr et al., 2013, Yarovinsky, 2013, Oliveira et al., 2017), and, consistent with our hypothesis, MyD88$^{-/-}$ mice infected with *H. polygyrus* have increased worm expulsion and IL-4 expression (Reynolds et al., 2014a). In addition, IFNγ$^{-/-}$ mice infected with *H. polygyrus* have reduced egg counts and worm burden, indicating an elevated Th2 response in the absence of Th1 cells and IFNγ (Reynolds and Maizels, 2012). Together these data show that reduced Th1 responses allow for the expansion of Th2 cells, supporting the idea that IL-10 suppression of IFNγ may promote the Th2 response.

4.4.3 The expression of the IL-10R as a measure of Th responsiveness to IL-10

To assess if *in vitro* and *in vivo* Th subsets respond differently to IL-10, we measured IL-10R expression on these cells. Blocking IL-10 signalling in the SILP, but not the MLN, resulted in increased Th2 cells. In keeping with this, IL-10R expression was higher overall in the SILP compared to the MLN. This is in line with our previous data that reports IL-10 expression being higher in the SILP compared to the MLN (Figure 3-11). The surface expression of the IL-10R reflects gene expression and surface binding of the cytokine, internalisation, and recycling (Cendrowski et al., 2016). I*n vitro*, when rIL-10 is added, active signalling can result in loss of surface detection of the receptors (Munitic et al., 2004, Perona-Wright et al., 2010). *In vivo*, active cytokine concentrations are lower and receptor stripping is less likely, and receptor downregulation is normally observed only in those cells that have become exhausted (Ingram et al., 2011). Furthermore, *in vivo*, the expression of the IL-10R1 is associated with IL-10 responsiveness (Shouval et al., 2014b, Liu et al., 1997). These factors are important to consider when drawing conclusions from our *in vivo* and *in vitro* IL-10R data.

We report that *in vitro* Th1 and Th0 cells downregulate IL-10R expression when treated with rIL-10 compared to Th2 cells. *In vivo*, we used CXCR3 to identify Th1 cells and B6 4get mice were used for these experiments which have GFP in IL-4 expressing cells, allowing the identification of Th2 cells (Mohrs et al., 2005b). CXCR3 is a chemokine receptor expressed by numerous cell types and is rapidly upregulated on activated T cells (Groom and Luster, 2011). CXCR3 expression only remains high on Th1 and $CD8^+$ T cells and facilitates trafficking of these effector T cells to sites of inflammation (Groom and Luster, 2011, Xie et al., 2003). For these experiments, Th cells were gated on both CD4 and TCRß expression, therefore giving a pure population of Th cells for further gating on CXCR3 and IL-4 expression. We found that in the SILP, $CXCR3^+$ "Th1" cells expressed higher levels of the IL-10R compared to IL-4^+ Th2 cells. The combination of our *in vitro* and *in vivo* data measuring IL-10R expression of Th subsets indicates that downstream effects of IL-10R signalling may be different in Th1 and Th2 cells. Further studies are required to understand the role of IL-10 signalling in different Th subsets.

In vitro experiments focusing on TF expression in Th1 and Th2 cells after treatment with IL-10, may highlight different signalling pathways in these cells. IL-10 has been reported to activate not only STAT3 but STAT5 and STAT1 also (Finbloom and Winestock, 1995, Moore et al., 2001, Weber-Nordt et al., 1996, Wehinger et al., 1996). Investigating which STATs are activated in response to IL-10R signalling in Th1 and Th2 cells may help to address the question of differences in downstream signalling in these cells. Furthermore, the TF Blimp-1 can promote Th2 polarisation and so investigating this TF in both Th1 and Th2 cells would also be interesting (He et al., 2020). As previously described, the cytokine environment at the time of IL-10R signalling is key for the immunological function of this cytokine. Perhaps, in our *in vitro* system, the presence of the polarising cytokines IL-4 and IL-12 for Th2 and Th1 cells respectively may also influence the outcome of IL-10R signalling. Assessing the expression of other cytokine receptors such as the IL-4 receptor and subsequent downstream signalling molecules induced would also contribute to unravelling IL-10R signalling in Th1 and Th2 cells. Together our data support the sensitivity of tissue-based effector cells to IL-10 signalling, and particularly intestinal Th1 cells.

4.4.4 Concluding remarks

In conclusion, we report that IL-10 promotes the Th2 response to a helminth infection in the SILP. We show that IL-10 can signal directly to $CD4^+$ T cells to increase Th2 differentiation *in vitro*, but that IL-10 production and IL-10R expression are both concentrated in the infected tissue rather than the MLN. High expression of the IL-10R by Th1 cells in the SILP indicates that these cells are sensitive to IL-10 signalling and subsequent IL-10 mediated suppression, providing an indirect mechanism for promoting the Th2 response. These data provide new insight into the complexity of tissue-based regulation during a Th2 immune response and suggest that IL-10 may be an interesting candidate for therapeutic targeting in Th2 dominated diseases such as allergy, asthma, and helminth infection.

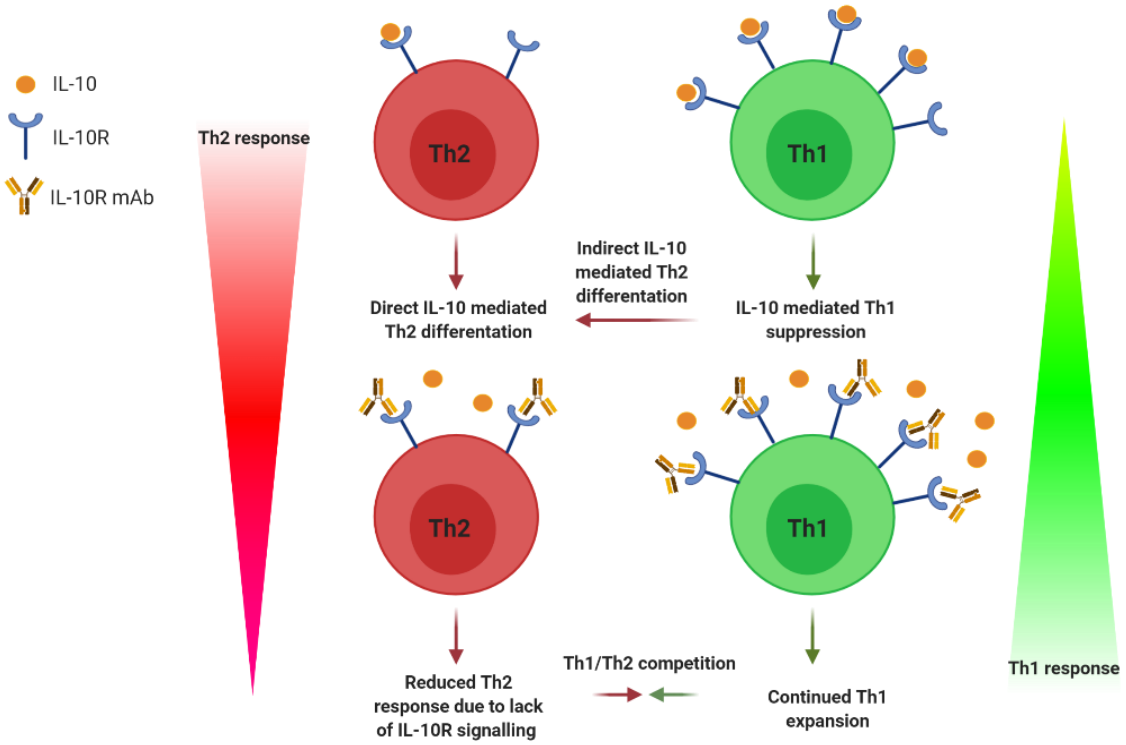

Figure 4-13 Schematic of proposed mechanism of IL-10 promoting Th2 responses

Chapter-5 Barrier breach and IFNγ responses during *H. polygyrus* infection

5.1 Introduction

The immune response to helminths is typically characterised by a strong Th2 response and the effector cytokines IL-13, IL-5 and IL-4 as previously described in section 1.3. Despite the well described Th2 response to *H. polygyrus* infection, there is some evidence in the literature suggesting IFNγ, a type 1 cytokine, may also play a role in this model (Gentile et al., 2020, Reynolds and Maizels, 2012, Filbey et al., 2014). IFNγ dependent NK cell recruitment is required for wound repair at the early stages of infection (Gentile et al., 2020). In addition, IFNγ secretion by MLN cells from *H. polygyrus* infected mice has been reported. IFNγ$^{-/-}$ mice infected with *H. polygyrus* have reduced worm burden, suggesting that IFNγ provides immune competition during infection (Reynolds and Maizels, 2012). Underlying IFNγ responses, specifically Th1 responses, are associated with the development of chronic helminth infections. Antigen-specific Th1 cells occur in susceptible mouse strains infected with *T. muris*, resulting in chronic infection (Klementowicz et al., 2012). In addition, injecting IFNγ into mice infected with *N. brasiliensis*, antagonised ILC2 responses (Moro et al., 2016). In this chapter, I aimed to characterise the timing and function of IFNγ responses to *H. polygyrus* and investigate the stimuli behind it.

Alterations of the microbiota are of interest as bacteria can drive IFNγ responses (Kaiko et al., 2008). Bacteria induced IFNγ responses require immune cell priming by bacterial antigens. Changes to barrier permeability or damage can result in translocation of bacteria or bacterial products, allowing immune cell access to these antigens (Ding et al., 2004). Helminth-induced changes in both the composition and abundance of the microbiome in the intestine have been reported. In *H. polygyrus* infection, both Lactobacillaceae and Enterobacteriaceae species are increased in the small intestine, although this change to the microbiota varies between mouse strains (Walk et al., 2010, Reynolds et al., 2014b, Rapin and Harris, 2018, Rapin et al., 2020, Rausch et al., 2013). Promotion of Lactobacillaceae species by *H. polygyrus* is reported to

increase host susceptibility to infection (Reynolds et al., 2014b). Similarly, in *T. muris* infection, there is an increase in Lactobacillaceae species and also a reduction in the diversity of the microbiota (Houlden et al., 2015). As well as changes to the microbiota, there have been a small number of reports of helminth-induced changes to tight junctions (TJs) which are required to maintain epithelial barrier integrity. *N. brasiliensis* infection alters the expression of E-cadherin resulting in loss of cell-cell adhesion in the small intestine (Hyoh et al., 1999). Co-infection with *H. polygyrus* and the pathogenic bacteria *Citrobacter rodentium* results in translocation of this bacteria to systemic tissues, suggesting an increase in the movement of luminal contents across the epithelial barrier (Chen et al., 2005). Although *H. polygyrus* infection is restricted to the small intestine, Th2 cells have been reported at distal sites, including the peritoneal cavity and omentum during infection, suggesting that *H. polygyrus* antigens may also be present at these sites or that cells home to these sites as well as the intestine (Jenkins et al., 2013, Mohrs et al., 2005a). We therefore hypothesised that bacterial antigens may also access the peritoneal cavity and omentum during *H. polygyrus* infection, and that these antigens might drive a local type 1, IFNγ-mediated immune response.

The omentum is an adipose tissue with immune properties found within the peritoneal cavity. This tissue contains clusters of leukocytes called milky spots (MS) (Meza-Perez and Randall, 2017). The MS of the omentum are exposed to antigen via the drainage of lymphatic fluid from the peritoneal cavity (Meza-Perez and Randall, 2017). This gives the omentum a filtration function for the peritoneal cavity and the capacity to generate immune responses to any pathogens or antigens found within the peritoneal cavity (Meza-Perez and Randall, 2017). In addition, mucosal homing ligands are expressed on the blood vessels of the omentum (Briskin et al., 1997, Berberich et al., 2008, Meza-Perez and Randall, 2017, Carlow et al., 2009). Lymphocytes express mucosal homing receptors, which facilitate their migration into areas where mucosal homing ligands are expressed (Bono et al., 2016, Hosoe et al., 2004, Denucci et al., 2010). As a result, immune cells that are programmed to home to the intestine may also home to the omentum and this presents an interesting question on the role of this tissue during mucosal immune responses. During *H. polygyrus* infection, Th2 cells accumulate in both the peritoneal cavity and the omentum (Rangel-Moreno et al., 2009) and similarly mice infected with influenza virus

accumulate $CD4^+$ and $CD8^+$ memory T cells at both these sites (Rangel-Moreno et al., 2009). This suggests an interesting role for the omentum and the peritoneal cavity as a homing site for mucosal immune cells from distal sites such as the intestine and lung. The potential combination of immune cells and bacterial antigens in the peritoneal cavity would have the theoretical ability to drive a type 1 component during *H. polygyrus* infection.

We aimed to unravel if an underlying type 1 response occurs during *H. polygyrus*, and whether that type 1 response is driven by translocating bacteria. We hypothesised that increased bacterial translocation may present an indirect mechanism by which IL-10 promotes Th2 responses as described in Chapter 2. To assess how strongly type 1 immunity features in *H. polygyrus* infection, we measured local IFNγ responses in both the SILP and MLN and also in the omentum, a tissue where mucosal immune cells have been reported to reside (Rangel-Moreno et al., 2009). Our initial IFNγ data suggested the possibility of an immune response initiated by bacteria or bacterial antigens. To assess intestinal permeability during *H. polygyrus* infection, faecal lipocalin-2 and albumin were measured. Furthermore, to understand how the epithelial barrier is held together during infection, TJ gene expression was analysed. Our preliminary experiments showed minimal evidence of bacterial translocation during infection, although further studies are required. In addition, our data do not provide evidence of bacterial-specific Th1 cells. Instead, our data suggest that proteins secreted by *H. polygyrus* may elicit a Th1 response. Together the data in this chapter suggest that although IFNγ secretion and alterations in TJs occur during *H. polygyrus* infection, we found little evidence of bacterial translocation. Instead IFNγ secretion may be directed against *H. polygyrus* itself, although further studies are required to address this.

5.2 Aims

- To investigate where and when IFNγ is expressed during *H. polygyrus* infection

- To determine if *H. polygyrus* infection results in a leaky intestinal barrier

- To examine if bacterial translocation and a subsequent bacteria-specific Th1 response occurs during *H. polygyrus* infection

5.3 Results

5.3.1 Expression of IFNγ during H. polygyrus infection

At the end of Chapter 3, we discussed the interactions between Th2 and Th1 cytokines, and in this chapter, we aimed to investigate the type 1 cytokine IFNγ

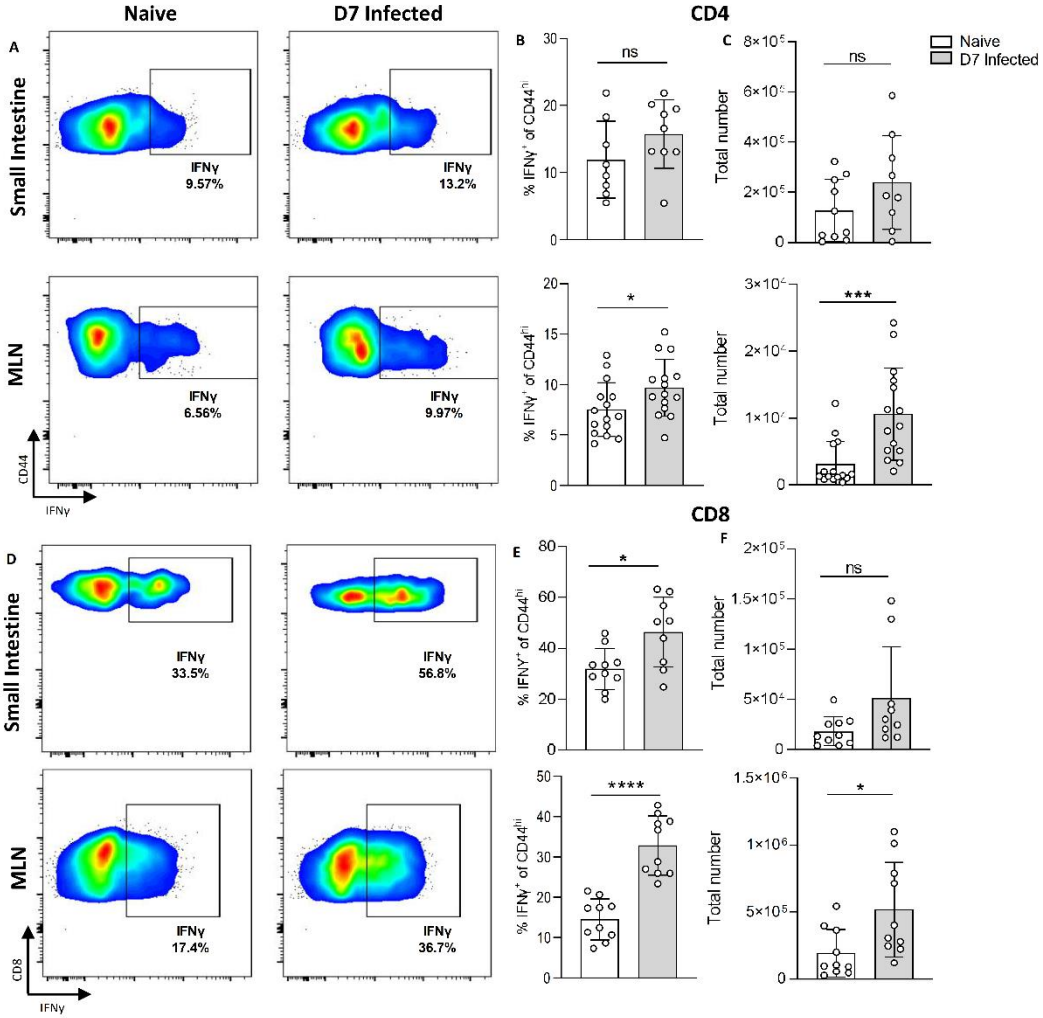

Figure 5-1 CD8⁺ T cells are the main IFNγ⁺ producing T cell subset in the MLN and SILP
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 7 days post-infection the small intestine and MLN removed. (A) Representative FACS plots of IFNγ⁺ CD4⁺ CD44^{hi} T cells from the small intestine (top) and MLN (bottom) from naïve (left) and D7 (right) infected mice. (B) % IFNγ⁺ of CD4⁺ CD44^{hi} T cells from the small intestine (top) and MLN (bottom) from naïve and D7 infected mice. (C) Total number of IFNγ⁺ CD4⁺ CD44^{hi} T cells from the small intestine (top) and MLN (bottom) from naïve and D7 infected mice. (D) Representative FACS plots of IFNγ⁺ CD8⁺ CD44^{hi} T cells from the small intestine (top) and MLN (bottom) from naïve (left) and D7 (right) infected mice. (E) percentage IFNγ⁺ of CD8⁺ CD44^{hi} T cells from the small intestine (top) and MLN (bottom) from naïve and D7 infected mice. (F) Total number of IFNγ⁺ CD8⁺ CD44^{hi} T cells from the small intestine (top) and MLN (bottom) from naïve and D7 infected mice. Gating for IFNγ⁺ cells was carried out

using an appropriate isotype control. Graphed data are shown with mean ± SD and are pooled from 3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by Mann Whitney U test where data were not normally distributed (C (MLN), F) and Student *t* test where data were normally distributed (B, C (SILP), E) (Significance **p*<0.05, ****p*< 0.001, *****p*< .0001).

during *H. polygyrus* infection further. As described previously, there has been some evidence of IFNγ being part of the immune responses triggered by *H. polygyrus* (Reynolds and Maizels, 2012, Filbey et al., 2014, Gentile et al., 2020). To investigate if this IFNγ was indicative of a T cell response, we first analysed T cell populations 7 days after *H. polygyrus* infection. IFNγ secretion by both $CD4^+$ and $CD8^+$ cells in the SILP and MLN was measured (Figure 5-1). As was previously shown in Figure 3-8, the number of Th1 cells ($TBET^+$ $CD4^+$ $TCR\beta^+$ $CD44^{hi}$) increased in the MLN and the SILP. Figure 5-1 shows that both percentage and number of $IFN\gamma^+$ $CD4^+$ $TCR\beta^+$ $CD44^{hi}$ cells again increased within the MLN but not the SILP (Figure 5-1A & B). This suggests that although $TBET^+$

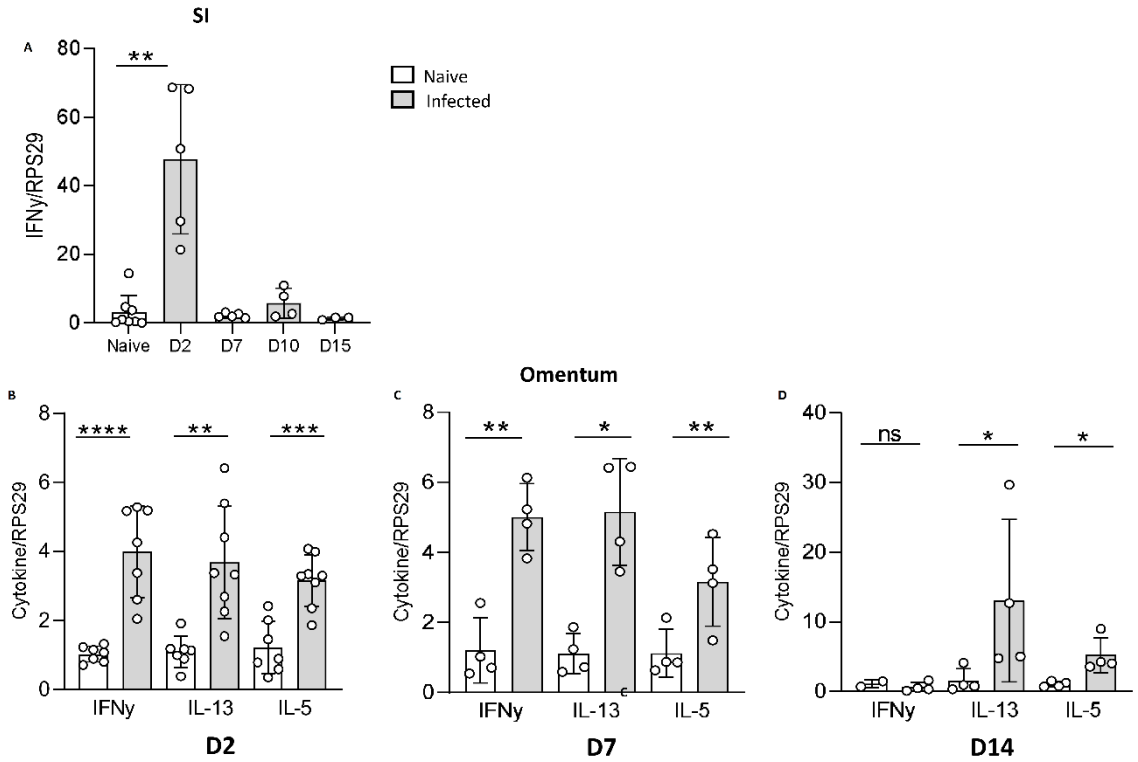

Figure 5-2 There is a spike in IFNγ gene expression in the duodenum and omentum at D2 of *H. polygyrus* infection

C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 2-, 7-, 10-, 14 and 15- days post-infection the small intestine collect for analysis. (A) Fold change of IFNγ gene expression in the duodenum at timepoints during *H. polygyrus* infection compared to housekeeping gene (RSP29) and normalised to an average of naïve samples. Samples for timepoints 10 and 15 were provided by Claire Drurey from the Maizels laboratory. Fold change of (B) IFNγ, (C) IL-13 and (D) IL-5 gene expression in the omentum compared to housekeeping gene (RPS29) and normalised to an average of naïve samples. Graphed data are shown with mean ± SD and are representative of 1-3 independent experiments with n=3-6 per experiment. Statistical significance was calculated by significance was calculated by Student *t* test where samples were normally distributed (B&C) and

Mann Whitney U test where data were not normally distributed (D) and Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups (A) (Significance *p<0.05, **p< 0.01, ***p< 0.001).

cells arrive in the SILP, they do not secrete IFNγ, and I hypothesise that this is perhaps due to IL-10 mediated suppression. When investigating $CD8^+$ T cells, there was a significant increase in percentage and number of $IFN\gamma^+$ $CD8^+$ $TCR\beta^+$ $CD44^{hi}$ cells in the MLN, and an increase in percentage, but not number, of these cells in the SILP. These data demonstrate that both $IFN\gamma^+$ $CD4^+$ and $CD8^+$ T cells expand in the MLN during *H. polygyrus* infection, but this is not reflected at the site of infection.

A recent report demonstrated a strong IFNγ signature at the earlier stages of *H. polygyrus* infection (Days 2-4) (Gentile et al., 2020). At this timepoint, IFNγ is unlikely to be T cell derived. We therefore used qPCR to capture IFNγ gene expression from all cell types. IFNγ gene expression was measured in the duodenum at early and later timepoints of *H. polygyrus* infection (Days 2, 7, 10 and 15). Samples from day 10 and 15 were kindly given to us by Claire Drurey from the Maizels laboratory, University of Glasgow. In keeping with the published data, we observed a clear spike in IFNγ gene expression at D2 in the duodenum compared to naïve mice and later timepoints (Figure 5-2). Although the IFNγ expression spike was very acute, only spiking at D2 of infection in the small intestine, when we examined surrounding tissues, such as the omentum (which is thought to filter the peritoneal cavity), we saw IFNγ expression at D2 and sustained to D7, although gone by D14 (Figure 5-2). In comparison, the Th2 tissue cytokines, IL-13, and IL-5, were also detectable in the omentum early and continued to increase till D14 (Figure 5-2). Overall, we observed an IFNγ signature from MLN T cells at D7 of *H. polygyrus* infection, which was not reflected in the SILP. We observed IFNγ gene expression in both the omentum and duodenum 2 days post-infection, which was sustained until D7 in the omentum only.

5.3.2 Changes in intestinal barrier integrity during H. polygyrus infection

As mentioned previously, a recent study observed an increase in IFNγ gene expression which was required for NK cell recruitment and subsequent limiting

of tissue damage during *H. polygyrus* infection (Gentile et al., 2020). The requirement for NK cells to limit tissue damage during infection suggests barrier breach may occur. To test this hypothesis, we aimed to assess the integrity of the intestinal barrier at D2 of infection. We used lipocalin-2 as a marker for loss of barrier integrity. Lipocalin-2 release has been well established as an indicator of intestinal leakiness and damage (Chassaing et al., 2012, Hsieh et al., 2016).

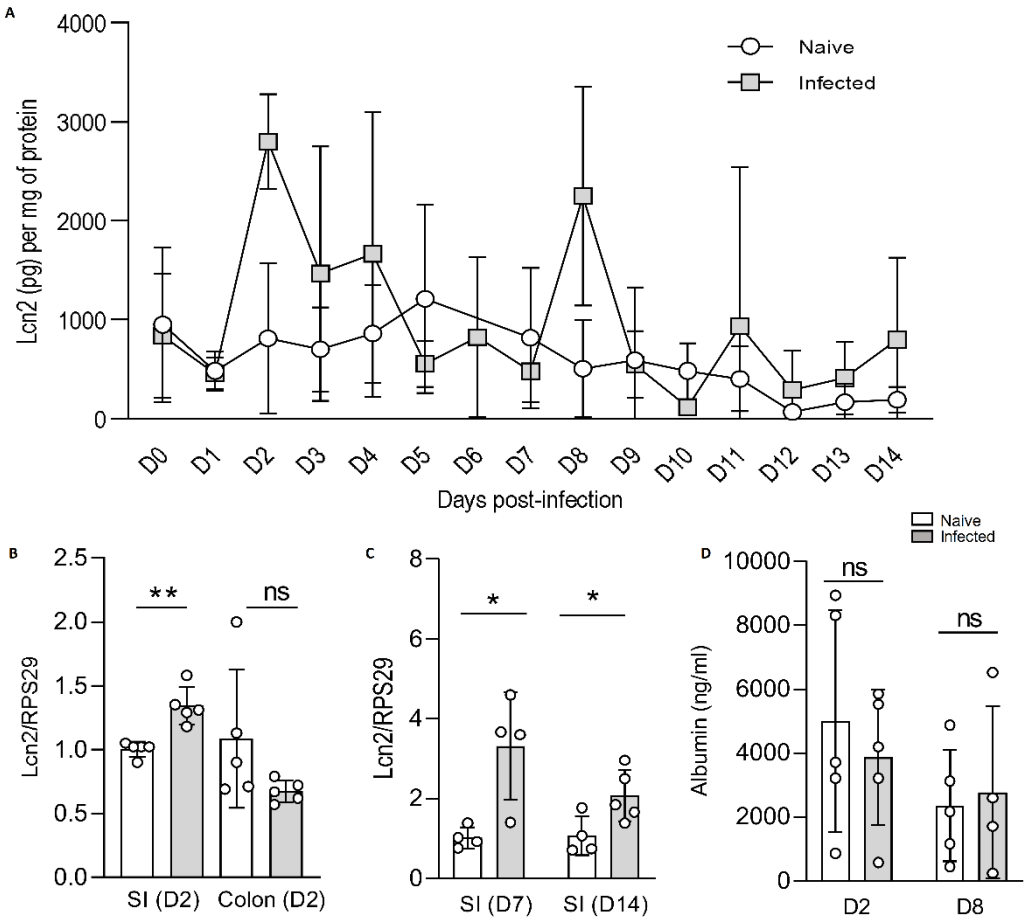

Figure 5-3 Spike in lipocalin-2 but not albumin in the faeces during *H. polygyrus* infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and fresh faecal samples collected daily for 14 days. (A) Lcn2 (pg) per mg of protein from faecal samples was measured by ELISA from naïve (circle) and *H. polygyrus* (square) infected mice. (B) Fold change of Lcn2 gene expression of in the duodenum and colon from naïve and D2 infected mice compared to housekeeping gene (RSP29) and normalised to an average of naïve samples. (C) Fold change of Lcn2 gene expression in the duodenum from naïve and D7 and D14 infected mice compared to housekeeping gene (RSP29). (D) Albumin (ng/ml) in faecal samples from naïve, D2 and D8 infected mice. Graphed data are shown with mean ± SD and are representative of 1-3 experiments with *n*=2-5 per experiment. Statistical significance was calculated by Mann Whitney U test where data were not normally distributed (B (Colon)) and Student *t* test where data were normally distributed (B (SI), C, D) (Significance *$p<0.05$, **$p< 0.01$).

We measured faecal lipocalin-2 daily over a 14-day infection with *H. polygyrus* to determine if intestinal leakiness occured throughout infection (Figure 5-3). Of interest, faecal samples taken from a mouse undergoing an acute, DSS-induced

colitis (provided by Madeline White from the Maizels laboratory, University of Glasgow), gave Lcn2 protein measurements higher than the detection limit of the ELISA. There was a clear increase in faecal lipocalin-2 at day 2 of *H. polygyrus* infection compared to naïve controls (Figure 5-3A). In addition, another peak was observed at day 8. These two timepoints match the days when *H. polygyrus* migrates through the wall of the gut (Figure 1-2). We further

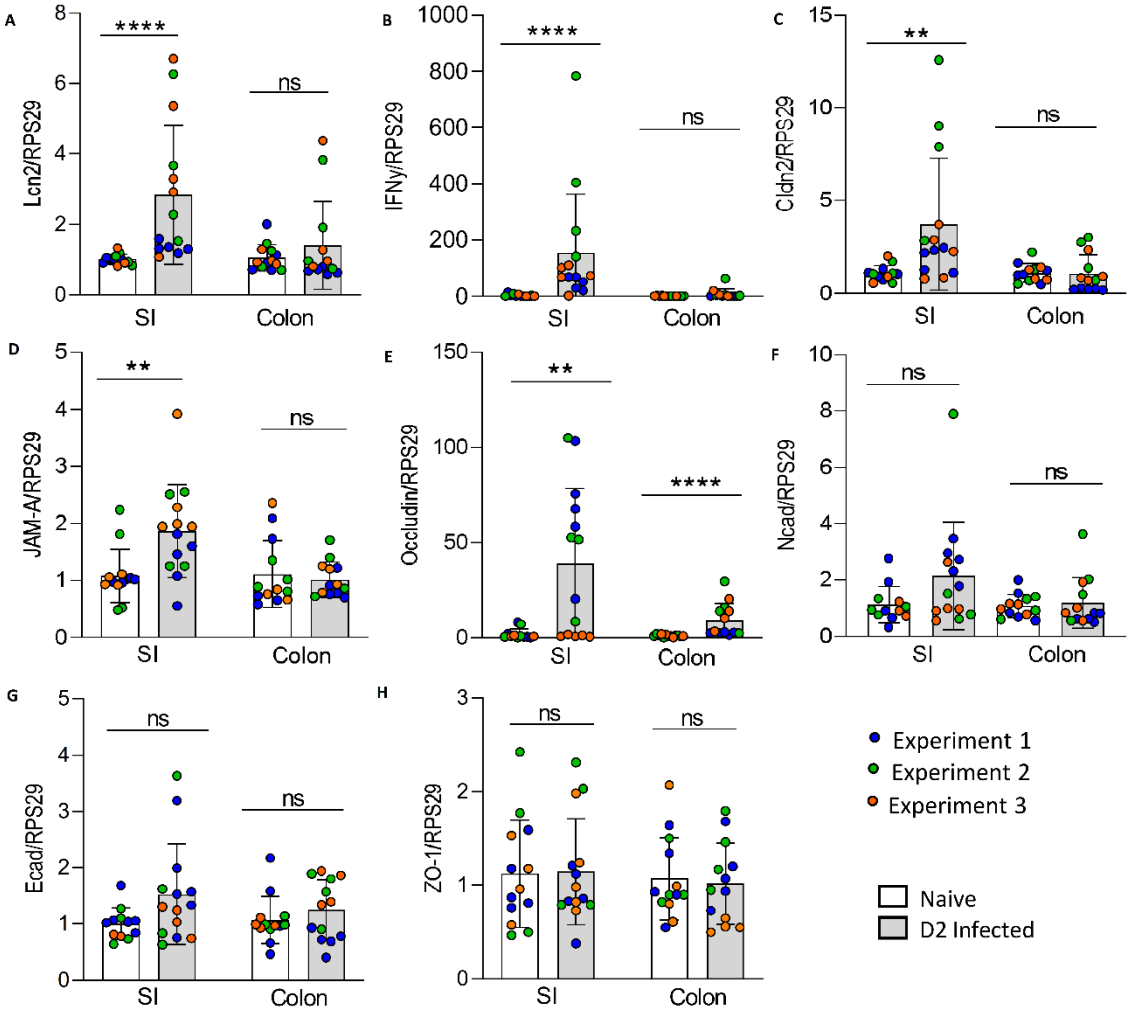

Figure 5-4 Changes in TJ protein gene expression in the duodenum at D2 of *H. polygyrus* infection
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 2 days post-infection the small intestine and colon removed. Fold change of (A) Lcn2, (B) IFNγ, (C) Cldn2, (D) JAM-A, (E) Occludin, (F) N-cadherin, (G) E-cadherin and (H) ZO-1 gene expression in the duodenum and colon compared to housekeeping gene (RSP29) and normalised to an average of naïve samples. Graphed data are shown with mean ± SD and are pooled from 3 independent experiments with *n*=4-5 per experiment. Statistical significance was calculated by Mann Whitney U test (Significance $^{**}p< 0.01$, $^{****}p< .0001$).

validated these data by examining Lcn2 gene expression in the duodenum and colon at day 2 of *H. polygyrus* infection (Figure 5-3B). An increase in Lcn2 gene expression was observed in the duodenum but not the colon, reflecting that *H. polygyrus* only migrates through the wall of the small intestine. In addition, an increase in Lcn2 gene expression was also observed at D7 and D14 of *H.*

polygyrus infection (Figure 5-3C). To confirm these suggestions of possible barrier breach at D2 and D8 of infection, we also used a second assay of barrier integrity. The presence of faecal albumin indicates severe inflammation, bleeding and barrier disruption, a marker of extreme barrier breach as compared to lipocalin-2 (Maccioni et al., 2020, Zhao et al., 2018, Khan et al., 2017). We measured faecal albumin at 2- and 8-days post-infection, but we saw no changes in this protein between naïve and infected mice (Figure 5-3D). Similarly, to our lipocalin-2 ELISA, albumin in faecal samples taken from a mouse undergoing an acute, DSS-induced colitis was above the detection limit of the assay. From these data we see some suggestion of barrier disruption at D2 and D8 of infection but is much milder than in DSS induced colitis for example and is not associated with total loss of barrier integrity or bleeding. The contrasting results between lipocalin-2 and albumin in the faeces suggests that intestinal leakiness and inflammation occurs at timepoints where *H. polygyrus* is moving through the wall of the small intestine. However, we hypothesise the severity of this is low due to the lack of faecal albumin.

We next aimed to understand what is keeping the barrier intact as *H. polygyrus* migrates through the wall of the intestine. Based on the spike in both IFNγ and Lipocalin-2 observed at day 2 post-infection, we therefore decided to focus on this timepoint. Alterations in TJ proteins are associated with intestinal barrier disruption and we therefore assessed the gene expression of key TJ proteins in the duodenum at day 2 post infection with *H. polygyrus* (Figure 5-4). In keeping with our previous data, both Lcn2 and IFNγ expression increased in the duodenum but not the colon (Figure 5-4A & B).The expression of the TJ proteins Cldn2, JAM-A and Occludin increased in the duodenum at day 2 of *H. polygyrus* infection, but, N-cad (N-cadherin), E-cad (E-cadherin) and ZO-1 did not change in comparison to uninfected controls (Figure 5-4C-H). These data suggest there are changes in cell-to-cell contacts during *H. polygyrus* infection. It is possible that the upregulation of some TJ proteins in the duodenal tissue represents a rapid restoration of TJ integrity, after disruption by a passing worm, contributing to consistent structural integrity of the gut epithelium during *H. polygyrus* infection.

5.3.3 *H. polygyrus* as a stimulus for IFNγ expression during infection

Although we hypothesise that barrier breach is mild during *H. polygyrus* infection, we do observe an increase in disruption, measured by lipocalin-2, repair through the upregulation of TJ proteins and some evidence of a type 1 response in the small intestine and more distal tissues. Based on these data it was possible that low level bacterial translocation may occur. To address the

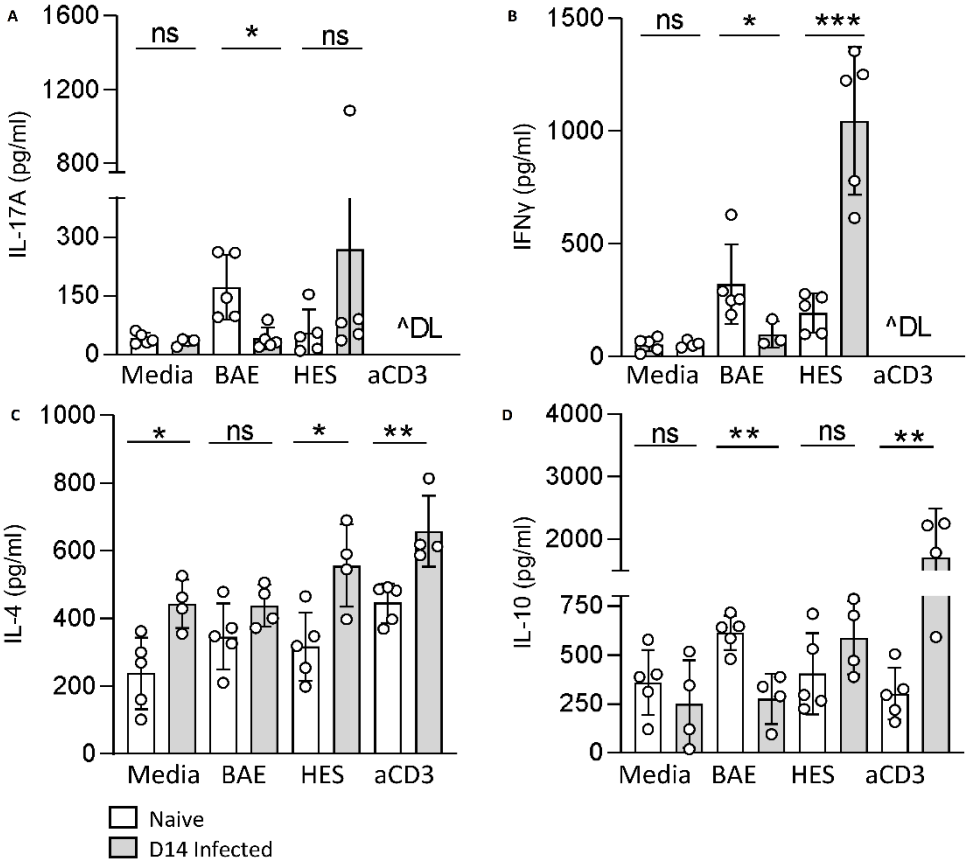

Figure 5-5 *Ex-vivo* re-stimulation with HES induces IFNγ secretion from MLN cells from *H. polygyrus* infected mice
C57BL/6 mice were infected with 200 L3 *H. polygyrus* and 14-days post-infection the MLN removed, MLN cells were re-stimulated *ex-vivo* with media/bacterial antigen extract (BAE)/HES or anti-CD3 for 3 days and cytokines measured in the supernatant. The concentration (pg/ml) of (A) IL-17A, (B) IFNγ, (C) IL-4 and (D) IL-10 in culture supernatants. ^DL = above the detection limit of the assay. Graphed data are shown with mean ± SD and are reprehensive of 1 independent experiment with n=3-5. Statistical significance was calculated by Mann Whitney U test where data were not normally distributed (A (HES), B (BAE), C (HES)) and Student *t* test where data were normally distributed (A (media, BAE), B (media, HES), C (media, BAE, aCD3), D) (Significance *$p<0.05$, **$p< 0.01$).

question of bacterial translocation as a result of intestinal leakiness during *H. polygyrus* infection, two attempts were made to detect systemic bacteria. We

first attempted to detect bacteria in the MLN and spleen, after 14 days of *H. polygyrus* infection by plating homogenised organs on to bacterial growth medium (data not shown). This pilot experiment showed bacterial growth in the MLN and spleen from both naïve and infected mice, indicating that sample contamination had occurred as SLOs from naïve mice should be sterile. This experiment requires optimisation of sterile techniques which due to time limitations could not be carried out. We next measured LPS in the serum of naïve and day 14 infected mice. This assay showed no detectable LPS in either set of samples, naïve or infected (data not shown). The assay itself worked well, as the standard curve was clear, but the lower detection limit of this assay was 0.01ng/ml. Serum concentrations of LPS at this level would be expected only in severe bacteraemia, and sepsis is not reported during *H. polygyrus* infection (Reynolds et al., 2012).

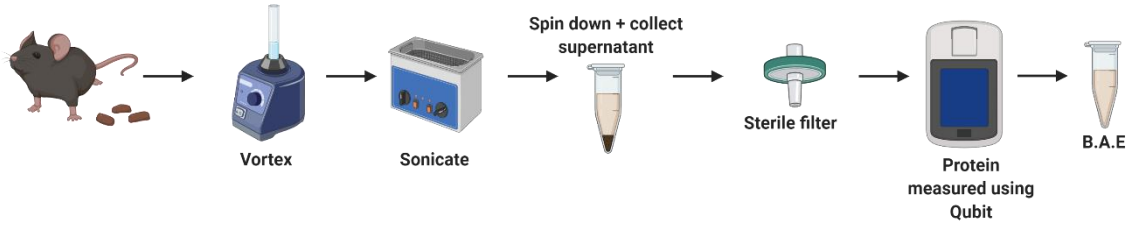

Figure 5-6 Collection of bacterial antigen extract
The collection of BAE is described in detail in section 2.12. Created with BioRender.com

It remained possible that low level bacterial translocation could occur during *H. polygyrus* infection and could be responsible for driving the IFNγ $CD4^+$ and $CD8^+$ T cell responses seen in Figure 5-1. To test whether T cell responses are directed against bacterial antigens during *H. polygyrus* infection, we aimed to determine if the IFNγ signature we observed during infection in different tissues was a result of bacteria specific Th1 cells. MLN cells from naïve and day 14 infected mice were re-stimulated *ex-vivo* with bacterial antigen extract (BAE) for 3 days and then cytokines measured in the supernatant (Figure 5-5). The collection of BAE is described in Figure 5-6 and is a crude method obtaining bacterial antigen from the faeces. We found that both IL-17A and IFNγ, two cytokines associated with type 1 responses against many pathogens, did not increase when stimulated with BAE but decreased (Figure 5-5A&B), we therefore could not conclude from this assay that there are T cells responding to bacteria during *H. polygyrus* infection. IL-10 also decreased in response to BAE compared to naïve controls,

and IL-4 remained unchanged (Figure 5-5C&D). MLN cells were also re-stimulated with HES, and interestingly both IL-4 and IFNγ but not IL-17A significantly increased in MLN cells from day 14 infected mice compared to naïve controls (Figure 5-5). These data demonstrate that IFNγ is expressed at early timepoints in the small intestine and omentum which persists to D7 in both the MLN and omentum. Our data also suggest that minor barrier breach occurs during *H. polygyrus* infection. Although we do not conclusively assess bacterial translocation during infection, we show that IFNγ may be released in response to *H. polygyrus* itself.

5.4 Discussion

5.4.1 Local and distal IFNγ expression during H. polygyrus infection

In this chapter I aimed to characterise the site and timing of IFNγ expression during *H. polygyrus* and to subsequently understand the stimulus behind it. Although both the percentage and number of IFNγ producing T cells increased in the MLN 7 days post *H. polygyrus* infection, this increase was not observed in the SILP. I hypothesise that the high concentrations of IL-10 found in the intestinal tissue, as seen in Figure 3-11, might act directly on tissue-based T cells to limit their IFNγ expression during upon *H. polygyrus* infection (Coomes et al., 2017, Joss et al., 2000). Indeed, a trend towards an increase in IFNγ gene expression was observed during IL-10R mAb blockade (Figure 4-4). When examining IFNγ production from distinct $CD4^+$ and $CD8^+$ T cells, we found an increase in the percentage but not number of $CD8^+$ IFNγ producing cells in the SILP. An expansion of CD8 regulatory T cells, which produce both IL-10 and IFNγ (Yu et al., 2018), has been reported in *H. polygyrus* infection (Metwali et al., 2006). I hypothesise that perhaps these cells may account for the observed increase of $CD8^+$ IFNγ producing cells in the SILP. Overall, these data at 7 days post-infection show little evidence of an increase in IFNγ expression by $CD4^+$ and $CD8^+$ T cells in SILP but do show a clear increase in IFNγ expression by both T cell subsets in the MLN. Therefore, suggesting that perhaps IFNγ expression by these cells is suppressed upon entering the helminth infected tissue.

To obtain a better overall understanding of when IFNγ is expressed in the small intestine, we assessed gene expression of this cytokine at different timepoints. We became increasingly interested in earlier timepoints of *H. polygyrus* infection as recent literature suggests a role for this cytokine at earlier stages of infection (Nusse et al., 2018, Gentile et al., 2020). IFNγ production by lymphocytes has been reported within granulomas and has been shown to be important for promoting epithelial turnover during *H. polygyrus* infection (Nusse et al., 2018). In addition, another recent study reported that the depletion of NK cells resulted in intestinal bleeding during *H. polygyrus* infection and demonstrated that IFNγ was essential for NK cell recruitment in this model. These data therefore show an early, at days 2-4, requirement for IFNγ during infection and that wound healing is required for epithelial barrier integrity also (Gentile et al., 2020). In keeping with the observations in these studies, we observed an increase in IFNγ gene expression at D2 of infection in the duodenum. This increase was not observed at later timepoints. These data coupled with the literature suggest that IFNγ may be important for wound healing at the larval and granuloma stages of infection, but other mechanisms of wound repair may be sufficient when adult worms migrate back to the lumen. Possible ways of testing this hypothesis would be to analyse IL-22 gene expression at early and later timepoints of infection. IL-22 is important for wound repair and perhaps may be more highly expressed at later timepoints when IFNγ is absent (Wei et al., 2020). Although, we did not report an increase in gene expression of IL-22 at D7 of infection (Figure 4-4F), this timepoint is slightly earlier than when worms are to migrate back to the lumen which is usually around day 8 (Monroy and Enriquez, 1992). In addition, blocking IFNγ signalling at the larval and granuloma stages may further elucidate the roles this cytokine plays during *H. polygyrus* infection. Overall, the role of IFNγ in the small intestine thus far from our data appears restricted to D2 of infection and from the literature early IFNγ expression contributes towards wound healing via NK cell recruitment and epithelial cell turnover(Gentile et al., 2020). However, we do report increased IFNγ in the MLN during *H. polygyrus* infection, suggesting that the role of this cytokine may not restricted wound healing in the intestine.

During *H. polygyrus* infection despite parasites only residing within the small intestine, systemic immune effects have been described. In a mouse model of airway inflammation, infection with *H. polygyrus* resulted in reduced eosinophil

recruitment to the lung (Rzepecka et al., 2007). The suppression of inflammation in the skin as a result of *H. polygyrus* infection has been demonstrated in a model of contact hypersensitivity (Filbey et al., 2020). In addition, as we show in Figure 3-1B and as previously published (Brailsford and Mapes, 1987) that splenomegaly occurs during *H. polygyrus* infection. In addition, Th2 cells are found in the spleen, as well as the lung, peritoneal cavity, and liver (Mohrs et al., 2005a). Interestingly, the number of Th2 cells in the peritoneal cavity were similar to those found in the SLOs, the MLN and spleen (Mohrs et al., 2005a). Furthermore, cells in the peritoneal cavity expressed high levels of the intestinal homing molecule α4ß7, rapidly produce IL-4 when stimulated *ex vivo* and persist with in the peritoneal cavity after worm clearance with anti-helminthics (Mohrs et al., 2005a). These data suggest that the peritoneal cavity is a site of Th2 memory cell accumulation, despite parasites themselves not being present at this site (Mohrs et al., 2005a).

In addition to being found in the peritoneal cavity, Th2 cells have also been identified in the omentum, a fatty tissue with immunological properties that drains fluid from the peritoneal cavity (Rangel-Moreno et al., 2009). We decided to investigate IFNγ responses in the omentum as we hypothesised that perhaps effector Th1 memory cells may also accumulate at this site or that as the omentum filters the peritoneal cavity, bacteria or bacterial products leaking from the intestine may become trapped in the omentum. In addition, we considered the idea that bacteria or bacterial products may also arrive in the omentum in the blood.

Our data measuring IFNγ and type 2 cytokine gene expression in the omentum suggested that IFNγ responses occur at this site at D2 and D7 of infection but this signature was lost at D14. These data suggest that the IFNγ gene expression in this tissue is not a result of Th1 effector memory cells at D2, as this timepoint is too early for the accumulation of these adaptive immune cells. However, IFNγ expression at D7 could be a result of Th1 cells beginning to home to the omentum. The blood vessels of the omentum express molecules such as mucosal addressin cell adhesion molecule 1 (MAdCAM-1) and peripheral lymph node addressin (PNAd) typically found on HEVs of Peyer's patches and the MLN (Briskin et al., 1997, Berberich et al., 2008, Meza-Perez and Randall, 2017). The intestinal homing integrin α4ß7 is also associated with cell migration from the

blood to the omentum (Carlow et al., 2009, Meza-Perez and Randall, 2017). This suggests that primed IFNγ$^+$ Th1 cells from the MLN (Figure 5-1) may home to the omentum as part of a memory response, although the stimulus behind this Th1 response remains unclear and the absence of an IFNγ signature at D14 could potential be a result of Th1 cells moving to the peritoneal cavity or low cytokine activity by these cells.

Our data also suggest that a local innate immune response occurs in the omentum during *H. polygyrus* infection due to the observed D2 cytokine signature. Interestingly, NK cells have been reported in the omentum (Sorensen et al., 2009, Sedlacek et al., 2013) and LPS stimulation of NK cells results in IFNγ expression (Kanevskiy et al., 2013). Low level LPS in the blood as a result of early barrier breach during *H. polygyrus* infection may arrive at the omentum and stimulate NK cells, presenting a possible reason for the early IFNγ signature in the omentum during *H. polygyrus* infection. We examined LPS in the serum and peritoneal wash at D7 of infection and found no detectable LPS (data not shown), it would be important to carry this experiment out at D2 of infection also. These data present an exciting question surrounding systemic responses to *H. polygyrus* in the omentum. A method to explore this question further would be to analyse innate cells with the capacity to produce IFNγ such as macrophages and NK cells, which have been previously identified in the omentum, by flow cytometry from naïve and D2 infected mice. Furthermore, the presence of IFNγ producing Th1 cells in the omentum and D7 of infection should also be analysed, as the D2 and D7 IFNγ signatures may be due to different responses. In addition, unravelling the stimuli behind IFNγ expression by cells is a key part of understanding this response in the omentum. To address this question, measuring the integrity of and changes to the intestinal barrier is important as a leaky gut may result in systemic bacteria or bacterial products.

5.4.2 Changes in epithelial barrier integrity during H. polygyrus infection

We hypothesised that due to the migration of *H. polygyrus* through the wall of the small intestine that barrier breach may occur during infection and that subsequent bacterial translocation could be a potential stimulus for IFNγ responses during infection. In keeping with this hypothesis, we observed in an

increase in lipocalin-2 in the faeces at day 2 of infection and also at day 8 of infection. Lipocalin-2 is an innate immune protein produced by epithelial cells and macrophages in response to inflammation. Neutrophils also store lipocalin-2 in granules (Meheus et al., 1993, Chan et al., 2009, Toyonaga et al., 2016). The primary role of lipocalin-2 is to starve and therefore prevent overgrowth of bacteria via sequestering iron (Goetz et al., 2002, Toyonaga et al., 2016) and $Lcn2^{-/-}$ mice have bacterial dysbiosis (Singh et al., 2020). Lipocalin-2 is a well-established marker of inflammation and barrier breach in IBD and murine colitis models (Chassaing et al., 2012, Hsieh et al., 2016), however, it is not exclusively a marker of a leaky intestinal barrier. We therefore measured albumin in the faeces, a protein found in the blood which increases in organs such as the intestine when bleeding and severe pathology occur (Khan et al., 2017, Powell-Tuck, 1986). There were no changes to faecal albumin at day 2 or day 8 of infection, indicating that there is no major barrier disruption during *H. polygyrus* but does not remove the possibility that minor barrier breach occurs.

To assess barrier integrity further, gene expression of TJ proteins in the duodenum were measured. We observed significant increases in numerous TJ proteins at 2 days post-infection with *H. polygyrus*. These data could be consistent with a process in which burrowing worms disrupt TJs and gene expression increases as the TJs are restored. Previously, *H. polygyrus* has been shown to increase distal colonic barrier permeability as a result of decrease in E-cadherin at the protein level and this was shown to be dependent on IL-4 signalling (Su et al., 2011). This decrease of protein E-cadherin may require increased gene expression to overcome loss of TJs, a similar process may occur in the duodenum and our data showing increased TJ gene expression supports the idea of renewing TJ proteins. To validate these observations at the protein level, a possible experiment would be to carry out immunofluorescence imaging of TJ proteins in the duodenum of naïve and D2 infected mice. In addition, although we observe clear changes at D2 of infection, it would be interesting to carry out these experiments at D8 of infection, where a second lipocalin-2 peak occurred and when adult worms are moving into the lumen. To gain a conclusive answer surrounding barrier breach during H. polygyrus infection, my proposed experiment would be to use FITC-dextran at day 2 and 8 of infection to assess movement across the intestinal barrier. This technique would require optimisation as a result of the high levels of mucus reported during *H. polygyrus*

infection. These data show that major barrier disruption does not occur in responses to *H. polygyrus* infection, but our data do indicate changes to the epithelial barrier do occur and we hypothesised that this may be sufficient for bacterial translocation and subsequent bacterial specific responses.

5.4.3 Potential stimuli of IFNγ expression in the immune response to H. polygyrus infection

The detection of bacterial translocation and bacteria-specific immune responses requires the optimisation of techniques that require sterile handling of samples to prevent contamination and false positive results. As described previously we carried out numerous pilot experiments to assess bacterial translocation during *H. polygyrus* infection. However, contamination was a common problem across experiments. However, using bacterial antigen extract (BAE) (Figure 5-6) as a crude bacterial *ex vivo* stimulus for MLN cells provided some interesting preliminary data surrounding IFNγ responses during *H. polygyrus* infection. Although this experimental set up does have some caveats. Firstly, the components of BAE are unknown and there may be a super-antigen within BAE samples. Secondly, BAE most likely contains a high concentration of bacterial antigen from the colon, and we hypothesise that any bacterial specific cells would respond small intestinal bacteria rather than those found in the colon. Despite these caveats, we did observe some interesting data from these experiments.

Our *ex-vivo* MLN stimulation data demonstrated that BAE did not induce the secretion of IFNγ from *H. polygyrus* infected MLN cells, instead secretion of this cytokine decreased. In addition, HES, the secretory product of *H. polygyrus* promoted IFNγ secretion by MLN cells from *H. polygyrus* infected mice. HES induction of IFNγ from MLN cells has also been shown at D7 and D28 of *H. polygyrus* infection (Filbey et al., 2014). The induction of IFNγ expression by HES may be a mechanism by *H. polygyrus* to create immune competition during infection, subsequently reducing the Th2 responses and allowing parasite persistence. An additional experiment to elucidate if IFNγ is derived from $CD4^+$ T cells would be to re-stimulate $CD4^+$ T cell from the MLN, rather than entire MLN cells, from *H. polygyrus* infected mice. In addition, HES promoting IFNγ expression is not described in BALB/C mice which are more resistant to *H.*

polygyrus infection (Filbey et al., 2014), suggesting that in the context, IFNγ responses are detrimental to parasite expulsion. These data suggest that perhaps *H. polygyrus* specific Th1 cell are primed in the MLN, but upon arrival at the SILP, IFNγ secretion by these cells is suppressed. A potential mechanism for this suppression is IL-10, the concentration of IL-10 in the intestine is overall higher and increases with infection (Figure 3-11), even in naïve mice, compared to the MLN. Therefore, when IFNγ producing cells arrives in the SILP, exposure to high concentrations of IL-10 may result in suppression of these responses. In addition, the strong type 2 cytokine environment in the SILP is not favourable for Th1 responses. A potential mechanism to test these hypothesise would be to block IL-10R signalling at the peak of $CD4^+$ T cell response, D14 of infection (Perona-Wright et al., 2010), and test if this results in an increase in IFNγ producing Th1 cells in the SILP and subsequent increased worm burden due to immune competition. Overall, our preliminary studies investigating bacterial translocation as an IFNγ stimuli do not show evidence of this during *H. polygyrus* infection. In addition, we hypothesise that *H. polygyrus* may also be a candidate for stimulating IFNγ responses, although further studies surrounding both bacterial translocation and *H. polygyrus* specific Th responses are required.

5.4.4 Concluding remarks

To conclude, we have demonstrated a slight increase in intestinal permeability and inflammation that may reflect minor barrier breach at the two points of infection where *H. polygyrus* moves through the wall of the intestine. However, our data do not indicate that this results in bacterial translocation. Further experiments and optimisation are required to confirm this preliminary observation. In addition, we show that both Th1 and Th2 cell cytokine signatures are found in the omentum, a site of possible mucosal effector cell homing or a previously undescribed site where *H. polygyrus* induces innate immune responses. Furthermore, we show that HES induces IFNγ production as well as IL-4 by MLN cells from *H. polygyrus* infected mice. Overall, we have demonstrated that during *H. polygyrus* infection, IFNγ is expressed in both local and distal sites and at earlier (D2) and later (D7) stages of infection. However, the stimulus behind this remains unclear, and may differ between tissues and at different timepoints. This suggests that IFNγ may play numerous, previously overlooked, roles in the immune response to *H. polygyrus* infection and further experiments

should focus on elucidating this. Understanding the functions of IFNγ and possible immune competition during *H. polygyrus* is key for understanding immunity to this parasite and may contribute towards anti-helminth treatment.

Chapter-6 Main discussion

Cytokines are pivotal in orchestrating and maintaining immune responses to pathogens such as helminth infections. The Th2 cytokines IL-4, IL-13 and IL-5 play a critical role in the immune response to *H. polygyrus* infection (Reynolds et al., 2012). Interestingly, location-specific functions of these cytokines have been described, with IL-4 being essential within the priming lymph node and IL-13 and IL-5 often described as tissue cytokines (Redpath et al., 2015). This emphasises the importance of investigating both of these sites during infection. There have been numerous studies using IL-10$^{-/-}$ mice to study the protective roles of *H. polygyrus* infection in models of colitis (Elliott et al., 2004, Setiawan et al., 2007, Blum et al., 2012, Metwali et al., 2006). However, these studies do not investigate the role of IL-10 in the small intestine in the context of *H. polygyrus* alone. Therefore, it remains unclear if IL-10 has a role in promoting Th2 responses to helminth infection and if this affects Th2 cells directly, indirectly or a combination of both. A possible mechanism for indirect promotion of Th2 responses by IL-10 is the suppression of Th1 cells, removing immune competition in this infection model (Figure 6-3). Although the idea of an underlying bacterial Th1 response during *H. polygyrus* has been hypothesised in the literature, due to the nature of the *H. polygyrus* life cycle, this has never been directly reported (Filbey et al., 2014, Ahmed et al., 2017). The overarching aims of this thesis were to understand the possible direct and indirect mechanisms of the cytokine IL-10 in promoting Th2 immunity to helminth infection and to explore immune competition during *H. polygyrus* infection.

6.1.1 Analysing immune responses in both the SILP and MLN is key for understanding immunity to H. polygyrus infection

The priming LN and tissue-based site of infection have distinct organisation, immune cells, and functions. The lymph node is a hub of lymphocytes comprising mostly B cells and T cells, however stromal cells and DCs are also key players at these sites (Jenkins et al., 2001, Martín- Fontecha et al., 2003). One of the primary functions of the LN is to orchestrate and initiate the adaptive immune response to infection, as described in section 1.4.2. The site of infection is where primed effector cells, such as activated Th2 cells and plasma cells, traffic to, in order to exert their effector functions (Maizels et al., 2012). At these

sites, there are a variety of immune cells that are abundant in the intestine but found in much fewer numbers or are absent from the MLN, including ILCs, macrophages, basophils and mast cells (Pelly et al., 2016, Reynolds et al., 2012). There are also almost no naïve lymphocytes found in the tissue, unlike the MLN (Jenkins et al., 2001). The difference in immune cells and the subsequent cytokine signatures found in the LN compared to the tissue create a distinct immune environment. Furthermore, commensal bacteria do not reside in SLOs such as the MLN, whereas in tissues such as the intestine, there is an abundance of commensal bacteria and food antigen, presenting the requirement for increased regulation of immune cells at these sites (Bowcutt et al., 2014). These differences mean that investigating both the LN and tissue-based site of infection are key when studying immune responses to infection. Much of the published literature that explores immune responses to *H. polygyrus* focuses on the MLN (Mosconi et al., 2015, Pelly et al., 2016, Perona-Wright et al., 2010). There is a lack of studies using techniques such as flow cytometry to analyse *H. polygyrus* infected small intestine samples at later time points of infection, such as 14 days post-infection, where Th2 expansion peaks (Perona-Wright et al., 2010). This is largely due to the technical challenge of isolating cells and maintaining cell viability from samples with high levels of mucus. Although studying the MLN is critical for understanding the priming of the adaptive response to *H. polygyrus* it does not allow for full understanding of subsequent effector responses in the small intestine. The aim of Chapter-3 was therefore to optimise a method to isolate leukocytes from *H. polygyrus* infected small intestine samples at timepoints where mucus levels are high. In addition, two further methods for isolating cells from the SILP during *H. polygyrus* infection were published quickly after our own method was published, emphasising the need for this technique to move the field forward (Webster et al., 2020, Mayer et al., 2020, Jarjour et al., 2020). The successful development of this protocol enabled us to carry out key experiments in both Chapter-4 and Chapter-5, allowing us to address our hypothesis surrounding the role of IL-10 in the Th2 response to helminth infection and immune competition in this model.

6.1.2 The source of IL-10 and surrounding immune environment are key for the immunological role of this cytokine

The role of IL-10 in maintaining gut homeostasis and suppression of Th1 responses is well described (Glocker et al., 2009, Moran et al., 2013, Fiorentino et al., 1989, Couper et al., 2008). However, the role of this cytokine in the Th2 response to helminths and other type 2 mediated disease, such as allergy, remains unclear. A factor that appears important for the role of IL-10 is the surrounding immune environment (Dennis et al., 2013, Fiorentino et al., 1989, Moore et al., 2001, Vieira et al., 1991). In addition, the cellular source of IL-10 also appears to be important for the resulting immunological role of IL-10. The importance of T cell derived IL-10 has been shown in mice lacking IL-10 specifically in T cells, using IL-10 $^{FL/FL}$ x CD4-cre mice, which develop spontaneous colitis, similar to that seen in global IL-10$^{-/-}$ mice (Roers et al., 2004). Infection of these mice with *T. gondii* resulted in a lethal Th1 response, due to the lack of IL-10 mediated regulation. This was characterised by high CD4^{+} T cell infiltrates in the liver and severe immunopathology (Roers et al., 2004). In contrast, these T cell specific IL-10$^{-/-}$ mice did not have exacerbated responses to cutaneous skin irritation or to LPS exposure (Roers et al., 2004). This suggests that in the skin, IL-10 production by other cells such as keratinocytes or macrophages may be key for regulating immune responses. Furthermore, in mice lacking B cells, the mouse model EAE is non-remitting. However, remission was restored by transfer of WT B cells and the same restoration was not found when transferring B cells from IL-10$^{-/-}$ mice. This study shows a specific role of B cell derived IL-10 for promoting remission in EAE (Fillatreau et al., 2002). Interestingly, B cell derived IL-10 has been reported to promote IL-10 secretion by T cells, so in this model the lack of IL-10 from B cells may result in a lack of IL-10 secretion by other immune cells also (Pennati et al., 2016). Another important factor to consider when studying IL-10 is what promotes the expression of IL-10 by different cell subsets. Cells such as macrophages and DCs have been shown to produce IL-10 in response to TLR ligation (Chanteux et al., 2007, Samarasinghe et al., 2006, Netea et al., 2004, Nguyen et al., 2020). Similarly, in B cells, ligation of TLR2, TLR4 and TLR9 have all been shown to induce IL-10 production by these cells (Sun et al., 2005, Sayi et al., 2011, Ma et al., 2015). B cells activated via TLR-2 can also activate Tr1 cells, and this TLR2 mediated activation of B cells was responsible for the

suppression of gastric immunopathology induced in *Helicobacter pylori* infection (Sayi et al., 2011). The induction of IL-10 by the ligation of certain TLRs may be a self-limiting mechanism, where excess release of cytokines such as IL-12 after TLR ligation is limited by IL-10 production. In addition, another study demonstrated that macrophages release IL-10 in response to phagocytosis of apoptotic cells, again suggesting the requirement for regulation when immune cells are stimulated (Chung et al., 2007). These studies combined emphasise the importance of the immune environment and cellular source of IL-10 when considering immunological functions of this cytokine.

6.1.3 Potential mechanism of IL-10 promoting Th2 responses

We have demonstrated that in the absence of IL-10R signalling, the Th2 response in the SILP, but not MLN, is decreased and in addition type 2 cytokine gene expression is dramatically reduced. This emphasises once again the importance of investigating both the MLN and SILP when studying *H. polygyrus* infection. In addition, it demonstrates an important role for IL-10 in maintaining Th2 responses and type 2 cytokines in the SILP. The absence of this effect in the MLN suggests a tissue-specific and perhaps even an intestinal specific role of IL-10 in promoting Th2 responses. The idea of the second touch hypothesis (also known as the two-hit hypothesis) states that for full T cell activation and differentiation, interactions with APCs such as DCs and macrophages at the site of infection where antigen was first acquired are needed (Ley, 2014, Mohrs et al., 2005b). In addition, ILC2s have also been shown to express MHCII and have been reported to interact with antigen specific $CD4^+$ T cells in the tissue (Oliphant et al., 2014). This hypothesis implies that Th cells retain some plasticity when arriving at the site of infection and interactions with APCs and the resulting cytokine environment in the tissue drives further polarisation of Th cells (Ley, 2014). IL-10 can act on DCs to prevent the secretion of IL-12 (Aste-Amezaga et al., 1998, Huang et al., 2001, Corinti et al., 2001, Ma et al., 2015). A potential mechanism for IL-10 support of Th2 responses in the small intestine may be due to IL-10 conditioning of APCs in the tissue to prevent the induction of Th1 cells and subsequent IFNγ production via inhibition of IL-12 (Figure 6-1). This is also in keeping with our observation in Chapter-5, where we show an increase in IFNγ producing $CD4^+$ T cells in the MLN but not the SILP with *H. polygyrus* infection. IL-10 conditioned DCs may skew any IFNγ producing $CD4^+$ T

cells to a Th2 phenotype once they arrive in the small intestine, explaining why we do not observe an increase in these cells in the SILP (Figure 6-1). In addition to this, in a model of allergic dermatitis induced by OVA sensitization in the skin, IL-10 derived from DCs was reported to be critical for induction of antigen specific Th2 responses (Laouini et al., 2003). In this model, adoptive transfer of

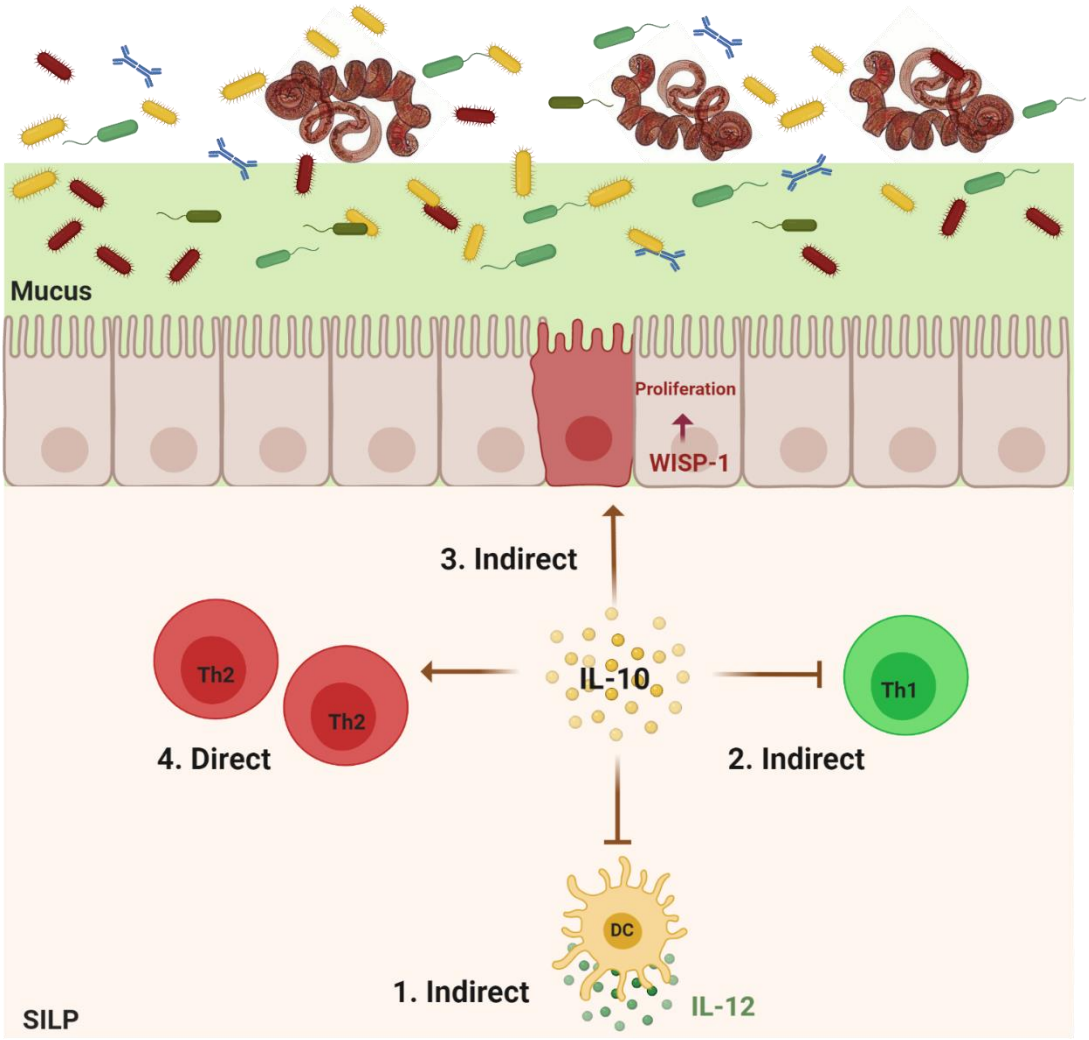

Figure 6-1 Proposed mechanisms of IL-10 promoting the Th2 response
Based on the literature and our data I propose that IL-10 (which increases during *H. polygyrus* infection) promotes Th2 responses through a combination of direct and indirect mechanisms. **Mechanism 1. (Indirect)** IL-10 inhibits IL-12 secretion by DCs, inhibiting Th1 responses. Based on the second touch hypothesis and the importance of the surrounding cytokine environment this removes immune competition and promotes Th2 differentiation in the SILP. **Mechanism 2. (Indirect)** The literature has described AAM derived IL-10 promoting wound healing during *H. polygyrus* infection via promoting epithelial proliferation via WISP-1. This mechanism may prevent or limit bacterial translocation during infection, preventing anti-bacterial Th1 responses which would act as immune competition for Th2 responses. **Mechanism 3. (Indirect)** Our data show that there is increase in $IFN\gamma^{+}$ $CD4^{+}$ T cells in the MLN but not the SILP at D7 of *H. polygyrus* infection. Indicating that IL-10 may suppress these cells upon entering the SILP, this is in keeping with higher IL-10R expression by Th1 cells in the SILP compared to Th2 cells. **Mechanism 4. (Direct)** We have shown *in vitro* that IL-10 can directly promote Th0 and Th2 cells to produce the type 2 cytokines IL-13 and IL-5. These direct and indirect mechanisms combined may explain the reduction in SILP Th2 cells we observe when blocking IL-10R signalling.

OVA-pulsed IL-10$^{-/-}$ DCs into WT and IL-10$^{-/-}$ mice resulted in equally poor IL-4 secretion by T cells (Laouini et al., 2003). This suggests an important role of DC derived IL-10 in promoting Th2 responses in the skin, although it is unclear if this is a direct or indirect response. These data demonstrate that IL-10 derived from DCs in the tissue may be an interesting target for further investigation in *H. polygyrus* infection.

I hypothesise that IL-10 may also act directly on T cells to promote a Th2 phenotype. Our data demonstrate that IL-10 can stimulate both Th0 and Th2 cells to produce the type 2 tissue cytokines IL-13 and IL-5 *in vitro*. This *in vitro* system is set up using purified CD4^{+} T cells, suggesting direct effects of IL-10 that are independent of APCs. Furthermore, this effect occurred independently of changes to activation and proliferation of the T cells but was partially dependent on IFNγ suppression. The suppression of IFNγ as a mechanism for IL-10 promoting Th2 cell skewing is supported further by our data demonstrating that in the SILP but not the MLN, CXCR3^{+} "Th1" cells express higher levels of the IL-10R compared to IL-4^{+} "Th2" cells. This would suggest that the increased levels of IL-10 reported during *H. polygyrus* infection may act on Th cells to further skew to a Th2 phenotype by eliminating immune competition (Figure 6-1). To test this hypothesis, I suggest blocking IFNγ signalling using a mAb during *H. polygyrus* infection. If an increase in Th2 cells was observed, this would indicate that, as we found *in vitro*, IL-10 promoting Th2 cells may be partially dependent on IFNγ suppression. To determine if IL-10 promoting Th2 cells is only partially dependent on IFNγ suppression *in vivo*, I suggest that carrying out a dual treatment where IFNγ signalling is blocked and rIL-10 is administered. Comparing these treatments together to treatments with either rIL-10 or IFNγ blockade alone would elucidate the extent of the importance of IL-10 mediated IFNγ suppression in promoting Th2 cells.

IL-10 derived from AAMs has been shown to promote intestinal wound repair by inducing WISP-1 signalling in epithelial cells, which promotes proliferation and subsequent wound repair (Gordon, 2003, Quiros et al., 2017, Morhardt et al., 2019). IL-10 has also been shown to promote intestinal stem cell proliferation (Biton et al., 2018). IL-10 mediated wound repair may prevent microbiota induced inflammation during *H. polygyrus* infection, preventing the recruitment

of bacteria-specific Th1/Th17 cells, which would otherwise become immune competition for Th2 cells (Figure 6-1). The combination of both

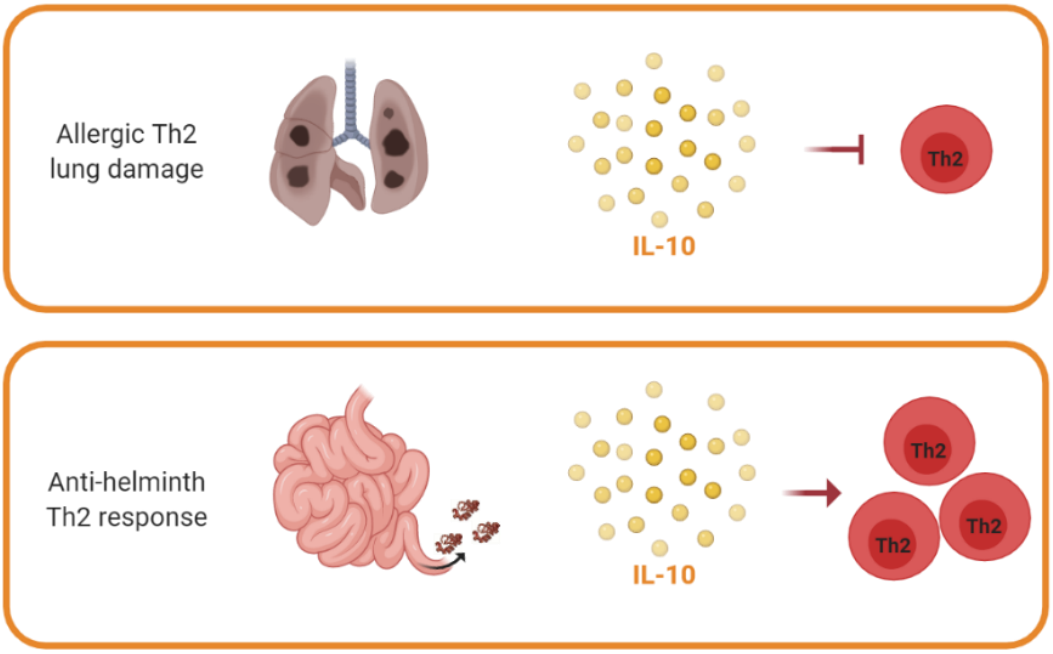

Figure 6-2 The role of IL-10 is dependent on the surrounding immune environment. In allergic airway inflammation, immunopathology occurs in the lung. Furthermore, in models of allergic airway inflammation IL-10 has been reported to suppress the Th2 response. Conversely, the Th2 response to *H. polygyrus* infection is host protective and our data demonstrate that IL-10 is important for an optimal Th2 response. I hypothesise that the function of IL-10 depends on the pathological potential of the target Th2 response.

the hypothesised indirect and direct mechanisms may support Th2 cell responses. To test these hypotheses, the generation of cell specific IL-10 or IL-10R knockout mice would facilitate and allow us to address each of these proposed mechanisms.

As discussed in section 1.3, there are numerous mechanisms that promote the clearance of *H. polygyrus*. The type 2 promoting role for IL-10 we describe may play a role in numerous clearance mechanisms at both the larval and adult stages of infection. AAMs can immobilise larvae within granulomas, as discussed in section 1.3.4, this effect is CD64 dependent and previous studies have shown that IL-10 induces the expression of CD64 on human monocytes (Te Velde et al., 1992, Bovolenta et al., 1998). Therefore, IL-10 acting on AAMs may promote larval immobilisation indirectly via the upregulation of CD64. Similarly, eosinophils also play an important role in larval killing at the granulomas stage of infection. Simultaneous stimulation of AAMs with IL-10 and IL-4 has been

shown to result in increased CCL24 production which subsequently promotes the migration of eosinophils (Makita et al., 2015). This mechanism could be a possible IL-10 and IL-4 dependant mechanism for eosinophil recruitment to granulomas. IL-10 may also act on ILC2s to enhance type 2 cytokine secretion, therefore furthering the secretion of the type 2 cytokines IL-4, IL-13 and IL-5 which in turn promote goblet cell hyperplasia, the function of AAMs and the Th2 response, all of which contribute to the clearance of *H. polygyrus*.

Adult worms reside in the lumen of the gut wrapped around villi (Maizels et al., 2012). As mentioned previously, IL-10 promotes epithelial cell proliferation (Gordon, 2003, Quiros et al., 2017, Morhardt et al., 2019). The subsequent epithelial shedding as a result of proliferation induced by IL-10 could perhaps provide a mechanical mechanism for dislodging worms from the villi, preventing their persistence and mating. The secretion of antibodies such as IgG and IgE by plasma cells occurs during *H. polygyrus* infection. IL-10 has been shown to promote class switching of human B cells to IgG secreting plasma cells (Heine et al., 2014). IgG "coating" of larvae is required for immobilisation and killing by innate immune cells (Esser-Von Bieren et al., 2015). A further potential mechanism for IL-10 mediated expulsion of *H. polygyrus* may be via promoting class switching in B cells to IgG. The direct effect of IL-10 in promoting Th2 cells could promote the expulsion of *H. polygyrus* via enhancing type 2 cytokine secretion, which promotes both the innate and adaptive arms of the type 2 immune response, all of which contribute to the clearance of parasites.

Most studies that have investigated the role for IL-10 in allergic disease in the lung report suppression of type 2 responses by this cytokine, conflicting with our data that IL-10 promotes Th2 cell responses. In these contexts, the type 2 immune response results in severe damage and fibrosis in the lung mediated by pathogenic Th2 cells. This level of inflammation is not described in *H. polygyrus* infection, where Th2 cells are host protective. I hypothesise that IL-10 may have a pro-/anti- Th2 switch, whereby IL-10 will promote Th2 responses unless severe inflammation occurs as a result, where the collateral damage of the Th2 response outweighs the protective benefits and so Th2 suppression is required (Figure 6-2). This concept may apply to other helminth infections, such as *N. brasiliensis,* where IL-10 secretion as a result of IL-4R signalling helps to maintain Th2 responses (Balic et al., 2006). In addition, another layer of

complexity when comparing the lung and intestine is the abundance of bacteria found at these mucosal sites, with the lung microbiota having a much lower biomass than that of the gut (Enaud et al., 2020). In addition, Tregs have been reported to be key for homeostasis within the lung, similarly to the gut. In the lung, Tregs achieve homeostasis by suppressing both Th1 and Th2 responses and this Treg mediated suppression in the lung depends on TGF-ß rather than IL-10, unlike in the gut where IL-10 is required for intestinal homeostasis (Rubtsov et al., 2008, Ray et al., 2010, Kühn et al., 1993). This suggests that although Tregs are involved in the regulatory responses in both of these tissues, the mechanisms of action differ. Therefore, the role of IL-10 may be context dependent. However, our data demonstrate that IL-10 promotes the anti-helminthic Th2 response in the intestine.

6.1.4 Immune competition is key for the outcome of helminth infection

The concept of mixed Th1/Th2 responses during helminth infection is well established in mouse models of helminth infection including *T. muris*, *T. spiralis* and *S. mansoni* (Pearce and Macdonald, 2002, Klementowicz et al., 2012, Ilic et al., 2011). Typically, in mouse strains that are more resistant to helminth infection, Th2 responses are dominant and result in lower worm burden (Cortés et al., 2017). However, in more susceptible mouse strains, Th1 responses limit host protective Th2 responses, resulting in parasite persistence (Cortés et al., 2017). It is important to note that this broad summary varies between helminth models and their respective life cycles (Cortés et al., 2017). Co-infection with *H. polygyrus* and *T. gondii*, a Th1 inducing protozoan parasite, prevents the induction of Th2 cells and promotes *H. polygyrus*-specific Th1 cells (Ahmed et al., 2017). This study supports the concept of *H. polygyrus* specific Th1 responses occurring during infection. Interestingly, C57BL/6 mice are described as susceptible to *H. polygyrus* and develop chronic infection, unlike BALB/C mice which are more resistant, although infection is still chronic (Reynolds et al., 2012, Scott, 1991). It would be interesting to examine if a similar decrease in Th2 cytokines is also observed in BALB/C mice when treated with an IL-10R blocking antibody. This strain has a more robust Th2 response overall and therefore I hypothesise the requirement for indirect support of Th2 responses by IL-10 mediated suppression of Th1 cells may be less (Reynolds et al., 2012). As

well as discussing the relevance of our observation in different laboratory mouse strains, it would also be important to address the concept of wild mice and the relevance our data has in this setting. A recent study developed 'wilding mice', where embryos from C57BL/6 mice were transferred into wild mice, which harness a natural microbiome, including many pathogens (Rosshart et al., 2019). These mice reflected human phenotypes in two pre-clinical trial studies, where laboratory C57BL/6 mice did not (Rosshart et al., 2019). These data suggest that using mice that have a naturally acquired microbiome may be more reliable for translational clinical studies. *H. polygyrus* has been described in the natural microbiome (Behnke et al., 2009). To test the importance of IL-10 for Th2 responses in mice with a natural microbiome, embryos from C57BL/6 mice could be transferred into mice naturally infected with *H. polygyrus* and IL-10R signalling blocking in the wilding offspring. I hypothesise that due to the presence of numerous pathogens in these mice, including *H. polygyrus,* that the requirement for IL-10 signalling would be more so than in "clean" laboratory mice, for both the suppression of Th1 responses to prevent intestinal inflammation and the promoting of Th2 responses against intestinal helminths. Therefore, although our data demonstrate a role for IL-10 in promoting Th2 responses in laboratory C57BL/6 mice, it would be important to investigate this in other strains of laboratory mice and in mice with a natural microbiota as the Th1/Th2 immune balance may differ.

Despite the increased susceptibility in C57BL/6 mice and our data demonstrating an increase in IFNγ producing Th1 cells in the MLN at D7 of infection, suggesting priming of a Th1 response in the MLN, IFNγ producing Th1 cells did not increase in the SILP at D7 of infection. To confirm the lack of Th1 cells in the SILP during *H. polygyrus* infection, further analysis is required at D14 to confirm that Th1 cells primed in the MLN do not migrate to and exert their effector functions in the SILP at this later timepoint. We hypothesise that the lack of IFNγ-producing Th1 cells in the SILP at D7 could be due to IL-10 mediated suppression of the cells in the infected tissue. This is in keeping with our data that demonstrate that Th1 cells express higher levels of the IL-10R compared to IL-4 producing Th2 counterparts. Therefore, the balance of Th1 and Th2 cells is key for helminth expulsion and it remains unclear if Th1 cells tip the balance in immunity to *H. polygyrus*. A potential stimulus for a Th1 response to *H. polygyrus* infection

could be bacterial translocation, although our preliminary studies showed little evidence for this.

In the omentum, a tissue where Th2 cells have been reported to reside during *H. polygyrus* infection (Rangel-Moreno et al., 2009), we observed mixed Th1/Th2 cytokine gene expression. We hypothesise that IFNγ expression in the omentum at different timepoints, D2 and D7, may be a result of different stimuli. Expression at D2 could perhaps be a result of a previously unknown innate response in the omentum to *H. polygyrus* infection. As described in Chapter 5, this could be a result of innate immune cells such as NK cells in the omentum responding to LPS in the blood, due to early barrier breach during infection. On the other hand, IFNγ expression at D7 of infection may be a result of IFNγ producing immune cells homing to the omentum. Further experiments are required to understand immune responses in the omentum during *H. polygyrus* infection. Targeting the movement of immune cells in and out of the omentum without preventing trafficking to mucosal sites is difficult due to the expression of gut homing ligands on blood vessels in the omentum (Carlow et al., 2009, Meza-Perez and Randall, 2017, Berberich et al., 2008). In-depth analysis of immune cells in the omentum prior to and during *H. polygyrus* infection using single-cell RNA sequencing would contribute to our understanding of immune cell subsets at this site. In addition, this would demonstrate how immune cell subsets in the SILP change as a result of cell proliferation or recruitment in the omentum during *H. polygyrus* infection. However, the stimulus for IFNγ responses during *H. polygyrus* infection are unclear, and we hypothesised that as we see mixed Th1/Th2 responses in the omentum, a distal site from the small intestine, that translocation of bacteria may occur in *H. polygyrus* infection.

The lack of severe barrier breach we observed during *H. polygyrus* does not reflect the conditions, such as in the DSS model of colitis, where prolonged destruction of the epithelial barrier results in bacterial translocation and subsequent Th1/17 driven immunopathology (Eichele and Kharbanda, 2017). Despite this, we hypothesise a potential reason for a lack of extensive barrier breach could be due to *H. polygyrus* promoting repair as it moves through the wall of gut. *H. polygyrus* may have evolved this mechanism to limit barrier breach during infection as this would result in a damaging immune response to the host, which could be detrimental to parasite persistence. The increase in TJ

proteins we found at the early timepoints of infection may be as a result of HES induced changes to increase gene expression and therefore limiting epithelial barrier disruption. In keeping with this concept, an effect of *H. polygyrus* in modulating TJ proteins in the colon, which is distal from the site of infection, has been described (Su et al., 2011). An experiment that would help to address this hypothesis, would be to carry out microinjections of HES into intestinal organoids and subsequently analyse TJ proteins at both the gene and protein level. Understanding mechanisms where *H. polygyrus* modulates the immune system and barrier sites are key for identifying proteins secreted by this parasite that may have therapeutic potential.

A recent concept depicted in the literature is the moulding of Th effector cell subsets in the SILP by the microbiota (Kiner et al., 2021). This is an interesting concept when considering *H. polygyrus* infection. Perhaps the changes induced in the microbiota by *H. polygyrus* is a detrimental mechanism of action, resulting in a pro-Th2 shift in the microbiota. One way to address this would be to treat Th cells from the MLN *ex vivo* with bacteria species such as Lactobacillaceae and Enterobacteriaceae. These species are expanded during *H. polygyrus* infection and may skew to a Th2 phenotype, although Lactobacillaceae species have been shown to promote Tregs *in* vivo (Morris et al., 2017, Walk et al., 2010, Reynolds et al., 2012). Another way to test this would be to treat mice with antibiotics, to remove most commensal bacteria, and reconstitute mice with a single bacterium, such as Lactobacillus. Subsequent infection of these mice with *H. polygyrus* and measuring the role of the resulting Th1/2 responses may elucidate precise roles of different commensal bacteria in skewing responses to *H. polygyrus* infection. Importantly, *H. polygyrus* infection of GF mice, which lack an intestinal microbiome, have an increased Th2 response (Russell et al., 2020). However, GF mice have underdeveloped SLOs and mucosal immune system, which would affect results when considering the role of bacteria in influencing Th responses in this model (Bauer et al., 1963, Macpherson and Harris, 2004). Furthermore, the effect of the microbiota may not be directly on T cells. Commensal bacteria could also perhaps modulate other immune subsets that indirectly result in changes to Th responses. Therefore, commensal bacteria are an interesting and important factor to consider when investigating immune competition in infections such as *H.*

polygyrus, particularly when the parasite itself alters the presence of bacterial species.

Our data and the literature suggest that a multitude of factors may induce IFNγ expression during *H. polygyrus* infection. However, an interesting concept which requires further investigation is the priming of *H. polygyrus* antigen specific IFNγ producing Th1 cells in the MLN. The first step in answering if *H. polygyrus* specific IFNγ response occur, would be to stimulate MLN cells from *H. polygyrus* infected mice with isolated proteins from HES, such as the venom allergen-like (VAL) proteins which have shown to be associated with the surface of *H. polygyrus* (Hewitson et al., 2011b). This experiment would be a first step in identifying *H. polygyrus* antigen specific Th1 and Th2 cells. Furthermore, the generation of *H. polygyrus* antigen tetramers would allow Th cells specific for *H. polygyrus* to be tracked during infection and allow us to determine if *H. polygyrus* specific Th1 cells are part of the immune response to infection. However, the immunogenic antigen has yet to be identified in *H. polygyrus* infection, although currently there are projects underway by the Perona-Wright and Maizels laboratories at the University of Glasgow that aim to address this. A potential alternative method would be to genetically modify the parasite itself, for example, to create a model where *H. polygyrus* surface proteins such as VAL proteins are fluorescently labelled. Using flow cytometry, DCs expressing fluorescent VAL antigen on MHCII in the MLN could be identified. This would also take the field closer to identifying the immunogenic antigen in *H. polygyrus* infection. Excitingly, a recent publication on *N. brasiliensis,* described a new method for genetically modifying helminths that could be applied to *H. polygyrus* (Hagen et al., 2021). Together, to further our understanding of immune competition and IFNγ secretion *in H. polygyrus* infection, further experiments using new techniques are required.

6.2 Conclusions

In this thesis, I aimed to answer questions surrounding the regulation of type 2 immune responses by IL-10 and to address if immune competition occurs in *H. polygyrus* infection. The optimisation of a method for the isolation of leukocytes from the SILP of *H. polygyrus* infected mice allowed us to address these questions. Through the analysis of both the SILP and MLN we have demonstrated

a tissue-specific role for IL-10 in promoting optimal Th2 responses in the SILP. We report evidence that IL-10 supporting Th2 responses is mediated through,

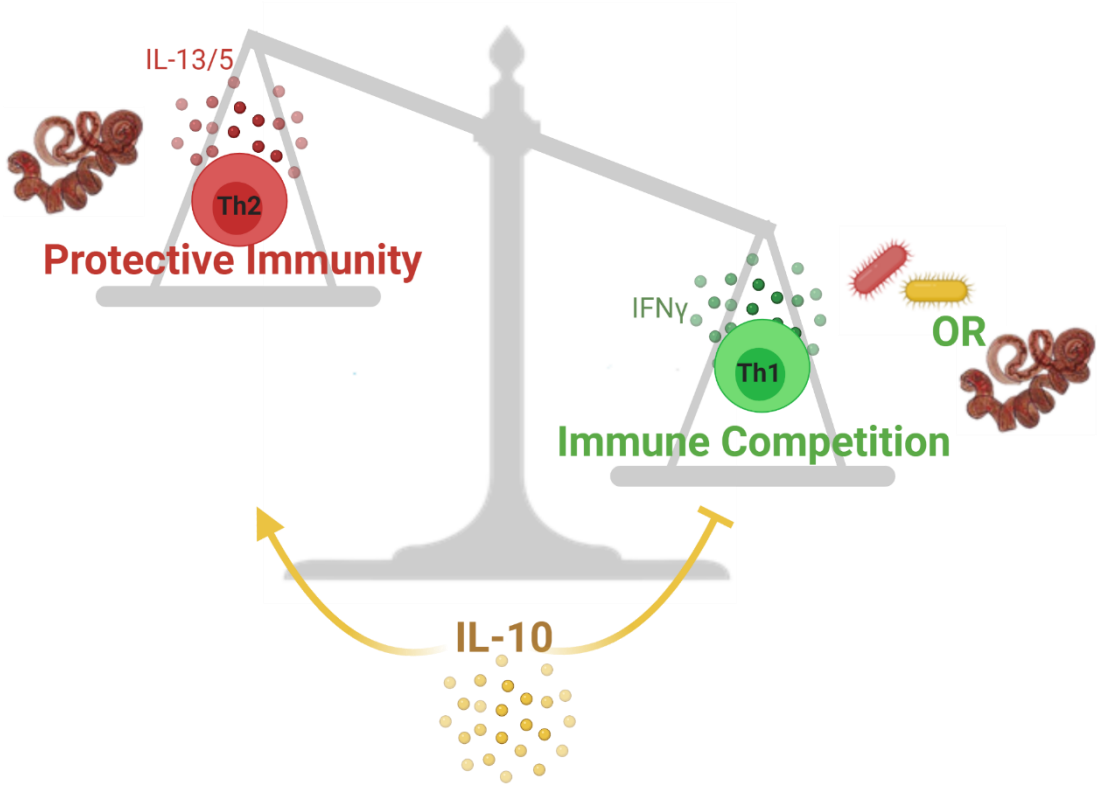

Figure 6-3 The role of IL-10 in promoting Th2 responses to *H. polygyrus* by limiting immune competition

direct instruction of Th2 cells and perhaps due to indirect suppression of Th1 cells to eliminate immune competition (Figure 6-3). These data demonstrate a previously unreported role for IL-10 in the intestine during helminth infection which contributes to the understanding of cytokine regulation during *H. polygyrus* infection. This finding would also be interesting to explore in other helminth models and type 2 mediated disease, although, comparing our data to published work, we hypothesise that IL-10 promotion of Th2 responses does not occur in setting of severe tissue damage.

The observation that IL-10 might be driving Th2 responses, in part through IFNγ suppression led us to examine IFNγ producing Th1 cells during *H. polygyrus* infection. From these studies, we have demonstrated that increased intestinal permeability and inflammation may result in minor barrier breach during *H. polygyrus* infection, but preliminary data indicates a lack of bacterial translocation in this model. However, we do report IFNγ producing $CD4^+$ T cells

in the MLN that are not present in the SILP, perhaps in part due to IL-10 mediated suppression in the infected tissue. The antigen stimulus for the induction of this Th1 response is unclear, but we hypothesise that these Th1 cells are specific for *H. polygyrus* itself, as HES stimulated cells released IFNγ as well as type 2 cytokines. The lack of severe barrier breach and little evidence for bacterial translocation that we report raises questions of the stimulus, mechanism, and reasoning for an underlying Th1 response, either to *H. polygyrus* itself or to bacteria. Together, these data advance our knowledge of immune competition and cytokine responses to *H. polygyrus* which may contribute towards the improvement of anti-helminth strategies. In addition, we have elucidated a role for IL-10 in promoting Th2 responses in the context of helminth infection. Future experiments should assess this mechanism in different mucosal tissues, in different helminth models and in other type 2 mediated disease as understanding cytokine regulation in these settings is essential.

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