CHARACTERIZATION OF IMMUNE RESPONSE AND EFFECTS OF ENERGY BALANCE ON SEVERITY OF COLITIS AFTER INFECTION WITH *HELICOBACTER HEPATICUS* IN THE SMAD3-/- MOUSE MODEL

By

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ABSTRACT

CHARACTERIZATION OF IMMUNE RESPONSE AND EFFECTS OF ENERGY BALANCE ON SEVERITY OF COLITIS AFTER INFECTION WITH *HELICOBACTER HEPATICUS* IN THE SMAD3-/- MOUSE MODEL

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The SMAD3-/- mouse model is used study the pathogenesis of ulcerative colitis and colon cancer development in humans. The absence of SMAD3 causes dysregulation in TGF-β signaling and impairment of T regulatory cell development; however, current information regarding alterations in immune cell populations in this model is lacking. Therefore, we designed a study to identify and characterize SMAD3-dependent changes in immune cell populations in response to infection with the pathogenic bacteria *H. hepaticus*. We found significant alterations in NK cells, T cells and T cell subsets in SMAD3-/- mice, indicating that these mice have an inherently increased susceptibility to colitis due to a decreased ability to respond infection with *Helicobacter hepaticus*. These novel data provide potential immunological changes by which genetic alterations can affect the severity of colitis and subsequent tumorigenesis.

We then used the SMAD3-/- model to examine potential effects of energy balance on the severity of colitis and dysplasia following infection. We found that calorie-restricted mice died shortly after infection, and that there was no difference in severity of colitis between high-fat and control mice. These results are likely due to the fact that we were unable to achieve significantly increased body weight or fat percentages in the high-fat mice compared to control, especially after 18 weeks of age. Future studies will be designed to account for age-related changes in body composition prior to infection.

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KEY TO SYMBOLS

Symbol	Meaning
*	Significant effect of genotype within each time point following infection ($p<0.05$)
**	Significant effect of genotype within each time point following infection ($p<0.01$)
Δ	Significant effect of time in SMAD3+/- infected mice compared to control ($p<0.05$)
#	Significant effect of time in SMAD3-/- infected mice compared to control ($p<0.05$)

Chapter 1 – Literature Review

Obesity and Cancer

Obesity, defined as a Body Mass Index (BMI) greater than 30, is the result of complex interactions between a variety of factors, including lifestyle (diet and physical activity), biological (genetics, metabolism) and environmental attributes, such as culture and socioeconomic status. Excess weight is gained via sustained energy imbalance, in that too many calories are consumed and/or not enough calories are expended through physical activity. The prevalence of obesity in adults, as well as the frequency of overweight in children and adolescents, has increased dramatically in the United States over the past several decades. Data obtained as a part of National Health and Nutrition Examination Survey in 2003-2004 indicated that 32.2% of adults in the United States were obese, as compared to 23% in 1988-1994 and 15% in 1976-1980 [1, 2]. At the same time, 17.1% of American children and adolescents were overweight [1, 2]. These trends are of particular concern due to the subsequent increase in comorbidities and related health consequences. Obesity increases the risk of heart disease, stroke, and many types of cancer, which are the leading three causes of death for Americans, and also increases risk of hypertension, osteoarthritis, dyslipidemia, diabetes mellitus type 2, gallbladder diseases, sleep apnea and respiratory difficulties [2]. In a global, systematic review, total adiposity was associated with increased risk of cancer in the esophagus, pancreas, colorectum (CRC), breast (postmenopausal), endometrium, and kidney. The association between obesity and cancer risk are increased when measured by and increased waist-to-hip ratio, as abdominal adiposity is also specifically associated with colorectal cancer risk [3-5].

One of the connections between obesity and increased risk of cancer involves the altered production of adipokines, cell-signaling peptides produced by adipose tissue. Historically, adipose tissue was primarily considered to be an organ used to store energy in the form of triacylglycerols, with secondary functions of insulation and shock absorption [6]. Now, adipose tissue is recognized as a functional endocrine organ with the ability to secrete a number of adipokines. These adipokines act both locally and distally in tissues, and have effects on a large number of bodily functions, including lipid metabolism, inflammation, atherosclerosis, insulin resistance, reproduction, vascular homeostasis, food intake, thermoregulation, angiogenesis and immune function [7]. Unlike other secretory organs, adipose tissue can be located in multiple areas throughout the entire body, with the contributions of metabolic secretions depending upon the location and size of fat pads [8]. Furthermore, because fat cells have the ability to "network" with other tissues and organs such as skeletal muscle and the brain via hormone circulation and sympathetic nervous system activation, adipose tissue is highly integrated into overall metabolism and physiological processes of the body [9].

Adipose tissue is unique as an organ because of its nearly limitless potential to grow, even throughout adulthood after all other developmental growth has ended. In the obese state, adipose tissue also becomes dysregulated as a result of an abnormal interaction between energy balance, vasculature and immune cell infiltration, which is associated with the progression of chronic inflammation [10]. All adipose tissue releases adipokines regardless of body fat percentage of the individual, thus the inference can be made that the release of adipokines will increase along with increases in amounts of adipose tissue. As a result, production and excretion of several adipokines and other

adipose tissue-related hormones, including insulin, leptin and interleukin-6, are significantly increased in obesity [6]. Several of these are pro-inflammatory cytokines that are released from adipose tissue even in the absence of acute injury or inflammation, supporting the characterization of obesity as a disorder of chronic mild inflammation. Intra-abdominal adipose tissue appears to produce many of the adipokines in amounts greater than that of fat deposits elsewhere in the body, causing android obesity to be both a symptom of and a diagnostic indicator for metabolic syndrome [11]. Metabolic syndrome in turn is a risk factor for the development of cardiovascular disease, diabetes mellitus, peripheral vascular disease and stroke. Multiple studies have also found circulating adipokines to be contributing factors for increased risk of many types of cancer, including cancers of the breast, colon, kidney and esophagus [12]. The influence of adipokines on insulin sensitivity, glucose metabolism, inflammation and atherosclerosis may provide the molecular link between increased adiposity and development of obesity-related cancers [13]. Therefore, adipokines may be useful as clinical biomarkers to assess increased risk of cancer associated with increased adiposity and related comorbidities.

Adipokines and Cancer

Insulin/IGF-1 and cancer.

Insulin is a hormone produced and released by the pancreas and is therefore not an adipokine by definition; however, obesity is known to cause insulin resistance, thereby leading to an increase in serum levels of insulin as a compensatory response to the resistance [14]. As such, insulin is included as a hormone that is markedly increased in conditions of obesity. Chronic hyperinsulinemia and insulin resistance has been found to

increase risk for many types of cancer, although it is unclear whether the tumor-proliferating results are due to the direct effect of insulin on pre-neoplastic cells or the indirect stimulation of insulin-like growth factor (IGF) and other hormones [15]. Aside from its role in the metabolism of glucose, insulin also increases serum levels of the structurally similar but more potent IGF, thereby acting indirectly through the IGF/IGF receptor system that is present on most cells throughout the body, which may also explain in part the relationship between obesity-related hyperinsulinemia and cancer risk [16]. IGF can be produced by the liver, brain, kidney, pancreas and muscle, and affects nearly every cell type in the human body, including liver, kidney, nerves, skin, lungs, muscle and bone [17]. When functioning normally, a signal transduced through IGF receptor leads to activation of several intracellular pathways to maintain cell growth and survival, and these pathways are tightly controlled. However, overexpression of IGF receptors may lead to neoplastic transformation, inhibition of apoptosis, metastasis and angiogenesis, all of which are cell alterations conducive to the development of tumors [18]. Downstream targets of IGF receptor comprise a signaling network that regulates cell growth, proliferation, survival and metabolism via energy availability and supply of growth factor [15].

Increased expression of IGF/IGFR is observed in carcinomas and malignancies, including cancers of the breast, colon, stomach, pancreas, liver, lung, thyroid and ovaries [19]. Although the exact role of IGF in the disease progression of some cancers (e.g. breast, prostate) remains controversial, multiple studies have found that higher levels of IGF are predictive of both stage and risk of metastasis for several types of tumors, including colorectal, gastric, liver, lung and gallbladder [20-24]. As a whole, these

studies suggest the significance of IGF/IGFR expression as a possible indicator of tumor progression and outcome, although the data varies somewhat between specific types of cancers.

Leptin and cancer.

The development that first established adipose tissue to be an endocrine organ was the discovery of the adipokine leptin in 1994 [6]. A product of the *ob* gene, leptin was discovered as a hypothalamic regulator of body weight and energy balance by promoting a sensation of satiety upon adequate nourishment [25]. Research has shown that mice with mutations in the gene encoding leptin become morbidly obese, infertile, hyperphagic, hyperthermic and diabetic due to an insatiable appetite caused by a lack of leptin production, but human obesity is in fact related to increased levels of leptin, leading to leptin resistance [26, 27]. Although leptin may also be secreted from other sources, such as gastric mucosa and mammary epithelia, plasma concentrations of leptin are largely influenced by adipose tissue stores [28]. Increased concentrations of leptin in obese individuals is associated with both greater amounts of adipose tissue as well as increased leptin release from adipocytes, these levels are higher in women than in men, and synthesis of leptin correlates with the presence of other hormonal factors, including insulin.

The association between leptin and cancer risk is a multifaceted relationship, as leptin is shown to have effects on several tumorigenic pathways. Leptin has been suggested to play a role vascular remodeling via angiogenesis, both by itself and coupled with vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), a necessary trait in the survival of tumors [29]. Leptin also increases endothelial cell

hyperplasia and suppresses apoptosis, both of which are contributing factors in tumorigenesis [30]. The stimulation of several types of pre-neoplastic and neoplastic cells *in vitro* and in animal models appears to promote angiogenesis and tumor invasion [31, 32]. Leptin has also been found to play more specific roles *in vitro* depending in the location of the cancer and type of tumors: increased cell proliferation has been associated with leptin in cancers of the breast, prostate, ovary and lung, suppression of apoptosis by leptin is seen in colorectal and prostate cancer, and increased pathway activation (e.g. ERK, STAT, PKC-α) in breast, colorectal, pancreatic, ovarian and lung cancers [28]. Because leptin can be secreted by both adipose tissue as well as tumors themselves, local concentrations of leptin are crucial for tumor development, and may prove to become a useful target for inhibition in cancer treatment and prevention.

IL-6 and cancer.

Interleukin-6 (IL-6) is a cytokine that is produced by many tissues in the body, including adipose tissue, thereby also being classified as an adipokine. Previous studies have demonstrated that IL-6 is secreted by subcutaneous adipose tissue and therefore triggers systemic effects, particularly in obese subjects [33]. IL-6 is also secreted from perivascular adipose tissue, which decreases the protective vasodilation of small arteries in obese subjects [34]. Adipose tissue has been found to contribute up to 35% of circulating levels of IL-6, leading to systemic effects as well as tissue-specific pleiotropic functions [35]. One of these effects is an increase in serum C-reactive protein (CRP), a blood protein released in response to inflammation, serving as a further indication that obesity is a disease of chronic inflammation. Park *et al.* found that IL-6 and CRP concentrations are significantly correlated with weight, BMI, waist/hip circumference

and waist/hip ratio [36]. IL-6 also has direct central actions, as evidenced by the discovery of IL-6 receptors on the hypothalamus, making IL-6 a possible vehicle of information transfer from adipocytes to the hypothalamus with respect to regulation of energy balance [6].

The mechanism of action for IL-6 begins with binding of the molecule to a hexametric receptor that includes an IL-6 receptor and a signal transducer; the attachment of IL-6 and its receptor causes phosphorylation of STAT3 proteins that directly bind to target genes, mediating the expression of a variety of genes that affect cellular processes such as cell growth and apoptosis [37]. The link between IL-6 and tumorigenesis has been proposed to involve the activation of this pathway [38-40]. A previous study of IL-6 and its implications specifically for colon cancer concluded that tumor-infiltrating macrophages have the ability to release IL-6, increasing levels of intratumor IL-6 that condition the cancer cells to secrete IL-6 themselves, thereby perpetuating a favorable environment for tumor growth and infiltration [41]. IL-6 is also been implicated in several other types of cancer; serum IL-6 levels were found in patients with advanced stage or metastatic cancers of the breast, gastrointestinal tract, lymph nodes, lung, skin, ovary, pancreas, prostate and kidney [42-51]. Conversely, other evidence in the literature indicates that IL-6 also acts as an inhibitor of cancer progression, for example, exhibiting the anti-cancer function of growth inhibition for some cancers of the prostate, colon and breast [52-54].

Adiponectin and cancer.

Adiponectin is another adipokine; however, unlike the previously aforementioned pro-inflammatory adipokines, adiponectin functions as an anti-inflammatory hormone by

suppressing the migration of inflammatory mediators, such as monocytes and macrophages, to inhibit inflammation [55]. Although it is secreted both as an active high-molecular weight (HMW) complex and a low-molecular weight (LMW) complex, the HMW complex is primarily associated with the known beneficial physiological and metabolic effects [56]. Adiponectin reduces plasma concentrations of triglycerides and fatty acids while increasing expression of proteins necessary for lipid metabolism, leading to an overall improvement of fatty acid catabolism, particularly in skeletal muscles [57]. Adiponectin may be involved in increased insulin sensitivity, possibly as a result of its roles in increased lipid oxidation, improvement of insulin signaling, inhibition of gluconeogenesis, or inhibition of TNF-α [55]. The combination of increased insulin sensitivity and improved lipid catabolism indicates that adiponectin has a strong effect on regulation of energy balance and body weight, similar to that of leptin. Although most other adipokines, including leptin, are upregulated and found in increased circulating levels in obesity, adiponectin expression and concentration is markedly reduced in overweight and obese subjects [58]. Therefore, the diminished synthesis of adiponectin as a result of obesity may lead to dysregulation of the production of pro-inflammatory cytokines, thereby leading to increased insulin resistance and inflammation [55]. A negative correlation between circulating levels of adiponectin and obesity (particularly android obesity), insulin resistance and type 2 diabetes has already been well established, and has been propagated by evidence that serum adiponectin levels increase when loss of adipose tissue occurs [59].

High circulating levels of adiponectin have several protective effects against the development of cancer. Adiponectin also negatively regulates hematopoiesis,

downregulates immune system response, induces apoptosis in certain cancer cell lines *in vitro*, and modulates expression of apoptosis-related genes [60]. Adiponectin exhibits an antiproliferative effect by binding several mitogenic growth factors, inhibiting superoxide production, and negatively regulating angiogenesis [61-63]. Given these protective effects, there are also strong indications that decreased adiponectin may have a causal role in the development of several types of cancer. Kelesidis *et al.* reported that adiponectin levels are inversely associated *in vivo* with increased risk of cancers associated with obesity and insulin resistance, including cancers of the endometrium, breast (particularly post-menopausal), colon, stomach and prostate, as well as leukemia [59]. This may be due in part to the relationship between adiponectin and insulin, in that hyperinsulinemia associated with decreased adiponectin may lead to an increase in circulating IGF1, which in turn promotes cellular proliferation, inhibition of apoptosis and increased secretion of VEGF, all of which are necessary components of tumorigenesis [64].

Colon Cancer

Colon cancer is the fourth most common cancer in the United States; it is estimated that 142,570 adults (72,090 men and 70,480 women) will be diagnosed with and 51,370 men and women will die of cancer of the colon and rectum in 2010 [65]. The rates of incidence and mortality of colon cancer vary by race and ethnicity as well, with the highest incidence of colon cancer in African Americans, followed by Caucasians, Native Americans, Asian/Pacific Islander and Hispanic Americans. Several migrant studies regarding cancer and mortality rates found that a migrant person's risk of many cancers, including colorectal cancer, changes to match that of the country to which

migration occurred, particularly those with Westernized cultures [66, 67]. Ethnic and racial differences in development of colon cancer indicate that environmental factors may heavily influence etiology of colon cancer and is therefore avoidable in theory. Although colon cancer is widely believed to be an environmental disease, risk may include a complex interaction of genetic, cultural, social and lifestyle practices [68].

Although the aforementioned "environmental factors" that are widely believed to contribute to colon cancer are less well-defined, there are several risk factors that are common throughout populations with a high incidence of colon cancer. Countries that are more developed, affluent and Westernized have much higher rates of colon cancer that are often attributed to greater frequency of obesity, physical inactivity, alcohol consumption and tobacco use, especially as compared to less-developed countries. Dietary risk factors include the consumption of a diet high in red and/or processed meats, saturated and trans fats, low intake of dietary fiber, and may include ingested carcinogens (e.g. heterocyclic amines from charred meat) [69, 70].

A majority of colon cancers (estimated at about 80%) develop from the transformation of sporadic adenomatous polyps [71]. Polyp formation is often caused by one or more mutations of the *adenomatous polyposis coli* (APC) gene, a known tumor suppressor. Mutations in this gene may cause dysfunction of the proteins produced by APC, rendering them ineffective in the suppression of uncontrolled cellular growth, and lead to the development of familial adenomatous polyposis (FAP). Individuals with FAP may develop anywhere from several hundred to several thousand polyps lining the large intestine; these polyps develop as benign growths, but eventually become malignant unless the colon is removed. In cases of classic FAP, the average age at which an

individual is diagnosed with colon cancer is 39 years [72]. Family history of colon cancer amplifies lifetime risk of death by colon cancer anywhere from a 2-4 fold increase, depending on number of first-degree relatives affected and at what age(s) the cancer presented.

A model for molecular determinants of colorectal tumorigenesis was first proposed by Fearon and Vogelstein in 1990 and outlined the successive genetic changes and the specific genes involved in the development of colorectal cancer, including APC, k-Ras, DCC and p53 [73]. Normal epithelial tissue becomes mutated to form regions of hyperproliferative monoclonal epithelial cells. This neoplastic tissue results in the successive formation of early, intermediate and late stage adenomas, and finally mutate into carcinomas. Although Fearon and Vogelstein stressed that the overall accumulation of genetic alterations was more important than the order in which the changes occurred, subsequent research revealed more information on the functions of key molecules in the original model.

In a 2000 review, Arends outlined the findings about the specific role of certain genes in the Vogelstein model. APC, originally thought to be involved in cell adhesion via binding with β-catenin, is bound to other molecules such as EB1, and was also shown to induce apoptosis in APC-deficient cell lines [74]. The k-Ras oncogene is a signal transduction molecule that becomes activated when paired with GTP and is inactivated in the presence of GTPase-activating proteins (GAPs). If the Ras product mutates, it becomes less sensitive to hydrolysis by GAPs, induces greater cellular proliferation, and inhibits apoptosis, all of which are factors essential to the growth of tumors. The cell adhesion-like molecule DCC (Deleted in Colorectal Cancer), an oncosuppressor gene that

was believed to have been involved in 18q deletions and influenced negatively on prognosis, was determined to be involved in axon guidance rather than deletions [75]. These genetic alterations, however, are much more conducive of the development of polypoid rather than nonpolypoid lesions.

A remaining minority of colon cancers are not attributable to polyp formation. Roughly 1-7% of all diagnosed cases of colon cancer are attributable to hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome [76]. The most common hereditary cause of colon cancer, HNPCC is caused by mutations in five specific genes that are involved in DNA mismatch repair: MLH1, MLH2, MSH6, PMS and PMS2. A hallmark implication in most cancers, microsatellite instability occurs when repeated sequences of DNA (microsatellites), normally of a set length, become longer or shorter than usual. These altered-length sequences are usually corrected via mismatch repair; however, if these genes become defective, stability of the genome is severely compromised. DNA replication errors may further alter major tumor suppressor genes or activate oncogenes, thereby greatly increasing the risk of developing malignancies. Individuals with HNPCC have roughly an 80% lifetime risk for colon cancer, compared to a less than 5% lifetime risk for those without HNPCC.

Furthermore, there is a clear association between inflammatory bowel diseases (such as Crohn's disease or ulcerative colitis [UC]) and colon cancer, indicating that patients with IBD, particularly UC, have up to a 20-fold higher risk of developing colon cancer than the general population. Eaden *et al.* conducted a meta-analysis of 116 studies and found that the prevalence of colon cancer in patients with UC is approximately 3.7%

(95% CI 3.2–4.2, $p<0.0001$), with the cumulative probability reaching 18% by 30 years, regardless of disease severity [77].

Ulcerative Colitis and Colon Cancer

Although the etiology of ulcerative colitis is uncertain, there are indications that the immune systems of individuals with UC react abnormally to bacteria in the digestive tract, and that these immune mechanisms are important in the pathogenesis of the disease and potential for subsequent dysplasia and tumor development [78-80]. In a manner similar to that in which it responds to bacterial and viral pathogens, the immune system also reacts to cancer cells, in that such cells are recognized as an invading foreign entity against which the body must be defended. The changes that occur to the surface of a cell during malignant transformation cause the antigens present on that cell to change as well, which in turn trigger the immune system into recognizing the cancerous cell as "non-self" and mounting an immune response. The theory of immunosurveillance suggests that lymphocytes, including macrophages, natural killer cells and cytotoxic T cells, constantly patrol the body and eliminate mutated cells [81]. When this surveillance system is either overwhelmed or dysfunctional, the cancerous cells can evade the immune system and tumors may develop.

Macrophages and ulcerative colitis.

Immune system dysregulation associated with UC includes dysfunction and/or imbalances of several types of immune cells. Macrophages are phagocytic white blood cells that play a role in both innate (non-specific) and humoral (specific) immunity by acting as antigen presenting cells. In an innate immune capacity, macrophages work to engulf and digest invading pathogens as well as the cellular debris remaining from other

immune cell response. The role of macrophages in humoral immunity stems from the release of chemokines to stimulate other lymphocytes to respond to the pathogen. The histopathological characterization of UC involves infiltration of activated macrophages within the colonic mucosa, and these cells are responsible for the release of inflammatory cytokines TNF-α, IL-1β, and IL-6, implicating pathogenesis of the disease [82]. In specific response to cancer cells, around which macrophages have been found to cluster, they partake in antitumor activity mediated by lytic enzymes and reactive oxygen or nitrogen intermediates, as well as the production of tumor necrosis factor (TNF), a chemokine that activates cytolysis of tumor cells [83].

Natural killer cells and ulcerative colitis.

Natural killer (NK) cells are capable of destroying foreign and dysfunctional cells via degranulation and induction of cell lysis. Unlike other lymphocytes, however, natural killer cells can recognize infected and cancerous cells non-specifically and without previous exposure or antigen presentation. Because NK cells have such marked cytolytic abilities as well as the potential for self-reactivity, the action of NK cells is strongly regulated. An NK cell must receive an activating signal, such as the binding of an antibody to its Fc receptor molecule, before it becomes fully activated and functional. The levels of NK cytotoxicity in UC are related to the clinical activity of the disease; in an active disease state, NK cells are present in normal numbers but are functionally defective, whereas NK cells exhibit normal cytotoxic activity in an inactive disease state [84]. The inflammation and damage caused by increased secretion of inflammatory cytokines during an active disease state may be triggered by false recognition and processing of the natural gut commensal bacteria by NK cells [85]. Neither macrophages

nor NK cells are MHC restricted and both express Fc receptors, indicating that these cells can bind antibody directly on the tumor cells through antibody-dependent cell-mediated cytotoxicity [83]. Recent studies have identified a specific population of NK cells that express the transcription factor RORγt^{+}NKp46^{+}; these cells provide an innate source of IL-22 and play a protective role in mucosal inflammation, in that a reduction or absence of this cell type results in worsened colitis [86-89].

T cells and ulcerative colitis.

Cytotoxic T cells, also known as T_C or killer T cells, are capable of killing cells that are infected, damaged or otherwise dysfunctional, including cancerous cells. T_C cells are particularly important in the pathogenesis of UC, as evidenced by extensive T_C cell infiltration within intestinal lesions and contribution to epithelial cell damage [90, 91]. In order to function, T_C cells must first be activated, a process that requires two signals to occur between an antigen presenting cell (APC) and the T_C cell. Peptide fragments (antigens) from a dysfunctional cell bind to MHC class I molecules on the surface of the APC. This antigen-bound MHC class I molecule must bind to the T_C cell antigen receptor (TCR) to provide the first signal of activation. The second signal arises from costimulation of the CD28 molecule on the T_C cell and either the CD80 or CD86 molecules on the APC [92]. After the second signal is received and the T_C cell becomes activated, it uses IL-2 as a growth and differentiation factor to commence clonal expansion, exponentially increasing the number of T_C cells that will specifically target

cells that express the same antigen [92]. Once bound to a target cell, the T_C cell releases perforin and granulysin, enzymes that perforate the membrane of the target cell and trigger caspase cascades that lead to apoptosis.

After the immune response has taken place and the invading or threatening cells are destroyed, regulatory T cells (T_{reg}) work to suppress immune activation, prevent self-reactivity and maintain homeostasis of the immune system by inhibiting the proliferation and effector functions of other T cell populations [93]. T_{reg} cells are uniquely defined from other thymus-derived immune cells by the transcription factor FoxP3, which controls the development and function of the regulatory cell [94]. T_{reg} cells also identified by the expression of CD25, a high-affinity IL-2 receptor α-chain that is also present on the surface of activated T_H cells [93]. The role of the T_{reg} cell in immune regulation is especially important in the pathology of inflammatory bowel diseases, as the loss of homeostasis between T_{reg} cells and proinflammatory T helper 17 (T_H17) cells is thought to trigger inflammation associated with these diseases. Specifically in the case of UC, there is a decrease in T_{reg} cells and an increase in T_H17 effector cells in peripheral blood, and consequently a significant decrease in the ratio of T_{reg}/T_H17 cells, which is associated with an increased proinflammatory state [93].

UC has historically been thought to be mediated by the T_H2 immune response, in which there is high antibody production relative to the activity of cytotoxic T cells, however recent studies strongly suggest that T_H17 cells play a more pivotal role in the

propagation of immune-mediated tissue damage [78, 95, 96]. There is not yet a clear consensus regarding the cytokines involved in the development of T_H17 cells; however initial *in vivo* mouse and *in vitro* human studies have identified IL-6, TGF-β, IL-1, IL-21 and IL-23 as differentiating factors [95, 97-99]. Developmentally distinct from other T helper cells, T_H17 cells are a unique subset of T_H cells that are defined by the production of IL-17A and IL-17F, cytokines implicated in the induction and mediation of proinflammatory responses, as well as IL-22 and IL-26.

IBD, Chronic Inflammation and Colon Cancer

The chronic inflammatory damage to intestinal epithelial cells that is characteristic of IBD is believed to make an individual more susceptible to cancer via accumulating mutations in rapidly dividing cells. If the mistake causes the dividing cell to experience microsatellite instability and become dysplastic, the cell may become cancerous. Histological differences between polypoid and nonpolypoid lesions render the latter to be more difficult to detect; rather than an obvious polyp, lesions are flat and more difficult to identify. Because these dysplastic cells do not develop in polyp form, rather in tissue that appears otherwise normal, an IBD-related flat lesion may only be found at later stages of cancer progression, thereby reducing the rate of survival [100]. Associated carcinomas are often infiltrative without obvious protruding masses; therefore, early screening for colon cancer is of utmost importance for patients with long-term IBD [101].

Patients with a family history of colon cancer (presenting at an early age), chronic UC or primary sclerosing cholangitis-associated neoplasia have an increased risk of developing colon cancer. Due to the fact that most colon cancers develop from a

malignant change in colon epithelial cells that may have developed decades prior, regular screening of asymptomatic individuals is a necessary measure to ensure that any potentially threatening lesions are identified as early as possible so as to improve treatment and prognosis. Screening techniques include fecal occult blood tests, stool tests, barium enemas, sigmoidoscopy and colonoscopy. Screening is conducted on patients who present with suspicious signs and symptoms, but is also necessary for people older than 40 and those with family history of colon cancer, as age and familial risk are the two most common risk factors for the development of colon cancer [102].

Symptoms of colon cancer depend on location and type of lesion(s), extent of lesion formation, and any complications associated with polyp formation. Initial signs and symptoms include bleeding upon defecation, abdominal pain, and fatigue and weakness, likely caused by anemia due to intestinal blood loss [103]. Patients may also experience changes in bowel habits, often alternating between constipation and diarrhea or increased stools. Some may first experience symptoms associated with metastatic disease, including ascites, swollen supraclavicular lymph nodes, and enlarged liver. Those with non-polypoid cancer may only experience gastrointestinal symptoms (pain, change in bowel habits) and weight loss from inadequate absorption of nutrients in the gut.

Altered cell signaling in colon cancer.

A shared attribute of both polypoid and nonpolypoid lesions is that of microsatellite instability, although it is more frequent in nonpolypoid cancers [104, 105]. As in the case of chronic inflammation in IBD, damaged cells develop defects in the DNA repair mechanism and thereby develop microsatellite instabilities. These

instabilities can either alter or inactivate tumor suppressor genes and signaling pathways that influence regulation of the cell cycle, ultimately affecting cell proliferation.

TGF-β is one such signaling pathway that is especially important in the process of tumorigenesis because it controls proliferation, differentiation, apoptosis, homeostasis and other functions of cells [106]. The process by which this control occurs, known as the TGF-β signaling pathway, takes place when one or more TGF-β superfamily ligands bind to a type II receptor, in turn phosphorylating a type I receptor. The phosphorylated type I receptor itself phosphorylates complexes SMAD2, SMAD3 and SMAD4, which accumulate in the nucleus, acting as transcription factors for the regulation of specific gene expression in the cell. When functioning normally, TGF-β decreases the likelihood of cell proliferation in all forms, most importantly cancer cells. Recent research has indicated that development of cancerous intestinal epithelial cells can be attributed to the loss of growth-inhibiting response to TGF-β due to one or more of three possible pathways: 1) the cancerous cells have lost expression of TGF-β receptors entirely, 2) the cells may only express a nonsignaling TGF-β receptor, or 3) the postreceptor signal-transducing mechanism in the cell may be defective [107].

Multiple studies of inactivating mutations in the TGF-β ligands, the TGF-β receptor, SMAD2 or SMAD4 complexes resulted in either no development of colon cancer (in heterozygous mice) or premature death of mice in utero or shortly after birth (in homozygous mice); nonetheless, the function of SMAD3 was not addressed in these studies [108]. Zhu *et al.* studied the potential role of SMAD3 in the development and progression of tumors by inactivating the SMAD3 gene in homologous recombination. As opposed to homozygous SMAD2 or SMAD4 mice, homozygous SMAD3 -/- mice

were found to be viable, but spontaneously presented with invasive colorectal adenocarcinomas throughout all layers of the intestinal wall that metastasized to lymph nodes. This spontaneous tumorigenesis demonstrated a clear role of SMAD3 in the development of nonpolypoid colorectal cancer, and provided a strong animal model for continued research. Recent epidemiologic and genetic studies in IBD-related animal models suggest that a combination of genetic susceptibility factors and altered immune response driven by microbial factors in the enteric environment contributes to the initiation and duration of inflammatory bowel diseases [109]. As previously reported by Maggio-Price *et al*, TGF-β dysregulation is involved in the development of colitis in *Helicobacter*-infected SMAD3-/- mice as well as humans with inflammatory bowel disease, conferring an increased risk for colon cancer; therefore, the induction of colon cancer in SMAD3-/- mice with *Helicobacter* is a relevant model to investigate the role of bacteria and inflammation in colon cancer in humans with disrupted TGF-β signaling [110]. The previous successes with the SMAD3-/- mouse model have established the ability of this model to investigate the relationship of diet and body composition to inflammation and the development of colon cancer.

Hypothesis and Specific Aims

We will use this model address the hypothesis that energy balance influences inflammatory bowel disease severity and subsequent colon cancer in the SMAD3-/- susceptible model. However, the model is poorly characterized. Therefore, we propose the following specific aims:

1. We will characterize the model for the following parameters a) weight, body composition and severity of colitis post-infection, and b) immune response after infection with *H. hepaticus*. We will assess immune response at 0, 3, 7 and 28 days post-infection by identifying alterations in baseline immune cell populations between SMAD3-/- and SMAD3+/- (control) mice, and determining how these populations change throughout the course of infection.
2. We will determine the effect of energy balance on the development of colitis and subsequent progression of colon cancer. Mice from each diet group (calorie-restricted, control and high-fat) will be infected with *H. hepaticus* and we will collect a) weight and body composition data, and b) colons and ceca at 4, 5, and 6 weeks post-infection for histopathological analysis.

These studies may be used as a basis of research upon which to investigate effective preventative measures against development of colon cancer.

BIBLIOGRAPHY

1. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM: Prevalence of overweight and obesity in the United States, 1999-2004. *Jama* 2006, 295(13):1549-1555.

2. National Health and Nutrition Examination Survey In.: National Center for Health Statistics, CDC, http://www.cdc.gov/nchs/; 2003-2004.

3. WCRF/AICR: Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. In. Washington DC; 2007.

4. Moayyedi P: The epidemiology of obesity and gastrointestinal and other diseases: an overview. *Dig Dis Sci* 2008, 53(9):2293-2299.

5. Nam SY, Kim BC, Han KS, Ryu KH, Park BJ, Kim HB, Nam BH: Abdominal visceral adipose tissue predicts risk of colorectal adenoma in both sexes. *Clin Gastroenterol Hepatol* 2010, 8(5):443-450 e441-442.

6. Trayhurn P, Wood I: Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004, 92(3):347-355.

7. Ronti T, Lupattelli G, Mannarino E: The endocrine function of adipose tissue: an update. *Clin Endocrinol (Oxf)* 2006, 64(4):355-365.

8. Guerre-Millo M: Adipose tissue and adipokines: for better or worse. *Diabetes Metab* 2004, 30(1):13-19.

9. Rayner D, Trayhurn P: Regulation of leptin production: sympathetic nervous system interactions. *J Mol Med* 2001, 79(1):8-20.

10. Suganami T, Ogawa Y: Adipose tissue macrophages: their role in adipose tissue remodeling. *J Leukoc Biol* 2010.

11. Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C: Definition of metabolic syndrome: report of the National Heart, Lung, and Blood

Institute/American Heart Association conference on scientific issues related to definition. *Arterioscler Thromb Vasc Biol* 2004, 24(2):e13-18.

12. Vainio H, Bianchini F: IARC handbooks of cancer prevention. Volume 6: Weight control and physical activity., vol. 6. Lyon, France: IARC Press; 2002.

13. Inadera H: The usefulness of circulating adipokine levels for the assessment of obesity-related health problems. *Int J Med Sci* 2008, 5(5):248-262.

14. Kahn B, Flier J: Obesity and insulin resistance. *J Clin Invest* 2000, 106(4):473-481.

15. Hursting S, Lashinger L, Wheatley K, Rogers C, Colbert L, Nunez N, Perkins S: Reducing the weight of cancer: mechanistic targets for breaking the obesity-carcinogenesis link. *Best Pract Res Clin Endocrinol Metab* 2008, 22(4):659-669.

16. Boyd D: Insulin and cancer. *Integr Cancer Ther* 2003, 2(4):315-329.

17. LeRoith D, Bondy C: Insulin-like growth factors. In: *Growth Factors and Cytokines in Health and Disease.* Edited by Derek L, Carolyn B, vol. Volume 1, Part 1: JAI; 1996: 1-26.

18. Moschos SJ, Mantzoros CS: The role of the IGF system in cancer: from basic to clinical studies and clinical applications. *Oncology* 2002, 63(4):317-332.

19. Samani A, Yakar S, LeRoith D, Brodt P: The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* 2007, 28(1):20-47. Epub 2006 Aug 2024.

20. Hakam A, Yeatman TJ, Lu L, Mora L, Marcet G, Nicosia SV, Karl RC, Coppola D: Expression of insulin-like growth factor-1 receptor in human colorectal cancer. *Hum Pathol* 1999, 30(10):1128-1133.

21. Jiang Y, Wang L, Gong W, Wei D, Le X, Yao J, Ajani J, Abbruzzese J, Huang S, Xie K: A high expression level of insulin-like growth factor I receptor is associated with increased expression of transcription factor Sp1 and regional lymph node metastasis of human gastric cancer. *Clin Exp Metastasis* 2004, 21(8):755-764.

22. Kornprat P, Rehak P, Ruschoff J, Langner C: Expression of IGF-I, IGF-II, and IGF-IR in gallbladder carcinoma. A systematic analysis including primary and corresponding metastatic tumours. *J Clin Pathol* 2006, 59(2):202-206.

23. Xie Y, Skytting B, Nilsson G, Brodin B, Larsson O: Expression of insulin-like growth factor-1 receptor in synovial sarcoma: association with an aggressive phenotype. *Cancer Res* 1999, 59(15):3588-3591.

24. All-Ericsson C, Girnita L, Seregard S, Bartolazzi A, Jager M, Larsson O: Insulin-like growth factor-1 receptor in uveal melanoma: a predictor for metastatic disease and a potential therapeutic target. *Invest Ophthalmol Vis Sci* 2002, 43(1):1-8.

25. Aloulou N, Bastuji-Garin S, Le Gouvello S, Abolhassani M, Chaumette M, Charachon A, Leroy K, Sobhani I: Involvement of the leptin receptor in the immune response in intestinal cancer. *Cancer Res* 2008, 68(22):9413-9422.

26. Huang L, Li C: Leptin: a multifunctional hormone. *Cell Res* 2000, 10(2):81-92.

27. Mark A, Correia M, Rahmouni K, Haynes W: Loss of leptin actions in obesity: two concepts with cardiovascular implications. *Clin Exp Hypertens* 2004, 26(7-8):629-636.

28. Garofalo C, Surmacz E: Leptin and cancer. *J Cell Physiol* 2006, 207(1):12-22.

29. Cao R, Brakenhielm E, Wahlestedt C, Thyberg J, Cao Y: Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proc Natl Acad Sci U S A* 2001, 98(11):6390-6395. Epub 2001 May 6398.

30. Artwohl M, Roden M, Holzenbein T, Freudenthaler A, Waldhausl W, Baumgartner-Parzer S: Modulation by leptin of proliferation and apoptosis in vascular endothelial cells. *Int J Obes Relat Metab Disord* 2002, 26(4):577-580.

31. Fenton JI, Hord NG, Lavigne JA, Perkins SN, Hursting SD: Leptin, insulin-like growth factor-1, and insulin-like growth factor-2 are mitogens in ApcMin/+ but not Apc+/+ colonic epithelial cell lines. *Cancer Epidemiol Biomarkers Prev* 2005, 14(7):1646-1652.

32. Bouloumie A, Drexler H, Lafontan M, Busse R: Leptin, the product of Ob gene, promotes angiogenesis. *Circ Res* 1998, 83(10):1059-1066.

33. Mohamed-Ali V, Goodrick S, Rawesh A, Katz D, Miles J, Yudkin J, Klein S, Coppack S: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997, 82(12):4196-4200.

34. Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates AP, Pemberton PW, Malik RA *et al*: Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* 2009, 119(12):1661-1670.

35. Kim J, Bachmann R, Chen J: Interleukin-6 and insulin resistance. *Vitam Horm* 2009, 80:613-633.

36. Park H, Park J, Yu R: Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. *Diabetes Res Clin Pract* 2005, 69(1):29-35. Epub 2004 Dec 2030.

37. Akira S: Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene* 2000, 19(21):2607-2611.

38. Syed V, Ulinski G, Mok SC, Ho SM: Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells. *J Natl Cancer Inst* 2002, 94(8):617-629.

39. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS: Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. *Cancer Res* 2003, 63(11):2948-2956.

40. Giri D, Ozen M, Ittmann M: Interleukin-6 is an autocrine growth factor in human prostate cancer. *Am J Pathol* 2001, 159(6):2159-2165.

41. Li YY, Hsieh LL, Tang RP, Liao SK, Yeh KY: Interleukin-6 (IL-6) released by macrophages induces IL-6 secretion in the human colon cancer HT-29 cell line. *Hum Immunol* 2009, 70(3):151-158.

42. Tempfer C, Zeisler H, Sliutz G, Haeusler G, Hanzal E, Kainz C: Serum evaluation of interleukin 6 in ovarian cancer patients. *Gynecol Oncol* 1997, 66(1):27-30.

43. Yokoe T, Iino Y, Takei H, Horiguchi J, Koibuchi Y, Maemura M, Ohwada S, Morishita Y: Changes of cytokines and thyroid function in patients with recurrent breast cancer. *Anticancer Res* 1997, 17(1B):695-699.

44. Goydos J, Brumfield A, Frezza E, Booth A, Lotze M, Carty S: Marked elevation of serum interleukin-6 in patients with cholangiocarcinoma: validation of utility as a clinical marker. *Ann Surg* 1998, 227(3):398-404.

45. Preti H, Cabanillas F, Talpaz M, Tucker S, Seymour J, Kurzrock R: Prognostic value of serum interleukin-6 in diffuse large-cell lymphoma. *Ann Intern Med* 1997, 127(3):186-194.

46. Dowlati A, Levitan N, Remick S: Evaluation of interleukin-6 in bronchoalveolar lavage fluid and serum of patients with lung cancer. *J Lab Clin Med* 1999, 134(4):405-409.

47. Mouawad R, Benhammouda A, Rixe O, Antoine E, Borel C, Weil M, Khayat D, Soubrane C: Endogenous interleukin 6 levels in patients with metastatic malignant melanoma: correlation with tumor burden. *Clin Cancer Res* 1996, 2(8):1405-1409.

48. Wierzbowska A, Urbanska-Rys H, Robak T: Circulating IL-6-type cytokines and sIL-6R in patients with multiple myeloma. *Br J Haematol* 1999, 105(2):412-419.

49. Okada S, Okusaka T, Ishii H, Kyogoku A, Yoshimori M, Kajimura N, Yamaguchi K, Kakizoe T: Elevated serum interleukin-6 levels in patients with pancreatic cancer. *Jpn J Clin Oncol* 1998, 28(1):12-15.

50. Nakashima J, Tachibana M, Horiguchi Y, Oya M, Ohigashi T, Asakura H, Murai M: Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin Cancer Res* 2000, 6(7):2702-2706.

51. Blay J, Rossi J, Wijdenes J, Menetrier-Caux C, Schemann S, Negrier S, Philip T, Favrot M: Role of interleukin-6 in the paraneoplastic inflammatory syndrome associated with renal-cell carcinoma. *Int J Cancer* 1997, 72(3):424-430.

52. Wang Q, Horiatis D, Pinski J: Interleukin-6 inhibits the growth of prostate cancer xenografts in mice by the process of neuroendocrine differentiation. *Int J Cancer* 2004, 111(4):508-513.

53. Poppenborg H, Knupfer M, Galla H, Wolff J: In vitro modulation of cisplatin resistance by cytokines. *Cytokine* 1999, 11(9):689-695.

54. Honma S, Shimodaira K, Shimizu Y, Tsuchiya N, Saito H, Yanaihara T, Okai T: The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells. *Endocr J* 2002, 49(3):371-377.

55. Beltowski J: Adiponectin and resistin--new hormones of white adipose tissue. *Med Sci Monit* 2003, 9(2):RA55-61.

56. Plaisance E, Grandjean P, Brunson B, Judd R: Increased total and high–molecular weight adiponectin after extended-release niacin. *Metabolism* 2008, 57(3):404-409.

57. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N *et al*: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 2001, 7(8):941-946.

58. Hu E, Liang P, Spiegelman BM: AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996, 271(18):10697-10703.

59. Kelesidis I, Kelesidis T, Mantzoros CS: Adiponectin and cancer: a systematic review. *Br J Cancer* 2006, 94(9):1221-1225.

60. Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, Kihara S, Funahashi T, Tenner AJ, Tomiyama Y *et al*: Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 2000, 96(5):1723-1732.

61. Goldstein BJ, Scalia R: Adiponectin: A novel adipokine linking adipocytes and vascular function. *J Clin Endocrinol Metab* 2004, 89(6):2563-2568.

62. Brakenhielm E, Veitonmaki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y: Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci U S A* 2004, 101(8):2476-2481.

63. Wang Y, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A: Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J Biol Chem* 2005, 280(18):18341-18347.

64. Calle EE, Kaaks R: Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004, 4(8):579-591.

65. Altekruse S, Kosary C, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H *et al*: SEER Cancer Statistics Review 1975-2007. In. Bethesda, MD: National Cancer Institute; 2010.

66. Keys A, Menotti A, Karvonen MJ, Aravanis C, Blackburn H, Buzina R, Djordjevic BS, Dontas AS, Fidanza F, Keys MH *et al*: The diet and 15-year death rate in the seven countries study. *Am J Epidemiol* 1986, 124(6):903-915.

67. Kune S, Kune GA, Watson L: The Melbourne colorectal cancer study: incidence findings by age, sex, site, migrants and religion. *Int J Epidemiol* 1986, 15(4):483-493.

68. Boyle P, Langman JS: ABC of colorectal cancer: Epidemiology. *Bmj* 2000, 321(7264):805-808.

69. Singh P, Fraser G: Dietary risk factors for colon cancer in a low-risk population. *Am J Epidemiol* 1998, 148(8):761-774.

70. Park Y, Hunter D, Spiegelman D, Bergkvist L, Berrino F, van den Brandt P, Buring J, Colditz G, Freudenheim J, Fuchs C *et al*: Dietary fiber intake and risk of colorectal cancer: a pooled analysis of prospective cohort studies. *JAMA* 2005, 294(22):2849-2857.

71. Livstone E: Colorectal Cancer. In: *Merck Manuals*. 2008.

72. Gordon PH NS: Principles and Practice of Surgery for the Colon, Rectum and Anus., 2nd edn. New York, NY: Informa Health Care; 2007.

73. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. *Cell* 1990, 61(5):759-767.

74. Arends J: Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol* 2000, 190(4):412-416.

75. Keino-Masu K, Masu M, Hinck L, Leonardo E, Chan S, Culotti J, Tessier-Lavigne M: Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* 1996, 87(2):175-185.

76. Silva FC, Valentin MD, Ferreira Fde O, Carraro DM, Rossi BM: Mismatch repair genes in Lynch syndrome: a review. *Sao Paulo Med J* 2009, 127(1):46-51.

77. Eaden JA, Abrams KR, Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001, 48(4):526-535.

78. Monteleone I, Pallone F, Monteleone G: Interleukin-23 and Th17 cells in the control of gut inflammation. *Mediators Inflamm* 2009:297645.

79. Yeung MM, Melgar S, Baranov V, Oberg A, Danielsson A, Hammarstrom S, Hammarstrom ML: Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-gammadelta expression. *Gut* 2000, 47(2):215-227.

80. Tlaskalova-Hogenova H, Tuckova L, Stepankova R, Hudcovic T, Palova-Jelinkova L, Kozakova H, Rossmann P, Sanchez D, Cinova J, Hrncir T *et al*: Involvement of innate immunity in the development of inflammatory and autoimmune diseases. *Ann N Y Acad Sci* 2005, 1051:787-798.

81. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD: Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002, 3(11):991-998.

82. Murakami H, Akbar SM, Matsui H, Onji M: Macrophage migration inhibitory factor in the sera and at the colonic mucosa in patients with ulcerative colitis: clinical implications and pathogenic significance. *Eur J Clin Invest* 2001, 31(4):337-343.

83. Kindt T, Goldsby R, Osborne B: Kuby Immunology, 6th edn. New York: W.H. Freeman; 2006.

84. Manzano L, Alvarez-Mon M, Abreu L, Antonio Vargas J, de la Morena E, Corugedo F, Durantez A: Functional impairment of natural killer cells in active ulcerative colitis: reversion of the defective natural killer activity by interleukin 2. *Gut* 1992, 33(2):246-251.

85. Baumgart D, Carding S: Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007, 369(9573):1627-1640.

86. Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz J, Doherty J, Mills J, Colonna M: A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 2009, 457(7230):722-725. Epub 2008 Nov 2002.

87. Sanos S, Bui V, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A: RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol* 2009, 10(1):83-91. Epub 2008 Nov 2023.

88. Luci C, Reynders A, Ivanov I, Cognet C, Chiche L, Chasson L, Hardwigsen J, Anguiano E, Banchereau J, Chaussabel D *et al*: Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol* 2009, 10(1):75-82. Epub 2008 Nov 2023.

89. Zenewicz L, Yancopoulos G, Valenzuela D, Murphy A, Stevens S, Flavell R: Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 2008, 29(6):947-957.

90. Ueyama H, Kiyohara T, Sawada N, Isozaki K, Kitamura S, Kondo S, Miyagawa J, Kanayama S, Shinomura Y, Ishikawa H *et al*: High Fas ligand expression on lymphocytes in lesions of ulcerative colitis. *Gut* 1998, 43(1):48-55.

91. Yonamine Y, Watanabe M, Kinjo F, Hibi T: Generation of MHC class I-restricted cytotoxic T cell lines and clones against colonic epithelial cells from ulcerative colitis. *J Clin Immunol* 1999, 19(1):77-85.

92. Lefrancois L, Obar JJ: Once a killer, always a killer: from cytotoxic T cell to memory cell. *Immunol Rev* 2010, 235(1):206-218.

93. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S: Foxp3(+) Regulatory T Cells, Th17 Effector Cells, and Cytokine Environment in Inflammatory Bowel Disease. *J Clin Immunol* 2009.

94. Zhang L, Zhao Y: The regulation of Foxp3 expression in regulatory CD4(+)CD25(+)T cells: multiple pathways on the road. *J Cell Physiol* 2007, 211(3):590-597.

95. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F *et al*: Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007, 8(9):950-957.

96. Bamias G, Cominelli F: Immunopathogenesis of inflammatory bowel disease: current concepts. *Curr Opin Gastroenterol* 2007, 23(4):365-369.

97. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B: TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006, 24(2):179-189.

98. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F: Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007, 8(9):942-949.

99. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH: Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 2009, 31(2):331-341.

100. Soetikno R, Kaltenbach T, Rouse R, Park W, Maheshwari A, Sato T, Matsui S, Friedland S: Prevalence of nonpolypoid (flat and depressed) colorectal neoplasms in asymptomatic and symptomatic adults. *JAMA* 2008, 299(9):1027-1035.

101. Kumar V AA, Fausto N: Robbins & Cotran Pathological Basis of Disease. In., 7th edn: Saunders; 2004.

102. Burt R: Inheritance of Colorectal Cancer. *Drug Discov Today Dis Mech* 2007, 4(4):293-300.

103. Gund S: Colon Cancer. In: *MedlinePlus Medical Encyclopedia.* NIH; 2008.

104. Korff S, Woerner S, Yuan Y, Bork P, von Knebel Doeberitz M, Gebert J: Frameshift mutations in coding repeats of protein tyrosine phosphatase genes in colorectal tumors with microsatellite instability. *BMC Cancer* 2008, 8:329.

105. Grady WM, Carethers JM: Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008, 135(4):1079-1099.

106. Munoz N, Baek J, Grady W: TGF-beta has paradoxical and context dependent effects on proliferation and anoikis in human colorectal cancer cell lines. *Growth Factors* 2008, 26(5):254-262.

107. MacKay S, Yaswen L, Tarnuzzer R, Moldawer L, Bland KI, Copeland Er, Schultz G: Colon cancer cells that are not growth inhibited by TGF-beta lack functional type I and type II TGF-beta receptors. *Ann Surg* 1995, 221(6):767-776; discussion 776-767.

108. Zhu Y, Richardson JA, Parada LF, Graff JM: Smad3 mutant mice develop metastatic colorectal cancer. *Cell* 1998, 94(6):703-714.

109. Wirtz S, Neufert C, Weigmann B, Neurath MF: Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007, 2(3):541-546.

110. Maggio-Price L, Treuting P, Zeng W, Tsang M, Bielefeldt-Ohmann H, Iritani BM: Helicobacter infection is required for inflammation and colon cancer in SMAD3-deficient mice. *Cancer Res* 2006, 66(2):828-838.

Chapter 2 - Identification of kinetic and tissue-specific changes in natural killer cell and T cell populations during *Helicobacter hepaticus* infection in SMAD3-/- mice

Introduction

Individuals with inflammatory bowel disease (IBD), particularly ulcerative colitis (UC), are at a higher risk of developing colon cancer than the general population [1]. A meta-analysis from 116 studies indicated that the prevalence of colon cancer in patients with UC is approximately 3.7% (95% CI 3.2–4.2, $p<0.0001$), with the cumulative probability reaching 18% by 30 years regardless of disease severity [2]. Although the etiology of ulcerative colitis is poorly understood, there are indications that the immune system of individuals with UC react abnormally to bacteria in the digestive tract. This altered immune response is important in the pathogenesis of IBD and of dysplastic epithelial changes leading to tumor development [3-5].

Imbalances in both innate and adaptive immune cells, such as natural killer cells (NK) and T cell subsets, including CD4+ T helper (T_H), T regulatory (T_{reg}) cells and CD8+ cytotoxic (T_C) cells, are associated with UC [2]. The inflammation and damage caused by increased secretion of inflammatory cytokines during an active disease state is thought to be triggered by false recognition and processing of the natural gut commensal bacteria by NK cells [6]. The levels of NK cytotoxicity in UC are related to the clinical activity of the disease [7]. In active disease states, NK cells are present in normal numbers, but are functionally defective, whereas NK cells exhibit normal cytotoxic activity in an inactive disease state [7]. Induction of inflammatory cytokines can also result from the disruption of the homeostatic balance between T_{reg} and effector T_H cells.

Elevated levels of proinflammatory CD4+ T cells lead to excess cytokine/chemokine production, thereby recruiting additional leukocytes and influencing the severity of the inflammatory response [2]. Under normal conditions, T_{reg} cells differentiate in the Peyer's patches (PP) and mesenteric lymph nodes (MsLN) via action of the cytokine transforming growth factor β (TGF-β). Defects in the TGF-β pathway affect the development and/or function of T_{reg} cells resulting in intestinal inflammation in mice [2, 8]. T_C cells are also important in the pathogenesis of UC in humans, as evidenced by extensive T_C cell infiltration within intestinal lesions contributing to mucosal damage [9, 10].

The SMAD3-/- mouse model was recently developed as an animal model of human UC due to similar histological and immune cell changes present during the course of colitis [11-14]. In previous studies, the absence of SMAD3 caused a significant reduction in the ability of TGF-β to induce expression of Forkhead box P3 (FoxP3) on naïve T cells, which is required for the development and function of T_{reg} cells [15]. However, there is currently insufficient data describing changes in immune cell populations during the course of infection or between infection-prone (SMAD3-/-) and resistant (SMAD3+/-) mice. Understanding differential responses to infection may lead to future targets for treating human disease. Therefore, the current study was conducted to characterize SMAD3-dependent alterations in immune cell populations (T, B and NK cells) at baseline and throughout the course of infection with *Helicobacter hepaticus*.

Materials and Methods

Murine model.

SMAD3+/- and SMAD3-/- breeder pairs of 129SvEv background were generously donated by Dr. Lillian Maggio-Price at the University of Washington. Homozygous males and heterozygous females were mated to obtain SMAD3-/- pups. Genotypes were confirmed by PCR. Animals were housed under specific pathogen-free conditions in 60 square inch plastic cages (maximum of 5 adult mice per cage) with microisolator lids in an AAALAC approved facility at Michigan State University. Animal rooms were maintained at 23.3±2.2°C with a 12-hour light-dark cycle. Mice were fed Harlan Teklad 7913 rodent chow and sterile water *ad libitum*. Animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Bacterial culture and infection.

The wild-type *H. hepaticus* strain 3B1 (ATCC 51488) was kindly donated by Dr. Vince Young at the University of Michigan. Isolates were aseptically streaked onto sheep blood agar and incubated at 36°C for 24-48 hrs inside GasPak^{TM} gas generating pouch systems (BD Diagnostic Systems, Sparks, MD). Mice were infected as previously described [12]. Briefly, bacteria were collected and resuspended in tryptic soy broth at an optical density $\geq$ 1.8 (600nm wavelength). Animals were then gavaged with 0.3 mL dosages of fresh bacterial suspension on two consecutive days.

Experimental Design.

In study 1, SMAD3-/- mice (n = 50) at 8-10 weeks of age were infected with *H. hepaticus* to determine onset and duration of colitis. Colons and ceca were collected and

processed for histopathology at 2, 3, 4, 5, 6, 7, and 8 wks post infection. In study 2, SMAD3+/- (n = 24) and -/- mice (n = 19) at 8-10 weeks of age were infected with *H. hepaticus*. Weights (g) were taken daily and body composition was assessed at days 0, 3, 7, 14, 21 and 28. Spleen, PP and MsLN were collected at sacrifice at day 28 post-infection and processed for lymphocyte isolation as described below. Colons and ceca were collected and regularly processed for histopathology.

Body composition.

Body composition was analyzed using an EchoMRI-100^{TM} quantitative nuclear magnetic resonance machine (Echo Medical Systems, Houston, TX).

Tissue fixation and histopathology.

At the time of necropsy, mice were asphyxiated with CO2 and exsanguinated via cardiac puncture. Intact colons and ceca were removed and flushed with PBS. Tissues were fixed in 10% formalin overnight, embedded in paraffin, then sectioned and stained with hematoxylin and eosin (H&E). Longitudinal sections were graded for inflammation and dysplasia by pathologist using a blinded scoring system adapted from Dr. Maggio-Price [16]. Ceca and colons were scored on a 1 to 4 scale both for inflammation (1, no inflammation; 2, mild inflammation; 3, moderate inflammation; 4, marked inflammation) and dysplasia (1, no dysplasia; 2, low grade dysplasia; 3, high grade dysplasia; 4, high grade dysplasia with invasion/adenocarcinoma). The two scores for colon and two scores for cecum tissue in each animal were combined such that a score of 4 indicated no inflammation or dysplasia and a score of 16 reflected maximal inflammation and neoplasia.

Lymphocyte isolation.

Spleens, PP and MsLN were removed and placed in ice cold RPMI at the time of necropsy. Spleens were processed with a dounce homogenizer, pelleted, and washed in RPMI. Cells were resuspended in ACK lysing buffer (Invitrogen, Carlsbad, CA) and washed twice in RPMI. PP were treated with 5 mL enzymatic digest (5% FBS, 0.5mg/mL collagenase, 0.05mg/mL DNase I) for 30 minutes at 37°C. PP and MsLN were passed through a 70 μm filter and washed with RPMI. Cell counts were performed with a hemocytometer using trypan blue exclusion and resuspended to a concentration of one million cells per mL of media.

Flow cytometry.

Lymphocytes were resuspended in FACS buffer (0.1% sodium azide, 1% FBS, in dPBS) blocked with anti-Fc receptor γII/III [CD16/CD32 (purified from clone 2.4G2 hybridoma, ATCC, Manassas, VA)] for 10 min on ice, and subsequently incubated with combinations of the following fluorochrome-conjugated antibodies (E-bioscience, San Diego, CA; or BD Bioscience, San José, CA) at concentrations ranging from 1:100 to 1:300 in FACS buffer: CD3 (PerCP-Cy5.5), CD4 (eFluor450), CD8 (PE-Cy7), CD25 (PE), FoxP3 (FITC or Alexa Fluor488), CD62 (APC), Nkp46 (FITC), DX5 (APC) and CD19 (PerCP-Cy5.5). Cells were incubated in staining cocktails (one million cells per cocktail) on ice in the dark for 30 min. Intracellular staining was performed using FoxP3 staining buffer set as per the manufacturer's instructions (eBioscience). Briefly, after surface staining, cells were washed twice in FACS buffer, fixed in 4% paraformaldehyde for 25 min, and permeabilized for 30 min. Permeabilization was followed by incubation for 30 min with the appropriate antibodies diluted in permeabilization diluent. Samples

were then acquired on a LSR II (BD Bioscience) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Statistics.

Data analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Data were represented as mean ± SEM unless otherwise noted. Two-way ANOVA was performed with Bonferroni's Multiple Comparison Test to determine differences between two groups within a parameter unless noted otherwise. *P* values < 0.05 were considered significant.

Results

Weight and body fat percentage change throughout course of infection.

SMAD3-/- mice weighed less than aged-matched heterozygous mice (SMAD3+/-) at the beginning of the study (16.4 ± 1.7g versus 24.6 ± 2.1g, respectively). These differences remained significant during the course of infection with *H. hepaticus* ($P<0.05$; Figure 2.1A). There were no significant changes in body weights within genotypes.

Average body fat percentages of SMAD3-/- mice were significantly lower than heterozygous mice at days 14, 21 and 28 after infection (Figure 2.1B). By day 28, control and infected SMAD3-/- mice lost body fat (-4% ± 5% and -2% ± 2%, respectively) compared to baseline values. No changes in percent body fat were observed in uninfected SMAD3+/- mice whereas SMAD3+/- mice regained body fat by 28 days following infection (Figure 2.1B).

Histological scoring of colitis and dysplasia.

Histopathology scores in infected SMAD3-/- mice (Figure 2.1C) were dramatically increased at week 4 post-infection, with an average colitis score of 7.5 ± 0.49. This value was significant compared to samples taken at all other time points; colitis scores returned to baseline values in SMAD3-/- mice by 8 weeks post-infection. No significant changes in colitis or dysplasia scores were observed in SMAD3+/- mice at any time point (data not shown).

Differences in lymphocyte populations between SMAD3-/- and SMAD3+/- mice at baseline (pre-infection).

B cell percentages were similar between SMAD3-/- and SMAD3+/- mice at baseline in all tissues examined (Table 2.1). However, when calculated based upon the number of live cells, B cell numbers differed significantly between genotypes ($P<0.05$). SMAD3-/- had average B cell numbers of $1.8 \pm 0.4 \times 10^4$, $1.6 \pm 0.2 \times 10^4$, and $1.5 \pm 0.1 \times 10^4$, compared to heterozygote values of $2.1 \pm 1.2 \times 10^7$, $2.7 \pm 1.7 \times 10^6$, and $1.9 \pm 1.3 \times 10^6$ in the spleen, MsLN, and PP, respectively (data not shown). The percentage of natural killer (NK) cells in the lymphocyte populations from all three tissues were significantly lower at baseline in SMAD3-/- compared to heterozygous mice in all three tissues (Table 2.1). However, the total numbers of NK cells were reduced in only the spleen of SMAD3-/- mice compared to heterozygous mice ($2.1 \pm 0.4 \times 10^5$ vs $5.0 \pm 1.0 \times 10^5$, data not shown).

There were no significant differences between the two genotypes in percentages of CD3+ T cells in spleens at baseline (Table 2.1). The percentage of CD3+ T cells in

both MsLN and PP of SMAD3-/- mice were significantly lower than SMAD3+/- mice at baseline. However, there were no differences in total number of T cells in any of the tissues analyzed (data not shown). SMAD3-/- mice had higher percentages of CD4+/CD3+ T cells in spleens at baseline compared with SMAD3+/- mice (Table 2.1). There was no significant difference in percentages of CD8+/CD3+ T cells between genotypes in any tissue. Differences in CD3+ T cell subset populations at baseline in MsLN and PP were not statistically significant (Table 2.1).

***Lymphocyte population changes in SMAD3-/- and SMAD3+/- mice following infection with* H. hepaticus.**

There were no significant differences in B cell populations of spleen, MsLN or PP between SMAD3-/- and SMAD3+/- mice during the course of infection. In all three tissues, significant differences ($P<0.05$) in the percentage of NK cells were observed between genotypes during at least one of the four time points in the study (Figure 2.2). While the percentage of NK cells in the spleens of SMAD3+/- mice were significantly greater at days 3 and 28 post-infection (Figure 2.2A), the total number of NK cells was significantly higher only on day 28 (Figure 2.2B). By comparison, the percentages of NK cells in SMAD3-/- mice were significantly lower on day 3 compared to all other time points. In MsLN, SMAD3+/- mice had increased NK cell percentages at day 28 compared to SMAD3-/- mice, and decreased percentages of NK cells at days 3 and 7 within the genotype (Table 2.2); however there were no other significant differences related to total NK cell counts (data not shown). SMAD3-/- mice had both decreased percentages (Table 2.2) and total numbers of NK cells (data not shown) that did not change significantly throughout the course of infection in MsLN. SMAD3+/- mice also

had greater percentages of NK cells in PP than SMAD3-/- at all time points. This was only significant at baseline and at day 28 post infection (Table 2.2).

CD3+ T cell population changes were not significant in the spleen or PP (data not shown); however, SMAD3-/- mice had significantly reduced T cell numbers in the MsLN at all time points during infection. Total CD3+ T cells were lower in SMAD3-/- mice compared to SMAD3+/- at days 3, 7 and 28 ($P<0.05$; Figure 2.3A). There were significantly fewer T_H cells (CD4+/CD3+) throughout infection, whereas cytotoxic T cells (CD8+/CD3+) were only significantly lower at days 3 and 7 (Figure 2.3B and C). In all three tissues, the SMAD3+/- mice displayed a uniform increase in T cells and subset populations, with a significant peak at 7 days post-infection. Populations of T cells in SMAD3-/- mice were largely unchanged throughout infection, increasing slightly after infection (day 7) with no differences across time within the group.

T_{reg} cell populations differ between genotypes and across time in MsLN.

Total numbers of activated T_{reg} cells (FoxP3+/CD25+/CD4+) in the MsLN were significantly lower in SMAD3-/- mice than in SMAD3+/- mice at days 3 and 7 post-infection (Figure 2.4A). There were no significant changes in numbers of T_{reg} cells in SMAD3-/- mice across time, whereas total numbers of T_{reg} cells in SMAD3+/- were significantly increased at day 7. T_{reg} populations throughout course of infection mirrored that which was seen in overall T cell populations, in that SMAD3+/- T_{reg} populations increased through day 7 and decreased at day 28, whereas SMAD3-/- T_{reg} populations

increased through day 7 with no further changes. T_{reg} populations were not significantly different in the spleen or PP at any time point.

Expression of $CD62L^{lo}$ on T_{reg} cells was significantly higher in SMAD3+/- than in SMAD3-/- mice throughout infection (Figure 2.4B). $CD62L^{lo}$+ T_{reg} cells in SMAD3+/- were, on average, 9.4% higher in MsLN, 13.9% higher in spleens, and 34.7% higher in PP than in SMAD3-/- mice (data not shown). These increased percentages did not translate into significantly different total $CD62L^{lo}$+ cells in spleens or PP; however, total $CD62L^{lo}$+ cells in MsLN averaged 3.2 times higher in heterozygous mice (Figure 2.4B). Within the SMAD3+/- mice, total $CD62L^{lo}$+ cells were significantly increased at day 7 post-infection; within the SMAD3-/- mice, total $CD62L^{lo}$+ cells were significantly increased at day 28 post-infection.

Discussion

Chronic inflammation associated with UC causes damage to intestinal epithelial cells and is thought to make an individual more susceptible to cancer by altering or inactivating tumor suppressor genes and signaling pathways that influence regulation of the cell cycle [17]. TGF-β is an important growth factor in the process of colon tumorigenesis because it controls proliferation, differentiation, and apoptosis [18]. When functioning normally, TGF-β decreases the likelihood of cell proliferation [19]. TGF-β dysregulation is involved in the development of colitis in helicobacter-infected SMAD3-/- mice as well as humans with inflammatory bowel disease. Therefore, SMAD3-/-

mouse model of colitis is highly relevant to investigate the role of bacteria and inflammation in the pathogenesis of colon cancer.

In the present study, we demonstrate for the first time significant alterations in NK cells, T cells and T cell subsets between SMAD3-/- and SMAD3+/- mice, both at baseline and throughout the course of infection. The differences in lymphocyte populations in SMAD3-/- mice, as a percentage of total lymphocytes and total cell number, indicate that these mice have an inherently increased susceptibility to colitis due to a decreased ability to respond infection of *H. hepaticus*. Total numbers of B cells in SMAD3+/- mice at baseline were at least 127-fold higher in PP and up to 1200-fold higher in the spleen than in SMAD3-/- mice. B cells have been implicated in the suppression of colitis through interaction with T_{reg} cell subsets in other murine models of IBD [20-24]. B cells possess a number of immunological functions, such as immunoglobulin (Ig) secretion, antigen presentation and cytokine production. Therefore, B cells or products thereof may play a regulatory role in the pathogenesis of colitis, likely via rapid removal of apoptotic cells, maintenance of epithelial barrier and/or control of enteric bacterial colonization [20, 22]. Studies by Mizoguchi *et al.* indicate that B cells directly suppress proliferation of CD4+ T cells through CD40/CD154 interactions suggesting the existence of a novel B cell phenotype ($CD1d^{high}$) in MsLN and colonic lamina propria that suppresses the severity of colitis by down-regulating pro-inflammatory cascades associated with IL-1β and IL-6 [22, 25]. In addition, Wei *et al.* found that MsLN B cells contribute to immunoregulation and protect mice from CD4+ T cell colitis through recruitment of T_{reg} cell subsets to the site of infection [24]. Because

the SMAD3-/- mice in our study had such drastically reduced populations of B cells at baseline, it is possible that these mice have an inability to respond to infection and effectively control colitis, either from inadequate production of immunoglobulins and cytokines or through reduced capacity to recruit necessary T cell subset populations.

Similar differences were observed in NK cell populations in our study. We found that the percentage of NK cells in spleen, MsLN and PP were all significantly reduced in SMAD3-/- compared to SMAD3+/-, however the total NK cell numbers only differed in the spleen. NK cells have an important role in the eradication of bacterial infections via NK cell-derived IFN-γ production, which has been demonstrated in multiple *in vivo* models [26-32]. Our results support findings from other murine models in which immunosuppressed animals with depleted populations of NK cells develop colitis more rapidly and with increased severity than controls [33, 34]. Fort *et al.* suggested that the NK cells play an inhibitory role in development of colitis by lysing inflammatory effector T cells via a perforin-dependent mechanism [34]. Other studies provide evidence that NK cells are in fact an innate source of IL-22 in the colon, a cytokine that has proinflammatory properties but is also proposed to protect tissues during inflammation.[35-37] Taken together, these mechanisms consistent with our findings suggest that a depleted population of NK cells at baseline may result in an inherent inability to properly respond to bacterial infection, leading to colitis.

We were particularly interested in the differences in T cell subset populations between SMAD3-/- mice and controls, and how these subsets changed throughout the course of infection. We found that the most remarkable changes in lymphocyte populations occurred in the MsLN. All three T cell populations examined in SMAD3+/-

mice increased in number up to 7 days post-infection and returned to baseline levels by day 28. SMAD3-/- mice did not elicit a comparable response to these T cell populations, remaining close to baseline values throughout the course of infection. These data are consistent with a previous study by Maggio-Price *et al.*, in which there was no significant T cell response to infection with *Helicobacter in vitro*, although it is important to note that only splenic lymphocytes were assessed and that both *H. hepaticus* and *H. bilis* were used for infection [12]. These data indicate that SMAD3-/- mice are unable to mount a sufficient T cell response to bacterial infection, either by failing to increase the total population of T lymphocytes or, more specifically, CD4+ T_H cells and CD8+ T_C cells, thus exacerbating the effect of *H. hepaticus* infection on colitis.

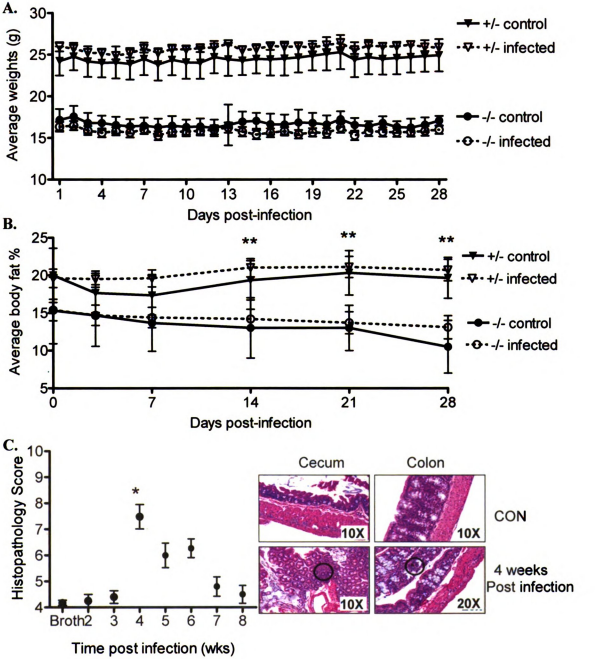
Data are lacking regarding immune cell population changes throughout the course of *H. hepaticus* infection and colitis in SMAD3-/- mice. Wang *et al.* investigated the changes of lymphocyte subpopulations, particularly CD4+/CD25+ T regulatory cells in SMAD3-/- mice. They reported significantly reduced levels of B cells in peripheral blood and spleen, as well as decreased population of CD8+ T cells in the thymus, compared to SMAD3+/+ littermate controls [13]. Our findings are somewhat consistent with this study, in that we also showed significantly decreased populations of B cells in SMAD3-/- mice at baseline as well as increased CD4+/CD25+ T cells. However, we did not find similar effects on T cell subsets through the course of infection, nor did Wang *et al.* observe any differences in MsLN, which was the site of most significant population differences in our study. The inconsistent findings between studies may reflect differences in experimental design: we compared SMAD3-/- to SMAD3+/- mice rather than wild type controls and we did not examine lymphocyte changes in blood or thymus

tissues. Furthermore, Wang *et al.* described the CD4+/CD25+ T cell subset population as T_{reg} cells, whereas we identified T_{reg} cells by using intracellular expression of FoxP3+ in addition to surface expression of CD4 and CD25, which better defines this population according to the recent literature. T_{reg} cells serve to reduce inflammation during infection, which is beneficial to the host [38]. Several studies show that depletion of T_{reg} cells in rodent models leads to an inflammatory colitis similar to human UC [39-42]. Furthermore, recent human studies have demonstrated reduced T_{reg} populations in patients with UC compared to controls both at baseline and during active stages of the disease, consistent with the patterns of immune response found in our studies [43, 44].

We also examined L-selectin (CD62L) expression on T_{reg} cells, which is required for the migration of T_{reg} cells and is cleaved from the surface upon activation [45, 46]. We found that, in addition to decreased numbers of T_{reg} cells, SMAD3-/- mice also had significantly decreased number of T_{reg} cells expressing CD62L in MsLN throughout infection. Our data suggests that absence of SMAD3 may decrease the ability of T_{reg} cells to migrate to lymph nodes in close proximity to the site of infection because L-selectin is important in lymphocyte migration to MsLN and PP [46]. Future studies will examine expression of homing receptors and on these lymphocytes and functional assays to further elucidate any differences in immune cell migration in SMAD3-/- mice during colitis.

In conclusion, we have characterized immune cell populations at baseline and during infection with *H. hepaticus*, as well as changes in colitis in the SMAD3 knockout model. We find that the changes in immune cells across time parallel the progression of the disease and are consistent with innate immune response to an acute infection. Importantly, baseline NK cells were lower in the SMAD3-/- and are a key part of the very early innate immune response to a pathogen, which may explain, in part, the exacerbation of colitis in this mouse model. The combination of significantly reduced T_{reg} populations and increased colitis and dysplasia scores in our model are consistent with previous findings and underscore the importance of elucidating potential alterations in the function of these immune cells. Our study indicates that it is imperative to determine if the difference in the timing of changes in immune cell populations between the SMAD3+/- vs SMAD3-/- contributes significantly to the impaired immune response and subsequent development of colitis in SMAD3-/- mice. Future studies will address these questions by evaluating temporal changes along with functional changes of these cell populations. These studies underscore the need to understand differential responses to infection in an effort to identify future targets for treating human disease.

Figure 2.1.



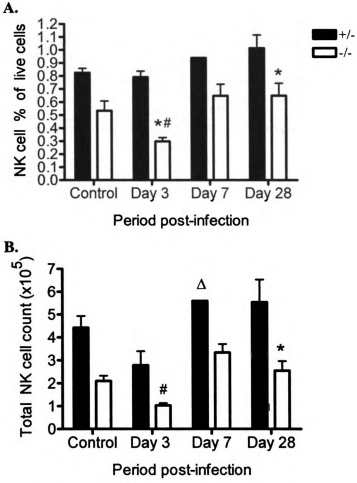
Average weight (A) and body fat percentage (B) of SMAD3-/- and SMAD3+/- mice throughout course of infection. (C) Average combined colitis and dysplasia scores of control (Broth) and infected mice through 8 weeks post-infection.

Table 2.1. Total baseline lymphocyte cell population as percentage of live cells by tissue and genotype, SMAD3+/- or -/-, prior to infection (Mean±SEM).

		Spleen	MsLN	PP
B cell	+/-	55 ± 3.9	32 ± 1.1	57 ± 1.6
	-/-	41 ± 3.1	36 ± 2.6	63 ± 2.3
NK cell	+/-	**0.8 ± 0.02***	**0.25 ± 0.02***	**0.22 ± 0.02***
	-/-	0.53 ± 0.07	0.07 ± 0.02	0.03 ± 0.01
CD3+	+/-	33 ± 1.3	63 ± 1.2	21 ± 1.0
	-/-	28 ± 6.3	**53 ± 5.2***	**15 ± 1.0***
CD4+ of CD3+	+/-	52 ± 15.0	67 ± 0.8	50 ± 2.7
	-/-	**63 ± 2.5***	65 ± 2.5	57 ± 1.2
CD8+ of CD3+	+/-	33 ± 0.8	29 ± 1.8	27 ± 3.6
	-/-	32 ± 1.9	31 ± 2.0	25 ± 4.8

* Significant effect of genotype within each time point following infection ($p<0.05$)

Figure 2.2.



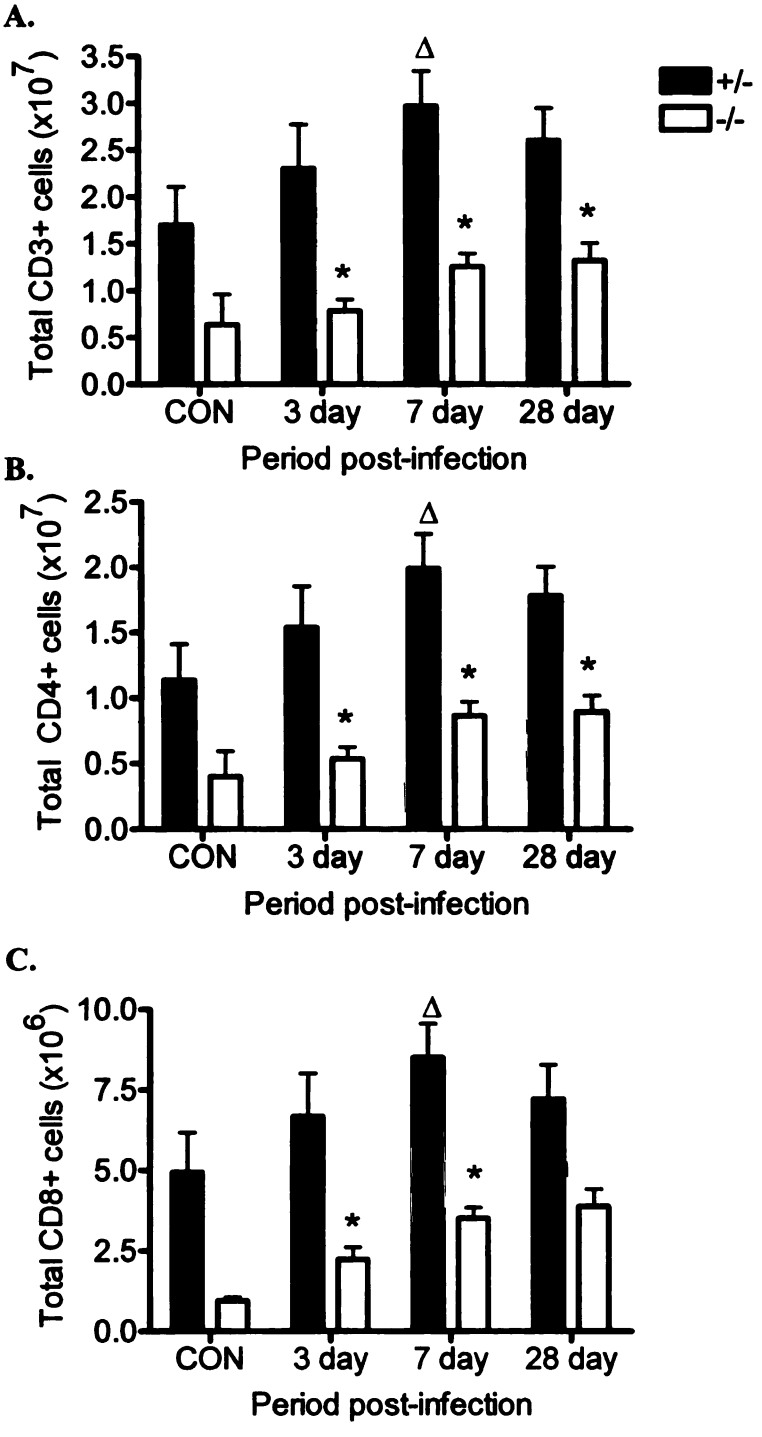
Total NK cell population changes throughout the course of infection in the spleen, expressed as both (A) percentage of live cells and (B) total NK cell number.

Table 2.2. NK cell populations as percentage of live cells by tissue and genotype through the course of infection (Mean±SD).

		Control		Day 3		Day 7		Day 28	
	genotype	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MsLN	+/-	0.176	0.091	0.075	0.057	0.066	0.033	0.280	0.206
	-/-	0.065	0.025	0.066	0.030	0.034	0.014	**0.042**	**0.018***
PP	+/-	0.264	0.052	0.072	0.014	0.072	0.012	0.372	0.164
	-/-	**0.030**	**0.011***	0.024	0.005	0.016	0.008	**0.016**	**0.007***

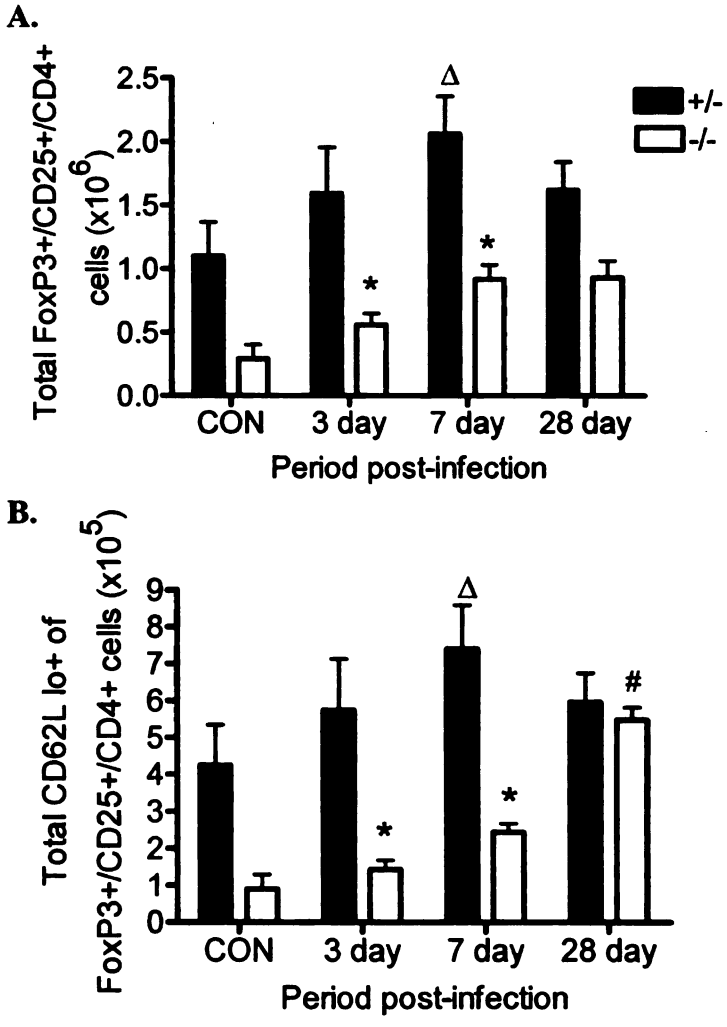
* Significant effect of genotype within each time point following infection ($p<0.05$)

Figure 2.3.



Total T lymphocyte cell number changes in the MsLN of SMAD3+/- and -/- mice at baseline and at 3, 7, and 28 days post infection. (A) Total CD3+ T cells. (B) Total CD4+/CD3+ T cells. (C) Total CD8+/CD3+ T cells.

Figure 2.4.



(A) Total FoxP3+ T regulatory lymphocyte cell number in the MsLN of SMAD3+/- and -/- mice post infection. (B) Total FoxP3+ T regulatory cells expressing CD62L lo.

BIBLIOGRAPHY

1. Clevers H: At the crossroads of inflammation and cancer. *Cell* 2004, 118(6):671-674.

2. Eaden JA, Abrams KR, Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001, 48(4):526-535.

3. Monteleone I, Pallone F, Monteleone G: Interleukin-23 and Th17 cells in the control of gut inflammation. *Mediators Inflamm* 2009:297645.

4. Yeung MM, Melgar S, Baranov V, Oberg A, Danielsson A, Hammarstrom S, Hammarstrom ML: Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-gammadelta expression. *Gut* 2000, 47(2):215-227.

5. Tlaskalova-Hogenova H, Tuckova L, Stepankova R, Hudcovic T, Palova-Jelinkova L, Kozakova H, Rossmann P, Sanchez D, Cinova J, Hrncir T *et al*: Involvement of innate immunity in the development of inflammatory and autoimmune diseases. *Ann N Y Acad Sci* 2005, 1051:787-798.

6. Baumgart D, Carding S: Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007, 369(9573):1627-1640.

7. Manzano L, Alvarez-Mon M, Abreu L, Antonio Vargas J, de la Morena E, Corugedo F, Durantez A: Functional impairment of natural killer cells in active ulcerative colitis: reversion of the defective natural killer activity by interleukin 2. *Gut* 1992, 33(2):246-251.

8. Nedjic J, Aichinger M, Emmerich J, Mizushima N, Klein L: Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 2008, 455(7211):396-400.

9. Ueyama H, Kiyohara T, Sawada N, Isozaki K, Kitamura S, Kondo S, Miyagawa J, Kanayama S, Shinomura Y, Ishikawa H *et al*: High Fas ligand expression on lymphocytes in lesions of ulcerative colitis. *Gut* 1998, 43(1):48-55.

10. Yonamine Y, Watanabe M, Kinjo F, Hibi T: Generation of MHC cla cytotoxic T cell lines and clones against colonic epithelial cells from colitis. *J Clin Immunol* 1999, 19(1):77-85.

11. Zanninelli G, Vetuschi A, Sferra R, D'Angelo A, Fratticci A, Contin Chiaramonte M, Gaudio E, Caprilli R, Latella G: Smad3 knock-out r useful model to study intestinal fibrogenesis. *World J Gastroenterol* 12(8):1211-1218.

12. Maggio-Price L, Treuting P, Zeng W, Tsang M, Bielefeldt-Ohmann BM: Helicobacter infection is required for inflammation and colon c SMAD3-deficient mice. *Cancer Res* 2006, 66(2):828-838.

13. Wang ZB, Cui YF, Liu YQ, Jin W, Xu H, Jiang ZJ, Lu YX, Zhang Dong B: Increase of CD4(+)CD25(+) T cells in Smad3(-/-) mice. *W Gastroenterol* 2006, 12(15):2455-2458.

14. Martinez G, Zhang Z, Chung Y, Reynolds J, Lin X, Jetten A, Feng Smad3 Differentially Regulates the Induction of Regulatory and In Cell Differentiation. *Journal of Biological Chemistry* 2009, 284(51 35286.

15. Zhang L, Zhao Y: The regulation of Foxp3 expression in regulator CD4(+)CD25(+)T cells: multiple pathways on the road. *J Cell Phy.* 211(3):590-597.

16. Maggio-Price L, Bielefeldt-Ohmann H, Treuting P, Iritani BM, Zer Tsang M, Shows D, Morrissey P, Viney JL: Dual infection with H and Helicobacter hepaticus in p-glycoprotein-deficient mdr1a-/- m colitis that progresses to dysplasia. *Am J Pathol* 2005, 166(6):179

17. O'Connor P, Lapointe T, Beck P, Buret A: Mechanisms by which i may increase intestinal cancer risk in inflammatory bowel disease. *Dis* 2010.

18. Munoz N, Baek J, Grady W: TGF-beta has paradoxical and conte effects on proliferation and anoikis in human colorectal cancer cel *Factors* 2008, 26(5):254-262.

19. MacKay S, Yaswen L, Tarnuzzer R, Moldawer L, Bland KI, Copeland Er, Schultz G: Colon cancer cells that are not growth inhibited by TGF-beta lack functional type I and type II TGF-beta receptors. *Ann Surg* 1995, 221(6):767-776; discussion 776-767.

20. Mizoguchi A, Mizoguchi E, Bhan AK: Immune networks in animal models of inflammatory bowel disease. *Inflamm Bowel Dis* 2003, 9(4):246-259.

21. Strober W, Ehrhardt RO: Chronic intestinal inflammation: an unexpected outcome in cytokine or T cell receptor mutant mice. *Cell* 1993, 75(2):203-205.

22. Mizoguchi A, Mizoguchi E, Smith RN, Preffer FI, Bhan AK: Suppressive role of B cells in chronic colitis of T cell receptor alpha mutant mice. *J Exp Med* 1997, 186(10):1749-1756.

23. Davidson NJ, Leach MW, Fort MM, Thompson-Snipes L, Kuhn R, Muller W, Berg DJ, Rennick DM: T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J Exp Med* 1996, 184(1):241-251.

24. Wei B, Velazquez P, Turovskaya O, Spricher K, Aranda R, Kronenberg M, Birnbaumer L, Braun J: Mesenteric B cells centrally inhibit CD4+ T cell colitis through interaction with regulatory T cell subsets. *Proc Natl Acad Sci U S A* 2005, 102(6):2010-2015.

25. Mizoguchi E, Mizoguchi A, Preffer FI, Bhan AK: Regulatory role of mature B cells in a murine model of inflammatory bowel disease. *Int Immunol* 2000, 12(5):597-605.

26. Byrne P, McGuirk P, Todryk S, Mills KH: Depletion of NK cells results in disseminating lethal infection with Bordetella pertussis associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur J Immunol* 2004, 34(9):2579-2588.

27. Dunn PL, North RJ: Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. *Infect Immun* 1991, 59(9):2892-2900.

28. Harty JT, Bevan MJ: Specific immunity to Listeria monocytogenes in the absence of IFN gamma. *Immunity* 1995, 3(1):109-117.

29. Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M: Immune response in mice that lack the interferon-gamma receptor. *Science* 1993, 259(5102):1742-1745.

30. Le-Barillec K, Magalhaes JG, Corcuff E, Thuizat A, Sansonetti PJ, Phalipon A, Di Santo JP: Roles for T and NK cells in the innate immune response to Shigella flexneri. *J Immunol* 2005, 175(3):1735-1740.

31. Sporri R, Joller N, Albers U, Hilbi H, Oxenius A: MyD88-dependent IFN-gamma production by NK cells is key for control of Legionella pneumophila infection. *J Immunol* 2006, 176(10):6162-6171.

32. Way SS, Borczuk AC, Dominitz R, Goldberg MB: An essential role for gamma interferon in innate resistance to Shigella flexneri infection. *Infect Immun* 1998, 66(4):1342-1348.

33. Takeda K, Dennert G: The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J Exp Med* 1993, 177(1):155-164.

34. Fort MM, Leach MW, Rennick DM: A role for NK cells as regulators of CD4+ T cells in a transfer model of colitis. *J Immunol* 1998, 161(7):3256-3261.

35. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA: Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 2008, 29(6):947-957.

36. Radaeva S, Sun R, Pan HN, Hong F, Gao B: Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* 2004, 39(5):1332-1342.

37. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA: Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. *Immunity* 2007, 27(4):647-659.

38. Essence of harmony. *Nat Immunol* 2005, 6(4):325.

39. Powrie F, Read S, Mottet C, Uhlig H, Maloy K: Control of immune pathology by regulatory T cells. *Novartis Found Symp* 2003, 252:92-98; discussion 98-105, 106-114.

40. Liu H, Hu B, Xu D, Liew FY: CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA4. *J Immunol* 2003, 171(10):5012-5017.

41. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA, Powrie F: T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2005, 201(5):737-746.

42. Singh B, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, Stepankova R, Tlaskalova H, Powrie F: Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001, 182:190-200.

43. Yokoyama Y, Fukunaga K, Fukuda Y, Tozawa K, Kamikozuru K, Ohnishi K, Kusaka T, Kosaka T, Hida N, Ohda Y *et al*: Demonstration of low-regulatory CD25High+CD4+ and high-pro-inflammatory CD28-CD4+ T-Cell subsets in patients with ulcerative colitis: modified by selective granulocyte and monocyte adsorption apheresis. *Dig Dis Sci* 2007, 52(10):2725-2731.

44. Kamikozuru K, Fukunaga K, Hirota S, Hida N, Ohda Y, Yoshida K, Yokoyama Y, Tozawa K, Kawa K, Iimuro M *et al*: The expression profile of functional regulatory T cells, CD4+CD25high+/forkhead box protein P3+, in patients with ulcerative colitis during active and quiescent disease. *Clin Exp Immunol* 2009, 156(2):320-327.

45. Venturi GM, Conway RM, Steeber DA, Tedder TF: CD25+CD4+ regulatory T cell migration requires L-selectin expression: L-selectin transcriptional regulation balances constitutive receptor turnover. *J Immunol* 2007, 178(1):291-300.

46. Grailer JJ, Kodera M, Steeber DA: L-selectin: role in regulating homeostasis and cutaneous inflammation. *J Dermatol Sci* 2009, 56(3):141-147.

Chapter 3 - Effect of energy balance on severity of colitis and dysplasia after infection with *Helicobacter hepaticus* in SMAD3-/- mice.

Introduction

Obesity increases the risk of many diseases and conditions, including cardiovascular disease, stroke, hypertension, osteoarthritis, dyslipidemia, diabetes mellitus type 2, and also increases risk of many types of cancer [1]. Total adiposity is associated with increased risk of cancer in the esophagus, pancreas, colon/rectum (CRC), breast (postmenopausal), endometrium, and kidney [2]. Some associations between obesity and cancer risk are even more pronounced when measured by waist-to-hip ratio (a marker of visceral adiposity), particularly the risk of colorectal cancer [3-5]. A potential connection between obesity and increased risk of cancer involves the altered production of adipokines. Adipokines are cell-signaling peptides produced by adipose tissue (AT) that have effects on a large number of bodily functions, including lipid metabolism, inflammation, atherosclerosis, insulin resistance, vascular homeostasis, angiogenesis and immune function [6].

AT dysregulation that occurs during obesity is characterized by an atypical interaction between energy balance, adipocyte size and number, extracellular matrix balance, vasculature and immune cell infiltration, which is associated with the progression of chronic inflammation [7]. Several pro-inflammatory cytokines are released from adipose tissue even in the absence of acute injury or inflammation, and production is increased in proportion to greater AT mass; therefore, the elevated circulating levels of these cytokines in obesity supports the characterization of obesity as a disorder of chronic inflammation [8]. In visceral AT, CD8+ cytotoxic T cells accumulate and cooperate with AT to recruit and induce macrophage differentiation,

initiating an inflammatory cascade [9]. Mast cells are also elevated in AT to produce interleukin-6 (IL-6) and interferon-γ (IFN-γ), thereby contributing to angiogenesis by stimulating the release of proangiogenic peptides by adipocytes [10]. There is also a decrease in proportions of anti-inflammatory T regulatory (T_{reg}) cells compared to proinflammatory T helper 1 (T_H1) cells in visceral AT, although it is unclear as to whether or not this decrease is a cause or consequence of AT inflammation [11]. These findings are consistent with known comorbidities of obesity associated particularly with visceral adiposity [12-14].

Visceral adiposity is an important component of metabolic syndrome, in that it is strongly associated with the other symptoms of the syndrome, including elevated blood pressure, insulin resistance and dyslipidemia (increased triglycerides and decreased HDL cholesterol) [15-17]. Obesity is also an important risk factor for colon cancer, specifically visceral obesity, as evidenced by increased risk associated more so with greater waist-to-hip ratio as opposed to overall BMI [18, 19]. It is unknown as to what impact an obesity-related proinflammatory state has on populations already at higher risk for cancer due to other inflammatory conditions, such as inflammatory bowel disease (IBD).

Individuals with IBD, particularly ulcerative colitis (UC), are at a higher risk of developing colon cancer than the general population [20]. A meta-analysis from 116 studies indicated that the prevalence of colon cancer in patients with UC is approximately 3.7% (95% CI 3.2–4.2, $p<0.0001$), with the cumulative probability reaching 18% by 30 years regardless of disease severity [21]. There is currently insufficient evidence to support a causal relationship between obesity and IBD; however, the conditions share

similar inflammatory characteristics, providing a potential link between excessive adiposity and pathogenesis of IBD.

Recent studies indicate that the constant low-grade inflammation associated with excess adipose tissue, as evidenced by elevated serum levels of C-reactive protein (CRP), IL-6 and TNF-α, may also contribute to the severity of IBD. A study of the phenotypic characteristics of individuals with IBD who had persistently lower levels of CRP found that these individuals also had significantly lower body mass indices than those with elevated CRP [22]. These data indicate a pathophysiologic triad between inflamed AT, liver and the colon.

Alternative theories that link obesity with IBD inflammation include increased oxidative stress and a decrease in the anti-inflammatory effects of insulin (e.g. suppression of inflammatory markers and attenuation of cytokine stimulation of acute-phase protein gene expression) caused by obesity-associated insulin resistance [23-26]. From an epidemiological perspective, there have been no studies to investigate a link between increasing rates of obesity and the rising incidence of IBD, but there is some evidence for the influence of obesity on the clinical course of IBD, indicating that obese patients have more severe disease activity as compared to lean patients [27]. In this study, we examine the effect of energy balance on severity of colitis and dysplasia following infection with *Helicobacter hepaticus* in the SMAD3-/- mouse model of colon cancer.

Materials and Methods

Mice.

Specimens of 129-*Smad3*$^{tm/Par}$/J (referred to hereafter as SMAD3 -/-) mice were generously donated by Lillian Maggio-Price at the University of Washington. The mouse colony was developed by pairing SMAD3-/- males with SMAD3+/- females; breeding cages contained one male and up to three females per breeding guidelines. Weaning and genotyping of subsequent litters was performed as described below at approximately 21 days after birth. Those pups determined to be SMAD3 -/- were separated by sex into cages with mice of similar age; all mice were housed in 60 square inch plastic cages with microisolator lids. Standard diet for breeding pairs and weaned pups was Harlan Teklad 22/5 Rodent Diet 8640 (22% crude protein, 5% crude fat). Genotyped heterozygous mouse pups were euthanized by CO2 asphyxiation and cervical dislocation as stipulated by the AVMA Panel on Euthanasia. All mouse procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Genotyping.

2 millimeter ear tissue samples were obtained and DNA extracted with REDExtract-N-Amp^{TM} Tissue PCR Kits (Sigma-Aldrich, St. Louis, MO) according to manufacturer's recommendations. Four primers were used for polymerase chain reaction: 1271 (GGA TGG TCG GCT GCA GGT GTC C) and 1272 (TGT TGA AGG CAA ACT CAC AGA GC) to recognize SMAD sequences at give a 130 bp product, and 506 (CGG CGA GGA TCT CGT CGT GAC CCA) and 507 (GCG ATA CCG TAA AGC ACG AGG AAG) to recognize vector sequences and give a 200 bp product. Thermal cycling of the samples was conducted with an initial denaturation at 94°C for 3

minutes, 40 cycles of denaturation-annealing-extension (respectively 20 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 70°C), a final extension of 72°C for 3 minutes, and holding at 4°C. Amplified DNA was immediately loaded onto a 2% agarose gel and separated via electrophoresis at 130-140V for 30-60 minutes.

Induction of lean, control and obese body weight phenotypes.

Weaned SMAD3-/- mice were randomly assigned to one of three OpenSource diets (Research Diets Inc, New Brunswick, NJ): control (CON; formula D12450B: 20% protein, 70% carbohydrate, 10% fat), 30% calorie-restricted (CR; formula D03020702B: 27% protein, 54% carbohydrate, 6% fat) or calorie dense (HF; formula D12492: 20% protein, 20% carbohydrate, 60% fat). Dietary fat in the diets was composed of 9.3% soybean oil and 90.7% lard. Three separate body weight phenotype induction studies were conducted: Group 1 included a total of 73 mice (24 HF, 29 CON, and 20 CR); Group 2 included 57 mice (20 HF, 18 CON, and 19 CR); Group 3 included 35 mice (16 HF and 19 CON) and excluded any CR subjects. All mice were fed *ad libitum*, and were weighed weekly to assess body weight changes between each diet formula. Mice were kept on assigned diets for a minimum of 20 weeks.

Preparation of blood agar plates.

Blood agar plates were prepared by autoclaving 500 mL of tryptic soy agar broth to sterilize and adding 25 mL of sheep's blood. 15 mL of the blood agar mixture was pippetted into sterile Petri dishes and left to dry overnight in the chemical fume hood. Prepared blood agar plates were stored at 4°C.

Helicobacter hepaticus culture and infection.

Isolates of *H. hepaticus* (strain 3B1, ATCC 51449) were kindly donated by Vince Young at University of Michigan. Bacteria were streaked onto blood agar plates and incubated at 36°C for 24-48 hours. Optimal environmental conditions for *H. hepaticus* growth were sustained using GasPak™ gas generating pouch systems (BD, Franklin Lakes, NJ) to produce an anaerobic atmosphere. After incubation, up to 2.5 ml of broth was added to each agar plate, and the broth/culture mixture was collected and optical density was assessed using a Bio-Tek Synergy HT multi-mode microplate reader (Bio-Tek, Winooski, VT) to ensure adequate bacterial population (≥1.8 at 600nm wavelength) Mice were gavaged with two separate 0.3mL dosages of either bacteria-free control Bacto™ Tryptic Soy Broth (BD, Franklin Lakes, NJ) or the *H. hepaticus*-broth mixture, one dosage per day on two consecutive days. Continued weight monitoring was conducted on the gavaged mice; any mice that exhibited a weight loss of >20% from one week to the next was euthanized following the aforementioned procedures.

Body composition analysis.

Body composition of Group 3 mice, including total fat mass and lean tissue mass, was analyzed using an EchoMRI-100™ quantitative nuclear magnetic resonance machine (Echo Medical Systems, Houston, TX).

Sample collection at necropsy.

At necropsy, mice were euthanized via carbon dioxide asphyxiation. Terminal bleeds were performed via cardiac puncture and blood was collected with a heparin-coated syringe. Blood samples were centrifuged at 1500 xg for 15 minutes at 4°C, and

plasma was isolated and frozen at -80°C. The entire lower gastrointestinal tract was isolated and removed. Ceca were incised and cleared of fecal material with ice-cold phosphate buffered saline (PBS). Colons were similarly cleared, rinsed with PBS and sectioned. Cecum and colon samples were placed into tissue biopsy cassettes and fixed for 24 hrs in a 10% formalin solution. Samples were then transferred to a 70% ethanol solution and processed for histological examination and scoring of colitis and adenocarcinomas.

Pathology.

Colon and cecum slides were scored by a blinded pathologist (Dr. Ingeborg Langohr) for degree of colitis and dysplasia adapted from Dr. Maggio-Price [28]. Grades were on a 1 to 4 scale both for inflammation (1, no inflammation; 2, mild inflammation; 3, moderate inflammation; 4, marked inflammation) and dysplasia (1, no dysplasia; 2, low grade dysplasia; 3, high grade dysplasia; 4, high grade dysplasia with invasion/adenocarcinoma). Briefly, low-grade dysplasia was characterized by thickened mucosa with branching and elongated crypts with normal epithelial differentiation including goblet cells, maintenance of polarity, and nuclear morphology. High-grade dysplasia was characterized by thickened mucosa with elongated, irregularly branching glands, cytological and nuclear atypia including loss of differentiation and polarity, bunched and enlarged nuclei, and numerous mitotic figures. The two scores for colon and two scores for cecum tissue in each animal were combined such that a score of 4 indicated no inflammation or dysplasia and a score of 16 reflected maximal inflammation and neoplasia.

Results

Changes in weight distribution and body fat composition between diet treatments.

Group 1 mice were kept on their respective diet treatments for longer than 20 weeks, as planned in the study design, due to insufficient weight gain compared to expected gains and a lack of significant differentiation in weights between groups receiving different diet treatments. HF mice in this group did have significantly increased body weight compared to CON and CR at week 14 and during weeks 16-27, however no difference was observed at week 28 (Figure 3.1A). There was no difference in weight between CON and CR diets at any point during the study. Group 2 mice were kept on diet for 20 weeks; HF mice had significantly higher average body weight at weeks 14, 15, 18 and 19, however there was no difference at the end of diet treatment (Figure 3.1B). There was no significant difference in weight between CON and CR mice in group 2 at any point during the study.

After at least 20 weeks on diet, selected mice from each diet treatment in groups 1 and 2 were infected with *H. hepaticus* (as described above) for a pilot examination of differences in histology scores at 28 days post-infection; however, CR mice had unexpectedly decreased survival before the end of the infection period (Figure 3.2). Because CR mice were unable to survive the full 28-day infection, the CR group was excluded from further study.

Group 3 HF and CON mice were fed respective diets for 20 weeks, with no significant difference in weights between groups at any point during diet treatment (Figure 3.3A). These mice were also assessed for body fat composition every other week beginning at week 8 on diet. Although there were no significant differences in weight,

HF mice had significantly greater body fat percentages at weeks 8, 11, 13 and 17 on diet (Figure 3.3B). There was no significance in body fat percentage between groups by the end of diet treatment and at the time of infection.

Effect of diet treatment on colitis and dysplasia scores at 28 days post-infection.

HF and CON mice were sacrificed 28 days after infection with *H. hepaticus* to assess differences in colitis and dysplasia scores between diet groups. There was no difference between combined colitis and dysplasia scores between CON (7.9 ± 1.9) and HF (7.8 ± 2.1) mice at 28 days post-infection (Figure 3.4A). In order to examine the potential effect of time, we allowed some mice to remain infected for up to 6 weeks, and compared combined colitis scores between diet groups at weeks 4, 5 and 6 post-infection. There was no difference between diet treatments at any time point, nor were there differences within diet treatment groups across time (Figure 3.4B).

Discussion

The results of this study indicate that there is no effect of HF diet on colitis or dysplasia in the SMAD3-/- mouse model. However, caloric restriction increased mortality of mice infected with *H. hepaticus*, similar to previous observations with CR mice infected with influenza [29-31]. Although CR without malnutrition is known to extend lifespan and postpone age-related changes in immunity in mice, multiple studies have found that CR mice exhibit increased mortality, decreased viral clearance and impaired innate immune response, particularly reduced natural killer (NK) cell cytotoxicity [29, 30, 32, 33]. CR and increased mortality post-infection represents a highly novel and important observation meriting further study. Future collaborations

between the Fenton and Gardner laboratories will investigate potential mechanisms for this significant observation.

Obesity increases cancer risk through a promotional influence on transformed cells compared to other models of diet-induced obesity (DIO). The mice in our studies were not obese, nor were we able to induce increased adiposity or weight gain compared to controls, possibly explaining the lack of effect on colitis scoring. SMAD3 is part of a family of proteins that serve as signal transduction intermediates for transforming growth factor beta (TGF-β), a family of ligands that influences a variety of cellular functions, including adipocyte differentiation [34-36]. Although TGF-β expression is increased in both cultured adipocytes and in adipose tissue, TGF-β has been shown to impede adipogenesis by inhibiting differentiation of preadipocytes both, *in vitro* and *in vivo* [37-39]. SMAD3 mediates these adipocyte differentiation/inhibition and proliferation responses because it is activated by and acts as an effector of TGF-β signaling [40]. Interference with SMAD3 signaling enhances both rate and extent of differentiation and inhibits cell proliferation, while the blockage of endogenous TGF-β signaling (via inhibition of SMAD3) increases adipogenesis [39, 40]. Given this information, we expected to see increased adiposity in the SMAD3-/- mice, especially those on HF diet, due to disrupted TGF-β functioning leading to increased adipocyte accumulation; however, any significant gains in body weight or fat percentage in these mice were lost by the end of diet treatment prior to infection with *H. hepaticus.*

Previous studies of DIO models report significant increases in weights and body fat percentages of high-fat fed mice compared to controls, and have identified a number of specific inbred mouse strains that are particularly prone to obesity with high-fat

feeding, including C57BL/6, AKR/J, DBA/2J, C57L/J, A/J, and C3H/HeJ [41, 42]. Of these models, C57BL/6 mice have been used routinely to study the effect of DIO on a number of conditions. Recently, Guo *et al.* able to achieve significant DIO in a C57BL/6 mouse model, with high-fat fed mice reaching body weights of approximately 55g and up to 40% body fat percentage, with obesity persisting after removal of obesigenic diet [43]. Furthermore, obesity in C57BL/6 mice fed a high-fat diet is not a product of hyperphagia or reduced activity, but of increased feed efficiency, accompanied by metabolic abnormalities such as altered inflammatory cytokine profiles, hyperglycemia and adipocyte hyperplasia and hypertrophy [44-50].

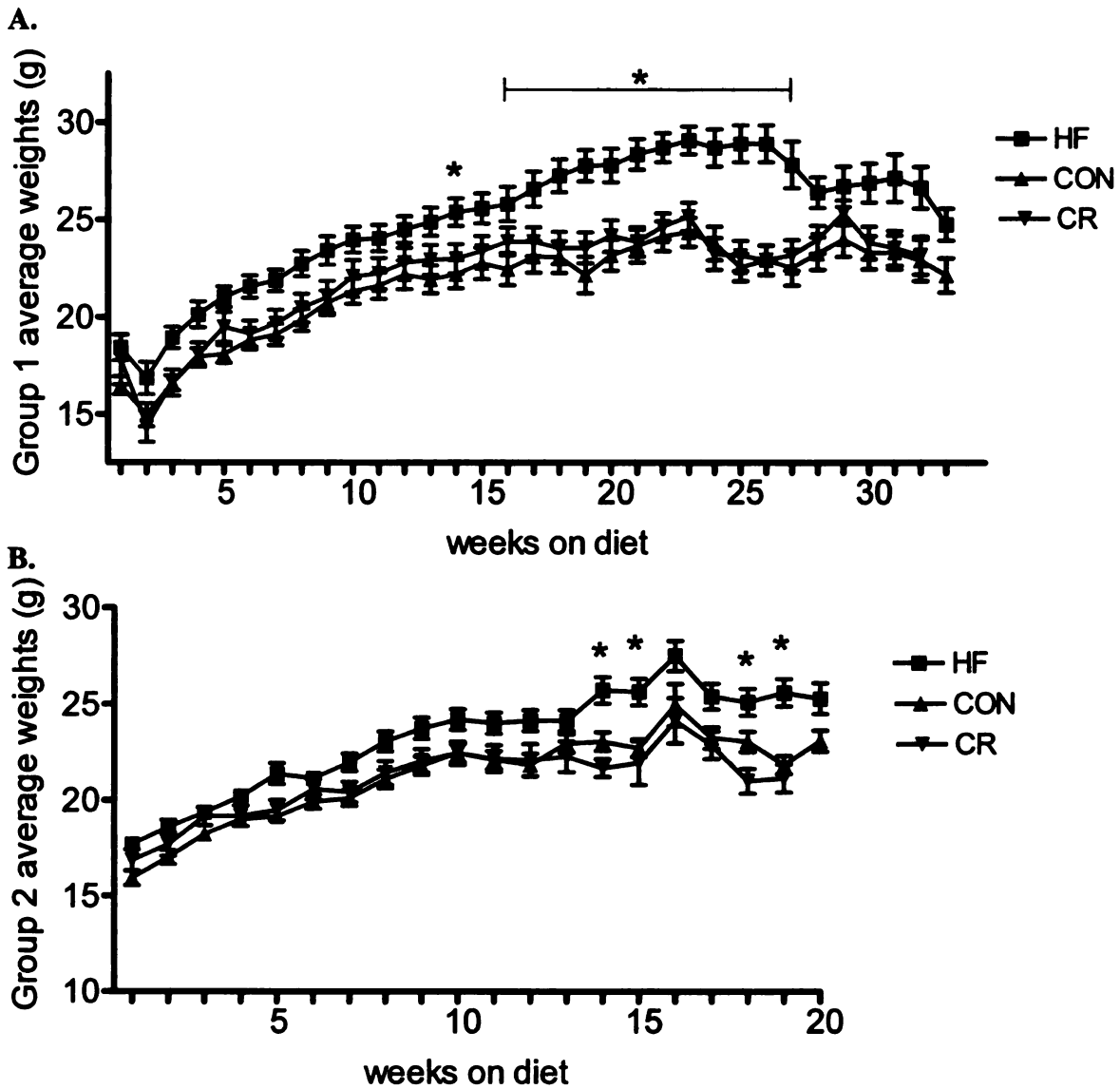
The lack of significant gains in body weight or fat percentage in our SMAD3-/- model may be due to the 129 genetic background of the colony. Multiple studies have shown that the 129 strain is resistant to DIO [51-53]. Our data is consistent with these studies, in that HF diet mice presented with body compositions similar to CON and CR mice, even after long term HF feeding. As such, we can surmise that the 129-*Smad3*$^{tm/Par}$/J mice in our study may have also been resistant to significant increases in body weight or fat percentage when placed on HF diet.

A phenotypical assessment of SMAD3-/- mice was first described by Zhu *et al.* to illustrate inherent physical differences between knockout mice and controls [54]. Notably, SMAD3-/- mice are 20-30% smaller than controls, with males exhibiting a greater size reduction than females, which may explain why we did not observe weight gains similar to those in other DIO studies with different mouse strains. Furthermore, SMAD3-/- mice show evidence of distress as they age beyond 18 weeks, and exhibit decreased life span and premature death, particularly in the 129 background [54].

Because our mice were started on respective diet treatments at 6-8 weeks of age and kept on diet for at least 20 weeks, the animals would have been a minimum of 26 weeks of age at the end of diet treatment. The observed plateaus and/or losses in body weight and fat percentage appear to coincide with the onset of age-related distress in the SMAD3-/-, which is another possible explanation for lack of significant differences between diet treatment groups after 20 weeks.

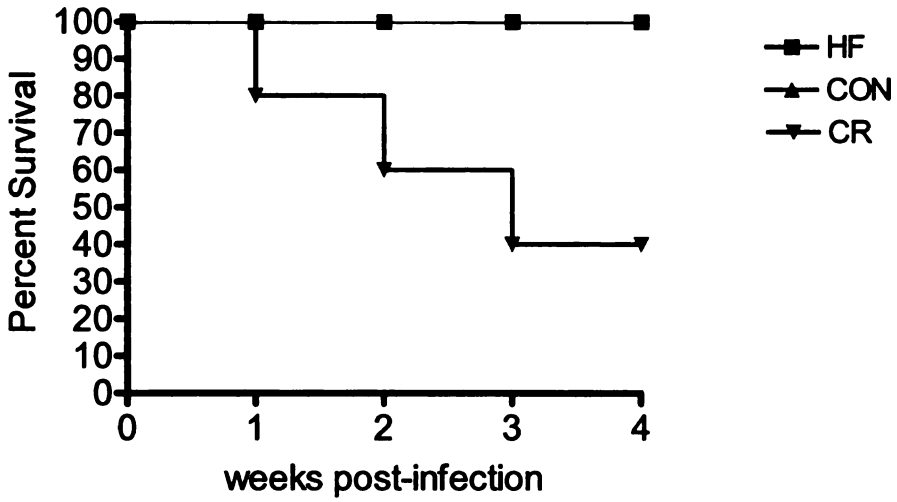
In the present study, we were only able to induce a maximum body fat percentage of roughly 25% in the SMAD3-/- mice. This percentage body fat may be insufficient to induce the chronic inflammation associated with obesity that promotes colon carcinogenesis, such as in other animal models of DIO in which animals with 40-50% body fat exhibit obesity-associated inflammation and tumor proliferation. It remains unclear as to why we were unable to induce a higher percentage of body fat consistent with DIO in the SMAD3-/- mice. Although the 129 strain background is DIO-resistant, the deletion of SMAD3-/-, as well as subsequent TGF-β disruption and increased adipogenesis, should be sufficient to supersede any strain-associated resistance to DIO. Pilot DIO studies with the SMAD3-/- mice conducted by Dr. Fenton were able to achieve greater levels of obesity; however, those mice were started on diet treatments immediately post-wean at 21 days of age as opposed to 6-8 weeks of age in the present study. As such, future DIO studies will be designed so that SMAD3-/- mice are placed on diet immediate after weaning, thereby allowing for an additional 3 to 5 weeks for mice to become obese before age-related distress and weight loss presents at 18 weeks of age.

Figure 3.1.



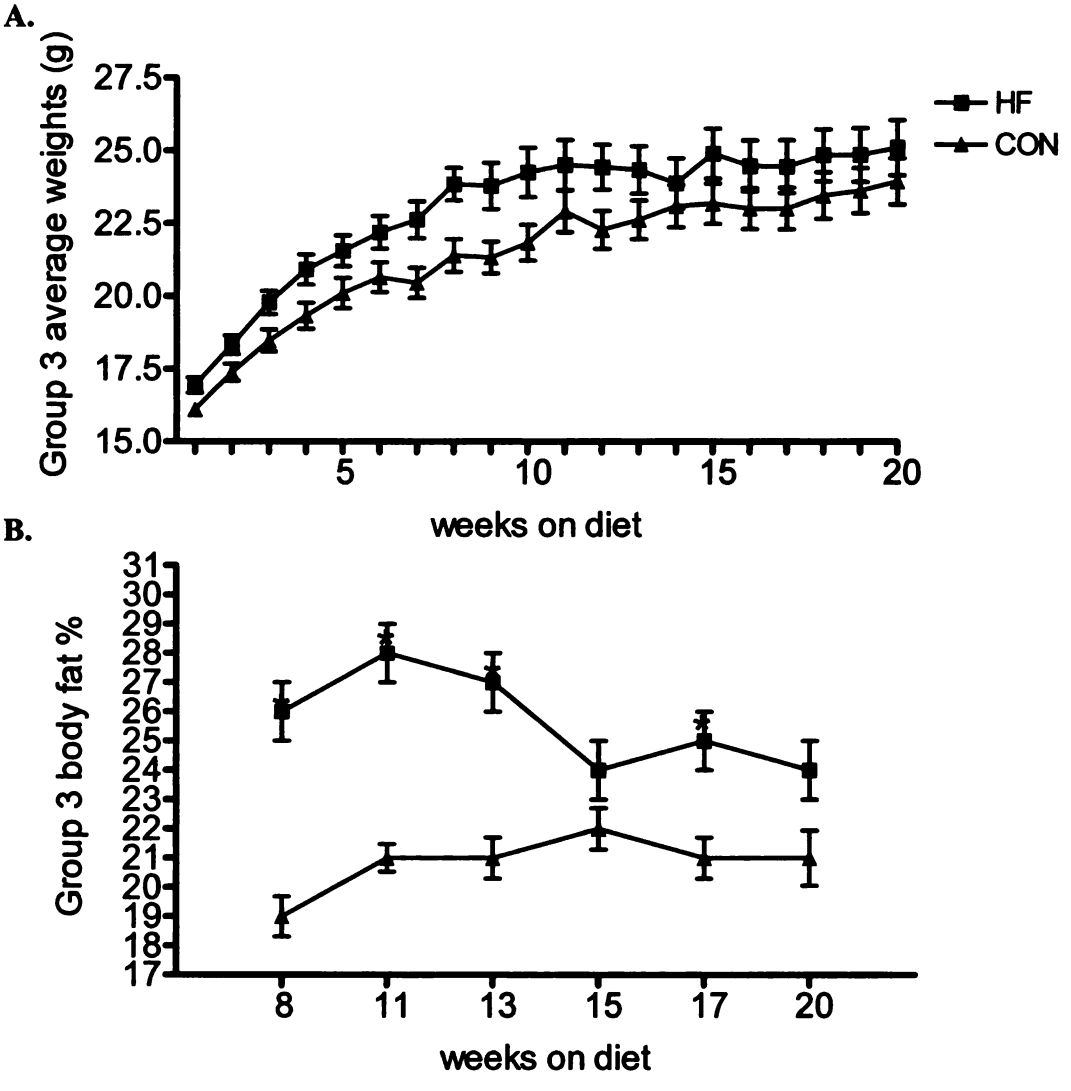
Average body weight (g) for HF, CON and CR diet groups throughout the course of diet treatment for (A) Group 1 and (B) Group 2.

Figure 3.2.



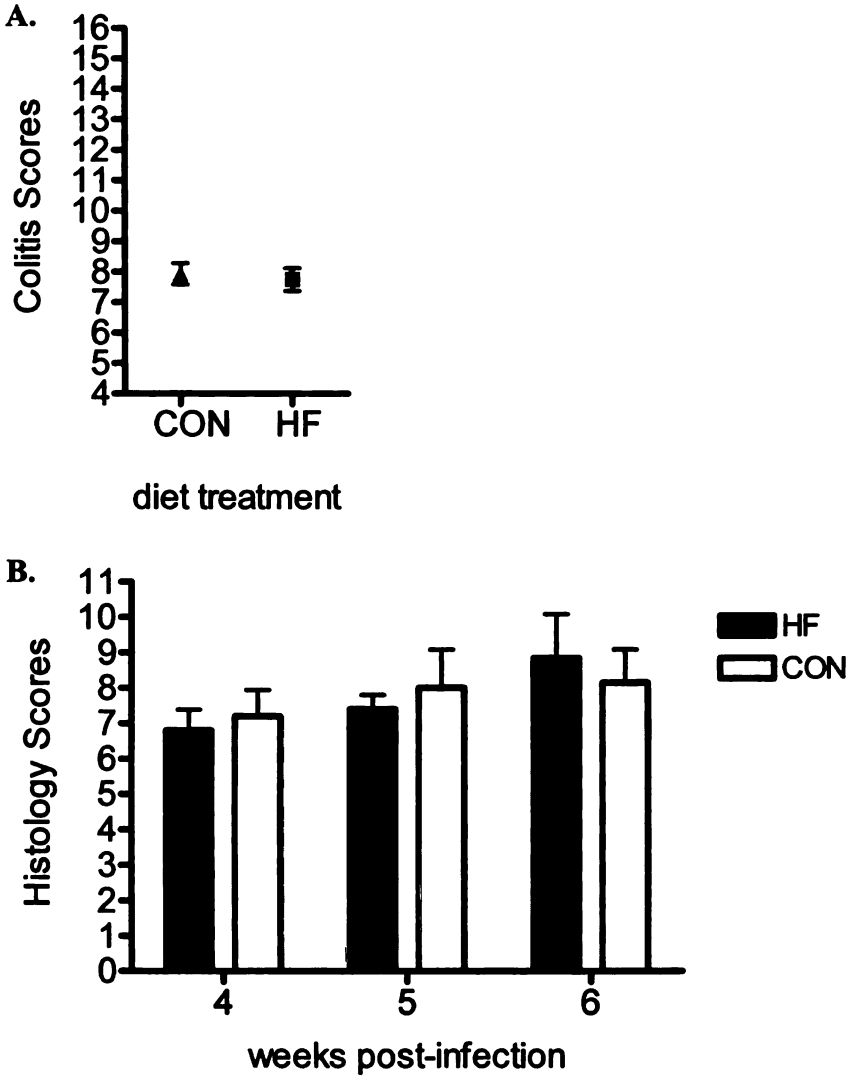
Survival curve of HF, CON and CR mice after infection with *H. hepaticus*.

Figure 3.3.



(A) Average body weight (g) of HF and CON diet groups throughout course of diet treatment. (B) Average body fat percentages of HF and CON diet groups.

Figure 3.4.



(A) Average combined colitis and dysplasia scores between diet groups at 4 weeks post-infection. (B) Average combined colitis and dysplasia scores between diet groups at 4, 5 and 6 weeks post-infection to assess potential effect of time.

BIBLIOGRAPHY

1. National Health and Nutrition Examination Survey In.: National Center for Health Statistics, CDC, http://www.cdc.gov/nchs/; 2003-2004.

2. WCRF/AICR: Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. In. Washington DC; 2007.

3. Moayyedi P: The epidemiology of obesity and gastrointestinal and other diseases: an overview. *Dig Dis Sci* 2008, 53(9):2293-2299.

4. Burke CA: Colonic complications of obesity. *Gastroenterol Clin North Am* 2010, 39(1):47-55.

5. Nam SY, Kim BC, Han KS, Ryu KH, Park BJ, Kim HB, Nam BH: Abdominal visceral adipose tissue predicts risk of colorectal adenoma in both sexes. *Clin Gastroenterol Hepatol* 2010, 8(5):443-450 e441-442.

6. Ronti T, Lupattelli G, Mannarino E: The endocrine function of adipose tissue: an update. *Clin Endocrinol (Oxf)* 2006, 64(4):355-365.

7. Suganami T, Ogawa Y: Adipose tissue macrophages: their role in adipose tissue remodeling. *J Leukoc Biol* 2010.

8. Trayhurn P, Wood I: Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004, 92(3):347-355.

9. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, Sugiura S *et al*: CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med* 2009, 15(8):914-920.

10. Liu J, Divoux A, Sun J, Zhang J, Clement K, Glickman JN, Sukhova GK, Wolters PJ, Du J, Gorgun CZ *et al*: Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat Med* 2009, 15(8):940-945.

11. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, Dorfman R, Wang Y, Zielenski J, Mastronardi F *et al*: Normalization of obesity-associated insulin resistance through immunotherapy. *Nat Med* 2009, 15(8):921-929.

12. Barbarroja N, Lopez-Pedrera R, Mayas MD, Garcia-Fuentes E, Garrido-Sanchez L, Macias-Gonzalez M, El Bekay R, Vidal-Puig A, Tinahones FJ: The obese healthy paradox: is inflammation the answer? *Biochem J* 2010.

13. Faber DR, van der Graaf Y, Westerink J, Visseren FL: Increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. *Atherosclerosis* 2010.

14. Koster A, Stenholm S, Alley DE, Kim LJ, Simonsick EM, Kanaya AM, Visser M, Houston DK, Nicklas BJ, Tylavsky FA *et al*: Body Fat Distribution and Inflammation Among Obese Older Adults With and Without Metabolic Syndrome. *Obesity (Silver Spring)* 2010.

15. Bruce KD, Byrne CD: The metabolic syndrome: common origins of a multifactorial disorder. *Postgrad Med J* 2009, 85(1009):614-621.

16. Demerath EW, Reed D, Rogers N, Sun SS, Lee M, Choh AC, Couch W, Czerwinski SA, Chumlea WC, Siervogel RM *et al*: Visceral adiposity and its anatomical distribution as predictors of the metabolic syndrome and cardiometabolic risk factor levels. *Am J Clin Nutr* 2008, 88(5):1263-1271.

17. Elks CM, Francis J: Central adiposity, systemic inflammation, and the metabolic syndrome. *Curr Hypertens Rep*, 12(2):99-104.

18. Pischon T, Nothlings U, Boeing H: Obesity and cancer. *Proc Nutr Soc* 2008, 67(2):128-145.

19. Wang Y, Jacobs EJ, Patel AV, Rodriguez C, McCullough ML, Thun MJ, Calle EE: A prospective study of waist circumference and body mass index in relation to colorectal cancer incidence. *Cancer Causes Control* 2008, 19(7):783-792.

20. Clevers H: At the crossroads of inflammation and cancer. *Cell* 2004, 118(6):671-674.

21. Eaden JA, Abrams KR, Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001, 48(4):526-535.

22. Florin T, Paterson E, Fowler E, Radford-Smith G: Clinically active Crohn's disease in the presence of a low C-reactive protein. *Scand J Gastroenterol* 2006, 41(3):306-311.

23. Aljada A, Mohanty P, Ghanim H, Abdo T, Tripathy D, Chaudhuri A, Dandona P: Increase in intranuclear nuclear factor kappaB and decrease in inhibitor kappaB in mononuclear cells after a mixed meal: evidence for a proinflammatory effect. *Am J Clin Nutr* 2004, 79(4):682-690.

24. Dandona P, Aljada A, Bandyopadhyay A: Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 2004, 25(1):4-7.

25. Campos SP, Baumann H: Insulin is a prominent modulator of the cytokine-stimulated expression of acute-phase plasma protein genes. *Mol Cell Biol* 1992, 12(4):1789-1797.

26. Dandona P, Aljada A: A rational approach to pathogenesis and treatment of type 2 diabetes mellitus, insulin resistance, inflammation, and atherosclerosis. *Am J Cardiol* 2002, 90(5A):27G-33G.

27. Chapman-Kiddell C, Davies P, Gillen L, Radford-Smith G: Role of diet in the development of inflammatory bowel disease. *Inflamm bowel dis* 2010, 16(1):137-151.

28. Maggio-Price L, Bielefeldt-Ohmann H, Treuting P, Iritani BM, Zeng W, Nicks A, Tsang M, Shows D, Morrissey P, Viney JL: Dual infection with Helicobacter bilis and Helicobacter hepaticus in p-glycoprotein-deficient mdr1a-/- mice results in colitis that progresses to dysplasia. *Am J Pathol* 2005, 166(6):1793-1806.

29. Ritz BW, Aktan I, Nogusa S, Gardner EM: Energy restriction impairs natural killer cell function and increases the severity of influenza infection in young adult male C57BL/6 mice. *J Nutr* 2008, 138(11):2269-2275.

30. Clinthorne JF, Adams DJ, Fenton JI, Ritz BW, Gardner EM: Short-Term Re-Feeding of Previously Energy-Restricted C57BL/6 Male Mice Restores Body Weight and Body Fat and Attenuates the Decline in Natural Killer Cell Function after Primary Influenza Infection. *J Nutr* 2010.

31. Ritz BW, Gardner EM: Malnutrition and energy restriction differentially affect viral immunity. *J Nutr* 2006, 136(5):1141-1144.

32. Gardner EM: Caloric restriction decreases survival of aged mice in response to primary influenza infection. *J Gerontol A Biol Sci Med Sci* 2005, 60(6):688-694.

33. Nogusa S, Ritz BW, Kassim SH, Jennings SR, Gardner EM: Characterization of age-related changes in natural killer cells during primary influenza infection in mice. *Mech Ageing Dev* 2008, 129(4):223-230.

34. Shi Y, Massague J: Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003, 113(6):685-700.

35. Derynck R, Zhang YE: Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003, 425(6958):577-584.

36. Zanninelli G, Vetuschi A, Sferra R, D'Angelo A, Fratticci A, Continenza MA, Chiaramonte M, Gaudio E, Caprilli R, Latella G: Smad3 knock-out mice as a useful model to study intestinal fibrogenesis. *World J Gastroenterol* 2006, 12(8):1211-1218.

37. Samad F, Yamamoto K, Pandey M, Loskutoff DJ: Elevated expression of transforming growth factor-beta in adipose tissue from obese mice. *Mol Med* 1997, 3(1):37-48.

38. Choy L, Derynck R: Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *J Biol Chem* 2003, 278(11):9609-9619.

39. Rosen ED, MacDougald OA: Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* 2006, 7(12):885-896.

40. Choy L, Skillington J, Derynck R: Roles of autocrine TGF-beta receptor and Smad signaling in adipocyte differentiation. *J Cell Biol* 2000, 149(3):667-682.

41. West DB, Boozer CN, Moody DL, Atkinson RL: Dietary obesity in nine inbred mouse strains. *Am J Physiol* 1992, 262(6 Pt 2):R1025-1032.

42. Alexander J, Chang GQ, Dourmashkin JT, Leibowitz SF: Distinct phenotypes of obesity-prone AKR/J, DBA2J and C57BL/6J mice compared to control strains. *Int J Obes (Lond)* 2006, 30(1):50-59.

43. Guo J, Jou W, Gavrilova O, Hall KD: Persistent diet-induced obesity in male C57BL/6 mice resulting from temporary obesigenic diets. *PLoS One* 2009, 4(4):e5370.

44. Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, Kuhn CM, Rebuffe-Scrive M: Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism* 1995, 44(5):645-651.

45. Brownlow BS, Petro A, Feinglos MN, Surwit RS: The role of motor activity in diet-induced obesity in C57BL/6J mice. *Physiol Behav* 1996, 60(1):37-41.

46. Parekh PI, Petro AE, Tiller JM, Feinglos MN, Surwit RS: Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism* 1998, 47(9):1089-1096.

47. Surwit RS, Petro AE, Parekh P, Collins S: Low plasma leptin in response to dietary fat in diabetes- and obesity-prone mice. *Diabetes* 1997, 46(9):1516-1520.

48. Black BL, Croom J, Eisen EJ, Petro AE, Edwards CL, Surwit RS: Differential effects of fat and sucrose on body composition in A/J and C57BL/6 mice. *Metabolism* 1998, 47(11):1354-1359.

49. Petro AE, Cotter J, Cooper DA, Peters JC, Surwit SJ, Surwit RS: Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse. *Metabolism* 2004, 53(4):454-457.

50. Fenton JI, Nunez NP, Yakar S, Perkins SN, Hord NG, Hursting SD: Diet-induced adiposity alters the serum profile of inflammation in C57BL/6N mice as measured by antibody array. *Diabetes Obes Metab* 2009, 11(4):343-354.

51. Almind K, Kahn CR: Genetic determinants of energy expenditure and insulin resistance in diet-induced obesity in mice. *Diabetes* 2004, 53(12):3274-3285.

52. Ishimori N, Li R, Kelmenson PM, Korstanje R, Walsh KA, Churchill GA, Forsman-Semb K, Paigen B: Quantitative trait loci that determine plasma lipids and obesity in C57BL/6J and 129S1/SvImJ inbred mice. *J Lipid Res* 2004, 45(9):1624-1632.

53. Bachmanov AA, Reed DR, Tordoff MG, Price RA, Beauchamp GK: Nutrient preference and diet-induced adiposity in C57BL/6ByJ and 129P3/J mice. *Physiol Behav* 2001, 72(4):603-613.

54. Zhu Y, Richardson JA, Parada LF, Graff JM: Smad3 mutant mice develop metastatic colorectal cancer. *Cell* 1998, 94(6):703-714.