Immune modulation by parasitic nematodes

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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.

The experiments described in this thesis involving MBD2-/- mice were part of a collaboration with Adrian Bird, and performed jointly with Aimee Deaton.

John Grainger

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ABSTRACT

Almost 2 billion people world-wide are infected with parasitic helminths. These complex multicellular eukaryotic organisms are capable of establishing long-term infections even in the face of an intact immune response. Typically, in these settings regulatory components of the immune response, such as Foxp3^{+} T regulatory cells (Tregs), become dominant, limiting protective effector responses towards the parasite. Helminths are thought to have evolved mechanisms, including release of immunomodulatory molecules termed excretory-secretory products (ES), to sway the balance between the regulatory and effector arms of the immune response to favour their persistence. In this thesis both the development of a protective immune response toward, and the potential manipulation of the immune response by, the rodent gastrointestinal nematode *Heligmosomoides polygyrus* have been studied.

Firstly, the effects of *H. polygyrus* ES (HES) on bone-marrow derived dendritic cells (DCs) were analysed. Although HES did not alter the phenotype of the DC it was found to be able to suppress the ability of the DC to respond to inflammatory stimuli. This activity was lost when HES was heat-inactivated (hiHES). After adoptive transfer, HES-pulsed DCs were able to induce a HES-specific T helper (Th)2-type response even if co-treated with an inflammatory stimulus. Th2-type responses are protective against *H. polygyrus* infection. Surprisingly, the ability of HES to generate a Th2-response in a co-treatment situation was not related to its anti-inflammatory properties; DCs co-treated with hiHES and an inflammatory stimulus were able to drive an equivalent Th2-response to HES in this situation.

Next, making use of mouse strains with different susceptibility phenotypes to primary *H. polygyrus* infection, potential mechanisms of resistance were characterised. Development of granulomas in the gut wall were found to be associated with reduced worm burdens. Furthermore, in highly susceptible C57BL/6 mice, production of IL-23 was shown to be counter-regulatory to this process, as mice on the same background but deficient in this cytokine have increased numbers of granulomas and dramatically enhanced resistance. Susceptibility to *H. polygyrus* was also considered at the level of epigenetic regulation. A protein that binds

specifically to methylated DNA, methyl-CpG binding domain protein (MBD)2, was found to affect the proportion of Foxp3^{+} Tregs within the CD4^{+} T cell population *in vivo*. Additionally, *in vitro* induction of Foxp3 in response to TGF-β was enhanced in MBD2$^{-/-}$ CD4^{+} T cells. MBD2$^{-/-}$ mice had a trend towards increased worm burdens when infected with *H. polygyrus,* suggesting that the difference in proportion of Tregs may limit generation of an effector response.

Finally, the ability of HES to directly affect the regulatory arm of the immune response was focussed upon. It was found that HES was able to induce Foxp3 expression in naïve peripheral T cells, and that this was mediated by stimulation of the TGF-β pathway. The TGF-β mimic was of parasite origin as a pan-vertebrate TGF-β antibody was unable to block its effects but sera from *H. polygyrus* infected animals was competent to do this. Activity of this type was not limited to HES as ES from the ovine helminth *Haemonchus contortus* was found to have the same property. These data imply that some helminth parasites have evolved mechanisms to support generation of Foxp3^{+} Tregs, thus favouring the regulatory arm of the immune response and hence their own persistence.

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ABBREVIATIONS

AAMϕ - alternatively activated macrophage

APC – antigen presenting cell

CAMϕ - classically activated macrophage

BSA – bovine serum albumin

BMDC – bone marrow derived dendritic cell

Con A – concanavalin A

DC – dendritic cell

FACS – fluorescence assisted cell sorting

FcεRI – high affinity IgE receptor

GALT- gut associated lymphoid tissue

HE_x – *Heligmosomoides polygyrus* adult extract

HES – *Heligmosomoides polygyrus* excretory-secretory products

hiHES – heat inactivated HES

ICCS – intracellular cytokine staining

IEC – intestinal epithelial cell

IEL – intraepithelial lymphocyte

IFN - interferon

IL - interleukin

IPSE – IL-4-inducing principle of *Schistosoma mansoni* eggs

LN – lymph node

MACS – magnet assisted cell sorting

mAb – monoclonal antibody

MBD – methyl-CpG binding domain protein

MLN – mesenteric lymph node

MMC – mucosal mast cell

NES – *Nippostrongylus brasiliensis* excretory-secretory products

LPL – lamina propria lymphocyte

Pa – *Propionibacterium acnes*

PAMP – pathogen associated molecular pattern

PBS – phosphate buffered saline

PRR – pattern recognition receptor

RA – retinoic acid

SEA – *Schistosoma mansoni* soluble egg antigen

St – *Salmonella typhimurium*

STAT – signal transducers and activators of transcription proteins

TCR – T cell receptor

Teff – T effector cell

Th – T helper cell

TGF – transforming growth factor

TNF – tumour necrosis factor

Treg – T regulatory cell

TSLP – thymic stromal lymphopoietin

WT – wild-type (mice)

1 Introduction

1.1 Overview of helminth infections.

Estimates suggest that almost 2 billion people worldwide harbour parasitic helminth infections. The majority of these individuals live in the developing world where a combination of inadequate public sanitation and poor health care provision combine to favour parasite transmission. Despite the high prevalence of helminth infections less than 1% of global research funding goes towards their study and as such they are considered to be neglected tropical diseases. In part this lack of scientific focus is reflective of the relatively low rates of mortality associated with these parasites, when compared to other global health concerns including malaria, HIV/AIDS and tuberculosis (reviewed by (Hotez et al., 2008)).

Parasitism by helminths, however, can incur a considerable cost to host fitness. Children and adolescents often have heaviest worm burdens and in this age group infection can cause growth stunting, decreased physical fitness, and impairment of normal cognitive function (reviewed by (Crompton and Nesheim, 2002). Another group at high risk from these diseases are pregnant women; helminth infection is associated with neo-natal prematurity, lower birth weights, and increased pregnancy-associated mortality. Moreover, although helminths elicit relatively minor symptoms in most individuals, the morbidity they can cause should not be underestimated. Onchocerciasis, and lymphatic filariasis are, respectively, leading causes of blindness, and limb and genital deformity, in some parts of the world. Parasitic worm infections are also frequently co-endemic with malaria, HIV/AIDS, and tuberculosis; diseases upon which they are capable of having a negative impact (Spiegel et al., 2003; Borkow and Bentwich, 2006; Resende Co et al., 2007). Indeed, even though they are not typically considered a major global health concern, it is difficult to quantify the immense detrimental effects that these pathogens have on the worker productivity, and hence economies of developing countries (reviewed by (Hotez et al., 2008).

Helminth refers to organisms belonging to two phyla: platyhelminths (flatworms) and nematodes (roundworms). Platyhelminths, which can be further sub-

divided into trematodes (flukes) and cestodes (tapeworms), are solely parasitic, whilst nematodes additionally include many free-living species. The commonly used model organism, *Caenorhabditis elegans*, is the best-known example of a free-living nematode.

Although a plethora of platyhelminth and parasitic nematode species are capable of establishing infection in human populations, a relatively small number are responsible for the majority of infections across the globe. The most prevalent platyhelminth species are the trematodes *Schistosoma haematobium*, *S. mansoni*, and *S. japonicum*, which are collectively estimated to infect 207 million people in the developing world. However, these numbers are overshadowed by the intestinal nematodes, *Ascaris lumbricoides*, *Trichuris trichuria*, and *Necator americanus*, infecting 807 million, 604 million, and 507 million, people, respectively (adapted from (Hotez et al., 2008). Other common parasitic helminths include filarial nematodes, such as *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus*, and the cestode *Taenia solium*.

Given the vast numbers of people harbouring helminth infections, it is not surprising to learn that a large proportion are host to several different species of worm. In order to compete in this world of polyparasitism, helminths have evolved to survive in a wide-variety of host niches, including the gut, lymphatic system and blood vessels, and to be transmitted in very different, and often highly complex, ways. Perhaps the most complex are those parasites whose lifecycles involve an intermediate host or vector. Examples of these include: schistosomes, which use freshwater snails; the lymphatic filarial nematodes, *W. bancrofti* and *B. malayi*, which use mosquitoes; and *O. volvulus*, which uses black flies of the genus *Simulium*.

Irrespective of their method of transmission or final location within the host, a key feature of most helminth infections is their ability to persist for long-periods of time in the face of an intact immune system. Moreover, protective immunity towards re-infection is frequently only elicited after many years, or even decades, of exposure. This is testimony to the highly effective mechanisms of immune evasion and immunomodulation used by parasitic worms (reviewed by (Maizels and Yazdanbakhsh, 2003). A lack of understanding of these mechanisms, combined with

a modest commercial market for anti-helminthics, means that currently tools available for control of these infections are limited.

The introduction to this thesis will firstly consider the immune mechanisms by which protection against helminth infection is induced, and effected, and then move on to assess how these mechanisms are regulated, and how parasites might manipulate host immunity through excretion-secretion of immunomodulatory molecules.

1.2 The role of T helper (Th) 2-type responses in helminth infections.

1.2.1 Failure to induce Th2-type responses can lead to Th1/Th17-mediated immune pathology.

In most human infections, and animal models, helminth parasites are associated with T helper (Th) 2-type responses classically characterised by production of cytokines that include interleukin (IL) -4, IL-5, IL-9, IL-13, and IL-21. Irrespective of infection routes and preferred niche occupied by the parasite, common features resulting from this type of response include, tissue eosinophilia, mucosal mastocytosis and production of immunoglobulin (Ig) E. Where helminth infection does not induce a Th2-type response, and subsequently a Th1-type response is favoured, severe immune mediated pathology is often found to occur. In human filarial infections, for example, Th1-type responses are thought to lead to inflammation of the lymphatic system. This causes the failure of drainage and increased susceptibility to opportunistic secondary infections that eventually result in elephantiasis. A similar situation occurs in schistosome infections where a correlation is observed between the presence of Th1-associated cytokines in the peripheral blood and hepatosplenic disease (reviewed by (Maizels and Yazdanbakhsh, 2003; Stadecker et al., 2004).

Another Th-cell subset that has been implicated in animal models as playing a non-redundant role in helminth-associated immune pathology is Th17 (Rutitzky and Stadecker, 2006; Rutitzky et al., 2008). Characteristic cytokines produced by Th17 cells are IL-17, IL-6, TNF-α (Park et al., 2005) and, in response to

environmental toxins, IL-22 (Veldhoen et al., 2008a). These cells are key mediators of the chronic inflammation associated with a number of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) (Cua et al., 2003), inflammatory bowel disease (Yen et al., 2006), and collagen-induced arthritis (Murphy et al., 2003).

Optimal induction of both Th1-, and Th17-, type responses are associated with production of IL-12-family members by accessory cells, mainly dendritic cells (DCs) and macrophages. Acquisition of a Th1 phenotype is dependent on IL-12 itself (Szabo et al., 2003), while the, relatively-recently discovered, cytokine IL-23 is required for stable expression of IL-17 by Th17-cells (Stritesky et al., 2008). IL-12 and IL-23 are heterodimeric proteins formed from the combination of a p35 or a p19 subunit respectively, and a common p40 subunit (Trinchieri et al., 2003). The shared usage of this p40 subunit meant that, prior to the discovery of IL-23, studies using p40-deficient mice, or mAb depletion of p40, incorrectly implicated Th1-type responses as being the sole drivers of inflammation in a variety of settings.

In an induced model of high-pathology schistosomiasis, using C57BL/6 mice, genetic-deficiency of IL-23(p19), but not IL-12(p35), was found to decrease liver granuloma size to background levels (Rutitzky et al., 2008). This reduction in pathology correlated with markedly lower lesional, but not local lymph node (LN), expression of IL-17. IL-23 is known to have IL-17-independent effects (Izcue et al., 2008), but in this setting the effects of IL-23 appeared to be predominantly mediated by IL-17, since mAb-depletion of this cytokine similarly decreased liver granuloma size (Rutitzky and Stadecker, 2006). Furthermore, it was shown that the requirement for IL-17 in development of pathology was not limited to the C57BL/6 induced model of disease, as mAb-depletion also decreased granuloma size in, naturally high-pathology, CBA mice.

At this time there is no direct evidence linking IL-17 to helminth-associated immune pathology in human populations. Indeed, in work focussing on hepatosplenic disease in *S. mansoni* infection, IL-17 was found to be low or not detectable in antigen stimulated whole blood cultures from all patients tested (Wilson et al., 2008). This data could be explained by local, but not systemic, pathology-related changes in IL-17, as has been observed in mouse models (Rutitzky et al.,

2008). As a result, in order to correlate Th17-type responses to helminth infection with pathology in human subjects, it may be necessary for future studies to take on the challenging task of focussing on Th-cell phenotype at the site of inflammation rather than in the periphery.

1.2.2 Induction of Th2-type responses and protection against helminth infections.

It is generally assumed that Th2-type responses are protective against all helminth infections. However, although this type of response is typically associated with protection from re-infection some ambiguities and exceptions still remain. In murine models of schistosomiasis, for example, the immune response against the tissue migrating larval stage is primarily of a Th1-type, and in vaccination studies this type of response can be associated with protection to challenge infection (Smythies et al., 1992). Similarly Th1-mediated protection has been implicated in experimental cysticercosis, where mice were found to be more susceptible to infection by the cestode, *Taenia crassiceps*, under an IFN-γ mAb-depleting regime (Terrazas et al., 1999), or when IL-12p35 was genetically-deficient (Rodriguez-Sosa et al., 2003). Whether Th1-type responses are protective towards certain helminth infections in human populations is less clear, but evidence suggests that natural immunity to the filarial nematodes, including *Wucheraria bancrofti* (Dimock et al., 1996) and *Onchocerca volvulus* (Elson et al., 1995), is associated with immune dominance of this kind.

Surprisingly, the only situation in which an unequivocally protective role for Th2-type responses has been substantiated, is for gastrointestinal nematode infections of rodents (reviewed by (Lawrence, 2003; Anthony et al., 2007). Perhaps the most clear-cut demonstration of this, is observed in respect to the resistance and susceptibility phenotypes of different mouse strains infected with *T. muris* (Cliffe and Grencis, 2004). Mice that are resistant to the parasite mount a strong Th2-type response in the site-draining mesenteric lymph nodes (MLNs), characterised by the presence of IL-4, IL-5 and IL-9 and a relative absence of IFN-γ. In contrast, chronically susceptible mice display characteristics of Th1-type polarisation, instead

producing large amounts of IFN-γ in the MLNs. Resistant mice can be converted to a susceptible phenotype by knocking-out, or mAb depleting, Th2-type cytokines, such as IL-4, whilst susceptible mice can be made resistant by administration of the same cytokines. This has proved to be an extremely useful tool to identify mechanisms involved in driving Th2-type resistance to gut-dwelling helminths, including IL-9 (Faulkner et al., 1997), IL-13 (Bancroft et al., 1998), and TNF-α (Artis et al., 1999), and more recently, IL-25 (Owyang et al., 2006) and IL-33 (Humphreys et al., 2008).

Another rodent gastrointestinal nematode, towards which Th2-type responses are known to be protective, is *Heligmosomides polygyrus*. If mAb to IL-4, or IL-4 receptor, are administered during challenge infections then protection is limited (Urban et al., 1991b), and if an IL-4 stabilising antibody is used during primary infection then worm expulsion is enhanced (Urban et al., 1995). Like the *T. muris* model there is variation in the susceptibility of different strains of mice to *H. polygyrus*. These variations are apparent in primary infection (Prowse and Mitchell, 1980; Zhong and Dobson, 1996), but are accentuated in secondary challenge, or trickle, infection with the parasite (Behnke et al., 2003; Menge et al., 2003). In part, as for other helminth parasites including *T. muris* (Else and Wakelin, 1988), susceptibility appears to be dependent on genes within the mouse major histocompatibility complex (MHC, or H-2 genes). The H-2^{q} and H-2^{s} haplotypes are associated with low susceptibility, H-2^{k} and H-2^{b} haplotypes with high susceptibility, and H-2^{d} with an intermediate response (Wahid and Behnke, 1993) (reviewed by (Monroy and Enriquez, 1992). Additionally, non-H-2 genes also appear to affect this parameter, as mice with the same haplotype but crossed onto an alternative strain background vary in their responses to *H. polygyrus* (Wahid and Behnke, 1993). Intriguingly, unlike the *T. muris* model, even susceptible strains generate a Th2-type response (Wahid et al., 1994) towards *H. polygyrus*, suggesting that a simple Th1/Th2 dichotomy to explain chronicity of infection is not applicable to all gut-dwelling helminths.

1.3 Innate cells as initiators of, and effectors in, Th2-type responses.

Irrespective of whether Th2-type responses are protective towards all helminth parasites, studying the activities of the various immune cell populations during the course of these infections has improved understanding of how the immune system is driven towards, and then functions in, this polarisation state. This section will concentrate on work that has characterised the role of innate immune cell-types in favouring generation of Th2-cells, and, when acted upon by Th2-associated cytokines, effecting putatively protective mechanisms against invading pathogens.

The potential cell-types involved in orchestrating clearance of intestinal nematodes will be a particular focus. Much of our knowledge relating to this subject has been gained from a small number of murine infection models, namely, *H. polygyrus*, *Nippostrongylus brasiliensis*, *T. muris*, and *Trichinella spiralis*. Although adult worms from all these species ultimately come to inhabit the gut, their lifecycles prior to colonisation of this site and the precise locations they inhabit within this environment vary markedly (Table 1.1). These differences are instrumental in distinguishing any common mechanisms invoked by the host to expel multiple helminth parasites from those that are effective only against individual species. Frequently, however, proposed mechanisms of expulsion have only been studied in one model making it difficult to ascertain whether they fall into the common, or species-specific category.

Table 1.1 – Overview of intestinal nematode models.

Species	Route of infection	Infective stage	Gastrointestinal location
H. polygyrus	Ingestion free-living larvae.	L_3 larvae	Small intestine
N. brasiliensis	Free-living larvae penetrate skin.	L_3 larvae	Small intestine
T. muris	Ingestion of eggs.	Egg	Large intestine
T. spiralis	Larvae ingested in muscle of prey eaten by carnivore.	L_1 larvae	Small intestine

One example of a common clearance mechanism is termed 'weep and sweep'; where fluid is increased in the intestinal lumen (weep), and smooth muscle contractility is altered (sweep) to literally wash the parasite away. This process is highly on dependent on mast cells (Section 1.3.6), goblet cells (Section 1.3.7), and direct effects of IL-4 and IL-13 on intestinal epithelial cells (Shea-Donohue et al., 2001). Since this has been found to be such a ubiquitous response to gut helminth infection, where relevant the role of each cell type in mediating this final outcome will be described.

1.3.1 Intestinal epithelial cells (IECs).

In order for a Th2-type response to be initiated the host must first recognise that a pathogen is invading. A key way in which this can occur is through the recognition and uptake of parasite antigens by immune cell populations. However, the essential barrier against pathogen attack provided by intestinal epithelial cells (IECs) , also create a physical block to this process. Specialised lymphoid structures, including Peyer's patches (PPs), present only in the small intestine, and isolated lymphoid follicles, found the entire length of the intestinal tract, are one way that this problem is circumvented. Within these structures, M (microfold) cells, which are interspersed between the IECs overlying the lymphoid tissue, mediate antigen exposure by sampling antigen and delivering it to, amongst other cell types, dendritic cells (DCs) situated beneath the epithelial layer (reviewed by (Artis, 2008). When exposed to helminth parasite antigen, these DCs are instrumental in activating the adaptive immune system and promoting Th2-differentiation of naïve antigen-specific T cells. Additionally, it is possible for some subsets of intestinal DCs to access antigen in the lumen by extending their dendrites between the tight-junctions connecting IECs (Rescigno et al., 2001).

Given their location, IECs are often in intimate contact with gut-dwelling helminths, and are, therefore, in a prime position to act as sentinels. IECs express a broad range of pattern recognition receptors (PRRs) and as a result are often able to respond to parasite antigens. When mice with an IEC-lineage specific deficiency in IκB kinase β (IKKβ) knockout mice were infected with *T. muris* this cell-type was

found to be integral to the development of a Th2-, rather than a Th1-, type response, via NF-κB-induced expression of thymic stromal lymphopoietin (TSLP). Lack of TSLP correlated with enhanced production of the Th1/Th17-associated cytokine IL-12/23p40 by DCs in this setting, suggesting a route by which Th2-type responses were no longer favoured (Zaph et al., 2007). Although not considered in this study, an additional way in which TSLP may support Th2-type polarisation is by stimulating mast cells to produce Th2-associated cytokines, including IL-5, and IL-13 (Allakhverdi et al., 2007a).

Along with TSLP, IECs are also major producers of IL-25, and IL-33, two cytokines that bias towards Th2-type responses. Genetic-deficiency in IL-25 leads to parasite specific Th1/Th17 polarisation in *T. muris* infected animals. One explanation for this is that IL-25, like TSLP, suppresses IL-23 production, and thus, when not present, Th17, rather than Th2-cells, become prominent (Zaph et al., 2008). IL-33, also plays a role in resistance to *T. muris* infection, as susceptible mice can be manipulated to expel the parasite by exogenous administration of this cytokine (Humphreys et al., 2008). It has been described as a chemoattractant to (Komai-Koma et al., 2007), and a potent inducer of, Th2-cells (Schmitz et al., 2005), as well as working in combination with TSLP to enhance Th2-associated cytokine release by mast cells (Allakhverdi et al., 2007b).

Aside from release of factors to predispose towards Th2-differentiation, IECs can be acted upon by Th2 cytokines to aid in worm expulsion. IL-4, and IL-13, in a STAT6-dependent fashion induce IECs to alter cell permeability, absorption, and secretion, to favour the intraluminal fluid increases associated with ‘weeping’ to flush parasites out of the gut (Madden et al., 2004). Furthermore, some nematodes, like *T. muris*, which reside in IEC syncytia, can be driven out into this fluid-rich environment by Th2 cytokine-driven acceleration of epithelial turnover, functioning as a kind of ‘escalator’ (Cliffe et al., 2005).

1.3.2 Basophils.

Basophils, which make-up less than 0.3% of leukocytes in the peripheral blood, are a poorly understood cell-type in regard to function and development (Min, 2008). In

mouse models of helminth infection, including *N. brasiliensis*, basophil numbers increase dramatically in a manner that is thought to be dependent on the Th2-associated cytokine IL-3 (Sokol et al., 2008). Ligation of the high affinity IgE receptor (FcεRI), or increases in intracellular calcium stores, stimulate significant IL-4 production by basophils (Seder et al., 1991). Basophils have, therefore, been postulated as a potential source of early IL-4, potentiating Th2-cell differentiation in response to helminths. Supporting this idea, when 'G4' IL-4-reporter mice (in which IL-4 is knocked out and GFP is expressed in the cells that would have expressed this cytokine) were infected with *N. brasiliensis*, basophils were found to be a prominent source of IL-4 (Min et al., 2004). Moreover, a glycoprotein from *S. mansoni* soluble egg antigen (SEA), IL-4-inducing principle of *S. mansoni* eggs (IPSE)/alpha-1, binds directly to basophils *in vitro* to trigger release of this cytokine (Haisch et al., 2001; Schramm et al., 2007).

Recent work has suggested that basophils can also be activated to induce Th2 differentiation by agents such as papain and bromalein, in a manner that is dependent on their cysteine protease activity (Sokol et al., 2008). No genetic-model of basophil deficiency is available, but using antibodys specific for FcεRIα it was possible to show that depletion of these cells inhibited papain-generated Th2-type responses. It was unclear, in this study, whether Th2-polarisation was directly attributable to IL-4 or another basophil produced cytokine such as TSLP. Given that helminths excrete/secrete proteases to gain access to the host, migrate through tissues, and to feed (Section 1.7.1), this mechanism of basophil stimulation may in future be found to be one route to Th2 differentiation in these infections. However, as the case of IPSE shows, other pathways of basophil stimulation may also be evoked by helminth parasites.

1.3.3 Macrophages.

Mirroring T cell subset classification, macrophages are described as becoming classically or alternatively activated in response to infection. Classically activated macrophages (CAMφs) protect against Th1-driving infectious agents, such as intracellular bacteria and viruses, in part through pathways that are dependent on

nitric oxide (NO) production. CAMϕs can be identified by their expression of pro-inflammatory cytokines, such as IL-12, and IL-23, and inducible nitric oxide synthase (iNOS), which is involved in converting L-arginine into NO. In contrast, alternatively activated macrophages (AAMϕs) are found at sites of inflammation in Th2-type settings. *In vivo*, induction of AAMϕs is dependent upon ligation of IL-4Rα. This receptor forms one half of heterodimeric receptors for both of the Th2-associated cytokines, IL-4 and IL-13, which *in vitro* generate AAMϕs (LaPorte et al., 2008). When mice, infected with *S. mansoni*, are genetically deficient in expression of this receptor specifically on Mϕs (LysM^{Cre}IL-4Rα$^{-/flox}$), CAMϕs develop rather than AAMϕs in this Th2-type setting (Herbert et al., 2004). Additionally, IL-21 seems to enhance alternative activation as, *in vitro*, it augments the activity of IL-4 and IL-13 on Mϕs (Pesce et al., 2006), and, suggesting that this is biologically important, *in vivo*, IL-21R$^{-/-}$ mice have attenuated Th2-type responses towards *H. polygyrus*, *N. brasiliensis*, and *S. mansoni* (Pesce et al., 2006; Frohlich et al., 2007).

Characteristics of AAMϕs are their high expression of IL-4 receptor α-chain (IL-4Rα), and the mannose receptor CD206, along with, arginase-1 (Arg-1), and low expression of iNOS (reviewed by (Reyes and Terrazas, 2007). This abundance of Arg-1 favours the metabolism of L-arginine towards prolines, polyamines, and urea production, rather than NO. Prolines play an important role in collagen production, whilst polyamines stimulate cellular proliferation. Together these metabolites are thought to confer a wound healing capacity that has been implicated as an important function of AAMϕs (Loke et al., 2007).

AAMϕs have now been identified in all phyla of helminth infections (Reyes and Terrazas, 2007), but have been particularly well-phenotyped using a murine peritoneal implant model of *B. malayi* infection. Macrophages isolated from this setting, termed nematode elicited macrophages (NeMϕs), displayed high Arg-1 activity, but were found to more dramatically express two other molecules, Ym1, and Resistin-like molecule (RELM) α (Loke et al., 2002). The role of these molecules, in regard to the function of AAMϕs, is still poorly understood.

Ym1 is a lectin that has an affinity for the abundant natural polymer, chitin, which is important to structural support in a wide-range of organisms including fungi, insects, and helminths. Approximately 5% of the dry weight of *H. polygyrus*

eggs is chitin (Arnold et al., 1993), and expression of chitin synthase genes is evident in the developing embryos of *B. malayi* (Harris et al., 2000). Despite its ability to bind chitin, in contrast to other homologous proteins, Ym1 has no chitinase activity. Some studies have implicated Ym1 as having chemotactic properties towards eosinophils (Falcone et al., 2001), whilst others have suggested that its ability to also bind heparin may be involved in mediating interactions between certain cell-types and the extracellular matrix (Chang et al., 2001).

RELMα, as its name suggests, bears resemblance, on the basis of an unusual C-terminal cysteine-rich domain, to resistin. Resistin itself is an adipocyte-derived hormone that antagonises glucose homeostasis, and insulin sensitivity. Alongside RELMα, two other family members have been identified, RELM-β, and RELM-γ, both of which also have increased expression in helminth infection (reviewed by (Artis, 2006; Anthony et al., 2007). Each RELM-protein has a different expression pattern: RELMα, aside from its expression in AAMϕs, is expressed in a diverse set of tissues including the lung, and adipose tissue; RELM-β has a much more restricted expression profile, and is most prominently produced by goblet cells in the intestinal tract. It has been shown to bind to nematodes (Section 1.3.7); RELM-γ, the least well characterised member of the family is found, like RELMα in the lung, but additionally in haematopoietic tissue, and the intestine during helminth infection (Wang et al., 2005). Although RELMα has multiple functions, including inhibition of adipocyte differentiation, its action when released by AAMϕs in Th2-type settings is unclear. RELMα has been shown to stimulate collagen production by myofibroblasts, so one possibility is that, like Arg-1, RELMα plays a role in aiding tissue repair in the face of the large amount of damage caused by large extracellular parasites (reviewed by (Anthony et al., 2007).

AAMϕs are essential to the development of resistance in secondary challenge infections to *H. polygyrus*. In primary inoculations, most strains of mice are to some extent susceptible to the parasite, with infections lasting between four weeks to eight months (reviewed by (Monroy and Enriquez, 1992). However, in secondary challenge infection some strains become resistant to re-infection, with clearance occurring by 14 days. This resistance is largely dependent on an immune response directed against the larval stage of the parasite, which dwells in the intestinal sub-

mucosa (Patel et al., 2008). A granuloma forms around the parasite, that is dependent on memory Th2 cells, made up of neutrophils directly in contact with the invading larvae and larger numbers of AAMϕs enclosing the parasites and the neutrophils (Fig. 1.1) (Anthony et al., 2006). If the host is clodronate depleted of macrophages, or the AAMϕ-associated enzyme Arg-1 is chemically inhibited, then resistance is compromised, implying that AAMϕs are mediators in the attack against larvae in the tissues. Furthermore, data from *N. brasiliensis* infection suggests that AAMϕs may be involved in favouring expulsion of the parasite once it is in the gut lumen. In this setting inhibition of Arg-1 blocked smooth muscle contractility, increased smooth muscle thickness and partially affected protective immunity of the host, whilst clodronate treatment prevented worm expulsion (Zhao et al., 2008).

1.3.4 Neutrophils

Alongside AAMϕs, another cell type apparent at sites of tissue invasion by helminth parasites is the neutrophil. When gerbils are intradermally inoculated with *Brugia pahangi* L_3s, neutrophils are the major cell-type observed surrounding the larvae near the injection site by 3 hrs post-injection (Porthouse et al., 2006). Similarly, in nodules encasing the adult worms of *O. volvulus* infected human patients, neutrophils are a predominant cell-type (Brattig et al., 2001). Filarial nematodes, like these, typically carry symbiotic bacteria and it appears that much of the neutrophilia can be attributed, not to recognition of the parasite, but to recognition of the bacteria. This is exemplified by the loss of neutrophils from *O. volvulus* nodules of individuals treated with bactericidal antibiotics (Brattig et al., 2001). As discussed previously (Section 1.3.3), neutrophils are also evident surrounding *H. polygyrus* larvae in the gut wall. Even in this setting it is possible that neutrophils are responding to resident gut bacteria, or potentially pathogenic bacteria carried by the parasite, as opposed to the parasite itself. Neutrophil recognition of bacteria, instead of direct recognition of the helminth, may still play a key role in favouring parasite clearance. Mice, genetically-deficient in CCL2, a chemokine important to neutrophil trafficking, were delayed in their ability to expel *N. brasiliensis*. This was associated with transient increases in levels of IFN-γ, implicating a role for neutrophil clearance of Th1-

driving bacteria in augmenting the Th2-type response to this nematode (Pesce et al., 2008).

The killing of the human threadworm, *Strongyloides stercoralis* (Galioto et al., 2006), by murine neutrophils, argues that there may still be a role *in vivo* for their direct interaction with helminths, albeit in defence against a non permissive infection. This could be dependent on release of molecules associated with, like macrophages, alternative activation. Alternatively activated neutrophils have not, as yet, been reported in helminth infection, but were identified in some strains of mice infected with methicillin-resistant *Staphylococcus aureus* (Tsuda et al., 2004). Although much work still needs to be done to better understand their role, it appears that neutrophils are an important component of the protective immune response to many helminths. However, their importance is clearly context dependent since neutrophil-depleted mice, infected with *S. mansoni*, did not have any discernable change in disease severity (Herbert et al., 2004).

1.3.5 Eosinophils

Dramatic eosinophilopoiesis, followed by eosinophil infiltration into tissue-sites local to the parasite, and subsequent release of toxic, eosinophil cationic protein (ECP), are a common feature of helminth infections. Despite their highly ubiquitous presence, conflicting data from various models has confounded efforts to define whether eosinophils are integral to host protection against these organisms (reviewed by (Meeusen and Balic, 2000). IL-5, unlike many other cytokines, does not appear to have pleiotropic roles but is predominantly involved in inducing eosinophil generation from bone-marrow precursors (reviewed by (Ovington and Behm, 1997). As a result, one approach to studying the function of eosinophils has been to target this cytokine, either by monoclonal mAb depletion or by generating genetically-deficient mice (reviewed by (Maizels and Balic, 2004). Using this technique it was found that IL-5 depletion had no effect on resistance to some helminths, including *T. muris* (Betts and Else, 1999), *H. polygyrus* (Urban et al., 1991b), and *S. mansoni* (Brunet et al., 1999), but led to greater worm recoveries in *S. stercoralis* (Rotman et al., 1996), and *O. volvulus* infections (Lange et al., 1994). An alternative method to address this issue was to infect transgenic mice that overexpress IL-5, and hence

have an overabundance of eosinophils, with various parasites. Once again results were wide-ranging; decreased susceptibility was observed in response to *N. brasiliensis* (Dent et al., 1999), but no change in protection was recorded with regard to the larvae of the canine roundworm, *Toxocara canis* (Dent et al., 1999), or *T. spiralis* (Hokibara et al., 1997).

One issue with these models is that IL-5 can have affects on other cell types, including neutrophils (reviewed by (Maizels and Balic, 2004), so there was a possibility that eosinophils were not directly responsible for the phenotypes observed. Furthermore, IL-5 depletion was not associated with complete loss of the eosinophil compartment. More recent studies have made use of eosinophil deficient mice, such as ΔdblGATA mice, which have a deletion within the GATA-1 promoter, and TgPHIL mice, which express a diptheria toxin receptor under control of the eosinophil peroxidase promoter (Humbles et al., 2004). These mice have corroborated some of the earlier data, including the lack of an apparent role for eosinophils in protection against schistosome infection (Swartz et al., 2006), and furthered understanding of how eosinophils may mediate resistance in other settings (Swartz et al., 2006). For example, ΔdblGATA mice had impaired resistance in primary and secondary infection with *N. brasiliensis*, which was found to be due to a lack of response in the skin towards the invading larvae, rather than an effect on the ability of the mice to expel adult worms from the gut (Knott et al., 2007). In addition to this, any protective effects of eosinophils may not be mediated by release of cationic proteins, as protection against *B. pahangi* in mice was not altered by genetic-deficiency of these proteins but was affected by total depletion of eosinophils (Ramalingam et al., 2005).

It is intriguing that eosinophils are recruited to tissue sites in large numbers in models of infection, even where they appear not to play a role in protection. For example, in the *H. polygyrus* granuloma, eosinophils are found in surrounding tissue but not directly in contact with the parasite (Anthony et al., 2006). Two main explanations have been proposed for this. Firstly, eosinophil recruitment has evolved as a constitutive response to all helminth infections, because in some settings it does favour parasite killing and host fitness. Secondly, eosinophils have an alternative non-protective role to play in some responses, perhaps in tissue remodelling or

resolution of damage associated with pathogen challenge. Future work will need to focus on identifying whether this second option is the case.

1.3.6 Mast cells.

A classic feature associated with helminth infections is mucosal mastocytosis, the peak of which is often correlative with worm expulsion. One example of this is in *T. spiralis* infection, where maximal mastocytosis occurs at day 14 of infection, the time at which adult parasites are rejected, returning to low levels, typically observed in uninfected mice, by 8 weeks post infection (Friend et al., 1996). It has been shown that stem cell factor (SCF), another protein secreted by IECs in the small intestine, is necessary for this process in response to *T. spiralis* infection (Donaldson et al., 1996). Other cytokines, identified from *in vivo* studies, which favour increases in the mucosal mast cell (MMC) population are the Th2-type cytokines IL-3 (Abe et al., 1988), IL-4 (Madden et al., 1991), and IL-9 (Faulkner et al., 1997). IL-9-transgenic mice that constitutively overexpress IL-9, have enhanced MMC hyperplasia (Faulkner et al., 1997). When infected with *T. spiralis* (Faulkner et al., 1997), or *T. muris* (Faulkner et al., 1998), increased resistance was noted.

MMC secrete many active mediators that are associated with increased fluid accumulation in the gut, some of which can be accounted for by the effects of prostaglandins and histamines on the IECs. There is also a suggestion that MMC serine proteases may be important in increasing this fluid accumulation. One such group are chymases, which are associated with epithelial paracellular permeability (reviewed by (Knight et al., 2008). If rats are infected with *N. brasiliensis* during infection macro-molecular leakage occurs due to mast cell chymase II (Scudamore et al., 1995). Mice lacking the mast cell protease Mcpt-1, were significantly delayed in their ability to expel *T. spiralis* despite normal numbers MMCs (Knight et al., 2008). These MMC proteases were not relevant to all infections though, as genetic-deficiency in Mcpt-1 has no effect on expulsion of *N. brasiliensis* (Knight et al., 2000).

The importance of mast cells in protection against a particular helminth may be dependent on its niche. *T. spiralis* and *T. muris*, both live in epithelial-cell

syncytia and as a result may be more susceptible to expulsion due to changes in vascular permeability at this site.

1.3.7 Goblet cells.

In response to gut nematode infection goblet cell hyperplasia is apparent. Enlarged goblet cells primarily release mucins, which cross-link to form a viscoelastic mucous gel that protects mucosal surfaces (reviewed by (Knight et al., 2008). The stimulation of goblet cells is dependent on IL-4 and IL-13, as shown in *T. spiralis* infection (Khan et al., 2001). As well as mucins IL-4 and IL-13 stimulate goblet cells to produce other effector molecules including RELM-β (Knight et al., 2004) (Section 1.3.3), and intelectin-2 (Pemberton et al., 2004).

RELM-β is increased in the gut when resistance is observed towards *N. brasiliensis*, *T. spiralis,* or *T. muris* (Artis et al., 2004). This protein may promote expulsion of the worm by directly binding to its lateral alae. These chemosensory organs are integral to the worm being able to target its movement. When *S. stercoralis* was treated *in vitro* with RELM-β it became disorientated and was no longer able to move towards host tissue extract (Artis et al., 2004). It is possible that intelectin-2, a lectin (sugar binding protein), may also play a similar role to RELM-β. Intelectins can bind to certain sugar-moieties in bacterial cell walls and it is a possibility that they may also bind directly to the nematode and influence its feeding or attachment to the gut wall. Alternatively, intelectins could raise the viscosity of the mucus in the gut by affecting cross-linking between mucins. This is observed with *Xenopus* egg lectin, XL35, which is responsible for egg hardening (Nishihara et al., 1986) (reviewed by (Artis, 2006). In this way it is possible that a sort of 'glycoprotein cement' forms around the nematode promoting its expulsion (suggested by (Artis, 2006).

1.4 Adaptive immune cells in Th2-type responses to helminth infection.

Although innate cells are important in initiating an appropriate response to an invading pathogen, it is only their action in concert with the adaptive immune system that can ultimately lead to parasite clearance. Of particular importance is the differentiation of Th2-cells, which expand to become a major source of the cytokines IL-4, IL-5, IL-9, IL-13, and IL-21, which, as discussed in the previous section (Section 1.3), mediate Th2-type effector mechanisms.

The importance of CD4 cells in generating protective responses to helminths is evidenced by the fact that their depletion, in either primary or memory responses, in a number of models, such as *H. polgyrus* and *T. muris*, favours worm persistence and increases fecundity (Urban et al., 1991a; Koyama et al., 1995).

1.4.1 Generation of Th2 cells.

Until recently, the prototypic Th2-type cytokine, IL-4, secreted by T and non-T cell sources over the course of a helminth infection was considered to be key to the generation of Th2-cells. This idea was propagated by *in vitro* studies in which IL-4, acting via STAT6-signalling, was an absolute requirement for Th2 differentiation (Le Gros et al., 1990; Kaplan et al., 1996). *In vivo*, however, there does not appear to be a necessity for IL-4 to induce and expand Th2-cells. This was shown by infecting homozygous IL-$4^{G4/G4}$ transgenic mice, which express GFP as a surrogate for IL-4, with *N. brasiliensis*, and comparing their ability to polarise Th2-cells with mice heterozygous for the G4-gene (IL-$4^{+/G4}$) (van Panhuys et al., 2008). Little difference was observed in initial appearance of IL-4/GFP expression between these groups, or additionally $STAT6^{-/-}$IL-$4^{G4/G4}$ mice.

Aside from IL-4, various co-stimulatory molecules on non-T cells have been implicated as playing a role in Th2-cell generation. These cell-surface proteins, which include members of the tumour necrosis factor (TNF), integrin, and immunoglobulin superfamilies, act in concert with TCR-ligation to modulate effector cell development. CD40, OX40L, and B7 (CD80/86), are the molecules currently

thought to be most important in mediating Th2-cell differentiation in helminth infections.

The role of CD40 begins at the very onset of the response. When CD40 deficient, bone-marrow derived, DCs are pulsed with SEA, *in vitro*, and then adoptively transferred into WT mice (Section 3.1) they fail to induce Th2-cells, unlike their WT counterparts (MacDonald et al., 2002). Not only is CD40 directly required to interact with its ligand CD154 on T cells, but it also mediates interactions with innate populations (Jenkins et al., 2008). The importance of this relationship *in vivo* is demonstrated by the failure of CD154$^{-/-}$ animals to induce a Th2-type response when injected with *S. mansoni* eggs (MacDonald et al., 2002).

OX40L, unlike CD40, has only small effects on primary T cell responses to helminths, including *N. brasiliensis* (Pippig et al., 1999) and *H. polygyrus* (Ekkens et al., 2003). A more important role for this molecule appears to be in the memory Th2-response. This is exemplified by the fact that OX40L$^{-/-}$ mice, given a secondary challenge infection of *H. polygyrus* have reduced IL-4 production and are not protected against the parasite (Ekkens et al., 2003). The role of CD80/86 (or B7) molecules, in driving Th2-induction, is interesting in that the necessity for their expression is context dependent even between different helminth infections. CD80/86 deficient animals are unable to make normal IL-4 responses to *H. polygyrus*, whereas when infected with *N. brasiliensis* production of this cytokine is unimpaired (Lu et al., 1994; Greenwald et al., 1997; Liu et al., 2002).

Another level of control, rarely considered in infection settings, which is integral to establishment of a specific Th-cell differentiation state, is epigenetic regulation. Current models suggest that all naïve T cells have the potential to become Th1, or Th2, cells. At this stage the accessibility of DNA to transcription factors at both the Th1-effector cytokine gene *Ifng*, and the Th2-effector cytokine gene *IL4*, is limited (reviewed by (Reiner, 2005). This lack of accessibility is associated with a condensed chromatin (heterochromatin) structure.

Chromatin is the term used to describe the combination of DNA, RNA, and protein, making up chromosomes. A major component of chromatin are histones; complexes of which, DNA wraps around to create a ‘beads on a string’ like arrangement. By making post-translational modifications to histones the packaging

of DNA can be altered to modulate access to, and ultimately transcription of, expressed genes. Certain modifications, such as acetylation are associated with loosening of chromatin and hence transcription activation, whilst others, such as methylation, are associated with compacted chromatin and transcription repression (reviewed by (Turner, 2000) .

Although histone modifications affect chromatin organisation, epigenetic changes to the DNA itself are important in directing this process. DNA can be methylated, by DNA methyltransferases (DNMTs), on cytosine nucleotides, typically in a CpG dinucleotide context. Proteins known as methyl-CpG binding domain proteins (MBDs) associate with these sites and recruit histone-modifying enzymes, including histone deacetylases, that alter histone modifications such that a compacted chromatin structure is favoured. In this manner MBDs act as transcriptional repressors (Fig. 1.2) (reviewed by (Klose and Bird, 2006).

Returning to the situation in naïve T cells, although the chromatin organisation at the *Il4* and *Ifng* loci is repressive, they are not entirely transcriptionally silent. Early after their activation, transcriptional activity is measurable at both of these sites (Grogan et al., 2001) along with histone acetylation (Avni et al., 2002). As cells continue to proliferate in an appropriate setting transcription patterns become more restricted, eventually leading to mature expression of one or other of these genes in the progeny of the original cell.

Integral to the polarisation of a naïve T cell are the master transcriptional activators, T-bet (for Th1-cells), and GATA-3 (for Th2-cells). These proteins have been described as having a role in chromatin remodelling at two of their target genes, *Ifng*, and *Il-4* (reviewed by (Reiner, 2005). This is demonstrated by experiments showing that if *Gata3* is deleted at the onset of Th2-development, transcriptional induction of IL-4 is lost, but if *Gata3* is deleted in mature Th2-cells it does not impair continued expression of IL-4 (Pai et al., 2004; Zhu et al., 2004). Thus, it seems that during T cell differentiation some genes, including *Ifng* and *IL-4*, become fixed in an open state that is independent of their original activator.

DNA-methylation was found to be a modification important to this process. T cells from mice lacking DNMT1, which are unable to maintain methylation, and T cells from MBD2-deficient animals, which are unable to normally translate DNA-

methylation into a repressive signal, have derepression of cytokine expression when polarised towards a Th1, or Th2, lineage (Hutchins et al., 2002; Makar et al., 2003). In particular misexpression of typically non-overlapping cytokines is observed, for example IFN-γ, and IL-4. Thus, epigenetic control of gene expression, at the level of DNA-methylation, is essential for generation of Th1, or Th2-cells.

1.4.2 B cells and antibody production.

The role of B cells in helminth infections, are much less well understood than for T cells. B cells may act to aid in parasite clearance through production of antibodies, but they also can have antibody independent effects. In order to induce T cell expansion, interactions first occur between the TCR on these cells and their cognate MHC class II-peptide complexes presented on DCs. This process takes place in the T cell zone of peripheral lymphoid tissues (reviewed by (Steinman et al., 1997). After becoming activated the $CD4^+$ T cells head towards the B cell zone where they interact with B cells at the border between T and B cell regions (Garside et al., 1998). B cells are capable of acting as APCs, and can express cytokines and co-stimulatory molecules. At this point it is thought they may be necessary to support the continued expansion of T cells, required to get appropriate effector and memory function (Linton et al., 2000). Additionally, it has been suggested that B cells may favour Th2 responses by downregulating the levels of IL-12 released by DCs, thus inhibiting IFN-γ production (Moulin et al., 2000).

When B cell deficient mice were infected with *T. muris*, the protective Th2-type response was inhibited and a Th1-type response was apparent (Blackwell and Else, 2001). Additionally, in an *N. brasiliensis* model it was shown that B cells, although not required for the initial activation of T cells were required for subsequent proliferation and differentiation of Th2-cells (Liu et al., 2007).

Production of IL-4 in a Th2-type setting stimulates B cell class switching to an IgE isotype. IgE is taken up by FcRεI on mast cells and basophils and in the presence of antigen is cross-linked. This stimulates the release of basophil and mast cell derived factors that ultimately favour weeping and sweeping.

Antibody isotypes other than IgE are important in helminth infections. IgG1 dramatically increases in Th2-type settings and recent work has shown that transfer of this isotype of antibody correlates with protection in *H. polygyrus* infections (McCoy et al., 2008). Moreover, IgM is protective against the larval stage of filarial nematodes (Rajan et al., 2005).

1.5 Regulatory mechanisms in helminth infection.

In order for the host to survive a parasite infection, without significant morbidity, not only the type, i.e. Th1 vs. Th2, but also the strength of the immune response must be carefully regulated. This is exemplified in murine models of schistosome infection where, as discussed previously (Section 1.2.1), Th1/Th17 rather than Th2 polarisation is associated with immunopathology, but where, additionally, failure to control the voracity of the Th2-type response can lead to a similar outcome (Taylor et al., 2006). The balance between Th1 and Th2-cells could be described as a form of regulation, since these cell-types have been shown in many settings to counter the activity of each other. However, what is more commonly defined as "regulation" within the immune system are the cells and cytokines that limit the effector response, irrespective of its Th-type

As will be discussed in the next section, the most well-studied cell-types involved in this arm of the immune response are Regulatory T cells (Tregs) whilst the characteristic cytokines are IL-10 and TGF-β.

1.5.1 Natural Foxp3$^+$ Tregs.

Broadly speaking regulatory T cells can be separated into two main subsets: 'natural' Foxp3$^+$CD4$^+$CD25$^+$ Tregs, and inducible Tregs. Natural Foxp3$^+$ Tregs arise in the thymus and have bias, within the pool, towards self-antigen. Their main role is to maintain peripheral tolerance by limiting proliferation of self-reactive T cells that have escaped thymic selection (reviewed by (Sakaguchi, 2005). This is particularly important when damage occurs to tissues, mediating self-ligand release, for example

during infection. The importance of FOXP3 in maintaining peripheral tolerance is apparent when considering the pleiotropic features associated with human IPEX (immune dysregulation, polyendocrinopathy, X-linked) syndrome. Patients with this syndrome often have deleterious mutations in the *FOXP3* locus, and can present with a wide-variety of conditions including: eczema, autoimmune enteropathy, thyroiditis, autoimmune haemolytic anaemia, and membranous nephropathy (reviewed by (van der Vliet and Nieuwenhuis, 2007)). A comparable phenotype, including dermal thickening, anaemia and lymphadenomegaly, is observed in scurfy mice (Brunkow et al., 2001), which have a mutation in *Foxp3*, and Foxp3-deficient animals (Lin et al., 2007).

Many different mechanisms by which $Foxp3^{+}CD4^{+}CD25^{+}$ Tregs mediate suppression of effector immune responses have been reported. These can be split into three main categories: Tregs directly influencing other T cells; Tregs changing APCs; Tregs releasing suppressor molecules (reviewed by (Tang and Bluestone, 2008).

Tregs directly influencing other T cells

The proliferation, and cytokine production, of T effector (Teff) cells *in vitro*, and *in vivo*, can be limited by Tregs. Moreover, Tregs are also able to inhibit cytolytic activity of $CD8^{+}$ T cells *in vivo* (Mempel et al., 2006). When *in vitro* systems are considered Tregs are able to act in the absence of APCs, suggesting that they are capable of directly affecting the Teff. One mechanism by which this may occur is by the release of molecules such as granzyme B, and perforin, killing the Teff cells (Grossman et al., 2004; Gondek et al., 2005). An alternative way that Tregs can function *in vitro* is by starving Teff cells of cytokines. A cytokine important in this regard is IL-2, which is consumed by Tregs due to their constitutive expression of CD25 (IL-2Rα). As a result availability of IL-2 for Teff proliferation and differentiation is reduced and eventually apoptosis occurs (Pandiyan et al., 2007).

Tregs changing APCs

In vivo it has been observed that Tregs, after transfer, can rapidly interact with DCs. The ability of these DCs to subsequently interact with other T cells is altered either

by inhibiting their ability to present antigen or by stimulating them to release suppressive factors. Tregs are able to upregulate production by DCs of indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenine, affecting Teff proliferation by reducing availability of this important amino acid (reviewed by (Puccetti and Grohmann, 2007).

CTLA-4 on Tregs is known to be important in these interactions with DCs. Mice genetically deficient in CTLA-4 develop severe inflammatory disease demonstrating the importance of this molecule in negatively regulating Teff activity (Waterhouse et al., 1995). In a recent study, mice with a Foxp3-cre specific deletion of CTLA-4 were found to develop similarly severe inflammatory disease to CTLA-$4^{-/-}$ animals implying that this molecule is essential for Treg function (Wing et al., 2008). In this system CTLA-4 was found to mediate down-regulation of the costimulatory molecule CD86 on DCs, suggesting that limiting expression of this molecule is prevented activation of Teff cells.

Tregs releasing suppressor molecules

Many different molecules have been suggested to be important in the function of Tregs, including IFN-γ, IL-9, cAMP, galectins, and IL-35, but only two have been well-characterised *in vivo*, IL-10 and TGF-β. When these cytokines are blocked in a number mouse models including IBD, type 1 diabetes, and *Leishmania*, it has been shown that Treg suppression is ablated (reviewed by (Tang and Bluestone, 2008). Most recently, a mechanism of infectious tolerance has been described by which activated $CD4^+Foxp3^+$ Tregs are able to induce Foxp3 expression in naïve T cells and convert them into new regulatory phenotype cells. This occurs via a TGF-β dependent mechanism that does not require APCs (Andersson et al., 2008). Interestingly, induction is associated with $Foxp3^+$ cells that express latency-associated peptide (LAP), a peptide product of TGF-β processing.

Beyond the *in vitro* setting, molecules that effect the localisation of Tregs are extremely important to their function in suppressing proliferation of Teff cells *in vivo*. One such molecule, expressed on Tregs is CD103, an α_e integrin that is known, in combination with β7, to bind to E-cadherin (Agace et al., 2000). This affinity may

aid in the retention of Tregs at specific tissue sites, for example sites of infection. Upregulation of CD103 is associated with a number of parasites including *H. polygyrus* (Finney et al., 2007). The role of CD103 during infection has perhaps been best demonstrated in response to *Leishmania major*, where CD103$^+$ cells accumulate at the host parasite interface. When CD103 is genetically deficient there is no accumulation of Tregs at the site of infection, and animals on a susceptible genetic background now become resistant to this infection (Suffia et al., 2005). Induction of CD103 is dependent on TGF-β (Robinson et al., 2001) and is, therefore, predominantly expressed in the mucosal tissues of naïve animals.

1.5.2 Induced Tregs.

As stated above, Tregs can also be generated in the periphery when naïve conventional CD4$^+$ T cells differentiate in an appropriate cytokine milieu. These Tregs, termed inducible Tregs, were initially characterised as T regulatory 1 (Tr1), or Th3 cells. More recently, it has also been discovered that Foxp3$^+$ Tregs can also be induced in the periphery.

Tr1 cells

Human and murine Tr1 cells can be induced *in vitro* by, chronic activation of CD4$^+$ T cells in the presence of IL-10 (Groux et al., 1997), co-culture with tolerogenic DCs (Enk et al., 1993; Steinbrink et al., 1997), or with a combination of the immunosuppressive drugs vitamin D3 and dexamethasone (Barrat et al., 2002). These cells are characterised by their limited proliferative capacity, high levels of IL-10 and TGF-β production, low levels of IL-2 production, and lack of IL-4 production. The ability of Tr1 cells to act as Tregs, *in vivo*, is demonstrated by the prevention of colitis development, in a murine model, when they are adoptively transferred (Groux et al., 1997).

Tr1 cells have now been identified in human studies in a number of settings, such as bacterial infection of the respiratory tract (McGuirk et al., 2002) malaria infection (Jangpatarapongsa et al., 2008), and during periods of high dose bee venom

exposure amongst beekeepers (Meiler et al., 2008). Moreover, it has been shown that steroid resistance in some asthmatics is associated with a failure of Tr1 cells to secrete IL-10 in response to dexamethasone treatment (Xystrakis et al., 2006).

Although not formally identified, given that elevated IL-10 production, chronic antigen exposure, and the presence of DCs with a tolerogenic phenotype, are all associated with helminth infection, it seems likely that some of the T cell derived IL-10 and TGF-β produced in response to these parasites is derived from induced Tr1 cells.

Th3 cells

Th3 cells are the least well understood of the induced Treg subsets. They can be generated *in vitro* in the presence of TGF-β, and IL-4, and are classified by production of high-levels of TGF-β but, unlike Tr1 cells, no IL-10 (reviewed by (Weiner, 2001). Their generation has been described at mucosal sites during oral tolerance regimes where it is hypothesised that clonal deletion, via apoptosis, leads to release of TGF-β from dying T cells, and perhaps DCs and macrophages (reviewed by (Faria and Weiner, 2005). Transfer of these cells, *in vivo*, can rescue IL-2 deficient animals from autoimmunity by a mechanism that involves induction of Foxp3$^+$ T cells (Carrier et al., 2007). It is currently unclear whether some Th3 cells may also represent peripherally induced Foxp3$^+$ Tregs.

Induced Foxp3$^+$ T cells

As mentioned previously, Foxp3 expression can be switched on in naïve CD4$^+$ T cells when they are stimulated to proliferate, *in vitro*, in the presence of TGF-β (Chen et al., 2003). This process can be augmented if, exogenous IL-2 (Davidson et al., 2007), or the vitamin A metabolite, retinoic acid (RA) is included in these cultures (Coombes et al., 2007; Sun et al., 2007). Foxp3 induction has now been demonstrated in several models, *in vivo*, including, antigen delivery under subimmunogenic conditions (Apostolou and von Boehmer, 2004), targeting of antigen to DEC205$^+$ DC subsets (Kretschmer et al., 2005), and adoptive transfer of TCR transgenic cells followed by antigen feeding at mucosal sites (Sun et al., 2007). RA may be especially important to induction in this final situation, as a subset of

CD103^{+} DCs in the lamina propria (LP) have been shown to favour vitamin A metabolism into RA (Sun et al., 2007). Interestingly, RA does not act directly on the naïve T cell to induce Foxp3 but indirectly modulates this process by limiting production of cytokines including IL-4, IL-9, IL-21, and IFN-γ, from a subset of Teff cells (Hill et al., 2008). Again as is the case for Tr1 cells, although Foxp3 induction has not been explicitly observed in helminth infection, it is probable that a combination of chronic low level antigen exposure, and high TGF-β expression, would support this process. It is as yet unclear whether induced Tregs are as stable as natural Tregs. *In vitro*, at least, induced Foxp3^{+} Tregs lose their expression of this marker when restimulated (Polansky et al., 2008). Furthermore, TGF-β in combination with IL-6 has been shown to generate Th17 cells (Bettelli et al., 2006). This may provide a mechanism *in vivo* of switching from a regulatory to an effector phenotype.

Foxp3^{+} T cells have been shown to expand in *H. polygyrus* (Wilson et al., 2005), *B. malayi* (McSorley et al., 2008), and *L. sigmodontis* infection (Taylor et al., 2008). It is not yet known to what extent this represents expansion of natural Foxp3^{+} Tregs, or *de novo* induction of Foxp3 in naïve T cells. Moreover, it is unclear whether some of this expansion represents manipulation by the parasite to favour its persistence. In *L. sigmodontis* infection, resistance to the helminth can be increased by depleting Foxp3^{+} Tregs (Taylor et al., 2005). In addition to increased Foxp3 Treg numbers the suppressive ability, *in vitro*, per unit number of Tregs is increased during *H. polygyrus* infection (Finney et al., 2007).

1.5.3 Other regulatory cell-types.

Despite much of the research regarding immunoregulation being focussed on the role of CD4^{+} Tregs, many other cell-types are capable of limiting effector responses. These include DCs, and Mϕs, through their production of TGF-β, and IL-10, and other adaptive cell populations CD8^{+} Tregs, and regulatory B cells (Bregs).

1.5.4 $CD8^+$ T cells

Although $CD8^+$ Tregs were first described almost 40 years ago (Gershon and Kondo, 1970) they have been much less studied than $CD4^+$ Tregs, in part because of the lack of a characteristic marker. $CD8^+$ Tregs, like $CD4^+$ Tregs, have similar surface receptor expression to activated T cells, including upregulation of CD25 and CD122 (IL-2Rβ). These Tregs have been shown to act via a similar array of mechanisms to $CD4^+$ Tregs including direct killing of the target cell, altering the APC population, and secreting immunosuppressive cytokines (reviewed by (Smith and Kumar, 2008). One subset of $CD8^+$ Tregs that have been examined in a number of models are the $CD8\alpha\alpha^+TCR\alpha\beta^+$ population (reviewed by (Smith and Kumar, 2008). These occur naturally in the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), and expand during the course of disease (Tang et al., 2006). In this situation mAb activation of this population can effectively prevent development of EAE (Tang et al., 2007).

Although $CD8\alpha\alpha^+TCR\alpha\beta^+$ T cells only make up a small proportion of the total T cell population in the lymph nodes and spleen, they make-up a major percentage of the intra-epithelial lymphocytes (IELs) in the gut (reviewed by (Lefrancois and Lycke, 2001). In a murine colitis model, transfer of these cells was able to protect against disease in an IL-10 independent manner (Poussier et al., 2002). Interestingly then, in respect to this, it has been suggested that during the course of *H. polygyrus* infection a $CD8^+$ Treg population is induced, and that these cells are important in the observed ability of this parasite to reduce colitis in $IL\text{-}10^{-/-}$ mice (Metwali et al., 2006). Given that this helminth dwells in the gut, and that the effects of $CD8\alpha\alpha^+TCR\alpha\beta^+$ are IL-10 independent, it is a distinct possibility that these $CD8^+$ Tregs are derived from this subset.

Generation of $CD8^+$ Tregs have also been implicated as inhibiting immune responses to a number of other helminth parasites including, *S. mansoni* (Pedras-Vasconcelos and Pearce, 1996), *Echinococcus multilocularis* (Kizaki et al., 1993), and *B. pahangi* (Owhashi et al., 1990).

1.5.5 Bregs

Bregs have been reported to play an important role in a number of mouse models of immune-related disorders, in particular EAE, colitis, and diabetes (reviewed by (Mauri and Ehrenstein, 2008). As is the case for $CD8^+$ Tregs, the Breg phenotype is also poorly defined. It is clear that certain B cell subsets are responsible for production of high levels of the immunosuppressive cytokine IL-10, such as B1 cells in the peritoneal cavity (O'Garra et al., 1992), and B2 lineage marginal zone B cells (Brummel and Lenert, 2005), and that in situations of chronic intestinal inflammation, IL-10 producing CD1d positive B cells are generated (Mizoguchi et al., 2002).

During *S. mansoni* infection a population of IL-10 producing B cells is evident that are proposed to have a regulatory role, since B cell deficiency in this model is associated with increased disease severity (Jankovic et al., 1998). The fact that these B cells are regulatory is further testified to, by transfer experiments in which they were shown to reduce allergic symptoms in an airway hypersensitivity model (Smits et al., 2007), and protect against experimentally induced anaphylaxis (Mangan et al., 2004).

B cells may additionally be important in aiding the peripheral induction or maintenance of Foxp3 expression in $CD4^+$ T cells. Delayed emergence of $Foxp3^+$ cells in the CNS during the recovery phase of EAE was associated with B cell deficiency (Mann et al., 2007), whilst in the resting state B cells have been shown to expand the $CD4^+CD25^+Foxp3^+$ T cell population via a TGF-β3 dependent mechanism (Shah and Qiao, 2008). Gut-dwelling helminth infections, including *H. polygyrus* and *T. muris*, are associated with influx of B cells to the sites of infection, as well as MLNs (Parker and Inchley, 1990; Little et al., 2005). An important aspect of future research will be to ascertain whether sub-populations of these cells have regulatory potential.

1.6 Regulatory cytokines.

1.6.1 IL-10.

Although first identified in Th2 cells, as a factor that suppressed synthesis of Th1 cytokines (Fiorentino et al., 1989), IL-10 has since been shown to be produced by a number of innate and adaptive cell-types including DCs, macrophages, and various B, and T cell subsets. IL-10 is known to regulate the immune response by targeting a number of cell populations. It is able to limit activation of DCs, and Mϕs, inhibiting, MHC class II and costimulatory molecule expression, along with secretion of the proinflammatory cytokines, IL-6, IL-12, and TNF-α (reviewed by (Moore et al., 2001), thus, indirectly affecting T cell priming. Additionally, IL-10 is able to act directly on Th1 and Th2 cells to both prevent their proliferation and cytokine production, and to induce Tr1 cells (Section 1.5.2).

In a number of situations, IL-10 has been reported to act in an autocrine manner to mediate its effects. For example, IL-10 released by DCs can further reduce their ability to make chemokines, inhibiting trafficking to lymph nodes, and hence abolishing productive interactions with T cells (Demangel et al., 2002). Also, the co-production of IL-10 by Th2 (Del Prete et al., 1993), and as has been more recently described, by Th1 cells (Jankovic et al., 2007), is likely to feed back to suppress their own effector functions.

Testifying to the importance of IL-10 as an immunoregulatory cytokine, depletion, or inhibition of activity, early in infection is frequently associated with improved parasite killing. This is demonstrated in *Mycobacterium avium* infection, where ablation of IL-10 signalling in BALB/c mice leads to better control of the pathogen in this normally susceptible strain (Roque et al., 2007). However, this is not the case in all settings, and particularly gut-helminth infection, as early mAb inhibition of IL-10R signalling, or genetic deficiency of IL-10, during *T. spiralis* establishment, favours adult worm persistence (Helmby and Grencis, 2003). This was associated with increases in Th1 and Th2 cytokine production, suggesting that IL-10 controls both of these effector cell subsets at mucosal surfaces. Failure to expel *T. spiralis* was explained by abnormal maturation and development of mast cells, a

process that requires IL-10. A similar situation is observed when IL-$10^{-/-}$ animals are infected with *T. muris*, where Th2 responses are ultimately inhibited and worm clearance does not occur, leading to severe Th1-mediated immune pathology (Schopf et al., 2002). Interestingly, in the case of *T. spiralis* the effects of IL-10 were tissue specific, as although adult worm burden was enhanced, tissue-dwelling larval load was decreased.

Targeting of IL-10 mediated effects, typically at later time points in infection, rather than aiding in resolution of infection can also enhance immune mediated pathology. This is exemplified in murine models of schistosome infection where Th1 or Th2 mediated pathology is significantly accentuated in the absence of IL-10 (Hoffmann et al., 2000). IL-10 production must, therefore, be temporally and spatially controlled to initially allow proinflammatory mechanisms to clear the pathogen but to eventually modulate this to prevent life-threatening immunopathology.

1.6.2 TGF-β.

TGF-β plays an important role in many different biological processes, including development, apoptosis, wound repair, and regulation of immune responses. In the immune system this importance is demonstrated by the severe autoimmune conditions developed by TGF-β1 knock-out mice (Kulkarni et al., 1993). TGF-β is produced by a wide variety of haematopoietic and non-haematopoietic cell types, such as DCs, macrophages, T cells, thymic epithelial cells, and smooth muscle cells. It is a member of a large superfamily of TGF-β-like molecules that include, bone morphogenetic proteins (BMPs), inhibins, and activins. TGF-β is originally translated as a pre-pro-protein that must undergo a number of different steps to become an active cytokine (Annes et al., 2003). These include removal of a signal peptide in the Golgi apparatus and subsequent cleavage at a tetrabasic protease site by furin. Although the pro- and active domains are cleaved separate units, the pro-domain (termed latency-associated peptide (LAP)), remains non-covalently associated with the TGF-β.

For TGF-β to be able to bind to its receptor, the LAP must be either degraded, or dissociated. *In vitro*, this is routinely achieved by heat, or acid treatment. However, *in vivo*, a number of enzymes, often those also associated with tissue damage, have been shown to mediate this process. These include, plasmin, matrix metalloprotease -2 and -9, and thrombospondin-1 (Lawler et al., 1998; Annes et al., 2003). Furthermore, activation of this latent TGF-β to its active form also requires integrin binding *in vivo*. Of particular relevance to the immune response is the integrin $\alpha_v\beta_8$, which, when specifically knocked-out on DCs, leads mice to develop a phenotype indistinguishable from TGF-βR deficiency (Travis et al., 2007).

Another interesting feature of TGF-β processing is that TGF-β isoforms (of which there are three in mammals) are able while in their latent form to homo- or hetero- dimerise and associate with latent TGF-β binding proteins, whilst in the Golgi. The resulting large latent complex (LLC) attaches to the extracellular matrix, sequestering latent TGF-β in tissues ready to be rapidly activated when needed (Annes et al., 2003). Once activated TGF-β binds to its heterodimeric receptor composed of TGF-βRI/ALK-5 and TGF-βRII to initiate a well-characterised signalling cascade (ten Dijke and Hill, 2004).

Aside from playing a role in generating Foxp3$^+$ Tregs in both the thymus and periphery, and hence suppressing effector responses, TGF-β can modulate the responses of many different immune cell types. Like IL-10, it is able to inhibit the ability of DCs (Fainaru et al., 2007), and macrophages (reviewed by (Ashcroft, 1999) to become activated, as well as limiting mast cell function by down-regulating FcεRI expression (Gomez et al., 2005).

In vivo during schistosome infection TGF-β and IL-10 may act redundantly to establish the balance point between parasite clearance and unwanted immunopathology. When mice were treated with mAb to TGF-β alone only marginal effects on hepatic inflammation were observed, but in tandem with mAb to IL-10R, severe pathology was apparent, which was far greater than that observed with IL-10R alone (Herbert et al., 2008).

1.7 Immunomodulation by helminth-derived products.

In order to favour their persistence helminths are thought to release a variety of products that modulate the immune response generated by the host. A common feature of chronic helminth infection is a skewing of the immune response towards its regulatory arm, suppressing the Th2-type response, and thus preventing expulsion of the parasite (reviewed by (Maizels and Yazdanbakhsh, 2003; Dzik, 2006). One group of helminth-derived products that are likely to be important in mediating this suppression are products released by the parasite at the host:pathogen interface termed Excretory-Secretory products (ES). Studies have, typically, focussed on either characterising the gross immunomodulatory effects of ES, or pin-pointing specific proteins present in ES and elucidating their function. This process has been aided recently by the burgeoning amount of helminth sequence data, making it possible for proteomic techniques to be used to rapidly identify the major ES components of many organisms.

Functional analysis of helminth-derived products has been undertaken in a wide-range of settings, and from this body of work, amongst other cell-types, specific modulation of DCs, basophils, macrophages, neutrophils, and mast cells, is apparent. One interesting, but rarely discussed issue, arising from phenotyping helminth-derived products in this manner, is whether the modulation of cellular activity observed represents a suppression of the effector response, supporting parasite persistence (as is usually assumed) or bias towards a Th2-type response, aiding in host expulsion. This concept is perhaps best exemplified by considering a common feature of helminth-derived products on DCs; inhibition of normal cytokine production in response to inflammatory stimuli. When BMDCs are stimulated with Th1/17-driving bacterial components in combination with helminth-derived products that include SEA (Cervi et al., 2004), *H. polygyrus* 24 hr ES (Segura et al., 2007), or *N. brasiliensis* ES (NES) (Balic et al., 2004) suppression of cytokine release, in particular IL-12p70, towards the bacterial components is apparent. This inhibition could be predictive of the parasite products pushing the DC into a hyporesponsive state, in which its ability to induce any type of response, including Th2-type response, is impaired. Alternatively, impairment in Th1/Th17-associated cytokine expression by the DC may be an appropriate host response upon recognition of

helminth molecules to decrease production of secreted mediators that oppose generation of a protective Th2-type response. A necessity for the latter response would seem plausible given that invasion of many helminth parasites is associated with simultaneous exposure to bacteria (Section 1.3.4). As yet there is no conclusive data to imply which of these possibilities is the case, or indeed whether both are concurrently at play.

Adding to the conceptual complexity of this situation is the prospect that polarisation of a Th2-type response could also be considered an immunomodulatory tactic by the worm. As discussed previously, Th2-type responses are not always protective to helminth parasites so in some situations products that bias towards this immune polarisation status may aid in parasite persistence. Moreover, Th1/17-type responses to helminths can be associated with severe immunopathology, modulation towards a Th2-type response may, therefore, improve host fitness providing a better long-term reservoir for parasite transmission. If considered from this perspective then the mixture of products generated by the helminth must integrate to strike a delicate balance between polarising a Th2-type response, whilst simultaneously inducing regulation such that expulsion does not occur.

In the next section the effects of numerous helminth-derived products, but particularly ES, in modulating the immune response will be discussed. Attention will be paid to the issue of which products are most likely to aid in host recognition and Th2 induction, to limit the Th2-type response directly, or to bias toward a dominant regulatory component.

1.7.1 Proteases and protease inhibitors.

Proteases hydrolyse peptide bonds to catalyse a broad spectrum of biological reactions, playing important roles in a diverse array of processes, including blood coagulation, fibrinolysis and tissue remodelling. Depending on the chemical group in their active site proteases can be separated into four major classes: metallo, aspartic, serine, and cysteine. Although proteases are integral to the development of free-living nematodes, for instance in digesting the cuticle during moulting, parasitic

helminths appear to have evolved to secrete proteases that aid in their establishment and persistence in the host (reviewed by (McKerrow et al., 2006).

A stage of helminth lifecycles during which proteases are commonly excreted-secreted is during invasion of the host. All major classes of protease are present in the ES of the infective L_1 larvae of *T. spiralis* (Moczon and Wranicz, 1999; Lun et al., 2003). These may aid larval penetration of epithelial cells in the absence of any obvious mechanical means of doing so, such as oral appendages or a stylet. In particular, metalloendoproteases, that can hydrolyse components of the extracellular matrix e.g. collagen, make-up a substantial proportion of the protease pool of this ES (Lun et al., 2003). Similarly, the L3 larvae of *O. volvulus* that migrate through cutaneous tissue after entry through the blackfly bite site, and the microfilariae that travel from subcutaneous nodules to the skin, have protease activity in their ES (Haffner et al., 1998). This ES contains both serine and metalloproteases that are capable of degrading components of the dermal extracellular matrix, which can be predicted to help in migration of these different lifecycle stages to their target destinations.

Aside from invasion, another important process to parasitic helminth survival, which requires proteases, is feeding. The human hookworm, *N. americanus*, and the ruminant nematode *Haemonchus contortus*, are known to feed on blood, and tissue exudates. The adult ES from *N. americanus* and *H. contortus* contain a number of proteases, some of which are thought to degrade host tissue whilst others have anti-coagulent properties (Karanu et al., 1993; Brown et al., 1995). Interestingly, *H. contortus* adult ES cysteine proteases may play an additional role in feeding of this parasite; extra corporeal digestion of the blood meal. This was implicated from *in vitro* experiments in which cysteine protease inhibitors were shown to block degradation but not uptake of radiolabelled haemoglobin by adult *H. contortus* (Fetterer and Rhoads, 1997).

In concert with proteases, parasitic helminths also release protease inhibitors. A probable explanation for this is that, although proteases often have a high degree of substrate specificity, their activity needs to be tightly regulated in order to prevent collateral damage to both the host and worm. The potential harm that uncontrolled protease activity can cause to the helminth is evidenced by the efficacy of plant-

derived cysteine proteases in destroying the cuticle of *H. polygyrus in vitro*, (Stepek et al., 2005) and bringing about its expulsion *in vivo* (Stepek et al., 2007). It seems, however, that parasitic helminths may release protease inhibitors as method to modulate aspects of the host environment that may be detrimental to their survival. Some of the earliest data suggesting this comes from extracts of the human parasitic nematode, *Ascaris lumbricoides*, which dwells in the intestinal tract, that were found to contain inhibitors of the common digestive proteases, pepsin and trypsin (reviewed by (Knox, 2007). Other protease inhibitors are thought to have effects on the immune response, such as cysteine protease inhibitors (CPIs), termed cystatins. A cystatin from *B. malayi*, Bm-CPI-2, was found to inhibit protease-dependent processing of antigen in the MHC class II presentation pathway, limiting the ability of APCs to present T cell epitopes (Manoury et al., 2001). Altering the presentation capability of APCs in this manner would presumably prevent worm antigens being displayed in an infection setting, thus favouring persistence by preventing a T cell response being generated against the parasite. Since basophil initiation of Th2-type responses can be activated in a manner that is dependent on cysteine protease activity (Sokol et al., 2008) an as yet untested mechanism by which cystatins could function is by blocking activation of this pathway. This could potentially not only inhibit parasite cysteine proteases, which has been suggested as a method used by the host to detect and respond to worms, but also inhibit host proteases that might further contribute to basophil activation.

Alongside cystatins, serine proteases known as serpins (SPNs) have also been described as having effects on immune cells. Again identified in *B. malayi*, Bm-SPN-2, which is secreted by the microfilarial stage of the parasite, inhibits two human neutrophil-derived serine proteases, cathepsin G, and elastase (Zang et al., 1999). Cathepsin G is a chemoattractant for T cells and monocytes (Chertov et al., 1997), whilst elastase may be able to break down parasite membrane proteins. Consequently, by producing an inhibitor of these proteases the helminth may limit immune cell recruitment to the host:parasite interface and prevent neutrophil-mediated parasite killing.

1.7.2 Chitin and chitinases.

Chitin, is an abundant polymer found in various fungi, bacteria, arthropods and helminths. It has been shown that intranasal administration of chitin leads to accumulation of eosinophils and basophils in the lung, as well as rapid alternative activation of macrophages, suggesting that detection of chitin may itself drive Th2-type polarisation (Reese et al., 2007).

Throughout development of the helminth parasite chitinases are reported to play important roles in breakdown of various structures, such as the egg-shell (Arnold et al., 1993). Whilst in the intermediate host L_3 filarial helminths stockpile chitinases, which are expelled upon entry into the vertebrate host (Adam et al., 1996). Previously, an implied function for these chitinases was to aid movement of the parasite through the tissues by breaking down the extracellular matrix. However, given the ability of chitin to induce the Th2-type response *in vivo*, an alternative possibility is that the helminth releases chitinases to break down residual chitin that may be stimulating to the immune system.

1.7.3 Acetylcholinesterase.

Acetylcholine is a neurotransmitter associated with the neuromuscular system of helminths. In order to terminate neuronal signals hydrolysis of acetylcholine is mediated by acetylcholinesterase (AchE). AchE has been identified in the ES of many gut-dwelling nematodes, including *H. polygyrus* (Lawrence and Pritchard, 1993), *N. brasiliensis* (Grigg et al., 1997), and *H. contortus* (Sutherland and Lee, 1993). It has been proposed that their secretion may hydrolyse acetylcholine from the host's enteric nervous system. Since acetylcholine mediated signalling is required to stimulate chloride secretion and mucus secretion in the intestine this production of AchEs may inhibit fluid increases in the gut that are detrimental to parasite persistence.

1.7.4 Metabolic enzymes and metabolite binding proteins.

Obviously, helminth parasites express a variety of enzymes involved in metabolic processes necessary for their growth and survival. Some metabolic enzymes seem to be selectively released into the ES, raising the question as to whether they may have immunomodulatory activity. One such enzyme is triose phosphate isomerase (TPI), which is a major component of *B. malayi* adult ES (Hewitson et al., 2008), and has also been idenitified in ES from schistosome larvae (Coustau et al., 2003).

Another glycolytic enzyme found in ES is enolase, and it has been suggested that this too may have immunomodulatory activity. Enolase from the trematode *Fasciola hepatica* is able to bind to human plasminogen *in vitro*, implying that a function of this enzyme, aside from glycolysis may be as a receptor for this molecule (Bernal et al., 2004). This has been suggested to enhance the activity of plasminogen and increased plasminogen-mediated proteolysis would favour breakdown of host extracellular matrix thereby aiding in establishment of the parasite.

Retinol appears to be an important vitamin for a variety of developmental processes in helminths. Several *in vitro* studies have demonstrated uptake of radiolabelled retinoic acid (RA), by helminths, including *B. malayi* (Wolff and Scott, 1995). Furthermore, *in vivo* depletion of vitamin A led to retarded development of Mfs in *L. sigmodontis* infected cotton rats (Storey, 1982). Interestingly, inside the onchocercal nodule the concentration of retinol is estimated to be around eight-fold higher than in the surrounding tissue environment. A way in which this differential may be achieved is by secretion of retinoid-binding proteins (RBP), in *O. volvulus* known as Ov-fatty acid receptor (FAR)-1. As well as sequestering retinol for the parasite these type of molecule could play another role in immunomodulation. As described (Section 1.5.2), RA can synergise with TGF-β to induce Foxp3$^+$ Tregs. It is an as yet unexplored possibility that RBPs could enhance vitamin A uptake by host tissues to favour conversion to RA and thus enhance Foxp3$^+$ Treg induction.

1.7.5 Glycans, lectins and lipids.

The ES from helminth parasites is rich in glycoproteins and lipids. These products have been described in a number of settings as interacting with PRRs, such as Toll-like receptors (TLRs), as well as C-type lectins in the case of glycoproteins. One well-characterised example of these is lacto-N-fucopentaose III (LNFPIII) a glycan found in SEA that is also present in milk (reviewed by (Thomas and Harn, 2004). LNFPIII interacts with TLR4 and specifically activates the mitogen-activated protein kinase (MAPK) protein, extracellular signal-related kinase (ERK) (Thomas et al., 2003). DCs stimulated in this way favour Th2 polarisation of naïve T cells. This interaction could be a method by which host recognition of parasite invasion occurs, biasing towards a Th2 response. Helminth glycans can also bind to C-TLs, in a calcium-dependent fashion. DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) is one such CT-L that interacts with *T. canis* (Schabussova et al., 2007) and schistosome sugar motifs (Meyer et al., 2007). Although binding to DC-SIGN by the peanut glycan allergen Ara h 1can favour differentiation of naïve T cells to a Th2-phenotype by the DC it is not clear whether this occurs in response to these helminth products (Shreffler et al., 2006).

As well as helminth glycans binding to host CT-Ls, parasite CT-Ls, and S-type lectins, also known as galectins, have been identified in helminth ES. *T. canis* L_2 larvae produce the CT-Ls, TES-32 and TES-70 (Page et al., 1992), whilst adult *B. malayi* ES contains galectins (Hewitson et al., 2008). Whether lectins are important in immune evasion is still unclear but many hypotheses have been put forward as to their function. These include: inhibiting binding of leukocytes to selectin ligands that are upregulated when tissue is damaged; limiting glycan-recognition dependent infiltration of immune cells into sites of inflammation (Loukas et al., 2000); altering the nature of the highly glycosylated mucin barrier in the gut; and tissue recognition allowing the helminth to establish itself at a particular site. Another intriguing possibility is that galectins may be able to directly affect T cells to drive toward a specific type of response. This is indicated by work on mouse T cells, in which galectin-1 was shown to selectively induce apoptosis of Th1-differentiated cells, but not Th2-cells, via its interaction with specific surface glycoproteins (Motran et al.,

2008). Whether helminth galectins are capable of mediating similar functions is still unknown.

Helminth-derived lipids, like glycoproteins, have also been demonstrated to interact with TLRs. Schistosome lysophosphatidyl-PS (lyso-PS), interacts with TLR2, and induces DCs to polarise IL-4/IL-10 producing T cells (van der Kleij et al., 2002). Surprisingly, if the number of acyl chains was increased then DCs could be conditioned to induce IL-10 secreting Treg cells rather than Th2 cells, thus swaying the immune system away from a protective response.

1.7.6 Phosphorylcholine.

A further modification that can occur to protein and carbohydrate molecules is addition of phosphorylcholine (PC). This occurs in both prokaryotes and eukaryotes, and frequently it is found on the cuticles of nematodes. Helminths also secrete molecules that have exposed PC moieties. The best-characterised molecule of this kind is ES-62, a glycoprotein from the filarial nematode *Acanthocheilonema viteae* (Harnett et al., 1989). Homologues of this protein have been found in other filarial nematodes including *B. pahangi* (Stepek et al., 2004) and *B. malayi* (Hewitson et al., 2008). However, in *B. malayi* ES-62 was not found to be PC-rich suggesting that heavy use of the PC modification is not always associated with this molecule.

Recognition of PC by DCs and macrophages seems to be associated the adoption of a Th2 phenotype. Macrophages, for example, when pre-exposed to ES-62, are impaired in their ability to produce IL-12, IL-6 and TNF-α when subsequently exposed to IFN-γ and LPS (Goodridge et al., 2004). To some extent these changes seemed to occur via interaction with TLR4 (Goodridge et al., 2005). However, there is also some suggestion that ES-62 may be able to impair Th2-type responses. It has been shown to make lymphocytes less able to proliferate in response to ligation of their antigen receptor (Marshall et al., 2005). Furthermore, ES-62-TLR4 complexes when internalised by mast cells inhibit their ability to degranulate in response to FcεRI ligation (Melendez et al., 2007). Interestingly, there is specific inhibition of TNF-α, IL-3 and IL-6, but not IL-5 and IL-13 perhaps suggesting they are favouring some kind of modified response.

1.7.7 Venom-allergen like proteins (VAL).

Another group of proteins that are present in the ES of several nematodes are the venom-allergen like proteins (VAL). They are related to proteins first described in *Ancylostoma caninum, Ancylostoma* secreted protein (ASP), and recently they were described as the most highly represented protein family in the ES of *A. caninum* (Mulvenna et al., 2008). Many of these appeared to be highly glycosylated. It is currently unclear as to the role of VALs in the parasitic lifecycle of the helminth, although some possible activities have been described. VAL proteins from *O. volvulus* have been reported to have angiogenic activity (Tawe et al., 2000), whilst *N. americanus* ASP-2 was found to induce neutrophil recruitment (Bower et al., 2008). Analysis of the structure of these proteins has revealed that they may have a wide-variety of functions. They consist of a cysteine-bonded structural framework but can have great variability in regions outside of the core, allowing for evolution of diverse activities.

1.7.8 Cytokine homologues.

There have been a number of reports of helminths secreting homologues of host cytokines. One example is from *T. muris*, which secretes a protein that is able to bind to the IFN-γ receptor on lymphocytes, inducing cellular changes similar to IFN-γ (Grencis and Entwistle, 1997). Favouring a Th1-type response could be a method of preventing expulsion of the parasite by Th2-type mechanisms.

Homologues of macrophage migration inhibitory factor (MIF) have been identified in the ES of a number of helminth parasites (reviewed by (Vermeire et al., 2008), and in many other groups of parasites. When primary structures of MIF from *T. spiralis* was compared to mouse and human MIF it was found to have (Tan et al., 2001) 44% and 42% amino-acid identity, including many of the conserved residues. Moreover, B. malayi MIF despite only having a 28% primary sequence identity had a crystal structure that was superimposable with human MIF (Zang et al., 2002). It is possible that MIF may favour parasite persistence by inhibiting moncyte migration

local to the parasite. MIF, however, has been shown to have a wide-variety of functions *in vivo* and the main purpose of excreting this molecule could not be associated with this its monocyte migration inhibiting capability. Most recently it has been shown that parasite MIF in the presence of IL-4 can enhance alternative activation of Mϕs (Prieto-Lafuente et al., in press).

The excretion of a TGF-β homologue has also been reported by *B. malayi* (Gomez-Escobar et al., 2000). Two homologues of this protein were identified although only one was found to be present in ES. This molecule was capable of activating a TGF-β responsive cell line suggesting that it would have immunomodulatory properties *in vivo*, although none were investigated. As discussed (Section 1.6.2), TGF-β has many effects on suppressing immune responses that could benefit parasite persistence.

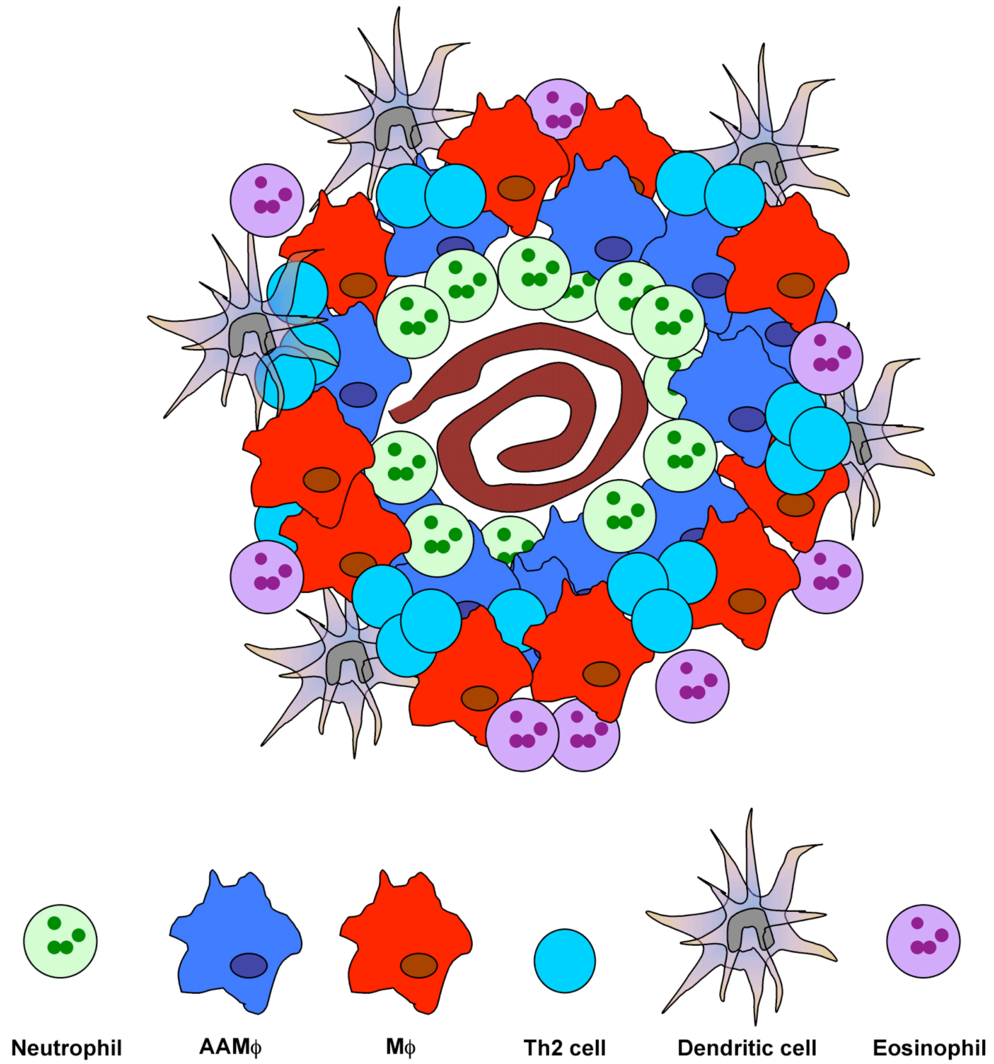


Figure 1.1 – Organisation of a Th2-type granuloma around *H. polygyrus* larvae at day 4 of a secondary challenge infection.

Adapted from (Patel et al., 2009).

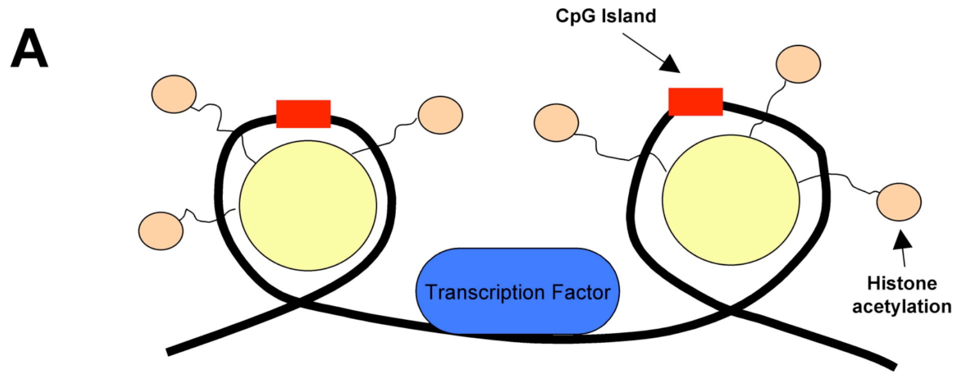
Figure 1.2 – Control of gene accessibility by MBD proteins.

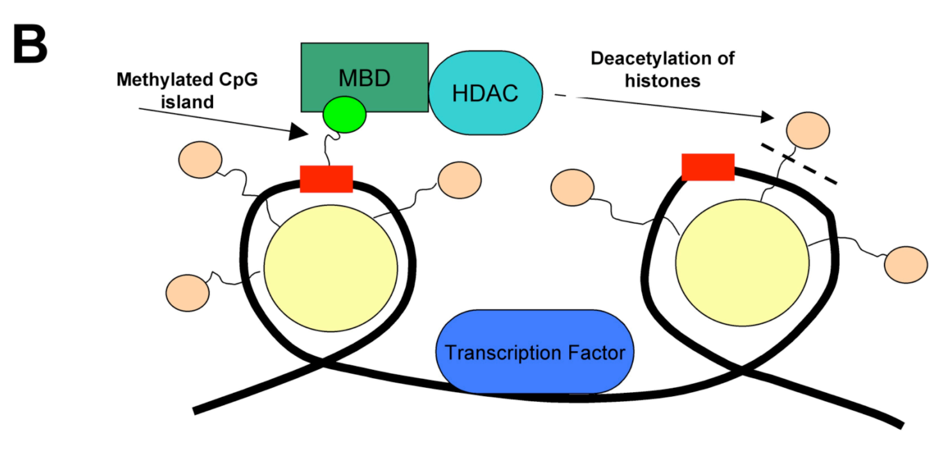
(A) CpG islands are not methylated and histone tails are acetylated. As a result DNA is loosely bound to histones and is accessible to transcription factors.

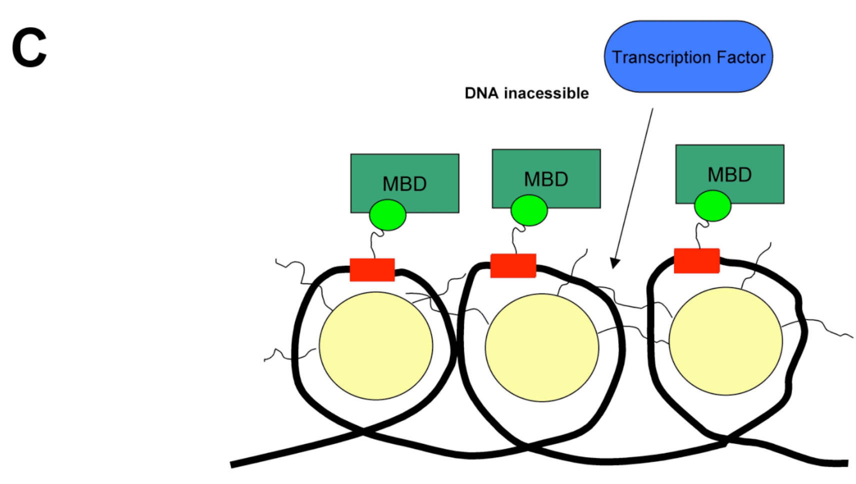
(B) CpG islands are methylated, resulting in MBD binding. MBDs recruit histone deacetylases (HDACs) that remove acetyl groups on histone tails.

(C) Loss of acetylation on histone tails leads to tight binding of the DNA around the histones and DNA becomes inaccessible to transcription factors.

Adapted from (Huehn et al., 2008).


A
CpG Island
Transcription Factor
Histone acetylation


B
Methylated CpG island
MBD
HDAC
Deacetylation of histones
Transcription Factor


C
Transcription Factor
DNA inacessible
MBD
MBD
MBD

2 Materials and Methods

2.1 Animals

CBA, C57BL/6, BALB/c, and SJL, mice used for all experiments in this thesis, were aged 6-12 weeks, and either purchased from Harlan (UK), or bred in-house. IL-12/23p40$^{-/-}$, IL-23p19$^{-/-}$, IFN-γ$^{-/-}$, TGF-βRII$^{-/-}$, MeCP2$^{-/-}$, and Foxp3-GFP knock-in, mice on a C57BL/6 background, and MBD2$^{-/-}$ mice on a BALB/c background, were also bred in-house. Mice were housed in individually ventilated cages (IVCs) during experiments. All experiments complied with UK Home Office guidelines.

2.2 General Reagents.

2.2.1 Complete RPMI.

RPMI medium, containing HEPES (Gibco), was supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), 10% heat-inactivated foetal calf serum (FCS) (Gibco), and 50 nM 2-mercaptoethanol (Gibco).

2.2.2 Serum free parasite culture media.

RPMI medium, containing HEPES (Gibco), was supplemented with 1% glucose (Sigma), 2mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco).

2.2.3 X-Vivo media.

X-Vivo 15 serum free media (BioWhittaker) was supplemented with 2 mM L-glutamine (Gibco).

2.2.4 Gut wash buffer.

Hanks' balanced salt solution (HBSS) (Sigma) was supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), and 5% heat-inactivated foetal calf serum (FCS) (Gibco).

2.2.5 Strip Buffer

Hanks' balanced salt solution (HBSS) (Sigma) was supplemented with 5 mM EDTA (Gibco), 1 mM dithiothreitol (DTT) (Sigma), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), and 5% heat-inactivated foetal calf serum (FCS) (Gibco).

2.2.6 DMEM/F12 media for MFB-F11 cell culture.

DMEM/F12+GLUTAMAX medium (Gibco) was supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), 15 μg/ml Hygromycin B (Invitrogen), and 10% heat-inactivated foetal calf serum (FCS) (Gibco) for MFB-F11 cell culture. For assaying TGF-β activity 10% FCS was replaced with 1% FCS.

2.2.7 Carbonate buffer for ELISA.

Solutions of Na_2CO_3 (Sigma) (1 M) and Na_2HCO_3 (Sigma) (1 M) were made up in distilled water. To make 0.06 M carbonate buffer, 45.3 ml of 1 M Na_2CO_3 and 18.2

ml of 1 M Na_2HCO_3 were added to 936.5 ml of distilled water and the solution was adjusted to pH 9.6.

2.2.8 FACS buffer.

FACS analyses were performed with PBS containing, 0.5% bovine serum albumin (BSA) (Sigma), and 0.05% sodium azide (Sigma).

2.2.9 ALK-5 inhibitor SB 431542.

The ALK5 inhibitor, SB 431542, was purchased from Tocris Bioscience, dissolved at 10 mM in DMSO, and used at a final concentration of 5 μM.

2.2.10 Lipopolysaccharide (LPS).

Escherichia coli LPS (Sigma) strain 0111:B4 was made up at 1 mg/ml in sterile PBS (Sigma) and used at a final concentration of 1 μg/ml.

2.3 *H. polygyrus* life cycle and excretory-secretory products (ES).

2.3.1 *H. polygyrus* lifecycle maintenance.

To maintain the *H. polygyrus* life cycle, CBA x C57BL/6 F1 mice were orally gavaged with 600 L_3 larvae. From day 14 post-infection faecal pellets were collected from these mice, and a slurry made by mixing with distilled water and charcoal. The faecal slurry was then thinly smeared on moist filter paper, which was placed in a Petri dish. Cultures were then left for 7 days in the dark, at room temperature, after which time L_3 larvae were collected by rinsing the filter paper with distilled water.

L_3 larvae were then stored at 4ºC for up to two months prior to use. Before use larvae were washed at least three times in distilled water.

2.3.2 Isolation of adult *H. polygyrus*.

The small intestine of day 14 *H. polygyrus* infected animals was removed and opened longitudinally using scissors. The opened small intestines were then gently scraped using the edge of a microscope slide to loosen worms tightly associated with the gut wall. Intestines were subsequently placed onto the upper surface of a coarse nylon mesh in a Baermann apparatus (Section 3.1), filled with HBSS (Sigma). The Baermann was incubated at 37ºC for 3 hrs after which time most of the motile parasites had become separated from the gut material and collected at the bottom of the apparatus. Typically, adults from 20 mice were then washed 12-times under unit gravity in 50 ml of RPMI (this is approx. 10x the packed volume of adult worms) (Gibco) supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma), and then incubated at room temperature for 20 min in RPMI supplemented with 1 mg/ml gentamycin (Gibco). After this time, parasites were washed a further 12-times in RPMI and were then cultured to generate *H. polygyrus* excretory-secretory product (HES).

2.3.4 *H. polygyrus* excretory-secretory product (HES).

Adult *H. polygyrus* worms were cultured in serum free parasite culture medium, at approximately 50 worms/ml for 21 days. After 24 hrs, and subsequently every 2-3 days, the supernatant was removed from the parasites and replaced with fresh media. After this period supernatants were pooled, excluding the first 24 hrs of supernatant, and concentrated and diafiltrated into PBS over a 3,000 MW filter (Millipore) in an Amicon stirred ultrafiltration device (Millipore). Routinely, 1 l of supernatant was diafiltrated to 5 ml of final product.

2.3.5 Other parasite products.

Propionibacterium acnes extract, *Salmonella typhimurium* extract, and *Schistosoma mansoni* soluble egg antigen were all kind gifts from Dr. A. MacDonald (University of Edinburgh). *Nippostrongylus brasiliensis* ES (NES) was produced in a similar fashion to *H. polygyrus* ES by Y. Harcus (University of Edinburgh). Live *Teladorsagia circumcinta* and *Haemonchus contortus* L_4 stage larvae, were supplied by Prof. D. Knox (Moredun Research Institute, Edinburgh) and subsequently cultured under the same conditions as *H. polygyrus* adults to obtain ES.

2.4 Cell isolation and culture.

2.4.4 Bone marrow derived DC culture and adoptive transfer.

Dendritic cells were derived by culturing bone marrow in the presence of GM-CSF. Briefly, PBS was used to flush the bone marrow from femurs and tibias and then homogenised by repeatedly passing through a 1.1 mm needle. Cells were plated at $2x10^6$ total cells in bacteriological petri dishes in 10 ml of complete RPMI supplemented with 20 ng/ml rGM-CSF (Peprotech). Media was replaced on days 3, 6 and 8 of culture. On day 10 cells were harvested by gently washing with media, thus removing contaminating cells that were stuck on the plates. Cells were then incubated for 18 h in the presence of antigen, in media containing 5 ng/ml GM-CSF. After this time supernatants were removed, cells washed once and $5x10^5$ cells transferred intraperitoneally (ip) into mice. Seven days later spleens were removed and $2x10^6$ splenocytes per well, in a 96-well plate, were cultured in X-vivo media (Cambrex), supplemented with 2 mM L-glutamine, in the presence of HES (10 μg/ml), SEA (10 μg/ml) or Pa (1 μg/ml) for 72 h (Section 3.2). Cytokine levels in 18 h DC supernatants or 72 h splenocyte cultures were measured by ELISA.

2.4.2 Mesenteric LN and splenocyte recovery.

MLNs, or spleens were mashed through 70 µm nylon filters (BD) to generate single cell suspensions in complete RPMI. Where necessary, red blood cells were lysed in suspensions using red blood cell lysis buffer (Sigma). Cells were then either used for FACS staining, or restimulated in complete RPMI with HES (1 µg/ml) or medium alone for 72 hrs at 37ºC, and antigen specific cytokine production measured by ELISA.

2.4.3 Peyer's patch, intraepithelial, and lamina propria, lymphocyte isolation

The small intestine was removed from naïve or *H. polygyrus* infected C57BL/6 mice and immediately placed into a Petri dish containing pre-warmed (37ºC) sterile PBS (Sigma). Peyer's patches were excised and stored in PBS on ice, until ready to process. Intestines were cut open longitudinally and washed with PBS to remove gut contents before being transferred into gut wash. Next intestines were sliced into approx. 1 cm pieces, which were then sucked up into a 25 ml stripette (Corning) in approx. 10 ml of gut wash using a Pipette aid. Media was bubbled through for 30 s, gut pieces decanted back into Petri dish and media disposed of. Process was repeated several times with fresh media until gut pieces were clean.

Peyer's patch lymphocyte isolation.

Peyer's patches were transferred into 15 ml of strip buffer, incubated for 20 min with shaking (300 rpm shaking incubator) to remove epithelial cells, and then washed in complete RPMI. Single cell suspensions of Peyer's patches were made by passing through a 70 µm filter (BD). Cells were resuspended in Percoll 30 (GE Healthcare) and then carefully layered on to a Percoll gradient in a universal tube (Sterilin) with an upper 5 ml Percoll 40 layer and a lower 2 ml Percoll 80 layer. Gradients were centrifuged for 20 min at room temperature, 400 g with the brake off. After this time cells were recovered at the 40/80 interface and washed twice in 20 ml complete RPMI before being stained for flow cytometry.

Intraepithelial lymphocyte (IEL) isolation.

Cleaned gut tissue was transferred into 15 ml of Strip Buffer in a 50 ml tube (Corning) and incubated for 15 min at 37ºC with vigorous shaking (300 rpm in shaking incubator). The supernatant was decanted and stored on ice. The process was repeated twice more, supernatants pooled and centrifuged at 200 g to pellet. Recovered cells were resuspended in 20 ml of complete RPMI and passed through a 100 μm cell strainer (BD) and then a 70 μm cell strainer (BD). IELs were then stained for flow cytometric analysis.

Lamina propria lymphocytes (LPL).

After stripping IELs, remaining gut tissue was washed in complete RPMI and then incubated for 20 min at 37ºC in 8 ml of compete RPMI containing 0.2 mg/ml liberase CI (Roche), again with shaking (300 rpm shaking incubator). Supernatants and remaining tissue pieces were then passed through a cell strainer (70 μm, BD) and then centrifuged at 200 g to pellet cells. Cells were resuspended in Percoll 30, and purified over a Percoll gradient as for Peyer's patches.

2.4.4 $CD4^+$ T cell isolation.

To purify $CD4^+$ T cells, typically $5x10^7$ red blood cell lysed splenocytes were incubated in 450 μl of complete RPMI with 25 μl of anti-CD4 (L3T4) microbeads (Miltenyi Biotech). Cells were then incubated at 4ºC for 15 min before being washed twice in 15 ml of complete media. Separation of cells that had bound beads then took place using a midi-MACS magnet in combination with a MACS LS separation column (Miltenyi Biotech). Positively selected cells were subsequently eluted from the column in complete RPMI, and cultured. Samples were also taken for surface staining to assess purity of sort by flow cytometry.

2.4.5 CFSE and CMTMR labelling.

MACS purified $CD4^+$ cells were washed and resuspended in PBS with 0.1% BSA (Sigma) at a concentration of $1x10^7$ cells/ml. CFSE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen) was then added to a final concentration of 5 μM, or CMTMR (5-(and-6)(4-chloromethyl-benzoyl-amino-tetramethyl-rhodamine)) (Invitrogen) to a final concentration of 20 μM, in the cell solution and thoroughly mixed. Cells were incubated for 15 min at 37ºC, and then washed once in complete RPMI before being resuspended in 5 ml of complete RPMI and incubated for a further 30 min at 37ºC to allow the labels to completely incorporate. Following this cells were washed twice more and then used for *in vitro* culture.

2.5 SDS-PAGE and silver staining.

For SDS-PAGE, 5 μl of 4x NuPAGE LDS sample buffer (Invitrogen) with 5% 2-mercaptoethanol (Sigma) was added to a 5 μg sample of HES made up to 15 μl with distilled water. Samples were heated for 5 min at 95ºC and then separated on a NuPAGE 4-12% Bis-Tris gel (Invitrogen). Gels were run in NuPAGE MES SDS running buffer (Invitrogen) at 200 V for 35 min.

After running, gels were fixed (40% ethanol (Fisher Scientific), 10% glacial acetic acid, Fisher Scientific, in distilled water) for 30 min, and then sensitised (30% ethanol, 5% w/v $Na_2S_2O_3$ (Sigma), 0.8 M sodium acetate (Sigma), in distilled water) for 30 min. Next the gel was washed with distilled water, and then incubated in silver reaction solution (2.5% w/v $AgNO_3$ (Sigma) in distilled water) for 30 min before being washed again. Gels were allowed to develop (200 mM Na_2CO_3 (Sigma), 0.01% formalin (Sigma), in distilled water) and then stopped (40 mM EDTA-Na_2 (Sigma) in distilled water).

2.6 FITC-dextran uptake.

After treating with antigen for 18 hrs, DCs were harvested, washed and replated. A final concentration of 1 mg/ml of FITC-dextran (Sigma) was added to the cultures and cells incubated at either 4ºC, or 37ºC for 20 min. After this time cells were harvested and washed 3x in ice cold FACS buffer before surface marker staining.

2.7 Foxp3$^+$ Treg induction assay.

CD4$^+$ cells were first MACS-purified from splenocytes, or if an initially Foxp3-GFP$^-$ population was required, stained for CD4-PerCP and sorted for GFP$^-$ population on a BD FACS Aria. Sorted cells were 5x10^5 cells per well in 24-well plates in complete RPMI. Cells were stimulated with plate-bound anti-CD3 (clone 145-2C11, 2 μg/ml), anti-CD28 (clone 37.51, 2 μg/ml) and recombinant murine IL-2 (20 μg/ml) (R+D, 402-ML-20) and either varying concentrations of rhTGF-β1 or 10 μg/ml HES. After 72 h cells were removed and stained for flow cytometric analysis.

2.8 Treg suppression assay.

Following culture under Treg inducing conditions, Foxp3-GFP positive and negative populations were FACS sorted; 5x10^4 of either population were cultured with the same number of FACS-sorted CD4$^+$Foxp3$^-$GFP$^-$ effector cells with 1x10^5 irradiated APCs and 2 μg/ml anti-CD3. Responder cells were stained with 1 ml of 20 μM CMTMR. Cells were then washed and cultured. After 90 h, cells were stained with CD4-PerCP for analysis on BD LSR II.

2.9 MFB-F11 bioassay.

MFB-F11 cells (Tesseur et al., 2006) are a mouse TGF-β1$^{-/-}$ fibroblast cell-line that have been stably transfected with a plasmid containing a TGF-β-responsive secreted alkaline phosphatase reporter construct. For the bioassay, cells were allowed to adhere to a 96-well flat-bottomed tissue culture plate at $4x10^4$ cells/well for 4 hrs in complete DMEM/F12. After the 4 hr period complete DMEM was removed, cells washed with PBS, and 50 μl samples added to each well, made-up in DMEM/F12 assay media (1% FCS. MFB-F11 cells were then incubated for 21 hrs with samples and after this time 10 μl of each supernatant removed to detect SEAP activity. SEAP was detected using the Great EscAPe SEAP chemiluminescence kit 2.0 (ClonTech) as per manufacturer's instructions and read on a LUMIstar luminometer (BMG Biotechnologies).

2.10 Detection of cytokines by ELISA.

Cytokine levels were detected in culture supernatants by enzyme-linked immunosorbent assay (ELISA). Plates were first coated with monoclonal capture antibodies by adding 50 μl of carbonate buffer containing antibody at concentration stated (Table 2.1) to each well of an ELISA plate (NUNC, Immunoplate MaxiSorp, NUNC) and incubating overnight at 4ºC. The next day carbonate buffer was flicked off and plates blocked for 2 hrs at 37ºC with 200 μl/well of TBS with 0.05% Tween and 10% FCS.

After blocking, plates were washed 5x in TBS with 0.05% Tween and then 50 μl of sample or standard was added to each well. The standard was a twelve-point standard of doubling dilutions starting at the concentrations stated (Table 2.1), made up in the same media that had been used for cell culture. Plates were then left to incubate overnight at 4ºC.

Following incubation, samples were flicked off and plates washed 5x with TBS with 0.05% Tween. Biotinylated detection antibodies were then added, 50 μl per well, and plates incubated at 37ºC for 1 hr (Table 2.1). After this time plates were

washed as previously, 50 μl of streptavidin-alkaline phosphatase (1:10,000)(Sigma) added to each well, and incubated for a further 45 min.

Finally, plates were washed 3x with TBS with 0.05% Tween, and 2x with distilled water, before adding 100 μl of p-nitrophenyl phosphate (pNPP) substrate to each well. After colour had developed optical densities were measured using an Emax precision microplate reader (Molecular Devices).

Table 2.1 – ELISA antibodies.

Cytokine	**Top Standard**	**Capture** Clone and Supplier	**Capture** mAb (coating conc.)	**Detection** Clone and Supplier	**Detection mAb** (coating conc.)
IFN-γ	50 ng/ml	R46A2, in house	2 μg/ml	BVD6-24G2, BD	5 μg/ml
IL-4	10 ng/ml	1B11, in house	2 μg/ml	TRFK4, BD	2 μg/ml
IL-6	100 ng/ml	MP5-20F3, BD	2 μg/ml	MP5-32C11, BD	0.5 μg/ml
IL-10	20 ng/ml	JES5-2A5, BD	4 μg/ml	SXC-1, BD	2 μg/ml
IL-12p40	100 ng/ml	C15.6, BD	2 μg/ml	C17.8, BD	0.5 μg/ml
IL-12p70	20 ng/ml	9A5, BD	4 μg/ml	C17.8, BD	0.5 μg/ml
IL-13	20 ng/ml	38213, R&D	2 μg/ml	Rabbit polyclonal, Peprotech	0.5 μg/ml
IL-17	20 ng/ml	TC11-18H10, BD	1 μg/ml	TC11-8H4.1, BD	0.25 μg/ml

2.11 Flow cytometric analysis.

2.11.1 Staining surface markers.

Cells were stained in 96-well round-bottomed plates. For DCs, typically $1x10^5$ cells were stained per sample, and for MLNs, or splenocytes typically $1.5x10^6$ cells. Prior to FACS antibody staining of cells, Fc receptors were blocked in 40 μl of FACS buffer containing 100 μg/ml of naïve rat IgG (Sigma) for 20 min at 4ºC. Samples were then washed in 200 μl of FACS buffer and stained for 20 min in 20 μl of FACS buffer containing antibodies at concentrations listed (Table 2.2), or appropriate isotype controls. After staining cells were washed twice in 200 μl of FACS buffer before acquisition on the LSR II flow cytometer (BD Bioscience), and subsequently being analysed using FlowJo (Tree Star).

Table 2.2 – FACS antibodies.

Antibodies	Clone and Supplier	Dilution	Isotype	Fluorochrome
B220	RA3-6B2, BD	1/200	Rat IgG2a	PerCP
CD4	RM4-5, BD, Biolegend	1/200	Rat IgG2a	FITC/PE/PerCP
CD8	53-6.7, Biolegend	1/200	Rat IgG2a	PE
CD11c	N418, Biolegend	1/200	Hamster IgG1	APC
CD25	PC61, Caltag	1/200	Rat IgG1	PE
CD40	3/23, BD	1/100	Rat IgG2a	PE
CD80	16-10A1, BD	1/100	Rat IgG2a	PE
CD86	GL1, BD	1/100	Rat IgG2a	PE
CD103	M290, BD	1/200	Rat IgG2a	Biotin
MHCII	M5/114.15.2	1/200	Rat IgG2a	PE

2.11.2 Intracellular cytokine staining.

To measure intracellular cytokines, cells were first stimulated for 4 hrs at 37ºC in the presence of PMA (50 ng/ml) (Sigma), Ionomycin (1 μg/ml) (Sigma), and Brefeldin A (20 μg/ml) (Sigma). Samples were then stained for surface markers. Following surface staining, cells were permeabilised for 30 min at 4ºC in fix/perm solution (BD Biosciences), and then washed twice in 200 μl of perm/wash (BD Biosciences). Cells were blocked and stained for intracellular cytokine expression (Table 2.3) in the same manner as for surface markers but substituting perm/wash for FACS buffer.

Table 2.3 – Intracellular cytokine antibodies.

Antibodies	Clone and Supplier	Dilution	Isotype	Fluorochrome
IFN-γ	XM6-1.2, Biolegend	1/200	IgG1	FITC/APC
IL-4	1B11, Biolegend	1/200	IgG1	PE
IL-10	JES5-16E3, BD	1/200	IgG1	APC
IL-17	TC11-18H10, BD	1/200	IgG1	PE

2.11.3 Foxp3 staining.

Samples were stained for surface markers. Following surface staining, cells were permeabilised for 18 hrs at 4ºC in fix/perm solution (eBioscience Foxp3 staining set), and then washed twice in 200 μl of perm/wash (eBioscience Foxp3 staining set). Cells were blocked and stained for Foxp3 expression (FJK-16s, isotype rat IgG2a, ebiosciences, 1/200 dilution) in the same manner as for surface markers but substituting perm/wash for FACS buffer.

2.12 Gene expression analysis by mRNA.

2.12.1 Gut mRNA extraction.

After removal, the small intestine of naïve or *H. polygyrus* infected mice was flushed with pre-warmed PBS (37ºC). Approximately 0.5 cm of gut (uppermost part of the duodenum) was removed, placed into 1 ml of Trizol (Invitrogen), and immediately stored at -80ºC. RNA was later extracted according to the manufacturer's protocol Briefly, tissue was disrupted using a TissueLyser (Qiagen). Samples were then centrifuged at 12,000 g for 15 min at 4ºC, and the upper aqueous layer removed. This was added to 500 μl of isopropanol, mixed, and left to incubate at room temperature for 10 min. After this time the sample was centrifuged again at 12,000 g for 10 min at 4ºC to pellet the RNA. RNA was washed once in 70% ethanol, and the pellet then allowed to air dry before being dissolved in 50 μl of DEPC-treated water. A DNA-free kit (Ambion) was used to degrade any remaining DNA. To do this, 2 μl of DNase with 2.4 μl of DNase buffer was added to 20 μl of dissolved RNA and incubated at 37ºC for 30 min. Subsequently, 5 μl of DNase inactivation reagent was added, and incubated at room temperature for 2 min before sample was spun down briefly and supernatant recovered. RNA concentration was determined using the Nanodrop 1000 (Thermo Scientific).

2.12.2 Reverse transcription

Reverse transcription was performed using 1-2 μg of RNA, 2 μl 10 reverse transcriptase buffer, 2 μl 25 mM dNTP mix, 1 μl of 50 U/μl MMLV reverse transcriptase, 0.5 μl of 40 U/μl RNAsin, 1 μl of 0.4 μg/ml Oligo dT primer, and made up to 20 μl with DEPC treated water. A PCR block (Peltier Thermal Cycler, MJ Research) was used for the transcription reaction, 20ºC for 10 min, 37ºC for 60 min, and 99ºC for 5 min.

2.12.3 Light cycler.

Relative quantification of the genes of interest were measured by real-time PCR using the Lightcycler 480 II (Roche). PCR amplifications were carried out in 10 μl total volume made up of 1 μl cDNA, 5 μl SYBR Green (Roche), 0.3 μl of each primer (10 μM) (Table 2.3), and 3.4 μl DEPC treated water (Ambion). The amplifications were performed using the following protocol: 30 s denaturation at 95ºC, 5 s for primers to anneal at 55ºC, 12 s elongation at 72ºC, for 40 cycles.

Gene	Forward Primer	Reverse Primer	Amplicon Length
Arg-1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	249
HPRT	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	90
RELMα	TATGAACAGATGGGCCTCCT	GGCAGTTGCAAGTATCTCCAC	107
Ym1	TCACAGGTCTGGCAATTCTTCTG	TTGTCCTTAGGAGGGCTTCCTC	435

2.13 Statistical analysis.

All statistical analyses were performed using Prism 4.0b (Graphpad Software Inc.). For comparisons of two groups Student’s t test was used. When three or more groups were analysed then a one-way ANOVA was used with a Tukey’s multiple comparison test. P values of <0.05 were considered to be significant.

3 HES alters DC responses to inflammatory stimuli.

3.1 Introduction

Dendritic cells (DCs) provide a key link between the innate and adaptive immune system. This cell-type acquires antigen in the peripheral tissues and then migrates to the draining LNs where it can initiate antigen-specific helper T cell expansion, and drive these cells towards a specific helper lineage. In response to Th1-driving pathogens, including bacteria, and protozoa, DCs take on what is termed a 'mature' or 'classically' activated phenotype, characterised by upregulation of major histocompatibility complex (MHC) II, and costimulatory molecules CD80, CD86, and CD40 (reviewed by (Pulendran, 2005)). These phenotypic changes are triggered by both release of inflammatory cytokines such as tumour necrosis factor (TNF)-α, and ligation of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs), for example lippopolysaccharide (LPS) detection by toll-like receptors (TLRs) (Fitzgerald et al., 2004).

When DCs are stimulated with helminth-derived products, including SEA (MacDonald et al., 2001), ES-62 (Whelan et al., 2000), and NES (Balic et al., 2004), *in vitro*, they become competent to stimulate Th2-polarisation. Unlike Th1-type pathogen products, no distinct Th2-favouring DC phenotype has been found. SEA-pulsed DCs have a phenotype that is almost indistinguishable from an unstimulated DC (MacDonald et al., 2001; Kane et al., 2004), whilst NES pulsed DCs upregulate expression of only a limited subset of the proteins typically associated with responses to Th1-driving pathogens (Balic et al., 2004).

Although DCs treated with helminth-derived products do not acquire a characteristic phenotype upon initial stimulation, they may be compromised in their ability to concurrently, or subsequently, respond to Th1-type PAMPs (Balic et al., 2004; Cervi et al., 2004; Kane et al., 2004; Goodridge et al., 2005). This was first demonstrated by co-pulsing bone-marrow derived (BM) DCs with SEA, and a preparation derived from the heat-killed gram-positive bacterium, *Propionobacterium acnes* (Pa). Production of the Th1-driving cytokine IL-12p70 was suppressed when DCs were co-pulsed with SEA/Pa compared to Pa alone, but

the anti-inflammatory cytokine IL-10 was enhanced (Cervi et al., 2004). This, and other similar work (Balic et al., 2004), suggested that helmith-derived products function by actively modulating the DC to drive a Th2-type response (Cervi et al., 2004). Given this, it is surprising that there have been no studies addressing whether suppression of DC activation towards Th1-driving stimuli correlates with the potency of the eventual helminth-specific Th2-type response. More recently, and in opposition to the idea of helminth-derived products actively driving Th2 polarisation, it was shown that 24 hr ES from *H. polygyrus* suppresses antigen-specific Th2-type induction by DCs, possibly as an immunomodulatory tactic to prevent its recognition (Segura et al., 2007).

Here the phenotype of BMDCs exposed, *in vitro*, to *H. polygyrus* ES (HES), and in co-treatments with inflammatory stimuli, are characterised. Subsequently, BMDCs are adoptively transferred, *in vivo*, and their ability to favour Th1, or Th2, polarisation assessed. Making use of a heat-inactivated form of HES (hiHES), the association between inhibition of phenotypic maturation and T cell differentiation is particularly focussed upon.

3.2 Results

3.2.1 Production of HES

Although there is published information on the ES from various lifecycle stages of several nematodes, including *B. malayi* (Hewitson et al., 2008), *T. canis* (Loukas et al., 2000) and *N. brasiliensis* (Holland et al., 2000), prior to the commencement of this project no work had been undertaken using the ES from adult *H. polygyrus*. Initially, therefore, methods had to be developed to both obtain adult worms in large enough numbers, and to maintain them for prolonged periods, to make sufficient quantities of *H. polygyrus* ES (HES) to be able to analyse its potential immune functions.

C57BL/6 x CBA F1 mice were used as hosts, since their large size and lack of resistance to primary infection (Chapter 4) meant that each animal could be infected with 600 *H. polygyrus* L_3 larvae by oral inoculation. Fourteen days post infection, when the worms are fecund and have been dwelling in the gut lumen for 5-7 days, mice were sacrificed and adult *H. polygyrus* isolated from the intestine using a modified Baermann technique (Fig. 3.1). After removal from the host, the worms were cultured in serum free media under similar conditions to *N. brasiliensis* (Balic et al., 2004). Typically, *N. brasiliensis* adults are cultured for 7 days. However, when assessed by microscopy, *H. polygyrus* were found to be viable and laying eggs up to day 28 of culture.

Every 2-3 days the supernatant from the worms was collected and replaced with fresh media. This was then diafiltrated and concentrated over a 3,000 MW filter, generating what will be referred to as *H. polygyrus* ES (HES). To assess whether the HES produced was broadly similar across the entire 4-week period, pooled supernatant taken at each week was visualised by silver-staining on a 1D gel (Fig. 3.1). Despite being a relatively crude way of comparing ES components, no dramatic differences in protein expression were noted between the various samples. On this basis, all subsequent batches of HES were made by concentrating pooled supernatants from several weeks of culture. Two further refinements were made to

the protocol: worms were only maintained for 3 weeks, to further ensure that products expelled by dying worms did not contaminate the cultures; and the first 24 hrs of supernatant was excluded, as in the case of *N. brasiliensis* some carry-over of host proteins has been noted (Healer et al., 1991). Samples of HES were subsequently run on 2D gels and the protein components were found to be very different to those present in 2D gels of adult worm homogenate (*H. polygyrus* extract (HE_x)). Implying that the HES did represent proteins selectively secreted by the parasite (K. Filbey, unpublished observation).

3.2.2 HES does not induce classical DC activation

The ability of HES to induce inflammatory cytokine production from, and alter surface phenotype of, BMDCs was first investigated. BMDCs were stimulated, *in vitro*, for 18 hrs (Fig 3.2) with either PBS, HES, heat-inactivated HES (hiHES – heat inactivation of HES was carried out for 30 min at 100ºC), or the TLR-4 ligand LPS, as a positive control. After this time, production of cytokines associated with classical DC activation (Fig. 3.3), and expression of surface markers (Fig. 3.4), were analysed. LPS, as anticipated, stimulated release of the pro-inflammatory cytokines IL-6, IL-12p40 and IL-12p70, and the regulatory cytokine IL-10. HES, in contrast, did not induce production of any of these cytokines (Fig. 3.3). Additionally, LPS triggered strong upregulation of the co-stimulatory molecules CD40, CD80 and CD86 as well as MHC class II. In comparison to LPS, HES treatment made very little difference to surface marker expression, although a small positive shift in MHC class II was apparent (Fig. 3.4).

hiHES allows any effects of heat-stable components in the HES, (possibly including LPS) to be identified, by relieving potential inhibition of inflammatory responses that may be caused by heat-labile parasite proteins. There are unlikely to be major inflammatory contaminants in HES, as no cytokine production was evident in response to hiHES (Fig. 3.3). Moreover, although greater differences in surface marker expression were apparent than with HES, these where marginal when compared to LPS (Fig. 3.4).

3.2.3 Treatment of DCs with HES does not affect antigen uptake.

Even though HES did not appear to classically activate the DC it was still possible that an alternative form of activation had occurred. If this was the case then one parameter that may be altered is the ability of the DC to subsequently take up antigen. This was quantified, *in vitro*, by assessing acquisition of fluorescently labelled dextran (FITC-dextran) as a measure of endocytic activity (Fig. 3.5). After 1 hr incubation at 37ºC, approx. 50% of the PBS-treated DCs had taken up FITC-dextran. This was unaltered in cells that had been pre-incubated with HES, or hiHES, for 18 hrs prior to addition of the fluorescent marker. LPS, however, markedly inhibited uptake, with only 19.6 % of DCs found to be FITC-dextran positive. Thus, HES does not inhibit endocytic uptake by DCs.

3.2.4 HES limits LPS induced DC cytokine release.

There was no evidence of HES activating the DC, in terms of classical activation phenotype. As discussed previously, this has been described for several helminth products including SEA, ES-62, and NES (Section 3.1). These helminth products did, however, affect the ability of the DC to respond to inflammatory stimuli. It was possible that HES would have a similar effect. DCs were co-treated with LPS and increasing amounts of HES for 18 hrs. Levels of the IL-12p70, and IL-10, were then measured in the supernatants. HES dramatically inhibited release of the Th1-driving cytokine IL-12p70, in a concentration dependent manner (Fig. 3.6 A, right panel). This was not a general suppression of cytokine production from the DC, little effect on IL-10 was detected, even with high concentrations of HES (Fig. 3.6 B, right panel). The inhibition was dependent on heat-labile components in the HES since hiHES, at the same concentration as the HES maximum, did not alter LPS stimulated cytokines (Fig. 3.6 A, B left panel).

3.2.5 HES modulates LPS stimulated changes to surface phenotype.

In concert with cytokine production, the ability of HES to alter LPS triggered surface marker upregulation on the DC was also analysed (Fig. 3.7). Although effects were not as striking as those observed for cytokine production, HES did limit, to some extent, upregulation of all surface markers in response to LPS (Fig. 3.8). Particularly affected were the co-stimulatory molecules CD40 ($CD40^+$ DCs, LPS mean 67.7%, HES/LPS mean 48.6%), and CD86 (CD86 high DCs, LPS mean 89.7%, HES/LPS mean 76.0%). Once again, the suppressive capabilities of HES were largely abrogated by heat-inactivation.

3.2.6 Inflammatory cytokine inhibition by HES is short-lived.

HES suppression of cytokine release could represent a long-term change to the DC phenotype, or a relatively transient effect. To distinguish between these two possibilities, DCs were pre-incubated for 18 hrs with HES, or hiHES, washed and then restimulated with LPS, again for 18 hrs, at various time points (Fig. 3.9 A). Interestingly, at day 0, when cells were pre-incubated with HES before addition of LPS, rather than co-pulsed, inhibition of IL-10, as well as IL-12p70 was observed (Fig. 3.9 B, C). Furthermore, pre-incubation with hiHES had some suppressive capacity, although not as great as that of HES.

The ability of HES to limit LPS stimulated IL-12p70 release was short-lived. At day 0 (LPS added after the initial 18 hrs pre-incubation with HES) a greater than two-fold reduction in IL-12p70 levels was recorded, however, by d1 IL-12p70 concentration was not different to the PBS control DCs (Fig. 3.9 B). Similar effects were apparent on IL-10 secretion, but some degree of suppression was still apparent at day 3 (Fig. 3.9 C). Surprisingly, IL-12p70 release was greater in HES treated DCs on day 3 than the PBS, or hiHES groups. This is possibly related to differential

protein expression as a result of previously being in an inhibited state. By day 5 cytokine levels were reduced in all groups, probably as a result of cell death.

3.2.7 HES inhibits DC activation by *P. acnes* extract.

It has been reported, that DCs are able to segregate both Th1 and Th2 antigens into separate compartments and thus simultaneously induce both Th1 and Th2 responses (Cervi et al., 2004). These studies were performed using *P. acnes* extract (Pa), and SEA.

To assess whether DCs were able to process HES, and a Th1-type antigen in a similar manner, adoptive transfer experiments were undertaken with BMDCs pulsed with HES, or hiHES, in combination with Pa. Groups of recipient mice were given intraperitoneally PBS BMDCs (A), HES pulsed BMDCs (B), hiHES pulsed BMDCs (C), Pa pulsed BMDCs (D), HES/Pa co-pulsed BMDCs (E), or hiHES/Pa co-pulsed BMDCs.

Prior to transfer, the cytokine production from (Fig. 3.10), and surface phenotype of (Fig. 3.11), the DCs were assessed to confirm that HES had anti-inflammatory properties when co-pulsed in the presence of Pa, as had been observed with LPS. It was possible that there would be differences in this situation since Pa is a complex mixture of PAMPs rather than a specific TLR-ligand. As previously HES and hiHES pulsed DCs showed no secretion of cytokines or upregulation of surface markers (data not shown).

Pa led to increases in IL-6, IL-10, and IL-12p70, levels, along with CD40, CD80, CD86, and MHC class II, expression. HES inhibition of Pa induced activation was phenotypically almost indistinguishable from LPS. In terms of cytokine secretion IL-12p70, and IL-6, were limited, but IL-10 was unaffected (Fig. 3.10), whilst downregulation was observed for all surface markers, particularly CD40, and CD86 (Fig. 3.11). Additionally, all inhibitory effects were decreased for hiHES.

3.2.8 HES/Pa co-pulsed DCs are able to maintain segregation of Th1, and Th2, but not Th17 antigen specific responses.

Having assessed that HES was able to alter the DC response to Pa, cells were adoptively transferred into recipient animals (Fig. 3.2). Seven days later, splenocytes from these mice were re-stimulated with HES or Pa and the production of cytokines associated with Th1, Th2 and Th17 polarisation measured (Fig. 3.12). Pa-pulsed DCs induced a Th1/Th17 response, as previously reported, whilst HES induced a Th2 response. DCs co-pulsed with HES and Pa developed a Pa-specific Th1 response and a HES-specific Th2 response. No evidence of HES-specific Th1 responses or Pa-specific Th2 responses could be observed. However, the strength of both the Th1 and the Th2 responses with co-pulsed DCs were reduced compared to those pulsed with each antigen alone.

Interestingly, Th17 cytokines did not follow the same pattern as those observed for Th1 and Th2. In comparison to Pa, DCs pulsed with HES had weak Ag-specific IL-17 production. However, in DCs co-pulsed with both Ags, HES-specific IL-17 responses developed that were of a similar intensity to the Pa-specific responses in DCs pulsed with this antigen alone. Additionally, unlike Pa-specific IFN-γ, Pa-specific IL-17 was not significantly reduced in co-pulsed DCs.

Antigen-specific secretion of IL-10, a cytokine associated with regulation in both Th1 and Th2 settings, was also measured in the restimulated supernatants. IL-10 was found to be produced at comparable levels in response to both HES-pulsed DCs and Pa-pulsed DCs. Surprisingly, given the strong IL-10 responses in the single Ag-pulse setting, HES-specific and Pa-specific IL-10 were both dramatically decreased when DCs were co-pulsed with these antigens.

3.2.9 SEA/Pa co-pulsed DCs are also unable to maintain segregation of Th17 antigen specific responses.

The cross-induction of IL-17 production towards HES could be a general feature of antigen co-pulse, or be specific to HES/Pa. The same experiment was, therefore, performed using PBS, SEA, Pa, or SEA/Pa co-pulsed DCs (Fig. 3.13). There was no evidence of Th1 polarisation towards SEA, nor Th2 polarisation towards Pa in any of

the groups. However, an IL-17 response developed towards SEA when products were co-pulsed together, as was found for HES/Pa.

3.2.10 Anti-inflammatory and Th2 driving properties of HES are separable.

Previously, it has been suggested that suppression of the ability of DCs to make inflammatory cytokines by helminth products is linked to their ability to drive a Th2 response (Cervi et al., 2004). hiHES is unable to suppress inflammatory cytokine production in response to Pa or LPS, and as a result it would be hypothesised to have reduced Th2 polarising potential. However, DCs pulsed with hiHES were able to drive IL-4 and IL-13 production to the same extent as HES. Moreover, there was no evidence of a deviation towards Th1 or Th17 polarisation and they stimulated a similar level of IL-10 production as HES (Fig. 3.12).

Although hiHES induces a comparable Th2 response to HES in a single pulse setting it is possible that the anti-inflammatory properties are important in generating a Th2 response in the face of a concurrent inflammatory stimulus. The effects of hiHES were studied when co-pulsed with Pa. hiHES/Pa co-pulsing of DCs, like HES/Pa, led to induction of HES-specific Th2 responses and Pa-specific Th1 responses. There was no discernable difference in levels of Th2 cytokines or IL-10. Additionally, there was no evidence of a significant Th1-specific HES response occurring in the hiHES/Pa co-pulsed DCs. There was, however, evidence of the emergence of a HES specific Th17 response that had been previously observed in HES/Pa co-pulsed DCs (Fig. 3.12). It appears, therefore, that the anti-inflammatory properties of HES are not related to the ability of HES to induce a Th2 response.

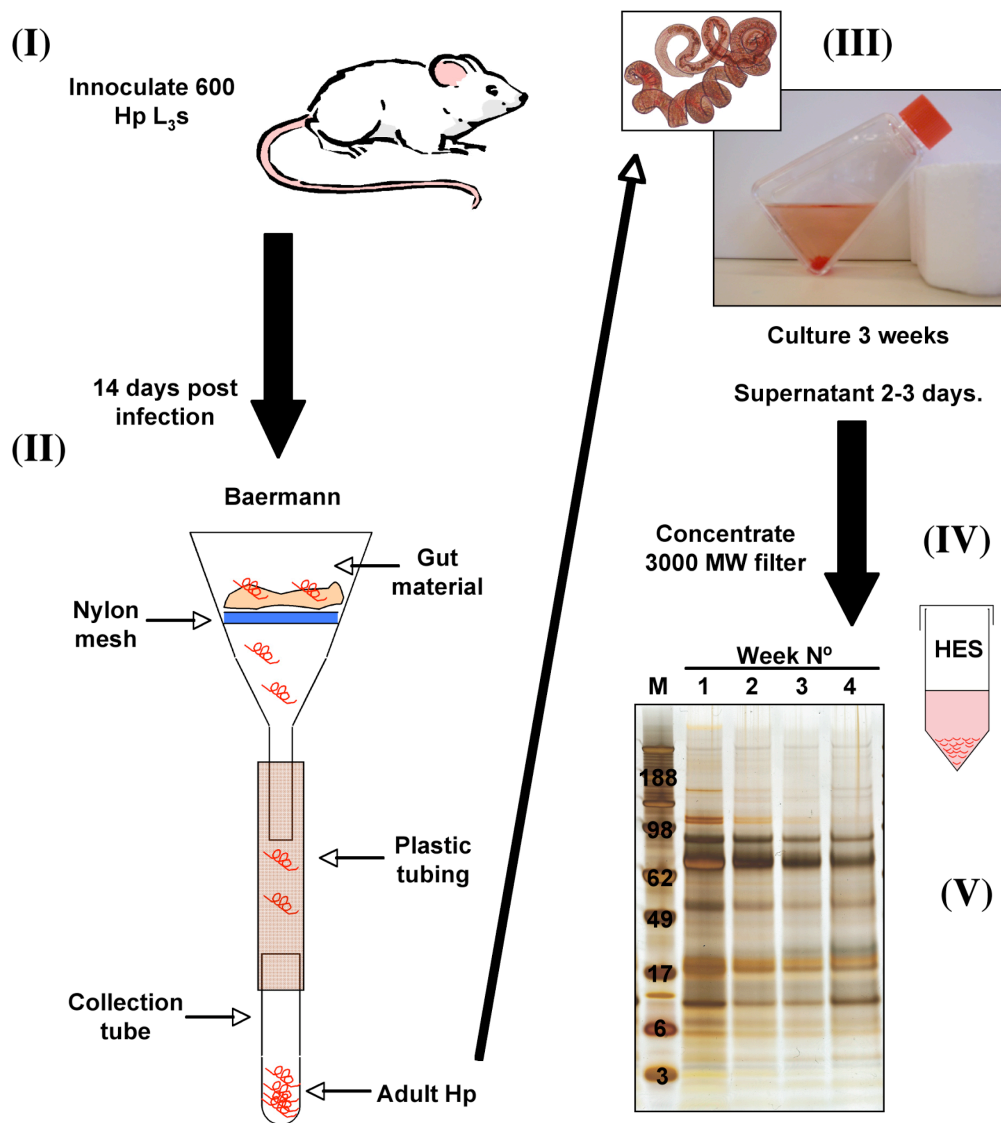


Figure 3.1 – Collecting *H. polygyrus* ES (HES).

(I) CBA (M) x C57BL/6 (F) mice were inoculated with 600 Hp L3s. **(II)** Fourteen days later mice were sacrificed and worms separated from gut material using a modified Baermann technique. **(III)** Worms were thoroughly washed and cultured at approx 50 worms/ml in vented flasks. Every 2-3 days supernatant was collected and replaced with fresh media. **(IV)** Worms were cultured for 21 days, after which time supernatant was concentrated down over a 3,000 MW filter. Concentration of HES was measured by Bradford assay. **(V)** One-dimensional silver stained gel showing different weeks of HES. M = molecular weight marker.

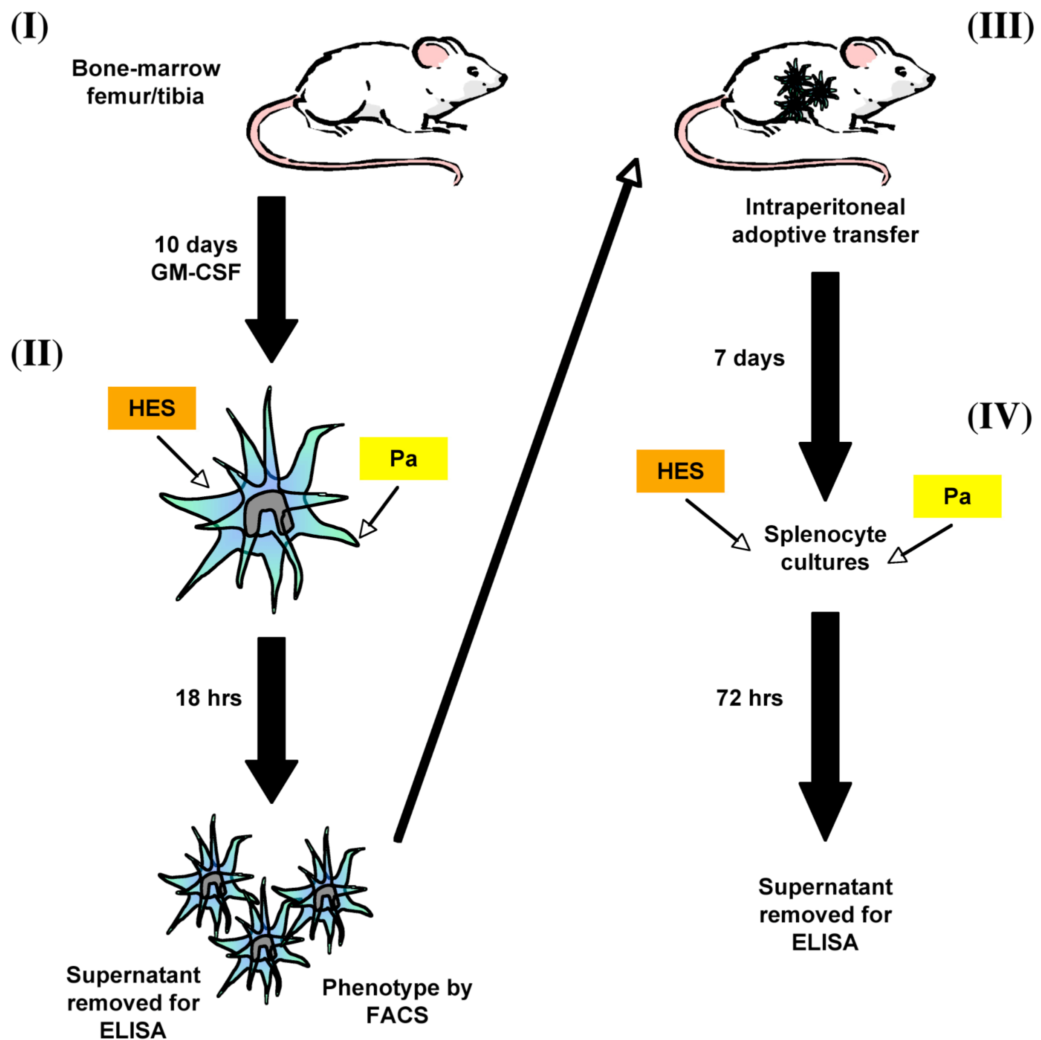


Figure 3.2 – BMDC transfer model.

(I) BMDCs were generated by culturing bone-marrow cells from C57BL/6 mice for 10 days in the presence of granulocyte macrophage colony stimulating factor (GM-CSF). **(II)** DCs were then stimulated with antigens, such as HES, or Pa, for 18 hrs, after which time supernatants were removed for cytokine ELISAs, and cells were washed and intraperitoneally transferred in recipient animals. **(III)** 7 days later mice were sacrificed and splenocyte cultures restimulated with antigens. After 72 hrs of culture supernatants were removed for cytokine ELISAs.

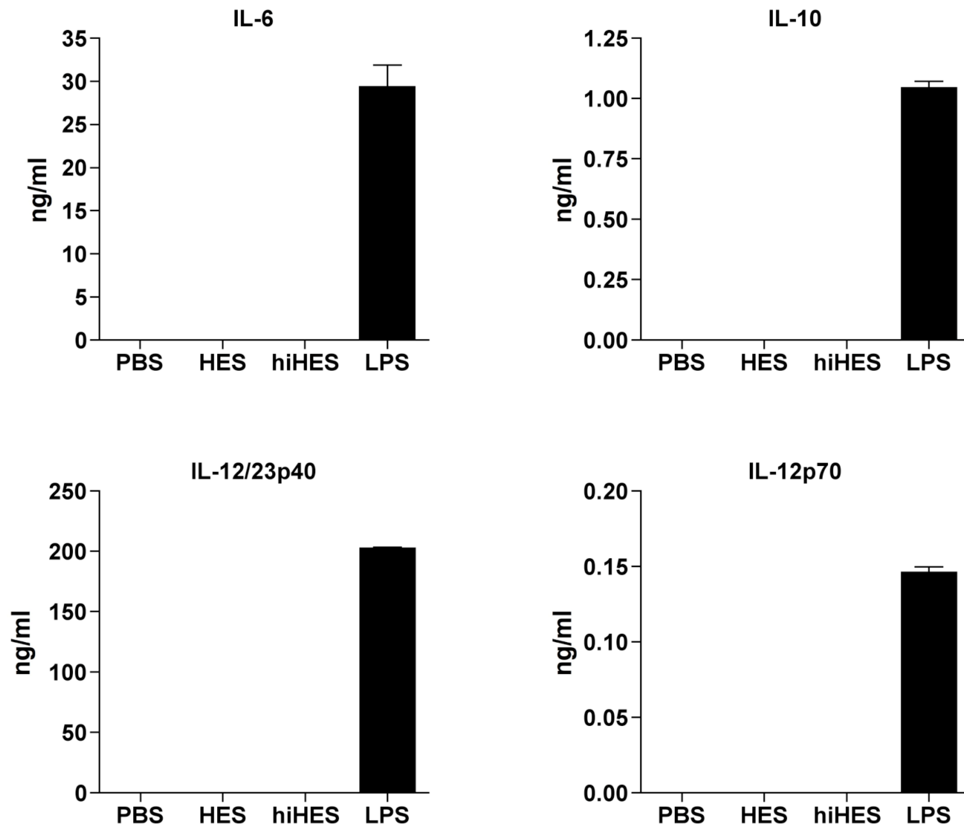


Figure 3.3 – HES does not induce inflammatory cytokine production by DCs.

DCs were stimulated, *in vitro*, with PBS, HES (25 μg/ml), hiHES (25 μg/ml), or LPS (1 μg/ml). After 18 hrs supernatants were removed and cytokines measured by ELISA, IL-6, IL-10, IL-12/23p40, and IL-12p70. Detection limits: IL-6, 400 pg/ml, IL-10, 20 pg/ml; IL-12p70, 400 pg/ml, IL-12p40, 40 pg/ml. Error bars represent SEM of triplicate wells plated from the same BMDC cultures in a single experiment. Data are representative of at least three similar experiments.

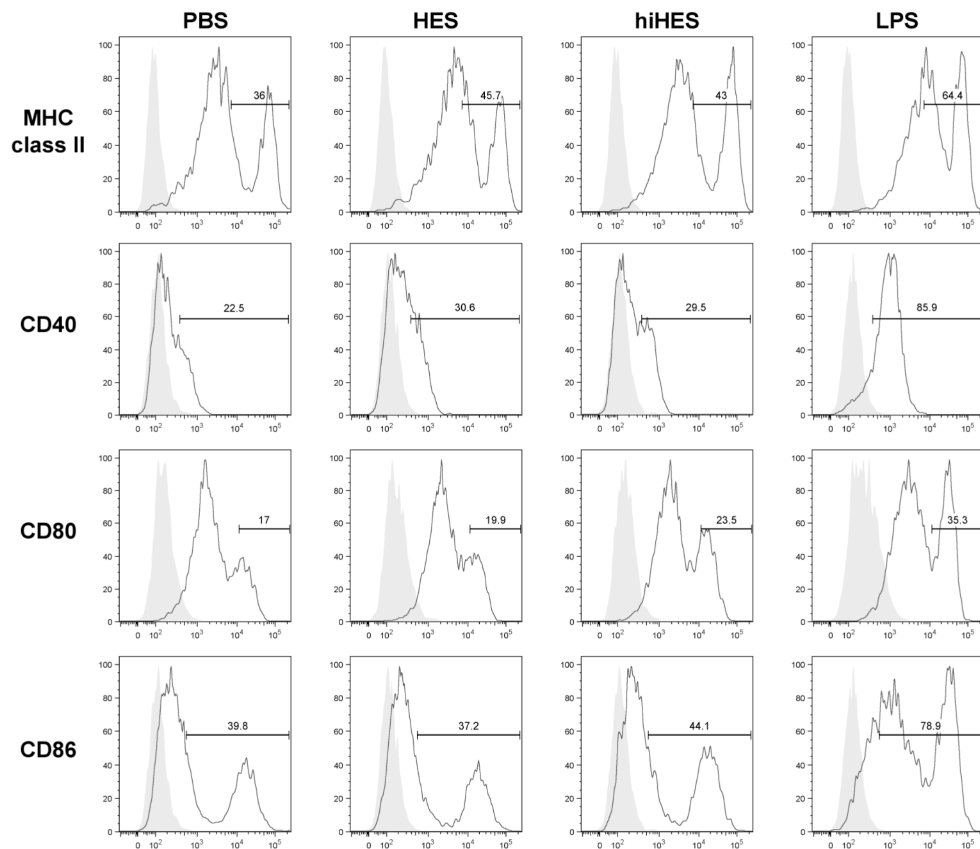


Figure 3.4 – HES does not upregulate surface expression of DC activation markers.

DCs were stimulated, *in vitro*, with PBS, HES (25 μg/ml), hiHES (25 μg/ml), or LPS (1 μg/ml). After 18 hrs cells were collected and their surface expression of CD40, CD80, CD86, and MHC class II, assessed by flow-cytometry. Black line, positive stain; solid grey, isotype. This is representative of at least three similar experiments.

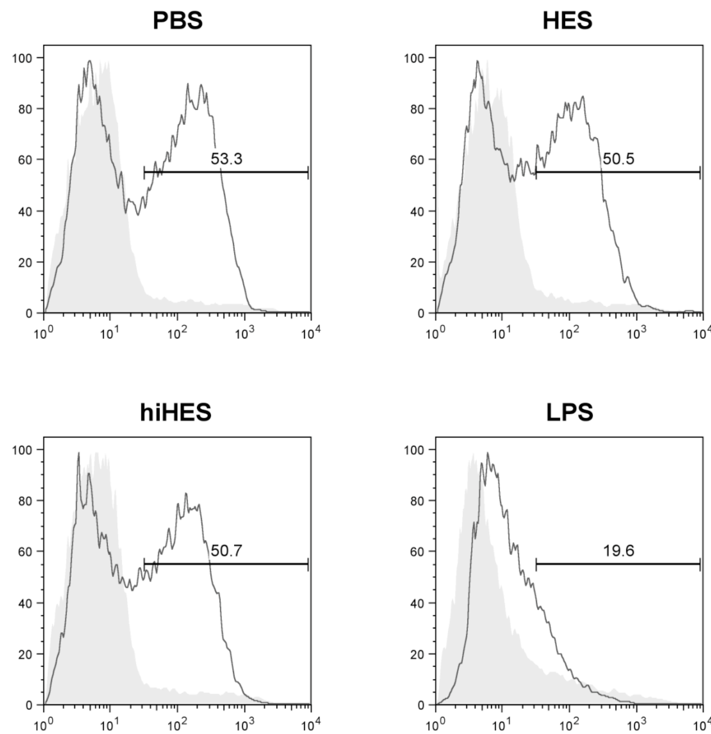


Figure 3.5 – HES does not alter endocytic activity of the DC.

DCs were stimulated, *in vitro*, with PBS, HES (25 μg/ml), hiHES (25 μg/ml), or LPS (1 μg/ml). After 18 hrs cells were washed and then incubated at 37ºC (Black line) with FITC-dextran, or at 4ºC as a negative control (Solid grey), for 1 hr. Uptake of FITC-dextran was then analysed by flow-cytometry.

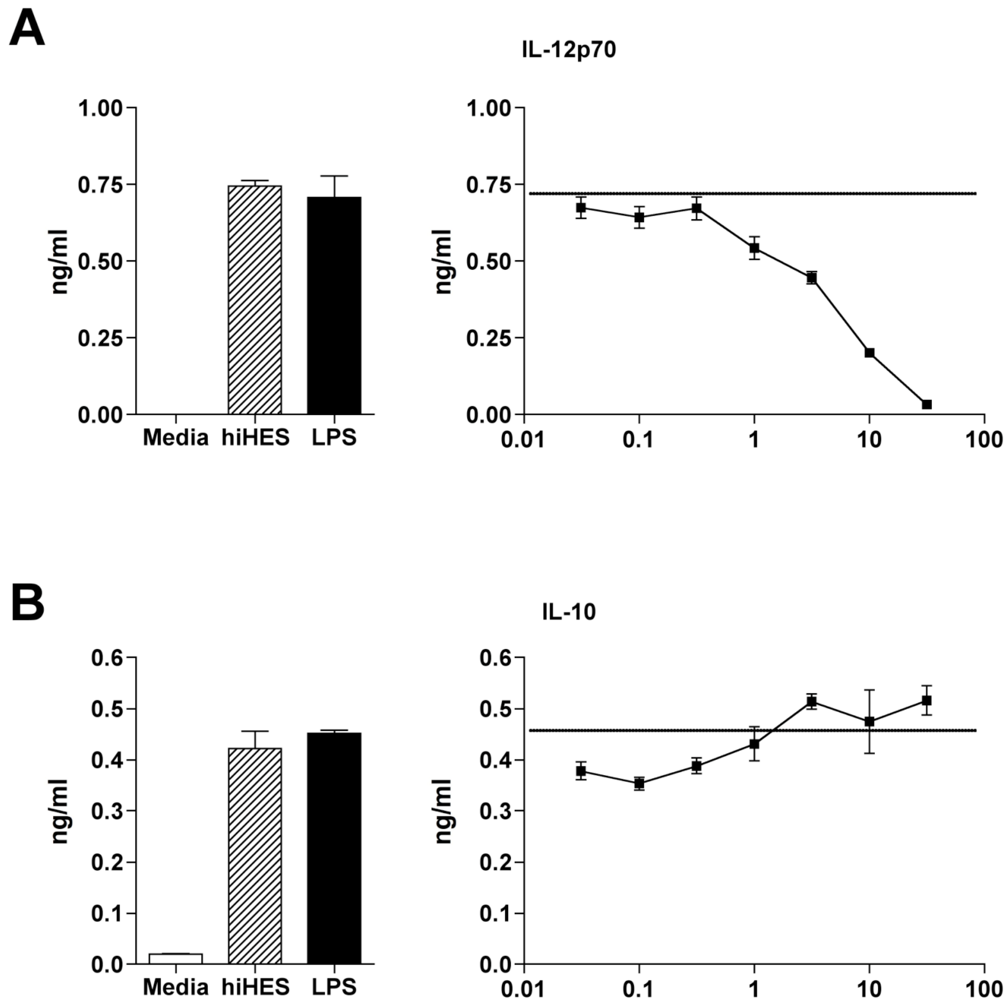


Figure 3.6 – HES inhibits LPS-induced inflammatory cytokine production from DCs.

DCs were stimulated, *in vitro*, with PBS, LPS (1 μg/ml), LPS (1 μg/ml)/hiHES (31.6 μg/ml) **(A, B, left panels)**, or LPS (1 μg/ml) with increasing concentrations of HES **(A, B right panels)**. IL-10, and IL-12p70, were measured by ELISA. Line on right panels represents cytokine released by LPS alone treated DCs. Error bars represent SEM of triplicate wells of DCs plated from the same original cultures. This is representative of two similar experiments.

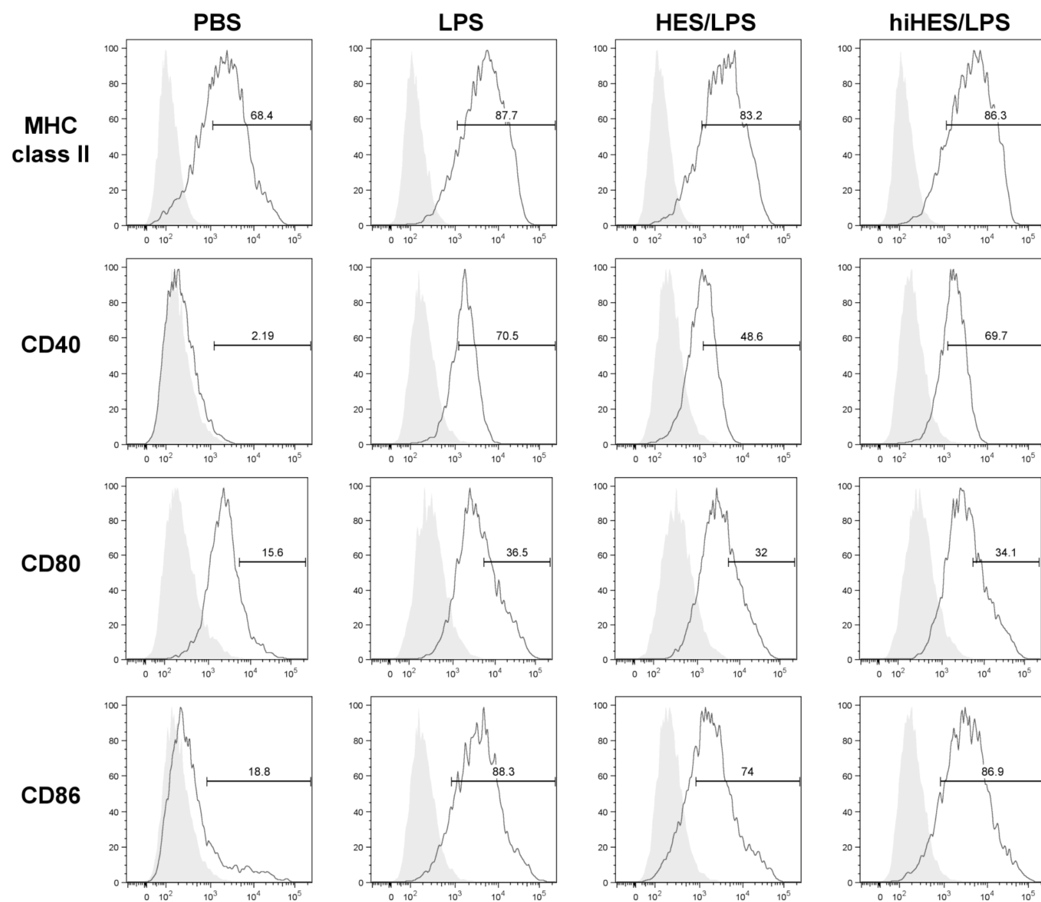


Figure 3.7 – HES inhibits upregulation of DC surface activation markers in response to LPS.

DCs were stimulated, *in vitro*, with PBS, LPS (1 μg/ml), HES (25 μg/ml)/LPS (1 μg/ml), or hiHES (25 μg/ml)/LPS (1 μg/ml). After 18 hrs cells were collected and CD40, CD80, CD86, and MHC class II, expression analysed by flow-cytometry. Black line, positive stain; solid grey, isotype. Representative plots are shown.

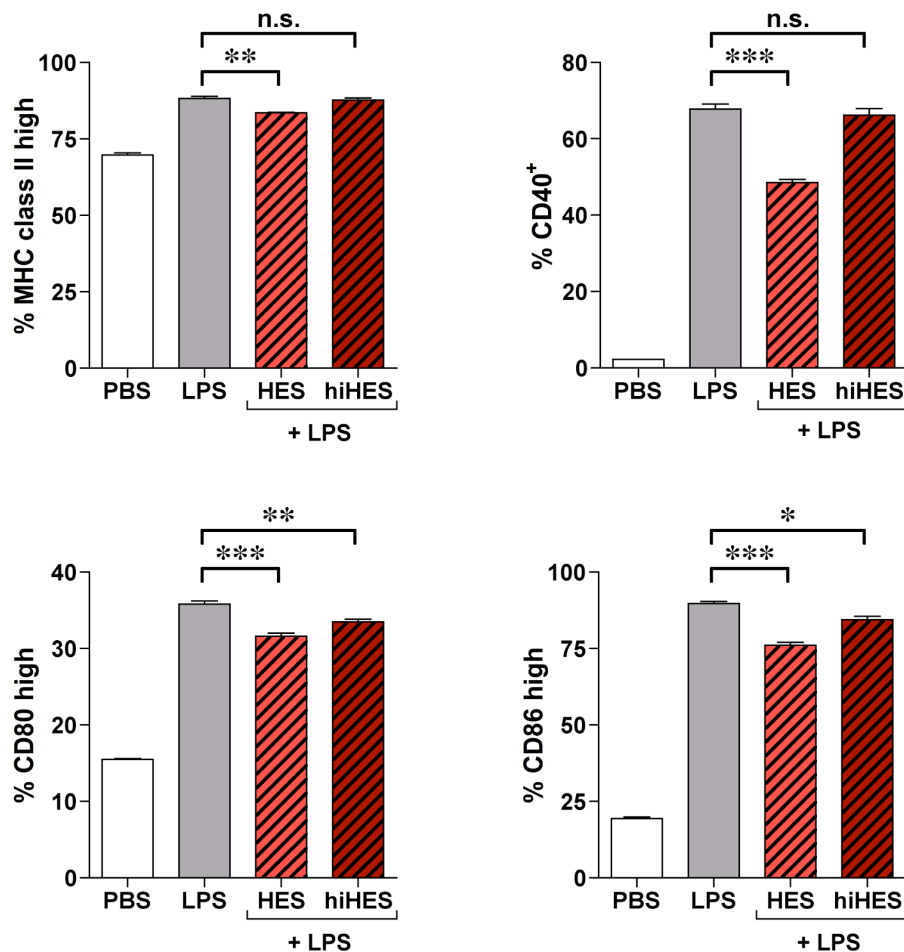


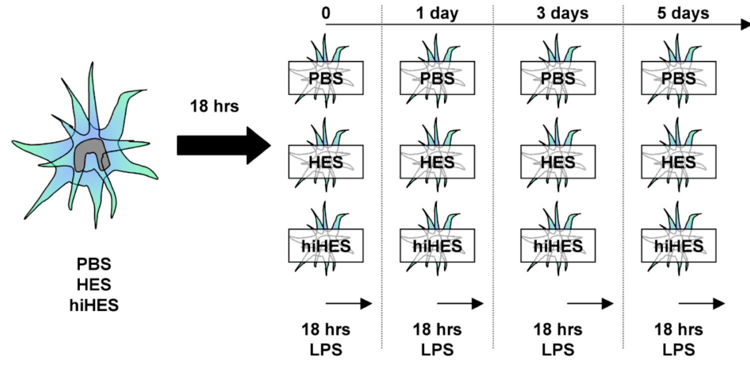
Figure 3.8 – HES inhibits upregulation of DC surface activation markers in response to LPS.

Flow-cytometry data (shown in Fig. 3.7), compiled from three separate wells, plated from the same DC cultures. Error bars represent the SEM. This is representative of at least two similar experiments. Results of one-way ANOVA and Tukey's test: n.s. = not significant; *, P < 0.05; **, P<0.01; ***, P<0.001.

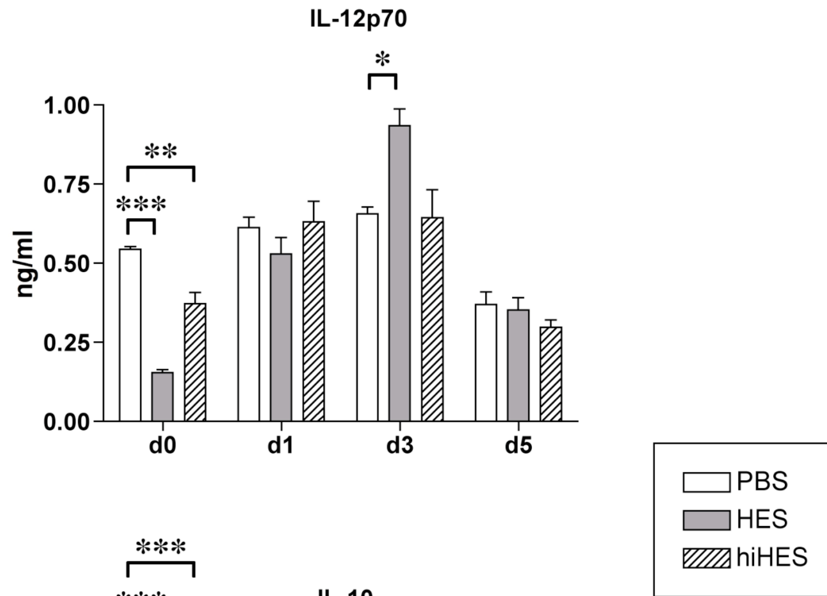
Figure 3.9 – HES inhibition of inflammatory cytokine production is short-lived.

(A) DCs were stimulated, *in vitro*, with PBS, HES (25 μg/ml), or hiHES (25 μg/ml) in 6 well plates. After 18 hrs cells were washed and replated in triplicate wells of a 96 well round-bottom plate. Cells were then stimulated with LPS (1 μg/ml) for 18 hrs at various time points, beginning straight after plating (day 0), and continuing up to day 5. **(B)** IL-12p70, and **(C)** IL-10, were then measured in the supernatant by ELISA. To ensure that cells remained viable fresh media was added to non-LPS treated wells daily. Error bars represent SEM of triplicate wells. Results of one-way ANOVA and Tukey's test: *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$.

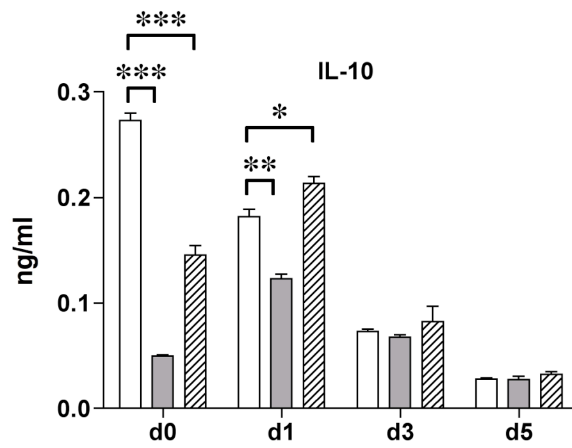
A



B



C



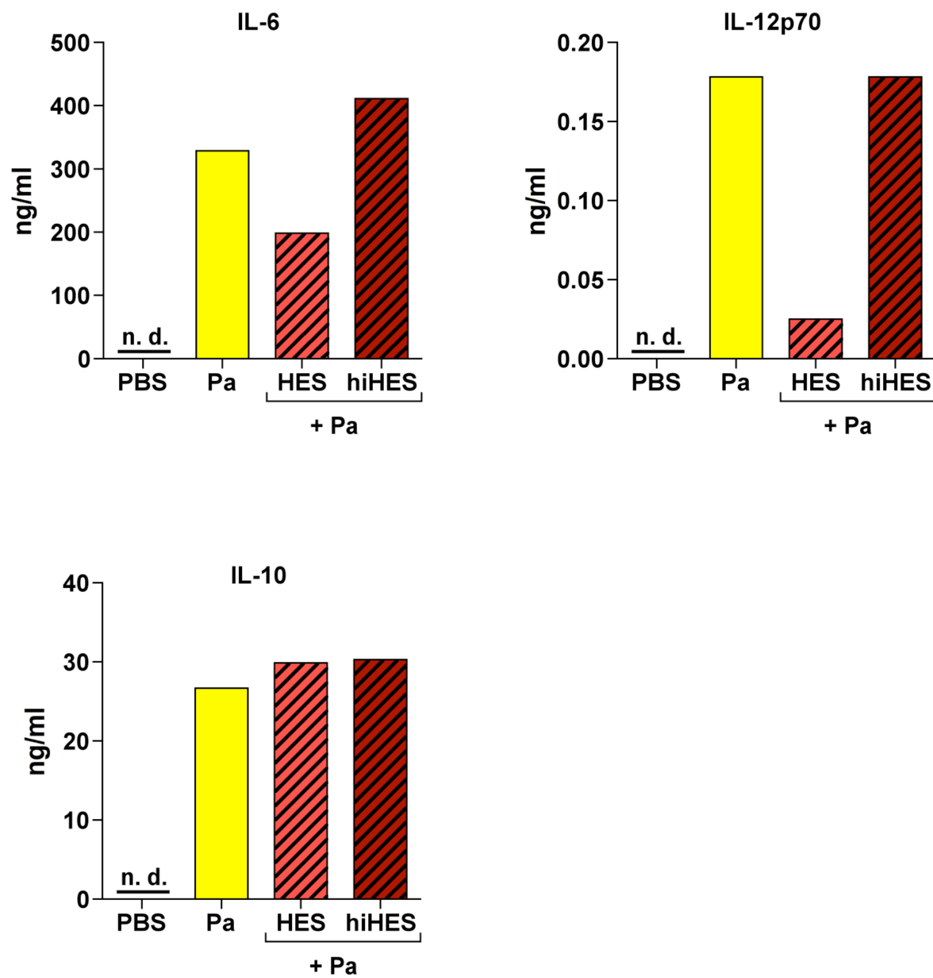


Figure 3.10 – HES inhibits Pa induced inflammatory cytokine production by DCs.

DCs were stimulated, *in vitro*, with PBS, Pa (10 μg/ml), HES (25 μg/ml)/Pa (10 μg/ml), or hiHES (25 μg/ml)/Pa (10 μg/ml). After 18 hrs supernatants were removed and cytokines measured by ELISA, IL-6, IL-10, and IL-12p70. n.d. = not detected. Detection limits: IL-6, 400 pg/ml, IL-10, 20 pg/ml; IL-12p70, 40 pg/ml. Data are representative of two similar experiments.

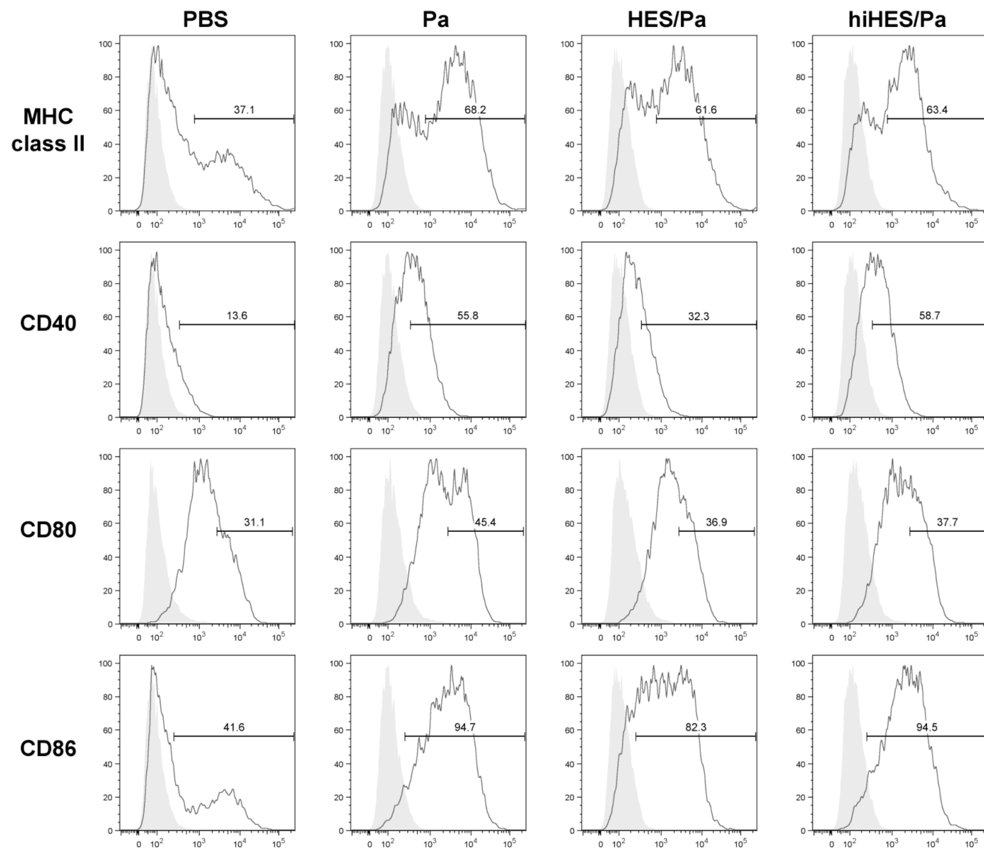


Figure 3.11 – HES limits DC activation associated surface marker upregulation by Pa.

DCs were stimulated, *in vitro*, with PBS, Pa (10 μg/ml), HES (25 μg/ml)/Pa (10 μg/ml), or hiHES (25 μg/ml)/Pa (10 μg/ml). After 18 hrs cells were collected and CD40, CD80, CD86, and MHC class II, expression analysed by flow-cytometry. Black line, positive stain; solid grey, isotype.

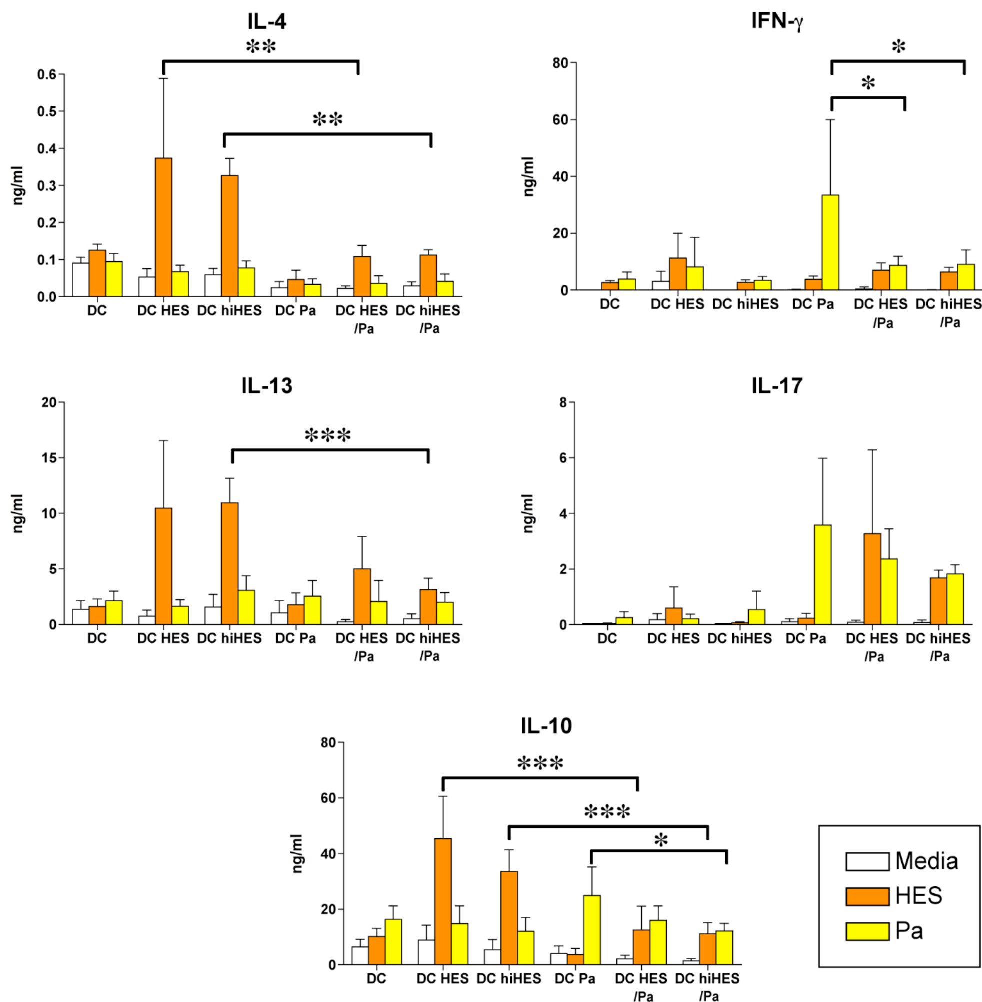


Figure 3.12 – Antigen specific responses driven by adoptively transferred HES, hiHES, Pa, and co-pulsed DCs.

Seven days after DC transfer (Fig. 3.2), splenocytes were restimulated with media alone, HES (1 μg/ml), or Pa (1 μg/ml). 72 hrs later supernatants were harvested and, the Th1-associated cytokine IFN-γ, the Th2-associated cytokines IL-4, IL-13, the Th17-associated cytokine IL-17, and the immunoregulatory cytokine IL-10, measured by ELISA. Error bars represent SD of splenocytes from five separate animals. Results of one-way ANOVA and Tukey's test: *, P < 0.05; **, P<0.01; ***, P<0.001.

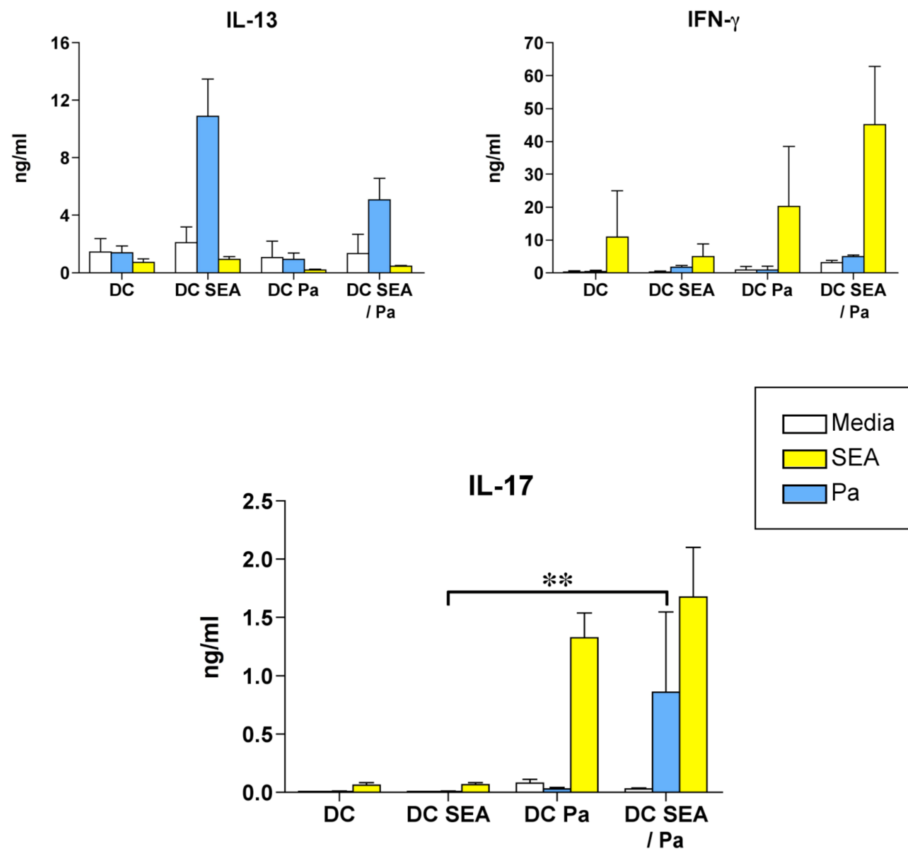


Figure 3.13 – Antigen specific responses driven by adoptively transferred SEA, Pa, and co-pulsed DCs.

Seven days after DC transfer (Fig. 3.2), splenocytes were restimulated with media alone, SEA (10 μg/ml), or Pa (1 μg/ml). 72 hrs later supernatants were harvested and, the Th1-associated cytokine IFN-γ, the Th2-associated cytokines IL-4, IL-13, the Th17-associated cytokine IL-17, and the immunoregulatory cytokine IL-10, measured by ELISA. Error bars represent SEM of splenocytes from five separate animals. Results of one-way ANOVA and Tukey's test: *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$.

3.3 Discussion

Treatment of DCs with helminth-derived products, and subsequent analysis of the type of immune response they induce, has proved a useful tool to study their immunomodulatory properties. Here, it has been shown that HES, as with other helminth-derived products, does not induce classical maturation of the DC. Corroborating data that have recently been published using ES released in the first 24 hrs of *H. polygyrus* culture (Segura et al., 2007), DCs pulsed, *in vitro*, with HES maintained an immature phenotype in regard to the surface markers analysed and cytokines measured. Additionally, HES pre-treatment did not alter the ability of the DC to uptake antigen, unlike inflammatory stimuli such as LPS. Despite this apparent lack of phenotypic alteration it remains a possibility that the effects of HES are subtle and an as yet un-described parameter is changed. This is evidenced by microarray studies of SEA-pulsed DCs in which only 29 genes were altered compared to unstimulated controls (Kane et al., 2004).

Although apparently inactivated, HES-pulsed DCs adoptively transferred *in vivo*, induced a Th2 response; characterised by production of antigen-specific IL-4 and IL-13. This is consistent with the phenotype of SEA-pulsed DCs, which are also maintained in an immature state and strongly polarise a Th2 response (MacDonald et al., 2001).

The lack of a signature phenotype for DCs that are capable of polarising Th2 responses has lead to the proposal of a 'default' hypothesis for their mode of action. In essence, the hypothesis suggests that Th2-type conditioning of the DC is not associated with a specific gene expression profile but rather on the absence of a classical maturation profile. This fits particularly well with data from *H. polygyrus* infections where DCs from the spleen exhibit an immature phenotype whilst a strong Th2 response is generated (Su et al., 2005). However, a number of studies have also been published which do not support this hypothesis. Perhaps the best example is that of DCs pulsed with *N. brasiliensis* ES (NES), which are able to polarise a Th2 response despite evident maturation (Section 3.1) (Balic et al., 2004). When phenotyped these DCs were described as being partially mature since they upregulated CD86 and OX40L, but not CD80, and secreted IL-6 and IL-12p40, but

not IL-12p70. The selective upregulation of genes associated with generation of Th2 immunity *in vivo* was suggested to be the mechanism by which these DCs were able to prime a response.

Other evidence that argues against the default hypothesis comes from co-pulse experiments using SEA and Pa. In this situation, transferred DCs are able to concurrently polarise Th1 responses towards Pa and Th2 responses towards SEA. Interestingly, these DCs, prior to transfer, shared some phenotypic similarity to NES-pulsed DCs, particularly in respect to the strong inhibition of Pa induced secretion of the inflammatory cytokine IL-12p70.

It was found that HES specifically inhibited production of the inflammatory cytokines IL-6, and IL-12p70, when co-administered with LPS, or Pa, whilst the immunoregulatory cytokine IL-10 was unaffected. HES also had inhibitory effects on LPS/Pa induced expression of the co-stimulatory receptors CD40, CD80 and CD86, as well as MHC class II. According to the default hypothesis of DC polarisation potential, this shift away from a classically activated DC phenotype towards a more immature state should favour Th2 responses.

Unlike previous studies, we were able to directly assess whether this was the case by comparing the effects of HES with its heat-inactivated form. Upon heating, HES lost its ability to modulate DC activation in the presence of LPS. However, hiHES-pulsed DCs adoptively transferred *in vivo* were still able to induce a robust Th2 response. This suggests that, in an immature DC, the factors that are required to modulate the phenotype of the DC towards inflammatory stimuli are not the same as those required to polarise a Th2 response.

Surprisingly, we found that these two effects of HES on DCs were also separable in the presence of a strong inflammatory stimulus. When HES/Pa, or hiHES/Pa, co-pulsed DCs were used in transfer experiments both induced comparable antigen-specific Th2 responses, even though hiHES was unable to significantly modulate DC maturation. This demonstrates, that at least for HES stimulated DCs, the default hypothesis does not hold true. DCs pulsed with components of HES are able to induce Th2 responses irrespective of their maturation status. It would seem, therefore, most likely that DC induced Th2-responses driven by HES, and possibly other helminth-derived products, are not dependent on the

attainment of a specific DC phenotype but are due to an inherent property of the antigen.

One possible mechanism by which this could occur is differential processing of the two antigens. This was observed previously in SEA/Pa co-pulsed DCs where, using fluorescently labelled antigens, SEA and Pa were found to traffic to different compartments after uptake. By studying the phenotype of these compartments it was suggested that Pa was more heavily processed than SEA and hence Pa-derived peptides could be displayed at a higher density on the surface of the DC. Since peptide concentration can affect T cell polarisation, this may explain how the DC is able to initiate appropriate responses to each antigen in a co-pulse setting.

Although this idea fits well for DCs co-pulsed with SEA/Pa, subtle differences in the ability of HES/Pa and hiHES/Pa co-pulsed DCs to induce T cell responses suggest that there may be an alternative explanation in this setting. Unlike SEA/Pa co-pulsed DCs, whilst there is no switching in type of response, HES/Pa and hiHES/Pa co-pulsed DCs polarise muted Pa-specific IFN-γ responses as well as muted HES-specific Th2 responses. Suppression is most clearly observed when considering antigen-specific IL-10 production. Although both antigens polarise similar IL-10 responses, when DCs are co-pulsed with HES/Pa or hiHES/Pa, IL-10 secretion stimulated by either antigen is dramatically suppressed.

Unexpectedly, the maintenance of polarisation phenotype in a DC co-pulse was not observed for Th17 generation. In HES/Pa and hiHES/Pa co-pulsed DC transfers a strong HES specific IL-17 response was observed, comparable to that of Pa alone. A possible explanation for this is that Th17 polarisation has little to do with the nature of the antigen and is more to with the local cytokine milieu.

If the suppression of DC activation by HES is unrelated to eventual T cell polarisation then, what is the purpose of this activity? One possibility is that modulation is important to limit inflammation at the site of infection. During an *H. polygyrus* infection damage caused to the gut wall is likely to mediate exposure of DCs to ligands from commensal bacteria or bacteria carried in by the helminth (Section 1.3.4). In this situation limiting production of cytokines such as IL-12/23 and allowing production of IL-10 would prevent inflammation at the site of infection. This concept is further backed-up by the short-period of time for which

HES inhibits the ability of the DC to make cytokine. As discussed (Section 1.7), this inhibition has been suggested as a form of parasite immunomodulation. However, it is equally possible that this represents an appropriate host response to recognition of parasite antigens. Down-regulation of inflammatory cytokines would favour Th2-type effector mechanisms local to the helminth, aiding in pathogen clearance.

Should this prove to be the case then there is little point in searching for a signature profile of a Th1 or Th2 phenotype in DCs. Indeed, our data suggests that the phenotype of the DC after pulsing tells you little about its polarisation potential but might be relevant to the kind of inflammation that will be induced at the site.

Future work to support the data in this chapter could focus on gaining a better understanding of the mechanism by which HES is able to suppress DC activation in response to inflammatory stimuli. Initially, the molecular pathways that HES is acting upon when co-pulsed with LPS could be characterised. Western blotting could be used to look for the stage at which inflammatory signalling is inhibited, for example mitogen-activated protein kinase (MAPK) phosphorylation. Once these pathways are identified it may be possible to predict receptors that HES may be binding to. Isolating molecules, perhaps using ion-exchange chromatography, from the HES, that are specifically responsible for the suppression could be another powerful tool for better understanding the process. Additionally, all the work presented here is looking at BM-derived DCs, it would be interesting to see whether different populations of DCs from the gut, such as lamina propia, or Peyer's patch, DCs respond in similar manner to HES.

4 Mechanisms of resistance to primary infection with *H. polygyrus*.

4.1 Introduction

H. polygyrus is reported to be capable of establishing a chronic primary infection (>4 weeks) in almost all strains of mice tested (Section 1.2.2). In contrast to this, a striking variation has been shown in response phenotypes of different strains to secondary challenge, or trickle infections (Section 1.2.2). This is exemplified by one study in which the final worm burdens, following a repeated weekly inoculation protocol, were in excess of 300 worms per animal in CBA mice at week 8 of infection, compared to a mean of 1.2 worms per animal in SWR mice (Behnke et al., 2003). As a result of the perceived minor strain distinctions in respect to resistance to primary infection, in relation to secondary or trickle infection, much of the work in this area has focussed on the latter setting. This has resulted in a clear mechanism being described by which secondary protection against *H. polygyrus* is mediated against the larval stage of the parasite, dwelling in the sub-mucosa of the small intestine. Memory Th2-cells induce rapid recruitment of AAMϕs that are involved in formation of granulomas around the invading pathogen, favouring its killing (Section 1.3.3).

For the reasons described above, little focus has been placed on exploring the well-documented disparities between rates of response in primary infection. Moreover, key aspects of the initial immune response to infection remain poorly understood. Correlations have been observed between helminth expulsion and a number of immunological parameters including levels, and type, of cytokine production (Behnke et al., 1993; Wahid et al., 1994), serum and luminal antibody concentrations (Ben-Smith et al., 1999), and mast cell metalloprotease concentrations (Behnke et al., 1993; Wahid et al., 1994). These studies did not analyse the early stages of *H. polygyrus* interaction with the host making it difficult to ascertain whether variation in any particular feature of the immune response was causative, or a result, of differential worm burdens.

One possible explanation for variation in resistance to primary infection is the balance between Th1 and Th2 responses, as strong Th2 polarisation is fundamental to the strain specific expulsion of *T. muris* (Section 1.2.2) (reviewed by (Cliffe and Grencis, 2004). The prospect of a similar balance existing in *H. polygyrus* infection, where some degree of Th2 polarisation occurs in both resistant and susceptible strains, has not been widely considered. If it is relevant then the relative strengths of Th1 and Th2 could be important in determining the response phenotype to this helminth too, or additional subtypes may be involved. For example, given its establishment in the gut, where Th17-cells are prevalent, there could be an as yet undescribed counter-regulatory relationship between this newly described subset and the Th2-cells required to aid in worm expulsion.

In this chapter, responses of slow and fast responder mice to *H. polygyrus* are studied in different strains of mice with particular focus on the very early responses, both in the LN and the gut tissue. Subsequently, the role of Th1, and Th17, counter-regulation in mediating any strain-specific differences is considered.

4.2 Results

4.2.1 Fecundity and worm burden is affected in different strains of mice, early in infection.

Based on the published literature, four different mouse strains were compared for susceptibility to primary infection. These strains were the slow responder strains, CBA, and C57BL/6, the intermediate responder strain, BALB/c, and the fast responder strain, SJL. Faecal egg counts were measured at day 14, day 21, and day 28, of infection to address how rapidly differences in worm fecundity were evident in primary *H. polygyrus* infections of different strains of mice. Reductions in eggs/g of faeces were apparent as early as day 14 of infection in BALB/c and SJL mice (Figure 4.1 A). At later time points egg counts continued to fall in these strains whilst remaining relatively constant in CBA and C57BL/6 mice. The reduced egg burden at day 14 represented a reduction in worm fecundity, as the total number of adult worms in the gut lumen were not significantly different between groups at this time point (Figure 4.1 B). By day 28 of infection, however, dramatic differences in worm burden were observed between the strains with, as expected, SJL, and BALB/c mice having the lowest worm burdens, while CBA and C57BL/6 mice carried the highest.

4.2.2 Phenotyping lymphocyte populations in different strains of mice.

It is possible that the increased rate of expulsion of *H. polygyrus* in different strains of mice is related to variation in the lymphocyte populations present in the MLNs. In the naïve state it was observed that, baseline cell numbers within MLNs were greater in the faster responding strains (Fig. 4.2 A, B). In order to broadly assess the cell types making up the lymphocyte pool, MLNs were isolated and stained for the helper T cell marker CD4, the cytotoxic T cell marker CD8, and the B cell marker B220. Initial percentages of $CD4^+$, $CD8^+$, and $B220^+$, cells were found to be different between strains. C57BL/6 mice in particular had low CD4 percentages but as a

result high CD8, and B220 percentages (Fig. 4.2 C). At day 7 of *H. polygyrus* infection there was no significant increase in the cellularity of MLNs, but there were changes in the lymphocyte populations (Fig. 4.2 C). Percentages of B220$^+$ cells, as has been previously reported for some strains of mice (Parker and Inchley, 1990), were increased in all groups. Increases were most striking in CBA mice, where B220$^+$ cell increased over four-fold, and in BALB/c mice where they increased two-fold. These B220$^+$ percentage increases were accompanied by decreases in CD4, but particularly CD8 percentages.

By day 14 post-inoculation, the MLNs of all mouse strains had expanded dramatically in response to infection. BALB/c mice had the largest overall cellularity rising from a mean of 2.5x10^7 cells, to 1.3x10^8 cells, whilst CBA, C57BL/6, and SJL mice (despite their different response phenotypes) all had similarly sized MLNs at this time point (Fig. 4.2 B). Percentages of lymphocyte populations, as defined by CD4, CD8, and B220 expression, were similar to those at day 7 (data not shown), although absolute numbers were greater due to the increases in MLN size.

4.2.3 CD4$^+$ T cell Foxp3 expression levels increase in different strains of mice at day 7 of *H. polygyrus* infection.

Previously it has been reported that early in *H. polygyrus* infection of BALB/c mice increased percentages of Foxp3$^+$ cells are present in the MLNs (Finney et al., 2007). These are most apparent at day 7, and reduced by day 14. Whether these changes also occur in the MLNs of mice with different responder phenotypes has not been investigated.

As for different lymphocyte populations, CD4$^+$ T cell expression of Foxp3 was also assessed at day 7 and day 14 of infection (Fig. 4.3). Differences were apparent in the percentage of Foxp3$^+$ cells in naïve animals from each strain. CBA mice had lowest Foxp3 expression, C57BL/6 had highest, whilst similar levels were evident in BALB/c, and SJL. Despite these baseline variations, all strains were found to have significantly upregulated proportions of CD4$^+$ Foxp3$^+$ T cells at day 7 of parasite infection (Fig. 4.3 A). The percentage-increase over naïve was markedly

different between strains and negatively correlated with susceptibility. CBA, and C57BL/6 had small mean percentage-increases, 5%, and 4%, but BALB/c, and SJL, had larger percentage-increases, 9%, and 11%. A similar effect was discernable if fold-change was calculated, to take into account the variations in the starting proportions of Foxp3^{+} cells (Fig. 4.3 B).

By day 14 of infection the situation was very different (Fig. 4.3 C). The increases in percentage of CD4^{+} Foxp3^{+} T cells had fallen back to baseline levels in CBA and C57BL/6 mice, whilst BALB/c mice had only a slight mean elevation of 4%. In contrast, SJL mice maintained an almost identically enhanced population to that observed at day 7 of infection (mean percentage change – 13%). As might be anticipated from the day 7 data, fold-change analysis at day 14 told a similar story to the percentage change (Fig. 4.3 D).

4.2.3 Cytokine production in different strains of mice.

Another factor that could affect the ability of a mouse strain to respond to primary infection with *H. polygyrus* is the strength, and type, of CD4^{+} T cell response. At days 7 and 14 of *H. polygyrus* infection, MLNs were isolated, polyclonally stimulated, and intracellularly stained for, the Th2-type cytokine IL-4, the Th1-type cytokine IFN-γ, and the Th17-type cytokine IL-17. First, the different Th-types were considered as a percentage of the total CD4^{+} T cell population. IL-4 producing CD4^{+} cells were enhanced in all strains at both day 7, and day 14, of infection (Fig. 4.4 A). The smallest percentage was in the susceptible CBA strain. However, the highest percentage was in the C57BL/6 strain, which is also susceptible. The two resistant strains, BALB/c and SJL, had intermediate percentages of CD4^{+} IL-4^{+} cells. Although Th1-type and Th2-type responses are considered to be in a counter-regulatory relationship at these early time-points trends in CD4^{+} IFN-γ production were similar to those observed with IL-4 (Fig. 4.4 B). CBA mice had the lowest percentage of IFN-γ producing cells, whilst C57BL/6 had the highest. No such pattern was apparent with IL-17-staining (Fig. 4.4 C). Indeed, only SJL mice displayed a clear increase in IL-17 over naïve at day 7 and day 14 of infection.

Interestingly, C57BL/6 mice appeared to have highest constitutive expression of IL-17, although this was unchanged in response to *H. polygyrus*.

Due to the variation in MLN cellularity (Fig. 4.2 A, B), and CD4 percentages (Fig. 4.2 C), of the various strains, when Th-cell responses were considered as absolute cell numbers a different relationship between groups was apparent (Fig. 4.5 A, B, C). BALB/c mice had the highest total number of Th2-type cells, 5-fold greater than CBAs, which had the lowest total number (Fig. 4.5 A). C57BL/6, and SJL, mice that have very different susceptibilities had comparable total Th2-type cell numbers. Once again, although there were smaller numbers of cells, the IFN-γ-producing (Fig. 4.5 B), and IL-17 producing (Fig. 4.5 C), $CD4^+$ populations followed a similar trend.

4.2.4 Resistance to primary infection is associated with granuloma formation.

As discussed (Section 1.3.3), in secondary infection, resistance to *H. polygyrus* is associated with granuloma formation around the tissue-dwelling larvae in the wall of the gut (Anthony et al., 2006). It was possible that susceptibility to primary infection was also dependent on this mechanism. Granuloma-like structures can be identified by eye, or using a light-microscope, in the wall of the gut at day 14 of primary *H. polygyrus* infection (Fig. 4.6 A). Even at this time-point parasites are evident in some of these structures (Fig. 4.6 B). These granuloma-like structures were counted under a light-microscope. Marked differences in their numbers were apparent between the slow and fast responding strains (Fig. 4.6 C). BALB/c and SJL mice had an over 5-fold greater mean granuloma count than CBA and C57BL/6 mice.

In secondary infection, alternative-activation of Mφs within the granulomas was implicated in larval killing (Anthony et al., 2006). mRNA levels of genes associated with AAMφ generation, Arginase-1, RELMα, and Ym1, were measured in the duodenal wall by real-time RT-PCR at day 7 of infection (Fig. 4.7). Expression of these genes varied between strains. In particular, BALB/c mice, despite having increased numbers of granulomas did not have enhanced expression of any of these AAMφ genes. CBA, and C57BL/6, mice had > 10-fold increased

expression of Arginase-1, and RELMα, but no significant change in Ym1. SJLs, in contrast had no change in Arginase-1 expression, but had increased RELMα, and Ym1 mRNA.

4.2.5 IL-12/23p40$^{-/-}$ C57BL/6 mice have increased resistance to *H. polygyrus* infection.

Although it was unclear from these data whether AAMϕs were associated with enhanced primary resistance to *H. polygyrus* it was possible that non-Th2-type cytokines such as IL-12, IFN-γ, IL-23, or IL-17, acting on this cell-type, may have a negative impact on the protective response to the parasite in some of the strains. To assess whether this was the case WT C57BL/6, or IL-12/23p40$^{-/-}$ animals on the same background were infected with *H. polygyrus* and their responses to the parasite characterised. At day 28 worm burdens were counted, and MLN cells isolated and re-stimulated with HES. A trend towards decreased infection intensity was evident at this time point (Fig. 4.8 A), which did not appear to be associated with increased cellularity of the MLNs (Fig. 4.8 B).

Enhanced clearance might be predicted to correlate with upregulation of IL-4 production and downregulation of IFN-γ production in response to parasite antigens. Little change was observed in the levels of either of these cytokines in supernatants when MLNs were restimulated with HES (Fig. 4.8 C). IL-10, which, as discussed (Section 1.6.1), may play a regulatory role in *H. polygyrus* infections, was also unaltered. There was, however, an over 2-fold decrease in IL-17 release, implicating the Th17 axis as playing an inhibitory role in resistance to primary *H. polygyrus* infection.

4.2.6 IL-23p19$^{-/-}$ mice have increased resistance to *H. polygyrus*, associated with granuloma development.

In order to ascertain whether the resistance to *H. polygyrus* observed in IL-12/23p40$^{-/-}$ animals might be associated with IL-17 production, mice with a specific deficiency in IL-23 (IL-23p19$^{-/-}$) were infected. This deficiency led to reduced fecundity at day 14 of infection (Fig. 4.9 A, left panel), and a comparable increase in resistance to the parasite as IL-12/23p40$^{-/-}$ at day 28 of infection (Fig. 4.9 A, right panel). In a repeat experiment, the lower egg count was associated with a reduced worm burden at day 14 of infection (Fig. 4.9 B). Interestingly, an increased presence of granuloma-like structures was observed in the gut wall of IL-23p19$^{-/-}$ animals, as was observed for naturally resistant BALB/c and SJL mice (Fig. 4.6).

4.2.7 IFN-γ-deficiency does not enhance resistance to *H. polygyrus* infection.

Since IL-17$^{-/-}$ animals were not available for use in this project, experiments were performed using IFN-γ$^{-/-}$ mice to assess whether resistance to *H. polygyrus* infection was dependent on Th1/Th2 dichotomy as is the case for *T. muris* (Section 1.2.2). At day 14 of infection, unlike IL-23p19$^{-/-}$ animals, there was no change in worm burden (Fig. 4.10 A) or fecundity of the parasites (Fig. 4.10 B). There was also no alteration in granuloma numbers in the wall of the gut (Fig. 4.10 C). Intracellular levels of the cytokines IL-4, IFN-γ, IL-17, and IL-10, were measured in response to polyclonal stimulation of the MLNs (Fig. 4.10 D). As expected, IFN-γ was not present, whilst the percentages of cells producing the other cytokines were not different between groups. These data again suggested that IL-23, perhaps acting via IL-17 induction, and not IFN-γ is important in defining primary susceptibility to *H. polygyrus*.

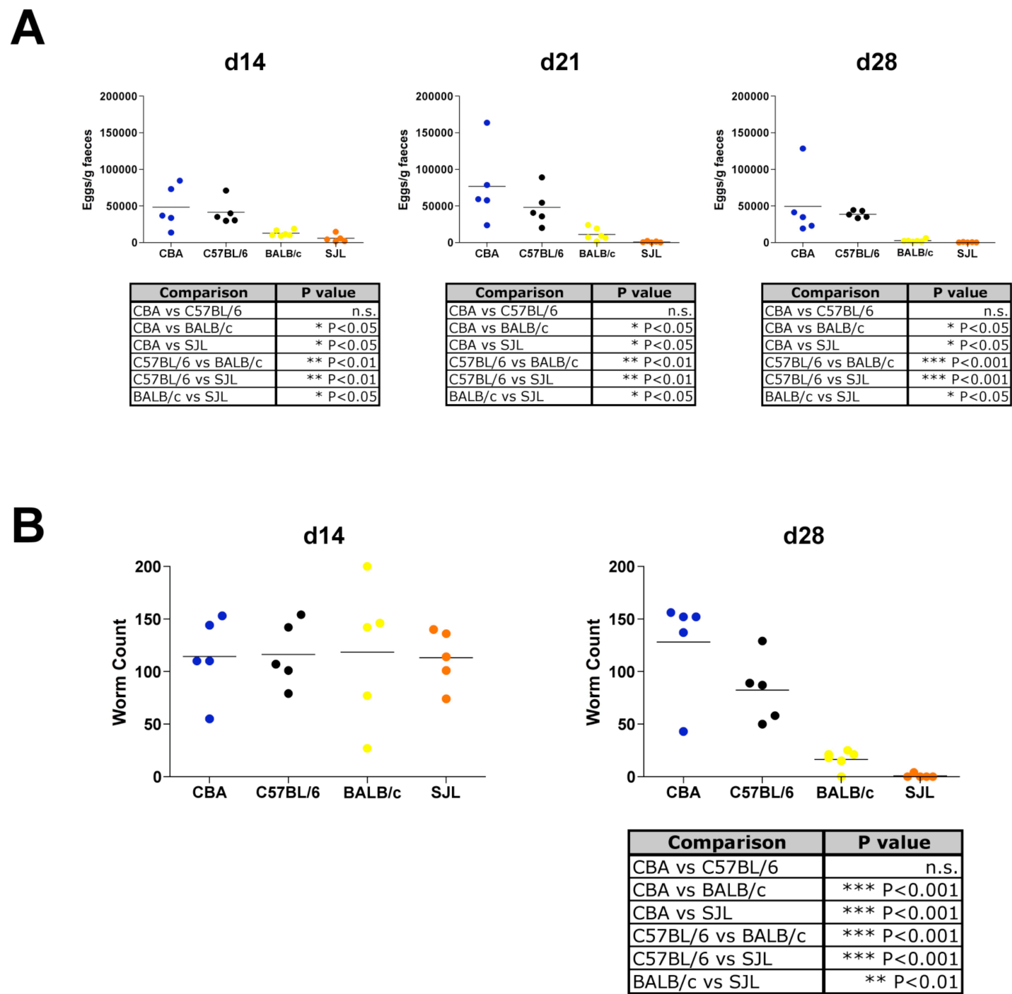
4.2.8 *H. polygyrus* infection leads to increases in IL-17 production that are abrogated in IL-23p19$^{-/-}$ animals.

H. polygyrus has been reported to inhibit IL-17 production in the MLNs at day 14 of infection in C57BL/6 mice (Elliott et al., 2008), however, it is possible that earlier in infection this cytokine is upregulated. This seems probable as damage mediated by the larval stage of the parasite is likely to expose the gut-associated lymphoid tissue (GALT) to antigens from commensal bacteria.

Polyclonal restimulation, followed by intracellular cytokine staining for IL-17, as well as IL-4, IFN-γ, and IL-10, was therefore performed on populations of lymphocytes extracted from the small intestinal tissue, the lamina-propria lymphocytes (LPLs), and Peyer's patches. These were isolated from naïve and day 7 *H. polygyrus* infected animals. Additionally, MLNs were restimulated and compared to the other lymphocyte populations. Isolation of the same populations was attempted from naïve and infected IL-23p19$^{-/-}$ mice. LPLs, could not be phenotyped from these genetically-deficient animals when infected with *H. polygyrus* due to the large amount of cell-death.

In the MLN at day 7 of infection a similar increase in CD4^{+} IL-17^{+} cells occurs in WT and IL-23p19$^{-/-}$ mice when compared to the naïve level of IL-17 production (Fig. 4.11). There are also increases in IL-4, IFN-γ, and IL-10, and again these are unchanged between the two groups. This situation is very different in sites closer to the parasite. The percentage of cells producing IL-17 in the Peyer's patches in the naïve state is around 60-fold higher than in the MLNs (Fig. 4.11). IL-17 does not increase at this site during infection, but IL-23p19$^{-/-}$ animals have approximately 2-fold less IL-17^{+} cells than WT. IFN-γ, and IL-10, production are similar between WT and IL-23p19$^{-/-}$ mice in the Peyer's patches, whilst IL-4 is significantly increased in the knock outs.

LPLs contained a greater percentage of CD4^{+} IL-17^{+} cells than the Peyer's patches (Fig. 4.11). Here also, IL-23p19$^{-/-}$ animals had decreased naïve IL-17 expression levels, but additionally were found to have increased levels of IL-10.



A, d14

Comparison	P value
CBA vs C57BL/6	n.s.
CBA vs BALB/c	* P<0.05
CBA vs SJL	* P<0.05
C57BL/6 vs BALB/c	** P<0.01
C57BL/6 vs SJL	** P<0.01
BALB/c vs SJL	* P<0.05

A, d21

Comparison	P value
CBA vs C57BL/6	n.s.
CBA vs BALB/c	* P<0.05
CBA vs SJL	* P<0.05
C57BL/6 vs BALB/c	** P<0.01
C57BL/6 vs SJL	** P<0.01
BALB/c vs SJL	* P<0.05

A, d28

Comparison	P value
CBA vs C57BL/6	n.s.
CBA vs BALB/c	* P<0.05
CBA vs SJL	* P<0.05
C57BL/6 vs BALB/c	*** P<0.001
C57BL/6 vs SJL	*** P<0.001
BALB/c vs SJL	* P<0.05

B, d28

Comparison	P value
CBA vs C57BL/6	n.s.
CBA vs BALB/c	*** P<0.001
CBA vs SJL	*** P<0.001
C57BL/6 vs BALB/c	*** P<0.001
C57BL/6 vs SJL	*** P<0.001
BALB/c vs SJL	** P<0.01

Figure 4.1 – Variation in susceptibility to primary infection with *H. polygyrus* is associated with early differences in parasite fecundity.

Female, 7 week-old, age-matched, CBA, C57BL/6, BALB/c, or SJL mice were infected with 200 *H. polygyrus* L_3 larvae by gavage. **(A)** Faecal egg counts were performed at day 14, 21, and 28 after infection. Data are representative of two separate experiments. **(B)** Mice were sacrificed at day 14, or day 28 after infection, and adult parasites in the lumen of the gut counted. Tables show results of one-way ANOVA and Tukey's test for each strain. Black lines represent mean.

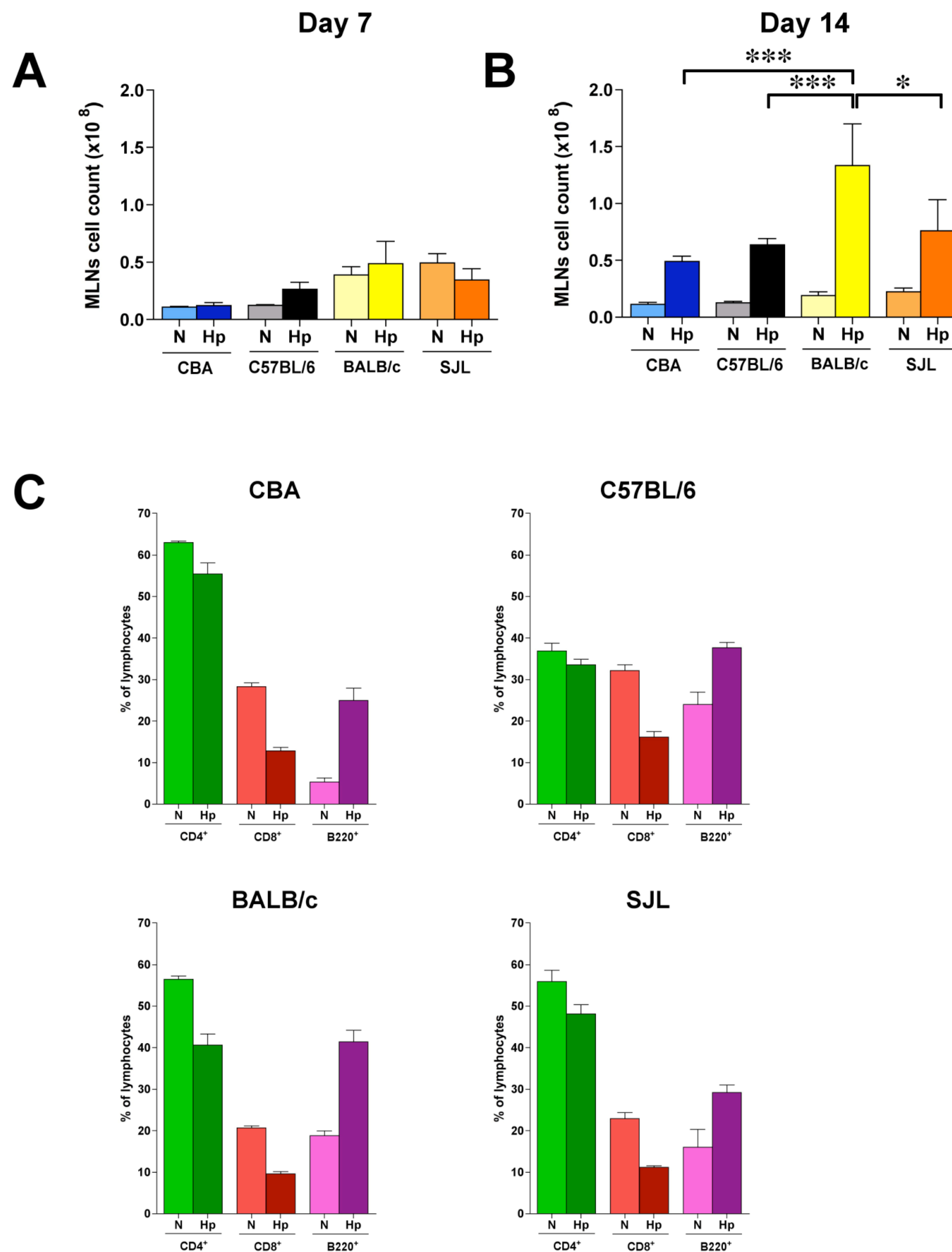
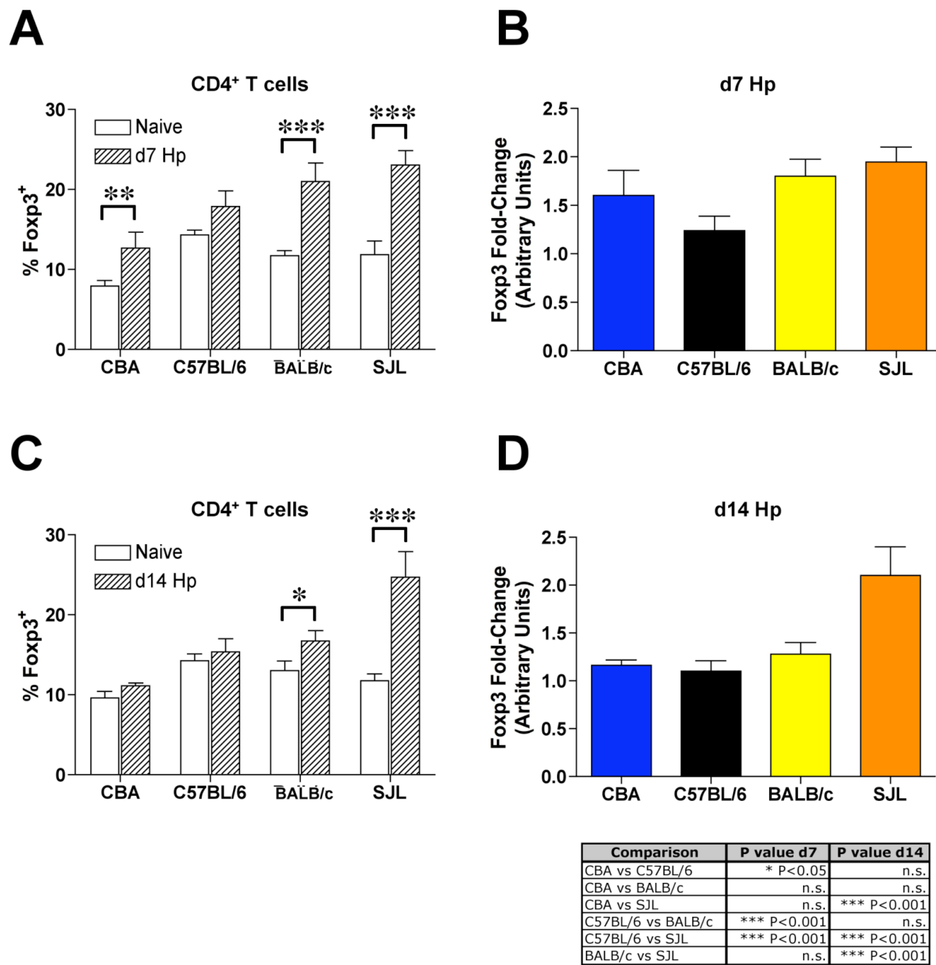


Figure 4.2 – B cell increases are apparent in MLNs early during *H. polygyrus* infection in all mouse strains studied.

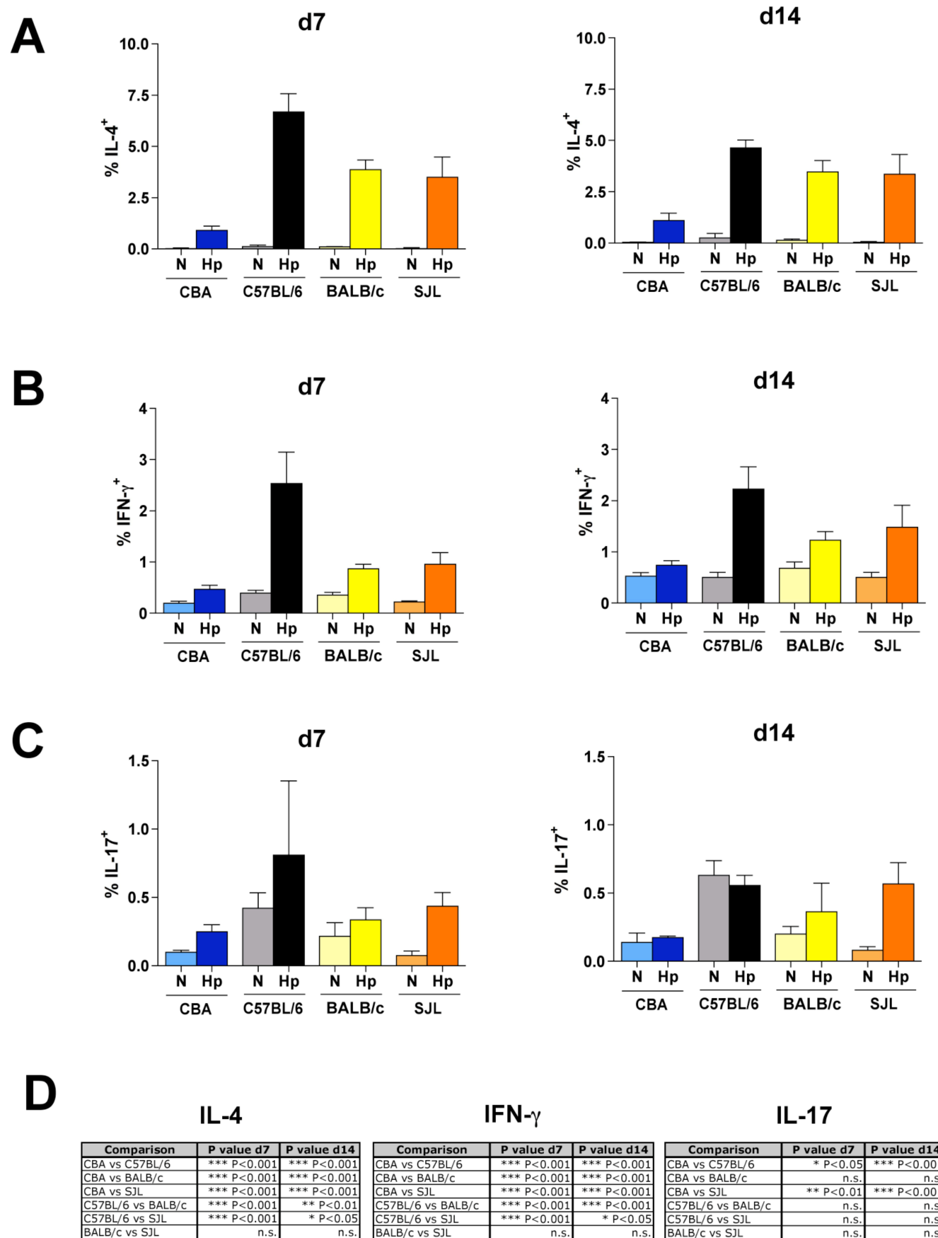
MLNs were isolated from naïve, day 7, or day 14, *H. polygyrus* infected, female, CBA, C57BL/6, BALB/c, or SJL mice. **(A)** Total MLN cell counts were calculated at day 7 and **(B)** day 14. Results of one-way ANOVA and Tukey's test comparing infected groups: *, P < 0.05; **, P<0.01; ***, P<0.001. **(C)** Composition of the MLNs were analysed by flow-cytometry. Percentages of $CD4^+$, $CD8^+$, and $B220^+$ cells, were measured within the lymphocyte gate. Day 7 data are shown but similar changes were observed at day 14 of infection.



Comparison	P value d7	P value d14
CBA vs C57BL/6	* P<0.05	n.s.
CBA vs BALB/c	n.s.	n.s.
CBA vs SJL	n.s.	*** P<0.001
C57BL/6 vs BALB/c	*** P<0.001	n.s.
C57BL/6 vs SJL	*** P<0.001	*** P<0.001
BALB/c vs SJL	n.s.	*** P<0.001

Figure 4.3 – Early stages of *H. polygyrus* infection are associated with percentage increases of Foxp3$^+$ cells, within the CD4$^+$ T cell population, in all mouse strains tested, irrespective of susceptibility phenotype.

Mesenteric lymph node (MLN) cells were isolated from naïve, or *H. polgyrus* infected, 7 week-old age-matched, female, CBA, C57BL/6, BALB/c, or SJL mice. The percentage of Foxp3$^+$ cells, within the CD4$^+$ T cell population, was assessed by flow-cytometry. **(A, C)** Percentage of CD4$^+$ Foxp3$^+$ T cells identified in naïve, and day 7, or day 14, infected mice for each strain. Both naïve and infected animals were sacrificed at the same time point. Error bars represent SD of at least four individual naïve, or five individual infected mice. Results of Student's t test: *, P < 0.05; ***, P<0.001. **(B, D)** Fold-change in Foxp3 expression at day 7, or day 14, for each infected animal, as compared to mean naïve Foxp3 percentage. Error bars represent SD of at least five individual infected mice. Table shows results of one-way ANOVA and Tukey's test for all groups.



IL-4

Comparison	P value d7	P value d14
CBA vs C57BL/6	*** P<0.001	*** P<0.001
CBA vs BALB/c	*** P<0.001	*** P<0.001
CBA vs SJL	*** P<0.001	*** P<0.001
C57BL/6 vs BALB/c	*** P<0.001	** P<0.01
C57BL/6 vs SJL	*** P<0.001	* P<0.05
BALB/c vs SJL	n.s.	n.s.

IFN-γ

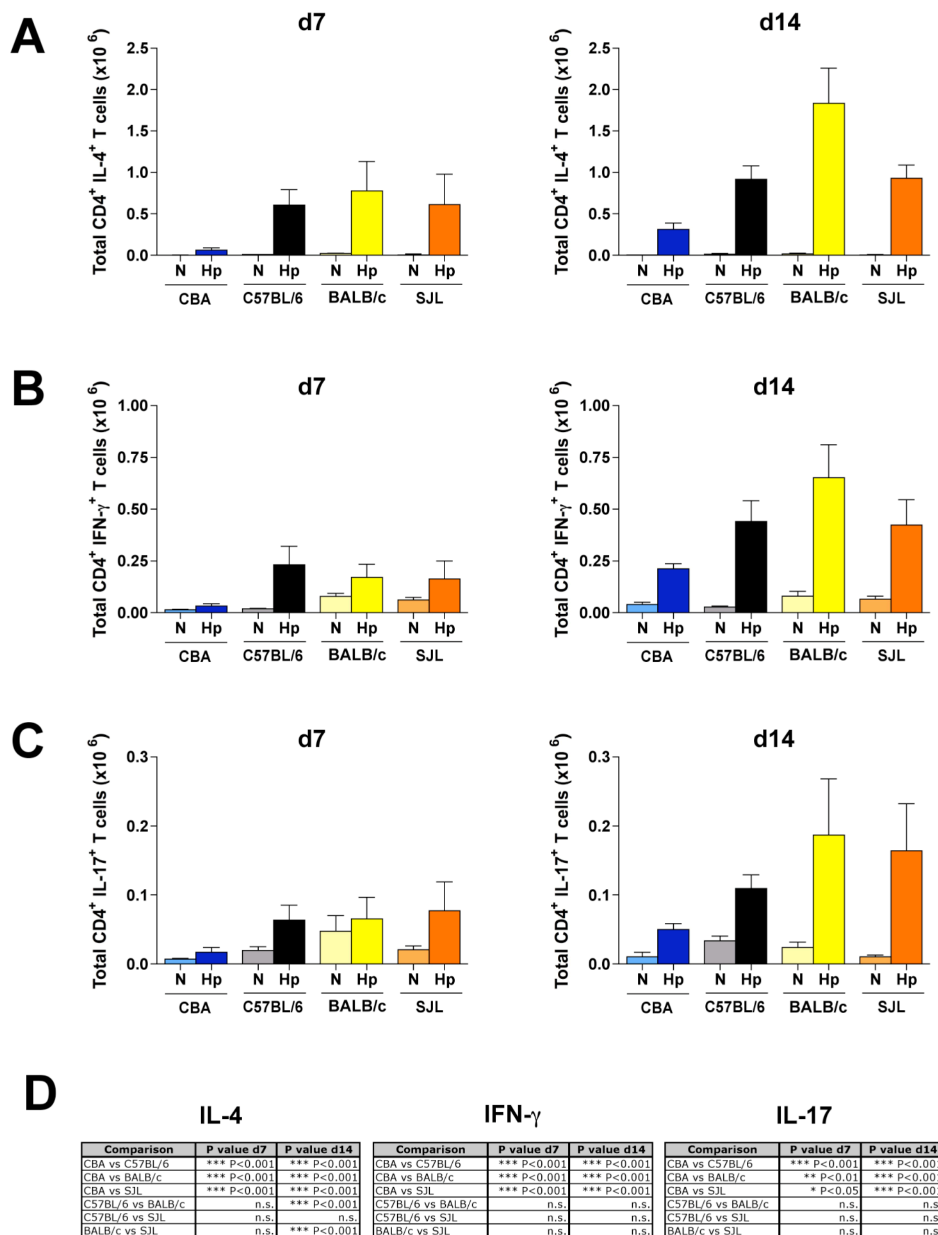
Comparison	P value d7	P value d14
CBA vs C57BL/6	*** P<0.001	*** P<0.001
CBA vs BALB/c	*** P<0.001	*** P<0.001
CBA vs SJL	*** P<0.001	*** P<0.001
C57BL/6 vs BALB/c	*** P<0.001	*** P<0.001
C57BL/6 vs SJL	*** P<0.001	* P<0.05
BALB/c vs SJL	n.s.	n.s.

IL-17

Comparison	P value d7	P value d14
CBA vs C57BL/6	* P<0.05	*** P<0.001
CBA vs BALB/c	n.s.	n.s.
CBA vs SJL	** P<0.01	*** P<0.001
C57BL/6 vs BALB/c	n.s.	n.s.
C57BL/6 vs SJL	n.s.	n.s.
BALB/c vs SJL	n.s.	n.s.

Figure 4.4 – Changes in $CD4^+$ T cell cytokine expression early during *H. polygyrus* infection.

After isolation MLNs from naïve, day 7, or day 14 *H. polygyrus* infected, female, CBA, C57BL/6, BALB/c, or SJL mice, were polyclonally stimulated and stained for intracellular cytokines. **(A)** Percentage $IL\text{-}4^+$ $CD4^+$ T cells at day 7, and day 14. **(B)** Percentage $IFN\text{-}\gamma^+$ $CD4^+$ T cells at day 7, and day 14. **(C)** Percentage $IL\text{-}17^+$ $CD4^+$ T cells. **(D)** Tables show results of one-way ANOVA and Tukey's test for infected groups. Error bars represent SD of at least four individual naïve, or five individual infected mice.



IL-4

Comparison	P value d7	P value d14
CBA vs C57BL/6	*** P<0.001	*** P<0.001
CBA vs BALB/c	*** P<0.001	*** P<0.001
CBA vs SJL	*** P<0.001	*** P<0.001
C57BL/6 vs BALB/c	n.s.	*** P<0.001
C57BL/6 vs SJL	n.s.	n.s.
BALB/c vs SJL	n.s.	*** P<0.001

IFN-γ

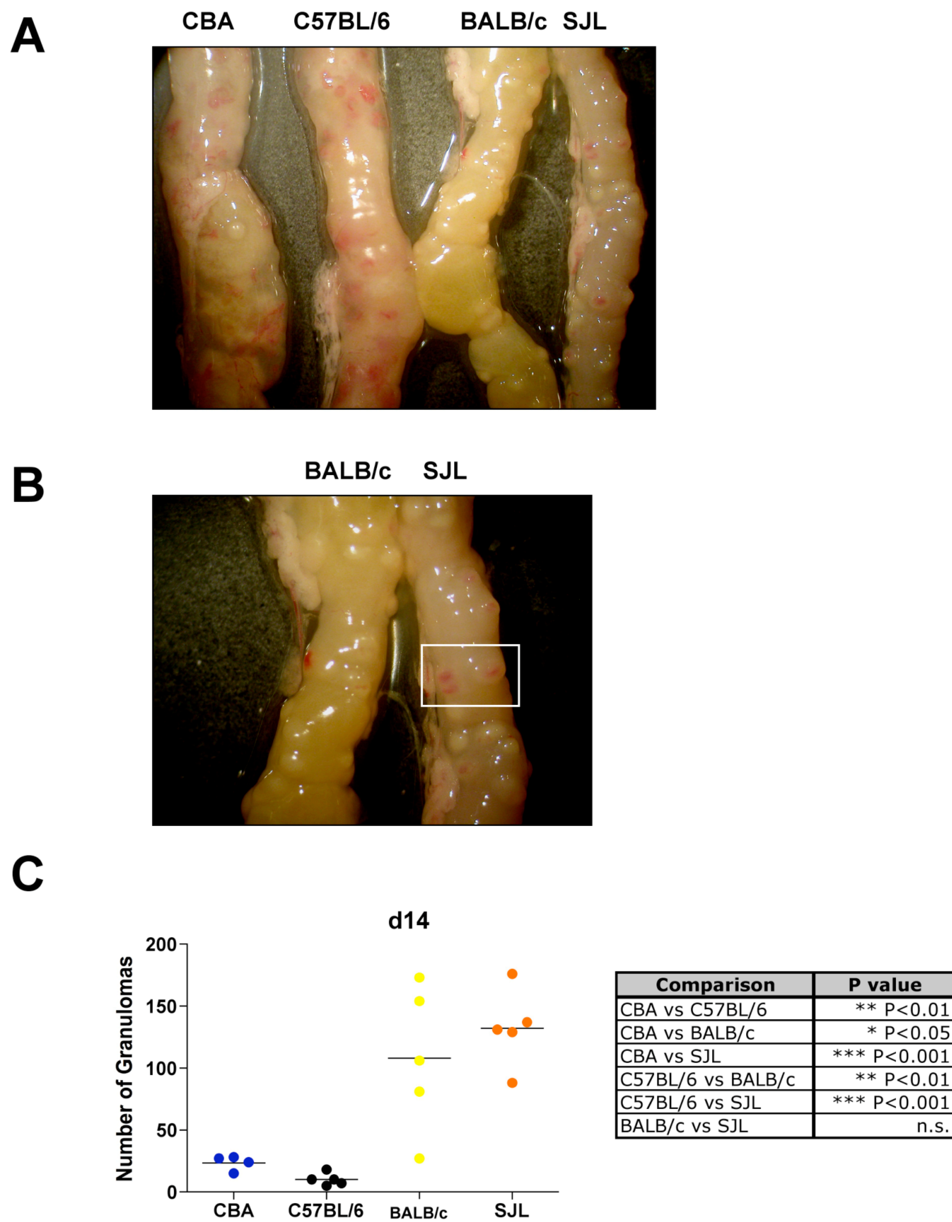
Comparison	P value d7	P value d14
CBA vs C57BL/6	*** P<0.001	*** P<0.001
CBA vs BALB/c	*** P<0.001	*** P<0.001
CBA vs SJL	*** P<0.001	*** P<0.001
C57BL/6 vs BALB/c	n.s.	n.s.
C57BL/6 vs SJL	n.s.	n.s.
BALB/c vs SJL	n.s.	n.s.

IL-17

Comparison	P value d7	P value d14
CBA vs C57BL/6	*** P<0.001	*** P<0.001
CBA vs BALB/c	** P<0.01	*** P<0.001
CBA vs SJL	* P<0.05	*** P<0.001
C57BL/6 vs BALB/c	n.s.	n.s.
C57BL/6 vs SJL	n.s.	n.s.
BALB/c vs SJL	n.s.	n.s.

Figure 4.5 – Changes in total number of CD4^{+} cytokine expressing T cells early during *H. polygyrus* infection.

After isolation MLNs from naïve, day 7, or day 14 *H. polygyrus* infected, female, CBA, C57BL/6, BALB/c, or SJL mice, were polyclonally stimulated and stained for intracellular cytokines. Total number of cells was calculated based on MLN cell count, percentage of CD4^{+} T cells, and percentage of cells producing each cytokine. **(A)** Total number of IL-4^{+} CD4^{+} T cells at day 7, and day 14. **(B)** Total number of IFN-γ^{+} CD4^{+} T cells at day 7, and day 14. **(C)** Total number of IL-17^{+} CD4^{+} T cells. **(D)** Tables show results of one-way ANOVA and Tukey's test for infected groups. Error bars represent SD of at least four individual naïve, or five individual infected mice.



Comparison	P value
CBA vs C57BL/6	** P<0.01
CBA vs BALB/c	* P<0.05
CBA vs SJL	*** P<0.001
C57BL/6 vs BALB/c	** P<0.01
C57BL/6 vs SJL	*** P<0.001
BALB/c vs SJL	n.s.

Figure 4.6 – Primary resistance to *H. polygyrus* infection is associated with granuloma-like formation in the gut wall.

(A) Image showing granuloma-like structures apparent in gut wall of SJL, and BALB/c, but not C57BL/6, or CBA mice, at day 14 post infection. **(B)** Larvae are apparent in granuloma-like structures from SJL mice (white box). **(C)** Number of surface granuloma-like structures identified along entire small intestine in different strains of mice. Tables show results of one-way ANOVA and Tukey's test for all groups.

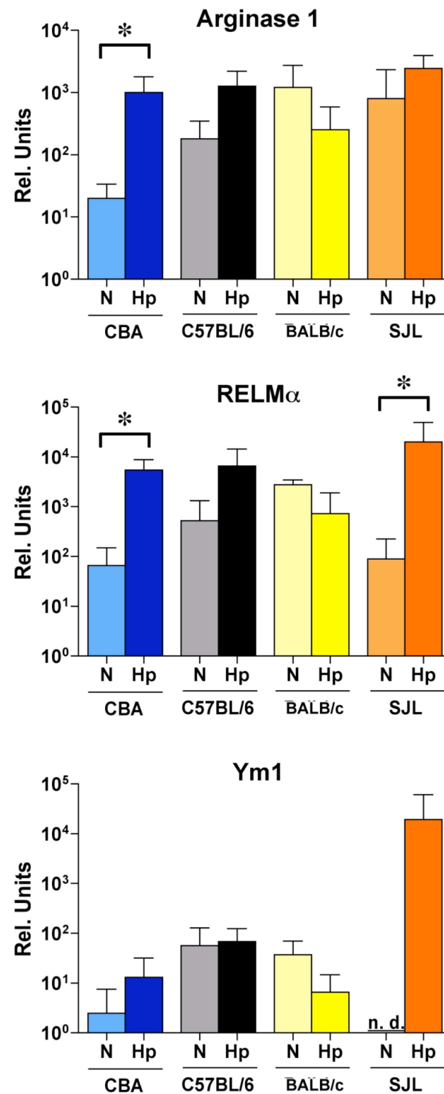


Figure 4.7 – Quantification by real time RT-PCR of AAMϕ associated genes in the gut wall.

cDNA was isolated from the duodenum of naïve, and day 7 *H. polygyrus* infected animals. Expression of Arginase-1, RELMα, and Ym1 were calculated relative to the housekeeping gene, hypoxanthine-guanine phosphoribosyl transferase (HPRT). Error bars represent SD of four individual mice in the naïve, and six individual mice in the infected group. Results of Student's t test comparing naïve to infected groups for each strain: *, $P < 0.05$.

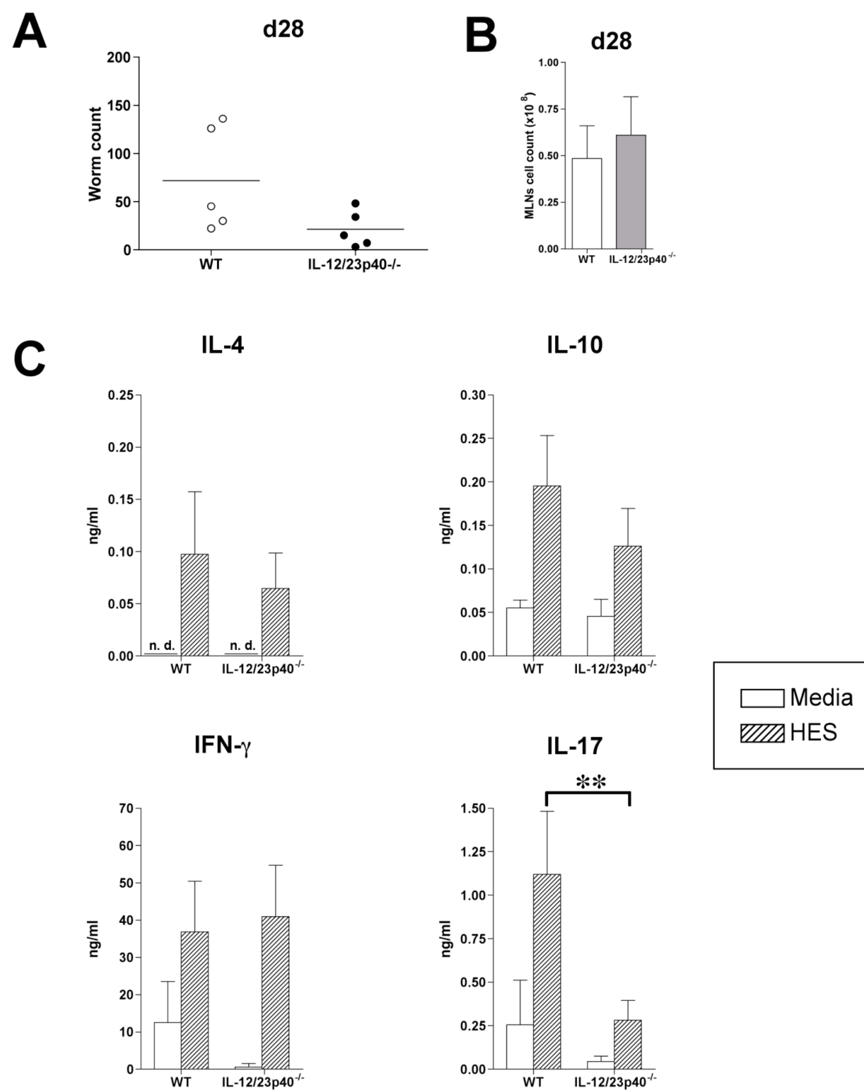


Figure 4.8 – IL-12/23p40$^{-/-}$ C57BL/6 mice have increased resistance to *H. polygyrus* infection.

C57BL/6 mice, or IL-12/23p40$^{-/-}$ animals were infected for 28 days with H. polygyrus. **(A)** Adult worms counts in lumen of the gut at day 28 of infection. **(B)** MLN were isolated and total cell numbers calculated. **(C)** Antigen specific IL-4, IL-10, IFN-γ, and IL-17, were measured in supernatants from $5x10^5$ MLN cells restimulated, *in vitro*, with 1 μg/ml of HES for 72 hrs, or medium alone. Error bars represent SD of cytokines measured in cultures from five individual mice in the WT, and four individual mice in the IL-12/23p40$^{-/-}$ group. Results of Student's t test: **, P<0.01.

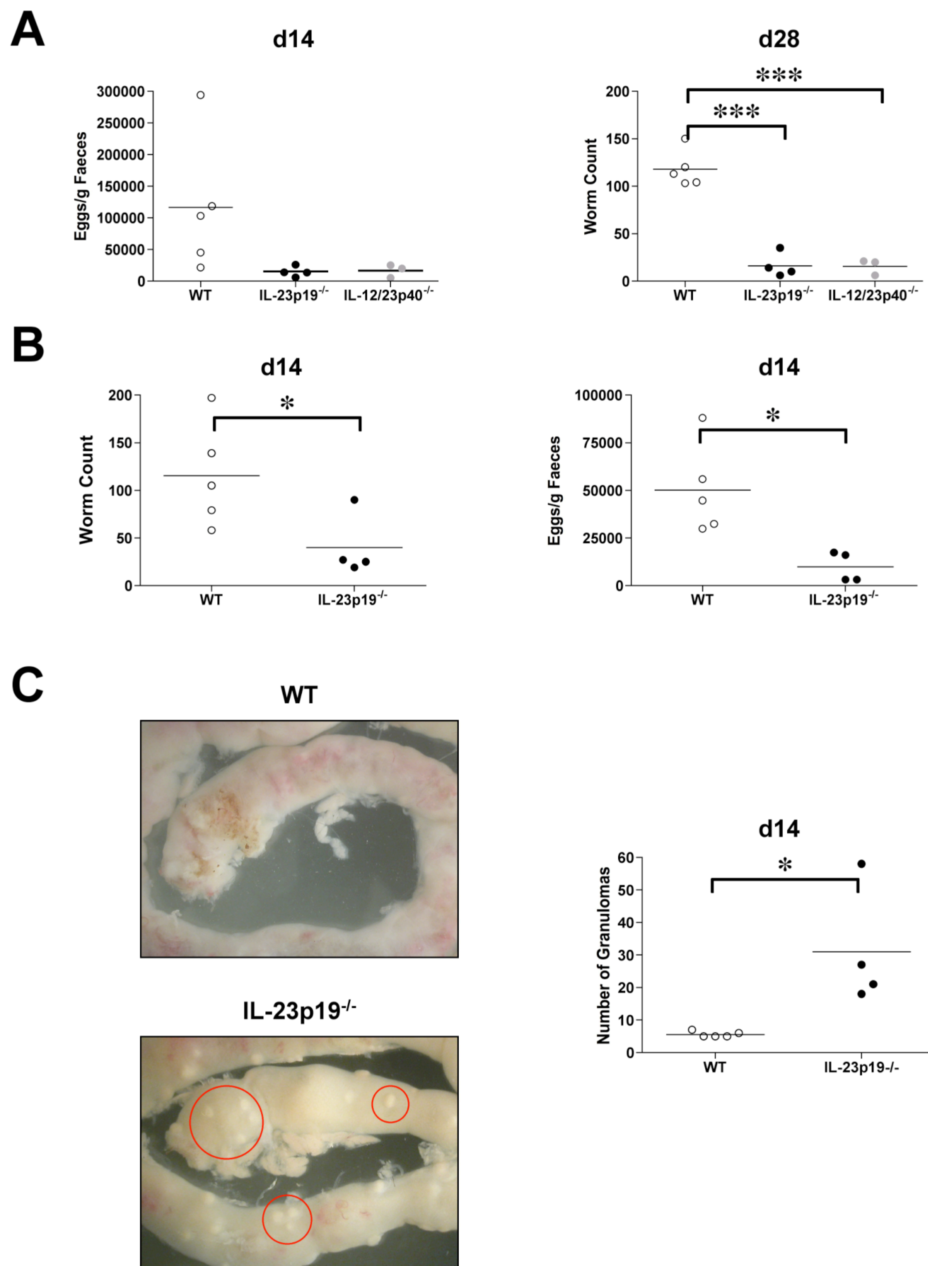


Figure 4.9 – IL-23p19$^{-/-}$ C57BL/6 mice are resistant to *H. polygyrus* and show increases in granuloma-like structures.

(A) C57BL/6 mice, IL-12/23p40$^{-/-}$, or IL-23p19$^{-/-}$, mice were infected for 28 days with *H. polygyrus*. Faecal egg counts were performed at day 14 of infection. Mice were sacrificed on day 28 of infection and luminal worms counted. **(B)** C57BL/6 mice, or IL-23p19$^{-/-}$ mice, were sacrificed at day 14 of infection and luminal worms counted. Faecal egg counts were also measured at the same time point. **(C)** Image showing granuloma-like structures in the wall of the gut from IL-23p19$^{-/-}$ mice **(red circles)** that are not present in WT mice. Counts of granuloma-like structures from WT and IL-23p19$^{-/-}$ animals. Results of Student's t test: *, $P<0.05$; ***, $P<0.001$.

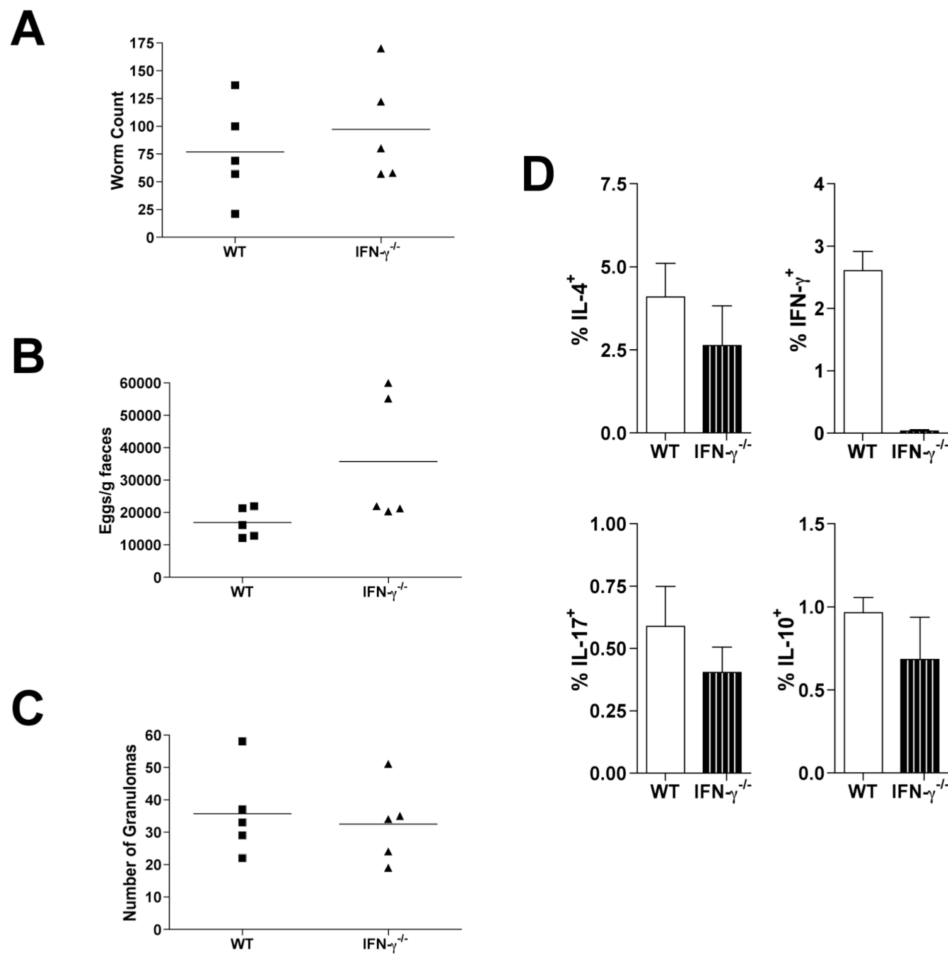


Figure 4.10 – IFN-$\gamma^{-/-}$ C57BL/6 mice do not have enhanced resistance to *H. polygyrus*.

C57BL/6, or IFN-$\gamma^{-/-}$, mice were infected with *H. polygyrus*. At day 14 mice were sacrificed and several parameters measured. **(A)** Luminal worm count. **(B)** Faecal egg counts. **(C)** Granuloma-like structures in the gut wall. **(D)** MLNs were polyclonally stimulated and stained for intracellular cytokines, IL-4, IFN-γ, IL-17, and IL-10. Error bars represent SD of five individual mice per group.

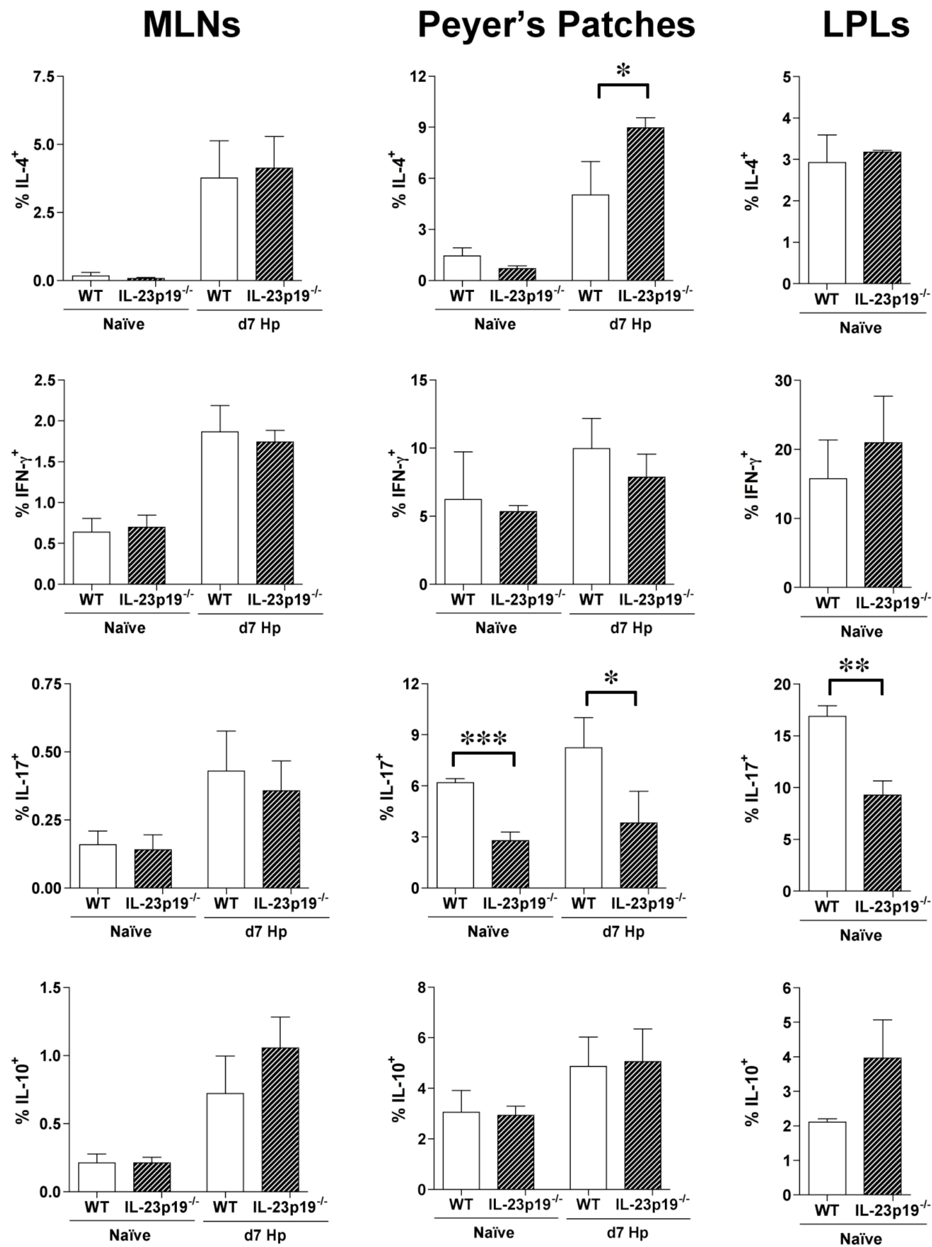


Figure 4.11 – Reduced IL-17 production by IL-23p19$^{-/-}$ mice compared to WT animals in the naïve setting, and at day 7 of *H. polygyrus* infection.

MLNs, Peyer's patches, or LPLs, were isolated from naïve, or day 7 *H. polygyrus* infected, C57BL/6, and IL-23p19$^{-/-}$ mice. Cells were polyclonally stimulated prior to intracellular staining for IL-4, IFN-γ, IL-17, and IL-10. Error bars represent SD of three mice per group. Results of Student's t test: *, P<0.05; **, P<0.01; ***, P<0.001.

4.3 Discussion

The studies in this chapter have addressed the mechanisms of primary resistance to *H. polygyrus* through comparisons of genetically different mouse strains. Although many previous studies had characterised SJL and BALB/c mice as being better able to control *H. polygyrus* establishment (Behnke et al., 1993; Lawrence and Pritchard, 1994; Wahid et al., 1994), it has not previously been shown that resistance is expressed at an early stage of infection. Indeed, by day 14 after inoculation dramatic differences were apparent in worm fecundity between the strains. Subsequently, between d14 and d28, resistant mice expelled the great majority of luminal worms. A key question this raises is whether expulsion follows from damage incurred while in the intestinal wall (explaining, for example, the poor fecundity), or if a separate and second phase of protective immunity is directed at *H. polygyrus* after it has emerged from its tissue dwelling phase. Alternatively, once they have emerged into the lumen, adult worms could develop immunomodulatory strategies that allow it them to neutralise host immunity.

Clear differences between strain susceptibility did not correlate with gross immunological measures such as total numbers or proportions of lymphocyte subsets in the mucosal lymphoid tissues. Thus, MLN cellularity did not correlate with susceptibility to primary infection, and indeed the highest and lowest cell counts were found in two susceptible strains, BALB/c and CBA. When lymphocyte subsets were compared, despite variations in naïve levels, the overall patterns of cell expansion were similar irrespective of resistance phenotype. B cell percentages increased rapidly, whilst $CD4^+$ and $CD8^+$ T cell populations were reduced, as had been previously reported in some strains (Parker and Inchley, 1990). This is of particular interest given that natural antibodies may be important in protecting aginst *H. polygyrus* infection (McCoy et al., 2008). A combination of ELISA and Western Blotting could be used to look at whether those mice that are less susceptible to infection have higher levels of natural antibody towards either HES or HE_x. There was, however, no clear relationship between increases in B cells and enhanced protection.

Foxp3$^+$ Tregs were also characterised, early in *H. polygyrus* infection the percentage of CD4$^+$ Foxp3$^+$ T cells rises in the MLNs of all strains of mice tested, irrespective of their susceptibility to this parasite. Differences are particularly striking at day 7, and only maintained to day 14, in resistant strains, suggesting that these increases in Foxp3 are favoured by the host immune system to control the burgeoning effector response. This inverse relationship, between response phenotype and Foxp3 expression, clearly highlights the problem of separating any parasite-mediated manipulation of the Foxp3$^+$ Treg compartment from appropriate expansion to limit immune-mediated damage to the host (Chapter 6).

The polarisation of Th cells towards a type-2 phenotype was then tested for correlation with resistance. At the simplest level, Th2 responses can be measured as a percentage of the CD4 population, or as an absolute number based on MLN cellularity. Using either measure, responses of all mice were dominated by IL-4-producing Th2 cells, with differences in absolute numbers appearing not to relate to susceptibility. For example, BALB/c mice had the greatest number of Th2 cells and CBA mice the least, while the absolute number of IL-4$^+$ CD4$^+$ T cells was comparabe in susceptible C57BL/6 mice and resistant SJL mice. In particular, counter-regulation of Th2-cells by Th1-cells did not appear to be of importance in this setting as mice with approximately equal susceptibility (CBA and C57BL/6) had, respectively, fewest and most IFN-γ producing CD4$^+$ cells.

A novel finding from this work is the development of granuloma-like structures, some containing larvae, in the small intestinal gut wall of the more resistant BALB/c and SJL mice. Moreover, few of these granulomas were observed in the susceptible CBA or C57BL/6 strains. The granulomas appeared to be similar to those described in secondary infection, within which it was suggested that AAMϕs played a key role in mediating parasite killing (Anthony et al., 2006).

To evaluate whether AAMϕ populations are involved in the gut wall reaction, duodenal samples were tested by RT-PCR for three major AAMϕ-associated transcripts, Arginase-1, RELMα and Ym1. BALB/c mice, which had many granulomas, failed to display upregulation at the mRNA level of any of these markers in the wall of the gut. In contrast, although CBA, and C57BL/6 had few granulomas, upregulation of the AAMϕ markers Arginase-1, and RELMα upon

infection were observed in these strains. There was, however, no increase in the chitinase-like protein Ym1. SJL mice strongly upregulated Ym1, and also RELMα, but not Arginase-1. This raises the possibility that perhaps Ym1, but not other AAMϕ products, might be important in conferring resistance to *H. polygyrus*, and/or that it is not the presence of AAMϕs per se that is important, but their mobilisation into granulomas around the tissue-phase larvae.

Further work will be required to investigate the granulomas in more detail. In particular histological examination can be used to ascertain the localisation of AAMϕs within the granulomas as well as in the gut wall generally. Furthermore, the involvement of neutrophils, and the microscopic state of the encapsulated larvae, will be important to determine.

If AAMϕs are responsible for resistance to *H. polygyrus*, at least in some strains of mice, they are likely to require IL-4 and/or IL-13, as it is known that AAM development is ablated in IL-4R$^{-/-}$ mice (Herbert et al., 2004). There may also be a requirement for certain counteracting factors, such as IFN-γ or IL-12. Release of these, along with IL-17, seems likely early in *H. polygyrus* infection where the gut wall is compromised perhaps leading to the GALT being exposed to antigens and PAMPs from commensal bacteria. Interestingly, resistance to *H. polygyrus* was enhanced in C57BL/6 mice genetically deficient in IL-23p19. No reports have as yet considered the effects of IL-23, or IL-17, on induction of the alternatively activated phenotype in Mϕs, but these data indicate that a component of the IL-23/IL-17 axis may directly inhibit AAM development. One way in which this could be tested is by adding IL-23, and IL-17, into BM-derived Mϕ cultures that contain factors to encourage alternative activation, and assessing whether they inhibit this process.

Surprisingly, an absence of IFN-γ did not alter the resistance of C57BL/6 mice, in acute infection, to *H. polygyrus*. This implies that the typically considered counter-regulatory balance of Th1/Th2 in gut helminth infection is not relevant to establishment of infection in this model. It contrasts sharply with work on *T. muris*, where a lack of IFN-γ drives parasite expulsion (Else et al., 1994). These data also indicate that the failure of susceptible mice to attack tissue phase larvae is not due to IFN-γ-mediated classical activation of macrophages, which could deplete the host's ability to generate AAMϕs.

The different roles of IFN-γ in *H. polygyrus* and *T. muris* may be explained by the variation in the load, and types of bacteria, that the host is exposed to when the small versus the large intestine is invaded by a helminth. Indeed, the data presented in this chapter strongly suggests that initial resistance to *H. polygyrus* is dependent on the Th2/IL-23 balance directly, or via the effects of IL-23 to enhance Th17, or a combination of both. To address the importance of IL-17 it will be necessary to either mAb deplete IL-17 during infection, or infect IL-17$^{-/-}$ animals. The latter idea seems preferable since in *S. mansoni* infection mAb depletion was only partially effective (Rutitzky and Stadecker, 2006). If IL-17 is found to affect response of the host to *H. polygyrus* then it will be tempting to speculate that neutrophils could be playing a negative role in parasite killing. Neutrophils are recruited by IL-17 and could disfavour alternative activation of Mϕs by responding in a pro-inflammatory fashion to commensal bacteria at the infection site. As mentioned, histological analysis of the granuloma-like structures could be used to look for neutrophil localisation.

Additionally, it would be interesting to see whether, as suggested previously, commensal bacteria are important in mediating the increases in IL-17 that are observed in the GALT at day 7 of *H. polygyrus* infection. This could be achieved by studying IL-17 responses, and resistance to the parasite, of either germ free mice, or antibiotic treated (to remove all commensals) WT mice. Although IL-23, and/or IL-17, have been defined as integral to the susceptibility status of C57BL/6 mice to *H. polygyrus*, these same cytokines are not necessarily key to the variation between all strains.

It is likely that a combination of different mechanisms, rather than one specific component, are at play in each strain to ultimately define their resistance or susceptibility to primary infection with *H. polygyrus*. BALB/c mice, for example, do not have enhanced expression of AAMϕ markers, despite the apparent presence of granulomas. Since killing of the larval stages of the parasite is thought to be driven by AAMϕs, this suggests that the relative resistance of BALB/c mice to *H. polygyrus* infection is mediated in another fashion. This could be in part directed at the adults once they enter the gut lumen, as BALB/c mice generated the largest number of Th2

cells by day 14 of infection. Alternatively, a different mechanism of damaging the larval stage of the parasite could be at play in the granulomas.

SJL mice on the other hand upregulated markers of AAMϕ induction at the mRNA level. Ym1 was particularly strongly increased but unaltered in any of the other mouse strains tested. It is a possibility, therefore, that much of the resistance of SJL mice is mediated at the tissue dwelling stage of *H. polygyrus*. At day 14 of infection SJL mice did not generate Th2-cells to any greater extent than the relatively susceptible C57BL/6 mice. CBA mice, which are most susceptible to the parasite were also unable to preferentially target *H. polygyrus* larvae in the wall of the gut, or induce a strong Th2 response in the MLNs. Much future work will be needed to better characterise how each strain of mice responds to *H. polygyrus* and which features of the response are important to resistance. This will require the depletion of candidate cell-types and cytokines across the course of infection, as has been presented here for IL-23 in C57BL/6 mice.

5 The role of methyl-binding domain (MBD) proteins in regulation of Foxp3 expression.

5.1 Introduction

DNA methylation, as discussed in the main introduction (Section 1.4.1), is thought to be a key epigenetic mechanism that plays a role in controlling the development of naïve T cells into differentiated effector subsets. One group of proteins that have been implicated in converting DNA methylation patterns into a phenotypic outcome are the methyl-CpG binding domain (MBD) proteins (Klose and Bird, 2006; Dhasarathy and Wade, 2008). As their name suggests the five members of this family, Methyl-CpG binding protein 2 (MeCP2), MBD1, MBD2, MBD3, and MBD4, all contain a conserved motif that directs interactions with methyl-CpG containing DNA fragments (Hendrich et al., 1999). It is currently considered that by virtue of these interactions and a concurrent ability to recruit histone deacetylases, MBD proteins are able to favour repressive chromatin organisation in regions of highly methylated DNA. As such they are repressive to gene expression, limiting DNA accessibility to transcriptional machinery.

Although the role of MBD proteins in regulation of the immune response has been relatively poorly characterised two studies have implicated MBD2 as playing a role in ensuring that T helper subsets maintain appropriate cytokine expression (Hutchins et al., 2002; Hutchins et al., 2005). *In vitro*, MBD2$^{-/-}$ T cells displayed disordered differentiation during polarisation towards Th1 or Th2 phenotypes (Hutchins et al., 2002). In particular, GATA-3 was found to be dispensable for IL-4 induction in MBD2$^{-/-}$ T cells leading to inappropriate IL-4 expression in otherwise Th1 polarised cells. Loss of MBD2 at GATA-3 binding-sites, resulting in relief of a repressive chromatin structure at the *il-4* locus and hence GATA-less expression of this gene, was implicated as the mechanism by which this misexpression occurred. Despite this aberrant IL-4 expression *in vitro*, unexpectedly *in vivo* MBD2$^{-/-}$ mice had a defect in Th2 polarisation and were susceptible to infection with *T. muris* (Hutchins et al., 2005). The suggested mechanism for this counter intuitive result was that MBD2$^{-/-}$ T cells as well as expressing GATA-less IL-4 were also capable of

expressing T-bet-less IFN-γ. However this data is reconciled it is clear that MBD2 is integral to the stringent control of gene expression patterns required to mount an appropriate T helper response.

Hand in hand with the T helper response the expression of genes associated with T regulatory cells must also be kept tightly in check. It is not surprising to learn then, that several recent reports have implicated DNA methylation as a key mechanism of controlling Foxp3 expression. When human natural killer (NK) T cells, a cell-type that do not normally express FOXP3, were treated with the demethylating agent 5-aza-2'deoxycytidine (5azaC) expression of this gene was observed in response to IL-2 (Zorn et al., 2006). In corroboration of this, bisulphite sequencing to determine DNA methylation patterns of murine T cells, revealed dramatically reduced levels of this modification at the *foxp3* locus in $CD4^{+}CD25^{+}$ cells compared to $CD4^{+}CD25^{-}$ cells (Floess et al., 2007). This reduction correlated with increased histone acetylation, a feature of a less condensed more accessible chromatin structure suggesting a mechanism by which Foxp3 expression is differentially regulated in these populations. Interestingly, in the same study, *de novo* induction of Foxp3 in $CD25^{-}$ T cells by TGF-β was associated with weak demethylation of regions of the *foxp3* locus.

Studies using chromatin immunoprecipitation (ChIP) to define binding-sites for MBDs have implicated MeCP2 as a candidate MBD family member that provides the link between methylation status of the Foxp3 locus and gene expression (Kim and Leonard, 2007). In CTLL-2 cells, a murine cytotoxic T cell line, MeCP2 has been found to bind to CpG islands in the promoter and also an intronic region of the *foxp3* gene. MeCP2 is perhaps the best characterised of the MBD proteins due to the fact that mutations at this locus are associated with the human autistic spectrum disorder Rett syndrome (Amir et al., 1999). However, there have as yet been no reports in humans, or animal models, of direct immunological defects associated with MeCP2.

This chapter focuses on the roles of MeCP2 and MBD2 in the control of Foxp3 expression and the implications of MBD2 deficiency in the development of an immune response towards *H. polygyrus*.

5.2 Results

5.2.1 MeCP2 deficiency does not affect *in vitro* Foxp3 induction.

Given that MeCP2 binds to the *foxp3* locus in CTLL-2 cells it seemed possible that a more open chromatin structure would be adopted in $CD4^+Foxp3^-$ cells from $MeCP2^{-/-}$ mice, and hence Foxp3 expression would be more easily induced. Induction of Foxp3 *in vitro* can be achieved by stimulating $CD4^+$ T cells with TGF-β, as described previously (Section 1.5.2). Purified WT and $MeCP2^{-/-}$ $CD4^+$ T cells were, therefore, stimulated for 72 hrs with increasing concentrations of recombinant human (rh)TGF-β1 in the presence of plate-bound anti-CD3/anti-CD28 and exogenous IL-2. After this time period, expression of Foxp3 in these cultures was assessed by flow cytometry (Fig. 5.1 A). Very little difference was observed in Foxp3 expression between the WT and $MeCP2^{-/-}$ T cells across a concentration curve ranging from 0.1 ng/ml to 10 ng/ml rhTGF-β1 (Fig. 5.1 A, B). Additionally, when stimulated to divide in the absence of TGF-β (Fig. 5.1 A, left panel) there was no evidence in $MeCP2^{-/-}$ $CD4^+$ T cells of aberrant switch on of Foxp3 expression.

5.2.2 *In vivo*, MeCP2 deficiency alters Foxp3 expression on a per cell basis.

Since there was no apparent change in the ability of TGF-β to induce Foxp3 expression in $CD4^+$ T cells *in vitro*, the impact of MeCP2 deficiency on *in vivo* expression of Foxp3 was next studied. To do this analysis of the *ex vivo* phenotype of $CD4^+Foxp3^+$ T cells from the MLNs of $MeCP2^{-/-}$ mice, or their $MeCP2^{+/+}$ littermates, was undertaken. MLNs were used, rather than other peripheral LNs, as they are a major site for accumulation of converted $CD4^+Foxp3^+$ T cells (Sun et al., 2007), allowing natural and induced Treg populations to be studied simultaneously.

Prior to phenotyping the T cells, it was apparent that there was a decrease in overall size of the MLNs from the $MeCP2^{-/-}$ animals (Fig. 5.2 A). If Foxp3

expressing cells are increased *in vivo* by MeCP2 deficiency, then one explanation for this effect would be that suppression of CD4 expansion is enhanced, leading to a reduction in LN size. There was, however, no change in the percentage of $CD4^+$ T cells in the MLNs of $MeCP2^{-/-}$ compared to $MeCP2^{+/+}$ animals, suggesting there was a concurrent proportional decrease in other lymphocyte populations (Fig. 5.2 B). Similarly the percentage of $CD4^+Foxp3^+$ cells in the MLNs was not altered (Fig. 5.2 C). This data is in agreement with the unchanged percentage of $Foxp3^+$ cells induced *in vitro* (Section 5.3.1).

Despite the lack of change in proportions of $Foxp3^+$ T cells, MeCP2 deficiency did have a more subtle effect on Foxp3 expression. Within $MeCP2^{-/-}$ $CD4^+Foxp3^+$ cells Foxp3 expression was enhanced. On average, the geometric mean Foxp3 intensity was found to be 1.5-fold higher than in $MeCP2^{+/+}$ cells (Fig. 5.2 D).

One result of increased Foxp3 expression may be upregulation of Treg associated markers, such as CD25 and CD103. When expression of these proteins was assessed on $CD4^+Foxp3^+$ T cells from $MeCP2^{-/-}$ mice no increase was evident compared to their $MeCP2^{+/+}$ counterparts (data not shown).

5.2.3 MBD2 deficiency enhances TGF-β-induced Foxp3 expression.

5azaC treatment of T cells suggested that the *foxp3* locus is heavily controlled by DNA methylation (Polansky et al., 2008). With this in mind, the relatively small enhancement of Foxp3 expression associated with MeCP2 deficiency implied that another MBD family member may be more important in binding to methylated sites, and repressing expression of this gene.

Based on published data (Su et al., 2004) MeCP2 is predominantly expressed in the brain whilst MBD2 is strongly expressed in immune cells (Fig. 5.3). Previously, it has been stated that MBD2 deficient animals do not have increased numbers of $CD4^+Foxp3^+$ cells in their naïve state (Hutchins et al., 2005). However, this did not exclude the possibility that induction of Foxp3 in $CD4^+Foxp3^-$ T cells may be altered. Foxp3 induction in WT compared to $MBD2^{-/-}$ $CD4^+$ T cells was assessed across a concentration curve of rhTGF-β1 from 0.01 ng/ml – 10 ng/ml (Fig.

5.4 A, B) in the same manner as for MeCP2$^{-/-}$ CD4^{+} T cells (Section 5.2.1). After 72 hrs, it was apparent that Foxp3 was induced far more frequently in MBD2$^{-/-}$ CD4^{+} T cells than WT. The most dramatic difference was at 1 ng/ml where over twice the number of MBD2$^{-/-}$ cells were expressing Foxp3 compared to WT (69% versus 34%). Interestingly, despite the dramatic changes in induction there was no evidence of inappropriate Foxp3 expression in the absence of TGF-β stimulation (Fig. 5.4 A, left panel).

5.2.4 Temporal expression of Foxp3 is altered in MBD2 deficient CD4^{+} T cells.

In light of the increases in Foxp3 expression observed in MBD2$^{-/-}$ T cells, it seemed possible that deficiency in this gene relieves normal restrictions on the *foxp3* locus. If so, then Foxp3 would be more strongly upregulated in early cell cycles when compared to WT. A similar effect on early protein expression has already been reported in respect to IL-4 production by MBD2$^{-/-}$ T cells under Th2 polarising conditions (Hutchins et al., 2002). CD4^{+} cells were labelled with the intracellular protein binding fluorescein derivative, carboxyfluorescein diacetate succinimidyl ester (CFSE), to allow quantitative assessment of cell divisions. Cells were then stimulated to divide in the presence of varying amounts of rhTGF-β1, or in the absence of this cytokine. After 72 hrs expression of Foxp3 in each round of cell division was measured by flow cytometry.

Proliferation of the entire CD4^{+} T cell population was first characterised. In the cultures that did not receive rhTGF-β1, very little change to the proliferation profile was apparent (Fig. 5.5 A, left panel, C). Upon addition of even low levels of rhTGF-β1, clear variations in proliferation were apparent (Fig. 5.5 A, B). At all concentrations of rhTGF-β1 there was an approximately 2-fold increase in proportion of cells still in generations 0 and 1, whilst there was a concurrent decrease in the proportion of cells in generations 3 and 4. A similar proportion of cells were found in generation 2 (Fig. 5.5 A, B).

The same analysis was perfomed within the gated Foxp3^{+} population from the same cell cultures (Fig. 5.6). As was observed previously the Foxp3^{+} population is almost doubled in the MBD2$^{-/-}$ cultures (Fig. 5.6 A). When the proliferation within this population was analysed a slight trend towards an increased proportion of cells within the earlier cell divisions was evident, although very small compared to that observed for the whole population (Fig. 5.5 B, Fig. 5.6 B).

5.2.5 Aberrant IFN-γ production, under Foxp3 inducing conditions, in MBD2 deficient cultures.

A feature described for MBD2$^{-/-}$ T cells when polarised towards a Th1 or Th2 phenotype was enhanced production of IFN-γ (Hutchins et al., 2002; Hutchins et al., 2005). When WT cells were cultured in the presence of TGF-β, IFN-γ production was reduced compared to untreated controls (Fig. 5.7, left panel). In contrast to this, in MBD2$^{-/-}$ cells IFN-γ was increased in the presence of TGF-β. Levels of IFN-γ were also higher in MBD2$^{-/-}$ cells under non-polarising conditions compared to WT.

As suggested from previous data, this increase in IFN-γ was not attributable to decreased production of the counter-regulatory cytokine IL-10 (Fig. 5.7, right panel). MBD2$^{-/-}$ T cells secreted higher levels of IL-10 than WT T cells when TGF-β was included in the cultures. However, IL-10 levels were reduced in both WT and MBD2$^{-/-}$ T cells in the presence of TGF-β, compared to untreated cultures.

5.2.6 Percentage of CD4^{+}Foxp3^{+} T cells are increased, *in vivo*, in MBD2$^{-/-}$ mice.

In vitro, MBD2 deficiency has a clear impact on Foxp3 induction and T cell proliferation. It seemed likely, therefore, that MBD2$^{-/-}$ mice would have an altered Treg compartment, particularly in sites with a high induced Treg component, such as the MLNs (Section 5.2.2). Total cell numbers from the MLNs of MBD2$^{-/-}$ mice were unaltered compared to WT (Fig. 5.8 A), but, in contrast to previous reports (Hutchins et al., 2002), the percentage of CD4^{+} T cells in the MLNs were found to be decreased

by approximately 40% (Fig. 5.8 B). Despite this decrease, when Tregs were measured as a percentage of the MLNs they were found to be unchanged (Fig. 5.8 C, D). This was due to a dramatic percentage increase of $Foxp3^+$ cells within the $CD4^+$ T cell population of approximately 30%.

5.2.7 Expression of CD103 is upregulated on $CD4^+$ T cells from $MBD2^{-/-}$ mice.

Next the activation state of the $CD4^+Foxp3^+$ cells in the MLNs was characterised. CD25 expression was reduced by a small but significant percentage in $MBD2^{-/-}$ cells compared to WT (Fig 5.9 A). This decrease was limited to the $CD4^+Foxp3^+$ population as within the $CD4^+Foxp3^-$ population a small increase in CD25 expression was evident (Fig. 5.9 B). No significant changes were observed in the $CD4^-$ cells (Fig. 5.9 C), which represent predominantly CD8 T cells, and B cells.

Changes in CD103 expression were very different to those observed for CD25. CD103 high cells were markedly increased within the $CD4^+Foxp3^+$ population increasing by around 40% (Fig. 5.10 A). Similarly striking increases in CD103 expression also occurred in the $CD4^+Foxp3^-$ compartment (Fig. 5.10 B). This was not a general increase in CD103 expression however, as the $CD4^-$ population had decreased expression of CD103 (Fig. 5.10 C).

Despite previous data stating that there was no difference in lymphocyte populations in $MBD2^{-/-}$ mice (Hutchins et al., 2002), a deficit in $CD4^+$ cells was also apparent in the spleens of these animals (5.11 B). However, while there was a trend towards increases in Foxp3 expression, it did not reach significance (Fig. 5.11 C). When the $CD4^+Foxp3^+$ cells in the spleens were phenotyped for CD25 and CD103 similar observations were made to these cells in the MLNs (Fig. 5.11 D, E). However, the CD103 increase was less dramatic whilst the CD25 defect was more apparent.

5.2.8 MBD2 deficiency is associated with reduced MLN cell numbers but normal percentages of $CD4^+$ T cells in response *H. polygyrus* infection.

The chronic persistence of *H. polygyrus* is associated with increases in $CD4^+Foxp3^+$ T cells at day 28 of infection. A possible implication of the increased CD103 expression and increased numbers of $Foxp3^+$ cells in $MBD2^{-/-}$ mice is that they will be more susceptible to infections such as *H. polygyrus* that are associated with a regulatory environment. To assess this, mice were sacrificed 14 days after oral inoculation with 200 *H. polygyrus* L_3 larvae, adult worm burdens counted and MLNs phenotyped. Although there was a trend towards increased worm burdens in MBD2-/- mice this did not reach statistical significance (Fig. 5.12 A).

$MBD2^{-/-}$ mice infected with *T. muris* were shown to have IBD-like gut pathology (Hutchins et al., 2005), which typically leads to weight loss, so mice were weighed at the beginning and end of the experiment to assess whether any weight loss occurred during *H. polygyrus* infection. $MBD2^{-/-}$ mice were slightly smaller than age-matched WT mice at the start of the experiment (Fig. 5.12 B) but over the course of the *H. polygyrus* infection there was no difference in weight change (Fig. 5.12 C).

Surprisingly, although cell numbers in MLNs were similar in naïve $MBD2^{-/-}$ and WT mice, at day 14 of *H. polygyrus* infection MLNs had expanded to a much greater extent in WT than $MBD2^{-/-}$ mice (Fig. 5.12 D). The T cell compartment had differentially expanded in the MLNs of the $MBD2^{-/-}$ mice during infection as they now made up a similar percentage of the LNs to WT (Fig. 5.12 E). There was also no longer a difference in the percentage of $CD4^+$ cells expressing Foxp3 (Fig. 5.12 F).

5.2.9 Difference in CD103 expression between WT and $MBD2^{-/-}$ mice is reduced by day 14 of *H. polygyrus* infection.

In infected mice CD25 expression on $CD4^+Foxp3^+$ cells was much the same as in the naïve setting (Fig. 5.13 A) but CD25 expression on the $CD4^-Foxp3^+$ cells was no longer different between the two groups (Fig. 5.13 B). On the $CD4^-$ cells there was no change between the very small percentage of $CD25^+$ cells (Fig. 5.13 C).

The difference between the percentage of CD103 high cells in both $CD4^+Foxp3^+$ (Fig. 5.14 A) and $CD4^+Foxp3^-$ (Fig. 5.14 B) populations was decreased between WT and MBD2-/- mice during infection. In the $CD4^-$ population CD103 expression was comparable in both WT and $MBD2^{-/-}$ animals (Fig. 5.14 C). Hence, infection appears to promote a similar phenotype in WT mice to that which initially exists in naïve $MBD2^{-/-}$ mice.

5.2.10 ICCS reveals no evidence of aberrant cytokine expression in Th1 or Th2 cells.

A possible explanation for increased antigen-specific IFN-γ observed in the MLNs from *H. polygyrus* infected $MBD2^{-/-}$ mice is that there is an inability to polarise a T helper cell response. This could either be due to misexpression of IFN-γ in IL-4 producing cells (Hutchins et al., 2002) or due to MBD2 playing a role in control of IFN-γ expression (Hutchins et al., 2005). ICCS was performed on cells directly *ex vivo* from day 14 *H. polygyrus* infection. IL-4 production was observed in both groups and no significant difference was detectable between them (Fig 5.15 A, B). There was also no evidence of incomplete polarisation as there was no change in the proportion of $IL\text{-}4^+IFN\text{-}\gamma^+$ double producing cells (Fig. 5.15 A, B). IFN-γ production, however, increased in the $CD4^+$ population of the $MBD2^{-/-}$ mice.

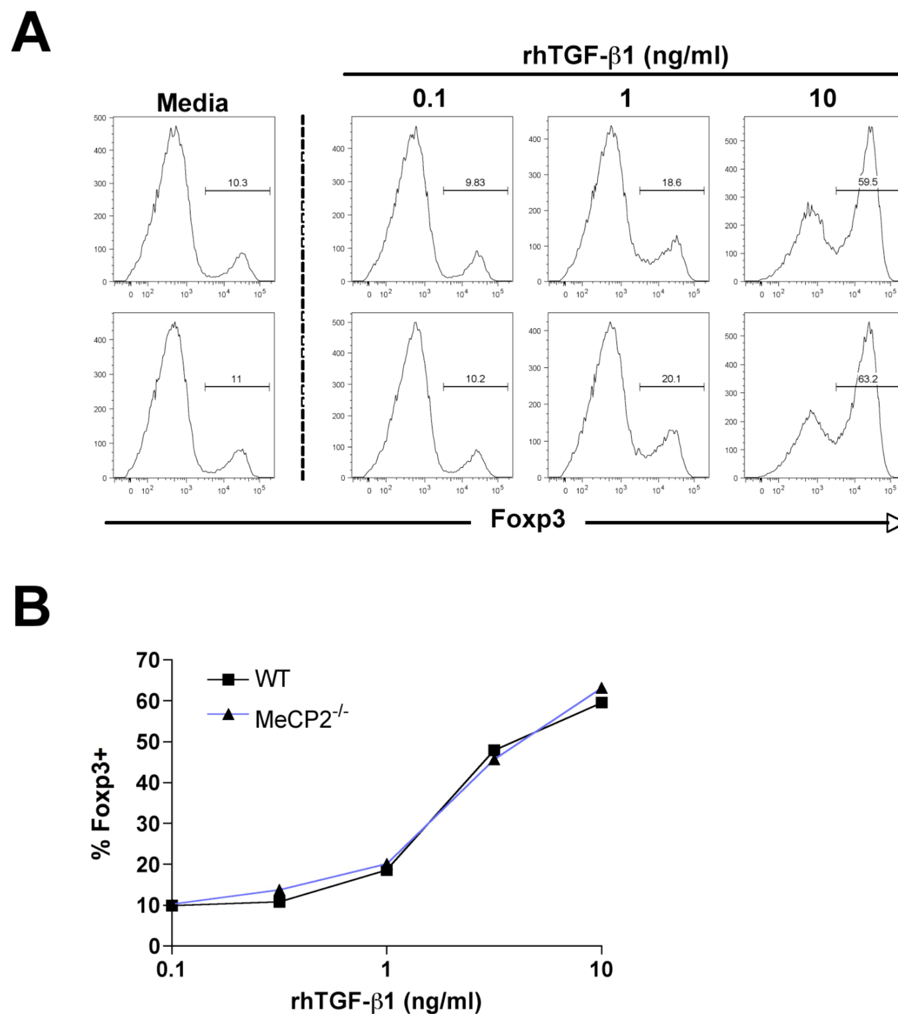


Figure 5.1 – MeCP2 deficiency does not affect *in vitro* Foxp3 induction.

MACS-sorted $CD4^+$ cells from $MeCP2^{-/-}$, or $MeCP2^{+/+}$ littermate controls, stimulated to proliferate with plate-bound anti-CD3/anti-CD28, were cultured in the presence of increasing concentrations of rhTGF-β1 and a constant amount of exogenous IL-2 for 72 hrs. As a control rhTGF-β1 was excluded from some cultures. **(A)** Representative plots of Foxp3 expression without rhTGF-β1, and selected concentrations of rhTGF-β1. **(B)** Graph showing Foxp3 expression in WT and $MeCP2^{-/-}$ cells, as concentration of rhTGF-β1 is increased. Data are taken from a single experiment using $CD4^+$ cells isolated from splenocytes of pooled animals.

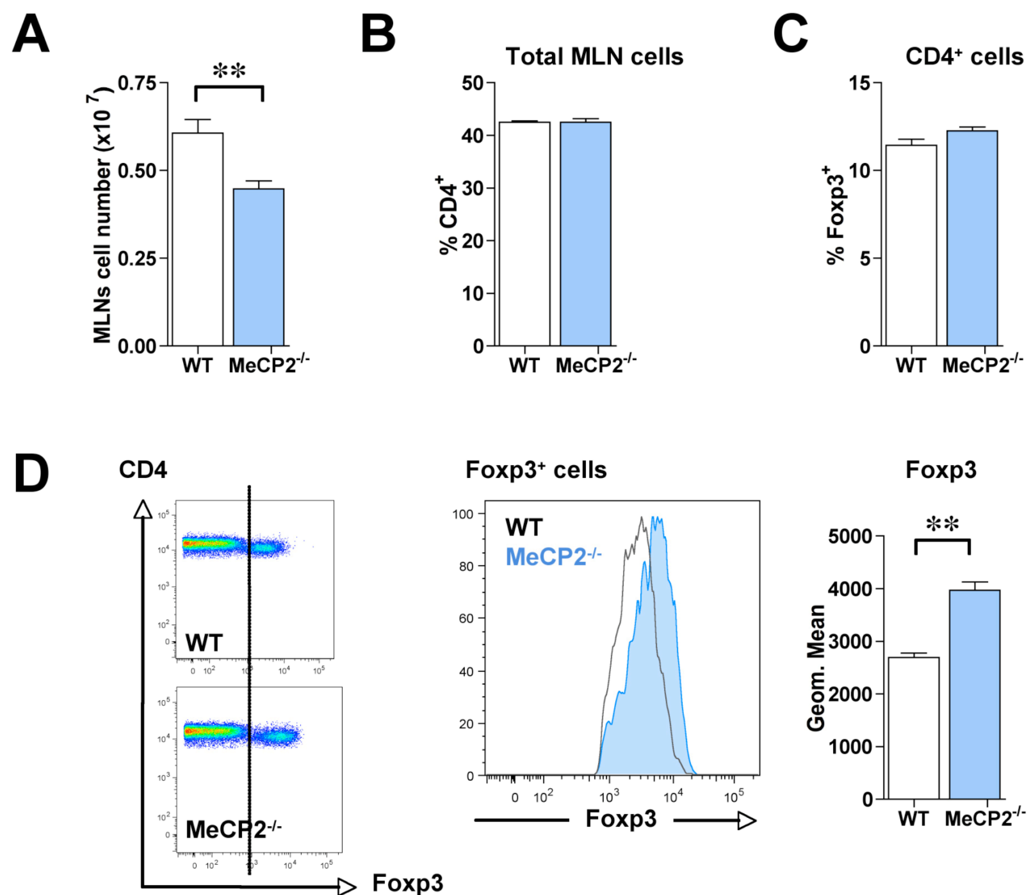


Figure 5.2 – *In vivo*, MeCP2 deficiency alters Foxp3 expression on a per cell basis.

MLNs were removed from naïve, MeCP2$^{-/-}$ mice, or MeCP2$^{+/+}$ littermates, and phenotyped. **(A)** Cellularity of MLNs. **(B)** FACS quantification of CD4^{+} lymphocytes as a proportion of total MLN cells. **(C)** FACS quantification of Foxp3^{+} cells as a proportion of CD4^{+} lymphocytes. **(D)** Representative FACS plots of CD4 vs Foxp3 on CD4^{+} population, and histogram showing intensity of Foxp3 geometric mean in MeCP2$^{-/-}$ CD4^{+}Foxp3^{+} T cells compared to MeCP2$^{+/+}$ cells **(left panels)**. Bar chart of mean Foxp3 geometric mean values for CD4^{+}Foxp3^{+} T cells from individual animals **(right panel)**. The experiment shown here is representative of two similar experiments. Five mice were analysed in the MeCP2$^{-/-}$ group and three mice in the MeCP2$^{+/+}$ group. Results of Student's t test: **, P<0.01. Error bars represent the SD.

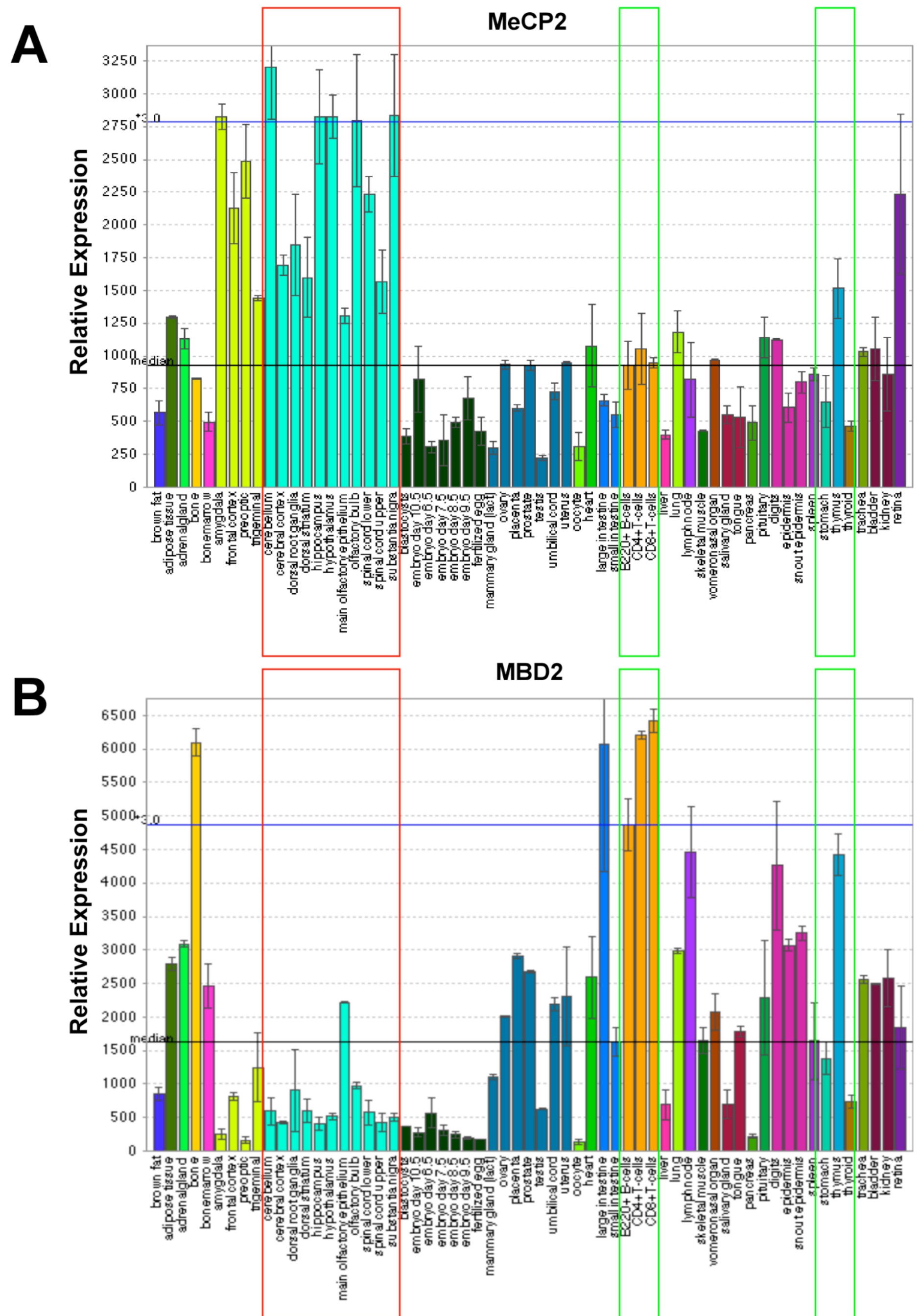


Figure 5.3 – Expression profiles of MeCP2 and MBD2.

Tissue specific mRNA expression profiles of **(A)** MeCP2 and **(B)** MBD2. **Black line**, median value calculated across all tissues. **Blue line**, 3x median value. Regions of the brain **(Red box)** and, lymphocyte populations and thymus **(Green Box)** are highlighted. Graph is adapted from, Symatlas (Genomics Institute of the Novartis Research Foundation) generated from a published data set (Su et al., 2002)

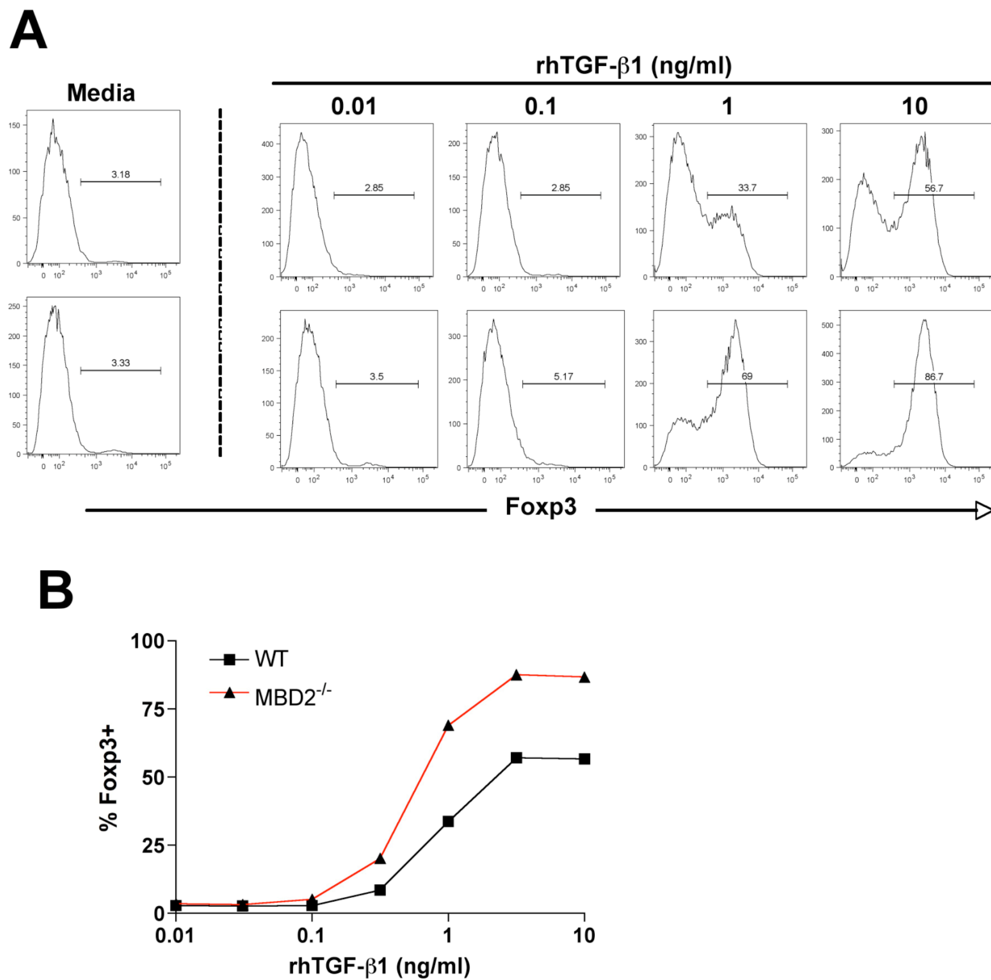


Figure 5.4 – MBD2 deficiency enhances TGF-β-induced Foxp3 expression.

MACS sorted $CD4^+$ spleen cells from WT BALB/c, and $MBD2^{-/-}$ mice, stimulated to proliferate with plate-bound anti-CD3/anti-CD28, were cultured in the presence of increasing concentrations of rhTGF-β1 and a constant amount of exogenous IL-2 for 72 hrs. As a control rhTGF-β1 was excluded from some cultures. **(A)** Representative plots of Foxp3 expression without rhTGF-β1 **(left panel)**, and selected concentrations of rhTGF-β1 **(right panel)**. **(B)** Graph showing Foxp3 expression in WT and $MBD2^{-/-}$ cells, as concentration of rhTGF-β1 is increased. Data shown are taken from a single experiment using $CD4^+$ cells isolated from splenocytes of pooled animals, but are representative of at least three similar experiments.

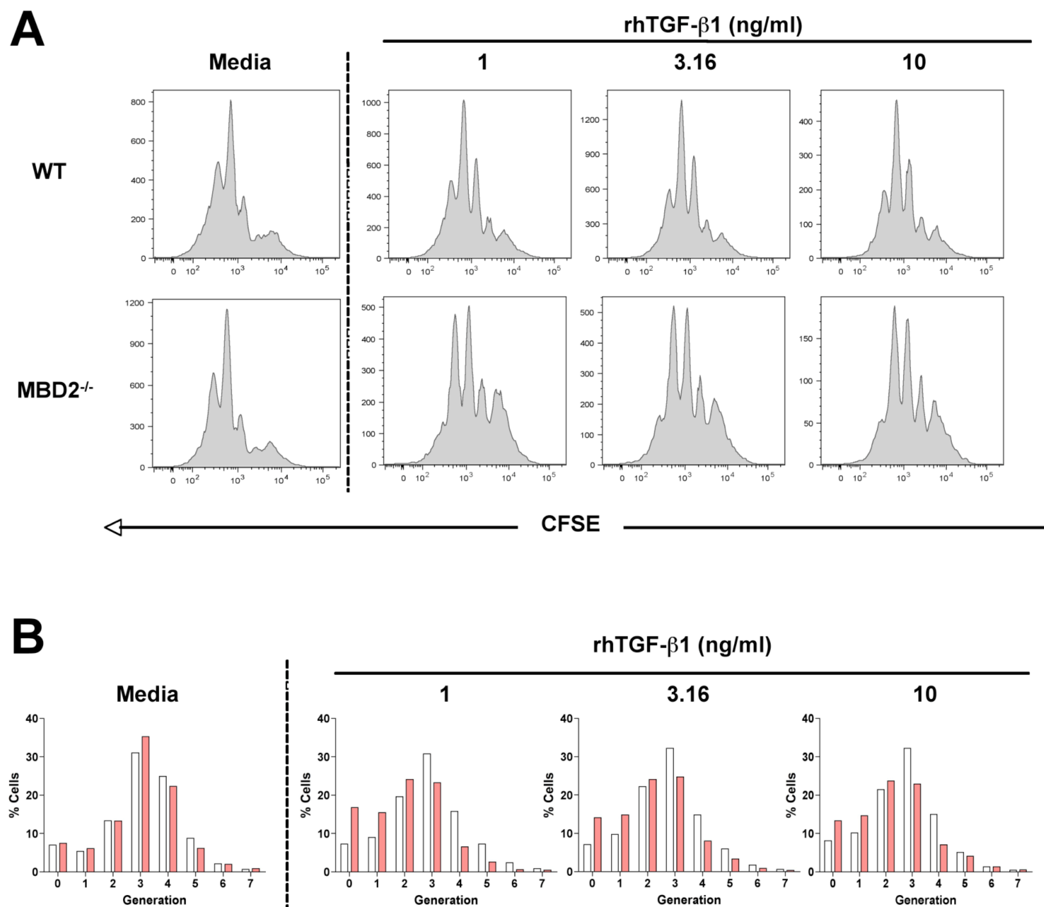
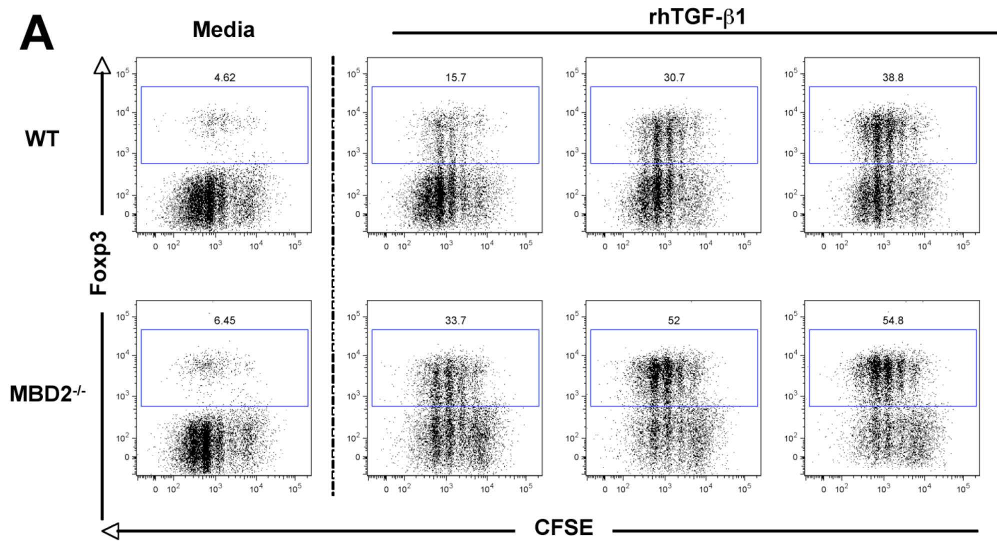


Figure 5.5 – Enhanced suppression of proliferation in MBD2$^{-/-}$ CD4^{+} T cells in response to rhTGF-β1.

MACS-sorted CD4^{+} T cells were labelled with CFSE and cultured under conditions favouring Foxp3 induction with increasing concentrations of rhTGF-β1. As a control rhTGF-β1 was not added to some cultures. **(A)** Histogram of cell division, as assessed by reduction in CFSE intensity, in both WT and MBD2$^{-/-}$ cultures. Cell generation gating calculated by Flow Jo. **(B, left panel)** Graphic representation of percentage of cells in each generation in WT and MBD2$^{-/-}$ cells in the absence of rhTGF-β1. **(B, right panel)** Graphic representation of percentage of cells in each generation in WT and MBD2$^{-/-}$ cells in the presence of increasing concentrations of rhTGF-β1. White bars = WT, pink bars = MBD2-/- cells. Data shown are from a single experiment but representative of two similar experiments.



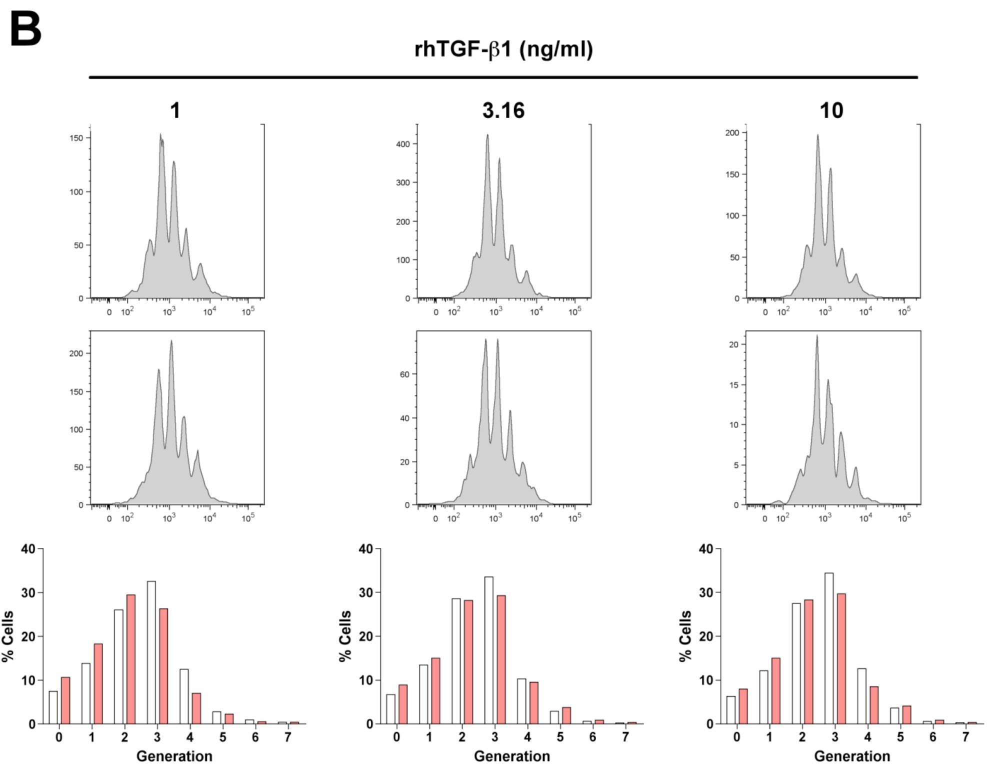


Figure 5.6 – Cell generation associated expression of Foxp3 is unchanged in MBD2 deficient CD4$^+$ T cells.

(A) Cells from Fig. 5.5 were costained with Foxp3. **(B)** Histograms and graphical representations of cell generations within Foxp3 expressing population. White bars = WT, pink bars = MBD2-/- cells.

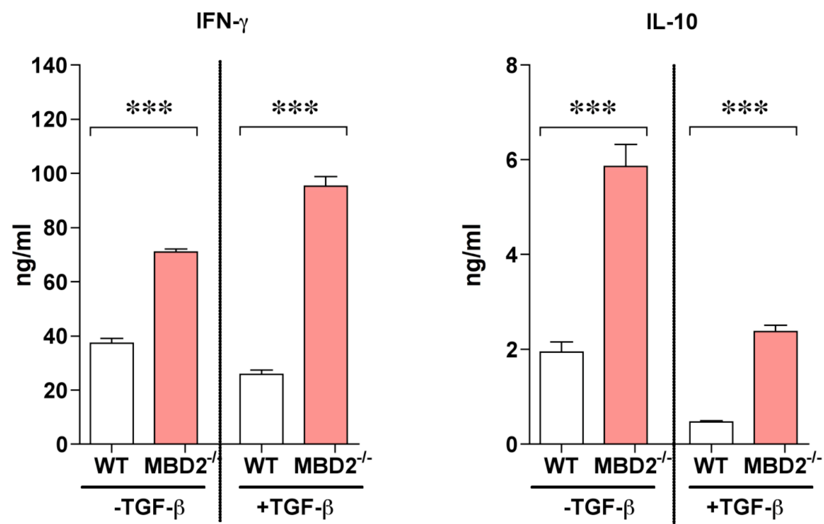


Figure 5.7 – Foxp3 expressing MBD2$^{-/-}$ CD4^{+} T cells aberrantly express IFN-γ.

Cytokines were measured by ELISA in supernatants from CD4^{+} cells cultured under non-polarising or Foxp3^{+} inducing conditions (+ TGF-β). IFN-γ, **(left panel)**. IL-10, **(right panel)**. Data shown are triplicate wells from a single experiment but representative of two similar experiments. Error bars represent the S.E.M. Results of Student's t test: ***, P<0.001.

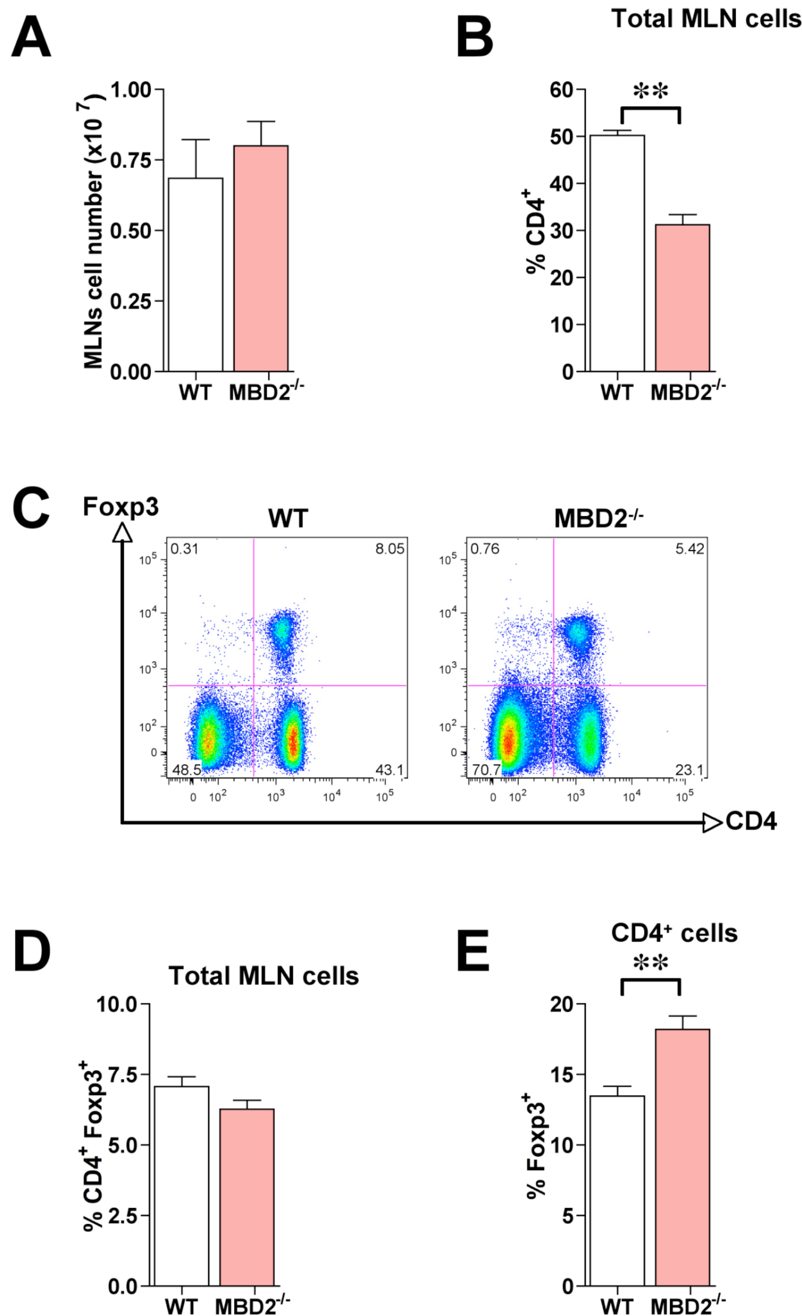


Figure 5.8 – Percentage of $CD4^{+}Foxp3^{+}$ T cells are increased, *in vivo*, in naïve $MBD2^{-/-}$ mice.

MLNs were removed from naïve, BALB/c WT, or $MBD2^{-/-}$ mice, and phenotyped. **(A)** Cellularity of MLNs. **(B)** FACS quantification of $CD4^{+}$ lymphocytes as a proportion of total MLN cells. **(C)** Representative FACS plots of Foxp3 against CD4. **(D)** FACS quantification of $CD4^{+}Foxp3^{+}$ T cells as a proportion of the total MLN cells. **(E)** FACS quantification of $Foxp3^{+}$ cells as a proportion of $CD4^{+}$ lymphocytes. The experiment shown here is representative of two similar experiments. Four mice were analysed in the each group. Results of student's t test: **, P<0.01. Error bars represent the SD.

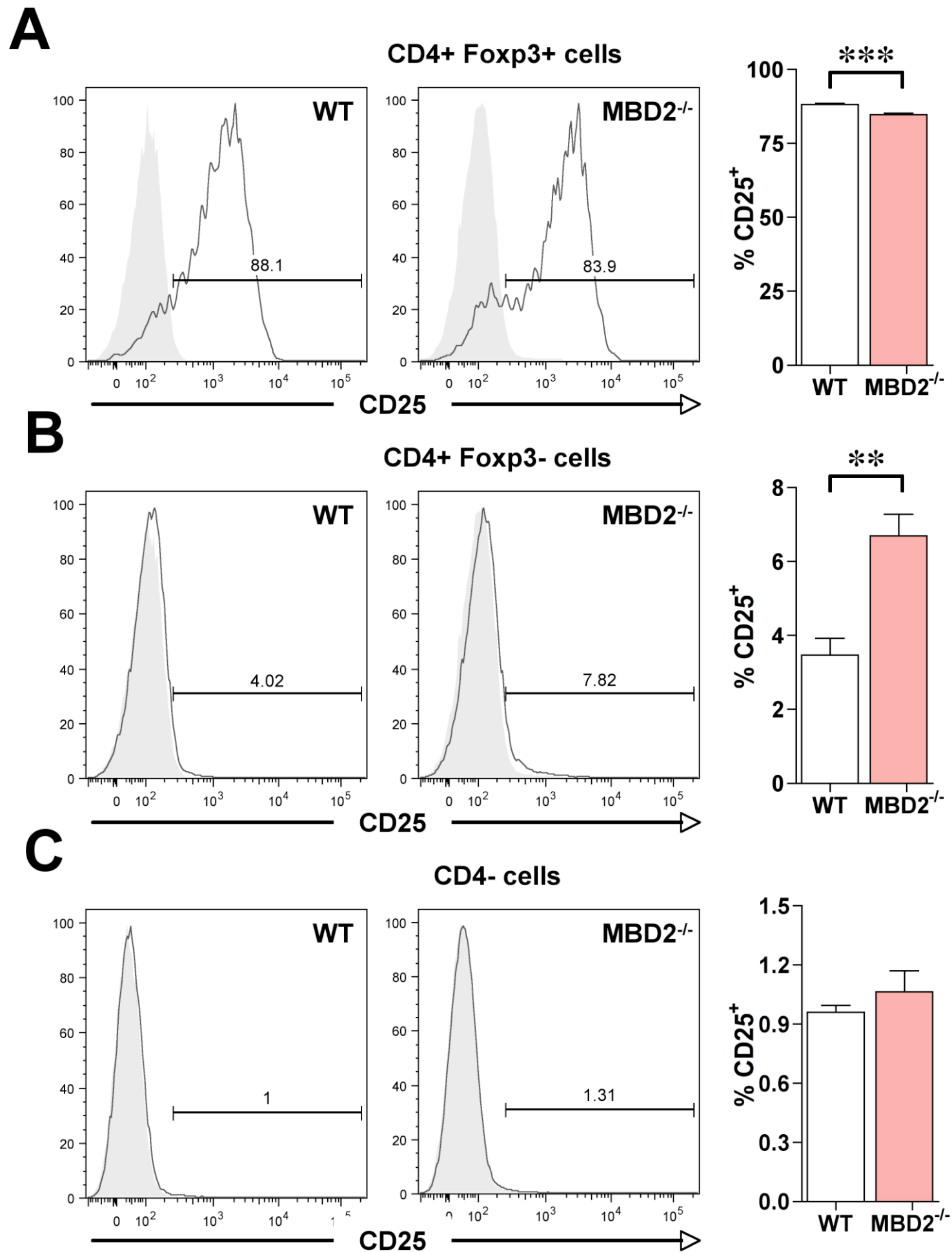


Figure 5.9 – Expression of CD25 on CD4$^+$ Foxp3$^+$ T cells from MBD2$^{-/-}$ mice is decreased.

CD25 was analysed by FACS on different cell populations from the MLNs of BALB/c WT, or MBD2$^{-/-}$ mice. Representative plots and bar charts of data from four mice per group showing: **(A)** CD25 expression on CD4$^+$Foxp3$^+$ T cells. **(B)** CD25 expression on CD4$^+$Foxp3$^-$ T cells. **(C)** CD25 expression on the total CD4$^-$ cell population. The experiment shown here is representative of two similar experiments. Results of Student's t test: **, P<0.01; ***, P<0.05. Error bars represent SD.

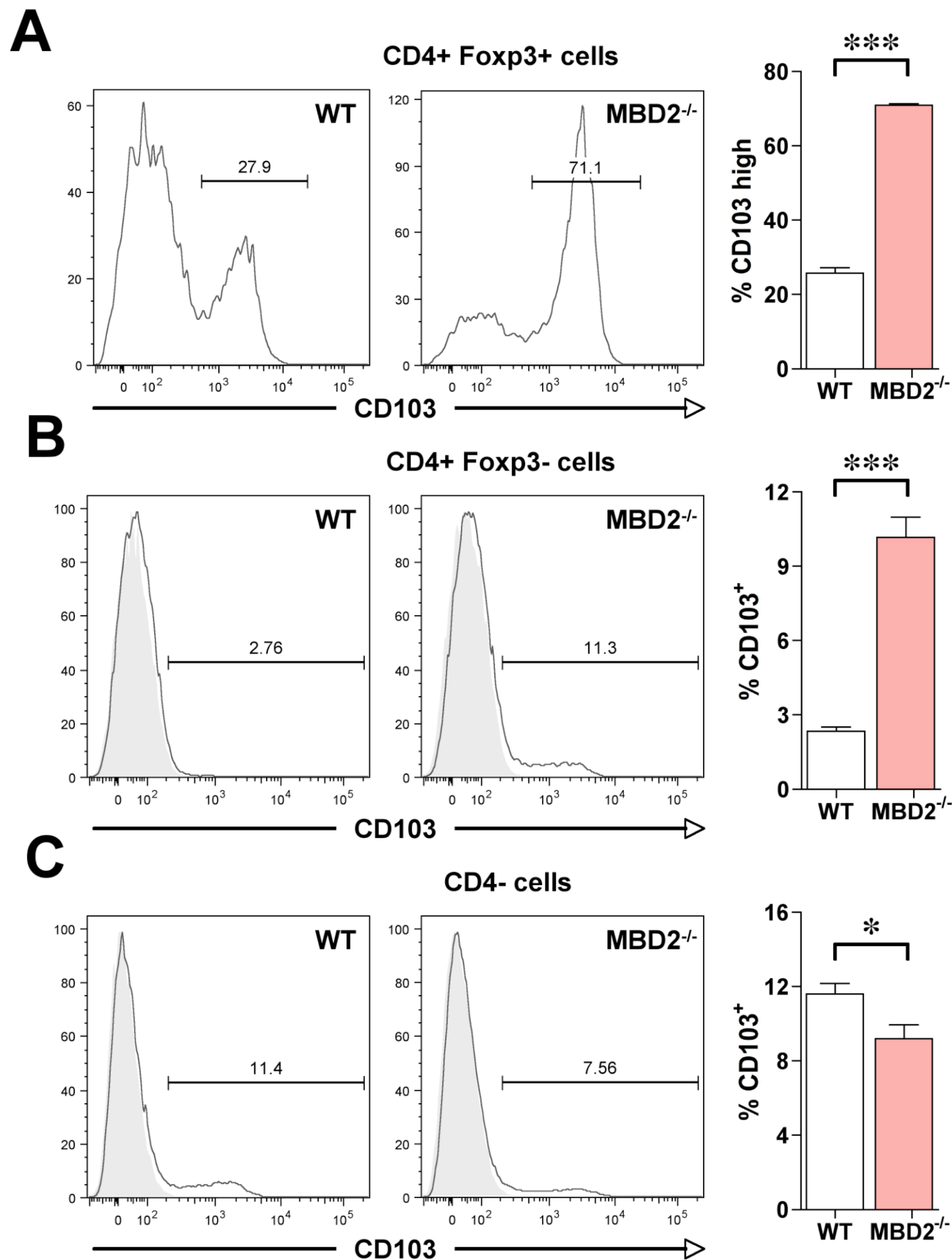


Figure 5.10 – Expression of CD103 is upregulated on $CD4^+$ T cells from $MBD2^{-/-}$ mice.

CD103 expression was analysed by FACS on different cell populations from the MLNs of BALB/c WT, or $MBD2^{-/-}$ mice. **(A)** CD103 expression on $CD4^+Foxp3^+$ T cells. **(B)** CD103 expression on $CD4^+Foxp3^-$ T cells. **(C)** CD103 expression on the total $CD4^-$ cell population. The experiment shown here is representative of two similar experiments. Results of Student's t test: *, P<0.05; ***, P<0.01. Error bars represent SD.

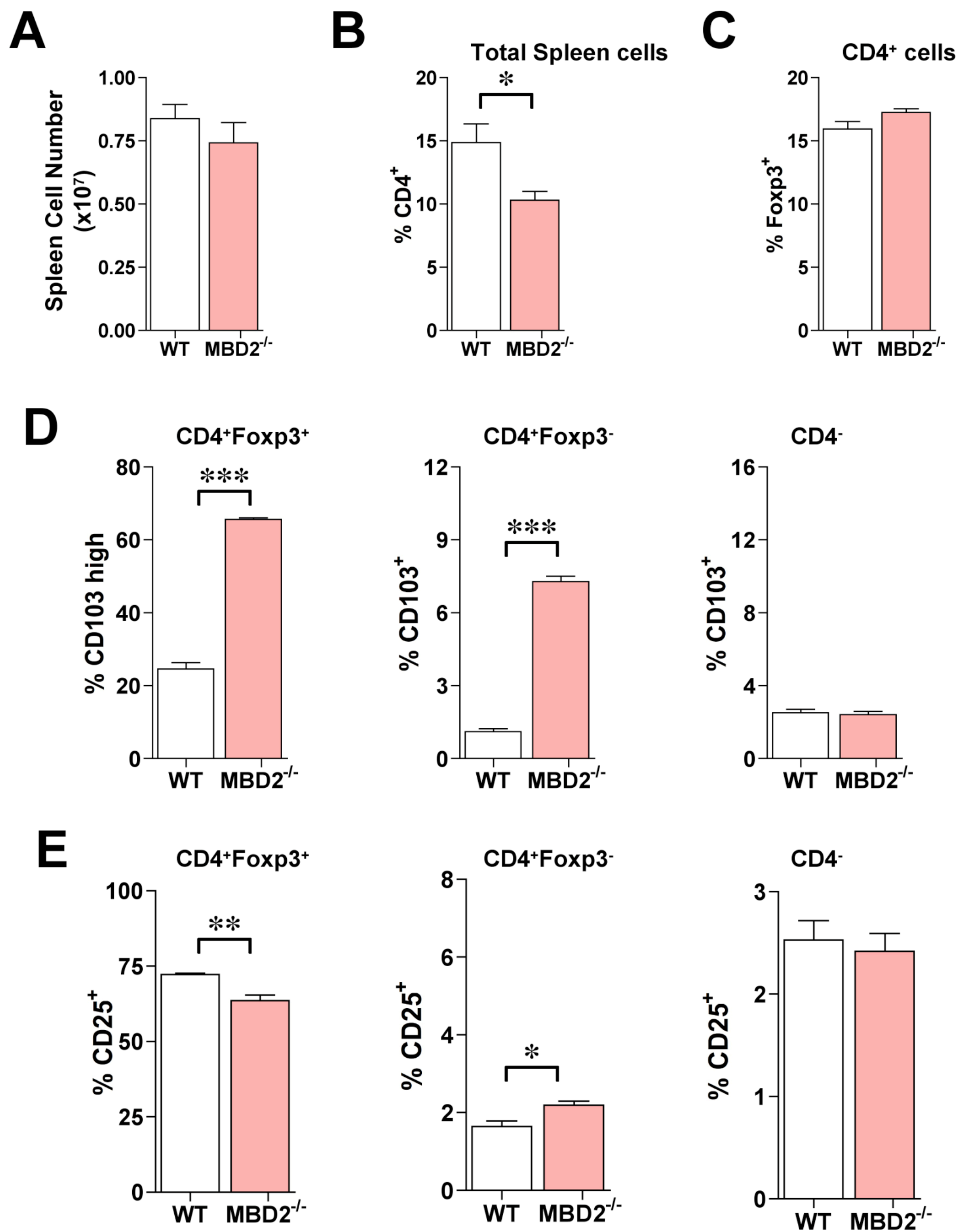


Figure 5.11 – Phenotypic changes to CD4$^+$ T cells are apparent in the spleen.

Spleen cells were taken from same animals as MLNs. **(A)** Total spleen cell counts. **(B)** Percentage of CD4$^+$ T cells in spleens. **(C)** Foxp3$^+$ cell as a percentage of the CD4$^+$ population. **(D)** CD103 expression on different spleen cell populations. **(E)** CD25 expression on different spleen cell populations. The experiment shown here is representative of two similar experiments. Results of Student's t test: *, P<0.05; **, P<0.01; ***, P<0.001. Error bars represent SD.

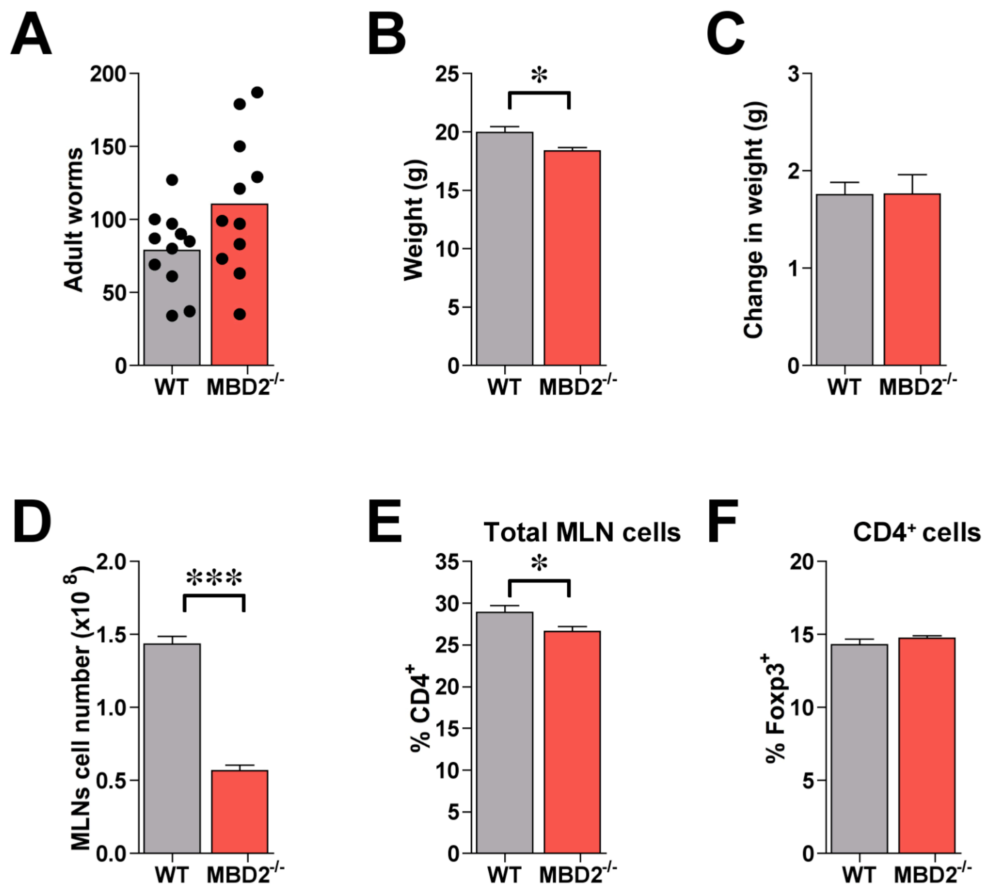


Figure 5.12 – MBD2 deficiency is associated with reduced MLN cell numbers but normal CD4$^+$ T cells in response to *H. polygyrus* infection.

BALB/c WT or MBD2$^{-/-}$ mice were infected orally with 200 *H. polygyrus* L_3 larvae and then sacrificed 14 days later. **(A)** Adult worm burden in the lumen of duodenum. **(B)** Initial weight of mice prior to infection. **(C)** Change in weight after 14 days infection with *H. polygyrus*. **(D)** Cellularity of MLNs on day 14 of *H. polygyrus* infection. **(E)** FACS quantification of the proportion of CD4$^+$ T cells in the total MLN cells. **(F)** FACS quantification of the proportion of Foxp3$^+$ cells within the CD4$^+$ compartment. Results of student's t test, *, P<0.05; *** P<0.001. Error bars represent SD.

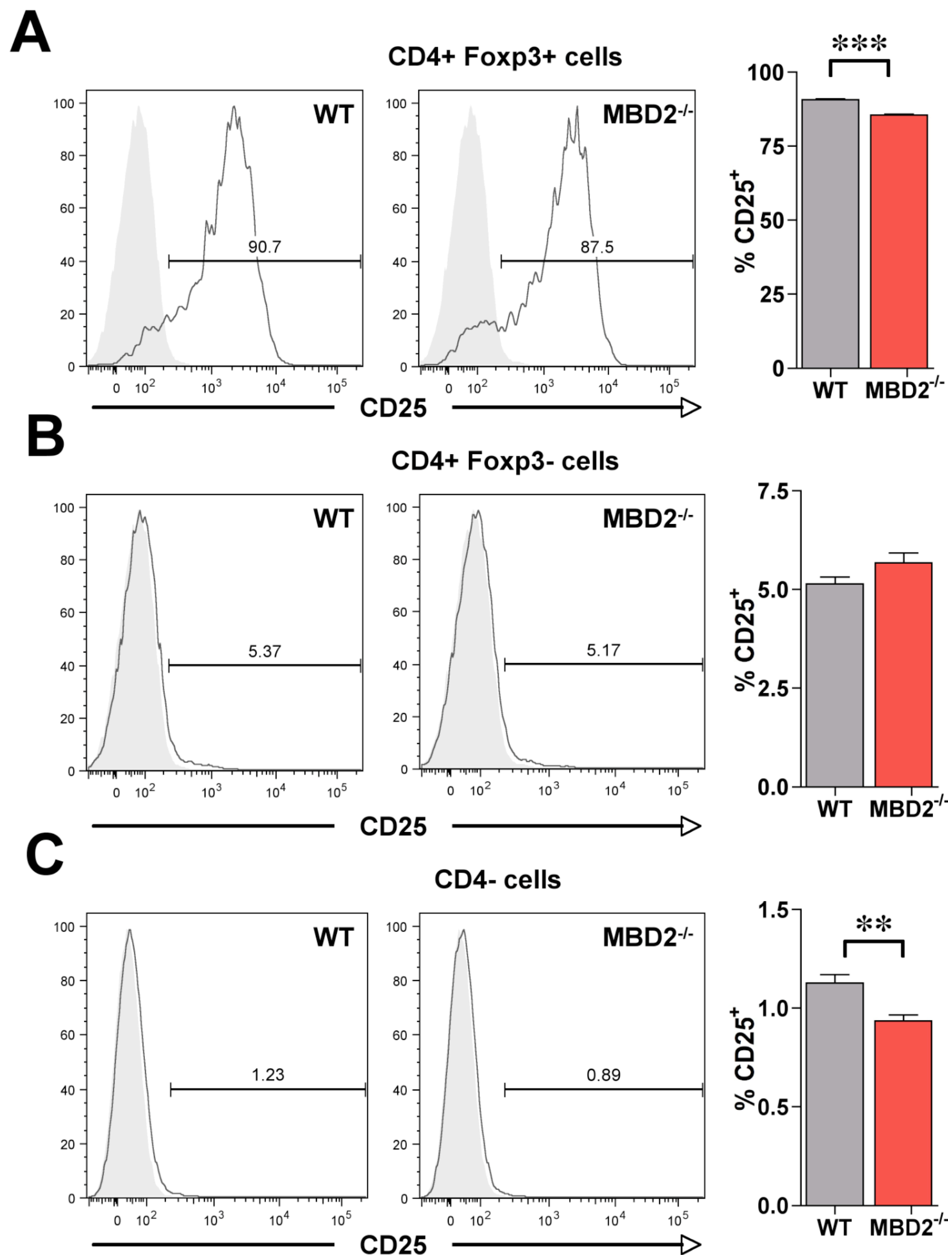


Figure 5.13 – Difference in CD25 expression between WT and MBD2-/- mice is maintained at day 14 of *H. polygyrus* infection.

CD25 expression was analysed by FACS on different cell populations from the MLNs of day 14 *H. polygyrus* infected BALB/c WT, or MBD2$^{-/-}$ mice. Representative plots and bar charts showing: **(A)** CD25 expression on CD4^{+}Foxp3^{+} T cells. **(B)** CD25 expression on CD4^{+}Foxp3^{-} T cells. **(C)** CD25 expression on the total CD4^{-} cell population. The experiment shown here is representative of two similar experiments. Results of student's t test: **, P<0.01; ***, P<0.001. Error bars represent SD.

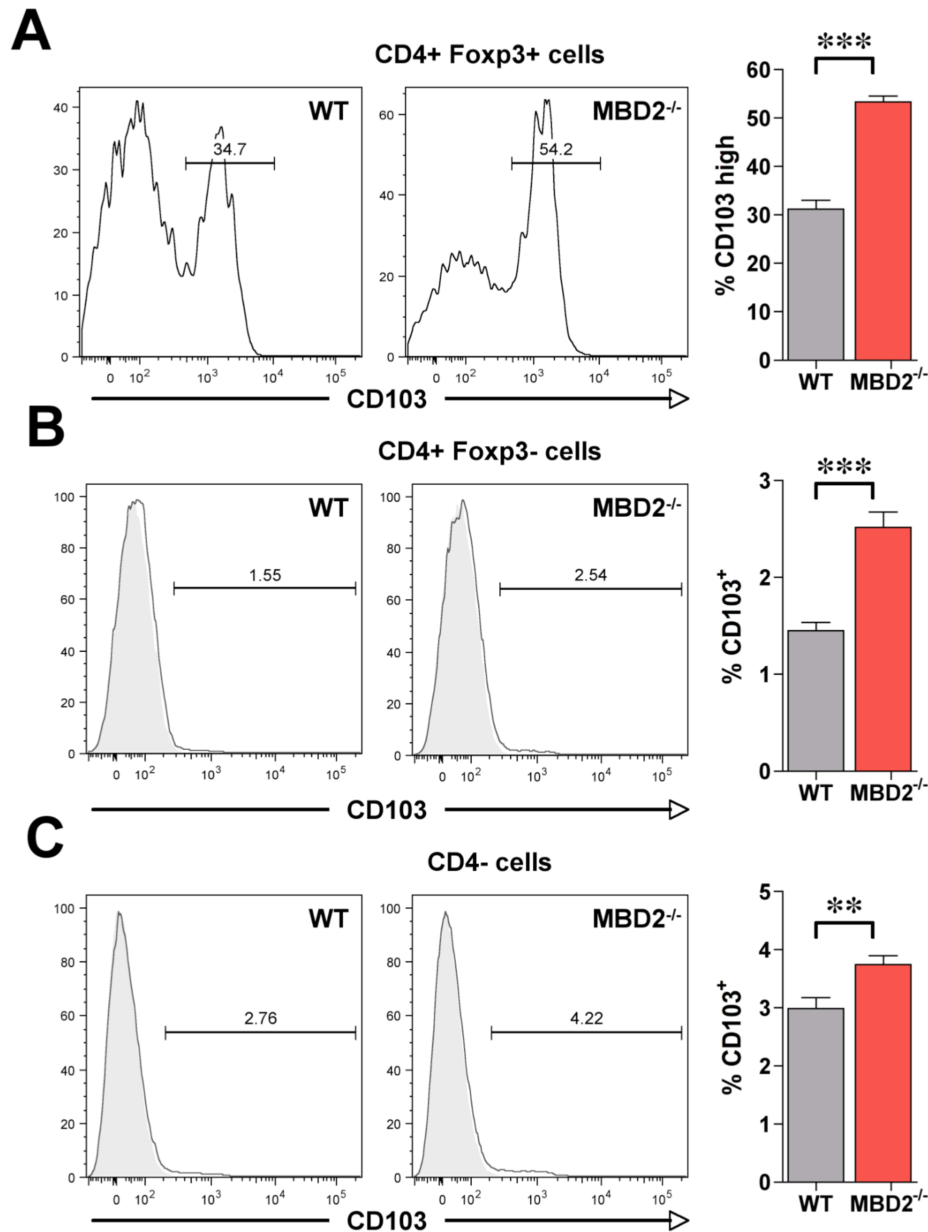
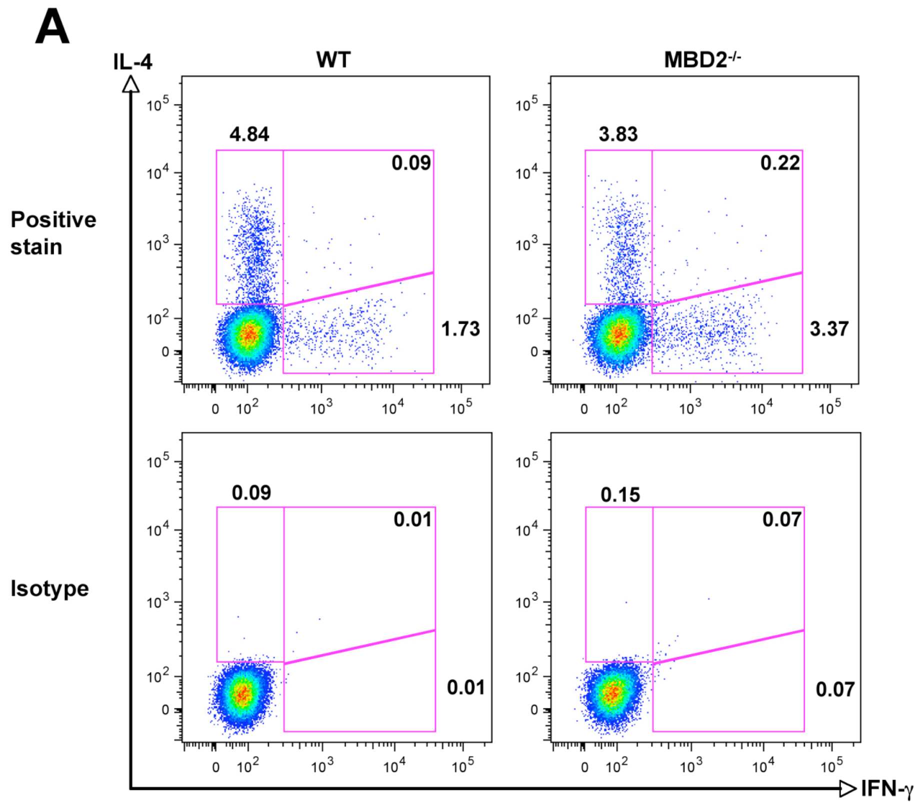


Figure 5.14 – Difference in CD103 expression between WT and MBD2-/- mice is reduced by day 14 of *H. polygyrus* infection.

CD103 expression was analysed by FACS on different cell populations from the MLNs of day 14 *H. polygyrus* infected BALB/c WT, or MBD2$^{-/-}$ mice. Representative plots and bar charts showing: **(A)** CD103 expression on CD4^{+}Foxp3^{+} T cells. **(B)** CD103 expression on CD4^{+}Foxp3^{-} T cells. **(C)** CD103 expression on the total CD4^{-} cell population. The experiment shown here is representative of two similar experiments. Results of student's t test: **, P<0.01; ***,P<0.001. Error bars represent SD.



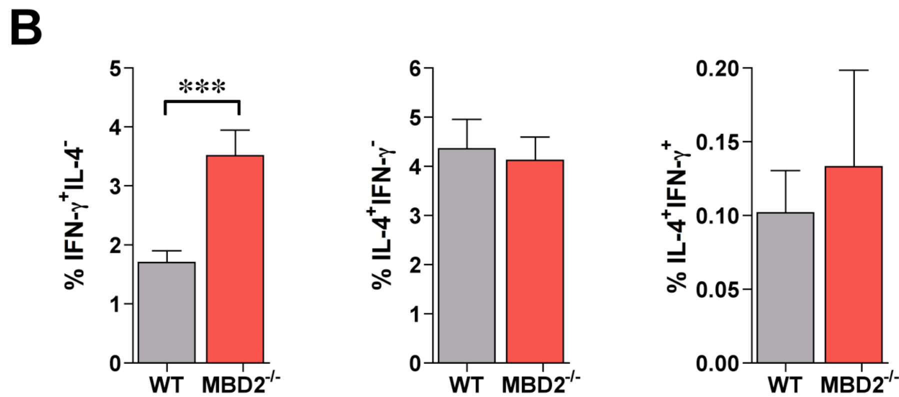


Figure 5.15 – ICCS reveals no evidence of aberrant cytokine expression in IL-4 or IFN-γ producing cells.

Ex vivo ICCS of the $CD4^+$ T cell population of MLNs from day 14 *H. polygyrus* infected BALB/c WT and $MBD2^{-/-}$ mice. **(A)** Sample plots of IL-4 against IFN-γ and their respective isotypes. Gates represent $IL\text{-}4^+IFN\text{-}\gamma^-$, $IL\text{-}4^+IFN\text{-}\gamma^+$, and $IL\text{-}4^-IFN\text{-}\gamma^+$ populations. **(B)** Bar charts of data from individual animals. The experiment shown here was performed once. The WT group contains six mice and the $MBD2^{-/-}$ group seven mice. Results of student's t test, *** P<0.01. Error bars represent SD.

5.3 Discussion

DNA methylation-mediated gene regulation plays an essential role in differentiation of T cells towards specific effector phenotypes. Whether this form of gene regulation is similarly important in differentiation of T regulatory cells, and if it impacts T effector : T regulatory cell balance, is less well understood. This chapter has begun to address this issue by characterising the role of two MBD family members, MeCP2, and MBD2, in control of Foxp3 expression.

Initially, an *in vitro* Treg induction assay was used to ascertain whether deficiency in either of these genes affected the ability of $CD4^+$ T cells to switch on Foxp3 in response to TGF-β. Using $MeCP2^{-/-}$ cells, no change was detected in this assay compared to WT. This suggested that if MeCP2 did have a role in repressing the *foxp3* locus it did not primarily affect regions involved in *de novo* expression of Foxp3 after exposure to TGF-β. In sharp contrast to this, induction of Foxp3 in $MBD2^{-/-}$ cultures was markedly increased compared to WT. At this time the mechanism behind this increase is not understood, however, two possibilities seem likely.

Firstly, given that the *foxp3* locus is methylated, MBD2 may bind directly to this site and inhibit TGF-β-activated transcription factor access. Alternatively, MBD2 may be acting indirectly, for example repressing components of the TGF-β pathway. It has already been shown that in certain situations components of the TGF-β pathway are controlled by DNA methylation. Breast (MCF-7L) and colon (GEO) cancer cell lines treated with 5azaC upregulated expression of TGF-βRI and TGF-βRII in a manner that is dependent upon downregulation of the transcription factor Sp3 (Ammanamanchi and Brattain, 2001).

Assessing whether TGF-β responsiveness is accentuated in an alternative setting, such as polarisation of Th17 cells, may be one method of distinguishing between the options presented above. Of course it is possible that both mechanisms are at play, in which case future work will need to focus on taking a genome wide approach to identify regions of MBD2 localisation, alongside changes in gene

expression patterns, in deficient $CD4^+$ T cells. Transcript analysis, for example by SOLEXA sequencing, in tandem with MBD2 ChIP and mRNA extraction would be a strategy by which this information could be acquired.

In concert with this *in vitro* data, the proportion of $Foxp3^+$ cells within the CD4 T cell compartment of the MLNs was unchanged in $MeCP2^{-/-}$, but significantly increased in $MBD2^{-/-}$, animals, when compared to WT controls. MeCP2 deficiency, however, was not without effect *in vivo* as the level of Foxp3 protein within individual Tregs was increased in this setting. The work in this chapter failed to address the implications of this finding. One outcome may be that these cells overexpress signature Treg genes known to be controlled by Foxp3 (Zheng et al., 2007), although the classical activation markers CD25 and CD103 were found to be unchanged. If this situation occurs then it seems probable that $MeCP2^{-/-}$ Tregs would have increased suppressive activity. This could easily be addressed *in vitro* by comparing the capacity of these cells from $MeCP2^{-/-}$ and WT animals to inhibit proliferation of a responder population.

The same assay should also be performed using $MBD2^{-/-}$ Tregs as evidence suggests that they too would have the same phenotype. *In vivo*, $MBD2^{-/-}$ $CD4^+Foxp3^+$, and $CD4^+Foxp3^-$, T cells had marked elevation of CD103 expression. Upregulation of CD103 has been associated with increased suppressive activity in a number of settings (Section 1.5.1). Interestingly, this observation regarding CD103 lends credence to the earlier suggestion that MBD2 may indirectly control Foxp3 induction by repressing a component of the TGF-β response pathway; CD103 expression is positively regulated by TGF-β (Section 1.5.1).

An additional characteristic of MBD2 deficiency was a deficit in $CD4^+$ T cells in the MLNs and spleen. It is plausible that this is not only explained by a change in the ratio between regulatory and responder cells but also an enhancement of the suppressive capacity of each regulatory cell. Alternatively, this could be reflective of effects of MBD2 on the non T cell compartment, for example B cells. MBD2 is expressed in B cells (Fig. 5.3), but its role in this cell-type has not been addressed.

Previously, it has been reported that $MBD2^{-/-}$ mice have increased susceptibility to *T. muris* (Hutchins et al., 2005). In this setting, an inappropriate

Th1-type response developed, although from analysis of IgG1 production, there was evidence of Th2 polarisation. To explain this result a competition between MBD2 and T-bet at the *ifn-γ* locus was suggested, leading to 'T-bet-less' IFN-γ expression in Th2 cells. In corroboration of this when T-bet was retrovirally transduced into $CD4^+$ cells, cultured under Th2 conditions, IFN-γ expression was increased in the $MBD2^{-/-}$ cells. It was not possible, however, using ChIP, to show direct binding of MBD2 at the *ifn-γ* locus, nor was there any *in vivo* evidence of the predicted IL-4^+IFN-γ^+ Th cells that should have been generated during *T. muris* infection.

Fourteen days after initial infection with *H. polygyrus* $MBD2^{-/-}$ mice, as was the case for *T. muris*, showed a trend towards increased adult worm burden. When the $CD4^+$ T helper phenotype in the MLNs was analysed at this time point no 'confused', IL-4^+IFN-γ^+, double-producing cells were apparent, and no change in the percentage of IL-4^+IFN-γ^- producing cells could be discerned. These data imply that in contrast to *in vitro* reports (Hutchins et al., 2002), a normally differentiated effector population can be derived in the presence of a Th2 driving pathogen *in vivo*. The lack of change in these cells could be reflective of the time point chosen but this seems unlikely as IL-4^+IFN-γ^+ cells were still found to be enhanced as late as 6 weeks when $MBD2^{-/-}$ mice were infected with a Th1-driving pathogen, *L. major* (Hutchins et al., 2005).

At day 14 of *H. polygyrus* infection if any trend towards decreased resistance is explained by cytokine misexpression, it is due to the increase in IFN-γ single positive cells. One source for the increased IFN-γ producing cells could be inappropriate expression of this gene by T cell subsets other than Th2 cells, such as Treg cells or Th17 cells. The aberrant secretion of IFN-γ by TGF-β induced $Foxp3^+$ $MBD2^{-/-}$ T cells *in vitro*, provides support to the concept that this may well occur *in vivo*. Whether this is the case, could be ascertained by co-staining intracellular IFN-γ with Foxp3, or previously uncharacterised lineage specific cytokines.

Another evident immunological defect in the absence of MBD2 was a failure, in the context of *H. polygyrus* infection, to appropriately expand the entire lymphocyte population. A defect in expansion of $MBD2^{-/-}$ $CD4^+$ cells was also observed *in vitro*. It is tempting to speculate that this occurs due to the increased proportion, and phenotypically aberrant, $Foxp3^+$ Tregs present in MBD2 deficiency,

keeping a tighter hold over proliferation of responder lymphocytes towards the pathogen. Both *in vitro* and *in vivo*, antibody depletion of $CD25^+$ cells, and hence the majority of $Foxp3^+$ cells, would help to assess whether $MBD2^{-/-}$ Tregs do in fact have a greater impact on lymphocyte proliferation. However, once again, this lack of expansion could be dependent on the non-$CD4^+$ T cell population. B cells are an obvious candidate, since they dramatically expand early in *H. polygyrus* infection (Parker and Inchley, 1990). In future work, it would not only be interesting to better phenotype this population but also to look for changes in serum antibody isotypes when MBD2 is deficient.

Clearly, a major limitation to the data regarding *H. polygyrus* infection discussed here is that despite striking immunological differences between WT and $MBD2^{-/-}$ animals at day 14 of infection, that are suggestive of decreased resistance, a significantly reduced adult worm burden was not recorded. Since worm expulsion continues to occur after day 14, if mice were sacrificed at a later stage of infection a more marked difference may be observed.

Overall, the data presented in this chapter, implicate for the first time interaction of MBDs with methylated DNA as an important mechanism to control Foxp3 expression within the Treg compartment. General understanding of how gene expression is controlled by epigenetic modifications, and specifically MBDs, is still in its infancy. One particularly interesting question is whether the different MBD family members have distinct or overlapping functions in control of gene expression. In regard to this it would have been instructive to characterise the response of $MeCP2^{-/-}$ mice to infection. The enhanced per cell expression of Foxp3 and the reduced MLN size of these mice, described here, suggest that deficiency of this gene may, like MBD2, have immunological consequences.

An important caveat when interpreting any data from both $MeCP2^{-/-}$, and $MBD2^{-/-}$, mouse models is that these deficiencies lead to non-immunological defects, which may impact on the immune system. $MeCP2^{-/-}$ mice have defects in motor activity and reduced body weight, whilst $MBD2^{-/-}$ animals have abnormal gut formation (Berger et al., 2007), perhaps bringing about changes to their GALT. In the long term, problems of this nature could be avoided by generating animals that

are deficient in specific MBDs within the Foxp3 compartment, whilst in the short term bone marrow chimaeras could also be used.

Recent work using inhibitors that broadly affect epigenetic mechanisms has shown how targeting the immune system at this level can dramatically alter the outcome of the final response (Tao et al., 2007). When mice were treated with HDAC inhibitors, Foxp3 was increased *in vivo* leading to suppression of colitis in a DSS-induced model. A better understanding of the mechanisms by which Treg cells are affected by epigenetic modifications, and the proteins responsible for their translation, such as MBDs, may in future allow development of new treatments that target very specific components of this network in order to modify the outcome of an immune response.

6 Helminth secretions induce *de novo* Foxp3 expression in T cells through the TGF-β pathway.

6.1 Introduction

Over the course of an immune response to an invading pathogen, Foxp3$^+$ Treg cells play a key role in controlling the voracity of the effector response (Section 1.5). The host aims to achieve a balance such that potentially harmful immune-mediated pathology is limited, whilst sufficient immune pressure is maintained against the infectious agent (reviewed by (Belkaid, 2007)). In some infection settings, including mycobacteria (Scott-Browne et al., 2007), leishmaniasis (Belkaid et al., 2002), and filariasis (Taylor et al., 2005), the Foxp3$^+$ Treg component is favoured and as a result the host fails to clear the infection. Currently, it is not clear whether this situation represents an intrinsic failure of the host to appropriately balance the regulatory and anti-pathogen response, or if pathogens have developed mechanisms to selectively enhance Foxp3$^+$ Treg suppression.

A problem associated with distinguishing between these two possibilities, is that in many systems it is difficult to separate Foxp3$^+$ Treg increases that may be due to parasite immunomodulation, from those mediated by an ongoing immune response. Models of helminth infection are ideally suited to addressing this problem as their immunomodulatory factors, ES (Section 1.7), can be screened for Foxp3$^+$ Treg enhancing activities in a naïve setting. *H. polygyrus* is one example of a parasite that might be predicted to harbour activity of this kind in its ES, since *in vivo* over the first 28 days of infection, levels of Foxp3 expression within the CD4$^+$ T cell population of the MLNs is significantly increased (Section 1.5).

In this chapter, changes to the Foxp3$^+$ Treg compartment early in *H. polygyrus* infection at sites local to the parasite are characterised. Furthermore, HES treatment of naïve T cells, *in vitro*, is used as a method to assess whether a helminth has evolved mechanisms to directly impact this key aspect of the regulatory arm of the immune response.

6.2 Results

6.2.1 Foxp3 increases occur at day 7 in lymphocyte populations local to the parasite.

Although C57BL/6 mice present a change in Foxp3 expression at day 7 of infection in the MLNs, it is unknown whether differences are also observed in lymphocyte populations local to the parasite. At this time point, *H. polygyrus* L_4 larvae are encysted in the sub-mucosal layer of the small intestine (Section 1.3.3). By sectioning and antibody-staining, it has been shown that $Foxp3^+$ cell numbers increase in the tissue surrounding the helminth at day 6 of infection (Rausch et al., 2008). However, these authors made no attempt to compare the frequency of $Foxp3^+$ cells in this environment to those in the MLNs, or to characterise differences in the specific lymphoid compartments of the small intestine.

IELs, LPLs, and Peyer's patches, were isolated from naïve C57BL/6 mice, or mice that had been inoculated 7 days earlier with *H. polygyrus* L_3 larvae. Cells were phenotyped by flow cytometry, and compared to MLNs. Naïve levels of $CD4^+$ T cells expressing Foxp3 varied depending on compartment. IELs had a lower mean-percentage, 6.2%, than either LPLs, 15.1%, Peyer's patches, 11.0%, or MLNs, 12.8% (Fig. 6.1). Within the IELs a subset of T cells co-express CD4 and CD8; as $Foxp3^+$ cells were only apparent in the CD4 single-positive group irrespective of infection status, all values were calculated based on this population alone. In the presence of *H. polygyrus*, mean Foxp3 expression increased in all compartments: IELs, mean 9.8%, LPLs, mean 17.7%, Peyer's patches, mean 16.8%, and MLNs, mean 15.8%. This confirms that in C57BL/6 mice at sites closest to the infection Foxp3 expression, as in the LN, is upregulated.

6.2.2 Induced Foxp3^{+} Tregs are present in the MLNs during *H. polygyrus* infection.

The expansion of Foxp3^{+} T cells over the course of *H. polygyrus* infection, could be due to, proliferation of natural Foxp3^{+} Tregs, conversion followed by expansion of naïve effector T cells, or a combination of both processes. *De novo* induction of Foxp3 has been described following adoptive transfer of a Foxp3^{-} cell population, from Foxp3^{eGFP} reporter mice, into recombination-activating gene 1-deficient (RAG-1$^{-/-}$) hosts (Sun et al., 2007), and after OVA-feeding of DO11.10 SCID mice (Coombes et al., 2007). There have been no data demonstrating whether Foxp3 induction occurs in WT animals over the course of any infection.

One day prior to infection with *H. polygyrus*, sorted Ly5.2^{+} CD4^{+} Foxp3-GFP^{-} T cells (from Foxp3-GFP knock-in animals (Fontenot et al., 2005)) were transferred IV into congenic Ly5.1^{+} C57BL/6 mice (Fig. 6.2 A). To distinguish GFP^{+} cells from any autofluorescence, at the end of the experiment, the same number of MACS sorted WT Ly5.2^{+} CD4^{+} cells were transferred at the same time into another group of Ly5.1^{+} C57BL/6 mice (Fig. 6.2 A). 28 days after inoculation with *H. polygyrus* larvae, MLNs were removed from infected mice, alongside naïve controls, and peripherally induced Tregs identified by their expression of Ly5.2 and Foxp3-GFP (Fig. 6.2 B). Due to the extremely low percentages of induced Tregs, prior to analysis, samples from each group were pooled, and CD4^{+} cells pre-enriched by MACS sorting (Fig. 6.2 A).

Induction of Tregs appeared to have occurred in both naïve and infected mice. Foxp3-GFP^{+} cells made-up 0.54%, and 0.85%, of the CD4 T cell population, respectively (Fig. 6.2 B, upper panels). In order to assess whether this represents an increase in proportion of induced Foxp3^{+} Tregs during infection, rather than experimental variation, the study will need to be repeated several times. What is clear, from these data, is that the absolute number of Foxp3-GFP^{+} cells from infected mice is far greater than from naïve animals, as the MLNs enlarged dramatically in response to the parasite (Fig. 6.2, lower panels).

6.2.3 HES increases percentage of Foxp3 expressing T cells in, naïve, mitogen stimulated splenocyte cultures.

As stated in the introduction to this chapter (Section 6.1), if any of the bias towards $CD4^+$ T cell expression of Foxp3 is due to manipulation by *H. polygyrus*, it is most probably mediated by the ES. To begin with, the ability of HES to enhance expression of Foxp3 within the $CD4^+$ T cell compartment of naïve C57BL/6 splenocyte cultures was assessed. Previously, it has been shown that a requirement for Foxp3 induction is T cell receptor (TCR) ligation. The T cell mitogen Concanavalin (Con) A was, therefore, included in some experimental groups. In cultures treated with HES (10 μg/ml) plus Con A, the percentage of $Foxp3^+$ $CD25^+$ cells within the $CD4^+$ T cell population was increased more than four-fold over a 48 hr period compared to Con A alone (Fig. 6.3 A, C). As expected, Con A alone strongly up-regulated CD25, consistent with polyclonal activation, but no evidence of Foxp3 enhancement was apparent (Fig. 6.3 A, C). HES acted in a dose-dependent manner in combination with Con A but failed to have any impact on Foxp3 in its absence (Fig. 6.3 C).

Although unlikely, it was plausible that the effects of HES were attributable to heat-stable contaminants, such as LPS. To address this, cultures were stimulated with hiHES plus Con A. In this situation, Foxp3 increases were abrogated, suggesting that this phenotype is most probably dependent on a heat-labile parasite component rather than a contaminant (Fig. 6.3 A, C).

This was the first time that a pathogen-associated factor had been demonstrated to interact with host cells to increase the percentage of T cells expressing Foxp3. There was a possibility that this was a general property of pathogen-derived products but had not been observed since they had not been assayed in this particular manner. Preparations from the gram-negative bacteria *Salmonella typhimurium* (Sa) and the gram-positive bacteria *P. acnes* (Pa) were found to be devoid of Treg cell-increasing activity. Similarly, the synthetic TLR-2 ligand PAM-3-CSK4, and the TLR-4 ligand LPS had no effect, along with the other helminth products NES and SEA (Fig. 6.3 B).

6.2.4 HES alters expression of cytokines released in response to Con A.

In addition to Foxp3 staining, supernatants from the same cultures were assayed, by ELISA, for the presence of IFN-γ, IL-4, and IL-10. When naïve splenocytes were stimulated with Con A alone, upregulation of all cytokines occurred (Fig. 6.4). The stimulation of IFN-γ secretion was particularly striking, rising from below detection limit to 32 ng/ml. Treatment with HES, in the presence of Con A, led to heightened IL-4, and IL-10, levels, but inhibition of IFN-γ, in a concentration dependent fashion. At 10 μg/ml HES inhibited IFN-γ over two-fold. As was observed in respect to Foxp3 percentage change, the effects of HES on cytokine production were heat-labile. Once again differences were only apparent when cells were co-stimulated with Con A; HES alone did not elicit IFN-γ, IL-4, or IL-10 production.

6.2.5 Foxp3 increases in response to HES are limited by strong TCR stimulation.

Since Con A is a lectin from the jack-bean *Canavalia ensiformis*, HES-driven increases in Foxp3 could be mediated specifically by this mitogen due to its interactions with HES glycans. To limit the likelihood of this occurring HES was added to splenocyte cultures 30 min prior to Con A. However, to totally exclude this possibility anti-CD3 was used as an alternative method to stimulate T cell expansion, anti-CD3. At 1 μg/ml anti-CD3, HES was able to increase Foxp3 to a comparable extent as with 2 μg/ml of Con A, showing that this effect is independent of the type of mitogenic stimulus (Fig. 6.5, upper panels).

As already described (Section 1.5.2), addition of exogenous TGF-β to proliferating T cell cultures induces Foxp3 expression *in vitro*. In this setting, Foxp3 induction is optimal when TCR stimulation is weak, decreasing as mitogen concentration increases (Kim and Rudensky, 2006). A similar sensitivity to mitogen dose was observed when splenocytes, incubated with HES, were treated with high (10 μg/ml), and low (1 μg/ml), concentrations of anti-CD3. At 1 μg/ml, 10% of the

$CD4^+$ T cells were $Foxp3^+$, whereas at 10 μg/ml, 4% were in this population, a <1% increase over anti-CD3 alone (Fig. 6.5).

6.2.6 HES induces *de novo* expression of Foxp3 in $CD4^+$ T cells.

The change in the proportion of $Foxp3^+$ $CD4^+$ T cells in response to HES may be a result of *de novo* Foxp3 induction, proliferation of natural $Foxp3^+CD4^+$ T cells in response to parasite antigens, or selective killing of $Foxp3^-CD4^+$ T cells. To ascertain which of these is the case, initially $Foxp3^-$ splenocyte cultures were stimulated with HES in the presence of an appropriate concentration of anti-CD3. $Foxp3^-$ splenocytes were sorted by flow-cytometry, as previously (Section 6.2), from Foxp3-GFP mice. After 48 hrs in the presence of HES, Foxp3 expression was evident in $CD4^+$ T cells derived from the originally $Foxp3^-$ cultures (Fig. 6.6). This demonstrates that HES is able to induce *de novo* Foxp3 expression in activated $CD4^+$ T cells.

6.2.7 $Foxp3^+$ T cells, induced by HES, are functionally suppressive.

Presence of Foxp3 typically correlates with T cells gaining regulatory activity (Fontenot et al., 2003). HES is a complex mixture of helminth products and it is feasible that in this situation $Foxp3^+$ cells do not take on a suppressor phenotype as a result of these other factors. The functional capacity of HES-induced $Foxp3^+$ $CD4^+$ T cells was, therefore, analysed. To test this, $Foxp3^-$ $CD4^+$ T cells were first purified from Foxp3-GFP mice (Fig. 6.7 A (I, II)), and then stimulated with plate-bound anti-CD3/anti-CD28 in the presence of either HES, or rhTGF-β1. 72 hrs later, substantial numbers of $Foxp3^+$ cells had been induced in both treatment groups (Fig. 6.7 A (III, IV). This demonstrated that HES, like TGF-β, acts on $CD4^+$ T cells to induce Foxp3 without necessity for APCs.

Using flow-cytometry, the $Foxp3^+$, and $Foxp3^-$, populations were isolated from these cultures, and added to a responder T cell population stained with the

quantitative cell division marker CMTMR. The responder T cell population was composed of polyclonally-stimulated, freshly isolated, Foxp3^{-}CD4^{-} cells, also sorted from Foxp3-GFP mice (Fig. 6.7 A (V)). HES- and rhTGF-β1- induced Foxp3^{+} cells were found to be equally suppressive, and exerted a comparable effect to freshly isolated Foxp3-GFP^{+} natural Tregs (Fig. 6.7 A (V), B). This was in contrast to the Foxp3^{-} cells taken from the same 72 hr cultures, which were only weakly suppressive.

6.2.8 HES contains a TGF-β-like activity that is not inhibited by a pan-vertebrate TGF-β antibody.

Given the phenotypic similarities between HES and TGF-β, and the reported release of a TGF-β homologue by the human filarial nematode *B. malayi* (Section 1.7.8), it seemed likely that HES contains a functional mimic of this molecule. To test whether HES did contain this activity, MFB-F11 cells were incubated with HES. MFB-F11 is a cell line derived from TGF-β1$^{-/-}$ fibroblasts that have been transfected with a TGF-β responsive alkaline phosphatase (AP) reporter construct. HES at 10 μg/ml was found, with batch to batch variation, to activate this assay to the same extent as between 0.5 and 5 ng/ml of rhTGF-β1 (Fig. 6.8 A).

Several studies of helminth ES products have revealed contamination with host material (DeMarco et al., 2007; Hewitson et al., 2008). Since *H. polygyrus* inhabits the TGF-β-rich environment of the duodenum, it is possible that the parasite sequesters host TGF-β *in vivo* and subsequently releases it *in vitro*. If this were the case, the contaminant would be a TGF-β, rather than a superfamily member, such as activins, nodals, or BMPs, as the MFB-F11 cell line is specifically responsive to TGF-βs (Tesseur et al., 2006). HES was pre-incubated for 1 hr with the pan-vertebrate monoclonal TGF-β antibody, 1D11, and its subsequent ability to activate the assay measured. 1D11 is known to interact with mammalian TGF-β1, 2, and 3, as well as amphibian TGF-β5. Even at 100 μg/ml of mAb, HES induction of AP was

not inhibited (Fig. 6.8 B, left panel), whereas stimulation by 5 ng/ml of rhTGF-β1 was abolished with as little as 1 μg/ml of 1D11 (Fig. 6.8 B, right panel).

The ability of HES to induce Foxp3 was similarly not inhibited by 1D11 in mitogenically stimulated splenocyte cultures, whilst the effect of rhTGF-β1 was abrogated. As 1D11 is continuously present in these cultures this additionally shows that HES does not function by stimulating an accessory cell population to release TGF-β (Fig. 6.8 C).

6.2.9 HES TGF-β-like activity is inhibited by *H. polygyrus* infection sera.

If the parasite molecule responsible for the TGF-β-like activity is dissimilar to mammalian TGF-β then it may stimulate an antibody response by the host. The ability of sera from day 28 *H. polygyrus* infected mice was tested for its potential to neutralise HES stimulation of the MFB-F11 assay. Over two-fold inhibition of TGF-β-like activity was observed with sera from several parasitised mice (Fig. 6.9 A). Naïve sera, used at the same concentration in this assay, was found to have no such effect (Fig. 6.9 A). Moreover, sera from infected mice did not alter the ability of rhTGF-β1 to stimulate the MFB-F11 cells, confirming that the host and parasite ligand are immunologically seperable entities (Fig. 6.9 B).

6.2.10 Other helminth-derived products have TGF-β-like activity.

Although there was no evidence of TGF-β-like activity in the ES from the acute rodent nematode *N. brasiliensis* (Fig. 6.3 B, right panel), it was possible that ES from chronic helminth infections may generally harbour this potential. ES was tested from the L_4 stage of two prominent ovine chronic gastrointestinal nematodes, *Haemonchus contortus* and *Teladorsagia circumcincta*. Using the MFB-F11 assay no detectable activity was present in *H. contortus* L_4 ES, but *T. circumcincta* L_4 ES was able to induce AP release (Fig. 6.10 A). Like HES, *T. circumcincta* L_4 ES activity was not inhibited by high concentrations of TGF-β antibody. *T. circumcincta*

ES was also able to induce Foxp3 expression in proliferating $CD4^+$ T cells (Fig. 6.10 B). Hence, it seems that *H. polygyrus* is not unique in its ability to release an active TGF-β mimic.

6.2.11 Foxp3 induction is dependent on TGF-β activity.

Having demonstrated that HES is able to induce Foxp3 in $CD4^+$ T cells, and that HES has TGF-β-like activity, experiments were then undertaken to ascertain whether these two effects are causally linked. A Foxp3 induction assay was performed, as previously, with MACS-sorted $CD4^+$ T cells from mice expressing a dominant negative (dn) form of TGF-β receptor (R) II. HES, like rhTGF-β1, was dependent on this receptor to induce Foxp3, as no increase in its expression was observed in these cultures (Fig. 6.11 A). This also confirmed, as was previously suggested (Section 6.2.7), that HES directly acts on $CD4^+$ T cells, as the dn construct is restricted to this cell type and is normal in other cells populations, such as APCs.

TGF-β signalling is dependent on heterodimerisation of TGF-βRII with ALK-5/TGF-βRI. If HES does contain a TGF-β mimic then Foxp3 induction would be dependent on both of these receptors. CD4 sorted T cells were, therefore, treated with an inhibitor of ALK-5 (SB 431542), or DMSO as a control, for 30 min prior to addition of HES or rhTGF-β1. After 72 hrs there was no evidence of Foxp3 induction in either of the cultures containing the inhibitor (Fig. 6.11 B). This data formally shows that the Foxp3 inducing activity of HES is dependent on it mediating a signal through the TGF-β receptor.

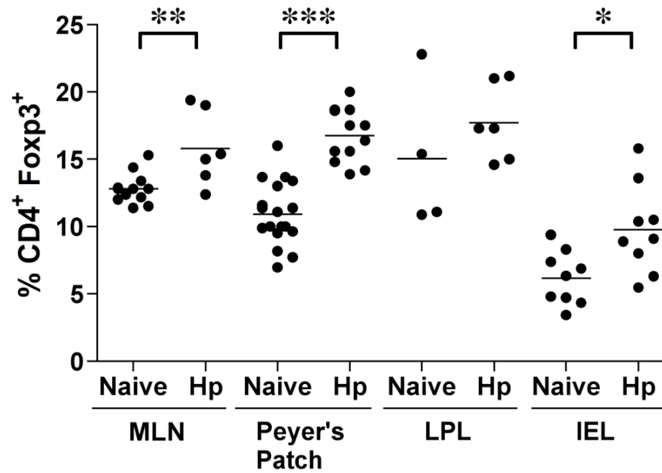


Fig. 6.1 – Percentage increases in $CD4^+$ $Foxp3^+$ cells occur in lymphocyte populations local to *H. polygyrus*.

Intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), Peyer's patches, and MLNs were isolated from naïve or day 7 *H. polygyrus* infected C57BL/6 mice. Foxp3 expression within the $CD4^+$ T cell population was assessed by flow cytometry. Graph represents pooled data from 3 identical experiments. Results of Student's t test: *, P<0.05, **, P<0.01, ***, P<0.001.

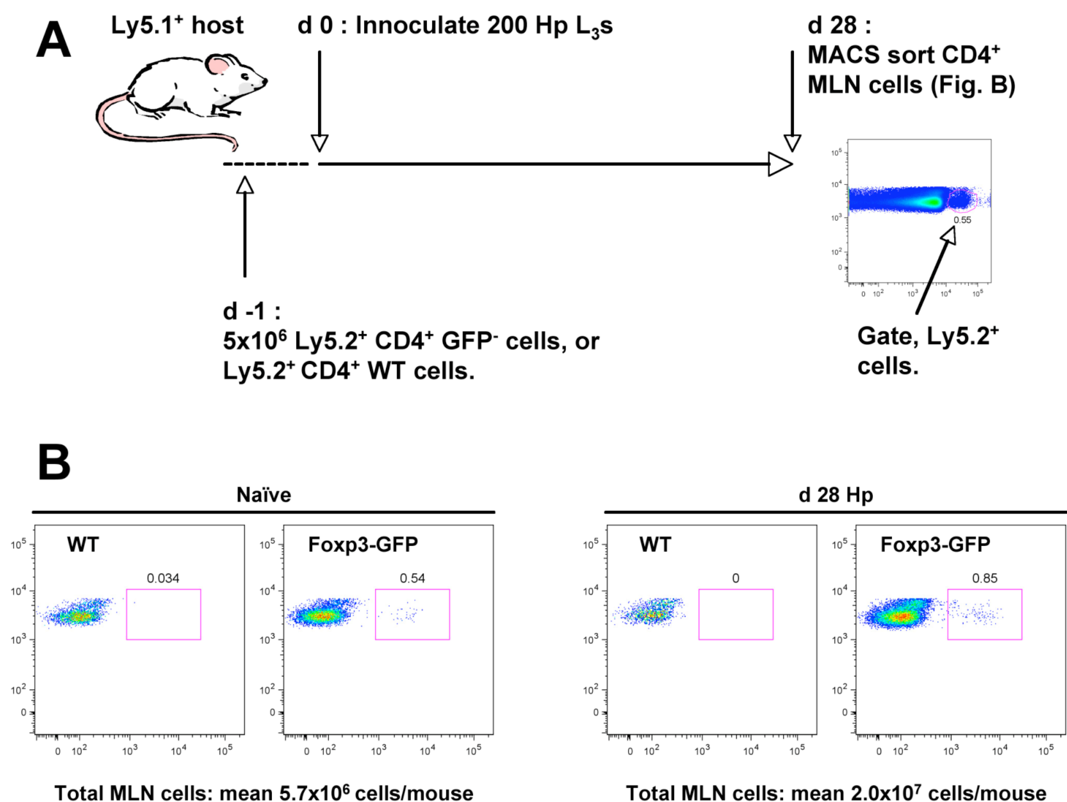
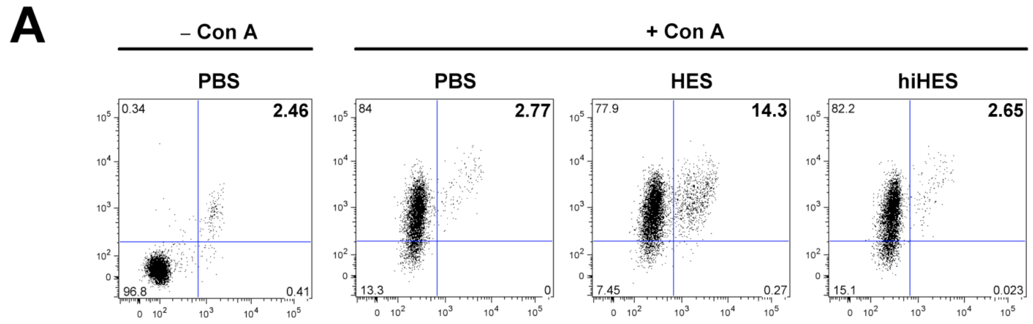


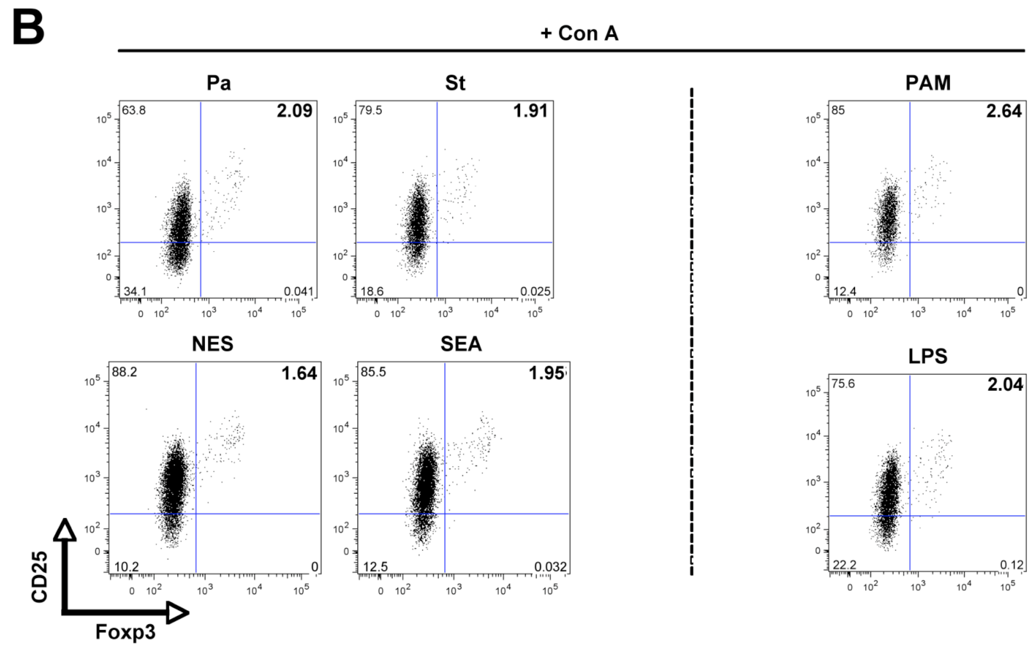
Figure 6.2 – Converted CD4$^+$ Foxp3$^+$ T cells are present in the MLNs at day 28 of *H. polygyrus* infection.

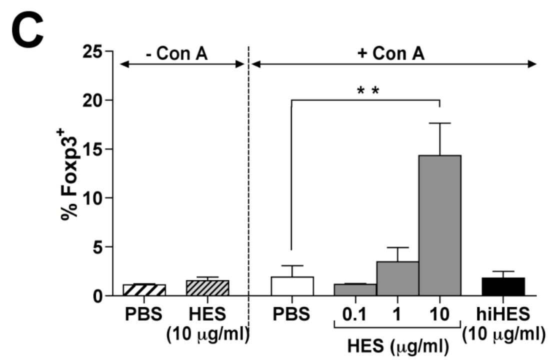
(A) Ly5.2$^+$ CD4$^+$ Foxp3$^-$ T cells were sorted from splenocytes of mice expressing a knocked-in Foxp3-GFP fusion protein. 5 x 10^6 of these cells, or the same number of WT MACS sorted CD4$^+$ cells, as a non-fluorescent control, were adoptively transferred by tail vein injection into congenic Ly5.1$^+$ hosts. 24 hrs later mice were inoculated with 200 *H. polygyrus* L_3 larvae, or left as an untreated group. After 28 days, MLNs were removed from all mice, cells from groups of mice pooled together, MACS sorted to enrich for CD4$^+$ cells, and Foxp3-GFP expression assessed by flow-cytometry. To aid in detection of Foxp3-GFP$^+$ cells, all analysis was performed on the gated Ly5.2$^+$ population. **(B)** Plots of WT non-fluorescent control, and Foxp3-GFP cells within the Ly5.2$^+$ CD4$^+$ T cell population of pooled MLN cells from naïve or day 28 *H. polygyrus* infected mice. Data are from one experiment in which MLNs from three mice were pooled per MACS sorted sample.

Figure 6.3 – HES increases the percentage of CD4$^+$ Foxp3$^+$ T cells in mitogen stimulated splenocyte cultures.

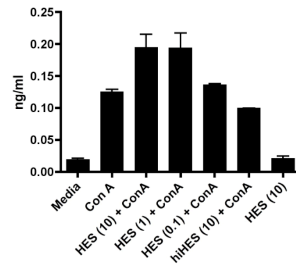
Sample plots of CD25 versus Foxp3 expression, gated on CD4$^+$ T cells, from C57BL/6 splenocyte cultures. Splenocytes were treated with PBS alone, Con A (2μg/ml), or combinations of Con A with pathogen products. Con A was added to cultures 30 min after pathogen products, and flow-cytometry performed 48 hrs later. **(A)** PBS alone, Con A plus: PBS, HES (10 μg/ml), heat-inactivated HES (hiHES) (10 μg/ml). Heat inactivation was carried out for 30 min at 100ºC. **(B)** Con A plus: *P. acnes* extract (Pa) (10 μg/ml), *S. typhimurium* extract (Sa) (10 μg/ml), the TLR-2-ligand Pam-3-CSK4 (1 μg/ml), the TLR-4-ligand LPS (1 μg/ml), *S. mansoni* soluble egg antigen (SEA) (10 μg/ml), or *N. brasiliensis* ES (NES) (10 μg/ml). **(C)** Percentage of Foxp3$^+$ cells within the CD4$^+$ T cell population of splenocytes exposed to indicated stimuli. Data represent the mean ± SD of three replicate cultures from individual mice. Results of Student's t test **, $P < 0.005$. All data in this figure are representative of at least three similar experiments performed using different batches of HES.


A
– Con A
+ Con A
PBS
PBS
HES
hiHES
0.34
2.46
96.8
0.41
84
2.77
13.3
0
77.9
14.3
7.45
0.27
82.2
2.65
15.1
0.023

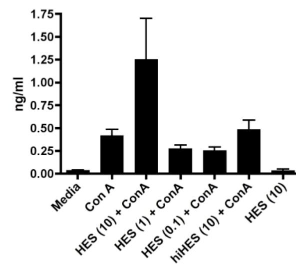

B
+ Con A
Pa
St
PAM
63.8
2.09
34.1
0.041
79.5
1.91
18.6
0.025
85
2.64
12.4
0
NES
SEA
LPS
88.2
1.64
10.2
0
85.5
1.95
12.5
0.032
75.6
2.04
22.2
0.12
CD25
Foxp3


C
- Con A
+ Con A
% Foxp3+
* *
PBS
HES
(10 µg/ml)
PBS
0.1
1
10
HES (µg/ml)
hiHES
(10 µg/ml)

IL-4



IL-10



IFN-γ

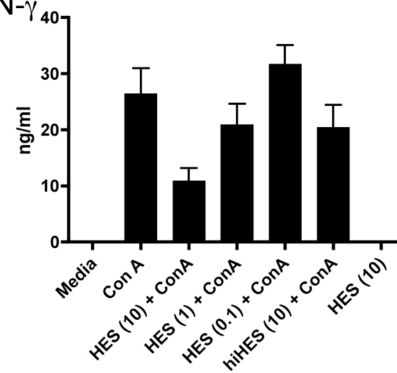


Figure 6.4 – Inhibition of IFN-γ, but heightened IL-4, secretion is observed in HES treated mitogen stimulated splenocyte cultures.

IL-4, IL-10, and IFN-γ (C), present in supernatants, taken from cultures described in Fig. 6.4 C, were measured by ELISA. Again, data represent the mean ± SD of three replicate cultures from individual mice. These data are representative of at least three similar experiments performed using different batches of HES.

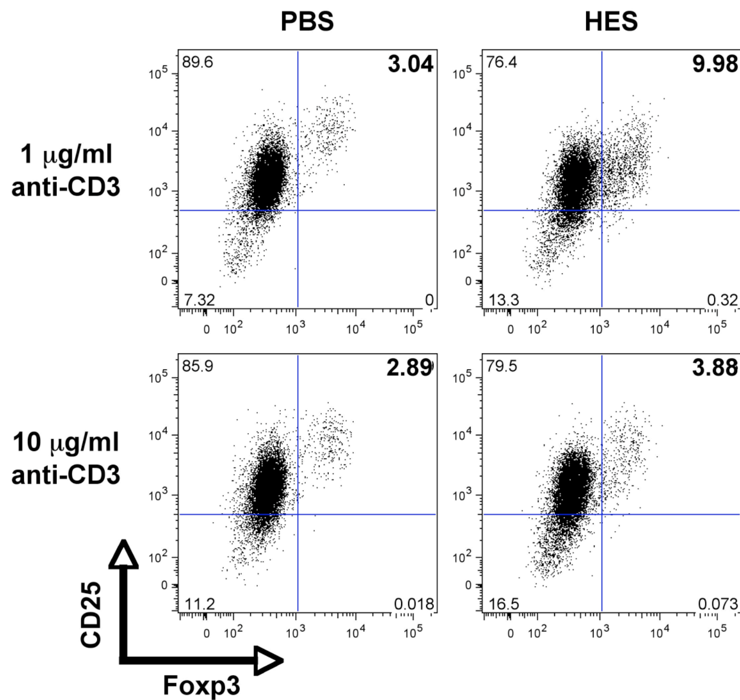


Figure 6.5 – HES mediated increases in percentage of CD4$^+$ Foxp3$^+$ T cells are inhibited by strong stimulation of the TCR.

Sample plots of CD25 versus Foxp3 expression, gated on CD4+ T cells, from naïve C57BL/6 mice splenocyte cultures exposed to: **Left panels**, PBS, or **right panels**, HES (10 μg/ml), in combination with a low (1 μg/ml), or high (10 μg/ml) concentration of anti-CD3. Data are representative of two similar experiments using different batches of HES.

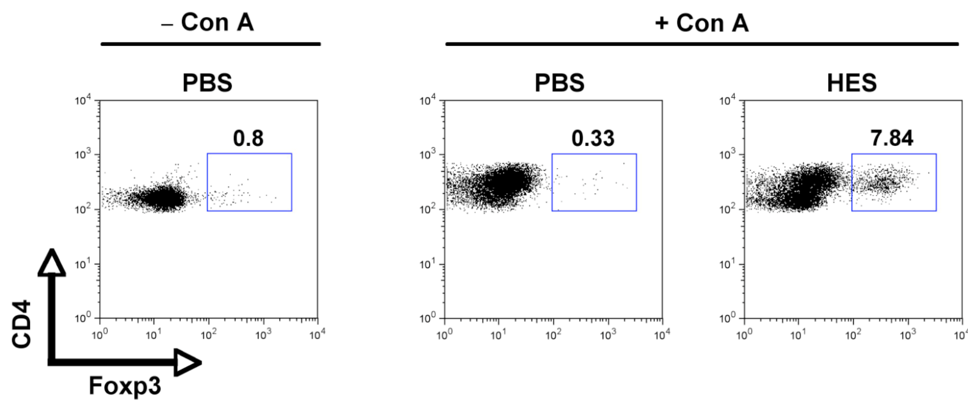
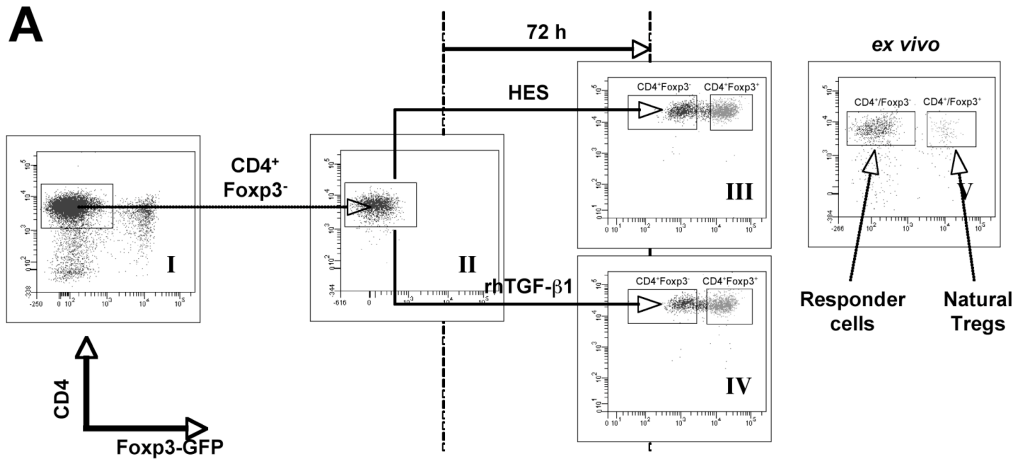


Figure 6.6 – Increases of CD4+ Foxp3+ T cells in response to HES occur by *de novo* induction of Foxp3.

Foxp3 expression in CD4+ T cells, after 48 hrs culture of flow-cytometry sorted GFP- splenocytes (from mice expressing a knocked-in Foxp3-GFP fusion protein) incubated with PBS alone, Con A (2 µg/ml), or HES (10 µg/ml) plus Con A (2 µg/ml).

Figure 6.7 – HES generated CD4$^+$ Foxp3$^+$ T cells have regulatory activity.

(A) Flow-cytometric analysis of, pre- (A I) and post- (A II) sort samples to obtain CD4+ Foxp3- T cells from a Foxp3-GFP mouse. These cells were subsequently expanded for 72 hrs, in an APC-free system, with plate-bound anti-CD3/anti-CD28, in the presence of HES (10 μg/ml) (A III), or rhTGF-β1 (2 ng/ml) (A IV). Cells from these cultures were sorted into CD4+ Foxp3+ and CD4+ Foxp3- populations (gates shown A III, IV), and their suppressive capacity assessed (see B). The responder population, used for this assessment, of CD4+ Foxp3- T cells, along with ex vivo CD4+ Foxp3+ T cells, was sorted from naïve Foxp3-GFP spleen and pLN cells (A V). (B) The proliferation of CD4+ Foxp3-, CMTMR-loaded, index responder T cells, stimulated with soluble anti-CD3 (1 μg/ml) in the presence of irradiated APCs for 4 days, and co-cultured with the different populations of 'suppressor' cells, was measured by flow-cytometry. Left panel, CD4+ Foxp3-, CMTMR-loaded, index responder T cells alone. Right upper panel, CMTMR-loaded responder cells mixed with equal numbers of induced Foxp3+ T cells from HES, or rhTGF-β1 treated cultures, or ex vivo, Foxp3+ T cells from a naïve Foxp3-GFP mouse. Right lower panel, CMTMR-loaded responder cells mixed with CD4+ T cells that did not express Foxp3 from HES and rhTGF-β1 treated cultures. Data are representative of two similar experiments.


A
72 h
HES
rhTGF-β1
CD4+
Foxp3-
CD4+Foxp3-
CD4+Foxp3+
I
II
III
IV
V
ex vivo
CD4+/Foxp3-
CD4+/Foxp3+
Responder cells
Natural Tregs
CD4
Foxp3-GFP

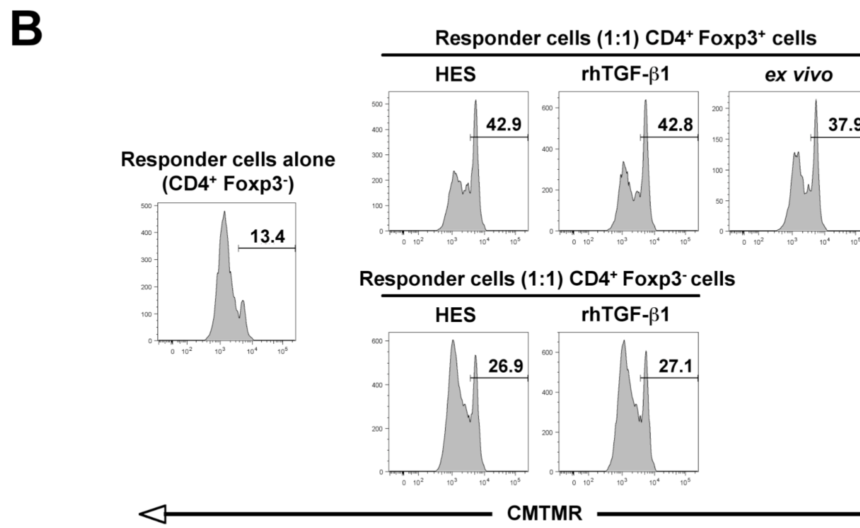
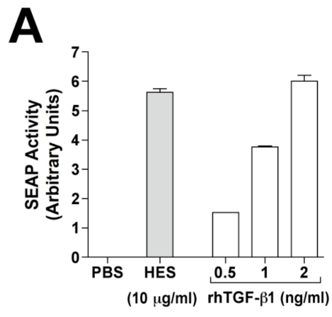

B
Responder cells (1:1) CD4+ Foxp3+ cells
HES
rhTGF-β1
ex vivo
42.9
42.8
37.9
Responder cells alone (CD4+ Foxp3-)
13.4
Responder cells (1:1) CD4+ Foxp3- cells
HES
rhTGF-β1
26.9
27.1
CMTMR

Figure 6.8 – HES contains a TGF-β-like activity, which is not blocked by a pan-vertebrate anti-TGF-β monoclonal antibody.

(A) HES activates the TGF-β responsive reporter cell-line, MFB-F11, inducing expression of alkaline phosphatase. **(B) Left panel**, HES TGF-β-like activity, as compared to a rhTGF-β1 standard curve, is not diminished when pre-incubated with up to 100 μg/ml of the pan-vertebrate TGF-β mAb, 1D11. **Right panel**, in contrast to HES, 5 ng/ml of rhTGF-β1 activity is abrogated by as little as 1 μg/ml of 1D11. **(C)** HES (10 μg/ml) induced Foxp3 expression in soluble anti-CD3 (1 μg/ml) stimulated splenocyte cultures is unchanged when pre-incubated with 1D11 when compared to isotype control. Foxp3 induction by 2 ng/ml rhTGF-β1 is, however, markedly inhibited. Sample plots of CD25 versus Foxp3, gated on $CD4^+$ T cells are shown.

Data shown in figure is representative of two similar experiments. Error bars represent SEM of triplicate wells of MFB-F11 cells stimulated with each treatment.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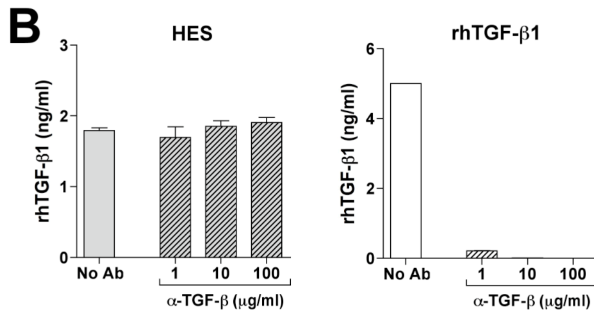
HES
(10 μg/ml)
0.5
1
2
rhTGF-β1 (ng/ml)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HES
rhTGF-β1 (ng/ml)
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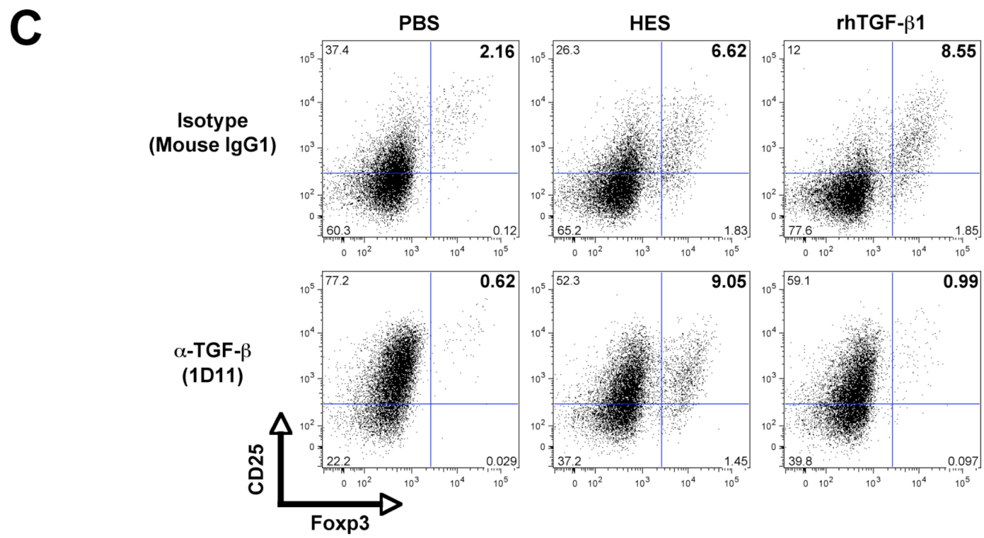
1
10
100
α-TGF-β (μg/ml)
rhTGF-β1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PBS
HES
rhTGF-β1
Isotype (Mouse IgG1)
α-TGF-β (1D11)
37.4
2.16
60.3
0.12
26.3
6.62
65.2
1.83
12
8.55
77.6
1.85
77.2
0.62
22.2
0.029
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0.097
CD25
Foxp3

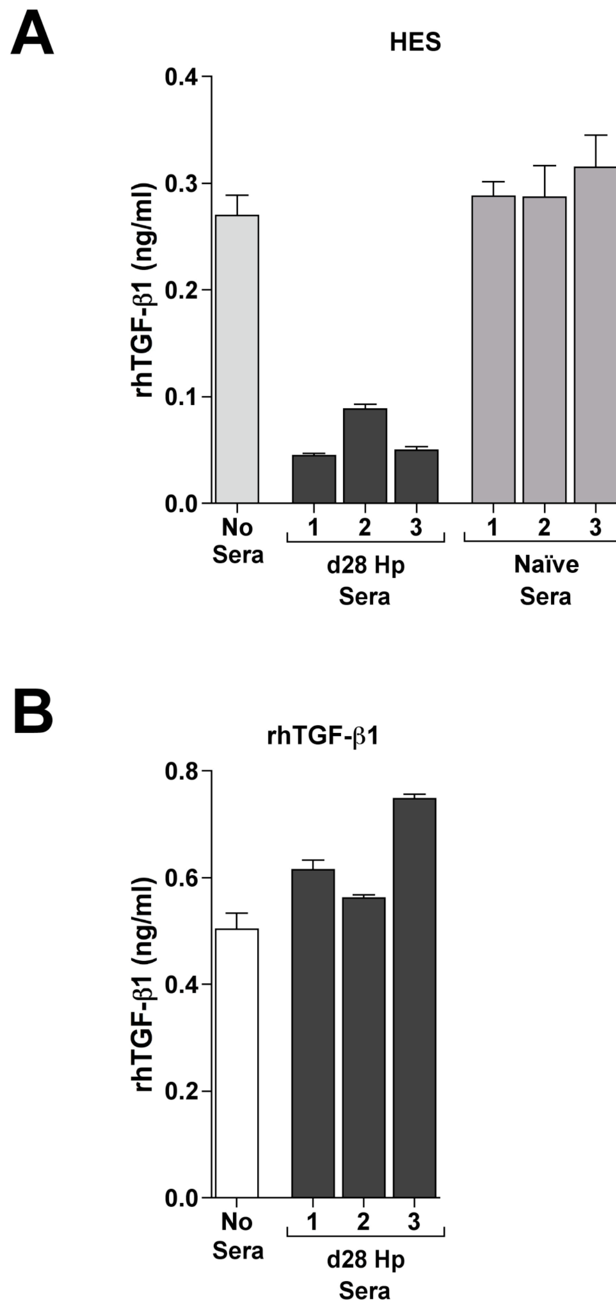

Figure 6.9 – Infection sera neutralises the TGF-β-like activity in HES, but has no effect on the efficacy of rhTGF-β1.

(A) Pre-incubation with sera (1:50) from day 28 *H. polygyrus* infected, but not naïve, mice blocks the ability of HES to induce reporter activity in the MFB-F11 cell line. **(B)** Activity of rhTGF-β1 is unaffected by treatment with the infection sera.

Data shown in figure is representative of two similar experiments. Error bars represent SEM of triplicate wells of MFB-F11 cells stimulated with each treatment.

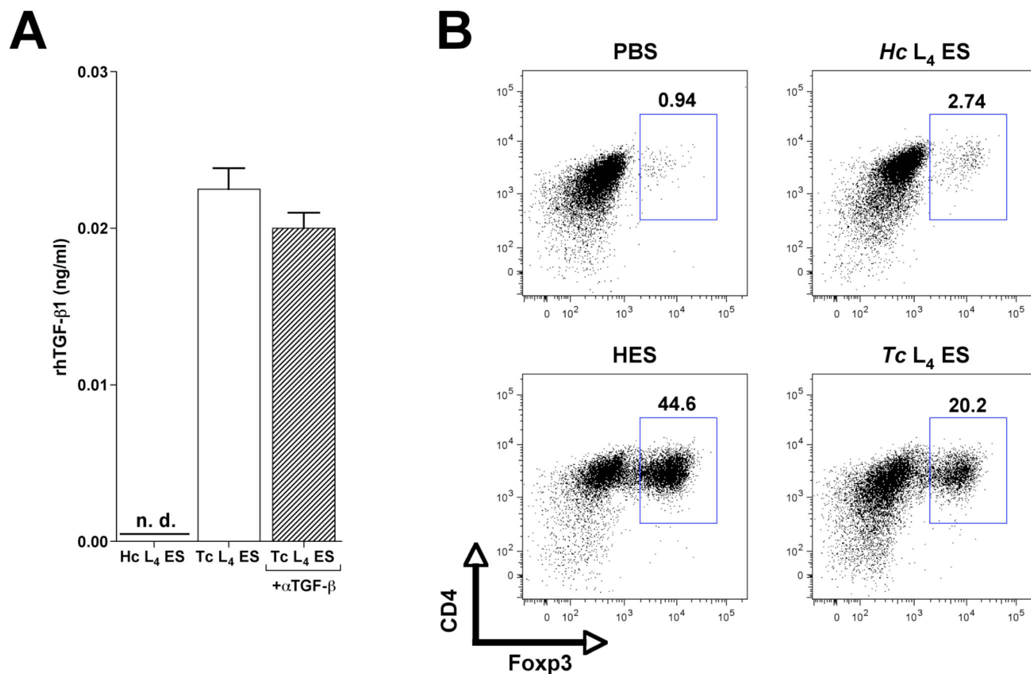

Figure 6.10 – *Teladorsagia circumcincta* L$_4$ ES, has TGF-β-like activity and is able to increase percentage of CD4$^+$ Foxp3$^+$ T cells.

(A) *T. circumcincta* (Tc)L$_4$ ES (30 μg/ml), but not *H. contortus* (Hc)L$_4$ES (30 μg/ml), stimulates alkaline phosphatase release by the MFB-F11 reporter assay. This activity is unaltered by high levels (100 μg/ml) of anti-TGF-β (1D11). Error bars represent SEM of triplicate wells of MFB-F11 cells stimulated with each treatment. **(B)** TcL$_4$ES (30 μg/ml) induces significant Foxp3 expression, after 72 hrs, in MACS purified CD4$^+$ cultures stimulated to expand with plate-bound anti-CD3/anti-CD28 and exogenous IL-2 (10 ng/ml). In contrast, HcL$_4$ES (30 μg/ml) had no effect.

Data in figure are representative of two identical experiments.

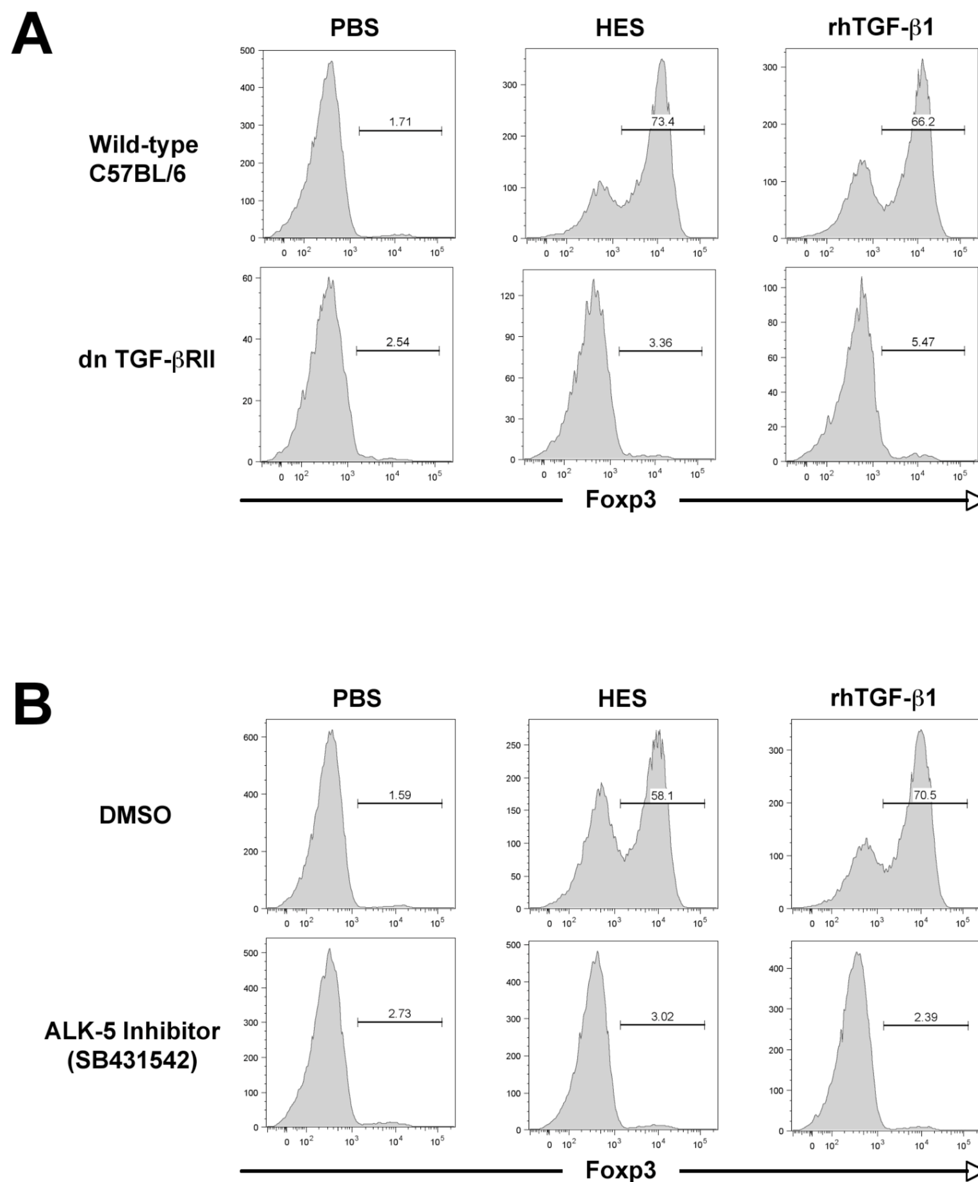

Figure 6.11 – HES induced Foxp3 expression is dependent on intact TGF-β-receptor signalling.

(A) MACS sorted $CD4^+$ cells were purified from WT C57BL/6, or dnTGF-βRII mice, and stimulated with plate-bound anti-CD3/anti-CD28, in the presence of IL-2 (10 ng/ml), with either 10 μg/ml HES, or 5 ng/ml rhTGF-β1. After 72 hrs Foxp3 expression was assessed. **(B)** $CD4^+$ cells were cultured as in A, but were treated with 5 μM of the ALK-5/THG-βRI inhibitor SB 431542, or DMSO as a control, along with HES or rhTGF-β1. Again, 72 hrs later expression of Foxp3 was analysed. Data in figure is representative of three similar experiment.

6.3 Discussion

Increased prevalence of Foxp3 expression within the CD4$^+$ T cell population is a common feature observed over the course of an infection (Section 1.5). If *H. polygyrus* is able to directly affect the Foxp3$^+$ Treg population, *in vivo*, it is probable that this would be most apparent within the tissue infiltrating lymphocytes, and Peyers patches, which are the local lymphoid structures. The proportion of CD4$^+$ Foxp3$^+$ cells was elevated, in all local populations analysed: IELs, LPLs, and Peyer's patches.

Production of ES is one method helminths are thought to use to modulate the host's immune response (Section 1.7). HES was found to have a component(s) that enhanced the Foxp3$^+$ Treg population when T cells were stimulated to proliferate, *in vitro*. As shown by stimulating an initially Foxp3-GFP$^-$ population, HES was able to induce Foxp3 expression in naïve peripheral T cells. This is the first report of a parasite product that is able to act in a direct fashion on the Foxp3$^+$ Treg component of the immune response. Despite HES being a complex mixture of products, perhaps with competing T effector and T regulatory activities (Section 1.7), the Foxp3$^+$ cells were functionally suppressive. Moreover, induction of Foxp3 was still favoured in whole splenocyte cultures, even though HES concurrently increased expression of IL-4, which has been shown to counteract this process (Wei et al., 2007). This implies that, *in vivo*, local to the parasite (where HES concentrations are highest) it would be possible for the immunological balance to be swayed such that Foxp3$^+$ Tregs are generated. As there is a requirement for TCR-ligation and subsequent expansion of T cells to switch on Foxp3 (Section 1.5.2), it seems probable, that these induced Tregs would have come from a naïve pool preferentially recognising HES antigens. Expansion of such HES-specific Tregs could ultimately lead to a preponderance of worm-responsive suppressor cells within the T cell pool, limiting development of Th2-cells, and hence Th2-type protective effector mechanisms. By this mechanism, *H. polygyrus* would eventually come to be recognised more like self than an invading pathogen.

Agreeing with this hypothesis, *de novo* expression of Foxp3 seems to occur during infection, as demonstrated by the conversion of a transferred Foxp3-GFP$^-$ cell population in this setting. Interestingly, a similar percentage of Foxp3-GFP$^+$ T cells

were found in the MLNs of naïve and infected mice, after 28 days, perhaps suggesting there is a homeostatic mechanism maintaining proportions of induced Tregs. There were, however, dramatic increases in the number of cells present in the infected LNs. This means that when responding to the parasite either more conversion to a Foxp3^{+} phenotype occurs, or else there is greater expansion of converted cells. A major caveat to this experiment is that a small number of Foxp3-GFP^{+} cells could have contaminated the original sample. If contaminants were present then over the relatively long time period of this study they may have expanded to become the GFP^{+} population described. Shortening the length of the experiment would be one way to avoid this problem, perhaps looking for evidence of induction at 7 days post-infection. Alternatively, a population of cells that do not contain natural Foxp3^{+} Tregs, such as those from DO11.10 SCID mice (in combination with OVA-feeding), could be transferred in the same setting and conversion assessed.

The activity in HES was defined as TGF-β-like since, using either genetic, or pharmaceutical, techniques to block TGF-β signalling, induction of Foxp3 could be abrogated. Additionally, HES was able to activate the TGF-β-specific-reporter cell-line, MFB-F11. Recently, in our lab, a TGF-β homologue has been identified from *H. polygyrus*, termed Hp-TGH-2 (McSorley et al., in submission). It has not been possible, at this time, to correlate the TGF-β-like activity in HES with the presence of this molecule, but given the high degree of structural similarity between TGF-βs from diverse organisms it would seem a plausible candidate. If a TGF-β homologue is responsible for the effects of HES on T cells, then its identification could prove to be difficult. This is because, it is likely be a minor component of the mixture, given the extremely low levels at which TGF-β is biologically active. A possible method to identify the molecule would to enrich using gel-filtration, perhaps followed by ion-exchange, chromatography, and then to use mass spectrometry to assign an identity to the active component. However, due to the very limited amount of sequence data available for *H. polygyrus*, unless this is a previously characterised protein, for example Hp-TGH, the approach could ultimately prove to be inconclusive.

Even in the absence of a defined parasite-product it was still possible to demonstrate that sera, derived from day 28 infected animals, were able to inhibit the

ability of HES to stimulate the MFB-F11 assay. This effect is specifically against HES, not altering recognition of rhTGF-β1, implicating antibodies as the likely mediators of suppression. To attempt to confirm this, the Ig component of the sera could be purified on a Protein A, or G, column and used in the same kind of blocking experiments. If antibodies do target this protein, *in vivo*, then it would be of interest to establish whether there is a correlation between its neutralisation and parasite expulsion. One obvious way of studying this would be simply to measure serum inhibition of TGF-β-like activity versus worm burden. This could be done for a single strain, but also between differently resistant strains.

There may of course be no role in protection for antibody-mediated inhibition of this molecule in resistant strains of mice, since the parasite is targeted whilst still in the sub-mucosa (Chapter 4), and it is unclear whether the TGF-β-like molecule(s) is released at this time. Although culture techniques would need to be optimised, ES from, exsheathed L_3, and L_4 stages of *H. polygyrus* could likely be obtained and TGF-β-like activity measured. Supporting the prospect that they would have this activity, L_4 ES from the ovine helminth *T. circumcincta*, which also dwell at this stage in the gut-mucosa was able to increase percentages of Foxp3$^+$ T cells. Indeed, it can be envisaged that excretion-secretion of products with this function early during infection, such as the L_3/L_4 stages, may be extremely important at a time when the parasite is potentially directly in contact with immune cells. A further question raised by the presence of TGF-β-like activity in TcL_4ES is: how common is this potentially immunomodulatory strategy amongst helminth species? In this chapter a number of helminth-derived products were found to be negative including, *H. contortus* ES, NES, and SEA. *N. brasiliensis*, unlike *H. polygyrus*, is unable to establish a chronic infection in the mouse, being expelled after 5-7 days. One intriguing possibility, is that the lack of TGF-β-like activity in NES is key to the rapid expulsion of the parasite. Better characterisation of TGF-β-like activity across a wide-variety of helminths is needed to ascertain both the prevalence of this feature, and whether it is related to chronicity of infection.

Until the molecule(s) has been identified it will be difficult to assess whether its primary role *in vivo* is to induce Foxp3$^+$ Tregs. TGF-β has been found to have anti-inflammatory effects on many different cell-types, including DCs, macrophages,

and mast cells, that could also aid worm persistence (in Section 1.6.2). Although poorly understood, feeding by *H. polygyrus* is thought to cause damage to the IEC barrier, an additional role for TGF-β-like activity, therefore, could be aiding in repair of this. Its breakdown could lead to systemic bacterial infiltration, negatively affecting host survival.

Although not parasite-derived, host TGF-β depletion, by mAb, reversed some of the immune suppression observed in *H. polygyrus* infection (Doligalska et al., 2006). Additionally, in work by our own lab, *in vivo* treatment of C57BL/6 mice with TGFβRI/ALK-5 inhibitor (SB 431542) at day 28 of *H. polygyrus* infection promoted worm expulsion (K. Smith, unpublished observation). This issue could be addressed further, specifically in respect to the effects of TGF-β on T cells, by comparing the response to infection of CD4dnTGFβRII mice. Other genetically-deficient mouse strains, that may be useful to shed light on any parasite-derived TGF-β-activity during the course of disease, are those which have deficiencies in TGF-β processing genes, such as furin (Pesu et al., 2008), or the integrin $\alpha_v\beta_8$ (Travis et al., 2007). If a pre-processed TGF-β-like molecule is released by *H. polygyrus* a predicted outcome of the infection would be a lessening of the severity of the colitic phenotype of these animals. It is worth note, at this point, that the benefits of TGF-β-like expression for *H. polygyrus* could be highly dependent on its appropriate temporal and spatial deployment. Recent work has revealed that in situations where IL-4 and TGF-β are co-expressed IL-9 producing T cells, Th9-cells, differentiate (Dardalhon et al., 2008; Veldhoen et al., 2008b). Generation of Th9-cells would plausibly be detrimental to the parasite, as IL-9 production has been linked to worm expulsion in other models (Section 1.3.6).

When considered in a broader context, an ES product with TGF-β-like activity could potentially be both a vaccine candidate, and/or a novel immunosuppressant. Again, identification of the molecule(s) is essential to assess whether either of these are the case, but preliminary studies could be undertaken using whole HES. No work has as yet been undertaken to ascertain whether HES is able to modulate autoimmune or allergic diseases. *H. polygyrus* is known to suppress a mouse model of allergy (Wilson et al., 2005), via expansion of the $CD4^+$ $CD25^+$ T cell compartment. This system would be an ideal candidate for any initial

characterisation of the abilities of HES to bias towards the regulatory arm of the immune response in the face of an ongoing inflammatory setting.

7 Final discussion

In order to establish a long-term infection, parasitic helminths must limit the host's immune response to favour their own persistence whilst preventing the development of unnecessary immunopathology. If Th1/Th17-type responses are generated towards helminths then pathology often ensues (Rutitzky and Stadecker, 2006), whilst Th2-type responses are more frequently associated with the relatively minor symptoms typical of these parasites. Driving a Th2-type response, however, although favouring host survival might lead to expulsion of the helminth. This means that the parasite must walk a dangerous line, allowing a Th2-type response to be generated against it but at the same time suppressing this response to such an extent that it will not be expelled. In the case of *H. polygyrus* infection, the precise mechanisms of expulsion are poorly understood, although generation of Th2 cells is clearly integral to the process (Urban et al., 1991a; Urban et al., 1991b). An important dimension which has yet to be fully explored is whether *H. polygyrus* and other helminths can selectively interfere with the Th2 response to neutralise only those components to which they are most vulnerable.

The work in this thesis has attempted to better describe how susceptibility to *H. polygyrus* is defined in a primary response to the parasite, both in terms of type and voracity of response, and in relation to how the ES products released by the parasite contribute to this process.

DCs are thought to be the main cell-type involved in initially presenting antigen to, and driving expansion of, the T cell pool during the initiation of an immune response to a helminth infection. Chapter 3 of this thesis examined this critical early phase of the immune response by characterising the effects of HES on BMDCs and their ability to drive Th2-type responses when transferred *in vivo*. Chapter 4 then moved on to consider how primary resistance to *H. polygyrus* was mediated in different strains of mice to elucidate general mechanisms of protection. Formation of granuloma-like structures in the duodenal wall, which have been reported to occur in secondary infection, seem to be related to early rejection of the parasite. Furthermore, the cytokine IL-23, plays a key role in counteracting this process in susceptible C57BL/6 mice.

A level at which polarisation and strength of the immune response is critically regulated, but often not considered, is the epigenetic level. Chapter 5 characterised the effects of genetic deficiency in a protein that binds to, and ultimately leads to repression of transcription in, regions of CpG methylation. Interestingly, despite having been reported as primarily affecting Th2 polarisation, it was found that MBD2 also regulated Foxp3 induction, altering the balance between Teff and Treg cells.

Finally, in Chapter 6 components of HES were identified which, rather than generating a Th2 response, are potentially important in downregulating the immune response against the parasite. It was found that *H. polygyrus* contains a TGF-β-like activity that is able to induce Foxp3^{+} Tregs, thus providing a means to directly enhance the regulatory arm of the immune response. In this discussion the implications of the results from each chapter will be considered in more detail, along with potential links between them (Fig. 7.1).

7.1 Recognition of HES and induction of Th2-type responses.

DCs are thought to be the primary cell-type involved in initiating immune responses to helminth infections. In the gut these cells are located in the gut mucosa and Peyer's patches. When infected with *H. polygyrus*, due to the damage caused to the epithelial layer, gut DCs will be concurrently exposed to parasite, and commensal bacterial, products. Inflammatory responses driven by the bacterial products could potentially lead to Th1/Th17-mediated immune pathology. Additionally, damage to the gut wall needs to be rapidly repaired to limit the risk of systemic bacterial infection, a process which Th2-type responses aid.

It is, therefore, of benefit to both host and parasite for a Th2-type response to balance Th1/Th17 responses. When BMDCs were co-treated with HES and LPS, as a bacterial stimulus, they were inhibited in their ability to release the pro-inflammatory cytokine IL-12p70, whilst maintaining production of the anti-inflammatory cytokine, IL-10. Previously, this phenotype has been suggested to be an immunosuppressive tactic used by the helminth to limit development of a response towards it (Segura et

al., 2007). DCs pulsed with HES were found to be competent to induce a Th2-type response, characterised by IL-4, and IL-13, production, with little IFN-γ, and no evidence of an IL-17 response. Additionally, HES pulsed DCs were able to drive production of the immunoregulatory cytokine IL-10. The ability of HES to generate a Th2-type response, however, was not related to its pro-inflammatory suppressive properties. DCs incubated with heat-inactivated (hi)HES still drove a comparable Th2-type response to HES-treated DCs. Moreover, the Th2-type response towards hiHES was no different to HES when DCs were co-treated with the Th1/Th17-driving stimulus, Pa. These data suggest that the role of the cytokine inhibition is not to directly induce a Th2-response.

One possibility is that its role is actually to locally suppress cytokine release to favour Th2-type effector responses (Fig. 7.1). This could include alternative activation of macrophages, which, as demonstrated in Chapter 4, may correlate with resistance to *H. polygyrus* in some strains of mice. Of course it is possible that if HES inhibits inflammatory cytokine production from DCs it could also act directly on Mφs and might be another route to favour their alternative activation. Although not tested it is possible that if HES is able to inhibit IL-12p70 production it could also inhibit IL-23 production (Fig. 7.1).

If the role of HES described here is to suppress cytokine production local to the helminth, then this could represent on appropriate response of the host to enhance parasite clearance, rather than a helminth immunosuppressive tactic. Of course, as described in the introduction, Th2-type polarisation could be thought of as an immunomodulatory response in itself. In Chapter 3 of this thesis no attempt was made to elucidate the mechanisms by which HES acts to inhibit pro-inflammatory cytokine production. A possibility is that the TGF-β-like activity described in Chapter 6 is responsible for this suppression (Fig. 7.1). Murine TGF-β has been reported to limit pro-inflammatory cytokine production from DCs (Fainaru et al., 2007). To further address whether this is the case Western-blotting could be used to study pathways typically associated with TGF-β-stimulation, such as SMAD-2/3-phosphorylation. Alternatively, DCs could be treated with the TGF-βRI inhibitor SB431542, prior to HES stimulation to see if IL-12p70 suppression is reversed in *H. polygyrus* infection.

7.2 Protection against primary infection with *H. polygyrus*.

In experimental infections of mice with *T. muris*, much work has focussed on the factors that affect the balance between Th1, and Th2, reponses towards the parasite. No such studies had been undertaken with *H. polygyrus*. The small and large intestine are very different immunological environments, particularly in regard to colonisation with bacteria. The data presented in Chapter 4 suggest that the factors counteracting protective immune responses in *H. polygyrus* infection may be very different to those in *T. muris* infection. In particular IFN-γ deficiency had no effect on primary susceptibility to *H. polygyrus* infection, but its depletion has previously been shown to have dramatic effects on susceptibility to *T. muris* (Else et al., 1994).

It was found that IL-23 deficiency is able to reduce susceptibility to primary *H. polygyrus* infection in C57BL/6 mice. It is not currently clear whether this is due to the actions of IL-23 directly, or whether this is mediated by its ability to stabilise IL-17 expression. This contrasts with the role of the IL-23/IL-17 axis in the lung where it promotes Th2-type responses in a mouse model of allergic airway hypersensitivity (Schnyder-Candrian et al., 2006; Wakashin et al., 2008). Whether IL-23 is specifically important to protection against helminth infection in the small intestine is not yet known as no studies have addressed the role of this cytokine in large intestinal dwelling helminths.

Additionally, it seems that resistance to *H. polygyrus*, even in primary infection, may be largely mediated against the tissue-dwelling larval stage of the parasite. Although, it was not clear whether this was associated with alternative activation of macrophages (as is the case for secondary infection), it did appear to be associated with granuloma formation. The importance of IL-23 in *H. polygyrus* infection could be due to the need for rapid effector cell activity at this early stage.

Once the worms emerge into the gut lumen, other mechanisms may become more important in clearance, but it is possible that damage sustained during the tissue phase shortens adult lifespan, and may account for reduced fecundity in the more resistant strains. Future, work should focus on better identifying these mechanisms

and in particular if the same balances are important. For example IL-23, IL-17, IFN-γ, depletion at later time points during the course of infection. Indeed, if bacterial status rather than just initial bacterial status of the host is important then perhaps antibiotic treatment may provide a novel therapeutic approach to favour the Th2 response, thus enhancing parasite expulsion.

7.3 Epigenetic regulation of Teff and Treg balance.

The importance of methyl-CpG binding domain proteins (MBDs) in maintenance of gene silencing during Teff polarisation has been described (Hutchins et al., 2002; Hutchins et al., 2005). Since MeCP2 has previously been found to bind to an intronic region of the Foxp3 gene, in Chapter 5, the role of MBDs in control of Foxp3 expression was assessed. It was found that genetic deficiency in MBD2, rather than MeCP2, enhanced Foxp3 induction, *in vitro*, in naïve T cells. When the $CD4^+$ T cell compartment was phenotyped, *ex vivo*, from these mice it was found that the percentage of $Foxp3^+$ cells was increased. $MBD2^{-/-}$ animals did, however, have reduced numbers of $CD4^+$ T cells, perhaps due to the increased ratio of Tregs to Teffs. When infected with *H. polygyrus* $MBD2^{-/-}$ animals had reduced MLN expansion, and hence a reduced number of $CD4^+$ IL-4 producing T cells. There was also a trend towards reduced expulsion of the parasite, although this did not reach statistical significance.

Specific polymorphisms in MBD proteins have been found to modify the risk of developing breast (Zhu et al., 2005), and lung cancer (Liu et al., 2008). The data from Chapter 5, gives further support to the possibility polymorphisms in these proteins might also alter susceptibility to certain types of infection. Future human studies should perhaps consider the role of these genes in affecting response to pathogens.

7.4 Immune suppression by helminth derived products.

Enhancement of the CD4$^+$ Foxp3$^+$ Treg compartment has been considered a likely mechanism by which parasites might manipulate the immune system to favour their persistence (Belkaid, 2007). Whether this is the case, has been of particular interest in helminth infections, since CD4$^+$ Foxp3$^+$ Tregs in these settings have been associated with the ability of these pathogens to abrogate allergic disease (Wilson et al., 2005). If the CD4$^+$ Foxp3$^+$ Treg increases are due to manipulation by helminth products, then it may be possible to derive novel therapeutics from these organisms. However, if the increases represent the host trying to return to a new homeostatic balance in the face of an infection that it cannot clear, then infection itself may be the only effective therapy. Infection with parasitic helminths as a treatment for allergic, or autoimmune, diseases, is far from ideal. There is a risk that the helminths themselves may cause pathology in some patients, whilst depending on the species used there is the possibility of transfer to other individuals. The work in Chapter 6 of this thesis demonstrates that, at least in some helminth infections, ES can contain activities that act to directly induce CD4$^+$ Foxp3$^+$ Tregs (Fig. 7.1). The identification of such an activity implies that at least some of the Treg enhancing capacity of these parasites could be recapitulated without the need for helminth infection.

Ultimately, in order to develop effective therapeutics from helminth products, it may be necessary to combine several different molecules identified from them. This concept is exemplified by the TGF-β-like activity characterised in Chapter 6 that could drive naïve T cells to take on a pathogenic Th17, rather than CD4$^+$ Foxp3$^+$ Treg, phenotype, in the presence of IL-6 (Bettelli et al., 2006). To prevent this unwanted result from occurring this TGF-β-like activity might need to be administered in the presence of IL-25 driving factors. IL-25 has been demonstrated to suppress Th17 generation (Zaph et al., 2008). Activities of this kind are likely present in ES, as IL-25 upregulation is associated with Th2-driving infections (Owyang et al., 2006).

Future work will be required to conclusively identify not only the TGF-β-like activity, but also additional helminth-derived products which (as the example of Treg induction illustrates) may be able to mimic the features of live helminth infections;

through this route, it is hoped that new and effective treatments of human and animal immunopathologies may be developed.

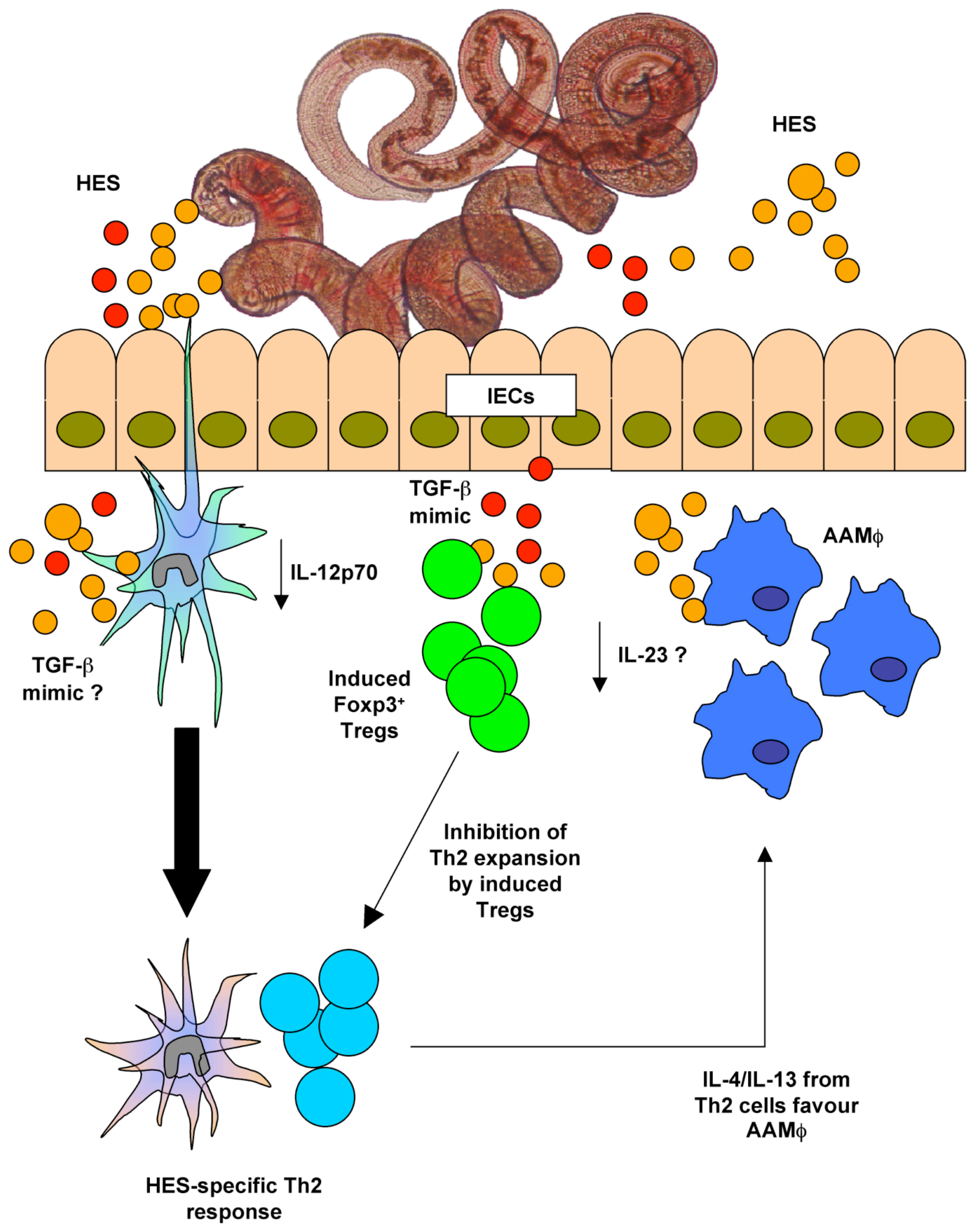

Figure 7.1 – Immune modulation by HES.

Proposed mechanisms by which HES is able to target different immune cell populations.

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