INTESTINAL CANCER: LINKING INFECTION, INFLAMMATION AND NEOPLASIA

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ABSTRACT

Cancer is a leading cause of death in the world. Much work has been done to study the role of inflammation in carcinogenesis. One hypothesis suggests that inflammation causes oxidative stress that induces damage to cellular targets, including DNA. The multistep model of cancer proposes that cancer is a genetic disease in which mutations are required in carcinogenesis. When this theory was championed, research focused on somatic mutations. The focus has broadened to include epigenetic mechanisms in changing gene expression.

The association between chronic infection, chronic inflammation and increased cancer risk has been supported by epidemiologic studies. Data link chronic inflammation associated with infectious disease to increased cancer risk. Some examples of such infectious agents include hepatitis B virus, *Schistosoma haemotobium*, and *Helicobacter pylori*. One objective of this thesis was to investigate the role of inflammation in self-limiting infection. Additional objectives focus on evaluating a novel model of intestinal and extraintestinal cancer, and using immune regulating cells as treatment for intestinal cancer.

For the first objective, a murine mutational analysis model was used to study infection with *Citrobacter rodentium*, an enteric bacterium that causes self-limiting hyperplasia and inflammation. Increased mutant frequency was observed in association with elevated levels of iNOS 13 days post infection. The second aim, to characterize a novel model of neoplasia, led to the discovery of basosquamous cancer in mice with intestinal tumors. Finally, Apc^{Min} mice, a model of intestinal neoplasia, were treated with T regulatory cells to investigate the role of these cells on tumor development. These cells were previously observed to have an anti-inflammatory and therapeutic effect on an infection-driven model of colon cancer. It was shown that T regulatory cells led to a decrease in the number of adenomas.

In conclusion, it has been shown that self-limiting infection can increase mutant frequency. In addition, a novel model of intestinal and basosquamous cancer has been characterized, and a promising therapy for intestinal cancer has been validated.

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Chapter 1: Background and literature review

This thesis focuses on the role of inflammation in cancer risk. Experimental studies encompass three different areas of research: bacterial infection as a risk factor for colorectal cancer, modeling of intestinal and extraintestinal cancer, and immunotherapy for intestinal cancer. Chapter 1 reviews pertinent literature relating directly to the studies reported in the subsequent chapters. The first part of this chapter briefly outlines colorectal cancer development. The second section describes intestinal carcinogenesis in terms of cells at risk and possible mechanisms. The last section focuses on the hypothesis that infection leading to inflammation increases the risk for cancer.

1.1 Intestinal cancer

1.1.1 Epidemiology, risk factors, detection, treatment

Colorectal cancer (CRC) is the third leading cause of cancer deaths in men and women in the United States [3]. Since the 1990's, incidence rates for CRC have stabilized, but an estimated 106,000 new cases and 57,000 deaths were projected in the United States for 2004 [3]. Worldwide, there were 944,717 cases and 492,411 deaths from CRC reported in 2000. The incidence rate in more developed countries is estimated at 48 per 100,000 persons at risk versus 7 per 100,000 persons at risk in developing countries. Though the incidence rate is significantly lower in developing countries, about forty percent of the deaths worldwide from CRC occur in

developing countries [4]. The lifetime risk for CRC in the United States is about 5 to 6 percent [5]. It is important to note that colon cancer and rectal cancer are separate diseases that are often studied together.

Environmental factors, especially diet, have been implicated in geographical differences in CRC incidence [6, 7]. The main factors that are studied as dietary risk factors are a high fat and high caloric diet, low unabsorbable vegetable fiber, high intake of refined carbohydrates, consumption of red meat, low levels of calcium, and low protective micronutrients such as the antioxidant vitamins A, C and E that may scavenge oxygen radicals (see section 1.2.2.1 "Oxidative stress") [8].

Family history of CRC is the leading risk factor for CRC, with twenty percent of patients having a first and/or second-degree family member with CRC. Approximately 5 to 10 percent of CRC patients have an autosomally dominant inherited pattern of CRC in their family (reviewed in [5].) The two major forms of hereditary CRC are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) [9].

Early diagnosis of CRC can be difficult because patients may remain asymptomatic for years. The peak incidence of CRC is at 60-79 years of age, with fewer than 20% of cases occurring before the age of 50 [10]. Cancer in the cecum and right side of the colon often cause fatigue, weakness, and iron deficiency, all due to anemia from bleeding lesions. Those in the left side of the colon tend to be associated with fecal occult blood, changes in bowel habit, and cramping in

the area of the lesion. If metastasis occurs, the sites of spreading are often regional lymph nodes, liver, lungs, and bones [11].

Screening is recommended for the general population by the American Cancer Society at regular intervals beginning at the age of 50. The following are in their recommended guidelines:

1. Yearly stool blood test (fecal occult blood test)
2. Flexible sigmoidoscopy every 5 years
3. Yearly stool blood test plus flexible sigmoidoscopy every 5 years

(Of the first three options, the ACS recommends the third option.)

Or :

4. Double contrast barium enema every 5 to 10 years
5. Colonoscopy every 10 years

Patients with risk factors such as a family history of CRC or IBD are recommended to start screening at an earlier age (http://www.cancer.org).

Prognosis and treatment is determined by the extent of local tumor invasiveness or metastasis assessed by pathologic staging. A widely used staging system is the Astler-Coller system which has five tumor stages ranging from neoplasms limited to the mucosa, to distant metastatic spread (reviewed in [11].) Once the stage of a tumor has been determined, treatment usually involves a combination of surgery, radiation therapy, and chemotherapy. Surgery for colon cancer that has not spread involves removal of all cancer and a margin of normal colon, then reanastomosis. Removal of regional lymph nodes is also recommended. Radiation is used to supplement surgery to kill remaining areas of cancer that were not detected, or to shrink the size of the tumor

for removal. Chemotherapy is often used after surgery as it has been shown to increase the survival rate at some stages of CRC (http://www.cancer.org).

1.1.1.1 Adenocarcinomas in the large intestine

Tumors of the intestine can be categorized into polyps (benign), epithelial tumors (benign and malignant), and mesenchymal lesions (benign and malignant). Adenocarcinomas constitute 98% of all cancers in the colon and rectum [10], based on tumor cell classification. The colon and rectum also harbor carcinoid tumors and lymphomas. Tumor types unique to the small intestine include smooth muscle tumors, lipomas, angiomas, and rare hamartomatous mucosal lesions [11].

The colon and rectum are highly susceptible to polyps in adults. James Crawford describes polyps in the Pathologic Basis of Disease [11]:

- A polyp is a tumorous mass that protrudes into the lumen of the gut. Presumably, all polyps start as small, sessile lesions without a definable stalk. In many instances, traction on the mass may create a stalked, or pedunculated, polyp.
- Polyps may be formed as a result of abnormal mucosal maturation, inflammation, or architectecture. These polyps are non-neoplastic and do not have malignant potential per se. An example is the hyperplastic polyp.
- Epithelial polyps that arise as the result of the proliferation and dysplasia are termed adenomatous polyps, or adenomas. They are true neoplastic lesions (new growth) and are precursors of carcinoma.

- Some polypoid lesions may be caused by submucosal or mural tumors. As with the stomach and small intestine, however, unless otherwise specified the term polyp refers to lesions arising from the epithelium of the mucosa.

Polyps can be described by morphologic type, staging, and location. Approximately 90% of polyps are non-neoplastic, with remaining adenomatous polyps described by three morphologic subtypes: tubular adenomas, villous adenomas, and tubulovillous adenomas. Carcinoid tumors, gastrointestinal lymphomas, and mesenchymal tumors are all rare tumors of the large intestine that will not be reviewed here. Cancerous lesions can be classified by their severity using staging systems, of which Astler-Coller is one of the most widely used. The Astler-Coller system focuses on the extent of invasion and metastasis (outlined in the previous section). The risk of malignant transformation of a polyp, however, positively correlates with three independent variables: large polyp size, high villous component, and severe dysplasia [12]. Lastly, determining location is especially important for successful removal of all cancers. Colorectal adenomas are most likely to develop in the cecum and ascending colon (38%), then the sigmoid (35%), the transverse colon (18%), and least likely to form in the descending colon (8%) [13].

1.1.1.2 Few tumors of the small intestine

Limited information is available on tumors of the small intestine because of their rarity in humans. In the United States, 5,260 new cases and 1,130 deaths from small bowel cancer were reported in 2004 [3]. Unlike colorectal cancer, approximately 40% of small bowel cancers are

adenocarcinomas, 40% carcinoids, 15% sarcomas, and the remaining are lymphomas (reviewed in [11]).

Based on data gathered from the National Cancer Data Base (http://www.facs.org/cancer/ncdb/index.html), 65% of small intestine adenocarcinomas arise in the duodenum. The high incidence of adenomas in the duodenum, which comprises less than 10% of the length of the small intestine, suggests that this region has a particular predisposition to cancer. It has been suggested that the duodenum is exposed to components found in bile or gastropancreatic secretions that may be involved in tumorigenesis, explaining the high level of cancer development [14].

Little is known about why small bowel cancer is so rare. Lowefels [15] wrote in 1973 that the scarcity of tumors was surprising considering three things: 1) the high frequency of tumors in the neighboring stomach and colon, 2) the large surface area at risk in the small intestine, and 3) the continued exposure of this area to ingested foodstuffs. He cited reasons generally proposed including the fluidity of ingested contents which may "reduce the intensity of exposure" to possible carcinogens, rapid transit of intestinal contents which limits contact with carcinogens, and the relative sterility of the small intestine compared to the colon which limits bacterial metabolism of chemicals to carcinogens. He hypothesized that a protective immune system exists in the small bowel, and cited anecdotal evidence from patients that had developed small bowel cancer after receiving immunosuppressive therapy. Bone and Wright [16] responded to Lowefels, pointing out the high turnover rate of the epithelial lining of the small intestine may lead to the rapid shedding of malignant cells.

1.1.1.3 Hereditary Syndromes: Hereditary nonpolyposis colorectal cancer & familial adenomatous polyposis

1.1.1.3.1 Hereditary Nonpolyposis Colorectal Cancer

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common autosomal dominant familial CRC syndrome. The phenotype of HNPCC can be limited to colorectal cancer (Lynch I) or can include extracolonic cancers of the endometrium ovary, stomach, small bowel pancreas, hepatobiliary tract, brain or upper uroepithelial tract (Lynch II). Patients diagnosed with HNPCC are characterized by five features: 1) early average onset (approximately 45 years of age), 2) a particular familial pattern of primary cancer which can include colonic and endometrial cancer, 3) survival rate that differs from the normal for the specific cancer, 4) distinguishing pathological features, and 5) identification of a germ-line mutation in all affected members of the family (reviewed in [5].)

Guidelines for diagnosis include the Amsterdam I and Amsterdam II criteria, created by the International Collaborative Group on HNPCC. The Amsterdam I criteria specifies: 1) Three or more relatives in two successive generations with histologically verified colorectal adenocarcinoma, one of whom is a first degree relative of the other two, 2) at least one of relative diagnosed before age 50, and 3) exclusion of familial adenomatous polyposis, either clinically or by DNA testing. Modifications to the Amsterdam I criteria resulted in the Amsterdam II criteria,

which adding that three or more relatives must have a "cancer associated with HNPCC", including extraintestinal cancers, and that "tumors should be verified whenever possible" [17].

A germ-line mutation in mismatch repair (MMR) genes is known to cause HNPCC. A database of mutations is maintained by the International Society for Gastrointestinal Hereditary Tumors at http://www.insight-group.org. Almost 90% of all mutations have been identified in MLH1 and MLH2, with few or no mutations identified in *MSH6*, *PMS2*, and *PMS1* (reviewed in [5].) Individuals carrying a heterozygous mutation in a MMR gene are prone to replication errors in 1-4 base repeat sequences, resulting in microsatellite instability (MSI) which is hallmark of HNPCC [18-20].

Mouse models of HNPCC have been developed by targeted inactivating mutations in MMR genes, resulting in mice that develop tumors and cancer [21]. MMR repair gene deficient mouse models develop upper gastrointestinal and skin cancer, but do not develop colorectal cancer. Like humans bearing homozygous mutations in MMR repair genes, these mice also have reduced lifespan and are prone to lymphomas [22-24]. Mice heterozygous for mutations in MMR genes have not been observed to be prone to gastrointestinal cancer, but some develop skin cancer later in life [25]. Murine models of MMR are further described in section 1.2.2.2.

1.1.1.3.2 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is a hereditary colorectal cancer syndrome that is characterized by the development of a hundred to thousands of adenomas by the second decade of life. Diagnosis for FAP is determined by an autosomally dominant pattern of inheritance and

the development of more than 100 colorectal adenomas. FAP was first described by Bussey as an "...inheritable condition in which the large intestine contains multiple adenomas, multiple being defined as more than 100" because patients in the St. Mark's Register all had more than 100 polyps in surgically resected specimens [26]. In this group of patients, the average onset of FAP was 22 years of age, and over 80% of the polyps were less than 5 mm in diameter. Though FAP was once considered a disease of the colon, adenomas have been reported throughout the gastrointestinal tract, especially in the stomach (4-44% of patients) and duodenum (46-100% of patients). Gastric or duodenal cancer is a common occurrence in patients who have undergone colon resection [27].

Germline mutations in *APC (*adenomatous polyposis coli), a gene on chromosome 5q21, were first identified in patients with FAP [28-31]. FAP patients show a spectrum of *APC* mutations, with most in the mutation cluster region (MCR). The MCR has been shown to be involved in interaction with β-catenin in vitro [32, 33]. β-catenin functions in epithelial cell adherens junctions, and as a transcriptional activator in the Wnt signal transduction pathway that regulates cell growth and survival (reviewed in [34]). Mutations in the MCR result in a truncated protein that retains β-catenin binding but not regulatory activity. Studies correlating the number of polyps to mutations in MCR have suggested that patients with numerous polyps show mutations in the MCR, whereas those with attenuated FAP (less than 100 polyps) show mutations outside of the MCR [35, 36]. This conclusion is controversial and no other modifying genes for tumor multiplicity have been identified.

Insight into tumor multiplicity has been gained through mouse models of FAP (reviewed by Boivin et al. [37]). The first FAP model to be described was the multiple intestinal neoplasia (Min) mouse that was generated by chemical induction of a germline mutation [38]. These mice are heterozygous for the Apc^{Min} (*Min*) allele, which is a mutation at codon 850 of the murine *Apc* homolog, resulting in a truncated APC protein [39, 40]. C57BL/6J (B6) Min mice develop an average of 29 ± 9.4 intestinal adenomas [41], with the majority in the small intestine, [38, 39]. B6 Min adenomas consistently exhibit loss of heterozygosity (LOH) at the *Apc* locus, defined as loss of the wildtype allele [42, 43]. The number of tumors in Min mice is influenced by *Modifier of Min-1* (*Mom1*) [44], which was identified by quantitative trait locus studies, and determined to be the gene for nonpancreatic secretory phospholipase A2 group IIA (*Pla2g2a*) that is a modifier of tumor multiplicity and size. It was discovered that *Pla2g2a* was not solely responsible for tumor multiplicity when congenic mice on other strains were developed that showed varying tumor multiplicity. No modifiers of intestinal tumor multiplicity have been identified in humans with FAP.

In addition to intestinal tumors, mouse models of FAP (reviewed in [37, 40]), and more importantly FAP patients, develop extraintestinal neoplasms. In 1962, Gardner published a report outlining the extracolonic syndromes of a group of patients with multiple intestinal polyposis. These patients were diagnosed with osteomas, fibromas, epidermal cysts, sebaceous cysts, and trichoepitheliomas [45]. Patients displaying these extraintestinal lesions with intestinal polyposis are now diagnosed as having Gardner Syndrome, a phenotypic variant of FAP.

1.1.1.4 Inflammatory bowel disease increases risk for intestinal cancer

Inflammatory bowel disease, which includes ulcerative colitis (UC) and Crohn's disease (CD), is characterized by an idiopathic chronic inflammation in the gastrointestinal tract of patients. Ulcerative colitis (UC) results in diffuse inflammation within the colon that extends proximally from the rectum. Crohn's disease (CD) can affect any portion of the gastrointestinal tract from the mouth to the anus, often causing focal areas of disease. Inflammation is transmural, involving both the mucosa and submucosa, and progresses over time, leading to structuring or fistulising complications [46]. General manifestations of both diseases include fatigue, loss of appetite, and sometimes fever. Specific symptoms relating to the bowel include irregular bowel movements containing mucus and/or blood, abdominal pain, nausea, or vomiting. In some cases, ulceration can result in blood loss leading to fecal blood and anemia. Patients are also diagnosed with inflammation at distant sites including the joints (arthritis) and eyes [11]. The peripheral arthritis predominantly affects the lower limbs [47].

The greatest risk factor for developing IBD is a positive family history, seen in 5-10% of IBD patients (reviewed in [48].) Orholm et al. [49] determined that first-degree relatives of UC patients have a population relative risk of 10, while those of CD patients have a relative risk of 14. Twin studies have given strong support for the involvement of genetic factors in CD. One such study determined that identical twins of IBD patients were more likely (11/63, 17%) to have IBD than non-identical twins (4/80, 5%) [50]. Because there is not a clear mendelian inheritance of IBD, it has been difficult to identify a gene responsible for UC or CD.

Significant progress has been made in identifying mutations in *NOD2* associated with CD. NOD2 is an example of a pattern recognition receptor (PRR) involved in recognizing pathogen-associated molecular patterns (PAMP's), which are highly conserved structural motifs in bacteria [51]. NOD2 is expressed in the cytoplasm of myeloid cells [52], and intestinal epithelial cells, and is involved in NF-κB activation in response to bacterial antigens [53].

Two laboratories implicated *NOD2,* within *IBD1,* a putative susceptibility locus for CD on chromosome 16q12 [54]. Hugot et al. [55] used microsatellite markers to perform linkage analysis on CD families, and identified alleles of *NOD2* that were associated with an increased risk for CD. Ogura et al. [56] identified a frameshift mutation in *NOD2* in association with CD, observed preferential transmission of this allele to affected children of heterozygous parents, and used case-control analysis to determine the high frequency of this allele in CD patients. Additional loci *IBD2*, *IBD3*, *IBD4* and *IBD5* are being investigated for their role in CD risk, and their interaction with NOD2 (reviewed in [57].)

Patients with IBD have an increased risk of developing CRC. Though IBD contributes to only 1-2% of all CRC cases, it is the third strongest risk factor for CRC, only behind the familial cancer syndromes HNPCC and FAP (reviewed in [58].) Early studies associating IBD to CRC were comprised of case reports and hospital series. Three major biases were introduced by the patient populations used these studies: 1) referral-center populations were not representative of the general population, (2) patients analyzed already had cancer at the time of referral, and thus increased the incidence of cancer, and (3) some patients had already had colectomies and thus were no longer at risk for colon cancer [59]. Population-based studies are considered the

standard for studying disease course and prognosis, but the cost of such studies and the availability of standardized records on large numbers of patients is limited. The following sections focus on the cancer risk associated with both UC and CRC based on epidemiological studies.

1.1.1.4.1 Cancer Risk in Ulcerative colitis

UC was first reported to be associated with CRC in 1925 by Crohn and Rosenberg in one case of carcinoma of the rectal wall in association with ulcers and long-term inflammation [60]. Numerous reports using case studies and hospital series have confirmed the association [61, 62], leading to a recommendation in the early 1980's that patients who had extensive UC for 7 to 10 years undergo a colonoscopy with multiple biopsies to help detect dysplasia every 1 to 3 years [59].

Estimates of the increased risk varied greatly in early studies using hospital series and referral centers. Efforts to address this have been made in analyzing larger populations based on geography. The first population-based study investigated 3117 patients from the Uppsala Health Care Region in central Sweden. The authors focused on extent of colon involvement and its association with increased CRC. When the ratio of observed to expected cases of CRC in UC patients was calculated, those with left-sided colitis had a ratio of 2.8, while those with pancolitis had a ratio of 14.8. This illustrated that more extensive disease correlated with increased incidence of CRC [63].

Lower rates of CRC in UC patients were found in a population study from Denmark [64] as compared to those found in the previously described study in Sweden. The majority of patients in this study, however, received 5-aminosalicylic acid as anti-inflammatory therapy, and it is hypothesized that the resulting decrease in inflammation partly accounted for the lower incidence of CRC.

More recently, a meta-analysis of 116 studies on the risk of CRC in UC was performed to better determine the increased risk of CRC in UC patients. Studies were chosen that were written in English, had a clear definition of the population studied, and defined criteria for UC and CRC [62]. The results from this analysis were consistent with most papers in the literature that have shown increased risk for CRC in UC patients, with a positive correlation between duration of disease and higher CRC incidence. It was found that the overall prevalence of CRC in UC patients is 3.7%, with the incidence rate of CRC in UC patients determined as 3 per 1000 person years duration (pyd). In the general population, the incidence rate of CRC is 0.6 per 1000 pyd. Thus, there is a 5-fold increase in CRC incidence in UC patients per pyd as compared to the general population. The cumulative risk of developing CRC for UC patients, stratified from time of diagnosis by decade in 19 reports, showed a 2/1000 pyd for the first decade, 7/1000 pyd for the second decade, and 12/1000 pyd for the third decade. This study also reported an increase in cases of CRC in UC patients over time by geographical region, but this finding was statistically insignificant. These data suggest that there may be a increase in CRC in UC patients that should continue to be monitored [62].

1.1.1.4.2 Cancer Risk in Crohn's Disease

The risk of CRC and small bowel cancer in patients with Crohn's disease (CD) has been studied less extensively, and results have been less clear on the extent to which Crohn's increases risk for CRC. Some studies have showed increase in relative risk (RR) for CRC in Crohn's patients. Ekbom et al. [65] showed an increased RR of 2.5 with a 95% confidence interval (CI) of 1.4-5.3, based on a study of 1655 Crohn's patients in Sweden from 1983 to 1984. Patients with a diagnosis of Crohn's before the age of 30 showed a RR of 20.9 (95% CI 6.8-48.7), while those who had been diagnosed at 30 or older showed a lower RR of 2.2 (95% CI of 0.6-5.7.) Gillen et al [66]. assessed RR for CRC cancer in 281 Crohn's patients in England. They followed the patients for 16 to 46 years, with a mean follow up time of more than 20 years. The RR for these Crohn's patients as compared to the general population was 3.4 (95% CI 1.5-6.7). Those with extensive colitis, however, were the only patients observed to develop colorectal cancer, and their RR for CRC was 18.2 (95% CI 7.8-35.6). There was also an increase in RR in patients with diagnosis of Crohn's before the age 25 as compared to those diagnosed at the age of 25 or thereafter.

Three population-based studies from Sweden [67] and Denmark [68, 69] have reported no increase in the incidence of CRC in Crohn's patients, but have reported a positive increase in incidence of small bowel cancer, as compared to the general population. Persson et al. [67] found no significant increase in the incidence of CRC in 1251 Swedish patients with CD, but did observe an increase in upper gastrointestinal tract cancers as compared to the general population. Jess et al. [68] found a significant increase in the incidence of small bowel adenocarcinoma in 374 Danish Crohn's patients diagnosed over a period of 25 years. They reported a 66-fold

increase (95% confidence interval 18-170), calculated by dividing the number of patients with small bowel cancer (4) by the number expected in the patient population (0.06). Since it is impossible to have 0.06 patients with cancer, the 66 fold increase is misleadingly high. Four cases of small bowel cancer, however, in this small study are significant (see section 1.1.1.2).

In conclusion, IBD is thought to contribute to less than 2% of CRC cases, but chronic inflammation caused by IBD is an important risk factor for CRC [61-63, 65, 66] as well as small bowel cancer [67-69]. As CRC is a leading cause of cancer death, understanding the carcinogenesis of CRC in the context of IBD may help elucidate important mechanisms of tumorigenesis. The risk factors for CRC in IBD include young age of onset of IBD, large extent of tissue involvement, and long duration of disease.

1.2 Intestinal Carcinogenesis

1.2.1 Cells at risk in intestinal cancer

Before constructing a model or hypothesizing mechanisms of intestinal carcinogenesis, it is necessary to understand the biology of the tissues involved and the type of tumors that may arise in the intestine.

In an adult human, the small intestine is approximately 6 meters in length and the colon is about 1.5 meters in length. The main function of the small intestine is to absorb degraded components of food. The first 25 cm of the small intestine is called the duodenum. The jejunum is approximately the next third, and the ileum comprises the remaining two thirds of the small

intestine. The colon absorbs water from the remaining waste, and holds feces before defecation. The proximal to distal colon is divided into the ascending, transverse, descending, and sigmoid colon [11].

The small intestine is characterized by its mucosal lining organized as villi, which are finger-like projections composed of epithelial cells. The major epithelial cell types are columnar absorptive cells with microvilli (brush border) and mucin-secreting goblet cells. Between the villar projections, crypts contain undifferentiated epithelial cells, goblet cells, endocrine cells, and a few Paneth cells. The core of the villi is comprised of lamina propria containing blood vessels, lymphatics and a small population of lymphocytes, eosinophils, mast cells, fibroblasts, and smooth muscle cells. Under these projections is a thin layer of muscle called the muscularis mucosae. Cells migrate from the base of the crypts to the tips of the villi, and the epithelial lining of the small intestine is renewed every 4 to 6 days [11]. The duration of mitosis in the human small intestine has been estimated to be 1-1.5 hours in vivo [70].

The colonic mucosa is relatively flat compared to the small intestine. The mucosa has crypts that extend to the muscularis mucosa and villi are absent. Crypts are comprised of columnar absorptive cells with scant, shorter microvilli, and mucinous goblet cells. Inside a crypt resides columnar absorptive cells, goblet cells, and endocrine cells. Paneth cells are occasionally found in the proximal large intestine. The epithelial surface of the colon is renewed every 3 to 8 days [11].

The immune system in the intestine is made up of nodules of lymphoid tissue and scattered cytotoxic T cells, helper T cells, and mature B cells. The nodules lie in the mucosa, or reside in the mucosa and submucosa, and are overlaid with columnar absorptive cells and M (membranous) cells. These M cells transcytose macromolecules from the lumen of the intestine and interact with underlying lymphocytes that serve as surveillance cells. In the ileum, these nodules can be quite large in size, and are referred to as Peyer's patches. These immunologic components join with the appendix and mesenteric lymph nodes (MLN's) to make up the mucosa-associated lymphoid tissue (MALT) [11].

1.2.2 Models/mechanisms

Cancer is thought to be a result of loss of genetic stability in tumor suppressor genes and oncogenes [1]. Colon cancer develops over the course of decades, making it possible to investigate the cellular changes involved in carcinogenesis. In 1990, Fearon and Vogelstein presented a multistep model for colon carcinogenesis (Figure 1-2), which points to genetic changes during the development of cancer [1]. An example of this is the observation of mutations in APC in the early stages of tumor development of sporadic colonic adenomas [71] (reviewed in [72]). Mutation or change in gene expression may arise from multiple mechanisms including nucleotide modification through oxidative stress during inflammation, microsatellite instability, and methylation [73, 74]. The following sections focus on what roles these mechanisms may have in carcinogenesis.

1.2.2.1 Oxidative stress

Chronic inflammation induced by biological, chemical or physical processes has been associated with increased cancer risk in humans. During chronic inflammation, there is an infiltration of damaged tissue by mononuclear cells/macrophages, lymphocytes, and plasma cells that attempt to resolve infections and repair the damage. Macrophages are key players in chronic inflammation and carcinogenesis because these cells release many products including cytokines, chemokines, growth factors, reactive metabolites of oxygen, and nitric oxide [75].

Inflammation can trigger cells to activate enzymes that generate oxidants. Such enzymes include inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO•) from L-arginine, NADPH oxidase and xanthine oxidase, which produce superoxide anion ($O•^-_2$), and myeloperoxidase (MPO), which produces hypochlorus acid (HOCl) from hydrogen chloride (H_2O_2) and chloride ion (Cl^-). These oxidants can target DNA, proteins, RNA and lipids. More specifically, these oxidants can lead to formation of DNA adducts and induction of somatic mutations [76, 77].

Chronic inflammatory conditions have been associated with cancer. IARC monographs evaluating human pathogens that lead to long-term inflammation have classified some infectious agents to be carcinogenic to humans [78-82]. More specifically, conditions such as schistosomiasis, papillomavirus infection, and hepatitis B virus infection all contribute to increased cancer risk (see section 1.3). In the gastrointestinal tract, chronic inflammation induced by noninfectious and infectious diseases have been associated with cancer. IBD, which

is the third leading risk factor for CRC (discussed in section 1.1.1.4), has been associated with mutations in the tumor suppressor gene *p53*. An increase in *p53* mutations and an increase in iNOS expression are found in inflamed tissues of UC patients [83, 84].

Helicobacter pylori-induced gastritis has also been associated with increased levels of nitrotyrosine [85]. It has been assumed that peroxynitrite (ONOO-), a product of NO• and $O•^-_2$, is the major causative agent of nitrotyrosine detected at sites of cellular damage. Initial support for ONOO- being associated with nitrotyrosine and tissue injury came from Beckman et al. [86] who hypothesized that reaction of NO• and $O•^-_2$ in vivo leads to production of ONOO-, which then causes tissue damage and leaves nitrated tyrosyl groups. This explained the observation that superoxide dismutase, which catalyzes the reaction of $O•^-_2$ to hydrogen peroxide, can inhibit tissue injury. The relevance of ONOO-, however, to nitrotyrosine formation has come into question. It has been argued that the requirement for equal amounts of NO• and $O•^-_2$ to produce ONOO- makes it unlikely that ONOO- could be a major source of nitrotyrosine in vivo (reviewed by [87].) Instead, it has been suggested that myeloperoxidase (MPO) may be a source of reactive nitrogen species involved in nitrotyrosine formation [88].

1.2.2.2 Microsatellite instability

Microsatellite instability (MSI), or mutations in repeat sequences of 1-4 basepairs, is a hallmark of tumors in HNPCC [5]. Inactivation of genes involved in the DNA mismatch repair (MMR) system is associated with MSI in tumors [21]. Thus, prokaryotic and eukaryotic models have been developed to study the process of MMR and tumorigenesis. Studies in yeast

(*Saccharomyces cerevisiae*), bacteria (*Echerichia coli)*, and mice deficient for genes involved in MMR have led to the current understanding of the types of mutations repaired by MMR, and the specific proteins involved with the resolution of these mutations.

The mammalian MMR system detects and repairs base substitution mutations and small nucleotide insertion/deletion mutations. It further signals apoptosis in response to DNA damaging agents and suppresses homologous recombination between similar but not identical sequences. Studies of the *E. coli* MMR system have led to the model of the MutHLS system which includes the proteins MutH, MutL and MutS. MutS binds to the base pair mismatch, then MutL binds to the MutS-mismatch complex in an ATP-dependent manner. MutH is activated, and this endonuclease excises the portion of the DNA strand that includes the base pair mismatch. The excised strand is replaced with a newly synthesized strand that pairs with the parental strand (reviewed in [89]).

In yeast, there are six MutS homologs, MSH1-MSH6. Mammals lack MSH1, which is used for repairing base substitution mutations in mitochondrial DNA. Yeast and mammals both utilize complexes of MSH2, MSH3, and MSH6 as homologues of MutS. MSH2 and MSH6 form a heterodimeric complex (Mut Sα) that initiates the repair of base-base mispairs and single base insertion/deletion mispairs. The MSH2-MSH3 complex (Mut Sβ) repairs insertion/deletion mispairs of 1 to 4 bases. The ability to repair even larger insertion/deletion mispairs up to 38 bases has been suggested [89]. Yeast and mammals also have similar MutL homologs. In yeast, MLH1-PMS1 (PMS2 in humans) complex interacts with MutSα and MutSβ. Yeast have two

additional MutL homologs MLH2 and MLH3. The latter interacts with MLH1, but MLH2 does not appear to be involved in MMR.

Inherited mutations in MMR genes have been shown in virtually all CRC's with HNPCC, and microsatellite instability has been detected in approximately 13% of sporadic CRC's [18, 20]. As previously mentioned (see "Hereditary Nonpolyposis coli" section), almost 90% of all mutations have been identified in MLH1 and MLH2, with few or no mutations identified in *MSH6*, *PMS2*, and *PMS1* (http://www.insight-group.org.) The redundancy in function of MSH3 and MSH6 may account for the low number of mutations in these genes [90]. Animals models deficient in MMR have led to models of HNPCC (reviewed in section 1.1.1.3).

1.2.2.3 Methylation

DNA methylation in CpG islands associated with promoters is an epigenetic mechanism regulating gene expression and silencing of repeat elements [74]. Several DNA methyltransferases have been implicated in playing a key role in maintaining methylation of cytosine in CpG islands. Research has focused on the DNA methyltransferases DNMT1 and DNMT3b, but it has not been clearly determined which DNA methyltransferases are essential for CpG methylation [91-93]. This is important as hypomethylation of CpG islands leading to activation of nearby oncogenes, or hypermethylation leading to silencing of tumor suppressor genes has been implicated in the development of cancer as outlined below.

In 1983, Feinberg and Vogelstein made the first report of a change in methylation levels in primary tumors. They showed that methylation at CpG nucleotides was substantially decreased in some genes in cells from primary tumors as compared to that from normal tissue in four out of five patients [94]. This led to the discovery that global hypomethylation was detected in the majority of metastatic neoplasms when measuring the 5-methylcytosine content of DNA from tumors by HPLC [95]. Specific genes shown to be hypomethylated in human cancers include *HRAS* [96] and *CAGE* [97] in gastric cancer. The gene *S100A4*, which has been associated with metastasis in colon cancer, has been shown to be hypomethylated in human colon adenocarcinoma cell lines [97].

Hypermethylation that leads to silencing tumor suppressor genes has also been investigated. Inactivation of genes by promoter region hypermethylation has been demonstrated for several tumor suppressor genes including *CDKN2/p16/MTS1* [98, 99], PTEN/MMAC1 [100], and *VHL* [101]. Many of these genes are involved in cell cycle progression including p16, which binds to cyclin-dependent kinase 4 (CDK4), inhibiting the ability of CDK4 to interact with cyclin D1 and stimulate cell cycling through the G1 phase. The role of *p16* as a tumor suppressor was once controversial because of the low rate of genetic mutations at this locus. Evidence that *p16* is hypermethylated in some tumors has illustrated that this could be an important pathway for *p16* inactivation.

Hypermethylation has been implicated in downregulating expression of both *APC* and *MLH1* in sporadic colon cancer. Though the majority of patients with sporadic colorectal cancer have been shown to have somatic mutations in *APC* as an early event in carcinogenesis [71],

methylation may be an important pathway to *APC* inactivation in some patients. Hiltunen et al. [102] investigated the methylation of CpG sites in the promoter region of *APC* in tissues from colorectal cancer patients. DNA from eighteen carcinomas showed approximately 3-fold more methylated cytosines compared to DNA from both twenty-four samples of normal mucosa and ten adenomas.

Kane et al. [103] investigated the expression of *MLH1* in 66 sporadic colorectal tumors and found that four of these tumors did not show *MLH1* expression, or showed mutations in the sequence of *MLH1*. All four tumors also showed methylation at the promoter site to *MLH1* [103]. The authors hypothesized that methylation is a common mechanism of mismatch repair inactivation.

1.3 Paradigm of chronic infection, inflammation and carcinogenesis

The association of cancer with chronically inflamed tissue has been recognized since 1863 when Rudolph Virchow observed leukocytes in neoplastic tissue [104]. Today, the causal relationship between oxidative stress that ensues from inflammatory cells and genetic damage that leads to cancer is widely accepted. The paradigm of chronic infection leading to inflammation being associated with cancer has been supported by the following examples of viral, helminth and bacterial infection.

1.3.1 Viral Hepatitis

Epidemiological and experimental studies have established chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) as major risk factors for hepatocellular carcinoma (HCC), the dominant form of liver cancer [79]. Chronic infection with HBV or HCV was determined a group 1 carcinogen [79]. The relative roles of HBV and HCV infection vary among populations. In the People's Republic of China where there is a high incidence of HCC, HBV is the leading risk factor [105]. HCV infection plays a minor role in HCC in China, but HCV infection has been implicated in the increasing incidence of HCC in the United States [106] as a result of high HCV infection in the 1960's and 70's. The association between HCV and HCC has also been made in Japan [107] and Europe where HCV was first linked to HCC [108, 109].

Individuals chronically infected with HBV have a 10-25% lifetime risk of dying from liver cancer [110]. The length and severity of inflammation are both important risk factors in HBV-associated liver cancer. The rate HCC occurs in an individual infected with HBV increases by 0.2-3% per year. This incidence rate is also dependent on the level of cirrhosis, an indicator of inflammation, with noncirrhotic patients having a rate as low as 0.2% per year, compared to cirrhotic patients that have a rate as high as 2-3% per year [111]. The time from infection to cirrhosis ranged from 13 to 23 yrs, and from infection to HCC is 17-31 years [112].

The mechanism by which HBV infection contributes to liver cancer has been studied by using an animal model with hepadnavirus woodchuck hepatitis virus (WHV), which shares genetic similarities to HBV [113]. This HBV-like virus causes chronic hepatitis in woodchucks,

resulting in HCC in almost all animals. Liu et al. [114] investigated the role of inflammation in these woodchucks by analyzing nitrate, a biomarker for inflammation, in the urine of experimentally infected animals. Urinary nitrate has been used as an indicator for nitric oxide production [115]. WHV-infected animals have 3-fold more endogenous nitrate than uninfected animals, and hepatocytes isolated from infected woodchucks formed twice as much nitrite as hepatocytes from noninfected animals [116]. These data show increased endogenous production of nitric oxide in association with HBV-induced inflammation. This is an important finding as it suggests that nitric oxide, a potent oxidizing agent, may play a role in HBV-associated cancer.

Efforts to create murine models of HBV and HCV infection have been difficult, as rodents cannot be infected with either virus. Several transgenic mouse models, however, have been developed using HBV or HCV genes. Many of these mouse models develop HCC after one year, but some do not due to immunotolerance to transgenes expressed during embryogenesis (reviewed in [117-119].) In an effort to create transgenic HBV models, transfer of immune cells from antigen-primed syngeneic wild type mice and other methods have been used to overcome immunotolerance (reviewed in [118].) Another approach has been to use transgenic mice that have conditional expression of viral genes.

Transgenic mouse systems and cell lines [120] have been developed to study the HCV core protein, E1, E2 and other HCV proteins (reviewed in [119]). Study of HCV proteins in both cell lines and mouse models have led to evidence that HCV plays a role in increasing oxidative stress. Cell line models showed that expression of HCV core protein increases cellular reactive oxygen species and lipid peroxidation products [121]. The same authors also reported that

transgenic mice expressing HCV core protein/E1/E2 showed a two fold increase in liver lipid peroxidation products when treated with CCl_4 by intraperitoneal injection as compared to animals treated with olive oil. These results suggest that HCV proteins may be involved in inducing oxidative stress in human infection.

1.3.2 Parasites

IARC monographs evaluating human pathogens that lead to long-term inflammation have classified the parasites *Schistomsoma haematobium* and *Opisthorchis viverrini* to be group 1 carcinogens based on epidemiologic data [78]. *S. haematobium* has been associated with bladder, *O. viverrini* with cholangiocarcinoma.

S. haematobium is a trematode that lives in the blood and matures in the veins that drain the bladder. Adult worms reside in the veins, producing eggs over a period of years. Most eggs leave the body, but some remain trapped in the bladder and result in hypersensitivity granulomas and chronic inflammation. Studies have shown that in Egyptian men, who have the highest rates of bladder cancer and *S. haemotobium* infection, urinary schistosomiasis accounts for an estimated 16% of bladder cancer cases [122]. Urothelial cells from Egyptian men were assayed for the presence of micronuclei, a quantitative indicator of chromosomal breakage [123]. Micronucleus frequencies were significantly higher in men infected with *S. haematobium* (mean frequency, 0.97 +/- 0.12%) than among controls (mean frequency, 0.12 +/- 0.04%, $p < 0.001$) [124]. Thus, it has been hypothesized that *S. haematobium* infection may lead to chromosomal breakage, a possible mechanism of carcinogenesis.

Infection with the helminth *O. viverrini* is thought to be responsible for the high incidence of cholangiocarcinoma in Thailand [125]. These parasites infect the bile duct, and sometimes the pancreatic ducts and gallbladder. Flukes mature and lay eggs in intrahepatic ducts, leading to biliary stones, gall-bladder stones, and inflammation in some hosts. Little is known about the mechanisms by which *O. viverrini* infection of humans leads to cancer, but some work has been done in animal models. Syrian golden hamsters experimentally infected with *O. viverrini* have significantly greater amounts of urinary nitrate, a stable oxidization product of NO, than uninfected hamsters (3.64 +/- 0.86 versus 2.64 +/- 0.60 umol/hamster/day, $P < 0.001$) [126]. *Fasciola hepatica*, a liver fluke of ruminants and a model for *O. viverrini*, has been shown to increase mutant frequency in Big Blue™ mice twenty-three days post infection [127]. Though these data are not conclusive, they support the hypothesis that helminth infection leading to inflammation can result in increased production of NO. This is important as increased NO production in vivo has been shown to lead to increased mutant frequency [128].

1.3.3 Helicobacter pylori

Helicobacter pylori was first isolated in 1982 from stomach biopsies of humans suffering from chronic superficial gastritis [129]. Infection with *H. pylori* is now implicated in the pathogenesis of gastritis [130] and is a risk factor for peptic ulcer disease. Epidemiological data has shown an increased risk of gastric cancer and gastric MALT lymphoma with chronic *H. pylori* infection, which has led to it being classified as a group 1 carcinogen by the World Health Organization [131].

There is mounting evidence that chronic infection induced by *H. pylori* induces somatic mutations that may lead to stomach cancer. Accumulation of nitrotyrosine has been observed in the inflamed tissue of individuals with *H. pylori*-associated gastritis. When these individuals were treated with a triple therapy of antibiotics to eradicate the infection, they showed significantly decreased levels of nitrotyrosine [85]. Nitrotyrosine has been used as a marker for oxidative stress, which is hypothesized to have a possible role in carcinogenesis [132] (see section 1.2.2.1). Furthermore, mutations in *p53* have been associated with infection by virulent strains of *H. pylori* [133, 134]. Infection by either *H. pylori* or *H. felis*, another gastric pathogen, can induce increased mutant frequency in Big Blue™ mice after six months of infection, and is associated with elevated levels of iNOS [135]. Thus, *H. pylori* has been associated with elevated NO• production concurrent with somatic mutations.

1.3.4 Mutational Analysis Systems

The association between infection leading to chronic inflammation and increased cancer risk has been established with epidemiological studies and animal models (see sections 1.3.2, 1.3.3). Many individuals have proposed that oxidative stress resulting from long-term inflammation infection induces mutations in key genes involved in carcinogenesis [136-139]. Recent experiments in mice involving *Fasciola hepatica* [127], *H. pylori* and *H. felis* [135] show that inflammation associated with infection can indeed lead to increased mutant frequency. This section discusses murine mutational analysis systems in which one can test such hypotheses.

1.3.4.1 Endogenous versus Exogenous DNA Mutational Analysis Systems

Genetic sequences are used as targets in mutational analysis systems to detect somatic mutations and assess the mutagenicity of chemical, biological, or physical agents. These in vivo models use endogenous genes or exogenous genes (transgenes) to measure mutations, with the assumption that results garnered from the target genes are indicative of mutations in the host genome. Both types of systems have advantages and disadvantages as outlined below.

Several transgenic systems have been developed in which lambda phage or plasmid DNA is used to detect mutations ex vivo by rescuing and expressing the transgene in *Escherichia coli* [140, 141]. Transgenic systems have two main disadvantages as compared to endogenous systems that use host DNA. The first disadvantage stems from the fact that transgenic DNA differs from many host genes in that it 1) is prokaryotic DNA, 2) is present in multiple tandem copies, 3) is methylated and therefore not expressed. Endogenous systems use host DNA that is expressed and remains in its native chromosomal conformation [142]. Second, transgenic systems rely on ex vivo expression of target genes, which makes the targets susceptible to mutations induced outside of the treated host. Endogenous genes have mutations that have been induced in vivo and are assessed in vivo.

Endogenous systems, however, are limited to certain cell types and can be cumbersome to perform [142]. An example is the *Dlb-1* mouse assay that allows the quantification of mutations in epithelial cells of the small intestine of mice. These mice have heterozygous *Dlb-1*^{a}/*Dlb-1*^{b} cells. The *Dlb-1*^{b} locus encodes the receptor for the lectin *Dolichos biflorus* agglutinin (DBA)

that is expressed on the villus surface. When mutations are induced in the *Dlb-1*^{b} allele, ribbons in villi appear unstained with DBA-peroxidase conjugate 5-7 days later after the mutated stem cell is allowed to produce mutant progeny [143]. Another endogenous system is the *Aprt* mouse model in which mutations can be detected in T lymphocytes and skin fibroblasts of *Aprt* heterozygous mice [144]. Unlike the Dlb-1 mouse assay, cells must be cultured in vitro to determine if they are mutant. The p^{un} mouse assay allows visual observation of intrachromosomal recombination by scoring black spots on light gray fur, or black cells on transparent retinal epithelium in offspring [145]. Transgenic systems were designed to be easier to perform and overcome the restriction of cell specific endogenous systems. The following section outlines common models of mutational analysis that use either endogenous or transgenic reporter genes.

1.3.4.2 Common Transgenic Mutational Analysis Models

Transgenic mutational analysis models that use lambda phage shuttle vectors and plasmid vectors have been used extensively to test various chemical, biological and physical agents. The BigBlue^{TM} and Muta^{TM}Mouse systems, that use *lacI* and *lacZ* respectively, were the first such models, and are now commercially available [146, 147]. The BigBlue^{TM} mouse model uses the λLIZα shuttle vector, comprised of the *lacI* gene as a reporter, the *lacI* promoter, the operator sequence *lacO*, a fragment of *lacZ*, and a gene for ampicillin resistance. This system is available on two mouse strains, C57BL/6 and B6C3F1 [146], and on the Fischer 344 rat [148] from Stratagene (La Jolla, CA, USA). Approximately 30-40 copies of the transgene construct are stably integrated head to tail on chromosome 4. After the rodents are treated with an agent,

genomic DNA is isolated, and single copies of the construct are packaged into phage heads. The phage are then incubated with *E. coli* SCS8, and infected bacteria are selected on media containing ampicillin, while mutants are assessed using X-gal in a colormetric assay. Those plaques expressing a nonfunctional repressor, as a result of a *lacI* mutation, have successful transcription of β-galactosidase. This enzyme allows the cleavage of X-gal to 5-bromo-4-chloro-indigo which gives rise to a blue plaque. The mutant frequency is calculated as the number of blue plaques, divided by the total plaque-forming units. This phage-based system can detect base substitutions, frameshift mutations, insertions and small deletions up to about 7.5 kilobases [148].

The Muta™Mouse system uses the λgt10*lacZ* shuttle vector that includes a *lacZ* reporter gene flanked by cos sites which enable rescue of the λ phage vector. The 40.6 mouse strain available from Covance Research Products (Princeton, NJ, USA) has approximately 40 integrated copies of the transgene on chromosome 3 [149]. Assessing mutant frequency follows the same principle as that for the BigBlue™ mouse, but the *lacZ* gene serves as the reporter in a colormetric screen.

An improved assay was developed for the Muta™Mouse system, using *E coli* lacking *galE* and *lacZ* , and selection media containing phenylgalactose (P-gal) [150] . When the *galE lacZ E. coli* are infected with phage carrying functional LacZ, P-gal is broken down by β-galactosidase to galactose. The galactose is eventually metabolized to UDP-galactose, which is toxic when accumulated. *E.coli* lacking *galE*, and thus UDP epimerase activity, cannot convert the toxic metabolite to UDP-glucose. When *galE lacZ E. coli* are infected with phage carrying a

functional mutation in *lacZ*, the *E. coli* hosts survive, and plaques can be enumerated to determine the mutant frequency.

An additional selection method can be used to assay for mutant frequency in the BigBlue™ and Muta™Mouse systems. The CII assay is a positive selection method that utilizes the CII repressor protein, involved in controlling the λ phage lysogenic/lytic cycle. When *E. coli* expressing Hfl protease are infected with phage with a functional CII protein, the Hfl protease degrades the CII protein and allows phages to enter the lytic cycle. In *E. coli* lacking *hfl*, phages with functional CII cannot enter the lytic cycle. Mutations in CII can be selected for by infecting hfl- *E. coli* with phage that do not encode for a functional CII protein and thus can make plaques. Due to a mutation in the CI genes of the BigBlue™ and Muta™Mouse mice, the CII assay must be carried out at 24°C for 2 days to select for mutants [151].

The pUR288 transgenic mouse system uses *lacZ* as a reporter gene similar to Muta™Mouse systems, but on a plasmid shuttle vector that is carried in 20 concatemerized copies [152]. This plasmid-based system was designed to address the limitation in phage-based systems to detect clastogens, such as x-ray treatment, which induce large deletions. The transgene construct contains *lacZ* and ampicillin resistance genes, and a HindIII site. The plasmid is isolated by digesting genomic DNA with HindIII to separate each plasmid copy. The digested DNA is incubated with anti-β-galactosidase antibody-coated magnetic beads to isolate each plasmid. To separate the plasmid from the beads, IPTG is added. Ligation is used to circularize the vector, and electroporation is used to infect an indicator *E. coli* strain in the same selection scheme discussed for the Muta™Mouse system [153].

1.3.4.3 The *gpt* delta transgenic mouse mutational analysis system

The *gpt* delta transgenic mouse has been developed by Nohmi et al. [2] to allow for the detection of point mutations and deletions less than 10 kb using 6-thioguanine and Spi- selection respectively, in the same animal. The mutational spectra, or composite of all the mutations, of the *gpt* gene in this system has been established for mice treated with ethylnitrosourea [154], 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) [154, 155], UV-B [156], aminophenylnorharman [157], and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) [158]. The following agents have been tested in the Spi- selection system: γ- [159] and X-rays [160], 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) [154, 155], mitomycin C [161], and aminopheylnorharman [157]. The following sections focus on utilizing the transgenic λEG10 phage vector (Figure 1-1) carried by the *gpt* delta transgenic mouse for assaying mutant frequency.

1.3.4.3.1 The gpt *assay*

The plasmid construct of the *gpt* delta transgenic mouse allows for selection of *gpt* mutants based on the purine salvage pathway. The 456 bp *gpt* gene encodes for guanine phosphoribosyltranstransferase (Gpt), which catalyzes the phosphoribosylation of guanine, a key step in the incorporation of guanine to DNA. The Gpt enzyme plays a role in the incorporation of 6-thioguanine (6-TG) that is lethal to cells. Thus, *E. coli* infected with plasmids expressing

functional *gpt* will not grow in media containing 6-TG, whereas *E. coli* carrying mutant *gpt* will form colonies.

To carry out the *gpt* assay, genomic DNA is isolated, and phage are circularized and packaged into phage heads. These phage are used to transduce *E. coli* YG6020 that express Cre recombinase, resulting in propogation of the pYG144 vector. *E. coli* carrying plasmids with mutant *gpt* are selected for on plates containing 6-TG and chloramphenicol (Cm). The total number of rescued plasmids is assessed by plating cells on plates with Cm alone [2].

1.3.4.3.2 Spi- assay

The Spi- assay selects for *E. coli* that are resistant to interference from the prophage P2. *E. coli* carrying prophage P2 inhibit the growth of wildtype λ phage, and thus this phenotype is called Spi+ (sensitive to P2 interference). Phages that are deficient in both *red* and *gam* gene function, and express *chiC* are resistant to P2 lysogens and therefore display a Spi- phenotype. These phage can create plaques in a *recA+ E. coli* strain that allow recombination events and the replication of phage DNA.

The *red*, *gam*, and *chiC* elements of the *gpt* delta construct, as well as proteins encoded by the *E. coli* host strain, work in concert to select for or against plaque formation. In wild-type phage, the protein encoded by the *gam* gene inactivates exonuclease V, encoded by the *recBCD* genes of *E. coli*. This results in blocking phage propagation because the *E. coli old* gene product kills the host cell when exonuclease V is inactivated. When phage lack functional products of *red* and

gam, exonuclease V is not inactivated. The phage DNA, however, must be protected from digestion by exonuclease V. The presence of *chiC* sequence prevents digestion by inactivating the *recD* gene product [2].

Information in this background review was used in designing and interpreting the experiments discussed in the following chapters. The *gpt* delta system is used in experiments outlined in the second chapter of this thesis to further investigate the paradigm of bacterial-induced infection associated with cancer. Observations made in human intestinal cancer and in mouse models of intestinal tumorigenesis guided the experimental design of experiments discussed in chapter three, which describes the characterization of a novel murine model of intestinal and skin cancer. The research presented in the last chapter, involving adaptive immunotherapy in a mouse model of familial colon cancer, is built on existing knowledge of cancer treatment in both humans and mice.

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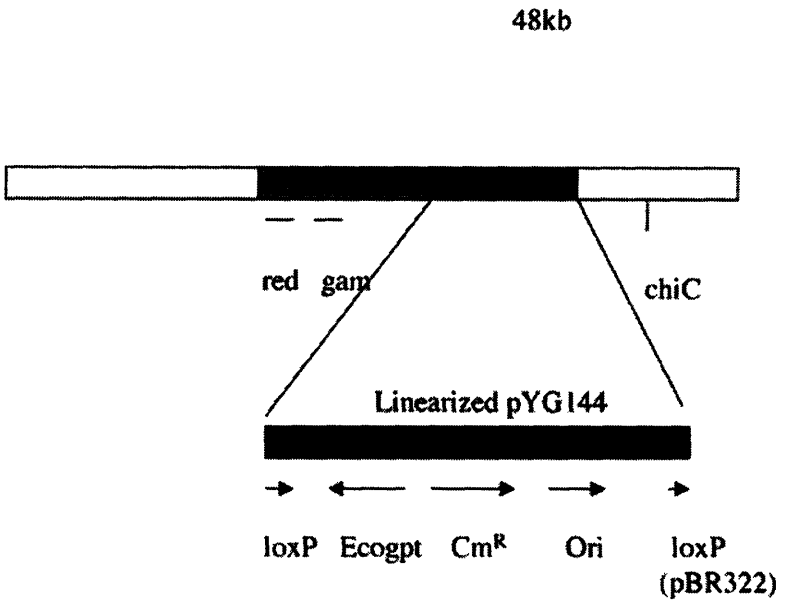
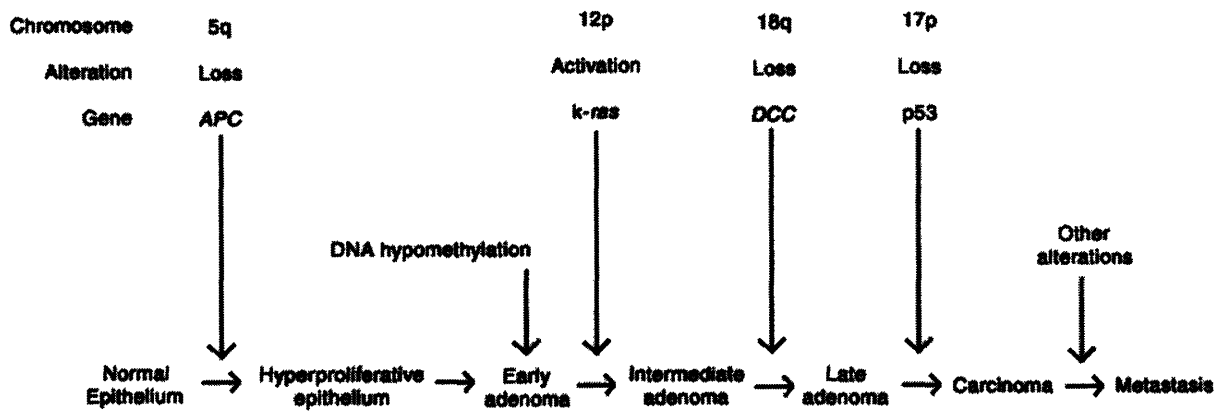
1.5 Figures



(Adapted from Nohmi et al [2])

Figure 1-1: The substrate of the *gpt* delta transgenic mouse

The transgene carried by the *gpt* delta transgenic mouse is a 48 kb λEG10 phage vector that has *red*, *gam* and *chiC*, as well as a linearized pYG144 vector carrying *E.coli gpt*, the chloramphenicol acetyltransferase gene (CAT), and the origin of replication for pBR322.



Adapted from Fearon and Vogelstein 1990 [1]

Figure 1-2: A genetic model for colorectal tumorigenesis

Chapter 2: Mutagenesis associated with *C. rodentium* infection in transgenic C57BL/6 mice

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2.1 Introduction

It is now recognized that chronic infection is a risk factor for certain types of cancer. IARC monographs evaluating human pathogens that lead to chronic inflammation have classified hepatitis B virus, *Schistosoma haemotobium*, *Ophisthorcis viverrini*, and *Helicobacter pylori* as group 1 carcinogens [1, 2]. It has been hypothesized that infection increases cancer risk by inducing inflammation that can trigger inflammatory cells to activate enzymes that generate oxidants. Such enzymes include inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO•) from L-arginine, NADPH oxidase and xanthine oxidase, which produce superoxide anion ($O\bullet^-_2$), and myeloperoxidase (MPO), which produces hypochlorus acid (HOCl) from hydrogen peroxide (H_2O_2) and chloride ion (Cl^-). These oxidants react to form reactive oxygen species (ROS) and reactive-nitrogen species (RNS), all of which can lead to various damage in host tissue including formation of DNA adducts and induction of somatic mutations [3, 4]. More specifically, peroxynitrite (ONOO-), causes both oxidative damage and nitration of DNA bases, and can diffuse within cells [5], making it physiologically relevant. Thus, it is hypothesized that infection that leads to inflammation can also lead to induction of oxidants and somatic mutation.

In vivo, infection that results in inflammation has been hypothesized to initiate carcinogenesis by causing changes in tumor suppressor genes and oncogenes by genetic change. Genetic change has been recognized in carcinogenesis [6]. Experiments in mouse models of mutational analysis have shown that both bacterial and parasitic agents can induce mutations. Transgenic reporter gene mice have been used to evaluate the mutagenicity of chemicals, and allow for physiologically relevant conditions. Mouse models using endogenous reporter genes limit target

tissue and are technically difficult in quantifying mutant frequency [7]. *Fasciola hepatica*, a model for the liver fluke *Opisthorchis viverrini*, has been shown to induce mutations in Big Blue™ mice twenty three days post infection [8]. *H. pylori* and *Helicobacter felis*, both gastric pathogens, can induce mutations in Big Blue™ mice after six months of infection [9]. Mutations in p53 in humans have been associated with infection by virulent strains of *H. pylori* [10, 11]. Furthermore, gastric adenocarcinoma can be induced in Mongolian gerbils by *H. pylori* [12, 13], and in C57BL/6 mice by *H. felis* [14]. The mutant frequency data in mice supports the hypothesis that liver flukes and gastric bacterial pathogens can induce mutations in humans.

C. rodentium, a mouse model of attaching and effacing pathogens enterohemorragic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC), has been shown to promote, but not initiate, colonic adenomas in a mouse model of chemically induced and hereditary cancer. In the presence of the carcinogen 1,2-dimethylhydrazine (DMH), *C. rodentium* infection promotes adenomas in the colon of outbred NIH Swiss mice [15], shown by reducing the latency period of tumor development and also increasing total tumor frequency. *Citrobacter rodentium* was also shown to promote colon tumors in $Apc^{Min/+}$ mice by increasing the number of colonic adenomas four-fold, compared to uninfected mice [16]. *C. rodentium* infection by itself, however, does not lead to adenomas, and thus has not been shown to be a complete carcinogen. In fact, *C. rodentium* infection is self-limiting and causes little inflammation. Mice experimentally inoculated with *C. rodentium* show hyperplasia with very little inflammation as early as 4 days after inoculation, which increases rapidly between weeks 2-3, and resolves around 5-8 weeks after inoculation [17] *C. rodentium* does not causes chronic inflammation, yet it increases tumor burden and decreases latency period.

The goal of this study was to determine the ability of hyperplasia and inflammation induced by *C. rodentium* infection to induce somatic mutations. To this end we infected 4 week old C57BL/6 *gpt* delta transgenic mice and determined the mutant frequency in infected mice versus uninoculated mice at 14 days post infection.

2.2 Materials and Methods

Animals. C57BL/6 *gpt* (guanine phoshoribosyltransferase) delta transgenic (tg) mice [18] were a kind gift from Dr. Takehito Nohmi at the National Institute of Health Sciences, Tokyo, Japan. The mice were rederived by embryo-transfer [19] to be specific pathogen free (for enterohepatic *Helicobacter spp.*, *Citrobacter rodentium*, *Salmonella spp.*, endoparasites, ectoparasites, and serum antibodies to murine viral pathogens). Progeny were bred to generate the mice used in the experiments reported here. The mice were maintained in microisolator cages in a barrier facility and were given LabDiet Prolab RPM 3000 mouse chow and water *ad libitium*. On days of urine collection, mice were placed in metabolic cages with air filters.

The following experiments were approved by the Committee on Animal Care of the Massachusetts Institute of Technology.

Experimental Infections and Bacterial Growth. *Citrobacter rodentium* strain DBS120, a Kanamycin resistant strain constructed using a Tn5 transposon and shown to have wildtype colonization [20], was grown overnight on MacConkey lactose agar containing Kanamycin (Kan

40mg/L). For inoculations, bacteria were grown overnight in Luria-Bertani, Miller broth (LB). Four-week-old mice were inoculated intragastrically with either 100 μL of LB containing approximately 10^9 CFU or 10^8 CFU of *C. rodentium*. Control mice were dosed with 100 μL of sterile LB.

Bacterial counts. Infection was confirmed and kinetics of infection were determined by enumerating colony-forming units of *C. rodentium* in feces on days 3, 6, and 9 days post inoculation as well as on the day of necropsy. Fresh fecal pellets were collected, weighed, and homogenized in phosphate-buffered saline (PBS pH 7.4). Homogenates were serially diluted in PBS and plated on LB agar containing Kan. Feces from all mice were plated prior to infection to confirm the absence of Kan^r commensal flora.

Urinary Nitrate. Urine was collected into tubes containing 0.5 mL 0.5 M NaOH to inhibit bacterial growth. As a measure total endogenous production of NO•, total urinary nitrate concentration was determined by the Greiss reaction as previously described by Green at al. [21] and normalized to body weight and total time of collection (24h).

Experimental Design

Experiment 1: Thirteen mice were inoculated with 10^9 CFU and sixteen mice were inoculated with 10^8 CFU of *C. rodentium*. Sixteen mice served as sham-dosed controls. All mice were euthanized by CO_2 overdose at 13 or 14 days post inoculation (p.i.). Tissues were formalin-fixed for hematoxylin and eosin (H&E) staining, and snap frozen for DNA extraction.

Experiment 2: Eight mice were inoculated with 10^9 CFU of *C. rodentium*, 8 were inoculated with 10^8 CFU, and 8 served as sham-dosed controls. Urine was collected on days 6 and 12 p.i. for 24 hours. All mice were euthanized at 14 days p.i. Tissues were formalin-fixed for hematoxylin and eosin (H&E) staining, and snap frozen for DNA extraction

Mutation Assay. The mutation assay was performed essentially as previously described by Nohmi et al. [18]. Briefly, genomic DNA was isolated from kidney and descending colon using the Big Blue™ DNA Extraction protocol (Stratagene) with the following modification. Tissues were disaggregated using sterile, disposable mortar and pestles (Kontes) in dounce buffer. Briefly, the homogenate was digested for 2.5-3 hrs at 55 °C with proteinase K, and DNA was extracted using phenol/chloroform, precipitated with ethanol, and suspended in 50-100 μL 10mM Tris•HCl, pH 7.5 1mM EDTA (TE).

Phage were packaged by using the Transpack Packaging Extract (Stratagene) to ligate concatamerized phage into phage heads. The *gpt* assay was carried out as previously described [18] by transducing *E. coli* YG6020 with λ phage. Cells were incubated at 37 °C on a stationary surface for 20 min, then 30 min with rotation. Suspensions were then plated on minimal media plates with chloramphenicol (Cm) and 6-thioguanine (6-TG) to select for cells expressing YG142 but functionally deficient for Gpt, and minimal media plates with only Cm to select for all cells expressing pYG142. Mutant frequency is expressed as the ratio of *gpt* mutants able to grow in the presence of 6-TG versus the number of total colonies on minimal media plates without 6-TG multiplied by a dilution factor.

Histopathological Analysis of Hyperplasia and Inflammation. At necropsy, colon and kidney were fixed in 10% formalin. Fixed tissues were processed routinely, embedded in paraffin, sectioned at 5 μM, and stained with hemotoxylin and eosin (H&E). Lesions were scored for hyperplasia and inflammation by a pathologist (ABR) blinded to sample identity. Scoring was done on a scale of 0 to 4 with increasing severity (0 = none, 1 = mild, 2 = moderate, 3 = moderate/severe, 4 = severe).

Immunohistochemical Analysis of Inflammation. Immunohistochemistry for inducible nitric oxide synthase (iNOS) was performed on formalin-fixed colon using antigen retrieval by steam and anti-iNOS rabbit IgG (M-19 sc-650 rabbit IgG Santa Cruz Biotech) at a dilution of 1:100 with vimentin as an indicator. The same sections were stained for the F4/80 using anti-F4/80 antibody to localize macrophages. Tissues were counterstained with hemotoxylin.

Fluorescent immunohistochemistry (FIHC) was also performed on tissue with antibodies for F4/80 (# m2915 Caltag Labs, Burlingame, CA) at a dilution of 1:100 and iNOS (same as above). Fluorescent indicators were Cy3 for F4/80 detection and FITC for iNOS detection. Cells were stained with DAPI to visualize nuclei.

Statistics. Student's T-test was used to analyze the kinetics of infection and urinary nitrate data. Mann-Whitney U Test was used to analyze histopathology scores between infected animals dosed with 10^8 versus 10^9 CFU. The Wilcoxon Signed-Rank Test was used to analyze histopathology scores of infected animals to the uninoculated animals by comparing scores of

infected animals to an assigned hypothetical mean value that corresponded to the actual mean value of histopathology scores for uninoculated animals. Data were considered significant if $P < 0.05$.

2.3 Results

Hyperplasia and Inflammation induced by *C. rodentium*. Susceptibility of C57BL/6 *gpt* delta transgenic animals to infection was confirmed. Based on plating of feces from animals in both experiments 1 and 2, all 21 animals that received 10^9 CFU were successfully infected, and 14 of the 24 mice (58%) that were dosed with 10^8 CFU became persistently infected (Figure 2-1). Of the 10 mice that did not show persistent infection, we observed low fecal counts of less than 10^3 CFU per mg of feces at only day 3 in three animals, and no counts in the remaining seven. All sham-dosed mice remained uninfected.

The colons from 14 successfully infected mice inoculated with 10^8 CFU, and 20 successfully infected mice inoculated with 10^9 CFU were evaluated for hyperplasia and inflammation. A comparison of lesions from the two dosage groups for hyperplasia ($p = 0.5$) and inflammation ($p = 0.6$), showed that severity of lesions was the same. We combined data from both groups, resulting in a total of 34 infected mice and 20 uninoculated mice that were assessed for lesion severity. Infected animals developed lesions with increased hyperplasia and inflammation as compared to uninoculated mice ($p < 0.0001$ for both categories). Overall, the mice showed mild inflammation and minimal to mild hyperplasia (Figure 2-2).

Nitric Oxide Production in Epithelial Cells. Urinary nitrate levels at 13 days p.i. showed that NO• production was approximately 2-fold higher in infected animals ($p = 0.025$), compared to uninoculated mice (Figure 2-3). This correlated with the moderate hyperplasia and inflammation that we observed in the descending colon of infected animals at 14 days p.i.

Based on the increase in urinary nitrate levels in infected mice 13 days p.i., and by *C. rodentium*-infected mice of similar age by Vallance et al. [22], immunohistochemistry was used to identify cells expressing iNOS at 14 days p.i. Epithelial cells were positive for iNOS in infected mice, with over 75% of positive cells toward the luminal surface of the crypts (Figure 2-4a). Some glands contained cells with iNOS throughout the length of the crypt (Figure 2-4a). At high power magnification, staining was distributed on the apical membrane of some epithelial cells (Figure 2-4b), and diffusely throughout the cytoplasm of others (Figure 2-4b). *C. rodentium* visualized at high magnification showed significantly lighter staining distinct from staining seen in epithelial cells positive for iNOS (data not shown). Epithelial cells in the colon of uninfected mice did not contain detectable iNOS (data not shown.)

FIHC for mice at 14 days p.i. showed that infected mice had colonic epithelial cells with positive fluorescent signal for iNOS, similar to the pattern shown with immunohistochemistry (Figure 2-5b, Figure 2-5d). Colonic cells of uninoculated mice had only light background levels of iNOS expression in the colonic crypts (Figure 2-5a, Figure 2-5c). No cells with positive fluorescence for F4/80 were detected in the crypts of either infected (Figure 2-5b, Figure 2-5d) or uninoculated animals (Figure 2-5a, Figure 2-5c).

The submucosa directly adjacent to the bottom of the crypts in infected mice showed cells with positive signal for F4/80. As expected, these cells also showed positive fluorescence for iNOS (Figure 2-5e, Figure 2-5f).

Mutant Frequencies. DNA was isolated from the colon of 5 infected and 5 uninoculated *gpt* delta transgenic mice in order to determine the induced and spontaneous mutant frequency. In addition, DNA was also isolated from the kidney of 3 infected and 3 control mice, to assess the mutant frequency in an organ that does not exhibit inflammation or hyperplasia following *C. rodentium*-infection (Figure 2-2). The colon of infected animals showed an 8-fold increase ($P < 0.05$) in mutant frequency as compared to the colon of uninoculated animals. Furthermore, there was no significant difference between the spontaneous mutant frequency of the colon and the mutant frequency in the kidney of infected or uninoculated mice (Figure 2-6). This demonstrates a positive association between *C. rodentium* infection and somatic mutations, and supports the hypothesis that *C. rodentium*-induced inflammation induces mutations.

2.4 Discussion

Bacterial infection has been recognized as a risk factor for cancer in humans and animals, and it has been hypothesized that chronic inflammation associated with infection leads to DNA damage through oxidative stress. Both *H. pylori* and *H. felis* have been shown to induce mutations in Big Blue™ mice concurrent with increased iNOS expression [9], supporting this hypothesis. We investigated whether *C. rodentium* infection, which has been associated with promotion of colonic adenomas in Min mice [16], can induce genotoxic events. *C. rodentium* induces a self-

limiting disease, characterized by mild hyperplasia and inflammation in this study, and a transient induction of iNOS expression [22]. Using *gpt* delta tg mice, we have demonstrated that *C. rodentium* is associated with an increase in mutant frequency at 14 days p.i.

We determined the mutant frequency in the descending colon of *C. rodentium*-infected mice as 8.62×10^{-5}, 8-times higher than that detected in the colon of uninoculated mice (Figure 2-5). We found spontaneous mutant frequencies of 0.54×10^{-5} in the colon and 0.68×10^{-5}in the kidney of uninoculated animals. The spontaneous mutant frequency in the colon is consistent with previously reported values by Masumura et al. [23, 24] testing for the mutagenicity of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP). The spontaneous mutation frequency in the kidney of C57BL/6 *gpt* delta tg mice has not been previously reported.

The 8-fold increase in mutant frequency due to *C. rodentium* is comparable to the 4-fold increase in C57BL/6 Big Blue™ mice infected with *H. pylori* for 6 months [9]. Touati et al. also detected a 1.7-fold increase in mutant frequency in the stomach of C57BL/6 Big Blue™ mice infected with *H. felis* for 6 months [9]. Interestingly, an increase in mutant frequency could no longer be detected at 12 months p.i. with *H. pylori*, suggesting that there is transient increase in mutant frequency at 6 months p.i. Evidence for the disappearance of mutants induced at 6 months was further provided by mutational spectra that showed a difference in infected versus uninfected mice at 6 months p.i., but were indistinguishable at 12 months p.i. One interpretation of these data is that the mutations first detected are important for initiating carcinogenesis, but that they are detrimental or are no longer crucial for further tumor formation, and are thus selected against.

This transient increase in mutant frequency of *H. pylori*-infected mice correlates with increased iNOS expression at 6 months p.i. [9].

Our studies continue to support the observation that increased mutant frequency is associated with iNOS expression. RNS play an important role in clearance of pathogens, but they may also cause genotoxic damage. In the case of NO•, it has been shown that elevated levels of iNOS and nitrotyrosine are associated with increased mutant frequency in the RCS-cell bearing SJL mouse model [26]. Almost concurrent with increased mutant frequency, we observed a 2-fold increase in urinary nitrate output, a biomarker for inflammation, at 13 days p.i. The increase in urinary nitrate is associated with secretion of NO• by epithelial cells, as indicated by iNOS positive staining cells that do not express F4/80 (Figure 2-2). Epithelial cells have previously shown to be positive for iNOS in C. *rodentium*, coinciding with increased expression of iNOS determined by mRNA levels [22]. We associated the increase in urinary nitrate and iNOS positive staining with inflammation found in *C. rodentium*-infected animals at 14 days p.i. No increase in urinary nitrate was detected at 7 days p.i. We believe that this is due to lower levels of colonization (Figure 2-1) and lower severity of lesions.

There are alternative hypotheses to oxidative stress being the mechanism by which chronic infections enhances tumorigenesis. *C. rodentium* infection causes hyperplasia and tissue injury, and cells undergoing increased turnover may be predisposed to replication error. Replication error resulting mutation in a tumor suppressor gene or oncogene may initiate tumorigenesis. NO• also has a role in many signaling pathways, including as an anti-apoptotic signal when expressed in low concentrations, depending on the tissue environment (reviewed in [27]). If NO• is indeed

having an anti-apoptotic effect, it may enable mutations to persist in cells that would otherwise undergo cell death. Inflammation may also act on cells by inducing cytokines that have a role in cell transformation. TNF-α has recently been shown to increase the migration of rat intestinal epithelial cells in vitro while elevating *Src* expression [28]. It remains to be determined if these mechanisms play a role in *C. rodentium* induced mutagenesis.

These results demonstrate that *C. rodentium* infection induces somatic mutations that can be detected in the colon of *gpt* delta tg mice. The results of these studies suggest that self-limiting infection with bacteria such as EHEC or EPEC may increase the risk for colon cancer in humans. Further studies are underway to elucidate alternative mechanisms by which *C. rodentium* infection enhances mutagenesis and tumorigenesis.

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2.5 References

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2.6 Figures

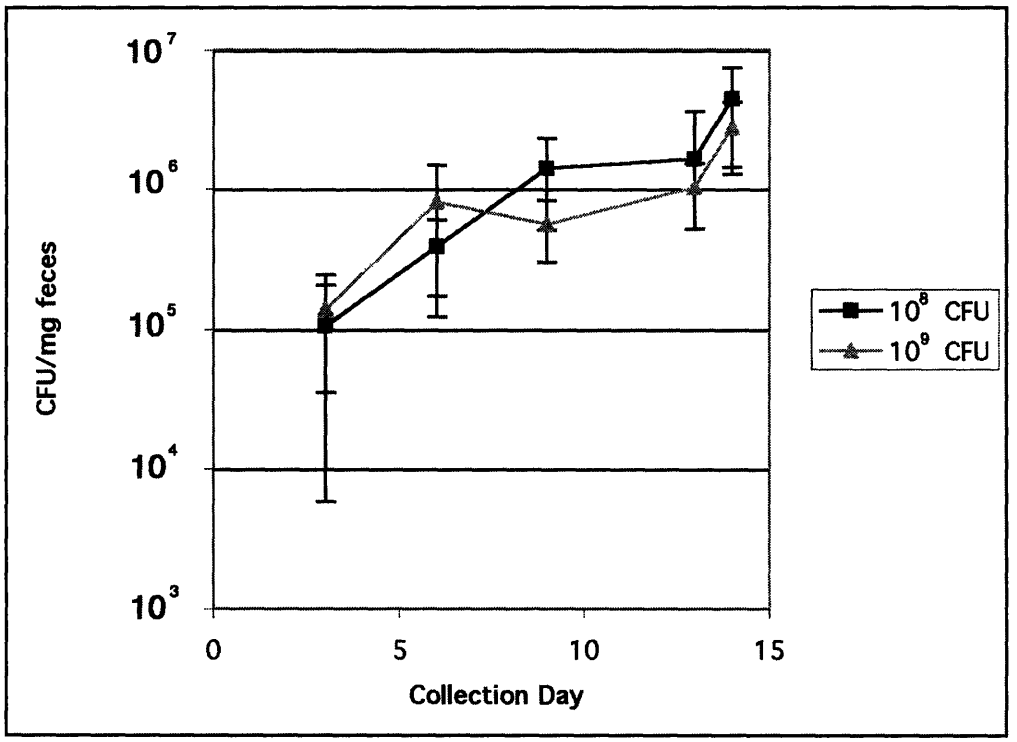

Figure 2-1: Kinetics of infection: *C. rodentium* infection in C57BL/6 transgenic

Mice inoculated with 10^8 CFU or 10^9 CFU of *C. rodentium* developed comparable ($P > 0.05$) bacterial loads as determined by fecal shedding.

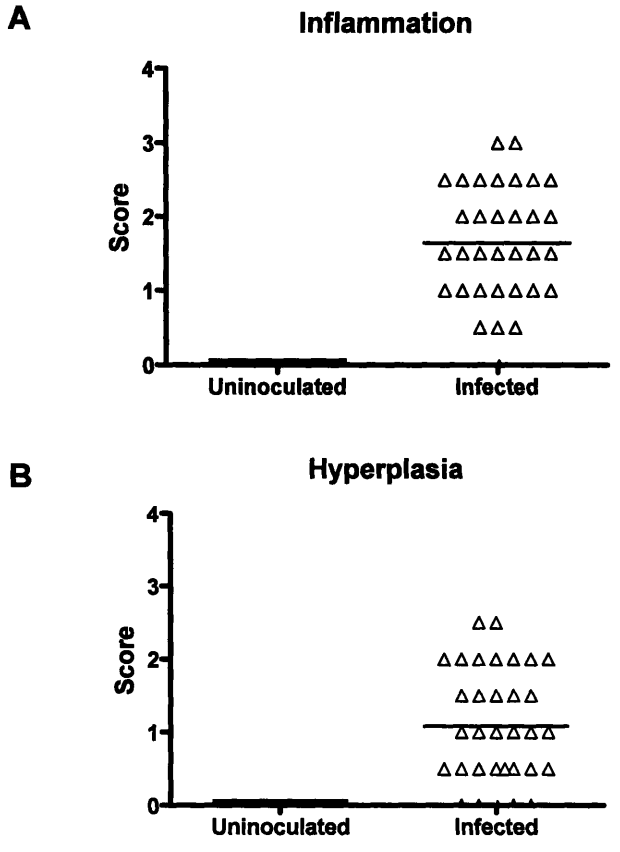

Figure 2-2: Histopathology scores for inflammation and hyperplasia in the colon of C57BL/6 *gpt* delta tg mice infected with *C. rodentium*

Hematoxylin and eosin stained fixed tissue from the descending colon of uninoculated and C. rodentium-infected mice was scored for hyperplasia and inflammation (ABR). Scoring was done on a scale of 0 to 4 (0 = none, 1 = mild, 2 = moderate, 3 = moderate/severe, 4=severe). Individual mice are represented by squares for uninoculated mice (n = 14), and triangles for infected mice (n = 20). Increased levels of inflammation ($p < 0.0001$) and hyperplasia ($p < 0.0001$) were identified in infected mice versus uninoculated controls. The median scores reflected mild inflammation, and minimal to mild hyperplasia in infected animals.

Infection status	Day 7 (μmol/gram/day)	Day 13 (μmol/gram/day)
uninoculated	0.0124 ± 0.0050	0.0153 ± 0.0081
infected	0.0131 ± 0.0089	0.0267 ± 0.0114

Figure 2-3 Urinary nitrate excretion in *C. rodentium*-infected mice at 7 and 13 days p.i.

Urinary nitrate, a biomarker for inflammation, was determined in uninoculated and infected mice at seven days, and at thirteen days p.i in μmol/gram/day. The mean urinary nitrate and standard deviation is shown above. Increased urinary nitrate was detected at 13 days p.i. ($p = 0.025$) in infected versus uninoculated mice. Urinary nitrate was also increased ($p = 0.006$) in infected mice at 13 days p.i. as compared to infected mice at 7 days p.i., showing a time-dependent increase.

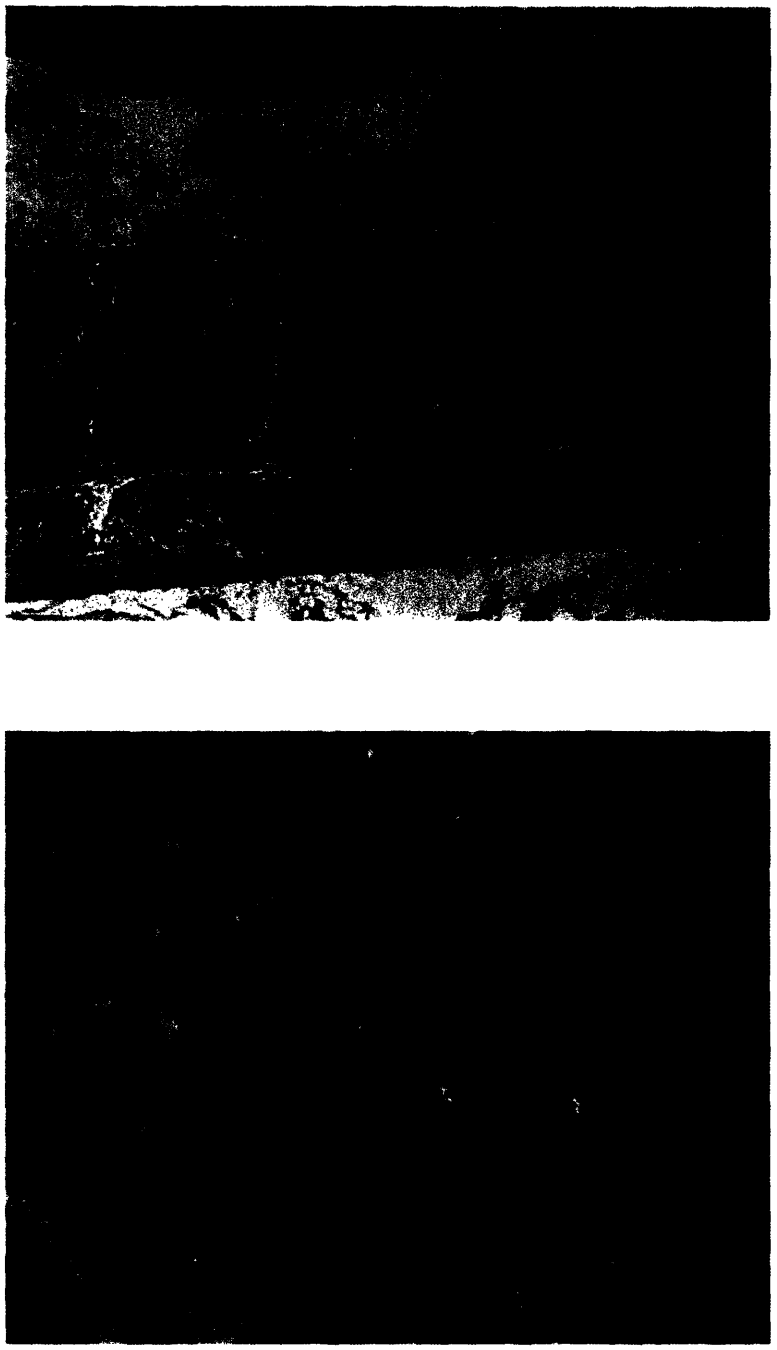

Figure 2-4 Immunohistochemical analysis of iNOS expression in colon of *C. rodentium* infected C57BL/6 *gpt* delta tg mice

Boxes A & B show immunohistochemistry of cells positive for iNOS (vimentin, brown) in sections of infected mice counterstained with hematoxylin (blue).

A) Longitudinal sections of the mucosa of infected animals showed that positive staining for iNOS was located mostly in the upper luminal portion of the crypt. Control mucosa showed no significant staining for iNOS (data not shown).

B) Cross-sections of crypts from infected animals showed both an annular pattern of iNOS expression, and a diffuse pattern of staining thoughout the cytoplasm.

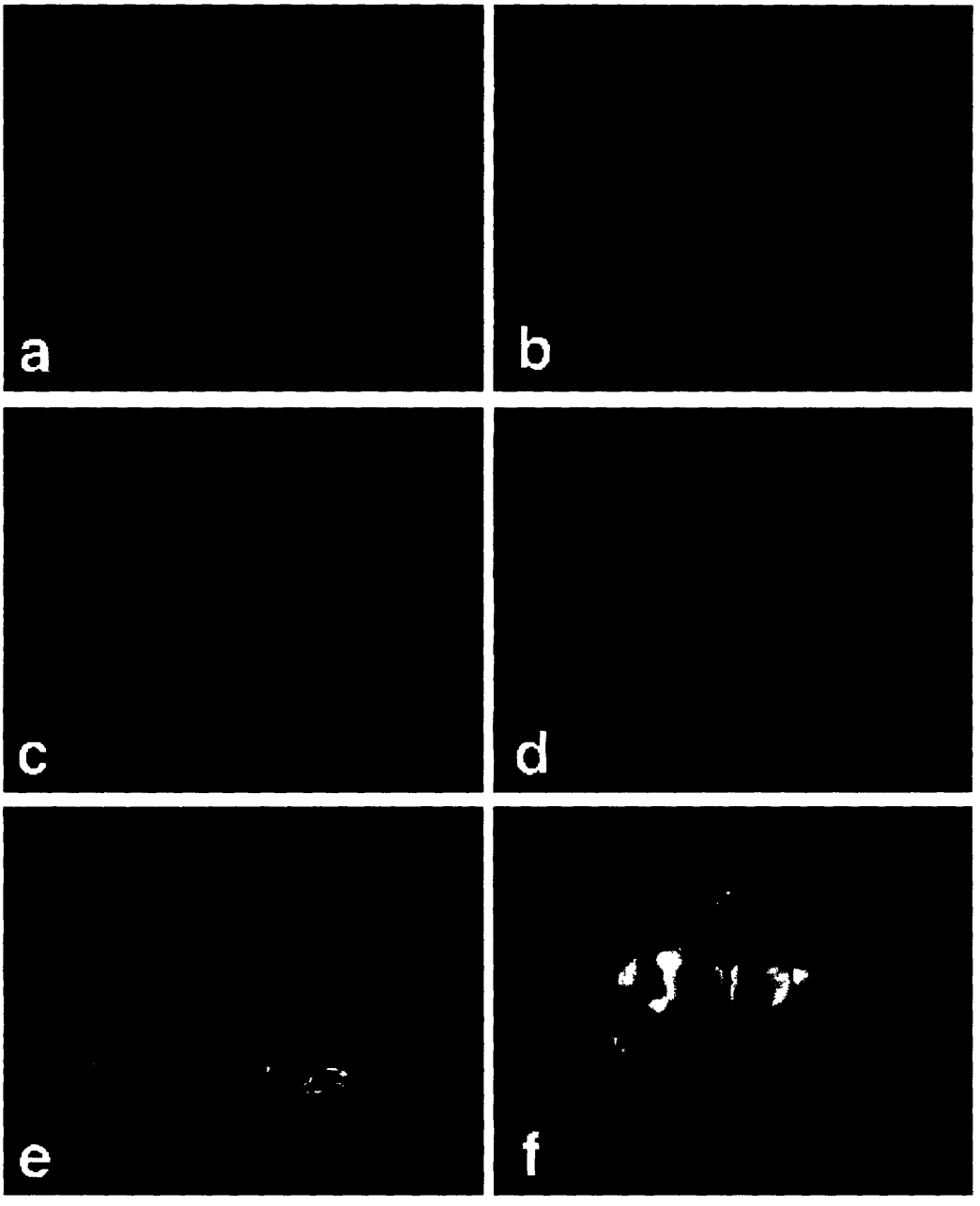

Figure 2-5: Fluorescent-immunohistochemical analysis of iNOS-positive cells in the colon of C57BL/6 *gpt* delta tg mice at 14 days p.i.

Fluorescent immunohistochemistry (FIHC) was performed on the descending colon *of C. rodentium*-infected and uninoculated mice for iNOS (red, Cy3), F4/80 for macrophages (green, Fitc), and nuclei (blue, DAPI).

A&C) Mucosa of uninoculated animals showed a low background of iNOS positive staining in epithelial cells.

B&D) Mucosa of infected animals showed an annular pattern of staining in epithelial glands. There were no cells that were positive for the macrophage marker F4/80.

E) This section from an infected mouse shows the area adjacent to the base of the crypts. Macrophages that are positive for F4/80 also show positive staining for iNOS in the submucosa.

F) This high magnification image of the section illustrated in box E shows an infected mouse with submucosal macrophages showing positive staining for iNOS.

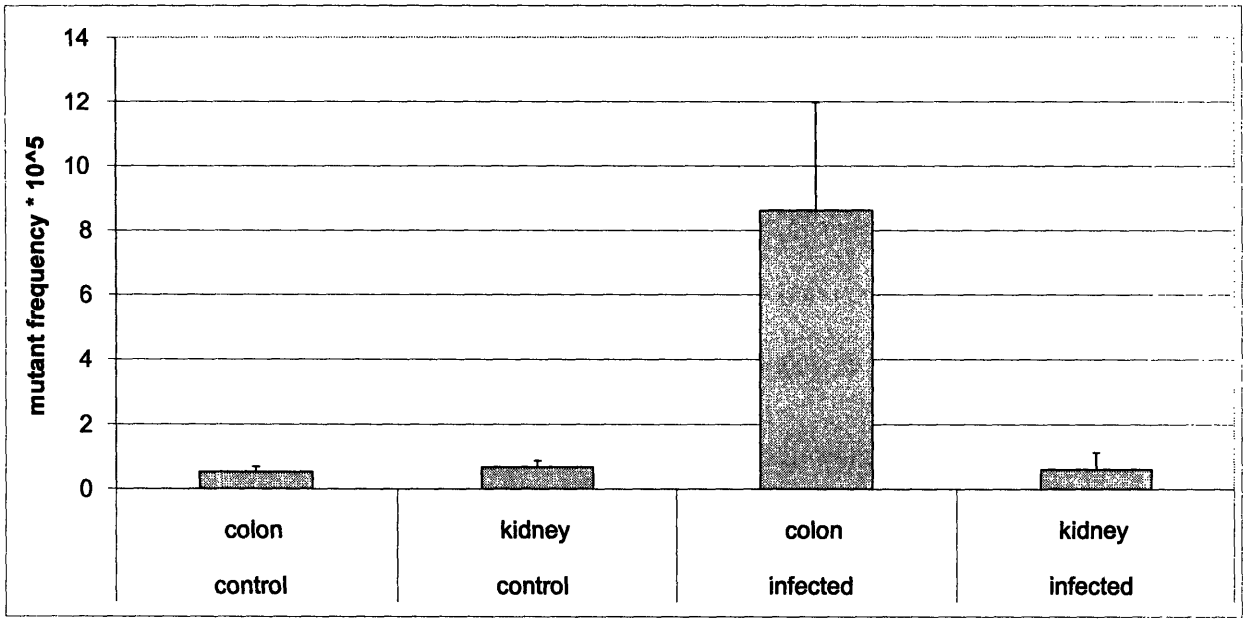

Figure 2-6: Mutation frequencies based on the *gpt* assay

DNA was extracted from target tissue (colon) and nontarget tissue (kidney) from three uninoculated and five infected animals. The mean mutant frequency and 95% confidence intervals are reported. DNA from target tissue of infected mice showed approximately an eight-fold increase in mutant frequency as compared to those found in nontarget tissue of the same animals, and target and nontarget tissue of uninoculated animals.

Treatment	Animal ID	No. of colonies (x10^5)	No. of mutants	Mutant frequency (X10^-5)
Control	1.2	324000	2	0.617283951
	1.3	349500	2	0.572246066
	1.4	238500	1	0.419287212
Infected	5.0	387000	20	5.167958656
	5.1	451500	32	7.087486157
	5.2	615000	58	9.430894309
	5.3	147000	12	8.163265306
	5.4	216000	21	9.722222222

Figure 2-7: Mutant frequencies in colonic tissues of *C. rodentium*-infected and uninoculated control mice as determined by the *gpt* assay

The *gpt* assay was used to determine mutant frequency in the colon of infected (n = 5) and uninoculated (n =3) mice. The number of colonies that were queried and the number of mutants selected on agar containing 6-TG is shown. Between 147,000 and 615,000 colonies per animal were queried in the *gpt* assay. A total of 148 *gpt* mutants were isolated.

Chapter 3: Characterization of BALB/c $Apc^{+/Min}$ mice: recognition of gastrointestinal and skin cancer

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3.1 Introduction

Colorectal cancer (CRC) is the third leading cause of cancer deaths in men and women in the United States [1]. A mendelian pattern of colorectal cancer is seen in 5 to 10 percent of patients with colorectal cancer [2]. Familial adenomatous polyposis (FAP) is a hereditary colorectal cancer syndrome that is characterized by the development of a hundred to thousands of adenomas by the second decade of life. Virtually all patients with FAP develop colorectal cancer if untreated [3]. Mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene are responsible for FAP [4-7] and also occur in many sporadic cases of colorectal cancer [8]. It is known that modifier genes influence the severity of FAP [9], but specific modifier genes have not yet been definitively identified [10-12]. There is some controversial evidence, however, that allelic differences in *APC* mutations influence phenotypic penetrance [13, 14].

Multiple intestinal neoplasia (Min) mice are one of the murine models for human FAP [15]. Min mice are heterozygous for the Apc^{Min} (*Min*) allele, which is a mutation at codon 850 of the murine *Apc* homologue, resulting in a truncated APC protein. C57BL/6J Min mice develop on average more than 70 intestinal adenomas, with the majority of tumors in the small intestine, and rarely survive beyond 150 days of age [16]. C57BL/6J Min adenomas consistently exhibit loss of heterozygosity (LOH) at the *Apc* locus, defined as chromosomal loss of the wildtype allele [17, 18].

The number and size tumors in Min mice is influenced by *Modifier of Min-1* (*Mom1*), which was identified as the gene for nonpancreatic secretory phospholipase A2 group IIA (*Pla2g2a*). B6

Min/+ mice carry mutant alleles of *Pla2g2a* [19], resulting in low levels of Pla2g2a expression in the small and large intestine. When C57/BL6J Min mice carrying wild type *Pla2g2a* alleles are generated, an average of 7.8 ± 3.4 tumors are observed at 120 days of age. Tumor burden is further decreased in AKR/J Min mice carrying wild type *Pla2g2a* alleles. Only 43 of 170 of AKR/J Min mice showed adenomas in the small and large intestine at approximately 138 days of age. Tumors on the AKR/J background show both LOH and also mutation of the wildtype *Apc* allele [20].

Unlike humans with FAP whose intestinal adenomas progress to adenocarcinomas, Min/+ mice have neither been reported to exhibit adenocarcinomas, nor features of invasion or metastasis. The short life span of C57BL/6J Min/+ mice, mostly likely due to anemia resulting from a high number of polyps, and the similiarly short life span of AKR/J Min/+, shortened by mortality from lymphoma [20], impair longitudinal studies to observe further progression of colonic adenomas from polyps as observed in humans. To determine if time-dependent progress of Min adenomas does occur, we transferred the Apc^{Min} allele from the C57BL/6J strain to the BALB/c strain, which is *Pla2g2a*+/+ and survives well beyond 6 months of age.

3.2 Materials and Methods

Animals. Male C57BL/6 (B6) Min mice were mated with wild type BALB/c (BALB) female mice, obtained from the Jackson Laboratory (Bar Harbor, Maine), and progeny were screened for the *Min* allele by PCR-RFLP [21]. BALB Min/+ mice used in these experiments were the result of a minimum of 12 generations of backcrossing. To confirm that the BALB Min mice were

congenic, genomic DNA from 7 BALB Min/+ mice were submitted to the Jackson Laboratory for the scanning of 110 markers spaced at 12-13 cM across the 19 murine autosomes. All the markers were consistent with BALB genotype.

Mice were housed in AAALAC approved facilities in static microisolator cages and maintained in specific pathogen free conditions (free of enterohepatic *Helicobacter spp.*, *Citrobacter rodentium*, *Salmonella spp.*, endoparasites, ectoparasites, and serum antibodies to murine viral pathogens). BALB wild type and Min mice were co-housed with same sex littermates. Mice were fed LabDiet Prolab RPM 3000 mouse chow and water ad libitium.

Gross and histomorphologic analysis. Mice at 7 and 12 months of age were euthanized by CO_2 asphyxiation. The gastrointestinal tract from the stomach to anus was excised. A total of 7-10 Min/+ mice and 9-10 wild type, at 7 and 12 months of age, were examined. The GI tract was opened lengthwise, rinsed with PBS, cut into sections representing the stomach, small intestine, cecum, and colon, then examined for grossly visible polyps. The location and diameter of each polyp at the widest point was recorded. The location of adenomas in the small and large intestine was determined based on distance from the pylorus and proximal end of cecum, respectively.

The small and large intestine, stomach and cecum were fixed overnight in 10% neutral buffered formalin, processed, and embedded in paraffin. Tissues were sectioned at 5μm and were stained with hemotoxylin and eosin (H&E). The intestinal mucosa was scored for inflammation and crypt hyperplasia on a scale of 0 to 4 (0 = none, 1 = mild, 2 = moderate, 3 = marked, 4 = severe)

by a board-certified veterinary pathologist (PRN). Lesions were scored during evaluation of the entire HE- stained intestine by light microscopy, evaluating by size and invasiveness. Lesion categories included gastrointestinal intraepithelial neoplasia (GIN) [15], adenocarcinoma, and invasive carcinoma. We defined GIN-1 as a focus of less than 5 dysplastic crypts (aberrant crypt foci), GIN-2 as 5-10 dysplastic crypts (microadenoma), GIN-2.5 as a focus of greater than 10 dysplastic crypts but not visible to the naked eye (microadenoma), and GIN-3 as a grossly visible adenoma. Adenocarcinoma was used to describe polyps protruding into lumen and pressing into the muscularis mucosa. Based on penetration of the intestinal wall by neoplastic cells and/or invasion of vasculature, lesions were classified as invasive adenocarcinomas.

The location and size of cutaneous nodules in the BALB Min/+ were noted. At necropsy, these cutaneous masses were excised and the widest diameter was recorded, followed by fixation in 10% neutral buffered formalin (NBF), paraffin-embedding, sectioning and H&E staining. To serve as additional controls, normal skin from corresponding locations (the mandible, lower abdomen, and hind leg) were also collected from BALB wild type and Min/+ littermates.

Statistical Analyses. The T-test with Welch's modification for different variances was used to analyze gross tumor multiplicity and tumor size between age groups of BALB Min mice. Comparisons were considered significant when $p < 0.05$.

Immunohistochemistry

Immunohistochemistry of formalin-fixed paraffin-embedded intestinal and skin tumor sections were stained for β-catenin with monoclonal mouse-anti-β-catenin antibody (#C-7207 Sigma-

Aldrich Inc., St.Louis, MO) at a dilution of 1:15000 in 5% rabbit serum, with a 2 hour incubation at 37 °C.

3.3 Results

Survival and Gross Pathology

BALB Min/+ consistently survived beyond 12 months of age. At necropsy, all BALB Min/+ mice had intestinal polyps (Figure 3-1). Solid subcutaneous masses were observed on the head and neck of 6 of 76 mice. One mass was observed on the hind limb, totaling 7/76 that presented with subcutaneous masses (9.21%) (Table 3-1). Upon necropsy, these subcutaneous nodular, discrete masses were 5-20 mm in diameter with central cores comprised of inspissated, viscous green material or liquefied necrotic cellular debris.

Tumor multiplicity in the small intestine of BALB/c Min mice. We examined the stomach, small intestine, cecum, and colon of 7 and 12 month old BALB wild type and Min mice. We recorded the size, and location of tumors. No tumors were observed in the tissues of BALB wild type mice. BALB Min mice had tumors primarily in the small intestine. There was no significant difference in average number or size of tumors in the total small intestine of BALB Min mice at 7 and 12 months of age ($p > 0.05$, Table 3-2).

The anatomic location of tumors in the small intestine was determined. Location of tumors was analyzed by quadrants of the small intestine labeled 1, 2, 3, or 4, starting from the stomach (pylorus) and ending at the distal small intestine (ileocecal junction). BALB Min/+ mice showed

different distribution of tumors at 7 and 12 months. (Figure 3-1). A possible explanation for the decrease ($p = 0.0477$) in the number of adenomas in quadrant 1of the small intestine between mice 7 and 12 months of age is continued growth of adjacent adenomas, with subsequent coalescing of the masses and an apparent reduction in the number of tumors. The significant increase in tumor diameter of masses in the most proximal quadrant of the small intestine between these mice supports this hypothesis.

Histopathology of lesions in the small intestine.

Several 12-month old BALB Min mice showed adenocarcinomas or invasive carcinoma. More specifically, five adenocarcinomas were found in the small intestine, and four invasive carcinomas were detected in the small intestine and cecum. No 7-month old BALB Min mice presented with adenocarcinomas or invasive carcinoma, but all these mice had GIN lesions. All age-matched BALB wild type control mice were free of GIN, adenocarcinomas, or invasive carcinomas (Figure 3-2). Of the 12-month old BALB Min mice, one presented with an invasive carcinoma in the ileum that extended through the lamina propria and muscularis mucosa into the serosa (Figure 3-3a, Figure 3-3b). One animal also showed vascular infiltration of dysplasic epithelial cells (Figure 3-3c, Figure 3-3d.)

Gross and microscopic appearance of extraintestinal neoplasms.

Subcutaneous masses on the head, neck, and limbs of BALB Min mice were identified as infiltrative basosquamous cell tumors (Figure 3-4). These neoplastic masses were often located within the hypodermis below the panniculus carnosus muscle and were well circumscribed,

unencapsulated, expansile, often compressing the overlying dermis and frequently infiltrative towards the ventral aspect of the hypodermis/panniculus. These nodular masses were frequently comprised of a thick peripheral layer of well-differentiated basaloid cells arranged in anastomosing cords and trabeculae that completely effaced the underlying tissue architecture. The central core was often multiloculated and lined by a peripheral 1-3 cell-layer of basaloid cells surrounding well differentiated stratified squamous epithelial cells with variable amounts of compact keratin deposits in the center. Multiple foci of scant to moderate squamous differentiation were also noted along the periphery of the masses. The basaloid cells were about 15-20 um diameter, with a large, oval to reniform finely vesiculated nuclei with minimal eosinophilic cytoplasm, high nuclear to cytoplasmic ratio and 3-4 mitotic figures for each high power field (40x). The neoplastic cells frequently infiltrated between the skeletal muscles, nerve fibers and blood vessels within the panniculus. A differential diagnosis to consider is trichoepithelioma, a tumor that arises from the pleuripotent cells in epidermis or follicular walls.

Adenomas from BALB Min mice showed significantly different staining for β-catenin from control tissue

Basosquamous cell tumors stained for β-catenin showed two patterns of staining. All seven tumors stained showed nuclear localization of β-catenin in basal cells (Figure 3-5a, Figure 3-5b) that is consistent with loss of binding activity of Apc. A small subset of tumors also showed nuclear localization in both basaloid cells and squamous cells, along with cytoplasmic and membranous staining (Figure 3-5c, Figure 3-5d).

3.4 Discussion

Large variation is seen in the number of polyps of FAP patients, and an attenuated form of FAP exists in which patients develop less than 100 adenomas throughout intestinal tract [9]. Some FAP patients also develop extraintestinal neoplasms, and are diagnosed as having the Gardner variant of FAP [22]. We observed that BALB Min mice have a lower multiplicity of tumors, as compared to B6 Min mice. BALB Min mice also developed basosquamous carcinomas, which showed nuclear localization of β-catenin. To our knowledge, this is the first report on the development of skin cancer in a mouse model of FAP.

Differences in intestinal tumor burden and extraintestinal neoplasia development between the B6 Min [23] and AKR Min [20] mice that both carry the Apc^{Min} allele has been previously demonstrated. AKR Min mice showed approximately a 95-fold decrease in average tumor multiplicity as compared to B6 Min mice [20]. BALB Min develop approximately 12 intestinal lesions at 1 year of age, compared to B6 Min mice in our facilities that develop approximately 85 polyps at 6 months of age (unpublished data), making a 7-fold difference in tumor multiplicity. It has been shown that the *Pla2g2a* allele from the AKR mouse on the B6 Min mouse results in a 4-fold decrease in tumor burden [24]. The discrepancy in tumor decrease between BALB Min and AKR Min mice suggests that Pla2g2a is not solely responsible for the difference in tumor burden.

We also observed several intestinal adenocarcinomas and invasive carcinomas in twelve-month old BALB Min mice. The most dramatic was vascular infiltration of epithelial cells in the

jejunum, as shown in Figure 3-3. We believe that invasiveness developed in a time-dependent manner based on the absence of invasive lesions in seven-month old BALB Min mice, and the presence of invasive carcinomas and vascular infiltration in twelve-month old mice. However, only a handful of 12-month old animals developed invasive tumors, with none developing more than 23 intestinal lesions. We hypothesize that BALB Min mice will show a higher incidence of invasive cancinomas if observed for more than one year.

We also identified invasive basosquamous carcinomas in approximately 10% of BALB Min mice. A subset of patients with FAP and hereditary nonpolyposis colorectal cancer (HNPCC) develop skin lesions. FAP patients with the Gardner variant develop epidermal cysts, often in conjunction with osteomas, fibromas, sebaceous cysts, and trichoepitheliomas [22]. The basosquamous tumors that we identified had similarities to trichoepitheliomas. Thus, the subset of BALB Min mice that develop basosquamous carcinomas may be analogous of some patients with the Gardner variant of FAP. It is interesting to also note that hereditary nonpolyposis colorectal cancer (HNPCC) patients with Muir-Torre Syndrome also develop skin tumors. These tumors, however, have a sebaceous cell component [25] which we did not observe in our mice.

ß-catenin nuclear localization was observed in association with the BALB Min basosquamous carcinomas. This is an important finding in light of data that shows that truncated ß -catenin expression in the skin of mice coincides with induction of the hair cycle and Lef-1 expression. Hair morphogenesis in these mice led to the creation of dermal pilla and sebaceous glands that subsequently produced a hair sheath and shaft [26]. This study led to the determination that ß -catenin is critical to skin development, especially the differentiation of stem cells into follicular

keratinocytes in the adult mouse [27]. We suggest that the BALB Min mouse may be a useful model for investigating the role of ß -catenin in basosquamous cancer development.

Our data indicate that the BALB Min mouse is a promising new model of FAP, especially in light of the development of invasive intestinal and skin carcinomas. This model may allow investigators to study tumorigenesis beyond adenoma formation and test therapies for tumor invasion in the intestine and skin. The BALB Min mouse is also unique in that it has a high number of duodenal adenomas. The popularity of prophylactic colectomy has decreased death by colon cancer in FAP patients, but these patients often go on to develop duodenal or desmoid lesions, which can be fatal. Duodenal adenocarcinoma surveillance and surgical intervention is often unsuccessful in preventing mortality in these FAP patients [28]. We propose that the prevalence of duodenal adenomas in the BALB Min mouse makes this model useful for developing therapeutic strategies to treat duodenal cancer.

3.5 References

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3.6 Tables and Figures

sex	genotype	age (months)	locus	size (cm)
f	Min/+	3	mandibular	1.5
f	Min/+	7	face	0.8
f	Min/+	N/A	mandibular	1.3
m	Min/+	6	mandibular	0.8
f	Min/+	8	below eye	0.5
f	Min/+	8	mandibular	1.5

Table 3-1: Subcutaneous tumors of the head and neck in BALB Min mice

BALB wild type and BALB Min littermates were observed for the development of subcutaneous masses. Six out of 76 BALB Min mice developed 5-20 mm diameter subcutaneous masses on the face or neck. The age of mice developing these subcutaneous tumors ranged from 3 to 8 months, with one mouse of unknown age. No such masses were observed in BALB wild type mice.

Timepoint (months)	Genotype	N	Number of adenomas in small intestine per animal	Average size of adenoma in small intestine
7	WT	10	0	0
7	Min/+	10	12 ± 6	0.5 ± 0.4
12	WT	9	0	0
12	Min/+	9	13 ± 6	0.6 ± 0.3

Table 3-2: Number and size (cm) of adenomas in the small intestine of BALB wild type and Min mice

BALB Min and wild type were examined for tumors in the gastrointestinal (GI) tract. The table shows a summary of the average tumor incidence and diameter, standard deviations. There was no statistical difference ($p > 0.05$) in average tumor number or size over the entire small intestine between BALB/c Min versus wild type mice at 7 and 12 months of age

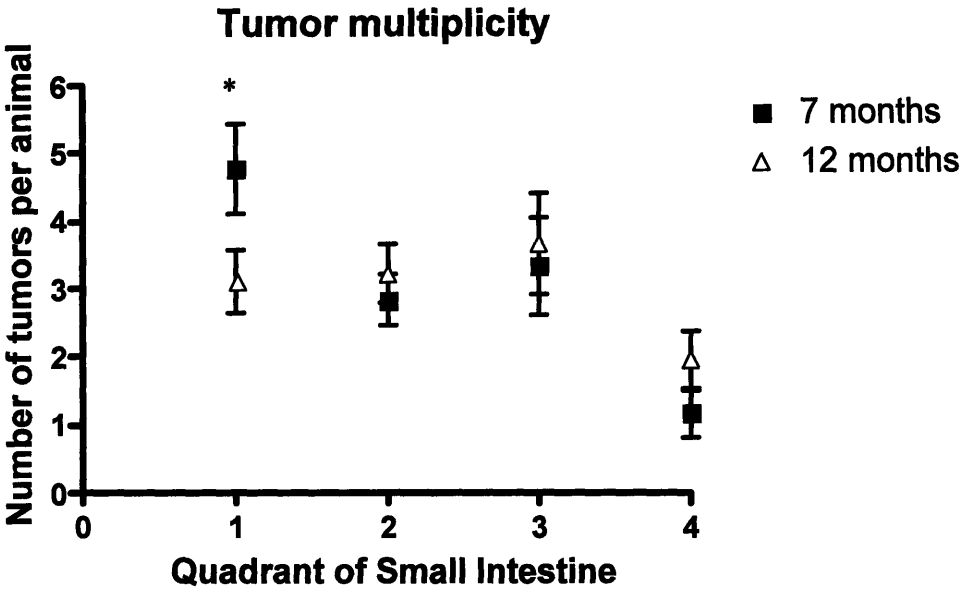

Figure 3-1: Tumor location in small intestine of 7 and 12 month old BALB Min mice.

The entire length of the small intestine of 7 and 12 month old BALB Min mice was scored for adenomas. Mean number and standard error is shown for each timepoint. Location of these adenomas was recorded as distance from pylorus, and then analyzed by quadrants numbered 1, 2, 3 and 4 from the proximal to distal small intestine. Tumor number in the first quadrant was higher in 7 month old mice as compared to 12 month old mice ($p = 0.0477$).

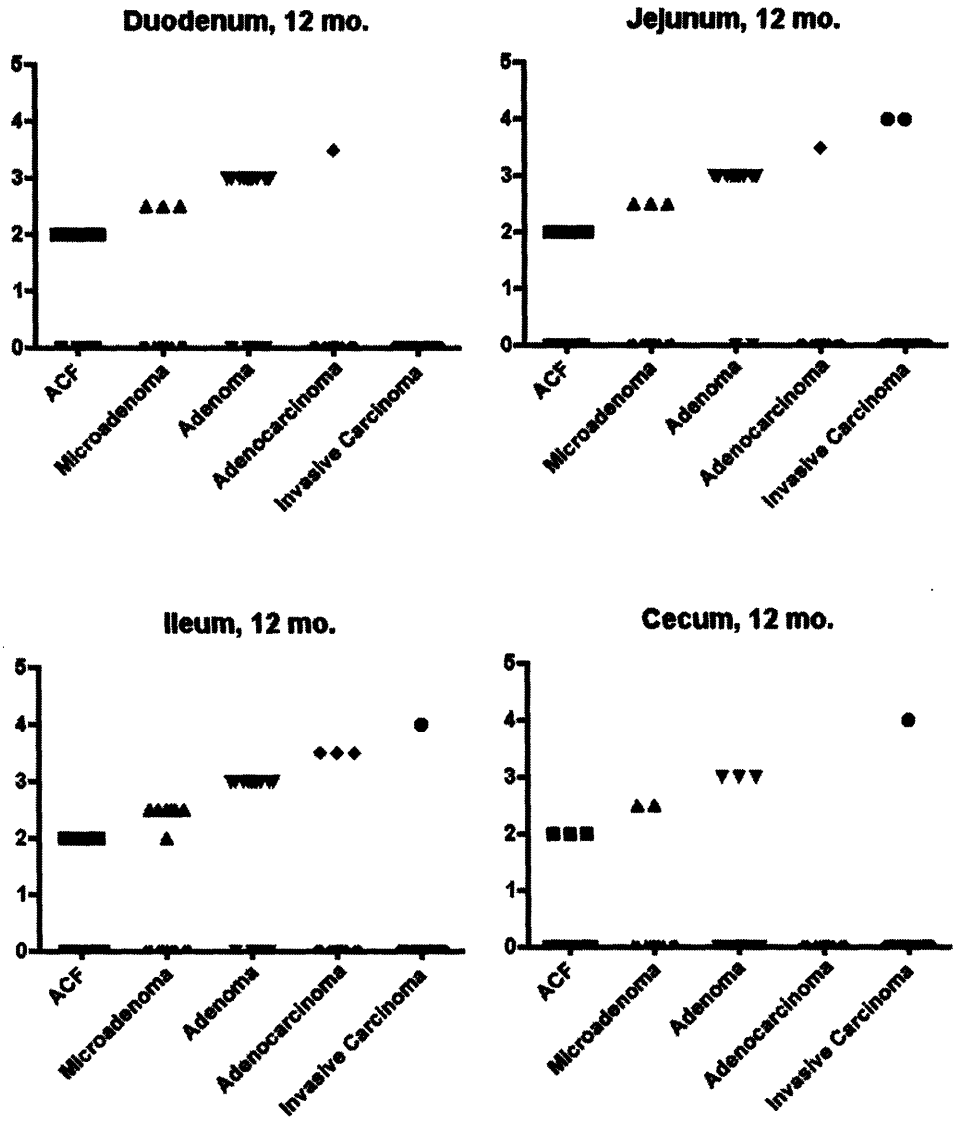

Figure 3-2: Evaluation of intestinal lesions of 12 month old BALB Min mice

Lesions in BALB Min mice were categorized based on size and invasiveness. Individual animals are represented by a shape corresponding to the category of the lesion. We defined aberrant crypt foci (ACF) as a focus of less than 5 dysplastic crypts, microadenoma as 5-10

dysplastic crypts, adenoma as a focus of greater than 10 dysplastic crypts but not visible to the naked eye, adenoma as a non-invasive tumor visible to the naked eye, adenocarcinoma as polyps protruding into lumen and pressing into the muscularis mucosa, and adenocarcinomas based on penetration of the intestinal wall by neoplastic cells and/or invasion of vasculature. Several 12 month old BALB Min mice showed adenocarcinomas or invasive carcinoma. Two BALB Min mice showed invasive carcinoma in the jejunum, three showed adenocarcinomas or invasive carcinomas in the ileum, and one mouse showed invasive carcinoma in the cecum.

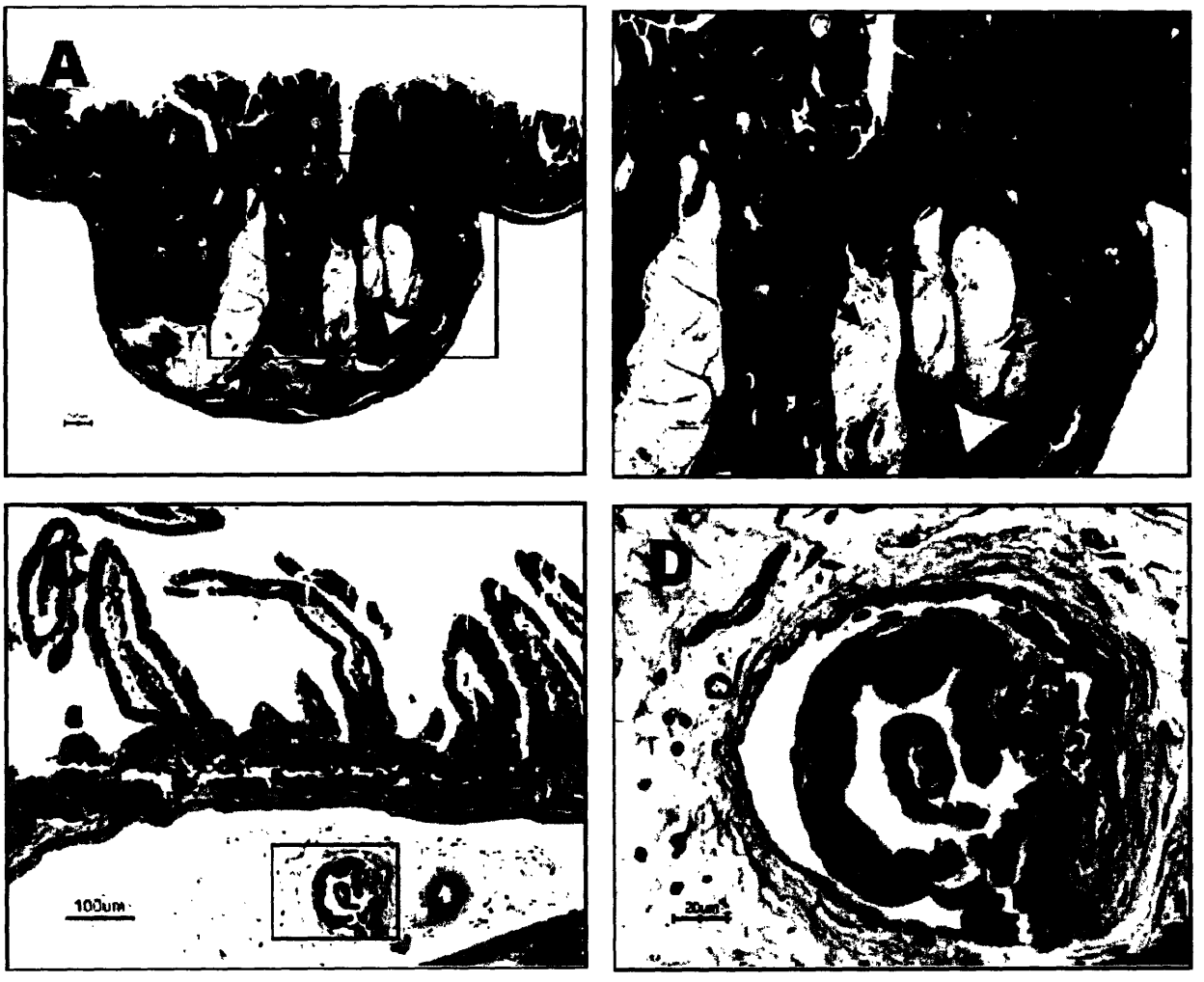

Figure 3-3: Histomorphology of an invasive carcinoma in the ileum, and vascular infiltration of epithelial cells in 12 month old BALB Min mice.

A) One twelve month old BALB Min mouse developed an invasive carcinoma in the ileum stained with hemotoxylin and eosin (H&E). Invasion of an adenoma through the lamina propria and muscularis mucosa into the serosa is shown. The bar represents 100 microns.

B) A higher magnification of the invasive carcinoma from box A is shown. The right side of the invasive carcinoma shows crypts filled with dysplastic cells that are hyperchromatic and

basophilic. The invading portion of the adenocarcinoma shows similarly dysplastic cells on the luminal side, but invading epithelial cells have lost crypt architecture. Interspersed are areas of muscle and pools of mucin (indicated by arrows). The bar represents 200 microns.

C) One twelve month old BALB Min mouse showed blood vessel invasion below the muscularis mucosa of the small intestine. The upper portion of the photomicrograph shows regular jejunum with intact villi (with exception of an artifact created by section of the fixed tissue). The bar represents 100 microns

D) A higher magnification of the blood vessel from box C showns a vein containing several red blood cells. Also inside the vein are ribbons of epithelial cells that are hyperchromatic and basophilic. As there are no overlying irregular epithelial cells in the villi above, and there is a space between the vein and the villi, this appears to be relocation of dysplastic epithelial cells. The bar represents 20 microns.

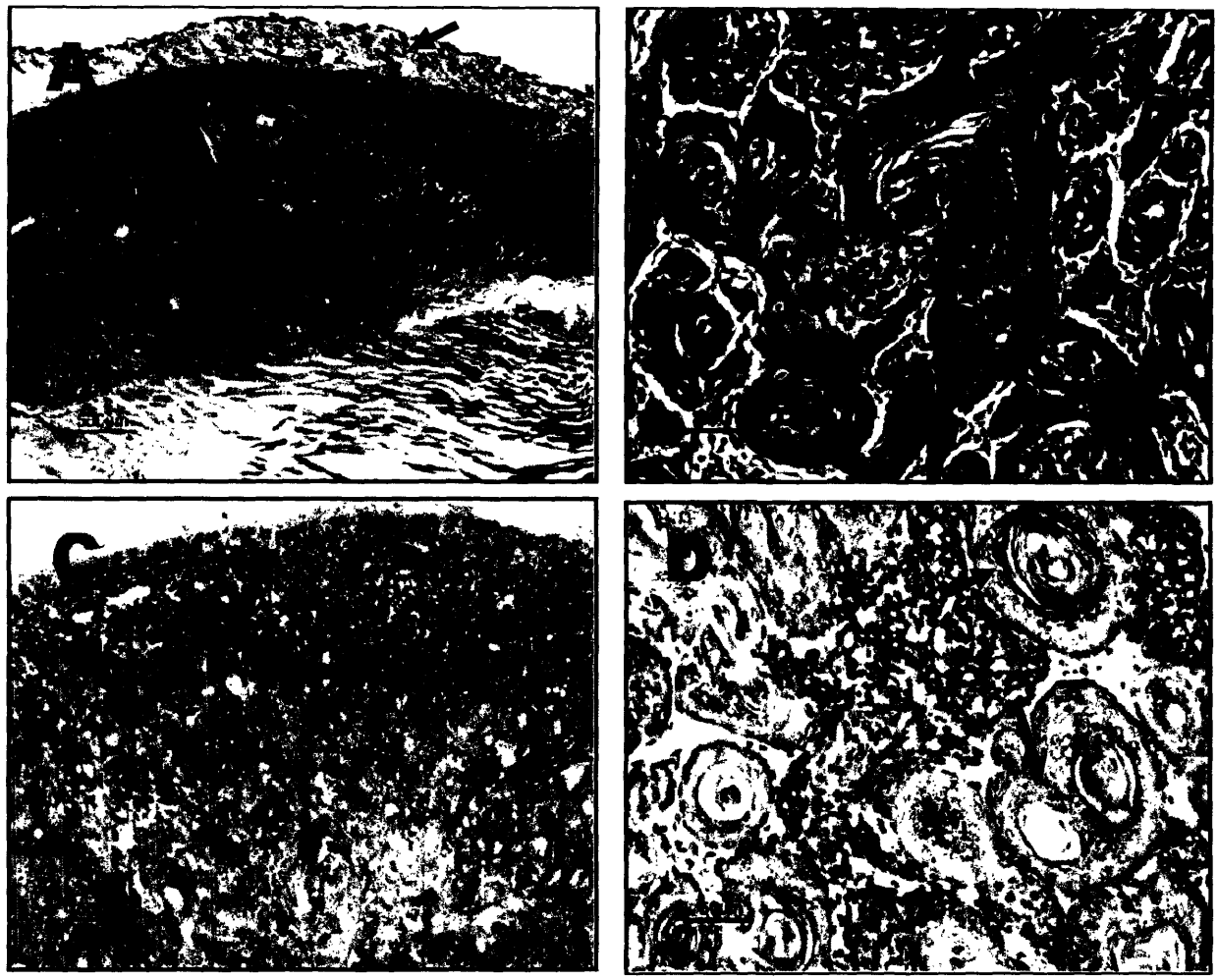

Figure 3-4: Histomorphology of basosquamaous carcinomas stained with hematoxylin and eosin

A & B) Basosquamous carcinomas were identified in BALB Min mice. Above in low magnification (A) is an example that developed in the hind leg. Basaloid cells are present with overlying normal skin (indicated by the arrow). At high magnification in box B, basaloid cells are shown to be dysplastic.

C & D) Basosquamous carcinomas were also observed (low magnification box C). Islands of squamous cells surrounding keratin are prominent among hyperchromatic basaloid cells (high manification box D).

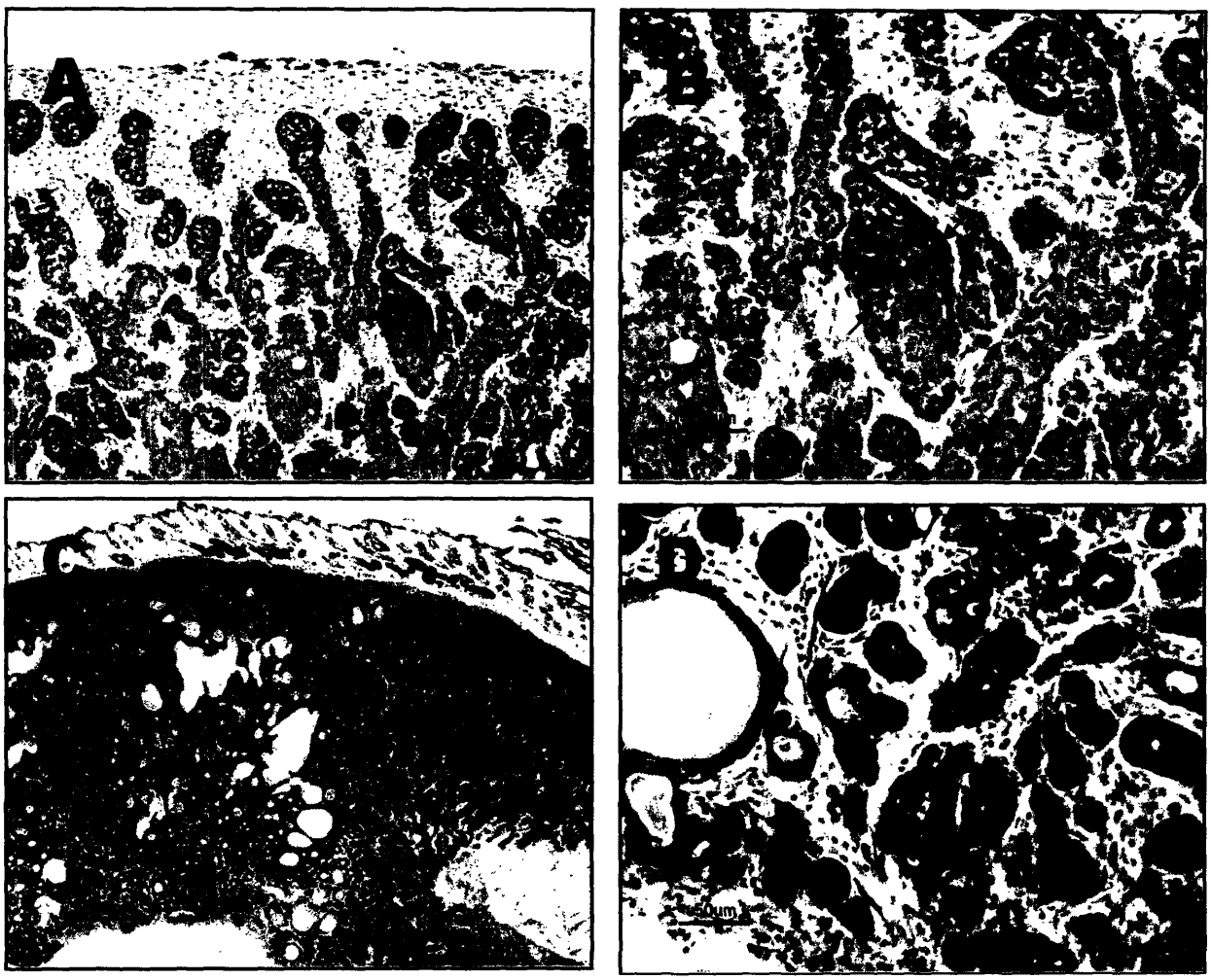

Figure 3-5: Immunohistochemistry for β-catenin localization in basosquamous tumors

A & B) Basal cells in this basal cell carcinoma show nuclear accumulation of β-catenin

C & D) Some basal cell carcinomas with squamous cell differentiation showed very unusual staining in that most squamous cells and some basal cells both showed nuclear localization of β-catenin. All cells showed strong cytoplasmic and membrane staining.

Chapter 4: $CD4^+CD25^+$ lymphocytes induce regression of intestinal tumors in $Apc^{Min/+}$ mice

This chapter is to be incorporated into the manuscript described below. Data included in this thesis was limited to experiments directly performed by Jane J Sohn.

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4.1 Introduction

Colorectal cancer is the third most common cause of cancer deaths in both men and women in the United States [Jemal, 2004 #322]. An inherited pattern of colorectal cancer is seen in less than 10% of patients with colorectal cancer. Of the inherited syndromes, familial adenomatous polyposis (FAP) is the most common. Mutations in the adenomatous polyposis coli (*Apc*) tumor suppressor gene are responsible for FAP [1-4] and also occur in many sporadic cases of colorectal cancer [5]. It is known that modifier genes influence the severity of FAP [6, 7].

C57BL/6J Min mice, a murine model of FAP, as well as other murine models of colon cancer have been used to test various therapies for their ability to prevent and treat intestinal tumorigenesis. Min mice are heterozygous for the Apc^{Min} (*Min*) allele, which is a mutation at codon 850 of the murine *Apc* homolog, resulting in truncated Apc. C57BL/6J Min mice develop numerous adenomas in the small intestine with lower numbers in the large intestine, and rarely live beyond six months of age [8].

Standard treatment for colorectal cancer focuses on surgery, chemotherapy, and radiation therapy (http://www.nci.nih.gov/cancertopics/pdq/treatment/colon/Patient/page4). Surgery involves removal of cancerous tissue by local excision, resection, radiofrequency ablation, or cryosurgery. Chemotherapy uses drugs to stop the growth of cancerous cells. Radiation therapy uses focused high-energy particles to target and kill cancer cells. The specific type of therapy used depends on the stage of the lesions, and alternative therapies may be available through clinical trials in the advanced stages of cancer.

Drugs that inhibit cyclooxygenases (COX) have been tested in clinical trials to determine their efficacy in both prevention and treatment. There are two COX enzymes: COX-1 is expressed constitutively in normal cells, and COX-2 is inducible and has been shown to be upregulated in colon tumors of humans and animals. Interest for COX as a therapeutic target has been supported by animal model and human epidemiologic studies using aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) that are known to inhibit COX expression. The first such large-scale, prospective study showed a significant reduction of colorectal cancer in both men and women with frequent aspirin use [9].

Prevention of colorectal cancer with COX inhibitors has led to mixed results. Treatment of colorectal adenomas in FAP patients to inhibit further progression of tumors with the NSAID sulindac showed a 44% decrease in the mean number of polyps, and 35% decrease in the mean diameter of polyps after 9 months of treatment [10]. During the study, however, a relative increase in the number of adenomas was observed between 6 and 9 months of therapy, suggesting the selection or development of polyps resistant to sulindac therapy. In addition, sulindac has been reported to be ineffective against some cases of rectal carcinoma [11-13], leading to mortality due to rectal cancer during treatment.

Alternative therapies have focused on using the immune system to target cancerous cells. Some studies have focused on vaccines targeting antigens overexpressed in tumors [Midgley, 2003 #526]. We are interested in investigating the role of lymphocyte populations, specifically $CD4^+CD25^+$regulatory T cells (Treg) on tumorigenesis, as this had not been examined

previously. These cells have been shown to have anti-inflammatory effects. In order to determine whether $CD4^+CD25^+$ Treg cells modulate progression of intestinal tumors, adoptive transfer of syngeneic $CD4^+CD45RB^{lo}CD25^+$ Treg cells was performed in 4-6 month old C57BL/6 Min mice that have numerous adenomas.

Prior studies using $CD4^+CD25^+$ Treg in Rag2-deficient mice have shown IL-10-dependent suppression of colitis-associated colon cancer [14, 15], suggesting that inhibition of enteric inflammation may be pivotal in intestinal tumorigenesis. While non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to decrease tumor latency and burden in Min mice and humans [16, 17], roles for immune cells that inhibit enteric inflammation have not been examined for either model. Thus, we examined whether $CD4^+CD25^+$ Treg may modulate development and progression of non-colitis associated intestinal tumors using adoptive transfer of purified wildtype $CD4^+CD25^+$ Treg in Min C57BL/6 mice.

4.2 Materials and Methods

C57BL/6 Min mice.

Animals were housed in AAALAC-approved facilities in static microisolator cages with mouse pathogen free health status. Mice had mouse pathogen free (MPF) health status as previously described [15]. There was no evidence of murine *Helicobacter* spp. in treated or untreated mice. Min mice on a C57BL/6 background were obtained from the Jackson Laboratories or bred in house.

Experimental design.

Overall, 24 Min mice served as untreated controls, 29 Min mice were treated with Treg from wild type C57BL/6 mice, and 6 Min mice were treated with IL-10-deficient Treg. Studies included slightly more males than females in both treatment and control groups. Two untreated Min control mice were found dead and were not used for tumor counts in the study. Some experiments were conducted using two to three separate trials each. Trials using age-matched animals were combined for statistical analyses.

Experiment 1 (Immunotherapy using $CD4^+CD25^+$ Treg in Min mice): To examine whether transfer of $CD4^+CD25^+$ Treg is able to induce regression of established intestinal adenomas, 9 Min mice aged 4.5-6 months received Treg from wild type mice. This experiment was performed in two separate trials: Four mice received wild type Treg in trial 1, and five mice received wild type Treg in trial 2. One mouse (age 6 months upon treatment) in trial 2 did poorly clinically, was euthanized 2 weeks post cell transfer (PCT), and was not included in the study. The remaining eight mice were euthanized 4 - 7 weeks after the initial transfer. Data from the two trials were combined for these analyses. Ten control Min mice were included.

In addition, to determine whether onset of regression was immediate or delayed, seven Min mice aged 6 months received Treg and were then euthanized 2-4 days PCT. There were three separate trials: two mice received wild type Treg in trial 1, two mice received wild type Treg in trial 2, and three mice received wild type Treg in trial 3. Data from these trials were combined for analyses.

Experiment 2 (Role of IL-10): In order to determine whether IL-10 was necessary in donor $CD4^+CD25^+$cells for therapeutic effect, twelve Min mice aged 3 - 4 months received Treg: six mice received wild type Treg and six mice received Treg from C57BL/6 mice lacking IL-10. All mice from both treatment groups underwent a second cell transfer 3 weeks later using the same numbers of cells to assure successful transfer of Treg. Six Min mice were left untreated and served as controls. One control mouse was found dead at age 4 months, and was not included in the study. Two recipients of IL-10-deficient $CD4^+CD25^+$Treg became moribund and were euthanized 4 weeks after onset of treatment (at age 4 months) due to rapidly declining body condition. The remaining mice were euthanized at 4.5 - 5 months of age.

Adoptive transfer of T cells in Min mice.

To examine the ability of T lymphocytes to modulate non-colitis associated intestinal adenomas, we transferred purified $CD4^+CD45RB^{lo}$ $CD25^+$ T lymphocytes from mouse pathogen free C57BL/6J donors into 4.5 –6 month old C57BL/6 Min mice. Half of the donor mice were males and half were females. To obtain viable and highly purified populations of lymphocytes, single cell suspensions from spleen and mesenteric lymph nodes from donor mice were prepared as described previously [14]. Re-analysis of these cells prior to transfer into mice indicated that they were >96% pure. Anesthetized mice were injected intravenously in the retro-orbital sinus with 3- 4 $\times 10^5$ T cells suspended in 0.2 ml of HBSS.

Quantitation of intestinal tumors.

Location and diameter of tumors was recorded using a stereomicroscope at 10× magnification. Location of tumors in the small intestine was recorded as distance from the pylorus, and in the colon as distance from cecocolic junction.

Histologic evaluation.

Formalin-fixed tissues were processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Lesions were evaluated by a board certified veterinary pathologist blinded to sample identity (PRN). Inflammatory lesions were graded on a scale of 0 to 4 with ascending severity as previously described [14, 18]. Non-parametric data are presented as median score and range (in parentheses) for each group (data not shown in this thesis).

Statistical analyses.

Tumor frequency between control and treatment groups was analyzed using ANOVA for normally distributed data, as determined by the Kolmogorov-Smirnov Test, then followed by a Dunnett's comparisons test. The gastrointestinal tract was analyzed by stomach, duodenum, jejunum, ileum, cecum and colon to investigate if specific areas showed significant change. The duodenum, jejunum, and ileum were defined as the first, second, and third sections of the small intestine, as determined using the length of small intestine for each animal. Tumor number was also analyzed for the entire gastrointestinal tract with the same statistical tests. Statistical analyses of inflammation scores were carried out with a non-parametric Kruskall-Wallis test with post-hoc Dunn's multiple comparison test. For apoptosis, the non-parametric Mann-Whitney test

was performed to test significance between control Min mice and treated Min mice. Comparisons of levels of cytokine transcript were also performed using Mann-Whitney U test.

4.3 Results

CD4+CD25+ cells induce regression of established adenomas in ApcMin/+ mice.

To examine whether $CD4^+CD25^+$ Treg are able to treat intestinal tumors in Min mice, we transferred these cells into nine C57BL/6 Min mice at 4.5 to 6 months of age (μ age 5.6 months). We found that the Min mouse recipients of $CD4^+$ Treg had 17 ± 13 tumors, significantly fewer ($p<0.01$) than untreated age-matched Min controls, which had 62 ± 15 tumors (Figure 4-1 "long-term treatment").

In addition, we found that Min mouse recipients of $CD4^+$ Treg ($n = 9$) had increased longevity and better body condition than age-matched untreated Min mice ($n =10$). Eight (8/9) Min mice treated with Treg were alert and active upon termination of the experiment at 6 to 7.5 months of age, whereas all ten (10/10) untreated mice had been euthanized due to morbidity prior to 6 months of age.

A reduction in tumor burden was evident as early as 2 to 4 days after adoptive transfer of Treg as animals that received this short-term treatment had 32 ± 17 tumors in the entire gastrointestinal tract ($p<0.01$; $n = 7$) (Figure 4-1 "short-term treatment"). The remaining intestinal tumors in

treated Min mice appeared grossly to be flattened with a depressed center, suggestive of cell death, in contrast to polypoid nodular tumors in untreated controls.

CD4+CD25+ Treg increase apoptosis in intestinal adenomas in Min mice.

To determine whether treatment with $CD4^+CD25^+$ cells induced regression of tumors through increased tumor cell death, apoptosis was quantified *in situ* on intestinal tissues from Treg-treated and untreated Min mice. We found a significant increase ($p = 0.011$) in apoptosis within tumors after treatment with Treg ($n = 7$), when compared with untreated control mice ($n = 5$) (data not shown in this thesis). Increased apoptosis was most evident within adenomas of mice euthanized 2-4 days after receiving Treg ($p = 0.002$) as compared to untreated mice. The remaining tumors in the intestine of treated mice appeared smaller (data not shown in this thesis), or demonstrated an umbilicated center characterized by central necrosis and ulceration with underlying granulation tissue (data not shown in this thesis). Two tumors within one of the treated animals demonstrated stromal vascular thrombosis (data not shown in this thesis). Two (2/15; 13%) untreated aged Min mice had localized neoplastic epithelial invasion involving the serosa (data not shown in this thesis). There was no evidence of malignant invasion (0/29) in mice that had been treated with $CD4^+CD25^+$ Treg (data not shown in this thesis). There were no differences in epithelial proliferation of crypts within treated versus untreated tumors at either interval post-treatment. These data suggested that adoptive transfer of $CD4^+CD25^+$ lymphocytes decreased tumor burden through rapid induction of apoptosis in intestinal tumors in Min mice.

CD4+CD25+ Treg modulate inflammatory gene expression Min mice.

While there were no significant morphological differences in inflammation within tumors of Treg-treated Min mice (n = 6) versus untreated Min mice (n = 6), there were significant decreases ($p = 0.07$) in pro-inflammatory cytokine expression within tumors after treatment with $CD4^+CD25^+$ cells. These findings demonstrate an association between tumor regression, increased apoptosis, and down-regulation of inflammatory gene expression in Min mice.

Interleukin 10 is required in CD4+CD25+ lymphocytes to suppress adenomas in Min mice.

To determine whether $CD4^+CD25^+$ cells lacking IL-10 were capable of reducing frequency of intestinal adenomas, Min mice received Treg from IL-10-deficient C57BL/6 donors (n = 6) or matched wild type mice (n = 6) (Figure 4-2). We found no significant differences (Figure 4-1) in recipients of $CD4^+CD25^+$ lymphocytes lacking IL-10, that showed 63 ± 18 tumors in the entire gastrointestinal tract, when compared with intestinal tumors from age-matched untreated Min mice (n = 5) that had 62 ± 19 tumors. In contrast, Min mouse recipients of wild type $CD4^+CD25^+$ cells (n = 6) had 13 ± 6 tumors in the entire gastrointestinal tract, significantly ($p < 0.01$) fewer tumors than age-matched untreated Min controls. Thus, IL-10 is necessary in $CD4^+CD25^+$ cells to suppress adenomas in Min mice.

4.4 Discussion

We have demonstrated that $CD4^+CD25^+$ Treg induce regression of established adenomas in the Min mouse model of human intestinal cancer. Tumor burden was significantly decreased

throughout all regions of the bowel in regulatory cell-treated mice. Adoptive immunotherapy using $CD4^+CD25^+$ cells induced apoptosis in tumors, coincident with tumor regression, within two to four days after lymphocyte transfer. We found that IL-10 was necessary in $CD4^+CD25^+$ cells for therapeutic effect.

$CD4^+CD25^+$ Treg have well-established abilities to suppress host inflammatory responses in humans and mice[23, 24]. Although IL-10 is most widely known for its anti-inflammatory properties in the lower bowel [25], it has previously been shown that IL-10 also modulates apoptosis and suppresses angiogenesis during tumor regression in other models [25, 26]. Whether IL-10 may modulate apoptosis or angiogenesis directly, or indirectly through suppression of chronic enteritis, has not been determined.

While it has been shown elsewhere that pro-inflammatory intestinal infections may facilitate development of intestinal adenomas in Min mice [27, 28], it is unclear whether inflammatory cytokines or associated growth factors were required to sustain epithelial tumors in the present study. The incongruity between enteric morphology and cytokine expression in Min mice in the present study suggests that contributions of chronic inflammation to intestinal pathogenesis may be underestimated using morphological criteria alone. The finding that interferon (INF)-γ - which has characterized anti-neoplastic activity in other systems [29] was not reduced at early intervals post-treatment requires further study. It was previously shown that IL-10-producing glioma-specific $CD4^+$ Tr1-like cells enhanced some pro-inflammatory cytokines during tumor rejection [30].

The recent observation of thymic depletion coincident with tumorigenesis in C57BL/6 Min mice [31] supports a pivotal role for lymphocytes in progression of adenomas in this model. There is accumulating evidence that $CD4^+CD25^+$ Treg are derived in the thymus of mice [32], and that functions of these cells may be compromised during thymic atrophy [33]. Indeed, preliminary data revealed fewer $CD4^+CD25^+$ cells in thymus, spleen and mesenteric lymph nodes of aging Min mice, when compared with age-matched wild type controls (data not shown in this thesis). Taken together, these data suggest that impaired regulatory cell functions may contribute to development of intestinal tumors in Min mice. Specific determinations of regulatory cell competency in aging Min mice are underway. In other studies, $CD4^+CD25^+$ Treg have been shown to promote epithelial cancer by inhibiting beneficial host anti-tumor responses [32, 34, 35].

Immunotherapy using competent Treg significantly diminished tumor counts in the colon, as well as the small intestine, whereas coxib therapy is most efficacious in the distal small intestine of mice, with increased frequency of colonic tumors in mice. This has been addressed with duel ursodiol and sulindac treatment, though long term efficacy of this combination therapy remains to be seen [36]. It has been shown that $CD4^+CD25^+$ Treg traffic to inflammatory foci throughout the body and demonstrate potent anti-inflammatory activity at these sites [32]. Recent studies suggest $CD4^+CD25^+$cell-mediated recruitment of other $CD4^+$ lymphocytes during inhibition of inflammation [37-39]. Possible roles for $CD4^+CD25^+$ Treg in amplifying tumoricidal activities of $CD4^+$ T regulatory (Tr)1 cells [30] in this setting need to be investigated further.

In humans, intestinal adenomas with mutations in *Apc* progress to malignancy and metastasis through a series of additional genetic mutations [1, 2]. Although Min mice on a C57BL/6 background generally have fewer invasive neoplastic foci than Min mice of other strain backgrounds [40], several aged untreated Min mice had localized neoplastic epithelial invasion in the present study. Whether anti-neoplastic activities of $CD4^+CD25^+$ lymphocytes extend beyond adenomatous polyps and *Apc* alone is unknown. The present data in Min mice suggest a potent anti-neoplastic role for these immunomodulatory lymphocytes in epithelial carcinogenesis.

In summary, we have demonstrated that adoptive transfer of $CD4^+CD25^+$ Treg rapidly induces regression of established adenomas in the Min mouse model of human intestinal cancer. The relevance of tumoricidal activities versus inhibition of beneficial anti-cancer surveillance activities in epithelial homeostasis requires further investigation. Because dysregulation of *Apc*-β-catenin signaling is a frequent early feature of non-colitis colon cancer in humans [5, 41], it will be important to consider $CD4^+$ Treg in immunotherapeutic strategies. Recent long-term trials using sulindac in humans have been discouraging [42], suggesting that adoptive immunotherapy may be a better candidate than aspirin, NSAIDs or coxibs for prevention and treatment of colon cancer.

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4.6 Figures

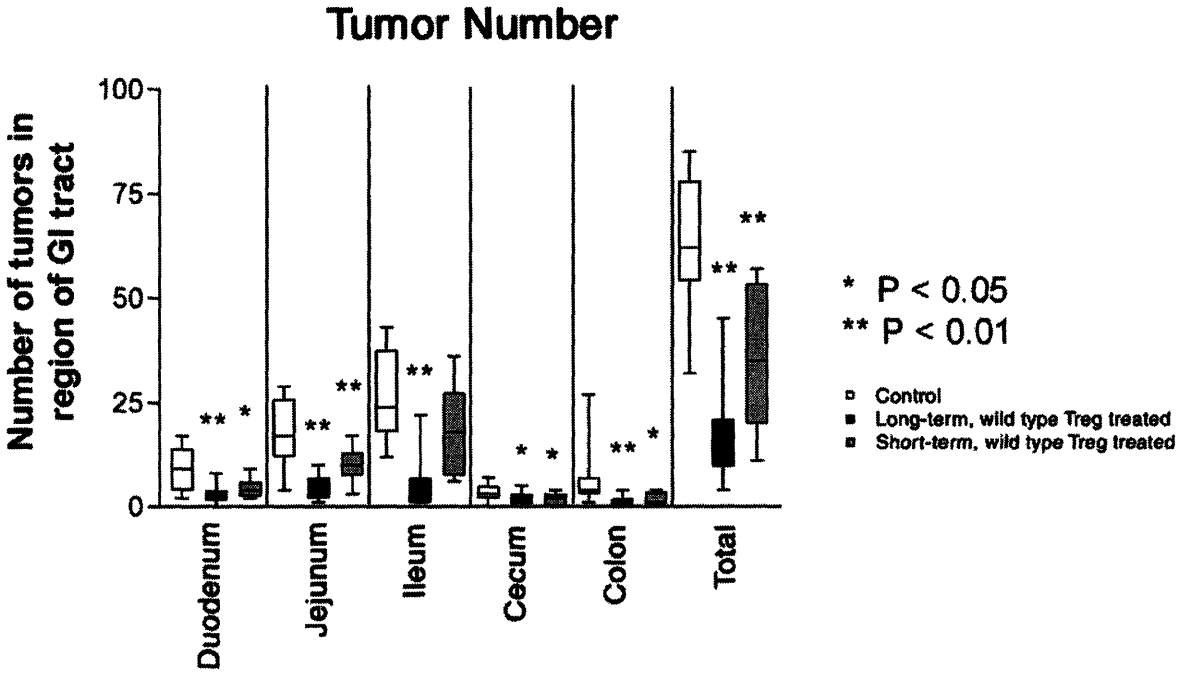

Figure 4-1: Effect of regulatory cell treatment on intestinal tumor number in Min mice.

Four to six-month old Min mice that received a long-term treatment of $CD4^+$ $CD25^+$ Treg had significantly fewer ($p<0.01$) intestinal adenomas than untreated age-matched Min controls. Moreover, a reduction in tumor burden was evident as early as 2 - 4 days (short-term treatment) after adoptive transfer of Treg ($p<0.01$; $n = 7$).

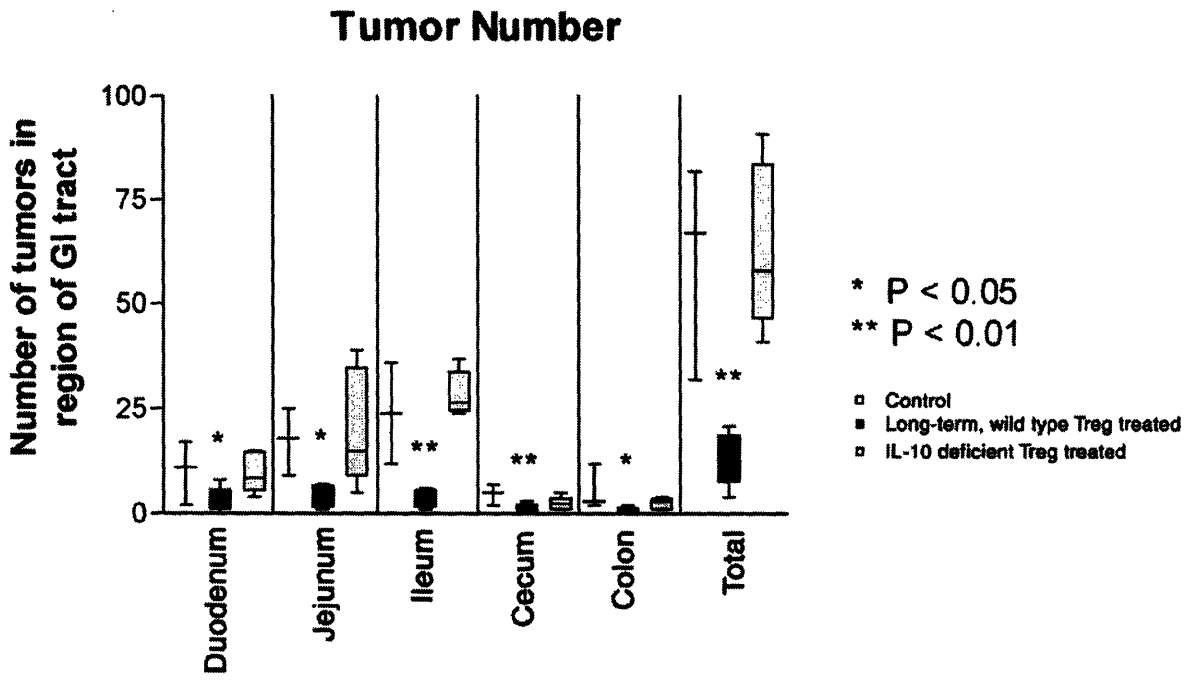

Figure 4-2: The role of IL-10 in tumor treatment of Min mice with Treg adoptive transfer.

Recipients of CD4$^+$CD25$^+$ lymphocytes lacking IL-10 showed comparable tumor burden when compared to age-matched untreated Min mice (n = 5). In contrast, Min recipients of wild type CD4$^+$CD25$^+$ cells (n = 6) had significantly ($p < 0.01$) fewer tumors than age-matched untreated Min controls.

Chapter 5: Conclusions

The main objective of this thesis was to investigate the role of inflammation in the development of intestinal neoplasia. To this end, three research goals were planned. The first was to investigate if self-limiting hyperplasia and inflammation with *C. rodentium* infection is associated with a change in mutant frequency. Indeed, increased mutant frequency was observed 14 days post infection, leading to the realization that self-limiting infection alone can lead to an increased number of cells bearing mutations. The second research aim was to characterize neoplasia in the BALB/c $Apc^{Min/+}$ (Min) model. This led to the discovery of basosquamous cancer of the skin that was only observed in BALB/c Min mice and not in BALB/c wild type littermates and cagemates. This supported the hypothesis that Apc was involved in the development of observed skin cancers. Further investigation of the β-catenin status of these tumors revealed evidence of a role of β-catenin dysregulation, and thus Apc inactivation in these tumors. Finally, $CD4^{+}CD25^{+}$ regulatory lymphocytes were tested as a treatment therapy for intestinal neoplasia in the C57BL/6 Min mouse model of intestinal neoplasia. This adoptive immunotherapy resulting in fewer number of adenomas in C57BL/6 Min mice treated at 3-4 months of age as compared to untreated C57BL6 Min mice. Furthermore, treatment of 4.5-6 month old C57BL6 Min mice appeared to decrease the number of adenomas. This suggests that Treg cells can cause tumors to regress.

The observation that self-limiting infection can induce mutant frequency raises questions about the necessity of chronic infection and chronic oxidative stress to induce somatic mutations.

Previous work by Touati et al. [1] has shown that increased mutant frequency in response to *Helicobacter felis* infection can be detected at 6 months post infection in association with increased iNOS expression. One year after infection, however, mutant frequency is no longer increased, and iNOS expression is not detected. In light of this result, it is important to investigate if the increased mutant frequency with *C. rodentium* infection can be detected after the infection is naturally cleared, and tissues are histologically normal. If the increase in mutant frequency is a transient event, this suggests that the induced mutations do not persist and that perhaps mutations associated with inflammation do not necessarily contribute to carcinogenesis.

The development of basosquamous cancer in the BALB/c Min mice is the first report of an invasive skin cancer in a model of familial adenomatous polyposis. β-catenin nuclear localization in the basosquamous cancers suggests β-catenin involved in the tumorigenesis of these lesions . This is supported by recent findings that ß -catenin is critical to skin development, especially the differentiation of stem cells into follicular keratinocytes in the adult mouse [2].

We do not know why C57BL/6 Min mice or other Min mice have not been reported to develop basosquamous tumors. Investigators may have failed to observe skin cancers in C57BL/6 Min mice because the relatively short lifespan [3] of this model may limit skin tumorigenesis to a small number of animals. We also hypothesize that BALB/c mice, may harbor a modifying gene that makes them more susceptible to basosquamous tumors. This is based on the observation that BALB/c mice show a higher number of squamous cell carcinoma than many other strains [4]. Treatment of BALB/c Min mice with a chemical that has been shown to increase the number of skin tumors in other models may increase the incidence of basosquamous tumors. Examples

of such chemicals include 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), which act as initiating and promoting agents in the SENCAR model [5].

Lastly, little is known about how Treg cells decrease the number of observable tumors, and if this therapy leads to long term suppression of tumorigenesis. A long-term study of Treg adoptive immunotherapy in the Min mouse would be an important step in helping to understand the extent to which Treg cells can decrease adenoma formation. The question of how Treg cells decrease tumor burden must continue to be addressed. Though we have presented evidence that this process is mediated by apoptosis, this may be an association and not a cause of the therapeutic affect. The studies presented here have contributed to the understanding of mutagenesis and tumorigenesis in the intestine.

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