

HELMINTH THERAPY IN A MURINE MODEL OF COLITIS

HELMINTH THERAPY IN A MURINE MODEL OF CHEMICALLY INDUCED
COLITIS

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Abstract

Helminth parasite infection induces a strong immune response in the host aimed at destroying the parasite and reducing any associated inflammation. In humans and rodents, this response is dominated by the Th2 cytokines and involves the activation of mast cells, B cells and eosinophils, as well as increased production of IgE. There is evidence from both murine and human studies that the helminth-induced anti-inflammatory response is also capable of protecting the host from co-existing autoimmune disease, including asthma, allergies and colitis. My hypothesis is that infection with the parasitic helminth *H. diminuta* can treat and/or prevent the symptoms of Th1-dominated inflammation. This anti-colitic effect is dependent on IL-10 and involves the recruitment of the alternatively activated macrophages.

Using the dinitrobenzene sulfonic acid (DNBS) model of colitis, I examined the ability of the rat tapeworm, *Hymenolepis diminuta*, to reduce inflammation in a non-permissive mouse host. *H. diminuta* was chosen as the ideal candidate for treating intestinal disease because it is non-invasive, does not have hooks or teeth which can damage the host, and can be easily maintained in the laboratory and controlled during experimental infection. Mice that received DNBS alone developed colitis within 72 hours. Mice that had been previously infected with five *H. diminuta* larvae were significantly protected from the colitis, as measured by reduced clinical disease and histological damage scores as well as reduced levels of myeloperoxidase (MPO) from colonic tissue samples. It was also determined that the anticolic effect was dependent on a viable parasite infection. Infection with *H. diminuta* induced an increase in colonic

IL-10 mRNA and IL-10 secretion by stimulated splenocytes – when IL-10 was blocked by administration of an anti-IL-10 antibody, the anticolitic effect of *H. diminuta* infection was reduced. *H. diminuta* infection also induced increased expression of the alternatively activated macrophage (AAM) markers arginase 1 and FIZZ1. Treatment with *in vitro*-derived AAMs reduced the symptoms of DNBS-colitis.

The effect of *H. diminuta* infection on oxazolone colitis, a chemically induced colitis characterized by increases in IL-4 and IL-13, was also examined. Infection with *H. diminuta* induced a significant increase in inflammation and inhibited recovery from oxazolone-colitis. Increases in IL-5 and eosinophils were also observed. Further examination revealed that increased IL-5, induced by administration of an adenovirus carrying the IL-5 gene, had a deleterious impact on the oxazolone colitis, exacerbating inflammation and increasing eosinophilia.

While the idea of helminth therapy may be unappealing, there is increasing interest in the use of helminth parasites for the treatment of inflammatory disease. There is some concern, however, that the Th2 response induced by *H. diminuta* infection could exacerbate some disorders involving increases in the Th2 cytokines. Thus, while this therapy may be beneficial for most, careful characterization of the immunological basis of any pre-existing disorders would be necessary in order to avoid any harmful side-effects.

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Several years ago, I read an interesting article about the effects of a parasite infection in the DSS-model of colitis. I contacted the senior author and told him how interesting I found the work – and was informed that I could come and see him, but that he wasn't taking on any new students. So, above all, I would like to thank my supervisor, Dr. Derek McKay, for changing his mind. He inspired me in the beginning of this project and continued to support me as my independence emerged and I began to take ownership of the work. I am also grateful to my committee members through the years, including Dr. Mary Perdue and Dr. Elena Verdu who took on supervisory roles after Derek moved the laboratory to the University of Calgary; Dr. Ken Rosenthal, whose immunological perspective was much appreciated; Dr. Mark Inman, whose difficult questions helped me explore unique angles of this work; and Dr. Waliul Khan, a recent member who has been very encouraging. I am also very thankful to the many people with whom I have shared bench space over the years. It is from collaboration and the sharing of skills that I have learnt the most – thank you for your patience and guidance.

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Publications

Most of the research presented in this thesis has been accepted for publication or presented at scientific conferences in the following form:

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Hunter MM, Wang A, and McKay DM (2006) Parasitic helminths to treat IBD – it may not be for everyone. *Canadian Journal of Gastroenterology* **20** (supplement A): 38A

Hunter MM, Green CL and McKay DM (2004) Tapeworm inhibition of DNBS colitis requires a viable infection and does not predispose to enhanced gut hypersensitivity reactions. *Canadian Journal of Gastroenterology* **18** (supplement A): 104A.

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Journal Articles:

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Hunter, MM, Wang A, Hirota CL, and McKay DM (2005) Neutralizing anti-IL-10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced colitis. *Journal of Immunology* **174**: 7368-75.

Hunter MM and McKay DM (2004) Review article: helminths as therapeutic agents for inflammatory bowel disease. *Alimentary Pharmacology and Therapeutics* **19**: 167-177.

List of Abbreviations

AAM, alternatively activated macrophages; Ad-IL-5, interleukin-5 adenovirus; anti-IL-10 Ab, anti-Interleukin-10 antibody; arg-1, arginase-1; CAM, classically activated macrophage; CCL3, CC chemokine ligand 3; conA, concanavalin A; DNBS, dinitrobenzene sulfonic acid; DNCB, dinitrochlorobenzene; DSS, dextran sodium sulphate; EPO, eosinophil peroxidase; EtOH, ethanol; GI, gastrointestinal; H.d., *Hymenolepis diminuta*; IBD, inflammatory bowel disease; IFN- γ , interferon gamma; iNOS, inducible nitric oxide synthase; IL-X, interleukin-X; ip, intraperitoneal; ir, intrarectal; iv, intravenous; LPS, lipopolysaccharide; MLN, mesenteric lymph node; MPO, myeloperoxidase; NF κ B, nuclear factor kappa B; TGF- β , transforming growth factor beta; TNBS, trinitrobenzene sulfonic acid

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Chapter 1: Introduction

1.1 The Mucosal Immune System

The mucosal immune system is faced with the challenging task of efficiently protecting the epithelial barrier from microbial invasion while at the same time avoiding any unnecessary responses to commensal bacteria or food proteins found in the lumen. When this fails, the response to antigenic stimuli from luminal bacteria can drive an inflammatory response (Tlaskalova-Hogenova et al., 2004.) The immune system of the GI tract is referred to as the gut-associated lymphoid tissue. Components of the GALT are localized in the Peyer's Patches (PP) and mesenteric lymph nodes (MLNs), and scattered intra-epithelially and throughout the lamina propria. The PP is the inductive site, and contains microfold (M) cells which are specialized for antigen uptake. The main function of the M cells is the transport of antigens from the lumen to the subepithelial lymphoid tissue. The sub-epithelial dome (SED) is found beneath the M cells and is rich in dendritic cells and T and B lymphocytes. The dendritic cells (DCs) are capable of migrating within the PPs: they can acquire antigen at the basolateral surface of the M cell and present it to the T cells. DCs also migrate to the MLNs, and the CD103⁺ DCs from the MLNs are actively involved in the generation of the FoxP3⁺ T-regulatory cells in the intestine (MacDonald, 2003). While commensal bacteria can induce an intestinal B and T cell response, this activation does not translate into inflammation as would be seen with invasion by a pathogen. DCs loaded with commensals are restricted to the inductive sites, which limits the systemic responses to these organisms (Macpherson and Uhr, 2004).

The production of IgA is dependent on the generation of a specific area within the PP, called the germinal centre (GC). Within the GC, T cells, B cells and antigens interact and B cells proliferate and switch to IgA production: PP DCs induce IgA production through the secretion of IL-6 and retinoic acid. IgA⁺ B cells develop in the PP then migrate to MLNs then through the circulation to the lamina propria (reviewed in Werchil and Furuta, 2008; Macpherson et al., 2000). IgA secreted into the lamina propria can bind with the polymeric immunoglobulin receptor expressed on the basolateral surface of the intestinal epithelial cells, after which the complex is transported to the apical surface of the cell where the IgA is released (reviewed in Tsuji et al., 2008).

IgA performs many protective functions, including preventing the luminal contents from penetrating the epithelial surface and neutralizing toxins to limit the extent of inflammation (Tlaskalova-Hogenova, 2002; Wershil and Furuta, 2008). *In vitro*, dimeric IgA can neutralize bacterial lipopolysaccharide (LPS) inside epithelial cells thereby preventing a proinflammatory response (Fernandez et al., 2003). IgA can also interact with the Fc receptor expressed on immune effector cells, including natural killer cells, macrophages, neutrophils and mast cells. This initiates a series of inflammatory reactions, including degranulation of eosinophils and increased phagocytic activity of monocytes, macrophages, neutrophils and eosinophils (Fagarasan and Honjo, 2003).

The effector sites (response sites) of the GALT include the lamina propria and the intraepithelial lymphocyte (IEL) cells. Lamina propria T cells proliferate weakly in response to antigen, but will express high levels of IL-4 and IFN- γ . IELs reside in the intestinal epithelium between adjacent cells. They appear to play a role in maintaining

the normal homeostasis of the intestinal epithelium (Wershill and Furuta, 2008; Tsuji et al., 2008). The intestinal epithelial cells also play a role in the mucosal immune system. In addition to transporting secretory IgA, intestinal epithelial cells can act as antigen presenting cells and can respond to bacteria by expressing Toll-like receptors (Dahan et al., 2007). Toll-like receptors (TLRs) are components of the innate immune system that recognize conserved molecules on microorganisms, such as LPS and peptidoglycans.

The overall homeostasis of the mucosal immune system appears to be a balancing act between effector cells (such as the Th1 and Th2 cells) and regulatory cells, such as CD4⁺CD25⁺ T cells. Dysregulation of the mucosal immune system contributes to the pathogenesis of IBD. Excessive inflammation can arise from either the increased activity of the effector cells or decreased function of the regulatory cells. CD4⁺CD25⁺ T cells secrete both TGF- β and IL-10: animal models demonstrate that TGF- β and IL-10 can prevent the differentiation of T cells into effector cells (reviewed in Kucharzik et al., 2006; Maul et al., 2005).

The Immune Response in Helminth Infections

The host-parasite relationship is unique, in that both species have co-evolved: the host attempting to reduce colonization by the intruder and the parasite attempting to evade the immune response of the host. Potential benefits of the parasitic association are rarely explored, as this relationship invariably imparts some degree of harm to the host. The mammalian immune response to parasitic helminths involves an increase in Th2 cytokines, aimed at eliminating the parasite or, at the least, limiting its colonization

(Grencis, 2003; Shin et al., 2007). The Th2 response typically involves increased secretion of IL-4, IL-5, IL-9 and IL-13, induction of IgE secreting plasma cells, and increases in goblet cells, mast cells and eosinophils. The goblet cell response results in increased mucin, which coats the luminal surface and limits contact with the parasite. Activated mast cells release a variety of mediators that increase epithelial permeability and the movement of water into the lumen, which can serve to flush parasite products out of the gut. Contractility is also increased, which will limit contact time with the parasite and speed its movement out of the gut (Dwinell et al., 1997; Barbara et al., 1997).

Helminth therapy for Inflammatory Bowel Disease (IBD)

The CD4⁺ T helper cell clones were first identified by Mosmann and colleagues (Mosmann et al., 1986) based on their secreted cytokine profile. T helper 1 (Th1) cells were described as producing IL-2, TNF- α and IFN- γ , while T helper 2 (Th2) cells produced IL-4, IL-5, IL-10, and IL-13. Most importantly, it was discovered that the Th1 and Th2 cytokines are mutually inhibitory, meaning that an increase in one set would be accompanied by a decrease in the other. Since infection with a parasitic helminth will initiate an immune response that involves an increase in Th2 cytokines, it should follow that this increase will protect the host from any unrelated Th1-type disease. Thus, the initial basis for attempting helminth therapy in models of Crohn's disease was the reciprocal nature of the Th1 and Th2 cytokines. While this is a useful model, the benefits being conferred by helminth infection extend far beyond promotion of a Th2 environment. There is substantial evidence indicating that helminth infection induces the

production of CD8⁺ T cells within the lamina propria that are capable of inhibiting the proliferation of other T cells (Metwali et al., 2006). As well, there is evidence to suggest that *Heligmosomoides polygyrus* infection will induce the expansion of T cells that express TLR4, the receptor for bacterial LPS, and will in fact secrete TGF- β when stimulated with LPS (Ince et al., 2006). These actions will be able to impede any excessive inflammation arising from other diseases or contact with bacterial products.

1.2 Structure and Function of the Colon

The most distal part of the gastrointestinal (GI) tract is made up of the large intestine and anus. The large intestine, in turn, comprises the colon, caecum and rectum. The main role of the colon is store indigestible waste, which is mixed with mucous and bacteria to form feces. The predominant function of the colon is the extraction of water from this feces – much of the fluid that is secreted into the lumen of the upper GI tract is reabsorbed in the colon.

In humans, the colon consists of 4 parts: the ascending colon, the transverse colon, the descending colon, and the sigmoid colon. The colon has a cylindrical shape composed of several layers. The space within the tube is called the lumen. It is surrounded by the innermost layer, the mucosa, which is responsible for water absorption and secretion. This is further divided into the epithelium, the lamina propria and the muscularis mucosae. The epithelium is a single layer of different cell types that lines the entire GI tract. Epithelial cells are closely associated with each other through intercellular adhesions called tight junctions. These adhesions result in the formation of a tight,

electrically polarized monolayer that acts as a barrier and restricts the uptake of material into the submucosa. As this is the primary physical barrier between the body and the external environment, it is essential to preventing the entry of antigens, toxins and pathogens (Mankertz and Schulzke, 2007). This epithelium also presents a barrier to fluid and electrolyte loss into the lumen.

The epithelium regenerates every 4-5 days. Renewal originates at the base of the intestinal crypt, a depressed pit within the mucosa. As they differentiate, epithelial cells move upwards towards the villus tip in the small bowel and the lumen in the colon, where they undergo apoptosis. The surface area of the epithelium is increased by the microvilli projections of enterocytes. Other cell types found within the epithelium include the goblet cells, whose primary role is mucous production, the enteroendocrine cells, which contain aminergic and peptidergic mediators that can act on other cells, and the intraepithelial lymphocytes (Okamoto and Watanabe, 2005).

The lamina propria lies underneath the epithelium, and is a loose layer of connective tissue including fibroblasts, capillaries and immune cells. This is underlain by a thin layer of smooth muscle cells, the muscularis mucosae. The “brain” of the gut is the enteric nervous system (ENS) which coordinates all of the functions of the GI system. Similar to the structure of the central nervous system, the ENS has both afferent and efferent neurons as well as interneurons. Though they are functionally separate, the CNS and ENS communicate through the vagus nerve as well as a network of nerves that enter the gut through the mesenteric ganglia (reviewed in Schemann, 2005).

The next layer is the submucosa, a dense layer of connective tissue with blood vessels, lymphatics and a network of nerves called the submucosal plexus. Movement of food waste through the GI tract is accomplished by peristalsis, the coordinated contractions of both the circular and longitudinal muscle layers. These two layers are adjacent to the submucosa, and together compose the muscularis externa. An extensive network of nerves, the myenteric plexus, is found between the two muscle layers and regulates the function of the outer muscle layers as well as providing a connection with the submucosa and the afferent neurons that project into the mucosa. The muscularis externae is covered by the serosa, a thin layer of cells which secrete a lubricating fluid that can reduce any friction from movement of the intestines within the peritoneal cavity (reviewed in Barrett, 2006).

Many species of bacteria have adapted their lifestyle to the environment of the human gut. While the acidic conditions of the upper GI tract allows only a few species of bacteria to thrive, within the colon the microbial concentration can reach 10^{12} cells/g of luminal contents. These non-pathogenic bacteria digest otherwise indigestible carbohydrates passing through the colon (Guarner and Malagelada, 2003). The bacteria break down some of the dietary fibre to create acetate, propionate and butyrate by-products, which in turn nourish the cells lining the colon. As enzymatic digestion is complete before the chyme reaches the colon, the large intestine does not produce its own digestive enzymes (reviewed in Barrett, 2006).

1.3 Inflammatory Bowel Disease

The human inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, are chronic disorders of the gastrointestinal tract. There is no cure for IBD, other than the possibility of surgery for ulcerative colitis to remove the affected area of the colon (Rutgeerts, 2003). According to the Crohn's and Colitis Foundation of Canada, IBD affects almost 200,000 Canadians, most of whom are diagnosed before the age of 30 (<http://www.cafc.ca/English/info/ibd.html>). This represents not only a great degree of human suffering, but also an immense cost to the health care system, both of which provide motivation to find new and innovative therapies that are effective at treating IBD (Fedorak, 2000).

Patients with Crohn's disease and ulcerative colitis present with different patterns of inflammation, though their symptoms, including diarrhea, vomiting, rectal bleeding and abdominal pain, are similar. Ulcerative colitis presents only in the colon, with inflammation starting in the rectum and continuing from there into the rest of the colon. This inflammation only penetrates to the mucosal layer. Crohn's disease can affect any part of the GI tract, and the inflammation is not continuous but occurs in patches bordered by healthy tissue. The inflammation of Crohn's disease extends through all layers of the intestinal tissue (Hanauer, 1996).

Although the precise etiology of IBD is unknown, it is a multifactorial disease. Susceptibility is determined by a combination of both genetics and a dysregulated immune response to environmental stimuli. There is a very strong bacterial influence, as both Crohn's disease and ulcerative colitis predominantly occur in areas of the gut which

contain the highest load of commensal bacteria. The importance of normal flora in animal models of IBD is highlighted by the finding that disease does not develop in germ-free conditions (Guarner and Malagelda, 2003). In terms of genetics, CARD15/NOD2 mutations are the best documented mutations related to greater susceptibility to Crohn's disease. Mutations of this gene are thought to disrupt sensing of bacterial components. In response to muramyl dipeptide (MDP), NOD2/CARD15 activates the intracellular NF- κ B, which is a major activator of transcription. As a result of the NOD2/CARD15 mutation, the resultant gene product is no longer able to interact with MDP, which results in a loss of bacterial sensing and inhibition of responses that would ordinarily limit bacterial invasion (reviewed in Kobayashi et al., 2005)

While the inflammation associated with Crohn's disease is traditionally thought to be due to an over-active Th1 cytokine response (Cobrin and Abreu, 2005), there is recent evidence indicating that Th17 cells are also involved (Elson et al., 2007). Th17 cells are a subset of T helper cells that produce IL-17, IL-21, and IL-22 (Ouyang, Kolls, and Zheng, 2008). They are thought to be involved in inflammation during autoimmune diseases, through the activation and recruitment of neutrophils, but also provide protection at mucosal surfaces by stimulating epithelial cells to produce anti-microbial proteins (Stockinger and Veldhoen, 2007; Steinman, 2007). Though the cytokine signals that lead to Th17 cell differentiation are not entirely known, IL-6 and IL-23 do play a role (Manel, Unutmaz, and Littman, 2008).

Non-surgical IBD treatments primarily address the symptoms of the disease. Antibiotics are used to control infections, while anti-inflammatories are used to control inflammation. The most commonly used medications are aminosalicylate anti-inflammatory drugs and corticosteroids. For more severe cases, immunosuppressive drugs, such as Azathioprine, can be prescribed, though this can be accompanied by the risk of exposing the patient to fatal infections (Podolsky, 2002). For this reason, surgery is better tolerated in patients with ulcerative colitis. Newer therapies target inflammatory cytokines, though they too can carry potentially fatal side-effects (Rutgeerts, 2006; Hanauer, 2006; Nakamura et al., 2006).

1.4 Animal Models of Colitis: DNBS and Oxazolone

Since there is no single known cause of IBD, numerous animal models have been developed to mimic the colonic inflammation and various underlying features of the disease. While these models offer the opportunity to manipulate treatment outcomes, there is no single model that can replicate all of the nuances of the human disease. The models that are commonly reported, however, present the main pathological processes involved in IBD. Most models are based on one of the following: naturally occurring genetic abnormalities; genetic changes that have been introduced either by targeting a specific gene or by introducing a transgene; colitis that is chemically induced; colitis that is induced by transferring T cell populations but not regulatory cells into Severe Combined Immunodeficient (SCID) mice; or models involving colitis induced by

exposure to a pathogen (reviewed in Bouma and Strober, 2003; Borenshtein et al., 2008). A summary of the common animal models of colitis can be seen in table 1.1.

My own studies primarily used the dinitrobenzene sulphonic acid (DNBS) model of colitis, which is a hapten model of colitis. In this model, the DNBS is administered in a single, intracolonic dose, dissolved in 50% ethanol (EtOH) (Morris et al, 1989). This results in the formation of colonic ulcers, as well as extensive transmural inflammation of the colon which has similarities to the histopathological presentation of Crohn's disease. The EtOH serves to break down the barrier of the colon, which allows the DNBS molecules to enter the lamina propria, where they can bind to the tissue and act as antigens (described in Wallace et al., 1995). While the DNBS/EtOH solution is corrosive and will cause physical damage as well as inflammation, an immune component is also indicated by the fact that prior sensitization to the DNBS will increase the severity of the inflammation. Over time, the immune response to DNBS is characterized by an increase in T helper 1 (Th1) cytokines similar to that seen in Crohn's disease (Elson et al., 1996). The damage induced by dinitrochlorobenzene (DNCB – DNBS without the sulfonic acid moiety) is indistinguishable from EtOH alone, indicating that the sulfonic acid is important to the induction of colitic symptoms (Wallace et al., 1995). Symptoms induced in both mice and rats by administration of DNBS can be seen in table 1.2

Not all haptening agents will induce the same profile of immune responses. In order to investigate a Th2-model of colitis, I used oxazolone, administered in a similar manner to DNBS. Similar to DNBS, oxazolone causes wasting, inflammation and damage to the distal part of the colon. The cytokine response to oxazolone is dominated

by Th2-mediated events and is characterized by an increase in IL-4 that is soon superseded by an increase in IL-13 produced by NK-T cells (Boirivant et al, 1998; Heller et al., 2002).

Table 1.1 Summary of the common animal models of colitis (reviewed in Bouma and Strober, 2003)

Basis for the Model	Model
<i>Genetically Based:</i> develop spontaneous colitis as a result of a genetic defect	Cotton-top tamarin SAMP1/Yit mice C3H/HeJ/Bir mice
<i>Genetically Manipulated:</i> develop colitis as a result of a targeted mutation or transgene	IL-10 ^{-/-} mice (regulatory cell defect) IL-2, IL-2R α or TGF- β -deficient mice (regulatory cell defect) Mdr1a-deficient mice (barrier dysfunction) Intestinal trefoil factor-deficient mice (barrier dysfunction) N-cadherin dominant negative mice (barrier dysfunction)
<i>Chemically Induced:</i> colitis induced by a chemical agent given orally or intra-rectally	Dextran sodium sulphate (DSS) Dinitrobenzene sulphonic acid / Trinitrobenzene sulphonic acid (DNBS / TNBS) Oxazolone Indomethacin Carrageenan Acetic acid Peptidoglycan polysaccharide
<i>Defective Induction of Regulatory Cells</i> <i>Infection with a Pathogen</i>	CD4 ⁺ CD45RB ^{hi} transfer into SCID mice <i>Citrobacter rodentium</i>

Table 1.2 Symptoms induced by intrarectal dinitrobenzene sulfonic acid (DNBS)

(Wallace et al., 1995)

Animal Species	Mouse, Rat
Area Involved	Distal colon
Pathology	Ulcers penetrating through the muscularis mucosae Transmural inflammation Goblet cell depletion
Symptoms	Weight loss Diarrhea Distress (ruffled fur; “hunched” appearance)

1.5 Helminth Parasites

Parasitism is a symbiotic relationship between two organisms of different species in which one, the parasite, benefits and the other, the host, is harmed to some degree. Helminth parasites are large, multicellular worms with complex life-cycles involving a definitive host, where the adult resides, and one or more intermediate hosts, where the larval stages are found. Infection normally occurs through contact with food, water or soil that has been contaminated with the infective stage of the worm, usually the immature larval form. The larval stage will invade the definitive host and migrate to its preferred niche where it will transform into the adult stage. Most species prefer to establish in either the intestine or blood, though some species aim for the lymphatic vessels, eyes, bladder, lungs or heart. These infections can be either chronic associations, with the worm surviving for years within the host, or acute infections that progress rapidly (Schmidt and Roberts, 1996). The life style of an individual parasite shows a high degree of specialization and most species cannot live outside of their preferred host.

Beyond the Th1/Th2 Paradigm

The interaction between the host and parasite is much more complex than a response to antigen: the parasite's very survival is dependent on its ability to evade the immune response of its host. The helminths themselves will act on their environment, and there is some evidence of immunosuppressive factors actually derived from the parasite. For example, *Nippostrongylus brasiliensis* secretes a VIP-like molecule which could interfere with the neuroimmune system of the host (Foster and Lee, 1995).

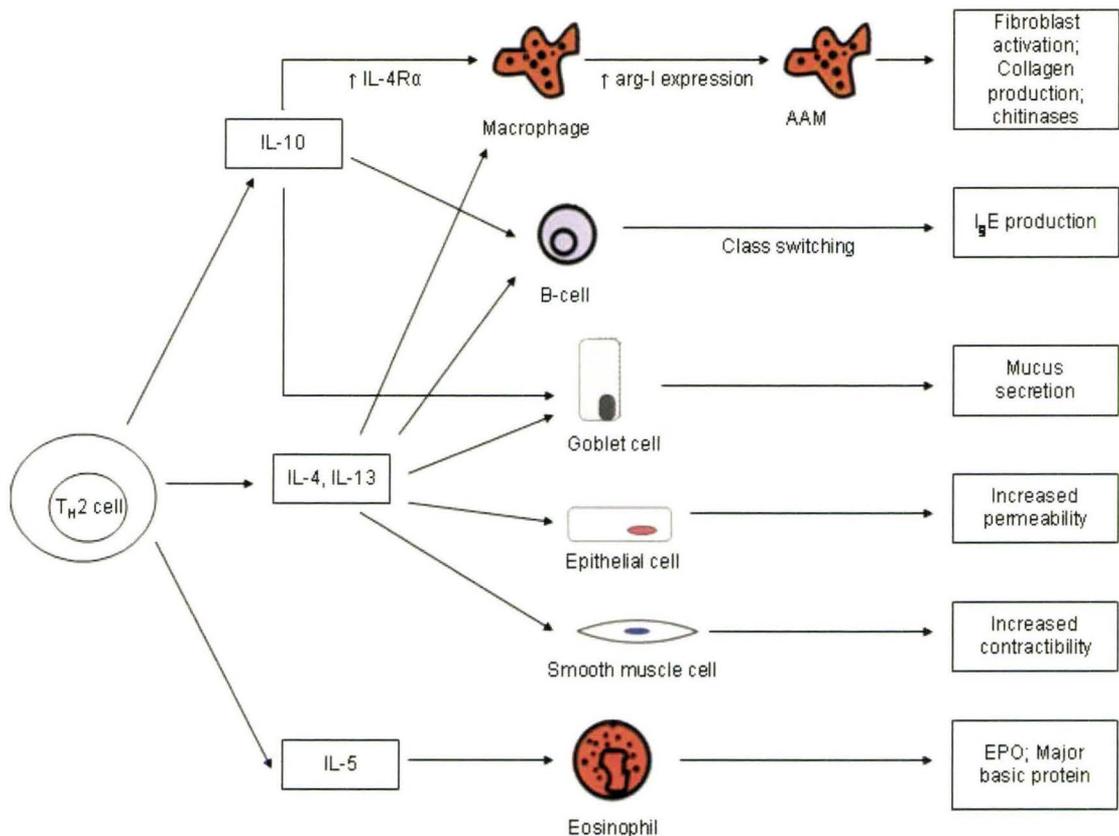
Additionally, a fraction of the tapeworm *Echinococcus granulosus* and a lysophosphatidylserine secreted by *S. mansoni* were both found to induce the production of IL-10 by the host as did a high molecular weight protein from *H. diminuta* (Dematteis et al., 2001; van der Kleij et al., 2002; Wang and McKay, 2004).

Helminth infection may promote a general anti-inflammatory environment, involving increased levels of IL-10 and TGF- β (Doetz et al., 2000). Within the infected gut, this increase in Th2 cytokines will promote an increase in mast and goblet cells (Stead et al., 1987; McKay et al., 1991). There is also an increased eosinophilia induced by helminth infection. The eosinophils migrate to the infected area where they will degranulate and release eosinophil secondary granule proteins (ESGPs). These actively attack the cuticle or tegument (outer covering) of the parasite (reviewed in Anthony et al., 2007). Thus, the intestinal worm is attacked, damaged, and flushed out of the host.

Overall, the host immune response to helminth infection has two goals: 1) to hasten the expulsion of the worm; and 2) to limit the inflammation induced by the infection (reviewed in Anthony et al., 2007). While the increased eosinophils and changes to the intestinal lumen will work to actively kill and eliminate the parasite, it is the response aimed at reducing inflammation that will have the most impact on any co-existing bowel disease. This will include factors that not only downregulate the inflammatory Th1 response but factors involved in healing and remodelling the tissue. Major responses initiated by the Th2 response to helminth infection are depicted in figure 1.1.

Figure 1.1 Major responses during intestinal helminth infections are mediated by the Th2 cytokines. These include, but are not limited to, the following effects: IL-5, increased eosinophilia; IL-10, increased proliferation of goblet cells and B cells as well as increased expression of the IL-4 receptor α ; IL-4/IL-13, alternative activation of macrophages, B cell switching to IgE production, increased proliferation of goblet cells, increased permeability of epithelial cells and increased contractility of smooth muscle cells.

(Hunter and McKay, 2004; Anthony et al., 2007)



Regulatory T Cells

Regulatory T cells are a subpopulation of T cells that are capable of inhibiting potentially harmful immune responses. Regulatory T cells come in many forms, including the CD4⁺CD25⁺ regulatory T cells that express CD4, CD25 and the transcription factor FoxP3 (Fontenot, Gavin and Rudensky, 2003). They are generated spontaneously *in vivo* in response to antigens presented via the mucosal surface (O'Garra, Steinman and Gijbels, 1997). Regulatory T cell activity in the intestine has been reported during helminth infection, including *H. polygyrus* and *H. diminuta* infection (Metwali et al., 2006; Setiawan et al., 2007; Rausch et al., 2008; Persaud et al., 2007). Is it possible that the immunosuppressive ability of these cells could also downregulate any co-existing bowel disease?

This seems to be the case for allergic airway disease. Infection with *H. polygyrus* was associated with reduced airway inflammation in response to ovalbumin and house dust mite allergens – a finding that was reversed by co-treatment with antibodies to CD25 (Wilson et al., 2005). Schistosoma egg antigens were also found to increase the number of CD4⁺CD25⁺ T cells and concomitantly reduce airway inflammation in a murine model of asthma (Yang et al., 2006). In terms of IBD, a case study of a patient with ulcerative colitis who was also infected with *Enterobius vermicularis* found an abundance of FoxP3⁺ regulatory T cells throughout the colonic mucosa except in areas

with active ulcerative colitis (Buening et al., 2008). As well, colitis induced by a *Leishmania major* infection in SCID mice could be reversed by the transfer of CD4⁺CD25⁺ T cells (Liu et al., 2003).

The immunosuppressive cytokines TGF- β and IL-10 have been implicated in regulatory T cell function. The study that assessed the effect of *H. polygyrus* on airway inflammation found that there was an increased expression of TGF- β and IL-10 (Wilson et al., 2005). The anti-colitic effect of the regulatory cells in the *L. major* model of colitis was eliminated by treatment with antibodies for either the IL-10 receptor or TGF- β (Liu et al., 2003).

The helminth-induced increase in TGF- β that is related to the T regulatory cells may play an important role in the protective effect of the helminths. TGF- β is a multi-functional cytokine with 3 isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. It is capable of regulating cell growth and differentiation, and has also been characterized as having anti-inflammatory effects, as demonstrated by the finding that blocking the production of TGF- β 1 from regulatory T cells inhibits their ability to suppress intestinal inflammation (Nakamura et al., 2004). Specifically, there is evidence that TGF- β plays an active role in the reduction of Th1-mediated events in experimental colitis (Powrie, 1996). The TGF- β family signals primarily through the SMAD pathway, forming a complex that enters the nucleus and acts as a transcription factor. The importance of TGF- β is again underscored by the fact that TGF- β ^{-/-} mice develop an autoimmune disease involving extreme inflammation that leads to death (Shull, 1992).

Interleukin 10 (IL-10)

IL-10 was initially identified in 1989 and characterized by its ability to inhibit cytokine secretion by Th1 cells (Florentino, Bond and Mosmann, 1989). Classified as a Type II cytokine, IL-10 is a member of a family of cytokines that function by activation of Janus kinase (JAK) and Signal transducer and activator of transcription (STAT) signalling pathways. The IL-10 receptor is a heterodimer of two subunits, IL-10R1 and IL-10R2. Both subunits are expressed on most cells allowing a diverse variety of cells to respond to IL-10 (reviewed in Mosser and Zhang, 2008).

Though initially identified as a product of Th2 lymphocytes, IL-10 production by B cells, mast cells and macrophages has also been described (reviewed in Taube and Stassen, 2008; Bouaziz, Yanaba and Tedder, 2008). While IL-10 is able to directly inhibit IFN- γ secretion by Th1-CD4⁺ cells, it also plays a role in inhibiting a variety of classical macrophage functions, including LPS and T-cell induced cytokine secretion and nitric oxide production. IL-10 also increases B-cell proliferation and antibody secretion, and enhances mast cell activation and proliferation (Moore et al., 1993; Schnoeller et al., 2008). During helminth infection, IL-10 plays an essential role in the response aimed at limiting colonization by the parasite. One study confirmed that IL-10 deficiency led to the development of a high IFN- γ response to adult *S. mansoni* infection, in sharp contrast to the solely Th2 response of wild-type mice (Hoffman, Cheever and Wynn, 2000).

It has also been observed that there is a decrease or absence of intestinal epithelial cell mucus in IL-10^{-/-} mice (Schopf et al., 2002), indicating that IL-10 plays a role in mucus production. Mucus coats the surface of the intestinal wall, providing protection

and limiting penetration. In terms of helminth infection, an increase in goblet cells is one of the responses to infection with *H. diminuta* (McKay et al., 1991).

IL-10 also plays an important role in the regulation of mucosal immunity as mice with a nonfunctioning IL-10 gene will develop enterocolitis (Kuhn, 1993), as will mice with macrophages that have a deletion of Stat-3, rendering them incapable of responding to IL-10 (Takeda et al., 1999). As well, normal mice will exhibit an IL-10 dependent response to *Helicobacter hepaticus* infection of the cecum in contrast to the overwhelmingly pathogenic Th1 response in IL-10^{-/-} mice (Kullberg, 1998). This supports the idea that the secretion of IL-10 suppresses inflammatory immune responses to intestinal antigens.

The precise mechanism of how IL-10 intercedes in an immuno-regulatory role is not entirely clear. IL-10 inhibits the recruitment of Th1 cells to the intestine, by acting on macrophages to prevent their activation and the release of proinflammatory molecules. IL-10 also influences the development of regulatory T cells (Tr-1) which in turn become IL-10 producing cells (Setiawan et al., 2007).

During the course of helminth infection, IL-10 plays an important role in limiting colonization by the parasite. *H. polygyrus* infection induced an increase in IL-10, with an accompanying decrease in production of IFN γ (Elliott, 2007). In terms of limiting the pathogenicity of parasitic infection, IL-10 was found to limit the inflammation associated with muscular penetration by *T. spiralis* (Beiting, 2004).

The immense load of commensal bacteria in the lower GI tract contains enormous amounts of immunostimulatory molecules that are separated from the remainder of the

body by a single layer of epithelial cells (Macpherson, Martinic and Harris, 2002). A subset of immunostimulatory enteric bacterial antigens will stimulate lamina propria CD4⁺ Th1 cells to produce IFN- γ which can then activate macrophages to produce proinflammatory cytokines. In the normal mucosa, this inflammation is limited by regulatory T cells that produce inhibitory cytokines, such as IL-10 and TGF- β (Elson and Cong, 2002). In human IBD, this balance is disrupted. Perhaps the increase in IL-10 associated with helminth therapy can return the tissue to a balance between proinflammatory and anti-inflammatory events.

Since IL-10 downregulates the production of pro-inflammatory responses, it was long thought to be a potential therapy for patients with IBD, especially those suffering from Crohn's disease. In a model of TNBS colitis in rats, the transfer of an adenovirus carrying the IL-10 gene was found to prevent the symptoms of TNBS colitis (Barbara, 2000). Unfortunately, human clinical trials have indicated that recombinant IL-10 therapy is of little benefit for patients with active Crohn's disease (Lindsay and Hodgson, 2001). In fact, the high levels of systemic IL-10 induced by the therapy were associated with several undesirable side-effects, including fever and headache (Li and He, 2004). These side-effects are most likely due to the widespread location of the IL-10 receptor: when the dose is administered, the cytokine does not target specific cells, but instead is able to act as a stimulus to a wide variety of cells that are neither immune cells or epithelial cells.

Alternatively Activated Macrophages

A number of effector mechanisms could potentially be mediate the anti-inflammatory benefits of helminth infection. One of these is the differentiation of the alternatively activated macrophages (AAM) (Gordon, 2003). While classically activated macrophages (CAM) are activated by LPS and IFN- γ , the AAM develops under the influence of IL-4 and IL-13 and is considered to be anti-inflammatory (Kreider et al., 2007). The pattern of gene expression of the AAMs is also different from classical macrophages, in that they express many novel genes, including chitinase-like molecules such as YM1, resistin-like molecule (RELM) α (or FIZZ-1) and arginase-1 in mice and the mannose receptor in humans. These macrophages are able to inhibit T cell proliferation via cell-to-cell contact and through the release of soluble mediators (Reyes and Terrazas, 2007). Arginase-1, which competes with iNOS for the substrate L-arginine, seems to be involved in protection from the parasite, as blocking its function also reduces the ability to eliminate the parasite (reviewed in Anthony et al., 2007). YM1 is among the most highly upregulated macrophage genes during nematode infections (Anthony et al., 2006). YM1 is a lectin with a chitin affinity and it also binds heparin (Chang et al., 2001), indicating a possible role in regulating interactions with the extracellular matrix. YM1 also shows the possibility of being a chemotactic factor for eosinophils (Falcone et al., 2001). FIZZ1 is involved in the stimulation of myofibroblast production of collagen, indicating a possible role in tissue repair (Nair, Guild and Artis, 2006). Together, upregulation of these proteins may play an essential role in wound repair after an inflammatory event.

Although it is known that the AAMs are recruited during the course of several conditions, including parasitic infection and allergy (Anthony et al., 2006; Kim et al., 2008), the extent to which they influence the severity of inflammation has not yet been defined. It is thought that they enhance host defense against parasite infection (Herbert et al., 2004) and promote tissue repair after injury (Loke et al., 2007). As well, deletion of the IL-4 receptor α in mice, which prevents IL-4 mediated development of AAMs, led to lethal intestinal inflammation following *Schistosoma mansoni* infection (Herbert et al., 2004). In terms of colitis, depletion of all of the intestinal macrophages by the administration of clodronate exacerbated DSS-induced inflammation (Qualls et al., 2006), a finding that has been repeated in our own laboratory using the DNBS model of colitis (experiments performed by Arthur Wang).

1.6 Helminth Therapy and the Hygiene Hypothesis

If helminth infection is anti-colitic, it follows that we should find a negative geographical relationship between the prevalence of human helminth infection and the incidence of IBD. This is supported by evidence from epidemiological studies. The prevalence of both Crohn's disease and ulcerative colitis continues to increase in industrialized countries, while remaining relatively rare in third world countries (Elliott et al., 2000; Buening et al., 2008). Specifically, IBD is seldom reported in Africa, Asia and South America (Hutt, 1979; Tan et al., 1992; Rolon, 1979), areas where helminth infection is epidemic. At the same time, the incidence of IBD is starting to rise in areas where it was once only rarely diagnosed (Loftus and Sandborn, 2002), perhaps as a result

of increased industrialization of the third world. Within the United States, Crohn's disease and ulcerative colitis are more prevalent in northern and urban regions (Sonnenberg, McCarty and Jacobsen, 1991). Genetic differences between these regions cannot explain these findings: while the incidence of IBD among black Africans is low, prevalence among the African-American and caucasian populations in the United States is similar (Farrokhyar, Swarbrick and Irvine, 2001).

The epidemiological findings, however, may be biased by variations in methodology and definitions. Surveys of third world countries may be affected by under-reporting, and the incidence rate may be affected by misdiagnosis and lower life-expectancies in these countries. As well, this may be an example of a correlation and not a cause-and-effect relationship as confounding factors, such as the impact of nutritional and life-style differences, cannot be dismissed.

Though the actual reason behind the differences in prevalence rates of IBD is unknown, this evidence does support the idea of the "hygiene hypothesis", which proposes a causal link between reduced exposure to infection and the increased prevalence of IBD, asthma and allergic diseases (Gale, 2002; Kemp and Bjorksten, 2003). This is supported by a finding that Crohn's disease is more common in patients whose infancies were spent in homes with hot water, a measure of increased hygiene in early childhood (Gent et al., 1994). Additionally, a survey of American veterans found a negative correlation between military duty in Vietnam, which would expose the veteran to a period of poor sanitation, and the incidence of Crohn's disease (Delco and

Sonnenberg, 1998). Regardless of these findings, evidence in the form of controlled experiments is also required.

1.7 Helminth Therapy for Human IBD

Clinical observations support the hypothesis that infection with parasitic helminths may reduce the symptoms of IBD. When given live *Trichuris suis* eggs, a group of Crohn's disease patients showed substantial improvement of their symptoms, as measured using the Crohn's Disease Activity Index (Summers et al., 2003). This improvement could be sustained with repeated doses of the parasite. Surprisingly, the same benefit was seen when *T. suis* was given to a group of ulcerative colitis patients, a disease that has been traditionally characterized as being Th2 in nature (Summers et al., 2005). While these results are promising, the sample sizes are small, with improvement of symptoms being reported in 21 out of 29 patients with Crohn's disease but only 13 out of 30 patients with ulcerative colitis. In order to maintain this improvement, repeated doses of the parasite were needed and there is a risk related to the invasiveness of *T. suis* and its ability to migrate into unintended parts of the body. The hookworm *Necator americanus* has been investigated as an alternative, as it has no risk of aberrant migration and adult worms will live in the small intestine for around five years negating the need for constant treatment. When five Crohn's disease patients were inoculated with *N. americanus*, they showed improvement of their symptoms 20 weeks after infection (Croese et al., 2006a). Again, this study involves a very small number of patients and requires verification in a controlled, large-scale randomized clinical trial.

By their very definition parasitic species are harmful to their hosts. There is a need for controlled experiments to examine any drawbacks that may arise from helminth therapy. Therapeutic use of helminths, and the subsequent promotion of a Th2 environment, could create a predisposition towards allergic disease. Though the immune response elicited by the helminth is similar to the enhanced Th2-allergic response, geographically the two do not coincide (Yazdanbakhsh, van den Biggelaar and Maizels, 2001). While the prevalence of allergic diseases, including asthma, is increasing in the western industrialized countries, the prevalence of helminth infections has substantially decreased (Cooper, 2002). Among Brazilian asthmatic patients, it was found that a milder case of asthma was associated with *S. mansoni* infection (Medieros et al., 2003), while infection with *H. polygyrus* was protective in a murine model of asthma (Wilson et al., 2005). *S. mansoni* infection was equally effective at attenuating airway hyperactivity (reviewed in Elliott, Summers and Weinstock, 2007).

While several studies promote the possible use of helminth therapy for treating, in addition to colitis, multiple sclerosis, diabetes, allergies, and asthma (Sewell et al., 2002; Cooke et al., 1999; Cooper, 2004), there is evidence that helminth infection could actually cause more damage than good. The hooks and teeth of the nematodes can inflict actual tissue damage. As well, the helminth-induced increase in IL-13 can induce tissue fibrosis, which could represent a pathological side-effect to the therapy (Wynn, 2004).

Due to their size, helminths cannot be eliminated from their host via macrophage phagocytosis. Instead, the host response is aimed at eliminating the parasite by destroying its outer surface. One tissue damaging molecule that is upregulated during

helminth infection is the eosinophil cationic protein. In the context of helminth therapy, there is a risk that the upregulation of this eosinophil response could lead to uncontrolled eosinophilia. Indeed, the study using *Necator americanus* therapy reported that patients developed eosinophilic enteritis (Croese et al., 2006a; Croese et al., 2006b).

Additional pathogenic side-effects related to helminth infection include effects on bacteria, as demonstrated by a case that examined increased pathogenicity of *Campylobacter jejuni* leading to acute renal failure during a co-existing *T. suis* infection (Shin et al., 2004). Indeed, *C. jejuni* infection in pigs has been shown to only be pathogenic when there is a pre-existing whipworm infection (Mansfield et al., 2003; Mansfield and Urban, 1996). As well, *Strongyloides stercoralis* infection has been associated with systemic sepsis, thought to originate from the overgrowth of small bowel bacteria (Ghoshal et al., 2002). Thus, any discussion of using helminth therapy must start with an understanding of the risks of increased penetration of bacteria as well as an assessment of eosinophil levels in the patient. Building animal models to test the efficacy of these treatments, as well as examining any deleterious side-effects, would be the safest way to approach this problem. The most common human helminth parasites are listed in table 1.3, while features that characterize the ideal candidate for helminth therapy are described in table 1.4.

Table 1.3 Helminth parasites that parasitize humans

Nematode Species (Roundworms)	Hook worms: <i>Necator Americanus</i> , <i>Ancylostoma duodenale</i> Pinworm: <i>Enterobius vermicularis</i> Filarial worms: <i>Wucharia bancrofti</i> , <i>Brugia malayi</i> , <i>Onchocerca volvulus</i> Guinea worm: <i>Dracanculus medinensis</i> Other: <i>Ascaris lumbricoides</i> , <i>Trichuris</i> <i>trichuria</i> , <i>Trichinella spiralis</i> , <i>Strongyloides stercoralis</i>
Platyheminth Species (Flatworms)	Trematode: <i>Schistosoma mansoni</i> , <i>S.</i> <i>japonicum</i> , <i>S. haematobium</i> , <i>Paraganimus</i> <i>sp.</i> , <i>Opisthorchis sinensis</i> , <i>Echinostoma sp.</i> , <i>Fasciola hepatica</i> , <i>Dicrocoelium</i> <i>dendriticum</i> , <i>Heterphyes sp.</i> Cestode: <i>Taenia saginata</i> , <i>Taenia solium</i> , <i>Diphyllobothrium sp.</i>

Table 1.4 Characteristics of the ideal candidate for helminth therapy (Elliott et al., 2007)

-
- Reduced potential for instigating a pathogenic response
 - Cannot autoinfect
 - Cannot spread to other hosts
 - Does not migrate within the host
 - Can easily be eradicated
 - Easy to administer
 - Easy to store / transport / maintain in large numbers suitable for treating multiple patients
-

1.8 Animal Studies

Animal models have established that an existing helminth infection can reduce the Th1 immune response to an unrelated bacterial or viral infection. *S. mansoni*, a fluke that promotes IL-4 production, was shown to reduce the secretion of IFN- γ normally induced in response to tetanus toxoid (Davidson et al., 1996). *S. mansoni* infection also reduced the production of IL-2 and IFN- γ in mice exposed to Sperm-Whale myoglobin (Kullberg et al., 1992).

The effect of helminth infection on experimentally induced colitis has also been explored. Models of intestinal inflammation have demonstrated the benefits of helminth therapy using the nematodes *T. spiralis* and *S. mansoni*. Among the genetically engineered mice, the IL-10^{-/-} mouse spontaneously develops colitis associated with an excessive production of Th1 cytokines, similar to Crohn's disease in humans (Fuss et al., 1996). After infection with live *Heligmosomoides polygyrus* larvae, these mice presented with reduced intestinal inflammation (Elliott et al., 2004). The effect of helminth therapy has also been shown in several chemically-induced models of intestinal inflammation. Infection with the nematode *Trichinella spiralis* decreased mortality and MPO activity in the DNBS model of colitis (Khan et al., 2002), while freeze-killed *S. mansoni* eggs decreased the production of IFN- γ in the colons of mice that had received TNBS (Elliott et al., 2003).

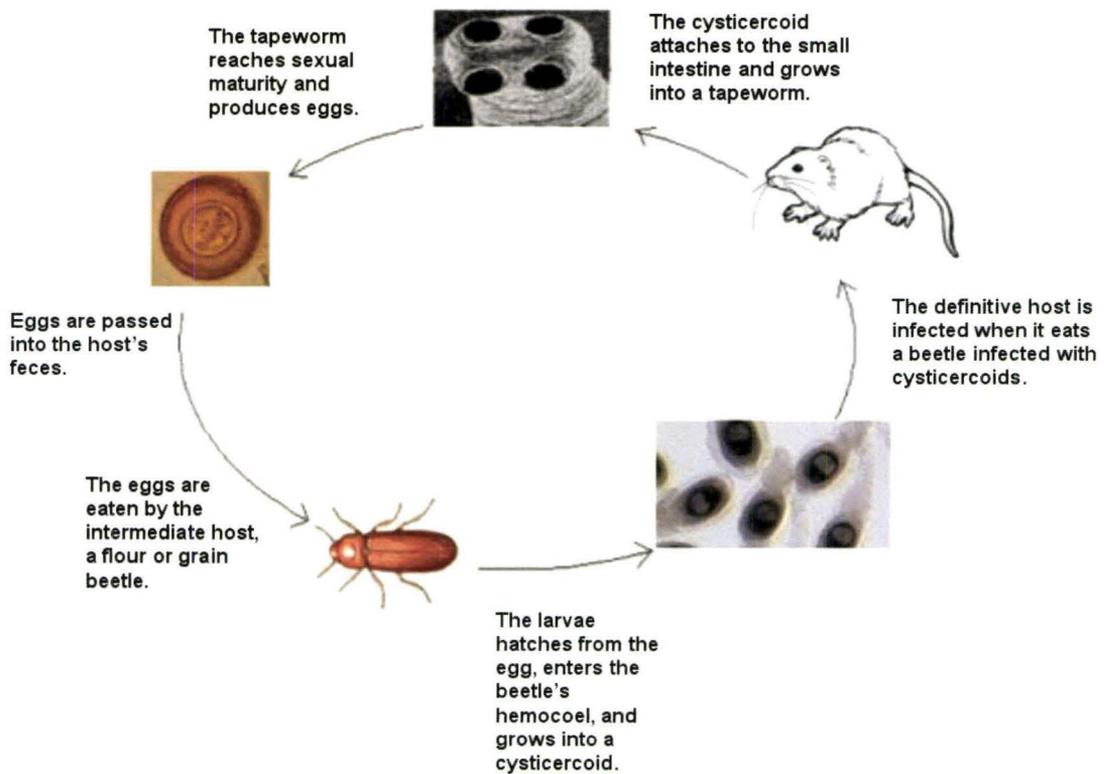
While the results from animal studies using helminth therapy are very promising, most of the helminths used in these studies are invasive parasites that will evoke varying degrees of pathology. Acute hypersensitivity to *S. mansoni* eggs that have migrated

through the bladder wall results in a disease called Schistosomiasis, while *T. spiralis* is the pathogenic agent in Trichinosis (Schmidt and Roberts, 1996). The rat tapeworm *H. diminuta* also elicits a Th2 response from its host, but is non-invasive. Instead of hooks or teeth, it is equipped with 4 suckers that allow it to attach to the villi of the small intestine. The specific life cycle of *H. diminuta* ensures that the worm can easily be maintained in the laboratory. In addition, this worm must pass through its intermediate beetle host in order to reach maturity, ensuring that there is no auto-infectivity. This allows us to precisely control the infectious burden when using *H. diminuta* as a therapeutic agent.

1.9 Hymenolepis diminuta

The rat tapeworm, *Hymenolepis diminuta*, lives and mates permissively in the small intestine of the rat host. The tapeworm is host species specific and does not cause any overt symptoms in the host. *H. diminuta* eggs are excreted within the rat droppings, which are then consumed by the flour beetle host. Once inside the beetle, the eggs mature into a cysticercoid, a juvenile tapeworm encased in a hard cyst. Once the rat ingests the beetle, the cyst is broken down by the rat digestive enzymes, and the juvenile migrates to the small intestine where it attaches to the villi of the small intestine and matures into its adult form (Fig. 1.2). This life cycle is extremely specific, as when the worms are ingested by the rat host they will mature, but when ingested by a mouse, which is a non-permissive host, they will be rejected within 12 days of infection (reviewed in McLauchlan et al., 1999).

Figure 1.2 The life cycle of *Hymenolepis diminuta* alternates between two hosts, a rodent final host and an arthropod intermediate host. Host preference is very specific: when ingested by the permissive rat host, the larvae will develop to maturity and produce eggs, but when ingested by the non-premissive mouse host, the worm will be rejected.



Within the small intestine of the permissive rat host, the presence of the parasite diminishes the rates of host absorption of glucose, salt and water, and will also alter Na^+ and HCO_3^- gradients (Podesta and Mettrick, 1977). Infection will also decrease the transit rate of materials travelling through the lumen and change the composition of commensal bacteria (Dwinell et al., 1997). Increases in mast cells in the mucosa of infected hosts have also been observed (Dwinell et al., 1998).

When the mouse is infected, the worm becomes destrobilated and is expelled. The mouse host retains some memory of the infection, as the first infection is rejected within 12 days, while a secondary infection will be rejected within 7 days (McCaigue, 1987). Infection within the non-permissive host is associated with changes in gross morphology of the intestine (McKay et al., 1990) as well as increases in intestinal goblet cells (McKay et al., 1990b) and mast cells (McLauchlan et al., 1999). More recently, it has been determined that infection with *H. diminuta* will increase the levels of IL-10 and AAM markers as measured using RT-PCR (Persaud et al., 2007). Thus, within the non-permissive mouse host, the immune events leading up to rejection may also have an effect on inflammation arising from an unrelated disorder.

1.10 Aims and Hypothesis

The focus of my Ph.D. research has been to define a novel model for treating the symptoms of DNBS-induced Th1-type colitis. In a previous study in our laboratory, *H. diminuta* infection, given both prophalactically and as a treatment, was found to improve the abnormalities in colonic epithelial ion transport of mice with dextran sodium sulphate

(DSS)-induced colitis (Reardon et al., 2001). Improvements to DSS-induced histopathology, however, were not seen. This may be due to the mixed Th1/Th2 nature of DSS colitis, hence the benefit in developing a model for helminth therapy using a purely Th1-driven colitis.

My hypothesis is that infection with the parasitic helminth *Hymenolepis diminuta* can treat and/or prevent the symptoms of Th1-dominated inflammation. This anti-colitic effect is dependent on IL-10 and involves the recruitment of the alternatively activated macrophages.

Aim I: To determine if infection with the rat tapeworm H. diminuta can prevent the symptoms of Th1-type colitis induced by an intra-rectal instillation of DNBS in the mouse; Aim II: To determine if infection with H. diminuta after the onset of DNBS-colitis enhances recovery from the disease.

There is evidence to suggest that a helminth infection will reduce the Th1-response to an unrelated infection. The host-parasite relationship is unique, however, in that both species have co-evolved in an evolutionary arms race, with the host attempting to reduce colonization by the intruder and the parasite evolving to evade the immune response of the host. An important question in this study is just how important the host-parasite interaction is to the modulation of colitis, and to what extent the anti-colitic effect is due to the rejection event that leads to the expulsion of the worm in the non-permissive host.

Aim III: To determine if a helminth-associated increase in IL-10 is crucial to the anti-colitic benefits of H. diminuta.

IL-10 plays an essential role in limiting colonization by parasites and plays a crucial role in the survival of *T. muris*, *Toxoplasma gondi* and *Trypanosoma cruzi*-infected mice (Schopf et al., 2002; Gazzinelli et al., 1996; Hunter et al., 1997). Though the precise mechanism of this protective effect is not clear, IL-10 plays a role in inhibiting the recruitment of Th1 cells to the intestine, by acting on macrophages to prevent their activation and the release of proinflammatory molecules (Asseman et al., 1999).

Aim IV: To determine if AAMs can mediate the anti-colitic effect of H. diminuta infection.

In fact, there are a number of potential effector mechanisms that could be mediating the anti-inflammatory benefits of helminth infection, including the differentiation and recruitment of type 2 or alternatively activated macrophages. These macrophages develop under the influence of IL-4 and IL-13 and participate in tissue repair after injury.

Aim V: To determine if H. diminuta infection will exaggerate the symptoms of chemically induced Th2-colitis.

There is some concern that helminth infection will promote a Th2 environment that could lead to a predisposition to allergic disease, and conflicting evidence over whether helminth infection will confer protection against allergic conditions.

Chapter 2: Materials and Methods

2.1 Helminth Infection

The life cycle of *H. diminuta* alternates between an arthropod intermediate host and a rodent final host. Within the laboratory, male Sprague-Dawley rats (Harlan Animal Suppliers, Indianapolis, IN) were infected with ten *H. diminuta* cysticercoids in 100 μ l of 0.9% NaCl by oral gavage. The rats were housed in filter-topped cages at McMaster University Central Animal Facility. Three months post-infection, rats were euthanized and the small intestine removed and rinsed with 0.9% NaCl to remove all adult worms. Adult worms were then mashed onto a filter paper and fed to mature flour beetles (sp. *Tribolium confusum*). The beetles had been without food for 48 hours before being placed in isolation with the mashed worm segments. After a 24 hour period, the beetles were returned to their normal diet. Two weeks after feeding, the beetles were dissected under a microscope and the hemocoel searched for mature cysticercoids. Mice received five infective *H. diminuta* cysticercoids in 100 μ l of 0.9% NaCl by oral gavage (McKay et al., 1990).

2.2 Induction of DNBS Colitis

Male Balb/c mice (7-9 weeks old, Harlan Animal Suppliers) were housed in filter-topped cages at McMaster University Central Animal Facility. Colitis was induced in lightly anaesthetized mice by an intrarectal injection of DNBS (3 mg) in 100 μ l of 50%

ethanol (EtOH). The colitic agent was delivered 3 cm into the colon with a polyethylene catheter (PE90 tubing, Clay Adams, Parsippany, New Jersey; Qui et al., 1999). In the prophylactic protocol, mice were infected with *H. diminuta* and colitis was induced 8 days later followed by autopsy at either 72 hours or 7 days post-DNBS. In the treatment protocol, mice received DNBS 48 hours before *H. diminuta* infection, with autopsy at 8 days post-infection. An additional experiment administered DNBS 1, 2 and 3 weeks post-*H. diminuta* infection followed by autopsy 72 hours after DNBS.

To measure the effectiveness of different preparations of *H. diminuta*, the prophylactic protocol was followed using one of the following preparations: (1) five cysticercoids that were killed by submersion in 70% EtOH that had been initially heated to 56⁰C, then left submerged in the EtOH for 14 hours; (2) an adult *H. diminuta* protein antigen preparation (1 mg/100 µl PBS) prepared by homogenizing whole worms in PBS using a polytron tissue homogenizer; or (3) five larvae that had been excysted by submersion in 1% pepsin for 10 minutes followed by a 1% trypsin/1% sodium tauroglycocholate solution for 30 minutes.

To examine the effect of a secondary infection, mice were infected with five *H. diminuta* larvae 28 days after the original infection. At this time-point, the colitis induced by DNBS had resolved and the original *H. diminuta* infection had been rejected.

In a subsequent experiment, male Sprague-Dawley rats (Harlan Animal Suppliers) were infected with 10 *H. diminuta* cysticercoids by oral gavage and assigned to either the chronic or acute experimental condition. In the chronic condition, colitis (22 mg DNBS) was induced 3 months after *H. diminuta* infection. In the acute condition, colitis was

induced 8 days after *H. diminuta* infection. The DNBS was delivered in 100 μ l of 50% EtOH 7 cm into the colon.

Controls consisted of naïve animals, EtOH only-treated animals, *H. diminuta* infected-only and DNBS-only. All animal experiments conformed to the Canadian guidelines for animal welfare as well as the regulations outlined by the animal care committee at McMaster University.

2.3 In Vivo Neutralization of IL-10

Following the prophylactic protocol, mice received five infective *H. diminuta* cysticercoids followed by DNBS 8 days later. Three days after *H. diminuta* infection, mice received 50 μ g of a neutralizing anti-IL-10 antibody (Pierce Endogen, Rockford, IL) via an intraperitoneal (ip) injection. Injections were repeated at day 7 (100 μ g) and day 9 (50 μ g) post-infection. The mice were autopsied 72 hours post-DNBS. Controls received an isotype-matched Ig (Pierce Endogen) following the same protocol.

2.4 In Vitro Development of AAMs

Peritoneal macrophages were labelled in vivo using a red fluorescent dye specific for phagocytic cells (PKH26PCL, Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. In brief, dye stock was mixed with absolute EtOH to make a 100 μ M stock solution. 25 μ l of stock was then mixed with 0.5 ml of PBS, which was

injected into the peritoneal cavity of naïve mice. Macrophages were harvested 48 hours after the dye was injected.

To harvest macrophages, mice were sacrificed and 5 ml of ice-cold PBS was forcefully injected into the abdomen. The abdomen was then massaged to loosen adherent cells and the fluid was withdrawn and centrifuged (4°C at 350 x g for 5 minutes). The cell pellet was then resuspended in 1 ml of Dulbecco's Modified Eagle Medium supplemented with 10% bovine calf serum, 1% penicillin/streptomycin and 2% sodium bicarbonate (Invitrogen Life Technologies, Burlington, ON). The peritoneal exudate cells were then seeded onto sterile 12-well plates (1 x 10⁶ cells/well) and differentiated into classical macrophages (CAM) or alternative macrophages (AAM) by the addition of either LPS (2.5 µg/ml; *E. coli*; Sigma-Aldrich) or mouse recombinant IL-4 and IL-13 (10 ng each; Invitrogen) for 48 hours. Macrophages were characterized by RT-PCR with primers specific for arginase 1 and FIZZ 1 (AAMs) or iNOS and CCL3 (CAMs). All primer sequences are listed in table 2.1.

2.5 Macrophage Transfer and Localization

Transformed macrophages were administered intravenously to naïve mice (1 x 10⁶ cells/mouse). Colitis was induced 48 hours after transfer. To determine localization of the transferred macrophages, segments of the mid-colon were fixed in Zamboni's fixative for 24 hours, then embedded in Tissue-Tek O.C.T. (Tissue-Tek Optimum Cutting Temperature; Sakura Finetek USA Inc., Torrance, CA), frozen in liquid nitrogen and stored at -20°C. Cryostat sections (4 µm) were collected on slides, air dried then mounted

Table 2.1 Primer sequences used to assess murine mRNA expression

Gene	Primer Sequence
β -actin	sense: 5'CCA GAG CAA GAG AGG TAT CC 3' antisense: 5' CTG TGG TGA AGC TGT AG 3'
IL-10	sense: 5' ATG CAG GAC TTT AAG GGT TAC TTG GGT T 3' antisense: 5' ATT TCG GAG AGA GGT ACA AAC GAG GTT T 3'
IL-4	sense: 5' ATG GGT CTC AAC CCC CAG CTA GT 3' antisense: 5' GCT CTT TAG GCT TTC CAG GAA GTC 3'
TNF- α	sense: 5' AGT CCG GGC AGG TCT ACT TT 3' antisense: 5' GCA CCT CAG GGA AGA GTC TG 3'
Arginase 1	sense: 5' CGC CTT TCT AAA AGG ACA 3' antisense: 5' CTG GTT GTC AGG GGA GTT TT 3'
FIZZ 1	sense: 5' CCC TTC TCA TCT GCA TCT CC 3' antisense: 5' CAG TAG CAG TCA TCC CAG CA 3'
iNOS	sense: 5' AGA CCT CAA CAG AGC CCT CA 3' antisense: 5' GCA GCC TCT CTT TGA CC 3'
CCL3	sense: 5' TGC CCT TGC TGT TCT TCT CT 3' antisense: 5' GAT GAA TTG GCG TGG AAT CT 3'
IL-5	sense: 5' ACG CAG GAG GAT CAC ATA CC 3' antisense: 5' GGC TCT CAT TCA CAC TGC AA 3'
Eotaxin	sense: 5' CTC CAC AGC GCT TCT ATT CC 3' antisense: 5' CTT CTT CTT GGG GTC AGC AC 3'

with H₂O. Images were recorded using a Zeiss LSM 510 laser scanning confocal microscope on an inverted stage (Carl Zeiss Canada Ltd., North York, ON).

2.6 Assessment of Colitis

(1) Macroscopic Assessment

Mice were weighed and examined daily for signs of disease, including diarrhea, altered behaviour and ruffled fur. Upon autopsy, the colon was removed, measured and examined for signs of loose stool, bleeding or damage. A clinical disease score was determined using a 5-point scale which accounted for both weight loss and damage to the colon. The parameters used to assess damage are listed in table 2.2. Colitis usually results in shortening of the colon, so the tissue was divided based on percentage of the total length: the distal 30% was discarded, the next 30% was snap-frozen in liquid nitrogen for an assay of myeloperoxidase (MPO), the next 10% was fixed in formalin for histology, and the next 10% was snap frozen in liquid nitrogen for RT-PCR analysis.

(2) Myeloperoxidase (MPO) Assay

The presence of myeloperoxidase (MPO), an enzyme found in the granulocytes, was assayed according to an established protocol (Diaz-Granados et al., 2000). The MPO activity was quantified by a kinetic assay in which H₂O₂ is broken down by the MPO released from tissue samples. The protocol was as follows: tissue samples were weighed before being suspended in 1 ml of potassium phosphate buffer (50 mmol/L) containing hexadecyltrimethylammonium bromide (5 mg/ml; Sigma Chemical Co.). A polytron

Table 2.2 Categories used to assign a clinical disease score; total score out of 5

Damage/Appearance Category	Allocated Score
Weight loss	0-1
Bloody anus; fecal blood	0-1
Soft stool; distal colon empty	0-1
Damage; ulcer	0 or 1
Death	1

tissue homogenizer was used to homogenize the tissue, then 1 ml of the homogenate was placed in a 1.5 ml Eppendorf tube. The homogenate was then centrifuged for 15 minutes at 4°C and 12,000 rpm. 200 µl of an o-dianisidine reaction mixture (Sigma Chemical Co.; 16.7 mg of o-dianisidine, 90 ml of distilled H₂O, 10 ml of potassium-phosphate buffer, and 50 µl of 1% H₂O₂) was added to each well of a 96 well plate. Each well also contained 7 µl of sample. Three absorbance readings at 450 nm were recorded at 30 second intervals on a plate scanner. The data are presented as units per mg of tissue, where 1 unit is equal to the amount of MPO required to degrade 1 µmol/L of H₂O₂ per minute.

(3) Histological Assessment

Segments of the colon were fixed in 10% neutral-buffered formalin, dehydrated in graded alcohols and cleared in xylene before being embedded in paraffin wax. Sections (3 µm thick) were collected on coded slides and stained with haematoxylin and eosin. A damage score was calculated on a 12-point scale and included an assessment of the loss of architecture, muscle thickening, the presence of inflammatory infiltrate, edema, ulceration and goblet cell depletion. Each slide was independently assessed by two investigators who assigned a score based on the scale presented in table 2.3. Both investigators were blind as to the treatment identity of each slide. Additional sections were stained with periodic acid-Schiff's reagent to identify goblet cells (McKay et al., 1990). In the small intestine, goblet cells were counted in five villus crypts; in the colon, goblet cells were counted in five fields of view under the 40x objective.

Table 2.3 Categories used to assign a histology damage score; total score out of 12

Damage/Appearance Category	Allocated Score
Loss of architecture	0-3
Cellular infiltrate	0-3
Goblet cell depletion	0-1
Ulcers	0-1
Edema	0-1
Muscle thickening	0-2
Crypt abscess	0-1

Following transfer of AAMs, collagen deposition was assessed using the Masson's Trichrome stain, where the presence of collagen was indicated by an increase in blue reaction product. Formalin-fixed sections were stained with three consecutive dyes (Weigert's Iron Hematoxylin, Biebrich Scarlet and Aniline Blue).

2.7 Colonic Cytokine mRNA

RNA was extracted from colonic tissue samples using the TRIZOL method (Invitrogen). cDNA was synthesized from 1 µg of total RNA using iSCRIPT reverse transcriptase (BioRad, Mississauga, ON). All primer sequences are listed in table 2.1. After an initial denaturation at 95°C for 3 minutes, there were 32 PCR cycles (95°C for 30 seconds, 55°C for 8 seconds and 72°C for 20 seconds), followed by a final 3 minute extension at 72°C. The products were electrophoresed through a 2% agarose gel containing 0.5 µg/ml ethidium bromide. The banding pattern was then visualised under UV light. The amplified products were compared against the β-actin housekeeping gene and densitometry was performed using the ImageJ software package provided by the National Institutes of Health (Bethesda, USA).

2.8 Cytokine Production

Spleens and mesenteric lymph nodes (MLNs) were removed, placed in RPMI media containing 5% bovine serum and mashed through a 200 µm nylon mesh screen. Pelleted cells were treated with a lysis buffer (0.15 M NH₄CL, 10 mmol/L KHCO₃, and

0.1 mmol/L Na₂EDTA) for two minutes in order to remove erythrocytes. Isolated cells were cultured at a concentration of 5×10^6 cells/ml with 2 µg/ml concanavalin A (conA; Sigma Chemical Co.). After 48 hours, the supernatants were collected and the levels of IL-4, IL-5, IL-10, IL-12, IL-13, and TGF-β determined by ELISA following the manufacturer's guidelines (detection limit 10 pg/ml; R&D systems, Minneapolis, MN.) In order to insure that the data fell within the range of the standard curve, a range of concentrations of each sample was used. Serial dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32 were made using the reagent diluent supplied with each kit. Two samples of each dilution were then pipetted onto the prepared plate.

For samples measuring TGF-β, cells were switched to serum-free media with 4 changes in media over a 12-hour period in order to reduce the background level of TGF-β arising from serum. The samples were then treated with 1 N HCl to activate latent TGF-β.

2.9 Gut Hypersensitivity Response

Following the prophylactic protocol, mice received five *H. diminuta* cysticercoids by oral gavage at the same time as an ip. injection of *Bordetella pertussis* toxin (50 ng/mouse dissolved in PBS; Connaught Laboratories, Mississauga, ON) and ovalbumin (100 µg/mouse dissolved in 10% (w/v) aluminum potassium sulphate solution; Grade V OVA was purchased from SIGMA; Yang et al., 1999). Two weeks later, a single, whole-thickness segment of midjejunum was mounted in an Ussing chamber, and basal ion conduction and short-circuit current were recorded under voltage clamp conditions

(McKay et al., 1996). Following a 20 minute equilibration period, the maximum change in the short circuit current response (ΔI_{sc}) in response to OVA (100 ug/ml added to the serosal side of the tissue) was compared from the OVA-sensitized and *H. diminuta*-infected mice. Controls consisted of OVA only-sensitized mice, *H. diminuta*-infected mice, and naïve mice.

2.10 Induction of Oxazolone Colitis

Mice were infected with *H. diminuta* as above and 8 days later received an intrarectal injection of oxazolone (3 or 4 mg) in 100 μ l of 50% EtOH (Boirivant et al., 1998). Prior to use, the oxazolone was dissolved overnight by rocking. Autopsy was at either 3 or 7 days post-oxazolone or when a predetermined end point was met (loss of \geq 20% of initial body weight and significant signs of distress). To check for worm infectivity, blood smears were stained using the Hema3 stain set (Fisher Scientific, Mississauga, ON) with successful infection defined as \geq 3% eosinophils. Assessment of colitis was as described above.

To assess the impact of IL-5, mice received an ip. injection of an IL-5 adenovirus (10^8 pfu/100 μ l PBS; Dr. Zhou Xing, McMaster University, Hamilton, ON) 24 hours before the induction of oxazolone colitis (3 mg in 100 μ l of 50% EtOH). The IL-5 vector was a recombinant, replication-deficient adenovirus containing the murine IL-5 gene. The control group consisted of mice that received the the vector without the IL-5 gene (Ad-delete). An assessment of the levels of IL-5 in the sera was done using ELISA (R &

D systems) at both 24 hours and 72 hours post-oxazolone. Autopsy was at 72-hours post-oxazolone, at which time sections of the colon were assessed as described above.

2.11 Eosinophil Assessment

The presence of Eosinophil Peroxidase (EPO) was assessed by repeating the MPO assay with the addition of 50 mmol/L 3-amino-1,2,4-triazol (AMT; Sigma Chemical Co.) to inhibit EPO. EPO activity was then calculated by subtracting the MPO + AMT value from the MPO-only value. In order to assess any increases in the number of eosinophils in the tissue, histological sections were stained with Congo red and haematoxylin. Five fields of view were randomly selected from slides that had been assigned a number in order to hide their treatment group. Positive cells were identified based on the appearance of a red granular cytoplasm and a bilobed nucleus, and were counted per section of colon examined under the 40x objective.

2.12 Statistical Analysis

Statistical comparisons for parametric data were performed via one-way ANOVA, followed by post-hoc pair-wise comparisons of the groups using Student's *t* test. Non-parametric data was analyzed using the Mann-Whitney test. The level of acceptable statistical difference was set at $p < 0.05$.

Chapter 3: Results

Preface

The author gratefully acknowledges the following contributions:

- Arthur Wang collaborated on all rat experiments presented in table 3.6
- Dr. Christina Hirota performed the Ussing chamber experiments presented in table 3.7
- Dr. Derek McKay did all eosinophil counts presented in figure 3.16

3.1 Prophylactic *H. diminuta* infection blocks DNBS-induced colitis

The viability of the *H. diminuta* infection was confirmed by an increase in goblet cells in jejunal tissue samples (Table 3.1). By 3 days post-DNBS, all mice had significant symptoms of disease including weight loss, diarrhea and the presence of ulcers in the colon. Mice that received DNBS alone also had significant reductions in the number of colonic goblet cells, which was normalized by previous infection with *H. diminuta* (Table 3.2). Mice previously infected with *H. diminuta* had significant reduction of these symptoms, as evidenced by reduced clinical disease scores (Fig. 3.1 A). Histological assessment revealed a loss of colonic architecture, transmural ulceration, inflammatory cell infiltrate and goblet cell depletion in DNBS-treated mice. Administration of DNBS also resulted in increases in MPO levels. Mice that had been infected with *H. diminuta* had improvements in histological appearance (Fig. 3.1 B and D) and MPO levels that approached the levels of the control animals (Fig. 3.1 C). Mice that received an intrarectal (ir.) injection of 50% EtOH alone (the vehicle for DNBS) did not show significant symptoms of colitis, though there was a small degree of damage that resulted in a small increase in clinical and histological damage scores, as well as a small elevation in MPO levels. The anti-colitic effect did not have a bias for gender, as female mice received equal protection when infected with *H. diminuta* (Table 3.3). A secondary *H. diminuta* infection, administered 28 days after the first infection, was equally effective at protecting against DNBS-induced colitis (Fig. 3.2).

Table 3.1 The presence of increased jejunal goblet cells confirms the viability of *H. diminuta* infection^{a, b}

Treatment	Goblet Cells/Villus Crypt
Control	11 ± 1
DNBS (3 mg ir.)	9 ± 1
<i>H. diminuta</i>	15 ± 1 ^c
<i>H. diminuta</i> + DNBS	17 ± 2 ^c

^a Male BALB/c mice. Values are the mean ± SEM (n=4)

^b Autopsy at 11 days post-*H. diminuta* infection

^c p < 0.05 compared with controls

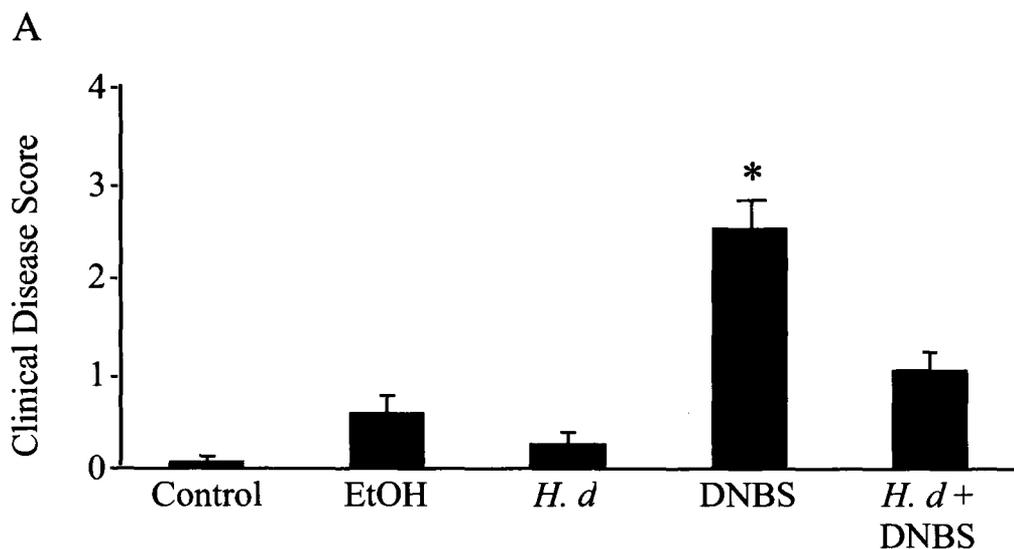
Table 3.2 Mice that received DNBS-only had significant reductions in the number of colonic goblet cells^a

Treatment	Goblet Cells/5 Fields of View
Control	102 ± 3
DNBS (3 mg ir.)	74 ± 6 ^b
<i>H. diminuta</i> + DNBS	105 ± 8

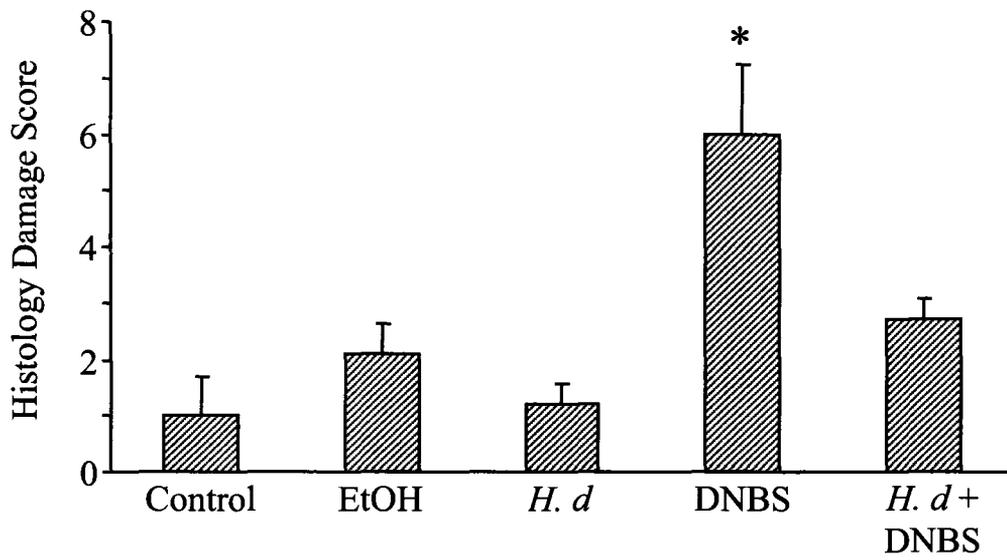
^a Male BALB/c mice. Values are the mean + SEM (n=6)

^b p < 0.05 compared with controls

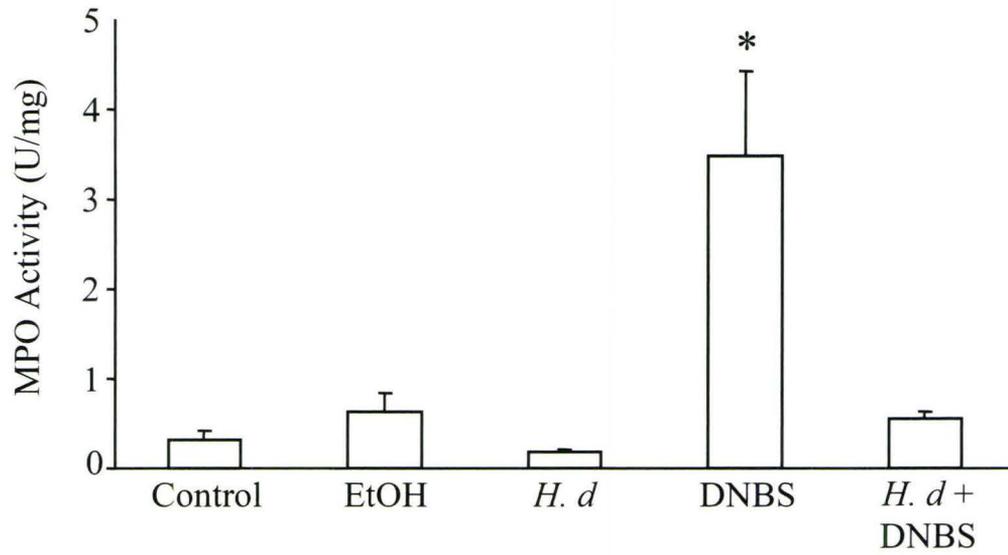
Figure 3.1 Infection with *H. diminuta* (H.d.) 8 days before the administration of DNBS (3 mg in 100 ul of 50% EtOH ir.) blocks colitis. Inhibition of colitis by prior *H. diminuta* infection was quantified by clinical disease scores (A), histology damage scores (B) and colonic MPO levels (C). D, Representative photomicrographs show the loss of colonic architecture that occurs with DNBS, while tissues from *H. diminuta*-infected + DNBS-treated mice have a more regular appearance (original magnification x200). Controls consisted of naïve, age-matched mice, mice that received 50% EtOH ir. and *H. diminuta*-only infected mice. Values are the mean \pm SEM (n=18-20 from 4-5 experiments). *, $p < 0.05$ compared to all other groups.



B



C



D

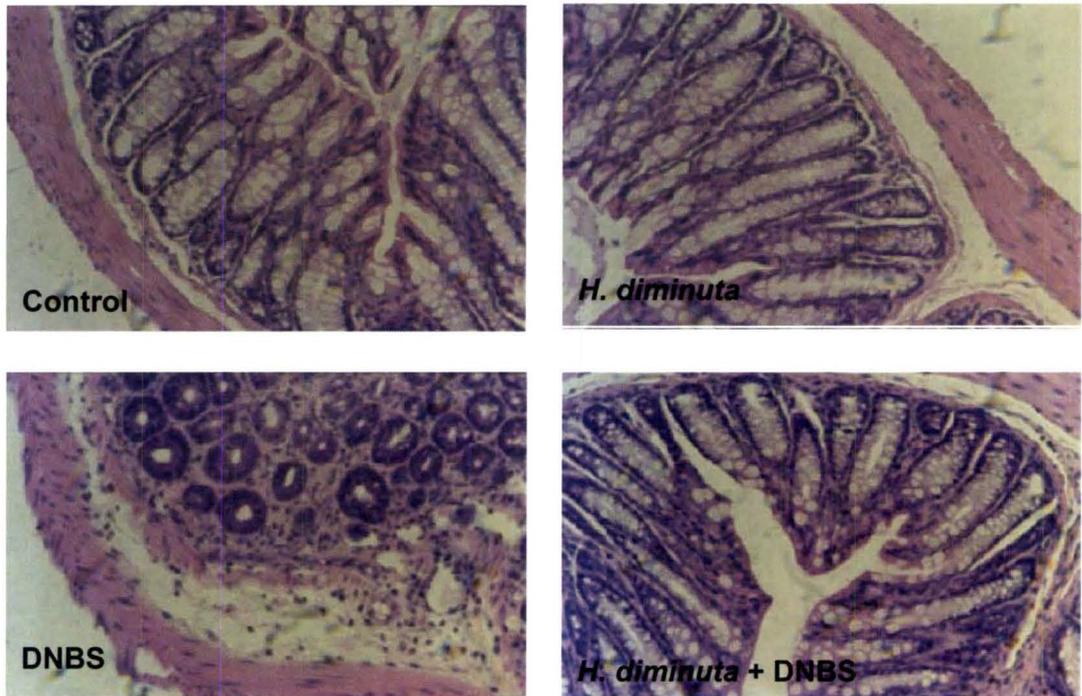
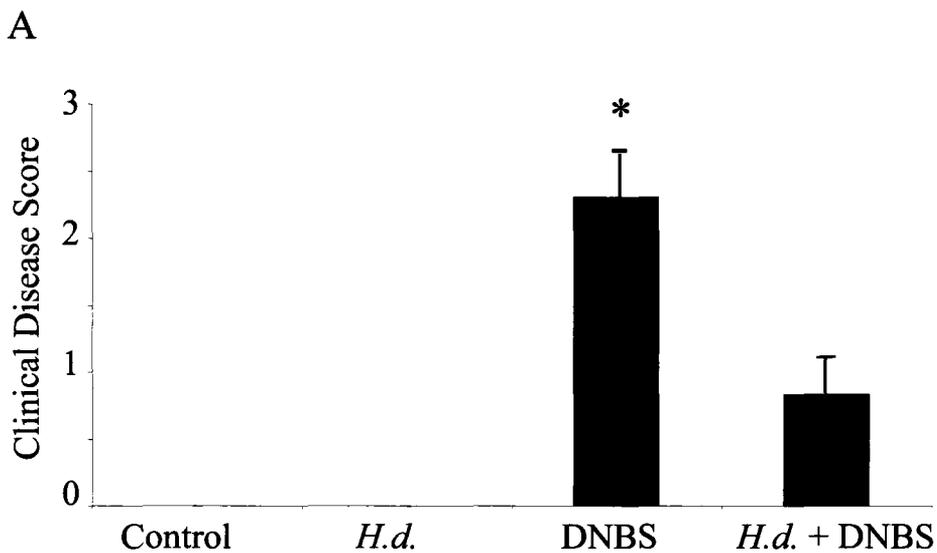


Figure 3.2 Secondary infection with *H. diminuta* (H.d.) 28 days after the primary infection blocks colitis, as quantified by clinical disease scores (A) and colonic MPO levels (B). Controls consisted of naïve, age-matched mice and *H. diminuta*-only infected mice. Values are the mean \pm SEM (n=4). *, $p < 0.05$ compared to all other groups.



B

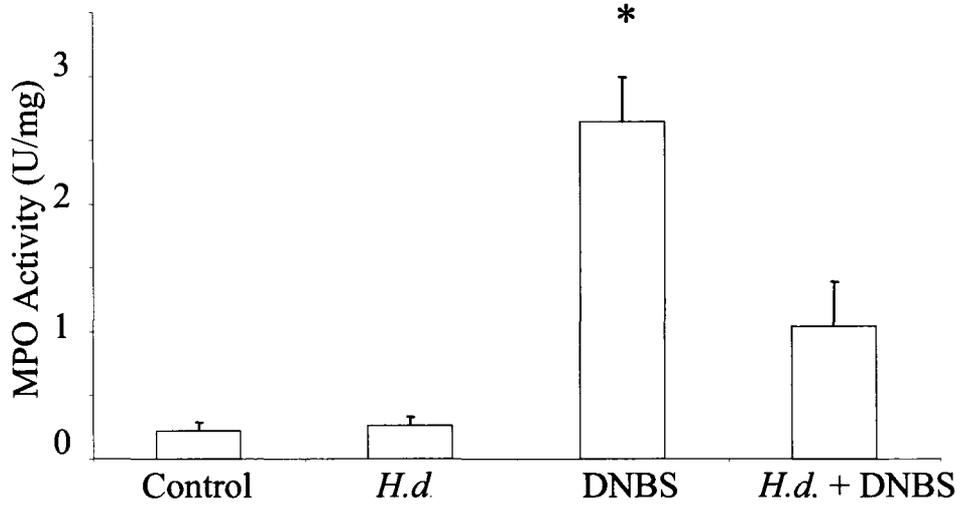


Table 3.3 *H. diminuta* infection was equally effective at treating the symptoms of DNBS colitis in female mice^a

Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score	Histology Damage Score
Control	0.7 ± 0.2	0.2 ± 0.1	110 ± 1	0 ± 0	0.7 ± 0.3
<i>H. diminuta</i>	0.4 ± 0.1	0.4 ± 0.1	109 ± 2	0 ± 0	0.8 ± 0.2
DNBS (3 mg ir.)	-2.3 ± 0.6 ^b	4.1 ± 1.2 ^b	81 ± 2 ^b	3.1 ± 0.8 ^b	6.2 ± 1.8 ^b
<i>H. diminuta</i> + DNBS	-0.3 ± 0.2	0.9 ± 0.3	101 ± 2 ^c	0.9 ± 0.4	2.4 ± 0.6 ^c

^a Female BALB/c mice. Values are the mean ± SEM (n=4)

^b p < 0.05 compared with control

^c p < 0.05 compared with both DNBS and control

The anticolitic effect of *H. diminuta* infection was still seen 7 days after DNBS treatment (Table 3.4). Subsequent experiments that examined the longevity of the anticolitic effect demonstrated that mice were protected for at least 2 weeks after infection, though this response waned by 3 weeks post-infection (Fig. 3.3): at this point, tissues from all mice that received DNBS were identical regardless of whether they had previously been infected with *H. diminuta*.

3.2 The anticolitic effect of *H. diminuta* is dependent on a viable infection and subsequent rejection response

The anticolitic effect of *H. diminuta* was dependent on the larvae being infective, as killed cysticercoids, excysted larvae, and a crude adult worm antigen preparation did not prevent the symptoms associated with DNBS-induced colitis (Table 3.5). Mice that lack the IL-4/IL-13 transcription factor STAT-6 are unable to expel *H. diminuta* and maintain mature worms in their small intestine. The STAT-6 knockout mice (C57 background) were not protected from colitis induced by DNBS as shown by elevated clinical disease scores and MO levels (Figure 3.4). While the permissive rat host is able to mount an immune response against *H. diminuta* (Webb, Hoque and Dimas, 2007), it is not sufficient to eradicate the helminth. There were no significant differences in symptoms between rats that received DNBS with or without a previous *H. diminuta* infection, established either 8 days or 3 months previously (Table 3.6; All rat experiments were performed in conjunction with Arthur Wang).

Table 3.4 The anti-colitic effect of *H. diminuta* infection is still evident at 7 days post-DNBS treatment^a

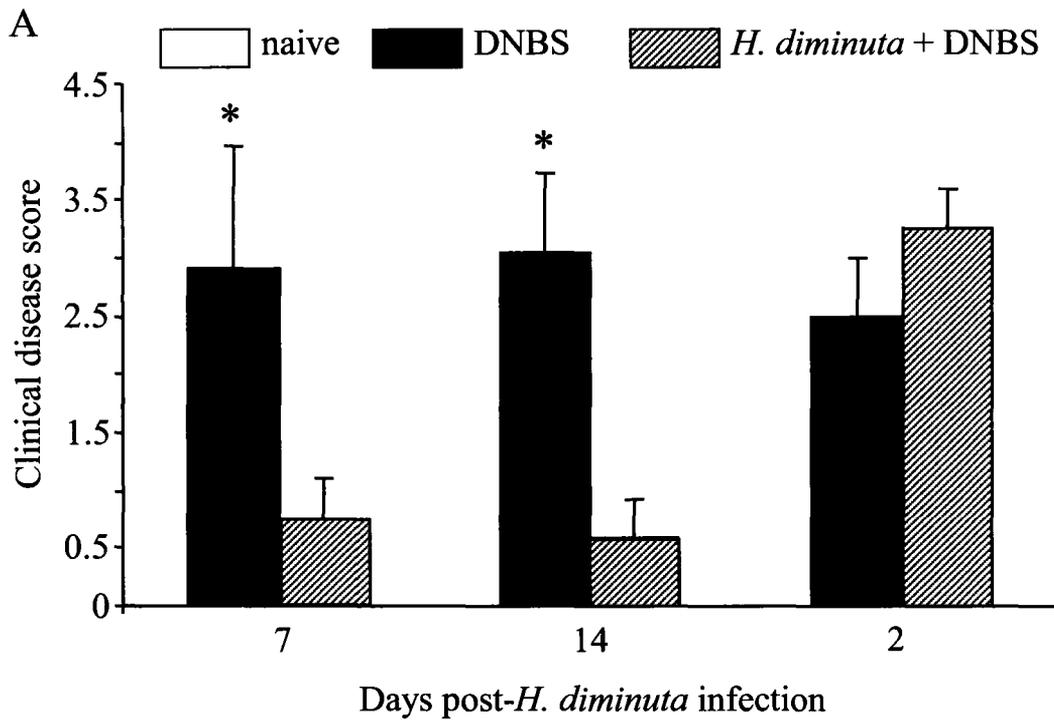
Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score	Histology Damage Score
Control	0.8 ± 0.3	0.3 ± 0.2	109 ± 1	0 ± 0	0.5 ± 0.2
<i>H. diminuta</i>	0.6 ± 0.2	0.2 ± 0.1	108 ± 2	0.2 ± 0.1	0.8 ± 0.3
DNBS (3 mg ir.)	-1.1 ± 0.9 ^b	3.4 ± 1.1 ^b	75 ± 4 ^b	2.3 ± 0.7 ^b	5.5 ± 1.9 ^b
<i>H. diminuta</i> + DNBS	-0.6 ± 0.3	0.9 ± 0.2	99 ± 3 ^c	0.6 ± 0.1	2.8 ± 0.5 ^c

^a Male BALB/c mice. Values are the mean ± SEM (n=4)

^b p < 0.05 compared with control

^c p < 0.05 compared with both DNBS and control

Figure 3.3 The protective effect of *H. diminuta* infection is no longer apparent 3 weeks after infection, as evidenced by clinical disease scores (A) and colonic MPO levels (B). Values are the mean \pm SEM (n=4-9 from three separate experiments). Controls consisted of naïve mice time-matched to the 21 days post-infection mice. A, clinical scores for naïve and *H. diminuta* only-infected mice reflected the finding of no pathology, *, p < 0.05 compared with DNBS only.



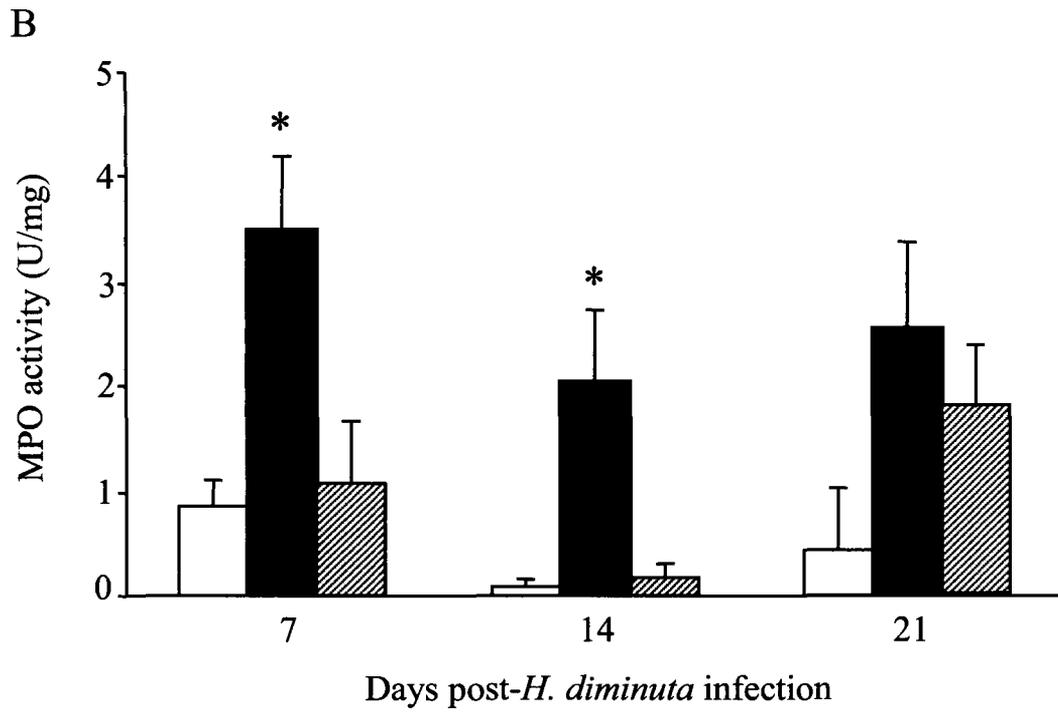


Table 3.5 The anti-colitic effect of *H. diminuta* infection is dependent on a viable infection^a

Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score
Control	+0.7±0.2	0.2±0.1	110±1	0±0
50% EtOH	+0.7±0.4	0.2±0.1	106±2	0.2±0.1
Five viable <i>H. diminuta</i> cysts	+0.6±0.2	0.1±0.1	100±3	0±0
Five killed <i>H. diminuta</i> cysts	+0.1±0.1	0.9±0.1	108±6	0.2±0.1
Adult worm homogenate (100 µg)	+0.8±0.3	0.7±0.1	111±4	0.1±0.1
DNBS (3 mg ir.)	-2.5±0.4 ^b	4.1±1.3 ^b	75±1 ^b	2.3±0.3 ^b
Viable <i>H. diminuta</i> cysts +DNBS	-0.4±0.2	0.6±0.1	102±3	0.5±0.2
Excysted <i>H. diminuta</i> ^c + DNBS	-3.4±1.2 ^b	10.3±4.0 ^b	83±5 ^b	2.4±0.5 ^b
Killed <i>H. diminuta</i> cysts + DNBS	-4.6±1.6 ^b	5.1±2.6 ^b	85±10 ^b	1.3±1.2 ^b
Adult worm homogenate + DNBS	-1.9±1.2 ^b	9.9±6.3 ^b	85±7 ^b	1.9±0.8 ^b

^a Male BALB/c mice. Values are the mean ± SEM (n=7 from 2 experiments)

^b p < 0.05 compared with controls

^c 5 excysted worms were gavaged into the stomach

Figure 3.4 Infection of STAT-6 knockout mice with *H. diminuta* 21 days before the administration of DNBS (3 mg in 100 μ l of 50% EtOH ir.) did not affect the symptoms of colitis, as assessed by clinical disease score and colonic MPO levels. Controls consisted of naïve, age-matched mice and *H. diminuta* only-infected mice. Autopsy was at 72 hours post-DNBS at which time mature worms were found in the small intestine. Values are the mean \pm SEM (n=4-7 mice from 2 experiments). *, p < 0.05 compared with controls.

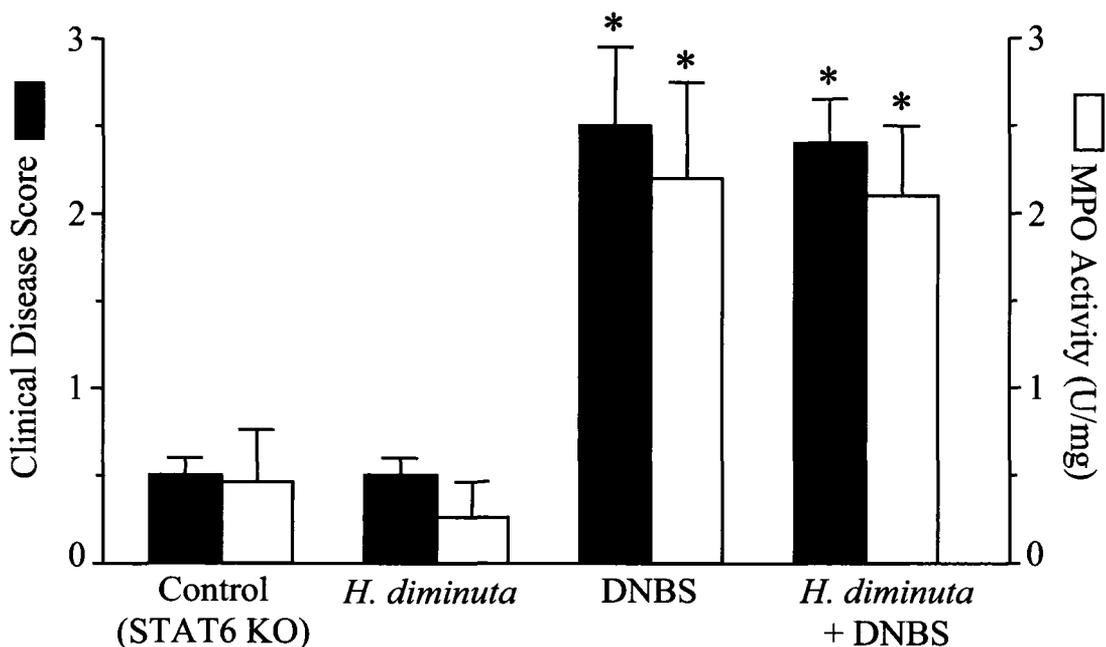


Table 3.6 *H. diminuta* infection in the permissive rat host does not reduce the symptoms of DNBS-induced colitis^{a,c}

Treatment	Weight Change (g)	MPO (U/mg)	Clinical Disease Score	Histology Damage Score
Chronic:				
Control	1.1 ± 8.8	0.6 ± 0.6	0.3 ± 0.3	2.0 ± 1.0
<i>H. diminuta</i>	2.0 ± 10.0	0.9 ± 0.1	0 ± 0	2.8 ± 0.8
DNBS (22 mg ir.)	-19.0 ± 7.9 ^b	5.6 ± 6.9 ^b	3.8 ± 0.4 ^b	6.0 ± 2.7 ^b
<i>H. diminuta</i> + DNBS	-10.4 ± 8.2 ^b	7.6 ± 8.0 ^b	2.4 ± 0.9 ^b	4.1 ± 3.3 ^b
Acute:				
Control	2.4 ± 1.3	0.4 ± 0.2	0 ± 0	ND
<i>H. diminuta</i>	2.3 ± 1.1	1.2 ± 0.2	0 ± 0	ND
DNBS (22 mg ir.)	-9.8 ± 2.0 ^b	2.3 ± 0.2 ^b	2.4 ± 0.2 ^b	ND
<i>H. diminuta</i> + DNBS	-6.2 ± 0.2 ^b	2.2 ± 0.18 ^b	2.0 ± 0.2 ^b	ND

^aMale Sprague-Dawley rats. Values are the mean ± SEM (n=7 from three experiments in the chronic condition and n=3 in the acute study). Rats received 10 *H. diminuta* cysticercoids. DNBS was administered 8 days (acute) or 3 months (chronic) after infection with autopsy at 72 hours post-DNBS.

^bp < 0.05 compared with controls

^cSpecial thanks to Arthur Wang who collaborated on the rat experiments

3.3 *H. diminuta* infection alters the cytokine profile in colitic animals

RT-PCR analysis of colonic tissue taken 72 hours after DNBS treatment showed an increase in TNF- α mRNA (Fig. 3.5 A). Tissues from *H. diminuta* only-infected mice had increased IL-4 and IL-10 mRNA, revealing a Th2 bias in infected mice (Fig. 3.5 B and C). Infection with *H. diminuta* 8 days before DNBS resulted in an increase in colonic levels of IL-4 and IL-10 mRNA compared with DNBS only tissue.

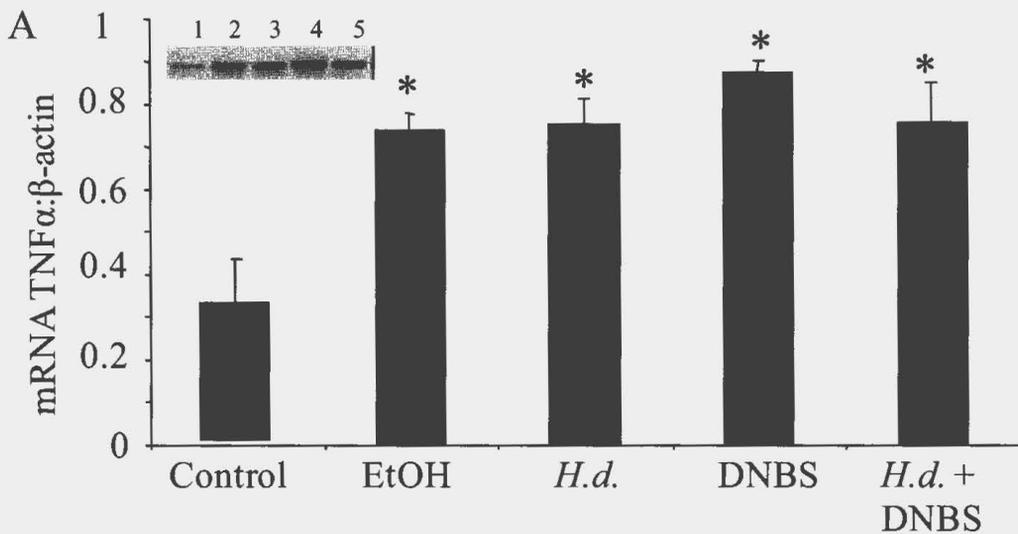
Spleen cells from control, 50% EtOH-treated or DNBS only-treated mice stimulated with a low dose of conA (0.5 μ g/ml) showed only negligible IL-10 production after 24 hours. In contrast, spleen cells from *H. diminuta*-infected mice produced small levels of IL-10 (Fig. 3.6 A) regardless of whether the mice received subsequent administration of DNBS. This effect was increased when the conA was increased to 2 μ g/ml for 48 hours (Fig. 3.6 B). In both experiments, unstimulated spleen cells produced very minimal amounts of IL-10 (< 50 pg/ml).

IL-12 (p40 subunit) production in response to conA was only significantly increased in spleen cells from EtOH or DNBS only-treated mice (Fig. 3.6 C).

3.4 IL-10 neutralization interferes with the anticolitic effect of *H. diminuta* infection

Mice infected with *H. diminuta* were treated with an anti-IL-10 antibody regimen where a total of 200 μ g was given ip. over three separate injections. In time-matched control mice who received an injection of an isotype matched Ig, DNBS elicited the

Figure 3.5 Infection with *H. diminuta* 8 days before DNBS treatment (3 mg/100 μ l 50% EtOH ir.) alters colonic TNF- α (A), IL-4 (B), and IL-10 (C) mRNA levels compared with DNBS only treatments. Cytokine mRNA was compared with β -actin as a housekeeper control gene. Insets: representative RT-PCR gels (1, control; 2, EtOH; 3, *H. diminuta*; 4, DNBS; 5, *H. diminuta* + DNBS). Values are the mean \pm SEM (n = 5 from two experiments). *, p<0.05 compared with all other groups. #, p<0.05 compared with *H. diminuta* only. ND, not detected.



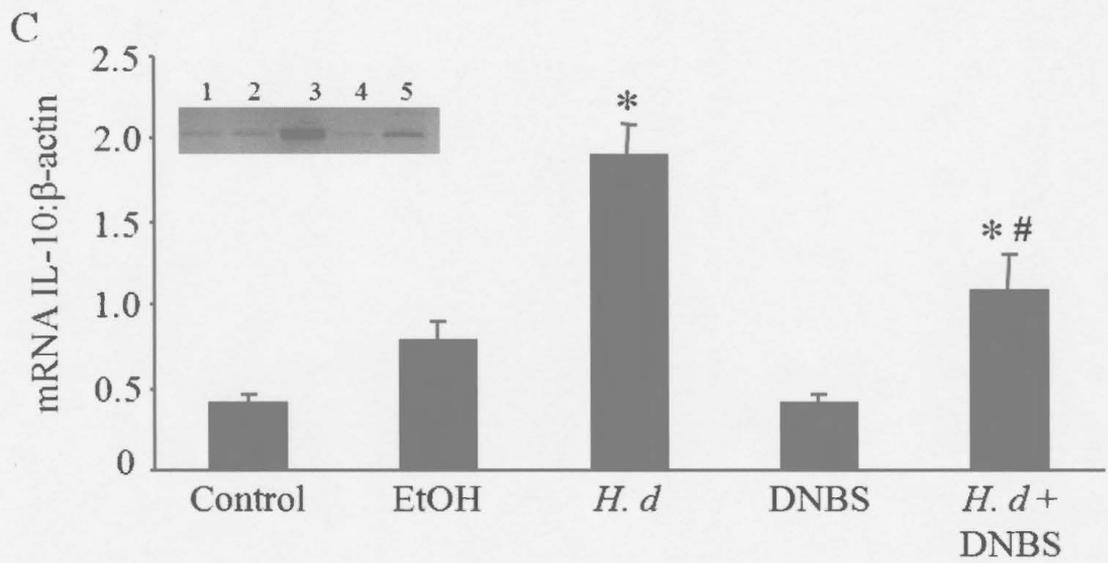
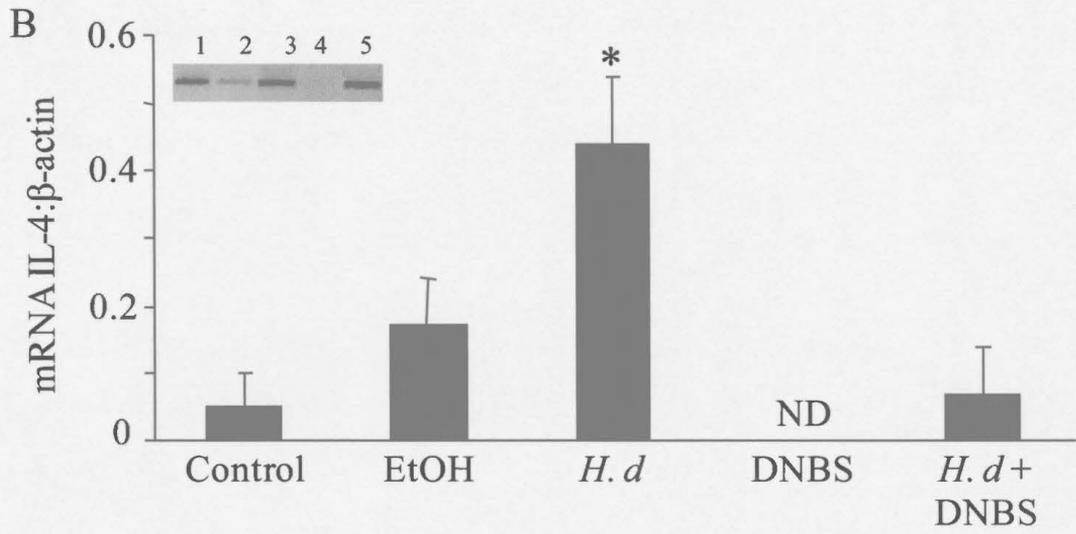
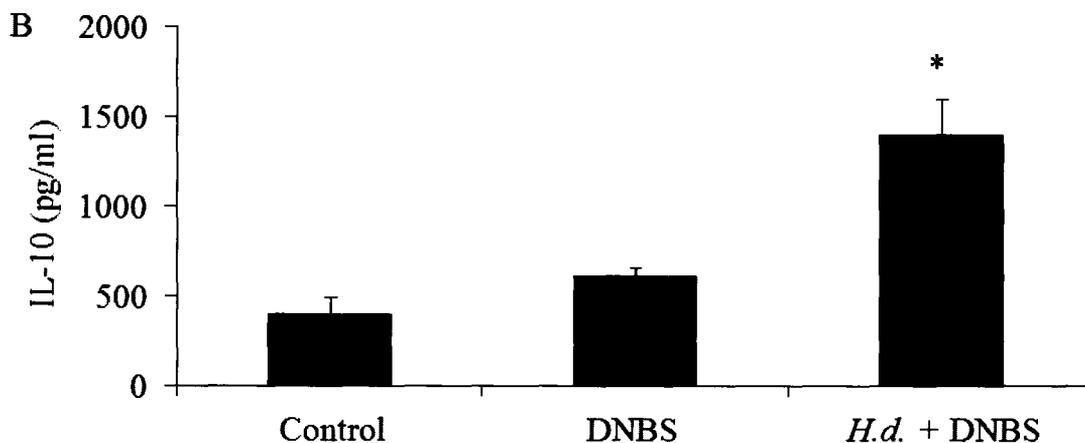
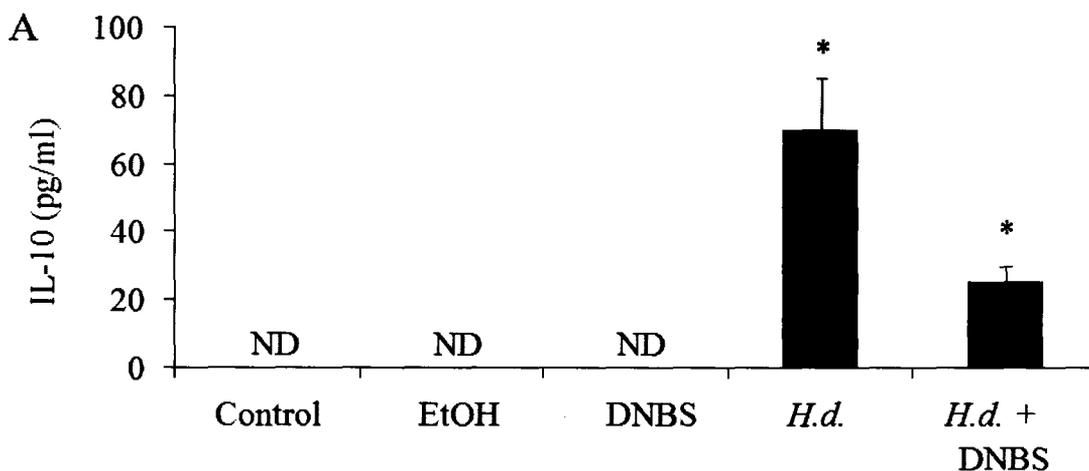
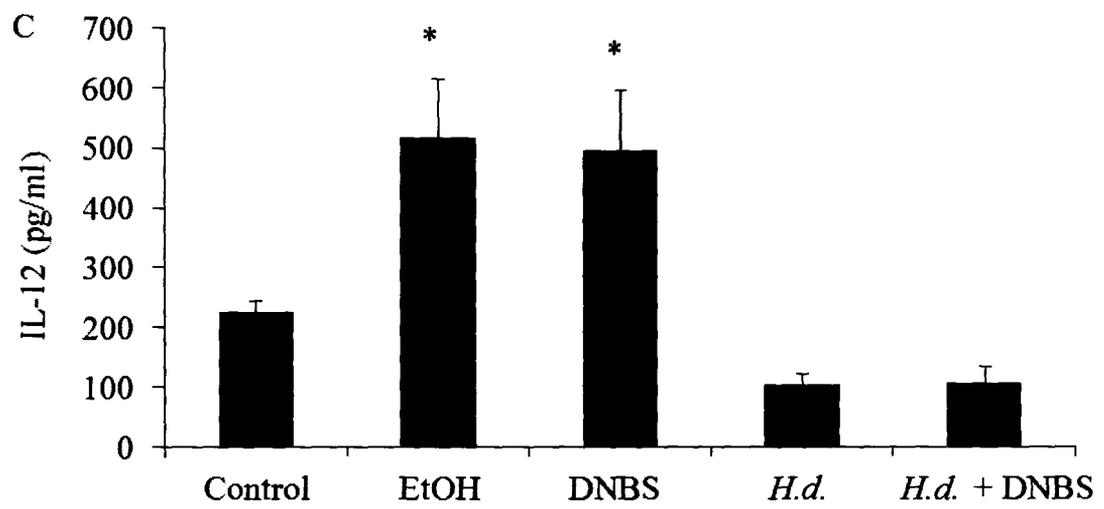


Figure 3.6 Spleen cells challenged *in vitro* with conA (0.5 µg/ml for 24 hours [A and C] or 2 µg/ml for 48 hours [B]) have increased secretion of IL-10 in *H. diminuta* (H.d.)-infected mice. IL-12 (p40 subunit) production was also increased in DNBS-treated mice and reduced in the co-treatment group. DNBS (3 mg) was given *ir.* 8 days after *H. diminuta* infection (five cysticercoids) with autopsy at 72 hours post-DNBS. Values are the mean \pm SEM (n=3-5 mice). *, p < 0.05 compared with controls.





expected colitis. As before, symptoms of disease were suppressed by previous infection with *H. diminuta*. Mice who received DNBS, *H. diminuta* and the anti-IL-10 antibody developed colitis that was similar to that seen in the DNBS-only mice (Fig. 3.7).

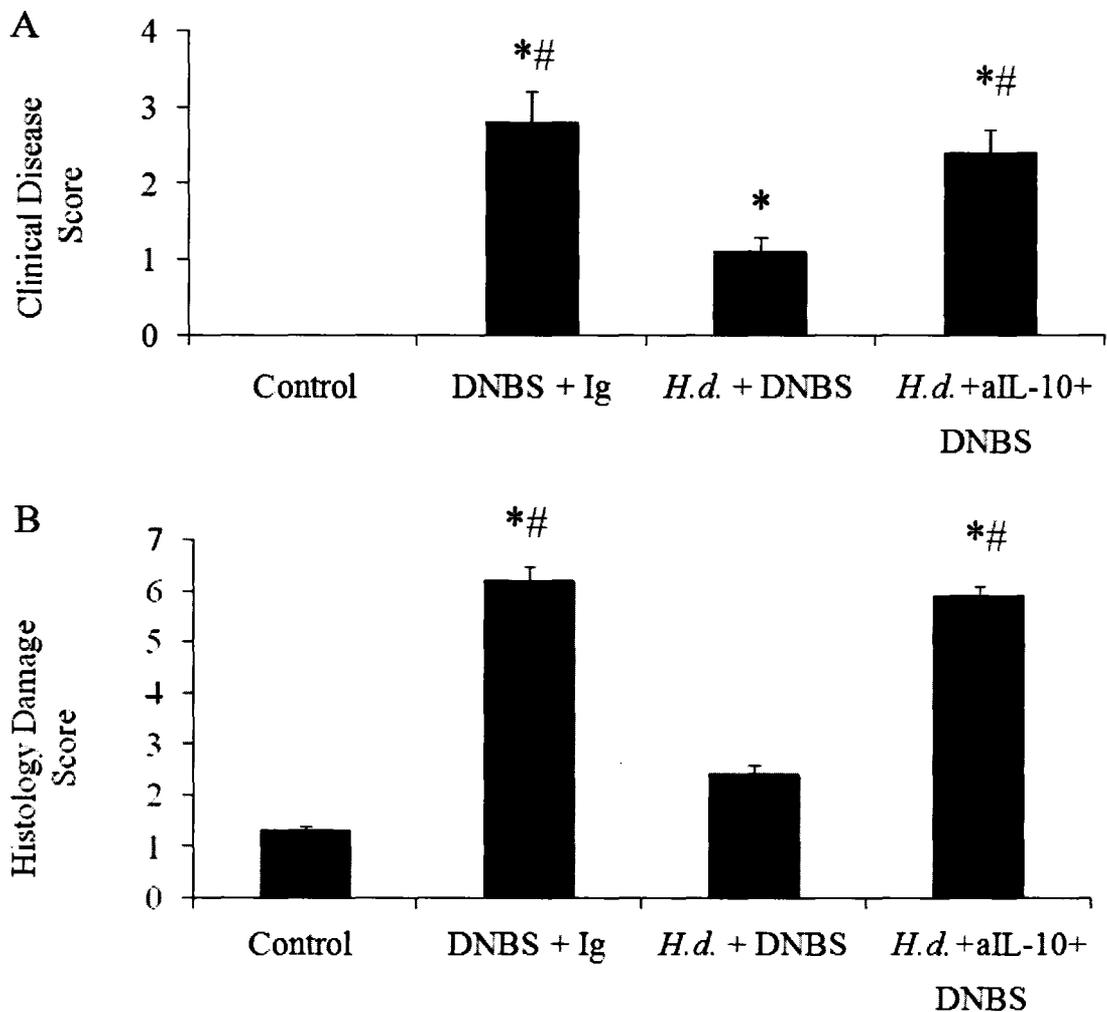
3.5 *H. diminuta* infection does not enhance murine gut hypersensitivity reactions

One of the risks that could potentially be associated with helminth therapy is the possibility of inducing allergic-type diseases. Jejunal segments from mice that were sensitized to OVA and infected with *H. diminuta* displayed no significant enhancement of OVA-induced, short-circuit current responses when challenged in vitro with OVA compared to samples from OVA only-sensitized mice. Tissue samples from naïve and *H. diminuta* only-infected mice were completely unresponsive to challenge with OVA (Table 3.7; Ussing chamber experiments were performed by Dr. Christina Hirota). Thus, *H. diminuta* infection did not enhance enteric hypersensitivity in response to a sensitizing antigen challenge.

3.6 Treatment with *H. diminuta* larvae after DNBS hastens recovery from colitis

Treatment with viable *H. diminuta* cysts 48 hours after the onset of DNBS colitis significantly increased recovery from the disease. Animals that received treatment gained weight at a faster rate than those that received DNBS alone (Fig. 3.8 A) and had reduced clinical disease scores and colonic MPO levels 9 days post-DNBS (Fig. 3.8 B).

Figure 3.7 Anti-IL-10 antibody (200 μ g/mouse ip.) blocked the anti-colitic benefits of *H. diminuta* (H.d.) as assessed by clinical disease score (A), histological damage score (B) and MPO activity from colonic tissue samples (C). DNBS (3 mg) was given ir. 8 days after infection with five *H. diminuta* cysticercoids, with autopsy at 72 hours post-DNBS. Values are the mean \pm SEM (n=5 mice). *, p < 0.05 compared with controls; #, p < 0.05 compared with co-treatment group. Ig, isotype matched Ig.



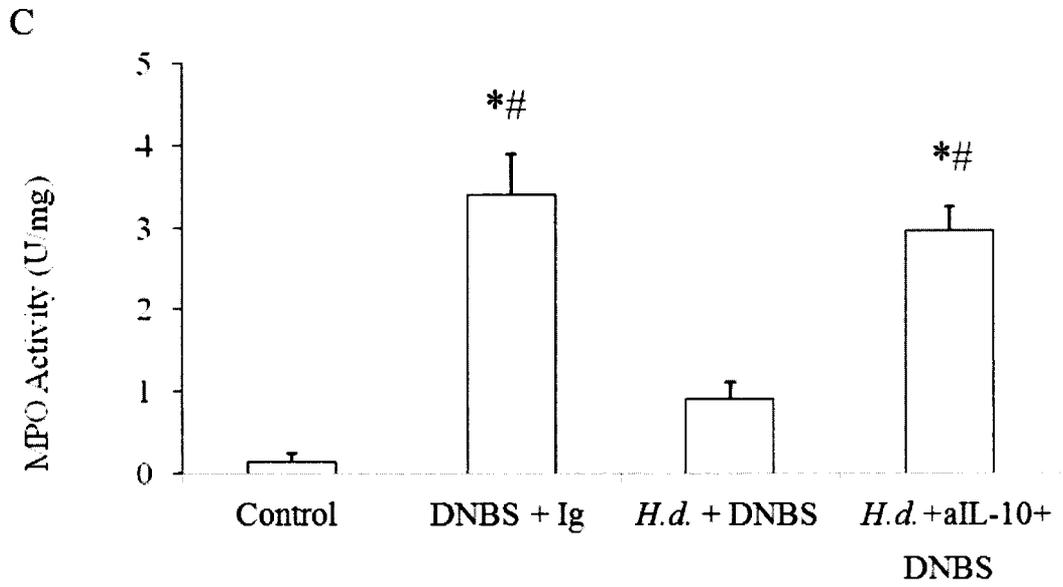


Table 3.7 *H. diminuta* infection does not enhance the response to ovalbumin (OVA) in OVA-sensitized mice^{a,c}

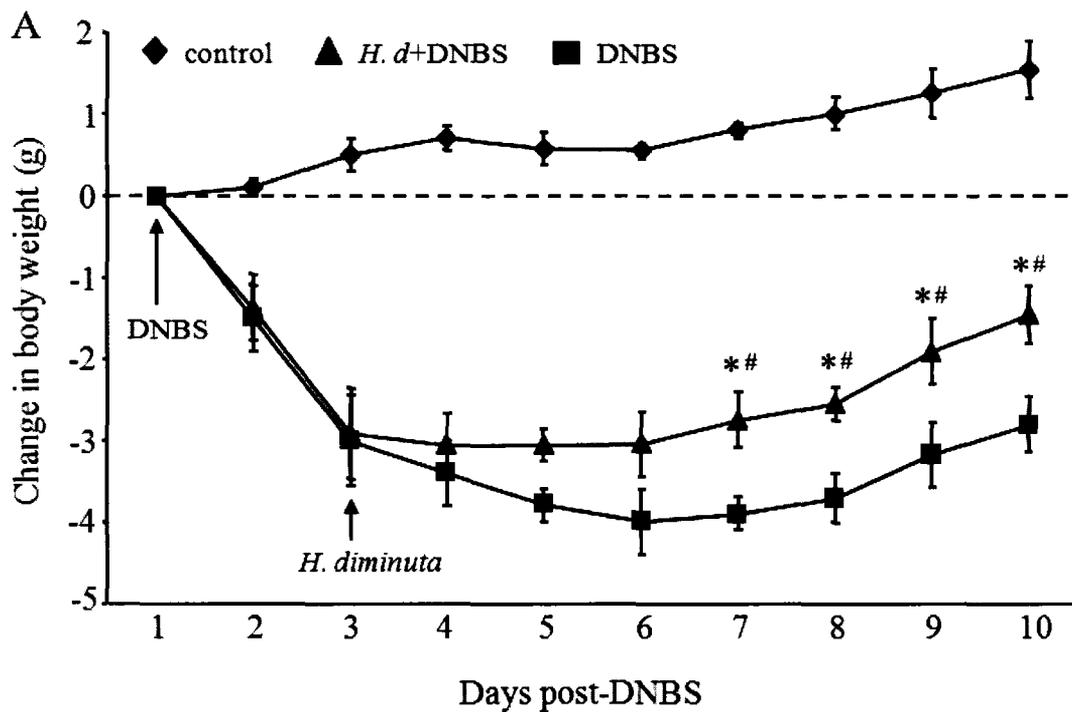
Treatment	ΔI_{sc}^b ($\mu A/cm^2$)
OVA sensitized	23.7 + 8.2
OVA sensitized + <i>H. diminuta</i>	25.4 + 4.3

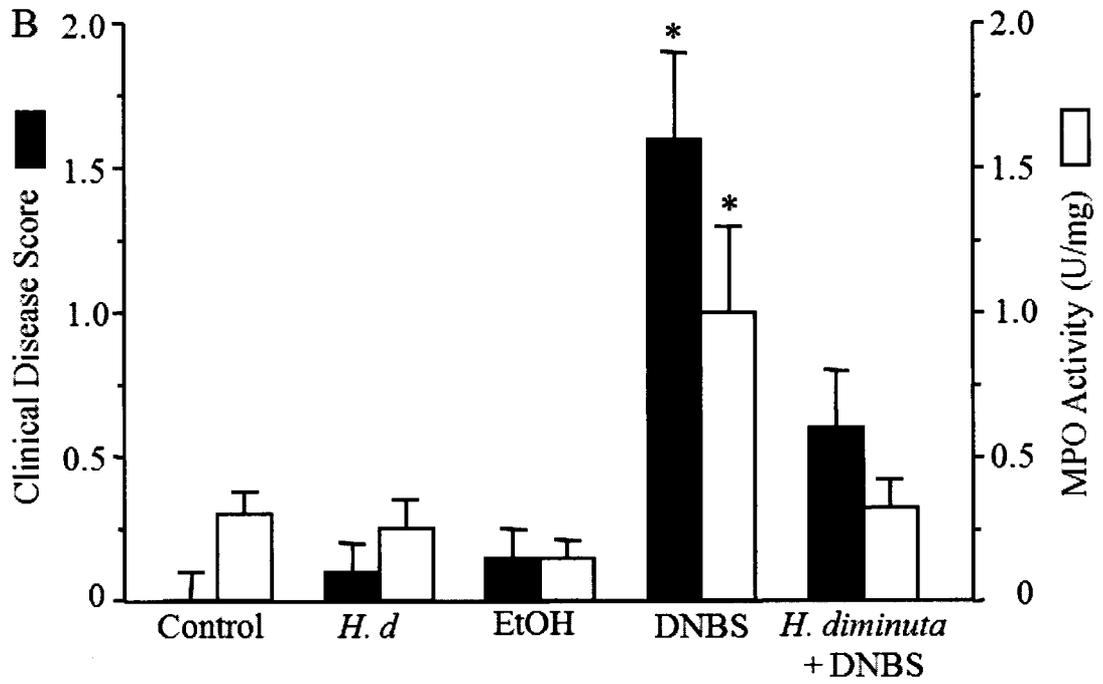
^aMale Balb/c mice. Values are the mean \pm SEM (n=8 mice)

^bChange in short-circuit current in response to challenge with OVA

^cSpecial thanks to Dr. Christina Hirota who performed the Ussing Chamber experiments

Figure 3.8 Male BALB/c mice infected with five *H. diminuta* cysticercoids (H.d.) 48 hours after DNBS (3 mg/100 ul 50% EtOH ir.) display an accelerated recovery of body weight (A) and evidence of less severe disease as evidenced by reduced clinical disease scores and colonic MPO levels 9 days after administration of DNBS. Values are the mean \pm SEM (n=8 from two experiments). *, p < 0.05 compared with control; #, p < 0.05 compared with DNBS only-treated mice.





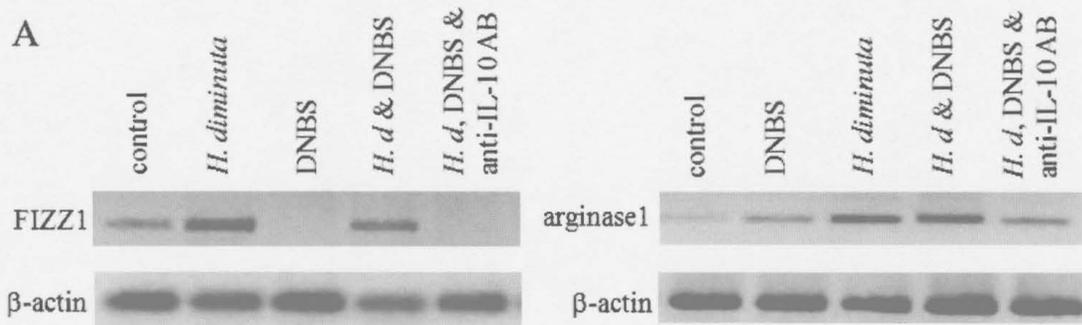
3.7 *H. diminuta* infection increases markers indicative of AAMs in the colon

Mice infected with *H. diminuta* showed increased expression of FIZZ1 and arg-1 mRNA in their colon. There was some variability in the expression of both markers in samples from DNBS-treated mice, as 3/5 mice examined had marked reductions in colonic expression of these markers while the other 2/5 had expression within the control range (Fig.3.9 A). Colon samples from *H. diminuta*+DNBS treated mice had significant increases in FIZZ-1 and arg-1 mRNA. This was reduced when a neutralizing anti-IL-10 antibody was administered following the same dosage regimen as used in section 3.4 (Fig. 3.9).

3.8 Adoptive transfer of alternatively activated macrophage reduces the symptoms of DNBS colitis

Macrophages were obtained from the peritoneal cavity of naïve Balb/c mice, differentiated into CAMs and AAMs *in vitro* (characterised by the expression of arg-1 or inducible nitric oxide (iNOS) (Fig. 3.10 A)) and injected (iv.) into mice 48h prior to intrarectal delivery of DNBS. Before injection, longevity of the cells was confirmed using trypan blue staining: 84% of the cells were still viable four weeks after alternative activation (n=6, SEM=0.8%). As expected, DNBS treatment resulted in inflammation in the colon. Co-treatment with CAMs had no effect on the severity of the colitis. Mice in the AAMs+DNBS group showed significant reductions in disease severity as compared to the DNBS-only or DNBS+CAM-treated mice (Fig.3.11 A-D). This was reflected in reduced clinical disease and histology damage scores, as well as MPO levels that

Figure 3.9 Infection with *H. diminuta* increases alternatively activated macrophage markers in the colon, as quantified using RT-PCR (A, Representative gels; B, densitometry analysis as compared to the β -actin housekeeping gene. Co-treatment with a neutralizing anti-IL-10 antibody (AB) inhibits the anti-colitic effect of *H. diminuta* and decreases the expression of FIZZ1 and arginase-1 (arg-1) mRNA in the colon. Values are the mean \pm SEM (n=5). *, p < 0.05 compared with control.



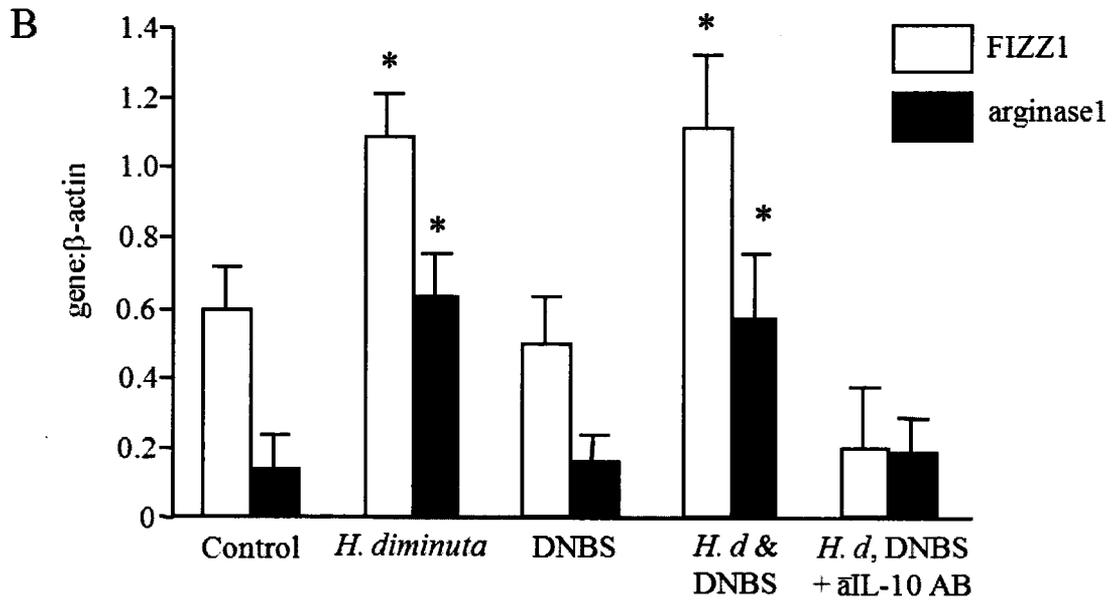


Figure 3.10 Representative gels show increased expression of arg-1 mRNA in IL-4/IL-13 treated cells (AAMs) and increased expression of iNOS in LPS treated cells (CAMs) (A, representative of n=5 samples; MO, non-differentiated macrophages). Five days after iv. administration of AAMs, increased arg-1 mRNA is seen in both colon and spleen tissue samples (B, representative of n=4 mice).

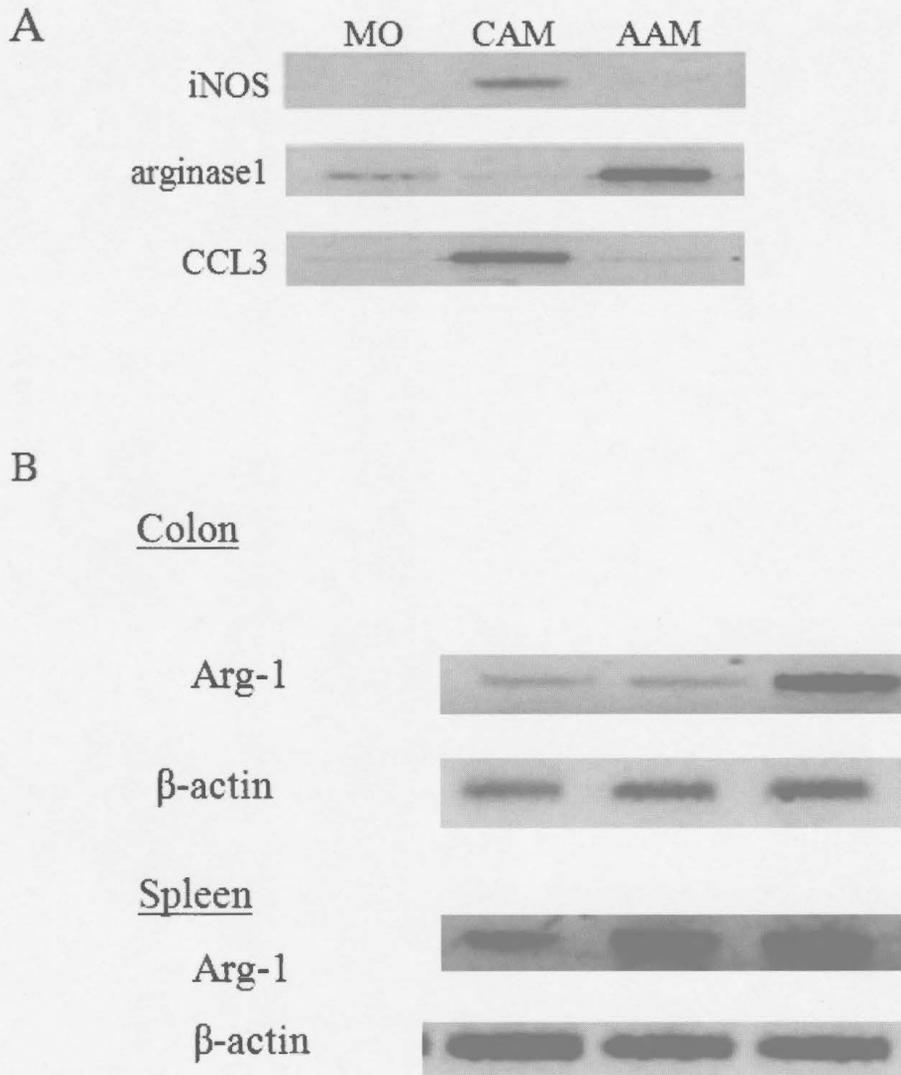
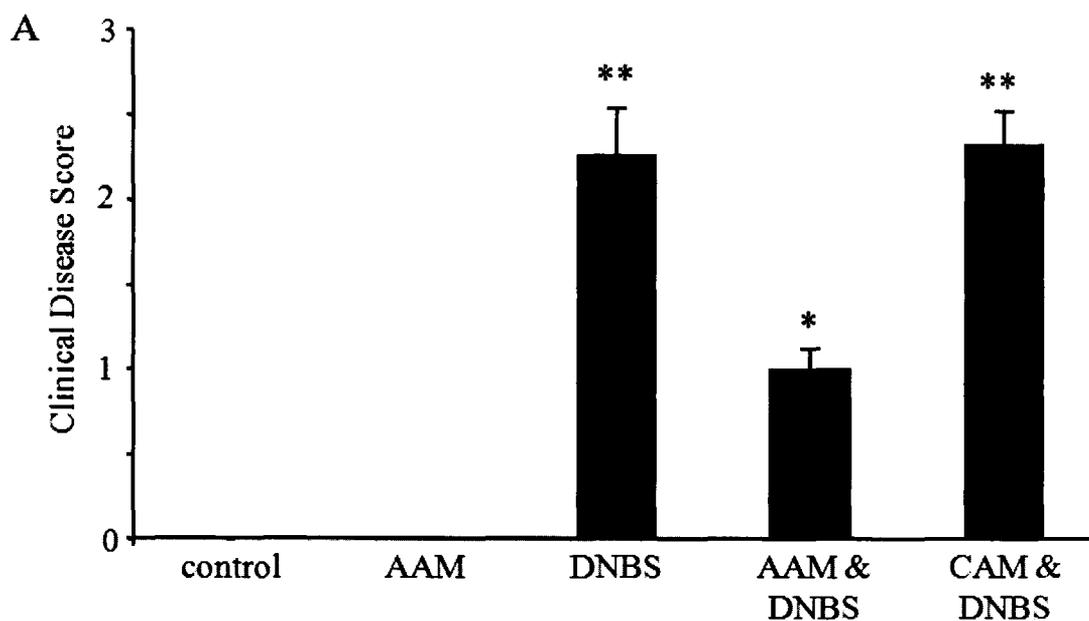
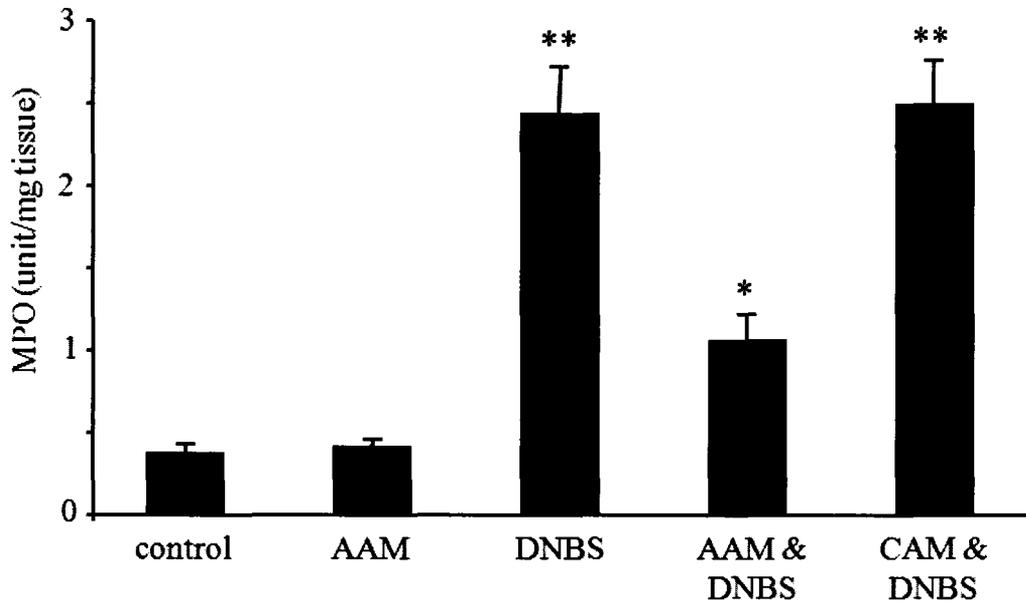


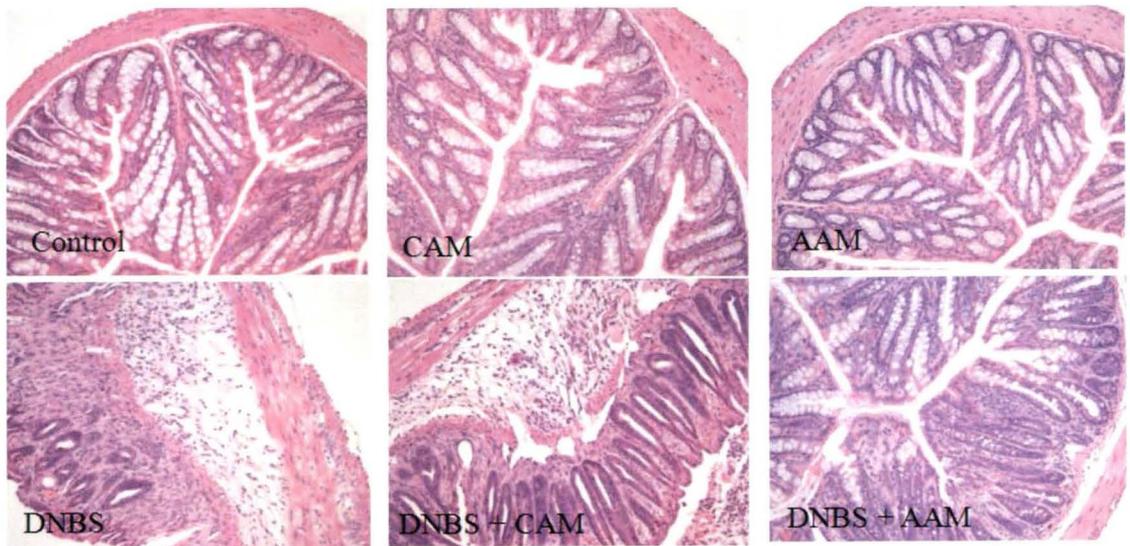
Figure 3.11 Injection of AAMs (iv., 10^6 /mouse) 48 hours after DNBS (3 mg ir.) resulted in significantly reduced symptoms of colitis, including improved clinical disease scores (A), reduced MPO levels (B) and improved histology damage scores (C). Histology sections revealed reduced tissue damage in the co-treatment group (D, magnification x100). Values are the mean \pm SEM (n=21 mice from 5 experiments except for CAM group, where n=8 mice from 2 experiments). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with all other groups



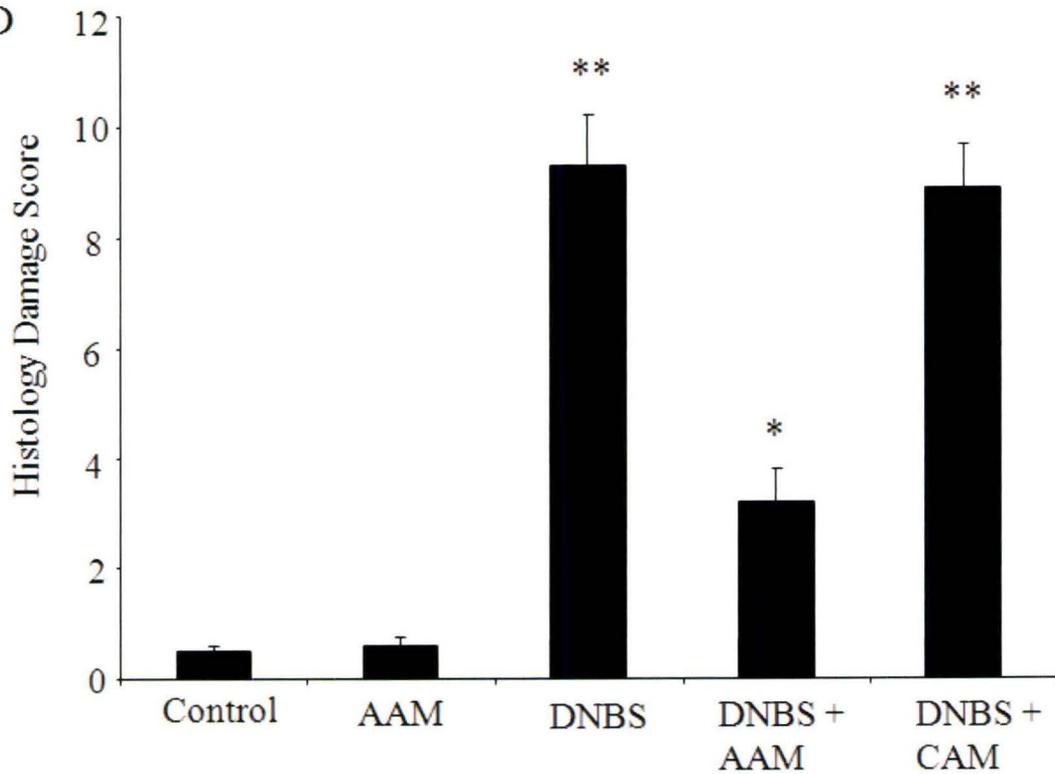
B



C



D



approached those of the control group. The appearance of the colon was improved and showed normalized architecture, less infiltration of inflammatory cells and normalized goblet cell numbers, as compared to DNBS-treated mice (Fig. 3.10 C). After transfer, there was an increased expression of arg-1 mRNA in the colon of mice that received both DNBS and AAMs as compared to the groups that received either treatment alone. Samples from the spleen, however, revealed that there was an increase in arg-1 mRNA from all groups that received AAMs, regardless of whether or not they also received DNBS (Fig. 3.10 B).

In an additional study, peritoneal macrophages were labelled *in vivo* with a red fluorescent dye prior to alternative activation and transfer to naïve mice. When visualized under the confocal microscope, it was revealed that the transferred AAMs had migrated to the colon. There was a concentration of red fluorescent cells found in the lamina propria – this most apparent in the crypt region (Fig. 3.12 A).

The anti-colitic effect of AAMs may be due to their ability to promote healing in injured tissue. Sections of colon that were stained for increased collagen deposition, where collagen absorbed a blue colour, revealed that mice that received both AAMs and DNBS had greater amounts of collagen in the submucosa, around the muscularis mucosae and extending into the lamina propria (Fig.3.12 B).

3.9 *In vivo* delivery of AAMs alters the cytokine profile

Spleen cells from control and DNBS only-treated mice stimulated *in vivo* with conA (2 µg/ml) for 48 hours showed low production of IL-10. This was significantly increased in spleen cells from mice given AAMs ± DNBS (Fig. 3.13 A) The levels of

Figure 3.12 Panel A shows representative images of AAM localization in the colon following iv. transfer (n=4 mice). Panel B shows representative images of collagen staining (Masson's trichrome stain, where a blue reaction product indicates the presence of collagen) in colon sections (n=4 mice; M, muscle; L, gut lumen; C, crypt; magnification x200).

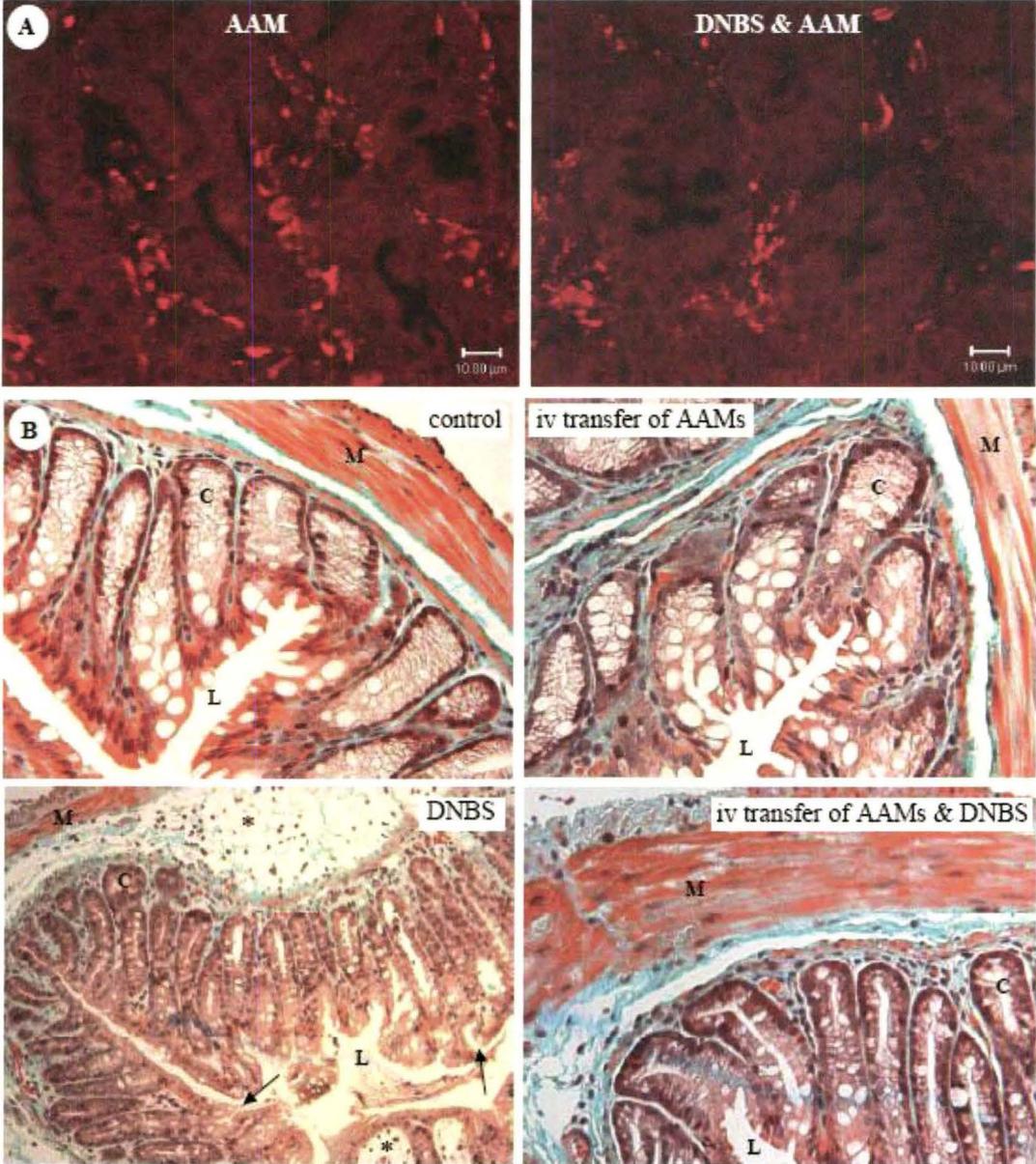
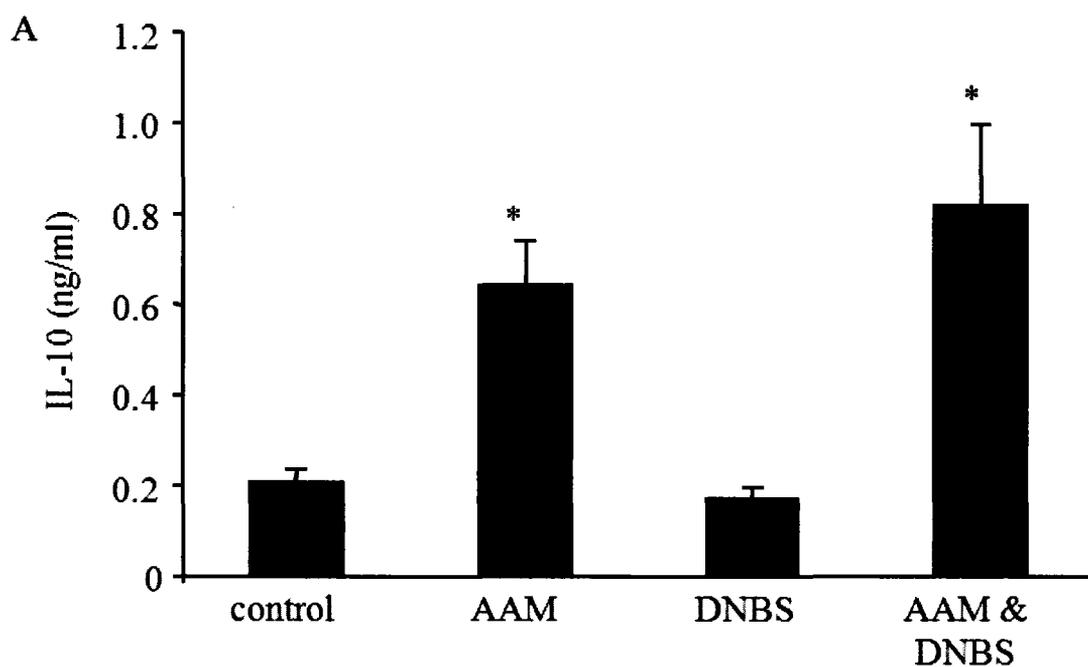
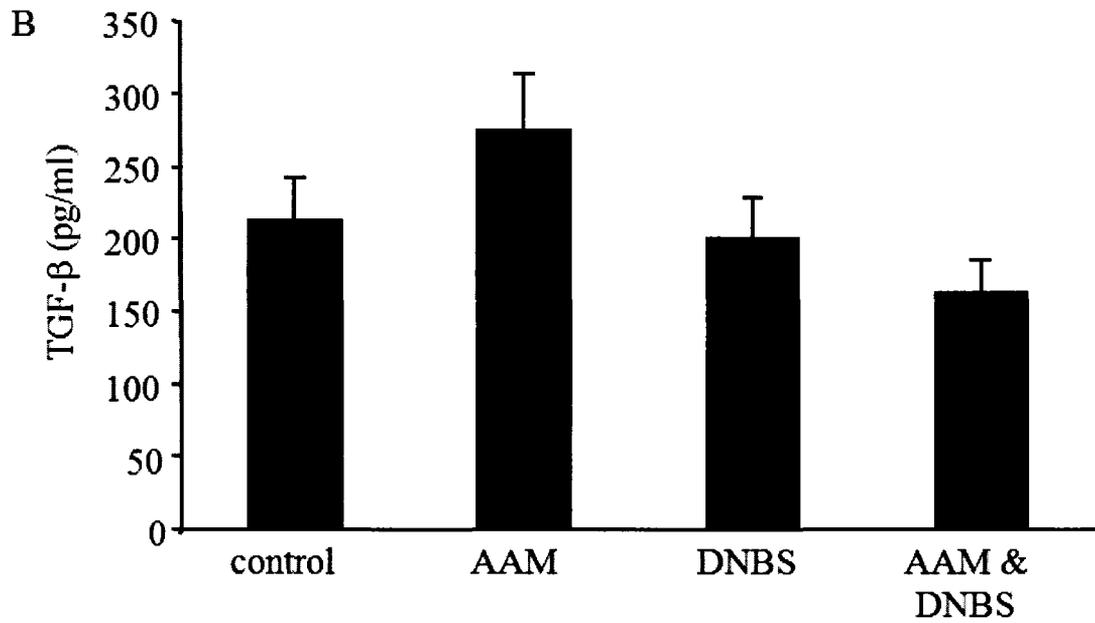


Figure 3.13 Spleen cells were isolated and incubated *in vitro* with conA (2 μ g/ml for 48 hours). Samples from AAM-treated mice showed enhanced production of IL-10 (A). There were no significant differences in TGF- β production between the groups (B). Values are the mean \pm SEM (n=8 mice from 2 experiments). *, p < 0.05 compared with control and DNBS groups.





total TGF β produced from stimulated spleen cells were not significantly different between the groups (Fig. 3.13 B).

3.10 *H. diminuta* infection increases the severity of oxazolone-induced damage to the colon

There is a concern that helminth therapy, and the accompanying increase in Th2 cytokines, could be harmful for patients whose colitis has a Th2 profile or individuals with atopic disorders. To assess the effect of helminth therapy in a Th2-model of colitis oxazolone was used as the chemical agent. Initial pilot studies found that *H. diminuta* infection before the onset of oxazolone colitis induced at a dose of 4 mg ir. resulted in 5 out of 8 mice reaching the experimental end point and being humanely sacrificed before the end of the 72 hours of the experiment. The remaining 3 out of 8 mice had disease that was significantly more severe than that seen in the oxazolone only group (Table 3.8). Due to the morbidity at this dose, subsequent experiments reduced the oxazolone to 3 mg. At this dose, the symptoms seen 72 hours post-oxazolone included weight loss, soft stool and fecal staining around the anus. Administration of oxazolone also induced an increase in MPO from colonic tissue samples.

Animals that received the prophylactic dose of 5 *H. diminuta* larvae showed exacerbated symptoms of colitis, including increased weight loss (Fig. 3.14 A), significantly increased clinical disease scores (Fig. 3.14 B), and rigid, shortened colons (Fig. 3.14 C) that were full of fluid and had visible ulceration. MPO levels also reflected the increased disease in the co-treatment group compared to the group that received oxazolone-only (Fig. 3.14 D). Histological assessment of samples from the colon

Table 3.8 *H. diminuta* infection prior to oxazolone (4 mg ir.) results in significantly more severe disease^a

Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score	Morbidity ^c
Control	+0.3±0.1	0.26±0.2	100±2	0±0	0/8
<i>H. diminuta</i>	+0.4±0.2	0.37±0.15	102±3	0±0	0/8
Oxazolone	-0.2±0.4	1.3±0.35 ^c	91±2 ^c	1.3±0.2 ^c	0/8
<i>H. diminuta</i> + Oxazolone ^b	-1.1±0.4 ^{c,d}	3.2±0.3 ^{c,d}	80±4 ^{c,d}	3.4±0.7 ^{c,d}	5/8

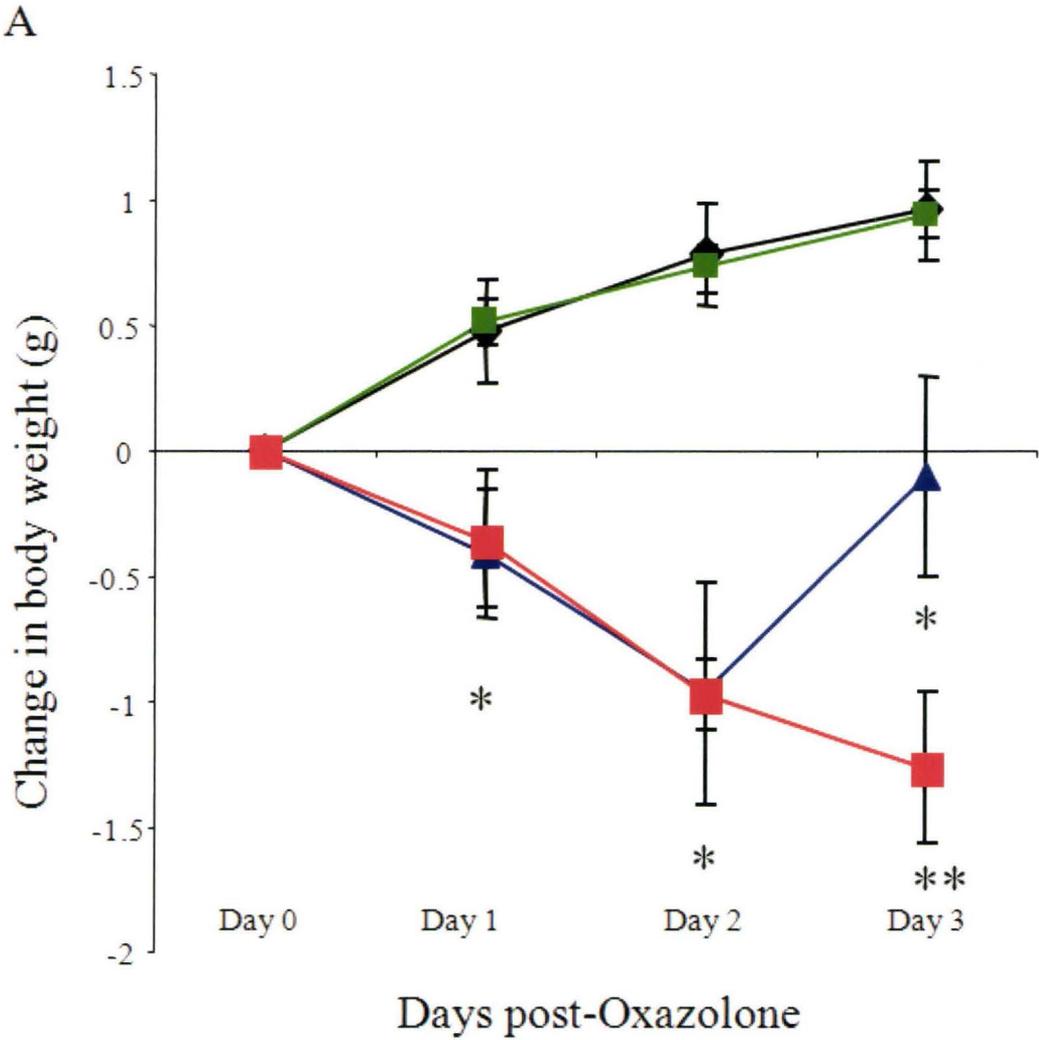
^aMale Balb/c mice. Values are the mean ± SEM (n=8 from 2 experiments)

^bn=3

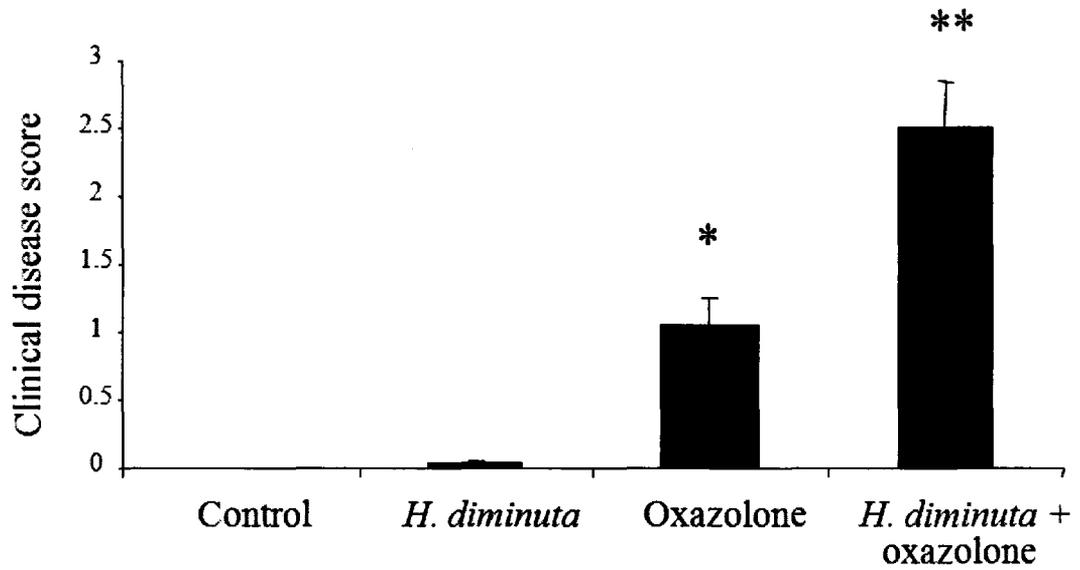
^{c,d}p<0.05 compared with control and oxazolone, respectively

^eMorbidity = number of animals humanely sacrificed because the severity of their disease reached a predetermined experimental end point (>20% weight loss and significant signs of distress)

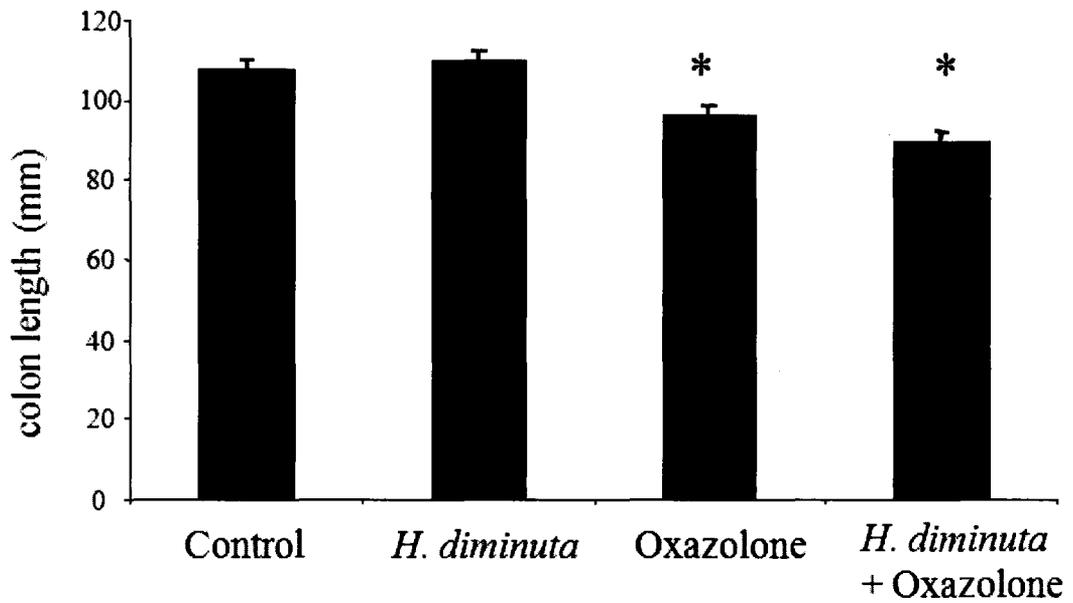
Figure 3.14 The severity of oxazolone-induced colitis is increased by co-treatment with *H. diminuta* as evidenced by increased weight loss (A), increased clinical damage scores (B), increased shortening of the colon (C), and elevated MPO levels (D). Mice were infected with five *H. diminuta* cysticercoids followed by oxazolone 8 days later (3 mg ir.). Autopsy was at 72 hours post-oxazolone. Values are the mean \pm SEM (n=16-20 mice from 5 separate experiments). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with oxazolone-only.



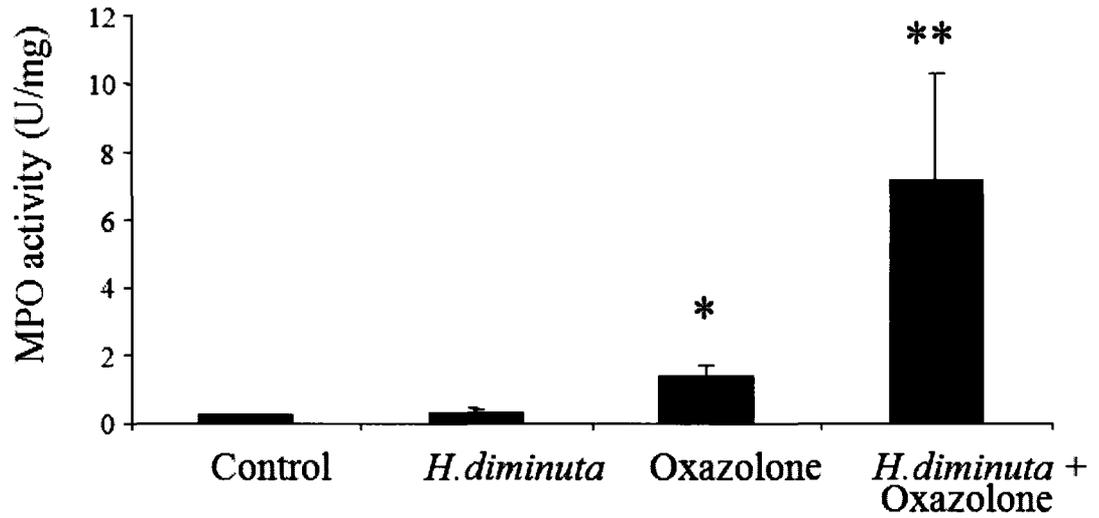
B



C



D



revealed a loss of colonic architecture, transmural ulceration, inflammatory cell infiltrate and goblet cell depletion in oxazolone-treated mice (Fig. 3.15).

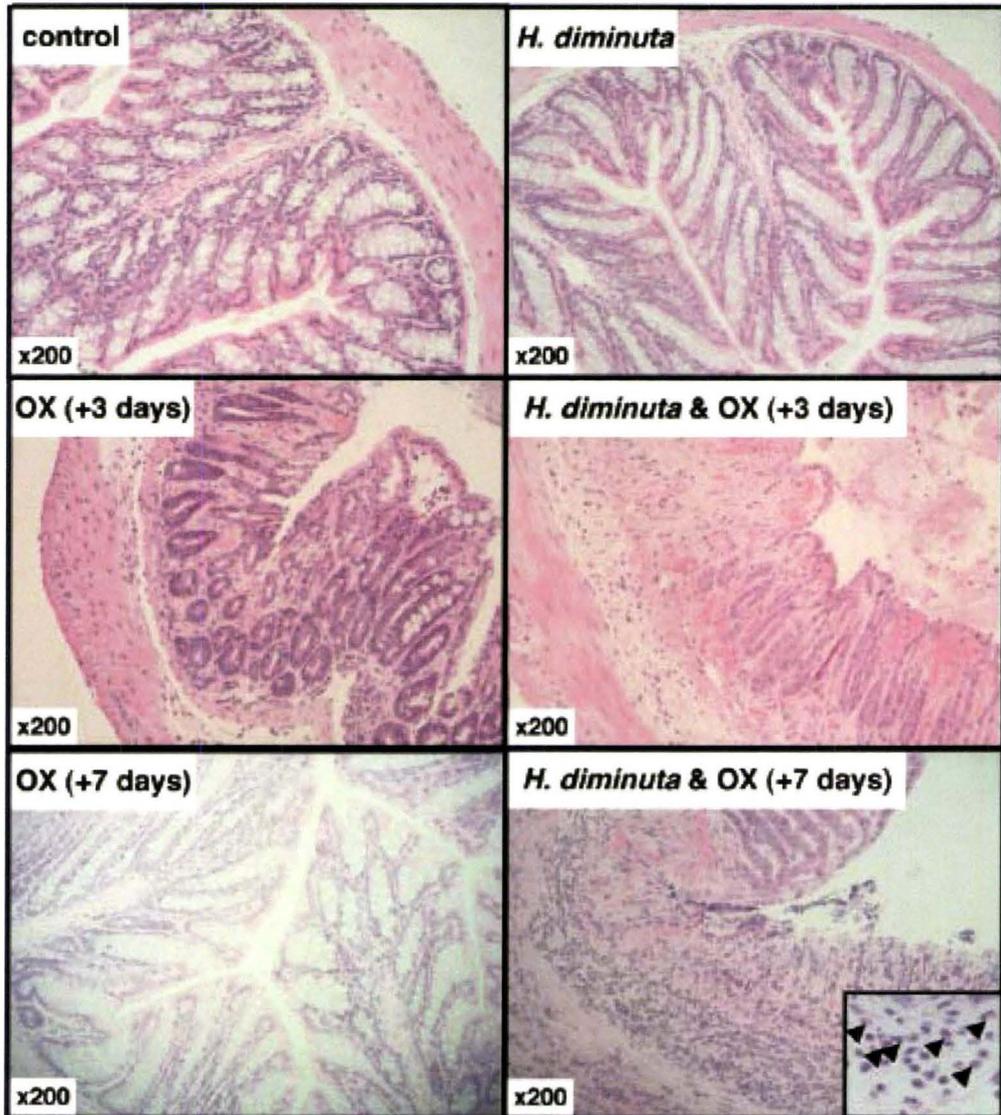
There was a small, but significant, increase in EPO levels from colonic tissue samples of mice that had been infected with *H. diminuta*. This was increased in the group that received oxazolone-only. The greatest EPO levels, however, were seen in the samples taken from the mice that received both oxazolone and *H. diminuta*. Eosinophil counts were also increased in the *H. diminuta* + oxazolone group, though there was more variability in this data (Fig. 3.16; Eosinophil counts were done by Dr. Derek McKay).

3.11 *H. diminuta* infection results in altered cytokine production in oxazolone-treated animals

Splenocytes and MLN cells from all groups had increased production of the Th2 cytokines IL-4, IL-5, IL-13, and IL-10 after stimulation with conA (2 µg/ml) for 48 hours. Samples taken from oxazolone only-treated mice showed increased production of all four cytokines as compared to the control, though the levels were significantly less than those seen with samples from *H. diminuta*-infected mice. In comparison, splenocytes and MLN cells from the co-treatment group showed increased production of IL-4 but decreased secretion of IL-10 in response to conA stimulation. Stimulated cells from groups that received oxazolone also released TGFβ. Significantly less TGFβ was secreted by spleen cells from the oxazolone + *H. diminuta* groups (while there was less TGFβ secreted by stimulated MLN cells, the difference did not reach statistical significance) (Fig. 3.17). RT-PCR analysis of colonic tissue extracts showed that there

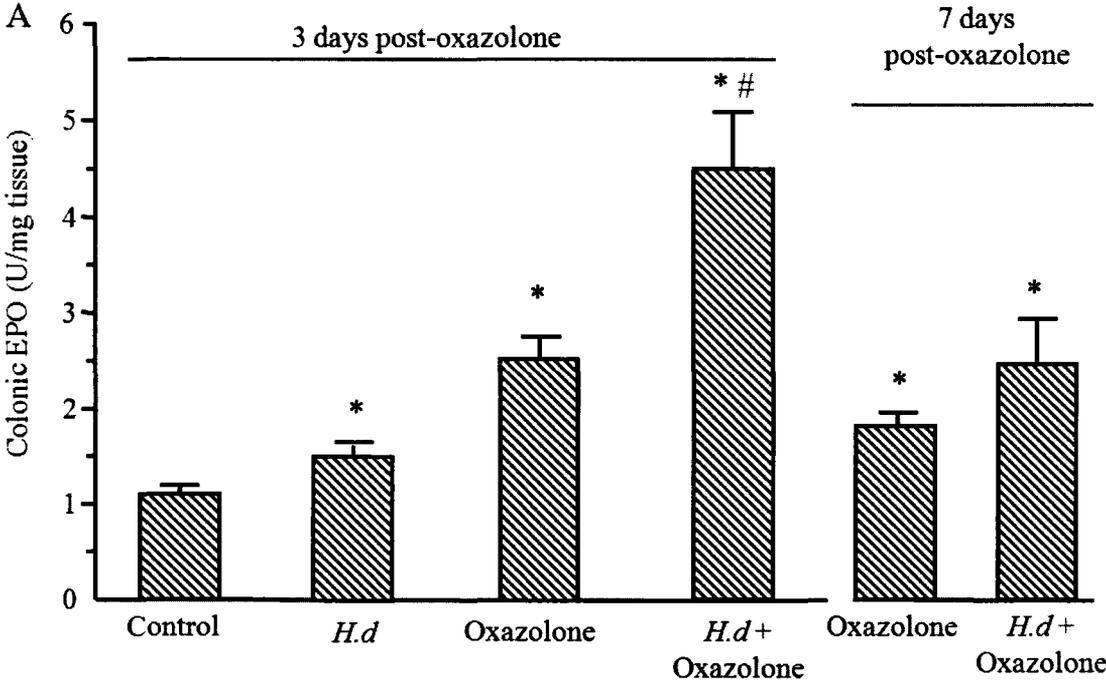
Figure 3.15 Panel A, infection with *H. diminuta* before the onset of oxazolone-induced colitis (OX; 3 mg ir.) increased the damage and inflammation in the colon. Representative photomicrographs show normal colonic architecture in control and *H. diminuta*-infected mice. Oxazolone results in loss of colonic architecture, edema, goblet cell depletion, and cellular infiltrate at 3 days post-oxazolone that has almost resolved by 7 days post-oxazolone. *H. diminuta* + oxazolone-treated mice have more severe inflammation which has not resolved by 7 days post-oxazolone. This inflammation is characterized by the presence of eosinophils (inset, arrows) (magnification x200; representative of n=8 mice/group).

A



(reproduced with permission from Hunter, Wang and McKay, 2007)

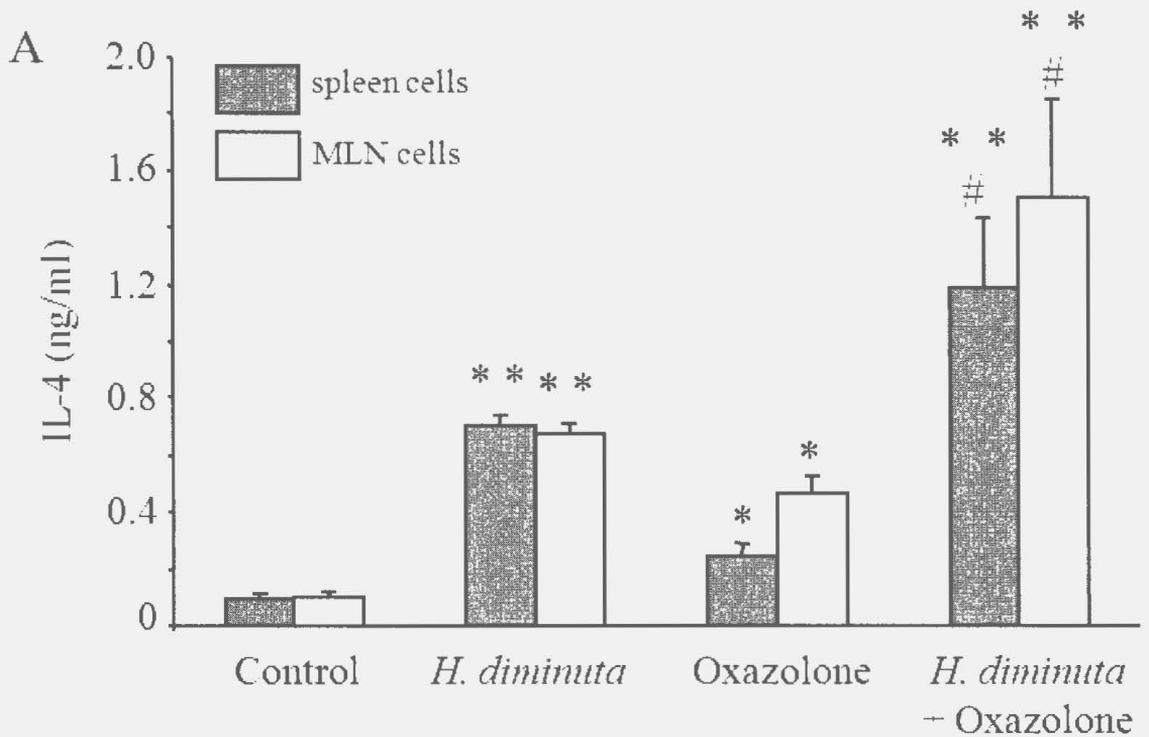
Figure 3.16 Colonic eosinophil peroxidase (EPO) activity was increased in samples from mice treated with either *H. diminuta* (H.d.) or oxazolone (3 mg ir.). This was further enhanced in the co-treatment group examined at 3 days post-oxazolone. The increase in EPO persisted at 7 days post-oxazolone (A). Values are the mean \pm SEM (n=9-13 mice from 3 separate experiments for the 3-day treatment and n=3-4 mice for the 7-day treatment). *, p < 0.05 compared with control; #, p < 0.05 compared with oxazolone-only. Eosinophils/section of examined tissue also revealed a significant increase in the co-treatment group that persisted at 7 days post-oxazolone (B). Values are the mean \pm SEM (n=4 mice for the 3-day experiment; for the 7-day experiment, co-treatment samples are from mice humanely sacrificed on days 5-7 post-oxazolone). Special thanks to Dr. Derek McKay for the eosinophil counts.

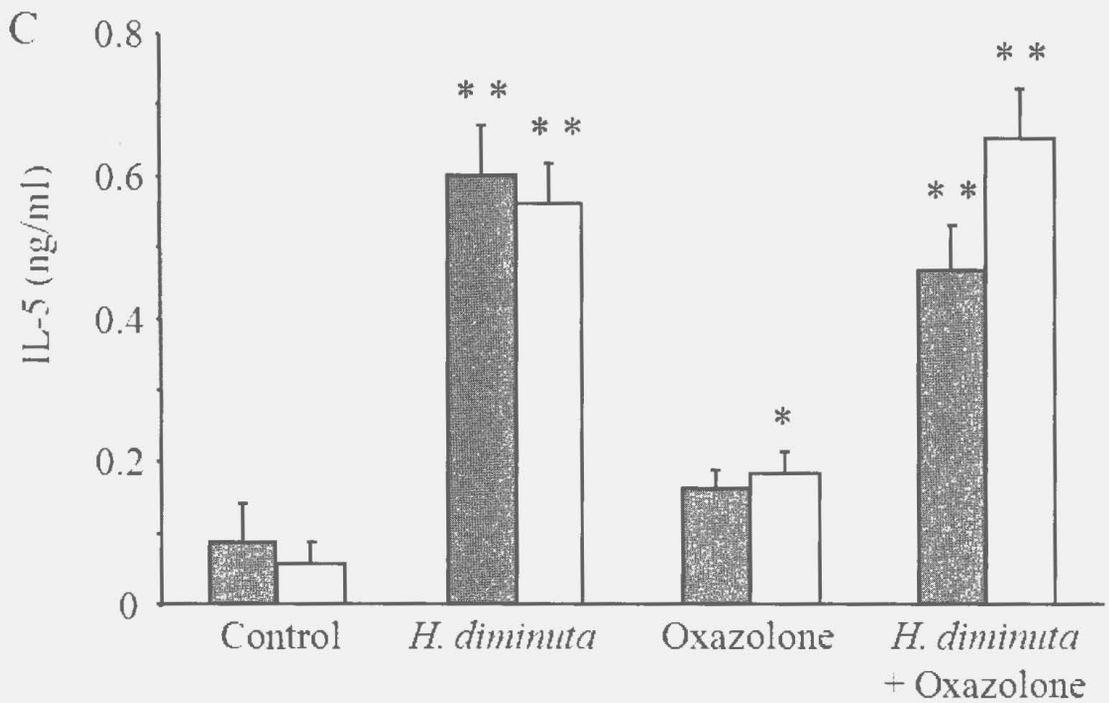
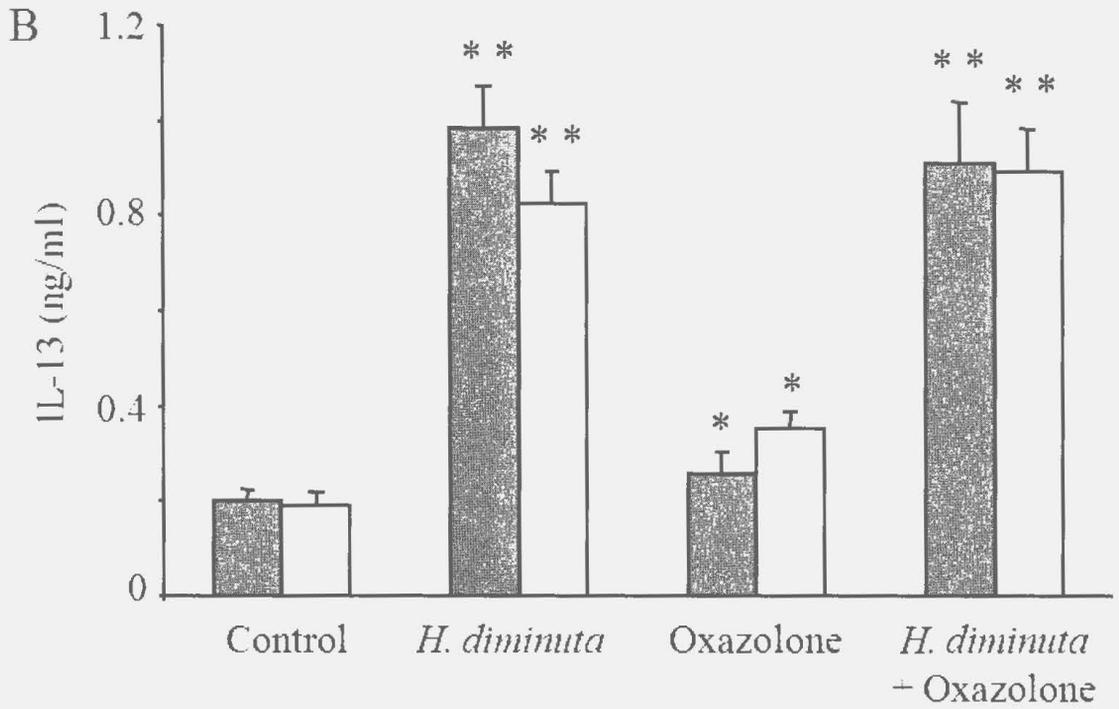


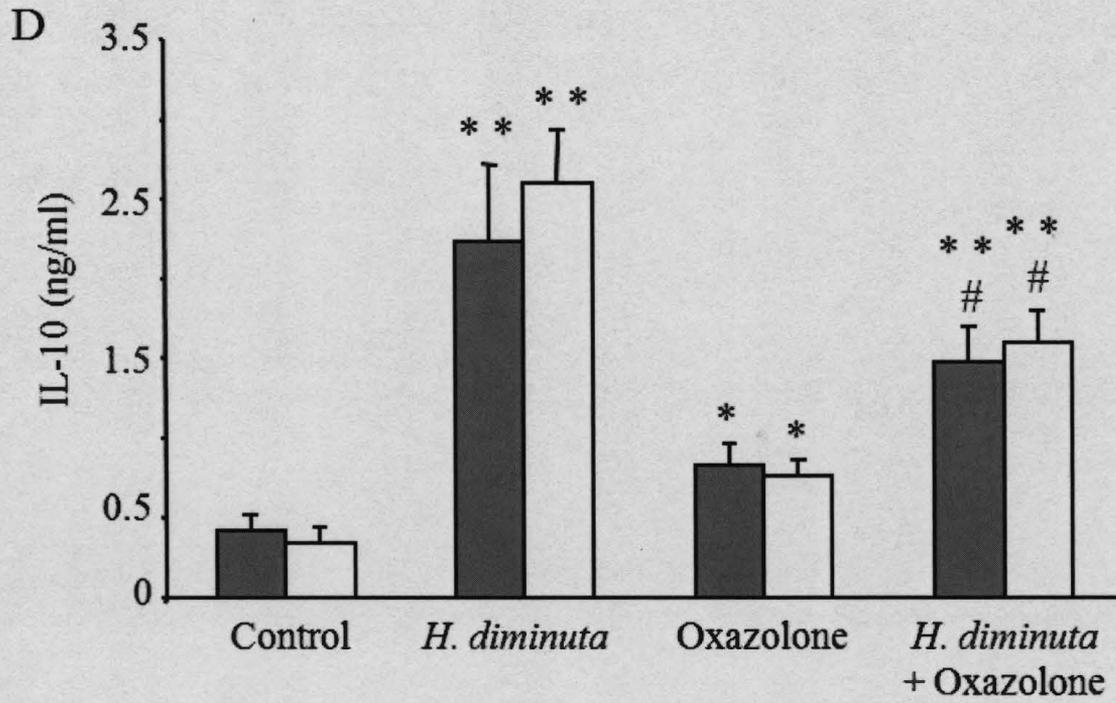
B Eosinophils / section

2±1	4±1	7±1	28±6	7±4	14±5
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Figure 3.17 Spleen (closed bars) and MLN cells (open bars) challenged *in vitro* with conA for 48 hours (2 μ g/ml) showed increased production of IL-4, -5, -10, and -13 in samples isolated from *H. diminuta* (H.d.) infected mice with or without co-treatment with oxazolone (A-D). Mice received five *H. diminuta* cysticercoids followed 8 days later by ir. oxazolone (3 mg), with autopsy at 3 days post-oxazolone. Values are the mean \pm SEM (n=8-9 mice from 3 separate experiments for the spleen cells, n=6-8 mice from 2 separate experiments for MLN cells). *, p < 0.05 compared with control; **, p < 0.05 compared with oxazolone-only; #, p < 0.05 compared with *H. diminuta*-only.







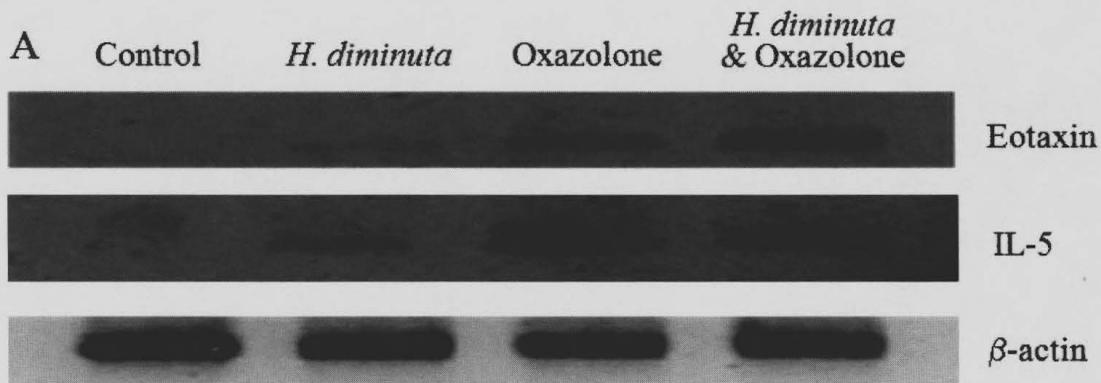
were significantly increased levels of IL-5 mRNA induced by *H. diminuta* infection. This was further increased by the addition of oxazolone. Eotaxin mRNA was significantly increased in the co-treatment samples versus samples obtained from all other groups (Fig. 3.18).

3.12 *H. diminuta* infection results in increased mortality at 7 days post-oxazolone

By 7 days post-oxazolone, mice appeared to have recovered from the inflammatory insult as seen by normalized clinical disease and scores and reduced colonic MPO levels. Histological damage, however, was still evident. Mice that received oxazolone in conjunction with *H. diminuta* infection, however, still had obvious symptoms of disease (Table 3.9). In fact, these mice had gotten progressively sicker over the 7 days and had increased eosinophil counts as well as increased EPO levels in colonic tissue samples (Fig. 3.16); 75% of the mice had to be humanely sacrificed before the end of the 7 day experiment because they had reached the experimental end point (Fig. 3.19).

At this time point, stimulated spleen cell samples from the oxazolone group had increased production of IL-4, though less than what was seen at the 3-day time point. On the other hand, the IL-13 response was increased compared to the 3-day time point. Mice with substantial morbidity resulting in early sacrifice also had a significant increase in IL-4 and IL-13 secretion from conA-stimulated splenocytes (Table 3.10).

Figure 3.18 Panel A shows representative RT-PCR gels indicating expression of eotaxin, IL-5 and β -actin mRNA in colonic tissue samples. Densitometric analysis compared each product to the β -actin housekeeper gene, and showed increased expression of both IL-5 (closed bars) and eotaxin (open bars) with both oxazolone and *H. diminuta* treatment (B). Values are the mean \pm SEM (n=4 mice). *, p < 0.05 compared with control; **, p < 0.05 compared with oxazolone-only; #, p < 0.05 compared with *H. diminuta*-only.



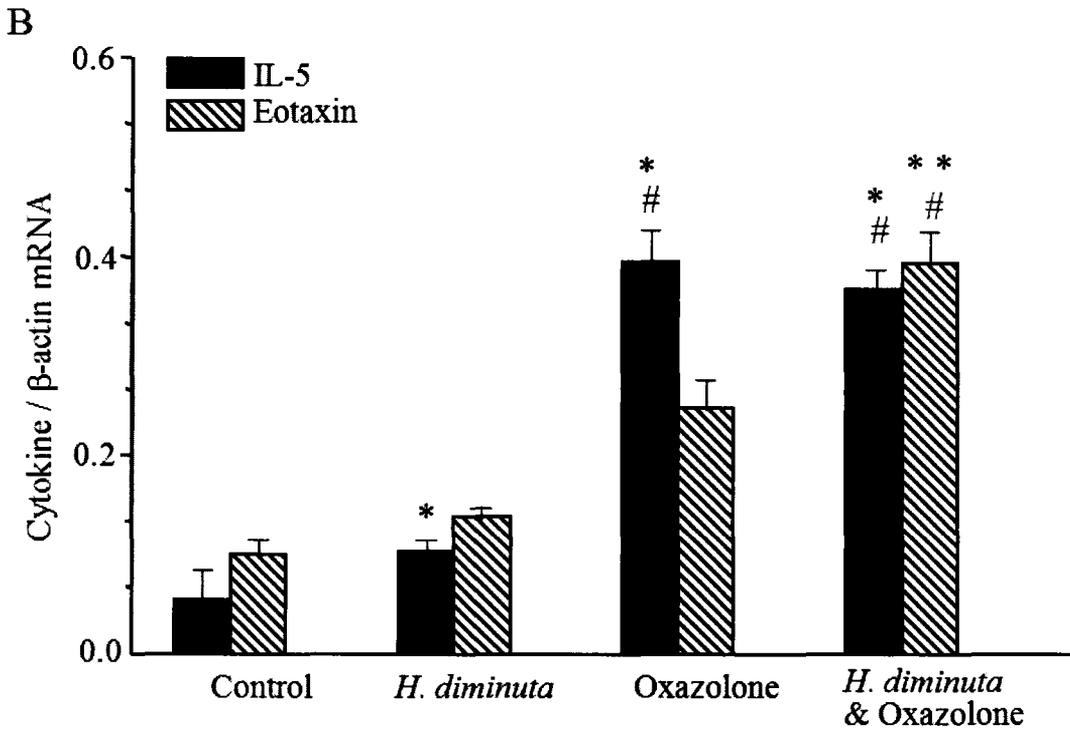


Table 3.9 Symptoms of colitis at 7 days post-oxazolone (3 mg ir.) are still exaggerated in mice that had been infected with *H. diminuta*^a

Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score	Morbidity ^c
Control	+0.2±0.4	0.21±0.3	108±1	0±0	0/4
<i>H. diminuta</i>	+0.4±0.2	0.41±0.15	111±3	0±0	0/4
Oxazolone	-0.4±1.8	0.9±0.67	105±3	0.2±0.1	0/4
<i>H. diminuta</i> + Oxazolone ^b	-2.5±0.9 ^c	4.1±0.2 ^c	90±4 ^c	2.9±1.2 ^c	3 of 4

^aMale Balb/c mice. Values are the mean ± SEM (n=4)

^bData were recorded at the time of animal sacrificed and combined

^cp<0.05 compared with other groups

^cMorbidity = number of animals humanely sacrificed because the severity of their disease reached a predetermined experimental end point (>20% weight loss and significant signs of distress)

Figure 3.19 *H. diminuta* infection inhibits recovery from oxazolone-induced colitis (3 mg ir.) as demonstrated by increased morbidity.

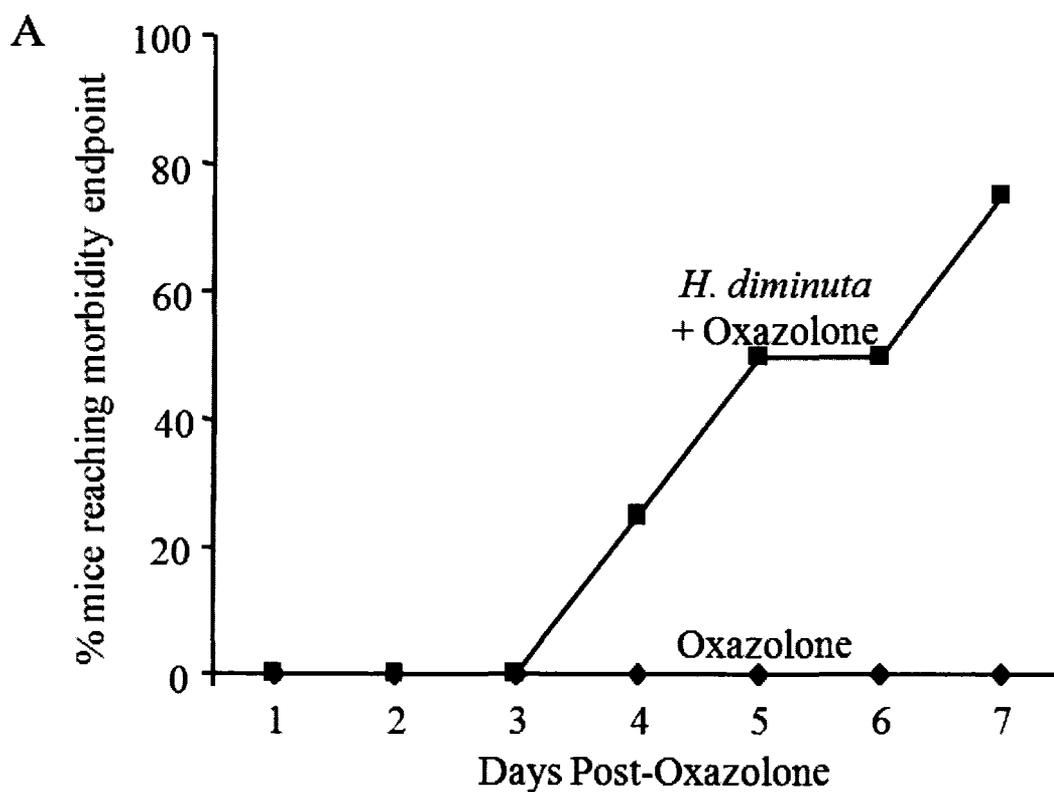


Table 3.10 Cytokine production (ng/ml) from conA stimulated splenocytes (2 µg/ml for 48 hours) at 7 days post-oxazolone^a

Treatment	cytokine	Days post-oxazolone (3 mg, ir.)			
		4	5	6	7
Control	IL-4	nd	nd	nd	0.08 ± 0.01
	IL-10	nd	nd	nd	0.12 ± 0.02
	IL-13	nd	nd	nd	0.1 ± 0.01
Oxazolone	IL-4	nd	nd	nd	0.39 ± 0.18 ^b
	IL-10	nd	nd	nd	0.04 ± 0.02
	IL-13	nd	nd	nd	1.2 ± 0.2 ^b
<i>H. diminuta</i> +	IL-4	0.7	0.4 ± 0.1	nd	0.8
Oxazolone	IL-10	1.3	1.0 ± 0.4	nd	1.04
	IL-13	0.9	1.2 ± 0.2	nd	1.1

^aMale BALB/c mice. Values are the mean ± SEM (n=4 for control and oxazolone groups; n=1, 2 and 1 at 4, 5 and 7 days post-*H. diminuta* + oxazolone, respectively due to humane sacrifice at those time points; nd = no data at this time point)

^bp < 0.05 compared with control

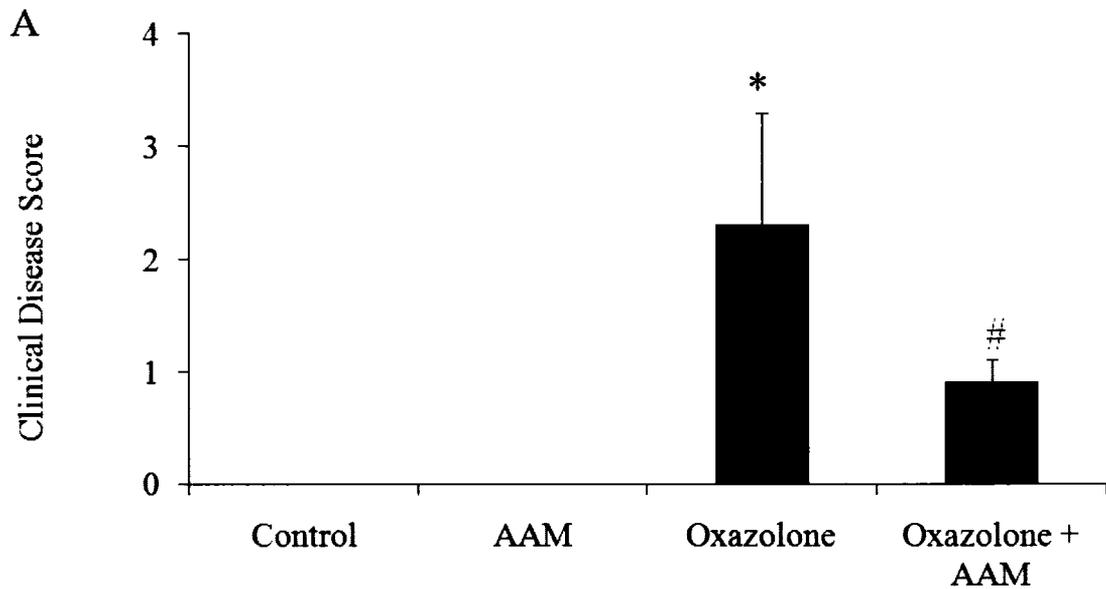
3.13 Adoptive transfer of alternatively activated macrophage reduces the symptoms of oxazolone-induced colitis

Peritoneal macrophages were differentiated in vitro into AAMs then injected (iv.) into mice 48h before delivery of oxazolone (5 mg ir.). As expected, oxazolone alone resulted in increased inflammation in the distal colon and symptoms of colitis including weight loss and diarrhea. Mice that received the AAMs prior to oxazolone showed significant reductions in the severity of disease, as reflected in reduced clinical disease scores and reduced MPO levels from colonic tissue samples (Fig. 3.20 A-C).

3.14 AD-IL5 exaggerates the symptoms of oxazolone-induced colitis

To further assess the importance of IL-5 in the oxazolone + *H. diminuta* group, mice were injected (ip.) with an adenovirus carrying the IL-5 gene (Ad-IL5). Serum IL-5 levels were examined 24 hours after injection, and found to be significantly increased in the group that received the Ad-IL5 (Fig. 3.21 A). This remained elevated at 72 hours post-injection, and was further increased in the oxazolone plus Ad-IL5 group (Fig. 3.21 B). Mice in the co-treatment group also had significant signs of disease as reflected in increased clinical disease scores and MPO levels (Fig. 3.22 A and B). EPO levels were also significantly increased (Fig. 3.22 C).

Figure 3.20 Alternatively activated macrophages (AAMs, 10^6 delivered iv. 48 hours before oxazolone, 5 mg ir.) reduce the symptoms of oxazolone-induced colitis, as seen with reduced clinical disease scores (A), normalized colon lengths (B) and decreased MPO from colonic tissue samples (C). Values are the mean \pm SEM (n=8 mice from 2 experiments). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with oxazolone-only.



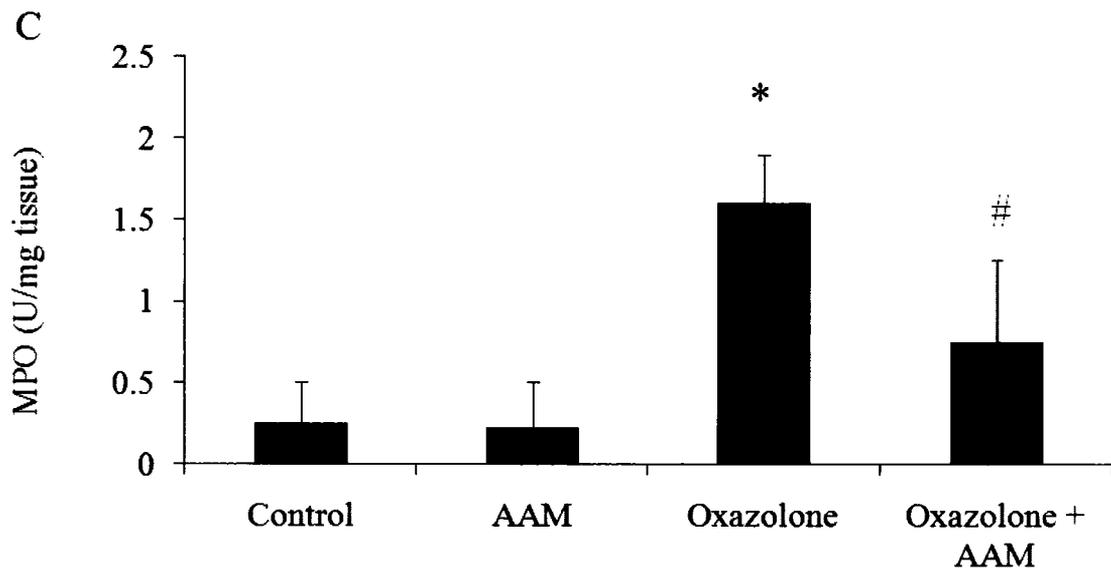
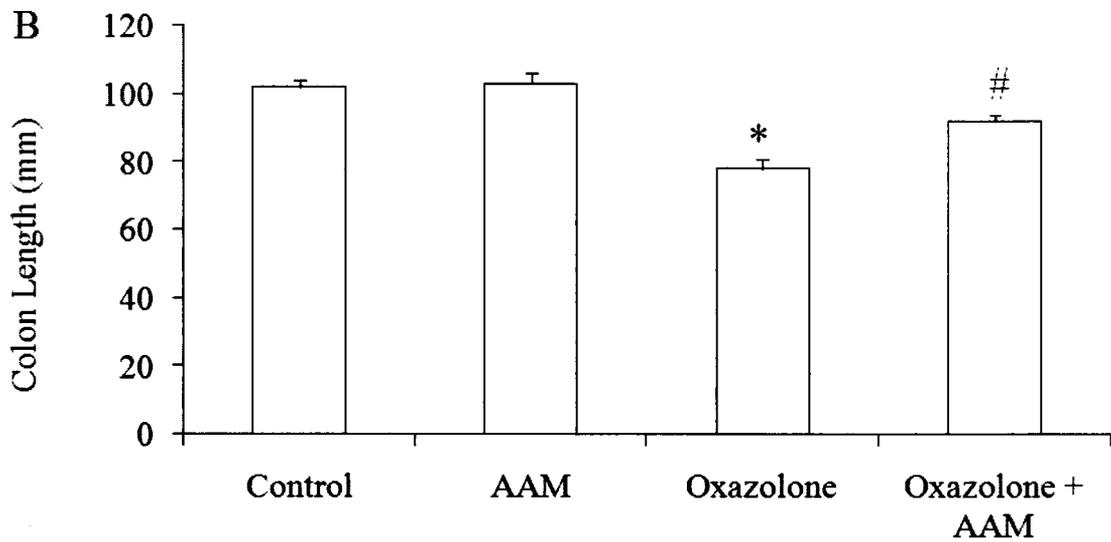
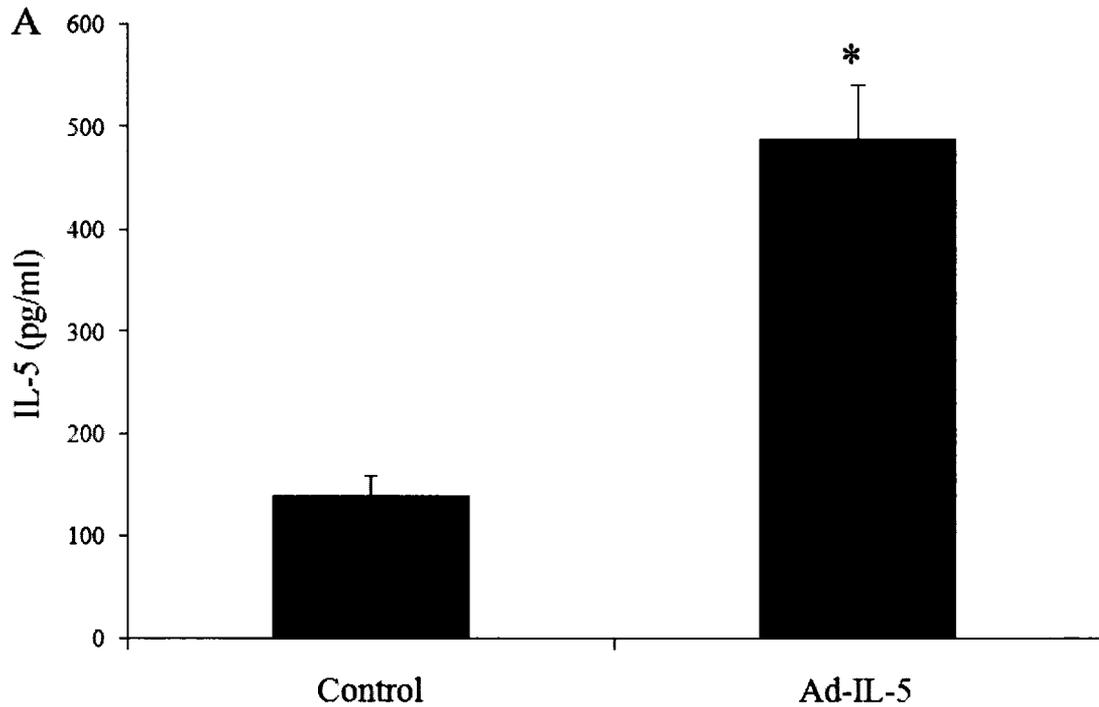


Figure 3.21 An IL-5 adenovirus (10^8 pfu/100 μ l PBS ip.) increased serum levels of IL-5 24 hours (A) and 72 hours (B) after injection. Values are the mean \pm SEM (n=4). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with oxazolone-only.



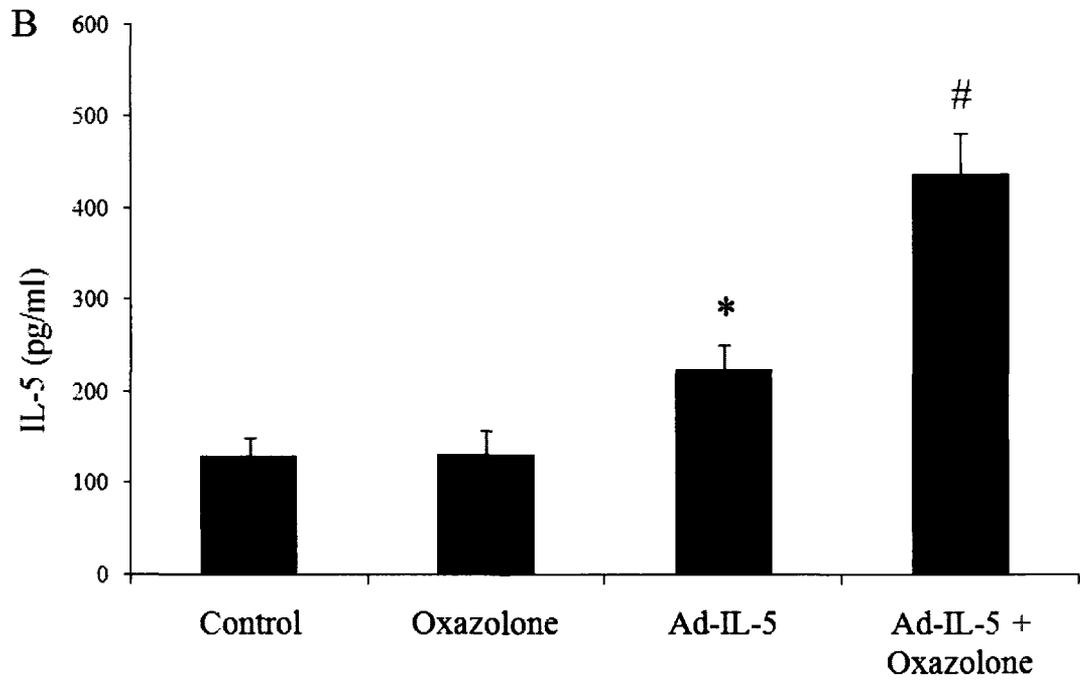
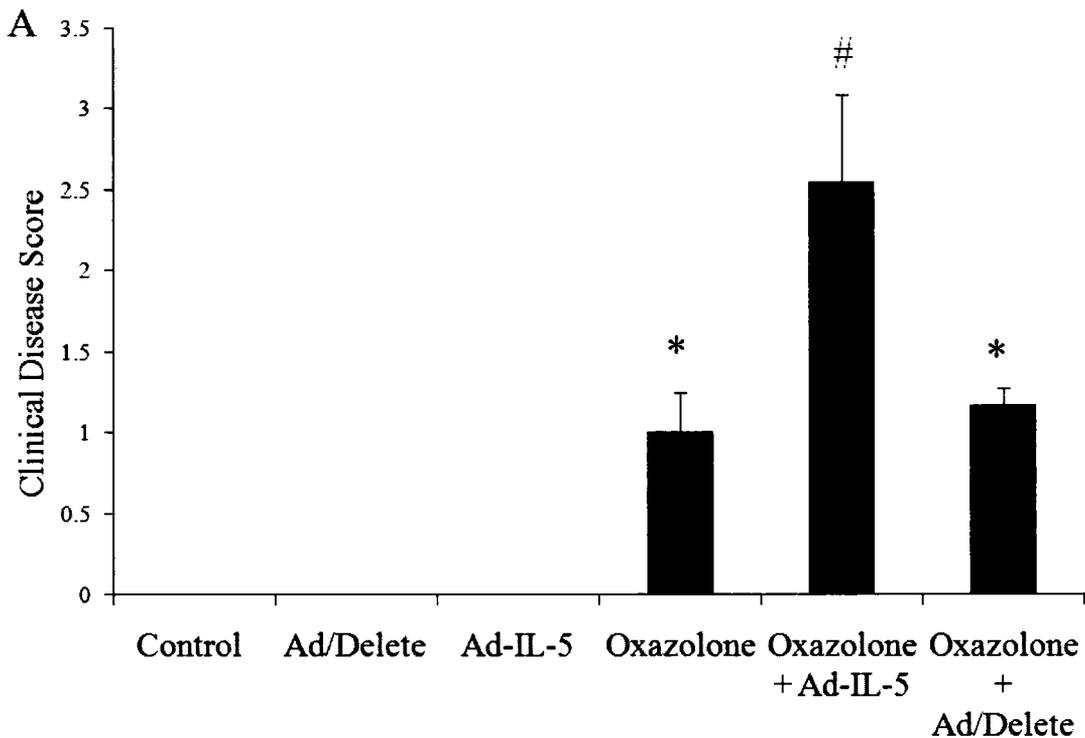
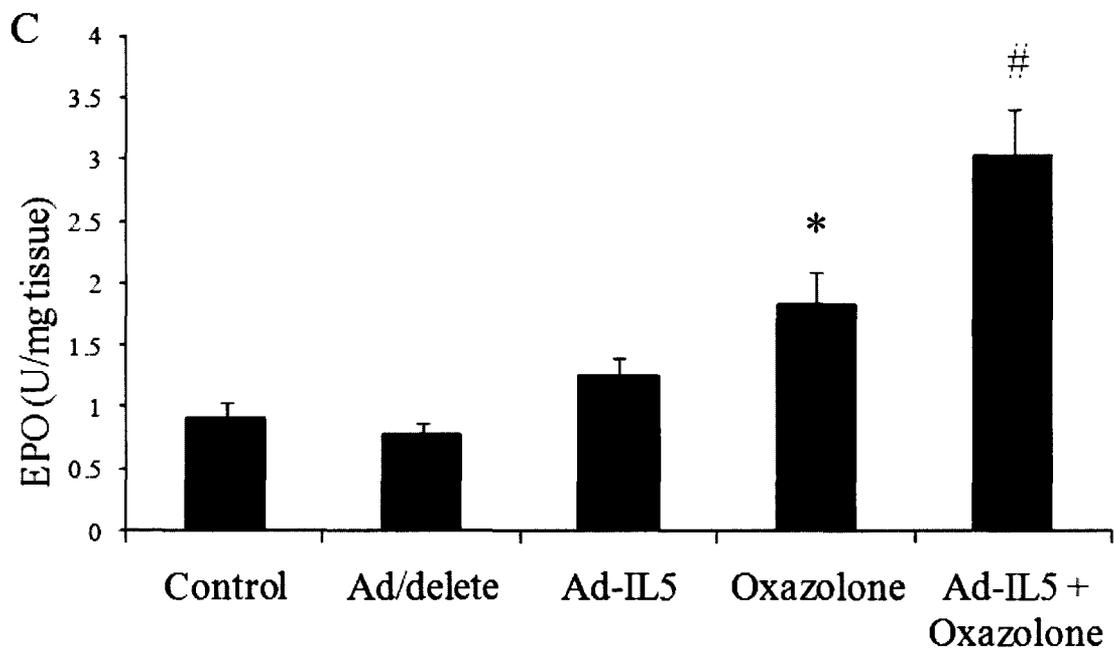
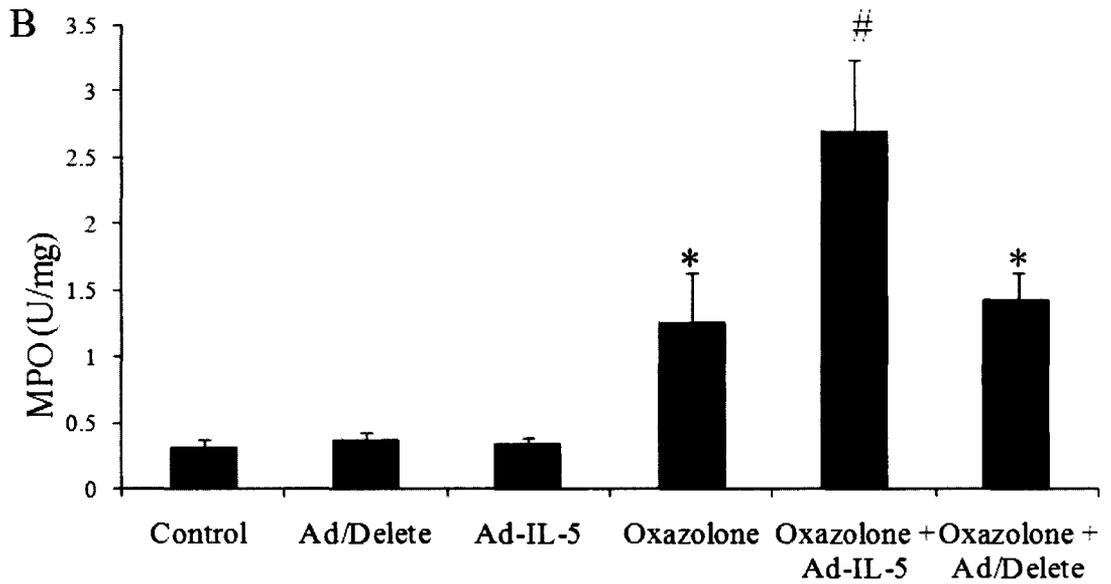


Figure 3.22 Increased IL-5 levels induced by administration of an IL-5 adenovirus (Ad-IL-510⁸ pfu/100 μ l PBS ip.) increased the symptoms of oxazolone-induced colitis. Oxazolone (3 mg ir.) was administered 24 hours after the ad-IL-5. Co-treatment resulted in increased clinical disease scores (A), MPO levels (B) and EPO levels (C). Ad-delete, the same adenovirus without the IL-5 gene insert, did not increase the symptoms of colitis. Values are the mean \pm SEM (n=12 mice from 3 experiments). *, p < 0.05 compared with control; #, p < 0.05 compared with all other groups.





Chapter 4: Discussion

The word parasitism brings to mind a malicious relationship, in which the parasite derives nutrients and a physical habitat from the host at the expense of the host's well-being. This has led to a co-evolutionary arms race, with the host attempting to mount an immune response that will destroy the parasite and the parasite attempting to evade the immune response of the host. The idea of an amicable parasite, which is actually imparting a benefit to the host, may seem strange, though it is certainly not unheard of. For example, the rodent nematode *Heligmosomoides polygyrus* has been shown to be protective against *Helicobacter*-induced gastritis in infected animals (Fox et al., 2000). There have also been cases of intestinal hookworm infections that confer resistance to bacterial infections (Desowitz, 1981). Conferring protection from other diseases also benefits the parasite as a dead host is not the ideal carrier.

There is substantial evidence that helminth infections will alter the course of other disease conditions. Epidemiological studies have shown that the prevalence of autoimmune disease is increased in areas where helminth infection is no longer endemic (Elliott et al., 2000; Buening et al., 2008). As well, current research examining animal models of allergies, asthma, diabetes, and other ailments have clearly shown the benefits of helminth infection (Sewell et al., 2002; Cooke et al., 1999; Cooper, 2004).

Though helminth therapy does show a lot of promise, it must not be forgotten that the parasitic relationship is defined by the fact that one of the organisms, the parasite, does take something from the host. Though it is sometimes difficult to show how the host

is harmed, parasites reduce host fitness in many ways and increase their own fitness by exploiting their hosts for food, habitat and as a means for dispersing their offspring.

Human Tapeworm Infections

The Cestoda is a class of parasitic flatworms, more commonly referred to as the tapeworms, whose adult forms live in the digestive tract of vertebrates. The juvenile forms inhabit a different host, which is species-specific. Live tapeworm larvae are often ingested by consuming undercooked food, after which the larvae can grow into a very large adult tapeworm (the beef tapeworm, *Taenia saginata*, can grow to be 12 m long). Many of the larvae themselves can also cause symptoms. For example, the human disease cysticercosis, which affects the central nervous system, develops after the ingestion of eggs or larvae of the tapeworm *Taenia solium*, from undercooked pork or water contaminated with infected feces. Symptoms of human tapeworm infection include constipation, diarrhea, abdominal pain and discomfort. The significant discomfort and potential dangers from parasitic tapeworm infections indicate that any attempts at treating human disease with helminths must be carefully monitored with great consideration being given to the candidate organism.

4.1 Helminth Therapy and the Th1 Model of Colitis

In the present study, DNBS was used to induce a colitis characterized by damage to the colon and clinical symptoms of disease including weight loss and diarrhea. *H. diminuta*, given both prophylactically and as a treatment, reduced all of the colitic

symptoms, as measured both clinically (i.e. damage scores and histology) and biochemically (i.e. MPO). The data also indicated that the anti-colitic effect is dependent on a viable infection and is not a simple response to the antigenic properties of the worm, as heat-killed and excysted larvae did not confer protection. The DNBS did induce an increase in IL-12 production from stimulated spleen cells, which was reduced in the DNBS + *H. diminuta* group. This is consistent with the idea that the anti-colitic effect of *H. diminuta* infection is due to its ability to reduce the Th1 response. However, we did not observe a similar DNBS-induced increase in IFN- γ and could not assess if this was also affected by *H. diminuta* infection. This is consistent with studies that have shown that the IFN- γ response is not seen until 1-2 weeks post-DNBS, a time-point which we did not examine (Sun et al., 2001).

The Role of IL-4

Similar to previous studies (Palmas et al., 1997), we found that *H. diminuta* infection initiated a Th2 response, characterized most notably by an increase in IL-4. An analysis of colonic tissue extracts revealed that while IL-4 mRNA was undetectable in animals that had received DNBS-only, it was significantly increased with *H. diminuta* infection. IL-4 induces the differentiation of naïve helper T cells to Th2 cells, after which these Th2 cells can produce additional IL-4. Important outcomes of an increased IL-4 response include the activation of anti-parasitic eosinophils as well as the induction of AAMs which potentially contribute to enhanced recovery from injury (reviewed in Anthony et al., 2007). Thus, IL-4 is important to the *anti-parasitic* response and may

have the additional effect of initiating the *anti-colitic* response. Many of the effects of IL-4 also overlap with the effects of IL-13: it has been observed that both cytokines will induce an increase in muscle contractility in mice infected with *T. spiralis* (Akiho et al., 2002), which could encourage parasite expulsion. Through the course of this study, we did not attempt to block the IL-4 response to determine if mobilization of this cytokine was absolutely crucial to the anti-colitic effect, though this is certainly something that could be investigated in the future using an anti-IL-4 antibody.

IL-4 is classified as an anti-inflammatory cytokine, though few studies have examined its role in colitis. The levels of IL-4 are reduced in IBD, which may play a part in the increased inflammation, as IL-4 is known for its inhibitory effect on inflammatory cytokines such as TNF α and IL-1 (reviewed in Rogler and Andus, 1998).

Rejection by the Non-Permissive Mouse Host

It was initially thought that the anti-colitic effect of *H. diminuta* infection is dependent on a full rejection response from the non-permissive mouse host, as the permissive rat host was not protected from the DNBS-induced damage. A subsequent finding in our laboratory, however, demonstrated that while CD4-knock-out mice rejected *H. diminuta*, they were not protected from the symptoms of DNBS-colitis (Persaud et al., 2007). From this, it would seem that another population of cells, perhaps NK cells or CD8⁺ cytotoxic T cells, is capable of orchestrating worm rejection. Without the CD4⁺ T cell response to the infection, however, there can be no protection from concurrent disease. Perhaps a closer look at chronic versus acute infections is required, as these can

be quite different. In infections of 10 or fewer *H. diminuta* cysticercoids in the rat host, the parasite will grow to its full adult size in the small intestine and remain there for the lifetime of the host. In infections greater than that, worms are gradually expelled until a chronic infection of only 5 worms remains: these large infections are also characterized by elevations in mucin and goblet cell hyperplasia, as well as an elevation in IL-13 (Webb et al., 2007). While the increased mucus will trap worms and reduce their contact with the surface of the host, the increased IL-13 is associated with smooth muscle contractility that will sweep the worms out of the intestine (Madden et al., 2002). An examination of the cytokine profile of stimulated mesenteric lymph nodes from *H. diminuta*-infected mice showed that IL-4 secretion peaked at days 6-9 post-infection, but returned to pre-infection levels by day 14 (Palmas et al., 1997). Subsequent *H. diminuta* infections, however, are expelled more rapidly, as the primary infection does induce immunological memory (McCaugue and Halton, 1987).

In terms of human infection, the prevailing pattern seems to indicate that there is an inverse relationship between the prevalence of helminth infection and the increased incidence of auto-immune and inflammatory conditions (Elliott et al., 2000). These would most likely be chronic infections, with the parasite burden remaining for years within the host, as opposed to the acute infections involving rejection that are currently being examined in human studies of helminth therapy (Summers et al., 2003 and 2005). This is an issue that requires more study, in order to determine if it is the rejection event that is important or only certain elements that are simultaneously seen in both chronic and

acute infections. The *H. diminuta*-rat infection model would be ideal for examining this, as we never went beyond the chronic infection to look at an acute 50 worm infection.

The protective effect of *H. diminuta* infection does not last forever, and mice challenged with DNBS three weeks after the primary infection were no longer protected. This is similar to the findings from human clinical trials, which showed that repeated doses of *T. suis* ova were needed in order to sustain remission from Crohn's disease (Summers et al., 2003).

Treatment vs. Prophylactic Administration

When used as a treatment rather than prophylactically, *H. diminuta* enhanced the recovery from injury after DNBS. Very few studies have examined helminth therapy as a treatment of a pre-existing condition, except the current investigations of the effect of *T. suis* in human IBD. Thus, an important animal model demonstrating the effectiveness of treatment was herein described. This is most important when considering the possible future applications of helminth therapy, as patients are much more likely to be undergoing treatment in response to a disorder rather than requesting a preventative dose of parasite.

4.2 The Important Role of IL-10

The very fact that *H. diminuta* is a small intestinal parasite that has an impact on disease and inflammation in the colon indicates that there is some modulation of communication between the large and small intestine. This could be via either the neuroendocrine or immune system, and could involve a migratory factor. RT-PCR

analysis of colonic tissue extracts revealed an increase in IL-10 in *H. diminuta*-infected mice regardless of whether they had also received DNBS. This was supported by evidence from stimulated splenocytes, which showed increased secretion of IL-10 from mice infected with *H. diminuta*, an increase which was still seen at 11 days post-infection (Persaud et al., 2007).

IL-10 has previously been recognized as having anti-colitic properties in both the TNBS and DNBS models of colitis (Barbara et al., 2000; Fuss et al., 2002). In fact, IL-10 has long been known for its strong anti-inflammatory activity with the capability of repressing the inflammatory cytokines IL-1, IL-6, IL-12, and TNF α (reviewed in Mosser and Zhang, 2008). It has also been established that the IL-10 knockout mouse develops spontaneous colitis (Berg et al., 1996). Thus, it was a logical choice to begin our investigations into the mechanism of *H. diminuta* therapy by examining the role of IL-10.

The current study described the induction of an IL-10 response, both locally in the colon and systemically in response to *H. diminuta* infection. Though initially characterized as a strictly Th2 cytokine, it is now recognized that IL-10 is involved in the generation of a more general, immunoregulatory environment that has the potential for modulating immune-mediated diseases beyond those that are defined as being strictly Th1 (reviewed in Hunter and McKay, 2004). Because of these properties, we considered further the role that IL-10 may be playing within the *H. diminuta*-DNBS model. Using three separate injections of a neutralizing IL-10 antibody, the anti-colitic effect of *H. diminuta* was abolished and the symptoms and inflammation induced by DNBS found to

be virtually identical to the DNBS-only group. Thus, IL-10 was determined to be critically important to the anti-colitic effect of this helminth.

A study showing that the nematode *H. polygyrus* was anti-colitic in the IL-10^{-/-} mouse (Elliott et al., 2004) contradicted this finding: these mice did not have the genetic means for upregulating IL-10 in response to infection. This could be interpreted to mean that there are redundant pathways by which helminth therapy is capable of reducing the symptoms of colitis. The same researchers found that transfer of T cells isolated from the mesenteric lymph nodes from *H. polygyrus*-infected IL-10^{-/-} mice was able to reduce inflammation in IL-10^{-/-} mice with active colitis, and that infection with the helminth resulted in increased expression of FoxP3, a natural regulatory T cell marker (Elliott et al., 2004; Elliott, Summers and Weinstock, 2005). Studies from our laboratory agree that there is an increase in both IL-10 and FoxP3 following *H. diminuta* infection (Hunter et al., 2005; Persaud et al., 2007).

IL-10 as Potential Therapy

IL-10 is a potent anti-inflammatory cytokine which functions via activation of the JAK/STAT signalling pathway. The IL-10 receptor subunits IL-10R1 and IL-10R2 are expressed on most cells. The main bioactivity of IL-10 is as an inhibitor of antigen presentation, which in turn prevents the production of pro-inflammatory Th1 cytokines (reviews in Mosser and Zhang, 2008). It was once thought that recombinant IL-10 would be a potent therapeutic for treating inflammatory disease. In the treatment of psoriasis,

IL-10 injections directly beneath the skin lesions were very effective at reducing their severity (reviewed in Numerof and Asadullah, 2006).

The pathogenesis of Crohn's disease is characterized by increased activation of pro-inflammatory Th1 cytokines (Elson et al., 1996). Since IL-10 downregulates the production of pro-inflammatory cytokines, it was once thought that recombinant IL-10 therapy would be effective for Crohn's disease patients. Unfortunately, clinical trials have indicated that therapeutic IL-10 injections have little benefit for patients with active Crohn's disease (reviewed in Kucharzik et al., 2006). While this is disappointing, the data presented here indicates that IL-10 does have potential value as a treatment based on this cytokine's ability to ameliorate disease in an animal model of chemically-induced colitis. IL-10 therapy should not be completely abandoned, as mode of delivery, as well as the stage and severity of the disease, may be affecting the treatment outcome.

One study looked at developing engineered T lymphocytes as a possible vehicle for directly delivering IL-10 to the intestinal mucosa. Human CD4⁺ cells were engineered to constitutively express recombinant IL-10: these cells would naturally hone to the intestinal mucosa and therefore specifically deliver IL-10 to this location. In vitro, the cells were capable of suppressing proliferation and IL-12 production by dendritic cells (von Montfrans et al., 2002). Another study used *Lactococcus lactis* to deliver IL-10 directly to the gastrointestinal tract and showed a significant decrease in the symptoms of murine DSS-induced colitis (Steidler et al., 2000). It has also been shown that IL-10 can be delivered directly to the GI tract using nanoparticles carrying the IL-10 gene (Bhavsar and Amiji, 2008).

The main advantage of directly targeting IL-10 to sites of inflammation is avoiding the side-effects, such as headache and fever, associated with high systemic levels of IL-10 (Li and He, 2004). Not only is the IL-10 receptor expressed on nearly all cells, but IL-10 can also have a stimulatory effect on the proliferation of subsets of CD8⁺ T cells and can also stimulate NK cell proliferation and B cell activation (Groux et al., 1998; Cai, Kastelein and Hunter, 1999). It is possible that the site-specific release of IL-10 would induce the development of native T regulatory cells, which would in turn prove to be anti-inflammatory. Unfortunately, testing this theory was beyond the scope of this project. We are, however, able to confidently promote the use of an alternative therapy with the same potential benefits. Infection with parasitic worms that preferentially migrate to the gastrointestinal tract will ensure site-specific delivery of IL-10, while at the same time proving to be a much more economical choice than specially engineered cells.

A significant danger of anti-IL-10 therapy is that by manipulating IL-10 we are also impacting the host's response to the parasitic infection. The host may no longer be able to reject the parasite, leading the model away from the rejection event that may be crucial to the anti-colitic effect (though this is still debated). This was not the case for these experiments, as there was no remaining evidence of the *H. diminuta* infection when mice treated with anti-IL-10 were examined at autopsy. Even more dangerous is the possibility that the lack of IL-10 could lead to the over-production of IFN- γ , which would not only endanger the experiment but could potentially endanger the life of the patient. For this reason, it will be important to maintain vigilant monitoring of the subjects and their response to the helminth infection.

4.3 The Role of Alternatively Activated Macrophages

When we blocked the production of IL-10 with an anti-IL-10 antibody, we no longer saw the anti-colitic effect of *H. diminuta* infection. Additionally, the expression of AAM markers was also blocked by the anti-IL-10 antibody, indicating a role for IL-10 in the development of these cells. Indeed, IL-10 is implicated in the definition of AAM subtypes: M2a, being the typical IL-4/IL-13 activated macrophage, M2b defined as a macrophage that releases high levels of IL-10, and M2c as a macrophage that is induced by IL-10 (Kreider et al., 2007). Based on this classification system, the AAM can act as either a source or a target for IL-10.

While macrophages were once thought to be pro-inflammatory (Kamada et al., 2005), they are now being characterized as having a more flexible phenotype, the expression of which is often determined by their environment (Gordon and Taylor, 2005; Gordon, 2003). The AAM is able to support tissue recovery after an injurious or inflammatory event due to the expression of genes that are capable of regulating interactions with the extracellular matrix and stimulating the production of collagen. Specifically, YM1 expression is induced by IL-4 via a STAT-dependent signalling pathway and binds heparin, indicating a role in interaction with the extracellular matrix (Falcone et al., 2001). YM1 is also chemotactic for eosinophils (Chang et al., 2001). FIZZ1, on the other hand, stimulates collagen production in myofibroblasts (Nair, Guild and Artis, 2006). Data showing that there is an increased expression of both FIZZ1 and arginase-1 mRNA in the colon following infection with *H. diminuta* larvae indicates the

likelihood that there is increased activation of AAMs in response to the infection. More interesting is the fact that once again this parasite that preferentially dwells in the small intestine is able to elicit a response in the colon which has the potential of being protective at this location.

When *in vitro* activated AAMs are transferred to naïve mice, they reduce the symptoms of DNBS-colitis. This coincides with an increased expression of arginase-1 mRNA in the colon - an increase in fluorescently labelled AAMs was also seen in the colon following transfer. Additional studies done in our laboratory have also shown that depleting macrophages using clodronate-containing liposomes during *H. diminuta* infection reduced the anti-colitic effect of the helminth infection (personal communication, Dr. Derek McKay). Together, these studies underline the importance of macrophages in mediating protection from chemically-induced colitis.

While mobilization of the AAMs may prove to be crucial to the anti-colitic effect of *H. diminuta* infection, other macrophage types may also be involved. For example, a study examining the effect of *S. mansoni* infection on the severity of DSS-induced colitis described an F4/80⁺ macrophage that was essential to recovery from the colitis, but did not show any of the characteristic gene expression of the AAMs (Qualls et al., 2006). An examination of DSS-induced colitis in mice also demonstrated a dependence on macrophages in the preventative effect of *Schistosoma mansoni* infection. The authors did not attribute this to AAMs, but to a population of F4/80⁺CD11b⁺CD11c⁻ macrophages that migrated into the colonic lamina propria (Smith et al., 2007). In terms of other

conditions, transfer of AAMs into mice with chronic, inflammatory renal disease reduced histological and clinical manifestations of the disease (Wang et al., 2007).

ConA stimulated spleen cells from mice that received *in vitro* activated AAMs produced 4 times more IL-10 than splenocytes from control or DNBS-only treated mice. This again highlights the relationship between AAMs and IL-10. As conA is a mitogen that predominantly stimulates T cells, these data suggest that the AAM is interacting with T cells and skewing their response to favour IL-10 production, or that the T cells are releasing IL-10 which is then affecting the alternative activation of the macrophages. A good follow-up to our current study would be to determine the source of increased IL-10 in response to *H. diminuta* infection and whether it is indeed a population of macrophages or another cell type, such as the T regulatory cells. Macrophages can be depleted in a non-specific manner using clodronate-containing liposomes, after which the levels of IL-10 can be measured. To more specifically inhibit the AAM population, arginase activity can also be inhibited using a daily injection of hydroxy-nor-L-of-arginine (Kropf et al., 2005). In a similar fashion, the CD4⁺CD25⁺ T regulatory cell population could be depleted using an anti-CD25⁺ antibody. This series of experiments could help to unravel the relationships between these cell types and whether the development of one is dependent on the development of the other.

Goals of the AAM response

The spectrum of responses associated with the induction of AAMs appears to fall into three main categories: conferring resistance to parasite infection, enhancing healing and recovery from injury, and regulating the immune response. AAMs are an important part of the host response to helminth infection as blocking arginase-1 or depleting the macrophages will also reduce protection against *H. polygyrus* (Anthony et al., 2006). The host's ability to expel the parasite is also compromised by blocking IL-4 (Gause, Urban and Stadecker, 2003), which makes sense considering that IL-4 drives alternative macrophage activation. In terms of the actual effects on the helminths, the AAMs secrete chitinase-like proteins, such as members of the YM1 family, which may target the glycan chitins that are expressed by helminths but not by their mammalian hosts (reviewed in Nair, Guild and Artis, 2006).

Most helminths are large and can cause tissue damage during their migration to their preferred location within the host. The nematodes are equipped with hooks and teeth, which allow them to attach and feed on the blood and tissue of the host. Some of the genes associated with the alternatively activated macrophages, such as YM1 which binds heparin and may mediate interactions with the extracellular matrix, are associated with wound healing. AAMs also hasten healing by releasing cytokines and growth factors, such as proline and TGF- β , which are involved in fibroblast recruitment and the production of collagen (Sakthianandeswaren et al., 2005). It is this effect that may be influencing the course of DNBS colitis in our model. *H. diminuta* infection may not be preventing the disease but instead enhancing recovery from the DNBS-induced damage to

the colon. Evidence for this is seen not only by fewer instances of damage and ulcer, but also by increased deposition of collagen in colonic tissue samples from *H. diminuta*-infected animals. This finding, however, may also be indicative of one of the negative side-effects of helminth therapy, as increased systemic fibrosis induced by infection could be very damaging to the host.

The final category of AAM responses are those that affect regulation of the immune system. AAMs have been shown to downregulate Th1 cytokines, a response that is mediated largely by TGF- β (Taylor et al., 2006). This study, however, failed to find a difference in TGF- β secretion from stimulated splenocytes following AAM transfer. We did, however, document a change in IL-10, possibly mediated by T regulatory cells under the influence of the AAMs. As well, an increase in arginase-1 was observed in spleen and colon samples from mice that received AAMs: as arginase-1 is a competitor for the precursors of the iNOS pathway (Hesse et al., 2001), this could be the mode by which inflammation is downregulated following AAM transfer.

Overall, it is evident that the AAMs induced by *H. diminuta* infection may play a key role in the recovery from concurrent chemically-induced colitis. This may be either via downregulation of inflammatory events, upregulation of IL-10 production from T regulatory cells, or through increased recovery from damage instigated by the colitic agent. Due to the unpalatable nature of helminth therapy, the identification of a cell type that is able reduce the symptoms of colitis in the absence of the actual helminth may prove to be very useful. Macrophages are dynamic cells with phenotypic plasticity, in that macrophages that have been classically or alternatively activated can be stimulated by a

secondary signal to express the opposite phenotype (Gratchev et al., 2006). The flexibility of this response allows the immune system to cope with different infections in a timely manner: for the purpose of a potential therapy, it ensures that the patient's own macrophages can be induced to switch phenotypes even after they have differentiated. This will negate the need for an active, ongoing infection, as the patient's own monocytes could be harvested, induced towards alternative activation, and re-injected back into the patient.

4.4 Helminth Therapy and the Th2 Model of Colitis

The predominant risk associated with promoting the helminth-driven Th2 response is the possibility that this will also predispose the patient to allergic-type diseases or exacerbate any pre-existing allergies or asthma. The study presented here found that *H. diminuta* infection did not enhance the enteric hypersensitivity response to challenge with OVA in sensitized mice. In fact the benefits of helminth infection have been shown in models of allergic airway sensitivity (Wilson et al., 2005), and epidemiological data indicate that there are fewer cases of allergy and asthma in areas where helminth infection is endemic. More specifically, *H. polygyrus* infection was actually found to reduce disease in a mouse model of asthma (Kitagaki et al., 2006). This indicates that the mechanism of helminth therapy may not rely on skewing the cytokine profile towards the Th2 end of the spectrum, but instead is generating a more immunoregulatory environment that is perhaps reliant on IL-10 and AAMs. In terms of human disease, *T. suis* ova were found to be effective at treating the symptoms of

ulcerative colitis, though to a lesser extent than Crohn's disease (Summers et al., 2005). While ulcerative colitis has been characterized as being a Th2-type disorder, this is in dispute as there is an increase in IL-5 but no corresponding increase in IL-4 (Sawa et al., 2003).

The oxazolone model of colitis, which is associated with an increase in IL-4 and IL-13 (Boirivant et al., 1998; Heller et al., 2002; Kojima et al., 2004), was used to determine if the Th2 response induced by *H. diminuta* infection would have an additive effect on the Th2 response mediating the colitis. After ir. administration, animals that received oxazolone-only had an inflammation that was concentrated in the distal part of the colon. This was accompanied by an initial increase in IL-4 secretion from conA-stimulated spleen cells that was soon superseded by an increase in IL-13 secretion. The symptoms of oxazolone colitis were exacerbated by concurrent infection with *H. diminuta*: while mice given a single dose of oxazolone eventually recuperated, the addition of the worm infection transformed it into a more severe and fatal disease. This is significantly different than what was observed in the DNBS model, where the *H. diminuta* infection reduced the severity of the disease.

The findings here also contrast with those of ongoing human studies, which showed that 13 out of 30 patients with ulcerative colitis derived some relief from their symptoms from *T. suis* treatment (Summers et al., 2005). This could be explained by the fact that oxazolone-induced colitis is an acute model of disease whereas the average duration of patients' disease in the clinical trial was 8-9 years. As well, the human patients did not report any relief until 12 weeks after they received their first dose of *T.*

suus ova, whereas the furthest time-point we examined was at 10 days post-infection. These findings could also reflect the differences between animal and human models of disease: even though oxazolone colitis is described as being similar to ulcerative colitis, no animal model can fully replicate all of the nuances of human disease. Additionally, the levels of IL-4 and IL-13 were not assessed in this group of patients, and it is unknown how closely the manifestation of their disease mirrored the well-characterized cytokine profile of oxazolone colitis.

Previous studies of the oxazolone model of colitis found that *in vivo* neutralization of IL-13 reduced the symptoms of the disease (Heller et al., 2002). Our own findings show that there is an increase in IL-13 secretion from conA stimulated spleen and MLN cells taken from *H. diminuta* + oxazolone-treated mice, which is far greater than the levels of IL-13 seen with oxazolone-only treated mice. One interpretation is that the IL-13 response generated by the oxazolone is being exacerbated by the *H. diminuta* infection, leading to the extreme severity of the disease. An examination of co-treated animals at 4-7 days post-oxazolone found that the increase in disease severity coincided with an increase in IL-13 secretion. In human patients with ulcerative colitis, an examination of lamina propria T cells from biopsy samples found that there was increased secretion of IL-13 which had a cytotoxic effect on the epithelium (Heller et al., 2005). IL-13 has also been associated with the parasite-induced damage, as blocking IL-13 during *T. spiralis* infection also reduced damage to the colon (McDermott et al., 2005). Spleen and MLN cells from animals that received *H. diminuta*-only, however, also had increased secretion of IL-13, yet the infection was not

accompanied by any signs of damage or inflammation in the colon. Thus, it seems unlikely that the IL-13 alone is responsible for the increased disease and inflammation. To determine if IL-13 is involved in this response, we could use a neutralizing IL-13 antibody, similar to that used in the anti-IL-10 study.

The increased risk of eosinophilia

One of the markers used to confirm *H. diminuta* infection is increased numbers of eosinophils in blood samples. In contrast to DNBS-treated mice, tissues from mice with oxazolone-induced colitis also had increased eosinophil counts (Boirivant et al., 1998). The increased eosinophilia is thought to play a role in the decreased barrier function involved in oxazolone-induced colitis (Furuta et al., 2005). Increased EPO was also seen in colonic tissue samples from *H. diminuta* + oxazolone-treated mice, and mRNA from the colon also showed increased eotaxin. Eotaxin (or CCL11) is a small cytokine that induces chemotaxis in eosinophils and thereby recruits them (Ponath et al., 1996). Taken together, this indicates that oxazolone colitis can develop into a severe eosinophilic colitis when combined with *H. diminuta* infection.

Eosinophils contain small cytoplasmic granules within which are found proteins such as eosinophil peroxidase (EPO) and Major Basic Protein. These are released via a process called degranulation and are toxic to both parasites and host tissues. Major Basic Protein in particular has been implicated in epithelial cell damage (Rothenberg and Hogan, 2006). In a study of Crohn's disease patients who received *N. americanus* treatment, all of the inoculated individuals developed eosinophilic enteritis (Croese et al.,

2006). This indicates that there is a very real risk associated with helminth therapy. Eosinophilia, defined as being more than 500 eosinophils/ml of blood, is often seen in patients with a helminth infection, rheumatoid arthritis, malignancies such as Hodgkin's disease, Addison's disease, and following treatment with some antibiotics such as penicillin (Tambo et al., 2008; Keresztes et al., 2007; Hyes, Anstead and Kuhn, 2007). The presence of any of these factors or of pre-existing, unexplained eosinophilia, must be carefully assessed before any attempt at helminth therapy is made.

The effect of increased IL-5

IL-5, released by T cells, mast cells or macrophages, will stimulate the production of eosinophils (Dubucquoi et al., 1994). Eosinophils are the most prominent cells that express the IL-5 receptor, IL-5R α , via which IL-5 can regulate the maturation and accumulation of eosinophils in the tissues. We observed increased expression of IL-5 mRNA in colonic tissue samples from mice in the *H. diminuta* + oxazolone group. As well, there was increased secretion of IL-5 from stimulated splenocytes. From these data we hypothesized that a helminth driven increase in IL-5 was responsible for the increased eosinophilia that was exaggerating oxazolone-colitis. This could be tested either by blocking IL-5 or by adding IL-5 to the oxazolone model and comparing the effects to that of tapeworm infection. We chose to take the later approach and add IL-5 to our oxazolone model in order to assess its impact on the colitis.

The Ad-IL-5 used for this experiment is a recombinant adenovirus which contains the murine IL-5 gene within the E3 region and expresses high levels of IL-5 protein *in*

vitro. Twenty-four hours after injection, there was increased levels of IL-5 in the sera of treated mice. Increased IL-5 in conjunction with oxazolone-colitis led to exaggerated disease as well as increased EPO and eosinophil counts in the colon. These data suggest that the exaggeration of oxazolone colitis by *H. diminuta* infection is likely via an IL-5-driven eosinophilia. This parasite-driven eosinophil response most likely has an additive effect on the response evoked by oxazolone alone. Thus, in order for helminth therapy to be seriously considered as a clinical option, patients must be carefully assessed to ensure that they do not have a condition that provokes an ongoing eosinophil response.

IL-10 and AAMs in the oxazolone model of colitis

Data arising from the DNBS model indicated the importance of both IL-10 and the AAMs to the anti-colitic effect of *H. diminuta* infection. IL-10 production from conA stimulated MLN cells was increased in the *H. diminuta* + oxazolone-treated group. While others have corroborated our findings that IL-10 is crucial to the anti-colitic effect in the DNBS model of colitis, (Fuss et al., 2002; Barbara et al., 2000), this is not the case for the oxazolone model. It is possible that the inflammation in the co-treated mice is so severe, that the anti-inflammatory effect of increased, *H. diminuta*-induced IL-10 is not enough to overcome it. We never examined other possibilities, however, such as reduced expression of the IL-10 receptor or an impact on the sensitivity of the receptor (Monteleone et al., 2005). In the absence of helminth infection, transfer of *in vitro* activated macrophages demonstrated that AAMs alone are able to reduce the symptoms of oxazolone-induced colitis. Thus, induction and transfer of AAMs could prove to be an

effective treatment for a multitude of disease-types regardless of their cytokine background. The protection conferred by the AAMs may transcend cytokine classification, as genes expressed by the AAMs, specifically YM1 and FIZZ1, offer protection from injury and the ability to repair tissue after an inflammatory event. Thus, the similarity in damage resulting from different diseases is more important than the dissimilarities in their underlying mechanisms.

Understanding the human context

The data presented here does conflict with results from ongoing human trials investigating the treatment of ulcerative colitis (Summers et al, 2005), which have shown an improvement in the symptoms of about 45% of patients in response to treatment with *T. suis* ova. While our evidence demonstrates the potential role eosinophils play in the development of the severe colitis induced by oxazolone in the context of *H. diminuta* infection, the eosinophil profile was not reported for this subset of patients. It is conceivable that the 45% of patients who responded had low levels of IL-4 and/or IL-13 and no persistent eosinophilia. Due to the severity of the side-effects we observed in our animal model, we would advocate caution before any human trials of this therapy. Negative side-effects arising from helminth therapy are not unheard of: *H. polygyrus*, while shown to be anticolitic, also increased the severity of a concurrent bacterial infection (Chen et al., 2005), as did infections with *T. suis* and *S. stercoralis* (Mansfield et al., 2003; Ghoshal et al., 2002). It may be that the actual parasite chosen as the

therapeutic agent proves to be important, with some being beneficial while others induce their own disease and damage.

While overall this therapy appears to be very promising, there are also many potential problems that could arise. The parasite must not cause damage in the host – while *S. mansoni* may prove valuable in animal models, in reality it can cause significant pathology in human hosts. As well, those patients who are immune compromised or who are receiving treatment, such as steroids, that suppress the immune system, may be unable to reject an otherwise non-permissive parasite. As shown with our data, there is also the possibility of enhancing existing disease states involving activation of eosinophils.

4.5 Helminth Therapy for Human Disease

There are conflicting reports about the efficacy of helminth therapy in the course of human conditions. One study of Gabonese schoolchildren found that while the prevalence of schistosome and filarial infections increased with age, there was a coincident decrease in reactivity to house dust mites (van den Biggelaar et al., 2001 and 2004). The same researchers determined that the reduced incidence of allergy in children infected with *S. haematobium* was due to increased concentrations of IL-10 as compared to uninfected children (van den Biggelaar et al., 2000). Indeed, IL-10 is thought to be the key to inhibiting the Th2-response that results in inflammation during the course of allergy and asthma. Cells from asthmatic patients infected with *S. mansoni* were found to produce lower levels of IL-5 and higher levels of IL-10 as compared to uninfected patients (reviewed in Araujo and de Carvalho, 2006). Not all epidemiological studies

agree, however, that infection with an intestinal parasite is protective against atopic conditions, and a meta-analysis of 33 studies determined that parasitic infection was actually associated with a small increase in asthma (though this finding was not significant). Specifically, *Ascaris lumbricoides* was associated with a significant increase in asthma, while hookworm infection was associated with a significant reduction that was related to the parasite burden (Leonardi-Bee, Pritchard and Britton, 2006).

Careful selection of the putative therapeutic agent is extremely important when considering helminth therapy. The above analysis determined that infection with *A. lumbricoides* actually increased the risk of asthma, while our own studies have shown an increase in symptoms when oxazolone-colitis is treated with *H. diminuta* (Hunter et al., 2007). An additional, complicating factor is the fact that almost all of the animal studies have provided evidence on acute infections which are rejected from the hosts, while the epidemiological studies are considering chronic infections that live for years within their hosts. These situations are not necessarily comparable, and though they may provide some similarities in the mobilization of immune cells, the differing mechanisms of each still need to be determined. This is especially important in the process of determining whether an antigenic “pill” will be a viable alternative to treatment with a live parasite. It is known that the acute parasite infection, once rejected, generates a memory that allows a subsequent infection to be resolved at a faster rate and the antigens that mobilize these immune events might be equally effective in treating disease in a patient that has resolved an acute infection.

To date, human clinical studies on the efficacy of helminth therapy for treating inflammatory bowel disease have had some success. It has been shown that a dose of viable *T. suis* ova provides relief from the symptoms of both Crohn's disease and Ulcerative Colitis (Summers et al., 2005 A and B). Although these studies did not report significant side-effects, a physician who subsequently prescribed the therapy for a patient with Crohn's disease reported that an active infection persisted and that the worms developed to the adult stage (Kradin et al., 2006). Thus, the claim that these infections will be resolved without harming the patient is not necessarily true, and may change on a case-by-case basis.

4.6 Summary and Conclusion

The work presented here describes the anti-colitic mechanism of helminth therapy in a model of chemically-induced DNBS colitis. The data demonstrates that the rejection response to a viable *H. diminuta* infection in the non-permissive mouse host can both prevent and treat the symptoms of DNBS-induced colitis. Furthermore, it was determined that this anti-colitic effect was dependent on IL-10 and could be mediated by the helminth-induced AAMs in the absence of worm infection. Overall, this study lends support to the emerging idea that biological therapies can be an effective treatment for IBD and, more specifically, that helminth therapy may prove to be beneficial for those patients who are refractory to more traditional treatments.

While we and others have shown that parasitic helminths can prevent colonic inflammation, there is a concern that this therapy may not be suitable for all IBD patients.

Helminth therapy may not be appropriate for patients who already have eosinophilia or high levels of IL-13 or IL-4. The potential benefits of this treatment, however, should not be ignored, as helminth therapy has proven to be safe in a variety of animal models of disease. Furthermore, this research has proven its overall value as the analysis of parasite-host interactions has furthered our understanding of anti-inflammatory and immunosuppressive mechanisms.

There are still several questions to consider that may guide future research in this field:

1) Which diseases are suitable for treatment with helminth therapy?

Each disease mediated by the immune system is unique, as is each patient's presentation of that disease. As mentioned previously, there are some conditions that involve increased eosinophilia that may not be appropriate for the treatment described here. As we learn more about the specific regulatory pathways that are manipulated by helminth infection, the list of diseases that are suitable for helminth therapy can be determined. A specific set of requirements that can be used by individual doctors when prescribing helminth therapy will also be useful in order to negate the risk of deleterious side-effects. There will also be inflammatory conditions that are neither negatively nor positively affected by exposure to helminths, such as was shown in a model of acetic-acid induced gastric ulcers in the rat host (McKay and Wallace, 2008).

2) Are all helminth species appropriate therapeutic candidates?

Many helminths are pathogenic to their host and could initiate disease that is actually more severe than the disease being treated. The ideal therapeutic helminth must have a minimal potential for pathogenicity, must not be able to self-replicate in the host and cannot be spread to other people that come in contact with the patient. As well, it is important that the helminth be easily eradicated by anti-helminthic treatment, in case it is able to evade the immune response of the host and establish itself permanently (Elliott et al., 2007). Some candidates that would be considered less than ideal include *S. mansoni*, where untreated human infection can lead to fibrosis of the liver and hepatosplenomegaly, and the filarial worms, which are also associated with significant human pathology. On the other hand, there are some tapeworms, such as *H. diminuta*, which are not associated with human disease and are easily rejected from their host. Consideration of the possible beneficial uses of helminths may also lead to a changing definition of parasitism as a whole, from a relationship that is detrimental to one of the species involved to instead being characterized as a more mutualistic association.

3) Can the anti-colitic effect of helminth infection be reproduced by administration of a factor isolated from the worm?

The unpalatable nature of helminth therapy may translate into limited use for treating actual human disease. For this reason, it may prove to be important to determine if the presence of the worm is a necessity, or whether the effect can be generated by administration of an antigenic factor derived from the worm. It has been shown that a secondary infection with *H. diminuta* will engender a more rapid rejection response than

the primary infection (McKay et al., 1991), indicating that memory T cells do develop in response to the worm infection. From this we could hypothesize that where there has been a previous and resolved *H. diminuta* infection, activation of these memory lymphocytes with an antigen preparation will elicit an anti-colitic effect when mice are challenged with DNBS. Of course, careful consideration of the appropriate dose will be needed, for there is a risk of inducing anaphylactic shock. As well, the appropriate source of the worm, whether it be larval or adult, will need to be determined. There is evidence that *S. mansoni* actually induces different immune responses from the host at different stages of its life cycle (Moreels et al., 2004). Should helminth therapy prove to be a popular alternative, treatment could become as simple as taking a pill composed of worm antigen.

The idea of antigen in a capsule does have promise, as several helminth-derived immunoregulatory molecules have been characterized. A carbohydrate-rich fraction from the tapeworm *Echinococcus granulosus* and an *S. mansoni* lysophosphatidylserine can both increase host synthesis of IL-10 (Dematteis et al., 2001; van der Kleij et al., 2002), while *Nippostrongylus brasiliensis* produces a neuropeptide VIP-like molecule (Foster and Lee, 1995). A whole-worm extract from the subject of the studies presented here, *H. diminuta*, was able to inhibit immune cell proliferation in response to Con A stimulation (Wang and McKay, 2005). More recently, mice treated with *Trichinella spiralis* antigens were found to be protected from DNBS-induced colitis (Motomura et al., 2008).

In conclusion, the potential of helminth therapy has been demonstrated, and the importance of both IL-10 and the AAMs has been defined. While several studies have shown the prophylactic benefits of helminth infection, this was one of the first to describe the anti-colitic effect from the treatment angle. This work was also the first demonstration of the ability of *in vivo* delivery of *in vitro* differentiated AAMs to block colitis in two separate models, defined as either Th1 or Th2. Thus, we have shown that AAM treatment is effective regardless of the immune profile of the disease. The success of helminth therapy in several animal models of disease, including but not limited to allergies, asthma, diabetes and MS, has led to an almost dogmatic belief that this treatment approach will be effective for all patients. Here, we provide an important warning that there may be circumstances in which this treatment is not appropriate.

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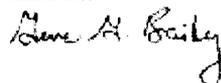
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