

INSIGHTS INTO THE ROLE OF INFLAMMATION IN COLITIS-ASSOCIATED
CANCER: TARGETING TUMOR NECROSIS FACTOR RECEPTORS

by

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This thesis is dedicated to my beautiful family,
Dr. Desmond Pink and Andrew Pink

And to those in my life, past and present, who made this research worthwhile

Pops (Robert Andrew Stillie) and Grandma (Marie Stillie)
My nephew Drake Stillie

And finally my wonderful parents,

Garry and Dorothy Stillie

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ABSTRACT

Inflammatory bowel diseases (IBD) are associated with an elevated risk of colorectal cancer that increases with disease duration and severity. Tumor necrosis factor (TNF) is a major therapeutic target in IBD, but long-term anti-TNF therapy is associated with increased risks of infection and lymphoma, therefore we asked whether TNF signaling through its receptors TNFR1 and TNFR2 could impact colitis and colitis-associated cancer (CAC). In acute dextran sulphate sodium (DSS)-colitis, no major inflammatory differences were found between wildtype (WT), TNFR1- and TNFR2-deficient mice, with the exception of reduced macrophage infiltration into inflamed tissue in TNFR1^{-/-} mice. Chronic colitis and tumor development was assessed in these mice using the carcinogen azoxymethane and 4 cycles of DSS. TNFR1^{-/-} mice were protected against colorectal tumor development compared to WT and TNFR2^{-/-} mice, while inflammation was similar between strains. Hematopoietic TNFR1 deficiency resulted in reduced inflammation and tumor incidence, while stromal/epithelial TNFR1 deficiency reduced indices of cancer without affecting inflammation. 8-OHDG was significantly lower in TNFR1^{-/-} mice compared to other strains, suggesting that TNF could contribute to oxidative stress within the colon. Mice lacking leukocyte NADPH oxidase were protected against clinical illness and CAC despite similar histological inflammation, indicating that inflammation-associated oxidative stress can play a role in CAC. In conclusion, TNF signaling through TNFR1 contributes significantly to the development of colorectal cancer in a model of CAC in a manner that involves both stromal/epithelial and hematopoietic TNFR1. This is significant because anti-TNF therapies may be effective at reducing CAC in the absence of a clinical reduction of IBD symptoms.

LIST OF ABBREVIATIONS USED

8-OHDG, 8-oxo-7,8-dihydro-2,-deoxyguanosine

AOM, Azoxymethane

APC, Adenomatous Polyposis Coli

CAC, Colitis-associated cancer

CD, Crohn's Disease

CIMP, CpG Island Methylator Phenotype

CIN, Chromosomal Instability

CRC, Colorectal Cancer

DISC, death inducing signaling complex

DSS, Dextran Sulphate Sodium

DTH, Delayed-type Hypersensitivity

FADD, Fas-associated Death Domain

FLIP, Flice Inhibitory Protein

IAP, Inhibitor of Apoptosis

IBD, Inflammatory Bowel Diseases

IEC, Intestinal Epithelial Cell

IKK, Inhibitor of Kappa B Kinase

IFN, Interferon

iNOS, inducible Nitric Oxide Synthase

i.p., Intraperitoneal

JNK, c-jun N-Terminal Kinase

KRAS, Kirsten rat sarcoma homolog

mAb, Monoclonal Antibody

MAPK, Mitogen activated protein kinase

MIF, Macrophage inhibitory factor

MMP, Matrix Metalloproteinase

MLC, Myosin Light Chain

MLH1, MutL homolog 1

MPO, Myeloperoxidase

MSI, Microsatellite Instability

NADPH, Nicotinamide Adenine Dinucleotide Phosphate

NF κ B, Nuclear Factor Kappa B

NO, Nitric Oxide

PI3K, Phosphatidyl Inositol 3 kinase

PLC, Protein Kinase C

PUMA, p53 upregulated modulator of apoptosis

RA, Rheumatoid Arthritis

RIP, Receptor Interacting Protein

ROS, Reactive Oxygen Species

SCC, Sporadic Colon Cancer

SCID, Severe Combine Immunodeficient

TACE, TNF-Converting Enzyme

TAK, TGF- β Activating Kinase

TGF, Transforming Growth Factor

TIMP, Tissue Inhibitor of Metalloproteases

TNBS, Trinitrobenzosulphonic acid

TNF, Tumor Necrosis Factor

TNFR1, Tumor Necrosis Factor Receptor Type 1

TNFR2, Tumor Necrosis Factor Receptor Type 2

TPA, 12-O-tetradecanoylphorbol-13-acetate

TTP, Tristetraprolin

TRAF2, TNF receptor associated factor 2

TRADD, TNF-associated death domain

UC, Ulcerative Colitis

VEGF, Vascular Endothelial Growth Factor

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CHAPTER 1: Introduction

An Overview

Tissue trauma as a result of infection, physical disruption, ischemic injury, or exposure to toxic substances can activate innate immunity. Trauma activates resident immune cells and/or serum factors such as complement or clotting proteins, which release signals that activate local endothelial cells, increasing the expression of selectins that promote the transendothelial migration of leukocytes from the blood into the tissues. This cascade of events leads to vasodilation, fluid exudation, migration of effector cells into the tissue from circulation, and an increased activation of pain receptors. Leukocytes, such as neutrophils and monocytes, can release toxic mediators that cause damage to the surrounding tissue, and can also activate tissue remodeling promoting healing. A sustained response might be propagated by the activation of the adaptive immune system with T and B-cell involvement. Normally, this cascade of events leads to pathogen clearance, healing and resolution; however, if the insult persists, or if there is an inappropriate response to the insult, chronic inflammation can result. During chronic inflammation, there is a sustained activation of signals that can lead to cell activation, proliferation, differentiation, migration, tissue remodeling and angiogenesis. These are all normal signals important in the reparation of damaged tissue. However, during chronic inflammation, a prolonged production of mediators such as proteases, reactive oxygen and nitrogen species can further injure the tissue. These mediators, normally serving to eliminate pathogens, can cause extensive damage to proteins, lipids and DNA, altering gene expression by a number of mechanisms. If DNA mutations and/or epigenetic changes in key genes involved in cell cycle control or DNA repair occur in tandem with enhanced immune activation and signals for wound healing and angiogenesis (activated during inflammation), the environment becomes conducive to carcinogenesis. As a consequence, tumors have been described as “wounds that never heal” [1].

Association Between Inflammation And Cancer

Rudolf Virchow was one of the first to describe the relationship between inflammation and cancer in the 1800's when he observed leukocytes within and

surrounding tumor tissue [2, 3]. Since then, epidemiological studies have reinforced this observation by demonstrating a positive relationship between chronic inflammation and risk of cancer development in several inflammatory disorders, including gastroesophageal reflux disease, Barrett's Esophagus and esophageal cancer [4], and chronic infections such as *Helicobacter pylori* [5]. A well-established association between intestinal inflammation and the development of intestinal tumors and CRC has been described consistently in the literature. IBD, including Ulcerative Colitis and Crohn's disease, is a significant risk factor for the development of CRC [13,14].

Inflammatory Bowel Diseases: Ulcerative Colitis and Crohn's Disease

Ulcerative colitis (UC) and Crohn's disease (CD) are remitting-recurring inflammatory bowel diseases of unknown etiology. The prevalence rate of IBD in Canada is about 468 per 100,000 amounting to 0.46% of the population. The incidence rate per 100,000 Canadians is approximately 16.3 for CD and 12.9 for UC [6]. This varies slightly per province but Nova Scotia is on the upper end of the spectrum of incidence and prevalence of IBD[6]. While genetics are theorized to play a role, especially genes involved in bacterial sensing [7] and autophagy [8], it is likely that a combination of genetics and environmental exposures contribute to the development of IBD [9]. While UC is limited to the colon, CD can affect the entire alimentary tract, commonly affecting the colon. Both diseases can result in extra-intestinal manifestations, including ankylosing spondylitis, primary sclerosing cholangitis (more common in UC), and uveitis [9].

In UC, colonic inflammation tends to be continuous in nature and results in granulocytic infiltration, disruption of the crypt architecture, and crypt abscesses [10]. UC-related inflammation involves mainly the superficial mucosal layer. Symptoms include cramping and bloody diarrhea with mucous [11]. Because UC affects the colon and rarely the terminal ileum, it can be cured with colectomy. Due to the superficial nature of the colonic inflammation, some of the complications observed in CD do not occur commonly in UC.

CD presently has no known cure, and it is difficult to treat due to the unpredictable and patchy nature of the inflammation. The disease presents itself as discontinuous inflammation in the bowel wall, and can become transmural leading to

stenosis, fistulas and abscesses [9]. Common intestinal and extra-intestinal pathologies associated with CD are abdominal pain, diarrhea, painful joints, fatigue, fever intestinal granulomas, anemia, sepsis, weight loss and malnutrition, growth retardation, and bone changes [11].

Inflammatory Bowel Diseases (IBD) and Cancer

Crohn and Rosenberg first described colon carcinoma in a case of UC in 1925 [12]. The evidence since then, that IBD is associated with cancer, has been extensive. The risk of cancer appears to be dependent on the duration, extent and anatomical involvement of the inflammation [13, 14].

It was estimated that 1% of all CRC cases are a consequence of UC [15]. Compared to colon cancer found in UC patients, CD-associated colon cancer is reported to be diagnosed more often at an advanced stage [16]. A recent meta-analysis of studies performed comparing the risk factor for intestinal cancer between CD patients and the background population determined that people with long-standing CD were at a higher risk of developing CRC. Conversely, there was no difference compared to the general population in rectal cancer relative risk. The role of anatomical location of disease was significant, as CRC relative risk in those with CD limited to the small bowel was similar to the general population. In contrast, those with CD limited to the colon had a much higher relative risk than in the general CD-affected groups. The relative risk of developing small intestinal cancer in those affected with CD was much higher compared to the general population. When anatomical location of disease was considered, this relative risk rose to 158 [17]. Those diagnosed before the age of 25 had a high risk of developing CRC [17-19]. It was recently estimated that the risk of CRC in those with CD after 10 years of disease was 2.9%, after 20 years it was 5.6%, and after 30 years it was 8.3% [20]. Overall, the risk of cancer is associated with the anatomical location, severity of disease, and duration of disease. Anatomical location of inflammation may be an important risk factor due to the destruction of tissue and regeneration that occurs. Local inflammatory signals may be important to this process and could potentially contribute to DNA damage locally as opposed to systemic inflammation.

An estimated 10% of those affected with IBD will develop CRC after 30 years of disease [21]. In UC specifically, the number is much higher, at 18% [22]. It was once

estimated that one sixth of all patients with UC will die of CRC [23], making this a serious secondary complication of the disease. Changes in therapies, such as colectomy in UC, have likely lowered this number [13]. It is possible that therapies limiting inflammation may reduce the need for colectomy [24] potentially leading to future susceptibility to colon cancer if separate mechanisms are involved. Crohn's colitis is still a factor and is unpredictable in nature, limiting the efficacy of colectomy in preventing pan-intestinal inflammation [25].

Extra-intestinal cancers have also been investigated in IBD patients with two meta-analyses addressing this issue. Both found that overall cancer risk in IBD is similar to the general population [26]. However, in site-specific analyses, CD patients had an increased risk of upper GI, lung, bladder and skin cancers. UC patients had an increased risk of liver cancer and leukemia [26]. Another meta-analysis found that death from lung cancer was greater in those affected by CD [25]. Extra-intestinal cancers in IBD associated with anti-TNF therapies, such as lymphoma have received attention in the past decade, however it was recently reported that no short-term risk of cancer is associated with any of the anti-TNF therapies [27]. Long-term risks associated with the use of anti-TNF therapies remain to be determined.

Sporadic Colon Cancer

The colonic epithelium is constantly being exposed to stressors from the environment. These include ingested bacteria, their by-products, environmental and endogenous toxins. Over time, these stressors may lead to damage to stem cells within the crypt from which the rapidly dividing epithelial cells originate. Damage to these cells could lead to an accumulation of DNA mutations and epigenetic changes that are passed to daughter cells. These changes can lead to dysplasia in a sequence of events referred to as the "adenoma-carcinoma sequence" [28]. Adenoma describes abnormal growth of the glands but without invasion of the surrounding tissue and is considered a pre-malignant event, while carcinoma can lead to invasion of the surrounding tissues resulting in potential metastasis [28]. While there are many mutations that have been implicated in colon cancer, there are a select few that are thought to be the main drivers of tumorigenesis [29, 30]. Fearon and Vogelstein proposed a model of genetic changes that characterized the alteration of the normal cell to the cancerous cell beginning with the

inactivation of the adenomatous polyposis coli (APC) gene [31]. After the loss of APC, KRAS mutations and the loss of genes found on chromosome 18q were associated with growth and progression of the adenoma, and the loss of the p53 gene was associated with the transition from adenoma to carcinoma [31]. While much has been learned since this paper was published, this general mechanism is still widely accepted.

There are three main pathways that colon cancer can arise from; Chromosomal instability (CIN), microsatellite instability (MSI), or the CpG island methylator pathway (CIMP) [32]. The MSI and CIMP pathway often overlap. In sporadic colon cancer (SCC), it has been suggested that an event occurs to create an accelerated “mutator phenotype” as the baseline mutation rate is normally low [33]. Mutations in genes whose products regulate chromosomal segregation during mitosis [34] can lead to abnormal DNA content, or aneuploidy, and loss of heterozygosity, contributing to genomic instability in daughter cells, leading to the CIN-associated SCC [32]. Each crypt is populated by stem cells that multiply and differentiate into enterocytes, enteroendocrine cells, goblet cells and Paneth cells as they move along the crypt. Damage that occurs to stem cells is followed by repopulation of the crypt with genetically or epigenetically altered daughter cells. Consequently, the altered daughter cells are more susceptible to further mutations, as are the stem cells. As mutations accumulate within stem cells, so does the clonal expansion of neoplastic daughter cells. Barker et al., identified a stem cell marker that is a leucine-rich repeat-containing G-protein coupled receptor called LgR5 [35]. When APC was deleted in LgR5⁺ cells of conditional knock-out mice, microadenomas were detected within eight days followed by macroscopic adenomas after three weeks, suggesting that tumors arise from mutations in these stem cells [36]. Deleting APC in differentiated cells in other areas of the crypt did not result in adenomas [36]. This would explain how tumors could arise among cells that typically only last a few days within the colon, which seems an insufficient amount of time for mutations to accumulate and change the behaviour of the cell.

The microsatellite instability pathway involves the silencing of genes involved in DNA repair, or mismatch repair genes. MSI⁺ tumors can arise via the CIMP phenotype via silencing of the mismatch-repair genes [37]. Approximately 15% of colorectal cancers show this phenotype, with 12% being associated with hypermethylated *MLHI* gene [38, 39], and 3% accounting for familial Lynch Syndrome, an inheritable mutation

in MLH1 leading to increased MSI and tumor development in affected individuals [40]. MSI⁺ tumors are usually diploid and do not show loss of heterozygosity. MSI unstable colorectal cancers have distinct features, such as being found primarily in the proximal colon [41]. Other features include poor differentiation, mucinous/ signet ring appearance and lymphocytic infiltration [42]. These tumours respond differently to chemotherapy compared to MSI-stable tumors [43], and have a better prognosis [44].

The third pathway, the CpG methylator phenotype, involves hypermethylation of the CpG-islands near genes important in colon cancer [37]. There is a subset of tumors that reportedly displays neither MSI nor CIN [45]. CpG islands, regions within the DNA enriched with cytosine-guanine dinucleotides, tend to be found near promoter regions, transcription start sites, and first exons of genes [46]. DNA methylation involves the addition of a methyl group to the 5' cytosine by methyltransferases via a methyl donor. CpG hypermethylation can silence gene transcription through various mechanisms, including steric hinderance of the transcriptional complex, through recruitment of histone deacetylases and by contributing to chromatin remodeling in a repressive manner [46]. Silencing genes involved in DNA repair and cell cycle control can contribute to both mutations and uncontrolled cell proliferation, respectively.

Global hypomethylation is a well-defined characteristic of progression in many cancers, and interestingly, is implicated in early stage colon cancer [47]. Certain DNA regions, such as satellite regions and repetitive sequences, normally have high levels of methylation in order to maintain genomic stability. Hypomethylation can have consequences such as chromosome instability and genetic mutation and can lead to the activation of oncogenes, such as RAS [46]. It is not known what is responsible for hypomethylation, as the activity of DNA methyltransferases remains normal [46]. A demethylating enzyme has not been described in this context, however it has been suggested that inactive variants of one of the DNA methyltransferases that are present in tumor cells could lead to competitive binding of the enzyme complexes and DNA [48]. These three pathways, including phenotypes where the three pathways overlap, are the basis of tumorigenesis in sporadic colon cancer and have been found to be involved in inflammation-mediated tumorigenesis as well. Other colon cancers can arise from inherited mutations, but will not be discussed.

Inflammation-mediated versus Sporadic Colorectal Cancer

Many of the genetic and epigenetic alterations observed in sporadic colorectal cancer also occur in colitis-associated cancer (CAC). Genomic changes in CAC, such as CIN and MSI appear to occur in similar proportions as in SCC [49]. In contrast, CpG island hypermethylation is common in the colon during UC [50, 51] but may not be a major route of tumor development. Age-related CpG-hypermethylation of the genes encoding p16 and the estrogen receptor, but not the *MLH1* (mismatch repair) gene, has been shown to occur before dysplasia in UC [52]. The lack of *MLH1* hypermethylation substantiated later studies that showed CpG hypermethylation was not a prominent phenotype in UC-associated cancers [53, 54].

Populations with more advanced CAC have poorer survival rates than those with advanced SCC [55]. In addition, those diagnosed with CAC tend to be younger [55]. CD especially is associated with detection of more advanced cancers [16]. As more is becoming known about CAC, better screening practices exist for those affected by IBD. Other phenotypical differences exist between sporadic colon cancer and CAC that may hinder surveillance. For example sporadic colon cancer forms visible polyps arising from a discrete precursor dysplastic lesion, while CAC may additionally present as flat dysplasia, diffuse throughout the colon, an observation first documented by Counsell and Dukes in 1952 [56].

The genetic changes in sporadic colon cancer similarly occur in CAC [57], although their appearance might follow a different sequence. In human CAC, DNA aneuploidy precedes dysplasia [58, 59]. p53 mutation is an early event in the dysplasia to carcinoma sequence, and the more advanced the dysplastic lesion is, the more likely it is to contain a p53 mutation [60-62]. APC and KRAS are commonly altered early in the adenoma-carcinoma sequence in SCC [63-66]. However, APC mutation in CAC is rare [65, 66]. Also, KRAS and p53 mutations were reported to be less frequent in colitis associated cancer compared to sporadic colon cancer [67]. This difference in sequence is summarized in (Figure 1.1), adapted from Itzkowitz et al., 2004 [13].

Animal Models of Colitis-Associated Cancer

There are several methods to study the role of intestinal inflammation in the development of CAC. There are chemical models of colitis such as the dextran sulphate

sodium salt (DSS) model. Others have used targeted mutations in mice to study the role of chronic colitis in the development of cancer, such as the IL-10 gene-deficient (IL-10^{-/-}) strain [68]. Adoptive transfer of colitis by administration of CD45RB⁺ cells is used less frequently in CAC models and will not be discussed here.

DSS has been used as a non-genotoxic carcinogen since the early 1990s, when it was found that chronic DSS administration could lead to dysplasia and carcinoma in rodents [69, 70]. Rodents are used to model chronic colitis but result in fairly low incidences of cancer over a long period of time making it difficult for effective statistical measurement of cancer incidence [71, 72]. In order to produce a more reliable and statistically measurable cancer incidence, an additional low dose carcinogen can be administered. One agent is supplemental dietary iron, which can exacerbate CAC, presumably through the oxidative stress that iron contributes to the diet [73]. Other carcinogens that are used include azoxymethane (AOM) and dimethylhydrazine. Dimethylhydrazine, an AOM precursor, is a hepatotoxin and extremely toxic to handle [74]. The carcinogen by itself is administered at a level that would not induce colon cancer on its own, and requires further promotion, which is effectively provided by inflammation.

The Azoxymethane/Dextran Sulphate Sodium Model of Colitis-Associated Cancer

A single, low dose of AOM (up to 12 mg/kg) followed by one or more cycles of DSS has proved adequate to study CAC. The addition of a sub-carcinogenic dose of AOM to the DSS cycle regime allows for a shorter time period to cancer development, and fewer animals per experiment. This model, like acute DSS colitis, is subject to strain differences. The two more common strains for targeted gene mutation are BALB/c and C57BL/6. While C57BL/6 mice are more susceptible to DSS colitis compared to BALB/c, BALB/c mice tend to be more sensitive to AOM [75]. AOM/DSS treatment results in mutations that affect similar pathways to what is observed in humans, such as APC/ β -catenin [76]. In accordance, in the AOM and DSS model, *C-MYC* is upregulated [77]. In CAC with DSS alone, the translocation of β -catenin from the membrane to the cytoplasm or nucleus was observed in precursor lesions, while p53 expression was not [78]. The AOM+DSS model has contributed greatly to the understanding of colitis in the

development of cancer, which will be discussed further in the following section.

AOM was first described for this use by Okayasu et al., 1996 [79]. It is administered intraperitoneally or subcutaneously in phosphate-buffered saline. It is a pro-carcinogen that is further metabolized into methoxyazoxymethanol by an isoform of cytochrome P450 called CYP2E1 [80]. Methoxyazoxymethanol further degrades into formaldehyde and an alkylating species that leads to DNA methylation and G:C-A:T transversion mutations in several key genes involved in colon cancer [81]. These include *KRAS*, *SRC-PI3K-AKT*, Wnt/ β -catenin, *TGF- β* and *TP53* [82]. In *KRAS* mutations for example, the transversion mutation at codon 12 results in glycine replacement by aspartic acid leading to a constitutively active form of KRAS. As one downstream signaling pathway of KRAS involves MAPK and PI3K/AKT, this can contribute to tumor development [81]. Interestingly in AOM-induced CRC, β -catenin mutations appear to be predominant where in human CRC the mutations tend to occur in the *APC* gene, affecting β -catenin signaling [76].

Other methods of inducing CAC involve using mice genetically predisposed to develop colorectal cancer and inducing colonic inflammation via chemical means[83]. Mice such as *Apc*^{min+/-} [83, 84], *Msh* [85], or *p53* gene-deficient mice [86] develop much higher incidences and tumor loads compared to gene-deficient mice that do not have colon inflammation. These models with targeted mutations in genes known to participate in colon cancer show that inflammation can greatly influence the transition from healthy epithelium to carcinoma. In summary, there are several models to evaluate the development of CAC. The most widely-used animal model of CAC is the AOM/DSS model. It is convenient, reproducible and minimally invasive. It is a good tool to evaluate drugs, dietary treatments and genes in CAC.

Chemical Induction of Colitis

It is important to understand the underlying mechanisms of inflammation in any experimental model, especially when disease etiology is not clear. DSS and trinitrobenzene sulphonic acid (TNBS) are chemicals administered to rodents that reproducibly induce colitis. TNBS is a hapten and is administered mixed in different concentrations of ethanol in enema form [87]. There are several disadvantages to this model. One of the drawbacks to this technique is that multiple enemas, each requiring withdrawing food, may produce excess stress on the animal. In addition, the manner in

which TNBS colitis is presently used often results in high mortality rates [88]. To develop a delayed-type hypersensitivity (DTH) response to TNBS, waiting 6 days following colitis administration is recommended, however, often animals are euthanized after 2-3 days, meaning the reaction is only to the acute sensitization phase, and not a true DTH response, as this method is often described as a “Th1” model of colitis [88]. Another limitation to this method is the number of ways in which it can be used to induce colitis. Different dose concentration and frequency, with timing of the sample collection might affect the mechanism by which TNBS is exerting its colitic effect [88]. While this method of chronic colitis induction has been described in combination with dimethylhydrazine to model CAC [89], it is not widely practiced.

A simpler, popular method is to combine multiple cycles of DSS colitis with AOM [90]. DSS is administered dissolved in the drinking water and produces a colon-specific inflammation that is considered T-cell independent as it occurs in athymic mice [91]. The inflammatory infiltrate in DSS colitis is, as in TNBS colitis, mainly neutrophils, macrophages and monocytes [92] with chronic colitis being associated with lymphocytic infiltrate [93]. Initial descriptions compared DSS-colitis to UC as it affects only the colon and cecum, does not involve fistulas or granulomas as in CD, and involves mainly the mucosa [93]. Chronic DSS colitis might be similar to UC in that Th17 cytokines are downregulated and IL-4 and IL-10 are upregulated [94]. However, acute DSS-colitis may demonstrate characteristics of CD as well, including transmural involvement and a Th17/Th1-skewed immune response, at least in its acute stage [94]. Measurements can be made to assess the extent of clinical illness, such as stool consistency, presence of blood in the stool and weight changes during DSS treatment [92].

The mechanisms of DSS colitis induction are not clear. DSS has been reported in Kupffer cells in the liver after the first day of DSS administration and is likely not substantially altered/digested in the small intestine [95]. This suggests that DSS can be absorbed, presumably in the small intestine, where it would be transported to the liver via the portal vein, potentially activating macrophages. It is not known whether activated macrophages could have originated in the gut and homed to the liver or if the DSS itself traveled through the portal vein, although DSS was reported in the mesenteric lymph nodes suggesting it was brought in by resident cells [95].

Many rodent models of colitis require the commensal microbiota for disease expression but the evidence surrounding the role of microflora in DSS colitis is conflicting. It has been reported that antibiotic treatment can ameliorate DSS-colitis [96], although incubation of DSS with cecal contents did not lead to appreciable changes in DSS chemical properties [97]. Supporting this, the microflora does not seem to be absolutely required for DSS-induced colitis as germ-free mice still develop inflammation in response to DSS, in fact were more severely affected by DSS administration than their conventionally-housed controls [98]. DSS is known to change the microbial flora of the gut during colitis but how much of this is post-inflammation and pre-inflammation is not known [99]. It is possible that the microflora exacerbates colitis once the epithelial layer has been breached as opposed to being modified in a harmful way by DSS treatment.

Immune activation and subsequent colitis in response to DSS administration in mice appears to be dose-dependent [100], molecular weight [101] and strain-dependent [102]. C57BL/6 mice are more susceptible to DSS colitis compared to BALB/c mice [102, 103] suggesting a role of genetics in this model. These two strains of mice are commonly used for targeted mutations. The chemical properties of DSS may affect disease expression. Feeding mice DSS of greater molecular weights (over 36,000 compared to 500) leads to a greater induction of colitis [101]. The mechanism for this is unknown, but it is possible that larger molecular weights have altered solubility, having implications for both immunogenicity and toxicity. In addition, larger molecular weight sulphated carbohydrates may lead to a yet unknown receptor cross-linking, enhancing signaling, if it is possible that the inflammatory properties of DSS are due to specific cell-surface signaling and not just endocytosis and actions within the cell.

DSS might cause colitis indirectly by activation of immune cells residing in the colon. It is known from early studies that it is stimulatory to B cells and intraepithelial and lamina propria lymphocytes residing in the gut [104-107], therefore making it possible for direct stimulation by DSS of immune cells in the gut. Indeed, treating dendritic cells with DSS resulted in inflammatory cytokine production and transfer of dendritic cells into mice subsequently challenged with DSS resulted in exacerbation of colitis [108]. This suggests that the dendritic cells can take in DSS and transport it to sites where there is immune activation, such as Peyer's patches or mesenteric lymph nodes. Interferon- γ ^{-/-} [109] and CXCR2^{-/-} [110] mice treated with DSS have very few ulcers,

suggesting that epithelial injury is indirectly related to the response to DSS, as it requires immune cell activation in order to cause damage.

Application of DSS to cell lines is not without cytotoxicity. Cytotoxicity was found in 5% of Caco-2 cells *in vitro* after DSS treatment [111]. In addition, adding DSS to these cells decreased transepithelial resistance [111], suggesting it interferes with barrier function. A decrease in electric resistance *ex vivo* through mucosal sections was reported in mice prior to histologically-detectable damage [112]. DSS induced the expression of pro-inflammatory cytokines by Caco-2 cells, such as IL-8 [111]. DSS treatment may directly affect gene expression as it was shown to prevent mRNA transcription by binding to DNA in the nucleus, having implications *in vivo* [111].

Colonic epithelial cell uptake of DSS directly in the colon has not been shown. Sulphated glycans are structurally similar to DSS and were shown to bind to a lectin domain on the epidermal growth factor receptor, possibly directly affecting colonic epithelial cells [113]. In addition, the sulphur content within the DSS preparation is associated with the severity of colitis [114]. Studying DSS in culture does not properly reflect the antigen-rich environment and does not account for other physical barriers such as mucins and bacteria.

The above studies indicate that DSS may exert colitic effects directly on the epithelium, and indirectly, through activation of resident immune cells. While the mechanism of DSS-colitis is not accurately determined at this time, it is likely a function of effects on both the epithelium and surrounding stroma, and resident immune cells within the colon. Why it affects only the large intestine as opposed to the entire intestinal tract may reflect the differences in receptor expression between intestinal epithelial cells and colonic epithelial cells that have not yet been determined. It is noteworthy that DSS alone can induce cancer after many cycles repeated over a long period of time [72], making it an ideal chemical means of studying colitis-mediated cancer.

TNF and Colitis-Associated Cancer

TNF is a major therapeutic target in both CD and UC, but paradoxically, whether TNF is a driving factor in the pathogenesis of IBD is not yet clear. TNF has more recently been implicated in CAC but the risks of long-term anti-TNF therapy are not fully understood. It is important to understand TNF biology and the role of TNF in colitis. The safety of anti-TNF therapy has been questioned due to a link with certain types of

cancer, such as lymphoma and susceptibility to opportunistic infection [115].

TNF is an intracellularly-assembled homotrimer with each subunit being 17kDa and is expressed as a type II transmembrane protein [116, 117]. It undergoes proteolytic cleavage by the TNF-alpha converting enzyme (TACE), resulting in a 51kDa soluble tripeptide [118]. LPS-induced TNF biosynthesis is regulated in part by MAP kinases, in particular p38, which phosphorylates downstream targets MK2 and MK3 [119, 120], resulting in their activation and subsequent phosphorylation of a protein called tristetraprolin (TTP). TTP binds the AU-rich element of TNF mRNA, which prevents TNF mRNA from being translated [121]. TTP phosphorylation by MK2 and to a lesser extent by MK3, results in TTP stabilization and release of TNF mRNA, freeing it for translation [122].

TNF Receptors and their Signaling

TNF can signal through two structurally distinct receptors, the TNFR1 and the TNFR2 [123]. TNFR1, a 55kDa protein first described in 1990 [124], is found on most cells in the body, while TNFR2 expression is thought to be limited to cells of lymphoid or myeloid lineage; however, TNFR2 has been reported on intestinal epithelial cells (IEC) during inflammation [125] hepatocytes [126] and endothelial cells. Both receptors contain similar cysteine-rich extra cellular domains that result in the formation of homotrimers [127]. TNFR1 possesses an 80-amino acid sequence on its intracellular domain that can activate apoptosis [128], whereas TNFR2 does not. While TNFR1 binds the soluble form of TNF, TNFR2 favors binding the membrane-bound form of TNF [129]. The low affinity interaction of soluble TNF and TNFR2 may augment signaling through TNFR1 via “ligand passing” of TNF from TNFR2 to TNFR1 [130].

Mice lacking the p55 TNFR1 gene have relatively normal physiology and unchanged T-cell development, but are unresponsive to LPS and superantigen challenge [131] and have been reported to fail to develop small intestinal Peyer’s patches [132]. A later study disputed this finding, reporting instead that the deficiency was in the formation of B-cell follicles within secondary lymphoid tissues [133]. In contrast, they are extremely susceptible to infection by intracellular bacterial infections such as *L. monocytogenes* [131, 134] and *Mycobacterium tuberculosis* [135].

TNF signaling can lead to two main cell responses. One is apoptosis through both

the extrinsic and intrinsic pathways and the other is cell survival/activation. When TNF signaling results in apoptosis, the TNFR1 trimerizes in response to TNF ligation. TNF ligation leads to the internalization of TNFR1 into clathrin-coated pits, which may mediate its pro-apoptotic effects, as blocking this step prevented apoptosis in several cell lines [136]. The internalization appears to be necessary for death inducing signaling complex (DISC) formation [137]. The TNFR1-associated adaptor protein, known as the TNFR-associated death domain (TRADD) [138], can interact with the E3 ubiquitin ligase, TNF receptor associated factor (TRAF) 2, receptor-interacting protein (RIP)-1 [139] or the fas-associated death domain (FADD), leading to activation or apoptosis, respectively [138, 140]. FADD then recruits procaspase-8 via the homologous death effector domain to form the DISC. Pro-caspase 8 is autolytically cleaved during DISC formation to caspase-8. Caspase-8 then activates the caspase cascade resulting in apoptosis. TNFR1-induced apoptosis can be regulated at several levels, including Flice-inhibitory protein (FLIP) which inhibits caspases, FLIP can also recruit activating adaptor proteins like TRAF 1-3 and RIP [141]. TNFR1 has been shown to activate apoptosis through the intrinsic pathway via the cleavage of the inactive Bid into a truncated form that can integrate into the mitochondrial membrane and lead to cytochrome C release [477].

In addition to apoptosis, NF κ B activation is a major consequence of TNFR1 signaling. RIP-1 can interact with mitogen activated protein kinase kinase kinase (MEKK-3), and transforming growth factor activated kinase (TAK)-1, leading to activation of inhibitor of κ B (I κ B) α kinase (IKK), which phosphorylates I κ B leading to the release and nuclear translocation of p65 and p50 together known as NF κ B. TRADD-TRAF2 association also leads to the nuclear translocation of NF κ B [142]. TRAF2 binds to inhibitor of cellular apoptosis proteins 1 and 2. This complex leads to the degradation of I κ B α and subsequent liberation of NF κ B [143-145]. RIP also directly links the IKK signalosome to TNFR1 [146]. TNF activation of NF κ B has also been shown to occur via PI3K and AKT [147]. AKT has been shown to phosphorylate IKK α at threonine 23 [147]. Rapidly after receptor ligation, PKC is activated [148] but NF κ B activation was demonstrated to occur independently of PKC [149]. TNF ligation can also activate phospholipase C [150].

As TNFR1 can result in two potential cell fates, how this decision is made is

important. During TNFR1 activation, MAPK are also activated and they can both promote [151] and inhibit apoptosis [152]. It has been proposed that TNFR1 activation occurs in two sequential processes [153]. First, after TNF stimulation, TRADD, RIP and TRAF2 are recruited to lipid rafts containing TNFR1 [154], forming a high molecular weight transient complex. Neither FADD nor Caspase-8 is detectable in this complex. This complex dissociates as TNFR1 is endocytosed and the TRADD death domains become available to bind to other death domains associated with TNFR1 such as FADD, where apoptosis may be activated via the association of another complex [153]. This appears to be the time that is important in whether or not apoptosis or cell survival will occur.

The activation of NF κ B by TNF upregulates the transcription of anti-apoptotic genes, counteracting the activation of the apoptosis. Mediators such as Bcl-2 and Bcl-XL are upregulated after NF κ B activation and can oppose apoptosis purportedly by binding up pro-apoptotic proteins within the mitochondrial membrane [478]. When components of the NF κ B pathway are blocked in intestinal epithelial cells, increased epithelial cell apoptosis has been reported [155, 156]. However, TNF-activated pathways independent of NF κ B may also decide the cell fate. TRAF2 possesses a domain that acts as an E3 ubiquitin ligase, and has been demonstrated to ubiquitinate RIP1 at lysine 63 [479]. This polyubiquitination of RIP1 leads to activation of NF κ B and inhibition of apoptosis. In the absence of RIP1 ubiquitination, RIP1 will interact with caspase-8 acting as a scaffold for DISC leading to activation and apoptosis, an event independent of NF κ B activation [479].

Despite the lack of a functional death domain, TNFR2 has been shown to contribute to apoptosis [157, 158]. Overexpression of TNFR2 in HeLa cells, which do not normally express TNFR2, led to TNF-induced apoptosis [159]. TNFR2 has been shown to activate c-jun NH2 terminal kinase (JNK) in HeLa and contribute to TNF-induced cell death, which was reduced when TNFR2 was blocked [160]. TNFR2 may also compete with TNFR1 for TRAF2 and IAP through recruitment during signaling rendering the cell more susceptible to apoptosis as the survival pathway is not being activated in TNFR1 [161].

This link between TNFR2 and apoptosis may be relevant to non-leukocytic expression of TNFR2 being associated with the pathology of several diseases. In

Pseudomonas exotoxin A-induced hepatitis, a model of T-cell-dependent hepatitis, mice that were irradiated and reconstituted with either WT or TNFR2^{-/-} bone marrow showed that a loss of TNFR2 on parenchymal cells but not bone-marrow-derived cells reduced the severity of hepatitis, despite an accumulation of TNFR2⁺ leukocytes. This suggests that TNFR2 expression elsewhere can contribute to inflammation. *In vitro*, TNFR2^{-/-} hepatocytes were resistant to apoptosis induced by antagonistic TNFR1 and TNFR2 antibodies [126], suggesting that TNFR2 can contribute to hepatocyte apoptosis and thus liver damage in this model. There is evidence to suggest that an alternatively spliced form of TNFR2 is expressed intracellularly that co-localizes with endogenous TNF in human cell lines [162]. This was proposed to protect TNF-expressing cells from apoptosis. TNFR2 was shown to be expressed in intestinal epithelial cells [125] during inflammation suggesting that this mechanism could potentially occur in the gut, although this has yet to be demonstrated. Other studies show induced expression of TNFR2 during inflammation [163].

TNF and Colitis

TNF is thought to play an important role in the pathogenesis of IBD, since TNF antagonization is a major route of therapy. This is supported by evidence that TNF is elevated in the plasma and mucosa in IBD [164-166], as are the levels of soluble TNF receptors [167, 168]. Paradoxically, soluble receptor levels are directly correlated with disease activity in IBD [168], although it is plausible that receptor cleavage is a method of limiting the inflammatory response [169]. However, despite the general consensus that TNF is pathogenic in IBD, there have been studies that have failed to detect elevation of TNF levels in the plasma [170] or the mucosa [171]. Whether elevated TNF is a product of inflammation or involved in the initiation of inflammation is not clear, although one study reported that experimental TNF overexpression resulted in spontaneous colitis in mice [121] that resembled the Th1 profile observed in CD [172]. Otherwise, experimental examination of the role of TNF in IBD has been made possible using antibodies that antagonize soluble and membrane-bound TNF, and using various genetic knockout models for both TNF and TNFRs.

Anti-TNF Therapy

Anti-TNF antibodies, such as Infliximab, Adalimumab and Certolizumab, may go

beyond simple TNF neutralization. The mechanisms by which Infliximab exerts its anti-colitic effects are not clear. It has been demonstrated that administration of anti-TNF antibodies leads to T-cell or leukocyte apoptosis [173]. In patients with steroid refractory CD, Infliximab was shown to induce lymphocyte apoptosis, specifically in activated T-cells [174] and mucosal T-cell apoptosis in CD patients [175]. It has been suggested that the ability of Infliximab to induce apoptosis in lymphocytes correlates to the reduction in disease activity in CD [176]. This cytotoxicity has been recapitulated in severe-combined immunodeficient (SCID) mice reconstituted with human THP-1 monocyte-like cells and Jurkat cells and then treated with Infliximab showed depletion of the THP-1 and Jurkat cells [173]. In the Samp1yt+ murine model of ileitis, anti-TNF treatment was associated with an increase in apoptosis of lamina propria mononuclear cell [177], attributing efficacy to specifically depleting mucosal effector cells. *In vitro* treatment of cells with Infliximab induced cell death which was abrogated by treatment with a caspase inhibitor, attributing cell death to apoptosis [173]. These studies support a model whereby anti-TNF has an indirect mechanism as an anti-inflammatory by inducing apoptosis of surface-TNF-bearing cells.

On the contrary, there are reports suggesting that outside-in signaling and subsequent apoptosis were not attributed to Infliximab. One paper in particular found no effect of Infliximab on monocyte apoptosis after Annexin V staining but did show that treatment of monocytes with Infliximab resulted in reduced production of TNF after stimulation [178]. Another study reported that while Infliximab and Adalimumab did induce complement and antibody-mediated cytotoxicity, Certolizumab, an anti-TNF PEGulated Fab' fragment effective in reducing IBD symptoms [179], did not share these properties [180]. It would seem there are multiple mechanisms responsible for the efficacy of anti-TNF therapy.

Etanercept, a soluble TNFR2 chimeric molecule that antagonizes TNF has not proven to be effective in IBD [181]. Etanercept, compared to Infliximab, was ineffective at inducing apoptosis in mucosal T-cells compared to Infliximab [175]. However, in rheumatoid arthritis, Infliximab and Etanercept both induced apoptosis in monocytes and macrophages from synovial biopsies but not lymphocytes [182]. It is known that while Etanercept can bind transmembrane TNF in its trimer form, unlike Infliximab, it cannot bind transmembrane TNF in its monomer form [183]. In addition, Infliximab was shown

to form more stable complexes with transmembrane TNF in transfected cells compared to Etanercept, and bind with more avidity [183]. This may facilitate outside-in signaling. Both Etanercept and Infliximab were shown to induce E-selectin expression in Jurkat cells stably-transfected with TNF. However, Infliximab, but not Etanercept, induced IL-10 expression by the transfected cells. Similarly, Infliximab, but not Etanercept, induced apoptosis and G0/G1 cell cycle arrest in these cells, which was associated with JNK phosphorylation [184]. This could act as a potential self-limiting response in mTNF-expressing cells but has yet to be confirmed *in vivo*. It has also been suggested that anti-TNF treatment could decrease IEC apoptosis [185], potentially aiding in healing from active disease, or by reducing bacterial translocation and access to underlying leukocytes.

A recent paper reported that three anti-TNF antibodies, Infliximab, Adalimumab, and to a limited extent, Certolizumab, effectively induced apoptosis in CD14+ peripheral blood monocytes but not CD4+ T-cells [186]. Co-culture of CD4+ lymphocytes with CD14+ monocytes resulted in significant apoptosis in UC CD4+ T-cells after *in vitro* Infliximab treatment. This suggests that Infliximab induces apoptosis indirectly through monocytes, which were found to express high levels of membrane-bound TNF [186]. CD4+ T-cells isolated from the lamina propria had increased expression of TNFR2, and a corresponding expression of TRAF2 and NFκB. Application of a neutralizing anti-TNFR2 antibody mimicked the apoptosis observed by the treatment of co-cultures with anti-TNF antibody [186], suggesting that TNFR2 is a mediator by which inflammation is exacerbated in IBD, and by targeting membrane-bound TNF with anti-TNF antibodies or knocking out TNFR2 within the CD4+ population, inflammation is effectively reduced.

Anti-TNF treatment reduced IL-6 production in the mucosa of IBD patients and blocking the IL-6 receptor along with anti-TNF treatment significantly increased the number of apoptotic CD4+ T-cells in the mucosa [186]. This paper is of particular significance because it highlights an indirect role in inducing T-cell apoptosis by anti-TNF therapy as opposed to direct activation of apoptosis cited previously [173-175]. This effectively reduces the number of activated T-cells during the inflammatory response. In addition, the efficacy of anti-TNF therapy might not be a function of outside-in signaling through membrane-bound TNF on T-cells, but a function of membrane-bound TNF on antigen-presenting cells interacting with TNFR2 on activated T-cells. More research is needed to further clarify the role of TNFR2 in colitis, as only a few studies have

addressed this.

In experimental colitis, data from anti-TNF treatment is equally conflicting. As in human studies, TNF upregulation in the colon and plasma is not consistently found [187]. While exacerbation of DSS colitis by anti-TNF antibodies has been reported [188], others have demonstrated a reduction in experimental colitis [189, 190]. Others yet have reported no effect of a neutralizing TNF antiserum on DSS colitis in mice [191]. As the conditions of each of these studies vary (different strains of mice, and doses/duration of DSS, polyclonal versus monoclonal antibodies) it is possible that there may be more than one underlying mechanism accounting for these conflicting outcomes. Blocking TNF using a small-purine-based molecule that inhibited the transcription of TNF did not decrease acute or chronic inflammation in mice treated with 3% DSS for seven days, despite the fact that it reduced TNF expression in the colon [192]. Overall, this supports the theory that anti-TNF treatment limits the inflammatory response by depleting effector cells rather than by neutralization of soluble TNF.

There are other potential mechanisms by which blocking TNF may reduce inflammation, in short, by acting as an anti-inflammatory. Anti-TNF therapy can reduce CD40/CD40L expression [193], and reduce TNF-mediated NO release, [194]. Infliximab may reduce pathogenic angiogenesis in CD [195], but at the same time promote effective angiogenesis by reducing endothelial dysfunction [196]. Infliximab use *in vivo* and *in vitro* was shown to induce regulatory macrophages, which could help in the resolution of inflammation [197]. These mechanisms outline some of the many actions that anti-TNF therapy has in reducing inflammation in IBD.

TNF in Acute and Chronic Models

The great number of research papers dedicated to unraveling the role of TNF in intestinal inflammation highlights the importance of this mediator. It has become evident that TNF could have a dual role in the inflammatory reaction during DSS colitis. TNF might exert a different action during acute colitis compared to chronic colitis. An early report demonstrated that during acute DSS colitis, TNF neutralization exacerbated colitis yet while during chronic colitis, TNF neutralization ameliorated colitis [188]. Additionally, TNF-deficient mice have an exacerbated and fatal response to acute DSS colitis [198]. During the acute phase of DSS-colitis there is an infiltration of leukocytes,

a T- and B-cell independent event [199]. Supporting a protective role for TNF during the acute phase of colitis, RAG^{-/-}TNFR1^{-/-} double-deficient mice, a strain lacking lymphocytes and TNFR1 in remaining myeloid-derived cells showed extensive mortality and disease severity compared to RAG^{-/-} controls and RAG^{-/-}TNFR2^{-/-} mice when treated with DSS [200]. In fact, RAG^{-/-}TNFR2^{-/-} mice were protected compared to RAG^{-/-} mice after DSS treatment, and this protection was found to be dependent on the expression of TNFR1. Bacterial translocation into distant organs such as the spleen and liver was increased in RAG^{-/-}TNFR1^{-/-} and triple knockout mice (RAG^{-/-}TNFR1^{-/-}TNFR2^{-/-}), suggesting a role for TNF receptors in preventing bacterial translocation or antibacterial immunity. When RAG^{-/-}TNFR1^{-/-} mice were irradiated and reconstituted with RAG^{-/-}TNFR1^{+/+} bone marrow, intestinal inflammation was attenuated. In addition, the presence of TNFR1 was associated with restitution of the colonic epithelium with increased BrDU uptake, and activation factors such as AKT expression [200]. Therefore, expression of TNFR1 on innate immune cells seemed to provide a protective effect in DSS-colitis.

TNF action via TNFR1 expression on IEC has also been proposed as a mechanism by which TNF can be protective in colitis. It has been proposed to support barrier function and prevent bacterial translocation. A mouse strain deficient in an intestinal epithelial cell-specific signaling molecule, TAK1, activated by TNF along the RIP-MEKK3 route, develops spontaneous inflammation and death shortly after birth. In the absence of TAK1, TNF signaling resulted in increased epithelial cell apoptosis. A double-deficient strain of TNFR1^{-/-}TAK1^{-/-} mice showed delayed onset of spontaneous inflammation associated with a reduction in intestinal epithelial apoptosis [156]. Deletion of intestinal epithelial NFκB essential modulator (NEMO), which forms a complex with IKKα and IKKβ was reported to sensitize these cells to TNF-mediated apoptosis, and leads to spontaneous intestinal inflammation and bacterial translocation [155]. *In vitro*, TNF has been shown to influence the uptake of protein antigens, but not affect transepithelial resistance in polarized T84 cells [201], whereas a decrease in transepithelial resistance has been attributed to TNF in T84 colon cancer cells reported elsewhere [202]. The above studies describing the action of TNF signaling early in intestinal inflammation suggest at least two mechanisms; that TNF is protective against inflammation by promoting innate antimicrobial responses, such as activating and mobilizing neutrophils and macrophages, and by contributing to barrier function of the

intestinal epithelium by promoting survival of the epithelial cells.

TNF in TNBS Colitis

Studies examining the role of TNF in TNBS-induced colitis have also yielded conflicting results. While TNF-deficient mice are extremely susceptible to DSS colitis [198], they are not as susceptible to TNBS [203]. In fact, TNFR1^{-/-}TNFR2^{-/-} double-deficient mice are also protected against TNBS colitis [204, 205]. TNBS-mediated colitis, when used with a sensitizing dose, is a delayed-type hypersensitivity (DTH) response, although as discussed, TNBS intracolonic can lead to a strong acute inflammatory response on first antigen exposure [88], confounding their interpretation. DSS and TNBS colitis have been reported to produce a very different gene expression profile during inflammation, at least on a BALB/c background [187] and so more work needs to be done to identify which elements of the response are modulated by TNF.

The involvement of TNFR1 and TNFR2 has also been investigated but animal studies have failed to yield a definitive role for TNF receptors in protection or damage in acute colitis. It was reported that TNFR1^{-/-} mice were more susceptible to colitis induced by TNBS, while TNFR2^{-/-} mice were less susceptible compared to WT mice [206]. In contrast, Nakai et al., reported that tissue damage was significantly reduced in TNFR1^{-/-} mice, associated with a reduced level of activated NFκB [204]. In agreement, blockade of TNFR1 attenuated TNBS colitis [207]. Interestingly in the study by Nakai, the concentration of TNBS administered was much higher at 6% compared to 1.75% by Ebach et al. In addition, in the study by Nakai, mice were killed at day 6 post-TNBS administration while in the other study they were killed at days 2 and 5. The different doses of TNBS might have an impact on the type of immunity activated during TNBS sensitization. While the volumes and concentrations of ethanol remained the same between the studies, the different results suggest different mechanisms of inflammation.

TNFR2 in Colitis

A pro-inflammatory effect in the colon has been also attributed to TNFR2. It is reportedly expressed in the colonic epithelium from inflamed mucosa in CD patients, but not in unaffected mucosa, suggesting that inflammation can trigger the expression of TNFR2 [125]. To further define a role for TNFR2 in the gut, it has been shown in vitro that IFN-γ can prime the human colon carcinoma cell line Caco-2 cells to express both

TNFR1 and TNFR2 however, TNFR2 expression by Caco-2 cells was associated with a loss of barrier function after TNF treatment. This loss of barrier function was associated with a disruption in tight junctions (measured by the expression of occludin and zonulin-1). In addition, TNFR2 signaling led to an increase in myosin light chain (MLC) phosphorylation and MLC kinase expression, demonstrated to contribute to the loss of barrier function by disrupting tight-junction-associated proteins [208]. Whether TNFR2-mediated barrier disruption significantly contributes to colitis *in vivo* remains to be determined.

TNFR2 is expressed on lamina propria and peripheral blood CD4⁺ T-cells from patients with CD compared to controls. Furthermore, adoptive transfer of CD4⁺CD62L⁺ T-cells that constitutively expressed TNFR2 led to a more severe colitis than adoptive transfer of WT cells [209]. Combined with this evidence for T-cell expression of TNFR2 associated with inflammation is evidence that TNFR2 can act as a costimulatory molecule for T-cells, particularly during clonal expansion during a primary T-cell response [210]. However, more recent evidence suggests that TNFR2 may be more important in regulatory T-cell function [211-213]. CD4⁺ T-cell deficiency of TNFR2 in cells reconstituted into RAG^{-/-} mice resulted in a more severe CD4⁺CD45RB^{hi} adoptive transfer model of colitis. Stromal TNFR2 did not influence the outcome of colitis in RAG^{-/-}TNFR2^{-/-} mice reconstituted with WT cells [214]. Studies examining the selective TNFR2 deficiency as opposed to whole animal deficiency, such as those by Ebach et al [206] can provide insights into the function of TNFR2 that contribute to more specific treatments for IBD. However, the conflicting roles reported for both receptors confound conclusions that can be made regarding the efficacy of selective targeting. Thus far, anti-TNF treatment appears to be successful regardless of specific receptor involvement in IBD.

TNF and Subepithelial Myofibroblasts

Along with both epithelial and bone-marrow-derived involvement of TNF in the development of colitis, there is a mesenchymal cell involvement as well. Intestinal myofibroblasts that overexpress TNF have greater MMP9 activity and expression [215]. This implicates intestinal myofibroblasts as targets for the action of TNF in the development of inflammation. In addition, mice that overexpress TNFR1 specifically within cells of mesenchymal origin, specifically the intestinal myofibroblasts, develop a

Crohn's like inflammation [215]. Inhibition of TNF signaling by Infliximab treatment was shown to reduce myofibroblast migration and collagen deposition [216]. Furthermore, TNF-induced expression of COX-2 and subsequent prostaglandin production by myofibroblasts could contribute to inflammation in IBD [217]. This suggests that TNF activation of these cells through TNFR1 is theoretically sufficient to initiate an inflammatory response within the intestine. As colonic myofibroblasts are often located close to the basement membrane of the colonic crypts in proximity to intestinal stem cells, and are purported to play an important role in maintaining the "stem cell niche" [218], their role in inflammation and development of cancer may be crucial.

After examining the involvement of TNF in IBD and a number of animal models, TNF does not have a distinct singular role. Rather, its effects on many different aspects of immunity, combined with differences in genetic make-up and initiating events may predict its effect on inflammation. In contrast, there is a consensus that TNF appears to be an important mediator in tumorigenesis.

TNF and Cancer

TNF was originally discovered as the mediator of endotoxin-induced tumor necrosis, hence its name [219]. Other studies that examined the effects of using anti-TNF antibodies in both rodents [220] and humans suggest that TNF may play a role in anti-cancer immunity, as cancers such as lymphoma [221, 222] and Kaposi's sarcoma [223, 224] have been associated with anti-TNF therapy. In contrast, other clinical studies failed to find an association with anti-TNF therapy with lymphoma [225-227]. This leads to the question of whether lymphoma could be a result of immunosuppression in general or targeted depletion of TNF-expressing lymphocytes that may have cytotoxic properties targeted at cancerous cells. It could reflect the age of the populations being studied; it is possible that earlier and longer-term treatment with anti-TNF therapies could increase the risk of lymphoma. This becomes of particular concern with anti-TNF therapy being used in the pediatric population [222, 228].

Many earlier studies examined TNF as a possible solid tumor therapy [229-231]. This strategy generally failed because systemic TNF treatment is extremely toxic [230, 232]. Some studies successfully showed tumor regression after intra-tumoral treatment via TNF-expressing adenovirus [233, 234]. Interestingly, one study found that intra-

tumoral injection of TNF-expressing adenovirus not only led to tumor shrinkage and disappearance, but also elicited a memory response against further application of tumor cells in mice [234]. Adenovirus targeting the tumor vasculature is another avenue by which TNF could function as a potential therapy, as TNF demonstrated angiostatic properties when injected within fusogenic liposomes into the femoral artery [235] and when administered via an adenoviral vector [236]. Together, these studies have found that TNF can be used as a potential adjunct therapy to aid in the shrinkage of tumors.

Although TNF therapy showed promising results in solid tumors, during the mid-2000's it became evident that TNF might actually play more of a role in promoting tumor growth when applied systemically. By blocking TNF *in vivo* or by using TNF-deficient cell lines or mouse strains it has been found that TNF plays a critical role in many cancer types and models [237, 238]. Anti-TNF therapies have been evaluated in phase II clinical trials for treatment of cancers with mixed success [239-241]. In renal cell carcinoma, it aided in stabilizing disease progression [239]. In breast cancer, Etanercept did not demonstrate a particular therapeutic effect within the studied parameters [241], and Infliximab may inhibit bone metastasis by breast cancer cells via a reduction in CXCR4 [242]. Whether this is biologically significant remains unknown. In ovarian cancer, there is some evidence that Etanercept may lead to disease stabilization, however, more study is required [241]. It appears that anti-TNF therapy may not be the magic bullet for cancer treatment as it appeared in several animal models [243]; however, it is possible that it may provide supportive therapy in combination with other drugs.

In animal models of cancer, blocking TNF or using TNF or TNFR deficient animals typically reduces cancer [163, 237, 238]. TNFR1, but not TNFR2 was shown to be involved in a reduction in hepatic neoplasia [237]. Applying a phorbol ester to the skin of wildtype and TNF-deficient mice showed that TNF-deficient mice were protected from skin tumor development [238]. TNFR1^{-/-} and TNFR2^{-/-} mice are less susceptible to 9,10-dimethyl-1,2-benzanthracene and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced skin tumors compared to WT mice [163]. TNFR1^{-/-} mice were the most resistant with the difference possibly attributed to differential expression of the receptors in the skin. Whereas TNFR1 expression was constitutive, TNFR2 mRNA was induced after TPA treatment in the epidermis. WT mice also had increased expression of activated c-jun after 24 hours of treatment compared to receptor knockout mice suggesting that TNF-

mediated tumorigenesis may occur via the AP-1 pathway. Interestingly, TNFR1 and TNFR2 differentially induced AP-1-responsive genes, granulocyte/macrophage colony-stimulating factor (GM-CSF) and matrix metalloproteinase-3 (MMP-3), with TNFR2^{-/-} mice having a markedly reduced epidermal GM-CSF expression and both TNFR1^{-/-} and TNFR2^{-/-} mice demonstrating reduced MMP-3 expression [163]. These studies show that TNF inhibition may be an effective means of targeting several different cancers, with the main effects being attributed to TNFR1. TNF signaling through TNFR1 as it pertains to tumorigenesis will be discussed in the next section.

Mechanisms of Colitis-Associated Carcinogenesis

Epithelial Cell Turnover

Colon epithelial cells are normally rapidly dividing cells arising from stem cells near the base of the crypt [244]. Inflammation can cause damage to the epithelium, stimulating a reaction to replace the epithelium and accordingly increased epithelial cell turnover has been described in experimental colitis [245], with an increase in both colonic epithelial cell apoptosis [246, 247] and proliferation [248] in IBD. NFκB-induced expression of anti-apoptotic proteins within the colonic epithelium, and cytokines from infiltrating cells, are associated with CAC [249]. Deletion of RelA, the gene encoding the p65 subunit of NFκB, specifically in intestinal epithelial cells, resulted in increased epithelial apoptosis and a low-grade spontaneous colonic inflammation [250], suggesting that an imbalance between proliferation and apoptosis might contribute to inflammation susceptibility. While NF-κB may be protective during acute inflammation in preserving the epithelium and maintaining barrier function, this may be detrimental in chronic inflammation. The administration of an anti-proliferative drug taurolidine did not affect the outcome of CAC in mice [251], suggesting that in addition to increased colonic epithelial turnover, other signals are required for progression to cancer, emphasizing the importance of cytokines produced during inflammation acting as growth factors for the regenerating epithelium.

Inflammation Activates Tumorigenic Pathways within Intestinal Epithelial Cells

During epithelial regeneration after injury there is an activation of signaling

pathways within the colonic epithelial cells that are associated with the development of cancer. Infiltrating cells produce cytokines, chemokines and lipid mediators that coordinate cellular efforts to prevent infection. These mediators are known to induce signaling that is involved in carcinogenesis. This section will discuss these mediators with an emphasis on what is currently known about the role of TNF in CAC. As TNF is reportedly upregulated in the colonic mucosa during inflammation [252], and is a major target in IBD treatment, it follows that it might play a substantial role in the development of colorectal cancer after prolonged periods of inflammation. TNF is a major activator of the NF κ B pathway, and several studies suggest this may be the main mechanism of activation that leads to the development of cancer.

NF κ B activation appears to be critical in the development of colorectal cancer subsequent to repeated bouts of colitis [249]. Mice with a selective myeloid or IEC-IKK β deficiency were treated with AOM and three cycles of DSS to induce CAC. Intestinal IKK β deficiency resulted in reduced tumor numbers and incidence without affecting inflammation severity. Meanwhile, myeloid-specific deletion of IKK β resulted in smaller tumors. Epithelial-specific IKK β deletion was associated with increased epithelial cell apoptosis while cytokines produced by myeloid-derived cell act as growth factors for tumors during inflammation [249]. While inflammation and cancer are related, there may be specific molecular events during an inflammatory response that lead to tumorigenesis. In addition, inflammatory signals within the epithelial cells may contribute just as importantly as those derived from infiltrating leukocytes.

One obvious mechanism of NF κ B activation in intestinal epithelial cells during active inflammation is through the presence of TNF in the inflammatory milieu; however TNF-deficient mice had similar NF κ B DNA-binding activity in the colon as WT mice after acute DSS-induced colitis [198], suggesting that other factors present during colonic inflammation can activate NF κ B in place of TNF. This raises the question whether TNF-mediated activation of NF κ B is the most important pathway of colonic epithelial cell protection in an acute response, as TNF deficiency is associated with high mortality during acute DSS colitis, or if tumorigenesis via TNF signaling occurs via the NF κ B pathway.

In contrast, treatment with an anti-TNF antibody reduced the colitis-associated tumor development in mice treated with AOM and DSS with no difference in

colitis severity after three cycles of DSS. Antibody treatment significantly reduced nuclear translocation of p65 in colonic tissue from treated mice, suggesting that blocking TNF can reduce NF κ B signaling without affecting inflammation [252]. Whether NF κ B nuclear translocation was reduced *in vivo* as a direct result of TNF signaling or an indirect effect on other inflammatory measures was not clear. TNFR1 expression directly on epithelial cells was not shown. Nevertheless, one mechanism by which TNF might contribute to cancer is through activation of NF κ B. The lack of difference in acute DSS colitis in TNF-deficient mice could be due to a complete TNF deficiency and a susceptibility to infection leading to a hyperinflammatory response [198]. This could mask any effect of TNF on NF κ B expression within the epithelium.

TNF signaling through TNFR1 may promote carcinogenesis through other mechanisms. The development of CAC was evaluated in BALB/c mice treated with AOM and DSS for three cycles. TNFR1^{-/-} mice had reduced severity of inflammation and reduced tumor development compared to WT mice. The culprit appeared to be cells of hematopoietic origin as bone marrow chimeras made from WT mice reconstituted with TNFR1^{-/-} bone marrow had a reduced tumor load compared to TNFR1^{-/-} mice reconstituted with WT bone marrow and WT mice reconstituted with WT bone marrow [243]. Therefore, in this specific model of colitis-mediated cancer, hematopoietic cells are the main contributors to TNF-specific inflammation-mediated cancer, as opposed to the activation of NF κ B via TNFR1 within epithelial cells [249]. TNFR1^{-/-} mice had a reduced infiltration of neutrophils, and this was associated with a decreased expression of CCL2 and CXCL1 in the colon measured by RT-PCR [243]. Despite reports that TNFR1 is expressed on epithelial cells, this study did not find TNFR1 protein in the epithelium via immunohistochemistry, and TNFR1 expression was limited to lamina propria cells [243]. This would support why TNFR1 expression within bone-marrow derived cells is more important in colitis mediated cancer.

The activation of NF κ B by TNF leads to increased expression of the enzyme COX-2, which is involved in the production of lipid mediators prostaglandins and leukotrienes. COX-2 is well-established as a contributor to CRC [253, 254]. Prostaglandins also activate signaling pathways within the cell that promote survival. Prostaglandin E₂ (PGE₂) has been shown to activate β -catenin signaling and subsequent activation of cyclinD1 and c-MYC, both considered important in tumorigenesis [255].

Accordingly, Joseph et al. showed that PGE₂ addition to detached intestinal epithelial cells could delay detachment-induced cell death *in vitro* [256]. Deletion of PGE₂ synthase, the last step in PGE₂ production, reduces development of tumors in *Apc*^{min+/-} mice. This is associated with a reduced accumulation of nuclear β-catenin in dysplastic precursor lesions, showing an *in vivo* effect of PGE₂ [257]. It should be noted that *Apc*^{min+/-} mice already have an increased nuclear translocation of β-catenin due to a mutated *Apc*, and prostaglandins further propagate this translocation by signaling through the EP2 G-protein coupled receptor, leading to the inhibition of glycogen synthase kinase 3β, which normally inhibits β-catenin translocation to the nucleus by phosphorylating it, enabling its ubiquitination and subsequent destruction [255]. Colonic epithelial cells could be a source of prostaglandins, but both infiltrating leukocytes [243], and subepithelial myofibroblasts [217] may be a more significant source during CAC. COX-2^{-/-} mice are not any more susceptible to CAC when compared to WT [258], but it is not known if cell selective deletion of COX-2 would have a different outcome in CAC. It was shown that epithelial loss of COX-2 was of no consequence in DSS-colitis, however, loss of COX-2 in endothelial cells and myeloid-derived cells exacerbated colitis, suggesting further that these cells are important in the protective effects of prostaglandins on the epithelium in acute colitis [259] and prostaglandins derived from these sources could promote tumorigenesis in CAC.

TNF-mediated PI3K/AKT activation may be involved in CAC. PI3K/AKT, a serine-threonine kinase, is involved in cell proliferation and protection from apoptosis and can activate NFκB [147]. AKT phosphorylation is detectable in UC biopsies [260], and it has been reported that wortmannin, a PI3K inhibitor, reduced colitis in DSS-treated mice [260]. In a study of *C. rodentium* infectious colitis, inhibition of PI3K signaling using wortmannin resulted in reduced phosphorylation of β-catenin at Ser552, leading to reduced epithelial cell proliferation [261]. TNF is known to activate the PI3K/AKT pathway [147]. While PI3K inhibition led to a susceptibility to *C. rodentium* infection, these results have implications that it is possible that TNF could lead to β-catenin signaling and thus another mechanism by which it could contribute to CAC.

Cytokines and chemokines may play an important role in the development of CAC due to their abilities to attract leukocytes and stimulate angiogenesis. Recently, Popivanova et al reported that CCL2, a major macrophage-attracting chemokine, was

crucial for the colonic infiltration of COX-2-expressing macrophages and blocking CCL2 resulted in reduced cancer and inflammatory indices and angiogenesis after AOM+DSS treatment [262].

In addition to TNF, IL-6 is another example of the impact of cytokines in CAC. IL-6 signaling through IL-6 receptor α /gp130 activating the signal transducer and activator of transcription (STAT) 3 pathway has recently garnered attention in CAC. It has previously been shown that suppression of TGF- β -mediated IL-6 was shown to prevent colon tumor growth via STAT3 [263], outlining the importance of this pathway in cancer. IL-6-deficient mice demonstrate a reduced number of tumors per mouse after AOM+DSS treatment [264]. This was associated with increased survival and proliferation markers in WT cells compared to IL-6-deficient colonic epithelial cells [264]. IL-6 deficiency specifically in hematopoietic cells, especially dendritic cells and CD11b⁺ macrophages, led to a decrease in colonic epithelial STAT3 activation. Furthermore, conditional STAT3 knockout in enterocytes reduced the levels of CAC [264]. STAT3 signaling is associated with many different stages in the progression of cancer and can promote tumorigenesis by influencing proliferation and survival of epithelial cells, and it can also perpetuate inflammatory signals by further promoting the production of cytokines [265].

IL-6 α , only expressed on a limited variety of cells, can be cleaved to become a soluble receptor. The gp130, which is widely expressed, can bind this IL-6/IL-6 α and induce STAT3 signaling known as IL-6 trans signaling. This may be important in the development of colitis in Sampliyit mice [266]. BALB/c mice treated with an antagonist to gp130 had a lower incidence and number of colitis-associated tumors, and this was associated with a decreased activation of epithelial STAT3 [267]. STAT3 signaling may also contribute to the expression of a DNA methyltransferase gene and subsequent methylation activity in colon cancer cells, which could have implications in gene silencing [268]. Other players in the IL-6/STAT3 signaling pathway that have been evaluated for their role in CAC include suppressor of cytokine signaling (SOCS) 3, where overexpression of SOCS3 inhibited the IL-6/STAT3 pathway, and the NF κ B pathway and reduced CAC in a mouse model of AOM+DSS-induced cancer [269]. As IL-6/STAT3 signaling has been deemed a major player in CAC, the relationship to TNF, already targeted in IBD, may be of importance. It has been demonstrated that TNFR1 co-

precipitated with Jak2, suggesting that TNF may lead to Jak/STAT signaling [270].

Inflammatory Mediators can Promote Angiogenesis

Angiogenesis is the formation of blood vessels within the tissue and is a normal physiological process that is tightly regulated. After tissue injury, angiogenesis occurs to re-establish blood flow to the injured tissue. When it occurs during chronic inflammation it may lead to blood vessels that are more permeable, disorganized, and activated with increased potential to further recruit inflammatory cells [271]. Consequently, angiogenesis is a feature of IBD [272, 273], and measures taken to reduce it have resulted in attenuation of inflammation [195]. Angiogenesis is also an important feature of tumor development as blood vessels are important for nutrient delivery for tumor growth and survival [271]. During inflammation-mediated tumorigenesis, increased angiogenesis may promote tumor growth by providing larger quantities of oxygen and nutrients to sites of tissue repair and regeneration. In addition, blood-derived immune cells, such as neutrophils and monocytes, gain access to regenerating tissue, where the release of more growth factors, cytokines and chemokines can further promote tumorigenesis. Infiltrating leukocytes, in particular neutrophils, may play a major role in angiogenesis. As described, they are known to be a source of COX-2 and prostaglandins within the inflamed colon [243]. Neutrophils are also a significant source of matrix metalloproteases, such as MMP-9.

Matrix metalloprotease-9 (MMP-9) is an important neutrophil-derived enzyme involved in angiogenesis. This promotes the “angiogenic switch” and triggers angiogenesis by liberating and activating the angiogenic factor vascular endothelial growth factor (VEGF) [274-276] that is constitutively expressed [277]. Neutrophil proMMP-9 is uniquely free of inhibitors of metalloproteinases (TIMP), leading to efficient proangiogenic action of zymogen proMMP-9, whereas, MMP-9 sources from other immune cells such as macrophages, do not have the same level of activity due to the presence of TIMP [278]. The proMMP-9 angiogenic switch is thought to involve both VEGF and basic fibroblast growth factor (FGF)2 [279].

Human umbilical cord endothelial cells (HUVEC) cultured in medium conditioned by neutrophils exposed to hepatocarcinoma cells readily formed tubes compared to non-tumor-exposed neutrophil-conditioned medium, and this tube formation

was abrogated by a blocking antibody to MMP-9 [276]. In a multi-stage transgenic model of insulinoma, where mice have simian-virus-40 Tag oncoproteins expressed under the control of the rat insulin promoter, neutrophils were shown to be instrumental in the earlier stage “angiogenic switch” associated with MMP-9 expressing neutrophils within the islets, whereas MMP-9-expressing macrophages remained outside the tumor. Gr-1-mediated neutrophil depletion led to a decrease in angiogenic switch within dysplastic regions [275]. Together, these studies highlight a role for leukocytes in promoting cancer growth through activation of angiogenesis.

TNF has been demonstrated to induce the expression of MMP-9 [280, 281]. Epithelial MMP-9 contributes to the severity of experimental colitis versus leukocyte MMP-9, which did not [282]. In addition, MMP-9^{-/-} mice are protected against CAC [283]. This was attributed to MMP-9 cleavage of Notch1, a transcription factor that prevents colonic stem cell differentiation within the crypt [284]. This study did not differentiate between stromal or bone-marrow-derived expression of MMP-9, sourcing the MMP-9 to either neutrophils or the colon stroma. TNF could potentially induce epithelial MMP-9 expression, leading to Notch1 activation, providing another mechanism that TNF can contribute to, leading to carcinogenesis within the intestine.

TNF has been implicated in angiogenesis during tumor formation. Etanercept was shown to be effective at reducing tumor size in the AOM+DSS model of CAC, even in established tumors, and was associated with fewer blood vessels within tumors [243]. In bladder cancer, tumor TNF levels correlate positively with microvascular density [285]. Reducing angiogenesis by TNF blockade could be another mechanism by which anti-TNF therapy is effective [193, 195], even in the absence of inflammation resolution.

In contrast, TNF has been shown to induce tumor necrosis in mice bearing fibrosarcomas, not through direct tumoricidal effects as might be expected, but through the influence on the tumor vasculature. WT and TNFR1^{-/-} mice used to make chimeras and bearing fibrosarcomas were treated with recombinant mouse TNF. In WT mice reconstituted with WT bone marrow there was significant tumor necrosis, but reconstitution of TNFR1^{-/-} mice with WT bone marrow inhibited tumor necrosis, suggesting that expression of TNFR1 on hematopoietic cells was not involved in tumor necrosis. TNF did not directly cause necrosis in these tumor cells. This led the authors to conclude that it was TNFR1 expression on endothelial cells that are the target causing

tumor necrosis, however, this was not directly shown [286].

TNF has been shown to contribute to angiogenesis in a TNFR2-dependent manner. Specifically, TNFR2 signaling in endothelial cells can cause activation of endothelial/epithelial tyrosine kinase (Etk), which is involved in cell migration, proliferation and survival. Etk could be a target for TNF-mediated angiogenesis. Specifically, Etk can form a complex independent of TRAF2 with TNFR2, meaning signaling through TNFR2 does not necessarily lead to NF κ B activation. Endothelial cells transfected with a constitutively active form of Etk showed increased cell migration and blood vessel formation when treated with TNF compared to cells transfected with a dominant-negative form of Etk [287]. This suggests that blocking TNF can inhibit angiogenesis through multiple mechanisms, but neutralizing TNF directly, and preventing membrane-bound TNF from interacting with TNFR2 may be effective.

Inflammation can Directly Damage Tissues through Reactive Oxygen Species Production

IBD is associated with a high level of oxidative stress, defined as an imbalance between the level of oxygen radicals produced and the enzymes and antioxidants available to neutralize them [288-292]. Oxidative stress can damage cellular components including proteins, lipids and DNA via the formation of reactive oxygen species (ROS). Most frequently, the most DNA-damaging ROS is the hydroxyl radical [293]. ROS can be produced via a number of pathways. First, there is oxidative phosphorylation, where ROS can be released from mitochondria during metabolic processes. The contribution of mitochondrial ROS to inflammatory-related oxidative stress is not clear. Inhibitors of the respiratory electron chain, such as rotenone, do appear to have some effect on reducing inflammation in the gut, specifically in ischemia-reperfusion injury [294]. Other sources include xanthine oxidases, involved in purine metabolism, lipoxygenases and cytochrome P450, involved in drug metabolism. Xanthine oxidase activity does not appear to be increased during UC [295]. Allopurinol, a xanthine oxidase inhibitor, is sometimes prescribed during IBD as an adjunct prophylactic drug for hyperuricemia that can occur with certain drugs used in IBD therapy such as 6-mercaptopurine [296] and is therefore not a major mode of reducing inflammation in itself. Inducible nitric oxide synthase (iNOS) is another significant source of ROS, leading to the production of nitric

oxide (NO) that can be further transformed to peroxynitrite by reaction with superoxide radicals [297]. iNOS produces NO using the terminal nitrogen of L-arginine and molecular oxygen [297].

NADPH oxidase is an enzyme complex found in various cell types that catalyzes the transfer of electrons from NADPH to O₂, resulting in the superoxide anion (O⁻). This reaction is important for anti-microbial immunity. Lacking a functional NADPH oxidase enzyme complex in cells of the immune system can result in an immunocompromised phenotype manifesting as Chronic Granulomatous Disease in humans [298]. Those affected are unable to mount an effective anti-bacterial response particularly at mucosal surfaces and as a result develop frequent bacterial infections and granulomas [298]. The NADPH complex is comprised of several proteins; the membrane associated flavocytochrome b, which is composed of two proteins, gp91 and p22phox, and cytosolic proteins Rac2, p47phox, p67phox and p40phox. Several isoforms of NADPH oxidase exist with different functions in different tissues. For example, Nox1 is found primarily in intestinal epithelial cells and is thought to play a role in host defense and regulating inflammation. Nox2 is found primarily in neutrophils and macrophages, but can also be found in T-cells and NK cells.

Just as reduced levels of NADPH oxidase results in disease, overproduction of reactive oxygen species produced by Nox enzymes have been implicated in the pathology of several inflammatory disorders including atherosclerosis [299], and ischemia-stroke [300].

Reactive Oxygen Species and Colitis

As leukocyte NADPH oxidase is critical in protecting the host from opportunistic infections, its role in IBD, where there is mass leukocyte infiltration, warrants examination. While it may play a role in worsening already established disease by causing tissue damage and further activating the immune system, there is also a possibility that it could protect against the development of disease by preventing infection in the gut. Interestingly, two cases of a Crohn's-like colitis were reported in a form of chronic granulomatous disease where there was an autosomal-recessive p47phox deficiency [301]. Other studies suggest that inhibitors of ROS production, including superoxide dismutase (SOD), improve colitis in mice treated with 2.5% acetic acid to induce colitis. In this model of colitis in particular, xanthine oxidase pathway

inhibitors did not affect the production of ROS during colitis, suggesting that another source of ROS, such as NADPH oxidase, is the major contributor [302]. One of the strongest indicators that high oxidative stress is a potent contributor to colitis comes from a mouse strain deficient in 2 isoforms of glutathione peroxidase, *Gpx1* and *Gpx2* an enzyme which functions to convert hydrogen peroxide to water. These mice develop spontaneous colonic inflammation, suggesting that antioxidant enzymes play a homeostatic role in the colon and that IBD could potentially result as an imbalance between oxygen radicals and efforts to quench them [321].

Less is known about NADPH oxidase in intestinal inflammation. One study examining the role of NADPH oxidase found that *p47^{-/-}* mice developed inflammation similar to WT mice [303]. SOD transgenic mice overexpressing SOD, on the other hand had increased measures of colitis compared to WT, while *iNOS^{-/-}* mice had attenuated colitis. When iNOS was inhibited in *p47^{-/-}* mice, there was an enhanced protection against colitis. *p47* deficiency was associated with an increased NO production in LPS+IFN- γ -stimulated neutrophils, but $O_2\bullet$ production by *iNOS^{-/-}* neutrophils was not different from WT mice [303]. In contrast, a recent paper that described DSS-induced colitis in mice deficient in the main catalytic subunit of NADPH oxidase, *gp91*, demonstrated that *gp91^{-/-}* mice have a reduced clinical and histopathological outcome and reduced MPO activity and IL-6 expression in the colon compared to WT [304]. While *p47* is a component of the NADPH oxidase enzyme, and *p47* deficiency is associated with chronic granulomatous disease, *p47* has the potential to interact with other NADPH oxidase isoforms [305] potentially masking a true deficiency, which could help explain the differences between the two studies.

There is little evidence connecting Nox2-derived ROS and colitis mediated cancer, despite the role of ROS in cellular damage. ROS can induce lipid peroxidation and protein/DNA damage. Lipid peroxidation can lead to the formation of aldehydes, such as malondialdehyde, which can further damage DNA and protein. Protein damage can alter the function of the protein, and this can contribute to cancer if proteins involved in DNA repair or cell cycle control are affected. DNA damage by ROS can manifest in single/double strand breakage, amongst other changes. There are many different DNA adducts that form as a result of oxidative damage. If this damage is not repaired, it could lead to DNA mutations, replication errors, genomic instability and cell death.

TNF and NADPH Oxidase

TNF can directly cause mutations through oxidative damage via ROS [306]. TNF has the ability to act as a mutagen as powerful as ionizing radiation [306]. In addition, normal lung epithelial cells showed DNA damage after TNF treatment [307] and long-term TNF treatment was shown to directly induce immortality in cell lines [308]. A relationship between TNF signaling and ROS production was first described in 1986 by Klebanoff et al. where TNF alone was shown to slightly increase phagocytosis and the production of H₂O₂ by human neutrophils [309]. TNF may enhance ROS production when added with chemotactic peptides such as fMLP and phorbol esters [309, 310]. TNF has been reported to upregulate transcription of NADPH proteins through NFκB in human macrophages [311], indicating one mechanism by which TNF can induce ROS production. TNF-induced ROS production by BALB 3T3 and L929 cells was abrogated by rotenone treatment, which inhibits mitochondrial-derived ROS, suggesting that the mitochondria-derived ROS could be induced by TNF [306]. L929 fibrosarcoma cells were shown to express Nox1, a TNF-activated ROS-producing isoform of NADPH oxidase [312], indicating that there are several different avenues of TNF-mediated ROS production.

Neutrophils are one of the most abundant sources of ROS during inflammation [313]. TNF alone was shown to activate NADPH oxidase in human neutrophils when cells were adhered to matrix proteins fibronectin and fibrinogen, inducing conformational changes in the α_Lβ₂ integrin [314]. Two studies evaluated the role of TNFR1 and TNFR2 in TNF-induced respiratory burst in matrix-adherent human neutrophils. Using monoclonal antibodies, the first study showed that antagonizing TNFR1 with a monoclonal antibody resulted in ROS production. An antagonizing antibody to TNFR2 did not, so it was proposed that TNFR1 was the mediator of TNF-activated ROS production. The second study used a different mAb to antagonize TNFR2, and found that when TNFR2 was also activated, ROS production was enhanced by 60% [315]. More recently, an association between TNFR1 and riboflavin kinase links TNFR1 to NADPH oxidase through p22phox. Riboflavin kinase aids in flavin adenine dinucleotide synthesis, which is an important cofactor in the function of NOX2 [316]. Therefore, it is possible that TNFR2 could contribute to enhancing TNF-activated ROS by passing the ligand to TNFR1. A mechanism for TNFR2-mediated ROS production has not been described.

It was shown that TNF stimulation induces serine phosphorylation of p47, an important event in the priming of neutrophils for increased sensitivity to inflammatory stimuli [317]. Therefore, TNF could prime cells for efficient ROS production, where they would be quickly induced by integrin activation when leukocytes arrive at the affected location. While this mechanism has not been described in neutrophils, it is possible that TNF can affect neutrophil-mediated ROS release in a similar fashion.

It has become clear that Nox2-derived ROS are not the only inflammation-related ROS. Kim et al., 2007 found that L629 cells that do not express Nox2 treated with TNF produced ROS. Knocking down Nox1, a Nox2 isoform expressed largely in the colon, resulted in a decrease in the TNF-stimulated ROS production by these cells [318]. Nox1 co-precipitated with TRADD and Rac1, suggesting a complex was formed in response to TNF. RIP1 was shown to associate to Nox1. TRADD was shown to associate with NoxO1 as well. The exact mechanism that linked TNFR1 and Nox1 is still poorly defined. While TRADD, Rac1 and Nox1 coprecipitated, it was unknown how Nox1 physically interacted with TRADD [318]. Nox1 is an isoform expressed in the colonic epithelium and may play a role in both IBD and CAC but has yet to be investigated experimentally. It is possible that TNF can contribute to oxidative stress within the colon. Whether this might occur through leukocyte Nox2, endothelial Nox2, or colonic epithelial Nox1 is not known.

Microbial Colonization and Colitis-Associated Cancer

IBD does not have a clear infectious origin, yet it is thought to be a function of an improper host response to microbial colonization, presumably by commensal organisms. Animal studies suggest that microbial colonization itself can affect the outcome of colitis-mediated cancer. It has been reported that germ-free AOM-treated IL-10^{-/-} mice monocolonized with *B. vulgatus* have reduced cancer development compared to conventionally-housed IL-10^{-/-} mice [319]. As these mice develop a more mild form of colitis, it is not clear if inflammation severity directly affects tumor growth. Inflammation in these mice is dependent on the presence of bacteria, therefore it will be difficult to delineate the contributions of each. Mice on a TGFβ1^{-/-}RAG2^{-/-} background that were exposed to a specific pathogen-free environment developed colitis, but not cancer, and *H. hepaticus* introduction resulted in colon cancer, suggesting that in this model cancer is associated with a specific infectious cause [320]. Whether this is due to the general

presence of bacterial antigens, or a specific molecular mechanism is not clear. Mice deficient in two glutathione peroxidase enzymes, *Gpx1* and *Gpx2*, develop spontaneous inflammation when exposed to commensal microflora [321] but not when mice were germ-free [322]. This was a function of enterocyte-specific gpx [323] and inflammation was associated with an increase in mutations in the colon [324] which in non-specific-pathogen-free conditions resulted in the development of invasive carcinoma [325]. Collectively, these studies suggest that specific bacterial ligands can trigger pro-tumorigenic pathways within the colon. Whether this is a function of enterocyte-microbial interactions or leukocyte-microbial interactions remains to be determined.

It has been proposed that genes involved in bacterial sensing might be involved in the inflammatory progression to cancer. In humans, several candidate genes, such as the nucleotide oligomerization domain (*NOD2*)/*CARD15* gene, are implicated as such a genetic factor in CD susceptibility [326, 327]. The *NOD2/CARD15* gene product is an intracellular receptor that senses the bacterial product muramyl dipeptide. We await epidemiological evidence on whether it is associated with any increase in risk of CAC, other than the innate risk that increased inflammation would contribute [328].

Mice with deficiency in *Nod1*, a NOD2-related receptor, showed a significant increase in tumor development compared to WT when administered AOM and DSS [329]. This was associated with a greater activation of NF κ B, MAPK and intestinal permeability leading to bacterial translocation. *Nod1* is an intracellular pathogen recognition receptor, specific for a moiety found on peptidoglycan. Treating these mice with antibiotics or germ-free conditions reduced tumor formation, suggesting that the commensal microflora potentiates tumorigenic signals in the absence of *Nod1* [329]. Toll-like receptor (*TLR2*)^{-/-} mice also develop more severe dysplasia and mortality when exposed to the AOM and repeated DSS regimen. Although inflammation was not scored, more *TLR2*^{-/-} mice died before the end of the regimen, and *TLR2*^{-/-} was associated with higher inflammatory cytokine levels, increased leukocyte infiltration and larger, more frequent tumors [330]. This suggests that inflammation is dependent on the *TLR2* loss, and might have confounded the role of *TLR2* on cancer development. Together, these studies suggest that *Nod1* and *TLR2* may be important in regulating colonic homeostasis and that the bacterial population within the gastrointestinal tract can potentiate protective signals against both inflammation and cancer.

In contrast to the outcomes in mice with compromised TLR function, mice with a constitutively active IEC-specific, TLR4 were found to have increased CAC [331]. In addition, bone marrow chimeras lacking hematopoietic TLR4 developed larger tumors compared to WT bone marrow, presumably due to recruiting COX-2⁺ macrophages to the colon [332]. TLR4-deficient mice were less susceptible to CAC owing to a reduction in COX2 expression and epidermal growth factor receptor signaling [333, 334]. Together, these studies ascribe a pro-tumorigenic function to TLR4 in CAC.

In contrast, studies have reported that a downstream molecule to TLR2 and TLR4, MyD88, may be protective against development of CAC after AOM and DSS treatment, as MyD88-deficient mice had more tumors compared to WT mice [335, 336]. MyD88^{-/-} mice develop a severe inflammation in response to DSS treatment [337]. However, in the absence of IL-10, MyD88 deficiency protected mice against the development of inflammation-mediated cancer [319]. Together, these studies suggest that IL-10 can modulate the tumorigenic response in a manner dependent on bacterial colonization. TLR agonists, at least those that signal via MyD88, may represent a normal commensal colonization, and may protect against the development of tumors during chronic inflammation. In addition, TLR2/MyD88 signaling may reduce inflammation severity, playing a regulatory role in the gut, which protects against tumor development. The contrast between the roles TLR4, TLR2/MyD88 play in colitis-mediated cancer suggest that the MyD88-independent pathway might be involved in the development of CAC, however there is no existing evidence to support this. Interestingly, MyD88-independent TLR2/TLR4 signaling is associated with anti-cancer immunity [338]. It is possible that cell-specific expression (epithelial vs. myeloid) of the receptors modulate the outcome of inflammation-mediated cancer, as this was not addressed in the TLR2/MyD88 studies. In addition, MyD88 is also involved in IL-1 and IL-18 signaling, which could be pro-tumorigenic and anti-tumorigenic, respectively. When IL-1 receptor or IL-18 deficient mice were treated with AOM+ DSS, it was found that the presence of IL-18, a cytokine that strongly induces IFN γ and influences anti-cancer immunity, was protective against CAC in this model [335].

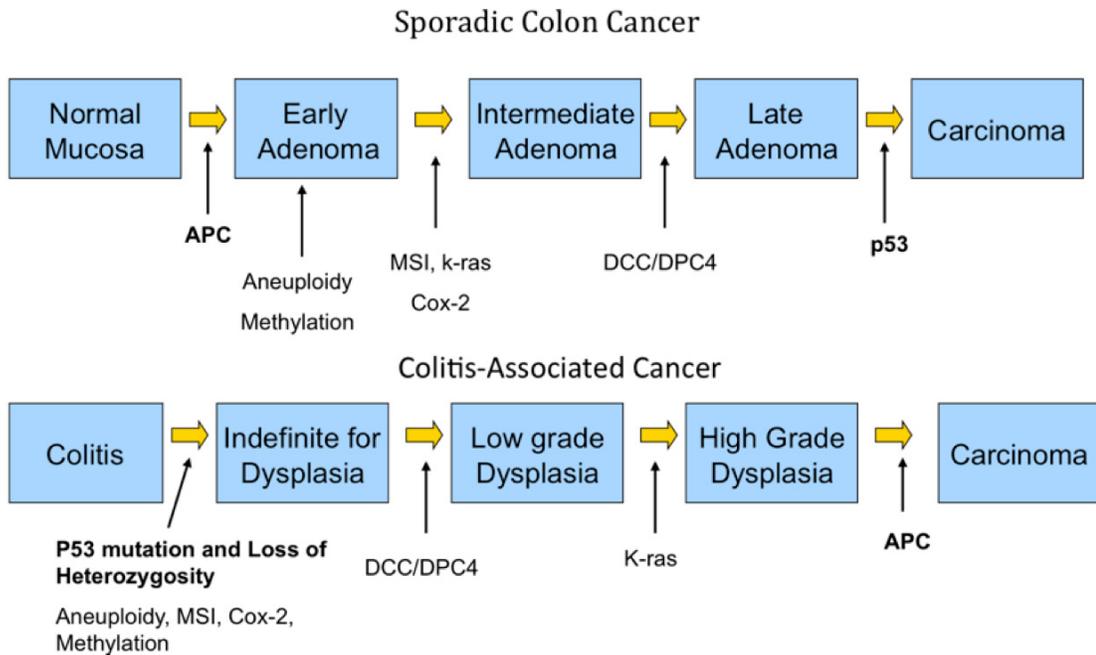
MyD88-dependent TLR activation leads to NF κ B activation, which is implicated in tumorigenesis [143]. In the context of protecting the epithelium against bacterial translocation, this NF κ B activation may also be protective against inflammation and

subsequent tumor development. When inflammation does occur, downstream of receptor activation is the production of inflammatory mediators, including TNF. As discussed, TNF may play an important role, specifically in innate immunity, in protecting the body against acute inflammation and infection, while activating pathways that can contribute to tumorigenesis when present during chronic inflammation.

Objectives and Rationale:

At the inception of this project, there were reports of extra-intestinal cancers associated with anti-TNF therapy [224, 339, 340] but the role of TNF and its receptors in chronic colitis and the development of cancer were not known. The influence of long-term anti-TNF therapy on the presentation of colitis-associated cancer had not yet been explored. With mixed outcomes observed after blocking TNF during experimental colitis, we sought to examine this question in DSS-colitis. The prevailing evidence is suggestive that TNF promotes tumor development so we sought to dissect any impact of TNF by manipulating receptor expression. The hypothesis was that TNF would contribute to inflammation and colitis-associated cancer via one, or both TNF receptors. Specifically, we asked if TNF signaling through its cognate receptors, TNFR1 and TNFR2, played a role in acute DSS-mediated colitis. As we were interested in the receptor deficiency in chronic CAC, we first had to establish a benchmark of inflammatory status after 1 cycle of DSS. Differences in the severity of inflammation could potentially confound findings during chronic colitis if the development of cancer is dependent on colitis severity. Other reports that TNFR1 may play a very important role in innate immunity during colitis [200] prompted us to ask whether the pro-tumorigenic effect of TNF signaling through TNFR1 occurred within the epithelium or infiltrating leukocytes. To achieve this, we created bone marrow chimeras in WT and TNFR1^{-/-} mice reconstituted with either WT or TNFR1^{-/-} bone marrow. Finally, we asked if TNF signaling could induce oxidative stress in a manner that could contribute to carcinogenesis. Oxidative damage via leukocyte Nox2 was examined in this model of CAC using mice lacking the gp91 subunit of the NADPH oxidase enzyme.

Figure 1.1 The Adenoma/Dysplasia to Carcinoma Sequence between Sporadic Colon Cancer and Colitis-Associated Cancer

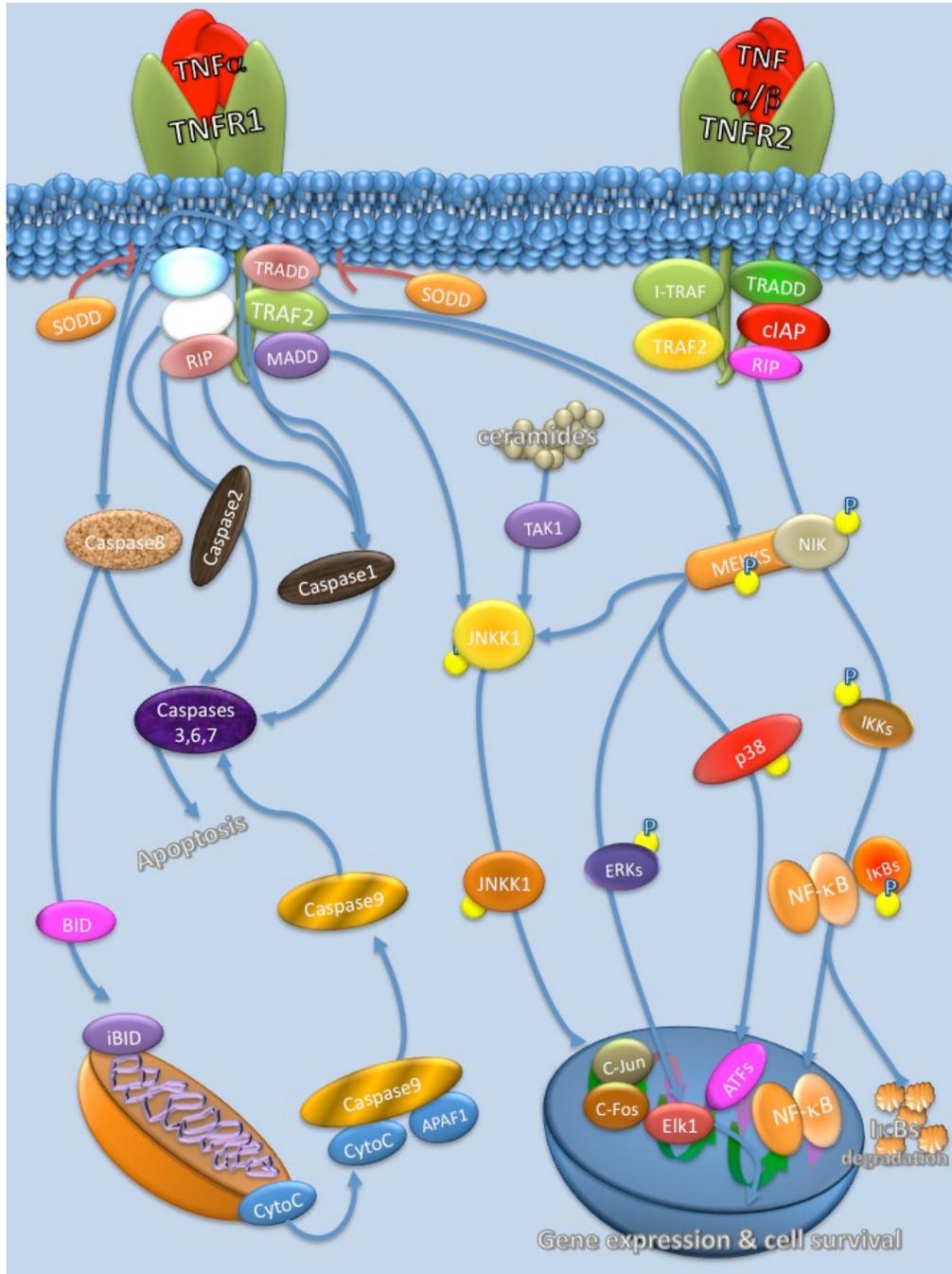


Itzkowitz, S. H. et al. Am J Physiol Gastrointest Liver Physiol 287: G7-G17 2004

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Molecular events that occur during the development of sporadic colon cancer (top) and colitis-associated cancer (bottom). Adenoma indicates a lesion arising from dysplastic glands in the colon, while dysplasia indicates abnormal growth of glands that could be a result of healing (indefinite) or tumorigenesis (high grade-carcinoma). MSI, microsatellite instability, APC, adenomatous polyposis coli, DCC, deleted in colon cancer, DPC, Deleted in pancreatic cancer.

Figure 1.2 TNF Signaling through TNFR1 and TNFR2



Depiction of TNFR1 and TNFR2 signaling pathways within the cell. TNFR1 signaling leads to cell survival via NFκB and MAP kinases while it activates extrinsic apoptosis via death domains and the caspase cascade. Caspase-8 can cleave Bid activating the intrinsic pathway of apoptosis. TNFR2 does not possess a death domain, but can activate both NFκB and apoptosis via other mechanisms.

CHAPTER 2: MATERIALS AND METHODS

Reagents and Antibodies

Azoxymethane (AOM) and DSS were purchased from Sigma, (St. Louis, MO), and MP Biomedicals (Irvine CA), respectively. Antibodies for Western Blotting and Immunohistochemistry were sourced as follows: sTNFR2 (R&D Systems, Minneapolis, MN), Ly6G (Clone 1A8, BD Biosciences, San Jose, CA), TNFR1 and TNFR2 (abcam, Cambridge, UK), Caspase-3 (Cell Signaling, Danvers, MA), F480 (clone CI:A3-1, Serotec, Raleigh, NC), 8-OHDG (clone N45.1 JaICA, Japan). MIP-2 ELISA TNF, IL-12, and IL-4 ELISAs were purchased from Peprotech (Rocky Hill, NJ),

Animals and Experimental Protocols

All protocols using mice were approved by the University Committee on Laboratory Animals, which is governed by the guidelines of the Canadian Council on Animal Care. Mice with targeted mutations, originally obtained from Jackson Laboratories (Bar Harbor, Maine), were bred in our facility. C57BL/6 mice were purchased from either Jackson Laboratories (Barr Harbor, MA), or Charles River (Saint-Constant, QC). Mice were housed conventionally in plastic shoebox cages on a 12-hour light/dark cycle with *ad libitum* access to both food and water. Mice with targeted mutations were on a C57BL/6 background backcrossed for 10 generations by the supplier. Genotypes were confirmed in tail snips/ear punches from random mice in the colony using primer sequences published by Jackson Labs.

Acute DSS Colitis

All mice (8-12 week-old, both sexes) were fed 3% w/v DSS (MW 36,000-50,000) in facility water *ad libitum* for five days then were switched back to regular water for two (all strains) or seven days (TNFR1^{-/-} and TNFR2^{-/-}). At the end of the experiment mice were anesthetized with isoflurane and killed by exsanguination via cardiac puncture. Serum was obtained by clotting the blood recovered by cardiac puncture for an hour at room temperature in a sterile microfuge tube followed by centrifugation, and was stored frozen at -20°C until analysis.

Azoxymethane and DSS-induced Chronic Colitis and Colon Cancer

Mice (12 weeks of age) (WT, TNFR1^{-/-}, TNFR2^{-/-}, Nox2^{-/-}) were subjected to a single intraperitoneal (i.p.) injection of AOM (10 mg/kg) dissolved in sterile 0.9% saline. Control mice were administered an equal volume of 0.9% saline alone. Mice were divided into the following groups, 0.9% saline alone (no treatment, NT), AOM alone (AOM only), 0.9% saline followed by four cycles of DSS (DSS only) and AOM followed by 4 cycles of DSS (AOM+DSS). One cycle of DSS consisted of 3% w/v DSS fed for five days, followed by 14 days of tap water (Figure 2.1). Weights and clinical scores were recorded daily during the first 12 days of the cycle unless mice were losing significant weight (>10% body weight). In this case, weights were monitored daily, and if the weight dropped more than 20% of the original weight the mouse was euthanized.

Assessing Disease Severity

Clinical Scores

Clinical illness scores were assigned based on the extent of weight loss, the presence of blood in the stool (occult and gross) and the severity of diarrhea in a modified clinical scoring system based on that described by Cooper et al. [90]. The criteria utilized for this score are outlined in Table 2.1. Stools were considered normal if they were small and hard, soft if they were wet and large, and diarrhea if there was residue left on the anus. The presence of occult blood was detected with a Hemocult kit (Beckman-Coulter, Mississauga, ON). Gross blood was noted if bright red blood was visible on the anus or within the stools.

Histopathological Scores

At necropsy the colon and cecum were removed and opened longitudinally along the mesenteric axis. The colon was divided in half longitudinally and one full-length half was prepared as a 'Swiss roll' and fixed in 10% neutral buffered formalin. The Swiss roll technique allows the observation of the entire colon length. The remaining half was weighed and snap frozen in liquid nitrogen followed by homogenization at full speed while frozen for 30 seconds (PowerGen 125, Fisher Scientific, Ottawa, ON) in 50mM HEPES buffer (4 µl/mg colon tissue) containing a protease and phosphatase inhibitor cocktail (Sigma). Homogenates were centrifuged (18,000 x g) and cell-free

supernatants were collected for analysis and frozen at -80°C.

The fixed tissue was paraffin-embedded and sections were stained with hematoxylin and eosin to ascertain histopathology after the treatment regime. Slides were coded and scored blindly by two individuals based on a scale that graded the extent of inflammatory infiltrate (0-5), crypt damage (0-4), ulceration (0-3) and the presence or absence of edema (0 or 1) (Table 2.2). The scores represent the histological damage associated with DSS treatment in the mid-colon (from the distal end of the plicae circulares to two thirds from the anus), where inflammation was the most severe. The sums of the scores in each category were used for statistical analysis.

Assessing Cancer

The severity of cancer development in mice on the chronic AOM+DSS regime was assessed by gross examination of the tissue and by microscopic examination of formalin-fixed Swiss rolled samples. Macroscopically, tumors were counted as solid, opaque, raised lesions. Tumor diameter was measured using the average of three diameter measurements in tumor-bearing mice. Microscopically, the presence or absence of dysplasia was used to determine dysplasia incidence in mice without visible tumor growths. Dysplasia was described as crypts (glands) that were irregularly shaped with an absence of goblet cells. In addition, nuclei in the dysplastic glandular epithelium were large, depolarized and hyperchromatic. Tumors and dysplasia in these experiments were confirmed by a clinical pathologist as intramucosal adenocarcinomas (Dr. Heidi Sapp, Department of Anatomical Pathology, QEII Health Science Centre, Halifax). Cancer severity was not quantified as a measurable unit, however, increased tumor number and size were considered more advanced and severe than flat dysplasia.

Enumerating Neutrophils and Eosinophils

Evidence suggests that innate immune cells play an important role in the pathology of DSS colitis via TNFR1 [200]. Therefore, we measured the presence of neutrophils, eosinophils and macrophages in the colon after colitis induction. To differentiate between neutrophils (Chapter 3) and eosinophils, tissue sections were stained with Congo red. Congo red stains the granules in eosinophils but not neutrophils. Congo red dye (Sigma) was dissolved in 50% ethanol and filtered. After

deparaffinization and rehydration, as described above, slides were incubated at ambient temperature for 2 hours in freshly prepared Congo red dye and counterstained with hematoxylin. In AOM+DSS experiments, the rat anti-mouse IgG2a antibody to neutrophil-specific Ly6G was used at a concentration of 1:1000, with a biotinylated goat anti-rat secondary as described above. Neutrophils and/or eosinophils were counted in the mucosa and submucosa in 5 high-power fields. If ulcers were present, neutrophils and eosinophils were counted within the ulcers to control for inflammation severity between strains, and where they were not present, they were counted in random selections of tissue. Mice in the no treatment or AOM-only groups had negligible numbers of neutrophils present in the gut.

ELISAs

TNF concentrations in Chapter 3 were determined in whole colon preparations by ELISA (sensitivity 41 pg/ml) as described elsewhere [341]. The ELISA was developed using the GIBCO amplification system (Life Technologies, Gaithersburg, MD). The capture and detection antibodies were obtained from R&D Systems (Minneapolis, MN) and Endogen (Woburn, MA), respectively. Mouse MIP-2 (sensitivity 32-2000 pg/ml), TNF (16-2000 pg/ml, Chapter 4), IL-12 (16-1000pg/ml), IL-4 (16-2000 pg/ml) concentrations were determined in whole colon preparations according to the manufacturer's instructions and was developed with ABTS substrate and read on a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA) at OD405 nm.

Soluble TNFR2 levels were determined in both serum and whole colon preparations using an ELISA specific for the extracellular region of the molecule (R&D Systems). Samples were loaded in triplicate at a dilution of 1:100 (serum) or 1:10 (whole colon) and ELISAs were performed according to manufacturer's instructions. Standards for the TNFR2 ELISA were spiked with serum from healthy TNFR2^{-/-} mice in the same concentrations as samples to verify that there were no confounding factors in serum that would affect the sensitivity of the assay plates developed with tetramethylbenzidine substrate were read at OD450 nm.

Western Blotting

Cell-free supernatants were added to 2x SDS buffer (62.5 mM Tris-HCl (pH 6.8),

2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, and 0.01% bromophenol blue) and boiled for 10 minutes [342]. Proteins were separated by SDS-polyacrylamide gel electrophoresis in 12% acrylamide then transferred to nitrocellulose membrane via electrophoresis. The membranes were then blocked with 5% skim milk in TBS buffer followed by incubation overnight with rabbit anti-human cleaved caspase-3 antibody or rabbit anti-human TNFR1 (Abcam, Cambridge, MA). For a protein loading control, anti-actin antibodies were used (Sigma, St. Louis, MO). Western blots were analyzed with software for densitometry (Unscan it, Silk Scientific, Orem, UT) and cleaved caspase-3 levels were calculated relative to actin levels.

Bone Marrow Chimeras

Six-12-week old recipient WT and TNFR1^{-/-} female mice were fed 0.2% neomycin sulphate in their drinking water for two weeks while being housed in conventional facility. The mice were then irradiated with two doses of 500 rads two hours apart with a gamma-irradiator to minimize the negative effects on the animal while maximizing bone marrow ablation [343]. Bone marrow was isolated from healthy donor male WT and TNFR1^{-/-} 6-12-week-old mice by aseptic removal of the femur and tibias, and rinsed in sterile HBSS without Ca²⁺ Mg²⁺. White blood cells were counted using a crystal violet stain, and recipient mice received 5x10⁶ cells via tail vein injection following the second dose of radiation. Following irradiation and bone marrow reconstitution, mice were housed in sterile HEPA-filtered cages and fed sterile food and water containing 0.2% neomycin sulphate for two weeks. Weights and health were monitored daily. Eight-12 weeks following the bone-marrow reconstitution, mice were subjected to the AOM-DSS regime described previously. By 8-12 weeks, most host immune cells are replaced [344]. Chimerism was detected immunohistochemically via *in situ* hybridization of the Y chromosome in the spleen and colon as per the manufacturer's instructions (Cambio, New York, NY). Briefly, 10 µm formalin-fixed, paraffin-embedded tissue sections (colon, spleen) were incubated in 1M sodium thiocyanate followed by protein digestion with pepsin at 37°C. Slides were then refixed in 4% paraformaldehyde and dehydrated with graded ethanols. The FITC-labeled Y-chromosome paint was applied under a cover slip and rubber cement and incubated overnight in a humidified chamber at 37°C. The following day, slides were washed in a

formamide solution, then citrate buffer and then a biotinylated antibody to FITC (Vector laboratories, Burlingame, CA) was applied for 1 hour at room temperature. Horseradish peroxidase-avidin complex was applied for 30 minutes (Vector laboratories), followed by chromagen development with diaminobenzidine (Vector Laboratories). Slides were counterstained with hematoxylin, dehydrated through graded ethanols and mounted. Cells with small brown spots within nuclei of infiltrating leukocytes were considered positive for the Y chromosome. Male gut sections were used as positive controls and female gut sections were used as negative controls.

Immunohistochemistry

Immunohistochemistry was performed on tissue sections fixed in 10% neutral buffered formalin for at least 24 hours. Sectioned (3 μm) samples were deparaffinized and rehydrated in xylene and graded ethanol, respectively. Endogenous peroxidase activity was blocked using 3% H_2O_2 for 20 minutes, and antigen retrieval was performed using 10mM citrate buffer, pH 6, microwaved for 2 minutes until boiling then maintained in a 95°C water bath for 20 minutes. Following that, a blocking step using 5% goat serum in PBS plus 0.1% tween-20 for 1 hour was performed. Primary antibody was applied at concentrations as per manufacturer's instructions overnight at 4°C diluted with blocking buffer. Biotinylated secondary antibody was also diluted in blocking buffer and applied to individual slides for one hour at ambient temperature. Slides were then subjected to avidin-bound horseradish-peroxidase (ABC elite, Vector Laboratories) for 30 minutes. Colour was developed using diaminobenzidine (Vector Laboratories) and slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich).

To measure a footprint of oxidative damage, formalin-fixed samples were stained immunohistochemically with an mouse antibody to 8-OHDG (1:100) with the following variations; antigen retrieval was performed using proteinase K for 20 minutes at 37°C, mouse seroblock (Serotec) was used to block Fc receptor binding in the blocking solution, and only positive nuclei within the colonic epithelial cells were evaluated to limit counting false positives due to mouse-on-mouse staining. Nitrotyrosine was also measured in paraffin-embedded samples to ascertain the level of oxidative and nitrosative damage to the cells and tissues. Antigen retrieval was performed in 10mm Citrate buffer as described previously and a goat anti-rabbit secondary antibody conjugated with biotin

was used. Primary antibody was applied at 1:500 for 24 hours at 4°C.

Apoptosis was measured by terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) and by activated Caspase-3 antibody. The TUNEL assay was performed as indicated by the manufacturer's instructions (Chemicon, Temecula, CA). Briefly, deparaffinized and rehydrated samples were treated with proteinase K for one hour. Slides were then treated with terminal deoxynucleotidyl transferase which adds digoxigenin-labeled nucleotides to ends of damaged DNA. For activated caspase-3, primary antibody (Cell signaling) was applied at 1:100 to rehydrated gut sections followed by a goat anti-rabbit biotinylated IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Positive-stained cells were counted per 100 crypts. Diaminobenzidine was used as a substrate to develop color and the slides were counterstained with methyl green. Slides were mounted and examined using light microscopy. To quantify the number of apoptotic cells per treatment group, positively-stained colonic epithelial cells were counted within 100 intact crypts per section per mouse in two sections per slide.

Macrophages and neutrophils were detected using a rat monoclonal antibody to F4/80 and Ly6G, respectively at a concentration of 1:1000 and incubated for 24 hours at 4°C (Serotec, Raleigh, NC). Biotinylated goat anti-rat IgG (Santa Cruz Biotechnology) was used as a secondary antibody at a concentration of 1:400 and applied for 1 hour at ambient temperature. Sections were incubated for 30 minutes at ambient temperature with a peroxidase-conjugate and diaminobenzidine was used to develop color. Slides were counterstained with hematoxylin and dehydrated and mounted as described previously. Macrophages were counted in ulcerated lesions in both the mucosa and submucosa. A total of 5 high-power fields were counted per section. To enumerate neutrophils and macrophages in tumors, cells were enumerated in at least 3 high power fields in tumor tissue in tumor-bearing mice.

Anti-rabbit polyclonal TNFR1 and TNFR2 antibodies (abcam) were used to detect the intracellular portion of receptor expression within the colon. They were used at concentrations of 1:100 and 1:50, respectively.

Statistics

Where data distribution was normal (determined by lack of skew or kurtosis), a two-way ANOVA was performed using main effects strain and treatment, with two or

three levels of strain and four levels of treatment. Non-parametric data, such as pathology and illness scores, were analyzed using the Kruskal-Wallis One-way ANOVA between strains or treatments. Weight loss data, where normal, was analyzed using a repeated-measures ANOVA over the treatment period. Chi square was used to detect differences in the tumor incidence between groups at a power of 0.8, with the n required for that power calculated previously. Where only two groups existed (ie. Nox2 vs. WT tumor size and multiplicity), two tailed- t-tests were used to detect differences. Prism GraphPad (Version 4.0a, 2003) was used to perform analyses.

Table 2.1 Clinical Illness Scoring System

Scores	Weight Loss (%)	Stool Consistency	Presence of Blood
0	None	Normal	None
1	None	Normal	Occult+
2	1-5	Normal	None
3	1-5	Soft	None
4	1-5	Soft	Occult+
5	1-5	Diarrhea	Occult+/Gross
6	5-10	Soft	Occult+/Gross
7	5-10	Diarrhea	Occult+/Gross
8	10-20	Soft	Occult+/Gross
9	10-20	Diarrhea	Occult+/Gross
10	>20	Diarrhea	Occult+/Gross

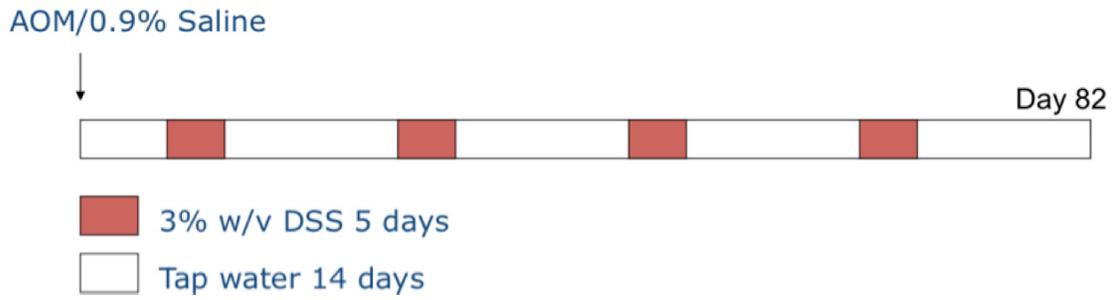
Clinical illness was scored using a combined score for weight loss, stool consistency and presence or absence of occult/gross blood in the stool. Normal stool was hard, while soft stool was soft and moist, and diarrhea was soft stool that left a residue on the anus of the mouse. Where gross blood was not detectable, occult blood was measured in the stool with a Hemacult kit.

Table 2.2 Histopathological Scoring System

Score	Inflammation	Crypt Damage	Ulceration	Edema
0	No infiltrate	None	None	Absent
1	Occasional cell limited to submucosa	Some crypt damage, spaces between crypts	Small, focal ulcers	Present
2	Significant presence of inflammatory cells in submucosa, limited to focal areas	Larger spaces between crypts, loss of goblet cells, some shortening of crypts	Frequent small ulcers	
3	Infiltrate present in both submucosa and lamina propria, limited to focal areas	Large areas without crypts, surrounded by normal crypts	Large areas lacking surface epithelium	
4	Large amount of infiltrate in submucosa, lamina propria and surrounding blood vessels, covering large areas of mucosa	No crypts		
5	Transmural inflammation (mucosa to muscularis)			

Histopathological scores were determined in the mid-colon by examining H&E-stained whole colon sections. Scores were determined by the sum for individual indicators of disease severity, such as the presence of inflammatory infiltrate (out of 5), ulceration (out of 4), crypt damage (out of 3) and the presence of submucosal edema (out of 1).

Figure 2.1 Regimen for Colitis-Associated Cancer



Mice were administered 10mg/kg AOM (Day 1) followed by 4 cycles of 3% DSS/14 days of tap water. Controls were administered either DSS alone or AOM alone.

CHAPTER 3: THE ROLE OF TNF RECEPTORS, TNFR1 AND TNFR2 IN DEXTRAN SODIUM SULPHATE-INDUCED COLITIS

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Introduction

Tumor necrosis factor (TNF) has long been appreciated as a mediator central to various inflammatory diseases in humans (recently reviewed in [345]). This includes the inflammatory bowel diseases, Crohn's disease and ulcerative colitis, during which the levels of TNF are elevated in the serum and intestinal mucosa [164-166]. These findings are consistent with the observations that neutralizing TNF is associated with improved disease, particularly in Crohn's disease [346-348]. A similar pattern of elevated TNF is associated with animal models of colitis, and transgenic mice that overexpress TNF develop a spontaneous ileitis [121]. However, whether neutralizing TNF reduces these model diseases has yielded mixed results and thus the roles TNF may play in colitis are not entirely understood [173, 188, 191, 349].

Studying the role of TNF in disease is complicated by the fact that there are two TNF receptors, TNFR1 (p55) and TNFR2 (p75), with TNFR1 but not TNFR2 possessing a death domain that can activate apoptotic signaling pathways [350]. TNFR1 is constitutively expressed by most cell types while constitutive expression of TNFR2 is mostly limited to cells of the immune system. TNFR2 may also share TNF by making it available to TNFR1 in the membrane of cells with both receptors. TNFR1 signaling can result in the activation of several pathways including MAP kinases, NF- κ B, and caspases, leading to apoptosis. TNFR2 does not activate the apoptotic pathways but otherwise signals similar to TNFR1, through the MAP kinases and NF- κ B. Both receptors can be proteolytically cleaved, becoming soluble TNF antagonists [351], and TNFR2 binds preferentially to membrane-bound TNF while TNFR1 binds with higher affinity to soluble TNF [129, 352]. Regarding TNFR expression during IBD, TNFR2 expression is reportedly upregulated on colonic epithelial cells during both Crohn's disease and ulcerative colitis [125]. TNFR2 but not TNFR1 is upregulated on lamina propria lymphocytes in active Crohn's disease [209]. Since TNFR1 and TNFR2 can signal through different pathways, and an upregulation of inducible TNFR2 occurs during

inflammation, it is possible that they play distinct roles in the pathogenesis of IBD.

Two murine models of IBD, in particular, have been used widely to examine the role of TNF in colitis. One model better resembles Crohn's colitis; 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induces a T cell-dependent colitis [88] while the second, dextran sodium sulphate (DSS) induces colitis that is lymphocyte-independent and more closely resembles ulcerative colitis [91, 92]. Studies using antibodies to block TNF in these models have provided mixed results. Treating mice with a rabbit antisera had no effect on DSS-colitis while treatment with a rat monoclonal antibody exacerbated acute DSS colitis [188]. These studies are contrasted with one in which antisense oligodeoxynucleotides and rat monoclonal anti-TNF antibodies both resulted in less DSS colitis [353]. Blocking TNF with antibodies resulted in reduced clinical illness and inflammation in TNBS colitis [173]. The inconsistencies observed with antibody treatments may be due to differences in the timing of their use, in the capacity of the antibodies to block cell-associated TNF or fix complement, or in the case of rabbit antisera may be due to host antibodies to the rabbit immunoglobulin.

A more definitive approach to address the role of TNF is to use TNF gene knockout mice. TNF gene knockout mice developed less inflammation in response to TNBS treatment [203], but experienced an exacerbated, potentially fatal colitis when treated with DSS [198]. These opposing results suggest that TNF may induce a harmful (TNBS) or protective (DSS) effects through different mechanisms, depending on the model of colitis induction. Considering the different signaling outcomes of the two TNF receptors, an alternate approach to using TNF deficient mice is to use mice deficient in either receptor.

TNFR1 and TNFR2 deficient animals have been used in TNBS colitis and have again yielded conflicting results. In one study, mice lacking TNFR2 were protected from weight loss compared to wildtype while TNFR1^{-/-} mice had severe weight loss and mortality despite similar histological damage observed after treatment [206]. This suggests that TNFR1 is protective and TNFR2 may be involved in the pathogenesis of TNBS colitis. However in a second study, no differences in inflammatory infiltrate between strains after TNBS treatment were reported, while tissue damage scores were lower in TNFR1^{-/-} mice and double receptor knockout mice compared to WT [204]. This second study suggests that TNFR1, but not TNFR2, plays a role in the pathogenesis of

TNBS colitis. Why the roles of TNFR in TNBS colitis are different between these two studies is unclear. Now, to advance on this understanding of the role of the TNF receptors in acute colitis we made it our objective to characterize the inflammation in DSS-treated mice lacking expression of each TNF receptor type.

Results

Mouse Characteristics

We first established whether the colons of untreated TNFR1^{-/-} and TNFR2^{-/-} mice were normal, as the characteristics of the intestines in these mice have not yet been described. Figure 3.1 shows using the Swiss roll technique that TNFR1^{-/-} and TNFR2^{-/-} mice have grossly normal colons, similar to WT mice. The proximal colon is distinguished by the plicae and is in the centre of the coil. Colons from untreated older (>60 weeks) receptor knockout mice were also histologically normal (data not shown), indicating that these mice do not experience any spontaneous colitis.

Mice in all groups began losing weight between days 4 and 5 of DSS treatment (Figure 3.2a) while untreated mice maintained their body weight throughout the experiment (data not shown). In DSS-treated groups observed out to day 12, WT mice had their highest percentage of weight loss at day 7 but weight was completely restored by day 8. Weight loss peaked at day 8 but was completely restored by day 11 in DSS-treated TNFR1^{-/-} mice. In DSS-treated TNFR2^{-/-} mice, weight loss peaked at day 6 but was regained by day 10. The percentage of total weight loss did not differ significantly between WT and TNFR2^{-/-} mice. In TNFR1^{-/-} mice, weight loss did not differ significantly from WT until day 8, when weight loss peaked and was restored more slowly. At day 11, the weight of both knockout strains was similar to WT mice. Weight loss did not differ between TNFR1^{-/-} and TNFR2^{-/-} mice. Compared to WT, TNFR1^{-/-} mice had more severe weight loss but even this strain recovered by day 12.

All strains developed soft, enlarged stools when treated with DSS, with evidence of diarrhea (stool residue apparent on anus). Occult blood appeared in the stools in all strains at day two of treatment. By day four, mice showed gross blood in their stools and some showed rectal bleeding. These features were not detectably different between strains and were not apparent in untreated mice. We observed that fecal occult blood could occur in the absence of weight loss and thus we combined weight loss, stool

consistency and the presence of fecal blood into a scale indicating clinical disease severity (maximum score = 10, Table 1). Clinical disease scores for WT mice were most severe at day 6, and returned to normal by day 10 (Figure 3.2b). In TNFR1^{-/-} mice clinical scores were most severe at day 8 but returned to near normal by day 12. TNFR2^{-/-} mice peaked by day 7 but it returned to normal by day 11. At days 8 and 9 of treatment, TNFR1^{-/-} mice had a significantly more severe score than both WT and TNFR2^{-/-} mice; however, no difference existed between WT and TNFR2^{-/-} mice.

Post-necropsy we evaluated the pathology associated with DSS colitis in each strain. Colon length, which is frequently reduced in DSS colitis, did not differ between strains of DSS-treated mice, but in all strains, colon length was significantly shorter compared to non DSS-treated strain controls at day 7 and in both knock-out strains at day 12. In addition, colon wet weight was significantly greater only in DSS-treated TNFR2^{-/-} mice compared to their strain controls at day 7. The ratio of colon weight:length was not different between strains but all strains treated with DSS had a significantly greater weight:length ratio compared to their untreated controls at day 7, while only TNFR2^{-/-} mice had a greater weight:length ratio at day 12 (Table 3.1). We also observed that the cecum was shrunken in mice treated with DSS but when measured, only TNFR2^{-/-} reached statistical significance. Cecum weight:length ratios were not different between strains and treatments. DSS-treated TNFR2^{-/-} mice had a significantly greater number of macroscopically visible ulcers at day 7 compared to WT and TNFR1^{-/-} mice, but the number of visible ulcers in DSS-treated TNFR2^{-/-} mice at day 12 were similar to the other strains. There were no differences between strains and treatments in spleen weights. We conducted one experiment using mice with combined TNFR1 and TNFR2 deficiency and these animals experienced severe clinical disease and consequently most were euthanized when their weight loss exceeded 20% of their initial weight (APPENDIX B).

Histological Inflammation Characteristics

As a random sampling from the colon could potentially lead to different levels of inflammation, we used the Swiss roll technique to evaluate the severity of inflammation along the entire colon length. We found that DSS treatment affected primarily the mid-colon (Figure 3.3), with some cellular infiltrate but almost no ulceration in the distal colon. In all strains the mid-colon colonic mucosa was completely damaged, with only a

layer of infiltrate and no remaining crypts. All strains showed submucosal edema, crypt damage, cellular infiltrate and ulceration. Crypt abscesses were also observed in non-damaged epithelium. We summarized histological inflammation using a scoring system that weighted the severity of cellular infiltrate, ulceration, crypt damage and edema (maximum score = 14). We did not detect a difference in the severity of inflammation between strains treated with DSS using this scoring system (Figure 3.4).

TNFR1 and TNFR2 Expression

To determine whether TNFR1 expression was affected by strain or treatment, we measured TNFR1 in the colon by Western blot. TNFR1 levels were similar between treatment groups within a strain (Figure 3.5a). Soluble TNFR2 levels have been found elevated in the serum of IBD patients, particularly in Crohn's disease [168, 354] and differences in concentration could confound any interpretation of the role TNF may play in the different strains. We therefore measured sTNFR2 in the serum and found that levels were not significantly elevated due to the colitis, nor were there significant differences between strains. sTNFR2 was not detected in TNFR2^{-/-} mice, as expected (Figure 3.5b). In contrast to the serum results, sTNFR2 levels in the colon were significantly higher in WT and TNFR1^{-/-} mice after DSS treatment at day 7 compared to untreated mice, and while this level remained high in WT mice at day 12, the levels in TNFR1^{-/-} mice were similar to untreated mice at day 12 (Figure 3.5c).

TNF and MIP-2 Levels in the Colon

TNF levels were measured in whole colon preparations of uninflamed and DSS inflamed mice. There were no significant differences between strains or treatment groups in the levels of TNF in the colons of mice (Figure 3.6a). TNF was not detected in the serum in any of the strains used (data not shown). We also measured levels of the neutrophil chemokine, MIP-2, in the colon since neutrophils feature prominently in the histopathology. Untreated mice did not have detectable levels of MIP-2 in colon tissue. TNFR1^{-/-} mice appeared to have lower levels of MIP-2 in the colon tissue however this was not significantly different than WT or TNFR2^{-/-} mice (Figure 3.6b).

Cellular Infiltrate

Neutrophils, eosinophils and macrophages were counted in ulcerated tissue in

both the submucosa and mucosa in 5 high-power fields per colon section. There were no differences in the number of neutrophils or eosinophils in the mucosa or submucosa between strains treated with DSS. However, macrophages were significantly less numerous in the mucosa of TNFR1^{-/-} mice compared with TNFR2^{-/-} and WT. There were no differences in the numbers of submucosal F4/80⁺ macrophages in areas directly below ulcers (Figure 3.7).

Measurement of Apoptosis in the Colon using TUNEL and Cleaved Caspase-3 Levels

TNF reputedly directly affects epithelial cell apoptosis in the gut and early extensive epithelial apoptosis has been suggested to underlie increased intestinal permeability [155]. Moreover, others have reported that both cell proliferation and apoptosis are different in the colons of mice lacking either TNFR1 or TNFR2, yet we observed no differences in the number of apoptotic (TUNEL⁺) cells between DSS-inflamed strains (Figure 3.8a). The number of TUNEL positive cells per 100 crypts in untreated controls of all strains was similar to DSS-treated mice (not shown). Cleaved caspase-3 is another indicator of apoptosis and levels in whole colon tissue were not significantly different among DSS-treated strains or treatments (Figure 3.8b). Cleaved caspase-3 levels did not differ between treatments in WT or TNFR1^{-/-} or TNFR2^{-/-} mice.

Discussion

TNF is widely appreciated to be part of the mechanism behind multiple inflammatory diseases, including IBD [345, 355] and rodent models of IBD. The DSS model of mouse colitis is appreciated to cause inflammation resembling ulcerative colitis, is responsive to a number of therapies used to treat IBD [356], and used acutely, includes increases in Th1 and Th17 cytokines [94]. Yet mice deficient in TNF, in contrast to what may have been predicted, experience exacerbated DSS colitis [198, 357]. Considering there are two receptor types, one approach to examining the role of TNF has been to induce colitis in mice deficient in one or both receptors. We therefore sought to examine whether the exacerbated DSS-induced colitis was attributable to a single TNF receptor type by using receptor deficient mice. We observed that mice deficient in either receptor have no evidence of spontaneous colitis, and experience clinical illness and colonic

inflammation that are for the most part similar to wildtype strain mice. Wildtype and both receptor-deficient strains show severe mid-colonic inflammation with striking ulcers, submucosal edema, and submucosal and mucosal cellular infiltrates after only 5 days of ingesting DSS followed by 2 further days of water only (e.g. day 7). All strains rebounded to a large extent, evident with the mice gaining weight and recovery of the colon crypt architecture at day 12, or 7 days after removing DSS from the drinking water. Some measures, albeit few, showed statistically significant differences between strains. TNFR1^{-/-} mice had the greater clinical illness scores at day 8 and 9 as well as fewer mucosal (but not submucosal) infiltrating macrophages. This pattern of reduced macrophages with increased illness contrasts with TNF deficient mice which reportedly experience greater macrophage infiltration [357] and in anti-macrophage inhibitory factor (MIF) treated inflamed mice, which had less inflammation [358]. It is noteworthy that the increase in MIF in colitis mice was TNF-independent since it was unaffected by anti-TNF antibodies [358]. TNFR1 deficient mice lacking lymphocytes (crossed with RAG1 deficiency) also showed increased macrophage infiltration associated with increased pathology [200]. There is, however, a precedent for a protective role for macrophages, as depleting macrophages prior to DSS treatment resulted in a more severe colitis associated with increased neutrophil infiltration [359]. Whether there is a unique inverse relationship directly between mucosal macrophage numbers and clinical illness in lymphocyte sufficient TNFR1^{-/-} mice will require further analyses.

Our finding that there was no striking difference in the colonic inflammation between the receptor knockout strains corroborates a similar lack of difference in colonic pathology scores reported for TNF receptor deficient mice assessed within 5 days of TNBS administration, although systemic indicators of illness and mortality were greater in this model for TNFR1^{-/-} mice compared to wildtype [206]. In a second study using TNBS, in which mice were assessed after one week, colon inflammatory cell scores were similar but tissue damage was reportedly less in TNFR1^{-/-} and double TNFR knockout mice [204]. These findings using TNBS colitis in receptor deficient mice contrast the result of TNBS in TNF^{-/-} mice, which experienced less inflammation [203]. Despite this apparent incongruence in the TNBS model, possessing either receptor protects mice from the exacerbated and often fatal colitis that double receptor deficient or TNF deficient mice experience due to DSS. An exception is the exacerbation of DSS-induced colitis in

double TNF receptor-deficient mice crossed with RAG1 deficiency and also TNFR1 deficiency crossed with RAG1 deficiency, which suggests that TNFR1 acting through innate mechanisms provides suppression from exacerbated colitis [200]. That single TNF receptor-type deficient mice experience similar levels of inflammation whether colitis is due to DSS or TNBS indicates that lymphocytes modulate the response, particularly by acting through TNFR2 to oppose the pro-inflammatory effects of TNF on innate effector cell mechanisms as suggested by the TNFR^{-/-}RAG^{-/-} double deficient model. Finally, we observed that TNFR1^{-/-} mice experienced higher clinical illness as did the TNFR1^{-/-} crossed with RAG deficiency [200]. As suggested by Mizogouchi and co-workers, this may be due to TNF acting through TNFR1 on hematopoietic cells to protect against the translocation of colonic bacteria.

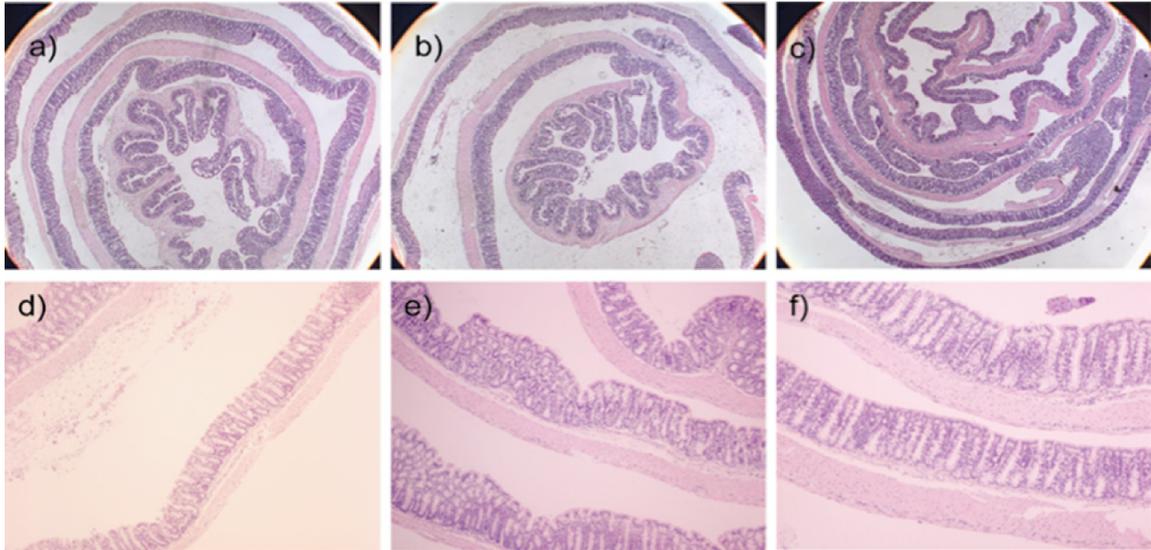
Another feature of TNF biology that may begin to explain how different mechanisms lead to similar inflammatory phenotypes in receptor deficient but lymphocyte normal mice, are levels of soluble receptors. IBD patients have elevated levels of both receptors in their urine correlating with disease activity [360] while TNFR1 levels are increased in serum whether disease is clinically active or not and TNFR2 levels were higher in Crohn's disease versus ulcerative colitis but both were higher than healthy control levels [168]. Despite higher than normal levels in IBD patients, one study of adsorptive granulocyte-monocyte apheresis found increased levels of soluble TNF receptors in the column eluate administered back to the patient and levels correlated with reductions in clinical disease suggesting there is a therapeutic benefit to high receptor levels [361]. Lymphocyte expression of TNFR2 but not TNFR1 is increased among cells recovered from the mucosa of Crohn's patients [209]. We similarly found no differences between healthy and inflamed mucosal homogenate concentrations of TNFR1; however, we did find mucosal concentrations of TNFR2 were increased in day 7 DSS-inflamed mice compared to uninflamed mice of the same strain. This means that mucosal TNFR2 in both wildtype and TNFR1^{-/-} mice is a better marker of the DSS-induced inflammation than TNFR1 in wildtype or TNFR2^{-/-}. This pattern also indicates that the upregulation of TNFR2 is independent of TNFR1. The elevated mucosal TNFR2 was sustained to day 12 in wildtype but not TNFR1^{-/-} mice which may indicate that the inflammation was abating in these mice but our histopathology scores did not support this hypothesis. Moreover, our detecting higher mucosal levels of TNFR2 in the absence of increased

serum levels may indicate that the molecule could be increased in its membrane-bound form. Whether mucosal cells secrete TNFR2 could be reconciled by measuring the supernatant of cultured mucosal explants. Whether the TNFR changes we observe are representative of rodent colitis cannot be definitively concluded as data for levels of TNFR in other models using single receptor deficient mice are not available.

During colitis TNF is widely presumed to directly result in increased epithelial cell apoptosis. For example, intestinal epithelial NEMO-deficient mice experience a severely exacerbated DSS-induced colitis which is prevented when the mice are crossed to also become TNFR1 deficient [155]. The authors suggested this was due to NEMO-deficient epithelial cells becoming particularly sensitive to TNF driving apoptosis. Yet the rate of apoptosis as detected by TUNEL in TNBS colitis were higher compared to untreated mice, although no effect of TNFR deficiency was observed [204]. Nor was any difference in the frequency of apoptotic epithelial cells detected in TNFR deficient mice versus wildtype strain in a T cell-dependent model (anti-CD3 injection) of small intestinal injury [362]. We likewise did not only find any difference in TUNEL-positive cell frequency between strains of mice but also between DSS-inflamed versus uninflamed. Other reports support a model whereby T-lymphocytes stimulated by TNF effect epithelial cell apoptosis, for example, the Crohn's-like SamP1/YitFc mouse strain. A single injection of TNF antagonizing antibodies into these mice prevents the spontaneous colitis these mice would otherwise experience concomitant with increased T lymphocyte apoptosis but decreased epithelial cell apoptosis [177]. Thus it would seem that the role of TNF in epithelial cell apoptosis could be context specific, showing considerable variability among the various models of mouse colitis.

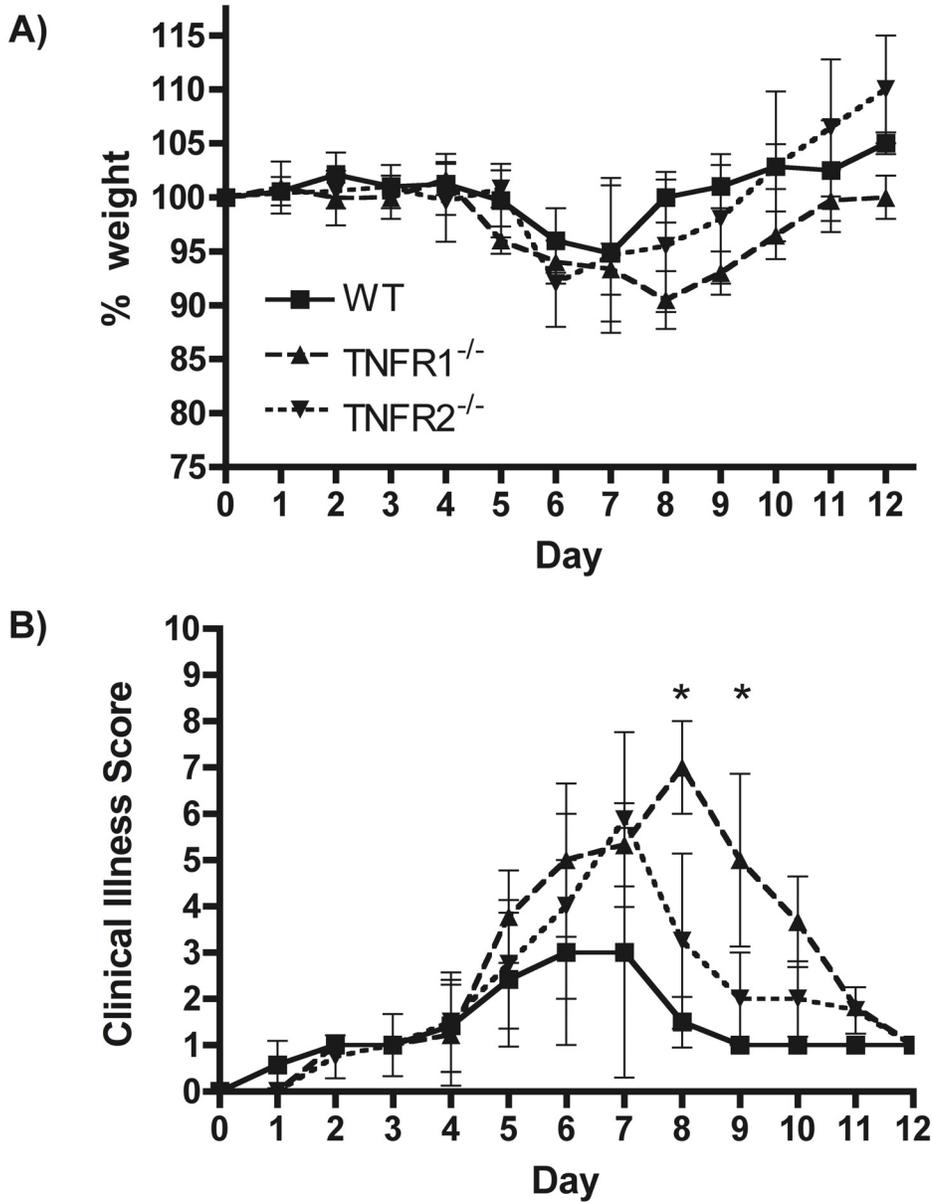
While deficiency of either receptor contributes to some measures of DSS colitis, the evidence from our experiments and others favors TNFR1 playing a protective role in the event of an innate-mediated colitis. Histopathological scores were similar between groups suggesting that TNF receptors either do not play a major role or are redundant in the pathology associated with DSS colitis. TNF may be playing a more significant role in the reaction to bacteria than in the early inflammatory events. This hypothesis is compatible with blocking TNF in humans not resulting in an exacerbation of colitis but perhaps increased risk of infections.

Figure 3.1 Untreated WT, TNFR1^{-/-} And TNFR2^{-/-} Mice Do Not Have Any Differences In Colon Pathology And Do Not Show Signs Of Spontaneous Inflammation



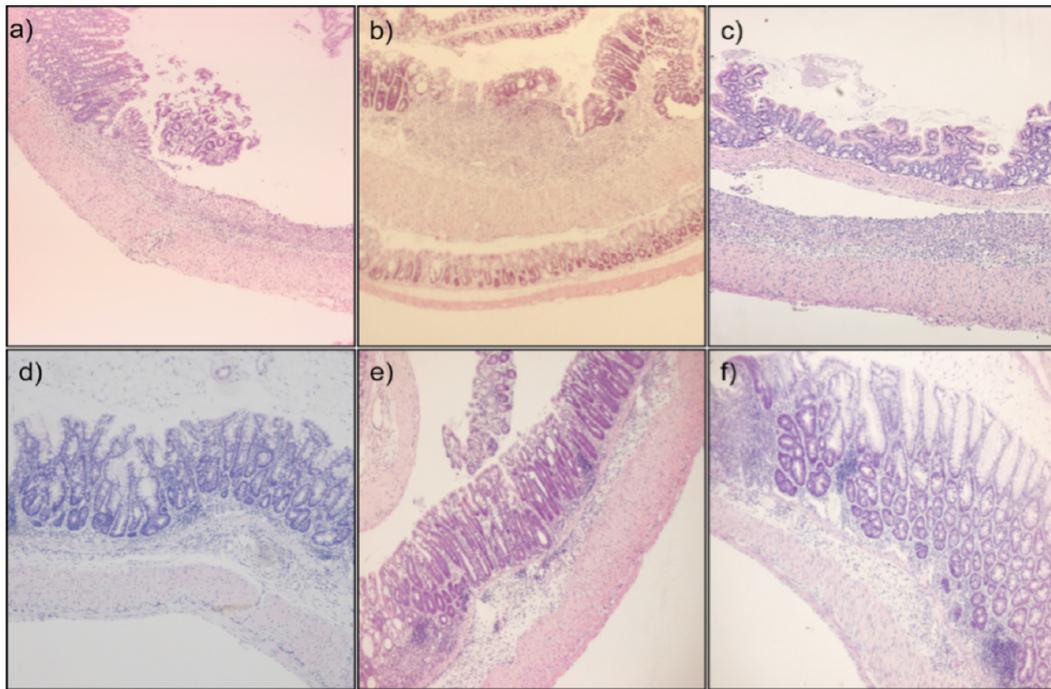
Hematoxylin and eosin-stained mouse colons prepared in the Swiss roll manner from the three strains without DSS treatment. a,d) Wildtype, b,e) TNFR1^{-/-}, c,f) TNFR2^{-/-}. Magnification 10x (a-c), 100x (d-f)

Figure 3.2 Weight Loss And Clinical Scores In WT, TNFR1^{-/-} And TNFR2^{-/-} Mice



Percent original weight (A) and clinical illness score summary (B) from wildtype (WT), TNFR1^{-/-}, TNFR2^{-/-} mice. * denotes a significant difference (p<0.05) between TNFR1^{-/-} and the other strains, WT and TNFR2^{-/-}. Data presented as mean ± standard deviation.

Figure 3.3 Histopathology of WT, TNFR1^{-/-} and TNFR2^{-/-} Mice at Days 7 and 12



Representative hematoxylin and eosin-stained mid-colon sections from DSS-treated wildtype (WT) (a, d), TNFR1^{-/-} (b,e) and TNFR2^{-/-} mice (c,f) showing inflammation at days 7 (a-c) and 12 (d-f). Original magnification 200x.

Figure 3.4 Histopathological Scores Are Not Significantly Different Between Strains

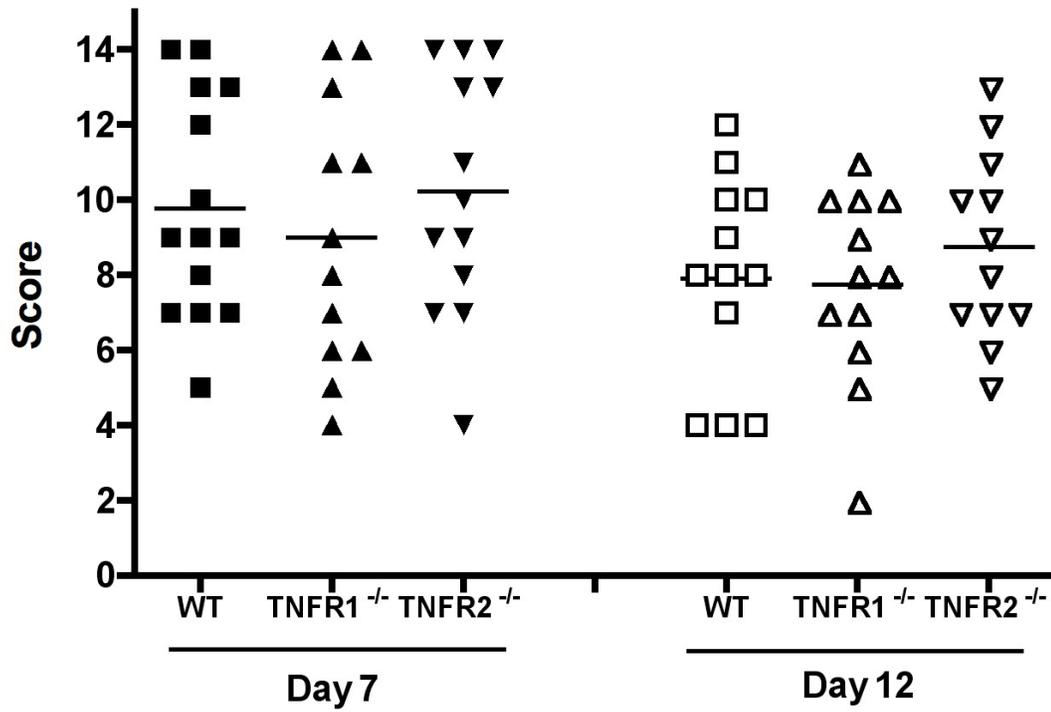
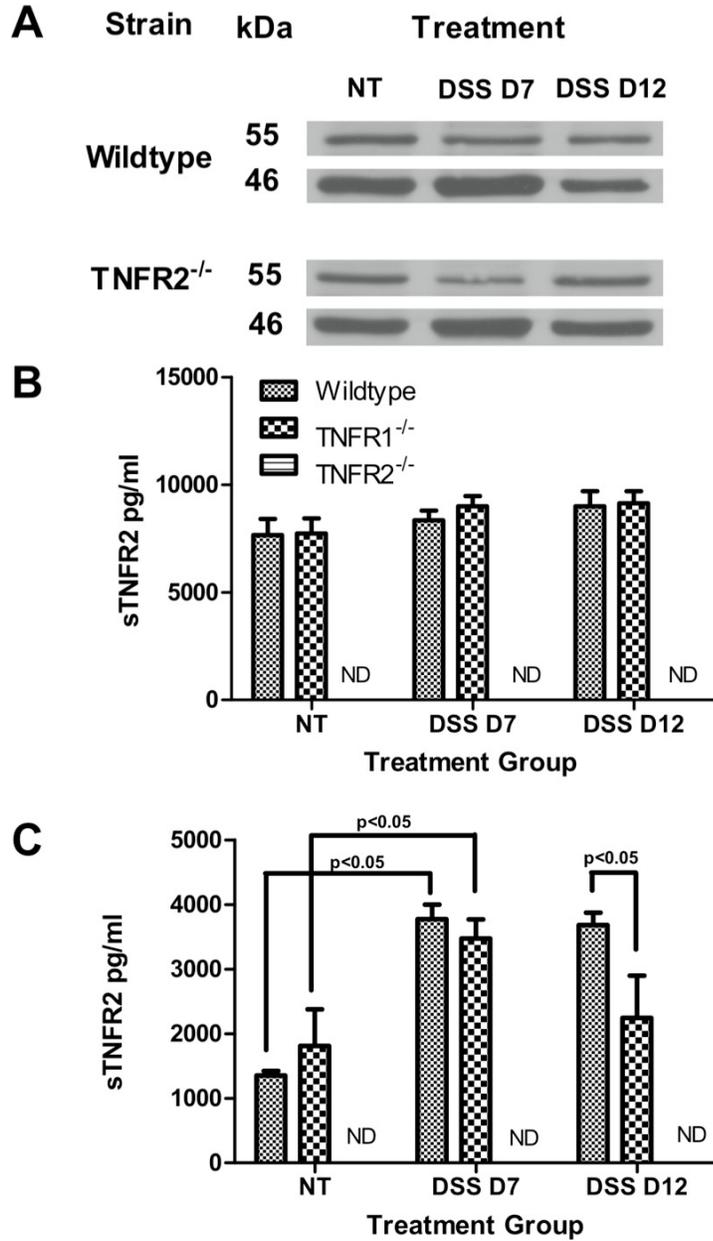


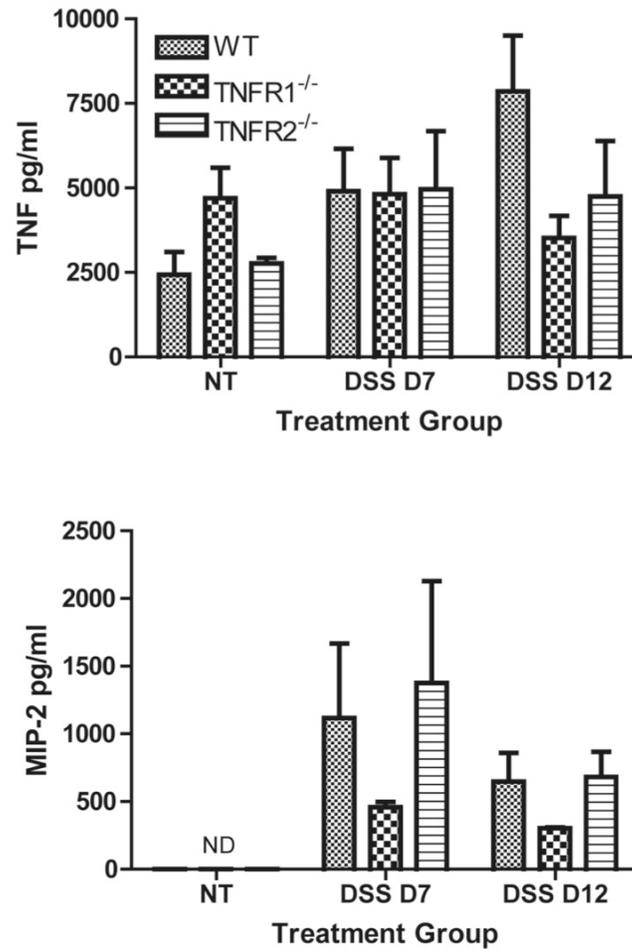
Figure 4. Histopathological disease severity scores of wildtype (WT), TNFR1^{-/-} and TNFR2^{-/-} mice treated with 3 % DSS for 5 days followed by 2 (day 7) and 7 (day 12) days of facility water.

Figure 3.5 TNFR1 And Soluble TNFR2 Expression In The Colon And Serum.



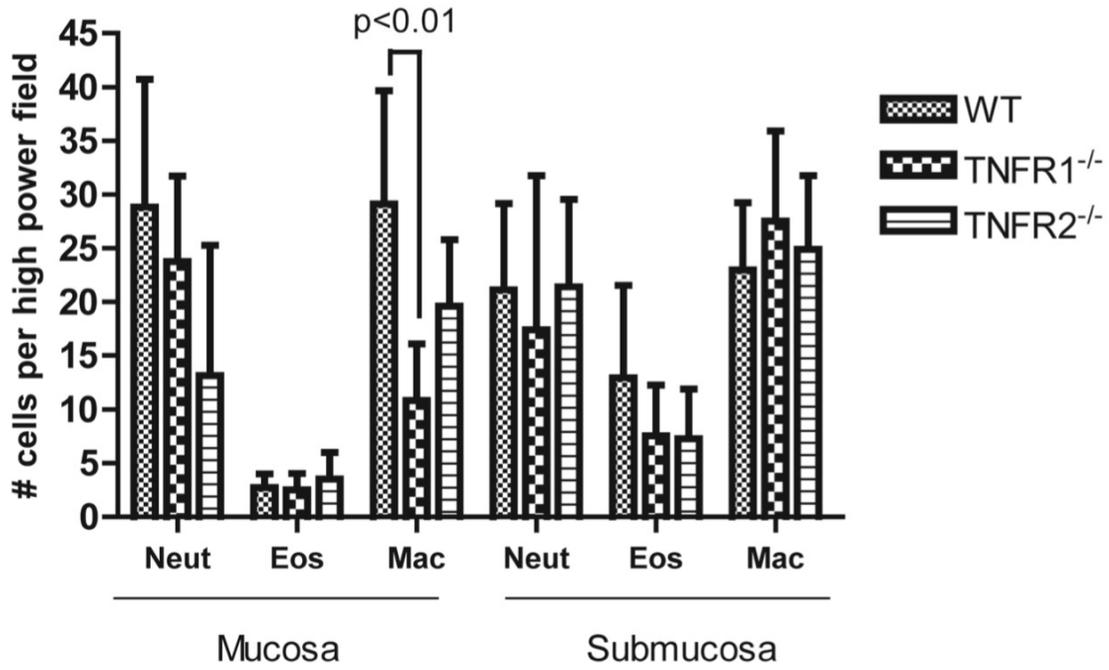
(A) Western blot detection of TNFR1 in whole colon preparations of wildtype (WT) and TNFR2^{-/-} mice (B) ELISA detection of sTNFR2 receptor levels in the serum (n=6/group) and (C) whole colon preparations of WT, TNFR1^{-/-} and TNFR2^{-/-} mice treated with 3% DSS for five days followed by two (day 7) and seven (day 12) days of regular water (n=6/group). NT, no treatment, ND, not detectable. Data are mean ± SEM.

Figure 3.6 Cytokine levels in colon homogenates from WT, TNFR1^{-/-} and TNFR2^{-/-} at days 7 and 12.



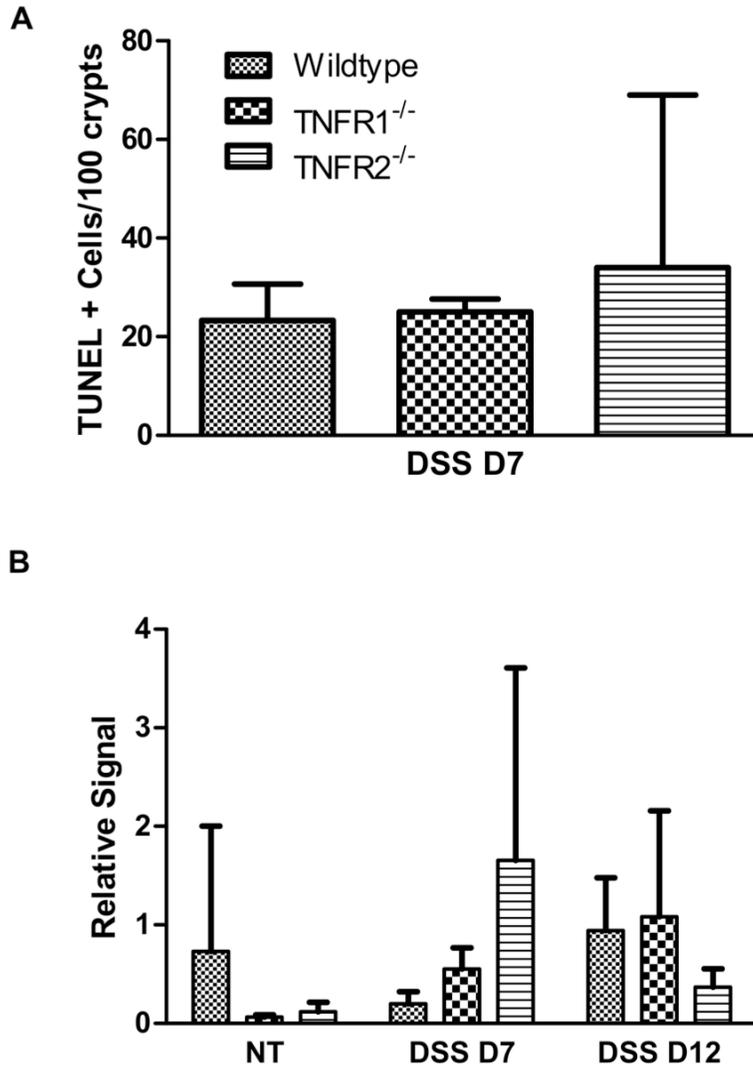
TNF (A) and MIP-2 (B) levels determined by ELISA in whole colon extracts at day 7 and day 12 (n=6/group). Data are mean ± SEM and analysed by Kruskal-Wallis ANOVA for both strain and treatment.

Figure 3.7 TNFR1^{-/-} Mice Have Fewer Colon Macrophages At Day 7



Inflammatory cell infiltrate in the colon at day 7. Formalin-fixed, paraffin-embedded sections from mice killed at day 7 were stained with congo red to differentiate between neutrophil (neut) and eosinophil (eos) granulocytes, and using an antibody to F4/80 to stain macrophages (mac). Cells were counted in 5 high power fields in ulcerated areas, distinguishing submucosa from mucosa, counted per sample (n=6/group). Data expressed as mean \pm standard deviation.

Figure 3.8 Apoptosis Is Not Different Between Strains In WT, TNFR1^{-/-} And TNFR2^{-/-} Colon Mucosa



Apoptosis in the colon. A) Formalin-fixed, paraffin-embedded sections from mice killed on day 7 were probed for TUNEL positive cells. Numbers were counted as the number of positively-stained cells per 100 intact crypts in DSS-treated mice (n=6/group). Data are expressed as mean \pm standard deviation.

B) Cleaved Caspase-3 levels in colonic tissue determined by SDS-PAGE and Western blot (n=4/group). Bars represent Cleaved Caspase-3 levels relative to actin levels. Data are expressed as mean \pm standard deviation.

Table 3.1 Mouse Characteristics
 Statistical differences were detected using a 2-way ANOVA and where an interaction occurred, Tukey's multiple comparison post-test was performed to detect differences between groups. Significant differences are denoted by superscripted letters across a row. Data presented as mean±SEM.

Measurement	WT			TNFR1 ^{+/+}			TNFR2 ^{+/+}			Significance		
	NT	Day 7	Day 12	NT	Day 7	Day 12	NT	Day 7	Day 12	Strain	Tx	Interaction
Colon Length (cm)	7.9±0.7	6.9±0.7*	7.3±0.8	8.7±0.3	7.1±0.5*	7.1±0.2*	8.7±0.4	6.4±0.7*	7.2±0.6*	NS	p<0.001	NS
Colon Wet Weight (mg)	251±78	326±69	288±40	302±94	354±61	310±60	235±16	357±36*	350±90*	NS	p<0.05	NS
Colon Weight/length Ratio (mg/cm)	32±13	47±10*	38±3	34±10	50±7*	44±9	27±0.7	56±7*	48±10*	NS	p<0.001	p<0.05
Cecum Length (cm)	2.4±0.6	1.9±0.6	2.6±0.2	2.7±0.5	2.3±0.6	2.2±0.4	3.1±0.2	2.1±0.3*	2.3±0.2*	NS	NS	p<0.05
Cecum Wet Weight (mg)	130±35	196±23*	148±27	128±27	156±19*	171±37*	136±12	173±11*	167±48*	NS	p<0.01	NS
Cecum Weight/Length Ratio (mg/cm)	55±10	108±30*	55±7**	27±23	94±11*	103±18*	44±5	90±22*	78±5*	NS	p<0.0001	p<0.05
Macroscopic Ulcers	0	2.8±1.2*	4.3±2.2*	0	2.6±0.9*	4.3±1.6*	0	7.5±3**	2.3±0.3*	p<0.05	NS	p<0.001

CHAPTER 4: TNFR1 DEFICIENCY PROTECTS MICE FROM COLITIS-ASSOCIATED CANCER AND IS ASSOCIATED WITH A DECREASED LEVEL OF OXIDATIVE DAMAGE WITHIN THE COLON.

Introduction

Crohn's Disease (CD) and Ulcerative colitis (UC) are inflammatory bowel diseases (IBD) associated with an increased risk of colorectal cancer [20, 22]. This risk increases with duration and severity of disease. Consequently, therapies that reduce inflammation often reduce the risk of colorectal cancer in affected individuals, but exactly which element of the inflammation is mechanistically contributing to cancer risk is not clear.

TNF is a major therapeutic target in IBD. TNF is a pleiotropic cytokine reportedly elevated in the serum [165, 166] and mucosa during IBD. TNF signaling through its cognate receptors, TNFR1 and TNFR2 can activate several pathways within cells of the immune system, such as NF κ B, PI3K/AKT, and MAPK/AP-1 [144]. The role of TNF in colonic inflammation remains controversial. Blocking TNF has yielded mixed success in rodent models [188]. Furthermore, genetic ablation of TNF or its receptors has not been shown to significantly reduce disease measures in several models of experimental colitis [203, 204, 363], and in some cases, exacerbated them, especially in dextran sulphate sodium (DSS) colitis [198, 200]. It is possible that TNF has different roles depending on the target cells. This is supported by the inefficacy of Etanercept, a soluble chimeric TNFR2 molecule, in IBD [181] while anti-TNF therapies targeting membrane-bound TNF on activated leukocytes are effective [348, 364, 365].

Originally, TNF was identified as the factor involved in LPS-induced tumor regression [219]. However, research in the past decade is challenging this [366], especially in models of colitis-associated cancer (CAC) [243, 252]. Using a TNF-deficient mouse model on a C57BL/6 background worsened DSS-induced colitis compared to WT controls [198] yet did not affect cancer development in a model of colitis-associated cancer in APC^{min+/-} model [367], suggesting that pathways involved in inflammation differ than those involved in cancer. In contrast, mice on a BALB/c background that lacked TNFR1 showed a reduced level of inflammation and

reduced colonic tumors after azoxymethane (AOM)+DSS-induced CAC. A tumorigenic effect was attributed to leukocyte-specific TNFR1 as chimeric WT mice irradiated and reconstituted with TNFR1^{-/-} bone marrow were protected against tumor development compared to mice reconstituted with WT bone marrow [243]. Previous studies have not addressed whether the presence of TNFR2 could affect CAC.

Components of the NFκB signaling pathway are considered integral to inflammation-mediated cancer. In a model of AOM+DSS CAC, with intestinal epithelial and leukocyte-specific IKKβ deletion, expression of IKKβ was considered crucial for tumor development [249]. Epithelial IKKβ was associated with more frequent tumors, while leukocyte IKKβ was associated with larger tumors [249]. It has been suggested that TNF-induced NFκB activation may play a significant role in a azoxymethane (AOM)+DSS model of CAC as Infliximab-treated mice showed reduced mucosal NFκB activation [252], however a direct cause-effect has yet to be directly shown *in vivo*, for example, if NFκB is activated by TNF directly in intestinal inflammation, or if it becomes activated in response to other signals as well. In addition to activating the NFκB pathway, TNF can contribute to oxidative stress by inducing the production of reactive oxygen species (ROS) via several isoforms of NADPH oxidase [316, 368]. TNF treatment can directly lead to oxidative damage and DNA mutations in cell culture [306]. ROS may contribute to tumorigenesis by damaging proteins, lipids and DNA. TNF-mediated oxidative damage has not been yet been evaluated *in vivo*.

In this study, we sought to further clarify the contribution of TNF in CAC. We previously reported no major histological differences during acute inflammation in C57BL/6 mice and TNFR1^{-/-} and TNFR2^{-/-} mice [363]. Here, we report that mice (on a C57BL/6 background) that are deficient in TNFR1 are less susceptible tumor development after chronic colitis compared to WT or TNFR2^{-/-} mice while inflammatory measures remain similar. Additionally, we evaluated colonic oxidative damage during chronic colitis in mice lacking either TNFR, on the premise that TNFR1 may be protective against the development of tumors due to the reduction of oxidative damage to sensitive cells within the crypt.

Results

We treated WT, TNFR1^{-/-} and TNFR2^{-/-} mice with 10 mg/kg AOM followed by

four cycles of 3% w/v DSS. The pathology of acute DSS colitis in these strains is described in detail in Chapter 3 [363]. Blood appeared in the stool (occult) after the second day of DSS treatment and by the fourth day, gross blood appeared in all strains treated with DSS in the first cycle. After the DSS treatment was discontinued in each cycle, the appearance of gross blood subsided by the end of the 14-day water period but occult blood was still detectable in the stool. There was no mortality associated with treatment in these strains. Three experiments, each using 8-12 mice per group, were conducted. There were no significant differences (interexperimental variation) between experiments with respect to measures of disease severity and cancer incidence therefore they were grouped together for statistical purposes. Percent weight change during each cycle was not significantly different between strain and DSS/AOM+DSS treated mice while each strain lost weight during each 5 day DSS exposure. Generally, weight lost was regained by the end of each cycle.

As the severity of inflammation has been linked to the development of colon cancer [13], we first looked at inflammation acutely in female mice (after 5 days 3% of DSS followed by 2 days of water). We previously established that histologically-detectable inflammation did not differ between WT, TNFR1^{-/-} and TNFR2^{-/-} mice [363].

In the present study, untreated mice and mice treated with 10 mg/kg of AOM alone did not have any detectable inflammation at the experiments' conclusion (Figure 4.1). In DSS-treated groups, we found that most inflammation occurred in the mid colon, while little to none occurred in the proximal colon. The distal colon showed less infiltrate and less damage and ulceration than the mid-colon, and was not significantly different among DSS and DSS+AOM-treated strains. Differences in the mid-colon inflammation scores were also not statistically significant between strains after four cycles of DSS in the DSS only and DSS+AOM groups (Figure 4.1b). Cellular infiltrate consisted of granulocytes (neutrophils and eosinophils), lymphocytes and macrophages. A cellular infiltrate was observed in the submucosa, mucosa and even the muscularis layers (Figure 4.1 d-f).

No treatment (NT), AOM-alone and DSS-alone control groups did not develop any detectable tumors or dysplasia, macroscopically or microscopically, respectively. Tumors were observed in all strains treated with DSS+AOM both macroscopically and microscopically (Figure 4.2). All three strains developed polyp-like tumors that were

non-invasive (did not penetrate into the submucosa) intramucosal adenocarcinomas, as determined by Dr. Heidi Sapp. Tumors were found exclusively in the mid-to distal colon. TNFR1^{-/-} mice had a significantly lower incidence of tumors and dysplasia after 4 cycles of DSS compared to WT and TNFR2^{-/-} both macroscopically and microscopically (Figure 4.2a), as determined by Chi-square analysis. Tumor incidence was not significantly different between WT and TNFR2^{-/-} mice. In addition, TNFR1^{-/-} mice had significantly fewer tumors per mouse compared to TNFR2^{-/-} and WT (Figure 4.2c), while WT and TNFR2^{-/-} mice had similar tumor counts per colon. Tumor size did not differ significantly between strains (Figure 4.2b).

There were no statistically significant relationships between the severity of inflammation and tumor size, or number (APPENDIX C). In addition, when the inflammation scores between mice with and without microscopically detectable dysplasia were compared, there were no significant differences (Figure 4.2d). This is an important observation as it further suggests differences in tumor development are a function of genotype and not a difference in inflammation severity.

Infiltration of Leukocytes during Inflammation in Mice Deficient in TNFR1 or TNFR2

To determine if lacking either TNFR1 or TNFR2 affects leukocyte infiltration into the colon, the expression of Ly6G (neutrophils), F480 (macrophages) were measured in the colon. We determined the extent of eosinophil infiltration because they are an innate immune cell that produces many inflammatory mediators. However, there is evidence that they play a protective role against the development of tumors [483]. No differences between strains treated with AOM+DSS were found in the number of infiltrating leukocytes (Figure 4.3).

Activated-Caspase-3

There is a precedent for TNFR1 promoting epithelial cell homeostasis in the intestine, especially when NFκB is inhibited [155, 156]. To determine the effect of treatment and genotype on colonic epithelial apoptosis, activated caspase-3 was measured by immunohistochemistry on tissue sections. We did not find any significant effect of strain on the presence of colonic epithelial cell apoptosis (Figure 4.4), although DSS-treated mice had a significantly greater number of activated caspase-3-positive cells per

100 crypts compared to untreated or AOM-treated mice.

TNFRs in the Colon

TNFR1 and TNFR2 are reportedly expressed in intestinal epithelial cells [125, 208, 369]. TNFR2 expression was demonstrated in the colonic epithelium during inflammation [125]. We tested for the presence of TNFRs in the colonic epithelium via immunohistochemistry to determine if TNF could directly affect the development of tumors by acting upon the epithelium. We found that TNFR1 was expressed within the colonic epithelium in DSS-treated and untreated mice (Figure 4.5). Because TNFR2 is so readily shed during inflammation [354], we measured soluble TNFR2 in the serum of these mice after the AOM+DSS regime. No differences were found between AOM+DSS WT and TNFR1^{-/-} mice in the level of serum sTNFR2. TNFR1^{-/-} mice treated with DSS alone had reduced levels of sTNFR2 (Figure 4.5a). No sTNFR2 was detected in TNFR2^{-/-} mice. We decided to measure sTNFR2 in the serum, and not the whole colon, as in Chapter 3, because of the availability of an anti-TNFR2 antibody that detected and localized TNFR2 within the epithelium. TNFR2 was expressed solely on infiltrating cells within the lamina propria and areas of inflammation, and was not detected in the epithelium in untreated (not shown) and treated mice (Figure 4.5d).

Colonic Cytokines

We measured cytokines present in homogenized colon by ELISA to determine if there were any differences between strains. TNF, MIP-2, and IL-4 levels in the whole colon were similar between treatment and strains when corrected for both tissue weight and protein content. Interestingly, IL-12 was significantly increased within the colon of AOM+DSS-treated TNFR1^{-/-} mice when adjusted for total protein compared to all other groups (Figure 4.6b).

Bone-Marrow Chimeras

To determine whether the tumor-promoting actions of TNF are due to the action of TNF through TNFR1 on the mesenchymal and stromal tissue, or an indirect effect due to activation of hematopoietic cells, we created bone marrow chimeras using WT and TNFR1^{-/-} mice and treated them with the AOM+DSS regime. Histological inflammation was similar between WT→WT mice and R1→R1 mice (Figure 4.7a). WT→R1

mice had similar histological inflammation to WT→WT and R1→R1. In contrast, WT mice reconstituted with TNFR1^{-/-} bone-marrow had a significantly decreased level of histologically-detectable inflammation compared to both WT→WT and WT→R1 mice. R1→WT mice showed a reduced Ly6G⁺ cellular infiltrate compared to R1→R1 mice, however, in other strains infiltration was similar (Figure 4.7b).

With regard to cancer development, WT→WT mice had an incidence similar to non-chimeric mice (87%), while R1→R1 mice failed to develop any tumors or dysplasia (incidence of 0%). It was found that mice lacking either stromal/mesenchymal (WT→R1) or bone-marrow-derived TNFR1 (R1→WT) had significantly reduced incidences of tumors compared to WT→WT (Figure 4.8a), and had incidences higher than R1→R1 mice. While tumor multiplicity was not affected by strain or bone-marrow in tumor-bearing reconstituted mice (Figure 4.8c), tumor diameter was significantly reduced in WT mice bearing TNFR1^{-/-} bone marrow compared to other groups (Figure 4.8b). These chimeric data suggest that in the C57BL/6 mice, selective leukocyte-derived TNFR1 deficiency can affect inflammation, but both hematopoietic and stromal TNFR1 expression can mediate carcinogenesis.

Role of TNFR1 in Oxidative Damage to the Colon

Whether TNF can promote tumor development through an effect of oxygen radicals has not been reported. We measured nuclear 8-OHDG, a DNA adduct formed after exposure to oxygen radicals that promotes DNA mutations favourable to tumorigenesis [371]. Untreated mice had few 8-OHDG⁺ nuclei/20 crypts (Figure 4.9a). We found a significant difference between strains treated with AOM+DSS in the level of oxidative damage to colonic epithelial cells, with TNFR1^{-/-} mice showing reduced numbers of 8-OHDG⁺ nuclei (Figure 4.9b) compared to WT and TNFR2^{-/-} mice (Figure 4.9c or d, respectively).

Discussion

A common finding is that the development of CAC is dependent on the severity and extent of inflammation [13]. In our study, despite a reduction in tumor development in TNFR1-deficient mice, inflammation was similar between strains treated with AOM+DSS. This has implications in IBD, as Etanercept was ineffective at reducing

intestinal inflammation [181], but due to its TNF-antagonizing actions, may reduce the risk of colorectal cancer.

The finding that TNFR1^{-/-} mice had reduced tumor/dysplasia incidence and tumor load is not surprising, as similar findings have been reported in other models [243, 252]. Etanercept [243] and Infliximab [252] have been shown to prevent CAC in a DSS mouse model without affecting inflammation. This has implications in clinical IBD where treatment might be stopped if it is ineffective. There may be a sub-threshold effect that could impact future cancer development.

Not all models of CAC show an effect of TNF-antagonism on the outcome of cancer. Sakai et al., showed that TNF deficiency in an *Apc*^{min+/-} on a C57BL/6 background did not lead to a reduction in chronic colitis or tumor development in mice treated with DSS [367]. In agreement with the present study, the level of colonic inflammation after DSS treatment was similar between TNF^{-/-} and TNF^{+/+} mice. However, DSS was only given once, not accounting for the remitting recurring course of the disease [367]. *Apc*^{min+/-} mice develop tumors throughout the small and large intestine, however, DSS further promotes the development of colonic tumors in these mice [83, 372]. Small intestinal tumor development was also not affected by the presence or absence of TNF. It is possible that the *Apc*^{min+/-} mutation affected any protection that TNF deficiency might have had in this model, as previous studies suggested that TNF deficiency was fatal in DSS colitis on a C57BL/6 background, the same background used in the study by Sakai et al. [198].

The finding that TNFR1^{-/-} mice develop less cancer than WT supports the prior study by Popivanova et al, 2008 [243]. In their paper, TNFR1 deficiency in BALB/c mice resulted in a reduced level of intestinal inflammation and AOM/DSS-induced cancer development within the colon. There are several distinctions between this previous study and our own. First, BALB/c mice are reportedly less sensitive to DSS treatment when compared to C57BL/6 mice [103]. It is possible that the reduced sensitivity to DSS made a difference between WT and TNFR1^{-/-} mice more evident. Conversely, BALB/c mice are much more sensitive to AOM when compared to C57BL/6 mice [75]. While Etanercept treatment lowered cancer indices without affecting inflammation, TNFR1 signaling in particular in BALB/c mice appears to be important for inflammation, while it is not in a complete TNFR1 deficiency in C57BL/6 mice.

Nevertheless, both studies concluded that TNF likely plays a pro-tumorigenic role in colitis-mediated cancer.

Despite a similar level of inflammation, tumor severity is reduced when TNFR1 is deficient in our study. This suggests that an element of TNF signaling that does not directly affect inflammation is responsible for the reduction in cancer susceptibility. It also suggests that inflammation severity may not be directly related to tumorigenesis. Supporting this finding, most of the tumors were noted in the mid-distal colon, with the majority of tumors located close to the anus. This suggests that anatomical location of the inflammation may not be as important as systemic inflammation in the development of tumors, as the most severe inflammation occurred in the mid colon in our hands. This has been reported in IBD, where microscopic colitis can influence tumor development as opposed to the presence of gross inflammation [373].

We generated bone marrow chimeras to determine whether the reduction in cancer incidence between WT and TNFR1^{-/-} mice was due to an indirect effect of TNFR1 deficiency in leukocytes versus a direct effect of TNFR1 present on epithelial cells and other stromal/mesenchymal tissue. According to our data, TNFR1 expression on both bone-marrow-derived cells and the stroma/epithelium are important for tumor development. Which cell subset expressing TNFR1 may be the contributing factor to tumor development remains to be determined. Unlike Papivanova et al., we found that epithelial/stromal TNFR1 deficiency could result in a significant reduction in tumor incidence, suggesting that epithelial expression of TNFR1 is an important determinant of cancer risk. BALB/c mice did not show epithelial TNFR1 expression, therefore it is possible that pro-tumorigenic signaling through TNFR1 did not occur [243].

In addition to reduced colon cancer, mice lacking TNFR1 expression in bone-marrow-derived cells had a significantly decreased histological pathology score compared to WT mice reconstituted with WT bone-marrow, and even TNFR1^{-/-} mice reconstituted with WT bone marrow, which is consistent with previous reports [243]. While TNFR1 deficiency itself did not lead to any inflammatory differences in non-chimeric mice, it was unexpected that leukocyte TNFR1 deficiency would be sufficient to reduce inflammatory measures. If TNFR1 is pathogenic in the bone-marrow derived tissue, it is possible that it is protective within the stromal/epithelial tissue. TNFR1 can activate NFκB and it has been suggested that NFκB activation can be beneficial in barrier

function within the colonic epithelium [155, 156], potentially affecting inflammation. TNFR1-mediated activation of hematopoietic cells may lead to the activation of COX-2 and production of pro-inflammatory lipid mediators such as prostaglandins [243]. These may also have implications in epithelial cell activation by TNF, as TNF and subsequent COX-2 activation within these cells can induce β -catenin translocation to the nucleus via prostaglandins. In addition, mice that lack COX-2 had no measurable differences in CAC indices although this did not address the targeted deletion of COX-2 in AOM+DSS CAC [258].

If TNF played a role in homeostasis of the colonic epithelium, then we would predict changes in the number of apoptotic cells within the colon. We measured activated caspase-3 as a measure of apoptosis. TNF in some circumstances can lead to IEC apoptosis [374, 375] and treatment with anti-TNF therapy may reduce colonic epithelial cell apoptosis [185]. Whether an alteration in apoptosis by TNFR deficiency would affect cancer incidence was unclear. There were no differences in the number of activated caspase-3+ cells within the colon, suggesting that TNFRs likely do not play a significant role in controlling the rate of colonic epithelial apoptosis, at least in the DSS model. While recent evidence implicates NF κ B signaling in colonic epithelial survival *in vivo* via TNFR1 signaling [155, 156], whether this occurs as a result of TNFR1 in the gut has not been directly shown. *In situ* NF κ B activation via TNFR1 has not been demonstrated *in vivo* in the colon. It is possible that other sources of NF κ B activation, such as microbial ligands, can induce survival signals within the epithelium.

In non-chimeric mice, the finding that pro-inflammatory cytokines such as TNF and MIP-2 were not different between treatments and strains is consistent with the lack of difference in inflammation between the strains. While it has been reported that serum and colonic TNF is elevated during intestinal inflammation [165], this is not always the case [170, 171]. MIP-2, or CXCL2, a product of macrophages chemotactic to neutrophils [376], signals through CXCR2. MIP-2 be important in DSS-colitis as treating mice with an antiserum to CXCR2 [110] or a genetic deficiency for CXCR2 [377] was successful in reducing measures of DSS-induced inflammation associated with a reduced neutrophil infiltration. IL-4 is reportedly elevated in the colon during chronic DSS-colitis [94]. Taken together, similarities between strains in IL-4 and MIP-2 levels in the colon confirm a similar extent of inflammation in our study.

The finding that IL-12 is elevated in TNFR1^{-/-} mice treated with AOM+DSS but not other strains is interesting. IL-12 is produced predominantly by dendritic cells inducing T-cell maturation and differentiation [378], and is also involved in the cytotoxic response by CD8⁺ T-cells and natural killer cells. An increased IL-12 production could be indicative of an increase in anti-cancer immunity [379], partially explaining a protective effect of TNFR1 deficiency in this model. In contrast, a similar increase in IFN- γ was not observed in our study (not shown), which would be expected in an anti-tumor response. Why IL-12 levels would be increased in AOM+DSS-treated mice, but not DSS-only-treated TNFR1^{-/-} mice is unclear. This elevated IL-12 expression only represents one point in time 83 days following AOM administration and chronic colitis, and it is unknown if this increased level was present earlier on, or throughout the study at some critical point in tumorigenesis.

Oxidative stress, reportedly increased in colitis [290, 380], has not previously been attributed to TNFR1 *in vivo* in colitis. TNFR1 has been shown to contribute to oxidative stress in an animal model of lung injury [381] therefore it is possible that it contributes to oxidative damage in colitis. TNFR1^{-/-} mice had significantly fewer 8-OHDG⁺ colonic epithelial nuclei compared to WT and TNFR2^{-/-} mice. 8-OHDG is a DNA adduct that occurs after oxidative damage, and is normally cleaved by an enzyme in DNA repair. 8-OHDG adducts can lead to translocations and alterations in gene expression. High levels of 8-OHDG are associated with tumorigenesis [382] and is elevated in UC patients with dysplasia [383]. As TNF has been shown to cause oxidative stress [306] and TNFR1 directly associates with Nox enzymes [316], it is possible that TNFR1 deficiency reduces oxidative stress and damage, potentially reducing CAC. Mice with a reduced capacity to produce oxygen radicals during DSS colitis show a similar level of intestinal inflammation [303] compared to WT mice suggesting that oxygen radicals may damage the epithelium independently of inflammation status. We sought to determine whether TNF could influence the production of oxygen radicals in WT and TNFR1^{-/-} neutrophils and macrophages, the most abundant source of oxygen radicals [313]. We were not able to detect TNF-mediated ROS production by either neutrophils or macrophages isolated from the bone marrow or peritoneal lavage from thioglycollate-induced peritonitis (APPENDIX E). While we cannot conclude that the reduction in oxidative damage within the epithelium of TNFR1^{-/-} mice is a result of reduced ROS

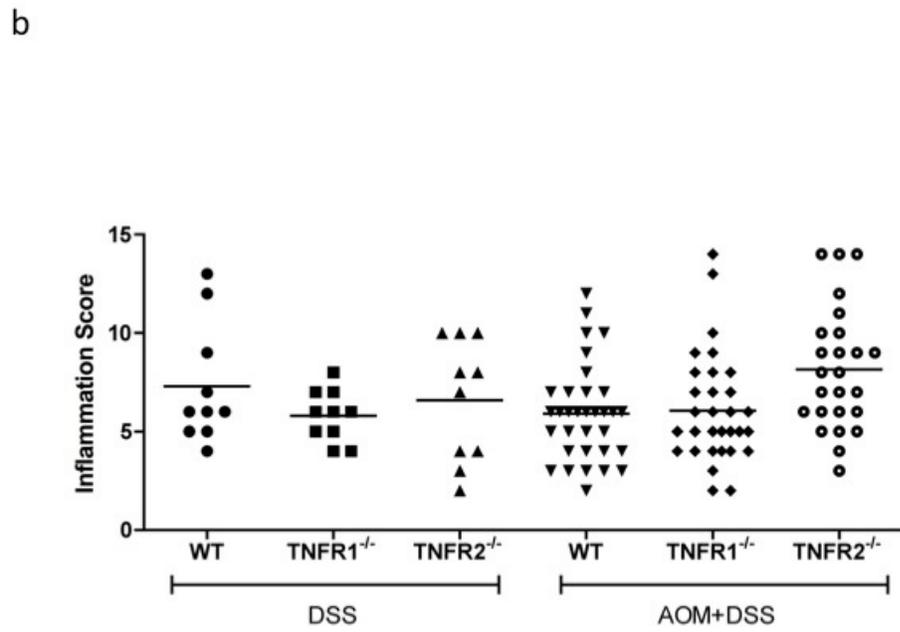
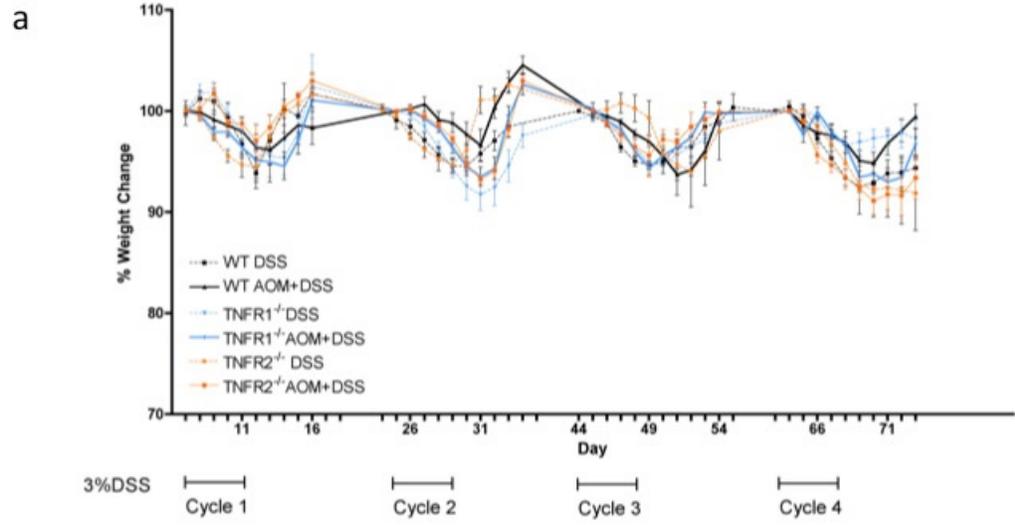
production by infiltrating leukocytes, we do know that PMA-stimulation of leukocytes showed that TNFR1^{-/-} mice were still able to produce ROS in the same capacity as WT mice (APPENDIX E).

It is possible that internal production of ROS by colonic epithelial cells, mediated by the NADPH oxidase isoform Nox1, was responsible for this difference in 8-OHDG levels. Nox1 can be directly activated [318] and upregulated by TNF [368], and it has been suggested that Nox1 may play an important role in inflammation-mediated tumorigenesis in the colon [384]. The role of ROS in CAC has been investigated combining the use of antioxidants which reduced DSS-mediated CAC [385]. Additionally, glutathione peroxidase-deficient mice develop spontaneous colitis when exposed to commensal bacteria and subsequent ileal and colon cancer [386]. In these mice the inflammation is associated with an increased number of mutations [324].

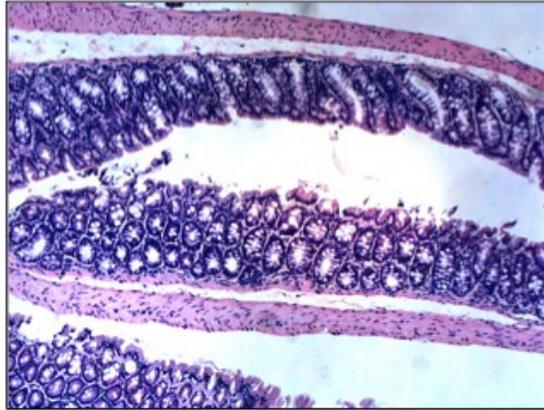
In conclusion, TNFR1 deficiency is protective against the development of colitis-associated cancer and this protective effect exists independent of the severity of inflammation. Both leukocytic and stromal TNFR1 expression are important in this protective effect. Finally, a reduction in oxidative damage may account for the protective effect of TNFR1 deficiency, however, more research is needed to determine the mechanisms by which this occurs.

Figure 4.1 Inflammation In WT, TNFR1^{-/-} And TNFR2^{-/-} Mice Treated With 10mg/Kg Azoxymethane Followed By 4 Cycles Of 3% DSS.

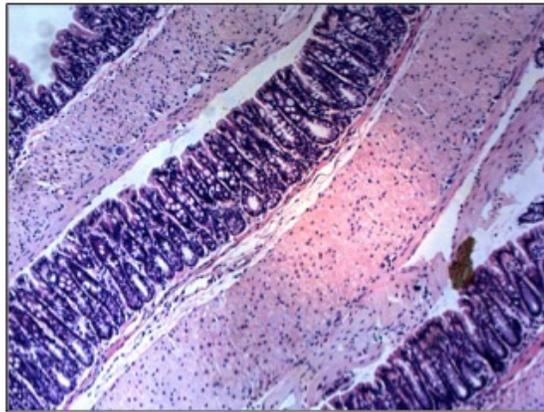
- a) Weight change during the 83-day azoxymethane (AOM) and dextran sulphate sodium (DSS) regime. Graph shows AOM+DSS treated wildtype (WT), TNFR1^{-/-} and TNFR2^{-/-} mice from days 1-12 of each cycle. Values are a percent change in weight from the beginning of each cycle as there was a net weight gain from the beginning to the termination of the study. Data are presented as mean ± SEM and statistics were performed using repeated measures ANOVA with a p<0.05 considered significant.
- b) Histologically-detectable inflammation in hematoxylin and eosin-stained colon sections after AOM+ 4 cycles of 3% DSS. Sections were scored based on a previously validated scale based on presence of inflammatory cells, ulceration, crypt damage and edema. Horizontal lines indicate the mean, and statistics were performed using non-parametric ANOVA.
- c) WT AOM only-treated mouse H&E-stained section 100x magnification
- d) TNFR1^{-/-} AOM only-treated mouse H&E-stained section 100x magnification
- e) TNFR2^{-/-} AOM only-treated mouse H&E-stained section 100x magnification
- f) WT DSS only-treated mouse H&E-stained section 100x magnification
- g) TNFR1^{-/-} DSS only-treated mouse H&E-stained section 100x magnification
- h) TNFR2^{-/-} DSS only-treated mouse H&E-stained section 100x magnification
- i) WT AOM+DSS-treated mouse H&E-stained section 100x magnification.
- j) TNFR1^{-/-} AOM+DSS-treated mouse H&E-stained section 100x magnification.
- k) TNFR2^{-/-} AOM+DSS-treated mouse H&E-stained section 100x magnification



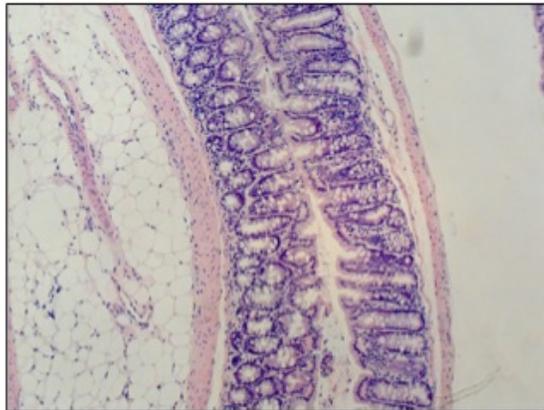
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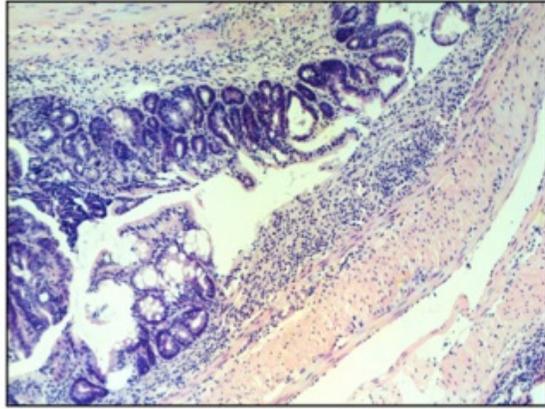
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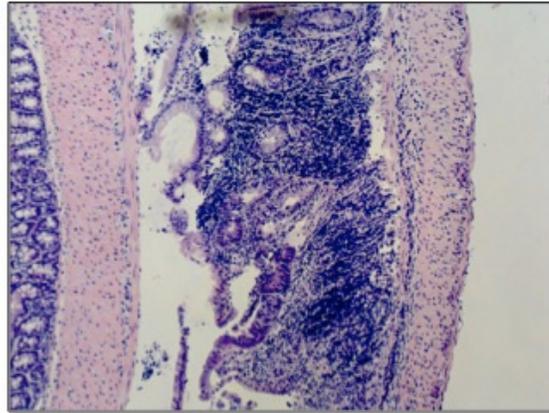
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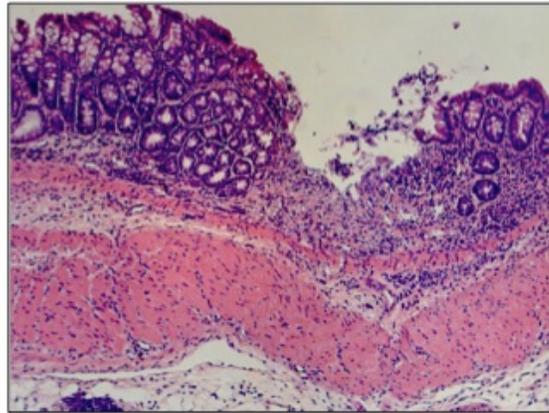
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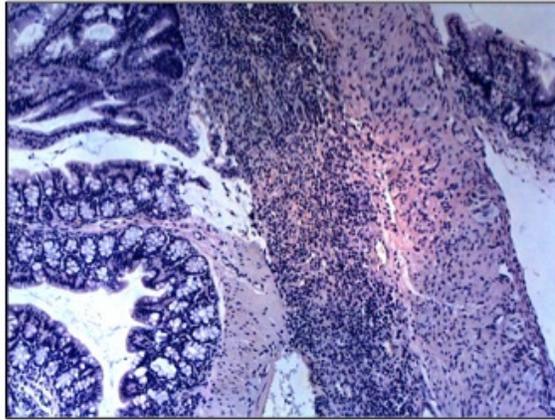
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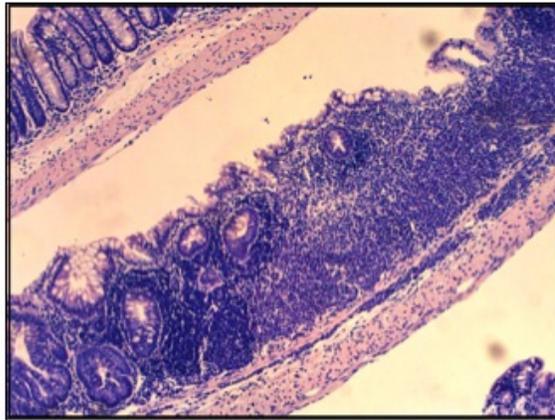
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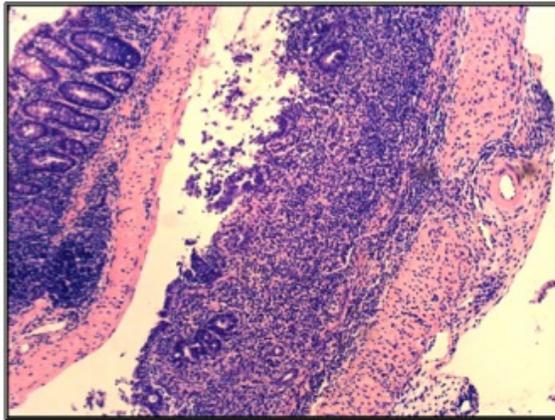


Figure 4.2 TNFR1^{-/-} Mice Have A Reduced Tumor And Dysplasia Incidence And Tumor Multiplicity After Treatment With 10mg/Kg Azoxymethane Followed By 4 Cycles Of 3% DSS.

a) Tumor (macroscopic) and dysplasia (microscopic) incidence (%) in WT (n=33), TNFR1^{-/-} (n=32) and TNFR2^{-/-} (n=25) mice treated with 10mg/kg AOM followed by 4 cycles of 3% w/v DSS. Significance (p<0.05) determined by χ^2 analysis with a power of 0.9.

b) Tumor size (average of 3 diameters in mm) in WT, TNFR1^{-/-} and TNFR2^{-/-} mice treated with AOM+DSS. No statistical differences were found with a one-way ANOVA between strains. Data presented as mean±SEM.

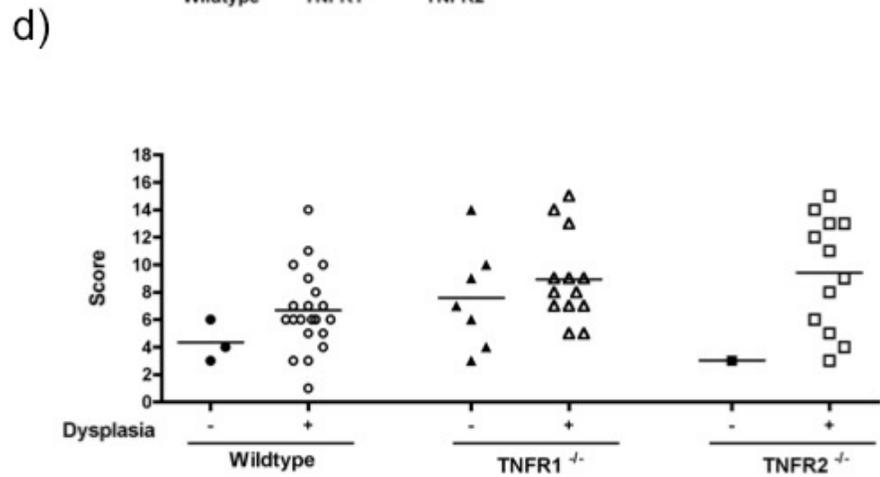
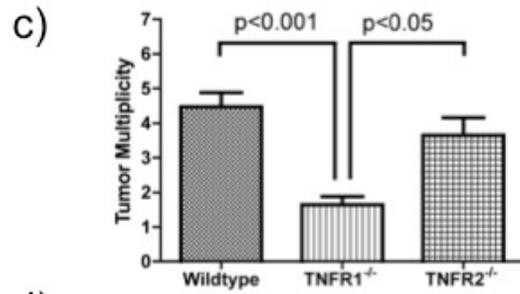
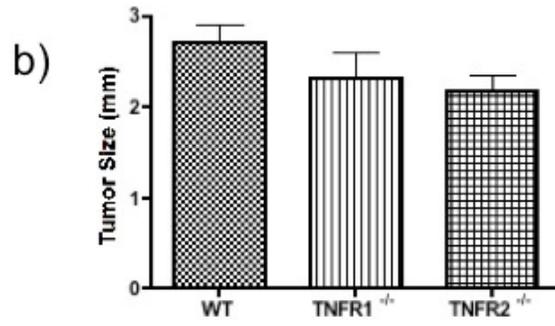
c) Tumor Multiplicity (gross tumor count) in WT, TNFR1^{-/-} and TNFR2^{-/-} mice treated with AOM+DSS. Gross tumors were counted in the whole colon under a dissecting microscope. A one way-ANOVA found significant differences between strains and differences between strains were determined with a Tukey's Multiple Comparison Post-test. Data presented as mean±SEM.

d) Inflammation score in mice with and without dysplasia/tumors to determine if there is a difference in level of inflammation based on tumor presence

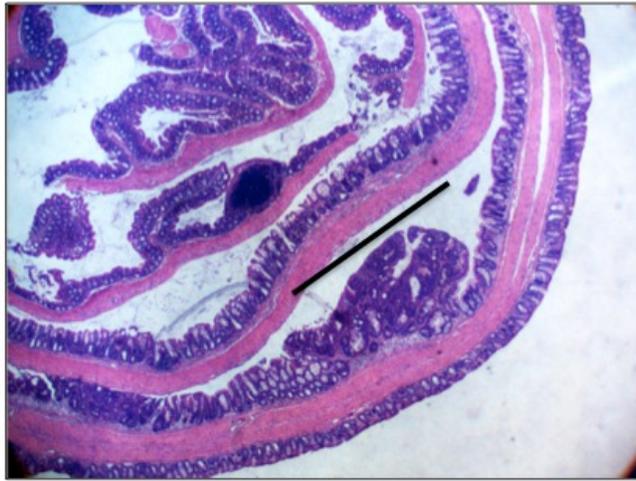
e-g) Hematoxylin and Eosin-stained tissue sections depicting adenocarcinomas in each of the three strains WT (e), TNFR1^{-/-} (f), TNFR2^{-/-} (g) at 25x magnification. Line denotes an adenocarcinoma.

a)

% Incidence	WT	TNFR1 ^{-/-}	TNFR2 ^{-/-}
Gross Tumors	82.60%	50%	89.40%
Dysplasia	87%	54%	100%



e



f



g

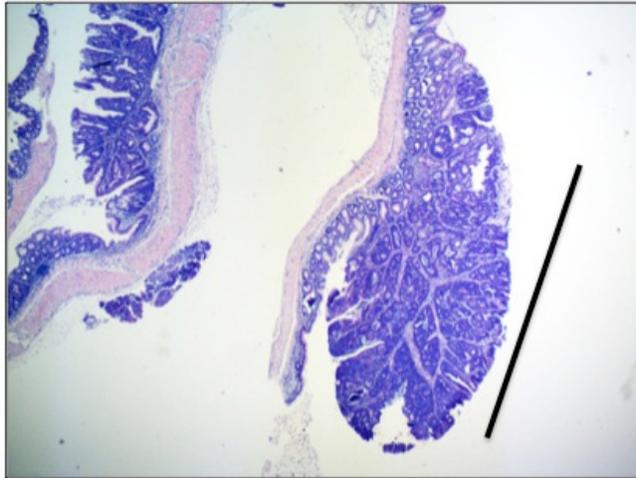
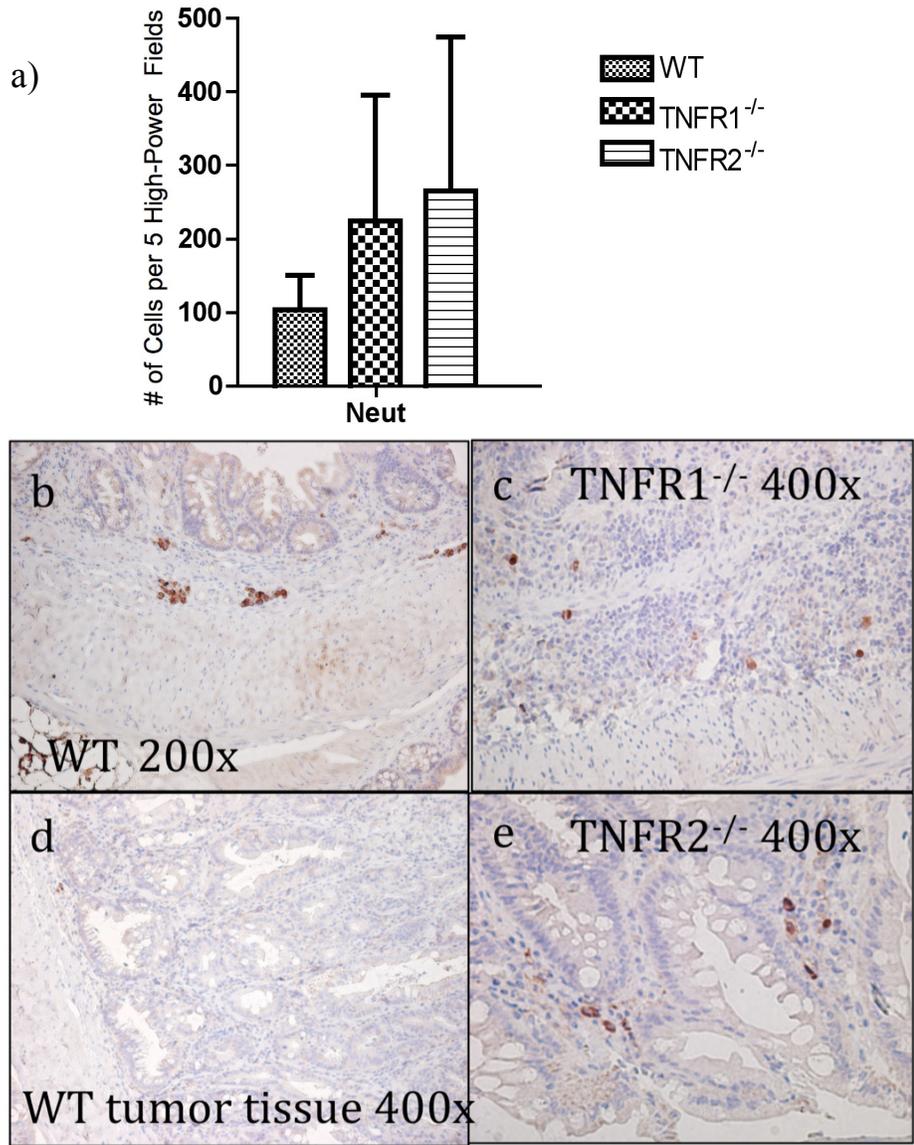


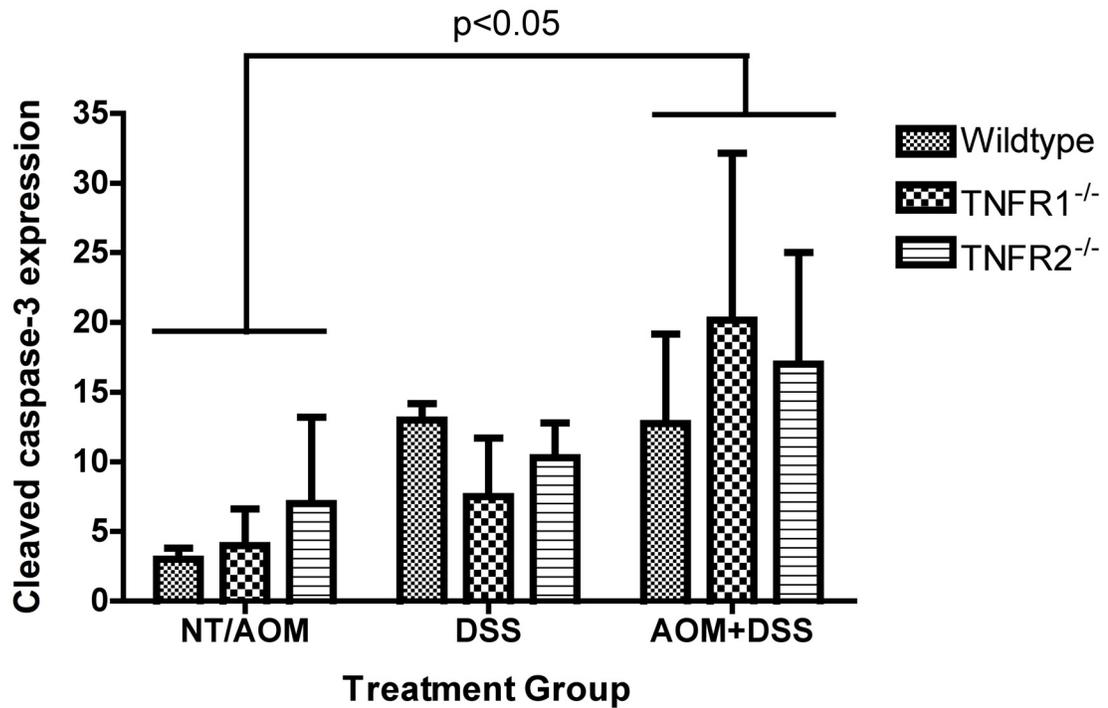
Figure 4.3 Infiltration Of Leukocytes After AOM+ 4 Cycles Of 3% DSS Treatment



a) Neutrophils (neut, Ly6G+) and Eosinophils (Eos, Congo red+) were counted in 5 high power fields in AOM+DSS-treated mice. Neutrophils were counted at 400x magnification in areas of inflammation with an inflammation score of at least 3 (out of 5) within that field. b-e) F480+ macrophages in AOM+DSS treated mice were not different between strains, as measured by Kruskal Wallis ANOVA. d) Tumor tissue showing an absence of F480+ cells.

Figure 4.4 Apoptosis In The Colon Of WT, TNFR1^{-/-} And TNFR2^{-/-} Mice Is Not

Different Between Strains



Activated (cleaved) Caspase-3+ cells measured by immunohistochemistry in No Treatment (NT)/AOM-treated groups (n=6/group, 3 NT, 3 AOM-only, pooled), DSS only-treated groups (n=6 per group), and AOM+DSS-treated groups (n=6 per group) were counted within 100 intact crypts within the colon of WT, TNFR1^{-/-} and TNFR2^{-/-} mice at a magnification of 400x. Mice treated with AOM+DSS had significantly greater activated Caspase-3+ cells per 100 crypts compared to untreated and AOM treated mice, as measured by a Kruskal-Wallis One-way ANOVA for both strain and treatment.

Figure 4.5 TNFR1 and TNFR2 Expression in the Epithelium

TNFR1 and TNFR2 expression was determined by immunohistochemistry in WT mice to determine localization of receptor expression in the colon. a) Negative control in an untreated WT mouse section with no primary antibody application (400x), b) TNFR1+ cells in the colon in untreated mouse colon section (400x), c) TNFR1 expression in a AOM+DSS-treated mouse section (400x) d) TNFR2 expression in AOM+DSS-treated mouse section, with TNFR2 expression only found in infiltrate (400x), e) soluble TNFR2 levels in the serum as measured by ELISA in WT, TNFR1^{-/-} and TNFR2^{-/-} mice. Significant difference denoted by *.

Figure 4.6 Colonic IL-12 is elevated in TNFR1^{-/-} mice, but other cytokines in the colon are not affected by strain

Cytokines were measured via ELISA in colon extracts from DSS-only and DSS+AOM-treated mice as outlined in Chapter 2. Data are presented as mean±SEM (for DSS-only group n=6-8, AOM+DSS groups n=10-15) and expressed per mg of protein as determined by Bradford assay of extracts. Statistics were determined by Kruskal-Wallis ANOVA for both strain and treatment. Significant differences denoted with a star (p<0.05).

- a) TNF levels pg/mg of protein in colon extracts
- b) IL-12 levels pg/mg of protein in colon extracts
- c) IL-4 levels pg/mg protein in colon extracts
- d) MIP-2 levels pg/mg protein in colon extracts

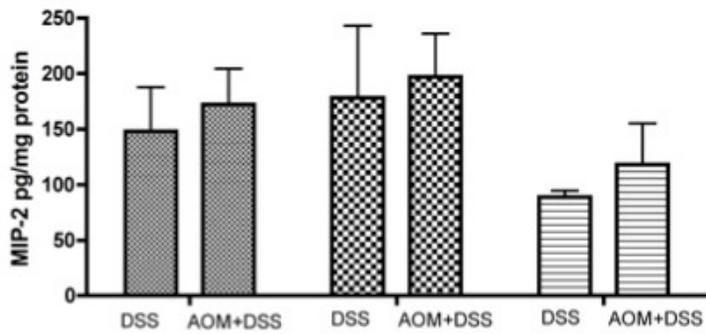
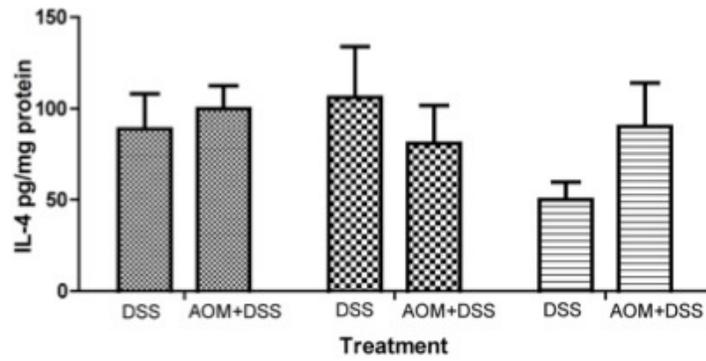
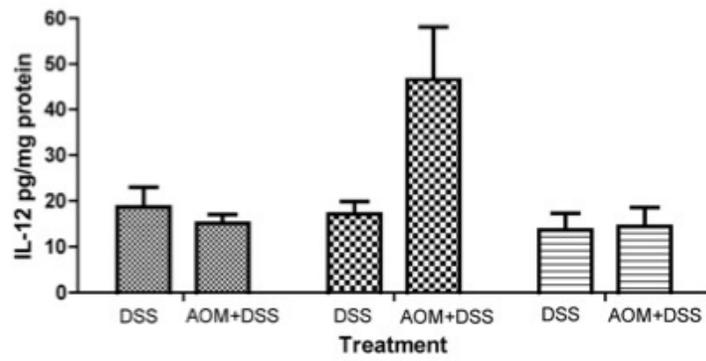
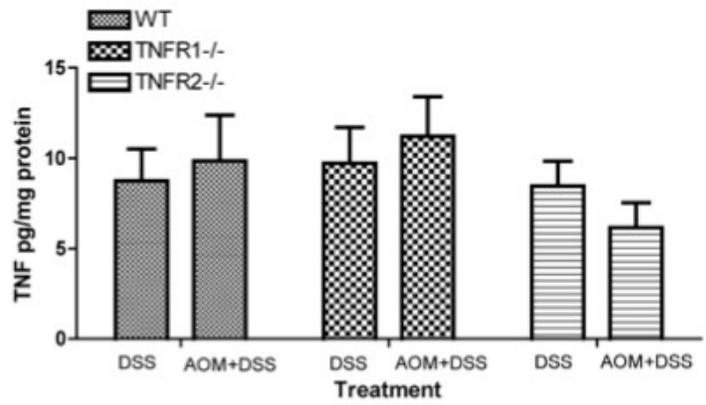
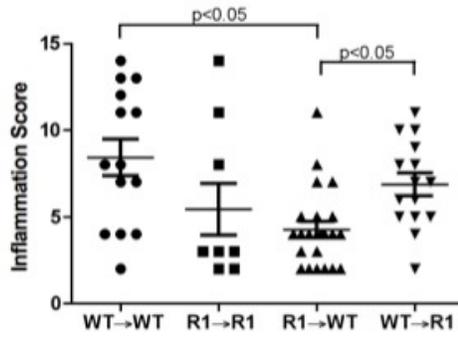


Figure 4.7 TNFR1 On Bone-Marrow-Derived Cells Contributes To Chronic Intestinal Inflammation

- a) Inflammation scores of chimeric mice treated with 10 mg/kg AOM+ 4 cycles of 3% w/v DSS for 4 cycles as previously described. Data presented as mean (denoted by horizontal line), and plotted as individual points. Statistics were performed as Kruskal-Wallis One-way ANOVA with a Dunn's post-test to determine differences between groups ($p < 0.05$). Number of animals per group: WT→WT n=14, R1→R1 n=8, R1→WT n=23, WT→R1 n=15.
- b) Ly6G⁺ cells within inflamed areas in 5 high-powered fields of colon sections from chimeric mice as measured by Kruskal-Wallis One-way ANOVA.
- c) –f) Hematoxylin and Eosin-stained sections from chimeric mice at 100x magnification showing areas of inflammation in each group.

a)



b)

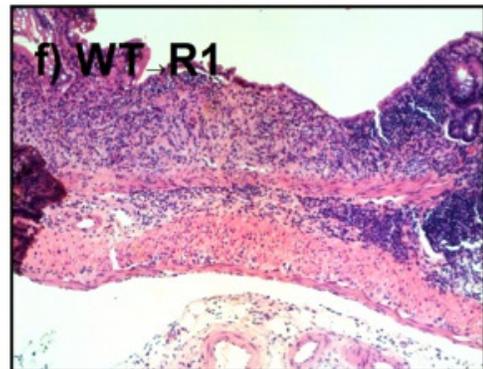
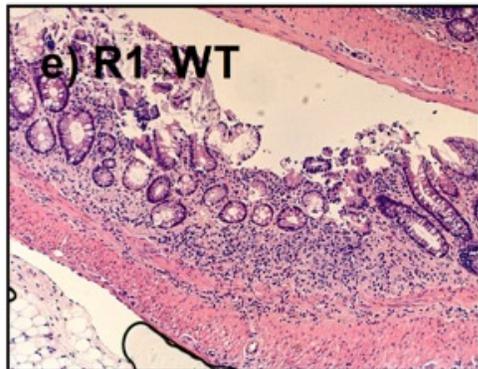
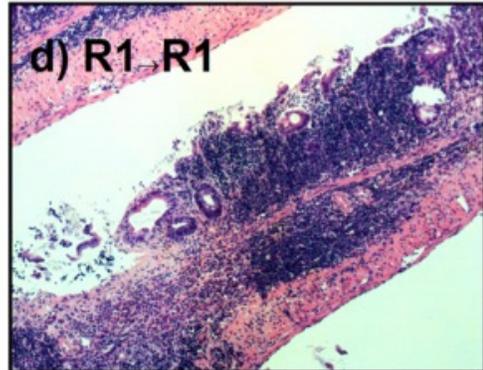
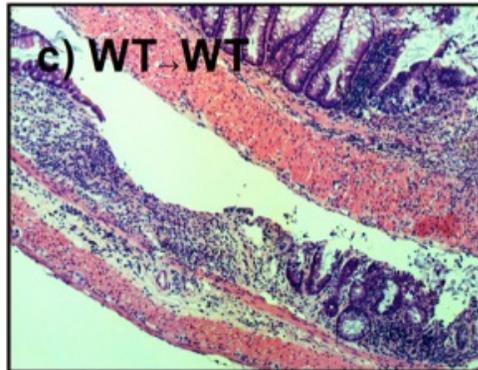
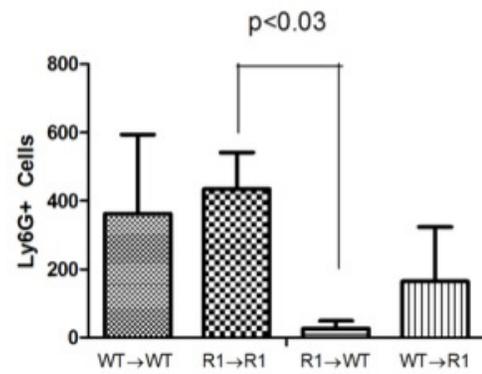


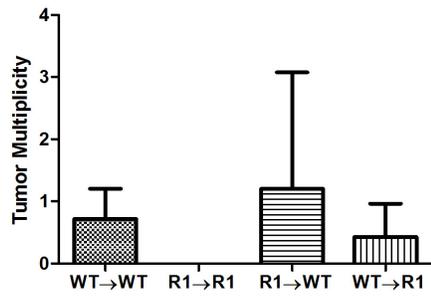
Figure 4.8 TNFR1 On Bone-Marrow-Derived Cells And Stromal Cells Play A Role In The Development Of Colitis-Associated Cancer.

- a) Incidence of tumors and dysplasia in chimeric mice treated with 10mg/kg AOM+ 3% w/v DSS for 4 cycles as previously described. χ^2 analysis was performed to detect statistical differences in incidence between groups (WT→WT n=14, R1→R1 n=8, R1→WT n=23, WT→R1 n=15).
- b) Tumor size from tumor-bearing mice measured as an average of 3 diameters (mm). A one-way ANOVA with a Tukey's Multiple Comparison post-test was used to determine differences between groups.
- c) Tumor multiplicity in tumor-bearing mice as determined by gross examination, with the exception of R1→R1 mice, which did not develop tumors. A one-way ANOVA was used to determine statistically significant differences between groups in tumor-bearing mice.
- d) Hematoxylin and eosin stained sections from WT→WT mouse (25x). Lines denote tumors.
- e) Hematoxylin and eosin stained sections from R1→R1 mouse (25x)
- f) Hematoxylin and eosin stained sections from R1→WT mouse (25x)
- g) Hematoxylin and eosin stained sections from WT→R1 mouse (25x)

a

% Incidence	WT→WT	R1→R1	R1→WT	WT→R1
Gross Tumors	86%	0%	41%	43%
Dysplasia	86%	0%	46%	43%

c



b

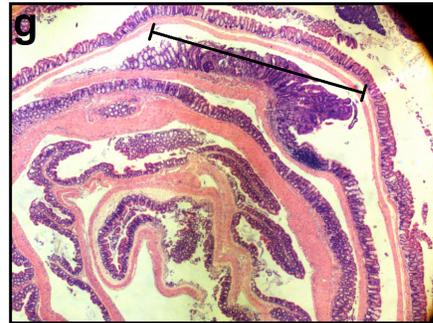
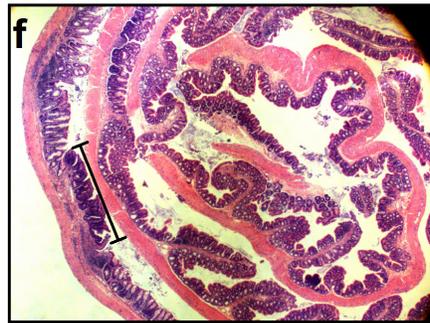
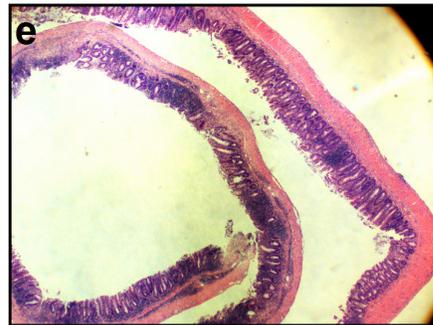
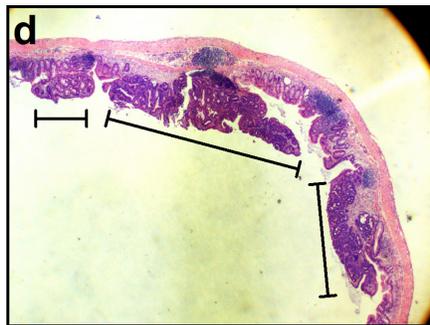
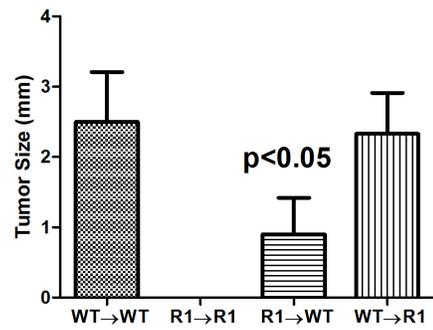
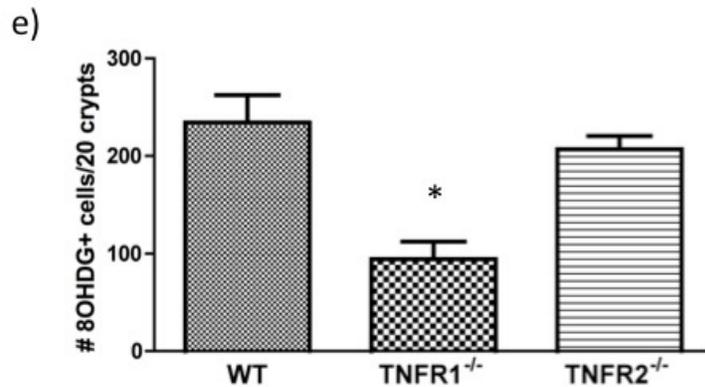
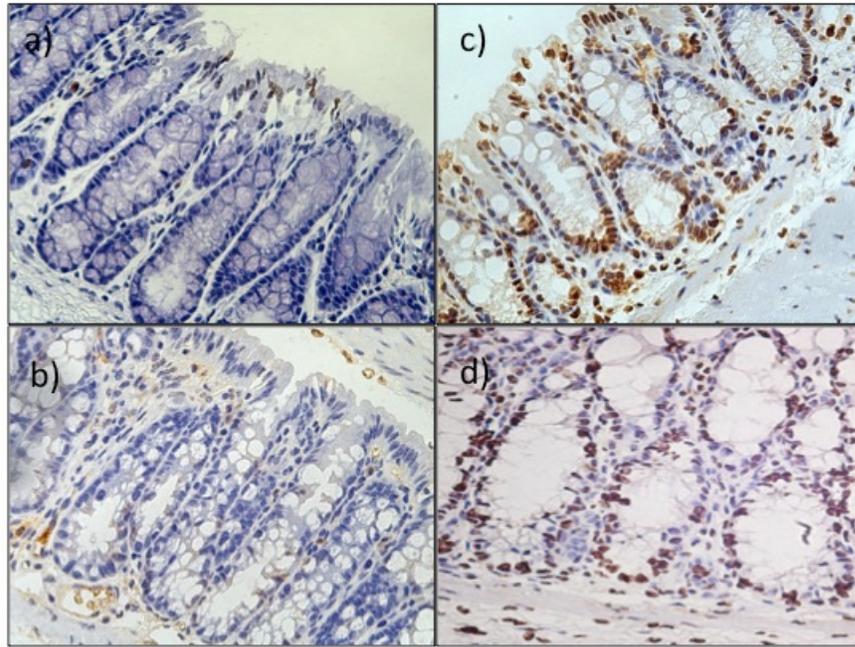


Figure 4.9 8-OHDG+ Colonic Epithelial Nuclei Are Reduced In TNFR1^{-/-} Mice, Suggesting A Reduction In Oxidative Damage In These Mice.



a-d) Photomicrographs of 8-OHDG+ cells within colonic epithelium of AOM-only-treated WT (a), AOM+DSS-treated TNFR1^{-/-} (b), and AOM+DSS-treated WT (c) and AOM+DSS-treated TNFR2^{-/-} mice (d). Magnification 400x, positive nuclei show brown staining.

e) The number of 8-OHDG+ cells within 20 intact colonic crypts from AOM+DSS-treated mice, * denotes statistically significant difference (p<0.05) determined by a Kruskal-Wallis one-way ANOVA with a Dunn's multiple comparison post-test.

CHAPTER 5: MICE LACKING A FUNCTIONAL NADPH OXIDASE 2 ARE PROTECTED AGAINST COLITIS-ASSOCIATED CANCER

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and Ulcerative colitis (UC), are conditions in the gastrointestinal tract associated with oxidative stress [288]. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the mechanisms to control these, namely, the ROS-scavenging system of antioxidants [288]. Several lines of evidence support an imbalance in the pathogenesis of IBD. First, the treatment of experimental colitis with antioxidants has shown success in reducing inflammation [387]. Next, impaired antioxidant defense systems have been shown in IBD [388-390] and animal models of colitis [391]. Whether this impairment is a result of an overwhelmed antioxidant defense system during inflammation, or a precursor to inflammation is not clear [304]. Third, measures of oxidative stress from the plasma are higher in IBD patients [290, 388, 392]. Collectively, this suggests that excess production of ROS or an impaired ability to neutralize them contributes to inflammation within the colon. Mice with a deficiency in two glutathione peroxidase enzymes develop a spontaneous colonic inflammation when exposed to conventional housing [322], suggesting that this balance is necessary for homeostasis in the colon.

ROS are produced as byproducts from a range of metabolic processes, including energy metabolism, and are also produced by cells of the immune system to kill bacteria [393]. Normally, extra ROS are scavenged and neutralized by antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase. Failure of these systems could lead to tissue and cell damage by ROS attack of proteins, lipids and nucleic acids. Damaging these components affect a wide range of cellular processes, including cell cycle control, and DNA repair, leading to mutations [393].

During IBD, ROS are produced in abundance and the resulting tissue and cellular damage and the accompanying molecular alterations likely contribute to carcinogenesis. Evidence supporting this idea comes from studies that show that the addition of a strong oxidant, iron, to the diet of mice treated with dextran sulphate sodium (DSS), increases the frequency of tumors in inflamed mice [73]. Impaired antioxidants in dysplastic tissue in DSS colitis has also been reported [394]. In addition, treatment with an

antioxidant can reduce the severity of colitis-associated cancer [385]. Finally, the double glutathione peroxidase-deficient murine model of colitis develops inflammation and cancer after exposure to commensal microflora in conventional housing [325]. The inflammation in this model is associated with a number of molecular alterations that could lead to tumorigenesis [324]. Together, these studies suggest a role for excess ROS and oxidative damage in CAC.

NADPH oxidase 2 (Nox2) is an enzyme complex the function of which is critical in the formation of ROS involved in phagocyte function [395]. Integral in this complex are protein subunits p47phox, p22phox, p67phox and the glycoprotein 91 (gp91) catalytic core. Other important members of this complex include Rac1, Rac2, which interact with the p67phox [395]. Several isoforms of NADPH oxidase exist, but the Nox2 isoform is the most prevalent form within the immune system particularly in neutrophils and macrophages [313], the frontline innate immune cells involved in clearing bacterial infection. Individuals lacking a functional Nox2 can develop chronic granulomatous disease, an immunodeficiency where leukocytes are unable to mount an effective response to bacteria, and instead surround the infection with numerous effector cells creating granulomas.

The contribution of the Nox2 enzyme in colitis has been investigated in three studies of DSS colitis. One study, using a strain of mouse deficient in the p47phox subunit, found no major differences in the severity of inflammation and illness compared to WT mice on a C57BL/6 background [303]. The second and third studies used a strain of mice lacking Nox2 [304, 396]. While one found a pathogenic effect of Nox2 in DSS colitis [304], the other did not report differences in clinical measures (such as weight loss and stool consistency) from the WT strain [396]. The study by Mori et al., [396] did not include histopathological data so comparisons are difficult. Notwithstanding differences in their protocol, these conflicting reports suggest that a role for Nox2 in colitis warrants further investigation. In addition, no data exists for the role of Nox2 in chronic colitis and its participation in tumorigenesis, despite Nox2^{-/-}-expressing cells purportedly contributing to the development of colitis-mediated cancer [396]. Here, we report that there is no significant difference in the severity of acute colitis between WT and gp91^{-/-} (Nox2) mice after 5 days of 3% DSS treatment. In addition, Nox2^{-/-} mice treated with 10mg/kg azoxymethane (AOM) and 4 cycles of 3% DSS were protected against tumor

formation compared to WT.

Results

Acute Colitis

Eight-12-week old male C57BL/6 (WT) and Nox2^{-/-} mice were fed 3% DSS for five days followed by two days of reverse osmosis-purified water. Expectedly, no signs of spontaneous inflammation were observed in untreated Nox2^{-/-} mice. In both strains, occult blood appeared in the stool at two days after DSS commencement. At the termination of the experiment, gross blood was visible within the colons of both strains. Both strains developed enlarged, soft stools, but did not develop diarrhea. Neither weight change over the seven-day period nor clinical scores differed significantly between groups (Figures 5.1a and 5.1b) with both groups losing as much as 2-5% of their original weight by day seven.

Inflammatory Measures

At the end of the seven-day experiment, colons were removed, and sections were formalin-fixed and paraffin-embedded then sectioned and stained with hematoxylin and eosin for assessment of inflammation based on the criteria outlined in Table 2.2. There were no significant differences found between Nox2^{-/-} and WT with respect to histopathological disease severity (Figure 5.1c). Both strains showed infiltration of leukocytes within the submucosa and ulceration of the epithelium (Figure 5.1d-e). In addition, the number of Ly6G⁺ cells were similar between strains (Figure 5.1f). Untreated mice did not show any Ly6G⁺ leukocyte infiltration into the colon (data not shown).

Chronic Colitis

To study colitis-associated cancer, mice were treated with 10mg/kg of AOM followed by 4 cycles of 3% DSS. Weight loss and clinical illness was assessed. During the first two cycles, weight loss and clinical illness scores did not differ significantly between strains. Unlike our previous experiments, WT mice were very sensitive to this regime and many had to be euthanized before the completion of the experiment. In contrast it was found that Nox2^{-/-} mice were significantly protected from weight loss

following the third and fourth cycles (Figure 5.2a). In addition, their clinical scores in these two cycles were lower compared to WT mice. A survival curve indicated that WT mice experienced a significantly greater mortality compared to Nox2^{-/-} mice, with mortality beginning following the third cycle (Figure 5.2b). When the animals that were euthanized early were assessed by necropsy, it was found that there were extremely large tumors causing obstructions in the bowel. The colons were distended and lacking muscle tone. Weight loss and clinical illness scores were not significantly different between DSS-only treated mice despite slightly greater weight loss and clinical scores in these mice.

At necropsy, colon lengths and weights were measured and it was found that in both strains, colon length was significantly shorter after DSS treatment, however, Nox2^{-/-} mice had significantly greater colon lengths after 4 cycles of AOM+DSS compared to WT mice (Table 5.1). In addition, AOM+DSS-treated WT mice had a significantly greater colon weight compared to untreated/AOM-treated WT mice, while AOM+DSS-treated Nox2^{-/-} mice did not. Weight: length ratios were also unchanged after AOM+DSS treatment in Nox2^{-/-} mice while WT mice had a greater weight:length ratio compared to untreated WT mice (Table 5.1).

Histopathological inflammation scores were not significantly different between the AOM+DSS-treated strains or the DSS-only strains at day 83 (Figures 5.2c-e). Both strains had significant influx of leukocytes including neutrophils and macrophages. In addition, both strains showed leukocyte extravasation from blood vessels in the muscularis layer tunneling up through to the submucosa, which could lead to the development of fistulas, often observed in severe CD [397]. It should be noted that histopathological scores were only performed on those mice that survived to the completion of the study, therefore they might not completely reflect the true scores as those mice that were euthanized earlier in the cycle were not included in the analysis.

Tumor Development in WT and Nox2^{-/-} Mice

When cancer was assessed in these two strains, it was found that Nox2^{-/-} mice were protected from colitis-associated cancer. While the incidence of macroscopic colon tumors and microscopic dysplasia was 100% in WT mice, only 58% of Nox2^{-/-} mice developed macroscopic tumors and 71% of Nox2^{-/-} mice developed dysplasia (Figure

5.3a). In addition, *Nox2*^{-/-} mice had significantly smaller and fewer tumors compared to WT mice, (Figures 5.3 b, c respectively). Tumors that developed in the mucosa in each strain were adenocarcinomas *in situ*, and neither strain displayed any evidence of breaching the basement membrane. Tumors within the colon of close to 50% of WT mice were very large, and several appeared to be obstructing the colonic lumen.

Ly6G+ Infiltrate into Inflammatory Lesions and Tumors in WT and *Nox2*^{-/-} Mice

Examination of blood smears revealed no differences in the proportions of white blood cells (polymorphonuclear cells, monocytes and lymphocytes) within the blood of these mice (Figure 5.2f), suggesting that *Nox2*^{-/-} deficiency does not affect the migration of leukocytes into the bloodstream. Ly6G⁺ cells, which are mostly neutrophils, were enumerated within the colonic mucosa and submucosa in areas with inflammation scores of at least 3. No difference between WT and *Nox2*^{-/-} mice treated with both AOM and DSS were found in the number of neutrophils (Figure 5.4). In addition, DSS-only-treated mice showed no strain-related differences in neutrophil numbers in 5 high power fields (not shown). Neutrophils can influence tumor growth and angiogenesis by secreting matrix metalloproteinases and other mediators [275, 398]. Significant neutrophil numbers were noted within tumors of mice, most abundant around the luminal surface of the tumor (Figure 5.5a,b). When Ly6G⁺ cells were enumerated within dysplastic tissue, it was found that *Nox2*^{-/-} mice had a reduced Ly6G⁺ cellular infiltration into tumors and dysplastic tissue compared to WT mice (Figure 5.5c). In areas of inflammation, Ly6G⁺ cell numbers were similar between AOM+DSS-treated mice.

The number of F480⁺ macrophages were not different between strains and treatments (Figure 5.5 d-e). They were present within both tumors and the lamina propria of the colon, as well as in areas of inflammation.

8-OHDG is not Different Between WT and *Nox2*^{-/-} Mice

8-OHDG was measured by immunohistochemistry to determine if *Nox2* deficiency affected the amount of oxidative damage detectable within the colon. Unexpectedly, the number of 8-OHDG⁺ nuclei within intact crypts was not different between WT and *Nox2*^{-/-} mice treated with AOM+DSS (Figure 5.5).

Discussion

This study examined acute and chronic colitis in a mouse strain lacking Nox2, the catalytic core of an important enzyme involved in antibacterial immunity. Acutely, mice were not protected against the development of colitis after DSS treatment. Chronically, Nox2^{-/-} mice were protected clinically against illness after AOM+ four cycles of 3% DSS treatment, with WT mice suffering significant morbidity and mortality. In addition to clinical measurements, post-necropsy measurements of colon length, weight, and weight:length ratio suggest that Nox2^{-/-} mice do not develop colitis as severely as do WT mice. Colon shortening has been reported in DSS colitis as a rough measure of damage and fibrosis [77], while weight:length ratio can give an indication of edema within the tissues. Nox2^{-/-} mice also developed a lower incidence of tumors and dysplasia compared to WT mice, while histological inflammation scores were not significantly different. This suggests that while Nox2^{-/-} may be protective against clinical illness, it does not prevent DSS-mediated histological damage and mechanisms independent of histological damage could contribute to tumor development. In addition, the weight loss tracks the illness throughout the experiment, while the histopathological scores evaluate one timepoint only. Therefore, while there is no evidence that histopathological inflammation was different between groups, important in considering tumor development, clinical illness scores suggest that Nox2 deficiency is protective. In keeping with the similar degree of inflammation, inflammatory cell infiltrate was not significantly different between strains. This includes both Ly6G+ and F480+ cells. When examining neutrophil infiltration into dysplastic tissue, WT mice had greater numbers of tumor-associated neutrophils compared to Nox2^{-/-} mice. As inflammatory lesion-associated and blood neutrophils were present at similar levels between strains, Nox2 may affect the migration of neutrophils into dysplastic tissue, but not normal epithelium. While not examined in this study, it is not unreasonable to speculate that some of these Ly6G+ cells possessed the myeloid-derived suppressor cell (MDSC) phenotype. On mechanism by which Nox2^{-/-} mice might be protected against tumor development is through impaired MDSC development. MDSC can utilize Nox2 to produce ROS involved in inhibiting the T-cell response [443], and thus anti-tumor immunity.

Nox2^{-/-} mice are used as a model of chronic granulomatous disease (CGD), however, even in aged mice granulomas were not detectable in the intestines (not shown).

In addition, no spontaneous inflammation was detected, which would be expected based on a previous report of DSS-colitis in Nox2^{-/-} mice [304], however, human CGD has been associated with hyperinflammation [399], and a mutation in the *NCF1* gene that encodes p47 is associated with intestinal inflammation in CGD [301], however, it is unlikely that it is related to IBD in the absence of CGD [400]. We suggest that despite the NADPH oxidase functional deficiency, this model does not mimic CGD and therefore is not confounded by spontaneous granulomatous disease.

Previous studies examining the deficiency of Nox2 on colitis are conflicting [303, 304, 396]. This research is consistent with the findings of Krieglstein et al. where acute inflammatory measures, such as clinical score, histopathological score, TNF, IL-1 and MIP-2 were similar between mice lacking Nox2^{-/-} and WT mice. Krieglstein used a model of NADPH deficiency where mice were lacking the p47^{Phox} subunit [303], while the studies by Bao et al looked at the same strain of mice as in the present study, finding protection in the Nox2 deficiency [304]. The p47 subunit may have roles in other NADPH oxidase isoforms in addition to Nox2, including Nox1 and Nox3 [305, 401], which may account for the difference.

While several measures of inflammation severity were slightly lower in Nox2^{-/-} mice during acute inflammation, they failed to reach statistical significance. Histologically-detectable inflammation was not significantly lower in Nox2^{-/-} mice treated with AOM+DSS. Whether multiple parameters, each slightly lower in Nox2^{-/-} mice, collectively contributed in a protective effect against tumor development is not clear. We did not find significant differences in histological inflammation scores between strains, but they were slightly lower in Nox2^{-/-} mice. While no significant differences between infiltrating neutrophils were detected, Nox2^{-/-} mice had slightly lower numbers in the mucosa and submucosa of the colon. WT mice put on the AOM+DSS regime may have experienced obstructions that are responsible for the decline in the animals' health. Mice treated with DSS-alone did not develop this degree of clinical illness. Therefore, we suggest that the clinical illness scores in these select mice are more likely linked to the severity of cancer and not a more severe histological inflammation.

A role for neutrophils in promoting cancer has been well-established [275, 278, 402]. Neutrophils secrete matrix metalloproteinase-9, activating vascular endothelial growth factor, driving the angiogenic switch in tumors and allowing the tumor to obtain

more nutrients from the host [275]. Whether neutrophils are involved in the initial stages of tumorigenesis is less clear. While Nox2 is expressed in several types of leukocytes, neutrophils remain the most abundant source of inflammation-derived oxygen radicals generated by Nox2 [313]. It has been shown that neutrophils can cause cell cycle arrest at the G2/M stage, leading to colonic epithelial replication errors *in vitro* and the activation of p53 and p21(waf1/cip1)[398]. However, the arrest and p53 activation were not prevented by the addition of catalase or superoxide dismutase, suggesting that these effects were not due to the superoxide radical or hydrogen peroxide alone [398]. While it remains to be determined which mediator was responsible for promoting these replication errors, the idea that ROS generation and oxidative stress can lead to genetic damage has been documented [324].

8-OHDG is a DNA adduct that is associated with increased levels of oxidative stress [403]. As much of the oxidative stress during inflammation occurs as a result of inflammatory cell infiltration, we hypothesized that 8-OHDG would be reduced in chronic colitis in Nox2^{-/-} mice. However, contrary to our hypothesis, there was no difference between WT and Nox2^{-/-} mice in the number of 8-OHDG⁺ nuclei detected immunohistochemically in mid-colon crypts. Several mechanisms could explain this finding. First, the level or capacity of anti-oxidant enzymes could be reduced in NADPH oxidase deficiency, therefore in mice genetically predisposed to produce fewer ROS, a reduced antioxidant capability could result in DNA damage. It has been reported that there is a reduced antioxidant capacity in IBD [403]. Next, there could be a reduced ability to cleave 8-OHDG adducts by the enzyme in Nox2^{-/-} mice, resulting in higher 8-OHDG expression. Further it is possible that within the intestinal epithelium, despite a lack of leukocyte Nox2, ROS are being produced by other mechanisms, in particular by Nox1. Activated Nox1 could account for the increased 8-OHDG expression at this timepoint. Earlier timepoints might have revealed differences in 8-OHDG staining and damage to the epithelium prior to tumor development. While 8-OHDG is a good representative of cumulative oxidative damage, it is constantly being cleaved, therefore, expression may have been different at different time points. To control for these factors, another measure of oxidative damage should be done, such as the measure of nitrosative damage in the colon epithelium by detecting nitrotyrosine expression via immunohistochemistry.

In conclusion, mice deficient in Nox2^{-/-} are protected against tumorigenesis and dysplasia after chronic inflammation, suggesting that Nox2 and subsequent inflammation-related ROS production can contribute to this process. Whether this process results from direct ROS-induced damage to the epithelium or indirectly through reducing inflammation was not clear in this study and warrants further investigation.

Figure 5.1 Acute Colitis is similar between WT and Nox2^{-/-} mice

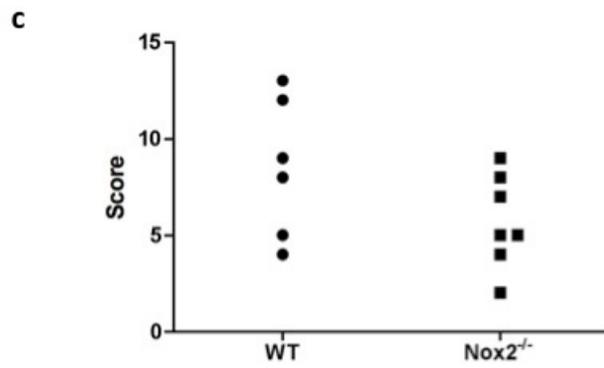
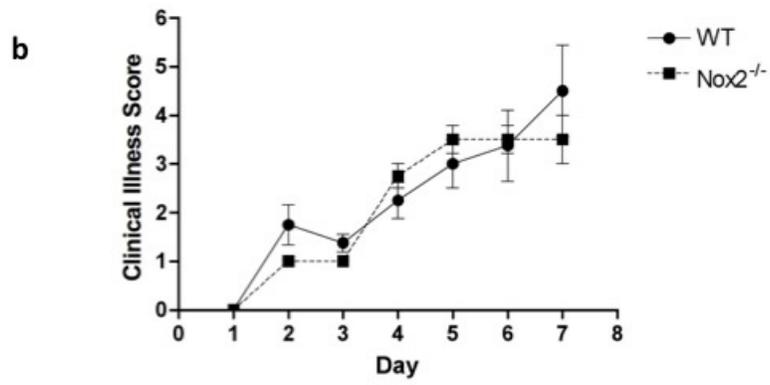
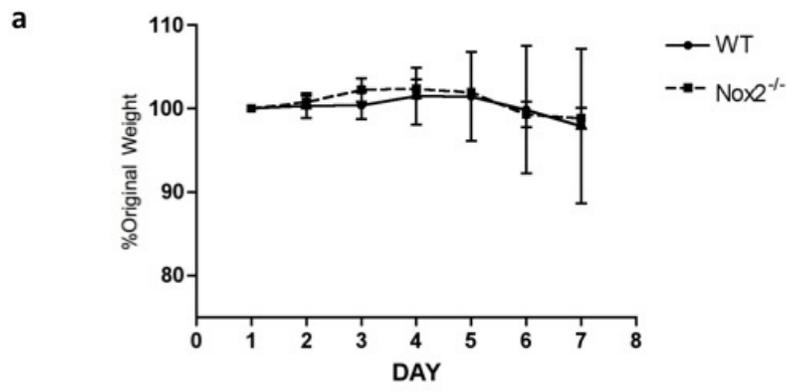
Figure 5.1a) Weight change in WT (n=6) and Nox2^{-/-} mice (n=7) fed 3% w/v DSS in drinking water for 5 days followed by 2 days of regular tap water was not significantly different between groups. Repeated measures ANOVA was used to determine differences between groups.

Figure 5.1b) Clinical illness scores in WT and Nox2^{-/-} mice fed 3% w/v DSS for 5 days followed by 2 days of regular tap water is not significantly different between strains. Repeated measures ANOVA was used to detect differences between groups.

Figure 5.1c) Histopathological scores in WT and Nox2^{-/-} mice fed 3% DSS for 5 days followed by 2 days of regular tap water are not significantly different between strains. Statistical differences were determined using Kruskal-Wallis one-way ANOVA for both strain and treatment.

Figure 5.1d-e) Hematoxylin and Eosin –stained sections (100x magnification) from the mid-colon of WT and Nox2^{-/-} mice, respectively, showing inflammation after DSS-administration.

Figures 5.1f) Ly6G⁺ cells from 5 high powered fields (1000x magnification) within areas of inflammation with a score of at least 3 (see Table 2.2). Differences between groups were determined by two-tailed t-test (p<0.05).



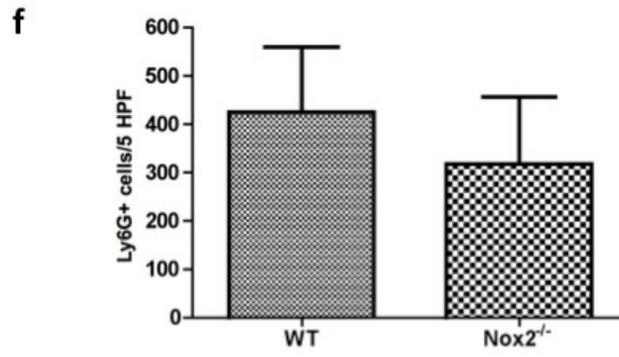
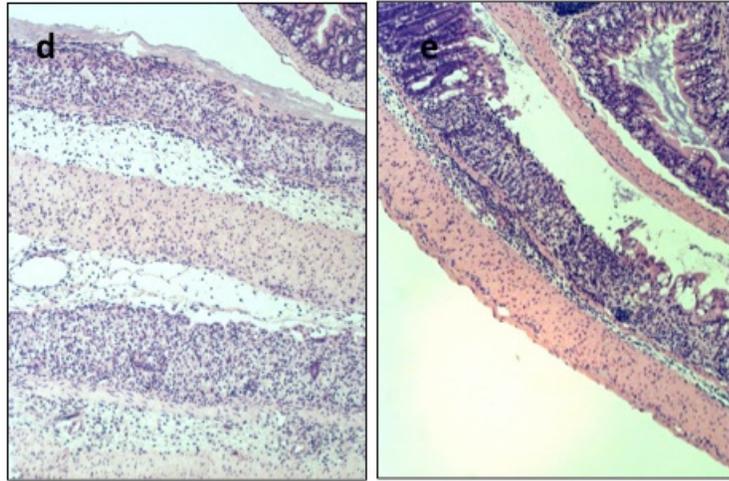


Figure 5.2 Histopathological Inflammation In Chronic Colitis In Wildtype And Nox2^{-/-} Mice Is Not Significantly Different Between Strains Despite Greater Clinical Illness In Wildtype Mice

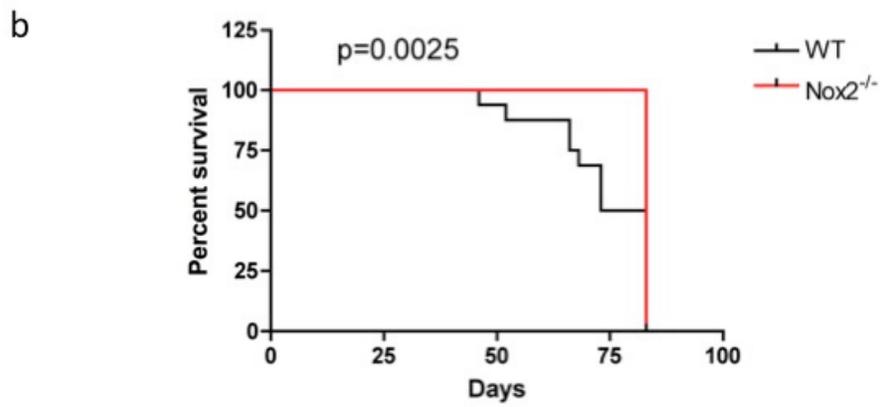
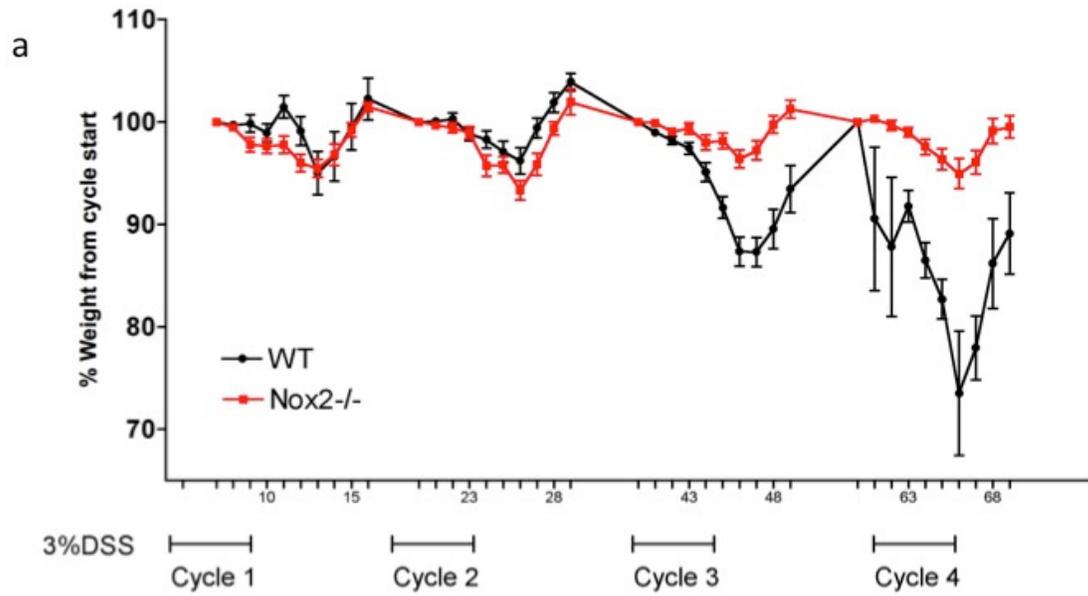
Figure 5.2a) Weight loss during the 83-day experiment in WT (n=22) and Nox2^{-/-} (n=24) mice treated with AOM+DSS is significantly greater in WT mice compared to Nox2^{-/-} mice as determined by a repeated measures ANOVA (p<0.05).

Figure 5.2b) WT mice treated with AOM+DSS develop significant illness and weight loss leading to mortality in WT mice after the third cycle.

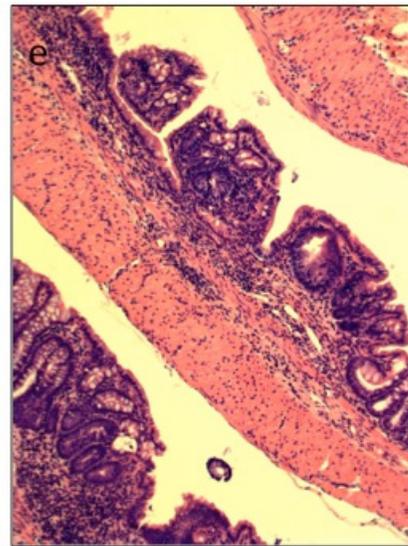
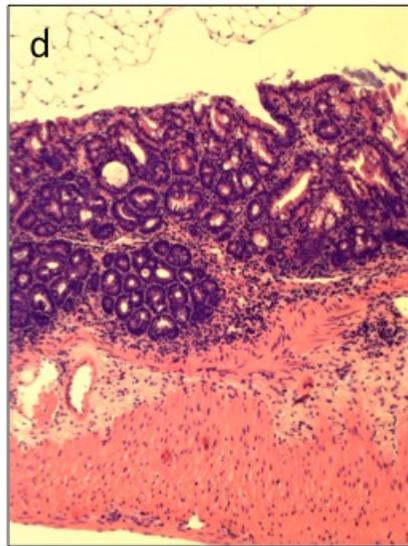
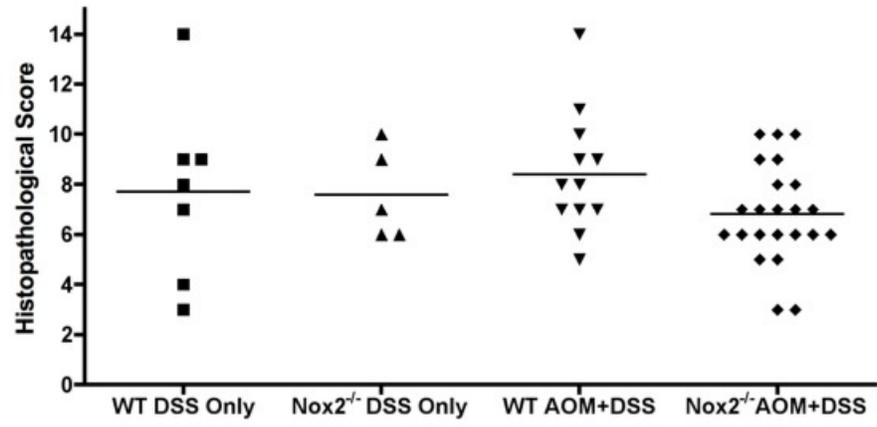
Figure 5.2c) Histopathological scores are not significantly different between WT (n=12) and Nox2^{-/-} (n=24) mice after treatment with AOM+ 4 cycles of 3% w/v DSS. Only those mice that were present at the end of the experiment were included in scoring.

Figure 5.2d-e) Representative hematoxylin and eosin-stained sections showing inflammation within the mid-colon (100x magnification) in both WT and Nox2^{-/-} mice, respectively.

Figure 5.2f) White blood cells counted in blood smears stained with crystal violet, counted as a proportion of total white blood cells in WT and Nox2^{-/-} mice treated with AOM+DSS.



C



f

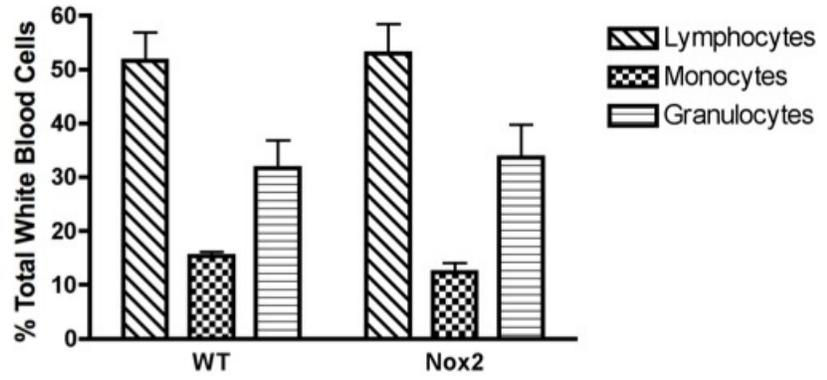


Table 5.1 WT and Nox2^{-/-} characteristics at necropsy

Legend: After the 83-day regimen, surviving mice were euthanized and post-necropsy measurements were made. Statistical differences detected using a 2-way ANOVA and where an interaction occurred, Tukey's multiple comparison post-test was performed to detect differences between groups. Significant differences are denoted by superscripted letters across a row. Data presented as mean±SEM.

Measurement	Wildtype			Nox2 ^{-/-}		
	NT	DSS	DSS+AOM	NT	DSS	DSS+AOM
Colon Length (cm)	7.9±0.2 ^a	5.7±0.2 ^b	5.3±0.4 ^b	9±0.7 ^c	5.8±0.6 ^b	7±1 ^a
Colon Wet Weight (mg)	314±12 ^a	444±17 ^b	524±113 ^b	392±68 ^{ab}	489±64 ^b	389±90 ^{ab}
Colon mg/cm	40±17 ^a	85±9 ^b	91±21 ^b	44±8 ^a	85±14 ^b	52±12 ^a
Cecum Wet Weight (mg)	157±17 ^a	230±24 ^b	152±50 ^a	189±42 ^a	230±24 ^b	193±30 ^{ab}
Spleen Wet Weight (mg)	93±6 ^a	225±22 ^b	221±73 ^b	128±38 ^a	211±28 ^b	265±94 ^b

Figure 5.3 Nox2^{-/-} Mice Are Protected From Tumor Development After Azoxymethane And 3 Cycles Of Dextran Sulphate Sodium

Figure 5.3a) Incidence of dysplasia and tumors within the colon of WT and Nox2^{-/-} mice treated with AOM+4 cycles of 3% w/v DSS. Chi-squared analysis was used to determine differences in incidence between strains (p<0.05).

Figure 5.3b) Tumor diameter was significantly reduced in Nox2^{-/-} mice. Tumor diameter was determined taking the average of three diameter measurements per macroscopic tumor. Significance was determined using a two-tailed t-test.

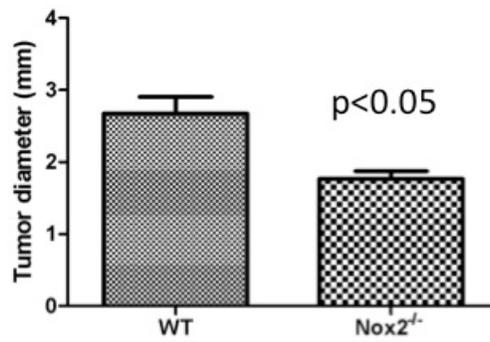
Figure 5.3c) Tumor multiplicity was significantly reduced in Nox2^{-/-} mice. Tumor multiplicity represents the number of macroscopically visible tumors per colon. Significance was determined using a two-tailed t-test.

Figure 5.3d-e) Hematoxylin and eosin-stained sections (100x magnification) showing a non-invasive adenocarcinoma in both WT (d) and Nox2^{-/-} (e) mice.

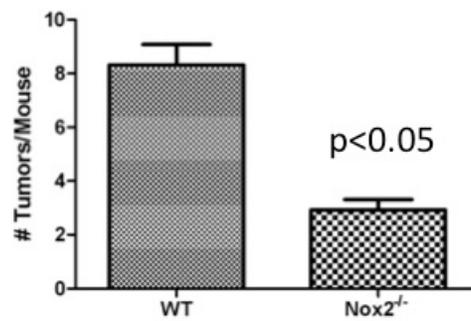
a

% Incidence	WT	Nox2 ^{-/-}	P-Value
Gross Tumors	100%	58%	0.0037
Dysplasia	100%	71%	0.02

b



c



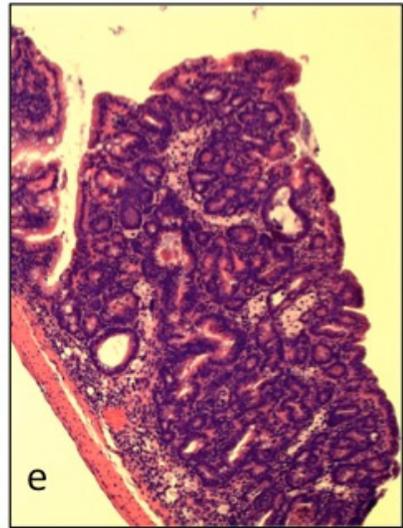
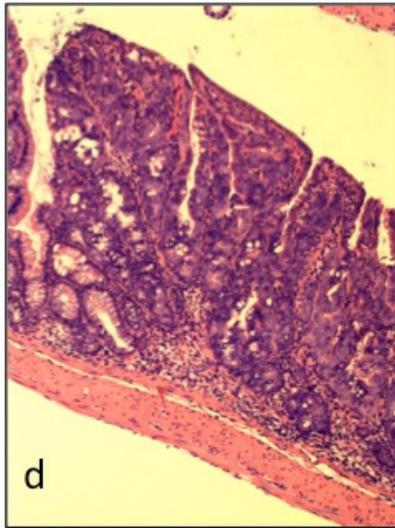


Figure 5.4 Ly6G + Cellular Infiltrate Into Tumors Is Reduced In Nox2^{-/-} Mice, But Not In Inflamed Tissue Compared To Wildtype

Figure 5.4a) High power field (1000x magnification) showing Ly6G+ cells within the tumor of a WT mice.

Figure 5.4b) High power field (1000x magnification) showing Ly6G+ cells within a tumor of a Nox2^{-/-} mouse.

Figure 5.4c) The number of Ly6G+ cells within 5 high power fields (400x magnification) in dysplastic tissue. Differences between strains were detected using a two-tailed t-test.

Figure 5.4d) The number of Ly6G+ cells within 5 high power fields (400x magnification) in inflamed tissue in the colon.

Figure 5.4e) F480+ cells within AOM+DSS-treated WT mice, 400x magnification

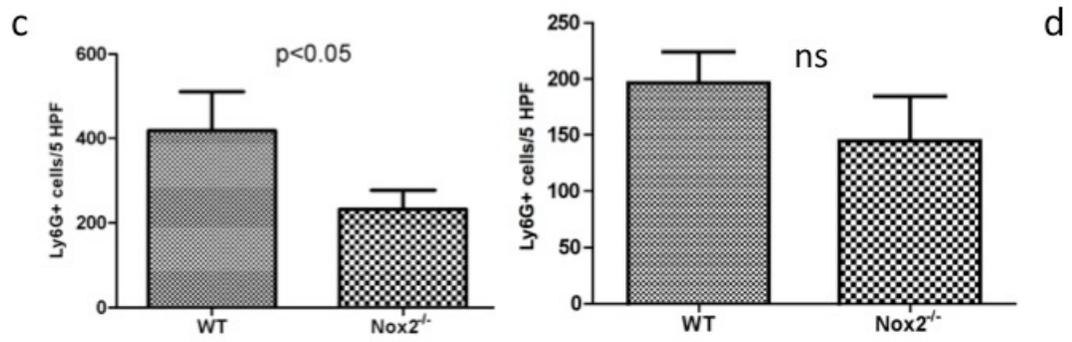
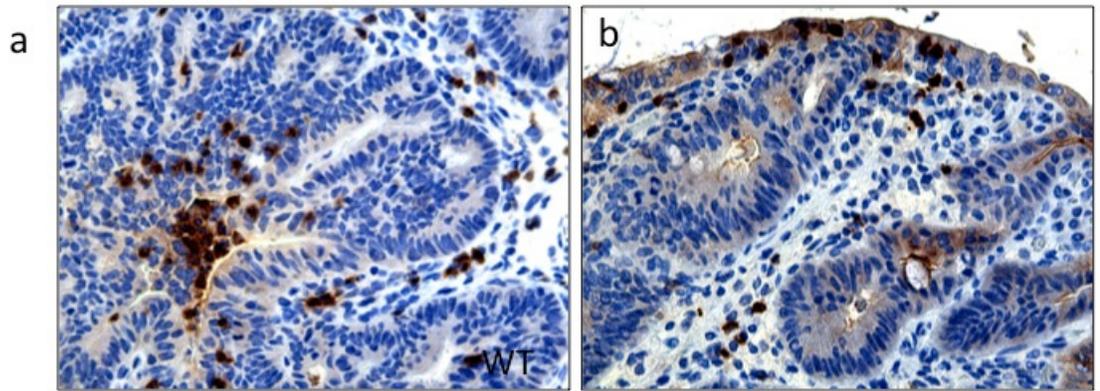
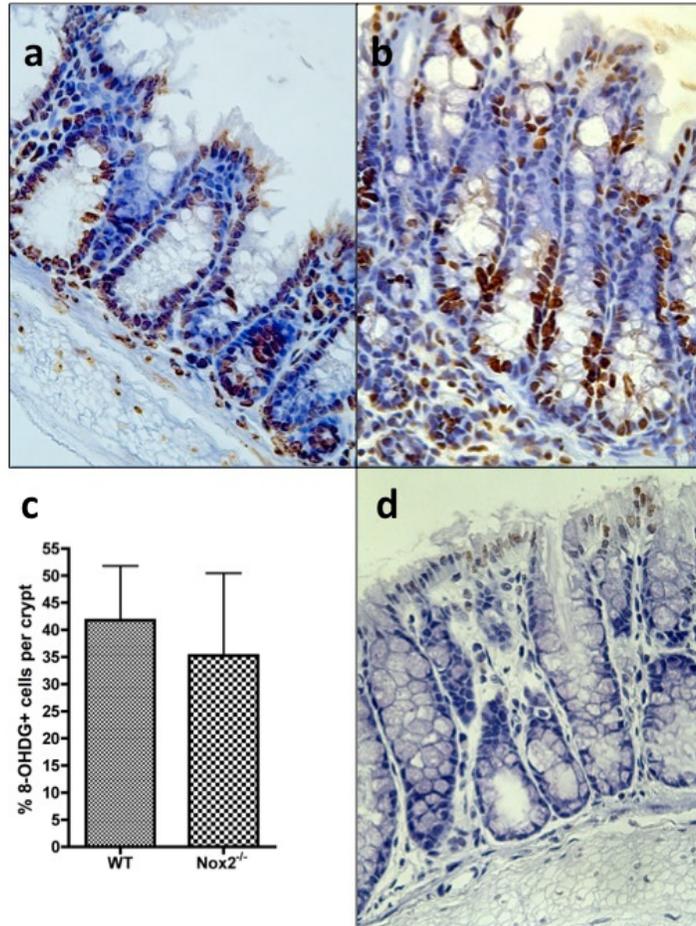


Figure 5.5 8-OHDG Staining Within Epithelial Cells Is Similar Between Wildtype And $Nox2^{-/-}$ Mice



$Nox2^{-/-}$ and WT mice were treated with 10mg/kg AOM+ 3% w/v DSS for 4 cycles, each cycle consisting of 5 days of DSS followed by 14 days of facility water. 8-OHDG, a marker of oxidative damage, was measured in sectioned colon. Positive nuclei within the crypt epithelium were counted and compared to total number of nuclei per crypt to determine proportion of 8-OHDG+ cells per crypt. 10 intact crypts per section were included per sample. a) 8-OHDG staining in $Nox2^{-/-}$ mice treated with AOM+DSS (400x) b) 8-OHDG staining in WT mouse treated with AOM+DSS (400x), c) Proportion of 8-OHDG+ nuclei per crypt (5 crypts counted per sample), d) AOM only-treated mouse section stained with 8-OHDG (400x)

CHAPTER 6: DISCUSSION

Anti-TNF therapy remains an important means of reducing inflammation in IBD. Research into the mechanisms of action of this therapy is necessary to both prevent complications and to develop newer, more effective therapies. Whether the efficacy of anti-TNF therapy is due to antagonizing TNF during inflammation, or a function of removing activated leukocytes expressing TNF is not yet clear. More importantly, the long-term outcome of the use of anti-TNF therapies on the risk of CAC is unknown. This research therefore aimed to further clarify the role of TNF signaling through its cognate receptors in an animal model of colitis and colitis-associated cancer. Initially, reports that anti-TNF therapy was associated with an increased risk of extra-intestinal cancers (which has since been disputed, but not disproven [27]), led us to ask questions about whether TNF might actually play an anti-tumor role in the intestine. At the initial stages of this research this question had not yet been asked. There is evidence that TNF can activate colonic epithelial cell apoptosis in the absence of NF κ B signaling [155, 156], which could potentially exacerbate colitis if there is an imbalance between cell death and regeneration and the epithelial layer becomes breached. In addition, TNF, as its name suggests, has been known to be toxic to tumors and can induce tumor regression, especially in solid tumors [404]. It has since become evident that TNF possesses pro-tumorigenic properties, and our research supports this.

Summary of Major Findings

We showed a lack of major differences between mice lacking TNFR1, TNFR2 and WT mice in the severity of acute inflammation following experimental DSS-mediated colitis. This was associated with no differences in measures of cytokines within the colon tissue and inflammatory cells between strains treated with DSS. TNFR1^{-/-} mice had a higher clinical score at days 6 and 7, suggesting a delay in healing, but this did not correspond with any increases in histopathological scores at days 7 and 12. Macrophage infiltration into the colon of TNFR1^{-/-} mice was significantly reduced at day 7 compared to other strains. Colonic epithelial cell apoptosis was not affected by strain after DSS treatment.

Using DSS combined with AOM to model remitting/recurring chronic colitis,

TNFR1 gene-deficient C57BL/6 mice developed a significantly reduced incidence of colorectal tumors and microscopically-detectable dysplasia compared to wildtype and TNFR2-gene-deficient mice. In addition, TNFR1^{-/-} mice had fewer macroscopic tumors compared to the other strains. There were no correlations between tumor size and number with inflammation severity in the mid-colon, nor did mice with or without dysplasia differ in their inflammation severity. This is important as overall inflammation scores showed great variability, possibly masking a potential statistical significance between strains. By subdividing the groups into categories based on disease outcome, we still observe no significant difference between groups in the inflammation severity. Measures of inflammation, such as cellular infiltrate and cytokine levels, remained similar between groups with the exception of elevated IL-12 protein levels in the colon of AOM+DSS-treated TNFR1^{-/-} mice. When mice were irradiated and made chimeric with donor bone marrow, we found in WT and TNFR1^{-/-} mice engrafted with TNFR1^{-/-} and WT bone marrow, respectively, there was a significantly decreased incidence of colonic dysplasia and cancer. This suggests that both stromal TNFR1 and bone-marrow-derived TNFR1 are important in the development of colitis-associated cancer. We showed that there were significantly fewer 8-OHDG+ cells within the colonic epithelium, corresponding to our hypothesis that TNF may play a role in regulating the production of ROS in the colon.

We then were interested in the role of leukocyte-derived ROS in the development of colitis-associated cancer. To determine the contribution of leukocytic production of ROS to colitis-mediated cancer we treated mice lacking a functional NADPH oxidase 2 with the same regime described previously. We did not find a significant difference between WT and Nox2^{-/-} mice with respect to inflammation severity when we measured acute colitis; however, Nox2^{-/-} mice tended to have lower inflammation parameters, and Ly6G-expressing cells compared to WT.

When treated with the AOM +DSS regime, Nox2^{-/-} mice were protected against the development of CAC and had fewer and smaller tumors compared to WT mice. While inflammatory measures were similar between groups (histopathological score, cellular infiltrate), clinical scores and weight loss were significantly greater in WT mice. This was associated with a significantly greater mortality. Nox2^{-/-}, while showing a similar Ly6G+ infiltrate into the inflamed epithelium, showed reduced infiltrate into dysplastic tissue. Interestingly, 8-OHDG was similar between WT and Nox2^{-/-} mice,

confounding the potential role of leukocyte-derived ROS in epithelial cell damage.

Relevance to Current Literature

Despite the success of anti-TNF therapies, the role of TNF as a soluble mediator driving colitis has not yet been definitively confirmed. Both Infliximab and Etanercept bind to the soluble TNF trimer, however, Infliximab binds with more affinity, while Etanercept appears to dissociate from soluble TNF readily [183]. This could potentially underscore the higher impact of Infliximab therapy on IBD compared to a lack of efficacy by Etanercept. However, evidence points to the presence of outside-in signaling by membrane-bound TNF on monocytes and lymphocytes as being a mechanism by which anti-TNF therapy exerts efficacy [173, 184]. Once these activated effector cells are depleted, the sustained chronic inflammatory response triggered by an unknown insult can be controlled. Therefore it is not surprising that single TNF receptor deficiency does not significantly alter the course of colitis during DSS treatment.

In both acute and chronic colitis, we did not see any differences in TNF protein levels between strains. In our hands, TNF levels, while slightly higher compared to untreated mice, were not significantly elevated in the soluble fractions from colon homogenates of DSS-treated WT mice compared to untreated mice. Other studies, as discussed, have both confirmed [243] or refuted [198] the role of TNF in the pathology of IBD. Therefore, an overall consensus has not been yet been achieved. We suggest that while in some cases TNF may be elevated in the mucosa during active inflammation it is not a major driver of inflammation and in fact may be important in limiting inflammation in the context of maintaining barrier function. To this end, TRAF2, a downstream molecule in TNFR1 signaling, is required in neonatal mice to prevent spontaneous colonic inflammation (BALB/c) and mortality in C57BL/6 mice [405]. There is also high mortality in DSS-treated TNF deficient mice [198], and in our own experience using double TNF receptor knock-out mice (Appendix B). These mice developed severe inflammation and colonic hemorrhage and splenic necrosis and 50% mortality after 1 cycle of DSS treatment. While we did not measure bacterial translocation, it is possible that without functioning TNF signaling, these mice were unable to mount an effective antibacterial response to bacterial translocation and sepsis, or TNF deficiency could have resulted in an impaired ability for epithelial regeneration. TNFR1^{-/-}RAG^{-/-} double

knockout mice treated with DSS show increased bacterial translocation to distant organs [200], suggesting that in the absence of T and B-cells, TNFR1 signaling on remaining innate immune cells is protective in the development of inflammation. Mice deficient for TRAF2 show high levels of colonic epithelial cell apoptosis [405], suggesting that TRAF2-mediated activation of the NF κ B pathway is protective by preventing colonic epithelial cell apoptosis. Another explanation is that TRAF2 deficient mice lack the ability to ubiquitinate RIP1, leading to the association of RIP1 with Caspase-8, providing a scaffold for the DISC [479]. In addition, epithelial TAK1 and NEMO are important for epithelial cell survival via TNFR1 [155, 156]. We did not see any differences in apoptosis between WT and TNFR-deficient mice either acutely or chronically. It is possible that a complete TNFR deficiency might have more impact on epithelial cell apoptosis.

TNF bioactivity is another confounder to consider when measuring TNF levels in our models. When we measured TNF in the colon extracts by ELISA we did not find any strain or treatment-dependent differences in TNF levels. The levels we did detect might not have taken into account total TNF levels in the colon, as soluble receptors could potentially have bound and masked TNF [481]. We did report high levels of soluble TNFR2 in the serum and colons of DSS-treated mice, confounding a possible difference between treatments or strains. This may not give an accurate indication of TNF bioactivity during inflammation. Indeed, Ebach et al., [206] reported that TNBS treatment did not significantly increase serum soluble TNF, which was essentially undetectable, but significantly increased total TNF (soluble plus receptor-bound) [206]. We were not able to detect TNF in the serum (not shown), but it is possible that with an ELISA kit detecting bound and unbound TNF, we would have been able to measure it more accurately.

That TNFR1 deficiency is associated with a decreased incidence and severity of cancer, broadly, is consistent with previous research. In a similar model using BALB/c mice, TNFR1 deficiency reduced inflammation-mediated colon cancer [243]. Where our findings differed; however, is that TNFR1-deficient BALB/c mice were protected against DSS-induced colitis as well as cancer, meaning the reduction in cancer could potentially be a function of the reduced inflammation. This was confirmed further with bone marrow chimeric mice, where TNFR1 expression on hematopoietic cells but not stromal cells was

associated with a decrease in the number of tumors per mouse [243]. When WT bone marrow was reconstituted in TNFR1^{-/-} mice, there was no significant difference in inflammation after AOM+DSS compared to WT mice reconstituted with WT bone marrow, but a significantly greater inflammatory score compared to WT mice reconstituted with TNFR1^{-/-} bone-marrow. This attributes a significant pro-inflammatory role to TNFR1 in hematopoietic cells that was not evident in whole-body TNFR1 deficiency. It is possible when TNFR1 is absent in all cells, the effect of hematopoietic-specific TNFR1 deficiency is lost. Therefore TNFR1-deficiency in other tissues is contributing to the inflammation, ascribing a protective role to TNFR1 expression in the mesenchyme and stroma. With this idea, it is expected that stromal TNFR1 deficiency would affect inflammation scores if it was indeed protective.

Papivanova et al., reported that stromal TNFR1 deficiency did not reduce cancer incidence, or tumor number [243], however, we found that it did. Unlike Papivanova et al., we found that epithelial cells expressed TNFR1, suggesting that a pro-tumorigenic signaling pathway could potentially be activated if TNF is being produced. In future studies, TNFR1-activated NFκB signaling should be explored to determine if this is the pro-tumorigenic pathway activated within epithelial cells and not mediators produced by cells in close proximity to the epithelium. In a mouse model of spontaneous Crohn's like ileitis, where TNF was overexpressed after the removal of the AU-rich regulatory elements, it was reported that specific intestinal epithelial expression of TNFR1, with TNF overexpression, did not lead to significant intestinal pathology, supporting the role of cells other than intestinal epithelial cells as targets of TNF signaling through TNFR1 [406]. Therefore the protective effect of TNF may not be through direct TNF signaling in the epithelium, but indirectly through the production of protective mediators by other cells.

In our experiments, TNFR1 deficiency in either the bone marrow or stroma/epithelium led to a reduction in tumor incidence and severity to a degree similar to TNFR1^{-/-} animals. As mice with hematopoietic TNFR1 deficiency had reduced inflammation, it leads us to conclude that hematopoietic TNFR1 deficiency is protective against tumor development in an inflammation-dependent manner. Therefore inflammation may contribute directly to tumorigenesis. In stromal/epithelial TNFR1 deficiency, inflammation was similar to WT mice with WT bone-marrow, suggesting that

tumorigenesis in these mice is occurring in a TNFR1-specific manner independently of inflammation. It leads us to question what role specifically TNFR1 has in tumorigenesis and which cells are involved, as our results suggest that TNFR1 is widely expressed.

Two examples of TNFR1-expressing cells within the stroma/mesenchyme are myofibroblasts and endothelial cells. Myofibroblasts can respond to inflammatory mediators, such as LPS, IL-1 and TNF. TNF-stimulated myofibroblasts can produce cytokines such as IL-6 and IL-8 [407] Cox-2 [217, 408] and MMP-3 [409]. These cells are positioned in close proximity to the epithelium, and are purported to be integral in maintaining the stem cell niche and homeostasis in colonic crypts [410]. Due to their proximity to the epithelium, it is plausible that TNF produced by infiltrating leukocytes such as macrophages and neutrophils can activate myofibroblasts to further produce inflammatory mediators, amplifying inflammation. In the mouse model of Crohn's-like ileitis induced by deletion of the AU-rich regulatory elements from TNF, intestinal myofibroblasts were shown to mediate inflammation via TNFR1 [215]. In fact, intestinal myofibroblasts may be activated by epithelial-derived TNF early in the course of inflammation [406].

The activation of myofibroblasts during inflammation can stimulate signals that lead to epithelial activation and proliferation. The production of mediators like IL-6 and IL-24 by colonic myofibroblasts after activation through TNFR1 could lead to the activation of STAT3 in colonic epithelial cells, although this has yet to be shown in primary epithelial cells, as IL-24-mediated STAT3 activation was observed in HT-29 colonic carcinoma cells [411]. IL-6 receptor expression has been shown on colonic epithelial cells *in vivo* [412]. STAT3 activation in CAC has been described and it is thought that STAT3 induces colonic epithelial proliferation, and also protects them from apoptosis [264]. Therefore, the activation of subepithelial myofibroblasts by TNF could be a mechanism by which stromal TNFR1 signaling contributes to tumor formation in CAC.

Endothelial cells play a crucial role in inflammation by expressing adhesion molecules and chemokines that allow leukocytes to extravasate to the site of inflammation. Many studies have shown that they possess bioactivity in response to TNF. Any alterations in the number of infiltrating leukocytes could be a function of changes in endothelial cells. Infliximab therapy can reduce inflammation by reducing angiogenesis

[195, 196]. This may have application in cancer therapy, as tumor blood vessels are often disorganized, leaky and phenotypically heterogeneous [271]. It has been demonstrated that anti-TNF therapy can reduce angiogenesis within tumors [243], and directly inhibit endothelial proliferation *in vitro* [196]. TNF has been shown to induce Pim-3 expression [413], a serine and threonine kinase involved in endothelial cell migration [414]. In tumors, TNF can induce vascular endothelial growth factor, a potent pro-angiogenic factor [415]. Whether this is a direct effect on endothelial cells *in vivo* by TNF or whether activation of infiltrating leukocytes leading to production of inflammatory mediators such as prostaglandins is not known. Stromal TNFR1 signaling contributes to tumor development without affecting inflammation in our model of CAC. As we did not find significant TNFR1 protein in colonic epithelial cells, it is likely that other cell types, including endothelial cells or subepithelial myofibroblasts contributed to tumorigenesis.

Popivanova et al., reported that during chronic colitis infiltrating leukocytes expressed COX-2 and hypothesized that subsequent PGE₂ production could influence angiogenesis [243]. They demonstrated that anti-TNF treatment reduced vascular density within tumors, and actually reduced the size of established tumors [243]. Reduction in angiogenesis has been reported in CD after Infliximab treatment [195]. It was not determined whether PGE₂ was actually the factor that led to tumorigenesis in this model, although it has been reported that *Cox-2*^{-/-} mice are as susceptible to experimental CAC as WT mice [258]. Complete deficiency may have a different impact than a targeted deficiency. EP4 deficiency, the prostanoid receptor shown to have most impact during DSS-colitis [416, 417], has not been evaluated in this model but epithelial-specific EP4 deficiency could provide some insight into the role of PGE₂ in CAC. If PGE₂ deficiency could lead to reduced tumorigenesis, then it is possible that TNF activation of PGE₂ production could contribute to CAC. PGE₂ has been shown to contribute to epithelial cell survival from detachment-induced apoptosis [256, 416] and has been implicated in other GI cancers [418, 419]. As PGE₂ has been implicated in the nuclear translocation of β -catenin followed by the expression of oncogenic c-myc and cyclinD1, it is possible that this is a mechanism by which tumorigenesis could occur.

As mentioned, a TNF-mediated signaling pathway that could potentially contribute to tumorigenesis is through the activation of NF κ B. Components of the NF κ B pathway, specifically IKK β , were shown to be important in the development of cancer

after chronic inflammation [249]. Through the production of conditionally IKK β -deficient mice, lacking IKK β either in the intestinal epithelium or myeloid-derived cells, it was determined that epithelial IKK β was important in the initiation of tumor development, while myeloid IKK β was important in the production of cytokines that could act as growth factors [249]. In addition, epithelial IKK β deficient mice had a much higher rate of epithelial cell apoptosis, suggesting that IKK β signaling resulted in greater survival [249]. Further supporting this mechanism, a role for TNF in activating the NF κ B pathway in intestinal epithelial cells during CAC was recently demonstrated by Onizawa et al., [252]. These two studies suggest that NF κ B signaling is very important in the development of CAC and that TNF can activate this signaling pathway in intestinal epithelial cells. Whether TNF signaling is directly involved in activating NF κ B in colonic epithelium, or if NF κ B activation is an indirect result of inflammation remains to be determined. Popivanova reported that TNFR1 expression was confined to infiltrating cells, and not on colonic epithelial cells [243]. It is possible that TNF activates mediators in other cells that can activate the NF κ B pathway in colonic epithelial cells, however our experiments did not directly measure this.

In both our acute and chronic colitis models, we were surprised that we did not detect differences in the number of apoptotic cells within the gut. Given that TNFR1 can trigger both apoptosis and survival, we expected to see alterations in apoptotic cells in TNFR1-deficient mice. Cleaved caspase-3 is a sensitive measurement for early events in apoptosis [484]. TUNEL, which measures DNA fragmentation, a later event, has been questioned for its reproducibility and reliability [484]. In acute DSS colitis, we confirmed both with immunohistochemical TUNEL and colonic extract cleaved caspase-3 that there were no differences between strains in the level of apoptosis. In our chronic AOM+DSS model, we looked at immunohistochemically-detectable cleaved-caspase 3 only. It would have been beneficial to examine another marker of apoptosis such as TUNEL, as DSS treatment reportedly increases the rate of apoptosis in the colon [245]. P53-upregulated modulator of apoptosis, or PUMA, is also upregulated during DSS colitis and IBD [485]. PUMA can promote apoptosis and is upregulated in inflamed tissues. Anti-TNF treatment reduced PUMA expression and colonic epithelial apoptosis. In addition, PUMA-deficient mice were protected against DSS-mediated colitis, suggesting that epithelial apoptosis could play a role in inflammation. TNF can

promote PUMA expression via NF κ B [486], thereby creating an alternate mechanism by which TNF can contribute to apoptosis independent of the death domains. Whether this has relevance to CAC remains to be determined, and it would be desirable to determine if TNFR1 mice have altered PUMA expression. While at the time point measured there was no difference in apoptosis between strains, it is possible that early in DSS treatment, or during the early healing response, differences in apoptosis could affect the development of tumors in knockout mice.

There is much emphasis on the activation of pro-carcinogenic transcription factors in CAC, however, TNF has other biological actions that could potentially contribute to tumor development. TNF has the ability to increase oxidative stress. TNF has been functionally linked, through the TNFR1, to NADPH oxidase [316]. TNF has been shown to prime neutrophils and induce ROS production [420]. Furthermore, TNF-induced activation of NF κ B can result in upregulation of Nox subunits, increasing the capacity of ROS production [311, 368]. Therefore, we hypothesized that TNF signaling would induce ROS production in infiltrating leukocytes, leading to oxidative damage within the tissues. While we were unable to source these ROS, we did find that TNFR1 mice had lower levels of an oxidative damage marker, 8-OHDG in the nuclei of colonic epithelial cells compared to WT.

TNF-mediated ROS production is not limited to Nox2. In the colon, ROS production can occur after a number of events. TNF can activate an enzyme called spermine oxidase [307], which is expressed and upregulated in infiltrating leukocytes in inflamed tissue in UC [421] along with the substrate polyamine, spermine [422]. Furthermore, exposure of HT-29 carcinoma cell line to *Bacteriodes fragilis* resulted in an increase in intracellular spermine oxidase, suggesting that it is present in both leukocytes and epithelial cells [423]. In addition to spermine oxidase, a second NADPH oxidase isoform, Nox1, has been implicated in colonic epithelial cell production of ROS [424]. Nox1 has been implicated in carcinogenesis and is overexpressed in colon cancer [425, 426]. TNF activates Nox1 during necrotic cell death in fibroblasts [318] and in the mouse colon [427]. Therefore, while oxidative stress can theoretically occur via TNF-activated ROS production by Nox2, there are other oxidative enzymes that may have contributed to the formation of 8-OHDG adducts in the nuclei of colonic epithelial cells.

The finding that TNFR1^{-/-} mice were protected from CAC and this was associated

with a reduced measure of oxidative damage prompted us to question directly the role of ROS produced by neutrophils and macrophages via the enzyme Nox2. Nox2^{-/-} mice lack the ability to produce ROS and are an animal model of chronic granulomatous disease. It was recently reported that Nox2^{-/-} mice had a markedly reduced colitis compared to WT [304]. This was associated with reduced tissue MPO expression in the early stages of colitis, suggesting that Nox2 contributes to inflammation. Our study suggested that there was a small, but statistically insignificant, difference in inflammation scores between WT and Nox2^{-/-} mice. We measured Ly6G⁺ cells in the colon as a measure of neutrophil infiltration, and again found a small but statistically insignificant reduction in neutrophil numbers. While not statistically significant, it is possible that it was biologically significant, as Nox2^{-/-} mice had reduced rates of cancer and less severe cancer measurements, such as tumor number. It is possible that in the absence of statistical significance, a slightly reduced inflammation in Nox2^{-/-} mice was enough to reduce the effect on cancer development after inflammation. A reduction in neutrophils could still have an impact on tumorigenesis despite a lack of Nox2, as neutrophils produce pro-inflammatory mediators such as cytokines and proteases that could contribute to tissue damage and epithelial proliferation [428].

In our Nox2^{-/-} experiments, colonic epithelial cells show similar levels of 8-OHDG between strains, suggesting that they encountered similar levels of oxidative damage. Thus, whether the inability of infiltrating leukocytes to produce ROS and a resultant reduced level of colonic epithelial damage is responsible for the decrease in cancer incidence is not clear. This is inconsistent with the idea that the main producers of oxidative radicals during inflammation are Nox2-expressing cells. There could be a number of possible explanations. First, there could be intrinsic ROS production as other ROS-producing enzymes are activated by inflammatory mediators within the colon, such as Nox1, abundant in the colon [384]. Intrinsic Nox1 activation is known to damage DNA [429, 430]. Second, there is potential for other ROS production to be increased in both infiltrating cells and epithelial cells, as p47^{-/-} neutrophils were reported as having a significantly increased stimulated nitric oxide production compared to WT cells [303]. iNOS has been shown to play an anti-inflammatory [431, 432] and a pro-inflammatory role [303] in experimental colitis. WT mice irradiated and reconstituted with iNOS^{-/-} bone marrow are protected against DSS and TNBS colitis, suggesting that leukocyte

iNOS may have a pathogenic role in colitis [433]. Neutrophils were found to be major producers of iNOS [433]. It is possible that leukocytic iNOS can lead to inflammatory damage while epithelial/stromal iNOS could be more protective, and leukocyte NO production in the case of Nox2 deficiency could compensate for oxidative damage in epithelial cells. iNOS expression has been correlated with 8-OHdG expression, however, no definitive link has been reported [434].

Similar to models of colitis, the role of iNOS in CAC is not clear. It was reported that a nitric-oxide inhibitor was effective at reducing CAC in APC^{+/-} mice treated with DSS [435]. A second study found that iNOS deficiency did not affect the expression of CAC [436]. Double-deficient IL-10^{-/-}iNOS^{-/-} mice did not develop inflammation differently than IL-10^{-/-} mice, however, IL-10^{-/-}iNOS^{-/-} mice had greater cancer severity compared to IL-10^{-/-} mice, suggesting that iNOS served a protective role in the absence of IL-10 [437]. Interestingly, a protective role of NO has been described in the ability of NO to reduce damage by ROS by inhibiting NADPH oxidase [438]. It is possible that in the absence of NO these radicals can further damage the mucosa. All three studies used different models to evaluate the role of iNOS in CAC. As there is a complex interplay of both genetic and environmental factors (with the method of colitis induction being an environmental factor), it is difficult to compare these studies in a common context. Measuring iNOS and NO in our model would help clarify the interplay between ROS and nitric oxide. In addition, measuring nitrotyrosine would give another indicator of both oxidative and nitrosative damage, as nitrotyrosine is an oxidation product of peroxynitrite and measurable via immunohistochemistry or Western blot.

In addition to ROS-induced oxidative damage, TNF may lead to DNA damage by other mechanisms. One mechanism of note is the activation of cytidine deaminase. This enzyme removes the amino group from the cytidine base, altering it to uridine [480]. This causes DNA repair mechanisms to recognize the former cytidine as thymidine, leading to mutations changing the C:G base pair to T:A base pairs. This enzyme is crucial in the development of antibody diversity [480]. In CAC, increased levels of cytidine deaminase were detected in both inflamed epithelium and tumors. In a colonic epithelial cell line, TNF induced cytidine deaminase and this led to an increase in p53 mutations [480]. Therefore, it is possible that TNFR1 deficiency could affect the expression of cytidine deaminase, however, this has yet to be determined.

During my research, I became interested in the role that neutrophils played in tumor development. We observed high levels of Ly6G⁺ cells within tumors in all of the mouse strains within the dysplastic glandular crypts and tumor periphery, where Ly6G⁺ cells appeared to be migrating out of the tissue into the lumen. The involvement of these cells in tumors has been well-documented [275, 398, 439]. As they are a major infiltrate during colitis [440], it is possible that they contribute to tumorigenesis. We began to evaluate the role of neutrophils in colitis by depleting Ly6G⁺ cells during DSS treatment in C57BL/6 mice. Due to high clinical scores and weight loss, we were hesitant to continue with repeated DSS treatment in case of extreme morbidity and mortality. Another barrier to this study was whether we were depleting mature neutrophils, or whether we would be depleting myeloid-derived suppressor cells (MDSC), which have been shown to express Ly6G when they are of the granulocytic MDSC phenotype in mice [441]. Upon reflection, it would have been interesting to look at depleted Ly6G⁺, or Gr-1⁺ cells in a modified AOM+DSS model to determine a role for neutrophils that infiltrate in chronic colitis and their function in tumor development. In addition, earlier time points could be used to determine how neutrophils could function in the initial process of tumor development. Complications of this regimen, especially depletion over several cycles could potentially lead to problems with sepsis, and neutralizing antibody production [442]. In addition, the depletion of neutrophils would be transient, occurring only during DSS treatment, as after the discontinuance of the antibody treatment, mice began to show neutrophils in the blood, bone marrow and intestine again. Nevertheless, the finding that tissue damage is abated, yet clinical illness is more severe is an interesting finding and supports a dual role for neutrophils in infection and colitis.

While TNFR deficiency did not affect Ly6G⁺ cell migration into tumors, Nox2^{-/-} mice showed a reduction in tumor-associated neutrophils. It is possible that the neutrophil infiltration into tumors is indicative of the presence of MDSC. Nox2^{-/-} MDSC demonstrate an inability to suppress T-cell activity, suggesting that anti-tumor immunity in Nox2^{-/-} mice may be more effective, potentially explaining the reduced cancer incidence and severity we observed [443].

Whether neutrophils play a role in the development of tumors from healthy tissue needs to be investigated further. It has been demonstrated in clinical studies that intratumoral neutrophils are associated with a shorter, recurrence-free survival in those

affected by clear cell renal-cell carcinoma [439]. It was also reported recently that neutrophils were present in high numbers in the peritumoral stroma in human hepatocarcinoma, and the number of neutrophils present was associated with a poor prognosis [276]. For example, head and neck tumor cells have been shown to produce macrophage inhibitory factor (MIF), leading to CXCR2-dependent neutrophil chemotaxis towards the tumor and MIF production was directly proportional to neutrophil infiltration into tumor tissue. Tumor-derived MIF led to MMP-9 production by neutrophils [274]. *In vitro* experiments revealed that neutrophils cultured in tumor-conditioned medium, as opposed to liver cell-conditioned medium, produced elevated levels of MMP-9. In addition, Ly6G⁺CD11b⁺ neutrophils can develop into a pro-tumorigenic phenotype dependent on TGF- β , demonstrated by reduced tumor growth after neutrophil depletion in mice [402]. Neutrophils can play a pro-tumorigenic role by secreting pro-inflammatory and angiogenic factors such as MMP-9 and several cytokines, as well as ROS that can damage cells and tissues, and contribute to oxidative stress and potentially contributing to DNA mutations. The contribution of neutrophil-derived ROS and oxidative stress remains unclear at present.

Relevance of the Model to Human Colitis-Associated Cancer

The model we have used to evaluate CAC has several advantages over other models. The use of DSS allows the control of the inflammation [77]. With this tool, we can mimic a remitting-recurring course of inflammation that occurs in both CD and UC. DSS is minimally invasive and does not place, other than inflammation and related complications, any extra stress on the animal. It does not require any genetic deficiencies, although genetics do play a role [75, 102, 444, 445], and can therefore be used to study the roles of different genes in the development of CAC without a pre-existing genetic deficiency confounding the results. An example of this in the context of this study was the use of *Apc*^{min/+} mice as a background strain to demonstrate CAC in TNF-deficient mice. The TNF deficiency did not alter the course of inflammation or cancer, which is contrary to what we, and others have found. In addition, *APC* mutations are a late occurrence in CAC, with the *APC* mutation being the major genetic change that occurs between dysplasia and carcinoma [13]. The *APC* gene product plays a role in cell-cell adhesion and regulation of β -catenin signaling, which could have an impact in inflammation as well as cancer development. One major difference between

AOM+DSS-induced CAC and IBD-associated cancer is mutations of β -catenin. IBD-associated cancer tends to present mutations in *APC*, which occur in advanced dysplasia and carcinoma, and is thus a late event [57, 63], while in AOM+DSS-induced cancers these mutations more commonly occur in β -catenin [76]. Other reports suggest that β -catenin and *APC* mutations are not common events in UC-associated cancers and are more involved in sporadic colon cancer [65]. Therefore differences between tumor phenotypes in the mouse model and human CAC may affect the efficacy of therapeutic interventions.

In AOM+DSS induced CAC, the relative frequency of chromosomal instable tumors has been reported as 81% [446], not that different from the 85% reported in IBD-associated cancers [49]. Another similarity between the mouse model and IBD-associated cancer is that in both our experiments and in IBD-associated cancers, dysplastic lesions and tumors tend to occur distally and not in the proximal colon [447]. In AOM+DSS associated cancers, several oncogenic genes are upregulated such as *MYC* and Wnt inhibitory factor [448]. IBD-associated cancers also show *KRAS* mutations [63], while *KRAS* mutations have not been reported in AOM+DSS. Inflammation-independent AOM-induced carcinogenesis, however, has been reported to produce mutations in many genes observed altered in IBD-associated cancer, specifically *KRAS*, suggesting that this is likely mutated in CAC as well [82]. Overall, much less is known about mutations that occur in the AOM+DSS model of colitis and if they reflect the genetic profiles of IBD-associated cancers.

In our experience, AOM+DSS-induced colitis does not cause invasive carcinomas at least during the regimen used in these studies. It is possible that with increased dose of AOM and longer periods between DSS treatments, this could occur. The molecular events determining invasive-carcinoma are not as well-understood in our model. The expression of p53 may be an indicator of advanced stage in this model, however mutations in p53 are not widely reported. Invasive carcinomas were described in AOM+DSS-treated mice lacking heat-shock protein 70, associated with an increased Cox-2 and iNOS expression as well as p53 mutations in 2/5 tumors [449]. Positive immunostaining for p53 is associated with the severity of dysplasia, with more advanced dysplasia and carcinoma being positive for p53 [450, 451]. In murine DSS-only CAC, p53 immunostaining is not as common within tumors [78, 452], suggesting that p53

positivity is associated with AOM-induced mutations/alterations. p53 is a tumor suppressor activated when DNA damage or oxidative stress occurs, and regulates the function of DNA repair enzymes and can halt the cell cycle [453]. There is evidence that p53 can regulate genes involved in tumor invasion and progression, and loss of function of this gene may be important in determining the invasiveness in CAC. How this may correlate to invasiveness and positive immunostaining in CAC is unclear, although it could mean that overexpression of p53 occurs in response to cell stress but mutations render this protein non-functional. The lack of invasiveness in the AOM+DSS model is definitely a disadvantage because tumors are not truly malignant until they can breach the submucosal barrier.

There are several limitations to this model that should be addressed. First, because water is fed *ad libitum*, the mice might receive variable doses of DSS. While group housing, and constant conditions will minimize this, this is a fact that we must consider and may explain some of the variation in inflammation observed within groups. In addition, the DSS batch could have variable composition. The molecular weights of the DSS polymers are variable, being 36,000-50,000, suggesting that a particular batch could have a more potent colitogenic action, as higher molecular weight DSS molecules are associated with a more robust inflammatory response [101]. Even the sulphur content of the DSS may be linked to the colitogenic properties of DSS [114] and this could explain the clinical illness differences between the TNFR^{-/-} and the Nox2^{-/-} studies in WT mice. It is possible that the sulphur content was different between lots, although this has not been specifically evaluated. Another limitation is that intestinal inflammation, after DSS administration, does not result in any measurable tumor development. AOM is required to induce DNA damage that can result in tumor development with inflammation acting as a promoter. Unlike inbred mice, humans are genetically heterogeneous, and our environment is not controlled therefore we are exposed to a number of toxins that could contribute to cancer development. Therefore, AOM essentially acts as that environmental factor.

Another difference between this model and IBD is that in IBD, anatomical location is an important risk factor for CAC [26]. In our model, despite lower levels of inflammation in the distal colon compared to the mid-colon, tumors were found in similar numbers in both the mid and distal colon. We would have predicted that due to the extent

of inflammation in the mid-colon, most of the tumors would have developed there. It is possible that the enzyme that catalyzes AOM was more bioactive or expressed at greater levels in the distal colonic epithelium, therefore requiring less inflammation to cause tumorigenesis. Another explanation is that inflammation was severe in the distal colon, but resolved at the time of our measurements. It is also possible that DSS feeding changed the microflora [99], changing the signaling patterns within the epithelium of the distal colon. A study evaluating colorectal cancer in MyD88-deficient mice found that in the absence of inflammation, this strain was less susceptible to tumorigenesis when crossed with the APC^{min/+} strain [486], suggesting that signals induced by the microflora may play a role in tumorigenesis.

During consideration of a CRC model in the study design, the AOM+DSS model provided minimal invasiveness and maximum consistency, and the tools were available (mice with targeted mutations) to study the genes of interest. Overall, this model provides a good foundation upon which to evaluate the mechanisms of human CAC.

Limitations of Research

We were interested in complete TNF deficiency in the development of colitis-associated cancer. We treated TNFR1/TNFR2 double receptor knockout mice with 3% DSS for 5 days followed by 2 days of regular tap water to determine extent of colitis (Appendix B). In agreement with previous research done using TNF deficient mice [198], these mice became very ill and half had to be euthanized before the termination of the experiment. Therefore we could not continue this experiment to evaluate chronic colitis in these mice. Upon reflection, the doses and timing of AOM and DSS could have been altered to measure the effect of complete TNF deficiency on CAC. It could also have been possible to use these mice in a chimera model to evaluate tissue-specific TNF deficiency. It is possible that TNFR2 could play a role in cancer development under different circumstances.

We measured neutrophil numbers (Ly6G⁺ cells) as an indicator of relative inflammation severity. These numbers appeared to confirm our more subjective measures of inflammation severity, such as clinical score and histopathological scores. Nevertheless, a second objective measure of inflammation severity would have helped strengthen the data and overcome any biases that were present. Certainly the scale and relative weighting of each element may have some impact on our conclusions, in

particular with respect to the scale used to measure histological inflammation. The scale used to measure acute colitis may not be suitable to characterize chronic inflammation. In the chronic studies, especially the Nox2^{-/-} study, there were some situations where dysplastic tissue spanned the whole mid-colon. It was difficult to ascertain inflammation severity in those cases because it is not clear if the infiltrate that is present is a result of the tumor cells or inflammation, and instead is responsible for tumorigenesis.

There is a possibility that the secreted homotrimer lymphotoxin $\alpha 3$, a membrane-bound member of the TNF superfamily, could affect TNF signaling, as it can interact with both TNFR1 and TNFR2. There is evidence that lymphotoxin $\alpha 3$ promotes inflammation through TNFR1, as shown in transgenic mice overexpressing lymphotoxin $\alpha 3$ in the kidney and pancreas then crossed with TNFR1 or TNFR2-deficient mice [482]. This has implications in our TNFR1-deficient model as the pathogenesis of AOM+DSS CAC could involve lymphotoxin $\alpha 3$. We did not consider whether or not the pro-tumorigenic effect of TNFR1 signaling is attributable to TNF or lymphotoxin $\alpha 3$.

With respect to the chimera data, given that chronic inflammation was affected by the strain of the host and graft, it would have been desirable to look at acute inflammation in these mice. We would have predicted based on our acute studies using single receptor knockout mice that inflammation would not have been different between groups. While a complete TNFR1 deficiency might have little effect on clinical and pathological outcomes in acute colitis, further delineating where exactly TNFR1 might be more important would help our understanding of how TNF antagonists reduce colitis.

The chimera model has a confounding effect related to the extent of chimerism. Because we used donor bone marrow from male mice, we looked at Y-chromosome via *in situ* hybridization within the nuclei in both the spleen and the gut from chimeric mice. This method may not accurately reflect the extent of chimerism due to differences in the way that the section is cut. While 10 μ m sections were used, it is quite possible the Y-chromosome was present in an area of the section not visualized on the slide. We were able to detect Y-chromosome in our samples, although we cannot accurately determine the proportion of cells that originate from donor cells. If we were to repeat this study, it would be more efficient and quantitative if we were to use congenic mice as wildtype mice and measure the congenic marker such as Thy 1.1 in bone-marrow-derived cells as a positive indicator of effective reconstitution.

Alternatives to bone marrow chimeras exist, such as creating a targeted mutation under the control of a tissue-specific promoter could be used to specifically knock out the receptors in myeloid versus lymphoid tissue, or in mesenchymal or epithelial tissue. This could be beneficial irradiation and reconstitution, especially if it a knock-out mouse, will lead to a deficiency of the specific receptor in all non-hematopoietic cells. With a conditional knockout model, we could specifically remove the TNF receptors from the colonic epithelium or sub-epithelial myofibroblasts. It is of significance that targeted deletion is not absolute and could result in a leaky model, similar to the production of chimeras.

Our models used mice with TNFR deficiencies. These deficiencies affected cells of all systems. This could be particularly important in TNFR1 deficiency, where TNFR1 is reportedly widely expressed in most cell types. While we looked at bone marrow versus stromal expression of TNFR1 in experimental CAC, we did not look at the contribution of individual cell types. For example, inhibiting TNF specifically in macrophages reportedly reduces both TNBS and CD45RB+ colitis [454]. This could have implications in IBD as specifically targeting cells rather than broadly inhibiting an important inflammatory mediator might result in reduced side effects such as immunodeficiency.

While the TNF-dependent oxidative damage is implicated in this research, these studies did not definitively conclude that it originates from neutrophil-derived ROS or that it induced colonic epithelial cell carcinogenesis. In addition, there is no causative link to suggest that ROS-mediated damage is the link between inflammation and cancer in this model. There are challenges to working with neutrophils given that they are terminally differentiated and do not survive long enough for effective transfection to study receptor function. Research examining TNF receptors on neutrophils utilizing blocking antibodies [315] may have unintended side effects such as Fc receptor activation, complement activation, or non-specific binding.

The use of a knock-out model somewhat overcomes some of the limitations of using blocking antibodies, providing that the targeted mutation does not affect leukocyte development within the bone marrow. We used both bone-marrow derived granulocytes and mature granulocytes from a model of thioglycollate-induced peritonitis to detect ROS production after TNF treatment. TNF signaling can induce phosphorylation the p47

subunit of Nox2, which in effect primes NADPH oxidase [317, 420]. A second stimulus is required for full activation, such as adherence of the neutrophil leading to integrin activation [315, 455]. We were not able to measure ROS production in mouse neutrophils or macrophages, neither in bone marrow derived granulocytes, whole bone marrow, nor from activated neutrophils and macrophages from thioglycollate-induced peritonitis. After using three different methods to detect ROS production, including the use of dichlorodihydrofluorescein diacetate (fluorescent), superoxide dismutase-inhibitable cytochrome C reduction (colorimetric), and the excitation of luminol (chemiluminescent), we were still unable to detect TNF-mediated production of ROS. Our positive control, PMA, was able to induce immediately detectable ROS production. In contrast to our findings, TNF-induced priming of murine bone-marrow-derived neutrophils via TNFR1 has been reported [456], therefore, it is still possible that TNF can contribute to neutrophil and macrophage-mediated oxidative stress. TNF-priming significantly enhanced superoxide radical production by a form of fMLP, a bacterial peptide that has a receptor in the mouse. The study by Onnheim et al., suggested that TNF priming through TNFR1 resulted in the mobilization of stored granular proteins such as the fMLP receptor in addition to the complement receptor 3 proteins [456], triggering a swift and strong ROS response after stimulation via the bacterial peptide. While we did not find an effect of TNF priming or priming via other mediators on neutrophil ROS production in response to fMLP this does not rule out a role for the mouse analogue.

Future Directions

While we implicated TNF in oxidative stress, more research is needed to clarify if TNF plays a role in the production of ROS during inflammation. WT and TNFR1^{-/-} mice could be treated with a dietary or injectable antioxidant. Caution would have to be taken that the antioxidant only acted to quench free radicals and did not have actions on inflammation. Several studies using antioxidants in similar models have found protection against the development of cancer after chronic inflammation [385, 387, 457, 458]. If the mechanism did involve TNF, one would expect that antioxidants would not be as effective in TNFR1^{-/-} mice as they would be in WT mice. Furthermore, using conditional knockouts could provide further insight into the role of TNFR1-mediated

oxidative stress via Nox2. A hematopoietic or mesenchymal Nox2/TNFR1 deficiency specifically in Nox2^{-/-} mice would provide a model in which we could investigate the contribution of bone-marrow-derived cells and/or subepithelial myofibroblasts in propagating oxidative damage to the epithelium.

Interestingly, TNFR2 deficiency did not greatly alter the course of colitis or colitis-associated cancer compared to WT mice. TNFR2 has been implicated as a costimulatory molecule on T-cells [210], in particular, a necessary mediator in the suppressive function of natural Treg [212, 213]. The involvement of Treg in colon carcinoma has not been extensively examined, although in contrast to other cancers, Treg may play a protective role. It has been shown that CD4⁺CD25⁺ T-cell adoptive transfer to RAG2^{-/-} mice treated with *H. hepaticus* not only reduced inflammation, but also colorectal cancer. In that case, Treg were protective [459]. Another study showed that CD4⁺CD25⁺ cells led to intestinal tumor regression in *Apc*^{min/+} mice in an IL-10-dependent manner [460]. It would be interesting to measure Treg in this model of CAC and to determine if TNFR2 would have any impact on the development of colitis and/or cancer. It is possible that in TNFR1^{-/-} mice, there could be an increase in this T-cell subset and this could potentially reduce cancer incidence. We did not measure T-cells after our treatment regimen, which would have been interesting given TNFR1^{-/-} mice have elevated IL-12 concentrations. IL-12 can reportedly alter the Treg phenotype, namely by inducing IFN expression by Treg, a phenotype associated with reduced colitis [461]. Whether this would have an effect on colitis-associated cancer is not known. It could be speculated that TNFR2 signaling could induce Treg development and in the presence of IL-12, Tregs could have an anti-tumor function in TNFR1^{-/-} mice.

Peroxisome proliferator activated receptor (PPAR) γ is a nuclear receptor that regulates cytokine production and is potentially protective in CAC [253, 462]. PPAR agonists are shown to be protective in rodent models as well as UC [463-465]. In addition, PPAR γ deletion specifically in macrophages exacerbates colitis, suggesting a tissue-specific role [466]. Similarly, PPAR γ deletion specifically in the epithelium exacerbates colitis [467]. Treating IL-10^{-/-} mice with anti-TNF antibody was shown to increase growth hormone signaling and STAT5b activation, and subsequent colonic epithelial PPAR γ expression [190]. PPAR agonism could explain lower levels of cancer in TNFR1^{-/-} mice, as PPAR γ is known to protect against tumorigenesis in rodent models

of CAC [253]. TNF was shown to inhibit PPAR γ by both reducing its transcription [468, 469] and reducing its DNA-binding activity in adipocytes [470], a function associated with NF κ B signaling. ERK and JNK have been shown to inhibit PPAR γ transcriptional activity, by phosphorylating sites directly on PPAR γ and both kinases are regulated by TNF [471]. One mechanism by which this may occur is through the TNF-induced nuclear translocation of histone deacetylase C3 (HDAC3), which is normally complexed with I κ B α , but becomes disassociated from HDAC3 upon activation-induced degradation of I κ B α . In 3T3-L1 adipocytes, treatment with TNF leads to HDAC3-mediated inhibition of PPAR γ transcription after nuclear translocation of HDAC3 [472].

As such, HDAC inhibitors are being studied for their use in cancer therapies. In experimental colitis, the oral administration of HDAC inhibitors valproic acid and suberyolanilide hydroxamic acid was shown to reduce measures of colitis in both TNBS and DSS-treated mice. This reduction in disease severity was associated with a hyperacetylation of histone within the inflamed colon [473]. HDAC inhibitors are described in the literature in the prevention of colitis-associated cancer [474, 475]. Measuring histone acetylation in our model would indicate whether HDAC inhibitor treatment would be beneficial. If TNFR1^{-/-} mice were treated with a HDAC inhibitor resulting in an increase in PPAR γ activity, then this could be a mechanism by which TNFR1 is contributing to both inflammation and cancer. Likewise a PPAR γ agonist could be used in TNFR1^{-/-} mice to determine if there is any additional benefit to activating PPARs and therefore any additional mechanisms by which TNFR1 contributes to cancer.

In Nox2^{-/-} mice, the mechanism by which Nox2 deficiency is protective against CAC development remains unknown. Given that Nox2^{-/-} tumors have a reduced Ly6G⁺ cell infiltration, it is tempting to speculate that MDSC are reduced, providing a mechanism for smaller tumors observed in this study. Measuring Gr-1⁺CD11b⁺ cells in these mice would be the first step in determining this, and I would hypothesize that they would be present in reduced numbers in Nox2^{-/-} mice. Then steps could be taken to reduce infiltration by these cells could be studied as a means of reducing tumors.

Significance of Research and Concluding Remarks

The importance of targeting TNF in IBD therapy is paramount, and numerous biological therapies have been developed to effectively reduce disease and improve

patient quality of life. Despite this, there is still a significant subset of IBD patients (between 25-40%) who do not respond or lose responsiveness to anti-TNF therapy [442]. This research based on a CAC model in the mouse, suggests that TNF antagonism might still be beneficial despite a lack of efficacy in reducing acute colitis symptoms in that it may reduce the risk of colon cancer in affected individuals. Colectomy is reportedly reduced in the year following treatment with Infliximab [24], yet with a reduction in colectomy rates, the risk of developing colorectal cancer could increase, especially if microscopic inflammation that might not produce clinical symptoms can contribute to cancer development.

We found that despite similar inflammation between WT and TNFR1^{-/-} mice, TNFR1^{-/-} mice were protected against cancer development. Likewise in BALB/c mice treated with Etanercept and Infliximab [243, 252], a reduction in tumor burden was found without a concurrent reduction in inflammation, respectively. Now, we confirmed that TNF blockade through TNFR1 independent of inflammation protects against cancer development, suggesting that the anti-tumor effect can be attributed to TNFR1. This lends significance to the continued use of Etanercept in IBD, which is reportedly ineffective [181], especially in patients that develop tolerance to other anti-TNF therapies. It also suggests that targeting TNFR1 specifically could be of clinical benefit in patients that elect not to get a colectomy, and this specific targeting may reduce the immunosuppressant properties of anti-TNF therapies.

This research is important because effective treatments are reducing the colectomy rate in ulcerative colitis [24] therefore even with reduced inflammation, there still might be a risk of cancer. There is evidence that Adalimumab can be used for maintenance therapy [476] and our data suggest might be beneficial in preventing CAC even if does not effectively treat inflammation in the subset of individuals who are unresponsive. More research is needed to clearly identify the cell types involved in this TNFR1-mediated response, as anti-TNF therapy may in the future be better able to specifically target cells to reduce undesirable side effects like increased susceptibility to infection and lymphomas associated with immunocompromised states.

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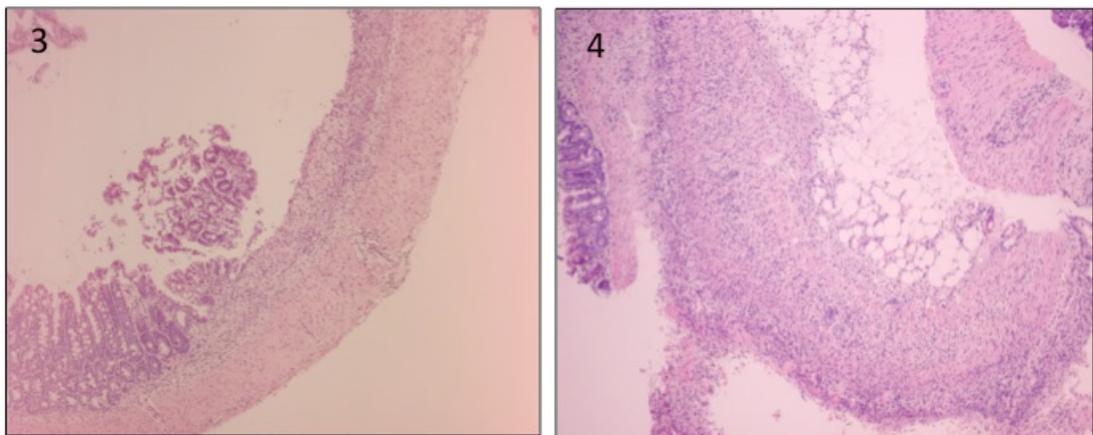
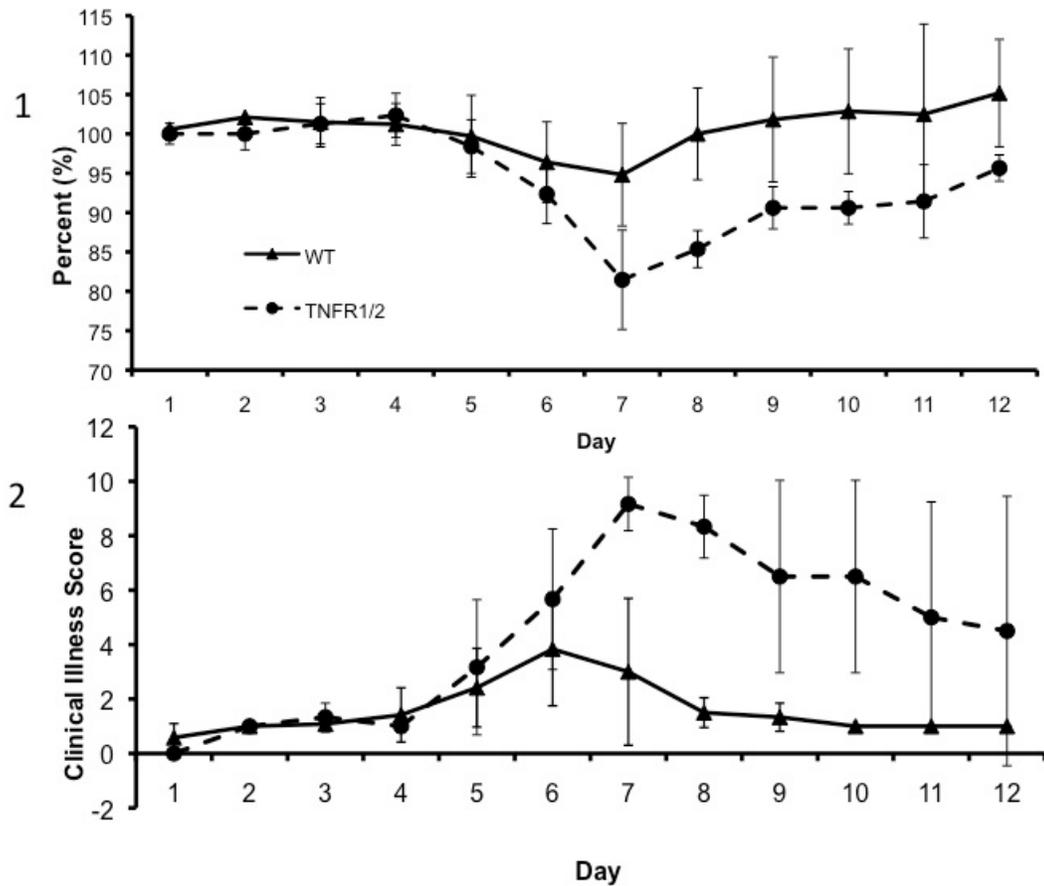
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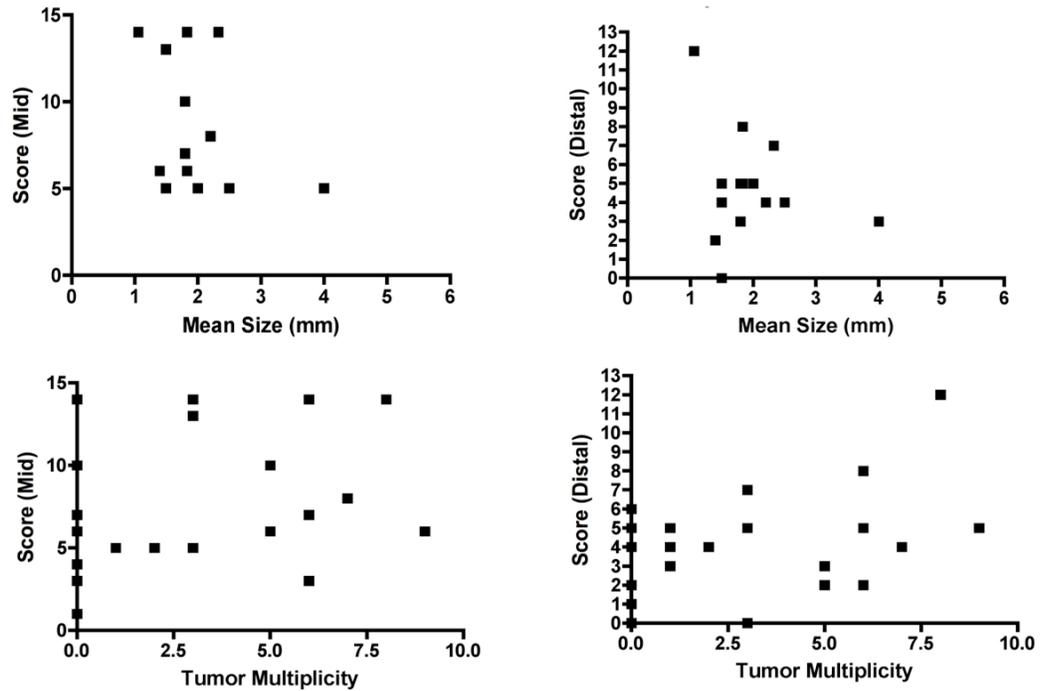
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APPENDIX B: Double-TNF Receptor-Deficient mice and Acute DSS Colitis



WT and TNFR double receptor knockout (TNFR) mice were subjected to the acute DSS colitis regime as described in Chapter 2. Figures 1 and 2 show the percent weight loss in both strains, with a significantly greater weight loss and clinical scores, respectively, occurring in TNFR^{-/-} mice. Figures 3 and 4 depict histological damage (magnification 100x) in the mid-colon in WT (3) and TNFR^{-/-} (4), respectively. These experiments were not included in Chapter 3 due to a potential difference in background strains. The TNFR mice were only backcrossed onto C57BL/6 mice for 3 generations.

APPENDIX C: Correlation Between Inflammation Severity and Tumor Sizes and Multiplicity in WT, TNFR1^{-/-} and TNFR2^{-/-} Mice

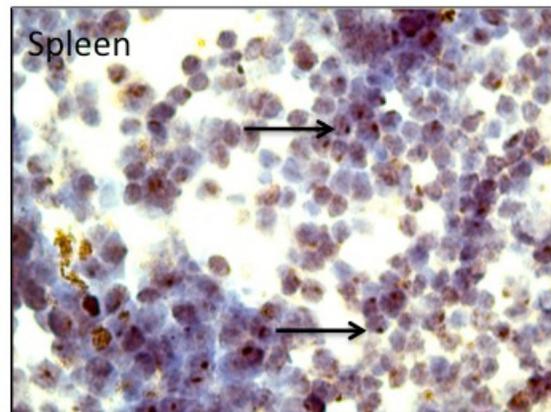
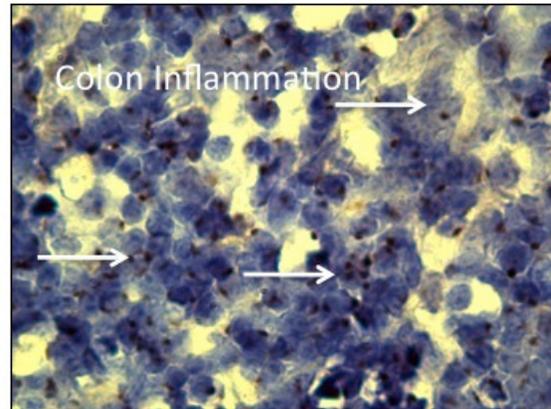


$r^2=NS$

The number of tumors per colon, and the size of tumors in the colon did not correlate with the severity of inflammation in the mid-colon or distal colons in WT, TNFR1^{-/-} and TNFR2^{-/-} mice. NS=not significant.

APPENDIX D: Y-Chromosome Detection in Bone-Marrow Chimeric Mice.

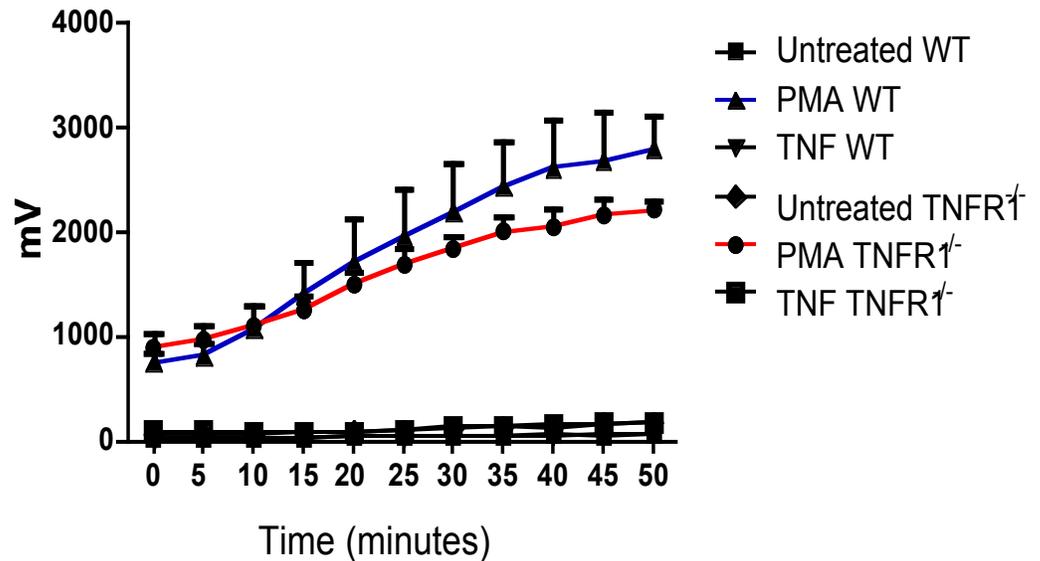
Magnification 1000x oil immersion



Female mice were irradiated and reconstituted with bone marrow derived from male donors. To determine engraftment, Y chromosome staining was detected by in situ hybridization and immunohistochemical methods (DAB). Nuclei positive for the Y-chromosome show dark brown spots (denoted by white and black arrows).

APPENDIX E: TNF-Stimulated Reactive Oxygen Species Production in Neutrophils

Oxygen radical production by mouse PMN adhered to fibrinogen derived from thioglycollate-induced peritonitis



TNF=50 ng/ml
PMN= 3×10^6 cells/ml

Mice were administered 1 ml of sterile thioglycollate I.P for 4 hours (neutrophils) or 4 days (macrophages) to induce peritonitis. After 4 hours or 4 days, mice were euthanized via CO₂ inhalation and a peritoneal lavage was performed to remove cells from the peritoneal cavity. The above figure depicts neutrophil (approximately 88% purity) ROS production after adherence to fibrinogen and PMA or TNF stimulation from WT or TNFR1^{-/-} mice in triplicate. Luminol was added prior to stimulation and chemiluminescence was measured on a Veritas Luminometre. This is a representative graph from at least 3 experiments. This experiment was also performed with fibronectin coated wells, and with different stimulants such as CXCL2, fMLP, LPS, combined with TNF or on their own. Except for PMA, we did not find any ROS production after stimulation. Significance was determined using a repeated measures ANOVA ($p < 0.05$) over time.

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