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The immune response and the intestinal microbiota in control of susceptibility to *Heligmosomoides polygyrus*

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

Sections of the Introduction have been previously published in a review article published in Seminars in Immunopathology, and data and text from Chapter 6 have been previously published in a research article in the Journal of Immunology. Both published papers are attached in the appendix; text and data reproduced here are my own work.

Lisa Anne Reynolds

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Abstract

The mammalian intestinal tract is highly colonised with a diverse bacterial microbiota. The importance of this bacterial presence is now recognised; these bacteria contribute both to the nutritional status of their hosts and are required for the development of a competent immune system. In addition, the composition of the microbiota is likely important in influencing how the immune system reacts to antigens, as the presence of specific bacterial species can promote differentiation of T cells towards specific effector or regulatory fates. Though the ability of the microbiota to influence infections with bacterial and viral agents has been reported, whether the microbiota can affect a parasitic infection has not yet been described. It is likely, due to millions of years of co-evolution within mammalian hosts, that helminths have co-opted mechanisms of the microbiota to manipulate the host's immune system, in order to promote their own survival.

In this thesis, the immune parameters required for expulsion of a primary infection with the murine gastrointestinal helminth parasite *Heligmosomoides polygyrus* are examined, and whether the microflora influence these parameters in order to modulate susceptibility is explored.

Firstly, a multiparameter analysis of *H. polygyrus* infection was performed in two mouse strains which differ in susceptibility to a primary infection, to identify both immune factors and microbial populations which correlate with susceptibility to infection. BALB/c mice exhibited a stronger T helper (Th)2-type response to *H. polygyrus* excretory-secretory antigen (HES), produced high numbers of intestinal granulomas following infection and were better able to expel *H. polygyrus*, whereas the more susceptible C57BL/6 strain produced higher levels of inflammatory Th1 cytokines in response to HES. High levels of duodenal *Lactobacillus/Lactococcus* species positively correlated with *H. polygyrus* persistence within the BALB/c host, as did high levels of Enterobacteriaceae in the C57BL/6 host. Furthermore, the abundance of both of these bacterial groups was elevated in *H. polygyrus*-infected C57BL/6 mice compared to naïve controls, and mice given antibiotic treatment to diminish these groups were rendered more resistant to *H. polygyrus*. Infection

persistence was prolonged in BALB/c mice which were administered the single species *Lactobacillus taiwanensis*, a normal component of the microbiota.

Next, the impact of a loss of microbiota signalling by immune cells during *H. polygyrus* infection was examined, through the use of Toll-like receptor (TLR)- and TLR adaptor protein-deficient mice. MyD88$^{-/-}$ mice were more resistant to *H. polygyrus* than wildtype (Wt) C57BL/6 mice and exhibited increased granuloma formation: phenotypes which were not recapitulated by individual deficiencies in TLR2, TLR4, TLR5 or TLR9, and not seen in TRIF$^{-/-}$ mice. When MyD88$^{-/-}$ mice were additionally deficient in TRIF, the increased granuloma formation phenotype of MyD88$^{-/-}$ mice was lost. Whether MyD88 controls susceptibility to *H. polygyrus* infection via a TLR-independent mechanism, and how MyD88 and TRIF antagonistically contribute to granuloma formation remains to be resolved.

Finally, the importance of TGF-β signalling during *H. polygyrus* infection was examined, using mice deficient in TGF-β signalling specifically in T cells (TGF-βRII DN mice). These mice were more susceptible to *H. polygyrus* than Wt C57BL/6 mice, which was explained by an attenuated Th2 response to infection accompanied by exuberant IFN-γ production. The increased susceptibility to *H. polygyrus* was lost in TGF-βRII DN IFN-γ$^{-/-}$mice, in which Th2 responsiveness was partly restored.

These data highlight the importance of both immune components, particularly IFN-γ, which promotes susceptibility, and the presence of specific intestinal bacterial populations in controlling the persistence of a primary *H. polygyrus* infection.

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Abbreviations

AID- activation-induced cytidine deaminase
APC- antigen-presenting cell
ASF- altered Schaedler flora
ATP- adenosine 5'-triphosphate
AU- arbitrary units
BAL- bronchoalveolar lavage
BSA- bovine serum albumin
bp- base pairs
Breg- B regulatory cell
CBA- cytokine bead array
CD- Crohn's disease
CFU- colony forming units
CLR- C-type lectin receptor
CpG- deoxy-cytidylate-phosphate-deoxy-guanylate
DC- dendritic cell
DSS- dextran sodium sulphate
DTR- diphtheria toxin receptor
ELISA- enzyme-linked immunosorbent assay
FCS- foetal calf serum
GF- germ-free
H&E- hematoxylin and eosin
HES- *H. polygyrus* excretory secretory antigen
IBD- inflammatory bowel disease
ICCS- intracellular cytokine staining
IFN- interferon
Ig- immunoglobulin
IL- interleukin
IL-4C- IL-4 complexed with α-IL-4
IL-4R- IL-4 receptor
ILC- innate lymphoid cell
IVC- individually ventilated cage
L3- third stage larvae
LCMV- lymphocytic choriomeningitis virus
LP- lamina propria
LPS- lipopolysaccharide
MCMV- mouse cytomegalovirus
MIF- macrophage migration inhibitory factor
MHC- major histocompatibility complex

MLN- mesenteric lymph node

mMCP- mouse mast cell protease

MMP7- matrix metalloproteinase 7

MMTV- mouse mammary tumour virus

MyD88- Myeloid differentiation primary response gene 88

NK- natural killer

NLR- nucleotide-binding oligomerisation domain protein-like receptor

Nod- nucleotide-binding oligomerisation domain (receptor)

NOD- non-obese diabetic (mice)

NOS-2- nitric oxide synthase 2

OD- optical density

PAMP- pathogen-associated molecular pattern

PAR- protease-activated receptor

PBS- phosphate buffered saline

PGE_2- prostaglandin E2

PNPP- p-nitrophenyl phosphate

PP- Peyer's Patch

PRR- pattern recognition receptor

PSA- polysaccharide A

rad- radiation absorbed dose

RAG- recombination activating gene

SCID- severe combined immunodeficient

SFB- segmented filamentous bacteria

SPF- specific pathogen free

$sPLA_2$- secretory phospholipase A_2

T1D- type 1 diabetes

TCR- T cell receptor

Tfh- T follicular helper

Th- T helper

TLR- toll-like receptor

TBSt- tris-buffered saline with 0.1% Tween

TFF2- trefoil factor 2

TNBS- trinitrobenzene sulfonic acid

TNF- tumour necrosis factor

Treg- Regulatory T cell

TRIF- Toll/IL-1 domain containing adaptor inducing IFN-β

TSLP- thymic stromal lymphopoietin

UC- ulcerative colitis

Wt- wildtype

Chapter 1. Introduction

1.1 Overview

The mammalian intestinal tract is highly colonised by bacteria, to the extent that the number of bacterial cells surpasses the number of the mammal's own cells approximately 10-fold. Historically, it was thought that the presence of these bacteria was simply tolerated by the mammalian host, without contributing to the host's fitness; hence they were originally named 'commensal bacteria'. A commensal organism is one that gains some benefit from living within a host, without positively or negatively affecting the host's fitness. It is now known that this nomenclature is misleading, as the presence of the intestinal microbiota is enormously beneficial to mammals. It is advantageous to the bacteria to ensure the survival of their host, and the ways in which they have contributed to our successful evolution are numerous.

As well as metabolising dietary components that would be unable to be digested otherwise [1-4], the high density of these bacteria contribute to the physical barrier preventing pathogenic bacteria from penetrating through to the gut mucosa. Furthermore, these bacteria are critical for normal maturation of the immune system, and for optimal responses to many infectious agents [3].

1.2 Microflora acquisition and diversity

1.2.1 Gut microbial composition

The intestinal tract of the foetus is sterile, with colonisation occurring immediately after birth. The bacteria which colonise the gut initially differ depending on the route of delivery; vaginally delivered infants acquire a flora similar to the mother's vaginal flora, whereas those delivered by Caesarean

section acquire a flora dominated by bacterial communities found on the skin [5].

More than 90% of the human intestinal flora is from two phyla: the majority are Firmicutes (including the families Lactobacillaceae, Eubacteriaceae and Clostridiaceae), and the remainder from the phylum Bacteroidetes (including Bacteroidaceae) [6]. Less abundant, but also present, are bacteria from the phyla Actinobacteria (including Bifidobacteriaceae) and Proteobacteria (including Enterobacteriaceae) [6]. This broad pattern of phyla abundance is similar across all mammals, including mice [7].

1.2.2 Variation in microflora between individuals

Although the core composition of bacterial phyla within the gut is stable between individuals, individual variation in species abundance is high [8], and can be driven by differences in host genetics, diet, inflammation and stress [9, 10]. At least three 'enterotypes' of human microbiota have been described, based on similarities between individual species and the functional capacity of gut bacteria between people [11]. Enterotypes do not appear to be defined by geographical location, age, or body mass index and it remains elusive whether environmental or genetic factors play the more critical role in determining microflora composition [11].

1.3 Immune recognition of microflora

1.3.1 Toll-like receptors

Toll-like receptors (TLR) are a family of pattern recognition receptors (PRR) that recognise highly conserved microbial products (Table 1.3.1). TLR2 [12-14], TLR4 [12], TLR5 [15-17] and TLR9 [18] are all involved in gut bacterial recognition. Excessive stimulation through TLRs is in part restricted by their limited expression. During steady state conditions, intestinal epithelial cells express low levels of TLR2 and TLR4 [19]. However, in inflammatory settings

such as during inflammatory bowel disease (IBD), expression of TLR4 is markedly upregulated [19]. TLR4 signalling requires the presence of MD-2 on the cell surface in order to confer responsiveness to lipopolysaccharide (LPS) [20], thus limiting the expression of MD-2 also limits TLR4 overactivation [21]. Additionally, in the event of constitutive stimulation with TLR agonists, tolerisation to receptor signalling can be induced, which likely limits damage from persistent inflammation [22].

Table 1.3.1 The TLRs involved in bacterial recognition and their ligands.

Receptor	Dimerises with	Ligand (not exhaustive)	Adaptor proteins involved	References
TLR2	TLR1 TLR6	Triacylated lipopeptides Diacylated lipopeptides	MyD88, MAL	[23-26]
TLR4	TLR4	LPS	MyD88, TRIF, MAL,TRAM, SARM	[23, 24, 27-29]
TLR5	TLR5	Flagellin	MyD88	[23, 30, 31]
TLR9	TLR9	deoxy-cytidylate-phosphate-deoxy-guanylate (CpG) DNA	MyD88	[23, 32]

1.3.2 TLR adaptor proteins and downstream signalling

Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein through which all above-mentioned TLRs signal [33]. TLR4 is unique amongst the bacteria-recognising TLRs, because as well as signalling through MyD88, it can also signal independently of MyD88 through the adaptor protein Toll/IL-1 domain containing adaptor inducing IFN-β (TRIF) [28]. Macrophages isolated from $MyD88^{-/-}TRIF^{-/-}$ mice do not upregulate any genes in response to LPS exposure [34].

TLR stimulation generally results in proinflammatory cytokine and chemokine transcription via activation of the transcription factor NF-κB [35]. Studies

using MyD88 and TRIF single or double-deficient murine macrophages have revealed that following LPS stimulation, transcription of downstream genes can be redundantly controlled by the two adaptor proteins, but some genes require the presence of the individual adaptors for their transcription [36]. For example, MyD88 is critically required for tumour necrosis factor (TNF) and interleukin (IL)-1β transcription, TRIF is required for the transcription of interferon (IFN)-inducible genes, and the presence of both adaptors is required for the transcription of IL-1α and IL-6 [34].

Other adaptor proteins involved in TLR signalling are TRAM, MAL and SARM [23, 37]. SARM is thought to be a negative regulator of TRIF-dependent transcription factor activation [37].

1.3.3 NLRs

Nucleotide-binding oligomerisation domain (Nod)-like receptors (NLR) are cytosolic sensors of bacterial pathogen-associated molecular patterns (PAMP) (Table 1.3.3) and danger signals. The mammalian NLRs Nod1 and Nod2 recognise distinct motifs of bacterial peptidoglycan, and recognition leads to transcription of the proinflammatory cytokines IL-1β and TNF via NF-κB activation [38].

Inflammasomes are cytosolic scaffolds of proteins that combine to cause caspase-1 activation, in response to bacterial or danger signals. Several inflammasome types have been described, which are named after the PRR they are dependent on, and they include NLRC4, NLRP1 and NLRP3 [36]. Caspase-1 activation causes cleavage of the proinflammatory cytokines pro-IL-1β, pro-IL-18 and pro-IL-33 to their active forms [39-41].

NLRs provide a further precaution against overactive TLR stimulation, as the proinflammatory cytokines induced by TLRs are not cleaved without the action of caspase-1 activity, mediated by the NLRs. The cytosolic location of NLRs results in stimulation only when the epithelial barrier has been breached, and an inflammatory response is necessary.

Table 1.3.3 The NLRs involved in bacterial recognition and their ligands.

Only NLRs for which a ligand has been identified are shown.

Receptor	Alternative names	Ligand (not exhaustive)	References
Nod1	CARD4, NLRC1, CLR7.1	γ-D-glutamyl-meso-diaminopimelic acid	[42]
Nod2	CARD15, CD, BLAU, IBD1, PSORAS1, CLR16.3	Muramyl dipeptide	[43, 44]
NLRC4	IPAF, CLAN, CARD12	Flagellin	[45, 46]
NLRP1	NALP1, NAC, CARD7	Muramyl dipeptide *Bacillus anthracis* lethal toxin	[47, 48]
NLRP3	NALP3, cryopyrin, CIAS1	Various microbial stimuli including: Muramyl dipeptide Bacterial RNA LPS	[36]

1.3.4 C-type lectin receptors

C-type lectin receptors (CLR) are transmembrane receptors expressed on epithelial cells, T cells, B cells, natural killer (NK) cells and antigen-presenting cells (APC). CLRs recognise carbohydrate structures (primarily mannose, fucose and glucan) on pathogens including bacteria, as well as on fungi, viruses and parasites [49].

CLRs recognising carbohydrates present on bacterial surfaces include DC-SIGN, MGL and the mannose receptor [50]. Following CLR ligand engagement, the receptor-carbohydrate complex is rapidly internalised by DCs for processing prior to presentation to T cells [51]. Signalling through CLRs can induce NF-κB expression leading to the production of proinflammatory cytokines independently of other PRRs [52]. Alternatively, CLR signalling can enhance and prolong transcription of proinflammatory cytokines induced by stimulation through other PRRs, by phosphorylating a subunit of NF-κB which enhances its DNA binding affinity [52].

1.4 Control of microflora

1.4.1 Compartmentalisation

The major defence against inappropriate immune responses to intestinal bacteria is segregation: the majority of the microbiota do not come into contact with gut epithelial cells. Between the intestinal epithelial cells themselves are tight junctions, through which permeability can be controlled [53]. The epithelial monolayer is comprised of absorptive enterocytes, hormone-producing enteroendocrine cells, antimicrobial factor-producing Paneth cells and goblet cells which secrete mucus.

The intestinal mucus layer, consisting of glycosylated proteins called mucins, provides the major barrier to bacteria. The structure of the mucus layers starkly differs between the small and large intestine, presumably to deal with the increased bacterial load in the large intestine. In the colon, mucus is in two major layers- the inner layer, adjacent to the epithelial cells, is impermeable to bacteria, and ranges from 50-100 μm across in mice [54]. The outer layer of mucus is able to be penetrated by bacteria, and it is in this layer alone that the intestinal microbiota are found under normal conditions [55]. In contrast, in the small intestine, the inner impermeable layer of mucus is not present; instead a layer exists which is similar to the consistency of the outer layer of the colon, which is penetrable by bacteria [54]. An additional 1 μm thick matrix of densely packed mucins, termed the glycocalyx, is thought to cover the apical surface of the gut epithelial cells in both the small and large intestine [54].

Muc2 is a major component of mucus in both the small and large intestine, and unsurprisingly, $Muc2^{-/-}$ mice are more easily colonised by pathogenic bacteria, and exhibit more severe pathology following dextran sulfate sodium (DSS; a sulphated polysaccharide which causes loss of epithelial barrier function in the colon [56]) treatment than wildtype (Wt) mice [57].

1.4.2 Innate defences

Paneth cells, localised predominantly in the small intestine, are key producers of antimicrobial factors within the gut. Under normal conditions Paneth cells constitutively express the enzymes lysozyme and secretory phospholipase A_2 ($sPLA_2$) [58]. Lysozyme kills bacteria by hydrolising glycosidic linkages between components of peptidoglycan, an essential component of the cell wall, and is more effective against Gram-positive bacteria, where the peptidoglycan is more exposed (as Gram-negative bacteria have an additional outer layer, providing some protection to the peptidoglycan layer) [58]. $sPLA_2$ kills by hydrolysing phospholipids in bacterial cell membranes, which it gains access to as its highly positive charge allows it to penetrate through bacterial cell walls [59].

Another class of antimicrobial peptides are the defensins; small (2 to 6 kDa) proteins which can be produced by Paneth cells (these proteins are termed cryptidins when derived from Paneth cells), neutrophils and macrophages [58]. Their positive charge allows them to form electrostatic bonds with negatively charged phospholipids in the bacterial membrane, in which they form pores [58]. A subtype of defensin, the α-defensins, are produced in the mouse in an inactive form, and must be activated by cleavage mediated by the enzyme matrix metalloproteinase 7 (MMP7; also known as matrilysin) [60]. Mice lacking MMP7 therefore lack mature α-defensins, and the small intestine of these mice harbours a higher density of Firmicutes, and a lower ratio of Bacteroidetes species than MMP7-sufficient mice, illustrating the role for defensins in shaping the normal intestinal flora [61].

Mouse Paneth cells also produce the antimicrobial protein angiogenin4, which is bactericidal to both Gram-positive and Gram-negative bacteria [62], though its mechanism of action remains unclear. A further class of the antimicrobial family are the cathelicidins, which are produced by neutrophils, macrophages and colonic epithelial cells, and kill bacteria by similar mechanisms to the defensins [58].

Finally, C-type lectins are a group of antimicrobial peptides that include RegIIIγ, which is expressed by Paneth cells and γδ T cells [63, 64]. RegIIIγ targets Gram-positive, and not Gram-negative bacteria, which is again thought to be as the peptidoglycan that it targets is more accessible in these bacteria [65]. *RegIIIγ* mRNA is expressed increasingly along the cephalocaudal axis of the small intestine, likely due to the increasing density of bacterial colonisation [65]. The presence of bacteria is necessary for stimulation of RegIIIγ production, as germ-free (GF) mice, which completely lack intestinal bacteria, show minimal expression of *RegIIIγ* mRNA [65], and mice lacking MyD88 on epithelial cells show greatly reduced expression of *RegIIIγ* mRNA compared to Wt mice [66]. In the absence of MyD88 on epithelial cells, or in RegIIIγ$^{-/-}$ mice, bacterial localisation is dysregulated, and bacteria are able to come into contact with the intestinal epithelial layer [66]. This results in adaptive immune response inflammation, characterised by an increase in immunoglobulin (Ig)A^{+} cells and IFN-γ production by small intestinal lamina propria (LP) CD4^{+} T cells [66].

1.4.3 Adaptive defences

Adaptive immune system responses to control the intestinal microflora first require the uptake and processing of bacterial antigens by APCs. Bacteria can survive in low numbers following uptake by dendritic cells (DCs), and this persistence may aid the immune response in inducing IgA production when they are transported to the gut-draining mesenteric lymph nodes (MLN) [67]. DC cells are able to open the tight junctions between epithelial cells, and may sample the luminal bacterial flora by extending their dendrites through the gaps they create [68]. Whether this mechanism plays a role during steady state remains unclear, as under normal conditions the microflora would not be in a close enough proximity to epithelial cells for DC sampling to occur in this way.

Almost 80% of Ig-secreting cells are located in the LP, and the vast majority of these cells are IgA producers [69]. IgA production controls the intestinal

microflora by two complementary strategies: by antigen-specific, and non-antigen specific mechanisms [70].

IgA specific for intestinal microflora antigens can be produced in the gut in a T cell-independent manner [71, 72], which may reflect an evolutionarily primitive form of defence against inhabiting bacteria, through neutralising bacterial toxins or preventing adherence to the epithelial monolayer [70]. Many bacteria bind epithelial cells via their Type 1 fimbrae, and non antigen-specific IgA can competitively inhibit this binding via the presence of glycans on IgA proteins, which agglutinate bacteria [70].

In the absence of gut IgA production, intestinal microflora populations are disrupted, with dramatic increases in the abundance of anaerobic bacteria, particularly segmented filamentous bacteria (SFB) in the small intestines of IgA-deficient mice [73, 74].

Antigen specific $CD4^+$ T cell responses are made to the intestinal microbiota in situations when the epithelial barrier is breached, as was recently shown to be the case during a *Toxoplasma gondii* infection [75]. In this study, $CD4^+$ cells from mice which expressed a transgenic T cell receptor (TCR) specific to a flagellin from a subset of *Clostridium* bacteria (CBir1 Tg mice) were shown to produce IFN-γ *ex vivo* in response to PMA/Ionomycin stimulation, only after infection with *T. gondii* [75].

1.4.4 Genetic control of the microflora

Genetic deficiencies can lead to an abnormal composition of intestinal bacteria populations, which can not be restored to a Wt composition even following co-housing with Wt mice.

MyD88 expression is essential for RegIIIγ and RegIIIβ expression by colonic epithelial cells, while it has also been found that the composition of the microflora significantly differs in $MyD88^{-/-}$ mice from that of Wt mice in the duodenum, jejunum and ileum [76]. It is possible, however, that some of the

differences between Wt and MyD88$^{-/-}$ microflora composition can be attributed to separate housing of the genotypes, as no differences were seen between Wt and MyD88 flora in the lumen of the terminal ileum after mice were co-housed [66].

ob/ob mice have a deficiency in the *leptin* gene, and become obese as a result of excessive food consumption. These mice show elevated Firmicutes and reduced Bacteroidetes species in their caeca compared to *ob/+* littermates [77]. Notably, transplanting faeces from *ob/ob* mice to *ob/+* recipient mice results in hyperphagia of recipient mice resulting in obesity, indicating that the altered microflora causes, rather than is an effect of, the obesity phenotype [77]. Interestingly, TLR5$^{-/-}$ mice exhibit a similar phenotype, which is also transferrable to Wt mice following co-housing, but they do not show the same deviation in Firmicutes:Bacteroidetes ratio as *ob/ob* mice [78]. TLR5$^{-/-}$ mice do exhibit significant species-level differences within both the Firmicutes and Bacteroides phyla, suggesting that the functional capacity of individual species dictates the metabolic phenotype of mice [78].

The role of the adaptive immune system in controlling normal immune homeostasis is also highlighted in mice deficient in the enzyme required for Ig class switching, activation-induced cytidine deaminase (AID), as these mice exhibit 100-fold higher levels of anaerobic bacteria in their small intestine, which could not simply be attributed to a lack of IgA [74]. Mice of the same background which express different major histocompatibility complex (MHC) haplotypes harbour differing faecal bacteria populations, suggesting that the immune detection and response to intestinal bacteria is at least in part antigen specific [79].

1.5 Immune development and the microflora

1.5.1 GF mice

The majority of laboratory mice are housed in specific pathogen free (SPF) conditions, whereby the presence of known pathogenic agents including bacterial pathogens is regularly tested for and eliminated when detected.

It is also possible to breed mice which are entirely devoid of bacteria: germ-free (GF) mice. These mice are delivered by sterile Caesarean section and housed in sterile isolators. Additionally, it is possible to rear mice with an entirely defined intestinal microbe composition, when previously GF mice are gavaged with a known mix or single bacterial species; these mice are termed gnotobiotic mice.

GF mice differ from SPF mice in several ways, both physiologically and immunologically. In the absence of an intestinal flora, many mammalian dietary components are unable to be metabolised, the morphology and pH of the intestinal tract is altered, and immune development is perturbed [3]. Specifically, GF mice develop less secondary lymphoid tissue, have fewer germinal centres, and produce less intestinal IgA [3, 80]. As a consequence, GF mice are generally more susceptible to infections with bacterial and viral pathogens [3] and they respond less well to administration of systemic antigen in vaccination models [81].

The gut T cell response is also dysregulated in the absence of microbiota. GF mice have a higher frequency of IL-17A producing cells in the colon than SPF mice, which has been shown to result from a lack of microbially-stimulated IL-25 from intestinal epithelial cells, which limits IL-17A production [82].

When previously GF mice are recolonised with the altered Schaedler flora [83] (ASF; a defined mix of 8 intestinal bacterial species), the production of IL-10 from $CD4^+$ T cells in the colonic LP is elevated [84]. This appears to contribute to the normal homeostasis of the gut, as if this IL-10 signalling is blocked using an IL-10R blocking antibody, T cells instead deviate towards production of T helper (Th)1 or Th17 cytokines [84].

A reversible colonisation model of GF mice has been developed, where the strain of *Escherichia coli* administered requires nutrients not available within the mammalian host, thus bacteria persist for no longer than 72 hours following introduction [85]. *E. coli*-specific IgA responses are induced in these mice, which persist for several weeks following bacterial exposure, highlighting the importance of bacterial colonisation in inducing protective immune responses [85].

1.5.2 Microflora influencing T cell differentiation

As well as stimulating immune development in general, recent reports have highlighted that certain species of intestinal microbes are necessary for the differentiation of specific T effector and regulatory cells.

The observation that Wt C57BL/6 mice purchased from different commercial vendors have different numbers of Th17 cells in their small intestinal LP lead to the hypothesis that specific microbes are driving Th17 development [86]. Indeed, co-housing mice from different vendors equalised Th17 cell numbers between mice [86]. Further work has demonstrated that the presence of the single bacterial species SFB, which is able to penetrate the mucus barrier and directly contact the intestinal epithelial cells, is sufficient for increased Th17 cell differentiation in the small intestine LP of GF mice and SPF mice [87, 88].

$MyD88^{-/-}TRIF^{-/-}$ mice, which lack all TLR signalling, do not have any fewer IL-17-producing $CD4^{+}$ LP cells than Wt mice, indicating that the induction of Th17 cells by bacteria is TLR independent [89]. Bacteria may promote the generation of Th17 cells through the generation of adenosine 5'-triphosphate (ATP); SPF mice have higher levels of ATP in their faeces than GF or antibiotic treated animals, and administering ATP to mice systemically or rectally results in increased LP Th17 cell numbers [89]. It is not yet clear why SFB in particular drive Th17 responses, though it is possible ATP generated by these bacteria has a greater effect on T cell differentiation due to the close proximity of SFB to the intestinal epithelial cells.

Th1 differentiation can also be modulated by a bacterial species. GF mice have reduced IFN-γ-producing $CD4^+$ splenocytes compared to SPF mice, and levels of these cells are restored when GF mice are monocolonised with *Bacteroides fragilis* [90]. Expression of the *B. fragilis* capsular polysaccharide A (PSA) is critical for the promotion of these cells, as GF mice colonised by PSA-null *B. fragilis* do not show elevated IFN-γ $CD4^+$ levels [90]. Whether this is a property solely of *B. fragilis*, or if it occurs after colonisation with any bacterial species remains to be determined.

Regulatory T cell (Treg) levels specifically in the colonic LP can be elevated by colonisation of GF mice with *Clostridium* species, and this Treg induction is MyD88 and Rip2 (an adaptor molecule for NOD receptors) independent [91]. *B. fragilis* monocolonisation can also promote IL-10 production from Tregs in the LP of previously GF mice, whereas colonisation with PSA-null *B. fragilis* lacks this effect [92]. Many of the colonic LP Tregs found in unmanipulated SPF mice appear to be specific for bacterial antigens [93], suggesting that in the steady state LP Tregs develop to promote tolerance towards the intestinal microflora.

Thus, the differentiation of T cell subsets can be modulated by specific bacteria, in a site specific fashion. Further work is necessary to determine the antigen specificity of the generated T cells, and to resolve the mechanisms by which different bacteria alter T cell subset ratios.

1.6 Microflora influencing immune diseases and response to infectious pathogens

Given that bacterial species can influence T cell differentiation, it is not surprising that recent studies have revealed that altering the microflora composition is able to influence susceptibility to immune diseases and infections.

1.6.1 Microflora and allergy

GF mice exhibit exaggerated airway pathology following OVA-challenge when compared to SPF mice, due to increased local Th2 cytokine and IgE production [94]. As well as heightened airway inflammation in the absence of intestinal bacteria, modifying the composition of the intestinal bacteria can modulate pulmonary inflammatory responses. Treatment with the antibiotic Vancomycin reduces the abundance of Bacteroidales and Clostridiales species in murine faecal pellets, and this correlates with a reduced proportion of $CD4^+CD25^+Foxp3^+$ cells in the colon compared to untreated mice [95]. Interestingly, Vancomycin-treated mice exhibit greater lung pathology, increased airway hyperresponsiveness and elevated eosinophilia in an OVA-induced model of asthma compared to untreated control mice [95].

Mice that have been tolerised to OVA exhibit a weaker IgG response to OVA compared with untolerised mice, however, following treatment with the antibiotic Erythromycin, this tolerance is lost [96]. In these experiments Erythromycin reduced the abundance of *Lactobacillus* populations in the faeces of mice, yet whether other bacterial populations were additionally altered, and the mechanism by which disease is modulated remains unclear [96].

1.6.2 Microflora and autoimmune diseases

Inflammatory bowel disease

IBD is characterised by an inappropriate inflammatory response of the gut to microbial antigens. There are two main forms of the disease: Crohn's disease (CD), which can affect the entire length of the gut, and ulcerative colitis (UC), which is localised only to the colon.

IL-10 appears important in the control of IBD, as $IL\text{-}10^{-/-}$ mice develop spontaneous colitis [97], and in humans IL-10 is a susceptibility locus for UC [98]. When IL-10 production is induced via *B. fragilis* administration, the

severity of colitis induced by intrarectal injection of trinitrobenzene sulfonic acid (TNBS) is reduced [99].

Signalling through TLRs appears to be required for intestinal homeostasis, as when colitis is induced in MyD88-deficient mice by DSS or *Helicobacter hepaticus*-administration, heightened morbidity and mortality is seen compared to Wt mice [12, 100, 101]. Under steady state conditions, intestinal microbiota stimulate the production of inflammatory and reparative mediators in Wt mice, including TNF-α, IL-6, and heat-shock proteins 25 and 72, and low levels of these mediators are seen in MyD88$^{-/-}$ mice at steady state [12]. Following DSS-damage, increased production of the inflammatory cytokines TNF-α and IL-6 is seen in Wt mice, yet no upregulation is seen in MyD88$^{-/-}$ mice, suggesting that the microbiota may contribute to intestinal protection by stimulating factors required to repair damage [12, 100]. Additionally, MyD88$^{-/-}$ have higher bacterial dissemination to the liver following DSS treatment than Wt mice, suggesting a lack of bacterial control in MyD88$^{-/-}$ mice is responsible for the increased pathology [101]. MyD88$^{-/-}$ mice are rescued from increased morbidity and mortality following DSS treatment when they are administered a broad-spectrum antibiotic cocktail [101].

The importance of NLRs in controlling intestinal homeostasis is highlighted as human polymorphisms of *NOD2* correspond with increased proinflammatory cytokine processing and incidence of IBD [102, 103]. In mice lacking components required for NLR signalling including NLRP6, caspase-1 and IL-18, there is an expansion of Bacteroidetes and the candidate phylum TM7 (to date unculturable bacteria, identified though 16S rRNA sequencing) species and an associated increased pathology in the DSS colitis model [104]. The altered microflora composition appears to be the cause rather than the consequence of the aggravated pathology, since Wt mice co-housed with the NLRP6 signalling-deficient mice acquired the latter's microbial flora populations and were also rendered more susceptible to DSS [104]. Furthermore, this effect seems specific to mice lacking NLRP6 signalling, as mice lacking other NLR family members including NLRP3, NLRC4, NLRP12

or NLRP10 did not confer increased susceptibility to DSS colitis to co-housed Wt mice [104].

The composition of the intestinal microbiota, as well as the total load of bacteria influences the severity of DSS-induced colitis. GF rats colonised with anaerobic bacteria show more severe pathology to those colonised with aerobic bacteria [105]. More specifically, when antibiotic treatment is given to mice to disrupt the composition of the microflora, there is a positive correlation between the presence of Bacteroidetes phylum members and resistance to *Salmonella typhimurium*-induced colitis [106]. Conversely, *Lactobacillus* species predisposed mice to worsened pathology in this colitis model [106].

The presence of *Bacteroides* species appears to have the opposite effect on pathology in a different model of colitis- mice which express a dominant negative form of the TGF-βRII in T cells crossed with IL-10-deficient mice develop spontaneous colitis, and when these mice are antibiotic-treated they do not show pathology [107]. When these mice are recolonised with *Bacteroides* species, colitis occurs, indicating that these species can drive disease in some models of IBD [107].

Colitis can also be caused following infection of mice with the murine bacterial pathogen *Citrobacter rodentium*. C3H/HeOuJ mice suffer 100% mortality as a result of colitis induced by *C. rodentium*, whereas C57BL/6 mice recover from the infection [108]. A role for the microbiota composition modulating susceptibility is clear in this model, as when C3H/HeOuJ mice are gavaged with faeces from C57BL/6 mice, *C. rodentium*-colitis no longer causes fatalities [108]. This protection is associated with an increase in Bacteroidetes species, and higher transcript levels of colonic IFN-γ, TNF-α and IL-22, which have been associated with *C. rodentium*-killing [108].

Thus, host genetics and the model of colitis being examined need to be considered, before conclusions can be drawn about the bacterial species which are protective or detrimental to disease onset.

Arthritis

K/BxN mice harbour transgenic TCRs which recognise a self-peptide derived from glucose-6-phosphate isomerase, resulting in almost 100% penetrance of arthritis beginning at approximately 4 weeks of age [109]. When K/BxN mice are housed in GF conditions, self-peptide antigen titres are reduced, as are the numbers of Th17 cells and the resulting arthritic disease phenotype [109]. Colonisation of GF K/BxN mice with SFB restores Th17 numbers to SPF-housed K/BxN mouse levels, and arthritic disease ensued within 3 days of colonisation [109]. Whether other bacterial species are able to modulate arthritic disease onset has not been investigated to date.

Type 1 diabetes

The presence and recognition of intestinal bacteria by the immune system also modulates the onset of Type 1 diabetes (T1D) [110]. Non-obese diabetic (NOD) mice spontaneously become diabetic (as measured by blood glucose levels of ≥ 200 mg/dl) by 25 weeks of age [111, 112]. MyD88$^{-/-}$NOD mice are completely protected from diabetes onset, however when MyD88$^{-/-}$NOD mice are treated with a broad-spectrum antibiotic cocktail, or rederived into GF conditions, the incidence of diabetes returns to that of MyD88-sufficient NOD mice [110]. This finding clearly demonstrates that it is not the lack of IL-1, IL-18, or MyD88-dependent TLR signalling that prevent diabetes onset in MyD88$^{-/-}$NOD mice, but a consequence of the microflora. Sequencing of the 16S rRNA gene in the caecum of MyD88-sufficient and -deficient NOD mice revealed that the MyD88-deficient NOD mice had higher levels of Lactobacillaceae species, as well as Rikenellaceae and Porphoromadaceae (both Bacteroidetes families) species [110]. The current hypothesis is that these bacterial species are able to promote tolerance towards an anti-islet T cell response in MyD88-sufficient mice [110], although the mechanism by which this is occurring remains unknown.

1.6.3 Microflora influencing infectious disease

The normal intestinal microflora compete for resources with some enteric pathogens, thus helping to prevent infection, yet paradoxically, other infecting agents appear to require the presence of the microflora for host colonisation.

Mouse mammary tumour virus (MMTV) induces unresponsiveness to viral antigens, and is unable to be transmitted in broad-spectrum antibiotic-treated or GF mice [113]. MMTV is able to bind bacterially-derived LPS, which triggers IL-10 production via TLR4 signalling [113]. Importantly, MMTV-bound LPS triggers more IL-10 production through the TLR4 signalling pathway than does MMTV or LPS alone [113]. This study suggests that MMTV exploits the presence of gut bacteria, to promote a regulatory response against its own antigens. Similarly, the susceptibility of mice to both poliovirus and reovirus is reduced following antibiotic-treatment, and poliovirus also binds LPS, suggesting that exploitation of the microflora is a common mechanism for survival of enteric viruses [114].

To add to the complexity of commensal-pathogen interactions, the presence of intestinal bacterial is required for early immune responsiveness to some viruses, including influenza, lymphocytic choriomeningitis virus (LCMV) and mouse cytomegalovirus (MCMV) [115, 116]. Both GF and broad-spectrum antibiotic-treated mice are more susceptible to these viral infections, which appears to be due to reduced NK cell cytotoxic activity, $CD8^+$ T cell expansion, antibody production and type 1 IFN production [115, 116]. Thus, presence of the bacterial microflora primes the immune response to respond to enteric viruses, yet some viruses can avoid immune attack via mechanisms dependent on the bacterial microflora.

Intestinal bacteria can also modulate the susceptibility to bacterial infections. Perturbing the microbiota using antibiotic treatment increases colonisation with *S. typhimurium* [106, 117], and susceptibility can also be modulated by administering single species of the normal intestinal bacteria to mice. For example, when mice are fed *Bifidobacterium infantis* prior to *S. typhimurium* infection, reduced pro-inflammatory cytokine secretion, T cell proliferation and

co-stimulatory molecule expression on DCs is seen, as well as a reduction in murine sickness and *S. typhimurium* numbers [118].

Resistance to *C. rodentium* varies between mouse strains, and susceptibility can be conferred by administering faeces from susceptible mice [108, 119]. Modifying the composition, but not altering total bacterial numbers using the antibiotic Metronidazole causes an increase in *Lactobacilli* species abundance and reduces populations of Porphyromonadaceae species in the colon, and an accompanying increase in *C. rodentium* colonisation is seen [120]. Increased colonisation of this bacterial pathogen could be explained by a thinning of the inner mucus layer in the colon following Metronidazole treatment, allowing for increased attachment of *C. rodentium* to the intestinal epithelium [120].

C. rodentium is less able to colonise SPF mice administered SFB than SFB-free SPF mice [87], and although the mechanism for this has not yet been determined, it is tempting to speculate that it is through the induction of antimicrobial peptides, promoted by Th17 cells which are induced by SFB.

1.7 *Heligmosomoides polygyrus*

Little is known about how parasitic infections are affected by the intestinal microflora. Parasitic helminth infections have co-evolved with the mammalian immune system in the presence of intestinal bacteria, and thus it is likely that mechanisms employed by intestinal helminths and bacteria to persist in the host share common pathways, and may be co-dependent.

1.7.1 Origin and taxonomy

H. polygyrus is an intestinal nematode parasite, common in wild mouse populations, and widely used as a laboratory model of helminth infections. This parasite is phylogenetically placed in the same suborder, Trichostrongylina, as the ruminant parasites *Haemonchus contortus* and

Teladorsagia circumcincta and within the same order, Strongylida, as the human hookworm parasites *Ancylostoma duodenale* and *Necator americanus* [121]. *H. polygyrus* is often used as a model of chronic helminthiases, as in susceptible strains of mice, a primary infection can last for many months. In contrast, *H. polygyrus* can also be used as a model of helminth expulsion, as the majority of mouse strains are resistant to a secondary infection, once a primary infection has been drug cured.

The strain of *H. polygyrus* used in laboratories worldwide is thought to have been isolated from wild Californian mice in the 1940s [122] and was known for some years as *Nematospiroides dubius*. The vast majority of the literature describing experiments with this isolate refer to the parasite as *H. polygyrus*. It was however suggested that this laboratory strain should be referred to as *H. polygyrus bakeri*, to differentiate it from wild strains of the parasite, considered to be *H. polygyrus polygyrus* (found in the wood mouse *Apodemus sylvaticus* in Europe), *H. polygyrus corsicus* (from the house mouse *Mus musculus* in Corsica) and *H. polygyrus americanus* (from the vole *Phenacomys intermedius* in North America) [123]. More recently, there has been an additional proposal of a name change for the laboratory isolate to *H. bakeri* [122], based on sequence divergence between laboratory and European wood mouse isolates [124]. This proposal has not received widespread support, due to the preliminary nature of the data, the sequence variation even within the laboratory strain, and the need to remain consistent with previous literature [125]. In this thesis, the laboratory strain of the parasite will be referred to as *H. polygyrus*.

1.7.2 Lifecycle

In an experimental setting, *H. polygyrus* is introduced by orally gavaging mice with infective third stage larvae (L3). Following ingestion, within 24 hours larvae have penetrated through into the submucosa of the small intestine. Here they undergo two developmental moults, before emerging back into the lumen as adult worms, which feed on host intestinal tissue [126]. The adult

worms coil around the small intestine villi to secure themselves, mate, and produce eggs, which are released in the faeces. In the external environment the eggs hatch and undergo two further moults to become infective L3s, and so the lifecycle continues (Figure 1.7.2). *H. polygyrus* secretes a variety of products, termed *H. polygyrus* excretory-secretory antigen (HES), both *in vivo*, and when adult worms are cultured *in vitro*, allowing for the collection and identification of components of the secreted material [127].

Immune responses to *H. polygyrus* have been studied after both primary and secondary infections. Different mechanisms of expulsion are likely employed in these two settings, as the components influencing expulsion between the setting often differ.

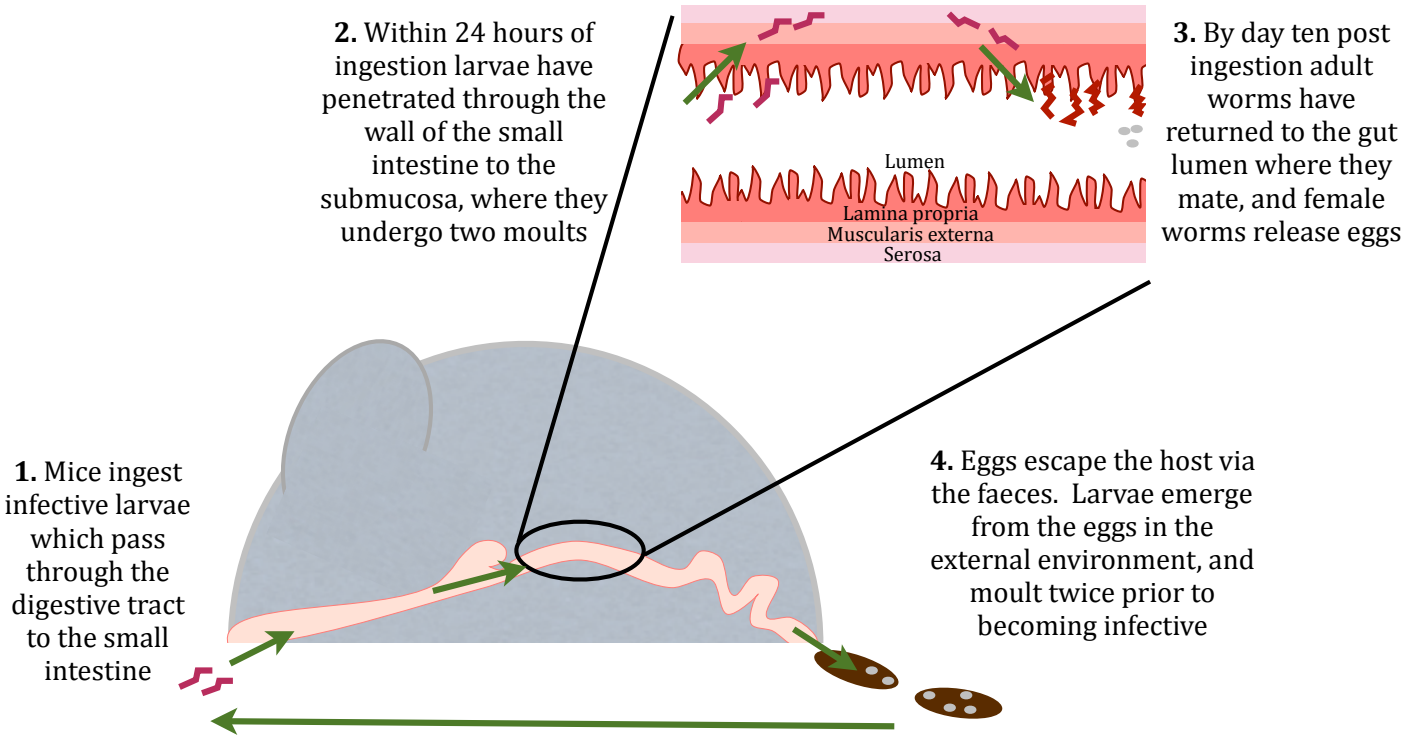

Figure 1.7.2 Lifecycle of *H. polygyrus*.

1.8 Adaptive immune response to *H. polygyrus*

The role of T and B cells during a *H. polygyrus* infection have been deduced by studying infections of severe combined immunodeficient (SCID), B cell-deficient and athymic mice.

1.8.1 T cells

Following a primary infection, both SCID and athymic mice show impaired expulsion of *H. polygyrus* compared to Wt controls [128, 129]. Furthermore, *H. polygyrus* is more fecund in mice administered α-CD4 antibody [130]. Transferring the effector T cell subset ($CD4^+CD25^-CD103^-$) from the MLNs of chronically *H. polygyrus*-infected mice to recipients prior to infection results in recipient mice harbouring lower worm burdens than those mice receiving PBS control injections, revealing the role of effector T cells in controlling *H. polygyrus* expulsion [131].

A primary infection with *H. polygyrus* results in elevation of IL-2, IL-3, IL-4, IL-5, IL-9, IL-10 and IFN-γ cytokines in the spleen, Peyer's Patches (PP) and MLN [132, 133]. *In vitro* restimulation of MLN cells with *H. polygyrus* antigen causes IL-4, IL-13, IL-10 and IFN-γ production [131, 133].

The single most important cytokine for promoting expulsion of *H. polygyrus* is IL-4 [129, 134], which is predominantly produced by $CD4^+$ T cells [132]. Using antibody treatment to block IL-4 partially abolishes protection from a secondary *H. polygyrus* infection, whereas blocking the IL-4 receptor (IL-4R) alongside IL-4 completely abolishes secondary protection [134]. This suggests that IL-13, which also signals through the IL-4R, can partially compensate for the loss of IL-4, but in the absence of signalling from both cytokines protection against re-infection with *H. polygyrus* is lost. When IL-4 is administered as a complex with α-IL-4 (IL-4C) to extend the activity time of this cytokine, Wt BALB/c mice expel *H. polygyrus* more rapidly than control mice [129]. This effect does not depend on the adaptive immune system, as

H. polygyrus expulsion is also seen in SCID mice and α-CD4 treated BALB/c mice given IL-4C [129].

T follicular helper (Tfh) cells promote proliferation, differentiation and isotype switching of B cells through the cytokine IL-21 [135]. Mice deficient in the IL-21αR do not produce the high IgG1 titres seen in Wt mice following a secondary infection with *H. polygyrus*, and are unable to clear adult worms from the gut [136]. During a primary *H. polygyrus* infection, the dominant source of MLN IL-4 is Tfh cells [137].

1.8.2 B cell and humoral responses

B cells appear important for controlling fecundity of *H. polygyrus* following a primary infection, as *H. polygyrus* egg production is higher in JHD mice (which lack mature B cells) than Wt controls, however a B cell deficiency does not impact on worm expulsion during a primary infection [138]. In contrast, B cells are absolutely required for worm expulsion following a secondary infection, as JHD mice, and B cell-deficient μMT mice are completely unable to expel challenge infections [138-140]. Immunity to a challenge *H. polygyrus* infection is restored in μMT mice following transfer of serum from immune mice, indicating a role for B cells in protective immunity [138, 139]. B cells are required for optimal T cell cytokine responses during secondary *H. polygyrus* infections, as memory T cells from MLNs of B-cell deficient mice produce less Th2 cytokines than those from B-cell sufficient mice [140].

A primary infection causes the production of polyclonal IgE and IgG1, with HES-specific antibodies not being produced until late time points (day 25 onwards) of infection [138], perhaps revealing why antibody-deficiency does not alter worm expulsion during a primary infection.

Selective antibody isotype deficient mice have been used to determine the key isotypes required for protection from a secondary *H. polygyrus* infection. IgG1 production appears to be the dominant isotype in conferring protection to challenge infections, as mice deficient in IgM or IgE production are still able to clear adult worms during secondary infections, and IgA-lacking mice are only

moderately impaired in their ability to expel worms [138, 140]. *H. polygyrus*-specific IgG1 is the only isotype which can reduce adult worm burdens following purification and administration to mice prior to primary infection [141].

1.9 Innate immune response to *H. polygyrus*

Innate immune cells are the initial responders to a *H. polygyrus* infection, and are also implicated in the end-stage expulsion of parasites. Innate cells release type 2 cytokines that can act directly to alter gut physiology and polarise the adaptive immune response, while themselves employing helminth-damaging or killing mechanisms [142, 143].

1.9.1 Dendritic cells

DCs are the predominant innate APC which are required to prime Th2 responses against helminths [144]. When $CD11c^+$ DCs are depleted using CD11c.DTR mice [145] that co-express CD11c with the human diphtheria toxin receptor (DTR), Th2 cytokine responses against HES are compromised [146].

1.9.2 Macrophages

The alternative activation of macrophages is a hallmark of helminth-elicited Th2 responses, and is associated with high expression of a characteristic set of gene products, including Ym1 (Chi3L3), RELM-α (FIZZ-1), arginase-1, IL-4Rα and the mannose receptor CD206 [142, 147]. Macrophages can differentially express the enzymes nitric oxide synthase 2 (NOS-2) and arginase-1, which compete for the common substrate L-arginine, and are competitively induced by IFN-γ and Th2 cytokines (IL-4, IL-10, IL-13 and IL-21) respectively [148-151].

Alternatively activated macrophages are critical to the protective immune response to secondary *H. polygyrus* infection, as mice lose the ability to reject challenge infections when depleted of macrophages via clodronate treatment, or when treated with a pharmacological inhibitor of arginase [152]. Arginase-1 activity may directly harm parasites, as *H. polygyrus* exhibits higher levels of cytochrome oxidase, a marker of a stress response, in a secondary infection compared to a primary infection, and this increase is lost following arginase inhibition [152]. In contrast to arginase, no anti-parasite function has been found for Ym1, a member of the chitinase-like family of proteins that lacks demonstrable chitinase activity [153]. Ym1 does bind heparin on cell surfaces and in the extracellular matrix [153], which may indicate a role for Ym1 and alternatively activated macrophages in mediating repair of tissue damage caused by *H. polygyrus* when migrating through the intestinal wall [154].

1.9.3 Granulocytes

Perhaps surprisingly, the role of the principal granulocyte cell types (neutrophils, eosinophils and basophils) has not been directly evaluated in *H. polygyrus* infection. Neutrophils are prominent in primary, and to a lesser extent, secondary granulomas during *H. polygyrus* infection [152, 155, 156]. The finding that neutrophils are less prevalent in a setting of heightened resistance may indicate that they are not a key cell type in immunity.

No role for eosinophils in *H. polygyrus* expulsion has yet been described. Eosinophilia in response to *H. polygyrus* is blocked when mice are administered α-IL-5 antibody [134] but this does not affect adult worm survival, and eosinophils within the gut wall have been reported to be inhibited during *H. polygyrus* infection in a manner reversible with α-TGF-β antibody treatment [157].

As with the other granulocytes, few studies have investigated the role of basophils in *H. polygyrus* infections. In other gastrointestinal nematode infections, basophilia is conspicuous, and their presence may be required for expulsion of the rat nematode *Nippostrongylus brasiliensis* [158]. Expulsion

of the colon-residing mouse nematode *Trichuris muris* is impaired when basophil numbers are depleted using MAR-1 antibody [159]; however this antibody targets the FcεRI which is also expressed by mast cells, so this does not conclusively prove a role for basophils alone.

1.9.4 Mast cells

Mast cells are major players in the intestinal immune response to infection with *H. polygyrus*, as expulsion correlates with epithelial mastocytosis [160, 161] and elevated intestinal fluid levels of mMCP-1 in different murine strains [162]. Mast cells may promote helminth damage by increasing the permeability of the gut via mMCP-1-mediated breakdown of epithelial tight junction proteins [163, 164], thereby increasing luminal flow and disrupting the niche of parasitic helminths.

The mast cell response to infections with *N. brasiliensis* or the nematode parasite *Trichinella spiralis* are ablated in mice carrying an *H. polygyrus* co-infection [165] arguing that *H. polygyrus* is able to suppress host mastocytosis to a significant degree.

Most *in vivo* studies on mast cells in helminth infection have involved the mast cell deficient mice $Kit^{W}/Kit^{W\text{-}v}$ which carry a mutated gene encoding the tyrosine kinase receptor c-kit. During *H. polygyrus* infections these mice produce higher egg numbers than Wt controls, indicative of impaired immunity [166]. Consistent with this, reduced egg production is seen in Tg2Rbeta mice [167], which exhibit mastocytosis.

In terms of protective immunity to adult worms, however, $Kit^{W}/Kit^{W\text{-}v}$ mice have been found to be similar to Wt animals in slowly expelling primary *H. polygyrus* infection between 4 and 9 weeks of infection [128]. However a more recent report has shown $Kit^{W}/Kit^{W\text{-}v}$ mice, and another mast cell deficient strain, $Kit^{W\text{-}sh}$ mice, do have impairments in *H. polygyrus* expulsion, as both strains had higher worm burdens than Wt mice after 3 weeks of a primary *H. polygyrus* infection [168]. The same authors also showed that $Kit^{W}/Kit^{W\text{-}v}$ mice

are not resistant to a secondary *H. polygyrus* infection [168]. The reason for the discrepancy between the reports on the ability of Kit^{W}/Kit^{W-v} mice to expel *H. polygyrus* is not clear, and more studies are required to confirm the importance of mast cells during infections. If mast cells do contribute to expulsion of *H. polygyrus*, it could be via their contributions towards priming a Th2 response early in infection, as well as their potential role as a later effector cell. Kit^{W}/Kit^{W-v} MLN cells do not show the high levels of *H. polygyrus*-antigen specific Th2 cytokines produced by Wt MLN cells in response to *H. polygyrus* [168].

It should be noted that both Kit^{W}/Kit^{W-v} and Kit^{W-sh} mice have defects that extend beyond a mast cell deficiency [169]. Many of the recently described subsets of lineage negative innate type 2 cells, discussed below, express c-kit, and so it is likely that some of the deficiencies of Kit^{W}/Kit^{W-v} and Kit^{W-sh} mice can be explained by the additional disruption of these cell types.

1.9.5 Innate lymphoid cells

Recently a number of studies have identified a population of lineage marker negative innate lymphoid cells (ILC), which produce type 2 cytokines (particularly IL-5 and IL-13) in response to epithelial cell-derived cytokines, including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (reviewed in [170]). IL-25 may also be derived from other cell types, such as mast cells [171], but the importance of IL-25 from this source is as yet unknown. Epithelial cells produce elevated levels of these cytokines in response to damage, thereby raising the first alarm leading to Th2 responses (reviewed in [172]). Trefoil factor 2 (TFF2) is a molecule involved in epithelial cell repair, which induces IL-33 production by epithelial cells in response to damage caused by *N. brasiliensis* [173]. $TFF2^{-/-}$ mice do not show the elevated epithelial IL-33 levels in response to *N. brasiliensis* seen in Wt mice, instead having lower serum IL-4 levels after 7 days of infection, and delayed worm expulsion [173]. Similarly, Th2 cytokine production is delayed and *N. brasiliensis* expulsion is impaired in IL-$25^{-/-}$ mice, which correlates with the

absence of a non-B non-T cell c-kit^{+} IL-4, IL-5, IL-13 producing population induced in infected Wt mice or mice administered rIL-25 or rIL-33 [174-176]. A role for ILCs has not yet been reported during a *H. polygyrus* infection, though it seems likely that these cell types are important inducers of Th2 responses during all intestinal helminth infections.

1.10 Gut physiology and intestinal epithelial function in response to *H. polygyrus*

IL-4 and IL-13, derived from innate or adaptive sources, are likely to have direct effects on the physiology of the gut as well as on effector cells that promote helminth expulsion. Although few changes in epithelial cell function are noted during primary *H. polygyrus* infection, in secondary infections increased mucosal permeability, decreased ion absorption, and increased pro-secretory effects in response to prostaglandin E2 (PGE_2) and histamine are seen [177, 178]. Moreover, these changes are dependent on the IL-4R and STAT6 and are reproduced by administration of IL-4C [177, 178]. These alterations to the worm's environment may interfere with its abilities to feed on the intestinal tissue [126] or remain wrapped around the villi in the small intestine.

1.10.1 Goblet cells

Goblet cell hyperplasia develops in response to intestinal helminth infections, including *H. polygyrus*, where hyperplasia is dependent on a functional T cell response [128]. Enhanced mucus production by goblet cells has been suggested to act against helminth establishment, and it may be that specific components within the mucus play a role in control of helminth expulsion.

RELM-β (FIZZ-2) is a cysteine-rich mediator expressed by goblet cells in response to IL-13, and is important for the normal control of epithelial cell barrier permeability [179, 180]. RELM-$\beta^{-/-}$ mice do not expel a secondary *H.*

polygyrus infection as rapidly as Wt mice, and adult *H. polygyrus* worms treated *in vitro* with rRELM-β prior to transfer to a new host survive less well than untreated adult worms [181]. This suggests RELM-β is an important factor in inhibiting worm survival, perhaps by interfering with worm chemotaxis and nutrition [179, 181]. Secretion of Muc2 is also upregulated during a *H. polygyrus* infection [128]. No evidence for a role for Muc2 in expulsion of *H. polygyrus* has yet been reported, however Muc2 production correlates with the expulsion of *T. muris* [182, 183] and *N. brasiliensis* [184].

1.10.2 Smooth muscle contraction

Both IL-4 and IL-13 enhance smooth muscle contractility in the small intestine [185], a mechanism which has been shown to be important for resistance to other helminth infections including *Schistosoma mansoni* [186], *T. spiralis* [187, 188] and *N. brasiliensis* [189]. Increased intestinal smooth muscle contractility has been shown after infection with *H. polygyrus* [190]. Both *N. brasiliensis* and *H. polygyrus* infections cause an upregulation of protease-activated receptor $(PAR)_2$ mRNA in the small intestine, and a PAR_2 agonist causes smooth muscle contractility, which is enhanced in both parasite-infected groups and, for *N. brasiliensis* at least, dependent on STAT6 [190]. The infection-induced hypercontractility in the presence of PAR_2 agonist is lost when nerve conduction is blocked using the neurotoxin TXX [190]. Whether smooth muscle hypercontractility plays a critical role in *H. polygyrus* expulsion has yet to be determined.

1.10.3 Granuloma formation

A striking phenomenon during *H. polygyrus* infection is the formation of granulomas around the site of larval invasion in the intestinal tract, which are more numerous in resistant strains of mice [191], particularly following secondary infections. While granuloma formation is Th2 dependent, their function has yet to be determined, either in damaging larval worms encysted

in the submucosal layer of the small intestine, or in tissue repair after *H. polygyrus* has departed into the lumen of the gut [192]. Granulomas in both primary and secondary infection consist of neutrophils, macrophages, dendritic cells, and eosinophils; in secondary infection $CD4^+$ Th2 cells and a high proportion of alternatively activated macrophages rapidly migrate to the site of infection to surround the larvae [152, 155, 156].

1.11 Regulatory cells during *H. polygyrus* infection

Many cell types are able to regulate excessive immune responses, including regulatory B cells (Bregs), proregulatory DCs, and most notably Tregs [146, 193-195]. Following *H. polygyrus* infection, there is a dramatic increase in the numbers of $CD4^+CD25^+Foxp3^+$ T cells in the MLN [131, 133, 196].

Expansion of these cells is likely to be advantageous to *H. polygyrus*, as the parasite is able to drive expression of Foxp3 in naïve T cells though the secretion of a TGF-β-like molecule in HES [197]. When a pharmacological inhibitor of TGF-β is administered to mice, adult worm burden is reduced, suggesting that the induction of Tregs by *H. polygyrus* is critical for worm persistence [197].

Treg induction during infection also limits the pathology caused by helminth presence, and the ensuing immune response. When Tregs are depleted early during *H. polygyrus* infection, MLN cells produce more IL-4 and IL-13, and gut pathology scores are significantly worse than Treg-competent animals, with more epithelial cell atrophy, crypt hyperplasia and cellular infiltrates in the lamina propria [196]. The expansion of Tregs during infection is thus also beneficial to *H. polygyrus* as they limit pathology so ensuring the survival of the host, increasing the likelihood that *H. polygyrus* will be transmit to a new host [198].

$CD8^+$ T cells also have a regulatory function during *H. polygyrus* infection, as $CD8^+$ T cells purified from the LP of infected mice are able to suppress proliferation of splenic cells [199].

1.12 *H. polygyrus*, autoimmunity and allergy

The immunomodulatory properties of *H. polygyrus* extend far beyond the site of infection alone. This has led to many investigations of the potential for, and mechanisms of, parasite downregulation of allergic and autoimmune conditions, as discussed below, as well as in the modulation of co-infections with other pathogens (reviewed in [200]).

1.12.1 *H. polygyrus* and allergy

H. polygyrus offers protection in several murine models of allergy, including intestinal, airway and cutaneous reactions. Mice fed peanut extract administered alongside the mucosal adjuvant cholera toxin produce peanut specific IgE, have elevated plasma histamine levels and exhibit systemic anaphylactic shock symptoms [201]. All phenotypes are diminished in *H. polygyrus*-infected mice [201]. In the presence of *H. polygyrus*, the peanut antigen-specific IL-13 levels are drastically reduced, and these dampened IL-13 levels, along with protection from peanut allergy, are lost when mice are treated with neutralising IL-10 antibody [201]. The source of IL-10, and the mechanism by which it acts to dampen allergic responses to peanut antigen during *H. polygyrus* infection, have not yet been determined.

H. polygyrus-infected mice have reduced inflammatory cell infiltrates and bronchoalveolar lavage (BAL) eosinophilia in experimentally induced airway allergy to both ovalbumin [202-204] and the house dust mite antigen Der p 1 [203]. Protection against these allergens can be transferred by MLN cells from infected mice, which contain a high proportion of $CD4^+CD25^+Foxp3^+$ T cells, or by transfer of sorted $CD4^+CD25^+$ cells from infected mice, implicating the action of Treg cells in protection [203]. *H. polygyrus*-infected IL-$10^{-/-}$ mice are not protected from ovalbumin-induced asthma [202], however MLN cells transferred from IL-$10^{-/-}$ *H. polygyrus* infected mice can still protect from allergy to these antigens, suggesting that IL-10 independent mechanisms can confer protection from allergy [203].

1.12.2 *H. polygyrus* and inflammatory bowel disease

There are many differing mouse models of IBD (reviewed in [205]), and the effect of *H. polygyrus* infection on controlling the disease has been examined in a number of these models.

IL-10$^{-/-}$ mice suffer from spontaneous chronic colitis [97] associated with excessive IFN-γ production [206]. Spontaneous colitis develops sporadically over several months, but piroxicam-treatment will induce rapid and uniform disease in IL-10-deficient mice [207, 208], which likely occurs as a result of increased colonic epithelial cell apoptosis causing a loss of barrier function to inflammatory microbial stimuli [208]. When *H. polygyrus* is given to piroxicam-treated IL-10$^{-/-}$ mice, the histological scores of colitis severity are drastically reduced within 14 days [209, 210]. LP mononuclear cells from uninfected colitic mice release the inflammatory cytokines IFN-γ, IL-12p40, and IL-17A, whereas cells from *H. polygyrus*-infected mice have significantly reduced levels of these cytokines [209, 210].

Severe colitis also develops when recombination activating gene (RAG)-deficient mice are reconstituted with IL-10$^{-/-}$ T cells and treated with piroxicam [211]. *H. polygyrus* colonisation reduces gut inflammation in this model, as shown by lower levels of IFN-γ and IL-17 production by restimulated LP mononuclear cells, and a drop in colonic histological score from an average of above 3 (some epithelial and muscle hypertrophy, mucus depletion, crypt abscesses and epithelial erosions) to less than 1 (some mononuclear cell infiltrates in the LP) [199, 212]. In RAG$^{-/-}$ mice that have been infected with *H. polygyrus*, and subsequently drug-cleared of the infection prior to transfer of the colitogenic IL-10$^{-/-}$ T cells and piroxicam administration, mice still show reduced levels of inflammation compared to those that had never been infected [212]. The authors reported that protection coincided with down-regulation of the co-stimulatory molecules CD80 and CD86 on DCs, thus inhibiting antigen presentation to T cells resulting in less inflammatory cytokine release [212].

When mice that had been infected with *H. polygyrus* for 10 days are given TNBS injection, they exhibit markedly reduced TNBS-induced colonic damage and inflammation, and decreased Th1 cytokine mRNA expression compared to uninfected control mice, which is accompanied by increased IL-10 secretion during *H. polygyrus* infection [213, 214].

In contrast to other models of colitis, *H. polygyrus* seems to intensify colitis caused by the *C. rodentium* [215-217]. Disease exacerbation could be due to the influx of alternatively activated macrophages during *H. polygyrus* infection, which are less able to kill bacteria than classically activated macrophages [215], or due to increased IL-10 production by DCs impairing mechanisms that kill *C. rodentium*, leading to more persistent infection and colitis [216]. These studies exemplify the need to understand the causes of colitis, and the mechanisms by which helminths modulate disease progression, before helminth therapy can be applied to human IBDs.

1.12.3 *H. polygyrus* and T1D

When NOD mice are infected with *H. polygyrus* at 5 weeks old, the onset of diabetes is completely blocked, at least until 40 weeks of age [111, 112]. Administering *H. polygyrus* when NOD mice are 7 and 12 weeks of age results in less effective protection from diabetes, yet onset is still delayed compared to untreated NOD mice [111]. The severity of insulitis (the infiltration of immune cells into the islets of Langerhans) was examined in mice aged 13 weeks, and is sharply reduced in NOD mice infected with *H. polygyrus* since the age of 5 weeks [111]. This reduction is maintained in *H. polygyrus* mice given α-CD25 antibody [111], suggesting that *H. polygyrus* modulates T1D onset in a Treg-independent manner, although whether this protection extends beyond the 13 week time point has not been examined.

1.13 *H. polygyrus* and the microflora

1.13.1 Are the immunomodulatory effects of *H. polygyrus* due to alterations in the microflora?

There is the possibility that the modulatory effects of *H. polygyrus* are due in part to changes in gut microbial composition during infection [218]. After a 14 day infection with *H. polygyrus* in C57BL/6 mice, the abundance of Lactobacillaceae family members was increased in the ileum compared to naïve mice [218]. Given that species within the Lactobacillaceae family have been reported to promote Treg differentiation [219-222], it is possible that this is the mechanism by which *H. polygyrus* exerts its immunomodulatory effects.

It has yet to be demonstrated whether this shift is a helminth-mediated mechanism that acts to promote the survival of *H. polygyrus* within the murine host, or if it is simply as a consequence of a changing immune environment, in which bacteria of the Lactobacillaceae family are better able to survive.

H. polygyrus may be actively secreting proteins which alter the intestinal microflora. HES contains putative antimicrobial lysozymes [127], yet whether these are functionally active against bacteria, and whether they are selective in their action, has yet to be tested.

1.13.2 Gut microflora in control of *H. polygyrus* survival?

Equally, composition or presence of microflora might influence the establishment and survival of a *H. polygyrus* infection.

Although GF mice are generally more susceptible to infections than SPF mice [3], *H. polygyrus* is more rapidly expelled in GF mice than in SPF mice [223-225], and female *H. polygyrus* worms harbour less eggs when in GF hosts [223], suggesting the intestinal microflora are required for optimal fecundity and establishment in the intestine. GF mice produce more granulomas along the intestinal tract in response to *H. polygyrus*-infection than SPF mice [223],

which could be a mechanism by which worms are damaged and thus more readily expelled.

The intestinal microbiota is exploited by *T. muris*, as presence of bacteria is critical for *T. muris* eggs to hatch when they reach the caecum of mice [226]. *H. polygyrus* eggs do not hatch in the caecum, but in faeces in the external environment. It has been shown that eggs do hatch even in the faeces of GF mice cultured in sterile conditions, however they do not mature to infective L3s [225]. If eggs from GF faeces are separated from the faeces, they develop normally, and if faeces from SPF mice are added to the mixture, eggs also develop normally, suggesting that the microflora are necessary to block an inhibitor of development found in faeces [225].

1.14 Aims

Broadly, the work in this thesis explores the immune mechanisms that are required for the expulsion of *H. polygyrus*, and investigates how the intestinal microflora act to influence these mechanisms and *H. polygyrus* expulsion.

Key questions addressed are:

What immunological factors are important for expulsion following a primary *H. polygyrus* infection?

- Do any immunological factors explain the variance in *H. polygyrus* expulsion between C57BL/6 and BALB/c mice?

- Do any immunological factors explain the variance in *H. polygyrus* expulsion *within* a cohort of C57BL/6 or BALB/c mice?

- Does the systemic immune response at day 7 following *H. polygyrus* expulsion predict the number of worms remaining in the host 28 days post-infection?

Does the intestinal microbial composition affect expulsion following a primary *H. polygyrus* infection?

- Does the microbiota composition prior to infection explain the variance in expulsion of *H. polygyrus* in C57BL/6 or BALB/c mice?

- Does the microbiota composition in the small intestine 28 days following *H. polygyrus* infection correlate with the number of worms remaining in the host at this timepoint?

-Does modifying the microbiota composition with selective antibiotic treatment alter the immune response or expulsion of *H. polygyrus*?

-Does administering single species of bacteria modify the immune response or expulsion of *H. polygyrus*?

Does *H. polygyrus* infection manipulate the microflora composition?

- Do intestinal microbiota populations shift following infection in C57BL/6 or BALB/c mice?

- At what timepoint following infection do microbiota population shifts occur?

Does ablation of intestinal microbiota signalling affect expulsion following a primary *H. polygyrus* infection?

- What effect does administering a broad-spectrum antibiotic cocktail to mice have on the immune response and expulsion of *H. polygyrus*?

- How is the immune response to *H. polygyrus* altered in TLR-deficient or TLR adaptor protein-deficient mice?

- How is expulsion of *H. polygyrus* affected in TLR-deficient or TLR adaptor protein-deficient mice?

Does TGF-β signalling modulate the murine response to a primary *H. polygyrus* infection?

- How is the differentiation of T helper subsets after *H. polygyrus* infection altered in mice that cannot respond to TGF-β?

- How is expulsion of *H. polygyrus* affected in mice that cannot respond to TGF-β?

Chapter 2. Materials and Methods

2.1 Animals

C57BL/6 and BALB/c mice were bred in-house at the University of Edinburgh. TGFβRII DN, TGFβRII DN IFNγ$^{-/-}$, IFNγ$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, MyD88$^{-/-}$, TRIF$^{-/-}$ and MyD88$^{-/-}$ TRIF$^{-/-}$ mice were on a C57BL/6 background, and also bred in-house. TLR5$^{-/-}$ mice were on a C57BL/6 background, and bred at the University of Birmingham, where the experiments were done in the laboratory of Adam Cunningham. Animals were age and sex matched within experiments. All mice were housed in individually ventilated cages (IVCs) or in an isolator in SPF conditions. For experiments comparing the susceptibility of Wt C57BL/6 and TLR- and TLR-adaptor protein-deficient mice to *H. polygyrus*, bedding was mixed between the experimental animals for two weeks prior to infection and throughout the course of infection for male mice; female mice were co-housed for two weeks prior to infection and throughout the course of infection. All experiments complied with UK Home office guidelines.

2.2 Reagents

2.2.1 Complete RPMI

RPMI 1640 (Gibco) was supplemented with 10% foetal calf serum (FCS;Hyclone), 2 mM L-glutamine (Gibco), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco).

2.2.2 Strip medium

HBSS (Sigma-Aldrich) was supplemented with 2mM EDTA (Gibco), 0.16 mg/ml DTT (Sigma-Aldrich), 10% FCS (Hyclone), 2mM L-glutamine (Gibco) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco).

2.2.3 Shake medium

RPMI 1640 (Gibco) was supplemented with 2mM EDTA (Gibco), 20mM HEPES (Sigma-Aldrich) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco).

2.2.4 3% medium

RPMI 1640 (Gibco) was supplemented with 3% FCS (Hyclone), 20mM HEPES (Sigma-Aldrich) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco).

2.2.5 0%+all medium

RPMI 1640 (Gibco) was supplemented with 20mM HEPES (Sigma-Aldrich), non essential amino acids (Gibco), 1mM sodium pyruvate (Gibco), 0.05mM β-mecaptoethanol (Gibco), 2mM L-glutamine (Gibco), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco).

2.2.6 DTT medium

3% medium was supplemented with 1% FCS (Hyclone), 0.5 mM EDTA (Gibco) and 14.5 µg/ml DTT (Sigma-Aldrich).

2.2.7 Liberase medium

0%+all medium was supplemented with 0.5 mg/ml Dexoyribonuclease I from bovine pancreas (Sigma-Aldrich) and 0.1 mg/ml Liberase TL (Roche).

2.2.8 *H. poly* medium

RPMI 1640 (Gibco) was supplemented with 1% glucose, 0.1 mg/ml Gentamicin (Gibco), 2mM L-glutamine (Gibco) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco).

2.2.9 Percoll

Percoll (GE Healthcare) was supplemented with 10% 10X phosphate buffered saline (PBS).

2.2.10 Carbonate buffer

1 M solutions of Na_2HCO_3 (Sigma-Aldrich) and $NaCO_3$ (Sigma-Aldrich) were made up in distilled water and combined at a 45.3 ml: 18.2 ml ratio, and the pH was adjusted to 9.6.

2.2.11 FACS buffer

0.5% bovine serum albumin (BSA;Sigma-Aldrich) and 0.05 % sodium azide (Sigma-Aldrich) were added to PBS.

2.2.12 *Lactobacilli* MRS broth

Lactobacilli MRS broth (Difco) was made up in distilled water according to the manufacturer's instructions.

2.3 *H. polygyrus* lifecycle, HES, parasitology measures and infections

2.3.1 *H. polygyrus* lifecycle maintenance and HES collection

F1 mice from a C57BL/6 x CBA cross were infected with 500 *H. polygyrus* L3s by oral gavage using a 24-gauge feeding needle. 14 days post-infection, mice were sacrificed and small and large intestines were collected.

Faeces was removed from the large intestine, mixed with charcoal until a sticky consistency was achieved, and a thin layer was spread over dampened filter paper (55mm pores; Whatman) and placed in a petri dish. Petri dishes were stored in a dark, dampened box at room temperature for two weeks, after which L3 larvae were collected from the edges of the dishes. L3s were washed three times in dH_2O, then stored at 4 °C in dH_2O until the time of use.

From the small intestine, adult *H. polygyrus* worms were collected by scraping the tissue with 2 glass slides. Worms were placed into muslin bags (1mm pores) in HBSS medium (Sigma-Aldrich), which were suspended over a collection tube for 2 hours at 37 °C, so that live worms penetrated through the muslin bags, and intestinal tissue remained behind. Worms were then washed 6 times in HBSS medium (Sigma-Aldrich) containing 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco), steeped in HBSS medium (Sigma-Aldrich) containing 1 mg/ml Gentamicin (Gibco) for 20 minutes at room temperature, and finally washed a further 6 times in HBSS medium (Sigma-Aldrich) containing 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco). Worms were then stored in 25 cm^2 flasks (Corning) in *H. poly* medium in 37 °C, 5% CO_2 incubators for three weeks. 24 hours after the beginning of culture, medium from the flasks was collected twice weekly (and

replaced with fresh *H. poly* medium) and processed to isolate HES. Medium was concentrated over a 3,000 MW filter (Millipore) in an Amicon stirred ultrafiltration device (Millipore). When roughly 1 L of medium had been concentrated to a 5 ml volume, the liquid was sterile-filtered using a syringe and a 0.22 µM pore filter unit (Millex), and stored at -80 °C prior to use.

2.3.2 Faecal egg counts

Faeces were weighed and left to form a homogenous solution in 2 ml of distilled H_2O. An equal volume of saturated NaCl was added, and the resulting solution loaded to fill one chamber of a egg counting slide (McMaster 2 cell counter; Hawksley). Eggs within the grid were enumerated using a light microscope, and eggs per gram of faeces were calculated using the following calculation:

$$\text{Eggs/gram of faeces} = \frac{\text{egg count} \times 26.67^{*}}{\text{faeces mass (g)}}$$

$$^{*} = \text{calculated as: } \frac{\text{volume of liquid faeces was dissolved in (4ml)}}{\text{chamber volume (0.15ml)}}$$

2.3.3 ATPlite assay

Individual *H. polygyrus* adult male worms were incubated at 37 °C, 5% CO_2 for 24 hours in eppendorfs containing 100 µl of *H. poly* medium with the indicated concentrations of Trimethoprim and Sulfadoxine (Borgal 24; Dunlops). Following incubation, worms were lysed by adding 50 µl lysis buffer (ATPlite kit; PerkinElmer), and shaken with a stainless steel bead (Qiagen) on

a TissueLyser II (Qiagen) machine (frequency 15; 5 minutes). The resulting solution was used in the ATPlite assay (PerkinElmer) by manufacturer's instructions.

2.3.4 Fecundity assay

Individual *H. polygyrus* adult female worms were incubated at 37 °C, with 5% CO_2 for 24 hours in individual wells of a 48 well plate (Costar) containing *H. poly* medium with the indicated concentrations of Trimethoprim and Sulfadoxine (Borgal 24; Dunlops). Following culture, adult worms were removed from wells, and the number of eggs per well was enumerated using a light microscope.

2.3.5 *H. polygyrus* experimental infections

Mice were infected with 200 *H. polygyrus* L3s in 200 µl of dH_2O by oral gavage, using a 24-gauge feeding needle. For experiments examining the effect of *H. polygyrus* infection on the microflora populations, and for TLR- and TLR-adaptor protein-deficient mouse experiments, naïve control mice were also gavaged with 200 µl of water that *H. polygyrus* L3 had been stored in, after L3s were filtered out using a 40 µM cell strainer.

2.4 Cell isolation, serum collection, irradiation and cell transfer

2.4.1 MLN cell isolation

MLN were isolated and single cell suspensions were made using a 70 µM cell strainer in Complete RPMI.

2.4.2 PP cell isolation

PP were manually dissected from the intestinal tract, and shaken at ~300 rpm at 37 °C in Strip medium for 20 minutes. Strip medium was then removed, and single cell suspensions were made by passing cells through a 70 μM, followed by a 40 μM cell strainer in Complete RPMI medium. Cells were spun down at 1500 rpm at 4 °C for 5 minutes, and resuspended in 30 % Percoll (GE Healthcare) in Complete RPMI. The 30 % Percoll/cell suspension was layered on top of a layer of 40 % Percoll in PBS, with a base of 80 % Percoll in Complete RPMI. After spinning at 1800 rpm for 20 minutes, cells were collected from the 80 %/40 % interface and washed twice in Complete RPMI.

2.4.3 Small intestine LP cell isolation

The small intestine was isolated, and PP manually dissected. The gut was longitudinally slit open in ice cold 3% medium, and the intestinal contents was removed by gently scraping along the length of the gut. Gut was then chopped up into ~1 cm pieces into a 50 ml tube containing 10 mls of Shake medium. Tubes were manually shaken for 30 seconds, after which contents was poured through a tea strainer, with liquid flow through draining through to a 250 ml beaker. Gut pieces were collected, and placed back into 50 ml tubes containing 10 mls of fresh Shake medium. The shake/draining process was repeated a further 2 times, after which gut pieces were placed into 50 ml beakers containing 20 mls of DTT medium. Stir bars were added, and guts were stirred on a Varigomag magnetic stir plate (Thermo Scientific) for 15 minutes at 37 °C. Following stirring, the mixture was poured through tea strainers, and gut pieces placed back into 50 ml tubes containing 10 mls of fresh Shake medium. Tubes were manually shaken/drained three times as described earlier, after which gut pieces were placed into clean 50 ml tubes containing 5 mls of Liberase medium. Guts were further diced manually, stir bars added, and stirred on the Varigomag magnetic stir plate (Thermo Scientific) for 24 minutes at 37 °C, with 5 additional mls of Liberase medium added after the first 12 minutes of incubation. Following stirring, the Liberase

reaction was stopped by adding 30 mls of 3% medium. Gut pieces were mashed through a 70 µM, then a 40 µM cell strainer in 3% medium. Cells were then spun for 5 minutes at 4 °C at 1500 rpm, and resuspended in 0%+all medium.

2.4.4 Serum collection

Blood was collected non-terminally via puncture of the submandibular vein using a 26-gauge needle. When blood was collected at experimental endpoints, mice were first anaesthetised by a 100 µl subcutaneous injection containing 50µl 100 mg/ml Vetlar (Pfizer) and 50µl 1 mg/ml Domitor (Janssen), and blood was taken from the brachial artery before mice were culled by a Schedule 1 method. Blood was collected into Microtainer tubes (BD), which were spun at 13,000 rpm at 4 ˚C for 10 minutes. Serum was removed from the pellets and frozen at -80 ˚C until use.

2.4.5 Bone marrow isolation, irradiation and cell transfer

Five days prior to irradiation, all recipient mice were placed on water containing 0.1 mg/ml Enrofloxacin (Baytril;Bayer), which they were kept on for four weeks following irradiation. Recipient mice were given a lethal radiation dose of 980 radiation absorbed dose (rad). Following irradiation, diet pellets were mashed in water and placed in the cage for two weeks. 24 hours following irradiation, mice were reconstituted with 2-5 million bone marrow cells by injection into the tail vein.

To prepare cells for reconstitution, donor mice were sacrificed, and bone marrow cells extracted into PBS. Red blood cells were lysed by incubating cell suspensions in red blood cell lysis buffer (Sigma-Aldrich) at room temperature for 5 minutes. Cells were washed twice in PBS, and depleted of $CD90^+$ cells using CD90.2 microbeads (Miltenyi Biotech) and MACS negative selection columns (Miltenyi Biotech) using the manufacturer's recommended

instructions. Following CD90 depletion, cells were counted and resuspended in PBS.

2.5 Cell culture and flow cytometry analysis

2.5.1 Cell restimulation

For antigen-specific restimulation, 1 x 10^6 cells were plated in duplicate in a 96-well flat bottom plate (Costar) in complete RPMI medium with or without 1 µg/ml HES, at 37 ˚C with 5% CO_2 for 72 hours. Supernatants were then collected and frozen at -20 ˚C prior to analysis.

For restimulation prior to intracellular cytokine staining, 2 x 10^6 cells were plated in a 96-well round bottom plate (Costar) and stimulated with 0.5 µg/ml PMA (Sigma-Aldrich) and 1 µg/ml Ionomycin (Sigma-Aldrich) for 3.5 hours, with 10 µg/ml Brefeldin A (Sigma-Aldrich) included for the final 2.5 hours, at 37 ˚C with 5% CO_2.

2.5.2 Live/dead markers and cell surface staining

Cells were washed in PBS and stained with LIVE/DEAD (Invitrogen) at a 1/1000 dilution in 100 µl of PBS, for 15 minutes at 4 °C. Cells were washed a further two times in PBS, and rat IgG (Sigma-Aldrich) was added to cells at 100 µg/ml in FACS buffer to block for 10 minutes at 4 °C. Surface markers were then added to cells in 20 µl of FACS buffer, for 20 minutes at 4 °C, at the concentrations indicated in Table 2.5.

2.5.3 Intracellular cytokine staining

For intracellular cytokine staining (ICCS), cells were first restimulated with PMA/Ionomycin/Brefeldin, and stained with LIVE/DEAD and surface markers as described above. Cells were washed three times in FACS buffer, and fixed for 20 minutes at 4 °C in Cytofix/Cytoperm (BD Bioscences). Following fixation, cells were washed three times in Perm/Wash (BD Biosciences) and stained with antibodies in 20 µl of Perm/Wash (BD Biosciences) at the concentrations indicated in Table 2.5 for 20 minutes at 4 °C. Isotype matched control antibodies were used on a pool of samples. Marker expression was measured on FACSCanto (BD Biosciences) or LSRII (BD Biosciences) flow cytometers and data were analysed using FlowJo software (Tree Star).

2.5.4 Transcription factor staining

For transcription factor staining, cells were taken directly *ex vivo* and stained with LIVE/DEAD and surface markers as described above. Cells were washed three times in FACS buffer, and fixed for 1 hour or overnight at 4 °C in Fix/Perm (eBioscience). Following fixation, cells were washed three times in Permeabilization buffer (eBioscience) and stained with antibodies in 20 µl of Permeabilization buffer (eBioscience) at the concentrations indicated in Table 2.5 for 20 minutes at 4 °C. Isotype matched control antibodies were used on a pool of samples. Marker expression was measured on FACSCanto (BD Biosciences) or LSRII (BD Biosciences) flow cytometers and data were analysed using FlowJo software (Tree Star).

Table 2.5 Antibodies used for flow cytometry.

Antibody name	Clone	Florochrome	Concentration used at	Supplier
TCR-β	H57-597	Pacific Blue	1/100	eBioscience
TCR-β	H57-597	APC-eFluor 780	1/100	BioLegend
CD4	GK1.5	PerCP	1/100	BioLegend
CD4	RM4-5	A700	1/100	BD Biosciences

CD4	RM4-5	PE	1/100	BioLegend
CD4	RM4-5	APC	1/100	BioLegend
CD8-α	53-6.7	PerCP	1/100	BioLegend
IL-4	11B11	PE	1/100	BioLegend
IL-13	eBio13A	Alexa Fluor 647	1/100	eBioscience
IL-9	RM9A4	PE	1/100	BioLegend
IL-17A	TC11-18H10.1	Pe-Cy7	1/100	BioLegend
IL-10	JES5-16E3	Pacific Blue	1/100	BioLegend
IFN-γ	XMG1.2	PerCp	1/100	BD Pharmingen
IFN-γ	XMG1.2	FITC	1/100	BioLegend
Foxp3	FJK-16S	APC	1/100	eBioscience
Helios	22F6	PE	1/25	BioLegend
Biotin-CD103	M290	-	1/100	BD Biosciences
Streptavidin	-	PerCP	1/100	BD Pharmingen

2.6 Enzyme-linked immunosorbent assay (ELISA)

2.6.1 Detection of cytokines by ELISA

96-well plates (Nunc) were coated overnight at 4 °C with 50 μl per well of capture antibody in carbonate buffer, at the indicated concentrations (Table 2.6.1). Plates were washed with Tris-buffered saline with 0.1% Tween 20 (Sigma-Aldrich;TBSt) and blocked for 90 minutes at 37 °C with 200 μl per well of TBSt with 10% FCS. Recombinant cytokine standards were prepared in the same medium as the test samples, and duplicate doubling dilutions were made from the top standard concentration shown in Table 2.6.1. Plates were washed in TBSt, and 50 μl per well of standards and samples were incubated at 4 °C overnight. Plates were washed in TBSt, and biotinylated detection antibodies were diluted in TBSt with 5% FCS, and 50 μl per well was plated at the indicated concentrations (Table 2.6.1) at 37 °C for 1 hour. Plates were washed in TBSt and 50 μl per well of alkaline phosphatase (Sigma-Aldrich) at a 1/12500 dilution in TBSt with 5% FCS was added and incubated at 37 °C for 45 minutes. Plates were washed in TBSt, then in distilled water, and 100 μl per well of the alkaline phosphatase substrate p-nitrophenyl phosphate (PNPP; 1 mg/ml; Sigma-Aldrich) was added. Plates were incubated at room temperature in the dark and the OD was measured at 405nm on a Precision

Microplate reader (Molecular Devices) as the assay developed. Softmax Pro (Molecular Devices) software was used to calculate a standard curve of OD 405 nm vs concentration.

Table 2.6.1 Antibodies used for cytokine ELISAs.

Cytokine	Top standard	Capture clone	Capture coating concentration	Detection clone	Detection concentration	Supplier
IFN-γ	50 ng/ml	R4-6A2	2 µg/ml	XMG1.2	1 µg/ml	BD Pharmingen
IL-4	8 ng/ml	11B11	2 µg/ml	BVD6-24G2	1 µg/ml	BD Pharmingen
IL-10	10 ng/ml	JES5-2A5	4 µg/ml	SXC-1	2 µg/ml	BD Pharmingen

2.6.2 Detection of HES-specific antibodies by ELISA

96-well plates (Nunc) were coated overnight at 4 °C with 50 µl per well of 1 µg/ml HES in carbonate buffer. Plates were washed with TBSt and blocked for 2 hours at 37 °C with 200 µl per well of TBSt with 2% BSA. Plates were washed in TBSt before samples and standards were added. Serum samples were made to a 1/100 dilution in TBSt with 2% BSA, and twelve 3-fold dilutions of serum samples were made. For standards, serum samples were pooled and diluted 1/50 in TBSt with 2% BSA, and twelve duplicate doubling dilutions were made. 30 µl per well of standards and samples were added to plates, which were incubated for 2 hours at 37 °C, or at 4 °C overnight. Plates were washed in TBSt, and HRP conjugated detection antibodies were diluted in TBSt with 2% BSA, and 50 µl per well was plated at the indicated concentrations (Table 2.6.2) at 37 °C for 1 hour. Plates were washed in TBSt and twice with dH_20. 50 µl per well of ABTS peroxidase substrate (KPL) was added. Plates were incubated at room temperature in the dark and the OD was measured at 405nm on a Precision Microplate reader (Molecular Devices) as the assay developed. End-point antibody titres shown indicate

the maximum serum dilution at which the reaction from each sample reached an OD of 0.1.

Table 2.6.2 Antibodies used for antibody ELISAs.

Isotype	Detection clone	Detection concentration	Supplier
IgG1	SB77e	0.125 µg/ml	Southern Biotech
IgA	11-44-2	0.125 µg/ml	Southern Biotech

2.7 Histology

Gut samples were isolated and stored in 4% formaldehyde (Sigma-Aldrich) in PBS, at 4 °C for 24 hours. Samples were then transferred to a 70% EtOH solution at 4 °C until the time of processing. Transverse sections were cut and sections were stained with hematoxylin and eosin (H&E) or toluidine blue. Images were captured on a compound microscope (Leica) and processed in ImageJ.

2.8 Cytokine bead array (CBA) assay

Cytokines present in sera were assayed using a CBA flex set (BD Biosciences), using cytokine-conjugated beads and PE-detection reagent at 1/5 of the manufacturer's recommended concentrations, by the manufacturer's recommended protocol. Samples were run on a FACSarray Bioanalyzer (BD) and data were analysed in FlowJo (TreeStar).

Table 2.8 Cytokine bead positions for CBA assay.

Cytokine	Bead position
IFN-γ	A4
IL-4	A7
IL-5	A6
IL-6	B4
IL-9	B5
IL-10	C4
IL-13	B8
IL-17A	C5

2.9 Bacterial Identification

2.9.1 Bacteria DNA extraction

DNA was extracted from intestinal tissue or faeces using the QIAamp DNA Stool Mini Kit (Qiagen). Tissue or faeces was homogenised using a stainless steel bead (Qiagen) and a TissueLyser II (Qiagen) machine. The resulting homogenate was treated following the manufacturer's recommended instructions, including the optional 95 °C lysis step, to lyse bacterial cell walls.

2.9.2 Real-time PCR for bacterial populations

DNA was first made to a 30 ng/μl concentration following quantification with a Nanodrop 200 (Thermo Scientific). 4 μl of the DNA solutions were mixed with 5 μl SYBR Green I (Roche), 0.4 μl DEPC-treated water, and 0.2 μl each of forward and reverse primers, at 10 μM (Table 2.9.2). Reactions were set up in triplicate with standards made from a mixture of undiluted DNA. The reactions were run on a LightCycler 480 II (Roche). 45 cycles of 95 ˚C for 10 seconds, 60 ˚C for 10 seconds, and 72 ˚C for 10 seconds were run. Concentrations are shown on graphs as arbitrary units (AU), and represent the mean concentration of each sample relative to the standard pool.

Table 2.9.2 Primers used for bacteria identification.

Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Purpose	Reference
mGAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG	House-keeping gene	Designed in-house
Lactobacillus/Lactococcus-specific primers	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	Amplifying part of the 16S rRNA gene specific to *Lactobacillus/Lactococcus* bacteria	[227]
Bacteroides-specific primers	GGTTCTGAGAGGAGGTCCC	GCTGCCTCCCGTAGGAGT	Amplifying part of the 16S rRNA gene specific to *Bacteroides* bacteria	[228]
Enterobacteriaceae	GTGCCAGCMGCCGCGGTAA	GCCTCAAGGGCACAACCTCCAAG	Amplifying part of the 16S rRNA gene specific to Enterobacteriaceae bacteria	[229]
Eubacterium rectale-Clostridium coccoides-specific primers	ACTCCTACGGGAGGCAGC	GCTTCTTTAGTCAGGTACCGTCAT	Amplifying part of the 16S rRNA gene specific to *Eubacterium rectale-Clostridium coccoides* bacteria	[230]
Lactobacillus taiwanensis-specific primers (GyrB2)	CAACGGATATAAGACAACACTCATGACCTTC	GGTAGACCGCGCATTTTCAGAAACC	Amplifying part of the GyrB gene specific for *L. taiwanensis*.	Designed in-house
T7F	TAATACGACTCACTATAGGG	-	Sequencing from the pGEM vector	Designed in-house
SP6	-	ATTTAGGTGACACTATAGAAT	Sequencing from the pGEM vector	Designed in-house

2.9.3 Sequencing of *Lactobacillus/Lactococcus* species

DNA extracted using the QIAamp DNA Stool Mini Kit was diluted to 350 ng/µl. 2.5 µl of this was added to 0.2 µl Taq polymerase (Qiagen); 2.5 µl 10X buffer (Qiagen), 1.5 µl $MgCl_2$ (Qiagen), 0.5 µl 1mM dNTPs; 15.3 µl distilled water and 1.25 µl each of 10 µM forward and reverse *Lactobacillus/Lactococcus*-specific primers (Table 2.9.2). The mix was incubated on a PCR machine (Bio-rad) with the following programme: 94 °C for 3 minutes; 39 cycles of 94 °C for 45 seconds, 50 °C for 45 seconds, 72 °C for 45 seconds; then 72 °C for

2 minutes. The resulting products were run on a 1% agarose gel, and the band visualised on a UV machine. The band formed at the expected product size of 342 base pairs (bp) was cut out, and DNA extracted using a QIAquick gel extraction kit (Qiagen), using the manufacturer's recommended instructions, with product eluted in a 50 µl volume. The *Lactobacillus/Lactococcus* amplicon was ligated into pGEM vectors (Promega) at a ratio of 6 µl gel extracted product: 1 µl pGEM by manufacturer's recommended guidelines. JM109 cells (Promega) were transformed with the ligation reaction by manufacturer's instructions, and colonies were left to grow overnight on agar plates containing 50 µg/ml Ampicillin in a 37 °C, 5% CO_2 incubator. Colonies were grown overnight in LB broth containing 50 µg/ml Ampicillin in a shaking 37 °C incubator, and DNA extracted using QIAprep Spin Miniprep kits (Qiagen). DNA was sequenced using pGEM-specific T7F and SP6 primers (Table 2.9.2) at 1 µM with a BigDye sequencing kit (AppliedBiosystems) by manufacturer's recommended protocol, and sequenced in-house. Resulting sequences were analysed on MacVector and Blast-searched against known microbial sequences.

2.10 Bacterial culture and faecal transfers

2.10.1 Culture of *Lactobacillus taiwanensis*

L. taiwanensis was obtained from the laboratory of Maria Yebra (Instituto de Agroquímica y Tecnología de Alimentos, Valencia, Spain). Static overnight cultures were set up at 37 ˚C with 5 % CO_2, in *Lactobacilli* MRS broth (Difco). Optical density (OD) of the culture was measured at 600 nm and the number of colony forming units (CFU) determined using the following equation, based on the standard growth curve for a closely related bacterial species, *L. casei*, calculated by the Yebra laboratory.

$$CFU/ml = (8 \times 10^8 \times OD) - 6 \times 10^7$$

2.10.2 Oral gavage of *L. taiwanensis*

L. taiwanensis cells were washed twice in dH_2O, resuspended in PBS and 10^{10} CFU were given by oral gavage, using a 24-gauge feeding needle.

2.10.3 Administration of *L. taiwanensis* in drinking water

L. taiwanensis cells were washed twice in dH_2O, and administered in UV-sterilised drinking water at a concentration of 2×10^8 CFU/ml.

2.10.4 Faecal transfers

Faecal pellets were collected from donor mice, weighed, and resuspended in saline solution at 1/50 w/v. Two doses of 5 mg faeces were administered to recipient mice at 24 hour intervals.

2.11 Antibiotic treatments

2.11.1 Broad-spectrum antibiotic cocktail

Mice were treated with either of the following antibiotic/antifungals made up in autoclaved UV-sterilised water for the indicated time periods. All antibiotics/antifungals were obtained from the University of Edinburgh Pharmacy, except where indicated.

Broad-spectrum cocktail 1: Metronidazole (1 g/L); Ciprofloxacin (200 mg/L); Imipenem (250 mg/L); Ampicillin (1 g/L); Vancomycin (500 mg/L); Fluconazole (1 g/L); 3 sweetener tablets/L (Canderel).

Broad-spectrum cocktail 2: Metronidazole (1 g/L); Ampicillin (1 g/L); Vancomycin (500 mg/L); Neomycin (1 g/L; Sigma-Aldrich); 3 sweetener tablets/L (Canderel).

Where mice were given these cocktails for periods of more than 2 weeks, to prevent dehydration and weight loss, diet pellets were mashed in water and placed in the cage, to give mice an additional source of water.

2.11.2 Trimethoprim and Sulfadoxine cocktail

Mice were maintained on 125 mg/L Trimethoprim and 25 mg/L Sulfadoxine (Borgal 24; Dunlops) made up in autoclaved UV-sterilised water, from weaning age until the end of the experiment.

2.12 Statistical analysis

Statistical analysis was performed using Prism 5 and JMP 10 software. Unless otherwise indicated in figure legends, data analysis was done as follows:

Where data from more than one experiment were pooled, the separate data sets were first compared to ensure no statistical differences between data sets existed. Data were next assessed for normality. For comparison between parametric data sets, an unpaired T test was used (if two groups were being compared) or a One-way ANOVA was used followed by a Tukey's multiple comparison test (if more than two groups were being compared). For comparison between non parametric data, a Mann–Whitney test (if two groups were being compared) or Kruskal-Wallis test followed by Dunn's multiple comparison test (if more than two groups were being compared) was used.

Average lines shown on graphs represent the mean value for parametrically analysed data, and median values for non parametrically analysed data.

For correlation analyses, Pearson r tests were done on parametric data, and Spearman r tests on non parametric data. The correlation co-efficient R value was added to graphs where correlations reached significance.

A p value of < 0.05 was considered significant and indicated by *; $p < 0.01$ by ** and $p < 0.001$ by ***.

Chapter 3. Multiparameter analysis of *Heligmosomoides polygyrus* infection

3.1 Introduction

H. polygyrus is often referred to as a chronic helminth infection, yet it is known that different inbred mouse strains display varied degrees of susceptibility to infection [231, 232]. Furthermore, within a cohort of an age-matched single strain of mice, there is often substantial variation in the ability of mice to expel *H. polygyrus*, indicating that factors beyond the influence of genetics affect susceptibility.

Susceptibility differences between strains of mice are partly explained by polymorphisms within the H-2 loci of the MHC. Mice with the genotypes H-2^d, H-2^q and H-2^s generally expel *H. polygyrus* more rapidly than those of H-2^b and H-2^k genotypes [232, 233]. BALB/c mice are H-2^d, and have been shown to be more resistant to *H. polygyrus* than H-2^b C57BL/6 mice, both after a primary infection and in a secondary challenge [234-236].

Susceptibility differences *within* a mouse strain may be attributed to gender differences; female mice across a variety of strains clear primary *H. polygyrus* infections more rapidly than their male counterparts, although the critical cell types affected by different gender environments remain unknown [237-239]. Additionally, differences in intestinal microbiota populations may be a factor leading to differing levels of susceptibility to infection. The composition of bacterial populations in the intestine is diverse between individuals, both in humans and mice [8, 9]. *H. polygyrus* colonises the mouse in the anterior small intestine, alongside a substantial microbial flora. The presence of specific species of bacteria within the microflora can polarise the differentiation of naïve T cells towards particular Th subset fates [86, 87, 91], and as the outcome of *H. polygyrus* infection is likely to be dependent on the immediate cytokine environment, it seems reasonable to imagine that

commensal microbes may alter the ability of the murine immune system to cause worm expulsion.

This study aimed to exploit the variation in mice to a *H. polygyrus* infection in both BALB/c, and the more susceptible C57BL/6 mice, and use it to find correlates between immunity to *H. polygyrus*, the murine microflora, and the immune response. Many studies examine only single factors that are associated with infection outcome, despite the fact that in a complex *in vivo* infection setting, multiple relationships between immune, environmental and parasitic factors are at play. The number of biological parameters that can now be measured allows for a more complex analysis of data sets, where relationships between variables, and their combined impact on infections can be examined [240]. The data presented here, therefore, measured a suite of parasitological, immunological and microbial factors in large cohorts of *H. polygyrus*-infected BALB/c and C57BL/6 mice, and identified factors which co-vary and which acted positively or negatively to affect the susceptibility to *H. polygyrus*.

Interestingly, the factors affecting the ability of the mouse to expel *H. polygyrus* differed between BALB/c and C57BL/6 mice, allowing the importance of different immune mechanisms to be examined in the context of different immune backgrounds. A major difference between the strains studied here was the formation of a high number of granulomas following infection in the more resistant BALB/c strain. Additionally, the absolute number of these granulomas negatively correlated with endpoint worm burden. BALB/c mice were generally more reactive to *H. polygyrus*-infection than C57BL/6 mice, in terms of systemic cytokine production, and favoured type 2 cytokine production when MLN cells taken *ex vivo* were restimulated with *H. polygyrus* antigens. In many settings, C57BL/6 mice are known to be predisposed towards Th1 responses [241], and it is shown here that high serum IFN-γ responses at day 7 of infection in these mice is predictive of in higher susceptibility to *H. polygyrus* by day 28 of infection.

A major finding of this study is a likely role for microflora in influencing susceptibility, as faecal Enterobacteriaceae levels prior to infection in BALB/c

mice appeared to be a predictor of immunity, correlating negatively with *H. polygyrus* worm burden after 28 days of infection. In both BALB/c and C57BL/6 mice microflora composition in the small intestine was altered after *H. polygyrus* infection, with the BALB/c and C57BL/6 mice which retained the highest *Lactobacillus/Lactococcus* and Enterobacteriaceae levels respectively post-infection being the mice which harboured the highest worm burdens.

This study highlights the importance of both the genetic background and the microflora composition in controlling immunity to *H. polygyrus*.

3.2 Results

3.2.1 BALB/c and C57BL/6 mouse strains show contrasting levels of susceptibility and granuloma development, but both show substantial intra-strain variability in response to infection

It has previously been reported that BALB/c mice are more resistant than C57BL/6 mice to a primary infection with *H. polygyrus* [234]. Here, this was confirmed, as after 28 days of *H. polygyrus*-infection BALB/c mice were better able to expel the worms than C57BL/6 mice (Figure 3.2.1 A). Interestingly, though all mice of each strain were age-matched and housed in similar conditions, both BALB/c and C57BL/6 mice showed extensive variation in their susceptibility to *H. polygyrus*, with some mice maintaining high worm burdens and some completely expelling the worms by 28 days post-infection (Figure 3.2.1 A).

Egg production by *H. polygyrus* in both BALB/c and C57BL/6 mice closely reflected the worm burden, with much reduced fecundity in the BALB/c host compared to C57BL/6 (Figure 3.2.1 B). Strong positive correlations were found between the number of worms present in the host and the amount of eggs released at the same time point, in both BALB/c (Figure 3.2.1 C) and C57BL/6 mice (Figure 3.2.1 D).

A striking feature of *H. polygyrus* infection in more resistant strains of mice is the formation of granulomas around the site of larval invasion in the intestinal tract [191]. BALB/c mice had a robust granuloma response to infection, whereas C57BL/6 mice produced few granulomas following infection (Figure 3.2.1 E). Granuloma formation was associated with reduced adult worm survival within BALB/c mice, as the number of granulomas produced negatively correlated with the number of worms remaining in the host 28 days post-infection (Figure 3.2.1 F).

Next, the immune response in the mice was explored, to look for factors which explained the variation in susceptibility within a strain, and to determine whether these factors also explained variation between strains.

3.2.2 Serum cytokines measured in the course of infection show strain differences and can correlate with infection outcome

The levels of cytokines circulating in the sera of mice were assayed at days 7, 14 and 28 of infection, both to assess how the cytokine environment altered throughout the infection, and to identify any parameter at days 7 or 14 which predicted infection outcome at day 28 (Figure 3.2.2 A-G).

All cytokines measured (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A and IFN-γ) were present at a higher level in naïve BALB/c mice than in naïve C57BL/6 mice (Figure 3.2.2); indeed, no IL-4, IL-10 or IL-13 was detectable in the serum of either naïve or infected C57BL/6 mice. In BALB/c mice, levels of the Th2 cytokines IL-4, IL-10 and IL-13 peaked in 28-day infected mice (Figure 3.2.2 A,B,D), as did levels of the inflammatory cytokines IL-6, IL-17A and IFN-γ (Figure 3.2.2 E,F,G). Levels of serum IL-9 were unchanged by infection in BALB/c mice, and not detectable in C57BL/6 mice (data not shown).

The largest increase in serum cytokine production in following infection was in IL-5, which peaked at day 7 post-infection in both BALB/c and C57BL/6 mice; at this point serum IL-5 levels were significantly higher in the more resistant strain (Figure 3.2.2 C). In contrast, the more susceptible C57BL/6 mice showed early elevated levels of the inflammatory cytokines IL-6 (Figure 3.2.2 F) and IFN-γ (Figure 3.2.2 G) 7 days after infection compared to naïve mice, whilst no infection-induced response in these cytokines was seen at the same timepoint in BALB/c mice. Mice deficient in these cytokines are known to show increased resistance to *H. polygyrus* infection [242, 243].

Interestingly, in the C57BL/6 strain, mice that mounted the strongest serum IFN-γ response at day 7 post-infection were the most susceptible to *H. polygyrus*, as measured by day 28 worm burden (Figure 3.2.2 H), indicating that this measure could be used as a non-terminal predictor of later immunity, at an early timepoint of infection. No other day 7 cytokines measured, including IL-6, correlated with day 28 worm burden in the C57BL/6 stain (data not shown).

In BALB/c mice, none of the measured serum cytokine levels at day 7 significantly correlated with the outcome of infection, in terms of day 28 worm burden (data not shown), despite there being a considerable spread within the infected mice in many of the cytokines measured, especially in IL-5 (Figure 3.2.2 C). The importance of serum cytokine responses in controlling immunity to *H. polygyrus* therefore varied between BALB/c and C57BL/6 mice.

3.2.3 Th1, Th2 and Th17 cytokine-producing cells are upregulated during *H. polygyrus*-infection in BALB/c and C57BL/6 mice

To investigate cytokine responses more proximal to the site of infection, MLNs, which drain the intestine, were isolated from naïve and 28-day infected mice, and cells were stained for a panel of cytokines representing key Th helper subsets. MLN cell numbers expanded dramatically with *H. polygyrus* infection equivalently in both BALB/c and C57BL/6 mice, with 4-5 fold higher cell numbers isolated in infected compared to naïve mice (data not shown).

H. polygyrus infection resulted in a higher proportion of $CD4^+$ T cells producing IL-4, IL-10, IL-13, IL-17A, and IFN-γ, and a higher proportion of $CD8^+$ T cells producing IFN-γ, in both strains of mice (Figure 3.2.3, left-hand panels). The total numbers of cytokine producing cells also increased with infection (Figure 3.2.3 right-hand panels), of which the total numbers of $CD8^+$ IFN-γ-producing cells dominated in both strains (Figure 3.2.3 L).

In both strains, a significant Th2 cytokine response developed, with expansion of IL-4, IL-10 and IL-13-secreting cells (Fig 3.2.3 A-F). Perhaps surprisingly, the magnitude of canonical Th2 cytokine production was if anything higher in the more susceptible C57BL/6 mice (Fig 3.2.3 C+D), but the more resistant BALB/c mice showed a greater frequency of IL-10-secreting cells (Fig. 3.2.3 E). A further notable difference in MLN cytokine production between strains was in the proportion and total numbers of IL-17A-producing $CD4^+$ cells, with higher levels in naïve and infected C57BL/6 mice than their BALB/c counterparts (Figure 3.2.3 G+H). Further differences were observed between the infected BALB/c and C57BL/6 mice, with BALB/c mice showing a lower

proportion, but similar total numbers, of $CD4^+$ IFN-γ producing cells (Figure 3.2.3 I+J). Both strains showed a marked expansion in $CD8^+$ IFN-γ producing cells (Figure 3.2.3 K+L).

Hence, *H. polygyrus* infection causes expansion of cells of Th1, Th2, and Th17 subsets in both BALB/c and C57BL/6 mice, however more defined differences in, for example the balance between IL-10 and IFN-γ responses may result in the different levels of immunity between strains.

3.2.4 Cytokine production at day 28 of infection principally reflects worm load in BALB/c and C57BL/6 mice

Whether differential cytokine production by Th subsets between BALB/c and C57BL/6 mice could explain the differences in worm burden 28 days post-infection was next examined, by correlating cytokine production by $CD4^+$ or $CD8^+$ MLN cells with the number of worms remaining in the intestinal tract. Total numbers of $CD4^+$ IL-4, IL-13, IL-17A, IL-10, IFN-γ and $CD8^+$ IFN-γ MLN cells all positively correlated with worm burden in both strains of mice, which was expected due to the total expansion of MLN cells following infection (data not shown). The proportion of $CD4^+$ or $CD8^+$ cells expressing each cytokine was therefore correlated with worm burden (Figure 3.2.4). In both strains, the proportion of $CD4^+$ cells expressing IL-4, $CD4^+$ cells expressing IFN-γ, and $CD8^+$ cells expressing IFN-γ all positively correlated with worm burden 28 days post-infection (Figure 3.2.4 A+B,I+J,K+L). Only in the more susceptible C57BL/6 mice did the proportion of $CD4^+$ cells producing IL-13 or IL-10 correlate with worm burden (Figure 3.2.4 C-F), and no correlation between the proportion of $CD4^+$ cells expressing IL-17A and worm burden was seen in either mouse strain (Figure 3.2.4 G+H), perhaps indicating that these cytokines are less important for promoting worm expulsion.

3.2.5 BALB/c mice mount a stronger HES-specific Th2 response and a poorer HES-specific Th1 response than C57BL/6 mice

To investigate to the specificity of these cytokine responses to *H. polygyrus*, the memory/effector T cell response to *H. polygyrus* antigen was next examined, by a recall assay to HES. MLN cells were cultured in the presence of absence of HES, and tested for their cytokine production.

MLN cells from infected mice responded to HES by releasing the Th2 cytokines IL-4 (Figure 3.2.5 A), IL-5 (Figure 3.2.5 B) IL-10 (Figure 3.2.5 C) and IL-13 (Fig 3.2.5 D). Interestingly however, HES-stimulated cells from infected BALB/c mice produced far higher IL-4, but less IL-13, than MLN cells from infected C57BL/6 mice (Figure 3.2.5 A+D). MLN cells from infected mice incubated with medium alone showed some IL-5 and IL-13 production that was not apparent in naïve MLN cells (Figure 3.2.5 B+D); whether this represented ILC contributions was not determined.

Levels of IL-17A production were unchanged in cells from naïve and infected animals following HES-stimulation in either strain, yet overall IL-17A production was higher in C57BL/6 mice than in BALB/c mice (Figure 3.2.5 E). All cell cultures, from naïve and infected mice, showed elevated IL-17A when co-incubated with HES (Figure 3.2.5 E), which could be attributed to the presence of a TGFβ-like activity in the parasite products [197].

In accordance with the serum cytokine (Figure 3.2.2) and MLN cell staining (Figure 3.2.3) data, MLN cells from infected C57BL/6 mice generated considerably more IFN-γ in response to HES-stimulation than cells from infected BALB/c mice, also producing a strong non-antigen specific IFN-γ response to infection (Figure 3.2.5 F). Additionally, infected C57BL/6 differed from BALB/c mice in that MLN cells from these mice produced significantly higher IL-9 *in vitro*, which was enhanced by HES stimulation (Figure 3.2.5 G).

3.2.6 HES-specific inflammatory cytokine production correlates with high worm burdens in BALB/c, but not C57BL/6 mice

Next, it was asked whether these levels of HES-specific cytokine production had any association with susceptibility to *H. polygyrus* infection. No correlation was found in either strain between worm burden and the levels of HES-specific IL-4 (Figure 3.2.6 E+F) or of IL-5, IL-9, IL-10 or IL-13 (data not shown).

In contrast, HES-specific IL-17A and IFN-γ-production by MLN cells positively correlated with worm burden in BALB/c mice (Figure 3.2.6 A+C), indicating that the production of these cytokines promotes worm survival. No such relationship was found with these cytokines and *H. polygyrus* susceptibility in C57BL/6 mice (Figure 3.2.6 B+D), indicating perhaps that the effect of inflammatory cytokines is only observed in the stronger Th2 setting of more resistant mice.

3.2.7 BALB/c mice show greater expansion of Helios$^+$ Tregs, and C57BL/6 of Helios$^-$ Tregs in response to infection

As well as the expansion of CD4$^+$ and CD8$^+$ effector T cells during *H. polygyrus* infection, the number of Tregs accumulating in the MLN increases over time [133]. HES can also cause the differentiation of naïve T cells into Foxp3-expressing Tregs *in vitro* [197], suggesting that the parasite favours a Treg-rich environment for its survival within the mouse. The proportion and total numbers of CD4$^+$ Tregs were therefore examined in the MLN cells of naïve and infected BALB/c and C57BL/6 mice at 28 days post-infection, to examine whether Treg expansion differs between the differentially susceptible strains.

The proportion of CD4$^+$ MLN cells expressing Foxp3 increases subtly following infection in BALB/c mice, and to a lesser extent in C57BL/6 mice (Figure 3.2.7 A), yet both strains show a clear increase in absolute number of

Foxp3$^+$CD4$^+$ cells following infection, with no differences between the strains (Figure 3.2.7 B).

Expression of the integrin CD103 by Tregs has been reported as a marker of Treg activation [244]. CD103$^+$ Tregs have been previously shown to release more IL-10 after *in vitro* stimulation with *H. polygyrus* antigen than CD103$^-$ Tregs, and CD103$^+$ Tregs were more effective at suppressing CD4$^+$ effector T cells *in vitro* [131]. Levels of CD103 expression were therefore examined within the total Treg population. BALB/c mice showed a marked elevation in CD103 expression amongst Foxp3$^+$CD4$^+$ cells following infection, which was not seen in C57BL/6 mice (Figure 3.2.7 C), however total numbers of CD103$^+$Fox3$^+$CD4$^+$ cells increased to similar levels following infection between the two strains (Figure 3.2.7 D).

Treg populations can be further subdivided into those that are thymically derived, and those induced in the periphery. The transcription factor Helios has been described as a marker of thymically derived, 'natural' Tregs [245], in contrast to peripherally 'induced' Tregs. Here, it was found that the increase in Foxp3$^+$ expression amongst CD4$^+$ cells after infection in BALB/c mice was not due to a rise in the Foxp3$^+$Helios$^-$ Treg compartment (Figure 3.2.7 E), but in the Foxp3$^+$Helios$^+$ Treg population (Figure 3.2.7 G). The total number of both Helios$^-$ and Helios$^+$ Tregs increased with *H. polygyrus* infection in both BALB/c and C57BL/6 mice, however interestingly, total numbers of Helios$^-$ Tregs were higher in C57BL/6 infected mice than in BALB/c infected mice, and total numbers of Helios$^+$ Tregs were higher in BALB/c infected mice than in infected C57BL/6 mice (Figure 3.2.7 F,H,M). Infected C57BL/6 mice not only had a higher proportion and number of Helios$^-$ Tregs than BALB/c mice (Figure 3.2.7 E,F,M), but they also may be more functionally active, as they showed higher expression levels of CD103 than BALB/c mice (Figure 3.2.7 I+J). Conversely, infected BALB/c mice had a higher proportion of CD103$^+$Helios$^+$ Tregs than infected C57BL/6 mice (Figure 3.2.7 K), though no differences were seen in total numbers of these cells (Figure 3.2.7 L).

3.2.8 The proportion of Helios$^+$ Tregs positively correlates with day 28 worm burden in BALB/c, but not C57BL/6 mice

Whether the differential expansion of Treg subsets correlated with worm burdens 28 days post-infection was next examined. For BALB/c mice, the proportion of MLN CD4$^+$ cells expressing both Foxp3 and Helios positively correlated with day 28 worm burden (Figure 3.2.8 C). The proportion of Foxp3$^+$Helios$^-$ cells in BALB/c mice also positively correlated with day-28 worm burden, though this did not reach statistical significance (p= 0.0784; Figure 3.2.8 A). Additionally, absolute numbers of these Treg subsets positively correlated with worm burden in this strain (data not shown).

In contrast, neither proportion nor total numbers of Foxp3$^+$Helios$^-$ or Foxp3$^+$Helios$^+$ CD4$^+$ MLN cells correlated with worm burden in C57BL/6 mice (Figure 3.2.8 B+D+data not shown). This perhaps indicates that Treg expansion is not as important for control of worm survival in C57BL/6 mice, or perhaps no relationship between these parameters is seen as the majority of C57BL/6 mice retain a high level of worms at this timepoint.

3.2.9 Microbial populations in the duodenum are altered during *H. polygyrus*-infection in BALB/c and C57BL/6 mice

Next, factors other than the immune response of the mice were examined, to see how they affected *H. polygyrus* survival within the host. *H. polygyrus* resides in the small intestine, where it is surrounded by hundreds of microbial species. Changes in the microflora composition have been previously noted after *H. polygyrus* infection [218], yet whether this change is beneficial for the survival of *H. polygyrus* has not been examined, and whether this change is activity induced by the worm is unknown.

In this study, the relative levels of the bacterial groups *Lactobacillus/Lactococcus*, *Bacteroides*, *Eubacterium/Clostridium* and Enterobacteriaceae were measured by real-time PCR using 16S rRNA gene-specific primers on samples from the duodenum and jejunum of naïve and

infected mice. Infected BALB/c mice showed elevated levels of *Lactobacillus/Lactococcus* in the small intestine at day 14 post-infection, though this did not reach statistical significance (Figure 3.2.9 A). The same trend occurred in C57BL/6 mice, with a significantly higher *Lactobacillus/Lactococcus* load in the duodenum of infected mice (Figure 3.2.9 B). By 28 days post-infection however, the duodenum *Lactobacillus/Lactococcus* abundance in BALB/c mice had dropped, to below the level of naïve mice (Figure 3.2.9 C). This was not the case for C57BL/6 mice, where *H. polygyrus*-infected animals maintained higher levels of *Lactobacillus/Lactococcus* in the duodenum 28 days post-infection (Figure 3.2.9 D).

Enterobacteriaceae species were also altered during infection, with a significant increase seen at day 14 post-infection in the jejunum of infected BALB/c mice (Figure 3.2.9 E), but no change in abundance with infection in C57BL/6 mice at this time point (Figure 3.2.9 F). Similarly to the pattern of *Lactobacillus/Lactococcus* abundance, Enterobacteriaceae levels are reduced in infected BALB/c mice compared to naïve controls by day 28 of infection (Figure 3.2.9 G), whereas the same bacterial group is more abundant in infected C57BL/6 mice compared to naïve controls at this time point (Figure 3.2.9 H).

No change in the abundance of *Bacteroides* or *Eubacterium/Clostridium* were seen at day 14 or day 28 post-infection, in either BALB/c or C57BL/6 mice (data not shown).

The shifts in bacterial populations between strains may result from differing immune environments in BALB/c versus C57BL/6 mice, as certain subgroups of bacteria are may vary in their tolerance for particular immune conditions. Alternatively, the reduction in *Lactobacillus/Lactococcus* and Enterobacteriaceae in BALB/c mice at day 28 may be a reflection of the fact that the majority of these mice have expelled all their worms, or have a very low worm burden at this time point (Figure 3.2.1 A).

3.2.10 Levels of duodenal *Lactobacillus/Lactococcus* species and Enterobacteriaceae positively correlate with adult worm survival 28 days post-infection, in BALB/c and C57BL/6 mice respectively

To investigate whether the shifts in *Lactobacillus/Lactococcus* and Enterobacteriaceae were linked to worm survival within the host, correlations were made between the abundance of these bacterial groups and worm burdens at day 28 post-infection. Interestingly, both groups of bacteria that were altered in abundance after *H. polygyrus* infection were significantly associated with *H. polygyrus* persistence in the host. Those BALB/c mice which maintained high *Lactobacillus/Lactococcus* levels in the duodenum 28 days post-infection had higher worm burdens at day 28 post-infection (Figure 3.2.10 A), though there was no relationship with *Lactobacillus/Lactococcus* and worm burdens in C57BL/6 mice at the same timepoint (Figure 3.2.10 B). Conversely, there was a strong positive correlation between duodenal levels of Enterobacteriaceae and worm burden at day 28 of infection in C57BL/6 mice (Figure 3.2.10 D) which was not evident in the BALB/c strain (Figure 3.2.10 C).

No correlations between day 28 duodenal *Bacteroides* or *Eubacterium/Clostridium* levels and worm burdens existed in either strain of mice.

Pre-infection microbiota levels were also determined from faecal samples taken on the day of infection, and compared with the day 28 worm burden. Surprisingly, pre-infection faecal Enterobacteriaceae levels are associated with stronger immunity in BALB/c mice 28 days post-infection (Figure 3.2.10 E). No other bacterial groups in pre-infection faeces correlated with day 28 worm burden, in either strain (data not shown). That initial Enterobacteriaceae levels in BALB/c mice are able to predispose mice towards differing susceptibility to *H. polygyrus* highlights the potential importance of the microflora, yet this finding made it necessary to further explore why the microflora populations differentially influence immunity between BALB/c and C57BL/6 mice.

3.2.11 Levels of duodenal *Bacteroides* species positively correlate with granuloma formation 28 days post-infection, in BALB/c mice

As previously shown here, the formation of granulomas along the intestinal tract after infection occurred in BALB/c mice, and this had a weak negative correlation with worm burden (Figure 3.2.1 E+F). Although the levels of duodenal *Bacteroides* species at 28 days post-infection did not correlate with worm burden in BALB/c mice (data not shown), *Bacteroides* levels at this time point did positively correlate with granuloma number (Figure 3.2.11 A), whereas no other bacterial group correlated with granuloma formation (Figure 3.2.11 B-D). This may indicate that granulomas form in response to exposure to a species of *Bacteroides*, perhaps after the intestinal mucosal barrier is disrupted during the lifecycle of *H. polygyrus*, allowing increased penetration of these bacterial species through the epithelial cell layer.

Granuloma formation following infection was very rare in C57BL/6 mice (Figure 3.2.1 E), and so bacterial correlations with granuloma formation could not be performed for this strain.

3.2.12 HES-specific cytokine production correlates differentially with the levels of duodenal *Lactobacillus/Lactococcus* and Enterobacteriaceae species in BALB/c and C57BL/6 mice

The intriguing contrast between how the abundance of duodenal *Lactobacillus*/*Lactococcus* and Enterobacteriaceae species affected *H. polygyrus* burden in BALB/c and C57BL/6 mice was further explored by examining how the levels of these bacterial groups were related to the cytokine response to *H. polygyrus*. IL-17A production by MLN cells in response to HES positively correlated with duodenal *Lactobacillus/Lactococcus* species abundance at day 28 of infection in BALB/c (Figure 3.2.12 A), but not C57BL/6 mice (Figure 3.2.12 B). IL-17A was the only cytokine measured which showed this positive correlation in BALB/c mice; no correlation with *Lactobacillus/Lactococcus* levels was seen with HES-specific IL-4, IL-5, IL-9, IL-10, IL-13 or IFN-γ, neither did any of

these cytokines correlate with *Lactobacillus/Lactococcus* levels in C57BL/6 mice (data not shown).

Furthermore, HES-specific production of the Th2 cytokines IL-4, IL-5 and IL-10 all positively correlated with duodenal Enterobacteriaceae levels in infected BALB/c mice (Figure 3.2.12 C,E,G), whereas these same cytokines negatively correlated with duodenal Enterobacteriaceae levels in infected C57BL/6 mice (Figure 3.2.12 D,F,G).

Neither *Bacteroides* nor *Eubacterium/Clostridum* species showed a relationship with HES-specific cytokine production in either BALB/c or C57BL/6 mice (data not shown).

3.2.13 Treg number correlates with the levels of *Lactobacillus/Lactococcus* and Enterobacteriaceae species in the duodenum differentially between BALB/c and C57BL/6 mice

To ascertain whether Enterobacteriaceae species inhibit the production of Th2 cytokines in C57BL/6 mice via promotion of a regulatory response, the relationship between Treg numbers and duodenal bacterial levels at day 28 of *H. polygyrus*-infection was examined. It was found that duodenal Enterobacteriaceae species in C57BL/6 mice did positively correlate with MLN Treg numbers (Figure 3.2.13 B), so it is conceivable that this is the mechanism by which Enterobacteriaceae species affect HES-specific Th2 cytokine production. Opposingly, Enterobacteriaceae species negatively correlated with MLN Treg number in BALB/c mice (Figure 3.2.13 A), perhaps as they preferentially promote Th2 responses in these mice (Figure 3.2.12 C,E,G).

Another mechanism by which *Lactobacillus/Lactococcus* species could be promoting *H. polygyrus* survival in BALB/c mice (Figure 3.2.10 A) is through the promotion of Treg differentiation which would act to dampen a worm-expelling Th2 response, as in these mice *Lactobacillus/Lactococcus* species abundance positively correlated with MLN Treg number (Figure 3.2.13 C). In

contrast no relationship between these variables was seen in C57BL/6 mice (Figure 3.2.13 D), where *Lactobacillus/Lactococcus* species abundance had no effect on worm burden (Figure 3.2.10 C).

It should be noted, however, that no correlations were found between proportions of any Treg subset and levels of duodenal bacteria (data not shown). Thus relationships between the total number of Treg subsets and bacterial group abundance may be an indirect relationship, as a result of the proportional increase in MLN size with worm burdens.

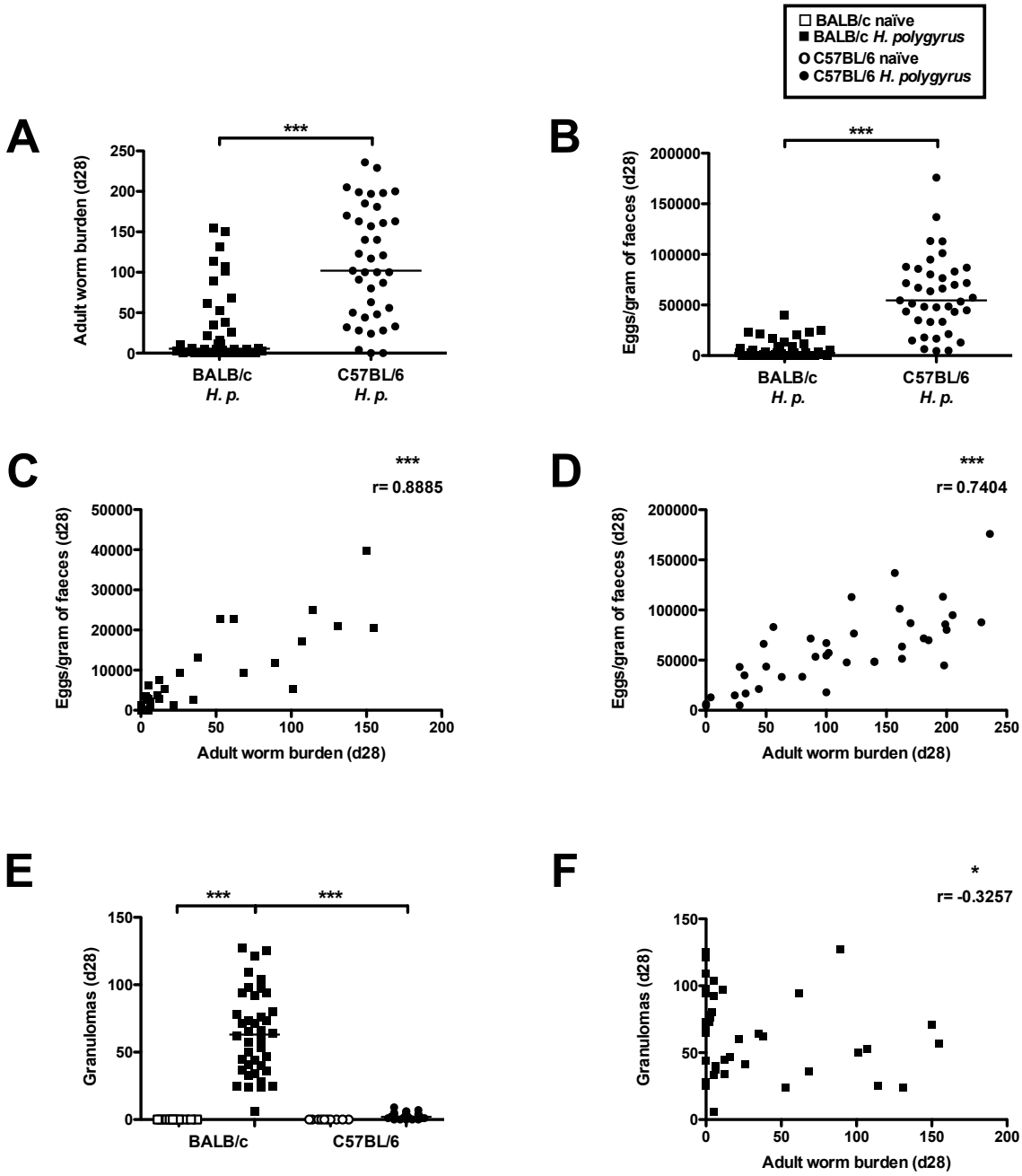

Figure 3.2.1 BALB/c and C57BL/6 mouse strains show contrasting levels of susceptibility and granuloma development, but both show substantial intra-strain variability in response to infection.

BALB/c or C57BL/6 female mice were left naïve or infected with 200 *H. polygyrus* L3s. Open symbols (BALB/c= □, C57BL/6= O) represent naïve mice; closed symbols (BALB/c= ■, C57BL/6= ●) represent *H. polygyrus*-infected mice. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown.

* indicates p= <0.05; *** indicates p= <0.001.

Data shown for each strain is from one experiment; strain experiments were performed separately.

(A) Adult *H. polygyrus* numbers recovered from the intestinal tract 28 days post *H. polygyrus*-infection.

(B) *H. polygyrus* eggs per gram of faeces taken 28 days post *H. polygyrus*-infection.

(C+D) Correlation of adult *H. polygyrus* numbers recovered from the intestinal tract 28 days post *H. polygyrus*-infection and *H. polygyrus* eggs per gram of faeces at the same timepoint, in **(C)** BALB/c and **(D)** C57BL/6 mice.

(E) Granuloma number along the intestinal tract of naïve or 28-day *H. polygyrus*-infected mice.

(F) Correlation of adult *H. polygyrus* numbers recovered from the intestinal tract 28 days post *H. polygyrus*-infection and number of granulomas along the intestinal tract at the same timepoint in BALB/c mice.

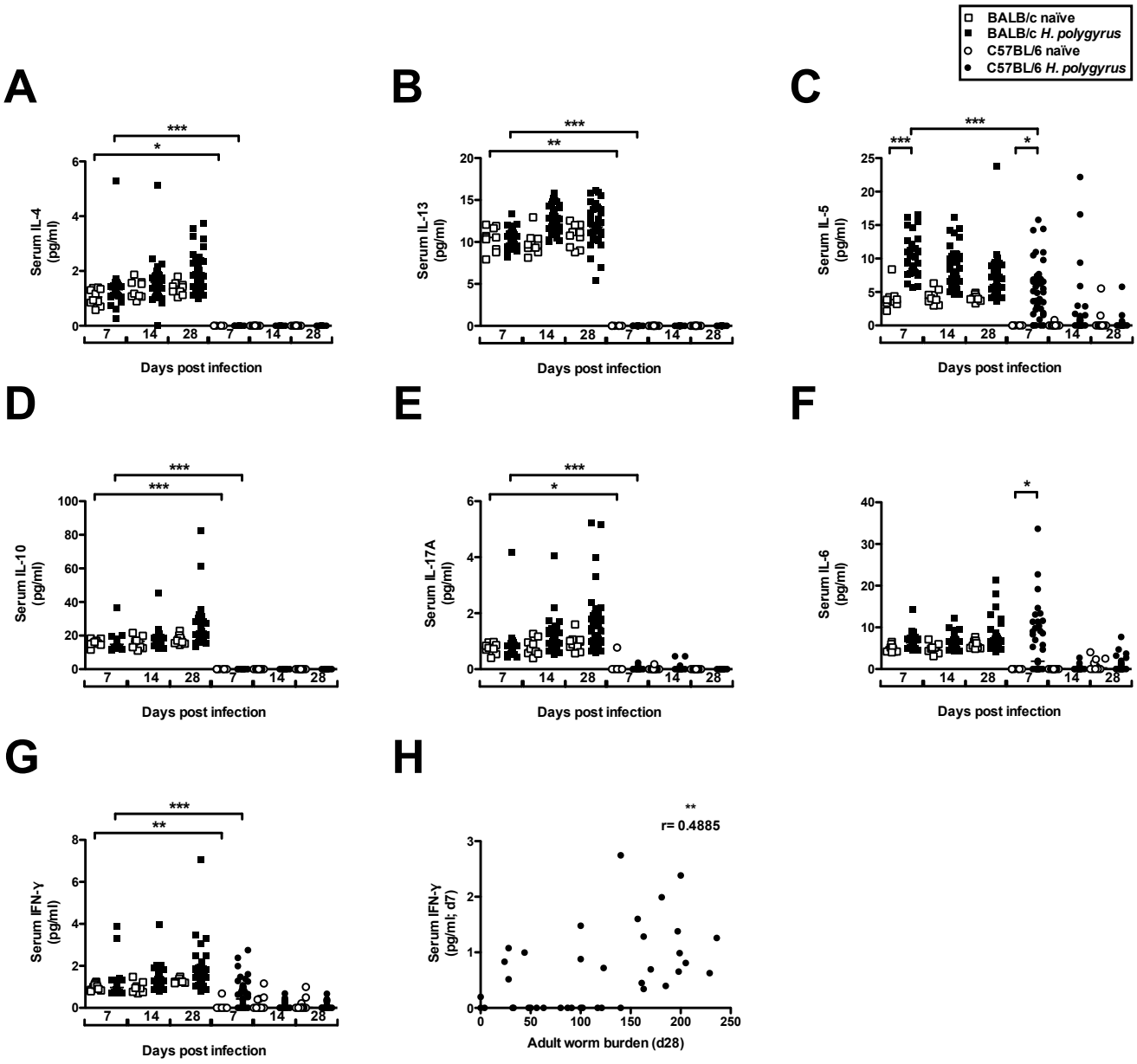

Figure 3.2.2 Serum cytokines measured in the course of infection show strain differences and can correlate with infection outcome.

BALB/c or C57BL/6 female mice were left naïve or infected with 200 *H. polygyrus* L3s. Blood was taken at days 7, 14 and 28 post-infection, and levels of cytokines in the sera were measured. Open symbols (BALB/c= □, C57BL/6= O) represent naïve mice; closed symbols (BALB/c= ■, C57BL/6= ●) represent *H. polygyrus*-infected mice. Data shown for each strain is from one experiment; strain experiments were performed separately.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

(A-G) Serum concentration of **(A)** IL-4 **(B)** IL-13 **(C)** IL-5 **(D)** IL-10 **(E)** IL-17A **(F)** IL-6 and **(G)** IFN-γ. Statistical comparisons only between naïve and 7-day infected BALB/c and C57BL/6 mice are shown, as analysed by Kruskal-Wallis tests followed by Dunn's Multiple Comparison tests.

(H) Correlation of adult *H. polygyrus* numbers recovered from the intestinal tract 28 days post *H. polygyrus*-infection and day 7 serum concentrations of IFN-γ in C57BL/6 mice. r= Spearman r correlation coefficient.

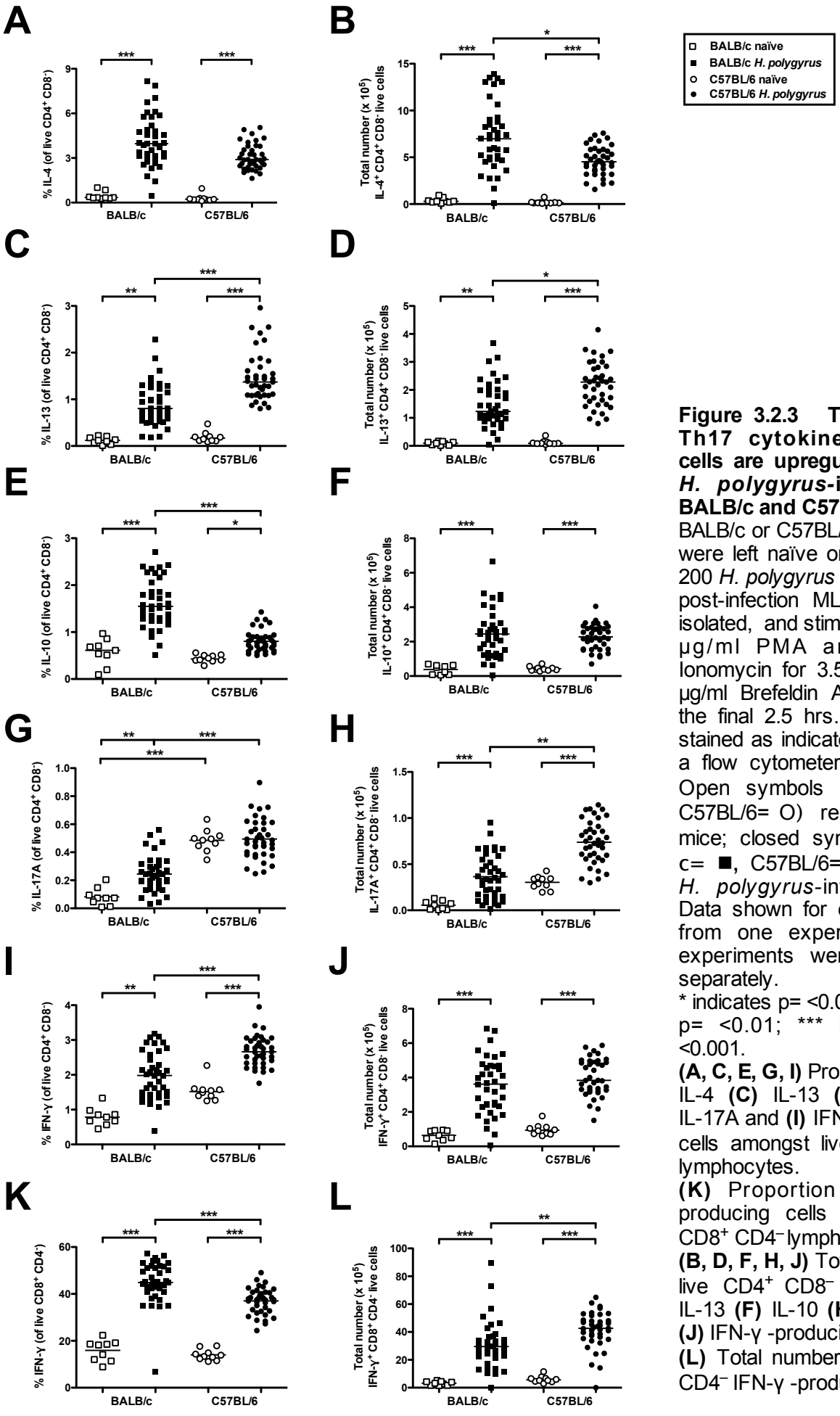

Figure 3.2.3 Th1, Th2 and Th17 cytokine-producing cells are upregulated during *H. polygyrus*-infection in BALB/c and C57BL/6 mice.
BALB/c or C57BL/6 female mice were left naïve or infected with 200 *H. polygyrus* L3s. 28 days post-infection MLN cells were isolated, and stimulated with 0.5 µg/ml PMA and 1 µg/ml Ionomycin for 3.5 hrs, with 10 µg/ml Brefeldin A included for the final 2.5 hrs. Cells were stained as indicated and run on a flow cytometer for analysis. Open symbols (BALB/c= □, C57BL/6= O) represent naïve mice; closed symbols (BALB/c= ■, C57BL/6= ●) represent *H. polygyrus*-infected mice. Data shown for each strain is from one experiment; strain experiments were performed separately.
* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.
(A, C, E, G, I) Proportions of **(A)** IL-4 **(C)** IL-13 **(E)** IL-10 **(G)** IL-17A and **(I)** IFN-γ -producing cells amongst live $CD4^+$ $CD8^-$ lymphocytes.
(K) Proportion of IFN-γ -producing cells amongst live $CD8^+$ $CD4^-$ lymphocytes.
(B, D, F, H, J) Total numbers of live $CD4^+$ $CD8^-$ **(B)** IL-4 **(D)** IL-13 **(F)** IL-10 **(H)** IL-17A and **(J)** IFN-γ -producing cells.
(L) Total number of live $CD8^+$ $CD4^-$ IFN-γ -producing cells.

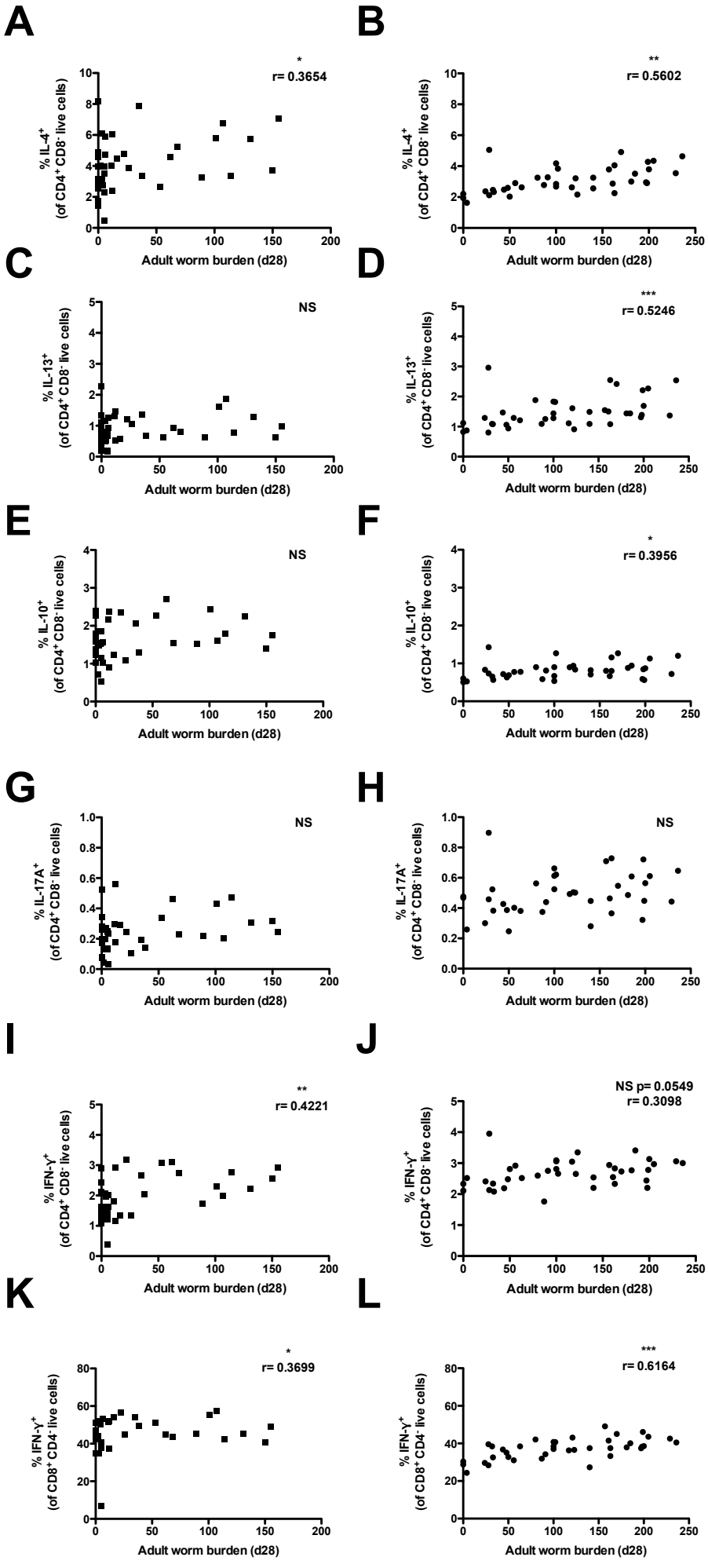

Figure 3.2.4 Cytokine production at day 28 of infection principally reflects worm load in BALB/c and C57BL/6 mice.

BALB/c (■) or C57BL/6 (●) female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection the number of adult worms along the intestinal tract were enumerated, and MLN cells were isolated and stimulated with 0.5 µg/ml PMA and 1 µg/ml Ionomycin for 3.5 hrs, with 10 µg/ml Brefeldin A included for the final 2.5 hrs. Cells were stained as indicated and run on a flow cytometer for analysis. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown for each strain is from one experiment; strain experiments were performed separately.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001; NS indicates a non significant correlation.

(A, C, E, G, I, K) Correlation of adult *H. polygyrus* numbers recovered from the intestinal tract of BALB/c mice 28 days post *H. polygyrus*-infection and the % of **(A)** IL-4 amongst $CD4^+$ **(C)** IL-13 amongst $CD4^+$ **(E)** IL-10 amongst $CD4^+$ **(G)** IL-17A amongst $CD4^+$ **(I)** IFN-γ amongst $CD4^+$ and **(K)** IFN-γ amongst $CD8^+$ MLN cells.

(B, D, F, H, J, L) Correlation of adult *H. polygyrus* numbers recovered from the intestinal tract of C57BL/6 mice 28 days post *H. polygyrus*-infection and the % of **(B)** IL-4 amongst $CD4^+$ **(D)** IL-13 amongst $CD4^+$ **(F)** IL-10 amongst $CD4^+$ **(H)** IL-17A amongst $CD4^+$ **(J)** IFN-γ amongst $CD4^+$ and **(L)** IFN-γ amongst $CD8^+$ MLN cells.

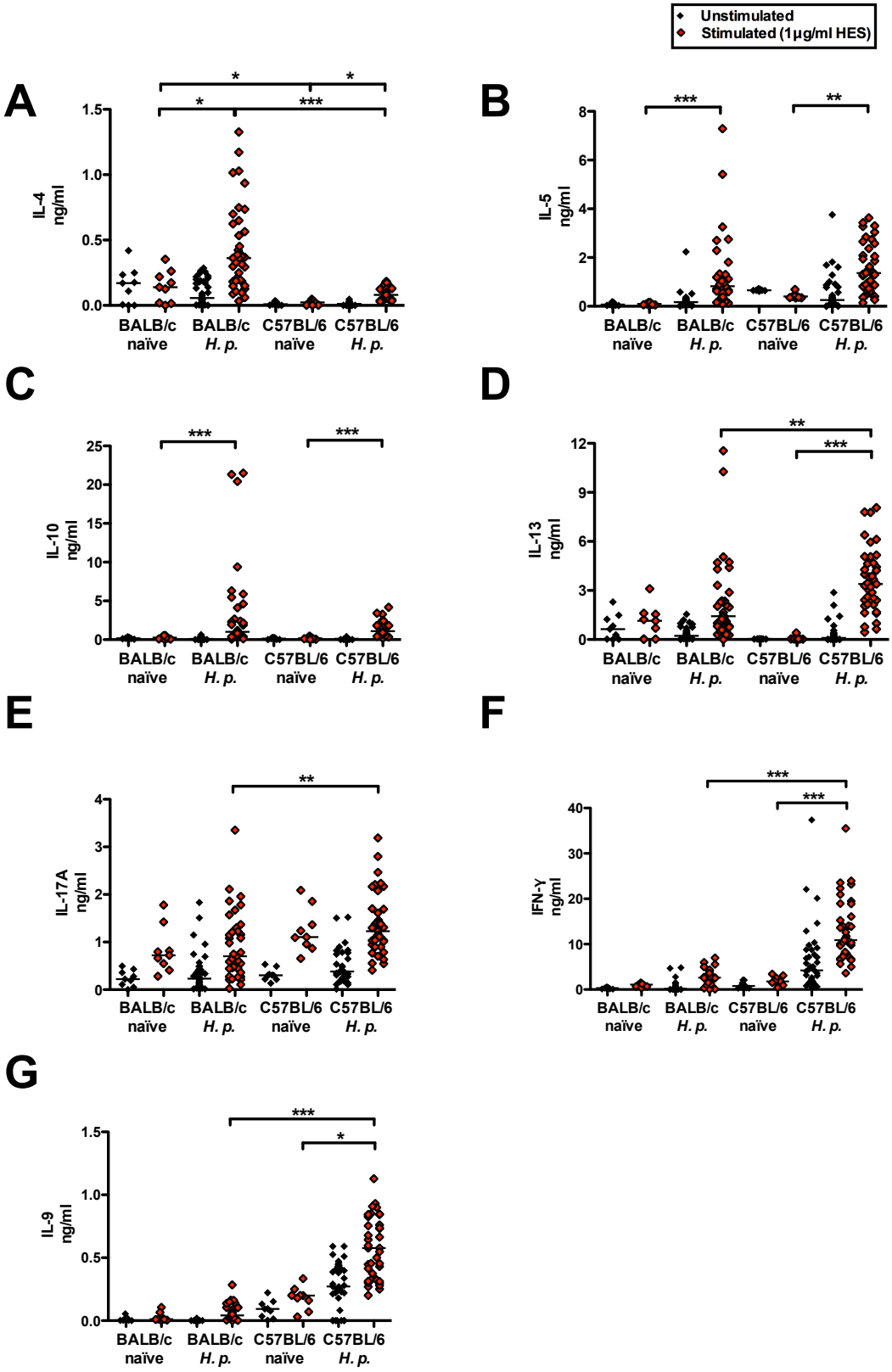

Figure 3.2.5 BALB/c mice mount a stronger HES-specific Th2 response and a poorer HES-specific Th1 response than C57BL/6 mice.
BALB/c female or C57BL/6 mice were left naïve or infected with 200 *H. polygyrus* L3s. 28 days post-infection, MLN cells were isolated and cultured in Complete RPMI media in the absence (◆) or presence (◆) of 1 µg/ml HES for 72 hrs. Levels of cytokines released into the media were then assayed by ELISA. Each data point represents the mean value from duplicate culture wells from one mouse. Kruskal-Wallis tests were performed between the HES-stimulated groups only. Data shown for each strain is from one experiment; strain experiments were performed separately.
* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.
Concentration of **(A)** IL-4 **(B)** IL-5 **(C)** IL-10 **(D)** IL-13 **(E)** IL-17A **(F)** IFN-γ and **(G)** IL-9.

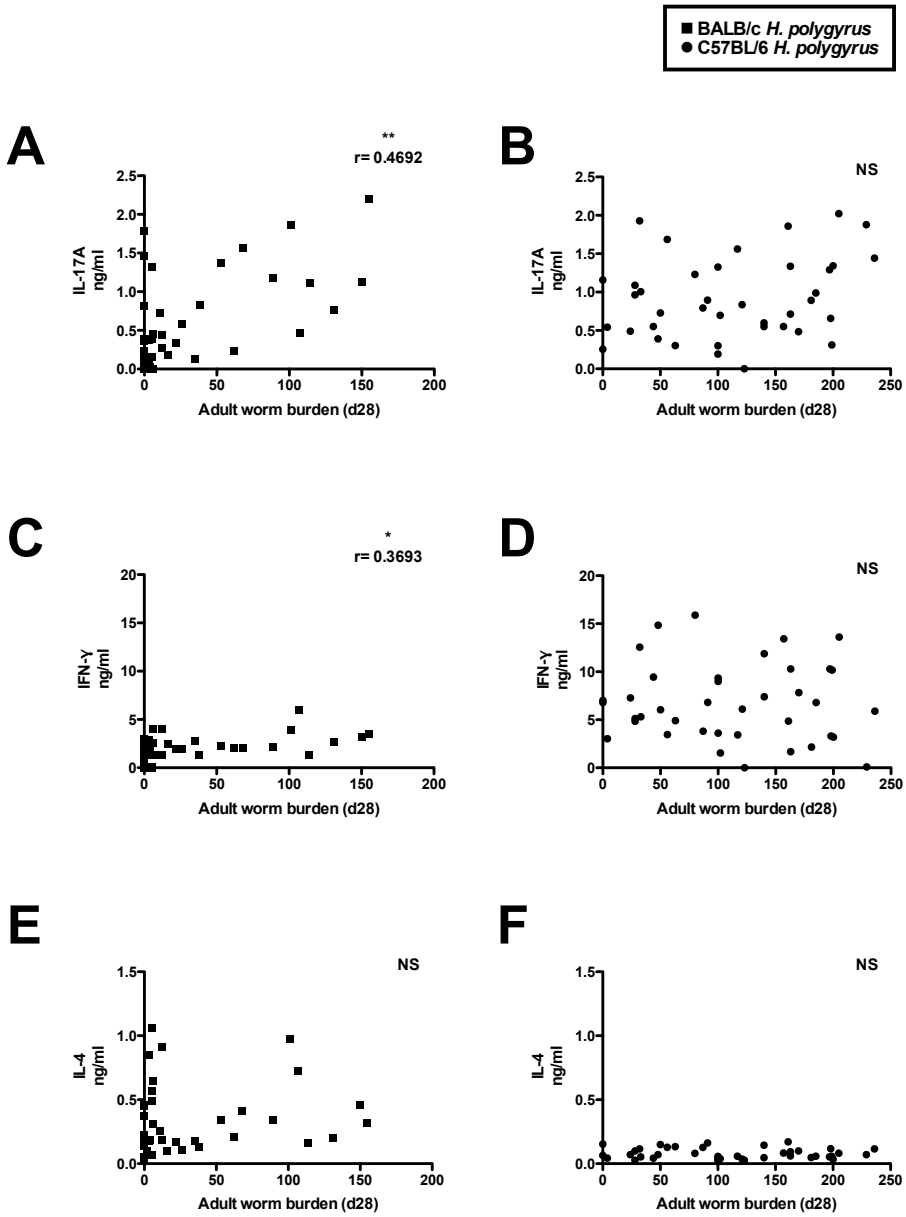

Figure 3.2.6 HES-specific inflammatory cytokine production correlates with high worm burdens in BALB/c, but not C57BL/6 mice.
BALB/c (■) or C57BL/6 (●) female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection, MLN cells were isolated and cultured in Complete RPMI media in the absence or presence of 1 µg/ml HES for 72 hrs. Levels of cytokines released into the media were then assayed by ELISA. Each data point represents the mean concentration from duplicate HES stimulated culture wells from one mouse minus the concentration of cytokine in the media of unstimulated cells. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown for each strain is from one experiment; strain experiments were performed separately.
* indicates p= <0.05; ** indicates p= <0.01; NS indicates a non significant correlation.
(A, C, E) For BALB/c mice, correlations were performed between the number of adult worms along the intestinal tract 28 days post-infection and the concentration of **(A)** IL-17A **(C)** IFN-γ and **(E)** IL-4 in the supernatant of restimulated MLN cells.
(B, D, F) For C57BL/6 mice, correlations were performed between the number of adult worms along the intestinal tract 28 days post-infection and the concentration of **(A)** IL-17A **(C)** IFN-γ and **(E)** IL-4 in the supernatant of restimulated MLN cells.

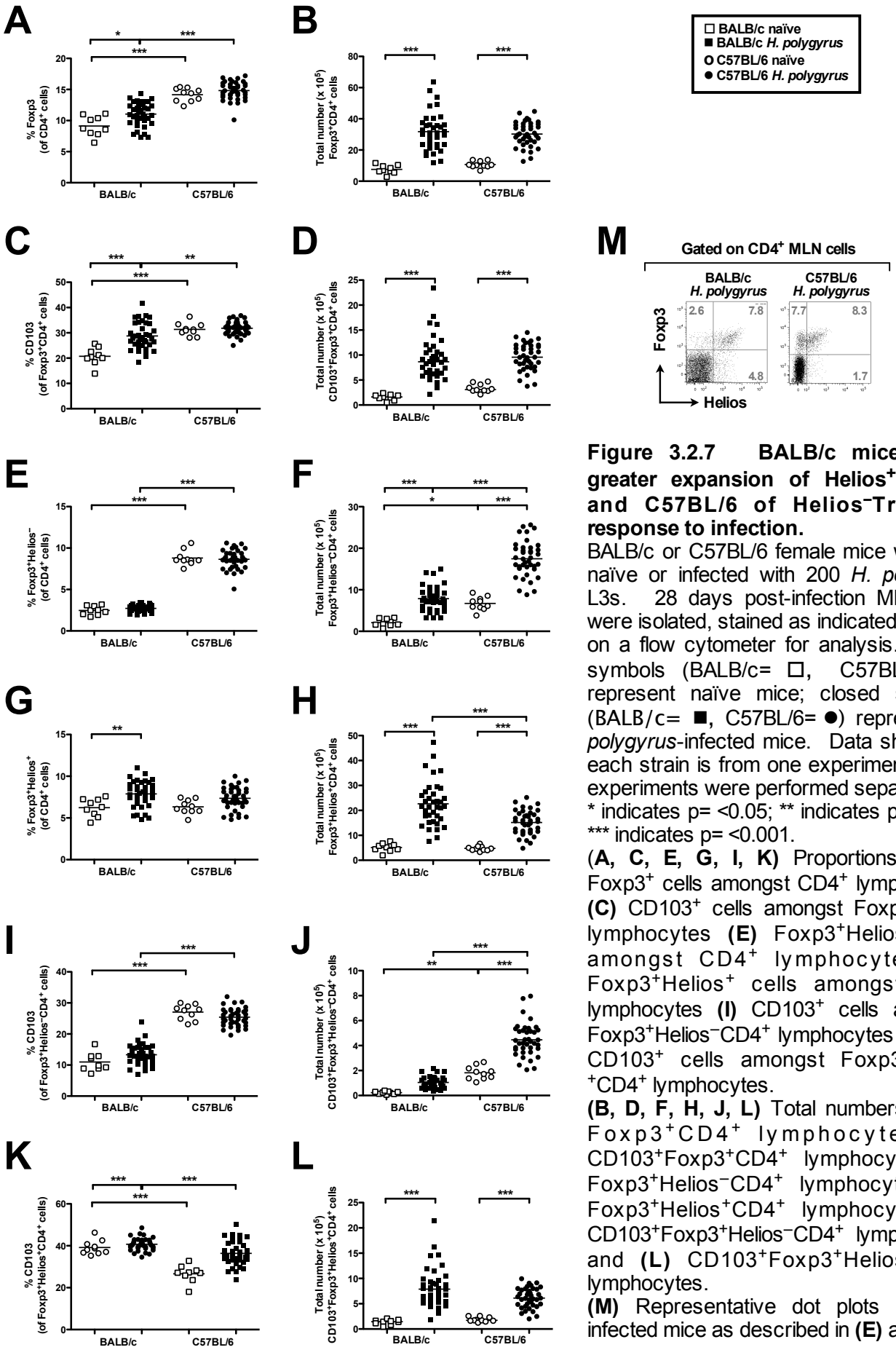

Figure 3.2.7 BALB/c mice show greater expansion of Helios$^+$ Tregs, and C57BL/6 of Helios$^-$Tregs in response to infection.

BALB/c or C57BL/6 female mice were left naïve or infected with 200 *H. polygyrus* L3s. 28 days post-infection MLN cells were isolated, stained as indicated and run on a flow cytometer for analysis. Open symbols (BALB/c= □, C57BL/6= O) represent naïve mice; closed symbols (BALB/c= ■, C57BL/6= ●) represent *H. polygyrus*-infected mice. Data shown for each strain is from one experiment; strain experiments were performed separately.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

(**A, C, E, G, I, K)** Proportions of **(A)** Foxp3$^+$ cells amongst CD4$^+$ lymphocytes **(C)** CD103$^+$ cells amongst Foxp3$^+$CD4$^+$ lymphocytes **(E)** Foxp3$^+$Helios$^-$ cells amongst CD4$^+$ lymphocytes **(G)** Foxp3$^+$Helios$^+$ cells amongst CD4$^+$ lymphocytes **(I)** CD103$^+$ cells amongst Foxp3$^+$Helios$^-$CD4$^+$ lymphocytes and **(K)** CD103$^+$ cells amongst Foxp3$^+$Helios$^+$CD4$^+$ lymphocytes.

(B, D, F, H, J, L) Total numbers of **(B)** Foxp3$^+$CD4$^+$ lymphocytes **(D)** CD103$^+$Foxp3$^+$CD4$^+$ lymphocytes **(F)** Foxp3$^+$Helios$^-$CD4$^+$ lymphocytes **(H)** Foxp3$^+$Helios$^+$CD4$^+$ lymphocytes **(J)** CD103$^+$Foxp3$^+$Helios$^-$CD4$^+$ lymphocytes and **(L)** CD103$^+$Foxp3$^+$Helios$^+$CD4$^+$ lymphocytes.

(M) Representative dot plots showing infected mice as described in **(E)** and **(G)**.

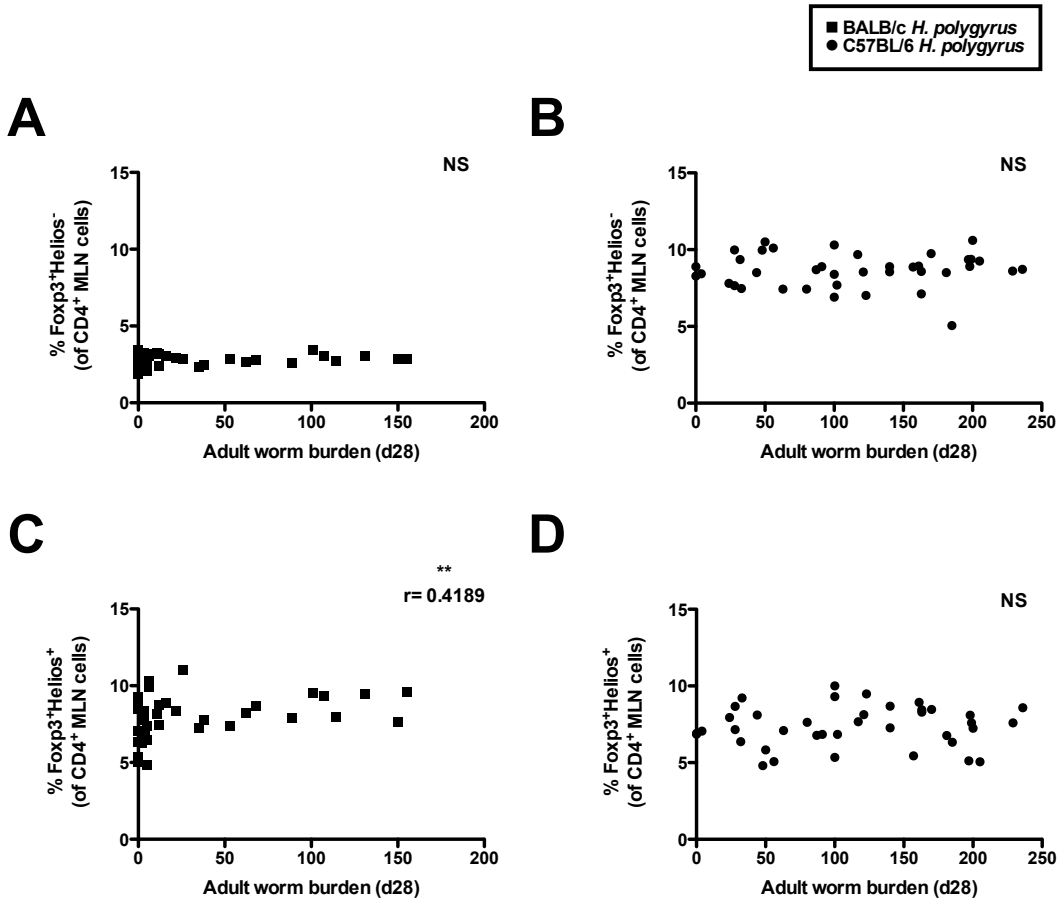

Figure 3.2.8 The proportion of Helios$^+$ Tregs positively correlates with day 28 worm burden in BALB/c, but not C57BL/6 mice.

BALB/c (■) or C57BL/6 (●) female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection MLN cells were isolated, stained as indicated and run on a flow cytometer for analysis. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown for each strain is from one experiment; strain experiments were performed separately.

** indicates p= <0.01; NS indicates a non significant correlation.

(A, C) Correlation of adult worm numbers along the intestinal tract at day 28 post-infection in BALB/c mice, and the proportion of **(A)** Foxp3$^+$Helios$^-$ and **(C)** Foxp3$^+$Helios$^+$ cells amongst CD4$^+$ MLN cells.

(B, D) Correlation of adult worm numbers along the intestinal tract at day 28 post-infection in C57BL/6 mice, and the proportion of **(B)** Foxp3$^+$Helios$^-$ and **(D)** Foxp3$^+$Helios$^+$ cells amongst CD4$^+$ MLN cells.

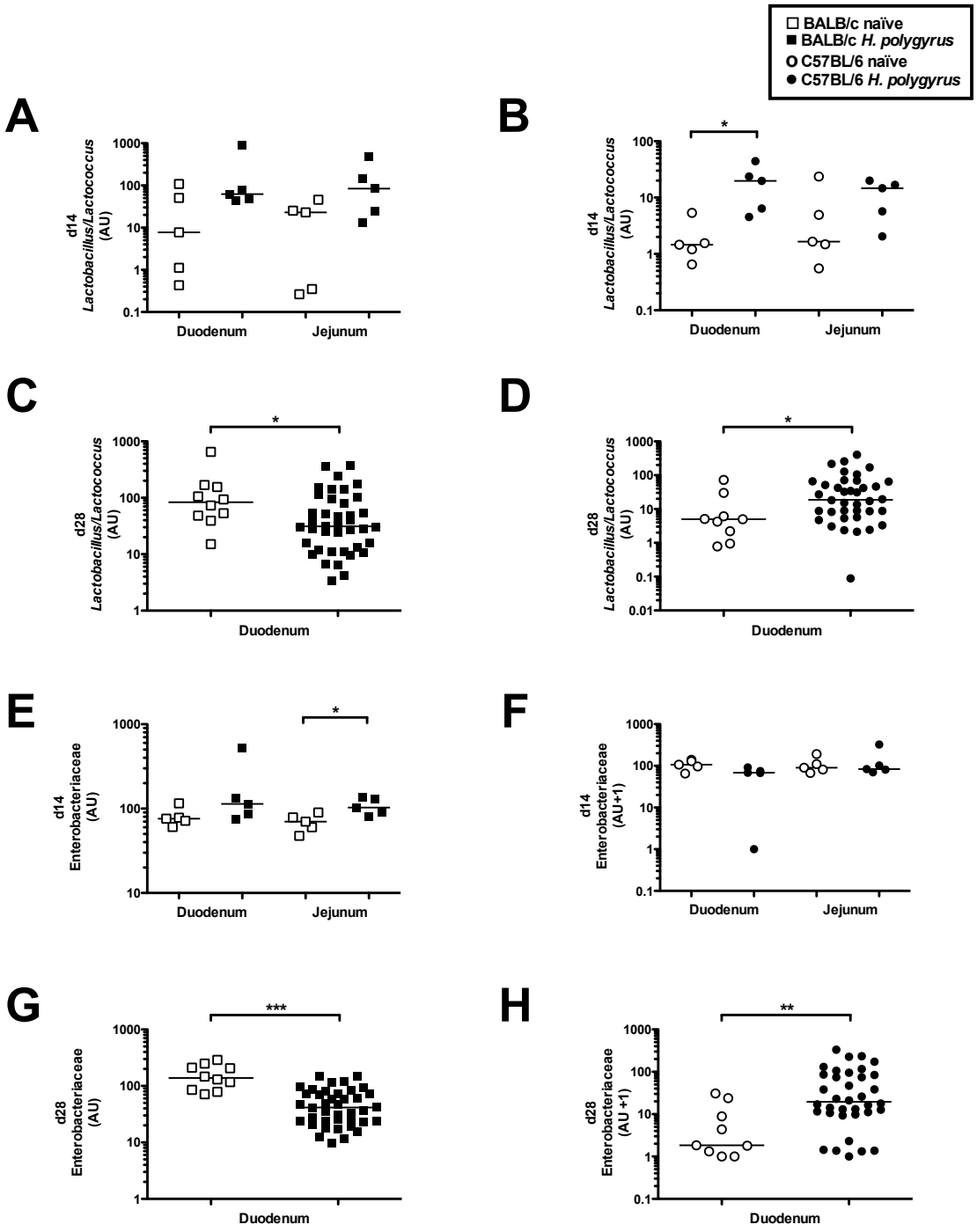

Figure 3.2.9 Microbial populations in the duodenum are altered during *H. polygyrus*-infection in BALB/c and C57BL/6 mice.

BALB/c or C57BL/6 mice were left naïve or infected with 200 *H. polygyrus* L3s. 14 days or 28 days post-infection, total DNA was extracted from the duodenum and jejunum. Relative levels of *Lactobacillus/ Lactococcus* and Enterobacteriaceae were determined by real-time PCR, using primers specific for the 16S rRNA gene of each bacterial group.

Open symbols (BALB/c= □, C57BL/6= O) represent naïve mice; closed symbols (BALB/c= ■, C57BL/6= ●) represent *H. polygyrus*-infected mice. * indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

(A+B) *Lactobacillus/Lactococcus* concentrations in the duodenum and jejunum of naïve or 14-day infected **(A)** BALB/c mice and **(B)** C57BL/6 mice. Data are representative of **(A)** one experiment and **(B)** four independent experiments for the duodenum; two for the jejunum.

(C+D) *Lactobacillus/Lactococcus* concentrations in the duodenum and jejunum of naïve or 28-day infected **(C)** BALB/c mice and **(D)** C57BL/6 mice. Data are representaive of one experiment.

(E+F) Enterobacteriaceae concentrations in the duodenum of naïve or 14-day infected **(E)** BALB/c mice and **(F)** C57BL/6 mice. Data are representative of one experiment for BALB/c mice; two experiments for C57BL/ 6 mice.

(G+H) Enterobacteriaceae concentrations in the duodenum of naïve or 28-day infected **(G)** BALB/c mice and **(H)** C57BL/6 mice. Data are representative of one experiment.

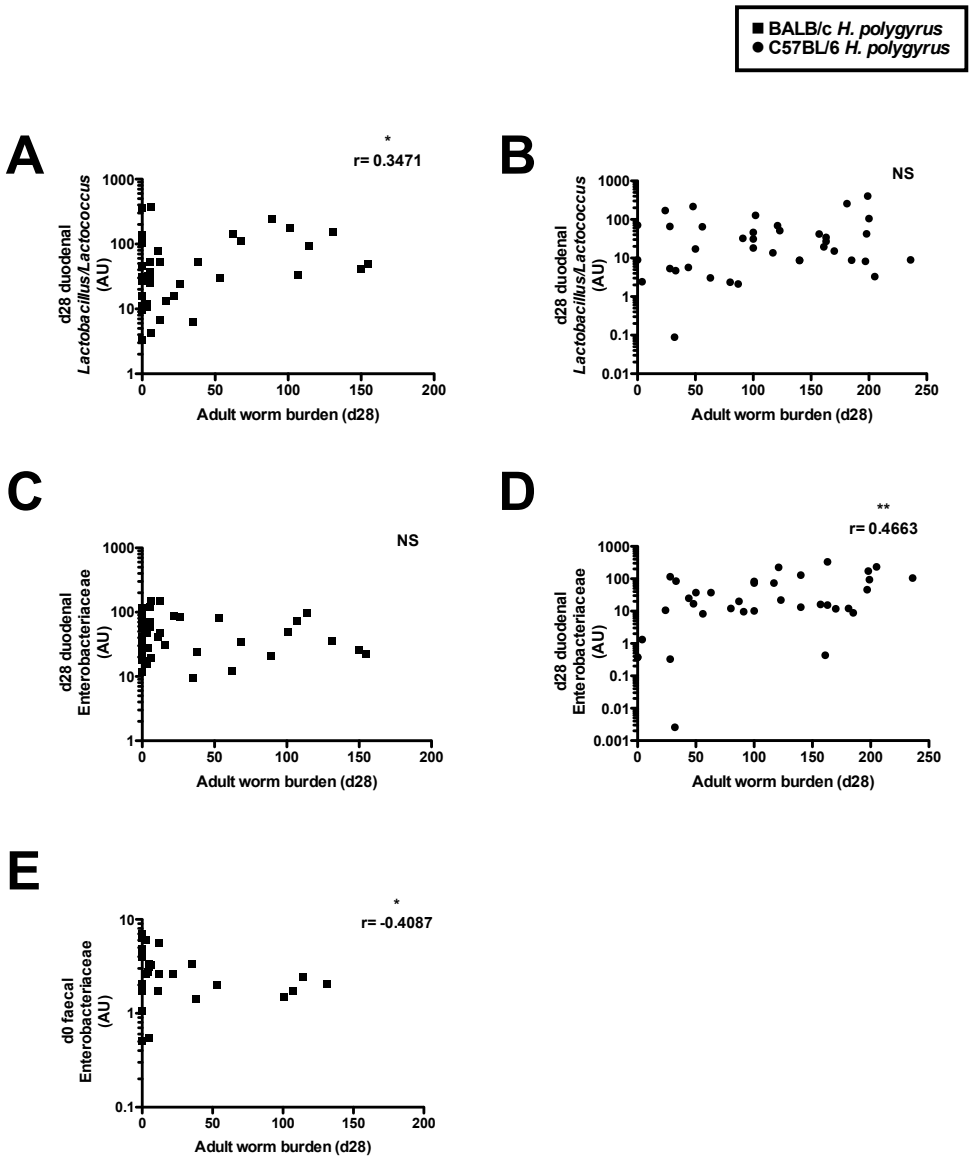

Figure 3.2.10 Levels of duodenal *Lactobacillus/Lactococcus* species and Enterobacteriaceae positively correlate with adult worm survival 28 days post-infection, in BALB/c and C57BL/6 mice respectively, and faecal Enterobacteriaceae can be used as a predictor of immunity in BALB/c mice.

Faeces were collected from BALB/c (■) or C57BL/6 (●) female mice prior to infection, and total DNA was extracted. Mice were then infected with 200 *H. polygyrus* L3s. 28 days post-infection, the number of adult worms along the intestinal tract were enumerated, and total DNA was extracted from the duodenum. Relative levels of the indicated bacterial groups present within faeces from day 0 prior to infection and within duodenums day 28 post-infection were determined by real-time PCR, using primers specific for the 16S rRNA gene of each bacterial group. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown for each strain is from one experiment; strain experiments were performed separately.

* indicates p= <0.05; ** indicates p= <0.01; NS indicates a non significant correlation.

(A, C) Correlation of adult *H. polygyrus* numbers and d28 duodenal levels of **(A)** *Lactobacillus/ Lactococcus* and **(C)** Enterobacteriaceae in BALB/c mice.

(B, D) Correlation of adult *H. polygyrus* numbers and d28 duodenal levels of **(A)** *Lactobacillus/ Lactococcus* and **(D)** Enterobacteriaceae in C57BL/6 mice.

(E) Correlation of adult *H. polygyrus* numbers and d0 faecal levels of Enterobacteriaceae in BALB/c mice.

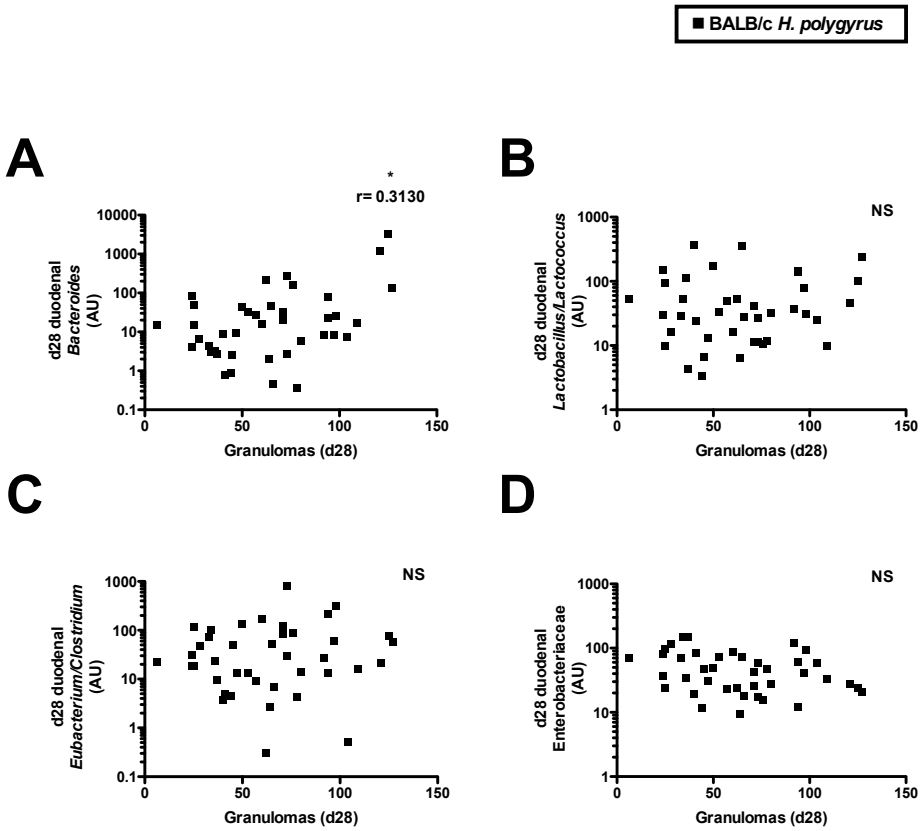

Figure 3.2.11 Levels of duodenal *Bacteroides* species positively correlate with granuloma formation 28 days post-infection, in BALB/c mice.

BALB/c (■) female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection, the number of granulomas along the intestinal tract were enumerated, and total DNA was extracted from the duodenum. Relative levels of the indicated bacterial groups present within the duodenum DNA were determined by real time PCR, using primers specific for the 16S rRNA gene of each bacterial group. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown is from one experiment.

* indicates p= <0.05; NS indicates a non significant correlation.

Correlation of intestinal granuloma numbers and duodenal levels of **(A)** *Bacteroides* **(B)** *Lactobacillus/Lactococcus* **(C)** *Eubacterium/Clostridium* and **(D)** Enterobacteriaceae**.**

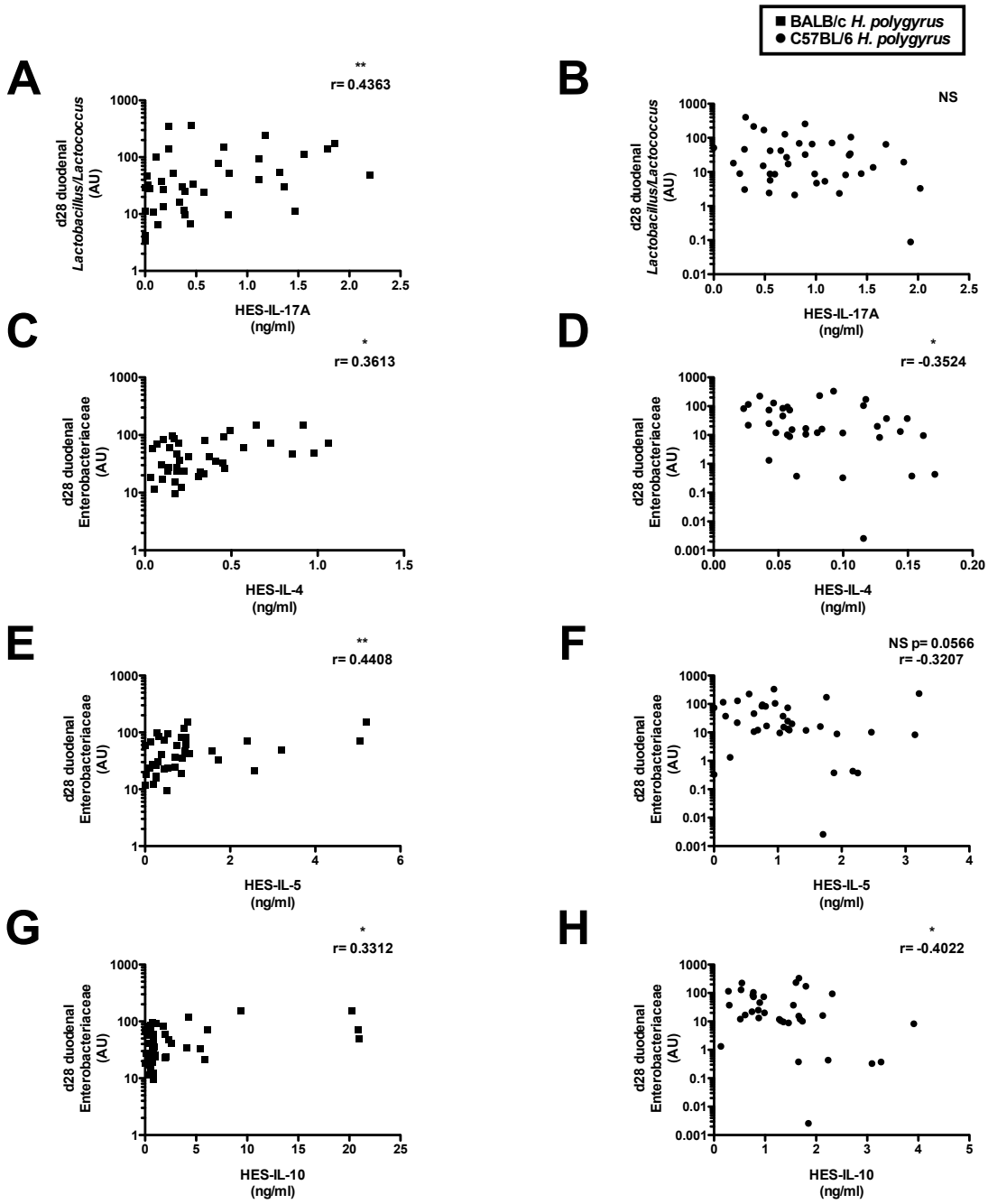

Figure 3.2.12 HES-specific cytokine production correlates differentially with the levels of duodenal *Lactobacillus/Lactococcus* and Enterobacteriaceae species in BALB/c and C57BL/6 mice.
BALB/c (■) or C57BL/6 (●) female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection, MLN cells were isolated and cultured in complete RPMI media in the absence or presence of 1 µg/ml HES for 72 hrs. Levels of cytokines released into the media were then assayed by ELISA. Each data point represents the mean concentration from duplicate HES stimulated culture wells from one mouse minus the concentration of cytokine in the media of unstimulated cells. Cytokine levels were correlated with the relative levels of duodenal *Lactobacillus/Lactococcus* and Enterobacteriaceae, which were determined by real-time PCR for the 16S rRNA genes of each bacterial group. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown for each strain is from one experiment; strain experiments were performed separately.
* indicates p= <0.05; ** indicates p= <0.01; NS indicates a non significant correlation.
(A+B) Correlations were performed between the level of duodenal *Lactobacillus/Lactococcus* and the concentration of HES-specific IL-17A in the supernatant of restimulated MLN cells in **(A)** BALB/c and **(B)** C57BL/6 mice.
(C+D) Correlations were performed between the level of duodenal Enterobacteriaceae and the concentration of HES-specific IL-4 in the supernatant of restimulated MLN cells in **(C)** BALB/c and **(D)** C57BL/6 mice.
(E+F) Correlations were performed between the level of duodenal Enterobacteriaceae and the concentration of HES-specific IL-5 in the supernatant of restimulated MLN cells in **(E)** BALB/c and **(F)** C57BL/6 mice.
(G+H) Correlations were performed between the level of duodenal Enterobacteriaceae and the concentration of HES-specific IL-10 in the supernatant of restimulated MLN cells in **(G)** BALB/c and **(H)** C57BL/6 mice.

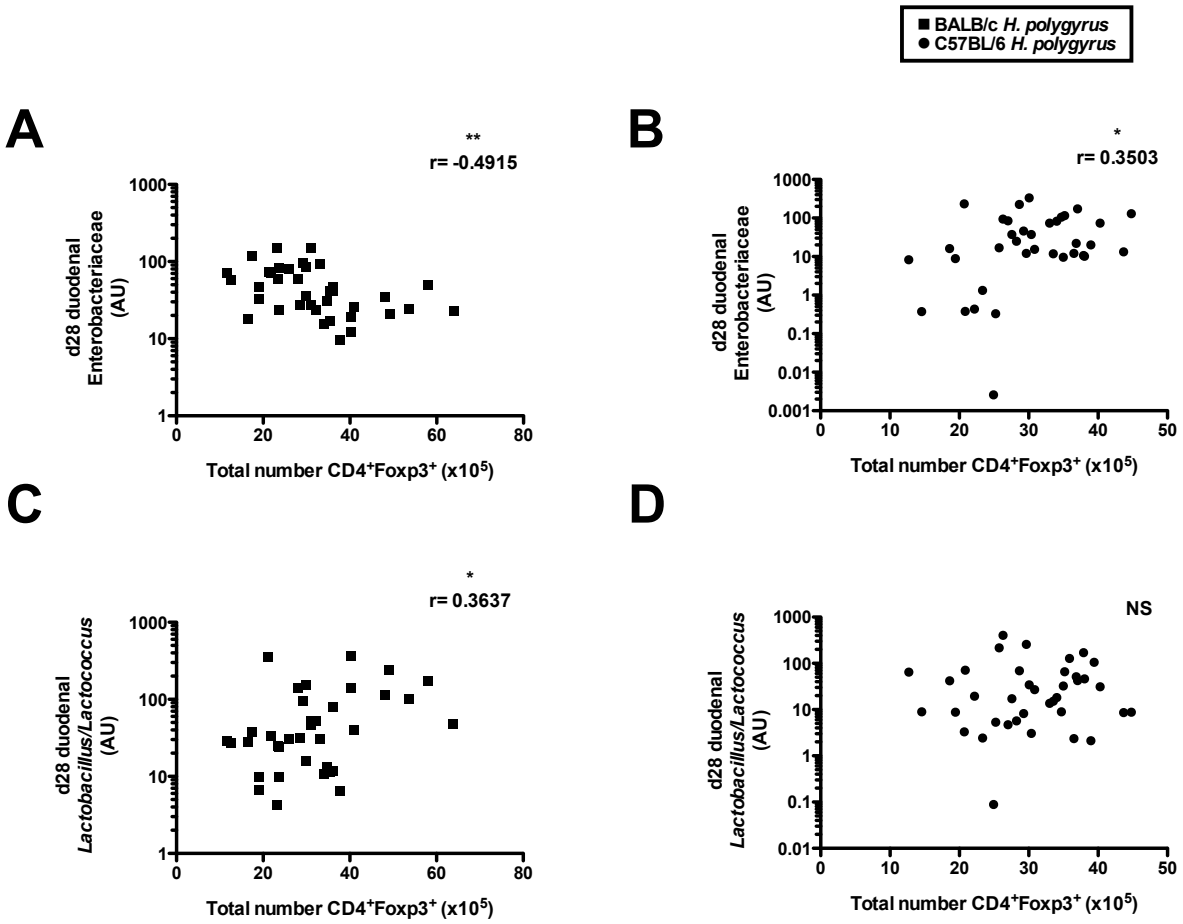

Figure 3.2.13 Treg number correlates with the levels of *Lactobacillus/Lactococcus* and Enterobacteriaceae species in the duodenum differentially between BALB/c and C57BL/6 mice.

BALB/c (■) or C57BL/6 (●) female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection, MLN cells were isolated, stained as indicated and run on a flow cytometer for analysis. Total numbers of Tregs were correlated with the relative levels of duodenal *Lactobacillus/Lactococcus* and Enterobacteriaceae, which were determined by real-time PCR for the 16S rRNA genes of each bacterial group. Where correlations are statistically significant, the Pearson r (for parametric data) or Spearman r (for non parametric data) correlation coefficients are shown. Data shown for each strain is from one experiment; strain experiments were performed separately.

* indicates p= <0.05; ** indicates p= <0.01; NS indicates a non significant correlation.

(A+B) Correlations were performed between the level of duodenal Enterobacteriaceae and the total numbers of $CD4^+Foxp3^+$ MLN cells in **(C)** BALB/c and **(D)** C57BL/6 mice.

(C+D) Correlations were performed between the level of duodenal *Lactobacillus/Lactococcus* and the total numbers of $CD4^+Foxp3^+$ MLN cells in **(A)** BALB/c and **(B)** C57BL/6 mice.

3.3 Discussion

Many effector mechanisms have previously been described which positively correlate with the degree of immunity to *H. polygyrus* shown by different mouse strains, including mastocytosis [162, 246, 247], eosinophilia [238, 248], monocytosis [249], neutrophilia [249], goblet cell hyperplasia [162, 246] and antibody production [162, 247, 250]. The study presented here not only compared the immune response to *H. polygyrus* in a relatively resistant (BALB/c) and relatively susceptible (C57BL/6) mouse strain, but took the unique approach of investigating what mediated the variation in worm expulsion within a large cohort of a single mouse strain. A full list of variables correlating significantly with day 28 worm burden in each strain of mice is included in the Appendix (Table 9.1).

Although helminth infections are traditionally thought to induce type 2 immune responses, it was found that cytokines of both Th1 and Th17 origin were additionally elevated after infection, albeit to different extents in BALB/c and C57BL/6 mice. Th1 and Th17 may be produced in response to inflammation caused by *H. polygyrus*-induced damage and microbial exposure, respectively, rather than to the parasite itself. Therefore, to investigate the worm-specific cytokine response independently of the current level of inflammation within the host, MLN cells were taken from naïve or 28 day-infected mice and examined *ex vivo* for their ability to response to HES. Importantly, the level of cytokines produced from *ex vivo* HES-stimulated MLN cells did not correlate with the level of PMA-Ionomycin stimulated MLN cells (data not shown), highlighting that these assays report very different aspects of the immune response.

HES-antigen specific Th1 and Th2 cytokines were produced in response to infection in both BALB/c and C57BL/6 mice, but in an intriguing pattern. Although BALB/c MLN cells produced much higher IL-4 responses than cells from C57BL/6 mice, HES-specific IL-5 production was comparable between strains, and HES-specific IL-9 and IL-13 production was actually higher in C57BL/6 mice. These data suggest that IL-4 production is the most critical cytokine for promoting resistance; indeed, previous work has shown that IL-4

administration is sufficient to cause *H. polygyrus* expulsion, whereas anti-IL-5 treatment does not affect expulsion [129, 134].

IFN-γ production did appear to be an inhibitor of immunity to *H. polygyrus* in C57BL/6 mice, as serum levels of IFN-γ at day 7 of infection negatively correlated with worm burden. We have shown previously that a lack of IFN-γ promotes immunity to *H. polygyrus* in these mice [242]. The fact that an IFN-γ response at day 7 following infection negatively correlates with subsequent expression of immunity suggests that IFN-γ might inhibit Th2 mechanisms early during the course of infection to modulate immunity.

Moreover, at day 28 HES-specific IL-17A and IFN-γ-production by *H. polygyrus*-infected MLN cells positively correlated with worm burden in BALB/c mice, indicating that the production of these cytokines promotes worm survival. No such relationship was found with these cytokines and *H. polygyrus* susceptibility in C57BL/6 mice, perhaps because the overall higher levels of these cytokines contribute to the overall susceptibility of the strain, and there is not enough variation in cytokine levels within the strain for a relationship between cytokine level and worm burden to be seen.

Substantial expansion of Tregs was seen following *H. polygyrus* infection in both BALB/c and C57BL/6 mice. Treg accumulation during *H. polygyrus* infection has been reported previously [197]; in fact, *H. polygyrus* may actively induce Treg formation, since HES is able to cause conversion of naïve T cells to Foxp3$^+$ Tregs *in vitro* [197]. The ability of *H. polygyrus* secretions to actively induce Tregs suggests that Treg expansion promotes its survival, which may be via controlling inflammation leading to host pathology, and/or by suppressing an effector type 2 response against the worm.

Evidence exists in other helminth infections that Tregs may reduce anti-parasite effector responses [251-254]. However, Tregs have also been found to reduce inflammatory pathology caused by parasites, as intestinal pathology was exacerbated by Treg depletion following both *T. muris* [255] and *H. polygyrus* [196] infections. Published experimental studies into the role of Tregs in susceptibility to *H. polygyrus* have not chosen optimal settings: for

example, the role of $CD4^+CD25^+CD103^+$ Tregs was tested by transfer into C57BL/6 recipients. Although no effect on adult worm burden 28 days post-infection was seen [131], control mice which received just PBS retained high worm burdens at this timepoint, as this strain is relatively susceptible. Treg depleted C57BL/6 mice showed no worm burden difference to controls after 14 days of infection [131], however, again, susceptibility differences may not be revealed until later time points of infection. It will be important to extend these studies into the BALB/c system.

Although total numbers of $CD4^+Foxp3^+$ cells increased equivalently following infection in BALB/c and C57BL/6 mice, the proportion of these cells expressing Helios differed between strains. Infected BALB/c mice accumulated a higher number of $CD4^+Foxp3^+Helios^+$ MLN cells than C57BL/6 mice; the opposite being true for $CD4^+Foxp3^+Helios^-$ MLN cells. Helios has been described as a marker for thymically derived natural Tregs, as opposed to those which are induced in the periphery [245] though the specificity of Helios as a marker for natural Tregs is controversial, as expression of Helios has been reported on *in vitro*-induced Tregs [256-258]. Helios has instead been proposed as a marker of more activated and proliferative Tregs [258], containing increased levels of TGF-β mRNA and having an increased ability to suppress in *in vitro* suppression assays [256]. Perhaps the elevated proportion of $CD4^+Foxp3^+Helios^+$ cells in BALB/c mice reflects the need to control the intestinal pathology in these mice, which have mounted a stronger, more effective parasite-clearing immune response.

A clear physiological difference between BALB/c and C57BL/6 mice in their response to *H. polygyrus*-infection was the production of a large number of granulomas along the intestinal tract in the former strain, which have previously described as being more prevalent in more resistant strains of mice [191]. Here, granuloma formation negatively correlated with worm expulsion, suggesting granuloma formation promotes worm expulsion, however since the correlation was weak, perhaps the quality of the granuloma, as well as their quantity, plays a role in determining immunity. In BALB/c mice, granulomas formed after a primary infection consist primarily of neutrophils, classically

activated macrophages, with some alternatively activated macrophages, eosinophils, and DCs [152, 156].

Although granuloma formation during *H. polygyrus* infection has been linked to increased resistance to the parasite, their actual function remains unclear. It is possible that granulomas form around *H. polygyrus* larvae as they migrate through the submucosal layer of the gut during their lifecycle, and serve to trap or damage them, thus reducing the fitness of the adult larvae when they reach the small intestinal lumen. Alternatively, granulomas may not play a direct role in parasite killing, but instead form to repair tissue damage and eliminate microbes [192] which may have translocated across the muscosal barrier during *H. polygyrus* migration. Since the composition of granulomas during a secondary challenge infection with *H. polygyrus* is altered, with a much higher proportion of alternatively activated macrophages and eosinophils, and fewer neutrophils [152, 156], it is possible that in a more immune mouse granulomas function primarily to kill or damage larvae, whereas in primary exposure granulomas function to limit microbial invasion and repair tissue damage.

Here, it was found that the number of granulomas formed by day 28 of a primary *H. polygyrus*-infection in BALB/c mice positively correlated with the abundance of co-existing duodenal *Bacteroides* species. This suggests the intriguing possibility that primary granulomas form in these mice as a result of *Bacteroides* infiltration into the submucosa of the small intestine. *Bacteroides* are a genus of Gram-negative bacteria, which are one of the most prevalent groups of anaerobes colonising the intestinal tract of mammals [7]. The most-studied, and clinically important member of this groups is *Bacteroides fragilis*, which produces several structurally distinct polysaccharide capsules, of which polysaccharide A (PSA) is the most abundantly expressed [259]. Remarkably, PSA possesses immunomodulatory abilities; reportedly promoting both proinflammatory and regulatory responses, presumably dependent on the context in which the antigen is seen [260]. Conceivably therefore, *Bacteroides* presence in the submucosa after disruption of the intestinal epithelial barrier during *H. polygyrus* larvae migration causes the

accumulation of inflammatory cells which form a granuloma. It has previously been shown that bacteria-specific immune responses can occur after the intestinal epithelial barrier is disrupted following *T. gondii* infection [75], though it remains to be determined whether *Bacteroides* or any microbe-specific responses are mounted following *H. polygyrus* infection.

In contrast, in C57BL/6 mice, few granulomas are formed in response to *H. polygyrus* infection. If granulomas are formed in response to infiltrating bacteria as proposed above, it is hard to imagine why they would not also form in this strain of mice. It is possible that primary granulomas persist as a result of continued bacterial presence in the submucosa of BALB/c mice, whilst C57BL/6 mice are better able to control and kill the commensal flora, due to their innate Th1 bias [241] and the higher basal Th17 levels in their MLN and IL-6 and IFN-γ in their sera compared to BALB/c mice as described here. Alternatively, the high levels of inflammatory cytokines in C57BL/6 mice may inhibit granuloma formation in these mice, if their formation and persistence is dependent on Th2 cytokines.

Perhaps the most striking of data described here was the finding that *H. polygyrus*-infection altered the microbial flora composition of the small intestine, and the abundance of different microbial groups was linked to *H. polygyrus* survival within the host. *Lactobacillus/Lactococcus* species expanded in the small intestine by day 14 of *H. polygyrus*-infection in both BALB/c and C57BL/6 mice, yet by day 28 in BALB/c mice the abundance of these bacteria had dropped to below the levels of naïve mice, whereas levels remained higher than naïve mice in C57BL/6 mice at this time point. This finding is in accordance with one previous study, which examined microflora population changes following *H. polygyrus*-infection, where it was found that C57BL/6 mice had a higher abundance of Lactobacillaceae family members in the ileum compared to naïve mice at day 14 of infection [218]. *Lactobacillus/Lactococcus* are a genus of Gram-positive, facultative anaerobic bacteria, most heavily studied for their ‘probiotic’ effects on the immune system.

Enterobacteriaceae bacterial species were also affected by *H. polygyrus* infection, showing an increase in abundance in BALB/c mice 14 days after infection, but a significant decrease in abundance by 28 days post-infection in BALB/c mice, and a significant increase in prevalence after 28 days in C57BL/6 mice. Enterobacteriaceae are a family of Gram-negative, facultative anaerobic bacteria, which include opportunistic pathogens such as *Salmonella* and *Citrobacter* as well as many harmless symbionts.

Two main hypotheses can be formulated to explain changes in abundance of these two bacterial groups:

1) Firstly, *H. polygyrus* could be actively modifying the microflora populations through its secretory products (collectively termed HES). For example, *H. polygyrus* could actively modify bacterial populations, as HES contains putative antimicrobial lysozymes [127]. These lysozymes may be selectively bactericidal, killing those species inhibitory towards *H. polygyrus* survival, or perhaps following lysozyme exposure, certain bacterial groups are more rapid at recolonising the gut, leading to alterations in the ratios of bacterial groups present.

2) Second, the change in microenvironment caused by helminth infection (the immune response elicited by infection, or the exposure of different niches caused by helminth disruption of the epithelial barrier) may allow certain bacterial species to better proliferate. Microflora populations in the colon are altered following *Trichuris suis* infection of the pig, and the authors suggest this is due to the availability of different nutritional sources for bacteria as a result of parasite damage to the gut epithelium [261].

Inducing microbial population changes is likely to influence *H. polygyrus* survival, as it was seen here that the abundance of duodenal *Lactobacillus/Lactococcus* species correlated positively with worm survival in BALB/c, though this correlation did not reach statistical significance in C57BL/6 mice, perhaps as *Lactobacillus/Lactococcus* levels remained high in the majority of C57BL/6 mice at this time point. The more resistant phenotype of BALB/c mice compared to C57BL/6 mice in general may be a result of the fact that *H. polygyrus* is unable to promote the survival of

Lactobacillus/Lactococcus species within the mouse, and when *Lactobacillus/Lactococcus* levels *do* remain high in these mice, worm survival is promoted. There have been many previous reports that *Lactobacillus* species are able to promote Treg expansion when administered to mice [219-222], and here, it was seen that duodenal *Lactobacillus/Lactococcus* levels positively correlated with MLN Treg number in BALB/c mice. Promoting a regulatory response, as well as the observation that *Lactobacillus/Lactococcus* levels positively correlate with HES-specific IL-17A release, may be a mechanism by which *Lactobacillus/Lactococcus* levels promote worm survival, as the immune response is biased towards a regulatory or Th17 phenotype, rather than a helminth-expelling Th2 response.

Paradoxically, high Enterobacteriaceae levels appeared to be detrimental to *H. polygyrus* survival in BALB/c mice, yet beneficial for survival in C57BL/6 mice. In BALB/c mice, high Enterobacteriaceae levels positively correlated with release of the HES-specific Th2 cytokines IL-4, IL-5, and IL-10, whereas in C57BL/6 mice, high Enterobacteriaceae levels *negatively* correlated with release of these cytokines. This leads to the intriguing hypothesis that species of Enterobacteriaceae elicit different modes of T cell responsiveness in different genotypes of mice. Many examples have been described of how bacterial species can polarise the immune response towards the production of different cytokines [87, 88, 91], yet to date it has not been examined how host genetic background affects this polarisation. The cytokine IL-18, a product of TLR signalling, causes splenocytes from BALB/c mice to produce Th2-cytokines, but stimulates Th1-cytokine production from C57BL/6 splenocytes [262]. Thus, the downstream products of TLR signalling could be a mechanism by which T cells differentially respond to bacterial species in mice of different genotypes. In addition, influential bacterial groups may be present which have not been measured during these experiments, thus a comprehensive sequencing of all intestinal microflora is required to address this concern.

A major caveat of the experiments described within this chapter is that relationships are correlative, and not causal. Relationships described here

may be indirect and as a result of an unknown factor, which has not been measured, thus further experimentation is necessary to test the hypotheses proposed here. Despite this limitation, these data are useful in broadly describing the factors important for *H. polygyrus* expulsion, and for exploring how factors differ in impacting on worm expulsion in two mouse strains with different initial immune biases.

Overall, these data suggest that the microflora composition prior to infection can impact the survival of *H. polygyrus* within the murine host, and furthermore, *H. polygyrus* may be actively modifying the microflora in order to promote its survival. It has yet to be resolved whether alteration of the microflora during infection is a helminth-mediated mechanism that acts to promote the survival of *H. polygyrus* within the murine host, or if it is simply as a consequence of a changing immune environment or physiological perturbation of the intestinal niche, in which certain bacterial groups are better able to survive. To investigate this further, survival of *H. polygyrus* was examined in mice whose microflora had been experimentally altered, as discussed in the following chapter.

Chapter 4. The effect of microbiota modification on *Heligmosomoides polygyrus* infection

4.1 Introduction

The presence of the intestinal microbiota is required for normal development of the immune system, as evidenced from studies of GF mice. GF mice display many deviations from conventionally housed SPF mice, such as a reduced size of secondary lymphoidal tissue, a lack of germinal centre formation, and low Ig levels [3]. GF mice are also generally less responsive to infection with bacterial or viral agents [3], and they respond less well than SPF mice to administration of systemic antigens [81], indicating that the presence of intestinal microbiota is necessary for normal immune responsiveness.

When GF mice are infected with *H. polygyrus*, however, they are not more susceptible to infection, and indeed *H. polygyrus* survival is markedly reduced within these hosts compared to in SPF mice [223-225]. In GF mouse studies, however, it is difficult to ascertain whether differences in the ability to clear infections are due to an abnormally developed immune system, or due to the absence of bacteria that may alter the immune response or physiology of the host at the time of infection. Therefore, many studies have adopted the approach of antibiotic-treating SPF mice, to examine the effects of depleting or modifying the microbiota in a developmentally normal setting.

Strikingly, establishment of *T. muris* within the murine host is dependent on the intestinal microbiota [226]. *T. muris* eggs are orally ingested by mice, and hatch when they reach the colon. Egg hatching was found to be induced by the high bacterial load of the colon relative to the small intestine, and adult worm number was markedly reduced after bacteria were depleted by antibiotic treatment [226]. It is as yet unclear whether specific bacteria are required for this process, or if the presence of any bacterial species in abundance is sufficient to trigger hatching.

In the studies presented in this chapter, the question of whether the outcome of *H. polygyrus* infection could be altered through antibiotic treatment to either delete or modify the intestinal microbiota was examined. When the entire microbiota was deleted through a broad-spectrum antibiotic cocktail, no impact on susceptibility to *H. polygyrus* was observed. However, when more restricted antibiotic treatment was used to modify the microbiota populations, *H. polygyrus* survival was impaired, suggesting that specific bacterial groups are required to alter the susceptibility to *H. polygyrus*.

A more defined approach was also taken, to examine the effects on *H. polygyrus* of feeding a single commensal bacteria species to mice. *Lactobacillus taiwanensis* was found to be the most abundant species of the *Lactobacillus/Lactococcus* family in highly infected BALB/c mice, and levels of this bacterium in the duodenum at day 28 of *H. polygyrus*-infection correlated with worm burden at the same timepoint. BALB/c mice that were administered drinking water containing *L. taiwanensis* did not expel *H. polygyrus* as rapidly as untreated control animals, suggesting that the presence of *L. taiwanensis* positively controls *H. polygyrus* persistence within the host, although the mechanisms behind this relationship remain to be explored. These data suggest therefore that it is the composition of the intestinal microbiota, rather than simply the presence of bacteria, which is able to influence the expulsion of *H. polygyrus*.

4.2 Results

4.2.1 Depleting microbiota by multiple broad-spectrum antibiotic treatment does not alter *H. polygyrus* survival after 14 days in BALB/c or C57BL/6 mice

Evidence from infections of GF mice has suggested that *H. polygyrus* survival is reduced in the absence of a commensal microbiota [223-225], implying that the presence of gut bacteria promotes *H. polygyrus* survival. However, as a result of a lack of bacterial stimulation during development, the immune system and gut physiology of GF mice differs markedly from SPF mice [3], and it may be alterations in the environment of the small intestine which discourages *H. polygyrus* survival in these mice. To address this, the gut commensal flora was depleted using antibiotics, so that *H. polygyrus* infection could be examined in the absence of a microbiota in developmentally normal mice. Mice were administered a broad-spectrum cocktail of antibiotics consisting of 1 g/L Metronidazole, 200 mg/L Ciprofloxacin, 250 mg/L Imipenem, 1 g/L Ampicillin and 500 mg/L Vancomycin (cocktail 1) in their drinking water, for 2 weeks prior to infection (Figure 4.2.1 A). 1 g/L of the antifungal compound Fluconazole was included in the cocktail to control any potential outgrowth of fungi after bacteria depletion.

Mice did not respond well to the antibiotic treatment, and exhibited severe weight loss, thought primarily to be due to dehydration, as mice did not drink as much of the antibiotic-treated water as unmanipulated water. Confirming that the antibiotic treatment was successful in depleting the microbiota of the gut, upon sacrifice, enlarged caeca were seen in antibiotic-treated mice, which has previously been observed both in GF mice [3] and following antibiotic-treatment [263], as a result of an accumulation of mucus in the caecum which is usually degraded by the intestinal bacteria [2, 264].

Due to the unexpected weight loss of the antibiotic-treated mice, all animals were sacrificed 14 days following *H. polygyrus*-infection. This was not the optimal timepoint for examining differences in parasitology, as often susceptible and resistant strains of mice have similar worm burdens 14 days

post-infection. Indeed, at this timepoint, antibiotic-treated and control mice harboured similar adult worm burdens, both in BALB/c (Figure 4.2.1 B) and C57BL/6 mice (Figure 4.2.1 E). No evidence for decreased worm fitness was seen in BALB/c antibiotic-treated mice, as *H. polygyrus* egg production was comparable with controls (Figure 4.2.1 D); egg production was not examined in C57BL/6 mice at this time point.

Interestingly, although antibiotic treatment had no impact on the already high number of granulomas produced along the intestinal tract in response to *H. polygyrus* in BALB/c mice (Figure 4.2.1 C), the treatment significantly increased granuloma formation in C57BL/6 mice (Figure 4.2.1 F), which may be indicative of a stronger anti-parasite response.

4.2.2 Depleting microbiota by multiple broad-spectrum antibiotic treatment does not alter *H. polygyrus* survival after 28 days in C57BL/6 mice

Because changes in immunity or susceptibility to *H. polygyrus* are not usually evident as early as 14 days following infection, the antibiotic treatment protocol was amended to give mice food mashed in untreated water alongside the antibiotic-treated drinking water. This prevented dehydration, thus allowing the outcome of *H. polygyrus*-infection to be examined at a later timepoint, 28 days post-infection (Figure 4.2.2 A). An additional cocktail of antibiotics, consisting of Metronidazole (1 g/L), Ampicillin (1 g/L), Vancomycin (500 mg/L) and Neomycin (1 g/L; Sigma) (cocktail 2) was also tested, to examine whether it had a less severe effect on weight loss. These experiments were performed in C57BL/6 mice, because as demonstrated in Chapter 3, the majority of untreated BALB/c mice are able to clear a *H. polygyrus* infection 28 days post-infection (Figure 3.2.1 A).

No significant differences in worm burden compared to untreated controls were seen 28 days post-infection, following treatment with either the broad-spectrum antibiotic treatment cocktail 1 (Figure 4.2.2 B) or cocktail 2 (Figure 4.2.2 E). The granuloma response to *H. polygyrus* in C57BL/6 mice appears

transient, as by 28 days post-infection mice had very few granulomas, and numbers were unaffected by antibiotic treatment with either cocktail (Figure 4.2.2 C+F). Furthermore, at this timepoint, *H. polygyrus* fecundity was unaffected by antibiotic treatment (Figure 4.2.2 D+G).

Microbiota depletion appeared effective following treatment with both antibiotic cocktails, as all mice exhibited enlarged caeca upon harvest. These data therefore suggest that depleting the entire gut microbiota has no net impact on *H. polygyrus* infection. Data from Chapter 3 however, suggested that specific microbial groups may be able to influence the outcome of infection. Next, therefore, it was examined whether modifying the composition of the microbiota, rather than completely deleting it, could affect *H. polygyrus* survival.

4.2.3 Trimethoprim and Sulfadoxine antibiotic treatment reduces *H. polygyrus* fitness in C57BL/6 mice

Mice were administered untreated drinking water or water containing just two broad-spectrum antibiotics, Trimethoprim and Sulfadoxine, for 4 weeks prior to *H. polygyrus*-infection, and throughout the duration of infection (Figure 4.2.3 A). This treatment did not result in any weight loss, and upon harvest, caeca looked no different to untreated control mice (data not shown). Treatment did, however, alter the outcome of *H. polygyrus*-infection, with Trimethoprim and Sulfadoxine-treated mice harboring significantly lower worm burdens after 28 days of infection in 3 out of 5 identical experiments (Figure 4.2.3 B). *H. polygyrus* fecundity was also reduced in Trimethoprim and Sulfadoxine treated mice at day 14 following infection (Figure 4.2.3 C). To investigate whether the antibiotic-treatment altered the immune response to *H. polygyrus*, MLN and PP cells were isolated 28 days following infection, and stained for cytokine production following PMA/Ionomycin stimulation. No evidence for an amplified IL-4-response was seen in antibiotic-treated mice, in either the MLN (Figure 4.2.3 D) or PP (Figure 4.2.3 E), and no deviation was seen from untreated control mice in IFN-γ, IL-10, IL-13 or IL-17A production at either of these sites (data not shown).

4.2.4 Trimethoprim and Sulfadoxine have no effect on adult *H. polygyrus* fitness *in vitro*, but alter the microbiota composition of C57BL/6 mice

It was possible that Trimethoprim and Sulfadoxine treatment affected expulsion of *H. polygyrus* either by directly damaging the worms, or by altering the composition of the gut microbial flora in a manner which causes increased expulsion of *H. polygyrus*. To address this, it was first examined whether Trimethoprim and Sulfadoxine could impair the survival of adult *H. polygyrus* worms *in vitro*. Adult female worms were isolated and incubated *in vitro* with these antibiotics at the indicated concentrations, and their egg production was recorded. No significant differences in egg production were seen with any concentration of Trimethoprim and Sulfadoxine tested (Figure 4.2.4 A). Equally, when male worms had been incubated with the same concentrations of Trimethoprim and Sulfadoxine, under no conditions did the antibiotics affect the ATP content of worms when compared to control untreated worms (Figure 4.2.4 B). When administered in the drinking water, Trimethoprim and Sulfadoxine were at concentrations of 125 mg/L and 25 mg/L respectively, much lower than the highest concentration in *in vitro* assays, arguing that it is unlikely that Trimethoprim and Sulfadoxine treatment directly damages *H. polygyrus*.

The possibility that Trimethoprim and Sulfadoxine treatment promotes *H. polygyrus* expulsion by altering the microbiota was next examined. Faeces were collected from mice after they had been treated with Trimethoprim and Sulfadoxine in their drinking water for 4 weeks (Figure 4.2.3 A). DNA was extracted from the faeces, and the presence of various bacterial groups was examined using PCR for the specific 16S rRNA gene regions of each group. In all three experiments examined, shifts from untreated mouse bacterial populations were seen (Figure 4.2.4 C). Whilst the effect that Trimethoprim and Sulfadoxine treatment had on the presence of *Bacteroides* and *Eubacterium/Clostridium* groups in the faeces was not consistent, *Lactobacillus/Lactococcus* and Enterobacteriaceae levels were consistently reduced following treatment (Figure 4.2.4 C). The positive or negative shift in abundance of each bacterial group did not correlate with the strength of the effect

that Trimethoprim and Sulfadoxine treatment had on *H. polygyrus* expulsion (Figure 4.2.3 B + 4.2.4 C).

4.2.5 Transfer of faeces from Trimethoprim and Sulfadoxine treated mice does not alter susceptibility to *H. polygyrus* in C57BL/6 mice

As an independent experimental approach to test whether microflora population changes following Trimethoprim and Sulfadoxine treatment cause increased *H. polygyrus* expulsion, faeces from untreated mice, or mice which had received Trimethoprim and Sulfadoxine treatment for 4 weeks were collected, homogenised, and administered to recipient experimental mice prior to *H. polygyrus*-infection (Figure 4.2.5 A). No significant differences were observed in how the faecal-recipient mice responded to *H. polygyrus*, in terms of egg production by the parasite 14 days post-infection (Figure 4.2.5 B), 28 days post-infection (Figure 4.2.5 C) or adult worm burden 28 days post-infection (Figure 4.2.5 D). This indicated that either the Trimethoprim and Sulfadoxine treatment does not alter *H. polygyrus* burden through changes in the microbiota population, or that the faecal transplant method used was not sufficient to replace the existing microbiota of recipient mice with a Trimethoprim and Sulfadoxine-altered microbiota.

4.2.6 *Lactobacillus taiwanensis* is the most common species of *Lactobacillus/Lactococcus* in heavily *H. polygyrus*-infected BALB/c mice

A different experimental approach was next adopted, which aimed to produce more defined alterations in the microbiota of mice. As described in Chapter 3, a positive correlation was found between the abundance of *Lactobacillus/Lactococcus* species in the duodenum of BALB/c mice and the number of adult worms remaining in the host 28 days post-infection. To investigate whether this relationship was causal, by directly administering *Lactobacillus/Lactococcus* species to mice and examining whether susceptibility to *H. polygyrus* was altered, it was first necessary to identify the most abundant

species of *Lactobacillus/Lactococcus* residing in the small intestine of these mice (Figure 4.2.6 A). To achieve this, DNA was extracted from the duodenum of those BALB/c mice that maintained the highest worm burdens 28 days following infection. Primers specific for the 16S rRNA gene of *Lactobacillus/Lactococcus* family members were used to amplify that region of the DNA, and amplicons were ligated into bacterial vectors, and transformed into *E. coli* hosts, which were cultured overnight. Single colonies harboring the vector were picked, and DNA from them was extracted. Inserts were sequenced and the results searched against a BLAST database. Of the 13 colonies screened, 12 were a 100% nucleotide match to the sequence of *Lactobacillus taiwanensis* described in [265] (Figure 4.2.6 B). The remaining sample sequenced showed one nucleotide change from the sequence described for *L. taiwanensis*, which was still the closest match.

L. taiwanensis was first identified from silage cattle feed [265] and has since been isolated from the intestinal contents of rats [266]. This species of bacteria is a member of the Gram-positive, facultatively anaerobic, lactic acid bacteria family, and has been highly characterised, at both the molecular and phenotypic levels [266].

4.2.7 *Lactobacillus taiwanensis* abundance in the duodenum 28 days post *H. polygyrus*-infection positively correlates with worm burden in BALB/c mice

It has previously been reported that *L. taiwanensis* is relatively highly divergent from closely related species at the gyrB gene locus, which encodes the subunit B protein of DNA gyrase [265], therefore this region of the genome was chosen to design primers which would amplify DNA from *L. taiwanensis*, but not its close family members (Figure 4.2.7 A+B). Levels of *L. taiwanensis* present in the duodenum of 28-day *H. polygyrus*-infected BALB/c mice positively correlated with the number of worms remaining in the host at the same timepoint (Figure 4.2.7 C). In addition, there was a strong positive correlation between the levels of *L. taiwanensis* and the total levels of duodenal *Lactobacillus/Lactococcus*

bacteria (Figure 4.2.7 D), arguing that *L. taiwanensis* is the dominant *Lactobacillus/Lactococcus* present in these mice. These data suggest that levels of this single species may play a role in controlling susceptibility to *H. polygyrus*.

4.2.8 Oral gavage of 2 x 10^{10} CFU of *L. taiwanensis* is not sufficient to alter the immune response to *H. polygyrus*

To test whether *L. taiwanensis* directly influences the outcome of *H. polygyrus* infection, cultures of this species were obtained from the laboratory of Maria Yebra, in Valencia, Spain. *L. taiwanensis* can be cultured in static aerobic cultures, at 37 °C [266]. 10^{10} CFU of *L. taiwanensis* were administered by oral gavage to BALB/c mice, the day prior to and the day following infection with *H. polygyrus* (Figure 4.2.8 A). 15 days following *H. polygyrus*-infection, there was no significant difference in worm burden (Figure 4.2.8 B) or egg production (Figure 4.2.8 C) between *L. taiwanensis*-treated and untreated mice, nor was there a difference in the number of granulomas formed in response to *H. polygyrus* (Figure 4.2.8 D).

Lactobacillus species have been previously shown to promote Treg expansion when administered to mice [219-222], thus the presence of $Foxp3^+$ cells in the MLN was examined (Figure 4.2.8 E). No difference in Treg differentiation was seen following *L. taiwanensis* treatment. Additionally, no alteration in the proportion of IL-4-producing cells was seen in either the PP (Figure 4.2.8 F) or MLN (Figure 4.2.8 G), nor were there differences in the proportions of IL-10, IL-13, IL-17A, IFN-γ $CD4^+$ or $CD8^+$ cells at either site (data not shown).

As no effect of the *L. taiwanensis* treatment was seen, the question of whether the administered bacteria were able to colonise the intestinal tract was raised. Real-time PCR for the gyrB gene of *L. taiwanensis* was performed on faeces collected throughout the course of the experiment. No detectable increase in the abundance of faecal *L. taiwanensis* was seen in the treated animals (Figure 4.2.8 H), suggesting that the dose of *L. taiwanensis* given either was not sufficient to elevate the basal levels of this bacteria within the BALB/c host, or that the administered bacteria were able to only transiently colonise the gut.

4.2.9 Administering *L. taiwanensis* in drinking water prolongs *H. polygyrus*-infection in BALB/c mice

To increase the likelihood that *L. taiwanensis* would be able to remain within the intestinal tract long enough to impact on *H. polygyrus* infection, a different treatment strategy was adopted (Figure 4.2.9 A). *L. taiwanensis* was placed into the drinking water of mice, at a concentration 2 x 10^8 CFU/ml. The average mouse consumes 5 ml of water per day [267], therefore it was expected that mice received approximately 1 x 10^9 CFU of *L. taiwanensis* per day. BALB/c mice received untreated drinking water or water containing *L. taiwanensis* for 1 week prior to *H. polygyrus*-infection, and throughout the course of infection.

By 21 days post-infection, *H. polygyrus*-infected untreated BALB/c mice had a significantly lower egg output than they did at 14 days post-infection (Figure 4.2.9 B). In contrast, BALB/c mice that had received *L. taiwanensis* did not show a significant reduction in egg output over the same period (Figure 4.2.9 B), suggesting that the presence of *L. taiwanensis* may be able to prolong a *H. polygyrus* infection in BALB/c mice. *L. taiwanensis*-fed BALB/c mice showed a strong trend towards an increased *H. polygyrus* burden 28 days post-infection compared to untreated mice, however, this did not reach statistical significance (Figure 4.2.9 C). No impact of *L. taiwanensis*-treatment was seen on granuloma formation along the intestinal tract in response to *H. polygyrus* (Figure 4.2.9 D), and no detectable change in Treg numbers was seen in treated mice, either in the LP (Figure 4.2.9 E) or the MLN (Figure 4.2.9 F).

The question was again addressed, of whether the continuous treatment regime led to increases in *L. taiwanensis* abundance which were detectable by real-time PCR. However, no difference between groups was seen in the levels of *L. taiwanensis* in the faeces 28 days post-infection (Figure 4.2.9 G) or in the duodenum (Figure 4.2.9 H), suggesting the possibility that administering 2 x 10^8 CFU/ml of *L. taiwanensis* in the drinking water did not cause a major shift in colonisation of the intestinal tract.

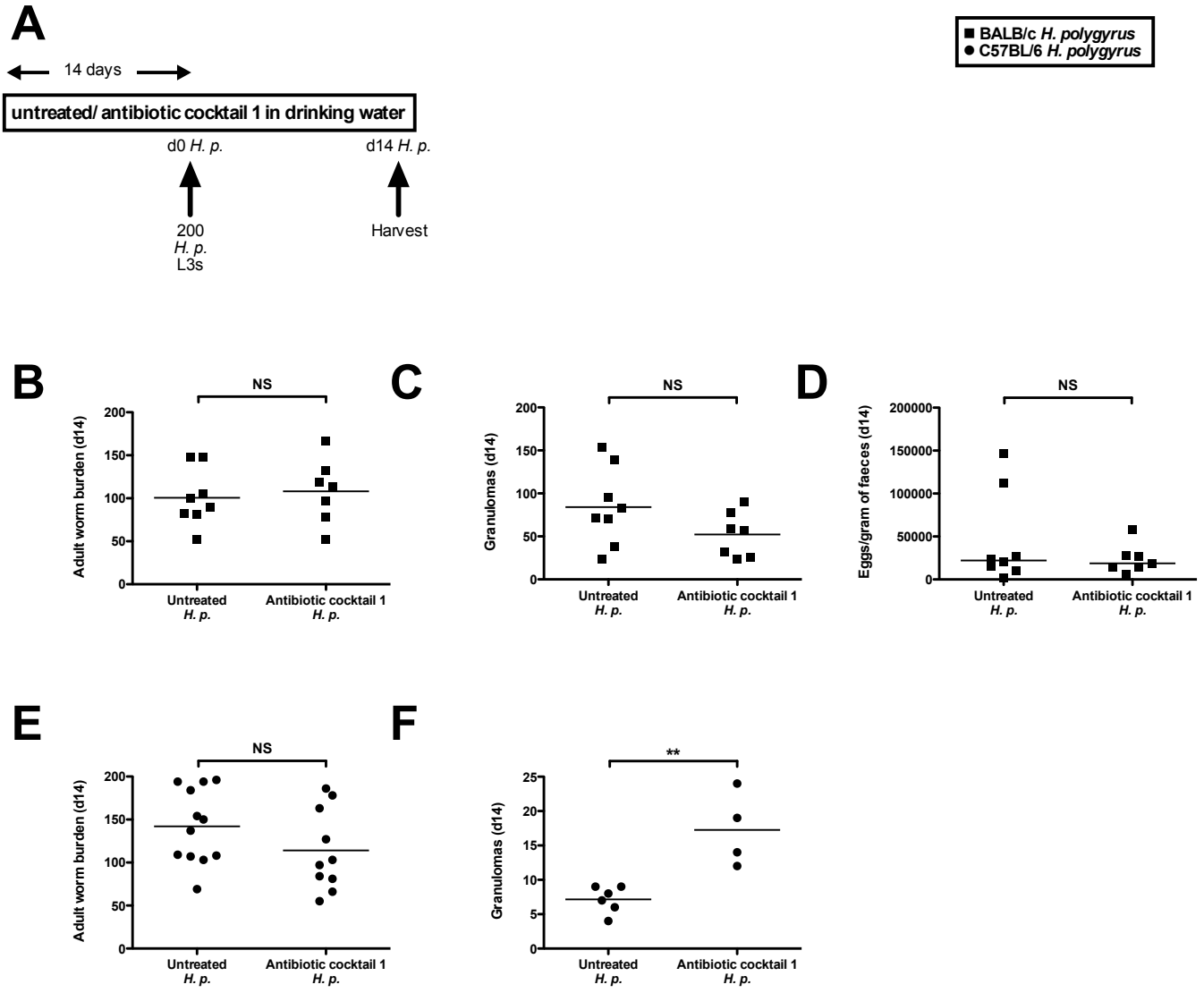

Figure 4.2.1 Depleting microbiota by multiple broad-spectrum antibiotic treatment does not alter *H. polygyrus* survival after 14 days in BALB/c or C57BL/6 mice.
(A) Treatment protocol. BALB/c (■) or C57BL/6 (●) male mice were administered untreated drinking water, or water containing antibiotic cockail 1 (1 g/L Metronidazole; 200 mg/L Ciprofloxacin; 250 mg/L Imipenem; 1 g/L Ampicillin; 500 mg/L Vancomycin; 1 g/L Fluconazole) for 14 days prior to infection with 200 *H. polygyrus* L3s, and throughout the *H. polygyrus* infection.
(B-D) 14 days post-infection, BALB/c mice were sacrificed and the number of **(B)** adult worms recovered from the intestinal tract **(C)** granulomas along the intestinal tract and **(D)** eggs released per gram of faeces were recorded. Data shown is from one experiment.
(E+F) 14 days post-infection, C57BL/6 mice were sacrificed and the number of **(E)** adult worms recovered from the intestinal tract and **(F)** granulomas along the intestinal tract were recorded. Data shown is **(E)** pooled from two independent experiments and **(F)** from one experiment.
** indicates p= <0.01; NS indicates a non significant result.

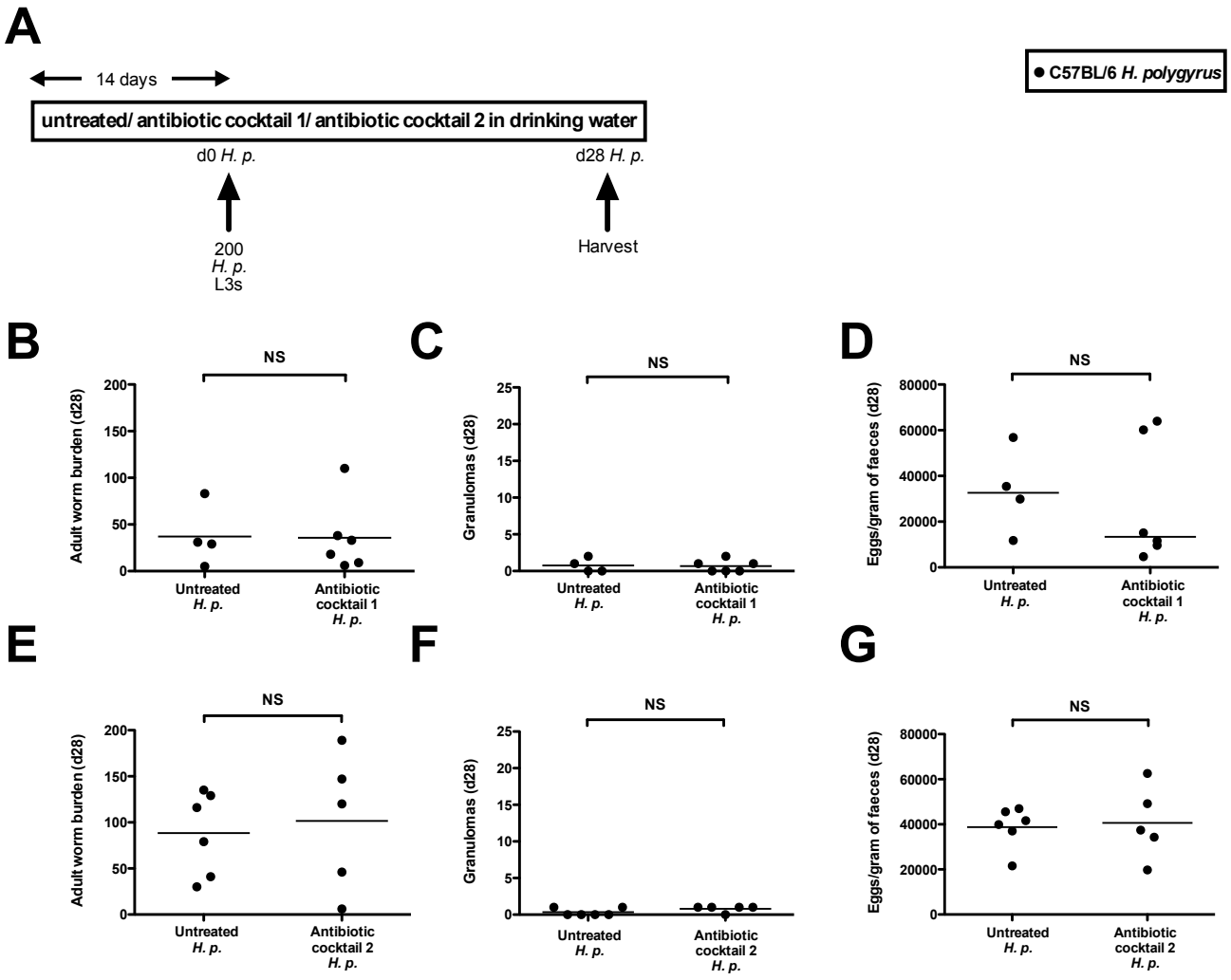

Figure 4.2.2 Depleting microbiota by multiple broad-spectrum antibiotic treatment does not alter *H. polygyrus* survival after 28 days in C57BL/6 mice.

(A) Treatment protocol. C57BL/6 male mice were administered untreated drinking water, or water containing antibiotic cockail 1 (1 g/L Metronidazole; 200 mg/L Ciprofloxacin; 250 mg/L Imipenem; 1 g/L Ampicillin; 500 mg/L Vancomycin; 1 g/L Fluconazole) or 2 (1 g/L Metronidazole; 1 g/L Ampicillin; 500 mg/L Vancomycin; 1 g/L Neomycin) for 14 days prior to infection with 200 *H. polygyrus* L3s, and throughout the *H. polygyrus* infection.

(B-D) Mice were administered untreated drinking water or water containing antibiotic cocktail 1. 28 days following *H. polygyrus*-infection, the number of **(A)** adult worms recovered from the intestinal tract **(B)** granulomas along the intestinal tract and **(C)** eggs released per gram of faeces were recorded. Data shown is from one experiment.

(E-G) Mice treated exactly as in **(B-D)** except antibiotic cocktail 2 was used. 28 days following *H. polygyrus*-infection, the number of **(A)** adult worms recovered from the intestinal tract **(B)** granulomas along the intestinal tract and **(C)** eggs released per gram of faeces were recorded. Data shown is from one experiment.

NS indicates a non significant result.

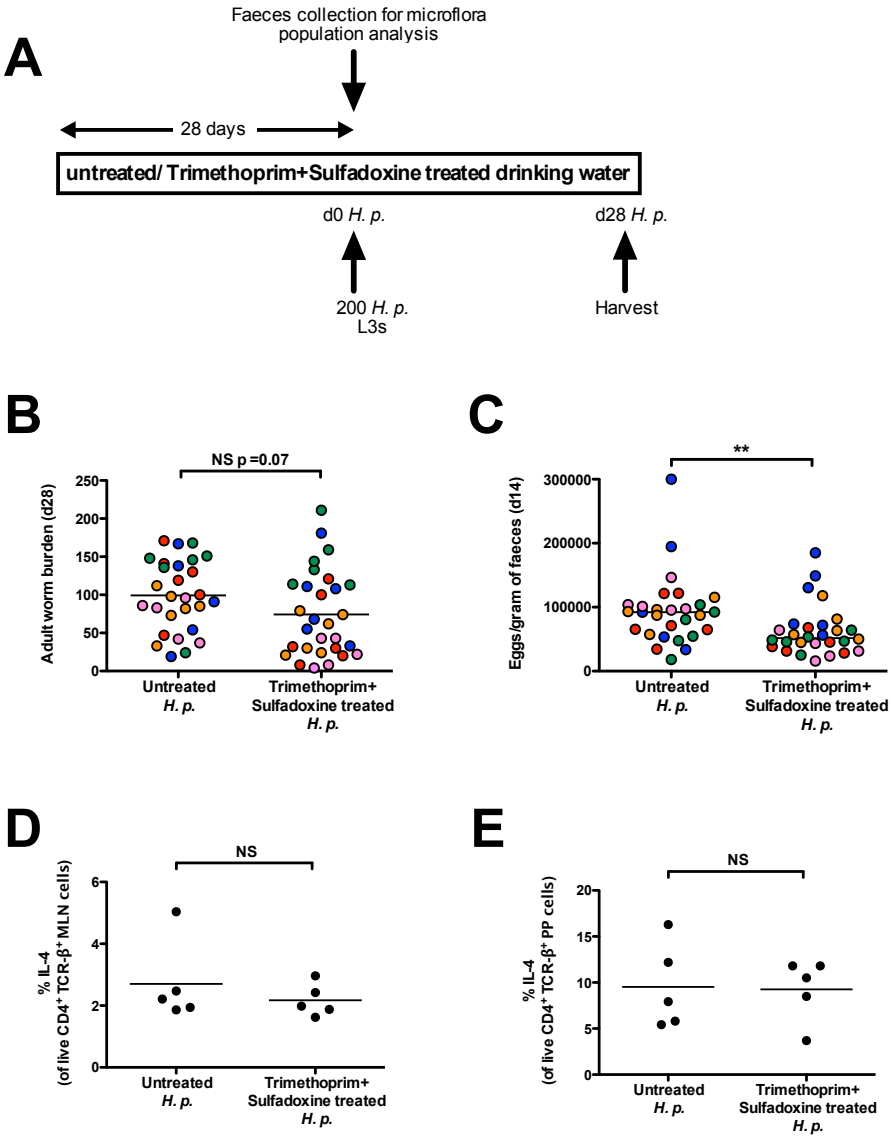

Figure 4.2.3 Trimethoprim and Sulfadoxine antibiotic treatment reduces *H. polygyrus* fitness in C57BL/6 mice.

(A) Treatment protocol. C57BL/6 female mice were administered untreated drinking water, or water containing 125 mg/L Trimethoprim and 25 mg/L Sulfadoxine for 28 days prior to infection with 200 *H. polygyrus* L3s, and throughout the *H. polygyrus*-infection.

(B+C) The number of **(B)** adult worms recovered 28 days post-infection and **(C)** eggs released per gram of faeces 14 days post-infection were recorded. Data shown are pooled from five independent experiments; data points from each experiment are represented in different colours.

(D+E) 28 days post-infection, MLN and PP cells were isolated and stimulated with 0.5 µg/ml PMA and 1 µg/ml Ionomycin for 3.5 hrs, with 10 µg/ml Brefeldin A included for the final 2.5 hrs. Cells were stained as indicated and run on a flow cytometer for analysis. **(D)** % IL-4 producing cells among $CD4^+TCR\text{-}\beta^+$ MLN cells. Data shown is from one experiment and representative of the results of four independent experiments. **(E)** % IL-4 producing cells among $CD4^+TCR\text{-}\beta^+$ PP cells. Data shown is from one experiment.

** indicates p= <0.01; NS indicates a non significant result.

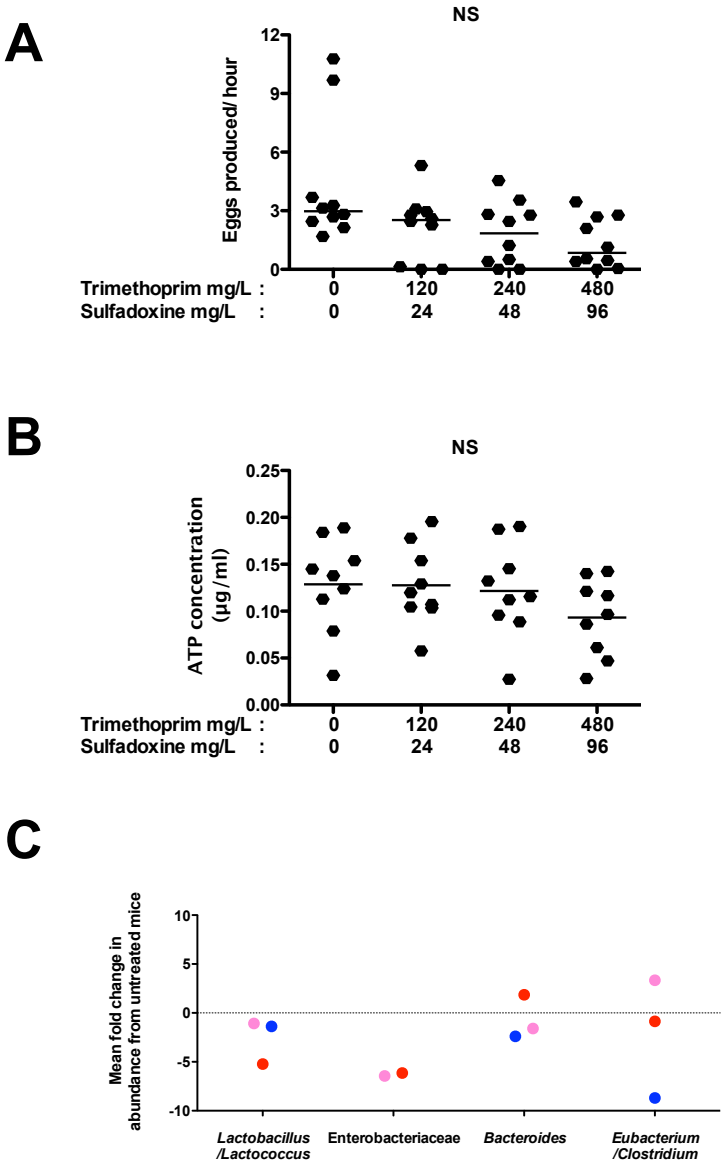

Figure 4.2.4 Trimethoprim and Sulfadoxine have no effect on adult *H. polygyrus* fitness *in vitro*, but alter the microbiota composition of C57BL/6 mice.
(A+B) *H. polygyrus* adult worms were extracted from the intestinal tracts of 14-day infected F1 mice from a C57BL/6 x CBA cross. **(A)** Female worms were incubated in individual culture wells for 24 hrs in '*H. poly* media' containing the indicated concentrations of Trimethoprim and Sulfadoxine. Following incubation, the number of eggs produced per worm was enumerated, and each data point represents the number of eggs produced per hour by an individual worm. Data is from one experiment. **(B)** Male worms were incubated in individual culture wells for 24 hrs in '*H. poly* media' containing the indicated concentrations of Trimethoprim and Sulfadoxine. Following incubation, ATPlite assays were performed on individual worms, and each data point represents the concentration of ATP from an individual worm. Data is from one experiment.
(C) C57BL/6 female mice were administered untreated drinking water, or water containing 125 mg/L Trimethoprim and 25 mg/L Sulfadoxine for 28 days. Following 28 days of Trimethoprim and Sulfadoxine treatment, faeces were collected from individual mice, from which DNA was extracted. The presence of the indicated bacterial groups was analysed by real-time PCR for the specific 16S rRNA genes of each group. Each data point shows the fold change of the mean concentration of each bacterial group in Trimethoprim and Sulfadoxine-treated mice from the mean concentration of the same bacterial group in untreated mice. Data from three independent experiments are shown, each with ≥ 5 mice per group. Colours indicate that faeces was taken from experiments represented in Figure 4.2.3 with matching colours.
NS indicates a non significant result.

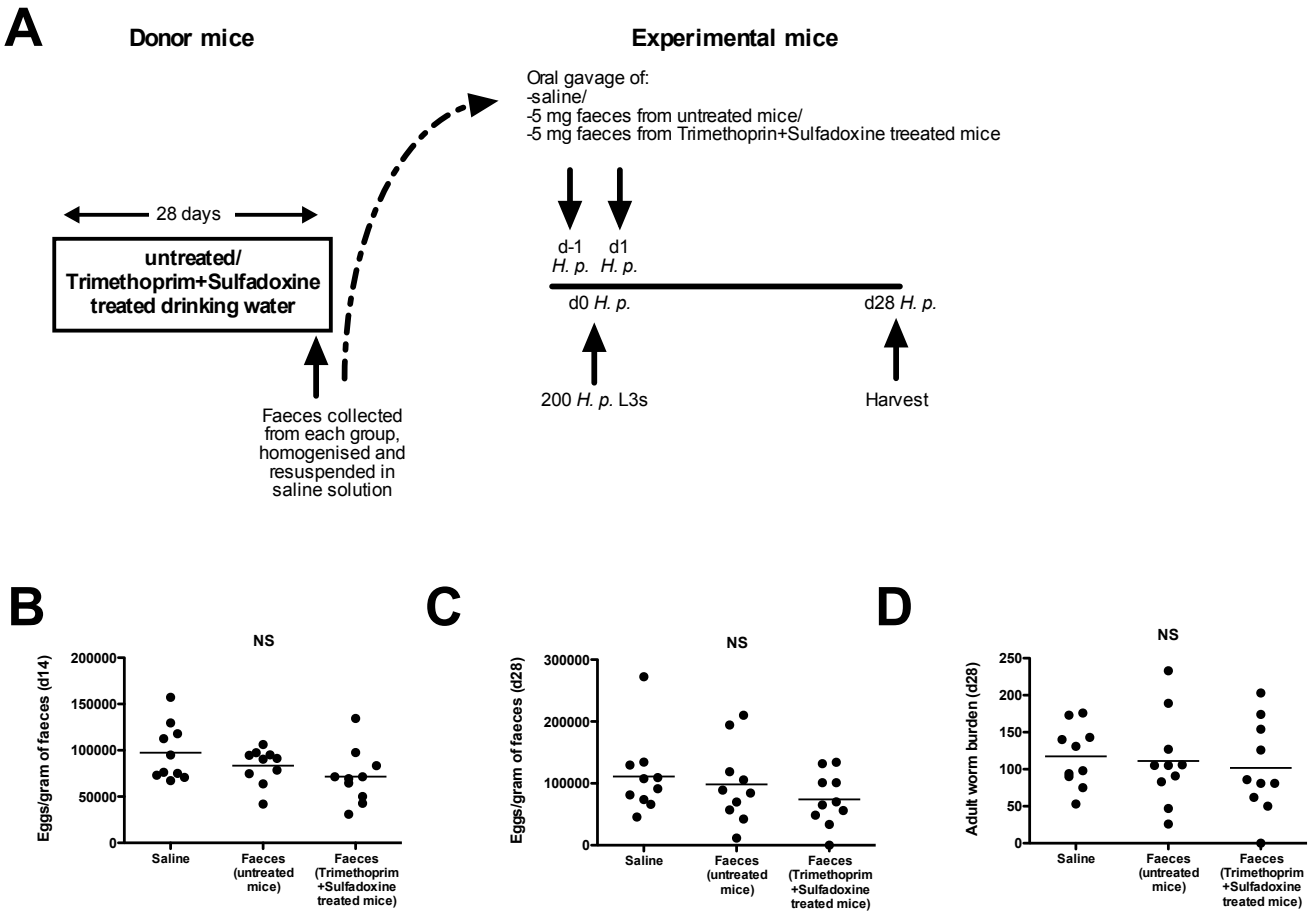

Figure 4.2.5 Transfer of faeces from Trimethoprim and Sulfadoxine treated mice does not alter susceptibility to *H. polygyrus* in C57BL/6 mice.
C57BL/6 'donor' mice were administered untreated drinking water, or water containing 125 mg/L Trimethoprim and 25 mg/L Sulfadoxine for 28 days. Faeces were then collected from these mice, homogenised and resuspended in saline solution. 'Experimental' mice received oral gavages at day -1 and day 1 of *H. polygyrus* infection of either saline solution, saline solution containing 5 mg of faeces from untreated donor mice, or saline solution containing 5 mg of faeces from Trimethoprim and Sulfadoxine treated mice. Experimental mice were infected with 200 *H. polygyrus* L3s on day 0, and sacrificed after 28 days of infection.
(A) Treatment protocol.
(B-D) The number of **(B)** eggs released per gram of faeces 14 days post-infection **(C)** eggs released per gram of faeces 28 days post-infection and **(D)** adult worms recovered from the intestinal tract 28 days post-infection was recorded. Data shown is pooled from two independent experiments.
NS indicates a non significant result.

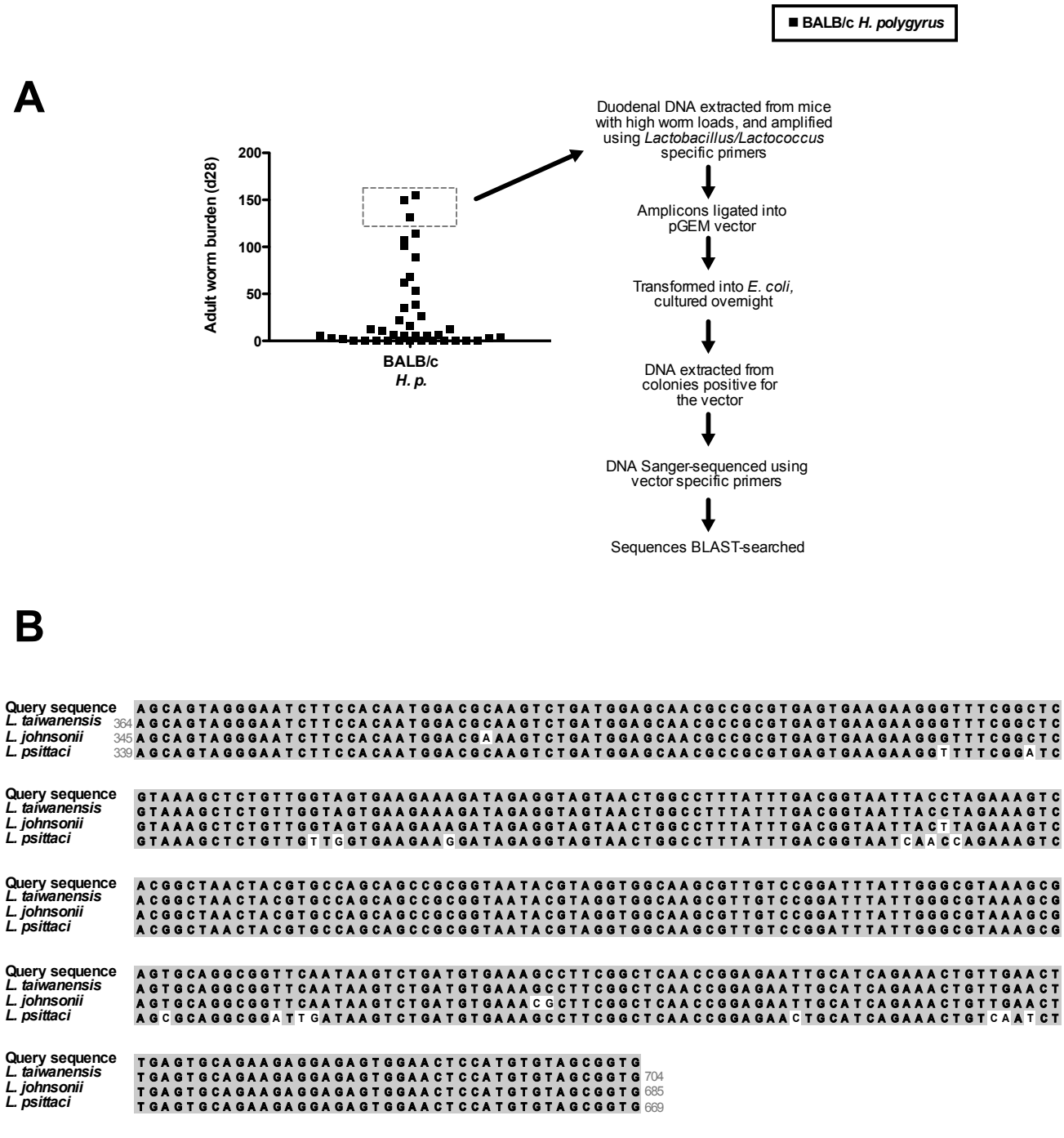

Figure 4.2.6 *Lactobacillus taiwanensis* is the most common species of *Lactobacillus/ Lactococcus* in heavily *H. polygyrus*-infected BALB/c mice.
(A) Experimental strategy. BALB/c female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection, mice were sacrificed and the number of adult worms remaining along the intestinal tract were recorded. DNA from the duodenum of the three most heavily infected mice was extracted, and primers specific for the 16S rRNA gene of *Lactobacillus/Lactococcus* family members were used to amplify this region within the duodenal DNA. Amplicons were ligated into the pGEM vector and transformed into *E. coli* which were then cultured overnight. 13 *E. coli* colonies contained the *Lactobacillus/Lactococcus* amplicons, which were sequenced, then BLAST-searched.
(B) Alignment of the sequencing results from amplicons described in **(A)**- 'Query sequence' with the matching 16S rRNA gene region from *L. taiwanensis,* the same region from a closely related species, *L. johnsonii* and the same region from a more distantly related *Lactobacillus* species- *L. psittaci.* Numbers shown indicate the nucleotide position within the 16S rRNA for each species.

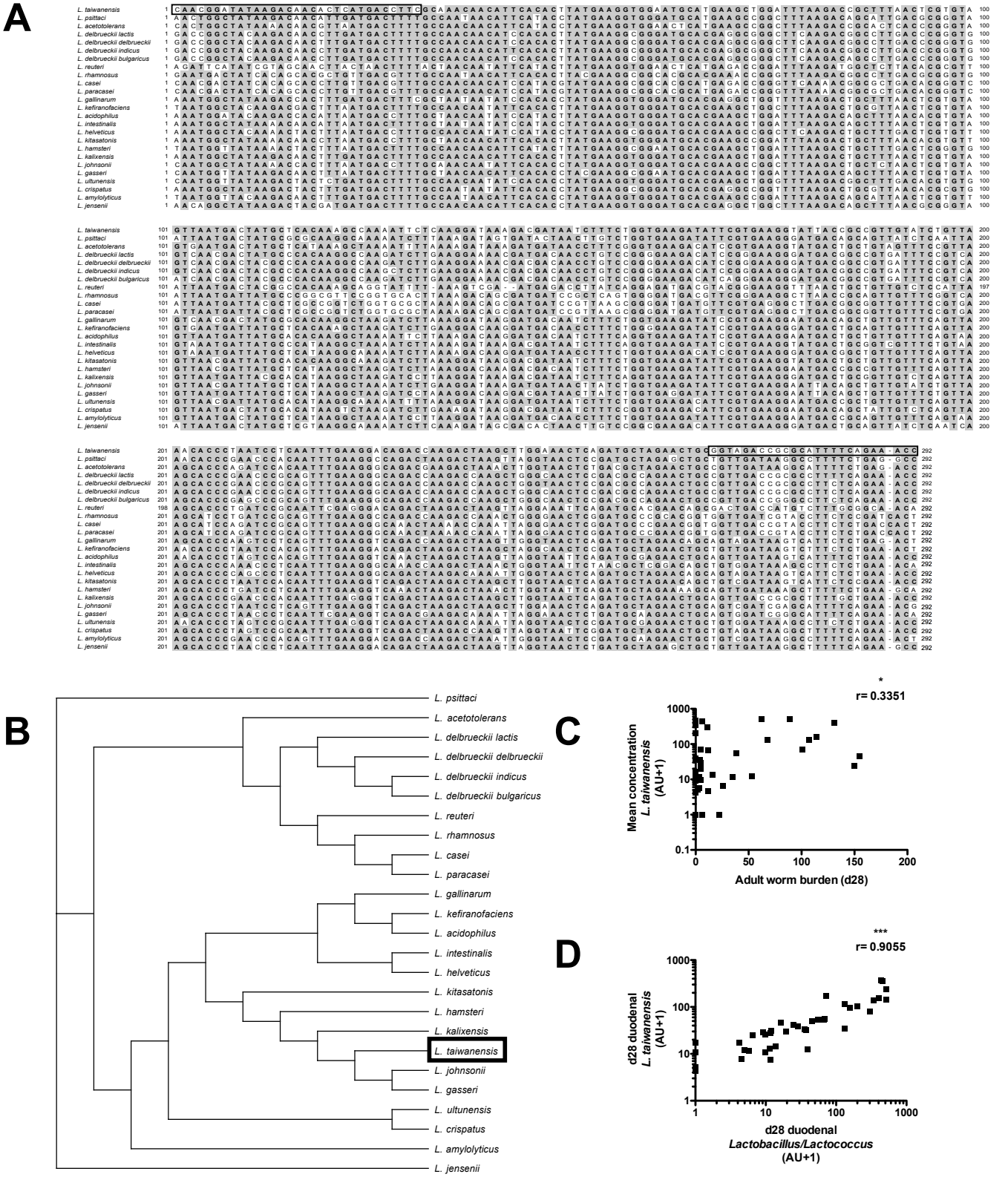

Figure 4.2.7 ***Lactobacillus taiwanensis*** **abundance in the duodenum 28 days post** ***H. polygyrus*****-infection positively correlates with worm burden in BALB/c mice.**

(A) Alignment of *L. taiwanensis* with related species of *Lactobacillus* at a region of the gyrB gene. Boxes indicate the sequence of primers used to amplify *L. taiwanensis*-specific DNA from this locus.

(B) Neighbour-joining tree showing relatedness of *Lactobacillus* species based on the similarity at the region of the gyrB gene shown in **(A)**.

(C-D) BALB/c female mice were infected with 200 *H. polygyrus* L3s. 28 days post-infection mice were sacrificed and the number of adult worms remaining in the intestinal tract was recorded. At the same timepoint, DNA was extracted from the duodenum and real-time PCR was performed using primers specific for the gyrB gene of *L. taiwanensis* described in **(A)**, and the 16S rRNA gene of the *Lactobacillus/Lactococcus* genera. **(C)** A correlation between the number of adult worms and the levels of duodenal *L. taiwanensis*. **(D)** A correlation between the levels of duodenal *L. taiwanensis* and the levels of duodenal *Lactobacillus/Lactococcus.* Spearman r correlation coefficient is shown. Data shown is from one experiment.

* indicates p= <0.05; *** indicates p= <0.001.

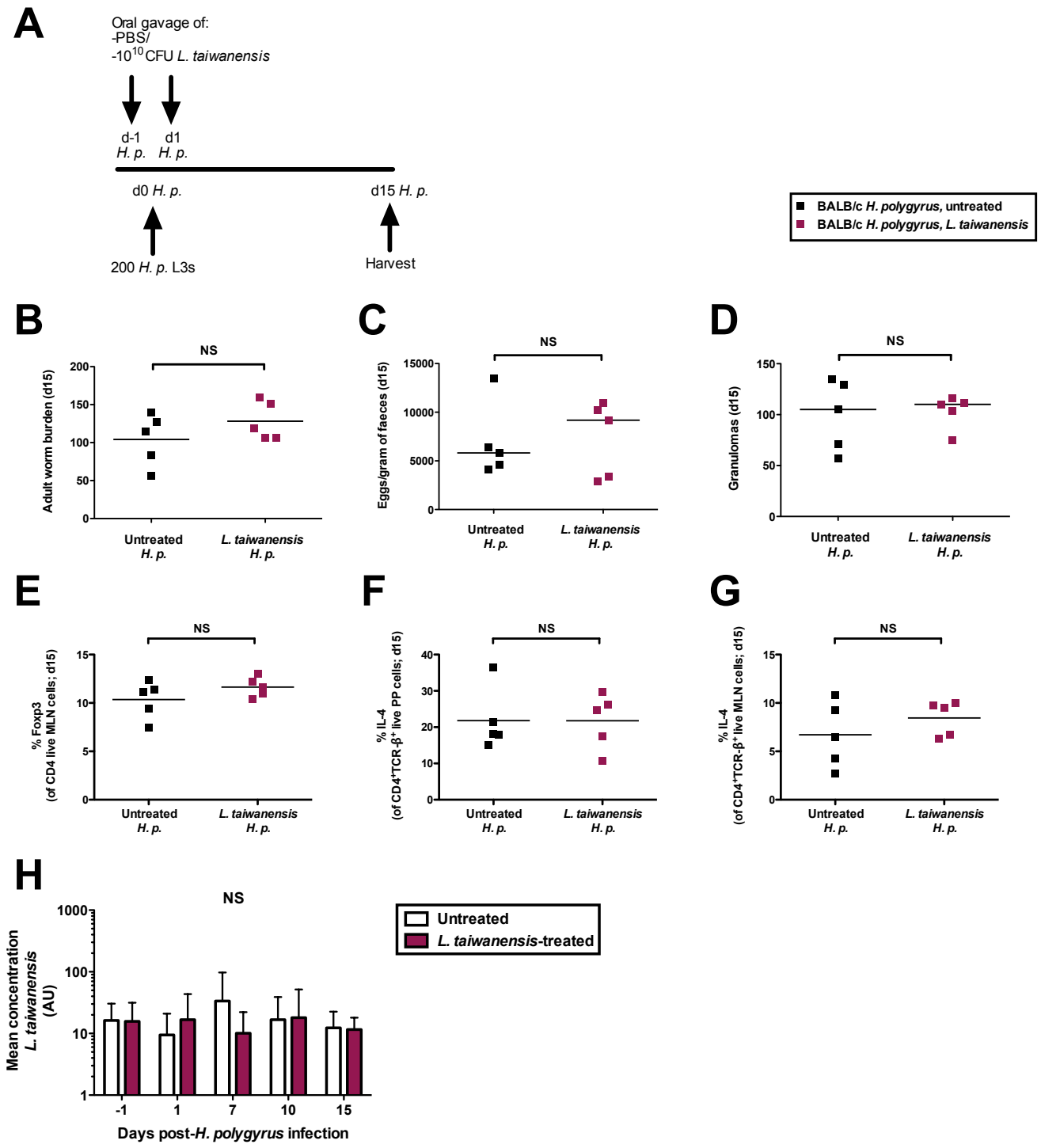

Figure 4.2.8 Oral gavage of 2 x 10^{10} CFU of *L. taiwanensis* is not sufficient to alter the immune response to *H. polygyrus.*

BALB/c female mice were orally gavaged with 10^{10} CFU of *L. taiwanensis* the day before and the day after being infected with 200 *H. polygyrus* L3s. Mice were sacrificed 15 days post-infection and examined for parasitology and immunology. Data shown is from one experiment.

(A) Treatment protocol.

(B-D) The number of **(B)** adult worms along the intestinal tract **(C)** *H. polygyrus* eggs in the faeces and **(D)** granulomas along the intestinal tract were recorded.

(E) MLN cells were isolated, stained directly as indicated and run on a flow cytometer for analysis. % Foxp3 among $CD4^+$ live cells.

(F+G) PP and MLN cells were isolated and stimulated with 0.5 µg/ml PMA and 1 µg/ml Ionomycin for 3.5 hrs, with 10 µg/ml Brefeldin A included for the final 2.5 hrs. Cells were stained as indicated and run on a flow cytometer for analysis. % IL-4 among $CD4^+TCR\text{-}\beta^+$ live **(F)** PP and **(G)** MLN cells.

(H) Faeces were collected at the indicated timepoints and examined for the presence of *L. taiwanensis*. DNA from faeces was isolated and real-time PCR was performed for the gyrB gene of *L. taiwanensis*. Relative mean concentrations in PBS treated and *L. taiwanensis* treated mice are shown, with error bars indicating the standard deviation from mean values. Data were analysed by two-way ANOVA.

NS indicates a non significant result.

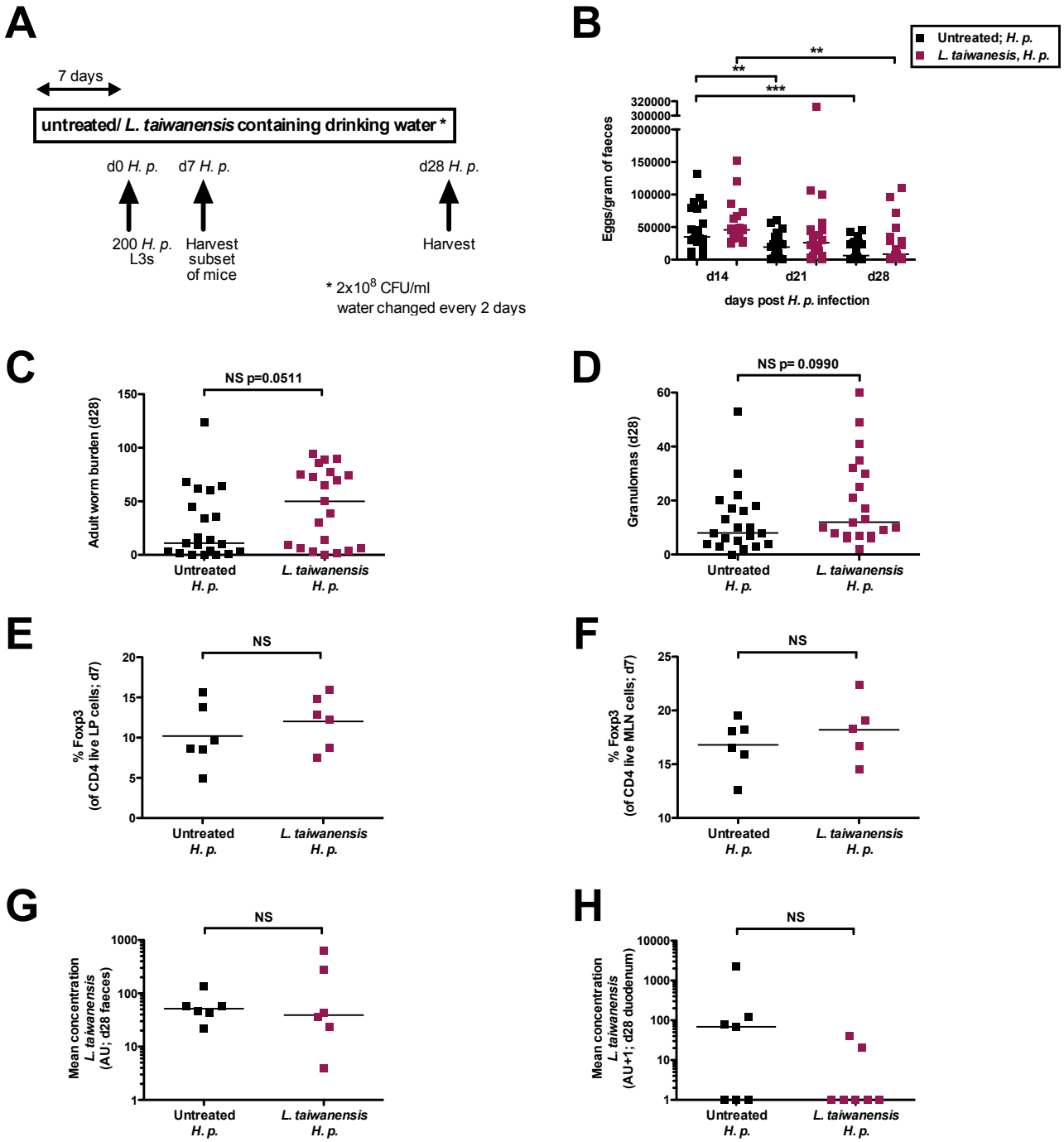

Figure 4.2.9 Administering *L. taiwanensis* in drinking water prolongs *H. polygyrus*-infection in BALB/c mice.

BALB/c female mice were given untreated drinking water or drinking water containing 2 x 10^8 CFU/ml of *L. taiwanensis* for one week prior to *H. polygyrus* infection and for the remainder of the experiment. Mice were infected with 200 *H. polygyrus* L3s. A subset of mice were sacrificed 7 days post-infection to examine the early immunological response; the remainder of mice were sacrificed 28 days post-infection.

(A) Treatment protocol.

(B) Number of *H. polygyrus* eggs released in the faeces at the indicated timepoints following infection. Statistics shown are results from Kruskal-Wallis tests for each treatment group, comparing the egg output at different timepoints.

(C+D) 28 days following infection, the number of **(C)** adult worms along the intestinal tract and **(D)** granulomas along the intestinal tract were recorded.

(E+F) 7 days post-infection, MLN and LP cells were isolated, stained directly as indicated and run on a flow cytometer for analysis. % Foxp3$^+$ cells among CD4$^+$ live **(E)** LP and **(F)** MLN cells.

(G+H) Faeces and duodenums were collected 28 days following *H. polygyrus* infection and examined for the presence of *L. taiwanensis*. DNA was isolated and real-time PCR was performed for the gyrB gene of *L. taiwanensis*. Relative mean concentrations of *L. taiwanesis* are shown in the **(G)** faeces and **(H)** duodenum.

Data shown are **(B-D)** pooled from three independent experiments, **(E-F)** pooled from two independent experiments and **(G+H)** from one experiment. * indicates p= <0.05; NS indicates a non significant result.

4.3 Discussion

The importance of the bacteria which colonise the gastrointestinal tract of mammals is now well recognised. These bacteria provide dietary metabolites, contribute to the intestinal barrier by competing with pathogenic bacteria, and stimulate normal immune development [9]. Many critical roles of the intestinal microflora have been elucidated through studying GF mice, which are entirely devoid of colonising bacteria. Although these mice are viable, GF mice require exogenous vitamins including B and K in their diet, and have greatly reduced lymphatic tissue, resulting in a heightened susceptibility to the majority of bacterial and viral infectious agents [3]. As well as the presence of an intestinal microflora being required to stimulate normal immune development, the composition of bacterial populations appears important, as specific species of bacteria are able to promote the differentiation of naïve T cells towards specific T effector or regulatory fates [87, 88, 90, 91]. Modulating the composition of the intestinal bacteria can alter the predisposition to autoimmune [99, 105-110] and allergic [95, 96] diseases, as well as influencing the outcome of both bacterial [87, 106, 108, 117-120] and viral [113-116, 268] infections. In this chapter, the composition of the microflora was altered, to investigate how the murine response to *H. polygyrus* was affected.

It is perhaps surprising that GF mice are more resistant to *H. polygyrus* infection [223-225], since due to their under-developed immune system, they are more susceptible to many other infectious bacterial and viral organisms [3]. This could result if the intestinal environment of GF mice is altered in such a way that *H. polygyrus* cannot inhabit the gut as readily. For example, GF mice produce higher amounts of mucin, and their gut pH is higher than SPF mice [3]- both of these conditions could provide a negative environment for *H. polygyrus*. Alternative explanations may be that immune responsiveness in GF mice is skewed towards pathways which are better suited for worm expulsion, or that the presence of the microbiota is actively required to promote *H. polygyrus* survival.

To study the effect of a lack of microbial flora in a setting where mice were developmentally normal, antibiotics were administered to mice using a range of regimens . Initial experiments showed that multiple broad-spectrum antibiotic

treatments (cocktails 1+2) did not affect *H. polygyrus* worm burden after 28 days of infection, however, replicating these experiments was decided against, due to the negative effects of treatment on the health of the mice. Although the use of antibiotic cocktail 2 has been reported in several papers without descriptions of adverse effects [12, 18, 108, 269], other groups have noted similar problems with weight loss [270] as was seen here. An alternative and effective strategy for microbiota depletion has been suggested by Reikvam et al [270], whereby mice are orally gavaged with antibiotics, to prevent dehydration and weight loss, which could be used for future confirmation as to whether microbiota depletion affects susceptibility to *H. polygyrus*.

Here, treatment of mice with Trimethoprim and Sulfadoxine, to modify, rather than delete the microbiota, resulted in a lower fecundity of *H. polygyrus*, and a reduced adult worm load in 3 out of 5 experiments. Trimethoprim and Sulfadoxine treatment did not produce a consistent modification of the bacterial groups measured, which may explain the inconsistent effect seen on altering *H. polygyrus* expulsion.

In vitro assays suggested that Trimethoprim and Sulfadoxine treatment did not directly damage adult *H. polygyrus* worms, however, it is still possible that Trimethoprim and Sulfadoxine directly damaged *H. polygyrus* larvae, thus inhibiting parasite establishment. Similar *in vitro* fitness assays need to be done with Trimethoprim and Sulfadoxine and larval *H. polygyrus* to rule out this possibility. However, since Trimethoprim and Sulfadoxine did not affect *H. polygyrus* survival in 2 out of the 5 experiments, it seems likely that these antibiotics are not directly damaging the parasite, and that the inconsistencies between experiments are due to differential effects of the antibiotics on the intestinal microbiota.

Shifts in any of the bacterial groups measured could not consistently explain the outcome of infection, thus it is likely that the worm burden-determining bacterial group was not being measured in these experiments. To resolve this, a thorough examination of intestinal microbiota changes following Trimethoprim and Sulfadoxine treatment is necessary, by high throughput pyrosequencing of the 16S rRNA gene.

Faecal transplants from Trimethoprim and Sulfadoxine treated mice were not sufficient to alter susceptibility of *H. polygyrus* in recipient mice, however, due to the ill-defined effects of Trimethoprim and Sulfadoxine treatment, it was difficult to measure whether the faecal transplants were sufficient to induce the relevant shifts in microbiota of recipient mice which would affect worm burden.

An additional concern with antibiotic treatment is that a disruption of the intestinal microbiota may affect the nutritional status of treated mice, as the intestinal microbiota are required for production of Vitamins B12, K, and folic acid [2], and in the absence of microbiota, increases in free amino acids and urea are found in the faeces [3, 4]. Protein-malnourished mice mount a weaker Th2 response to *H. polygyrus*, and maintain higher worm burdens than control mice at day 28 post-infection [271]. The absence or modification of intestinal microbiota may thus affect a *H. polygyrus* infection indirectly by altering the nutritional status of mice, as well as by potentially modifying the immune response of the mouse.

A more defined approach than antibiotic treatment was next taken, whereby the most abundant species of *Lactobacillus/Lactococcus* in highly infected BALB/c mice was identified as *L. taiwanensis*. The immunomodulatory abilities of *L. taiwanensis* have not been previously investigated, however other members of the *Lactobacillus/Lactococcus* family, including *Lactobacillus rhamnosus, Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Lactobacillus casei* have all been shown to promote Treg expansion [219-222] when administered to mice.

When GF mice were monoassociated with *Lactobacillus*, *H. polygyrus* survival was increased compared to GF mice, yet survival was not as great as in SPF mice [223]. This may be taken as evidence that *Lactobacillus* species are able to improve *H. polygyrus* survival, however the effect may not be specific to *Lactobacillus*; it may be that the presence of any bacterial species in previously GF mice can increase *H. polygyrus* persistence.

Interestingly, when BALB/c mice were administered *L. taiwanensis* in their drinking water, egg production by *H. polygyrus* had not significantly diminished 21 days post-infection, in contrast to untreated mice. *L. taiwanensis*-fed mice

tended to harbor more adult worms 28 days post-infection than untreated mice, though this did not reach statistical significance. One concern with these experiments was the inability to detect an increase in *L. taiwanensis* abundance in the duodenum or faeces following treatment with *L. taiwanensis*-containing drinking water. It is possible that the administered *L. taiwanensis* may not be able to colonise the intestinal tract, as it has been previously reported that oral administration of *Lactobacillus fermentum* (two doses of 1 x 10^8 CFU) can only be detected in the faeces of SPF C57BL/6 mice for 24 hours [272]. However, given that *L. taiwanensis* was being continuously administered in the drinking water of mice, it was surprising that no detectable differences in *L. taiwanensis* levels were seen here. If, as these data suggest, *L. taiwanensis* is already the dominant *Lactobacillus* species in the intestinal tract of the BALB/c colony used here, the difference in levels upon *L. taiwanensis* administration may be too small to detect by PCR.

The implication that feeding of a single microbe could alter the outcome of a *H. polygyrus* infection is striking, and in concordance with the findings of Chapter 3. Further experiments with differing doses of *L. taiwanensis*, and administration of different components of the microbiota, are necessary to confirm these findings, and to determine whether other microbiota have detrimental effects on *H. polygyrus* survival. An early report has also alluded that the microbiota composition may affect the efficacy of a *H. polygyrus* infection; Lewis et al reported low *H. polygyrus* establishment and egg production until mice were treated with the antibiotic Oxytetracycline hydrochloride, suggesting that Oxytetracycline hydrochloride-susceptible bacteria are able to negatively influence *H. polygyrus* survival [273].

If the intestinal microbiota are able to modulate susceptibility to *H. polygyrus*, it is necessary to understand the mechanisms by which bacteria are detected by the immune system during infection. Next, therefore, TLR deficient mice were examined for their capacity to respond to *H. polygyrus* infection.

Chapter 5. Examining the response to *Heligmosomoides polygyrus* in Toll-like receptor-deficient mice

5.1 Introduction

One of the challenges faced by the mammalian immune system is tolerating the existence of commensal or symbiotic bacteria along the intestinal tract, yet maintaining the ability to respond rapidly to opportunistic bacterial pathogens. The major way this problem is addressed is by compartmentalising the gut microflora, so that under normal circumstances commensal bacteria do not trigger an immune response. Mucus production [54, 55, 57], IgA secretion [70-74] and the release of antimicrobial peptides [58, 63, 64] are the predominant methods by which bacteria are segregated from intestinal epithelial cells.

The immune system recognises highly conserved bacterial components through PRRs, which include the TLRs, NLRs and CLRs (see 1.3). Recognition of the intestinal bacteria by these PRRs is required to maintain gut homeostasis in conditions where the epithelial cell barrier function is lost. Deficiencies in PRR signalling pathways lead to heightened morbidity and mortality after DSS-treatment to induce colitis [12, 101, 104]. Following epithelial cell damage, inflammatory and reparative cytokines including TNF and IL-6 are induced to mediate repair, and it is the recognition of intestinal microbes that provide the trigger for production of these cytokines [12].

Given that during the *H. polygyrus* lifecycle, the epithelial cell barrier is disrupted (Figure 1.7.2), it is likely that ligation of PRRs by intestinal bacteria occurs as *H. polygyrus* L3s enter the submucosa. A recent study has shown that when the integrity of the gut epithelium is disrupted during *T. gondii* infection, a microflora-specific T cell response is mounted [75]. Thus, it is reasonable to assume that the presence of the microflora will influence the immune environment surrounding infective *H. polygyrus* L3s. Data discussed

in the previous two chapters suggested that the composition of the intestinal microbiota can influence the susceptibility of mice to *H. polygyrus*. Here, whether recognition of the microflora by PRRs alters susceptibility to *H. polygyrus* is addressed.

Previous studies have reported that MyD88$^{-/-}$ mice possess a differing microflora to Wt mice [76], however no differences in microflora composition are seen following co-housing of the two genotypes [66]. To eliminate differences in microflora populations as far as possible, for experiments described in this chapter all mice were co-housed, or bedding was frequently mixed between cages for two weeks prior to infections, and throughout the course of the experiments.

Interestingly, it was found that mice lacking the TLR adaptor protein MyD88 are more resistant to *H. polygyrus* than Wt mice. This was associated with high numbers of granulomas being produced following *H. polygyrus* infection. This increased expulsion could not be attributed to a lack of signalling through TLR2, TLR4, TLR5, or TLR9, leading to the possibilities that either these TLRs signal redundantly, or that signalling through other receptors which utilise MyD88 are able to modulate immunity.

5.2 Results

5.2.1 MyD88-deficiency renders mice more resistant to *H. polygyrus* than Wt C57BL/6 mice

Data from Chapters 3 and 4 suggested that the presence of bacteria may modulate the outcome of a *H. polygyrus* infection. To investigate this further, mice lacking the adaptor protein MyD88, through which TLRs signal [33, 274], were compared to Wt C57BL/6 for their susceptibility to *H. polygyrus*.

It was found that MyD88$^{-/-}$ mice expel *H. polygyrus* more rapidly than Wt C57BL/6 controls, with a significantly lower egg output seen at day 14 and day 27 post-infection, and a significantly lower number of adult worms remaining in the host 28 days following infection (Figure 5.2.1 A-C). A striking phenotype of MyD88$^{-/-}$ mice was the heightened number of granulomas that form along the intestinal tract in response to *H. polygyrus* infection (Figure 5.2.1 D), which rarely form on the C57BL/6 background (Figure 5.2.1 D + 3.2.1 E).

5.2.2 Individual TLR$^{-/-}$ mice harbour similar *H. polygyrus* burdens to Wt C57BL/6 mice

While MyD88 is utilised for signalling by TLRs [33], a number of additional receptors including the IL-1R [274-276], IL-18R [274] and the IL-33 receptor T1/ST2 [277] are also dependent on MyD88 for signalling transduction. To address whether the increased resistance to *H. polygyrus* of MyD88$^{-/-}$ mice is due to a lack of signalling through a specific TLR, mice deficient in individual TLR receptors were assessed for their susceptibility to *H. polygyrus*. However, the absence of TLR2, TLR4, TLR5 or TLR9 did not reproduce the phenotype of the MyD88-deficient mouse. Thus, although *H. polygyrus* showed reduced fecundity by day 28 post-infection in TLR2$^{-/-}$ mice (Figure 5.2.2 A), adult worm numbers remaining in the gut at this time point were equivalent to those in Wt mice (Figure 5.2.2 B). Moreover, neither TLR4$^{-/-}$

(Figure 5.2.2 C+D), TLR5$^{-/-}$ (Figure 5.2.2 E+F) nor TLR9$^{-/-}$ mice (Figure 5.2.2 G+H) showed significant differences in *H. polygyrus* fecundity or expulsion compared to Wt controls.

5.2.3 MyD88$^{-/-}$TRIF$^{-/-}$ mice, but not TRIF$^{-/-}$ mice, are more resistant to *H. polygyrus* than Wt C57BL/6 mice

MyD88$^{-/-}$ mice retain the ability to respond to certain bacterial signals, as TLR4, which recognises LPS [29], can signal independently of MyD88 through the adaptor protein TRIF [28]. Thus, only mice deficient in both MyD88 and TRIF are unable to respond to bacterial ligands through TLRs, and hence MyD88$^{-/-}$TRIF$^{-/-}$ doubly-deficient mice were tested for susceptibility to *H. polygyrus* infection.

MyD88$^{-/-}$TRIF$^{-/-}$ mice were similar to MyD88$^{-/-}$ in their response to *H. polygyrus* infection, with reduced *H. polygyrus* fecundity and adult worm survival compared to Wt mice by day 28 post-infection (Figure 5.2.3 A+B). However, the MyD88$^{-/-}$TRIF$^{-/-}$ phenotype was slightly weaker than that of MyD88-singly deficient mice, in terms of fecundity, as egg production was not reduced at day 14. Hence, the loss of TRIF appeared to weaken resistance to infection.

In contrast to MyD88$^{-/-}$TRIF$^{-/-}$, mice deficient only in TRIF displayed no differences in susceptibility to *H. polygyrus* from Wt mice (Figure 5.2.3 C+D), confirming that MyD88 is the key adaptor protein responsible for the resistant phenotype of MyD88$^{-/-}$TRIF$^{-/-}$ mice.

5.2.4 Increased granuloma formation in MyD88$^{-/-}$ mice requires the presence of TRIF

In these experiments, individual TLR-deficient and TRIF-deficient mice were also examined for the development of granulomas following *H. polygyrus* infection. No mice deficient in individual TLR or TRIF molecules produced the high number of granulomas seen in MyD88$^{-/-}$ mice (Figure 5.2.4).

Surprisingly, MyD88$^{-/-}$TRIF$^{-/-}$ mice examined did not produce as many granulomas (mean 10.6; 95% confidence intervals 2.5-18.8) as mice only deficient in MyD88 (mean 38.4; 95% confidence intervals 28.9-47.9; Figure 5.2.4), suggesting that TRIF is required for the high level of granuloma formation observed in the absence of MyD88, and that these two adaptor proteins act in opposing directions with respect to the granulomatous response.

5.2.5 Irradiated MyD88$^{-/-}$ mice do not form high granuloma numbers following *H. polygyrus* infection

As Wt C57BL/6 mice are susceptible to a primary *H. polygyrus*-infection, and produce few granulomas in response to infection, it was hypothesised that in Wt mice signalling through MyD88 inhibits granuloma formation and *H. polygyrus* expulsion. To test which cells need to express MyD88 to prevent granuloma formation, bone marrow chimeras were generated, in which either only haematopoietic or non-haematopoietic cells expressed MyD88. Mice were lethally irradiated, and reconstituted with bone marrow from Wt or MyD88$^{-/-}$ donors. Eight weeks following bone marrow reconstitution all mice were infected with *H. polygyrus*.

As expected, MyD88$^{-/-}$ recipients reconstituted with MyD88$^{-/-}$ bone marrow (MyD88$^{-/-}$(MyD88$^{-/-}$) mice) showed reduced *H. polygyrus* egg output compared to Wt recipients reconstituted with Wt bone marrow (Wt(Wt)) mice 14 days post-infection (Figure 5.2.5 A). However, by 28 days post-infection, the reduced levels of egg output and adult worm burdens in MyD88$^{-/-}$(MyD88$^{-/-}$) mice did not reach significance when compared to Wt(Wt) mice (Figure 5.2.5 B+C). Wt(MyD88$^{-/-}$) and MyD88$^{-/-}$(Wt) mice had equivalent egg and worm burdens to Wt (Wt) mice at all time points examined (Figure 5.2.5 A-C). Most notably, very few granulomas formed following infection with *H. polygyrus* in any of the mice subjected to irradiation, including the MyD88$^{-/-}$(MyD88$^{-/-}$) mice (Figure 5.2.5 D). Thus, it was difficult to conclude whether MyD88 is required on haematopoietic or non-haematopoietic cells to inhibit granuloma formation,

as in this reconstitution model, the ability of MyD88$^{-/-}$(MyD88$^{-/-}$) mice to form granulomas in response to *H. polygyrus* was lost. Further, these data indicate that an irradiation-sensitive stromal cell in the intestinal tissue may be required for the generation of granulomas in the MyD88-deficient setting, and that granulomas contribute to reduced fitness, fecundity and survival of *H. polygyrus.*

5.2.6 MyD88$^{-/-}$ mice have high basal IFN-γ levels but normal early Th2 responsiveness to *H. polygyrus*

To determine immunological correlates of increased *H. polygyrus* expulsion and granuloma formation in intact MyD88$^{-/-}$ mice, the early cytokine response to infection was examined. No difference to Wt mice was seen in CD4^{+} T cell IL-4 production 5 days following *H. polygyrus* infection, with increased IL-4 production in the PP and MLN following infection in both MyD88-sufficient and -deficient mice (Figure 5.2.6 A+D). MyD88$^{-/-}$ mice had higher basal production of IFN-γ from CD4^{+} T cells than naïve Wt mice, both in the PP and MLN (Figure 5.2.6 B+E), although following infection in the MLN IFN-γ levels were comparable to Wt mice (Figure 5.2.6 E). Although IL-17A production in the PP was not elevated following infection in MyD88$^{-/-}$ mice, whereas it was in Wt mice at this site, overall levels of this cytokine were similar between the two genotypes (Figure 5.2.6 C+F). Thus, although MyD88$^{-/-}$ mice show dysregulation in T cell IFN-γ and IL-17A production, the early IL-4 response to *H. polygyrus* is unaltered.

5.2.7 MyD88$^{-/-}$ mice mount a stronger T cell cytokine response 28 days following *H. polygyrus* infection than Wt C57BL/6 mice

Cytokine production at later timepoints following infection (28 days post-infection) were next examined. Despite MyD88$^{-/-}$ mice having fewer worms at this timepoint (Figure 5.2.1 C) and thus a lower antigen load, levels of IL-4,

IFN-γ and IL-17A being produced by $CD4^+$ T cells in the MLN of *H. polygyrus*-infected mice were higher than in Wt control mice (Figure 5.2.7 A-C).

To test whether the dysregulated cytokine repertoire of $MyD88^{-/-}$ mice influenced antibody class switching, the anti-HES antibody response was examined 28 days post-infection. $MyD88^{-/-}$ mice mounted an equivalent HES-specific IgG1 serum response to that seen in infection of Wt mice (Figure 5.2.7 D), yet some $MyD88^{-/-}$ mice failed to increase IgA production following *H. polygyrus* infection (Figure 5.2.7 E).

5.2.8 TLR signalling contributes differentially to Treg proportions following *H. polygyrus* infection

As $MyD88^{-/-}$ mice produced more cytokines of Th1, Th2 and Th17 subsets than Wt mice 28 days post-infection, the proportions and activation status of Tregs were examined in these mice. Surprisingly, no deficiencies in $Foxp3^+CD4^+$ T cell proportions, or in their expression of CD103, were seen in the MLN of $MyD88^{-/-}$ mice (Figure 5.2.8 A+B), or in $MyD88^{-/-}TRIF^{-/-}$ mice (data not shown).

Dysregulation of Treg proportions were, however, seen in other TLR-signalling deficient mice. *H. polygyrus*-infected $TLR2^{-/-}$, $TLR5^{-/-}$ and $TRIF^{-/-}$ mice had a lower proportion of $Foxp3^+CD4^+$ MLN T cells than infected Wt mice (Figure 5.2.8 C,E,I). While the proportion of $Foxp3^+CD4^+$ MLN T cells expressing CD103 was equivalent to Wt in $TLR5^{-/-}$ mice (Figure 5.2.8 F), these cells expressed more CD103 both in $TLR2^{-/-}$ and $TRIF^{-/-}$ mice (Figure 5.2.8 D+J). Conversely, $TLR9^{-/-}$ mice had a higher proportion of $Foxp3^+CD4^+$ MLN T cells following *H. polygyrus* infection than Wt mice (Figure 5.2.8 G), and expression of CD103 by these cells was also higher than in Wt mice (Figure 5.2.8 H). $TLR4^{-/-}$ mice displayed no differences to Wt mice in $Foxp3^+CD4^+$ MLN T cell proportions or CD103 expression following *H. polygyrus* infection (data not shown).

Thus, independent TLR signalling contributes differentially to Treg proportions and marker expression following infection, resulting in no overall differences in Treg phenotype to Wt mice when TLR signalling is ablated in MyD88$^{-/-}$ and MyD88$^{-/-}$TRIF$^{-/-}$ mice.

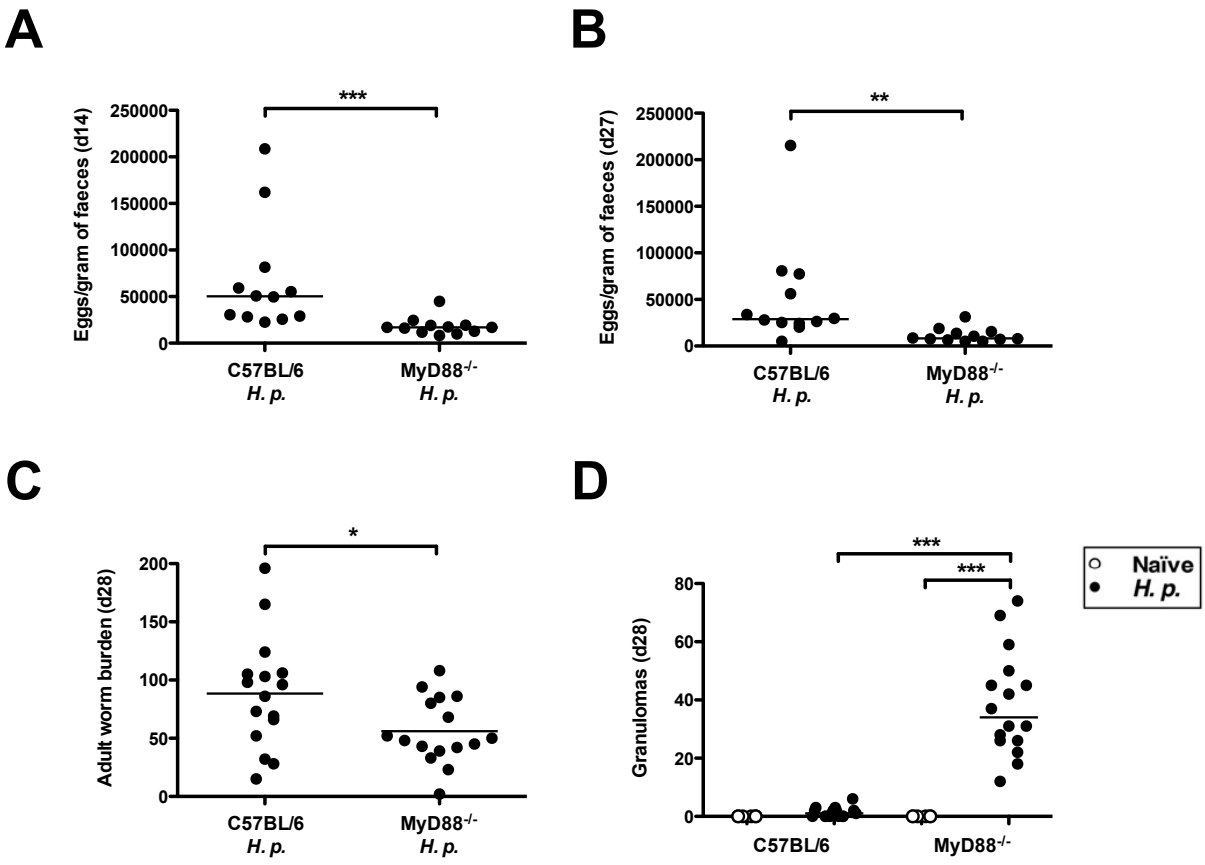

Figure 5.2.1 MyD88-deficiency renders mice more resistant to *H. polygyrus* than Wt C57BL/6 mice.

Bedding was mixed between C57BL/6 and MyD88$^{-/-}$ male mouse cages, and female C57BL/6 and MyD88$^{-/-}$ mice were co-housed for at least two weeks prior to *H. polygyrus* infection and throughout the experiment. Mice were left naïve or infected with 200 *H. polygyrus* L3s. Open symbols (O) represent naïve mice; closed symbols (●) represent *H. polygyrus*-infected mice. Data shown is pooled from three independent experiments.

(A+B) *H. polygyrus* eggs per gram of faeces taken **(A)** 14 days and **(B)** 27 days following *H. polygyrus* infection.

(C) Adult *H. polygyrus* numbers recovered from the intestinal tract 28 days following *H. polygyrus* infection.

(D) Granuloma number along the intestinal tract of naïve or 28 day *H. polygyrus*-infected mice.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

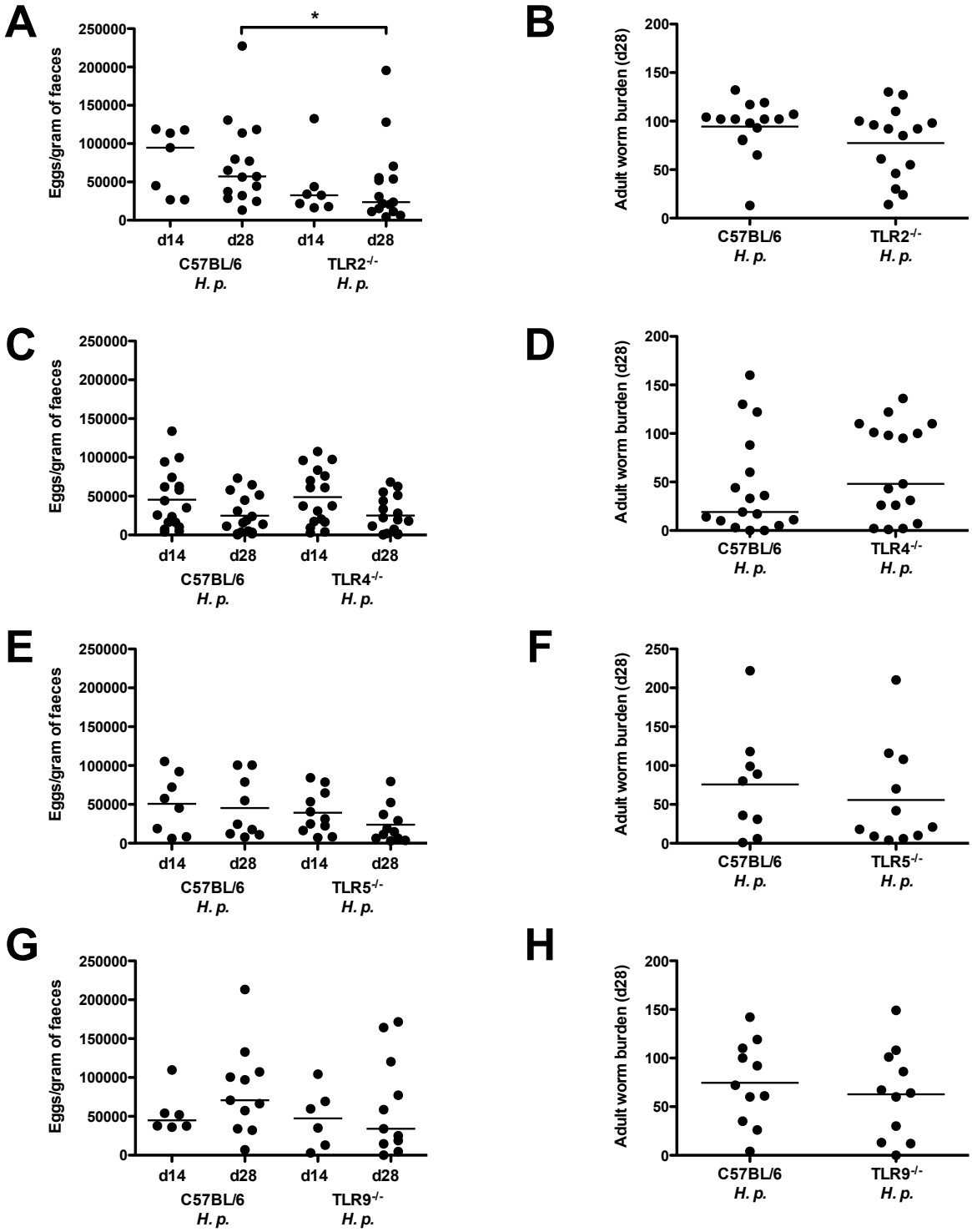

Figure 5.2.2 Individual TLR$^{-/-}$ mice harbour similar *H. polygyrus* burdens to Wt C57BL/6 mice.

Bedding was mixed between C57BL/6 and gene-deficient male mouse cages, and female C57BL/6 and gene-deficient mice were co-housed for at least two weeks prior to *H. polygyrus* infection and throughout the experiment. Mice were infected with 200 *H. polygyrus* L3s.

(A,C,E,G) *H. polygyrus* eggs per gram of faeces were enumerated at 14 and 28 days post-infection. Statistical comparisons were made between genotypes at the same timepoint.

(B,D,F,H) Adult *H. polygyrus* numbers were assessed 28 days following *H. polygyrus* infection.

Data is shown for Wt C57BL/6 mice and **(A+B)** TLR2$^{-/-}$ mice; data pooled from four independent experiments **(C+D)** TLR4$^{-/-}$ mice; data pooled from three independent experiments, **(E+F)** TLR5$^{-/-}$ mice; data pooled from two independent experiments and **(G+H)** TLR9$^{-/-}$ mice; data pooled from two independent experiments and representative of the results from five independent experiments.

* indicates p= <0.05.

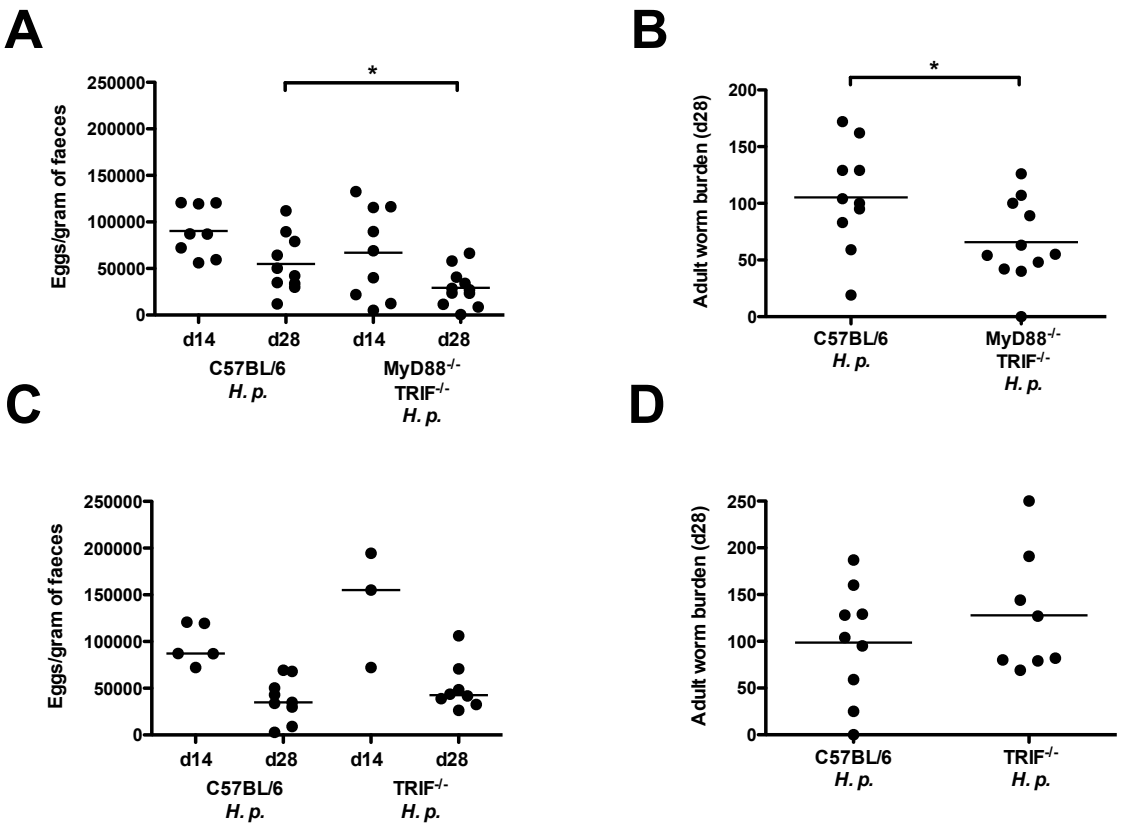

Figure 5.2.3 MyD88$^{-/-}$TRIF$^{-/-}$ mice, but not TRIF$^{-/-}$ mice, are more resistant to *H. polygyrus* than Wt C57BL/6 mice.

Bedding was mixed between C57BL/6 and gene-deficient male mouse cages, and female C57BL/6 and gene-deficient mice were co-housed for at least two weeks prior to *H. polygyrus* infection and throughout the experiment. Mice were infected with 200 *H. polygyrus* L3s.
(A,C) *H. polygyrus* eggs per gram of faeces were enumerated at 14 and 28 days post-infection. Statistical comparisons were made between genotypes at the same timepoint.
(B,D) Adult *H. polygyrus* numbers were assessed 28 days following *H. polygyrus* infection.
Data is shown for wildtype C57BL/6 mice and **(A+B)** MyD88$^{-/-}$TRIF$^{-/-}$ mice; data pooled from three independent experiments **(C+D)** TRIF$^{-/-}$ mice; data pooled from two independent experiments.
* indicates p= <0.05.

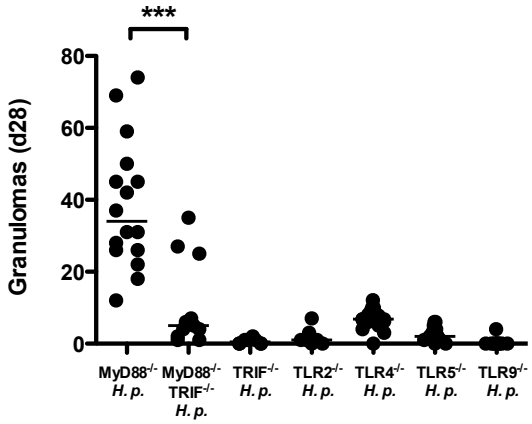

Figure 5.2.4 Increased granuloma formation in MyD88$^{-/-}$ requires the presence of TRIF.
Bedding was mixed between C57BL/6 and gene-deficient male mouse cages, and female C57BL/6 and gene-deficient mice were co-housed for at least two weeks prior to *H. polygyrus* infection and throughout the experiment. Mice were infected with 200 *H. polygyrus* L3s.
Granuloma number along the intestinal tract of naïve or 28 day *H. polygyrus*-infected mice. Data shown for each genotype is pooled from two or three independent experiments, and experiments for each genotype were performed seperately.
Statistical comparisons are shown only between MyD88$^{-/-}$ and MyD88$^{-/-}$TRIF$^{-/-}$ mice; *** indicates p= <0.001.

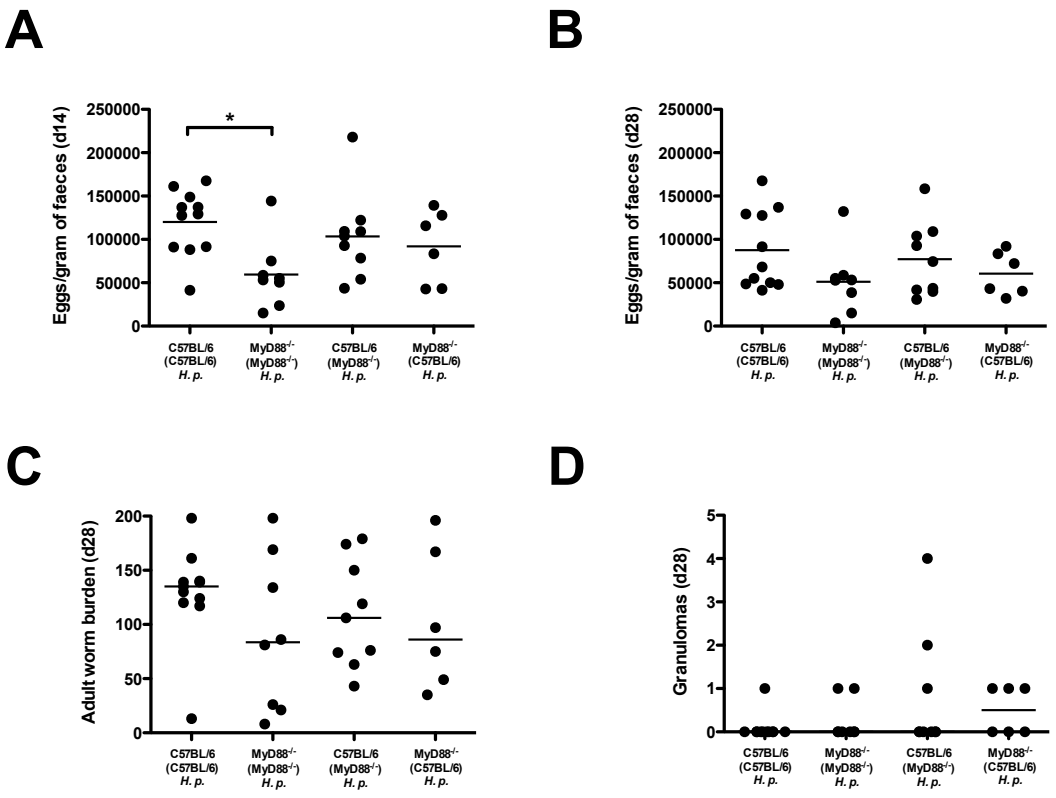

Figure 5.2.5 Irradiated MyD88$^{-/-}$ mice do not form high granuloma numbers following *H. polygyrus* infection.
C57BL/6 or MyD88$^{-/-}$ male mice were given a lethal radiation dose of 980 rad, and reconstituted the following day with bone marrow cells from C57BL/6 or MyD88$^{-/-}$ donors. Eight weeks following reconstitution, all mice were infected with 200 *H. polygyrus* L3s. The genotypes of bone marrow cells used for reconstitution are shown in parentheses.
(A+B) *H. polygyrus* eggs per gram of faeces taken **(A)** 14 days and **(B)** 28 days following *H. polygyrus* infection.
(C) Adult *H. polygyrus* numbers recovered from the intestinal tract 28 days following *H. polygyrus* infection.
(D) Granuloma number along the intestinal tract of 28 day *H. polygyrus*-infected mice.
Data shown is pooled from two independent experiments; * indicates p= <0.05.

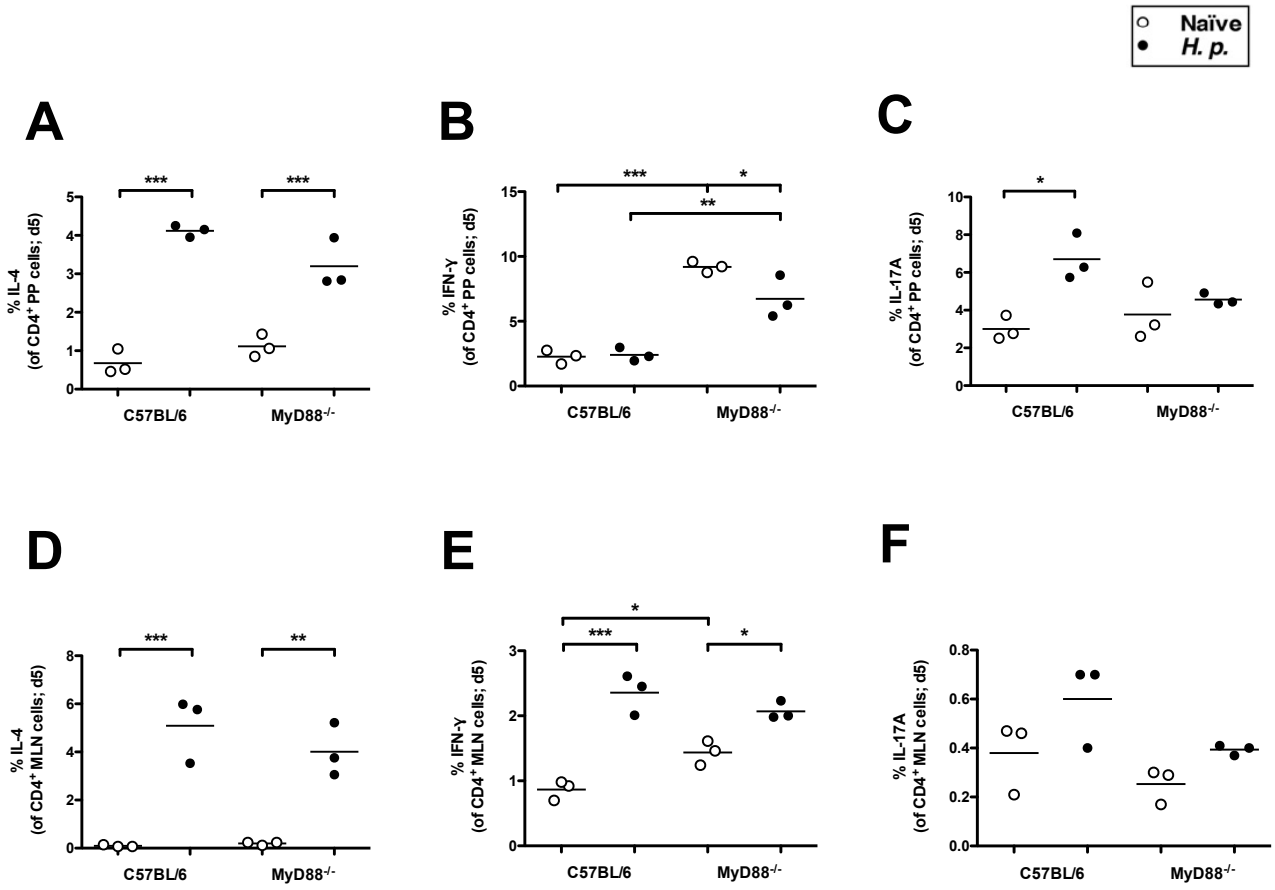

Figure 5.2.6 MyD88$^{-/-}$ mice have high basal IFN-γ levels but normal early Th2 responsiveness to *H. polygyrus*.

C57BL/6 or MyD88$^{-/-}$ male mice were left naïve or infected with 200 *H. polygyrus* L3s. 5 days following infection PP and MLN cells were isolated and stimulated with 0.5 µg/ml PMA and 1 µg/ml Ionomycin for 3.5 hrs, with 10 µg/ml Brefeldin A included for the final 2.5 hrs. Cells were stained as indicated and run on a flow cytometer for analysis. Open symbols (O) represent naïve mice; closed symbols (●) represent *H. polygyrus*-infected mice. Data shown is from one experiment.

(A) Proportion of IL-4 producing cells amongst CD4$^+$ PP cells.
(B) Proportion of IFN-γ producing cells amongst CD4$^+$ PP cells.
(C) Proportion of IL-17A producing cells amongst CD4$^+$ PP cells.
(D) Proportion of IL-4 producing cells amongst CD4$^+$ MLN cells.
(E) Proportion of IFN-γ producing cells amongst CD4$^+$ MLN cells.
(F) Proportion of IL-17A producing cells amongst CD4$^+$ MLN cells.
* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

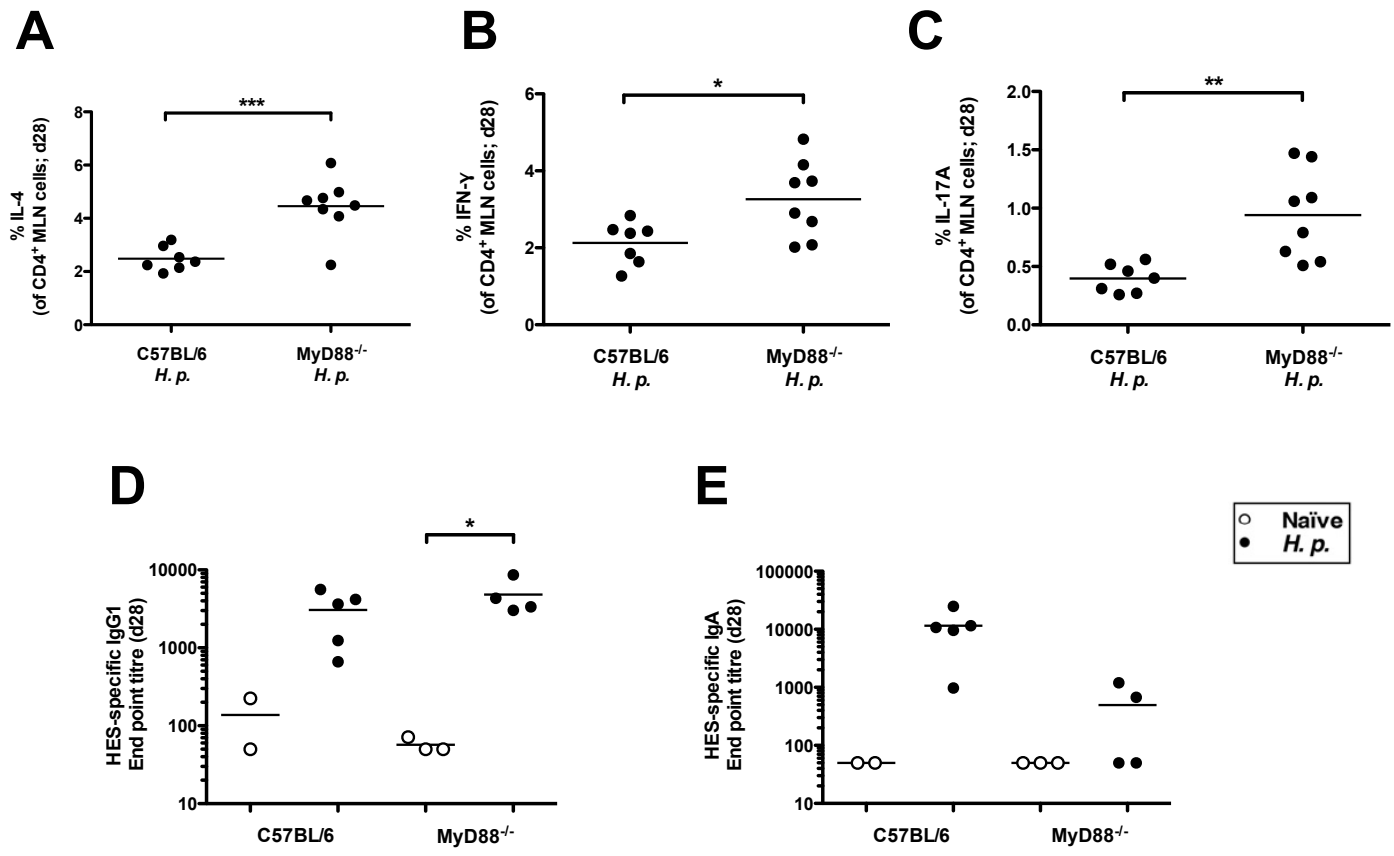

Figure 5.2.7 MyD88$^{-/-}$ mice mount a stronger T cell cytokine response 28 days following *H. polygyrus* infection than Wt C57BL/6 mice.

Bedding was mixed between C57BL/6 and MyD88$^{-/-}$ male mouse cages, and female C57BL/6 and MyD88$^{-/-}$ mice were co-housed for at least two weeks prior to *H. polygyrus* infection and throughout the experiment. Mice were left naïve or infected with 200 *H. polygyrus* L3s. Open symbols (O) represent naïve mice; closed symbols (●) represent *H. polygyrus*-infected mice.

(A-C) 28 days following infection MLN cells were isolated and stimulated with 0.5 µg/ml PMA and 1 µg/ml Ionomycin for 3.5 hrs, with 10 µg/ml Brefeldin A included for the final 2.5 hrs. Cells were stained as indicated and run on a flow cytometer for analysis. Data shown are from one experiment and representative of the results of three independent experiments. Proportion of **(A)** IL-4 **(B)** IFN-γ and **(C)** IL-17A producing cells amongst CD4^{+} MLN cells.

(D+E) End point HES-specific **(D)** IgG1 and **(E)** IgA titres in the sera of naïve and 28-day *H. polygyrus* infected mice. Data are from one experiment.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

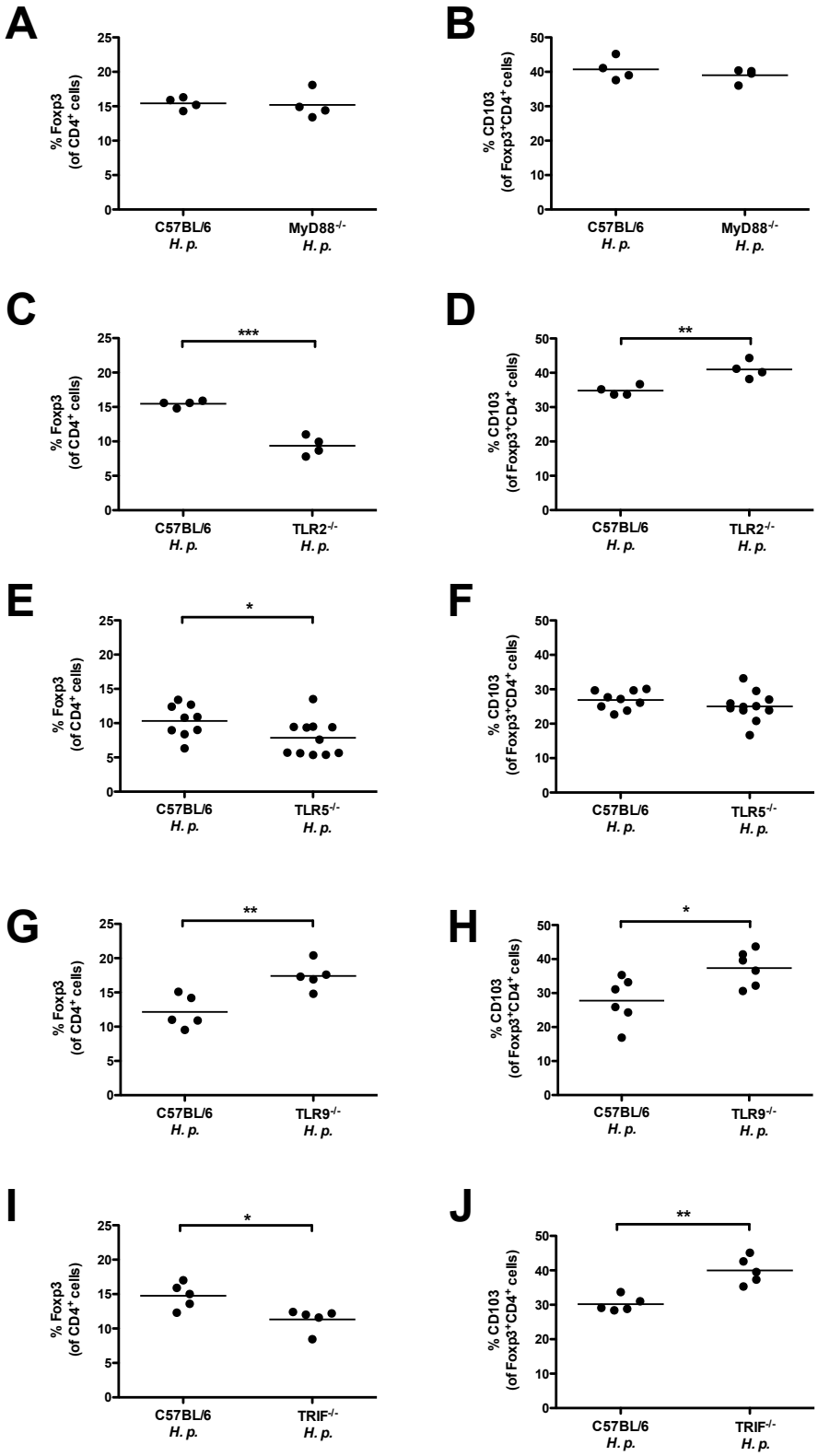

Figure 5.2.8 TLR signalling contributes differentially to Treg proportions following *H. polygyrus* infection.

Bedding was mixed between C57BL/6 and gene-deficient male mouse cages, and female C57BL/6 and gene-deficient mice were co-housed for at least two weeks prior to *H. polygyrus* infection and throughout the experiment. Mice were infected with 200 *H. polygyrus* L3s, and 28 days post-infection MLN cells were isolated, stained as indicated and run on a flow cytometer for analysis.

(**A, C, E, G, I)** Proportions of $Foxp3^+$ cells amongst $CD4^+$ lymphocytes in C57BL/6 and **(A)** $MyD88^{-/-}$ **(C)** $TLR2^{-/-}$ **(E)** $TLR5^{-/-}$ **(G)** $TLR9^{-/-}$ and **(I)** $TRIF^{-/-}$ mice.

(**B, D, F, H, J)** Proportions of $CD103^+$ cells amongst $CD4^+Foxp3^+$ lymphocytes in C57BL/6 and **(B)** $MyD88^{-/-}$ **(D)** $TLR2^{-/-}$ **(F)** $TLR5^{-/-}$ **(H)** $TLR9^{-/-}$ and **(J)** $TRIF^{-/-}$ mice.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

5.3 Discussion

Many helminth parasites are able to undergo chronic infections in mammals, which is largely attributed to their excretory-secretory proteins manipulating host immune responses [278]. One of the least explored pathways by which helminths modulate host signalling is though interference with mammalian TLR signalling.

Helminth products can bind to TLRs and mediate signalling through these receptors: double stranded RNA from *S. mansoni* eggs binds TLR3 leading to NF-κB activation [279]; the lipid lysophosphatidylserine extracted from *S. mansoni* eggs or adult worms stimulates TNF-α and IL-10 production via TLR2 [280]; ES-62 from the rodent filarial nematode *Acanthocheilonema viteae* signals via TLR4 to cause IL-12 and TNF-α production [281] and the *S. mansoni* egg carbohydrate lacto-N-fucopentaose III signals through TLR4 to cause MAP kinase activation and IL-4 production [282]. LPS signalling through TLR4 does not typically induce IL-4 production, and how helminth products cause differential cytokine production to bacterial TLR agonists is yet to be resolved. A potential mechanism by which helminth products can skew downstream responses of TLR signalling is through the additional engagement of co-receptors [283]. As well as the potential that the secretory molecules in HES may ligate TLRs, *H. polygyrus* infection may trigger TLR signalling by increasing the exposure of bacterial ligands to immune cells. In particular, when *H. polygyrus* L3s disrupt the epithelial cell barrier during migration through to the submucosa, they likely facilitate bacterial translocation and contact with host cells through to the serosal layer of the gut (Figure 1.7.2).

Here, it was found that mice deficient in MyD88 signalling are more resistant to a primary *H. polygyrus* infection than Wt C57BL/6 mice, and these mice produce large numbers of granulomas along the intestinal wall in response to infection. GF mice are also more resistant to *H. polygyrus* infection [223-225], and importantly, GF mice also produce many more granulomas in response to infection than SPF control mice [223]. These findings lead to the intriguing

hypothesis that a component of the microflora, signalling through MyD88, may function to inhibit granuloma formation in the C57BL/6 background.

Accordingly, if the stimulation for granuloma formation is the appearance of bacteria in the intestinal mucosa, MyD88$^{-/-}$ mice and GF mice may form greater numbers of granulomas due to reduced innate defences against bacteria in these mice, including the reduced IgA secretion seen here, which increases the proximity between the microflora and intestinal cells [3, 66, 284]. The increased contact between intestinal cells and bacteria may explain the high IFN-γ levels produced by MLN and PP CD4^{+} T cells in the MyD88$^{-/-}$ mice studied here.

Granulomas formed following *H. polygyrus* infection are made up of neutrophils, dendritic cells, eosinophils and alternatively activated macrophages [152, 156]. MyD88-deficiency has not been shown to alter the alternative activation of macrophages in the context of filarial parasite infections [285], however it will be interesting to characterise the basal numbers and expansion of these cell types in MyD88$^{-/-}$ mice at early time points following *H. polygyrus* infection. Further work is necessary to determine the key cell types in granulomas and the mechanisms by which they contribute to helminth expulsion. It seems probable that a key component of the *H. polygyrus*-induced granuloma is irradiation-sensitive, and does not reconstitute within 8 weeks, as irradiated MyD88$^{-/-}$ mice reconstituted with MyD88$^{-/-}$ bone marrow cells produced very few granulomas following infection.

It has previously been shown that MyD88$^{-/-}$ mice are more resistant to a primary *T. muris* infection than Wt mice [286]. This may be attributed to a lack of signalling though TLR4, as TLR4$^{-/-}$ mice are also more resistant to *T. muris* [286]. The increased *H. polygyrus* expulsion phenotype of MyD88$^{-/-}$ mice was not replicated in any individual TLR-deficient mouse examined, although *H. polygyrus* was less fecund by day 28 post-infection in TLR2$^{-/-}$ mice, suggesting that signalling through this receptor partially regulates immunity to *H. polygyrus*. Deficiencies in individual TLRs did, however, alter Treg proportions following infection. Consistent with the results described here,

TLR2 and TLR5 engagement has been previously shown to expand Tregs [287-289], whereas TLR9 stimulation has been shown to limit Treg function [18]. TLR4 stimulation enhances Treg survival and proliferation *in vitro* [290], but in these *in vivo* experiments, TLR4-deficiency did not affect Treg proportions. Infection status likely determines the importance of TLR signalling in controlling Treg frequencies; $TLR2^{-/-}$ mice have equal proportions of $Foxp3^{+}CD4^{+}$ cells in their MLNs at steady state [291], yet during the *H. polygyrus* infections described in this chapter, and during *Candida albicans* infection [292] proportions are reduced compared to Wt mice. This highlights that TLR control of Treg expansion and proliferation is site and context dependent [293].

It should be noted that as well as mediating TLR signalling, MyD88 is also required for signalling through the IL-1, IL-18, and IL-33 receptors, and thus a lack of signalling through these receptors may be a key factor in the phenotype of $MyD88^{-/-}$ mice (Figure 5.3). IL-1, IL-18 and IL-33 are all members of the IL-1 superfamily, and as such the processing and signalling paths of these cytokines are similar. Caspase-1 is required for cleavage of all three cytokines to their active form, which occurs following NLR signalling in response to intracellular damage or recognition of PAMPs [41].

Both IL-1 and IL-18 have been widely studied with regard to their role in mediating inflammatory diseases, including IBD, arthritis and T1D [41]. Blocking IL-1β is effective in reducing inflammation in many models of inflammatory diseases [41], which may be due to reduced stimulation of IL-17A-production from both ILC and T cells [294]. The role of IL-1 during helminth infections is less well explored. $IL\text{-}1\alpha^{-/-}$ and $IL\text{-}1\beta^{-/-}$ mice are impaired in type 2 cytokine production in response to *T. muris* infection, and are more susceptible to this infection than Wt mice [295, 296], yet mice deficient in the IL-1R are no different to Wt mice in their ability to expel *T. muris* infection [296], suggesting the existence of an alternative signalling pathway for IL-1.

When IL-18 was originally identified, it was named IFN-γ-inducing factor [297]. Following TLR agonist stimulation, the production of IFN-γ is greatly reduced upon treatment with α-IL-18 antibody or in mice deficient in caspase-1 [298],

however alone IL-18 does not induce IFN-γ production from T cells [299]. Depending on the cytokine environment, IL-18 can either induce IFN-γ (in the presence of IL-12), or IL-4 (in the absence of IL-12) from T and NK cells, mast cells or basophils [300].

The final cytokine dependent on MyD88 for signal transduction is IL-33, which signals through the T1/ST2 receptor and IL-1RAP [277, 301, 302]. T1/ST2 is expressed on Th2 cells and in the absence of this receptor Th2 cytokine responses are attenuated [303, 304]. IL-33 has been shown to promote type 2 cytokine production and expulsion of other helminth parasites, including *T. muris* [305] and *N. brasiliensis* [175]. It is therefore not expected that loss of signalling by this cytokine is responsible for the increased resistance of MyD88$^{-/-}$ mice to *H. polygyrus* infection.

No deficiencies in Th2 or Th1 cytokine production were seen in the MyD88$^{-/-}$ mice studied here, and the effect of individual deficiencies in IL-1, IL-18 or IL-33 signalling during *H. polygyrus* infection has not been investigated to date.

A surprising finding was that whilst MyD88$^{-/-}$ mice form a high number of granulomas following infection, when TRIF is additionally deficient (MyD88$^{-/-}$ TRIF$^{-/-}$), mice do not produce the same high numbers of granulomas. This suggests that both MyD88 and TRIF control granuloma formation, with TRIF promoting, and MyD88 inhibiting granuloma formation (Figure 5.3). Several genes are differentially regulated by MyD88 and TRIF following stimulation with LPS [34, 306]. A major product of TRIF signalling are type 1 IFN family members; TRIF signalling, but not MyD88 signalling, leads to the activation of IFN-β [306].

Type 1 IFNs, including IFN-α and IFN-β signal through a two chained receptor, consisting of IFN-αR1 and IFN-αR2c (IFNαβR) [307]. Future work could test whether granuloma formation is type 1 IFN dependent by crossing MyD88$^{-/-}$ mice with IFNαβR$^{-/-}$ mice and investigating whether doubly deficient offspring mount a granuloma response to *H. polygyrus* infection. Cross regulation between type 1 interferon and IL-1 signalling has been reported in other systems, with type 1 interferons inhibiting IL-1α and IL-1β production

[308, 309], thus whether this is a mechanism by which granuloma formation is controlled during *H. polygyrus* infection should also be investigated.

The previous chapters have largely focused on how commensal bacteria may influence an infection with *H. polygyrus*. Many other factors and pathways have been shown to modify the murine response to *H. polygyrus*, including Treg induction via the TGF-β pathway [197]. The following chapter continues to explore the importance of TGF-β signalling during a *H. polygyrus* infection.

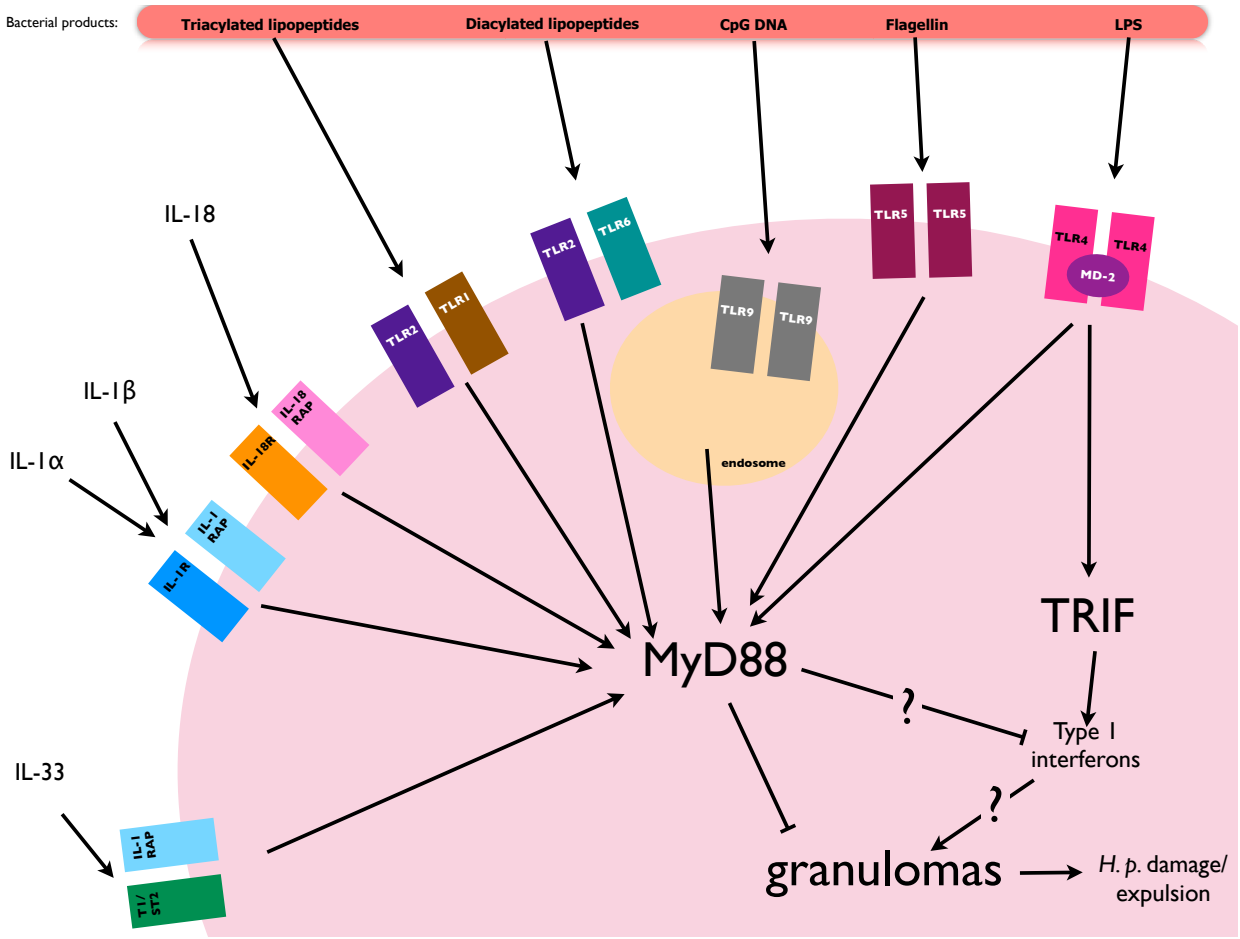

Figure 5.3 Ligands signalling through MyD88 and TRIF and potential downstream interactions.
Triacyclated and diacyclated lipopeptides ligate TLR2 dimerised with TLR1 and TLR6 respectively, and signalling is mediated by MyD88 [23-26]. TLR5 forms homodimers and recognises the bacterial flagellin protein, and also requires MyD88 for functional signalling [23, 30, 31]. TLR9 is not located on the cell surface, but in intracellular endosomes, where it forms homodimers, recognises CpG DNA and signals through MyD88 [23, 32]. TLR4, the receptor for LPS, is unique amongst the bacteria-recognising TLRs in that it can signal via MyD88, or independently of MyD88 through the adaptor protein TRIF [23, 24, 27-29].
IL-18 signals through the hetrodimer receptor consisting of IL-18R and IL-18RAP, with downstream signalling dependent on MyD88 [274, 310]. IL-1α and IL-1β signalling is dependent on MyD88, after they have bound their receptor comprised of IL-1R and IL-1RAP [274-276, 311]. Finally, IL-33, which signals through the T1/ST2 receptor and IL-1RAP, is dependent on MyD88 for downstream signal transduction [277, 301, 302].
Proposed downstream signalling consequences are presented, where MyD88 inhibits granuloma formation by unknown mechanisms, and TRIF potentiates granuloma formation, through the production of Type I IFNs leading to *H. polygyrus* damage or expulsion.

Chapter 6. The role of TGF-β signalling during *Heligmosomoides polygyrus* infection

6.1 Introduction

Immunity to gastrointestinal helminths is dependent on Th2 mechanisms [142, 147], which are controlled by Tregs [197] and antagonised by Th1 effector cells [312]. TGF-β is a widely distributed cytokine that has multiple effects on many cell types [313]. In T cells, TGF-β signalling contributes both to the differentiation of effector T cell subsets, and to the differentiation and survival of Treg cells, particularly in the periphery [314]. TGF-β signalling is likely important in modulating immune responses during *H. polygyrus* infection, as when TGF-β signalling is disrupted using the inhibitor SB431542, immunity to *H. polygyrus* is enhanced [197].

TGF-β signalling is necessary for mediating the immunomodulatory abilities of *H. polygyrus* in models of colitis, where *H. polygyrus* can alleviate disease severity in the absence of IL-10 [212], but not when T cell responsiveness to TGF-β is abrogated [315]. Infection with *H. polygyrus* causes naïve T cells to express Foxp3 *in vivo*, and HES is able to induce Foxp3 expression in naïve T cells *in vitro*, but not in the absence of TGF-β signalling [197].

TGF-β also participates in generating IL-9- [316, 317] and IL-17-producing [318] Th subsets in the presence of IL-4 and IL-6 respectively. IL-9 is important for mast cell survival and proliferation [319], and mast cells have been implicated in having a role in *H. polygyrus* expulsion [160, 166, 167]. In the setting of *H. polygyrus* infection, however, both IL-9-dependent mast cell responses [320] and Th17 cells [210] have previously been reported to be blocked.

As a global inhibition of TGF-β signalling reduces *H. polygyrus* survival [321], it was surprising that mice whose T cells expressed a dominant negative

TGF-β receptor II (TGF-βRII DN) [322] are not more resistant to this parasite [315]. The experiments presented here not only confirmed this phenotype, but established that TGF-βRII DN mice are in fact more susceptible to infection than Wt C57BL/6 animals, in terms of increased *H. polygyrus* fecundity.

It is known that TGF-β signalling can act to downregulate multiple cell types [313, 321], and among the conspicuous phenotypes of TGF-βRII DN mice is potentiation of IFN-γ and Th1 responses [323, 324]. The question was raised therefore, whether TGF-βRII DN mice were more susceptible to *H. polygyrus* due to uninhibited Th1 cytokine release, which was tested in mice deficient in IFN-γ.

The results show that when IFN-γ release is abrogated in the absence of TGF-β signalling in T cells, the increased fecundity of *H. polygyrus* within the host is lost. Infected TGF-βRII DN mice show no deficiencies in the differentiation of IL-9 and IL-17 producing $CD4^+$ T cells, and display equivalent mast cell and HES-specific IgG1 responses to Wt mice following infection. These data therefore support the hypothesis that the increased susceptibility of TGF-βRII DN mice to *H. polygyrus* is due to elevated IFN-γ release, and not to a loss of Th9 or Th17 effector responses, or antibody production.

In addition, the balance of Treg frequencies and subsets in the presence or absence of TGF-β signaling and the consequent outcome of infection was investigated. These data show that within the $Foxp3^+$ Treg population of TGF-βRII DN and TGF-βRII DN IFN-$\gamma^{-/-}$ mice, CD103 expression is low on both $Helios^+$ and $Helios^-$ cells, but a compensatory increase in the proportion of $Foxp3^+Helios^+$ Tregs may account for the continuing susceptibility of mice expressing the mutated TGF-β receptor.

6.2 Results

6.2.1 Ablation of TGF-β signalling in T cells does not confer resistance to *H. polygyrus* infection

C57BL/6 mice have a high level of susceptibility to *H. polygyrus* (Figure 3.2.1) [234], yet immunity can be enhanced by pharmacological inhibition of TGF-β signalling [197]. TGF-βRII DN mice express a dominant-negative form of the TGF-β receptor type II in both $CD4^+$ and $CD8^+$ T cells [322], resulting in an almost complete lack of TGF-β signalling in either of these cell types, so they may be expected to be more resistant to *H. polygyrus* than their Wt littermates, and may lack inducible Tregs to inhibit effector responses against the worm. Surprisingly, however, these mice are not more resistant to *H. polygyrus* [324]. In fact, *H. polygyrus* was more fecund in TGF-βRII DN mice than in Wt C57BL/6 counterparts, both at 14-days (Figure 6.2.1 A) and 28-days (Figure 6.2.1 B) post-infection. Adult worm numbers remaining in the gut were broadly similar to Wt at 14-days following infection (Figure 6.2.1 C), but were slightly elevated in TGF-βRII DN mice 28-days post-infection, although this did not reach statistical significance (Figure 6.2.1 D).

6.2.2 TGF-βRII DN mice mount a reduced Th2 response to *H. polygyrus* compared to Wt C57BL/6 mice

In addition to a heightened susceptibility to *H. polygyrus*, TGF-βRII DN mice showed diminished Th2 cytokine responses to *H. polygyrus*-infection, failing to generate the significant increase in proportion of $IL\text{-}4^+CD4^+$ or $IL\text{-}13^+CD4^+$ lymphocytes in the MLN by day-14 of infection shown by Wt mice (Figure 6.2.2 A+B). Furthermore, at day-7 post-infection, IL-5 was detectable in the serum of *H. polygyrus*-infected Wt mice, but completely absent in all but one of the TGF-βRII DN mice examined (Figure 6.2.2 C). No IL-4 or IL-13 was detectable in the sera of either strain.

A trend for reduced IL-4 responsiveness by MLN cells in *H. polygyrus*-infected TGF-βRII DN mice was also seen by antigen-specific recall to HES *in vitro* (Figure 6.2.2 D). IL-10 plays a role in promoting Th2 responsiveness in gastrointestinal helminth infections [142], and reduced IL-10 production in response to HES-stimulation *in vitro* was also found in *H. polygyrus*-infected TGF-βRII DN mice (Figure 6.2.2 E).

Hence, T cell-specific loss of TGF-β signalling did not recapitulate the effects of global pharmacological inhibition [197], and the phenotype of the TGF-βRII DN mice did not equate to the Th2-boosting effects of broader interference with Treg function in nematode infections [196, 252, 325].

6.2.3 *H. polygyrus*-infected TGF-βRII DN mice have a higher proportion of Tregs than infected Wt C57BL/6 mice, but they express less CD103

As TGF-β signalling promotes Treg differentiation, particularly in the periphery [314], Treg proportions were next examined in *H. polygyrus*-infected TGF-βRII DN mice. Surprisingly, infected TGF-βRII DN mice had a significantly higher proportion of Foxp3 expressing $CD4^+$ lymphocytes in their MLN cells compared to Wt animals (Figure 6.2.3 A). The increased frequency of $Foxp3^+$ cells was accounted for by a greater proportion of $CD4^+$ cells expressing the transcription factor Helios (Figure 6.2.3 B+C), which has been associated with thymic or natural Tregs [245], while the frequencies of $Foxp3^+Helios^-CD4^+$ (considered to be peripherally induced Tregs) were not significantly different between the two genotypes (Figure 6.2.3 B+D). Although the $Foxp3^+$ Treg compartment as a whole was thus not numerically diminished in TGF-βRII DN mice, the expression of the activation and memory marker CD103, known to be inducible by TGF-β [326] was substantially reduced (Figure 6.2.3 E+F) on both $Helios^-$ and $Helios^+$ $Foxp3^+CD4^+$ lymphocytes (data not shown).

6.2.4 Th17, Th9, mast cell responses and IgG1 responses to *H. polygyrus* are not compromised in TGF-βRII DN mice

Next, whether a loss of TGF-β signalling impacted on other effector functions within the immune response was examined. As TGF-β signalling promotes the differentiation of IL-9- producing cells in the presence of IL-4 [316, 317] and IL-17-producing cells in the presence of IL-6 [318], we investigated the generation of these cell types following infection, as it was feasible that a failure to generate either of these cell types in TGF-βRII DN mice was responsible for the increased susceptibility to *H. polygyrus*.

Few Th17 cells identified by intracellular IL-17A staining develop in the MLN in either genotype following infection (Figure 6.2.4 A), suggesting that the conditions for optimal Th17 expansion are not generated at this site during *H. polygyrus* infection.

The frequency of $CD4^+$ lymphocytes producing IL-9 was, however, altered in TGF-βRII DN mice, with a significantly greater, rather than lower, proportion of IL-9-expressing $CD4^+$ lymphocytes compared to Wt mice (Figure 6.2.4 B). IL-9 is important for the survival and proliferation of mast cells [319], which have been proposed as an important effector subset in the expulsion of *H. polygyrus* [160, 166, 167]. The numbers of mast cells in the jejunum were therefore quantified, but it was found that numbers increase significantly and equivalently after *H. polygyrus* infection in both C57BL/6 and TGF-βRII DN mice (Figure 6.2.4 C-E).

As *H. polygyrus* was more fecund in TGF-βRII DN mice (Figure 6.2.1 A+B), and it has previously been reported that antibodies function to limit egg production by *H .polygyrus* [138], TGF-βRII DN mice were examined for any defects in an anthelmintic antibody response. Both Wt and TGF-βRII DN mice had equivalent levels of HES-specific IgG1 (Figure 6.2.4 F) and total IgG (data not shown) in their serum following *H. polygyrus* infection, suggesting that *H. polygyrus* is not more fecund in TGF-βRII DN mice because of a failure of the host to produce appropriate antibodies.

6.2.5 Naïve and *H. polygyrus*-infected TGF-βRII DN mice produce exuberant IFN-γ

Expulsion of helminth parasites in general requires a functional Th2 response [142, 147]. TGF-βRII DN mice, however, were highly polarised towards a Th1 phenotype in naïve animals, and this was exacerbated following *H. polygyrus* infection (Figure 6.2.5). Levels of circulating IFN-γ at day 7 post-infection were elevated in TGF-βRII DN mice compared to Wt mice (Figure 6.2.5 A), and MLN cells from both naïve and *H. polygyrus*-infected TGF-βRII DN mice produced more IFN-γ after being cultured *ex vivo* in the presence or absence of HES than Wt mice (Figure 6.2.5 B). The increased IFN-γ production in naïve and infected TGF-βRII DN mice was from both $CD4^+$ and $CD8^+$ lymphocytes (Figure 6.2.5 C-F).

6.2.6 Increased susceptibility of TGF-βRII DN mice is reversed in the absence of IFN-γ

To investigate whether the substantial IFN-γ response in TGF-βRII DN mice inhibited Th2 cytokines required to control *H. polygyrus*, double-transgenic mice were bred, which had the TGF-βRII DN mutation together with the IFN-$\gamma^{-/-}$ genotype on a C57BL/6 background (TGF-βRII DN IFN-$\gamma^{-/-}$ mice). At both 14 and 28 days post-infection, TGF-βRII DN mice lacking IFN-γ had lower faecal egg burdens than IFN-γ-sufficient TGF-βRII DN mice (Figure 6.2.6 A+B), and by day 28, lower worm counts (Figure 6.2.6 C).

6.2.7 Early Th2 response to *H. polygyrus* in TGF-βRII DN mice is partially restored in the absence of IFN-γ

Serum cytokine analysis confirmed the absence of IFN-γ in gene-targeted mice (Figure 6.2.7 A), and showed that serum IL-5 levels were partially restored to the levels of Wt mice following *H. polygyrus* infection (Figure 6.2.7 B). However, intracellular staining of MLN cells showed broadly similar levels of Th2 cytokine

production by day-28 of infection between all four genotypes of mice (data not shown), indicating that the suppression of Th2 responses is only relieved at early timepoints during infection in the absence of both IFN-γ and TGF-β signalling in T cells.

6.2.8 Treg proportion and CD103 expression in TGF-βRII DN mice is unaffected by loss of IFN-γ

Analysis of Treg populations showed that the proportion of $Foxp3^+$ cells within the $CD4^+$ lymphocyte compartment was elevated in *H. polygyrus*-infected TGF-βRII DN mice irrespective of their IFN-γ status (Figure 6.2.8 A), and this was due to an increase in the proportion of Helios-expressing Tregs (Figure 6.2.8 B), rather than due to an increase in $Helios^-$ Tregs (Figure 6.2.8 C). Moreover, CD103 expression was reduced on $Foxp3^+$ cells in both IFN-γ-deficient and -sufficient TGF-βRII DN mice (Figure 6.2.8 D), within both the $Helios^+$ and $Helios^-$ subsets (data not shown).

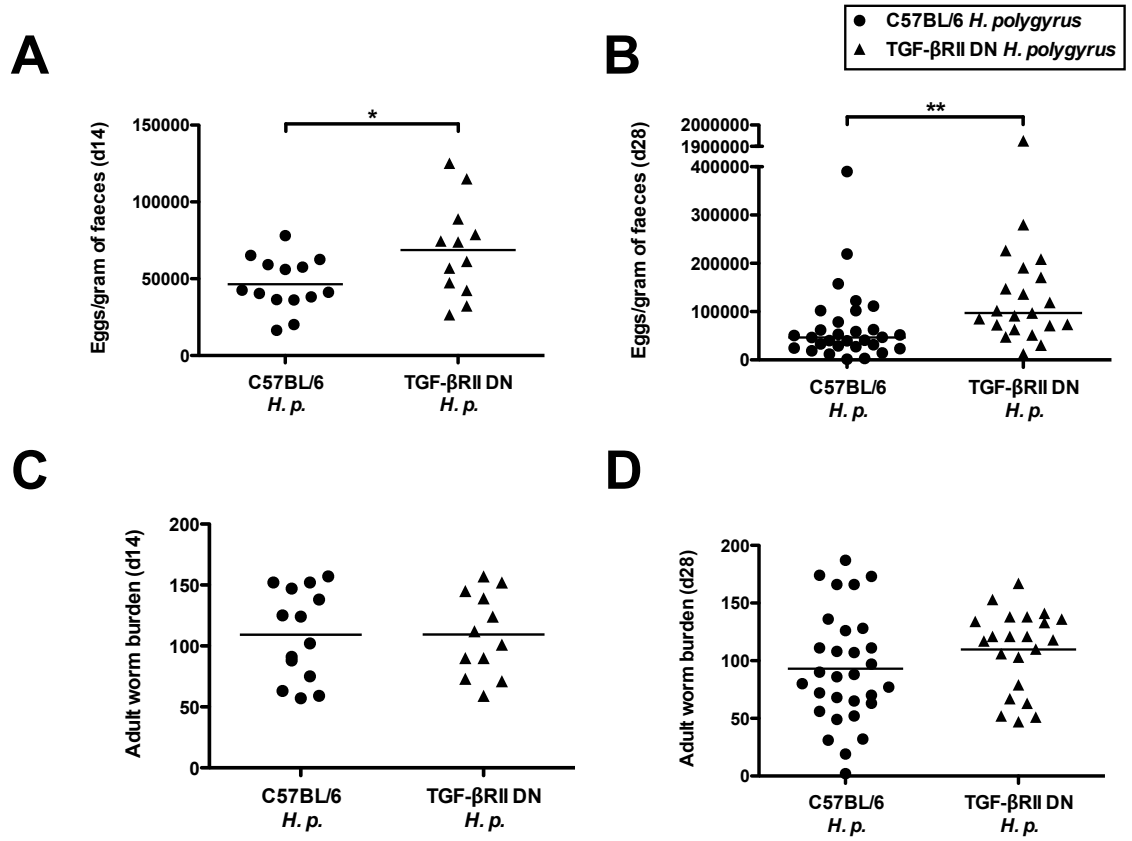

Figure 6.2.1 Ablation of TGF-β signaling in T cells does not confer resistance to *H. polygyrus* infection.
Wt C57BL/6 or TGF-βRII DN mice were infected with 200 *H. polygyrus* L3s.
(A+B) Numbers of *H. polygyrus* eggs released per gram of faeces at (**A)** 14 and **(B)** 28 days post infection.
(C+D) Numbers of adult *H. polygyrus* worms along the intestinal tract **(C)** 14 and **(D)** 28 days post infection.
Data pooled from **(A+C)** 3 and **(B+D)** 5 independent experiments.
* indicates p= <0.05; ** indicates p= <0.01.

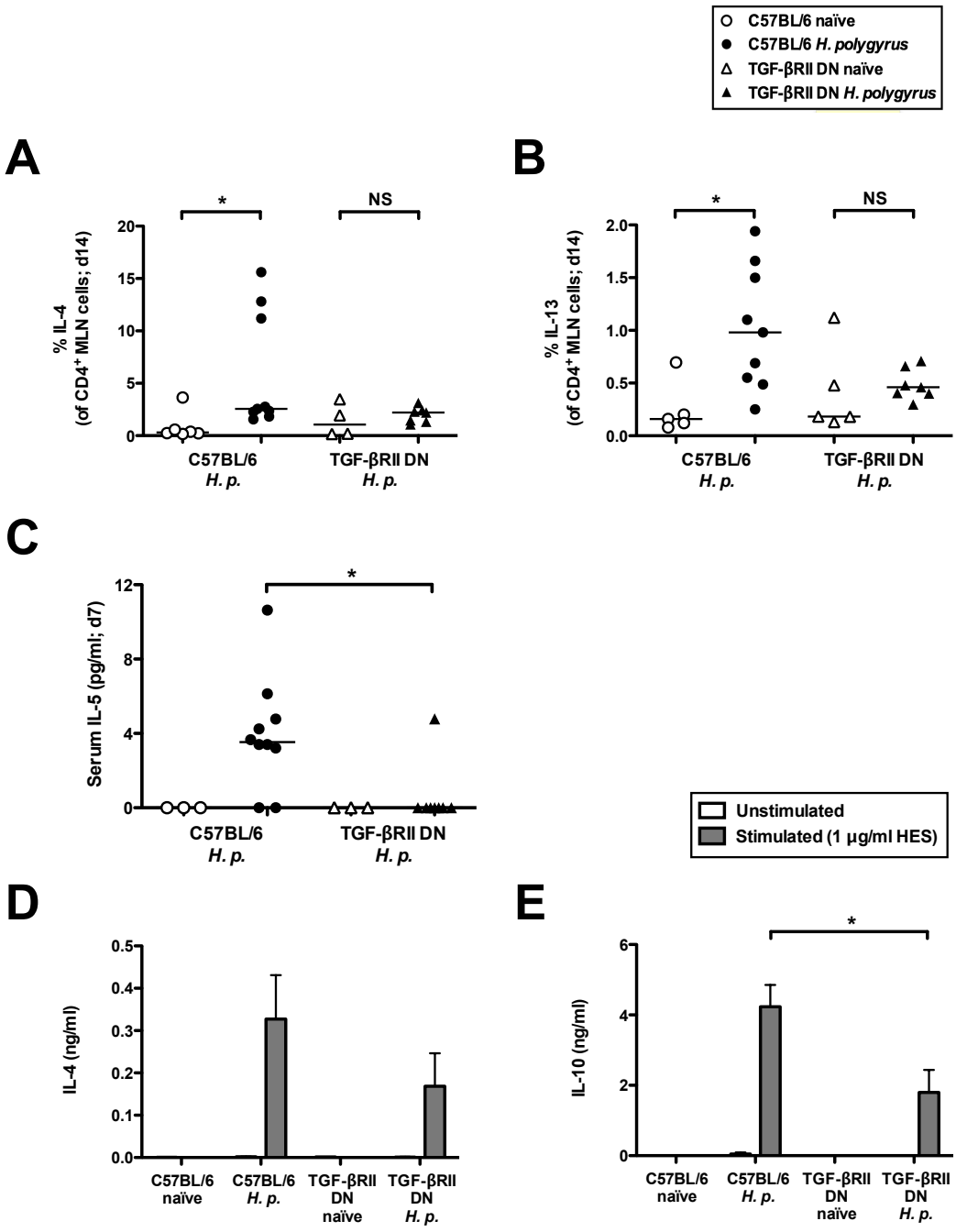

Figure 6.2.2 TGF-BRII DN mice mount a reduced Th2 response to *H. polygyrus* compared to Wt C57BL/6 mice.
Wt C57BL/6 or TGF-βRII DN mice were left naïve or infected with 200 *H. polygyrus* L3s.
(A+B) % of **(A)** IL−4 or **(B)** IL-13 producing cells among PMA/Ionomycin-stimulated $CD4^+$ MLN lymphocytes isolated from naïve or 14-day infected mice. Data are pooled from two independent experiments.
(C) Levels of serum IL-5 in naïve or 7-day infected mice. A Mann-Whitney test was performed between infected groups. Data are pooled from two independent experiments; naïve mice were examined in one of these experiments.
(D) IL-4 and **(E)** IL-10 production by MLN cells from naïve and 14-day infected mice, cultured *ex vivo* for 72 hrs with 1 µg/ml HES or medium alone. T tests were performed between HES-stimulated *H. polygyrus*-infected groups. Data are from one experiment and are representative of two independent experiments each with ≥ 2 mice per group.
* indicates p= <0.05; NS indicates a non significant result.

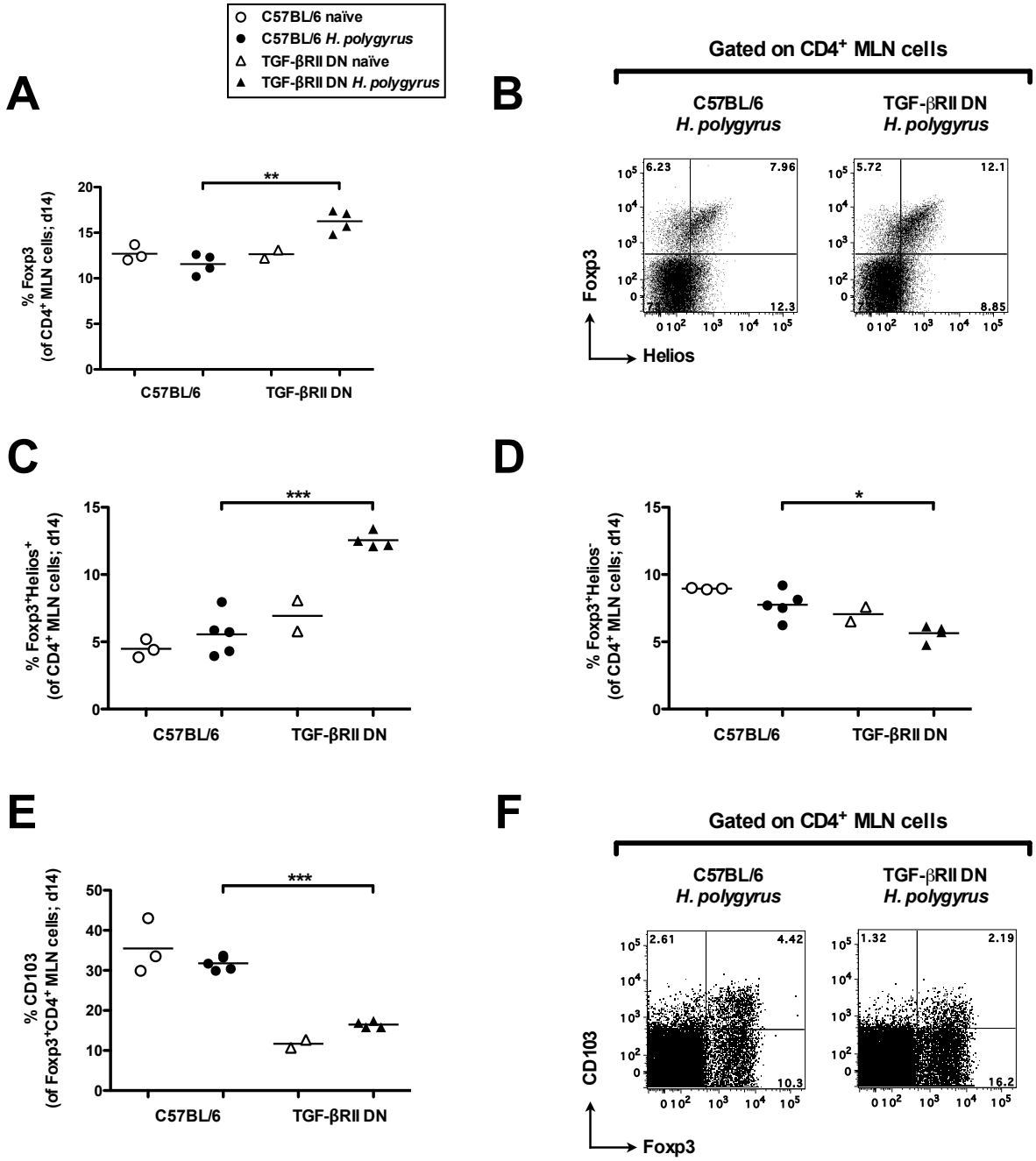

Figure 6.2.3 ***H. polygyrus*-infected TGF-βRII DN mice have a higher proportion of Tregs than infected Wt C57BL/6 mice, but they express less CD103.**

Wt C57BL/6 or TGF-βRII DN mice were left naïve or infected with 200 *H. polygyrus* L3s. Data are from one experiment and representative of the results from two independent experiments each with ≥ 2 mice per group. T tests were performed between infected groups.

(A) % Foxp3 cells of $CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 14-day infected mice.

(B) Representative Foxp3 and Helios staining of $CD4^+$ MLN cells stained directly *ex vivo* in 14-day infected mice.

(C) % $Foxp3^+Helios^+$ cells of $CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 14-day infected mice.

(D) % $Foxp3^+Helios^-$ cells of $CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 14-day infected mice.

(E) % CD103 cells of $Foxp3^+CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 14-day infected mice.

(F) Representative CD103 and Foxp3 staining of $CD4^+$ MLN cells stained directly *ex vivo* in 14-day infected mice.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

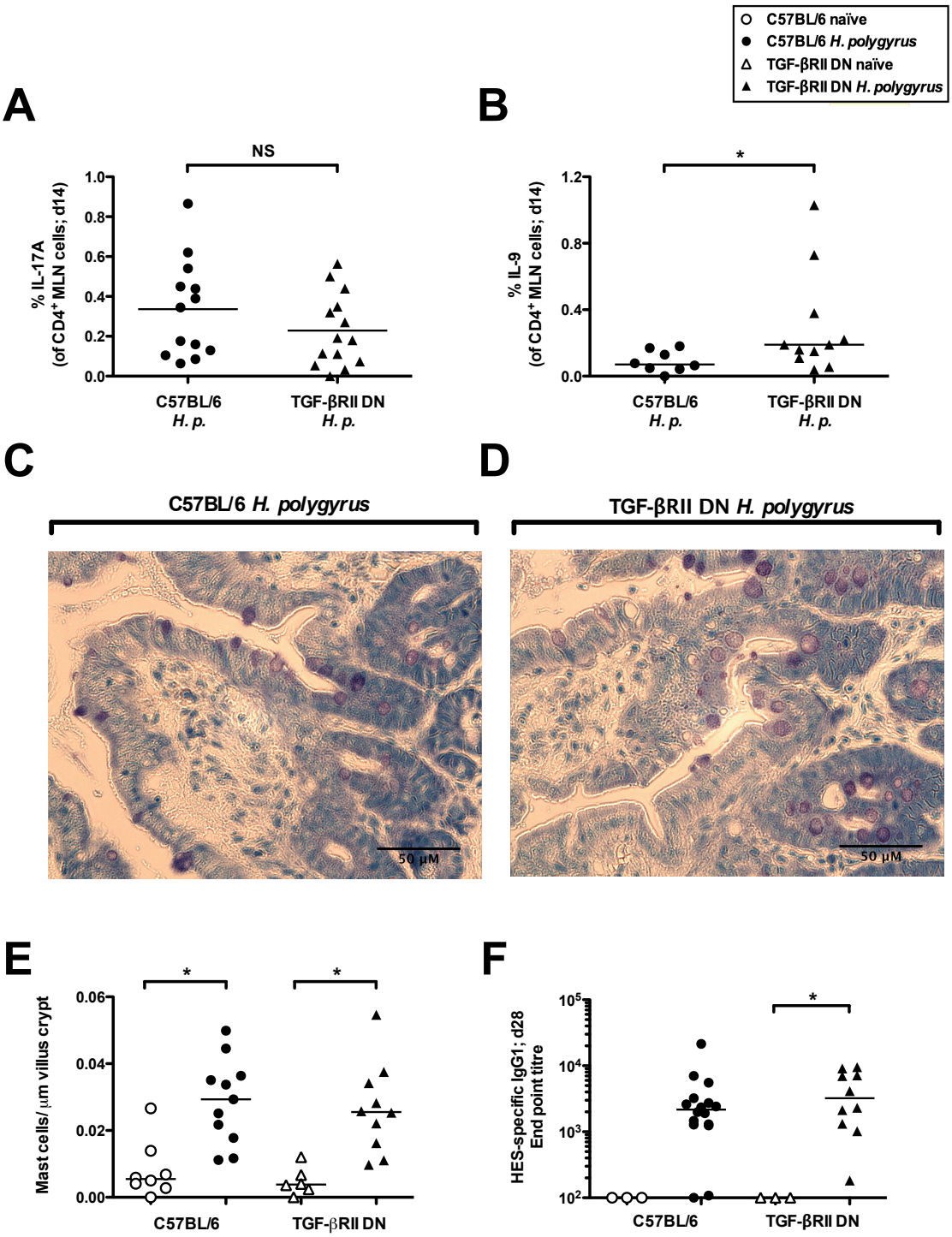

Figure 6.2.4 Th17, Th9, mast cell responses and IgG1 responses to *H. polygyrus* are not compromised in TGF-βRII DN mice.
Wt C57BL/6 or TGF-βRII DN mice were left naïve or infected with 200 *H. polygyrus* L3s
(A) % IL-17A or **(B)** IL-9 produced by PMA/Ionomycin-stimulated $CD4^+$ MLN lymphocytes isolated from 14-day infected mice. Data are pooled from four independent experiments.
(C-E) Jejunums were sectioned and stained for mast cells with Toluidine Blue. Representative images are shown from 14-day infected **(C)** C57BL/6 and **(D)** TGF-βRII DN mice.
(E) Number of mast cells per µm of villus crypt in naïve and 14-day infected mice. Data are pooled from two independent experiments; naïve mice were examined in one of these experiments.
(F) End point HES-specific IgG1 titres in the sera of naïve and 28-day infected mice. Data are pooled from two independent experiments; naïve mice were examined in one of these experiments.
* indicates p= <0.05; NS indicates a non significant result.

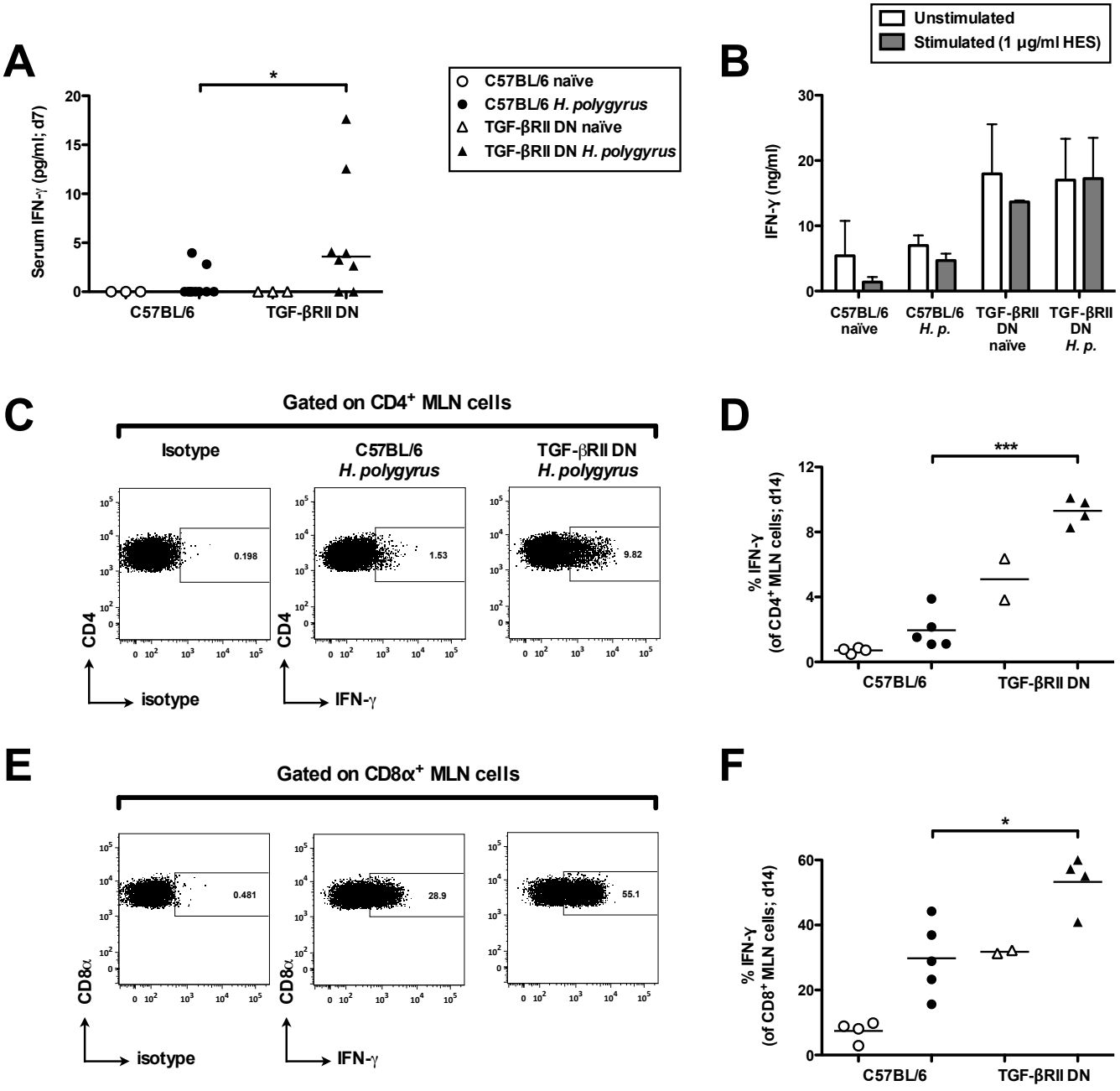

Figure 6.2.5 Naïve and *H. polygyrus*-infected TGF-βRII DN mice produce exuberant IFN-γ.
Wt C57BL/6 or TGF-βRII DN mice were left naïve or infected with 200 *H. polygyrus* L3s
(A) Levels of serum IFN-γ in naïve or 7-day infected mice. A Mann-Whitney test was performed between infected groups. Data are pooled from two independent expeirments; naïve mice were examined in one of these experiments.
(B) IFN-γ production by MLN cells from naïve and 14-day infected mice, cultured *ex vivo* for 72 hrs with 1 µg/ml HES or medium alone. Data are from one experiment and are representative of two independent experiments each with ≥ 2 mice per group.
(C) Representative IFN-γ staining of $CD4^+$ PMA/Ionomycin stimulated MLN cells from 14-day infected mice.
(D) % IFN-γ producing cells among PMA/Ionomycin-stimulated $CD4^+$ MLN lymphocytes isolated from naïve or 14-day infected mice. Data are from one experiment and representative of the results from four independent experiments each with ≥ 2 mice per group.
(E) Representative IFN-γ staining of $CD8\alpha^+$ PMA/Ionomycin stimulated MLN cells from 14-day infected mice.
(F) % IFN-γ producing cells among PMA/Ionomycin-stimulated $CD8\alpha^+$ MLN lymphocytes isolated from naïve or 14-day infected mice. Data are from one experiment and representative of the results from four independent experiments each with ≥ 2 mice per group.
* indicates p= <0.05; *** indicates p= <0.001.

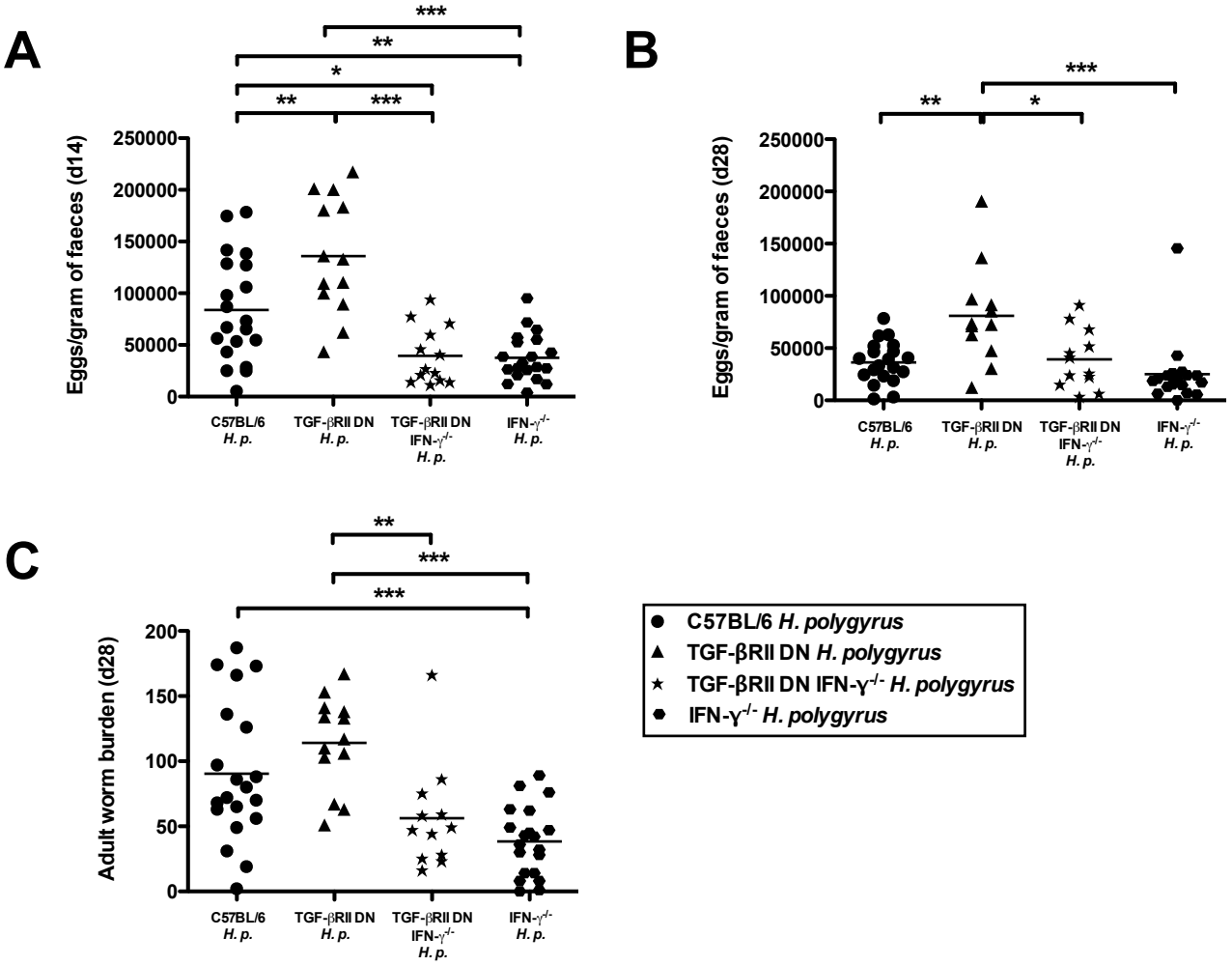

Figure 6.2.6 Increased susceptibility of TGF-βRII DN mice is reversed in the absence of IFN-γ.

Wt C57BL/6, TGF-βRII DN, TGF-βRII DN IFN-$\gamma^{-/-}$ and IFN-$\gamma^{-/-}$ mice were infected with 200 *H. polygyrus* L3s. Data are pooled from three independent experiments.

(A+B) Numbers of *H. polygyrus* eggs released per gram of faeces in **(A)** 14-day infected and **(B)** 28-day infected mice.

(C) Numbers of adult *H. polygyrus* worms along the intestinal tract 28 days post-infection.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

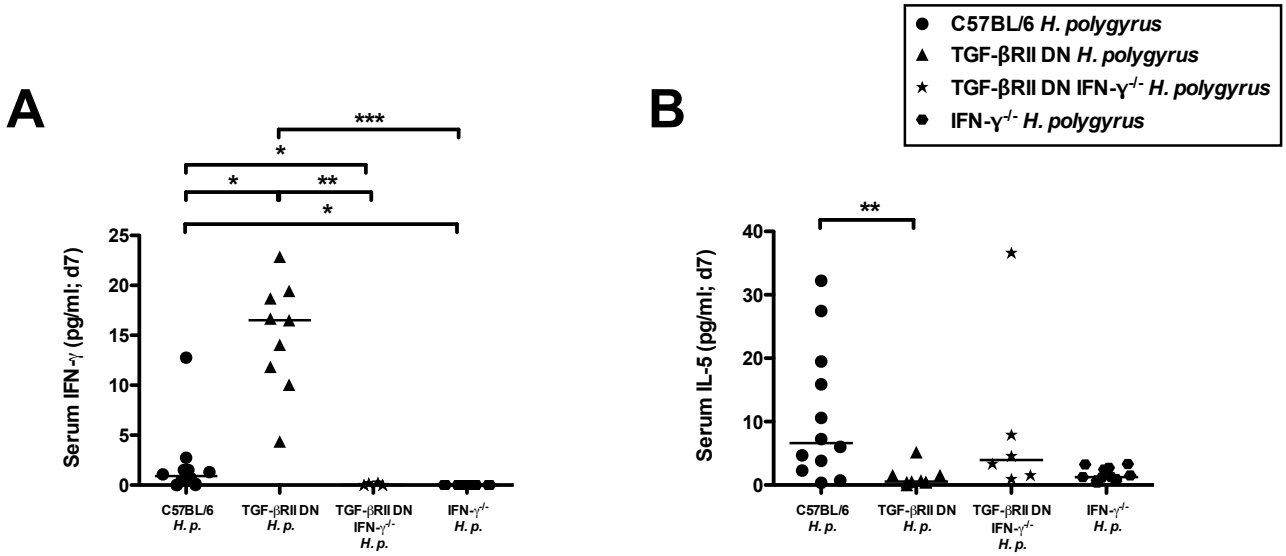

Figure 6.2.7 Early Th2 response to *H. polygyrus* in TGF-βRII DN mice is partially restored in the absence of IFN-γ.
Wt C57BL/6, TGF-βRII DN, TGF-βRII DN IFN-$\gamma^{-/-}$ and IFN-$\gamma^{-/-}$ mice were infected with 200 *H. polygyrus* L3s. Data are pooled from two independent experiments.
(A+B) Levels of serum **(A)** IFN-γ and **(IL-5)** in 7-day infected mice.
* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

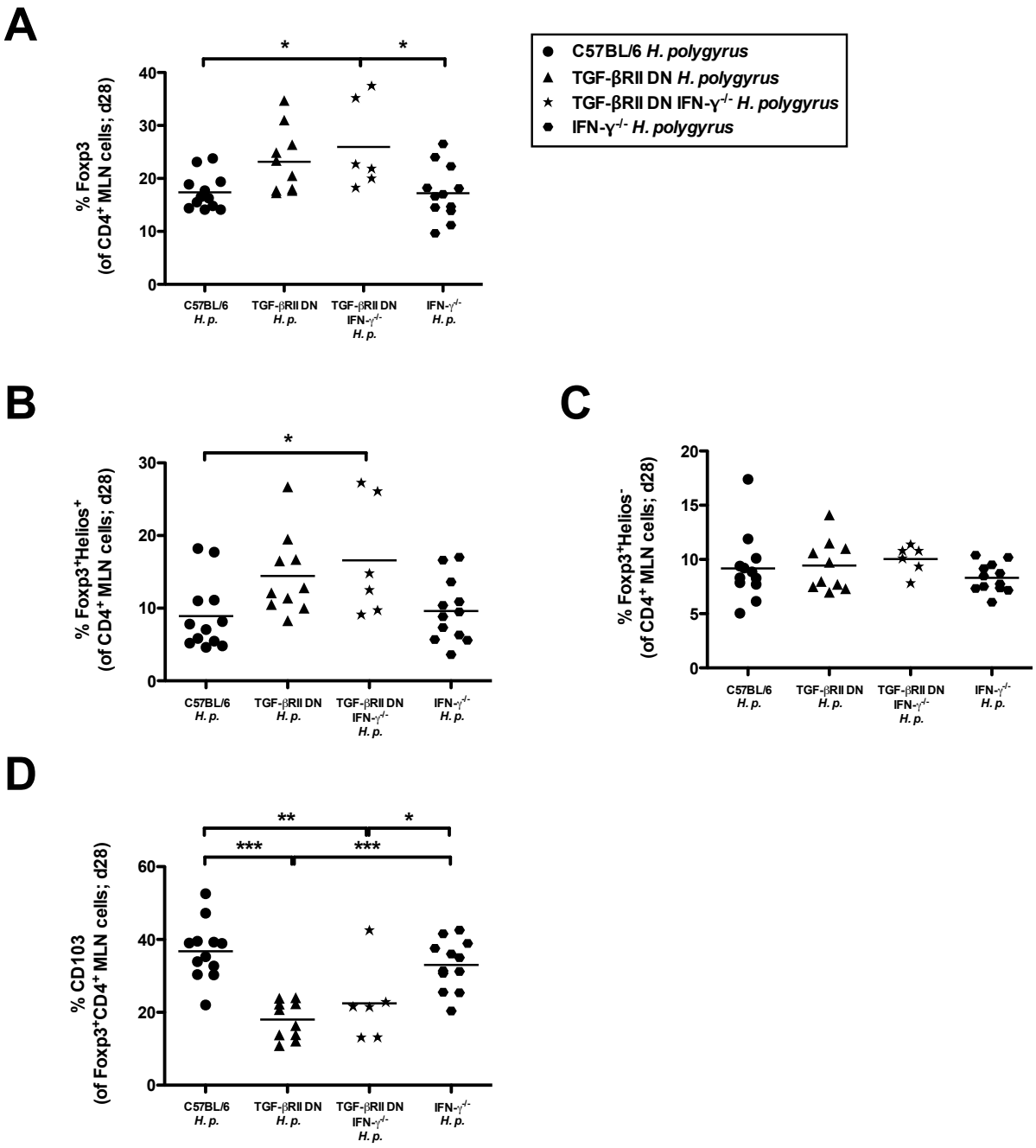

Figure 6.2.8 Treg proportion and CD103 expression in TGF-βRII DN mice is unaffected by loss of IFN-γ.

Wt C57BL/6, TGF-βRII DN, TGF-βRII DN IFN-$\gamma^{-/-}$ and IFN-$\gamma^{-/-}$ mice were infected with 200 *H. polygyrus* L3s. Data are pooled from two independent experiments.

(A) % Foxp3 cells of $CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 28-day infected mice.

(B) % $Foxp3^+Helios^+$ cells of $CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 28-day infected mice.

(C) % $Foxp3^+Helios^-$ cells of $CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 28-day infected mice.

(D) % CD103 cells of $Foxp3^+CD4^+$ MLN lymphocytes stained directly *ex vivo* in naïve or 28-day infected mice.

* indicates p= <0.05; ** indicates p= <0.01; *** indicates p= <0.001.

6.3 Discussion

TGF-βRII DN mice, which are impaired in TGF-β signalling specifically in CD4 and CD8 lymphocytes [322], are not resistant to *H. polygyrus* infection; in fact *H. polygyrus* was more fecund in these mice than in Wt counterparts. This was surprising, as inhibition of total TGF-β signalling using a pharmacological inhibitor caused mice to become more resistant to *H. polygyrus* [197].

It was feasible that that the increased susceptibility to *H. polygyrus* in TGF-βRII DN mice was due to a lack of generation of TGF-β dependent Th effector subsets, as TGF-β is involved in the generation of both IL-17- [318] and IL-9- [316, 317] producing cells. Although there is no current evidence for Th17 cells being anthelmintic, many reports have implicated IL-9 in worm expulsion, primarily by inducing and maintaining mast cell populations [160, 166, 167]. However, no difference to Wt mice in IL-17A production was seen during *H. polygyrus* infection in TGF-βRII DN mice, and significantly more, rather than less, IL-9 producing cells accumulated in the MLN during infection. These data suggest that in the context of a *H. polygyrus*-infection, T cell TGF-β signalling is not required for generation of these subsets, and rules out the hypothesis that a lack of generation of these subsets is a reason for the increased fecundity of *H. polygyrus* in TGF-βRII DN mice.

In TGF-βRII DN mice, a high proportion of splenic $CD4^+$ and $CD8^+$ lymphocytes develop into IFN-γ-producing cells *in vitro* [322], and constitutive IFN-γ levels in naïve mice are markedly elevated [324]. *H. polygyrus* infection in these mice is therefore initiated in an environment intrinsically unfavourable to Th2, indeed, early Th2 responses to *H. polygyrus* in terms of circulating IL-5 were almost absent in TGF-βRII DN mice. Reduced Th2 effectors were also seen at day 14 of infection in these mice, as no IL-4 or IL-13 response to infection was seen in the MLN by intracellular cytokine staining, and MLN cells had a reduced IL-10 recall response to HES. This is consistent with a report that IL-10 release by LP cells is inhibited in *H. polygyrus*-infected TGF-βRII DN mice [324].

The absence of a functional IFN-γ gene in TGF-βRII DN mice abrogated the increased fecundity of TGF-βRII DN mice compared to Wt mice, and partially restored the early IL-5 response to *H. polygyrus*. Double-transgenic mice were not however, able to fully clear infection by day 28 of infection. Thus, while over-expression of IFN-γ is responsible for the heightened susceptibility of TGF-βRII DN mice, IFN-γ itself is not solely responsible for the failure of mice to expel the parasite. In this manner, control of *H. polygyrus* appears to be more complex than *T. muris*, in which neutralisation of IFN-γ is sufficient to convert a susceptible genotype to a resistant phenotype [312]. Hence, the previously reported greater susceptibility of TGF-βRII DN mice to *T. muris* [327] may not simply be due to a lack of Th9-driven mast cell responses, as the authors suggest, but predominantly due to high intrinsic IFN-γ in this model.

TGF-βRII DN mice, whether IFN-γ-deficient or -sufficient, had a higher proportion of Helios^{+} Tregs than TGF-β Wt mice, and had lower levels of CD103 on all Tregs, confirming that CD103 expression is regulated by TGF-β signalling [328]. The levels of CD103 therefore do not correspond to the susceptibility of the mouse strain, suggesting that CD103 is not required for functional suppression of the anti-helminth response. However, as CD103 is important for effector T cell migration and retention in the gut [329], and as the TGF-βRII DN Foxp3^{-} effector population also fails to express as high CD103 levels as Wt mice (Figure 6.2.3F and data not shown), the susceptibility of this genotype could reflect a diminished presence of CD103^{+} effector cells at the site of infection.

Overall, these data argue that neither IFN-γ nor TGF-β-induced Tregs are essential for repression of the protective Th2 response to *H. polygyrus*, since although TGF-βRII DN IFN-γ$^{-/-}$ mice are more resistant to *H. polygyrus* than TGF-βRII DN mice, worms still persist in the host 28 days post-infection.

It is possible that the greater expansion of Helios^{+} Tregs in TGF-βRII DN mice accounts for their continued susceptibility. As discussed previously (3.3), it is not yet clear whether Helios can be used as a reliable marker of natural Tregs [256-258], as it has been suggested that Helios is simply expressed by a more activated subset of Foxp3^{+} cells [258]. Either way, TGF-βRII DN mice may have outgrowth of these Helios^{+} cells as a homeostatic compensation for the paucity

of $CD103^+$ adaptive Tregs and/or outgrowth to control a greater muscosal inflammatory response in the absence of TGF-β-inducible $CD103^+$ adaptive Tregs [330].

Alternatively, in the absence of TGF-β induced Tregs to inhibit a protective Th2 response against helminths, other regulatory populations may play a Th2-suppressing role in TGF-βRII DN mice. Bregs, DCs, eosinophils and macrophages can suppress inflammatory immune responses in some settings [142].

Finally, it should be noted that significant non-lymphoid populations are responsive to TGF-β [313]. The efficacy of global TGF-β inhibition [197] and the TGF-β dependent effects in *H. polygyrus*-infected RAG-deficient hosts [212] implies that there are critical non-T cell targets of this suppressive cytokine which affect immunity to *H. polygyrus*. Epithelial cells are responsive to TGF-β, and TGF-β signalling in these cells is required to mediate repair following intestinal damage caused by DSS treatment [331]. It is likely that following treatment with the global TGF-β-signalling inhibitor, epithelial cell repair following *H. polygyrus*-induced damage is inhibited, which may result in an inhospitable intestinal environment which does not support *H. polygyrus* survival.

In conclusion, although inducible Tregs control mucosal inflammation [330], these data support the idea that control of protective immunity in the intestinal setting may be regulated by $Helios^+$ and not $Helios^-$ Tregs. This intriguing and unexpected division of labour between Treg subsets remains to be further explored.

Chapter 7. Final discussion

Intestinal bacteria and helminth parasites have co-evolved within mammalian hosts for millions of years. In the modern developed world, the relationship between these intestinal dwellers and the human immune system has been disrupted, through improved sanitation, the use of anthelmintics and antibiotics. The absence of intestinal helminth parasites and the disruption of microbial populations in the gut has correlated with an increased prevalence of autoimmune and allergic diseases [332]. Direct studies using murine models have recognised both helminth parasites and intestinal microbes for their contributions to mammalian immune homeostasis, both in the intestinal tract and at more distal sites, such as the lung [9, 333].

The intestinal helminth parasite *H. polygyrus* has been widely studied due to its immunomodulatory properties; murine studies have shown that *H. polygyrus*-infection reduces pathology in many models of allergic and autoimmune diseases, which in several cases is attributed to increased IL-10 production in infected mice [333]. Similarly, specific populations of intestinal bacteria can modulate autoimmune and allergic disease pathology, which has been associated with changes in Treg proportions and IL-10 production [95, 99], although many of the mechanisms by which pathology is altered remain elusive. Both *H. polygyrus* and the microbiota have likely evolved ways of modulating immune responses in order to promote tolerance towards themselves, allowing for colonisation of the intestinal tract [197, 334]. However, whether the mechanisms they employ are co-dependent remains to be determined. The work here aimed to investigate how the presence of the intestinal bacteria influences the establishment and persistence of *H. polygyrus* in the host, and vice versa, and how modifying either organism altered intestinal immune responses.

7.1 The abundance of specific bacterial groups correlates with *H. polygyrus*-persistence, in a manner dependent on host genotype

Both antibiotic treatment experiments and correlation studies performed here indicate the importance of *Lactobacillus/Lactococcus* and Enterobacteriaceae species in modulating the persistence of *H. polygyrus* infection. Reducing the abundance of both these bacterial groups by treatment with the antibiotics Trimethoprim and Sulfadoxine resulted in a reduction in *H. polygyrus* fecundity in C57BL/6 mice, and a strong trend for increased adult worm expulsion. Interestingly, the abundance of these bacterial groups in the duodenum, where *H. polygyrus* localises, was elevated following infection in C57BL/6 mice, which maintained high worm burdens 28 days post-infection. In contrast, in BALB/c mice, the majority of which had expelled *H. polygyrus* 28 days post-infection, levels of *Lactobacillus/Lactococcus* and Enterobacteriaceae species were reduced following *H. polygyrus* infection. Differential shifts in microflora abundance following infection could be a factor explaining host genotype variation in susceptibility to *H. polygyrus* infections.

Relationships between bacterial species abundance were also seen *within* cohorts of both BALB/c and C57BL/6 mice, with *Lactobacillus/Lactococcus* levels positively correlating with worm burden in BALB/c mice, and Enterobacteriaceae abundance positively correlating with worm burden in C57BL/6 mice. To confirm this correlation in BALB/c mice, the most abundant species of *Lactobacillus/Lactococcus* was identified as *Lactobacillus taiwanensis*, and administering this species to BALB/c mice was sufficient to extend the persistence of *H. polygyrus* infection within these mice.

These experiments confirm mutualistic relationships between intestinal microbes and *H. polygyrus*; modifying the composition of the intestinal microflora is able to alter the susceptibility of mice to *H. polygyrus*, and *H. polygyrus*-infection changes the composition of intestinal bacteria populations. In addition, the genotype of the host influences the direction of these relationships.

Host genotype is a major factor in controlling intestinal microflora populations. Different strains of Wt inbred mice have distinct repertoires of antimicrobial peptides, and differing numbers of Paneth cells [335]. Importantly, this results in divergent compositions of intestinal microbes, when GF mice from different strains are recolonised with identical bacteria populations [335]. A direct comparison between the initial microbial composition of BALB/c and C57BL/6 mice, and a comparison of antimicrobial peptide production in each strain has not yet been carried out.

Many studies have reported that components of the microflora are able to polarise naïve T cells towards specific regulator or effector phenotypes. Colonisation of GF BALB/c and IQI mice with a defined mix of *Clostridium* species increases the abundance of $Foxp3^+$ Tregs in the colonic LP [91]; administration of SFB to GF IQI and C3H/HeN mice induces IL-17A production from $CD4^+$ cells in the LP [87, 88] and recolonisation of Swiss-Webster GF mice with *B. fragilis* results in IFN-γ production by $CD4^+$ splenocytes. However, it is important not to draw broad conclusions from these studies, as in many cases the influence on T cells is dependent on the host genotype.

Although administration of *Clostridium* species to both GF BALB/c and IQI mice increases the proportions of $Foxp3^+$ $CD4^+$ cells, administration of a mixture of 16 strains of *Bacteroides* species also significantly elevates $Foxp3^+$ $CD4^+$ cell proportions in the colonic LP in IQI, but not BALB/c mice [91]. Similarly, whilst SFB administration to GF IQI mice increases IL-17A production from $CD4^+$ small intestine LP cells without elevating the proportion of IFN-γ- or Foxp3- expressing cells [87], administration of the same bacterial species to GF C3H/HeN mice results in increased proportions of Th1, Th2, Th17 and Treg subsets at the same sites [88]. It is therefore unsurprising that in the experiments described here, the bacterial groups that positively correlate with *H. polygyrus* survival are different between BALB/c and C57BL/6 mice.

7.2 C57BL/6 mice deficient in MyD88 are more resistant to *H. polygyrus* than Wt C57BL/6 mice

A striking finding during these studies was that C57BL/6 mice are rendered more resistant to *H. polygyrus*-infection when they are deficient in the signalling adaptor protein MyD88. C57BL/6 MyD88$^{-/-}$ mice had a lower egg output at day-14 and day-28 post-infection, and harboured significantly fewer worms 28 days post-infection than C57BL/6 Wt mice. Although the fecundity of *H. polygyrus* in TLR2$^{-/-}$ mice was also reduced 28 days post-infection compared to Wt mice, adult worm expulsion was not significantly enhanced in these mice, and the day-28 worm burdens of mice deficient in TLR4, TLR5 or TLR9 did not significantly differ from Wt mice. Therefore other signals through MyD88 in Wt mice may contribute to the control of immunity to *H. polygyrus*.

As well as mediating signals from TLRs, MyD88 is required for signalling of IL-1 family members including IL-1α, IL-1β and IL-18 [274], thus it is possible that a lack of signalling by these cytokines in MyD88$^{-/-}$ mice contributes to the increased resistance of these mice to *H. polygyrus*. Cleavage of pro-IL-1 and pro-IL-18 to their active forms occurs following NLR signalling in response to intracellular damage or recognition of PAMPs [41]. It is likely that production of these cytokines is triggered following *H. polygyrus*-mediated damage of the gut epithelium, although levels of cytokines have thus far not been directly measured following *H. polygyrus* infection.

Further experiments are necessary to determine whether the critical signal through MyD88 which controls immunity to *H. polygyrus* is from bacteria signalling through TLRs, or damage-mediated signalling through IL-1 or IL-18 receptors. To resolve this, mice deficient in IL-1R or IL-18R should be examined for their susceptibility to *H. polygyrus*. If the critical signal is bacterially-derived, it is tempting to speculate that MyD88$^{-/-}$ mice lack the ability to detect *Lactobacillus/Lactococcus* or Enterobacteriaceae species, which in Wt C57BL/6 mice appear able to promote *H. polygyrus* survival. It will be particularly interesting to test whether *Lactobacillus/Lactococcus* or

Enterobacteriaceae modulate immunity to *H. polygyrus* in a TLR-dependent manner, by examining whether administering or depleting these bacterial species in MyD88$^{-/-}$ mice further modulates immunity.

7.3 Mechanisms by which the intestinal microflora could alter *H. polygyrus*-infection outcome

7.3.1 Modulation of granuloma formation by the microflora

In three experimental settings, the absence of intestinal bacteria (or signalling via intestinal bacteria) correlates with the production of high numbers of intestinal granulomas following *H. polygyrus* infection. *H. polygyrus*-infected GF mice produce more granulomas than *H. polygyrus*-infected SPF mice [223], *H. polygyrus*-infected mice treated with a cocktail of broad-spectrum antibiotics produce more granulomas than untreated *H. polygyrus*-infected mice, and *H. polygyrus*-infected MyD88-deficient mice produce more granulomas than *H. polygyrus*-infected MyD88-sufficient mice. These findings raise the possibility that a component of the microbiota which signals through MyD88 may inhibit granuloma formation in conventionally reared mice. Alternatively, in the absence of immune stimulation by intestinal bacteria, a reduction in type 1 inflammatory cytokines may allow for greater formation of granulomas.

Granuloma formation has been linked to increased *H. polygyrus* expulsion as more resistant strains of mice produce higher granuloma numbers than susceptible strains [191]. Further strength to this argument is provided by the observation that within a cohort of BALB/c mice, there was a negative correlation between granuloma numbers and adult *H. polygyrus* survival at day 28 post-infection. However, there are many cases where high granuloma number does not dictate worm survival. Notably, BALB/c mice deficient in macrophage migration inhibitory factor (MIF) produce similar high granuloma numbers following *H. polygyrus*-infection to Wt BALB/c mice, yet are more susceptible to infection [336]. Thus, it is likely that the quality of the

granuloma is important to control immunity to *H. polygyrus*, and a more detailed analysis of the key cell types within granulomas in these different settings is required.

7.3.2 Modulation of goblet cell function by the microflora

Enhanced mucus production by goblet cells has been suggested as a mechanism of helminth expulsion [337], and goblet cell hyperplasia occurs following infections with many different intestinal helminth parasites, including *H. polygyrus* [128]. A role for enhanced mucus production has not been directly shown to promote *H. polygyrus* expulsion, although Muc2 production correlates with the expulsion of *T. muris* [182, 183] and *N. brasiliensis* [184]. In addition, RELM-β production by goblet cells can modulate immunity to *H. polygyrus*, most likely by interfering with worm chemotaxis and nutrition [179, 181].

Interestingly, modulating the composition of intestinal bacteria populations by specific antibiotic treatment can alter the production of these goblet cell-derived factors [120, 338]. Treatment with the antibiotic Metronidazole reduces the colonic abundance of Porphoromadaceae species, and increases the abundance of *Lactobacilli* species [120]. Metronidazole treatment also results in a thinning of the inner colonic mucus layer from an average of 23 μm in untreated mice to an average of 13 μm, with depleted levels of Muc2 and RELM-β mRNA in the colon, suggesting that specific species within the intestinal flora control secretion of these factors [120]. Whether production of these factors was altered during the microflora modification experiments described in this thesis was not examined, and future work could examine whether this is a mechanism by which intestinal bacteria influence immunity to *H. polygyrus*.

7.3.3 Modulation of immune responsiveness to *H. polygyrus* by the microflora

Importantly, levels of Enterobacteriaceae species in the duodenum correlated with HES-specific cytokine production, differentially between the more resistant BALB/c mice, and the more susceptible C57BL/6 mice. As described above, the presence of the same species of intestinal bacteria can influence the differentiation of T cells in distinct ways in hosts of different genotypes. Here, in the BALB/c host, duodenal Enterobacteriaceae positively correlated with HES-specific Th2 cytokines, including IL-4. IL-4 is the most important cytokine for promoting *H. polygyrus* expulsion; Wt BALB/c mice which are given IL-4C expel *H. polygyrus* more rapidly than control mice [129].

In C57BL/6 mice, however, duodenal Enterobacteriaceae levels negatively correlated with HES-specific Th2 cytokines, although there was no corresponding positive correlation of these species with HES-specific cytokine production from other Th subsets. A reduction in HES-specific Th2 cytokines in these mice may be a result of reduced antigen sampling by DCs in these mice; a number of Enterobacteriaceae species have previously been shown to reduce DC activation status in *in vitro* studies [339, 340]. Specific bacterial species may therefore also act to interfere with antigen sampling, and well as by directing differential cytokine production. Direct experiments in which single commensal species are administered to *H. polygyrus*-infected mice will be important to verify these correlations, and to examine the antigen-specificity of cytokine production during infection.

It is likely that as well as influencing the differentiation of T cells specific to *H. polygyrus* antigens, specific bacterial species elicit responses against their own epitopes. In the case of *T. gondii*, the epithelial barrier is breached during infection, and intestinal bacterial translocate to the MLN, liver and spleen [75]. Following this infection, an elevation in the frequency of IFN-γ-producing $CD4^+$ cells in the small intestine LP is seen, the majority of which are specific to intestinal bacteria antigens, rather than to *T. gondii* [75]. Similarly to *T. gondii*, *H. polygyrus* disrupts the integrity of the intestinal

epithelium, as infective larvae migrate through to the submucosa following infection. *H. polygyrus* infection has previously been shown to cause increases in IFN-γ production [132, 133], and in the experiments described in this thesis, the highest change in cytokine-producing cell numbers seen following infection was in $CD8^+$ cells producing IFN-γ, both in BALB/c and C57BL/6 mice. It seems likely that a large proportion of this IFN-γ is produced in response to intestinal bacteria antigens, although this has not yet been experimentally addressed.

In C57BL/6 TGF-βRII DN mice, which are impaired in TGF-β signalling by $CD4^+$ and $CD8^+$ cells, IFN-γ production in the MLN, as well as systemically in the sera, is vastly increased compared to Wt C57BL/6 mice following *H. polygyrus* infection [324]. Tregs from TGF-βRII DN mice expressed lower levels of the activation marker CD103 both on $Helios^+$ and $Helios^-$ $Foxp3^+CD4^+$ cells, and it may be that these Tregs are less able to suppress an IFN-γ response to intestinal bacteria antigens in these mice [242].

7.4 IFN-γ modulates susceptibility to *H. polygyrus*

IFN-γ has been identified as a critical cytokine in modulating immunity to *H. polygyrus* in two settings. Firstly, the levels of serum IFN-γ at day-7 following *H. polygyrus* infection could be used as a predictor of day-28 immunity in C57BL/6 mice; high early IFN-γ levels resulted in heightened susceptibility to *H. polygyrus*. Additionally, the high susceptibility of TGF-βRII DN mice was attributed to exuberant IFN-γ production, from both $CD4^+$ and $CD8^+$ T cells, which blocked early Th2 cytokine production during *H. polygyrus*-infection [242]. When TGF-βRII DN were rendered deficient in IFN-γ, early Th2 responsiveness was partly restored, and mice became more resistant to *H. polygyrus* than Wt or TGF-βRIIDN IFN-γ-sufficient animals [242]

7.5 Future considerations

To further examine the interactions between the intestinal microflora and murine immune responses during *H. polygyrus*-infection it is necessary to characterise microflora population changes at the single species level, by high throughput sequencing of the 16S rRNA gene [341]. Once single species are defined, correlative relationships identified here can be verified by examining the immune response and effect on *H. polygyrus* persistence after single bacterial species are administered to mice. As colonising GF mice with single species of bacteria is not physiologically relevant, it may be more informative to recolonise GF mice with a defined mix of bacteria, such as the 8 species of bacteria defined as the ASF [83], and investigate how modulating the ratio of these species affects the immune response and immunity to *H. polygyrus* infection.

The work described here highlights that it is important to acknowledge the role of the microbiota when examining the susceptibility of different mouse strains to *H. polygyrus*. Mice of the same genotype that are housed separately or bred in different facilities can have differing microflora compositions, which can result in marked differences in the composition of immune cells in a naïve state [86]. Ideally, when comparing the response of mice with differing genotypes to *H. polygyrus*, littermate controls should be used, and where this is not possible mice should be co-housed prior to infection. Co-housing appears to eliminate microflora population differences seen between many genotypes of mice when they are housed separately [66, 86, 104].

As well as further defining the species of bacteria that are present in the mammalian intestinal tract, future work should also consider the functional capacity of the microflora. Metatranscriptomic, metaproteomic and metabolomic studies will help to further define the roles of the microflora [342], and determine whether different bacterial species can act redundantly to influence the immune response to infections such as *H. polygyrus*.

7.6 Conclusions

The known and hypothesised interactions between the intestinal bacteria, *H. polygyrus* and the murine immune cells discussed in this thesis are presented in Figure 7.1. It is clear from these studies that *H. polygyrus* infection modulates the composition of the intestinal microflora, and the presence of specific species of intestinal bacteria in turn alters the persistence of *H. polygyrus* infection. These mutual effects are dependent on the genotype of the murine host.

Both the intestinal microflora and *H. polygyrus* have immunomodulatory effects on autoimmune and allergic diseases [9, 333], which raises the possibility that the modulatory effects of *H. polygyrus*-infection are due in part to changes in intestinal microbial composition during infection [218]. Understanding the interactions between intestinal microbes, helminth parasites and the host immune system will allow for a more considered approach when using antibiotics and anthelmintics, and when designing treatments for autoimmune and allergic conditions.

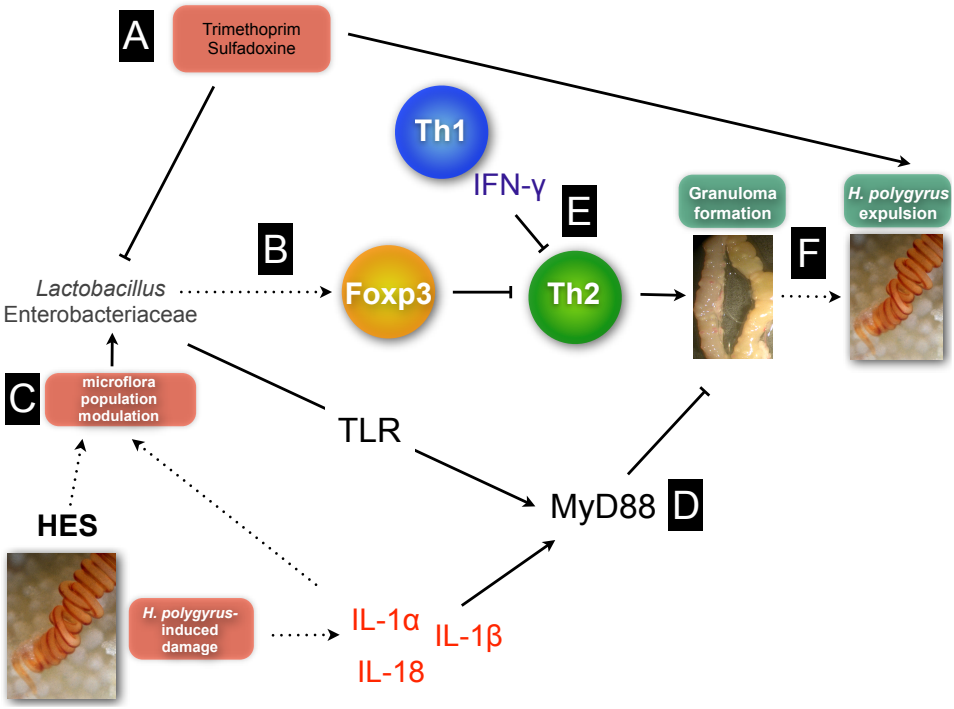

Figure 7.1 Interactions between the intestinal microflora, *H. polygyrus* and murine immune cells.
Solid lines indicate known interactions; dotted lines indicate hypothesised or likely interactions.
(A) Treatment of C57BL/6 mice with the antibiotics Trimethoprim and Sulfadoxine results in reductions in faecal *Lactobacillus/Lactococcus* and Enterobacteriaceae species, a decrease in *H. polygyrus* fecundity and a strong trend for reduced *H. polygyrus* adult worm burden, implying these species may promote the persistence of *H. polygyrus* within the host.
(B) *Lactobacillus* species abundance in the duodenum 28 days post-infection with *H. polygyrus* positively correlates with $CD4^{+}Foxp3^{+}$ total numbers in the MLN in BALB/c mice, and previous studies have shown that administration of single *Lactobacillus* species is able to promote expansion and survival of Tregs [219-222]. Treg induction by *Lactobacillus* species may be a mechanism by which a Th2 response against *H. polygyrus* is suppressed, resulting in reduced *H. polygyrus* expulsion.
(C) The abundance of both *Lactobacillus* and Enterobacteriaceae species is altered following *H. polygyrus* infection in both BALB/c and C57BL/6 mice, with a reduced duodenal presence of both groups following infection in BALB/c mice, and increased abundance post-infection in C57BL/6 mice. These shifts in abundance may be a result of a changing inflammatory environment [343], or due to modulation via lysozymes or other proteins present in HES [127].
(D) C57BL/6 mice deficient in the signalling adaptor protein MyD88 produce high numbers of granulomas following *H. polygyrus*-infection, and are more resistant to *H. polygyrus* than Wt C57BL/6 mice. MyD88 mediates through TLRs as well as through the IL-1 and IL-18 receptors [33]. It is unclear whether it is the loss of TLR or IL-1 family member signalling in $MyD88^{-/-}$ mice that is responsible for the phenotype of these mice with *H. polygyrus*.
(E) High serum IFN-γ levels 7 days post-infection in C57BL/6 mice correlate with increased susceptibility to *H. polygyrus*. Additionally, IFN-γ limits early Th2 responsiveness to *H. polygyrus* in TGF-βRII DN mice. When IFN-γ is deficient in these mice, early Th2 responsiveness is partly restored, and mice are rendered more resistant to *H. polygyrus* [242]. IFN-γ may be produced in response to intestinal bacteria stimulation, as well as to *H. polygyrus* antigens.
(F) BALB/c mice produce high granuloma numbers and are relatively resistant to *H. polygyrus*, whereas C57BL/6 mice produce few granulomas and retain high worm burdens 28-days post-infection. Negative correlations between granuloma number and worm burdens have been noted across a variety of mouse strains [191]. Furthermore, within a cohort of BALB/c mice, there is a negative correlation between granuloma number and worm burden. It is likely that the quality of cells within a granuloma dictate the impact on worm expulsion, rather than simply the presence of a granuloma.

Chapter 8. References

1. Antunes, LC, Han, J, Ferreira, RB, Lolic, P, Borchers, CH, and Finlay, BB. 2011. Effect of antibiotic treatment on the intestinal metabolome. *Antimicrob Agents Chemother*. 55: 1494-503.
2. Wostmann, BS. 1981. The germfree animal in nutritional studies. *Annu Rev Nutr*. 1: 257-79.
3. Smith, K, McCoy, KD, and Macpherson, AJ. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol*. 19: 59-69.
4. Gordon, HA and Pesti, L. 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev*. 35: 390-429.
5. Dominguez-Bello, MG, Costello, EK, Contreras, M, Magris, M, Hidalgo, G, Fierer, N, and Knight, R. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A*. 107: 11971-5.
6. Eckburg, PB, Bik, EM, Bernstein, CN, Purdom, E, Dethlefsen, L, Sargent, M, Gill, SR, Nelson, KE, and Relman, DA. 2005. Diversity of the human intestinal microbial flora. *Science*. 308: 1635-8.
7. Ley, RE, Hamady, M, Lozupone, C, Turnbaugh, PJ, Ramey, RR, Bircher, JS, Schlegel, ML, Tucker, TA, Schrenzel, MD, Knight, R, and Gordon, JI. 2008. Evolution of mammals and their gut microbes. *Science*. 320: 1647-51.
8. Huse, SM, Ye, Y, Zhou, Y, and Fodor, AA. 2012. A core human microbiome as viewed through 16S rRNA sequence clusters. *PLoS One*. 7: e34242.
9. Honda, K and Littman, DR. 2012. The Microbiome in Infectious Disease and Inflammation. *Annu Rev Immunol*. 30: 759-95.
10. Bailey, MT, Dowd, SE, Parry, NM, Galley, JD, Schauer, DB, and Lyte, M. 2010. Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium. Infect Immun*. 78: 1509-19.
11. Arumugam, M, Raes, J, Pelletier, E, Le Paslier, D, Yamada, T, Mende, DR, Fernandes, GR, Tap, J, Bruls, T, Batto, JM, Bertalan, M, Borruel, N, Casellas, F, Fernandez, L, Gautier, L, Hansen, T, Hattori, M, Hayashi, T, Kleerebezem, M, Kurokawa, K, Leclerc, M, Levenez, F, Manichanh, C, Nielsen, HB, Nielsen, T, Pons, N, Poulain, J, Qin, J, Sicheritz-Ponten, T, Tims, S, Torrents, D, Ugarte, E, Zoetendal, EG, Wang, J, Guarner, F, Pedersen, O, de Vos, WM, Brunak, S, Dore, J, Antolin, M, Artiguenave, F, Blottiere, HM, Almeida, M, Brechot, C, Cara, C, Chervaux, C, Cultrone, A, Delorme, C, Denariaz, G, Dervyn, R, Foerstner, KU, Friss, C, van de Guchte, M, Guedon, E, Haimet, F, Huber, W, van Hylckama-Vlieg, J, Jamet, A, Juste, C, Kaci, G, Knol, J, Lakhdari, O, Layec, S, Le Roux, K, Maguin, E, Merieux, A, Melo Minardi, R, M'Rini, C, Muller, J, Oozeer, R, Parkhill, J, Renault, P, Rescigno, M, Sanchez, N, Sunagawa, S, Torrejon, A, Turner, K,

Vandemeulebrouck, G, Varela, E, Winogradsky, Y, Zeller, G, Weissenbach, J, Ehrlich, SD, and Bork, P. 2011. Enterotypes of the human gut microbiome. *Nature*. 473: 174-80.
12. Rakoff-Nahoum, S, Paglino, J, Eslami-Varzaneh, F, Edberg, S, and Medzhitov, R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*. 118: 229-41.
13. Cario, E. 2008. Barrier-protective function of intestinal epithelial Toll-like receptor 2. *Mucosal Immunol*. 1 Suppl 1: S62-6.
14. Round, JL, Lee, SM, Li, J, Tran, G, Jabri, B, Chatila, TA, and Mazmanian, SK. 2011. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*. 332: 974-7.
15. Vijay-Kumar, M, Sanders, CJ, Taylor, RT, Kumar, A, Aitken, JD, Sitaraman, SV, Neish, AS, Uematsu, S, Akira, S, Williams, IR, and Gewirtz, AT. 2007. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest*. 117: 3909-21.
16. Michelsen, KS and Arditi, M. 2007. Toll-like receptors and innate immunity in gut homeostasis and pathology. *Curr Opin Hematol*. 14: 48-54.
17. Vijay-Kumar, M, Aitken, JD, and Gewirtz, AT. 2008. Toll like receptor-5: protecting the gut from enteric microbes. *Semin Immunopathol*. 30: 11-21.
18. Hall, JA, Bouladoux, N, Sun, CM, Wohlfert, EA, Blank, RB, Zhu, Q, Grigg, ME, Berzofsky, JA, and Belkaid, Y. 2008. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity*. 29: 637-49.
19. Cario, E and Podolsky, DK. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun*. 68: 7010-7.
20. Shimazu, R, Akashi, S, Ogata, H, Nagai, Y, Fukudome, K, Miyake, K, and Kimoto, M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med*. 189: 1777-82.
21. Vamadevan, AS, Fukata, M, Arnold, ET, Thomas, LS, Hsu, D, and Abreu, MT. 2010. Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. *Innate Immun*. 16: 93-103.
22. Carvalho, FA, Aitken, JD, Vijay-Kumar, M, and Gewirtz, AT. 2012. Toll-Like Receptor-Gut Microbiota Interactions: Perturb at Your Own Risk! *Annu Rev Physiol*. 74: 117-98.
23. Kenny, EF and O'Neill, LA. 2008. Signalling adaptors used by Toll-like receptors: an update. *Cytokine*. 43: 342-9.
24. Yamamoto, M, Sato, S, Hemmi, H, Sanjo, H, Uematsu, S, Kaisho, T, Hoshino, K, Takeuchi, O, Kobayashi, M, Fujita, T, Takeda, K, and Akira, S. 2002. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature*. 420: 324-9.
25. Yoshimura, A, Lien, E, Ingalls, RR, Tuomanen, E, Dziarski, R, and Golenbock, D. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol*. 163: 1-5.

26. Kang, JY, Nan, X, Jin, MS, Youn, SJ, Ryu, YH, Mah, S, Han, SH, Lee, H, Paik, SG, and Lee, JO. 2009. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity*. 31: 873-84.
27. Carty, M, Goodbody, R, Schroder, M, Stack, J, Moynagh, PN, and Bowie, AG. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat Immunol*. 7: 1074-81.
28. Yamamoto, M, Sato, S, Hemmi, H, Hoshino, K, Kaisho, T, Sanjo, H, Takeuchi, O, Sugiyama, M, Okabe, M, Takeda, K, and Akira, S. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*. 301: 640-3.
29. Poltorak, A, He, X, Smirnova, I, Liu, MY, Van Huffel, C, Du, X, Birdwell, D, Alejos, E, Silva, M, Galanos, C, Freudenberg, M, Ricciardi-Castagnoli, P, Layton, B, and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 282: 2085-8.
30. Gewirtz, AT, Navas, TA, Lyons, S, Godowski, PJ, and Madara, JL. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol*. 167: 1882-5.
31. Lu, J and Sun, PD. 2012. The structure of the TLR5-flagellin complex: a new mode of pathogen detection, conserved receptor dimerization for signaling. *Sci Signal*. 5: pe11.
32. Hemmi, H, Takeuchi, O, Kawai, T, Kaisho, T, Sato, S, Sanjo, H, Matsumoto, M, Hoshino, K, Wagner, H, Takeda, K, and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature*. 408: 740-5.
33. Medzhitov, R, Preston-Hurlburt, P, Kopp, E, Stadlen, A, Chen, C, Ghosh, S, and Janeway, CA, Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell*. 2: 253-8.
34. Hirotani, T, Yamamoto, M, Kumagai, Y, Uematsu, S, Kawase, I, Takeuchi, O, and Akira, S. 2005. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. *Biochem Biophys Res Commun*. 328: 383-92.
35. Kawai, T and Akira, S. 2007. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*. 13: 460-9.
36. Franchi, L, Munoz-Planillo, R, and Nunez, G. 2012. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol*. 13: 325-32.
37. O'Neill, LA and Bowie, AG. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol*. 7: 353-64.
38. Bonardi, V, Cherkis, K, Nishimura, MT, and Dangl, JL. 2012. A new eye on NLR proteins: focused on clarity or diffused by complexity? *Curr Opin Immunol*. 24: 41-50.
39. Thornberry, NA and Molineaux, SM. 1995. Interleukin-1 beta converting enzyme: a novel cysteine protease required for IL-1 beta production and implicated in programmed cell death. *Protein Sci*. 4: 3-12.

40. Dinarello, CA. 1999. IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol*. 103: 11-24.
41. Dinarello, CA. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol*. 27: 519-50.
42. Chamaillard, M, Hashimoto, M, Horie, Y, Masumoto, J, Qiu, S, Saab, L, Ogura, Y, Kawasaki, A, Fukase, K, Kusumoto, S, Valvano, MA, Foster, SJ, Mak, TW, Nunez, G, and Inohara, N. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol*. 4: 702-7.
43. Mo, J, Boyle, JP, Howard, CB, Monie, TP, Davis, BK, and Duncan, JA. 2012. Pathogen sensing by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is mediated by direct binding to muramyl dipeptide and ATP. *J Biol Chem*. 287: 23057-67.
44. Girardin, SE, Boneca, IG, Viala, J, Chamaillard, M, Labigne, A, Thomas, G, Philpott, DJ, and Sansonetti, PJ. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem*. 278: 8869-72.
45. Miao, EA, Alpuche-Aranda, CM, Dors, M, Clark, AE, Bader, MW, Miller, SI, and Aderem, A. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol*. 7: 569-75.
46. Franchi, L, Amer, A, Body-Malapel, M, Kanneganti, TD, Ozoren, N, Jagirdar, R, Inohara, N, Vandenabeele, P, Bertin, J, Coyle, A, Grant, EP, and Nunez, G. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol*. 7: 576-82.
47. Boyden, ED and Dietrich, WF. 2006. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet*. 38: 240-4.
48. Hsu, LC, Ali, SR, McGillivray, S, Tseng, PH, Mariathasan, S, Humke, EW, Eckmann, L, Powell, JJ, Nizet, V, Dixit, VM, and Karin, M. 2008. A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide. *Proc Natl Acad Sci U S A*. 105: 7803-8.
49. van Kooyk, Y and Rabinovich, GA. 2008. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol*. 9: 593-601.
50. Davicino, RC, Elicabe, RJ, Di Genaro, MS, and Rabinovich, GA. 2011. Coupling pathogen recognition to innate immunity through glycan-dependent mechanisms. *Int Immunopharmacol*. 11: 1457-63.
51. Engering, A, Geijtenbeek, TB, van Vliet, SJ, Wijers, M, van Liempt, E, Demaurex, N, Lanzavecchia, A, Fransen, J, Figdor, CG, Piguet, V, and van Kooyk, Y. 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol*. 168: 2118-26.
52. Geijtenbeek, TB and Gringhuis, SI. 2009. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol*. 9: 465-79.
53. Moens, E and Veldhoen, M. 2012. Epithelial barrier biology: good fences make good neighbours. *Immunology*. 135: 1-8.
54. Johansson, ME, Ambort, D, Pelaseyed, T, Schutte, A, Gustafsson, JK, Ermund, A, Subramani, DB, Holmen-Larsson, JM, Thomsson, KA,

Bergstrom, JH, van der Post, S, Rodriguez-Pineiro, AM, Sjovall, H, Backstrom, M, and Hansson, GC. 2011. Composition and functional role of the mucus layers in the intestine. *Cell Mol Life Sci*. 68: 3635-41.
55. Johansson, ME, Phillipson, M, Petersson, J, Velcich, A, Holm, L, and Hansson, GC. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A*. 105: 15064-9.
56. Kitajima, S, Takuma, S, and Morimoto, M. 1999. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. *Exp Anim*. 48: 137-43.
57. Bergstrom, KS, Kissoon-Singh, V, Gibson, DL, Ma, C, Montero, M, Sham, HP, Ryz, N, Huang, T, Velcich, A, Finlay, BB, Chadee, K, and Vallance, BA. 2010. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog*. 6: e1000902.
58. Mukherjee, S, Vaishnava, S, and Hooper, LV. 2008. Multi-layered regulation of intestinal antimicrobial defense. *Cell Mol Life Sci*. 65: 3019-27.
59. Beers, SA, Buckland, AG, Koduri, RS, Cho, W, Gelb, MH, and Wilton, DC. 2002. The antibacterial properties of secreted phospholipases A2: a major physiological role for the group IIA enzyme that depends on the very high pI of the enzyme to allow penetration of the bacterial cell wall. *J Biol Chem*. 277: 1788-93.
60. Wilson, CL, Ouellette, AJ, Satchell, DP, Ayabe, T, Lopez-Boado, YS, Stratman, JL, Hultgren, SJ, Matrisian, LM, and Parks, WC. 1999. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science*. 286: 113-7.
61. Salzman, NH, Hung, K, Haribhai, D, Chu, H, Karlsson-Sjoberg, J, Amir, E, Teggatz, P, Barman, M, Hayward, M, Eastwood, D, Stoel, M, Zhou, Y, Sodergren, E, Weinstock, GM, Bevins, CL, Williams, CB, and Bos, NA. 2010. Enteric defensins are essential regulators of intestinal microbial ecology. *Nat Immunol*. 11: 76-83.
62. Hooper, LV, Stappenbeck, TS, Hong, CV, and Gordon, JI. 2003. Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat Immunol*. 4: 269-73.
63. Karlsson, J, Putsep, K, Chu, H, Kays, RJ, Bevins, CL, and Andersson, M. 2008. Regional variations in Paneth cell antimicrobial peptide expression along the mouse intestinal tract. *BMC Immunol*. 9: 37.
64. Ismail, AS, Severson, KM, Vaishnava, S, Behrendt, CL, Yu, X, Benjamin, JL, Ruhn, KA, Hou, B, Defranco, AL, Yarovinsky, F, and Hooper, LV. 2011. γδ intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *Proc Natl Acad Sci U S A*. 108: 8743-8.
65. Cash, HL, Whitham, CV, Behrendt, CL, and Hooper, LV. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science*. 313: 1126-30.
66. Vaishnava, S, Yamamoto, M, Severson, KM, Ruhn, KA, Yu, X, Koren, O, Ley, R, Wakeland, EK, and Hooper, LV. 2011. The antibacterial

lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. *Science*. 334: 255-8.
67. Macpherson, AJ and Uhr, T. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science*. 303: 1662-5.
68. Rescigno, M, Urbano, M, Valzasina, B, Francolini, M, Rotta, G, Bonasio, R, Granucci, F, Kraehenbuhl, JP, and Ricciardi-Castagnoli, P. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol*. 2: 361-7.
69. van der Heijden, PJ, Stok, W, and Bianchi, AT. 1987. Contribution of immunoglobulin-secreting cells in the murine small intestine to the total 'background' immunoglobulin production. *Immunology*. 62: 551-5.
70. Mestecky, J and Russell, MW. 2009. Specific antibody activity, glycan heterogeneity and polyreactivity contribute to the protective activity of S-IgA at mucosal surfaces. *Immunol Lett*. 124: 57-62.
71. Peterson, DA, McNulty, NP, Guruge, JL, and Gordon, JI. 2007. IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell Host Microbe*. 2: 328-39.
72. Macpherson, AJ, Gatto, D, Sainsbury, E, Harriman, GR, Hengartner, H, and Zinkernagel, RM. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science*. 288: 2222-6.
73. Suzuki, K, Meek, B, Doi, Y, Muramatsu, M, Chiba, T, Honjo, T, and Fagarasan, S. 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A*. 101: 1981-6.
74. Fagarasan, S, Muramatsu, M, Suzuki, K, Nagaoka, H, Hiai, H, and Honjo, T. 2002. Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science*. 298: 1424-7.
75. Hand, TW, Dos Santos, LM, Bouladoux, N, Molloy, MJ, Pagan, AJ, Pepper, M, Maynard, CL, Elson, CO, 3rd, and Belkaid, Y. 2012. Acute Gastrointestinal Infection Induces Long-Lived Microbiota-Specific T Cell Responses. *Science*. 337: 1553-6.
76. Larsson, E, Tremaroli, V, Lee, YS, Koren, O, Nookaew, I, Fricker, A, Nielsen, J, Ley, RE, and Backhed, F. 2012. Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88. *Gut*. 61: 1124-31.
77. Turnbaugh, PJ, Ley, RE, Mahowald, MA, Magrini, V, Mardis, ER, and Gordon, JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. 444: 1027-31.
78. Vijay-Kumar, M, Aitken, JD, Carvalho, FA, Cullender, TC, Mwangi, S, Srinivasan, S, Sitaraman, SV, Knight, R, Ley, RE, and Gewirtz, AT. 2010. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science*. 328: 228-31.
79. Toivanen, P, Vaahtovuo, J, and Eerola, E. 2001. Influence of major histocompatibility complex on bacterial composition of fecal flora. *Infect Immun*. 69: 2372-7.
80. Yamanaka, T, Helgeland, L, Farstad, IN, Fukushima, H, Midtvedt, T, and Brandtzaeg, P. 2003. Microbial colonization drives lymphocyte accumulation and differentiation in the follicle-associated epithelium of Peyer's patches. *J Immunol*. 170: 816-22.

81. Lamouse-Smith, ES, Tzeng, A, and Starnbach, MN. 2011. The intestinal flora is required to support antibody responses to systemic immunization in infant and germ free mice. *PLoS One*. 6: e27662.
82. Zaph, C, Du, Y, Saenz, SA, Nair, MG, Perrigoue, JG, Taylor, BC, Troy, AE, Kobuley, DE, Kastelein, RA, Cua, DJ, Yu, Y, and Artis, D. 2008. Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *J Exp Med*. 205: 2191-8.
83. Dewhirst, FE, Chien, CC, Paster, BJ, Ericson, RL, Orcutt, RP, Schauer, DB, and Fox, JG. 1999. Phylogeny of the defined murine microbiota: altered Schaedler flora. *Appl Environ Microbiol*. 65: 3287-92.
84. Geuking, MB, Cahenzli, J, Lawson, MA, Ng, DC, Slack, E, Hapfelmeier, S, McCoy, KD, and Macpherson, AJ. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity*. 34: 794-806.
85. Hapfelmeier, S, Lawson, MA, Slack, E, Kirundi, JK, Stoel, M, Heikenwalder, M, Cahenzli, J, Velykoredko, Y, Balmer, ML, Endt, K, Geuking, MB, Curtiss, R, 3rd, McCoy, KD, and Macpherson, AJ. 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science*. 328: 1705-9.
86. Ivanov, II, Frutos Rde, L, Manel, N, Yoshinaga, K, Rifkin, DB, Sartor, RB, Finlay, BB, and Littman, DR. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. 4: 337-49.
87. Ivanov, II, Atarashi, K, Manel, N, Brodie, EL, Shima, T, Karaoz, U, Wei, D, Goldfarb, KC, Santee, CA, Lynch, SV, Tanoue, T, Imaoka, A, Itoh, K, Takeda, K, Umesaki, Y, Honda, K, and Littman, DR. 2009. Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell*. 139: 485-98.
88. Gaboriau-Routhiau, V, Rakotobe, S, Lecuyer, E, Mulder, I, Lan, A, Bridonneau, C, Rochet, V, Pisi, A, De Paepe, M, Brandi, G, Eberl, G, Snel, J, Kelly, D, and Cerf-Bensussan, N. 2009. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity*. 31: 677-89.
89. Atarashi, K, Nishimura, J, Shima, T, Umesaki, Y, Yamamoto, M, Onoue, M, Yagita, H, Ishii, N, Evans, R, Honda, K, and Takeda, K. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature*. 455: 808-12.
90. Mazmanian, SK, Liu, CH, Tzianabos, AO, and Kasper, DL. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell*. 122: 107-18.
91. Atarashi, K, Tanoue, T, Shima, T, Imaoka, A, Kuwahara, T, Momose, Y, Cheng, G, Yamasaki, S, Saito, T, Ohba, Y, Taniguchi, T, Takeda, K, Hori, S, Ivanov, II, Umesaki, Y, Itoh, K, and Honda, K. 2011. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*. 331: 337-41.
92. Round, JL and Mazmanian, SK. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A*. 107: 12204-9.
93. Lathrop, SK, Bloom, SM, Rao, SM, Nutsch, K, Lio, CW, Santacruz, N, Peterson, DA, Stappenbeck, TS, and Hsieh, CS. 2011. Peripheral

education of the immune system by colonic commensal microbiota. *Nature*. 478: 250-4.
94. Herbst, T, Sichelstiel, A, Schar, C, Yadava, K, Burki, K, Cahenzli, J, McCoy, K, Marsland, BJ, and Harris, NL. 2011. Dysregulation of allergic airway inflammation in the absence of microbial colonization. *Am J Respir Crit Care Med*. 184: 198-205.
95. Russell, SL, Gold, MJ, Hartmann, M, Willing, BP, Thorson, L, Wlodarska, M, Gill, N, Blanchet, MR, Mohn, WW, McNagny, KM, and Finlay, BB. 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep*. 13: 440-7.
96. Lambert, SE, Kinder, JM, Then, JE, Parliament, KN, and Bruns, HA. 2012. Erythromycin treatment hinders the induction of oral tolerance to fed ovalbumin. *Front Immunol*. 3: 203.
97. Kuhn, R, Lohler, J, Rennick, D, Rajewsky, K, and Muller, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 75: 263-74.
98. Franke, A, Balschun, T, Sina, C, Ellinghaus, D, Hasler, R, Mayr, G, Albrecht, M, Wittig, M, Buchert, E, Nikolaus, S, Gieger, C, Wichmann, HE, Sventoraityte, J, Kupcinskas, L, Onnie, CM, Gazouli, M, Anagnou, NP, Strachan, D, McArdle, WL, Mathew, CG, Rutgeerts, P, Vermeire, S, Vatn, MH, Krawczak, M, Rosenstiel, P, Karlsen, TH, and Schreiber, S. 2010. Genome-wide association study for ulcerative colitis identifies risk loci at 7q22 and 22q13 (IL17REL). *Nat Genet*. 42: 292-4.
99. Mazmanian, SK, Round, JL, and Kasper, DL. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*. 453: 620-5.
100. Asquith, MJ, Boulard, O, Powrie, F, and Maloy, KJ. 2010. Pathogenic and protective roles of MyD88 in leukocytes and epithelial cells in mouse models of inflammatory bowel disease. *Gastroenterology*. 139: 519-29, 529 e1-2.
101. Kirkland, D, Benson, A, Mirpuri, J, Pifer, R, Hou, B, DeFranco, AL, and Yarovinsky, F. 2012. B cell-intrinsic MyD88 signaling prevents the lethal dissemination of commensal bacteria during colonic damage. *Immunity*. 36: 228-38.
102. Hugot, JP, Chamaillard, M, Zouali, H, Lesage, S, Cezard, JP, Belaiche, J, Almer, S, Tysk, C, O'Morain, CA, Gassull, M, Binder, V, Finkel, Y, Cortot, A, Modigliani, R, Laurent-Puig, P, Gower-Rousseau, C, Macry, J, Colombel, JF, Sahbatou, M, and Thomas, G. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 411: 599-603.
103. Maeda, S, Hsu, LC, Liu, H, Bankston, LA, Iimura, M, Kagnoff, MF, Eckmann, L, and Karin, M. 2005. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science*. 307: 734-8.
104. Elinav, E, Strowig, T, Kau, AL, Henao-Mejia, J, Thaiss, CA, Booth, CJ, Peaper, DR, Bertin, J, Eisenbarth, SC, Gordon, JI, and Flavell, RA. 2011. NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. *Cell*. 145: 745-57.
105. Rath, HC, Schultz, M, Freitag, R, Dieleman, LA, Li, F, Linde, HJ, Scholmerich, J, and Sartor, RB. 2001. Different subsets of enteric

bacteria induce and perpetuate experimental colitis in rats and mice. *Infect Immun*. 69: 2277-85.
106. Ferreira, RB, Gill, N, Willing, BP, Antunes, LC, Russell, SL, Croxen, MA, and Finlay, BB. 2011. The intestinal microbiota plays a role in *Salmonella*-induced colitis independent of pathogen colonization. *PLoS One*. 6: e20338.
107. Bloom, SM, Bijanki, VN, Nava, GM, Sun, L, Malvin, NP, Donermeyer, DL, Dunne, WM, Jr., Allen, PM, and Stappenbeck, TS. 2011. Commensal *Bacteroides* species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. *Cell Host Microbe*. 9: 390-403.
108. Ghosh, S, Dai, C, Brown, K, Rajendiran, E, Makarenko, S, Baker, J, Ma, C, Halder, S, Montero, M, Ionescu, VA, Klegeris, A, Vallance, BA, and Gibson, DL. 2011. Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression. *Am J Physiol Gastrointest Liver Physiol*. 301: G39-49.
109. Wu, HJ, Ivanov, II, Darce, J, Hattori, K, Shima, T, Umesaki, Y, Littman, DR, Benoist, C, and Mathis, D. 2010. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity*. 32: 815-27.
110. Wen, L, Ley, RE, Volchkov, PY, Stranges, PB, Avanesyan, L, Stonebraker, AC, Hu, C, Wong, FS, Szot, GL, Bluestone, JA, Gordon, JI, and Chervonsky, AV. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature*. 455: 1109-13.
111. Liu, Q, Sundar, K, Mishra, PK, Mousavi, G, Liu, Z, Gaydo, A, Alem, F, Lagunoff, D, Bleich, D, and Gause, WC. 2009. Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. *Infect Immun*. 77: 5347-58.
112. Saunders, KA, Raine, T, Cooke, A, and Lawrence, CE. 2007. Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. *Infect Immun*. 75: 397-407.
113. Kane, M, Case, LK, Kopaskie, K, Kozlova, A, MacDearmid, C, Chervonsky, AV, and Golovkina, TV. 2011. Successful transmission of a retrovirus depends on the commensal microbiota. *Science*. 334: 245-9.
114. Kuss, SK, Best, GT, Etheredge, CA, Pruijssers, AJ, Frierson, JM, Hooper, LV, Dermody, TS, and Pfeiffer, JK. 2011. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science*. 334: 249-52.
115. Ganal, SC, Sanos, SL, Kallfass, C, Oberle, K, Johner, C, Kirschning, C, Lienenklaus, S, Weiss, S, Staeheli, P, Aichele, P, and Diefenbach, A. 2012. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity*. 37: 171-86.
116. Abt, MC, Osborne, LC, Monticelli, LA, Doering, TA, Alenghat, T, Sonnenberg, GF, Paley, MA, Antenus, M, Williams, KL, Erikson, J, Wherry, EJ, and Artis, D. 2012. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*. 37: 158-70.

117. Sekirov, I, Tam, NM, Jogova, M, Robertson, ML, Li, Y, Lupp, C, and Finlay, BB. 2008. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect Immun*. 76: 4726-36.
118. O'Mahony, C, Scully, P, O'Mahony, D, Murphy, S, O'Brien, F, Lyons, A, Sherlock, G, MacSharry, J, Kiely, B, Shanahan, F, and O'Mahony, L. 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog*. 4: e1000112.
119. Willing, BP, Vacharaksa, A, Croxen, M, Thanachayanont, T, and Finlay, BB. 2011. Altering host resistance to infections through microbial transplantation. *PLoS One*. 6: e26988.
120. Wlodarska, M, Willing, B, Keeney, KM, Menendez, A, Bergstrom, KS, Gill, N, Russell, SL, Vallance, BA, and Finlay, BB. 2011. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated *Citrobacter rodentium*-induced colitis. *Infect Immun*. 79: 1536-45.
121. Gouy de Bellocq, J, Ferte, H, Depaquit, J, Justine, JL, Tillier, A, and Durette-Desset, MC. 2001. Phylogeny of the Trichostrongylina (Nematoda) inferred from 28S rDNA sequences. *Mol Phylogenet Evol*. 19: 430-42.
122. Behnke, J and Harris, PD. 2010. *Heligmosomoides bakeri*: a new name for an old worm? *Trends Parasitol*. 26: 524-9.
123. Behnke, JM, Keymer, AE, and Lewis, JW. 1991. *Heligmosomoides polygyrus* or *Nematospiroides dubius*? *Parasitol Today*. 7: 177-9.
124. Cable, J, Harris, PD, Lewis, JW, and Behnke, JM. 2006. Molecular evidence that *Heligmosomoides polygyrus* from laboratory mice and wood mice are separate species. *Parasitology*. 133: 111-22.
125. Maizels, RM, Hewitson, JP, and Gause, WC. 2010. *Heligmosomoides polygyrus*: one species still. *Trends Parasitol*. 27: 100-1.
126. Bansemir, AD and Sukhdeo, MV. 1994. The food resource of adult *Heligmosomoides polygyrus* in the small intestine. *J Parasitol*. 80: 24-8.
127. Hewitson, JP, Harcus, Y, Murray, J, van Agtmaal, M, Filbey, KJ, Grainger, JR, Bridgett, S, Blaxter, ML, Ashton, PD, Ashford, DA, Curwen, RS, Wilson, RA, Dowle, AA, and Maizels, RM. 2011. Proteomic analysis of secretory products from the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of venom allergen-like (VAL) proteins. *J Proteomics*. 74: 1573-94.
128. Hashimoto, K, Uchikawa, R, Tegoshi, T, Takeda, K, Yamada, M, and Arizono, N. 2009. Depleted intestinal goblet cells and severe pathological changes in SCID mice infected with *Heligmosomoides polygyrus. Parasite Immunol*. 31: 457-65.
129. Urban, JF, Jr., Maliszewski, CR, Madden, KB, Katona, IM, and Finkelman, FD. 1995. IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice. *J Immunol*. 154: 4675-84.
130. Urban, JF, Jr., Katona, IM, and Finkelman, FD. 1991. *Heligmosomoides polygyrus*: CD4+ but not CD8+ T cells regulate the

IgE response and protective immunity in mice. *Exp Parasitol*. 73: 500-11.
131. Rausch, S, Huehn, J, Kirchhoff, D, Rzepecka, J, Schnoeller, C, Pillai, S, Loddenkemper, C, Scheffold, A, Hamann, A, Lucius, R, and Hartmann, S. 2008. Functional analysis of effector and regulatory T cells in a parasitic nematode infection. *Infect Immun*. 76: 1908-19.
132. Svetic, A, Madden, KB, Zhou, XD, Lu, P, Katona, IM, Finkelman, FD, Urban, JF, Jr., and Gause, WC. 1993. A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *J Immunol*. 150: 3434-41.
133. Finney, CAM, Taylor, MD, Wilson, MS, and Maizels, RM. 2007. Expansion and activation of CD4(+)CD25(+) regulatory T cells in *Heligmosomoides polygyrus* infection. *Eur J Immunol*. 37: 1874-86.
134. Urban, JF, Jr., Katona, IM, Paul, WE, and Finkelman, FD. 1991. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc Natl Acad Sci U S A*. 88: 5513-7.
135. King, C, Tangye, SG, and Mackay, CR. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol*. 26: 741-66.
136. King, IL, Mohrs, K, and Mohrs, M. 2010. A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. *J Immunol*. 185: 6138-45.
137. King, IL and Mohrs, M. 2009. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med*. 206: 1001-7.
138. McCoy, KD, Stoel, M, Stettler, R, Merky, P, Fink, K, Senn, BM, Schaer, C, Massacand, J, Odermatt, B, Oettgen, HC, Zinkernagel, RM, Bos, NA, Hengartner, H, Macpherson, AJ, and Harris, NL. 2008. Polyclonal and specific antibodies mediate protective immunity against enteric helminth infection. *Cell Host Microbe*. 4: 362-73.
139. Liu, Q, Kreider, T, Bowdridge, S, Liu, Z, Song, Y, Gaydo, AG, Urban, JF, Jr., and Gause, WC. 2010. B cells have distinct roles in host protection against different nematode parasites. *J Immunol*. 184: 5213-23.
140. Wojciechowski, W, Harris, DP, Sprague, F, Mousseau, B, Makris, M, Kusser, K, Honjo, T, Mohrs, K, Mohrs, M, Randall, T, and Lund, FE. 2009. Cytokine-producing effector B cells regulate type 2 immunity to *H. polygyrus. Immunity*. 30: 421-33.
141. Pritchard, DI, Williams, DJ, Behnke, JM, and Lee, TD. 1983. The role of IgG1 hypergammaglobulinaemia in immunity to the gastrointestinal nematode *Nematospiroides dubius*. The immunochemical purification, antigen-specificity and *in vivo* anti-parasite effect of IgG1 from immune serum. *Immunology*. 49: 353-65.
142. Allen, JE and Maizels, RM. 2011. Diversity and dialogue in immunity to helminths. *Nat Rev Immunol*. 11: 375-88.
143. Neill, DR and McKenzie, AN. 2011. Nuocytes and beyond: new insights into helminth expulsion. *Trends Parasitol*. 27: 214-21.
144. MacDonald, AS and Maizels, RM. 2008. Alarming dendritic cells for Th2 induction. *J Exp Med*. 205: 13-7.

145. Hochweller, K, Striegler, J, Hammerling, GJ, and Garbi, N. 2008. A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells. *Eur J Immunol*. 38: 2776-83.
146. Smith, KA, Hochweller, K, Hammerling, GJ, Boon, L, MacDonald, AS, and Maizels, RM. 2011. Chronic helminth infection promotes immune regulation *in vivo* through dominance of CD11cloCD103- dendritic cells. *J Immunol*. 186: 7098-109.
147. Anthony, RM, Rutitzky, LI, Urban, JF, Jr., Stadecker, MJ, and Gause, WC. 2007. Protective immune mechanisms in helminth infection. *Nat Rev Immunol*. 7: 975-87.
148. Modolell, M, Corraliza, IM, Link, F, Soler, G, and Eichmann, K. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur J Immunol*. 25: 1101-4.
149. Hesse, M, Modolell, M, La Flamme, AC, Schito, M, Fuentes, JM, Cheever, AW, Pearce, EJ, and Wynn, TA. 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines *in vivo*: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol*. 167: 6533-44.
150. Pesce, J, Kaviratne, M, Ramalingam, TR, Thompson, RW, Urban, JF, Jr., Cheever, AW, Young, DA, Collins, M, Grusby, MJ, and Wynn, TA. 2006. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest*. 116: 2044-55.
151. Park-Min, KH, Antoniv, TT, and Ivashkiv, LB. 2005. Regulation of macrophage phenotype by long-term exposure to IL-10. *Immunobiology*. 210: 77-86.
152. Anthony, RM, Urban, JF, Jr., Alem, F, Hamed, HA, Rozo, CT, Boucher, JL, Van Rooijen, N, and Gause, WC. 2006. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med*. 12: 955-60.
153. Chang, NC, Hung, SI, Hwa, KY, Kato, I, Chen, JE, Liu, CH, and Chang, AC. 2001. A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. *J Biol Chem*. 276: 17497-506.
154. Loke, P, Gallagher, I, Nair, MG, Zang, X, Brombacher, F, Mohrs, M, Allison, JP, and Allen, JE. 2007. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *J Immunol*. 179: 3926-36.
155. Morimoto, M, Whitmire, J, Xiao, S, Anthony, RM, Mirakami, H, Star, RA, Urban, JF, Jr., and Gause, WC. 2004. Peripheral CD4 T cells rapidly accumulate at the host: parasite interface during an inflammatory Th2 memory response. *J Immunol*. 172: 2424-30.
156. Patel, N, Kreider, T, Urban, JF, Jr., and Gause, WC. 2009. Characterisation of effector mechanisms at the host:parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. *Int J Parasitol*. 39: 13-21.
157. Doligalska, M, Rzepecka, J, Drela, N, Donskow, K, and Gerwel-Wronka, M. 2006. The role of TGF-beta in mice infected with *Heligmosomoides polygyrus. Parasite Immunol*. 28: 387-95.

158. Ohnmacht, C and Voehringer, D. 2009. Basophil effector function and homeostasis during helminth infection. *Blood*. 113: 2816-25.
159. Perrigoue, JG, Saenz, SA, Siracusa, MC, Allenspach, EJ, Taylor, BC, Giacomin, PR, Nair, MG, Du, Y, Zaph, C, van Rooijen, N, Comeau, MR, Pearce, EJ, Laufer, TM, and Artis, D. 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol*. 10: 697-705.
160. Behnke, JM, Wahid, FN, Grencis, RK, Else, KJ, Ben-Smith, AW, and Goyal, PK. 1993. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms. *Parasite Immunol*. 15: 415-21.
161. Wahid, FN, Behnke, JM, Grencis, RK, Else, KJ, and Ben-Smith, AW. 1994. Immunological relationships during primary infection with *Heligmosomoides polygyrus*: Th2 cytokines and primary response phenotype. *Parasitology*. 108: 461-71.
162. Ben-Smith, A, Lammas, DA, and Behnke, JM. 2003. The relative involvement of Th1 and Th2 associated immune responses in the expulsion of a primary infection of *Heligmosomoides polygyrus* in mice of differing response phenotype. *J Helminthol*. 77: 133-46.
163. Snoek, SA, Dhawan, S, van Bree, SH, Cailotto, C, van Diest, SA, Duarte, JM, Stanisor, OI, Hilbers, FW, Nijhuis, L, Koeman, A, van den Wijngaard, RM, Zuurbier, CJ, Boeckxstaens, GE, and de Jonge, WJ. 2012. Mast cells trigger epithelial barrier dysfunction, bacterial translocation and postoperative ileus in a mouse model. *Neurogastroenterol Motil*. 24: 172-e91.
164. McDermott, JR, Bartram, RE, Knight, PA, Miller, HR, Garrod, DR, and Grencis, RK. 2003. Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci U S A*. 100: 7761-6.
165. Dehlawi, MS, Wakelin, D, and Behnke, JM. 1987. Suppression of mucosal mastocytosis by infection with the intestinal nematode *Nematospiroides dubius*. *Parasite Immunol*. 9: 187-94.
166. Hashimoto, K, Uchikawa, R, Tegoshi, T, Takeda, K, Yamada, M, and Arizono, N. 2009. Immunity-mediated regulation of fecundity in the nematode *Heligmosomoides polygyrus*-the potential role of mast cells. *Parasitology*. 137: 881-7.
167. Morimoto, M and Utsumiya, K. 2011. Enhanced protection against *Heligmosomoides polygyrus* in IL-2 receptor beta-chain overexpressed transgenic mice with intestinal mastocytosis. *J Vet Med Sci*. 73: 849-51.
168. Hepworth, MR, Danilowicz-Luebert, E, Rausch, S, Metz, M, Klotz, C, Maurer, M, and Hartmann, S. 2012. Mast cells orchestrate type 2 immunity to helminths through regulation of tissue-derived cytokines. *Proc Natl Acad Sci U S A*. 109: 6644-9.
169. Grimbaldeston, MA, Chen, CC, Piliponsky, AM, Tsai, M, Tam, SY, and Galli, SJ. 2005. Mast cell-deficient *W-sash c-kit* mutant *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice as a model for investigating mast cell biology *in vivo*. *Am J Pathol*. 167: 835-48.

170. Barlow, JL and McKenzie, AN. 2011. Nuocytes: expanding the innate cell repertoire in type-2 immunity. *J Leukoc Biol*. 90: 867-74.
171. Ikeda, K, Nakajima, H, Suzuki, K, Kagami, S, Hirose, K, Suto, A, Saito, Y, and Iwamoto, I. 2003. Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood*. 101: 3594-6.
172. Paul, WE and Zhu, J. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol*. 10: 225-35.
173. Wills-Karp, M, Rani, R, Dienger, K, Lewkowich, I, Fox, JG, Perkins, C, Lewis, L, Finkelman, FD, Smith, DE, Bryce, PJ, Kurt-Jones, EA, Wang, TC, Sivaprasad, U, Hershey, GK, and Herbert, DR. 2012. Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. *J Exp Med*. 209: 607-22.
174. Fallon, PG, Ballantyne, SJ, Mangan, NE, Barlow, JL, Dasvarma, A, Hewett, DR, McIlgorm, A, Jolin, HE, and McKenzie, AN. 2006. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med*. 203: 1105-16.
175. Neill, DR, Wong, SH, Bellosi, A, Flynn, RJ, Daly, M, Langford, TK, Bucks, C, Kane, CM, Fallon, PG, Pannell, R, Jolin, HE, and McKenzie, ANJ. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 464: 1367-70.
176. Saenz, SA, Siracusa, MC, Perrigoue, JG, Spencer, SP, Urban, JF, Jr., Tocker, JE, Budelsky, AL, Kleinschek, MA, Kastelein, RA, Kambayashi, T, Bhandoola, A, and Artis, D. 2010. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature*. 464: 1362-6.
177. Shea-Donohue, T, Sullivan, C, Finkelman, FD, Madden, KB, Morris, SC, Goldhill, J, Pineiro-Carrero, V, and Urban, JF, Jr. 2001. The role of IL-4 in *Heligmosomoides polygyrus*-induced alterations in murine intestinal epithelial cell function. *J Immunol*. 167: 2234-9.
178. Madden, KB, Yeung, KA, Zhao, A, Gause, WC, Finkelman, FD, Katona, IM, Urban, JF, Jr., and Shea-Donohue, T. 2004. Enteric nematodes induce stereotypic STAT6-dependent alterations in intestinal epithelial cell function. *J Immunol*. 172: 5616-21.
179. Artis, D, Wang, ML, Keilbaugh, SA, He, W, Brenes, M, Swain, GP, Knight, PA, Donaldson, DD, Lazar, MA, Miller, HR, Schad, GA, Scott, P, and Wu, GD. 2004. RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci U S A*. 101: 13596-600.
180. Hogan, SP, Seidu, L, Blanchard, C, Groschwitz, K, Mishra, A, Karow, ML, Ahrens, R, Artis, D, Murphy, AJ, Valenzuela, DM, Yancopoulos, GD, and Rothenberg, ME. 2006. Resistin-like molecule beta regulates innate colonic function: barrier integrity and inflammation susceptibility. *J Allergy Clin Immunol*. 118: 257-68.
181. Herbert, DR, Yang, JQ, Hogan, SP, Groschwitz, K, Khodoun, M, Munitz, A, Orekov, T, Perkins, C, Wang, Q, Brombacher, F, Urban, JF, Jr., Rothenberg, ME, and Finkelman, FD. 2009. Intestinal epithelial cell secretion of RELM-β protects against gastrointestinal worm infection. *J Exp Med*. 206: 2947-57.

182. Hasnain, SZ, Wang, H, Ghia, JE, Haq, N, Deng, Y, Velcich, A, Grencis, RK, Thornton, DJ, and Khan, WI. 2010. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology*. 138: 1763-71.
183. Hasnain, SZ, Evans, CM, Roy, M, Gallagher, AL, Kindrachuk, KN, Barron, L, Dickey, BF, Wilson, MS, Wynn, TA, Grencis, RK, and Thornton, DJ. 2011. Muc5ac: a critical component mediating the rejection of enteric nematodes. *J Exp Med*. 208: 893-900.
184. Inagaki-Ohara, K, Sakamoto, Y, Dohi, T, and Smith, AL. 2011. gammadelta T cells play a protective role during infection with *Nippostrongylus brasiliensis* by promoting goblet cell function in the small intestine. *Immunology*. 134: 448-58.
185. Zhao, A, McDermott, J, Urban, JF, Jr., Gause, W, Madden, KB, Yeung, KA, Morris, SC, Finkelman, FD, and Shea-Donohue, T. 2003. Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. *J Immunol*. 171: 948-54.
186. Marillier, RG, Brombacher, TM, Dewals, B, Leeto, M, Barkhuizen, M, Govender, D, Kellaway, L, Horsnell, WG, and Brombacher, F. 2010. IL-4R{alpha}-responsive smooth muscle cells increase intestinal hypercontractility and contribute to resistance during acute Schistosomiasis. *Am J Physiol Gastrointest Liver Physiol*. 298: G943-51.
187. Vallance, BA, Blennerhassett, PA, and Collins, SM. 1997. Increased intestinal muscle contractility and worm expulsion in nematode-infected mice. *Am J Physiol*. 272: G321-7.
188. Vallance, BA, Blennerhassett, PA, Deng, Y, Matthaei, KI, Young, IG, and Collins, SM. 1999. IL-5 contributes to worm expulsion and muscle hypercontractility in a primary *T. spiralis* infection. *Am J Physiol*. 277: G400-8.
189. Zhao, A, Urban, JF, Jr., Anthony, RM, Sun, R, Stiltz, J, van Rooijen, N, Wynn, TA, Gause, WC, and Shea-Donohue, T. 2008. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. *Gastroenterology*. 135: 217-225 e1.
190. Shea-Donohue, T, Notari, L, Stiltz, J, Sun, R, Madden, KB, Urban, JF, Jr., and Zhao, A. 2010. Role of enteric nerves in immune-mediated changes in protease-activated receptor 2 effects on gut function. *Neurogastroenterol Motil*. 22: 1138-e291.
191. Menge, DM, Behnke, JM, Lowe, A, Gibson, JP, Iraqi, FA, Baker, RL, and Wakelin, D. 2003. Mapping of chromosomal regions influencing immunological responses to gastrointestinal nematode infections in mice. *Parasite Immunol*. 25: 341-9.
192. Martin, P and Leibovich, SJ. 2005. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol*. 15: 599-607.
193. Maizels, RM and Smith, KA. 2011. Regulatory T cells in infection. *Adv Immunol*. 112: 73-136.
194. Blum, AM, Hang, L, Setiawan, T, Urban, JP, Jr., Stoyanoff, KM, Leung, J, and Weinstock, JV. 2012. *Heligmosomoides polygyrus bakeri*

induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses. *J Immunol*. 189: 2512-20.
195. Wilson, MS, Taylor, MD, O'Gorman, MT, Balic, A, Barr, TA, Filbey, K, Anderton, SM, and Maizels, RM. 2010. Helminth-induced CD19+CD23hi B cells modulate experimental allergic and autoimmune inflammation. *Eur J Immunol*. 40: 1682-96.
196. Rausch, S, Huehn, J, Loddenkemper, C, Hepworth, MR, Klotz, C, Sparwasser, T, Hamann, A, Lucius, R, and Hartmann, S. 2009. Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3+ cells. *Eur J Immunol*. 39: 3066-77.
197. Grainger, JR, Smith, KA, Hewitson, JP, McSorley, HJ, Harcus, Y, Filbey, KJ, Finney, CA, Greenwood, EJ, Knox, DP, Wilson, MS, Belkaid, Y, Rudensky, AY, and Maizels, RM. 2010. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway. *J Exp Med*. 207: 2331-41.
198. Belkaid, Y and Tarbell, K. 2009. Regulatory T cells in the control of host-microorganism interactions. *Annu Rev Immunol*. 27: 551-89.
199. Metwali, A, Setiawan, T, Blum, AM, Urban, J, Elliott, DE, Hang, L, and Weinstock, JV. 2006. Induction of CD8+ regulatory T cells in the intestine by *Heligmosomoides polygyrus* infection. *Am J Physiol Gastrointest Liver Physiol*. 291: G253-9.
200. Maizels, RM, Hewitson, JP, Murray, J, Harcus, YM, Dayer, B, Filbey, KJ, Grainger, JR, McSorley, HJ, Reynolds, LA, and Smith, KA. 2012. Immune modulation and modulators in *Heligmosomoides polygyrus* infection. *Exp Parasitol*. 132: 76-89.
201. Bashir, ME, Andersen, P, Fuss, IJ, Shi, HN, and Nagler-Anderson, C. 2002. An enteric helminth infection protects against an allergic response to dietary antigen. *J Immunol*. 169: 3284-92.
202. Kitagaki, K, Businga, TR, Racila, D, Elliott, DE, Weinstock, JV, and Kline, JN. 2006. Intestinal helminths protect in a murine model of asthma. *J Immunol*. 177: 1628-35.
203. Wilson, MS, Taylor, MD, Balic, A, Finney, CAM, Lamb, JR, and Maizels, RM. 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med*. 202: 1199-212.
204. Hartmann, S, Schnoeller, C, Dahten, A, Avagyan, A, Rausch, S, Lendner, M, Bocian, C, Pillai, S, Loddenkemper, C, Lucius, R, Worm, M, and Hamelmann, E. 2009. Gastrointestinal nematode infection interferes with experimental allergic airway inflammation but not atopic dermatitis. *Clin Exp Allergy*. 39: 1585-96.
205. Whelan, RA, Hartmann, S, and Rausch, S. 2011. Nematode modulation of inflammatory bowel disease. *Protoplasma*.
206. Berg, DJ, Davidson, N, Kuhn, R, Muller, W, Menon, S, Holland, G, Thompson-Snipes, L, Leach, MW, and Rennick, D. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest*. 98: 1010-20.
207. Berg, DJ, Zhang, J, Weinstock, JV, Ismail, HF, Earle, KA, Alila, H, Pamukcu, R, Moore, S, and Lynch, RG. 2002. Rapid development of

colitis in NSAID-treated IL-10-deficient mice. *Gastroenterology*. 123: 1527-42.
208. Hale, LP, Gottfried, MR, and Swidsinski, A. 2005. Piroxicam treatment of IL-10-deficient mice enhances colonic epithelial apoptosis and mucosal exposure to intestinal bacteria. *Inflamm Bowel Dis*. 11: 1060-9.
209. Elliott, DE, Setiawan, T, Metwali, A, Blum, A, Urban, JF, Jr., and Weinstock, JV. 2004. *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. *Eur J Immunol*. 34: 2690-8.
210. Elliott, DE, Metwali, A, Leung, J, Setiawan, T, Blum, AM, Ince, MN, Bazzone, LE, Stadecker, MJ, Urban, JF, Jr., and Weinstock, JV. 2008. Colonization with *Heligmosomoides polygyrus* suppresses mucosal IL-17 production. *J Immunol*. 181: 2414-9.
211. Blum, AM, Metwali, A, Elliott, DE, Berg, DJ, and Weinstock, JV. 2004. CD4+ T cells from IL-10-deficient mice transfer susceptibility to NSAID-induced Rag colitis. *Am J Physiol Gastrointest Liver Physiol*. 287: G320-5.
212. Hang, L, Setiawan, T, Blum, AM, Urban, J, Stoyanoff, K, Arihiro, S, Reinecker, HC, and Weinstock, JV. 2010. *Heligmosomoides polygyrus* infection can inhibit colitis through direct interaction with innate immunity. *J Immunol*. 185: 3184-9.
213. Sutton, TL, Zhao, A, Madden, KB, Elfrey, JE, Tuft, BA, Sullivan, CA, Urban, JF, Jr., and Shea-Donohue, T. 2008. Anti-Inflammatory mechanisms of enteric *Heligmosomoides polygyrus* infection against trinitrobenzene sulfonic acid-induced colitis in a murine model. *Infect Immun*. 76: 4772-82.
214. Setiawan, T, Metwali, A, Blum, AM, Ince, MN, Urban, JF, Jr., Elliott, DE, and Weinstock, JV. 2007. *Heligmosomoides polygyrus* promotes regulatory T-cell cytokine production in the murine normal distal intestine. *Infect Immun*. 75: 4655-63.
215. Weng, M, Huntley, D, Huang, IF, Foye-Jackson, O, Wang, L, Sarkissian, A, Zhou, Q, Walker, WA, Cherayil, BJ, and Shi, HN. 2007. Alternatively activated macrophages in intestinal helminth infection: effects on concurrent bacterial colitis. *J Immunol*. 179: 4721-31.
216. Chen, CC, Louie, S, McCormick, BA, Walker, WA, and Shi, HN. 2006. Helminth-primed dendritic cells alter the host response to enteric bacterial infection. *J Immunol*. 176: 472-83.
217. Chen, CC, Louie, S, McCormick, B, Walker, WA, and Shi, HN. 2005. Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric *Citrobacter rodentium* and enhances *Citrobacter*-induced colitis in mice. *Infect Immun*. 73: 5468-81.
218. Walk, ST, Blum, AM, Ewing, SA, Weinstock, JV, and Young, VB. 2010. Alteration of the murine gut microbiota during infection with the parasitic helminth *Heligmosomoides polygyrus. Inflamm Bowel Dis*. 16: 1841-9.
219. Kim, HJ, Kim, YJ, Kang, MJ, Seo, JH, Kim, HY, Jeong, SK, Lee, SH, Kim, JM, and Hong, SJ. 2012. A novel mouse model of atopic dermatitis with epicutaneous allergen sensitization and the effect of *Lactobacillus rhamnosus. Exp Dermatol*. 21: 672-5.

220. Jang, SO, Kim, HJ, Kim, YJ, Kang, MJ, Kwon, JW, Seo, JH, Kim, HY, Kim, BJ, Yu, J, and Hong, SJ. 2012. Asthma Prevention by *Lactobacillus Rhamnosus* in a Mouse Model is Associated With CD4(+)CD25(+)Foxp3(+) T Cells*. Allergy Asthma Immunol Res*. 4: 150-6.
221. Shah, MM, Saio, M, Yamashita, H, Tanaka, H, Takami, T, Ezaki, T, and Inagaki, N. 2012. *Lactobacillus acidophilus* strain L-92 induces CD4(+)CD25(+)Foxp3(+) regulatory T cells and suppresses allergic contact dermatitis*. Biol Pharm Bull*. 35: 612-6.
222. Smits, HH, Engering, A, van der Kleij, D, de Jong, EC, Schipper, K, van Capel, TM, Zaat, BA, Yazdanbakhsh, M, Wierenga, EA, van Kooyk, Y, and Kapsenberg, ML. 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin*. J Allergy Clin Immunol*. 115: 1260-7.
223. Chang, J and Wescott, RB. 1972. Infectivity, fecundity, and survival of *Nematospiroides dubius* in gnotobiotic mice*. Exp Parasitol*. 32: 327-34.
224. Wescott, RB. 1968. Experimental *Nematospiroides dubius* infection in germfree and conventional mice*. Exp Parasitol*. 22: 245-9.
225. Weinstein, PP, Newton, WL, Sawyer, TK, and Sommerville, RI. 1969. *Nematospiroides dubius*: development and passage in the germfree mouse, and a comparative study of the free-living stages in germfree feces and conventional cultures*. Trans Am Microsc Soc*. 88: 95-117.
226. Hayes, KS, Bancroft, AJ, Goldrick, M, Portsmouth, C, Roberts, IS, and Grencis, RK. 2010. Exploitation of the intestinal microflora by the parasitic nematode *Trichuris muris. Science*. 328: 1391-4.
227. Rinttila, T, Kassinen, A, Malinen, E, Krogius, L, and Palva, A. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR*. J Appl Microbiol*. 97: 1166-77.
228. Dore, J, Sghir, A, Hannequart-Gramet, G, Corthier, G, and Pochart, P. 1998. Design and evaluation of a 16S rRNA-targeted oligonucleotide probe for specific detection and quantitation of human faecal Bacteroides populations*. Syst Appl Microbiol*. 21: 65-71.
229. Lane, DJ, ed. *rRNA sequencing*. Nucleic acid techniques in bacterial systematics, ed. E.G. Strackebrant, M. 1991, John Wiley & Sons: New York, NY. 115-175.
230. Franks, AH, Harmsen, HJ, Raangs, GC, Jansen, GJ, Schut, F, and Welling, GW. 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes*. Appl Environ Microbiol*. 64: 3336-45.
231. Robinson, M, Wahid, F, Behnke, JM, and Gilbert, FS. 1989. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): dose-dependent expulsion of adult worms*. Parasitology*. 98 (Pt 1): 115-24.
232. Behnke, JM and Robinson, M. 1985. Genetic control of immunity to *Nematospiroides dubius*: a 9-day anthelmintic abbreviated immunizing regime which separates weak and strong responder strains of mice*. Parasite Immunol*. 7: 235-53.

233. Behnke, JM and Wahid, FN. 1991. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): H-2 linked genes determine worm survival*. Parasitology*. 103: 157-64.
234. Prowse, SJ, Mitchell, GF, Ey, PL, and Jenkin, CR. 1979. The development of resistance in different inbred strains of mice to infection with *Nematospiroides dubius. Parasite Immunol*. 1: 277-88.
235. Cypess, RH and Zidian, JL. 1975. *Heligmosomoides polygyrus* (=*Nematospiroides dubius*): the development of self-cure and/or protection in several strains of mice*. J Parasitol*. 61: 819-24.
236. Enriquez, FJ, Zidian, JL, and Cypess, RH. 1988. *Nematospiroides dubius*: genetic control of immunity to infections of mice*. Exp Parasitol*. 67: 12-9.
237. Dobson, C and Owen, ME. 1978. Effect of host sex on passive immunity in mice infected with *Nematospiroides dubius. Int J Parasitol*. 8: 359-64.
238. Van Zandt, PD, Cypess, RH, and Zidian, JL. 1973. Development of age and sex resistance to *Nematospiroides dubius* in the mouse following single and multiple infections*. J Parasitol*. 59: 977-9.
239. Wahid, FN and Behnke, JM. 1993. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): parasite specific IgG1 antibody responses and primary response phenotype*. Parasite Immunol*. 15: 401-13.
240. Genser, B, Cooper, PJ, Yazdanbakhsh, M, Barreto, ML, and Rodrigues, LC. 2007. A guide to modern statistical analysis of immunological data*. BMC Immunol*. 8: 27.
241. Heinzel, FP, Sadick, MD, Holaday, BJ, Coffman, RL, and Locksley, RM. 1989. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets*. J Exp Med*. 169: 59-72.
242. Reynolds, LA and Maizels, RM. 2012. Cutting Edge: In the Absence of TGF-β Signaling in T Cells, Fewer CD103+ Regulatory T Cells Develop, but Exuberant IFN-γ Production Renders Mice More Susceptible to Helminth Infection*. J Immunol*. 189: 1113-7.
243. Smith, KA and Maizels, RM. 2012. IL-6 controls susceptibility to helminth infection by promoting natural regulatory T cell function *in vivo. Manuscript submitted*.
244. Lehmann, J, Huehn, J, de la Rosa, M, Maszyna, F, Kretschmer, U, Krenn, V, Brunner, M, Scheffold, A, and Hamann, A. 2002. Expression of the integrin αEβ7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells*. Proc Natl Acad Sci U S A*. 99: 13031-6.
245. Thornton, AM, Korty, PE, Tran, DQ, Wohlfert, EA, Murray, PE, Belkaid, Y, and Shevach, EM. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells*. J Immunol*. 184: 3433-41.
246. Behnke, JM, Lowe, A, Clifford, S, and Wakelin, D. 2003. Cellular and serological responses in resistant and susceptible mice exposed to repeated infection with *Heligmosomoides polygyrus bakeri. Parasite Immunol*. 25: 333-40.

247. Lawrence, CE and Pritchard, DI. 1994. Immune response profiles in responsive and non-responsive mouse strains infected with *Heligmosomoides polygyrus. Int J Parasitol*. 24: 487-94.
248. Wakelin, D and Donachie, AM. 1983. Genetic control of eosinophilia. Mouse strain variation in response to antigens of parasite origin*. Clin Exp Immunol*. 51: 239-46.
249. Ali, NM, Behnke, JM, and Manger, BR. 1985. The pattern of peripheral blood leucocyte changes in mice infected with *Nematospiroides dubius. J Helminthol*. 59: 83-93.
250. Ben-Smith, A, Wahid, FN, Lammas, DA, and Behnke, JM. 1999. The relationship between circulating and intestinal *Heligmosomoides polygyrus*-specific IgG1 and IgA and resistance to primary infection*. Parasite Immunol*. 21: 383-95.
251. Ricci, ND, Fiuza, JA, Bueno, LL, Cancado, GG, Gazzinelli-Guimaraes, PH, Martins, VG, Matoso, LF, de Miranda, RR, Geiger, SM, Correa-Oliveira, R, Gazzinelli, A, Bartholomeu, DC, and Fujiwara, RT. 2011. Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation*. PLoS Negl Trop Dis*. 5: e1383.
252. Blankenhaus, B, Klemm, U, Eschbach, ML, Sparwasser, T, Huehn, J, Kuhl, AA, Loddenkemper, C, Jacobs, T, and Breloer, M. 2011. *Strongyloides ratti* infection induces expansion of Foxp3+ regulatory T cells that interfere with immune response and parasite clearance in BALB/c mice*. J Immunol*. 186: 4295-305.
253. Taylor, MD, van der Werf, N, Harris, A, Graham, AL, Bain, O, Allen, JE, and Maizels, RM. 2009. Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection*. Eur J Immunol*. 39: 192-206.
254. Taylor, MD, LeGoff, L, Harris, A, Malone, E, Allen, JE, and Maizels, RM. 2005. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance *in vivo. J Immunol*. 174: 4924-33.
255. D'Elia, R, Behnke, JM, Bradley, JE, and Else, KJ. 2009. Regulatory T cells: a role in the control of helminth-driven intestinal pathology and worm survival*. J Immunol*. 182: 2340-8.
256. Zabransky, DJ, Nirschl, CJ, Durham, NM, Park, BV, Ceccato, CM, Bruno, TC, Tam, AJ, Getnet, D, and Drake, CG. 2012. Phenotypic and functional properties of Helios+ regulatory T cells*. PLoS One*. 7: e34547.
257. Gottschalk, RA, Corse, E, and Allison, JP. 2012. Expression of Helios in peripherally induced Foxp3+ regulatory T cells*. J Immunol*. 188: 976-80.
258. Akimova, T, Beier, UH, Wang, L, Levine, MH, and Hancock, WW. 2011. Helios expression is a marker of T cell activation and proliferation*. PLoS One*. 6: e24226.
259. Krinos, CM, Coyne, MJ, Weinacht, KG, Tzianabos, AO, Kasper, DL, and Comstock, LE. 2001. Extensive surface diversity of a commensal microorganism by multiple DNA inversions*. Nature*. 414: 555-8.

260. Surana, NK and Kasper, DL. 2012. The yin yang of bacterial polysaccharides: lessons learned from *B. fragilis* PSA. *Immunol Rev*. 245: 13-26.
261. Li, RW, Wu, S, Li, W, Navarro, K, Couch, RD, Hill, D, and Urban, JF, Jr. 2012. Alterations in the porcine colon microbiota induced by the gastrointestinal nematode *Trichuris suis. Infect Immun*.
262. Xu, D, Trajkovic, V, Hunter, D, Leung, BP, Schulz, K, Gracie, JA, McInnes, IB, and Liew, FY. 2000. IL-18 induces the differentiation of Th1 or Th2 cells depending upon cytokine milieu and genetic background. *Eur J Immunol*. 30: 3147-56.
263. Savage, DC and Dubos, R. 1968. Alterations in the mouse cecum and its flora produced by antibacterial drugs. *J Exp Med*. 128: 97-110.
264. Carlstedt-Duke, B, Midtvedt, T, Nord, CE, and Gustafsson, BE. 1986. Isolation and characterization of a mucin-degrading strain of *Peptostreptococcus* from rat intestinal tract. *Acta Pathol Microbiol Immunol Scand B*. 94: 293-300.
265. Wang, LT, Kuo, HP, Wu, YC, Tai, CJ, and Lee, FL. 2009. *Lactobacillus taiwanensis* sp. nov., isolated from silage. *Int J Syst Evol Microbiol*. 59: 2064-8.
266. Sarmiento-Rubiano, LA, Berger, B, Moine, D, Zuniga, M, Perez-Martinez, G, and Yebra, MJ. 2010. Characterization of a novel *Lactobacillus* species closely related to *Lactobacillus johnsonii* using a combination of molecular and comparative genomics methods. *BMC Genomics*. 11: 504.
267. JacksonLaboratory, *Biology of the Laboratory Mouse*. 2 ed, ed. E.L. Green. 1966, New York: Dover Publications.
268. Ichinohe, T, Pang, IK, Kumamoto, Y, Peaper, DR, Ho, JH, Murray, TS, and Iwasaki, A. 2011. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci U S A*. 108: 5354-9.
269. Carvalho, BM, Guadagnini, D, Tsukumo, DM, Schenka, AA, Latuf-Filho, P, Vassallo, J, Dias, JC, Kubota, LT, Carvalheira, JB, and Saad, MJ. 2012. Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice. *Diabetologia*. 55: 2823-34.
270. Reikvam, DH, Erofeev, A, Sandvik, A, Grcic, V, Jahnsen, FL, Gaustad, P, McCoy, KD, Macpherson, AJ, Meza-Zepeda, LA, and Johansen, FE. 2011. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PLoS One*. 6: e17996.
271. Ing, R, Su, Z, Scott, ME, and Koski, KG. 2000. Suppressed T helper 2 immunity and prolonged survival of a nematode parasite in protein-malnourished mice. *Proc Natl Acad Sci U S A*. 97: 7078-83.
272. Plant, L, Lam, C, Conway, PL, and O'Riordan, K. 2003. Gastrointestinal microbial community shifts observed following oral administration of a *Lactobacillus fermentum* strain to mice. *FEMS Microbiol Ecol*. 43: 133-40.
273. Lewis, JW and Mathers, PD. 1988. The microbial environment and intestinal nematode infections of *Heligmosomoides polygyrus* in laboratory mice. *Lab Anim*. 22: 109-16.
274. Adachi, O, Kawai, T, Takeda, K, Matsumoto, M, Tsutsui, H, Sakagami, M, Nakanishi, K, and Akira, S. 1998. Targeted disruption of the MyD88

gene results in loss of IL-1- and IL-18-mediated function. *Immunity*. 9: 143-50.
275. Muzio, M, Ni, J, Feng, P, and Dixit, VM. 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science*. 278: 1612-5.
276. Wesche, H, Henzel, WJ, Shillinglaw, W, Li, S, and Cao, Z. 1997. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*. 7: 837-47.
277. Brint, EK, Xu, D, Liu, H, Dunne, A, McKenzie, AN, O'Neill, LA, and Liew, FY. 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol*. 5: 373-9.
278. Hewitson, JP, Grainger, JR, and Maizels, RM. 2009. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Mol Biochem Parasitol*. 167: 1-11.
279. Aksoy, E, Zouain, CS, Vanhoutte, F, Fontaine, J, Pavelka, N, Thieblemont, N, Willems, F, Ricciardi-Castagnoli, P, Goldman, M, Capron, M, Ryffel, B, and Trottein, F. 2005. Double-stranded RNAs from the helminth parasite *Schistosoma* activate TLR3 in dendritic cells. *J Biol Chem*. 280: 277-83.
280. van der Kleij, D, Latz, E, Brouwers, JF, Kruize, YC, Schmitz, M, Kurt-Jones, EA, Espevik, T, de Jong, EC, Kapsenberg, ML, Golenbock, DT, Tielens, AG, and Yazdanbakhsh, M. 2002. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem*. 277: 48122-9.
281. Goodridge, HS, Marshall, FA, Else, KJ, Houston, KM, Egan, C, Al-Riyami, L, Liew, FY, Harnett, W, and Harnett, MM. 2005. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. *J Immunol*. 174: 284-93.
282. Thomas, PG, Carter, MR, Atochina, O, Da'Dara, AA, Piskorska, D, McGuire, E, and Harn, DA. 2003. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *J Immunol*. 171: 5837-41.
283. van Riet, E, Hartgers, FC, and Yazdanbakhsh, M. 2007. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology*. 212: 475-90.
284. Frantz, AL, Rogier, EW, Weber, CR, Shen, L, Cohen, DA, Fenton, LA, Bruno, ME, and Kaetzel, CS. 2012. Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides. *Mucosal Immunol*. 5: 501-12.
285. Mylonas, KJ, Hoeve, MA, Macdonald, AS, and Allen, JE. 2012. Alternative activation of macrophages by filarial nematodes is MyD88-independent. *Immunobiology*.
286. Helmby, H and Grencis, RK. 2003. Essential role for TLR4 and MyD88 in the development of chronic intestinal nematode infection. *Eur J Immunol*. 33: 2974-9.
287. Crellin, NK, Garcia, RV, Hadisfar, O, Allan, SE, Steiner, TS, and Levings, MK. 2005. Human CD4+ T cells express TLR5 and its ligand

flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J Immunol*. 175: 8051-9.
288. Liu, H, Komai-Koma, M, Xu, D, and Liew, FY. 2006. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci U S A*. 103: 7048-53.
289. Sutmuller, RP, den Brok, MH, Kramer, M, Bennink, EJ, Toonen, LW, Kullberg, BJ, Joosten, LA, Akira, S, Netea, MG, and Adema, GJ. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest*. 116: 485-94.
290. Caramalho, I, Lopes-Carvalho, T, Ostler, D, Zelenay, S, Haury, M, and Demengeot, J. 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med*. 197: 403-11.
291. Boulard, O, Asquith, MJ, Powrie, F, and Maloy, KJ. 2010. TLR2-independent induction and regulation of chronic intestinal inflammation. *Eur J Immunol*. 40: 516-24.
292. Netea, MG, Sutmuller, R, Hermann, C, Van der Graaf, CA, Van der Meer, JW, van Krieken, JH, Hartung, T, Adema, G, and Kullberg, BJ. 2004. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol*. 172: 3712-8.
293. Grainger, JR, Hall, JA, Bouladoux, N, Oldenhove, G, and Belkaid, Y. 2010. Microbe-dendritic cell dialog controls regulatory T-cell fate. *Immunol Rev*. 234: 305-16.
294. Coccia, M, Harrison, OJ, Schiering, C, Asquith, MJ, Becher, B, Powrie, F, and Maloy, KJ. 2012. IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *J Exp Med*. 209: 1595-609.
295. Helmby, H and Grencis, RK. 2004. Interleukin 1 plays a major role in the development of Th2-mediated immunity. *Eur J Immunol*. 34: 3674-81.
296. Humphreys, NE and Grencis, RK. 2009. IL-1-dependent, IL-1R1-independent resistance to gastrointestinal nematodes. *Eur J Immunol*. 39: 1036-45.
297. Nakamura, K, Okamura, H, Wada, M, Nagata, K, and Tamura, T. 1989. Endotoxin-induced serum factor that stimulates gamma interferon production. *Infect Immun*. 57: 590-5.
298. Fantuzzi, G, Puren, AJ, Harding, MW, Livingston, DJ, and Dinarello, CA. 1998. Interleukin-18 regulation of interferon gamma production and cell proliferation as shown in interleukin-1beta-converting enzyme (caspase-1)-deficient mice. *Blood*. 91: 2118-25.
299. Dinarello, CA, Novick, D, Puren, AJ, Fantuzzi, G, Shapiro, L, Muhl, H, Yoon, DY, Reznikov, LL, Kim, SH, and Rubinstein, M. 1998. Overview of interleukin-18: more than an interferon-gamma inducing factor. *J Leukoc Biol*. 63: 658-64.
300. Nakanishi, K, Yoshimoto, T, Tsutsui, H, and Okamura, H. 2001. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev*. 12: 53-72.

301. Lingel, A, Weiss, TM, Niebuhr, M, Pan, B, Appleton, BA, Wiesmann, C, Bazan, JF, and Fairbrother, WJ. 2009. Structure of IL-33 and its interaction with the ST2 and IL-1RAcP receptors--insight into heterotrimeric IL-1 signaling complexes. *Structure*. 17: 1398-410.
302. Schmitz, J, Owyang, A, Oldham, E, Song, Y, Murphy, E, McClanahan, TK, Zurawski, G, Moshrefi, M, Qin, J, Li, X, Gorman, DM, Bazan, JF, and Kastelein, RA. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 23: 479-90.
303. Xu, D, Chan, WL, Leung, BP, Huang, F, Wheeler, R, Piedrafita, D, Robinson, JH, and Liew, FY. 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med*. 187: 787-94.
304. Townsend, MJ, Fallon, PG, Matthews, DJ, Jolin, HE, and McKenzie, AN. 2000. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med*. 191: 1069-76.
305. Humphreys, NE, Xu, D, Hepworth, MR, Liew, FY, and Grencis, RK. 2008. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol*. 180: 2443-9.
306. Yamamoto, M, Sato, S, Mori, K, Hoshino, K, Takeuchi, O, Takeda, K, and Akira, S. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J Immunol*. 169: 6668-72.
307. Pestka, S, Krause, CD, and Walter, MR. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev*. 202: 8-32.
308. Mayer-Barber, KD, Andrade, BB, Barber, DL, Hieny, S, Feng, CG, Caspar, P, Oland, S, Gordon, S, and Sher, A. 2011. Innate and adaptive interferons suppress IL-1α and IL-1β production by distinct pulmonary myeloid subsets during *Mycobacterium tuberculosis* infection. *Immunity*. 35: 1023-34.
309. Novikov, A, Cardone, M, Thompson, R, Shenderov, K, Kirschman, KD, Mayer-Barber, KD, Myers, TG, Rabin, RL, Trinchieri, G, Sher, A, and Feng, CG. 2011. *Mycobacterium tuberculosis* triggers host type I IFN signaling to regulate IL-1β production in human macrophages. *J Immunol*. 187: 2540-7.
310. Born, TL, Thomassen, E, Bird, TA, and Sims, JE. 1998. Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. *J Biol Chem*. 273: 29445-50.
311. Wesche, H, Korherr, C, Kracht, M, Falk, W, Resch, K, and Martin, MU. 1997. The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). *J Biol Chem*. 272: 7727-31.
312. Else, KJ, Finkelman, FD, Maliszewski, CR, and Grencis, RK. 1994. Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med*. 179: 347-51.
313. Li, MO, Wan, YY, Sanjabi, S, Robertson, AK, and Flavell, RA. 2006. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol*. 24: 99-146.

314. Li, MO and Flavell, RA. 2008. TGF-beta: a master of all T cell trades. *Cell*. 134: 392-404.
315. Ince, MN, Elliott, DE, Setiawan, T, Metwali, A, Blum, A, Chen, HL, Urban, JF, Flavell, RA, and Weinstock, JV. 2009. Role of T cell TGF-β signaling in intestinal cytokine responses and helminthic immune modulation. *Eur J Immunol*. 39: 1870-1878.
316. Veldhoen, M, Uyttenhove, C, van Snick, J, Helmby, H, Westendorf, A, Buer, J, Martin, B, Wilhelm, C, and Stockinger, B. 2008. Transforming growth factor-β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. 9: 1341-6.
317. Schmitt, E, Germann, T, Goedert, S, Hoehn, P, Huels, C, Koelsch, S, Kuhn, R, Muller, W, Palm, N, and Rude, E. 1994. IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J Immunol*. 153: 3989-96.
318. Korn, T, Bettelli, E, Oukka, M, and Kuchroo, VK. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol*. 27: 485-517.
319. Townsend, JM, Fallon, GP, Matthews, JD, Smith, P, Jolin, EH, and McKenzie, NA. 2000. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity*. 13: 573-83.
320. Behnke, JM, Wahid, FN, Grencis, RK, Else, KJ, Ben-Smith, AW, and Goyal, PK. 1993. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms. *Parasite Immunology*. 15: 415-421.
321. Li, MO and Flavell, RA. 2008. TGF-β: a master of all T cell trades. *Cell*. 134: 392-404.
322. Gorelik, L and Flavell, RA. 2000. Abrogation of TGFβ signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity*. 12: 171-81.
323. Gorelik, L, Constant, S, and Flavell, RA. 2002. Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J Exp Med*. 195: 1499-505.
324. Ince, MN, Elliott, DE, Setiawan, T, Metwali, A, Blum, A, Chen, HL, Urban, JF, Flavell, RA, and Weinstock, JV. 2009. Role of T cell TGF-β signaling in intestinal cytokine responses and helminthic immune modulation. *Eur J Immunol*. 39: 1870-8.
325. Taylor, MD, Harris, A, Babayan, SA, Bain, O, Culshaw, A, Allen, JE, and Maizels, RM. 2007. CTLA-4 and CD4+ CD25+ regulatory T cells inhibit protective immunity to filarial parasites *in vivo. J Immunol*. 179: 4626-34.
326. Huehn, J, Siegmund, K, Lehmann, JC, Siewert, C, Haubold, U, Feuerer, M, Debes, GF, Lauber, J, Frey, O, Przybylski, GK, Niesner, U, de la Rosa, M, Schmidt, CA, Brauer, R, Buer, J, Scheffold, A, and Hamann, A. 2004. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med*. 199: 303-13.

327. Veldhoen, M, Uyttenhove, C, van Snick, J, Helmby, H, Westendorf, A, Buer, J, Martin, B, Wilhelm, C, and Stockinger, B. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. 9: 1341-6.
328. Robinson, PW, Green, SJ, Carter, C, Coadwell, J, and Kilshaw, PJ. 2001. Studies on transcriptional regulation of the mucosal T-cell integrin alphaEbeta7 (CD103). *Immunology*. 103: 146-54.
329. El-Asady, R, Yuan, R, Liu, K, Wang, D, Gress, RE, Lucas, PJ, Drachenberg, CB, and Hadley, GA. 2005. TGF-β-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J Exp Med*. 201: 1647-57.
330. Josefowicz, SZ, Niec, RE, Kim, HY, Treuting, P, Chinen, T, Zheng, Y, Umetsu, DT, and Rudensky, AY. 2012. Extrathymically generated regulatory T cells control mucosal Th2 inflammation. *Nature*. 482: 395-9.
331. Beck, PL, Rosenberg, IM, Xavier, RJ, Koh, T, Wong, JF, and Podolsky, DK. 2003. Transforming growth factor-beta mediates intestinal healing and susceptibility to injury *in vitro* and *in vivo* through epithelial cells. *Am J Pathol*. 162: 597-608.
332. Rook, GA. 2009. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology*. 126: 3-11.
333. Reynolds, LA, Filbey, KJ, and Maizels, RM. 2012. Immunity to the model intestinal helminth parasite *Heligmosomoides polygyrus. Semin Immunopathol*. 34: 829-846.
334. Lee, YK and Mazmanian, SK. 2011. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*. 330: 1768-73.
335. Gulati, AS, Shanahan, MT, Arthur, JC, Grossniklaus, E, von Furstenberg, RJ, Kreuk, L, Henning, SJ, Jobin, C, and Sartor, RB. 2012. Mouse background strain profoundly influences Paneth cell function and intestinal microbial composition. *PLoS One*. 7: e32403.
336. Filbey, KJ, Hewitson, JP, Ketalaars, E, Harcus, Y, Reynolds, LA, Smith, KA, and Maizels, RM. 2012. Macrophage migration inhibitory factor (MIF) is required for effective type 2 innate immunity to intestinal helminths. *Manuscript in preparation*.
337. Lawrence, CE. 2003. Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunol*. 25: 271-81.
338. Wlodarska, M and Finlay, BB. 2010. Host immune response to antibiotic perturbation of the microbiota. *Mucosal Immunol*. 3: 100-3.
339. Mittal, R, Bulgheresi, S, Emami, C, and Prasadarao, NV. 2009. *Enterobacter sakazakii* targets DC-SIGN to induce immunosuppressive responses in dendritic cells by modulating MAPKs. *J Immunol*. 183: 6588-99.
340. Schoppet, M, Huppertz, HI, Simm, A, and Bubert, A. 2000. Infection of dendritic cells by enterobacteriaceae. *Med Microbiol Immunol*. 188: 191-6.

341. Petrosino, JF, Highlander, S, Luna, RA, Gibbs, RA, and Versalovic, J. 2009. Metagenomic pyrosequencing and microbial identification. *Clin Chem*. 55: 856-66.
342. Ottman, N, Smidt, H, de Vos, WM, and Belzer, C. 2012. The function of our microbiota: who is out there and what do they do? *Front Cell Infect Microbiol*. 2: 104.
343. Lupp, C, Robertson, ML, Wickham, ME, Sekirov, I, Champion, OL, Gaynor, EC, and Finlay, BB. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe*. 2: 204.

Chapter 9. Appendix

9.1 Parameters correlating with day 28 worm burden in BALB/c and C57BL/6 mice

Table 9.1 Parameters correlating with day 28 worm burden in BALB/c and C57BL/6 mice

All parameters measured which had correlations with day 28 worm burden with a p value of less than 0.1 are presented.

Data was first assessed for normality. If both parameters were normally distributed, Pearson r tests were used; if either parameter was non parametrically distributed, Spearman r tests were used. Data for each genotype were analysed separately.

BALB/c worm burden (d28) vs:	Test	R value	P value	Significance
Serum IL-5 (d28)	Spearman r	0.5182	0.0006	***
MLN cell HES-recall IL-17A	Spearman r	0.4692	0.0026	**
ICCS %IFN-γ^+ of $CD4^+$ MLN cells (d28)	Spearman r	0.4221	0.0074	**
% $Foxp3^+Helios^+$ of $CD4^+$ MLN cells (d28)	Spearman r	0.4189	0.0099	**
% $Foxp3^+$ of $CD4^+$ MLN cells (d28)	Spearman r	0.4175	0.0101	*
Serum IL-5 (d14)	Spearman r	-0.3982	0.0133	*
ICCS %IFN-γ^+ of $CD8^+$ MLN cells (d28)	Spearman r	0.3699	0.0205	*
MLN cell HES-recall IFN-γ	Spearman r	0.3693	0.0207	*
ICCS %IL-4^+ of $CD4^+$ MLN cells (d28)	Spearman r	0.3654	0.0222	*
Faeces Enterobacteriaceae (d0)	Spearman r	-0.4087	0.0277	*
Duodenum *Lactobacillus/coccus* (d28)	Spearman r	0.3471	0.0282	*
Duodenum *L. taiwanensis* (d28)	Spearman r	0.3351	0.0345	*
Granulomas (d28)	Spearman r	-0.3257	0.0403	*
Serum IL-4 (d7)	Spearman r	0.3161	0.0604	
% $Foxp3^+Helios^-$ of $CD4^+$ MLN cells (d28)	Spearman r	0.2931	0.0784	
Serum IL-6 (d28)	Spearman r	0.2781	0.0823	
ICCS %IL-13^+ of $CD4^+$ MLN cells (d28)	Spearman r	0.2796	0.0847	
ICCS %IL-10^+ of $CD4^+$ MLN cells (d28)	Spearman r	0.2732	0.0925	
Serum IL-17A (d14)	Spearman r	0.2753	0.0943	

C57BL/6 worm burden (d28) vs:	Test	R value	P value	Significance
ICCS %IFN-γ$^+$ of CD8$^+$ MLN cells (d28)	Pearson r	0.6154	<0.0001	***
ICCS %IL-4$^+$ of CD4$^+$ MLN cells (d28)	Pearson r	0.5602	0.0002	***
ICCS %IL-13$^+$ of CD4$^+$ MLN cells (d28)	Spearman r	0.5246	0.0006	***
Serum IFN-γ (d7)	Spearman r	0.4885	0.0016	**
Duo Enterobacteriaceae (d28)	Spearman r	0.4663	0.0042	**
ICCS %IL-10$^+$ of CD4$^+$ MLN cells (d28)	Spearman r	0.3956	0.0127	*
ICCS %IFN-γ$^+$ of CD4$^+$ MLN cells (d28)	Pearson r	0.3098	0.0549	*
ICCS % IL-17A$^+$ of CD4$^+$ MLN cells (d28)	Pearson r	0.2815	0.0826	

9.2 In the absence of TGF-β signaling in T cells, fewer CD103^{+} regulatory T cells develop, but exuberant IFN-γ production renders mice more susceptible to helminth infection

Reynolds LA, Maizels RM.

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Cutting Edge

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Cutting Edge: In the Absence of TGF-β Signaling in T Cells, Fewer CD103$^+$ Regulatory T Cells Develop, but Exuberant IFN-γ Production Renders Mice More Susceptible to Helminth Infection

Lisa A. Reynolds and Rick M. Maizels

Multiple factors control susceptibility of C57BL/6 mice to infection with the helminth *Heligmosomoides polygyrus*, including TGF-β signaling, which inhibits immunity in vivo. However, mice expressing a T cell-specific dominant-negative TGF-β receptor II (TGF-βRII DN) show dampened Th2 immunity and diminished resistance to infection. Interestingly, *H. polygyrus*-infected TGF-βRII DN mice show greater frequencies of CD4$^+$Foxp3$^+$Helios$^+$ Tregs than infected wild-type mice, but levels of CD103 are greatly reduced on both these cells and on the CD4$^+$Foxp3$^+$Helios$^-$ population. Although Th9 and Th17 levels are comparable between infected TGF-βRII DN and wild-type mice, the former develop exaggerated CD4$^+$ and CD8$^+$ T cell IFN-γ responses. Increased susceptibility conferred by TGF-βRII DN expression was lost in IFN-γ–deficient mice, although they remained unable to completely clear infection. Hence, overexpression of IFN-γ negatively modulates immunity, and the presence of Helios$^+$ Tregs may maintain susceptibility on the C57BL/6 background. *The Journal of Immunology*, **2012, 189: 1113–1117.**

Immunity to gastrointestinal helminth infection is mediated by Th2-dependent mechanisms (1, 2), which are impaired by regulatory T cells (Tregs) (3) and cross-regulated by conventional IFN-γ Th1 effector populations (4). In the case of the murine nematode parasite *Heligmosomoides polygyrus*, immunity is boosted by interference with TGF-β signaling associated with the induction and activation of Foxp3$^+$ Tregs (3), a well-established property of this cytokine (5). *H. polygyrus* is a broadly immunomodulatory parasite that can alleviate colitis in the absence of IL-10 (6) but not when T cell responsiveness to TGF-β is abrogated (7). TGF-β also participates in generating IL-9– (8) and IL-17–producing (9) Th subsets in the presence of IL-4 and IL-6, respectively, although in the setting of *H. polygyrus* infection, both IL-9–dependent mast cell responses (10) and Th17 cells (11) have previously been reported to be blocked.

Because TGF-β signaling was found to promote Tregs and prolong *H. polygyrus* infection, it was surprising that mice whose T cells expressed a dominant-negative TGF-β receptor II (TGF-βRII DN) were not more resistant to this parasite (7). Our laboratory not only confirmed this phenotype but also established that TGF-βRII DN mice are in fact more susceptible to infection than wild-type animals. However, TGF-β can actively downregulate multiple cell types (5, 12), and among the conspicuous phenotypes of TGF-βRII DN mice is potentiation of IFN-γ and Th1 responses (7, 13). We therefore investigated, using mice lacking IFN-γ, whether ablation of TGF-β signaling increased susceptibility because of uninhibited Th1 cytokine release.

Our results show that when IFN-γ stimulation is abrogated in the absence of TGF-β signaling in T cells, the increased fecundity of *H. polygyrus* within the host is lost. Infected TGF-βRII DN and wild-type mice show equivalent differentiation of IL-9– or IL-17–producing CD4$^+$ T cells and display no differences in mast cell expansion following infection. Our data therefore support the hypothesis that the increased susceptibility of TGF-βRII DN mice to *H. polygyrus* is due to elevated IFN-γ release and not to a loss of Th9 or Th17 effector responses.

In addition, TGF-β plays a central role in the induction and maintenance of Tregs, particularly in the periphery (5, 14). Because of the importance of Tregs in modulating responses to pathogens in general (15), and *H. polygyrus* in particular (3), we also investigated the balance of Treg frequencies and subsets in the presence or absence of TGF-β signaling and the consequent outcome of infection. These studies show that within the Foxp3$^+$ Treg population of TGF-βRII DN mice, CD103 expression is low on both Helios$^+$ and Helios$^-$ cells, but a compensatory increase in Helios$^+$ Tregs may account for the continuing susceptibility of mice expressing the mutated receptor.

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Abbreviations used in this article: MLNC, mesenteric lymph node cell; TGF-βRII DN, dominant-negative TGF-β receptor II; Treg, regulatory T cell.

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Materials and Methods

Animals and parasites

C57BL/6, TGF-βRII DN [T cell-specific TGF-βRII DN (16)], IFN-$\gamma^{-/-}$, and doubly transgenic mice were housed in individually ventilated cages. Both transgenic lines were on a C57BL/6 background. Mice were infected by oral gavage with 200 *H. polygyrus bakeri* third-stage larvae, obtained from fecal cultures (3); 14 and 28 d later, small intestinal adult worms and fecal pellet eggs were enumerated.

Restimulation, flow cytometry, and cytokine measurements

Mesenteric lymph node cells (MLNC) were stained directly ex vivo (for Foxp3 and Helios measurements) or restimulated with 0.5 μg/ml PMA and 1 μg/ml ionomycin for 3.5 h, with 10 μg/ml brefeldin A included for the final 2.5 h (for intracellular cytokine measurements). Cells were stained with Abs to surface CD4 (RM4-5; BD), CD8α (53-6.7; BioLegend), TCR-β (H57-597; eBioscience), and CD103 conjugated to biotin (M290; BD Pharmingen), followed by PerCP–streptavidin (BD). Cells were fixed according to the manufacturer's instructions with Cytofix/Cytoperm (BD) or Fix/Perm (for Foxp3 and Helios staining; eBioscience) and then stained with Abs to intracellular IFN-γ (XMG1.2; BioLegend), IL-9 (RM9A4; BioLegend), IL-13 (eBio 13A; eBioscience), IL-17A (TC11-18H10.1; BioLegend), Foxp3 (FJK-16s; eBioscience), and Helios (22F6; BioLegend). Cells were analyzed using FACSCanto or LSRII flow cytometers (BD) and FlowJo software (Tree Star). Serum cytokines were assayed by CBA flex set (BD) with a minimum detection limit of 2.5 pg/ml.

Histology

Transverse sections of jejunum were fixed in 4% formaldehyde and stained with H&E and toluidine blue. Mast cell counts per micrometer of villus crypt were recorded.

Statistical analysis

Statistical tests were applied according to data normality and group numbers. Normally distributed two-way comparisons used unpaired *t* tests, and multiple comparisons used one-way ANOVA, followed by Tukey's test. If normality was not achieved, Mann–Whitney (for two-way comparisons) and Kruskal–Wallis tests (for multiple comparisons, followed by Dunn's test) were used. Data from multiple experiments were pooled only where no statistical differences existed between separate data sets.

Results and Discussion

The C57BL/6 mouse strain has a high level of susceptibility to the gastrointestinal helminth *H. polygyrus* (17), but immunity can be enhanced by pharmaceutical inhibition of TGF-β signaling (3). Because TGF-βRII DN mice have deficient TGF-β signaling in T cells, they may be expected to be more resistant to *H. polygyrus* than their wild-type littermates and may lack inducible Tregs to inhibit effector responses against the worm. Surprisingly, however, *H. polygyrus* shows heightened fecundity in TGF-βRII DN mice (Fig. 1A), and mice have similar adult worm burdens to wild-type mice (Fig. 1B), consistent with an earlier report (7). Furthermore, TGF-βRII DN mice show diminished Th2 cytokine responses, failing to generate a significant population of IL-13$^+$CD4$^+$ T cells in the MLNC (Fig. 1C). Although a degree of IL-4 responsiveness was maintained in TGF-βRII DN mice (data not shown), we also found that serum IL-5 responses to infection were absent in all but one gene-targeted animal (Fig. 1D). The IL-5 serum response at day 7 of infection in wild-type mice was transient and had returned to naive levels by day 14 of infection (data not shown). No IL-4 or IL-13 was detectable in the sera of either mouse strain. Reduced IL-10 production measured by Ag-specific recall responses to *H. polygyrus* excretory–secretory (HES) Ags in vitro was also found in TGF-βRII DN mice (data not shown), consistent with its role in promoting Th2 responsiveness in gastrointestinal helminth infections (2) and with a report that IL-10 release by lamina propria cells is

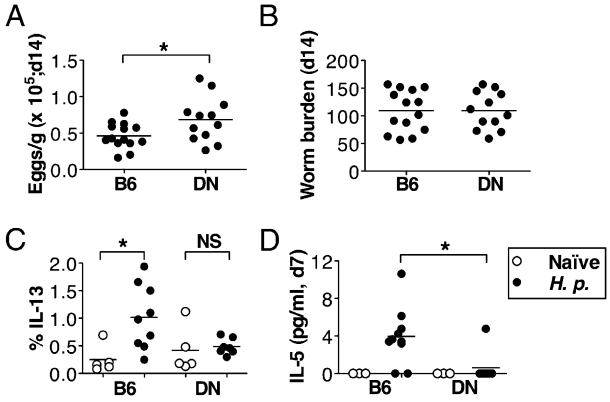

FIGURE 1. Ablation of TGF-β signaling in T cells does not confer resistance in mice to *H. polygyrus* infection. (**A**) Fecal *H. polygyrus* egg counts after 14 d of infection in C57BL/6 and TGF-βRII DN mice. Data are pooled from three experiments, with mice aged 7–18 wk. Mice were age matched between groups, and no age effect on *H. polygyrus* susceptibility was seen. (**B**) Adult *H. polygyrus* counts from the same experiments and time point as (A). (**C**) Percentage of IL-13$^+$ cells of CD4$^+$ lymphocytes in MLNC from naive (○) and 14-d postinfected (●) C57BL/6 and TGF-βRII DN mice. MLNC were stimulated with PMA/ionomycin and stained for flow cytometry. Data are pooled from two experiments with 6- to 9-wk-old mice. (**D**) Levels of serum IL-5 in naive (○) and 7-d postinfected (●) mice. Data are pooled from two experiments; naive mice were examined in one of these experiments. Mice were 7–12 wk old and age matched between groups. (A) and (B) were analyzed by unpaired *t* test, and (C) and (D) were analyzed by Kruskal–Wallis test. B6, C57BL/6; DN, TGF-βRII DN; *H. p.*, *H. polygyrus*-infected. $^*p < 0.05$.

inhibited in *H. polygyrus*-infected TGF-βRII DN mice (7). Hence, T cell-specific ablation of TGF-β signaling does not recapitulate the effects of global pharmaceutical inhibition (3), and the phenotype of the TGF-βRII DN mice does not equate to the Th2-boosting effects of broader interference with Treg function in nematode infection (18–20).

Because TGF-β signaling promotes Treg differentiation, particularly in the periphery, we next examined Treg fre-

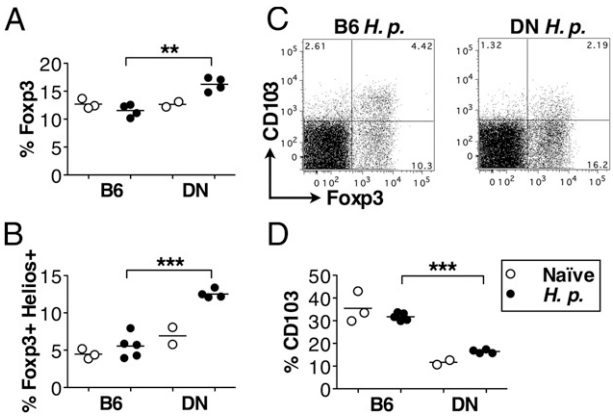

FIGURE 2. TGF-βRII DN mice have more Tregs but fewer CD103$^+$ Tregs than C57BL/6 animals during *H. polygyrus* infection. C57BL/6 and TGF-βRII DN mice were left naive (○) or *H. polygyrus*-infected for 14 d (●), and MLNC were isolated and stained directly ex vivo. Data are representative of the results from two experiments each with two to five mice per group. Mice were 7–9 wk old. (**A**) Percentage of Foxp3$^+$ cells among CD4$^+$ lymphocytes. (**B**) Percentage of Foxp3$^+$Helios$^+$ cells among CD4$^+$ lymphocytes. (**C**) Representative CD103 and Foxp3 staining among CD4$^+$ lymphocytes from 14-d *H. polygyrus*-infected C57BL/6 and TGF-βRII DN mice. (**D**) Percentage of CD103 among CD4$^+$Foxp3$^+$ lymphocytes. *H. polygyrus*-infected groups in (A), (B), and (D) were analyzed by unpaired *t* test. Abbreviations used as in Fig. 1. $^{**}p < 0.01$, $^{***}p < 0.001$.

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quencies in *H. polygyrus*-infected TGF-βRII DN mice. Surprisingly, we found a significantly higher proportion of $CD4^+Foxp3^+$ T cells in MLNC of infected TGF-βRII DN mice compared with wild-type C57BL/6 animals (Fig. 2A). The increased frequency of $Foxp3^+$ cells was accounted for by a greater proportion of $CD4^+$ cells expressing the transcription factor Helios (Fig. 2B), which is associated with thymic or natural Tregs (21), whereas the frequencies of $Foxp3^+Helios^-$ T cells (considered to be peripherally induced Tregs, known to be more dependent on TGF-β signaling) were not significantly different between the two genotypes (data not shown). Although the Treg compartment was not thus numerically diminished in TGF-βRII DN mice, their expression of CD103 [an activation/memory marker known to be inducible by TGF-β (22)] was substantially reduced (Fig. 2C, 2D), with low levels in both $Helios^+$ and $Helios^-$ subsets (data not shown).

We next addressed the question of whether a loss of TGF-β signaling impacts on other effector functions in the immune response. Because TGF-β signaling promotes differentiation of Th17 cells in the presence of IL-6 (9), and Th9 in the presence of IL-4 (8), we investigated the generation of these cell types following infection. Few Th17 cells identified by intracellular IL-17A staining develop in the MLNC in either genotype (Fig. 3A), suggesting that the conditions for optimal Th17 expansion are not generated at this site during *H. polygyrus* infection.

The frequency of $CD4^+$ T cells producing IL-9 was, however, altered in TGF-βRII DN mice, with a significantly greater, rather than lower, proportion of IL-9^+ T cells compared with wild-type mice (Fig. 3B). IL-9 is important for mast cell survival and proliferation (23), and mast cells have been suggested as an effector population for *H. polygyrus* expulsion (10, 24, 25). We therefore quantified the extent of jejunal mast cells but found their numbers increased significantly and equivalently after *H. polygyrus* infection in both C57BL/6 and TGF-βRII DN mice (Fig. 3C).

By day 14 postinfection, effector responses in wild-type mice are predominantly Th2 type (26). TGF-βRII DN mice, however, display strong Th1 IFN-γ production (Fig. 3). Notably, a high proportion of splenic $CD4^+$ and $CD8^+$ T cells develop into IFN-γ–producing cells in vitro (16), and constitutive IFN-γ levels in naive animals are markedly elevated (7). Infection is hence initiated in an environment intrinsically unfavorable to Th2. Following *H. polygyrus*-infection, this trend is exacerbated with TGF-βRII DN mice

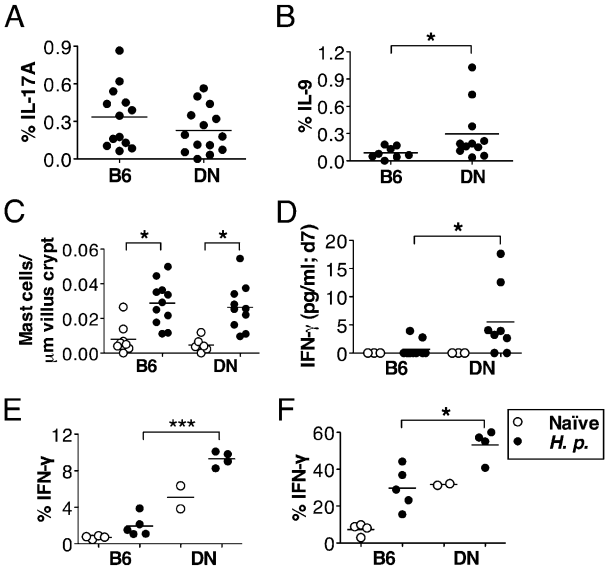

FIGURE 3. TGF-βRII DN mice have exaggerated IFN-γ responses yet Th17, Th9, and mast cell responses are not compromised. (**A**, **B**, **E**, **F**) MLNC were isolated from 14-d *H. polygyrus*-infected C57BL/6 and TGF-βRII DN mice, stimulated with PMA/ionomycin, and stained for flow cytometry. (A and B) Data are pooled from three experiments, with 6- to 18-wk-old mice age matched between groups. Percentage of IL-$17A^+$ cells (A) or percentage of IL-9^+ cells (B) of $CD4^+$ lymphocytes. (**C**) Jejunums were sectioned and stained for mast cells with toluidine blue. The number of mast cells per micrometer of villus crypt is shown in naive (○) and 14-d *H. polygyrus* infected (●) mice. Data shown are from 7- to 9-wk-old mice and are representative of two experiments each with four to five mice per group for infected mice; naive mice were included in one of these experiments. (**D**) Levels of circulating IFN-γ in the sera of naive (○) or 7-d *H. polygyrus*-infected C57BL/6 and TGF-βRII DN mice (●). Data are pooled from two experiments with 7- to 12-wk-old mice. (E and F) Percentage of IFN-γ^+ cells among $CD4^+$ (E) or $CD8\alpha^+$ (F) TCR-β^+ lymphocytes. Data shown are from 7- to 9-wk-old mice and are representative of four experiments each with two to five mice per group. (A) was analyzed by unpaired *t* test; (B) and (D) by Mann–Whitney; (C) by Kruskal–Wallis; and (E) and (F) by unpaired *t* test between *H. polygyrus*-infected groups. Abbreviations used as in Fig. 1. $^*p < 0.05$, $^{***}p < 0.001$.

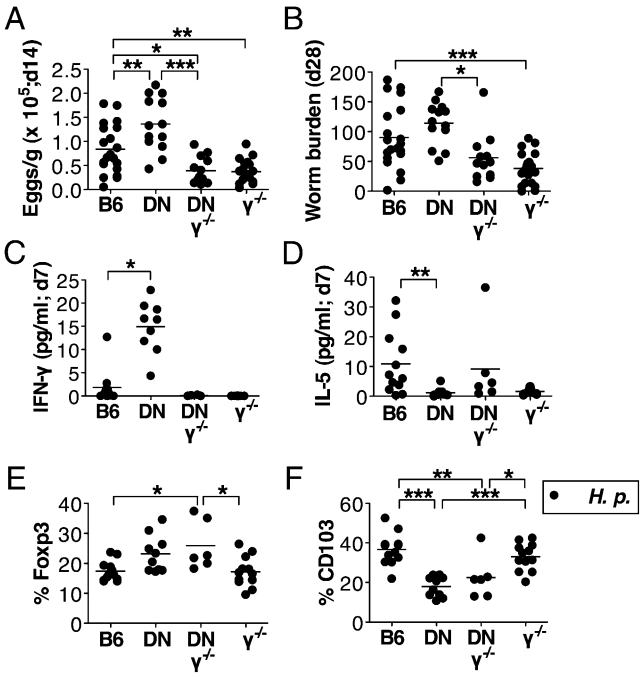

FIGURE 4. Increased susceptibility of TGF-βRII DN mice is reversed in the absence of IFN-γ. (**A**) Fecal egg counts after 14 d of infection in C57BL/6, TGF-βRII DN, TGF-βRII DN IFN-$\gamma^{-/-}$, and IFN-$\gamma^{-/-}$ mice. Data are pooled from three experiments with 6- to 14-wk-old mice age matched between groups. (**B**) Adult worm counts from the same experiments after 28 d of infection. (**C** and **D**) Levels of circulating IFN-γ (C) and IL-5 (D) in the same experiments after 7 d of infection. Data are pooled from two experiments with 7- to 14-wk-old mice. (**E**) Percentage of $Foxp3^+$ T cells among total $CD4^+$ lymphocytes. After 28 d of infection, MLNC were stained directly ex vivo. Data are pooled from two experiments with 7- to 14-wk-old mice. (**F**) Percentage of CD103 among $Foxp3^+CD4^+$ lymphocytes, in the same experiments as (E). (A), (B), (E), and (F) were analyzed by ANOVA; (C) and (D) by Kruskal–Wallis test. B6, C57BL/6; DN, TGF-βRII DN; DN$\gamma^{-/-}$, TGF-βRII DN IFN-$\gamma^{-/-}$; $\gamma^{-/-}$, IFN-$\gamma^{-/-}$; *H. p.*, *H. polygyrus* infected. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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showing elevated serum levels of IFN-γ compared with C57BL/6 mice (Fig. 3D), with an ~5-fold higher frequency of IFN-γ production among $CD4^+$ T cells in the MLNC (Fig. 3E) and a parallel increase in $CD8^+$ IFN-γ^+ T cells in the same mice (Fig. 3F).

To investigate whether the substantial IFN-γ in TGF-βRII DN mice inhibits Th2 cytokines required to control *H. polygyrus*, we bred double-transgenic mice with the TGF-βRII DN mutation together with the IFN-$\gamma^{-/-}$ genotype on the C57BL/6 background (TGF-βRII DN IFN-$\gamma^{-/-}$ mice).

After 14 d of infection, TGF-βRII DN mice lacking IFN-γ had a lower fecal egg burden than IFN-γ–sufficient TGF-βRII DN mice (Fig. 4A) and, by day 28, lower worm counts (Fig. 4B). However, the double-transgenic mice were not able to fully clear infection. Thus, although overexpression of IFN-γ is responsible for the heightened susceptibility of TGF-βRII DN mice, IFN-γ itself is not solely responsible for the failure of mice to expel the parasite. In this manner, control of *H. polygyrus* appears to be more complex than *Trichus muris*, in which neutralization of IFN-γ is sufficient to convert a susceptible genotype to a resistant phenotype (4). Hence, the reported greater susceptibility of TGF-βRII DN mice to *T. muris* may be due to high intrinsic IFN-γ in this model rather than lack of Th9-driven mast cell responses (8).

Serum cytokine analysis confirmed the absence of IFN-γ in gene-targeted mice (Fig. 4C) and showed that serum IL-5 levels were restored partially (Fig. 4D). However, intracellular staining of MLNC showed broadly similar levels of Th2 cytokine production by day 28 of infection in C57BL/6 and double-transgenic genotypes (data not shown), indicating that the suppression of Th2 responses is only partly relieved in the absence of both IFN-γ and TGF-β signaling in T cells. HES-specific IgG1 responses were equivalent in all strains at day 28 of infection.

Analysis of Treg populations showed that the proportion of $Foxp3^+CD4^+$ T cells are increased in TGF-βRII DN mice, irrespective of their IFN-γ status (Fig. 4E). Moreover, CD103 expression is reduced on both $Helios^+$ and $Helios^-$ $Foxp3^+CD4^+$ T cells in both IFN-γ–deficient and –sufficient TGF-βRII DN mice (Fig. 4F), confirming that CD103 expression is regulated by TGF-β signaling (27). Levels of CD103 therefore do not correspond to the susceptibility of the mouse strain, suggesting that CD103 is not required for functional suppression of the antihelminth response. However, because CD103 is important for effector T cell migration and retention in the gut (28), and because the TGF-βRII DN $Foxp3^-$ effector population also fails to express high CD103 levels (Fig. 2C; data not shown), the susceptibility of this genotype could reflect a diminished presence of effector cells at the site of infection.

Overall, these data argue that neither Th1 nor TGF-β–induced adaptive Tregs are essential for repression of the protective Th2 response to *H. polygyrus*. Several interesting alternatives can now be considered. First, the greater expansion of natural Tregs in TGF-βRII DN mice may account for their continued susceptibility. The outgrowth of natural or $Helios^+$ Tregs in vivo may result from a homeostatic compensation for the paucity of $CD103^+$ adaptive Tregs (moderated by cytokines such as IL-2) and/or outgrowth to control a greater mucosal inflammatory response in the absence of TGF-β–inducible $CD103^+$ adaptive Tregs (29). Second, although Th2 effectors would be inured from TGF-β–mediated inhibition (30), Tregs operate through other suppressive pathways including coinhibitors such as CTLA-4 and programmed cell death-1 known to be important in other helminth systems (18). Thirdly, many other regulatory subsets are known to arise in *H. polygyrus* infection including DCs, macrophages and regulatory B cells (reviewed in Ref. 2), which may account for the susceptibility of these mice. Finally, it should noted that significant nonlymphoid populations are responsive to TGF-β, and the efficacy of global TGF-β inhibition (3) and the TGF-β–dependent effects in *H. polygyrus*-infected RAG-deficient hosts (6), implies that there are critical non-T cell targets of this suppressive cytokine.

In conclusion, although inducible Tregs control mucosal inflammation (29), our data support the idea that control of protective immunity in the intestinal setting may be regulated by natural and not inducible Tregs. This scenario has been suggested by recent studies of the IL-6–deficient BALB/c mouse, which are highly resistant to *H. polygyrus* infection (K.A. Smith and R.M. Maizels, submitted for publication). This intriguing and unexpected division of labor between Treg subsets remains to be further explored.

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Disclosures

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References

1. Anthony, R. M., L. I. Rutitzky, J. F. Urban, Jr., M. J. Stadecker, and W. C. Gause. 2007. Protective immune mechanisms in helminth infection. *Nat. Rev. Immunol.* 7: 975–987.
2. Allen, J. E., and R. M. Maizels. 2011. Diversity and dialogue in immunity to helminths. *Nat. Rev. Immunol.* 11: 375–388.
3. Grainger, J. R., K. A. Smith, J. P. Hewitson, H. J. McSorley, Y. Harcus, K. J. Filbey, C. A. M. Finney, E. J. D. Greenwood, D. P. Knox, M. S. Wilson, et al. 2010. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway. *J. Exp. Med.* 207: 2331–2341.
4. Else, K. J., F. D. Finkelman, C. R. Maliszewski, and R. K. Grencis. 1994. Cytokine-mediated regulation of chronic intestinal helminth infection. *J. Exp. Med.* 179: 347–351.
5. Li, M. O., and R. A. Flavell. 2008. TGF-β: a master of all T cell trades. *Cell* 134: 392–404.
6. Hang, L., T. Setiawan, A. M. Blum, J. Urban, K. Stoyanoff, S. Arihiro, H. C. Reinecker, and J. V. Weinstock. 2010. *Heligmosomoides polygyrus* infection can inhibit colitis through direct interaction with innate immunity. *J. Immunol.* 185: 3184–3189.
7. Ince, M. N., D. E. Elliott, T. Setiawan, A. Metwali, A. Blum, H. L. Chen, J. F. Urban, R. A. Flavell, and J. V. Weinstock. 2009. Role of T cell TGF-β signaling in intestinal cytokine responses and helminthic immune modulation. *Eur. J. Immunol.* 39: 1870–1878.
8. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* 9: 1341–1346.
9. Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 cells. *Annu. Rev. Immunol.* 27: 485–517.
10. Behnke, J. M., F. N. Wahid, R. K. Grencis, K. J. Else, A. W. Ben-Smith, and P. K. Goyal. 1993. Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms. *Parasite Immunol.* 15: 415–421.
11. Elliott, D. E., A. Metwali, J. Leung, T. Setiawan, A. M. Blum, M. N. Ince, L. E. Bazzone, M. J. Stadecker, J. F. Urban, Jr., and J. V. Weinstock. 2008. Colonization with *Heligmosomoides polygyrus* suppresses mucosal IL-17 production. *J. Immunol.* 181: 2414–2419.
12. Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan. 1988. Deactivation of macrophages by transforming growth factor-β. *Nature* 334: 260–262.

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13. Gorelik, L., S. Constant, and R. A. Flavell. 2002. Mechanism of transforming growth factor β-induced inhibition of T helper type 1 differentiation. *J. Exp. Med.* 195: 1499–1505.
14. Marie, J. C., J. J. Letterio, M. Gavin, and A. Y. Rudensky. 2005. TGF-β1 maintains suppressor function and Foxp3 expression in $CD4^+CD25^+$ regulatory T cells. *J. Exp. Med.* 201: 1061–1067.
15. Maizels, R. M., and K. A. Smith. 2011. Regulatory T cells in infection. *Adv. Immunol.* 112: 73–136.
16. Gorelik, L., and R. A. Flavell. 2000. Abrogation of TGFβ signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12: 171–181.
17. Prowse, S. J., G. F. Mitchell, P. L. Ey, and C. R. Jenkin. 1979. The development of resistance in different inbred strains of mice to infection with *Nematospiroides dubius*. *Parasite Immunol.* 1: 277–288.
18. Taylor, M. D., A. Harris, S. A. Babayan, O. Bain, A. Culshaw, J. E. Allen, and R. M. Maizels. 2007. CTLA-4 and $CD4^+CD25^+$ regulatory T cells inhibit protective immunity to filarial parasites in vivo. *J. Immunol.* 179: 4626–4634.
19. Rausch, S., J. Huehn, C. Loddenkemper, M. R. Hepworth, C. Klotz, T. Sparwasser, A. Hamann, R. Lucius, and S. Hartmann. 2009. Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of $Foxp3^+$ cells. *Eur. J. Immunol.* 39: 3066–3077.
20. Blankenhaus, B., U. Klemm, M. L. Eschbach, T. Sparwasser, J. Huehn, A. A. Kühl, C. Loddenkemper, T. Jacobs, and M. Breloer. 2011. *Strongyloides ratti* infection induces expansion of $Foxp3^+$ regulatory T cells that interfere with immune response and parasite clearance in BALB/c mice. *J. Immunol.* 186: 4295–4305.
21. Thornton, A. M., P. E. Korty, D. Q. Tran, E. A. Wohlfert, P. E. Murray, Y. Belkaid, and E. M. Shevach. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced $Foxp3^+$ T regulatory cells. *J. Immunol.* 184: 3433–3441.
22. Huehn, J., K. Siegmund, J. C. U. Lehmann, C. Siewert, U. Haubold, M. Feuerer, G. F. Debes, J. Lauber, O. Frey, G. K. Przybylski, et al. 2004. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like $CD4^+$ regulatory T cells. *J. Exp. Med.* 199: 303–313.
23. Townsend, J. M., G. P. Fallon, J. D. Matthews, P. Smith, E. H. Jolin, and N. A. McKenzie. 2000. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity* 13: 573–583.
24. Hashimoto, K., R. Uchikawa, T. Tegoshi, K. Takeda, M. Yamada, and N. Arizono. 2010. Immunity-mediated regulation of fecundity in the nematode *Heligmosomoides polygyrus*—the potential role of mast cells. *Parasitology* 137: 881–887.
25. Morimoto, M., and K. Utsumiya. 2011. Enhanced protection against *Heligmosomoides polygyrus* in IL-2 receptor lygyrusumiya. *J. Vet. Med. Sci.* 73: 849–851.
26. Finney, C. A. M., M. D. Taylor, M. S. Wilson, and R. M. Maizels. 2007. Expansion and activation of $CD4^+CD25^+$ regulatory T cells in *Heligmosomoides polygyrus* infection. *Eur. J. Immunol.* 37: 1874–1886.
27. Robinson, P. W., S. J. Green, C. Carter, J. Coadwell, and P. J. Kilshaw. 2001. Studies on transcriptional regulation of the mucosal T-cell integrin $\alpha_E\beta_7$ (CD103). *Immunology* 103: 146–154.
28. El-Asady, R., R. Yuan, K. Liu, D. Wang, R. E. Gress, P. J. Lucas, C. B. Drachenberg, and G. A. Hadley. 2005. TGF-β-dependent CD103 expression by $CD8^+$ T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J. Exp. Med.* 201: 1647–1657.
29. Josefowicz, S. Z., R. E. Niec, H. Y. Kim, P. Treuting, T. Chinen, Y. Zheng, D. T. Umetsu, and A. Y. Rudensky. 2012. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* 482: 395–399.
30. Fahlén, L., S. Read, L. Gorelik, S. D. Hurst, R. L. Coffman, R. A. Flavell, and F. Powrie. 2005. T cells that cannot respond to TGF-β escape control by $CD4^+CD25^+$ regulatory T cells. *J. Exp. Med.* 201: 737–746.

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9.3 Immunity to the model intestinal helminth parasite *Heligmosomoides polygyrus*

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REVIEW

Immunity to the model intestinal helminth parasite *Heligmosomoides polygyrus*

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Abstract *Heligmosomoides polygyrus* is a natural intestinal parasite of mice, which offers an excellent model of the immunology of gastrointestinal helminth infections of humans and livestock. It is able to establish long-term chronic infections in many strains of mice, exerting potent immunomodulatory effects that dampen both protective immunity and bystander reactions to allergens and autoantigens. Immunity to the parasite develops naturally in some mouse strains and can be induced in others through immunization; while the mechanisms of protective immunity are not yet fully defined, both antibodies and a host cellular component are required, with strongest evidence for a role of alternatively activated macrophages. We discuss the balance between resistance and susceptibility in this model system and highlight new themes in innate and adaptive immunity, immunomodulation, and regulation of responsiveness in helminth infection.

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Introduction

Heligmosomoides polygyrus: a model organism

Chronic helminth infections remain a huge global health problem, causing extensive morbidity in both humans and livestock. Many of the most prevalent helminth parasites are difficult to study in the laboratory, as they have co-evolved with, and are closely adapted to, their definitive host species. However, model organisms such as *Heligmosomoides polygyrus*, a natural mouse parasite, offer tractable and informative systems to explore the mechanisms of immunity and immune evasion in helminth infections [1, 2].

H. polygyrus (previously named *Nematospiroides dubius*) is an intestinal nematode parasite in wild mouse populations that has successfully been transferred to the laboratory. It is phylogenetically placed in the same Suborder, Trichostrongylina, as the ruminant parasites *Haemonchus contortus* and *Teladorsagia circumcincta* and within the same Order, Strongylida, as the human hookworm parasites *Ancylostoma duodenale* and *Necator americanus* [3]. *H. polygyrus* is an appropriate model of these chronic helminthiases, as primary infections can persist for many months in susceptible strains of mice.

In an experimental setting, *H. polygyrus* is introduced by orally gavaging mice with infective L3 larvae. Following ingestion, within 24 h, larvae have penetrated through into the submucosa of the small intestine. Here they undergo two developmental molts, before emerging back into the lumen as adult worms, which feed on host intestinal tissue [4]. The adult worms coil around the small intestine villi to secure themselves, mate, and produce eggs, which are excreted in the feces. In the external environment, the eggs hatch and undergo two molts to become infective L3s, and so the lifecycle continues (Fig. 1).

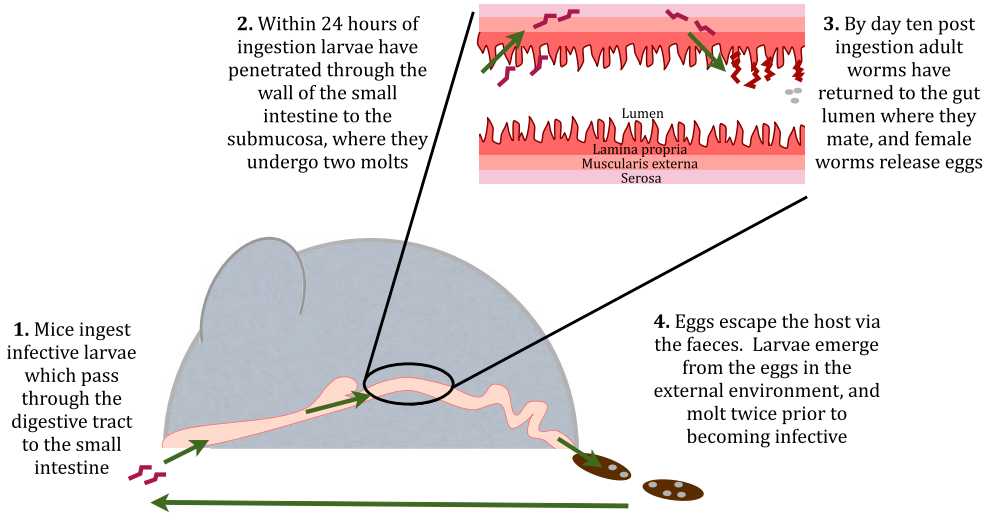

Fig. 1 Lifecycle of *Heligmosomoides polygyrus* in mice

The persistence of *H. polygyrus* within the murine host can be measured by determining the number of eggs that are released in the feces, or by enumerating adult worms remaining in the small intestine. As described below, the wide range of reagents available for assaying and manipulating the murine immune system cells in mice are being effectively applied to investigate responsiveness and immunity. The mechanisms behind helminth expulsion in mice can therefore be studied in order to make predictions about similar interactions between helminths and the immune system in livestock and humans, with a view to developing much-needed vaccines for control of these infections.

A further advantage of *H. polygyrus* is that the mammalian stages can be cultivated in vitro, where its secretory products, *H. polygyrus* excretory–secretory antigens (HES), can be collected, and individual components can be purified and identified [5]. This provides a fruitful strategy to test defined parasite molecules in vitro and in vivo for immunomodulatory functions and as candidate vaccine antigens.

Genetics of susceptibility to *H. polygyrus*

In primary infections of different mouse strains, the length of time *H. polygyrus* can persist and the degree of response it provokes shows considerable variation, and some genotypes are also poor at rejecting challenge infections following immunisation.

Table 1 shows a summary of "responsiveness" to *H. polygyrus* in different mouse strains, based on adult worm survival and fecundity after primary and secondary infection. The genetic factors controlling strain differences in resistance to infection include the major histocompatibility complex (MHC) H-2 loci, with weak responders among the H-2^k and H-2^b genotypes and the H-2^q or H-2^s genotypes associated with a rapid response [6, 7].

Experiments in H-2 congenic C57BL/10 mouse strains show that although establishment of *H. polygyrus* larvae is equal between all strains (shown by worm counts 2 weeks postinfection), by week 9, egg and adult worm numbers differ strikingly. Those with H-2^s and H-2^q haplotypes expelled the parasites more rapidly [7, 8], while mice carrying H-2^b or H-2^k haplotypes backcrossed into the fast-responding BALB/c background were unable to expel worms quickly [7]. Resistance was shown to be conferred by more than one gene, as F1 hybrids of fast responders, SJL and SWR, display heightened abilities to expel worms, and is inherited in a dominant fashion as C57BL/10xSJL hybrids are as rapid in expulsion as the SJL parental strain [7, 9].

More recently, a study mapping quantitative trait loci in fast responding (SWR, H-2^q) versus slow responding (CBA, H-2^k) strains found significant effects on resistance to *H. polygyrus* trickle infection from positions on chromosomes 1, 2, 13, and 17 [10]. Several candidate resistance genes were identified, including as expected MHC (on chromosome 17), and also interleukin-9 (IL-9; on chromosome 13), both of which correlate with worm expulsion [10].

A notable gender bias in susceptibility is also observed, with female mice of all strains clearing primary infections faster than their male counterparts as also apparent to a lesser degree following secondary exposure [11–13]. The greater susceptibility of male mice correlates with higher fecundity of worms recovered from male hosts and a larger adult worm body size [14], indicating that the parasites are fitter than those from a female host.

Concurrent pregnancy and worm infection imposes increased physiological demands on the mother in terms of the energy required to fight infection and to nourish the fetus. This can lead to immunosuppression (diminished Th2 responses) [15] and adverse reproductive outcomes (small pup size) [15, 16]. The effects of pregnancy on maternal serum cytokines during *H. polygyrus* infection include

Table 1 Strain-specific immunity to *H. polygyrus*

Responsiveness	Strain	Immune mechanisms investigated
Slow (>20 weeks to expel worms)	CBA C3H SL A/J	Primary response involves significantly lower cell numbers in the MLN than other strains [193, 194], very few mast cells in the gut [193], low levels of mMCP in serum and intestinal lavage [51, 53], and low eosinophilia [14] Have no, or very weak, protective response to re-challenge [6, 53, 195]
Intermediate (8–20 weeks)	C57BL/6 C57BL/10 129/J	C57BL/10 mice show less rapid and lower eosinophilia levels in circulation, after both primary *H. polygyrus* infection or injection of parasite antigens, than NIH mice [196]
Fast (6–8 weeks)	DBA/2 BALB/c NIH	NIH mice produced a higher peak of lymphocytosis, neutrophilia and monocytosis in the circulation than C57BL/10 mice after primary infection [197]
Rapid (4–6 weeks)	SJL	SJL and SWR have quicker and stronger antibody responses than other strains, involving stronger recognition of a larger number of antigens on a Western blot of HES [198] and adult homogenate [193], and higher titers of parasite-specific antibody of different isotypes in serum [51, 193, 198]
	SWR	Infected SWR MLN cells produced higher levels of IL-3, IL-4 and IL-9 after ConA stimulation than NIH and CBA [52] Both strains show early peaks of serum tumor necrosis factor alpha, mMCP-1, intestinal mast cells and goblet cells, which precede the expulsion of the worms [51, 53]

increased levels of IL-1β and IL-6 at day 20 postinfection [15] and lower concentrations of IL-4, IL-5, IL-13, and mucosal mast cell protease (mMCP-1) [15]. Pregnant mice also show a small but significant increase in adult worm burdens [15].

Models of resistance

Immunity to *H. polygyrus* can be studied in three separate settings each with distinct implications for human infection, namely, genetically determined, drug-induced, and vaccine-elicited immunity. In each of these contexts, the availability of numerous gene-targeted mouse strains and immunological reagents are being used to define immune system components and parameters required for immunity to infection.

As stated above, the outcome of primary *H. polygyrus* infection is strongly influenced by the genetic background of mice, with strains differing in their susceptibility to chronic infection. Studying how the immune response differs between those strains that endure chronic infections and those that are able to clear a primary infection has been highly instructive in defining the immune mechanisms the host must promote in order to clear the parasite.

If primary infection with *H. polygyrus* is cleared using antihelmintic drugs such as pyrantel pamoate or ivermectin [17], most mouse strains display a highly effective memory response, which provides immunity to reinfection [18]. Genetic background also impacts on resistance to reinfection, as BALB/c mice display significantly lower worm numbers postchallenge compared to C57BL/6 [19, 20].

Finally, HES administered in alum adjuvant has been shown to induce sterilizing immunity to *H. polygyrus* infection [21], and studies are ongoing to identify the specific components of HES and the immune mechanisms critical for this immunity.

Challenge infection/Trickle infection

Although most laboratory studies employ a single bolus infection, doses used are far from physiological or representative of field conditions. Hence, some investigators have developed trickle infection regimes, for example, administering twice weekly low doses of infective larvae. Under these conditions, different mouse strains show a gradation of resistance patterns similar to those seen with single-bolus primary infection, in that NIH and SWR strains resolve infection (showing an initial increase in adult worm burden, a period of stability, and then finally expulsion) while CBA and C57BL/10 mice continue to accumulate increasing adult worm burdens over the course of repeated infections [22].

Variation and adaptation by *H. polygyrus*

The strain of *H. polygyrus* used in laboratories worldwide is thought to have been isolated from wild Californian mice in the 1940s [23] and was known for some years as *Nematospiroides dubius*. The vast majority of the literature describing

experiments with this isolate refers to the parasite as *H. polygyrus*. It was, however, suggested that this laboratory strain should be referred to as *H. polygyrus bakeri*, to differentiate it from wild strains of the parasite, considered to be *H. polygyrus polygyrus* (found in the wood mouse *Apodemus sylvaticus* in Europe), *H. polygyrus corsicus* (from the house mouse *Mus musculus* in Corsica) and *H. polygyrus americanus* (from the vole *Phenacomys intermedius* in North America) [24]. More recently, there has been an additional proposal of a name change for the laboratory isolate to *H. bakeri* [23], based on sequence divergence between laboratory and European wood mouse isolates [25]. This proposal has not received widespread support due to the preliminary nature of the data, the sequence variation even within the laboratory strain, and the need to remain consistent with previous literature [26]. Here, we refer to the laboratory strain of the parasite as *H. polygyrus*.

In proteomic studies on *H. polygyrus* secreted antigens (see below), extensive sequence variation was observed in some gene families [5], indicating that despite many years of laboratory propagation, the parasite strain remains highly polymorphic. Moreover, there are indications that antigen expression by *H. polygyrus* may vary or adapt according to the host strain of mouse [27, 28], with proteomic differences in adult worms recovered 4 weeks postinfection in either C57BL/10 (slow responder) or SWR (fast responder) hosts [28]. Phosphatidylethanolamine-binding protein and several nematode globins are overexpressed in worms from C57BL/10 compared to worms from SWR mice, and myosin, troponin, actin, and several unidentified proteins are overexpressed in the worms from fast responder mice compared to slow [28]. Differential expression of worm products in different host strains may shed light on pathways targeted by the immune system that impact on worm survival or death.

Host immune responses

The critical requirement for the adaptive immune response in control of the parasite is illustrated when B- and T-cell responses are lacking. Severe combined immunodeficient (SCID; B- and T-cell deficient) and athymic mice show impaired expulsion of adult worms, maintaining high worm burdens several weeks post infection by which stage wild-type counterparts had expelled the majority of their worms [29, 30]. Treatment with anti-CD4 results in higher fecundity of female worms in a primary infection [31] and transfer of the effector T-cell subset from chronically infected animals significantly reduced worm burdens when transferred to naive mice before infection [32]. Although B-cell deficiency does not affect the outcome of primary *H. polygyrus* infection, B-cell or antibody deficiency significantly compromises the ability to expel a secondary challenge infection [33].

T-cell responses

H. polygyrus infection induces a strongly polarized Th2 response, which has been shown to be critical in control and expulsion of the worm [31]. A primary *H. polygyrus* infection induces IL-3, IL-4, IL-5, and IL-9 gene expression in the mesenteric lymph nodes (MLN) and Peyer's patches [34] and elicits the release of high concentrations of IL-4, IL-5, IL-9, IL-10, and IL-13 protein from MLN, spleen, and lamina propria mononuclear cells (LPMC) cultured with parasite antigens [32, 35, 36]. IL-4 is the most critical single cytokine for protection against primary and secondary *H. polygyrus* infection (both in expulsion of adult worms and inhibiting their egg production) [31]. Immunity to secondary infection is diminished by blocking antibody to IL-4 but completely abolished when the IL-4R is also blocked [31]. This suggests that IL-13, which also signals through IL-4Rα, can partially compensate for the loss of IL-4, but in the absence of signaling from both cytokines, protection against reinfection with *H. polygyrus* is lost. Blocking of IL-5 by antibody treatment had no effect on worm expulsion [31]. When IL-4 is administered as a complex with anti-IL-4 (IL-4C) to extend the activity time of this cytokine, wild-type BALB/c mice expelled *H. polygyrus* more rapidly [29]. This effect did not depend on the adaptive immune system, as *H. polygyrus* expulsion was also seen in SCID mice and anti-CD4-treated BALB/c mice, which were given the IL-4 complex [29]. After primary infection, $CD4^{+}IL\text{-}4^{+}$ T cells disseminate around the body to lymphoid and nonlymphoid organs, such as airways, peritoneal cavity, and liver, and have a lower apoptotic potential [37, 38]. These findings may be illustrative of peripheral reservoirs of long-lived memory Th2 cells primed to respond to subsequent infection challenges by the worm.

Further studies have delineated the costimulatory signals required to mount Th2 responses to *H. polygyrus* infection. By blocking signaling through both CD80 (B7-1) and CD86 (B7-2) with specific antibodies or a CTLA4-Ig construct, IL-4 expression, Th2 expansion, and IgE production in response to *H. polygyrus* were ablated [39, 40]; interestingly, an innate IL-5 response remained intact when T-cell costimulation was inhibited. Blocking antibodies against CD80 or CD86 alone had little effect [40], and while early (day 6 postinfection) responses to *H. polygyrus* were unaltered in CD86-deficient mice [41], by day 14 postinfection, CD86-deficient mice had higher parasite egg burdens and decreased Th2 responses [41]. This showed that, while CD86 was not required for the initiation of the antiworm response, it was necessary for its progression and persistence.

Although both CD80 and CD86 normally ligate to T-cell CD28, CD28-deficient mice were found to have no impairment in the early $CD4^{+}IL\text{-}4^{+}$ response, indicating an alternative mechanism for Th2 costimulation in *H. polygyrus*

infection [42]. Moreover, while the primary T-cell response to infection is CD80/CD86-dependent as outlined above, on secondary infection, memory helper T cells do not require CD80 or CD86 costimulation for their activation to protect against challenge [43, 44]. Studies into an additional costimulatory molecule, OX40L (CD143), showed that it is specifically required to promote IL-4 production from T cells (and the associated rise in IgE), without affecting Th2-cell expansion, migration, germinal center formation, or IgG1 levels [45].

T-follicular helper cells are now recognized as the instrumental subset, which induces germinal center formation and isotype switching in B cells, by migrating to B-cell follicles and releasing cytokines including IL-21 [46], while also producing IL-4 in the MLN of *H. polygyrus*-infected mice [47]. IL-21 plays key roles by stimulating multiple cell types across a range of infections [48]. In *H. polygyrus* infection, IL-21 deficiency results in reduced intestinal granuloma formation, impaired T-cell expansion and survival, and lower numbers of circulating basophils and eosinophils [49]. IL-21 also provides a critical signal for the differentiation of B cells into plasma cells and for protection against secondary challenge infection with *H. polygyrus* [50].

B-cell and humoral responses

In general, the intensity and speed of parasite-specific antibody responses are greater in more resistant mouse strains such as SJL and SWR than in susceptible strains. Specifically, the IgG1 and IgE responses (to adult worm homogenate and HES) negatively correlate across strains with worm survival after a primary infection [12, 51, 52]. However, after repeated low-dose ("trickle") infections, there was little difference between slow and fast responder strains in any antibody isotype measured to larval and worm antigens [53].

B cells, as well as secreting antibodies, also produce cytokines and costimulatory molecules that promote and amplify the T-cell response in a selective manner [54, 55]. Interestingly, the greatest increase in cell number in MLN after *H. polygyrus* infection is in the B-cell compartment [32, 56].

The protective response to secondary challenge with *H. polygyrus* is dependent on B cells, as μMT and JHD mice (both of which lack B cells) cannot clear the parasites [57–59]. Defective immunity in B-cell-deficient mice is not due to an impairment of Th2 responses, or to T regulatory cell (Treg) activation, development, or differentiation, as a pronounced local Th2 response in the intestinal tissues occurred with or without B cells, in both primary and secondary infection [58, 59]. However, a separate study showed impairment of the Th2 response in B-cell deficient mice, with significantly lower T-cell expansion and cytokine production [57]. These authors showed that a sufficient T-cell memory response was B-cell dependent and that immunity required B cells to produce the cytokines IL-2 and tumor necrosis factor alpha [57]. This discrepancy remains unclear, and B cells seem to have differing roles in other helminth infections. During a primary infection with the colon-residing murine nematode parasite *Trichuris muris*, B cells are required for resistance and the development of a Th2 response [60]; however, in primary and secondary infection with another gastrointestinal nematode, *Nippostrongylus brasiliensis*, Th2 responses and worm expulsion are B-cell independent [58].

The specific role of antibody in mediating protection against *H. polygyrus* has also been investigated using mice with targeted deficiencies within the B-cell compartment. For example, μs mice have secretory IgM-deficient B cells, but can produce parasite-specific class-switched IgG1 and IgE, and are able to clear secondary infection [57]. However, when crossed with a null activation-induced deaminase transgene, the resultant mice are unable to undergo affinity maturation or secrete antibody of any isotype and are not protected from secondary challenge with the parasite [57]. Using selective isotype knockout mice given a secondary *H. polygyrus* infection, it was found that IgE had no role in protection, and IgA had a minor role, leaving IgG as the major class-switched isotype leading to protection [59]. Indeed, it has been long known that the humoral response is dominated by parasite-specific IgG1 and that serum fractions with highest parasite-specific IgG1 activity afford greater protection when transferred to an infected animal [61]. Even transfer of whole serum from immune wild-type donors to JHD-recipient mice can significantly reduce the number of adult worms left in the intestine after a challenge infection [58].

Primary *H. polygyrus* infection also elicits an extraordinary increase in nonspecific serum IgG1 levels (hypergammaglobulinemia) [59, 61, 62], and following repeated trickle infections over 4 weeks, serum IgG1 concentrations can reach 30 times the normal level seen in uninfected mice [62, 63]. Despite these high concentrations, transfer of serum from 28-day infected mice does not protect naive animals from infection [59, 64]. In contrast, serum raised after multiple *H. polygyrus* infections is protective against adult worm survival when transferred into naive recipients [64, 65], presumably reflecting the higher ratio of parasite-specific to nonspecific IgG following repeated infection. The mechanisms through which such high levels of polyclonal IgG1 are produced in response to *H. polygyrus* (and other parasitic worms [63]) remain to be explored.

Innate immune responses

Innate immune cells are the initial responders to a *H. polygyrus* infection and are also implicated in the end-stage expulsion of parasites. Innate cells release type 2 cytokines

that can act directly to alter gut physiology and polarize the adaptive immune response, while themselves employing helminth-damaging or killing mechanisms [66, 67].

Dendritic cells

Dendritic cells (DCs) are the predominant innate antigen-presenting cell that are required to prime Th2 responses against helminths [68]. DCs loaded with helminth products in vitro can be transferred to naive animals to induce a Th2 response [69] and have been shown to inhibit allergic airway inflammation when transferred from a helminth-infected animal, resulting in increased numbers of Tregs and a downregulation of Th2-mediated inflammation [70]. When $CD11c^{+}$ DCs are depleted using CD11c.DTR mice [71] that coexpress CD11c with the human diphtheria toxin receptor (DTR), a Th2 response against several helminths (including *H. polygyrus*) is severely compromised [72, 73].

Macrophages

The alternative activation of macrophages is a hallmark of helminth-elicited Th2 responses and is associated with high expression of a characteristic set of gene products, including Ym1, RELM-α (FIZZ-1), arginase-1, IL-4Rα, and the mannose receptor CD206 [66, 74]. Macrophages can differentially express the enzymes nitric oxide synthase 2 (NOS-2) and arginase-1, which compete for the common substrate L-arginine, and are competitively induced by interferon gamma (IFN-γ) and Th2 cytokines (IL-4, IL-10, IL-13, and IL-21), respectively [75–78]. The activation state of macrophages in helminth infections is sufficiently plastic to respond to changing stimuli, as helminth-induced alternatively activated macrophages restimulated ex vivo with lipopolysaccharide and IFN-γ switch to a classically activated phenotype [79], suggesting that such plasticity may also occur in vivo.

Alternatively activated macrophages are critical to the protective immune response to secondary *H. polygyrus* infection, as mice lost the ability to reject challenge infections when depleted of macrophages via clodronate treatment, or when treated with *S*-(2-boronoethyl)-l-cysteine (BEC), a pharmaceutical inhibitor of arginase [80]. Arginase-1 may directly harm parasites, as *H. polygyrus* exhibited higher levels of cytochrome oxidase, a marker of a stress response, in a secondary infection compared to a primary infection, and this increase was lost following BEC administration [80]. In contrast to arginase, no antiparasite function has been found for Ym1, a member of the chitinase-like family of proteins that lacks demonstrable chitinase activity [81]. Ym1 does bind heparin on cell surfaces and in the extracellular matrix [81], which may indicate a role for Ym1 and alternatively activated macrophages in mediating repair of tissue damage caused by *H. polygyrus* when migrating through the intestinal wall [82].

Alternatively activated macrophages may also be important mediators of the smooth muscle hypercontractility response to intestinal helminth infections, at least in the context of a *N. brasiliensis* infection, as depleting macrophages via clodronate-treatment blocked smooth muscle hyperactivity and impaired worm expulsion [83].

Neutrophils

Perhaps surprisingly, the role of the principal granulocyte cell types (neutrophils, eosinophils, and basophils) has not been directly evaluated in *H. polygyrus* infection. Neutrophils are prominent in primary and, to a lesser extent, secondary granulomas during a *H. polygyrus* infection [80, 84, 85]. The finding that neutrophils are less prevalent in a setting of heightened resistance may indicate that they are not a key cell type in immunity. To date, a protective function for neutrophils during helminth infections has only been reported for infections of mice with tissue-migrating larvae of the human nematode parasite *Strongyloides stercoralis*, although even in this case killing was more effective when eosinophils were present alongside neutrophils [86, 87].

Eosinophils

No role for eosinophils in *H. polygyrus* expulsion has yet been described. In a genetic model of eosinophil deficiency, in which an eosinophil-specific site in the GATA-1 promoter is deleted [88], mice showed impaired resistance to challenge infections with *N. brasiliensis* [89]; significantly, in the absence of eosinophils, greater numbers of tissue larvae migrated to the lung, but expulsion of those parasites that subsequently reached the gut was unimpaired in the eosinophil-deficient mice. Eosinophilia in response to *N. brasiliensis* infection was blocked when mice were administered anti-IL-5 antibody [90, 91], but this had no impact on adult worm recovery [90], providing additional evidence that eosinophils are not a critical mediator of expulsion in this system. Anti-IL-5 treatment during *H. polygyrus* infection also had no impact on worm burden [92], and eosinophils within the gut wall have been reported to be inhibited during *H. polygyrus* infection in a manner reversible with anti-transforming growth factor beta (anti-TGF-β) antibody treatment [93].

Basophils

As with the other granulocytes, few studies have investigated the role of basophils in *H. polygyrus* infections. In other gastrointestinal nematode infections, basophilia is conspicuous, and their presence may be required for optimal *N.*

brasiliensis expulsion [94]. Worm expulsion of *T. muris* was impaired when basophil numbers were depleted using MAR-1 antibody [95]; however, this antibody targets the FcεRI, which is also expressed by mast cells, so this does not conclusively prove a role for basophils alone.

Mast cells

Mast cells are major players in the intestinal immune response to infection with *H. polygyrus*, as expulsion correlates with epithelial mastocytosis [52, 96] and elevated intestinal fluid levels of mMCP-1 in different murine strains [51]. Mast cells may promote helminth damage by increasing the permeability of the gut via mMCP-1-mediated breakdown of epithelial tight junction proteins [97, 98], thereby increasing luminal flow and disrupting the niche of parasitic helminths. Increased permeability of the gut in response to *Trichinella spiralis* is blocked in mMCP-1-deficient mice, which were less effective at clearing the worms than wild-type counterparts [98]. The mast cell response to *T. spiralis* (and *N. brasiliensis*) infection, however, is ablated in mice carrying an *H. polygyrus* coinfection [99] arguing that the latter parasite is able to suppress host mastocytosis to a significant degree.

Most in vivo studies on mast cells in helminth infection have involved the mast-cell-deficient mice $Kit^{W}/Kit^{W\text{-}v}$ which carry a mutated gene encoding the tyrosine kinase receptor c-kit. During *H. polygyrus* infections, these mice produce higher egg numbers than wild-type controls, indicative of impaired immunity [100]. Consistent with this, reduced egg production was seen in Tg2Rbeta mice [101], which exhibit mastocytosis.

In terms of protective immunity to adult worms, however, $Kit^{W}/Kit^{W\text{-}v}$ mice were found to be similar to wild type in slowly expelling primary *H. polygyrus* infection between 4 and 9 weeks of infection [100]. However, a more recent report has that shown $Kit^{W}/Kit^{W\text{-}v}$ mice and another mast cell deficient strain, $Kit^{W\text{-}sh}$ mice, do have impairments in *H. polygyrus* expulsion, as both strains had higher worm burdens than wild-type mice after 3 weeks of a primary *H. polygyrus* infection [102]. The same authors also showed that $Kit^{W}/Kit^{W\text{-}v}$ mice were not resistant to a secondary *H. polygyrus* infection, yet control wild-type mice were able to clear the infection [102]. The reason for the discrepancy between the reports on the ability of $Kit^{W}/Kit^{W\text{-}v}$ mice to clear *H. polygyrus* is not clear, and more studies are required to confirm the importance of mast cells during infections. If mast cells do contribute to expulsion of *H. polygyrus*, it could be via their contributions towards priming a Th2 response early in infection, as well as their potential role as a later effector cell. $Kit^{W}/Kit^{W\text{-}v}$ MLN cells did not show the high levels of *H. polygyrus*-antigen-specific Th2 cytokines produced by wild-type MLN cells in response to *H. polygyrus* [102].

It should be noted that both $Kit^{W}/Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$ mice have defects that extend beyond a mast cell deficiency [103]. Many of the recently described subsets of lineage negative innate type 2 cells, discussed below, express c-kit, and so it is likely that some of the deficiencies of $Kit^{W}/Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$ mice can be explained by the additional disruption of these cell types.

Innate lymphoid cells

Recently, a number of studies have identified a population of lineage marker negative innate lymphoid cells (ILC), which produce type 2 cytokines (particularly IL-5 and IL-13) in response to epithelial cell-derived cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin (reviewed in [104]). IL-25 may also be derived from other cell types, such as mast cells [105], but the importance of IL-25 from this source is as yet unknown. Epithelial cells produce elevated levels of these cytokines in response to damage, thereby raising the first alarm leading to Th2 responses (reviewed in [106]). Trefoil factor 2 (TFF2) is a molecule involved in epithelial cell repair, which induces IL-33 production by epithelial cells in response to damage caused by *N. brasiliensis* [107]. $TFF2^{-/-}$ mice did not show the elevated epithelial IL-33 levels in response to *N. brasiliensis* that wild-type mice did, instead having lower serum IL-4 levels after 7 days of infection, and delayed worm expulsion [107]. Similarly, Th2 cytokine production is delayed, and *N. brasiliensis* expulsion is impaired in IL-$25^{-/-}$ mice, which correlates with the absence of a non-B non-T-cell c-kit^{+} IL-4, IL-5, and IL-13 producing population induced in infected wild-type mice or mice administered rIL-25 or rIL-33 [108–110]. A role for ILCs has not yet been reported during a *H. polygyrus* infection though it seems likely that these cell types are important inducers of Th2 responses during all intestinal helminth infections.

Gut physiology and intestinal epithelial cell function

IL-4 and IL-13, derived from innate or adaptive sources, are likely to have direct effects on the physiology of the gut as well as on effector cells that promote helminth expulsion. Although few changes in epithelial cell function are noted during primary *H. polygyrus* infection, in secondary infections, increased mucosal permeability, decreased ion absorption, and increased prosecretory effects in response to prostaglandin E2 and histamine were seen [111, 112]. Moreover, these changes were dependent on the IL-4R and STAT6 and were reproduced by IL-4C administration [111, 112]. These alterations to the worm's environment may interfere with its abilities to feed on the intestinal tissue [4] or remain wrapped around the villi in the small intestine.

Goblet cell function

Within the intestinal epithelial layer are specialized goblet cells that secrete innate defence proteins as well as large quantities of mucins, the key components of mucus. Goblet cell hyperplasia develops in response to intestinal helminth infections, including *H. polygyrus*, where hyperplasia is dependent on a functional T-cell response [30].

Enhanced mucus production has been suggested to act against helminth establishment, and it may be that specific components within the mucus play a role in control of helminth expulsion. RELM-β (FIZZ-2) is a cysteine-rich mediator expressed by goblet cells in response to IL-13 and is important for the normal control of epithelial cell barrier permeability [113, 114]. RELM-$\beta^{-/-}$ mice do not expel a secondary *H. polygyrus* infection as rapidly as wild-type mice and adult *H. polygyrus* worms treated in vitro with recombinant RELM-β prior to transfer to a new host survived less well than untreated adult worms [115]. This suggests that RELM-β is an important factor in inhibiting worm survival, perhaps by interfering with worm chemotaxis and nutrition [113, 115]. Secretion of MUC2, a major component of mucus in both the small and large intestine, is also upregulated during a *H. polygyrus* infection [30]. No evidence for a role for MUC2 in expulsion of *H. polygyrus* has yet been reported; however, MUC2 production correlates with the expulsion of *T. muris* [116, 117] and *N. brasiliensis* [118].

Smooth muscle contraction

Both IL-4 and IL-13 enhance smooth muscle contractility in the small intestine [119], a mechanism that has been shown to be important for resistance to other helminth infections including *Schistosoma mansoni* [120], *T. spiralis* [121, 122], and *N. brasiliensis* [123]. Increased intestinal smooth muscle contractility has been shown after infection with *H. polygyrus* [124]. Both *N. brasiliensis* and *H. polygyrus* infections cause an upregulation of protease-activated receptor $(PAR)_2$ messenger RNA in the small intestine, and a PAR_2 agonist caused smooth muscle contractility, which was enhanced in both parasite-infected groups and, for *N. brasiliensis* at least, was dependent on STAT6 [124]. The infection-induced hypercontractility in the presence of PAR_2 agonist was lost when nerve conduction was blocked using the neurotoxin TXX [124]. Whether smooth muscle hypercontractility plays a critical role in *H. polygyrus* expulsion has yet to be determined.

Granuloma formation

A striking phenomenon in infection is the formation of granulomas around the site of larval invasion in the intestinal tract, and they are more numerous in resistant strains of mice [125], particularly following secondary *H. polygyrus* infection. While granuloma formation is Th2 dependent, their function has yet to be determined, either in damaging larval worms encysted in the submucosal layer of the small intestine or in tissue repair after *H. polygyrus* has departed into the lumen of the gut [126]. Granulomas in both primary and secondary infection consist of neutrophils, macrophages, dendritic cells, and eosinophils; in secondary infection, $CD4^+$ Th2 cells and a high proportion of alternatively activated macrophages rapidly migrate to the site of infection to surround the larvae [80, 84, 85].

Immuno-regulatory cells in chronic infection

Regulatory T cells

Several categories of T cells exert suppressive or immunomodulatory effects, most prominently the subset of Tregs expressing the transcription factor Foxp3. Sustained expression of Foxp3 is required to maintain Treg suppressive function, as in its absence Tregs acquire effector T-cell functions [127], and conversely, the forced expression of Foxp3 confers suppressor function to $CD4^+CD25^-$ T cells [128]. Tregs are essential during infection to protect against immune-mediated pathology while still allowing a sufficiently robust response to clear the pathogen [129]. Indeed, when $Foxp3^+$ T cells are removed at early stages of an infection with *H. polygyrus*, pathology of the small intestine is significantly worse, with higher numbers of effector T cells, IL-4, and IL-13 [130].

Foxp3 is constitutively expressed in a subset of regulatory cells termed natural Tregs, but expression can also be induced in resting $Foxp3^-$ peripheral T cells. Natural Tregs develop in the thymus to limit autoreactive T cells, while inducible Tregs leave the thymus as conventional T cells and are converted through TGF-β, IL-10, and retinoic acid stimulation [129]. Treg induction is particularly favored in the intestine and gut-associated lymphoid tissues, where *H. polygyrus* resides and where TGF-β is highly enriched [32, 131]. Tregs, which express the integrin CD103 ($CD4^+CD25^+CD103^+$), are more suppressive of $CD4^+$ effector cells in vitro and release significantly more IL-10 into culture supernatants after stimulation with *H. polygyrus* primed dendritic cells than $CD4^+CD25^+CD103^-$ Tregs [32].

A strong Treg response develops in the MLN and spleen of *H. polygyrus*-infected mice, peaking at day 28 postinfection [35]. $CD25^+CD103^+$ cells are the subset in the $CD4^+$ compartment that shows the greatest increase in cell number (compared to $CD25^-CD103^-$ effector cells and $CD25^+CD103^-$ cells) [32, 35]. Most significantly, Foxp3 can also be induced in naive T cells by HES in vitro, in a manner analogous to TGF-β, due to

parasite-derived TGF-β-like activity [132] (discussed below). Inhibition of TGF-β signaling during *H. polygyrus* infection using the inhibitor SB431542 reduces adult worm burden and results in an increased Th2 response [132], while administration of anti-TGF-β neutralizing antibody has also been reported to result in lower worm numbers [93]. When TGF-β signaling is lost only on $CD4^+$ T cells, in TGF-βRII DN mice [133], there was no reduction in adult *H. polygyrus* burden compared to wild-type mice; in fact, *H. polygyrus* is more fecund [134, 135]. This is likely due to excessive IFN-γ production in the absence of $CD4^+$ TGF-β signaling, as when IFN-γ-deficient TGF-βRII DN mice were infected with *H. polygyrus*, fewer adult worms survived after 28 days than in IFN-γ-sufficient TGF-βRII DN mice [134], illustrating the importance of both TGF-β and IFN-γ in determining susceptibility to *H. polygyrus*.

H. polygyrus infection also induces $CD8^+$ Tregs in the lamina propria of the small intestine, which can inhibit T-cell proliferation in vitro in an IL-10 and TGF-β independent manner [36, 136].

Regulatory B cells

In addition to Tregs, regulatory B cells (Bregs) have also been described that produce IL-10 and TGF-β, and can dampen potentially harmful immune responses [137]. Bregs induced during helminth infections can not only downregulate pathology elicited by schistosome eggs [138] but also ameliorate immunopathologies such as multiple sclerosis [139] and anaphylaxis [140] in humans and mice. While the role of Bregs in parasite persistence has not been directly investigated in *H. polygyrus* infection, suppressive B cells expand in the MLN of infected C57BL/6 mice, which on transfer to uninfected hosts, suppress airway allergy and inflammation in experimental autoimmune encephalomyelitis [56].

Proregulatory DCs

Different subsets of DCs have been identified, which are markedly altered during helminth infection. In the MLN of *H. polygyrus*-infected mice, the proportion of $CD11c^{high}CD8\alpha^{intermediate}$ DCs declines in infection, indicating a reduced migration of cells from the lamina propria [141]. Moreover, there is a sharp increase in the proportion of tolerogenic $CD11c^{lo}$ DCs in the MLN; this cell type responds suboptimally to Toll-like receptor stimulation, is unable to prime a strong Th2 response from T cells, but induces much higher proportions of $CD4^+CD25^-$ to express Foxp3 than the conventional $CD11c^{hi}$ subset [142, 143].

This effect was mirrored in an in vitro setting when OVA-pulsed bone marrow-derived DCs (BMDCs) were cultured with HES and showed lower costimulatory molecule expression and cytokine output compared to untreated OVA-pulsed BMDCs [144]. These cells also induced IL-10-secreting $CD4^+CD25^+Foxp3^+$ cell generation from $CD4^+$ cocultures [144], indicating a potential regulatory pathway initiated by *H. polygyrus* products.

Vaccine-induced immunity

Irradiated *H. polygyrus* larvae given orally stimulate protection against subsequent challenge [62, 145–148]. Notably, the efficacy of this irradiated larval vaccine is diminished by the coadministration of unirradiated larvae, indicating that the development of adult worms is able to inhibit development and/or expression of protective immunity against subsequent reinfection [146, 147]. The ability of adult worms to suppress protective immunity was further demonstrated by vaccine failure in mice given irradiated larvae before or after receiving adult parasites by intraintestinal laparatomy [146] or oral gavage [149].

As well as infective (L3) stage larvae, live L4 larvae isolated on days 4 or 6 postinfection from the intestinal wall of donor-infected animals given subcutaneously, elicit an ~95–100 % reduction in worms present 3 weeks after challenge compared to unimmunized controls [150]. When immunization was performed with late-stage L3 (isolated 2 days postinfection) or L5 larvae (isolated 8 days postinfection) a lower degree of protective immunity was induced (~60 and ~70 % reduction respectively compared to unimmunized controls) [150].

Recently, an effective nonliving vaccine against *H. polygyrus* has been developed, in the form of total HES administered with alum adjuvant, which induced sterile immunity against infection [21]. Earlier work had shown that mice immunized with a 60,000 mol wt HES-derived glycoprotein isolated from HES prior to infection had lower egg burdens than control mice, indicating an antifecundity effect of vaccination with this component [151].

Molecular basis of chronic infection

Parasite excretory–secretory products

The ability of helminth parasites to persist in the host for many months or years, evading host immunity, is most likely due to the secretion of active immunomodulatory molecules [152]. The secretome of a parasite is likely to continually mediate interactions with the host, through direct contact with host cells in proximity to the worm, and potentially systemically. Helminth-secreted immunomodulators have been intensely studied, with some candidates now being tested for treatments of other diseases and as targets for antiparasite drugs [153].

Early investigation of HES found immunomodulatory factors that suppressed proliferation of mitogen-stimulated lymphocytes [154] and Th2-dependent antibody production to a bystander antigen through effects on T cells [155]. More recently, HES has been shown to display a wide range of immunomodulatory activities, including inhibiting activation of DCs [144, 156], induction of Tregs [132], and suppression of airway allergic inflammation [157].

The individual components of the complex HES mixture from adult worms have recently been defined through proteomic and sequencing technology with the identification of several hundred proteins in HES [5, 158]. Most prominent and numerous among the HES products are >20 members of the Venom allergen/*Ancylostoma* secreted protein-like (VAL) multigene family, which show extensive sequence variation between genes [5]. VALs have also been found to be highly immunodominant as indicated by recognition of primary and secondary infection serum, and monoclonal antibodies [21], although their function is as yet undefined [5, 158]. These studies also found acetylcholinesterases and proteases to be abundant in HES [5] along with apyrases, lipid-binding proteins, lysosymes, globins, and vitellogenin homologues [5, 158].

Stage and sex specificity of HES

A small number of studies have identified lifecycle-stage-specific expression patterns of certain HES antigens. Infective larval stages of *H. polygyrus* secreted the highest levels of proteolytic enzymes and acetylcholinesterase [159, 160], which may be involved in migration through host tissues directly after infection. Calreticulin has been shown to be highly expressed in L4 larval stages and is localized in areas associated with excretory–secretory processes [161]. A TGF-β homologue has been shown to be abundantly expressed in adults compared to larval stages, which may indicate an immunomodulatory function when the adults reside in the lumen of the intestine for long periods of time [162]. A limited number of sex-specific adult antigens in both HES and on the cuticle of the worms have also been found [163].

H. polygyrus, autoimmunity and allergy

The immunomodulatory properties of *H. polygyrus*, which extend far beyond the site of infection alone, have led to many investigations of the potential for and mechanisms of parasite downregulation of allergic and autoimmune conditions, as discussed below, as well as in the modulation of coinfections with other pathogens (reviewed in [1]).

Allergy

H. polygyrus offers protection in several murine models of allergy, including intestinal, airway, and cutaneous reactions. Mice fed peanut extract administered alongside the mucosal adjuvant cholera toxin produced peanut specific IgE had elevated plasma histamine levels and exhibited systemic anaphylactic shock symptoms. All phenotypes were diminished in *H. polygyrus*-infected mice [164]. In the presence of *H. polygyrus*, the peanut antigen-specific IL-13 levels were drastically reduced, and these dampened IL-13 levels along with protection from peanut allergy were lost when mice were treated with neutralizing IL-10 antibody [164]. The source of IL-10 and the mechanism by which it acts to dampen allergic responses to peanut antigen during *H. polygyrus* infection have not yet been determined.

H. polygyrus-infected mice had reduced inflammatory cell infiltrates and bronchoalveolar lavage eosinophilia in experimentally induced airway allergy to both ovalbumin [165–167] and the house dust mite antigen Der p 1 [166]. Protection against these allergens could be transferred by MLN cells from infected mice, which contained a high proportion of $CD4^+CD25^+Foxp3^+$ T cells, or by transfer of sorted $CD4^+CD25^+$ cells from infected mice, implicating the action of Treg cells in protection [166]. *H. polygyrus*-infected IL-$10^{-/-}$ mice were not protected from ovalbumin-induced asthma [165]; however, MLN cells transferred from IL-$10^{-/-}$ *H. polygyrus* infected mice could still protect from allergy to these antigens, suggesting that IL-10 independent mechanisms can confer protection from allergy [166].

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterized by an inappropriate inflammatory response of the gut to microbial antigens. In humans, there are two main forms of the disease: Crohn's disease (CD), which can affect the entire length of the gut, and ulcerative colitis (UC), which is localized only to the colon. There are many differing mouse models of IBD (reviewed in [168]), and the effect of *H. polygyrus* infection on controlling the disease has been examined in a number of these models.

IL-$10^{-/-}$ mice suffer from spontaneous chronic colitis [169] associated with excessive IFN-γ production [170]. Spontaneous colitis develops sporadically over several months, but piroxicam treatment will induce rapid and uniform disease in IL-10-deficient mice [171, 172], which likely occurs as a result of increased colonic epithelial cell apoptosis causing a loss of barrier function to inflammatory microbial stimuli [172]. When *H. polygyrus* was given to piroxicam-treated IL-$10^{-/-}$ mice, the histological scores of colitis severity were drastically reduced within 14 days [173, 174]. LPMC from uninfected colitic mice released

the inflammatory cytokines IFN-γ, IL-12p40, and IL-17A, whereas LPMC from *H. polygyrus*-infected had significantly reduced levels of these cytokines [173, 174].

Severe colitis also develops when $RAG^{-/-}$ mice are reconstituted with $IL\text{-}10^{-/-}$ T cells and treated with piroxicam [175]. *H. polygyrus* colonization reduced gut inflammation in this model, as shown by lower levels of IFN-γ and IL-17 production by restimulated LPMC cells, and a drop in colonic histological score from an average of above 3 (some epithelial and muscle hypertrophy, mucus depletion, crypt abscesses, and epithelial erosions) to less than 1 (some mononuclear cell infiltrates in the lamina propria) [136, 176]. In $RAG^{-/-}$ mice that had been infected with *H. polygyrus*, and subsequently drug-cleared of the infection prior to transfer of the colitogenic $IL\text{-}10^{-/-}$ T cells and piroxicam administration, mice still showed reduced levels of inflammation compared to those that had never been infected [176]. The authors reported that protection coincided with downregulation of the costimulatory molecules CD80 and CD86 on DCs, thus inhibiting antigen presentation to T cells resulting in less inflammatory cytokine release [176].

TGF-βRII DN mice develop spontaneous colitis, which is unable to be suppressed by *H. polygyrus* infection [135]. The inability of *H. polygyrus* to suppress colitis in this model is likely due to the exacerbated IFN-γ levels seen in TGF-βRII DN mice, which are not dampened during *H. polygyrus* infection [135] and are known to aggravate intestinal inflammation.

Intrarectal injection of trinitrobenzene sulfonic acid (TNBS) administration also induces severe colitis in wild-type mice. When mice that had been infected with *H. polygyrus* for 10 days were given TNBS injection, they exhibited markedly reduced TNBS-induced colonic damage and inflammation and decreased Th1 cytokine mRNA expression compared to uninfected control mice, which was accompanied by increased IL-10 secretion during *H. polygyrus* infection [36, 177].

In contrast to other models of colitis, *H. polygyrus* seems to intensify colitis caused by the murine bacterial pathogen *Citrobacter rodentium* [178–180]. Disease exacerbation could be due to the influx of alternatively activated macrophages during *H. polygyrus* infection, which are less able to kill bacteria than classically activated macrophages [178], or due to increased IL-10 production by DCs impairing mechanisms that kill *C. rodentium*, leading to more persistent infection and colitis [180]. These studies exemplify the need to understand the causes of colitis, and the mechanisms by which helminths modulate disease progression, before helminth therapy can be applied to human inflammatory bowel diseases.

H. polygyrus is not the only parasitic nematode shown to have modulatory effects on the onset of colitis; both *T. spiralis* and excretory–secretory products from the hookworms *Ancylostoma caninum* and *Ancylostoma ceylanicum* can also ameliorate colitis progression in murine models, as reviewed in [168]. In human clinical trials, ova from the pig intestinal helminth parasite *Trichuris suis* reduced the severity of disease in some patients with UC and CD [181–183]. Although initial clinical trials with *T. suis* are promising, the fact that the therapy is not effective in 100 % of patients illustrates the need for further studies to understand the immunomodulatory actions of these helminths in murine models of IBD.

Type 1 diabetes

Nonobese diabetic (NOD) mice spontaneously become diabetic (as measured by blood glucose levels of ≥ 200 mg/dl) by 25 weeks of age [184, 185]. When these mice are infected with *H. polygyrus* at 5 weeks old, the onset of diabetes was completely blocked, at least until 40 weeks of age [184, 185]. Administering *H. polygyrus* when NOD mice were 7 and 12 weeks of age resulted in less effective protection from diabetes, yet onset was still delayed compared to untreated NOD mice [185]. The severity of insulitis (the infiltration of immune cells into the islets of Langerhans) was examined in mice aged 13 weeks, and was sharply reduced in NOD mice infected with *H. polygyrus* since the age of 5 weeks [185]. This reduction was maintained in *H. polygyrus* mice given anti-CD25 antibody [185], suggesting that *H. polygyrus* modulates type 1 diabetes (T1D) onset in a Treg-independent manner, although whether this protection extends beyond the 13-week time point has not been examined.

There is the possibility that the modulatory effects of *H. polygyrus* are due in part to changes in gut microbial composition during infection [186]. Studies to modify the microbial flora could address this, perhaps using fecal transplants, which would allow transfer of the microflora from *H. polygyrus* infected or naive mice to recipient mice using methods described in [187].

Role of the microbiota

H. polygyrus is localized in the anterior small intestine alongside a substantial microbial flora. The presence of specific species of bacteria within the gut is known to polarise naive T cells towards particular Th subset fates [188–190], and as the outcome of *H. polygyrus* infection is dependent on the immediate cytokine environment, it seems reasonable to imagine that commensal microbes may alter the ability of the murine immune system to cause worm expulsion.

Care must therefore be taken when performing experiments to compare the susceptibility to *H. polygyrus* in different mice, as the mice may initially differ in their microbial flora. Variation in microbial flora may be due to the source of mice, as mice of the same strain acquired from different vendors can

harbor different gut microbes [188], perhaps due to diet or housing conditions. The genotype of mice can also control microbial populations, as has been shown to be the case for MyD88-deficient mice, which have an altered microbial flora compared to MyD88-sufficient mice as MyD88 controls the release of some antimicrobial peptides [191].

After a 14-day infection with *H. polygyrus* in C57BL/6 mice, the abundance of *Lactobacillaceae* family members was increased in the ileum compared to naive mice [186]. It has yet to be demonstrated whether this shift is a helminth-mediated mechanism that acts to promote the survival of *H. polygyrus* within the murine host, or if it is simply as a consequence of a changing immune environment, in which bacteria of the *Lactobacillaceae* family are better able to survive. To resolve this, further studies to investigate the interplay among parasitic helminths, the microbial flora, and the immune system are necessary.

Conclusions and implications for human infections and disease

Parasitic helminth infections in humans and livestock are still responsible for unacceptably high levels of morbidity and economic loss worldwide. Understanding the mechanisms necessary for expulsion of the model gastrointestinal parasite *H. polygyrus* is likely to define new pathways, which target the immune system to provide the best protection against other helminth infections. The increasing prevalence of autoimmune diseases in the Western world correlates with the increasing absence of such helminth infections [192]. Our immune systems have evolved to develop in the presence of helminth parasite antigens, and it is vital to understand whether the human immune system can function optimally without this presence. Studies to isolate and understand how immunomodulatory factors secreted by helminths such as *H. polygyrus* act to maintain gut homeostasis are ongoing and will be invaluable both in understanding the interactions between helminths and the immune system and in the development of new pharmaceutical therapies for autoimmune and allergic diseases worldwide.

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References

1. Maizels RM, Hewitson JP, Murray J, Harcus Y, Dayer B, Filbey KJ, Grainger JR, McSorley HJ, Reynolds LA, Smith KA (2012) Immune modulation and modulators in *Heligmosomoides polygyrus* infection. Exp Parasitol 132:76–89
2. Maizels RM, Hewitson JP, Smith KA (2012) Susceptibility and immunity to helminth parasites. Curr Opin Immunol 25:459–466
3. Gouy de Bellocq J, Ferte H, Depaquit J, Justine JL, Tillier A, Durette-Desset MC (2001) Phylogeny of the *Trichostrongylina* (Nematoda) inferred from 28S rDNA sequences. Mol Phylogenet Evol 19:430–442
4. Bansemir AD, Sukhdeo MVK (1994) The food resource of adult *Heligmosomoides polygyrus* in the small intestine. J Parasitol 80:24–28
5. Hewitson JP, Harcus Y, Murray J, van Agtmaal M, Filbey KJ, Grainger JR, Bridgett S, Blaxter ML, Ashton PD, Ashford DA, Curwen RS, Wilson RA, Dowle AA, Maizels RM (2011) Proteomic analysis of secretory products from the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of Venom Allergen-Like (VAL) proteins. Journal of Proteomics 74:1573–1594
6. Behnke JM, Robinson M (1985) Genetic control of immunity to *Nematospiroides dubius*: a 9-day anthelmintic abbreviated immunizing regime which separates weak and strong responder strains of mice. Parasite Immunol 7:235–253
7. Behnke JM, Wahid FN (1991) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius): H-2 linked genes determine worm survival. Parasitology 103(Pt 1):157–164*
8. Enriquez FJ, Zidian JL, Cypess RH (1988) *Nematospiroides dubius*: genetic control of immunity to infections of mice. Exp Parasitol 67:12–19
9. Wahid FN, Behnke JM (1993) Immunological relationships during primary infection with *Heligmosomoides polygyrus*. Regulation of fast response phenotype by H-2 and non-H-2 genes. Parasitology 107:343–350
10. Behnke JM, Iraqi F, Menge D, Baker RL, Gibson J, Wakelin D (2003) Chasing the genes that control resistance to gastrointestinal nematodes. J Helminthol 77:99–110
11. Dobson C, Owen ME (1978) Effect of host sex on passive immunity in mice infected with *Nematospiroides dubius*. Int J Parasitol 8:359–364
12. Wahid FN, Behnke JM (1993) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius): parasite specific IgG1 antibody responses and primary response phenotype. Parasite Immunol 15:401–413*
13. Van Zandt PD, Cypess RH, Zidian JL (1973) Development of age and sex resistance to *Nematospiroides dubius* in the mouse following single and multiple infections. J Parasitol 59:977–979
14. Zhong S, Dobson C (1996) *Heligmosomoides polygyrus:* resistance in inbred, outbred, and selected mice. Exp Parasitol 82:122–131
15. Odiere MR, Koski KG, Weiler HA, Scott ME (2010) Concurrent nematode infection and pregnancy induce physiological responses that impair linear growth in the murine foetus. Parasitology 137:991–1002
16. Kristan DM (2002) Effects of intestinal nematodes during lactation: consequences for host morphology, physiology and offspring mass. J Exp Biol 205:3955–3965
17. Crump A, Omura S (2011) Ivermectin, 'wonder drug' from Japan: the human use perspective. Proc Jpn Acad Ser B Phys Biol Sci 87:13–28
18. Finkelman FD, Shea-Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden KB, Gause WC, Urban JF Jr (1997) Cytokine

regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. Annu Rev Immunol 15:505–533
19. Scott ME (1991) *Heligmosomoides polygyrus* (Nematoda): susceptible and resistant strains of mice are indistinguishable following natural infection. Parasitology 103:429–438
20. Prowse SJ, Mitchell GF, Ley PL, Jenkin CR (1979) The development of resistance in different inbred strains of mice to infection with *Nematospiroides dubius*. Parasite Immunology 1:277–288
21. Hewitson JP, Filbey KJ, Grainger JR, Dowle AA, Pearson M, Murray J, Harcus Y, Maizels RM (2011) *Heligmosomoides polygyrus* elicits a dominant nonprotective antibody response directed at restricted glycan and peptide epitopes. J Immunol 187:4764–4777
22. Brailsford TJ, Behnke JM (1992) The dynamics of trickle infections with *Heligmosomoides polygyrus* in syngeneic strains of mice. Int J Parasitol 22:351–359
23. Behnke J, Harris PD (2010) *Heligmosomoides bakeri:* a new name for an old worm? Trends Parasitol 26:524–529
24. Behnke JM, Keymer AE, Lewis JW (1991) *Heligmosomoides polygyrus* or *Nematospiroides dubius* ? Parasitology Today 7:177–179
25. Cable J, Harris PD, Lewis JW, Behnke JM (2006) Molecular evidence that *Heligmosomoides polygyrus* from laboratory mice and wood mice are separate species. Parasitology 133:111–122
26. Maizels RM, Hewitson JP, Gause WC (2011) *Heligmosomoides polygyrus*: one species still. Trends Parasitol 27:100–101
27. Tang J, Dobson C, McManus DP (1995) Antigens in phenotypes of *Heligmosomoides polygyrus* raised selectively from different strains of mice. Int J Parasitol 25:847–852
28. Morgan C, LaCourse EJ, Rushbrook BJ, Greetham D, Hamilton JV, Barrett J, Bailey K, Brophy PM (2006) Plasticity demonstrated in the proteome of a parasitic nematode within the intestine of different host strains. Proteomics 6:4633–4645
29. Urban JF Jr, Maliszewski CR, Madden KB, Katona IM, Finkelman FD (1995) IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice. J Immunol 154:4675–4684
30. Hashimoto K, Uchikawa R, Tegoshi T, Takeda K, Yamada M, Arizono N (2009) Depleted intestinal goblet cells and severe pathological changes in SCID mice infected with *Heligmosomoides polygyrus*. Parasite Immunol 31:457–465
31. Urban JF Jr, Katona IM, Finkelman FD (1991) *Heligmosomoides polygyrus:* CD4+ but not CD8+ T cells regulate the IgE response and protective immunity in mice. Exp Parasitol 73:500–511
32. Rausch S, Huehn J, Kirchhoff D, Rzepecka J, Schnoeller C, Pillai S, Loddenkemper C, Scheffold A, Hamann A, Lucius R, Hartmann S (2008) Functional analysis of effector and regulatory T cells in a parasitic nematode infection. Infect Immun 76:1908–1919
33. Harris N, Gause WC (2011) To B or not to B: B cells and the Th2-type immune response to helminths. Trends Immunol 32:80–88
34. Svetić A, Madden KB, di Zhou X, Lu P, Katona IM, Finkelman FD, Urban JF Jr, Gause WC (1993) A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. J Immunol 150:3434–3441
35. Finney CAM, Taylor MD, Wilson MS, Maizels RM (2007) Expansion and activation of $CD4^{+}CD25^{+}$ regulatory T cells in *Heligmosomoides polygyrus* infection. Eur J Immunol 37:1874–1886
36. Setiawan T, Metwali A, Blum AM, Ince MN, Urban JF Jr, Elliott DE, Weinstock JV (2007) *Heligmosomoides polygyrus* promotes regulatory T-cell cytokine production in the murine normal distal intestine. Infect Immun 75:4655–4663
37. Mohrs K, Harris DP, Lund FE, Mohrs M (2005) Systemic dissemination and persistence of Th2 and type 2 cells in response to infection with a strictly enteric nematode parasite. J Immunol 175:5306–5313
38. Doligalska M, Donskow-Schmelter K, Rzepecka J, Drela N (2007) Reduced apoptosis in BALB/c mice infected with *Heligmosomoides polygyrus*. Parasite Immunol 29:283–291
39. Lu P, di Zhou X, Chen S-J, Moorman M, Morris SC, Finkelman FD, Lionsley P, Urban JF, Gause WC (1994) CTLA-4 ligands are required to induce an in vivo interleukin 4 response to a gastrointestinal nematode parasite. Journal of Experimental Medicine 180:693–698
40. Greenwald RJ, Lu P, Halvorson MJ, Zhou X, Chen S, Madden KB, Perrin PJ, Morris SC, Finkelman FD, Peach R, Linsley PS, Urban JF Jr, Gause WC (1997) Effects of blocking B7-1 and B7-2 interactions during a type 2 in vivo immune response. J Immunol 158:4088–4096
41. Greenwald RJ, Urban JF, Ekkens MJ, Chen S, Nguyen D, Fang H, Finkelman FD, Sharpe AH, Gause WC (1999) B7-2 is required for the progression but not the initiation of the type 2 immune response to a gastrointestinal nematode parasite. J Immunol 162:4133–4139
42. Gause WC, Chen SJ, Greenwald RJ, Halvorson MJ, Lu P, Zhou XD, Morris SC, Lee KP, June CH, Finkelman FD, Urban JF, Abe R (1997) CD28 dependence of T cell differentiation to IL-4 production varies with the particular type 2 immune response. J Immunol 158:4082–4087
43. Gause WC, Lu P, Di Zhou X, Chen S-J, Madden KB, Morris SC, Linsley PS, Finkelman FD, Urban JF (1996) *H.polygyrus*: B7-independence of the secondary type 2 response. Exp Parasitol 84:264–273
44. Ekkens MJ, Liu Z, Liu Q, Foster A, Whitmire J, Pesce J, Sharpe AH, Urban JF, Gause WC (2002) Memory Th2 effector cells can develop in the absence of B7-1/B7-2, CD28 interactions, and effector Th cells after priming with an intestinal nematode parasite. J Immunol 168:6344–6351
45. Ekkens MJ, Liu Z, Liu Q, Whitmire J, Xiao S, Foster A, Pesce J, VanNoy J, Sharpe AH, Urban JF, Gause WC (2003) The role of OX40 ligand interactions in the development of the Th2 response to the gastrointestinal nematode parasite *Heligmosomoides polygyrus*. J Immunol 170:384–393
46. King C, Tangye SG, Mackay CR (2008) T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol 26:741–766
47. King IL, Mohrs M (2009) IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. J Exp Med 206:1001–1007
48. Yi JS, Cox MA, Zajac AJ (2010) Interleukin-21: a multifunctional regulator of immunity to infections. Microbes Infect 12:1111–1119
49. Fröhlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, Kopf M (2007) IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood 109:2023–2031
50. King IL, Mohrs K, Mohrs M (2010) A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. J Immunol 185:6138–6145
51. Ben-Smith A, Lammas DA, Behnke JM (2003) The relative involvement of Th1 and Th2 associated immune responses in the expulsion of a primary infection of *Heligmosomoides polygyrus* in mice of differing response phenotype. J Helminthol 77:133–146
52. Wahid FN, Behnke JM, Grencis RK, Else KJ, Ben-Smith AW (1994) Immunological relationships during primary infection with *Heligmosomoides polygyrus:* Th2 cytokines and primary response phenotype. Parasitology 108:461–471
53. Behnke JM, Lowe A, Clifford S, Wakelin D (2003) Cellular and serological responses in resistant and susceptible mice exposed to

repeated infection with *Heligmosomoides polygyrus bakeri*. Parasite Immunol 25:333–340
54. Alugupalli KR, Abraham D (2009) B cell multitasking is required to control nematode infection. Immunity 30:317–319
55. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE (2000) Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 1:475–482
56. Wilson MS, Taylor MD, O'Gorman MT, Balic A, Barr TA, Filbey K, Anderton SM, Maizels RM (2010) Helminth-induced $CD19^{+}CD23^{hi}$ B cells modulate experimental allergic and autoimmune inflammation. Eur J Immunol 40:1682–1696
57. Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, Kusser K, Honjo T, Mohrs K, Mohrs M, Randall T, Lund FE (2009) Cytokine-producing effector B cells regulate type 2 immunity to *H. polygyrus*. Immunity 30:421–433
58. Liu Q, Kreider T, Bowdridge S, Liu Z, Song Y, Gaydo AG, Urban JF Jr, Gause WC (2010) B cells have distinct roles in host protection against different nematode parasites. J Immunol 184:5213–5223
59. McCoy KD, Stoel M, Stettler R, Merky P, Fink K, Senn BM, Schaer C, Massacand J, Odermatt B, Oettgen HC, Zinkernagel RM, Bos NA, Hengartner H, Macpherson AJ, Harris NL (2008) Polyclonal and specific antibodies mediate protective immunity against enteric helminth infection. Cell Host Microbe 4:362–373
60. Blackwell NM, Else KJ (2001) B cells and antibodies are required for resistance to the parasitic gastrointestinal nematode *Trichuris muris*. Infect Immun 69:3860–3868
61. Pritchard DI, Williams DJL, Behnke JM, Lee TDG (1983) The role of IgG1 hypergammaglobulinaemia in immunity to the gastrointestinal nematode *Nematospiroides dubius.* The immunochemical purification, antigen-specificity and in vivo antiparasite effect of IgG1 from immune serum. Immunology 49:353–365
62. Prowse SJ, Ey PL, Jenkin CR (1978) Immunity to *Nematospiroides dubius*: cell and immunoglobulin changes associated with the onset of immunity in mice. Aust J Exp Biol Med Sci 56:237–246
63. Chapman CB, Knopf PM, Hicks JD, Mitchell GF (1979) IgG1 hypergammaglobulinaemia in chronic parasitic infections in mice: magnitude of the response in mice infected with various parasites. Aust J Exp Biol Med Sci 57:369–387
64. Williams DJ, Behnke JM (1983) Host protective antibodies and serum immunoglobulin isotypes in mice chronically infected or repeatedly immunized with the nematode parasite *Nematospiroides dubius*. Immunology 48:37–47
65. Harris NL, Spoerri I, Schopfer JF, Nembrini C, Merky P, Massacand J, Urban JF Jr, Lamarre A, Burki K, Odermatt B, Zinkernagel RM, Macpherson AJ (2006) Mechanisms of neonatal mucosal antibody protection. J Immunol 177:6256–6262
66. Allen JE, Maizels RM (2011) Diversity and dialogue in immunity to helminths. Nat Rev Immunol 11:375–388
67. Neill DR, McKenzie ANJ (2011) Nuocytes and beyond: new insights into helminth expulsion. Trends Parasitol 27:214–221
68. MacDonald AS, Maizels RM (2008) Alarming dendritic cells for Th2 induction. J Exp Med 205:13–17
69. Balic A, Harcus Y, Holland MJ, Maizels RM (2004) Selective maturation of dendritic cells by *Nippostrongylus brasiliensis* secreted proteins drives T helper type 2 immune responses. Eur J Immunol 34:3047–3059
70. Liu JY, Li LY, Yang XZ, Li J, Zhong G, Wang J, Li LJ, Ji B, Wu ZQ, Liu H, Yang X, Liu PM (2011) Adoptive transfer of DCs isolated from helminth-infected mice enhanced T regulatory cell responses in airway allergic inflammation. Parasite Immunol 33:525–534
71. Hochweller K, Striegler J, Hammerling GJ, Garbi N (2008) A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells. Eur J Immunol 38:2776–2783
72. Phythian-Adams AT, Cook PC, Lundie RJ, Jones LH, Smith KA, Barr TA, Hochweller K, Anderton SM, Hämmerling GJ, Maizels RM, MacDonald AS (2010) CD11c depletion severely disrupts Th2 induction and development in vivo. J Exp Med 207:2089–2096
73. Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, Voehringer D (2009) Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J Exp Med 206:549–559
74. Anthony RM, Rutitzky LI, Urban JF Jr, Stadecker MJ, Gause WC (2007) Protective immune mechanisms in helminth infection. Nat Rev Immunol 7:975–987
75. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K (1995) Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur J Immunol 25:1101–1104
76. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, Pearce EJ, Wynn TA (2001) Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: Granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol 167:6533–6544
77. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF, Cheever AW, Young DA, Collins M, Grusby MJ, Wynn TA (2006) The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J Clin Invest 116:2044–2055
78. Park-Min KH, Antoniv TT, Ivashkiv LB (2005) Regulation of macrophage phenotype by long-term exposure to IL-10. Immunobiology 210:77–86
79. Mylonas KJ, Nair MG, Prieto-Lafuente L, Paape D, Allen JE (2009) Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. J Immunol 182:3084–3094
80. Anthony RM, Urban JF Jr, Alem F, Hamed HA, Rozo CT, Boucher JL, Van Rooijen N, Gause WC (2006) Memory T_H2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med 12:955–960
81. Chang N-CA, Hung S-I, Hwa K-Y, Kato I, Chen J-E, Liu C-H, Chang AC (2001) A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. J Biol Chem 276:17497–17506
82. Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M, Allison JP, Allen JE (2007) Alternative activation is an innate response to injury that requires $CD4^{+}$ T cells to be sustained during chronic infection. J Immunol 179:3926–3936
83. Zhao A, Urban JF Jr, Anthony RM, Sun R, Stiltz J, van Rooijen N, Wynn TA, Gause WC, Shea-Donohue T (2008) Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. Gastroenterology 135 (217–25):e1
84. Morimoto M, Morimoto M, Whitmire J, Xiao S, Anthony RM, Mirakami H, Star RA, Urban JF Jr, Gause WC (2004) Peripheral CD4 T cells rapidly accumulate at the host: parasite interface during an inflammatory Th2 memory response. J Immunol 172:2424–2430
85. Patel N, Kreider T, Urban JF Jr, Gause WC (2009) Characterisation of effector mechanisms at the host: parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. Int J Parasitol 39:13–21
86. Galioto AM, Hess JA, Nolan TJ, Schad GA, Lee JJ, Abraham D (2006) Role of eosinophils and neutrophils in innate and adaptive protective immunity to larval strongyloides stercoralis in mice. Infect Immun 74:5730–5738
87. O'Connell AE, Hess JA, Santiago GA, Nolan TJ, Lok JB, Lee JJ, Abraham D (2011) Major basic protein from eosinophils and

myeloperoxidase from neutrophils are required for protective immunity to *Strongyloides stercoralis* in mice. Infect Immun 79:2770–2778
88. Yu C, Cantor AB, Yang H, Browne C, Wells RA, Fujiwara Y, Orkin SH (2002) Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J Exp Med 195:1387–1395
89. Knott ML, Matthaei KI, Giacomin PR, Wang H, Foster PS, Dent LA (2007) Impaired resistance in early secondary *Nippostrongylus brasiliensis* infections in mice with defective eosinophilopoeisis. Int J Parasitol 37:1367–1378
90. Khan WI, Abe T, Ishikawa N, Nawa Y, Yoshimura K (1995) Reduced amount of intestinal mucus by treatment with anti-CD4 antibody interferes with the spontaneous cure of *Nippostrongylus brasiliensis*-infection in mice. Parasite Immunology 17:485–491
91. Rennick DM, Thompson-Snipes L, Coffman RL, Seymour BW, Jackson JD, Hudak S (1990) In vivo administration of antibody to interleukin-5 inhibits increased generation of eosinophils and their progenitors in bone marrow of parasitized mice. Blood 76:312–316
92. Urban JF Jr, Katona IM, Paul WE, Finkelman FD (1991) Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proceedings of the National Academy of Sciences USA 88:5513–5517
93. Doligalska M, Rzepecka J, Drela N, Donskow K, Gerwel-Wronka M (2006) The role of TGF-β in mice infected with *Heligmosomoides polygyrus*. Parasite Immunol 28:387–395
94. Ohnmacht C, Voehringer D (2009) Basophil effector function and homeostasis during helminth infection. Blood 113:2816–2825
95. Perrigoue JG, Saenz SA, Siracusa MC, Allenspach EJ, Taylor BC, Giacomin PR, Nair MG, Du Y, Zaph C, van Rooijen N, Comeau MR, Pearce EJ, Laufer TM, Artis D (2009) MHC class II-dependent basophil-CD4$^+$ T cell interactions promote TH2 cytokine-dependent immunity. Nat Immunol 10:697–705
96. Behnke JM, Wahid FN, Grencis RK, Else KJ, Ben-Smith AW, Goyal PK (1993) Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms. Parasite Immunology 15:415–421
97. Snoek SA, Dhawan S, van Bree SH, Cailotto C, van Diest SA, Duarte JM, Stanisor OI, Hilbers FW, Nijhuis L, Koeman A, van den Wijngaard RM, Zuurbier CJ, Boeckxstaens GE, de Jonge WJ (2012) Mast cells trigger epithelial barrier dysfunction, bacterial translocation and postoperative ileus in a mouse model. Neurogastroenterol Motil 24:172–191
98. McDermott JR, Bartram RE, Knight PA, Miller HR, Garrod DR, Grencis RK (2003) Mast cells disrupt epithelial barrier function during enteric nematode infection. Proc Natl Acad Sci U S A 100:7761–7766
99. Dehlawi MS, Wakelin D, Behnke JM (1987) Suppression of mucosal mastocytosis by infection with the intestinal nematode *Nematospiroides dubius*. Parasite Immunology 9:187–194
100. Hashimoto K, Uchikawa R, Tegoshi T, Takeda K, Yamada M, Arizono N (2010) Immunity-mediated regulation of fecundity in the nematode *Heligmosomoides polygyrus*—the potential role of mast cells. Parasitology 137:881–887
101. Morimoto M, Utsumiya K (2011) Enhanced protection against *Heligmosomoides polygyrus* in IL-2 receptor β-chain overexpressed transgenic mice with intestinal mastocytosis. J Vet Med Sci 73:849–851
102. Hepworth MR, Danilowicz-Luebert E, Rausch S, Metz M, Klotz C, Maurer M, Hartmann S (2012) Mast cells orchestrate type 2 immunity to helminths through regulation of tissue-derived cytokines. Proc Natl Acad Sci U S A 109:6644–6649
103. Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ (2005) Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. Am J Pathol 167:835–848
104. Barlow JL, McKenzie AN (2011) Nuocytes: expanding the innate cell repertoire in type-2 immunity. J Leukoc Biol 90:867–874
105. Ikeda K, Nakajima H, Suzuki K, Kagami S, Hirose K, Suto A, Saito Y, Iwamoto I (2003) Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. Blood 101:3594–3596
106. Paul WE, Zhu J (2010) How are T_H2-type immune responses initiated and amplified? Nat Rev Immunol 10:225–235
107. Wills-Karp M, Rani R, Dienger K, Lewkowich I, Fox JG, Perkins C, Lewis L, Finkelman FD, Smith DE, Bryce PJ, Kurt-Jones EA, Wang TC, Sivaprasad U, Hershey GK, Herbert DR (2012) Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. J Exp Med 209:607–622
108. Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, McIlgorm A, Jolin HE, McKenzie ANJ (2006) Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. J Exp Med 203:1105–1116
109. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, Bucks C, Kane CM, Fallon PG, Pannell R, Jolin HE, McKenzie ANJ (2010) Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature 464:1367–1370
110. Saenz SA, Siracusa MC, Perrigoue JG, Spencer SP, Urban JF Jr, Tocker JE, Budelsky AL, Kleinschek MA, Kastelein RA, Kambayashi T, Bhandoola A, Artis D (2010) IL25 elicits a multipotent progenitor cell population that promotes T_H2 cytokine responses. Nature 464:1362–1366
111. Shea-Donohue T, Sullivan C, Finkelman FD, Madden KB, Morris SC, Goldhill J, Pineiro-Carrero V, Urban JF Jr (2001) The role of IL-4 in *Heligmosomoides polygyrus*-induced alterations in murine intestinal epithelial cell function. J Immunol 167:2234–2239
112. Madden KB, Yeung KA, Zhao A, Gause WC, Finkelman FD, Katona IM, Urban JF Jr, Shea-Donohue T (2004) Enteric nematodes induce stereotypic STAT6-dependent alterations in intestinal epithelial cell function. J Immunol 172:5616–5621
113. Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD (2004) RELMβ/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. Proceedings of the National Academy of Sciences USA 101:13596–13600
114. Hogan SP, Seidu L, Blanchard C, Groschwitz K, Mishra A, Karow ML, Ahrens R, Artis D, Murphy AJ, Valenzuela DM, Yancopoulos GD, Rothenberg ME (2006) Resistin-like molecule beta regulates innate colonic function: Barrier integrity and inflammation susceptibility. J Allergy Clin Immunol 118:257–268
115. Herbert DR, Yang J-Q, Hogan SP, Groschwitz K, Khodoun MV, Munitz A, Orekov T, Perkins C, Wang Q, Brombacher F, Urban JF Jr, Rothenberg ME, Finkelman FD (2009) Intestinal epithelial cell secretion of RELM-β protects against gastrointestinal worm infection. J Exp Med 206:2947–2957
116. Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010) Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. Gastroenterology 138:1763–1771
117. Hasnain SZ, Evans CM, Roy M, Gallagher AL, Kindrachuk KN, Barron L, Dickey BF, Wilson MS, Wynn TA, Grencis RK, Thornton DJ (2011) Muc5ac: a critical component mediating the rejection of enteric nematodes. J Exp Med 208:893–900
118. Inagaki-Ohara K, Sakamoto Y, Dohi T, Smith AL (2011) gamma-delta T cells play a protective role during infection with *Nippostrongylus brasiliensis* by promoting goblet cell function in the small intestine. Immunology 134:448–458
119. Zhao A, McDermott J, Urban JF Jr, Gause W, Madden KB, Yeung KA, Morris SC, Finkelman FD, Shea-Donohue T (2003)

Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. J Immunol 171:948–954
120. Marillier RG, Brombacher TM, Dewals B, Leeto M, Barkhuizen M, Govender D, Kellaway L, Horsnell WG, Brombacher F (2010) IL-4Rα-responsive smooth muscle cells increase intestinal hypercontractility and contribute to resistance during acute Schistosomiasis. Am J Physiol Gastrointest Liver Physiol 298: G943–G951
121. Vallance BA, Blennerhassett PA, Collins SM (1997) Increased intestinal muscle contractility and worm expulsion in nematode-infected mice. Am J Physiol 272:G321–G327
122. Vallance BA, Blennerhassett PA, Deng Y, Matthaei KI, Young IG, Collins SM (1999) IL-5 contributes to worm expulsion and muscle hypercontractility in a primary *T. spiralis* infection. Am J Physiol 277:G400–G408
123. Zhao M, Brown DM, Maccallum J, Proudfoot L (2009) Effect of *Nippostrongylus brasiliensis* L3 ES on inflammatory mediator gene transcription in lipopolysaccharide lung inflammation. Parasite Immunol 31:50–56
124. Shea-Donohue T, Notari L, Stiltz J, Sun R, Madden KB, Urban JF Jr, Zhao A (2010) Role of enteric nerves in immune-mediated changes in protease-activated receptor 2 effects on gut function. Neurogastroenterol Motil 22:1138–e291
125. Menge DM, Behnke JM, Lowe A, Gibson JP, Iraqi FA, Baker RL, Wakelin D (2003) Mapping of chromosomal regions influencing immunological responses to gastrointestinal nematode infections in mice. Parasite Immunol 25:341–349
126. Martin P, Leibovich SJ (2005) Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol 15:599–607
127. Williams LM, Rudensky AY (2007) Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol 8:277–284
128. Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat Immunol 4:330–336
129. Belkaid Y, Tarbell K (2009) Regulatory T cells in the control of host-microorganism interactions. Annu Rev Immunol 27:551–589
130. Rausch S, Huehn J, Loddenkemper C, Hepworth MR, Klotz C, Sparwasser T, Hamann A, Lucius R, Hartmann S (2009) Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of $Foxp3^+$ cells. Eur J Immunol 39:3066–3077
131. Murai M, Krause P, Cheroutre H, Kronenberg M (2010) Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. Mucosal Immunol 3:443–449
132. Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Harcus Y, Filbey KJ, Finney CAM, Greenwood EJD, Knox DP, Wilson MS, Belkaid Y, Rudensky AY, Maizels RM (2010) Helminth secretions induce *de novo* T cell Foxp3 expression and regulatory function through the TGF-β pathway. J Exp Med 207:2331–2341
133. Gorelik L, Flavell RA (2000) Abrogation of TGFβ signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity 12:171–181
134. Reynolds LA, Maizels RM (2012) Cutting edge: in the absence of TGF-β signaling in T cells, fewer CD103+ regulatory T cells develop, but exuberant IFN-gamma production renders mice more susceptible to helminth infection. J Immunol 189:1113–1117
135. Ince MN, Elliott DE, Setiawan T, Metwali A, Blum A, Chen HL, Urban JF, Flavell RA, Weinstock JV (2009) Role of T cell TGF-β signaling in intestinal cytokine responses and helminthic immune modulation. Eur J Immunol 39:1870–1878
136. Metwali A, Setiawan T, Blum AM, Urban J, Elliott DE, Hang L, Weinstock JV (2006) Induction of $CD8^+$ regulatory T cells in the intestine by *Heligmosomoides polygyrus* infection. Am J Physiol Gastrointest Liver Physiol 291:G253–G259
137. Mizoguchi A, Bhan AK (2006) A case for regulatory B cells. J Immunol 176:705–710
138. Jankovic D, Cheever AW, Kullberg MC, Wynn TA, Yap G, Caspar P, Lewis FA, Clynes R, Ravetch JV, Sher A (1998) $CD4^+$ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by B cell-dependent mechanism requiring Fc receptor signaling. Journal of Experimental Medicine 187:619–629
139. Correale J, Farez M, Razzitte G (2008) Helminth infections associated with multiple sclerosis induce regulatory B cells. Ann Neurol 64:187–199
140. Mangan NE, Fallon RE, Smith P, van Rooijen N, McKenzie AN, Fallon PG (2004) Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. J Immunol 173:6346–6356
141. Balic A, Smith KA, Harcus Y, Maizels RM (2009) Dynamics of $CD11c^+$ dendritic cell subsets in lymph nodes draining the site of intestinal nematode infection. Immunol Lett 127:68–75
142. Smith KA, Hochweller K, Hämmerling GJ, Boon L, Macdonald AS, Maizels RM (2011) Chronic helminth infection mediates tolerance in vivo through dominance of $CD11c^{lo}$ $CD103^-$ DC population. J Immunol 186:7098–7109
143. Li Z, Liu G, Chen Y, Liu Y, Liu B, Su Z (2011) The phenotype and function of naturally existing regulatory dendritic cells in nematode-infected mice. Int J Parasitol 41:1129–1137
144. Segura M, Su Z, Piccirillo C, Stevenson MM (2007) Impairment of dendritic cell function by excretory–secretory products: a potential mechanism for nematode-induced immunosuppression. Eur J Immunol 37:1887–1904
145. Chaicumpa V, Prowse SJ, Ey PL, Jenkin CR (1977) Induction of immunity in mice to the nematode parasite, *Nematospiroides dubius*. Australian Journal of Experimental Biology and Medicine 55:393–400
146. Behnke JM, Hannah J, Pritchard DI (1983) *Nematospiroides dubius* in the mouse: evidence that adult worms depress the expression of homologous immunity. Parasite Immunology 5:397–408
147. Pleass RJ, Bianco AE (1996) Irradiated larval vaccination and antibody responses evaluated in relation to the expression of immunity to *Heligmosomoides polygyrus*. Parasitol Res 82:445–453
148. Hagan P, Behnke JM, Parish HA (1981) Stimulation of immunity to *Nematospiroides dubius* in mice using larvae attentuated by cobalt 60 irradiation. Parasite Immunology 3:149–156
149. Pleass RJ, Bianco AE (1994) The role of adult worms in suppressing functional protective immunity to *Heligmosomoides polygyrus bakeri* challenge infections. Parasite Immunology 16:619–628
150. Larrick KS, Semprevivo LH, Maloney MD, Tritschler JP 2nd (1991) Immunity to *Heligmosomoides polygyrus* induced by subcutaneous vaccination with post-infection larvae. Int J Parasitol 21:321–328
151. Monroy FG, East IJ, Dobson C, Adams JH (1989) Immunity in mice vaccinated with a molecular weight 60,000 glycoprotein secreted by adult *Nematospiroides dubius*. Int J Parasitol 19:71–76
152. Hewitson JP, Grainger JR, Maizels RM (2009) Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol 167:1–11
153. Harnett W, Harnett MM (2010) Helminth-derived immunomodulators: can understanding the worm produce the pill? Nat Rev Immunol 10:278–284
154. Monroy FG, Dobson C, Adams JH (1989) Low molecular weight immunosuppressors secreted by adult *Nematospiroides dubius*. Int J Parasitol 19:125–127

155. Telford G, Wheeler DJ, Appleby P, Bowen JG, Pritchard DI (1998) *Heligmosomoides polygyrus* immunomodulatory factor (IMF), targets T- lymphocytes. Parasite Immunology 20:601–611
156. Massacand JC, Stettler RC, Meier R, Humphreys NE, Grencis RK, Marsland BJ, Harris NL (2009) Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. Proc Natl Acad Sci U S A 106:13968–13973
157. McSorley HJ, O'Gorman MT, Blair N, Sutherland TE, Filbey KJ, Maizels RM (2012) Suppression of type 2 immunity and allergic airway inflammation by secreted products of the helminth. Heligmosomoides polygyrus. Eur J Immunol. doi:10.1002/eji.201142161
158. Moreno Y, Gros PP, Tam M, Segura M, Valanparambil R, Geary TG, Stevenson MM (2011) Proteomic analysis of excretory-secretory products of *Heligmosomoides polygyrus* assessed with next-generation sequencing transcriptomic information. PLoS Negl Trop Dis 5:e1370
159. Lawrence CE, Pritchard DI (1993) Differential secretion of acetylcholinesterase and proteases during the development of *Heligmosomoides polygyrus*. Int J Parasitol 23:309–314
160. Ey PL (1988) *Heligmosomoides polygyrus*: excretory/secretory antigens released in vitro by exsheathed third-stage larvae. Exp Parasitol 67:210–220
161. Rzepecka J, Rausch S, Klotz C, Schnöller C, Kornprobst T, Hagen J, Ignatius R, Lucius R, Hartmann S (2009) Calreticulin from the intestinal nematode *Heligmosomoides polygyrus* is a Th2-skewing protein and interacts with murine scavenger receptor-A. Mol Immunol 46:1109–1119
162. McSorley HJ, Grainger JR, Harcus Y, Murray J, Nisbet AJ, Knox DP, Maizels RM (2010) daf-7-related TGF-β homologues from Trichostrongyloid nematodes show contrasting life-cycle expression patterns. Parasitology 137:159–171
163. Adams JH, East IJ, Monroy FG, Dobson C (1988) Sex-specific antigens on the surface and in the secretions of *Nematospiroides dubius*. Int J Parasitol 18:999–1001
164. Bashir ME, Andersen P, Fuss IJ, Shi HN, Nagler-Anderson C (2002) An enteric helminth infection protects against an allergic response to dietary antigen. J Immunol 169:3284–3292
165. Kitagaki K, Businga TR, Racila D, Elliott DE, Weinstock JV, Kline JN (2006) Intestinal helminths protect in a murine model of asthma. J Immunol 177:1628–1635
166. Wilson MS, Taylor M, Balic A, Finney CAM, Lamb JR, Maizels RM (2005) Suppression of allergic airway inflammation by helminth-induced regulatory T cells. Journal of Experimental Medicine 202:1199–1212
167. Hartmann S, Schnoeller C, Dahten A, Avagyan A, Rausch S, Lendner M, Bocian C, Pillai S, Loddenkemper C, Lucius R, Worm M, Hamelmann E (2009) Gastrointestinal nematode infection interferes with experimental allergic airway inflammation but not atopic dermatitis. Clin Exp Allergy 39:1585–1596
168. Whelan RAK, Hartmann S, Rausch S (2011) Nematode modulation of inflammatory bowel disease. Protoplasma doi:10.1007/s00709-011-0342-x
169. Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75:263–274
170. Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G, Thompson-Snipes L, Leach MW, Rennick D (1996) Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest 98:1010–1020
171. Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG (2002) Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology 123:1527–1542
172. Hale LP, Gottfried MR, Swidsinski A (2005) Piroxicam treatment of IL-10-deficient mice enhances colonic epithelial apoptosis and mucosal exposure to intestinal bacteria. Inflamm Bowel Dis 11:1060–1069
173. Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF Jr, Weinstock JV (2004) *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. Eur J Immunol 34:2690–2698
174. Elliott DE, Metwali A, Leung J, Setiawan T, Blum AM, Ince MN, Bazzone LE, Stadecker MJ, Urban JF Jr, Weinstock JV (2008) Colonization with *Heligmosomoides polygyrus* suppresses mucosal IL-17 production. J Immunol 181:2414–2419
175. Blum AM, Metwali A, Elliott DE, Berg DJ, Weinstock JV (2004) CD4+ T cells from IL-10-deficient mice transfer susceptibility to NSAID-induced Rag colitis. Am J Physiol Gastrointest Liver Physiol 287:G320–G325
176. Hang L, Setiawan T, Blum AM, Urban J, Stoyanoff K, Arihiro S, Reinecker HC, Weinstock JV (2010) *Heligmosomoides polygyrus* infection can inhibit colitis through direct interaction with innate immunity. J Immunol 185:3184–3189
177. Sutton TL, Zhao A, Madden KB, Elfrey JE, Tuft BA, Sullivan CA, Urban JF Jr, Shea-Donohue T (2008) Anti-Inflammatory mechanisms of enteric *Heligmosomoides polygyrus* infection against trinitrobenzene sulfonic acid-induced colitis in a murine model. Infect Immun 76:4772–4782
178. Weng M, Huntley D, Huang IF, Foye-Jackson O, Wang L, Sarkissian A, Zhou Q, Walker WA, Cherayil BJ, Shi HN (2007) Alternatively activated macrophages in intestinal helminth infection: effects on concurrent bacterial colitis. J Immunol 179:4721–4731
179. Chen CC, Louie S, McCormick B, Walker WA, Shi HN (2005) Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric *Citrobacter rodentium* and enhances *Citrobacter*-induced colitis in mice. Infect Immun 73:5468–5481
180. Chen C-C, Louie S, McCormick BA, Walker WA, Shi HN (2006) Helminth-primed dendritic cells alter the host response to enteric bacterial infection. J Immunol 176:472–483
181. Summers RW, Elliott DE, Qadir K, Urban JF Jr, Thompson R, Weinstock JV (2003) *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. Am J Gastroenterol 98:2034–2041
182. Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV (2005) *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology 128:825–832
183. Summers RW, Elliott DE, Urban JF Jr, Thompson R, Weinstock JV (2005) *Trichuris suis* therapy in Crohn's disease. Gut 54:87–90
184. Saunders KA, Raine T, Cooke A, Lawrence CE (2007) Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. Infect Immun 75:397–407
185. Liu Q, Sundar K, Mishra PK, Mousavi G, Liu Z, Gaydo A, Alem F, Lagunoff D, Bleich D, Gause WC (2009) Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. Infect Immun 77:5347–5358
186. Walk ST, Blum AM, Ewing SA, Weinstock JV, Young VB (2010) Alteration of the murine gut microbiota during infection with the parasitic helminth *Heligmosomoides polygyrus*. Inflamm Bowel Dis 16:1841–1849
187. Gillilland MG 3rd, Erb-Downward JR, Bassis CM, Shen MC, Toews GB, Young VB, Huffnagle GB (2012) Ecological succession of bacterial communities during conventionalization of germ-free mice. Appl Environ Microbiol 78:2359–2366
188. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR (2008) Specific microbiota

direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 4:337–349
189. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139:485–498
190. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, Honda K (2011) Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science 331:337–341
191. Larsson E, Tremaroli V, Lee YS, Koren O, Nookaew I, Fricker A, Nielsen J, Ley RE, Backhed F (2012) Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88. Gut 61:1124–1131
192. Weinstock JV, Elliott DE (2009) Helminths and the IBD hygiene hypothesis. Inflamm Bowel Dis 15:128–133
193. Lawrence CE, Pritchard DI (1994) Immune response profiles in responsive and non-responsive mouse strains infected with *Heligmosomoides polygyrus*. Int J Parasitol 24:487–494
194. Parker SJ, Inchley CJ (1990) *Heligmosomoides polygyrus* influence of infection on lymphocyte subpopulations in mouse mesenteric lymph nodes. Exp Parasitol 71:249–258
195. Behnke JM, Wakelin D (1977) *Nematospiroides dubius:* stimulation of acquired immunity in inbred strains of mice. J Helminthol 51:167–176
196. Wakelin D, Donachie AM (1983) Genetic control of eosinophilia. Mouse strain variation in response to antigens of parasite origin. Clin Exp Immunol 51:239–246
197. Ali NM, Behnke JM, Manger BR (1985) The pattern of peripheral blood leucocyte changes in mice infected with *Nematospiroides dubius*. J Helminthol 59:83–93
198. Ben-Smith A, Wahid FN, Lammas DA, Behnke JM (1999) The relationship between circulating and intestinal *Heligmosomoides polygyrus*-specific IgG1 and IgA and resistance to primary infection. Parasite Immunol 21:383–395