Investigating the immunomodulatory properties of hookworm excretory/secretory (ES) products

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"If we knew what it was we were doing, it would not be called research, would it?" – Albert Einstein

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Statement of Contributions

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Abstract

Chronic inflammatory conditions, such as inflammatory bowel diseases, are becoming increasingly common around the world. Concurrently there is a decrease in childhood exposure to microbes and parasites due to increased sanitation in industrialised nations and urban settings. The hygiene hypothesis, suggests that there may be a link between reduced exposures to pathogens during early childhood and chronic inflammatory conditions later in life. The 'old friends' hypothesis further suggests that parasites and microbes that were common during early human evolution, have played a role in the formation of the immune system and by removing these stimuli (with increasingly sanitary living conditions) we are perhaps removing an essential part of the immune education pathway. Parasites themselves have had to evolve certain mechanisms which allow them to evade immune surveillance from their hosts. Based on these concepts I hypothesized that the excretory/secretory products of the dog hookworm *Ancylostoma caninum* (*Ac*ES) could be utilized for modulating immune responses in mice that were concurrently exposed to chemicals which induce an inflammatory colitis.

*Ac*ES when administered to naïve mice, was able to skew the immune response towards a type 2 phenotype. Mice treated with *Ac*ES exhibited high levels of IL-4, IL-5 and IL-10 as well as eosinophilia, demonstrating that a live hookworm infection was not necessary to induce a type 2 immune response common to many helminth infections. Furthermore, under the pro-inflammatory setting of chemically induced mouse models of colitis, *Ac*ES administration significantly reduced weight loss, tissue damage and pro-inflammatory cytokines IL-17A and IFNγ. The work present herein highlights the potential involvement of myeloid derived suppressor cells (MDSCs) in mediating *Ac*ES induced amelioration of colitis. It further demonstrates that in the DSS (dextran sulphate sodium) model of colitis, the mechanism of action of *Ac*ES is not dependent on IL-10 nor the presence of T and B cells.

A single recombinantly expressed protein that is naturally found in *Ac*ES is also shown to be extremely potent at reducing inflammation in the TNBS model of colitis. These results suggests that the hookworm is actively secreting compounds that can alter the host's immune response,

providing a multitude of naturally occurring immunomodulators that could be harnessed for use as therapeutics in a range of different inflammatory conditions.

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List of Abbreviations

AAMacs = alternatively activated macrophages

***Ac*ES** = *Ancylostoma caninum* excretory/secretory products

***Ac*-AIP-1** = *Ancylostoma caninum* anti-inflammatory protein 1

AHR = airway hyper-responsiveness

Arg-1 = arginase-1

bES = boiled and trypsin digested *Ac*ES

Breg = regulatory B cell

DALYs = disability adjusted life years

DC = dendritic cell

DSS = dextran sulphate sodium salt

ELISA = enzyme linked immunosorbent assay

ES = excretory/secretory products

***Fh*ES** = *Fasciola hepatica* excretory/secretory products

FIZZ-1 = found in inflammatory zone-1 = RELMβ (resistin like molecule beta)/*retnlb*

GWAS = genome wide association studies

IBD = inflammatory bowel disease

IFNγ = interferon gamma

ILC = innate lymphoid cells

IL-(x) = interleukin

LPS = lipopolysaccharide

KO or $^{-/-}$ = gene knockout

MDSC = myeloid derived suppressor cell

Mo-MDSC = monocytic MDSC

PMN-MDSC = polymorphonuclear (granulocytic) MDSC

MIF = macrophage migration inhibitory factor

MLN = mesenteric lymph nodes

MMP = matrix metalloprotease

NIF = neutrophil inhibitory factor

NK = natural killer

NOS-2/iNOS = inducible nitrous oxide synthase

NTDs = neglected tropical diseases

OVA = ovalbumin

PBMC = peripheral blood mononuclear cells

PBS = phosphate buffered saline

PCR = polymerase chain reaction

SEA = *Schistosoma mansoni* egg antigen

Th = T helper

TIMP = tissue inhibitor of metalloproteases

TGF-β = transforming growth factor beta

TNF = tumour necrosis factor alpha

TNBS = 2,4,6-trinitro benzene sulfonic acid

Treg = regulatory T cell

TSLP = thymic stromal lymphopoietin

UC = ulcerative colitis

WT = wild type

1 Chapter 1 – Introduction

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a blanket term used to refer to different inflammatory diseases of the gastrointestinal tract. The three main types of IBD are Crohn's disease, ulcerative colitis (UC) and coeliac disease, however the latter is often regarded separately to Crohn's or UC due to the stimulating factor being a food derived peptide (1, 2). Crohn's disease presents as an ubiquitous transmural non-caseating granulomatous reaction with deep abscesses and fistulas whereas UC presents with Arthus-like lesions marked by crypt abscesses and confined to the superficial colonic mucosa (3), however the term IBD can relate to any spectrum of disease in between the two (4). Coeliac disease is caused by an unregulated response to the gluten antigen gliadin, resulting in both antigen specific antibodies towards gliadin and auto-antibodies directed towards the enzyme tissue transglutaminase 2, which leads to villous atrophy and other symptoms similar to Crohn's and UC (1). Conversely, the aetiologies of both Crohn's and UC are currently unknown but appear to be due to a chronic dysregulated inflammatory responses rather than by antigen-specific immunity as is the case for coeliac disease. For the purpose of this thesis and to maintain consistency with the literature, unless otherwise stated the term "IBD" refers to Crohn's and UC only.

1.1.1 Epidemiology

IBD is a common condition of industrialised nations, affecting over 1.4 million people in the United States of America and another 2.2 million people in Europe (3). In the past few decades however there has been a gradual increase in the incidence and prevalence of IBD in developing nations (5-7). The first documentation of UC appeared in the mid 1800's (8) when diagnosis was only possible post-mortem. It was not until the introduction of the colonoscopy technique in 1969 (9) that the incidence of IBD could be more accurately defined. The late acknowledgement of IBD combined with a lack of defined diagnostic criteria means that few studies have attempted to determine the incidence and prevalence

of this disease. Of the data that is now available we can conclude that both incidence and prevalence of IBD is on the increase. In developed countries, where the prevalence of the disease is already high, the incidence appears to be reaching a plateau. Alternatively, in countries where the prevalence is still low there is a trend towards a steady increase in the incidence in recent years (5). **Figure 1.1.1** provides a short summary of the published results of incidence and prevalence of both UC and Crohn's between the years 1993 - 2008.

IBD does not appear to show a sex distribution bias (10), with only one study noting a predominance for Crohn's in males in Asia (11). The peak age for IBD onset is between the second and third decade of life (10), however an emerging trend is the decreasing age of diagnosis in industrialised countries, with a rising incidence of Crohn's in children from northern Europe between 2005-2007 (7, 12). There also appears to be a north-south gradient in Europe with one study depicting 40% and 80% higher occurrences of UC and Crohn's respectively in northern European countries compared to southern European countries (13). Interestingly, an east to west gradient has also been reported, with double the occurrence of IBD in the western European countries compared to eastern European countries (14). These observations suggest that there are likely environmental factors at play.

Despite these trends for increasing numbers of affected individuals one must remain conscious of the research and diagnostic bias. The research is biased due to most IBD registry being performed in hospitals, where only very severe cases of IBD transpire. The diagnosis may be biased due to:

- The introduction of endoscopy/colonoscopy as a diagnostic tool likely influenced the increase in the number of new cases in developed countries
- A shortage of endoscopy/colonoscopy apparatus in developing countries limits their diagnostic capabilities
- Limited available healthcare to persons from developing countries suggests that some cases may go unnoticed

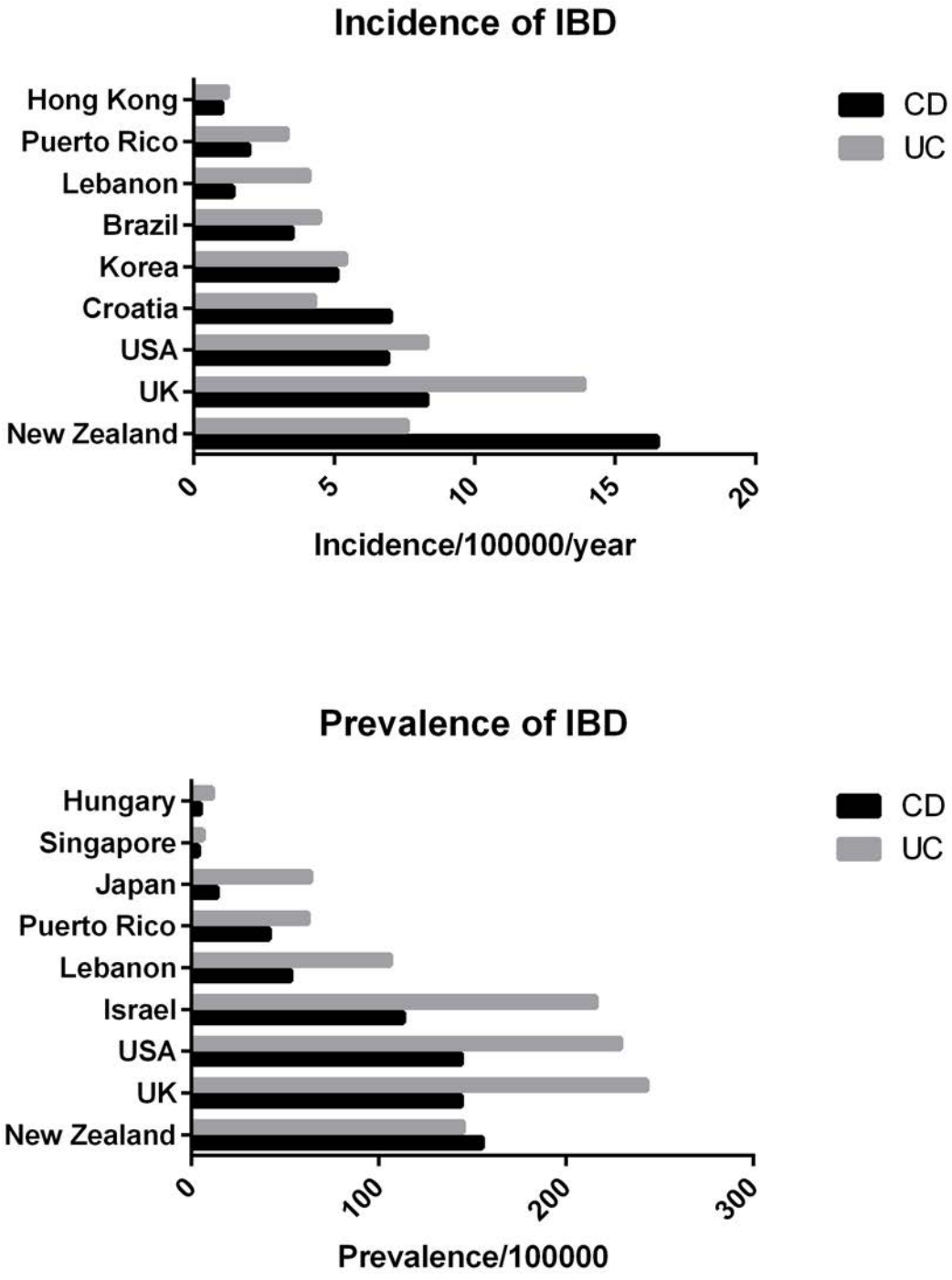


Figure 1.1.1: Incidence and prevalence of IBD around the world.
Compiled results on the incidence and prevalence of Crohn's disease (CD) and ulcerative colitis (UC) from various studies in the years 1993-2008 (15-33).

1.1.2 Aetiology

The exact mechanisms behind the manifestation of IBD are not yet known, however it is generally accepted that IBD occurs as a result of an inappropriate interaction between the patient's immune system and their commensal bacteria (34). Tolerance occurs frequently in the human body and comes in three forms, central, peripheral and acquired tolerance. Central tolerance is imperative for the education of T and B cells to self-antigens and occurs in the primary lymphoid tissues - the thymus and the bone marrow respectively (35). Peripheral tolerance acts as a safety net for any T or B cells that escape the negative selection in the primary lymphoid organs and still have some weak affinity for self-antigens - this occurs in the secondary lymphoid tissues such as the lymph nodes and the spleen (36, 37). Acquired tolerance is the tolerance that is obtained through exposure (education) to external antigens encountered in everyday life. Perhaps the most important aspect of acquired tolerance is oral tolerance, which is tolerance to antigens that come into contact with the mucosal system, namely food, bacterial and air borne antigens that are inhaled (38). Current hypotheses on the aetiology of IBD both relate to oral tolerance. One hypothesis suggests IBD is an immune/structural deficiency in the intestinal epithelial barrier that leads to a breakdown in oral tolerance to commensal bacteria (commonly referred to as leaky gut syndrome) (39). The other school of thought is based on the hygiene hypothesis and suggests that the breakdown in oral tolerance occurs through lack of external stimulus or lack of educational tolerance (40); this will be further discussed in **section 1.2** below.

A large body of current literature suggests that epithelial barrier dysfunction is the initiating factor in IBD (39, 41-43). The mucosal intestinal barrier is made up of epithelial cells that are connected through cellular junctions (41). These junctions include gap junction, desmosomes and tight junctions. Tight junctions are important for maintaining a physical barrier between the intestinal lumen and the cells of the intestine (42). Dysfunction of tight junctions allows bacterial solutes to make their way through the epithelial barrier where they are recognised by antigen presenting cells such as dendritic cells (DCs) and macrophages, resulting in inflammatory cytokine and chemokine expression

that attracts both leukocytes and granulocytes, further propagating the inflammatory response (44). Alterations to epithelial tight junction integrity is thought to be dependent on the cytokine IL-13 (45). Up-regulation of the IL-13 receptor, IL-13Rα2, on natural killer (NK) T cells in response to bacterial glycolipids (45) combined with expression of IL-13 (either by epithelial cells, T helper 2 (Th2) cells or autocrine expression from NK T cells) forms a feedback loop, augmenting IL-13-mediated NK T cell cytotoxicity which could further lead to epithelial apoptosis as well as increased expression of the pore-forming tight junction protein claudin-2 (45).

Epithelial barrier dysfunction is only one of many possible initiating factors in IBD. Other possible candidates include mucus production, regulatory mechanisms, autophagy, changes in microbiota or a combination of these pathways (34, 46-48). Many studies have identified a dysbiosis in IBD patients, however it is not yet know if these changes in the microbiome are a cause or consequence of the condition (49-51). Genome-wide association studies (GWAS) have suggested most associated genes seem to be related only to a specific population (52). Furthermore, it is unlikely, given the rapid increase in prevalence, that genetic drift is the cause of IBD. It is more likely that environmental factors such as diet, pathogenic/symbiotic interactions, pollutants and lifestyle choices influence the occurrence of IBD in populations (52). As previously mentioned, the presence of an East-West as well as a North-South prevalence gradient in Europe suggests either genetic and/or environmental factors are at play. Interestingly, epidemiological studies show that immigrants who have moved from areas of low to high IBD prevalence do not have an increased risk of IBD. However, the children of these immigrants do have an increased risk (53, 54). These studies suggest that environmental exposures early in life are instrumental in determining the susceptibility to IBD.

The inflammatory profile of IBD has been a big focus for both clinical and therapeutic research. Traditionally it was believed that Crohn's patients demonstrated a T helper 1 (Th1) skewing of the cytokine response with high levels of IFNγ, TNF and IL-12, whereas UC demonstrates a non-classical Th2 response with high levels of IL-5 and IL-13 but not IL-4 (55, 56). However, more recently it has become clear that there are many overlapping cytokines

between the two diseases such as IL-6, IL-8, IL-21, IL-23 and many others (57-59). Cytokines are popular targets for treatment of IBD and will be further discussed in **section 0 below**.

1.1.3 Diagnosis

IBD are chronic diseases with patients experiencing relapses for many years (60). The disease usually manifests in patients around 20-30 years of age, however reports of childhood IBD are increasing and this form of the disease is usually more severe than late onset IBD (12). Diagnosis of IBD is not straightforward. Patients generally present to doctors with abdominal discomfort that may continue for years until the disease progresses to more chronic stages, characterised by diarrhoea and presence of mucus and/or blood in the stools (61). At this stage, samples are collected to rule out any possible pathogens and if none are found, an endoscopy is performed to assess the level of inflammation present (62). UC begins from the distal colon (near the anus) and spreads upwards through the colon (57) (**Figure 1.1.2**). As only very few people will seek medical help for intestinal discomfort, patients usually only present once the symptoms become unbearable and has therefore progressed beyond the initial (and less complicated) stage of distal colitis (57). As a result only one-third of UC patients present with distal colitis at the time of diagnosis and two-thirds present with the more serious left-sided colitis or pan-colitis (63). From patients presenting with left-sided colitis, extension of the disease to pan-colitis will occur in 15-30% of these cases (63). Almost 50% of UC patients will have part of their colon removed during the course of the disease. Many of these patients will also develop colorectal cancer due to the chronic inflammation associated with UC (57). Quality of life is also severely impaired for UC patients, who report an average of 8 flare-ups per year (57).

Crohn's disease further complicates diagnosis of IBD due to the patchy nature of the disease. During endoscopic assessment it can be classified as a "discontinuous involvement with anal lesions and cobble stoning" (62). Several biopsies are retrieved during an endoscopic assessment from both the affected and non-affected sites to rule out any malignant abnormalities and for further histological assessment of the disease (44, 64). Crohn's disease patients can also be diagnosed via serological tests which can help to

distinguish between Crohn's and irritable bowel syndrome (IBS), however colonoscopy remains the number one diagnostic tool for both Crohn's and UC (65).

Diagnosis becomes increasingly complicated as many patients (up to 15%) also experience extra-intestinal manifestations, including but not limited to the joints (peripheral arthropathy), skin (erythema nodosum and pyoderma gangrenosum) and eyes (uveitis and episcleritis) (56). IBD sufferers also experience a range of psychological issues, with sufferers reporting significantly more embarrassment and depression than patients with other chronic illnesses (57). No single specific genetic mutation has yet been definitively linked with IBD although various GWAS have identified mutations in the NOD2, IL-23 and the Th17 axis of the immune system with strong correlation to the disease (7, 66). There are currently 163 associated risk loci as determined by GWAS for IBD, with 110 of these overlapping between the two diseases, 30 being specific to Crohn's and 23 being specific to UC (67-69). Without a distinct marker of disease this problem of diagnosis will continue, especially in developing countries where comprehensive healthcare is not available to a majority of the population.

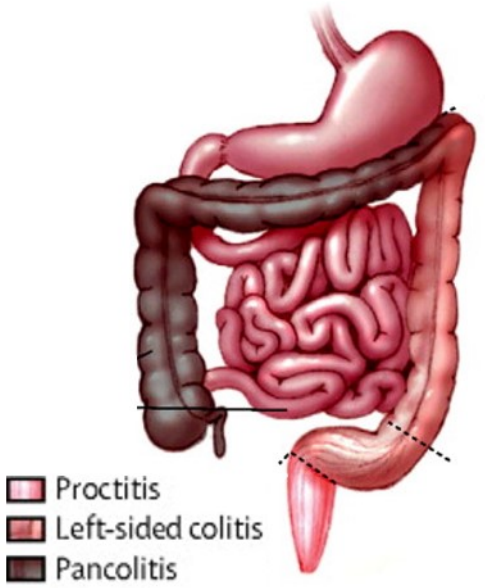


Figure 1.1.2: Classification of ulcerative colitis.
UC begins from the distal colon (near the anus) as proctitis and spreads upwards through the colon becoming left-sided colitis and can further develop into pancolitis, involving the entire length of the colon. Modified from (70).

1.1.4 Treatment

IBD is currently considered an incurable disease (71). Traditional treatment options include aminosalicylates, antibiotics, corticosteroids and other immunosuppressive agents such as methotrexate and azathioprine, however treatment options vary greatly between countries (7). Usually the first course of action will be treatment with aminosalicylates, and when this fails, glucocorticoid treatment and immunosuppressive agents originally manufactured for cancer chemotherapy is the next viable option (72). The final treatment regime is centred around the biologics, typified by the anti-TNF antibodies such as infliximab or adalimumab (73). Sulfasalazine or its metabolites, 5-aminosalicylic acid (5-ASA) and sulphapyridine (SP), were traditionally used as therapies for arthritis (74), however the compound is poorly absorbed into the blood stream and was found to work better locally within the mucosa (75). This drug can be administered either orally or rectally but appears to be better at inducing remission in UC rather than Crohn's patients (76). Unfortunately, even in UC patients the remission rate for 5-ASA alone is only an optimistic 50% (76). The mechanism of action of 5-ASA is not completely understood, however several reports suggest a function through the NFκB pathway (77) and maintenance of the mucosal barrier integrity via promoting cell-cell contacts (78). As with any drug however, 5-ASA has serious side effects, the most pronounced of which is myocarditis (79).

Glucocorticoids, the second line treatment option, are immunosuppressive and therefore characterized by numerous unwanted side effects. The glucocorticoid receptor is present on most cells, making this treatment option non-specific and leaving patients susceptible to infections (80). Even more alarming is the fact that in some countries corticosteroids are still being used as maintenance therapy when remission is achieved (11). Similarly, other commonly used drugs for remission and maintenance such as methotrexate and azathioprine (Immuran) can have detrimental side effects due to the overall immune suppression. Methotrexate is an anti-folate and antimetabolite drug initially manufactured and still currently used for treatment of cancers (81). Patients on methotrexate may experience side effects of chemotherapy such as nausea, dizziness and increased risk of liver damage (82) and the drug is not suitable for pregnant women due to its anti-folate activities

(83). Azathioprine is another immunosuppressive agent commonly used in transplant patients (84) and patients with autoimmune conditions including lupus and rheumatoid arthritis (85, 86). The most alarming side effect of this drug is the increased risk of developing lymphoma especially in people suffering from IBD (87). Both methotrexate and azathioprine are commonly used for steroid dependent IBD patients as the risk factors are significantly less than that of high dosage glucocorticoids alone (72, 82).

There is much hope for more targeted monoclonal antibody therapies that target TNF, such as infliximab and adalimumab. Through blocking TNF, infliximab limits inflammation by inhibiting expression of pro-inflammatory cytokines and mediators, inducing apoptosis of pathogenic T cells, limiting apoptosis of regulatory T cells (Tregs), inducing wound healing via alternatively activated macrophages (AAMacs), decreasing enterocyte apoptosis, limiting expression of adhesion molecules and regulating the balance of matrix metalloproteinases (MMPs) and tissue-inhibitors of metalloproteinases (TIMPs) (summarized in (88)). However, being relatively new on the market (approved by US FDA in 1998 (88)), many countries will only offer this course of treatment as an alternative to surgery (57). Surgery still remains the number one choice of treatment for UC in many countries because it is curative in some cases, however surgeries can lead to a range of complications such as fistulas, sexual or bladder dysfunction, infertility and persistent pain (44). Unfortunately for Crohn's patients surgery is not always an option, leaving them at the mercy of these partially efficacious drugs. There is therefore a huge unmet need for more targeted and safer therapies for IBD sufferers.

1.2 Hygiene and Old Friends Hypotheses

The hygiene hypothesis encompasses a mixture of theories that attempt to explain the sudden rise in autoimmunity and other immune related disorders over the past decades. Although the theories range from use of toothpaste (89) to the installation of sewage systems (90), they all correlate the increase in inflammatory disorders with a reduction in foreign stimuli to the immune system as a result of improved sanitation and

hygienic practices. The emergence of this hypothesis dates back to 1989 when Strachan first described differences in atopy in children from large families compared to smaller families in rural versus urban settings (91). Strachan's work suggested that children from larger families in rural areas have more exposure to foreign antigens. He hypothesised that the smaller family size and increased hygiene seen in urban settings contributed to the increased prevalence of allergic and autoimmune conditions such as hay fever, eczema and asthma in children (91). Although controversial at first, (following the discovery of lymphocyte differentiation into Th1 or Th2 phenotypes), the hygiene hypothesis took new ground in suggesting that a balance between Th1/Th2 responses was necessary for a healthy immune system (92, 93). It is now known that a Th1/Th2 balance is only part of the story, with the discovery of other T helper cell types (Th17 and Th9) and Tregs demonstrating that the interplay between these adaptive immune cells is much more complex than previously believed.

Epidemiological studies have demonstrated a correlation between low microbial/parasitic burden and high levels of autoimmune/allergic conditions in more urbanized settings (94, 95). Although epidemiological studies do not provide mechanistic data, they can help with generating new hypotheses. The correlation between parasitic burden and allergic conditions that was derived from the epidemiological data further bolstered the hygiene hypothesis and gave rise to the 'old friends' hypothesis, which implicates changes in microbiota (96) and loss of parasitic and microbial infections with faulty induction of immunoregulation (40). The 'old friends' hypothesis encompasses the critical concept of 'evolved dependence' (97). One of the first evolutionary concepts that is taught to students in a cellular biology class is that the mitochondria was once a symbiont which became integrated into our genome (98). The same concept can be applied to the evolution of the mammalian immune system, which formed in the era before medicines, chemicals and purified water, when exposure to various microbes and parasites was commonplace. With the recent urbanisation, commencing in the industrial era (from the early 19th century), exposure to these environmental saprophytes, microbes and helminths has dropped dramatically. It is possible that lack of exposure to these 'old friends' has

interrupted an educational pathway necessary for appropriate immune function (97). Indeed, studies have shown that the microbiota can affect various aspects of a person's wellbeing, from basic gastrointestinal homeostasis (99), to body fat composition (100, 101) and mental health (102), demonstrating that these 'old friends' do play an important role in shaping our lives.

The hygiene hypothesis has further grown to encompass changes in food manufacturing (103) and epigenetic modifications (104, 105). Over the years, many research groups have attempted to confirm these hypotheses through various studies involving both humans and animal models of disease. Some of these will be discussed in the following sub-sections, with a major focus on helminth infections and helminth derived-molecules.

1.2.1 Observational studies supporting the old friends hypothesis

Helminth is a term used to describe parasites with worm-like characteristics, including cestodes (flatworms), trematodes (flukes) and nematodes (roundworms). Observational studies of helminth infections in endemic areas enhance the 'old friends' hypothesis. A large study involving 520 Gabonese school children determined that the children with chronic *Schistosoma haematobium* infection had a lower prevalence of skin reactivity to house dust-mite (106). The authors further correlated the skin reactivity to increased presence of serum IL-10 induced by chronic schistosomiasis (106). Similar studies were undertaken in Brazil (107) and Ecuador (108) with consistent results. More recently, similar findings have been reported for infections with *Ascaris lumbricoides* (109, 110), *Trichuris trichuria* (110) and *Brugia malayi* (111). *A. lumbricoides* infections have also been associated with reduced risk of atopic dermatitis (112) and hookworm infections may afford protection against asthma (113, 114).

These immuno-epidemiological observations are reinforced by interventional studies which demonstrated that anthelmintic treatment increased the risk of atopic reactivity (115, 116), suggesting that a live parasitic infection was needed for associated immunomodulatory activity. Moreover, in a randomised, double-blinded, placebo

controlled trial involving 2,507 expectant mothers in Uganda, anthelmintic treatment led to an increased risk of atopic dermatitis or wheeze development in the infant (117). This result further suggests that the benefits of helminth infection may also be passed on to the infant *in utero* or possibly through breast milk.

Helminth infections have also been shown to protect against progression of multiple sclerosis (MS), an autoimmune inflammatory disease that causes destruction of myelin sheaths surrounding the nerve cells of the central nervous system (118). In this study, 12 uninfected relapsing/remitting MS patients in remission, matched for age, sex, and disease duration, served as control subjects for 12 MS patients who showed signs of current helminth infections (118). Patients were followed for a total of 4.6 years. Remarkably, the helminth infected group had a significantly lower numbers of relapses and new lesions assessed by MRI compared to the control groups. The authors correlated this finding to increased levels of IL-10 and TGF-β in the helminth infected group (118). Four of the twelve patients were later cleared of the infection by anthelmintic therapy, which led to a drop in the levels of IL-10 and TGF-β and exacerbation of MS symptoms (119). Recently, a small TSO trial was undertaken with MS patients, the trial was described as safe but non-effective (120).

1.2.2 Clinical trials of helminth therapy in humans

Clinical trials assessing the anti-inflammatory efficacy of experimental helminth infections in humans have to overcome many challenges. For example, experimental infections are generally limited in terms of the numbers of parasites administered for safety reasons. Furthermore, initial safety studies are considerably hampered by sample size due to ethical considerations, which may impact statistical significance due to a lack of power. Despite these challenges, results obtained generally trend towards improvement in the symptoms or clinical parameters of the disease in question. For example hookworm therapy for allergic rhinitis yielded no significant clinical differences between hookworm and placebo treated groups, however there was a reduced need for hookworm infected participants to take 'rescue medication' during the hay fever season (121, 122). Likewise,

hookworm infection was used to treat coeliac disease sufferers, and while no significant differences between treatment (hookworm) and control groups were detected for immunological and histological outcomes, the patients who received hookworms reported higher scores on their well-being surveys (123).

The initial studies that paved the way for helminth therapy utilised viable ova from *Trichuris suis* (TSO) to treat patients with IBD. The initial safety study consisted of 4 Crohn's disease patients and 3 UC patients, all of whom received a single dose of 2,500 TSO orally and were monitored for a total of 12 weeks (124). Remission from disease was achieved in 6 out of the 7 subjects, however there were no control subjects included in the trial (124). A larger trial consisting of 29 Crohn's disease patients given 2,500 TSO orally every 3 weeks for 12 weeks demonstrated improved disease activity index in 79% of patients, but also lacked control subjects (125). Nonetheless, these initial trials were paramount in demonstrating both the safety and effectiveness of helminth therapy. A subsequent study in which 59 UC patients ingested 2500 TSO or placebo every 2 weeks for 12 weeks, also demonstrated a significant reduction in disease activity score in the TSO treatment group (126), but the most recent TSO phase II clinical trials for Crohn's disease failed to meet their clinical endpoints (127). It is possible that since the previous trials did not include controls the potency of the placebo effect may have been overlooked. Moreover, TSO is not adapted to survival in humans and is too short-lived to induce a chronic infection, and thereby less likely to induce a potent regulatory response compared to other parasites.

The most recent clinical trial exploring the role of human hookworm infection in improving tolerance to gluten in coeliac disease patients represents a significant advance in the field. As previously mentioned, initial studies in this area demonstrated that hookworm infection is safe, with relatively low adverse side effects (123). It was further shown that the hookworm infection suppressed production of the pro-inflammatory cytokines IFNγ and IL-17A and increased production of the regulatory cytokines IL-10, TGF-β and IL-22 from duodenal biopsies of coeliac disease patients (128, 129). The authors noted that the initial trial, in which patients (previously on a gluten-free diet) ingested 4 slices of bread per day for 5 days, was an aggressive gluten challenge (130) which likely overwhelmed the

potentially beneficial effects of the hookworms. A follow-up trial was therefore conducted, where patients were exposed to escalating doses of gluten starting at just 10 mg daily and escalating gradually over 12 months to 3 grams of gluten per day (the equivalent of a medium sized bowl of pasta) (131). This trial demonstrated that combined hookworm infection and incremental exposure to gluten did not cause changes in mucosal pathology and promoted oral tolerance. Moreover, patients had reduced levels of anti-tissue transglutaminase antibodies and IFNγ producing cells as well as increased numbers of Tregs and improvement in quality of life indicators (131). Due to the toxicity of gluten to coeliac disease patients, it was deemed unethical to include a placebo control (non-infected) group in this study. This highlights yet another major challenge for human clinical trials; as the principal concept of bioethics is 'first do no harm', trials involving diseases which require priming (e.g. allergic diseases) will always require negotiations between ethical considerations and experimental design.

Other autoimmune diseases that have the potential to benefit from helminth therapy include MS, where initial pilot trials have shown favourable outcomes for TSO therapy in newly diagnosed MS patients (132, 133). There are also clinical trials currently underway for psoriasis, nut allergies and rheumatoid arthritis (134, 135). Hopefully following successful trials in animal models we may soon see diabetes mellitus added to this list. However it should be noted that there are obvious deterrents to helminth therapy. For example helminths are parasites and most parasites come with their own range of symptoms ranging from urticaria (136), to intestinal upsets and even cancer (cholangeocarcinoma directly linked with *Opisthorchis viverrini* infection) (137). Moreover not all helminths or helminth derived molecules necessarily ameliorate inflammatory conditions. For example, *Anisakis simplex* a fish parasite, has been known to cause severe allergic reactions when ingested by humans (138). In mouse models of disease, *Hymenolepis diminuta* infection led to exacerbation of oxazolone-induced colitis (139), persistent *Trichuris muris* was shown to aggravate colitis in IL-$10^{-/-}$ mice (140) and infection with *Toxocara canis* (141) and *Nippostrongylus brasiliensis* (142) leads to a exacerbation of experimental allergic airway inflammation. This highlights the importance of screening for

potential helminths/helminth derived molecules in mouse models of disease before undertaking human studies. Current research on rodent models and helminth therapy will be discussed in the following subsections.

1.2.3 Rodent models addressing helminth regulation of inflammatory diseases

Rodent models are indispensable for understanding the immune interaction between host and parasite and how the bystander effects may be useful in combating inflammatory disorders. The following section will focus on rodent models of disease in which helminth infections have been used as a therapeutic tool. Where possible, references to the most up to date reviews on each model are included.

Asthma: (see (143) for comprehensive review)

Asthma is a chronic inflammatory disease of the airways and many animal models mimicking asthma exist, with the airway hyper-responsiveness (AHR) mouse model being the most commonly used. In this model, mice are sensitized to an antigen such as ovalbumin (OVA) in combination with alum via intraperitoneal injections and are later challenged with the same antigen via aerosol to induce local allergic response in the airways. Disease development is measured through bronchoalveolar lavage (BAL) infiltrate and levels of Th2 cytokines. A recent review (143) has listed more than 30 studies of AHR that have utilised helminths to inhibit development of the disease. These studies have identified *Angiostrongylus costaricensis* (144), *Heligmosomoides polygyrus* (145-147), *Schistosoma japonicum* (148, 149), *Schistosoma mansoni* (150, 151), *Strongyloides stercoralis* (152) *and Trichinella spiralis* (153) infections as being protective in this model, whereas *Toxocara canis* (141) exacerbated the allergic response. Only 3 studies have attempted to use helminth infections as a treatment for already established AHR; of those, *H. polygyrus* (147) and *Strongyloides venezuelensis* (154) demonstrated protection, whereas *Nippostrongylus brasiliensis* (155) did not.

The mechanism of protection is not yet entirely known, but protected mice exhibit fewer infiltrating cells in the BAL as well as reduced levels of type 2 cytokines compared to

the controls (143). The contenders in this model seems to be cells that have regulatory properties, such as helminth induced Tregs and regulatory B cells (Bregs), as likely candidates for immune regulation (143, 146, 148, 150, 156).

Multiple Sclerosis: (See (157) for comprehensive review)

Multiple sclerosis is a chronic human autoimmune inflammatory disease in which the myelin sheaths surrounding the neurons of the central nervous system are progressively destroyed. The mouse model for multiple sclerosis is experimental autoimmune encephalomyelitis (EAE), which is a CD4 T cell-mediated disease that can be induced by immunisation with myelin proteins (158). Similar to the AHR model, studies show that a pre-established helminth infection can delay onset and reduce severity of EAE (157), however the effect of helminths on ameliorating pre-existing inflammation has not yet been addressed. The helminths used in this model have included *S. mansoni* (159)*, F. hepatica* (160), *T. spiralis* (161), *H. polygyrus* (146), *Taenia crassiceps* (162) *and Trichinella pseudospiralis* (163). Most of these studies related protection with decreased pro-inflammatory cytokines IFNγ and IL-17A and increased levels of IL-10 and IL-4 (159, 161-163). Interestingly, *F. hepatica*-mediated protection was independent of IL-10 but dependent on TGF-β, suggesting the involvement of regulatory cells. For *H. polygyrus*, protection was achieved by transferring B cells from infected mice into mice undergoing the induction phase of EAE (146).

Type 1 Diabetes: (See (164) for comprehensive review)

In type 1 diabetes the insulin producing beta-cells of the pancreas are destroyed by an autoimmune response. This disease previously carried a life sentence before insulin therapy became available to maintain glucose homeostasis (165). Diabetes is now manageable, however the incidence of type 1 and type 2 diabetes has exploded in recent years in what could only be described as an epidemic (166). The non-obese diabetic (NOD) mouse strain is widely used to study type 1 diabetes, and as in human disease, the development of diabetes in these mice is under complex genetic control (164). Helminth infections shown to be protective in this model include *S. mansoni* (167)*, H. polygyrus* (168,

169)*, T. spiralis* (168) *and Litomosoides sigmodontis* (170), all of which were attributed to a type 2 shift in immune responses. For *L. sigmodontis* significant increases in Tregs were also noted (170).

Rheumatoid Arthritis: (see (171) for review)

Rheumatoid arthritis is an autoimmune disease of the joints, causing chronic joint inflammation which can lead to stiffness, swelling and pain around the joints and eventually total destruction of joint structures (172). There are many mouse models available for studies of rheumatoid arthritis, and these fall into 3 categories: induced, genetically engineered and spontaneous arthritis (173). Induction of the disease can be achieved either through adjuvants, antigens, collagen, antibodies or by direct injection of pro-inflammatory cytokines into the joint (173). Genetically engineered models including IL-1 receptor antagonist and TNF transgenic mice (173) add to the long list of immune mediated arthritis models in mice. Similar to previous examples of helminth-driven regulation of autoimmune models, positive results are attributed to down regulation of pro-inflammatory cytokines, in this case TNF and IL-17A, along with increased levels of type 2 cytokines IL-4 and IL-10 (171). Helminth parasites that have been shown to be beneficial in mouse models of arthritis include *S. mansoni* and *S. japonicum* in collagen induced arthritis (174, 175) and *Hymenolepis diminuta* in the Freund's complete adjuvant model of mono-arthritis (176). Interestingly *H. diminuta* exacerbated inflammation in the K/BxN genetically engineered model of polyarthritis (177) and *T. crassiceps* did not affect disease progression in an OVA-specific model of arthritis (178).

IBD: (see (179) for comprehensive review)

There are currently over 50 mouse models that mimic human IBD (180). The simplest models rely on a chemical stimulant that directly affects the integrity of the colonic mucosa, creating a local inflammatory response. Two of these widely used models of colitis, DSS (dextran sulphate sodium) and TNBS (2,4,6-trinitrobenzenesulfonic acid), will be discussed in more detail in **section 1.3** below. Other mouse models of colitis include spontaneous models such as IL-$10^{-/-}$ mice, T cell transfer models of colitis and many more

(see (181) and (180) for review of models). Helminth therapy in mouse models of IBD has been intensively researched with *H. diminuta* (182, 183)*, T. spiralis* (184)*, H. polygyrus* (185, 186), *S. mansoni* (187, 188), *Trichinella papuae* (189) and *S. japonicum* ova (190), all functioning as preventative treatment against chemically-induced models of colitis. Furthermore, *H. polygyrus* (191) and *H. diminuta* (182) have also been shown to be curative in ameliorating pre-existing inflammation in chemically-induced colitis.

Most of the examples cited above suggest that helminth infections shift the cytokine response away from a type 1 towards a type 2 response, therefore suggesting that a balance between type 1 and type 2 responses is necessary for homeostasis. This of course is not true for the asthma and allergy models where an unregulated type 2 response is the hallmark of disease, suggesting that apart from skewing the cytokine environment helminth infections also lead to induction of regulatory cells. For example, it has been shown that mice infected with *H. polygyrus* have an expansion of Treg cells (192) that confer resistance against allergic airway inflammation (147). The same parasite is also capable of regulating immune function in mouse models of colitis (186, 193), demonstrating that the effects of these cells are systemic, at least in terms of the mucosa. Apart from Tregs, helminth infections have also been linked with induction of Bregs (194) and regulatory macrophages (195), demonstrating the multiple adaptations each parasite has achieved in order to survive within their hosts. The following section will briefly review some of the identified helminth molecules and their mechanisms of immuno-regulation.

1.2.4 Therapeutic helminth molecules: mechanisms of action

The concept of helminth therapy is marred with obstacles, as the re-introduction of parasites into the countries from which they have been eradicated will never be conceded, and the prescription of live worms seems equally unachievable. A viable alternative to live helminth therapy appears to be the use of excretory/secretory (ES) products to mimic (immunologically at least) the effects of a worm infection. This is an area where mouse models are far more advanced than that of human trials, with candidate antigens from *S. mansoni* and *Acanthocheilonema viteae* already showing promise. From *S. mansoni* egg

antigen (SEA), a glycosylated T2 ribonuclease named Omega-1 primes DCs for Th2 activation by inhibiting protein synthesis within the cytosol. It is likely that this causes the lack of co-stimulatory molecule expression seen when DCs are stimulated with SEA. A lack of co-stimulatory molecule expression is also noted when DCs are exposed to ES-62 from the filarial nematode *A. viteae,* as is the ability to induce a Th2 response (196). ES-62 also induces production of the regulatory cytokine IL-10 from B cells (197), as does lacto-N-fucopentose III from SEA (198) and *Dirofilaria immitis*-derived antigen (DiAg) (199). Cystatins from *A. vitea* also increase IL-10 production, but the cellular source is macrophages rather than B cells (200).

The regulation of T cell responses by nematode proteins is further propagated by induction of Tregs. Omega-1 again shows its versatility in that it is able to induce $Foxp3^+$ Tregs via induction of TGF-β (201). Similarly, increases in expression of TGF-β, necessary for Treg induction, were also seen in mice treated with recombinant *T. spiralis* protein rTs-P53 (202), a galectin-9 homologue (rTI-GAL) from *Toxascaris leonine* (203) and macrophage migratory inhibitory factor (MIF)-like protein (rAs-MIF) from *Anisakis simplex* (204). Although the exact moiety is not yet known, ES products from *H. polygyrus* are also capable of *de novo* $Foxp3^+$ induction in naïve $CD4^+$ T cells by acting as a TGF-β mimic (205).

The immunomodulatory properties of these helminth derived proteins have also been shown to be protective against mouse models of inflammation. For example ES-62 was deemed not suitable for therapeutic development, however recently two small molecule analogues, based around its active phosphorylcholine-moiety, have been shown to be effective against collagen induced arthritis (206) and suppressed generation of antinuclear antibodies (ANA) and kidney pathology in a mouse model of systemic lupus erythematosus (207). Lacto-N-fucopentose III, which is also found in breast milk, improves glucose tolerance and insulin sensitivity in diet-induced obese mice (208) and reduces the severity of central nervous system inflammation in EAE (209). Finally, both rTI-GAL and rAs-MIF have been shown to be protective in mouse models of colitis (203, 204).

Despite being incredibly diverse, helminths have all been extremely successful in adapting to their hosts and as such, convergence of immunomodulatory mechanisms can be found. One parasite which is particularly well adapted to its host is the hookworm. Unlike many other helminths, mild hookworm infections do not have major adverse side effects unless the host is already in a malnourished state. The specifics of the hookworm will be further discussed in **section 1.4** below.

1.3 Mouse models of colitis

Animal models of disease are imperative for understanding the pathogenesis of novel therapeutics for inflammatory conditions. There are many murine models of IBD available that usually fall into 3 different classes: gene knockout models, cell transfer models and chemical/diet induced models of disease. Gene knockout models include the IL-$10^{-/-}$ and IL-$2^{-/-}$ mice, where the mice develop a spontaneous colitis over time (210-212). These mice have proven valuable for determining the specific roles of these cytokines with different treatment modalities, but are otherwise limited to interactions with the specific knockout.

Cell transfer models utilise specific T cells (either IL-$10^{-/-}$ T cells, $CD4^{+}CD25^{-}CD62L^{+}$ T cells or $CD45RB^{hi}CD4^{+}$ T cells) that are transferred into immunocompromised hosts to induce a more progressive pathology (213, 214). The cell transfer models are more comprehensive models, which are thought to be most similar to human autoimmune conditions. However, because of the requirement of both donor and host mouse strains, cell sorting and cell transfers to initiate the disease, these models are the most costly and time-consuming to set up.

The most widely used models are the chemically induced models of IBD, including DSS, TNBS, DNBS (dinitrobenzene sulfonic acid) and oxazolone models. These models are highly popular, because they are easy to set up, have short time frames and are cost-effective. I will further discuss the two models used in this thesis, the DSS and TNBS models below.

1.3.1 DSS model of colitis

The DSS model of colitis is one of the easiest models to establish within a lab environment. It requires the substitution of normal drinking water with a 3-5 % solution of 36-50 kDa DSS (215). The length of the experimental cycle varies with each animal house environment, mouse strain/sex/age, batch and concentration of DSS used, so these factors have to be determined prior to commencement of a study (216). Other factors that may also affect the progression of disease include the type of water used (tap water vs deionised water) and the frequency in which the DSS solution is refreshed (215). Normally weight loss begins at day 3 post-first administration of DSS, with high levels of pathology being achieved by days 8/9, at which point the body weight has dropped to 70% of initial starting weight (217). Depending on the weight/age of the mice, presence of blood in the stools can be detected as early as day 2 and intensifies during the course of DSS treatment. The DSS model was chosen for these studies because of the involvement with the innate immune response and for its relatively small level of variability due to user input.

The DSS model does not require the addition of a solvent and preferentially affects the colon despite being administered orally. DSS is known to inhibit reverse transcriptase activity but how this translates to induction of colitis is still unclear. In 2012, Laroui *et al* concluded that DSS complexes with medium chain fatty acids to form 200 nm vesicles that can fuse with colonocytes, providing an explanation for the route of entry of DSS into cells (218). They further demonstrated that the inflammatory capacity of DSS is dependent on the dextran moieties (218). It is still unknown whether DSS-mediated interruption of fundamental mechanisms (e.g. reverse transcriptase activity) *in vivo* initiates the inflammatory process or if it initiates inflammation by some other signalling pathway. There is some evidence to suggest that pathways of mucus depletion (219, 220), epithelial cytotoxicity (221) and macrophage activation (222, 223) could also be involved. What is known, is that DSS-induced colitis is independent of T and B cell presence (215), making this model ideal for the study of innate immune responses.

1.3.2 TNBS model of colitis

The TNBS model of colitis requires more expertise than the DSS model of colitis, because the TNBS must be administered in an ethanol mixture intra-rectally whilst mice are sedated with mild anaesthetics. The required dosage of TNBS can vary depending on mouse strain/sex/age and batch of TNBS, and needs to be determined on a case by case basis (224). Generally, the dosage falls between 0.5-4.0 mg of TNBS in 45-50% ethanol for 6-8 week old male mice (224). It is important to note that the C57BL/6 strain is not susceptible to TNBS induced colitis, meaning that these mice will eventually regain the initial weight lost and will also show healing of the mucosa (215, 225). Although not ideal, this strain can be used for investigating acute inflammation and is beneficial as a screening model for novel therapeutics due to its short time frame (4 days) and the requirement of only a single dose of the therapeutic agent given around the same time as TNBS administration. However, these limitations must be considered and further studies utilising a mouse strain that is susceptible to TNBS should also be undertaken if a comprehensive analysis of the therapeutic in question is required.

The mechanism of action of TNBS-induced colitis is much better documented than that of DSS-induced colitis. TNBS is a haptenizing agent, which means that the trinitrophenyl groups interact with host proteins and render the proteins immunogenic, causing a delayed hypersensitivity reaction (226). Ethanol is a solvent which helps to break down the mucus in the colon and allows the TNBS to access the colonic epithelium (215). The immune response to TNBS is mediated by $CD4^+$ T cells and has been shown to be strongly skewed towards a type 1 inflammatory response, and inflammation can be neutralised with anti-IL-12 antibodies (226). However the $CD4^+$ T cell response is only evident in the chronic stages of pathology and the initial colitis like symptoms seen in the first 4 days following TNBS administration are likely due to mucus breakdown by the ethanol component. The dual response makes the TNBS model useful as both a screening model in the acute inflammatory setting and as a chronic model for investigating the role of effector $CD4^+$ T cells and their interactions with the desired therapeutics.

1.4 Hookworms

Hookworms are soil-transmitted nematodes that possess well developed buccal cavities with upper and lower sets of teeth (**Figure 1.4.1**). The human hookworm, *Necator americanus,* has cutting plates instead of teeth and uses these to bury its mouth parts deep into the host's mucosa to gain access to its nutrient source - blood. Hookworms belong to the order Strongylida and there are many different species which infect animals worldwide. *N. americanus* and *Ancylostoma duodenale* are the two main human hookworms. *Ancylostoma caninum* and *Ancylostoma braziliense* infect dogs, whereas *Ancylostoma tubaeforme* infects cats. *Ancylostoma ceylanicum* is known to commonly infect dogs and cats but has also been recently shown to cause significant human infections in some parts of Asia (227, 228). Many other species of hookworms also exist in the wild, each infecting its own specifically suited mammal, from native rats (229) to sea lions (230, 231).

Hookworm infection falls under the soil-transmitted helminthiases of the neglected tropical diseases (NTDs), with an estimated 500 million people infected with hookworms worldwide (232). As of 2010 hookworms were estimated to account for 3.23 million disability adjusted life years (DALYs) (232). One DALY is one year of healthy life lost and is a measure of disease burden. However, a recent study showed that the NTDs combined were only attributable to 1% of total worldwide DALYs (233), making research funding into the NTDs even more scarce. Moreover, due to the difficulty of maintaining the hookworm life cycle in the laboratory and its emphasis on morbidity rather than mortality, hookworm research is less advanced compared to that of some other NTDs.

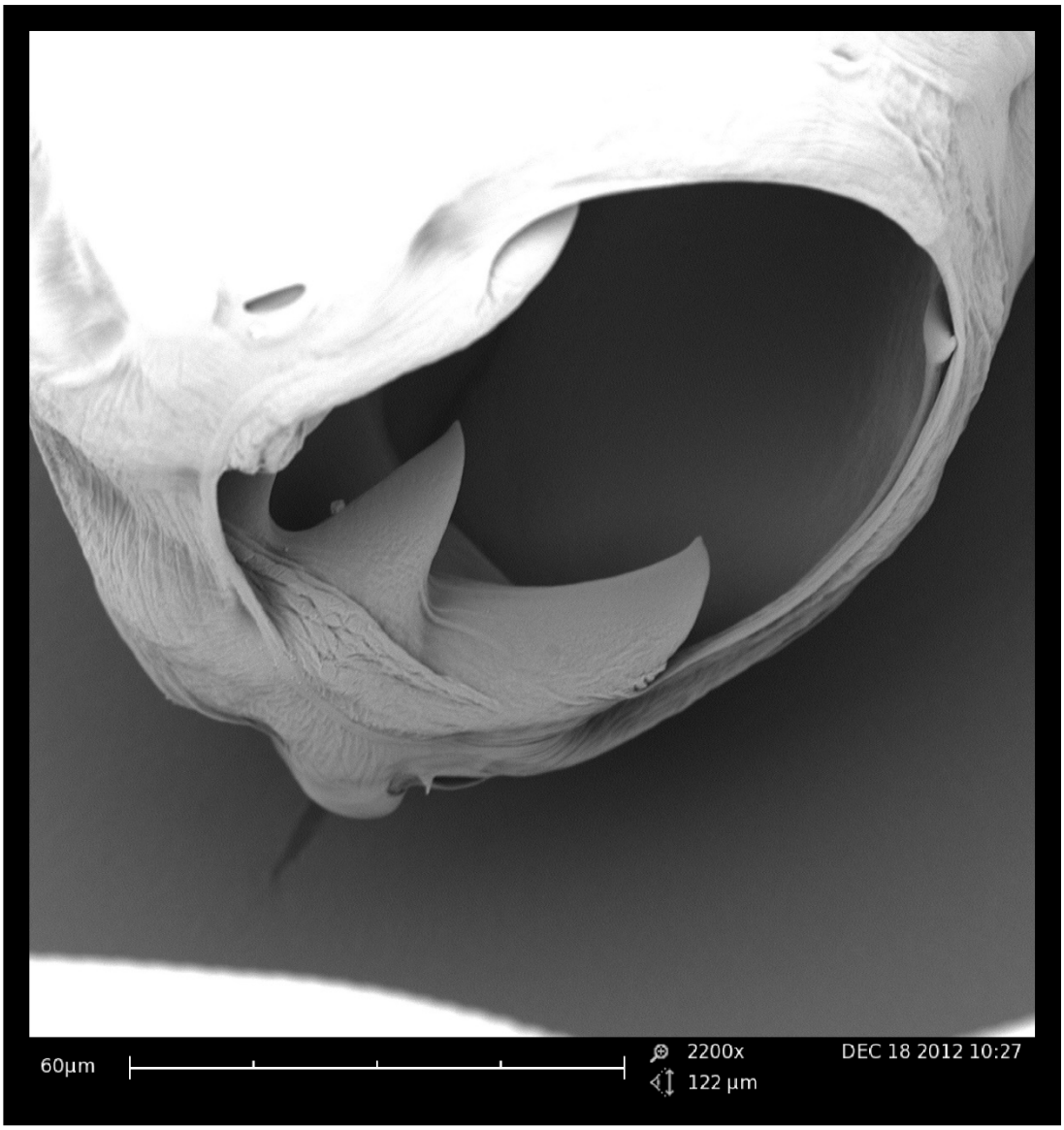


Figure 1.4.1: Scanning electron micrograph of *Ancylostoma caninum* buccal cavity. Scanning electron micrograph of the buccal cavity of *A. caninum* showing a top row of large sharp teeth, with smaller teeth also visible on the lower aspect of the buccal opening.

1.4.1 Life cycle

Hookworms are dioecious nematodes and mating between a male and female is necessary for fertilisation. A female hookworm can produce more than 10,000 eggs per day, which will eventually get passed in the host's faeces (234-236). If/when these eggs find themselves in a suitably moist soil environment they will hatch into first stage larvae (L1). Whilst in their larval stage the hookworms will feed on bacteria in the soil in order to grow and moult into the L2 stage, and finally the infective L3 stage. L3 make their way onto the tips of blades of grass in order to find a potential host (237). L3 infect the host percutaneously and enter into the circulatory system (237). During this stage of infection the larvae produce ES products, which allow them to not only migrate through host tissues but also modulate the immune response (238, 239). Once in the circulatory system the worms make their way into the alveolar spaces of the lungs where they creep up the trachea and are swallowed, ultimately residing in the small bowel (234). The maturation process from initial infection to sexually mature adult worms can take between 5–8 weeks and is called the pre-patent period (237). An infection is said to be patent when eggs can be found in the host's excrement.

When a hookworm infects a non-suitable host it is thought that many of the worms become arrested in the lung and musculature and never make it to the intestine. The mechanisms of host-specific migration and development have not been thoroughly elucidated, but specific host-parasite interactions likely occur in the lungs and elsewhere to provide migrating larvae with the required cues to reach their final destination (240). The finely tuned host-parasite interactions do not stop there - hookworms are extremely long-lived nematodes and have been known to survive for decades inside a single host. In order to achieve this longevity they produce a milieu of ES products which help them evade the host's immune system (238). Their adaptability to their hosts is demonstrated not only by the diverse range of hookworm species but also due to their continued existence, with reports of hookworms infecting humans since 294 BC (241). The decline in human hookworm infections began with the industrial revolution and the introduction of flushing toilet systems in the mid 1800's (242). It was further accelerated with the introduction of

anthelmintic mass drug administration, and now has been practically eradicated from most developed countries (234).

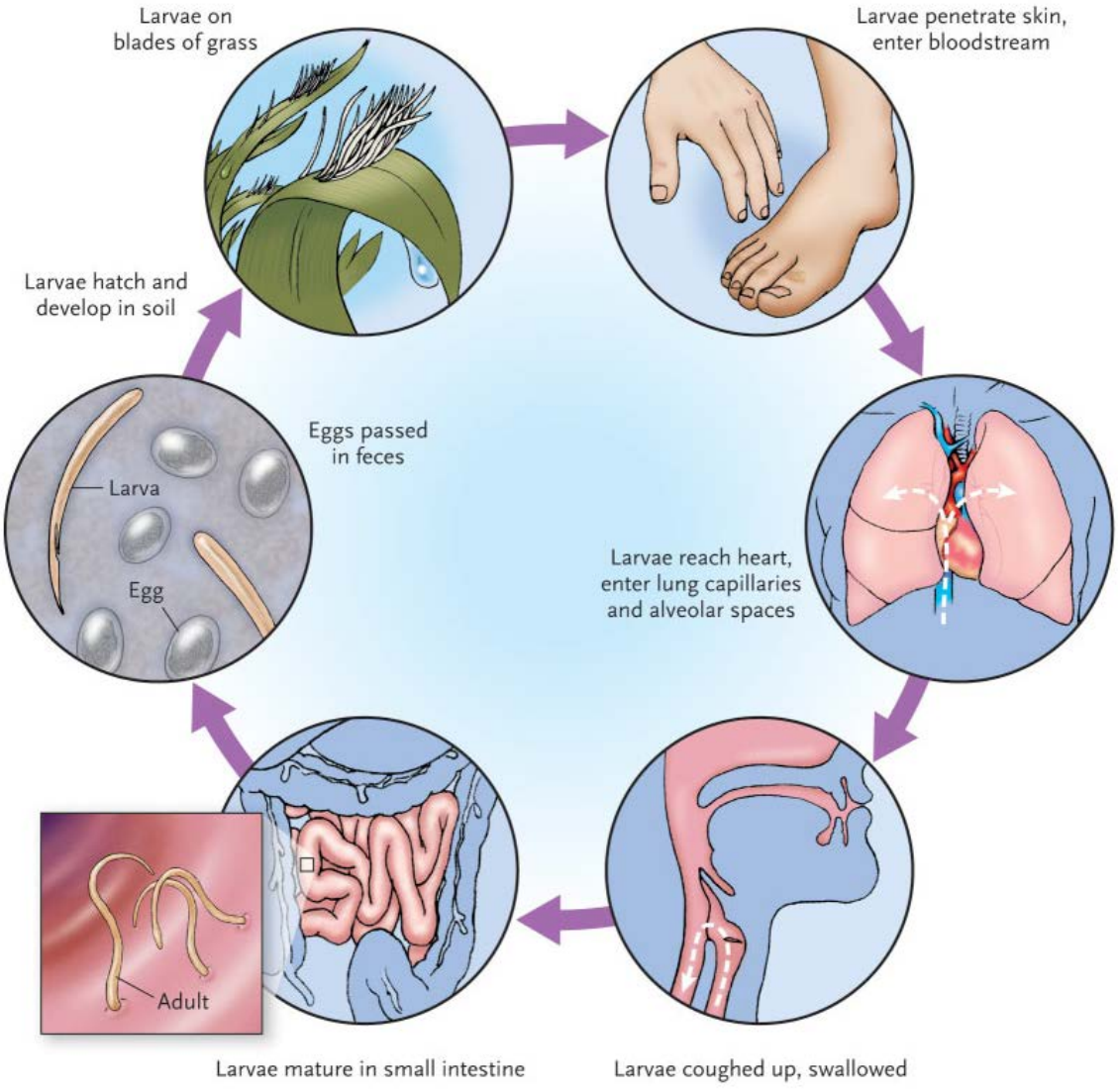


Figure 1.4.2 Hookworm life cycle.
Diagram duplicated from *(243)* without any modifications.

1.4.2 Pathogenesis

Hookworm infections can cause significant morbidity and occasionally mortality in heavily parasitised and malnourished individuals (234). Although not pathogenic in healthy subjects, moderate to heavy hookworm burdens combined with malnutrition can have severely detrimental effects, particularly to a growing child. As the hookworm feeds on blood, a malnourished child harbouring a heavy hookworm infection will quickly become anaemic (234). Anaemia leads to further complications such as stunted growth, delayed development, mental retardation and even death (234). However, cases like these are uncommon in industrialised countries since the introduction of proper sanitation. Because hookworms do not replicate inside their mammalian host, high numbers of hookworms can only be achieved through multiple rounds of re-infections via ongoing exposure to contaminated soil.

Experimental infection studies have shown that a bolus of up to 50 larvae during a single infection is well tolerated, however 100 larvae are less well tolerated and subjects may experience urticaria with papular eruptions at the site of infection and gastrointestinal pain (244). The immunological profile of a person infected with hookworm is one of high eosinophilia and IgE, but if the person is otherwise healthy and the number of worms is not excessive, no other symptoms or signs of infection will emerge (237). The increase in eosinophil numbers and IgE levels can be detected at around 4-9 weeks post-infection, which coincides with patency (122, 244, 245). Furthermore, peripheral blood mononuclear cells (PBMCs) from experimentally infected individuals re-stimulated with hookworm antigens *ex vivo* produce the type 2 cytokines IL-4, IL-5, IL-10 and IL-13 (246). Interestingly, these experimental infection studies have also shown that time to patency and peak eosinophilia reduces upon re-infection, even if patients have been treated with anthelmintics (237). This suggests that immunomodulatory changes from the first infection reconcile the host immune response allowing for faster and more efficient subsequent infections. Unlike many other parasitic infections, acquired immunity against hookworm infection (in terms of reduced parasitic burden over time) is uncommon, with the most

intense infections often seen in the elderly population (237), emphasising the unequivocal adaption of this parasite to its host.

1.4.3 Hookworm infections and interactions

One of the specific concerns regarding helminth therapy is that of overall immune suppression. This overall dampening of the immune system may be detrimental in some aspects when a strong immune response is required, as is the case for vaccination (247). This problem can, however, be easily overcome by deworming children prior to vaccination, to allow for better efficacy of the vaccine. Furthermore, this issue is only present in parasite-endemic countries where inflammatory disorders are not highly prevalent. Of the few studies that have focused on hookworm co-infections some encouraging results have been noted. For example a study in 2004 found that HIV patients co-infected with helminths did not have higher viral loads or advanced progression of the disease as might be expected if helminth infection caused overall immune suppression (248). Furthermore the authors found a positive association of hookworms and increased $CD4^+$ cell counts as well as a decreased mortality rate compared to the other parasitic infections (248). More recently, a study of Bolivians co-infected with hookworm and *Giardia lamblia* showed a negative association between the two infections, with those people infected with hookworm having a lower chance of contracting a *Giardia* infection (249). These studies suggest that hookworm therapy may not have as many undesirable side effects in terms of exposing patients to other infections as one might expect.

Observational studies in endemic areas have also demonstrated the beneficial aspects of hookworm immunoregulation. Fascinatingly, before the emergence of the hygiene hypothesis the initial assumption was not that helminths reduced risk of atopy but rather that atopy reduced the chances of contracting helminth infections (250). An early review article by David Grove highlighted 3 distinct theories; 1: worms cause asthma, 2: worms ameliorate asthma, and 3: asthma inhibit worms (236); Grove concluded that the most likely scenario was that asthma inhibited the establishment of helminth infections, most likely due to his own earlier study demonstrating that asthmatics in Papua New Guinea

had lower hookworm burdens than non-asthmatics (250). Grove further supported his claims with similar studies from Salako & Sofowara, which again demonstrated that fewer asthmatics had hookworm infections (14%) compared to non-asthmatics (44%) (251). Grove did however go on to suggest that further studies were necessary to either prove or disprove these 3 hypotheses.

Henceforth, observational studies began to take a different turn, most notably with the seminal study from Strachan in 1989 which triggered the hygiene hypothesis. Most of the key studies involving hookworms occurred in the early 2000's, with Scrivener *et al* showing that the risk of wheeze in an Ethiopian population was independently reduced by hookworm infection (114). A large-scale study on Ecuadorian schoolchildren aged between 5 and 19 years of age also associated hookworm infection with reduced reactivity to an allergen skin test (108). A further study of Vietnamese schoolchildren showed a decreased risk of house dust mite sensitisation in those children with higher hookworm burden (252). The authors further showed that the risk of atopy was higher for those using flushing toilet systems, which further reduced the risk of hookworm re-infection (252). Meta-analysis studies have indicated that hookworms can halve the risk of asthma (253), and more recently it was shown that hookworm infections, along with 3 other helminths, have protective effects against allergen sensitisation (254). These results suggest that hookworms likely produce immunomodulatory compounds that may have beneficial bystander effects in the host. Some of the candidate hookworm proteins of interest will be discussed in the next section.

1.4.4 Therapeutic hookworm proteins

The majority of studies focused on hookworm protein identification have been centred on identifying suitable vaccine antigens. Nonetheless, a number of hookworm proteins that show promise as anti-inflammatory therapeutics have been identified. The first hookworm protein to be of interest in a clinical setting was Nematode Anticoagulant Peptide C2 (NAPc2) from *A. caninum* soluble worm extract (255). NAPc2 was found to inhibit Factor VIIa (tissue factor), the initiating factor in the extrinsic coagulation cascade, by

binding to downstream Factor Xa (256). Recombinant NAPc2 proceeded to phase IIb clinical trials in humans as a preventative measure against deep vein thrombosis (DVT) following knee arthroplasty (257). Initial results were promising with fewer cases of DVT following administration of NAPc2 compared to previous data on the use of low molecular weight heparin (257). Unfortunately due to organisational changes in the pharmaceutical company NAPc2 clinical development did not progress further (258). Anticoagulant peptides have also been identified in a number of other hookworm species (255, 259, 260). *A. duodenale* secretes an anticoagulant peptide that has been shown to inhibit both factors Xa and XIa from the intrinsic coagulation cascade (260). To the best of my knowledge this peptide has not gone into clinical trials but the potential for the development of a novel anticoagulant for use in the medical field exists.

Around the same time that NAPc2 clinical trials were underway, the same pharmaceutical company was undertaking clinical trials for another hookworm protein, neutrophil inhibitory factor (NIF). This 41 kDa glycoprotein from *A. caninum* binds to the I-domain of CD11b/CD18 complex (also called complement receptor 3 (CR3), macrophage-1 antigen (Mac-1) or integrin $\alpha_m\beta_2$) found on innate cells (261-263). CD11b/CD18 is highly expressed on the surface of neutrophils and is involved in chemotaxis, adhesion and transmigration across epithelia (264). Binding of NIF to the CD11b/CD18 complex inhibits these processes (265), making *Ac*-NIF a suitable candidate for treating acute destructive inflammatory processes, such as cerebral ischemic injury. *Ac*-NIF was used in a double-blinded, randomised, dose response study for stroke patients but was disregarded due to a lack of significant results (266). Since then, various animal models have shown that *Ac*-NIF could be beneficial in other settings of acute inflammation such as allergic lung inflammation (267) and diabetic retinopathy (268). Importantly, the presence of NIF did not compromise the ability of mice to clear a bacterial challenge, demonstrating that its binding to CD11b/CD18 complex does not alter critical immune surveillance mechanisms (268).

A MIF homologue has also been identified from the ES products of *A. ceylanicum*. *Ace*MIF has been shown to bind to the human MIF receptor CD74 (269). Although initially identified as a possible hookworm vaccine candidate, *Ace*MIF may also be a useful

therapeutic. A MIF homologue from *B. malayi* has human macrophage chemotactic capacity *in vitro* (270). Furthermore a MIF homologue from *A. simplex* protects mice against experimental AHR (271) and DSS-induced colitis (204). More recently the same *A. simplex* MIF (r*As*-MIF) was shown to reduce IL-4 and IL-5 production and increase IL-10 production from PBMCs derived from asthmatic subjects and treated *ex vivo*, prompting the authors to propose its potential use in treating asthma (272). Human and mouse MIFs themselves have a wide range of actions and are instrumental in protecting against liver fibrosis in a chronic liver injury model (273) and cardiomyopathy resulting from the use of the cancer chemotherapeutic doxorubicin (274).

The above examples are likely only a subset of hookworm proteins with therapeutic potential. More than 100 secreted proteins were identified by mass-spectrometry analysis of adult *A. caninum* ES (*Ac*ES) products (275), and there are likely many more molecules of carbohydrate and lipid origins. The recent sequencing of the genome of *N. americanus* is a major step forward for the field of hookworm therapy (259) and will provide the framework for identification of novel molecules that can be harnessed for development of therapeutics.

1.5 Hypothesis underpinning this thesis

Parasites have co-evolved with their host species for centuries and in doing so have developed sophisticated immunomodulatory mechanisms to gain entry and survive within their hosts. I believe that the hookworm is one of the most advanced parasites - it can survive for decades within the host, completely unperturbed by any immune surveillance mechanisms and without causing major detriment to well-nourished hosts. Therefore, it is hypothesised that hookworms actively secrete immunomodulatory molecules that are able to interact with the host immune system to dampen inflammatory immune responses. I hypothesise that these hookworm molecules may be beneficial in managing certain inflammatory conditions, such as IBD, which occupy a similar niche to the adult stage hookworm. This thesis aims to investigate the immunomodulatory properties of hookworm

ES products with the view of translating this research into potential therapeutics for inflammatory diseases in the distant future.

2 Chapter 2 – Materials and Methods

2.1 Animals

Mice aged 5-10 week old (C57BL/6, BALB/c and RAG1$^{-/-}$ [BL/6 background]) were purchased from the Animal Resource Centre (ARC) in Perth, Western Australia. Mice were housed according to Australian animal rights and regulations standards with food and water available *ad libitum* in a specific pathogen free (SPF) facility at James Cook University (JCU), Cairns. All animal experiments were covered by ethics application number A1484 (see **10.1 Appendix A: Ethics approval**) approved by the JCU animal ethics committee.

2.2 Production of worm derived products

2.2.1 Collection of *Ancylostoma caninum* excretory/secretory (*Ac*ES) products

Ancylostoma caninum adult worms were manually extracted from the small intestines of recently euthanized stray dogs. The worms were placed in a petri dish and washed 3 times with RPMI 1640 medium (Gibco) at 37°C containing 5x antibiotic-antimycotic (Gibco). Next, the worms were transferred to a new petri dish and incubated at 37˚C for 2 hours at which point the media (containing worm vomitus enriched in engorged host tissues) was discarded. Worms were then washed another three times in media containing 5x antibiotic-antimycotic and transferred to a new petri dish containing media with 2x antibiotic-antimycotic. The worms were finally incubated for approximately 16 hours. After the initial 16 hour incubation, the media was collected and frozen at -20°C, fresh media was added and the process was repeated for a total of 3 days. The media now containing *A. caninum* excretory/secretory (*Ac*ES) products was pooled and concentrated using a 10 kDa spin column (Amicon, Merk-Millipore) according to the manufacturer's instructions and buffer exchanged into PBS (phosphate buffered saline).

Approximately half of the *Ac*ES used for this thesis was personally collected at JCU. The remaining *Ac*ES was collected by our collaborator, Associate Professor

Thewarach Laha at Khon Kaen University in Thailand using the same protocol as that employed at JCU, and shipped in non-concentrated form on dry ice to JCU for concentration and further processing.

2.2.2 Endotoxin removal

Endotoxins were removed by either Endotrap Blue (Hyglos) column separation according to manufacturer's instructions or by phase separation with Triton X-114 (Sigma). Briefly, *Ac*ES was incubated with 5% Triton X-114 at 4°C under gentle rotation for 30 min followed by heating to 37°C for 10 min. After a 15 min centrifugation at 16,000 *g,* the upper endotoxin-depleted phase was collected and the process was repeated twice. *Ac*ES was deemed suitable for use when lipopolysaccharide (LPS) contents were below 5 ng/mg of protein as determined using the Limulus Amoebocyte Lysate (LAL) chromogenic assay (Lonza). Finally, *Ac*ES protein content was quantified using the Pierce micro BCA kit and read on a BMG Omega Polarstar at 562 nm.

2.2.3 Production of bES

Heat and protease denatured *Ac*ES (bES) was produced by digesting 1-2 mg of *Ac*ES with 1 µg/ml of trypsin for 24 hours followed by heating to 95°C for 15 min to denature both the trypsin and *Ac*ES proteins.

2.2.4 Production of *Schistosoma mansoni* Soluble Egg Antigen (SEA)

Schistosoma mansoni soluble egg antigen (SEA) was produced using *S. mansoni* eggs extracted from livers of infected mice as previously described in (276). After eggs were harvested, they were homogenised in ice-cold sterile PBS using a Tenbroeck 7ml tissue grinder. An inverted microscope was used to check that eggs were completely disrupted. The homogenate was transferred to a 15ml Falcon tube and centrifuged at 2600 *g* for 15 minutes at 4°C. Supernatants were collected and transferred to 1ml eppendorfs and further centrifuged for 10 minutes at 16,000 *g* at 4°C. Supernatants were sterilised through a 0.45 µm syringe filter and protein content was quantified using the Pierce micro BCA kit and read on a BMG Omega Polarstar at 562 nm.

2.3 Production of recombinant *Ac*-AIP-1 and *Ac*-NIF

Recombinant *Ac*-AIP-1, *Ac*-AIP-1_{N119Q} and *Ac*-NIF were expressed as secreted proteins in the yeast *Pichia pastoris* using methods described elsewhere (277). The cDNA encoding the mature sequence of *Ac*-AIP-1 (amino acids 17-140) was cloned in frame into pPICZαA (Invitrogen) using *Eco*RI and *Xba*I restriction sites. The predicted *N*-linked glycosylation site of *Ac*-AIP-1, asparagine^{119} (N119), was mutated to glutamine (Q) by site directed mutagenesis using overlapping extension PCR. This mutant sequence *Ac*-AIP-1_{N119Q} was cloned in frame into pPICZαA (Invitrogen) using *Eco*RI and *Xba*I restriction sites. The mature coding sequence of *Ac*-NIF (Genbank accession L27427.1) was codon optimised for *P. pastoris* expression (GeneArt) and sub-cloned into pPICZαA (Invitrogen) using *Eco*RI and *Xba*I restriction sites. All expression sequences were cloned in frame with the alpha mating signal sequence (N-terminus) and the c-myc and hexa-his tag sequences (C-terminus) of the expression vector pPICZαA to target secretion of the recombinant protein into the culture media and to aid purification and detection respectively. The correct reading frame was confirmed by sequencing recombinant plasmids using the α-factor and 3' AOX1 vector-derived primers.

The recombinant plasmids were linearized by *Sac*I digestion and transformed into *P. pastoris* strain X-33 by electroporation according to the manufacturer's instructions (Invitrogen). The transformants were selected on yeast extract-peptone-dextrose plates containing zeocin and assessed for expression of recombinant protein via Western blot using anti-hexa His monoclonal antibody (Invitrogen). A Western blot-positive clone was grown in a shaker flask, and expression of the recombinant 6×His tagged protein was induced with methanol, as per the manufacturer's instructions (Invitrogen). The recombinant fusion protein was purified with a nickel affinity column and eluates containing recombinant protein were concentrated using Amicon Ultra Centrifugal concentrators and buffer exchanged into PBS pH 7.4. The production of both recombinant *Ac*-NIF, *Ac*-AIP-1_{N119Q} and *Ac*-AIP-1 was entirely performed by Darren Pickering.

2.4 Experimental colitis

2.4.1 TNBS induced colitis

7-10 week old male C57BL/6 mice were anaesthetized with xylazine (5 mg/kg) and ketamine (50 mg/kg). Once unresponsive to stimuli mice were administered 2.5 mg of TNBS (Sigma) diluted in 50% ethanol intra-rectally using a 20 gauge soft catheter (Terumo). In the acute model of TNBS colitis mice were monitored daily for weight loss over 4 days. Clinical scoring (macroscopic score) was performed on the colon upon euthanasia. Scoring of clinical pathology included adhesion (0-3), oedema (0-3), ulceration (0-3), thickening (0-3) faecal consistency (0-3), and colon shortening for a maximum total score of 15.

2.4.2 DSS induced colitis

The DSS model of colitis involves the administration of a 3.5% DSS (36 – 50 kDa MW, MP Biomedicals) solution to female C57BL/6 mice (6-8 week old) via the drinking water for a period of 6 – 14 days. Mice were monitored daily for changes in weight, appearance, faecal consistency and rectal bleeding for a maximum total pathology score of 13. Individual scoring parameters were as follows:

Weight loss:

No loss = 0; ≤5% loss = 1; 5-10% loss = 2; 10 – 20% loss = 3; ≥ 20% loss = 4

General appearance:

Normal = 0; Piloerection only = 1; Lethargy and piloerection = 2; Motionless/sickly = 3

Faecal consistency:

Normal/dry = 0; Loss of form = 1; Some blood visualised = 2; Bloody liquid = 3; Mucus, only/unable to defecate for longer than 10 minutes = 4

Rectal bleeding:

No blood = 0; Blood = 1; Blood spreading to fur = 2.

Macroscopic scoring upon euthanasia was performed as per TNBS clinical score in **section 2.4.1** above.

2.5 Cell preparations

2.5.1 Euthanasia

Mice were euthanized by either asphyxiation in a CO_2 chamber or by lethal dose of ketamine/xylazine (100 µl of a 1:1 ratio of undiluted ketamine/xylazine). After passing a lack of responsiveness test by squeezing of the footpads, cervical dislocation was performed to ensure mice were dead prior to commencement of organ retrieval.

2.5.2 Organ and cell collection

Blood was collected via cardiac puncture. Peritoneal exudate cells (PECs) were collected by washing out the peritoneal cavity with approximately 10 ml of ice-cold complete media (see **2.5.3** below). Spleens and mesenteric lymph nodes (MLN) were collected and cells were obtained by either gentle maceration through a 70 µM cell strainer (BD Biosciences) or by tissue digestion. Briefly, organs were incubated for 15 min (MLN) or 30 min (spleens) at 37˚C in 2 ml of RPMI 1640 containing 2% Foetal Bovine Serum (FBS) (Gibco), 400 U collagenase type I (Life technologies) and 0.1 mg/ml DNase I (Roche) and further dissociated on a GentleMACS Dissociator (Miltenyi Biotec). Cells were further washed, erythrocytes were lysed when needed with red cell lysis buffer (Sigma) and cell viability was assessed by Trypan Blue (Sigma) exclusion.

Colons were removed from the caecum to the anus. Faecal matter was removed by flushing the colon with ice-cold PBS using a blunt 18-gauge needle. For colon cultures, a small piece (0.5-1.0 cm) of the colon was removed, weighed and placed in 1 ml of complete media (see **2.5.3** below) and cultured overnight at 37˚C. Supernatants were collected the next day and placed at -80˚C for cytokine analysis. For colon homogenates a small piece of colon was removed, weighed and placed in a 2 ml Eppendorf tube containing 0.5-1.0 ml of PBS with a cocktail of protease inhibitors (Complete Ultra tablets - Roche) and a metal bead. Samples were lysed on a TissueLyser (QIAGEN) at 30 shakes per second for 8 min. Supernatants were collected and stored at -80˚C for cytokine analysis. For RNA extraction, a 0.5 cm piece of colon was placed directly in either 1 ml of Trizol or 1 ml RNA later solution then stored at -80˚C.

2.5.3 Media and buffers

Complete media: RPMI 1640 + 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) and 2 mM L-Glutamine (Invitrogen) was used for any application that required cell culture for longer than 2 hours.

Digestion media for lymph nodes: RPMI 1640 or HBSS containing 2% FBS, 1x collagenase (Roche) and 1x DNAse (Roche) at a volume of 5 ml per gut or spleen or 2 ml per set of MLN.

Wash media: RPMI or Hank's balanced salt solution (HBSS) with 5 mM ethylenediaminetetraacetic acid (EDTA) and 2% FBS.

DC culture medium: RPMI 1640, 10% heat-inactivated FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-Glutamine and 20 ng/ml GM-CSF (Invitrogen).

DC stimulation medium: RPMI 1640, 10% heat-inactivated FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-Glutamine and 5 ng/ml GM-CSF.

FACS buffer: Sterile PBS + 0.05% sodium azide + 0.5% bovine serum albumin

MACS buffer: Sterile PBS + 1% FBS + 2 mM EDTA

2.6 Purified cell populations

2.6.1 Macrophage purification

Peritoneal exudate cells from 3-5 mice were collected as described above. Cells were pooled, centrifuged at 300 *g* for 10 minutes at 4°C. Cells were counted and stained with F4/80-APC, CD45RB-PE and Siglec-F-PE at a 1:200 dilution in MACS buffer (see **2.5.3** above) at 4°C for 20 minutes. Cells were washed twice in MACS buffer prior to addition of anti-PE magnetic beads (Miltenyi Biotec) at a concentration of 20 µl of beads per 10^7 cells. Cells were incubated for 15 minutes at 4°C, washed in MACS buffer and centrifuged at 300 *g* for 10 minutes at 4°C. Magnetic columns (Miltenyi Biotec) were washed with MACS buffer prior to use. Cells were seeded through magnetic column with the PE- flow through population being collected. Following this purification, purity of macrophages

were assessed by flow cytometry. Purification was deemed successful if the macrophage ($F4/80^+$) population was above 95%.

2.6.2 Production of bone marrow derived dendritic cells (BMDCs)

Bone marrow derived dendritic cells were harvested as previously described in (278). Briefly, bone marrow was removed from tibias and femurs of mice using sterile ice-cold PBS. Cells were seeded at 5-7×10^6 cells in 100×20 mm non-treated cell culture plates in 10 ml of DC culture medium (see **2.5.3** above) and incubated at 37°C, 5% CO_2 for 72 hours. On days 4 and 7, 5 ml of pre-warmed (37°C) DC culture medium was added to the plates. On day 9 or 10, non-adherent cells were harvested and were assessed for purity by flow cytometry. Dendritic cells were deemed suitable if purity was above 95% for $CD11c^+$.

2.6.3 BMDCs stimulation and transfer

After assessing BMDC purity, BMDCs were transferred to 6-well plates in DC stimulation medium (see **2.5.3** above) at a concentration of 2x10^6 cells/ml. Cells were stimulated with either 10 µg/ml *Ac*ES, 100 ng/ml LPS or left unstimulated for 16-24 hours at 37°C, 5% CO_2. Cells were washed in sterile PBS and 1x10^5 cells were transferred into naïve mice using a 23 gauge needle. Mice were sacrificed 7 days post BMDC transfer for analysis.

2.7 Flow cytometry

Cells were stained for various surface markers (see **Table 8.1.1** in **Supplementary Material**) at a 1:200 dilution in FACS buffer for 30 min on ice. For intracellular cytokine stains, cells were incubated for 4 h at 37°C in complete medium containing 500 ng/ml phorbol myristate acetate (PMA), ionomycin (1 µg/ml) and brefeldin-A (10 µg/ml). Cells were next stained for surface markers then permeabilised with Fix/Perm buffer (BD biosciences) as per the manufacturer's instructions. Cells were then incubated with intracellular antibodies at a 1:100 dilution (**Table 8.1.1** in **Supplementary Material**) at 4˚C for 30 min. Acquisition was performed on either a BD FACS Canto II or a BD FACS Aria III flow cytometer (BD Biosciences), and analysed using FlowJo software (Treestar).

2.8 Cytokine measurement

Splenocytes were cultured in triplicate in flat-bottom 96 well plates (1 x 10^6 cells/well) in medium alone or medium supplemented with *Ac*ES (10 μg/ml), ovalbumin (OVA; Sigma) (10 μg/ml) or anti-CD3 (1 μg/ml), for 72 h at 37°C, 5% CO_2. Cell-free supernatants were collected and concentrations of IL-1β, IL-4, IL-5, IL-10, IL-12p70, IL-13, IFNγ, IL-17A, TSLP, TGF-β and TNF were measured by use of sandwich ELISA (OptEIA kits, BD Biosciences and/or Ready-Set-Go ELISA kits from eBioscience) or by cytometric bead arrays (BD Biosciences) as per the respective manufacturers' instructions.

2.9 Histology

Tissues were fixed by one of two methods; cryopreservation in O.C.T. compound (TissueTek) or fixed in 10% neutral buffered formalin solution (Sigma) for a maximum of 24 hours. Samples were then transported for processing by specialist pathology units (Cairns Base Hospital Pathology Unit or QIMR Pathology Laboratory). Samples fixed in formalin were embedded in paraffin and 6-10 μm sections were cut for analysis. Hemotoxylin and eosin (H&E) or periodic acid Schiff (PAS) + Alcian Blue staining were performed on sections for histological analyses.

Histological scoring was performed using a modified scoring system (279). Briefly colon cross-sections were assessed for number of ulcers, where no ulcers = 0, 1 ulcer = 1, 2 ulcers = 2, 3 ulcers = 3 and >3 ulcers = 4. Each ulcer should be about 200 μM in length; where ulceration occurred in large patches scoring was performed in 200 μM intervals. Epithelium integrity was assessed as 0 = normal morphology, 1 = loss of goblet cells in one area, 2 = loss of goblet cells in more than one area, 3 = loss of crypts in one area, 4 = loss of crypts in more than one area. Cellular infiltrate was scored based on 0 = no infiltrate, 1 = infiltrate around crypt bases, 2 = infiltrate extending to the muscularis mucosa, 3 = extensive infiltration reaching the muscularis mucosa, 4 = infiltration of the submucosa with oedema. Lymphoid follicles were scored as none = 0, 1 = 1, 2 = 2, 3 = 3, >3 = 4, for a maximum possible histological score of 16.

2.10 Real time PCR

2.10.1 Total RNA extraction

For cells in suspension: 2 million cells were pelleted by centrifugation at 500 *g* for 5 min and resuspended in 1 ml of Trizol (Invitrogen). For colon samples, a small (<0.5 cm) piece of colon was collected into 2 ml of Trizol and a metal bead and were dissociated on a TissueLyser (QIAGEN) at 30 shakes per second for 15 min. Total RNA extraction was performed by phenol:chloroform separation as per the manufacturer's instructions. Briefly, cells were lysed in Trizol and centrifuged to remove cellular material. Chloroform was added at 20% v/v of initial Trizol volume and mixed by shaking violently. After centrifugation (1200 *g* for 15 min), the clear upper phase containing the RNA was removed into a new tube. Isopropanol was added at 50% initial Trizol volume and allowed to interact with the upper phase for 10 min at room temperature before centrifugation. The resulting RNA pellet was washed in 75% ethanol and then re-solubilised in RNase free water. RNA quantities were assessed on a Nanodrop 2000 (Thermo Scientific). If necessary, degradation of RNA was assessed on an Agilent 2100 Bioanalyzer.

2.10.2 cDNA synthesis

Following treatment of RNA with RQ1 DNase (Promega), first strand cDNA was produced with random hexamers (Invitrogen) from 0.5 – 1.0 µg of total RNA by using SuperScript III reverse transcriptase (Invitrogen) as per the manufacturer's instructions on a Veriti PCR machine (ABI).

2.10.3 Semi-quantitative PCR

Levels of transcription were measured by comparing cross-threshold values to a standard curve made of a pool of all samples. Samples were tested in dilutions of up to 1:600 using SYBR Green (QIAGEN). A Rotor Gene 6000 (QIAGEN) was used for real time thermal cycling. Melting curve analysis was used to check that a single product had been amplified. All genes were normalised for levels of transcription relative to the housekeeping gene β-actin.

Primers used were:

β-actin sense: TGGAATCCTGTGGCATCCATGAAAC, **antisense:**

TAAAACGCAGCTCAGTAACAGTCCG;

FIZZ-1 sense: GTCCTGGAACCTTTCCTGAG, **antisense:** AGCTGGATTGGCAAGAAGTT;

YM1 sense: CTGAGAAGCTCATTGTGGGA, **antisense:** CTCAGTGGCTCCTTCATTCA;

Arg-1 sense: CAGAAGAATGGAAGAGTCAG, **antisense:** CAGATATGCAGGGAGTCACC;

NOS-2 sense: ACCTTGTTCAGCTACGCCTT, **antisense:** CATTCCCAAATGTGCTTGTC;

IL-6 sense: CCGGAGAGGAGACTTCACAG, **antisense:** TCCACGATTTCCCAGAGAAC;

IL-17A sense: CCTCCAGAATGTGAAGGTCA, **antisense:** CTATCAGGGTCTTCATTGCG;

IFNγ sense: AGCTCTTCCTCATGGCTGTT, **antisense:** TTTGCCAGTTCCTCCAGATA.

2.10.4 QIAGEN gene arrays

100-well gene arrays were purchased from QIAGEN for analysis of gene up/down regulation in the colons. Two different gene arrays were utilised in this thesis, the mouse wound healing array (PAMM-121ZR) and the allergy and asthma array (PAMM - 067ZR). Arrays were run and analysed as per the manufacturer's instructions with the excel analysis file provided by QIAGEN.

2.11 Statistical analyses

All data were analysed with GraphPad (Version 6; Prism). When three or more groups were compared, a one-way ANOVA was used with a Bonferroni post-test with a 95% confidence interval to compare all groups. When the effect of a treatment over time was compared for different treatment groups, a two-way ANOVA was used with a Bonferroni post-test to compare replicate means over time. P-values lower that 0.05 were considered significant unless otherwise stated. When only two groups were compared, a Mann-Whitney test was used. All results stated in the text are mean ± standard error of the mean. Both representative figures and pooled results are shown throughout this thesis; figure legends will contain details for each figure.

3 Chapter 3 – *Ac*ES induces an antigen specific Th2 response in mice

3.1 Introduction

Helminth parasites have the potential to cause pathological symptoms in the host that can lead to severe morbidity and mortality. However, helminths such as hookworms are finely adapted to living within their hosts while causing minimal pathology in low numbers, as only the heaviest infections will lead to anaemia in healthy subjects. Although several studies have focussed on the immunomodulatory capabilities of the excretory/secretory (ES) products of various helminth species, the molecular, parasitological and cellular mechanisms by which helminths interact with the host immune system to mediate immunoregulatory effects remain incompletely defined. It has already been demonstrated that helminth ES products are able to: induce alternative activation of macrophages (280, 281), stimulate inducible Tregs (205, 282), modulate DCs to induce a Th2 response (283, 284) and inhibit T cell proliferation (285) (reviewed in (286)). Hence, diverse species of helminths have evolved the capacity to regulate distinct arms of the immune system. The following chapter describes the molecular and cellular immune responses that occur following administration of *Ac*ES products into naïve mice.

3.2 Results: *Ac*ES induces a polarized type 2 cytokine response in mice.

As mentioned above, numerous studies have already described potent immunomodulatory functions of various helminth ES products, but it was clear from the literature that no two helminth species were alike, each with its own unique methods for modulating its environment (the host). Thus, it was necessary to first characterise the response elicited by *Ac*ES in a steady state model, as to the best of my knowledge, this had not previously been published. For these experiments, mice (BALB/c or C57BL/6) received 10 µg injections of *Ac*ES intra-peritoneally (i.p.) every second day for a period of 2-3 weeks. Control mice received either PBS or 10 µg OVA. At necropsy, spleens and MLN were harvested and *Ac*ES-specific immune responses were assessed

by *in vitro* stimulation with 10 μg/ml *Ac*ES or OVA for 72 hours and subsequent cytokine ELISA analyses. While stimulation of cells with media alone or OVA resulted in a minimal cytokine response, addition of *Ac*ES resulted in robust production of the type 2 cytokines IL-4, IL-5 and IL-10 specifically in mice that had been previously exposed to *Ac*ES (**Figure 3.2.1**). The production of the proinflammatory type 1 cytokines IFNγ and TNF was not affected by *in vitro* stimulation with *Ac*ES (**Figure 3.2.1**), further emphasising the skewing toward a type 2 cytokine phenotype. Similar responses were observed in the MLN with characteristic increases in type 2 cytokine production (**Table 3.2.1**).

While cell numbers in the spleen did not increase compared to control mice, injection of *Ac*ES resulted in significant cellular expansion in the MLN and peritoneal cavity (**Figure 3.2.2A**). When total cytokine expression from all cells in the peritoneal cavity was assessed there was a significant decrease in IL-10 and significant increase in IL-4 in the *Ac*ES group compared to controls (**Figure 3.2.2 B-C**). However, when the cellular composition was considered, a different picture emerged, with a significant decrease in IFNγ$^+$ CD4$^+$ cells and a significant increase in IL-10$^+$ CD4$^+$ cells in the *Ac*ES group (**Figure 3.2.2 C-D**). Greater than 60% of IL-4$^+$ cells were CD4$^+$ T cells in both the *Ac*ES and the PBS control groups (**Figure 3.2.2 C-D**). There was no difference in the frequency of cells expressing IFNγ or IL-17A between the groups. Similar expression profiles were obtained with MLN and spleen cells.

Interestingly, there was a distinct population of CD4$^+$ T cells that co-expressed IL-4 and IL-10 and was present exclusively in the *Ac*ES treated mice (**Figure 3.2.3A**). These cells were most highly represented at the site of injection, however increased frequency of these cells was also observed in the draining lymph nodes and in the spleen (**Figure 3.2.3B**).

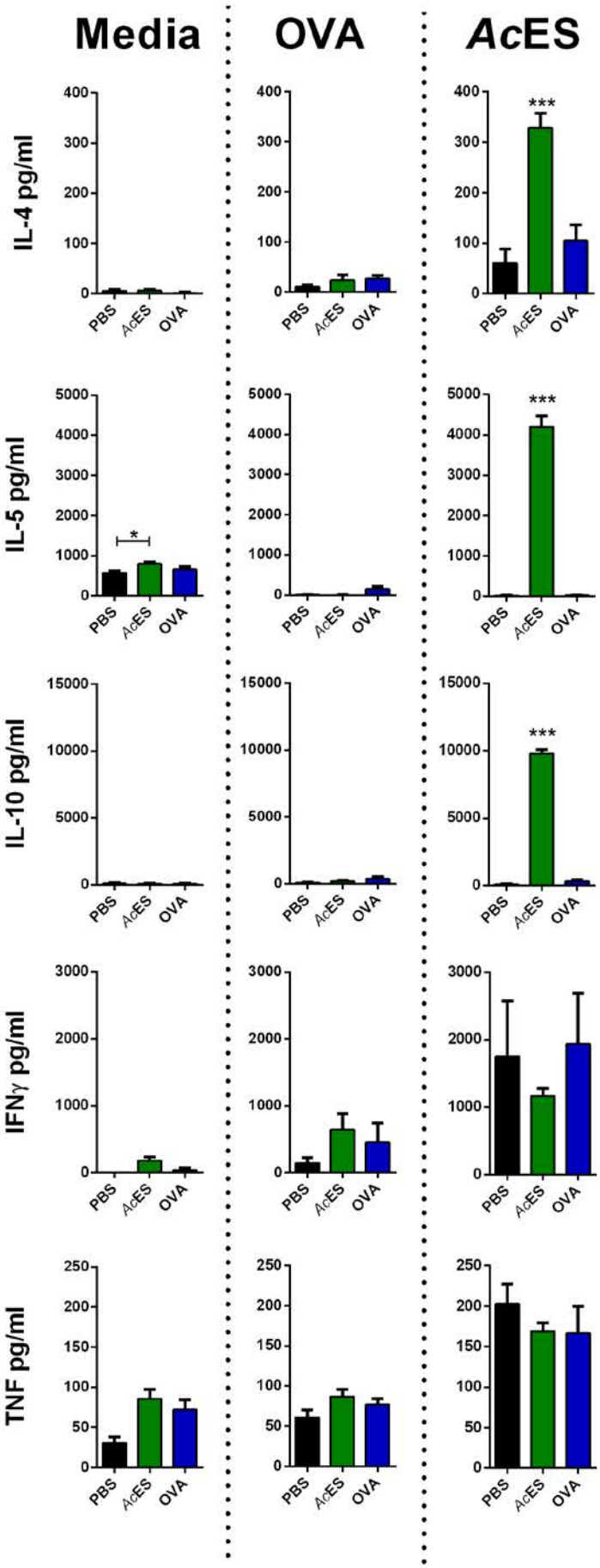
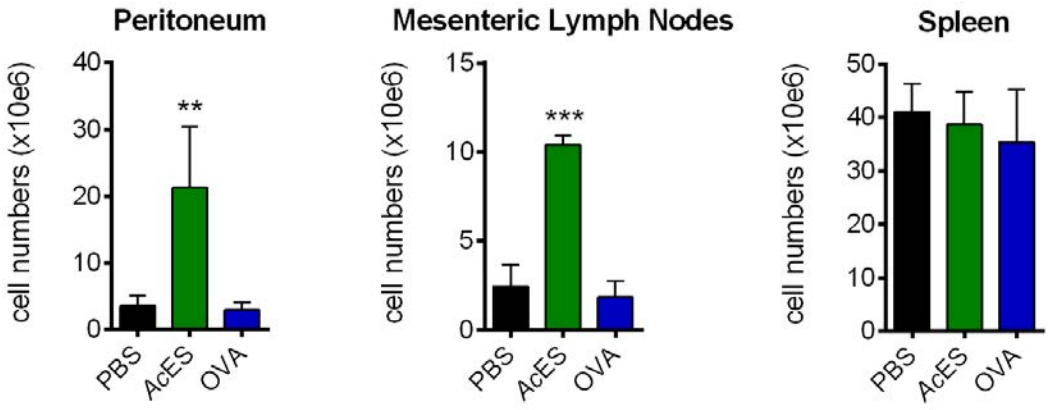

Media
OVA
AcES
IL-4 pg/ml
IL-5 pg/ml
IL-10 pg/ml
IFNγ pg/ml
TNF pg/ml
PBS
AcES
OVA
*
***

Figure 3.2.1: *Ac*ES induces a skewed type 2 cytokine response in mice.
Mice (BALB/c or C57BL/6) received 10 µg injections of *Ac*ES or OVA intra-peritoneally (i.p.) every second day for a period of 2-3 weeks (green and blue bars respectively). Control mice received mock injections of 100 µl of PBS (black bars). Splenocytes were re-stimulated with media, 10 µg/ml of *Ac*ES or 10 µg/ml of OVA (columns) *in vitro* for 72 hours. Cytokine levels were measured by ELISA. Sensitivity of ELISAs were IL-4 & IL-5 = <1 pg/ml, IL-10 = < 20 pg/ml, IFNγ = < 5 pg/ml and TNF = < 15pg/ml. * = $p<0.05$, ** = $p<0.01$ and *** = $p < 0.001$. Graphs are representative of at least three repeat experiments, n = 5 mice per group per experiment.

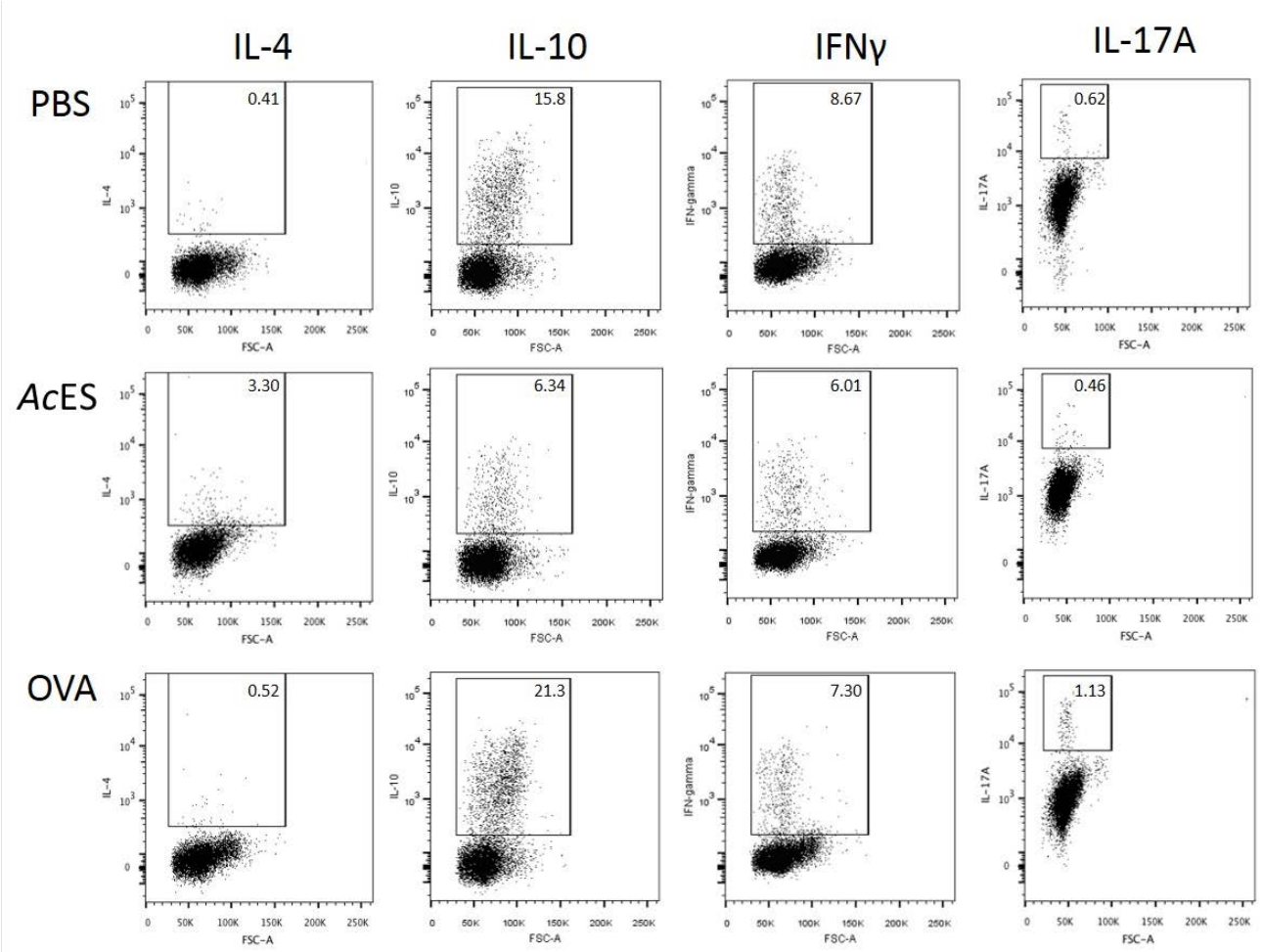
Stimulation	**Cytokine**	**PBS**	***Ac*ES**	**OVA**
***Ac*ES**	IL-4 (pg/ml)	0.1053	66.84	0.158
OVA	IL-4 (pg/ml)	0.0	0.0	0.0
***Ac*ES**	IL-5 (pg/ml)	0.3333	186.9	2.371
OVA	IL-5 (pg/ml)	0.1103	36.32	0.08275
***Ac*ES**	IL-10 (pg/ml)	5.382	1832.0	0.8065
OVA	IL-10 (pg/ml)	0.0	14.31	0.0

Table 3.2.1: Cytokine ELISAs from mesenteric lymph nodes re-stimulated with either *Ac*ES or OVA *ex vivo*.
Mice (BALB/c or C57BL/6) received 10 µg injections of *Ac*ES or OVA intra-peritoneally (i.p.) every second day for a period of 2-3 weeks (columns). Control mice received mock injections of 100 µl of PBS. Mesenteric lymph node cells were re-stimulated with 10 µg/ml of *Ac*ES or 10 µg/ml of OVA (rows) *in vitro* for 72 hours. Cytokine levels were measured by ELISA. Each column represents the test groups of mice (and the protein or buffer they received) and each row represents the mean cytokine response to re-stimulation with *Ac*ES or OVA performed *ex vivo* shown in pg/ml. Sensitivity of ELISAs were IL-4 & IL-5 = <1 pg/ml, IL-10 = < 20 pg/ml. Levels shown lower than this were extrapolated from the standard curve, using a line of best fit model on a BMG Omega Polarstar microplate reader. Results are representative of at least three repeat experiments, n = 5 mice per group per experiment.

A


Peritoneum
Mesenteric Lymph Nodes
Spleen
cell numbers (x10e6)
**
***
PBS
AcES
OVA

B


IL-4
IL-10
IFNγ
IL-17A
PBS
AcES
OVA
0.41
15.8
8.67
0.62
3.30
6.34
6.01
0.46
0.52
21.3
7.30
1.13
FSC-A

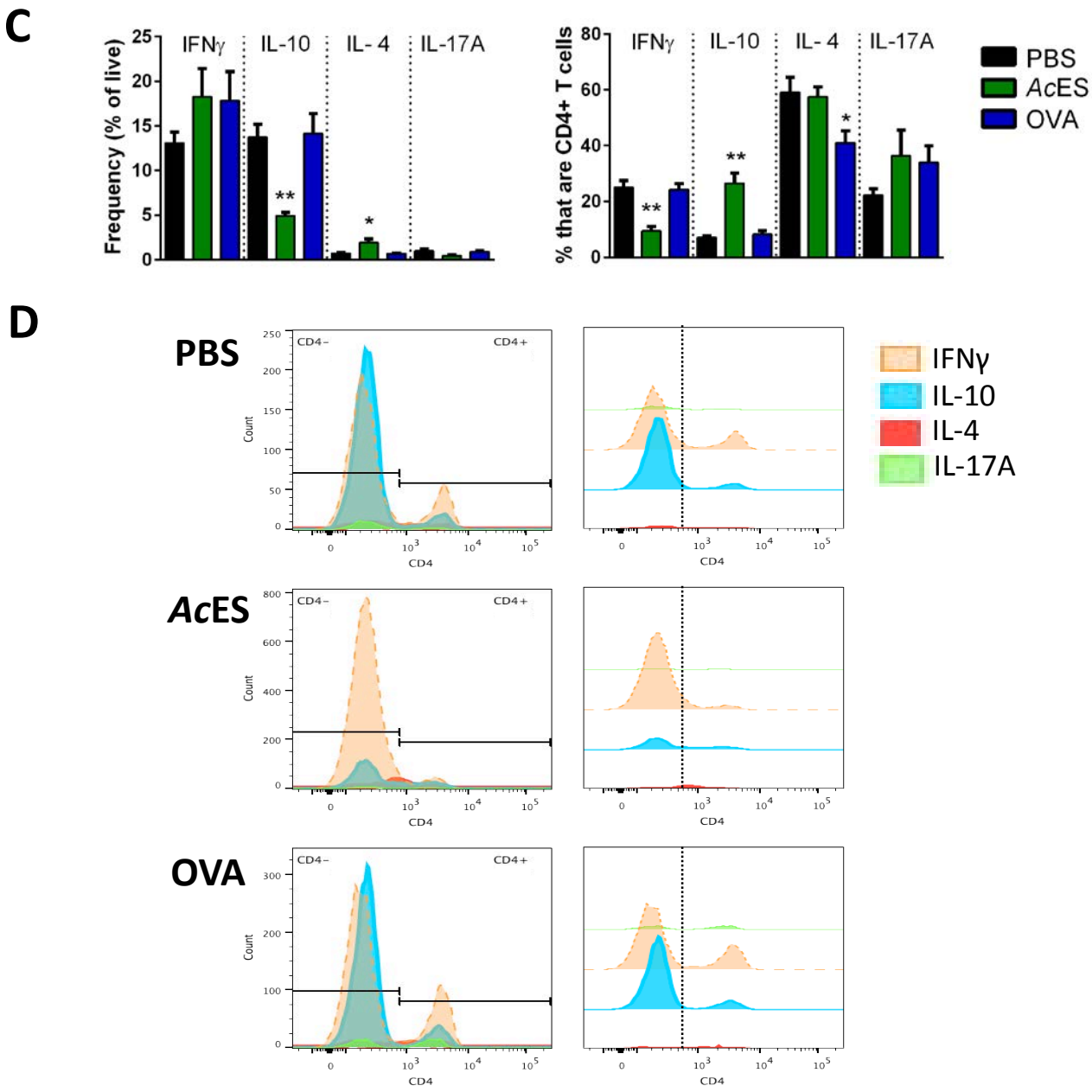


Figure 3.2.2: *Ac*ES treated mice have increased levels of total IL-4 and significantly lower frequency of CD4⁺ IFNγ⁺ cells.

Mice (BALB/c or C57BL/6) received 10 µg injections of *Ac*ES or OVA intra-peritoneally (i.p.) every second day for a period of 2-3 weeks. Control mice received mock injections of 100 µl of PBS. Total cell counts from the peritoneum, mesenteric lymph nodes and spleen of mice treated with PBS, *Ac*ES or OVA (A). Representative flow cytometry plots gated on live single cells in the peritoneum showing cytokine expression on the y-axis vs forward scatter on the x-axis for PBS, *Ac*ES and OVA groups (B). Frequency of IFNγ, IL-10, IL-4 and IL-17A producing cells in the peritoneum and the percentage of those cells that were CD4⁺ T cells (C). Representative histograms of the CD4⁺ and CD4⁻ populations of the IFNγ, IL-10, IL-4 and IL-17A producing cells in the peritoneum (D). Graphed results are pooled from three different experiments, n = 4 mice per group per experiment for a total number of 12 pooled mice per group. Error bars show mean ± S.E.M. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

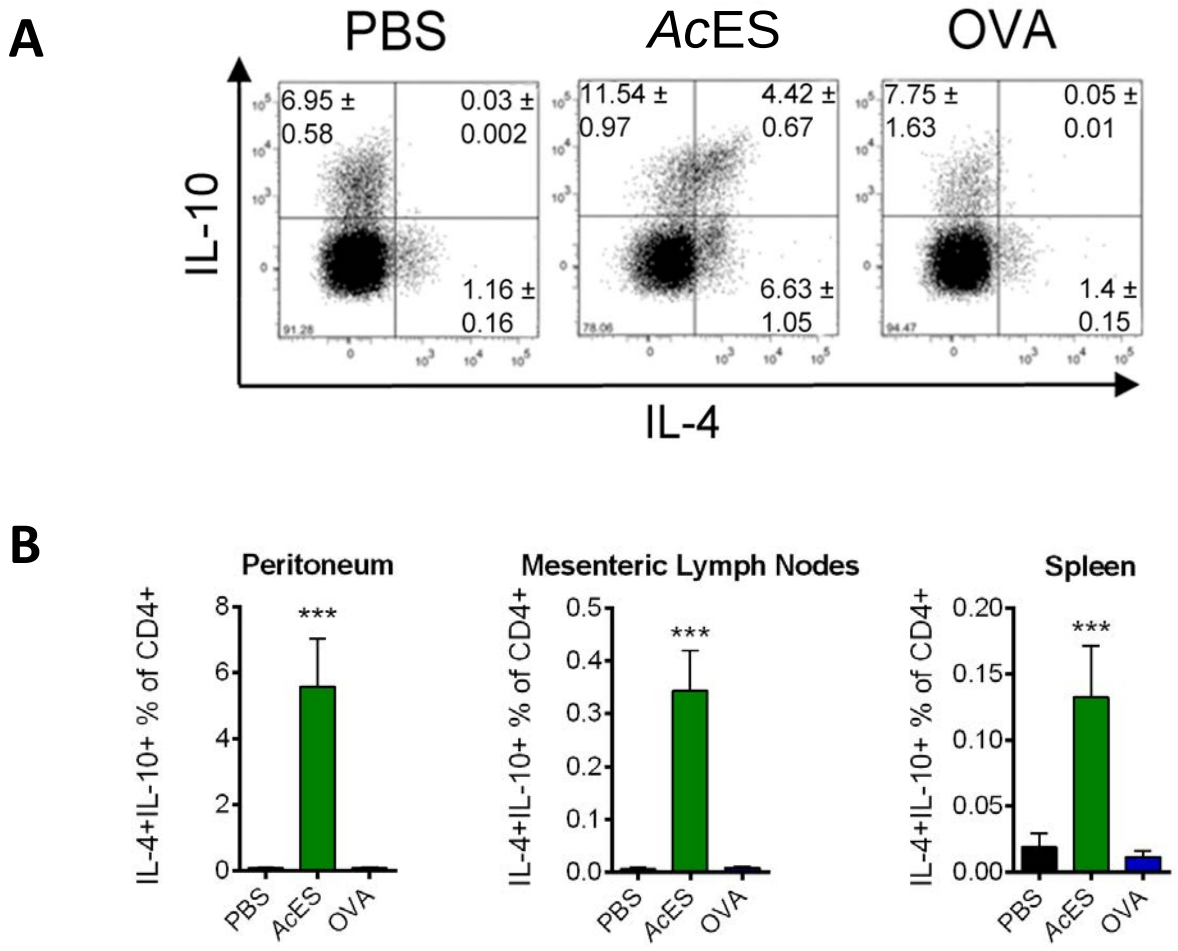


Figure 3.2.3: *Ac*ES injection induces a distinct population of CD4⁺ T cells that co-express IL-4 and IL-10.

Mice (BALB/c or C57BL/6) received 10 µg injections of *Ac*ES or OVA intra-peritoneally (i.p.) every second day for a period of 2-3 weeks. Control mice received mock injections of 100 µl of PBS. Representative flow cytometry plots gated on $CD4^+$ T cells in the peritoneum showing expression of IL-10 (y-axis) and IL-4 (x-axis) (A). Frequencies of $CD4^+$ T cells that co-express IL-4 and IL-10 in the peritoneum, mesenteric lymph nodes and spleen. Graphed results are representative from at least three repeat experiments, n = 6 mice per group. Error bars show mean ± S.E.M. * = p<0.05, ** = p<0.01, *** = p<0.001.

3.3 *Ac*ES injection leads to high numbers of eosinophils and AAMacs.

Induction of a type 2 immune response is typically accompanied by effector leukocyte responses, including eosinophilia and the alternative activation of macrophages. Hence, I next assessed the downstream effector leukocyte responses to *Ac*ES injection. Injection of *Ac*ES resulted in significant population expansion of eosinophils and macrophages to the site of injection (**Figure 3.3.1A-C**)**.** Analysis of the activation status of these macrophages by real-time PCR revealed significant up-regulation of alternative activation markers *Fizz-1* (*relm-α*), *YM1* (*chi3l3*) and *Arg-1* (*arginase-1*) (**Figure 3.3.1D-F**).

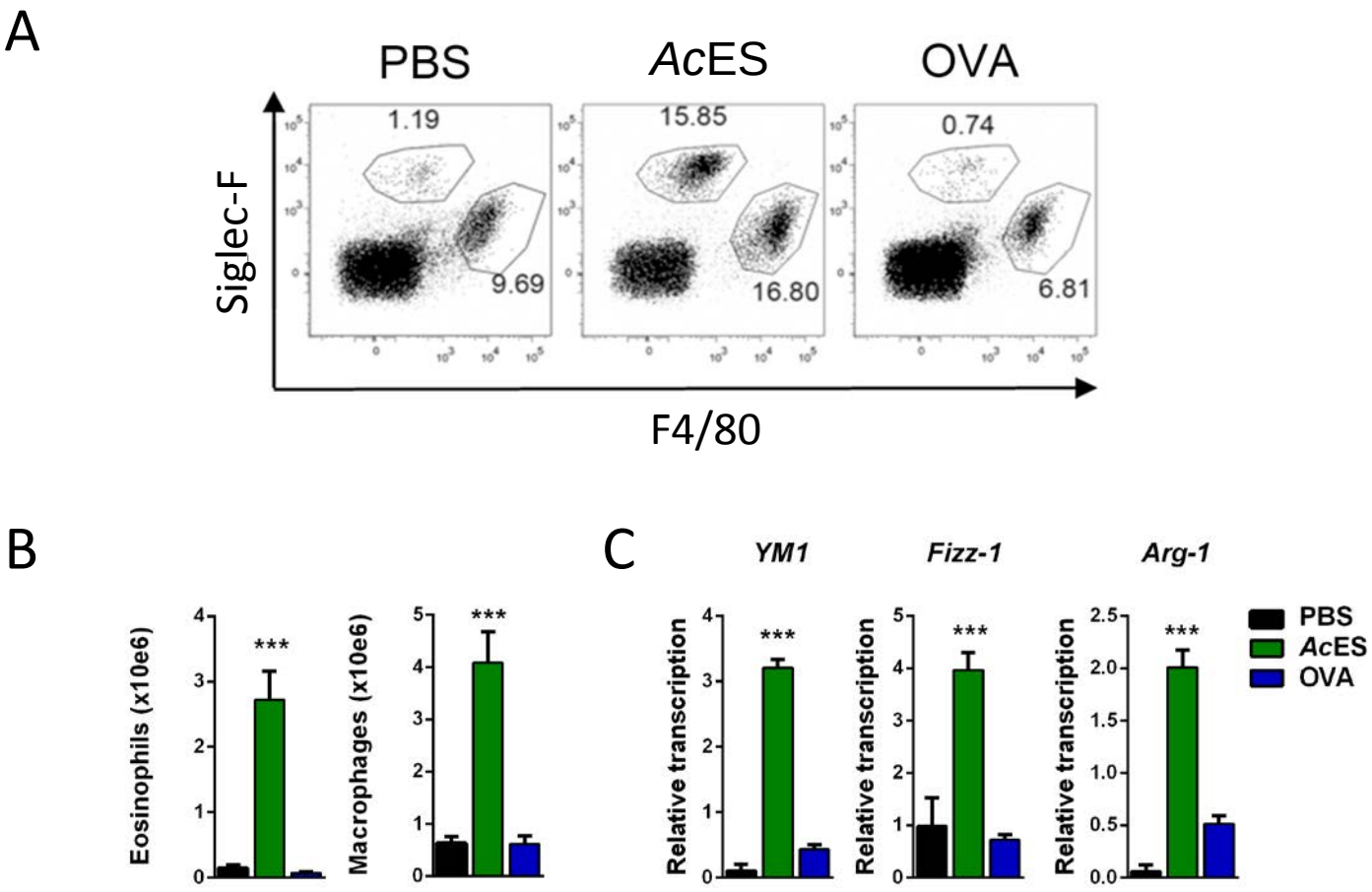


Figure 3.3.1: High numbers of eosinophils and alternatively activated macrophages at the site of *Ac*ES injection.

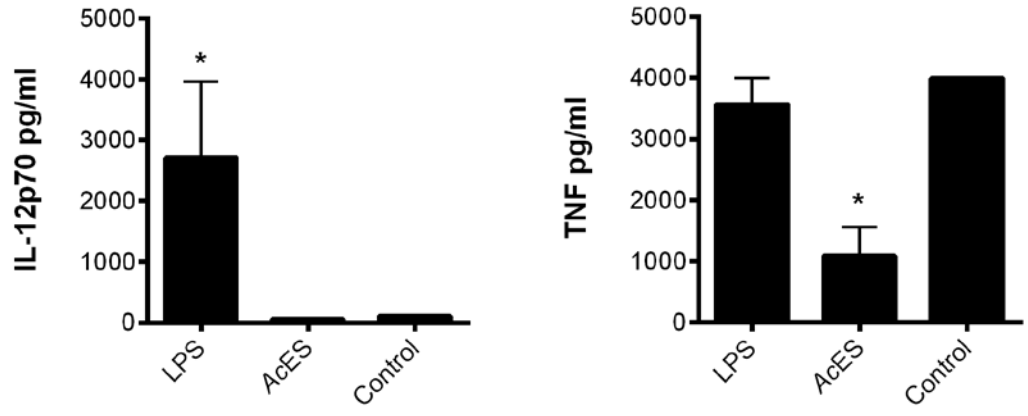
Mice (BALB/c or C57BL/6) received 10 µg injections of *Ac*ES or OVA intra-peritoneally (i.p.) every second day for a period of 2-3 weeks. Control mice received mock injections of 100 µl of PBS. Representative flow plots of peritoneal cells stained for Siglec-F (y-axis) and F4/80 (x-axis) (A). Total numbers of eosinophils and macrophages in the peritoneum (B). Transcript levels of *arginase-1*, *fizz-1* and *ym1* in peritoneal exudate cells was measured by real-time PCR and analysed relative to the transcription of the housekeeping gene β-*actin* (C). Error bars show the standard error of the mean, results are pooled from 3 repeat experiments, n = 5 mice per group per experiment for a total of 15, *** = $p<0.001$.

3.4 Dendritic cells stimulated with *Ac*ES *in vitro* induce a type 2 response in mice and have a non-activated profile

Dendritic cell (DC) numbers were monitored but differences between the groups were either not detected or were highly variable between experiments. Nonetheless, the DC is considered by some to be the most important antigen presenting cell, so particular attention was paid to addressing the ability of *Ac*ES to activate DCs *in vitro*. Bone marrow derived DCs were grown for 10 days *in vitro* and were stimulated overnight with LPS (100 ng/ml), *Ac*ES (10 µg/ml) or left un-stimulated. *Ac*ES-stimulated DCs produced significantly lower levels of the pro-inflammatory cytokines IL-12 and TNF (**Figure 3.4.1A**). When expression of the co-stimulatory molecules CD86 and MHCII were assessed by flow cytometry, it was apparent that *Ac*ES-treated DCs were more similar to the non-treated controls, whereas LPS-treated DCs had significantly higher expression of these co-stimulatory molecules (**Figure 3.4.1B**).

Considering the lack of MHCII and CD86 up-regulation it was hypothesised that these *Ac*ES loaded DCs would be unable to induce any kind of immune response in mice. However, when transferred into naïve mice, *Ac*ES-loaded DCs were capable of inducing a type 2 response similar to that induced by direct injection of *Ac*ES into the peritoneum, with high frequencies of IL-4 producing $CD4^+$ T cells and IL-4/IL-10 co-expressing $CD4^+$ T cells in the peritoneum and the spleen (**Figure 3.4.2**).

A



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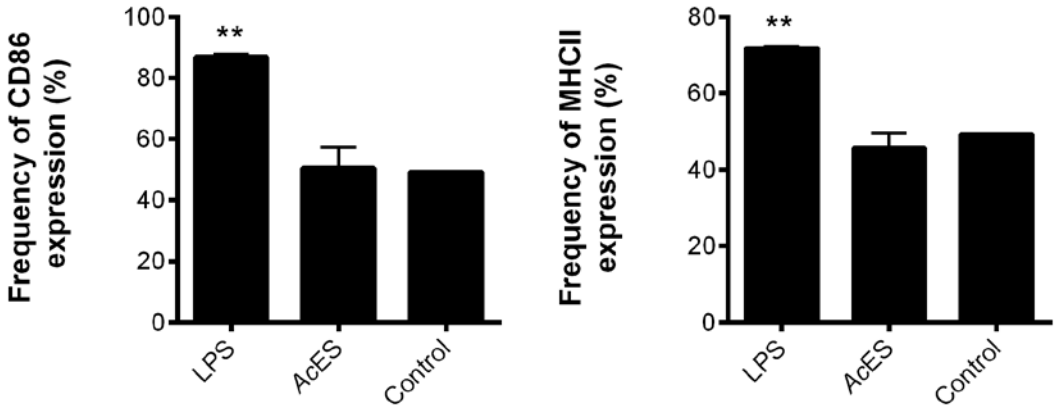


Figure 3.4.1: DCs exposed to *Ac*ES *in vitro* have a non-activated phenotype.
Bone marrow derived DCs were stimulated with LPS (100 ng/ml) or *Ac*ES (10 µg/ml) *in vitro* overnight. IL-12p70 and TNF cytokine production in supernatants were measured by ELISA (A). Sensitivity of ELISAs were 62.5 pg/ml and 15.6 pg/ml respectively. Frequency of CD86 and MHCII expression assessed by flow cytometry (B). Graphed results are representative of at least 2 repeat experiments, n = 5 wells in total with $3x10^6$ BMDCs in each well. Error bars show mean ± S.E.M. * = $p<0.05$, ** = $p<0.01$.

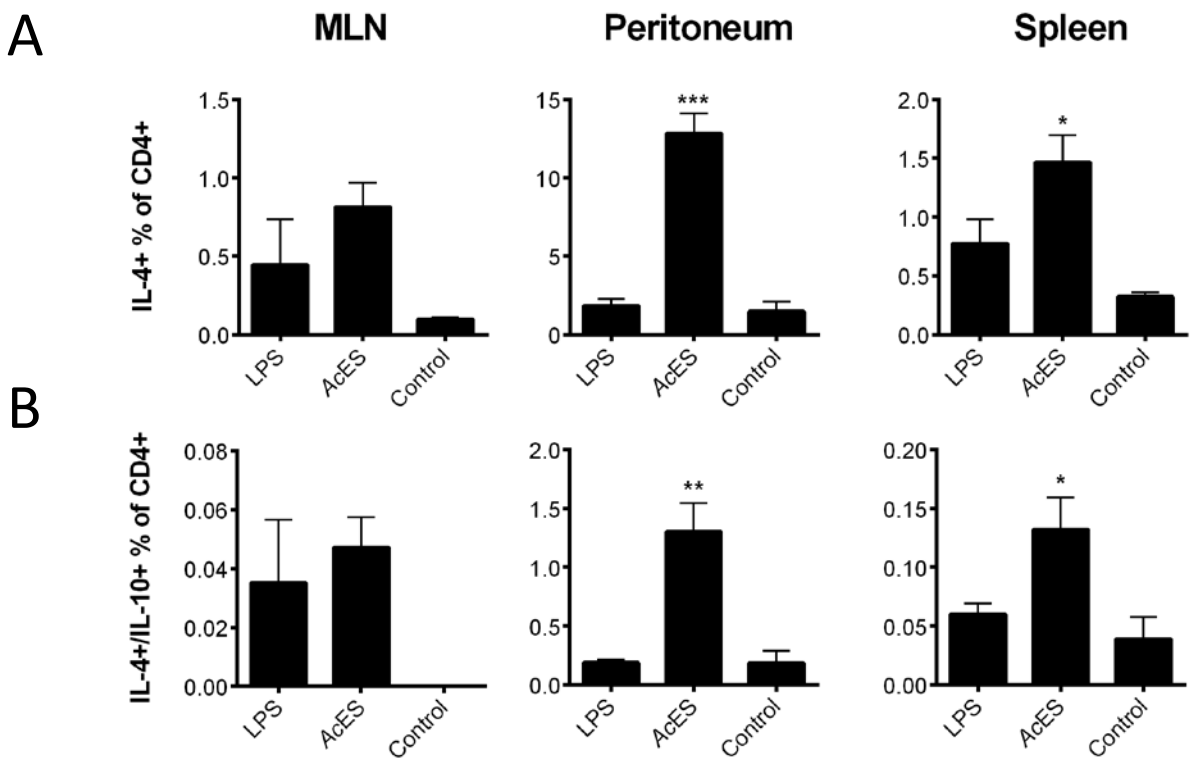


Figure 3.4.2: DCs pre-loaded with *Ac*ES can induce a type 2 response in mice.
Bone marrow derived DCs were stimulated with LPS (100 ng/ml), *Ac*ES (10 µg/ml) or left unstimulated overnight and then injected into naïve mice (10^5 cells i.p.). Seven days later mesenteric lymph node (MLN), spleen and peritoneal exudate cells were analysed by flow cytometry. Frequency of IL-4 producing $CD4^+$ T cells in MLN, PL and spleen (A). Frequency of $IL\text{-}4^+/IL\text{-}10^+$ co-expressing $CD4^+$ T cells in MLN, PL and spleen (B). Graphed results are representative of at least 2 repeat experiments, n = 4 mice per group. Error bars show mean ± S.E.M. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

3.5 Chapter 3 Summary

This chapter has described the immune responses that occur when *Ac*ES is administered to naïve mice in a steady state setting. This was an important first step to determine the phenotype of immune response induced and whether that response was similar to that induced by active hookworm infection. *Ac*ES induced a type 2 antigen-specific response in mice with high levels of IL-4, IL-5 and IL-10 produced by re-stimulated splenocytes. Although total levels of IL-10 production were low compared to controls, IL-10 production specifically by $CD4^+$ T cells was significantly increased when compared to controls, and this response was evident not only at the site of injection but also in the spleen. The similarities between *Ac*ES-induced cytokine expression profiles from two different tissue compartments suggests that components of *Ac*ES are able to diffuse throughout the body and are not limited to the site of injection or the draining lymph nodes. Whether this movement of ES products is due to passive movement via vascular flow or through active pathways by cell uptake and subsequent migration is still unknown, but the systemic effects of *Ac*ES are encouraging for the development of hookworm-derived anti-inflammatory biologics targeting diseases of different tissue compartments.

A distinct population of IL-4/IL-10 co-expressing $CD4^+$ T cells was identified in this study in mice that were treated with *Ac*ES. The role of these cells is unknown, and it is unclear why a cell would produce both an effector cytokine and a regulatory cytokine at the same time. However, it is tempting to speculate that in the human host the balance between IL-4 and IL-10 is necessary for B cell class switching to IgG4 and IgE (287). It may be in the hookworm's interest to skew class switching to IgG4 by increasing levels of IL-10 production, particularly given the role of IgE/IgG4 ratios in protection vs susceptibility to human helminthiases (288-290). Another potential role for these double positive cells may be to limit any type 1 response that may occur during hookworm feeding, whilst at the same time skewing towards a type 2 response that may facilitate wound healing (291) in response to mucosal damage and associated inflammation caused by attached worms. Moreover, IL-4 might be necessary for the production of AAMacs that aid in the healing process at the attachment site.

The alternative activation of macrophages is a common theme amongst helminth infections. Administration of ES products from some helminths, and even recombinant forms of defined ES proteins from the liver fluke *F. hepatica* (292) can directly induce the production of AAMacs. Administration of *Ac*ES to mice resulted in the production of AAMacs, but it was not clear whether this was due to direct or indirect effects of *Ac*ES on macrophages and requires further investigation.

The mechanisms behind the induction of the type 2 response by *Ac*ES are yet to be fully determined. However, my results show that bone marrow derived DCs adopt a non-activated phenotype when exposed to *Ac*ES *in vitro*. This is consistent with recent work with other helminth extracts describing a lack of expression of co-stimulatory molecules on DCs and the ability to induce a Th2 response *in vivo* (196, 282, 284, 293). Most of these reports also describe a reduced ability of helminth extract-primed DCs to produce IL-12p70, a finding that is consistent with my results. It has long been suggested that this lack of IL-12p70 results in a default switch to Th2 development, however this view has recently been challenged in a commentary by MacDonald and Maizels (294), where they refer to the reliance on such a default mechanism as 'unlikely'. The reduction in TNF production by *Ac*ES-primed DCs seen here is also interesting, as TNF has been shown to enhance eosinophil toxicity to *S. mansoni* larvae (295), suggesting another mechanism by which *Ac*ES may be working to protect the worm from innate expulsion mechanisms.

The presence of eosinophilia has always been used as a marker of helminth infection (296, 297), but the role of the eosinophil remains elusive. It has been widely reported that eosinophil granules are toxic to helminth larvae *in vitro* (298-301) and perhaps aid in their death/expulsion, but in hookworm infections, expulsion rarely occurs yet eosinophilia is generally always present (302). Another possible explanation for the presence of large numbers of eosinophils is antigen presentation, as has been described in eosinophils pulsed with *Strongyloides* antigen (303). In terms of helminth therapy and immunomodulation, eosinophils have been largely overlooked, and might play much more influential roles than currently appreciated.

In summary, the results in this chapter indicate a general systematic shift towards a type 2 immune response in mice that receive i.p. injections of a low dose of *Ac*ES for a

period of one week. The predominant Th2 response, accompanied by eosinophilia and type 2 macrophages, is similar to that observed in both experimental (129, 245) and natural (302) human hookworm infections at both mucosal and systemic levels. The conclusion that can be drawn from this chapter is that at least some of the immunomodulatory properties of hookworms reside within their ES products, suggesting that the worm is actively modifying its environment to promote its own survival.

4 Chapter 4 – *Ac*ES protects mice against DSS induced colitis and TNBS induced colitis

4.1 Introduction

IBD in humans is characterised by impaired maintenance of epithelial barrier function, resulting in leakage of commensal bacteria into the intestinal lamina propria and induction of chronic proinflammatory cytokine responses that induce additional epithelial tissue damage (39, 52, 55, 304). The use of rodent experimental models of IBD, such as DSS- and TNBS-induced colitis, has been invaluable in aiding our understanding of the aetiology and pathology of human IBD. Consequently, these well-characterised models were used in the next part of my project to understand the potential influence of hookworm ES proteins in regulating inflammation during IBD. My hypothesis is that *Ac*ES will aid in the amelioration of pathology in mouse models of colitis due to its ability to skew the immune system towards a type 2 and/or regulatory phenotype.

4.2 DSS model of colitis

DSS-induced murine colitis is an experimental model for human UC and involves the feeding of various concentrations of DSS to the mice via the drinking water for up to 14 days. The first sign of pathology can be seen as early as day 1 in younger mice presenting as soft stools, however the mice may not appear physically sick (piloerection, lethargy) until as late as day 6. For my experimental studies, a 3.5% mixture of 36-50 kDa DSS was used and rapid weight loss began at day 3-4 and coincided with more gradual onset of clinical pathology such as occult blood in stools and rectal bleeding. Mice were weighed and clinically scored daily, with the ethical limit of weight loss being 70% of initial weight, at which point mice were euthanased. The mechanism of how DSS induces colitis in mice is not completely understood, however elements of the innate immune system appear to play a role in the ensuing inflammatory response (203, 217, 305-308). Results from my previous chapter highlighted a potent ability of *Ac*ES to regulate both innate (AAMacs and eosinophils) and adaptive ($CD4^+$ T cells) immune

responses, and the DSS model of colitis is ideal to further interrogate the function of *Ac*ES in an innate model of IBD.

4.3 Results: Protection from DSS-induced weight loss by *Ac*ES is dose-dependent and correlates with reductions in type 1 cytokines

To assess the ability of *Ac*ES to limit DSS induced pathology, DSS-treated mice were subjected to various doses of *Ac*ES and weight loss was used as the primary outcome. C57BL/6 wild type (WT) mice were provided with 3.5% DSS solution via the drinking water and received daily i.p. injections of 1 µg/ml, 10 µg/ml, 25 µg/ml *Ac*ES, or PBS control. DSS-treated/PBS-injected control mice lost approximately 20% of their weight by day 8, and mice injected with 1 µg/ml *Ac*ES displayed similar weight loss (**Figure 4.3.1A**). While there was a trend for improvement in weight loss with a 10 µg dose of *Ac*ES, a 25 µg dose was necessary to achieve statistical significance (**Figure 4.3.1A**). Consistent with improvements in DSS-related colitic symptoms, there was a dose-dependent decrease in the production the pro-inflammatory cytokines IFNγ and IL-17A from polyclonally stimulated MLN cells (**Figure 4.3.1B-C**). Consistent with my hypothesis, these results suggest that *Ac*ES has a potent ability to limit disease symptoms in a murine model of colitis. Given its optimal therapeutic benefit, a dose of 25 µg would be utilised for any further DSS experiments.

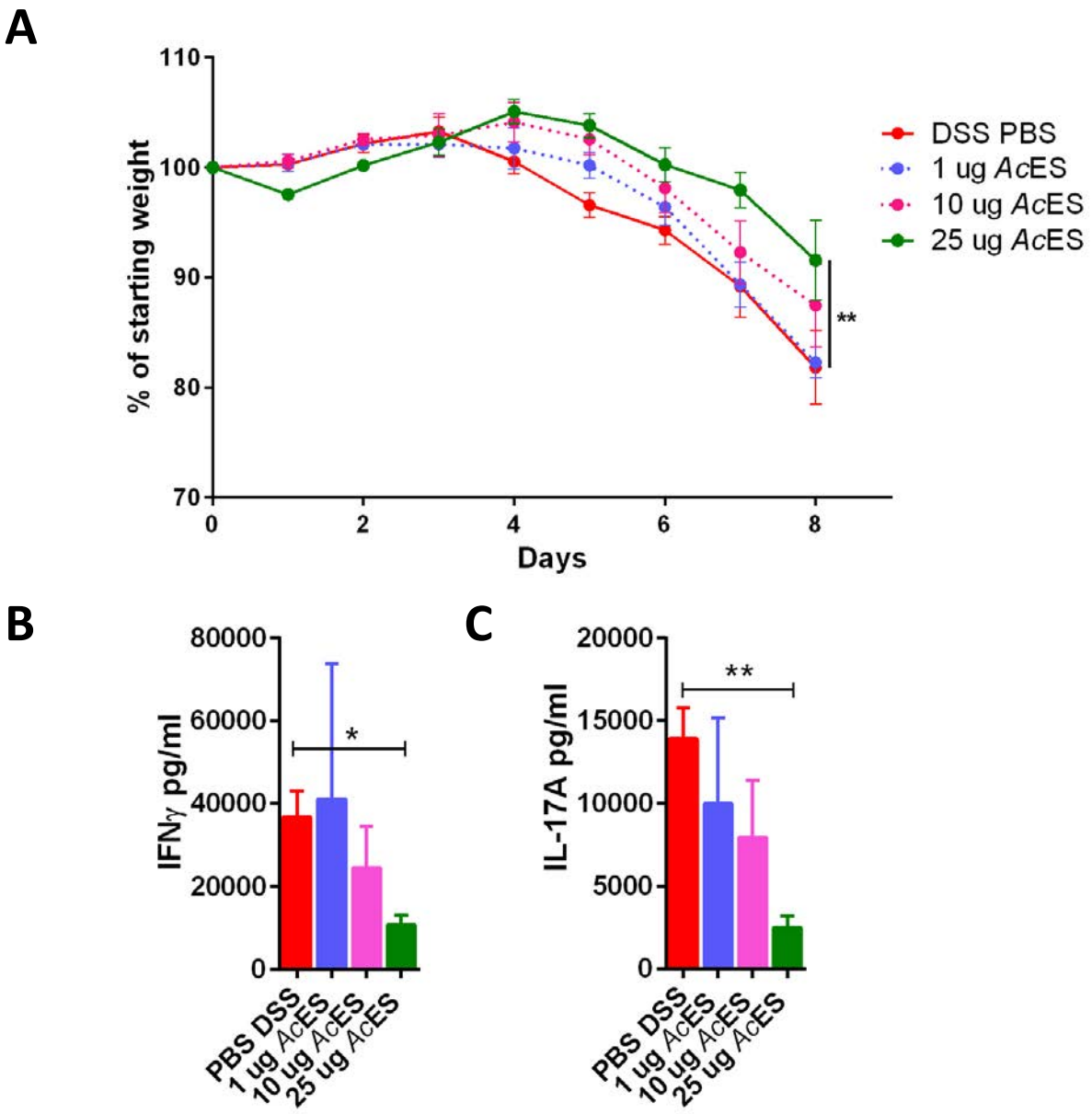
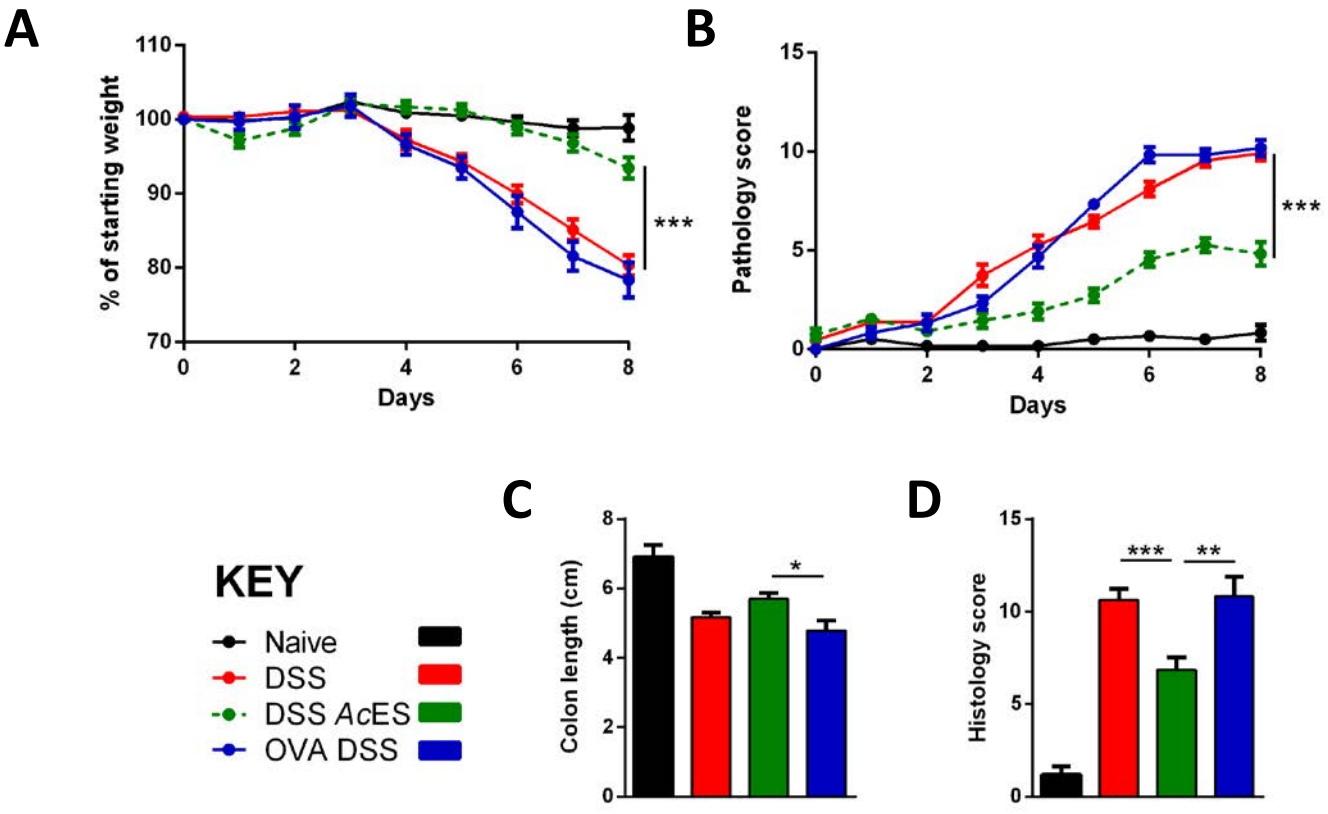


Figure 4.3.1: *Ac*ES provides dose-dependent protection against DSS-induced colitis. Percentage of starting weight for mice receiving 1 µg, 10 µg or 25 µg of *Ac*ES daily in the presence of *ad libitum* 3.5% DSS solution (A). IFNγ (B) and IL-17A (C) production by MLN cells polyclonally stimulated with αCD3 for 72 hours. Error bars show the standard error of the mean, results are one pilot experiment, n = 3-5 mice per group. * = $p<0.05$ and ** = $p<0.01$.

4.4 *Ac*ES protects against DSS-induced intestinal pathology

I next aimed to assess whether *Ac*ES prevented the development of other clinical symptoms and pathology of DSS-induced colitis, including general appearance, consistency of stools, rectal bleeding, colon shortening and intestinal histopathology. Colitis was induced by feeding a 3.5% DSS solution to 6-8 week old female C57BL/6 WT mice via their drinking water for 8 days. Mice received daily i.p. injections with PBS or 25 µg of OVA as a protein-based negative control, or 25 µg of *Ac*ES. Mice were weighed daily and assessed for disease progression as outlined in the methods (see **2.4.2 DSS induced colitis** on pg. **51**). As expected, mice fed DSS and treated with either PBS or OVA demonstrated rapid weight loss from day 3 without recovery (**Figure 4.4.1 A**). Mice who were administered *Ac*ES displayed an initial reduction in weight at day 1, however the mice quickly recovered and maintained a steady weight thereafter, similar to naïve mice that did not receive DSS (**Figure 4.4.1A**). The control mice achieved close to the maximum possible disease score by day 8, taking into account the general appearance of the mice, visible symptoms, and weight loss. Alternatively, mice receiving *Ac*ES maintained a significantly reduced severity of pathology throughout the course of the experiment (**Figure 4.4.1B**). The length of the colon (from caecum to anus) is often used as an indicator of disease progression (215, 217, 309). It has been shown that as the disease progresses the colon becomes increasingly shorter, possibly due to increased inflammation in the area leading to tissue fibrosis (2). Although there was a trend for reduced shortening in the *Ac*ES group, the difference was not significant when compared to the DSS control group. However, there was a significant difference between the *Ac*ES/DSS and OVA/DSS groups (**Figure 4.4.1C**). A more conspicuous indicator of pathology in DSS-exposed and PBS or OVA-treated mice was observed following analysis of histological sections of the colon. Histological analysis showed reduced appearance of sub-mucosal oedema and inflammatory cell infiltrates as well as less apparent epithelial damage and ulceration in the mice treated with *Ac*ES (**Figure 4.4.1D-E**).


A
% of starting weight
110
100
90
80
70
0
2
4
6
8
Days
***
B
Pathology score
15
10
5
0
0
2
4
6
8
Days
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KEY
Naive
DSS
DSS AcES
OVA DSS
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Colon length (cm)
8
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0
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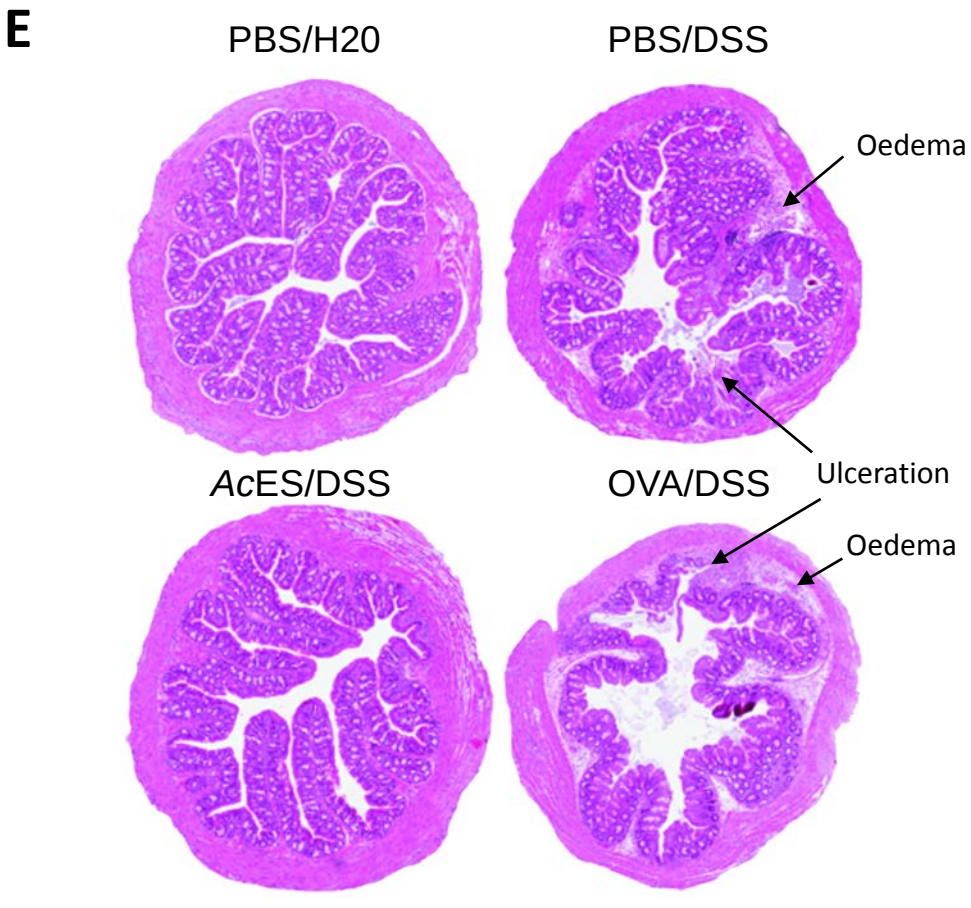

E
PBS/H20
PBS/DSS
Oedema
Ulceration
AcES/DSS
OVA/DSS
Oedema

Figure 4.4.1: *Ac*ES protects mice from intestinal pathology in the DSS model of colitis. Colitis was induced by feeding a 3.5% DSS solution to 6-8 week old female C57BL/6 WT mice via their drinking water for 8 days. Mice received daily i.p. injections with PBS, 25 µg of OVA or 25 µg of *Ac*ES. Percentage of starting weight (A) and clinical pathology (B) assessed daily. Colon length in centimetres (C) and histology scores (D) from colons on day 8. Representative H&E stained histology micrographs of colons (x20 magnification) (E). Error bars show the standard error of the mean, graphical results are pooled from 3 repeat experiments, n = 6-15 (non-DSS control group contained 2 mice per experiment for a total of 6 control mice, all treatment groups had 5 mice per experiment for a total of 15). Histology pictures are representative. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

4.5 *Ac*ES treatment of mice exposed to DSS results in a shift from a type 1 to type 2 cytokine response.

The above results highlighted a significant reduction in IL-17A and IFNγ production by MLN cells when mice were administered 25 μg of *Ac*ES. This was consistent with results from Chapter 3, where I demonstrated reduced IFNγ expression in naive mice treated with *Ac*ES, which corresponded with enhanced type 2 or regulatory cytokine responses. Thus, I next aimed to further characterise the pattern of cytokine expression in DSS-treated/*Ac*ES-injected mice. Intracellular cytokine staining was performed on MLN cells after stimulation with PMA and ionomycin in the presence of brefeldin A. Frequencies of cell populations were analysed by flow cytometry. Consistent with ELISA data from restimulated lymph nodes (**Figure 4.3.1B-C**), there was a trend for reduced frequencies of IFNγ$^+$ (**Figure 4.5.1A**) and IL-17A$^+$ (**Figure 4.5.1B**) producing CD4$^+$ T cells. A significant increase in the frequencies of both IL-4$^+$ (**Figure 4.5.1C**) and IL-4$^+$ IL-10$^+$ co-expressing (**Figure 4.5.1E**) CD4$^+$ T cells was observed in the mice that received *Ac*ES, as previously seen in the absence of DSS in **section 3.2**. Interestingly levels of IL-10 single expressing CD4+ T cells were not elevated compared to controls (**Figure 4.5.1D**) at this site. No differences in cytokine expression by CD8$^+$ T cells were seen (data not shown).

Next, I explored the antigen specific cytokine response to *Ac*ES in DSS-treated mice. To do this, splenocytes were re-stimulated with *Ac*ES *ex vivo* for 72 hours and cytokine production was measured by cytometric bead array. Consistent with the intracellular cytokine staining results in the MLN, and results from **section 3.2**, the *Ac*ES-specific cytokine profile showed a skewing towards a type 2 cytokine response, with increased levels of IL-13, IL-10 and IL-5 and a trend towards reduction in TNF, IL-6 and IFNγ (**Figure 4.5.2**).

While *Ac*ES was able to modulate cytokine responses at the site of injection, draining lymph nodes and spleens, it was important to determine whether these responses would translate to the site of DSS-induced pathology - the colon. Transcription levels of inflammatory mediators iNOS, IL-6, IL-17 and IFNγ in colonic tissue homogenates were determined by real-time PCR (**Figure 4.5.3A**). A significant decrease in transcription of each of these cytokines was noted in the mice receiving *Ac*ES when compared to PBS controls. Additionally, levels of the type 2 cytokines IL-10 and IL-

4 were increased, as measured by ELISA from colon tissue homogenates (**Figure 4.5.3B**). These results further suggest that *Ac*ES modulates the cytokine profile toward a skewed type 2 response that in turn may be helping to ameliorate the pathology in DSS-induced colitis by limiting the effect of inflammatory cytokines like IFNγ.

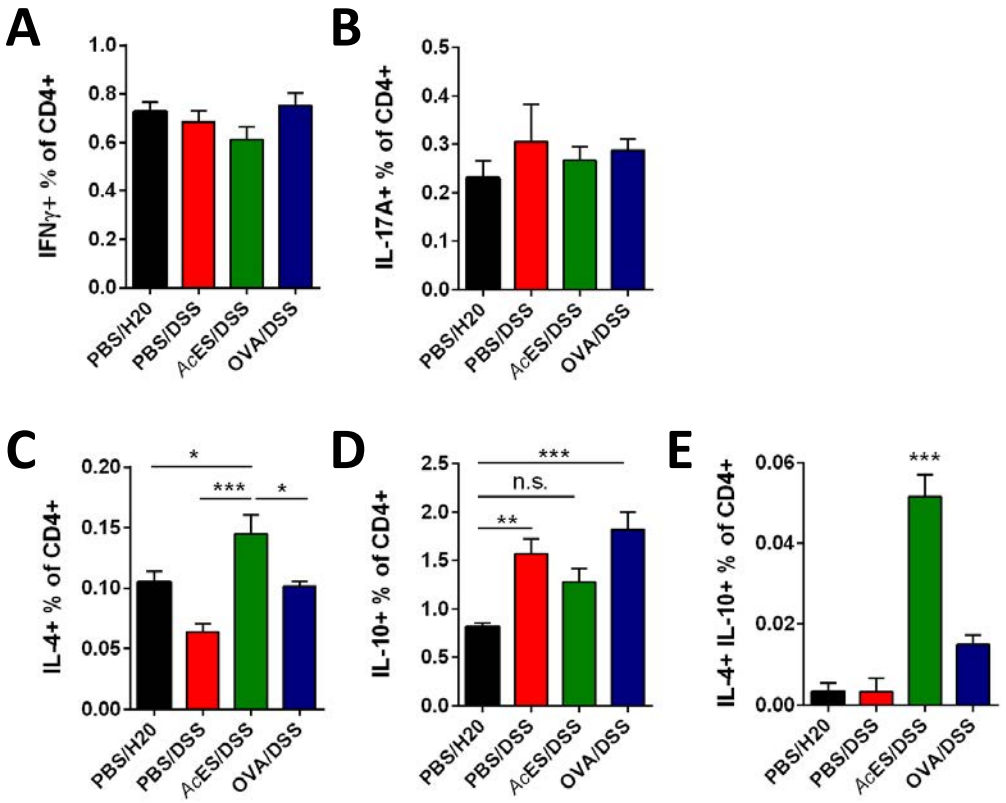


Figure 4.5.1: Intracellular cytokine staining in the MLN shows a shift towards a type 2 response in *Ac*ES-treated mice.

Colitis was induced by feeding a 3.5% DSS solution to 6-8 week old female C57BL/6 WT mice via their drinking water for 8 days. Mice received daily i.p. injections with PBS, 25 µg of OVA or 25 µg of *Ac*ES. Intracellular cytokine staining gated on $CD4^+$ cells showing frequency of $IFN\gamma^+$ (A), IL-$17A^+$ (B), IL-4^+ (C), IL-10^+ (D) and IL-4^+/ IL-10^+ (E) cells. Error bars show the standard error of the mean, graphical results are representative of 3 repeat experiments, n = 6 mice per group. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

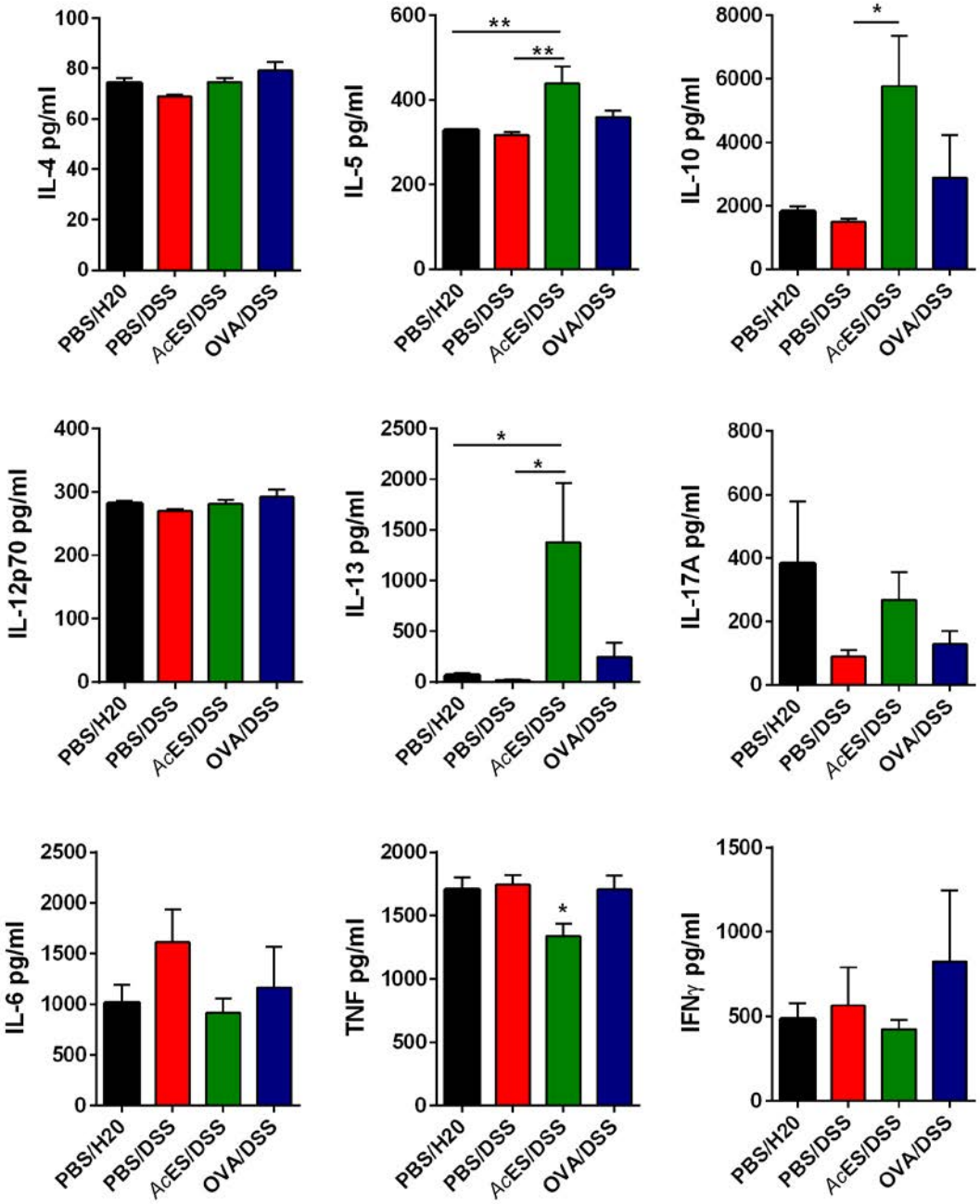


Figure 4.5.2: Antigen specific response to *Ac*ES shows a trend towards a type 2 cytokine response.

Colitis was induced by feeding a 3.5% DSS solution to 6-8 week old female C57BL/6 WT mice via their drinking water for 8 days. Mice received daily i.p. injections with PBS, 25 µg of OVA or 25 µg of *Ac*ES. Splenocytes were removed and re-stimulated with 10 µg/ml of *Ac*ES *ex vivo* for 72 hours. Levels of IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IL-6, TNF and IFNγ were measured concurrently using cytometric bead arrays. Error bars show the standard error of the mean, graphical results are representative of at least 3 repeat experiments, n = 6 mice per group. * = $p<0.05$ and ** = $p<0.01$.

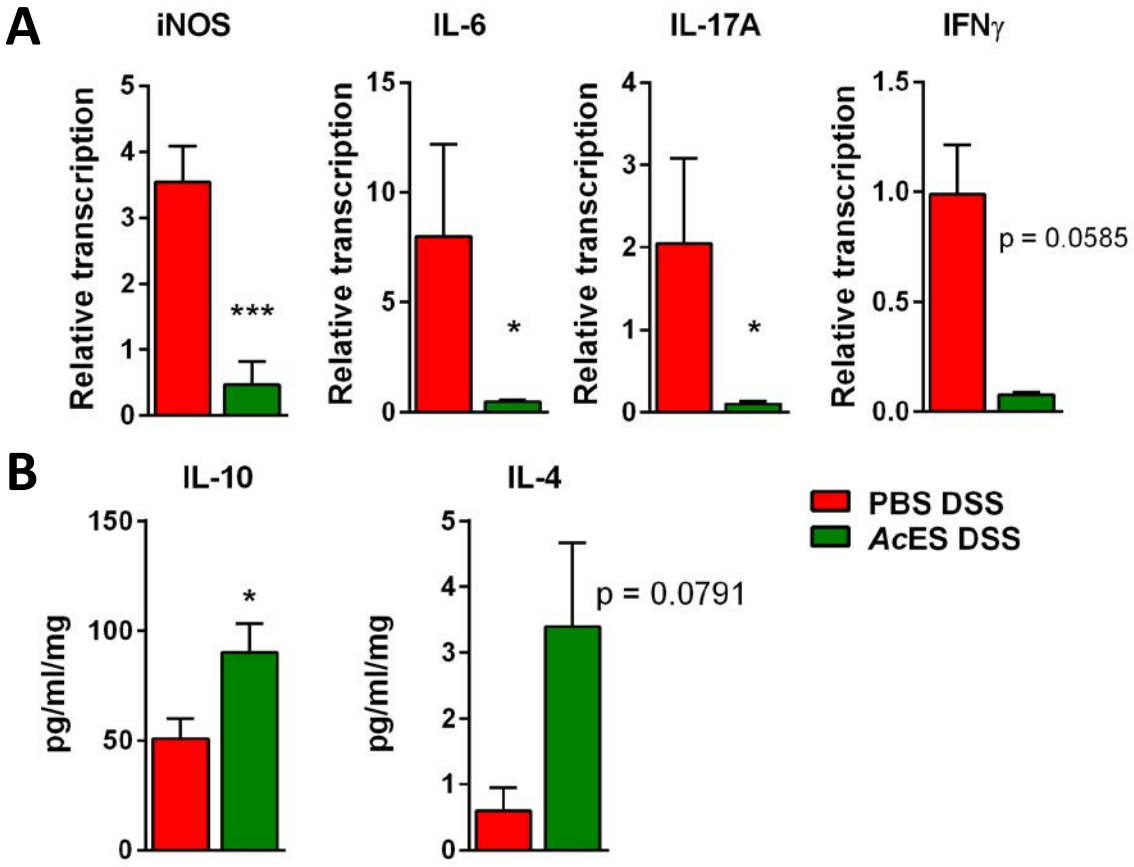


Figure 4.5.3: Cytokine expression in the colon suggests a reduced type 1 response. Mice received 3.5% DSS solution *ad libitum* for 8 days to induce colitis. Mice were administered daily i.p. injections with PBS or 25 µg of *Ac*ES. Relative transcription of type 1 associated genes *iNOS, IL-6, IL-17A and IFNγ* in the colon was measured by real-time PCR relative to β-*actin* expression (A). Production of type 2 cytokines IL-10 and IL-4 was measured in the colon homogenates by ELISA (B). Lowest level of detection of ELISAs were 30 pg/ml for IL-10 and 0.819 pg/ml for IL-4. Error bars show the standard error of the mean, graphical results are representative of at least 3 repeat experiments, n = 6 mice in each group. * = $p<0.05$ and *** = $p<0.001$

4.6 *Ac*ES administration leads to increased numbers of eosinophils and myeloid derived suppressor-like cells at the site of injection

Innate immune cells are important first responders to foreign antigen and can also function as downstream effector cells. In the previous chapter, mice receiving *Ac*ES had increased numbers of eosinophils and AAMacs, both hallmarks of type 2 inflammation, in the peritoneal cavity. I next aimed to determine if this response would also occur during DSS-induced colitis. Results indicated that *Ac*ES mice did have increased numbers of total cells in the peritoneum (data not shown), however the frequency of macrophages was variable (**Figure 4.6.1A**). *Ac*ES induced significant eosinophilia within the peritoneum (**Figure 4.6.1 A**), consistent with that observed in naïve mice treated with *Ac*ES (**Chapter 3 – *AcES* induces an antigen specific Th2 response in mice**). Furthermore, I noted a distinct population of F4/80^{+} CD11b^{hi} GR1^{hi} cells (**Figure 4.6.1B**) that was selectively expanded in the mice receiving *Ac*ES. The surface phenotype of these cells matched the description in the literature of macrophage-like myeloid derived suppressor cells (MDSCs) (310). While the function(s) of MDSCs in this model is unclear, the identification of this cell type following *Ac*ES injection provokes the hypothesis that MDSCs may contribute to immune regulation during hookworm infections.

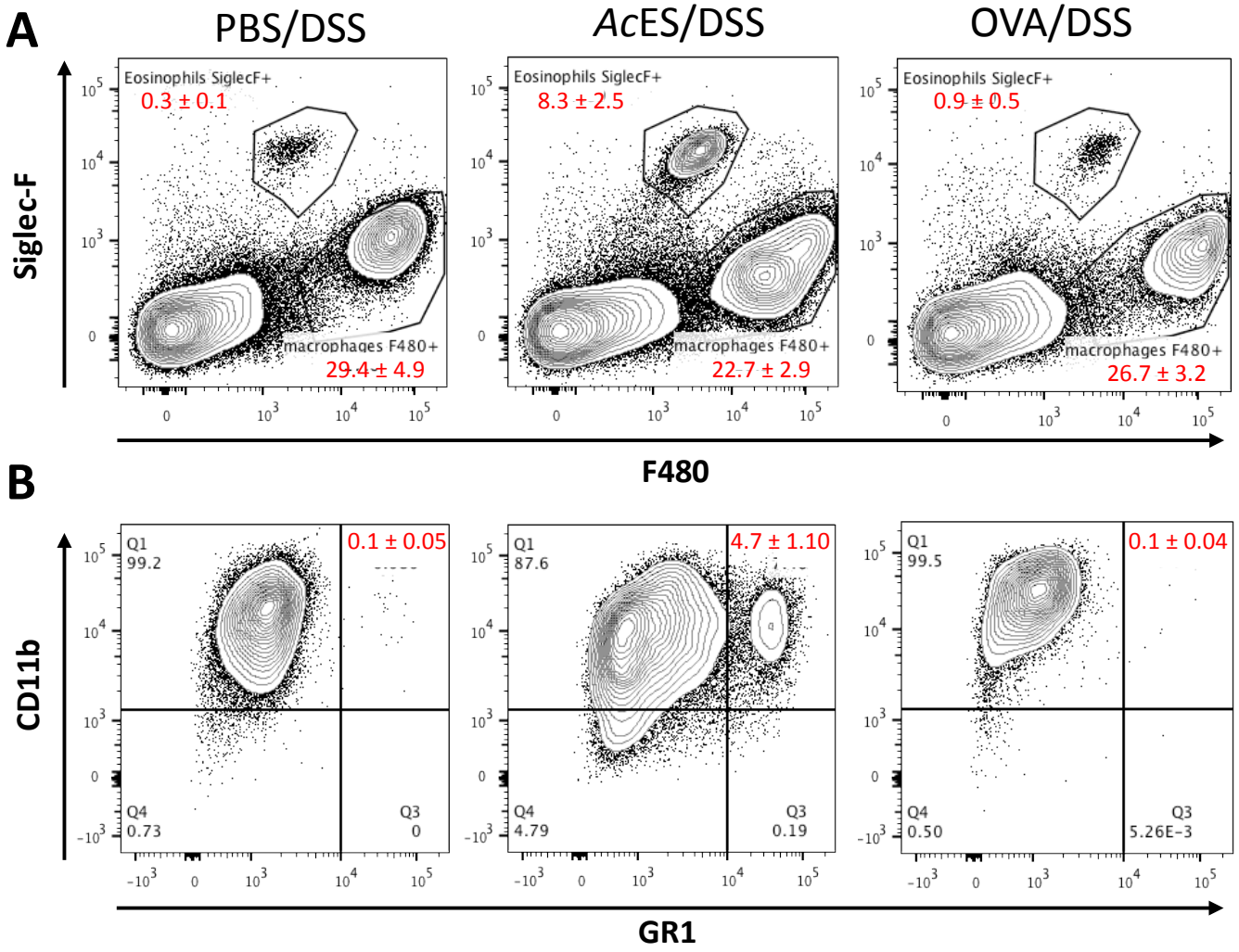


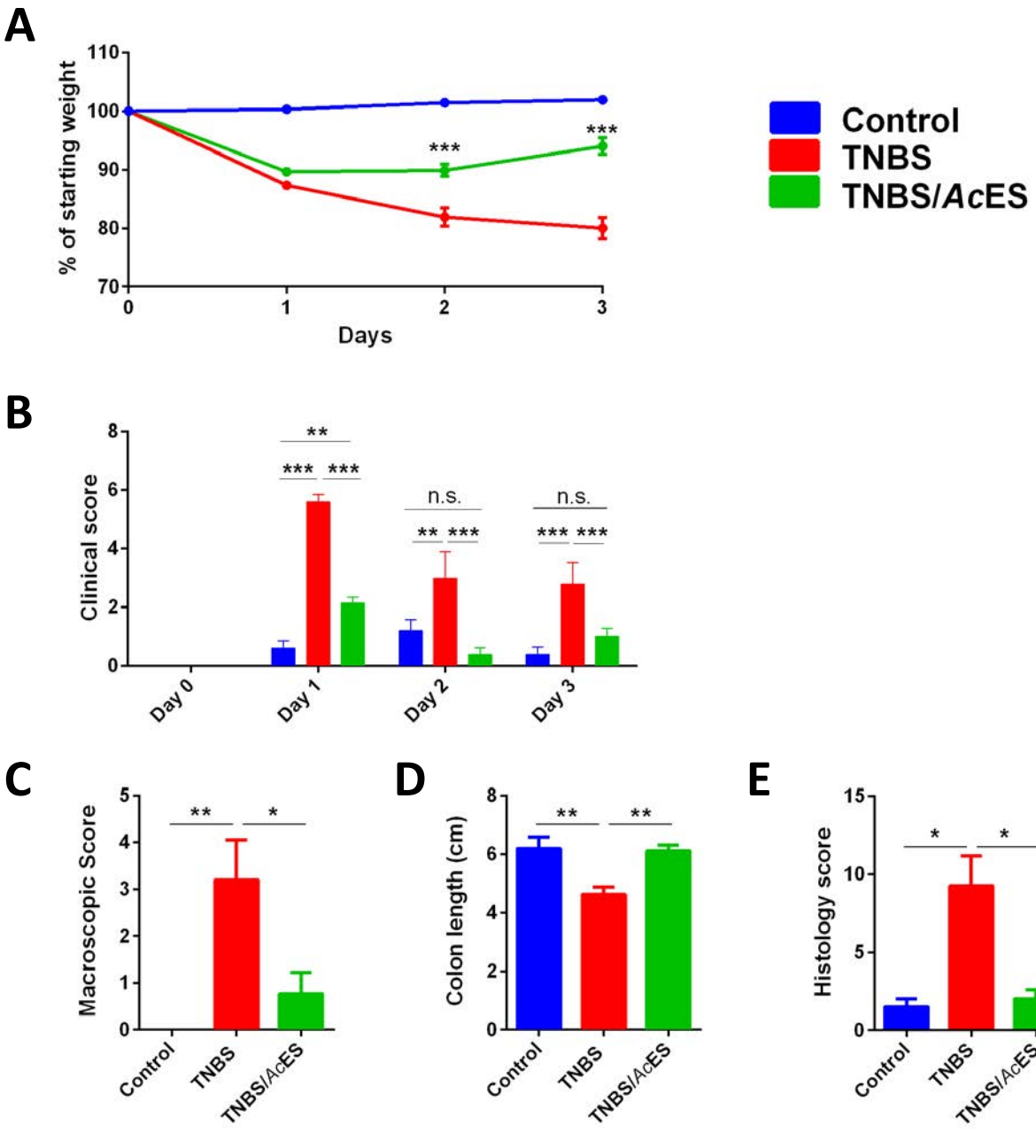
Figure 4.6.1: *Ac*ES administration leads to increased numbers of eosinophils and myeloid derived suppressor-like cells at the site of injection.

Mice received 3.5% DSS solution *ad libitum* for 8 days to induce colitis. Mice were administered daily i.p. injections with PBS, 25 µg OVA or 25 µg of *Ac*ES. Flow cytometry plots showing F4/80$^+$ (x-axis) and Siglec-F$^+$ (y-axis) peritoneal lavage cells, gated on live cells (A). Flow cytometry plots showing GR1$^+$ (x-axis) and CD11b$^+$ (y-axis) peritoneal lavage cells, gated on F4/80$^+$ cells (B). Red numbers in flow plots show the mean ± standard error of the mean, results are representative of 4 repeat experiments, n = 6 mice per group.

4.7 *Ac*ES protects mice from weight loss and overt pathology in the TNBS model of colitis

The DSS model of colitis induces a pathology in mice that is similar to that which is seen in human UC, with a superficial inflammation generally limited to the epithelium and submucosa (311). A pathology similar to Crohn's disease can be achieved with the TNBS (2, 4, 6-trinitrobenzene sulfonic acid) model of colitis, which presents with deeper transmural inflammation (311). The other major difference between these two models of colitis is their dependence on T cells; the DSS model seems to be independent of the adaptive immune system (312), whereas the TNBS model of colitis is largely dependent on T cells (226). In order to assess the suitability of *Ac*ES as a broad-spectrum therapy for multiple intestinal inflammatory disorders, it was of interest to determine whether *Ac*ES also has immunomodulatory capabilities in the TNBS model.

The TNBS model of colitis involves the intra-rectal administration of a pre-determined dose of TNBS mixed with 50% ethanol. The ethanol serves to strip the mucosal layer and allow for the haptenizing TNBS to be in contact with the mucosal cells (313). An acute 4-day TNBS protocol was utilised for maximum inflammation as previously published (314). This acute model differs from the DSS model of colitis as the mice only receive a single dose of *Ac*ES at day 0. Mice were weighed daily and upon euthanasia the colons were assessed for levels of oedema, thickening and ulceration as per **2.4.1 TNBS induced colitis** (pg. **51**). While all mice receiving TNBS lost between 10-20% of initial body weight at day 1, only the mice that received *Ac*ES began to recover their body weight after day 1 (**Figure 4.7.1A**). Clinical pathology scoring indicated that mice receiving *Ac*ES had a better general appearance and were producing well-formed faecal pellets (**Figure 4.7.1B**) compared to untreated mice that received TNBS. The macroscopic score showed that the mice receiving *Ac*ES had significantly less oedema, ulceration and shortening of their colons when compared to the TNBS-only mice (**Figure 4.7.1C**). The length of the colon in the mice receiving *Ac*ES was also significantly longer than that of the TNBS mice, indicating that there was less thickening/fibrosis in these mice (**Figure 4.7.1D**). Differences in the condition of the colon are shown in the histology scores (**Figure 4.7.1E**) and can be clearly seen in the representative histology micrographs (**Figure 4.7.1F**).


A
% of starting weight
110
100
90
80
70
0
1
2
3
Days
***
***
Control
TNBS
TNBS/AcES
B
Clinical score
8
6
4
2
0
**
*** ***
n.s.
** ***
n.s.
*** ***
Day 0
Day 1
Day 2
Day 3
C
Macroscopic Score
5
4
3
2
1
0
** *
Control
TNBS
TNBS/AcES
D
Colon length (cm)
8
6
4
2
0
** **
Control
TNBS
TNBS/AcES
E
Histology score
15
10
5
0
* *
Control
TNBS
TNBS/AcES

F

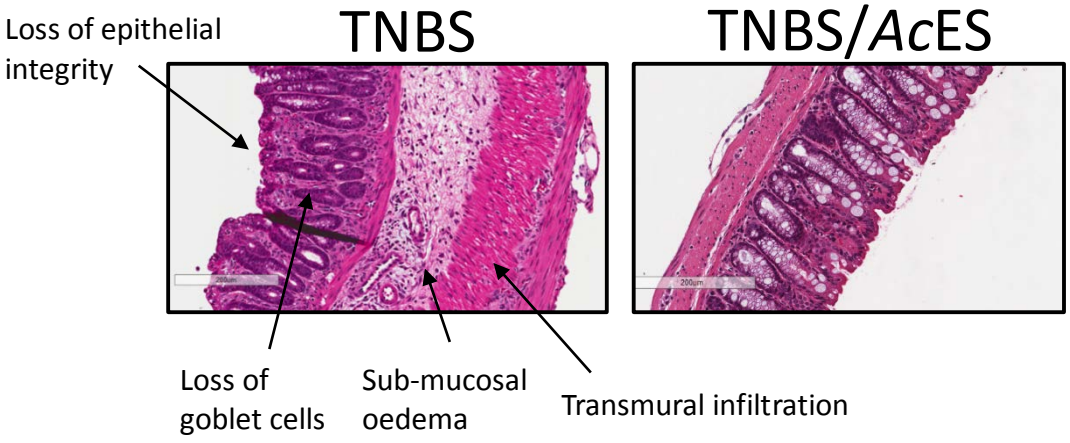

Loss of epithelial integrity
TNBS
TNBS/AcES
Loss of goblet cells
Sub-mucosal oedema
Transmural infiltration

Figure 4.7.1: *Ac*ES protects mice from severe pathology in TNBS-induced colitis.
Mice were given a single injection of 20 µg of *Ac*ES or mock PBS injection 4 hours before TNBS administration. Percentage of weight loss (A) was recorded daily along with clinical pathology score (B). Macroscopic score (C) was assessed upon euthanasia as well as colon length (D). Longitudinal H&E stained histology micrographs of the colon were assessed for histology scores (E). Representative histology micrographs showing loss of epithelial integrity, loss of goblet cells, sub-mucosal oedema and transmural infiltration in TNBS mouse colons compared to *Ac*ES treated colons (F). Bar along lower left corner of histology micrographs indicate 200 µm in length. Error bars show the standard error of the mean, graphical results are pooled from 4 repeat experiments, n = 3-4 mice per group per experiment for a total of 12-16. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$

4.8 *Ac*ES treatment leads to reduced frequencies of IL-17$^+$ CD4 cells, lowered IL-17 production and a down-regulation in *rorc* expression in the TNBS model of colitis

In both the steady state and during DSS-induced colitis it was shown that *Ac*ES had the potential to reduce type 1 cytokine responses and shift the response towards a type 2 phenotype. However, in this acute model of TNBS-induced colitis a shift towards a type 2 phenotype was not detected. Frequencies of IFNγ$^+$ CD4$^+$ cells remained unchanged between groups (**Figure 4.8.1A**), there was also no significant reduction in IL-17A$^+$ CD4 T cells in the MLN (**Figure 4.8.1B**). Results showed that levels of IL-17A (**Figure 4.8.1C**) and TNF production in the colon cultures (**Figure 4.8.1D**) were equally non-significant. However there was a trend for reductions in these inflammatory mediators, leading to the query of changes at the transcription level.

To assess in greater detail the impact of *Ac*ES injection on the expression of a range of different cytokines and type 2 related genes in the colon, I undertook high-throughput gene profiling using a QIAGEN PCR array (see **2.10.4 QIAGEN gene arrays** pg.57). As a result of the array's restricted sample size (n = 3), it was decided to change the significance cut-off to $p<0.1$. All of the genes presented in **Figure 4.8.2** were either up- or down-regulated in *Ac*ES-treated mouse colons in respect to the TNBS mouse colons using β-*actin* as a reference gene. Expression of both *IL-17A* and the transcription factor *rorc* was reduced in the colon of mice treated with *Ac*ES compared to non-treated mice (**Figure 4.8.2**). There was downregulation of several inflammation associated genes in the *Ac*ES treated mice, which may reflect the decreased level of inflammation in the *Ac*ES-treated mice compared to the non-treated controls. There was however, upregulation of *retnlg* (*RELM-γ/Fizz-3*), a gene from the same family as *retnla* (*RELM-α/Fizz-1*) that is commonly associated with AAMacs (315). *RELM-γ* is produced by colonic epithelial cells (316), and its upregulation in *Ac*ES-treated mice might just reflect a more intact epithelium capable of producing *retnlg* compared to TNBS-only mice which had lost all colonic epithelium. *Ac*ES treated mice had more than 15-fold downregulation of the integrin alpha subunit CD49D (alpha 4 subunit/*ITGA4*), which when combined with CD29 (beta-1 subunit/ITGB1) becomes a homing the receptor for lymphocytes (very late antigen-4), and is thus highly upregulated during inflammatory reactions (317). CD49D

can also combine with ITGB7 (beta 7 integrin) to form LPAM (lymphocyte payer patch adhesion molecule), which is necessary for T cell homing into lymphoid tissues in the intestines (318). There was also downregulation of *OX-40*, which is a co-stimulatory molecule on T cells that has been associated with promoting expression of apoptosis inhibitor BCL2, which in turn prevents T cells from undergoing apoptosis and thereby produce more cytokines (319). Of interest, two other genes, *postn and adam33* which are prominent in allergic responses including asthma (320), were also downregulated, suggesting an immunoregulatory role for *Ac*ES outside of the intestines. A graph of the remaining results that did not reach statistical significance can be found in section **8.2 Supplementary material from Chapter 4** (**Figure 8.2.1**).

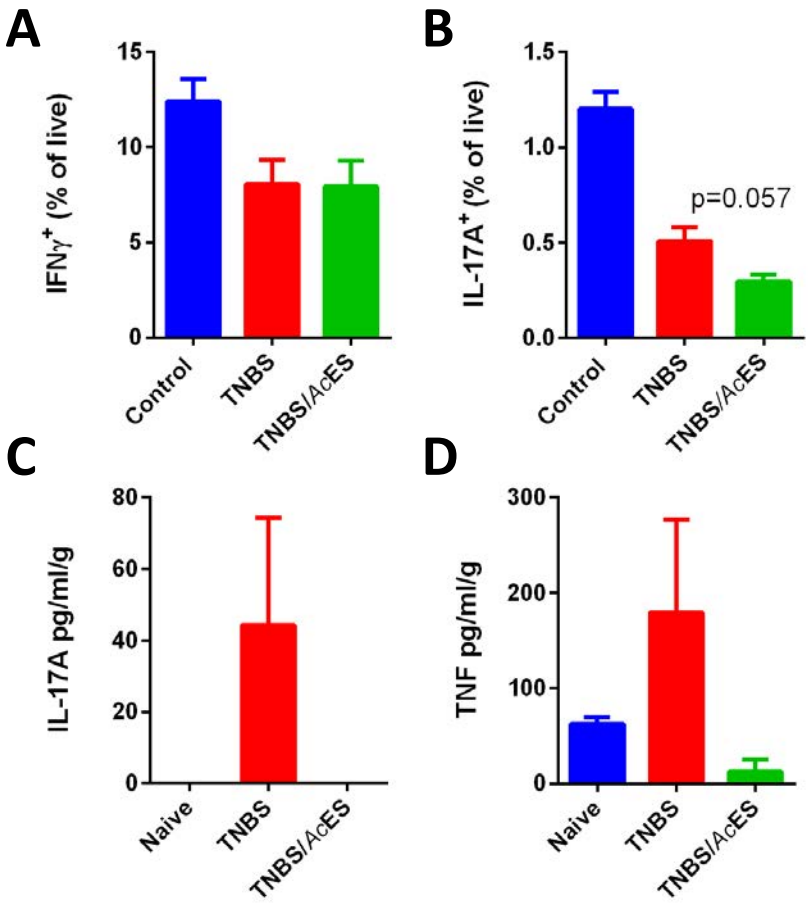


Figure 4.8.1: *Ac*ES limits IL-17A production in the TNBS colitis model.
Mice were given a single injection of 20 µg of *Ac*ES or mock PBS injection 4 hours before TNBS administration. Intracellular cytokine staining of MLN cells shows no change in IFNγ$^+$ CD4$^+$ frequencies (A) but did show a trend for reduced frequency of IL-17$^+$ CD4$^+$ cells (B). Production of IL-17A (C) and TNF (D) in colon cultures. Error bars show the standard error of the mean, graphical results are representative of at least 3 repeat experiments, n = 4.

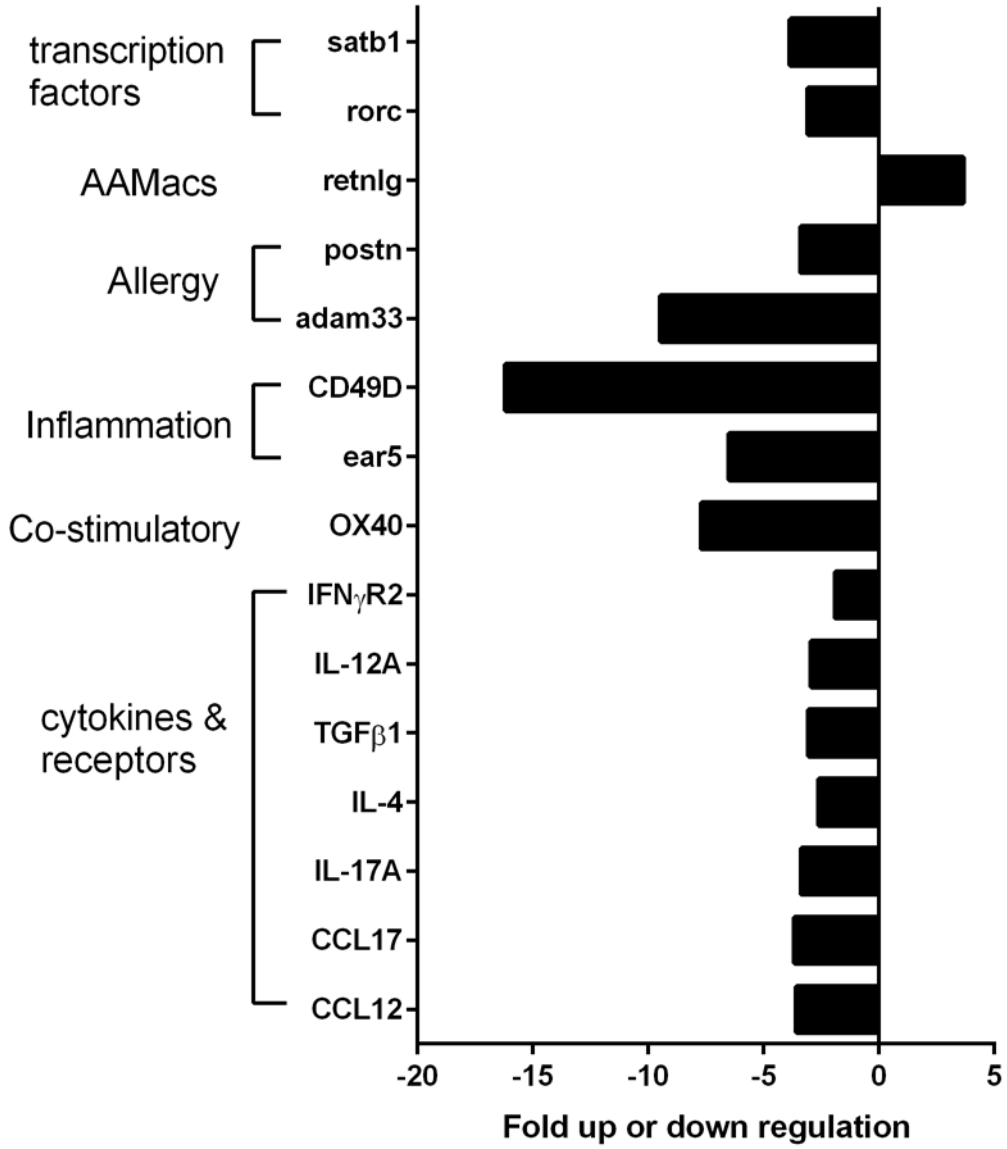


Figure 4.8.2: Type 2 related gene array in mouse colon tissue during TNBS-induced colitis.

Mice were given a single injection of 20 µg of *Ac*ES or mock PBS injection 4 hours before TNBS administration. Graph shows genes that are significantly up or down regulated in colons of *Ac*ES-treated mice compared to mock PBS injection controls during TNBS-induced colitis, with $p<0.1$, n = 3 mice per group.

4.9 Chapter 4 Summary

In this chapter I have explored the immunomodulatory capacity of *Ac*ES in two different mouse models of colitis. The first model involves the use of DSS in the drinking water to induce a slow but aggressive form of colitis that seems to be independent of the adaptive immune system (312). Daily injections of 25 µg of *Ac*ES diminished the pathology and associated weight loss in this model of colitis. Moreover, I found that the skewing towards a type 2 response that was shown in the previous chapter was also present within this model and was consistent with reduced levels of type 1 cytokines.

In the second model of colitis, intra-rectal administration of TNBS in an ethanol solution induced a very acute form of colitis that is highly dependent on T cells (226). In the C57BL/6 strain of mice, the acute onset of colitis gradually fades over a period of 4-7 days, where mice eventually regain their body weight. In this model, a single injection of 20 µg of *Ac*ES resulted in a very rapid recovery of these mice at day 2 post-TNBS with little to no signs of pathology. The skewing towards a type 2 phenotype was not evident in this model, probably due to a combination of the short time frame and lack of repeated antigenic presentation as the mice only received a single *Ac*ES injection at day 0. Nonetheless there was a trend for reduction in IL-17A responses in the *Ac*ES treated mice versus the mock treated mice, including down-regulation of the transcription factor *rorc*, thought to be involved in the differentiation of Th17 cells (321).

It has been previously shown that *S. mansoni* soluble worm proteins (*Sm*SWP) and *Ac*ES were protective against TNBS induced colitis in male Swiss OF1 mice (322). While SmSWP-induced protection was attributed to decreased IFNγ and IL-17A and increased IL-10 and TGF-β mRNA expression, no mechanism of action was specified for *Ac*ES (322). The DNBS model of colitis is very similar to TNBS, showing similar induction of pathology and progression of disease. *Trichinella spiralis* ES was shown to be protective in the DNBS model, with mice expressing increased levels of IL-13 and TGF-β and decreased IL-1β in the colons compared to the control mice (323). *H. diminuta* high molecular mass extract was also protective in the DNBS model with a similar type 2 cytokine skewing, and reduced TNF production and increased IL-4 and IL-10 production (324).

The results presented herein demonstrate that *Ac*ES has protective components that work within two very different models of colitis. Within the DSS model, which is more readily associated with the innate response, *Ac*ES is capable of delaying the onset of pathology. These results are supported by a recent study in which the ES products of the closely related *A. ceylanicum* was protective against DSS-induced colitis, attributed to diminished Th1 and Th17 responses (325). However, the decrease in the Th1 and Th17 cytokines in mice treated with *A. ceylanicum* ES was not related to increased type 2 cytokines IL-4 and IL-10 (325) as seen here. I further showed increased numbers of eosinophils in *Ac*ES-treated mice as seen previously in the absence of DSS, as well as an expansion of $F4/80^{+}$ $CD11b^{hi}$ $GR1^{hi}$ cells in these mice compared to controls.

In the TNBS model of colitis, which is highly dependent on T cells, it appears that *Ac*ES promotes rapid recovery from TNBS-induced pathology. It is pertinent to note that both of these models relied on administration of *Ac*ES prior to induction of pathology, demonstrating the effects of *Ac*ES as a prophylactic. It would be of interest to further investigate the use of *Ac*ES as a therapeutic substance in a chronic setting where colitis is already established. These experiments would highlight the translatability of *Ac*ES as a therapeutic compound.

The consistent results achieved with *Ac*ES were in themselves surprising, because *Ac*ES is a crude extract and cannot therefore be monitored for batch-to-batch variation. Although slight differences were noted, with some batches performing better than others, an overall reduction in pathological parameters was always achieved in the *Ac*ES-treated groups. An increase in type 2 cytokines was also a consistent outcome, but was not always followed by a decrease in levels of the pro-inflammatory cytokines IFNγ and IL-17A. This result, although contradictory to the hypothesis of a paradigm shift in T-helper responses as a potential treatment for inflammatory diseases, is supportive of the notion that *Ac*ES is not causing a general systemic immunosuppression.

The presence of IL-4/IL-10 co-expressing cells in the *Ac*ES-treated mice, has been a consistent result throughout this thesis, both in the presence and absence of the DSS inflammatory stimulus. These cells are intriguing due to the regulatory nature of IL-10 in other mouse models of colitis (IL-10 knockout), as well as its role in Tr1 cells that secrete high levels of IL-10 and low levels of IL-4 (326). In the next chapter I will further

explore the role of IL-10 in the DSS model of colitis, and explore the possible anti-inflammatory mechanisms of *Ac*ES.

5 Chapter 5 – Protection by *Ac*ES in the DSS model of colitis is not dependent on IL-10 signalling, B and T cells or TSLP.

5.1 Introduction

The previous two chapters have explored the immunomodulatory properties of *Ac*ES in both the steady state and in models of disease. The aim of the present chapter is to use loss-of-function studies to elucidate the potential immunological mechanism(s) by which *Ac*ES evokes immunomodulation in models of IBD. Results derived from chapters 3 and 4 suggested that *Ac*ES-mediated induction of a biased type 2 immune response, in particular the expansion of IL-4/IL-10-producing T cells, may be a factor that regulates intestinal inflammation. To investigate the relative role of IL-10 in this context, I used blocking antibodies against the IL-10 receptor (αIL-10R). Next, I assessed the relative importance of the adaptive immune system (B and T lymphocytes) by using mice deficient in *Rag1* (recombination activating gene 1), which is essential for the development of mature T and B cells (327). Lastly, I investigated the role of the epithelial alarmin thymic stromal lymphopoietin (TSLP), a key upstream regulator of type 2 immunity, and found that the common denominator involved in *Ac*ES-mediated immunomodulation may lie in an atypical population of innate myeloid cells.

5.2 Results: Blocking IL-10 receptor signalling alters *Ac*ES-induced cytokine responses

*Ac*ES administration leads to a skewing towards a type 2 immune response in naïve mice and under inflammatory conditions of DSS-induced colitis, and is characterised by the expression of IL-4, IL-5 and IL-10. Critically, a distinct population of $CD4^+$ T cells that co-express IL-4 and IL-10 was found in the mice receiving *Ac*ES. IL-10 is a potent regulatory cytokine; IL-10 deficient mice are prone to developing spontaneous colitis (328) and many other studies with parasitic worms have identified this as an important cytokine in parasite-mediated protection (182, 194, 329, 330). Subsequently, I hypothesised that induction of IL-10 expression may be responsible for the suppression of intestinal immunopathology in DSS-treated mice.

To test this hypothesis, mice were subjected to 3.5% DSS as described previously and received injections with either 0.5 mg of neutralising αIL-10R (1B1.3a; BioXcell) or control rat IgG on days 0, 3 and 6. I included another control group exposed to SEA (25 µg) from *S. mansoni*, also known to induce a type 2 response (331), instead of OVA as it was determined to be a more biologically relevant control. Intracellular cytokine staining of cells from the intestinal draining lymph nodes and the peritoneal cavity was performed to assess cytokine responses. As demonstrated previously, injection of *Ac*ES into DSS-treated mice caused marked reductions in the frequency of $CD4^+$ T cells expressing IFNγ (**Figure 5.2.1A**), modest reductions in IL-17A expression (**Figure 5.2.1B**) and increases in IL-4/IL-10 co-expressing cells (**Figure 5.2.1C**) compared to untreated mice that were administered DSS. Intriguingly, mice receiving αIL-10R had higher frequencies of IFNγ, IL-17A and IL-4/IL-10 co-producing $CD4^+$ T cells, particularly in the intestinal draining lymph nodes (**Figure 5.2.1**). As expected, SEA induced a biased type 2 cytokine response, with high frequencies of type 2 cytokines and IL-4/IL-10 co-expressing $CD4^+$ T cells (**Figure 5.2.1C**).

In vitro re-stimulation of MLN cells with αCD3 similarly revealed that αIL-10R-treated mice exhibited significant increases in IFNγ production as compared to controls (**Figure 5.2.2**). A noticeable increase in IL-17A and TNF production was also observed after IL-10R blockade, but failed to reach statistical significance (**Figure 5.2.2**). These results indicate that IL-10R blockade leads to a general increase in type 1 cytokine production, as well as increased frequencies of these cells in the draining lymph nodes. There was also an increased frequency of IL-4/IL-10 co-expressing $CD4^+$ cells upon IL-10R blockade, both at the site of injection and in the draining lymph nodes, in both the presence and absence of *Ac*ES. This suggests that IL-10R signalling influences the presence of these cells and that the stimulus of *Ac*ES amplifies this signal.

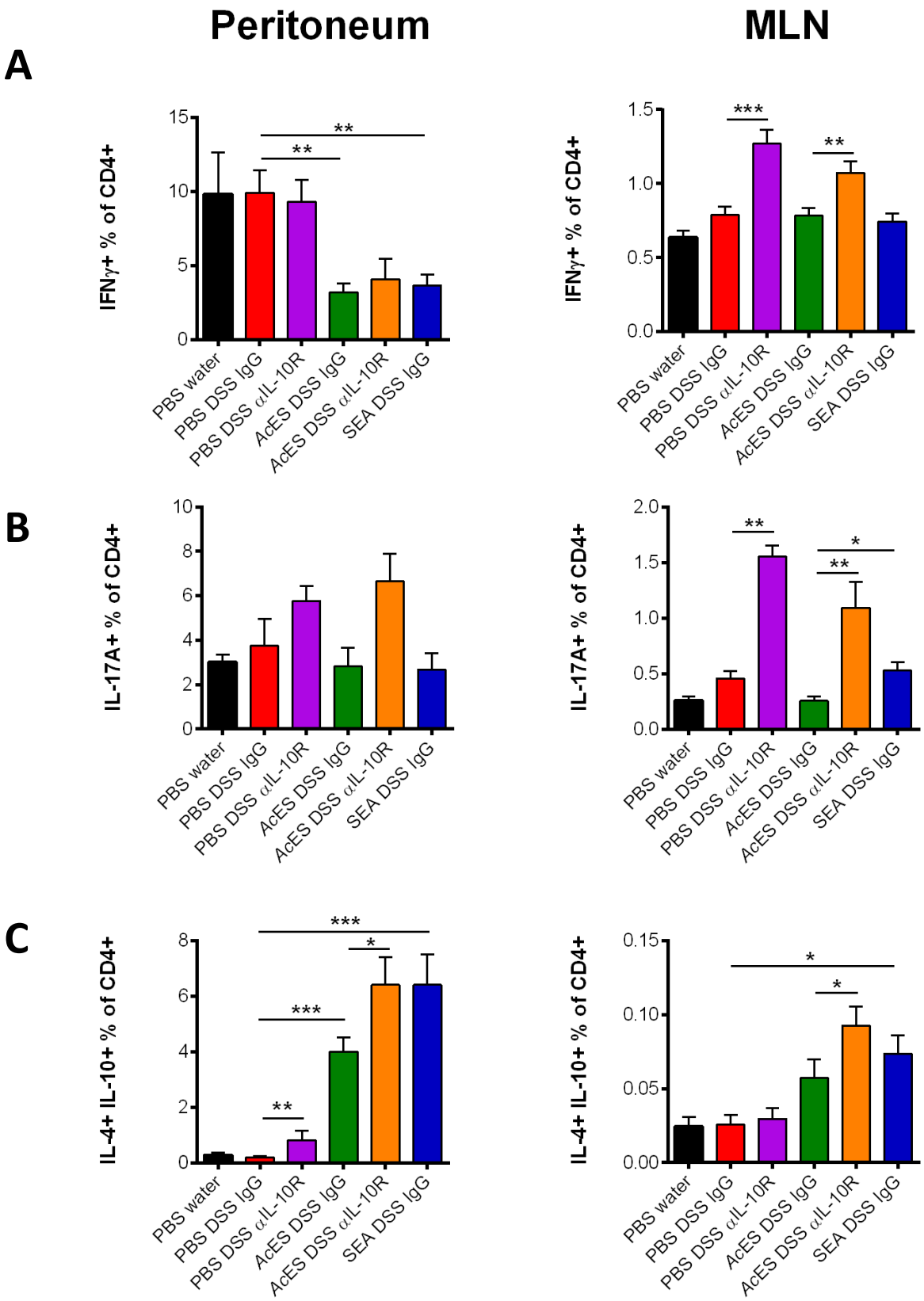
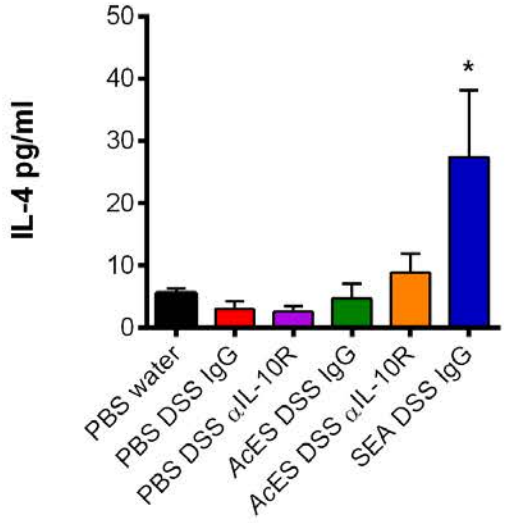

Peritoneum
MLN
A
B
C
IFNγ+ % of CD4+
IL-17A+ % of CD4+
IL-4+ IL-10+ % of CD4+
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG

Figure 5.2.1: IL-10 receptor blocking leads to increased frequencies of cytokine producing cells.
Mice were subjected to 3.5% DSS *ad libitum* for 8 days. Mice received daily i.p. injections of PBS, AcES (25 µg) or SEA (25 µg) and further received injections with either 0.5 mg of neutralizing αIL-10R or control rat IgG on days 0, 3 and 6. Intracellular cytokine staining shows frequencies of $IFN\gamma^+$ (A), $IL\text{-}17A^+$ (B) and $IL\text{-}4^+/IL\text{-}10^+$ (C) producing $CD4^+$ T cells in the peritoneum and the mesenteric lymph nodes (MLN). Statistical significance between the groups receiving control IgG was assessed by one-way ANOVA, statistical significance between PBS/DSS/IgG vs. PBS/DSS/αIL-10R or *Ac*ES/DSS/IgG vs. *Ac*ES/DSS/αIL-10R was assessed by 2-tailed t-test. Error bars show the standard error of the mean; graphical results are pooled from 3 experiments; n = 6-12 (non-DSS control group contained 2 mice per experiment, test groups contained 3-4 mice). *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$.


IL-4 pg/ml
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40
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20
10
0
*
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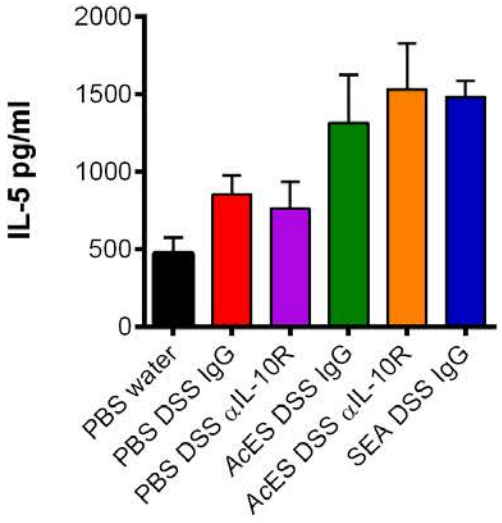
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG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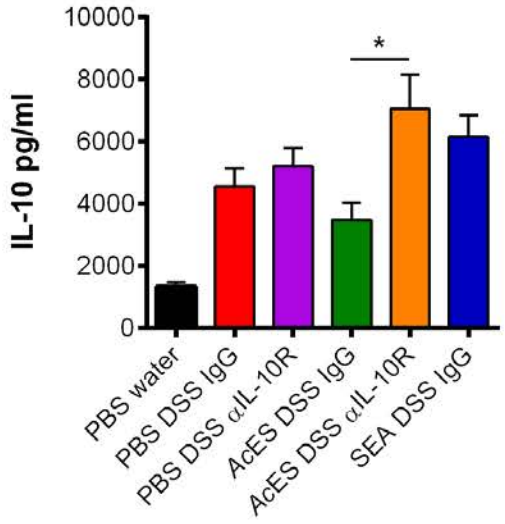
IL-10 pg/ml
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4000
2000
0
*
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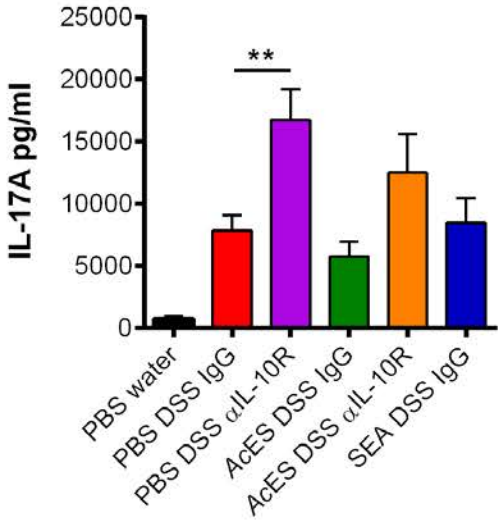
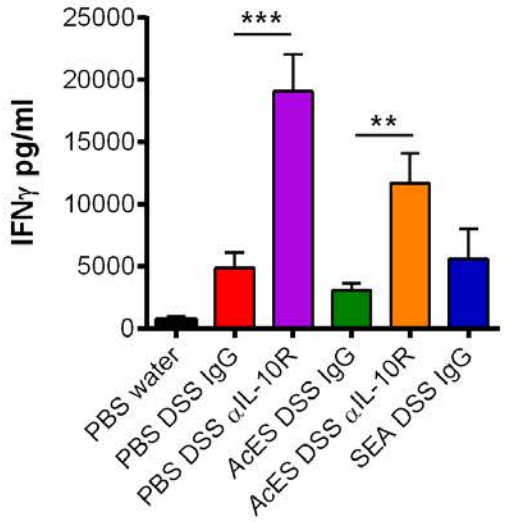
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG


IL-17A pg/ml
25000
20000
15000
10000
5000
0
**
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG


IFNγ pg/ml
25000
20000
15000
10000
5000
0
***
**
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG

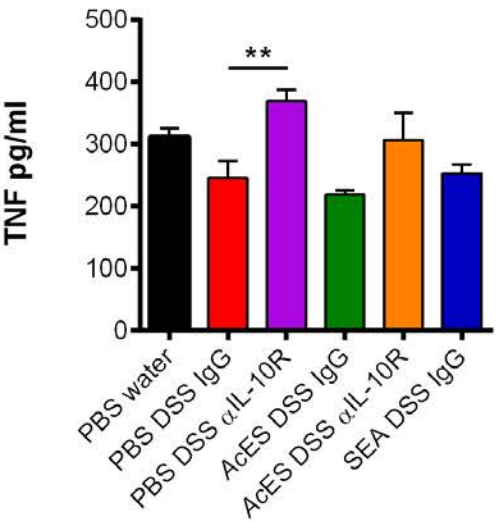
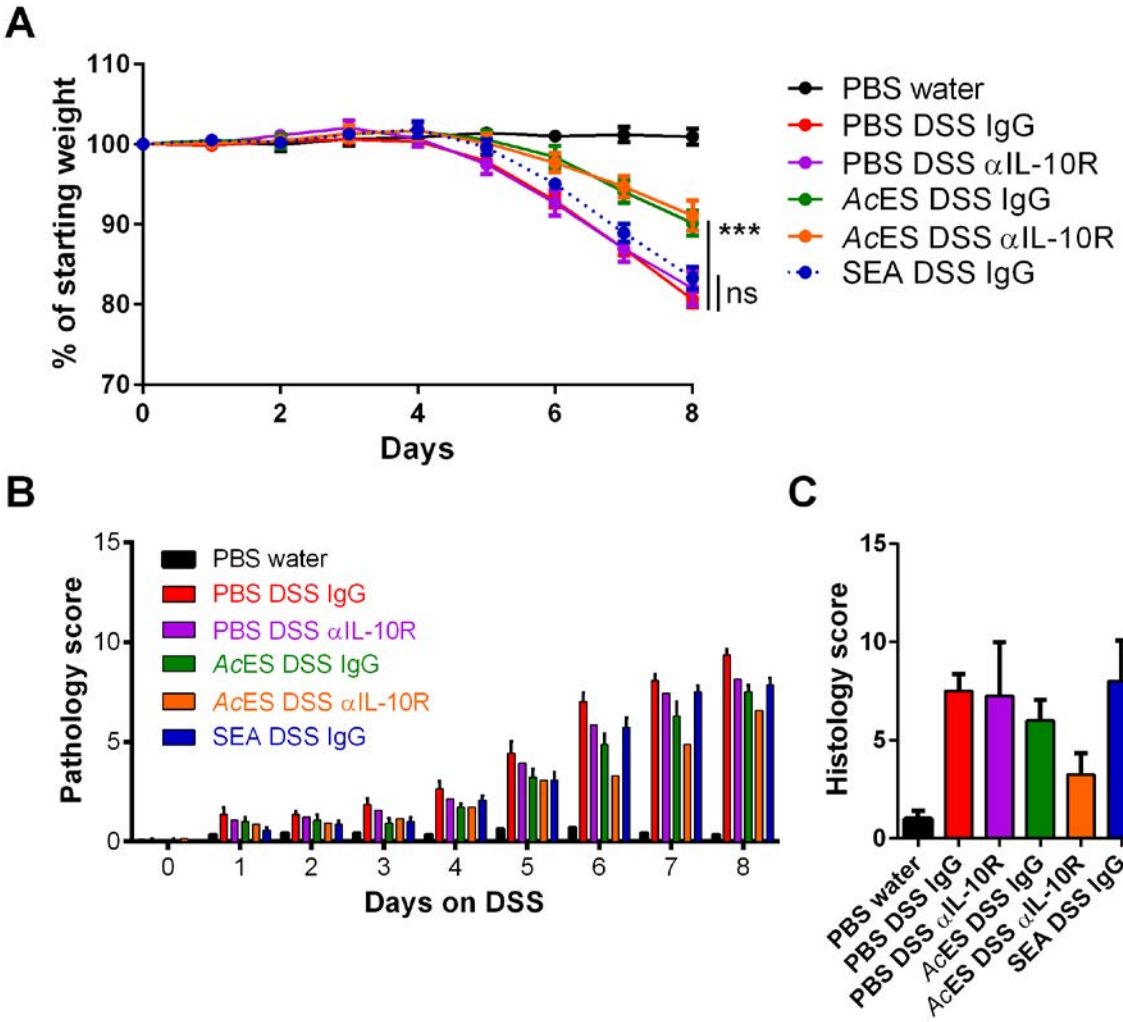

TNF pg/ml
500
400
300
200
100
0
**
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG

Figure 5.2.2: Blocking of IL-10 receptor leads to increased pro-inflammatory cytokines production.
Mice were subjected to 3.5% DSS *ad libitum* for 8 days. Mice received daily i.p. injections of PBS, AcES (25 μg) or SEA (25 μg) and further received injections with either 0.5 mg of neutralizing αIL-10R or control rat IgG on days 0, 3 and 6. MLN cells were cultured with 1 μg/ml of αCD3 for 72 hours. Supernatants were collected and analysed by ELISA for the presence of IL-4, IL-5, IL-10, IL-17A, IFNγ and TNF cytokines. Statistical significance between the control IgG-treated groups was assessed by one-way ANOVA. Statistical significance between PBS/DSS/IgG vs. PBS/DSS/αIL-10R and *Ac*ES/DSS/IgG vs. *Ac*ES/DSS/αIL-10R was assessed by 2-tailed t-test. Error bars show the standard error of the mean; graphical results are pooled from 3 experiments; n = 6-12 (non-DSS control group contained 2 mice per experiment, test groups contained 3-4 mice). *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$.

5.3 *Ac*ES-mediated protection in DSS-induced colitis is not dependent on IL-10 signalling

Given the profound effect of IL-10R blockade on *Ac*ES-induced cytokine responses, including the pro-inflammatory cytokines IFNγ and IL-17A that are commonly associated with colitis pathology (332), I hypothesised that IL-10R blockade would exacerbate DSS-induced pathology. Surprisingly however, IL-10R blockade had no effect on *Ac*ES-mediated protection against weight loss and gross pathology during DSS colitis (**Figure 5.3.1A-B**). Histopathology scoring further emphasised this result, as the blocking of IL-10R in mice receiving *Ac*ES produced diminished signs of histopathology (**Figure 5.3.1C-D**). SEA did not protect mice against DSS induced pathology or weight loss (**Figure 5.3.1**), which confirms recently published results (188). This is of interest as SEA induced high frequencies of IL-4^+/IL-10^+ co-expressing $CD4^+$ T cells (**Figure 5.2.1C**), the same cell type currently being investigated as a potential candidate for *Ac*ES-mediated protection against colitic inflammation.


A
% of starting weight
110
100
90
80
70
0
2
4
6
8
Days
***
ns
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG
B
Pathology score
15
10
5
0
0
1
2
3
4
5
6
7
8
Days on DSS
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG
C
Histology score
15
10
5
0
PBS water
PBS DSS IgG
PBS DSS αIL-10R
AcES DSS IgG
AcES DSS αIL-10R
SEA DSS IgG

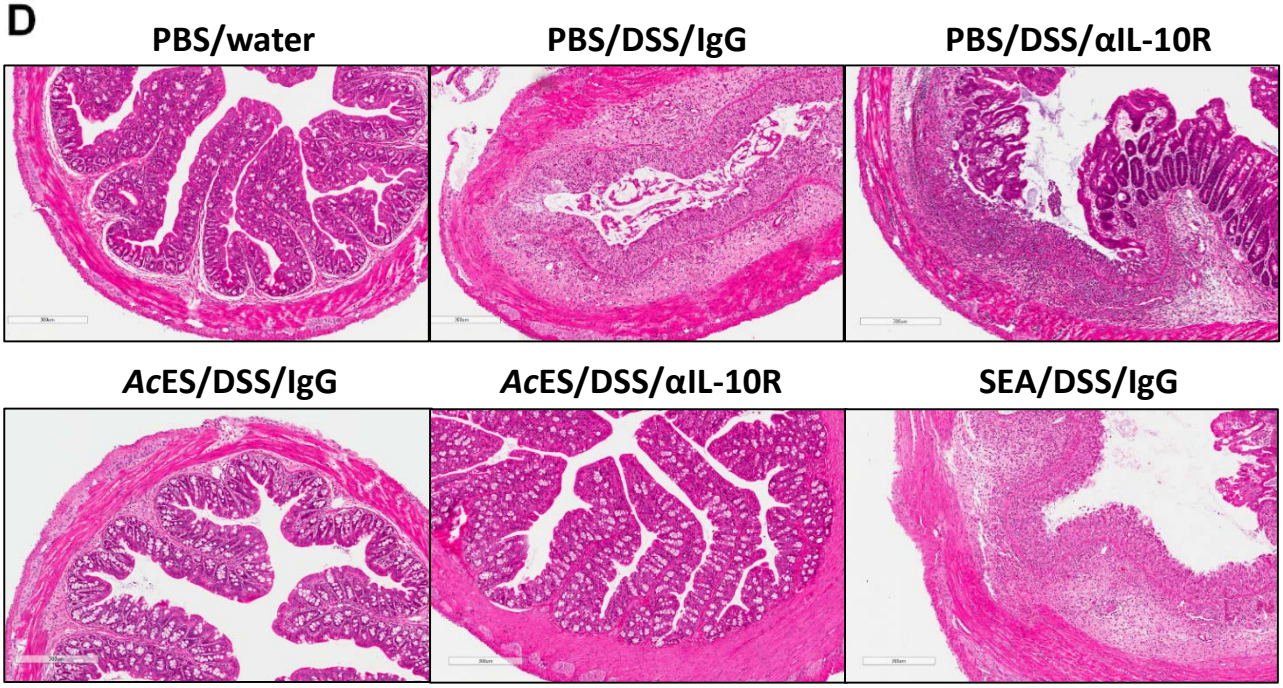

D
PBS/water
PBS/DSS/IgG
PBS/DSS/αIL-10R
AcES/DSS/IgG
AcES/DSS/αIL-10R
SEA/DSS/IgG

Figure 5.3.1: *Ac*ES mediated protection in DSS induced colitis is not dependent on IL-10 signalling.
Mice were subjected to 3.5% DSS *ad libitum* for 8 days. Mice received daily i.p. injections of PBS, *Ac*ES (25 μg) or SEA (25 μg) and further received injections with either 0.5 mg of neutralizing αIL-10R or control rat IgG on days 0, 3 and 6. Percentage of starting weight (A) and pathology score (B) of mice during DSS induced colitis model. Histology scores (C) and representative H&E stained colon micrographs (box on lower left corner indicates 300 μm) (D). Error bars show the standard error of the mean; graphical results are pooled from 3 repeat experiments; n = 6-12 (non-DSS control group contained 2 mice per experiment, test groups contained 3-4 mice); histology pictures are representative. *** = $p<0.001$, ns = non-significant.

5.4 IL-10R blockade causes a significant increase in the frequency of myeloid derived suppressor cells.

The results from the previous chapter suggest that there is little correlation between levels of type 1 and type 2 cytokines and disease outcome, because mice that received αIL-10R with *Ac*ES showed increased production of pro-inflammatory cytokines, but were still protected from DSS-induced pathology. Therefore, *Ac*ES-induced protection in DSS colitis may be mediated by other factors independent of the shift in Th1/2 profile. Consequently, I analysed the cellular composition at the site of *Ac*ES injection and how this was altered by IL-10R blockade. Peritoneal lavages from *Ac*ES/DSS/αIL-10R mice were analysed by flow cytometry for eosinophil and macrophage content (**Figure 5.4.1A**). The flow cytometry analysis revealed a shift in the $F4/80^{+}$ population towards a F4/80-low monocyte-like phenotype ($F4/80^{lo}$). Gating inside this population and assessment of CD11b and Gr1 surface expression showed that more than 50% of these cells were $CD11b^{hi}$ $Gr1^{hi}$ (**Figure 5.4.1B**), a phenotype consistent with MDSCs. The classification of these cells was determined by looking at the FSC-A vs. SSC-A and noting that these cells did not contain a high SSC-A profile which typifies neutrophils (see **Figure 8.3.1** in **section 8.3**). Interestingly, MDSCs were expanded in *Ac*ES/IgG treated mice when compared to PBS/IgG controls. This cellular expansion was dramatically increased when IL-10R signalling was neutralised in the *Ac*ES/DSS/αIL-10R group, with slight expansion also seen in the PBS/DSS/αIL10R group (**Figure 5.4.1C**). These findings suggest that IL-10R signalling in the presence of *Ac*ES may have a role in maintaining homeostasis in MDSCs, because when it was blocked there was a dramatic rise in the frequency and number of these cells.

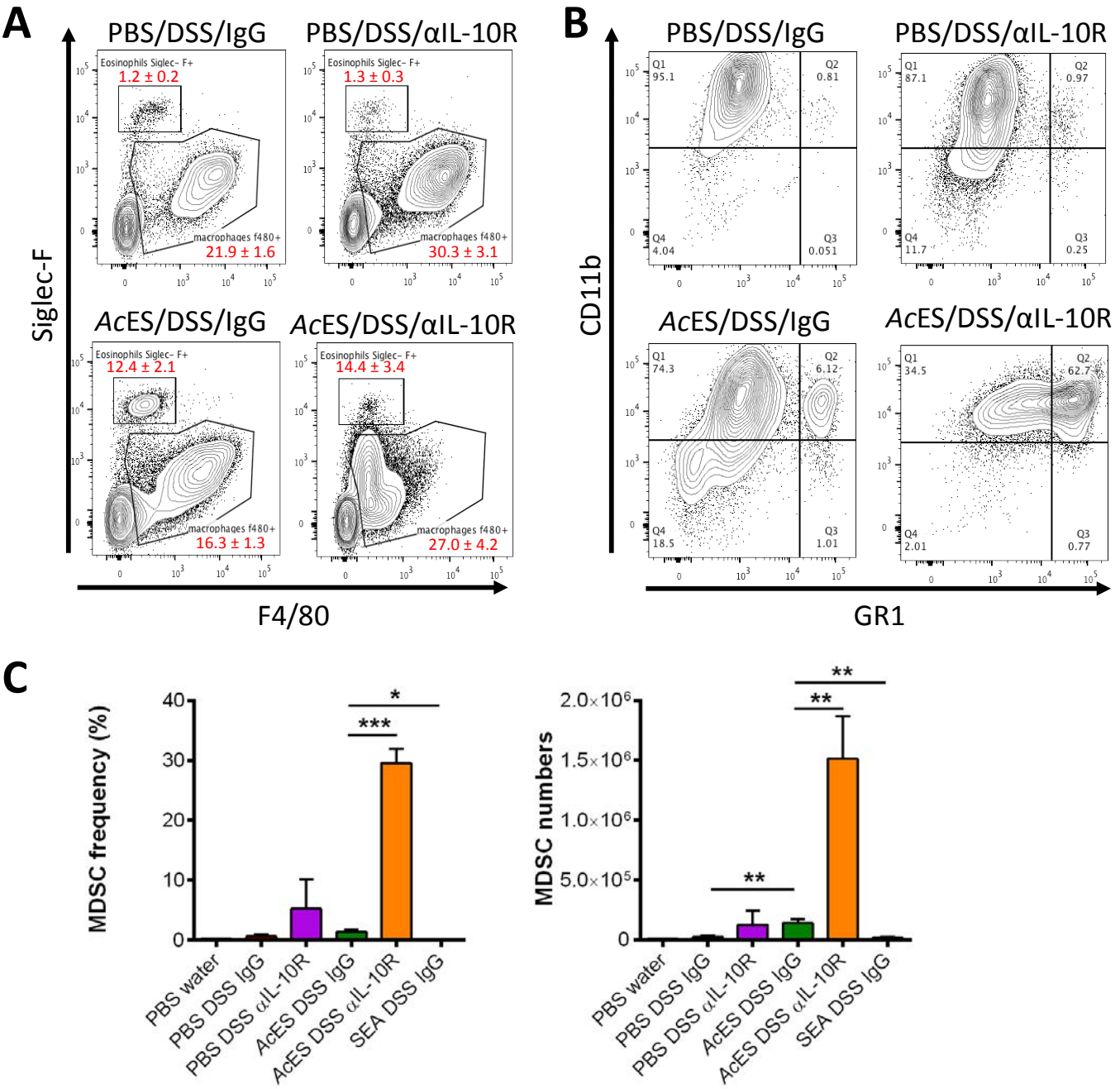


Figure 5.4.1: Blocking of IL-10R causes significant increases in MDSC frequencies.
Mice were subjected to 3.5% DSS *ad libitum* for 8 days. Mice received daily i.p. injections of PBS, *Ac*ES (25 µg) or SEA (25 µg) and further received injections with either 0.5 mg of neutralizing αIL-10R or control rat IgG on days 0, 3 and 6. Peritoneal lavage cells were stained with monoclonal antibodies against Siglec-F, F4/80, CD11b and GR1. Representative flow plots showing gating strategy for eosinophils (Siglec-F$^+$) and macrophages (F4/80$^+$) (A), showing mean frequencies of live cells ± standard error of the mean. Within the F4/80$^+$ gate, cells were further analysed for CD11b vs GR1 (B), showing frequencies of the F4/80$^+$ population. GR1$^+$ CD11b$^+$ frequency of live cells and total cell numbers (C). Statistical significance between the groups receiving control IgG was assessed by one-way ANOVA. Statistical significance between PBS/DSS/IgG vs. PBS/DSS/αIL-10R and *Ac*ES/DSS/IgG vs. *Ac*ES/DSS/αIL-10R was assessed by 2-tailed t-test. Error bars show the standard error of the mean; results are pooled from 3 experiments; n = 6-12 (non-DSS control group contained 2 mice per experiment, test groups contained 3-4 mice); * = p<0.05, ** = p<0.01 and *** = p<0.001.

5.5 T and B cells are dispensable for *Ac*ES mediated protection in DSS induced colitis

Results presented throughout this thesis have demonstrated that *Ac*ES induces a biased type 2 immune response, including a distinct population of IL-4/IL-10 co-expressing $CD4^+$ T cells. However, I subsequently demonstrated that IL-10R signalling was dispensable for the ability of *Ac*ES to limit the pathology associated with DSS induced colitis. Further, I showed that *Ac*ES injection is associated with the expansion of various innate immune cell subsets, including the recently discovered MDSC, raising the hypothesis that adaptive immunity may not be essential for *Ac*ES-induced immunoregulation. Given that DSS-induced colitis is mostly reliant on the innate immune system (217), I next aimed to determine whether *Ac*ES could protect $Rag1^{-/-}$ mice (which lack mature B and T cells) against DSS-induced colitis.

Firstly it was important to determine the dose of DSS to ensure the results would be comparable to that of the WT strain. $Rag1^{-/-}$ mice responded in a similar fashion to WT mice when fed 3.5% DSS (see **Figure 8.3.2** in **section 8.3**). I then treated either WT or $Rag1^{-/-}$ mice with *Ac*ES or vehicle control in the presence or absence of DSS in the drinking water. As expected, DSS-treated WT and $Rag1^{-/-}$ mice exhibited characteristic weight loss, and delivery of *Ac*ES to WT mice partially limited this weight loss (**Figure 5.5.1A**), gross pathology (**Figure 5.5.1B**) and histopathology (**Figure 5.5.1C**). Interestingly, *Ac*ES-treated $Rag1^{-/-}$ mice were similarly protected from weight loss and had significantly lower pathology and histology scores than the PBS/DSS/ $Rag1^{-/-}$ mice (**Figure 5.5.1A-D**). These data indicate that the mechanism of action of *Ac*ES, in this particular model of colitis, works independently of T and B cells and is likely affecting the innate immune system.

I next examined the profile of cytokine and innate immune cell responses in *Ac*ES-treated WT and $Rag1^{-/-}$ mice. Increased frequencies and number of eosinophils were detected in WT mice when treated with *Ac*ES as seen in the previous chapter (**Figure 5.5.2A**). Results also showed high frequencies of eosinophils in the $Rag1^{-/-}$ mice that diminished with DSS treatment (**Figure 5.5.2C**). Interestingly, *Ac*ES treatment in these mice did not restore the eosinophil numbers to non-DSS-treated levels. Analysis of the peritoneal exudate cells revealed a significant increase in MDSC frequencies and

numbers in both the WT and the Rag1$^{-/-}$ mice treated with *Ac*ES (**Figure 5.5.2B/D**). These results indicate that the expansion of MDSCs in the presence of *Ac*ES occurs independently of T and B cell influences.

Cytokine responses at the site of pathology were assessed by ELISA. Colon pieces were cultured in media overnight and cytokine production was measured in the supernatants. There was a trend for increased type 2 cytokine production from colons of *Ac*ES treated Rag1$^{-/-}$ mice, which reached significance for IL-4 and TSLP (**Figure 5.5.3**), further highlighting that *Ac*ES can elicit immunomodulation even in the absence of the adaptive immune system.

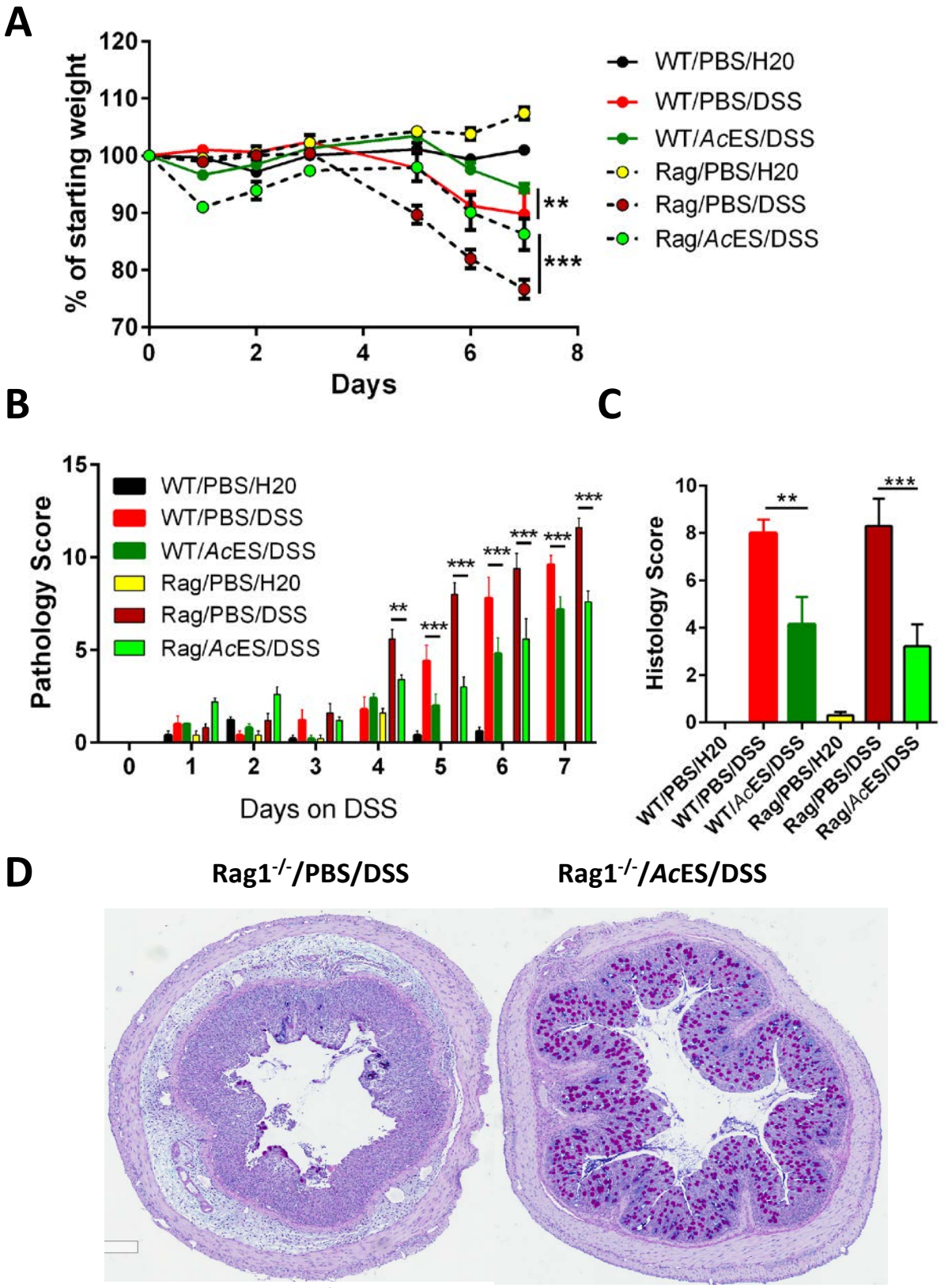
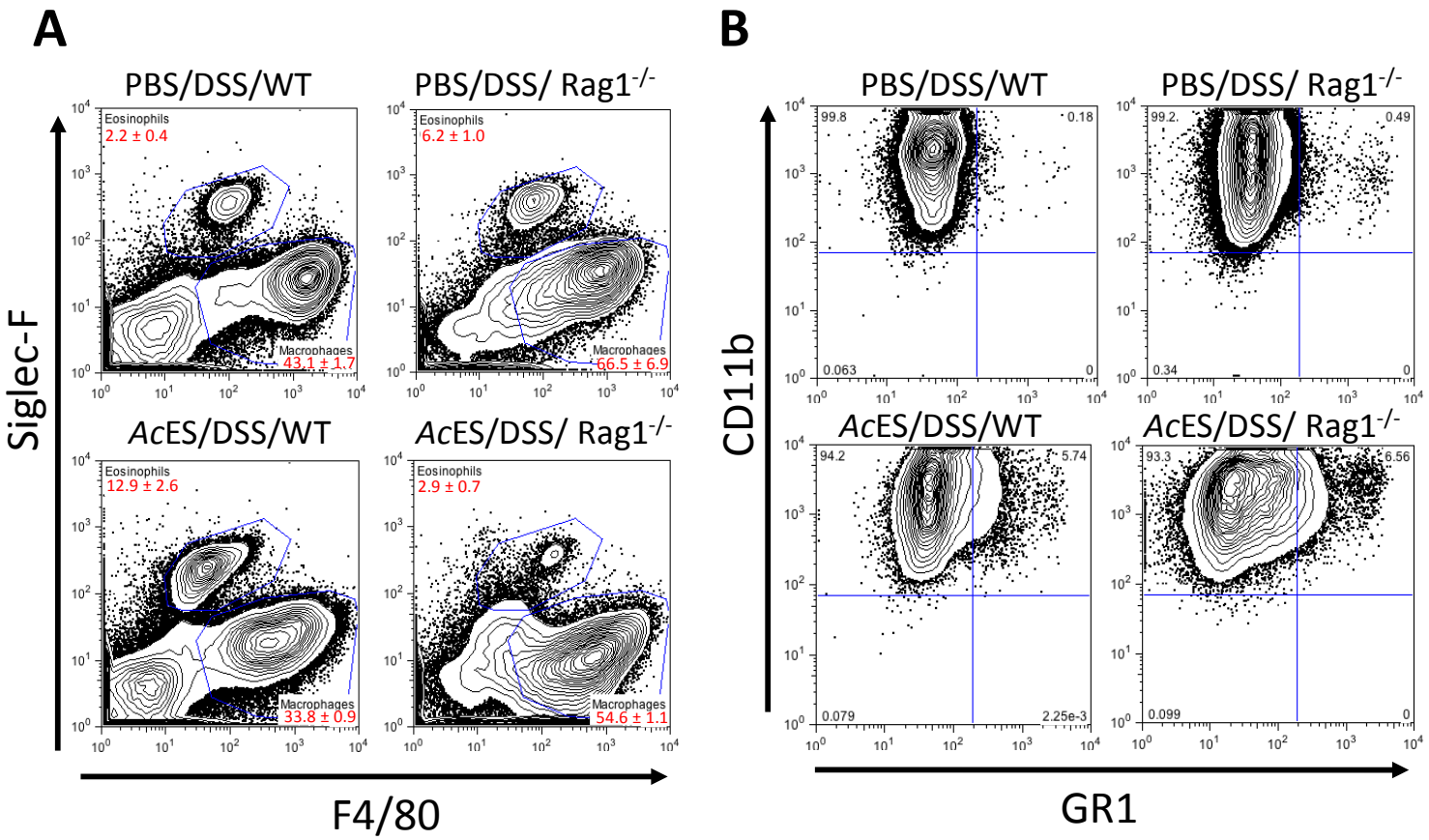
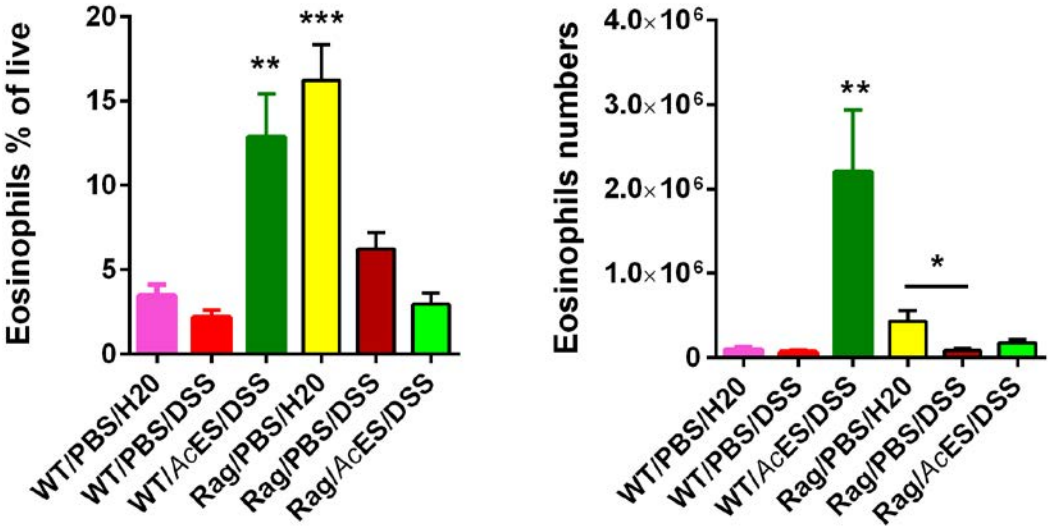


Figure 5.5.1: *Ac*ES protects Rag1$^{-/-}$ mice from DSS-induced colitis.
Wild type and Rag1$^{-/-}$ mice were given 3.5% DSS as a substitute for drinking water for 7 days. Mice received daily i.p. injections of PBS or 25 µg *Ac*ES. Percentage of starting weight (A), clinical pathology (B) and histology (C) of mice in the DSS-induced colitis model. Representative histology micrographs of mouse colons (x20 magnification) stained with PAS and Alcian Blue (D). Error bars show the standard error of the mean; results are representative of 2 repeat experiments; n = 5 mice per group. * = p<0.05, ** = p<0.01 and *** = p<0.001


A
PBS/DSS/WT
PBS/DSS/ Rag1-/-
AcES/DSS/WT
AcES/DSS/ Rag1-/-
Eosinophils
2.2 ± 0.4
6.2 ± 1.0
12.9 ± 2.6
2.9 ± 0.7
Macrophages
43.1 ± 1.7
66.5 ± 6.9
33.8 ± 0.9
54.6 ± 1.1
Siglec-F
F4/80
B
PBS/DSS/WT
PBS/DSS/ Rag1-/-
AcES/DSS/WT
AcES/DSS/ Rag1-/-
99.8
0.18
0.063
0
99.2
0.49
0.34
0
94.2
5.74
0.079
2.25e-3
93.3
6.56
0.099
0
CD11b
GR1

C


Eosinophils % of live
20
15
10
5
0
**
***
WT/PBS/H20
WT/PBS/DSS
WT/AcES/DSS
Rag/PBS/H20
Rag/PBS/DSS
Rag/AcES/DSS
Eosinophils numbers
4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
0
**
*

D

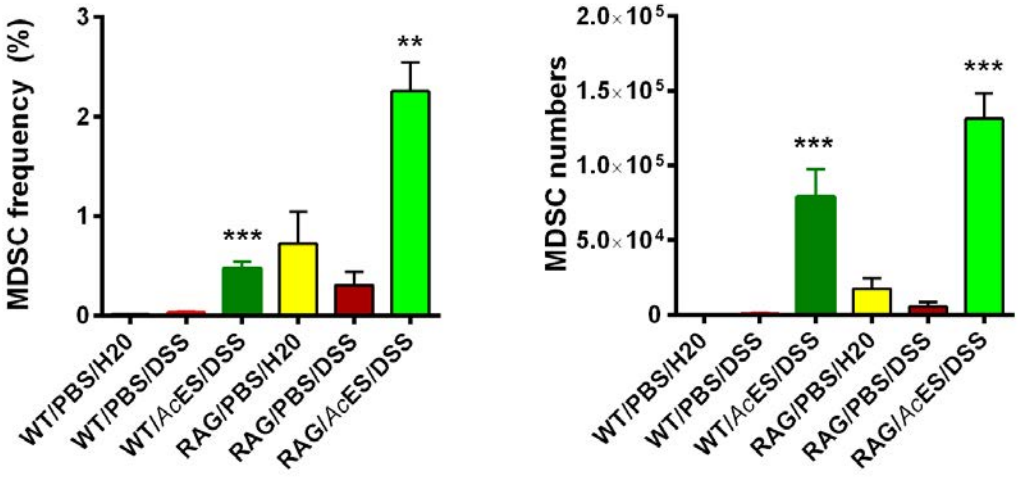


Figure 5.5.2: Rag1$^{-/-}$ mice receiving *Ac*ES have significantly higher frequencies of MDSC.
Wild type and Rag1$^{-/-}$ mice were given 3.5% DSS as a substitute for drinking water for 7 days. Mice received daily i.p. injections of PBS or 25 µg *Ac*ES. Peritoneal lavage cells were stained with monoclonal antibodies against Siglec-F, F4/80, CD11b and GR1. Representative flow plots showing gating strategy for eosinophils (Siglec-F$^+$) and macrophages (F4/80$^+$) (A), showing frequencies of live cells. Within the F4/80$^+$ gate, cells were further analysed for CD11b vs GR1 expression (B), showing frequencies of the F4/80$^+$ population. Frequencies and total cell numbers of eosinophils (Siglec-F$^+$) (C). GR1$^+$ CD11b$^+$ F4/80$^+$ (MDSC) frequency of live cells and total MDSC numbers (D). Error bars show the standard error of the mean; results are representative of 2 experiments; n = 5 mice per group. Statistical significance analysed by one-way ANOVA within the WT mice and the Rag1$^{-/-}$ mice separately; * = p<0.05, ** = p<0.01 and *** = p<0.001.

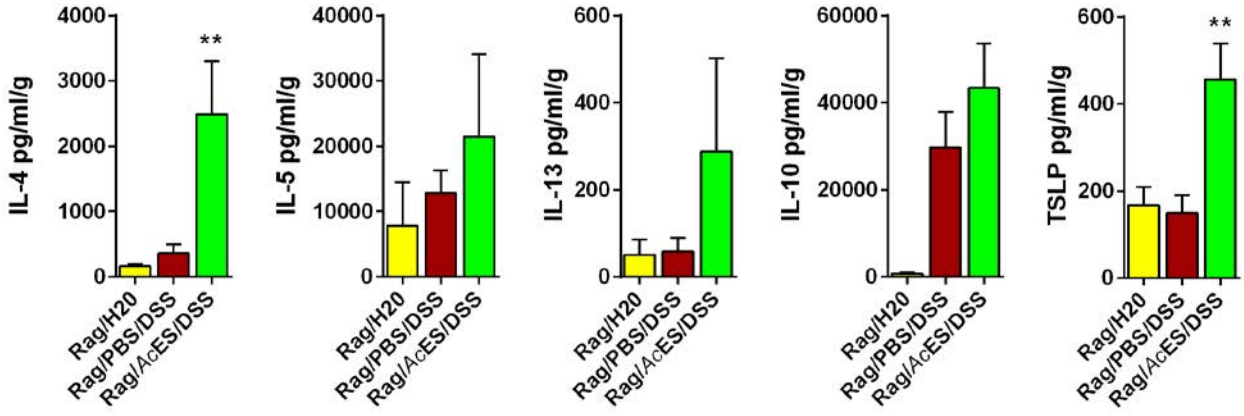


Figure 5.5.3: Colon cytokine production shows trend for increased type 2 cytokine responses in the absence of T and B cells.
Rag1$^{-/-}$ mice were given 3.5% DSS as a substitute for drinking water for 7 days. Mice received daily i.p. injections of PBS or 25 µg *Ac*ES. Colon pieces were cultured in media overnight and cytokine production was measured in the supernatant. Error bars show the standard error of the mean, graphical results are from a single experiment; n = 5 mice per group, ** = p<0.01.

5.6 Neutralising TSLP does not affect *Ac*ES mediated protection.

Results thus far indicated that *Ac*ES-mediated protection from DSS-induced colitis is neither dependent on mature T and B cells nor IL-10R signalling. This led me to hypothesise that *Ac*ES might act upstream of the effector responses, at the level of both innate and adaptive response initiation. TSLP is a cytokine produced mainly by keratinocytes, DCs and intestinal epithelial cells (333). It is thought to be pivotal in the maintenance of type 2 responses at barrier surfaces, and reduced expression of TSLP within the gastrointestinal tract is correlated with increased risk of IBD (334). Consistent with a potential role for TSLP in *Ac*ES-induced immunomodulation, I observed augmented TSLP production within the intestine of $Rag1^{-/-}$ mice following *Ac*ES administration (**Figure 5.5.3**). In order to investigate the functional role of TSLP in the protection induced by *Ac*ES, I used neutralising anti-TSLP monoclonal antibody (αTSLP - Amgen) or control isotype in $Rag1^{-/-}$ mice treated or not with *Ac*ES and exposed to DSS. I included an *Ac*ES group fed normal drinking water to verify that *Ac*ES was not having an adverse effect on $Rag1^{-/-}$ mice. *Ac*ES in the absence of DSS did not have any adverse effects on the $Rag1^{-/-}$ mice. As shown earlier, *Ac*ES protected mice from DSS induced weight loss, however neutralising TSLP did not significantly impact weight loss (**Figure 5.6.1A**). Neither pathology (**Figure 5.6.1B**) nor histology (**Figure 5.6.1C**) scores were affected in *Ac*ES-treated mice fed DSS by neutralising TSLP. Taken together, these results suggest that TSLP does not play an active role in *Ac*ES-induced protection of DSS mediated pathology. It should be noted that the anti-TSLP antibody (kindly provided by Amgen) is relatively new and further investigation is required to confirm these results. It is possible that the antibody did not reach the colon or possibly did not fully neutralise the TSLP and further experiments with appropriate controls are required to validate the data presented herein.

Flow cytometry analysis of the peritoneal lavages also confirmed that TSLP neutralisation did not affect the differential cell counts. Both the spleen and the peritoneum showed similar frequencies and numbers of eosinophils, macrophages and MDSCs in both the *Ac*ES/DSS/IgG and *Ac*ES/DSS/αTSLP groups (**Figure 5.6.2**), suggesting that the induction and/or expansion of these three cell populations are independent of TSLP. All these results have highlighted MDSCs as a common cell type that is expanded

in the presence of *Ac*ES. Their expansion was affected by blocking of IL-10 receptor signalling but not impacted by the absence of an adaptive immune response or the neutralising of TSLP.

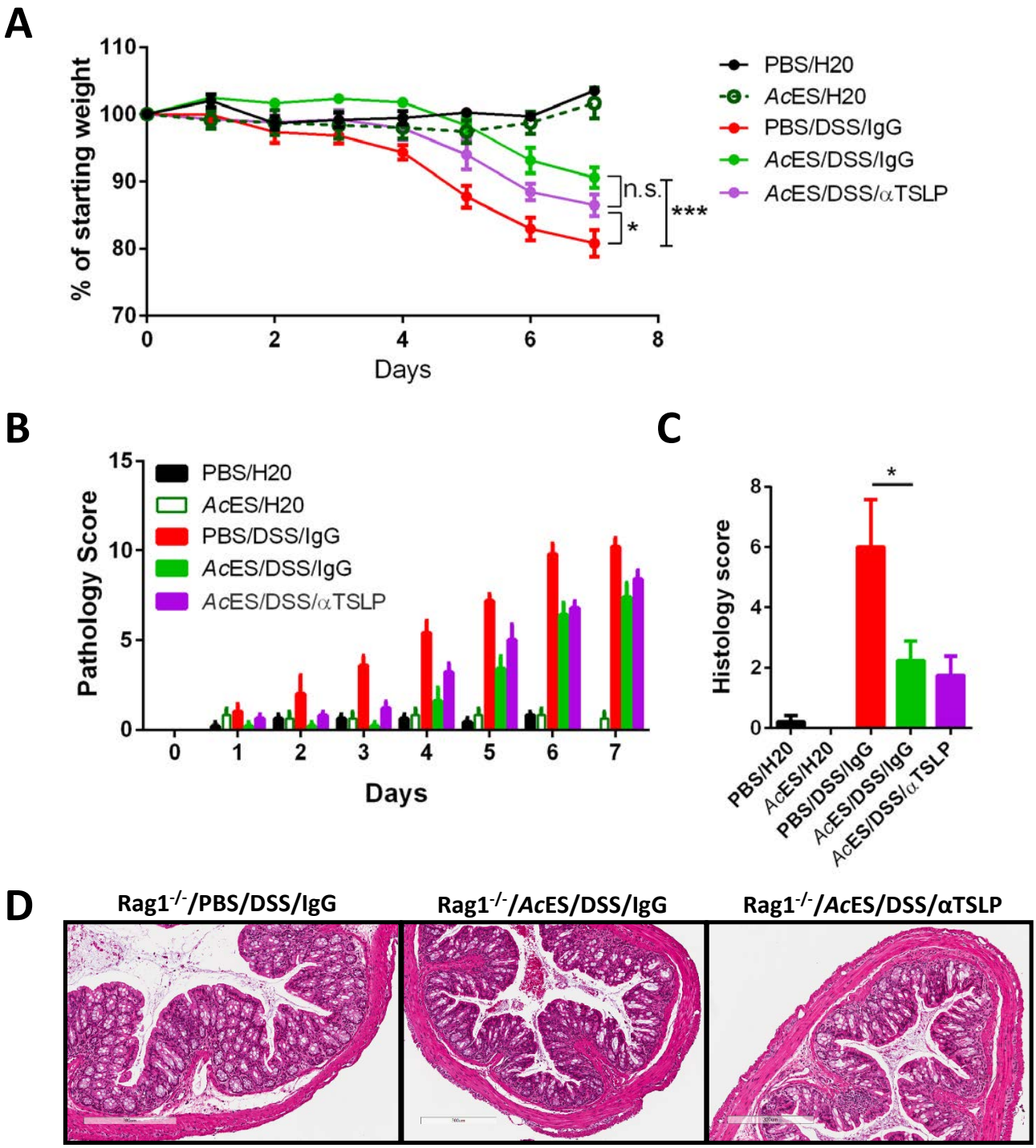
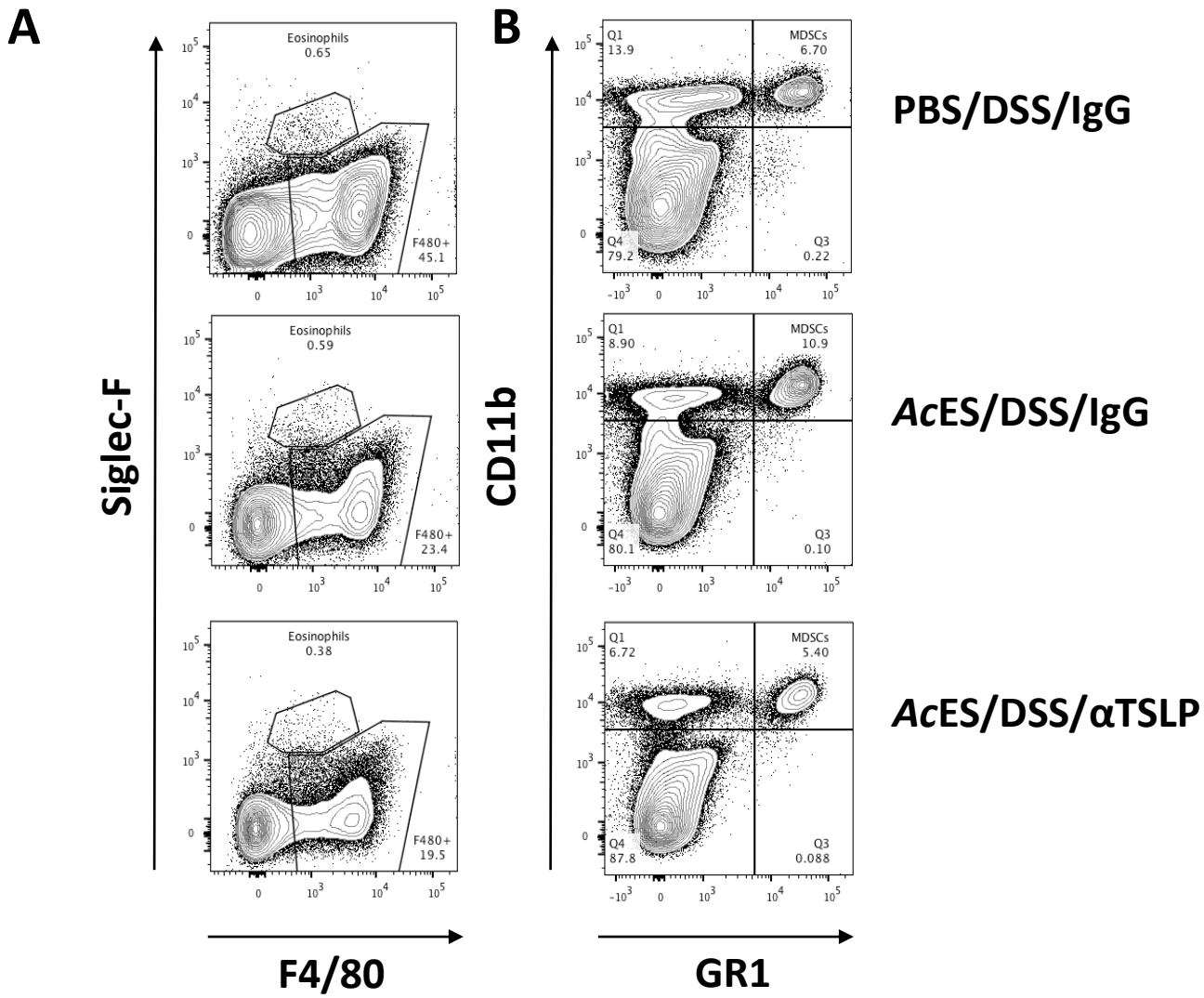


Figure 5.6.1: Neutralising TSLP does not affect *Ac*ES-mediated protection.
Rag1$^{-/-}$ mice were given 3.5% DSS as a substitute for drinking water for 7 days. Mice received daily i.p. injections of PBS or 25 µg *Ac*ES. Additionally, mice were administered thymic stromal lymphopoietin (αTSLP 0.5 mg) antibody or control IgG on days 0, 3 and 6. Percentage of starting weight (A), pathology (B) and histology scores (C) of mice during DSS-induced colitis. Representative H&E stained histology micrographs of colons (D) (box at lower left corner indicates 300 µm). Error bars show the standard error of the mean; graphical results are from a single experiment; n = 5 mice per group, histology micrographs are representative. * = $p<0.05$, *** = $p<0.001$, n.s. = non-significant.


A
B
Eosinophils
0.65
F480+
45.1
Eosinophils
0.59
F480+
23.4
Eosinophils
0.38
F480+
19.5
Siglec-F
F4/80
Q1
13.9
MDSCs
6.70
Q4
79.2
Q3
0.22
Q1
8.90
MDSCs
10.9
Q4
80.1
Q3
0.10
Q1
6.72
MDSCs
5.40
Q4
87.8
Q3
0.088
CD11b
GR1
PBS/DSS/IgG
AcES/DSS/IgG
AcES/DSS/αTSLP

C

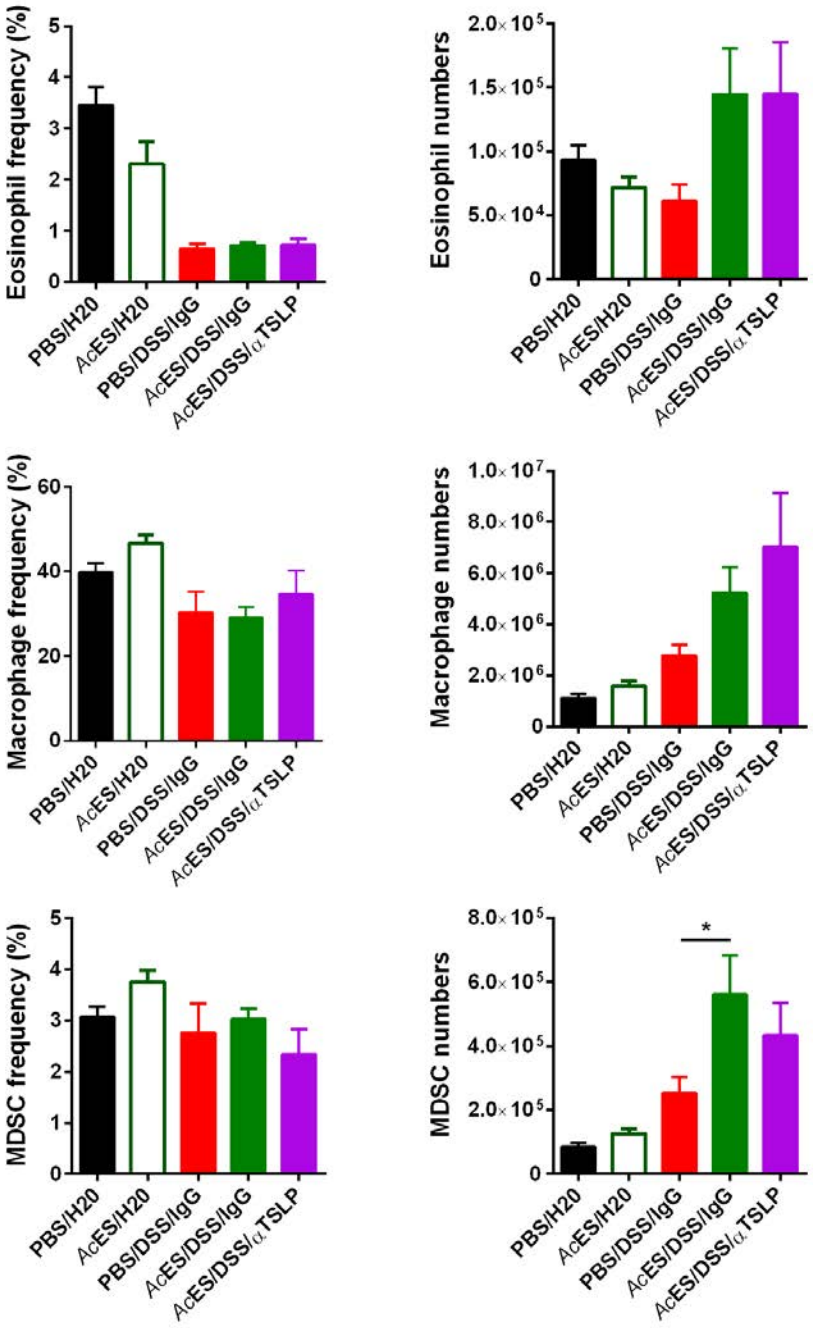


Figure 5.6.2: Neutralising TSLP has no significant effect on eosinophils, macrophages or MDSCs. Rag1$^{-/-}$ mice were given 3.5% DSS as a substitute for drinking water for 7 days. Mice received daily i.p. injections of PBS or 25 µg *Ac*ES. Additionally, mice were administered thymic stromal lymphopoietin (αTSLP 0.5 mg) antibody or control IgG on days 0, 3 and 6. Representative flow cytometry plots from splenocytes stained for surface markers Siglec-F, F4/80, GR1 and CD11b. Representative flow plots showing gating strategy for eosinophils (Siglec-F$^+$) and macrophages (F4/80$^+$) (A), showing frequencies of live cells. Within the F4/80$^+$ gate, cells were further analysed for CD11b vs GR1 expression (B), showing frequencies of the F4/80$^+$ population. Overall frequency of live cells and total numbers of eosinophils, macrophages and MDSCs in the spleen (C). Error bars show the standard error of the mean; graphical results are from a single experiment; n = 5 mice per group; * = $p<0.05$, ** = $p<0.01$.

5.7 Chapter 5 Summary

In this chapter, I have attempted to determine the molecular and cellular mechanisms by which *Ac*ES exerts immunomodulation in a mouse model of IBD. I focused on three key facets of the innate or adaptive immune system: the regulatory cytokine IL-10, T and B cells and the epithelial alarmin TSLP. None of these mediators were deemed to be major contributors to the protective response achieved by *Ac*ES administration during DSS-induced colitis.

IL-10 is a regulatory cytokine, which has been implicated in the regulation of a range of inflammatory conditions (335-338). IL-10 signalling was also of interest because of its association with spontaneous colitis in IL-$10^{-/-}$ mice (339). I originally postulated that blocking IL-10R signalling would interfere with the protection induced by *Ac*ES in DSS-induced colitis. However, the absence of weight loss or any overt pathological parameters, despite the increase in pro-inflammatory cytokines, indicated that *Ac*ES protects against colitic inflammation via mechanisms that do not depend on IL-10 signalling. It was interesting to note that blocking of IL-10R signalling did increase production of pro-inflammatory cytokines as expected, however it was surprising that despite the increase in pro-inflammatory mediators, colitic pathology was not altered in the course of the experiment.

Throughout the previous chapters, I have identified a distinct population of IL-4/IL-10 co-expressing $CD4^+$ T cells that were only present in the *Ac*ES treated mice. Interestingly, IL-4/IL-10 co-expressing $CD4^+$ T cells were also identified in the mice treated with SEA, but these mice were not protected from DSS. To more formally determine whether these cells were responsible for the protection induced by *Ac*ES, $Rag1^{-/-}$ mice, which lack mature B and T cells, were fed DSS therefore eliminating the involvement of lymphocytes as effector cells in this model. Results indicated that the presence of mature T and B cells was dispensable for the protection associated with administration of *Ac*ES. These results suggest that *Ac*ES is acting independently of the adaptive immune system, and is instead inducing regulatory cells or generating a suppressive environment associated with innate immunity. Another possibility is that there is an innate effector cell that is acting in a wound healing fashion to repair the damage that is caused by DSS. Indeed, it has often been postulated that helminths are

capable of inducing wound healing macrophages (291, 340, 341) and developing research into innate lymphoid cells (ILC) also suggests a role for tissue repair (342). Finally, it is also possible that *Ac*ES is directly inhibiting the mechanism of action of DSS, which is not yet completely understood.

Lastly this chapter explored the involvement of a cytokine that is primarily expressed by epithelial cells that line the intestinal tract (343, 344), hence is at the front-line during colitis. Because of its ability to induce and maintain Th2 responses, TSLP has been implicated as an instigator of allergic disease (345, 346). Recently, TSLP has also been shown to be crucial for maintaining homeostasis and aiding the healing process during DSS-induced colitis (347, 348). It was of interest to assess the role of TSLP in *Ac*ES mediated protection during DSS-induced colitis, as it has been reported that *H. polygyrus* and *N. brasiliensis* are still able to induce type 2 responses independently of TSLP, whereas in *Trichuris muris* infection, TSLP seems to be crucial for worm expulsion (349). To the best of my knowledge, the involvement of TSLP in response to hookworms has never been explored. Results showed that at least in $Rag1^{-/-}$ mice, neutralising TSLP did not significantly affect the protective capacity of *Ac*ES, however further tests need to be performed to obtain a conclusive answer.

One unifying factor throughout this chapter has been the increased population of $F4/80^{+}$ $GR1^{hi}$ $CD11b^{hi}$ cells that are present in mice that receive *Ac*ES, which I believe to be MDSCs. This population was present in high number in both $Rag1^{-/-}$ and WT mice and significantly expanded when IL-10R signalling was blocked, suggesting that IL-10 may be necessary to keep this cell population stable. Another possibility is that MDSCs work in concert with IL-10 to maintain homeostasis, and the blocking of IL-10 signalling may have induced an expansion in this cell type as a compensatory response. Regardless of how these cells interact with IL-10, it is likely they are playing a role in the mechanism of action of *Ac*ES-induced protection, and needs to be further investigated. Recent advances in MDSC biology has identified targeted peptides that specifically bind to MDSCs, both granulocytic and monocytic (350). The use of such peptides to deplete MDSCs before treatment with *Ac*ES would shed light on the role of these cells in mediating *Ac*ES-induced protection in the DSS model of colitis.

In summary, I have attempted to understand the mechanisms of protection generated by *Ac*ES in DSS-induced colitis. While no clear mechanism was identified, IL-10 signalling can be discounted as an *Ac*ES-mediated regulatory pathway, and more importantly protection against DSS-induced colitis seems to be independent of mature T and B cells.

6 Chapter 6 – *Ac*-AIP-1, a protein from *Ac*ES, protects mice against TNBS induced colitis.

6.1 Introduction

In chapter 5 I attempted to determine the anti-inflammatory mechanism of action of the crude *Ac*ES mixture. While I was able to demonstrate several key findings, i.e. that *Ac*ES-mediated protection is partially independent of the adaptive immune system, TSLP or IL-10R signalling, I was unable to definitively demonstrate a single, clear mechanism of action. Given the complexity of *Ac*ES, which is made up of a multitude of proteins, glycans and lipids (275), it was perhaps unsurprising that a single mechanism of action could not be elucidated. In order to better understand the anti-inflammatory mechanism of action of hookworm-derived proteins, I decided to identify a specific individual component of *Ac*ES that was protective in mouse IBD models, with a view to synthesising the particular component in recombinant form. Achieving this goal would have two major benefits - unlimited supply of the candidate protein and quality control of the material being used, as batch-to-batch variation was inevitable with native *Ac*ES. Here, I ascertain the protective components of *Ac*ES to be of protein origin, and go on to explore the therapeutic properties of some recombinant versions of individual *Ac*ES proteins in mouse models of colitis.

6.2 Results: Trypsin digestion and heat inactivation causes *Ac*ES to lose its protective capabilities

In order to determine if the protective component of *Ac*ES was due to protein constituents, *Ac*ES was enzymatically digested with trypsin overnight followed by heat-denaturation for 30 minutes – this material was termed bES. As described previously, mice were treated with either *Ac*ES or bES and exposed to DSS in the drinking water to induce colitis. While I observed significant protection against weight loss in *Ac*ES-treated mice as shown previously, mice treated with bES were not protected against weight loss (**Figure 6.2.1A**) or colon inflammation (**Figure 6.2.1C-D**). Furthermore, bES-treated mice showed increased frequency of $IFN\gamma^+$ $CD4^+$ T cells (**Figure 6.2.2A**) and reduced eosinophil

numbers (**Figure 6.2.2C**) when compared to *Ac*ES-treated mice. Macrophage numbers (**Figure 6.2.2D**) remained high in the bES group, however the expression of AAMac markers (**Figure 6.2.2E-G**) decreased significantly compared to the *Ac*ES group. bES treated mice also exhibited a significantly lowered frequency of MDSCs (**Figure 6.2.3**).Finally, the percentage of IL-4/IL-10 co-expressing $CD4^+$ T cells (**Figure 6.2.2B**) was reduced but did not reach significance, suggesting that these are not the cells responsible for the protective effects of *Ac*ES. Additionally, when mice were administered SEA in section **5.2,** levels of IL-4/IL-10 co-expressing $CD4^+$ T cells were equally elevated to levels similar to the *Ac*ES treated group, however the SEA treated mice were not protected from DSS induced colitis. However, it remains a possibility that under different conditions these cells may play a role in the protection against DSS-induced colitis and this needs to be directly investigated by either transferring these cells into DSS treated mice or through selective deletion of this cell type.

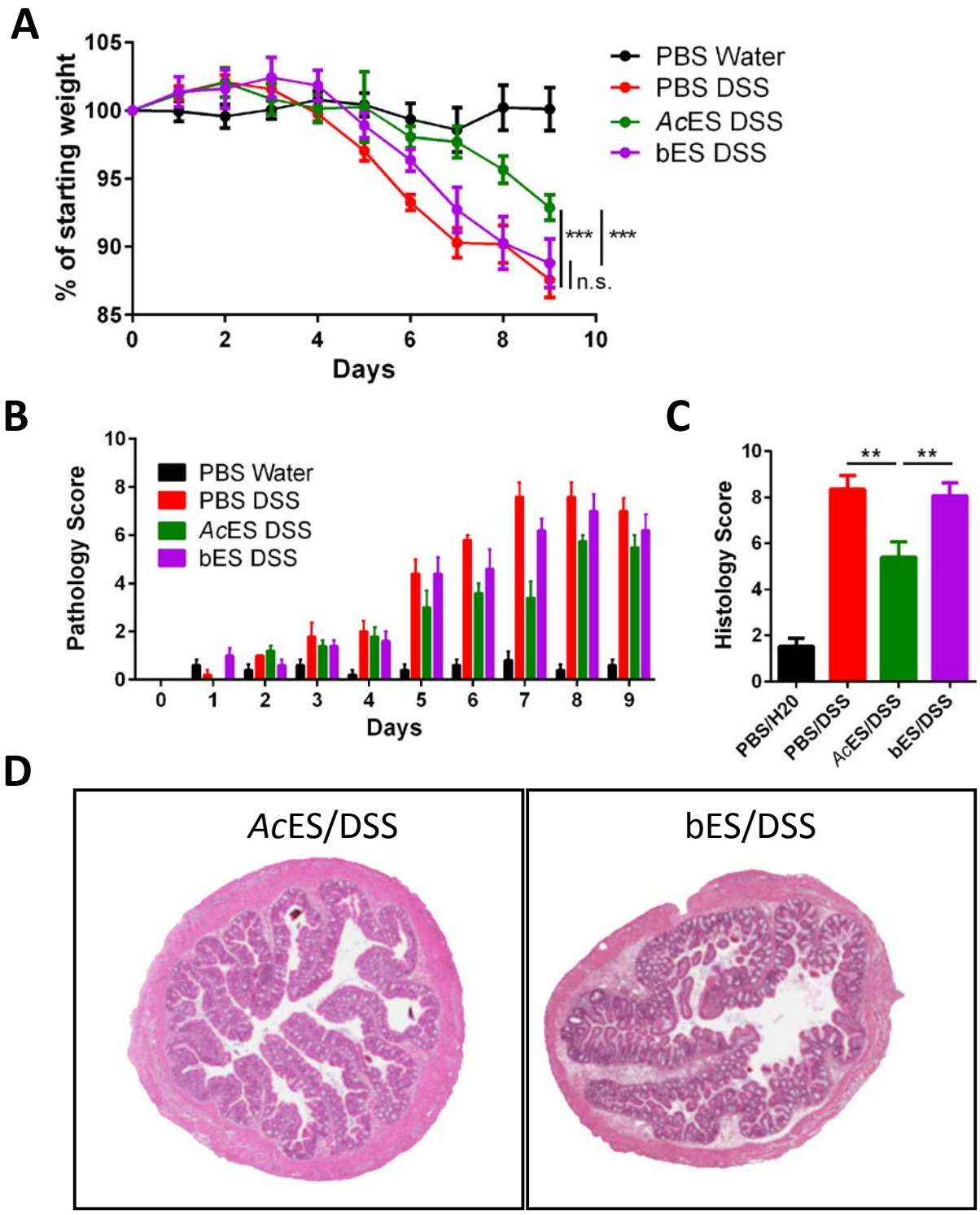


Figure 6.2.1: Trypsin digestion causes *Ac*ES to lose its protective properties.
Mice received 3.5% DSS *ad libitum.* Daily injections of PBS, 25 µg *Ac*ES or equivalent bES were administered i.p. Percentage of starting weight (A) and pathology score (B) of mice during DSS induced colitis model. Histology scores (C) and representative H&E stained colon micrographs (x20 magnification) (D). Error bars show the standard error of the mean, graphical results are pooled from 4 repeat experiments, n = 16 mice per group total (4 mice per group per experiment), histology pictures are representative. *** = $p<0.001$, ** = $p<0.01$ and n.s. = non-significant.

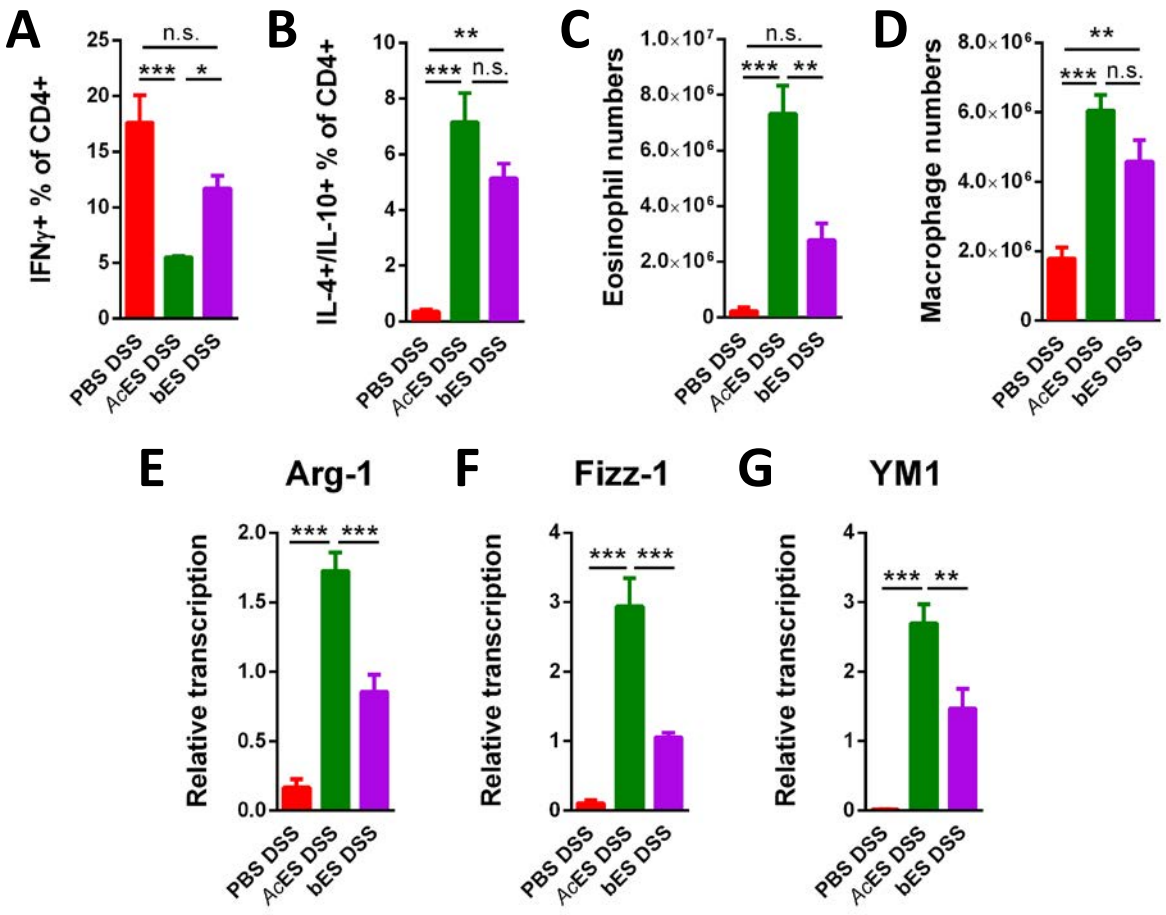


Figure 6.2.2: bES-treated mice have a lowered type 2 response compared to *Ac*ES-treated mice.

Mice received 3.5% DSS *ad libitum*. Daily injections of PBS, 25 µg *Ac*ES or equivalent bES were administered i.p. Intracellular cytokine staining showing frequency of IFNγ$^+$ (A) and IL-4$^+$/IL-10$^+$ (B) CD4$^+$ T cells in peritoneum of bES treated mice compared to *Ac*ES. Eosinophil (C) and macrophage (D) numbers in the peritoneum. Expression of alternative activation markers, *arg-1* (E), *fizz-1* (F) and *ym1* (G) from peritoneal macrophages. Error bars show the standard error of the mean, graphical results are representative from 3 repeat experiment, n = 5 mice per group, * = p<0.05, ** = p<0.01, *** = p<0.001, n.s. = non-significant.

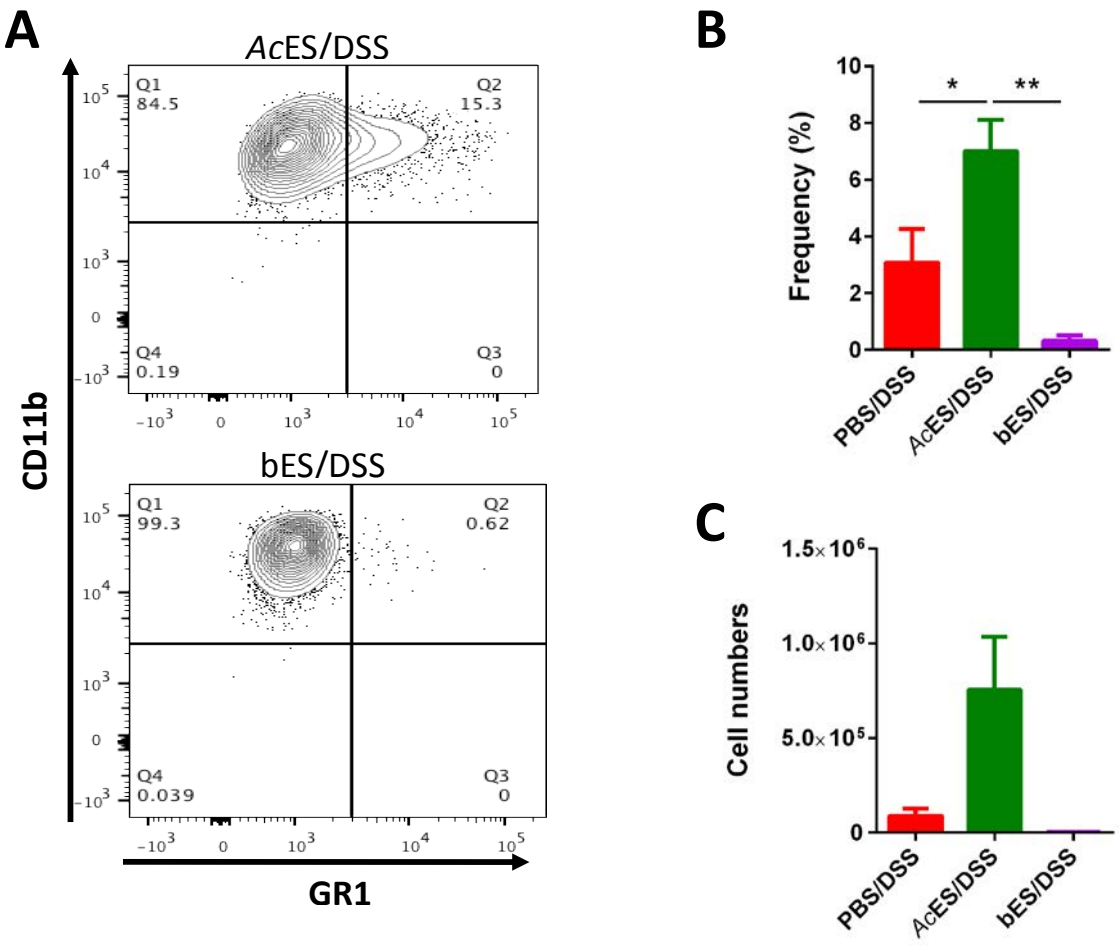


Figure 6.2.3: Trypsin digestion of *Ac*ES causes a significant reduction in myeloid derived suppressor cell (MDSC) recruitment.

Mice received 3.5% DSS *ad libitum.* Daily injections of PBS, 25 µg *Ac*ES or equivalent bES were administered i.p. Representative flow cytometry plots from peritoneal lavage cells stained for surface markers Siglec-F, F4/80, GR1 and CD11b. Plots were gated for F4/80 vs Siglec-F then were further analysed within the F4/80^{+} gate by looking at CD11b vs GR1 (A), showing frequencies of the F4/80^{+} population. MDSC frequency of live cells (B) and total MDSC cell numbers in the peritoneum (C). Error bars show the standard error of the mean, graphical results are representative of 3 repeat experiments, n = 5 mice per group, * = $p<0.05$, ** = $p<0.01$.

6.3 Recombinant Neutrophil inhibitory factor (rNIF) is not protective in models of chemically induced colitis

*Ac*ES contains at least 105 identified proteins (275), most with unknown functions. Neutrophil Inhibitory Factor (NIF) has been shown to bind to CD11b I-domain (351) and thereby inhibit the adhesion and transmigration of neutrophils across the epithelial layer (352). Furthermore, this protein could be readily synthesised in *P. pastoris* (353), which was already established in our lab (277). I therefore sought to assess the potential role of rNIF in protection against colitis in mice. rNIF (20 μg) was administered either the day before (D -1), the same day (D 0) or the day after (D +1) administration of TNBS and weight loss and pathology were assessed. A modest protective effect was observed when rNIF was administered the day before induction of TNBS induced colitis (**Figure 6.3.1**).

I next sought to assess whether administration of rNIF could regulate DSS-induced colitis in mice, particularly given the important role for neutrophils in UC (354-356). rNIF was administered daily from either day 0 or day 4 after the first delivery of DSS in drinking water. Daily administration of rNIF from the beginning of DSS treatment did not have any effect on weight loss or pathology outcomes (**Figure 6.3.2**). However, administration of rNIF from day 4 onwards significantly reduced pathology scores and colon shortening (**Figure 6.3.2B-C**). Together, these results indicated that rNIF may be partially effective at limiting some aspects of pathology during models of colitis, but the effects were not as conspicuous as the results achieved with native *Ac*ES.

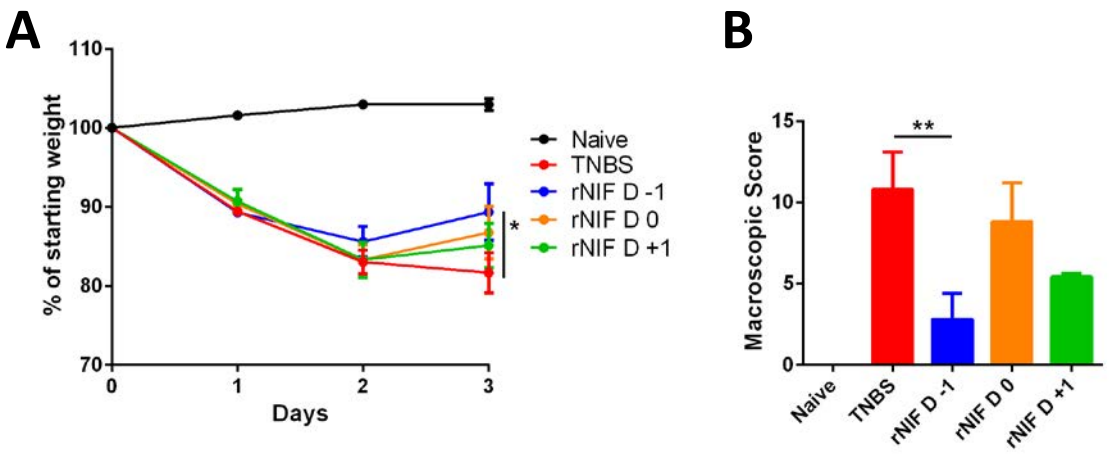


Figure 6.3.1: rNIF may prevent severe pathology if administered before induction of TNBS colitis.

Mice were administered 20 µg of rNIF i.p. either the day before (D -1), the day of (D 0), or the day after (D +1) administration of TNBS. Percentage of starting weight (A) and macroscopic score (B) of mice during TNBS induced colitis model. Error bars show the standard error of the mean, graphical results are representative of 2 repeat experiments, n = 5 mice per group. ** = $p<0.01$, * = $p<0.05$.

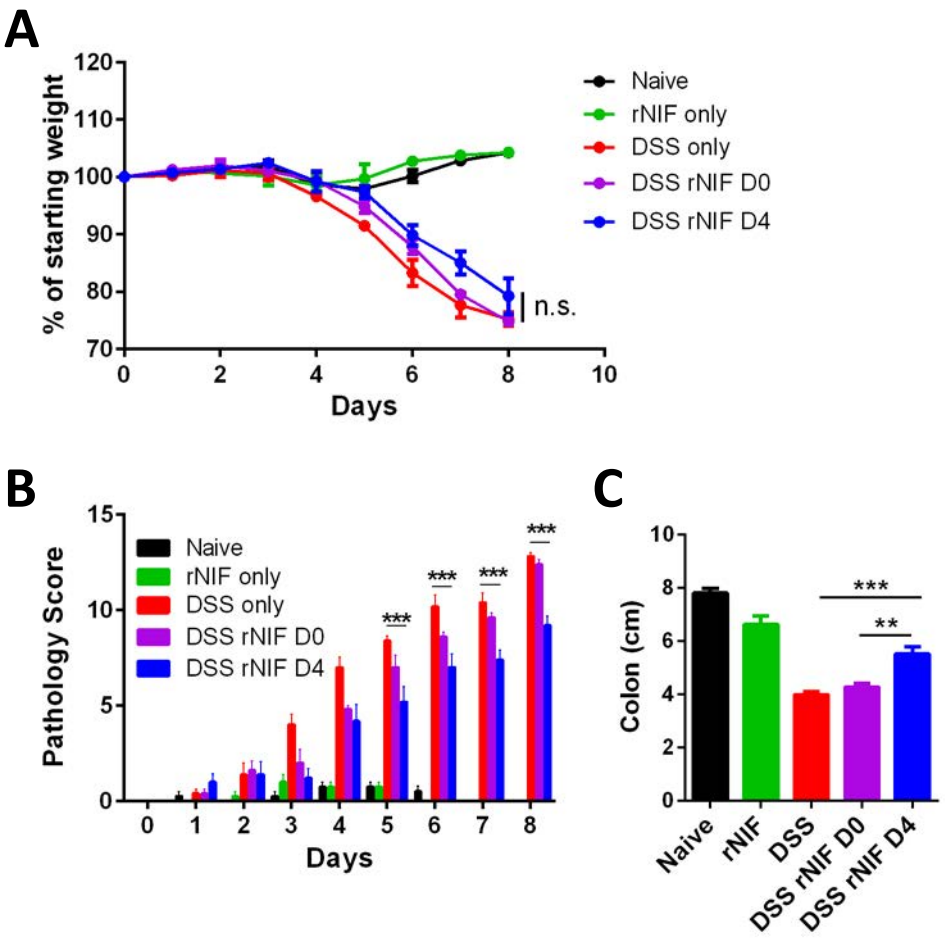


Figure 6.3.2: rNIF shows mild protection against DSS-induced pathology.
Mice were given 3.5% DSS *ad libitum*. Mice were administered 20 µg of rNIF i.p. on a daily basis from either day 0 or day 4 onwards. Weight was assessed daily and is represented as a percentage of initial weight at day 0 (A). Pathology scores were assessed daily (B). Colon length was measured upon euthanasia (C). Error bars show the standard error of the mean, graphical results are representative of 2 repeat experiments, n = 5 mice per group. *** = $p<0.001$, ** = $p<0.01$.

6.4 *Ac*-AIP-1, a recombinant protein from hookworm ES, is protective against TNBS-induced colitis in mice

Given the relative lack of therapeutic efficacy of rNIF in mouse models of IBD, I next explored the potential role for a different hookworm ES protein. Proteomics analysis of *Ac*ES revealed abundant expression (57% of *Ac*ES) (275) of two distinct Tissue Inhibitor of Matrix Metalloprotease (TIMP)-like proteins, one of which was *Ac*-TMP-1 (357, 358). In the absence of apparent metalloprotease-inhibitory properties for *Ac*-TMP-1 (359), and to avoid confusion about its proposed function, the protein was renamed *A. caninum* Anti-Inflammatory Protein-1 (*Ac*-AIP-1, or AIP-1) based on its anti-inflammatory properties described herein.

Ac-AIP-1 (20 µg) was administered i.p. five hours prior to TNBS injection as described previously. Control mice received PBS only, or *Ac*ES (20 µg). As expected, *Ac*ES-treated mice exhibited significantly reduced weight loss, macroscopic pathology score and histology scores (**Figure 6.4.1A-C**). Intriguingly, mice treated with *Ac*-AIP-1 exhibited significantly reduced weight loss by day 2 and returned to 95% of their initial body weight by day 3 (**Figure 6.4.1A**). Macroscopic and histological analysis also showed a dramatic reduction in inflammation in mice that received *Ac*-AIP-1 as compared to controls (**Figure 6.4.1B-C**). Histological micrographs further demonstrate the intact epithelial barrier and goblet cell production and no sign of sub-mucosal oedema in the mice that were treated with *Ac*-AIP-1 (**Figure 6.4.1D**).

The T cell cytokine-specific response in the MLN was assessed by FACS analysis. Production of IL-17A and IFNγ by MLN cells was diminished in *Ac*-AIP-1 treated mice compared to controls (**Figure 6.4.2A-B**). This data highlights the potential of *Ac*-AIP-1 to regulate cytokine responses during TNBS-induced colitis. To assess in greater detail the impact of *Ac*-AIP-1 on the expression of a range of different cytokines and genes related to TNBS induced colitis, I performed high-throughput gene profiling using a QIAGEN gene array. Significant up-regulation was observed for *cxcl11* (**Figure 6.4.3**), which is chemotactic factor for activated T lymphocytes. I also noted significant down-regulation of the gene encoding for colony stimulating factor-2 (*csf-2/GM-CSF*), which is important for the growth and differentiation of granulocytes and monocytes (360, 361). Matrix metalloproteinase 2 (MMP2), fibroblast growth factor 7, fibrinogen alpha, cathepsin K

and collagen type 4 were also up-regulated in *Ac*-AIP-1 treated mice compared to controls.

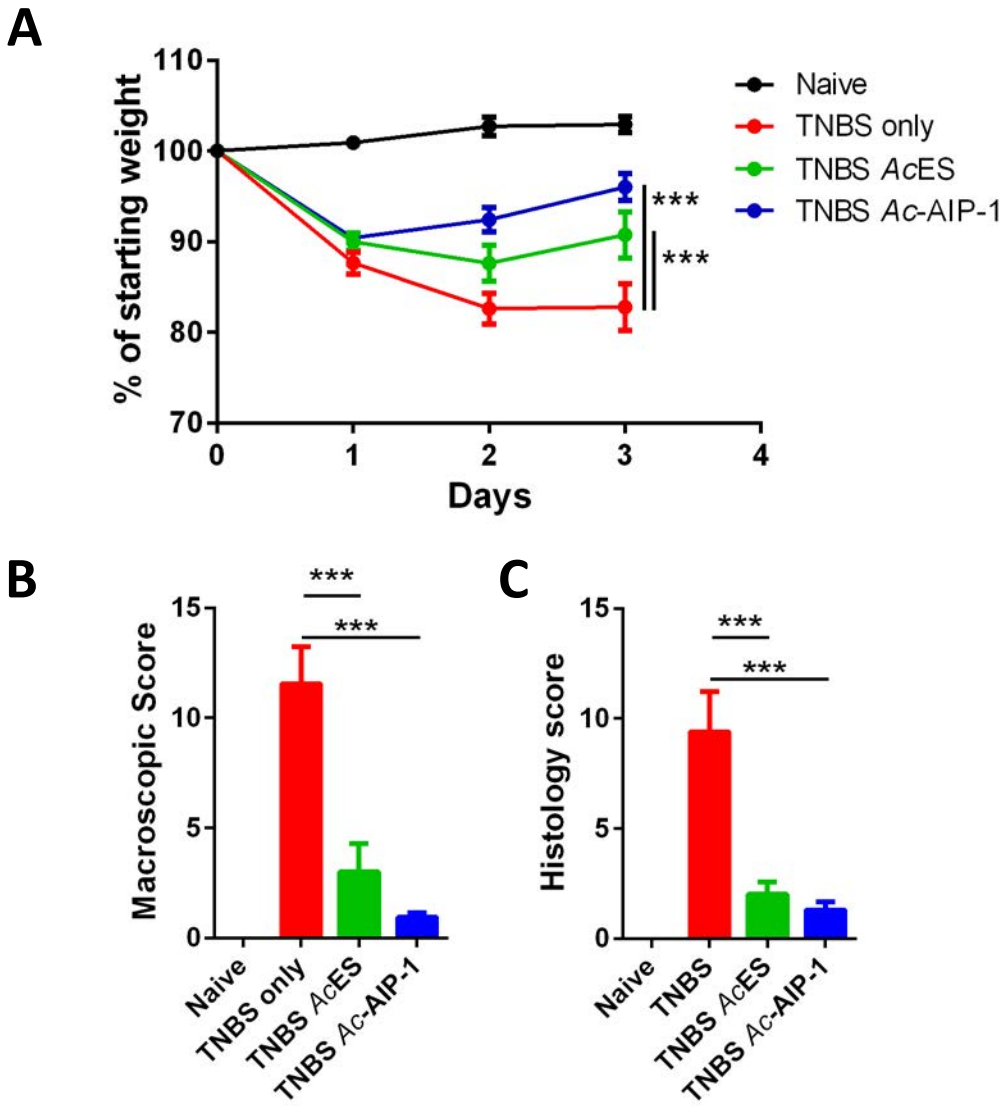


Figure 6.4.1: *Ac*-AIP-1 protects mice from TNBS-induced colitis.
Mice were given a single 20 µg injection of either *Ac*ES, *Ac*-AIP-1 or 100 µl of PBS 5 hours prior to TNBS administration. Weight was measured daily and represented as a percentage of starting weight (A). Macroscopic signs of inflammation were assessed upon euthanasia (B). Microscopic signs of inflammation were assessed on histology preparations (C). Representative histology micrographs stained with PAS and Alcian Blue (D) (box on lower left corner indicates 200 µm). Error bars show the standard error of the mean; graphical results are pooled from multiple experiments, n = 12-15 (4 – 5 mice per group from 3 repeat experiments); * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

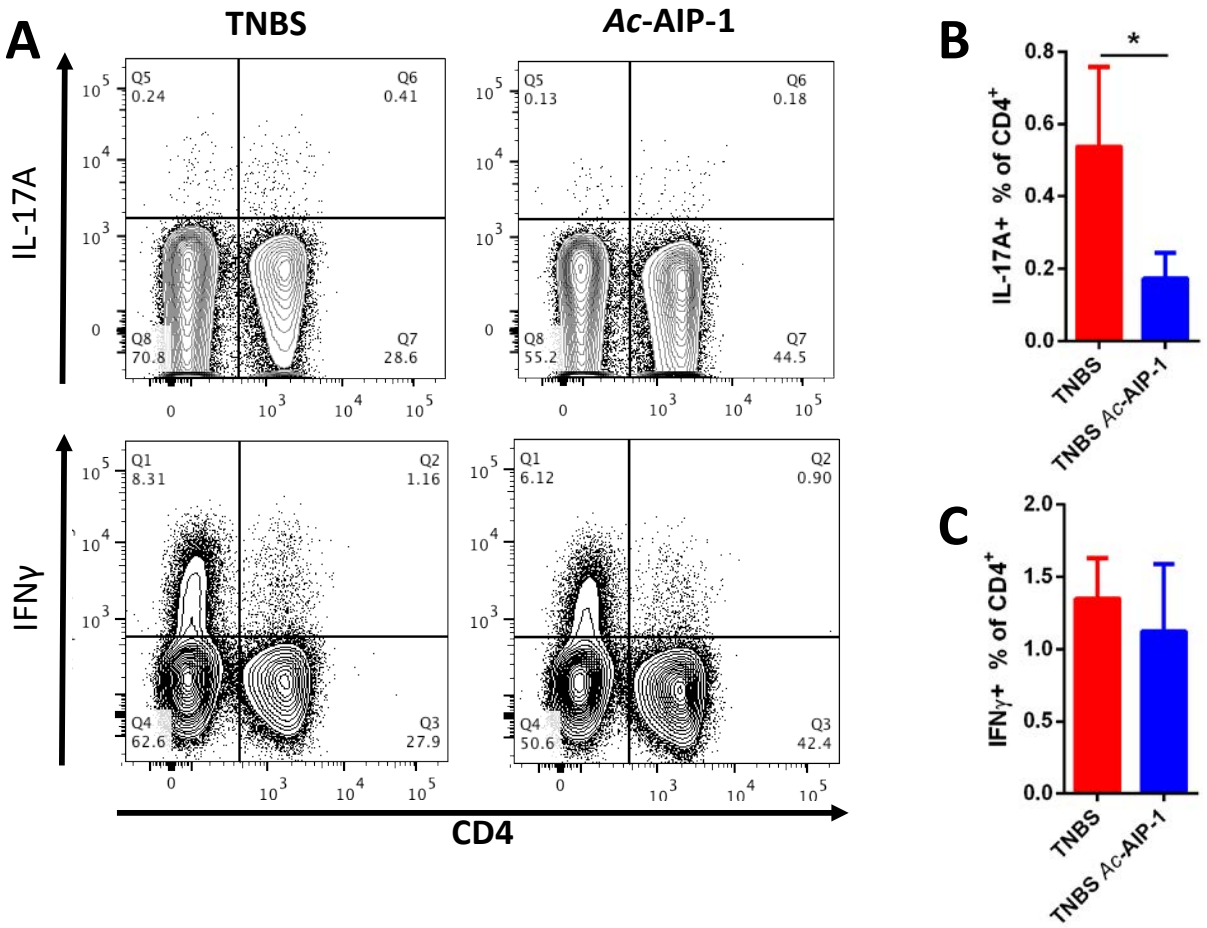


Figure 6.4.2: *Ac*-AIP-1 treated mice have lower frequencies of IL-17A producing CD4⁺ T cells. Mice were given a single 20 µg injection of either *Ac*ES, *Ac*-AIP-1 or 100 µl of PBS 5 hours prior to TNBS administration. Representative flow plots of intracellular cytokine staining, showing mesenteric lymph node cells producing either IL-17A or IFNγ (y-axis) versus CD4 (x-axis) expression (A). Graphical results of frequency of IL-17A (B) and IFNγ (C) producing CD4⁺ T cells. Error bars show the standard error of the mean; graphical results are representative of 3 repeat experiments, n = 5 mice per group; * = $p<0.05$.

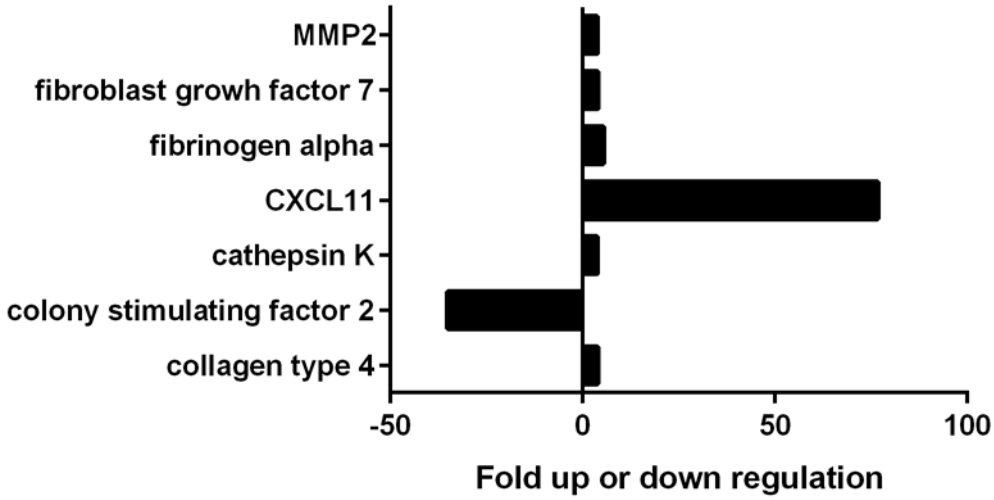


Figure 6.4.3: *Ac*-AIP-1 treatments results in up-regulated expression of cxcl11 and down-regulated expression of csf-2 in the colon during TNBS induced colitis.
Mice were given a single 20 µg injection of either *Ac*ES, *Ac*-AIP-1 or 100 µl of PBS 5 hours prior to TNBS administration. Graph shows genes that are either up or down regulated in *Ac*-AIP-1 treated mouse colons compared to TNBS only controls using *hPRT* as a reference gene with $p < 0.1$, n = 3 mice per group.

6.5 Chapter 6 Summary

Hookworms are obligate parasites that require entry into a suitable host in order to continue their life cycle. As such, hookworms and other helminths have developed an armoury of ES products that they use to gain access to the host and remain camouflaged from immune surveillance. *Ac*ES has been shown using mass spectrometry to contain over 100 identified proteins (275) and it likely contains many more. In my previous chapters, I have shown the protein content of *Ac*ES has the potential to inhibit inflammation in models of colitis. Two of these proteins have been expressed and their protective abilities against colitis have been assessed in this chapter.

NIF was tested first because it is known to bind to CD11b (351, 362). NIF had been previously trialled as a treatment for stroke patients due to its ability to limit transmigration of neutrophils and thereby limit cytotoxic damage to blood vessels (266, 363). Recently it has also been shown to limit transmigration of eosinophils (267). Given these reports, I hypothesised that NIF might be able to limit the inflammatory response in chemically-induced models of murine colitis. Despite the minor effect on weight loss and cytokine production when mice received rNIF the day prior to TNBS administration, or on day four of the DSS model, the protective effects were minimal compared to that of *Ac*ES. It would be expected that a purified protein with anti-inflammatory properties would provide better protection than the same quantity of a crude mixture containing only a fraction of that protein. Hence, rNIF was not further pursued during this thesis.

Due to its relative abundance in *Ac*ES, the next protein to be tested in the TNBS model of colitis was *Ac*-AIP-1. In comparison to the vehicle-treated TNBS group, mice treated with AIP-1 were significantly protected from weight loss and colon inflammation. Interestingly, I did not observe any protective effect of AIP-1 in the DSS model of colitis (**Figure 8.4.1** in **section 8.4**). This difference may be due to the types of responses induced; the DSS model colitis induces inflammation via innate immune responses as opposed to the TNBS model, which in contrast is mainly T cell-mediated. In fact, my gene expression data revealed elevated transcription of *cxcl11*, a chemotactic factor for activated T cells, suggesting that *Ac*-AIP-1 has an effect on the T cell population. Moreover, AIP-1 treatment resulted in reduced transcription of *csf-2*, which

is necessary for growth and differentiation of granulocytes, suggesting that AIP-1 may inhibit innate cells responses.

In summary, I have shown here that there are protein constituents within *Ac*ES that have anti-inflammatory properties. I have also shown that a particular protein, *Ac*-AIP-1, can be readily produced in yeast and shows potent anti-inflammatory properties in the TNBS model of colitis. It is hypothesised that *Ac*-AIP-1 may be acting via a T cell-dependent mechanism, possibly through activation of regulatory T cells which are able to regulate pathologic effector T cell function, and may therefore be an excellent candidate for other inflammatory conditions. Further investigation into which cells are expanded and activated by *Ac*-AIP-1 and the cell mediated uptake of *Ac*-AIP-1 are necessary to confirm this hypothesis.

7 Chapter 7 - Discussion

The immune system is likely the most complex system in the human body, evolving to protect us from the many foreign substances that we encounter in our everyday lives, be it airborne, ingested or forcibly introduced through trauma of our barrier surfaces. The immune system has, and continues to be, shaped by both pathogenic and non-pathogenic influences. These interactions are essential to the development and maintenance of the immune system, and the rise in immune related disorders worldwide is perhaps a sign that something has gone awry. We have embraced cleaner living conditions, sanitation, water treatment and better healthcare, as well as an explosion in antimicrobial and chemical compounds (364). The prevalence of soil-transmitted helminths has been severely affected by improved sanitary conditions, having been practically wiped out in most developed countries (365), beginning with the introduction of the flushing toilet system (242). Coincident with the improved hygiene is the rise in inflammatory conditions, both autoimmune and allergic, which begs the question, 'have we done away with a vital component of our immune system's education?'

Soil transmitted helminths have been co-evolving with vertebrates for millions of years. Helminths are evident from the very earliest of recorded human history, and helminth eggs have even been found within fossilised dinosaur coprolites (366). Not only has our immune system evolved to be able to deal with helminths, but the helminths themselves have also evolved in such a way to be able to live within their vertebrate hosts. This is particularly evident in human hookworms, which can live for decades inside their hosts without undergoing any form of developmental arrest or causing overt pathology in small numbers (237). The immunological profile of a person with hookworm infection is mostly skewed towards a type 2 response with high levels of IL-4, IL-5, IL-10 and IL-13, increased eosinophilia and IgE, but also less dramatic increases in IFNγ and IL-2 (237). There is now convincing evidence that the hookworm itself produces immunomodulatory molecules to skew the immune response in its favour, and that its ES products may contain molecules that could be harnessed for use as therapeutics for inflammatory diseases (129, 258, 275).

Initially I sought to investigate the responses resulting from the injection of *Ac*ES products into healthy adult mice. As described in Chapter 3, *Ac*ES alone is sufficient to stimulate a polarised type 2 response typical of a helminth infection. This was validated not only by the production of cytokines such as IL-4, IL-5, IL-10 from the antigen-specific restimulation of splenocytes of these mice, but also by the increased numbers of eosinophils and AAMacs recruited to the site of injection. There is an ongoing debate on whether the Th2 response is beneficial to the worms or to the host. Many papers have described a link between helminth-induced Th2 responses and enhanced luminal fluid secretions (367) and gut epithelial cell turnover (368) as a means of worm expulsion. In this case, one might speculate that it is the hookworm's sharp "teeth" or cutting plates and their ability to bury themselves deep into the host mucosa that keeps them in place and able to withstand these Th2 associated responses. An alternative explanation is that the worm itself induces a Th2 repsonse that is beneficial for its survival within the host. This is particularly evident during schistosomiasis when the initial Th1 response switches to a Th2 response upon initiation of egg laying by female worms (369). It has been shown that SEA can directly modulate DC function, whereby DCs maintain an immature-like phenotype and have a reduced capacity to produce type 1 cytokines, further skewing the immune response towards a type 2 phenotype (369). Similarly, *Ac*ES also drives DCs towards an immature-like phenotype, with lowered expression of activation markers and the type 1 cytokines IL-12p70 and TNF, as well as the ability to induce a type 2 response when transferred back into mice (see **section 3.4**).

Another way in which helminths may modulate their environment is through induction of AAMacs which have been implicated in wound healing (370, 371). Due to the hookworm's feeding strategy, it is plausible that they secrete factors which help the host to heal wounded tissues. Indeed the human liver fluke *Opisthorchis viverrini,* has been shown to produce a protein that stimulates cell proliferation, likely as a damage control mechanism to accelerate repair of the wounds it causes during feeding (372). Studies by Donelly *et. al* using ES products from a platyhelminth liver fluke, *Fasciola hepatica*, suggest that the AAMacs arise first and regulate the cytokine environment, which in turn leads to a Th2 response (292). However my own results presented herein are more in agreement with Flynn *et. al*, who suggest that there is a possible link

between *F. hepatica*-induced IL-4 and subsequent AAMac induction (373). Interestingly, helminth-stimulated AAMacs have also been implicated with maintaining glucose tolerance and insulin sensitivity in mice fed a high fat diet (374). It is known that AAMacs can utilise the fatty acids for energy via the oxidative metabolic pathway, whilst classically activated macrophages undertake glycolysis for the production of energy, adding to the body's fat stores (375). This highlights yet another facet of how helminth infections can be beneficial for humans, potentially helping to combat the rising incidence of metabolic diseases like diabetes. Indeed Lund *et al.* recently demonstrated that *F. hepatica* ES (*Fh*ES) considerably delays or even prevents the onset of type 1 diabetes in non-obese diabetic (NOD) mice (376).

After determining that *Ac*ES did have immunomodulatory properties, I wanted to determine whether this translated into a clinical benefit in an animal model of gastrointestinal inflammation. I chose to focus on mouse models of IBD, primarily because the ongoing human clinical trials in our lab were focused on this area, but also because the hookworm occupies the same niche as this group of diseases, the gastrointestinal tract. Yet, it is clear from our own work and that of others that molecules from parasitic worms are able to affect a range of different organs and have protective effects on a variety of inflammatory conditions (119, 128, 188, 272, 376, 377).

IBD are chronic and debilitating diseases, and current treatments are designed to manage the disease rather than cure it. Moreover, of the currently available therapies only glucocorticoids or anti-TNF antibody therapy, provide benefits to the patients, yet both have the downside of general immunosuppression, and the latter is extremely costly (378). There is a clear need for a therapy that is readily synthesized and is safe and well tolerated by patients. Human studies utilising live worm therapy have already indicated a beneficial effect of hookworms on Crohn's disease (379) and coeliac disease (131), however neither the protective mechanisms nor the hookworm molecules involved are known. This thesis is the first step in attempting to answer these questions.

Data from the DSS model of colitis indicated that *Ac*ES was able to reduce pathology scores and weight loss in mice. The immunological profile of mice receiving *Ac*ES in the DSS model was similar to that achieved by administration of *Ac*ES in the absence of DSS,

and included increased eosinophils, AAMacs and type 2 cytokine production. There was also a distinct group of IL-4/IL-10 co-expressing $CD4^+$ cells, which were expanded in mice treated with *Ac*ES. This cell type was of particular interest due to the secretion of IL-10, a cytokine that has long been associated with immune regulation, particularly in colitis (211, 328). However, attempts at neutralising IL-10 proved that the immunoregulation afforded by *Ac*ES is not dependent on IL-10 signalling as seen in **section 5.3**.

Without access to IL-4 reporter mice, and in order to determine whether IL-4/IL-10 co-expressing $CD4^+$ T cells were involved in the *Ac*ES-mediated protection, I used a system in which both T and B cells were absent - $Rag1^{-/-}$ mice. Induction of colitis with DSS in $Rag1^{-/-}$ mice indicated that *Ac*ES mediated protection in the DSS model of colitis is more reliant on the innate immune response and is not dependent on T or B cells, as seen by the protection against weight loss and pathology. These results strongly suggested that IL-4/IL-10 co-expressing $CD4^+$ T cells were not involved in *Ac*ES-mediated protection against colitis, and deterred any further investigation into potential T cell populations such as Tregs, which have been shown to be stimulated by other parasitic worms (380, 381). That is not to say that *Ac*ES does not induce Tregs - this matter still requires further investigation, and there is evidence that defined components of *Ac*ES can induce Treg expansion in mice (382). Given the multifaceted nature of *Ac*ES it is likely that there are many pathways at play, especially when considering its effect in both the DSS and the TNBS models of colitis, which have different aetiologies.

These last experiments have revealed the importance of the innate component of *Ac*ES-induced responses that have been shown to interact with epithelial cells via the secretion of TSLP, a potent inducer of IL-4 production (334). TSLP has been shown to be a master regulator of Th2 responses and has been implicated in exarcebating a range of allergic diseases (343, 345, 346, 383, 384). It is mainly expressed by epithelial cells and keratinocytes but is also expressed by DCs, smooth muscle cells and fibroblasts (333, 385, 386). Moreover, TSLP is constituitively expressed by intestinal epithelial cells in both mice and humans (334, 347) and its expression is reduced in people suffering from IBD (334, 387). Due to the strong Th2 polarisation induced by *Ac*ES and in order to limit the involvement of the adaptive immune response in the DSS model, I chose to neutralise TSLP in $Rag1^{-/-}$ mice. However, neutralising TSLP did not have any significant

effect on the capacity of *Ac*ES to protect Rag1$^{-/-}$ from DSS induced colitis. Interestingly, a recent paper from Spadoni *et. al* showed that TSLP produced by CD103^{+} DCs can directly influence IL-17A production by T cells (333). By directly acting on T cells, TSLP is able to reduce IL-17A production and establish a favourable environment for induction of Tregs which possibly affects the overall inflammation induced by DSS exposure. Therefore, it is possible that had the experiment been perfomed in wild type (WT) mice instead of Rag1$^{-/-}$, different results may have been achieved and should be investigated in future studies.

Another interesting cell type seen throughout these experiments was only observed in the *Ac*ES group and defined as F4/80^{+}CD11b^{hi}GR1^{hi}, which are believed to be MDSCs. To this day, these cells have been poorly described but they seem to appear in response to chronic inflammation, and putatively function to dampen the immune response to avoid overt pathology (388). MDSCs are described as a heterogenous population of both monocytic and granulocytic myeloid cells in different stages of differentiation (389). Due to their complexity in morphology and differentiation states, characterisation of these cells has been a challenge. Several markers such as CD124 (IL-4Rα), CD115, CD40 and CD80 have been shown to be expressed by MDSCs, however expression of these markers may differ depending on the experimental model (310, 389-391). The only concrete markers expressed by all MDSCs is a combination of CD11b and Gr-1 protein isoforms, LY6C and LY6G (390). If triple staining with separate LY6C and LY6G isoforms is available, MDSCs can be further classified into monocytic-like MDSCs (Mo-MDSCs: CD11b^{+}Ly6G^{-}Ly6C^{high}) and polymorphoneuclear-like (granulocytic) MDSCs (PMN-MDSCs: CD11b^{+}Ly6G^{+}Ly6C$^{low/int}$) (392).

In my experiments I have shown that a consistent population of F480^{+}CD11b^{hi}GR1^{hi} cells is expanded in mice treated with *Ac*ES. I have labelled these as MDSCs based on their characteristics, however functional suppression studies would need to be perfomed in order to ascertain if they are indeed MDSCs. Further classification of these cells was not possible during the course of my thesis, but should definitely be performed in future experiments. A simple addition of LY6C and LY6G isoforms into the staining panel instead of the combined GR1 isoform, would help to further classify these cells into Mo-MDSCs and/or PMN-MDSCs. Since these are all surface markers the presumed

MDSCs could be purified and tested for suppression of T cell responses by CFSE dilution. Furthermore, it would be intriguing to test their function within a model of disease by either transfering these cells into a colitic mouse or neutralising these cells with an anti-GR1 antibody. However, neither of the suggested experiments may give definitive results, as MDSCs are known to be incredibly sensitive to sorting techniques (393) and lose their suppressive abilities 2 days after adoptive transfer (394). The downside to the anti-GR1 antibody is that it would also bind to other cells such as neutrophils, and to some extent DCs and macropahges as well (395). However, a recently published paper by Qin *et al.* describes a new peptide that depletes MDSCs in tumour bearing mice with better specificity than the anti-GR1 antibody (350). If this 'peptibody' becomes commercially available soon, it would be a superior method of investigating the role of MDSCs in *Ac*ES-mediated protection against DSS. It is important to note however, that MDSCs are induced in both DSS (396, 397) and TNBS (396) models of colitis, peaking around the time when inflammation is highest. The peak in MDSCs has been shown to occur just before an amelioration of pathology in TNBS colitis and adoptive transfer of MDSCs (either *in vitro* derived or sorted) is capable of ameliorating pathology (396). Interestingly, resveratrol (a naturally occuring polyphenol of plants) has been shown to induce MDSCs when administered orally, which correlated with a reduction of $CXCR3^{+}$ T cells and amelioration of chronic colitis in IL-$10^{-/-}$ mice (394). It would be of interest to search for a resveratrol homologue within *Ac*ES and other parasites, to see if there are any similar compounds that could be potentially inducing MDSCs.

Although MDSCs have mostly been described in a pathological sense during cancer, it is clear that they also play a beneficial role in decreasing inflammation in mouse models of colitis (394, 396, 397) and may also play a non-pathological role in the response to helminth parasites. MDSCs are also known to be recruited during protozoan infections such as leishmaniasis and Chagas disease (398, 399). Interestingly, MDSC function has been linked with susceptibility to *Leishmania* infection in Balb/c vs C57BL/6 mice (398). Reports of MDSCs from nematode infections are sparse, with only one report found from *N. brasiliensis infection* (400). The mechanisms by which MDSCs exert their suppressive functions are varied, but most commonly it is noted that production of

reactive oxygen/nitrogen species is necessary to inhibit the funtion of T cells; in a manner similar to that employed by AAMAcs in metabolising L-arginine (401, 402).

The other possible player in *Ac*ES-mediated protection is the newly described innate lymphoid cells type 2 (ILC2). ILCs are cells which have morphological characteristics of lymphoid cells, and do not carry any rearranged antigen receptors commonly found on cells of the adaptive immune system (403), meaning that they do not act in an antigen-specific manner. There are three currently recognized ILC groups which have similar properties to cells of the T helper cell subtypes (404). The ILC1s produce type 1 cytokines, notably IFNγ and TNF and includes NK cells (404); the ILC2s produce type 2 cytokines and are thought to be the main source of initial IL-5 and IL-13 production (404); and the ILC3s produce cytokines associated with Th17 cells such as IL-22 and IL-17A (404). Furthermore, ILC2s have been shown to be involved in a range of type 2 inflammatory conditions, from responses to helminth parasites (405) to allergies (406, 407). It is thought that ILCs provide many of the early signals necessary to induce a type 2 immune response (404). More recently these cells have also been implicated in various homeostatic conditions such as atherosclerosis (408) and tissue repair (342, 409) through induction of AAMacs and eosinophils (410). Unfortunately, I did not get the opportunity to investigate the incidence of these cells in response to *Ac*ES, but the immunological profile of ILC2s indicates that they are likely to be involved in the immune response to *Ac*ES in mice, characterised by high levels of IL-5 and IL-13, involvement of eosinophils and AAMacs. The protection observed against DSS in $Rag1^{-/-}$ mice treated with *Ac*ES also supports the idea that ILC2s may be present. The functionality of this cell type as mediators of protection needs to be investigated in future studies. It is possible that the ILC2s set up a conditional environment for the MDSCs and AAMacs, which can dampen any pro-inflammatory type 1 responses whilst the ILC2s and AAMacs limit the disease progression by aiding in tissue repair.

Due to the multicomponent nature of *Ac*ES it is likely that there are many molecules working in concert to both modulate the host immmune response and to aid in the nutrition and survival of the hookworm. Attempting to pinpoint a singular mechanism of action from such a complex mixture proved to be difficult, hence identifying singular molecules with immunomodulatory properties was a more realistic option.

Denaturation of *Ac*ES identified the protective component in the DSS model to be proteinaceous, but there are also likely roles for glycans and lipids. Indeed many carbohydrates from other parasitic worms have been shown to have immunomodulatory properties. For example when metaperiodate-treated SEA (mSEA) was administered to mice itranasally there was a marked reduction in IgE response and eosinophil recruitment compared to untreated SEA (411). Furthermore, lymphocytes from mSEA treated mice were unable to produce type 2 cytokines when re-stimulated *ex vivo*, suggesting that the carbohydrates within the SEA were necessary to induce this type 2 response (411). In *T. crassiceps,* metaperiodate treatment of its ES products alters its ability to induce MDSCs (412). Similarly for *Ac*ES, trypsin digestion abolished its capacity to induce MDSCs, however it would be of interest to find out if sodium metaporiodate treatment of *Ac*ES would also affect expansion of this cell type.

Previous studies had already identified *Ac*-NIF as a protein of interest due to its binding capacity to CD11b/CD18 (263) and potential to inhibit neutrophil transmigration (265). However, clinical trials exploring the use of *Ac*-NIF in stroke patients stalled due to lack of clinical significance (266). Data from both the DSS and TNBS models of colitis showed that *Ac*-NIF provided only mild protection against pathology in both models. Because the goal was to find a protein that was at least comparable to *Ac*ES in its protective properties, no further studies were performed with *Ac*-NIF.

Another *A. caninum* protein *Ac*-AIP-1 did show significant improvement of TNBS-induced colitis but not against DSS-induced colitis. The reason for this discrepancy might be due to the cell types with which *Ac*-AIP-1 interacts. Data from the gene array identified substantial upregulation of *cxcl11*, which is a prominent chemokine in wound healing (413). More importantly, CXCL11 was recently shown to polarize naïve T cells into a Tr1 phenotype with increased expression of IL-10 and reduced inflammation in a mouse model of EAE (414). The TNBS model of colitis is dependent on T cells, whereas the DSS model seems to be mostly dependent on the innate immune system. Therefore it is possible that *Ac*-AIP-1 is having a direct effect on T lymphocytes, which mediate TNBS induced colitis, but is not a major component in *Ac*ES-mediated protection against DSS colitis. Moreover, *Ac*-AIP-1 treatment resulted in downregulation of the granulocyte differention gene *csf-2* compared to TNBS treated mice. CSF-2 may be a marker of

decreased inflammation; it triggers the expansion of MDSC (415) which are maximally recruited during peak inflammation of TNBS-induced colitis (396), suggesting that *Ac*-AIP-1-treated mice had significantly lower inflammation than untreated mice. Unfortunately the presence of MDSCs was not assessed in this model but it would be interesting to see if they respond to *Ac*-AIP-1 in future studies. It is plausible that MDSCs are induced much earlier and therefore mice can recuperate from the short TNBS insult. Another possibility is that the reduction of CSF-2 keeps these cells in their immature state where they are most suppressive, as CSF-2 in combination with the correct cytokine environment is required for the maturation of these cells (416).

In summary I have shown that *Ac*ES contains molecules that can initiate a type 2 response in mice similar to that observed with a live hookworm infection. *Ac*ES contains therapeutic properties and can ameliorate pathology in both DSS and TNBS models of colitis whilst limiting Th1 and Th17 responses. This finding has helped include hookworm proteins into the library of helminth derived molecules available for the mining of therapeutics. The protection against DSS colitis was dependent on protein/s structure within *Ac*ES and was independent of IL-10R signalling and T and/or B cells. This was an important finding as the mechanism of action of many helminth molecules has been attributed to Tregs. Although it is still to be determined if *Ac*ES is capable of inducing Tregs, it is valuable to know that *Ac*ES is able to modulate inflammatory pathways in the innate immune system. I identifed a population of $F480^{+}CD11b^{hi}GR1^{hi}$ cells which is expanded in response to *Ac*ES and further expanded when IL-10R is blocked, and is diminished if *Ac*ES is digested with trypsin. This is a novel and exciting concept for the field of helminth therapeutics as MDSCs have not been specifically correlated with helminth infections or with positive immune modulation. If these MDSCs are indeed capable of limiting dysregulated pro-inflammatory immune responses they pose a novel target to combat these inflammatory conditions. Finally, a candidate immunoregulatory protein was identified - recombinant *Ac*-AIP-1 displayed significant protection against TNBS induced pathology. This protein is a promising candidate for therapeutics, especially if it is found to target T cells specifically, as many inflammatory conditions are exarcebated by dysregulated effector T cell function. Further work should be aimed at elucidating the mechanism of action of *Ac*-AIP-1 and other potential anti-inflammatory

hookworm proteins that could be developed as therapeutics for inflammatory conditions.

8 Supplementary Material

8.1 Supplementary material from Chapter 2

Table 8.1.1: Antibodies used for FACS analysis.

Colour	*Name*	*Company*	*Cat no.*	*Clone*
FITC	CD4	BD	553047	RM4-5
	CD11c	BD	553801	HL3
	CD8	BD	553031	53-6.7
	CD14	BD	553739	rmC5-3
PE	MHCII	BD	557000	M5/114
	CD45RB	BD	553101	16A
	IL-10	BD	554467	JES5-16E3
	IFN-g	BD	554412	XMG1.2
	IL-4	BD	554435	11B11
	SIGLEC-F	BD	552126	E50-2440
	CTLA-4	BD	553720	UC10-4F10-11
	TNF	BD	554419	MP6-XT22
	IL-5	BD	554395	TRFK5
APC	F4/80	Caltag	MF48005	BM8
	IL-10	E-bio	17-7101	JES5-16E3
	CD4	BD	553051	RM4-5
	FOXP3	E-bio	17-5773	FJK-16s
	CD40	E-bio	17-0401	1C10
EF450	IFN-g	E-bio	48-7311	XMG1.2
	CD8	E-bio	48-0081	53-6.7
	CD4	E-bio	48-0042	RM4-5
	GR1	E-bio	48-5931	RB6-8C5
PERC-P cy5.5	IL-17A	E-bio	45-7177	eBio17B7
	CD11b	E-bio	45-0112	M1/70
	IFN-g	E-bio	45-7311	XMG1.2
	CD25	E-bio	45-0251	PC61.5
Biotin	CD103	E-bio	13-1031	"2E7
	CD45RB	E-bio	13-0455	C363.16A
	CD16/32	BD	553143	2.4G2
	CD86	Caltag	RM7015	RMMP-2
	OX40L	E-bio	13-5905	RM134L

8.2 Supplementary material from Chapter 4

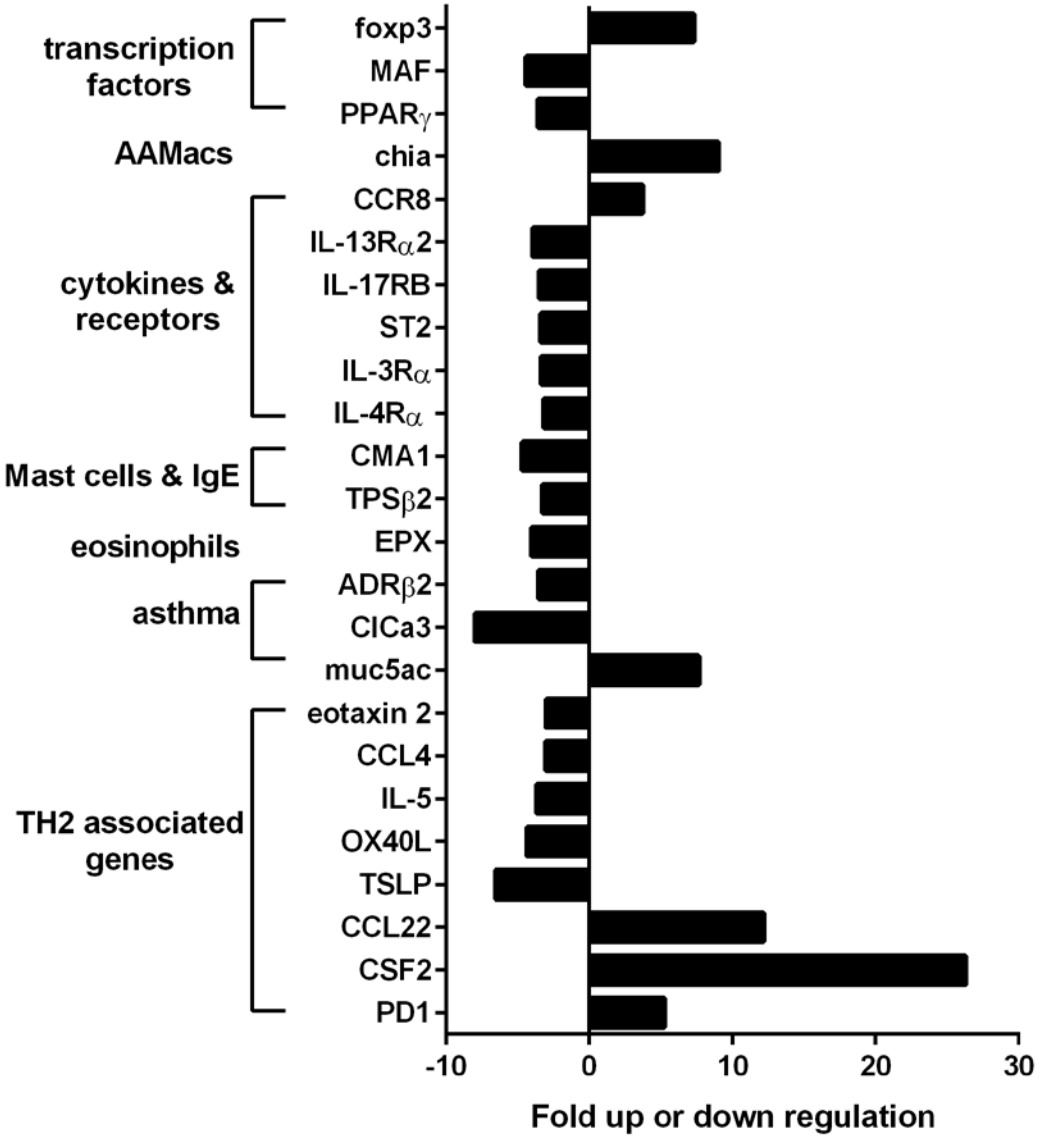


Figure 8.2.1: Type 2 related gene array in mouse colon tissue during TNBS induced colitis.

Mice were given a single injection of 20 µg of *Ac*ES or mock PBS injection 4 hours before TNBS administration. Graph shows genes that are up or down regulated in colons of *Ac*ES treated mice compared to mock PBS injection controls during TNBS induced colitis, with a p-value greater than 0.1, n=3 mice per group.

8.3 Supplementary material from Chapter 5

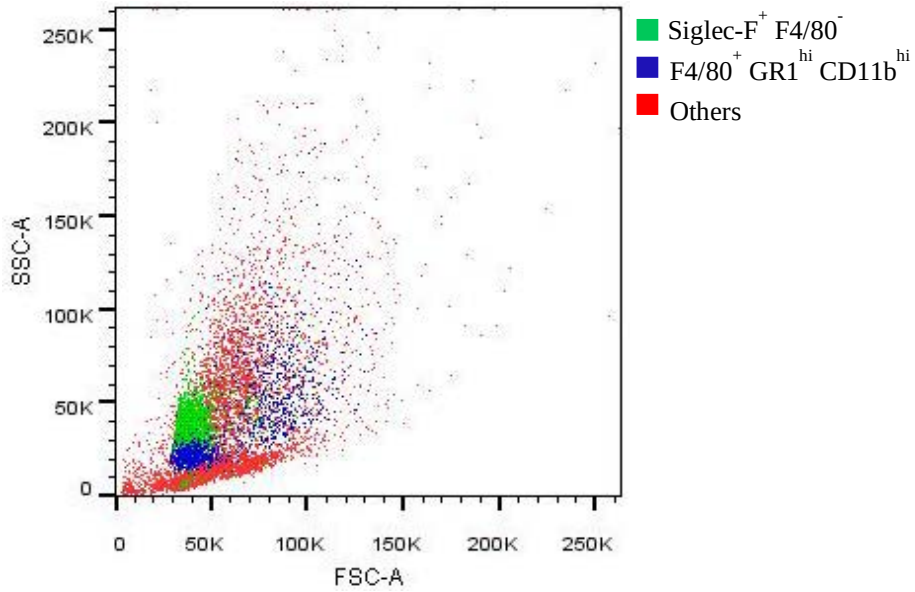


Figure 8.3.1: F4/80^{+}GR1^{hi}CD11b^{hi} cells have a low FSC vs SSC compared to granulocytes.

Forward scatter (FSC) vs. side scatter (SSC) flow plot of cell population. Green population is gated on Siglec-F^{+} and F4/80^{-} eosinophils. Blue population is gated on Siglec-F^{-} and F4/80^{+}, then further gated for CD11b^{hi} GR1^{hi} inside the F4/80 population. Red cells represent all other cells.

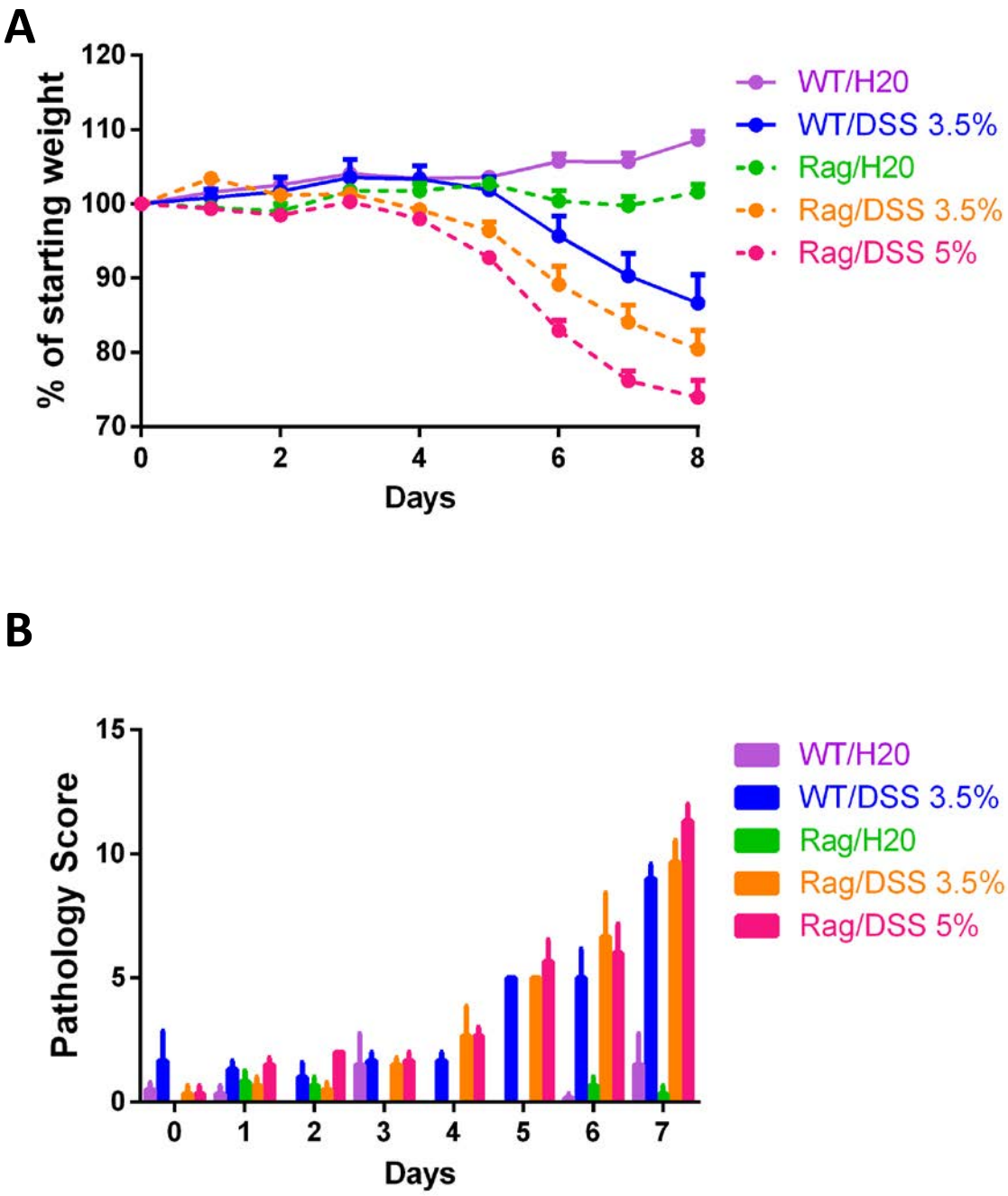


Figure 8.3.2: A 3.5% DSS dose is equally as effective in Rag1$^{-/-}$ mice as WT mice. Wild type (WT) C57BL/6 and Rag1$^{-/-}$ (on a C57BL/6 background) received 3.5% w/v DSS solution in their drinking water; another group of Rag1$^{-/-}$ mice also received a 5% w/v DSS solution. Weight loss as a percentage of starting weigh (A). Pathology score (B). Error bars show the standard error of the mean; n = 5 mice per group.

8.4 Supplementary material from Chapter 6

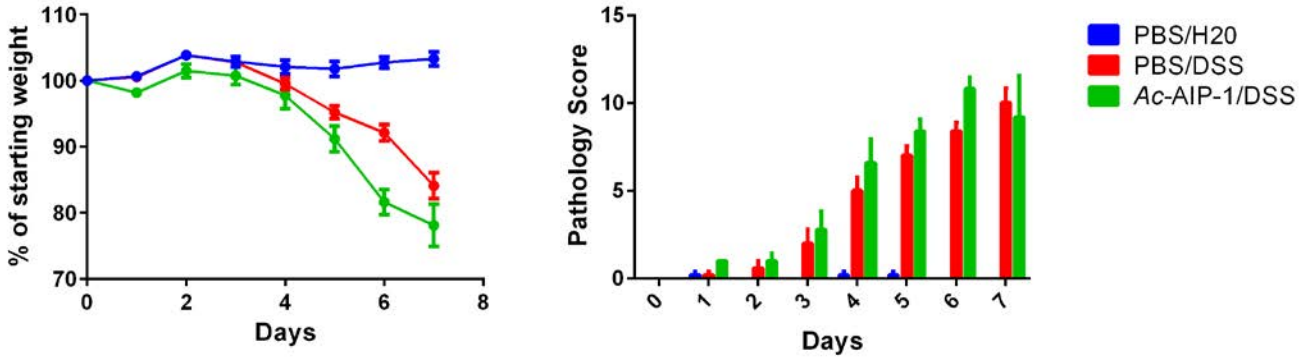


Figure 8.4.1: *Ac*-AIP-1 does not protect mice against DSS induced colitis.
Mice received 3.5% DSS *ad libitum*. Mice received daily injections of either 20 µg of *Ac*-AIP-1 or PBS mock injection. Percentage of starting weight (A) and pathology score (B) of mice during DSS induced colitis model. Error bars show the standard error of the mean, results are representative from 2 repeat experiments, n = 5 mice per group.

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10 Appendices

10.1 Appendix A: Ethics approval

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10.2 Appendix B: Published papers directly relevant to this thesis

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Current Opinion

The hookworm pharmacopoeia for inflammatory diseases

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ABSTRACT

In the developed world, declining prevalence of parasitic infections correlates with increased incidence of allergic and autoimmune disorders. Current treatments for these chronic inflammatory conditions have little to no effect on their prevalence and are referred to as "controllers" rather than cures. There has been limited success in therapeutically targeting allergic and autoimmune pathways, leaving an unmet need for development of effective anti-inflammatories. We discuss the benefit of hookworm infections and the parasite's ability to condition the immune system to prevent allergic asthma and inflammatory bowel diseases. We then examine the immunomodulatory properties of selected hookworm-derived proteins in these two models of inflammation. While hookworm protein therapy has yet to be fully exploited, the identification of these proteins and the mechanisms by which they skew the immune system will provide new avenues for controlling and optimally reversing key pathological processes important in allergic and inflammatory bowel diseases.

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Hookworm Excretory/Secretory Products Induce Interleukin-4 (IL-4)$^+$ IL-10$^+$ CD4$^+$ T Cell Responses and Suppress Pathology in a Mouse Model of Colitis

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Evidence from human studies and mouse models shows that infection with parasitic helminths has a suppressive effect on the pathogenesis of some inflammatory diseases. Recently, we and others have shown that some of the suppressive effects of hookworms reside in their excretory/secretory (ES) products. Here, we demonstrate that ES products of the hookworm *Ancylostoma caninum* (AcES) suppress intestinal pathology in a model of chemically induced colitis. This suppression was associated with potent induction of a type 2 cytokine response characterized by coexpression of interleukin-4 (IL-4) and IL-10 by CD4$^+$ T cells, downregulation of proinflammatory cytokine expression in the draining lymph nodes and the colon, and recruitment of alternatively activated (M2) macrophages and eosinophils to the site of ES administration. Protease digestion and heat denaturation of AcES resulted in impaired induction of CD4$^+$ IL-4$^+$ IL-10$^+$ cell responses and diminished ability to suppress colitis, indicating that protein component(s) are responsible for some of the immunosuppressive effects of AcES. Identification of the specific parasite-derived molecules responsible for reducing pathology during chemically induced colitis could lead to the development of novel therapeutics for the treatment of human inflammatory bowel disease.

The existence of parasitic helminths predates that of humans (1), and it is believed that some parasite-host interactions, such as those of gastrointestinal helminths and their vertebrate hosts, have developed in such a way that benefits both the parasite and the host. This phenomenon is embodied by the "hygiene hypothesis," which suggests that the elimination of pathogens such as helminths from people living in the developed world has predisposed the immune system to respond inappropriately to self and otherwise innocuous environmental antigens, culminating in increased incidences of allergic and autoimmune diseases (2).

Inflammatory bowel disease (IBD) is a term used to describe two chronic inflammatory diseases, ulcerative colitis (UC) and Crohn's disease (CD). IBD is characterized by a dysregulation of the mucosal immune response to intestinal bacteria, resulting in chronic inflammation of the gastrointestinal tract, pain, diarrhea, and vomiting (3). At present, treatment methods range from lifelong use of immunomodulatory drugs (e.g., corticosteroids) to surgery; however, therapies involving deliberate human infection with helminths have been proposed as an alternative treatment method for these chronic diseases. Multiple clinical trials in humans have demonstrated that exposure to gastrointestinal parasites can significantly reduce the severity of intestinal inflammation in humans with UC (4) and CD (5). These clinical trials utilized the pig whipworm *Trichuris suis*, where infection is short-lived in humans and requires repeated administration of larvae to maintain the infection (6). Recent clinical trials performed by our laboratory have focused on the potential use for helminths that persist within the intestine, such as the hookworm *Necator americanus*, to alleviate intestinal inflammation associated with celiac disease (5, 11, 21). This hookworm-based therapy resulted in the suppression of proinflammatory anti-gliadin immune responses (21) and the induction of systemic and mucosal type 2 cytokine responses (7), although overt suppression of clinical disease was not observed (8). Although the potential benefits of parasite-derived therapies for IBD and other autoimmune diseases are apparent, the safety of such approaches has been questioned (9, 10), and a "fear factor" reaction by the public, as well as logistical concerns for scale-up, may preclude their widespread use.

The mechanism of parasite-mediated suppression of inflammatory immune responses has been investigated in a number of mouse models of disease, with roles described for cross-regulation of inflammatory Th1 responses by parasite-derived Th2 responses (11), regulatory T cells (12–14), and suppressive macrophages (15, 16). Helminths secrete proteins that modulate and/or skew immune responses (17, 18), suggesting that "helminth therapy" for autoimmunity could take the form of soluble molecules derived from helminths rather than an active infection (19).

Here we show that the administration of *Ancylostoma caninum* excretory/secretory products (AcES) limits intestinal pathology and proinflammatory cytokine expression during dextran sodium sulfate (DSS)-induced colitis. Injection of AcES in mice induces a

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robust antigen-specific type 2 cytokine response, including the emergence of a distinct $CD4^+$ T cell population that coexpresses IL-4 and IL-10 and the recruitment of macrophages and eosinophils to the site of injection. Denaturation of AcES resulted in a loss in the protective effect during colitis, suggesting that the immunomodulatory properties of AcES are at least partly due to a protein constituent.

MATERIALS AND METHODS

Production of *A. caninum* ES. *A. caninum* adult worms were cultured in serum free medium containing 100 U of penicillin/μl and 100 μg of streptomycin/ml (pen/strep) for 24 h. The supernatant (AcES) was collected, filter sterilized through a 0.22-μm-pore-size filter (Pall), and concentrated and buffer exchanged to phosphate-buffered saline (PBS) using a 10-kDa spin column (Pall). Removal of lipopolysaccharide from AcES and ovalbumin (OVA; Sigma) was then performed using one of two methods, Endotrap Blue (Hyglos) according to the manufacturer's instructions or Triton X-114 (Sigma) as previously described (20) with some minor changes. Briefly, AcES was incubated with 5% Triton X-114 at 4°C on a rotating wheel for 30 min, followed by heating to 37°C for 10 min and centrifugation at 1,600 × *g* for 15 min at room temperature. The upper endotoxin-depleted phase was collected, and the process was repeated twice to ensure thorough removal of endotoxin. A *Limulus* amebocyte lysate (Lonza) assay was used to ascertain the adequate removal of endotoxin, and the protein concentration was calculated by using a micro-BCA protein assay kit (Pierce). Some experiments used boiled and trypsinized AcES (bES) as a control. Briefly, AcES was digested with 1 μg of trypsin (Sigma)/ml at 37°C for 24 h, followed by boiling at 95°C for 15 min to denature both trypsin and the AcES protein constituents.

Mice. Female 6- to 10-week-old C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia) and were housed according to Australian animal rights and regulations standards. Mice received food and water *ad libitum*. All injections were administered via the intraperitoneal (i.p.) route without adjuvant. In some experiments, mice received i.p. injections of PBS or 1 to 25 μg of AcES, bES, or OVA at various time points as indicated in the text. All procedures involving mice were approved by the James Cook University Animal Ethics Committee.

DSS-induced colitis. A 3.5% (wt/vol) solution of dextran sodium sulfate (DSS; 36,000 to 50,000 molecular weight; MP Biomedicals) was administered to mice as a substitute for normal drinking water. The mice were weighed and scored daily to assess disease progression based on a modified scoring system (21). Mice were scored on weight (percent change; 0 to 4), the level of fecal consistency (0 to 4), rectal bleeding (0 to 2), and general appearance (0 to 3) for a daily score out of a total of 13.

Histopathology. Upon termination of the experiment the mouse colons were given a macroscopic score for severity of adhesion (0 to 3), ulceration (0 to 3), wall thickening (0 to 3), and edema (0 to 3) for a total possible score of 12 as previously described (22). A small piece of the proximal colon was fixed in 4% formaldehyde for histological processing. Cross-sections of the colons were stained with hematoxylin and eosin (H&E) for microscopic visualization of inflammation. Histological scoring of the cross-sections was performed in a blinded fashion using a modified scoring system (23). Colon cross-sections were assessed for number of ulcers (no ulcers = 0, 1 ulcer = 1, 2 ulcers = 2, 3 ulcers = 3, and >3 ulcers = 4). Each ulcer was ~200 μm in length; where ulceration was bigger than this, scoring was performed in 200-μm intervals. Epithelium integrity was scored follows: 0 = normal morphology, 1 = loss of goblet cells in 1 area, 2 = loss of goblet cells in more than one area, 3 = loss of crypts in 1 area, and 4 = loss of crypts in more than one area. Cellular infiltrate was scored as follows: 0 = no infiltrate, 1 = infiltrate around crypt bases, 2 = infiltrate reaching to muscularis mucosae, 3 = extensive infiltration reaching the muscularis, and 4 = infiltration of the submucosa with edema. Finally, lymphoid follicles were scored as none = 0, 1 = 1, 2 = 2, 3 = 3, >3 = 4. Together, these criteria could achieve a total possible score of 16.

Cell preparation and cytokine analysis. Peritoneal cells were collected by washing the peritoneal cavity with 10 ml of ice-cold complete medium (RPMI 1640 plus 10% heat-inactivated fetal calf serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine; Invitrogen). Splenocyte restimulations and cytokine assays were performed as previously described (24). Briefly, spleens were macerated through 70-μm-pore-size nylon filters (BD Biosciences), and red blood cells were lysed using red blood cell lysis buffer (Sigma). Splenocytes were cultured in triplicate in flat-bottom 96-well plates (10^6 cells/well) either in medium alone or in medium supplemented with AcES (10 μg/ml), OVA (10 μg/ml), or anti-CD3 (1 μg/ml) for 72 h at 37°C and 5% CO_2. Colon lysates were produced by flushing colons with PBS and placing a small piece of known weight into 1 ml of PBS and lysing on a TissueLyser (Qiagen) with the use of a metal bead. Cell-free supernatants were removed and concentrations of IL-4, IL-5, IL-10, gamma interferon (IFN-γ), IL-17A, and tumor necrosis factor alpha (TNF-α) were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA; OptEIA kits; BD Biosciences).

Flow cytometry. Peritoneal cells were stained for CD11c-FITC (clone HL3), SIGLEC-F-PE (clone E50-2440) (BD Biosciences), and F4/80-APC (clone BM8; Caltag/Invitrogen), acquired on a FACSCanto flow cytometer (BD Biosciences), and analyzed using FlowJo software (TreeStar). Intracellular cytokine stains were performed on spleen and lymph node cells. Prior to staining, the cells were cultured for 4 h at 37°C and 5% CO_2 in complete medium containing phorbol myristate acetate (500 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml). The cells were stained for CD4-FITC (clone RM4-5; BD Biosciences) and then permeabilized with Fix/Perm buffer (BD Biosciences) and stained for IL-4-PE (clone 11B11; BD Biosciences), IL-10-APC (clone JES5-16E3; eBioscience), and IFN-γ-eF450 (clone XMG1.2; eBioscience).

RNA extraction and real-time PCR. For peritoneal cells, 10^6 cells were pelleted by centrifugation and resuspended in 1 ml of TRIzol (Invitrogen). Similarly, a small 0.5-cm piece of colon was washed in PBS, placed in 1 ml of TRIzol, and macerated on a TissueLyser (Qiagen) for 10 min with the use of a metal bead. Total RNA extraction was performed by phenol-chloroform separation according to the manufacturer's instructions. After treatment of RNA with RQ1 DNase (Promega), first-strand cDNA was produced with random hexamers (Invitrogen) from 0.5 to 1 μg of total RNA by using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The levels of transcription were measured by comparing cross-threshold values to a standard curve made of a pool of all samples. Samples were tested in dilutions of up to 1:600 using SYBR green (Applied Biosystems/Qiagen). A Rotor Gene 6000 (Qiagen) was used for real-time thermal cycling. Melting-curve analysis was used to confirm that a single product had been amplified. All genes were normalized for levels of transcription relative to the housekeeping gene β-actin. All primers were purchased from Sigma-Aldrich and were diluted to a 10 μM final concentration. The primers used were as follows: β-actin, sense (TGGAATCCTGTGGCATCCATGAAAC) and antisense (TAAAACGCAGCTCAGTAACAGTCCG); Fizz-1, sense (GTCCTGGAACCTTTCCTGAG) and antisense (AGCTGGATTGGCAAGAAGTT); Ym1, sense (CTGAGAAGCTCATTGTGGGA) and antisense (CTCAGTGGCTCCTTCATTCA); Arg-1, sense (CAGAAGAATGGAAGAGTCAG) and antisense (CAGATATGCAGGGAGTCACC); NOS-2, sense (ACCTTGTTCAGCTACGCCTT) and antisense (CATTCCCAAATGTGCTTGTC); IL-6, sense (CCGGAGAGGAGACTTCACAG) and antisense (TCCACGATTTCCCAGAGAAC); IL-17A, sense (CCTCCAGAATGTGAAGGTCA) and antisense (CTATCAGGGTCTTCATTGCG); and IFN-γ, sense (AGCTCTTCCTCATGGCTGTT) and antisense (TTTGCCAGTTCCTCCAGATA).

Statistical analyses. All data were analyzed with GraphPad (version 5; Prism). When three or more groups were compared, one-way analysis of variance (ANOVA) was used with a Bonferroni post-test with a 95% confidence interval to compare all columns. When the effect of a treatment over time was compared for different treatment groups, two-way ANOVA was used with a Bonferroni post-test to compare replicate means over

time. P values of <0.05 were considered significant. When only two groups were compared, a Mann-Whitney test was used. All results stated in the text are means ± the standard errors of the mean (SEM). None of the figures presented here are pooled from multiple runs, and all data are representative of at least three repeat experiments.

RESULTS

AcES products protect mice from DSS-induced colitis. Ingestion of DSS via the drinking water by mice injected i.p. with vehicle (PBS) or a control protein (OVA) caused rapid weight loss beginning at day 5 posttreatment compared to mice receiving normal drinking water (Fig. 1A). In contrast, i.p. administration of AcES protected against DSS-mediated weight loss (Fig. 1A). Upon euthanasia, the colons were scored for pathology on a macroscopic level (Fig. 1B), and H&E-stained tissue sections scored at a microscopic level (Fig. 1C), showing that AcES-treated mice had significantly lower colon pathology than control mice. Representative transverse sections of the colons demonstrate that mice receiving DSS and either PBS or OVA control treatments had increased cellular infiltrate and edema in the submucosa, whereas mice that had been treated with AcES had markedly less infiltrate and swelling (Fig. 1D).

AcES reduces levels of proinflammatory cytokines associated with pathology in the draining lymph nodes and the colon. We next addressed the impact of AcES administration on the expression levels of proinflammatory cytokines associated with DSS-induced pathology. Mice received 3.5% DSS in their drinking water for 8 days and received either a vehicle injection of PBS or 1, 10, or 25 μg of AcES daily i.p. Mesenteric lymph node (MLN) and spleen cells were polyclonally stimulated with anti-CD3 *in vitro*, and cytokine production was measured by ELISA. The results showed a dose-dependent suppression of IFN-γ and IL-17A expression by MLN cells (Fig. 2A) and splenocytes (data not shown).

We next compared the levels of gene expression at the site of inflammation, the colon, in mice treated with either vehicle or 25 μg of AcES. Critically, expression of the proinflammatory mediators iNOS, IL-6, and IL-17A was significantly reduced by AcES treatment (Fig. 2B), and the levels of IFN-γ tended to be lower but did not reach statistical significance ($P = 0.0585$). In contrast, IL-4 and IL-10 protein levels in the colon were increased in AcES-treated mice compared to control-treated mice (Fig. 2C). Together, these data indicate that AcES causes downregulation of proinflammatory type 1/type 17 responses potentially by inducing a bias toward a type 2 or regulatory cytokine.

AcES products induce a biased Th2 cytokine response in the absence of any adjuvant or live infection. To further investigate the induction of a type 2 cytokine response in mice receiving AcES, mice were injected with either PBS alone or 10 μg of AcES every 2 days for a total of 2 weeks in the absence of any other stimulus. A 10-μg dose was chosen as pilot studies had indicated that 10 μg produced a similar Th2 response as a 25-μg dose in the absence of DSS. Analysis of intracellular cytokine staining in peritoneal lavage cells demonstrated that injection of AcES into mice caused significantly reduced frequencies of $CD4^+$ T cells that expressed IFN-γ (Fig. 3A) and significantly increased frequencies of $CD4^+$ T cells expressing IL-4 and IL-10, including a prominent population expressing both of these cytokines (Fig. 3B). AcES injection also induced populations of IL-4/IL-10 double-positive CD4 T cells in the spleen and MLN (data not shown). Significant increases in AcES-specific IL-4, IL-5, and IL-10 production by

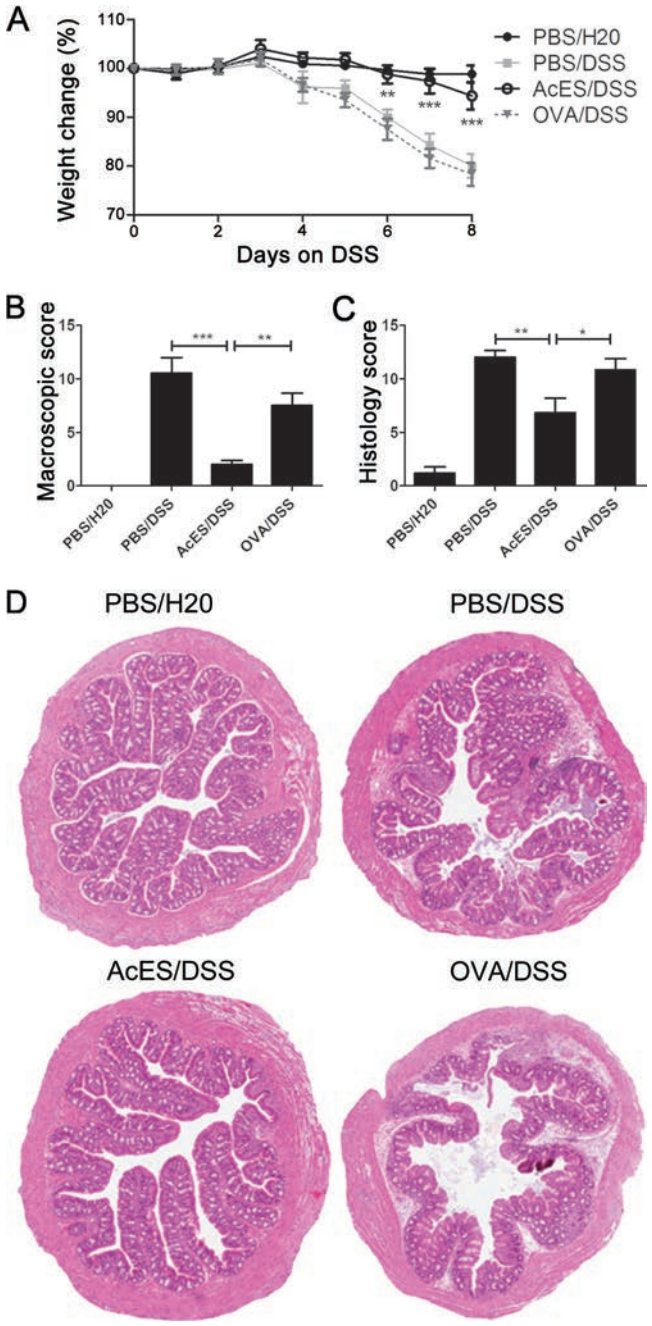

FIG 1 AcES protects mice from DSS induced colitis. Mice received 3.5% DSS in drinking water to induce colitis; test groups received 25 μg of AcES or OVA i.p. every day. (A) Weight loss graph, showing the percentages of mean weight lost compared to day 0. (B) Colons from mice were removed and assessed for levels of macroscopic inflammation based on adhesion, ulceration, edema, and wall thickening for a total possible score of 12. (C) Histology sections were assessed for levels of inflammation based on ulceration, epithelial integrity, lymphoid follicles, and cellular infiltrate for a total possible score of 16. (D) Representative transverse sections (×20 magnification) of mouse colons stained with H&E. All graphs show mean ± the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 6$). The data are representative of at least three repeat experiments.

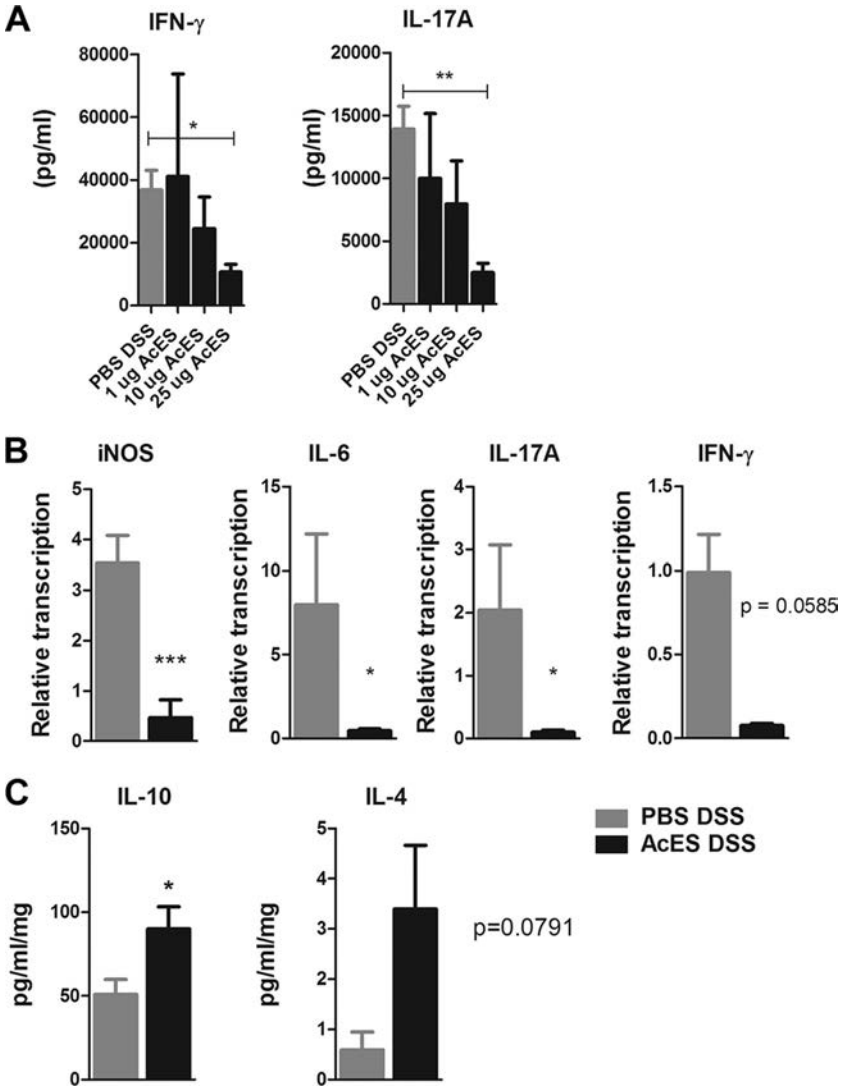

FIG 2 AcES reduces levels of proinflammatory cytokines both in the draining lymph nodes and the colon. (A) IFN-γ and IL-17A levels in culture supernatants of polyclonally stimulated MLN cells. (B) Transcription of inducible nitrous oxide synthetase (iNOS), IL-6, IL-17A, and IFN-γ by RT-PCR in the colonic tissue of mice. (C) IL-10 and IL-4 levels in colon lysates. Gray bars indicate control mice, and black bars indicate mice that received AcES. Mice received 3.5% DSS in drinking water to induce colitis; test groups received AcES i.p. daily. Where not indicated on the graph, the amount of AcES was 25 μg. All graphs show means ± the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 3$ to 6). The data are representative of at least three repeat experiments.

restimulated splenocytes were observed in AcES-treated mice compared to PBS- or OVA-treated control mice (Fig. 3C). Splenocytes from OVA-injected animals restimulated with OVA did not produce significantly elevated levels of IL-4, IL-5, or IL-10 (IL-4, 25.34 ± 4.49 pg/ml; IL-5, 0.0; IL-10, 302.1 ± 151.8 pg/ml). Finally, no significant differences were noted in the production of either TNF or IFN-γ.

AcES recruits eosinophils and M2 macrophages to the site of injection. Given the ability of AcES to induce a type 2 cytokine bias, we explored whether AcES elicits a downstream innate effector eosinophil and M2 (alternatively activated) macrophage response at the site of injection. Mice injected with AcES had significantly more cells at site of injection than control-treated animals [$(20.33 \pm 3.69) \times 10^6$ versus $(4.40 \pm 0.74) \times 10^6$, $P = 0.0055$]. Flow cytometric analysis revealed that AcES injection resulted in significantly higher frequencies and total numbers of F4/80$^+$ macrophages ($P < 0.001$) and Siglec-F$^+$ eosinophils ($P < 0.001$) in the peritoneal cavity compared to mice injected with OVA or PBS (Fig. 3D-3F).

M2 macrophages are associated with suppression of T cell responses, and anti-parasite responses (25). Therefore, we analyzed the expression of various M2 macrophage markers in peritoneal cells by reverse transcription-PCR (RT-PCR). Consistent with the macrophages recruited to the peritoneal cavity after AcES injection being of an M2 macrophage phenotype, we detected a significant increase ($P < 0.001$) in *YM1*, *FIZZ-1*, and *Arg-1* expression (Fig. 3G) in cells from mice that were injected with AcES compared to control groups. Together, these data indicate that the injection of AcES alone is able to potently modulate the immune status of mice toward a type 2 cytokine response, thereby limiting proinflammatory Th1 and Th17 cytokine responses.

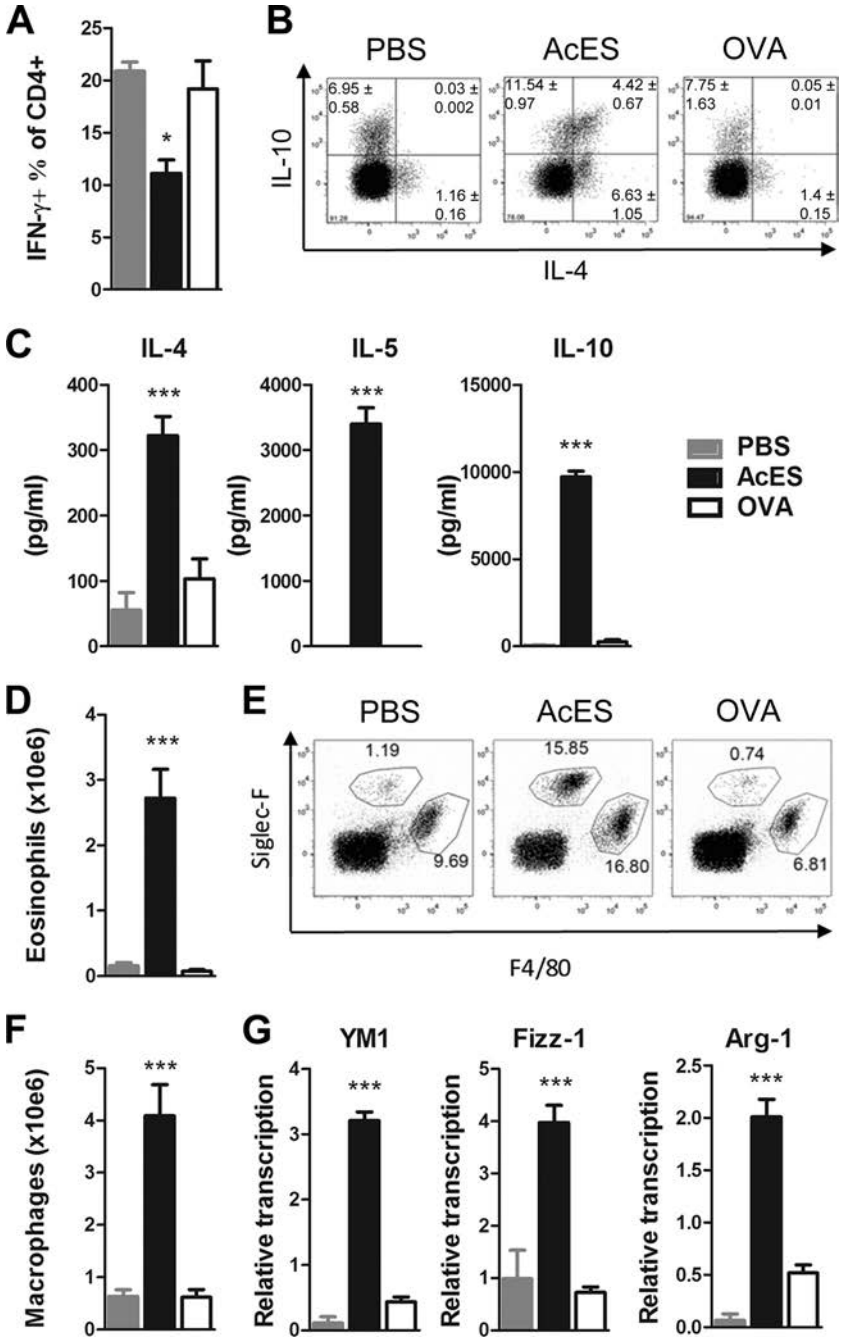

FIG 3 AcES induces an antigen-specific type 2 response, as well as a distinct population of IL-4/IL-10 double-positive CD4 T cells. (A) Frequencies of IFN-γ-producing $CD4^+$ cells in the peritoneum. (B) Representative flow cytometry plots of intracellular cytokine staining of IL-4 (*x* axis) and IL-10 (*y* axis) production by peritoneal exudate cells, gated on $CD4^+$ T cells. (C) ELISAs were performed for IL-4, IL-5, and IL-10 from supernatants of splenocytes restimulated *ex vivo* with 10 μg of AcES/ml for 72 h. (D) Number of eosinophils in the peritoneum. (E) Representative flow cytometry plots of peritoneal exudate cells, showing F4/80 (*x* axis) versus Siglec-F (*y* axis). (F) Number of macrophages in the peritoneum. (G) Transcription of M2 markers in peritoneal macrophages. Mice were injected with PBS, AcES (10 μg), or OVA (10 μg) every second day for a total of 2 weeks. Gray bars represent the PBS vehicle control group, black bars represent the AcES group, and white bars represent the OVA control group. All graphs show means ± the SEM. *, $P < 0.05$; ***, $P < 0.001$ ($n = 5$). The data are representative of at least three repeat experiments.

Denaturation and tryptic digestion of AcES diminished the induction of Th2 responses. To determine whether the factor(s) within AcES responsible for inducing type 2 cytokine responses are of a protein nature, we digested AcES with trypsin, followed by heat denaturation, a preparation we termed "boiled ES" (bES). Although the injection of AcES in DSS-treated mice resulted in characteristic reductions in IFN-γ expression and increased IL-4 and IL-10 coexpression by $CD4^+$ T cells, bES administration had a significantly diminished effect (Fig. 4A). Consistent with an impaired ability to induce a Th2 cytokine response, bES administra-

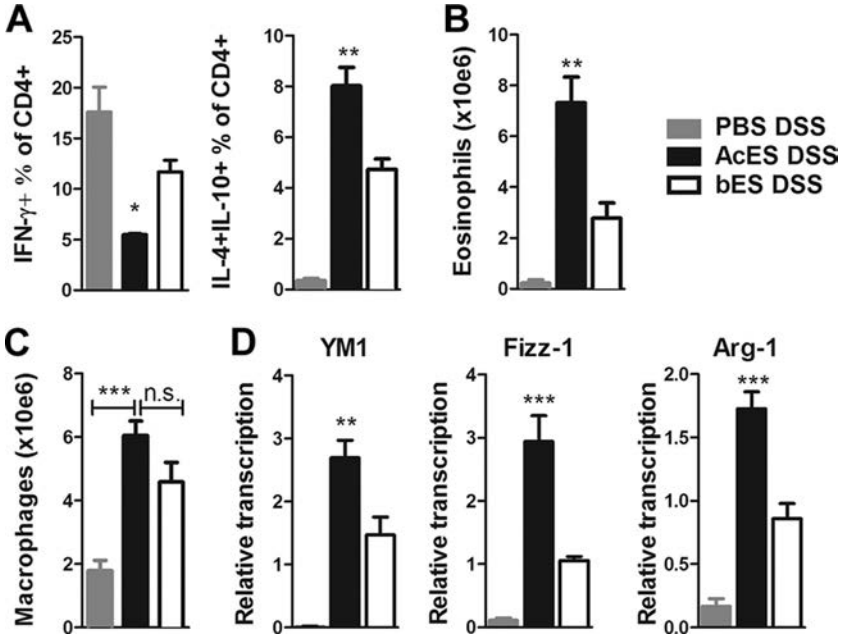

FIG 4 Denaturation and trypsin digestion of AcES leads to reduced Th2 profile. (A) Frequencies of IFN-γ-producing $CD4^+$ cells and IL-4^+ IL-10^+ CD4 T cells in the peritoneum. (B) Number of eosinophils present in the peritoneum. (C) Number of macrophages in the peritoneum. (D) Transcription of M2 markers in the peritoneum measure by RT-PCR. Mice received normal water or a 3.5% DSS solution for a period of 9 days and daily injections of either PBS, 25 μg of AcES, or 25 μg of boiled and trypsinized AcES (bES). Gray bars represent the PBS vehicle control group, black bars represent the AcES group, and white bars represent the bES group. All graphs show the mean SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 4$ to 6). The data are representative of at least three repeat experiments.

tion resulted in a less-pronounced eosinophil response (Fig. 4B) but still induced significant expansion of macrophages in the peritoneum (Fig. 4C). However, the transcription of M2 macrophage activation markers was significantly reduced in the bES mice (Fig. 4D), suggesting a reduction in alternative activation of macrophages in these mice. Similar results to these were seen when bES was administered to mice in the absence of DSS (data not shown). Hence, the ability of AcES to induce optimal type 2 cytokine responses in mice is predominantly due to heat-labile protein factors.

Protein denaturation of AcES abrogates its protective effect during DSS colitis. To assess whether the impaired ability of bES to induce a type 2 cytokine bias results in a reduced capacity to limit disease severity during colitis, we assessed weight loss and intestinal pathology in DSS-treated mice coadministered either AcES or bES. Although AcES treatment resulted in less pronounced weight loss than when mice were treated with PBS, mice injected with bES lost significantly more weight by day 7 than did mice treated with AcES (Fig. 5A). Histological analysis showed that bES-treated mice had significant edema and cellular infiltrate in the submucosa of the colon, whereas the colons of AcES-treated mice appeared relatively healthy (Fig. 5B). These data demonstrate that the component(s) of AcES that mediate protection against DSS-induced colitis is likely a protein.

DISCUSSION

Hookworms have been known survive for more than 10 years in their human hosts, and their longevity is attributed at least in part to the exquisitely refined immune-evasive strategies that they have evolved to ensure their long-term survival and propagation. While human hookworm infections exhibit some of the hallmark features of protective T helper type 2 (Th2) immune responses, including IgE and local and systemic eosinophilia, these immune responses clearly fail to protect most people from reinfection (26–28). The reason for the observed lack of an effective anti-hookworm response remains unknown, although the production of immunomodulatory ES proteins that skew or dampen immune responses to promote the long-term survival of the parasite is a likely contributing factor (29–32). In the present study, we inves-

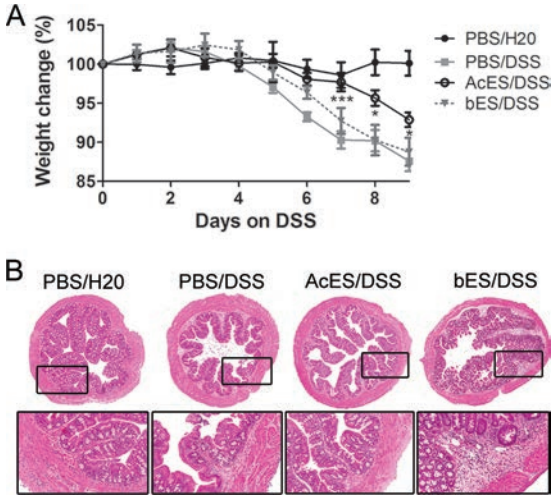

FIG 5 bES fails to protect mice against DSS-induced colitis. Mice received normal water or a 3.5% DSS solution for a period of 9 days. Mice received daily injections of either PBS, 25 μg of AcES, or 25 μg of boiled and trypsinized AcES (bES). (A) Weight loss graph, showing percentage of mean weight lost compared to day 0. (B) Low (×20)- and high (×400)-magnification histological images of H&E-stained colonic tissue. The data are representative of a minimum of three repeat experiments. Graphs show means ± the SEM. *, $P < 0.05$; ***, $P < 0.001$ ($n = 4$ to 6).

tigated the potentially beneficial properties of hookworm ES products on inflammation and the suppression of pathology associated with IBD.

Infections with live helminths from phylogenetically distant groups can protect mice against a range of autoimmune or allergic diseases (33). A growing body of literature suggests that much of this protection against inflammation is mediated by soluble molecules released by the parasites. Some examples include protection in the dinitrobenzene sulfonic acid (DNBS) model of colitis utilizing soluble somatic extracts from *Trichinella spiralis* (34) and *Hymenolepis diminuta* (35), as well as the use of ES products from *Ancylostoma ceylanicum*, a relative of *A. caninum*, in the DSS model of colitis (36). Furthermore, AcES and *Schistosoma mansoni* somatic proteins alleviated pathology associated with TNBS-mediated colitis (22). Administration of *S. mansoni* somatic proteins tended to be associated with reduced colonic transcription of inflammatory cytokines (IFN-γ, IL-12, and IL-17), increased Th2 cytokines (IL-4 and IL-5), and increased regulatory cytokines (IL-10 and transforming growth factor β) (22).

In the present study, we show that the administration of AcES in a mouse model of DSS-induced colitis prevented weight loss and significantly reduced colon pathology. The treatment of AcES alone induced a robust type 2 immune response in the draining lymph nodes and colon of mice, characterized by coexpression of IL-4 and IL-10 by $CD4^+$ T cells. Although it has been shown that the administration of AcES in a mouse model of TNBS-induced colitis protected against inflammation, no further characterization of the immunological responses were conducted (22). Here we show that both Th1 and Th17 responses characterizing colitis-induced inflammation were significantly decreased upon treatment with AcES. TNBS-mediated colitis is largely T cell dependent, whereas DSS colitis is primarily mediated by the innate cell response (21). The suppression of pathology by AcES in both models of colitis suggests a role in regulating both innate and adaptive immune responses. Indeed, we show that AcES affects elements of the adaptive (expansion of $IL\text{-}4^+IL\text{-}10^+$ $CD4^+$ T cells) and innate (expansion of M2 macrophages and eosinophils) immune responses in both diseased and healthy mice. Although we did not provide definitive proof that the IL-4/IL-10 double-positive $CD4^+$ T cells were responsible for the suppression of colitis, previous studies have shown that neutralization of both IL-4 and IL-10, but not IL-10 alone, restores IL-17 production in mice infected with *Heligmosomoides polygyrus*, suggesting a possible synergistic role of these cytokines in promoting optimal immunosuppressive activity (7).

There are numerous parallels that can be drawn between our observed effects of AcES on cytokine production in mice and in previous studies involving experimental human hookworm infections. In a recent placebo-controlled clinical trial assessing the therapeutic effect of experimental *N. americanus* infection on the immunopathogenesis of celiac disease, hookworms reduced the systemic and mucosal expression of IL-17A and IFN-γ, which are signature cytokines involved in the pathogenesis of IBD, and caused elevated Th2 cytokine responses and eosinophilia (7, 37). We observed a similar immune phenotype in mice injected with AcES, with reduced production of IL-17A and IFN-γ and elevated levels of type 2 cytokines and eosinophilia.

The ability of AcES to provoke enhanced M2 macrophage responses could also play a role in the protection against colitis due to their potential suppressive and wound healing effects (38, 39). *In vitro*-derived M2 macrophages can reduce inflammation in dinitrobenzene sulfonic acid (DNBS)-induced colitis (15), and their numbers correlate with remission of Crohn's disease in humans (15). Tapeworm extracts can also suppress classical activation of macrophages *in vitro* and suppress DNBS-mediated colitis (35). Moreover, protection against DSS-induced colitis in schistosome-infected mice was shown to be macrophage dependent; however, it did not appear to be due to M2 macrophages (16). Thus, although the suppression of M1 (inflammatory) macrophage activation and M2 macrophage activation may act through separate pathways, macrophages are clearly pivotal for exacerbation and suppression of colitis. However, denaturation of AcES (bES) did not affect recruitment of macrophages but did ablate the protection against colitis, suggesting that these cell types are not required for the anti-inflammatory properties of AcES in DSS-induced colitis. We believe that the transcription of M2 markers is dependent on IL-4 expression, and thus the reduced numbers of IL-4 expressing cells in the bES mice leads to reduced expression of M2 markers.

Finally, the protection induced by AcES in our model of colitis seemed to be entirely due to protein moieties that are sensitive to denaturation. AcES is comprised of more than 100 different proteins (40), as well as uncharacterized lipids and carbohydrates. Our study suggest that the protective properties of AcES is likely due to one or several protein components and that some of these are also necessary for the induction of the Th2 response. Ultimately, our goal is to find therapeutic specificities for each of the identified proteins of interest. However, in order to develop such molecules as therapeutics for human inflammatory diseases, the active product(s) first needs to be identified through thorough testing in animal models of disease. Some excretory molecules from other helminths, including a protein from *Trichinella spiralis* (41), phosphorylcholine from *Acanthocheilonema viteae* (42), and a glycan from *S. mansoni* (43) have already been identified as potential candidates to treat inflammatory diseases. We anticipate that AcES will be a reservoir of novel therapeutic targets for treatment of mammalian diseases.

In summary, we present data showing that a protein constituent of AcES suppresses pathology in a mouse model of IBD, correlating with a reduced inflammatory response in the intestine and a robust type 2 cytokine response, characterized by a distinct population of IL-4/IL-10 double-positive $CD4^+$ T cells. Future work will focus on defining the protective mechanisms and the specific protein components of AcES that are responsible for these effects. The recent characterization of the AcES proteome using tandem mass spectrometry will help in the identification of potential immunosuppressive factors (40). Helminths are masterful immunomodulators, and we show here that much of their suppressive capacity lies within their secreted proteins, so it is reasonable to assume that these organisms are a prime source of novel anti-inflammatory therapeutics for human diseases.

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10.3 Appendix C: Published papers relating to but not constituting part of this thesis

Experimental hookworm infection and gluten microchallenge promote tolerance in celiac disease

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Background: Celiac disease (CeD) is a common gluten-sensitive autoimmune enteropathy. A gluten-free diet is an effective treatment, but compliance is demanding; hence, new treatment strategies for CeD are required.
Objective: Parasitic helminths hold promise for treating inflammatory disorders, so we examined the influence of experimental hookworm infection on the predicted outcomes of escalating gluten challenges in CeD subjects.
Methods: A 52-week study was conducted involving 12 adults with diet-managed CeD. Subjects were inoculated with 20 *Necator americanus* larvae, and escalating gluten challenges consumed as pasta were subsequently administered: (1) 10 to 50 mg for 12 weeks (microchallenge); (2) 25 mg daily + 1 g twice weekly for 12 weeks (GC-1g); and (3) 3 g daily (60-75 straws of spaghetti) for 2 weeks (GC-3g). Symptomatic, serologic, and histological outcomes evaluated gluten toxicity. Regulatory and inflammatory T cell populations in blood and mucosa were examined.
Results: Two gluten-intolerant subjects were withdrawn after microchallenge. Ten completed GC-1g, 8 of whom enrolled in and completed GC-3g. Primary outcomes: median villous height-to-crypt depth ratios (2.60-2.63; $P = .98$) did not decrease as predicted after GC-1g, and the mean IgA-tissue transglutaminase titers declined, contrary to the predicted rise after GC-3g. Secondary outcomes: quality of life scores improved (46.3-40.6; $P = .05$); celiac symptom indices (24.3-24.3; $P = .53$), intra-epithelial lymphocyte percentages (32.5-35.0; $P = .47$), and Marsh scores were unchanged by gluten challenge. Intestinal T cells expressing IFNγ were reduced following hookworm infection (23.9%-11.5%; $P = .04$), with corresponding increases in $CD4^+$ $Foxp3^+$ regulatory T cells (0.19%-1.12%; $P = .001$).
Conclusions: *Necator americanus* and gluten microchallenge promoted tolerance and stabilized or improved all tested indices of gluten toxicity in CeD subjects. (J Allergy Clin Immunol 2015;135:508-16.)

Key words: *Celiac disease, gluten, hookworm, autoimmunity, helminth therapy, desensitization, mucosal immunology, regulatory T cells, intra-epithelial lymphocytes*

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