Characterising Trickle Infection and Polyparasitism of Gastrointestinal Helminths

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine, & Health

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“Hence, though my philosophical studies have been pursued with more zeal than profundity, I have nevertheless given myself all possible trouble and have taken the greatest care to convince myself of facts with my own eyes by means of accurate and continued experiments before submitting them to my mind as matter for reflection. In this manner, though I may not have arrived at perfect knowledge of anything, I have gone far enough to perceive that I am still entirely ignorant of many things the nature of which I supposed was known to me, and when I discover a palpable falsehood in ancient writings or in modern belief, I feel so irresolute and doubtful of my own knowledge that I scarcely dare attack it without first consulting some learned and prudent friends.”

Francesco Redi - *Esperienze Interno alla Generazione Deglinsetti*
1 Introduction

1.1 Gastrointestinal Helminths: A Global Health Problem

1.1.1 Epidemiology of GI Helminths

1.1.2 Helminths and Co-infection

1.2 Human Immunity to Gastrointestinal Helminths

1.3 Murine Models of Helminth Infection

1.3.1 *Trichuris muris*

1.3.2 *Heligmosomoides polygyrus*

1.4 Immune Responses to *T. muris*

1.4.1 The Innate Response

1.4.2 The Adaptive Response

1.4.3 Effectors of Expulsion

1.5 Immune Responses to *H. polygyrus*

1.5.1 The Innate Response
1.5.2 The Adaptive Response ........................................... 38
1.5.3 Physiological Effectors of Parasite Expulsion .......... 40
1.6 Helminth-mediated Immunomodulation ......................... 41
  1.6.1 T. muris ......................................................... 41
  1.6.2 H. polygyrus ..................................................... 42
1.7 Modelling Natural Infection ....................................... 44
  1.7.1 Trickle Infection ............................................... 45
  1.7.2 Co-Infection ..................................................... 46
  1.7.3 Diet ............................................................. 46
1.8 The Gut Microbiome ............................................... 47
  1.8.1 Helminths and the Microbiota ............................... 50
1.9 Project Aims and Objectives .................................... 51

2 Materials & Methods .................................................. 53
  2.1 Animals ............................................................ 54
    2.1.1 Procedures ................................................... 54
    2.1.2 Measurement of Bodyfat ................................. 55
  2.2 Parasites ........................................................... 55
    2.2.1 Trichuris muris .............................................. 55
    2.2.2 Heligmosomoides polygyrus ............................. 57
    2.2.3 Faecal Egg Counts ........................................ 59
  2.3 Cytokine Analysis ................................................ 60
    2.3.1 Collection and Re-stimulation of Mesenteric Lymph Nodes 60
    2.3.2 Cytometric Bead Array .................................. 60
2.4 ELISAs ................................................................. 61
  2.4.1 Serum IgG ELISA ............................................. 61
  2.4.2 Serum mMCP-1 ELISA ....................................... 61
2.5 Flow Cytometry ..................................................... 62
2.6 Histology .............................................................. 63
  2.6.1 Periodic Acid Schiff .......................................... 64
  2.6.2 Alcian Blue ....................................................... 64
  2.6.3 Immunofluorescence Microscopy ............................. 64
2.7 Microbial Metagenomics .......................................... 65
  2.7.1 Extraction of DNA from Faecal Samples .................. 65
  2.7.2 16S Library Preparation ...................................... 66
  2.7.3 DNA Sequencing and Data Analysis ......................... 67
2.8 Quantification of Gene Expression by PCR ..................... 68
  2.8.1 RNA Extraction ................................................ 68
  2.8.2 cDNA synthesis ............................................... 68
  2.8.3 Quantitative PCR .............................................. 69
2.9 Statistical Analysis and Data Presentation .................... 70

3 Characterisation of the Immune Response to \textit{H. polygyrus} Trickle Infection ................................................................. 71
  3.1 Introduction ......................................................... 72
  3.2 Results ............................................................... 75
    3.2.1 Trickle infection results in protection against subsequent infection and drives expulsion of established parasites. .. 75
3.2.2 Cytokine expression in the Mesenteric Lymph Node during trickle and single dose infections 78

3.2.3 Gene expression in the duodenum during trickle and single dose infections 82

3.2.4 Cytokine expression in the lung during trickle and single dose infections 85

3.2.5 Mucosal mast cell response during *H. polygyrus* infection 85

3.2.6 IgG1 Response During *H. polygyrus* infection 89

3.2.7 αβ-T cells are essential for the expulsion of *H. polygyrus* during a trickle infection 91

3.2.8 Diet induced obesity does not affect expulsion of *H. polygyrus* 95

3.2.9 The effect of co-infection with *H. polygyrus* on the dynamics of *T. muris* trickle infection 99

3.3 Discussion 106

3.3.1 *H. polygyrus* trickle induces both resistance and parasite expulsion 106

3.3.2 Trickle infection drives a potent Th2 response in the duodenum and draining lymph nodes 107

3.3.3 Trickle-induced expulsion of *H. polygyrus* is dependent on αβ-T cells 108

3.3.4 DIO does not appear to affect resistance to *H. polygyrus* 109

3.3.5 Co-infection with *H. polygyrus* does not prevent trickle-induced immunity to *T. muris* 111

4 Chronic Helminthiases and the Microbiota 113

4.1 Introduction 114

4.2 Results 116
4.2.1 Low dose *T. muris* infection causes chronic non-resolving dysbiosis ........................................ 116

4.2.2 The microbiota changes dynamically in response to changes in immunity to *T. muris* ........................................ 123

4.2.3 *H. polygyrus* has little effect on the faecal microbiota ...... 132

4.2.4 Diet and the Microbiota ........................................ 136

4.3 Discussion .......................................................... 144

4.3.1 *T. muris* and the Microbiota .................................. 144

4.3.2 *H. polygyrus* and the Microbiota .................................. 146

4.3.3 Diet and the Microbiota ........................................ 148

4.3.4 Microbial Hallmarks of *T. muris* Infection ................. 149

5 The Effect of Co-infection on the Expulsion of *Trichuris muris* 153

5.1 Introduction .......................................................... 154

5.2 Results ............................................................. 156

5.2.1 Co-infection with *H. polygyrus* prevents expulsion of a high-dose *T. muris* infection ........................................ 156

5.2.2 Effect of co-infection of levels of parasite-specific serum IgG isotypes ........................................ 156

5.2.3 Cellular immune responses in the MLN during co-infection 160

5.2.4 Pathophysiological responses in the caecum during co-infection ........................................ 166

5.2.5 Co-infection in immune-deficient mice ....................... 170

5.2.6 Infection with *H. polygyrus* does not affect immunisation-induced immunity to infection with *T. muris* .............. 172

5.2.7 HpES can recapitulate the effect of co-infection on *T. muris* high-dose infection ........................................ 175
5.3 Discussion .......................................................... 178

5.3.1 Co-infection with *H. polygyrus* prevents expulsion of a high-dose *T. muris* infection .................................................. 178

5.3.2 Immune-independent helminth-helminth interactions .......... 180

5.3.3 Co-infection does not appear to inhibit immunisation-induced immunity to *T. muris* .................................................. 182

5.3.4 HpES prevents resistance to high dose *T. muris* infection .. 183

6 Discussion ...................................................................... 185

6.1 Trickle infection and the development of resistance to helminth infection ................................................................. 187

6.2 Co-infection and immunity to parasitic infection ................. 190

6.3 Helminths and the microbiota ........................................... 192

6.4 Concluding remarks ...................................................... 194

References ........................................................................ 195

Word Count: 42,000
# List of Figures

1.1 *H. polygyrus* and *T. muris* are enteric parasites that transmit via the faecal-oral route. .............................. 26

1.2 CD4+ Cell Differentiation ................................................................. 33

1.3 Gastrointestinal Mucosal Responses to *T. muris* Infection ............. 35

1.4 Interactions Between the Host, Helminths & the Microbiota .......... 49

3.1 Schematic representation of Infection Strategy and Treatment Groups 74

3.2 Intestinal Worm Burden of Mice Infected with *H. polygyrus* ........ 76

3.3 Faecal Egg Burden of Mice Infected with *H. polygyrus* ............... 77

3.4 Cytokine expression in the Mesenteric Lymph Nodes of *H. polygyrus* infected mice .................................. 81

3.5 Gene expression in the duodenum of *H. polygyrus* infected mice. . 84

3.6 Cytokine expression in the lung of *H. polygyrus* infected mice. . . 87

3.7 Serum levels of mMCP-1 in *H. polygyrus* infected mice. ............... 88

3.8 Serum levels of parasite-specific IgG1 in *H. polygyrus* infected mice. 90

3.9 Expulsion of *H. polygyrus* in *Tcrα-/-* mice ................................. 92

3.10 Serum levels of *H. polygyrus*-specific IgG1 in *Tcrα-/-* mice .......... 93

3.11 Gene expression *Tcrα-/-* mice ....................................................... 94

3.12 Diet induced weight change during *H. polygyrus* infection ........... 98

3.13 Effect of diet induced obesity on *H. polygyrus* infection ............. 98

3.14 The effect of single dose *H. polygyrus* co-infection on *T. muris* trickle infection ................................................. 100
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.15</td>
<td>The effect of trickle <em>H. polygyrus</em> co-infection on <em>T. muris</em> trickle infection</td>
<td>101</td>
</tr>
<tr>
<td>3.16</td>
<td>Histology of caecal tissue</td>
<td>103</td>
</tr>
<tr>
<td>3.17</td>
<td>Analysis of Caecal Histology</td>
<td>105</td>
</tr>
<tr>
<td>4.1</td>
<td>16S Sequencing of stool from low dose <em>T. muris</em> infection.</td>
<td>117</td>
</tr>
<tr>
<td>4.2</td>
<td>NMDS analysis of global microbiota composition during <em>T. muris</em> low dose infection</td>
<td>118</td>
</tr>
<tr>
<td>4.3</td>
<td>Phyla-level analysis of faecal microbiota during low dose <em>T. muris</em> infection</td>
<td>120</td>
</tr>
<tr>
<td>4.4</td>
<td>Differential expression analysis of significantly different OTUs during <em>T. muris</em> low dose infection</td>
<td>122</td>
</tr>
<tr>
<td>4.5</td>
<td>16S Sequencing of stool from <em>T. muris</em> trickle infection.</td>
<td>124</td>
</tr>
<tr>
<td>4.6</td>
<td>NMDS analysis of global microbiota composition during <em>T. muris</em> trickle infection</td>
<td>125</td>
</tr>
<tr>
<td>4.7</td>
<td>Phyla-level analysis of faecal microbiota during low dose <em>T. muris</em> infection</td>
<td>126</td>
</tr>
<tr>
<td>4.8</td>
<td>Relative abundance of highly represented genera during <em>T. muris</em> Trickle infection</td>
<td>131</td>
</tr>
<tr>
<td>4.9</td>
<td>Relative abundance of selected genera during <em>T. muris</em> Trickle infection</td>
<td>131</td>
</tr>
<tr>
<td>4.10</td>
<td>Differential expression analysis of OTUs during <em>T. muris</em> trickle infection</td>
<td>131</td>
</tr>
<tr>
<td>4.11</td>
<td>16S Sequencing of stool from <em>H. polygyrus</em> infection.</td>
<td>135</td>
</tr>
<tr>
<td>4.12</td>
<td>16S Sequencing of stool from <em>H. polygyrus</em> trickle infection.</td>
<td>135</td>
</tr>
<tr>
<td>4.13</td>
<td>16S Sequencing of stool from low dose <em>T. muris</em> infection on a HFD.</td>
<td>138</td>
</tr>
<tr>
<td>4.14</td>
<td>NMDS analysis of faecal microbiota during HFD and <em>T. muris</em> infection</td>
<td>139</td>
</tr>
</tbody>
</table>
4.15 Alpha diversity of the faecal microbiota in mice infected with *T. muris* on a HFD. .................................................. 140

4.16 Relative abundance of highly represented genera during *T. muris* infection in mice on specialised diets. .......................... 141

4.17 Differential expression analysis of OTUs during *T. muris* infection on a LFD. .................................................. 143

5.1 Co-infection with *H. polygyrus* inhibits *T. muris* expulsion ...... 157

5.2 *H. polygyrus* burden does not correlate with *T. muris* burden in co-infected mice .................................................. 158

5.3 Co-infection with *H. polygyrus* changes the ratio of anti-*T. muris* serum IgG isotypes .................................................. 159

5.4 Enumeration of cMLN cells during co-infection ......................... 161

5.5 Cytokine production by MLN cells during co-infection ............... 162

5.6 Gating strategies for flow cytometry of MLN cells .................... 164

5.7 Flow cytometric analysis of MLN cells during co-infection .......... 165

5.8 Gene expression analysis in the Caecum and Duodenum during co-infection .................................................. 167

5.9 Histological analysis of the caecum during co-infection .............. 168

5.10 pSmad2/3 positive cells during co-infection .......................... 169

5.11 *T. muris* fitness during co-infection in SCID mice .................. 171

5.12 The effect of *H. polygyrus* infection on immunisation against *T. muris* .......................... 173

5.13 The effect of *H. polygyrus* infection on immunisation-induced caecal responses to *T. muris* .................................................. 174

5.14 The effect of HpES on infection with *T. muris* ...................... 176

5.15 The effect of HpES on caecal responses to *T. muris* ................ 177
List of Tables

2.1 List of Antibodies used for Flow Cytometry .................................. 63
2.2 List of Antibodies used for Immunofluorescence Microscopy ... 65
2.3 List of primers used for qPCR ...................................................... 70
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Abstract

Infection with gastrointestinal helminths is a significant cause of global morbidity. Given the challenges of investigating helminth infection in humans, murine models of infection have been central in defining the mechanisms that determine the outcome of infection. Two of the most well-characterised model helminths are the whipworm *Trichuris muris* and the hookworm *Heligomosomoides polygyrus*. The use of these parasites has cemented a critical role for Th2 immunity in the expulsion of parasitic nematodes. However, the majority of these studies have utilised experimental models in which single large boluses of an individual parasite are given, and analysis of subsequent immune responses are measured over a relatively narrow window of time. However, *in situ* individuals are more likely to be exposed to frequent re-infection, and are likely to be co-infected with multiple species. In this thesis both re-infection and co-infection were experimentally modeled. Trickle infection of *H. polygyrus* revealed that through multiple infections a more potent Th2 immune response develops that not only confers anti-larval immunity but also allows for a more rapid expulsion of adult parasites. This effect was dependant on $\alpha\beta$-T cells. Co-infection of *H. polygyrus* and *T. muris* demonstrated that co-infection reduced resistance to *T. muris*. This correlated with the reduced cellularity of the colonic mesenteric lymph node and an increase in the fitness of adult female *Trichuris* worms. However, overt changes in canonical markers of resistance to *T. muris* remained unaffected suggesting the presence of as yet undefined modes of susceptibility and resistance to infection. During infection, an intimate relationship exists between the host, the parasite and microbiota, and it is thought that the microbiota plays an important role in directing host immune responses. However, there is a limited amount of data on the effect of helminth infection on the microbiome. Here a range of infection models were used to assess changes in the microbiota driven by helminths, these were then correlated with resistance and susceptibility to define bacteria groups connected to the outcome of infection. These include the *Bacteroides* and *Escherichia-Shigella* genera. Collectively the data presented here highlight a need for the adaptation of conventional infection models to better reflect natural modes of infection.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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List of Abbreviations

aaMφs Alternatively Activated Macrophages
BAL Bronchoalveolar Lavage
BCG Bacillus Calmette-Gurin
BCR B cell Receptor
BrdU 5-bromo-2’-deoxyuridine
CBA Cytometric Bead Array
CCP Complement Control Protein
d.p.i. days post infection
DC Dendritic Cell
DIO Diet-induced Obesity
DSS Dextran Sulphate Sodium
ELISA Enzyme Linked Immunosorbant Assay
EV Extracellular Vesicle
FCS Foetal Calf Serum
GI Gastrointestinal
HpAg *H. polygyrus* Whole Worm Homogenate
HpARI *H. polygyrus* Alarmin Release Inhibitor
HpES *H. polygyrus* Excretory Secretory Products
HS Heparan Sulphate
IBD Inflammatory Bowl Disease
IF Immunofluorescence
IFN Interferon
Ig Immunoglobulin
IL Interleukin
ILC Innate Lymphoid Cell
LN Lymph Node
MHC Major Histocompatibility complex
miRNA micro RNA
MLN Mesenteric Lymph Node
mMCP mucosal Mast Cell Protease
NBF Neutral Buffered Formalin
NMDS Non-metric MultiDimensional Scaling
NOD Nucleotide Oligomerization Domain
OTU Operational Taxonomic Unit
PAMP Pattern Associated Molecular Patterns
PBMC Peripheral Blood Mononuclear Cell
PBS Phosphate Buffered Saline
PCR Polymerase Chain Reaction
qPCR quantitative PCR
RELM Resistin-like Molecule
SCID Severe Combined Immunodeficient
SEM Standard Error of the Mean
TCR T cell Receptor
TGF Transforming Growth Factor
Th T helper
TLR Toll Like Receptor
TmES *T. muris* Excretory Secretory Products
TNF Tumor Necrosis Factor
Treg Regulatory T cell
TSLP Thymic Stromal Lymphopoietin
w.p.i. weeks post infection
Chapter 1

Introduction
1.1 Gastrointestinal Helminths: A Global Health Problem

Gastrointestinal (GI) helminths remain one of the most prevalent sources of human morbidity globally. It is estimated that somewhere between 1 and 1.5 billion people are infected with at least one species of GI helminth\textsuperscript{1,2}. The collection of helminth species that infect humans is diverse, however, four species are responsible for the majority of the associated morbidity: the whipworm \textit{Trichuris trichiura}, the hookworms \textit{Necator americanus} and \textit{Ancylostoma duodenale}, and the large roundworm \textit{Ascaris lumbricoides}. These infections disproportionately impact those of low socioeconomic status, particularly children and pregnant women, and place a considerable medical, social and economic burden on the regions in which they are endemic\textsuperscript{3,4}.

Whilst protective immunity to infection does develop, it rarely results in complete protection against infection\textsuperscript{5}. Further, the most severe health effects of chronic helminthiasis occur in young children, meaning that the generation of partial immunity in late adolescence is likely too late to limit helminth-associated morbidity\textsuperscript{6}. Whilst effective anthelminthics are available, wide spread drug administration programmes have been largely ineffective in controlling infection. This is due to the inability of anthelminthics to confer protective immunity. As such rapid re-infection, from long-lived environmental reservoirs of the parasites, occurs once treatment is removed\textsuperscript{4}.

1.1.1 Epidemiology of GI Helminths

GI helminths are broadly distributed across the globe with all four of the major species present in Asia, Africa, Latin America, and Oceana\textsuperscript{7}. These infections have been largely eradicated from Europe and North America through industrialisation and improved infrastructure for sanitation, although hookworm infection is still present in the southern parts of the United States. As such, helminth infection is largely a disease of poverty impacting most severely on developing nations. The highest prevalences are found in the tropics where moist warm conditions are optimal for the transmission stages of each helminth.
Within a given population, parasitic infection correlates strongly with age. *Trichuris* burdens rapidly build in early childhood peaking around 10 years of age. Burden then steadily declines and plateaus after adolescence, leaving individuals with a chronic low-level infection throughout the rest of their lives\(^6,8,9\). Similarly, hookworm infections rapidly building during childhood, but plateau from adolescence into adulthood as resistance develops. This is problematic as it creates a reservoir of largely asymptomatic individuals generating new infectious stages. The dynamic relationship between age and intensity of infection likely demonstrates the slow development of protective immunity and is remarkably robust across different populations.

### 1.1.2 Helminths and Co-infection

Due to their overlapping geographical distribution, and shared ecological niche, a common feature of individuals in endemic areas is concurrent infection with multiple species of helminth. In fact, in most study cohorts, co-infection appears to be the most common infection status, with mono-infected individuals being quite rare\(^10–13\). Interestingly, co-infection was associated with higher burden of infection for each individual species\(^11,12\). What is unclear is whether this relationship is simply correlative – i.e. individuals susceptible to one species may simply be more likely to be susceptible to another – or if synergistic relationships exist between helminths where by infection with one promotes the survival of another. Co-infection puts individuals at greater risk of disease-associated morbidity, and can have significant implications for the generation of therapeutics which typically begin development in single-parasite models of infection. This has been a particular challenge in treating Onchoceriasis, caused by the filarial worm *Onchocerca volvulus*, where use of standard anti-parasitics results in severe complications in individuals co-infected with *Loa loa* (another filarial helminth)\(^14,15\).
1.2 Human Immunity to Gastrointestinal Helminths

Within an infected population considerable variation in burden of infection and severity of disease can be observed. As discussed above, age is one key factor in determining susceptibility to infection. However, little is known about what determines whether an individual will be resistant to infection or not. Studies in humans are largely limited to observations from easily collected peripheral tissue such as the blood or the stool, and are often confounded by a large range of variables such as sex, previous infection history, co-infection with other pathogens etc.

What is well understood is that helminth infection results in the induction of a potent type 2 (Th2) immune response. *Ex vivo* analysis of peripheral blood mononuclear cells (PBMCs) showed that those derived from individuals infected with helminths produced significant levels of Th2-associated cytokines, such as Interleukin (IL)-4, IL-5, & IL-13. Infection was also associated with an increase in peripheral blood eosinophils, which are known to be regulated by IL-5. Further, individuals with higher levels of IL-5 prior to antihelminthic treatment suffered lower worm burden on subsequent re-infection with *A. lumbricoides*, *T. trichiura*, and *N. americanus*. These data show a clear correlation between Th2-associated immunity and infection, and suggest that an individual’s capacity to generate a strong Th2 cytokine response is at least predictive of their resistance to subsequent reinfection. In accord with the notion that immunity to infection increases with age, the frequency of individuals responding to parasite antigen by producing Th2-associated cytokine increases with age.

GI helminth infections exist primarily at mucosal barrier sites. Analysis of rectal biopsies from children suffering from Trichuriasis showed significant mastocytosis, an increased presence of IgE+ cells, and spontaneous production of histamine, indicating an immediate hypersensitivity reaction was occurring in the rectal mucosa. Increased levels of serum IgE have been negatively correlated with hookworm weight and fecundity. Additionally, *T. trichiura* infected individuals show increased rates of epithelial cell proliferation.
Of considerable interest in recent years is the inverse correlation between the prevalence of GI helminths and rates of allergic disease\textsuperscript{27,28}. This has led to the hypothesis that there is a causal relationship between the absence of helminth infection and the acquisition of allergies and autoimmune disorders\textsuperscript{29}. One proposed explanation for this is that helminths directly induce immunosuppressive pathways in their host in order to promote their own survival, the side effect of which is a global suppression of aggressive inflammatory conditions. Indeed a wealth of studies have shown considerable reduction in allergic skin reactivity in individuals infected with helminths\textsuperscript{30–32}. This effect is lost upon the termination of infection by treatment with antihelminthics\textsuperscript{33,34}. Resulting from these observations has been a considerable effort in attempting to use controlled helminth infections as a therapeutic, so called ‘helminth therapy,’ particularly for inflammatory bowel disease (IBD). Both \textit{Trichuris suis} and \textit{N. americanus} have been repeatedly trialed, however, success has been mixed with some studies finding an amelioration of symptoms\textsuperscript{35–37} while others saw no effect over the placebo\textsuperscript{38–40}. What has emerged, however, is the capacity of experimental helminth infection to reduce levels of cytokine release in response to allergen\textsuperscript{40,41}. This appears to correlate with a helminth-induced increase in immunosuppressive cytokines IL-10 and TGF\textsubscript{β}\textsuperscript{19,41}.

Together these data demonstrate a role for Th2 immunity in resistance to helminth infection. This response was associated with physiological changes at the GI mucosal barrier. However, they also indicate a reciprocal interaction with the parasite whereby helminths may functions as immuno-modulators in the host, regulating host immunity to promote their own survival.
Figure 1.1: *H. polygyrus* and *T. muris* are enteric parasites that transmit via the faecal-oral route. (A) [1] The embryonated eggs of *T. muris (Tm)* are ingested and make their way into the digestive system. [2] When they reach the caecum, interaction with the bacterial flora initiates hatching. The L1 larvae burrow into the epithelial layer where they undergo four moults. As they grow they extend into the lumen of the gut where the adults worms mature and begin to reproduce 32 days p.i. [3] As the adults mate they release eggs into the faeces which are egested into the soil [4] where the eggs embryonate over the course of two months. [5] These eggs are then ready for ingestion by a new host. (B) [1] The free-living L3 stage of *H. polygyrus* larvae are ingested by the host – under experimental conditions this is achieved by oral gavage. [2] When the larvae reach the proximal duodenum they penetrate the epithelium and encyst within the sub-mucosa for seven to nine days. [3] Having undergone a further two moults the now adult worms emerge into the lumen of the duodenum. Here they coil around the intestinal villi and mate. [4] Eggs produced by mated females are released into the faeces which are then egested by the host. [5] The eggs hatch in the faeces and the newly released L1 larvae undergo several moults into L3 larvae capable of infecting a new host.
1.3 Murine Models of Helminth Infection

Given the limitations of studying helminthiases in humans, robust laboratory models of infection are essential in elucidating mechanisms of immunity to infection. Of considerable advantage is the existence of a large number of naturally occurring rodent-specific helminths, many of which are relatively evolutionarily close to human helminths. A number of these have been adapted to the laboratory and have become essential to our understanding of immune responses to parasitic infection. Here we will focus on two of the most well characterised model species, the whipworm *Trichuris muris* and the hookworm *Heligmosomoides polygyrus*.

1.3.1 *Trichuris muris*

Closely related to the human *T. trichiura*, *T. muris* has been a robust model for human trichuriasis. They share highly similar morphologies and lifecycle. In addition, of the roughly five thousand directly orthologous genes shared between the two, these genes show 79% similarity in amino acid sequence on average. Infecting most mouse strains with a low dose of *T. muris* (< 25 eggs) results in a chronic infection that persists for several months and thus provides a model for natural chronic trichuriasis. Additionally, resistance can be generated in mice by infecting with a large dose of eggs allowing for investigation into modes of expulsion and immunity.

*T. muris* follows a strictly enteric lifecycle being transmitted through the faecal-oral route (Fig. 1.1 A). Infection begins when embryonated eggs are ingested. In *situ* this would be through the consumption of contaminated food or water, but under laboratory conditions accurate dosing can be achieved by oral gavage. When the eggs reach the caecum direct contact with the caecal microbiota initiates hatching. Released L1 stage larvae then rapidly migrate to the base of the intestinal crypts where they burrow into the epithelial layer. Here they form a syncytial tunnel through the epithelium, never breaking past the basement membrane into the lamina propria. The larvae then undergo four moult before reaching adulthood at 32 days post infection. During development the parasites move from occupying...
cells at the base of the crypt to cells higher up the crypt, eventually occupying cells at the crypt apex. As they moult the anterior end remains firmly inside its syncytial tunnel anchoring the parasite to the host. The posterior end slowly extends into the gut lumen to allow for reproduction. Once mating has occurred the females release eggs into the caecal content which are then passed in the faeces. The eggs then require approximately two months of incubation to become embryonated and thus infective.

1.3.2 Heligmosomoides polygyrus

*H. polygyrus* is a widely prevalent helminth of wild mice and has been adapted as a laboratory model for chronic helminth infection. It is used as representative of human hookworm infection as it has a tissue stage and a long lived presence in the lumen of the duodenum. Patent infection with *H. polygyrus* is easily established, regardless of dose, with mice being highly susceptible to a primary infection. Immunity to *H. polygyrus* can be modelled using secondary challenge infection in which a strong protective response is generated against newly invasive larvae.

Infection begins when L3 stage larvae are ingested (Fig. 1.1 B). As they reach the duodenum they rapidly penetrate through the submucosa into the lamina propria. Here they encyst for seven to nine days, in which time they transition through two moults to become adults. The adults then break back through the submucosa into the luminal space where they use their coiled forms to wrap around the intestinal villi. Secured to the epithelium they mate. Eggs can be detected in the faeces as shortly as two weeks post infection. Under damp conditions they hatch in the external environment and undergo two moults to reach L3 stage where they developmentally arrest until ingested.

1.4 Immune Responses to *T. muris*

The dynamics of infection with *T. muris* are dependent on both infective dose and genetic background of the mouse. In both C57BL/6 and BALB/c mice, infection
with a high dose of eggs (i.e. usually greater than 200 eggs) results in expulsion of the parasite before they reach patency. In BALB/c mice this occurs by 21 days post infection, and by 27 days post infection in C57BL/6s\textsuperscript{46,47}. However, when the same strains are given a low dose infection, the parasites reach sexual maturity and may persist for several months\textsuperscript{46}. Why there is a dose dependent response to infection remains an elusive question. However, it appears that there is dose-dependent polarisation between Th1 and Th2 immune responses, with Th2 immunity required for successful expulsion.

1.4.1 The Innate Response

Throughout infection dramatic changes in immune cell populations occur. This is true of the innate compartment, in which considerable eosinophilia, mastocytosis and basophilia have been described following high dose \textit{T. muris} infection\textsuperscript{48–50}. Recruitment and activation of these populations is characteristic of Th2-mediated inflammation, and their potential to serve as reservoirs of rapidly releasable IL-4\textsuperscript{51} make them likely candidates for driving Th2-specific adaptive responses.

Excessive eosinophilia is associated with Th2 driven allergic inflammation leading to wide speculation that its function is as an effector in Th2 responses to parasites\textsuperscript{21}. Indeed eosinophils accumulate following infection with \textit{T. muris} and produce IL-4\textsuperscript{48}. Mast cells have the capacity to release vast amounts of mucosal Mast Cell Proteases (mMCPs) which may regulate barrier integrity by degradation of tight junction proteins. Levels of mMCP increase following infection which correlates with an increase in intestinal permeability\textsuperscript{49,52,53}. Despite a strong response, a functional role for these cell types during infection has yet to be established. Direct depletion of mast cells or eosinophils has no effect on expulsion\textsuperscript{54}. However, the role these cells play subsequent to infection as mediators of repair to the epithelial barrier and resolution of inflammation remains largely understudied.

By way of contrast, adoptive transfer of basophils into normally susceptible \textit{Tslpr}\textsuperscript{-/-} mice resulted in a partial restoration of resistance to \textit{T. muris} high dose infection, reinstating Th2 cytokine production and goblet cell hyperplasia\textsuperscript{50}. Further, deple-
tion of basophils resulted in an increase in susceptibility to infection\textsuperscript{55}. As well as being potent sources of IL-4, they are capable of expressing MHC-II. Indeed, the restriction of MHC-II to dendritic cells (DCs) results in the failure to generate an effective response against \textit{T. muris}\textsuperscript{55}, suggesting that basophils may play a vital role in antigen presentation. However, in other models of helminth infection, specifically \textit{Nippostrongulus brasiliensis}\textsuperscript{56} and \textit{Schistosoma mansoni}\textsuperscript{57}, depletion of basophils had no effect on generation of Th2 responses to infection. Thus, it remains unclear whether a non-redundant role for basophils is specific to \textit{T. muris} infection, or the model employed by Perrigoue \textit{et al}\textsuperscript{55}.

Recently there has been a strong focus on the role of innate lymphoid cells (ILCs), particularly ILC2s, in initiating immune responses to helminths. They can act as sources of the alarmins IL-25 and IL-33, Th2 cytokines like IL-13, and express MHC-II\textsuperscript{58}. Indeed, a non-redundant function has been demonstrated for ILC2s in generating a strong Th2 response to infection with \textit{N. brasiliensis}\textsuperscript{58,59}. However, there is little change in ILC2 number following \textit{T. muris} infection, and their depletion had no impact on resistance to high-dose infection\textsuperscript{60}. Further, there appears to be no requirement for IL-33 signalling in resistance to high-dose infection, although addition of exogenous IL-33 could enhance parasite expulsion in immunocompetent mice\textsuperscript{61,62}. This may be due to the distinct niche \textit{T. muris} occupies. Where most other model helminths colonise the small intestine, \textit{T. muris} is restricted to the caecum and proximal colon. Tuft cells, potent sources of IL-25 and thus key drivers of ILC2 expansion, exist at very low frequency in the caecum and don’t appear to respond to \textit{T. muris} infection\textsuperscript{60}. In contrast rapid expansion of Tuft cells is evident following infection with other helminths\textsuperscript{63}.

\subsection*{1.4.2 The Adaptive Response}

Whilst identifying clear essential roles for elements of the innate response has remained challenging in \textit{T. muris} infection, the non-redundant roles of the adaptive response are well characterised. Early experiments using immunocompromised mice identified a critical role for CD4\textsuperscript{+} T cells in resistance to \textit{T. muris}. Severe combined immunodeficiency (SCID) mice – which are deficient in the develop-
ment of T and B cells – are highly susceptible to infection with *T. muris*. It is only when reconstituted with CD4+ cells, not B cells or CD8+ T cells, that resistance is restored in these mice. Further, specific depletion of CD4+ cells using neutralising antibodies, in otherwise immunocompetent mice, was sufficient to induce susceptibility to infection. Indeed, during infection there is considerable increase in the number of CD4+ cells in the large intestinal mucosa. Blocking the homing of cells to the mucosa by treating mice with anti-β7-integrin and anti-MAdCAM-1 antibodies, results in loss of resistance to infection. Further, in resistant strains of mice, CD4+ cell recruitment peaks around the time of worm expulsion. However, in susceptible strains CD4+ cell numbers are highest after a chronic infection is already established.

CD4+ T cells are a highly heterogenous group of cells with a wide range of subsets. They can be divided based upon the transcription factors they express and their secreted cytokine profile (Fig. 1.2). Resistance to *T. muris* is reliant on the dominance of Th2 cells over Th1 cells, and whether a particular strain of mouse is resistant or susceptible to infection appears to be dependent on its propensity to skew towards Th1 or Th2 differentiation.

Secretion of IL-4 and IL-13 by Th2 cells has been defined as the essential factor in generating resistance to *T. muris*. Blocking the IL-4 receptor, through which both IL-4 and 13 signal, causes otherwise resistant mice to become susceptible. Whereas depletion of IFNγ, associated with Th1 cells, conferred resistance in susceptible mice. Interestingly, whilst IL-4−/− C57BL/6 mice are susceptible to infection IL-4−/− BALB/c mice are still resistance to infection. However, when IL-13 was neutralised in these mice susceptibility to infection was induced. Supporting this observation, IL-13−/− mice are susceptible to infection whilst their wild type litter-mates remain resistant. Thus, IL-13 is the crucial cytokine required in the expulsion of *T. muris*. Highlighting the essential role for Th2 polarisation in resistance to infection is the fact that chronically infected mice produce high levels of IFNγ and preferentially class switch to IgG2a/c, characteristic of a Th1 response. Additionally, treatment of normally resistant mice with recombinant IL-12 is sufficient to generate a chronic infection. These data demonstrate an
essential role of Th2 cell-derived IL-4 and IL-13 in resistance to *T. muris*, with induction of Th1 cells responsible for chronicity.

The role of the regulatory immune response remains uncertain during *T. muris* infection. Chronic infection is associated with an increase both in FoxP3+ cells and IL-10 production\(^74,75\). Whilst, IL-10\(^{-/-}\) mice are susceptible to high-dose infection\(^76\), antibody depletion of FoxP3+ cells has had either no effect\(^75\) or increased resistance to infection\(^77\). However, use of DEREG mice to deplete Tregs showed no effect on worm burden during chronic infection\(^77\). Although, mice in which Tregs were depleted showed higher levels of intestinal pathology\(^75\). Therefore, whilst a regulatory response may have limited consequences for parasite expulsion it may play a crucial role in limiting infection-associated immunopathology.

There has been recent interest in the role of IL-17A as a modulator of immune responses. However, as yet a critical role for IL-17A or indeed the Th17 CD4+ T cell subset in infection with parasitic nematodes has yet to be published. An increase in IL-17A during chronic *T. muris* infection has been observed, however, depletion of IL-17A appears to have no effect on *T. muris* expulsion during high-dose infection\(^74\). Nor do IL-17A null mice fail to expel *T. muris* [personal unpublished observations].
**Figure 1.2: CD4+ Cell Differentiation.** CD4+ T cells, also known as T helper (Th) cells, are capable of differentiating into a wide variety of different sub-types. This occurs in response to the cytokines the naive CD4+ cell is exposed to upon activation. Cytokine signalling induces the expression of transcription factors that define the sub-type of that Th cell. These cells can also be defined by the profile of cytokines they secrete. These cytokines are associated with the particular immune function associated with that Th cell sub-type.
1.4.3 Effectors of Expulsion

Given the essential role for IL-13 in expulsion of *T. muris* it follows that responses downstream of IL-13 signalling are responsible for parasite clearance. Unlike a Th1-driven response that induces potent intracellular inflammatory responses, IL-13 drives extracellular physiological changes that alter the environment in which *T. muris* finds itself (Fig. 1.3).

The first contact the larval stages have with their host is at the mucus barrier. This gel-like structure is comprised of highly glycosylated proteins known as mucins, and it provides a thick coat along the large intestine creating a barrier between the epithelium and the lumenal environment. The predominating mucin in the GI tract is Muc2, but the composition of the mucus layer can adjust readily in response to enteric pathogens; under the regulation of the immune system⁷⁸,⁷⁹. Muc2 is secreted by goblet cells which undergo a dramatic hyperplasia in response to infection with *T. muris*⁸⁰. A clear role for mucins has been demonstrated with Muc2 deficient mice which exhibit a delay in expulsion when infected with a high dose of parasites, although expulsion is eventually achieved suggesting increase in Muc2 alone is insufficient to expel worms⁸¹. Indeed, resistant strains of mice (C57BL/6 and BALB/c) are capable of secreting another mucin in the gut, Muc5ac – normally present only in the lungs, eyes and stomach – when infected⁸². Muc5ac was present in IL-4-deficient but not IL-4R-deficient mice, indicating it is under regulation by IL-13. Knocking-out Muc5ac resulted in complete failure of normally resistant mice to expel their infection as well as an increase in the permeability of the mucus barrier⁸². Moreover, *T. muris* secretes serine proteases which are capable of degrading Muc2. Muc5ac, on the other hand, is resistant to the effects of *T. muris*-derived proteases⁸³. Thus a compositional change in the mucus barrier during acute infection appears to be protective against *T. muris* by limiting their capacity to colonise the intestinal mucosa.
Figure 1.3: Gastrointestinal Mucosal Responses to T. muris Infection. During infection considerable remodelling occurs in the mucosal environment. IL-13 secreted by Th2 cells induces both an increase in epithelial cell proliferation and a hyperplasia of goblet cells, the so-called “weep and sweep” response. Additionally, goblet cells begin secreting T. muris resistant Muc5ac in addition to Muc2, they also begin producing other proteins such as RELMβ and TFFs. Lymphocytes accumulate in the lamina propria space, and degranulation by mast cells and eosinophils occurs.
Once through the mucus layer, \textit{T. muris} anchors itself to its host by embedding its anterior end in the epithelium. When stimulated by IL-13, the gut epithelium begins to increase its rate of turnover\textsuperscript{84}. Proliferative cells at the base of epithelial crypts differentiate as they move towards the gut lumen. Eventually these cells apoptose and are shed into the lumen. The induction of Th2 responses upon a high dose \textit{T. muris} infection increases the rate of turnover, causing the cells to which the worms are anchored to be more rapidly shed. This creates a treadmill effect, in which the parasites are attempting to maintain purchase on an epithelium that is rapidly acting to expel them into the lumen. The significance of this mechanism was elegantly demonstrated in Setd7\textsuperscript{-/-} mice which have a constitutively accelerated epithelial cell turnover\textsuperscript{85}. These mice were also able to expel a low-dose infection which established chronic infection in their wild type litter mates\textsuperscript{85}. In concert with this, mice deficient for Amphiregulin – an epidermal growth factor (EGF) family member – show a reduction in epithelial turnover and inhibition of parasite expulsion\textsuperscript{86}.

As well as alterations to the mucus and epithelial barriers, Th2 responses can increase the contractility of the smooth muscle of the intestine. Treating mice with IL-9 during early infection resulted in hyper-contractility and early expulsion of \textit{T. muris}\textsuperscript{87}. In accord with this, depletion of IL-9 resulted in a reduction in both muscle contractility and resistance to infection\textsuperscript{87}.

1.5 Immune Responses to \textit{H. polygyrus}

\textit{H. polygyrus} is highly infective in most strains of mice irrespective of dose, generally resulting in a long lived patent infection. There is mouse strain variation, with BALB/c mice beginning to clear their infection around 28 days post infection, where C57BL/6 mice will sustain their infection for several months\textsuperscript{45}. However, it is highly immunogenic and a primary infection typically results in a potent protective response against subsequent challenge infections following anthelminthic removal of the primary infection\textsuperscript{88}. 

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36
1.5.1 The Innate Response

Upon invasion, *H. polygyrus* migrates through the epithelium into the submucosa where it encysts. This encystation is, in-part, mediated by innate cells which form granulomatous structures around the larvae. These granulomas are comprised primarily of neutrophils and macrophages\(^8^9\). The presence of visible granulomas is more apparent in mouse strains that are more resistant to primary infection, and is dependent on signalling through the IL-4R\(^9^0\).

The alternative activation of macrophages (aaMΦ) is characteristic of a helminth-induced Th2 immune response\(^4^5\). aaMΦs are characterised by expression of a variety of factors including Ym1, RELM\(\alpha\), and arginase-1. A role for aaMΦs in protective immunity against secondary *H. polygyrus* challenge has been shown. aaMΦs accumulate during both primary and challenge infection, and this is dependent on IL-4\(^9^0–9^2\). Depletion of aaMΦs, or the inhibition of arginase, results in loss of resistance to challenge\(^9^2\). Alternative activation is dependent on the ST2 domain of the IL-33R, and ST2\(^{-/-}\) mice show significant impairment in alternative activation, loss of resistance to primary infection, and an inability to generate protective immunity in response to vaccination\(^9^3\). aaMΦs appear to mediate larval killing during secondary challenge through adhering to the worm and blocking migration back into the intestinal lumen\(^9^4\). Adherence to the larvae is greatly enhanced by addition of larvae-specific antibody, and FcR\(\gamma^{-/-}\) macrophages showed a severe reduction in ability to adhere to the larvae\(^9^4\). Larval trapping appears to be arginase-dependent with the use of arginase inhibitors resulting in increase larval motility in macrophage-larval co-culture\(^9^4\).

Roles for other myeloid cells have been harder to define. Depletion of basophils or mast cells had no effect on the ability to generate a Th2 response to primary infection nor to vaccine-induced immunity to secondary challenge\(^9^1,9^5\). Eosinophil-deficient BALB/c Δdbl-GATA-1 mice have higher worm burdens than their wild-type counterparts suggesting a role for eosinophils in controlling a primary infection\(^9^1\). However, eosinophil-deficient C57BL/6 IL-5\(^{-/-}\) mice are fully protected against infection by vaccination\(^9^1\), suggesting they are not important in a memory
response. Similarly, a robust accumulation of neutrophils can be observed following infection\textsuperscript{92,96,97}. Neutrophilia during primary infection appears to be important, with antibody depletion resulting in reduced resistance to infection\textsuperscript{91}. However, they appear dispensable for a memory response with their absence having no effect on vaccine-induced protection\textsuperscript{91}. The mechanisms through which these cell types are capable of limiting primary \textit{H. polygyrus} infection remain unknown. Current models are limited to complete ablation rather than targeted disruption of specific effector functions employed by these cells. In the case of eosinophils, the mechanism does not appear to be through adherence to the larvae as they showed almost no capacity to bind\textsuperscript{94}. Nor do they appear essential sources of IL-4, as their depletion had no effect on the capacity to generate Th2 immunity\textsuperscript{98}.

Unlike \textit{T. muris} infection, a considerable expansion of ILC2s can be observed during \textit{H. polygyrus} infection\textsuperscript{99,100}. These ILC2s are capable of secreting IL-4 during infection, and their loss results in a stunted Th2 response\textsuperscript{99}. ILC2s are activated through IL-33 signalling, \textit{Spib}\textsuperscript{-/-} mice which (due to a bias in the development of myeloid progenitor cells) generate significantly higher number of granulocytes, including mast cells, produce significantly higher levels of IL-33, show increased ILC2 numbers and are more resistant to infection with \textit{H. polygyrus}\textsuperscript{101}. Supporting this is evidence that mast cell-deficient mice show increased susceptibility to infection\textsuperscript{102}. These data suggest a model whereby mast cell-derived IL-33 drives ILC2 expansion to induce a Th2 response to infection. In concert with this \textit{H. polygyrus} invests heavily in secreting inhibitors of IL-33\textsuperscript{103,104} (discussed below) suggesting that IL-33-driven responses exert considerable selective pressure upon the parasite. However, a non-redundant role for ILC2s in immunity \textit{H. polygyrus} has yet to be demonstrated.

1.5.2 The Adaptive Response

As with \textit{T. muris}, an essential role for adaptive immunity is well established for immunity to \textit{H. polygyrus}. SCID mice remain infected significantly longer than their immunocompetent counterparts following primary infection, are unable to up-regulate goblet cell responses, and show more severe tissue pathology\textsuperscript{105}. De-
pletion of CD4+ cells rendered mice susceptible to challenge infection, however, depletion of CD8+ cells had no effect\textsuperscript{90,92,106}. It was previously demonstrated that blocking the co-stimulatory molecule CD86 resulted in the failure to sustain a long-term Th2 response, however, the initial induction of Th2 cytokines was present\textsuperscript{107}. This would suggest an essential hand-off from the innate compartment to the adaptive response, requiring T cells for consolidating a Th2 response.

Resistance to infection correlates strongly with a polarisation towards Th2 immunity\textsuperscript{90}. Treatment with exogenous IL-4 is sufficient to cure BALB/c mice of a chronic infection whilst IL-4R\textsubscript{α}−/− mice are highly susceptible to infection\textsuperscript{91,108}. However, treatment of SCIDs with IL-4, whilst partially reducing worm burden and fecundity, does not result in complete parasite expulsion suggesting IL-4 alone is insufficient for immunity and that a complete adaptive response is required\textsuperscript{108}.

Unlike \textit{T. muris} infection, B cells have been shown to be indispensable in immunity to infection, particularly in response to secondary challenge. Mature B cell deficient J\textsubscript{H}−/− mice and JHD mice are highly susceptible to secondary challenge infection\textsuperscript{94,109}. This was specific to the loss of adaptive memory as adoptive transfer of memory B cells, but not naive B cells, was able to restore resistance to challenge infection\textsuperscript{109}. This protective effect appears to be antibody-dependent, with transfer of parasite-specific IgG to a naive animal being sufficient to reduce worm burdens upon infection\textsuperscript{91,96}. Indeed, IgG1 had been previously shown to be the isotype protective against \textit{H. polygyrus}\textsuperscript{110}.

Immunity to \textit{H. polygyrus} can be induced by vaccination with its excretory/secretory products (HES)\textsuperscript{91}. B cell-deficient μMT mice are unable to generate vaccine-induced immunity\textsuperscript{91}. Both MD4 mice (which have a fixed B cell receptor (BCR)) and CD40−/− mice (which can’t class switch to IgG1) gain no resistance from vaccination with HES\textsuperscript{91}. Given that vaccination against HES is sufficient to provide protective immunity, it suggests that neutralisation of excreted proteins enables host-protective mechanisms to facilitate parasite killing. Indeed, there has been wide interest in the roles that \textit{H. polygyrus}-derived products have in suppressing immune responses (discussed below), with its secretome being a vital
1.5.3 Physiological Effectors of Parasite Expulsion

Whilst a fundamental role of antibody has been demonstrated for protection against the L3 larval stage of *H. polygyrus* there is no evidence that antibodies are efficacious against adult parasites. However, given that *H. polygyrus* is expelled at considerably different time points depending on mouse strain, it suggests that mechanisms exist for parasite expulsion before they simply pass at the point of their natural death.

As with *T. muris* infection, *H. polygyrus* induces an intestinal goblet cell hyperplasia that is lost in SCID mice. These mice show a severely compromised mucosal architecture during infection. A specific role for mucins has yet to be investigated during *H. polygyrus* infection. However, as well as mucins, goblet cells secrete other protein mediators into the mucus layer. One such component is RELMβ, an IL-13-induced member of the resistin-like family that regulates the integrity of the intestinal epithelium. RELMβ-/- mice show reduced resistance to secondary challenge, and treatment of IL-4Rα-/- mice with recombinant RELMβ is sufficient to induce resistance to infection, as was pre-treatment of the parasite. This effect was independent of the adaptive immune response, as RELMβ pre-treated larvae were unable to establish a chronic infection in RAG2-/- mice. Indeed, it appears RELMβ directly impairs the ability of the parasite to feed, as pre-treated parasites showed considerably reduced ATP content and an inability to ingest host epithelium. However, the detailed mechanism through which this effect is mediated remains unclear. Studies in other helminths have shown that RELMβ binds directly to the surface of nematodes, and can reduce their capacity to chemotax *in vitro*. Of note, RELMβ-/- mice show no delay in expulsion of *T. muris*, a parasite that is unlikely to rely on chemotaxis for feeding given that it remains embedded in the epithelium throughout its lifespan. This would suggest that RELMβ blocks *H. polygyrus*’s ability to locate the epithelium reducing its feeding and perhaps ability to remain adhered to the gut mucosa.
There is little evidence for other physiological effector mechanisms being essential in resistance to *H. polygyrus*. Increased epithelial turn-over has no effect on parasite burden\(^85\). This is consistent with *H. polygyrus*’s free-living lifestyle in the lumen, where remaining in the duodenum is not dependent on strictly adhering to the epithelium, but through coiling around the vili for support. Similarly, an increase in gut contractility is observed during infection\(^115\), however, a functional role in expulsion has yet to be demonstrated.

1.6 Helminth-mediated Immunomodulation

A considerable amount of work has been done to understand how the host interacts with helminths to generate an effective response. By comparison, there is relatively little understood about how the parasites interact with their host. This is in part due to the genetic intractability of the majority of model helminths and the dearth of detailed information about the parasite genome. However, what has proven accessible are the excreted/secreted components produced by these organisms, termed the ES. Both *T. muris* ES (TmES) and *H. polygyrus* ES (HpES) have been the subject of scrutiny which has identified molecules capable of interacting with host proteins and modulating the immune response.

1.6.1 *T. muris*

TmES is a complex mixture containing over 460 proteins\(^116\). These proteins are sufficiently immunogenic to generate vaccine-induced immunity to infection\(^116\). GO term analysis predicted that these proteins were likely associated with ‘protein binding,’ ‘metal-ion binding,’ and ‘serine type peptidase activity’\(^117\). The most abundant component of TmES is the protein p43\(^118\). Not only is it the most abundant TmES protein, but it is the tenth most highly expressed protein in the adult *T. muris* genome\(^42\). p43 was shown to bind to both IL-13 and heparan sulphate (HS), a proteoglycan component of the extracellular matrix\(^118\). p43 is able to inhibit IL-13-induced responses *in vitro* and *in vivo*, preventing the induction of RELM\(\alpha^+\) macrophages in the lung when co-administered intranasally with IL-13\(^118\). This
presents the possibility that p43 is produced in such abundance to actively dampen the IL-13 response to infection. By binding IL-13 and tethering it to the extracellular matrix, through interaction with HS, p43 maybe sequestering IL-13 away from its receptor preventing induction of Th2 immunity.

TmES also contains exosome-like extracellular vesicles (EVs). These can be internalised by mammalian cells in organoid culture and are sufficient to generate immunity when used as a vaccine\textsuperscript{117,119}. These EVs may be a crucial mechanism through which \textit{T. muris} secretes proteins, as the majority of peptides identified from EVs lack a localisation signal\textsuperscript{119}. Also contained within \textit{T. muris}-derived EVs are a large number of miRNAs. Computational analysis of the sequences of these miRNAs predicted interactions with mouse genes associated with the immune system and disease pathways\textsuperscript{117}. This indicates that \textit{T. muris} may actively modulate immune responses through down-regulation of specific pathways through miRNA-mediated gene silencing. However, a functional effect of these miRNAs has yet to be demonstrated \textit{in vitro} or \textit{in vivo}.

1.6.2 \textit{H. polygyrus}

HpES is complex mixture that has long been a source of interest for identifying immunomodulatory peptides. It is well established that infection with \textit{H. polygyrus} is associated with a degree of immunosuppression that can be largely attributed to its excreted components. Previous work has shown that HpES has the capacity to block induction of Th2-associated cytokines, and suppress markers of alternative activation, during allergic challenge\textsuperscript{103}. These effects can be abrogated by heat-treatment of HpES suggesting that a protein component is responsible\textsuperscript{103}.

During infection, despite the induction of a robust Th2 response, a considerable expansion in Tregs is observed\textsuperscript{120,121}. Tregs derived from \textit{H. polygyrus} infected mice were able to suppress CD4+ cell activation \textit{in vitro}\textsuperscript{121}. This induction of a regulatory response is thought to be a critical parasite-induced mechanism that allows for the establishment of a chronic infection by delaying a host-protective Th2 response. The suppressive effects of \textit{H. polygyrus} could be recapitulated \textit{in vitro} with
HpES\textsuperscript{122}. Follow up proteomic analysis of HpES identified a 49-kDa protein that exhibited activity that mimicked TGF-\(\beta\), termed \textit{Hp-TGM}\textsuperscript{123}. \textit{Hp-TGM} is able to bind directly to TGF-\(\beta\) receptor and is able to induce Treg differentiation\textsuperscript{123}. Subsequently, a large group of related TGM proteins have been identified in HpES, a number of which share the capacity to activate the TGF-\(\beta\) pathway\textsuperscript{124}. These proteins show little homology with mammalian TGF-\(\beta\) family members\textsuperscript{124}, but act as effective mimetics and support the notion of parasite driven immunomodulation.

Despite a role for Treg induction during infection being well defined, in acute models of allergy, treatment with TGF-\(\beta\) has been shown to be unable to recapitulate some of the effects of HpES\textsuperscript{103}. This suggests that in more acute Th2 inflammation activation of TGF-\(\beta\) signalling must not be solely responsible for immunosuppression. Indeed, during allergic challenge HpES has also been shown to block the release of IL-33, and thus limit the early secretion of ILC2-derived IL-5 and IL-13 that may be required for the induction of host-protective immunity\textsuperscript{103}. At least one component responsible for this blockade of IL-33 signalling has been identified. \textit{H. polygyrus} Alarmin Release Inhibitor (HpARI) has been recently identified as capable of binding directly to both IL-33 and DNA\textsuperscript{104}. HpARI functions to retain IL-33 in the nucleus of necrotic cells, which are a potent source of IL-33 during tissue damage, sequestering it away from its receptor.

Interestingly, conserved between both the TGM proteins and Hp-ARI is the presence of complement control protein (CCP) domains\textsuperscript{104,124}. The CCP domain is highly conserved from the origins of multicellular animals\textsuperscript{125}, and is well represented in the \textit{H. polygyrus} genome\textsuperscript{124}. It appears that \textit{H. polygyrus} utilises this simple domain in a modular fashion to construct more complex proteins. This versatility may have allowed for the development of an abundance of unique immunomodulatory proteins. It also demonstrates the presence of a powerful selective force acting on \textit{H. polygyrus} that necessitates the production of a diverse array of proteins responsible for manipulating the host immune system.

Like \textit{T. muris}, \textit{H. polygyrus} produces EVs containing a heterogenous group of miRNAs and proteins\textsuperscript{126}. These are actively internalised by mammalian cells,
and uptake can be abrogated by treatment with Cytochalasin D\textsuperscript{93,126}. EVs are able to reduce ILC expression of IL-5 and IL-13 as well as the overall level of IL-33R\textsuperscript{126}. \textit{H. polygyrus}-derived EVs are also able to suppress alternative activation of macrophages \textit{in vitro}\textsuperscript{93}. There is some evidence that these effects may be mediated in-part by the miRNA component with \textit{H. polygyrus}-derived miRNAs resulting in down-regulation of host genes\textsuperscript{126}, however, as with \textit{T. muris} the role of parasite miRNAs remains largely unexplored.

1.7 Modelling Natural Infection

Our current level of understanding about immune responses to infection have come largely from experimental models that rely on a single bolus infection, given to immunologically naive animals, in otherwise pathogen free environments. This is in stark contrast to the realities of infection in humans, or rodents for that matter, where an array of environmental variables are likely to be responsible for the outcome of infection. Indeed, studies using laboratory mice that have been re-introduced to a semi-natural environment, so called rewilded mice, show considerable differences in the immune composition and responses to helminth infection compared to those retained under laboratory conditions\textsuperscript{127,128}. But even these studies have relied on solely increasing environmental diversity, but rarely increase the complexity or the nature of the infectious challenge. Individuals living in environments endemic for helminth infection are being constantly re-exposed to infectious stages of the parasite and thus are likely to be re-infected multiple times. On top of this, given that most helminths occupy similar environmental niches, individuals are likely to be concurrently infected with multiple different species. Additionally, in humans movement from rural to urban environments has resulted in considerable lifestyle changes for people in endemic regions, in particular a shift to diets high in fat. These variables are likely to have a considerable impact upon the dynamic of infection, however, presently remain relatively understudied.
1.7.1 Trickle Infection

Models of regular re-infection, termed trickle infection, were employed many decades ago and the kinetics of the infectious burden of several model helminths were documented\textsuperscript{129–131}. However, despite the interest at the time, the experiments were inherently limited by the available technology and there was a resultant absence of immunological information.

The earliest trickle infections of \textit{T. muris} demonstrated that repeated low dose infections could result in similar worm burdens compared to mice that have received a single high dose infection\textsuperscript{131}. Subsequent work confirmed this with a cumulative effect of repeated infections in C57BL/6 mice, although, the more naturally resistant BALB/c strain did not accumulate parasites under these conditions\textsuperscript{132}. However, these experiments were conducted under a relatively narrow time frame (35 days) and showed little difference in immune response between trickle infected mice and low dose infected ones in terms of immunophenotype\textsuperscript{132}. More recent work has investigated the effect of long term \textit{T. muris} trickle infection in C57BL/6 mice. Analysis over a three month period showed that whilst early during trickle infection worm burden builds in a stepwise fashion with each subsequent challenge, resistance can be generated to infection over time\textsuperscript{60}. This is associated with a transition from a Th1-dominated immune response to a Th2-driven one at the point where resistance to re-infection develops. Under these conditions resistance was dependent upon the presence of CD4+ cells, consistent with data from single dose infections that have established these cells as key mediators of resistance to \textit{T. muris}\textsuperscript{60}. These data suggest that resistance to infection can develop after a period of susceptibility, dependent on a critical threshold of parasite burden, and better reflect data from the field that show in human populations resistance develops after a period where worm burden rapidly accrues in early childhood\textsuperscript{6,8,9}.

Information for trickle infections with \textit{H. polygyrus} are similarly limited. Investigation into the dynamics of \textit{H. polygyrus} worm burden during trickle infection showed that trickle infection, where L3 larvae were administered twice weekly, resulted in an initial increase in worm burden followed by expulsion, with the rate
of expulsion dependent on the number of larvae given per infection\textsuperscript{133}. However, data from frequent challenge infections with high doses of the parasite showed no evidence of an increase in worm burden following the primary dose, but a steady decline in burden was observed over time\textsuperscript{134}. Repeat infection induced a much greater level of parasite-specific serum IgG1 relative to a single infection\textsuperscript{134}. These data indicate that repeated challenge results in a cumulative induction of immune responses, although this remains to be fully characterised.

1.7.2 Co-Infection

Co-infection with multiple GI helminths is a common feature of infection in humans. However, as with trickle infection, this facet of natural infection has remained largely under-investigated. Given that most model helminths are potent drivers of Th2 immunity, it would be natural to assume that co-infection would result in a greater levels of Th2 activation and thus more rapid parasite expulsion. However, as discussed above, chronicity with \textit{T. muris} and \textit{H. polygyrus} is determined by different mechanisms, and each parasite has evolved unique methods to subvert the host immune response. How these mechanisms interact has received little attention. Very early work looking at co-infection with these two species identified a dramatic effect on resistance to a \textit{T. muris} high dose infection; mice that were co-infected with \textit{H. polygyrus} became susceptible to a high dose infection\textsuperscript{135}. This has also been observed when \textit{T. muris} is co-infected with \textit{Trichinella spiralis}\textsuperscript{136}. The functional reason for this dramatic phenotypic shift remains unidentified.

1.7.3 Diet

Of recent interest is the effect diet may play on the immune response. With regards to helminth infection, in endemic countries a sharp increase in the proportion of individuals living in urban environments has resulted in an increase in obesity\textsuperscript{137}. Data from animal models of obesity, using mice fed on high-fat diets, has demonstrated a change in the profile of cytokines secreted in response to stimulation. In particular an increase in the expression of pro-inflammatory cytokines like IFN\textgreek{y}
and IL-1β. However, the relationship between a change in diet and the effect on helminth infection remains largely under-investigated. There is evidence that infection with *H. polygyrus* is capable of mitigating some of the effects of diet-induced obesity (DIO) in mice, reversing the pro-inflammatory phenotype developed. However, the authors did not assay the effect that DIO had on the kinetics of worm infection. Data from a study looking at the effect of reduced dietary protein on *T. muris* infection demonstrated that reduced protein content resulted in higher levels of body fat that were associated with a reduced IL-13 response to infection and a minor increase in worm burden. However, this model did not specifically investigate DIO and the effects may be more specific to the levels of dietary protein.

### 1.8 The Gut Microbiome

A crucial component of the GI tract is the community of commensal microorganism that reside there. The gut microbiota is responsible for a major component of mammalian digestion, breaking down plant-derived polysaccharides that would be otherwise inaccessible. An increase in the use of high-throughput sequencing has led to a rapid advancement in our ability to characterise the composition of this population; and it is now well understood that the intestines are a highly diverse ecosystem containing over a thousand species. Remarkably, within individuals the composition of the microbiota remains largely consistent across time. This suggests the presence of active regulation of the community, either from within or externally from the host to prevent stochastic changes that would threaten homeostasis within the intestine.

Despite this tight regulation within a given host, there is considerable variation between individuals especially across geographic lines, likely due to variation in diet, genetic background, presence of enteric pathogens, and access to antibiotics. It has therefore been highly challenging to define a ‘healthy’ microbiota, although efforts have been made to define this in western populations. However, it has been consistently observed that those living in urban environments on western diets have a significant reduction in the diversity of their microbial communities relative
to peoples in regions where parasitic nematodes are endemic\textsuperscript{146,147}. This is supported by experimental data from mice that show rewilding results in an overall increase in microbial diversity\textsuperscript{128}.

As well as a role in digestion, it is now understood that interaction with the microbiota is essential for the healthy development and maintenance of the intestinal barrier, particularly in the mucosal immune systems\textsuperscript{149}. A dysbiosis, namely a loss in microbial diversity or presence of a pathogenic species, in the gut has been linked to a wide range of immune disorders both enteric and systemic including IDB\textsuperscript{150,151}, rheumatoid arthritis\textsuperscript{152,153}, and allergic diseases\textsuperscript{154,155}. It is thought that constitutive signalling from a homeostatic biota, via production of metabolites and microbial components, drives a state of physiological inflammation essential for barrier maintenance\textsuperscript{149}. Mice treated with antibiotics show a marked increase in susceptibility to dextran sulphate sodium (DSS)-induced colitis, due to the absence of constitutive TLR signalling normally maintained by the intestinal microbiota\textsuperscript{156}. Induction of innate signalling also feeds back onto the microbiota with activation of Nucleotide oligomerization domain (NOD) receptors by microbes required for homeostasis in the biota itself. For example, \textit{Nod2}\textsuperscript{-/-} mice are more susceptible to infection with opportunistic pathogens and show higher loads of commensal bacteria\textsuperscript{157}.

To limit bacterial-epithelial contact and to prevent disease, the host restricts gut bacteria to the mucus barrier. In the colon bacteria are restricted to a clearly defined upper layer, however, in the small intestine no such stratification exists\textsuperscript{158,159}. Maintaining this compartmentalisation is dependent on the production of antibacterial proteins by epithelial cells, such as \textit{RegIII}\(\gamma\), and the secretion of bacteria-specific IgA by B cells\textsuperscript{160,161}. Additionally, mucins themselves have a function in protecting against disease by regulating the phenotype of opportunistic pathogens\textsuperscript{162}. A breach in this barrier requires rapid phagocytosis to prevent dissemination of invasive species, however, it also provides an opportunity for antigen presentation by DCs for induction of anti-bacterial antibody responses\textsuperscript{163}.
Figure 1.4: Interactions Between the Host, Helminths & the Microbiota. During infection there is constant interaction between the host, its commensal microbiota, and the invasive helminth species present. Interactions between each party are understood to occur either directly or indirectly. Interactions between the helminth and the host, or the host and the biota are reasonably well characterised and involve secreted mediators and physical effects. However, less well characterised are the interactions that occur between helminths and the microbiota.
1.8.1 Helminths and the Microbiota

Given that GI helminths are primarily lumen-dwelling, it is reasonable to assume that as they have evolved with their host they have also co-evolved with the microbiota of that host. This can be seen clearly with *Trichuris* species that require interaction with gut bacteria to initiate hatching\(^44\). There is now growing interest in the possibility that helminths either directly influence the host microbiota or that their presence has indirect consequences for bacterial community homeostasis\(^164\). A complex and poorly defined relationship between host, helminth and microbiota exists and appears intrinsic in the outcome of infection (Fig. 1.4).

Data from field studies in humans is severely limited and has been largely conflicting, likely due to the presence of a considerable number of confounding factors. Evidence suggests that infection with *T. trichiura* either has no effect on the composition of the microbiota\(^165\), or results in a small positive increase in species richness that could be reversed through treatment with anthelmintics\(^146\). However, given that these two studies were performed in different locations with different methodologies it is hard to draw any concrete conclusions.

Data from murine models on the other hand has been more forthcoming. A low dose chronic *T. muris* infection has been consistently shown to result in a dysbiosis consistent with an overall decrease in the diversity of the gut microbiota\(^166–168\). This effect was somewhat reversible when mice were treated with anthelmintics to remove the infection\(^166\). Chronic *T. muris* infection was associated with a considerable increase in abundance of the *Lactobacillaceae* family\(^168\). Expansion of *Lactobacillaceae* family members, particularly those in the *Lactobacillus* genus, appears to be a consistent trend across infection with multiple helminth species\(^168–171\) and correlates with susceptibility to *H. polygyrus* infection\(^172\). In fact, probiotic treatment of mice with members of the the *Lactobacillus* genus is sufficient to increase susceptibility to infection with both *T. muris* and *H. polygyrus*\(^172,173\). This effect may be mediated through suppression of Th2 immune responses by an increase Tregs which positively correlates with the abundance of *Lactobacillus*\(^172\). This is consistent with models of allergic inflammation, where treatment with *Lacto-
*bacillus* species has been demonstrated to induce Tregs and attenuate the allergic response \(^{174–176}\).

These data hint that helminth induced effects on the biota are capable of enhancing persistence in the host, although a mechanistic understanding of how the parasite drives changes in the microbiota remains elusive. It may be through direct manipulation by secretion of peptide mediators into the lumen, but also likely is that drastic physiological changes induced by infection alter the mucosal environment and favour the survival of different species. For example, an increase in the abundance of mucus would favour species adapted colonising the mucus layer such as *Mucispirillum* species which have been observed to increase during *T. muris* infection \(^{168}\). A significant hinderance to our understanding of how infection alters the microbiome is the absence of longitudinal studies on the biota in mouse models. Typically studies have looked at a relatively narrow time frame where the effects are likely to be most drastic, and thus fail to give a dynamic picture of the changing microbial landscape throughout infection. Additionally, the infectious models employed thus far are restricted to comparing naive animals to a static chronic infection and therefore provide little information as to how transition between immune states influences the microbiota.

### 1.9 Project Aims and Objectives

Developing our understanding of helminth infection demands an increase in the complexity of the models used in order to better reflect natural infection. Laboratory models to date focus primarily on single bolus infection of individual helminth species over a relatively narrow time frame. This has resulted in a detailed understanding of a simple binary – susceptible vs resistant – that whilst informative is inherently limited. However, data from field studies suggests that immunity under natural conditions develops over time but is rarely complete. This project aimed to characterise the effects of trickle infection and co-infection on the development of immunity to infection over long periods of infection in order to better reflect infection *in situ*. It also aimed to characterise dynamic changes in the microbiota during helminth infection.
Specific objectives:

- Characterise the dynamics of *H. polygyrus* trickle infection.
- Assess the effects of co-infection on *T. muris* trickle infection.
- Characterise changes in the gut microbiome induced by helminth infection.
- Investigate how *H. polygyrus* co-infection alters *T. muris* high dose infection.
Chapter 2

Materials & Methods
2.1 Animals

C57BL/6 mice were purchased from Envigo. Mice arrived at six weeks of age and were allowed one week prior to experimentation to acclimatise in the University of Manchester biological services facility (BSF). SCID and TCR\(x^{-/}\) mice were bred in-house. Mice were maintained in accordance with the Animals (Scientific Procedures) Act (ASPA) under pathogen free conditions. Mice were housed in groups of five. Dosing with parasites was achieved by oral gavage. Animals were sacrificed by CO\(_2\) asphyxiation, and death was confirmed by cardiac puncture.

2.1.1 Procedures

Diet

Following a week of acclimatisation following arrival at the BSF, mice were placed on either a high fat (HFD 60 % Kcal from fat, Research Diets Inc. USA, D12492) or low fat (LFD 12% Kcal from fat, Research Diets Inc, USA, D16011009) diet. Mice were maintained on diet for 12 weeks prior to infection, and were kept on diet throughout infection.

Fasted Glucose Measurement

Mice were fasted overnight by removing food from their enclosure. The following morning blood was drawn from the tail vein by needle prick and was quantified immediately using an Accu-Chek Aviva blood glucose meter. Food was then returned.
Injection of Parasite ES products

30 μg of HpES in 200 μl of PBS was injected intraperitoneally, every second day following the primary injection. The side on which injection was performed was alternated between injections.

For immunisation with TmES, 50 μg of TmES was resuspended in 200 μl of Alum (Thermo scientific 77161) and stirred using a magnetic stirrer until thoroughly homogenised. Once mixed the TmES + Alum solution was kept in suspension, and then injected subcutaneously.

2.1.2 Measurement of Bodyfat

At autopsy, subcutaneous fat from the left flank was collected by scraping with forceps. Fat was stored in plastic dishes until dissection was completed. The fat was then weighed immediately following autopsy on a standard bench-top balance.

2.2 Parasites

2.2.1 Trichuris muris

Maintenance of the T.muris Lifecycle and Generation of TmES

SCID mice were infected with 300 embryonated eggs by oral gavage and sacrificed at day 42 post infection. The large intestine was removed intact immediately at autopsy, cut longitudinally and manually washed in warm RPMI 1640 medium supplemented with 5x penicillin/streptomycin (500U/ml penicillin, 500g/ml streptomycin, 500ml RMPI 1640 medium). Adult worms were removed from the gut using forceps and collected into 6 well plates containing 4 ml of 5x penicillin/streptomycin RPMI 1640 medium. Plates were then incubated in a humidity chamber at 37°C for 4 hours. Each well was then split into two new wells con-
taining fresh 5x penicillin/streptomycin RPMI 1640 medium, and incubated again for 24 hours under the same conditions. The media from each incubation was collected and centrifuged for 15 minutes at 2000 g. The resulting supernatant was removed, and the pellet (comprised of eggs) was resuspended in 40 ml dH2O. The eggs were filtered through a 100 μm sieve into a 75 ml cell culture flask. Eggs were allowed to embryonate for at least 8 weeks. SCID mice were then infected with a high dose infection to determine infectivity of each batch. To generate TmES the supernatant was filtered through a 0.22 μm filter. It was then concentrated by centrifugation through an Amicon Ultra 15 Centrifugation 10 KDa filter for 15 min at 4°C. The remaining solution was then dialysed against PBS for 24 hours at 4°C. Protein concentration was determined using a Pierce BCA Protein Assay Kit and the resultant TmES was stored at -20°C until use.

**T. muris High and Low Dose Infections**

A stock solution from a batch of eggs was placed in a 5 ml bijou tube. Eggs were kept in suspension by the addition of a magnetic stirrer. The volume was then adjusted to achieve a known concentration, typically 20 eggs per 20 μl. Concentration was confirmed by counting five samples from the tube and calculating the average value. Eggs were then aliquoted into glass tubes, for low dose infection this was 20 eggs, and for high dose infection this was 200 eggs. The volume was then adjusted to 200 μl. Mice were then infected by oral gavage.

**Assessing T. muris Worm Burden, Length, and Fecundity**

At autopsy the caecum and proximal colon were removed and stored at -20°C. The tissue was thawed at room temperature and opened longitudinally in a dish containing dH2O. The contents were washed out and the tissue transferred to a fresh dish containing dH2O. Worms were removed gently from the tissue and intestinal content using forceps and counted by eye under dissecting microscope.
Upon dissection the whole caecum and proximal colon were opened longitudinally and placed into 5x penicillin/streptomycin RPMI 1640 medium. The tissue was gently washed to remove the luminal contents and then placed in a dish containing fresh 5x penicillin/streptomycin RPMI 1640 medium. Individual female worms were removed from the caecum gently with forceps and placed into individual wells of a 48 well plate containing 1 ml 5x penicillin/streptomycin RPMI 1640 medium. The worms were then incubated in a humidity chamber at 37°C for 4 hours. Following this the culture media was removed and the number of eggs present were assessed by eye under dissecting microscope.

Worm size was determined by removing the worms from the caecum immediately following autopsy. The worms were then fixed in Bles medium and mounted on glass slides using PERTEX mounting media. The worms were then imaged under a Leica Stereomicroscope microscopy at 7x magnification. Worm length was measured using ImageJ.

2.2.2 Heligmosomoides polygyrus

Maintenance of the H. polygyrus Lifecycle

L3 larvae were prepared from faecal pellets collected from infected C57BL/6 females maintained in-house. Faecal pellets were re-hydrated in tap water, ground into a paste, and smeared onto filter paper placed in glass petri-dishes. This was left for seven days in the dark allowing the larvae to hatch and crawl out of the faeces. The larvae were collected by washing off of the filter paper with tap water. The collected larvae were then stored at 4°C until use. Larvae for gavage were counted by eye and agitated by vortex or pipetting up-and-down to confirm they were still alive.
**Assessing* H. polygyrus* Worm Burden**

Worm burden was determined upon sacrifice of infected mice. The duodenum was dissected and placed in a dish of warm PBS prior to worm collection. The duodenum was then cut along its length exposing the lumen. This was then placed in a gauze mesh affixed to the top of a 50 ml falcon tube filled with PBS. The falcon tube was placed in a water-bath set to 37°C and incubated for 4 hrs. This allowed the worms time to leave the duodenum and migrate towards the source of heat causing them fall through the gauze mesh and accumulate at the base of the tube. The temperature of the water-bath was then increased to 57°C for 90 mins in order to untangle the worms. The parasites were transferred to a Petri-dish for counting under dissecting microscope at 0.63X magnification. The remaining duodenum was searched for any parasites that had remained trapped.

**Generation of HpAg and HpES**

*H. polygyrus* whole worm homogenate (HpAg) was produced from live adults collected three weeks post infection. Worm were collected as described above, and washed five times in sterile PBS. The worms were then stored in 1 ml of PBS overnight at -80°C. Worms were then thawed and homogenised by pestle and mortar. The homogenate was then allowed to incubate at 4°C overnight. The homogenate was then centrifuged for 20 min at 15,700 g and the HpAg supernatant was collected. Protein concentration was determined using a Pierce BCA Protein Assay Kit. HpAg was then stored at -20°C until use.

To generate *H. polygyrus* Excretory/Secretory Products (HpES) the duodenum from infected mice were removed at autopsy, cut longitudinally and placed into a 6 well plate containing 5 ml Hank’s Solution. Worms were collected by placing the dissected intestines into a gauze bag affixed to the top of a 50 ml falcon tube filled with Hank’s Solution, and incubated for 2 hr at 37°C. The worms that collected at the bottom of the tube were then transferred to a fresh 50 ml tube using a Pasteur pipette, and washed six times with fresh Hank’s Solution. The falcon tube
was then transferred to a laminar flow hood and the worms were then washed six times with Hank’s Solution supplemented with penicillin/streptomycin (100 U/ml penicillin, 100 μg/ml streptomycin) (Hanks + P/S). Worms were then transferred to a 6 well plate and soaked in RPMI 1650 Medium supplemented with 10% Gentamycin for 20 mins. Worms were then washed a subsequent six times in Hanks + P/S. Worms were then aliquoted into 25 ml tissue culture flasks containing 15 ml *H. polygyrus* culture medium (500 ml RPMI 1640 + 11.1 ml 45% glucose + 100 U/ml penicillin, 100 μg/ml streptomycin, + 2 mM L-glutamine, + 100 μg/ml Gentamycin) with no more than 1000 worms/flask. Worms were then cultured for 10 days at 5% CO₂ and 37°C. Media from the first 24 hours was discarded and replaced with fresh culture media. Media was then collected and replaced every 3 days. The collected media was centrifuged at 400 g for 5 min and the supernatant was filtered through a 0.22 μm filter and stored at 4°C. Media from multiple days was pooled and concentrated by centrifugation through an Amicon Ultra 15 Centrifugation 3 KDa filter for 45 min at 4°C. Protein concentration was determined using a Pierce BCA Protein Assay Kit and endotoxin levels were assayed using a Pierce LAL Chromogenic Endotoxin Quantitation Kit. HpES was stored at -20°C until use.

### 2.2.3 Faecal Egg Counts

Faecal eggs counts were performed by collecting fresh pellets from infected mice. The pellets were weighed then placed in water for 1 hr at room temperature. The pellets were then vortexed until a homogenous solution formed. The sample was then diluted in saturated NaCl solution and loaded into a McMaster egg counting chamber. Eggs were counted using a dissecting microscope (Leica MZ75) at 5X magnification. Two counts were made per sample and the following equation was used to calculate eggs per mg faeces: 

\[ \frac{((\text{Vol H}_2\text{O} + \text{Vol NaCl} + \text{grams faeces})/\text{grams faeces}*0.15))}{1000} \times \text{mean egg count} \]
2.3 Cytokine Analysis

2.3.1 Collection and Re-stimulation of Mesenteric Lymph Nodes

MLNs were collected at autopsy either whole or separated in colonic MLNs (cMLN) and small intestinal MLNs (sMLN). MLNs were collected into 15 ml tubes containing chilled 2% FCS 1x penicillin/streptomycin in RPMI 1640 medium and placed onto ice. MLN cells were homogenised by forcing them through a 70 μm cell strainer, washed twice by centrifugation (400 g for 5 min) replacing the media each time, and resuspended in complete RPMI 1640 medium (10% FCS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin). Cell number was determined by CASY cell counter and then the concentration was adjusted to 5x10⁶ cells/ml. 200 μl of cells were then seeded into a 96 well plate and re-stimulated with 50 μg/ml TmES or HpAg. The cells were then incubated for 36 hours in an incubator at 5% CO₂ and 37°C. The supernatant was then collected and stored at -20°C prior to being assayed.

2.3.2 Cytometric Bead Array

The concentration of cytokines in the MLN culture supernatant was measured by Cytometric Bead Array (CBA) (BD Biosciences). IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, TNF, and IFNγ were quantified. 16.6 μl of thawed supernatant was transferred to a round bottomed 96 well plate. Standards were diluted serially 2-fold from 2500 pg/ml to 10 pg/ml and added to the same plate in separate wells. 16.6 μl of a solution containing capture beads for each cytokine (0.33 μl of beads per cytokine diluted in Capture Diluent ) to each well. The samples were then incubated at room temperature on an orbital shaker for 5 min. 16.6 μl of detection antibody solution (0.33 μl of antibody per cytokine diluted in Detection Diluent ) was then added to each well and the samples were incubated at room temperature for a following 60 minutes on an orbital shaker. Following this 150 μl of wash buffer was added to each well, and the plate was then centrifuged at 400 g for 5 min to pellet the beads. The supernatant was discarded and 70 μl of fresh wash
buffer was added to each well. The assay plate was then read using a MACSQuant Analyzer 10 (Miltenyi Biotech) and quantification of the data was performed using FCAP array v3.01 (BD Biosciences).

2.4 ELISAs

2.4.1 Serum IgG ELISA

Whole blood was collected from sacrificed animals by cardiac puncture. The blood was allowed to clot at room temperature. It was then spun for 10 mins at 15,000 g. The serum was collected from the top and stored at -20°C until use. For IgG ELISAs, BRANDplates 96-well immunoGrade plates were incubated overnight at 4°C with HpAg or TmES (10 μg/ml) diluted in 0.05 M Carbonate/Bicarbonate buffer. Following this (and all subsequent steps) the wells were washed three times with PBS-T. Wells were next blocked for one hour with 3% bovine serum albumin (BSA). The wells were then incubated with serum diluted to 1:40, 1:80, 1:160 & 1:320 for each sample for 90 mins. Following this, biotinylated antibodies, diluted in PBS-T, were added against either IgG1 (1:2000) or IgG2c (1:1000) and incubated for one hour. A secondary anti-mouse Ig antibody conjugated to streptavidin peroxidase was added for 60 mins (1:1000). ABTS substrate was added to the wells and optical density was read at 405 with a reference filter of 495 nm.

2.4.2 Serum mMCP-1 ELISA

mouse mMCP-1 ELISAs were performed using the ‘Mouse MCPT-1 (mMCP-1) ELISA Ready-SET-Go! Kit’ (eBiosciences) as directed by the manufacturer. PBS-T washes were performed between each step. In brief, immunoGrade plates were coated with anti-mouse mMCP-1 capture antibody overnight. Wells were blocked for 60 mins, following which, serum and standards were added and incubated for 120 min. Anti-mouse mMCP-1 biotin detection antibody was added and incubated for 1 hr. Next, avadin-HRP was added and incubated for 30 mins. HRP substrate was added and incubated for 15 mins. The reaction was stopped using 1M H₃PO₄.
The plate was read at 450 nm with a correction read at 570 nm.

### 2.5 Flow Cytometry

MLN cells were isolated as described above for re-stimulation assays. Cells were re-suspended in PBS then centrifuged at 400 g for 5 min. Cells were then blocked in 50 μl of anti-CD16/CD32 (1:100) for 15 min at 4°C. A 50 μl solution containing surface staining antibodies was then added (antibodies listed in table 2.1) giving a concentration of 1:200 for all extracellular antibodies. Cells were then incubated for 25 mins at 4°C. Flowing extracellular staining cells were washed twice with PBS and incubated in 1 ml of Live/Dead stain (1:1000) for 30 mins at 4°C. Cells were then washed twice with 1 ml of PBS. For intracellular staining cells were fixed and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) as directed by the manufacturer. Cells were then washed in 0.5 ml permeabilisation buffer. Following this cells were incubated in 50 μl of permeabilisation buffer containing intracellular antibodies at a 1:100 dilution (except for FoxP3 which was used at 1:66). Cells were then washed twice in permeabilisation buffer then twice in PBS. Flow cytometry was performed on a BD LSR II flow cytometer and analysis was performed in FlowJo.
### Table 2.1: List of Antibodies used for Flow Cytometry

<table>
<thead>
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<th>Target</th>
<th>Flurochrome</th>
<th>Cat. Reference</th>
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<tbody>
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<tr>
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<td>eFluor660</td>
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<tr>
<td>IL-17</td>
<td>PerCP/Cy5.5</td>
<td>506920</td>
</tr>
<tr>
<td>Live/Dead</td>
<td>DAPI</td>
<td>L34961</td>
</tr>
</tbody>
</table>

#### 2.6 Histology

Tissue samples were taken at autopsy and immediately placed in either 4% Neutral Buffered Formalin (NBF) or Carnoy’s Fixative (60% Ethanol, 30% Chloroform, 10% Glacial Acetic Acid). Tissue was stored in 70% Ethanol or 100% Ethanol respectively before being processed into paraffin blocks. 5 μm sections were cut using a HM325 MICROM microtome and mounted on glass slides. Sections were allowed to dry before staining.
2.6.1 Periodic Acid Schiff

Samples were de-waxed for 20 min in Citroclear then rehydrated by incubation through an ethanol series (100%, 100%, 90%, 70%, 50%, dH₂O, 2 min per concentration). Samples were then incubated in 1% periodic acid for 5 min, washed for 1 min in dH₂O, then rinsed for 1 min in tap water, and washed again for 1 min in dH₂O. Sections were then incubated for 10 min in Schiff’s reagent, washed for 1 min in dH₂O, then rinsed for 5 min in tap water. Samples were counter stained with Mayer’s Haematoxylin for 20 sec, then rinsed in tap water for 5 mins. Sections were then dehydrated by reversing the ethanol series above. Samples were mounted using Pertex mounting media. Samples were imaged using a Nikon Eclipse Ci Upright Microscope and images were analysed using ImageJ.

2.6.2 Alcian Blue

Samples were de-waxed and rehydrated as described above. Samples were then incubated in Alcian blue solution (1% Alcian blue, 3% Acetic acid) for 10 mins. Samples were then rinsed in tap water for 5 mins, counterstained with Mayer’s Haematoxylin for 20 sec, then washed again for 5 min in tap water. Samples were then dehydrated and mounted as described above. Samples were imaged using a Nikon Eclipse Ci Upright Microscope and images were analysed using ImageJ.

2.6.3 Immunofluorescence Microscopy

Samples were de-waxed and rehydrated as described above. They were then washed twice with PBS. Following washing antigen retrieval was performed by boiling the samples in citrate buffer (10 mM Citric Acid, 0.05% Tween, pH 6) for 3 min, then left for 20 min at room temperature to cool. They were then washed three times with PBS then blocked with 5% serum (species of origin dependent on which animal the secondary antibody was raised in) for 30 min at room temperature. Samples were then incubated overnight at 4°C with primary antibody diluted in blocking solution (List of antibodies and dilutions used in table 2.2) in a
humidity chamber. Samples were then washed three times with PBS and incubated with secondary antibody for 2 hrs at room temperature, then washed three times in PBS. Samples were then incubated with DAPI (1:1000) for 5 mins, washed twice with PBS and once in dH₂O. Following staining samples were allowed at least 1 hour to dry before mounting with Prolong Gold Antifade Mountant. Samples were imaged using a Nikon Eclipse Ci Upright Microscope and images were analysed using ImageJ.

**Table 2.2:** List of Antibodies used for Immunofluorescence Microscopy

<table>
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<th>Target</th>
<th>Dilution</th>
<th>Cat. Reference</th>
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</thead>
<tbody>
<tr>
<td>Alex Fluor 594 anti Rabbit</td>
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<td>A21207</td>
</tr>
<tr>
<td>Alexa Fluor 488 anti Rabbit</td>
<td>1:1000</td>
<td>A11008</td>
</tr>
<tr>
<td>Muc2</td>
<td>1:500</td>
<td>sc-15334</td>
</tr>
<tr>
<td>pSmad2 (S467)</td>
<td>1:200</td>
<td>ab53100</td>
</tr>
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</table>

2.7 Microbial Metagenomics

2.7.1 Extraction of DNA from Faecal Samples

The final stool was collected at autopsy and stored at 4°C prior to DNA extraction. Faecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). Stool samples were placed into 2 ml tubes containing 1 ml of InhibitEX buffer and ceramic beads, the samples were then homogenised using a tissue homogeniser. Samples were then heated to 95°C for 30 min. Following this the samples were centrifuged at 20,000 g. 400 μl of the supernatant was then transferred to a fresh 1.5 ml eppendorf containing 30 μl Proteinase K. 400 μl of buffer AL was then
added and the sample was vortexed to create a homogenous lysate. The lysate was
incubated at 70°C for 10 min. 400 μl of ethanol was then added and the sample was
vortexed. The lysate was then passed through QIAamp spin column, washed with
500 μl of buffer AW1 then buffer AW2. DNA was then eluted from the column by
the addition of ATE buffer. DNA concentration was measured by NanoDrop.

2.7.2 16S Library Preparation

The 16S V3/V4 region was targeted using the following 16S amplicon
PCR primers F: 5’ TCGTCGAGCAGTCGAGATGTATGCTTATAAGAGACAGCC-
TAGGGGACWGCAG & R: 5’ GTCTCGTGGGCTCGGAGATGTATGCTTATAA-
GAGACAGGACTACHVGGGTATCTAAATCC. For each sample the following re-
action was set up 2.5 μl faecal DNA, 2.5 μl 1 μM Forward primer, 2.5 μl 1 μM
Reverse primer, & 12.5 μl 2x KAPA HiFi HotStart ReadyMix. The reaction was
run in a thermal cycler using the following cycling conditions:

- 95°C for 3 min
- 25 Cycles of:
  - 95°C for 30 sec
  - 55°C for 30 sec
  - 72°C for 30 sec
- 72°C for 5 min
- Hold at 4°C

PCR clean up was performed using AMPureXP beads. 20 μl of beads were added
to each well containing the 16S amplicon. The sample was mixed, then incubated
at room temperature for 5 min. Beads were isolated using a magnetic stand and the
supernatant was discarded. The beads were then washed twice with 80% ethanol,
the supernatant was removed and the beads were then allowed to air dry for 10 min.
The beads were then incubated for 2 min with 10 mM Tris pH 8.5. The supernatant
was then separated from the beads and collected into a fresh 96 well plate.

Individual samples were indexed using Nextera XT Index Primers in the following
reaction: 5 μl DNA, 5 μl Nextera XT Index Primer 1, 5 μl Nextera XT Index Primer 2, 25 μl 2x KAPA HiFi HotStart ReadyMix, & 10 μl Nuclease-free water. The reaction was run in a thermal cycler using the following cycling conditions listed above with the exception of 8 cycles instead of 25.

PCR clean up was repeated as described above. Samples were then stored at 4°C prior to sequencing.

2.7.3 DNA Sequencing and Data Analysis

Sequencing was performed by technicians at the University of Manchester Genomic Technologies core facility using an Illumina MiSeq System. In brief, the final libraries were diluted to 4 nM with 10 mM Tris pH 8.5 and pooled using 5 μl of each indexed sample. 5 μl of pooled DNA was denatured with 5 μl of 0.2 N NaOH for 5 min. The samples were then diluted with 990 μl chilled hybridisation buffer. A PhiX control library was included and the samples were then loaded onto a MiSeq flow cell for sequencing.

Raw FASTQ files were sent to the University of Manchester Bioinformatics core facility for trimming and quality control. Operational taxonomic units (OTUs) were assigned at a 97% sequence similarity threshold and identified against a 16S rRNA gene data base (SILVA 132, released December 2017) using Quantitative Insights into Microbial Ecology (QIIME) software. Statistical analysis was performed by the author using R software. Analysis was performed using the DESeq2, Vegan, and Pheatmap packages. Graphs were either generated directly in R, using the ggPlot2 package, or raw data was exported and plotted in GraphPad Prism version 8.00.
2.8 Quantification of Gene Expression by PCR

2.8.1 RNA Extraction

Tissue was dissected from the mesenteric lymph node and small intestine, placed in 1 ml of TRIzol reagent, and then immediately frozen on dry ice. Samples were stored at -80°C prior to extraction. RNA extraction was performed as described by the manufacturer. Briefly, each sample was homogenised using a FastPrep-24 (MPBio), 0.2 ml chloroform (Sigma) was added, and samples were centrifuged for 15 min at 12,000 x g. The aqueous phase containing the RNA was removed, 0.5 ml isopropanol was added, and and centrifuged at 12,000 x g for 10 min pelleting the RNA. The isopropanol was removed and the RNA was washed in 75% ethanol (Fisher). The RNA was then resuspended in 20μl. RNA concentration was determined on a Nanodrop ND-1000 (Labtech international, UK).

2.8.2 cDNA synthesis

Extracted RNA was converted to cDNA using the Promega GoScript reverse transcriptase kit following the manufacturers instructions. In short, 2 μg of RNA and 0.5 μg Oligo(dT) were combined and RNase-free water was added to make a final volume of 5 μl. This solution was heated to 70°C for 5 min and then chilled on ice for a further 5 mins. An reverse transcription (RT) master-mix – consisting of GoScript reaction buffer, MgCl₂, 0.5 mM dNTPs, recombinant RNasin, GoScript RT and Nuclease-free water – was prepared. 15 μl of (RT) master-mix was added to the RNA and briefly centrifuged. The samples were allowed to anneal for 5 min at 25°C; the samples were then heated to 42°C for 1 hr for extension to occur; the samples were then heated to 70°C for 15 mins to inactivate the the RT. Heating occurred in a Veriti thermal cycler (Applied Biosystems). The resultant cDNA was stored at 4°C prior to use.
2.8.3 Quantitative PCR

qPCR was performed using the SensiFAST SYBR Hi-ROX kit (Bioline). A reaction mix was prepared combining 10 μl of 2x SensiFAST SYBR Hi-ROX mix, 0.8 μl of 10 μM forward primer, 0.8 μl of 10 μM reverse primer, 6.4 μl of RNase-free H₂O, and 2 μl of cDNA. The reaction mix was combined by centrifugation. Using a StepOnePlus Real-Time PCR system (ThermoFisher), samples were heated to 95°C for 2 mins and then run for 40 cycles using the following cycling conditions: 95°C for 5 secs, 60°C for 10 sec, 72°C for 20 sec. Following the final cycle the samples were heated to 95°C for 15 sec, cooled to 60°C for 1 min and heated to 95°C for a further 15 secs. Data was analysed using the 2^−δCT method with β-actin used as a reference gene. RNA levels are reported as a proportion of the total β-actin. Primer sequences are listed in table 2.3.
### Table 2.3: List of primers used for qPCR

<table>
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<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>F – 5’ TCT TGG GTA TGG AAT GTG GCA</td>
</tr>
<tr>
<td></td>
<td>R – 5’ ACA GCA CTG TGT TGG CAT AGA GGT</td>
</tr>
<tr>
<td>IL-4</td>
<td>F – 5’ ACA GGA GAA GGG ACG CCA T</td>
</tr>
<tr>
<td></td>
<td>R – 5’ GAA GCC CTA CAG ACG AGC TCA</td>
</tr>
<tr>
<td>IL-10</td>
<td>F – 5’ TGG CCC AGA AAT CAA GGA GC</td>
</tr>
<tr>
<td></td>
<td>R – 5’ CAG CAG ACT CAA TAC ACA CT</td>
</tr>
<tr>
<td>IFNγ</td>
<td>F – 5’ TCA ACG CTA CAC ACT GCA TCT TGG</td>
</tr>
<tr>
<td></td>
<td>R – 5’ CGA CTC CTT TTC CGC TTC CTG AG</td>
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<tr>
<td>RELMα</td>
<td>F – 5’ TCC CAG TGA ATA CTG ATG AGA</td>
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<td>R – 5’ CCA CTC TGG ATC TCC CAA GA</td>
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<td>RELMβ</td>
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<td></td>
<td>R – 5’ AAC ACA GTG TAG GCT TCA TGC TGT</td>
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### 2.9 Statistical Analysis and Data Presentation

Statistical tests were performed, and graphs were produced, using GraphPad Prism version 8.00 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com.
Chapter 3

Characterisation of the Immune Response to *H. polygyrus* Trickle Infection
3.1 Introduction

The natural mouse parasite *H. polygyrus* has long been used as a model for the evaluation of immune responses to gastrointestinal helminths, and extensively as a tool used for the study of Th2 immune responses in general. Infection with *H. polygyrus* results in a patent chronic infection in most strains of mice irrespective of dose, and can persist for many months in C57BL/6 mice. Resistance to infection can be modelled using re-challenge models where a primary infection is cleared by treatment with anthelminthics. Resistance to infection is primarily associated with a robust IL-4-driven Th2 response that activates effector mechanisms such as larval trapping by aaMΦs and secretion of anti-parasitic peptides from the epithelium such as RELMβ. *H. polygyrus* combats these mechanisms by secreting immunomodulatory peptides that inhibit IL-33 signalling and promoting Treg expansion through induction of TGFβ pathways.

These data have been derived from models using single dose infections and relatively short windows. However, under natural conditions individuals are regularly re-infected; likely prior to expelling any previous parasites. Under these conditions, in humans, immunity appears to develop slowly and is only partially effective. There has been little work undertaken to model this process under laboratory conditions, and it is therefore unknown how repeated challenge alters the dynamics of immune responses to infection. Previous work only sought to characterise the kinetics of *H. polygyrus* burden under repeated challenge conditions, so called “trickle infections”, however, results were conflicting. What is apparent is that trickle infection alters the kinetics of worm expulsion, but the immunological mechanisms that underly this process remain undetermined.

Using *T. muris* it has been demonstrated that there are clear differences between single dose models of infection and trickle infection. Resistance develops slowly and requires a dose-dependent transition from a Th1 dominant immune response to a Th2 response, in a CD4+ T cell-dependent fashion. The employment of such a model has been crucial in characterising the processes that occur when individuals transition from one immune state to another, and establishes a more representative
model of natural infection.

To gain a comprehensive understanding of how immunity to helminths develops it is essential that laboratory model better reflect natural modes of infection. To build on previous work, here mice were repeatedly infected with different doses of *H. polygyrus*. The immune response was characterised and compared to more traditional single dose infections. This regime of *H. polygyrus* infection was then combined with a previously published *T. muris* trickle infection to investigate the effect of co-infection in long-term dynamic helminthiases.

Specific objectives:

- Develop a model of *H. polygyrus* trickle infection and contrast this with traditional single-dose infections.

- Assess the role of T cells in the development of resistance to *H. polygyrus*.

- Use this novel model of *H. polygyrus* infection to investigate the effect of diet on resistance to infection.

- Investigate the effect of *H. polygyrus* co-infection on a defined trickle infection model of *T. muris*. 
Figure 3.1: Schematic representation of Infection Strategy and Treatment Groups. C57BL/6 male mice were infected with *H.polygyrus* L3 larvae by oral gavage. Mice were given either a single dose at the beginning of the experiment or re-infected weekly. Mice were culled at 3, 7 and 12 weeks post infection.
3.2 Results

3.2.1 Trickle infection results in protection against subsequent infection and drives expulsion of established parasites.

To characterise the dynamics of infection to single and trickle infections mice were treated with one of four infection regimes: a single dose of 70 L3 larvae (Single dose\textsuperscript{Lo}), a single dose of 350 L3 larvae (Single dose\textsuperscript{Hi}), trickled with 10 L3 larvae once per week (Trickle\textsuperscript{Lo}), or trickled with 50 larvae once per week (Trickle\textsuperscript{Hi}) (Figure 3.1). Groups were culled at three, seven or twelve weeks post infection (w.p.i.), at each point the worm burden was determined.

Trickle\textsuperscript{Lo} infected mice showed a low level of worm burden at 3 w.p.i. that decreased at 7 w.p.i. where it remained out to 12 w.p.i. (Fig. 3.2A). This moderate decrease from 3 w.p.i. was not statistically significant at either time point. Despite repeated challenge infections worm burden did not accrue over time indicating the development of resistance to new larvae. In addition, at both 7 and 12 w.p.i., worms could not be identified in the small intestine of several mice suggesting a mild ability to expel established adult parasites. Trickle\textsuperscript{Hi} infected mice showed an initially moderate burden at 3 w.p.i., which rapidly decreased at 7 w.p.i.. By 12 w.p.i. the majority of mice had cleared their infection entirely (Fig. 3.2B). These data indicate that not only do Trickle\textsuperscript{Hi} mice develop a protective response against challenge infection, but that they are also capable of mounting an effective response against the existing primary infection.

Mice infected with either a Single Dose\textsuperscript{Lo} or Single Dose\textsuperscript{Hi} regime maintained their infection throughout the length of the experiment. Within groups there was no significant difference between time points (Fig. 3.2 C&D). These patterns of worm burden were mirrored by faecal egg counts, although for single dose groups there was an evident peak of egg output at 4 weeks post infection not evident from worm burden alone (Fig. 3.3).
Figure 3.2: Intestinal Worm Burden of Mice Infected with H. polygyrus. Male C57BL/6 mice were infected with H. polygyrus by oral gavage. Adult worm burden was assessed at 3 (n = 5), 7 (n = 10), and 12 (n = 10) weeks p.i. (A) Worm burden of trickle$^{L_0}$ infected mice. (B) Worm burden of trickle$^{H_1}$ infected mice. (C) Worm burden of single dose$^{L_0}$ infected mice. (D) Worm burden of single dose$^{H_1}$ infected mice. Mice are represented as individual data points with line and error bars denoting the median and interquartile range respectively. * indicates significance (P < 0.05), *** indicates significance (P < 0.001) as calculated by Kruskal-Wallis test with post hoc Dunn’s comparisons.
Figure 3.3: Faecal Egg Burden of Mice Infected with *H. polygyrus*. Male C57BL/6 mice were infected with *H. polygyrus* by oral gavage. Faecal egg burden was assessed at every two weeks p.i. (A) Faecal egg burden of trickle$^{\text{Lo}}$ infected mice. (B) Faecal egg burden of trickle$^{\text{Hi}}$ infected mice. (C) Faecal egg burden of single dose$^{\text{Lo}}$ infected mice. (D) Faecal egg burden of single dose$^{\text{Hi}}$ infected mice. Mice are represented as individual data points with line and error bars denoting the mean and SEM respectively.
3.2.2 Cytokine expression in the Mesenteric Lymph Node during trickle and single dose infections

Resistance to GI helminth infection is associated with the Th2 immune response. In particular expression of Th2 cytokines such as IL-4 correlates with resistance. Loss of the IL-4R results in an increase in susceptibility to *H. polygyrus* infection. To determine whether trickle infection induced a different pattern of cytokine expression compared to single dose infection, the expression of IL-4, IL-10 and IFN-γ in the MLN was measured by qPCR.

For all infection regimes, expression of IL-4 was increased compared to naive controls at 3 w.p.i. (Fig 3.4 A,D,G&J). There was no significant difference between infected groups at this time point (P = 0.3916). However, for single-dose infected groups expression of IL-4 did not change between 3 and 7 w.p.i. and began to decline by 12 w.p.i. with some individual mice returning to naive levels of expression (Fig 3.4 A&D). There was no significant difference between single dose infection regimes at any time point (P > 0.05) suggesting parasite burden did not effect expression of IL-4.

By way of contrast, expression of IL-4 continued to increase out to 7 w.p.i. in mice trickled with *H. polygyrus* (Fig 3.4 G&J). At this time point expression of IL-4 in TrickleLo and TrickleHi infection groups was not significantly different (P = 0.4488). However, both groups expressed significantly greater levels of IL-4 than both Single DoseLo and Single DoseHi groups (P = 0.0004). At 12 w.p.i. expression of IL-4 began to decrease in trickle infected mice, however, there was not a significant difference between trickle regimes (P = 0.2282), and both groups remained significantly higher than single dose infection groups (P < 0.0001).

Expression of both IL-10 and IFN-γ did not change considerably during the course of infection for either single dose infection group (Fig 3.4 B, C, E & F). Conversely, both trickle infected groups saw subtle but statistically significant decreases in expression of both IL-10 and IFN-γ at 7 w.p.i. (Fig 3.4 H, I, K & L). This correlated with the strong peak of IL-4 expression observed in these groups at this time point.
By 12 w.p.i. expression of these genes had returned to median levels comparable with the naive control.

Together these data show a clear difference in the dynamics of cytokine expression between single dose and trickle infections. Single dose infections cause an increase of IL-4 expression which plateaus and slowly decreases over time, whereas, trickle infection causes IL-4 expression to continue to increase peaking around 7 w.p.i. but remaining high at 12 w.p.i. Further, there is little change in other regulatory cytokines during single dose infection, whereas in trickle infection there is a dynamic reduction in expression of cytokines associated with susceptibility to helminth infection. Therefore, the data suggests that elevated IL-4 expression, and reduction in IFNγ and IL-10, may be responsible for the development of resistance to established *H. polygyrus* infection.
Figure 3.4: Cytokine expression in the Mesenteric Lymph Nodes of *H. polygyrus* infected mice. Male C57BL/6 mice were infected with *H. polygyrus* by oral gavage. Cytokine expression in the MLN was assessed using quantitative PCR. Values are given as expression relative to β-actin as calculated by the 2^ΔΔCT method. (A - C) Expression of [A] IL-4, [B] IL-10, & [C] IFNγ in Single Dose^Lo^ infected mice. (D - F) Expression of [D] IL-4, [E] IL-10, & [F] IFNγ in Single Dose^Hi^ infected mice. (G - I) Expression of [G] IL-4, [H] IL-10, & [I] IFNγ in Trickle^Lo^ infected mice. (J - L) Expression of [J] IL-4, [K] IL-10, & [L] IFNγ in Trickle^Hi^ infected mice. Naive n = 23; 3 w.p.i. n = 5; 7 & 12 w.p.i. n = 8 - 10. Mice are represented as individual data points with line and error bars denoting the median and interquartile range respectively. * indicates significance (P <0.05), ** indicates significance (P < 0.01) *** indicates significance (P < 0.001), **** indicates significance (P < 0.0001). Significance values are as calculated by Kruskal-Wallis test with post hoc Dunn’s comparisons from the naive controls.
3.2.3 Gene expression in the duodenum during trickle and single
dose infections

The role of cytokines during helminth infection is to induce changes in gene expression in the gut mucosa which lead to physiological changes responsible for parasite expulsion. To determine whether increased IL-4 expression in the MLN correlated with changes to gene expression in the gut, qPCR was performed on RNA extracted from the duodenum. The resistin-like molecules (RELMs) are a family of immune-modulatory molecules strongly up-regulated during helminth- and allergen- induced immune responses. Both RELM\(\beta\) and RELM\(\alpha\) are directly regulated by IL-4 and IL-13 through the STAT6 pathway.

All infected groups demonstrated strong up-regulation of both RELM\(\beta\) and RELM\(\alpha\) in response to infection with *H. polygyrus* (Fig 3.5). At 3 w.p.i. there was no significant difference in expression of either gene between infected groups (P > 0.05). However, at 7 w.p.i. median expression of RELM\(\beta\) and RELM\(\alpha\) had continued to increase in trickle infected groups (Fig 3.5 E-H), where in single dose infected groups expression had plateaued or decreased relative to 3 w.p.i. (Fig 3.5 A-D). Expression of these genes continued to plateau in single dose infected groups at 12 w.p.i.. There was no significant difference between single dose infected groups for either gene at any time point (P > 0.05). For trickle infected groups there was a small decrease in expression of RELM\(\beta\) and RELM\(\alpha\) from 7 to 12 w.p.i.. However, expression of these genes was still significantly higher for the Trickle\(\text{Hi}\) group compared to single dose infected groups at this time point (P = 0.0085).

Collectively, these data demonstrate that the pattern of Th2-induced gene expression in the duodenum mirrors the expression of IL-4 in the MLN. Single dose infected mice experience an increase in gene expression at 3 w.p.i. which either plateaus or decreases across the rest of the time course. Whereas, trickle infected mice continue to increase gene expression out to 7 w.p.i. with a small decrease by 12 w.p.i.. Again, parasite burden did not appear to affect gene expression, with no significance difference between different doses of trickle infection or between different single dose infections.
Figure 3.5: Gene expression in the duodenum of *H. polygyrus* infected mice. Male C57BL/6 mice were infected with *H. polygyrus* by oral gavage. Cytokine expression in the duodenum was assessed using quantitative PCR. Values are given as expression relative to β-actin as calculated by the 2^−ΔΔCT method. (A & B) Expression of [A] RELMβ & [B] RELMα in Single DoseLo infected mice. (C & D) Expression of [C] RELMβ & [D] RELMα in Single DoseHi infected mice. (E & F) Expression of [E] RELMβ & [F] RELMα in TrickleLo infected mice. (G & H) Expression of [G] RELMβ & [H] RELMα in TrickleHi infected mice. Naive n = 22; 3 w.p.i. n = 5; 7 & 12 w.p.i. n = 9 - 10. Mice are represented as individual data points with line and error bars denoting the median and interquartile range respectively. * indicates significance (P < 0.05), ** indicates significance (P < 0.01) *** indicates significance (P < 0.001), **** indicates significance (P < 0.0001). Significance values are as calculated by Kruskal-Wallis test with post hoc Dunn’s comparisons from the naive controls.
3.2.4 Cytokine expression in the lung during trickle and single dose infections

There is growing understanding that responses in one mucosal site have long distance effects on separate mucosal sites. Indeed infection with *H. polygyrus* has been shown to affect immune responses in the lung. To assess whether infection with *H. polygyrus* was inducing changes in lung mucosa RNA was extracted from the lung tissue at autopsy and the expression of RELMα was measured by qPCR.

Expression of RELMα increased during infection in all treatment groups (Fig 3.6). For both single dose infected groups there was a decrease in median expression at 7 w.p.i., however, this recovered by 12 w.p.i. (Fig 3.6 A&B). Trickle infected groups on the other hand sustained RELMα up-regulation throughout the course of infection (Fig 3.6 C&D). Despite the small decreased expression in single dose infected groups at 7 w.p.i., there was no statistically significant difference between any infected groups at any time point.

3.2.5 Mucosal mast cell response during *H. polygyrus* infection

Mucosal mastocytosis is characteristic to helminth infection, and a role for mast cells in directing Th2 responses has been described. To investigate the mast cell response during *H. polygyrus* infection levels of mMCP-1 in the serum were measured by ELISA. mMCP-1 is specific to mucosal mast cells and serum concentration of this protein correlates with mast cell frequency in the gut mucosa.

Throughout the course of infection levels of mMCP-1 remained elevated in all infected groups relative to naive mice (Fig 3.7). mMCP-1 concentration in infected groups was greatest at 3 w.p.i. with an average concentration of around 200 ng/μl and no significant difference between groups (P = 0.2307). In all groups there was a steady decline in the median concentration of mMCP-1 through 7 and 12
w.p.i.. However, this decline was more pronounced in single dose infected groups. At these time points trickle infected groups had statistically significantly greater levels of protein in the serum than single dose infected mice (Fig 3.7).
Figure 3.6: Cytokine expression in the lung of *H. polygyrus* infected mice. Male C57BL/6 mice were infected with *H. polygyrus* by oral gavage. Cytokine expression in the lung was assessed using quantitative PCR. Values are given as expression relative to β-actin as calculated by the 2^−ΔΔCT method. (A - D) Expression of RELMα in [A] Single Dose^Lo, [B] Single Dose^Hi, [C] Trickle^Lo & [D] Trickle^Hi infected groups. Naive n = 14; 3 w.p.i. n = 3 - 5; 7 & 12 w.p.i. n = 5. Mice are represented as individual data points with line and error bars denoting the median and interquartile range respectively. * indicates significance (P < 0.05), ** indicates significance (P < 0.01) *** indicates significance (P < 0.001). Significance values are as calculated by Kruskal-Wallis test with post hoc Dunn’s comparisons from the naive controls.
Figure 3.7: Serum levels of mMCP-1 in *H. polygyrus* infected mice. Male C57BL/6 mice were infected with *H. polygyrus* by oral gavage. Serum concentration of mMCP-1 was measured by sandwich ELISA. Naive n = 24; 3 w.p.i. n = 3 - 4; 7 w.p.i. n = 9 - 10; 12 w.p.i. n = 10. Mice are represented as individual data points with line and error bars denoting the median and interquartile range respectively. The dotted grey line indicates the median expression of mMCP-1 in naive mice. * indicates significance (P < 0.05), ** indicates significance (P < 0.01). Significance values are as calculated by Kruskal-Wallis test with *post hoc* Dunn’s comparisons.
3.2.6 IgG1 Response During *H. polygyrus* infection

Characteristic of GI helminth infection is Ig class switching to the IL-4-driven IgG isotype, IgG1\(^1\). Production of IgG has been shown to be protective against *H. polygyrus*. IgG null mice fail to develop resistance to *H. polygyrus* in challenge infection models\(^9\). Given that trickle infected mice expressed significantly higher levels of IL-4 in the MLN, the levels of parasite-specific IgG1 were measured to determine whether isotype switching had been altered by trickle infection. Levels of parasite-specific serum IgG1 were determined by indirect-ELISA.

At 3 w.p.i. There was little production of IgG1 by any of the infected groups. However, by 7 w.p.i. all groups showed strong expression of parasite-specific IgG1. In accord with the gene expression data discussed above, both trickle infected groups produced significantly more IgG1 than the single-dose infected groups. A similar pattern was seen at 12 w.p.i. with trickle infected groups having greater mean levels of IgG1 than the single-dose groups (Fig. 3.8).

These data indicate that increased IL-4 expression by trickle infection is likely driving Th2 IgG isotype switching.
Figure 3.8: Serum levels of parasite-specific IgG1 in *H. polygyrus* infected mice. Male C57BL/6 mice were infected with *H. polygyrus* by oral gavage. Serum concentration of parasite-specific IgG1 was measured by indirect ELISA. n = 5 mice per group. Mice are represented as individual data points with line and error bars denoting the mean and SEM respectively. ** indicates significance (P <0.01), **** indicates significance (P < 0.0001). Significance values are as calculated by 2-way ANOVA test with post hoc Holm-Sidak’s comparisons.
3.2.7 \( \alpha \beta \)-T cells are essential for the expulsion of *H. polygyrus* during a trickle infection

A role for the adaptive immune response is well established in resistance to *H. polygyrus* infection\textsuperscript{105}. Depletion of CD4+ cells results in mice becoming susceptible to challenge infection\textsuperscript{106}. However, there is growing interest in the roles for innate cells in being key drivers of resistance to infection, particularly ILC2s\textsuperscript{99,100}. To determine whether T cells were essential for trickle-induced parasite expulsion, *Tcrα/-* mice, which fail to generate mature \( \alpha \beta \) T cells, were infected with a Trickle\textsuperscript{Hi} infection regime for 7 weeks.

Where WT mice were able to reduce their worm burden, *Tcrα/-* mice showed higher worm burdens at both 3 and 7 w.p.i. (Fig. 3.9). This increase in worm burden was highly statistically significant (\( p < 0.0001 \)). *Tcrα/-* mice were also unable to induce production of *H. polygyrus*-specific IgG1 (Fig. 3.10), suggesting that IL-4 from \( \gamma \delta \)-T cells or innate cells is insufficient to drive class switching.

Analysis of gene expression showed that whilst WT mice were able to significantly increase level of IL-4 expression in the MLN between 3 and 7 w.p.i., *Tcrα/-* mice were unable to increase IL-4 expression over time (Fig. 3.11 A). In accord with this, *Tcrα/-* mice showed reduced levels of RELM\( \alpha \) and RELM\( \beta \) in the duodenum (Fig. 3.11 B&C).
Figure 3.9: Expulsion of *H. polygyrus* in *Tcrα*⁻/⁻ mice. *Tcrα*⁻/⁻ mice or wildtype (WT) controls were infected with 50 L3 *H. polygyrus* larvae weekly for three or seven weeks. At autopsy worm burden was assessed by eye under dissecting microscope. Points represent individual mice and bars indicate the mean. * indicates significance (P < 0.05), **** indicates significance (P < 0.0001). Significance values are as calculated by 2-way ANOVA test with *post hoc* Tukey’s comparisons.
Figure 3.10: Serum levels of *H. polygyrus*-specific IgG1 in *Tcrα*−/− mice. *Tcrα*−/− mice or wildtype (WT) controls were infected with 50 L3 *H. polygyrus* larvae weekly for three or seven weeks. Serum concentration of parasite-specific IgG1 was measured by indirect ELISA. Points represent individual mice and bars indicate the mean. **** indicates significance (P < 0.0001). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons.
Figure 3.11: Gene expression $Tcr\alpha^{-/-}$ mice. $Tcr\alpha^{-/-}$ mice or wildtype controls were infected with 50 L3 $H. polygyrus$ larvae weekly for three or seven weeks. (A) IL-4 expression in the MLN and (B) RELM$\alpha/\beta$ expression in the duodenum was assessed using quantitative PCR. Values are given as expression relative to $\beta$-actin as calculated by the $2^{\delta CT}$ method. Points represent individual mice and bars indicate the mean. * indicates significance (P < 0.05). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons.
3.2.8 Diet induced obesity does not affect expulsion of *H. polygyrus*

There has been recent interest in the interaction between diet and helminth infection. Previous work has suggested that *H. polygyrus* is able to protect against some of the effects of DIO\textsuperscript{140}. However, to date, there has been no published assessment of the effect DIO has on resistance to infection. To investigate this mice were placed on either a LFD or HFD for 12 weeks then infected with a trickle infection (20 L3 larvae/week) or given a single high dose infection (200 L3 Larvae), mice were then culled at 7 weeks post infection. Prior to infection, mice on a HFD gained significantly more weight than those on a LFD (p < 0.0001) (Fig. 3.12 A). Infection did not appear to affect body weight with mice in all groups maintaining a consistent body weight across the course of infection (Fig. 3.12 B). Infection also had no effect on markers of DIO with all groups showing significantly raised levels of blood glucose following fasting (Fig. 3.12 C) and increased levels of subcutaneous fat (Fig. 3.12 D) when compared to mice on a low fat diet.

The effect of DIO on infection was assessed following autopsy. There was no significant difference in worm burden between mice on HFD or LFD for either single dose infection or trickle infection (Fig. 3.13 A). Both single dose and trickle infected mice on a HFD had a small but significant increase in the level of parasite-specific serum IgG1 relative to those on a LFD (Fig. 3.13 B). However, there was no effect of HFD diet on levels of RELM\textalpha{} expression in the duodenum (Fig. 3.13 C).
Figure 3.12

Panel A: Graph showing weight (g) over weeks pre-infection.

Panel B: Graph showing weight (g) over weeks post-infection.

Panel C: Bar graph showing blood glucose levels (pg/mL) across different groups.

Panel D: Bar graph showing subcutaneous fat weight (g) across different groups.
Figure 3.12: Diet induced weight change during *H. polygyrus* infection. Mice were placed on specialised diets either high in fat (HFD) or low in fat (LFD) for 12 weeks. Mice were then infected with either a single dose of *H. polygyrus* or given a trickle infection for 7 weeks. (A & B) Body weight was measured throughout the course of the experiment. Points and bars indicated the mean, error is given as the SEM. (C) Fasted blood glucose was measured at 6 weeks post infection by fasting mice overnight for 16 hr. (D) Subcutaneous fat was collected from the left subcutaneous fat pad at autopsy and weighed. n = 5, * indicates significance (P <0.05), ** indicates significance (P <0.01), **** indicates significance (P <0.0001). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons.

Figure 3.13: Effect of diet induced obesity on *H. polygyrus* infection. Mice were placed on specialised diets either high in fat (HFD) or low in fat (LFD) for 12 weeks. Mice were then infected with either a single dose of *H. polygyrus* or given a trickle infection for 7 weeks. (A) Worm burden was then assessed at autopsy. (B) Serum concentration of parasite-specific IgG1 was measured by indirect ELISA. (C) RELMα expression in the duodenum was assessed using quantitative PCR. Values are given as expression relative to β-actin as calculated by the 2^-ΔΔCT method. n = 5, * indicates significance (P <0.05), ** indicates significance (P <0.01). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons.
3.2.9 The effect of co-infection with *H. polygyrus* on the dynamics of *T. muris* trickle infection

We have previously shown that trickle infection with *T. muris* results in the steady acquisition of immunity over time, with mice susceptible to initial infections but resistant to later infections beginning around 11 w.p.i.\(^60\). Given that *in situ* infected individuals are likely to be concurrently infected with multiple species of GI helminth we sought to investigate whether co-infection with *H. polygyrus* affected the ability of mice to generate resistance during *T. muris* trickle infection.

Mice were infected with either a single dose of *H. polygyrus* (200 L3 Larvae), a single low dose of *T. muris* (20 eggs), trickle infected with *T. muris* (20 eggs/week), or co-infected (20 *T. muris* eggs/week + single dose of 200 L3 *H. polygyrus* larvae). As expected mice trickle infected with *T. muris* showed a significant increase in worm burden between 3 and 9 w.p.i., and then a significant decrease in worm burden at 11 w.p.i. indicating the development of resistance to infection (Fig. 3.14 A). Co-infection with *H. polygyrus* did not appear to affect the development of resistance to *T. muris* at week 11 with the co-infected group showing a comparable decrease in *T. muris* worm burden (Fig. 3.14 A). There also did not appear to be any effect on *H. polygyrus* burden as the co-infected group had similar levels of *H. polygyrus* present at each time point (Fig. 3.14 B). There was also no significant correlation between *H. polygyrus* and *T. muris* burdens in the co-infected group at any time point (Fig. 3.14 C).

To see if a ‘co-trickle’ infection would instead have an effect, mice were either, trickle infected with *H. polygyrus* (20 L3 Larvae/week), given a single low dose of *T. muris* (20 eggs), trickle infected with *T. muris* (20 eggs/week), or co-trickle infected (20 *T. muris* eggs/week + 20 L3 *H. polygyrus* larvae/week). There was no apparent effect on the ability of mice to generate resistance to *T. muris* at 11 w.p.i. when co-infected with *H. polygyrus* (Fig. 3.15 A). The ability of mice to reduce their *H. polygyrus* burden also appeared to be unaffected, although due to low numbers it was harder to resolve (Fig. 3.15 B). In co-trickle infected mice there was also no significant correlation between *H. polygyrus* and *T. muris* burdens at any time point (Fig 3.15 C).
Figure 3.14: The effect of single dose *H. polygyrus* co-infection on *T. muris* trickle infection. Mice were infected with either a single low dose of *T. muris* (20 eggs), a single dose of *H. polygyrus* (200 L3 larvae), trickle infected with *T. muris* (20 eggs/week), or co-infected with a trickle infection of *T. muris* (20 eggs/week) and a single dose of *H. polygyrus* (200 L3 larvae). Mice were sacrificed at 3, 9 & 11 weeks post infection. Worm burden was assessed at autopsy by eye under dissecting microscope for (A) *T. muris* and (B) *H. polygyrus*. Bar represents the mean and point represent individual replicates. n = 5, * indicates significance (P < 0.05), ** indicates significance (P < 0.001), *** indicates significance (P < 0.0001). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons. (C) For the co-infected group *T. muris* burden was correlated against *H. polygyrus* burden. Linear regression analysis was performed to identify significant correlations.
Figure 3.15: The effect of trickle dose *H. polygyrus* co-infection on *T. muris* trickle infection. Mice were infected with either a single low dose of *T. muris* (20 eggs), a trickle infection of *H. polygyrus* (20 L3 larvae/week), trickle infected with *T. muris* (20 eggs/week), or co-trickle infected *T. muris* (20 eggs/week) and *H. polygyrus* (20 L3 larvae/week). Mice were sacrificed at 5, 9 & 11 weeks post infection. Worm burden was assessed at autopsy by eye under dissecting microscope for (A) *T. muris* and (B) *H. polygyrus*. Bar represents the mean and point represent individual replicates. n = 4, ** indicates significance (P < 0.01). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons. (C) For the co-infected group *T. muris* burden was correlated against *H. polygyrus* burden. Linear regression analysis was performed to identify significant correlations.
To assess whether co-infection was impacting pathology during infection tissue sections were taken from the caecum of mice that had been co-trickle infected and histological analysis was performed following alcian blue staining (Fig. 3.16). Naive mice, and those trickle infected with *H. polygyrus* alone, showed little change in caecal architecture over time indicating the absence of inflammation or pathology. Mice given a single low dose of *T. muris* showed increased crypt length, goblet cell hyperplasia, and a minor increase in the width of the submucosa (Fig. 3.17). Mice that received a *T. muris* trickle infection showed comparable levels of goblet cell and crypt hyperplasia to low dose infected animals at 5 w.p.i., however, at 9 w.p.i. showed a sharp increase in these parameters, followed by a decrease at 11 w.p.i. (Fig. 3.17 A&B). Interestingly, co-infected mice also showed an increase in goblet cell and crypt hyperplasia at 9 w.p.i., however at 11 w.p.i. there was no reduction in either parameter. Additionally, mice trickle infected with *T. muris* alone showed a sharp increase in submucosal width early during infection which returned to naive levels by 11 w.p.i.. By way of contrast, co-infected mice maintained a consistent low level of increased mucosal thickness (Fig. 3.17 C).

These data suggest that whilst co-infection with *H. polygyrus* does not prevent trickle infection-induced resistance to *T. muris*, it does prevent co-infected mice from resolving chronic infection-associated inflammation.
Figure 3.16: Histology of caecal tissue. Male C57BL/6 mice were infected with a low dose of *T. muris* or trickled weekly with either *T. muris*, *H. polygyrus*, or both by oral gavage. At autopsy, caecal tissue was collected, fixed, and 5 μm sections were stained with Alcian Blue. Images were taken at x10 magnification.
Figure 3.17: Analysis of caecal histology. Male C57BL/6 mice were infected with a low dose of *T. muris* or trickled weekly with either *T. muris, H. polygyrus*, co-trickle infected with both. Mice were sacrificed at 5, 9 and 11 weeks post infection, and caecal tissue was collected for histological analysis. Histological sections were stained with Alcian Blue and analysed for signs of histopathology, these are; (A) crypt hyperplasia, measured as crypt length from the base of the crypt to the gut lumen; (B) goblet cell hyperplasia, measured as the number of goblet cells per crypt; and (C) the degree of oedema, measured as distance from base of the crypt to tunica muscularis. Symbols denote the mean (n = 4 mice/group), and error bars denote the SEM. * indicates significance (P < 0.05), ** indicates significance (P < 0.01), *** indicates significance (P < 0.001), **** indicates significance (P < 0.0001). Significance values are as calculated by 2-way ANOVA test with post hoc Tukey’s comparisons.
3.3 Discussion

Murine models of helminth infection have been crucial in defining the mechanisms that govern resistance and susceptibility to infection. However, these models have relied primarily on single-bolus infections, that fail to mimic both the natural state of exposure as well as dynamic changes that can occur in host immune status. Previous work has shown that using a trickle infection of *T. muris* a more representative phenotype can be observed, whereby partial immunity develops slowly with recurrent infections\(^{60}\). Here the dynamics of *H. polygyrus* trickle infection were characterised using two different doses. This infection regime was then combined with a *T. muris* trickle infection to observed how co-infection impacted the ability to generate trickle-induced immunity.

3.3.1 *H. polygyrus* trickle induces both resistance and parasite expulsion

Infection of C57BL/6 mice with a single bolus of *H. polygyrus* is understood to result in a long-lived infection with mice still being heavily infected after several months\(^{45}\). The data presented here are consistent with that notion, mice given single doses of *H. polygyrus* had consistent worm burdens throughout infection which did not decrease significantly even after 12 w.p.i. irrespective of infectious dose. By way of contrast mice that received a trickle infection showed a consistent decrease in worm burden between each time point, although this effect was more muted for the Trickle\(^{L0}\) group. Whilst the data presented here is not definitive, it indicates that a single infection is sufficient to induce sterilising immunity to subsequent challenges in C57BL/6 mice, but it requires multiple challenges to drive a sufficient response to expel existing parasites. This is in contrast to previously published data that showed an initial increase in worm burden during trickle infection that peaked between 5 and 6 weeks post infection\(^{133}\). However, the authors administered larvae twice weekly in NIH mice, the combination of different dosing regime and mouse strain may account for the different kinetics observed here. Indeed there is considerable differences in response to helminth infection between mouse strains\(^{45}\). This also presents a considerably different dynamic to
trickle infection with *T. muris* where there is an extended period of susceptibility to infection with resistance only developing after nine weeks\textsuperscript{60}. However, both models suggest that successive challenge infections are able to drive parasite expulsion during infections that would otherwise be chronic.

*H. polygyrus* begin mating and producing eggs shortly after reaching maturity (around 10 days post infection). In both single dose and trickle infected groups faecal eggs burden was observed to peak at four w.p.i.. It is interesting that this was also the case in mice infected with a single dose as worm burden remained consistent throughout infection. This suggests that overall fecundity is decreasing as the infection progresses, although it is unclear from data presented here whether this is a direct immune mediated-effect of the host on the parasite, or whether it is simply a natural feature of the *H. polygyrus* lifecycle. However, it has previously been shown that transplantation of adult *H. polygyrus* into naive mice resulted in significantly higher faecal egg burdens than transplantation into mice that had previously been infected then treated with anthelminthic\textsuperscript{182}. This suggests the presence of an immune-mediated mechanism that limits parasite fecundity when immunity develops. This is consistent with the notion that the Th2-driven secretion of anti-parasitic molecules, such as RELM\textsubscript{β}, by the epithelium is capable of reducing parasite fitness by limiting their ability to feed\textsuperscript{111}.

3.3.2 Trickle infection drives a potent Th2 response in the duodenum and draining lymph nodes

Resistance to *H. polygyrus* is dependent on a robust Th2 driven immune response. Key to this is signalling through the IL-4R, with IL-4R\textsubscript{α}−/− mice highly susceptible to infection and administration of exogenous IL-4 sufficient to cure immunocompetent mice of an ongoing infection\textsuperscript{90,108}. Levels of IL-4 expression were measured in the MLN by qPCR. In mice receiving a single dose infection levels of IL-4 expression peaked at 3 w.p.i. and then slowly declined with some mice returning to wildtype levels. In contrast to this, trickle infected mice also showed increased IL-4 expression at 3 w.p.i. but continued to increase expression at 7 w.p.i. to levels significantly higher than peak expression in mice infected with a single dose. Additionally, whilst mice that had received a single dose infection
showed no change in IL-10 or IFNγ expression relative to naive controls, trickle infected groups had significantly reduced levels of these cytokines at 7 w.p.i..

Cytokine expression in the MLN correlated with expression of RELMα and RELMβ in the duodenum with single dose infected mice showing peak expression at 3 w.p.i. whilst trickle infected mice continued to increase gene expression at 7 w.p.i. RELMα and RELMβ are regulated by Th2 cytokines via the IL-4R112. RELMα is a marker of alternative activation which is essential for resistance to infection92, and RELMβ has direct anthelminthic properties111,113. The data presented here suggest that trickle infection is capable of inducing higher levels of Th2 activation that drives the expression of anti-parasite effector genes. Consistent with the notion of increased Th2 immunity was the increased levels of serum mMCP-1 levels which are indicative of mast cell activation in the gut49. Whilst mast cells are not thought to be essential in the expulsion of H. polygyrus they have been linked to reduced parasite fecundity182 and mast cell deficient mice suffer increased worm burdens102.

A strong antibody response is crucial for resistance to H. polygyrus in re-challenge models94,109. Cytokine-driven class switching is essential in this process, this has been demonstrated in class-switching impaired CD40−/− mice which are cannot generate vaccine-induced immunity to H. polygyrus91. IgG1 has been shown to be the essential Ig isotype in resistance to infection110. Here, trickle infection was able to induce higher levels of IgG1 than single dose infection at both 7 and 12 w.p.i.. Interestingly, the TrickleLo had comparable levels of IgG1 to the TrickleHi, both single dose groups also had similar levels of IgG1. This would suggest that levels of IgG1 are not driven by antigenic load but rather the degree of systemic Th2 activation.

3.3.3 Trickle-induced expulsion of H. polygyrus is dependent on αβ-T cells

A requirement for CD4+ T cells has previously been demonstrated in resistance to secondary challenge infection with H. polygyrus106. This is unsurprising as an effective antibody response is dependent on T cell-directed activation of B cells.
However, cells in the innate compartment, particularly αMΦs, are also effective mediators of resistance and can be potent sources of Th2 cytokines that may drive secretion of anthelminthic peptides by the epithelium\textsuperscript{93,94}. Thus it is possible that resistance to infection and expulsion of existing adults is driven either by an adaptive response, or through a steady accumulation of innate cells stimulated by frequent challenges.

Here Tcr$\alpha$-/- mice, which fail to develop mature $\alpha$β-T cells, were given a Trickle$^{\text{Hi}}$ infection. Where wildtype mice showed resistance to infection and the ability to expel parasite, Tcr$\alpha$-/- mice had dramatically higher worm burdens indicating that parasites were accumulating with each subsequent infection. These mice also failed to produce parasite-specific IgG1 and had reduced levels of RELM$\alpha$ and RELM$\beta$ expression in the duodenum indicating an impaired Th2 response was occurring. These data demonstrate not only an essential role for $\alpha$β-T cells in resistance to H. polygyrus trickle infection, but that $\gamma$δ-T cells are unable to compensate for their loss. This is unlikely due to an inability of $\gamma$δ-T cells to respond to helminth infection. Indeed, $\gamma$δ-T cells respond to N. brasiliensis infection more rapidly than $\alpha$β-T cells, and are capable of driving host protective responses in the small intestine\textsuperscript{183,184}. Tcr$\delta$-/- mice are unable to expel N. brasiliensis and suffer more severe histopathology\textsuperscript{184}. Whilst the data here does not rule out a role for $\gamma$δ-T cells as contributors to the immune response during H. polygyrus infection, it indicates that alone they are insufficient to drive resistance.

3.3.4 DIO does not appear to affect resistance to H. polygyrus

Transition from rural environments to urban ones in countries endemic for helminthiases has resulted in the adoption of westernised diets and an increase in obesity\textsuperscript{137}. The effects of diet on the immune response is poorly understood although it appears to increase the propensity for the production of pro-inflammatory cytokines such as IFN$\gamma$\textsuperscript{138,139}. Therefore, it would seem likely that obesity may increase susceptibility to helminth infection as obese individuals have a basal skew towards a Th1 phenotype. However, evidence from T. muris suggests the opposite is true. Mice placed on a HFD prior to infection were resistant to a low dose T. muris infection expelling their infections by 35 days post infection whilst mice
on a LFD remained chronically infected\textsuperscript{185}. The mechanism underlying these observations remains unclear. However, mice on a HFD had reduced levels of pro-inflammatory cytokines and Th1 cells, and had increased levels of parasite-specific IgG1 indicating an increased Th2 phenotype\textsuperscript{185}.

To determine whether this was a general feature of obesity on helminth infection, mice were placed on either a HFD or LFD for 12 weeks then given a Single Dose\textsuperscript{Hi} or Trickle\textsuperscript{Hi} \textit{H. polygyrus} infection. Whilst there was a small but significant increase in parasite-specific IgG1 in mice on a HFD, there was no difference in worm burdens between mice on different diets. There was also no difference in the expression of RELM\textgreek{a} in the duodenum between diets suggesting the level of Th2 activation was unaffected by diet. The inconsistencies in these results may be explained by the mode in which these two parasites drive chronic infection. \textit{T. muris} relies on promoting a Th1 response to reach chronicity\textsuperscript{73}, whereas \textit{H. polygyrus} uses direct suppression of Th2 responses and activation of regulatory responses\textsuperscript{103,122,123}. Therefore, whilst a slight skew towards a Th2 response may be sufficient to drive immunity to \textit{T. muris}, \textit{H. polygyrus} is inherently capable of subverting these responses.

A recent publication presented data that suggested \textit{H. polygyrus} protects against DIO. The authors showed a dramatic suppression of weight gain, prevention in the accumulation of subcutaneous body fat, reduced blood glucose levels and an enhanced Th2 phenotype\textsuperscript{140}. Whilst induction of Th2 immunity during helminth infection is unsurprising, the data presented in this thesis suggests that infection with \textit{H. polygyrus} has little to no effect on mouse weight, blood glucose levels, or subcutaneous fat weight. Here, following 12 weeks of diet mouse body weight largely plateaued and increases in weight in subsequent weeks were mild (roughly 1-2 g, i.e. 2-4 \%). The data from Su and colleagues suggests that, following an initial 65 day HFD, control mice were capable of increasing their body weight by 40\% in the same 7 week time frame presented in this thesis or by 80\% over 100 days and that infection with \textit{H. polygyrus} was able to almost entirely prevent this weight gain\textsuperscript{140}. This is highly inconsistent with the findings prevented here.
3.3.5 Co-infection with *H. polygyrus* does not prevent trickle-induced immunity to *T. muris*

In regions endemic for GI helminths, epidemiological data indicates that co-infection with multiple parasites is likely the normal situation for infected individuals\(^{10–12}\). An interesting observation from these studies has been that those infected with multiple species of helminth have greater burdens of infection for each individual species\(^{11,12}\). What has remained unclear is whether there is a cause effect relationship, where infection with one species promotes the survival of another, or whether this is simply correlative. Given the ability of helminths to directly modulate their host’s immune response\(^ {93,103,118,122}\) it is possible that these mechanisms overlap and have indirect effects on coexisting parasites. Indeed, in early work using single dose infections, co-infection with *H. polygyrus* resulted in delayed expulsion of a *T. muris* high dose\(^ {135}\).

Given that trickle infection is a more representative model of acquired immunity to *T. muris*\(^ {60}\), the effect of co-infection on trickle-induced immunity to *T. muris* was investigated. Mice received a *T. muris* trickle infection and were co-infected with either a single dose of *H. polygyrus* or co-trickle infected. Resistance to infection is known to develop around 11 w.p.i. during *T. muris* trickle infection\(^ {60}\). In either model of co-infection this was not altered with co-infected groups showing significant decreases in worm burden at 11 w.p.i.. Co-infection also did not appear to increase susceptibility to infection during the first 9 weeks as the peak worm burden was not significantly different between single infection and co-infection groups.

Histological analysis of the gut show that whilst mice infected with *T. muris* alone were able to reduce levels of inflammation once resistance had developed. Those co-infected still showed an increased goblet cell hyperplasia, crypt hyperextension, and increased thickening of the submucosa. This suggests that co-infection resulted in a failure to resolve immuno-pathology with the onset of resistance to infection. However, the mechanism through which this is occurring remains unclear. This, however, presents a model whereby the resolution of inflammation during ongoing infection can be interrogated.
Summary:

- Trickle infection of *H. polygyrus* results in parasite expulsion whilst single dose treatment result in chronic infection.

- Trickle infection is associated with increased IL-4 expression in the MLN and activation of systemic and local Th2 responses.

- Resistance to infection during trickle infection is dependent on αβ-T cells.

- DIO does not affect resistance to *H. polygyrus* nor does *H. polygyrus* abrogate the effects of a HFD.

- Co-infection with *H. polygyrus* does not prevent the development of resistance to *T. muris* during trickle infection, but does inhibit the resolution of inflammation in the caecum.
Chapter 4

Chronic Helminthiases and the Microbiota
4.1 Introduction

The gut microbiota is a major component of health. It plays a vital role in the digestion of plant polysaccharides\textsuperscript{142}, and has been implicated in the development of the gut immune system\textsuperscript{186}. Evidence from field studies on the effect of helminths on the microbiota or vice versa has been conflicting. One publication suggested there is no effect of helminths on the biota\textsuperscript{165}, whilst another indicates that infection may increase microbial diversity\textsuperscript{146}. However, the considerable number of confounding factors present in field studies makes it hard to disentangle the impact of infection from other variables.

There is a limited amount of data from murine models of infection on changes in the microbiota following infection. However, there is consistent evidence that during chronic infection the overall diversity of the microbiota is reduced\textsuperscript{166–168}. These effects are reversible following expulsion of the parasite\textsuperscript{166}. Also consistent with chronic infection is the expansion of members of the \textit{Lactobacillus} genus\textsuperscript{168–171}. Increased abundance of this genus is associated with susceptibility to both \textit{T. muris} and \textit{H. polygyrus} infection\textsuperscript{172,173}, as well as suppression of Th2 responses during allergic responses\textsuperscript{174–176}.

However, these studies have relied on traditional single dose models of infection over short time frames. As such, there is an absence of longitudinal studies investigating how the microbiota changes over longer periods of time. These studies have also been restricted to comparing naive mice to relatively immunologically static chronic infection, and thus lack detail on how the microbiota responds to dynamic changes in immune status.

Here a range of models of infection, including \textit{T. muris} and \textit{H. polygyrus} trickle infections, were used to assay how the microbiota responds over long periods of infection. Using the \textit{T. muris} trickle of infection microbial responses to changes in immune status were investigated. The interaction between infection and diet was also tested.
Specific objectives:

- Investigate how the development of resistance to *T. muris* during trickle infection alters the microbiota.

- Characterise changes in the microbiota during chronic *H. polygyrus* infection.

- Look at the effect of diet and helminth infection on the microbiota.
4.2 Results

4.2.1 Low dose *T. muris* infection causes chronic non-resolving dysbiosis

Previous work has shown that chronic infection with *T. muris* causes a dysbiosis in the gut microbiome that persists throughout infection\(^{166}\). Houlden *et al* demonstrated this dysbiosis was reversible upon treatment with anthelminthics, but otherwise remained constant\(^{166}\). To confirm that *T. muris* infection caused dysbiosis in the gut flora, mice were infected with a low dose of *T. muris* and at autopsy the final stool was collected. 16S sequencing was performed on DNA extracted from each sample (Fig. 4.1 A). Sequences with 97% identity were clustered into Operational Taxonomic Units (OTUs) which were then assigned taxonomic identities. Samples with less than 12,000 reads were excluded from downstream analysis. Two independent experiments were performed, in experiment 1 samples were collected at 9 and 11 w.p.i., in experiment 2 samples were collected at 3, 9 & 11 w.p.i. Rarefaction analysis was performed on all samples to confirm that sequencing had been performed to a sufficient depth i.e. the rarefaction curve nears or reaches plateau (Fig. 4.1 B&C).

Differences in overall microbial community composition were analysed using Non-metric Multidimensional Scaling (NMDS). In brief NMDS analysis plots complex multidimensional space in fewer dimensions. Here Bray-Curtis dissimilarity scores were calculated then converted into rank-order distances which were then plotted in two dimensions. The NMDS co-ordinates for each sample are arbitrary, and represent the dissimilarly between samples i.e. the further two points are from one another the less similar they are. In both experiment 1 (Fig 4.2 A) and experiment 2 (Fig 4.2 B) infected mice appeared to cluster away from naive mice, testing by permutational multivariate analysis of variance (PERMANOVA) revealed the effect of infection to be statistically significant in both experiments (p = 0.004 & p = 0.001 respectively). Time had a weak effect in experiment 1 (p = 0.026) but no effect in experiment 2 (p = 0.084).
Figure 4.1: 16S Sequencing of stool from low dose *T. muris* infection. (A) C57BL/6 male mice were infected with a low dose of *T. muris* by oral gavage. The final stool was collected at sacrifice at 3, 9 and 11 weeks post infection, DNA was extracted, the 16S locus amplified by PCR, and the product sequenced by Illumina MiSeq. Samples were collected from two independent experiments. (B & C) Rarefaction curves for individual samples from [B] experiment 1, and [C] experiment 2.
Figure 4.2: NMDS analysis of global microbiota composition during *T. muris* low dose infection. NMDS analysis was performed on samples from (A) experiment 1 and (B) experiment 2. NMDS scores are a measure of rank order distance relative to a centre point of zero. Stress is a measure of quality of fit (< 0.2 indicates a good fit)
To identify differences in community composition, analysis was performed at the phyla level. The relative abundance of each phylum remained largely consistent between time point and between those infected or not infected (Fig. 4.3 A). The Bacteroidetes and Firmicutes phyla represent the majority of the overall microbial community. Total microbial diversity, and diversity within specific phyla, was measured by calculating Shannon index scores. In naive mice there was strong consistency in microbial diversity within and between timepoints. Infection with *T. muris* resulted in a small but statistically significant decrease in overall community diversity (p < 0.001) (Fig. 4.3 B). This decrease in diversity was present in both the Bacteroidetes and Firmicutes phyla, but not in much less abundant Proteobacteria which had much less overall diversity and a higher degree of variance within time points.

To identify specific OTUs that changed during infection, differential expression analysis was performed. Significantly differentially expressed OTUs were identified as those where p < 0.01, the 15 with the lowest p values were ordered and displayed as a heat map (Fig. 4.4). The majority of the most significantly differentially expressed OTUs were significantly down-regulated in infected mice. Clustering using these OTUs did not cluster individual samples by week post infection. Differentially regulated OTUs came primarily from the *Lachnospiraceae* and *Muribaculaceae* families, however, species level identification of these OTUs was not possible.
Figure 4.3: Phyla-level analysis of faecal microbiota during low dose *T. muris* infection. (A) Bar graphs representing the relative abundance of different Phyla. Phyla representing less that 1% of overall abundance were grouped into one group titled ‘other.’ (B) Alpha diversity was measured by calculating Shannon Index scores for the total microbiota and at the level of individual Phyla. Significant differences were calculated by 2-way ANOVA. *** indicated significance (p < 0.001).
Figure 4.4: Differential expression analysis of significantly different OTUs during *T. muris* low dose infection. Significantly differentially expressed OTUs were identified by differential expression analysis performed using the DESeq2 package in R where significance is identified as those OTUs where p < 0.01. The 15 OTUs with the lowest p values were ordered and displayed as a heat map. Clustering of individual samples is shown as a dendrogram.
4.2.2 The microbiota changes dynamically in response to changes in immunity to *T. muris*

Comparison between low dose infection and naive mice is a binary situation in which both groups are largely static in terms of immune status. Glover et al previously developed a *T. muris* trickle infection model where repeated low dose infections were given on a weekly basis. T. *muris* trickle infection results in the slow acquisition of partial immunity. Crucially there is a threshold between 9 and 11 w.p.i. where the immune response shifts from Th1 dominated to Th2 dominated. Mice remain infected with adult parasites but are resistant to subsequent doses. Here this model was used to investigate whether the microbiota responded to a change in immune status whilst still chronically infected.

Mice received a *T. muris* trickle infection and stool samples were collected at autopsy at 3, 9 & 11 weeks post infection (Fig. 4.5 A). DNA was extracted, the 16S V3/4 region amplified by PCR and the amplicon sent for sequencing. Sequences with 97% identity were clustered into Operational Taxonomic Units (OTUs) which were then assigned taxonomic identities. Samples with less than 12,000 reads were excluded from downstream analysis. Two independent experiments were performed, in experiment 1 samples were collected at 9 and 11 w.p.i., in experiment 2 samples were collected at 3, 9 & 11 w.p.i.. Rarefaction analysis was performed on all samples to confirm that sequencing had been performed to a sufficient depth i.e. the rarefaction curve nears or reaches plateau (Fig. 4.5 B&C).

NMDS analysis showed naive mice clustered closely together at each time point. Infection had a significant effect in both experiments as calculated by PERMANOVA (*p* < 0.001). Interestingly mice that had been trickle infected for 11 weeks – where resistance develops – clustered more closely to the naive mice than those that had been trickle infected for only 9 weeks – where mice are still susceptible to infection (Fig 4.6 A&B).
Figure 4.5: 16S Sequencing of stool from *T. muris* trickle infection. (A) C57BL/6 male mice were trickle infected with *T. muris* (20 eggs/week) by oral gavage. Dosing was stopped two weeks prior to sacrifice. The final stool was collected at sacrifice at 3, 9 and 11 weeks post infection. DNA was extracted, the 16S locus amplified by PCR, and the product sequenced by Illumina MiSeq. Samples were collected from two independent experiments. (B & C) Rarefaction curves for individual samples from [B] experiment 1, and [C] experiment 2.
Figure 4.6: NMDS analysis of global microbiota composition during *T. muris* trickle infection. Mice were trickle infected with *T. muris* and sacrificed at 3, 9 & 11 w.p.i. The final stool was collected for metagenomic analysis. NMDS analysis was performed on samples from (A) experiment 1 and (B) experiment 2. NMDS scores are a measure of rank order distance relative to a centre point of zero. Stress is a measure of quality of fit (< 0.2 indicates a good fit).
Figure 4.7: Phyla-level analysis of faecal microbiota during low dose *T. muris* infection. Mice were trickle infected with *T. muris* and sacrificed at 3, 9 & 11 w.p.i. The final stool was collected for metagenomic analysis. (A) Bar graphs representing the relative abundance of different Phyla. Phyla representing less than 1% of overall abundance were grouped into one group titled ‘other.’ (B) Alpha diversity was measured by calculating Shannon Index scores for the total microbiota and at the level of individual Phyla. Significant differences were calculated by 2-way ANOVA. * indicates significance (p < 0.05), ** indicates significance (p < 0.01), *** indicates significance (p < 0.001), **** indicates significance (p < 0.0001).
To assess differences in microbiota composition were occurring phyla-level comparisons were made. Infection had little effect on the overall abundance of different phyla with Bacteroidetes and Firmicutes dominating throughout. However, Verrucomicrobia, which was fairly abundant in naive mice at 9 and 11 w.p.i., was virtually absent during infection (Fig. 4.7 A). Analysis of overall sample diversity by measuring Shannon Index scores demonstrated that mice trickled infected then culled at 9 w.p.i. had a strong significant reduction in overall diversity ($p < 0.0001$). Interestingly the diversity of trickle infected mice culled at 11 w.p.i. increased significantly relative to mice culled at 9 w.p.i. (Fig. 4.7 B). The decrease in diversity at 9 w.p.i. followed by a recovery of diversity at 11 w.p.i. was observed in both the Bacteriodetes and Firmicutes Phyla.

To identify where loss and recovery of diversity was occurring within the microbiota the relative abundance of different genera was assessed. The 20 most abundant genera were identified as those with the greatest mean abundance across all mice in the dataset. These genera are displayed in Fig 4.8. It is apparent that the relative abundance of these genera in naive mice between and within time points remains largely consistent. Across the entire dataset an unknown genera in the *Muribaculaceae* family predominated. Trickle infected mice sacrificed at 3 w.p.i. showed little difference in overall composition of these genera. However, those infected then culled at 9 and 11 w.p.i. showed considerable changes in microbial composition (Fig. 4.8). A number of these genera showed interesting dynamic changes during infection which are highlighted in Fig 4.9. These could grouped into four categories: (i) genera which decreased during infection and remained at low levels throughout such as *Muribaculum* and *PrevotellaceaeUCG-001*; (ii) genera which increased during infection and remained increased throughout such as *Bacteroides*, and *Escherichia-Shigella*; (iii) genera which decreased at 9 w.p.i. but then showed a relative increase at 11 w.p.i. such as *LachnospiraceaeNK4A136group*; (iv) genera which increased at 9 w.p.i. but then showed a relative decrease at 11 w.p.i. such as *Parasutterella* and *Bifidobacterium* (Fig. 4.9).
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| Proteobacteria   | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | Citrobacter         | Citrobacter salmonicolaæs

Figure 4.10
**Figure 4.8: Relative abundance of highly represented genera during *T. muris* Trickle infection.** Mice were trickle infected with *T. muris* and sacrificed at 3, 9 & 11 w.p.i. The final stool was collected for metagenomic analysis. Bubble plot representing the abundance of different genera. The 20 most represented genera were identified as those with the greatest mean abundance across all mice included in the data set. Each column represents an individual mouse. Circle size indicates the relative abundance of each genus within an individual sample. Blank spaces indicate that genus could not be identified in that sample.

**Figure 4.9: Relative abundance of selected genera during *T. muris* Trickle infection.** Mice were trickle infected with *T. muris* and sacrificed at 3, 9 & 11 w.p.i. The final stool was collected for metagenomic analysis. Scatter graph of the relative abundance scores for different genera determined by 16S sequencing. Points represent individual mice and line and error bars indicate mean and SEM respectively. * indicates significance (p < 0.05), **** indicates significance (p < 0.0001).

**Figure 4.10: Differential expression analysis of OTUs during *T. muris* trickle infection.** Mice were trickle infected with *T. muris* and sacrificed at 9 w.p.i. The final stool was collected for metagenomic analysis. Differential expression analysis was performed to identify significantly differentially regulated OTUs. Significance was determined as OTUs where p < 0.01. OTUs were ranked by p value and those with the 15 lowest values are graphed as a heatmap. Samples were clustered and this clustering is represented by dendrogram.
Differential expression analysis was performed to identify specific OTUs that were significantly differentially regulated between naive mice and trickled infected mice culled at 9 w.p.i. OTUs were considered significantly differentially expressed if \( p < 0.01 \). The 15 most significantly differentially expressed OTUs were plotted as a heat map (Fig. 4.10). A mix of OTUs significantly increase and significantly reduced in trickle infected mice. Significantly reduced OTUs came primarily from the *Muribaculaceae* family and *Lachnospiraceae NK4A136 group* genus. Consistent with relative abundance data significantly increased OTUs came from the *Bacteroides*, *Bifidobacterium*, and *Escherichia-Shigella* genera among others. Species-level identification, and in some cases genus-level, identification was not possible for most OTUs with the exception of *Citrobacter citrobacter amalonaticus* which was significantly increased in trickle infected mice (Fig 4.10).

### 4.2.3 *H. polygyrus* has little effect on the faecal microbiota

Given the profound effects of *T. muris* infection on the faecal microbiota, and the considerable differences in dynamics between single-dose and trickle infection, the question arose as to whether this was specific to *T. muris* or was a general feature of helminth infection. For comparison mice were given either a single dose of 200 L3-stage *H. polygyrus* larvae or trickle infected with 20 L3 per week. Mice were culled at 3, 9 and 11 w.p.i., the final stool was collected, and 16S sequencing analysis was performed on extracted faecal DNA.

For the single dose infection rarefaction analysis showed that sequencing was performed to sufficient depth (Fig. 4.11 A). NMDS analysis showed a mild effect of infection with some individuals separating from the naive cluster, however, PERMANOVA analysis showed that this was not significant (\( p = 0.366 \)) (Fig. 4.11 B). Concurrent with the NDMS analysis was an absence of change in overall diversity between timepoint or infection status (Fig. 4.11 C). There was also no apparent change in the overall abundance of different Phyla between samples (Fig. 4.11 D).

This analysis was repeated on samples from trickle infected mice. Rarefaction analysis confirmed that sequencing depth was sufficient (Fig. 4.12. A). NMDS
analysis showed no effect of infection on the composition of the stool microbiota (p = 0.028) (Fig. 4.12 B). There was also no apparent difference in the diversity of infected samples relative to naive mice (Fig. 4.12 C) or in the relative abundance of different phyla (Fig. 4.12 D).

**Figure. 4.11**

A

![Graph showing total OTUs vs read counts](image)

B

![NMDS plot with stress value](image)

C

![Bar chart showing Shannon diversity](image)

D

![Heatmap showing phylum abundance](image)
Figure 4.11: 16S Sequencing of stool from *H. polygyrus* infection. Mice were infected with a single dose of 200 L3-stage *H. polygyrus* larvae. Final stool samples were collected at 3, 9 & 11 w.p.i. Faecal DNA was analysed by 16S sequencing. (A) Rarefaction curves from individual samples. (B) NMDS analysis of samples. NMDS scores are a measure of rank order distance relative to a centre point of zero. Stress is a measure of quality of fit (< 0.2 indicates a good fit). (C) Scatter graph representing Shannon Index scores. Points represent individual mice, bar represents mean score. (C) Bubble plot representing the abundance of different phyla. Phyla that represented less than 1% of the dataset were grouped into ‘other.’ Circle size indicates the relative abundance of each phyla within an individual sample.

Figure 4.12: 16S Sequencing of stool from *H. polygyrus* infection. Mice were trickle infected with L3-stage *H. polygyrus* larvae (20 larvae/week). Final stool samples were collected at 9 & 11 w.p.i. Faecal DNA was analysed by 16S sequencing. (A) Rarefaction curves from individual samples. (B) NMDS analysis of samples. NMDS scores are a measure of rank order distance relative to a centre point of zero. Stress is a measure of quality of fit (< 0.2 indicates a good fit). (C) Scatter graph representing Shannon Index scores. Points represent individual mice, bar represents mean score. (C) Bubble plot representing the abundance of different phyla. Phyla that represented less than 1% of the dataset were grouped into ‘other.’ Circle size indicates the relative abundance of each phyla within an individual sample.
4.2.4 Diet and the Microbiota

A major factor in determining the composition of the microbiota is the composition of an individual’s diet. Funjika recently demonstrated that mice on a HFD are resistant to a low dose *T. muris* infection\(^{185}\). Given the effect of the microbiota on mucosal immunity, and the effect of diet on the microbiota, here the composition of the microbiota was assessed during low dose *T. muris* in mice fed on a high fat diet.

Mice were placed on a specialised diet for 12 weeks prior to infection one either high in fat (HFD) or low in fat (LFD). Mice were then infected with a low dose of *T. muris* (20 eggs). Mice were sacrificed at 21 and 42 days post infection (d.p.i.) (Fig. 4.13 A). At autopsy the final stool was collected, faecal DNA extracted and the samples metagenomically analysed by 16S sequencing. Rarefaction analysis showed that samples were sequenced to a sufficient depth (Fig. 4.13 B&C).

NMDS analysis showed a clear separation between samples on different diets at both time points with this division being highly statistically significant (p < 0.001 for both time points) (Fig. 4.14). Interestingly mice on a LFD appeared to cluster separately at both time points. On the other hand, mice on a HFD appeared to cluster closely together regardless of whether they were infected or not.

Analysis of Shannon Index scores indicated that neither diet nor infection had any effect on the overall diversity of the microbiota (Fig. 4.15). To identify compositional changes in the microbiota the relative abundance of different genera were calculated. Genera were then ordered by greatest mean abundance across the entire dataset to identify the 15 most abundant genera present (Fig. 4.16). This analysis demonstrated that there were considerable differences in community composition between mice on a LFD and HFD. Relative to mice on a LFD, HFD mice showed decreases in the *Bifidobacterium*, *Faecalibaculum* genera, and an uncultered genus from the *Muribaculaceae* family. They also showed an increase in the *Lactobacillus* and *Ruminiclostridium* genera. Infection seemed to have little impact on the top 15 genera for both diets. However, infected LFD mice showed a mild increase in *RikenellaceaeRCPgutgroup* at both time points, and strong in-
crease on D42 in *Bacteroides*, relative to naive LFD mice.

To identify specific OTUs that changed during infection in the LFD mice differential expression analysis was performed. OTUs where $p < 0.05$ were considered significantly differentially expressed. Those with the lowest $p$ values from each time point were plotted as a heat map (Fig. 4.17 A). Combining both time points, a total of 44 OTUs were identified that were significantly different from naive mice. At D21 only five OTUs were significantly different, but at D42 there were a total of 41 OTUs that were significantly different (Fig. 4.17B). Of these only two OTUs were shared between both infected groups, an unidentified species from the *Blautia* genus which was significantly increased at both D21 and D42 ($\text{padj} = 0.009$ & $\text{padj} = 0.015$ respectively), and an OTU from the *Ruminococcaceae* family whose genus and species are unknown which was significantly reduced at both time points ($\text{padj} = 0.0002$ & $\text{padj} = 0.0005$ respectively).

Of the OTUs most significantly differentially regulated during infection in the LFD mice at D42 several stand out. An additional unidentified species from the *Blautia* genus was identified. Additionally, there were significant increases in two OTUs assigned to different unidentified species in the *Bacteroides* genus, and an increase in an OTU assigned to an unknown species in the *Escherichia-Shigella* genus. These genera stand out as they were also associated with the biota of mice in the susceptible phase of *T. muris* trickle infection.
Figure 4.13: 16S Sequencing of stool from low dose *T. muris* infection on a HFD. (A) C57BL/6 male mice were placed on specialised diets for 12 weeks prior to infection. At D0 mice were infected with a low dose of *T. muris* (20 eggs) by oral gavage. The final stool was collected at sacrifice at D21 and D42, DNA was extracted, the 16S locus amplified by PCR, and the product sequenced by Illumina MiSeq. Rarefaction curves for individual samples from (B) D21, and (C) D42.
Figure 4.14: NMDS analysis of faecal microbiota during HFD and *T. muris* infection. C57BL/6 male mice were placed on specialised diets for 12 weeks prior to infection. At D0 mice were infected with a low dose of *T. muris* (20 eggs) by oral gavage. Mice were culled at D21 and D42 post infection and stool samples were taken from metagenomic analysis. NMDS analysis was performed on samples from (A) D21 and (B) D42. NMDS scores are a measure of rank order distance relative to a centre point of zero. Stress is a measure of quality of fit (< 0.2 indicates a good fit)
Figure 4.15: Alpha diversity of the faecal microbiota in mice infected with *T. muris* on a HFD. C57BL/6 male mice were placed on specialised LFD 12 weeks prior to infection. Mice were infected with a low dose of *T. muris*. Stool was collected at D21 and D42 post infection and metagenomic analysis was performed on extracted bacterial DNA. Alpha diversity was measured by calculating Shannon index scores for the total faecal microbiota. There were no significant differences between groups as calculated by 2way ANOVA.
Figure 4.16: Relative abundance of highly represented genera during *T. muris* infection in mice on specialised diets. C57BL/6 male mice were placed on specialised LFD 12 weeks prior to infection. Mice were infected with a low dose of *T. muris*. Stool was collected at (A) D21 and (B) D42 post infection and metagenomic analysis was performed on extracted bacterial DNA. Bubble plots represent the relative abundance of different genera. The 15 most abundant genera were determined as those with the greatest mean abundance across all mice in the dataset. Each column represents an individual mouse. Circle size indicates the relative abundance of each genus within an individual sample. Blank spaces indicate that genus could not be detected in that sample.
Figure 4.17: Differential expression analysis of OTUs during *T. muris* infection on a HFD. C57BL/6 male mice were placed on specialised LFD 12 weeks prior to infection. Mice were infected with a low dose of *T. muris*. Stool was collected at D21 and D42 post infection and metagenomic analysis was performed on extracted bacterial DNA. (A) Heatmaps comparing the most significantly differentially regulated OTUs, where \( p < 0.05 \), between naive and infected mice at both time points. OTUs were ranked by \( p \) value. Samples were clustered and this is represented as a dendrogram. (B) Venn diagram comparing the total number of significantly differentially regulated OTUs in infected mice between D21 and D42.
4.3 Discussion

GI helminths colonise a similar niche and come in close contact with the GI microbiota. However, the relationship between microbiota and GI helminths remains poorly characterised. Data from field studies in humans is largely inconclusive suggesting either no relationship between helminth infection, or an associated increase in population diversity\textsuperscript{146,165}. Data from mouse models, where conditions can be controlled, has been somewhat more forthcoming. Chronic infection with \textit{T. muris} has been linked to a decrease in microbial diversity\textsuperscript{166,167}. More generally chronic helminth infection has been associated with an increase in members of the \textit{Lactobacillus} genus\textsuperscript{168–171}, a genus whose abundance has been linked to induction of regulatory immune responses\textsuperscript{172,173}.

However, previous studies have been limited in several ways. Firstly they tend to focus on a binary comparison between naive mice that either are uninfected or infected with a static chronic infection, this means that they do not assay how the microbiota responds to changes in immune status. Secondly, these studies have been conducted over relatively short periods of time and thus miss potential long-term changes in microbial composition.

Here metagenomic analysis was performed on faecal samples collected from a variety of model helminth infections including those designed to mimic more natural modes of infection i.e. trickle infection and alterations to diet. Samples were assayed for community composition, diversity, and differential expression between groups.

4.3.1 \textit{T. muris} and the Microbiota

Chronic infection with \textit{T. muris} has been previously associated with a dysbiosis of the faecal microbiota\textsuperscript{166–168}. To validate this observation, mice were infected with a low dose of \textit{T. muris} and the faecal microbiota was assessed longitudinally. Consistent with previous findings a loss in microbial diversity was seen that persisted throughout infection (up to 11 w.p.i.). Whilst the overall balance of different Phyla remained largely unchanged as a result of infection their richness decreased,
particularly within the Bacteroidetes and Firmicutes groups. In accord with this the most significantly differentially expressed OTUs were assigned to these Phyla, the majority of which were significantly reduced. In particular a large number of these were found in the LachnospiraceaeNK4A136 group and the Muribaculaceae family. Curiously, changes in either of these groups had not previously been connected with helminth infection.

Infection with a low dose of T. muris results in a chronic infection that does not resolve until the parasites are naturally lost at the end of their lives. This effect on the immune system is a persistent regulated Th1 response throughout infection. Thus, observations on the microbiota during low dose infection only provides data from the context of a managed Th1 immune response. Recently, Glover and colleagues developed a model of T. muris trickle infection where giving frequent low dose infection initially results in a Th1-dominated chronic infection. Followed by the development of a Th2 response at, around 11 w.p.i.; whilst mice are still infected with adult parasites they are resistant to subsequent challenges. Therefore, this model provides a system in which changes in immune status can be correlated with changes in the microbiota during an ongoing infection.

Mice were trickle infected with 20 T. muris eggs on a weekly basis and metagenomic analysis was performed on stool samples collected at 3, 9, and 11 weeks post infection. Clustering analysis showed that by 9 w.p.i. trickle infected mice had separated significantly from naive mice in terms of overall community composition. However, by 11 w.p.i., when Th1 immunity has given way to a Th2-dominated phenotype, mice clustered closer to the naive group relative to mice at 9 w.p.i.. This was reflected in assessment of overall community diversity which was strongly reduced in trickle infected mice at 9 w.p.i., but had largely recovered by 11 w.p.i.. These data suggest that, in part, the composition of the microbiome is influenced by the immune phenotype present during infection. In particular, it highlights that even when mice are still infected, that the microbiota can change in response to other variables.

The susceptible phase of T. muris trickle infection (i.e. 9 w.p.i.) was associated with drastic changes in some of the most abundantly represented genera in
the dataset. Expansion in the *Bacteroides, Bifidobacterium, Lactobacillus, Parasutterella*, genera. *Escherichia-Shigella* appeared very rarely, and at low abundance, in naive mice but was highly prevalent in infected mice. There was also a loss of certain genera such as *Muribaculum, LachnospiraceaeNK4A136group* and *PrevotellaceaeUCG-001*. However, following the development of resistance to infection, a number of these genera changed in abundance closer to naive levels. Although, a full return to the homeostatic state was rare for most genera, it is possible that given enough time a full restoration to the naive state would occur. The association of an increase in *Bacteroides, Bifidobacterium, Lactobacillus*, and *Escherichia-Shigella* with *T. muris* infection is consistent with previous analyses.

Data from a field study failed to find an effect of *T. trichiura* on the microbiota. The data presented here hints at an explanation for this finding that accounts for laboratory data suggesting that *Trichuris* can induce dysbiosis. Infection with *Trichuris* may initially be associated with dysbiosis, driven by a Th1-dominated inflammatory response, however, upon the acquisition of partial resistance to infection microbial diversity recovers and returns to a homeostatic phenotype. This dynamic relationship between the microbiota, the helminth and the immune system would be lost when observing static time points in infected individuals.

### 4.3.2 *H. polygyrus* and the Microbiota

Infection with *H. polygyrus* has been widely associated with changes to the microbiota. This has been linked to direct antimicrobial properties of secreted proteins from the adult parasite, and modulation of the microbiota have been connected with some of *H. polygyrus’* immunomodulatory properties. In particular, expansion of *Lactobacillus* during infection appears to increase susceptibility to infection.

To expand on this body of work mice were infected with either a single dose or trickle infected with *H. polygyrus*. Data presented in this thesis suggests that trickle infection induces a more potent Th2 response (Chapter 3), and therefore
a comparison between single dose and trickle infected mice may have yielded information on the contribution of Th2 immunity in determining the composition of the microbiome.

Metagenomic analysis of the faecal microbiome was performed. However, no significant differences were observed between naive and infected mice regardless of infection regime. Given the weight of evidence that shows a strong relationship between *H. polygyrus* and the microbiome, this confounding result is likely a product of the methodology. Faecal samples were used as previous evidence, from infection with *T. muris*, had suggested that stool samples were a good proxy for the microbiota at sites where collection of bacterial DNA is more challenging. Thus, it was hoped that the faecal DNA would be sufficient as a gauge of the meta-state of the entire intestinal microbiota. However, a recent publication demonstrated that whilst the stool, colon and caecum are relatively similar in terms of microbial ecology, they differ substantially from the small intestine. Given that *H. polygyrus* resides at the top of the duodenum it is likely that analysis of faecal microbiota is insufficient to capture changes occurring in the small intestine.

In work made available shortly before the submission of this thesis Rapin et al performed a comprehensive characterisation on the effect of infection with *H. polygyrus* on the microbiota at multiple sites throughout the GI tract including each segment of the small intestine, the caecum, colon and faeces. Rapin and colleagues demonstrated that infection with *H. polygyrus* had systemic and consistent effects on the microbiota throughout the GI tract, including the caecum and colon, with the exception of the faeces where they detected no significant alteration in microbial composition. In concert with the data presented here it suggests that *H. polygyrus* mediated effects on the microbiota cannot be detected in the stool. However, the authors identified trends that both mirror and fail to align with observations presented here concerning infection with *T. muris*. The authors correlated infection with *H. polygyrus* with increased abundance of the *Bacteroides* and *Escherichia* genera, data presented here shows that these genera are associated with susceptibility to infection during *T. muris* trickle infection and with dysbiosis in mice on a LFD. These data suggests that expansion of these groups may be a common feature of chronic helminthiases. By way of contrast, they linked a reduction in *Bifidobac-
terium to *H. polygyrus* whereas the data shown here indicates that *Bifidobacterium* expands during *T. muris* infection and that a decrease in *Bifidobacterium* induced by a HFD correlates with resistance to infection.

4.3.3 Diet and the Microbiota

Little is understood about the relationship between diet and helminth infection. Two publications have suggested that infection with *H. polygyrus* is protective against some of the effects of a HFD such as increased levels of body fat\(^ {140,193}\). A relatively superficial analysis of the microbiota by Shimokawae *et al* in *H. polygyrus* infected mice on a HFD suggested that both diet and infection could have effects on overall composition of the microbiota, highlighting genera that increase specifically in mice on a HFD such as *Escherichia* and *Bacillus*\(^ {193}\). However, in this system *H. polygyrus* appears unaffected by changes in diet composition, and therefore this does not tell us about how changes in the microbiota may influence the outcome of infection.

In her recently submitted thesis, Funjika demonstrated that placing mice on a HFD resulted in protection against low dose infection with *T. muris*\(^ {185}\). The mechanism underlying this remains unclear, however, mice on a HFD appeared to have an enhanced Th2 response, contrary to previously held dogma that fatty tissue is inherently pro-inflammatory. However, it provides a model in which mice on different diets have distinct outcomes during helminth infection.

Mice were placed on either a LFD or HFD for 12 weeks prior to infection (mice were maintained on diet throughout the experiment), following this they were infected with a low dose of *T. muris* and faecal samples were collected at autopsy at D21 and D42. Metagenomic analysis showed a distinct difference in the composition of the microbiota between mice on a LFD and HFD demonstrating the major role diet has in shaping the microbiota. Despite this, mice on different diets had comparable species diversity. What was particularly interesting was that, in clustering analysis, infected mice on a LFD appeared to cluster away from naive mice. However, infected mice on a HFD remained closely associated with one another. This would imply that a dysbiosis in the microbiome may play a role in suscepti-
ility to infection, particularly as this effect was seen at D21 where worm burdens are similar between mice on different diets.

Analysis was performed to identify changes in the microbiota associated with HFD relative to LFD. Mice on a HFD showed reduction in *Bifidobacterium*, *Faecalibaculum*, and an unidentified genus in the *Muribaculaceae* family. HFD mice also showed an increase in *Lactobacillus* and *Ruminiclostridium*. The increase in *Lactobacillus* is confounding as there is a strong base of evidence from the literature that suggests members of this genus are associated with susceptibility to infection and suppression of Th2 immune responses. This result may imply that effects mediated by species of *Lactobacillus* require a particular context, and that the overall composition of the microbiota may influence their behaviour.

To identify OTUs associated with susceptibility to infection differential expression analysis was performed on mice on LFD at both timepoints. This yielded a total of 44 OTUs that were significantly differentially expressed. Interestingly, there were only 5 significant OTUs at D21 but 41 were found at D42. Of these only 2 OTUs were found to overlap, an unknown species of *Blautia* increased in abundance and an unidentified member of the *Ruminococcaceae* family was reduced. This suggests that dysbiosis occurs in a cumulative fashion during infection but that early hallmarks can be identified. Consistent with other data presented here chronic infection in the LFD mice was associated with significant increases in members of the *Bacteroides* and *Escherichia-Shigella* genera.

Given that *Bacteroides* and *Escherichia-Shigella* are linked with susceptibility in data presented both in this thesis and in previous publications it is interesting to speculate that these genera might be contributing factors in chronicity. That HFD appears to prevent these species from expanding during infection with *T. muris* raises the possibility that diet has the potential to provide a protective effect against parasites by removing the niche for bacteria that would promote infection.

4.3.4 Microbial Hallmarks of *T. muris* Infection

Whilst the *T. muris* trickle infection model differs substantially from the HFD/LFD model, comparison between trickle infected mice a 9 w.p.i. (i.e. during Th1-
driven susceptibility) and infected mice on a LFD reveal consistent trends in the microbiota. In particular increases in members of the *Bacteroides* and *Escherichia-Shigella* genera are observed in both systems, these groups have previously been identified in association with chronic *T. muris* infection. In addition, here an increase in *Bifidobacterium* is consistently observed in mice susceptible to infection. Also observed was a consistent decrease in members belonging to the *Lachnospiraceae* NK4A136 group genus and *Ruminococcaceae* family. However, species-level identification of the OTUs assigned to these genera was not possible, indicating a vast absence of knowledge about key bacteria associated with helminth infection.

Expansion of both *Bacteroides* and *Escherichia-Shigella* have been associated with dysbiosis in humans resulting in diarrheal disease\(^{194,195}\). The expansion of these genera during infection with *Trichuris* may account for commonly observed *Trichuris* dysentery syndrome\(^{196}\). *Ruminococcaceae* family members have been linked to antibiotic biosynthesis\(^{197}\) and thus their diminution has implications for host pathogen defence. *Bifidobacterium*, which was consistently observed to increase upon infection, has been associated with protection against inflammatory and diarrheal disease in humans\(^{198}\). This presents the possibility of host-mediated promotion of *Bifidobacterium* to protect against inflammatory effects of chronic *Trichuris* infection. However, it may also be that the human context is highly divergent from the mouse and that *Bifidobacterium* may be playing a potentially pathogenic role here.

What remains unclear is whether these genera are simply opportunists that arise as an indirect consequence of infection, or if *Trichuris* actively modulates the biota selecting for groups that contribute to its survival. However, the models presented here provide characterised systems that can now be manipulated with probiotic treatment of certain species or removal of components of the biota via antibiotic treatment that may yield new insight into the contribution of the microbiota to helminth infection.
Summary:

- Low dose infection results in a low level dysbiosis that persists throughout infection.
- The microbiota responds dynamically during *T. muris* trickle infection, correlating with changes in immune status.
- Infection with *H. polygyrus* had no detectable effect on the faecal microbiota.
- High fat diet protects against changes in the microbiota associated with chronic infection.
Chapter 5

The Effect of Co-infection on the Expulsion of *Trichuris muris*
5.1 Introduction

The vast majority of publications addressing immunity to experimental helminth infection focus on an individual model parasite. This has been undeniably illuminating in terms of defining fundamental mechanisms of resistance and susceptibility to infection. However, it is made abundantly clear by epidemiological data that infection with a single helminth is incredibly rare\textsuperscript{10–13}. It is far more common for individuals to be concurrently infected with multiple GI helminths. An interesting observation from several of these studies is that co-infection with several GI helminth correlates with a high burden of infection of each individual species\textsuperscript{11,12}. There are two possible explanations for this observation; (i) individuals that are co-infected with a greater number of species are inherently more susceptible to infection than those with single species infection and thus more likely to be polyparasitised; or (ii) infection with one species renders the infected individual more susceptible to subsequent infection with other GI helminths.

The mechanisms with which GI helminths may influence the kinetics of each others infection have not been investigated in depth. However, historic experiments have shown that co-infection of \textit{T. muris} with \textit{H. polygyrus} results in otherwise resistant mice becoming susceptible to a high dose \textit{T. muris} infection\textsuperscript{135}. Given the limitations present at the time, the authors did not interrogate the mechanisms responsible for this observation. \textit{H. polygyrus} is now known to exert potent immunomodulatory properties suppressing the IL-33 response\textsuperscript{103,104} and promoting Treg induction\textsuperscript{120,121}. Interestingly, these mechanisms have not been thought to play crucial roles in resistance or susceptibility to \textit{T. muris}. Therefore, this model of infection presents an interesting system to investigate how infection with one helminth can affect the outcome of infection with another.

To investigate the relationship between co-infecting helminths mice were simultaneously infected with \textit{T. muris} and \textit{H. polygyrus}, these mice were then immunophenotyped to identify changes in the immune response during co-infection. Ability of \textit{H. polygyrus} to influence immunization-induced immunity to infection was also investigated.
Specific objectives:

- Characterise the effect of co-infection on resistance to *T. muris*.
- Phenotype the immune response during co-infection.
- Assay the effect of *H. polygyrus* infection on immunization against *T. muris*.
- Investigate the impact of HpES on immunity to *T. muris*. 
5.2 Results

5.2.1 Co-infection with *H. polygyrus* prevents expulsion of a high-dose *T. muris* infection

It has been previously observed that infection with *H. polygyrus* delays expulsion of *T. muris*\textsuperscript{135}. To confirm this female C57BL/6 mice were co-infected with *H. polygyrus* and *T. muris* and worm burden was assessed at 19 and 35 d.p.i. (D19 and D35 respectively). At D19 co-infected mice had comparable *T. muris* burdens to mice that had received a single-dose infection. However, at D35 where single-dose infected mice had most expelled their infection, co-infected mice remained heavily infected with *T. muris* (Fig. 5.1 A). The kinetics of low-dose *T. muris* infection, which typically drives a chronic infection via activation of Th1 immune responses, was unaffected by co-infection with *H. polygyrus* (Fig. 5.1 B). Co-infection with either a high or low dose of *T. muris* did not have a significant effect on *H. polygyrus* worm burden, although there was a trend to higher numbers of *H. polygyrus* in mice co-infected with a high-dose of *T. muris* (Fig. 5.1 C&D). Interestingly, in co-infected mice that had received a high-dose of *T. muris* there appeared to be no correlation between *T. muris* burden and *H. polygyrus* burden, suggesting that the effect on *T. muris* expulsion was not dose dependent (Fig. 5.2).

5.2.2 Effect of co-infection of levels of parasite-specific serum IgG isotypes

IgG class-switching is under the control of cytokines with IL-4 inducing IgG1 whilst IFN\textgreek{G} induces IgG2c\textsuperscript{181}. Thus, the relative levels of these isotypes represent the balance between Th1 and Th2 immunity. Serum IgGs were measured by ELISA. Levels of *T. muris*-specific IgG1 (Fig. 5.3 A) and IgG2c (Fig. 5.3 B) were not significantly different in mice that had been infected with *T. muris* alone or co-infected with *H. polygyrus*. However, when the ratio of IgG1 to IgG2c was compared between these mice, those that were co-infected showed a significant reduction in the ratio of IgG1:IgG2c (Fig. 5.3 C). Co-infection did not appear to affect the levels of *H. polygyrus*-specific IgG isotypes (Fig. 5.3 D&E).
Figure 5.1: Co-infection with *H. polygyrus* inhibits *T. muris* expulsion. Female C57BL/6 mice were infected with a high (200 eggs) or low (20 eggs) dose of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 19 and 35 days post-infection and worm burden was assessed by eye under dissecting microscope for (A & B) *T. muris* and (C & D) *H. polygyrus*. Symbols denote individual mice and bars represent the group mean. ** indicates significance (p < 0.01), **** indicates significance (p < 0.0001) as calculated by 2way ANOVA with *post-hoc* Sidak’s multiple comparisons.
Figure 5.2: *H. polygyrus* burden does not correlate with *T. muris* burden in co-infected mice. Female C57BL/6 mice were co-infected with a high dose of *T. muris* (200 eggs) and 200 L3 *H. polygyrus* larvae. Mice were culled 35 days post-infection and worm burden was assessed by eye under dissecting microscope. Worm burdens were plotted as a scatter graph. Points represent individual mice. Linear regression analysis was performed and goodness of fit ($R^2$) and significance value were calculated.
Figure 5.3: Co-infection with *H. polygyrus* changes the ratio of anti-*T. muris* serum IgG isotypes. Female C57BL/6 mice were infected with a high (200 eggs) or low (20 eggs) dose of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 35 days post-infection and serum was collected from the blood at autopsy. Levels of (A -C) *T. muris*-specific and (D & E) *H. polygyrus*-specific serum IgG1 and IgG2c were measured by ELISA. ELISA was performed using serum diluted 1:120 on plates coated with either TmES or HpAg. Values are given as the optical density of the substrate read at 405 nm. ** indicates significance (p < 0.01) as calculated by Mann-Whitney test.
5.2.3 Cellular immune responses in the MLN during co-infection

The draining lymph nodes for the intestines are the MLNs. This chain of lymph nodes can be separated based into those that drain the small intestine (sMLNs) and those that drain the large intestine (cMLNs). To determine if co-infection with *H. polygyrus* was influencing the cellular immune response to *T. muris* the cMLNs were collected and analysed.

Enumeration of cMLN size by counting total numbers of cMLN cells revealed that infection with *T. muris* alone results in an initially small increase in cMLN cell number, relative to naive animals, between 3 and 14 days post infection followed by a strong increase in cell number at 20 days post infection. Co-infection on the other hand resulted in an initial decrease in cMLN number and a delay in cMLN hyperplasia (Fig. 5.4 A). Mice infected with *H. polygyrus* alone had a significant reduction in cMLN cell number relative to naive animal during infection (Fig. 5.4 B).

To investigate whether co-infection was affecting the ability of cells to respond to *T. muris* antigen, cMLN cells were cultured *ex vivo* in the presence of TmES. The supernatant was collected and a panel of cytokines were assessed by cytometric bead array. Naive mice, and those infected with *H. polygyrus* showed little response to re-stimulation with TmES (Fig. 5.5). Mice infected with *T. muris* alone or co-infected with *H. polygyrus* showed comparable temporal dynamics (Fig. 5.5 A) in cytokine production as well as similar overall levels of all cytokines when cytokine levels peaked at 20 days post infection (Fig. 5.5 B).

cMLN and sMLN cells from mice culled at 20 days post infection were further analysed by flow cytometry to assess T cell numbers and intracellular cytokine levels (Fig. 5.6). There was no obvious differences between *T. muris* alone and co-infected mice in the cMLN with both groups showing similar increases in CD4+ cells and FoxP3+ T cell numbers relative to the naive control (Fig. 5.7 A). Infection with *H. polygyrus* alone did not appear to increase CD4+ cell number in the cMLN (Fig 5.7 A).
Figure 5.4: Enumeration of cMLN cells during co-infection. Female C57BL/6 mice were infected with a high (200 eggs) of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 3, 7, 14, & 20 days post-infection the cMLNs were collected. cMLN cells were homogenised and counted using an automated cell counter. (A) Line graph and (B) scatter graph of cMLN cell number. [A] points represent the mean and error bars indicated SEM. [B] points represent individual mice, bar and error indicate mean and SEM. Dotted lines indicate the mean (black dots) and SEM (red dots) of the naive group. * indicates significance (p < 0.05), *** indicates significance (p < 0.001), **** indicates significance (p < 0.0001) as calculated by 2way ANOVA with post-hoc Dunn’s multiple comparisons when compared to the naive control.
Figure 5.5: Cytokine production by MLN cells during co-infection. Female C57BL/6 mice were infected with a high (200 eggs) of T. muris either alone or co-infected with 200 L3 H. polygyrus larvae. Mice were culled at 3, 7, 14, & 20 days post-infection the cMLNs were collected. cMLN cells were homogenised, and re-stimulated with TmES for 36 hours in culture. Cytokines levels were measured by cytometric bead array. (A) Line graph of cytokine levels for IFNγ, IL-6, IL-10, IL-13, IL-17A & TNF. (B) Bar graphs representing the log2 normalised values of cytokines at 20 days post-infection.
As expected the number of CD4+ cells in the sMLN increased during infection with *H. polygyrus* and this increase was not affected by co-infection with *T. muris*. Infection with *T. muris* alone did not induce expansion of sMLN cells (Fig 5.7 B).

To identify the difference in cytokine production specifically in CD4+ cells, cMLN cells were cultured *ex vivo* in the presence of PMA/ionomycin to re-stimulate them and Brefeldin A to prevent secretion. Cells were stained with antibodies against CD4, IFNγ, IL-10, IL-13, and IL-17A then analysed by flow cytometry. Co-infection did not appear to affect the number of CD4+ producing any of the measured cytokines relative to mice infected with *T. muris* alone (Fig 5.7 C).
Figure 5.6: Gating strategies for flow cytometry of MLN cells. Representative gating strategies of MLN cells for identification of T cell subsets and staining of intracellular cytokines. (A) Cells were gated first on size followed by gating of live cells. T cells were identified as CD45+CD3+ cells, these were then subsetted into CD4+ or CD8+ cells. Tregs were identified as FoxP3+CD4+ cells. (B) To analyse intracellular cytokine levels cells were first gated on size, Th cells were then identified as CD45+CD3+CD4+ cells. CD4+ T cells were then gated on either IFNγ, IL-10, IL-13, or IL-17A.
Figure 5.7: Flow cytometric analysis of MLN cells during co-infection. Female C57BL/6 mice were infected with a high (200 eggs) of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 20 days post-infection the MLNs collected and seperated in cMLNs and sMLNs. Cells were stained and analysed by flow cytometry. (A) Percentage of T cell subsets in the cMLN. (B) Percentage of T cell subsets in the sMLNs. (C) Levels of intracellular cytokines in CD4+ cells from cMLN. Points represent individual mice, bar represent the mean.
5.2.4 Pathophysiological responses in the caecum during co-infection

Given that expulsion of *T. muris* is mediated by effector mechanisms in the caecum physiological responses were measured during infection. The resistin-like molecules RELMα and RELMβ are known to be produced in response to Th2 signalling at mucosal sites. Expression of these genes in the intestine was measured by qPCR on RNA extracted from whole small intestinal and whole caecal tissue. There was little induction of RELMα in the caecum during infection (Fig. 5.8 A). On the other hand expression of RELMβ in the caecum increased steadily through infection in mice infected with *T. muris*, this was not affected by co-infection (Fig. 5.8 B). Infection with *H. polygyrus* alone did not appear to affect gene expression in the caecum.

In the small intestine infection with *H. polygyrus* increased levels of both RELMα and RELMβ at day 20 post infection. This increase in expression was not impaired by co-infection with *T. muris* (Fig. 5.8 C&D).

Both caecal crypt hyperplasia and goblet cell hyperplasia, under the control of Th2 signalling, have been linked with expulsion of *T. muris*80,84. To analyse histological changes in the caecum caecal tissue was collected, fixed in NBF, embedded in paraffin, cut in to 5 μm sections and PAS stained (Fig. 5.9 A). Infection with *T. muris* resulted in an increase in both the number of goblet cells per crypt and the length of the caecal crypts (Fig. 5.9 B&C). Co-infection did not impair these increases, nor did infection with *H. polygyrus* alone have any effect on caecal architecture when compared to naive animals (Fig. 5.9).

Infection with *H. polygyrus* is thought to induce a potent regulatory response in part by induction of Treg through activation of TGF-β signalling120,121,123. To determine whether *H. polygyrus* was inducing TGF-β signalling in the caecum the number of phospho-Smad2/3 positive cells (pSmad2/3+ve) were measured by immunofluorescence (Fig. 5.10 A). Phosphorylation of Smad2/3 is associated with TGF-β signalling200.

166
Figure 5.8: Gene expression analysis in the Caecum and Duodenum during co-infection. Female C57BL/6 mice were infected with a high dose (200 eggs) of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 7, 14, 20 & 35 days post-infection and tissue from the (A & B) caecum and (C & D) small intestine was collected. RNA was extracted by phenol-chloroform extraction and a cDNA library was generated. Expression of (A & C) RELMα and (B & D) RELMβ was determined by qPCR. Values are given as relative expression to a β-actin control calculated by the $2^{-\delta CT}$ method. Points represent individual mice, bar and error represent mean and SEM.
Figure 5.9: Histological analysis of the caecum during co-infection. Female C57BL/6 mice were infected with a high dose (200 eggs) of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 20 & 35 days post-infection and the caecal tip was collected, fixed in NBF, embedded in paraffin and cut into 5 μm sections. Sections were stained with Periodic-acid-Schiff. (A) Representative tissue sections from 35 d.p.i. Enumeration of (B) mean number of goblet cells per crypt and (C) mean crypt length. Points represent individual mice, bar and error represent mean and SEM.
Figure 5.10: pSmad2/3 positive cells during co-infection. Female C57BL/6 mice were infected with a high dose (200 eggs) of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 20 & 35 days post-infection and the caecal tip was collected, fixed in NBF, embedded in paraffin and cut into 5 μm sections. Tissue sections were stained with DAPI (blue) and anti-pSmad2/3 (red). (A) Representative tissue sections from 35 d.p.i. (B) Enumeration of pSmad2/3+ve cells. Points represent individual mice, bar and error represent mean and SEM.
Naive mice showed almost no phosphorylation of Smad2/3. Mice infected with *H. polygyrus* alone showed a small increase in pSmad2/3+ve cells. However, infection with *T. muris* was a much more potent inducer of pSmad2/3+ve cells in the caecum and this induction of TGF-β signalling did not appear to be enhanced by co-infection (Fig. 5.10 B).

### 5.2.5 Co-infection in immune-deficient mice

To determine whether there were direct helminth-helminth effects independent of the adaptive immune system female SCID mice were infected with *T. muris* either alone or co-infected with *H. polygyrus*. Unlike immunocompetent mice, SCID mice fail to expel a high-dose *T. muris* infection. Mice were culled at 33 days post infection and *T. muris* fitness was assessed. Total *T. muris* burden did not appear to be affected by co-infection (Fig. 5.11 A). Measuring worm length showed no difference in the length of male worms between those collected from mice infected with *T. muris* alone and those co-infected. However, female worms showed a small but significant increase in length in the co-infected group (Fig. 5.11 B). Despite this increase these worms were no more fecund than those in the single infection group (Fig. 5.11 C).
Figure 5.11: *T. muris* fitness during co-infection in SCID mice. Female SCID mice were infected with a high dose (200 eggs) of *T. muris* either alone or co-infected with 200 L3 *H. polygyrus* larvae. Mice were culled at 33 days post-infection. At autopsy the caecum was collected and the worms were isolated under dissecting microscopes, co-infection was confirmed by dissection. (A) Total *T. muris* burdens for each mouse were determined. (B) 4-5 male (♂) and female (♀) worms were collected whole from each mouse, fixed, and imaged under dissecting microscope. The length of each worm was measured in ImageJ. (C) 5 female worms from each mouse were collected, washed, and cultured for 4 hours. The number of eggs released into the medium was counted by eye. Points represent values of individual [A] mice or [B-C] worms. Line represents the mean, error bars indicate the SEM. Significance was calculated by either [B] 2way ANOVA with post hoc Sidak’s comparisons or [C] by Mann-Whitney test.
5.2.6 Infection with *H. polygyrus* does not affect immunisation-induced immunity to infection with *T. muris*

Immunisation of mice with TmES via subcutaneous injection is sufficient to generate potent protective immunity against infection\(^{201}\). Immunised mice generate a rapid antibody response, enhanced cytokine production and a stronger goblet cell hyperplasia than sham injected individuals\(^{201}\). Despite extensive work a vaccine to *T. trichiura*, and indeed any parasitic nematode of humans, remains elusive. One factor that may contribute is the impact of co-infection with other immunomodulatory helminths which may suppress vaccine-induced immune responses.

To test this hypothesis female C57BL/6 mice were infected with *H. polygyrus*, and subsequently immunised with TmES in Alum by subcutaneous injection (or given a sham PBS + Alum injection) and challenged with a high dose of *T. muris*. Mice were culled 19 days post infection when non-immunised mice would still be infected (Fig. 5.12 A).

Prior infection with *H. polygyrus* has no effect on immunisation-induced resistance to *T. muris*. Both the control group that received *T. muris* only and the group that was infected with *H. polygyrus* were completely resistant to infection following immunisation (Fig. 5.12 B). There was also no effect on generation of *T. muris* IgG1 which was strongly increased over the PBS control groups in both the *T. muris* only and co-infected groups (Fig. 5.12 D).

To determine whether infection with *H. polygyrus* was having any effect on immunisation-induced responses caecal tissue was collected for histological and gene expression analysis. Immunisation increased goblet cell hyperplasia, and RELM\(\alpha\) and RELM\(\beta\) expression. This response was not affected by infection with *H. polygyrus* (Fig. 5.13).
Figure 5.12: The effect of *H. polygyrus* infection on immunisation against *T. muris*. (A) Female C57BL/6 mice were either left naive or infected with 200 L3 *H. polygyrus* larvae, after 3 weeks both groups mice were immunised with 50 μg of TmES in Alum, or given a PBS + Alum control by subcutaneous injection. 10 days after immunisation mice with infected with 200 *T. muris* eggs. 19 days after infection mice were culled. (B) *T. muris* burden was assessed by eye under dissecting microscope. (C) *H. polygyrus* burden. (D) Levels *T. muris*-specific IgG1 in the serum were measured by ELISA.
Figure 5.13: The effect of *H. polygyrus* infection on immunisation-induced caecal responses to *T. muris*. (A) Female C57BL/6 mice were either left naive or infected with 200 L3 *H. polygyrus* larvae, after 3 weeks both groups mice were immunised with 50 μg of TmES in Alum, or given a PBS + Alum control by subcutaneous injection. 10 days after immunisation mice with infected with 200 *T. muris* eggs. 19 days after infection mice were culled. (B) *T. muris* burden was assessed by eye under dissecting microscope. (C) *H. polygyrus* burden. (D) Levels *T. muris*-specific IgG1 in the serum were measured by ELISA.
5.2.7 HpES can recapitulate the effect of co-infection on *T. muris* high-dose infection

It has been previously demonstrated that many of the immunomodulatory effects associated with *H. polygyrus* are, at least in part, controlled by secreted factors\(^{103,122,124}\). To determine whether HpES was sufficient to recapitulate the delayed expulsion of *T. muris* conferred by co-infection, mice were infected with a high dose of *T. muris* and injected every other day with HpES starting at different points post infection (Fig. 5.14 A).

Mice that had been infected with *T. muris* alone had expelled their infections by 35 days post infection. However, co-infected mice, and those that had been treated with HpES remained infected, although some HpES treated individuals were able to expel their infection (Fig. 5.14 B). The later treatment with HpES began the less efficacious it appeared to be with a trend to lower worm burdens in those mice that began treatment at 28 days post infection compared to mice that had been co-infected. Treatment with HpES was also able to mimic the effect of co-infection of IgG isotype switching with mice that were co-infected or treated with HpES having a lower ratio of IgG1 to IgG2ac than those mice that were infected with *T. muris* alone (Fig. 5.14 C). Neither co-infection or treatment with HpES appeared to have a strong effect on numbers of CD4+ cells or the number of FoxP3+ve cells in the cMLN (Fig 5.14 D&E).

Analysis of the caecum showed that induction of goblet cell and caecal crypt hyperplasia were comparable between mice that were co-infected and those that had been treated with HpES (Fig. 5.15 A-C). However, mice that were co-infected appeared to show a suppression of expression of RELMα and RELMβ relative to mice that had been infected with *T. muris* alone, this affect on gene expression was not recapitulated by treatment with HpES (Fig. 15.15 D&E).
Figure 5.14: The effect of HpES on infection with *T. muris*. (A) Schematic representation of the experimental protocol. Mice were infected with a high dose of *T. muris* (200 eggs). Following infection, mice were injected with HpES every second day starting at 7, 14, 21, or 28 days post infection. Mice were culled at 35 days post infection. (B) *T. muris* burden was assessed by eye under dissecting microscope. (C) Levels of *T. muris*-specific IgG1 in the serum were measured by ELISA. Proportion of (D) CD4+ T cells and (E) FoxP3+ CD4+ cells in the cMLN measured by flow cytometry.
Figure 5.15: The effect of HpES on caecal responses to T. muris. (A) Representative images of caecal sections stained with DAPI (blue) and anti-Muc2 (red). (B) Quantification of mean caecal crypt length per mouse. (C) Enumeration of number of Muc2+ve cells per mouse. Expression of (D) RELMα and (E) RELMβ was determined by qPCR. Values are given as relative expression to a β-actin control calculated by the 2^(-ΔΔCT) method. Points represent individual mice, bar and error represent mean and SEM.
5.3 Discussion

The vast majority of literature on immune responses to helminth infection have focused on single-species infections. However, epidemiological data consistently shows that infection with a single species of helminth is incredibly rare, and that co-infection is the norm in situ\textsuperscript{10–13}. Thus, both helminths and their hosts have likely evolved under the selection of polyparasitism and the modes through which resistance to infection develop may be influenced by this.

It was previously reported that co-infection with a high dose of \textit{T. muris} and \textit{H. polygyrus} results in a delay in the expulsion of \textit{T. muris}\textsuperscript{135}. However, no characterisation of the immune response under these conditions was detailed. Therefore, this system presents a possible avenue to interrogate how co-infection impacts the development of immunity to \textit{Trichuris}.

Here female C57BL/6 mice were infected with a high dose of both \textit{T. muris} and \textit{H. polygyrus} simultaneously. Worm burdens were assessed as were immune responses and physiological changes in the caecum.

5.3.1 Co-infection with \textit{H. polygyrus} prevents expulsion of a high-dose \textit{T. muris} infection

Mice that were infected with a high dose of \textit{T. muris} alone showed typical expulsion kinetics, with mice still highly infected at 19 days post infection and free of parasitism at 35 days post infection. Consistent with a previous report\textsuperscript{135}, mice that were co-infected with \textit{H. polygyrus} remained highly infected at 35 days post infection. The ability of \textit{H. polygyrus} to increase susceptibility to other nematodes does not appear to be limited to \textit{T. muris}. Indeed, infection with \textit{H. polygyrus} has been shown to also delay expulsion of \textit{T. spiralis}\textsuperscript{136}. Interestingly, the data presented here suggests that this effect is not dose-dependent, as, in co-infected mice, \textit{H. polygyrus} burden did not correlate positively with that of \textit{T. muris}. This would support the notion that there is a narrow threshold between resistance and susceptibility to \textit{T. muris} with mice being either fully susceptible to chronic infection or entirely resistant.
The ability of *H. polygyrus* to drive susceptibility to concurrent infections does not appear to be limited to other GI helminths. Infection with *Leishmania donovani* is exacerbated by co-infection with *H. polygyrus*\(^{202}\), as is infection with *Toxoplasma gondii*\(^{203}\), and the immune response to infection with *Bacillus Calmette-Gurin* (BCG) is compromised by co-infection\(^{204,205}\). Interestingly, the cellularity of the skin-draining lymph nodes was seen to be reduced, and their expansion impaired, during infection with BCG in co-infected mice\(^{204}\). This is consistent with a previous publication that demonstrated that infection with *H. polygyrus* results in the redistribution of lymphocytes from peripheral LNs, such as inguinal, axillary, and brachial LNs, to the sMLNs\(^{206}\). Here, an atrophy in the cMLN was observed in mice infected with *H. polygyrus*, and co-infected mice showed a delay in expansion of cMLN in response to infection with *T. muris*. Thus, one mechanism through which *H. polygyrus* induces susceptibility to *T. muris* may be through inducing a more potent immune response that outcompetes that against *T. muris* for available lymphocytes. Counter to this argument, however, was the ability of HpES to also induce susceptibility to infection. Injection of antigen alone would not result in expansion of the sMLN and thus redistribution of lymphocytes alone is unlikely to account for delayed *T. muris* expulsion.

It has been posited that co-infection with *H. polygyrus* can alter the outcome of infection with protozoan parasites such as *L. donovani* by skewing the immune response towards a Th2-dominated response, suppressing the Th1 response required for resistance\(^ {202}\). However, expulsion of *T. muris* is dependent on a robust Th2 immune response\(^ {74}\). It would therefore follow that if *H. polygyrus* was a driving systemic skew towards Th2 immunity that infected mice would be more, not less, resistant to infection with *T. muris*. Therefore, it seems unlikely that *H. polygyrus* is mediating its effect by altering the cytokine profile of infected mice. Indeed, assaying cells from the cMLN showed no effect on cytokine production in response to *T. muris*-derived antigen in co-infected mice relative to those infected with *T. muris* alone, at least for the cytokines measured. Nor was the abundance of CD4+ cells or FoxP3+ cells affected by co-infection, although the flow cytometric analysis presented here is superficial and requires deeper investigation.

Assaying the caecum for effects on gene expression local to the site of infection,
and on key histological changes required for expulsion of \textit{T. muris} showed that expression of characteristic Th2-associated genes was not impaired by co-infection, nor was the induction of goblet cell hyperplasia diminished. \textit{H. polygyrus} has been suggested to be highly immunomodulatory, and it has been argued that it induces a strong regulatory immune response in order to promote its own longevity in the host\textsuperscript{120,121}. This, along with strong interest in the hygiene-hypothesis, has led to idea that infection with a GI helminth can have long-distance immunosuppressive effects at distal sites, although efforts to employ this therapeutically have often been unsuccessful\textsuperscript{38–40}. The data presented here suggests that infection with \textit{H. polygyrus} has little to no effect on the immune response to \textit{T. muris} either in the draining lymph nodes, or at the site of infection. There was also no evidence of an induction of a regulatory response occurring in the caecum with levels of TGF-\textit{\beta} signalling appearing similar between the mono-infected and co-infected groups.

The data presented in this thesis focused on assaying key markers, and effectors, of resistance to \textit{T. muris} i.e. the cytokine profile and histological changes in the caecum. Previous investigations in single parasite models of infection have borne out only a limited number of mechanisms as being essential for resistance: (i) production of IL-13 by CD4+ cells\textsuperscript{70,72} and (ii) maintenance/plasticity of the mucus barrier\textsuperscript{81,82}. From these investigations it appears that these mechanisms remain intact during co-infection. Therefore, it seems likely that co-infection does not drive susceptibility to infection by suppressing essential effectors of resistance, nor were traditional drivers of susceptibility enhanced i.e. activation of a Th1 response. This suggests that unknown mechanisms of susceptibility to infection with \textit{Trichuris} exist, and may be specific to the context of co-infection. Identifying responsible genes/proteins will require non-biased approaches such as RNA sequencing or mass spectroscopy. However, these data highlight that using highly reductionist models, like single-parasite infections, obscures such facets of infection.

\subsection{5.3.2 Immune-independent helminth-helminth interactions}

Given the key role the immune system and the downstream effector mechanisms play in resistance to \textit{T. muris} this chapter, and indeed this thesis, focused on the immune response to infection. However, often overlooked are the direct, or indirect-
non-immune, pathogen-pathogen effects that may occur in co-infection models. To an extent this is due to the genetic intractability of helminths versus the relative ease of manipulating the host. As such, the persistence of *T. muris* during co-infection may not be due to a reduction in the ability of the host to generate resistance but rather and enhancement in fitness of the parasite under such conditions. To see if such an effect could be observed, SCID mice were infected either with *T. muris* alone, or co-infected with *H. polygyrus*. Mice were culled at 33 days post infection, the point at which *T. muris* reaches patency, and worm fitness was assessed.

Co-infected mice had *T. muris* burdens comparable to the single-parasite infection controls. Assessing worm size showed that while male parasites were of a comparable size between both groups, the female *T. muris* were significantly longer in the co-infected group when compared to the control, there was also a trend to these worms being more fecund although this was not statistically significant. These data suggest that *H. polygyrus* may have effects on *T. muris* that are independent of the immune response. However, how this is occurring remains unclear although several possibilities present themselves: (i) *T. muris* can detect secreted factors from *H. polygyrus* and increases its own growth in response; (ii) *H. polygyrus*-derived changes to the intestinal environment, such as a modulation of the microbiota, have downstream effects in the caecum that favour *T. muris* growth; (iii) Infection with *H. polygyrus* reduces elements of the host’s fitness, independent of the immune system, that reduces constraints on *T. muris* size or growth rate.

Given the preliminary nature of these data further investigation is required. A more detailed time course of *T. muris* growth in this system would illuminate whether the rate at which the parasites grow and mature is enhanced during co-infection. Additionally, now that the *T. muris* genome has been sequenced, RNA-seq experiments are possible and would allow an investigation into whether *T. muris* changes its gene expression profile in response to co-infection. This approach may reveal genes that are crucial to *T. muris* growth and maturation and, therefore, provide novel targets for anthelminthic therapeutics.
5.3.3 Co-infection does not appear to inhibit immunisation-induced immunity to *T. muris*

One of the most challenging aspects of controlling helminthiases *in situ* is the absence of vaccines against any human helminth. Data from the administration of existing vaccines against bacterial/viral infection has suggested that infection with helminths reduces the efficacy of these vaccines\(^{207,208}\). Therefore, co-infection with other helminths may offer one explanation as to why vaccine candidates that appear efficacious *ex situ* fail to survive the transition into clinical application. Previous work has shown that infection with *H. polygyrus* resulted in a reduced efficacy of infection with *Plasmodium falciparum*\(^{209}\). *H. polygyrus* may therefore be useful to model helminth-mediated suppression of anthelminthic vaccines.

To attempt to model this, mice were infected with *H. polygyrus* which were allowed three weeks to establish a chronic infection. Mice were then immunised with 50 μg of TmES in Alum via subcutaneous injection, 10 days later mice were infected with a high dose of *T. muris*. This immunisation regime has previously been shown to result in the expulsion of *T. muris* by 19 days post infection\(^{201}\). Dixon and colleagues had shown that immunisation in this manner resulted in an elevated antibody response to *T. muris* and an enhancement in goblet cell responses in the caecum. Infection with *H. polygyrus* did not appear to inhibit the efficacy of immunisation. Both immunised groups were highly resistant to infection with *T. muris*, showed a robust parasite-specific IgG1 response, and had an enhanced goblet cell hyperplasia and Th2 gene expression relative to mice that had received the adjuvant alone.

Whilst this model suggests that co-infection does not impair immunisation-derived immunity it may require refinement to better reflect data from the field. In particular, whilst this immunisation regime confers robust short-term protection, there is no evidence that it is efficacious long-term. Therefore, if the time between immunisation and infection with *T. muris* was increased, to a point at which the immunisation is less effective, an effect of co-infection may be observed. However, what this data suggests is that whilst co-infection may enhance survival of *T. muris* in the absence of sensitisation against *Trichuris*, once an immune response is estab-
lished, in this case via immunisation, *H. polygyrus*-derived effect of *T. muris* are insufficient to prevent expulsion.

### 5.3.4 HpES prevents resistance to high dose *T. muris* infection

A considerable amount of research has been conducted on the properties of secreted proteins produced by *H. polygyrus*. This stems from observations that certain aspects of *H. polygyrus* infection could be recapitulated with HpES alone, i.e. induction of Tregs\textsuperscript{122}, and suppression of Th2-associated allergic inflammation\textsuperscript{103}. Thus far, this has resulted in the identification of a family of TGF-β mimics\textsuperscript{124}, and an IL-33 inhibitor\textsuperscript{104}. All of the proteins currently identified contain CCP domains arranged in diverse confirmations. It is also likely that there are as yet other uncharacterised immunomodulatory CCP domain proteins in the *H. polygyrus* secretome.

Given the potent immunomodulatory potential of HpES it is possible that *H. polygyrus*-induced susceptibility to high dose *T. muris* infection is in-part driven by HpES. To investigate this mice were infected with *T. muris* then (starting at 7, 14, 21, and 28 days post-infection) injected with HpES intraperitoneally. Injection with HpES prevented expulsion of *T. muris* in the majority of treated mice. HpES was able to prevent expulsion regardless of the timepoint at which treatment began, although the later HpES administration began the fewer worms were recovered from infected mice relative to the co-infected control.

How this effect is mediated is unclear. As with co-infection HpES was able to reduce the ratio of IgG1 to IgG2ac in the serum suggesting some reduced Th2-induced class switching. However, an antibody response is thought to be dispensable for resistance to *T. muris* infection\textsuperscript{210}. There was also no evidence that injection with HpES was inducing significant changes in the gut mucosa.

The protein HpARI, isolated from HpES, has been shown to be a potent IL-33 blocker and is capable of reducing allergic inflammation\textsuperscript{104}. However, it is unlikely that suppression of IL-33 is responsible for the induction of susceptibility to infection, as previous work has shown IL-33 signalling to be dispensable in the expulsion of *T. muris*\textsuperscript{61}. Indeed, whilst administration of exogenous IL-33 is able to
enhance the adaptive response to *T. muris* it is unable to induce parasite expulsion in SCID mice\textsuperscript{62}.

Thus, the mechanism through which HpES may result in susceptibility remains unclear. However, a more comprehensive assessment of the gut mucosa response is required as the data presented here focuses on known markers of resistance to infection, and therefore is liable to miss as yet unidentified mediators of resistance/susceptibly to infection. It does, however, raise the possibility that proteins exist in HpES that modulate the immune response via mechanisms not currently associated with *H. polygyrus* separate from IL-33 and TGF-β signalling.

**Summary:**

- Co-infection with *H. polygyrus* results in a failure to expel a high dose *T. muris* infection in C57BL/6 mice.
- Infection with *H. polygyrus* is associated with atrophy of the cMLN.
- *H. polygyrus* has effects on the growth of *T. muris* independent of the immune system.
- Infection with *H. polygyrus* does not suppress immunisation-induced immunity to *T. muris*.
- Treatment with HpES is sufficient to prevent expulsion of *T. muris*. 
Chapter 6

Discussion
Infection with GI helminths exerts an incredible burden of morbidity upon the regions in which they are endemic. Additionally, the effect of helminths on the health of livestock presents significant economic concerns. Resistance to infection is thought to develop over time, however, it is rarely complete and the mechanisms through which resistance builds over time remain obtuse. A comprehensive understanding of the underlying immune responses during infection is critical in order to develop better therapeutic strategies, especially vaccines.

Traditional murine models of infection have been crucial in elucidating fundamental components of resistance to infection. In particular *T. muris* and *H. polygyrus* have been widely used models, due to the ability to maintain a robust lifecycle in the laboratory, the ability to modulate the immune response via dose or re-challenge, and their ability to generate long-lived patent infections in mice. Through experiments with these model parasites, the importance of an effective type 2 immune response in driving resistance to infection has been demonstrated, with a central role for Th2 cells as orchestrators.

However, the vast majority of experimental infections have relied on single bolus infection with a large dose of one species. However, *in situ* it is far more likely that individuals are frequently exposed to small doses of a parasite, rather than a one-off high dose. There is also a significant amount of data that demonstrates that infection with a single species of helminth is rare and that co-infection is far more common\(^\text{10–13}\). Along with this difference in the mode of infection, there is a discrepancy between the kinetics of infection observed in humans and those observed in experimental mice. Humans appear to develop resistance over time, with individuals experiencing a phase in which they are susceptible to infection followed by a plateau or decrease in worm burden when resistance develops\(^6,8,9\). Infection models of mice on the other-hand tend to produce binary states where mice are either entirely susceptible to infection, or entirely resistant, rather than developing immunity over time.

Therefore, to bridge the gap between what is observed in the field and what is observed in the laboratory, the models used must be modified to better reflect infection *in situ*.  

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186
6.1 Trickle infection and the development of resistance to helminth infection

One option is the ‘trickle’ infection. Under this regime, rather than being infected with a single bolus, mice are infected on a regular basis. Previous efforts to use a *H. polygyrus* trickle infection regime suggested that the kinetics of infection were altered by trickle infection, and that immunity to challenge infection developed over time\textsuperscript{133,134}. However, these studies provided little immunological insight and were performed in strains of mice no longer widely used rather than the now more standard C57BL/6 and BALB/c strains.

The data presented here shows that in C57BL/6 mice a single infection is sufficient to protect mice against subsequent challenges during trickle infection, and unlike the *T. muris* trickle infection model\textsuperscript{60} worm burden does not initially increase before resistance develops. Interestingly when trickle infected mice were compared to mice that had received a single dose infection there appeared to be a difference in the longevity of the infecting adult parasites. Throughout the 11 week protocol mice that had been single dose infected had consistent worm burdens between time points, but those that had been trickle infected lost the majority of their parasites by the end of the experiment. This suggests that trickle infection promotes resistance to the adult parasite not induced by a single dose infection.

This increase in resistance correlated with higher levels of IL-4 expression in the MLN of trickle infected mice, as well as a reduction in IFN\textsubscript{γ} expression, increased RELM\textsubscript{α/β} expression in the small intestine, increased mastocytosis, and heightened parasite-specific IgG1 levels in the serum. Collectively these data support the notion that trickle infection drives a stronger Th2 response than a single dose infection. This is consistent with Th2 immunity being required for resistance to *H. polygyrus*. BALB/c mice, which are naturally more prone to a Th2 immune response, expel *H. polygyrus* infection more rapidly than C57BL/6 mice which tend produce a weaker Th2 response\textsuperscript{45}.

The means through which adult *H. polygyrus* are expelled is still poorly defined. A great deal of work has characterised the role of IgG and aaMΦs in the trapping
of the L3 stages\textsuperscript{90–92,94,96,109}. However, these mechanisms are unlikely to be effective once the parasite has reached its luminal adult stage where trapping by macrophages would pose little risk. There is some evidence that secreted factors in the small intestinal mucus may facilitate parasite killing. RELM\textsubscript{β} appears to reduce \textit{H. polygyrus} fitness by impairing its ability to feed \textit{in vivo}\textsuperscript{113}. Indeed, trickle infected mice showed higher level of RELM\textsubscript{β} expression in the small intestine and this may account for the increased resistance. However, the use of RELM\textsubscript{β}\textsuperscript{−/−} mice would be required to define an essential role for trickle-driven resistance to \textit{H. polygyrus}.

Comparing the \textit{H. polygyrus} trickle regime presented here to the previously reported \textit{T. muris} trickle protocol, by Glover and colleagues\textsuperscript{60}, differences in the kinetics of infection and associated immune responses can be observed. Trickle infecting mice with \textit{T. muris} results in a relatively sustained period of susceptibility to infection, during which a step-wise increase in worm burden occurs, followed by the generation of resistance to infection and a concomitant reduction in worm burden. This correlates with a change in immune phenotype from Th1-dominated to Th2-dominated\textsuperscript{60}. In contrast, during \textit{H. polygyrus} trickle infection, mice only appear to be susceptible to the first infection and become almost entirely immune to subsequent challenges. A strong Th2 immune response is rapidly established, however, whereas in a single-dose model this response plateaus, during trickle infection subsequent doses appear to enhance this response and drive a phenotype sufficient for the expulsion of the existing adult parasites.

The reason for this difference in kinetics is likely manifold. One possibility is the difference in size of the parasite. \textit{T. muris} is ingested as eggs and progresses slowly to adulthood, thus, it’s likely to produce less initial damage and provoke a weaker initial immune response. This notion is supported by the fact that anti-\textit{T. muris} IgG isn’t detected until several weeks post infection. On the other hand, \textit{H. polygyrus} is ingested already at the L3 stage and is a mature adult within eight days post infection. It would follow that \textit{H. polygyrus} creates more damage and exerts a higher metabolic cost more rapidly, and therefore would necessitate expulsion over tolerance. \textit{T. muris} on the other hand, producing less damage and exerting a lower cost, can be tolerated for longer, but once the burden has built to a threshold.
mechanism develop to prevent further increase in parasite burden.

Where the *T. muris* trickle model better reflects the kinetics seen in human *Trichuris* infection\textsuperscript{6,60}, trickle infection with *H. polygyrus* does not appear to reflect human hookworm kinetics, in which worm burden builds progressively over time followed by a plateau when resistance is established\textsuperscript{6}. This difference does not appear to simply reflect a difference in lifecycle strategy by *H. polygyrus* relative to other hookworm species; analysis of wild mice shows a similar relationship between age of host and *H. polygyrus* burden as is seen in human hookworm infection\textsuperscript{211}. Whilst the reasons for the discrepancy between field and laboratory may be manifold, one possibility is that the artificial nature of laboratory conditions impacts the trade-off between resistance to infection and tolerance of infection. Mice maintained in animal facilities are kept at an optimal temperature, with an excess of food (which, whilst nutritionally balanced, does not reflect the variety of food consumed by wild counterparts), and an absence of predators. Wild mice must first allocate resources to maintenance/repair, then to growth, and finally to the combat of infectious agents such as parasites\textsuperscript{141}. In a study on wild unmanaged Soay sheep, natural selection appeared to favour tolerance of nematode infection rather than resistance, with tolerant animals loosing less weight during infection and having higher breeding success\textsuperscript{212}. A similar observation was made in field voles, where mature individuals that showed evidence of good body condition tolerated infection by macro-parasites\textsuperscript{213}. Further, in wild mice *H. polygyrus* burden is lowest (although not to expulsion) in the summer months when nutritional resources are most abundant\textsuperscript{211}. This may also explain recent observations that mice on HFD are more resistant to infection with *T. muris* than those on LFD\textsuperscript{185}. In laboratory conditions, where resources are in effect limitless, there may no longer exist a pressure for mice to tolerate infection, and mice may therefore invest more resources in the expulsion of nematodes.

What both trickle infection regimes highlight is that there is a distinct difference between the immune response generated by single doses and those generated from repeated infections. Single dose infections appear to generate relatively static immune responses that rapidly plateau in intensity shortly following infection then remain at a constant level until the infection is resolved. Trickle infections on the
other hand drive dynamic immune responses that either change or grow with each subsequent infection. Understanding how this plasticity in magnitude or phenotype of response is controlled may provide useful insight when considering how to improve vaccine candidates or adjuvants, which has been notoriously challenging for helminths\textsuperscript{214}.

6.2 Co-infection and immunity to parasitic infection

As well as frequent re-infection, another facet of natural helminth is presence of multiple infecting species. Helminths tend to occupy similar ecological niches and share vast overlapping geographical distributions. This likely indicates a long-shared evolutionary history with the migration of vertebrates extending the ranges of their nematode parasites. Whilst a fair amount of attention has been paid to the significance of the relationship between helminth and host, especially with regard to the hygiene hypothesis\textsuperscript{29,164} and the potential application of helminth therapy\textsuperscript{36,37,39}, little attention has been given to the relationships that may exist between different helminth species that occupy the same host.

Previous investigation had demonstrated that co-infection with \textit{H. polygyrus} was sufficient to induce susceptibility to different species of GI helminth in otherwise immunocompetent mice including \textit{T. muris} and \textit{T. spiralis}\textsuperscript{135,136}. Despite these observations being made over 40 years ago, the mechanisms responsible for this delay have remained elusive. Co-infection of \textit{H. polygyrus} with protozoan parasites or bacteria has also revealed an ability of this parasite to increase susceptibility to infection with other pathogens\textsuperscript{202,204,215}. In the cases of co-infection with \textit{L. donovani} and \textit{Cryptosporidium parvum}, it is thought that \textit{H. polygyrus} drives a systemic Th2 response that prevents the induction of a Th1 response sufficient to resist the protozoan parasites\textsuperscript{202,215}. However, given that a robust Th2 response is required to drive expulsion of \textit{T. muris} it seems unlikely that this skew in the immune response is responsible for delayed expulsion. Indeed, co-infection with \textit{S. mansoni}, which also provokes a strong Th2 response at a distal site, induced expulsion of a low-dose \textit{T. muris} infection\textsuperscript{216}. Here, in re-stimulation assays of the cMLN, there appeared to be no difference in the profile of measured cytokines produced between the single-infected and co-infected groups.
Relatively recent observations have shown that infection with *H. polygyrus* results in a reduced cellularity of peripheral LNs such as the brachial and inguinal LNs\(^{206}\). It is thought that this is due to the existence of a limited lymphocyte pool available to all LNs that are competed for, and that infection with *H. polygyrus* results in a sequestration of lymphocytes at the sMLN\(^{206}\). This sequestering of lymphocytes at the sMLN has been shown to result in reduced cellularity of the skin-draining LNs which exacerbates infection with BCG\(^{204}\). Here it was observed that infection with *H. polygyrus* results in the reduced cellularity of the cMLN, and a delay in its expansion during co-infection. It is therefore possible that whilst no difference in production of cytokine or cell number was observed in assays such as the re-stimulation of cMLN cells, where cell number is normalised between samples for comparison, the overall production of Th2 cytokines is lower due to a reduced pool of lymphocytes available to direct anti-*T. muris* responses.

However, whilst a model in which a reduced lymphocyte pool results in an impaired response to *T. muris* is attractive, it does not account for all the observations presented here. No discernible effect on goblet cell hyperplasia or gene expression in the caecum was observed, suggesting an unimpaired Th2 response was occurring at the mucosa. Also, injection with HpES (which is unlikely to result in cMLN atrophy) was sufficient to prevent expulsion of *T. muris*, and co-infection in SCIDs appeared to increase female *T. muris* fitness in the absence of an effect on the adaptive immune response.

The effect of co-infection on the fitness of female *T. muris* is particularly interesting. A great deal of focus in research into nematode parasitology focuses on the immune responses of the host, this is largely due to a greater ability to genetically and experimentally manipulate and measure the host. The biology of helminths by contrast is virtually unexplored. Given that GI helminths will have likely evolved in the evolutionary context of co-infection with other GI nematodes mechanisms may exist, independent from the host immune system, that affect parasite growth and survival. One possibility is that, in co-infected mice, helminths compete for resources that must be acquired from the host. The presence of multiple parasites exerts a fitness cost on the host, it would therefore be advantageous for *T. muris* to increase its growth rate in order to either outcompete *H. polygyrus*, or to reach
maturity and sexually reproduce before the host can no longer sustain the burden of combined infection. Alternatively, helminths may act synergistically, *H. polygyrus* reshapes the host intestinal microbiota not only at the site of infection but throughout the GI tract\(^{192}\). This may result in a change in microbial composition that favours *T. muris* growth and survival perhaps via the production of essential metabolites.

The mechanisms through which helminth promote the survival of co-infecting species remains an open question. However, the data presented here suggests that it may not be entirely through manipulation of the immune response. Further investigation into helminth-helminth co-infection will likely yield new insights into fundamental aspects of helminth biology.

### 6.3 Helminths and the microbiota

GI helminths exist in close contact with the host intestinal microbiota. *T. muris* cultivates it own microbiota distinct from the host\(^{167}\) and requires the presence of gut flora for hatching\(^{44}\). However, limited and conflicting field data has left unclear the relationship between biota composition and parasite\(^{146,165}\). Experimental data from murine models has indicated that helminth infection results in a dysbiosis characterised by a decrease in alpha diversity and an increase in the abundance of the *Lactobacillaceae* family\(^{166,168–171}\). Whilst these observations are interesting they are generally derived from models which compare naive mice with infected ones over a relatively short period of time. This results in a relatively binary comparison i.e. infected vs un-infected. Under these conditions the status of the immune system is virtually static as is the relative resistance/susceptibility of a given mouse to infection. This means that whether dynamic changes in the microbiota occur in response to changing immune status is unknown. Also undetermined is how having a different starting microbiota affects the outcome of infection. Here trickle infection was used to observed how changing immune responses affect the microbiota during *T. muris* infection. Additionally, manipulation of the diet was used to observe how different microbiotas change during infection and may affect outcome of infection.
Using the *T. muris* trickle infection model clear dynamic shifts in the microbiota that correlate with change in the immune response could be observed. The advantage of this system is that, whilst mice generate resistance to subsequent infection (at a defined time point), they retain adult parasites, meaning that changes in the biota can be directly linked to changes in the immune response. Throughout the period in which mice were still susceptible to infection a significant decrease in the diversity of the microbiota was observed as well as an increase in genera associated with susceptibility to infection. Once a Th2-dominated phenotype had been established, despite the ongoing presence of parasites, the diversity of the microbiota increased. These data suggests that the microbiota responds directly to the immune response. What remains an intriguing question, is how these effects are mediated, and if there are functional consequences to this. It is possible that the recovery of the microbiota is simply a consequence of the development of resistance, however, a more interesting possibility is that a diverse microbiota is required for resistance to infection. Mice that were placed on a HFD developed a microbiota that was more robust to challenge with *T. muris*, it may be that the resistance to infection observed in these mice is a direct consequence of their microbiota not succumbing to dysbiosis.

Reduced microbial diversity in the gut has been linked to increased susceptibility to bacteria infection\(^217\text{-}219\). Combined with the data presented here this points to a direct protective effect of a stable and diverse microbiota against pathogenic agents. An exciting prospect is that the host actively promotes microbial diversity to combat parasitic infection. Whilst significant weight has been placed on the role the microbiota plays in suppressing Th2 associated inflammatory responses in the gut\(^220\), and on the ability of the immune system to prevent microbial colonisation of the submucosa\(^160,161\), there is little data on the immune system actively promoting specific microbes. Alternatively, it may be that during the susceptible phase, where the constant damage to the intestinal epithelial increases the risk of a breach in the mucosal barrier which could lead to sepsis, that the production of anti-microbial peptides is up-regulated and when resistance to infection is acquired the concomitant Th2 response promotes barrier repair and reduces the necessity for these proteins.
Another possibility is that helminths are directly responsible for alteration to the microbiota, not through induction of specific immune responses or changes to the mucosa, but through secretion of factors that directly manipulate gut microbes. Both *T. muris* and *H. polygyrus* produce secretomes highly diverse in both peptides and miRNAs\textsuperscript{103,116,117,123,126}. The focus thus far has been on the potential immunomodulatory properties of these molecules\textsuperscript{104,118,124}, however, the vast majority of these proteins remain uncharacterised and unexplored are their potential non-immunomodulatory properties. There is some evidence that HpES has antimicrobial properties\textsuperscript{189}, however, investigation of purified peptides or miRNAs within HpES and TmES is likely to provide more convincing data of specific antimicrobial activity. Further, data from experimental infection with *H. polygyrus* and observations in field mice suggest that infection results in the up-regulation of TLRs and responsiveness to TLR ligands, which may impact the way in which the host regulates its microbiome\textsuperscript{221,222}.

Going forward it will be crucial to integrate microbiome data with other omics-style data sets. Defining connections between the microbiota, gene expression of the host/parasite, and the metabolome of all three parties will shed light on the fascinating complexity of the multi-dimensional interactions that exist in the intestinal ecosystem.

### 6.4 Concluding remarks

Highly reductionist models have been the mainstay of experimental immunoparasitology for nearly half a century. Whilst these models have been undeniably crucial in revealing fundamental information about the immune response to helminth infection, there is a limitation in what can be learnt from simple knock down/out experiments. What data in this thesis indicates is that by increasing complexity in the model system we see that there is still a great deal unknown about the development of resistance to helminths. Taken together, they highlight the need for increased complexity in our experimental systems.
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