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A Biologically Active Tnf-Alpha Inhibitor Fails To Suppress Colitis In Balb/C Mice

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A BIOLOGICALLY ACTIVE TNF-ALPHA INHIBITOR FAILS TO SUPPRESS COLITIS IN BALB/C MICE

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By

Stephanie Elyse Biel

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ABSTRACT

Tumor necrosis factor α (TNFα), a potent inflammatory cytokine, has long been established as a major driving force for pathologic inflammation. Currently, anti-TNFα therapies are the standard in Inflammatory Bowel Disease (IBD) management; however, one-third of IBD patients fail to respond to anti-TNFα therapies. Previous data from this lab indicate that TNFα Converting Enzyme (TACE) inhibition does not ameliorate colitis in BALB/C mice. Thus, we hypothesized that TNFα is not a critical component in the BALB/C model of colitis. To test this, acute colitis was induced in BALB/C mice by consumption of 5% dextran sulfate sodium (DSS) in drinking water for 7 days. TACE inhibition was achieved through twice daily intraperitoneal injection of DPC-333 (10 mg/kg; *BSM, Inc.*) To determine the effects of TACE inhibition during colitis, BALB/C mice received the following experimental treatments: Group 1) H_2O + vehicle; Group 2) DSS + vehicle; Group 3) DSS + DPC-333. Although TACE inhibition significantly reduced colon TNFα levels ($p = 0.0172$), no significant improvement in disease activity was observed ($p = 0.74$), as determined by clinical scoring of bodyweight loss, rectal bleeding, and diarrhea. Thus, colitis in BALB/C mice does not appear to be TNFα-driven and an alternative pathway must exist. It is possible that BALB/C mice could represent a pre-clinical model of primary non-responders to anti-TNFα therapies. Future studies may use this model to better understand mechanisms of primary non-response in IBD patients.

KEYWORDS: inflammatory bowel disease, tumor necrosis factor alpha, tumor necrosis factor alpha converting enzyme, ulcerative colitis, crohn's disease

This abstract is approved as to form and content

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Inflammatory Bowel Diseases

Overview. Inflammatory bowel diseases (IBDs), such as Ulcerative Colitis (UC) and Crohn's Disease (CD), have been estimated to affect about 2.8 million people in the United States (Kappelman et al., 2007). These diseases are characterized by recurring episodes of acute intestinal inflammation resulting in diarrhea, abdominal pain, fever, blood in the feces, and weight loss. Currently, the etiology of IBDs is unclear, although the CDC has observed relationships between IBDs and gender, smoking status, socioeconomic status, and diet, suggesting a role for epigenetics in disease development (Jenke, 2012). Research attributes disease pathology to an inappropriately activated immune system leading to increased permeability of the intestinal wall in response to oxidative stress (Obermeier et al., 1999).

Although research has led to the innovation of therapies and improved diagnostics, a steady rise in the occurrence of IBDs has been documented in Western Europe and the United States since the 1960s. Furthermore, IBD patients are more likely to develop pernicious anemia and lactose intolerance due to intestinal degradation (Podolsky, 1991). In childhood IBDs, malnutrition is a major cause of stunted growth (Murch et al., 1991). Likewise, severe UC inflammation has been proven to be related to neoplasm formation in the colon (Rutter et al., 2004). Treatments aimed at reduction of inflammation may improve these complications.

However, treating IBDs has proven to be a substantial economic burden. In 2004, the mean cost of treatments per patient for CD and UC were $8265 and $5066 per year,

respectively. For both diseases, over 30% of costs were attributed to hospitalization. Current pharmaceutical therapies are limited by efficacy and cost, and surgical management via colectomy has been reported to occur in 13.0-16.5% of UC patients at 20 years after diagnosis (Targownik et al., 2012). In 1991, it was reported that approximately 30% of CD patients required surgical intervention within a year of diagnosis, while the remainder required surgery at a rate of 5% per year (Podolsky, 1991). Surgical intervention often results in the required use of an ileostomy bag and increased risk of surgical complications, such as sepsis, colorectal cancers, fistula formation, intra-abdominal bleeding, and death (Tulchinsky et al., 2003).

IBD Pathogenesis. As earlier stated, the mechanisms of IBD pathogenesis are poorly understood, yet it is thought that the immune system is inappropriately activated (Figure 1). In healthy intestines, a luminal stimulus can incite inflammation through either an antigen-specific immune response or an antigen-nonspecific inflammatory response. The intestinal immune system will eliminate the stimulus, which halts the inflammatory response and preserves the integrity of the epithelial barrier. However, in the disease state, an inflammation develops in the same manner, but stimulus elimination will either not occur or occur inappropriately, leading to sustained inflammation and tissue damage. The mucosal barrier integrity is compromised as it becomes more permeable to intestinal flora and infiltrating inflammatory cells. Thus, a positive feedback loop ensues, perpetuating the inflammatory response and characterizing the disease state (Podolsky, 1991). We do not yet understand why IBD patients are being thrown out of homeostasis, but we think it is because of a dysregulation of the inflammatory response and the gut microbiota (Figure 2) (Xavier and Podolsky, 2007).

Genetic Factors in IBD Development. Throughout the study of IBDs, family aggregation has long been established. First-degree relatives of IBD patients have a relative risk of 5-fold or greater. Likewise, IBDs are more prevalent in Ashkenazi Jews (Podolsky, 1991); however, these conditions are not inherited in a simple Mendelian pattern, instead, substantial evidence supports a multigenic mode of inheritance (Pokorny et al., 1997). Furthermore, it is thought that epigenetic alterations may underlie gene-environmental modifications and constitute causal disease variants.

During chronic inflammation, inflammatory mediators such as cytokines and reactive oxygen species can induce methylation of various IBD-associated genes through various mechanisms (Chiba et al., 2012). The investigation of epigenetic mechanisms in IBD was fueled by the identification of DNMT3A, a methyltransferase gene, as a CD susceptibility gene. Consequently, altered methylation of a series of genes involved in the production of cytokines in response to IL-17 has been observed in IBD patients. IL-17 is secreted by Th17 cells, a subset of CD4+ T cells that are strongly implicated in intestinal inflammation (Nimmo et al., 2012).

Thus, recent genome-wide searches for IBD susceptibility loci have been successful in identifying genes that contribute to disease susceptibility (Figure 3) (Biancheri et al., 2013). These genes have been defined as those whose products may contribute to functional or structural abnormalities in the GI tract that cause it to be more susceptible to attack by infection, toxins, or autoimmune activity (Podolsky, 1991).

Novel associations exist between IBD and two genetic markers within the DNA mismatch repair (MMR) gene *MLH1*, which has been localized to human chromosome 3p21, a region recently identified as an IBD locus. The protein encoded by *MLH1*, MutL

Homolog 1, is one component in a system of seven DNA MMR proteins (Pal et al., 2008). The DNA MMR system provides fidelity in replication by correcting any post-replication errors that have escaped the proofreading function of DNA polymerase (Chang et al., 2000). Thus, mutations in DNA MMR genes impart a mutator phenotype to affected cells in UC, increasing genomic instability. In fact, the lack of efficient DNA MMR has been shown to lead to carcinoma as well as the production of aberrant proteins that could incite a localized or systemic autoimmune response (Pokorny et al., 1997).

Other studies have suggested that alterations in the matrix of mucin glycoproteins may be associated with UC. The dense layer of glycoproteins that coat the colonic surface is an important component in maintaining the integrity of the intestinal epithelial barrier (Podolsky and Isselbacher, 1983). One study found a selective reduction of one population of glycoprotein in UC patients; this alteration persists independently of inflammatory activity. Similarly, these glycoprotein alterations have been observed in unaffected monozygotic twins of patients with UC, supporting the idea that genetics may present a pre-disposing factor in IBDs (Podolsky, 1991).

Furthermore, altered expression of key immunoregulatory genes, such as TNFα and NOD2, has been reported in the peripheral blood of IBD patients. The *TNF* gene maps to the *IBD3* susceptibility locus on chromosome 6p21. Deletion of 3' regulatory elements from the *TNF* transcript in mice has been shown to induce a model of CD in mice due to increased TNFα biosynthesis (van Heel et al., 2002). Likewise, a frameshift mutation in the *NOD2* gene causes increased expression of NOD2, a protein responsible for inciting an inflammatory response following recognition of bacterial cell wall components; this up-regulation of NOD2 is associated with susceptibility to CD (Ogura

et al., 2001). Additionally, a 2012 study of IBD-associated genes provided evidence of differential methylation of CpG sites within these and other genes that are plausible candidates in CD pathogenesis (Nimmo et al., 2012).

Diagnosis of IBD. Because the etiology of IBDs remains unclear, the major forms of IBD, CD and UC, are defined by their clinical, pathologic, radiologic, endoscopic, and laboratory features. While these diseases share many signs and symptoms, increasing evidence exists to suggest that CD and UC are two distinct diseases (Podolsky, 1991).

Inflammation in UC presents continuously throughout the large intestine and rectum, although it is mainly confined to the mucosa and superficial submucosa of the intestinal wall (Figure 4) (Gore et al., 1996). In contrast, CD inflammatory processes can occur at any location along the GI tract, including the small intestine. However, inflammatory lesions are less continuous than those of UC and can penetrate deeper layers of the intestinal wall (Figure 5) (Podolsky, 1991). Another unique feature of CD is the development of small, discrete ulcers, termed aphthoid ulcerations. These ulcers have been discovered prior to onset of inflammatory relapse, give a more accurate assessment of disease severity, and may herald a relapse in established quiescent disease (Simpkins, 1977).

As stated earlier, diagnosis of IBD is accomplished via assessment of disease activity, primarily through patient-reported symptoms (i.e. abdominal pain, diarrhea, and weight loss), laboratory testing, and radiographic and endoscopic evaluation of the colon mucosa. Endoscopy of the colon, or colonoscopy, along with tissue biopsy, allows for histological assessment of the colon mucosa. This is pertinent in differentiating between UC and CD (Leighton et al., 2006). Laboratory biomarkers correlating with leukocyte

migration into the colon also prove useful as a secondary diagnostic tool (Schoepfer et al., 2013).

Once an IBD is diagnosed, pharmacological management begins. Results of disease assessment dictate which pharmacological therapy to use; this is determined by two general parameters: anatomical location of inflammation and inflammatory severity. UC practice guidelines, released in 2010 by the Practice Parameters Committee of the American College of Gastroenterology, establish a classification system for UC inflammatory severity as mild, moderate, or severe. UC disease severity can be determined by stool frequency, fecal blood content, erythrocyte sedimentation rate, and signs of toxicity such as fever, anemia, or tachycardia (Kornbluth and Sachar, 2010).

Current Treatments of Ulcerative Colitis. Along with changes in diet and pain management, UC patients are usually prescribed medication to reduce inflammation at the primary site of tissue injury and prevent recurrence. A variety of pharmaceuticals are currently available for physicians to choose from based on the specific benefits to individual patients and potential obstacles. Current therapies include anti-inflammatories, immunosuppressants, and pharmaceuticals aimed at blocking TNFα signaling.

Mesalamine, also known as mesalazine or 5-aminosalicylic acid (5-ASA), belongs to the aminosalicylate family of anti-inflammatories. Oral administration of the prodrug sulfasalazine allows for delivery of mesalamine directly to the large intestine (Azad Khan et al., 1977). In the colon, sulfasalazine is reduced to mesalamine by intestinal bacteria (Peppercorn and Goldman, 1972). Safety of these pharmaceuticals is currently under debate, as there are concerns regarding nephrotoxicity and side effects such as abdominal pain, nausea, and diarrhea (Reviewed by Böhm and Kruis, 2014).

Corticosteroids prove a more flexible treatment, as they can be administered orally, by enema, or topically. Corticosteroid medications are commonly used to control acute inflammation; however, adverse effects include steroid dependency, weight gain, cataracts, osteoporosis, myopathy, and increased susceptibility to infections (Reviewed by Lichtenstein et al., 2006). Thus, corticosteroid use has a limited duration and is determined a failure if resultant in steroid-dependent colitis remission (Benchimol et al., 2008).

Azathioprine is an oral immunosuppressant currently used to prevent transplant rejection and as a treatment for rheumatoid arthritis and dermatologic disorders (Reviewed by el-Azhary, 2003). Azathioprine is also used to induce remission for steroid-dependent UC patients (Ardizzone et al., 2006). While this steroid-sparing effect may reduce the need for subsequent surgical resolution, the optimal effects of this treatment can be delayed for up to four months, making UC management difficult (Present et al., 1980). Furthermore, there is evidence that treatment with azathioprine may increase the risk of lymphoma (Kandiel et al., 2005).

Another means of treating steroid-dependent UC is by CsA, an immunosuppressant that inhibits calcineurin, a protein phosphatase which plays a role in T cell development; inhibition of calcineurin by CsA blocks T cell function, thus reducing the immune response (Bram et al., 1993). Like azathioprine, CsA can increase the risk of malignancy, as well as damage the renal system (Grossman et al., 1996). Because of these adverse effects, CsA is regarded as a “last-ditch” attempt, reserved for severe colitis episodes when reduction of inflammation and prevention of colectomy is urgent.

Most recently, IBD treatments focus on reducing inflammation by blocking TNFα signaling. Currently, Infliximab, a monoclonal antibody against TNFα, is the standard in anti-TNFα therapies. In mild to moderate colitis, Infliximab resolves acute inflammatory episodes and improves remission time (Rutgeerts et al., 2006). It has also been shown to improve cases of severe inflammation, although it is less effective in these cases (Olsen et al., 2009). One study demonstrated that long-term Infliximab treatment induces mucosal healing, improves long-term outcomes, and reduces the need for surgical intervention (Figure 6) (Schnitzler et al., 2009). While Infliximab is the current standard in IBD treatment, its effectiveness may be hindered by development of host antibodies, resulting in undetectable levels of Infliximab in serum and increased risk of allergic reaction to Infliximab infusions (Baert et al., 2003).

A review of the current IBD treatments reveals that a quick-acting, long-term treatment aimed at increasing remission time and reducing inflammatory episodes are imperative. Anti-TNFα therapies may allow for long-term IBD management, however, these therapies are last-resort pharmacological approaches to steroid- and thiopurine-resistant patients. Furthermore, studies show that anti-TNFα therapies are only effective in about two-thirds of IBD patients; the remaining one-third of patients demonstrate no response or a loss of response during treatment (Figure 7) (Papadakis et al., 2005). Further medical options are necessary to effectively treat this cohort of colitis patients.

Tumor Necrosis Factor α

Overview. Tumor Necrosis Factor α, or TNFα, is a potent inflammatory mediator originally observed as a host-derived mediator required for endotoxin-induced

necrosis of tumor cells (Carswell et al., 1975). Since its identification in 1975, over-expression of TNFα has been implicated in many inflammatory pathologies; novel methods of disease treatment through TNFα inhibition are continuously being explored.

TNFα Structure and Biology. TNFα, a cytokine primarily produced by activated macrophages and monocytes, plays an important role in the initiation, regulation, and perpetuation of the inflammatory response (Koss et al., 2000). Over-expression of TNFα has been implicated in many chronic inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, insulin-dependent diabetes mellitus, and IBDs (Plevy et al., 1997).

TNFα is a member of the TNF superfamily, the largest known family of cytokines. It is initially expressed as an insoluble 26 kDa homotrimeric type II transmembrane protein (mTNFα). Upon cell stimulation, TNFα Converting Enzyme (TACE) cleaves the extracellular domain of mTNFα into a 17 kDa soluble isoform (sTNFα) (Solomon, 1999). Binding of TNFα to a TNFα receptor (TNFR) ultimately activates downstream signaling pathways that can lead to inflammation (Pasparakis, 1996).

TNFα Receptors. Members of the TNF superfamily act as ligands to activate corresponding transmembrane receptors of the TNF receptor superfamily. Highly conserved among TNF ligands, the TNF homology domain (THD) interacts with the cysteine-rich domains of the TNF receptors (TNFR) (Reviewed by Bodmer et al., 2002). Activation of TNFRs generally results in one of two opposing signaling pathways: cell death via apoptosis or cell survival and inflammation (Dempsey et al., 2003).

Cell survival or death in response to ligand binding to the receptor depend on the presence of a death domain in the TNFR and affinity of that receptor for intracellular signaling proteins. Apoptotic signaling is induced by caspase interaction with TNFR death domains, while cell survival or inflammation result from TNFR interaction with TNF receptor associated factor (TRAF) proteins (Dempsey et al., 2003).

So far, two TNFRs that are activated by TNFα have been identified, TNFR1 and TNFR2 (Brockhaus et al., 1990), both of which bind soluble and insoluble, membrane-bound TNFα (Locksley et al., 2001). These receptors consist of hydrophobic cysteine-rich repeats that form a pre-ligand binding assembly domain (PLAD), which results in trimerization of the receptors in the absence of TNFα activation (Chan et al., 2000). Upon interaction with TNFα, the extracellular domains of TNFR1 and TNFR2 are cleaved into soluble TNFR1/2, a protein that sequesters sTNFα in order to attenuate the inflammatory response (Wallach et al., 1991).

However, TNFR1 and TNFR2 differ in their activation and downstream signaling. TNFR1 is fully activated by sTNFα, while TNFR2 is primarily activated by mTNFα and only partially activated by sTNFα. Also, TNFR1 is expressed ubiquitously across cell types, while TNFR2 is mainly expressed in immune cells (Grell et al., 1995).

TNFR Signaling. Signal transduction from TNFR1 can result in either cell death via apoptosis or cell survival and inflammation (Figure 8) (Gupta et al., 2006). Cell death signaling involves the interaction of TNFR1's death domain and pro-caspases 8 and 10 to induce apoptosis. This paper will focus on TNFR signaling for survival and inflammation, which results in the activation of the transcription factor nuclear factor

kappa B (NF-κB) and ultimately induces further production of TNFα and other inflammatory cytokines (DasGupta et al., 2008).

Prior to TNFR1 activation by TNFα, a silencer of death domain (SODD) protein associates with TNFR1 to mask its death domain, preventing the recruitment of signaling proteins (Jiang et al., 1999). Upon TNFR1 activation, SODD dissociates and TNFR1-associated death domain (TRADD) protein serves as a scaffold for TNFR-associated factors 1 and 2 (TRAF1/2) and receptor-interacting kinase (RIP) (Hsu et al., 1996). TRAF2 recruits inhibitor of apoptosis proteins (IAPs) and IκB kinase (IKK). IAPs are essential for the polyubiquitination of RIP, which is required for IKK activation (Ea et al., 2006). Subsequently, RIP activates IKK, which in turn phosphorylates the regulator domain of inhibitor kappa B (IκB) protein, marking it for recognition by an E3 ubiquitin ligase and degradation (Devin et al., 2000). Initially, IκB sequesters NF-κB in the cytoplasm by masking its nuclear localization signal (NLS). Degradation of IκB results in exposure of the NF-κB NLS, allowing the protein to translocate to the nucleus where it interacts with κB response elements to promote expression of pro-inflammatory cytokines and anti-apoptotic genes (Wang et al., 1998).

TNFR2 signaling is very similar to that of TNFR1, except that TNFR2 does not have a death domain, thus it does not require interaction with TRADD and can directly recruit TRAF proteins upon interaction with TNFα (Rothe et al., 1995). Furthermore, association of TRAF proteins with TNFR2 allows for induction of either the traditional or alternative pathways of NF-κB signaling (Rauert et al., 2010). Both pathways result in nuclear translocation of NF-κB, however, the alternative pathway activates a NF-κB-

inducing kinase (NIK) mediator to generate free cytoplasmic NF-κB from an inhibitory precursor.

TNFR signaling via NF-κB acts as a positive feedback mechanism to propagate the inflammatory response. TNFα activates TNFR which in turn activates the NF-κB pathway. The NF-κB pathway ultimately promotes expression of pro-inflammatory cytokines, including TNFα. Newly synthesized TNFα can activate more TNFRs, perpetuating the inflammatory cycle (Collart et al., 1990).

Soluble TNFRs. In addition to a membrane-associated form (mTNFR), TNFRs also exist in a soluble form (sTNFR) which is released from mTNFR via the proteolytic cleavage of its extracellular domains. Previous research has demonstrated that sTNFRs neutralize the function of TNFα without invoking an inflammatory response, thus playing a role in the regulation of TNFα activity under physiologic conditions (Spoettl et al., 2007).

Recent studies suggest that sTNFRs also exhibit immunomodulatory functions. Through reverse mTNFα signaling, sTNFR is able to induce apoptosis in monocytes independent of death receptor pathways. It is thought that sTNFR production may mediate the host response to pathologic states via interacting with TNFα, thus sequestering it from mTNFRs and preventing induction of inflammatory signaling pathways. Incidentally, quantification of sTNFRs in plasma provides information about immune processes leading to a better understanding of various diseases. In fact, sTNFR levels demonstrate high accuracy in disease prognosis. sTNFR concentrations have been used as predictive values as they are strongly associated with the clinical stage and progression of pathologies such as HIV infection and sepsis (Spoettl et al., 2007).

A 2007 study by Spoettl et al. demonstrated that sTNFR concentrations are higher in the serum of IBD patients than in healthy controls. sTNFR1 levels were significantly upregulated in both CD and UC patients, while sTNFR2 concentrations were significantly increased in CD patients and slightly increased in UC patients. Furthermore, sTNFR concentrations have been associated with disease activity. A correlation between increased sTNFR levels in the urine and clinical activity index has been observed (Hadziselimovic et al., 1995). A 1998 study by Noguchi et al. described a failure to upregulate sTNFRs in response to enhanced TNFα secretion in the lamina propria mononuclear cells of IBD patients. This study concluded that an imbalance in concentrations of TNFα and its natural inhibitors, such as sTNFR, may play a role in the pathogenesis of IBD. Thus, a better understanding of the mechanisms involved in both sTNFα and sTNFR release could lead to novel approaches to IBD treatment.

Tumor Necrosis Factor α Converting Enzyme

Overview. Tumor necrosis factor α Converting Enzyme (TACE) is responsible for cleaving membrane-bound TNFα to create a soluble, mature protein (Figure 9) (Reviewed by DasGupta et al., 2008). A variety of inflammatory disorders have been characterized by aberrant TACE activity leading to over-expression of TNFα. As increased TNFα expression is characteristic of IBDs, it can be inferred that TACE is also involved in IBD pathology.

TNFα Converting Enzyme. TACE, also known as ADAM17, is a member of the adamalysin family of metalloproteases, enzymes which require interaction with a metal ion for activation of the catalytic site (Hooper, 1994). A Disintegrin and Metalloprotease

(ADAM) proteins are a subtype of adamalysins which contain a metalloprotease and disintegrin domain (Edwards et al., 2008).

Specifically, TACE contains a pro-domain, catalytic (metalloprotease) domain, a disintegrin and cysteine-rich region, a transmembrane segment, and a cytoplasmic segment. The pro-domain acts as an inhibitor of metalloprotease activity. This inactivity is maintained via interaction of a cysteine residue in the pro-domain with an essential zinc ion located in the catalytic domain (Van Wart and Birkedal-Hansen, 1990). Removal of the pro-domain is a pre-requisite for TACE activity (Milla et al., 1999).

The metalloprotease domain of ADAM proteins contains a zinc-binding motif, which coordinates the zinc with histidine residues, creating the active site of the enzyme. Although the function of the disintegrin domain in human ADAM proteins is poorly characterized, it is thought to be responsible for substrate recognition and TACE maturation (Reddy et al., 2000).

Mechanisms for the regulation of TACE activity are poorly understood. Research has shown that inhibitors of the MAPK pathway block aberrant increases in TNFα shedding rate by TACE, however, the mechanism for this is unclear (Fan and Derynck, 1999).

TACE Involvement in Pathologic Inflammation. As stated earlier, a variety of inflammatory disorders have been characterized by aberrant TACE activity. For example, increased TACE expression in peripheral monocytes has been observed in patients with early systemic sclerosis, while rheumatoid arthritis patients exhibit increased TACE mRNA levels in cartilage (Bohgaki et al., 2005; Patel et al., 1998).

The pivotal role of TACE in inflammatory signaling was clarified in a study of mice with cleavage-defective mTNFα. These mice displayed attenuated inflammation, similar to TNFα knockout mice, demonstrating that mTNFα shedding is necessary for the propagation of inflammation (Ruuls et al., 2001). Further research has shown that reducing sTNFα levels through TACE inhibition allows for regulation of the inflammatory response. TACE inhibition was also shown to improve survival of mice against a lethal dose of lipopolysaccharide (LPS), a component in bacterial cell walls that induces inflammation (Figure 10) (Newton et al., 2001).

TACE has also been shown to play a role in IBD pathology. UC patients display increased TNFα cleavage potential, indicating increased functional TACE capacity (Brynskov et al., 2002). Furthermore, biopsies of inflammatory lesions in the intestinal epithelia of CD patients exhibited increased TACE expression (Cesaro et al., 2009). Thus, TACE inhibition, with the purpose of reducing TNFα levels may prove a viable method of IBD treatment.

History of TACE Inhibition. In the attempt to discover an effective TACE inhibitor, a variety of chemicals have been proposed and developed. Initially scientists began testing matrix metalloproteinase (MMP) inhibitors and found that, because TACE shares catalytic site structure with MMPs, MMP inhibitors also inhibited TACE activity. Because both TACE and MMPs are involved in normal physiological processes, the lack of TACE-specificity caused these drugs to exhibit toxicity (Yocum et al., 1999). Thus, the next step was to observe the electronic, structural, and kinetic differences between TACE and MMPs to design TACE-specific inhibitors.

Indeed, comparison of the crystal structures of TACE and MMPs led to the discovery of structural differences. The fact that the S1' pocket of TACE is longer and narrower than that of MMPs has formed a basis for designing selective TACE inhibitors, such as anthranilate-based compounds (Duan et al., 2002). Furthermore, modifications to the P1' and P2' residues have been found to confer specificity on TACE inhibitors. For example, macrocyclic inhibitors, formed by joining the P1' and P2' groups of succinate-based TACE inhibitors to form a cyclic structure, were found to be more selective towards TACE over MMP by 100-fold (Holms et al., 2001).

Based on these discoveries, many compounds based on various chemical classes have been developed as selective TACE inhibitors and TACE inhibition during inflammatory disorders has been explored in pre-clinical animal models of rheumatoid arthritis (Reviewed by Moss et al., 2008). In a study of murine rheumatoid arthritis models, TACE inhibition significantly suppressed release of TNFα into serum. Furthermore, histological evaluation of the joints showed normal joint structure following TACE inhibition; prior to TACE inhibition, joints were plagued with lesions and inflammation (Newton et al., 2001).

Although TACE inhibition was successful in pre-clinical models, concerns about liver toxicity and lack of efficacy have blocked progression of TACE inhibitors past Phase II clinical trials (Reviewed by DasGupta et al., 2008).

TACE Inhibition Through DPC-333. DPC-333, also known as BMS-561392, is a TACE-specific, small molecule inhibitor developed in 2002 (Grootveld and McDermott, 2003). Upon finding that DPC-333 was 100-fold more selective of TACE over MMPs, Bristol-Myers Squibb Company claimed that DPC-333 was unlikely to have

many adverse effects due to its non-antigenic structure and oral administration; although they did warn that close monitoring was required with treatment. Pre-clinically, DPC-333 was found to have good bioavailability in primates and dogs (54%) and reasonable bioavailability in rats (16%) (Grootveld and McDermott, 2003).

In Phase I clinical trials, DPC-333 was found to be well tolerated by humans at a dose rage of 15 to 530 mg (Grootveld and McDermott, 2003); however, 53% of DPC-333 recipients (n=6) and 40% of the placebo group (n=20) reported adverse effects, taste disturbance being the most commonly reported adverse experience (Qian et al., 2007). Although the compound demonstrated high potency and selectivity toward TACE, DPC-333 caused liver toxicity issues, resulting in withdrawal from Phase II clinical trials (Reviewed by Moss et al., 2008).

While adverse effects were observed in humans, continued study of the effects of DPC-333 on TNFα production in mice could lead to advances in inflammatory disease research. Oral administration of DPC-333 to mice inhibited LPS-induced TNFα production (Figure 11) (Sharma et al., 2012). Although mice have low oral bioavailability (<20%), they have 98% bioavailability after intraperitoneal (IP) injection of 11 mg/kg bodyweight. Furthermore, IP injection of DPC-333 in mice is rapidly absorbed and maximum plasma concentration is reached at 0.1 hours after injection (Qian et al., 2007).

Selecting a Mouse Model of Colitis

Overview. Although the etiology of IBD remains unclear, current knowledge of the mechanisms that underlie IBD pathology can be attributed largely to the study of colitis in mice. Mice represent a readily available and flexible model of study; the many

methods of disease induction alone provide a large variety of study designs. The multiple mouse strains available to choose from add further variety as each strain exhibits differences in response to disease induction methods and disease activity. Murine models of colitis have been organized into four main categories based on method of disease induction (Blumberg et al., 1999).

Spontaneous Colitis Models. Currently, only two mouse models that spontaneously develop colitis have been described: the SAMP1/Yit model and the C3H/HeJBir mouse. SAMP1/Yit mice were originally developed from AKR mice, which were bred for the purpose of studying senescence. By chance, these mice also develop mucosal inflammation with features comparable to those of CD. The C3H/HeJBir mouse lacks the toll-like receptor 4, and so is unresponsive to LPS. These mice spontaneously develop colitis at 3-4 weeks of age (Hoffman et al., 2002). Although spontaneous colitis is uncommon in mice, with knowledge of their genetic background these models offer the possibility of defining the genetic factors that lead to IBD (Blumberg et al., 1999).

Genetically Engineered Colitis Models. The category of genetically engineered colitis models consists of those with genetic alterations produced by gene targeting or the induction of a transgene. The major advantage of these models is that they allow the study of a particular gene product and its role, or lack thereof, in colitis development and progression (Blumberg et al., 1999). Study of these mice allowed for characterization of the immune network of IBD-associated genes (Mizoguchi & Mizoguchi, 2010). Currently, several KO mouse strains have been developed which lack IBD-associated genes, some of which spontaneously develop colitis while others require additional immune or environmental factors to fully elicit colitis development (Mizoguchi &

Mizoguchi, 2010). For example, Mdr1a$^{-/-}$ mice lack the murine multiple drug resistance gene for P-glycoprotein 170 and spontaneously develop colitis at around 12 weeks of age, highlighting a role for MDR genes in IBD pathology (Wilk et al., 2005). Models that do not develop colitis spontaneously allow for in-depth study of a desired gene product. For example, administration of DSS to a MMP9$^{-/-}$ mouse revealed a role for MMP9 activation in colitis-associated neutrophil infiltration of the intestinal mucosa (Munakata et al., 2015).

Transfer Models of Colitis. A third category of colitis models involves the development of colitis following transfer of particular cell populations into a neutral host. It has long been established that shifts in microbiota composition can induce colitis (Bloom et al., 2011). Studies involving mice with IBD-associated defects further demonstrated the importance of microbiota composition in colitis development when KO mice raised under germ-free conditions failed to develop colitis. This discovery led to the development of colitis transfer models to differentiate between bacterial populations associated with colitis development or prevention. For example, transfer of various *Lactobacillus* species prevented colitis development in IL-10-deficient mice living under specific pathogen-free conditions. In contrast, these mice develop colitis following introduction of *Helicobacter hepaticus* (Blumberg et al., 1999).

Exogenously-induced Colitis Models. The development of colitis following exposure of wild type mice to an exogenous agent that induces an immune response comprises the fourth category of colitis model. The advantage of this category is the ability to observe relationships between particular immune responses and histopathologic reaction, as well as immunopathogenesis and treatment (Blumberg et al., 1999).

Commonly, colitis is induced by two different chemicals: dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS). Consumption of DSS in drinking water results in a model of colitis relevant to clinical UC, while intra-rectal administration of TNBS induces a model of colitis comparable to CD (Scheiffele & Fuss, 2002). Both chemicals are thought induce colitis by causing direct injury to intestinal epithelia (Perse & Cerar, 2012).

Additionally, a model of colitis comparable to clinical UC can be induced by IP injection of an agonistic CD40 monoclonal antibody to B- and T-cell-deficient mice (Munakata et al., 2015). CD40 is a type I transmembrane protein that belongs to the TNFR superfamily and is ubiquitously expressed on the surface of immune cells. The CD40/CD40L system allows for signaling between immune cells that, when stimulated in excess, results in an autoimmune inflammatory response dependent on the release of pro-inflammatory signaling molecules (Danese et al., 2004).

A DSS-Induced Colitis Mouse Model

Overview. Previous research has demonstrated that consumption of dextran sulfate sodium (DSS) in a murine model produces a clinically relevant model of colitis; the induced disruption of the colon mucosa is similar to clinical colitis in humans (Figure 12) (Perse and Cerar, 2012). Supplementing the animal's drinking water with DSS for 7 days results in erosion of the colon mucosa, dysplasia, shortening of the colon, diarrhea, fecal blood content, and weight loss (Okayasu et al., 1990). The mechanism by which DSS induces inflammation is not well understood, however, mucosal barrier degradation may result from direct toxicity to intestinal epithelia (Dieleman et al., 1994). It has also

been reported that TNFα and interferon-γ (IFN-γ), cytokines involved in the perpetuation of chronic DSS-induced colitis, cause excessive nitric oxide activity which could be the effector mechanism (Obermeier et al., 1999). Due to the ease of administration and rapid development of colitis, the DSS murine model is beneficial in studying IBDs (Wirtz et al., 2007). Furthermore, severity of colitis in response to DSS is strain dependent, which could highlight a role for genetic background in the development of inflammatory diseases (Melgar et al., 2005).

Tissue Destruction in Colitis Development. The primary function of the intestinal epithelium is to serve as a selectively permeable barrier between internal and external environments through which nutrients, ions, and water are absorbed and secreted. When the barrier is intact, tight junctions limit solute flux to create trans-epithelial gradients which drive passive paracellular transport of ions and water (Turner, 2009). Absorption of nutrients occurs through coupling of organic solutes (i.e. sugars and amino acids) to Na^+ transport across the small-intestinal epithelial lining (Figure 13). The organic solutes then move from enterocytes to blood via basolateral membrane carriers operating independently of ion transport (Field, 2003).

Physical features of the epithelial barrier, such as crypts, villi, and surface cells, are responsible for absorption and secretion (Field, 2003). The direct damage to the epithelial lining caused by DSS exposure disrupts these features, resulting in diarrhea and bodyweight loss due to malabsorption. This correlates to human IBD in that bacterial flora, food products, and inflammation can alter secretion and absorption through disrupting the integrity of crypts, villi, and surface cells (Field, 2003). Additionally,

apoptosis in response to cell damage reduces intestinal surface area and thus, decreases the capacity for absorption (Weber & Turner, 2007; Owens & Greenson, 2007).

Furthermore, the epithelial damage caused by DSS consumption results in increased intestinal permeability, a characteristic observed in human IBD. Physiologically, tight junctions seal the paracellular pathway between epithelial cells, restricting molecule passage based on size and charge. Destruction of tight junctions opens the paracellular pathway, increasing intestinal permeability and allowing infiltration of immune cells into the colon mucosa. Thus, inflammation of the colon mucosa, or colitis, results (Field, 2003).

Pro-inflammatory Effects of TNFα in Colitis Development. Previous research has demonstrated that, while DSS causes direct tissue damage, the over-expression of TNFα drives colon inflammation in DSS-induced colitis. It has long been established that TNFα plays a critical role in pathologic inflammation by recruiting inflammatory cells, inducing edema, promoting granuloma formation, and activating the coagulation cascade. TNFα has been shown to induce intestinal epithelia to express cell adhesion molecules and secrete chemokines; these processes result in the influx of inflammatory cells. Once recruited to the mucosal epithelium, monocytes and T-cells are further activated by TNFα to secrete pro-inflammatory cytokines and tissue degrading enzymes (Baugh & Bucala, 2001).

The importance of TNFα in the propagation of inflammation was highlighted by the $TNF^{\Delta ARE/WT}$ mouse model. Deletion of a repeated AU-rich motif in the 3'-untranslated region of the TNF encoding gene enhanced mRNA stability of TNFα, resulting in mice that spontaneously develop a severe CD8+ T cell ileitis resembling

human CD. Studies of this mouse attribute pathology to local expression of pro-inflammatory cytokines, such as TNFα, likely produced by infiltrating immune or epithelial cells (Baur et al., 2011).

Additionally, multiple studies by Wang et al. have characterized a mechanism by which TNFα contributes to barrier dysfunction by up-regulating myosin light chain kinase (MLCK) expression (Figure 14). Phosphorylation of myosin II regulatory light chain (MLC) by MLCK has been shown to be involved in physiological and pathological tight junction regulation. Treatment of intestinal epithelial monolayers with IFN-γ and TNFα increased MLC phosphorylation and increased intestinal permeability, leading to the conclusion that barrier dysfunction is inducible through IFN-γ and TNFα production (Wang et al., 2005).

Cytokine Expression in Two Models of DSS-Induced Colitis. The C57/BL6 and BALB/C mouse models have both been used in the pre-clinical study of colitis. After 5 days of consuming 5% DSS in drinking water, both mouse models developed acute colitis; however, when no longer exposed to DSS, C57/BL6 mice progressed to severe chronic inflammation, while BALB/C mice were symptom-free within two weeks (Melgar et al., 2005).

Cytokine quantification revealed that IL-1β, IL-12 p70, and IL-17 were progressively upregulated following chronic development in C57/BL6 mice; these cytokines are involved in T cell-mediated immunity, suggesting that the development of chronic colitis is T cell-driven. In contrast, during the acute phase of the disease, BALB/C mice demonstrated up-regulation of cytokines involved in macrophage activation (IL-1, IL-6, IL-18, and G-CSF); these levels were decreased coinciding with

disease resolution. Furthermore, production of IFN-γ, a macrophage activator produced by T cells, was low in BALB/C during the acute phase while high in C57/BL6, suggesting that the acute response in BALB/C mice is macrophage-driven (Figure 15) (Melgar et al., 2005).

As it has been previously established that multiple loci affect the disease in both mice and humans, Melgar et al. concluded that these results demonstrate the effect of genetics on the development and severity of colitis (Melgar et al., 2005).

TACE Inhibition in Colitis Mouse Models. As TACE has become a target of interest in colitis treatment, TACE inhibition has been studied in both C57/BL6 and BALB/C mice. A study by Sharma et al. demonstrated dose-dependent improvement of colitis in C57/BL6 mice through administration of a selective TACE inhibitor. The authors were able to show improved disease activity index (DAI) of colitis through evaluation of percent bodyweight loss, colon length, fecal blood content, and stool consistency (Figure 16). Following a 5-day regimen of 3.5% DSS paired with oral administration of the TACE inhibitor to female C57/BL6 mice for 7 days, the cytokine quantification of colon-cultured media exhibited a decrease in serum TNFα levels (Sharma et al., 2014).

Concurrently with Sharma et al., Maddox and Haines examined the effects of TACE inhibition in a BALB/C model of colitis (5% DSS consumption for 7 days). These results show no significant difference in disease activity between DSS mice that received the TACE inhibitor versus a control group (Figure 17); however, Maddox and Haines did demonstrate bioactivity of the TACE inhibitor in a model of systemic inflammation due to LPS injection (10 mg/kg bodyweight) (Figure 18) (Maddox, 2015). Further study is

necessary to determine the cause of failure of TACE inhibition to improve disease activity in BALB/C mice. It is possible that the acute colitis observed in BALB/C mice could effectively act as a pre-clinical model for studying IBD patients (1/3) who do not respond to anti-TNFα therapies.

Efficacy of Anti-TNFα Therapies for IBD Treatment

Overview. Modulating TNFα activity has been shown to ameliorate disease in many inflammatory pathologies, such as rheumatoid arthritis and IBD. Down-regulation of TNFα activity has been accomplished in three main ways: antibody-induced neutralization of TNFα, non-specific TNFα inhibition, and inhibition of TNFα processing through blockage of TACE activity. While initially successful, these treatments have demonstrated a loss of response over time, as well as potentially serious side effects. The likelihood of these approaches to improve long-term outcomes of IBD depends upon whether TNFα plays a critical role in the condition (Baugh & Bucala, 2001). Efficacy, or the ability of a therapy to produce the desired result, is a major determinant of the therapy's success in treating disease. Anti-TNFα therapies have been shown to be efficacious in inflammatory pathologies in which TNFα is the driving force; however, a decrease or lack of efficacy, as seen in loss of response and non-response, could be explained should an alternative pathway drive inflammation in some cases of IBD.

Mechanisms for Loss of Response. It has been observed that approximately one-third of IBD patients exhibit a lack of response to anti-TNFα therapies (Papadakis et al., 2005). In a Danish study of 759 IBD patients, ~70% were responders, ~13% were partial

responders, and ~17% were non-responders (Bank et al., 2015). Furthermore, 23-46% of IBD patients demonstrate a loss of response to anti-TNFα treatments over time (Roda et al., 2016).

The definition of loss of response, or secondary non-response, is initial response to therapy after an induction regimen followed by loss of response during maintenance treatment. A common mechanism behind loss of response to anti-TNFα is the development of immunogenicity due to the development of host antibodies against the TNFα agonists. These antibodies either prevent binding of TNFα to TNFR or hasten drug clearance. Studies have demonstrated that the use of immunosuppressive therapy along with Infliximab results in reduced anti-drug antibody formation. Furthermore, pre-treatment with corticosteroids led to significantly less patients who developed antibodies (26%) compared with those that did not receive steroid treatment prior to Infliximab therapy (42%) (Roda et al., 2016).

The accepted clinical definition of primary non-response (PNR) is a lack of improvement of clinical signs and symptoms with induction therapy. Several factors seem to negatively influence the risk of PNR, such as disease longer than 2 years, small intestinal involvement, smoking, C reactive protein, and genetic mutations. Evidence suggests that optimization of the dosing regimen and combination therapy can reduce PNR occurrence (Figure 19); however, this involves escalating dosage and close drug monitoring. Current opinions on mechanisms of PNR involve symptoms other than active inflammation, early immunogenicity, or non-TNFα-mediated inflammation (Roda et al., 2016).

Hypothesis and Rationale

The involvement of TNFα in acute IBD in BALB/C mice will be studied in this research project as a continuation of the research in the Morris lab. Based upon previous data in the Morris lab, the hypothesis states that TNFα is not a critical component in the development of colitis in BALB/C mice. It has been established that one-third of IBD patients do not respond to anti-TNFα therapies, thus, if our hypothesis is correct, BALB/C mice could represent a pre-clinical model for primary non-responders.

In studies conducted by Maddox, very low levels of TNFα were measured at 2-6 pg/mg total protein; however, colon tissue was homogenized ex-vivo in the absence of tissue culture (Figure 20). Previous studies utilized a tissue culture technique to allow for further cytokine production which resulted in more significant TNFα levels. Thus, in order to determine whether TNFα production is involved in this model, this study will utilize a tissue culture system to determine the impact of the TACE inhibitor.

Only the distal portion of the colon will be used for quantification of TNFα levels, as Maddox and Haines demonstrated that inflammation is most active in this region, especially regarding IL-6 production. The distal colon will be removed and cultured for 24 hours to allow for further production of TNFα. Plasma TNFα levels will then be quantified using the MagPix system.

Acute colitis will be induced by administration of 5% DSS in drinking water for 7 days. The TACE inhibitor DPC-333 will be supplied by Bristol-Myers Squibb, Inc.; approval for use of DPC-333 by Bristol-Myers Squibb was acquired April 8, 2013. The drug, dissolved in 25 mM citric acid saline, will be delivered twice daily via IP injection, using a 1 mg/kg bodyweight ratio.

As demonstrated by Melgar et al., the mechanisms of acute colitis differ from those of chronic disease. Thus, it may be these differences that cause one-third of IBD patients to be unresponsive to anti-TNFα treatments. Determining the involvement of TNFα in acute colitis through study of BALB/C mice may lead to a better understanding of the disease and provide insights into mechanisms of treatment in human colitis.

Figures

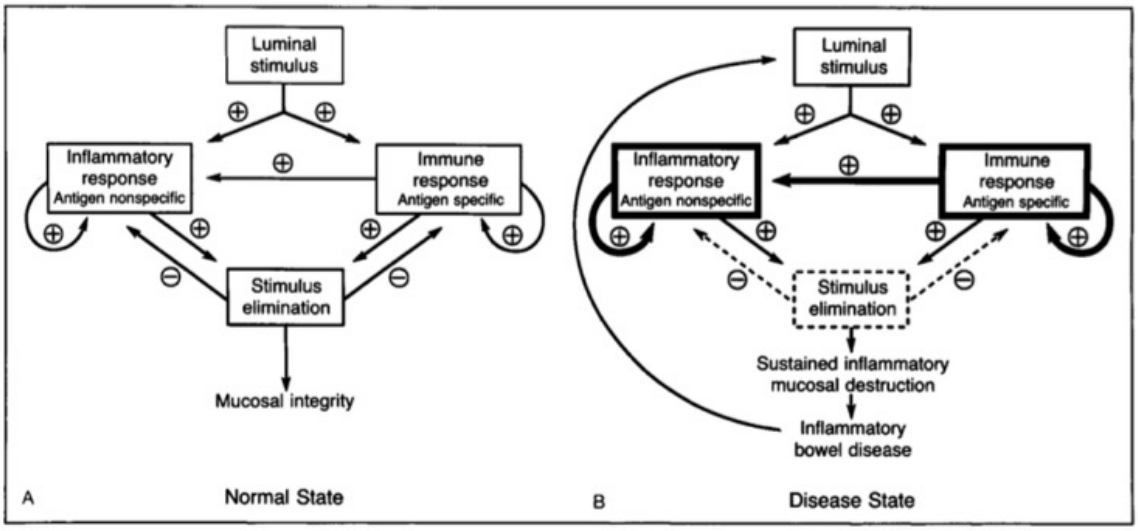

Figure 1. Potential pathogenic relations in Inflammatory Bowel Disease. In the normal state, shown in Panel A, the intestinal lumen comes into contact with a variety of substances that might stimulate either a specific immune response, a nonspecific inflammatory response, or both. This mechanism has the potential to auto-amplify, but is counterbalanced by the mucosal barrier, which limits access of luminal constituents to the intestinal epithelium, and by feedback mechanisms that down-regulate the immune and inflammatory responses after eliminating the stimulus. In the disease state, shown in Panel B, fundamental differences in the integrity of the mucosal barrier and/or in the regulatory mechanisms of the immune system may contribute to sustained inflammation, regardless of stimulus elimination; this results in tissue destruction and subsequent inflammatory bowel disease (Reproduced with permission from The New England Journal of Medicine, Podolsky, 1991. Copyright Massachusetts Medical Society).

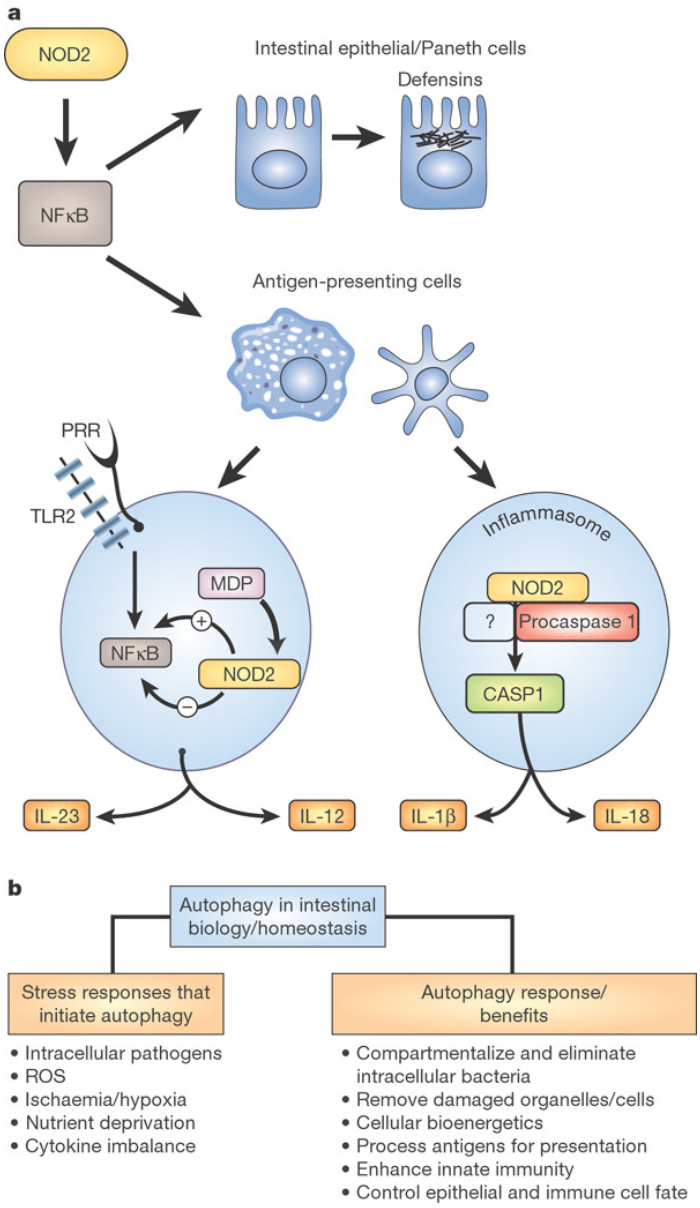

Figure 2. Several IBD susceptibility gene products modulate host-cell functional responses to microbial flora. Panel (a) illustrates cell-specific NOD2/CARD15 signaling pathways. In intestinal epithelium, bacterial cell wall components are recognized by the leucine-rich repeats domains of NOD2, which leads to activation of the NF-κB pathway. Panel (b) denotes potential roles for autophagy in IBD. Autophagy provides a mechanism of response among cells to limit the harmful effects of exogenous and endogenous stressors and thus, is essential for homeostasis. This diagram denotes the stages at which autophagy may play a role in acute inflammation and the pathogenesis of IBD (Reprinted with permission from Macmillan Publishers Ltd: Nature, Xavier RJ and Podolsky DK, 2007).

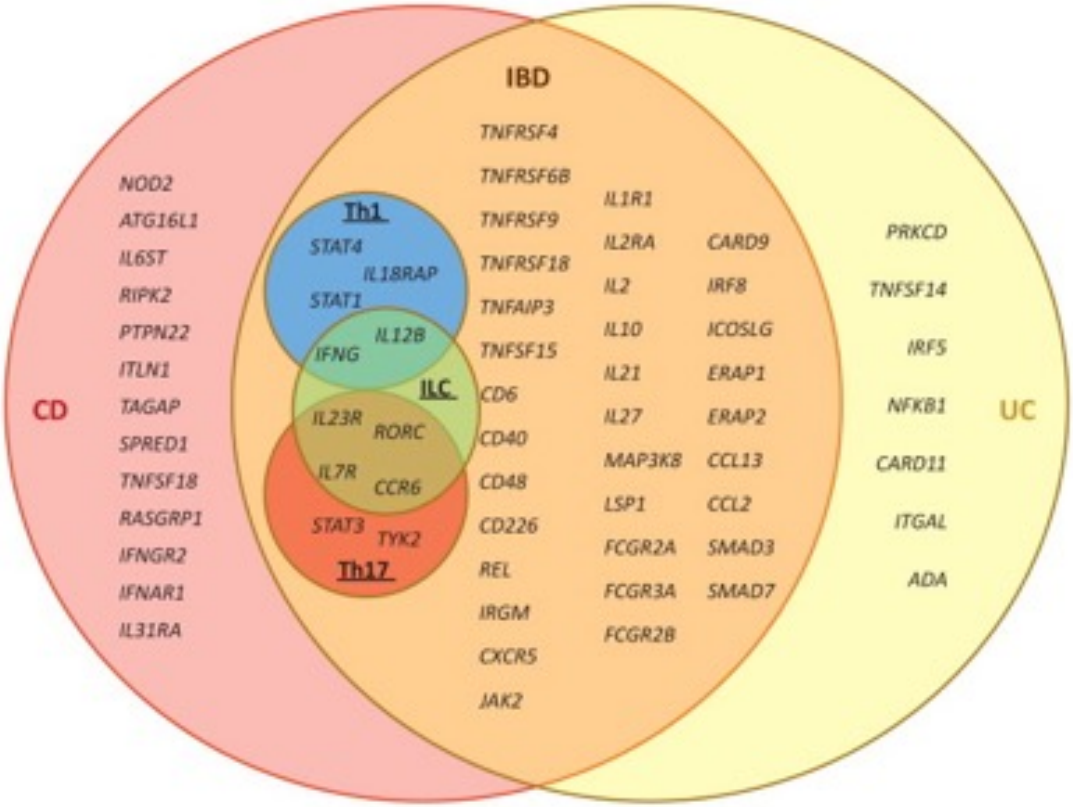

Figure 3. IBD-associated genes. Many genes have been identified as IBD susceptibility loci, including genes for cytokine production, DNA mismatch repair, mucin glycoproteins, and immunoregulation (Reprinted from Trends in Immunology 34(11), Biancheri et al., The challenges of stratifying patients for trials in inflammatory bowel disease, 2013, with permission from Elsevier).

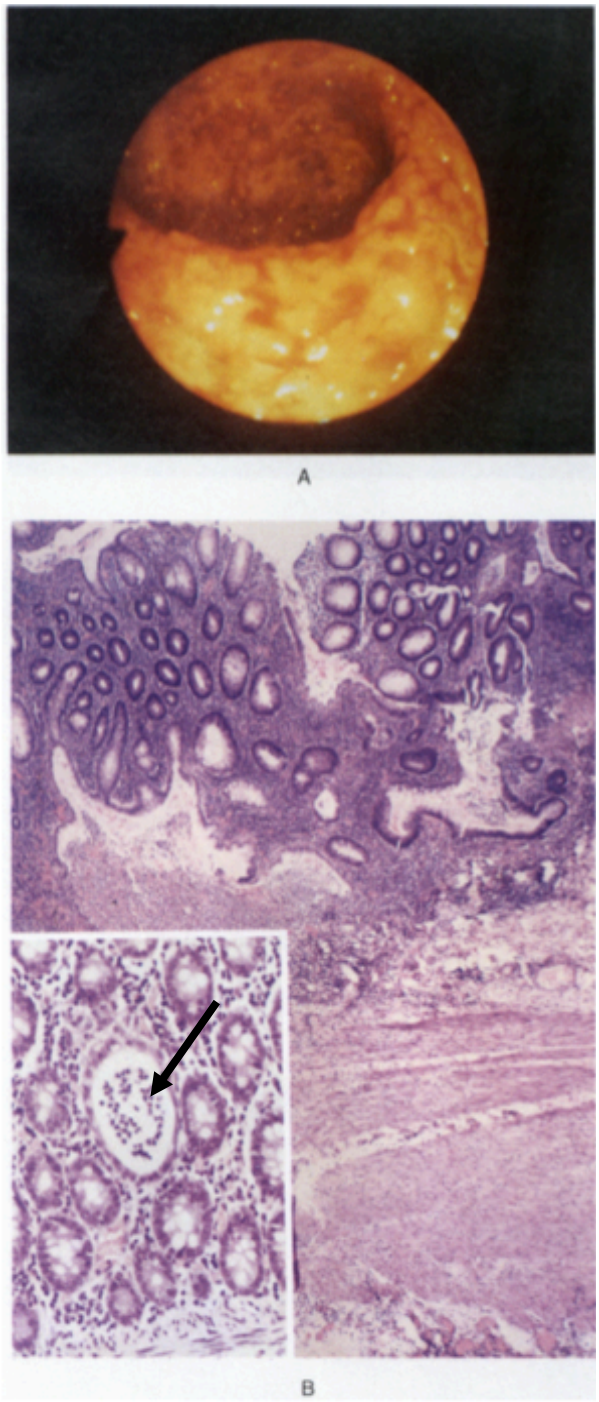

Figure 4. Endoscopic and histologic features of Ulcerative Colitis. In panel A, colonoscopy of the sigmoid colon of a patient with severe UC depicts diffuse mucosal ulceration associated with mucopurulent exudate and mucosal bleeding. In panel B, histologic analysis demonstrating typical features of UC, including mucosal ulceration, formation of crypt abscesses (arrow indicates a typical crypt microabscess) and depletion of goblet cells. Additionally, chronic inflammation is present, confined to the mucosal and submucosal layers (Reproduced with permission from The New England Journal of Medicine, Podolsky, 1991. Copyright Massachusetts Medical Society).

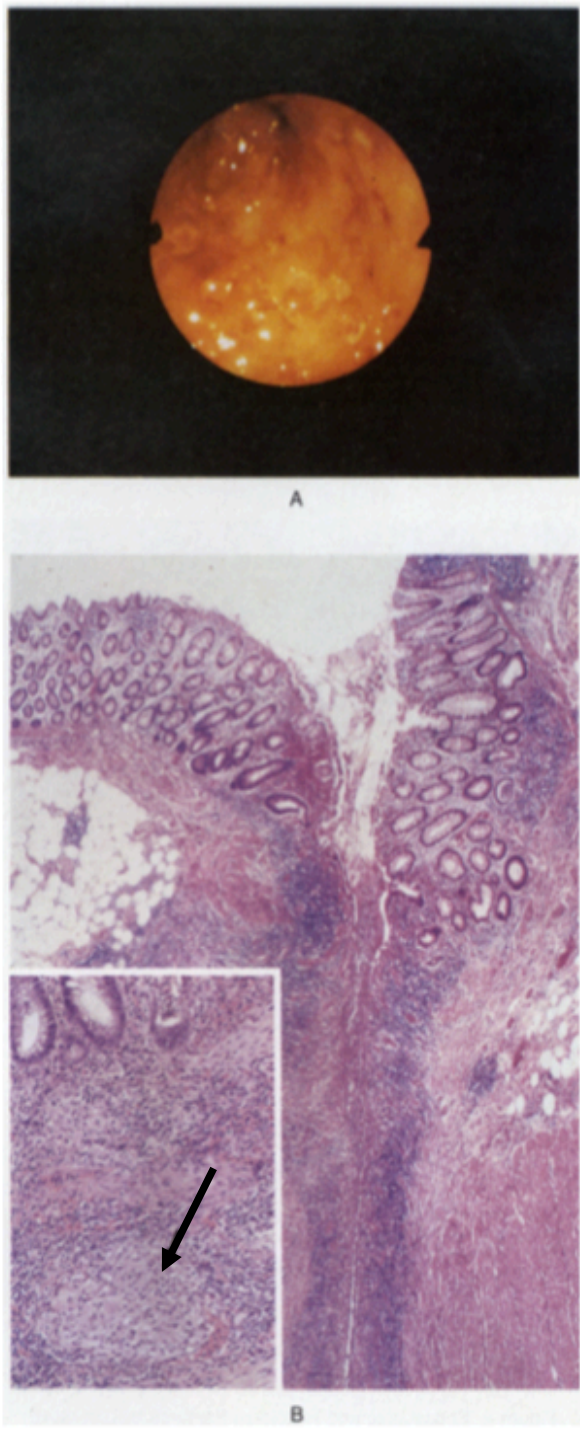

Figure 5. Endoscopic and histologic features of Crohn's Disease. In panel A, colonoscopy of the sigmoid colon of a patient with CD depicts the patchy nature of the inflammatory process; irregular ulcers are separated by mucosa that has been relatively spared. In panel B, histologic analysis illustrates transmural inflammation with the formation of deep linear ulcers (arrow in inset denotes a typical non-caseating granuloma (Reproduced with permission from The New England Journal of Medicine, Podolsky, 1991. Copyright Massachusetts Medical Society).

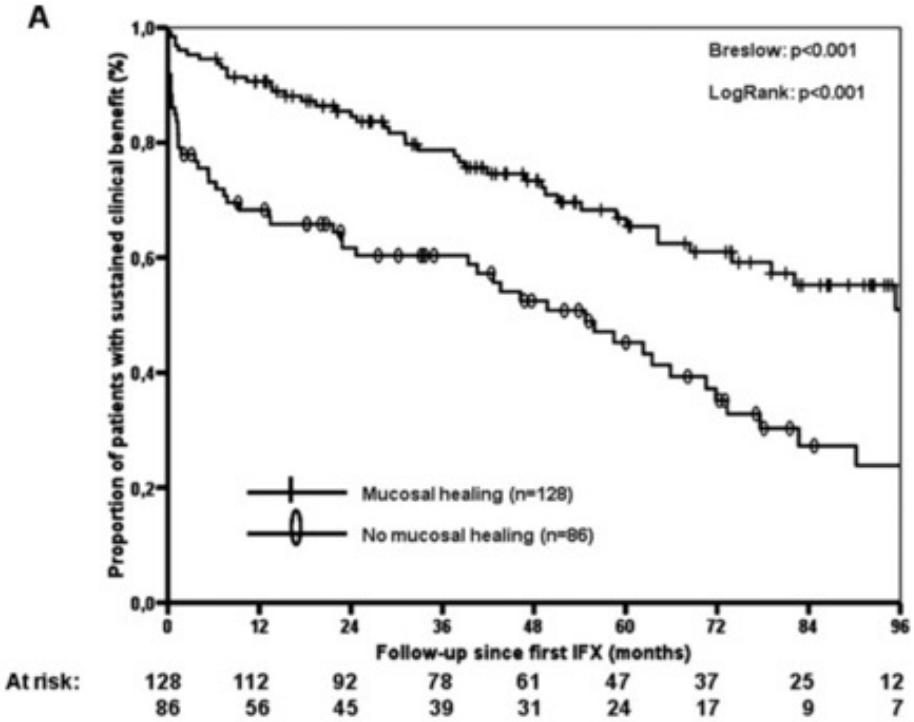

Figure 6. Long-term clinical benefit of Infliximab treatment. Panel A illustrates the long-term clinical benefit with respect to mucosal healing during Infliximab treatment (n=214) (Reproduced with permission from Inflammatory Bowel Disease, Schnitzler et al., 2009).

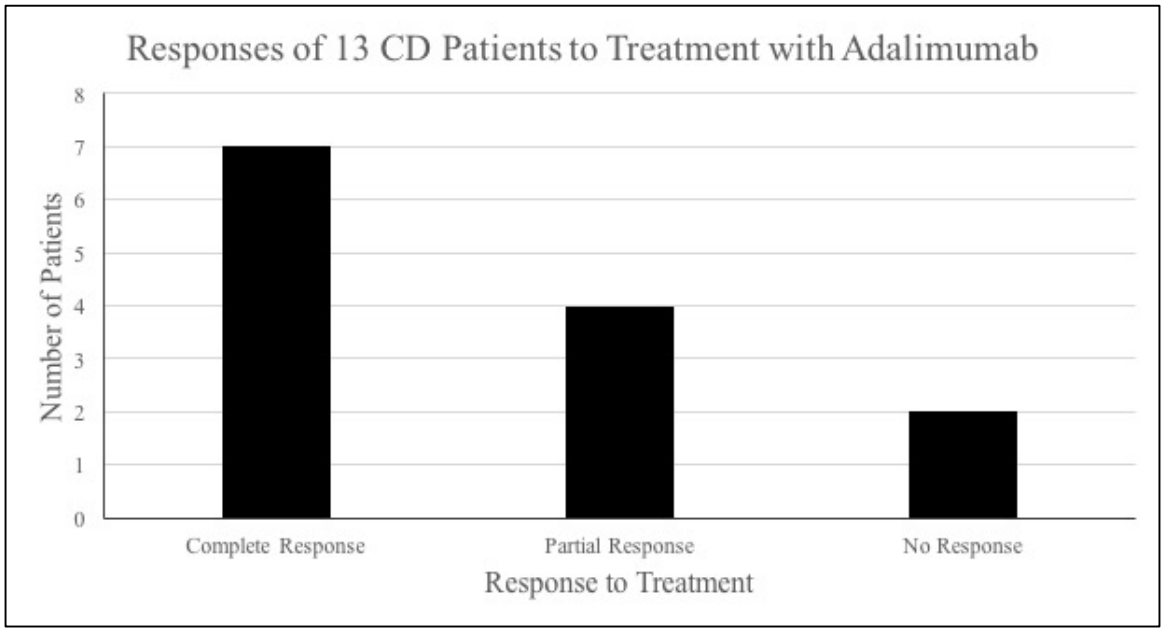

Figure 7. Responses to Adalimumab treatment among CD patients with attenuated response to Infliximab. Approximately one-third of CD patients demonstrate no response to anti-TNFα therapies (Adapted from Papadakis et al., 2005 with permission from Blackwell Publishing: American Journal of Gastroenterology).

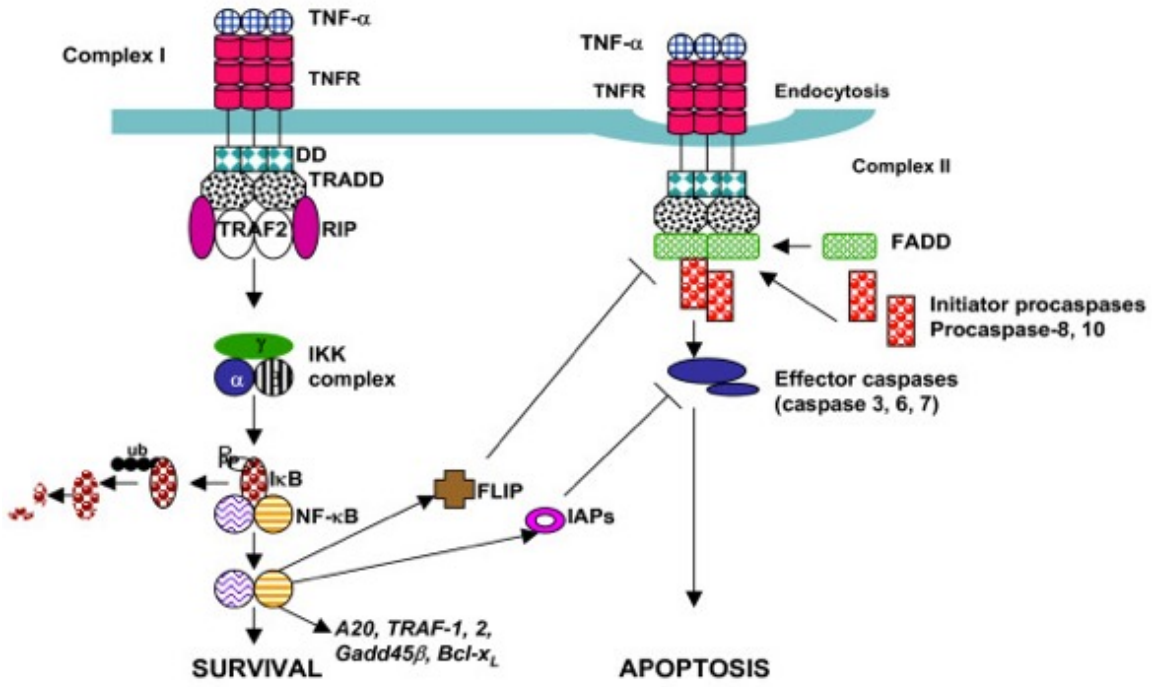

Figure 8. TNFR1-induced pathways. Formation of complex I upon activation by TNFα leads to NF-κB activation and, ultimately, cell survival through inflammation. Formation of complex II upon activation by TNFα leads to cell death via apoptosis (Reproduced with permission from BioMed Central Ltd: Immunity and Ageing, Gupta et al., 2006).

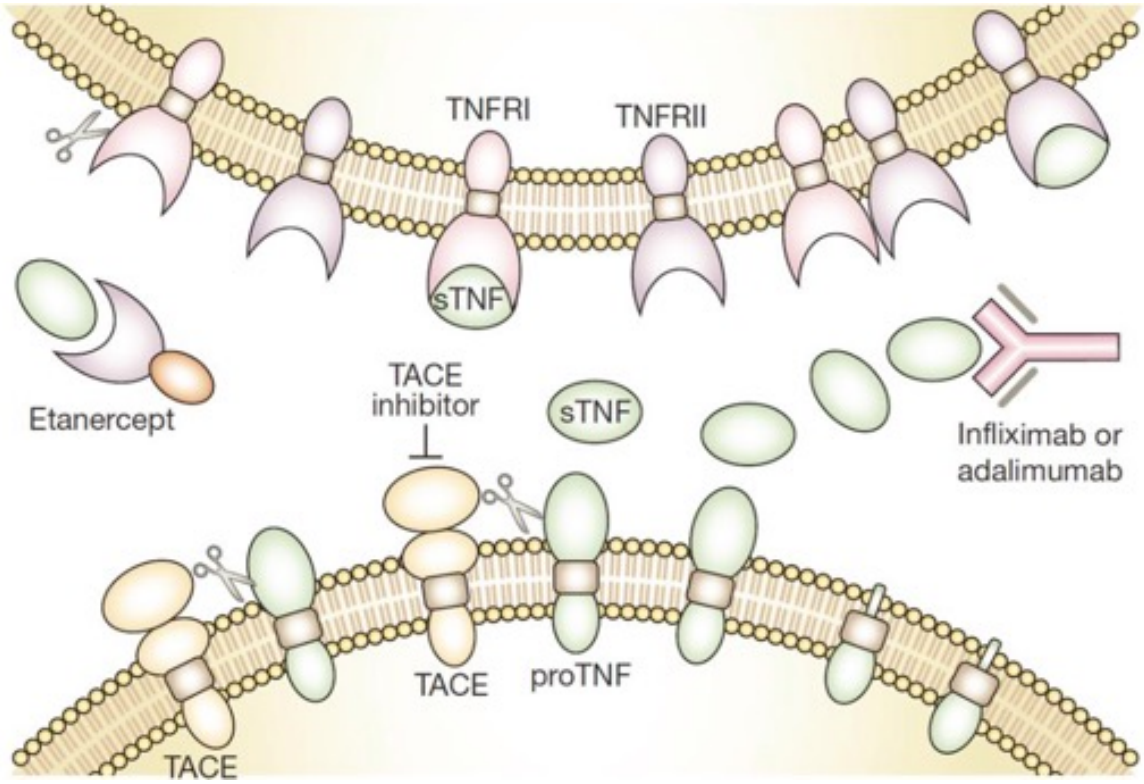

Figure 9. Activation and inhibition of TNFα and TNFR. Membrane-bound proTNFα is cleaved by TACE, releasing mature, soluble TNF (sTNFα). Infliximab/adalimumab and Etanercept represent current pharmaceuticals directed at reducing sTNFα levels, thus preventing activation of TNFR1/2 (Reprinted from Bioorganic & Medicinal Chemistry, 17(2), DasGupta et al., Current perspective of TACE inhibitors: A review, 2009, with permission from Elsevier).

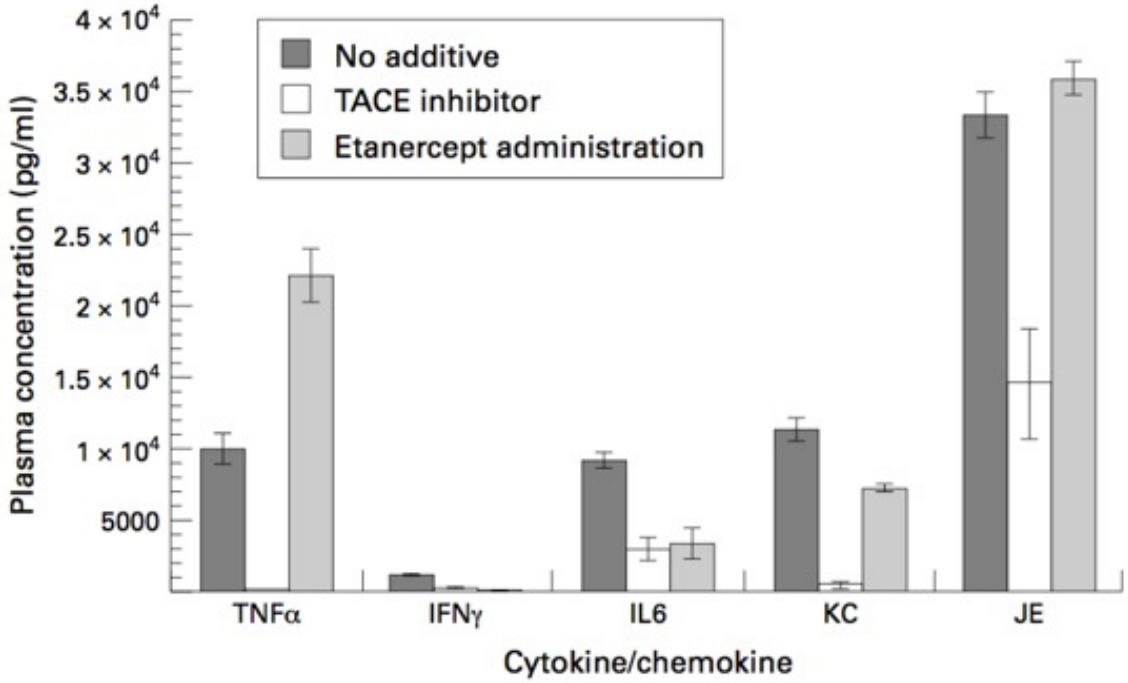

Figure 10. Effects of TACE inhibition or Etanercept administration on LPS-induced cytokine production. TACE inhibition resulted in significant reduction of plasma TNFα levels, suggesting TACE inhibition may prove a viable method of treatment for inflammatory pathologies. Etanercept is a decoy receptor that binds and neutralizes TNF-alpha. However, it has been demonstrated to increase plasma TNF-alpha concentrations by prolonging the half-life of TNF-alpha and reducing its clearance. Blocking TNF-alpha upstream of membrane shedding via TACE inhibition would eliminate this problem by preventing soluble isoforms in the plasma (Reproduced from Biology of TACE inhibition, Newton et al., 60:30, 2001, with permission from BMJ Publishing Group Ltd).

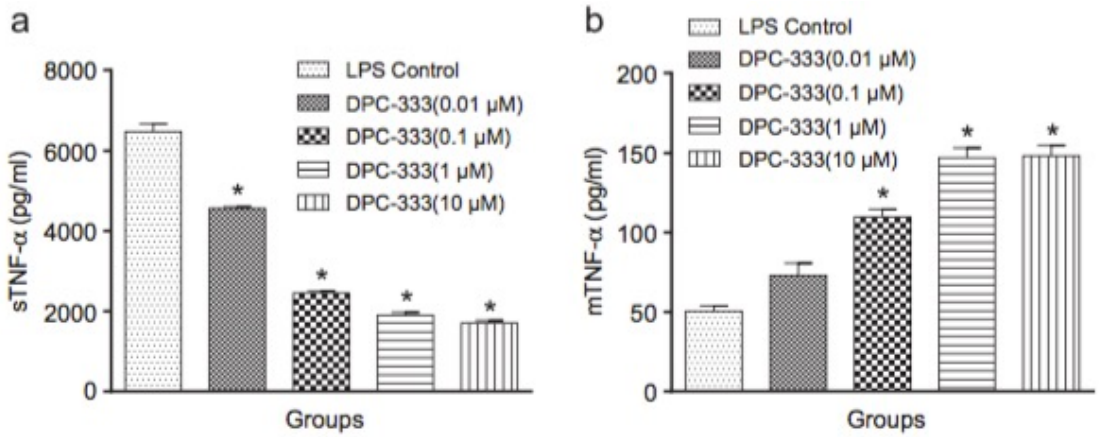

Figure 11. Effect of various concentrations of DPC-333 on LPS-induced TNFα levels in mice. Panel A illustrates significant reduction in sTNFα with increasing DPC-333 concentrations. Panel B illustrates significant increases in mTNFα levels with increasing DPC-333 concentrations. Taken together, this data indicates reduction in TNFα processing and successful inhibition of TACE (Reprinted from European Journal of Pharmacology, 701(1), Sharma et al., Blockade of tumor necrosis factor-alpha converting enzyme (TACE) enhances IL-1B and IFN-gamma via caspase-1 activation: A probable cause for loss of efficacy of TACE inhibitors in humans?, 2012, with permission from Elsevier).

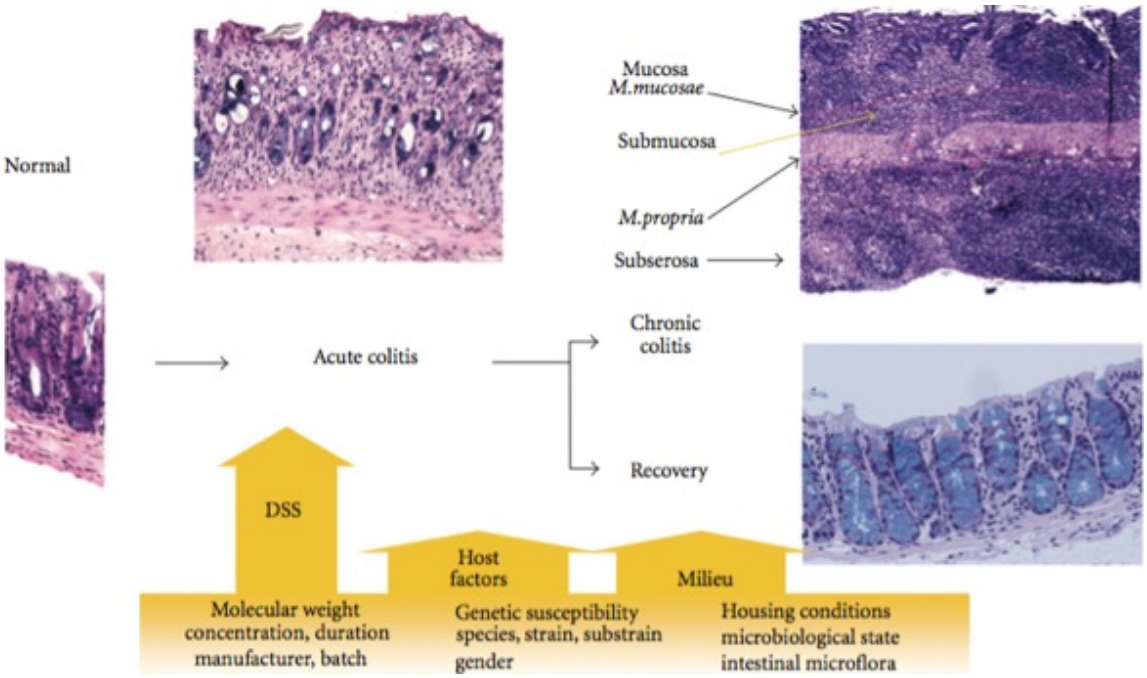

Figure 12. Schematic of DSS-induced colitis. Various factors regarding the DSS itself, the host, and the environment can influence the susceptibility, onset, severity, and responsiveness to DSS-induced colitis (Reproduced from *Journal of Biomedicine and Biotechnology*; Perse M, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. 2012, with permission from Hindawi Publishing Corporation).

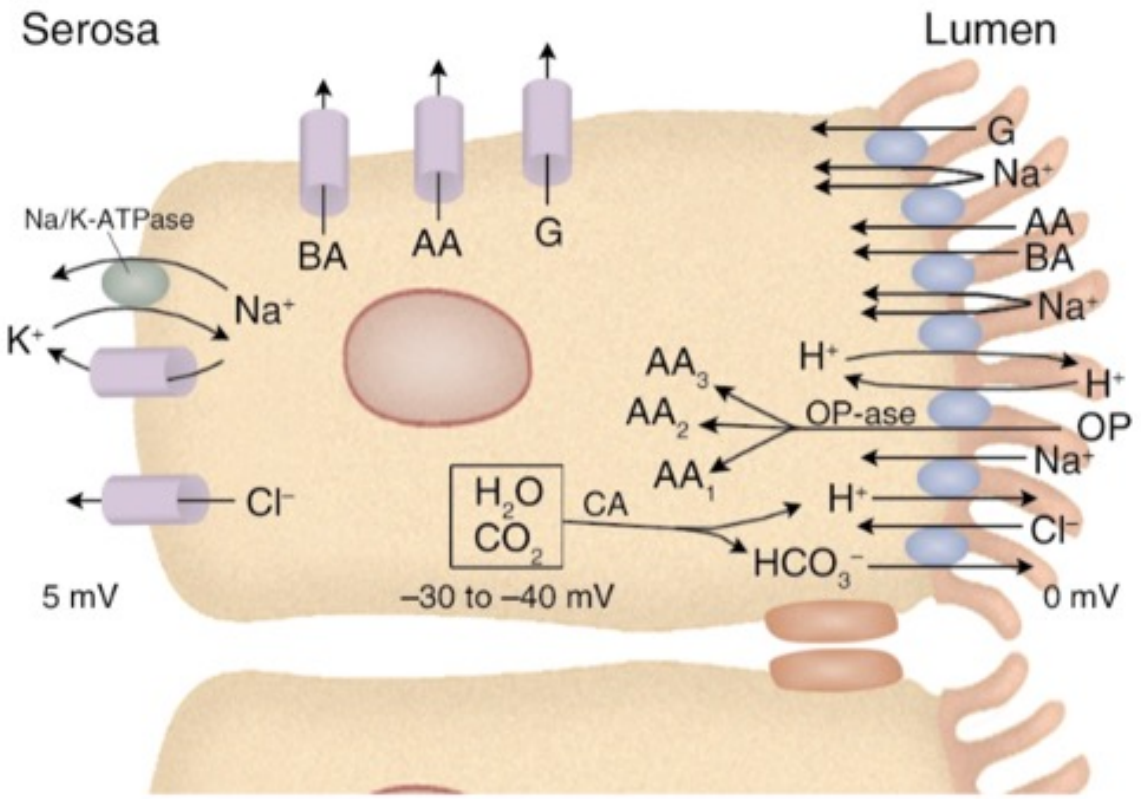

Figure 13. Intestinal absorptive cell. Nutrient absorption is coupled to sodium transport across the intestinal epithelial lining (Republished with permission of The American Society for Clinical Investigation from Intestinal ion transport and the pathophysiology of diarrhea, Field M, vol 111, 2003; permission conveyed through Copyright Clearance Center, Inc).

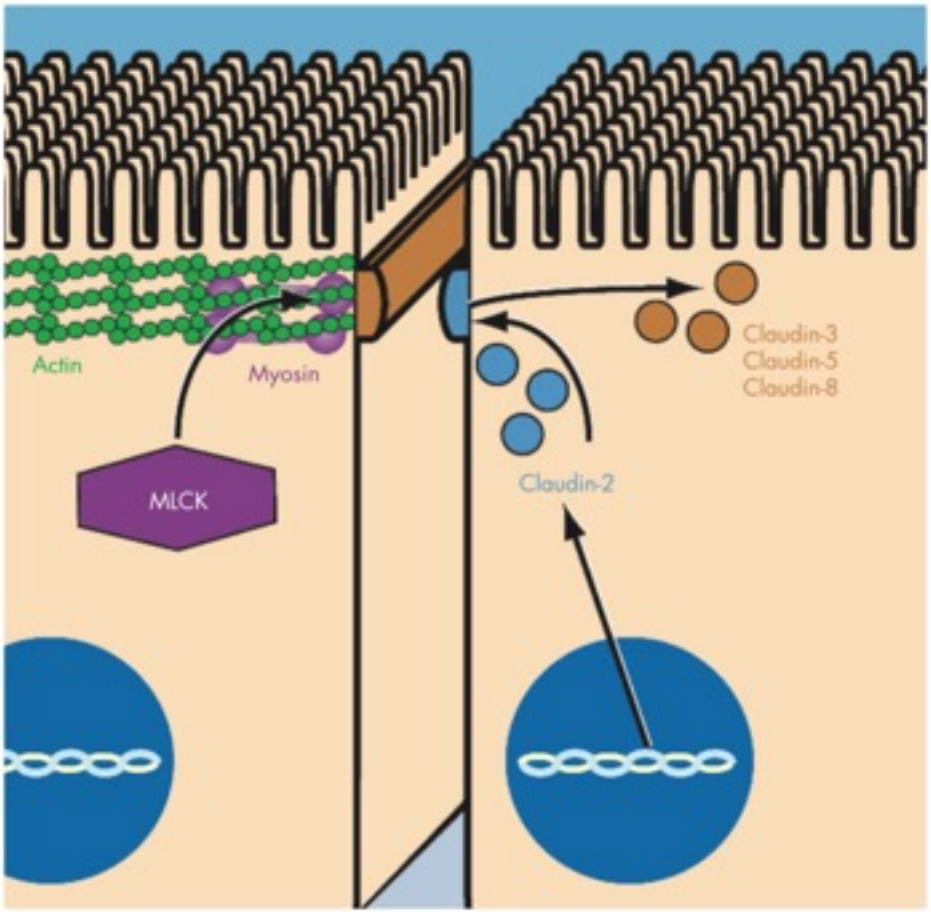

Figure 14. Pathogenesis of barrier dysfunction in IBD. TNFα affects transcriptional and enzymatic activation of myosin light chain kinase (MLCK) resulting in acute and rapidly reversible intestinal epithelial barrier defects (Reproduced from Inflammatory bowel disease: is it really just another break in the wall?, Weber CR, Turner JR, 56:6-8, 2007 with permission from BMJ Publishing Group Ltd).

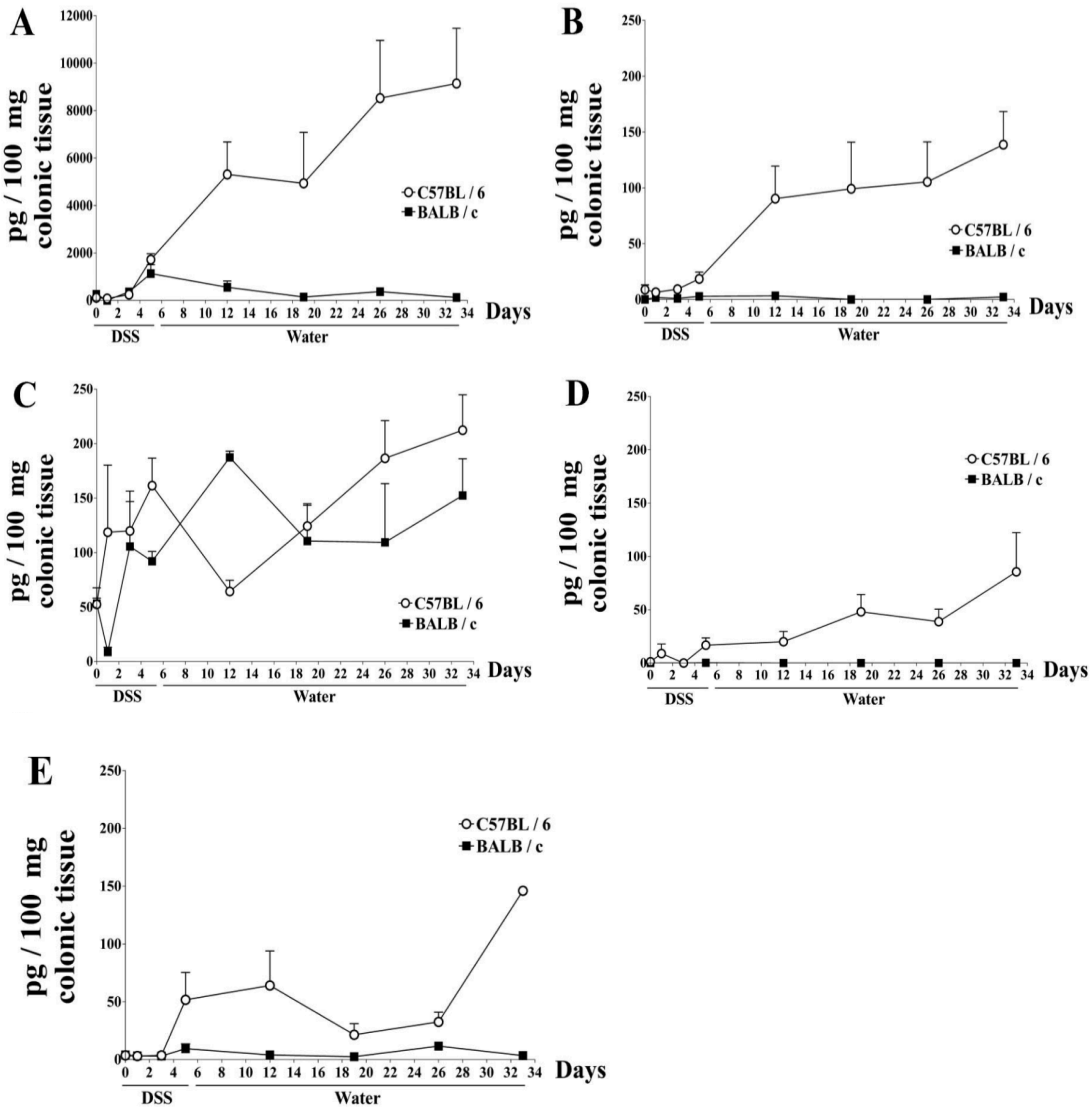

Figure 15. Cytokine production in acute, chronic and recovery phases of DSS-induced colitis in C57/BL6 and BALB/C mice. Levels of IL-1β (A), IL-12 p70 (B), IL-12 p40 (C), IL-17 (D), and IFN-γ (E) were analyzed for each strain (Reproduced with permission from The American Journal of Gastroenterology and Liver Physiology, Melgar et al., 2005).

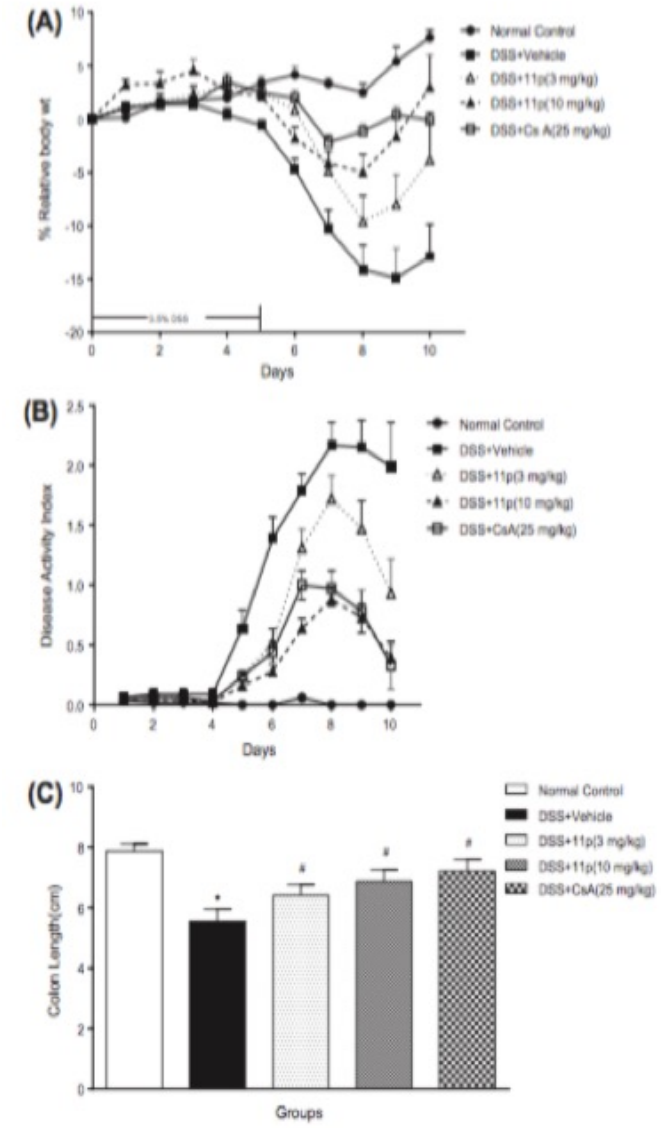

Figure 16. Effect of TACE inhibition on DSS-induced colitis. Colitis was induced in C57/BL6 mice by consumption of 3.5% DSS in drinking water for 5 days. Compound 11p, CsA, or vehicle treatments were administered daily starting on day 1 of DSS administration and ending on day 7. Animals were observed daily for body weight and DAI. On day 10 animals were sacrificed and colon lengths measured. These data demonstrate overall TACE inhibition reduces disease symptoms when compared with diseased control groups (Reprinted from Involvement of TACE in colon inflammation : A novel mechanism of regulation via SIRT-1 activation, 66(1), Sharma et al., 2014, with permission from Elsevier).

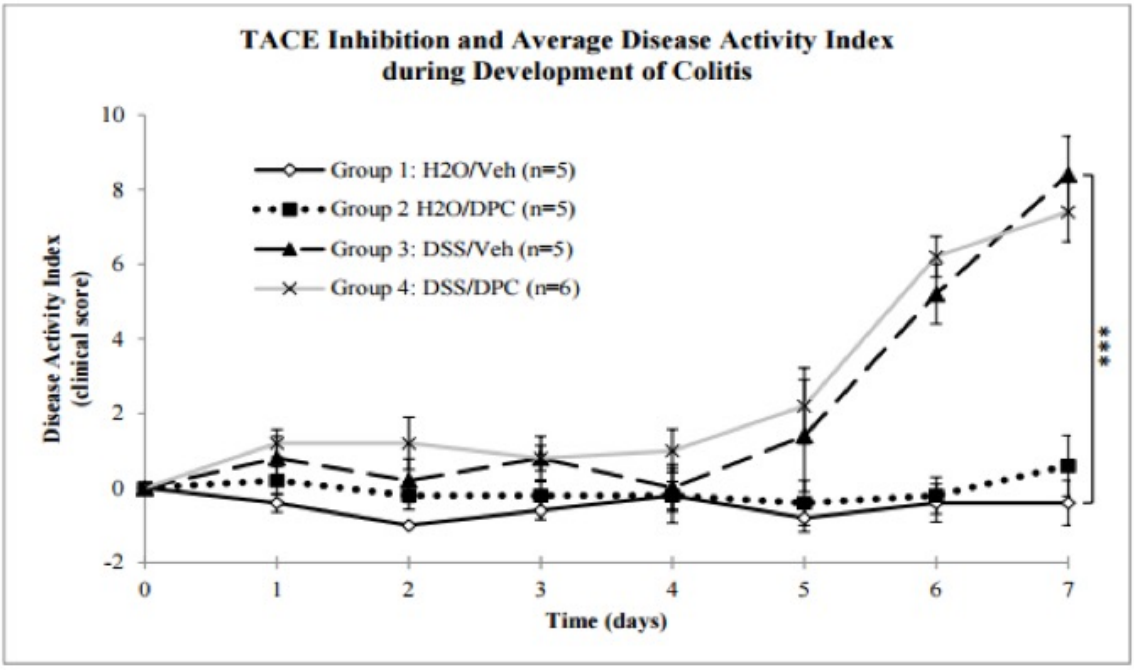

Figure 17. TACE inhibition and average disease activity index during development of colitis in BALB/C mice. No significant difference in DAI was observed between DSS mice that received the TACE inhibitor versus the control group (Reproduced with permission from Missouri State University, Maddox, 2015).

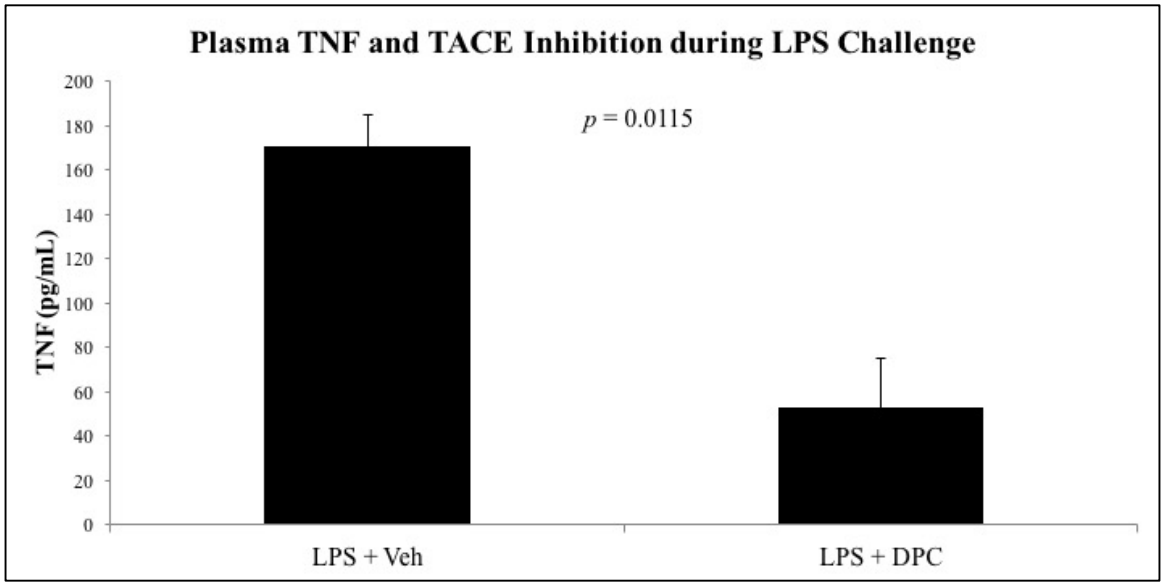

Figure 18. Plasma TNF and TACE inhibition during LPS challenge in BALB/C mice. Bioactivity of the TACE inhibitor was confirmed in a model of LPS-induced systemic inflammation (Reproduced with permission from Missouri State University, Maddox, 2015).

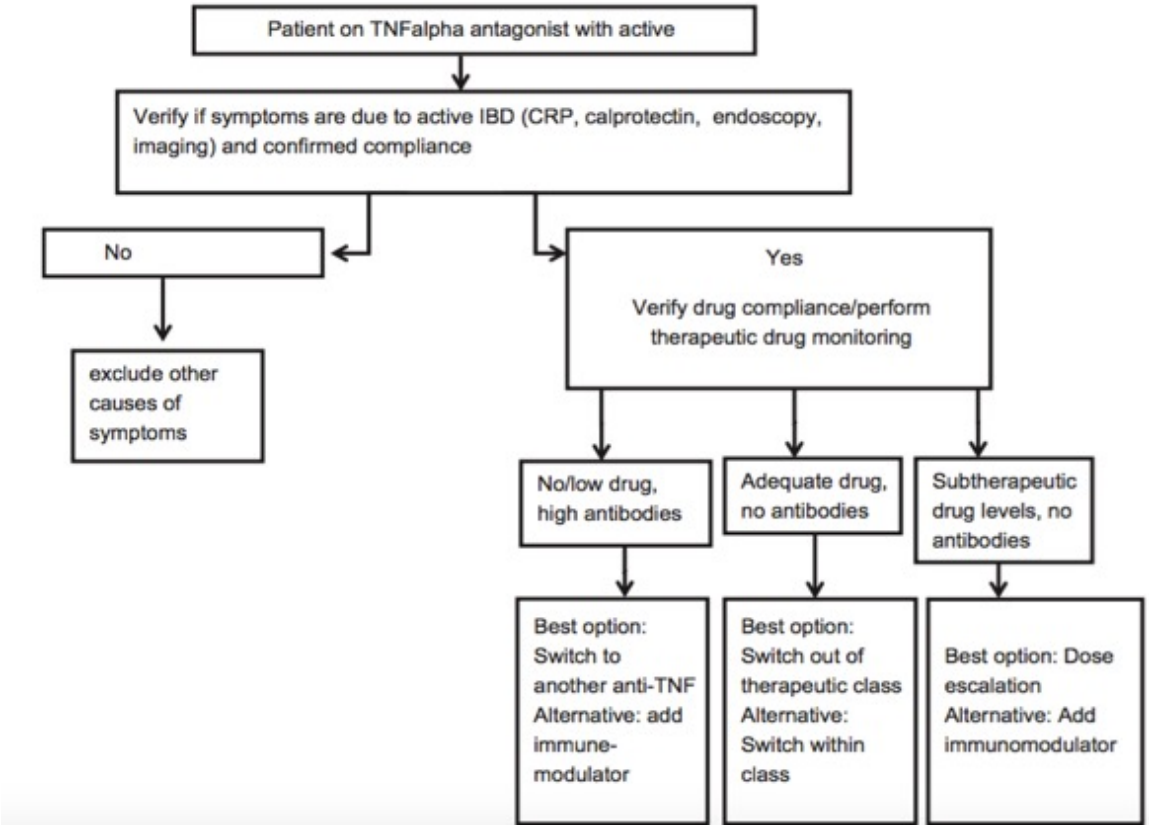

Figure 19. Management of patients with loss of response to anti-TNF therapies (Reproduced from Roda G, Loss of response to anti-TNFs: definition, epidemiology, and management; 2016, with permission from Nature Publishing Group).

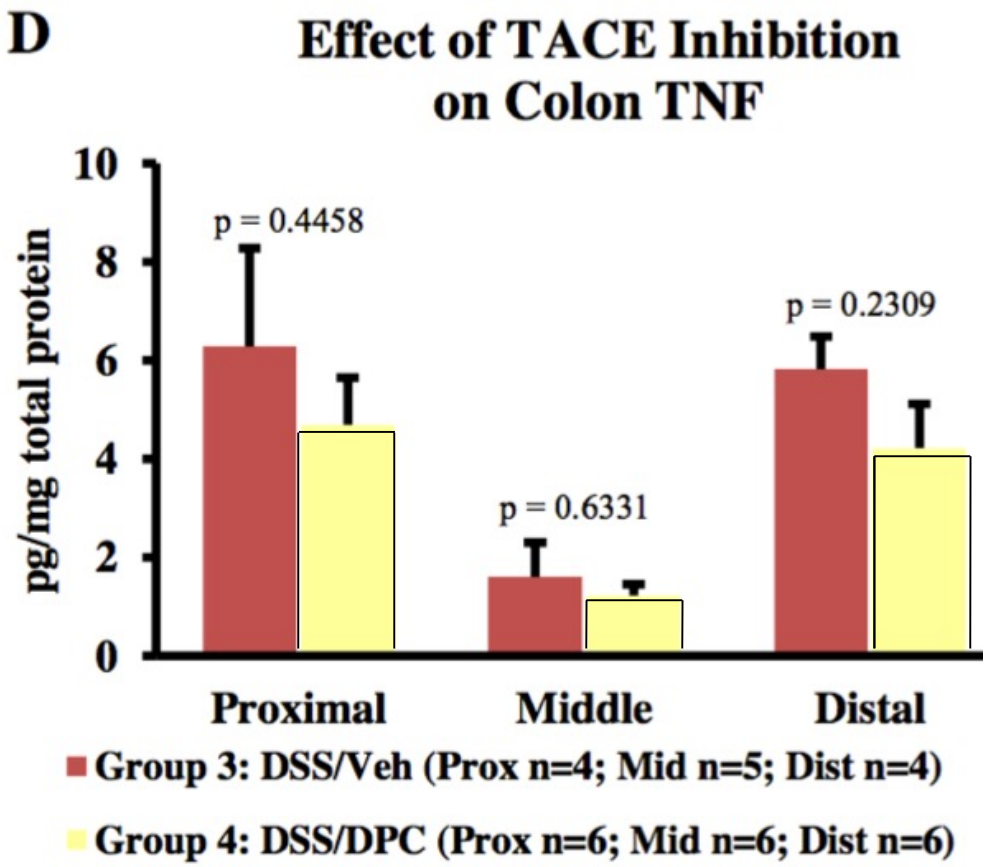

Figure 20. Effect of TACE inhibition on colon TNF without tissue culture. TACE inhibition had no effect on colon TNF-alpha concentrations. Maddox removed colon tissue, homogenized it, then took the supernatant for quantification. Colon TNF-alpha concentrations were quantified at 2-6 pg/mg total protein. These levels are so low as to be insignificant and represent a weakness of the Maddox and Haines study (Reprinted from Tumor necrosis factor alpha converting enzyme during acute colitis in mice: a regional analysis, Maddox, 2015, with permission from Missouri State University).

MATERIALS AND METHODS

Cohort Establishment and Induction of Colitis

Animal care followed the Missouri State University guidelines for experimentation with animals and was approved by the Institutional Animal Care and Use Committee of Missouri State University on April 29, 2016 (ID# 16-027.0-A). Acute colitis was induced in male BALB/C mice by supplementing drinking water with 5% DSS weight/volume. The 5% DSS water was replaced with fresh DSS solution every two days.

Dosing of DPC-333

DPC-333 was dissolved in 25 mM citric acid (CA) saline at 1 mg DPC-333 per 1 mL CA saline. Previous studies established CA saline as the vehicle for DPC-333 and performed qualitative solubility tests to determine appropriate CA concentrations (Kim et al., 2008; Maddox, 2015). DPC-333 was administered at 10 mg/kg bodyweight by intraperitoneal (IP) injections of 1 mg/mL DPC-333. Previous investigation of pharmacodynamics of DPC-333 demonstrated that 10 mg/kg bodyweight IP injection was found to effectively block TACE activity and production of sTNFα in vivo (Figure 21) (Qian et al., 2007).

Study Design

Three cohorts (n=6-7) of mice were used in this study to generate one control group and two disease groups: 1) H2O + vehicle, 2) 5% DSS + vehicle, 3) 5% DSS +

inhibitor (Figure 22). A second control group, H2O + inhibitor, was not generated due to previous study in this lab (Maddox, 2015). Each mouse received twice daily IP injections of either DPC-333 (10 mg/kg bodyweight) or an equivalent volume of vehicle (25 mM CA saline). DSS was administered for 7 days before tissue collection under isoflurane anesthetization and animal sacrifice by isoflurane overdose and cervical dislocation. Food and water consumption were monitored daily. Additionally, clinical scoring parameters, including bodyweight, stool consistency, and presence of blood in the stool and at the anus, were evaluated daily.

Single Blinding of Control and Experimental Groups

Cohort treatments were blinded from assessor. Each mouse was randomly assigned to a cohort. Conical vials were filled with either 5% DSS in drinking water or tap water by an assistant and installed in the appropriate cage without assessor knowledge of vial contents. Syringes were filled with either inhibitor or vehicle by assistants and labeled with mouse identifier. The assessor then injected the appropriate mouse with the corresponding syringe without knowledge of contents.

Assessment of Disease Activity

Disease activity was evaluated through daily recording of bodyweight, food and water consumption, stool consistency, and rectal bleeding. A disease activity index (DAI), adapted from Cooper et al., was calculated by assigning scores for parameters analogous to clinical presentation of IBD in humans.

DAI was calculated as the sum of DAI scores resulting in the total DAI score ranging from 0 (unaffected) to 12 (severe colitis) (Table 1). Score parameters included weight loss (-1 = 1-5% weight gain, 0 = none, 1 = 1-5% weight loss, 2 = 5-10% weight loss, 3 = 10-15% weight loss, and 4 = >15% weight loss), stool consistency (0 = normal, 2 = loose stools, 4 = watery diarrhea), and bleeding (0 = no bleeding, 2 = slight bleeding, 4 = gross bleeding).

Tissue Sample Collection and Culture

Distal colon specimens were collected from mice anesthetized under isoflurane. Specimens were placed in cold saline, opened longitudinally, and cleared of fecal matter. Specimens then underwent three washing steps with ice cold saline, placed in 1 mL of complete RPMI medium 1640 [10% FBS vol/vol, 100 IU penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine], and minced. Medium containing minced specimen was then transferred to culture plates and incubated for 24 hours at 37°C. Following incubation, culture media was transferred to a microcentrifuge tube and centrifuged at 16.2 RCF for 10 minutes at 4°C. Aliquots of supernatant were then taken for TNFα quantification (Protocol adapted from Sharma et al., 2014).

TNFα Quantification

Tissues were homogenized in lysis buffer compatible with EMD Millipore Milliplex Mouse TNFα Magnetic Bead Panel kit for use with the Luminex MagPix singleplex system. Protocol for plasma and tissue preparation as outlined by the kit instructions were followed.

Luminex xMAP kits provide magnetic beads conjugated with bead-specific antibodies for use in a multiplex immunoassay similar to the enzyme-linked immunosorbent assay (ELISA) concept of protein quantification (Luminex, 2011). The bead provides a solid surface for building the assay. A bead-conjugated primary antibody is incubated with sample containing an unknown concentration of analyte. Subsequent incubations are performed to attach a secondary antibody followed by a reporter-conjugated tertiary antibody (streptavidin-phycoerythrin).

The MagPix multiplex system is a LED-based system which detects individual bead identity through excitation at 635 nm and identification of bead-specific fluorescence (Figure 23). Primary antibodies for TNFα are conjugated to beads with a specific fluorescence range. Simultaneously, the fluorescent label of the immunoassay is excited with a green LED at 525 nm and the intensity of fluorescent emission is quantified. In this manner, the MagPix system is capable of quantifying TNFα; median fluorescence intensity (MFI) for TNFα is then used to interpolate assay sample concentration from a standard curve.

For the quantification of TNFα, 25 µL of colon tissue homogenate samples diluted to 2-5 µg/mL were analyzed. Standards were generated from serial dilution of 10,000 pg/mL reconstituted Mouse Cytokine Standard, resulting in 6 standards down to 3.2 pg/mL. Experimental and standard samples were incubated overnight with 25 µL antibody conjugated beads. Tris lysis buffer was used as a background matrix with tissue homogenate assays. Samples were then incubated with a secondary antibody for one hour, followed by an hour-long incubation with a streptavidin-phycoerythrin conjugate (reporter). Standard, control, and experimental samples were then run in duplicate.

Standard curve analysis was performed with Milliplex Analyst software. Curve fit was produced with 5-parameter non-linear regression analysis and assay sample TNFα values were interpolated from the standard curve. Results were normalized to total protein of the original tissue homogenate, previously determined in BCA assay.

Statistical Analysis

Disease activity data was analyzed using independent t-tests within GraphPad Prism analysis software. Analysis for successful development of colitis was performed via comparison between water control and DSS colitis group (Group 1 versus Group 2). DSS colitis groups receiving vehicle control injections were compared to DSS colitis groups receiving the inhibitor (DPC-333) to determine the effects of the inhibitor (Group 2 versus Group 3).

Quantified TNFα levels were analyzed using independent t-tests within GraphPad Prism analysis software. Analysis for successful development of colitis, and thus induction of TNFα production, was performed with t-tests between water control and DSS colitis groups receiving vehicle control injections (Group 1 versus Group 2). Effect of the inhibitor on TNFα levels was determined through t-test between DSS colitis groups receiving either vehicle control or DPC-333 injections (Group 2 versus Group 3).

Tables

Table 1. Clinical Scoring Guide for Disease Activity Index determination. Disease Activity Index (DAI) was calculated as the daily sum of clinical scoring parameters including percent bodyweight change, stool consistency, and bleeding (Reproduced with permission from Missouri State University, Maddox, 2015).

	Clinical Scoring Guide	
	Range	Score
Percent Bodyweight Change	5-10% Gain	-2
	1-5% Gain	-1
	0	0
	1-5% Loss	1
	5-10% Loss	2
	10-15% Loss	3
	>15% Loss	4
Stool Consistency	Normal	0
	Loose	2
	Diarrhea	4
Bleeding	None	0
	Slight	2
	Gross	4

Figures

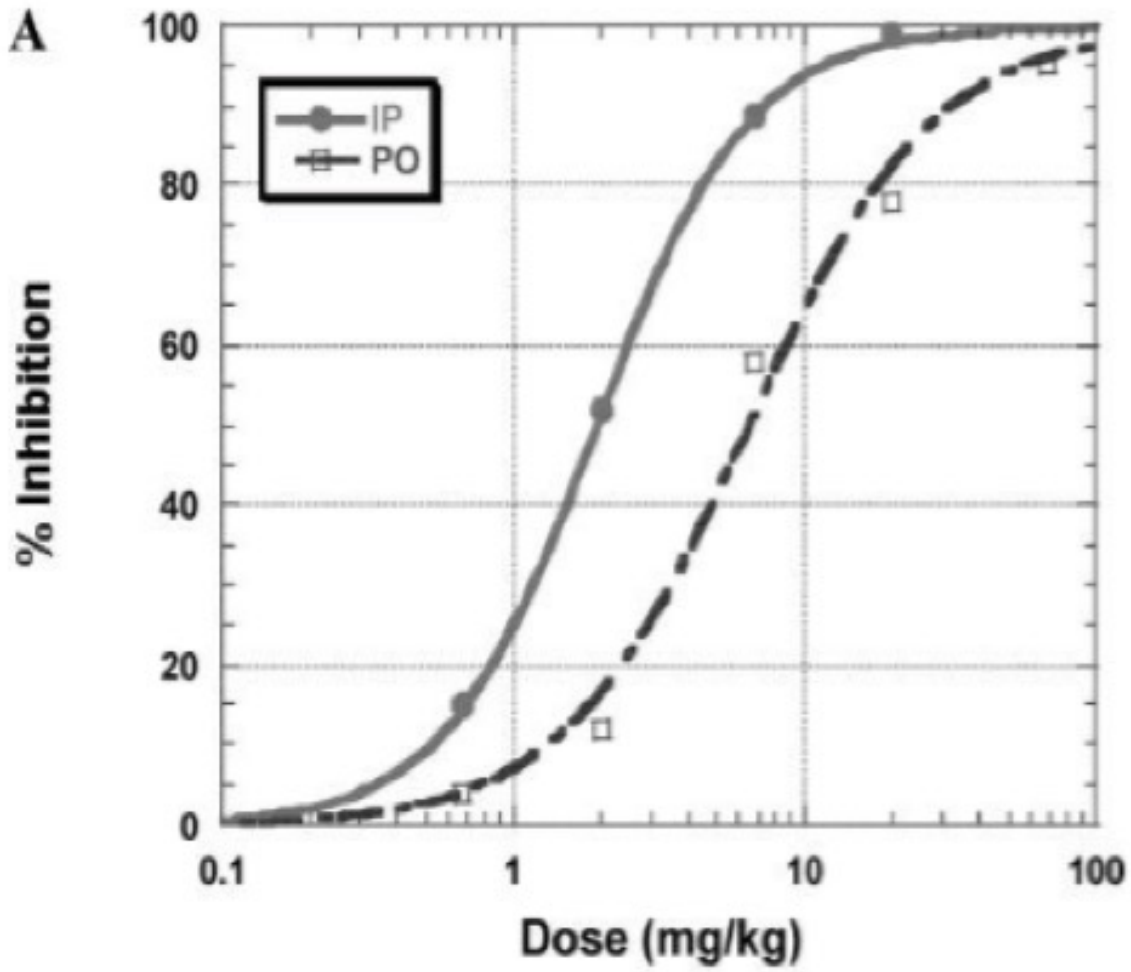

Figure 21. Dose-dependent inhibition of sTNFα production in vivo. Dose dependent sTNFα inhibition with DPC-333 was determined in male BALB/C mice challenged with 10 μg/mouse LPS IP injection. Determination of percent inhibition of sTNFα release was achieved through comparison of plasma sTNFα concentrations following IP (solid line) and oral (dotted line) doses of DPC-333 to vehicle control (Reproduced with permission from The American Society for Pharmacology and Experimental Therapeutics: Drug Metabolism and Disposition, Qian et al. 2007).

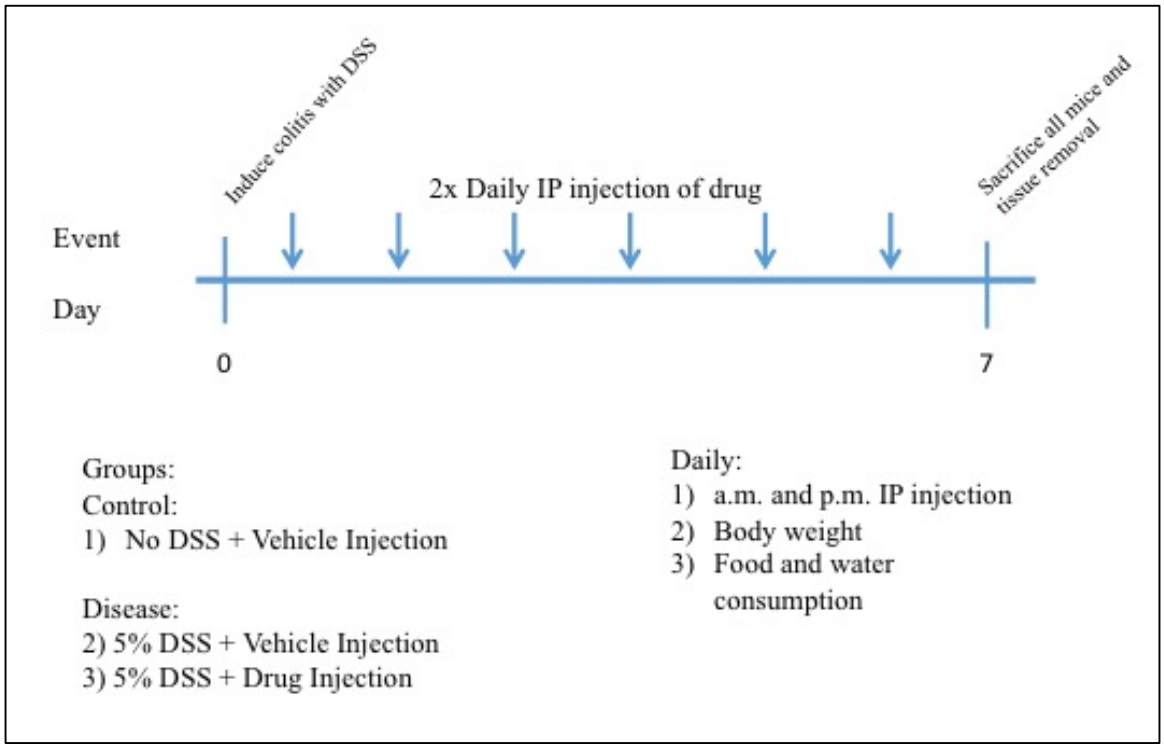

Figure 22. Study Design. Mice were randomly assigned to one of three groups: a water control (Group 1), a disease control (Group 2), and an experimental group (Group 3). For 7 days, mice consumed either untreated drinking water or 5% DSS in drinking water and received twice daily injections of either the TACE inhibitor DPC-333 or its vehicle 25 mM citric acid saline. Every morning, food and water consumption as well as bodyweight were recorded and clinical parameters were scored to create a disease activity index. Following morning routine, mice were sacrificed and the distal 1 cm of colon was removed.

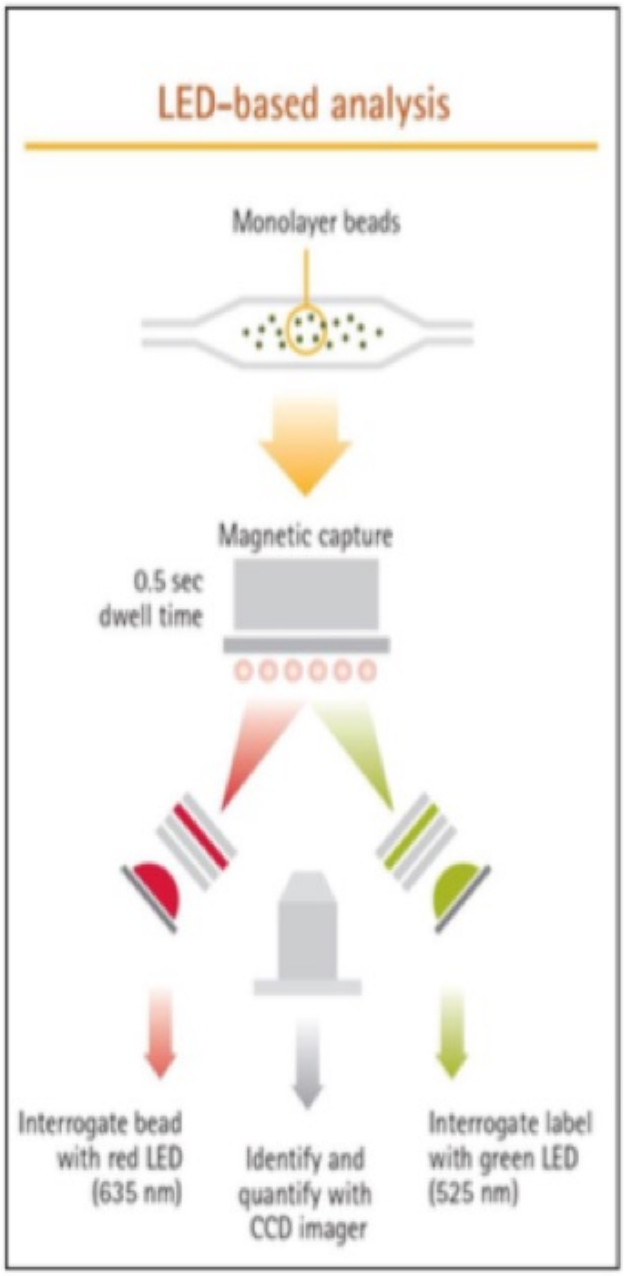

Figure 23. Luminex xMAP Technology and MagPix Multiplex System. The Luminex MagPix system is a LED-based fluorescent detection system for protein quantification. Antibody-conjugated magnetic beads are passed through a magnetic capture field and excited with 635 nm LED. Resulting fluorescence intensity is used to quantify analyte concentrations (Reprinted with permission from EMD, 2015).

RESULTS

Clinical Scoring of 5% DSS Colitis

Effects of DSS Consumption on Colitis Development. The effects of 5% DSS colitis were assessed via scoring of clinical parameters to create a disease activity index (DAI). The results of this clinical assessment are summarized in Table 2. To evaluate the successful induction of 5% DSS colitis, independent t-tests were performed to compare groups 1 and 2 (Group 1: H_2O + vehicle injection; Group 2: DSS + vehicle injection). The effect of the TACE inhibitor during 5% DSS colitis was evaluated via independent t-test between groups 2 and 3 (Group 2: DSS + vehicle injection; Group 3: DSS + DPC-33 injection). Consumption of 5% DSS in drinking water for 7 days significantly increased the DAI, indicating successful induction of colitis ($p < 0.0001$; Group 1 vs Group 2 on day 7) (Figure 24). Diseased mice exhibited clinically relevant signs of colitis, such as significant bodyweight loss, rectal bleeding, and diarrhea. TACE inhibition had no significant effect on DAI and did not improve colitis ($p = 0.74$; Group 2 vs Group 3 on day 7) (Figure 24). Diseased mice receiving the TACE inhibitor exhibited clinical signs of colitis comparable to those that received only vehicle injections.

Bodyweight Loss in Response to DSS Consumption. The effects of DSS consumption on bodyweight loss was evaluated via an independent t-test comparing groups 1 and 2 (Group 1: H_2O + vehicle injection; Group 2: DSS + vehicle injection). Mice that consumed 5% DSS in drinking water exhibited significant bodyweight loss compared to control mice ($p = 0.0002$; Group 1 vs Group 2 on day 7). An independent t-test between groups 2 and 3 (Group 2: DSS + vehicle injection; Group 3: DSS + DPC-

333 injection) revealed that TACE inhibition had no effect on bodyweight loss ($p = 0.45$; Group 2 vs Group 3 on day 7) (Figure 25); the final bodyweights of these mice were comparable to diseased mice that received vehicle injections.

Effects of TACE Inhibition on Rectal Bleeding. An independent t-test comparing groups 1 and 2 (Group 1: H_2O + vehicle injection; Group 2: DSS + vehicle injection) revealed that 5% DSS consumption induced significant gross bleeding at the rectum as early as day 5 and continuing through day 7 ($p < 0.0001$; Group 1 vs Group 2 on day 7). To evaluate the effects of TACE inhibition on rectal bleeding, an independent t-test between groups 2 and 3 (Group 2: DSS + vehicle injection; Group 3: DSS + DPC-333 injection) was performed. No significant difference in rectal bleeding was observed between these groups, indicating that TACE inhibition had no effect on the clinical bleeding score ($p = 1.00$; Group 2 vs Group 3 on day 7) (Figure 26).

Alterations in Stool Consistency Following DSS Consumption. Occurrence of loose stools and watery diarrhea in response to 5% DSS consumption were evaluated via independent t-test between groups 1 and 2 (Group 1: H_2O + vehicle injection; Group 2: DSS + vehicle injection). Mice receiving DSS passed loose stools as early as day 5, though a significant increase in loose stool scores was not seen until day 7 ($p < 0.0001$; Group 1 vs Group 2). An independent t-test between groups 2 and 3 (Group 2: DSS + vehicle injection; Group 3: DSS + DPC-333 injection) revealed that TACE inhibition had no effect on stool consistency scores ($p = 0.15$; Group 2 vs Group 3) (Figure 27). In fact, diseased mice receiving the TACE inhibitor exhibited signs of diarrhea comparable to those that received only vehicle injection.

Impact of DSS Consumption on Food and Water Consumption. Food and water consumption were recorded daily for each group of mice in order to evaluate the effects of DSS consumption and TACE inhibition on food and water intake. Quantitative analysis of this data indicates that neither consumption of 5% DSS in drinking water nor twice daily DPC-333 or vehicle injections had an effect on food and water intake (Figures 28 and 29). This suggests that mice consumed food and water regardless of the stressors associated with experimental protocols.

Quantification of Colon TNFα Levels

Overview. Following 24-hour tissue culture, colon TNFα levels were measured through multiplex immunoassay quantification of distal colon tissue homogenate and normalized to total protein (pg/mg total protein acquired by BCA assay).

Effect of DSS Colitis on Colon TNFα Levels. The effect of 5% DSS administration for 7 days on colon TNFα levels was evaluated via an independent t-test between groups 1 and 2 (Group 1: H_2O + vehicle injection; Group 2: DSS + vehicle injection) (Figure 30). Although not statistically significant, consumption of 5% DSS for 7 days increased colon TNFα levels by 51.9% ($p = 0.11$; Group 1 vs Group 2) (Table 3), suggesting that DSS-induced colitis up-regulates colon TNFα.

Colon TNFα Levels in Response to TACE Inhibition. The effect of TACE inhibition on colon TNFα levels during 5% DSS colitis was evaluated through an independent t-test between groups 2 and 3 (Group 2: DSS + vehicle injection; Group 3: DSS + inhibitor injection) (Figure 30). TACE inhibition significantly reduced TNFα levels by 56.2% ($p = 0.017$; Group 2 vs Group 3) (Table 3). These findings confirm that

the TACE inhibitor DPC-333 is bioactive in the colon and that TACE inhibition successfully reduces colon TNFα levels.

Tables

Table 2. Analysis of clinical scoring in colitis development. p values were obtained from an independent t-test comparing parameters on day 7 of 5% DSS. Group 1 (G1): H2O + vehicle injection; Group 2 (G2): DSS + vehicle injection; Group 3 (G3): DSS + inhibitor injection.

Analysis of Clinical Scoring in Colitis Development		
	p-value	
Parameter	G1 vs G2	G2 vs G3
Disease Activity Index (DAI)	<0.0001	0.74
% Bodyweight Loss Score	0.0002	0.45
Bleeding Score	<0.0001	1.0000
Diarrhea Score	<0.0001	0.15

Table 3. TACE Inhibition during Colitis and Percent Difference in TNFα Concentration. Results are independent t-test values of TNFα concentrations after 24-hour tissue culture. Group 1 (G1): H2O + vehicle injection; Group 2 (G2): DSS + vehicle injection; Group 3 (G3): DSS + inhibitor injection.

TACE Inhibition during Colitis and Percent Difference in TNFα Concentration			
		Group 1 vs Group 2	Group 2 vs Group 3
TNFα Concentration	% Difference	+51.9	-56.2
	p-value	0.11	0.017

Figures

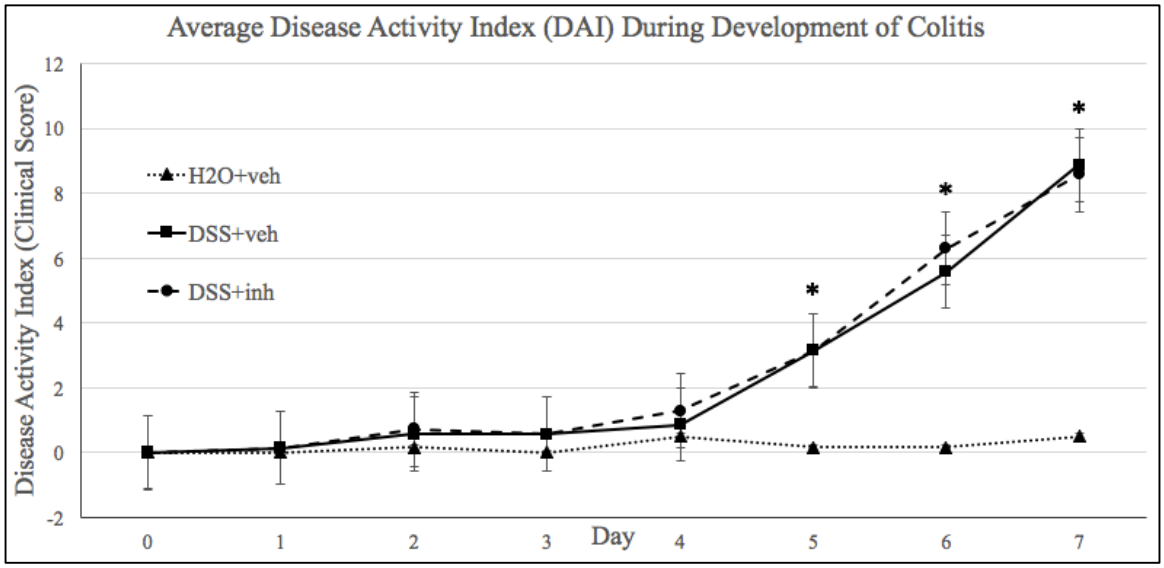

Figure 24. Average disease activity index (DAI) during development of 5% DSS-induced colitis. DAI was quantified through scoring of clinical parameters as described in the text. Values are group means ± SEM; * $p<0.05$ (DSS + vehicle versus respective H2O+vehicle day). No significant difference between DSS + vehicle versus DSS + inhibitor.

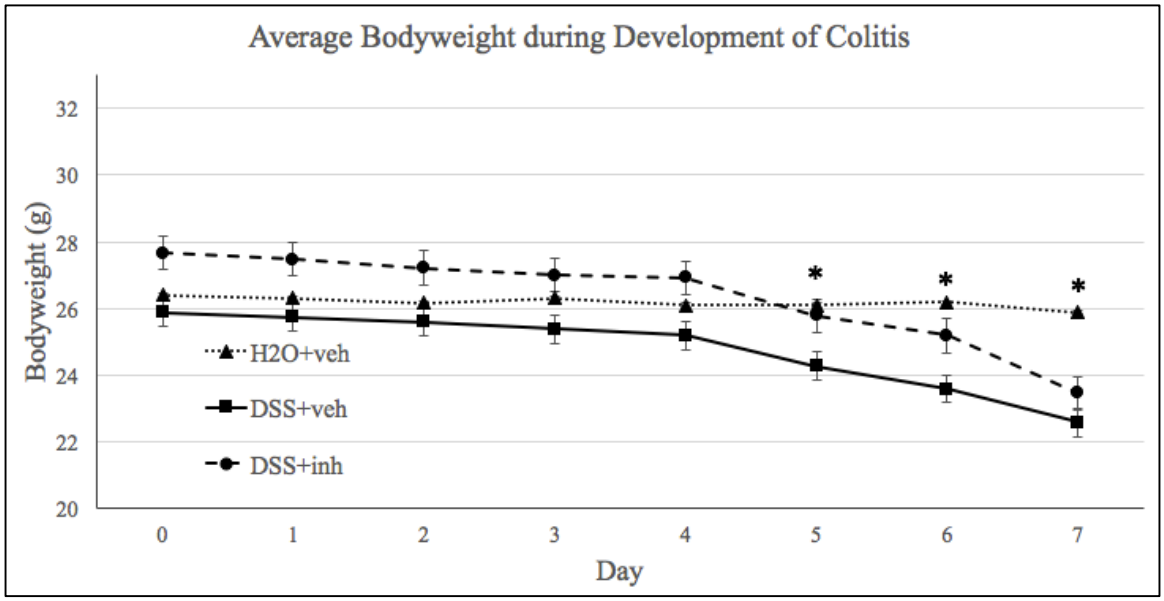

Figure 25. Average bodyweight during development of colitis. Values are group means ± SEM; * $p<0.05$ (DSS + vehicle versus respective H2O + vehicle day). No significant difference between DSS + vehicle versus DSS + inhibitor.

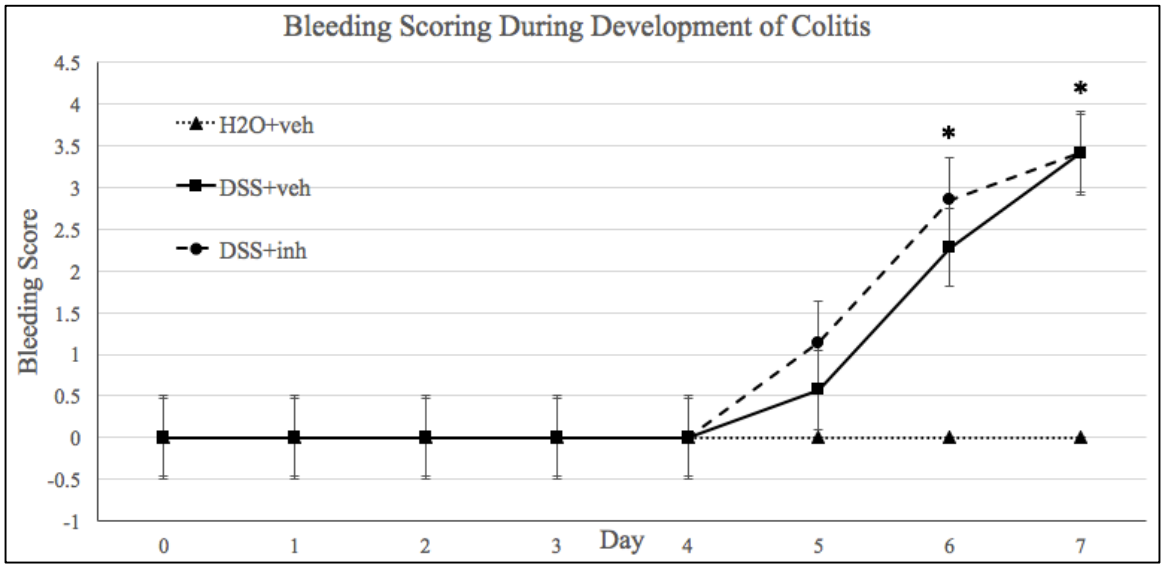

Figure 26. Bleeding scoring during development of colitis. Values are group means ± SEM; * $p<0.05$ (DSS + vehicle versus respective H2O+vehicle day). No significant difference between DSS + vehicle versus DSS + inhibitor.

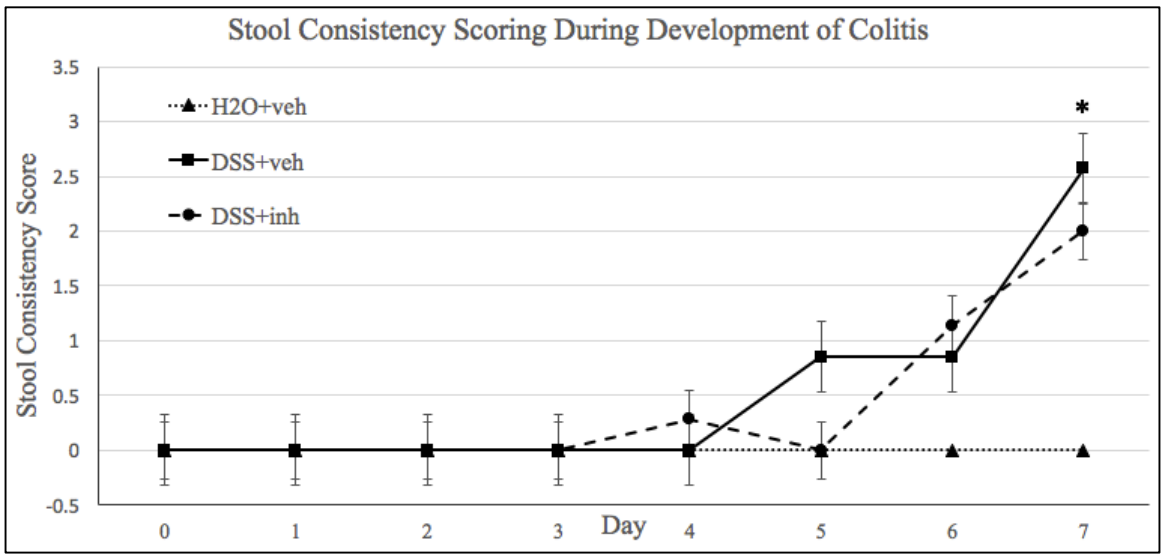

Figure 27. Stool consistency scoring during development of colitis. Values are group means ± SEM; * $p<0.05$ (DSS + vehicle versus respective H2O+vehicle day). No significant difference between DSS + vehicle versus DSS + inhibitor.

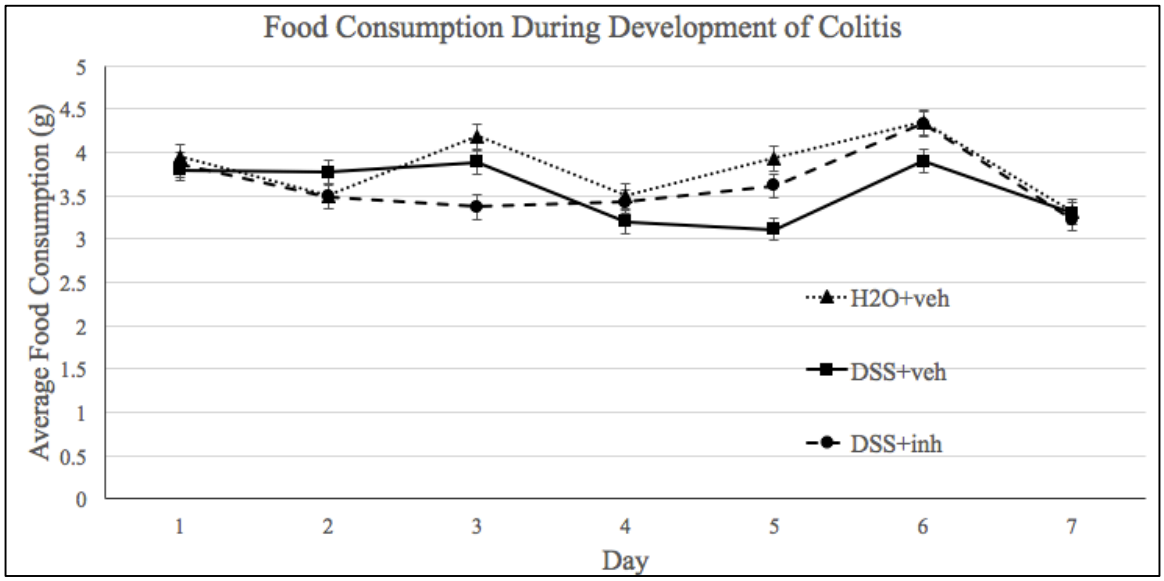

Figure 28. Food consumption during development of colitis. Values are group means ± SEM. No significant difference between groups.

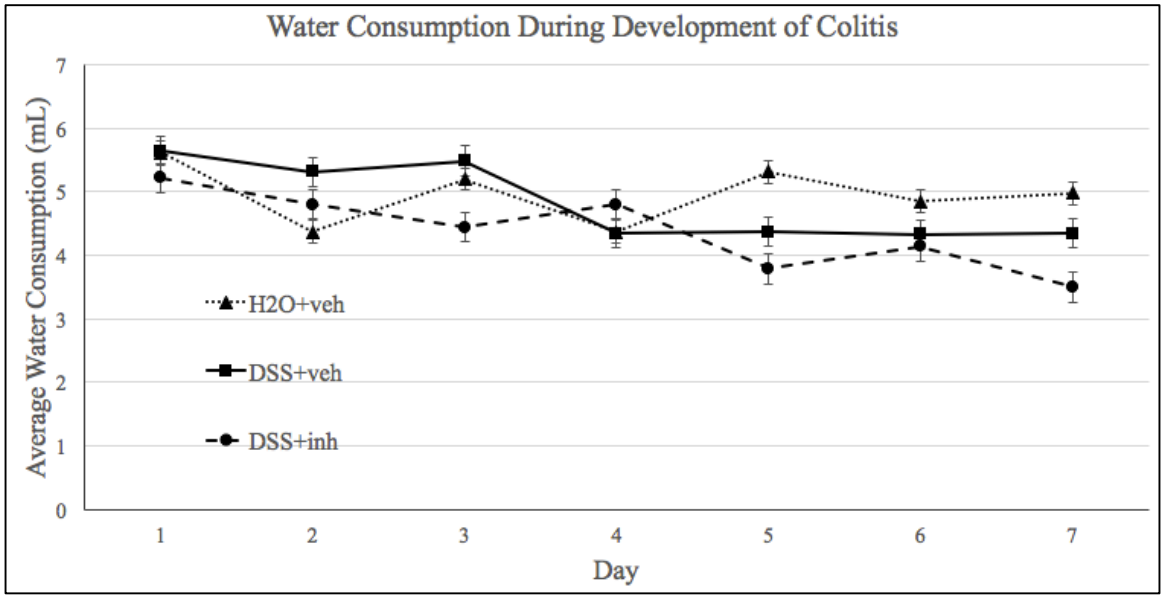

Figure 29. Water consumption during development of colitis. Values are group means ± SEM. No significant difference between groups.

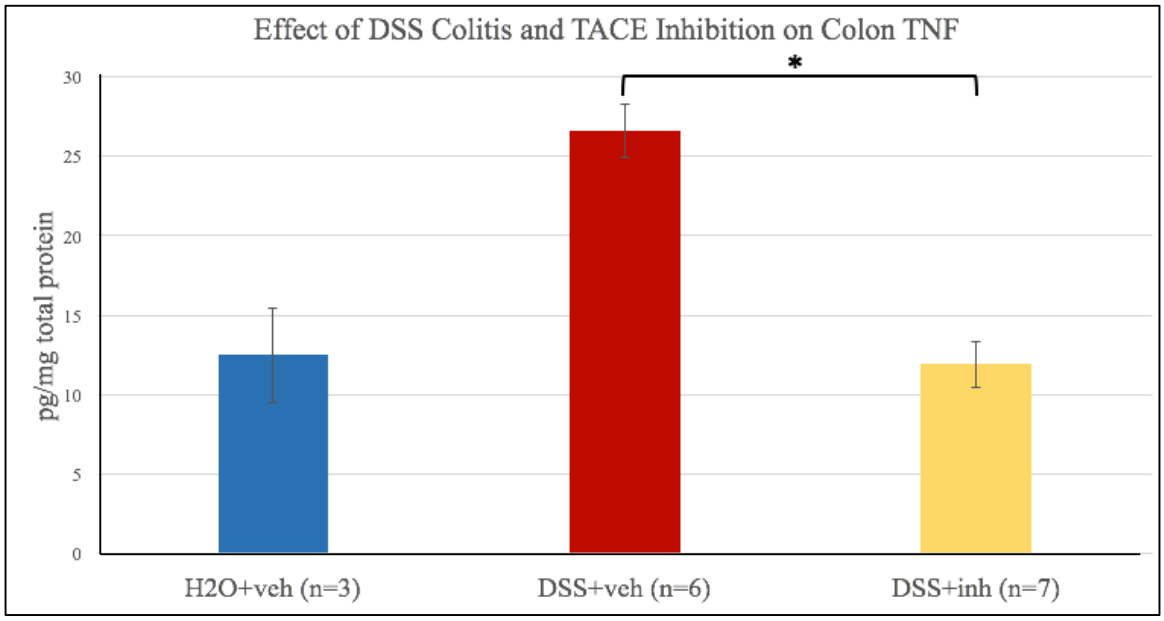

Figure 30. Effect of DSS colitis and TACE inhibition on colon TNFα. Tissue TNFα concentrations were quantified after 7 days of 5% DSS. Groups received twice daily IP injections of either 10 mg/kg DPC-333 or an equivalent volume of vehicle (25mM citric acid saline). Values are group means ± SEM; *$p<0.05$ (DSS + vehicle versus DSS + inhibitor).

DISCUSSION

Novel Impact of Data Collected and Rejection of Hypothesis

BALB/C Mouse Model as Primary Non-Responders to Anti-TNFα Therapy.

Current literature and research on the DSS model of clinical colitis demonstrate that the disease model and findings vary between mouse strain, thus establishing difference in susceptibility to DSS-induced colitis are due to genetic background. A 2005 study by Melgar et al. observed the differences in response to DSS between C57/BL6 and BALB/C mice (Table 4). While BALB/C mice recover after termination of DSS administration for 7 days, C57/BL6 progress to chronic disease. These two models of colitis also differ in the concentration of DSS tolerated and the inflammatory cytokines produced. Identification and quantification of inflammatory cytokines revealed that the acute colitis in BALB/C may be macrophage-driven, while the progression to chronic disease in C57/BL6 mice may be T-cell-driven.

Additionally, studies of TACE inhibition in DSS colitis models vary widely in methods (DasGupta et al., 2008). One study provided evidence for TACE inhibition improving disease phenotype in C57/BL6 mice by giving twice daily IP injections of the TACE inhibitor DPC-333 during 7 days of DSS administration (Sharma et al., 2014). Concurrent with the methods of Sharma et al., Maddox demonstrated that administration of DPC-333 does not improve the disease state in a BALB/C model of DSS colitis. This study improved upon the methods of Maddox by conducted a single-blind randomized study to prevent bias in phenotyping of clinical symptoms. Additionally, a 24-hour tissue incubation step was included to allow for continued cytokine production and resulted in

more significant readings of colon TNFα Furthermore, DPC-333 was observed to be bioactive in the target tissue, knocking down colon TNFα levels to concentrations comparable to those of healthy animals. These results suggest that TNFα is not involved in the development of DSS colitis in the BALB/C model and that an alternative pathway may exist.

Further research is necessary to determine the mechanism behind primary non-response to anti-TNFα treatments and characterize an alternative pathway in colitis development. In light of the failure of TACE inhibition to resolve the disease phenotype in BALB/C mice, it is possible that the BALB/C colitis model could serve as a pre-clinical model of study for IBD patients who do not respond to anti-TNFα treatments.

Future Directions and Preliminary Data in C57/BL6 Mice

TNFα Involvement in BALB/C Colitis Models. It has long been established that TNFα is a key player in the development and progression of IBD. However, current research suggests that TNFα may not be the driving force in some forms of IBD. Further research is necessary to determine an alternative driving force for inflammation and an animal model that does not respond to anti-TNFα therapies could provide insight in these cases. We have confirmed that BALB/C mice fail to respond to TACE inhibition during DSS colitis, thus, we find it pertinent to provide further evidence for the lack of involvement of TNFα in BALB/C colitis models by conducting an identical study in C57/BL6 mice. Although our methods were adopted from the study of TACE inhibition in the C57/BL6 model of DSS colitis conducted by Sharma et al., we are currently recreating this study to generate data with which we can compare the response of these

two mouse models to TACE inhibition. Although preliminary data shows no statistically significant difference in disease activity between the three groups (Group 1: H_2O + vehicle, n=4; Group 2: DSS + vehicle, n=4; Group 3: DSS + inhibitor, n=4), there appears to be a trend for improvement of disease activity with TACE inhibition ($p = 0.0758$) (Figure 31).

If a difference in response to TACE inhibition is observed between C57/BL6 and BALB/C mice, it would be necessary to investigate BALB/C response to TACE inhibition in colitis induced by other methods as well as response to other forms of anti-TNFα therapy. Lack of response to all forms of anti-TNFα treatments in multiple colitis models would provide sufficient evidence to suggest that BALB/C mice are a pre-clinical model of study for a form of IBD in which TNFα is not involved.

Alternative Driving Forces in IBD. Evidence suggests that forms of IBD exist in which TNFα is not the driving force for inflammation, thus, alternative pathways of colitis development need to be investigated. Determination of an alternative driving force might prove a difficult feat due to the variety of factors involved in IBD development, such as genetics, signaling pathways, diet, and microbiota composition. Because BALB/C and C57/BL6 mice vary in their response to DSS-induced colitis, study of the differing pro-inflammatory cytokines between the two models might illuminate an alternative cytokine as the driving force for inflammation in BALB/C mice. Additionally, comparison of the genetic backgrounds of the two mouse strains might shed light on genetic factors behind non-TNFα-mediated colitis.

Impact of Results on Current Directions in IBD Research- Alternative Therapeutics for Non-Responders

Overview. The failure of current pharmaceuticals to completely ameliorate colitis has led current IBD research to broaden its horizons. Traditionally, the focus has been on anti-inflammatory agents that modulate the immune system, with the intestinal immune response as the key focus of developing therapies (Bernstein, 2015). Research has now shifted its focus to developing new therapeutics, investigating the role of the gut microbiota in IBD development, and improving clinical assessment protocols and clinical goals to optimize personalized patient treatments (Löwenberg & Haens, 2015; Marchesi et al., 2015; Levesque et al., 2015).

Novel Therapeutics for IBD. Recently, various novel drugs have been evaluated in clinical trials, showing promising outcomes in IBD patients. These drugs include small molecules interfering with intracellular signaling pathways and therapeutic antibodies directed against extracellular targets.

Golimumab, a novel anti-TNFα monoclonal antibody, has recently been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for UC patients with moderate to severe IBD (Löwenberg & Haens, 2015). Induction treatment with golimumab in UC patients naïve to biologic treatment significantly increased clinical response, remission, and mucosal healing rates at week 6 compared to a placebo (Sandborn et al., 2014).

Several agents interfering with leukocyte trafficking have been developed to selectively target cell adhesion molecules and interfere with T-cell trafficking. The recently developed therapies vedolizumab, etrolizumab, and PF-00547659 are more gut

selective than previous leukocyte trafficking drugs, and have so far not led to progressive multifocal leukoencephalopathy (PML) (Löwenberg & Haens, 2015).

Orally active small molecules represent novel therapeutics that interfere with intracellular signaling and have lower production costs than therapeutic antibodies. So far, Janus kinase (JAK) inhibitors are the most developed small molecules, one of which has already been approved and marketed for treatment of rheumatoid arthritis. JAKs play a major role in regulation of cell proliferation, differentiation, and immune cell function. JAK-dependent signaling pathways are known to be involved in chronic inflammatory pathologies, such as rheumatoid arthritis and IBD. Thus, JAK inhibition is a novel approach to IBD treatment as an anti-inflammatory therapy (Löwenberg & Haens, 2015).

Gut Microbiota Modulation as an IBD Therapy. Early studies of intestinal bacteria in IBD pathogenesis focused on identifying a specific cell population that could potentially initiate colitis. Recently, the realization that the gut microbiota as a whole is altered in IBD has shifted the focus to microbiota modulation as a therapy. Changes in gut microbiota composition, such as a reduction in Firmicutes coincident with an increase in Bacteroidetes, have been reported in patients with IBD. Furthermore, certain changes have been clearly linked to either CD or UC.

Several clinical trials have investigated modulation of the microbiota in IBD through antibiotics, probiotics, prebiotics, enteral nutrition, and fecal transfer. Randomized controlled trials of antibiotic combinations in CD and UC have demonstrated beneficial effects and antibiotics are a popular treatment for perineal fistulizing CD. While proof of safety and efficacy are lacking for the use of probiotics, there are promising results for *E. coli* Nissle 1917 as a maintenance therapy for UC.

Prebiotics are food substances that are not digested in the small intestine and promote selective growth of beneficial bacteria in the colon. To date, no proof of benefit from prebiotic treatment exists for IBD patients, although their use is still being investigated.

Modulation of enteric nutrition through specialized dietary formulations has demonstrated effectiveness in treatment of pediatric CD. Thus, this approach has been widely adopted by pediatric gastroenterologists. Finally, fecal transfer has been considered in the treatment of IBD; however, in IBD, the microbiota is altered very permanently and preliminary data on randomized controlled trial of fecal transplantation by enema in UC was negative (Bernstein, 2015). Thus, investigation of definitive microbiota abnormalities in IBD is ongoing with the hope that better understanding will lead to novel therapeutic approaches.

Improvement of Clinical Assessment and Practices. Therapeutic advances and better understanding of IBD have led to a shift in the assessment of disease activity, as remission targets are more achievable (Walsh et al., 2016). IBD management goals have been based on composite indices which incorporate symptoms, signs, laboratory test results, and endoscopic assessments. Because these indices are complex and not intuitive to clinicians, or individual patient phenotypes, there is a disconnect between clinical trials and practice. Consideration of patient-reported outcomes, in addition to use of these composite indices, could provide a clinically meaningful and scientifically valid assessment of disease activity (Levesque et al., 2015). Thus, focus has shifted to fine-tuning disease activity assessment protocols and combination therapies with the hopes of personalizing IBD management plans to individual patients.

One aspect of this new goal is to predict patient response to anti-TNFα therapies so as to design a practical management plan to optimize response prior to treatment. One recent study formulated clinical algorithms that employ therapeutic drug monitoring to determine the underlying cause of primary non-response and secondary loss of response (Figure 32). Ultimately, these algorithms should allow clinicians to identify which patients are most likely to respond to anti-TNFα therapies and optimize drug therapy for those who are losing response (Ding et al., 2015).

Additionally, new serological markers are being utilized in the prediction of IBD patient outcomes. So far, only anti-neutrophilic cytoplasmic antibodies (ANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) have demonstrated diagnostic ability and their simultaneous use allows for distinction between CD and UC. More recently, antibodies such as anti-glycoprotein 2 (anti-GP2) and anti-granulocyte macrophage colony-stimulating factor (anti-GM-CSF) have been identified in IBD patients. GP2 is thought to play an immunomodulatory role in the intestinal immune system, while GM-CSF promotes myeloid cell maturation and is necessary for homeostatic responses to tissue damage. Both proteins have been demonstrated to be neutralized in the serum of IBD patients, while their antibodies are up-regulated (Bonneau et al., 2014). Thus, identification and use of serological markers could improve IBD diagnosis and optimize personalization of management plans.

Limitations of this Study

Type of Murine Colitis Model. The DSS model represents only one type of colitis mouse model and presents potential limitations. Because the mechanism behind

DSS-induced colitis is poorly understood, no potential systemic effects have been identified (Dieleman et al., 1994); thus, we cannot control for any underlying issues that might skew our study. Furthermore, chemically-induced colitis models receiving a drug or vehicle treatment could be experiencing drug interactions that researchers are unaware of. Ideally, a model of colitis would be generated in which BALB/C mice spontaneously develop intestinal inflammation as a result of genetic and environmental factors. However, the only known murine models that develop spontaneous colitis are the SAMP1/Yit or C3H/H3JBir models which were referenced in the introduction (Hoffman et al., 2002). Induction of spontaneous colitis in BALB/C mice would require mutations in IBD-associated genes similar to those observed in human IBD, the exact details of which would take time and extensive resources to determine.

Method of TACE Inhibition. TACE inhibition is a novel approach to TNFα blockade in inflammatory pathologies. Reducing TNFα upstream of membrane shedding prevents soluble TNFα production, thus effectively down-regulating the amount of TNFα that can activate TNFRs (Ruuls et al., 2001). However, TACE inhibition did not progress past phase II clinical trials due to liver toxicity concerns (Reviewed by DasGupta et al., 2008). It remains to be seen whether liver toxicity occurs in response to TACE inhibitor use in mice and if the effects of liver toxicity could affect our colitis model.

As mentioned earlier, we do not know of any drug interactions between DSS and DPC-333; if an interaction exists, this could also affect our study. Additionally, DPC-333 was solubilized in 25 mM citric acid (CA) saline; although our water control group accounted for any adverse effects to CA saline, we do not know what those effects might

be. Furthermore, administering DPC-333 through twice daily IP injection caused stress to the mice, as well as abdominal bruising. For this study, an ideal TACE inhibitor would be bioactive only in the target tissue (i.e. the colon) following oral administration, thus eliminating the pain and stress associated with needle injections.

Confirmation of Role for TNFα in Colitis Development. Blocking TNFα signaling by TACE inhibition is effective in preventing soluble TNFα production, thus reducing the amount of TNFα that could activate membrane-bound TNFRs on other inflammatory cells (Ruuls et al., 2001). However, TACE inhibition does not completely prevent TNFα signaling; it is possible for membrane-bound TNFα to interact with TNFRs on neighboring inflammatory cells that come into contact with the plasma membrane (Figure 33) (Campbell et al., 2003).

Generation of a TNFα knockout (KO) or a TNFR KO mouse, in which TNFα signaling is completely ablated, would allow for conclusive study of the role of TNFα in the BALB/C colitis model. Convincing evidence that TNFα is not involved in the development of colitis in BALB/C mice would be provided if TNFα is effectively knocked out in these mice yet colitis development still occurs. Ideally, a mouse would have to be generated in which TNFα knockout only occurs in the colonic epithelium and mucosal tissues. One major limitation to this approach is that infiltrating inflammatory cells would still produce TNFα; thus, ablation of TNFα signaling in leukocytes would be necessary. However, this systemic ablation of TNFα signaling would induce systemic side effects, such as immunosuppression and prolonged recovery from injury that could skew the study (Bruce et al., 1996). Developing a perfect model in which to study the role of TNFα in the development of colitis in BALB/C mice may be impossible. Further

study of current models is necessary to understand all of the interactions involved and ensure that future colitis studies can control for unwanted systemic and local side effects.

Overall Conclusions

Management and treatment of IBD proves difficult due to the complexity of the mechanisms involved in pathologic inflammation. In fact, it is possible that there are factors yet to be discovered. Currently, anti-TNFα therapies are the standard in IBD treatment as it has been established that TNFα drives inflammation in many inflammatory diseases; however, one-third of IBD patients do not respond to anti-TNFα therapies and many primary responders exhibit secondary loss of response (Papadakis et al., 2005). This study demonstrates the failure of BALB/C mice to respond to anti-TNFα therapy via TACE inhibition in a DSS-induced model of colitis, suggesting that forms of colitis exist in which TNFα is not the driving force for inflammation. This finding highlights the need for new approaches to IBD management in unique patient cohorts and supports the endeavors of current research to formulate personalized management plans for IBD patients.

Tables

Table 4. Two colitis models emphasize role for genetics in colitis development. During DSS administration, BALB/C mice develop an acute colitis characterized by the up-regulation of cytokines involved in a macrophage-mediated inflammatory response. Following DSS termination, BALB/C mice enter a recovery phase in which they are symptom-free within 2 weeks and completely recovered by day 28. In contrast, C57/BL6 mice progress to a severe chronic colitis following DSS termination characterized by the up-regulation of cytokines involved in T cell-mediated inflammation. This study concluded that genetic background plays a role in the development of colitis (Adapted from the American Journal of Physiology, Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57/BL6 mice but not in BALB/C mice: correlation between symptoms and inflammation, Melgar et al., 2005, with permission from the American Physiological Society).

C57/BL6		**BALB/C**
Severe chronic colitis	**After DSS removal**	Symptom free in 2 weeks
IL-1B, IL-12 p70, IL-17	**Up-regulated cytokines**	IL-1, IL-6, IL-18, G-CSF
T cells	**Inflammatory effector**	Macrophages

Figures

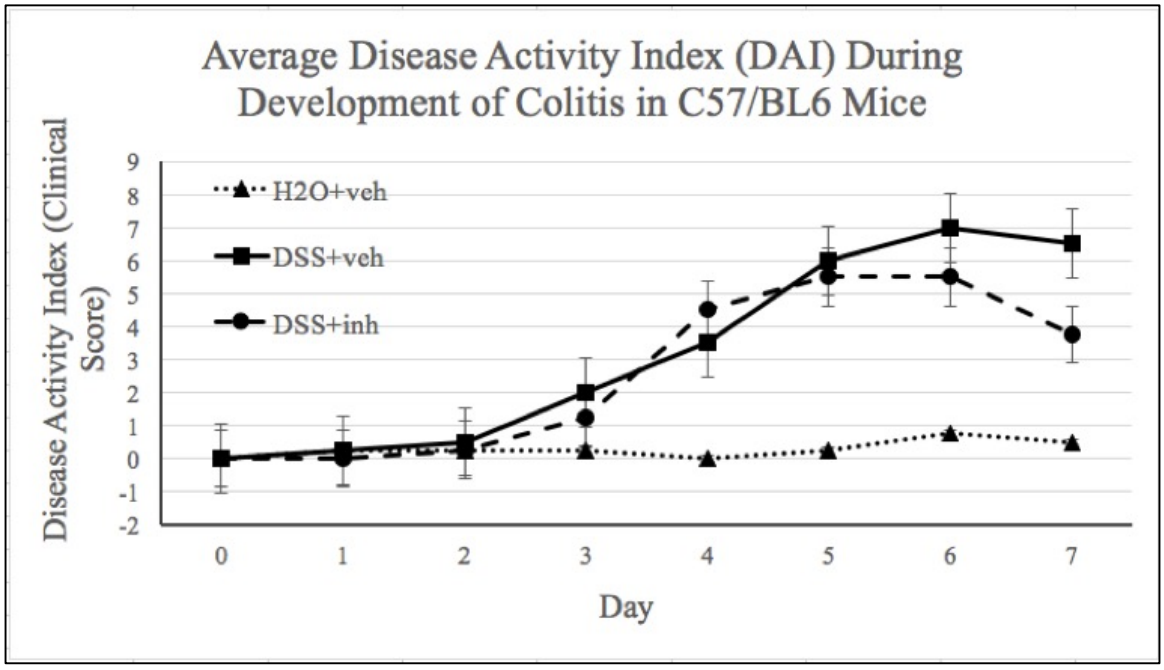

Figure 31. Average disease activity index (DAI) during development of colitis in C57/BL6 mice. DAI was quantified through scoring of clinical parameters, such as percent bodyweight loss, rectal bleeding, and stool consistency. Values are groups means ± SEM. DSS consumption significantly increased DAI (*$p < 0.05$; DSS + veh versus respective H2O + veh day). Although not statistically significant, there appears to be a trend for improvement in DAI following TACE inhibition ($p = 0.0758$).

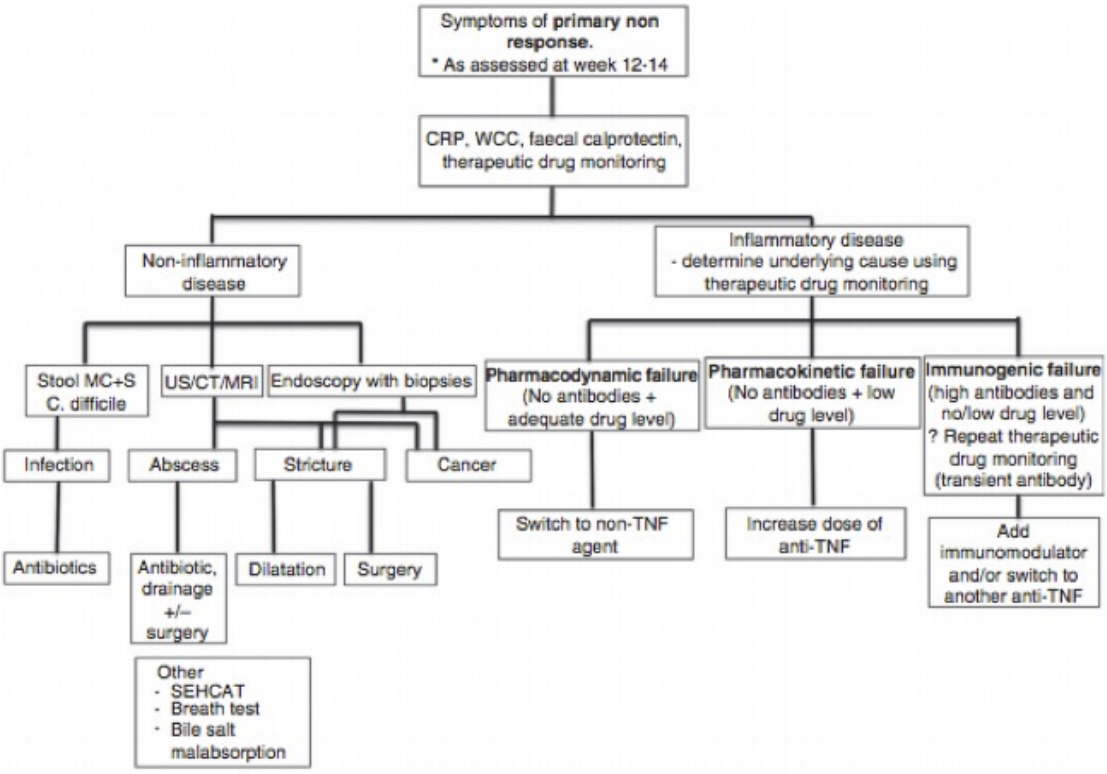

Figure 32. Proposed algorithm for managing primary nonresponse to anti-TNF therapy in Crohn's disease. These algorithms employ therapeutic drug monitoring to determine the underlying cause of nonresponse or loss of response to anti-TNF therapies. This strategy should allow clinicians to identify patients that are most likely to respond to anti-TNF treatments and optimize therapies for those who are losing response (Reproduced from Alimentary Pharmacology & Therapeutics, Systematic review: predicting and optimising response to anti-TNF therapy in Crohn's disease - algorithm for practical management, Ding et al., 2015, with permission from John Wiley and Sons).

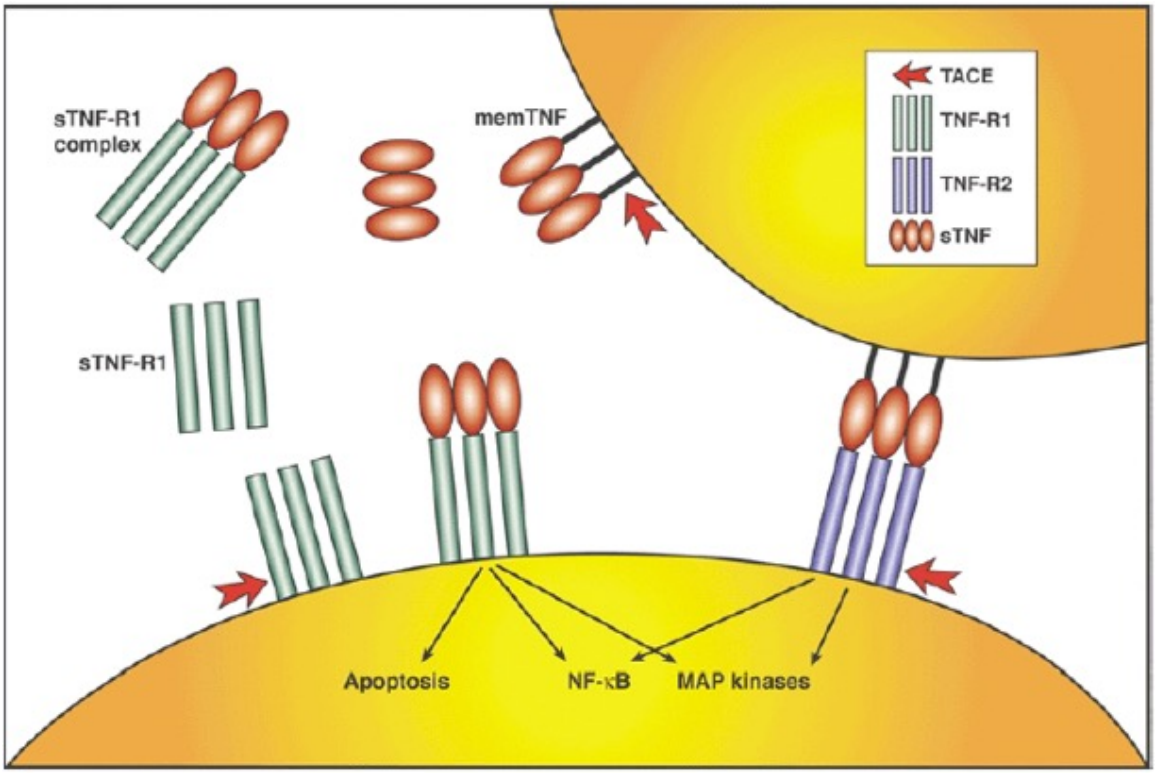

Figure 33. Membrane-bound TNF-alpha can interact with TNF receptors on neighboring cells. TNF-alpha signaling can occur through either soluble or membrane-bound TNF-alpha binding to membrane-bound receptors on other inflammatory cells (Reprinted by permission from Macmillan Publishers Ltd: Immunology and Cell Biology, Campbell et al., copyright 2003).

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APPENDIX A

Application to Use Live Vertebrate Animals

PI: Robert Tyler Morris
Dept: Biomedical Sciences
IACUC ID: 16-027.0 Web ID: 248

Title: Impact of TNF Converting Enzyme During Colitis Development in BALB/c and C57BL/6 Mice
Species: Mouse
Application Type: Continuation - 13-015.0
Multiple Species No
Total Animal Number: 40 (ORC,Non-ORC - Other Source:Charles River Laboratories & Jackson Laboratory)

Office Use Only

IACUC ID:	16-027.0-A
Renewal Date:	05/2019

Yes 4.1 REQUIRED - Check this box in order to access Section 4.1, Alternatives to Proposed Procedures. Failure to check this box may result in protocol review delays.

Submission History for Continuation:

04/06/2016 - Submitted
04/08/2016 - Under Review
04/22/2016 - Reopened
04/25/2016 - Revised
04/25/2016 - Under Review
05/03/2016 - Approved
05/03/2016 - Complete
05/02/2019 - Renewal Date

Approval Date: 5/03/2016

1. Personnel Information

Personnel		Roles	Techniques
Name:	**Stephanie Biel**	Student Investigator	Anesthesia - Administering Anesthesia - Monitoring Anesthesia - Vaporizer CO2 with Physical Euthanasia Handling and Restraint Instrument Prep Intraperitoneal Injection Scrubbing and Gloving Sexing Weighing and Measuring
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Campus Box:	901 S National Ave Springfield MO 65897-0027		
Phone:			
Email:	stephanie180@live.missouristate.edu		
Name:	**Robert Tyler Morris**	Email Contact Laboratory Coordinator Official Contact Principal Investigator	Adding and Removing Data Loggers Anesthesia - Administering Anesthesia - Monitoring Anesthesia - Vaporizer CO2 with Physical Euthanasia Handling and Restraint Identification - Ear Notch Instrument Prep Intraperitoneal Injection Scrubbing and Gloving Sexing Weighing and Measuring
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Name:	**Ellen Reusch**	Student Investigator	Anesthesia - Monitoring Anesthesia - Vaporizer Handling and Restraint Identification - Ear Notch Instrument Prep Intraperitoneal Injection Scrubbing and Gloving Sexing Weighing and Measuring
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Name:	**Fatima Alfadhul**	Student Investigator	Anesthesia - Monitoring Anesthesia - Vaporizer Handling and Restraint Identification - Ear Notch Intraperitoneal Injection Scrubbing and Gloving Sexing Weighing and Measuring
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Email:	fatima8@live.missouristate.edu		
Name:	**Hailee Marino**	Student Investigator	Anesthesia - Monitoring

Dept: -
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Anesthesia - Vaporizer
Handling and Restraint
Identification - Ear Notch
Instrument Prep
Intraperitoneal Injection
Scrubbing and Gloving
Sexing
Weighing and Measuring

2. Funding

Funding Source	Agency Deadline	Funding Period	Grant Number
National Institutes of Health			

3. Scientific Justification for Animal Species

1. Justify the species to be used by indicating:

This is a new model. (Veterinarians available for consultation on new model development.) **No**

The results will be directly applicable to the health, care or welfare of this species. **Yes**

Other Justification? **No**

If Yes, Explain:

The Morris lab has preliminary data that the BALB/c mouse strain is resistant to the beneficial effects of TNF-alpha blockade. However, literature suggests the C57BL/6 mouse is responsive to this treatment. A comparison between these pre-clinical models has not been established.

2. Features of the species (e.g. anatomic, physiologic, genetic, etc.) that make it desirable for this model.

The mouse serves as a relevant pre-clinical model to test the effects of TNF-alpha inhibition during disease states, such as acute inflammation. Many published research studies have used this model.

3. Will the PI conduct the same experiment in multiple species? No

If Yes, Explain:

4. Reduction, Refinement, Replacement, and Animal Numbers

1. Reduction, Refinement, and Replacement

a. Replacing vertebrate animals

No Are there computer simulation, non-living, or in vitro alternatives to the proposed use of animals described in your application?

If Yes, Explain:

b. Refining experimental procedures to minimize pain or distress

Yes Did you consider the use of pain-relieving drugs, or procedures that avoid or minimize discomfort, distress and pain, and humane endpoints in the design of the experiment?

If No, Explain:

c. Reduction in the number of animals

Specify the methods used for reducing the number of animals that were considered in the design of the proposed experiments.

Yes Rational selection of group size (e.g., pilot studies to estimate variability, power analysis)

Yes Careful experimental design (e.g., appropriate choice of control groups)

Yes Maximize use of animals (e.g., selecting the minimal number of animals per group required for statistical verification, sharing tissues with other investigators)

Yes Minimize the loss of animals (e.g., good post-operative care, avoidance of unintended breeding)

For any of the above items not checked, please provide a brief comment about why the option is not appropriate.

2. Using the specifics of your experimental plan, justify the number of animals requested for each pain category (B, C, D, E).

The investigator has proposed to use 40 male mice: 20 BALB/c and 20 C57BL/6. The pain category is C and D.

BALB/c study n=20 total

1) Control group- 7 days of drinking water with vehicle (citric acid saline) intraperitoneal injections 2x daily (n=6)

2) Colitis group- 7 days of Dextran Sodium Sulfate (DSS 5 percent- Colitis) in drinking water with vehicle (citric acid saline) intraperitoneal injections 2x daily (n=7)

3) Colitis group with TNF blockade (DPC-333)- 7 days of Dextran Sodium Sulfate in drinking water (DSS 5 percent- Colitis) plus DPC-333 (10mg/kg) intraperitoneal injections 2x daily (n=7)

C57BL/6 study n=20 total

1) Control group- 7 days of drinking water with vehicle (citric acid saline) intraperitoneal injections 2x daily (n=6)

2) Colitis group- 7 days of Dextran Sodium Sulfate (DSS 5 percent- Colitis) in drinking water with vehicle (citric acid saline) intraperitoneal injections 2x daily (n=7)

3) Colitis group with TNF blockade (DPC-333)- 7 days of Dextran Sodium Sulfate in drinking water (DSS 5 percent- Colitis) plus DPC-333 (10mg/kg) intraperitoneal injections 2x daily (n=7)

3. Estimate the following animal number totals required for this study during the three-year approval period.

Pain Category	Animals
B	0
C	12
D	28
E	0
	Total = 40

Justification for Category E:

4. Transfer of Existing Animals: Yes

If Yes, Indicate the IACUC ID: **13-015.0**

4.1 Alternatives to Proposed Procedures

1. Details about the search for alternatives

a. Names of searched databases and the date when the search was conducted:

No Agricola:
No AWIC:
No Biosis:
No Cab Abstracts:
No Crisis:
No Embase:
Yes Pub Med: **04/06/16**
No Medline:
No NTIS:

No Psychlit:
No Scisearch:
No Toxline:
No Pascal:
No Other:
If Yes, Explain:

b. Keywords used in the database searches:

Keywords

- **colitis**
- **TNF converting enzyme**

Summary of Literature Searched

With the exception of the following study, no other publications have begun to evaluate TNF converting enzyme during colitis in the mouse. No study has evaluated sensitivity vs resistance toward TNF converting enzyme inhibition in the mouse as a pre-clinical model.

Involvement of TACE in colon inflammation: a novel mechanism of regulation via SIRT-1 activation, Cytokine. 2014 Pubmed ID: 24548422

c. Years Searched:

1960-2016

d. Resources used in addition to the computer database search:

Information Services and other Literature Sources:

No Animal Welfare Information Center
No Lab Animal Welfare Bibliography (NLM)
No Laboratory Animal Science Journal
No Alternatives to Laboratory Animals Journal (FRAME, U.K.)
No Quick Bibliography Series (AGRICOLA)
No Peer Review
If Yes, Explain:
No Other
If Yes, Explain:

Other Methods or Sources Used:

No Direct contact with investigators in field
If Yes, Explain:
No Consultation with expert in the area of investigation
If Yes, Explain:
No Other methods or sources
If Yes, Explain:

5. Details of Animal Use:

1. **Goals and objectives of your research**

Inflammatory Bowel Disease (IBD), such as Crohn's Disease or Ulcerative Colitis, is characterized by a lifelong, relapsing condition that disrupts normal digestive outcomes. It affects ~1.4 million people in the U.S. with clinical symptoms including abdominal pain, rectal bleeding, diarrhea, and weight loss. The pathophysiology of IBD is considered a multifactorial interaction between genetic, environmental, and immunologic factors leading to inflammation of the intestines. However, the specific inflammatory events and interactions promoting poor function in this patient population are still unclear. A growing body of evidence suggests the synthesis of pro- and anti-inflammatory signals responding to the microbiota (i.e. host bacterial community) of the digestive tract regulate the interface between health and disease. Tumor Necrosis Factor-α (TNF-α) is a crucial pro-inflammatory cytokine that regulates the underlying immune response during many inflammatory diseases,

including IBD. Anti-TNF-α treatments, such as infliximab (TNF antibody) and etanercept (soluble TNF receptor), have been tested in some clinical trials of IBD. However, the effectiveness of such therapeutics was less than optimal and some patients either lose responsiveness over time or are resistant to TNF inhibition. Thus, other alternative treatments like TNF-α converting enzyme (TACE) inhibition could have clinical benefit in IBD. TACE is a central TNF-α cleaving enzyme and serves as a novel target for reducing the over production of TNF-α during IBD. The investigator hypothesizes that treatment of mice with TACE inhibitors (DPC-333) will reduce the local, inflammatory response in the intestines of C57BL/6, but not BALB/c. If the hypothesis is accepted, BALB/c mice may represent a pre-clinical model to help improve therapeutics in patients who do not respond to TNF inhibition.

2. If this application is a continuation of an ongoing project, state concisely how these goals differ from those in the original application and what was accomplished during the prior approval period. If this is a new project, please indicate so.

The previous IACUC protocol did not attempt to compare sensitivity or resistance toward TNF inhibition in different mouse strains. Also, a tissue culture method will be added to improve detection of TNF production (ex-vivo).

Note: the previous protocol did perform some studies in the transgenic NF-KB luciferase mouse on a BALB/c background strain. However, most of the work done on the previous protocol was using wild type BALB/c strain. The continuation of this protocol will attempt to determine differences in sensitivity towards TNF blockade in wild type BALB/c and wild type C57BL/6 mice.

3. Provide a concise overview of the experimental manipulations and treatments conducted on animals. This description should allow the IACUC reviewer to understand exactly what will be done to all animals from entry into the experiment to the endpoint of the study.

1)Dextran Sodium Sulfate in drinking water (Days 0-7) to induce colitis. Delivery via a 15 ml conical tube. This allows easy measurement of amount of water consumed.

2) 2x daily intraperitoneal injections of vehicle (citric acid saline) or TNF inhibitor (DPC-333)

3) Disease Activity Index (Days 0-7):

In response to DSS (colitis) treatment using either vehicle or TNF inhibition, a disease activity index will be measured. This index include body weight loss (scale of 1-4), stool consistency (scale 1-4), and blood in stool (scale 1-4). Each will recorded daily on a scale before and during colitis to monitor overall colitis phenotype of the mouse.

4) Euthanasia

On day 7 mice will be anesthetized by Isoflurane and the distal colon will be removed for analysis. Mice will then by euthanized by cervical dislocation while under Isoflurane anesthesia.

6. Animal Care

1. Animal ID Method:

Yes Ear Tag
No Ear Punch
No Microchip
No Not Applicable
No Tattoo
No Toe Clip
No Other

If Yes, Explain:

2. How will animals be monitored and maintained?

Animals will be kept in a Centrally Managed Missouri State University (MSU) Vivarium. They will be monitored daily by Vivarium staff and researchers and at least weekly by the Attending Veterinarian (AV). Husbandry practices will follow the MSU - Laboratory Animal Standard Operating Procedures book. Environmental conditions and husbandry include:

- **Light/dark cycle 12h/12h**
- **Temperature between 74 - 76°F**
- **Air changes between 10-15 per hour**
- **Humidity between 30-70%**
- **Food and water *ad libitum***
- **Animals housed 1/cage with cages changed at minimum once/week**

If special monitoring has been arranged with DLAM facility supervisor, provide DLAM contact name:

3. Should ORC contact the PI or the emergency contact if animals are found dead? Yes

4. Indicate requests for special handling of sick and dead animals.

Sick animals: AV and PI will be contacted and discuss possible treatment or euthanasia options.

Dead animals: AV and PI will be contacted and animals will be bagged to be placed in a refrigerator to be saved for necropsy (if needed).

5. Special Housing

Will any special housing or care be necessary? No

If Yes, describe and list any deviations from standard ORC husbandry procedures, Guide recommendations or special animal care needs.

6. Special Diets

Are special diets, additives to food and/or water, or antibiotics needed? Yes

If Yes, Describe and List Agents:

Administation of Dextran Sodium Sulfate (5%) in water for induction of colitis.

Anesthetic Agent:	**Dextran Sodium Sulfate**
Dosage:	**5%**
Route:	**Drinking water**
Schedule:	**7 days**
Volume:	**3-5 ml / day**

7. Describe endpoints (time points, tumor sizes etc.) and/or the maximum time length of study.

Day -2: Solo housed. Begin acclimation to 15ml conical tube delivery of drinking water
Day 0-7: Administration of Dextran Sodium Sulfate (5 percent) in drinking water for induction of colitis or control drinking water
Day 0-7: Provide 2x daily intraperitonal injection of TACE inhibitor or control saline
Day 7: Sacrifice time point (Colitis and Control)

8. Describe the criteria used to determine when an animal should be removed from the study prior its endpoint.

The investigator will monitor body weights throughout the study to ensure that a mouse with excessive disease (greater than 20 percent body weight loss) is identified. However, this is not anticipated based upon Wirtz S et al, Nature Protocols, 2007. If mice display greater than 20 percent body weight loss, they will be removed from the study and sacrificed.

If a mouse looks sick or unhealthy, the investigator or assistant will closely observe the mouse for fur ruffling, "crusty eyes", or inactive behavior. The Attending Vet will be notified of any adverse events and will be used for consultation throughout this study.

9. Will animals be euthanized as part of the study? Yes

If No, Describe the final disposition:

If Yes, Answer all of the following questions:

Euthanasia Methods

No **CO2-compressed carbon dioxide gas in cylinders and a physical method**

No **Barbiturate overdose**

If Yes, Specify Dosage and Route:

Yes **Overdose of Gas Anesthetic**

If Yes, Specify Agent:

Isoflurane

No **Anesthesia - followed by physical euthanasia**

If Yes, Specify Agent, Dosage, and Route:

If any of the above have been checked, indicate the physical methods that are used to ensure animals are dead:

Cervical Dislocation

No **Cervical Dislocation performed with no anesthesia**

If Yes, Justify:

No **Decapitation performed with no anesthesia**

If Yes, Justify:

No **Other Methods**

If Yes, Describe:

10. Would the PI be willing to make available extra animal tissues or organs to other PI's?

Yes

7. Anticipated Animal Pain & Distress

1. Are there any clinical, behavioral, or physiological manifestations expected to result from experimental manipulation?

Yes

If Yes, Answer all questions in this section.

a. Expected clinical and/or behavioral signs of pain and distress in animals:

Yes **Decreased weight**
Yes **Changes in food/water consumption**
Yes **Decreased ambulation**
No **Ruffled fur**
No **Skin abnormality**
No **Urinary problems**
Yes **Hunched posture**
No **Paw guarding**
No **Porphyrin Staining**
Yes **Lethargy**
Yes **Diarrhea**
No **Other**

If Yes, Explain:

b. Methods of dealing with the above complications:

No **Analgesics**
No **Anesthetics**
No **Sedation or tranquilization**
No **Increased bedding**
Yes **Other**

If Yes, Explain:

The amount of body weight loss is an important consideration for the overall health of the mouse. It is anticipated that the experimental mice may reduce food consumption and could lose ~2-4 g of body mass (or ~10 percent) of body mass. Some diarrhea and minimal dehydration is also an anticipated outcome of the colitis. If body mass lost exceeds 20 percent, the mouse will likely be excluded from the study and sacrificed due to the excessive consequences of the inflammatory stress. For mice experiencing 10 percent loss of body mass, the investigator will not provide intervention as this is a potential outcome of the acute colitis model.

Agents used in dealing with complications:

2. Animals experiencing unrelieved pain or distress prior to the endpoint, as defined by institutional policy, must be humanely euthanized, unless an exception to policy is requested and approved. Is exception required?

No

If Yes, Answer all questions in this section.

a. Criteria for euthanasia that will be used in this exception:

b. Scientific justification for not using an earlier endpoint:

12. Items not covered in other parts of the application

Potential Hazardous Materials used:

- Isoflurane
- Acetone
- Ethanol
- Dextran sodium sulfate (DSS)
- 10 percent formalin
- Hematoxylin and Eosin