**A study on three aspects of Inflammatory Bowel Disease's Triad of
Susceptibility: Hormones, Helminths and T regulatory cells, in the
Helicobacter hepaticus mouse model for IBD**

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

**A STUDY ON THREE ASPECTS OF INFLAMMATORY BOWEL DISEASE'S
TRIAD OF SUSCEPTIBILITY: HORMONES, HELMINTHS AND T
REGULATORY CELLS, IN THE *HELICOBACTER HEPATICUS* MOUSE
MODEL FOR IBD**

presented by Lydia Crystal Cook,

a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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Professor Daniel Hassett

Professor Helen Mullen

Professor Matthew Myles

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*This work is dedicated to all those that have inspired
& supported my pursuit of the question*

“Why”

In particular:

My Family, both immediate and extended,

&

Captain Benjamin Cassutto, DVM (1960-2012)

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***“A soul in tension that's learning to fly
Condition grounded but determined to try
Can't keep my eyes from the circling skies
Tongue-tied and twisted, just an earth-bound misfit, I”
~Pink Floyd, A Momentary Lapse of Reason***

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ABSTRACT

Over 1.4 million American's have been diagnosed with an Inflammatory Bowel Disease (IBD). The etiology of IBDs is unknown, however, it is believed that these diseases develop due to an inappropriate immune response to the microbiota driven by the complex interactions of an individual's genetics, environment and immune system. The research presented here evaluates one aspect from each of the three susceptibility categories (genetics, environment and immune system) utilizing the *H. hepaticus* mouse model for IBD. In this model of IBD, the opportunistic bacterium *H. hepaticus* induces a T_H1 mediated inflammation in the ceca of susceptible mouse strains such as A/J mice. All described experiments utilized cytokine mRNA expression and histologic evaluation.

First, the effects of estrogen signaling on the development of IBD were evaluated in the A/J mouse. Previous studies within our laboratory have demonstrated increased severity of disease in female mice. Here, experiments utilized estrogen receptor (ER) α and β knock-out mice, ER α and ER β specific agonists and the adoptive transfer of CD4⁺ lymphocytes into RAG2^{-/-}, RAG2^{-/-} ER α ^{-/-} and RAG2^{-/-} ER β ^{-/-} mice. Collectively these experiments suggest when estrogen signaling is limited to ER β on a non-CD4⁺ cell population, disease severity is decreased.

Next, the use of a helminth based therapy in this microbiota driven IBD disease model was evaluated. A/J mice with ongoing *H. hepaticus* induced

disease were treated with *Trichuris muris*. The data demonstrate that disease severity is acutely exacerbated and chronically unaltered by *T. muris* therapy. Additionally, we demonstrated that the response to *T. muris*, including the A/J mouse's ability to clear worms, is altered when ongoing T_H1 mediated inflammation exists. These data collectively suggest that helminth therapeutics should be used with caution to treat chronic T_H1 mediated inflammation.

Lastly, the role T regulatory cells in the resistance to *H. hepaticus* induced inflammation was examined. By utilizing neonatal thymectomies and diphtheria toxin treatments, natural T regulatory cells and Foxp3⁺ T regulatory cells were selectively depleted in the C57BL/6 mouse. As this strain of mice is resistant to *H. hepaticus* induced disease, depletion of these cellular populations allowed for evaluation of their role in protection from disease. We demonstrate that neither the depletion of natural or inducible T regulatory cells at the time of *H. hepaticus* inoculation results in disease development in the C57BL/6 mouse. Additionally we present data that suggests *H. hepaticus*, in this mouse strain, drives a protective immune response which may include the induction of inducible T regulatory cells.

Chapter 1 - Inflammatory Bowel Disease

Disease Presentation

Members of the medical community identified the two major forms of Inflammatory Bowel Disease (IBD) over 150 years ago - Ulcerative Colitis (UC) was first described in 1859 by Sir Samuel Wilks¹, followed by Crohn's Disease (CD) in 1932 by Dr. Burril Crohn². Unfortunately, despite major advances in diagnostics and pharmacological treatments available, neither the etiology nor cure for IBD has been discovered. Presently, the focus of IBD research is extremely variable and includes investigations into the influence of genetics, the microbiota, the environment and the immune system on disease development, as well as the identification of viable treatment options. Overall, the scientific and medical communities agree that IBD is the result of an exaggerated and inappropriate immune response, most likely instigated by the commensal microbiota population³.

The Crohn's and Colitis Foundation of America (CCFA) estimates that there are approximately 1.4 million people living with an IBD in the United States. While IBDs are typically diagnosed early in adulthood⁴, it has been estimated that approximately 10% of those patients living with an IBD in the United States are children⁵. As there is no cure for IBDs and the response to available therapeutics is unpredictable, the associated fiscal and emotional costs

throughout an individual's life are high. It is estimated that in the United States alone approximately 1.7 billion dollars a year are spend on IBD associated health care expenses⁵. Furthermore, individuals living with active IBDs have been reported to have a high incidence of depression^{6,7}, and an overall lower quality of life⁸⁻¹¹ than those not living with an IBD.

Although both CD and UC are described as IBDs, their clinical presentations are quite dissimilar. The hallmark clinical sign for an UC patient is presentation with bloody diarrhea with or without accompanying abdominal pain and tenesmus. The clinical presentation of a CD patient is less specific; the most common clinical signs associated with CD are abdominal pain, weight loss and diarrhea. CD patients are more likely to experience fever, malaise and anorexia than their UC counterparts.⁴

Making a diagnosis of either CD or UC can be a long and frustrating process, as the etiology of IBD is unknown and there is not a specific test for diagnosis. Before a diagnosis is made, a general examination of the patient, including blood counts and the evaluation of general organ function must be done. Additionally the patient must be screened for infectious agents such as *Clostridium difficile* which can cause similar clinical signs as those seen in IBD. Often, patients undergo radiography and endoscopic imaging to evaluate the extent of intestinal dilation, to perform tissue biopsies, and to check for strictures, fistulas and abscesses.⁴ The development of serologic marker diagnostics for IBD patients which test for the presence of specific antibodies shown to be up-

regulated in either CD or UC has allowed for more exact disease diagnoses and may eventually aid in the development of a more individualized treatment regimen for patients¹².

Histology and anatomic location of disease can further help distinguish a diagnosis of CD from UC. In general, CD lesions are found predominately in the ileum and proximal colon, but can be found anywhere in the gastrointestinal (GI) tract between the mouth and anus. Lesions are transmural and found in patchy distributions with healthy bowel interspersed with diseased bowel. The development of fistulas between the GI tract and itself, other organs or the skin is almost exclusively found in CD patients. The inflammation found in CD patients is characterized by lymphocytic infiltration and the development of granulomatous inflammation. In contrast the inflammation found in most UC patients usually begins and extends proximally from the rectum and is predominated by neutrophils within the lamina propria (LP) and intestinal crypts.^{3,4,13}

Defining the type of inflammatory response can also help distinguish between CD and UC. Historically it was thought that CD was mediated by a T_H1 type inflammatory response with the over production of IL-12 p40 and IFN- γ ¹³⁻¹⁶, while UC was driven by a T_H2 type inflammatory response including increased production of IL-13^{13,14,17}. Recent data demonstrate that the immune response driving the development of CD also includes the T_H17 inflammatory axis¹⁸⁻²¹. Of vast importance was finding that IL-23, which is involved in the maintenance of

T_H17 mediated inflammation¹⁴, is composed of the p19 and p40 subunits^{22,23}. Previous IBD research which demonstrated the therapeutic effects of IL-12 p40 subunit neutralization in human IBD patients²⁴⁻²⁶ and in animal IBD models²⁷, which were thought to be acting upon IL-12 alone, had to be re-evaluated. Recent literature has demonstrated that T_H17 inflammation, plays a substantial role in human IBD patients^{28,29}. To further complicate matters, the vast reports documenting the apparent involvement and influence of multiple cell populations such as dendritic cells, T regulatory cells, epithelial cells, and T effector cells. The role of these cell populations are further discussed later on in this chapter.

The Triad of Susceptibility

As previously stated, the etiology of IBD is unknown; however, medical and scientific communities alike agree that the development of IBD is due to the interaction of multiple factors and not the result of one particular dysfunctional gene, molecular incident or pathogen exposure. Instead, it is suspected that the interplay between factors such as an individual's sex, microbiota, overall hygiene, early-exposure to antigens, genetics, lifestyle, and physical environment can all affect the likelihood disease development^{3,18,30-32}. For the purposes of this discussion, these factors have been grouped into three main categories: (1) genetics, (2) environment, and (3) the immune system (Figure 1-1).

Genetics

The genetic inheritance from your ancestors can dictate your sex, appearance, anatomical attributes and your susceptibility to the development of diseases, including IBD. The evidence for a genetic influence on the development of UC and CD has been demonstrated repeatedly through epidemiologic studies which evaluated the importance of sex^{33,34} and ethnicity³⁵. Furthermore, identification of numerous susceptibility loci for IBD in general and for UC and CD specifically, has solidified the role of genetics in disease development³⁶.

Familial Predispositions

The influence of race and ethnicity on IBD has been extensively studied in many regions of the world³⁷. Within the United States, Caucasians have the highest incidence of IBD; they are closely followed by African Americans, then Hispanics and Asians^{35,38}. Interestingly, individuals of Hispanic or Asian origin were more likely to develop UC, and African Americans and Caucasians were more likely to develop CD^{35,39,40}. A study done in British Columbia supports these findings – Caucasians were more frequently diagnosed with an IBD and were more likely than either of the Asian populations (Pacific and South Asian) evaluated to develop CD⁴¹. Interestingly, the authors identified differences in disease presentation between the two Asian populations – the South Asian population was more likely to be diagnosed with UC than their Pacific counterparts⁴¹. The occurrence of IBDs within families has also been shown to

be variable dependent on the ethnicity of the family for example, within Caucasians those of Jewish descent are more likely to develop either CD and UC than non-Jewish individuals⁴². A compounding factor of these studies are the environmental factors which may predispose to the development of IBD; because of this the region of the world from which study samples are taken must be considered. This concept is further explored in the “Environmental Influences” section of this chapter.

In regards to familial inheritance of an IBD, it was shown in the early 1990s that your risk of developing IBD was ten times greater than the general population if you had a relative with either UC or CD, suggesting a genetic component to disease development⁴³. Studies done in twins have demonstrated a higher incidence of IBD as a whole in both individuals when the twins are monozygotic, as opposed to dizygotic^{44,45}. Overall, twins have proven to be an invaluable resource for the division of environmental and genetic influences on IBD development⁴⁶.

Sex Bias

Although not nearly as severe as the female sex biases reported in many autoimmune diseases such as Sjogren’s syndrome, rheumatoid arthritis or multiple sclerosis⁴⁷⁻⁵⁰, a slight female sex bias has been reported for CD³³. Furthermore, when severity of disease is evaluated, it has been shown that female IBD patients are more likely to experience severe disease than their male

counterparts³⁴. Interestingly, more female than male patients are affected by other diseases which involve the deregulation of the gastrointestinal (GI) mucosal immune system, such as Irritable Bowel Syndrome⁵¹, colon cancer⁵² and microscopic colitis^{53,54}. Furthermore there are reports of IBD severity being influenced by gonadal hormone related events such pregnancy^{55,56}, estrogen-based hormone replacement therapy⁵⁷ or either the use or cessation of oral contraceptives⁵⁸⁻⁶¹. Overall the literature has presented evidence that gonadal sex hormones could be involved in the development and regulation of IBDs, particularly CD.

Susceptibility Loci

Since the early 2000s when the first CD susceptibility gene NOD2 (CARD15)^{62,63} was identified, the existence of IBD susceptibility loci, has been extensively evaluated. To date, over 160 distinct genetic loci have been identified which can influence the development of IBDs^{36,64-68}. One-hundred and ten of the 163 identified loci are associated with both UC and CD patients, with the remaining 53 split relatively equally between CD (30) and UC (23)⁶⁵. Interestingly, about 70% of the susceptibility loci identified for IBD have also been identified as susceptibility loci for other immune modulated diseases^{36,69}. This overlap supports the influence of other factors such as the environment and microbiota on disease when a genetic modification which has the potential to predispose for IBD development, is present.

Further complicating the matter was the realization that different ethnicities may have different IBD susceptibility loci. The infamous NOD2 loci identified in Caucasian IBD patients, is not important in Asian patients^{62,63}. Research focused on Japanese IBD patients has identified novel susceptibility loci in Asians not previously identified in Caucasian case studies^{70,71}.

Environment

The environment in which you choose to live contains a variety of factors which may affect your immune system in a positive or negative way. Exposure to helminths, cigarette smoking, your microbiota and overall hygiene all have the potential to affect the development of IBD.^{30,32,72}

Microbiota

When discussing IBD, the role of microbiota in disease development is undisputed. For the purposes of this discussion, microbiota is defined as all the bacteria living within the GI tract of an individual person or mouse⁷³. As the number of bacteria living within us vastly outnumber the number of cells in our own bodies⁷³, it is not unexpected that they have the capability to alter intestinal homeostasis⁷⁴⁻⁷⁶. The influence of the microbiota has been documented in both IBD patients^{77,78} and in mouse models⁷⁹⁻⁸¹ of IBD. In general, the microbiota has been shown to be integral in the development of a functional immune system⁸². Interestingly, several of the genetic susceptibility loci identified for IBD, such as NOD2 and ATG16L1 are involved in the recognition and response to the

microbiota⁷⁸. A recent publication which has correlated the use of antibiotics in children with the development of IBDs also lends support to the need for an unaltered and healthy microbiota for proper immune development³¹.

The microbiota has been shown to interact with multiple cell types involved in the maintenance of GI homeostasis. For example, the induction of inducible T regulatory cells, a set of regulatory cells which can suppress inflammatory processes such as colitis, has been shown to be influenced by the microbiota⁸³⁻⁸⁶. Furthermore, adoptive transfer experiments in germfree mice have demonstrated that the microbiota is necessary for the induction of T cells responsible for colitis development⁸⁷⁻⁸⁹. The discovery that dendritic cells (DC) penetrate the epithelial barrier to sample luminal contents further solidified the involvement of the microbiota in gut homeostasis^{90,91}. As the gut epithelium is the physical barrier between members of the microbiome and the immune system, is not surprising that the expression of cytokines by the epithelium can influence dendritic cell luminal sampling⁹².

It is likely that the influence of microbiota on the development of IBD is dependent upon the pre-existence of other risk factors however, the possibility of pathogenic bacteria being responsible for IBD cannot be completely ruled out at this time. The development of advanced microbiome analysis techniques will help shed light on these unanswered questions.

The hygiene hypothesis

In the early 1990s it was proposed that excessive cleanliness can deprive the developing immune system of the antigen exposure needed to prime appropriate immune responses. This phenomenon is now known as the hygiene hypothesis and simply suggests that lack of antigen exposure early in life can predispose children to the development of immune-related diseases, such as IBD⁹³. In the time since, the hygiene hypothesis has been extensively evaluated and not yet been disproved⁹⁴⁻⁹⁷.

Developed versus Developing Countries

As previously mentioned, IBDs are a diagnosis historically made in the developed countries of North America and Europe¹⁸ however, the rate of diagnosis in other regions of the world such as Asia and Africa has slowly increased^{37,98}. It was once believed that race, due to familial genetics, was an important factor in the development of IBD however, recent studies have provided data that lifestyle and environment may have more of an influence than genetics as incidence of disease appears to be altered with a change in geography. For example, a study that compared the incidence of UC in South Asian immigrants to the United Kingdom demonstrated that second generation immigrants had a higher incidence of UC, in comparison to first generation immigrants⁹⁹. Furthermore the incidence of UC in the second generation population was comparable to that seen in the indigenous European population,

further lending support to the hygiene hypothesis and the importance of environment in development of IBD⁹⁹.

Helminths

When considering exposure to antigens capable of affecting gut homeostasis in developed and developing countries, gastrointestinal parasites immediately come to mind. In developed countries where the incidence of IBD is high, Westernized living's hygiene standards have included the eradication of gastrointestinal parasites^{95,96}. Interestingly, reports have emerged that suggest the integration of western lifestyle practices, such as the use of anti-helminthics, could be linked to increased IBD diagnosis rates in regions such as Asia, Africa and South America³⁷. For example, in South Africa, where western hygiene standards are being practiced by some individuals, exposure to helminths during childhood appears to be protective against IBD development¹⁰⁰. Similar trends have been noted in China, where a rise in IBD diagnoses correlates with the introduction of Westernized living practices⁹⁸.

Smoking

A relationship between IBD development and tobacco use has been identified¹⁰¹⁻¹⁰⁴. As approximately 1 out of every 5 adults in westernized countries smoke, the effects on the immune system and inflammation in general have been extensively researched¹⁰². As cigarette smoking has been repeatedly shown to having systemic pro-inflammatory effects, it is not surprising that it has

an effect on the development of IBD. What was surprising, however, was the finding that while smoking increases the risk for CD, it is protective for the development of UC¹⁰¹⁻¹⁰⁴.

Interestingly, a recent publication demonstrated that cessation of cigarette smoking correlated with a considerable shift in the microbiota population, that include an increase in bacterial diversity¹⁰⁵. Findings such as these demonstrate the complex relationship between factors previously thought to independently effect disease development. It is yet to be determined if the protective effects of cigarette smoking seen in UC are due to the decreased stimulation by a less complex microbiota or decreased levels a particular microbe responsible for inciting inflammation.

The Immune System

Although IBDs are believed to develop when a disturbance in normal immune function occurs¹⁰⁶, they are not classified as an autoimmune disease as the immune response is not targeted towards self-antigens¹⁰⁷. Rather, the immune response is most likely targeted towards endogenous, non-pathogenic bacteria. Mouse models and human research have identified many members of the immune system as being involved in the development and resulting inflammation seen in IBDs³⁶.

Effector T cells

Following thymic development and selection, naïve T cells capable of differentiating into effector T cell populations, migrate to the periphery¹⁰⁸. Their differentiation into T_H1, T_H2, or T_H17 effector cells is mediated by the expression of IL-12, IL-4 and IL-6/TGF β , respectively^{14,109-112}. Originally it was thought that CD was driven by the expansion of T_H1 T cells and UC by T_H2 T cells. However, recent advances in the understanding of T_H17 inflammatory pathways suggest the involvement of these effector cells in CD as well^{14,113,114}.

Following differentiation, T effector cells secrete pro-inflammatory cytokines such as IFN- γ , IL-13 and IL-17. It has been shown in IBD that the elevated levels of these cytokines are predominately produced by activated T cells^{3,14,17,108}. Mouse models of IBD have demonstrated the importance of lymphocytes in the development of colitis and general intestinal inflammation through adoptive transfer experiments and studies utilizing lymphocyte deficient mice^{16,115-119}. Elevated production of the T_H2 cytokine IL-13 in UC patients^{15-17,120} and the T_H1 and T_H17 cytokines IFN- γ and IL-17 in CD patients^{3,14-16,21,121} has further supported the involvement of lymphocytes in disease progression. Antibody neutralization therapies which target the actions of lymphocytes by inhibiting IFN- γ and IL-12/23 have had promising results^{25,122-124}. T effector cells are suppressed endogenously by IL-10 and TGF β which are secreted and expressed by T regulatory cells.

T regulatory cells

T regulatory cells are a type of lymphocyte generally identified by the expression of the transcription factor *Foxp3* and are capable of suppressing the immune system through the secretion of anti-inflammatory cytokines such as IL-10 and TGFβ¹²⁵⁻¹³⁷. The concept of a suppressive cell that could control the immune system had existed within the scientific community for decades¹³⁸ and was truly implied in the mid-1900s with the observation that removal of the thymus causes an uncontrolled immune response which ultimately results in autoimmunity¹³⁹⁻¹⁴². Since this time, thousands of articles attempting to characterize and describe this cell population have been published, including one in 2003 by Bluestone and Abbas which suggested the existence of not one but two distinct CD4⁺ regulatory cell populations: a natural and an inducible T regulatory cell¹⁴³. Their speculations have since been proven^{132,133,143-153} and it is now known that inducible T regulatory cells can develop from CD4⁺ naïve T cells in the periphery^{127,128,138-148,154-158}. Interestingly, the microbiota which is believed to be involved in IBD development, has been shown to play an important role in the induction of inducible T regulatory cells which can control the development of colitis^{84,86}.

Humans without a functional *Foxp3* gene lack T regulatory cells and many develop severe intestinal inflammation due to the uncontrolled infiltration of lymphocytes¹⁵⁹⁻¹⁶². In a similar fashion, mice without functional T regulatory cells have been shown to develop colitis^{163,164}. It has not been shown that those with

IBD have dysfunctional T regulatory cells. However, it has been demonstrated that a decreased proportion of T regulatory cells are present in IBD patients compared to healthy controls¹⁶⁵, which may be due to an increased rate of T regulatory cell apoptosis¹⁶⁶. As T regulatory cells have been shown to suppress lymphocyte proliferation *in vitro* via IL-10 and TGF β ¹⁶⁷⁻¹⁶⁹, it is not surprising that they can suppress colitis development *in vivo*^{118,170,171}. Furthermore, T regulatory cells have been shown to be capable of both preventing and reversing colitis in mouse IBD models^{116-119,155,172-178}.

Dendritic Cells

Dendritic cells (DCs), are an antigen presenting cell (APC) which are present in the lamina propria of the gut and are responsible for directing immune responses to pathogens. DC have been shown to penetrate the epithelium in order to monitor the microbiota makeup^{90,91}. Following antigen recognition, either an inflammatory immune response or the induction of tolerance can be orchestrated by DCs¹⁷⁹⁻¹⁸¹. In CD patients, intestinal DC have been shown to produce elevated levels of the cytokine IL-12, which drives T_H1 lymphocyte differentiation, in comparison to healthy controls¹⁸². Additionally, the expression of toll-like receptors (TLRs) on DCs has been shown to be elevated in IBD patients compared to controls¹⁸².

Mouse models of IBD have further demonstrated the involvement and importance of DC in the maintenance of intestinal homeostasis. In the dextran

sulfate sodium (DSS) mouse model of colitis, selective depletion of dendritic cells decreases inflammation, while the adoptive transfer of DC increases disease severity¹⁸³. Clustering of DC and lymphocytes has been observed prior to the onset of colitis and suggests these two cell types interact during disease development¹⁸⁴. Interestingly, in both adoptive transfer and DSS mouse models of IBD, active colitis resulted in a decreased number of CD103⁺ DCs¹⁸⁵, which have been reported to be required for the induction of inducible T regulatory cells in the periphery¹⁸⁶⁻¹⁸⁸.

The Epithelium

The gastrointestinal epithelium is not only a physical barrier between the gut lumen and the body, but also a highly dynamic system intricately involved in the maintenance of gut homeostasis in which commensal bacteria are tolerated and pathogens incite an immune response¹⁸⁹. Epithelial cells routinely utilize pattern recognition receptors (PRR), such as toll-like receptors, to survey luminal contents¹⁸⁹⁻¹⁹². Although specific TLR defects have not been identified in IBD patients, several susceptibility loci contain TLR signaling components and some believe alterations in TLR capabilities may contribute to differences in disease severity between patients¹⁹³. NOD2 mutations identified in IBDs^{62,63} can affect the PRR activity of a specialized epithelium cell known as the Paneth cell, which in addition to pathogen recognition also secretes defensins that have antimicrobial activities¹⁹⁴.

In addition to pathogen recognition, epithelial cells are capable of directing dendritic cell activities through the production of cytokines⁹² and have been shown to play a role in the activation of T cells¹⁹⁵. Disruption of the nuclear factor- κ B (NF- κ B) signaling pathway, which drives the transcription of cytokines when unaltered, within epithelial cells has shown to disrupt GI homeostasis and can result in increased susceptibility to colitis in mouse IBD models^{196,197}.

Not to be overlooked is the mucus component of the GI epithelium, which is secreted by goblet cells, and covers the epithelial cell luminal surface serving as an additional layer of defense against bacteria¹⁹⁸. While UC patients have been shown to have decreased colonic mucus layers, CD patients do not appear to share this defect¹⁹⁹.

Therapeutics for IBD

While not classified as a disease with high mortality, IBDs require life-long medical attention as there is no known cure. In general, the treatment options available to patients are designed to suppress clinical signs and include surgery, pharmaceuticals and alternative therapies. Special consideration in treatment plans must be made for children^{5,200-202}, the elderly^{203,204} and women currently or planning on becoming pregnant^{4,205}. Additionally, the mental health of IBD patients must be taken into consideration when a treatment plan is being developed^{206,207}. Overall the treatment of IBD is complicated by the “sub-groups” of patients which respond differently to treatment, suggesting the need for

personalized medicine in which treatment is tailored to each patient individually, rather than to the disease diagnosis in general²⁰⁸⁻²¹⁰.

Surgery

Today, there is a high surgical resection rate among IBD patients, approximately 20-30% of UC patients and 70-80% of CD patients will undergo a surgical resection during their lifetimes as a result of uncontrolled disease⁴. The decision to undergo surgery for an IBD patient is most often due to the persistence of uncontrollable clinical signs. UC patients are typically treated more aggressively in regards to surgical resection of the bowel due to the consistent anatomical location of lesions⁴. Often, those with UC, will have a total proctocolectomy instead of a partial colectomy due to the high probability that disease will re-develop in the remaining colon²⁰³. While UC patients who have undergone a total proctocolectomy will often live disease free postoperatively, this is not the case for CD patients who have undergone bowel resections²⁰³.

For a CD patient, surgical resections are more conservative and are recommended to be limited to areas of symptomatic macroscopic disease⁴ such as obstructions, abdominal abscesses, bleeding, the development of cancer, and extreme cases of stricture or dilation²⁰³. The use of laproscopic surgery has made IBD related procedures less invasive, thus making surgery a viable option for patients who would not be considered surgical candidates due to the risks associated with traditional surgery techniques²⁰³.

Pharmaceuticals

Pharmaceutical treatment for IBD patients include the use of: aminosalicylates, corticosteroids, immune modulators and biological therapies^{4,211-214}. Unfortunately, widely variable remission rates are reported with these treatments^{211,215} and can be difficult to interpret due to IBD's cyclic patterns of exacerbation and remission⁴. Also, many IBD clinical trials lack evaluation of a true placebo group, which further complicates the ability to identify the true efficacy of proposed treatments²¹⁵.

Aminosalicylate treatment utilizes 5-ASA derivatives for the treatment of mild to moderate UC and CD²¹⁶. Its main target are GI epithelial cells⁴ where it is suspected to limit the release of inflammatory mediators such as prostaglandins and leukotrienes which ultimately, through decreased release of cytokine and reactive oxygen metabolites, inhibit activation of NF- κ B²¹⁶.

Corticosteroids can be administered orally (prednisolone, prednisone and budesonide), intravenously (hydrocortisone or methylprednisolone) or topically (hydrocortisone and prednisolone)⁴. These drugs are generic anti-inflammatories that suppress the induction of inflammatory pathways such as I κ B, which is upstream of NF- κ B activation. Additional mechanisms of action include: decreased interleukin transcription and the induction of lymphocyte apoptosis within the GI lamina propria.⁴ The side effects associated with corticosteroid use and their lack of efficacy beyond suppression of acute inflammation limit their long-term use in IBD patients^{4,216,217}. Immune modulators which target either

particular cell populations or mechanisms required for cell life can also be used in IBD patients. Examples include methotrexate which inhibits DNA and RNA synthesis through dihydrofolate reductase inhibition and ciclosporin (CsA) which inhibits calcineurin which limits lymphocyte expansion⁴. As with corticosteroids, generic immunosuppression can occur with long term treatment²¹⁶.

Tailored biological therapies target specific signaling molecules and receptors involved in the IBD inflammatory response. Treatments which neutralize TNF α are popular and include adalimumab, certolizumab pegol and infliximab^{4,122,212,216}. Therapies targeting CD3⁺ and CD25⁺ T cell populations utilize the antibody based drugs visilizumab and daclizumab, respectively^{122,212}. Also available are treatments which neutralize or block the receptors of IL-12, IL-6, IFN- γ and IL-2¹²². Interestingly, responses to supplemented recombinant IL-10, which was thought to be integral in the suppression of IBD development, have been contradictory and sometimes unsuccessful in clinical trials²¹⁸⁻²²¹.

Alternative Therapies

The pharmaceuticals previously described have not been developed to target the etiology of IBD but rather the resulting inflammatory processes associated with disease and clinical signs. As previously stated, the remission rates achievable with these therapeutics are low²¹¹ and has led to investigations into non-traditional therapeutic options for the treatment of IBD such as helminths, cell based therapeutics and the use of probiotics or antibiotics to alter the microbiota²¹².

Helminths

As previously discussed, the hygiene hypothesis suggests that in an attempt to achieve cleanliness, people are depriving their immune systems of antigen stimulation needed for a balanced immune system. One such exposure situation is helminths. As a result, the use of helminths as a therapeutic is currently being examined in IBD patients²²²⁻²²⁶. Preliminary data generated in clinical trials which administered *Trichuris suis* to patients with active IBD demonstrates a decrease in disease related symptoms and/ or the induction of remission in approximately 70% of patients^{227,228}. A study documenting the long-term effects of *T. suis* in humans without IBD demonstrated that within four months of continued treatment there was a lack of clinical signs associated with helminthosis²²⁹. Although results from these clinical trials have been promising, patient reluctance to be treated with worms and the documented negative effects of acute parasitism with *T. suis*, has limited this method of treatment²²⁹. Further complicating the use of helminths as a therapeutic option is the lack of quality control protocols for the generation of medical-grade helminths^{230,231}. To circumvent the negative attributes of helminth therapy, worm derived proteins and/or molecules is also being considered²³⁰⁻²³².

Antibiotics & Probiotics

As the microbiota has been repeatedly demonstrated to be integral in the development of IBD, it is logical that antibiotics and probiotics, both of which

have the potential to alter the microbiota population, would be considered therapeutically. Like antibiotics, which are drugs that target specific classifications of bacteria, the administration of probiotics, which typically consist of live bacteria, to the IBD patient has the potential to alter the microbiota makeup by preventing the overgrowth of pathogenic bacteria^{74,77,233,234}.

It has been demonstrated that both antibiotics and probiotics can have positive effects in UC patients with pouchitis^{77,235}. In practice, the antibiotics metronidazole and ciprofloxacin are often used in conjunction with the traditional pharmaceuticals discussed earlier, and their use in CD patients seems to be beneficial in achieving and maintaining remission^{77,216,235}. Whether antibiotics and probiotics have the capability to act upon a specific bacterial population which, unbeknownst to us, is responsible for disease development or are merely beneficial in restoring a healthy microbiome is unknown. Controlled clinical trials are needed to further evaluate the effects of both in IBD patients.

Cell-Based Therapeutics

Cell based therapeutics are an interesting alternative to traditional therapeutics for IBD and involve treatment with or targeting of a particular cell population. Treatment with T regulatory cells is currently being examined as an IBD therapy and involves the isolation and/or induction of T regulatory cells from a patient to be used in their own treatment regimen^{208,236,237}. Supporting the possible applications of this new therapy, is the recent discovery that T regulatory

cells can be isolated from patients with CD, cultured, propagated in vitro and then used to suppress T cell proliferation²³⁸. Despite the political controversy associated with stem cell therapy, preliminary clinical trials have produced promising results in IBD patients^{122,208,212}.

Clinical trials involving selective cell depletion, accomplished with apheresis through either centrifugation or column filtration appear to have beneficial effects in IBD patients¹²². Granulocytes, monocytes, lymphocytes and platelets can all be removed from IBD patients with this technique; the cells targeted and extent of depletion depends upon the apheresis technique utilized^{122,212}.

Unfortunately, despite the extensive array of traditional and non-traditional therapeutic options available for IBD patients, a panacea has not been found. Overall, managing IBD is a difficult task considering the extreme variations in remission rates reported within IBD patients^{211,215}, the unpredictable cyclic patterns of exacerbation and remission⁴ and the general lack of balanced clinical trials utilizing placebo controls²¹⁵. As previously stated, the need for a personalized medicine approach in which patients undergo treatments specifically tailored for them following biomarker and genetic analysis, rather than to the disease diagnosis in general, is needed²⁰⁸.

The Helicobacter hepaticus model of IBD

Attributes of the model

There are a multitude of mouse models utilized to study IBD, all of which utilize different “triggers” to induce IBD-like inflammation; examples of which include: adoptive cell transfers, the administration of haptening agents and genetically modified mice²³⁹⁻²⁴¹. The trigger used in our model of IBD is the bacterium *Helicobacter hepaticus*. The genus *Helicobacter* includes multiple bacteria, all of which are motile, gram-negative, microaerophilic organisms. This genus is most known for *H. pylori* which has been repeatedly shown to be associated with the development of gastritis, gastric ulcers and gastric cancer, such as adenocarcinoma, in humans²⁴²⁻²⁴⁴. Multiple helicobacter species have been identified which infect mice including, but not limited to: *H. hepaticus*, *H. mastomyrinus*, *H. bilis*, *H. ganmani*, *H. felis*, *H. muridarum* and *H. thyphoni*²⁴⁵⁻²⁵⁰. As many rodent vendors and institutional research colonies have been shown to harbor *Helicobacter spp.*, the role these bacteria play in disease modeling and overall animal health has been questioned^{245,247,251}.

When the prevalence of *H. hepaticus*, the species utilized in our IBD model, was evaluated it was found that many wild-type mice harbor the bacteria but do not exhibit disease²⁴⁵. Because of the overall lack of pathogenic evidence in most mouse strains, *H. hepaticus* it is not thought of as an infectious pathogen, but rather as an opportunistic member of the murine microbiota. In the

genetically susceptible A/J mouse, however *H. hepaticus* is capable of inducing inflammation of the cecum which can be utilized as an IBD model^{25,252-254}.

Although the exact mechanism of action is unknown, the presence of *H. hepaticus* is thought to catalyze an immune response towards other members of the microbiota, which results in the development of inflammation. The presence of *Helicobacter spp.* in general, and *H. hepaticus* specifically, has been demonstrated to have immune modulating capabilities within the GI system^{89,255-264}. Furthermore, several models of IBD, have reported the need for *H. hepaticus*, in combination with the endogenous microbiota, for the development of consistent, severe disease^{79,265-267}. Although the role of the microbiota is undisputed in human IBD, helicobacters have not been linked to disease development to date²⁶⁸.

Overall, the A/J *H. hepaticus* model for IBD recapitulates the hypothesized dysfunctional interaction between an individual's immune system and microbiota, which may or may not contain pathogenic members^{75,80}. As previously discussed, a genetic susceptibility loci has not been identified that is consistent for all IBD patients, thus suggesting the importance of other factors such as the microbiota^{74,75,80} and environmental influence^{30,32}. Unlike other mouse IBD models which require a genetic modification or exposure to a haptening agent²³⁹⁻²⁴¹, this model is triggered by a microbiota member.

One distinction of the *H. hepaticus* IBD model is that it incorporates a genetic susceptibility component as many inbred mouse strains infected with *H. hepaticus* exhibit no evidence of disease development^{25,245,247}. For example, A/J mice administered *H. hepaticus* develop histologic inflammatory lesions in the cecum three months post inoculation while C57BL/6 mice do not^{25,254}. It is unknown as to why these two mouse strains ultimately respond differently to the addition of *H. hepaticus* to the endogenous microbiome. Despite knowing that both strains are persistently colonized, data on the effect of *H. hepaticus* bacterial loads on the immune response has been contradictory^{25,269}. Differences in genetics between the two strains has also been evaluated with Quantitative Trait Loci (QTL) and loci have been identified on chromosomes 3 and 17 which are thought to be linked to disease susceptibility, as identified by the production of IL-12/23 p40. Several of the potential genes identified in this QTL analysis have been shown to be involved in disease development in human IBD patients, thus giving further support for the use of this mouse model for the study of IBD.²⁷⁰

An additional shared attribute of the *H. hepaticus* IBD model and IBD is the existence of both a sex bias and differences in disease severity between the sexes. CD has a documented slight female sex bias³³ and, female IBD patients are more likely to experience severe disease than their male counterparts³⁴. In a similar fashion, female A/J mice inoculated with *H. hepaticus* develop a more severe histologic disease than inoculated male A/J mice²⁵². Lastly, the

inflammation seen in female A/J mice recapitulates several aspects of that seen in CD including the up-regulation of IL-12/23 p40 and IFN- γ ^{25,253}.

Disease Presentation

Acute Immune Response (4 days post-inoculation)

Four days following inoculation with *H. hepaticus* both A/J and C57BL/6 mice have elevated expression of pro-inflammatory cytokine and chemokine genes²⁵. At this time point, no histologically detectable changes exist. Included in the list of elevated cytokines are the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines IL-12/23 p40 and IFN- γ ^{25,252-254}.

The cytokine IL-12 is comprised of two subunits: p40 and p70 and is produced by APCs such as DCs and other phagocytotic cells, like macrophages, which are involved in the initial immune response to bacteria²⁷¹. The production of IL-12 drives T_H1 differentiation of naïve T cells, while simultaneously inhibiting T_H2 differentiation, which is typically mediated through IL-4^{110,114,272,273}. Naïve T cells differentiate into T_H1 lymphocytes following stimulation with IL-12 and IFN- γ ; the initial production of IFN- γ is attributed to NK cells following bacterial stimulation¹⁰⁸. The resulting T_H1 lymphocytes, after activation of the JAK/STAT1 and T-bet signaling pathways, secrete more IFN- γ ^{108,274,275}, which acts in a positive feedback fashion to further induce the production of IL-12 by APCs²⁷⁶. This perpetuates the differentiation of more naïve T cells into T_H1 lymphocytes¹⁰⁸. This positive feedback loop could potentially be involved in the uncontrolled

inflammation seen in IBD, as neutralization of IFN- γ can prevent disease development in the *H. hepaticus* IBD mouse model^{25,123,124}.

IL-10 involvement in IBD development is also logical, as IL-10 knock-out mice exhibit cecal inflammation^{265,277,278}, as do humans with IL-10 production or receptor defects²²⁰. IL-10 is produced by multiple cell types, including T regulatory cells and suppresses inflammation by inhibiting the differentiation of lymphocytes through the down regulation of IL-12 production by dendritic cells and macrophages^{108,172}. *H. hepaticus* IBD mouse models have repeatedly demonstrated the importance of T regulatory cells and IL-10 in abrogation or prevention of disease development^{118,123,124,176}.

Chronic Immune Response and Disease Development (90 days post-inoculation)

Despite both mouse strain's immediate response to *H. hepaticus*, only the A/J mouse has a detectable immune response 90 days post-inoculation. At this time, in addition to the elevations in mRNA expression seen 4 days post-inoculation, A/J mice inoculated with *H. hepaticus* will have detectable typhlitis (inflammation of the cecum). This inflammation can be described as an infiltration of lymphocytes within the lamina propria of the cecum^{25,252-254} and is characterized by a chronic T_H1 (IL-12/23 p40 and IFN- γ) driven intestinal inflammation^{25,253} which is similar to the distribution and degree of inflammation seen in CD patients^{15,16}. C57BL/6 mice evaluated at this time point will not have elevations in mRNA gene expression, nor will they have histologic disease⁸.

Prologue to Chapters 2-4

The research presented here evaluates one aspect from each of the **three susceptibility categories (genetics, environment and immune system) utilizing the *H. hepaticus* mouse model for IBD**. First, in Chapter 2, the effects of **estrogen signaling** on the development of IBD is evaluated. Previous studies within our laboratory have demonstrated increased severity of disease in female mice. The experiments described in Chapter 2 utilized estrogen receptor (ER) knock-out mice, ER specific agonists and adoptive transfers to demonstrate that decreased severity of disease occurs when estrogen signaling is limited to ER β on a non-CD4⁺ cell population. This data was generated through the collaborative efforts of both the author and Dr. Andrew Hillhouse.

In Chapter 3, the use of a **helminth based therapy** in this microbiota driven IBD disease model was evaluated. Our results suggest that the use of helminths, when disease is driven by a particular member of the microbiota, is not recommended and has the potential to exacerbate disease severity. Furthermore, we demonstrated that the ability to clear the helminth *T. muris* is hindered by the presence of *H. hepaticus*, thus suggesting that an individual's immune response to helminths can be altered by their microbiota.

Next, in Chapter 4, we evaluated the role of **natural and inducible T regulatory cells** in immune response to *H. hepaticus*, which serves as our

model's trigger for IBD-like inflammation. With the use of neonatal thymectomies and selective depletion of Foxp3⁺ cells the role of inducible and natural T regulatory cells were evaluated. We demonstrated that inducible T regulatory cells, but not natural T regulatory cells, have a protective function in microbial driven inflammation. The final chapter briefly outlines the author's unpublished experiments which often served as preliminary data for those experiments described in Chapters 3-5.

Figure 1-1. The Inflammatory Bowel Disease Triad of Susceptibility.

The development of IBD has not been linked to any one particular pathogen, gene or exposure incidence. The development of this disease is believed to be driven by the complex interactions of an individual's environment, immune system and genetics.

Chapter 2 - The Role of Estrogen Signaling in a Mouse Model of Inflammatory Bowel Disease: A *Helicobacter hepaticus* model

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are classified as inflammatory bowel diseases (IBDs) and are diagnosed primarily in North America and northern Europe³. There are approximately one million people in the U.S. living with IBDs⁵, and while the cause is unknown, differences in intestinal microbiota, hygiene, genetics, geography, and the environment can contribute to disease development³. There is no known cure for IBDs and current treatments are predominantly pharmaceutical, merely suppressing clinical symptoms. When pharmaceutical intervention fails, the final option for treatment is bowel resection.

ER α and ER β modulate gene expression by directly interacting with estrogen response elements or other transcription factors²⁷⁹, and have been implicated in the modulation of several immune-mediated diseases⁴⁸. For example, physiologic events that result in elevated estrogen levels are associated with increased disease severity in systemic lupus erythematosus (SLE) patients, but decreased disease severity in rheumatoid arthritis (RA) and multiple sclerosis (MS) patients^{47,49,50}. Furthermore, there are distinct sex biases in colon cancer⁵² and inflammatory intestinal disorders such as Irritable Bowel Syndrome

(IBS)⁵¹ and microscopic colitides^{53,54}, suggesting a role for gonadal sex hormones (GSHs), including estrogen, in the modulation of intestinal health. GSH-related events, such as menopause, pregnancy and the use of oral contraceptives can modulate IBD. In particular, the development of IBD has been associated with oral contraceptive use^{58,59} and the cessation of oral contraceptive use has been associated with the resolution of clinical signs and symptoms^{60,61}. Furthermore, women are partially protected from disease flare-ups during pregnancy⁵⁵ and estrogen-based hormone replacement therapies are associated with decreases in disease severity in post-menopausal patients⁵⁷.

Animal models of IBD also support the use of estrogen therapies to modulate disease. For example, in an IBD model utilizing the HLA-B27 transgenic rat, estrogen administration decreases diarrhea and intestinal inflammation²⁸⁰. Further work with this model has demonstrated decreased disease severity when NF- κ B signaling is inhibited by ERB-041, a selective ER β antagonist²⁸¹⁻²⁸³. Furthermore, estrogen administration decreases disease in other IBD models such as dinitrobenzene sulfonic acid (DNB)-induced and acetic acid-induced colitides^{284,285}. Finally, estrogen signaling has also been shown to protect against the development of colitis-associated diseases, such as neoplasia, in the azoxymethane/dextrane sodium sulfate (DSS)-induced colorectal cancer model²⁸⁶.

In the *Helicobacter hepaticus*-induced model of IBD, susceptible mouse strains, such as the A/J mouse, develop histologically observable disease in the

cecum three months post-inoculation²⁵. While the exact mechanism by which *H. hepaticus* initiates disease is unknown, it is believed that *H. hepaticus* colonizes the cecum and serves as a provocateur for a CD-like inflammatory response against endogenous microbiota. Findings by Cahill *et al.* and Maggio-Price *et al.* demonstrated that the presence of *H. hepaticus* modulates mucosal immune responses^{89,256-258}. *H. hepaticus*-induced typhlitis is characterized by lymphocytic infiltration of the lamina propria and increased expression of the T_H1 cytokine genes IL-12/23 p40 and IFN- γ , as well as TNF- α and the chemokines CXCL9 and CXCL10^{25,252-254}. *H. hepaticus*-infected female A/J mice develop more severe intestinal inflammation and have significantly higher T_H1 cytokine gene expression than infected males²⁵². This data and the previously reported associations between estrogen and IBD led to the hypothesis that estrogen signaling modulates the development of *H. hepaticus*-induced inflammation. To test this hypothesis, *H. hepaticus*-infected ER α - and ER β -knockout mice and mice treated with ER specific agonists were used. Additionally, an adoptive transfer model was used to identify the cell type through which estrogen-mediated events occur.

Results

Estrogen administration decreases disease severity

To evaluate the role of estrogen, female mice were ovariectomized at 3-4 weeks of age and implanted with either a placebo (control) or continuous-release

hormone pellet (1.5 mg - supraphysiologic dose of 17 β -estradiol, E2). All mice were orally inoculated with *H. hepaticus* one week post-operatively. Ninety days post-inoculation, mice were sacrificed and ceca collected for the evaluation of histologic disease and mRNA expression.

The ceca of placebo-treated animals had an increase in cellular infiltration and thickening of the cecal mucosa (Figure 2-1A, top), whereas the E2-treated animals had little to no inflammatory infiltrates (Figure 2-1A, bottom). Evaluation of cecal histologic sections revealed that E2-treated animals had significantly lower lesion scores than those that received the placebo treatment (Figure 2-1B).

We previously demonstrated that mRNA expression of IL-12/23 p40, IFN- γ and CXCL9 is elevated during *H. hepaticus*-induced intestinal inflammation²⁵. Estrogen treatment significantly reduced the cecal expression of all three inflammatory mediators compared to placebo-treated animals (Figure 2-1C). The decreases in inflammation and inflammatory gene expression suggest estrogen could effectively reduce disease severity.

Decreased disease severity when estrogen signaling is limited to ER β

To determine the relative role of the two estrogen receptors, ER α and ER β , in modulating disease, A/J ER α ^{-/-} and ER β ^{-/-} mice were inoculated with *H. hepaticus* at 3-4 weeks of age. To serve as controls, heterozygotes (ER α ^{+/-} or ER β ^{+/-}) and wild-type littermates were also inoculated. Ninety days post-inoculation, mice were necropsied and their ceca assessed for histologic

inflammation and mRNA expression. Mean lesion scores of *ERβ*-deficient mice did not differ from those of the control mice, however *ERα* deficiency resulted in a significant decrease in disease (Figure 2-2A). Because female IBD patients are more likely than male patients to experience severe disease³⁴, the incidence of severe disease in this model (defined as a lesion score ≥ 6) was evaluated. When estrogen signaling was limited to *ERβ* (*ERα*-deficient mice) a significantly lower incidence of severe disease was observed in comparison to control and *ERβ*-deficient mouse groups (Figure 2-2B). Analyses of the ceca of *ERα*-deficient mice showed significantly decreased mRNA expression of *CXCL9* and a trend of decreased *IFN-γ* expression levels when compared to littermate controls; there were no differences in the expression of *IL-12/23 p40* (Figure 2-2C).

***ERβ* signaling decreases the development of inflammation and inflammatory cytokine expression**

Although data generated using knockout mice suggest that lack of functional *ERα* decreases disease development, it is possible that these changes are the result of preferential estrogen signaling through *ERβ*, rather than the lack of *ERα*. To better identify the mechanism by which disease development is modulated, ER-specific agonists were utilized.

Wild type A/J mice were ovariectomized at 3-4 weeks of age, implanted with an *ERβ* agonist (DPN), *ERα* agonist (PPT), or placebo continuous-release

pellet, and then inoculated with *H. hepaticus* one week post-operatively. Ninety days post-inoculation, mice were sacrificed and ceca collected for evaluation.

Histologic evaluation demonstrated that treatment with the ER β agonist (DPN) was associated with significantly less cecal inflammation compared to the control mice (Figure 2-3A), while treatment with the ER α agonist (PPT) did not alter intestinal inflammation compared to control mice. The percentage of mice with severe disease (severe lesion score ≥ 6) in the group treated with DPN was significantly less than the control group (Figure 2-3B). Cecal expression of *IL-12/23 p40* and *CXCL9* was significantly less than in placebo-treated control mice. Again, there was a trend of decreased *IFN- γ* expression in the DPN-treated mice when compared to the control and PPT-treated mouse groups (Figure 2-3C). Collectively, these results indicate that signaling through ER β reduces the expression of *CXCL9*, and *IL-12/23 p40*, and that this ultimately results in reduced intestinal inflammation.

Estrogen signaling on non-CD4⁺ lymphocytes suppresses disease development and decreases IFN- γ production

Previous studies in our laboratory have demonstrated that *H. hepaticus* induces a T_H1 inflammatory response, suggesting a role for CD4⁺ T cells in disease development. Administration of the ER β agonist decreases expression of genes expressed in CD4⁺ and non-CD4⁺ cellular subsets. To investigate the influence estrogen receptors have on individual cellular subsets, adoptive

transfer experiments were carried out using controlled expression of estrogen receptors on CD4⁺ T cells and non-CD4⁺ T cells.

In the first experiment, splenic CD4⁺ lymphocytes were isolated from mice deficient for either *ERα* or *ERβ* and adoptively transferred to *RAG2*^{-/-} A/J mice. As a control, CD4⁺ lymphocytes isolated from wild type A/J mice were adoptively transferred to *RAG2*^{-/-} recipients. Ninety days after adoptive transfers, all mice were sacrificed and ceca collected. Histologic evaluation revealed no significant differences in mean lesion scores between mice that received CD4⁺ lymphocytes from wild type, *ERα*-deficient, or *ERβ*-deficient donors (Figure 2-4A). Analysis of the cecal expression of *IL-12/23 p40*, *IFN-γ* and *CXCL9* revealed no significant differences between the experimental and control mice (Figure 2-4B-D), implying that estrogen signaling on a CD4⁺ cell population is not responsible for altering disease development.

In the second adoptive transfer experiment, the role of *ERα* and *ERβ* on non-CD4⁺ lymphocyte cell populations was examined by adoptively transferring CD4⁺ lymphocytes from wild type donors into mice inoculated with *H. hepaticus* and dually deficient for *RAG2* and either *ERα* or *ERβ* expression. Again, *H. hepaticus*-inoculated *RAG2*^{-/-} mice with functional *ERα* and *ERβ* were adoptively transferred wild type CD4⁺ lymphocytes as controls.

Adoptive transfer of CD4⁺ lymphocytes into mice lacking *ERα* resulted in significant decreases in disease severity (Figure 2-5A). Interestingly, among the

cecal cytokines and chemokines studied, *IFN-γ* mRNA levels were significantly less in both the group missing ERα and the group missing ERβ on the non-CD4⁺ cellular subsets (Figure 2-5C). There were no differences in *IL-12/23 p40* and *CXCL9* expression (Figure 2-5B & D). The reduction in disease development along with the decreased expression of *IFN-γ* in the mice lacking ERα signaling capabilities on their non-CD4⁺ cells provides further support that estrogen signaling through ERβ can modulate mucosal inflammation.

Discussion

The regulation of autoimmune diseases by gonadal sex hormones (GSHs) is complex and multifaceted. There are many theories as to why women are more likely to develop an autoimmune disease. Many of these recognize the influence of GSHs; additionally most acknowledge that females, due to their evolutionary role to bear offspring, have an immune system programmed to accept exposure to allogeneic antigens, such as a developing fetus during pregnancy^{47,49}. Many diseases, such as MS, RA and SLE, demonstrate a female sex bias^{48,50}. In SLE, elevations in estrogen are associated with increased disease severity, while in MS and RA, increases in estrogen levels, such as occurs during pregnancy, have been shown to decrease disease severity^{50,287}. Additionally, animal models of MS²⁸⁸⁻²⁹⁰ and RA²⁹¹ have recapitulated the beneficial effects of estrogen therapies.

The mucosal immune system of the gastrointestinal track also appears to be highly influenced by estrogen signaling. A female sex bias is seen in both IBS⁵¹ and Crohn's disease, a subset of IBD³³, although it is small relative to the sex-bias of many other autoimmune-related diseases⁴⁸. Furthermore, there is evidence that estrogen can modulate disease severity in IBD patients⁵⁵⁻⁵⁷ and in animal models of IBD²⁸⁰⁻²⁸³. The influence of gonadal sex hormones is also apparent in our model as female *H. hepaticus*-infected A/J mice develop more severe intestinal inflammation than their male counterparts²⁵². Additionally, there is a greater increase in the expression of inflammatory T_H1 cytokines and chemokines in *H. hepaticus*-infected female mice, relative to male mice²⁵². In light of these observations, we sought to investigate the effects of estrogen signaling on disease development and the associated cytokine responses.

As mentioned, in other animal models of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis and DNB-induced colitis, high doses of estrogen effectively decrease disease severity^{280,284,285}. Our findings that administration of estrogen decreases disease severity are consistent with these reports. The immune response to *H. hepaticus* infection in A/J mice is a T_H1-type immune response characterized by an increase in cecal expression of IL-12/23 p40, IFN- γ , TNF α , IP-10 and CXCL9^{25,252}. In these studies, we assessed the mRNA expression of IL-12/23 p40, CXCL9 and IFN- γ as markers for the immune response and demonstrated that estrogen administration in *H. hepaticus*-infected mice was associated with

significantly decreased expression of all three. Decreases in *IL-12/23p40*, *IFN-γ* and *CXCL9* gene expression and histologic disease suggest that estrogen therapy affects microbe-induced T_H1-type inflammation of the cecum.

The exact mechanism by which estrogen alters disease has yet to be identified and although chronic estrogen treatment reduces disease severity, these alterations may be secondary to estrogen's systemic effects. Evaluation of estrogen-treated animals did confirm the presence of systemic changes. When compared to placebo treated animals, estrogen-treated mice had reduced numbers of circulating red blood cells (RBCs), reduced weight gain, and changes in urogenital anatomy (data not shown). Anatomical changes associated with estrogen administration included swelling and hardening of the cervix, as well as urinary abnormalities such as the inability to urinate normally and bladder distension. Secondly, urine leakage and perianal swelling and inflammation were observed in some mice (data not shown). Although it is unclear if chronic estrogen treatment directly decreases intestinal inflammation or if the change is a secondary effect, the other clinical effects associated with chronic estrogen therapies negate its therapeutic viability.

To circumvent the unwanted side effects of chronic estrogen administration, while still utilizing the anti-inflammatory properties of an estrogen-based therapy, selective estrogen receptor (ER) modulation was evaluated. Although the signaling pathways initiated by ERs have been shown to elicit distinct immune responses in other models of autoimmune disease^{281,292}, the

relative expressions of ER α and ER β vary between the intestinal cellular populations and thus induce a variety of immune responses²⁹³⁻²⁹⁹. Further investigation was required to identify both the specific cell type and estrogen receptor responsible for decreased disease severity following estrogen administration.

To assess the role of specific ERs, mice lacking either ER α or ER β were inoculated with *H. hepaticus*. ER α ^{-/-} mice had decreased severity of disease that appeared to be mediated through both the innate and adaptive immune response as expression of *IL-12/23 p40* did not significantly vary, but *IFN- γ* and *CXCL9* were decreased. Although these results are somewhat inconclusive, it is reasonable to hypothesize that ER α signaling promotes a pro-inflammatory immune response, and removal of this signaling pathway decreases disease severity. Alternatively, removal of ER α may simply redirect most estrogen signaling through ER β , and preferential signaling through this receptor modulates the inflammatory response and decreases disease severity.

We subsequently demonstrated decreases in both disease development and inflammatory mediator gene expressions when mice were treated with the ER β agonist DPN. Other studies have also suggested a role for ER β in modulating intestinal inflammation⁵⁸, in particular the publication by Looijer-van Langen *et al.* that demonstrates decreased ER β expression in both human IBD patients, and in rodent models of IBD, including the IL-10^{-/-} mouse and the HLA-B27 rat³⁰⁰. The recent work by Saleiro *et al.* has further highlighted the protective

properties of intestinal ER β signaling by demonstrating the ability of ER β signaling to inhibit the development of colitis-associated cancers in mice²⁸⁶. These findings were further supported by an epidemiological study in which the authors demonstrated that females with IBD are less likely to develop colorectal cancer than male IBD patients³³. Our results also suggest that ER β signaling may be important in suppressing the development of *H. hepaticus*-induced typhlitis. ER β is expressed primarily on intestinal epithelial cells, suggesting its signaling would function primarily through the innate immune response. Signaling through ER β increases the development of tight junctions in the intestinal epithelium and decreases intestinal permeability³⁰⁰⁻³⁰², possibly resulting in decreased sampling of the luminal bacteria. Furthermore, treatment of colonic cells in culture with either 17 β Estradiol or DPN, the ER β agonist used in this study, results in increased epithelial resistance³⁰⁰. Increased epithelial resistance could decrease the ability of bacterial antigens to stimulate the immune system and could explain the decreased expression of *IL-12/23 p40* seen in the ER β agonist (DPN)-treated mice.

The finding that loss of ER α , which results in preferential signaling through ER β , decreased disease severity, coupled with the finding that an ER β agonist also decreased disease severity, initially suggests that signaling through ER β abrogates the immune response and ultimately prevents development of inflammation. However, given these findings, we expected ER β -deficient mice to have increased disease severity, but this was not the case. Given the complexity

of the mucosal immune system there are several plausible explanations for our findings. The first being that this model's maximum disease severity had been reached and depletion of ER β was insufficient to further exacerbate disease beyond this plateaued level. Conversely, it is also plausible that the immune response, in addition to being down-regulated by ER β signaling as demonstrated by our ER β agonist treatments, is up-regulated when estrogen signaling occurs solely through ER α . Further experiments were needed to identify the cell types in which ER α and β was occurring in our model. Previously published findings have shown intestinal epithelial cells express ER β ^{52,301} and CD4⁺ T cells express ER α ³⁰³ making it necessary to evaluate the involvement of both CD4⁺ and non-CD4⁺ cellular populations in disease modulation.

To identify the cell type through which ER signaling modulates disease, an adoptive transfer model was utilized. When estrogen signaling on CD4⁺ T cells was altered, no differences in histologic disease or mRNA expression were observed. However, when non-CD4⁺ cellular populations lacked ER α signaling, disease severity was decreased and *IFN- γ* mRNA expression was lowered. These results suggest that the development of *H. hepaticus*-induced inflammation is independent of estrogen signaling on CD4⁺ T cells, and is dependent on estrogen signaling in a non-CD4⁺ cellular population. Interestingly, although the development of inflammation was not affected when there was a lack of ER β signaling on non-CD4⁺ cells, there was a significant decrease in the amount of *IFN- γ* mRNA present in the cecum. These results suggest that,

although both ER α and ER β signaling is linked to IFN- γ production, only signaling through ER β is capable of changing histologic disease development.

It is possible that, in this experimental model, the changes in *IFN- γ* gene expression are not due to the direct effects of estrogen signaling. For example, estrogen signaling in dendritic cells can decrease antigen presentation and, in turn, indirectly affect the resulting immune response. Reports on the expression of ERs on dendritic cells are somewhat contradictory and although it is generally accepted that ER α is constitutively expressed on dendritic cells²⁹⁶, there are reports of co-expression of ER β ^{304,305}. Additionally, *in vitro* studies have shown that a lack of ER α results in decreased dendritic cell differentiation. Furthermore, these populations can be restored with high doses of E2, thus demonstrating the ability of ER β to induce dendritic cell differentiation independently of ER α ³⁰⁴.

The findings reported herein also suggest that ER β -mediated immune modulation is occurring through a non-CD4⁺ cell population which expresses ER β , such as epithelial cells or dendritic cells. Epithelial cells with increased ER β signaling could be increasing tight junctions and consequently decreasing the overall antigen pool presented to the immune system. Alternatively, ER β signaling on dendritic cells may be altering antigen presentation to the overall immune system, ultimately resulting in decreased disease development.

Overall, this research suggests that when estrogen signals solely through ER β in a non-CD4⁺ cell population, an immunosuppressive effect occurs. This is

supported by data from knock-out animals, ER agonists, and adoptive transfer experiments, all of which demonstrated decreased histologic disease development when estrogen signaling is limited to ER β . This research supports further investigation of the potential uses of targeting estrogen signaling pathways, particularly ER β , to modulate the development of pathology in diseases such as IBD.

Materials & Methods

Ethics Statement

This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the University of Missouri Animal Care and Use Committee [ACUC Protocol Number: 7749]. All surgical procedures were performed under isoflurane anesthesia and all mice received post-operative buprenorphine for analgesia.

Mice

Wild type A/JCr mice were obtained from Frederick National Laboratory for Cancer Research.

Generation of estrogen receptor (ER) and RAG2 deficient A/J Mice

B6;129-*Esr1*^{tmUNC} (ER α knockout)³⁰⁶ and B6;129-*Esr2*^{tmUNC} (ER β knockout)³⁰⁷ mice were kindly donated by Dr. Dennis Lubahn of the University of Missouri. A marker-assisted congenic breeding scheme was used to transfer the ER mutations to the A/J mouse strain. Resulting strains (A.129(P2)-*Esr1*^{tmKsk}/*Mmmh* and A.129P2(B6)-*Esr2*^{tmUNC}/*Mmmh*) strain were subsequently submitted to the Mutant Mouse Regional Resource Center (MMRRC 029880 and 029908 respectively). C57BL/6 mice containing a disruption in the *Rag2* (recombination activating gene 2) gene were obtained from Taconic laboratories (B6.129S6-*Rag2*^{tm1Fwa}). Again, a marker-assisted congenic breeding scheme was used to

generate *Rag2*-deficient mice on an A/JCr background (A.129S6(B6)-*Rag2*^{tm1Fwa}). The generation of mice with mutations in both *Rag2* and either ER α or ER β was accomplished by crossing the A.129S6(B6)-*Rag2*^{tm1Fwa} strain with either A.129(P2)-*Esr1*^{tmKsk}/*Mmmh* or A.129P2(B6)-*Esr2*^{tmUNC}/*Mmmh* mice.

ER α , ER β , and RAG2 PCR

At 3-4 weeks of age, all mice were ear-tagged (National Band & Tag, Newport, KY) and 3-5 millimeter tail snips were collected for genotyping. DNA was extracted using a Qiagen DNeasy DNA extraction kit (Valencia, CA) per manufacturer's protocol. The resulting DNA was used in PCR reactions to determine each individual mouse's genotype. Each reaction contained 0.2 μ l FastStart *Taq* (5 U/ μ l) (Roche, Indianapolis, IN), 0.3 μ l of each 25 μ M primer (IDT Coralville, IA), 3.2 μ l of 1.25 mM dNTPs (Roche) and 2 μ l 10 $\times$ PCR buffer with MgCl₂ (Roche). Thermal cycling conditions were: 1 cycle at 94⁰C for 4 min, 45 cycles of 15 at 94⁰C , 45 sec at the appropriate annealing temperature (*Table 1*), 1 min at 72⁰C and 1 cycle at 72⁰C for 10 min. PCR reactions were analyzed by gel electrophoresis with 3% 1 $\times$ TBE agarose gels and amplicons were visualized with ethidium bromide (Bio-rad Hercules, CA). Primer sequences, product sizes and annealing temperatures are listed in *Table 2-1*.

Bacterial Culture

Culture of *Helicobacter hepaticus* strain MU-94 was previously described^{25,252}. Mice were inoculated via gastric gavage with 0.5 ml of culture containing approximately 5×10^8 bacteria/mL. Colonization was confirmed 2-4 weeks post-inoculation and at necropsy with *H. hepaticus*-specific PCR of feces²⁴⁷.

Ovariectomy

Mice were anesthetized with isoflurane, clipped dorsally, and aseptically scrubbed. A single longitudinal skin incision was made on the dorsal midline. The ovaries were visualized, the muscle wall was incised, and ovaries were removed. After a continuous-release hormone pellet (described below) was inserted rostrally into the subcutaneous space between the scapulae through the initial incision, the incision was closed with sterile skin staples. Mice were given 0.0015 mg buprenorphine IP for analgesia and recovered on a heating pad. Seven to ten days post-operatively, the skin staples were removed. At the time of necropsy, mice with detectable ovarian remnants were removed from the study.

Compounds

In initial studies, a 90-day continuous-release hormone pellet containing 1.5 mg/pellet of 17 β -estradiol (E2) or a placebo (Innovative Research of America, Sarasota, FL) was administered. The secondary studies utilized 90-day continuous-release pellets containing either 2.5 mg of the ER α agonist, PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol), or 10 mg of the ER β agonist, DPN (diarylpropionitrile 2,3-bis(4-hydroxyphenyl)-propionitrile) (Tocris Bioscience Ellisville, MO and Innovative Research of America). At the time of necropsy, the presence of a pellet was confirmed and mice without a visible, intact pellet were removed from the study.

Isolation of CD4⁺ Lymphocytes and Adoptive Transfers

CD4⁺ T cells were isolated from the spleens of 8-12 week old donor mice. CD4⁺ T cells were negatively selected using a CD4⁺ T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA), and purified with a Miltenyi Biotec autoMACS cell separator. After isolation, cells were suspended in sterile PBS and 5×10^5 cells were injected into the tail vein of recipient mice. All mice were monitored for weight loss; loss of more than 20% of the starting body weight resulted in removal from the study.

Necropsy

In all experiments, the cecum was removed and cecal contents collected to confirm *H. hepaticus* colonization. The cecum was then sectioned longitudinally into two equal parts. One half was rinsed with sterile PBS and flash frozen in liquid nitrogen for use in mRNA expression analyses. The other half was fixed in zinc fixative and embedded in paraffin for histologic evaluation.

Histological Evaluation

Cecal histologic sections were stained with hematoxylin and eosin (H&E) stain and scored for inflammatory lesions using a previously described protocol^{246,252,253}. In short, all cecal sections were scored in a blinded fashion and assigned a category score for each of the following: inflammation intensity (0 = none, 1 = mild, 2 = moderate, 3 = severe); longitudinal extent of inflammation (0 = none, 1 = 1-2 small foci, 2 = patchy, 3 = diffuse); vertical extent of inflammation (0 = none, 1 = basal mucosal inflammation, 2 = full thickness mucosal inflammation, 3 = transmural inflammation); and hyperplasia (0 = none, 1 = focal, 2 = patchy hyperplasia, 3 = diffuse hyperplasia). The lesions scores depicted in figures are the sum of the four category scores; any lesions score greater than 2 were decreased by 2 to minimize inflation of scores by inflammation alone, which is described by three of the four categories evaluated.

RNA Isolation and Reverse Transcription

Frozen cecal sections were thawed in TRIzol (Invitrogen, Carlsbad, CA) and homogenized using a TissueLyser (Qiagen, Valencia, CA). Total RNA was isolated per manufacturer's (Invitrogen) instructions. Five micrograms of total RNA was reverse-transcribed using the Super Script II kit (Invitrogen, Carlsbad, CA) utilizing the manufacturer's oligo(dT) primer protocol. The resultant cDNA was diluted to a final concentration of 20 ng/ μ L.

Real-Time PCR

Real-time quantitative PCR was performed either with a Roche Light Cycler 2.0 or a Qiagen Rotor-Gene Q using Qiagen Quanti-tect SYBR Green (Qiagen, Valencia, CA). The run conditions used for real-time PCR have been previously described ²⁵ and primers can be found in *Table 2-2*.

Statistical Analysis

Two-tailed Student's T tests, one-way ANOVAs with Dunnett's post-hoc tests and Chi-Squared tests were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). When appropriate, Log2 transformations were used to correct for failures in normality. Non-parametric analysis, Mann-Whitney Rank Sum Test and Kruskal-Wallis One Way Analysis of Variance on Ranks were used when failures in normality could not be corrected for by a Log2 transformation. p values < 0.05 were regarded as significant. For lesion score

analysis of knockout mice, preliminary studies revealed that mean lesion scores of wild type, $ER\alpha^{+/-}$, and $ER\beta^{+/-}$ mice did not differ statistically. These groups were therefore combined as controls. All graphs and dot plots were generated using GraphPad Prism 5.0 software (GraphPad Prism Software Inc., La Jolla, CA).

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Figure 2-1. Estrogen treatment decreases disease severity and the expression of pro-inflammatory cytokines and chemokines.

(a) Representative histologic images of ceca from ovariectomized A/J mice sacrificed 90 days after *Helicobacter hepaticus* inoculation and implantation with a subcutaneous pellet containing either (top) placebo or (bottom) 17 β -estradiol (1.5 mg/pellet). (b) Cecal lesion scores of placebo (circles) and estradiol (squares) treated mice (n = 8, 8 respectively). Each point represents the lesion score for an individual animal. Mean lesion scores in the estrogen-treated mice are significantly lower than in the placebo-treated mice. Lesion score data analyzed with a Mann-Whitney Rank Sum Test; horizontal lines represent mean lesion score for each group and error bars correspond with s.d. (c) Real-time quantitative PCR measurements of cecal cytokine and chemokine mRNA expression levels in mice receiving placebo or estrogen treatment (n = 8). IL-12/23 p40 (left), IFN- γ (middle) and CXCL9 (right) mRNA expression was normalized to the expression of the housekeeping gene HPRT. For all three cytokines, IL-12/23 p40, IFN- γ and CXCL9, mice receiving estrogen treatment had significantly lower mRNA expression levels than mice receiving placebo treatment. All mRNA expression data analyzed by two-tailed Student's t tests and presented as group means $\pm$ s.d.

Figure 2-2. Absence of ER α expression decreases disease severity and mRNA expression of IFN- γ and CXCL9.

A/J female mice deficient for either ER α or ER β expression and heterozygous/wild type littermate controls were inoculated with *H. hepaticus* and evaluated 3 months post-inoculation. (a) Cecal lesion scores of inoculated ER α (squares), ER β (triangles) and control (circles) mice (n = 20, 11, and 37 respectively); each point represents the lesion score for an individual animal. ER $\alpha^{-/-}$ mice with normal ER β expression had significantly lower lesion scores when compared to controls. Lesion score data analyzed with a Kruskal-Wallis one way ANOVA on ranks with a Dunnett's post-hoc analysis. Horizontal bars represent group means $\pm$ s.d. (b) Percentage of animals with severe cecal inflammation (lesion score ≥ 6) within each genotype group. The percentage of severe disease was significantly lower in ER $\alpha^{-/-}$ mice when compared to controls and ER $\beta^{-/-}$ mice. Data analyzed with chi-squared analysis. (c) Real-time quantitative PCR measurements of cecal cytokine and chemokine mRNA expression levels in mice deficient for ER α or ER β and littermate controls (n = 13, 9, and 27 respectively). IL-12/23 p40 (left), IFN- γ (middle) and CXCL9 (right) mRNA expression levels were all normalized to HPRT. Expression of CXCL9, but not IFN- γ and IL-12/23 p40 were significantly less in ER $\alpha^{-/-}$ mice when compared to control mice. Additionally, a trend of decreased IFN- γ expression was noted in the ER $\alpha^{-/-}$ mice. Data analyzed with one-way ANOVAs with Student-Newman-Keuls post-hoc tests; all data presented as group means $\pm$ s.d..

Figure 2-3. Treatment with an ER β agonist decreases disease severity and the expression of pro-inflammatory cytokines.

Female A/J mice were ovariectomized, administered continuous-release pellets containing a placebo, ER α agonist (PPT) or ER β agonist (DPN), inoculated with *H. hepaticus*, and necropsied three months later. (a) Cecal lesion scores of mice treated with a placebo (circles), PPT (squares) or DPN (triangles) pellet ($n = 19, 22$, and 21 respectively). Points represent the lesion scores of individual animals. Administration of the ER β agonist significantly reduced the mean lesion scores when compared to the placebo- and ER α agonist-treated mice. Data analyzed by one-way ANOVA with a Student-Newman-Keuls post-hoc test; data presented as group means $\pm$ s.d (b) Depiction of the percentage of mice with severe disease (lesion score ≥ 6). The incidence of severe disease in mice treated with DPN was significantly less than the control and PPT-treated mice. Data analyzed with chi-squared analysis. (c) Real-time quantitative PCR measurements of cecal mRNA expression levels in mice treated with an ER α agonist, ER β agonist, or placebo control ($n = 8, 9$, and 9 respectively). IL-12/23 p40 (left), IFN- γ (middle) and CXCL9 (right) mRNA expression levels are normalized to HPRT expression. Expression of IL-12/23 p40 and CXCL9 is significantly lower in ER β agonist-treated mice when compared to placebo-treated mice. A trend of decreased IFN- γ expression was noted in the ER β agonist-treated mice. Data analyzed with one-way ANOVA with a Dunnett's post-hoc test (control = placebo treated mice); data presented as group means $\pm$ s.d.

Figure 2-4 Lack of ER signaling in CD4⁺ cellular populations does not alter cecal inflammation or cytokine and chemokine expression.

(a) Cecal lesion scores in female *H. hepaticus*-inoculated *RAG2*^{-/-} mice adoptively transferred *ERα*^{-/-} (n = 8) or *ERβ*^{-/-} (n = 9) CD4⁺ lymphocytes. As a control group, *RAG2*^{-/-} female mice were adoptively transferred wild type CD4⁺ lymphocytes (n = 20). Points represent the lesion scores of individual animals in each experimental group and horizontal bars indicate mean lesion scores; error bars correlate with s.d. No significant differences in lesion scores were noted between any of the experimental groups. (b-d) Real-time quantitative PCR measurements of cecal cytokine and chemokine mRNA expression levels in *RAG2*^{-/-} mice adoptively transferred CD4⁺ lymphocytes deficient for ERα or ERβ expression. *IL-12/23 p40* (b), *IFN-γ* (c) and *CXCL9* (d) mRNA expression levels were normalized to *HPRT* mRNA expression and data is represented as group means ± s.d. No differences in expression were noted. All data analyzed by one-way ANOVA and presented as group means ± SD.

Figure 2-5. ER β signaling on non-CD4 $^{+}$ cellular populations reduces cecal inflammation and IFN- γ expression.

(a) Cecal lesion scores in female *H. hepaticus*-inoculated ER α $^{-/-}$ RAG2 $^{-/-}$ (n = 11) (circles) or ER β $^{-/-}$ RAG2 $^{-/-}$ (n = 13) (squares) female mice adoptively transferred wild type CD4 $^{+}$ lymphocytes. As a control group (triangles), RAG2 $^{-/-}$ female mice were adoptively transferred wild type CD4 $^{+}$ lymphocytes (n = 20). Points represent the lesion scores of individual animals in each experimental group and horizontal bars indicate mean lesion scores. The adoptive transfer of WT CD4 $^{+}$ lymphocytes into ER α $^{-/-}$ RAG2 $^{-/-}$ recipients significantly reduced the mean cecal lesion scores when compared to the control group. Lesion score data analyzed with a Kruskal-Wallis one way ANOVA on ranks with a Dunnett's post-hoc analysis. Horizontal bars represent group means $\pm$ s.d. (b-d) Real-time quantitative PCR measurements of cecal cytokine and chemokine mRNA expression levels in RAG2 $^{-/-}$ mice deficient for either ER α or ER β and adoptively transferred wild type CD4 $^{+}$ lymphocytes. IL-12/23 p40 (b), IFN- γ (c) and CXCL9 (d) mRNA expression levels are normalized to HPRT expression. Expression of IFN- γ is significantly lower in both experimental groups in which mice dually deficient for RAG2 and either ER α or ER β received wild type CD4 $^{+}$ lymphocytes. All data analyzed by one-way ANOVA with a Dunnett's post-hoc test (RAG2 $^{-/-}$ mice adoptively transferred WT CD4 $^{+}$ used as controls); all data presented as group means $\pm$ s.d.

Table 2-1. Genotyping PCR Primers, Products, & Annealing Temperatures.

PCR Primers	Forward (5'-3')	Reverse (5'-3')	Fragment size (bp)	Annealing Temp (°C)
ER α WT	TCTACGGCCAGTCGGGCATC	TAGGCGACACGCTGTTGAGC TG	102	66
ER β WT	TGACAATGGCTGTGTTCTCTGCTC	TTAAAGTTAAACGCCAGCCCA CCG	566	66
neo cassette	5'-TGCCTGCTCTTTACTGAAGGCTCT-3'		α – 363 β - 430	66
RAG2 WT	GGGAGGACACTCACTTGCCAGTA	AGTCAGGAGTCTCCATCTCAC TGA	263	62
neo cassette	5'-CGGCCGGAGAACCTGCGTGCAA-3'		350	62

Table 2-2. Real-time PCR Primers & Annealing Temperatures

PCR Primers	Forward (5'-3')	Reverse (5'-3')	Annealing Temp (°C)
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA	60
CXCL9	ACTCGGCAAATGTGAAGAAG	CCCATTAAGATTCAGGGTGC	58
IFN-γ	GCCATCAGCAACAACATAAGC	TGGGACAATCTCTTCCCCAC	58
IL-12/23 p40	ACTCACATCTGCTGCTCCAC	GGGAACTGCTACTGCTCTTGA	60

Chapter 3 - Trichuris muris therapy in a Helicobacter hepaticus-induced IBD mouse model

Introduction

The development of an Inflammatory Bowel Disease (IBD) such as Crohn's disease (CD) or Ulcerative Colitis (UC) is a multifaceted process driven by an individual's mucosal immune system. The disruption in mucosal homeostasis appears to be driven by the complex interactions of an individual's immune system, the environment in which they live, their genetic predisposition to disease development, and their microbiota^{3,13,67,106,216}.

Due to the complexity of the events which lead to the development of IBD, attempts to develop an effective pharmaceutical treatment for patients have been less than ideal. Traditional pharmaceutical treatments for IBD patients include, but are not limited to: aminosalicylates (5-ASA derivatives), corticosteroids (prednisolone, prednisone), immune modulators (thiopurines, methotrexate, cyclosporine A), antibiotics (metronidazole, ciprofloxacin) and biological therapies (Adalimumab, Certolizumab pegol, Infliximab and Natalizumab)^{4,211,212}.

Unfortunately, remission rates reported with these treatments are widely variable^{211,215} and can be difficult to interpret due to IBD's unpredictable cyclic patterns of exacerbation and remission⁴. Also, many IBD clinical trials lack evaluation of a true placebo group, which furthers complicates the ability to identify the true efficacy of proposed treatments²¹⁵. The lack of effective

pharmaceuticals for some patients is not surprising considering that IBDs develop through a variety of interactions, many of which are potentially unique to each individual. The concept of personalized medicine in which therapeutics are tailored to individuals using biomarker and genetic analysis may help pair patients with the most effective therapeutics²⁰⁸⁻²¹⁰. Overall, the need for additional treatment options is highlighted by the high surgical resection rates found among IBD patients: 20-30% of UC patients and 70-80% of CD patients will undergo a surgical resection during their lifetimes as a result of uncontrolled disease⁴.

Due to the ineffectiveness of traditional treatment regimens, a need for novel treatment options has been identified^{122,208,212}. In an attempt to produce non-traditional therapies, risk factors involved in disease development, such as an individual's environment, were scrutinized. Historically IBDs are predominantly diagnosed within the developed regions of North America and Europe¹⁸. The importance of environment, rather than genetic predispositions been examined epidemiologically by evaluating the diagnosis rates of IBD in first and second generation immigrants in Europe originally from regions of the world with a traditionally low IBD diagnosis rate. A study done by Carr et al. examined the rate of UC in first and second generation Asians that had immigrated to Europe and demonstrated that the incidence of UC in the immigrant populations normalized to the indigenous European population after only one generation⁹⁹.

The importance of exposure to environmental risk factors in combination with a genetic predisposition was further highlighted by the finding that some Asian populations actually had a higher potential to develop IBD than the indigenous European population following immigration and exposure to novel environmental factors present in western cultures³⁰⁸. When examining the influence of westernization on IBD development, one must also consider what is being lost within these environments⁹⁷. The exposure to helminths has been identified as an immunological event which could potentially protect from disease development within developing regions of the world^{93-96,100,309}.

Elliott *et al.* proposed an IBD hygiene hypothesis in the 1990s that suggested children would be predisposed to the development of immune-related diseases, such as IBD, if their immune system lacked appropriate antigen exposure during development⁹³. Since then, the hygiene hypothesis and IBD development has been extensively examined and considered⁹⁴⁻⁹⁷. As previously mentioned, one aspect of westernized living which has been identified in the minimization of antigen exposure is the eradication of gastrointestinal parasites^{95,96}. In the early 1900s the Rockefeller Foundation's International Health Board (formally the Rockefeller Sanitary Commission) began improving living conditions in and outside of the United States with programs that encouraged routine deworming of citizens, particularly schoolchildren³¹⁰. Today, organization such as the World Health Organization (WHO), World Health

Assembly and United Nations Children's Fund (UNICEF) are working to improve accessibility to anthelmintics in developing regions of the world³¹⁰.

With the increased emphasis on decreasing helminth exposure, it is interesting that recent reports have shown increased IBD diagnosis rates in regions such as Asia, Africa and South America³⁷. A recent report by Chu *et al.* demonstrated that in South Africa, where western hygiene standards are being integrated into everyday life for some citizens, exposure to helminths during childhood appears to be protective against IBD development¹⁰⁰. The authors also demonstrated that environmental conditions such as shared housing and consumption of raw meat both of which have the potential to increase helminth exposure were protective in disease development, while urban living and parental tertiary education was associated with IBDs¹⁰⁰.

In individual patients diagnosed with an IBD, the issue of pre-exposure to helminths is a moot point as this variable cannot be changed. However, it has been speculated that exposure to helminths, even after disease development, may be beneficial due to the interactive nature of the T_H1/T_H2/T_H17 signaling pathways active in IBDs and helminth infections^{223,225,231,311}. Following migration from the thymus, native CD4⁺ cells can be driven toward T_H1, T_H2 or T_H17 effector cell differentiation by IL-12, IL-4 and IL-6/TGFβ, respectively^{14,109-112}. As each of these differentiation pathways are suspected to act in a negative fashion on the others^{114,312}, the T_H1/T_H17 response in CD potentially could be minimized by the induction of T_H2 mediated inflammation by helminth exposure^{14,113,114}.

Our laboratory utilizes a microbial driven IBD model in which A/J mice develop a lymphocytic infiltrative histologic disease that is detectable in the cecum²⁵ three-months after gastric administration of *Helicobacter hepaticus*. Additionally, these mice have significantly elevated cecal mRNA gene expression of IL-12/23 p40, IFN- γ and IL-10^{25,252-254}. The mechanism by which *H. hepaticus* induces disease is unknown however, one hypothesis is that once established in the cecum, helicobacters act as a provocateur instigating an immune response against certain endogenous microbiota^{248,249}. While data from *Citrobacter rodentium* IBD models suggests helminth therapy exacerbates disease^{313,314}, there is need to further evaluate the efficacy of helminth therapy in an IBD model triggered by bacteria.

As it is reasonable to speculate that inflammation instigated by an opportunistic member of the microbiota, such as *H. hepaticus*, may respond differently to the presence of helminths we sought to investigate the effects of *Trichuris muris* in the *H. hepaticus* IBD model. *T. muris* was chosen for evaluation as it is closely related to *Trichuris suis*, the helminth utilized in the human trials^{36,37} and because it localizes to the cecum³¹⁵, the site of inflammation in this model. Our data demonstrates that *T. muris* is not only ineffective at reversing inflammation induced by *H. hepaticus* in the A/J mouse, but actually acutely exacerbates disease severity. Furthermore, our findings demonstrate that the immune response necessary to expel *T. muris* is affected by T_H1 inflammation, such as that seen in the *H. hepaticus* IBD model. In conclusion,

our data presents evidence that helminth therapeutics in IBD patients should be used with caution as there is a potential to cause exacerbation of disease and persistence of helminth colonization if disease is due to a microbiota driven T_H1 inflammation. .

Results

A/J mice mount a T_H2 cytokine response following exposure to *T. muris* which correlates with worm expulsion.

Some mouse strains have been demonstrated to mount an inappropriate T_H1 immune response to *T. muris* which results in chronic colonization by the parasite³¹⁶. Prior to evaluating *T. muris*'s therapeutic potential, the A/J mouse's ability to mount a T_H2 immune response must be confirmed. As worm expulsion has been linked to a T_H2 cytokine response, this was initially utilized to evaluate the immune response to the helminth. Five-month old female mice were inoculated with *T. muris* ova. Mice (n=4/time point) were necropsied at either twelve or twenty-eight day post-inoculation and examined for the presence larvae or adult worms, respectively. As shown in Figure 3-1A, at twelve days post-inoculation live larvae (Figure 3-1B) were detected in the cecal digests of all four mice. At twenty-eight day post-inoculation, one mouse (25%) had detectable adult worms at necropsy (<10 worms).

The results from this experiment suggested that the A/J mouse mounts a T_H2 cytokine response when exposed to *T. muris* based on the overall

resistance to worm colonization. To best evaluate if the T_H2 immune response to *T. muris* is capable of blunting or reversing the T_H1 mediated inflammation induced by *H. hepaticus*, the immune response needs to be further defined. To accomplish this, four-month old female A/J mice were inoculated with *T. muris* ova or PBS (vehicle control). Five mice were necropsied every week starting one week post-inoculation and continuing for a total of six weeks (Figure 3-2A). At necropsy, mice were evaluated for the appropriate life stage of *T. muris* (larvae at weeks 1-3 and adults at weeks 4-6). Live *T. muris* larvae were detected in all *T. muris* inoculated mice at the week 1 and 2 necropsy time points; 4 of the 5 mice examined at week 3 had live larvae. Adult worms were not found in any mice at week 4 and 6, however two mice evaluated at week 5 had adult worms (<10 worms/mouse) (Figure 3-2B). In addition, mRNA analyses of inflammatory cytokines were completed for: IL-12/23 p40, IFN- γ , IL-10, IL-4, IL-5, IL-6 and IL-13. mRNA expression of IL-4 and IL-13 was significantly elevated in *T. muris* inoculated mice when compared to PBS inoculated mice beginning at week 2 and continued through week 6. The expression of IL-5 was elevated at weeks 2-3 and the expression of IL-6 was elevated between weeks 3-5. IL-10 and IFN- γ mRNA expression was elevated at week 1, however both returned to the level of PBS inoculated mice by the weeks 3 and 5 necropsy time points, respectively. Expression of IL-12/23 p40 mRNA was elevated in *T. muris* inoculated mice only at the week 2 time point. Collectively these data demonstrate that two weeks

following exposure to *T. muris* A/J mice have mounted a T_H2 cytokine response which correlates with helminth expulsion.

***T. muris* acutely exacerbates *Helicobacter hepaticus* induced mucosal inflammation.**

Our laboratory has previously demonstrated that three months following *H. hepaticus* inoculation A/J mice develop a T_H1 driven mucosal inflammation which recapitulates several histologic aspects of CD. As it is now known that A/J mice mount a T_H2 response when exposed to *T. muris*, we sought to evaluate the therapeutic effects of this helminth in our model of IBD. To accomplish this, A/J mice, 3-4 weeks of age, were inoculated with either brucella broth (sham) or *H. hepaticus*. Three months following these inoculations mice were treated with either live *T. muris* ova or PBS (vehicle control). This resulted in a total of four experimental groups (n = 8 mice/group): *H. hepaticus*(-) *T. muris*(-), *H. hepaticus*(-) *T. muris*(+), *H. hepaticus*(+) *T. muris*(-), and *H. hepaticus*(+) *T. muris*(+). Mice from each group were evaluated 3 weeks after treatment with either PBS or *T. muris* ova for changes in histologic disease, presence of histologically detectable *T. muris* and mRNA expression of: IL-12/23 p40, IFN- γ , IL-10, IL-4, IL-5, IL-6, IL-13, IL-17a and IP-10.

Cecal lesion scores were significantly elevated in the *H. hepaticus* inoculated mice treated with *T. muris* when compared to *H. hepaticus* inoculated mice treated with PBS (Figure 3-3A). Representative histologic images of mice

from all four experimental groups are depicted in Figure 3-3B. During histologic evaluation of cecal sections, the presence of histologic *T. muris* was documented. 75% of the *H. hepaticus* inoculated mice treated with *T. muris* had histologically detectable helminths (Figure 3-3B, arrow heads); *T. muris* was not detected histologically in any of the other experimental groups.

The mRNA gene expression of cytokines thought to be involved in *H. hepaticus* induced inflammation (IL-12/23 p40, IFN- γ , IL-17a and IL-10) and the immune response to helminths (IL-4, IL-5, IL-6, IL-13 and IP-10) were evaluated. Significant elevations in gene expression of IFN- γ , IL-4, IL-5, IL-6, IL-12/23 p40, IL-13, and IP-10 were seen within the *H. hepaticus* inoculated mice when *T. muris* ova and PBS treated mice were compared (Figure 3-4). Although the A/J mice mounted the appropriate T_H2 immune response following *T. muris* treatment it failed to counteract the ongoing T_H1 inflammation and ultimately exacerbated disease severity.

Exposure to *T. muris* has no long-term effects on *Helicobacter hepaticus* induced mucosal inflammation.

To evaluate the long-term effects of *T. muris* exposure on *H. hepaticus*-induced inflammation, mice were inoculated and treated as described for the previous experiment. In short, mice were inoculated with either brucella broth (sham) or *H. hepaticus* and then treated with either live *T. muris* ova or PBS (vehicle control) three months later. As with the previous experiment, there were

four experimental groups (n = 8 mice/group): *H. hepaticus*(-) *T.muris*(-), *H. hepaticus*(-) *T.muris*(+), *H. hepaticus*(+) *T.muris*(-), and *H. hepaticus*(+) *T.muris*(+). Mice from each group were evaluated 8 weeks after PBS or *T. muris* ova treatment for severity of histologic disease, the presence of histologically detectable *T. muris*, the presence of adult worms in the cecum lumen and mRNA expression of: IL-12/23 p40, IFN- γ , IL-10, IL-4, IL-5, IL-6, IL-13, IL-17a, and IP-10.

Evaluation of cecal lesion scores revealed no significant differences in disease severity when *H. hepaticus* inoculated mice treated with *T. muris* were compared to *H. hepaticus* inoculated mice treated with PBS (Figure 3-5A). As with the previous group, histologic sections were evaluated for the presence of *T. muris*. One mouse in the *H. hepaticus* inoculated, *T. muris* treated experimental group had detectable helminths histologically. To further evaluate the effect of *H. hepaticus* on the development of adult worms, the ceca of all mice necropsied were evaluated for adult worms. Two of the mice inoculated with *H. hepaticus* and later treated with *T. muris* were found to have live *T. muris* adult worms within the cecum. The number of adult worms found in the two mice varied greatly, one mouse had 1 live worm and the other had 24 live worms. (Figure 3-5B) None of the other mice evaluated had either histologically detectable or live *T. muris* within the cecum.

In regards to cecal cytokine mRNA expression, when *H. hepaticus* inoculated mice treated with *T. muris* were compared to those treated with PBS

the expression of IL-12/23 p40 and IL-10 was significantly decreased and the expression of IL-4, IL-5 and IL-13 was significantly elevated (Figure 3-5C). Ultimately, despite the chronic elevation of T_H2 cytokine mRNA expression and the decreased IL-12/23 p40 mRNA expression, *T. muris* treatment did not alter *H. hepaticus*-induced disease severity.

Discussion

As previously discussed, the rate of intestinal resection for UC and CD patients is 20-30% and 70-80%, respectively⁴. These statistics, in combination with the reported remission rates for the pharmaceutical agents currently available for the treatment of IBDs^{211,215}, suggest a need for evaluation of non-traditional therapies, such as helminth ova. A variety of gastrointestinal parasites have been shown to have protective capacities in disease development in murine IBD models such as TNBS-induced colitis^{317,318}, DNBS-induced colitis^{319,320}, DSS-induced colitis^{321,322}, C57BL/6 IL-10^{-/-} mice⁹³ and cell transfer-induced colitis³²³⁻³²⁵. Additionally, data has been produced which demonstrates the capabilities of helminth derived antigens, such as killed eggs³²⁶⁻³²⁸ and helminth derived proteins³²⁹⁻³³⁵, to inhibit colitis development. While these correlations certainly do not imply causation, the findings in animal models of IBD are also consistent with the hygiene hypothesis's theory that exposure to gastrointestinal parasites can be protective for IBD development.

Research utilizing animal models of IBD has also yielded preliminary evidence that helminths can reverse ongoing colitis in both C57BL/6 IL-10^{-/-} mice^{277,336} and cell transfer-induced colitis³³⁷. Preliminary data from clinical trials conducted in human patients with active IBD have demonstrated that treatment with *T. suis* can cause a decrease in disease related symptoms and/ or induce remission rates in approximately 70% of patients^{227,228,338}.

The efficacy of helminth therapeutics to reverse inflammation in mouse IBD models is contradictory as the literature includes studies that demonstrate improvement of disease severity with helminth treatments, as well as studies that demonstrate exacerbation of colitis following helminth exposure^{313,314,339-341}. The variable responses to helminth therapy between mouse IBD models could be due to differences in the mechanism by which disease is induced and supports the need for helminth therapeutics to be further evaluated.

H. hepaticus induces mucosal inflammation in the cecum of A/J mice approximately three months post-inoculation. Although it is unknown how the addition of *H. hepaticus* to the microbiota induces disease development, it is suspected that it triggers an inflammatory reaction to the endogenous microbiota^{248,249}. Furthermore, its presence is suspected to be pivotal in other IBD models²⁴⁰, highlighting potential pro-inflammatory effects on the immune system. As previously stated, *T. muris* was selected for evaluation as a therapeutic agent because it localizes to the cecum³¹⁵ where *H. hepaticus*

induced inflammation occurs and, most importantly, is related to *T. suis*, the helminth currently being used in human IBD clinical trials^{36,37}.

Early on, scientists recognized a variability in resistance to *T. muris* as susceptibility in mice could be manipulated with selective breeding³⁴² and thus was attributed to a genetic influence^{316,343,344}. Mouse strains with a BALB genetic background are generally resistant to *T. muris*, while those on a B10 or AKR genetic background are generally susceptible^{344,345}. The immune response which develops following helminth exposure has been extensively documented^{198,346-351} and it now know that the induction of a T_H1 mediated immune response results in *T. muris* colonization while the induction of a T_H2 mediated immune response is protective³¹⁶. Although helminth expulsion correlates with expression of the T_H2 cytokine IL-4³⁵², the action is ultimately dependent upon the downstream production of IL-13^{353,354}. The involvement of IL-5, also produced by activated lymphocytes, has also been suggested as its expression is up-regulated during the response to *T. muris*^{355,356}.

As the A/J mouse strain used in our *H. hepaticus* IBD model is genetically related to the BALB mouse it was suspected that these mice would mount a protective T_H2 immune response following *T. muris* infection. However, before utilizing a helminth therapeutically in the A/J mouse, this speculation had to be confirmed. The results demonstrated in Figure 3-2 confirm that A/J mice generate a T_H2 immune response to *T. muris* that, in general, results in expulsion of the worm. Of all the mice evaluated for the presence of adult worms (4 weeks

post inoculation and beyond), only three had live *T. muris* adult worms within the cecum. Of importance is the fact that the three mice had a reduced worm burden (<10) compared to that typically seen in susceptible mouse strains (>100 worms). Collectively these data indicate that when exposed to *T. muris*, A/J mice mount an immune response dominated by T_H2 cytokines.

The ultimate goal of these studies is to evaluate the therapeutic potential of *T. muris* to reverse T_H1 mediated inflammation induced by *H. hepaticus*. To best accomplish this, the peak production periods of T_H2 cytokines were identified, as they would have the best potential to decrease IL-12/IFN- γ driven T_H1 inflammation^{14,113,114} and ultimately decrease disease severity. Four month old female A/J mice were inoculated with *T. muris* and subsets of 5 mice were necropsied weekly for 6 weeks. mRNA expression of IL-4 and IL-13 were significantly elevated within 2 weeks of *T. muris* ova inoculation (Figure 3-2C). Interestingly these cytokines remained elevated through the week 6 time point, suggesting that even after clearance of the worms a T_H2 cytokine response persists. This finding was not entirely unexpected as the T_H2 responses which develops following exposure to helminths has been shown to prevent the development of colitis^{18,47-55} in mouse IBD models and thought to be protective in human IBD development¹⁰⁰. As IL-5 has also been shown to be integral for expulsion of *T.muris*^{355,356}, the elevation of IL-5 seen during weeks 2 and 3 in the *T. muris* treated mice was not unexpected. As IL-6 expression, in combination with TGF β , has been linked to the induction of T_H17 inflammatory

responses^{14,109-112}, now known to play a role in CD^{14,113,114}, it was not surprising that this cytokine was also up-regulated. Additionally, epithelial cells have been shown to express elevated levels of IL-6 following exposure to *T. suis*³⁵⁷. The elevation in IL-6 levels documented between weeks 3 and 5 could be due to either epithelial cell production and/or the induction of a T_H17 cytokine response.

The elevation in mRNA expression detected of the non-T_H2 cytokines, IL-10, IL-12/23 p40 and IFN- γ at weeks 1-2, week 2 and weeks 1-4, respectively, were relatively short lived in comparison. Increased expression of these cytokines are typically observed in susceptible mouse strains³¹⁶ however, clearance of *T. muris* was documented in a majority of these mice. As T_H1 responses often occur following exposure to intracellular microbes, not helminths, it is possible that these elevations of mRNA expression occur as a secondary effect to *T. muris* exposure. Upon arrival at the cecum *T. muris* ova hatch into the L1 larval stage, penetrate the epithelium at the base of the crypts and undergo 4 larval molts within the epithelial layer before emerging into the lumen of the cecum^{343,358}. It is logical to suspect that the initial invasion of the mucosa by *T. muris* larvae and the entrance of adult worms into the cecal lumen could cause a temporary breach in the epithelium, which normally serves as a barrier between the microbiota and the immune system¹⁸⁹. This could result in a brief up regulation of a T_H1 immune response involving IL-12/23 p40 and IFN- γ . The immediate IFN- γ response could be due to the up-regulation of this cytokine by epithelial cells as this has been shown to occur within 24 hours of *T. muris*

exposure in mice³⁵⁹. IL-10 is an anti-inflammatory cytokine known have the ability to down regulate both T_H1 and T_H2 mediated inflammation^{14,147}. As T regulatory cells have been shown to be involved in the immune response required for helminth clearance^{360,361}, it is possible that the elevations in IL-10 are due to an increase T regulatory cell activity. Alternatively, either epithelial cells or macrophages could be responsible for the IL-10 elevations, as both of these cell types have been documented to produce the IL-10 in response to antigens^{362,363}.

To evaluate the therapeutic potential of a helminth to reverse T_H1 driven inflammation, A/J mice with ongoing *H. hepaticus* induced-inflammation were treated with *T. muris* ova and evaluated at one of two time points. As human trials have reported some adverse clinical signs during the first months of *T. suis* therapy²²⁹, despite the long term benefit of disease remission^{227,228,338}, it is important to evaluate changes in disease severity at both an acute and chronic time point following *T. muris* treatment. Week 3 post-treatment was chosen for the short-term evaluation time point as changes in disease severity could potentially be caused by the up regulation of the anti-inflammatory cytokine IL-10 and the T_H2 cytokines IL-4 and IL-13. The second time point was chosen to evaluate the long term effects of helminth therapy.

Interestingly, at three weeks post-*T. muris* treatment disease was exacerbated in the *H. hepaticus* inoculated mice (Figure 3-3A). This finding may simply be a side effect of the inflammatory response involved in helminth clearance^{316,358} as it coincides with the reports that helminth therapy in humans

can cause adverse clinical signs²²⁹. The findings at the week 8 time point (Figure 3-5A) support this hypothesis, as no long term effects on disease were observed. When the lesion scores of mice treated with *T. muris* ova were compared to those treated with PBS, no significant differences in cecal lesion scores were found within the *H. hepaticus* inoculation group.

Next, the ability of *T. muris* treatment to alter *H. hepaticus* induced cytokine mRNA expression was evaluated. Significant elevations were found for all cytokines, with the exception of IL-10 and IL-17a, when comparisons were made between *H. hepaticus* inoculated mice treated with *T. muris* and PBS at the three week time point. Interestingly, when the same comparisons were made at the 8 week time point, IL-4, IL-5 and IL-13 remained elevated while IL-12/23 p40 and IL-10 expression were significantly decreased. As previously discussed, despite decreased mRNA expression of these cytokines, there was not a significant reduction in disease severity (Figure 3-5A).

These observations are contrary to reports in other mouse models of IBD^{277,336,337} in which helminth exposure can suppress ongoing disease. These data do, however, agree with the findings in the *Citrobacter rodentium* model of IBD, in which treatment with helminths increased histologic disease^{313,314}. The discrepancy between these findings may be due to differences in disease induction between the mouse models being examined. For example, reversal of disease following helminth therapy appears to be limited to animal models induced by a disruption of the epithelium (TNBS, DSS and DNBS), the adoptive

transfer of T cells into an immunocompromised mouse or the use of a genetically modified mouse (C57BL/6 IL-10^{-/-}). Both the *C. rodentium* and *H. hepaticus* models rely on a bacteria for the induction of disease. Ultimately, these data demonstrated that, despite the induction of a T_H2 predominated cytokine response to *T. muris*, the ongoing T_H1 mediated inflammation induced by *H. hepaticus* in the A/J mouse was ultimately unaltered after an acute period of exacerbated disease severity.

In addition to the finding that *T. muris* is incapable of reversing *H. hepaticus*-induced inflammation, we demonstrated that the immune response to *T. muris* is altered by an ongoing T_H1 inflammation. During evaluation of disease, the presence of *T. muris* could be detected histologically as indicated by the arrows in Figure 3-3B. Interestingly, when this was examined at the week 3 time point, a significant difference in *T. muris* detection was found between mice inoculated with *H. hepaticus* and treated with *T. muris* and mice inoculated with brucella broth and treated with *T. muris*. As the immune modulation capabilities of *Helicobacter spp.*^{255,261,364,365} have been documented it is not surprising that *H. hepaticus* could delay the clearance of *T. muris*.

Comparisons between *T. muris* treated mice that had previously been inoculated with brucella broth or *H. hepaticus* allowed for evaluation of any effects an ongoing T_H1 inflammatory process may have on the immune response to *T. muris*. At 3 weeks post-inoculation, significant elevations in IFN- γ , IL-6, IL-10, IL-12/23 p40, and IP-10 were found in *T. muris* treated mice previously

inoculated with *H. hepaticus*, which exceeded the expression levels caused by *T. muris* alone. The same was found at the week 8 time point with the addition of significant elevations for IL-4, IL-5, IL-13 and IL-17a. The previous data presented in Figure 3-3C suggested that *H. hepaticus* inoculated mice have delayed clearance of *T. muris* as indicated by the significant increase of histologically detectable *T. muris* larvae. IP-10 has been demonstrated to inhibit the increased turnover of epithelial cells caused by IL-13^{366,367} and neutralization of IP-10 has been shown to decrease colitis in other mouse models of IBD^{366,368}; the up-regulation of IP-10 by *H. hepaticus* could potentially explain the delayed clearance of *T. muris* in these mice. Furthermore, the expression of IFN- γ and associated cytokines such as IL-12 have been identified as being up-regulated in mice susceptible to *T. muris* infection³⁴³. The previous induction of these cytokines by *H. hepaticus* could be interfering with worm expulsion.

While several mouse models of IBD have demonstrated the therapeutic effects of helminth treatments, our data highlights an important consideration when using helminth based therapeutics in human patients – microbiota driven inflammation. The influence of the microbiota have been well documented in both IBD patients^{77,78} and in mouse models⁷⁹⁻⁸¹ of IBD, and in general, have been shown to be integral in the development of a functional immune system⁸². As demonstrated by our data, the inverse situation in which inflammation driven by the microbiota could effect the immune response to worms, must also be considered. Changes in the microbiota have been shown to be responsible for

delays in immune response to *T. muris* in B10Br mice treated with the probiotic *Lactobacillus casei*³⁶⁹. *T. muris* burden has been shown to be decreased if antibiotics are given prior to helminth inoculation, suggesting the importance of an unaltered microbiota for helminth development³⁷⁰. In this model, the immune response to *H. hepaticus* following its addition to the A/J mouse's endogenous microbiota clearly affected not only the cytokine response, but also the severity of inflammation association with the presence *T. muris* and the timeframe in which the helminth was expelled. In regards to the delayed expulsion in the *H. hepaticus* infected mice, we hypothesize this could be due to the significant elevation in IP-10, which inhibits IL-13 induced epithelial cell turn over^{108,109} and was found to be induced by *H. hepaticus* at both necropsy time points. As depicted in Figure 3-7, instead of the helminth induced T_H2 inflammation suppressing the *H. hepaticus* associated T_H1/T_H17 inflammation, the *H. hepaticus* associated T_H1/T_H17 inflammation interfered with the T_H2 response required to expel *T. muris*.

These data demonstrate that T_H1/T_H17 inflammation instigated by *H. hepaticus*, which serves as a model for IBD, is acutely exacerbated and chronically unaffected by treatment with *T. muris*, despite the induction of a protective T_H2 cytokine response. Additionally, we demonstrated that the ability to clear *T. muris* was delayed in the presence of a chronic T_H1 inflammation. Overall our data suggests that helminth therapies should be used with caution when used to treat microbiota driven T_H1 inflammation.

Materials and Methods

Mice

This research described in this manuscript was approved by the University of Missouri's Animal Care and Use Committee (ACUC) and performed in accordance with the *Guide for the Care and Use of Laboratory Animals*. All wild type A/JCr mice used were obtained from Frederick National Laboratory for Cancer Research and either used directly in experiments or to create breeding pairs. Pups from breeding pairs were used in experiments as described.

Trichuris muris

Trichuris muris inoculum was kindly provided by Dr. Joseph Urban of the United States Department of Agriculture (Beltsville, MD). Mice were inoculated via gastric gavage with a volume (between 0.07 – 0.15 ml, dependent upon batch) of inoculum shown to result in a heavy worm burden (approximately 100 adult worms) in a susceptible mouse strain.

Helicobacter hepaticus

The MU-94 *Helicobacter hepaticus* strain was cultured as previously described^{25,252}. Mice were inoculated at 3-4 weeks of age with 0.5 mL of culture broth which contains approximately 5×10^8 bacteria/ml. One week later mice were re-inoculated with the same dose of *H. hepaticus*. A *H. hepaticus* specific

PCR was utilized to confirmed colonization within one month of inoculation and again at necropsy²⁴⁷.

Necropsy

Following euthanasia through CO₂ inhalation, the cecum was removed and cecal contents were collected. The cecum was then longitudinally sectioned into two equal portions and used for two of the following: verification of *T. muris* presence, mRNA expression analysis or histologic examination.

Verification of *Trichuris muris* larvae presence

Ceca were rinsed in warm PBS to remove cecal contents and then placed in a 50-ml conical tube filled with 10 mls of 10 mM EDTA. Following a two hour incubation in a 37⁰ C shaking incubator, tubes are vortexed on high speed for 15 seconds and all contents poured into a petri dish. The supernatant and cecal tissues are examined with an inverted microscope (40x) for larvae.

Verification of *Trichuris muris* adult worm presence

At necropsy, ceca were inspected with a dissecting microscope for the presence of adult worms.

Histologic Evaluation

Cecal portions utilized for histologic evaluation were fixed in zinc fixative, paraffin embedded, sectioned and then stained with hematoxylin and eosin

(H&E). Using a previously described protocol,^{246,252,253} the cecal sections were then scored for inflammatory lesions in a blinded fashion.

Isolation of RNA and Reverse Transcription

Cecal sections, stored at -80 °C until processing, were thawed in TRIzol (Invitrogen, Carlsbad, CA) and homogenized with a TissueLyser (Qiagen, Valencia, CA). Isolation of RNA was performed per manufacturer's (Invitrogen) instructions. A Super Script II kit (Invitrogen) was next used to reverse-transcribe RNA (5 µg total/ sample) into cDNA (final concentration 20 ng/µL) following the oligo(dT) primer protocol provided by the manufacturer.

Real Time PCR

A Qiagen Rotor-Gene Q was used to perform real time quantitative PCR with Qigen Quanti-tect SYBR Green. Table 3-1 contains the primers and annealing temperatures utilized. Run conditions for HPRT, IFN-γ, IL-4, IL-10, IL-12/23 p40, IL-17a and IP-10 have been previously published^{25,252,371}.

Statistical Analysis

Two-way ANOVAs with Student-Newman-Keuls post-hoc tests and Fisher exact tests were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). Due to the exponential nature of real-time PCR, Log2 transformations were used to correct for failures in normality. For all tests, p values < 0.05 were regarded as significant. All graphs and dot plots were generated using either

SigmaPlot 11.0 or GraphPad Prism 5.0 software (GraphPad Prism Software Inc., La Jolla, CA).

Figure 3-1. A/J mice expel *Trichuris muris*.

Eight five-month old female A/J mice were gastrically gavaged with *T. muris* ova. (A) Mice were sacrificed and evaluated for the presence of larvae at 12 days post-inoculation or adult worms at 28 days post-inoculation (n = 4 / necropsy time point). Data is presented as total percentage of each experimental group with detectable *T. muris* larvae (white striped bars) or adult worms (gray striped bars). (B) Images of *T. muris* larvae isolated from mice examined 12 days post-inoculation.

A **Percentage of mice with detectable *Trichuris muris* larvae (Day 12) or adult worms (Day 28)**

B

Figure 3-2. A/J mice mount a protective T_H2 immune response to *Trichuris muris*.

Two groups of four-month old A/J mice (30 mice/group) were gastrically gavaged with either *T. muris* ova or PBS (vehicle control). Five mice per group were necropsied weekly and examined for the presence of *T. muris* and IL-12/23 p40, IFN- γ , IL-10, IL-4, IL-5, IL-6 and IL-13 mRNA gene expression. (A) Experimental Design. (B) Percentage of mice positive for *T. muris* at necropsy; weeks 1-3 were evaluated for the presence of larvae (white striped bars) and weeks 4-6 were evaluated for adult worms (white bars). (C) mRNA analysis of cecal gene expression. mRNA expression was significantly elevated in *T. muris* (gray bars) treated mice when compared to PBS control (black bars) treated mice as indicated. All data was analyzed with a two-way ANOVA with Student-Newman-Keuls post-hoc test; $p \leq 0.05$ considered significant and indicated by lower case letters. Error bars represent $\pm$ S.D.

Figure 3-3. Treatment with *Trichuris muris* exacerbates intestinal inflammation.

3-4 week old female A/J mice were inoculated with either *H. hepaticus* or brucella broth (control). Three months later the mice were treated with either *T. muris* ova or PBS. (A) Cecal lesion scores of mice from all four experimental groups three weeks post-treatment. Data was analyzed with a two-way ANOVA with Student-Newman-Keuls post-hoc test; $p \leq 0.05$ considered significant and indicated by horizontal capped lines. Error bars represent $\pm$ S.D. (B) Representative depictions of inflammation from all four experimental groups. Black arrowheads indicate *T. muris* cross-sections. (C) During examination of cecal histology the presence or absence of *T. muris* was recorded. Data from mice treated with *T. muris* analyzed with a Fisher exact test; $p \leq 0.05$ considered significant and indicated by lower case letters.

Figure 3-4. Treatment with *Trichuris muris* significantly elevates T_H1 and T_H2 cytokine and chemokine expression.

3-4 weeks old female A/J mice were inoculated with either *H. hepaticus* or brucella broth (control). Three months post-inoculation the same mice were treated with either *Trichuris muris* ova or PBS (vehicle control); three weeks later mice were sacrificed and mRNA expression in cecal tissue was evaluated with real-time PCR analysis. All data was analyzed with a two-way ANOVA with Student-Newman-Keuls post-hoc test; $p \leq 0.05$ considered significant and indicated by lower case letters. Error bars represent $\pm$ S.D.

Figure 3-5. *Trichuris muris* treatment does not abrogate *H. hepaticus* induced intestinal inflammation.

3-4 week old female A/J mice were inoculated with either *H. hepaticus* or brucella broth (control). Three months post-inoculation the same mice were treated with either *T. muris* ova or PBS; eight weeks later mice were sacrificed. (A) Cecal lesion (B) Percentage of mice with *T. muris* detectable histologically and during necropsy. (C) Cecal mRNA gene expression evaluated with real-time PCR analysis. All data analyzed with a two-way ANOVA with Student-Newman-Keuls post-hoc tests or a Fisher exact test; $p \leq 0.05$ considered significant and indicated by lower case letters. Error bars represent $\pm$ S.D.

Figure 3-6. The immune response to *Trichuris muris* is altered by ongoing Th1 inflammation.

Table 3-1. Real-time PCR primers and annealing temperatures.

	Forward	Reverse	Anneal Temp (°C)
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA	60
IFN-γ	GCCATCAGCAACAACATAAGC	TGGGACAATCTCTTCCCCAC	58
IL-4	ATGGGTCTCAACCCCCAGCTAGT	GCTCTTTAGGCTTTCCAGGAAGTC	57
IL-5	TGACCGCCAAAAAGAGAAG	GGTATCCCTTGCAATTGCAC	53.7
IL-6	AGAGACTTCCATCCAGTTGC	GAATTGCCATTGCACAACTCTT	54.5
IL-10	CGGGAAGACAATAACTG	CATTTCCGATAAGGCTTGG	58
IL-12/23 p40	ACTCACATCTGCTGCTCCAC	GGGAACTGCTACTGCTCTTGA	60
IL-13	ATCACACAAGACCAGACTCC	GTTACAGAGGCCATGCAATATC	54
IL-17a	CAGAAGGCCCTCAGACTACC	CTTCCCTCCGCATTGACAC	56.6
IP-10	TCCCCATCAGCACCATGAAC	ACTTGAGCGAGGACTCAGAC	53

Chapter 4 – Depletion of natural and inducible Foxp3⁺ cell populations alters the immune response in a microbiota-dependent manner.

Introduction

Crohn's Disease (CD) and Ulcerative Colitis (UC) are types of Inflammatory Bowel Diseases (IBDs) which affect approximately 1.5 million Americans. Regardless of the type of IBD, it is assumed that disease develops as the result of inappropriate interactions between an individual's genetics, environment and microbiota which lead to an uncontrolled inflammatory response. As IBDs are thought to be the result of uncontrolled inflammation, it is logical to investigate the role of cellular subsets known to be capable of suppressing inflammatory reactions.

The concept of a suppressive cell that can control the immune system has existed within the scientific community for decades¹³⁸. The existence of this cellular population was first implied in the mid-1900s when scientists observed that the removal of the thymus caused an uncontrolled immune response which resulted in massive autoimmunity¹³⁹⁻¹⁴². However, the concept of a T regulatory cell was not in the limelight of science until 1995 when Sakaguchi observed that CD4⁺CD25⁺ cells prevented the development of autoimmunity in athymic mice³⁷². Since then, thousands of experiments attempting to characterize this cell population have been completed and published. One of the most important and

controversial concepts derived from these publications was discussed in 2003 by Bluestone and Abbas. Upon reviewing the published literature, they proposed the novel idea that not one, but two distinct CD4⁺ regulatory cell populations, a natural and an inducible T regulatory cell, exist and although both have regulatory actions, they develop and function in different manners.¹⁴³ As the distinction between inducible and natural T regulatory cells was made after the initial discovery of T regulatory cells, many of the early studies on T regulatory cells may actually reflect the synergistic effects of these two regulatory subsets.

The existence and function of these two subsets of T regulatory cells has been extensively documented by the numerous reviews^{132,133,143-153}. Natural T regulatory cells arise in the thymus early in life, are programmed to recognize self-antigens³⁷³ and can suppress the actions of auto-reactive lymphocytes, which, if left unchecked, could cause the development of autoimmunity³⁷³. The importance of natural T regulatory cells in preventing autoimmunity and their thymic origin has been extensively demonstrated in vivo through the use of the thymectomy model^{372,374,375}. Natural T regulatory cell's suppressive abilities have been found to be cell-contact dependent³⁷⁶⁻³⁷⁸. The ability of inducible T regulatory cells to develop from CD4⁺ naïve T cells in the periphery has been demonstrated by the development of T regulatory cells after either surgical thymectomy or the adoptive transfer of non-regulatory cells into a mouse lacking endogenous T regulatory cells¹⁵⁴⁻¹⁵⁸. The exact mechanism by which they are induced in vivo is yet to be determined, but in vitro studies suggest that IL-10,

TGF- β and IL-2 are critical for their development and suppressive actions³⁷⁹⁻³⁸². Additionally, it seems that specialized antigen presenting cells, such as CD103⁺ dendritic cells, may be required for inducible T regulatory cell induction in the periphery¹⁸⁶⁻¹⁸⁸. The requirement of CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4), for peripheral induction also differentiates inducible T regulatory cells from their thymic-derived counterparts which do not require CTLA-4 for the development of suppressive abilities^{383,384}.

The most commonly used marker for T regulatory cell identification is *Foxp3*, a transcription factor that has been shown to control T regulatory cell development^{164,385}. *Foxp3* is encoded on the X chromosome and genetic defects result in a fatal disorder known as IPEX (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome in humans¹⁵⁹⁻¹⁶², and scurfy in mice^{134,386,387}. Although *Foxp3* is, in general, accepted as a means to identify T regulatory cells, its use for in vitro and ex vivo studies are limited by its intracellular expression.

As a means to study T regulatory cells, a variety of scientific methods have been developed to deplete mice of these cells including: anti-CD25 antibody administration^{388,389}, adoptive transfers in RAG1^{-/-} and RAG2^{-/-} knock-out mice^{390,391}, the development of transgenic mice targeting specific T regulatory cell related genes such as *Foxp3*^{134,386,392,393} and the use of surgical thymectomies^{374,375,394,395}. Of these, the surgical thymectomy is unique in that it anatomically depletes the body of natural T regulatory cells and results in

autoimmunity^{372,374}, which can be reversed by the adoptive transfer of CD4⁺CD25⁺ cells³⁷⁵. Inducible T regulatory cells are not affected by day-3 neonatal thymectomies, as multiple groups have documented the development of a T regulatory cell population either after surgical thymectomy or when naïve T cells were adoptively transferred into mice lacking endogenous T cell populations^{154,155,157,158,396-398}.

Due to the therapeutic potential for T regulatory cells, protocols for the *in vitro* induction of these cells, from naïve T cell populations, have been described in the literature for cells isolated from both mice and humans^{169,379,380,382,383,399,400}. It has been shown that TGF- β is required for the development and maintenance of inducible T regulatory cells in the periphery^{169,379,380,383,401-403}, and that surface-bound TGF- β may be important for T regulatory cell suppressive functions^{168,404,405} in humans and mice. In addition to TGF- β , IL-2 also appears to be required for the induction of T regulatory cells from a naïve CD4⁺ cellular population^{154,382,406,407}. The inducible T regulatory cell's developmental independence from the thymus has been further documented *in vivo* by the peripheral expansion of T regulatory cells from naïve T cells after adoptive transfer to recipient animals^{154,408,409}.

Our laboratory utilizes a *Helicobacter hepaticus*-induced mucosal inflammation model which serves as a for IBD model. In this model, three months after inoculation with *H. hepaticus*, the ceca of A/J mice develop a lymphocytic infiltrative histologic disease²⁵ and have significant elevations in mRNA gene

expression of IL-12/23 p40, IFN- γ and IL-10^{25,252-254}. It is hypothesized that once established in the cecum, *Helicobacter spp.* act as a provocateur which instigate immune responses against certain endogenous microbiota^{248,249}. The importance of T regulatory cells in the prevention of mucosal inflammation induced by *H. hepaticus* is suggested by the development of typhlitis in C57BL/6 mice deficient for IL-10^{265,410}. While the C57BL/6 strain of mouse is known to be resistant to *H. hepaticus* induced disease²⁵, removal of functional IL-10 renders these mice susceptible to disease²⁶⁵. Furthermore, adoptive transfer experiments have demonstrated that only CD4⁺ cells with functional IL-10 isolated from a donor previously infected with *H. hepaticus* have the ability to protect against *H. hepaticus* induced-disease development¹¹⁷. As IL-10 is involved in the suppressive mechanism of T regulatory cells, it is logical to assume that these cells are involved in preventing inflammation following *H. hepaticus* exposure.

As previously discussed, it is impossible to differentiate between the two subsets of T regulatory cells in vivo. Instead, coordinated experiments which removed either just natural T regulatory cells or both subsets were utilized to dissect out the role of these cells in mucosal inflammation. First, the protective capabilities of inducible T regulatory cells in this disease model were evaluated by utilizing neonatal thymectomies to remove natural T regulatory cell populations. Next, both T regulatory cell subsets were removed utilizing C57BL/6- *Foxp3*^{DTR} mice which allow for the depletion of all Foxp3⁺ cells

following injection with diphtheria toxin (DT). This allowed us to evaluate the role of both T regulatory cells populations in the prevention of *H. hepaticus* induced disease development. The results from these experiments demonstrate that depletion of natural and inducible T regulatory cells had no effect on the C57BL/6 mouse strain's resistance to *H. hepaticus* induced disease. Interestingly, however, the data suggests that in the C57BL/6 mouse, *H. hepaticus* causes a protective immune response, which potentially includes the development of inducible T regulatory cell population, which drives maintenance of mucosal homeostasis.

Results

Depletion of natural T regulatory cells results in decreased IFN- γ response to *H. hepaticus* in the C57BL/6 mouse four days post-inoculation

C57BL/6 mice inoculated with *H. hepaticus* are resistant to the development of the T_H1 inflammation seen in susceptible mouse strains. When functional IL-10 is removed in this strain, disease develops following *H. hepaticus* inoculation, thus suggesting the importance of IL-10 and cells like T regulatory cells which utilize IL-10 to suppress inflammation. These findings, in combination with published reports of the ability of adoptively transferred T regulatory cells to reverse *H. hepaticus* induced inflammation, prompted us to more thoroughly evaluate the role of these cells in the C57BL/6 mouse's resistance to disease. To start, the capabilities of natural T regulatory cells to alter the acute response

to *H. hepaticus* were evaluated. In short, C57BL/6^{DTR} female mice were either thymectomized (removal of natural T regulatory cells) or sham thymectomized (control) three days following birth and then inoculated with either *H. hepaticus* or brucella broth at 3-4 weeks of age. Four days after inoculation, the mice were necropsied and evaluated for mRNA expression of IL-12/23 p40, IFN- γ , IL-10, IL-17a and IL-17f (Figure 4-1). When *H. hepaticus* inoculated mice depleted of natural T regulatory cells (thymectomized) were compared to those with T regulatory cells (sham thymectomy) the only significant difference noted was a decrease in IFN- γ mRNA expression. When the same comparison was made in brucella broth inoculated mice a significant decrease in IL-12/23 p40 mRNA expression and a significant increase in IL-10 mRNA expression was noted in the mice depleted of natural T regulatory cells. When thymectomized mice inoculated with *H. hepaticus* were compared to those inoculated with brucella broth significant increases in IFN- γ , IL-12/23 p40, IL-17a and IL-17f were seen. Finally when control thymectomized mice inoculated with *H. hepaticus* were compared to those inoculated with brucella broth, significant elevations were noted in IFN- γ , IL-10, IL-17a and IL-17f. The same experiment was repeated in male mice and, in general, similar trends were observed (Figure 4-5). These data demonstrate that the initial IFN- γ response generated to *H. hepaticus* is suppressed in the absence of a natural T regulatory cell population.

Depletion of natural T regulatory cells does not result in *H. hepaticus*-induced disease.

Next we sought to determine if the absence of natural T regulatory cells would affect the C57BL/6 mouse's resistance to the development of *H. hepaticus* induced disease. To accomplish this, C57BL/6-*Foxp3*^{DTR} female mice were either thymectomized or sham thymectomized three days following birth and then inoculated with either *H. hepaticus* or brucella broth at 3-4 weeks of age. Ninety days after inoculation the mice were necropsied and evaluated for the development of cecal inflammation and mRNA expression of IL-12/23 p40, IFN- γ , IL-10, IL-17a and IL-17f. When cecal inflammation was evaluated, no significant differences in disease severity were noted among the four groups, regardless of inoculum or natural T regulatory cell status (Figure 4-2A). The same observations were made when male mice were evaluated (Figure 4-6A).

When cecal mRNA expression in *H. hepaticus* inoculated mice depleted of natural T regulatory cells were compared to those with an intact natural T regulatory cell population no significant differences were noted in any of the cytokines evaluated. When the same comparisons were made of brucella broth inoculated mice, IFN- γ was significantly elevated. A significant decrease in the mRNA expression of IFN- γ and a significant increase in IL-10 mRNA expression were noted when *H. hepaticus* inoculated thymectomized mice were compared to brucella broth inoculated thymectomized mice. When *H. hepaticus* mice with unaltered T regulatory cells populations were compared to their brucella broth

counterparts, a significant increase in IL-10 was detected. (Figure 4-2B) As with the day 4 experiments, this experiment was repeated in male mice. Similar trends were noted, with lack of disease development in all four treatment groups (Figure 4-6A). In general, the trends observed for the cytokines in the female mice were also seen in the male mice. (Figure 4-6B) Collectively the data from the experiments involving natural T regulatory cell depletion demonstrate the despite some changes in mRNA cytokine expression, the absence of natural T regulatory cells does not alter the C57Bl/6 mouse's resistance to *H. hepaticus* induced disease.

Acute depletion of Foxp3⁺ cells results in decreased IL-10 expression in *H. hepaticus* inoculated mice.

The previous experiments demonstrate that natural T regulatory cells are not involved in the protective immune response which prevents the development of *H. hepaticus* induced disease in the C57BL/6 mouse. The next logical step was to evaluate the role of inducible cells in the resistance to *H. hepaticus* induced disease. As it is impossible to only deplete inducible T regulatory cells, the role of this cell population was extrapolated by comparing mice depleted of both T regulatory cells subsets (natural and inducible) to those only depleted of natural T regulatory cells. First, the acute immune response to *H. hepaticus* was evaluated by treating 3-4 week old C57BL/6-*Foxp3*^{DTR} mice with either diphtheria toxin (DT) or PBS (control), inoculating those mice with either *H. hepaticus* or brucella broth and then necropsying all mice 4 days post-inoculation. Again, the

expression of cytokine mRNAs were evaluated with real-time PCR (Figure 4-3). When *H. hepaticus* inoculated mice depleted of Foxp3⁺ cells were compared to those with intact T regulatory cell populations, a significant increase was noted in IL-12/23 p40 and a significant decrease was noted in IL-10. When the same groups were compared within brucella broth inoculated mice, significant increases were noted in IL-12/23 p40, IFN- γ , IL-10 and IL-17a. When *H. hepaticus* inoculated mice depleted of Foxp3⁺ cells were compared to brucella broth inoculated mice also depleted of this cell group, significant elevations were noted in IL-10 and IL-17a. When comparisons were made between the control treatment mice inoculated with brucella broth or *H. hepaticus*, significant elevations were found for IFN- γ , IL-10, IL-17a and IL-17f. The experiment was repeated in male mice and again similar trends were noted (Figure 4-5). This experiment suggests that when Foxp3⁺ cells are removed in the presence of *H. hepaticus*, a more pro-inflammatory cytokine environment persists as IL-12/23 p40 expression is up-regulated and the anti-inflammatory cytokine IL-10 is down regulated four days post-inoculation. .

Depletion of Foxp3⁺ T regulatory cells did not change susceptibility to *H. hepaticus* induced disease.

Finally, we sought to investigate if the pro-inflammatory immune response documented during the acute response to *H. hepaticus* would result in the development of disease. As with the prior experiment, C57BL/6-Foxp3^{DT^R} mice were depleted of Foxp3⁺ cell populations with DT treatments and inoculated with

either *H. hepaticus* or brucella broth. Ninety days post-inoculation mice were necropsied and their ceca used to evaluate the development of histologic disease and mRNA expression of IL-12/23 p40, IFN- γ , IL-10, IL-17a, and IL-17f. Interestingly, when histologic sections were evaluated a significant increase in disease was noted in female mice depleted of Foxp3⁺ cells and administered brucella broth, but not *H. hepaticus*. In fact, when the mice depleted of Foxp3⁺ cells were compared between the two inoculation groups, those inoculated with brucella broth had more severe lesion scores than those inoculated with *H. hepaticus*. (Figure 4-4A) When the experiment was repeated with male mice, these differences were not noted (Figure 4-8A).

When cytokine mRNA expression was evaluated, minimal changes were seen. No significant changes were seen among the four groups for IL-12/23 p40, IL-17a or IL-17f. IFN- γ expression was elevated only when *H. hepaticus* inoculated, Foxp3⁺ depleted mice were compared to the *H. hepaticus* inoculated sham thymectomized mice. Significant elevations in IL-10 were found within both T regulatory cell groups, depleted and intact, when *H. hepaticus* inoculated mice were compared to their brucella broth inoculated counterparts. (Figure 4-4B) Similar trends were seen in the repeat experiment which utilized male mice (Figure 4-8B). Collectively the experiments involving depletion of Foxp3⁺ T regulatory cells demonstrate that despite an acute pro-inflammatory response, the absence of these cells at the time of *H. hepaticus* inoculation does not alter the C57BL/6 mouse's resistance to disease.

Discussion

As the development of IBD is thought to be due to an inappropriate immune response to the bacterial flora, it is logical to assume that T regulatory cells, which are typically responsible for the regulation of the body's immune responses, are involved. Humans without functional *Foxp3* lack T regulatory cells and many develop severe intestinal inflammation due to the uncontrolled infiltration of lymphocytes¹⁵⁹⁻¹⁶². In a similar fashion, mice without functional T regulatory cells have been shown to develop colitis^{163,164}. Although it has not been shown that those with IBD have dysfunctional T regulatory cells, it has been demonstrated that a decreased proportion of T regulatory cells are present in IBD patients compared to healthy controls¹⁶⁵, which may be due to an increased rate of T regulatory cell apoptosis¹⁶⁶. It has been shown that T regulatory cell's suppressive abilities are mediated by both IL-10 and TGF β ¹⁶⁷⁻¹⁶⁹ and that this cell subset is capable of both preventing and reversing colitis in mouse IBD models^{116-119,155,170-178}.

Experiments in IL-10^{-/-} mice have demonstrated the importance of these T regulatory cells products in the prevention of colitis development. For example, when C57BL/6 mice lack functional IL-10, they develop a chronic enterocolitis which is thought to be driven by members of the microbiota such as *H. hepaticus*^{411,412}. Furthermore, it has been shown that the protective abilities of T regulatory cells in adoptive transfer models of colitis are negated when IL-10 signaling is not intact⁴¹³. As wild-type C57BL/6 mice do not develop disease

following inoculation with *H. hepaticus*²⁵, it is logical to suspect that disease resistance in this strain is mediated in some fashion by T regulatory cells.

First, we aimed to identify the T regulatory cell population responsible for resistance to *H. hepaticus*-induced disease in the C57BL/6 mouse. While both populations are suppressive in nature, natural and inducible T regulatory cells have been shown to develop through different pathways^{132,133,143-153}. Natural T regulatory cells develop within the thymus and inducible T regulatory cells develop from CD4⁺ naïve T cells in the periphery^{154-158,127,128,138-148}. In the following experiments, neonatal thymectomies were utilized to permanently deplete natural T regulatory cells^{83,158,374,414}, while still allowing for the development of an inducible T regulatory cell population^{21,30,100,101,415}. The use of a C57BL/6 mouse with a diphtheria toxin (DT) receptor linked to *Foxp3* expression (C57BL/6-*Foxp3*^{DTR}) allowed for the temporary depletion of all Foxp3⁺ cell populations, including both natural and inducible T regulatory cells³⁹². Injection with DT reduced T regulatory cell populations by 75% and 98% in the cecum and spleen, respectively, within 2 days following the last treatment (data not shown). It was at this time point that mice were inoculated to best evaluate the role of Foxp3⁺ T regulatory cells in the response to *H. hepaticus*.

For both natural T regulatory cell and Foxp3⁺ T regulatory cell depletion, mice were evaluated at both an acute (4 day) and chronic (90 day) time point to access changes in mRNA expression of IL-12/23 p40, IFN- γ , IL-10, IL-17a and IL-17f. These cytokines were chosen so as to evaluate both T_H1 and T_H17 driven

aspects of inflammation. Human CD patients have been documented to have elevated T_H1 and T_H17 cytokines such as IFN- γ and IL-17^{3,14-16,21,121}.

Furthermore mouse models of IBD involving *H. hepaticus* have demonstrated elevations in IL-12/23 p40, IFN- γ , IL-10 and IL-17a^{25,253,416}.

First, the role of natural T regulatory cells in the response to *H. hepaticus* was evaluated. To accomplish this, *H. hepaticus* inoculated, thymectomized mice were compared to *H. hepaticus* inoculated, sham thymectomized mice. Evaluation of mRNA expression with real-time PCR at the acute time point (four days post-inoculation) revealed a significant decrease in IFN- γ expression in the thymectomized female mice compared to their respective controls (Figure 4-1). At the ninety day time point no significant differences in cecal cytokine mRNA expression were noted among female mice (Figure 4-2B). The acute shift in mRNA expression of IFN- γ , although statistically significant, does not appear to be physiologically relevant as there were no differences in the development of histologic disease (Figure 4-2A). These data suggest that natural T regulatory cells are not involved in the resistance to *H. hepaticus* induced disease. These findings were not entirely unexpected, as susceptibility to neonatal thymectomy-induced autoimmunity has been reported to be strain dependent. The C57BL/6 mouse is cited as a “resistant” strain⁴¹⁷ and has been shown to only develop autoimmunity after complete removal of Foxp3⁺ cellular subsets¹⁶⁴.

These findings further support the importance of inducible T regulatory cells in C57BL/6 mouse and led to the evaluation of these cells. Unfortunately,

the selective depletion of inducible T regulatory cells is physiologically impossible with current techniques. To evaluate the immune response without the influence of Foxp3⁺ regulatory T cells (natural and inducible), mice were treated with either diphtheria toxin or PBS (vehicle control) at 3-4 weeks of age and then inoculated with *H. hepaticus* two days later. As with the prior experiment, mice were necropsied and evaluated either four or ninety days post inoculation. Evaluation of cecal mRNA with real-time PCR at the early (4 day post-inoculation) time point revealed increased IL-12/23 p40 expression and decreased IL-10 expression when Foxp3⁺ female depleted mice were compared to controls (Figure 4-3).

When *H. hepaticus* inoculated mice depleted of Foxp3⁺ cellular subsets were evaluated ninety days post-inoculation, the only shift observed in cytokine mRNA cytokine expression was a significant elevation of IFN- γ expression in female DT treated mice compared to controls (Figure 4-4B). Despite the elevation in IFN- γ there were no differences in histologic lesions, and neither the DT treated nor control mice had an average lesion score which correlated with disease development in this model (Figure 4-4A).

Collectively these experiments demonstrate that depletion of neither natural nor inducible T regulatory cells at the time of *H. hepaticus* renders the C57BL/6 mouse susceptible to *H. hepaticus* induced inflammation. These findings are contradictory to the implication derived from studies utilizing C57BL/6 IL-10^{-/-} mice. C57BL/6 mice which lack functional IL-10 develop disease when infected with *H. hepaticus*^{413,414}, thus suggesting a possible role for

T regulatory cells in disease resistance. The importance of T regulatory cells in disease resistance is further implied by studies which demonstrate that the adoptive transfer of T regulatory cells lacking functional IL-10 negates the typical protective properties of these cells⁴¹³. With this in mind, it must be considered, that the ability of the mice evaluated in this study to re-generate an inducible T regulatory cell population was not affected by the experimental protocols. Unlike C57BL/6 IL-10^{-/-} mice which have persistent loss of the cytokine's anti-inflammatory properties, the inducible T regulatory cell populations in these mice were only temporarily depleted. The continued resistance in these mice to disease development could be due to the regeneration of an inducible T regulatory cell population. These findings are in line with a previous study that demonstrated that protection from *H. hepaticus* induced disease following the adoptive transfer of T regulatory cells is dependent upon the *H. hepaticus* status of donor mice, which presumably drives the induction of an inducible T regulatory cell population¹¹⁷.

To evaluate the role of these T regulatory cell populations in the general maintenance of gut homeostasis without the influences of a bacteria provocateur such as *H. hepaticus*, the comparisons made in *H. hepaticus* inoculated mice were repeated in those inoculated with brucella broth. First we sought to evaluate the role of natural T regulatory cells in the immune response to the endogenous microbiota of the C57BL/6 mouse. To accomplish this, mice depleted of natural T regulatory cells and inoculated with brucella broth were

compared to controls inoculated with brucella broth. Evaluation of mRNA cytokine expression with real-time PCR at the acute time point (four days post-inoculation) revealed a significance decrease in IL-12/23 p40 expression and a significant increase in IL-10 expression when female mice depleted of natural T regulatory cells were compared to controls (Figure 4-1).

When the same treatment groups of female mice were evaluated ninety-days post-inoculation, there was a significant elevation in IFN- γ expression when thymectomized mice were compared to their control counterparts (Figure 4-2B). Interestingly, in the female mice, despite the elevation in IFN- γ expression, a cytokine associated with the T effector cell populations capable of disease induction^{25,253}, no differences in disease severity were noted in the female mice (Figure 4-2A).

Next the immune response to the microbiota, without the influence of Foxp3⁺ regulatory T cells (natural and inducible), was evaluated. At 3-4 weeks of age C57BL/6-*Foxp3*^{DTR} mice were treated with either diphtheria toxin or PBS (vehicle control) and then inoculated with *H. hepaticus* two days later. Evaluation of cecal mRNA with real-time PCR at the acute time point (four days post-inoculation) revealed a significance increases in IL-12/23 p40, IFN- γ , IL-10 and IL-17a expression in female DT treated mice compared to controls (Figure 4-3). The acute elevation of cytokine mRNA expression when both natural and inducible T regulatory cells are depleted, but not when only natural T regulatory cells were depleted, suggests that inducible T regulatory cells are more involved

in controlling the immune response to the microbiota. As it has been shown that development of this population of T regulatory cells is driven by the microbiota^{84,86}, these findings are not surprising.

At the late time point (90 days post inoculation), no significant differences in cecal mRNA expression of cytokines were found between the T regulatory cell depleted and control mice within the female mouse groups evaluated (Figure 4-4B). Interestingly, however there was a significant elevation in disease severity between female mice depleted of their Foxp3⁺ cellular subsets and the control mice (Figure 4-4A). The lack of long term changes in mRNA expression following the depletion of Foxp3⁺, is most likely due to the regeneration of T regulatory cell populations. Although mice depleted of their Foxp3⁺ cells had a significant elevation in lesions scores when compared to the control mice, the level of inflammation (mean = 4.4) is less severe than that typically seen in our IBD model^{25,252,253}. The development of this inflammation, although not severe, does point to the role of inducible T regulatory cells in establishing a threshold of physiologic inflammation early in life. As inflammation developed only when both inducible and natural T regulatory cells were depleted, but not when natural T regulatory cells alone were depleted, this data further supports the importance of inducible, not natural, T regulatory cells in the maintenance of gut homeostasis.

Interestingly, while our data did not demonstrate a lapse in protection from *H. hepaticus* induced inflammation when T regulatory cells are depleted, it did demonstrate that the removal of inducible T regulatory cells in the absence of *H.*

hepaticus resulted in an increased level of physiologic inflammation presumably driven by the microbiota. To further investigate this finding, we evaluated the differences in mRNA cytokine expression and severity of inflammation in mice depleted of T regulatory cells and inoculated with either *H. hepaticus* or brucella broth.

Four days after inoculation with either *H. hepaticus* or brucella broth removal of natural T regulatory cells resulted in elevated IFN- γ , IL-12/23 p40, IL-17a and IL-17f mRNA expression in female mice inoculated with *H. hepaticus* (Figure 4-1). At ninety-days post-inoculation depletion of natural T regulatory cells resulted in increased IL-10 and decreased IFN- γ mRNA expression when *H. hepaticus* inoculated female mice were compared their brucella broth inoculated counterparts (Figure 4-2B). No differences in inflammation were found between the two inoculation groups when they were depleted of natural T regulatory cells, suggesting that despite the shifts in IFN- γ a cytokine known to be up regulated in *H. hepaticus* driven inflammation^{25,253}, natural T regulatory cells are not required for the C57BL/6 mouse's protective immune response towards *H. hepaticus* (Figure 4-2A). The elevated levels of IL-10 mRNA expression does, however, lend further support to the possibility that inducible T regulatory cells, which produce IL-10, are up-regulated in the *H. hepaticus* inoculated mice.

Next the combined effects of *H. hepaticus* inoculation and the absence of Foxp3⁺ T regulatory cells were evaluated. When female mice depleted of Foxp3⁺ cells were compared at four days post-inoculation, elevations in the mRNA

expression of both IL-10 and IL-17a were found in *H. hepaticus* inoculated mice (Figure 4-3). At ninety days post-inoculation *H. hepaticus* inoculated mice depleted of Foxp3⁺ cells had a significant elevation in IL-10 mRNA expression compared to their brucella broth inoculated counterparts (Figure 4.4B). This finding correlated with the significant decrease in histologic lesion scores seen in *H. hepaticus* mice depleted of Foxp3⁺ cellular populations when compared to those inoculated with brucella broth (Figure 4.4A). It should be noted, however, that the lesion scores found in the DT treated, brucella broth inoculated mice are considered mild when compared to those typically seen in mice susceptible to the *H. hepaticus* model of IBD^{25,253}. The lack of inflammation in the *H. hepaticus* inoculated mice and the elevated levels of IL-10 mRNA expression further supports the hypothesis that this bacteria promotes the development of an inducible T regulatory cell population capable of suppressing inflammatory reactions in the gut.

When data collected from male mice were evaluated in a similar fashion as the females, similar trends were identified overall. The development of inflammation in the female mice, a finding not seen their male counterparts, may simply be due to female mice being more responsive to the microbiota than their male counterparts. This finding supports a previous observation that male mice develop a less severe, inconsistent microbiota driven inflammation compared to their female counterparts²⁵².

Collectively all of these experiments suggest the *H. hepaticus* supports the maintenance of mucosal homeostasis in the C57BL/6 mouse, possibly through the induction of an inducible T regulatory cell population. These findings help explain why the addition of *H. hepaticus* to the microbiota of C57BL/6 mice prevents, rather than induces, the development of inflammation. Without the presence of *H. hepaticus*, mice depleted of Foxp3⁺ T regulatory cells, but not natural T regulatory cells alone, developed a mild cecal inflammation further lending support to the ability of *H. hepaticus* to drive a protective immune response that ultimately helps maintain mucosal homeostasis.

Material's and Methods

Mice

This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the University of Missouri Animal Care and Use Committee. A breeding pair of C57BL/6- *Foxp3*^{DTR} was obtained from the laboratory of Alexander Y Rudensky (Department of Immunology, University of Washington, Seattle) and the offspring of these mice were utilized for all described experiments.

Bacterial Culture

The cultivation of *Helicobacter hepaticus* strain MU-94 has been previously described^{25,252}. For inoculations, mice were gastrically gavaged with 0.5 ml of culture containing approximately 5×10^8 bacteria/ml. 2-4 weeks following inoculation and at the time of necropsy, colonization was confirmed with a *H. hepaticus*-specific PCR of feces²⁴⁷. Brucella broth inoculations were utilized as the vehicle control for the *H. hepaticus* inoculum.

Diphtheria Toxin

Diphtheria toxin was purchased from Sigma (Saint Louis, MO) and all mice treated with diphtheria toxin received two 1 µg intraperitoneal injections; all mice were monitored for weight loss and any sign of malaise. Mice who lost more than 20% of their original body mass or who were exhibiting clinical signs

were immediately removed from the study and euthanized.

Thymectomy

The surgical protocol utilized was adapted from the protocol published in the *Current Protocols in Immunology*³⁷⁴. The pups were anesthetized by placing them on ice, with a plastic glove placed between the mice and the ice to prevent direct contact. After anesthesia, the pups were placed in dorsal recumbency, secured with limb restraints and scrubbed with ethanol and chlorahexadine surgical scrub. A skin incision was made from mid-sternum to the suprasternal notch and the thoracic cavity opened by cutting the first 3 ribs at the site of sterna attachment. . A pasteur pipette connected to a suction pump was then used to remove both thymic lobes and the skin incision closed with tissue glue. All mice received 0.05 mg/kg buprenorphine subcutaneously for post-surgical analgesia. At necropsy all mice were blindly evaluated for the presence of a thymus; any mouse with a thymic remnant was removed from the study.

Necropsy

For all experiments, ceca were removed and sectioned longitudinally into two equal parts. For all experiments, one half was rinsed with sterile PBS and flash frozen in liquid nitrogen for use in mRNA expression analyses. The other cecal half was either fixed in zinc fixative and embedded in paraffin for histologic evaluation.

Histological Evaluation

Following staining with hematoxylin and eosin (H&E), cecal sections were evaluated for inflammatory lesions with a previously described protocol^{246,252,253}. All cecal sections were blindly scored and given a category score for each of the following: inflammation intensity (0 = none, 1 = mild, 2 = moderate, 3 = severe); longitudinal extent of inflammation (0 = none, 1 = 1-2 small foci, 2 = patchy, 3 = diffuse); vertical extent of inflammation (0 = none, 1 = basal mucosal inflammation, 2 = full thickness mucosal inflammation, 3 = transmural inflammation); and hyperplasia (0 = none, 1 = focal, 2 = patchy hyperplasia, 3 = diffuse hyperplasia). All of the lesions scores reported are the sum of the four category scores; any lesions score greater than 2 were decreased by 2 to minimize score inflation by inflammation, which is represented by three of the four categories evaluated.

RNA Isolation and Reverse Transcription

The ceca snap frozen for mRNA analysis were thawed in TRIzol (Invitrogen, Carlsbad, CA) and then homogenized with a TissueLyser (Qiagen, Valencia, CA). Per the manufacturer's (Invitrogen) instructions, total RNA was isolated. Five micrograms of the isolated RNA was reverse-transcribed using a Super Script II kit (Invitrogen, Carlsbad, CA) per the manufacturer's oligo(dT) primer protocol. All resulting cDNA was diluted to 20 ng/ μ L.

Real-Time PCR

Real-time quantitative PCR was performed with a Qiagen Rotor-Gene Q and Qiagen Quanti-tect SYBR Green (Qiagen, Valencia, CA). All run conditions utilized for real-time PCR have been previously described^{25,371} and can be found in *Table 4-1*.

Statistical Analysis

Two-way ANOVAs with Student-Newman-Keuls post-hoc tests were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). Due to the exponential nature of real-time PCR, Log2 transformations were used to correct for failures in normality. p values ≤ 0.05 are regarded as significant. All graphs and dot plots were generated using either SigmaPlot 11.0 or GraphPad Prism 5.0 software (GraphPad Prism Software Inc., La Jolla, CA).

Figure 4-1. Depletion of natural T regulatory cells in female mice results in decreased IFN- γ production in *H. hepaticus* inoculated mice.

Female C57BL/6-*Foxp3*^{DTR} mice were thymectomized (nTreg Depleted) or sham thymectomized (control) at three days of age to remove natural T regulatory cells and then administered *H. hepaticus* (Hh) or brucella broth (BB) at 3-4 weeks of age. Four days later the mice were necropsied and ceca collected. Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters. .

Figure 4-2. Depletion of natural T regulatory cells in female mice does not result in disease development following *H. hepaticus* inoculation.

Female C57BL/6-*Foxp3*^{DTR} mice were thymectomized (nTreg Depleted) or sham thymectomized (control) at three days of age to remove natural T regulatory cells and then administered *H. hepaticus* (Hh) or brucella broth (BB) at 3-4 weeks of age. Ninety days later the mice were necropsied and ceca collected. (A) Cecal inflammation was evaluated histologically; each point represents an individual mouse's lesion score. No significant differences were found between the four groups. (B) Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters.

Figure 4-3. mRNA expression of IL-10 is reduced following Foxp3⁺ cell population depletion in *H. hepaticus* inoculated female mice.

Female C57BL/6-*Foxp3*^{DTR} mice were administered diphtheria toxin (Treg depleted) or PBS (Control) at 3-4 weeks of age to remove natural T regulatory cells and then administered *H. hepaticus* (Hh) or brucella broth (BB) at 2 days following the last DT treatment. Four days following inoculation, mice were necropsied and ceca collected. Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters.

Figure 4-4. Depletion of Foxp3⁺ cells in female mice does not result in *H. hepaticus*-induced disease development.

Female C57BL/6-*Foxp3*^{DTR} mice were administered diphtheria toxin (T reg depleted) to remove Foxp3⁺ cells (natural and inducible T regulatory cells) or PBS (control) at 3-4 weeks of age. Two days following DT treatments, they were administered *H. hepaticus* (Hh) or brucella broth (BB). Ninety days post-inoculation, the mice were necropsied and ceca collected. (A) Cecal inflammation was evaluated histologically; each point represents an individual mouse's lesion score. (B) Real-time PCR was utilized to evaluate the mRNA expression of cytokines. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized to evaluate all data; p values ≤ 0.05 are regarded as significant and indicated by either horizontal capped bars (A) or lowercase letters (B).

Figure 4-5. Depletion of natural T regulatory cells in male mice results in decreased IFN- γ production in *H. hepaticus* inoculated mice.

Male C57BL/6-*Foxp3*^{DTR} mice were thymectomized (nTreg Depleted) or sham thymectomized (Control) at three days of age to remove natural T regulatory cells and then administered *H. hepaticus* (Hh) or brucella broth (BB) at 3-4 weeks of age. Four days later the mice were necropsied and ceca collected. Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters.

Figure 4-6. Depletion of natural T regulatory cells in male mice does not result in disease development following *H. hepaticus* inoculation.

Female C57BL/6-*Foxp3*^{DTR} mice were thymectomized (nTreg Depleted) or sham thymectomized (control) at three days of age to remove natural T regulatory cells and then administered *H. hepaticus* (Hh) or brucella broth (BB) at 3-4 weeks of age. Ninety days later the mice were necropsied and ceca collected. (A) Cecal inflammation was evaluated histologically. Each point represents an individual mouse's lesion score and is presented as mean $\pm$ S.D. (B) Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized to evaluate all data; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters. .

Figure 4-7. IL-10 mRNA expression levels are reduced in *H. hepaticus* inoculated male mice depleted of Foxp3^{DTR} cell populations.

Male C57BL/6-*Foxp3*^{DTR} mice were administered diphtheria toxin (Treg depleted) or PBS (control) or at 3-4 weeks of age to remove natural T regulatory cells and then administered *H. hepaticus* (Hh) or brucella broth (BB) at 2 days following the last DT treatment. Four days following inoculation, mice were necropsied and ceca collected. Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters.

Figure 4-8. Depletion of Foxp3⁺ cells in male mice does not result in disease development.

Male C57BL/6-*Foxp3*^{DTR} mice were either administered PBS (Control) or diphtheria toxin (Treg Depleted) to remove Foxp3⁺ cells (natural and inducible T regulatory cells) at 3-4 weeks of age. Two days following DT treatments, they were administered *H. hepaticus* (Hh) or brucella broth (BB). Ninety days post- inoculation, the mice were necropsied and ceca collected. (A) Cecal inflammation was evaluated histologically and no significant differences were found. Each point represents an individual mouse's lesion score; data presented as mean $\pm$ S.D. (B) Real-time PCR was utilized to evaluate the mRNA expression of cytokines. All cytokine expression normalized to the housekeeping gene HPRT and presented as mean $\pm$ S.D. Two-way ANOVA with Student-Newman-Keuls post-hoc tests were utilized; p values $\leq$ 0.05 are regarded as significant and indicated by lowercase letters. .

Table 4-1. Real-time PCR primers and annealing temperatures.

	Forward	Reverse	Anneal Temp (°C)
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA	60
IFN-γ	GCCATCAGCAACAACATAAGC	TGGGACAATCTCTTCCCCAC	58
IL-10	CGGGAAGACAATAACTG	CATTTCGATAAGGCTTGG	58
IL-12/23 p40	ACTCACATCTGCTGCTCCAC	GGGAACTGCTACTGCTCTTGA	60
IL-17a	CAGAAGGCCCTCAGACTACC	CTTTCCTCCGCATTGACAC	56.6
IL-17f	ACTTGCCATTCTGAGGGAGG	TGATGCAGCCTGAGTGTCTG	57.1

Chapter 5 - The Forgotten Projects

Part I: Exploratory Research in TNBS-induced colitis with emphasis on the respective influences of gonadal sex hormones and previous infection with *Helicobacter hepaticus*.

Part II: Preliminary investigations of the immune response to *Trichuris muris* in the young A/J mouse and the helminth's ability to reverse *H. hepaticus* induced inflammation.

Part III: Alternative explorations in the role of T regulatory cells in *Helicobacter hepaticus* induced inflammation.

Exploratory Research in TNBS-induced colitis with emphasis on the respective influences of gonadal sex hormones and previous infection with Helicobacter hepaticus.

Introduction and Research Aims

The documented influences of gonadal sex hormones in IBD have been extensively discussed in Chapters 1 and 2. In short, females develop more autoimmune diseases than their male counterparts⁴⁷⁻⁵⁰ and the severity of disease in female IBD patients is higher than that seen in male IBD patients³⁴. Additionally, a slight female sex bias has been reported for CD³³. The work presented here utilizes another IBD model in which TNBS (2,4,6-trinitrobenzene sulfonic acid) is administered intra-rectally and induces a colonic inflammation that histologically resembles CD⁴¹⁸.

When TNBS is administered in a solution of 50% ethanol into the lumen of the colon, it causes haptenization of colonic proteins, which are viewed as antigenic by the immune system. A resulting inflammatory response develops within two weeks and is typically accompanied by diarrhea, rectal prolapse, abnormal hunched posture, anorexia and a scruffy hair coat. Additionally, animals typically lose significant amounts of weight which correlate with mortality.⁴¹⁸ Numerous studies utilizing TNBS have identified the importance of IL-12 and IFN- γ signaling in the development of this IBD model^{27,419-421}. Furthermore, it has been repeatedly demonstrated that oral feeding of haptenized proteins negates the effects of rectally administered TNBS^{278,422-424}.

The research presented here addresses two independent research aims. In the first, the ability of gonadal sex hormones to modulate the development of TNBS-induced colitis was evaluated. Female mice were ovariectomized, implanted with either an estrogen or placebo pellet and then administered either a high or low dose of TNBS. Male mice were orchiectomized, implanted with either a testosterone or placebo pellet and administered the high dose of TNBS. Following TNBS administration, mice were weighed daily as a secondary means of evaluating disease development and then necropsied one week post-administration. Colons were collected and evaluated for the development of disease. The second experiment evaluated the effects of prior infection with *H. hepaticus* on the development of TNBS-induced colitis. Ninety days following inoculation with *H. hepaticus* mice were administered TNBS and evaluated for the development of both colitis and typhlitis. Control mice received only *H. hepaticus* or only TNBS, along with the appropriate vehicle controls.

Results and Discussion

TNBS-induced colitis in the female A/J mouse is not modulated by estrogen signaling.

5-6 week old A/JCr mice were ovariectomized and, during the surgery, a pellet containing either 17 β estradiol or placebo was placed subcutaneously (n=10/treatment group). Mice were administered 0.5 mg TNBS seven days post-operatively and weighed daily for one week as a secondary indicator of disease.

At this point, mice were necropsied and the colon evaluated for histologic disease. The scoring system utilized to evaluate colitis can be found in Figure 5-1A; images depicting the range of disease severity can be found in Figure 5-1B . As depicted in Figure 5-1C (top) there were no statistical differences in the lesion scores between the two treatments.

To determine if the effects of estrogen signaling on TNBS-induced colitis were lost by the low concentration of TNBS used in the initial experiment (0.5 mg), the experiment was repeated with a higher dose of TNBS (2.0 mg). 5-6 week old A/JCr mice were ovariectomized and, during the surgery, a pellet containing either 17β estradiol or placebo placed subcutaneously (n=10/treatment group). Mice were administered 2.0 mg TNBS 7 days post-operatively and weighed daily for one week as a secondary indicator of disease. At this point, mice were necropsied and the colon evaluated for histologic disease. Even with the higher dose of TNBS, there were no statistical differences between the test treatments (Figure 5-1C, middle).

As stated, the mice from both experiments were weighed daily and the percent change in weight was calculated for each mouse. For both experiments, no significant differences in weight gain/loss were noted between the placebo and estrogen treated groups (Figure 5-2A & B).

Despite estrogen's proven immune-modulatory effects in prior experiments involving a T_H1 mediated mouse model²⁵², it does not appear to modulate TNBS-induced colitis in the A/J mouse. However these results must be interpreted cautiously as the validity of this disease model in the A/J mouse has not been evaluated previously. There is an identified strain susceptibility to TNBS induced-colitis and it was predicted that A/J mice would have a similar disease presentation as the closely related Balb/c mouse strain which is susceptible to disease induction⁴¹⁸. However, the overall disease severity seen in the female A/J mice treated with TNBS was lower than expected and did not significantly differ between the two TNBS doses administered, suggesting that the strain is not an ideal model for evaluation of estrogen signaling in TNBS-induced colitis.

TNBS-induced colitis in the male A/J mouse is not modulated by testosterone signaling.

Unlike other mouse models, many studies involving TNBS utilize male mice^{278,425-427} and the *Current Protocols in Immunology* actually recommends the use of male SJL/J mice⁴¹⁸. As testosterone has been shown to be a natural immunosuppressive agent⁴²⁸, we sought to determine if testosterone modulates the severity of TNBS-induced colitis. To accomplish this, twenty 3-4 week old male A/JCr mice were orchietomized. During surgery, a pellet containing either 5 α -dihydrotestosterone or a placebo was placed subcutaneously (n=10/treatment group). Mice were administered 2.0 mg TNBS seven days post-operatively and

weighed daily for one week as a secondary indicator of disease. At this point, mice were necropsied and the colon evaluated for histologic disease. There were no statistical differences between the severity of disease seen in the two treatment groups (Figure 5-1C, bottom). As with the female mice, no significant differences in weight gain/loss were noted (Figure 5-2C).

***H. hepaticus* colonization does not alter TNBS-induced colitis**

The IBD model used most frequently by our laboratory utilizes *H. hepaticus* to cause a T_H1 type inflammatory response in the cecum ninety days post-inoculation. Experiments involving flora and TNBS-induced colitis have demonstrated that the oral tolerance achievable in this model is limited to one's own flora⁴²⁹. Furthermore studies have shown probiotics to be protective against TNBS-induced colitis, suggesting a role for the microbiota in disease susceptibility⁴³⁰. To evaluate the affect *H. hepaticus* has upon the development of TNBS colitis in the A/J mouse, 3-4 week females were inoculated with either brucella broth or *H. hepaticus*. Three months post-inoculation, eight mice inoculated with *H. hepaticus* and seven mice inoculated with brucella broth were administered TNBS intracolonicallly. As a control, eight additional mice previously inoculated with *H. hepaticus* were administered a solution of 50% ethanol intracolonicallly as a vehicle control for the TNBS treatment. This resulted in three experimental groups: sham inoculated, TNBS treated (n=7); *H. hepaticus* inoculated, sham treated (n=8); and *H. hepaticus* inoculated, TNBS treated (n=8). These groups will be referred to as: TNBS, *H. hepaticus* and *H.*

hepaticus + TNBS in all further discussions, respectively. Mice were weighed daily for one week and then necropsied. The colon and the cecum were evaluated separately for disease severity.

The cecal lesion scores of both the *H. hepaticus* + TNBS mice and the *H. hepaticus* mice were significantly elevated when compared to the TNBS treated mice. The colon lesion scores of the TNBS mice and the *H. hepaticus* + TNBS mice were significantly elevated when compared to the *H. hepaticus* mice. (Figure 5-3A). The lack of colonic lesions in the *H. hepaticus* mice and cecal lesions in the TNBS mice suggest there is not an anatomical overlay of disease. This was further supported by the lack of differences in either cecal or colonic disease severity between the *H. hepaticus* + TNBS mice and either the *H. hepaticus* or TNBS treated mice. Although both diseases produce a T_H1 mediated inflammatory response characterized by the production of IL-12/23 p40 and IFN- γ , neither appeared to be exacerbated by the addition of the other. The lack of interaction may be simply due to the lack of anatomical similarity, or may be due to the non-responsiveness of the A/J mouse strain in general to TNBS induced colitis.

As mentioned, daily weights were taken to monitor a known secondary sign of TNBS-induced colitis⁴¹⁸. No significant differences in weight change were found between the groups (Figure 5-3B)

Conclusions

Despite the known genetic similarity of the A/J and BALB/c mouse strains, the lack of severe disease development suggests that the A/J mouse strain is not ideal for this IBD model. Although our experimental results suggest that neither GSH or a prior infection with *H. hepaticus* is capable of modulating TNBS-induced colitis, these results must be interpreted with caution as they may simply be a result of limited response to disease induction and the effects of either treatment may be lost due to the limited severity of disease presentation.

Materials & Methods

Mice

The University of Missouri's Animal Care and Use Committee (ACUC) approved all experiments involving the use of animals. All experiments were planned and performed in accordance with the Guide for the Care and Use of Laboratory Animals. The A/J mice were obtained from Frederick National Laboratory for Cancer Research were used in experiments as described.

Ovariectomy

Mice were anesthetized with isoflurane, placed in ventral recumbency, clipped dorsally, and aseptically scrubbed. A single longitudinal skin incision was made on the dorsal midline. The ovaries were visualized, and following incision of the muscle wall, were removed. Next, a continuous-release hormone pellet (described below) was inserted rostrally into the subcutaneous space between the scapulae through the initial incision and the incision closed with sterile skin staples. Mice were given an intraperitoneal injection of buprenorphine for analgesia and recovered on a heating pad. Seven to ten days post-operatively, the skin staples were removed. At the time of necropsy, mice with detectable ovarian remnants were removed from the study.

Orchiectomy

Male mice were anesthetized with isoflurane and placed in ventral recumbence. Scrotal hair was removed with clippers and the scrotum prepped with alternating betadine and alcohol surgical scrubs. The scrotum was incised with a scalpel and the testicle exteriorized and removed via gentle dissection from the testicular fat pad. To prevent herniation of abdominal contents, the testicular fat pad was left intact and the scrotum sealed with tissue adhesive. For placement of the continuous-release pellet, the dorsal thorax was clipped, and aseptically scrubbed. A single longitudinal skin incision was made on the dorsal midline and the pellet placed subcutaneously. Following pellet placement the incision was closed sterile skin staples. Mice were given buprenorphine IP for analgesia and recovered on a heating pad. Seven to ten days post-operatively, the skin staples were removed.

Compounds

Female animals were administered a 90-day continuous-release hormone pellet containing either 1.5 mg 17β -estradiol (E2) or 1.5 mg placebo (Innovative Research of America, Sarasota, FL Catalog # NE-121 and NC-111, respectively). Male animals were administered a 21-day continuous-release hormone pellet containing either 10 mg 5α -dihydrotestosterone or 12.5 mg testosterone placebo (Innovative Research of America, Sarasota, FL Catalog #A-161 and NC-111, respectively). At the time of necropsy, the presence of a pellet was confirmed and mice without a visible, intact pellet were removed from the study.

Induction of TNBS ((2,4,6-trinitrobenzene sulfonic acid)

Mice were lightly anesthetized with isoflurane. TNBS (Sigma Chemical, St. Louis, MO), diluted in 50% ethanol was slowly administered into the lumen of the colon through the tip of a 3.5 french catheter inserted approximately 4 cm proximal to the anus. Control mice received a solution of 50% ethanol. For either TNBS dose, 0.5 or 2 mg, the total injection volume was 100µl. Following TNBS administration, mice were kept in a vertical position for 30 seconds and then returned to their cages. Animals were weighed daily and euthanized seven days post-treatment. All mice were weighed daily and monitored for the development of colitis (diarrhea, rectal prolapse). Any mouse which lost >20% starting body weight or developed clinical signs was removed from the study and euthanized.

Helicobacter hepaticus

The culture protocol for the MU-94 *Helicobacter hepaticus* strain has been previously described^{25,252}. At 3-4 weeks of age, mice were gastrically gavaged with 0.5 mL of culture broth. The estimated concentration of *H. hepaticus* is approximately 5×10^8 bacteria/mL. A previously described PCR assay was used to confirm colonization with *H. hepaticus* at necropsy²⁴⁷.

Necropsy

CO₂ inhalation was utilized to achieve euthanasia. For all experiments, the cecum was removed and sectioned longitudinally into two equal parts. One half was rinsed with sterile PBS and flash frozen in liquid nitrogen (these cecal sections were not utilized further). The other half was fixed in 10% formalin and embedded in paraffin for histologic evaluation. For those experiments involving *H. hepaticus*, cecal contents were collected to confirm *H. hepaticus* colonization. The colon was also removed, flushed with PBS and sectioned into three portions identified as: distal, middle and proximal. These sections were then sectioned longitudinally - one half of each colon section was flash frozen in liquid nitrogen (these sections were not utilized further). The other halves were fixed in 10% formalin and embedded in paraffin for histologic evaluation.

Histological Evaluation

Cecal histologic sections were stained with hematoxylin and eosin (H&E) stain and scored for inflammatory lesions using a previously described protocol^{246,252,253}. In short, all cecal sections were scored in a blinded fashion and assigned a category score for each of the following: inflammation intensity (0 = none, 1 = mild, 2 = moderate, 3 = severe); longitudinal extent of inflammation (0 = none, 1 = 1-2 small foci, 2 = patchy, 3 = diffuse); vertical extent of inflammation (0 = none, 1 = basal mucosal inflammation, 2 = full thickness mucosal inflammation, 3 = transmural inflammation); and hyperplasia (0 = none, 1 = focal, 2 = patchy hyperplasia, 3 = diffuse hyperplasia). The lesions scores depicted in figures are the sum of the four category scores; any lesions score greater than 2

were decreased by 2 to minimize inflation of scores by inflammation alone, which is described by three of the four categories evaluated.

The three colonic histologic sections collected were stained with H&E stain and individually scored for inflammation utilizing the rubric provided in figure 5-2A which was adapted from the *Current Protocols in Immunology*⁴¹⁸. Each section (distal, middle and proximal) was scored individually on a scale of 0-4 (please see figure for corresponding pathology descriptions). The scores from all three sections were then summed for an overall colonic lesion score (minimum = 0, maximum = 12).

Statistical Analysis

Student's t-tests and one-way ANOVAs with a Student-Newman-Keuls post-hoc analysis were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). p values ≤ 0.05 were regarded as significant. All graphs and dot plots were generated using either Microsoft Office Professional Plus 2010 Excel software (Microsoft Corporation, Redmond, WA) or GraphPad Prism 5.0 software (GraphPad Prism Software Inc., La Jolla, CA).

Figure 5-1. GSH signaling in the A/J mouse does not affect the development of TNBS-induced colitis.

(A) Rubric used to score colon sections. (B) Histologic representations of disease severity. Corresponding lesion score indicated on individual images. (C) Mice were ovariectomized and administered either an estrogen or placebo pellet. All mice were administered TNBS at either a low (0.5 mg) or high (2.0 mg) dose. Seven days later the mice were necropsied and their colon's accessed for disease development. Dot plots represent individual mice's lesion scores of mice who received the low TNBS (top) and high TNBS (bottom) dose. (D) Male mice were orchietomized and administered either a testosterone or placebo pellet. All mice were administered a 2.0 mg dose of TNBS. Seven days later the mice were necropsied and their colon's accessed for disease development. Lesion scores were compared with a t-test, no significant differences were found. All data shown analyzed with a t-test; $p \leq 0.05$ considered significant. No significant differences were found for any of the comparisons made.

A

Colonic lesion scoring scale	
0	No evidence of inflammation.
1	Low level of leukocyte infiltration with infiltration seen in <10% HPF. No structural changes.
2	Moderate leukocyte infiltration with infiltration seen in 10% to 25% HPF, crypt elongation, bowel wall thickening that does not extend beyond the mucosal layer, and no ulcerations.
3	High level of leukocyte infiltration seen in 25% to 50% HPF, crypt elongation, infiltration beyond the mucosal layer, thickening of the bowel wall and superficial ulcerations.
4	Marked degree of transmural leukocyte infiltration seen in >50% HPF, elongated and distorted crypts, bowel-wall thickening, and extensive ulcerations.

B

C

D

Figure 5-2. GSH do not affect TNBS-associated weight changes in the A/J mouse.

(A) Mice were ovariectomized and administered either an estrogen or placebo pellet. All mice were administered TNBS at either a low (0.5 mg) or high (2.0 mg) dose and weighed daily for seven days. Average percentage of weight change was compared for both treatment groups. (B) Male mice were orchietomized and administered either a testosterone or placebo pellet. All mice were administered a 2.0 mg dose of TNBS and then weighed daily for seven days. Average percentage of weight change was compared for both treatment groups. All data shown analyzed with a t-test; $p \leq 0.05$ considered significant. Data plotted as mean percentage $\pm$ SD. No significant differences were found for any of the comparisons made.

Figure 5-3. The development of typhlitis and colitis in mice previously inoculated with *H. hepaticus* and then administered TNBS.

Ninety days following inoculation with *H. hepaticus* mice were administered TNBS (n=8) and evaluated for the development of both typhlitis (A) and colitis (B). Control mice received only *H. hepaticus* (n = 8) or only TNBS (n=7), with the appropriate vehicle controls. Dot plots indicate the lesion scores of individual mice, error bars indicate mean $\pm$ SD. (C) Average percent weight loss of each treatment group from the day of TNBS treatment; presented as the mean percentage $\pm$ SD. All data shown analyzed with a one-way ANOVAs with a Student-Newman-Keuls post-hoc analysis; $p \leq 0.05$ considered significant.

Preliminary investigations of the immune response to Trichuris muris in the young A/J mouse and the helminth's ability to reverse H. hepaticus induced inflammation.

Introduction and Research Aims

Helminths are being considered as a therapeutic treatment option for IBD patients for several reasons. First, the immune responses generated towards helminths for the purpose of worm clearance are typically T_H2 in nature^{198,346-351}. As T_H1/T_H17 immune responses are typically seen in CD patients^{14,113,114}, it has been speculated that the T_H2 response induced by helminth exposure could counter balance an ongoing T_H1 response and ultimately decrease inflammation. Second, regions of the world with an increased incidence of helminth infestations have a relatively low incidence of IBD diagnosis^{37,100}. In mouse models of IBD, pre-exposure to helminths prior to the administration of a trigger capable of causing IBD-like inflammation such as TNBS^{317,318}, DNBS^{319,320}, DSS^{321,322}, and adoptive cell transfers³²³⁻³²⁵ has been shown to be protective. Whether pre-exposure to helminths during childhood development is crucial, is yet to be determined. Furthermore, there has been published evidence supporting the ability of helminths to reverse inflammation seen in mouse models of IBD^{277,336,337} and improve clinical signs in human IBD patients^{227,228}. Overall, these data suggest that helminths are capable of modulating future and ongoing immune responses. We sought to determine if this was the case in the *Helicobacter hepaticus* model of IBD.

Previous studies have demonstrated that when A/J mice are administered *H. hepaticus* there is an immediate response to the bacterial addition to the microbiota by four days post-inoculation characterized by elevations in mRNA gene expression of several cytokines such as IL-12/23 p40, IFN- γ and IL-10²⁵. At ninety days post inoculation the elevations in gene expression persist, with the addition of histologic disease development which is localized to the cecum and characterized by lymphocytic infiltration of the lamina propria^{25,252-254}. *T. muris* is a whipworm of mice and was chosen for evaluation as a potential therapeutic in this model of IBD for two reasons. First, it is related to the helminth, *Trichuris suis*, used in human therapeutic trials^{227,228} and second, because disease development in our *H. hepaticus* model occurs in the cecum, where these worms predominantly reside³¹⁵.

The projects described here provide a closer examination of the effects of exposure to *T. muris* during and after exposure to *H. hepaticus*, known to incite mucosal inflammation. First, the A/J mouse's resistance to *T. muris* colonization was re-examined. A previous experiment (Figure 3-1) had demonstrated that 5 month old A/J mice are capable of expelling *T. muris*. To confirm this is also true in a young mouse, the susceptibility of 4 week old A/J mice to *T. muris* colonization was investigated. Next, a series of weekly necropsies were used to identify changes in cytokine expression following *T. muris* exposure in a young mouse. During the time course study, the presence of detectable *T. muris* was also documented. Following these studies, the effects of *T. muris* exposure at

the time of *H. hepaticus* inoculation were examined at both four and ninety days post-inoculation. Finally, the ability of *T. muris* to reverse ongoing disease induced by *H. hepaticus* was evaluated by administering the helminth to *H. hepaticus* infected mice. Changes in histologic disease and mRNA gene expression were then analyzed at both 3 and 5.5 weeks post-helminth treatment. The findings in these later experiments, lead to the projects presented in Chapter 3.

Results and Discussion

Young A/J mice are capable of *T. muris* expulsion.

To investigate the young A/J mouse's susceptibility to *T. muris*, four week old female mice were inoculated with *T. muris* ova. Four mice were then necropsied at either twelve or twenty-eight days post-inoculation. At the time of necropsy mice were examined for either the presence larvae (12 day necropsy time point) or adult worms (28 day necropsy time point). As shown in Figure 5-4, twelve days post-inoculation one-hundred percent (n=4) of the mice had detectable live larvae present in their cecal digests. In contrast, by twenty-eight day post-inoculation none of the mice had adult *T. muris* within their ceca. These findings are consistent with those previously described in Chapter 3, when the susceptibility in a mature 5 month old A/J mouse was examined, and further support the findings that A/J mice are resistant to *T. muris* colonization.

Characterizing the immune response to *T. muris* in the young A/J mouse

A/J mice were inoculated with *T. muris* ova or PBS (vehicle control) at 14 days of age. Eight mice (4 male and 4 female) from each inoculation group were necropsied at weeks 1, 2, 3, 4, 5 and 6 post-inoculation. The week 14 necropsy time point consisted of 5 male and 3 female PBS inoculated mice and 7 male and 3 female *T. muris* inoculated mice. At necropsy the cecum was collected for cytokine mRNA analysis and examined for the presence of live *T. muris*. Mice included in the week 1-3 necropsies were examined for larvae, while the mice included in the week 4-6 and 14 necropsies were examined for adult worms.

As shown in Figure 5-5 all mice examined at weeks 1 and 2 were found to have live *T. muris* larvae. At week 3, only three of the eight mice examined (62.5%) were positive for the helminth, suggesting the immune response required to clear the worms had begun by this time. One mouse was found to have a worm burden of one adult worm at the week six time point. Although this mouse did have an adult worm, it must be noted that the dose of *T. muris* ova given to the mice was sufficient to yield over 100 worms in a susceptible mouse strain. After examining the data it was concluded that this experiment's results concurred with the previously presented data and ultimately suggest that the A/J mouse is resistant to colonization by the helminth *T. muris*.

Analysis of mRNA expression was completed to further evaluate the immune response to *T. muris* in the young A/J mouse; all comparisons described

refer to mRNA expression levels in *T. muris* inoculated mice compared to the control PBS inoculated mice. As in the older mice (Figure 3-2), IL-12/23 p40 was elevated only at the week 2 necropsy time point. The expression of IFN- γ was elevated between weeks 1-4; this is one week longer than the IFN- γ expression pattern seen in the older A/J mice. Interestingly, the expression pattern of IL-10 was dissimilar to that seen in the older mice in which a brief elevation was seen during weeks 1 and 2; in the young mice significant elevations were seen during weeks 3 and 5. The cytokines associated with helminth clearance, IL-4 and IL-13, were initially elevated at week 2 in the young and old mice, however the elevation in expression in young mice was shorter than that seen in older mice. IL-4 was elevated during weeks 2 and 3, while IL-13 was elevated during weeks 2-4 and 6. IL-5 expression was identical in the young and old mice – elevations were seen only at weeks 2 and 3. Like IL-10, the elevations in IL-6 differed in the younger mice and occurred during weeks 2, 3 and 6.

In general, the cytokine expression seen in young A/J mice mirrored that documented in the mature A/J mice, with a few exceptions. The discrepancies in IL-10 could be due to the development of inducible T regulatory cells towards the developing microbiota in the young mice. It is plausible that the immediate IL-10 response seen in the older mice (Figure 3-2) was generated by a population of established T regulatory cells; while the delayed IL-10 response could be due to the ongoing development of this cell subset in the younger mice. This speculation is supported by the known involvement of T regulatory cells in

helminth clearance^{349,361} as well as the known induction of T regulatory cells in the periphery to endogenous microbiota^{84,117,145,431}. Overall, the general pattern of cytokine elevation is comparable between the two age groups evaluated in this experiment and the one described in Chapter 3.

The immediate effects of co-exposure to *T. muris* and *H. hepaticus*

To evaluate if the immediate immune response to *H. hepaticus* is altered by concurrent exposure to *T. muris* ova, 3-4 week old female A/J mice were inoculated with both agents (n=8). Four days later, the mice were sacrificed and cecal mRNA gene expression of IFN- γ , IL-4, IL-5, IL-6, IL10, IL-12/23 p40 and IL-13 was evaluated. As a control, age matched A/J females were inoculated with *H. hepaticus* and PBS, the vehicle control for the *T. muris* ova inoculum (n=8).

When gene expression in *H. hepaticus* inoculated mice treated with *T. muris* ova were compared to PBS treated mice, no significant differences were found (Figure 5-6). These data suggest that the initial cytokine response to *H. hepaticus*, typically defined by increased expression of IL-12/23 p40 and IFN- γ is not affected by the concurrent immune response to *T. muris*. As we had previously demonstrated that exposure to *T. muris* does not elicit a change in either of these cytokines, this was not unexpected. Furthermore, the previous experiment demonstrated elevations in the T_H2 cytokines IL-4, IL-5, IL-6 and IL-13 gene expression two week post-inoculation, which is well beyond the time point evaluated here (Figure 5-5). Although it is reasonable to conclude that the

immediate response to *H. hepaticus* is not altered by the concurrent exposure to *T. muris*, the long term effects must be evaluated.

The long term effects of co-exposure to *T. muris* and *H. hepaticus*

Next we sought to evaluate if the immune response and subsequent disease development triggered by *H. hepaticus* is altered by a concurrent initial exposure to *T. muris* ova. To accomplish this, groups of 3-4 week old female A/J mice were inoculated with *H. hepaticus* and either *T. muris* ova or PBS (n=8/group). Ninety days later, the mice were sacrificed and cecal mRNA gene expression of IFN- γ , IL-4, IL-5, IL-6, IL10, IL-12/23 p40 and IL-13 was evaluated. To document if changes in *H. hepaticus* induced inflammation occurred following exposure to *T. muris* ova cecal, histology was evaluated.

When the severity of *H. hepaticus*-induced disease was compared between mice treated with *T. muris* ova and PBS, no significant changes were found. However, it must be noted that the disease severity seen in the *H. hepaticus*, PBS treated mice are lower than historically observed in this model; typically inoculation with *H. hepaticus* yields average lesion scores between 5-7. The most logical explanation for the dissimilarity between the current and past data, is the *H. hepaticus* culture used to inoculate the mice. Although the mice were confirmed to be positive for *H. hepaticus* with PCR at the time of necropsy, the initial bacterial culture used for inoculation could have potentially been at a lower concentration or viability than normally used. Unfortunately, the data must

be considered inconclusive to avoid missing changes potentially masked by a lack of disease severity.

When gene expression of cytokines was compared between the two treatment groups, significant elevations in both IL-4 and IL-12/23 p40 were noted in the *T. muris* ova treated mice. Both of these findings were unexpected. While the previous experiment clearly showed IL-4 to be initially elevated in response to *T. muris* ova exposure, the increase had subsided within four weeks post-inoculation. Considering the findings presented in Chapter 3 which demonstrate that *H. hepaticus* is capable of delaying *T. muris* exposure, it is reasonable that the persistence of IL-4 gene expression could simply be attributable to the delayed clearance of *T. muris*. There were, however, no adult worms noted at necropsy or detected histologically. The elevations in IL-12/23 p40 cannot, however, be explained by persistence of the helminth as none of the previous experiments demonstrated an IL-12/23 p40 response to *T. muris*. Furthermore, elevations in IL-12/23 p40 in this model are typically associated with inflammation; as previously discussed the lesion scores in these mice were lower than typically seen in *H. hepaticus* infected A/J mice. It is possible that the discrepancies noted here are simply due to an in-effective *H. hepaticus* culture and, as previously stated, the data must be considered inconclusive.

The effects of *T. muris* treatment on *H. hepaticus* infected mice

Overall, the investigation into the effects of prior and concurrent helminth infections and inflammatory triggers is merely academic as helminth therapies for IBD patients would only be administered following the onset of inflammation, making pre-exposure to helminths an impossibility. Here it was done to document the immune response and susceptibility to *T. muris* in the A/J mouse, which had yet to be done in the literature. Therefore, we next sought to instead focus on the therapeutic potential of *T. muris* to reverse *H. hepaticus* induced inflammation. To accomplish this all mice in this study were inoculated with *H. hepaticus* at 3-4 weeks of age. Ninety days post inoculation the same mice were treated with either *T. muris* ova or PBS; animals treated with PBS serve as the control animals for all experiments described here. Equal groups of mice from the two treatment groups were necropsied at either 3 or 5.5 weeks following treatment.

The severity of disease documented at both necropsy time points was more consistent with the disease severity seen in previous experiments involving experimentally inoculated *H. hepaticus* A/J mice. A significant elevation in lesion scores were detected in *T. muris* ova treated mice when compared to PBS control treated mice at both time points (Figure 5-8A). This data supports the findings in Chapter 3 which suggest *T. muris* ova therapeutics exacerbate ongoing microbial driven disease, rather than alleviating inflammation. Also

consistent with the previously reported findings is the observation of live *T. muris* adults during the 5.5 week necropsy time point. The data from *H. hepaticus* negative mice inoculated with *T. muris* presented in Figures 3-1 and 5-3 demonstrate that both weanling and adult A/J mice are fairly capable of clearing *T. muris* infestations by 28 days (4 weeks). The data from the current experiments show 60% of the mice treated with *T. muris* had live adult worms present at the 5.5 week time point. Furthermore, when the rate of histologically detectable *T. muris* was scrutinized, over 50% of the *T. muris* treated mice had detectable helminths during both time points evaluated. Together, with the data presented in Figure 3-5, these data suggest that the presence of *H. hepaticus* delays clearance of *T. muris*.

The mRNA gene expression of IFN- γ , IL-4, IL-5, IL-6, IL-10, IL-12/23 p40 and IL-13 was compared between both treatment groups for each necropsy time point. Comparisons in expression levels were not made between necropsy time points. Significant elevations in IFN- γ , IL-4, IL-5, IL-6 and IL-13 were noted in the *T. muris* ova treated mice at both time points when compared to PBS treated controls. An elevation in *T. muris* treated mice was also noted for IL-10, but only during the week 3 time point. The elevations seen in Th2 cytokines (IL-4, IL-5 and IL-13) are likely due to the persistence of *T. muris* discussed previously. The literature has extensively documented the immune response in mice towards helminths^{346,348,350,432} and towards *T. muris* in particular^{316,344,351,355,356,358,359,433,434}. Considering these reports and our findings reported in Figure 5-5, it is not

surprising that these cytokines were elevated at the 3 week time point, however it was unexpected that their elevation persisted through the 5-5 week necropsy time point. The extended period of expression is most likely attributable to the persistence of *T. muris* in these *H. hepaticus* infected mice. Once again, the data suggests that persistent *T. muris* colonization has detectable effects on cytokine mRNA expression.

Conclusions

These data provide further support for the findings presented in Chapter 3 which suggest that the microbiota can alter the immune response to helminths. Inoculation with *H. hepaticus* has been demonstrated to delay the expulsion of the helminth *T. muris*. Furthermore neither co-exposure to *T. muris* at the time of *H. hepaticus* inoculation or treatment with *T. muris* following the onset of inflammation improved disease severity. In fact, the *H. hepaticus* induced disease was initially exacerbated following helminth treatment. As stated in Chapter 3, these findings highlight the importance of the microbiota in the immune response and suggest that helminth therapies may not be appropriate for IBD patients with specific microbiota profiles.

Materials & Methods

Mice

The University of Missouri's Animal Care and Use Committee (ACUC) approved all experiments involving the use of animals. All experiments were planned and performed in accordance with the Guide for the Care and Use of Laboratory Animals. The A/J mice were obtained from Frederick National Laboratory for Cancer Research and either used to create breeding pairs or for experiments. The pups resulting from the created breeding pairs were used in experiments as described.

Trichuris muris ova

Dr. Joseph Urban of the United States Department of Agriculture (Beltsville, MD) kindly provided the *Trichuris muris* ova inoculum used in all the experiments. The volume of inoculum was calculated to result in a heavy worm burden (approximately 100 adult worms) in a susceptible mouse strain. Gastric gavage was used to inoculate mice with the appropriate volume (between 0.07 – 0.15 ml, dependent upon batch) of inoculum.

Helicobacter hepaticus

The culture protocol for the MU-94 *Helicobacter hepaticus* strain has been previously described^{25,252}. At 3-4 weeks of age, mice were gastrically gavaged with 0.5 mL of culture broth. The estimated concentration of *H. hepaticus* is

approximately 5×10^8 bacteria/mL. A PCR assay was used to confirm colonization with *H. hepaticus* at necropsy²⁴⁷.

Necropsy

CO₂ inhalation was utilized to achieve euthanasia. The cecum was then removed, cecal contents collected and sectioned longitudinally. The two cecal portions was used for two of the following, dependent upon the experimnt: verification of *T. muris* larvae or adult worms, examination of histologic disease or cytokine mRNA expression analysis.

Verification of *T. muris*

To evaluate for the presence of *T. muris* larvae, warm PBS was used to rinse cecal section of all cecal contents and debris. The sections were then placed in a 50 ml conical tube containing 10 mls of 10 mM EDTA and left in a shaking incubator for two hours at 37° C. Following this incubation tubes were vortexed at high speed for 15 seconds. Next, the digested cecum and all associated fluids poured into a petri dish and examined with an inverted microscope (40x) for live, moving larvae.

To check for adult *T. muris* worms, the cecum was examined at necropsy for adult worms, which can be visualized without the aid of microscopy. To further evaluate the cecum for worms, the cecum was placed in warm PBS and evaluated with a dissecting microscope.

Histologic Evaluation

Cecal portions designated for histologic evaluation were paraffin embedded following fixation with zinc fixative. Sections were stained with Hematoxylin and eosin (H&E) stain and blinded scored for inflammatory lesions using a previously described protocol^{246,252,253}.

Isolation of RNA and Reverse Transcription

Cecal sections were stored at -80° C until processing. To begin RNA isolation, cecal sections were thawed in TRIzol (Invitrogen, Carlsbad, CA) and then homogenized with a TissueLyser (Qiagen, Valencia, CA). RNA isolation was done per the manufacturer's (Invitrogen) instructions. An Invitrogen Super Script II kit was used to reverse-transcribe RNA (5 µg total/ sample) into cDNA (final concentration 20 ng/µL) per the oligo(dT) primer protocol provided.

Real Time PCR

Real time quantitative PCR with Qigen Quanti-tect SYBR Green was done with a Qiagen Rotor-Gene Q. Table 3.1 contains the primers and annealing temperatures utilized. Run conditions for HPRT, IFN-γ, IL-4, IL-10 and IL-12/23 p40 had been previously published^{25,252}.

Statistical Analysis

Student's t-tests and two-way ANOVA with Student-Newman-Keuls post-hoc test were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). Due to the exponential nature of real-time PCR, Log2 transformations were used to correct for failures in normality. p values ≤ 0.05 were regarded as significant. All graphs and dot plots were generated using either SigmaPlot 11.0 or GraphPad Prism 5.0 software (GraphPad Prism Software Inc., La Jolla, CA).

Figure 5-4. The percentage of mice positive for *T. muris* at 12 and 28 days post-inoculation.

4 week old A/J female mice were gastrically gavaged with *T. muris* ova. Mice were sacrificed and examined for the presence of *T. muris* at one of the two time points. Mice necropsied 12 days post inoculated were examined for larvae, while the mice necropsied at day 28 were examined for adult worms.

Figure 5-5. The immune response to *Trichuris muris* in the 14 day old A/J mouse.

A/J mice were gastrically gavaged with either *T. muris* ova or PBS (vehicle control) at 14 days of age. Eight mice from each inoculation group were necropsied each week and their ceca evaluated for mRNA expression and examined for the presence of *T. muris*. (A) Mice sacrificed at the week 1-3 time points were evaluated for the presence of larvae (white striped bars) and mice sacrificed at the week 4-6 time points were evaluated for adult worms (gray striped bars). (B) IFN- γ , IL-4, IL-5, IL-6, IL-10, IL-12/23 p40 and IL-13 expression was significantly elevated in *T. muris* (gray bars) treated mice when compared to PBS control (black bars) treated mice. All data was analyzed with a two-way ANOVA with Student-Newman-Keuls post-hoc test; $p \leq 0.05$ considered significant. Error bars represent $\pm$ S.D.

Figure 5-6. Co-inoculation with *T. muris* does not alter the initial immune response to *H. hepaticus*.

3-4 week old A/J mice were inoculated with *H. hepaticus* and either *T. muris* ova or PBS (control). Mice necropsied four days post-inoculation and the ceca collected for the mRNA gene expression analysis of IFN- γ , IL-4, IL-5, IL-6, IL-10, IL-12/23 p40 and IL-13. No significant differences were found between the *T. muris* ova and PBS inoculated mice. All data was analyzed with a student's t-test; $p \leq 0.05$ considered significant. Error bars represent $\pm$ S.D.

Figure 5-7. Co-inoculation with *T. muris* has minimal effects on the persistent immune response to *H. hepaticus*.

3-4 week old A/J mice were co-inoculated with *H. hepaticus* and either *T. muris* ova or PBS (control). Mice were necropsied ninety days post-inoculation and the ceca collected for histologic disease evaluation and mRNA gene expression analysis of IFN- γ , IL-4, IL-5, IL-6, IL-10, IL-12/23 p40 and IL-13. (A) No significant differences in disease severity were found between the *T. muris* ova (squares) and PBS (circles) inoculated mice. (B) Gene expression of IL-4 and IL-12/23 p40 were significantly elevated in the mice inoculated with *T. muris* ova (Gray bars) compared to those inoculated with PBS (black bars). No significant differences were found for any of the other cytokines evaluated. All data was analyzed with a student's t-test; $p \leq 0.05$ considered significant. Dot plots represent individual mice. Error bars represent $\pm$ S.D.

Figure 5-8. Treatment of *H. hepaticus* induced inflammation with *T. muris* ova exacerbates disease.

3-4 week old A/J mice were inoculated with *H. hepaticus*. Ninety days later the mice were treated with either *T. muris* ova or PBS (control). Mice were necropsied either 3 or 5.5 weeks following treatment and the ceca collected for both histologic disease evaluation and mRNA gene expression. (A) At both time points, significant elevations in disease severity were found in mice treated with *T. muris* (squares) when compared to those treated with PBS (circles). (B) Sixty percent of the mice treated with *T. muris* and necropsied at the 5.5 week time point (gray checker), had visible adult worms in the cecum at necropsy. (C) During histologic evaluation, the percent of mice with histologically detectable *T. muris* was tabulated. (D) The level of gene expression in the ceca of *T. muris* treated mice were compared to PBS treated mice separately for each necropsy time point. Data for each time-point was analyzed with a student's t-test; $p \leq 0.05$ considered significant. Dot plots represents an individual mice. Error bars represent $\pm$ S.D.

Alternative explorations in the role of T regulatory cells in *Helicobacter hepaticus* induced inflammation.

Introduction and Research Aims

The role of T regulatory cells in the regulation of immune responses in general has been extensively discussed in Chapter 4. The novel data presented and discussed there suggests that inducible T regulatory cells in particular are involved in the management of inflammatory responses driven by *H. hepaticus*. The studies presented here explore the role of T regulatory cells defined by the expression of either CD25 or CD45RB in inflammation induced by the bacterium *H. hepaticus*, and should be considered a prequel to those experiments presented in Chapter 4.

As previously discussed, the lack of an extracellular marker that is exclusive to T regulatory cells is one of the greatest challenges of working with this cellular subset. Because of this, data generated from studies using a specific marker to identify T regulatory cells for targeted depletion or adoptive transfer must be carefully considered as other cell types may be unintentionally manipulated and ultimately affect the disease or condition being studied. The studies presented here utilize CD25 and CD45RB as markers for T regulatory cell identification.

CD25 seemed to be a logical marker for T regulatory cells after Sakaguchi and Asano demonstrated the suppressive abilities of CD4⁺CD25⁺ cells^{372,375};

however, subsequent research demonstrated that the expression of CD25 is found on almost all activated T cells, not just T regulatory cells⁴³⁵. Several other extracellular markers were linked to T regulatory cells including: CTLA-4, CD45, CD62L and GITR^{131,143,436,437}; however, it soon became apparent that there is not a unique surface marker available that can be used to distinguish the two subsets of T regulatory cells from one another or from other CD4⁺ T cells. To further complicate the study of T regulatory cells, combinations of these markers are often used to identify T regulatory cells and, because all laboratories do not use the same combinations to isolate and evaluate the suppressive capabilities of T regulatory cells, the literature is often confusing and difficult to interpret.

CD25 was identified as a marker for T regulatory cells shortly after the discovery of a regulatory lymphocyte subset. CD25 is a part of the receptor for IL-2⁴³⁸, which was first identified as a proliferation factor for T lymphocytes⁴³⁹. This marker is convenient for the study of T regulatory cells, because unlike *Foxp3*, it is expressed externally on the cell thus allowing for the live isolation of CD25⁺ cells with flow cytometry or magnetic bead separation. Adoptive transfer experiments have demonstrated that the transfer of CD4⁺CD25⁻ cells to mice which lack an endogenous T cell population results in disease development. Interestingly, the co-transfer of CD4⁺CD25⁺ cells prevents disease development^{155,173,174,178}.

CD45RB is another external marker that has been utilized to differentiate T regulatory cells. In adoptive transfer experiments, it has been shown that cell

populations with low levels of CD45RB expression have the capability to suppress inflammation, while the adoptive transfer of cells with high expression of this marker cause disease development^{116,119,155,173,174,178}. Unfortunately due to the physiologic distinction between high and low expression of CD45RB, cell populations must be isolated using flow cytometry based cell separation.

Several of CD25 and CD45RB based adoptive transfer based experiments have looked at the importance of *H. hepaticus* infection in the development of disease in these models. The results have been contradictory – while some models suggest that the adoptively transferred cells do not need prior exposure to *H. hepaticus*¹¹⁸, others clearly demonstrate the opposite¹¹⁷. Furthermore, several groups have demonstrated that the recipient mouse must be colonized with *H. hepaticus* for the induction of disease following an adoptive transfer⁸⁹. Of note is the unknown *H. hepaticus* status of the mice utilized in many published studies utilizing adoptive transfer.

For the following experiments, adoptive transfer and antibody depletion techniques were utilized to either remove or supplement the T regulatory cell populations in the A/J and C57BL/6 mouse strains which are known to be susceptible and resistant, respectively, to *H. hepaticus* induced inflammation. Additional experiments utilizing flow cytometry allowed for the characterization of T regulatory cell populations in the cecum of these mice.

Results and Discussion

Chapter 5 Part IIIA: Characterization of T regulatory cell populations in the A/J mouse

Inoculation with *H. hepaticus* does not alter the percentage of CD4⁺CD25⁺FoxP3⁺ T regulatory cells in the A/J mouse

As administration of *H. hepaticus* in A/J mice results in the development of typhlitis approximately 90 days later, it is reasonable to suspect that either the T regulatory cells induced by the bacteria are unable to control inflammation or *H. hepaticus* exposure simply does not result in the induction of a T regulatory cell population. To investigate this, the T regulatory cell populations, here defined as CD4⁺CD25⁺FoxP3⁺ cells, found within the spleen and the cecum were evaluated. The cecal T regulatory cell populations were evaluated following independent isolation of intraepithelial (IEL) and lamina propria (LPL) lymphocytes.

Forty 3-4 week old A/J mice were gastrically gavaged with *H. hepaticus*; four months later these mice were grouped into four groups of ten, necropsied and the ceca and spleens collected for analysis. Three groups of age matched *H. hepaticus* negative A/J mice were also necropsied and their tissues used as controls (n = 5 mice/group). The cells isolated from the spleen, IEL and LPL were stained with CD4, CD25 and Foxp3 fluorochromes and analyzed via flow cytometry. The data presented in Figure 5-9 demonstrates the percentage of CD25⁺ cells which are CD4⁺Foxp3⁺.

As demonstrated in Figure 5-9 no significant differences in the percentage of CD4⁺ CD25⁺Foxp3⁺ cells were found between the *H. hepaticus* and control inoculated mice. This data suggests that inoculation with *H. hepaticus* does not result in a detectable change in the number of CD4⁺CD25⁺Foxp3⁺ cells. It does not, however, provide any insight on if T regulatory cells are induced following exposure to *H. hepaticus* – it is plausible that the T regulatory cell population induced by *H. hepaticus* is negligible when considering all of the other T regulatory cells within the three cellular compartments evaluated.

Chapter 5 Part IIIB: Depletion of CD25⁺ Cell Populations in the A/J and C57BL/6 Mouse

1 mg of anti-CD25 significantly decreases the percentage of CD4⁺CD25⁺ splenocytes in the A/J and C57BL/6 mouse.

An antibody targeting CD25 can be utilized to deplete T regulatory cells in the mouse^{388,389}. As previously discussed, however its use is limited to the pre-inflammatory period, as T effector cells have been shown to upregulate the expression of CD25 following activation and thus could also be depleted with this antibody. Before any experiments utilizing this antibody can be completed, an effective dosage regime must be established. As it is possible, although unlikely, that A/J and C57BL/6 mice will require different doses of anti-CD25 to achieve optimal depletion of CD25⁺ cells, this pilot study was completed to ensure maximal depletion of cells in both strains.

Twelve female A/J and twelve female C57BL/6 mice 3-4 weeks of age were utilized for this study. The mice from each strain were divided into four equal groups, with three mice per group. Three of the groups underwent an experimental dosing regimen, while the fourth mouse group acted as the control and was not treated. Mice in the experimental groups received one of the following treatments: one treatment of 1 mg, two treatments of 1 mg or two treatments of 0.5 mg (Figure 5-10B). All treatments were administered by intraperitoneal (IP) injection; those groups given antibody twice were treated every three days. All mice were necropsied and evaluated three days following the last treatment. For an experimental timeline, see Figure 5-10A.

At necropsy, mice were euthanized and spleens were collected for cell population evaluation. The splenic cells isolated were stained for CD4 and CD25 and evaluated with flow cytometry. As shown in Figure 5-10C a significant decrease in the percentage of CD4⁺CD25⁺ occurred in both strains with all three treatment regimens. No significant differences between the A/J and C57BL/6 mouse strains were found for either the percentage of CD4⁺CD25⁺ cells in the control groups or in the responses seen in the experimental groups.

Because no differences were seen between the three treatment regimens, the regimen utilizing the lowest dose and smallest number of treatments was chosen. For all subsequent experiments, unless otherwise noted, mice were treated once with 1 mg of anti-CD25 antibody IP to accomplish depletion of CD25⁺ cellular subsets.

anti-CD25 antibody administration prior to *H. hepaticus* inoculation may deplete other cell types in addition to CD25⁺ T regulatory cells.

While A/J mice are known to develop lesions three-months following *H. hepaticus* administration, C57BL/6 mice administered the same dose of *H. hepaticus* are resistant to disease development. The extracellular marker, CD25, is known to be associated with a T regulatory cell subset. As previously discussed, the adoptive transfer (AT) of CD4⁺CD25⁺ cells has been shown to suppress the development of colitis caused by the AT of CD4⁺CD25⁻ cells^{155,173,174,178}. CD25 is the receptor for IL-2, which is a cytokine associated with lymphocyte proliferation⁴³⁹. Interestingly, CD25 is up-regulated on activated T effector cells following an immune stimulus⁴³⁵.

The utilization of an anti-CD25 antibody will allow for the selection depletion of CD25⁺ cells; however because both T regulatory and T effector cells will express CD25 following antigen stimulation, the timing of antibody administration must be considered to avoid depletion of both cell types. By administering the anti-CD25 antibody prior to antigen stimulation T regulatory cells, but not the T effector cell populations initiated by the presence of *H. hepaticus*, will be depleted.

The overall aim of this project is to determine the role of T regulatory cells, defined here as a CD4⁺CD25⁺ cellular subset, in the development of *H. hepaticus* induced inflammation. To accomplish this, both a resistant and susceptible

mouse strain, C57BL/6 and A/J respectively, were administered anti-CD25 antibody and then three days later, were gastrically gavaged with *H. hepaticus*. At the time of *H. hepaticus* administration it is assumed, based on the previous study depicted in Figure 5-10, that the T regulatory cell population has been significantly depleted and that the immune response initiated by the presence of *H. hepaticus* will develop without the full suppressive influences of a T regulatory cell population. As a control, both A/J and C57BL/6 mice were administered rat IgG, the isotype control of the anti-CD25 antibody, in the same manner as those mice treated with the depletion antibody and similarly inoculated with *H. hepaticus*. Groups of mice were necropsied at 4, 45 and 90 days post-inoculation. Those mice necropsied at day 4 were utilized to verify the depletion of CD25⁺ cells in addition to evaluation of mRNA gene expression of IP-10, MIG and IL-12/23 p40. Those mice necropsied at 45 and 90 days post inoculation were also evaluated for the mRNA gene expression of these there cytokines, as well as the expression of IL-10. Additionally, the ceca of the mice necropsied at the two later time points were evaluated for the development of inflammation.

Four days following inoculation with *H. hepaticus* five mice from each of the four groups (A/J mice administered anti-CD25, A/J mice administered the IgG isotype control, C57BL/6 mice administered anti-CD25 and C5BL/6 mice administered the IgG isotype control) were necropsied and their spleens and ceca collected for evaluation. To evaluate the depletion of CD25⁺ cells, the five mice from each group were split into two sub-groups, one with two and one with

three mice. The splenocytes from these mice were pooled and strained for CD4 and CD25. A representation of the staining patterns seen for the anti-CD25 and IgG isotype treated mice can be found in Figure 5-11A. On average, for the isotype control treated mice, 2.7% (range 2.1% – 3.4%) of all splenocytes were CD4⁺CD25⁺ compared to the anti-CD25 treated mice in which on average 1.3% (1.0% – 1.6%) of all splenocytes were CD4⁺CD25⁺. It should be noted that the percentage of CD4⁺CD25⁺ cells seen in the control groups here are slightly lower than those presented in Figure 5-10C, in which the range of CD4⁺CD25⁺ was 3.4% to 6.5%. This variation may be simply due to the low animal numbers evaluated in these studies. Ultimately, the data allows for the conclusion that a significant depletion of CD25⁺ cells occurred with the administration of anti-CD25 antibody and was still detectable six days following antibody administration.

Those mice necropsied 45 and 90 days post-*H. hepaticus* inoculation were evaluated for the development of typhlitis. As expected, at the early time point, the average lesions scores seen were below those considered as diseased in this model and there were no significant differences between the average lesion scores of the four treatment groups. As disease induced by *H. hepaticus* is not typically seen until 90 days post inoculation, the development of or changes in disease was not necessarily expected. However, as it was plausible that the absence of T regulatory cells during the initial response to *H. hepaticus* could result in the accelerated development of disease, it was necessary to evaluate inflammation at this early time point. (Figure 5-11B)

As previously seen in this model, at the 90 day necropsy time point, the A/J mice, regardless of antibody treatment, developed cecal inflammation while the C57BL/6 mice did not. There were no significant differences between lesion scores of the A/J mice administered anti-CD25 and the control isotype antibody; the same was observed in the C57BL/6 mice. Interestingly, there were significant differences between the A/J and C57BL/6 mice administered anti-CD25, but not those administered the IgG control antibody. Again, the low n for each of the treatment groups combined with the individual variability in disease development of this model may be contributing to the lack of significant differences between the treatment groups, especially in the case of the IgG treated mice.

As previously stated mRNA analysis of several cytokines was evaluated at all three necropsy time points. No significant differences were detected in the mRNA gene expression of IP-10, MIG or IL-12/23 p40 at the day 4 necropsy time-point (Figure 5-11C) At the day 45 time point these three cytokines and IL-10, were significantly elevated in the A/J mice when compared to C57BL/6 mice within both antibody treatment groups. Furthermore A/J mice who received the anti-CD25 antibody had a significantly lower mRNA expression of IL-10, IP-10 and MIG when compared to the A/J control mice. At the day 90 time point the trends in MIG expression were the same as that seen at day 45. For both IP-10 and IL-10 the only significant difference identified was an elevation in mRNA gene expression in the A/J mice administered the IgG control antibody when

compared to C57BL/6 control mice. Interestingly, when IL-12/23 p40 was evaluated at this time point in addition to the significant increases in mRNA gene expression in A/J mice compared to the C57BL/6 mice within both antibody treatment groups, there was a significant increase in IL-12/23 p40 expression in the control A/J mice compared to the aCD25 treated A/J mice.

The differences in mRNA cytokine gene expression at the two later time points within the IgG treated A/J and C57BL/6 mice were not unexpected; previous publications have demonstrated a significant elevation in these cytokines in A/J mice compared to C57BL/6 mice inoculated with *H. hepaticus*²⁵. The decrease mRNA expression of IL-10 in the CD25 antibody treated A/Js compared to the control antibody treated antibodies at the day 45 time point could be due to a decreased number of T regulatory cells, which produce IL-10, however the decreased expression of the pro-inflammatory cytokines IP-10, MIG and IL-12/23 p40 could indicate that T effector cells, known to produce or induce the expression of these cytokines, were also depleted with the administration of the anti-CD25 antibody.

As previously discussed activated T effector cells upregulate the expression of CD25, and thus could be susceptible to depletion by the antibody utilized in this study. The flow data presented in Figure 5-11A suggests that the depletion of CD25⁺ cells persisted at least six days post antibody administration and thus, it is rationale to hypothesize that residual anti-CD25 antibody may have affected the T effector cell population induced by the introduction of *H. hepaticus*.

The suppressed pro-inflammatory mRNA gene expression in the anti-CD25 antibody treated mice lends further support to this hypothesis. Before any further experiments could be completed, a kinetics study to evaluate the duration of CD25⁺ depletion in both A/J and C57BL/6 mice must be completed.

Identification of the time-point at which anti-CD25 antibody achieves maximum depletion of CD25⁺ cells.

The data from the previous experiment has lead us to believe that the ability of the anti-CD25 antibody to deplete CD25⁺ cells extends beyond three days. Furthermore, the residual effects of the anti-CD25 treatment is suspected to have depleted CD4⁺CD25⁺ T regulatory cells, as well as activated CD25⁺ T effector cells which developed in response to *H. hepaticus* exposure. The experiments described here attempt to document the duration of depletion following a single 1 mg dose of anti-CD25. Unfortunately immediately following the previously described experiment it was realized that the anti-CD25 antibody utilized was being administered at a less concentrated dose than originally intended. This was due to the presence of IgG in the FBS used in cell culture for antibody production; it was estimated that almost half of the IgG in the anti-CD25 isolates was not the intended antibody (data not shown). For this reason, subsequent experiments will be completed at a dosing regimen of one 0.5 mg treatment of anti-CD25. In addition to evaluating the residual effects of anti-CD25 antibody administration these experiments will confirm that the new dose of anti-CD25 antibody is sufficient for cellular depletion.

To accomplish this, A/J and C57BL/6 mice were administered 0.5 mg anti-CD25 antibody IP; mice not administered anti-CD25 were used as controls. On days 5, 7, 9 and 11 post-antibody three anti-CD25 treated and one control mouse was euthanized and spleens collected for flow cytometry analysis. Isolated splenocytes were stained for CD4, CD25 and Foxp3; cells were initially gated on CD4 expression and then the co-expression of CD25 and Foxp3. To allow for easier interpretation of the data, the percentage of CD4⁺CD25⁺Foxp3⁺ cells obtained were normalized to the percentage seen in the control mouse. In short, the control mouse's percentage of triple positive cells became the baseline to which the experimental, anti-CD25 treated mice were compared. A/J and C57BL/6 mice were analyzed separately.

As shown in Figure 5-12A, in both strains and at all four time points, those mice administered anti-CD25 had a reduced level of CD4⁺CD25⁺Foxp3⁺ compared to the control mice. As this experiment suggests that suppression of CD25⁺ cells extends beyond 11 days post-administration, the experiment was repeated as described below.

Again A/J and C57BL/6 mice were administered 0.5 mg anti-CD25 antibody IP; mice not administered anti-CD25 were used as controls. At days 3, 9, 11, 13, 15 17 and 19 post-antibody administration mice were euthanized and spleen again collected for flow cytometry analysis. The same staining, gating and analysis protocol described in the previous experiment was followed. The

day 3 time point was included to ensure an initial depletion of CD25⁺ did indeed occur in both mouse strains.

Subsequent studies will again aim to evaluate the immune response to *H. hepaticus* when T regulatory cells are depleted, but without altering the resultant T effector cell populations. Thus it is necessary to identify the time period immediately before CD25⁺ populations begin to return in an attempt to have both a decreased CD4⁺CD25⁺ T regulatory population and minimal antibody effects upon developing T effector cell populations. Figure 5-12B depicts the depletion of CD25⁺ cells in the A/J mouse. From this data it was concluded that by day 15 post-injection some of the mice had begun to regain their CD25⁺ cell populations. At the previous time point, however, the proportion of CD4⁺CD25⁺FoxP3⁺ cells in the antibody treated mice was still depressed compared to the control mouse. Interestingly in the C57BL/6 mouse (Figure 5-12C) the threshold for CD25 depletion appeared to be around 9 days post-inoculation. By the day 11 time-point the portion of CD4⁺CD25⁺Foxp3⁺ cells in the antibody treated mice was fairly similar to the control mice.

Based on these results it was determined that the optimal time for *H. hepaticus* administration following anti-CD25 antibody treatment would be 13 and 9 days post-treatment for the A/J and C57BL/6 mouse strains, respectively.

The administration of anti-CD25 antibody had short-term, but lacked long term effects on *H. hepaticus* induced inflammation.

In light of these recent findings, the original experiment in which anti-CD25 antibody was utilized to deplete CD25⁺ cells was repeated. However instead of administering antibody 3 days prior to *H. hepaticus* inoculation, the mice received antibody treatments according to the findings just discussed. A/J mice were administered 0.5 mg anti-CD25 or the rat IgG isotype control 14 days before *H. hepaticus* inoculation. The C57BL/6 mice were administered their antibody treatments 9 days prior to *H. hepaticus* inoculation.

Flow cytometry was used to confirm that CD4⁺CD25⁺Foxp3⁺ cell populations were detected at the time of *H. hepaticus* inoculation (data not shown). Lesion scores were evaluated at 45 and 90 days post-*H. hepaticus* inoculation. At the day 45 necropsy time point significant decreases in LS were noted when mice treated with anti-CD25 were compared to control, IgG treated mice; this trend was observed within both the A/J and C57BL/6 mice (Figure 5-13A). By the 90 day time point these differences were no longer observed, however control A/J mice had significantly higher lesion scores than the control C57BL/6 mice. Interestingly there were no differences in inflammation noted between the A/J and C57BL/6 mice administered anti-CD25.

The decreased histologic disease in both mouse strain groups treated with anti-CD25 could still be the result of residual anti-CD25 antibody depleting T

effector cells capable of causing disease. As the IgG treated A/J mice developed significantly increased disease compared to the C57BL/6 controls at the day 90 time point, there is no reason to suspect the *H. hepaticus* inoculum administered failed to produce disease. There were, however no statistical differences between the two A/J treatment groups suggesting that lacking T regulatory cells at the time of *H. hepaticus* does not affect disease development in the A/J mouse. Interestingly, there were no differences in lesion scores between the A/J and C57BL/6 mice administered anti-CD25 (Figure 5-13A). This could suggest that lack of T regulatory cells at the time of *H. hepaticus* administration in the C57BL/6 mouse makes the strain more susceptible to disease, however this cannot be definitely concluded to the lack of significant differences between these mice and those administered the control antibody.

Due to the lack of significant conclusions drawn from the lesions scores, which ultimately are the indicator of disease, mRNA gene analysis of IL-12/23 p30, MIG and IP-10 was completed only for the A/J mice necropsied at the day 45 and 90 time points (Figure 5-13B). At the 45 day necropsy time point mice administered anti-CD25 had significantly decreased mRNA expression of three cytokines/chemokines examined when compared to those mice administered the control antibody. At the 90 day time point, no significant differences were identified between the A/J mice administered anti-CD25 and control antibodies. The decreased pro-inflammatory mRNA expression at the day 45 time point further supports the lesion score observation that anti-CD25 antibody does

indeed have residual effects on disease progression, but ultimately does not affect disease development.

Due to the suspicion that the use of anti-CD25 antibody is affecting the T effector cell response to *H. hepaticus*, it will not be used for T regulatory cell depletion in further studies. The use of neonatal thymectomies and diphtheria toxin (DT) administration in C57BL/6-*Foxp3*^{DTR} were used to further evaluate the role of T regulatory cell in *H. hepaticus* induced-inflammation.

Chapter 5 Part IIIC: The Adoptive Transfer of T regulatory cells

The adoptive transfer of CD4⁺CD25⁺ T cells from H. hepaticus naïve A/J mice does not reverse H. hepaticus induced-inflammation.

These experiments sought to evaluate the ability of CD4⁺CD25⁺ T regulatory cells to reverse existing *Helicobacter hepaticus* induced inflammation. To accomplish this, A/J mice were inoculated with *H. hepaticus* and, three months later, adoptively transferred 1 x 10⁶ CD4⁺CD25⁺ T regulatory cells weekly for either 2 or 3 total transfers. The transferred cells were isolated from 4-6 week old, naïve A/J mice. Three days following the last adoptive transfer, mice were necropsied and their ceca collected for histologic evaluation. Evaluation of lesion scores revealed (Figure 5-14) no significant differences between any of the three treatment groups.

This data suggests that in this experiment the adoptive transfer of CD4⁺CD25⁺ T cells is not capable of reversing *H. hepaticus* induced disease.

These results are contradictory to published experiments which demonstrate that the adoptive transfer of T regulatory cells are capable of reversing colitis^{155,173,174,178}. The discrepancy between this data and that previously published by other laboratories may be due to the *H. hepaticus* status of the donor mice, the age of the donor mice and or a combination of both.

Neither the adoptive transfer of CD4⁺CD25⁻ or CD4⁺CD25⁺ cell populations effects the development of H. hepaticus induced disease.

The previous experiment evaluated the ability of CD4⁺CD25⁺ cell populations to reverse *H. hepaticus* induced inflammation in the A/J mouse. In those experiments adoptive transfers were completed when inflammation had already developed and did not evaluate the protective ability of these cells to prevent disease development. The experiment presented here, in addition to including non-treated controls and the adoptive transfer of CD4⁺CD25⁻, mice are treated prior to, during and at the development of *H. hepaticus*-induced inflammation. This experiment sought to better evaluate both the curative and preventative properties of CD4⁺CD25⁺ T regulatory cells in this inflammatory model. In addition, the potential for CD4⁺CD25⁻ T effector cells to exacerbate disease will be evaluated.

To accomplish these goals 3-4 week A/J mice were inoculated with *H. hepaticus*. The same mice received adoptive transfers of either 1 million CD4⁺CD25⁺ or CD4⁺CD25⁻ cells weekly for three weeks starting one, two or

three months post-*H. hepaticus* inoculation. Control mice were inoculated with *H. hepaticus* but received no cellular transfers. Mice administered cells one month following inoculation are referred to as “Group 1”, mice administered cells two months following inoculations are referred to as “Group 2” and mice administered cells three months following inoculations are referred to as “Group 3.” All cells used in adoptive transfers were isolated from six week old *H. hepaticus* free A/J mice. Three days following Group 3’s third adoptive transfer, all mice in the study were necropsied. At the time of necropsy ceca were collected for histologic evaluation and mRNA gene expression analysis of MIG, IP-10, IL-12/23 p40 and IL-10.

As shown in Figure 5-15A no significant differences were found between the disease severities of any two treatment groups. It should be noted, however that the disease seen in these mice is slightly less severe than that normally seen following *H. hepaticus* administration. When considering the mRNA gene expression, no significant differences were found in either MIG or IP-10 (Figure 5-15B). Significant decreases in IL-12/23 p40 expression were seen in those mice adoptively transferred CD4⁺CD25⁻ T effector cells in groups 1 and 2 compared to those in group 3. This finding was contrary to the hypothesis that the transfer of T effector cells would cause increased disease, accompanied by increased expression of IL-12/23 p40. Mice in group 1 adoptively transferred CD4⁺CD25⁻ cells had elevated IL-10 gene expression compared to all the other mice in Group 1 as well as the mice in groups 2 and 3 transferred CD4⁺CD25⁻

cells. This finding was unexpected and could potentially be due to the induction of induced T regulatory cells from the transferred CD4⁺CD25⁻ cell populations; however the lack of effect on disease development would suggest that if the elevations in mRNA cytokine expression were due to T regulatory cells, this cell population ultimately had minimal effects on disease development.

The lack of disease alteration in this experiment and those described in Figure 5-14 following the adoptive transfer of either CD25⁻ or CD25⁺ cells suggests that the addition of these two cell groups does not alter the progression of *H. hepaticus* induced inflammation in the A/J mouse. It does not, however, mean that these cells are not involved in the prevention or development of *H. hepaticus* induced inflammation.

The adoptive transfer of CD4⁺CD25⁺ cells causes significant elevations in histologic disease.

To further investigate the role of these two cell groups, A/J mice with a deficiency in RAG2 were utilized. As these mice lack functional T and B cells, specific T cell populations can be adoptively transferred and their role in the development of inflammation more thoroughly examined. While the protective effects of CD4⁺CD25⁺ have been demonstrated in the C57BL/6 RAG2^{-/-} mouse adoptive transfer model of colitis^{117,178} this mouse strain is resistant to the development of *H. hepaticus*²⁵. As the discrepancy in susceptibility between A/J and C57BL/6 mice to *H. hepaticus*-induced inflammation may be due to T

regulatory cell function, it is important to evaluate the ability of T regulatory cells to suppress inflammation in general in the A/J mouse.

As with the previous experiments, this experiment aims to determine the role of T regulatory cells ($CD4^+CD25^+$) in the development of inflammation in the A/J mouse. To accomplish this 8-12 week old A/J $RAG2^{-/-}$ mice (A.129S6(B6)- $Rag2^{tm1Fwa}$) were adoptively transferred 5×10^5 $CD4^+CD25^-$ or $CD4^+CD25^+$ or a 1 to 2 ratio of these cells (2.5×10^5 $CD4^+CD25^-$: 1.25×10^5 $CD4^+CD25^+$). Twelve weeks later these mice were necropsied and cecal inflammation evaluated. This experiment was completed with female donors/female recipients, male donors/male recipients and female donors/male recipients. Each experimental group contained 3-5 mice per group.

As demonstrated in Figure 5-16A, in $RAG2^{-/-}$ female mice the adoptive transfer of $CD4^+CD25^+$ cells from female A/J donors caused disease that was significantly more severe than that seen in the other two cell treatment groups. This was surprising, as the $CD4^+CD25^+$ cellular subset was originally predicted to contain T regulatory cells, which were predicted to suppress, not exacerbate disease. In the C57BL/6 adoptive transfer models previously cited, this subset of cells was capable of suppressing inflammation compared to the adoptive transfer of $CD4^+CD25^-$. As the opposite was seen here, it is logical to speculate if the population of cells isolated here in the A/J mouse contained a higher proportion of T effector cells, which would also be expressing $CD25^+$. When comparisons

were made in the male to male and female to male adoptive transfer no differences in lesion scores were noted (Figure 5-16B & C).

Based on this experiment and the previous experiments described in this chapter, CD25 does not appear to be the optimal extracellular marker for the isolation or depletion of T regulatory cells. All further experiments exploring the ability of T regulatory cells to alter disease development will utilize CD45RB expression as the criteria for isolation of the T regulatory and T effector cell populations adoptively transferred.

The adoptive transfer of cells sorted on CD45RB expression does not alter *H. hepaticus* disease development.

As an alternative to CD25, CD45RB was chosen for the selection of T regulatory and effector cells. CD45 is known to be highly up-regulated on T effectors cells (CD45RB^{high}), while T regulatory cells have minimal levels of expression (CD45RB^{low}). As cell selection is based on degree of expression, rather than simply expression or lack of expression a different method of cellular isolation was utilized than that used in previous experiments. Isolated lymphocytes were stained for CD4 and CD45RB, then flow cytometry was used to sort CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} cellular subsets. To ensure the development of disease in these mice, all mice were inoculated with *H. hepaticus*.

8-12 week old male mice were inoculated with *H. hepaticus* and then adoptively transferred 5×10^5 CD4⁺CD45RB^{high} cells, 5×10^5 CD4⁺CD45RB^{low} cells or a ratio of 1:2 of both cellular subsets (2.5×10^5 CD4⁺CD45RB^{high} cells: 1.25×10^5 CD4⁺CD45RB^{low}). 12 weeks later the mice were necropsied and ceca collected for evaluation of inflammation. As shown in Figure 5-17 no significant differences were found between the three groups, although there is a trend of elevated disease noted in those mice adoptively transferred T effector cells (CD4⁺CD45RB^{high}). It should be noted, however that following the adoptive transfers, it was found that the cell isolates were contaminated with bacteria. All mice were monitored for weight loss, a secondary skin of systemic bacteremia, however none of mice were noted to loss more than 20% of their body weight (data not shown).

Conclusions

Ultimately, it was determined that neither the use of anti-CD25 antibody nor the adoptive transfer of either CD25⁺ or CD45RB^{low} cells was effective at specifically selecting a T regulatory cell population. These findings led to the experiments described in Chapter 4 which utilized both neonatal thymectomies and DT administration in the C57BL/6-*Foxp3*^{DTR} mice to further evaluate the roles of natural and inducible T regulatory cells in *H. hepaticus* induced-inflammation.

Materials & Methods

Mice

The University of Missouri's Animal Care and Use Committee (ACUC) approved all experiments involving the use of animals. All experiments were planned and performed in accordance with the Guide for the Care and Use of Laboratory Animals. The A/J and C57BL/6 mice were obtained from Frederick National Laboratory for Cancer Research.

Helicobacter hepaticus

The culture protocol for the MU-94 *Helicobacter hepaticus* strain has been previously described^{25,252}. At 3-4 weeks of age, mice were gastrically gavaged with 0.5 mL of culture broth. The estimated concentration of *H. hepaticus* is approximately 5×10^8 bacteria/mL. A PCR assay was used to confirm colonization with *H. hepaticus* at necropsy²⁴⁷.

Necropsy

Following euthanasia through CO₂ inhalation, ceca were removed and cecal contents and feces collected for verification of *H. hepaticus* colonization. The cecum was then longitudinally sectioned into two equal portions and used for mRNA expression analysis and histologic examination.

Splenocyte Isolation

Following removal from the mouse, the spleen is placed in a petri dish containing 2 ml complete RPMI-10 and left on ice until processing. The spleen is then minced with a scalpel blade. Each piece is thoroughly flushed by drawing up the media into a 5 ml syringe and expelling it through a 20G needle into each piece. The supernatant and remaining spleen pieces are transferred to a 70 μ m cell strainer affixed atop a 15 ml conical tube. The plunger of the previously used 5 ml syringe is then used to pulverize the remaining spleen pieces in the cell strainer until only fibrous tissue remains. The cell strainer is rinsed with 5 additional mls of RPMI-10.

To remove red blood cells, BD Pharm Lyse™ buffer is used per manufactures protocol (BD Biosciences, San Jose, CA). The suspension is then centrifuged at 200 g for 5 minutes. The resulting cell pellet is suspended in 1 ml staining buffer (Biolegend, San Diego, CA) and the passed over a clean 70 μ m cell strainer. Cells are counted using trypan blue and a hemacytometer and, following centrifugation at 350 x g for 5 minute, suspended at the desired concentration in cold staining buffer.

IEL and LPL isolation

Following removal from the mouse, the cecum is cleaned of all cecal contents, stripped of all fat and mesenteric tissue and placed in a petri dish containing 5 mls of cold RPMI + 5% FBS. The cecum is then evaluated and all

peyers patches are dissected out. The cecum is then rinsed in fresh media and cut into 0.5 – 1.0 cm pieces. First, to isolate IELs, cecal pieces are placed in a 250 ml Erlenmyer flask containing 25 mls of RPMI + 5% FBS + 1 mM DTT and shook at 200 rpm, 37° C for 20 minutes. Next, the supernatant was filtered through a nylon mesh into a 50 ml conical tube and centrifuged at 1200-1400 rpm for 10 minutes. The cecal tissue collected in the nylon mesh is set aside for LPL isolation. The resulting cell pellet is then suspended in 3 mls of RPMI + 5% FBS. These steps (filter, centrifuge, suspend) are repeated twice for a total of three repetitions. Lympholyte® M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada), per the manufactures instructions, is used to isolate viable cells which is the IEL fraction used for the described experiments.

To isolate the LPLs, the cecal tissue previously collected is minced with a razor blade, suspended in 25 mls of RPMI + 5% PBS + 100 units/mls collagenase and placed in a 250 Erlenmyer flask. The suspension is shook at 37° C at 200 rpm for 1 hour and then decanted through nylon mesh into a 50 ml conical tube and spun at 1400 rpm for 10 minutes. Lympholyte-M (Cedarlane Laboratories Limited), per the manufactures instructions, is used to isolate viable cells which serves as the LPL fraction used for the described experiments.

Cell Staining, Analysis and Sorting

Cells isolated from the spleen and ceca were stained for CD4, CD25 and Foxp3 utilizing a Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego,

CA) per the manufactures instructions, along with the recommended isotype controls. For cell sorting, PE-conjugated CD45RB and FITC-conjugated CD4 antibodies were used per manufactures instructions (eBioscience) along with recommended isotype control. Following staining, cells were washed, suspended in an appropriate volume of staining buffer and analyzed with either a FACScan or FACSCalibur flow cytometer (BD Biosciences). A FACSdiva flow cytometer was used for all CD4/CD45RB cell sorts. For all experiments, side and forward scatter used to exclude dead cells and all gates were set upon isotype controls.

Magnetic Bead Isolation

CD4⁺CD25⁺ T cells were isolated from the spleens of donor mice. First CD4⁺ T cells were negatively selected using a CD4⁺ T Cell Isolation Kit per manufactures instructions (Miltenyi Biotec, Auburn, CA) and purified with either a Miltenyi Biotec autoMACS™ cell separator or an LS Column/MidiMACS™ Separator (Miltenyi Biotect). Then, from the isolated CD4⁺ cellular subset, CD25⁺ cells were positively selected using a CD25 Microbead Kit per manufactures instructions (Miltenyi Biotec) and purified in the same fashion as the first isolate.

Adoptive Transfer of lymphocytes

After isolation with either flow cytometry or magnetic bead separation, T cells were suspended in sterile PBS and 100 µl injected into the tail vein of

recipient mice. All mice were monitored for weight loss; loss of more than 20% of the starting body weight resulted in removal from the study.

anti-CD25 antibody production and administration

PC61 cells were generously donated by Dr. Habib Zaghouani. Cells were grown in an UltraDOMA serum-free hybridoma media (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) + 10% FCS at 37° C. Cell suspensions are centrifuged at 3000 RPM and the supernatant passed through Whatman 20.0 cm filter paper (GE Healthcare, Piscataway, NJ); if necessary supernatant was frozen and stored at -20° C until isolation. HiTRAP IgG columns (GE Healthcare) were used per manufacture's protocol for antibody isolation. All isolated fractions were dialyzed in PBS overnight and then concentrations determined using spectrophotometry (280nm); if necessary Amicon ® Ultra-15 centrifugal filter devices (Millipore, Billerica, MA) were used per manufacturer's instructions to concentrate antibody fractions. For the last experiment, anti-CD25 antibody (PC-61.5.3) was purchased from BioXCell (West Lebanon, NH). The isotype control rat IgG used in all experiments was purchased from Sigma-Aldrich (Saint Louis, MO). Anti-CD25 and control IgG antibodies were administered to all mice IP with a 25G needle.

Isolation of RNA and Reverse Transcription

Cecal sections were stored at -80° C until processing. To begin RNA isolation, cecal sections were thawed in TRIzol (Invitrogen, Carlsbad, CA) and

then homogenized with a TissueLyser (Qiagen, Valencia, CA). RNA isolation was done per the manufacturer's (Invitrogen) instructions. An Invitrogen Super Script II kit was used to reverse-transcribe RNA (5 µg total/ sample) into cDNA (final concentration 20 ng/µL) per the oligo(dT) primer protocol provided.

Real Time PCR

Real time quantitative PCR with Qigen Quanti-tect SYBR Green was done with a Qiagen Rotor-Gene Q. Table 4-1 contains the primers and annealing temperatures utilized. Run conditions for HPRT, MIG, IP-10, IL-10 and IL-12/23 p40 had been previously published^{25,252}.

Histologic Evaluation

At necropsy, cecal portions utilized for histologic evaluation were fixed in zinc fixative, paraffin embedded and then stained with hematoxylin and eosin (H&E) stain. Using a previously described protocol^{246,252,253}, the cecal sections were then scored for inflammatory lesions in a blinded fashion.

Statistical Analysis

Student's t-tests, two-way ANOVA with a Student-Newman-Keuls post-hoc analysis and two-way ANOVA with a Student-Newman-Keuls post-hoc analysis were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). When appropriate, Log2 transformations were used to correct for failures in normality. p values ≤ 0.05 were regarded as significant. All graphs and dot

plots were generated using either SigmaPlot 11.0 or GraphPad Prism 5.0 software (GraphPad Prism Software Inc., La Jolla, CA).

Figure 5-9. *H. hepaticus* does not alter the percentage of CD25⁺ which are CD4⁺FoxP3⁺.

Pooled splenic, IEL and LPL samples from A/J mice inoculated with *H. hepaticus* at 3-4 weeks of age were collected four months post-inoculation (n= 10 mice/group). Three groups of age matched A/J mice (*H. hepaticus* free) were used as controls. Data presented the percentage of CD25⁺ also CD4⁺FoxP3⁺; bars indicate group means $\pm$ SD. Data statistically analyzed with student t-tests; $p \leq 0.05$ considered statistically significant.

Figure 5-10. 1 mg of anti-CD25 antibody administered IP is sufficient to deplete CD25⁺ cellular populations in the A/J and C57BL/6 mouse.

(A) 3-4 week old A/J and C57BL/6 mice were administered one of the four treatment regimens listed. (B) To allow for necropsy of all mice on the same day, antibody was administered as outlined. (C) Following necropsy splenocytes were isolated and stained for CD4 and CD25. Following evaluation with flow cytometry, the percentage of CD4⁺CD25⁺ cells for all four treatment groups was determined; mice within each group were individually evaluated. Data statistically analyzed with a two-way ANOVA with a Student-Newman-Keuls post-hoc analysis; $p \leq 0.05$ considered statistically significant.

Figure 5-11. Administration of anti-CD25 antibody potentially depletes T effector cells generated in response to *H. hepaticus*.

15 A/J and 15 C57BL/6 mice were treated with 1 mg anti-CD25 antibody IP 3 days prior to inoculation with *H. hepaticus*. The same number of control mice were treated in the same manner with 1 mg rat IgG isotype control antibody. (A) Four days post-inoculation 5 mice from each group (20 total) were necropsied and evaluated for the presence of CD4⁺CD25⁺ cells; images are representative of those seen within the two treatment groups. A significant decrease in this cell population was noted in the anti-CD25 treated mice when compared to the controls. (B) At 45 and 90 days post-inoculation the remaining mice were necropsied (n=5 for each group at each time point) and evaluated for the development of cecal inflammation. Each dot plot represents the lesion score of an individual mouse. (C) Mice necropsied at the 45 and 90 day time points were evaluated for the mRNA expression of IP-10, MIG, IL-12/23 p40 and IL-10. Data statistically analyzed with either a student's t-test or a two-way ANOVA with a Student-Newman-Keuls post-hoc analysis; $p \leq 0.05$ considered statistically significant. All data presented as mean $\pm$ S.D..

Figure 5-12. 0.5 mg of anti-CD25 antibody suppresses CD25⁺ cell populations for 9 days in the C57BL/6 mouse and 13 days in the A/J mouse.

A/J and C57BL/6 mice were treated with 0.5 mg anti-CD25 antibody IP. Mice were necropsied on the indicated days and splenocytes isolated and evaluated for the expression of CD4, CD25 and Foxp3. Data from experimental mice is normalized to the control mouse for each time point. (A) CD4⁺CD25⁺Foxp3⁺ expression was evaluated at 5, 7, 9 and 11 days post-antibody administration. (B) CD4⁺CD25⁺Foxp3⁺ expression was evaluated at 3, 9, 11, 13, 15, 17 and 19 days post-antibody administration.

Figure 5-13. Administration of anti-CD25 has no long term effects on *H. hepaticus* induced-disease development in the A/J mouse.

A/J mice were treated with 0.5 mg anti-CD25 antibody IP 13 days prior to inoculation with *H. hepaticus*. C57BL/6 mice were treated with 1 mg anti-CD25 antibody IP 9 days prior to inoculation with *H. hepaticus*. A/J and C57BL/6 control mice were treated with 0.5 mg rat IgG isotype control antibody prior to inoculation. (A) At 45 and 90 days post-inoculation mice were necropsied (n= 5, 10 per group for the 45 and 90 day necropsy time points, respectively) and evaluated for the development of cecal inflammation. (C) A/J mice necropsied at both time points were evaluated for the mRNA expression of IL-12/23 p40, MIG and IP-10. All data statistically analyzed with a two-way ANOVA with a Student-Newman-Keuls post-hoc analysis; $p \leq 0.05$ considered statistically significant. Each dot plot represents the lesion score of an individual mouse; error bars indicate mean $\pm$ S.D..

Figure 5-14. The adoptive transfer of CD4⁺CD25⁺ cells does not reverse *H. hepaticus* induced inflammation.

3-4 week old female A/J mice were inoculated with *H. hepaticus*; ninety days later the mice were administered either 0, 2 or 3 treatments of 1×10^6 CD4⁺CD25⁺ cells intravenously. Treatments were done once every seven days. Three days following the last adoptive transfer for the mice which received three treatments, all mice were necropsied and ceca evaluated for histologic disease. Each dot plot represents the lesion score of an individual mouse. Data statistically analyzed with a one-way ANOVA; $p \leq 0.05$ considered statistically significant. No significant differences were identified.

Figure 5-15. The adoptive transfer of CD4⁺CD25⁺ during disease development does not ultimately inflammation severity.

3-4 week A/J mice were inoculated with *H. hepaticus*. The same mice received adoptive transfers of either CD4⁺CD25⁺ or CD4⁺CD25⁻ cells weekly for three weeks starting one (group one), two (group two) or three (group 3) months post-*H. hepaticus* inoculation. Control mice were inoculated with *H. hepaticus* but received no cellular transfers. All cells used in adoptive transfers were isolated from 6 week old A/J mice. Three days following group 3's third adoptive transfer, all mice in the study were necropsied. (A) Cecal histologic lesion scores of experimental and control mice. (B) mRNA gene expression analysis of MIG, IP-10, IL-12/23 p40 and IL-10. . Each dot plot represents the lesion score of an individual mouse. All data statistically analyzed with a two-way ANOVA with a Student-Newman-Keuls post-hoc analysis; $p \leq 0.05$ considered statistically significant. All data presented as mean $\pm$ S.D..

Figure 5-16. The adoptive transfer of CD4⁺CD25⁺ cells exacerbates disease.

8-12 week old A/J RAG2^{-/-} mice were adoptively transferred CD4⁺CD25⁻ or CD4⁺CD25⁺ or a 1 to 2 ratio of these cells. 12 weeks later these mice were necropsied and cecal inflammation evaluated. These adoptive transfer groups were completed with (A) female donors/female recipients (n = 4, 4 and 3), (B) male donors/male recipients (n = 3, 3 and 3) and (C) female donors/male recipients (n = 4, 4 and 5). Data statistically analyzed with a one-way ANOVA; p ≤ 0.05 considered statistically significant. Each dot plot represents the lesion score of an individual mouse; error bars indicate mean ± S.D..

Figure 5-17. The adoptive transfer of CD4⁺CD45RB^{low} T regulatory cells does not significantly decrease inflammation.

8-12 week old male A/J RAG2^{-/-} mice were inoculated with *H. hepaticus* and then adoptively transferred CD4⁺CD25⁻ or CD4⁺CD25⁺ or a 1 to 2 ratio of these cells from naïve male A/J mice. 12 weeks later these mice were necropsied and cecal inflammation evaluated. Each dot plot represents the lesion score of an individual mouse. Data statistically analyzed with a one-way ANOVA; $p \leq 0.05$ considered statistically significant. Each dot plot represents the lesion score of an individual mouse; error bars indicate mean $\pm$ S.D..

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VITA

Lydia Crystal Cook was born on the Ides of March in the year of 1982 to Charles W. Cook and Lydia Naomi Fern Cook in Landstuhl, West Germany. Her educational K-12 training was completed within the Department of Defense Dependent Schools on Pirmasens Army Depot (Germany), Guantanamo Bay Navy Station (Cuba) and Kadena Air Force Base (Okinawa, Japan). While enrolled at Kadena High School she participated annually in a regional science exposition known as Science Symposium. During this time, Lydia completed a research project with Cpt. B. Cassutto, an Army Veterinarian stationed on Kadena AFB, who mentored and encouraged Lydia to not only pursue a DVM, but also a PhD. After graduating as Kadena High School's Class of 2000

salutatorian, Lydia began her collegiate journey at the University of Missouri – Columbia. While completing her undergraduate studies Lydia held a variety of jobs including: student worker and summer extern for the Offices of Residential Life, cage washer for Office of Animal Resources and student laboratory worker for Dr. Craig Franklin. Her work in Dr. Franklin’s laboratory lead to the development of a dual DVM-PhD through the University’s College of Veterinary Medicine which she began following the completion of a bachelor of sciences degree in Animal Sciences and an Honors College certificate, in the spring of 2004.

Lydia’s path through the College of Veterinary Medicine was less than normal, as it required her to leave the Class of 2008 in 2005 to spend two years in the laboratory and then join the Class of 2010 in 2007. Within this new class Lydia found many good friends and has nothing but fond memories of her experiences with them at the VMTH. Her research in the Franklin Lab covered a number of topics, many of which were dead-ends, but from each she gained at least one skill, bit of knowledge or experience that has made her a better scientist. Her dissertation research ultimately focused on the role of gonadal sex hormone signaling, T regulatory cells and helminth therapies in Inflammatory Bowel Disease. The model utilized for a majority of her experiments utilizes the bacterium *H .hepaticus* to induce disease in the A/J mouse.

Lydia learned early on that a family is so much more than blood and genetic inheritance. She has approached life with this mentality and because of

this, in addition to her immediate family consisting of her partner in crime (Pat), her parents, sister, brother-in-law and nephew, but also an extended family that encompasses people she has met and who have helped shape her into the person she is today.

Lydia found the love of her life Patrick McNulty in 2011 - he's a fellow lab-rat who was working at the time as a research technician down the hall. They bonded over their love of Pink Floyd and now are living "happily ever after" with Dexter (dog) and four cats: Ragusa, Angel, Pumpkin and Cinco. They hope to travel the world and enjoy life to the fullest. She spends her free time participating in a bookclub which she helped found in 2006, camping and hiking with Pat and Dexter, tailgating with the Big-O-Bago Tailgaters during the MIZZOU football season, practicing as a local relief veterinarian and spending time with her friends and family.