HORMONAL AND GENETIC REGULATION OF *HELICOBACTER HEPATICUS*-INDUCED INTESTINAL INFLAMMATION

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by

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

HORMONAL AND GENETIC REGULATION OF *HELICOBACTER*

HEPATICUS-INDUCED INTESTINAL INFLAMMATION

presented by Andrew E. Hillhouse,

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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To my mother and father, Ben Ed and Lee Ann Hillhouse, my brother, Samuel, my wife, Katrina, and son, Phoenix, I love you all very much. Thank you for being so supportive of me throughout this work.

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Abbreviations and Terms

Gastrointestinal ..... GI

Inflammatory Bowel Disease ..... IBD

Prostaglandin E receptor 4 ..... EP4 or PTGER4

Dextran Sulfate Sodium ..... DSS

Nucleotide Oligermization Domain 2 ..... NOD2

Caspase Activating and Recruitment Domain 15 ..... CARD15

Nuclear Factor kappa B ..... NF-κB

Mucin 19 ..... MUC19

Mucin 2 ..... MUC2

Autophagy related protein 16 Like 1 ..... ATG16L1

Immunity Related GTPase family, M ..... IRGM

Interferon Gamma ..... IFNγ

Interleukin 1 beta IL-1β

Interleukin 18 ..... IL-18

Interleukin 12 ..... IL-12

Interleukin 17 ..... IL-17

T helper 1 ..... Th1

T helper 17 ..... Th17

Tumor Necrosis Factor alpha ..... TNFα

Interleukin 23 ..... IL-23

Interleukin 10 ..... IL-10

Signal Transducer and Activator of Transcription 3 ..... STAT3

Major Histocompatibility Complex ..... MHC

C-C Chemokine Receptor 6 ..... CCR6

Macrophage Stimulating 1 ..... MST1

Systemic lupus erythematosus ..... SLE

Rheumatoid Arthritis ..... RA

Multiple Sclerosis ..... MS

Estrogen Receptor alpha ..... ERα

Estrogen Receptor beta ..... ERβ

Dendritic Cell ..... DC

Lipopolysaccharide ..... LPS

Severe Combined Immunodeficient ..... SCID

Dinitrobenzenesulphonic acid ..... DNB

Selective Estrogen Receptor Modulator ..... SERM

Phosphate Buffered Saline ..... PBS

Diethyl Pyrocarbonate ..... DEPC

Monokine Induced by Gamma Interferon ..... MIG or CXCL9

4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol ..... PPT

Diarylpropionitrile 2,3-bis(4-Hydroxyphenyl)-propionitrile ..... DPN

17β-Estradiol E2

Experimental Autoimmune Encephalomyelitis ..... EAE

Quantitative Trait Locus ..... QTL

Centimorgan ..... cM

Single Nucleotide Polymorphism ..... SNP

Logarithm of Odds ..... LOD

Chromosome Substitution Strain ..... CSS

G-protein α Inhibitory 2 chain *Gnai2*

G-protein deficiency-induced colitis 1 ..... *Gpdc1*

Hormonal and Genetic Regulation of *Helicobacter hepaticus*-Induced Intestinal Inflammation

Chapter 1

Introduction to Inflammatory Bowel Disease

The human gastrointestinal (GI) tract is the largest exposed surface of the body with an approximate total surface area of 300 square meters. It is tasked with digestion and nutrient uptake and acts as the first line of defense against over 400 bacterial species and greater than 10^{14} total microbes inhabiting the gastrointestinal tract.[1, 2] The GI tract is therefore populated with a variety of cells set to aid in digestion and respond to possible infections. To this end, the human GI tract is the largest immune organ in the human body, and its duty is to preserve the fine balance of tolerance to food antigens and commensal bacteria that aid in digestion while maintaining an alert immune system capable of responding to pathogenic organisms. When this balance is shifted, a variety of digestive diseases can occur.

Inflammatory Bowel Diseases (IBDs) encompass two main disorders, Crohn's Disease and ulcerative colitis. Collectively, IBDs are characterized by chronic relapsing intestinal inflammation resulting from a dysregulated immune response in genetically predisposed individuals.[3] Both diseases have related characteristics but each disorder has distinct clinical features. Inflammation in Crohn's Disease can occur in any portion of the gastrointestinal tract from the mouth to the anus. Inflammatory lesions are not always continuous, can be transmural (extending throughout the thickness of the intestine), are

often flanked by normal tissue, and are characterized by aggregation of macrophages that form granulomas. Lesions in ulcerative colitis typically begin near the rectum and are continuous throughout the colon. Inflammation and ulceration is limited to the mucosa and is rarely transmural with an increase in neutrophil accumulation in the lamina propria.[4] Despite their differences, these two disorders are thought to share many of the same factors responsible for causing disease.

The pathogenesis of IBDs is complex and multifactoral in nature requiring a combination of genetic mutations, environmental cues, and an exaggerated immune response in the intestine to result in intestinal inflammation. Development of IBD has traditionally been thought of as segregating to western populations with a prevalence of approximately 100-150 per 100,000 individuals of European descent.[5] Disease rates in other ethnic groups are lower than those of Caucasian populations, but recent studies have shown a drastic increase in Asian populations, particularly those of Japan, China, and Korea.[6] Development of IBDs is also closely associated with populations in urban versus rural areas. This increase in disease incidence seems to correlate with the increase in socioeconomic development in these countries.[7] Among other risk factors associated with IBD, tobacco use in IBD patients is very important. Smoking has been shown to increase the risk of Crohn's Disease while smoking decreases the risk of developing ulcerative colitis.[8]

Genetics of Inflammatory Bowel Disease

One of the key discoveries in IBD research was the implication of genetic factors in the pathogenesis of disease. Patients with Crohn's Disease are 15-20% more likely to

have an affected family member with an inflammatory bowel disease, and monozygotic (identical) twins are 50% more likely to develop IBD if they have an affected sibling.[9] This knowledge has led to multiple studies to identify genes responsible for IBD. Linkage analysis, and more recent whole-genome association studies in Crohn's Disease have implicated a total of no less than 70 genes or genetic loci, and another 30 loci have been associated with ulcerative colitis.[5] Roughly half of the loci identified to be associated with Crohn's Disease are also associated with ulcerative colitis, adding weight to the argument that Crohn's and ulcerative colitis share factors responsible for the development of disease. The overlapping genes identified to date may play an over-arching role in intestinal inflammation, while the disease specific genes may play a role in the distinct clinical presentation of either Crohn's Disease or ulcerative colitis.[10] The genes associated with these two disorders indicate the complex nature of the pathogenesis and can be divided between genes involved in intestinal barrier function, microbial sensing (innate immunity), and adaptive immune response.

Genes in Intestinal Epithelium

The intestinal epithelium is the first line of defense between the luminal contents of the gut and the rest of the body. Genes associated with inflammatory bowel disease and intestinal integrity are important in intestinal repair, ER stress, or maintenance of cellular architecture.[10, 11] Prostaglandin E receptor 4 (*EP4*) is a gene responsible for mucosal repair and barrier function. Mice deficient in *EP4* develop more severe colitis than wild type controls, and in humans, IBD locus 5 is closely associated with PTGER4 the gene that encodes EP4 protein. Patients with inflammatory bowel disease and first-

degree relatives of IBD patients have increased intestinal permeability compared to non-related control populations.[12]

A major component of the intestinal barrier, mucin, is produced by goblet cells in the intestine. A recent study identified a Crohn's Disease associated locus in a region that includes the human mucin gene, *MUC19*.[13] Administration of dextran sulfate sodium (DSS) in the drinking water of mice causes colitis in my by disrupting intestinal epithelial integrity. Mice with mutations in *MUC2*, another mucin gene, spontaneously develop mild colitis, and have increased sensitivity to DSS-induced colitis.[14] Polymorphisms in these genes could be responsible for a decrease in intestinal epithelial integrity removing a key functional component of the GI tract. Further studies will help to fully elucidate the role these genes play in IBD pathogenesis.

Genes in Microbial Detection

The gastrointestinal tract is continuously exposed to microbial antigens. Many genes identified are important for host-microbe interactions in stimulating or maintaining intestinal immune responses. *NOD2/CARD15*, the first Crohn's Disease associated gene identified, is an intracellular pathogen recognition receptor that recognizes the bacterial cell wall component muramyl dipeptide and signals through the transcription factor NF-κB.[15] *NOD2* is expressed in a variety of cell types including intestinal epithelial cells (particularly Paneth cells), granulocytes, macrophages, and dendritic cells. The presence of any combination of three disease associated variants can increase the risk for developing ileal Crohn's Disease up to 40-fold.[4] Mice deficient in *NOD2*, however, do not develop spontaneous colitis or show significant increase in susceptibility to DSS-

induced colitis.[16] How polymorphisms in *NOD2* increase susceptibility to Crohn's Disease is still unclear, but a prevailing hypothesis is that *NOD2* mutations result in a hyporesponsive innate immune response that then requires a much more aggressive adaptive immune response after contact with a bacterium. Another gene in the same family, *CARD9* has also been associated with Crohn's Disease and ulcerative colitis, and has been shown to be essential for innate immune signaling in response to intracellular and extracellular pathogens.[17] Intestinal microbial flora have been implicated in the pathogenesis of Inflammatory Bowel Diseases and identification of mutations in bacterial response genes in IBD patients lends weight to this hypothesis.

The autophagy pathway has also recently been shown to play an important role in Crohn's and ulcerative colitis. Autophagy, literally means "self eating," and is a means of degrading large cellular components, intracellular pathogens, and antigen processing.[18, 19] Polymorphisms in two autophagy genes, *ATG16L1* and *IRGM*, have been associated with Inflammatory Bowel Disease and are important in response to intracellular pathogens, antigen presentation to T cells, and IFNγ signaling during the immune response.[18, 19] Mouse models have shown that defects in *ATG16L1* cause Paneth cell abnormalities resulting in alterations in the secretion of antimicrobial peptides in the gut similar to Paneth cell abnormalities seen in patients with Crohn's Disease.[20] In intestinal macrophages with *ATG16L1* polymorphisms, there is an increased expression of the inflammatory molecules IL-1β and IL-18 in response to stimulation with lipopolysaccharide.[21]

Genes in the Adaptive Immune Response

The adaptive immune response is also important in the development and maintenance of intestinal inflammation. A key pathological feature of active IBD is the infiltration of antigen presenting cells as well as T and B cells into affected portions of the gut. CD4+ T cells have been shown in mouse models and in IBD patients to be important in the development of colitis. Specific pathogen free mice, deficient in T and B cells do not develop spontaneous colitis, but adoptive transfer of effector populations of CD4+ T cells can trigger intestinal inflammation.[22] In intestinal biopsies from Crohn's Disease patients there are increased levels of IL-12 and IL-17 mRNAs, key cytokines in the Th1 and Th17 pathways, along with an increased expression of IFNγ and TNFα.[23] Mutations in both of these pathways have been associated with Crohn's Disease and ulcerative colitis. Polymorphisms in the *IL12B* gene have been associated with both ulcerative colitis and Crohn's Disease[24, 25] *IL12B* codes for the p40 subunit of IL-12 and is necessary for the formation of the heterodimeric cytokines IL-12 and IL-23, Th1 and Th17 cytokines respectively. A recent study has also identified a polymorphism in the receptor for IL-23 (*IL23R*), a key molecule involved in sustaining Th17 responses. This polymorphism was shown to be protective against Crohn's Disease lending weight to the role of the Th17 pathway in Crohn's.[26] Other polymorphisms associated with the adaptive immune response in Crohn's and ulcerative colitis are involved in immune suppression (IL-10), cell signaling (STAT3), antigen specificity (MHC), and chemotaxis (CCR6, MST1).[11, 27]

To date, more than 30 genes or genetic loci have been identified that play a role in the pathogenesis of either Crohn's Disease or ulcerative colitis. Understanding the

complex interactions of these and other, yet to be discovered genes, along with interactions with various environmental factors associated with IBD will aid in the understanding of disease pathogenesis and will provide novel targets for future therapy.

Inflammatory Bowel Diseases can be successfully managed with a combination of therapies. A majority of patients respond positively to combination antibiotic and anti-inflammatory therapy, but these treatments are not curative, and patients require life-long treatment. While rarely fatal, roughly 80% of Crohn's Disease patients will require major abdominal surgery as a result of the disease.[28] Other treatment options have shown varying amounts of success in controlling disease, but further understanding into the pathogenesis of IBD will lead to better treatment options and eventually a cure.[29]

Estrogen in Inflammatory Bowel Disease

While the genetic makeup of an individual greatly affects the chances of developing an inflammatory bowel disease, there are other factors known to contribute to disease development including intestinal microbial flora, geographic location, smoking status, and other environmental factors. In many cases, IBDs can be considered an autoimmune disease to the endogenous microbial flora of the gut. During IBD, the intestinal immune response changes from a tolerogenic state to hyper-responsive resulting in uncontrolled intestinal inflammation.

Multiple autoimmune diseases have been shown to have a strong female bias in the development of disease. Systemic lupus erythematosus (SLE) has a 9:1 female/male ratio, rheumatoid arthritis 3:1, Sjogren's syndrome up to 20:1, and multiple sclerosis

2:1.[30] Estrogen has been shown to play an important, yet complex role in the pathogenesis of many of these diseases.

While Inflammatory Bowel Diseases have not been shown to have as distinct a sex bias as other autoimmune diseases, there is evidence that supports a role for sex hormones in disease development. In some cases, pregnancy or menstruation has been reported to increase the severity of symptoms associated with IBDs, while other reports have noted a protective role for pregnancy.[31, 32] The affect of pregnancy on the course of disease depends on the state of disease at the time of conception. Patients with active disease at conception often develop more severe inflammation throughout the pregnancy, while patients in remission at conception tend to remain disease free throughout pregnancy.[31, 33, 34] Along with natural production of estrogen, exogenous estrogen treatment has also been shown to have effects on IBD severity. The use of oral contraceptive agents and their role in inflammatory bowel disease has been addressed by many studies. A modest association between the use of oral contraceptives and the development of inflammatory bowel diseases has been observed in a number of studies and cessation of oral contraceptive use has been shown to be beneficial, resulting in less severe disease.[35-37] Hormone replacement therapy in post-menopausal female IBD patients can be effective in decreasing the severity of IBD.[38] Together, these data indicate a complex role for sex hormone signaling in the modulation of intestinal inflammation.

To understand the role that estrogen plays in inflammatory bowel disease, it is important to understand how and through what cell types estrogen signals. Estrogen receptors are nuclear hormone receptors that can either directly bind to estrogen response

elements on gene promoters or serve as cofactors with other transcription factors.[39] The first estrogen receptor, estrogen receptor alpha, was identified and cloned in humans in 1985, and in rats two years later.[40, 41] Estrogen receptor beta was first described in 1996.[42] ERα is the dominant estrogen receptor in the uterus, kidney, and areas of the brain controlling the hypothalamic pituitary-ovarian axis while ERβ is expressed more in the genitourinary, cardiovascular, respiratory, gastrointestinal and central nervous systems.[43] Signaling through the different estrogen receptors has been characterized in a variety of mouse models of autoimmune diseases, and ER signaling has been shown to play divergent and frequently competing roles in these diseases.

Estrogen receptors are expressed to varying degrees on a wide variety of cell types involved in the pathogenesis of IBD. Levels of estrogen receptor alpha and beta expression in the rat intestines are similar, but cell type specific expression has been demonstrated to vary greatly.[44] To understand the role that estrogen and estrogen receptor signaling plays in inflammatory bowel disease, it is important to understand in which cell types the estrogen receptors are expressed, and what actions these receptors are performing.

In the innate immune response, the intestinal epithelium is the first line of defense in the intestine. Intestinal epithelial cells in the colon have been shown to primarily express estrogen receptor beta and ER beta signaling has been shown to be important in intestinal epithelial function.[45] Intestinal permeability is decreased after exposure to estrogen and estrogenic compounds such as bisphenol A.[46, 47] Colonic myeloperoxidase activity and macrophage migration inhibitory factor are also significantly decreased upon exposure to estrogenic compounds resulting in decreased inflammation.[46] In other cells

of the innate immune system, estrogen and estrogen receptors play a different role. Bacterial sensing and antigen presentation are important parts of the innate immune response and estrogen has been shown to play a role in antigen presenting cell function. *In vitro* studies in dendritic cells have shown differential responses to estrogen. Dendritic cells (DC) require estrogen for differentiation. This appears to be ERα mediated since the differentiation of estrogen receptor alpha deficient cells is inhibited.[48, 49] The effects of estrogen on DC populations is also seen *in vivo.* In a mouse model of multiple sclerosis, supra-physiologic doses of E2 decrease DC numbers and decrease dendritic cell responses upon LPS stimulation. E2 treatment also showed profound defects in DC's abilities to present antigen and a downstream result of lower inflammatory molecules TNFα and IFNγ.[50] This effect has also been shown in macrophages. High levels of estrogen attenuates LPS-induced TNFα expression in macrophages by preventing NF-kB signaling.[51]

Estrogen is also important in the control of the adaptive immune response. ERα and ERβ are both expressed on primary T and B cells.[52] Estrogen has been shown to stimulate B cells and immunoglobulin production. ERα deficiency or blocking of estrogen signaling reduces antibody production.[53] In the NZB/NZW mouse model of lupus signaling through ERα increased production of self-reacting antibodies and significantly shortened the survival time of the mice. Signaling through ERβ was shown to be moderately protective for disease.[54] In T cells, expression levels of both estrogen receptors are equal and upon activation, in the presence of estrogen, T cells increase IFNγ production most likely through NF-κB activation.[52] Estrogen has also been shown to increase antigen specific CD4+ T cells numbers and decrease apoptosis in an estrogen

receptor alpha dependent manner.[55, 56] Increases in estrogen levels are also associated with increased numbers of FoxP3+ CD4+CD25+ regulatory T cells and increased expression of the immunosuppressive cytokine IL-10.[57] While effector T cells increase their expression of inflammatory molecules in the presence of estrogen, regulatory T cells increase their expression of immunosuppressive cytokines to maintain the immunologic balance. Understanding the role of estrogen, estrogen receptors, and the downstream signaling elicited by these molecules will help in the development of new treatment options for Inflammatory Bowel Disease.

Helicobacter hepaticus-Induced Mouse Model

Mouse models of intestinal inflammation are valuable tools for the investigation of the factors crucial to the development of Inflammatory Bowel Diseases. In 1992, ten years after the characterization of *Helicobacter pylori*, a spiral-to-curve shaped gram-negative bacterium was identified in the gastrointestinal tracts and livers of severe combined immunodeficient (SCID/NCr) and A/JCr mice. The isolates were originally associated with hepatitis and were identified as *Helicobacter hepaticus*.[58] This species was found to be morphologically similar to previously identified helicobacters, growing under microareophilic conditions, and having strong urease activity. It was also found to be motile with a single, bipolar, and sheathed flagella, different from previously identified helicobacters that have multiple flagella.

Helicobacter hepaticus has been implicated in causing inflammatory bowel diseases in both immunocompromised and immunocompetent mouse strains. Various experimental and genetic modifications in mice have resulted in susceptibility to

Helicobacter hepaticus. In an adoptive transfer of $CD45RB^{high}$ $CD4^{+}$ T cells to C.B-17 *scid* mice, IBD is induced in the presence of *H. hepaticus*, but mice do not develop colitis in germfree conditions.[22] Immunodeficient SCID/NCr mice develop colitis when infected with *H. hepaticus*.[59] *Helicobacter* infection in interleukin-10 or T cell receptor alpha/beta deficient mice leads to increased IBD severity.[60] Characterization of the immune response that accompanies inflammation shows an increase in production in IL-12, FNγ, and TNFα. Treatment with antibodies that block these molecules has been effective in ameliorating disease.[24, 61] In the *Helicobacter hepaticus*-induced mouse model of intestinal inflammation, we have observed a distinct difference in *Helicobacter hepaticus*-induced inflammation between male and female A/JCr mice. We suspect that these differences are moderated by sex hormones.[62] We also see distinct differences in susceptibility and immune response between A/J and C57BL/6 inbred mice.[24] We therefore chose to investigate the hormonal and genetic factors involved in the modulation of *Helicobacter hepaticus*-induced intestinal inflammation.

Chapter 2

The Role of Estrogen Receptors in Modulation of *Helicobacter hepaticus*-Induced Intestinal Inflammation

Background and Aims

The two major forms of Inflammatory Bowel Disease (IBD) Crohn's Disease and ulcerative colitis affect approximately 1 million people in the United States.[63] No definitive cause for these diseases has been identified, but it is widely accepted that the development of Inflammatory Bowel Diseases results from a dysregulated immune response against normally harmless commensal intestinal flora in genetically susceptible individuals.[4, 64-66] While rarely fatal, those affected with IBD often require a lifetime of treatment, and disease is seldom cured. Often, patients with IBDs will require surgical intervention to remove the portions of inflammed GI tract. Occurrence of IBD also increases a patient's risk for the development of colon cancer and increases the risk of developing other autoimmune diseases.[67-71] It is therefore important to develop novel, more effective therapeutics to treat IBD.

Estrogen and estrogen signaling has long been recognized as a factor involved in mediating various autoimmune diseases.[72] Eighty percent of autoimmune disease patients are women, and increased estrogen levels have been associated with either increasing disease severity in diseases such as lupus or decreasing disease severity in rheumatoid arthritis and multiple sclerosis [30, 73, 74] In IBDs, there is an equal incidence of disease in men and women, but a distinct sex bias has been reported in other intestinal

disorders such as Irritable Bowel Syndrome, microscopic colitides, and colon cancer suggesting a possible role for sex hormones in modulating intestinal health.[33, 75-78] Oral contraceptive use has been linked to the development of inflammatory bowel diseases in a number of studies and cessation of oral contraceptive use has been shown to be beneficial in the resolution of disease.[35-37] Studies investigating the effect of pregnancy on Inflammatory Bowel Disease are inconclusive, some show that pregnancy can help prevent the reoccurrence of disease flares, while others report that pregnancy can exacerbate disease.[31, 33, 34] It has been reported however, that hormone replacement therapy can be effective in decreasing the severity of IBD in post-menopausal female patients, suggesting that estrogen may be an effective therapy for IBDs.[38]

Animal models of IBD have shown encouraging results as to the effectiveness of estrogen therapy. Estrogen administration to the HLA-B27 transgenic rat model of IBD decreased diarrhea in animals and significantly decreased intestinal inflammation.[79] Estrogen is effective in decreasing disease severity in DNB and also acetic acid-induced colitis.[80, 81] Further work in the HLA-B27 rat model of IBD has shown that inhibition of NF-κB through either estrogen receptor alpha or beta (ERα or ERβ) signaling decreased intestinal disease severity and that use of the selective estrogen receptor modulator (SERM) against ERβ, ERB-041, was effective in decreasing disease.[82-84]

In humans, IBD is thought to be an over active immune response to normal gut flora. In mice, the bacterium *Helicobacter hepaticus* colonizes the gut and in some mouse strains acts as a commensal organism while in other, susceptible mouse strains, causes a typhlocolitis that is similar in presentation to Crohn's Disease. When infected with *H. hepaticus*, A/J mice develop cecal inflammation characterized by lymphocytic

infiltration and increased production of the Th1 cytokines IL-12, IFNγ, TNFα, and the chemokines CXCL9 and CXCL10.[24, 62, 65, 85] We have previously observed that *Helicobacter hepaticus* infected female mice develop more severe intestinal inflammation and significantly higher Th1 cytokine expression than infected male A/J mice.[62] Given the difference between male and female disease susceptibility and the results of estrogen administration in other models, we hypothesized that gonadal sex hormones may play a role in the development of *Helicobacter hepaticus*-induced inflammation. We therefore sought to address the role of estrogen in the *Helicobacter hepaticus*-induced model of inflammatory bowel disease, and further assess the role of specific estrogen receptors in disease pathogenesis and as a potential target for novel therapeutics.

Methods

Mice

The following study was conducted in accordance with guidelines set forth by the *Guide for the Care and Use of Laboratory Animals* and approved by the University of Missouri-Columbia Animal Care and Use Committee. Female four-week-old A/JCR mice were obtained from Frederick Cancer Research and Development Center, Frederick, MD.

Generation of estrogen receptor deficient A/J Mice

Estrogen receptor deficient mice B6;129-*Esr1*^{tmUNC} (ERα knockout), B6;129-*Esr2*^{tmUNC} (ERβ knockout) have previously been generated on the C57BL/6 background.[86, 87] This mouse strain is resistant to *H. hepaticus*-induced inflammation.[24] To generate A/J mice congenic for either of the specific knockouts we utilized a marker-assisted congenic breeding scheme. B6;129-*Esr1*^{tmUNC} and B6;129-*Esr2*^{tmUNC} were donated by Dr. Dennis Lubahn, University of Missouri. Using marker assisted selection, B6;129-*Esr1*^{tmUNC} and B6;129-*Esr2*^{tmUNC} mice were backcrossed onto the susceptible A/JCr mouse strain. Each backcross was genotyped using a genome wide panel of microsatellite markers polymorphic between A/J and C57BL/6 mouse strains. PCR assays for the neomycin cassette confirmed the presence of the estrogen receptor knockout. Offspring from each backcross generation that were PCR positive for the knockout with highest percentage of markers homozygous for the A/J genotype were selected as breeders for the next generation for a total of 7 backcross generations. Homozygous deficient mice for either ERα or ERβ are infertile, so a colony of heterozygous brother-sister matings (ERα$^{+/-}$ x ERα$^{+/-}$ or ERβ$^{+/-}$ x ERβ$^{+/-}$) were maintained to generate A.B6;129-*Esr1*^{tmUNC} (A/JCr ERα Knockout) and A.B6;129-*Esr2*^{tmUNC} (A/JCr ERβ Knockout) mice for study.

ER alpha and beta 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 DNA extraction and genotyping. Tail DNA was extracted using the DNeasy DNA extraction kit (Qiagen) as per

manufacture's protocol. Polymerase chain reaction was carried out in 10x reaction buffer, 1.25 mM dNTP's, Fast Start Taq 5 U/μL (Roche) and WT forward and reverse primers (25 μM) and the primer to detect the neomycin cassette in the knockout insert (25 μM). Thermocycler conditions were as follows 94^0 C for 30 seconds, 66^0 C for 30 seconds and 72^0 C for 30 seconds for 35 cycles in a thermocycler (ABI 2720). All primer sequences and annealing temperatures can be found in Table 1. Mice were genotyped at weaning and retested at the time of necropsy to confirm the initial PCR results

Bacterial Culture

Isolation and growth of *Helicobacter hepaticus* strain MU-94 has been previously described.[24, 62] A 1.5 mL glycerol stock solution with ~5 x 10^8 bacteria/mL of *H. hepaticus* was diluted in 15 mL of brucella broth (Becton Dickinson, Franklin Lakes, N.J.), divided equally between three sheep's blood agar plates and grown for 24 hours at 37^0 C in a microaerobic environment with 90% N_2 / 5% H_2 / 5% CO_2. After 24 hours the remaining broth containing the *Helicobacter* was transferred to a 250 mL Erlenmeyer flask along with 35 mL of fresh brucella broth supplemented with 10% of fetal bovine serum (Sigma-Aldrich Co., St. Louis, Mo.). The culture was grown for another 24 hours at 37^0 C in a microaerobic environment with 90% N_2 / 5% H_2 / 5% CO_2 with constant stirring. Mice were inoculated via oral gavage with 0.5 ml of culture containing 5 x 10^8 bacteria/mL. Colonization was confirmed 2-4 weeks post inoculation and at time of necropsy via a *Helicobacter hepaticus* specific PCR.[88]

Ovariectomy

Ovariectomies were performed by trained personnel under veterinary supervision. Mice were anesthetized with isoflurane throughout the duration of the procedure. A single incision was made through the skin longitudinally along the center of the back. The ovaries of the mice were removed leaving the uterus intact. Continuous release hormone pellets (described below) were then inserted subcutaneously between the shoulder blades through the initial incision site. The skin was closed with skin clamps. Mice were given buprenorpherine to diminish pain and allowed to recover on a heating pad. Seven to ten days after surgery skin clamps were removed. At sacrifice, the uteri of the mice were collected, weighed, and normalized to the body weight of the mouse. Any mice with detectable ovarian remnants were removed from the study. Increased uterine/body weight confirmed the presence of ovarian remnants, estrogen, or estrogen receptor alpha agonists in study mice.

Compounds

In our initial study we administered 90-day continuous releasing hormone pellets containing 1.5 mg/pellet of 17β-estradiol (E2) or a placebo (Innovative Research of America, Sarasota, FL). Further studies included 90-day continuous release pellets containing the estrogen receptor α agonist PPT (4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol), 2.5 mg/pellet and the estrogen receptor β agonist DPN (diarylpropionitrile 2,3-bis(4-Hydroxyphenyl)-propionitrile),10 mg/pellet both from Tocris Bioscience (Ellisville, MO).

Histological Evaluation

At the time of sacrifice, ceca were removed, laid out on note cards, and opened along the mesenteric border. The cecal contents were scraped from the cecum and saved for confirmation of *Helicobacter hepaticus* colonization. The cleaned cecum was then split longitudinally. One half was rinsed with sterile PBS then flash frozen in liquid nitrogen for use in RNA expression analyses. The other half was fixed in zinc-fixative and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin stain and scored for severity of cecal lesions. Cecal lesions were evaluated using a modified protocol as described previously.[62, 65, 89] Lesions were scored for intensity of inflammation (0 = none, 1 = mild, 2 = moderate, and 3 = severe), longitudinal extent (1 = one or two small foci, 2 = patchy, and 3 = diffuse), and vertical extent of inflammation (1 = basal mucosal inflammation, 2 = full-thickness mucosal inflammation, and 3 = transmural inflammation). In addition, lesions were scored for hyperplasia by using the following criteria: the presence of basophilic staining "crypt" epithelial cells in at least the lower two-thirds of the gland or at least a doubling of the height of the mucosal crypt epithelium. Focal hyperplasia was given a score of 1, and diffuse hyperplasia was given a score of 2. The scores for intensity of inflammation, longitudinal and vertical extents of inflammation, and hyperplasia were added together to give a total score for each animal. Because the minimum inflammation score using this system is 3 (mild, focal, and basal), inflammation and hyperplasia were not on comparable scales. To rectify this bias, lesion scores of >2 were adjusted by subtracting 2 from the total score to give a total adjusted score.

RNA Isolation and Reverse Transcription

Portions of the ceca frozen in liquid nitrogen were thawed in a solution of phenol and guanidine isothiocyanite (TRIzol reagent, Invitrogen, Carlsbad, CA). Cecal sections were homogenized using a Tissuelyser (Qiagen, Valencia, CA) for 2 minutes at 30 Hz. Total RNA was isolated as per manufacture's instructions. The extracted RNA was then dissolved in 75μL of DEPC treated water (Sigma-Aldrich, St. Louis, MO). Quantity and quality of RNA used for reverse transcription was assessed by measuring the absorbance at 260 nm and 280 nm (Nanodrop-1000 Spectrophotometer, Nanodrop, Wilmington, DE). Five micrograms of total RNA was reverse-transcribed using the Super Script III kit (Invitrogen, Carlsbad, CA) by following the oligo(dT) primer protocol. The resultant cDNA was diluted with DEPC treated water to a final expected concentration of 20 ng/μL.

Real Time PCR

Real time RT-PCR was performed on the Roche Light Cycler 2.0 using Qiagen Quanti-tect SYBR Green (Qiagen, Valencia, CA). Primers (Table 1) and run conditions for real time PCR have been previously described.[24]

Statistical Analysis

Significant differences ($p<0.05$) between lesion scores of placebo and estrogen treated animals as well as mRNA expression levels of placebo and estrogen treated mice were assessed using Student's T-test. Dot plots were generated using GraphPad Prism software (Version 5.0, GraphPad Prism Software Inc., La Jolla, CA). Lesion scores and

mRNA expression levels of estrogen receptor knockout mice and estrogen receptor agonist treated mice were evaluated for significance one-way ANOVA (Sigma Plot, Version 11, San Jose, CA) using Dunnett's *post-hoc* test to compare experimental groups to controls. Differences were considered statistically significant if $p<0.05$.

Table 1: Primers for PCR and Quantitative Real Time PCR

PCR Primers	Forward	Reverse	Product Size	Anneal Temp (^{0}C)
ER α WT	TCTACGGCCAGTCGGGCATC	TAGGCGACACGCTGTTGAGCTG	102	66
ER β WT	TGACAATGGCTGTGTTCTCTGCTC	TTAAAGTTAAACGCCAGCCCACCG	566	66
knockout neomyacin cassette	TGCCTGCTCTTTACTGAAGGCTCT		α - 363 β - 430	66
HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA	177	60
IFNγ	GCCATCAGCAACAACATAAGC	TGGGACAATCTCTTCCCCAC	238	58
CXCL9	ACTCGGCAAATGTGAAGAAG	CCCATTAAGATTCAGGGTGC	194	58
IL-12/23p40	ACTCACATCTGCTGCTCCAC	GGGAACTGCTACTGCTCTTGA	183	60

Results

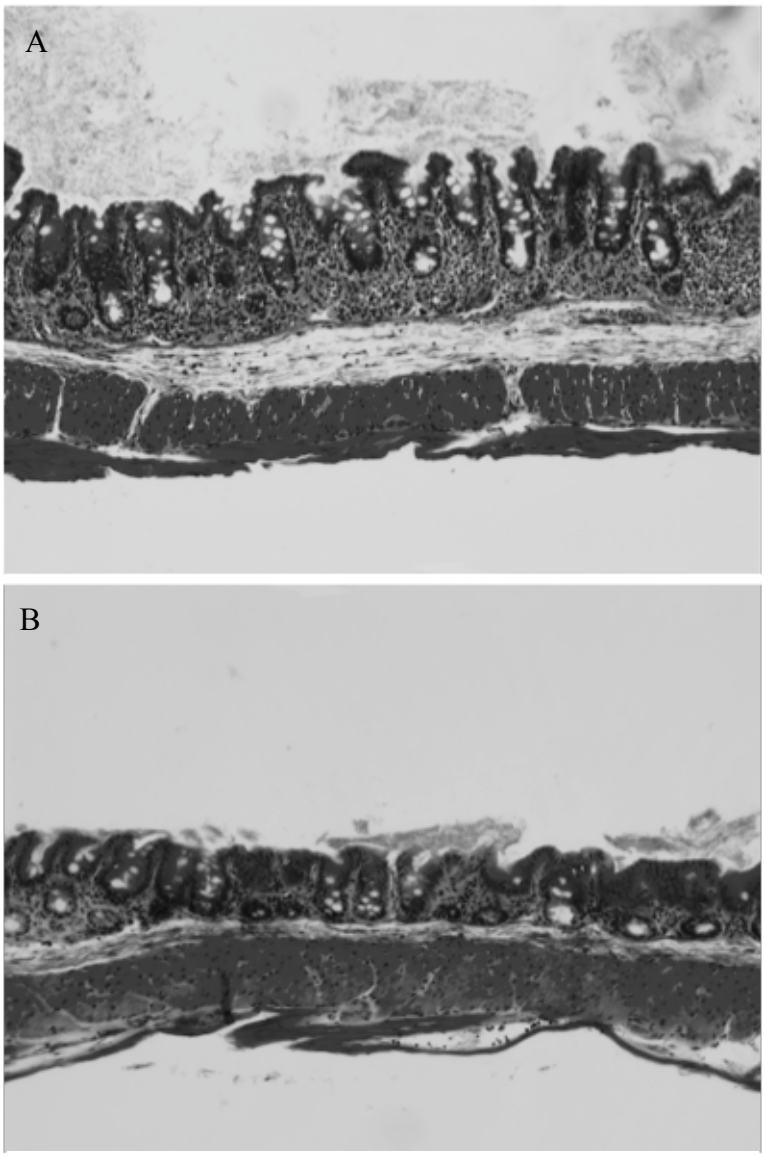
Estrogen Administration Throughout Infection Decreases Disease Severity

Previous studies in our lab have demonstrated a sex bias in *Helicobacter hepaticus*-induced typhlitis. Infected female mice develop more severe disease than do infected males.[62] Other autoimmune diseases including, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis, have similar sex biases. In animal models of multiple sclerosis and rheumatoid arthritis, administration of supraphysiologic doses of 17β-estradiol has been shown to be effective in decreasing disease.[90-92] Given these results and the effects of estrogen and estrogen receptor modulator administration in other animal models of IBD, we hypothesized that disease in our model was modulated through the action of gonadal sex hormones.

To test this, female mice were ovariectomized to remove endogenous estrogens and implanted either with a hormone pellet meant to continuously administer a supraphysiologic dose of 17β-estradiol for up to 90 days (E2 dose 1.5 mg/pellet) or a placebo pellet. Mice were then inoculated with *H. hepaticus*, and 90 days post inoculation, cecal inflammation was assessed histologically. Representative micrographs show an increase in cellular infiltration and thickening of the ceca in placebo treated animals, whereas E2 treatment resulted in little to no inflammatory infiltrates (Figure 2.1 A and B). When quantified, lesion scores in placebo treated animals were significantly higher than those of estrogen treated animals indicating that continuous administration of estrogen is effective in modulating disease (Figure 2.1 C). This is consistent with

Figure 2.1 Estrogen treatment in mice decreases disease severity –Representative photomicrographs from ovariectomized A/JCr mice, infected with *Helicobacter hepaticus*, and given a subcutaneous pellet containing (A) placebo or (B) 17β-estradiol (1.5 mg/pellet). In estradiol treated animals, decrease in disease is evident by decreased thickening of the ceca, decreased cellular infiltration, and less hyperplasia when compared to placebo treated animals. (C) Cecal lesion scores of placebo and estradiol treated mice. Each point represents the lesion score for an individual animal. Horizontal lines represent mean lesion score for each group. Mean lesion scores in E2 treated mice are significantly lower than in placebo treated animals. Significance between groups was determined by Student's T-test where significance was $p<0.05$.

Figure 2.1


A
B
C
8
6
4
2
0
p<0.05
Cecal Lesion Score
Placebo
17β-estradiol (E2)
Treatment

previous reports in other models of inflammatory bowel disease that estrogen administration is effective in decreasing disease severity.[79-81]

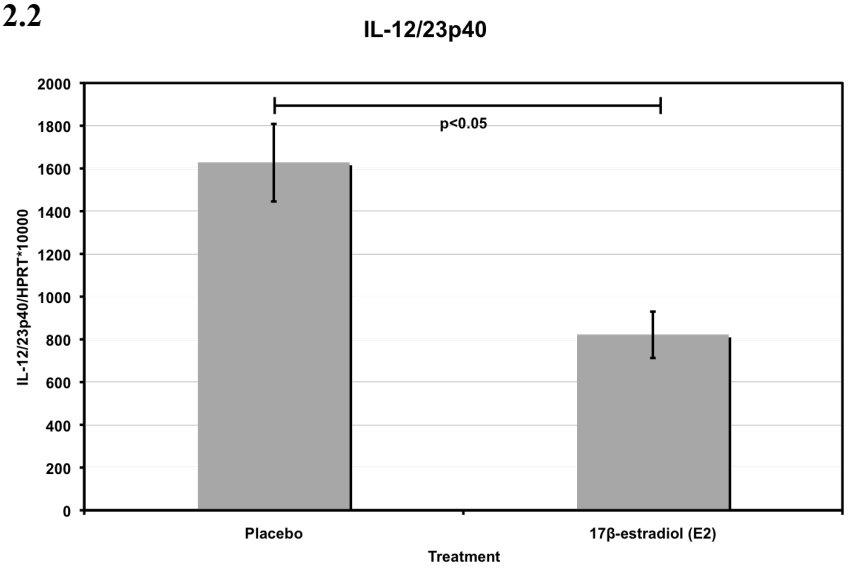
We have previously identified *H. hepaticus*-induced inflammation as a Th1 mediated disease. Upon infection with *H. hepaticus* A/JCr female mice develop cecal lesions and increase expression of inflammatory cytokines and chemokines including IFNγ, TNFα, IL-12/23p40, CXCL9, CXCL10, and CCL3.[24, 62] We have also shown that antibody blocking of either IL-12/23p40 or IFNγ is effective in ameliorating disease. IL-12 is the canonical Th1 driving cytokine and cecal expression of the p40 subunit is significantly increased in the *Helicobacter hepaticus*-induced model of inflammatory bowel disease. In the presence of IL-12, T helper cells will express IFNγ, which in turn acts on intestinal epithelial cells to upregulate the chemoattractant molecule CXCL9 (monokine induced by gamma interferon, MIG). We therefore chose to evaluate the effect of estrogen administration on cecal gene expression of IL-12/23p40, IFNγ, and CXCL9 as a means of investigating the role of estrogen in both innate and adaptive immune signaling. Administration of E2 in *Helicobacter hepaticus* infected mice significantly reduced the cecal expression levels of IL-12/23p40 compared to placebo treated animals (Figure 2.2 A). Cecal expression of IFNγ and CXCL9 (MIG) was also significantly reduced in mice that received estrogen treatment (Figure 2.2 B and C). Together, the decrease in inflammation and inflammatory gene expression indicate that estrogen is effective at modulating disease and that E2 treatment decreases expression of signals necessary for Th1 type inflammation.

This may be an effect of the E2 acting directly on the inflammatory population or it may be a result of more systemic effects of the estrogen treatment over such a long

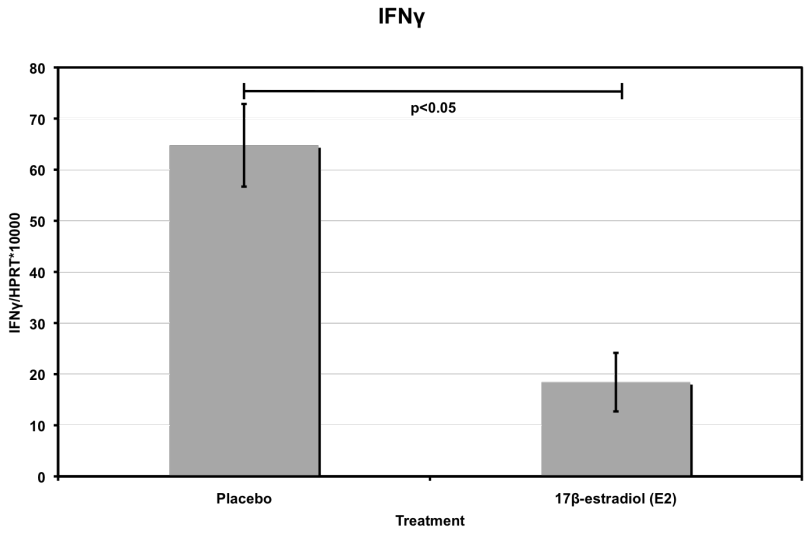
Figure 2.2 Estrogen treatment decreases inflammatory cytokine expression - Measurements of cecal cytokine and chemokine mRNA expression levels in mice receiving placebo or E2 treatment. Levels of (A) IL-12/23p40, (B) IFNγ, and (C) CXCL9 were measured to identify the effects of estrogen on both the innate and the adaptive immune response to *Helicobacter hepaticus* infection. Data are reported as mean number of molecules normalized to levels of the housekeeping gene HPRT. Data are expressed as mean ± SEM. Significance between treatment groups was determined by Students T-test at $p<0.05$

Figure 2.2

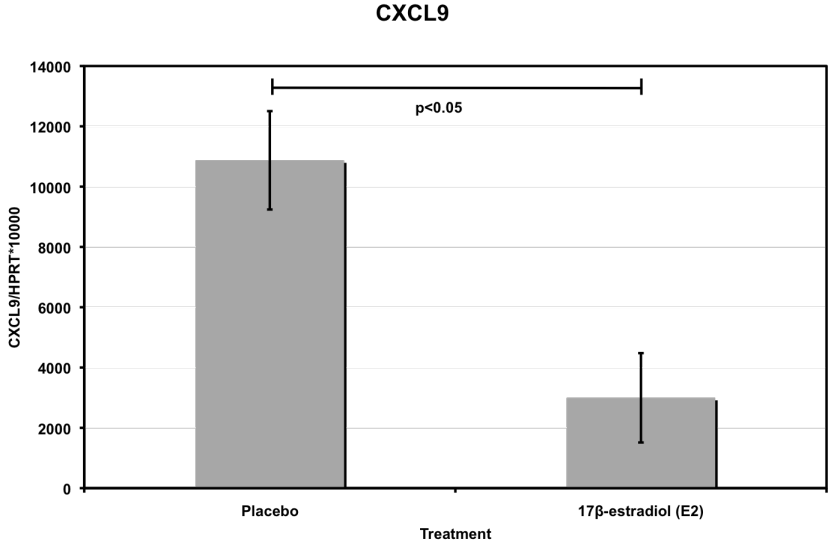
A



B



C



period of time. Estrogen treated mice had significantly lower weight gain throughout the study compared to placebo treated animals. Estrogen treated animals were also highly anemic due to osteopetrosis-mediated bone marrow depletion, which would also cause fewer circulating immune cells and immune suppression. Estrogen administration also caused severe cervical swelling and hardening, resulting in urinary difficulties in estrogen treated mice. Many estrogen treated mice were unable to urinate effectively and developed distended bladders. As a result of the estrogen effects on the urinary tract, many mice also developed perianal inflammation and swelling due to a constant urinary leakage (data not shown). Estrogen treatment alone is therefore not a viable therapeutic candidate. To assess if specific estrogen receptor signaling could have an effect on intestinal inflammation, we assessed the role of specific estrogen receptors in disease development.

Estrogen Receptor Alpha Deficiency Decreases Disease Severity

The observation that estrogen administration resulted in a decrease in disease severity led us to next ask which estrogen receptor was responsible for modulating disease. The two best characterized receptors are estrogen receptor alpha and beta (ERα; ERβ). Both estrogen receptors are expressed in the intestines of mice, but on different intestinal cell types, and signaling through the different receptors has been shown to elicit different immune responses.[44, 45, 48, 49, 51, 52, 93]

Mice deficient in the genes encoding ERα and ERβ (*Esr1* and *Esr2* respectively) were originally generated on the C57BL/6 background mouse strain.[86, 87] However, C57BL/6 mice are resistant to *H. hepaticus*-induced inflammation, and do not exhibit

perturbations in the expression of inflammatory cytokines.[24] We therefore generated A/J mice congenic for the ERα and ERβ knockouts. Both ERα and ERβ deficient mice were inoculated at 3-4 weeks of age along with heterozygous and wild type littermate controls. Ninety days after inoculation, mice were necropsied and their ceca were assessed for inflammation. Lesion scores of wild type and mice heterozygous for either ERα or ERβ did not differ statistically, and those groups were therefore combined and used as controls. Mean lesion scores of ERβ deficient mice were not significantly different from those of the controls, however ERα deficiency resulted in a significant decrease in disease (Figure 2.3 A). Upon further examination it was apparent that a large subset of control mice had very high lesion scores. Comparison of the incidence of severe disease (lesion score $\geq$6) revealed a dramatic decrease in severe disease in ERα deficient mice whereas a large percentage of control and ERβ deficient mice developed severe disease (Figure 2.3 B). While lesion scores were significantly decreased in ERα deficient mice, the cecal expression of IL-12/23p40 between ERα, ERβ, and controls was not statistically different unlike IL-12/23p40 expression in E2 treated animals (Figure 2.4 A). Expression of IFNγ and CXCL9 however, was significantly decreased in ERα deficient mice when compared to littermate controls (Figure 2.4 B and C). In ERβ deficient mice, there was no significant difference in IL-12/23p40, IFNγ or CXCL9 expression between knockout and control animals.

Figure 2.3 Deficiency in Estrogen receptor α decreases disease severity - A/J females deficient in either ERα, ERβ, or littermate controls were inoculated with *H. hepaticus* and assessed for cecal inflammation 3 months post-inoculation. (A) Each point represents the lesion score for an individual animal of the indicated genotype and horizontal bars are the mean for each group. Mice deficient in ERα, but not ERβ had significantly less intestinal inflammation when compared to littermate controls. (B) Severe intestinal inflammation in *H. hepaticus*-induced model of IBD is defined as an animal with a cecal lesion score of 6 or above. Estrogen receptor β deficient mice had a similar incidence of animals with severe intestinal inflammation as WT/Het controls whereas half as many ERα deficient mice had severe disease. Significance was determined with a one-way ANOVA comparing lesion scores from the estrogen receptor knockout groups to the WT/Het controls.

Figure 2.3

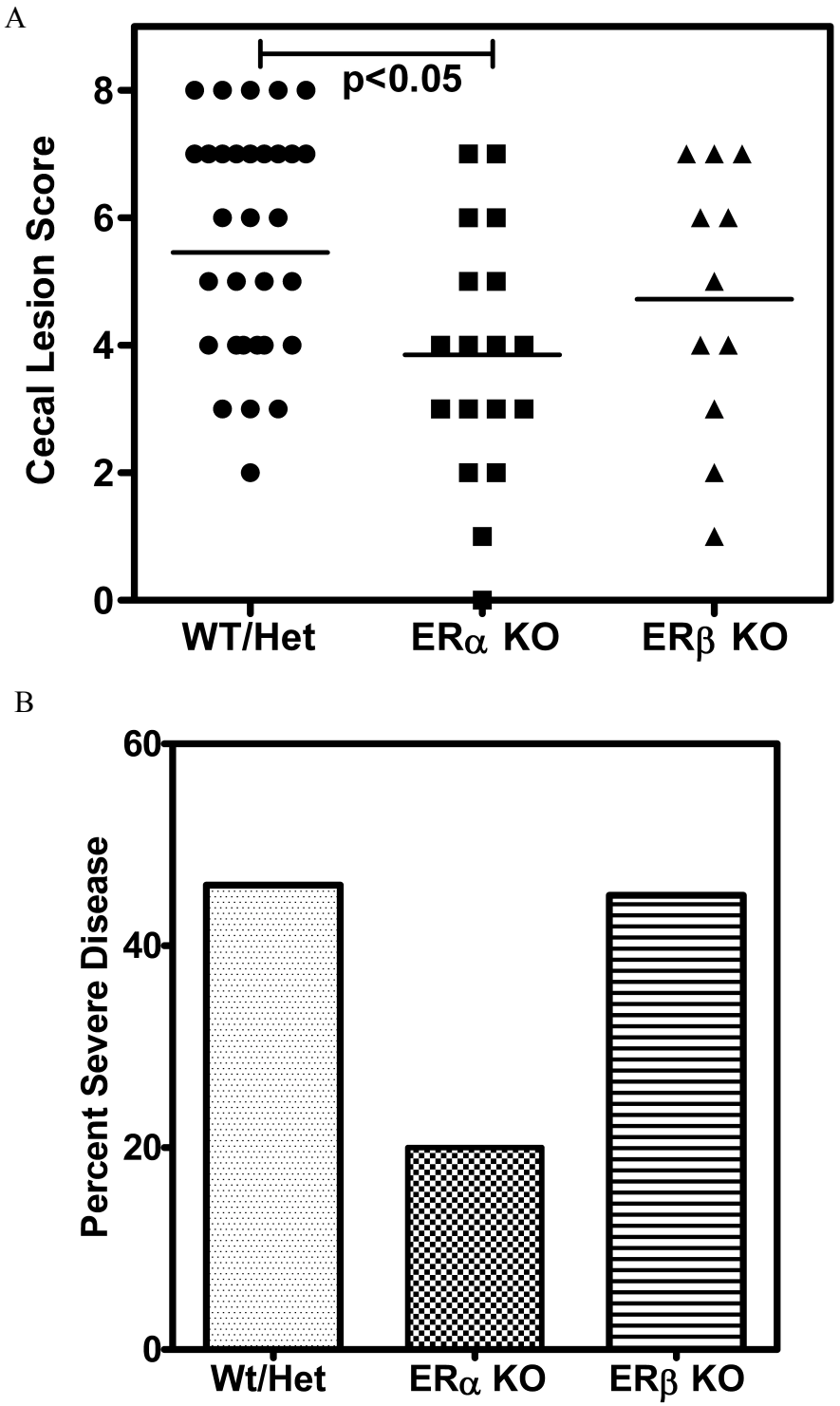
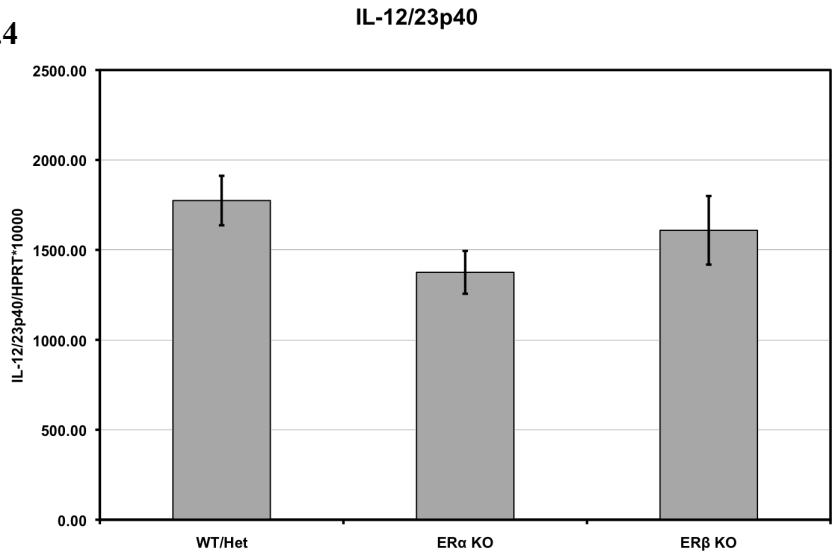

A
p<0.05
Cecal Lesion Score
0
2
4
6
8
WT/Het
ERα KO
ERβ KO
B
Percent Severe Disease
0
20
40
60
Wt/Het
ERα KO
ERβ KO

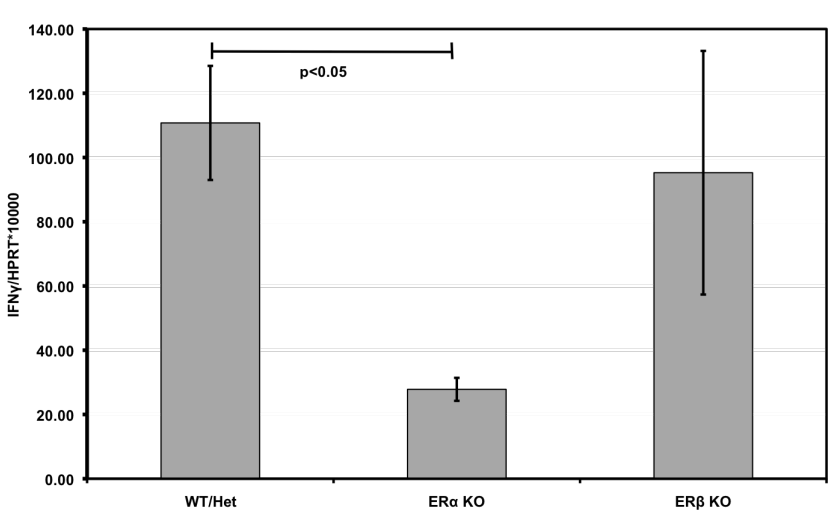
Figure 2.4 Deficiency in Estrogen receptor α expression of adaptive immune cytokines and chemokines – Measurement of cecal cytokine and chemokine mRNA expression in mice deficient for ERα, ERβ, or littermate controls using quantitative real time PCR. (A) IL-12/23p40, (B) IFNγ, and (C) CXCL9 expression levels were normalized to HPRT. Data are represented as mean ± SEM. Expression levels of IL-12/23p40 (A) were not affected by deficiency in either ERα or ERβ. Expression of IFNγ (B), and CXCL9 (C) were significantly reduced in ERα deficient mice. Significance was determined with a one-way ANOVA using Dunnett's *post-hoc* test to compare ER knockout groups to the WT/Het controls.

Figure 2.4

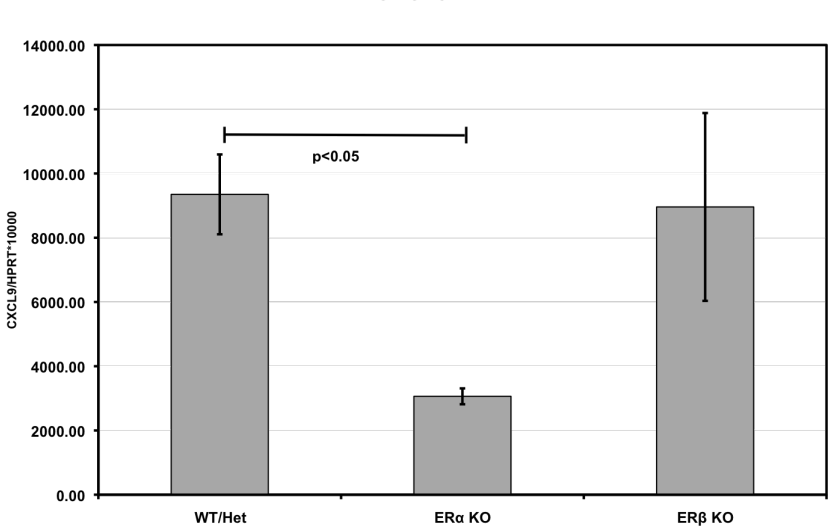
A



B



C



Collectively these results suggest one of two possibilities. Either estrogen receptor alpha is necessary for the promotion of intestinal inflammation, and removal of estrogen receptor alpha prevents the expression of IFNγ and CXCL9, preventing the development of inflammation, or that estrogen receptor beta is necessary to control inflammation, and by removing estrogen receptor alpha, all endogenous estrogen signals through estrogen receptor beta decreasing the disease severity. The lack of an effect on IL-12/23p40 expression suggests that whichever estrogen receptor is responsible for the decrease in disease, the effects may only be seen through the adaptive immune response, and may not affect the innate immune response.

Estrogen Receptor Beta Signaling Decreases Intestinal Inflammation

Given the differential expression patterns observed between estrogen treatment and estrogen receptor deficiency, we hypothesized that estrogen receptors alpha and beta could be playing opposing roles in regulating the immune response. We have shown that ERα deficiency decreases disease (Figure 2.3), but it appears only to affect the adaptive immune response, while estrogen administration affected both adaptive and innate components. A previous report has shown that administration of agonists specific for estrogen receptor beta is effective at decreasing intestinal inflammation in the HLA-B27 transgenic rat model of IBD.[83] Therefore, we assessed specific estrogen receptor modulators in controlling *Helicobacter hepaticus*-induced inflammation.

To address a possible role for estrogen receptor β in our model we treated ovariectomized female mice with continuous releasing pellets containing agonists that

signal specifically through either ERα or ERβ (PPT or DPN, respectively) and inoculated with *H. hepaticus*. After 90 days the animals were sacrificed and the ceca were assessed for inflammation and the expression of inflammatory molecules IL-12/23p40, IFNγ, and CXCL9. Evaluation of intestinal inflammation revealed that treatment with estrogen receptor alpha agonist PPT had no effect on disease severity as lesions scores were not significantly different from controls. Treatment with the estrogen receptor beta agonist DPN was effective in significantly decreasing intestinal inflammation (Figure 2.5 A). When the incidence of severe disease (mice with a lesion score >6) was assessed, DPN treated animals had less than half as many animals with severe disease when compared to PPT or placebo treated mice (Figure 2.5 B). These results indicate that selectively signaling through estrogen receptor beta is effective in preventing the development of intestinal inflammation in *H. hepaticus* infected mice.

Estrogen Receptor Beta Signaling Decrease Inflammatory Cytokine Expression

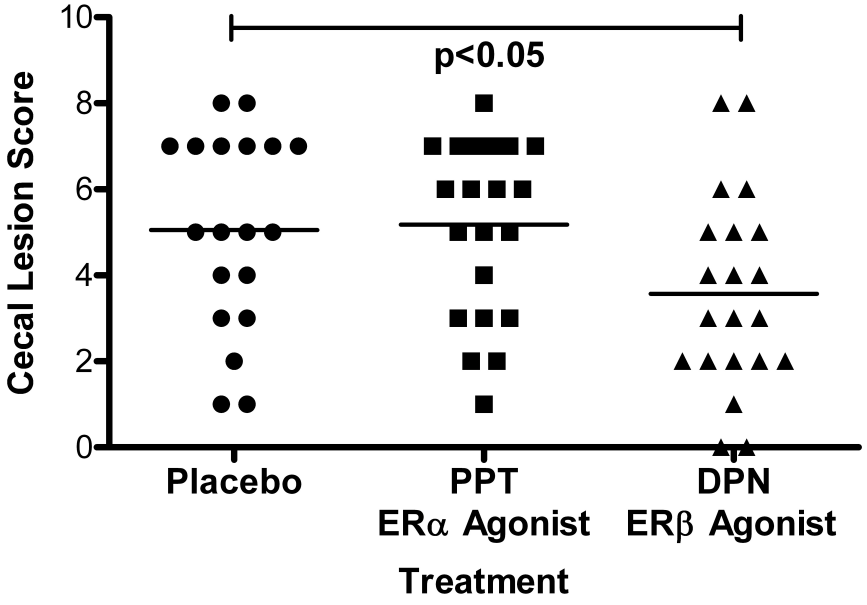
Treatment with the ERβ agonist DPN decreased intestinal inflammation in infected animals. Administration of the ERα agonist PPT did not significantly affect the development of intestinal inflammation, nor did it affect the expression of IL-12/23p40, IFNγ, or CXCL9 (Figure 2.6 A-C). However, as with E2 administration, expression of IL-12/23p40 was significantly decreased in mice that received ERβ agonist treatment (Figure 2.6A). The expression of cecal IFNγ and CXCL9 was also significantly decreased in DPN treated animals when compared to placebo treatment (Figure 2.6 B and

C). These results indicate that signaling through estrogen receptor beta acts not only to reduce expression of the adaptive immune signals (IFNγ and CXCL9) but also reduces the expression of IL-12/23p40.

Figure 2.5 Estrogen receptor β agonist treatment decreases disease severity – To test the effect of specific estrogen receptor signaling in intestinal inflammation, A/J mice were ovariectomized and implanted with subcutaneous, continuous releasing pellets containing either estrogen receptor alpha agonist (PPT), estrogen receptor beta agonist (DPN) or a placebo. (A) Cecal lesion scores in mice that received specific estrogen receptor agonists. Points represent lesion scores of individual animals and horizontal bars indicate mean lesion scores. Administration of the ERβ agonist DPN significantly reduced the mean cecal lesion scores when compared to either placebo controls or ERα agonist treated animals $p<0.05$ (B) Incidence of severe disease in animals that received continuous doses of ERβ agonist was lower than the incidence in either the placebo or ERα agonist treated animals. Significance was determined by two-way ANOVA taking into account treatment and a replicate experiment.

Figure 2.5

A


10
8
6
4
2
0
Cecal Lesion Score
p<0.05
Placebo
PPT
ERα Agonist
DPN
ERβ Agonist
Treatment

B

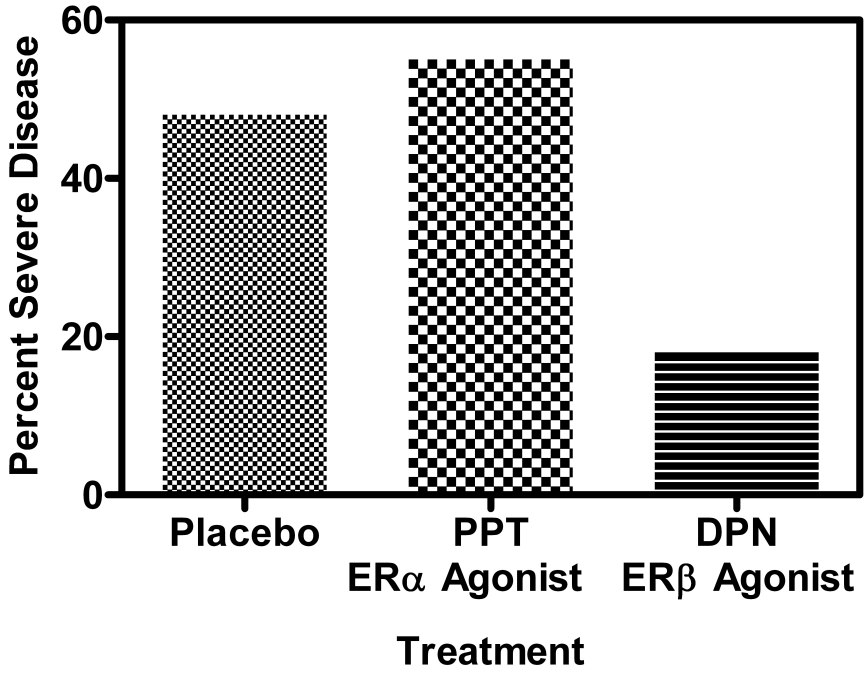
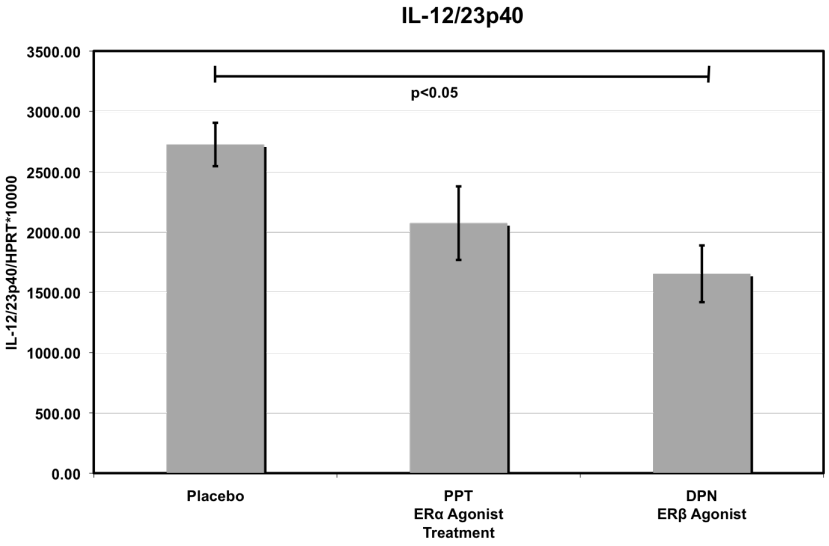

60
40
20
0
Percent Severe Disease
Placebo
PPT
ERα Agonist
DPN
ERβ Agonist
Treatment

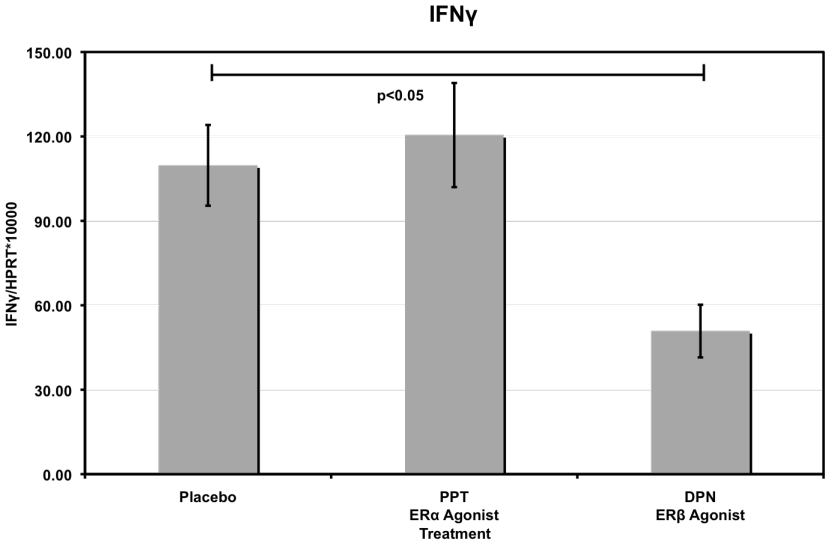
Figure 2.6 Estrogen receptor β agonist treatment decreases expression of innate and adaptive cytokines and chemokines – Cecal cytokine and chemokine mRNA expression in *Helicobacter hepaticus* infected mice that received subcutaneous, continuous releasing pellets containing either estrogen receptor alpha agonist (PPT), estrogen receptor beta agonist (DPN) or placebo. (A) IL-12/23p40, (B) IFNγ, (C) CXCL9 expression was normalized to the expression of HPRT. Data are represented as mean and SEM. Expression of (A) IL-12/23p40 (B) IFNγ and (C) CXCL9 were significantly lower in mice that received ERβ agonist treatment when compared to placebo treated control animals. Significance was determined with a one way ANOVA using Dunnett's *post-hoc* test to compare expression levels from either of the ER agonist treated groups to the placebo treated controls.

Figure 2.6

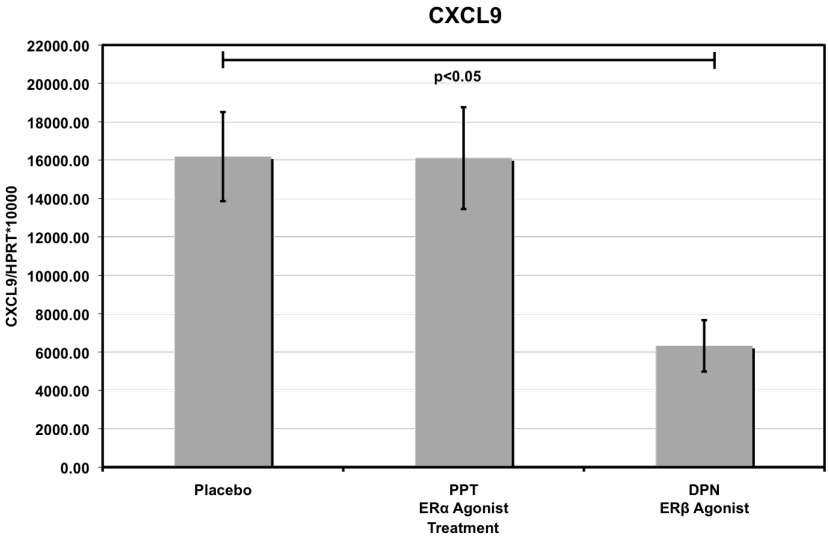
A



B



C



Discussion

The role of sex hormones in regulation of autoimmune diseases is complex and multifaceted.[94] Many diseases show a sex bias with a higher incidence of females developing disease than males. [72, 73] In many cases, paradoxically, an increase in female hormone levels has been shown to decrease disease severity. Pregnancy has been shown to ameliorate multiple sclerosis and rheumatoid arthritis.[73, 95] Animal models of MS (experimental autoimmune encephalomyelitis, EAE) and RA (collagen-induced arthritis) have shown that administration of estrogen is beneficial in treating disease.[90, 92, 96]

Unlike other autoimmune diseases, Inflammatory Bowel Diseases, Crohn's Disease and ulcerative colitis have relatively equal occurrences of disease between males and females.[72] Other intestinal disorders such as microscopic colitis, lymphocytic colitis, and irritable bowel syndrome have shown elevated rates of disease in females when compared to males.[76-78] While Crohn's Disease and ulcerative colitis do not have a sex bias, there are several reports that female hormones can modulate disease severity in IBD.[31, 34, 38] The affect of female hormones is also seen in animal models of disease as well.[79] We have previously noted a sex bias in disease development in a *Helicobacter hepaticus*-induced mouse model of intestinal inflammation. Female mice develop more severe intestinal inflammation characterized by an increase in histologic signs of inflammation and an increase in inflammatory Th1 cytokines and chemokines.[62] We therefore sought to investigate the roles of sex hormones in modulating the immune response in this model.

In animal models of autoimmune diseases, EAE, collagen-induced arthritis, and in DNB-induced colitis, high doses of estrogen were effective in decreasing disease

severity. In *H. hepaticus*-induced inflammation this was also the case. Administration of supraphysiologic doses of estrogen throughout the course of *H. hepaticus* infection significantly decreased disease severity.

We have previously characterized the immune response of A/J mice to *Helicobacter hepaticus* infection as a Th1 type immune response. This is characterized by an increase in cecal mRNA expression in IL-12/23p40, IFNγ, TNFα, IP-10, and CXCL9.[24, 62] We assessed IL-12/23p40 expression as a marker for the innate immune response, and IFNγ and CXCL9 as markers for the downstream adaptive immune response to elucidate the role of estrogen and estrogen receptor signaling. Administration of estrogen in *H. hepaticus* infected mice significantly decreased the expression of IL-12/23p40, IFNγ, and CXCL9, indicating that estrogen administration is down-regulating the Th1 immune response.

There are two estrogen receptors, ERα and ERβ. These molecules are estrogen activated transcription factors that modulate gene expression by directly interacting with estrogen response elements or interacting with other transcription factors.[97] Each has been shown to elicit different effects on various models of autoimmune diseases.[83, 98] ER alpha is known to be expressed on CD4+ T cells and ER beta is expressed on intestinal epithelial cells.[75, 99, 100] Both the location of expression and the downstream targets of estrogen receptor signaling could be responsible for the modulation of disease. We therefore sought to dissect out the roles of each estrogen receptor in *H. hepaticus*-induced intestinal inflammation.

Utilizing mice deficient for either ERα or ERβ, we discovered that deficiency in ER alpha resulted in significantly lower disease severity and an >50% reduction in the

number of animals with severe intestinal inflammation. Decrease in disease appeared to be mediated through the adaptive immune response as expression levels of IL-12/23p40 were not significantly different between groups, but the molecules downstream of IL-12 signaling in the adaptive response, IFNγ and CXCL9 were significantly decreased. As ERα is expressed in CD4+ T cells, it is reasonable to conclude that signaling through ER alpha may be immuno-stimulatory, and that removing that signal results in a decrease in the CD4+ T cell response to *H. hepaticus*.

Other studies have pointed to a role for ER beta in modulating intestinal inflammation.[83] This may also be the case in our model of intestinal inflammation. While removing ERα decreased disease severity and expression of IFNγ and CXCL9, it did not decrease expression of IL-12/23p40. This difference could point to a role of ERβ in modulating disease. As in other models of intestinal inflammation, administration of ER beta agonists DPN to *H. hepaticus* infected mice significantly decreased histologic lesion scores. ERβ is expressed primarily on intestinal epithelial cells, indicating its signaling would affect the innate immune response. Signaling through ER beta has been shown to increase the development of tight junctions in the intestinal epithelium and decrease intestinal permeability. An increase in tight junction formation and decrease in permeability may allow less sampling of the intestinal luminal bacteria. With less bacterial antigens, this could explain why expression of IL-12p40 was significantly decreased in ERβ treated mice. Lower IL-12/23p40 expression may also result in less antigen presentation to effector T cells in the lamina propria decreasing the expression of IFNγ.

Taken together, these data indicate that during intestinal inflammation, signaling through ER alpha is immunostimulatory, most likely acting through the adaptive immune response, while signaling through ER beta down-modulates the epithelial or innate immune response, decreasing the severity of intestinal inflammation. Further studies will be necessary to identify the exact cell types and interactions that are responsible for specific estrogen receptor signaling. The current work offers intriguing targets for the development of future estrogen receptor based therapies for the intestinal inflammation associated with Inflammatory Bowel Disease.

Chapter 3

Identification of Quantitative Trait Loci on Mouse Chromosome 3 and 17 Associated with *Helicobacter hepaticus*-Induced Intestinal Inflammation

Background and Aims

The Inflammatory Bowel Diseases (IBDs) are complex disorders with multiple factors contributing to disease pathogenesis. It is widely accepted that IBD results from a dysregulated intestinal mucosal immune response in genetically susceptible individuals and that inflammation is induced or exacerbated by various environmental factors including, but not limited to, smoking status, diet, and intestinal microbial flora.[3] In recent years, much attention has been devoted to understanding the roles that various genes play in the pathogenesis of the two most common forms of IBD, Crohn's Disease and ulcerative colitis. Through multiple genome-wide association studies in humans, at least 70 loci have been associated with susceptibility to Crohn's Disease, and 30 loci have been associated with ulcerative colitis.[5, 10, 13, 18, 101] Roughly half of the loci associated with ulcerative colitis are also associated with susceptibility to Crohn's Disease, suggesting that some genes play and over-arching role in intestinal inflammation, while others are more specific to the pathogenesis of either Crohn's Disease or ulcerative colitis.[10] The identification of so many putative IBD genes with relatively small affects suggests that interactions between multiple genes may be of critical importance to IBD pathogenesis.[101] Investigations using mouse models of IBD have also helped identify loci and candidate genes associated with intestinal

inflammation.[102, 103] Importantly, because mice of varying genetic makeup can be produced in short time spans, these models are amenable to studies not possible with human populations such as studies of complex interactions between genes.

The composition of the intestinal microbial flora is another key factor involved in the development of IBDs. Multiple bacterial species have been implicated in disease pathogenesis, but there has yet to be a single pathogenic agent identified. Several mouse models have shown a requirement for the intestinal flora in development of disease. To this end, when mice are rendered germ-free, these models do not develop intestinal inflammation, but when subsequently colonized under "specific pathogen free" conditions, or with specific bacterial species such as *Helicobacter hepaticus,* they develop inflammation.[104, 105] Given the likely complex interaction between genetic susceptibility and intestinal flora, we sought to investigate a gene or genes involved in susceptibility in a bacterially induced mouse model of inflammatory bowel disease.

We and others have observed a distinct difference between the response of A/J and C57BL/6 mouse strains to infection with the bacterium *Helicobacter hepaticus*.[24, 85, 106, 107] A/J mice develop chronic cecal inflammation and increased cecal expression of Th1 type inflammatory cytokines and chemokines, whereas C57BL/6 mice do not develop inflammation nor do they upregulate cytokine and chemokine expression.[24, 65] Like human IBD, cytokine genes dysregulated in this model include IFNγ, TNFα and IL-12/23p40, which is shared by IL-12 and IL-23. Importantly, blockage of IFNγ or IL-12/23p40 abrogates disease in susceptibility individuals indicating a definitive role in disease pathogenesis.[108] Moreover, IL-12/23p40 expression is elevated in susceptible

strains as early as 4 days post-inoculation, making it an ideal biomarker for disease susceptibility.

We sought to use quantitative trait locus (QTL) analysis to identify genes that contribute to differential disease susceptibility between the A/J and C57BL/6 mouse strains. This analysis identified two loci on chromosomes 3 and 17 associated with an increase in expression of IL-12/23p40 in F_2 mice homozygous for A/J alleles at these loci. We further confirm, through the use of the C57BL/6-Chr3$^{A/J}$ chromosome substitution mouse strain, that the presence of a chromosome 3 from the susceptible A/J mouse on an otherwise resistant C57BL/6 mouse strain results in increased IL-12/23p40 expression, but that this alone is insufficient to result in intestinal inflammation upon infection with *Helicobacter hepaticus*, suggesting that other loci are also necessary to reach the threshold for inflammation. Thus, this model will be ideal for future studies investigating the genetic interactions necessary for disease susceptibility.

Materials and Methods

Mice

This study was conducted in accordance with guidelines set forth by the *Guide for the Care and Use of Laboratory Animals* and approved by the University of Missouri-Columbia Animal Care and Use Committee. C57BL/6Cr and A/JCr mice were obtained from Frederick Cancer Research and Development Center, Frederick, MD. Reciprocal F_1 crosses were generated by mating female A/JCr mice to male C57BL/6 mice (AB6 F_1) and C57BL/6 females to A/J males (B6A F_1). Reciprocal F_2 mice were generated by

brother × sister matings of F_1 animals (AB6 × AB6 F_2 or B6A × B6A F_2) or by crossing AB6 females with B6A males and vice versa (AB6 × B6A and B6A × AB6). Breeding trios of C57BL/6J-Chr3$^{A/J}$/NaJ and C57BL/6J-Chr17$^{A/J}$/NaJ (JAX stock number 004381 and 004395, respectively) were obtained from the Jackson Laboratory, Bar Harbor, ME and breeding colonies were established.

Bacterial Culture and Mouse Inoculation

Isolation and growth of *Helicobacter hepaticus* strain MU-94 has previously been described.[24, 62] 1.5 mL of a stock solution containing approximately 5 x 10^8 bacteria/mL of *H. hepaticus* was diluted in 15 mL of brucella broth (Becton Dickinson, Franklin Lakes, N.J.), divided equally between three sheep's blood agar plates and incubated for 24 hours at 37^0 C in a microaerobic environment with 90% N_2 / 5% H_2 / 5% CO_2. After 24 hours the remaining broth containing bacteria was transferred to a 250 mL Erlenmeyer flask along with 35 mL of fresh brucella broth supplemented with 10% of fetal bovine serum (Sigma-Aldrich Co., St. Louis, Mo.). The culture was incubated for another 24 hours at 37^0 C under the same microaerobic conditions with constant stirring. Three to four week old mice of both sexes were inoculated via gastric gavage with 0.5 ml of culture containing 5 x 10^8 bacteria/mL. Colonization was confirmed 2-4 weeks post-inoculation and at time of necropsy via a *Helicobacter hepaticus* specific PCR.[88]

Necropsy and Sample Collection

Depending on the study, mice were euthanized for sample collection at either 4 days or 4 months post-inoculation. At the time of sacrifice, ceca were removed, laid on

note cards, and opened longitudinally along the mesenteric border. The cecal contents were removed and used to confirm *Helicobacter hepaticus* colonization by PCR.[88] The cleaned cecum was then split longitudinally. One half was rinsed with sterile PBS then flash frozen in liquid nitrogen for use in cytokine gene expression analysis. The other half was fixed in zinc-fixative and embedded in paraffin. Five micron sections were prepared and stained with hematoxylin and eosin stain for histologic examination and lesion scoring.

IL-12/23p40 Cecal Expression

Frozen ceca from all mice were thawed in a solution of phenol and guanidine isothiocyanite (TRIzol reagent, Invitrogen, Carlsbad, CA). Cecal sections were homogenized using a Tissuelyser (Qiagen, Valencia, CA) for 2 minutes at 30 Hz. Total RNA was isolated as per manufacturer's instructions. The extracted RNA was then dissolved in 75 μL of DEPC treated water (Sigma-Aldrich, St. Louis, MO). Quantity and quality of RNA were assessed by measuring the absorbance at 260 nm and 280 nm (Nanodrop-1000 Spectrophotometer, Nanodrop, Wilmington, DE).

Five micrograms of total RNA was reverse-transcribed using the Super Script III kit (Invitrogen, Carlsbad, CA) by following the oligo(dT) primer protocol. The resultant cDNA was diluted with DEPC treated water to a final expected concentration of 20 ng/μL.

Real time RT-PCR was performed on the Roche Light Cycler 2.0 using Qiagen Quanti-tect SYBR Green (Qiagen, Valencia, CA). All expression was normalized to cecal levels of HPRT. Run conditions for real time PCR have been previously described and

primers used included 1) HPRT Forward – 5 GTAATGATCAGTCAACGGGGGAC 3 2) HPRT Reverse – 5 CCAGCAAGCTTGCAACCTTAACCA 3 3) IL-12/23p40 Forward – 5 ACTCACATCTGCTGCTCCAC 3 4) IL-12/23p40 Reverse – 5 GGGAACTGCTACTGCTCTTGA 3.[24]

Genotyping

Tail snips were collected upon the necropsy of F_2 animals. Tail snips were digested in Proteinase K and DNA was isolated with the Qiagen DNeasy spin columns following the manufacturer's protocol (Qiagen, Valencia, CA).

A total of 86 fluorescently labeled microsatellite primers with an average spacing of 16 cM were obtained from Applied Biosciences (ABI, Carlsbad, CA). Microsatellites were amplified via PCR following the manufacture's protocol and analyzed using an ABI 3100 Genetic Analyzer and Gene Mapper software version 4.0 (Applied Biosciences, Carlsbad, CA). Additional SNP genotyping was performed using the Illumina LD Linkage Panel consisting of 377 SNP markers (Illumina, San Diego, CA) following manufacturer's protocol. Samples were genotyped on the Illumina BeadArray Reader. Analysis was carried out using the Illumina BeadStudio Software. Genotyping calls were based on A/J, C57BL/6, and F_1 control samples. A total of 244 SNPs informative between A/J and C57BL/6 were used for subsequent analysis.

Lesion Scoring

Cecal lesions in mice sacrificed at 3 months post-inoculation were evaluated using a modified protocol as previously described.[62, 65, 89] Lesions were scored for

intensity of inflammation (0 = none, 1 = mild, 2 = moderate, and 3 = severe), longitudinal extent (1 = one or two small foci, 2 = patchy, and 3 = diffuse), and vertical extent of inflammation (1 = basal mucosal inflammation, 2 = full-thickness mucosal inflammation, and 3 = transmural inflammation). In addition, lesions were scored for hyperplasia by using the following criteria: the presence of basophilic staining "crypt" epithelial cells in at least the lower two-thirds of the gland or at least doubling of the height of the mucosal epithelium. Focal hyperplasia was given a score of 1, and diffuse hyperplasia was given a score of 2. The scores for intensity of inflammation, longitudinal and vertical extents of inflammation, and hyperplasia were added to give a total score for each animal. Because the minimum inflammation score using this system is 3 (mild, focal, and basal), inflammation and hyperplasia were not on comparable scales. To rectify this bias, lesion scores of >2 were adjusted by subtracting 2 from the total score to obtain a total adjusted score.

Data Analysis

IL-12/23p40 expression and cecal lesion scores were compared via one-way ANOVA and differences between groups were considered significant if $p<0.05$ (SigmaPlot version 11, San Jose, CA). QTL analysis of Log_2 transformed IL-12/23p40 levels from F_2 mice was carried out utilizing R version 2.9, R/QTL, and J/QTL software package version 1.3.0 (Jackson Labs, Bar Harbor, ME).[109, 110] In J/QTL, a parametric one QTL scan (Multiple Imputation model) was used to calculate the logarithm of odds (LOD) score, and 10,000 phenotype permutations were run to determine the significance threshold. LOD scores were considered significant if genome-wide $p<0.05$.

Results

Susceptibility to *Helicobacter hepaticus* in F_1 crosses

Our lab and others have previously reported a distinct, strain-dependent response to *Helicobacter hepaticus*.[24, 85, 106, 107] *Helicobacter hepaticus* infected A/J mice develop increased inflammatory cytokine expression (IL-12/23p40 and IFNγ) within 4 days of inoculation and histologic signs of disease accompanied by increased inflammatory cytokine expression seen by 90 days post-inoculation. C57BL/6 mice are resistant to *H. hepaticus* and do not develop inflammation or increased cytokine expression despite colonization levels equivalent to A/J mice.[24] To determine the mode of inheritance for susceptibility to *Helicobacter hepaticus*-induced inflammation, reciprocal male and female F_1 mice (AB6 and B6A F_1) were inoculated with *H. hepaticus*. Cecal expression of IL-12/23p40 mRNA was measured in animals at 4 days and 90 days post-inoculation. Cecal inflammation was also assessed in F_1 mice 90 days post-inoculation.

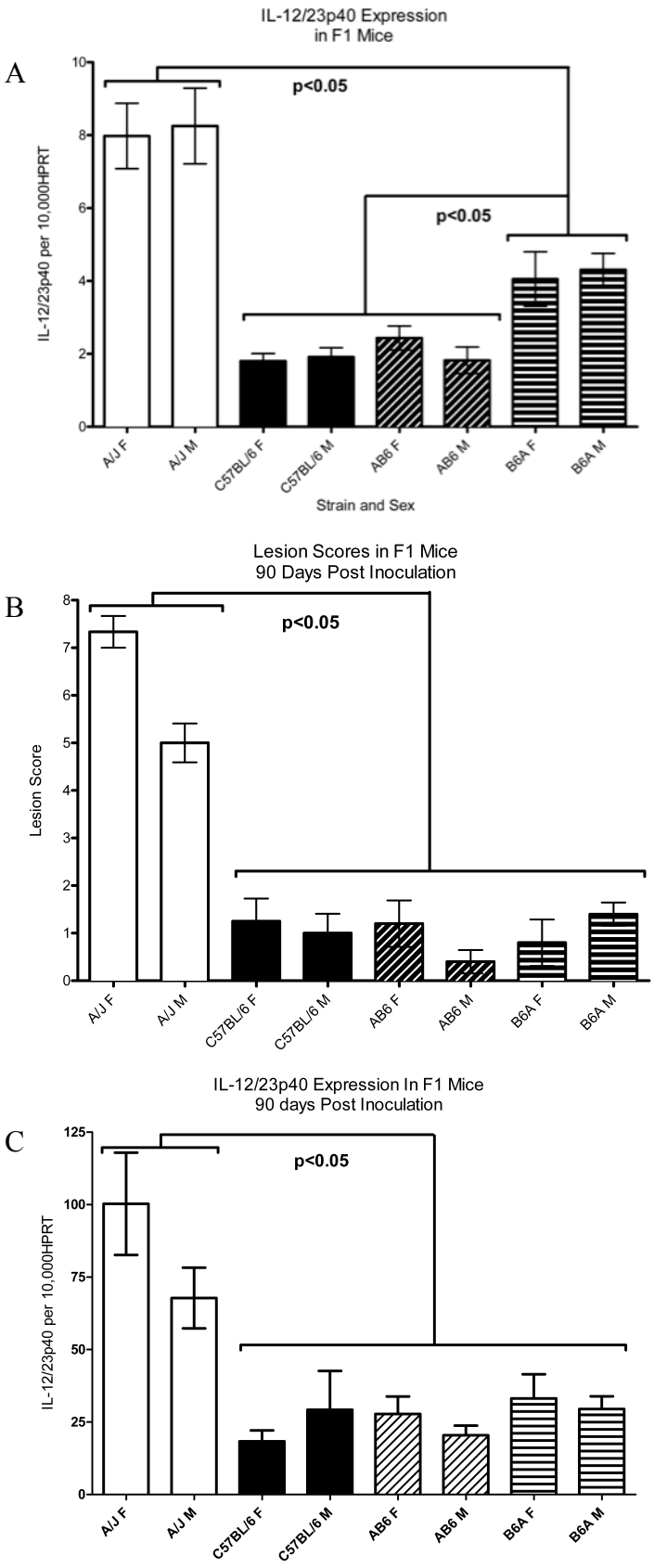
At 4 days post-inoculation (Figure 3.1 A) A/J mice had significantly higher cecal expression of IL-12/23p40 than the infected C57BL/6 mice. IL-12/23p40 expression levels in B6A F_1 mice were significantly lower than in the A/J controls, but significantly higher than in the C57BL/6 controls, whereas IL-12/23p40 expression levels in AB6 F_1 mice were not significantly different from the expression in C57BL/6 mice.

By 90 days post-inoculation, as expected, A/J mice had significantly elevated IL-12/23p40 expression and lesion scores when compared to C57BL/6 mice, and as previously reported, female A/J mice had higher expression levels and lesion scores when

compared to males.[24, 62] Lesion scores and cytokine expression levels of infected F_1 mice of all crosses were similar to those of the C57BL/6 parental strain without exhibiting a sex bias seen in A/J mice (Figure 3.1 B and C). Collectively, these results suggest that resistance to *H. hepaticus* is dominantly inherited.

Figure 3.1 Susceptibility to *Helicobacter hepaticus* in F_1 crosses at 4 and 90 days post-inoculation – (A) Consistent with previous studies A/J mice expressed significantly higher mean levels of IL-12/23p40 ($p<0.05$) than did C57BL/6 mice at 4 days post-inoculation with *Helicobacter hepaticus*. Mean expression levels of IL-12/23p40 in AB6 F_1 mice were similar to those seen in C57BL/6 mice. In contrast, B6A F_1 mice had a slight, but significant ($p<0.05$) increase in p40 expression when compared to C57BL/6 controls. At 90 days post-inoculation, A/J mice had significantly higher lesion scores (B) and IL-12/23p40 expression (C) when compared to all other strains examined with female A/J mice having higher lesion scores and cytokine expression than males.

Figure 3.1


A
IL-12/23p40 Expression
in F1 Mice
p<0.05
p<0.05
IL-12/23p40 per 10,000HPRT
Strain and Sex
A/J F
A/J M
C57BL/6 F
C57BL/6 M
AB6 F
AB6 M
B6A F
B6A M
B
Lesion Scores in F1 Mice
90 Days Post Inoculation
p<0.05
Lesion Score
C
IL-12/23p40 Expression In F1 Mice
90 days Post Inoculation
p<0.05
IL-12/23p40 per 10,000HPRT

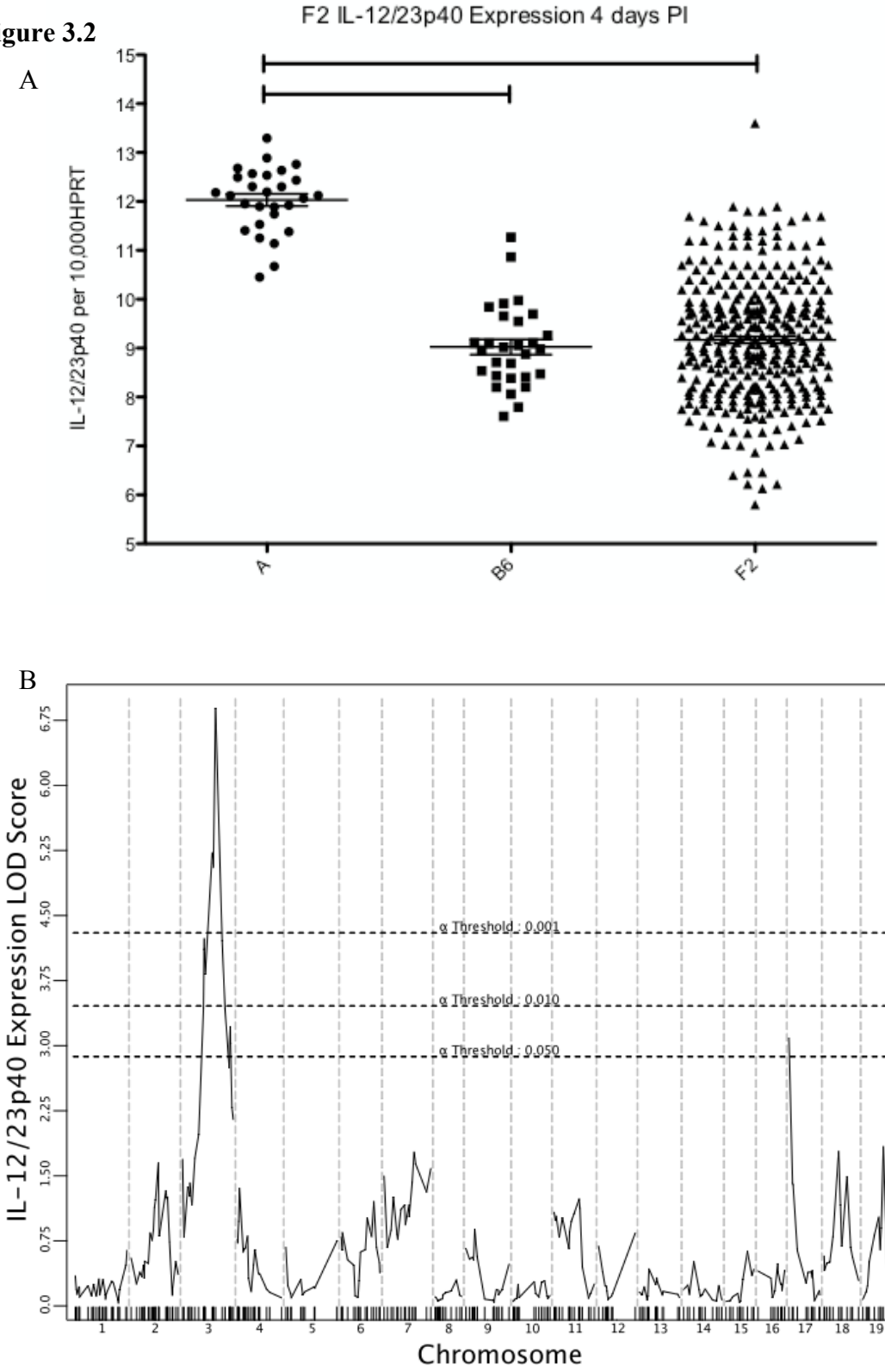
QTL Analysis Reveals Two Loci Associated with Increased Early IL-12/23p40 Expression

To map the loci responsible for susceptibility to *H. hepaticus*-induced inflammation, we examined the expression of IL-12/23p40 in F_2 mice at 4 days post-inoculation as a biomarker for disease susceptibility. Since there was a difference in the early expression of p40 between the AB6 and the B6A F_1 mice, we generated F_2 animals from all possible parental strain combinations (AB6 × AB6, B6A × B6A, AB6 × B6A, and B6A × AB6). A total of 314 F_2 mice were inoculated with *H. hepaticus* and assessed for cecal IL-12/23p40 expression 4 days post-inoculation (Figure 3.2 A). Expression of p40 within the F_2 population varied widely from high (A/J-like) to low (C57BL/6-like).

The 93 F_2 mice with the highest and the 93 mice with the lowest IL-12/23p40 expression were genotyped using a combination of 86 microsatellite and 244 SNP markers. Linkage analysis of all F_2 animals identified two quantitative trait loci with significant LOD scores associated with expression levels of IL-12/23p40 (Figure 3.2 B). The QTL with the largest LOD score of 6.89 was on chromosome 3. A second QTL with a LOD score of 3.09 was located on the proximal end of chromosome 17. The QTL on chromosome 3 explained 10% of the total variance of the IL-12/23p40 expression in the F_2 population. The peak signal for this QTL was at SNP *mCV23483645* located at 123 Mbp. The flanking markers defined a 20 Mbp region from 116.7 to 136 Mbp. The QTL on chromosome 17 explained 4% of the variance in IL-12/23p40 expression in the F_2 population. The peak signal for this QTL was at SNP *rs6239530* at 3.8 Mbp and the flanking region consisted of a 12.7 Mbp region on the proximal end of chromosome 17.

Figure 3.2 Early expression of IL-12/23p40 in F_2 mice and QTL analysis - Expression of IL-12/23p40 at 4 days post-inoculation was used as a biomarker for disease susceptibility in 314 *Helicobacter hepaticus* infected F_2 mice. A) Distribution of phenotypes in the F_2 population ranged from high IL-12/23p40 expression (A/J-like) to low IL-12/23p40 expression (C57BL/6-like). B) QTL analysis of 314 F_2 mice revealed significant QTL on chromosomes 3 and 17 with LOD scores of 6.89 and 3.09, respectively, associated with increased expression of IL-12/23p40 upon exposure to *Helicobacter hepaticus*.

Figure 3.2


A
F2 IL-12/23p40 Expression 4 days PI
IL-12/23p40 per 10,000HPRT
15
14
13
12
11
10
9
8
7
6
5
A
B6
F2
B
IL-12/23p40 Expression LOD Score
6.75
6.00
5.25
4.50
3.75
3.00
2.25
1.50
0.75
0.0
α Threshold: 0.001
α Threshold: 0.010
α Threshold: 0.050
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X
Chromosome

Influence of sex on IL-12/23p40 expression-associated QTL

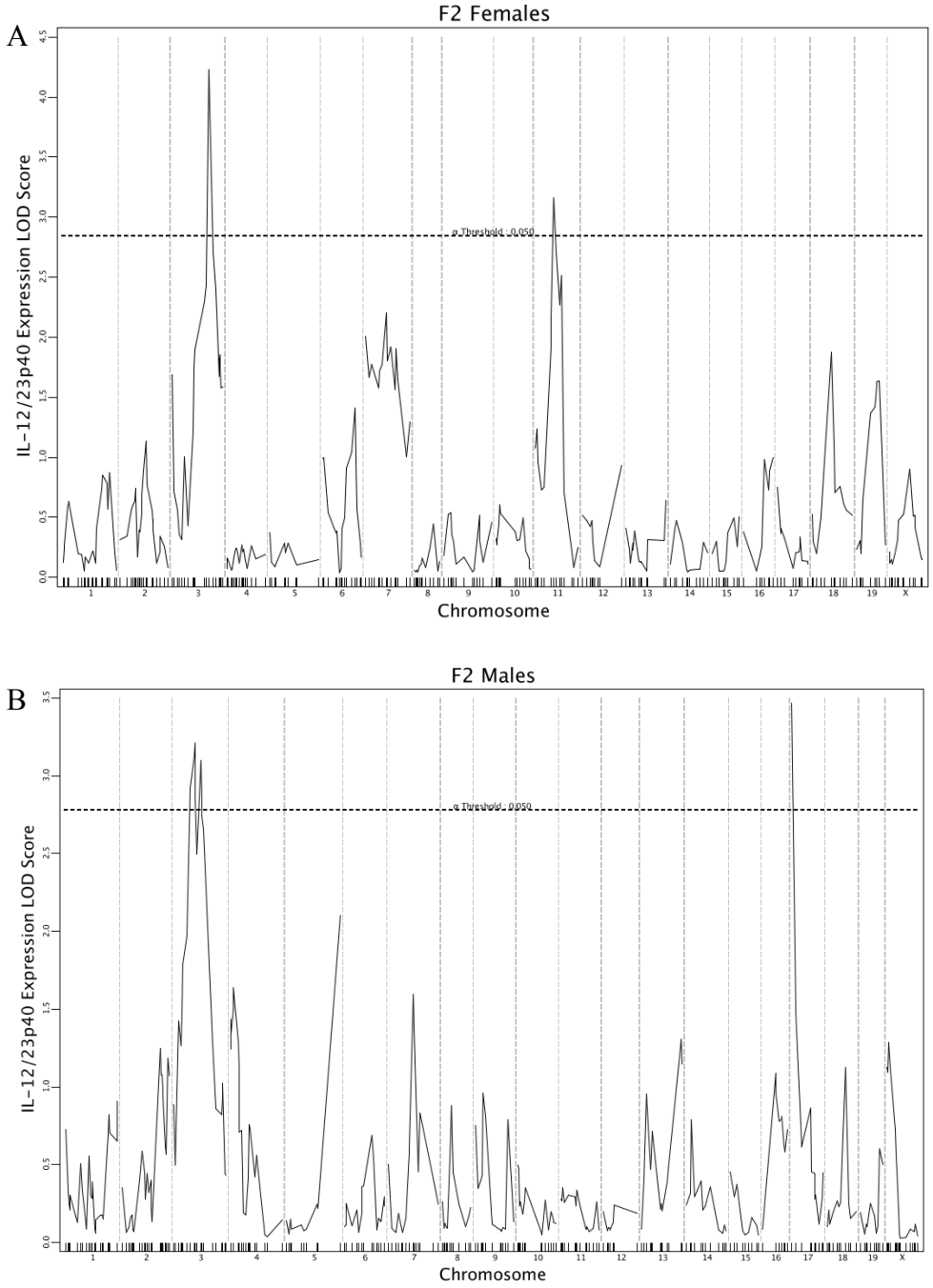
We have previously observed a sex bias in the intestinal inflammation of *Helicobacter hepaticus*-infected A/J mice. Infected female mice consistently develop more severe intestinal inflammation and increased expression of inflammatory cytokines in the cecum.[62] To test for a sex influenced QTL in the F_2 population, mice were partitioned based on sex and analyzed separately. Genotypes for 97 female mice were included in this analysis, which revealed a strong QTL on chromosome 3 with a significant LOD score of 4.23 in the same position (*i.e.* SNP *mCV23483645*) identified when males and females were jointly analyzed (Figure 3.3 A). Another, previously unidentified QTL at approximately 50 Mbp on chromosome 11, was detected in the female population. This QTL was at SNP *rs13481045* and had a LOD score of 3.16.

Analysis of 88 genotyped male F_2 mice revealed two QTL peaks on chromosome 3. The QTL with the highest LOD score of 3.13 was at marker *D3Mit98* (86 Mbp). Another QTL at marker *D3Mit57* (116 Mbp) also reached significance with a LOD score of 3.09. The second locus is within 8 Mbp of *mCV23483645*, the SNP identified in the joint analysis of all F_2 animals. Given its proximity to SNP *mCV23483645,* it is likely that the locus at *D3Mit57* is the same locus detected in the original analysis of the entire F_2 population. It is also possible that the QTL at marker *D3Mit98* may be a false positive due to a reduced sample size from the original analysis. Analysis of the male population also identified a significant QTL on chromosome 17 at SNP *rs6239530* (3.8 Mbp) with a

LOD score of 3.47. This QTL was previously identified in the analysis of all F_2 animals. Comparison of the male and female analyses shows that the QTL on chromosome 3 is conserved and not influenced by the sex of the mice. The previously identified QTL on chromosome 17 appears to influence the inflammatory response only in males while a previously unidentified QTL on chromosome 11 appears to only influence the female response.

Figure 3.3 Influence of sex on IL-12/23p40 expression-associated QTL – (A) Linkage analysis of the 97 F_2 female mice revealed two QTL with significant LOD scores, a QTL on chromosome 3 at SNP of *mCV23483645* with a LOD score of 4.23 and a QTL on chromosome 11 at SNP *rs13481045* with a LOD score of 3.16. (B) Analysis of 88 F_2 male mice revealed a two QTL on Chromosome 3 on at marker *D3Mit98* and a second at *D3Mit57* with LOD scores of 3.13 and 3.09 respectively.

Figure 3.3

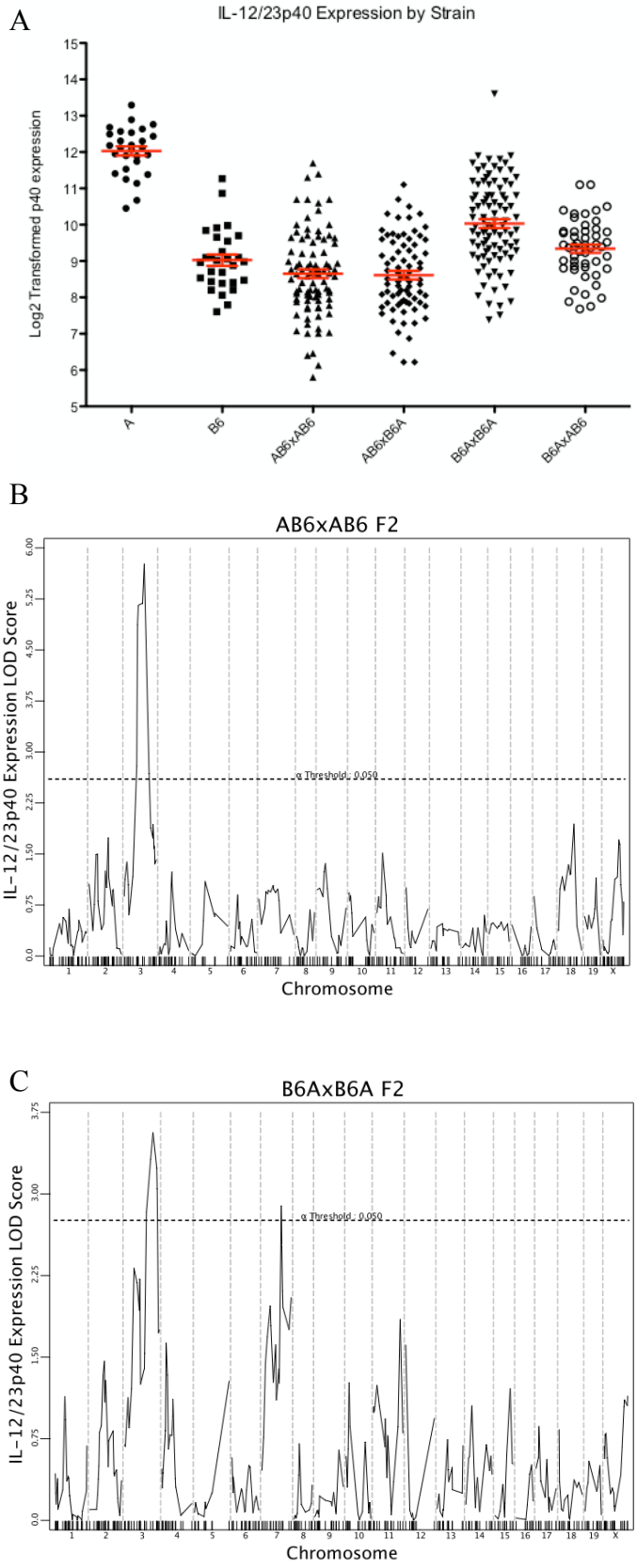

A
F2 Females
IL-12/23p40 Expression LOD Score
Chromosome
B
F2 Males
IL-12/23p40 Expression LOD Score
Chromosome

Influence of cross on IL-12/23p40 expression-associated QTL

Because the IL-12/23p40 expression in the reciprocal F_1 crosses differed between B6A and AB6 F_1 mice, the influence of the direction of the cross on susceptibility to *Helicobacter*-induced gene expression was further assessed. F_2 mice with a B6A mother (B6A × B6A and B6A × AB6 F_2 mice) had significantly higher IL-12/23p40 expression than mice with an AB6 mother (AB6 × AB6 and AB6 × B6A F_2 mice) (Figure 3.4 A). To assess the presence of genetic loci with parent of origin affects, AB6 × AB6 and the B6A × B6A F_2 mice were individually analyzed (Figure 3.4 B and C). Analysis of 78 AB6 × AB6 mice identified the QTL previously seen in the global analysis (chromosome 3 at SNP *mCV23483645*) with a LOD score of 5.76. Analysis of the reciprocal B6A × B6A F_2 population (n= 80) also identified a significant QTL on chromosome 3 at marker *D3Mit320* (LOD 3.56), 20 Mbp downstream of the locus identified in the original scan. In the B6A × B6A cross, a previously unidentified QTL on chromosome 7 at SNP *mCV24269234* was detected with a LOD score of 2.89 was also observed. This QTL may contribute to the differences in IL-12/23p40 expression observed in the F_1 and F_2 populations of mice as this QTL was only identified in the B6A maternally derived population.

Figure 3.4 Early expression of IL-12/23p40 in subgroups of F_2 mice and QTL analysis – QTL analysis was performed on F_2 population separated according to the maternal cross from which they were derived. A) The mean IL-12/23p40 expression in F_2 mice derived from a B6A F_1 mother was significantly higher than the mean expression in F_2 mice derived from an AB6 F_1 mother. B) Analysis of individual F_2 crosses of AB6 × AB6 F_2 mice, and C) B6A × B6A F_2 mice which consistently identified the locus on chromosome 3, but not the chromosome 17 QTL. Analysis of the B6A × B6A F_2 cross identified a QTL on chromosome 7 that was not identified in the original scan.

Figure 3.4

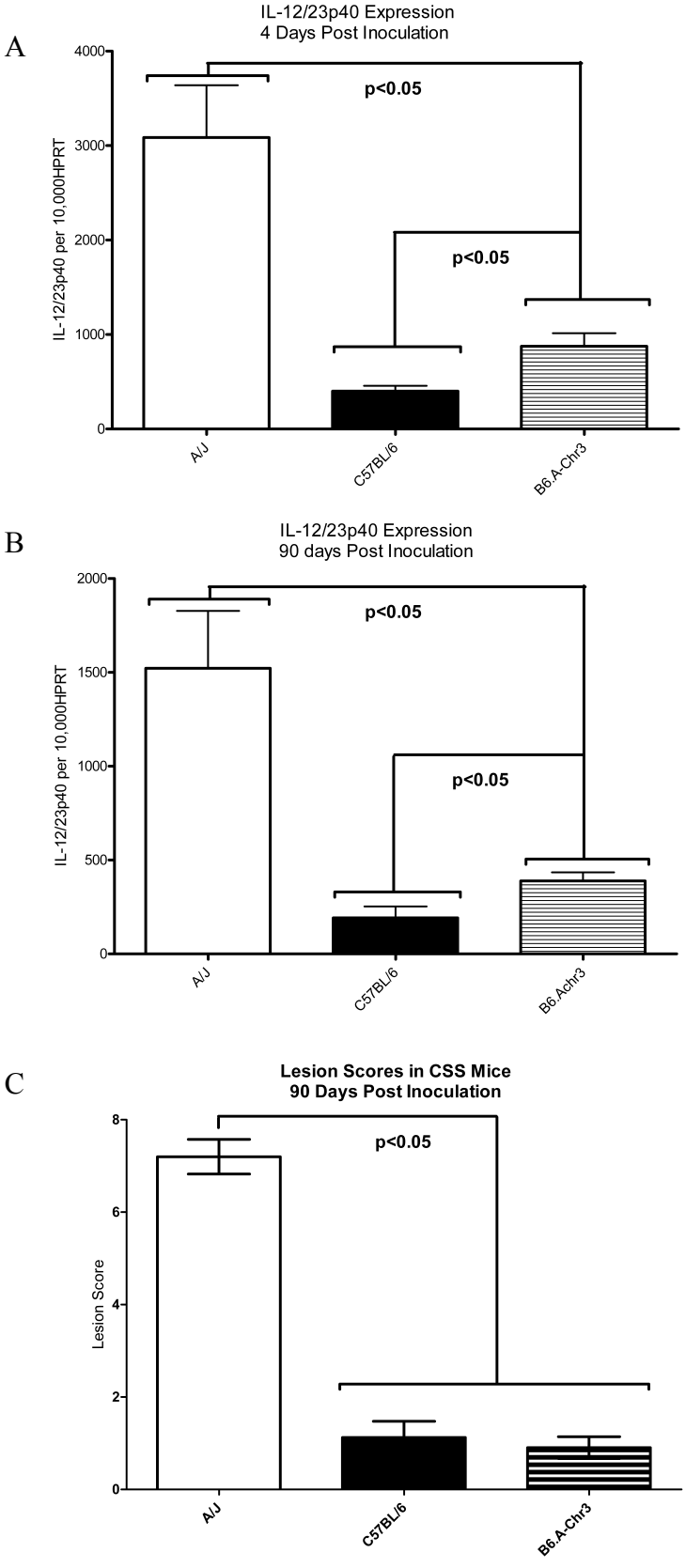

A
IL-12/23p40 Expression by Strain
Log2 Transformed p40 expression
A
B6
AB6xAB6
AB6xB6A
B6AxB6A
B6AxAB6
B
AB6xAB6 F2
IL-12/23p40 Expression LOD Score
α Threshold, 0.050
Chromosome
C
B6AxB6A F2
IL-12/23p40 Expression LOD Score
α Threshold, 0.050
Chromosome

Presence of A/J alleles on chromosome 3 increases cecal IL-12/23p40 expression

Because the QTL on chromosome 3 was consistently detected regardless of the cross used to produce the F_2, we sought to test its affect on susceptibility to *Helicobacter hepaticus* utilizing the C57BL/6 chromosome substitution mice with an A/J chromosome 3 (C57BL/6J-Chr3$^{A/J}$/NaJ) developed in the laboratory of Dr. Joe Nadeau.[111] These mice were infected with *Helicobacter hepaticus*, and assessed for cecal IL-12/23p40 expression and/or inflammation at 4 days and 90 days post-inoculation. At 4 days post-inoculation IL-12/23p40 expression in infected B6.A-Chr3 mice was significantly higher compared to expression in C57BL/6 mice and significantly lower than expression in the A/J mice (Figure 3.5A). At 90 days post-inoculation, the IL-12/23p40 expression in B6.A-Chr3 mice was again intermediate to the two parental strains (Figure 3.5 B). However, even with the moderate increase in IL-12/23p40 expression, B6.A-Chr3 mice failed to develop cecal inflammation and lesion scores in these mice were not significantly different from C57BL/6 controls (Figure 3.5 C). Taken together, these data show that the presence of A/J alleles at this susceptibility locus on chromosome 3 on an otherwise resistant background moderately increases IL-12/23p40 expression, but these alleles are not sufficient to cause disease. This suggests that multiple genes including those located on other chromosomes contribute to disease susceptibility.

Figure 3.5 Response of C57BL/6-Chr3$^{A/J}$ mice to *Helicobacter hepaticus* at 4 days and 90 days post-inoculation - To test the effect of the QTL identified on chromosome 3, C57BL/6J-Chr3$^{A/J}$/NaJ chromosome substitution mice were inoculated with *Helicobacter hepaticus* and assessed for IL-12/23p40 expression at 4 and 90 days post-inoculation and cecal inflammation 90 days post-inoculation. A) At 4 days post-inoculation, cecal IL-12/23p40 expression was significantly increased in B6.A-Chr3 mice compared to the resistant C57BL/6 controls. Expression of IL-12/23p40 was also significantly lower in the B6.A-Chr3 mice than in the susceptible A/J controls. B) Expression of IL-12/23p40 was also increased in B6.A-Chr3 mice 90 days post-inoculation, but not to the levels of the susceptible A/J controls. C) While IL-12/23p40 expression was greater than in the C57BL/6 mice, the increase was not sufficient to increase the overall inflammation in the ceca of infected animals. Inflammation scores in the B6.A-Chr3 mice were not significantly different than in the resistant C57BL/6 controls.

Figure 3.5

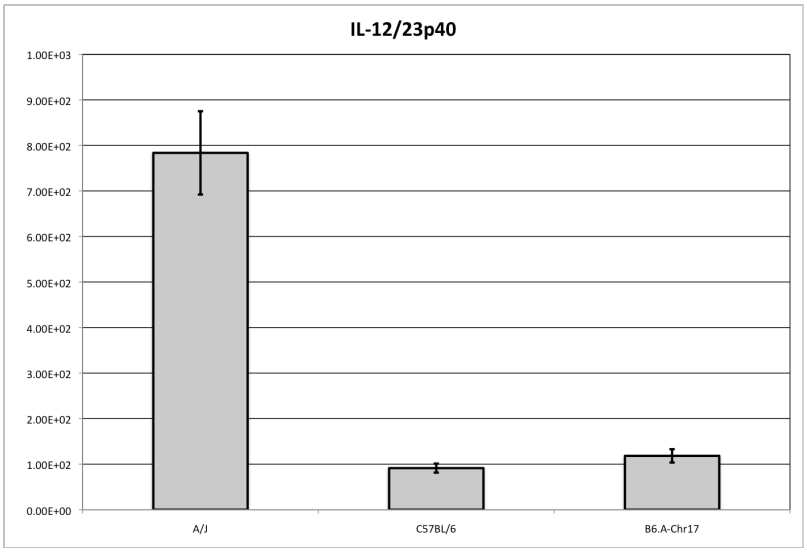

A
IL-12/23p40 Expression
4 Days Post Inoculation
IL-12/23p40 per 10,000HPRT
4000
3000
2000
1000
0
p<0.05
p<0.05
A/J
C57BL/6
B6.A-Chr3
B
IL-12/23p40 Expression
90 days Post Inoculation
IL-12/23p40 per 10,000HPRT
2000
1500
1000
500
0
p<0.05
p<0.05
A/J
C57BL/6
B6.Achr3
C
Lesion Scores in CSS Mice
90 Days Post Inoculation
Lesion Score
8
6
4
2
0
p<0.05
A/J
C57BL/6
B6.A-Chr3

We also sought to investigate the role of the A/J allele on chromosome 17 using the C57BL/6J-Chr17$^{A/J}$/NaJ chromosome substitution mice. Expression of IL-12/23p40 in *Helicobacter hepaticus* infected C57BL/6J-Chr17$^{A/J}$/NaJ was similar to control C57BL/6 mice (Figure 3.6 A). The QTL identified was at the very proximal end of chromosome 17. During the generation of the B6.A-Chr17 mice, the marker used for genotyping in the selection was telomeric of the detected QTL leaving the upstream region to be of unknown genotype (Figure 3.6 B). We therefore genotyped the B6.A-Chr17 mice using the microsatellite marker *D17Mit164* located approximately 100 kbp downstream of the SNP at which the peak signal was identified in our QTL scan. Genotypes for the C57BL/6J-Chr17$^{A/J}$/NaJ mice at this marker were the same as for a C57BL/6 control at this marker, indicating that at least a 3.9 Mbp region of residual C57BL/6 genome resides in this region of chromosome 17 in the C57BL/6J-Chr17$^{A/J}$/NaJ mice (Figure 3.6 C). These mice could therefore not be used to further study the QTL identified on chromosome 17.

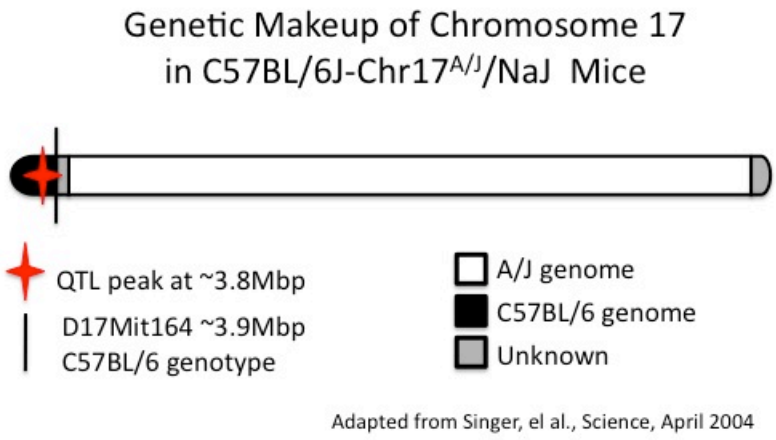
Figure 3.6 Response of C57BL/6-Chr17$^{A/J}$ to *Helicobacter hepaticus* and genetic analysis of centromeric genetic markers - To test the effect of the QTL identified on Chromosome 17, chromosome substitution mice were obtained that contained the A/J Chromosome 17 on a C57BL/6 background. (A) 4 days post inoculation, expression of IL-12/23p40 was not significantly higher in C57BL/6-Chr17$^{A/J}$ mice when compared to C57BL/6 controls. (B) Originally these mice were developed by genotyping selected markers on Chromosome 17 with the most proximal marker, *D17Mit143*, located at ~8.9 Mbp. Regions upstream of this marker were of unknown strain origin. (C) Analysis of *D17Mit164* located near the Chromosome 17 QTL (3.9 Mbp) reveals that there are portions of residual C57BL/6 genome upstream of the original marker used and it is likely that the QTL region in these mice is contained in this area of residual C57BL/6 genome.

Figure 3.5

A



B



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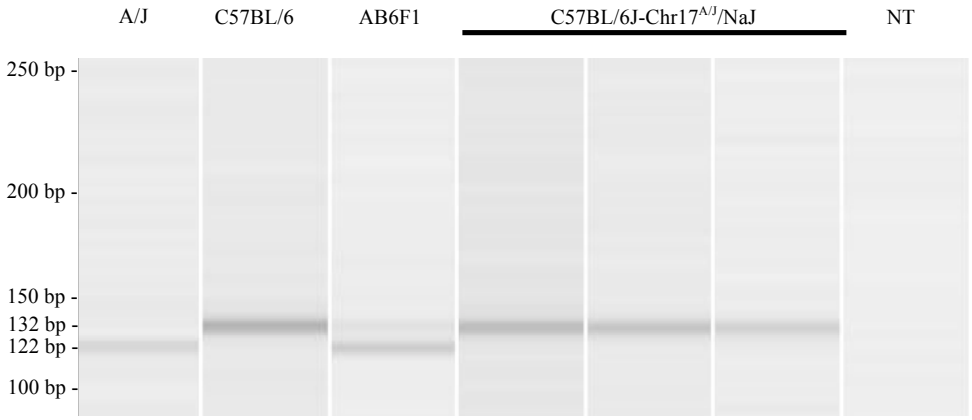


Discussion

Inflammatory Bowel Diseases result from a dysregulated intestinal mucosal immune response in genetically susceptible individuals and that inflammation is induced or exacerbated by various environmental factors. Recent genome wide association studies have implicated over 70 genes in the pathogenesis of Crohn's disease and 30 genes in the pathogenesis of ulcerative colitis, the two most prevalent forms of IBD.[101] Like in humans, A/J mice infected with *Helicobacter hepaticus* have delayed onset of intestinal inflammation. Infected A/J mice develop increased cecal cytokine gene expression as early as 4 days post-inoculation and inflammation by 90 days post-inoculation. C57BL/6 mice are resistant to *H. hepaticus*-induced gene dysregulation and intestinal inflammation.[24] Using early expression of IL-12/23p40 as an early biomarker for disease susceptibility, these studies identified at least 2 major QTL associated with susceptibility to a bacterially induced intestinal inflammation between these two strains.

Like their C57BL/6 parents, *Helicobacter hepaticus* infected F_1 mice did not develop intestinal inflammation or dysregulated gene expression 90 days post inoculation, indicating that resistance to inflammation is a dominantly inherited trait. In analyzing the early IL-12/23p40 expression in response to *H. hepaticus* in F_1 mice, an unexpected observation was made. B6A F_1 mice expressed IL-12/23p40 intermediate to the controls, significantly higher than the resistant C57BL/6 mice, but significantly lower than A/J mice. On the other hand, the reciprocal AB6 F_1 mice had the same expression levels as the C57BL/6 controls. Parental effects were also detected in the F_2 mice. Animals derived from B6A mothers had higher mean expression of IL-12/23p40 expression than F_2 mice derived from AB6 mothers. As there were no differences in

expression detected between males and females within strains in either generation, we cannot attribute these changes in expression to a sex-linked locus.

Utilizing a reciprocal F_2 population of mice from crosses between A/J and C57BL/6 mice, two QTL associated with increased early expression of IL-12/23p40 in response to *Helicobacter hepaticus* infection were identified. The two loci are located on

the distal end of chromosome 3 in a region of approximately 20 Mbp in size from 116.7 to 136 Mbp and within a region on the proximal end of chromosome 17 of 12.7 Mbp in size. The QTL on chromosome 3 was detected in all F2 subpopulations regardless of sex or direction of cross. Individual analysis of male and female F_2 mice not only identified the QTL on chromosome 3, but a very strong QTL on chromosome 17 was detected in males while females had a QTL on chromosome 11. Analysis of the different F_2 crosses also detected a QTL on chromosome 3 in both the AB6 × AB6 population as well as the B6A × B6A populations. Subsequent tested of *Helicobacter hepaticus* infected C57BL/6J-Chr3$^{A/J}$/NaJ chromosome substitution mice showed that the presence of the susceptible A/J chromosome 3 in an otherwise resistant C57BL/6 mouse leads to increased IL-12/23p40 expression after inoculation, but this chromosome alone did not cause inflammation.

Several studies in other mouse models have also identified loci on chromosome 3 associated with intestinal inflammation, strengthening the evidence of the role that this locus plays in intestinal inflammatory responses (Table 3.1). C3H/HeN mice deficient for *Gnai2*$^{-/-}$ develop severe colitis while C57BL/6 mice with this deficiency do not. Analysis of an F_2 cross between *Gnai2*$^{-/-}$ C3H/HeN and *Gnai2*$^{-/-}$ C57BL/6 mice revealed multiple QTL responsible for the development of colitis. Included was a major QTL on

chromosome 3 (*Gpdc1)* located at 59-61 cM between markers *D3Mit316-D3Mit348* respectively, or from 120-127 Mbp, similar to the location of the chromosome 3 locus identified in the present study.[112] In the IL-10 deficient mouse model of colitis, QTL analysis has revealed at least 10 quantitative trait loci.[113] A major QTL on chromosome 3 (*Cdcs1*) was originally detected in an F_2 population of IL-10 deficient C3H/HeJBir and IL-10 deficient C57BL/6 mice and subsequently confirmed in an N_2 population of the same IL-10 deficient mice.[114] This locus was associated with cecal and colonic inflammation and overlaps at the same location as the QTL identified in the *Gnai2*$^{-/-}$ mice and the studies described herein.

The finding of a conserved region among three separate mouse models of IBD suggests that this region is vital in the control of intestinal inflammation. The region on chromosome 3 harboring the QTL is large, and there are many good candidate genes. *Nfkb1* is an excellent candidate gene located at 135 Mbp. This gene encodes NF-κB, a transcription factor responsible for controlling various immune responses, such as the response to pathogenic organisms. Mice deficient in NF-κB are highly susceptible to *Helicobacter*-induced inflammation. Studies of the human syntenic region with *Cdcs1* have revealed certain human *Nfkb1* haplotypes in Crohn's Disease patients. These patients may have polymorphisms in the NF-κB gene, or a gene that is closely linked to the NF-κB locus. Patients with these haplotypes have increased circulating anti-flagellin antibodies and decreased NF-κB expression and function, resulting in an inability to appropriately respond to pathogenic organisms in the gut.[115]

Microarray analysis of the C3H/HejBir and C57BL/6 parental strains used to form the IL-10 deficient model, which differ in severity of intestinal disease that they develop,

revealed no differences in *Nfkb1* expression. Rather, differences in the expression of the *Gbp1* gene on chromosome 3 were seen.[103] Microarray analysis of *Helicobacter hepaticus* infected parental strains used in the present study (A/J and C57BL/6) also showed dysregulation in the *Gbp1* and *Gbp2* genes.[24] Genes in the guanylate binding protein family (GBP) are known to be induced by interferon gamma signaling and mediate the anti-proliferative effect of proinflammatory cytokines.[103, 116] Knockdown of *Gbp1* with siRNA increased permeability in intestinal epithelium and epithelial apoptosis.[117] Mutations in this gene could lead to increase intestinal permeability and susceptibility to intestinal inflammation.

The role of *Cdcs1* in the pathogenesis of intestinal inflammation of IL-10 deficient mice has been further assessed in reciprocal congenic mice containing the *Cdcs1* locus. These studies showed that the presence of the disease locus in resistant mice was found to be sufficient to confer susceptibility to disease, and to alter the immune response of cells stimulated *in vitro*.[118] However, this is not the case for *Helicobacter hepaticus*-induced inflammation. *Helicobacter* infected C57BL/6J-Chr3$^{A/J}$/NaJ chromosome substitution mice developed moderately increased IL-12/23p40 expression, but they did not develop intestinal inflammation. This may be due to the presence of intact IL-10 in the chromosome substitution mice working to suppress intestinal inflammation unlike in the IL-10 deficient model, or it may be due to multiple loci interacting in the *Helicobacter*-induced model.

Further analysis of congenic mouse strains generated between the IL-10 deficient C3H/HeJBir and C57BL/6 suggests that *Cdcs1* may actually consists of at least three distinct regions involved in disease susceptibility (*Cdcs 1.1, Cdcs 1.2,* and *Cdcs 1.3*) and

that the presence of *Nfkb1* alone does not confer disease susceptibility. Through the microarray analysis of susceptible congenic mice, attractive candidate genes in this region were identified, including: *Fcgr1*, *Cnn3*, *Larp7*, and *Alpk1*.[113] Future analysis of the expression and function of these genes in the *Helicobacter hepaticus*-induced model of intestinal inflammation will elucidate if any of these genes are responsible for disease susceptibility. The identification of an overlapping locus on chromosome 3 amongst multiple models of intestinal inflammation indicates the importance of this region in controlling intestinal inflammation. It is reasonable to speculate that this locus is fundamental in regulating the immune response in all three cases of inflammation (IL-10 deficiency, Gnai2 deficiency, and *H. hepaticus*-induced intestinal inflammation).

A QTL on chromosome 17 was also identified in the main scan of all F_2 mice and in the male subpopulation. This is novel locus that has not been identified in any previous mapping studies in mouse models. One of the most interesting genes in this region is the CC chemokine receptor 6 (*CCR6*). *CCR6* encodes for a chemokine receptor often expressed on dendritic cells and memory T cells and is expressed on these cells in order to home to sites of inflammation. Polymorphisms in this gene have been identified as being associated with Crohn's Disease, adding weight to the prospects that *CCR6* is the gene underlying this QTL.[13] Additionally in the female F_2 population, at QTL on chromosome 11 was identified. This is in a similar location to a QTL identified in dextran sulfate sodium induced colitis.[119] This QTL is very close to both the interleukin 5 and the interleukin 3 genes. Both of these molecules have been shown to be important in eosinophil survival and have been implicated in ulcerative colitis pathogenesis.[120]

Another interesting observation made in this study was that reciprocal F_1 and F_2 populations responded differently to infection with *Helicobacter hepaticus*. B6A F_1 mice and F_2 mice derived from a B6A mother had higher levels of IL-12/23p40 than their reciprocal counter parts. This may be evidence of a paternally imprinted gene increasing susceptibility in mice that inherited a gene from their father or grandfather. However, with an F_2 population, we are unable test for the presence of imprinted loci. Future studies utilizing backcross (N_2) populations will be useful in understanding this phenomenon as it will allow for the distinction of the origin of the parental alleles. Environmental factors have also been demonstrated to impact parental effects and could conceivably modulate a difference in susceptibility among offspring of different parents. For example, studies of other autoimmune disease models have shown that the postnatal maternal environment can affect disease phenotype. In mouse models of autoimmune ovarian disease and multiple sclerosis, cross fostering of A/J pups to C57BL/6 mothers resulted in increased disease severity in both models likely due to differences is factors in the maternal milk.[121] In a mouse model of colitis, T-bet and Rag double knockout mice develop spontaneous colitis that requires the presence of microbial flora. Cross-fostering of wild type mice to double knockout mothers transferred the maternal flora to the cross fostered pubs resulting in the development of colitis.[122] Pilot studies have shown differences in the culturable intestinal flora between A/J and C57BL/6 mice. Susceptibility to *Helicobacter hepaticus* induced IL-12/23p40 expression increases in A/J pups cross-fostered to C57BL/6 mothers (Data not shown). The difference in postnatal environment could lead to the increased IL-12/23p40 expression observed at 4-day post-inoculation in both the F_1 and F_2 crosses. This observation has resulted in new targets for

investigation to determine factors associated with susceptibility to intestinal inflammation.

The complex nature of intestinal inflammation is apparent from this study. A major QTL on chromosome 3 contributes only 10% of the phenotypic variance across all F2 mice analyzed. Another two sex specific QTL were identified in subpopulations of the F2 mice, and an apparent environmental factor or factors may also be contributing in part to disease susceptibility. This study recapitulates the complex nature of human Inflammatory Bowel Diseases. Utilizing the *Helicobacter*-induced model will allow for the future investigation into the complex interactions between genetics and environment that result in intestinal inflammation. To this end, future studies will focus not only on identifying the gene or genes on chromosome 3, but they will also yield congenic mice that can investigate the role of multiple susceptibility loci together.

Table 3.1 Colitis models and previously identified quantitative trait loci associated with intestinal inflammation.

Colitis Model	Strains Used	Loci Identified (chromosome)	QTL Location	Candidate Gene(s)	Citation
IL-10$^{-/-}$	C57BL/6 X C3H/HejBir (F_2 and N_2)	***Cdcs 1* (Chr 3)**	**61.8 cM**	***Nfkb1, Egf, Gbp1***	Farmer, et al. 2001[114]
		Cdcs 2 (Chr 1)	32.8 cM	*Casp8, Cd28, Ctla4*	
		Cdcs 3 (Chr 2)	65 cM	*Il1, Pcna,*	Mähler, et al. 2002[123]
		Cdcs 4 (Chr 8)	21 cM	*Defb, Scc8*	
		Cdcs 5 (Chr 17)	19.06 cM	*H2-Ea, Tnf, Hsp1a, Hsp1b, Ly6g6c*	Beckwith, et al. 2005[118]
		Cdcs 6 (Chr 18)	32 cM	*Dcc, Apc, Mad2, CD14*	de Buhr, et al. 2006[103]
		Cdcs 7 (Chr 12)			
		Cdcs 8 (Chr 12)			de Buhr, et al. 2009[102]
		Cdcs 9 (Chr 4)		*Pla2g2a, Gnb1*	
		Cdcs 10 (Chr 5)		*Areg*	
Gnai2$^{-/-}$	C57BL/6 X C3H/HeN (F_2)	***Gpdc1* (Chr 3)**	**62 cM**	***Nfkb1, Egf, Gbp1***	Borm, et al. 2005[112]
		Gpdc2 (Chr 1)	47 cM		
		Gpdc3 (Chr 9)	42 cM		
Dextran Sulfate Sodium (DSS)	C57BL/6 X C3H/HeJ (F_2 and N_2)	*Dssc1* (Chr 5)	72 cM	*Selp1, Pdgfa*	Mähler, et al. 1999[119]
		Dssc2 (Chr 2)	47 cM	*Itga4, Il1a, Il1b*	
Spontaneous	C57BL/6 X SAMP1/YitFc (F_2)	*Ibdq1* (Chr 9)	34 cM	*Il18, Il10ra*	Kozaiwa, et al. 2003[124]
Trinitrobenzene Sulfonic Acid (TNBS)	C57BL/6 X SJL/J (F_2)	*Tnbs1* (Chr 9)	48 cM		Bouma, et al. 2002[125]
		Tnbs2 (Chr 11)	17 cM	*Il12b*	

Chapter 4

Work in Progress: Maternal Influence in *Helicobacter hepaticus* Induced Inflammation

Background and Aims

The pathogenesis of Inflammatory Bowel Diseases is complex and multifactoral. Development of intestinal inflammation is thought to be due to an exaggerated and uncontrolled intestinal immune response to normal commensal gut flora in genetically predisposed individuals. The importance of the intestinal bacterial flora in the development of Inflammatory Bowel Disease is only now being sufficiently investigated.

The human gastro-intestinal tract is estimated to contain over 400 species and greater than 10^{14} total microbes, of which only a small fraction can be identified through standard culture techniques.[1, 2] Recent advances in high throughput genetic sequencing have allowed for more in depth analysis and characterization of the microbial communities that inhabit the intestinal tract. These studies have revealed a distinct difference in the bacterial communities inhabiting patients with inflammatory bowel disease compared to control populations. Dysbiosis, or an imbalance between "good" and "bad" bacteria in the gut is thought to be key in the pathogenesis of Inflammatory Bowel Disease. Bacterial flagellin has been shown to be the dominant antigen in Crohn's Disease, and antibodies against *Clostridium* flagellin, *Pseudomonas*, *E. coli,* and *Sarcharomyces cerevisiae* have been associated with disease.[115, 126] These studies highlight that in Crohn's Disease patients, there is a response against normal inhabitants

of the gut. The role for bacteria in the development of human IBDs is further supported by the fact that antibiotic treatment or diversion of the fecal stream, in essence removing the antigenic stimulation, in IBD patients is effective in controlling disease and aids in resolution of inflammation in affected patients.[127, 128]

Many mouse models have demonstrated a direct need for intestinal commensal bacteria in the development of intestinal inflammation. Mice in various models of IBD rendered axenic (germ free) or treated with broad spectrum antibiotics to reduce the bacterial load fail to develop colitis, indicating a direct interaction between commensal bacteria and the host.[122, 129] Germ-free IL-10-deficient mice do not develop colitis whereas IL-10-deficient mice reared in SPF conditions, or colonized with specific bacteria known to induce colitis in other models do, indicating a direct need for intestinal bacteria for the development of intestinal inflammation.[25, 105]

In multiple inbred mouse strains, colonization with the bacterium *Helicobacter hepaticus* triggers a Th1 type immune response and intestinal inflammation similar to Crohn's Disease. Characterization of the A/J response to *Helicobacter hepaticus* has revealed that as early as four days post inoculation there is upregulation of inflammatory cytokine mRNA expression including IL-12/23p40, IFNγ and the chemokines CXCL9 and CCL3. Histologic signs of disease appear ~90 days post inoculation and are also accompanied by high levels of inflammatory cytokines. In C57BL/6 mice, *Helicobacter hepaticus*, fails to elicit inflammation, and inflammatory cytokine levels are only briefly, and mildly upregulated. In previous studies to identify QTL associated with *Helicobacter hepaticus*-induced inflammation in C57BL/6 and A/J mouse strains, loci on Chromosomes 3 and 17 were associated with an early increase in IL-12/23p40 expression

in susceptible mice. There were also distinct differences in both F_1 and F_2 populations derived from different maternal lines in early IL-12/23p40 expression in response to *Helicobacter* infection. F_1 and F_2 mice with a C57BL/6 mother or grandmother, respectively, had significantly higher IL-12/23p40 expression than mice derived from an A/J mother/grandmother, or from control C57BL/6 parents. We suspect that this maternal effect may be in part due to the different specific maternal environments to which F_1 and F_2 mice were exposed. To evaluate a possible maternal effect on *Helicobacter hepaticus*-induced inflammation, we undertook a cross-fostering experiment to determine if C57BL/6 mothers could influence disease susceptibility or if A/J mothers could render C57BL/6 pups more susceptible to *Helicobacter hepaticus*. We suspect that the majority of the contributing maternal effects are due to differences in intestinal microbial flora. We therefore also assessed the culturable bacterial flora colonizing these two mouse strains to assess any differences between A/J and C57BL/6 flora.

Materials and Methods

Mice

Male and female A/JCr and C57BL/6 mice were obtained from Frederick Cancer Research and Development Center, Frederick, MD. Timed breedings were performed so that pups from both strains would be born at the same time. Litters born within one day of each other were cross fostered; half of C57BL/6 pups were left with original mother (as controls) and the other half were cross-fostered with an A/J mother. This procedure

was repeated for the A/J litters. Mixed litters were reared by their respective mothers until 3-4 weeks of age at which time they were weaned.

Helicobacter hepaticus Culture

Isolation and growth of *Helicobacter hepaticus* strain MU-94 has been previously described.[24, 62] A 1.5 mL glycerol stock solution with ~5 x 10^8 bacteria/mL of *H. hepaticus* was diluted in 15 mL of brucella broth (Becton Dickinson, Franklin Lakes, N.J.), divided equally between three sheep's blood agar plates and grown for 24 hours at 37^0 C in a microaerobic environment with 90% N_2 / 5% H_2 / 5% CO_2. After 24 hours, the remaining broth containing the *Helicobacter* was transferred to a 250mL Erlenmeyer flask along with 35 mL of fresh brucella broth supplemented with 10% of fetal bovine serum (Sigma-Aldrich Co., St. Louis, Mo.). The culture was grown for another 24 hours at 37^0 C in a microaerobic environment with 90% N_2 / 5% H_2 / 5% CO_2 with constant stirring. Mice were inoculated via oral gavage with 0.5 ml of culture containing 5 x 10^8 bacteria/mL. Colonization was confirmed 2-4 weeks post inoculation and at time of necropsy via a *Helicobacter hepaticus* specific PCR.[130]

Histological Evaluation and Lesion Scoring

Ninety days post inoculation, mice were sacrificed, their ceca were removed, laid on note cards, and opened longitudinally. The cecal contents were scraped from the cecum and saved for confirmation of *Helicobacter hepaticus* colonization. The cleaned cecum was then split longitudinally. One half was rinsed with sterile PBS then flash frozen in liquid nitrogen for use in RNA expression analysis. The other half was fixed in

zinc-fixative and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin and scored for severity of cecal lesions.

Cecal lesions were evaluated using a modified protocol as previously described.[62, 65, 89] Lesions were scored for intensity of inflammation (0 = none, 1 = mild, 2 = moderate, and 3 = severe), longitudinal extent (1 = one or two small foci, 2 = patchy, and 3 = diffuse), and vertical extent of inflammation (1 = basal mucosal inflammation, 2 = full-thickness mucosal inflammation, and 3 = transmural inflammation). In addition, lesions were scored for hyperplasia by using the following criteria: the presence of basophilic staining "crypt" epithelial cells in at least the lower two-thirds of the gland or at least doubling of the height of the mucosal epithelium. Focal hyperplasia was given a score of 1, and diffuse hyperplasia was given a score of 2. The scores for intensity of inflammation, longitudinal and vertical extents of inflammation, and hyperplasia were summed to give a total score for each animal. Because the minimum inflammation score using this system is 3 (mild, focal, and basal), inflammation and hyperplasia were not on comparable scales. To rectify this bias, lesion scores of >2 were adjusted by subtracting 2 from the total score to give a total adjusted score.

RNA Isolation and Reverse Transcription

Portions of the ceca frozen in liquid nitrogen were thawed in a solution of phenol and guanidine isothiocyanite (TRIzol reagent, Invitrogen, Carlsbad, CA). Cecal sections were homogenized using a Tissuelyser (Qiagen, Valencia, CA) for 2 minutes at 30 Hz. Total RNA was isolated as per manufacture's instructions. The extracted RNA was then dissolved in 75μL of DEPC treated water (Sigma-Aldrich, St. Louis, MO). Quantity and

quality of RNA used for reverse transcription was assessed by measuring the absorbance at 260 nm and 280 nm (Nanodrop-1000 Spectrophotometer, Nanodrop, Wilmington, DE).

Five micrograms of total RNA was reverse-transcribed using the Super Script III kit (Invitrogen, Carlsbad, CA) by following the oligo(dT) primer protocol. The resultant cDNA was diluted with DEPC treated water to a final expected concentration of 20 ng/μL.

Real Time PCR

Real time RT-PCR was performed on the Roche Light Cycler 2.0 using Qiagen Quanti-tect SYBR Green (Qiagen, Valencia, CA). All expression was normalized to cecal levels of HPRT. Primers HPRT Forward – 5` GTAATGATCAGTCAACGGGGGAC 3` HPRT Reverse - 5` CCAGCAAGCTTGCAACCTTAACCA 3` produce a product of 177 bp at an annealing temperature of 60^0 C, and IL-12/23p40 Forward – 5` ACTCACATCTGCTGCTCCAC 3` Reverse – 5` GGGAACTGCTACTGCTCTTGA 3` produce a produce of 183 bp and run conditions for real time PCR have been previously described.[24]

Isolation of Microbial Flora

Cecal contents from young mice (3-4 weeks) and old mice (6-7 months) were collected upon necropsy. A total of 0.06 g of cecal content from each mouse were diluted in 1 ml of PBS, homogenized in a tissue lyser (Qiagen) and centrifuged at 200 x g for 1 minute. Serial dilutions were performed by transferring 100 μL of the slurry supernatant into 900 μL of PBS. 100 μL of a 1:1000 dilution were transferred onto blood agar plates

and spread evenly. The plates were incubated for 2 days at 37° C in both aerobic and anaerobic conditions. Individual bacterial colonies were streaked for isolation and were speciated using standard biochemical methodologies.

Data Analysis

IL-12/23p40 expression and cecal lesions scores were compared via two-way ANOVA comparing strain and foster group, and sex within each strain and foster group. Results were considered significant when $p<0.05$. Columns labeled with the same letter indicate no significant difference between those groups. Columns with different letters did have a significant difference $p<0.05$.

Results

Affect of Cross-Foster on Disease Susceptibility

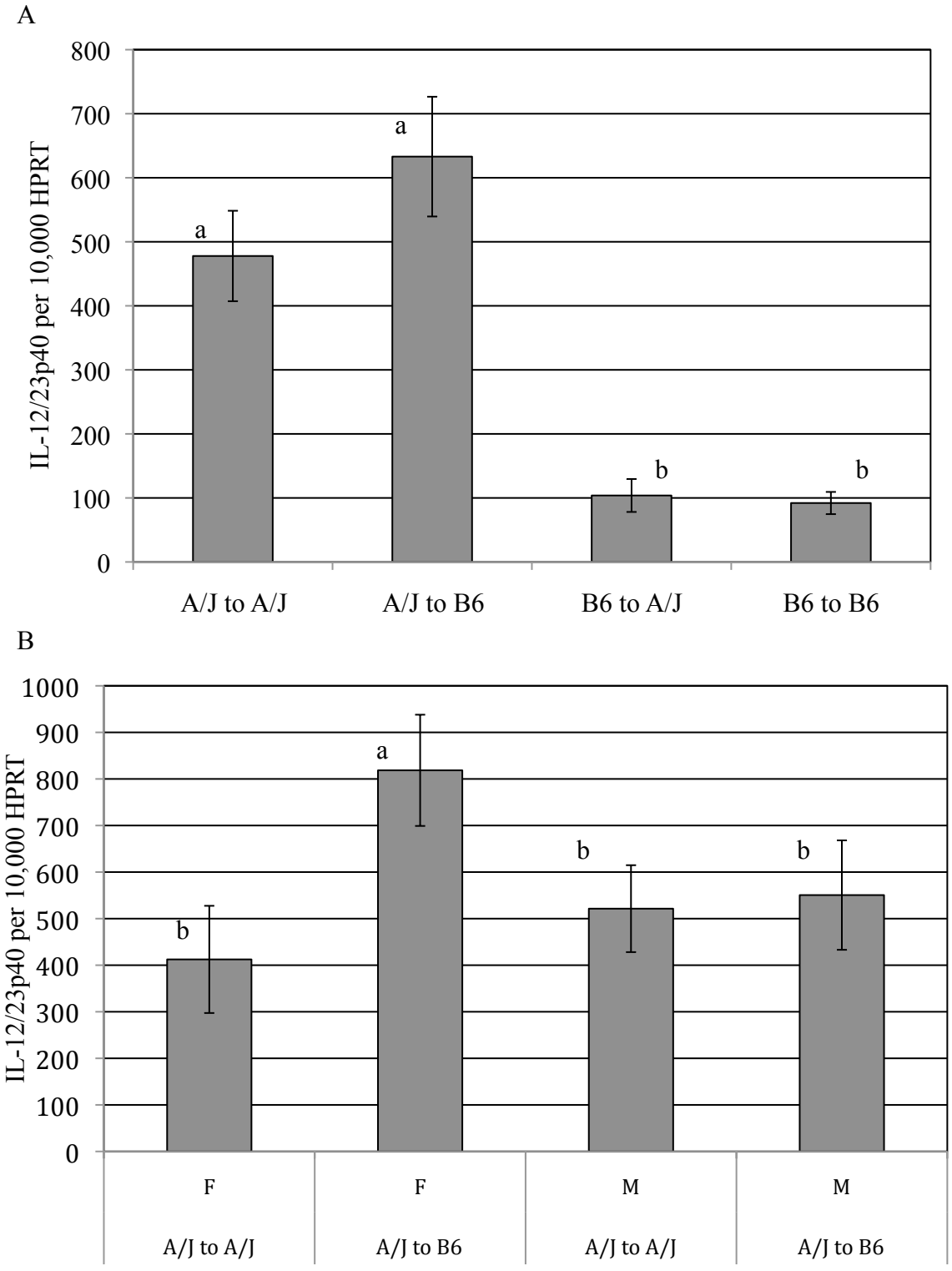
To assess maternal effects on *Helicobacter hepaticus*-induced intestinal inflammation, 1-day-old A/J mouse pups were cross fostered to C57BL/6 mothers and C57BL/6 pups were fostered to A/J mothers. Upon weaning, mice were inoculated with *Helicobacter hepaticus* and assessed at either 4 days post inoculation for cecal IL-12/23p40 or at 90 days post inoculation for cecal inflammation and IL-12/23p40 expression. At 4 days post inoculation, both fostered and unfostered A/J mice had significantly higher IL-12/23p40 expression than either group of C57BL/6 mice (Figure 4.1 A). Cross fostering within either the A/J or the C57BL/6 groups showed no significant affect on IL-12/23p40 expression. Interestingly, there was a trend toward

increased IL-12/23p40 expression in A/J mice fostered to C57BL/6 mothers. Stratification of the expression data between the sexes within the A/J groups revealed that A/J female mice cross fostered to C57BL/6 mothers had significantly higher IL-12/23p40 expression than either A/J females fostered to A/J mothers or either of the two groups of male A/J mice (Figure 4.1 B).

Ninety days post inoculation, A/J mice exhibited increased cytokine expression and cecal inflammation compared to C57BL/6 mice (Figure 4.2 and data not shown). Cross fostering of C57BL/6 mice to A/J mothers did not affect expression of IL-12/23p40 when compared to unfostered C57BL/6 mice. Cross fostering A/J mice to C57BL/6 mothers also did not significantly affect IL-12/23p40 expression. However, there was a distinct trend of increased IL-12/23p40 expression in both males and females cross fostered to C57BL/6 mothers. Preliminary ranking of cecal inflammation showed an increase in inflammation in both fostered and unfostered A/J mice compared to C57BL/6. There was no difference within strains between fostered and unfostered groups (data not shown).

Figure 4.1 Early *Helicobacter hepaticus*-induced IL-12/23p40 expression in cross fostered mice - (A) A/J mice fostered to B6 had elevated IL-12/23 p40 expression when compared to A/J mice fostered to A/J mice; however this difference was not statistically significant. Since we saw a trend towards increased cecal IL-12/23 p40 expression in A/J mice fostered to B6 mice, we assessed whether the sex of the progeny influenced the phenotype. (B) This analysis revealed that female A/J mice fostered to B6 mother had significant elevations in IL-12/23 expression when compared to female A/J mice fostered to A/J mothers ($p<0.05$). No difference was detected in male mice.

Figure 4.1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500
400
300
200
100
0
IL-12/23p40 per 10,000 HPRT
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a
b
b
A/J to A/J
A/J to B6
B6 to A/J
B6 to B6
B
1000
900
800
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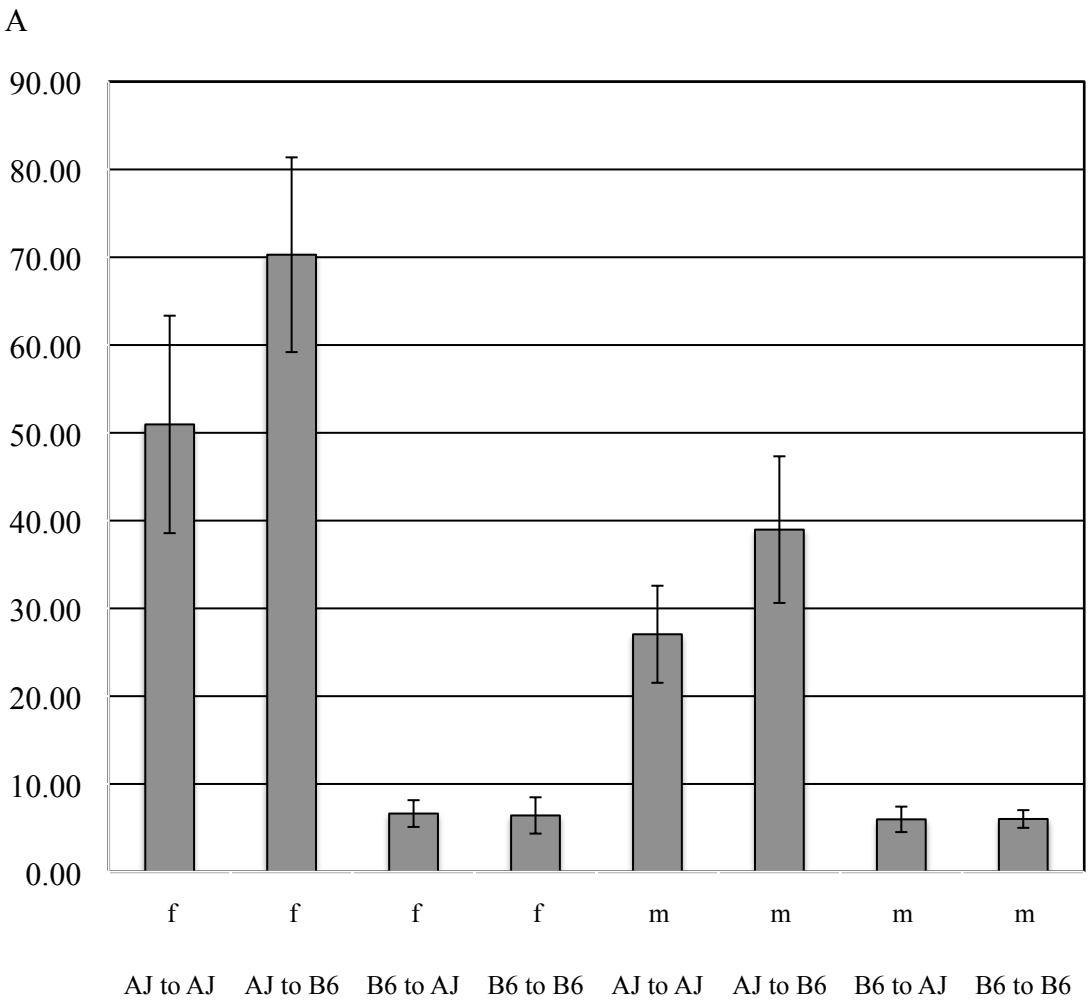
100
0
IL-12/23p40 per 10,000 HPRT
b
a
b
b
F
F
M
M
A/J to A/J
A/J to B6
A/J to A/J
A/J to B6

Figure 4.2 *Helicobacter hepaticus*-induced IL-12/23p40 expression in cross fostered mice 90 days post inoculation - A/J mice fostered to B6 had elevated IL-12/23p40 after 90 days compared to A/J mice fostered to A/J mice; these results however were not statistically significant.



Differences in Cecal Bacterial Makeup

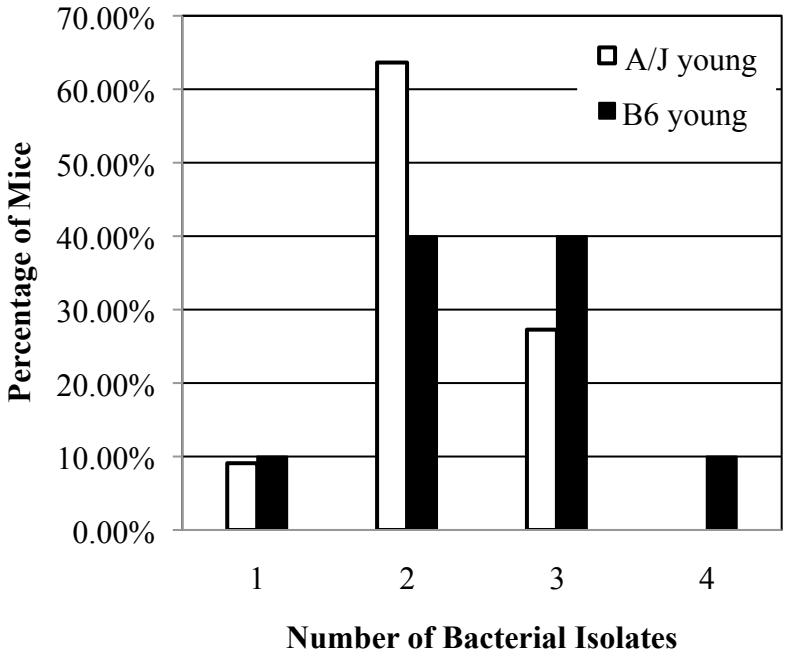
Upon infection with *Helicobacter hepaticus,* A/J mice develop cecal inflammation accompanied by an increase in inflammatory cytokine and chemokine expression. C57BL/6 mice infected with *Helicobacter* do not develop inflammation and express significantly lower levels of inflammatory cytokines. Endogenous microbial flora have been implicated in the pathogenesis of Inflammatory Bowel Disease, and differences within the microbial populations have been observed between IBD patients and controls. Given the differential susceptibility to *Helicobacter hepaticus*, we sought to assess the differences in endogenous bacterial species between A/J and C57BL/6 mouse strains at the time of weaning (3-4 weeks of age), and in fully mature mice. The composition of the culturable flora from C57BL/6 mice appeared to be more complex than from A/J mice. Weanling and mature C57BL/6 mice consistently had more culturable bacterial species than did A/J mice. Only 2 bacterial isolates were identified in the majority of A/J mice whereas weanling C57BL/6 mice had 2-3 bacterial isolates (Figure 4.3 A). The divergence between the strains was increased in the mature A/J and C57BL/6 mice. A majority of mature A/J mice only had 2 identifiable bacterial isolates, whereas the highest percentage of mature C57BL/6 contained 4 (Figure 4.3 B). A total of 6 bacterial species were identified in cecal contents of A/J and C57BL/6 mice. Amongst

the weanling aged A/J and C57BL/6 mice, 82% of young A/J mice were positive for alpha hemolytic *Streptococcus* species while only 40% of C57BL/6 mice were positive. Conversely more C57BL/6 mice (80%) were positive for *Enterococcus faecalis* than were A/J mice (9%). Increased *Enterococcus faecalis* was also observed more in mature C57BL/6 mice compared to mature A/J mice (78% vs. 56%). Another interesting finding was a complete absence of *Escherichia coli* in mature A/J mice whereas 67% of mature C57BL/6 mice were positive for this bacterium (Table 4.1).

Figure 4.3 Percentage of mice positive for bacterial isolates - (A) The number of bacterial isolates was similar between young A/J and B6 mice, however, there were more B6 mice with 3 or more bacterial isolates than there were for A/J mice. (B) Only two bacterial isolates were found in the majority of old A/J mice, whereas 4 bacterial isolates were found in the majority of B6 mice.

Figure 4.3

A


70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Percentage of Mice
A/J young
B6 young
1
2
3
4
Number of Bacterial Isolates

B

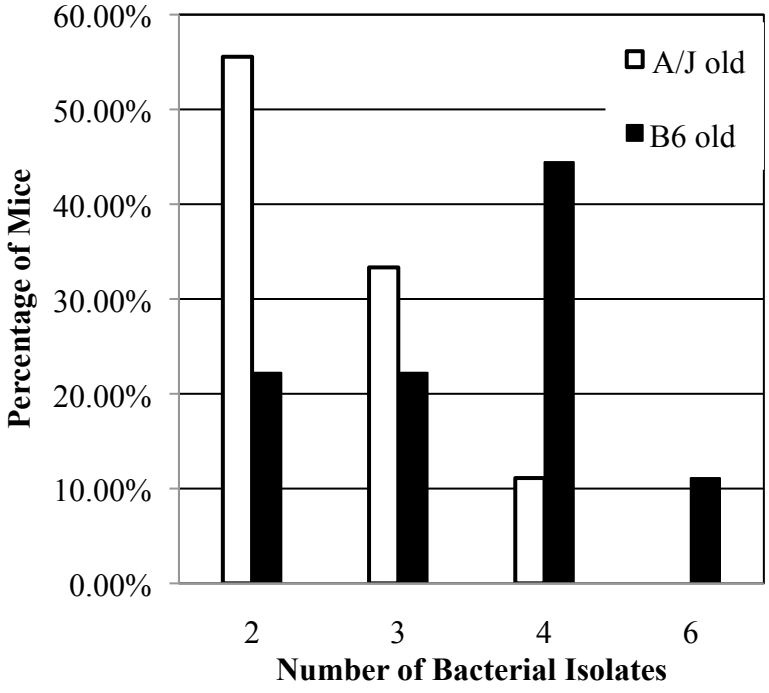

60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Percentage of Mice
A/J old
B6 old
2
3
4
6
Number of Bacterial Isolates

Table 4.1 Bacterial isolates identified between A/J and C57BL/6 mice at weaning and in mature mice – Bacterial isolates from cecal contents of weanling age and mature A/J and C57BL/6 mice were speciated and charted as a percentage of positive mice out of the total number tested within each strain.

Bacterial Isolate	Young A/J	Young B6	Adult A/J	Adult B6
***Lactobacillus* sp.**	**11/11 (100%)**	**10/10 (100%)**	**6/9 (67%)**	**7/9 (78%)**
Enterococcus faecalis	**1/11 (9%)**	**8/10 (80%)**	**5/9 (56%)**	**7/9 (78%)**
Staphylococcus xylosus	**3/11 (27%)**	**3/10 (30%)**	**4/9 (44%)**	**4/9 (44%)**
Alpha hemolytic *Streptococcus* sp.	**9/11 (82%)**	**4/10 (40%)**	**7/9 (78%)**	**7/9 (78%)**
Escherichia coli	**0/11 (0%)**	**0/10 (0%)**	**0/9 (0%)**	**6/9 (67%)**
***Bacteriodes thetaiotamicron* (anaerobic)**	**11/11 (100%)**	**10/10 (100%)**	**7/9 (78%)**	**7/9 (78%)**

Discussion

We sought to identify maternal contributions to disease in *Helicobacter hepaticus*-induced inflammation. We previously identified a possible maternal effect that increases early IL-12/23p40 in F_1 and F_2 mice derived from crosses between susceptible A/J and resistant C57BL/6 mice. F_1 and F_2 mice with a C57BL/6 mother or grandmother, respectively had increased early cecal IL-12/23p40 in response to *Helicobacter hepaticus* when compared to C57BL/6 controls.

To assess the maternal environment as a factor affecting the response to *Helicobacter hepaticus,* A/J and C57BL/6 pups were cross-fostered to mothers of the opposing mouse strain. Upon weaning, mice were inoculated with *Helicobacter hepaticus* and assessed for early IL-12/23p40 expression 4 days post inoculation or assessed for inflammation and IL-12/23p40 expression at 90 days post inoculation. The only significant effect was an increase in IL-12/23p40 expression at 4 days post inoculation in A/J females fostered to C57BL/6 mothers. At 90 days post inoculation, there was a trend toward increased expression in both male and female A/J mice reared by a C57BL/6 mother, but this was not significant. These data, while preliminary, suggest that there is a factor or factors in the maternal environment that can affect disease phenotype in *Helicobacter hepaticus*-induced inflammation.

There are many possible maternal factors that could be contributing to the observed increase in response to *Helicobacter*, from milk quality between strains, different maternal ability in rearing the pups, and maternal microbial flora. We sought to investigate the differences in the maternal flora between the two mouse strains as a possible explanation to the observed maternal effect. From our results, the microbial

flora of C57BL/6 appears to be more complex with a higher number of bacterial isolates identified in a large percentage of C57BL/6 mice. The two most striking differences were the high numbers of C57BL/6 mice positive for *Enterococcus faecalis* compared to A/J mice, and the complete absence of *Escherichia coli* in mature A/J mice while C57BL/6 had 67% incidence. Both of these bacteria have been implicated in the development of Inflammatory Bowel Disease in humans and in mouse models.[131, 132] Their presence in C57BL/6 mice may help to explain why there was an increased IL-12/23p40 response in the more disease prone, cross fostered A/J mice.

Together these data indicate that the microbial flora of A/J and C57BL/6 differ in regards to identity and number of species present, and it suggests that these differences may contribute to increasing the inflammatory response to *Helicobacter hepaticus*. Future studies using molecular biological techniques to characterize the intestinal microbiome will be necessary to fully understand the extent of the differences between these two mouse strains. Understanding the microbial population differences between these two strains will also enable an understanding of the complex interactions between known genetic factors and the intestinal flora that contribute to disease.

Chapter 5

Conclusions

In conclusion, my work has focused on three distinct factors associated with *Helicobacter hepaticus*-induced inflammation as a mouse model for Inflammatory Bowel Disease. I have sought to identify hormonal, genetic, and microbial factors important in the regulation of *Helicobacter hepaticus*-induced intestinal inflammation. I have found that signaling through ERβ appears to be the key immunomodulatory estrogen receptor. Treating *Helicobacter* infected mice with an agonist that signals preferentially through ERβ significantly decreased intestinal inflammation along with expression of IL-12/23p40 and downstream IFNγ and CXCL9. This work indicates that ERβ acting through the innate immune system is effective in decreasing disease. From our studies in estrogen receptor deficient mice, we have evidence that supports the idea that ERα signaling works primarily through the adaptive immune response. Mice deficient for ERα did not have significant decreases in IL-12/23p40, but inflammation was still decreased along with expression of IFNγ and CXCL9. Further studies into the specific cell types involved in ER signaling will help reveal the exact mechanism that specific estrogen receptors use to modulate the intestinal immune response.

I have also sought to identify genetic factors associated with susceptibility to *Helicobacter hepaticus*-induced intestinal inflammation. QTL analysis of over 300 F_2 mice revealed two major QTL on Chromosomes 3 and 17 associated with increased IL-12/23p40 expression in response to *Helicobacter* infection. The QTL on Chromosome

17 is novel, and contains the CCR6 gene. Polymorphisms in this gene have been recently associated with Crohn's Disease. The QTL identified on Chromosome 3 overlaps with other QTL identified in other rodent models of IBD, lending weight to the importance of this locus in regulating intestinal inflammation. *Helicobacter hepaticus* infection in C57BL/6-Chr3$^{A/J}$ chromosome substitution mice shows that the presence of the A/J alleles on Chromosome 3 in an otherwise resistant mouse strain is sufficient to significantly increase the expression of IL-12/23p40 upon infection with *Helicobacter hepaticus*. However, the presence of the susceptibility alleles at this locus were not sufficient to cause cecal inflammation. With genetic tools such as the C57BL/6J-Chr3$^{A/J}$ chromosome substitution mouse strains, it will be possible, through the development of congenic mouse lines, to narrow the previously identified QTL regions and eventually identify the causative gene or genes on Chromosome 3 that contribute to intestinal inflammation. With the newly available reciprocal A/J-Chr3$^{C57BL/6}$ and A/J-Chr17$^{C57BL/6}$ chromosome substitution mouse strains, it will also be possible to investigate the role of multiple interacting loci in the resistance to intestinal inflammation.

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VITA

Andrew "Drew" Hillhouse grew up in his parents Vet Clinic where he developed an interest in biology early on. Throughout high school he excelled in the sciences and upon graduation, enrolled at McMurry University in Abilene, Texas as a Biology major with the intention of becoming a physician.

After a research internship at Texas Tech University the summer before his junior year, he changed his mind and focused more on research. That summer he also began pursuing a second degree in History. With an interest in science and history, Drew began an honors project under Dr. Donald Frazier researching and editing a medical recipe book from a Civil War doctor in the confederate army. This work has been included in the recent publication Love and War: The Civil War Letters and Medicinal Book of Augustus V. Ball. He also studied genetic variation in iron uptake genes in the pea plant with Dr. Brian Waters. Drew finished McMurry and was awarded a Bachelor of Arts in History and a Bachelor of Science in Biology.

From there, Drew moved on to graduate work in Molecular Microbiology and Immunology at the University of Missouri. Working in the lab of Dr. Craig Franklin, Drew investigated hormonal and genetic factors that regulated intestinal inflammation. He presented his findings at many local and national meetings and has been recognized as the outstanding presenter multiple times.

During his first year of graduate school Drew married Katrina Keith and their son Phoenix was born a short time after. They currently live in Raleigh, North Carolina where Drew has taken a postdoctoral research position in the lab of Dr. David Threadgill. His current projects are focused on understanding genetic factors that contribute to susceptibly to colon cancer.