Summer 2015

Tumor Necrosis Factor Alpha Converting Enzyme Inhibition during Acute Colitis in Mice: A Regional Analysis

Brian Maddox

TUMOR NECROSIS FACTOR ALPHA CONVERTING ENZYME INHIBITION DURING ACUTE COLITIS IN MICE: A REGIONAL ANALYSIS

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Cell and Molecular Biology

By

Brian Maddox

July 2015

TUMOR NECROSIS FACTOR ALPHA CONVERTING ENZYME INHIBITION DURING ACUTE COLITIS IN MICE: A REGIONAL ANALYSIS

Biomedical Sciences

Missouri State University, July 2015

Master of Science

Brian Maddox

ABSTRACT

Tumor Necrosis Factor-α Converting Enzyme (TACE) induces active TNFα and may contribute to the development of colitis in humans. I hypothesized that pharmacological blockade of TNFα production would improve colitis scoring through decreased expression of inflammatory biomarkers. Acute colitis was induced in wild type BALB/c mice using 5% dextran sulfate sodium (DSS) in drinking water for 7 days. TACE inhibition was accomplished through twice daily intraperitoneal injection of DPC-333 (10mg/kg; *BSM Inc*.) To determine the effects of TACE blockade during colitis, the following experimental groups (n=6-7/group) were tested: 1) vehicle; 2) DPC-333; 3) 5% DSS and vehicle; and 4) 5% DSS and DPC-333. Twice daily TACE inhibition did not significantly improve overall colitis scoring index as determined by the presence of diarrhea, rectal bleeding, and percent weight loss. TNFα, IL-6, Il-1β, IL-10, and MPO in regional colon tissue were not reduced following DPC-333 in this model ($p<0.05$). Thus, TACE inhibition does not appear to reduce colitis scoring, TNFα production, or other regional biomarkers of inflammation in this model of experimental colitis. Future studies may ascertain the role of TACE during systemic inflammatory diseases.

KEYWORDS: TNFα converting enzyme, colitis, inflammation

This abstract is approved as to form and content

_____

Tyler Morris
Chairperson, Advisory Committee
Missouri State University

TUMOR NECROSIS FACTOR ALPHA CONVERTING ENZYME INHIBITION DURING ACUTE COLITIS IN MICE: A REGIONAL ANALYSIS

By

Brian Maddox

A Masters Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Cell and Molecular Biology

July 2015

Approved:

_____
Tyler Morris, PhD

_____
Paul Durham, PhD

_____
Jianjie Wang, MD, PhD

_____
Julie Masterson, PhD: Dean, Graduate College

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Morris, for insight, encouragement, and understanding throughout completion of this project and writing of this thesis. I would also like to thank my committee members, Dr. Paul Durham and Dr. Jainjie Wang for their expert advice and input on the project and thesis. In addition, I would like to thank my project collaborator Tricia Haynes. Our laboratory undergraduates Zach Harter, James Norris, and Kate Sanders have been an indispensable addition to the project, making an overwhelming workload manageable. This project was made possible through funding from Missouri State University, by a Faculty Research Grant and thesis research funding from the Graduate College. Finally, I would like to thank my friends and family; their personal support has truly made this work possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Clinical Inflammatory Bowel Disease

Overview of Inflammatory Bowel Disease. Inflammatory bowel diseases (IBDs) are chronic conditions characterized by relapsing events of aberrant inflammation in the gastrointestinal tract and gut-associated organs. The two most prevalent IBDs are Crohn's Disease (CD) and Ulcerative Colitis (UC). As reported by the Centers for Disease Control and Prevention, CD and UC occur in 201 and 238 adults per 100,000 in the United States (Kappelman et al., 2007). In 2008, the mean cost of IBD treatment was reported as $8265 per year for CD patients and $5066 per for UC patients, and IBD-associated treatment costs in the United States were estimated at 6.3 billion dollars (Kappelman et al., 2008).

Current pharmaceutical treatments of IBD are limited by efficacy and cost, and colectomy has been reported to occur in 13.0-16.5% of UC patents at 20 years after diagnosis (Targownik et al., 2012). Surgical management of UC often results in required use of an ileostomy bag, and increases risk for complications, which include sepsis, fistula formation, rectal cancer, intra-abdominal bleeding, and death (Tulchinsky et al., 2003). Severity of UC inflammation is a predictor of neoplasm formation in the colon, and reduction of inflammation may improve oncogenic effects of the condition (Rutter et al., 2004). Ongoing research characterizing inflammatory signaling in animal models and identifying novel therapeutic targets is necessary to improve UC patient outcome.

Signs and Symptoms of IBD. CD and UC present with some similar signs and symptoms, including abdominal pain and cramping, diarrhea, gastrointestinal (GI)

bleeding, and weight loss. Differences between CD and UC include location of lesions along the GI tract and their depth within the intestinal wall. Sites of inflammation in CD can occur at any location along the GI tract, most often at the terminal ileum and proximal colon (Gore et al., 1996). Aphthoid ulcers are indicative of CD, and present in small, discrete formations, often penetrating transmurally across the intestinal wall (Simpkins, 1977).

In contrast, ulceration in UC tends to be more diffuse than CD, with more continuous patches of inflammation effecting the mucosa of the rectum and colon (Gore et al., 1996). Although not universal, UC generally begins from the rectum and progresses to more proximal regions of the colon, in a diffuse and continuous pattern (D'Haens et al., 1997). Compared to CD, UC presents discontinuous lesions less often, but these skip-lesions are routinely associated with the appendiceal orifice (D'Haens et al., 1997). Severe conditions of UC may result in ulcers with observable transmural inflammation.

Diagnosis of Clinical IBD. In addition to patient-reported symptoms, IBD disease activity assessment for diagnosis and disease monitoring is accomplished primarily through radiographic and endoscopic evaluation of colon mucosa. Endoscopy of the colon, or colonoscopy, accompanied with tissue biopsy allows for histological evaluation of the colon mucosa to be performed, an important step in differentiating IBD colitis from CD and other non-inflammatory colitis conditions such as toxic megacolon, infectious colitis, ischemic colitis, and radiation colitis (Leighton et al., Standards of Practice Committee, American Society for Gastrointestinal Endoscopy, 2006). Secondary diagnostic tools also improve diagnosis of inflammatory UC, such as laboratory

biomarkers found to correlate with leukocyte migration into the bowel lumen, including C-reactive protein and calprotectin (Schoepfer et al., 2013).

Following assessment of the patient and determination that an acute flare-up of inflammatory colitis is occurring, pharmacological management begins. Disease assessment dictates which pharmacologic approach to utilize, as primarily determined by two general parameters: anatomical location of UC inflammation and assessment of inflammatory severity. In 2010, the Practice Parameters Committee of the American College of Gastroenterology (ACG) released UC practice guidelines for diagnosis and treatment of adult UC (Kornbluth et al., 2010). The guidelines establish classification of UC severity: mild, moderate, or severe. These classifications were determined using Truelove-Witts disease activity scoring parameters, first established in 1954 for evaluation of corticosteroid treatment of UC (Truelove and Witts, 1954). According to ACG, UC disease severity can be determined according to stool frequency, presence of blood in the stool, erythrocyte sedimentation rate, and signs of toxicity such as fever, tachycardia, or anemia. Progression of UC beyond the distal colon (i.e. more proximal than the descending colon) increases likelihood of steroid-resistance and worsened prognosis, including increased rates of surgery and neoplasia (Etchevers et al., 2009). Therefore, some conditions of UC are categorized as "distal colitis" or "left-sided colitis."

Current Pharmacological Treatments of Ulcerative Colitis. Current medical treatments for UC attempt to avoid surgical intervention by reducing inflammation at the primary site of tissue injury. Multiple pharmaceutical approaches are available for clinicians and patients, and specific benefits and obstacles dictate their administration on

a patient-by-patient basis. These options are represented by the drug families of aminosalicylates, corticosteroids, thiopurines, antibiotics, cyclosporine (CsA), and anti-TNFα treatment.

Mesalamine belongs to the aminosalicylate family, and is also referred to as mesalazine or 5-aminosalicylic acid (5-ASA). Oral administration of sulfasalazine, a prodrug for mesalamine, allows for targeted delivery of mesalamine to the large intestine (Azad Khan et al., 1977). Sulfasalazine reaches the colon and is reduced to 5-ASA, at least in part by intestinal bacteria (Peppercorn and Goldman, 1972) Other formulations of pro-mesalamine have been developed in order to reduce side effects. Safety of the various aminosalicylate pharmaceuticals is currently being debated in the literature, with concern of nephrotoxicity and GI side effects such as abdominal pain, nausea, and diarrhea (Böhm and Kruis, 2014).

Corticosteroids can be administered either orally or topically, by enema or foam. Corticosteroid treatment is primarily used to control acute colitis. Although systemic corticosteroids are efficacious, some patients respond to treatment with steroid dependency, regarded as "medical treatment failure," and surgical management will often occur (Munkholm et al., 1994). Adverse effects of corticosteroids are substantial, and include cosmetic effects such as weight gain, cataracts, osteoporosis, myopathy, and susceptibility to infections (Lichtenstein et al., 2006). These side effects limit the duration of their use, and steroid dependent colitis remission is considered treatment failure (Benchimol et al., 2008).

Mercaptopurine (6-MP) is a thiopurine used in UC treatment. Azathioprine, a 6-MP prodrug, is effective in inducing remission for steroid dependent UC patients

(Ardizzone et al., 2006). The benefit of this treatment is primarily for steroid-dependent UC patients. The steroid sparing effect may result in subsequent avoidance of a surgical resolution. Despite the steroid sparing benefit, acute UC management is difficult with thiopurine treatment because of delayed time until optimal effect, which can be greater than four months (Present et al., 1980).

Cyclosporine (CsA) therapy was reported in 1994 as an effective non-surgical approach to treatment of corticosteroid-resistant UC (Lichtiger et al., 1994). CsA acts as an immunosuppressant by inhibiting calcineurin, a protein phosphatase. Calcineurin plays an integral role in T-cell development, and inhibition of calcineurin by CsA blocks T-cell function (Bram et al.,1993). CsA treatment carries serious adverse effects, and can damage the renal system and increase risk of malignancy (Grossman et al., 1996). Because of the adverse effects, CsA treatment is reserved for severe acute colitis, when medical urgency to reduce inflammation and prevent colectomy is apparent.

Tumor necrosis factor-α (TNFα) is a proinflammatory cytokine involved in inflammatory colitis. Infliximab is a monoclonal antibody administered via intravenous infusion, and is the current standard in anti-TNFα approaches to UC treatment. Anti-TNFα treatment is the most recent advancement in pharmaceutical approach to IBD management. In mild-moderate colitis, Infliximab is effective in resolving acute inflammation flare-up and improving remission time (Rutgeerts et al., 2005). Response to treatment may be hindered by development of antibodies against Infliximab by the patient, resulting in undetectable levels of Infliximab in the serum (Baert et al., 2003). Additionally, development of antibodies increases risk of reaction to Infliximab infusions.

Current Medical Guidelines of UC Treatment. For mild to moderate left-sided UC, the preferred treatment option is topical mesalamine administered by enema, although oral aminosalicylate or topical steroids are also effective (Cohen et al., 2000). While most patients respond to these treatments, oral corticosteroids or Infliximab may be required if the condition does not improve.

Extensive UC is a condition of inflammation progression proximal to the left colic flexure. For these patients, topical treatments such as enema, foams, and suppositories lose efficacy because of an inability to reach sites of injury (Kornbluth and Sachar, 2010). Oral sulfasalazine (or other mesalamine prodrug variations) have become the gold standard for extensive UC ever since its clinical studies were performed in the 1960s (Dick et al., 1964). Resistance to initial treatment in extensive UC is more common than left-sided UC. As a result, a variety of treatment options are practiced for patients who are unresponsive to sulfasalazine treatment or unable to tolerate the side effects. Oral administration of corticosteroids may be effective in these cases (Baron et al., 1962). Steroid approach may also be appropriate if there appears to be medical urgency in improvement (Kornbluth et al., 2010). If complete resolution of disease is not accomplished with corticosteroids, thiopurines may be administered. Following thiopurine treatment, Infliximab infusion may be necessary for steroid-refractory and steroid-dependent patients.

Acute severe UC also presents with increased incidence of steroid resistance and colectomy. Treatment guidelines for acute severe colitis dictate intravenous steroids such as methylprednisolone for 7 to 10 days. If the patient does not respond to steroid

treatment, rescue therapy with CsA or Infliximab occurs, and colectomy occurs with inadequate response (Fig. 1).

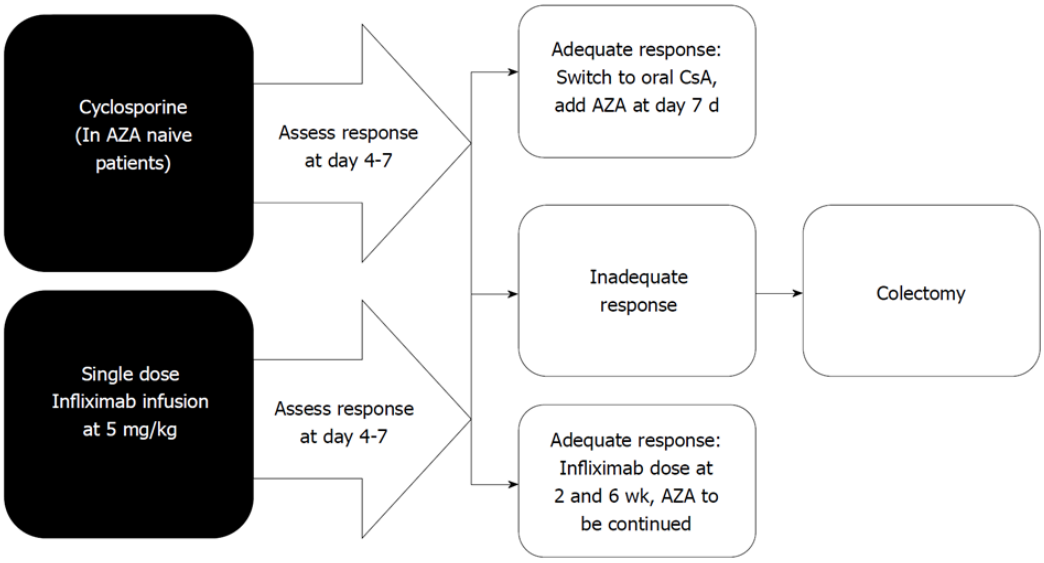

Figure 1. Methods of Medical Rescue Therapy for Severe Acute Ulcerative Colitis. Medical rescue therapies used in patients after failure of response to intravenous steroids include cyclosporine or inhibition of tumor necrosis factor with Infliximab. Inadequate response to rescue therapy is resolved with colectomy (Kedia et al., 2014).

Quick acting, long-term treatments aimed at increasing remission time and reducing flare-ups are necessary in treating inflammatory colitis. Steroid-sparing treatments, such as anti-TNFα treatments, may provide long-term solutions to IBD management. Current anti-TNFα treatments are primarily last-resort medical approaches to steroid and thiopurine resistant IBD patients, and patients not responding to these therapies will undergo colectomy (Nelson el al., 2014). Novel methods of TNFα inhibition in UC may increase patient response to treatment, decrease cost of manufacture, and decrease side effects. Through alternative methods of TNFα inhibition, a more universal treatment approach to inflammatory colitis may be possible.

Tumor Necrosis Factor-α and Inflammatory Signaling

Tumor Necrosis Factor-α. In 1975, while researching inhibitory effects of bacterial products on human cancer, Carswell et al. demonstrated endotoxin-induced necrosis of tumor cells required a host-derived mediator (Carswell et al., 1975). This factor was deemed tumor necrosis factor (TNF). TNF was originally observed in its soluble isoform, and in 1988, the membrane-bound precursor of TNF was observed (Kriegler et al., 1988). Pro-TNF is cleaved and activated by a metalloprotease, shedding the soluble ectodomain of TNF from the cell surface (McGeehan et al., 1994). The protein responsible for TNFα activation, TNFα converting enzyme (TACE), was isolated and identified in 1997 (Moss et al., 1997). Since its identification, TNFα has been implicated as a master regulator of inflammation, and novel methods of disease treatment through TNFα reduction are continuously being explored (Fig. 2).

The physiological role of TNFα and its ability to drive the inflammatory response is important for the body's defense against invading pathogens. As a result, current use of anti-TNFα approaches in treating pathological inflammation impart an increased risk of infection, particularly from latent infections such as with *M. tuberculosis* and opportunistic infections such as with *C. difficile* (Ali et al., 2013). Additionally, anti-TNFα treatment approaches are withheld during active infection by a variety of pathogens including bacterial, fungal, viral, and parasitic. TNFα driven inflammation produces a rigorous defense mechanism against pathogens, however, this protection comes at a cost. Pathological inflammatory conditions, such as during the development of sepsis, produce elevated levels of TNFα, IL-1β, and IL-6 and result in excess tissue damage and impairment of organ function in response to infection (Munoz et al., 1991).

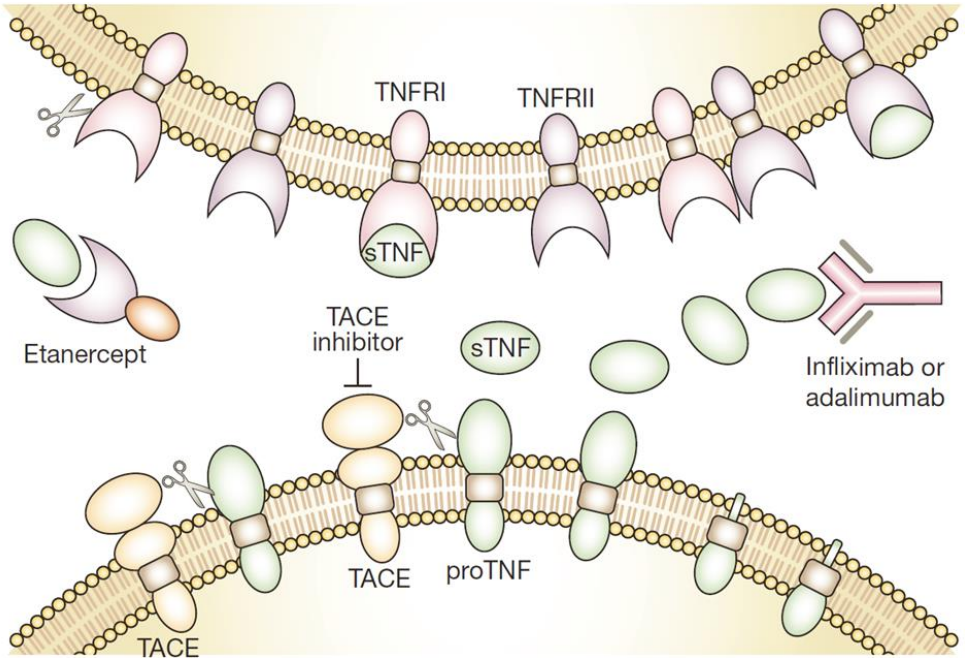

Figure 2. Activation and Inhibition of Tumor Necrosis Factor (TNF) and TNF receptor (TNFR). Membrane-bound proTNF is cleaved by TNF alpha converting enzyme (TACE) to release soluble TNF (sTNF). Current pharmaceuticals for inhibiting TNFR1/2 activation are monoclonal antibodies (Infliximab/adalimumab) and the fusion protein Etanercept. Reduction in sTNF through TACE inhibition and effects on inflammatory pathology is currently being investigated (Moss et al., 2008).

Other physiological roles of TNFα may not be primarily to initiate an inflammatory response. For example, acute vigorous exercise increases circulating levels of TNFα, without increasing expression of TNFα mRNA (Bernecker et al., 2013). In the central nervous system, TNFα is involved in a number of neurodegenerative and pathological conditions such as multiple sclerosis and Alzheimer's disease (Kruglov et al., 2011). However, TNFα is also promotes oligodendrocyte proliferation and re-myelination of neurons (Arnett et al., 2001). Additionally, TNFα is involved in maintaining neuronal cell survival and modulation of neuronal pathways during the proper development of the nervous system (Davies, 2003).

Physiological levels of circulating TNFα vary widely, and are impacted significantly with increased age. One study found circulating TNFα in healthy adults to range from < 1 pg/mL to 10 ng/mL (Mózes et al., 2011). Besides variation due to age, circulating TNFα levels may follow a circadian pattern, as TNFα stimulates the release of corticotropin-releasing hormone and activation of the hypothalamic-pituitary-adrenal axis and corticosterone secretion (Bernardini et al., 1990).

TNFα and Receptor Families: Structure and Biology. TNFα is a member of the TNF superfamily (TNFSF), the largest known family of cytokines, and TNF ligands activate corresponding transmembrane receptors of the TNF receptor superfamily (TNFRSF) superfamily. As of December 2011, 18 TNFSF and 29 TNFRSF genes have been identified in humans (Wiens and Glenney, 2011). The TNF homology domain is conserved across the TNFSF ligands, and interacts with cysteine-rich domains of the TNFRSF receptors (Bodmer et al., 2002).

In general, activation of TNFRSF members result in two opposing signal transduction pathways: cell death via apoptosis and necrosis or pro-inflammatory cell survival (Dempsey et al., 2003). Cell death signaling is induced by TNFR death domains, while cell survival and inflammation results from receptor interaction with TNF receptor associated factor (TRAF) proteins. The survival or death result of ligand binding is dependent on the presence of the death domain in the receptor and affinity of the receptor to intracellular signaling proteins promoting either cell death or inflammation (Dempsey et al., 2003). Two TNFRs have been identified for TNFα interaction and activation, TNFR1 (TNFRSF1A, CD120a, and p55) and TNFR 2 (TNFRSF1B, CD120b, and p75) (Brockhaus et al., 1990).

TNFα is initially expressed as a 26kDa homotrimeric type II transmembrane protein (mTNFα) (Kriegler et al., 1988). Upon cell stimulation, the extracellular domain of mTNFα is cleaved into a 17kDa soluble isoform (sTNFα) by TNFα converting enzyme (TACE), a disintegrin and metalloproteinase (ADAM) protein, specifically ADAM17 (Black et al., 1997). Both mTNFα and sTNFα are biologically active, and promote different signal transduction through TNFR1/2 activation (see below).

TNFα Receptors. TNFR1 and TNFR2 bind soluble and membrane-bound TNFα (Locksley et al., 2001). TNFR1/2 contain hydrophobic cysteine-rich repeats, which form a pre–ligand binding assembly domain (PLAD), resulting in trimerization of the receptors in the absence of TNFα activation (Chan et al., 2000). TNFR activity is not exclusively pro-inflammatory, as TNFR1 and TNFR2 extracellular domains are cleaved into soluble TNFR1/2, and act to attenuate the inflammatory response by sequestering sTNFα (Wallach et al., 1991). For example, mutations in the TNFR1 extracellular domain can prevent shedding and result in autosomal dominant periodic fever syndrome, characterized by relapsing episodes of localized inflammation and fever (McDermott et al., 1999). TNFR2 is primarily expressed in immune cells and TNFR1 expression is mostly ubiquitous across cell types. While TNFR1 is fully activated by sTNFα, TNFR2 is primarily activated by mTNFα and is not fully activated by sTNFα (Grell et al., 1995).

TNFR1 Signaling. TNFR1 activation is capable of activating either cell survival signaling or cell death. Survival and pro-inflammatory signal transduction occurs through activation of the transcription factor, nuclear factor kappa B (NF-κB) (Fig. 3). Production of TNFα and other inflammatory cytokines is activated by NF-κB. NF-κB is sequestered to the cytoplasm by inhibitor of kappa B protein (IκB) through masking of

NF-κB's nuclear localization signal. Inflammatory stimulation of the cell activates a IκB kinase (IKK) complex, which phosphorylates the regulatory domain of IκB (Mercurio et al., 1997). This phosphorylation marks IκB for recognition by E3 ubiquitin ligase and subsequent degradation. With its nuclear localization signal exposed, NF-κB translocates to the nucleus where it interacts with κB response elements.

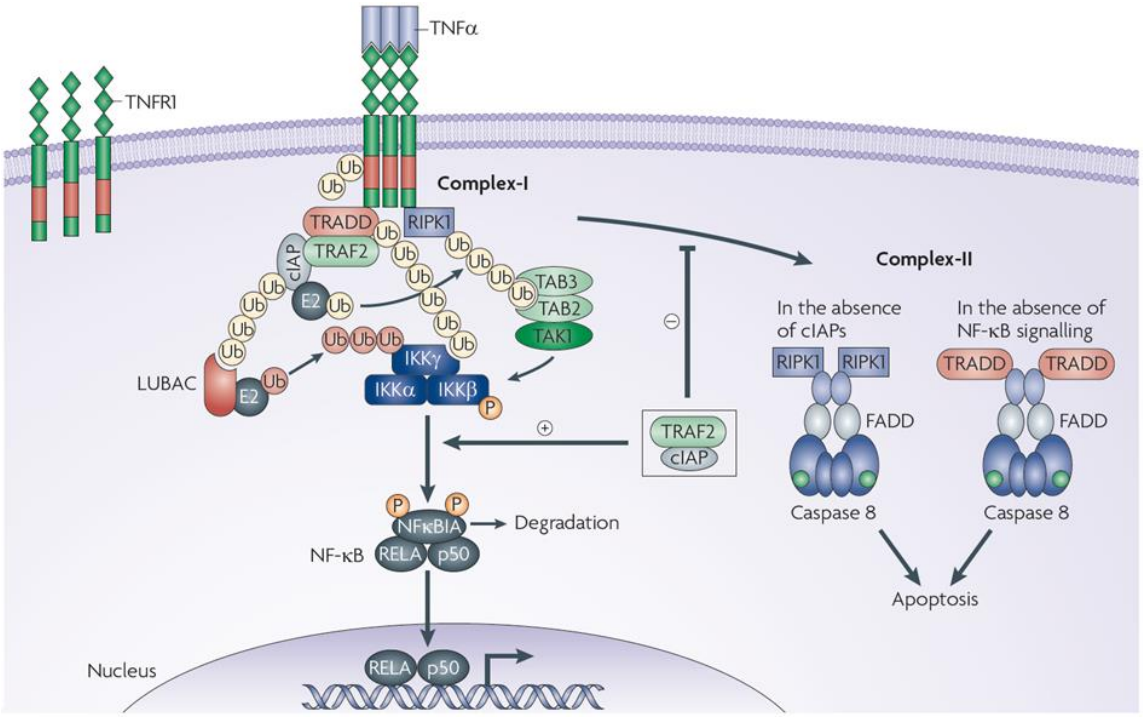

Figure 3. Tumor Necrosis Factor Receptor 1 Signaling. Proinflammatory signaling from TNF activation of TNFR1 results in translocation of Nuclear Factor-kB (NF-kB) to the nucleus. The receptor complex (complex-I) consists of TNFR-associated via death domain (TRADD), RIPK1, TNFR-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis 1 (cIAP1). Complex I activates linear ubiquitin chain assembly complex (LUBAC) and ubiquitin (Ub) chains are attached to IkB kinase (IKK) proteins. The activated IKK complex phosphorylates inhibitor of kappa B (IkB/NFkBIA), resulting in IkB degradation. Degradation of IkB exposes the nuclear localization signal contained in NF-kB, and translocation to the nucleus occurs. TNFR1 activation by TNF is also capable of initiating apoptosis, determined by the presence of pro-survival cIAPs and NF-kB transcriptional products (Gyrd-Hansen and Meier, 2010).

Signal transduction from TNFR1 is initiated by its intracellular death domain (DD), which shares critical amino acid residues with the Fas receptor (Tartaglia et al., 1993). The DDs of self-associated mTNFR1 complexes are capable of NF-κB activation, independent of ligand presence. However, a silencer of death domain (SODD) protein associates with TNFR1 and masks the DD, preventing recruitment of signaling proteins (Jiang et al., 1999). TNFα activation n of TNFR1 results in SODD dissociation and recruitment of TNFR1-associated death domain protein (TRADD) (Fig. 3) (Hsu et al., 1995). The TRADD-TNFR1 complex serves to scaffold the association of TNFR associated factors 1 and 2 (TRAF1/2) and receptor-interacting kinase (RIP), a serine-threonine kinase (Hsu et al., 1996). The TRAF protein family also interacts with pro-inflammatory receptors IL-1 receptor (IL-1R) and toll-like receptors (TLRs) (Wajant et al., 2001).

TNFR1 activation of IKK requires both TRAF2 and RIP. TRAF2 is essential in recruitment of IKK to the activated receptor complex, and RIP is essential in IKK activation (Devin et al., 2000). RIP mediated activation of NF-κB may also be indirect, as inhibition of the mitogen-activated protein kinase (MAPK) pathway attenuates NF-κB activity (Yang et al., 2001). TRAF also scaffolds the receptor to regulators of MAPK signaling pathway, therefore inducing cell survival signal transduction.

NF-κB activation requires receptor-associated TRAF1/2 recruitment of inhibitor of apoptosis protein (IAP) (Fig. 3) (Rothe et al., 1995). IAP proteins are essential for RIP ubiquitinaton (Varfolomeev et al., 2008) and RIP poly-ubiquitinaton at Lys-377 is required for IKK activation (Ea et al., 2006). A linear ubiquitin chain assembly complex (LUBAC) is responsible for attaching ubiquitin chains to RIP and also IKK (Haas et al.,

2009). IKK is recruited to the protein complex through interaction of the polyubiquitin chains and the IKK regulatory subunit, NF-kappa-B essential modulator (NEMO), also known as inhibitor of nuclear factor kappa-B kinase subunit gamma (IKK-γ) (Ea et al., 2006).

After recruitment to the receptor, IKK phosphorylates IκB. Phosphorylated IκB is ubiquitinated by beta-transducin repeat containing protein (βTrCP1), an E3 ubiquitin ligase (Fig. 3) (Kanarek et al., 2010). Ubiquitinated IκB is degraded through the ubiquitin-proteasome pathway. Degradation of IκB exposes the nuclear localization signal of NF-κB, allowing it to translocate to the nucleus.

Transcriptional activity of NF-κB promotes expression of anti-apoptotic genes, including TRAF1/2 and IAPs, which act to block caspase-8 activation and apoptosis initiation (Wang et al., 1998). Regulation of NF-κB transcriptional activity is achieved in part through autoregulation by NF-κB –inducible gene expression. For example, NF-κB activation increases protein A20 expression, an inhibitor of TRAF2 and NF-κB activation (Song et al., 1996).

TNFR2 Signaling. TNFR2 does not contain a death domain, and is fully activated by mTNFα (Grell et al., 1995). TNFα binding to TNFR2 recruits TRAF proteins to the receptor, and these TRAF proteins recruit IAP proteins to form a receptor complex similar to TNFR1 (Rothe et al., 1995). Similar to TNFR1, formation of the TNFR2 complex promotes cell survival and inflammation through activation of MAP kinase pathways and nuclear translocation of NF-κB (Marchetti et al., 2004). However, the TRAF proteins associated with TNFR2 may be capable of inducing two different NF-κB pathways: canonical (traditional) and noncanonical (alternative) (Rauert H. et al.,

2010) . Both pathways induce translocation of cytoplasmic NF-κB to the nucleus. However, the alternative pathway activates a NF-κB-inducing kinase (NIK) mediator to generate free cytoplasmic NF-κB from an inhibitory precursor (Fig. 4).

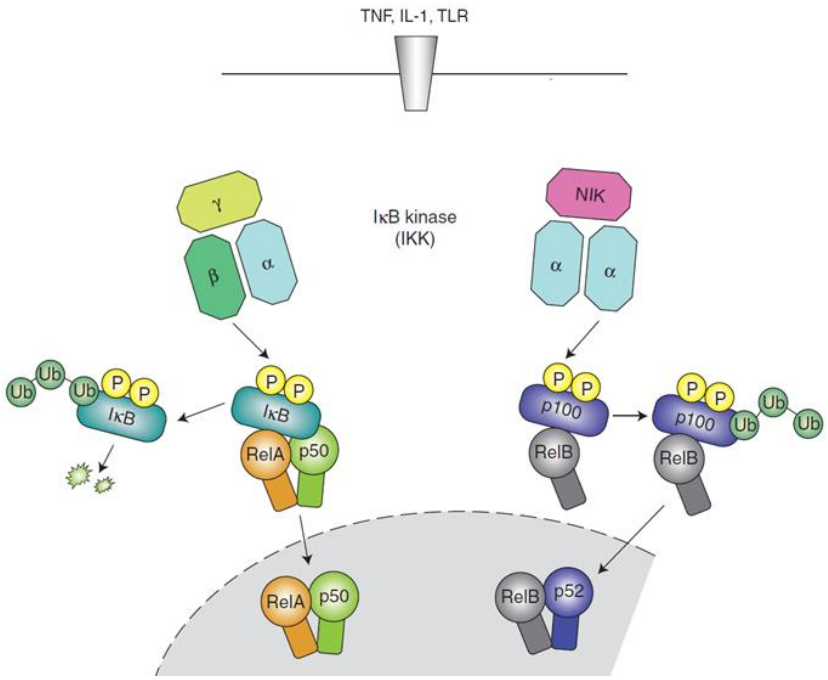

Figure 4. Canonical and Alternative NF-kB Activation Pathways. Activation of TNFR1/2 primarily results in NF-kB (RelA-p50) translocation to the nucleus through proteasomal degradation of Inhibitor of kB (IkB) and exposure of the nuclear localization signal within NF-kB. TNFR2 may also activate the alternative NF-kB pathway, through activation of NF-κB–inducing kinase (NIK) and processing of p100. Cleavage of p100 results in a p52 NF-kB subunit, which translocates to the nucleus (Lawrence, 2009).

The *nk-kb2* gene produces a larger p100 protein, which acts to sequester NF-κB to the cytosol, similar to the traditional IκB proteins. NIK action on the p100 protein results in ubiquitylation and proteasome-mediated cleavage of p100 into a p52 protein, a subunit of the NF-κB heterodimer (Coope et al., 2002). In this fashion the p100 protein acts as both an inhibitor of NF-κB activity and an NF-κB precursor within the alternative NF-κB pathway.

Signaling cascades initiated through TNFR2 activation are less characterized than its TNFR1 counterpart. For example, TNFR2 activates serine-threonine and tyrosine kinases. However, the resulting effects are still unknown. sTNFR2 is shed by TACE, and mutation of the catalytic domain in TACE results in decreased shedding of both sTNFα and sTNFR2 (Solomon et al., 1999). Importantly, sTNFR2 has been found to retain its biological activity and interact with sTNF, resulting in formation of inactive TNFα-TNFR2 complexes (Balcewicz-Sablinska et al., 1998).

The ability of sTNFR2 to sequester sTNFα has been utilized pharmacologically with the development of Etanercept, a fusion protein produced with the TNFR2 extracellular receptor-binding domain and immunoglobulin G regions (Fig. 2) (Peppel et al., 1991). Etanercept gained FDA approval in 1998 for treatment of rheumatoid arthritis (CDER, 2009). However, clinical trials of Etanercept in IBD have shown poor efficacy (Sandborn et al., 2001). Although Etanercept and Infliximab are both effective in reducing free TNFα, only Infliximab also interacts with and induces apoptosis of activated leukocytes (Van den Brande et al., 2003). In addition, administration of Etanercept in treatment of rheumatoid arthritis has been associated with inducing IBD, although this effect requires further investigation (Oh et al., 2005). Current applications of Etanercept administration are limited. In 2008 the FDA updated the package insert for Etanercept with a "black box" warning of the increased risk of infection associated with its administration. As a result of safety issues and lack of efficacy with Etanercept, the standard anti-TNFα approach to treatment of IBD is Infliximab.

Cell Survival or Death. TNFR1 activation results in formation of two different signaling complexes, complex I and complex II, also known as death-inducing signaling

complex (DISK) (Fig. 3) (Micheau and Tschopp, 2003). Complex I activates the NF-κB pathway, resulting in pro-inflammatory and cell-survival gene expression, and is comprised of TRADD, RIP, TRAF2, and c-IAP1 (Micheau and Tschopp, 2003). The second complex, complex II, initiates apoptosis via interactions of TNFR1's death domain and procaspases 8 and 10.

Complex II localizes within the cytosolic compartment of the cell, indicating dissociation from TNFR1. In contrast, complex I isolation experiments show it is associated with the cell membrane (Micheau and Tschopp, 2003). A balance between cell survival with pro-inflammatory cytokine expression or cell death is accomplished through these signaling systems and is determined by a complex system of input parameters, including cell type, location, and ligand activation of the cell. This system allows for spacial and temporal regulation of the cellular response to stress.

Molecular Roles in Inflammatory UC. NF-κB translocation to the nucleus results in transcription of proinflammatory cytokines IL-6 and IL-1β (Hiscott et al., 1993; Libermann and Baltimore, 1990). IBD pathology presents with increased IL-6 expression in the intestinal tissue, which promotes survival of inflammatory T cells and stimulates additional inflammatory signaling (Mudter and Neurath, 2007). IL-1β is also elevated in the plasma and colonic tissues of IBD patients (Ludwiczek et al., 2004). Additionally, NF-κB activation induces TNFα transcription (Collart et al., 1990). Inflammatory activation of NF-κB promotes the positive feedback nature of the inflammatory response through transcription of additional proinflammatory cytokines.

IL-10 is an anti-inflammatory cytokine, and mutations in the IL-10 receptor have been observed in patients with early-onset inflammatory enterocolitis (Glocker et al.,

2009). Expression of IL-10 decreases transcription of inflammatory cytokines, independent of NF-κB activation (Murray, 2005). IL-10 activates signal transducer and activator of transcription 3 (STAT3), which indirectly reduces cytokine transcription by promoting transcription of cytokine transcription regulators (Murray, 2005). IL-10 expression is also induced by NF-κB activation (Cao et al., 2006), although numerous other transcription factors also regulate IL-10 expression, such as cAMP-responsive-element-binding protein (CREB), musculoaponeurotic fibrosarcoma oncogene homolog (Maf), and c-jun (Saraiva and O'Garra, 2010).

Tumor Necrosis Factor-α Converting Enzyme

A Disintegrin and Metalloprotease Proteins. The zinc protease superfamily is a broad group of enzymes requiring zinc interaction for activation of the catalytic site. The member families are classified according to the primary sequence of the catalytic site, and include gluzincin, metzincin, inverzincin, carboxypeptidase, and DD-carboxypeptidase families (Hooper, 1994). Metzincins contain a methionine amino acid adjacent to the zinc active site in a "Met-turn" motif, and include serralysin, astacin, matrixin, and adamalysin subgroups (Stöcker et al., 1995). The adamalysins are proteins containing metalloprotease and disintegrin domains, and ADAM proteins (a disentigrin and metalloprotease) are a subtype of adamalysins (Fig. 5). There are 21 known ADAM genes in humans, 13 with functional protease activity (Edwards et al., 2008).

Localization of mature ADAM proteins to the membrane is achieved with the secretory pathway, from the rough endoplasmic reticulum, through the Golgi, and then incorporation into the plasma membrane (Lum et al., 1998). ADAM proteins are

expressed with a prodomain that inhibits the metalloprotease activity. A "cysteine-switch" maintains inactivity through interaction of a prodomain cysteine residue and the essential zinc ion in the catalytic site (Van Wart and Birkedal-Hansen, 1990). Removal of the inhibitory prodomain primarily occurs in the Golgi, by pro-protein convertase enzymes, resulting in predominately active forms of membrane-bound ADAM proteins (Lum et al., 1998).

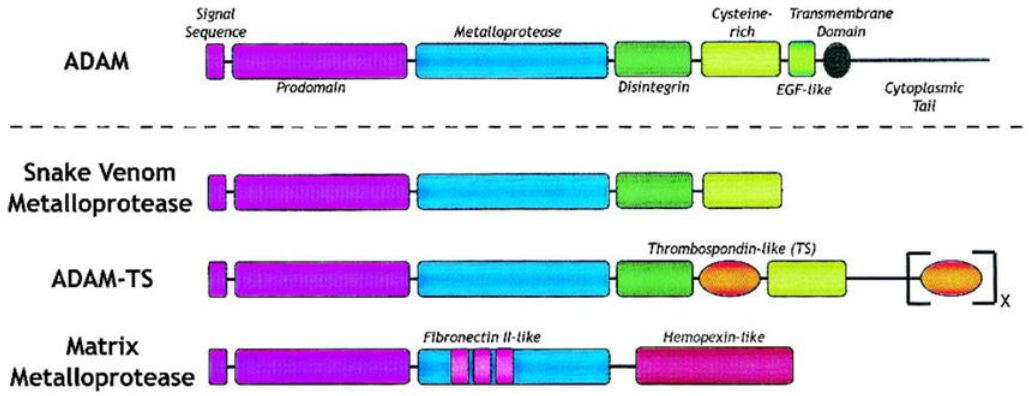

Figure 5. Functional Domains of A Disintegrin and Metalloprotease (ADAM) and Related Proteins. TACE (ADAM17) and the other ADAM proteins share domains with Matrix Metalloproteases (MMPs), Snake Venom-MMPs (SV-MMP), and Thrombospondin-like ADAM (ADAM-TS) proteins. ADAM proteins are directed to the plasma membrane with a signal sequence and anchored by a transmembrane domain. The prodomain regulates activation of the catalytic site within the metalloprotease domain. The disintegrin domain is known to interact with integrin receptors. The cysteine-rich and EGF-like domains may facilitate substrate interaction (Seals and Courtneidge, 2003).

The defining features of the ADAM proteins are its namesake metalloprotease and disintegrin domains. The metalloprotease domain contains the proteolytic active site. This site contains zinc atoms and water molecules essential for protein hydrolysis

(Maskos et al., 1998). Three histidine residues and the Met-turn coordinates the cofactors and substrate for cleavage.

The disintegrin domain is named after its activity in another adamalysin protein, snake venom metalloproteases (SVMPs), found in viper snake venom (Gutiérrez et al., 2005). These proteins block platelet aggregation and thrombus formation through interaction with platelet integrin receptors, resulting in hemorrhage (Huanget al., 1987). For human ADAM proteins, the type and function of integrin interaction is varied and poorly understood. Specific cell type and individual ADAM proteins determine integrin interaction and produce different cell adhesion and mobility effects (Cal et al., 2000; Nath et al., 2000; Zhang et al., 1998).

TACE Biology

The enzyme responsible for shedding soluble TNFα from the plasma membrane is ADAM17, commonly referred to as tumor necrosis factor-α converting enzyme (TACE) (McGeehan et al., 1994; Moss et al., 1997). The critical role of TACE in inflammatory signaling was highlighted in a study of mice with cleavage-defective mTNFα (Ruuls et al., 2001). These mice displayed attenuated inflammatory responses, similar to TNFα knockout mice, indicating that mTNFα shedding is essential to propagation of inflammatory signaling. Manipulating sTNFα levels through TACE inhibition allows for modulation of inflammatory response to stimulus, and TACE inhibition improves survival of mice against a lethal dose of the endotoxin lipopolysaccharide (Mohler et al., 1994).

In addition to its pivotal role during inflammation, the complete biological role of TACE is surprisingly complex. This was first observed in mice with non-functioning TACE, where TACE disruption limited the viability of most mice to the embryonic stage or to a few days of age (Peschon et al., 1998). Mice without TACE activity were born with stunted vibrissae (whiskers) and open eyelids (mice are usually born with closed eyelids), as a result of failed eyelid fusion. A small number of mice survived for several weeks. However, they displayed reduced bodyweight, disrupted eye development, and abnormal hair follicle structure and function. Defects in proper development of epithelial tissues and therefore digestive, respiratory, and hormonal systems were observed.

Peschon et al. also observed cells without functional TACE activity displayed reduced TNFR2, L-selectin, and transforming growth factor-α (TGFα). The phenotypes of the TACE-defective fetuses were similar to TGFα knockout mice (Sibilia and Wagner, 1995), thereby implicating TACE as a sheddase for TGFα and critical to embryologic development. Over thirty TACE substrates have since been identified, with involvement in development (TGFα), neuroprotective protein clearance (amyloid precursor protein), inflammation (TNFα, TNFR1/2, IL6R), and tumor development and proliferation (EGF, TGFα, CD44) (Pruessmeyer and Ludwig, 2009).

TACE Activity during Inflammatory Pathology. Aberrant TACE activity has been observed in a variety of inflammatory disorders. Rheumatoid arthritis patients have increased cartilage TACE mRNA levels (Patel et al., 1998), and chondrocyte expression (Amin, 1999). Peripheral monocyte expression of TACE is increased in patients with early systemic sclerosis (Bohgaki et al., 2005). Psoriasis patients experience increased TACE mRNA expression in keratinocytes, endothelium, and mast cells contained within

the psoriatic lesions (Kawaguchi et al., 2005). *Staphylococcus aureus* and *Pseudomonas aeruginosa* stimulate TACE transcription of lung epithelial cells (Gómez et al., 2005).

TACE is also involved in IBD pathologies. Cell membrane detergent extracts of UC patients display increased TNFα cleavage potential, indicating increased functional TACE capacity (Brynskov et al., 2002). Biopsies of inflammatory lesions of intestinal epithelia in CD patients showed increased TACE expression, during the early development of and active CD lesions (Cesaro et al., 2009). Regulation of TNFα levels through TACE inhibition may prove to be a viable method of disease treatment and maintenance of remission in IBD.

History of TACE Inhibition and DPC-333. A common model of septic endotoxemia utilizes lipopolysaccharide (LPS), a component of the cell wall in gram negative bacteria. After TACE inhibition was shown to protect mice against lethal LPS dosing, preclinical trials for TACE inhibition during inflammatory pathologies in animal models was explored (Mohler et al., 1994). Many of these early studies utilized intra-joint inflammatory models for rheumatoid arthritis, and later clinical trials in humans (Moss et al., 2008). Despite early success in preclinical models, the furthest progression of a TACE inhibitor to-date is phase II of clinical trials for treatment of rheumatoid arthritis, where issues with liver toxicity and/or lack of efficacy ended the trials.

There are four classes of ADAM metalloprotease inhibitors: denaturing inhibitors, Zn chelators, small molecule inhibitors, and tissue inhibitor of metalloproteinase proteins (TIMPS) (Moss et al., 2001). The TACE-specific inhibitor DPC-333 [(2R)-2-((3R)-3-Amino-3{4-[2-methyl-4-quinolinyl) methoxy] phenyl}-2-oxopyrrolidinyl)-N-hydroxy-4-methylpentanamide)], is a small molecule inhibitor invented in 2002 by Irma Benedek

and Michael Fossler and is owned by the Bristol-Myers Squibb Company (Fig. 6) (Benedek and Fossler, 2003). Small molecule inhibitors have traditionally exhibited poor specificity, and resulted in broad inhibition of all proteins with metalloprotease domains, and inhibition of additional metalloprotease enzymes may have contributed to increased adverse effects during previous clinical trials (Moss et al., 2001).

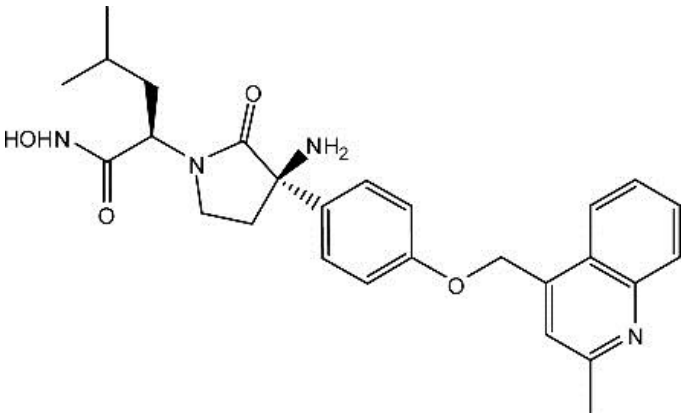

Figure 6. Chemical structure of DPC-333 (Qian et al., 2007)

DPC-333 is largely considered a partially selective inhibitor of TACE (Bahia and Silakari, 2010; Moss et al., 2008), reported by Bristol-Meyers Squib (BMS) to have a dissociation constant (Ki) of 0.18 nM to TACE, and Ki of 2, 10, and 12 nM for MMP-12, ADAMTS-4, and MMP-3 (product sheet - BMS). Previously, DPC-333 has been evaluated in clinical trial for rheumatoid arthritis treatment, reaching phase II before liver toxicity issues resulted in discontinuation of the trial (Moss et al., 2008).

Pharmacokinetics and pharmacodynamics data for DPC-333 in animals and humans were published in 2007 by Qian et al. Orally administered DPC-333 is biologically active, and results in decreased plasma TNFα levels in the blood of mice and

humans. The i.v. bolus dose of DPC-333 in mice had a half-life of approximately 1 hour. The mean systemic plasma clearance was 6.2 1/h/kg. Mice have low oral bioavailability of less than 20%, but 98% after IP injection of 11mg/kg bodyweight. The free fraction of DPC is 2 to 3 times greater in rodents than in humans. IP injection of DPC-333 in mice is rapidly absorbed and maximum plasma concentration is reached at 0.1 hours after injection. TNFα response to LPS challenge in mice is inhibited by IP DPC-333 in a dose-dependent manner, with ED50 of 1.9 mg/kg dose. In this clinical safety trial, DPC-333 was found to be safe and tolerated in humans. Adverse effects were reported in 53.3% of DPC-333 recipients (n=60) and 40% of the placebo group (n=20), with taste disturbance as the most commonly reported adverse experience.

Neutrophil Recruitment and Myeloperoxidase

A signature feature of the inflammatory response is chemotactic recruitment of polymorphonuclear leukocytes (neutrophils) and other leukocytes to the site of inflammatory stimulus (Ajuebor et al., 1999). Constitutive expression of L-selectin on the surface of leukocytes supports neutrophil rolling and resulting emigration through the vessel endothelium (Smith, 1993). Broad inhibition of metalloprotease activity and subsequent decreased L-selectin cleavage, has been observed to inhibit lymphocyte emigration across the capillary wall during inflammation (Faveeuw et al., 2001). TACE has been identified as a sheddase of L-selectin in-vivo, and TACE-deficient neutrophils and other leukocytes expressed increased L-selectin on the cell membrane (Li et al., 2006). TACE inhibition during colitis may disrupt neutrophil emigration to the primary site of inflammation in the colon, decreasing inflammation.

Neutrophils are phagocytic leukocytes and utilize mechanisms of oxidative stress to destroy engulfed pathogens. Azurophilic granules within the neutrophil store the enzyme myeloperoxidase (MPO). Pro-inflammatory signaling stimulates neutrophil degranulation and MPO activity (Schultz and Kaminker, 1962). MPO activity increases the oxidative potential of hydrogen peroxide by generating hypochlorous acid and tyrosyl radicals (Heinecke, 1999). Quantification of MPO levels in inflamed tissues and organs correlates to neutrophil recruitment and increased inflammatory conditions (Pulli et al., 2013).

A Colitis Mouse Model with Dextran Sulfate Sodium

Model Overview. A murine model of intestinal inflammation using the polymer dextran sulfate sodium (DSS) is an accepted method of investigating UC (Strober et al., 2002). Supplementing the animal's drinking water for 7 days results in erosion of the colonic mucosa, dysplasia, shortening of the large intestine, diarrhea, gross rectal bleeding, and weight loss (Okayasu et al., 1990). Although, the mechanism of DSS-induced inflammation is not completely understood, mucosal barrier degradation during DSS-induced colitis may result from direct toxicity to the intestinal epithelia (Dieleman et al., 1994). Severity of response to DSS is strain dependent, and C57BL/6 mice develop a more severe phenotype with less DSS than BALB/c mice (Melgar et al., 2005). The DSS colitis model is beneficial in studying inflammation due to rapid development of colitis, ease of administration, and dosing control (Wirtz et al., 2007). Inhibition of TNFα with intraperitoneal injection of Infliximab during the development of DSS colitis

reduces disease activity index, histology scoring of mucosal degradation, and intestinal epithelial apoptosis (Qiu et al., 2011)

The DSS induction of mucosal degradation occurs in the absence of T and B cell development (Dieleman et al., 1994). Therefore, DSS colitis may be a poor model of investigating aspects of inflammation involved with cell-mediated adaptive responses. However, induction of NF-κB activity and production of inflammatory cytokines allows the DSS model of colitis to be a good investigatory tool in evaluating the innate immune response during inflammation and colitis.

Cytokine and Neutrphil Response to DSS Colitis. Concentrations of inflammatory cytokines IL1β, IL-6, and TNFα increase in the colon during development of DSS colitis (Dieleman et al., 1994). After blood samples are allowed to clot for 30 minutes, the serum of DSS colitis mice also show increased pro-inflammatory cytokine concentrations (Alex et al., 2009). There is a larger increase of pro-inflammatory cytokines in the colon tissue of C57BL/6 than in BALB/c mice, corresponding to increased sensitivity of C57BL/6 mice to DSS (Melgar et al., 2005). For C57BL/6 mice, expression of IL1β, IL-6, and TNFα mRNA in the colon increases in response to DSS treatment (Yan et al., 2009). Increased TNFα mRNA predominately occurs in the proximal colon and IL-6 mRNA is more increased in the distal colon.

IL-10 emerged as a key suppressive mediator of intestinal inflammation when IL-10 knockout mice were found to spontaneously develop chronic colitis (Kühn et al., 1993). In contrast, overexpression of IL-10 in chemical colitis mouse models attenuated disease activity and reduced proinflammatory markers, such as IL-1β and TNFα (Lindsay et al., 2002). For DSS colitis models, high variability of IL-10 concentration has been

reported, dependent on strain type and duration of DSS treatment. Increased serum IL-10 concentration for chronic, but not acute DSS colitis in C57BL/6 mice has been reported (Alex et al., 2009). In C57BL/6 mice, colon tissue IL-10 mRNA expression increases with DSS administration until day 5, and expression may become consistent (Yan et al., 2009).

Acute DSS models also produces increased MPO activity in the colon tissues, indicating increased neutrophil recruitment to the primary site of injury (Alex et al., 2009). This increase may primarily occur in the proximal colon (Yan et al., 2009). An increase of MPO activity has been observed in the cecum and rectum of BALB/c mice after 7 days of 5% DSS treatment (Hamamoto et al., 1999).

Hypothesis and Rationale

My study will investigate the following hypotheses: 1) induction of acute colitis in BALB/c mice results in regional differences in colon inflammation, affecting analysis of the model; and 2) TACE inhibition will improve the disease state of DSS-induced colitis in mice. Colitis will be induced in BALB/c mice through consumption of 5% DSS for 7 days. TACE inhibition will be accomplished with twice daily intraperitoneal injections of the TACE inhibitor DPC-333. Effect of TACE inhibition will be performed through systemic and local analysis of the colon. Disease activity will be assessed with established parameters, including bodyweight loss, rectal bleeding, presence of diarrhea, and colon length. Concentrations of IL-1β, IL-6, IL-10, and TNFα cytokines will be quantified in whole unstimulated plasma. The local site of tissue injury in the colon will be evaluated through a regional analysis. Cytokines will be quantified in proximal,

middle, and distal colon tissues. MPO concentration will be quantified in the proximal and distal colons. Proximal, middle, and distal regions of the colon will be investigated to determine variation in cytokine concentration.

MATERIALS AND METHODS

Animals and Induction of Colitis

Animal care followed the Missouri State University guidelines for experimentation with animals and was approved on April 28, 2014 (ID# 13-015.0) by the Institutional Animal Care and Use Committee of Missouri State University. Acute colitis was induced in male BALB/c mice by supplementing drinking water 5% DSS weight/volume and allowing mice to drink *ad libitum* for 7 days. Administration of 5% DSS for 7 days is known to produce a model of severe acute inflammation in BALB/c mice (Kitajima et al., 2000). The 5% DSS water was replaced with fresh DSS solution every two days.

Dosing of DPC-333

DPC-333 was solubilized in 25 mM citric acid (CA) saline at 1 mg DPC-333 per 1 mL CA saline. In a previous study, DPC-333 was solubilized with CA saline (Kim et al., 2008). Qualitative solubility tests were performed to determine appropriate CA concentration. DPC-333 was administered at 10 mg DPC-333 per 1 kg bodyweight by intraperitoneal (IP) injections of 1mg/mL DPC-333. Pharmacokinetics and pharmacodynamics of DPC-333 were investigated by Qian et al., and 10 mg/kg bodyweight IP injection was found to effectively block TACE activity and production of sTNFα in vivo (Fig. 7) (Qian et al., 2007). Fresh DPC-333 CA saline was made every 4 days and stored at 4º C.

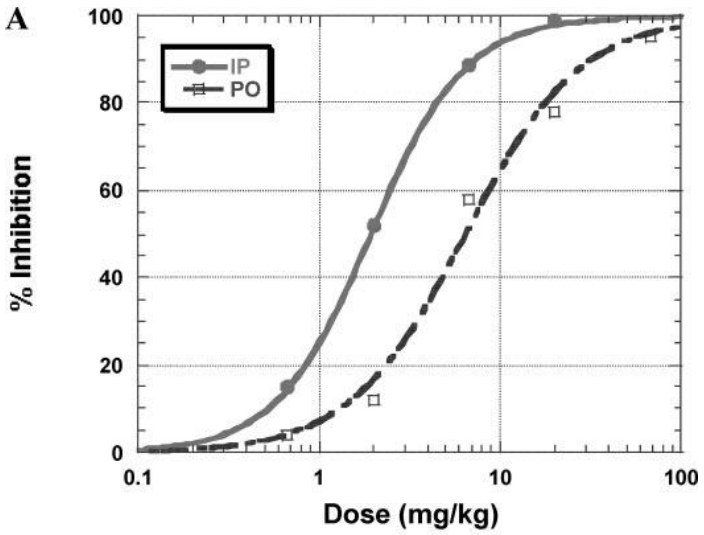

Figure 7. Dose-Dependent Inhibition of sTNFα Production In-Vivo. Dose-dependent TNFα inhibition with DPC-333 was determined with male BALB/c mice challenged with 10 µg/mouse LPS IP injection. Plasma sTNFα concentrations of IP (solid line) and oral (dotted line) doses of DPC-333 were compared to vehicle control to determine percent inhibition of sTNFα release (Qian et al., 2007).

Study Design

Two cohorts of 10 and 11 mice were used in this study. Two control groups and two disease groups were generated to account for the two independent variables of drinking water with or without DSS and injection of DPC-333 or vehicle. Therefore, the four groups generated included: 1) H_2O + vehicle, 2) H_2O + inhibitor, 3) DSS + vehicle, 4) DSS + inhibitor (Fig. 8). The mice received twice daily IP injections of 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 and animal sacrifice with isoflurane overdose and cervical dislocation. Food and water consumption were monitored and clinical scoring parameters of the disease activity index were evaluated daily.

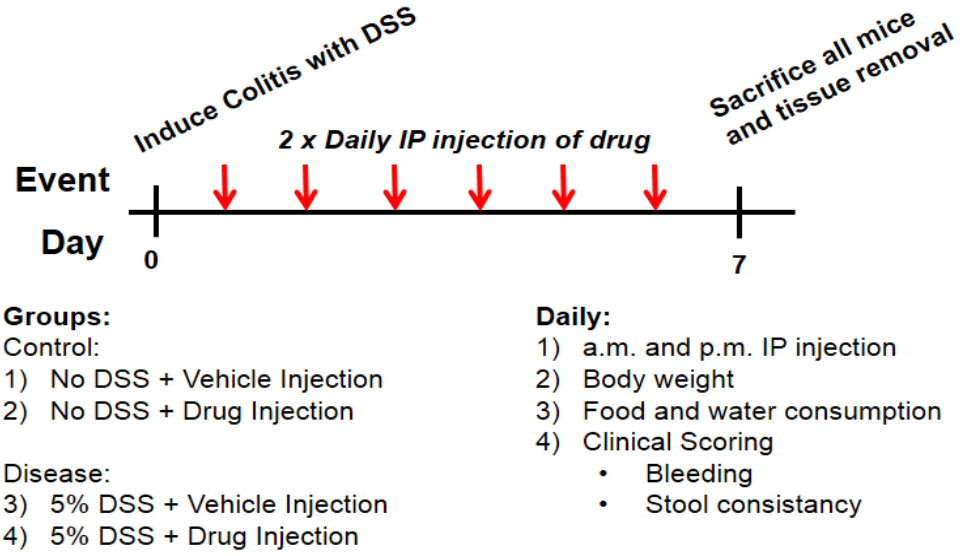

Figure 8. Study design. Two control groups and two disease groups were generated by consumption of water only or 5% DSS treated drinking water for seven days (days 0-7). One control group and one disease group received twice daily 1 mg/kg bodyweight injections of DPC-333 (groups 2 and 4) and the other received an equivalent volume of 25 mmol citric acid saline vehicle injections (groups 1 and 3). Bodyweight, food and water consumption, and clinical scoring were recorded daily.

Disease Activity Index (DAI) Methodology and Scoring

Animals were observed twice daily for body weight, water/food consumption, morbidity, stool consistency, and the presence of gross blood in feces and at the anus. DAI was calculated by assigning well-established and validated scores for parameters analogous to the clinical presentation of human IBD (Cooper et al., 1993).

The 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 (0 point = none, 1 point = 1–5% weight loss, 2 points = 5–10% weight loss, 3 points = 10–15% weight loss and 4 points- more than 15% weight loss, , -1 point = 1–5% weight gain, -2 points = 5–10%), stool consistency/diarrhea (0 points = normal, 2 points

= loose stools, 4 points = watery diarrhea), and bleeding (0 points = no bleeding, 2 = slight bleeding, 4 points = gross bleeding).

Table 1. Clinical Scoring Parameters and Scoring Guide of Disease Activity Index Determination. Disease Activity Index (DAI) was calculated as a sum of clinical scoring parameters including percent bodyweight change, stool consistency, and bleeding.

	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

Blood and Tissue Sample Collection

Cardiac blood samples and colon specimens were collected from anesthetized mice under isoflurane. Tissue samples were snap frozen in liquid nitrogen and stored at -80^{o}C. Blood samples were obtained via heart stick with heparinized syringe. Plasma was

separated by centrifugation at 4°C at 16.2 relative centrifugal force (RCF) for 10 minutes and stored at -80°C. Prior to freezing, colon samples were cleared of fecal matter with ice-cold saline and dissected to generate separate segments for regional analysis of cytokine and MPO concentrations (Fig. 9).

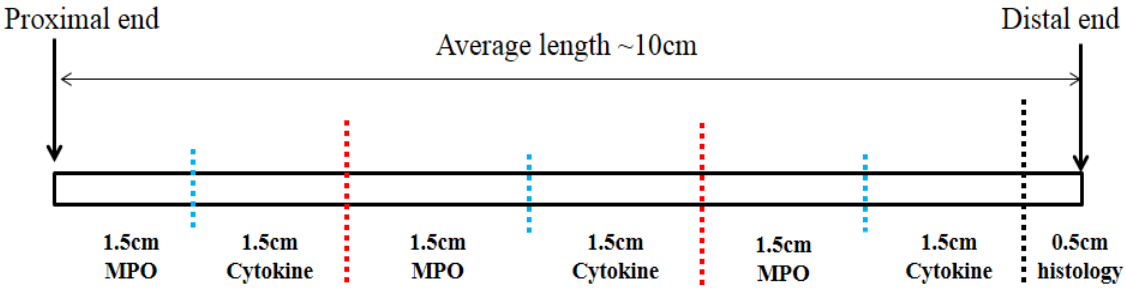

Figure 9. Colon Dissection Map and Regional Analysis. The large intestine was removed under isoflurane anesthesia and sectioned into proximal, middle, and distal regions. Each region was further divided into tissue samples for either MPO or cytokine analysis.

Tissue Homogenization

Tissue samples for both MPO and cytokine analysis were homogenized following suggested protocol supplied by Millipore (cytokine quantification kit) and within Hycult guidelines (MPO quantification kit). While kept frozen atop ceramic cooled with liquid nitrogen, colon tissue samples were massed and broken into approximately 50 mg pieces. Lysis buffer cocktail was added to each sample, at 20 µL cocktail per 1 mg tissue (e.g. 50 mg tissue/1000µL cocktail). The lysis buffer cocktail included Tris-lysis buffer with 1:100 protease inhibitor cocktail (Sigma-Aldrich P8340). Homogenization of tissue samples was performed with surgical scissors, and introduction of air bubbles was avoided. Samples were centrifuged at 16.2 RCF for 10 minutes at 4°C and supernatant removed. Analysis of MPO and cytokine portions of colon tissue, aliquots of sample

were made to provide sample for assay and protein quantification without unnecessary freeze-thaw cycles. All tissue samples underwent 3 cycles of freeze-thaw: 1) whole tissue removal from animal; 2) homogenization; and 3) analysis (total protein, cytokine, or MPO quantification).

Pierce Bicinchonicic Acid Assay

Prior to cytokine or MPO quantification, total protein of each sample was determined with bicinchonicic acid (BCA) assay – a colorimetric two-component assay measuring reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline environment. Standards of bovine serum albumin (BSA) were generated with 1:2 serial dilution of 1200 μg/mL BSA in phosphate buffered saline (PBS). Samples were included in the assay at1:40 dilutions of sample:PBS, and were ran in duplicate. Working reagent was generated by mixing of proprietary reagents A and B at 50:1. Standards and samples were added to 96-well plate, followed by working reagent with multichannel pipette (100μL each). Plates were incubated at room temperature for 1 hour with agitation. Absorbance was read on SpectraMax Paradigm Multi-Mode Detection Platform at 562 nm.

Standard curve analysis was performed with GraphPad Prism 6 graphing software. Curve fit was produced with 4-parameter non-linear regression analysis and assay sample protein values were interpolated from the standard curve. Central variance (CV) of duplicate assay samples was determined, and samples with CV greater than 20% were rejected. Total protein of original tissue homogenate were calculated through multiplying assay total protein value by the dilution factor (40x).

Cytokine Quantification

For plasma and proximal, middle, and distal colon tissue homogenates, concentrations of inflammatory mediators IL-1β, IL-6, IL-10, and TNFα were quantified with the Luminex MagPix multiplex system (Luminex Corporation; Austin Texas) and MilliPlex Mouse Cytokine/Chemokine Magnetic Bead Panel Kit (EMD Millipore; Billerica, Massachusetts). The MilliPlex kits provide magnetic bead microspheres conjugated with bead-specific antibodies for use in a multiplex sandwich immunoassay similar to the enzyme-linked immunosorbent assay (ELISA) concept of protein quantification (Luminex, 2011). However, unlike traditional ELISA assays, where the analyte-specific primary antibody is conjugated to a 96-well plate, the primary antibody is conjugated to magnetic, fluorescent beads (Fig. 10). The analyte of interest is captured by the primary antibody during incubation with the sample. The bead-antibody-analyte construct is incubated with a biotinylated, analyte-specific detection antibody, generating an immunoassay sandwich similar to traditional ELISA techniques. Finally an incubation with a streptavidin-phycoerythrin (PE) conjugate is performed. The streptavidin protein strongly interacts with biotin to anchor the PE conjugate to the immunoassay construct. PE is a fluorescent protein complex that functions as the reporter.

The MagPix system is a LED-based system that detects individual bead identity through interrogation at 635nm and identification of bead-specific fluorescence (Fig. 11). Simultaneously, the PE fluorescent label of the immunoassay sandwich is interrogated with a green LED at 525 nm and fluorescence intensity is quantified. In this manner, the MagPix system is capable of identifying bead/analyte type and the fluorescence intensity of conjugated analyte to the bead. Median fluorescence intensity (MFI) for each

bead/analyte type are then used to interpolate assay sample concentration from a standard curve.

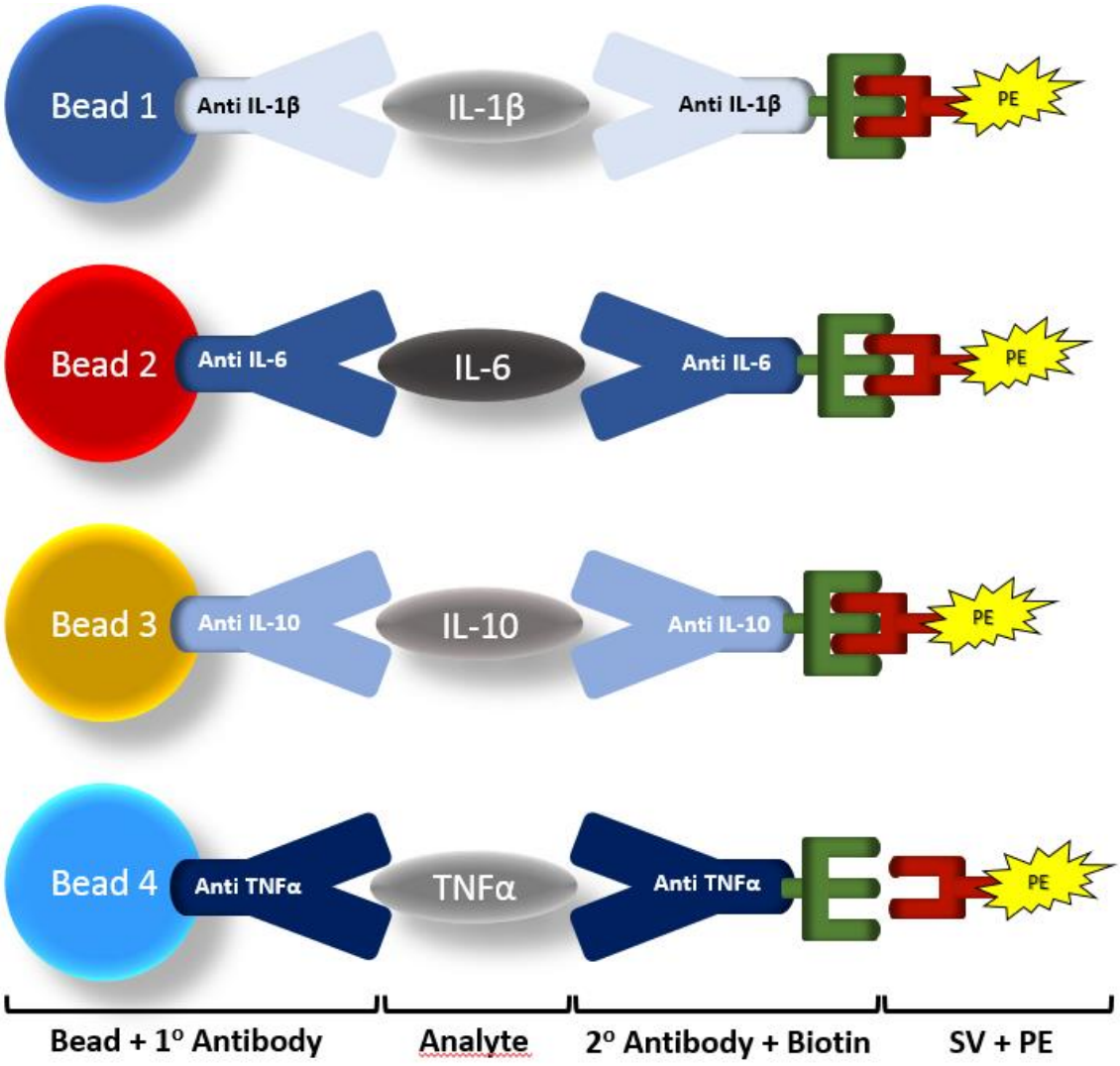

Figure 10. MilliPlex Magnetic Bead Panel Kit for Multiplex Immunoassay Protein Quantification. The kit consists of a primary (1^{o}) antibody conjugated to a magnetic bead. Bead fluorescence values are specific to the 1^{o} antibody, and therefore also to the analyte of interest. Incubation of the construct with sample is performed to capture the respective analyte. Next, a subsequent incubation is performed to conjugate the construct to a biotinylated secondary (2^{o}) antibody. A final incubation with a streptavidin-phycoerythrin (SV + PE) construct anchors the fluorescent PE molecule to the biotin of the 2^{o} antibody, completing the construct.

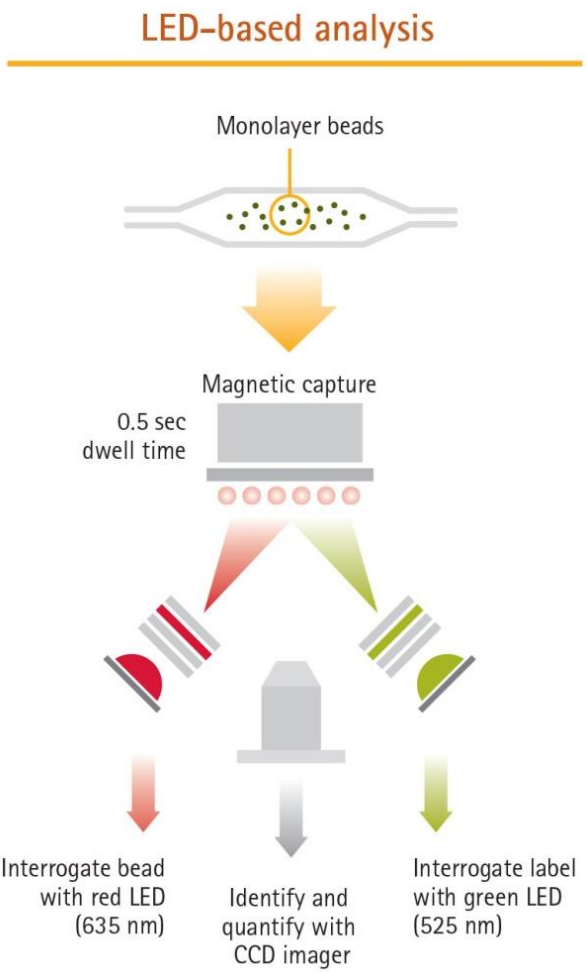

Figure 11. Luminex xMAP Technology and MagPix Multiplex System. The Luminex MagPix is an LED-based fluorescent detection system for protein quantification. Magnetic, antibody-conjugated beads are passed through a magnetic capture field and identified with 635 nm excitation LED. Prior to assay, beads incubated with an analyte of interest are "sandwiched" with a reporter antibody. During magnetic capture, the reporter is excited with a 525 nm LED. By combining a range of bead fluorescence values and multiple primary analyte antibodies, the multiplex system is capable of identifying and quantifying multiple analytes simultaneously (EMD, 2015).

For IL-1β, IL-6, IL-10, and TNFα quantification, 25 µL of whole, undiluted plasma and 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 antibody conjugated beads (25µL). Tris lysis buffer was used as background matrix with tissue homogenate assays, and serum matrix was used as background with the plasma assay. The samples were then biotinylated with a detection (secondary) antibody for one hour, and finally incubated for one hour with a streptavidin-phycoerythrin conjugate (reporter). Standard, control, and experimental samples were run in duplicate. Assay results of samples with greater than 20% coefficient of variation between the duplicate wells were rejected. Standard curve analysis was performed with Milliplex Analyst software. Curve fit was produced with 5-parameter non-linear regression analysis and assay sample protein values were interpolated from the standard curve. Results of assays with tissue homogenate were normalized to total protein of the original tissue homogenate, previously determined by the BCA assay. Prior to performing any assay, performance verification and calibration was performed with Millipore calibration (MPX-CAL-K25) and performance verification (MPX-PVER-K25) kits (EMD Millipore; Billerica, Massachusetts) to ensure optimal output.

MPO Quantification

Hycult Biotech HK210 mouse MPO ELISA kit was used to quantify MPO in tissue homogenates of proximal and distal colon segments. The kit supplied strips of microtiter wells coated with primary antibody, and 100µL of diluted standard and samples were incubated for one hour in the wells. Captured MPO was then incubated with biotnylated tracer antibody (second antibody) for one hour. Finally, a one hour incubation with a streptavidin-peroxidase conjugate (third antibody) was performed. The

oxidative capacity of the peroxide conjugate was determined through reduction and colorimetric change in tetramethylbenzidine (TMB) solution after a 30 minute incubation. The reduction of TMB was stopped with the addition of oxalic acid.

Mouse MPO standards were generated through serial dilution of a 100 pg/mL stock solution, producing 7 standards from 100 to 1.6 pg/mL. Standard curve analysis was performed with GraphPad Prism 6 graphing software. Curve fit was produced with 5-parameter non-linear regression analysis and assay sample protein values were interpolated from the standard curve. Central variance (CV) of duplicate assay samples was determined, and samples with CV greater than 20% were rejected. Total MPO levels of original tissue homogenate were calculated through normalizing assay MPO value by the total protein of the sample.

Statistics

Results from my study were analyzed with independent *t*-tests between group means and were performed with GraphPad Prism analysis software. Analysis for successful development of colitis was performed with *t*-tests between water control and DSS colitis groups receiving vehicle control injections (group 1 vs group 3). Effect of the inhibitor (DPC-333) was determined through *t*-test between DSS colitis groups receiving either vehicle control or DPC-333 injections (group 3 vs group 4). F-Test of equality of variances was used to determine if the variances between groups were significantly different ($p < 0.05$). For groups without equal variance, Welch's correction was performed. For group 3 (DSS/vehicle injection), a one-way between-subjects ANOVA was performed to compare the regional effect of DSS administration on IL-1β

and IL-6 concentrations in proximal, middle, and distal regions of the colon. Equal variance was verified with Brown-Forsythe test ($p > 0.05$). Post hoc comparisons between group means using the Tukey test were performed if the ANOVA indicated a significant regional effect on cytokine concentration.

RESULTS

Clinical Presentation of 5% DSS Colitis

To evaluate successful development of colitis, groups 1 and 3 were compared with an independent t test (Group 1: H_2O /Vehicle Injection; Group 3: 5% DSS/Vehicle Injection). Effect of TACE inhibition during DSS-induced colitis was evaluated with an independent t test between groups 3 and 4 (Group 3: 5% DSS/Vehicle Injection; Group 4: 5% DSS/DPC-333 Injection). Administration of 5% DSS for 7 days significantly increased the disease activity index (DAI) ($p < 0.0001$) (Fig. 12). TACE inhibition reduced DAI by 12%, although this reduction was not significant ($p = 0.1031$).

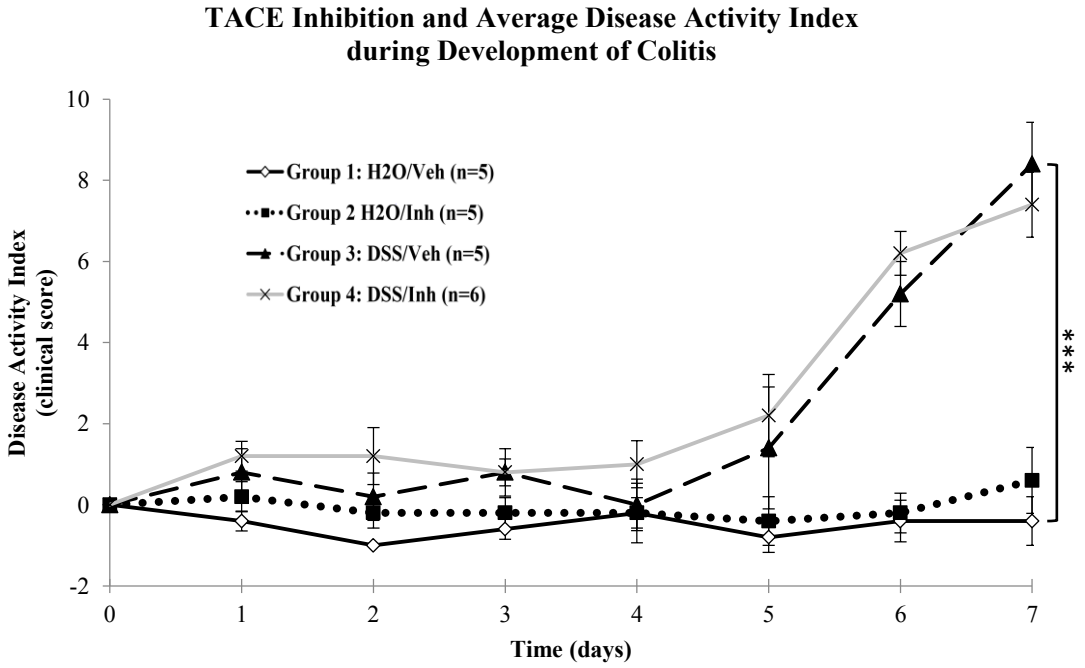

Figure 12. TACE Inhibition and Average Disease Activity Index during Development of Colitis. DAI quantified through evaluation and scoring of bodyweight loss, rectal bleeding, and diarrhea as described in the text. Values are group means ± SEM; ***$p < 0.0001$.

The contributing parameters to DAI were analyzed with the same methodology. DSS administration induced significant bodyweight loss on day seven ($p < 0.0001$) (Fig. 13). DSS drinking mice also receiving TACE inhibitor exhibited lower bodyweights on day seven (-2.4%), although this difference was not significant ($p = 0.3961$). DSS consumption significantly increased gross bleeding at the rectum and clinical bleeding score ($p < 0.0001$) (Fig.14). TACE inhibition during colitis significantly reduced gross rectal bleeding and bleeding clinical score on day seven ($p = 0.0113$). Watery stools and diarrhea were increased with DSS consumption and stool consistency score was significantly increased on day seven ($p < 0.0001$) (Fig. 15). Stool consistency scores were significantly improved on day seven by administration of TACE inhibitor ($p = 0.0014$). Semi-quantitative estimates of daily food and water consumption indicate healthy consumption of food and water (Fig. 16 and 17).

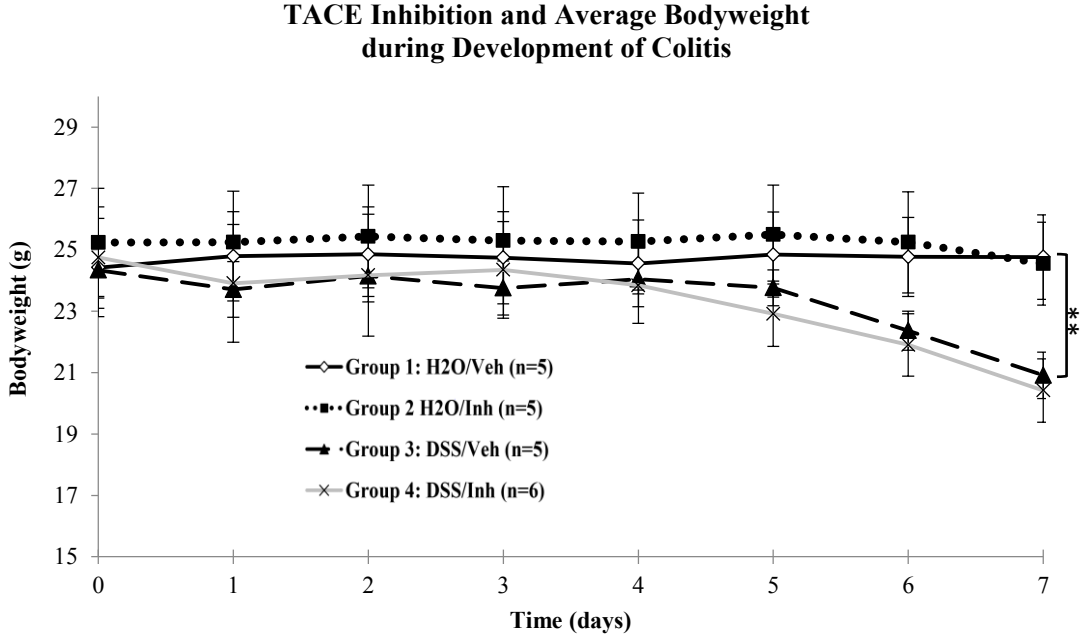

Figure 13. TACE Inhibition and Average Bodyweight during Development of Colitis. Values are group means ± SEM; **$p < 0.001$ (Group 1:Group 3).

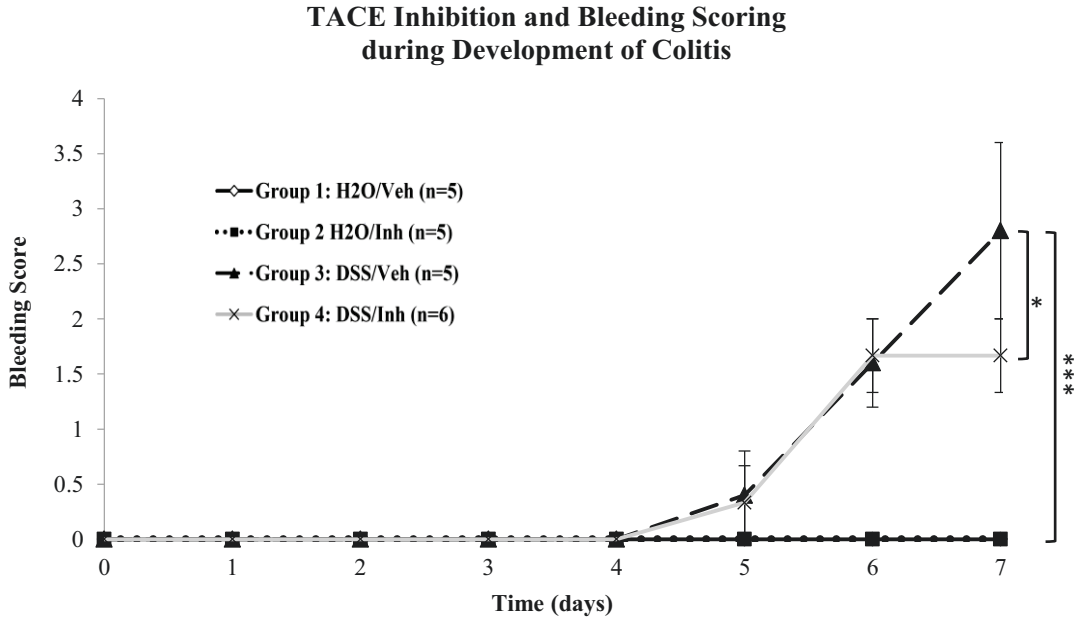

Figure 14. TACE Inhibition and Bleeding Scoring during Development of Colitis. Scoring methodology: no bleeding (0 points), slight bleeding (2 points), gross bleeding (4 points). Values are group means ± SEM; *p < 0.05, ***p < 0.0001 (Group 1:Group 3).

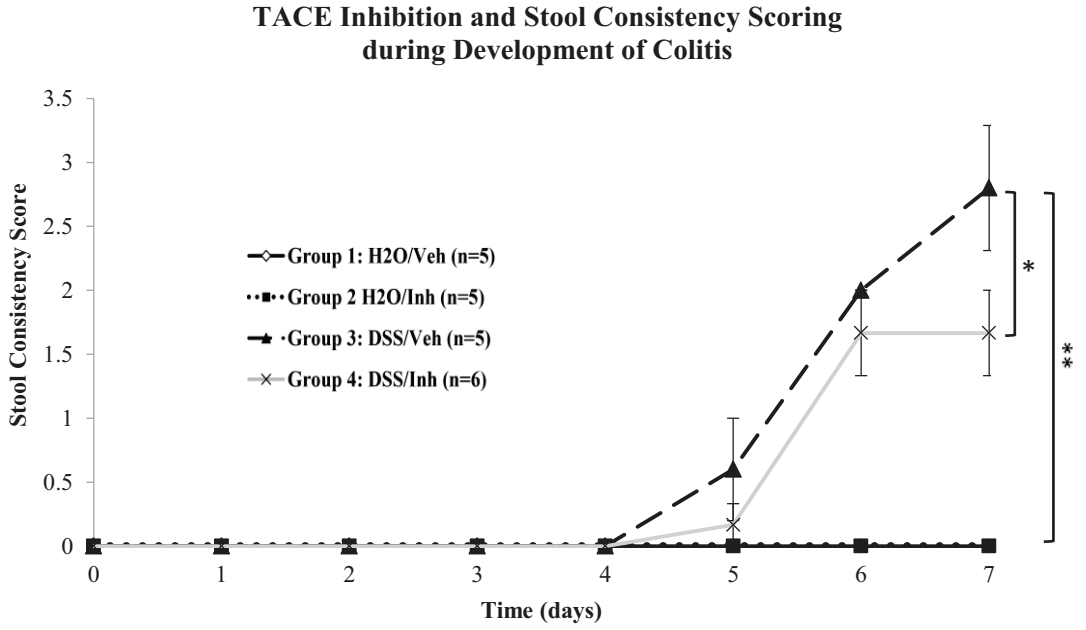

Figure 15. TACE Inhibition and Stool Consistency Scoring during Development of Colitis. Scoring methodology: normal (0 points), loose stools (2 points), watery diarrhea (4 points). Values are group means ± SEM; *p < 0.01, ***p < 0.0001 (Group 3:Group 4).

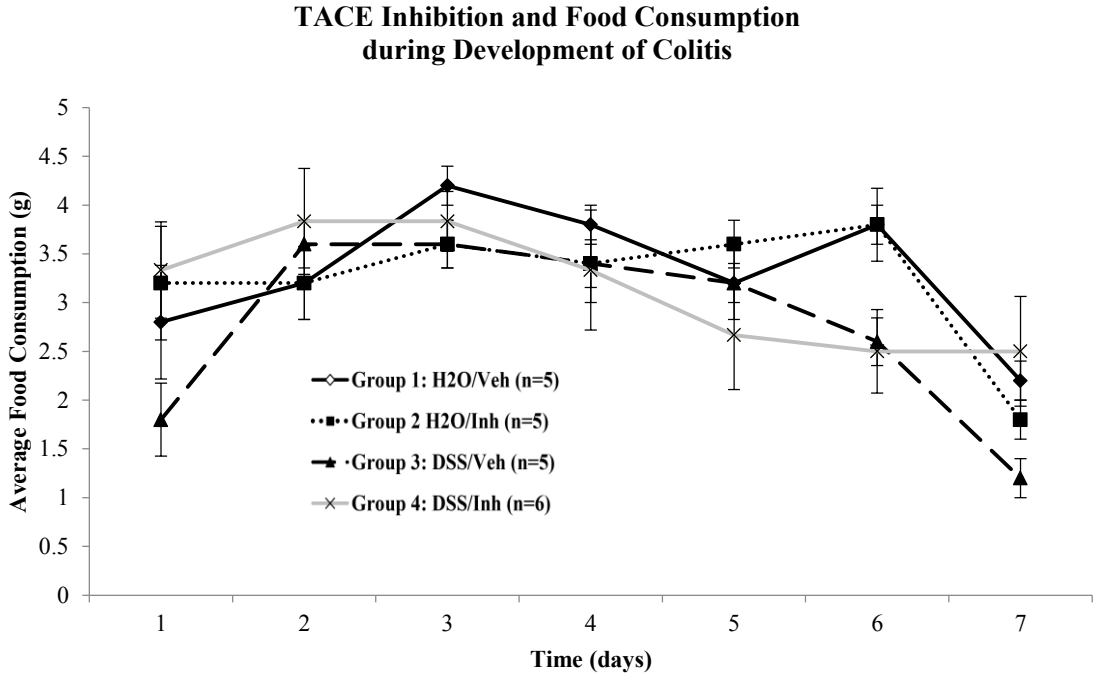

Figure 16. TACE Inhibition and Food Consumption during Development of Colitis. Semi-quantitative estimates of daily food consumption. Values are group means ± SEM.

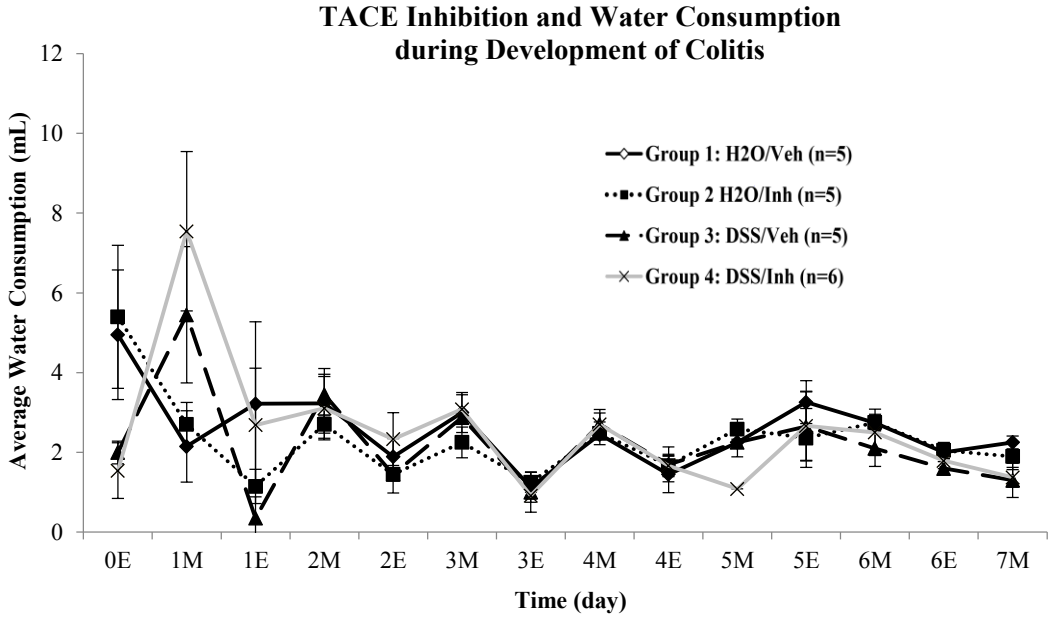

Figure 17. TACE Inhibition and Water Consumption during Development of Colitis. Semi-quantitative estimates of daily food consumption. Values are group means ± SEM.

DSS administration resulted in significantly decreased colon lengths ($p < 0.0001$) (Fig. 18). Injections of TACE inhibitor decreased colon lengths further, although the difference was not significant. For example, the colons of Group 4 (DSS/DPC-333) 4.7% shorter than Group 3 (DSS/Vehicle), however, the reduction was not significant ($p=0.2967$). The clinical results of 5% DSS administration for seven days with and without administration of the TACE inhibitor DPC-333 are summarized in Table 2.

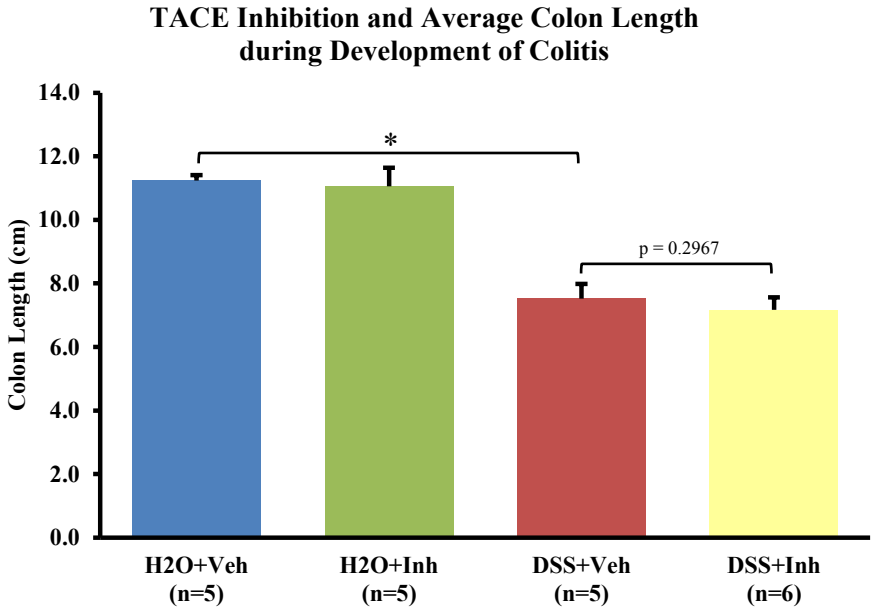

Figure 18. TACE Inhibition and Average Colon Length during Development of Colitis. Excised colon organs were measured from the ileocecal junction to the end of the rectum. Values are group means ± SEM; *$p < 0.0001$.

Plasma Cytokine Levels

Systemic inflammatory spillover from the colon tissue was evaluated through multiplex immunoassay quantification of IL-1β, IL-6, IL-10, and TNFα levels in whole plasma (Fig. 19). Plasma derived from cardiac blood samples did not exhibit significantly increased levels of any of the 4 cytokines after 7 days of 5% DSS

consumption (p = 0.0676, 0.7551, 0.8510, and 0.1307, respectively). Administration of DPC-333 during development of colitis did not significantly alter plasma levels of IL-1β, IL-6, or IL-10 (p = 0.5153, 0.3847, and 0.4768, respectively). Between the DSS groups (groups 3 and 4), plasma TNFα levels were significantly reduced by 7.9% with injections of TACE inhibitor (p = 0.0245) (Fig. 19D).

Table 2. Statistical Analysis of Clinical Parameters in Colitis Development. Results of independent *t*-test of clinical parameter on day 7 of 5% DSS. Equal variance was verified with F test to compare variances ($p < 0.05$). Group 1 (G1): H_2O +Vehicle injection; Group 3 (G3): DSS+Vehicle Injection; Group 4 (G4): DSS+DPC Injection. Values are expressed as group means ± SEM.

Parameter	G1 (n = 5)	G3 (n = 5)	G4 (n = 6)	*p* value	
				G1 vs G3	G3 vs G4
DAI	-0.4 ± 0.6	8.4 ± 1.0	7.4 ± 0.8	< 0.0001	0.1031
Ave Bodyweight (g)	24.8 ± 1.4	20.9 ± 0.8	20.4 ± 1.0	0.0006	0.3961
Bleeding Score	0.00 ± 0.0	2.80 ± 0.8	1.67 ± 0.33	< 0.0001	0.0113
Diarrhea Score	0.00 ± 0.0	2.80 ± 0.5	1.67 ± 0.33	< 0.0001	0.0014
Colon Length	11.2 ± 0.2	7.5 ± 0.5	7.2 ± 0.4	< 0.0001	0.2967

Colon Cytokine Levels

Regional Effect of DSS Colitis on Colon Cytokine Levels. The effect of 5% DSS administration for 7 days on regional colon cytokine concentrations was evaluated through an independent *t*-test between cytokine concentrations of groups 1 and 3 (Group 1: H_2O /Vehicle Injection; Group 3: 5% DSS/Vehicle Injection) (Fig. 20). A trend of DSS administration increasing cytokine concentration of each analyte (IL-1β, IL-6, IL-

10, and TNFα) was observed. However, a significant increase was observed in the distal colon concentrations of IL-10 (p = 0.0296) and TNFα (p = 0.007). Although not statistically significant, large percent increases between groups 1 and 3 of IL-1β (*p* = 0.1448) and IL-6 (*p* = 0.1152) concentrations in the distal colon were also observed (464.0% and 398.0%).

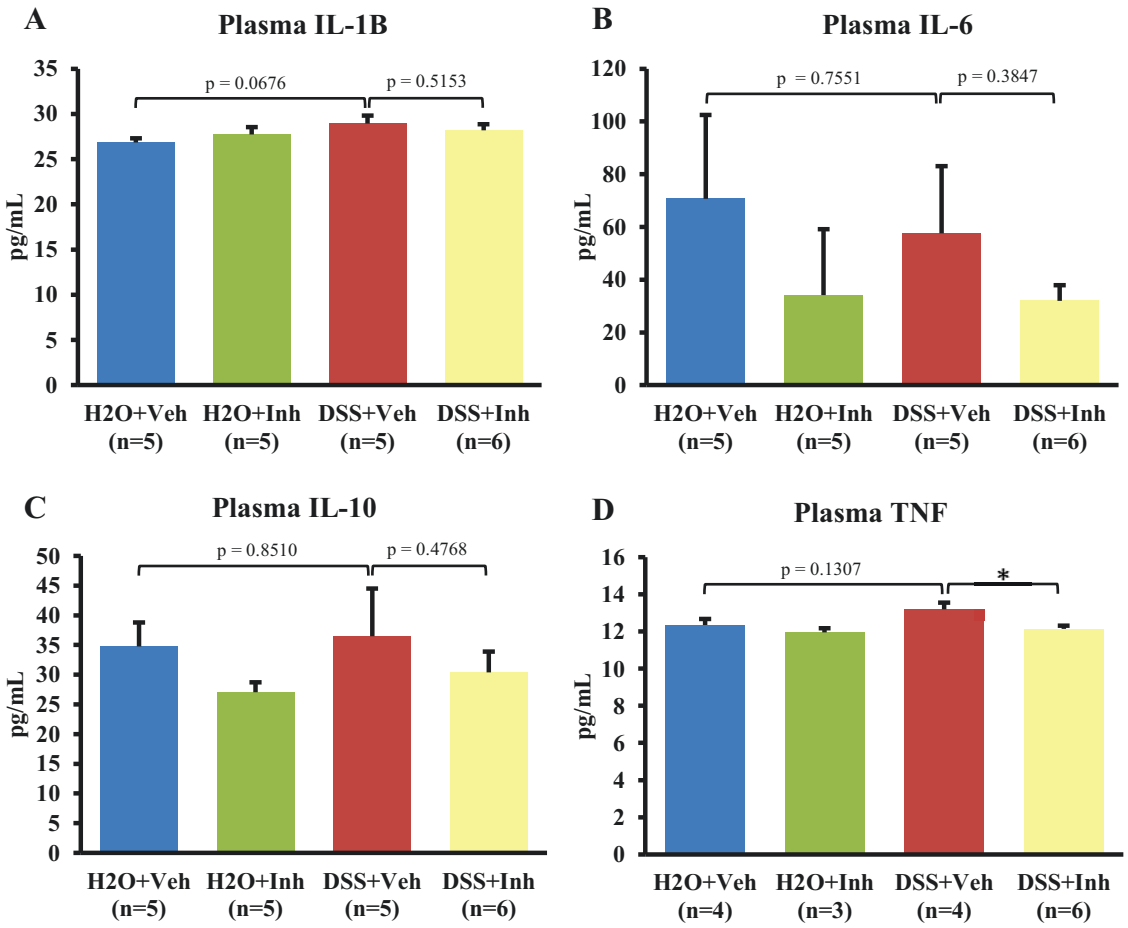

Figure 19. Plasma Concentration of Inflammatory Cytokines. Plasma concentration of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNFα were quantified from cardiac blood after 7 days of 5% DSS (or H_2O control) and 2x daily DPC-333 injections (or vehicle control). Values are group means ± SEM; *p < 0.05.

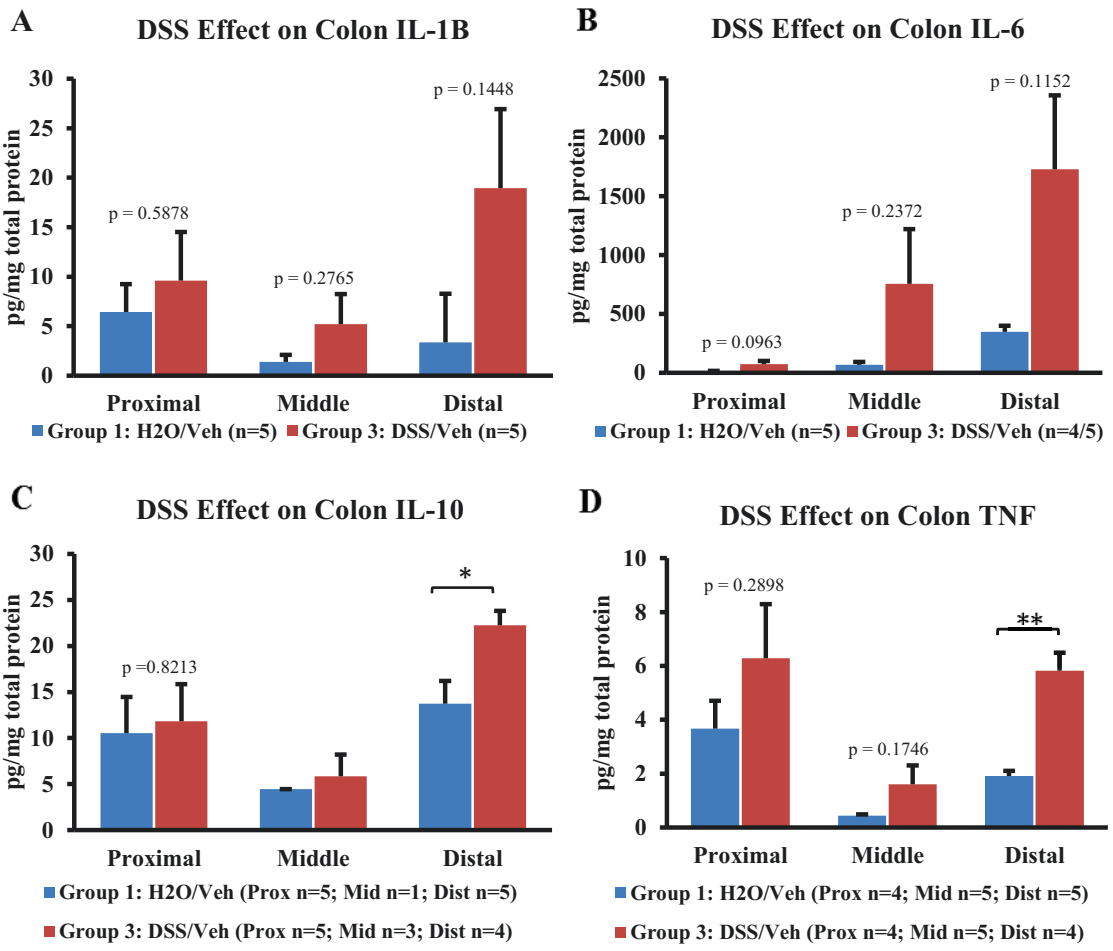

Figure 20. DSS Effect on Regional Cytokine Concentration in the Colon. Tissue concentrations of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNFα were quantified from proximal, middle, and distal regions of the colon after 7 days of 5% DSS or H_2O control. Both groups received 2x daily IP injections of vehicle (25mMol citric acid saline) for comparison to TACE inhibition groups (Fig. 20, 21). Values are group means ± SEM. *p = 0.0296; **p = 0.007.

TACE Inhibition during DSS Colitis and Colon Cytokine Levels. Effect of twice daily IP injections of the TACE inhibitor DPC-333 on colon tissue cytokines during the development of 5% DSS colitis was determined. Concentrations of IL-1β, IL-6, IL-10, and TNFα in tissue homogenate from proximal, middle, and distal colon regions were quantified with multiplex immunoassay (Fig. 21). Analysis of DPC-333 effect on 5% DSS colitis was performed through an independent t test between experimental

groups 3 and 4 for each cytokine (Group 3: 5% DSS/Vehicle Injection; Group 4: 5% DSS/DPC-333 Injection). No significant difference was observed for any of the analytes in any of the colon regions. The largest effect of TACE inhibition was observed in the distal colon, where DPC-333 injections during colitis development increased IL-10 concentrations by 81.7% ($p = 0.0746$). Administration of DPC-333 consistently decreased colon TNFα levels in the proximal, middle, and distal regions, although this decrease was not significant (Table 3).

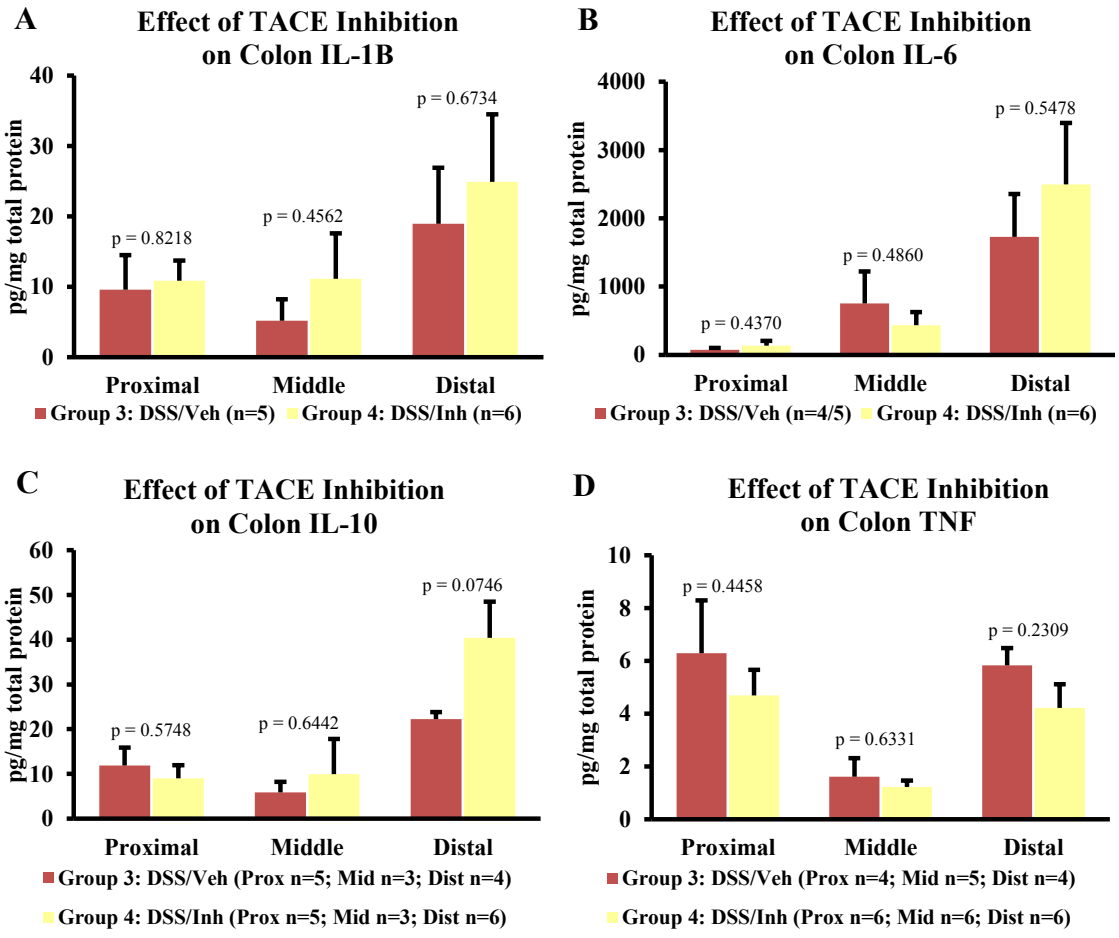

Figure 21. Effect of TACE Inhibition on Regional Cytokine Concentration in the Colon. Tissue concentrations of (A) IL-1β, (B) IL-6, (C) IL-10, and (D) TNFα were quantified from proximal, middle, and distal regions of the colon after 7 days of 5% DSS. Groups received 2x daily IP injections of either 10mg/kg BW DPC-333 or equivalent volume of vehicle (25mMol citric acid saline). Values are group means ± SEM.

Table 3. TACE Inhibition and Percent Difference in TNFα Levels. Percent difference in TNFα levels between Group 3 (DSS+Vehicle injection) and Group 4 (DSS+DPC-333 injection) in plasma, and proximal, middle, and distal colons (P Col, M Col, and D Col). Results are independent *t*-test *p* values of TNFα concentrations on day 7 of 5% DSS. Equal variance was verified with F test to compare variances ($p < 0.05$) and Welch's Correction performed if $p < 0.05$. Values are expressed as group means ± SEM.

		Plasma	P Col	M Col	D Col
TNFα Concentration (pg/mg tissue)	Group 3	13.19 ± 0.34	6.29 ± 2.00	1.60 ± 0.71	5.82 ± 0.66
	Group 4	12.15 ± 0.17	4.69 ± 0.97	1.22 ± 0.23	4.22 ± 0.90
	% Difference	-7.8	-25.4	-23.6	-27.6
	p value	0.0245	0.445	0.633	0.230

Overview of Colon Cytokine Levels during DSS Colitis and TACE Inhibition

Concentrations of individual cytokines in the proximal, middle, and distal colons of groups 1-4 are graphically overviewed in Fig. 22 and 23. In the absence of statistical significance between groups, trends of cytokine levels can be observed. Without stimulation from DSS (Groups 1 and 2), basal levels of IL-1β, IL-10, and TNFα may be elevated in the proximal and distal colons. In contrast, Il-6 concentrations may increase in more distal portions of the colon tissues. A trend of increased cytokine concentration in the distal colon with consumption of DSS can be observed. The highest IL-1β, IL-6, and IL-10 concentrations are observed in the distal colon. Regional TNFα release did not follow this trend, as proximal and distal levels were elevated.

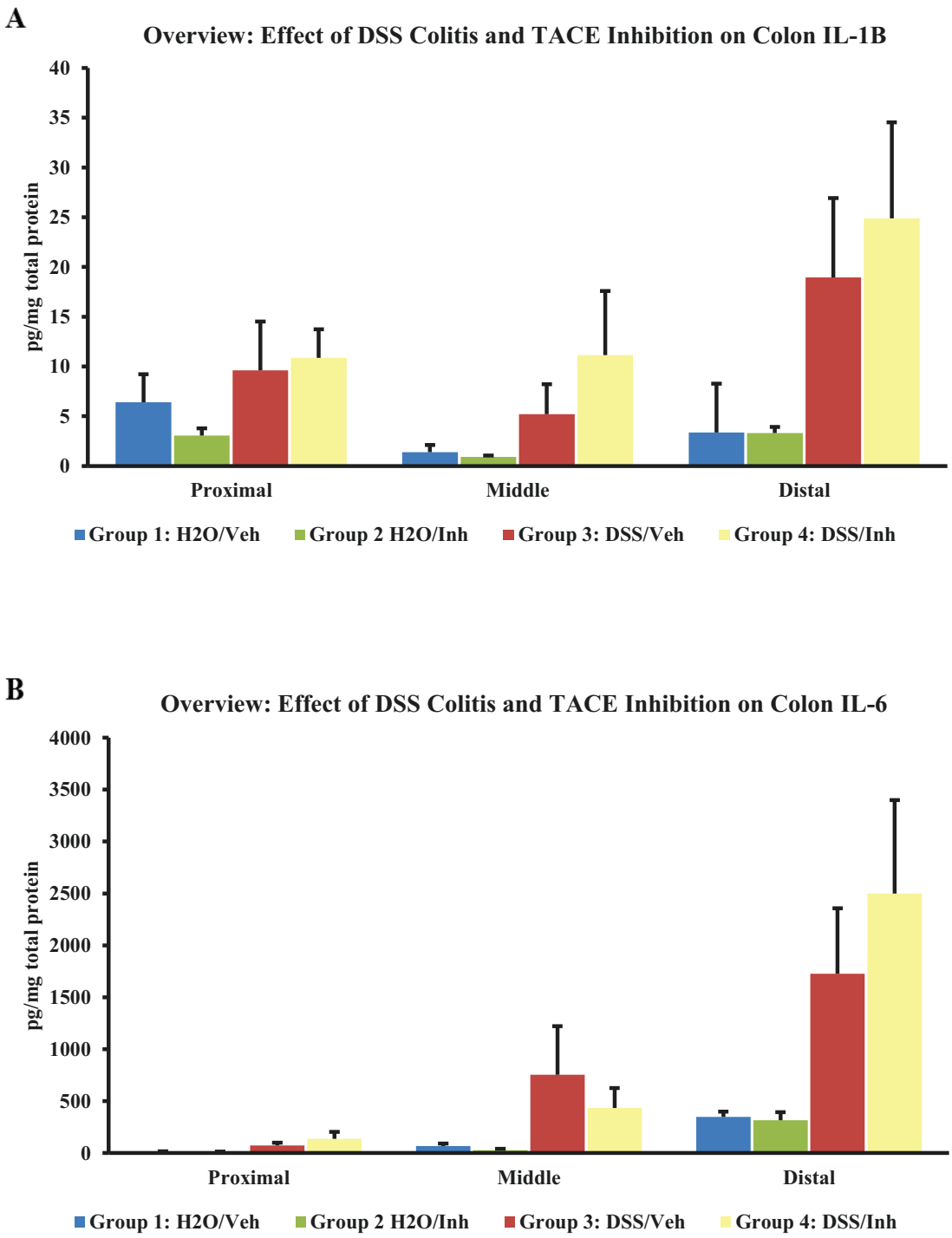

Figure 22. Overview of Colon IL-1β and IL-6 Levels during DSS Colitis and TACE Inhibition. Tissue concentrations of (A) IL-1β and (B) IL-6 were quantified from proximal, middle, and distal regions of the colon after 7 days of 5% DSS (or H_2O control). Groups received 2x daily IP injections of either 10mg/kg BW DPC-333 or equivalent volume of vehicle (25mMol citric acid saline). Values are group means ± SEM; *$p < 0.05$.

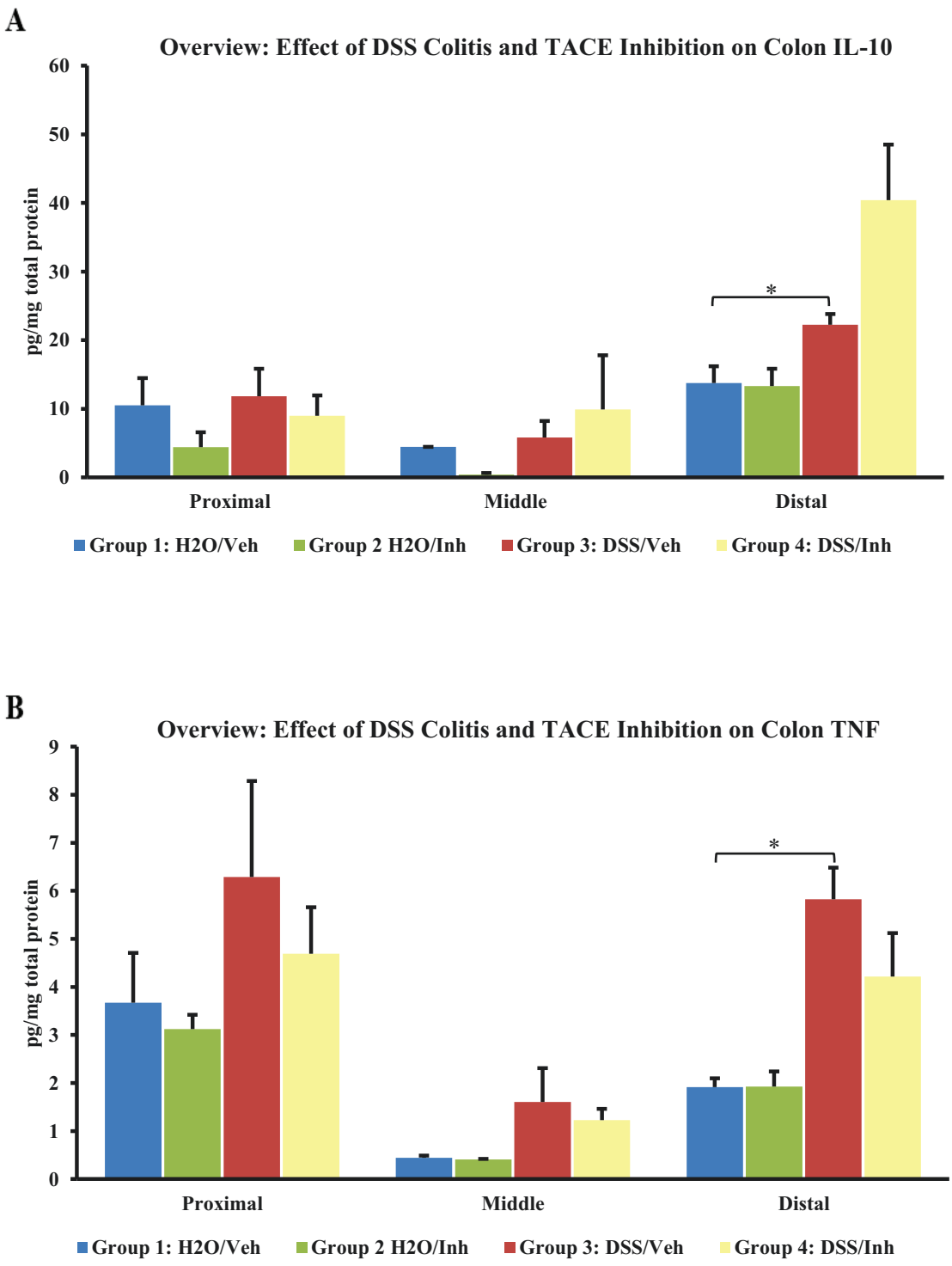

Figure 23. Overview of Colon IL-10 and TNFα Levels during DSS Colitis and TACE Inhibition. Tissue concentrations of (A) IL-10 and (B) TNFα were quantified from proximal, middle, and distal regions of the colon after 7 days of 5% DSS (or H_2O control). Groups received 2x daily IP injections of either 10mg/kg BW DPC-333 or equivalent volume of vehicle (25mMol citric acid saline). Values are group means ± SEM; *$p < 0.05$.

Colon Tissue Myeloperoxidase Levels

To evaluate neutrophil recruitment to the colon in response to inflammation, MPO concentration in colon tissue was quantified (Fig. 24). ELISA assays were performed to

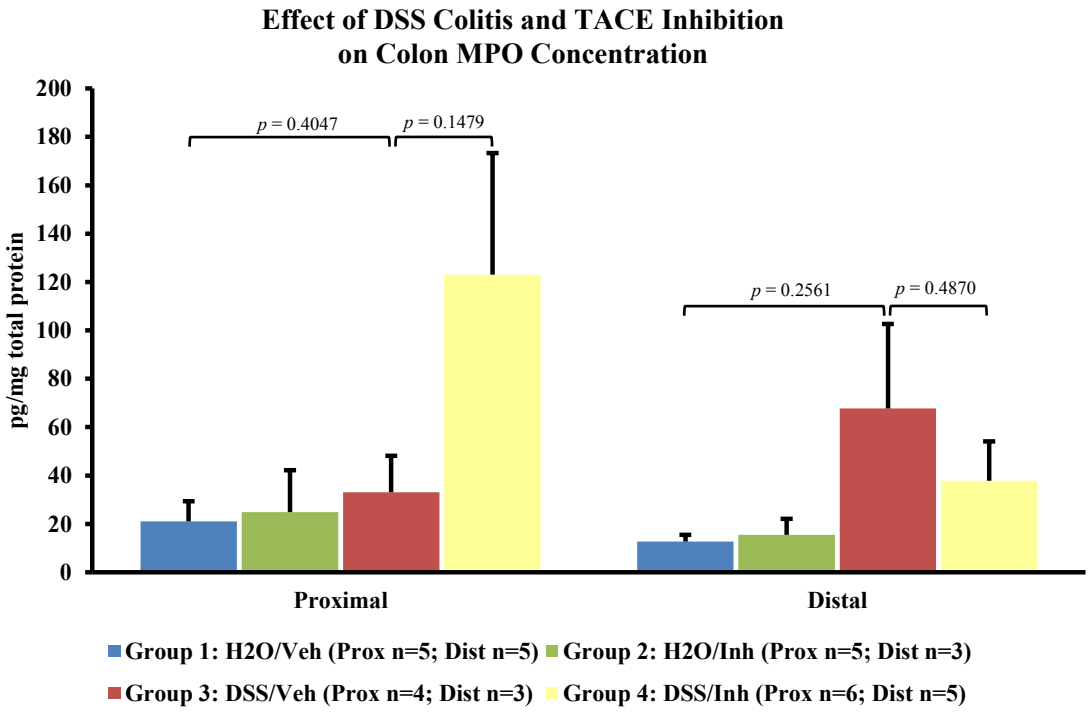

Figure 24. Effect of DSS Colitis and TACE Inhibition on Colon MPO Concentration. MPO concentration in proximal and distal regions of colon tissue after 7 days of 5% DSS (or H_2O control). Groups received 2x daily IP injections of either 10mg/kg BW DPC-333 or equivalent volume of vehicle (25mMol citric acid saline). Values are group means ± SEM.

quantify MPO levels in tissue homogenate from proximal and distal colon regions. Effect of 5% DSS for 7 days was determined through comparison between groups 1 and 3 (Group 1: H_2O /Vehicle Injection, Group 3: 5% DSS/Vehicle Injection) and effect of DPC-333 injections was determined through comparison of groups 3 and 4 (Group 3: 5% DSS/Vehicle Injection; Group 4: 5% DSS/DPC-333 Injection). Consumption of 5% DSS

did not significantly change MPO levels in the proximal or distal colon. TACE inhibition during the development of colitis did not significantly change MPO concentration in proximal or distal colons. Although the results of MPO quantification are not significant, DPC-333 administration during the development of colitis (group 4) produced a large percent increase of MPO (272.5%) in the proximal colon (Table 4). In contrast, the distal colon displayed increased MPO concentration with administration of 5% DSS (434.9%) and this increase was diminished with DPC-333 administration (-44.2%).

Table 4. Effect of 5% DSS and TACE Inhibition on Colon MPO Concentrations. Percent difference in MPO levels between groups 1:3 and groups 3:4. Results are independent *t*-test *p* values of TNFα concentrations on day 7 of 5% DSS. Equal variance was verified with F test to compare variances ($p < 0.05$) and Welch's Correction performed if $p < 0.05$.

				% Difference (*p* value)	
Region	G1(n = 5)	G3(n = 5)	G4(n = 6)	G1 vs G3	G3 vs G4
Proximal	21.0 ± 8.4	33.0 ± 15.2	123.0 ± 50.4	57.4 (0.405)	272.5 (0.148)
Distal	12.7 ± 2.9	67.8 ± 34.9	37.8 ± 16.3	434.9 (0.256)	-44.2 (0.487)

DISCUSSION

Clinical Evaluation of DSS Colitis and Effect of TACE Inhibition

Administration of 5% DSS for seven days in BALB/c mice produced a disease model consistent with experimental colitis literature (Chassaing et al., 2014). Bodyweight loss, rectal bleeding, incidence of diarrhea were all significantly increased with high confidence. Additionally, colon lengths of colitis mice were significantly shortened, also consistent with the DSS colitis model. TACE inhibition did not improve the overall disease state of 5% DSS colitis for seven days. Although a significant reduction in diarrhea and bleeding scores was observed, bodyweight loss was increased in the TACE inhibition experimental group. As a result, overall disease activity index (DAI) was not improved. Additionally, TACE inhibition did not attenuate shortened colon lengths in the DSS groups, instead resulting in a further non-significant decrease in length. Estimates of average food and water consumption by the animals indicate all groups continued to consume food and water throughout the trial. Abrupt decrease in food and water consumption leads to quick onset of bodyweight loss and would have impacted the DAI results. However, no such effect was observed in my study.

Effect of DSS on Inflammatory Cytokines

Administration of 5% DSS for 7 days did not significantly alter the plasma concentrations of IL-1β, IL-6, IL-10, or TNFα. This indicates the local inflammatory response had limited spillover into the systemic circulation. The blood samples were obtained from the heart, and therefore may not be representative of blood prior to

metabolic action of the liver. Samples from the hepatic portal vein may have increased levels of the inflammatory cytokines which are metabolized and reduced in the liver.

Regional analysis of cytokine concentration after 7 days of 5% DSS administration implicate the distal colon as the most impacted region. Distal colon IL-10 and TNFα were significantly increased compared to non-DSS controls. Large fluctuation of distal colon IL-1β and IL-6 concentrations were also observed, although due to high variance, these results were not significant.

A one-way between-subjects ANOVA was performed on group 3 (DSS/vehicle injection) to compare the regional effect of DSS administration on IL-1β and IL-6 concentrations in proximal, middle, and distal regions of the colon (Table 5). There was a significant effect of DSS administration on IL-6 concentration ($p < 0.05$) between the colon regions [$F(2, 10) = 4.136$, $p = 0.0491$]. There was not a significant effect on regional IL-1β concentrations [$F(2, 11) = 0.4983$, $p = 0.2346$]. Post hoc comparisons between group means using the Tukey test indicated the mean IL-6 concentrations between the proximal and distal colons (mean difference = -1655, SE of difference = 575.9) were significantly different ($p = 0.0402$) (Table 5C). This ANOVA of regional IL-6 concentration during DSS colitis suggests IL-6 concentrations are higher in the distal colon. This analysis is not ideal because the cytokine concentration is not compared to basal level concentrations in a non-DSS control. However, because the clinical data indicates successful induction of colitis, the ANOVA analysis serves as limited evidence further implicating the distal colon as the more affected region during DSS-induced colitis, particularly with regard to IL-6 concentration.

Table 5. ANOVA and Post Hoc Results of Colon IL-1β and IL-6 Concentrations in the Colon. Analysis of variance of IL-1β (A) and IL-6 (B) concentrations in proximal, middle, and distal colon regions after 7 days of 5% DSS administration was performed on group 3 (DSS/Vehicle injection). Post hoc comparisons (C) were made using Tukey's multiple comparisons test.

A) IL-1B ANOVA Results

Comparison	df	F	Sig
Between Groups	2	1.659	$p = 0.2346$
Within Groups	11		
Total	13		

B) IL-6 ANOVA Results

Between Groups	2	0.0491	$p = 4.136$
Within Groups	10		
Total	12		

C) IL-6 Post Hoc

	Mean diff.	SE of diff.	Adjusted *p* Value
Proximal vs Middle	-682.2	575.9	0.488
Middle vs. Distal	-1655	575.9	0.0402
Distal vs. Middle	-973	607	0.2889

Differences in relative levels of individual cytokines in the colon (e.g. IL-6 vs TNFα) corresponds to previous investigation quantifying mRNA expression of IL-1β, IL-6, and TNFα (Yan et al., 2009). These data indicate a 23-fold increase in colon IL-6 expression in C57BL/6 mice, while TNFα expression increased 9-fold (Fig. 25).

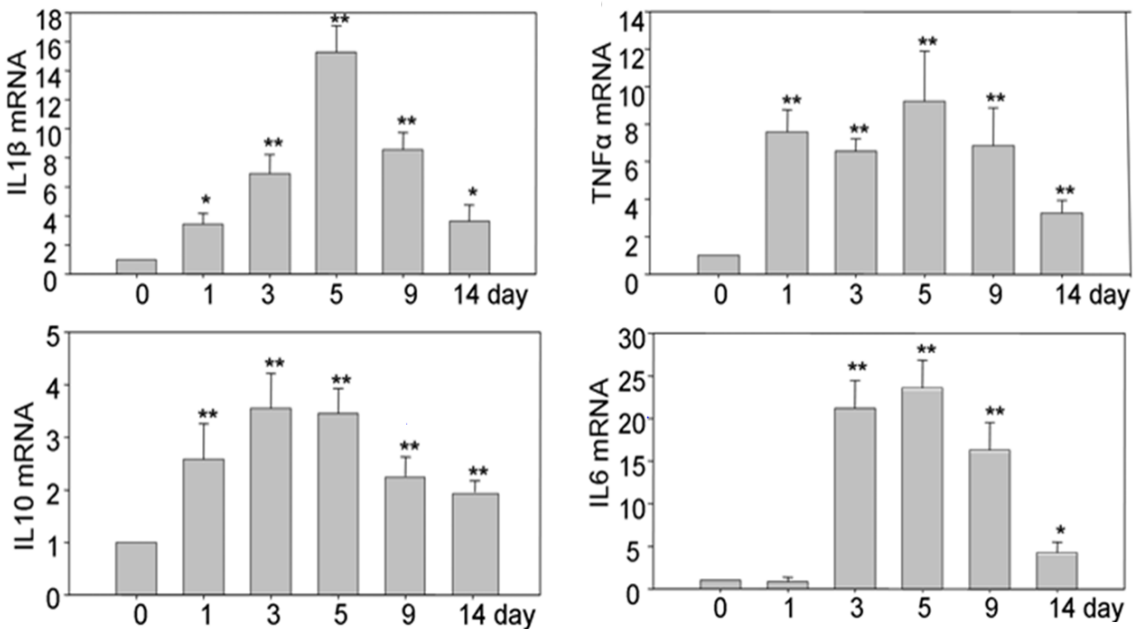

Figure 25. Colon Cytokine Production during 3.5% DSS in C57BL/6 Mice. Mice were administered 3.5% DSS for five days, followed by regular water after day five. Real time PCR was used to determine mRNA expression of inflammatory cytokines in colon tissue (Yan et al., 2009).

Additionally, the relative levels of individual cytokine concentrations in my study (TNFα < IL-1β < IL-6) also correspond to cytokine concentrations derived from mononuclear cell extracts in UC patients (Reinecker et al., 1993) (Fig. 26). Because of elevated IL-6 concentration relative to other cytokines, inhibition of IL-6 during pathologic inflammation has been investigated previously. Tocilizumab (a monoclonal antibody against IL-6) is currently used to treat rheumatoid arthritis (Smolen et al., 2008). However, direct inhibition of IL-6 during colitis may not be advantageous due to an important role of IL-6 in regenerating intestinal epithelium through the signal transducer and activator of transcription 3 (Grivennikov et al., 2009). Clinical trial of Tocilizumab administration during active CD was effective in reducing disease severity. However, only 2 of 10 patients experienced disease remission (Ito et al., 2004). Additionally,

Tocilizumab may increase risk for diverticulitis, relative to other rheumatoid arthritis treatments (Gout et al., 2011). Because of these concerns, Tocilizumab is currently avoided as treatment in rheumatoid arthritis when IBD conditions are already present.

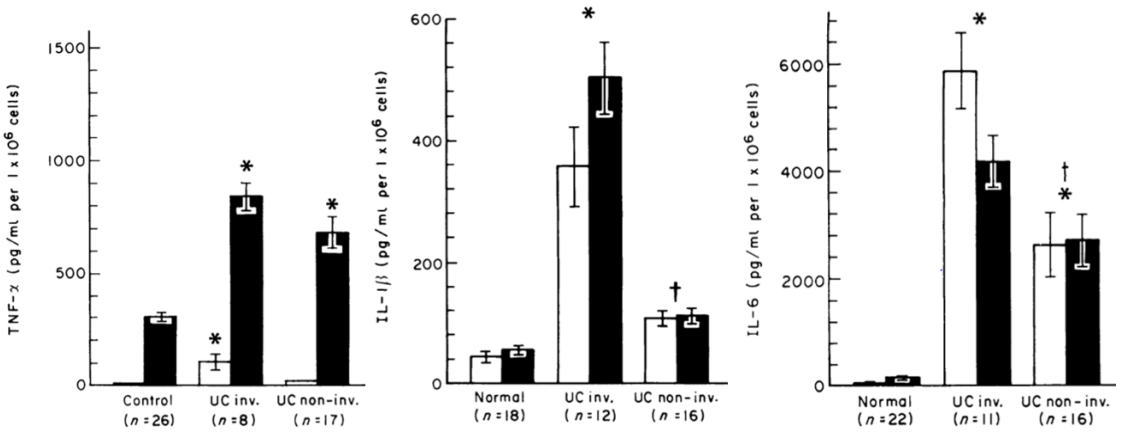

Figure 26. Inflammatory Cytokine Concentrations in Mononuclear Cells from UC Patient Colon Tissues. Lamina propria mononuclear cells were isolated from colon tissue biopsies in healthy and UC patients and cultured for 48 hours. Samples from UC patients were taken from involved (inv.) and non-involved (non-inv.) areas of mucosal inflammation. The samples were either unstimulated (white bars) or cultured with pokeweed mitogen (black bars), an agonist of toll-like receptors and activator of NF-κB (Reinecker et al., 1993).

Effect of TACE Inhibition on Inflammatory Cytokine Concentrations

A significant reduction in plasma sTNFα levels was observed with twice daily IP injections of DPC-333 (10mg/kg bodyweight) during the 7 day development of 5% DSS colitis. This result is indicative of systemic inhibition of TACE activity and is evidence of biologically active circulating levels of DPC-333. However, the activity of DPC-333 was not significantly extended to the colon tissue. Although there was a trend for reduction in sTNFα during the development of DSS colitis, these decreases were not statistically significant.

The largest impact DPC-333 administration had on the colon tissue cytokines was observed in the IL-10 concentrations of the distal colon (81.7% increase, $p = 0.0746$). Conclusions from this observation are speculative. Several studies using IL-10 knockout mice have implicated this cytokine as protective against colitis development (Kühn et al., 1993; Marlow et al., 2013; Scheinin et al., 2003). However, IL-10 expression also increases proportionally with increasing inflammatory cytokine signaling and NF-κB transcriptional activity. Because there was not an observable benefit to the DAI during TACE inhibition, a conclusion cannot be made that increased IL-10 levels are indicative of an anti-inflammatory effect by TACE inhibition.

Overview of Colon Cytokine Concentrations

The graphical overview of each cytokine (Fig. 22 and 23) highlights the role of the distal colon in the development of colitis in this model. The highest concentrations of IL1β, IL-6, and IL-10 were consistently observed in the distal colons of the DSS experimental groups (groups 3 and 4). An interesting exception to this trend was observed in the proximal colon levels of TNFα. Basal and DSS-stimulated TNFα concentrations of the proximal colon were more similar to the distal colon than the middle region. However, increased TNFα levels from DSS administration were only significant in the distal colon, also implicating the distal colon as the most affected region.

If the distal colon is the primary site of inflammation in this DSS colitis model, then anti-inflammatory effects on the cytokine expression profile in this region would likely be the most beneficial. TACE inhibition, although non-significant, increased the

levels of IL-1β and IL-6, suggesting a pro-inflammatory effect. Coupled with lack of improvement in the clinical parameters from TACE inhibition, increased IL-10 in the distal colon from TACE inhibition cannot be concluded as an anti-inflammatory effect of TACE inhibition.

Despite an overall trend (non-significant) of decreased colon TNFα levels with TACE inhibition, my evidence suggests other pro-inflammatory compensatory cytokine signaling may compensate for any reduced TNFα levels from providing a beneficial clinical effect. The bioavailability of IP injected DPC-333 to the colon tissue may be limited, and therefore not effective in reducing TNFα levels. Increasing the duration of TACE inhibition prior to DSS administration may not improve the outcome, as inhibition of TNFα with Infliximab during the development of DSS colitis is accomplished with rapid onset of TNFα inhibition. For example, three days of 10mg/kg bodyweight IP injection of Infliximab at the onset of DSS administration is effective at reducing clinical parameters of colitis after seven days of DSS consumption (Qiu et al., 2011). Therefore, IP injections of DPC-333 in conjunction with DSS administration should reduce TNFα levels if it is capable reaching the colon tissues. If TACE inhibition with DPC-333 was more effective in reducing colon TNFα, there may have been an improved response during development of DSS colitis.

Effect of DSS on Colon Neutrophil Recruitment as Determined by MPO Levels

On day seven of 5% DSS administration, MPO concentrations were not significantly increased in either the proximal or distal colon (Fig. 24). However, MPO in the distal colon was 434.9% greater ($p = 0.2561$) and increasing sample size in future

study may provide significance by reducing standard deviation from the mean (Table 4). The percent elevation in proximal colon MPO was much less (57.4%, $p = 0.4047$). A more pronounced increase of MPO levels in the distal colon during appears to correlate with increased cytokine concentrations in the distal colon. The MPO data suggests neutrophil recruitment predominately impacts the distal colon in this model of colitis.

Effect of TACE Inhibition on Colon MPO Levels during Development of Colitis

The most unexpected data of this study can be observed in the effect of TACE inhibition on MPO levels in the proximal region (Fig. 24). In the distal colon, TACE inhibition reduced MPO concentration of the DSS experimental groups (groups 3 and 4) by 44.7% (Table 4). Although this decrease was not significant ($p = 0.230$), it could be a result of decreased L-selectin cleavage by TACE, and impaired emigration of neutrophils out of the capillaries and into the colon tissue. Surprisingly, TACE inhibition during development of DSS colitis increased MPO concentrations by 434.9% in the proximal region for unexpected reasons. The data from this study suggests TACE inhibition may increase sensitivity or susceptibility to neutrophil recruitment to the proximal colon in this DSS model of colitis.

Conclusions

Clinical Colitis Model and Effect of TACE Inhibition. Current literature and research on chemical models of colitis in mice vary widely in multiple aspects which alter the disease model and findings (Melgar et al., 2005). These parameters include type of inflammatory stimulus, duration and concentration of stimulus administration, and

strain of mouse. Additionally, TACE inhibition studies also vary in duration of inhibition, timing of administration relative to induction of colitis, and specific TACE inhibitor used (Sharma et al., 2014). The current study shows twice daily IP injections of the TACE inhibitor DPC-333 does not provide a clinically-relevant protective effect during the development of colitis for 7 days with administration of 5% DSS in BALB/c mice. Considering the potent reduction of TNFα levels during TACE inhibition in LPS models of sepsis and a lack of reduction in colon TNFα levels in my study, other delivery methods of DPC-333 may improve bioavailability to the colon and result in significant reduction of TNFα levels (Qian et al., 2007).

Inhibition of TACE and effect on conditions of intestinal inflammation is a complex physiological process. Chalaris et al. published work in 2010 in the Journal of Experimental Medicine implicating decreased TACE activity with increased susceptibility to DSS inflammation (Chalaris et al., 2010).. Although complete disruption of TACE activity results in an unviable animal (Peschon et al., 1998), Chalaris et al. developed a novel method for producing mice with severely (although not completely) impaired TACE activity in all cells of the mouse. Subsequently, the presence of soluble products from TACE sheddase activity were reduced. For example, decreased levels of soluble TNFR2 were detected and these animals presented with EGF-R and EGF-R ligand-type mutations such as eye, hair, and skin defects. Despite these affects, the intestinal epithelia did not display any "overt abnormalities." The knockdown mice displayed dramatically increased susceptibility to DSS-induced inflammation and colitis. A 2% DSS regimen resulted in severe weight loss and a mortality rate greater than 50%. These results demonstrate increased susceptibility to DSS resulted from

impaired regeneration of epithelial cells in the absence of STAT3 activation by soluble EGF-R ligands.

In contrast, encouraging results of TACE inhibition and a protective effect against colitis were recently published (Sharma et al., 2014). The study demonstrated the ability of a novel TACE inhibitor (termed 11p) to improve the recovery phase of C57BL/6 mice from 3.5% DSS (Fig. 27). DSS was administered for 5 days and replaced with water

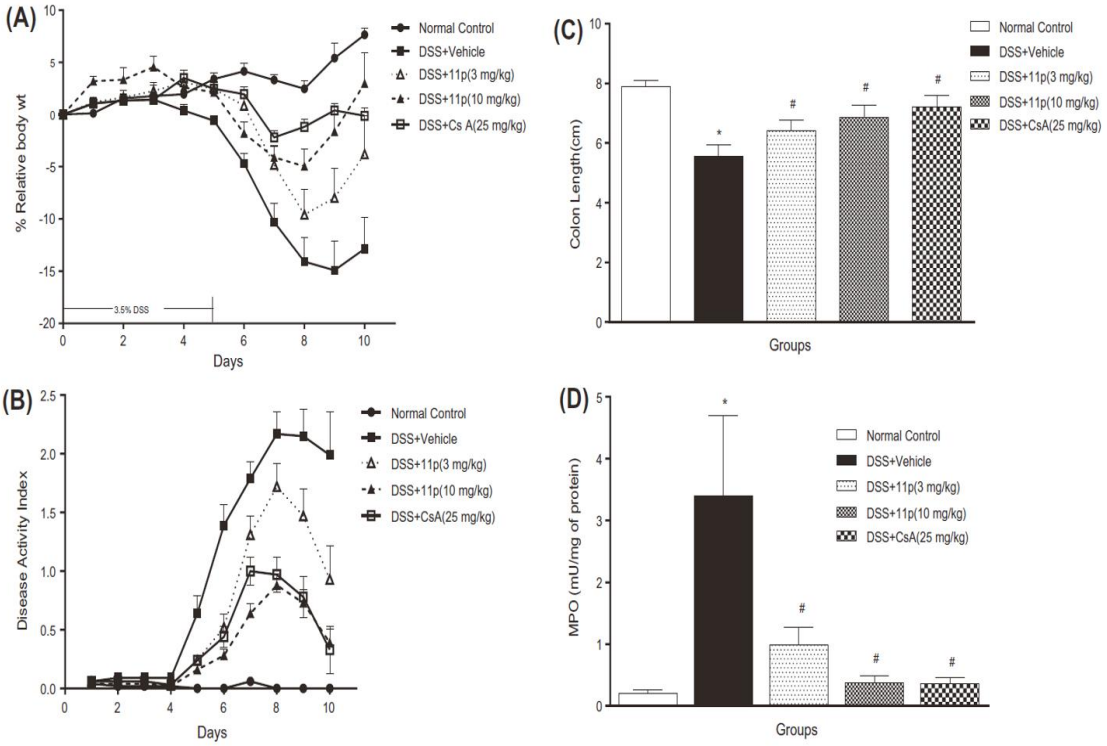

Figure 27. TACE Inhibition and Recovery from DSS Colitis. Data reported by Sharma et al. demonstrating a positive effect of TACE inhibition on DSS-induced colitis in C57BL/6 mice. 3.5% DSS was administered for 5 days and TACE inhibitor (11p) was orally administered daily for 7 days. Colon lengths were determined on day 10 (C). MPO activity was assayed on day 8 (D). TACE inhibition decreased percent bodyweight loss (A), improved DAI (B), attenuated colon shortening (C), and decreased colon MPO activity (D) (Sharma et al., 2014).

only for subsequent days. The TACE inhibitor 11p was orally administered daily from day 0 to day 7. Through day 10 (5 days post DSS), TACE inhibition improved DSS-induced DAI, bodyweight loss, colon lengths (day 10), and MPO activity (day 8). In addition, concentration of inflammatory cytokines IL-1β, IL-6, and TNFα in the colon were significantly decreased on day 5.

Regional Analysis of Colitis and TACE Inhibition. Regional between-group analysis of the H_2O vs 5% DSS administered mice and analysis of variance between colon regions indicates a disproportionate regional effect on the distal colon. DSS administration altered the inflammatory cytokine concentrations profile, particularly in the distal region of the colon. In support of this data, a non-significant elevation (45%) in MPO also occurred in the distal colon relative to the water control. Taken together, these finding implicate the distal colon as the most impacted region of the colon during the development of DSS colitis in mice.

This observation is clinically relevant and improves the validity of the DSS model of colitis, because clinical ulcerative colitis in humans also progresses in a distal to proximal manner along the colon. In addition, progression of clinical human colitis from "left sided" to "extensive" colitis drastically alters the delivery approach and response to current UC treatments. Therefore, this information is particularly relevant for the investigation of drug delivery methods of any current and potential pharmaceutical therapy to improve UC. In addition to clinical relevance, this data impacts the study of DSS experimental colitis. Whole-colon approaches investigating colitis with a DSS model must consider a loss of effect due to inclusion of colon tissue which is not in a state of inflammation.

The results of this study display striking similarities to DSS treatment of the TACE knockdown model generated by Chalaris et al. In the TACE knockdown mice, although L-selectin and TNFα shedding was "completely abrogated," DSS treated animals displayed increased MPO activity in the colon (Chalaris et al., 2010). Expression of IL-10 and IL-11 (a member of the IL-6-type cytokine family) were also up-regulated. These previous results correlate well with the surprising increase of MPO of the proximal colon and IL-10 of the distal colon during TACE inhibition. In addition, a lack of effect on clinical parameters is also explained. Charlaris et al., observed decreased soluble TNFR2 in the TACE knockdown model. Preloading the tissue with TACE inhibitor may sensitize the cell to soluble and membrane TNFα and prevent sTNFα sequestering effects of soluble TNFR2 (Fig. 28). This effect would compound other pro-inflammatory effects of impaired epithelial regeneration from inhibited EGF signaling during TACE inhibition. In contrast, oral administration of a TACE inhibitor instead of IP injection may allow for greater reduction in colon TNFα levels and improved recovery from DSS induced colitis (Sharma et al., 2014).

Concluding Remarks and Summary. TACE inhibition did not decrease clinical scoring parameters or reduce concentration of inflammatory biomarkers in the colon during experimental colitis. Regional analysis of the colon in the DSS colitis mouse model indicates a disproportionate inflammation of the distal aspect. These data provide novel information to field of experimental colitis due to DSS.

Limitations of Data

These data are limited in a number of ways, primarily by sample size. The most

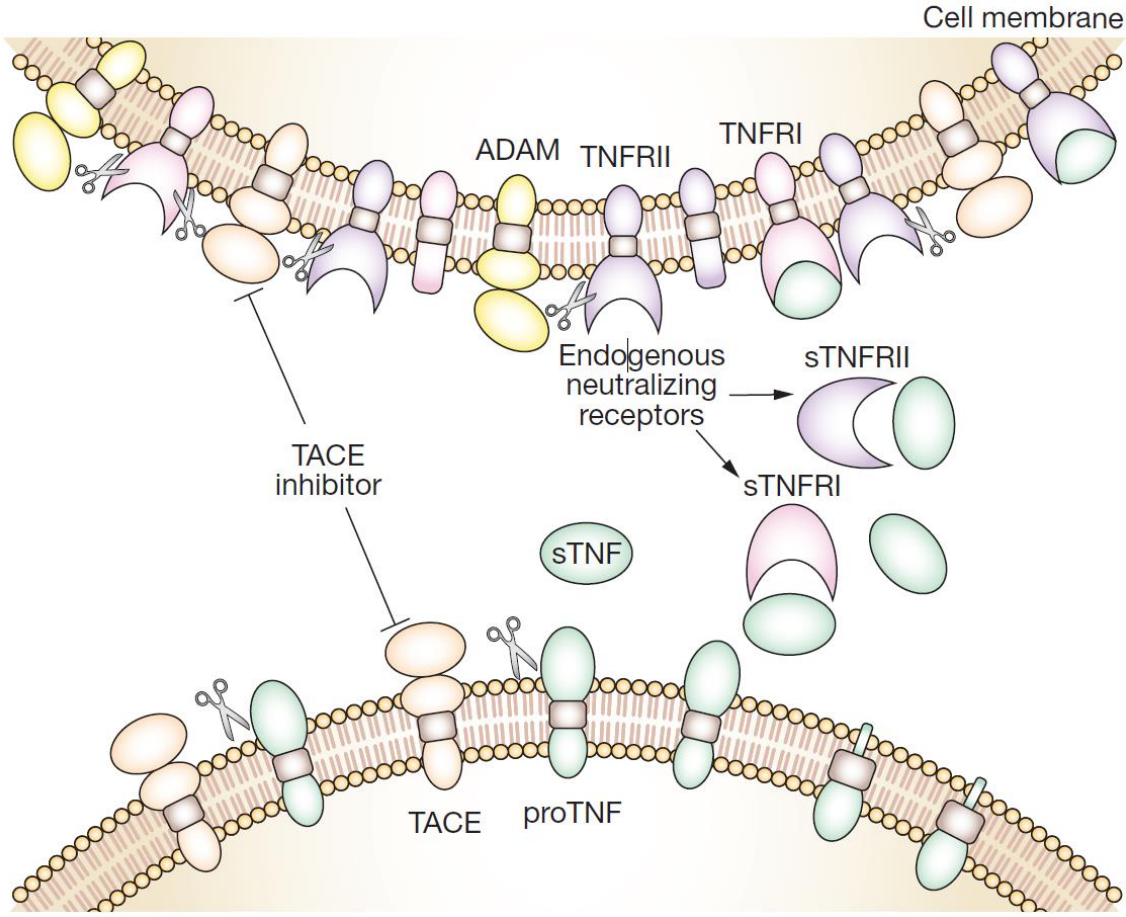

Figure 28. Implications of TACE Inhibition and Receptor Cleavage. In addition to TNFα, TACE is a principle sheddase of TNFR2. Inhibition of TACE may increase cell surface TNFR and sensitize the cell to sTNFα. Decreased receptor cleavage may also be pro-inflammatory as a consequence of lost neutralization of sTNF by soluble TNFR (Moss et al., 2008).

appropriate statistical analysis of this study design would be a 2-way ANOVA to determine effect of the two independent variables (DSS consumption, DPC-333 injections). However, because of small sample size, independent t tests were used to compare groups for induction of colitis and effect of DPC-333 injections during colitis. Because of this, effect of the inhibitor alone (Group 2: H_2O /DPC-333 Injection) was not statistically analyzed. However, TACE inhibition alone, including in the TACE knockdown model of Chalaris et al. has not previously presented pro-inflammatory

effects to date. In addition, large and non-significant percent differences are a common aspect of these results. Increased sample size may provide statistical significance in a number of comparisons.

The pre-clinical data is limited by lack of blinding during evaluation of the clinical parameters. A previous trial with TACE inhibition during development of 3% DSS colitis indicated TACE inhibition would not improve clinical parameters or DAI (data not shown). Because of this, blinding was omitted to increase confidence in administration of vehicle and DPC-333 to respective mice during twice daily injections. Although the bleeding and stool consistency data shows statistical significance, overall DAI is not reported to improve with TACE inhibition. In future studies, if TACE inhibition can be tailored to positively affect the clinical parameters of the DSS colitis, the clinical data will need to be blinded to ensure elimination of any unforeseen potential bias. Evaluation of colitis in future study would benefit from histological evaluation of the colon mucosa to investigate tissue morphology and impact of treatment on structure.

The effects of TACE inhibition preloading on colon MPO are supported by the regional cytokine analysis. However, much more support would have been provided through quantification of chemotactic factors, such as the chemokine IL-8. Inclusion of chemokines in the immunoassay would have allowed evaluation of signaling received by circulating neutrophils to undergo the process of emigration. The current conclusions hypothesized for this study would be strengthened if TACE inhibition during the development of colitis increased IL-8 in the proximal colons.

Future Study

Long-term treatment of IBDs such as UC and CD with TACE inhibition may not be beneficial due to side effects from reduced cleavage of other TACE (ADAM17) substrates such as EGF, angiotensin converting enzyme, and amyloid precursor protein (Allinson et al., 2004). This study and previous research also suggest TACE inhibition prior to the presence of an inflammatory stimulus may be detrimental. The clinical relevance of the protective effects of TACE inhibition in acute systemic inflammation models with LPS challenge are unknown (Qian et al., 2007). Preliminary data showing DPC-333 solubilized in 250 mM citric acid saline maintains biological activity and is capable of attenuating elevated plasma TNFα levels during LPS challenge (Fig. 29).

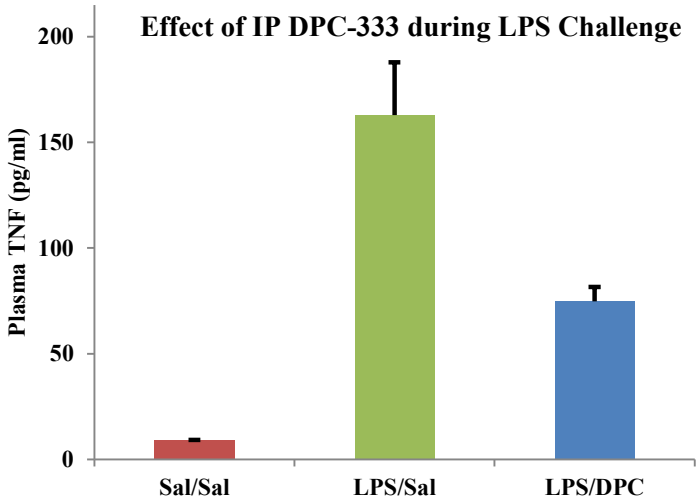

Figure 29. TACE Inhibition with DPC-333 in a Sepsis Model. Preliminary data (n=2 per group) of 10 mg/kg bodyweight IP injection of DPC-333 solubilized in 250 mM citric acid saline. DPC-333 injection was performed 1 hour prior to challenge with LPS (10 mg/kg bodyweight). At t = 5 hours, plasma samples were collected and TNFα quantified with multiplex immunoassay.

Based upon these preliminary data and previous literature, DPC-333 appears to be a more effective plasma inhibitor of TACE activity such as during the onset of sepsis. However, DPC-333 does not appear to positively impact the local tissue response during acute inflammation in this model (Table 4). If substrate-dependent inhibition of TACE is possible, benefit may arise from specific inhibition of sTNFα cleavage. However, more research into TACE substrate recognition is needed. If specific inhibition of mTNFα cleavage is possible, the possibility of cell accumulation of mTNFα and signaling effects would need to be investigated.

REFERENCES

Ajuebor, M.N., Das, A.M., Virág, L., Flower, R.J., Szabó, C., and Perretti, M. (1999). Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10. J. Immunol. *162*, 1685–1691.

Alex, P., Zachos, N.C., Nguyen, T., Gonzales, L., Chen, T.-E., Conklin, L.S., Centola, M., and Li, X. (2009). Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm. Bowel Dis. *15*, 341–352.

Ali, T., Kaitha, S., Mahmood, S., Ftesi, A., Stone, J., and Bronze, M.S. (2013). Clinical use of anti-TNF therapy and increased risk of infections. Drug Healthc Patient Saf *5*, 79–99.

Allinson, T.M.J., Parkin, E.T., Condon, T.P., Schwager, S.L.U., Sturrock, E.D., Turner, A.J., and Hooper, N.M. (2004). The role of ADAM10 and ADAM17 in the ectodomain shedding of angiotensin converting enzyme and the amyloid precursor protein. Eur. J. Biochem. *271*, 2539–2547.

Amin, A.R. (1999). Regulation of tumor necrosis factor-α and tumor necrosis factor converting enzyme in human osteoarthritis. Osteoarthritis and Cartilage *7*, 392–394.

Ardizzone, S., Maconi, G., Russo, A., Imbesi, V., Colombo, E., and Porro, G.B. (2006). Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. Gut *55*, 47–53.

Arnett, H.A., Mason, J., Marino, M., Suzuki, K., Matsushima, G.K., and Ting, J.P.-Y. (2001). TNFα promotes proliferation of oligodendrocyte progenitors and remyelination. Nat Neurosci *4*, 1116–1122.

Azad Khan, A.K., Piris, J., and Truelove, S.C. (1977). An experiment to determine the active therapeutic moiety of sulphasalazine. Lancet *2*, 892–895.

Baert, F., Noman, M., Vermeire, S., Van Assche, G., D' Haens, G., Carbonez, A., and Rutgeerts, P. (2003). Influence of Immunogenicity on the Long-Term Efficacy of Infliximab in Crohn's Disease. New England Journal of Medicine *348*, 601–608.

Bahia, M.S., and Silakari, O. (2010). Tumor necrosis factor alpha converting enzyme: an encouraging target for various inflammatory disorders. Chem Biol Drug Des *75*, 415–443.

Balcewicz-Sablinska, M.K., Keane, J., Kornfeld, H., and Remold, H.G. (1998). Pathogenic Mycobacterium tuberculosis Evades Apoptosis of Host Macrophages by Release of TNF-R2, Resulting in Inactivation of TNF-α. J Immunol *161*, 2636–2641.

Baron, J.H., Connell, A.M., Kanaghinis, T.G., Lennard-Jones, J.E., and Jones, F.A. (1962). Out-patient Treatment of Ulcerative Colitis. Br Med J *2*, 441–443.

Benchimol, E.I., Seow, C.H., Steinhart, A.H., and Griffiths, A.M. (2008). Traditional corticosteroids for induction of remission in Crohn's disease. Cochrane Database Syst Rev CD006792.

Benedek, I., and Fossler, Michael (2003). DPC 333 formulation having unique biopharmaceutical characteristics.

Bernardini, R., Kamilaris, T.C., Calogero, A.E., Johnson, E.O., Gomez, M.T., Gold, P.W., and Chrousos, G.P. (1990). Interactions between Tumor Necrosis Factor-α, Hypothalamic Corticotropin-Releasing Hormone, and Adrenocorticotropin Secretion in the Rat. Endocrinology *126*, 2876–2881.

Bernecker, C., Scherr, J., Schinner, S., Braun, S., Scherbaum, W.A., and Halle, M. (2013). Evidence for an exercise induced increase of TNF-α and IL-6 in marathon runners. Scand J Med Sci Sports *23*, 207–214.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., et al. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature *385*, 729–733.

Bodmer, J.-L., Schneider, P., and Tschopp, J. (2002). The molecular architecture of the TNF superfamily. Trends in Biochemical Sciences *27*, 19–26.

Bohgaki, T., Amasaki, Y., Nishimura, N., Bohgaki, M., Yamashita, Y., Nishio, M., Sawada, K.–., Jodo, S., Atsumi, T., and Koike, T. (2005). Up regulated expression of tumour necrosis factor α converting enzyme in peripheral monocytes of patients with early systemic sclerosis. Ann Rheum Dis *64*, 1165–1173.

Böhm, S.K., and Kruis, W. (2014). Long-term efficacy and safety of once-daily mesalazine granules for the treatment of active ulcerative colitis. Clin Exp Gastroenterol *7*, 369–383.

Bram, R.J., Hung, D.T., Martin, P.K., Schreiber, S.L., and Crabtree, G.R. (1993). Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. Mol. Cell. Biol. *13*, 4760–4769.

Van den Brande, J.M.H., Braat, H., van den Brink, G.R., Versteeg, H.H., Bauer, C.A., Hoedemaeker, I., van Montfrans, C., Hommes, D.W., Peppelenbosch, M.P., and van Deventer, S.J.H. (2003). Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology *124*, 1774–1785.

Brockhaus, M., Schoenfeld, H.J., Schlaeger, E.J., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990). Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci U S A *87*, 3127–3131.

Brynskov, J., Foegh, P., Pedersen, G., Ellervik, C., Kirkegaard, T., Bingham, A., and Saermark, T. (2002). Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. Gut *51*, 37–43.

Cal, S., Freije, J.M., López, J.M., Takada, Y., and López-Otín, C. (2000). ADAM 23/MDC3, a human disintegrin that promotes cell adhesion via interaction with the alphavbeta3 integrin through an RGD-independent mechanism. Mol. Biol. Cell *11*, 1457–1469.

Cao, S., Zhang, X., Edwards, J.P., and Mosser, D.M. (2006). NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J. Biol. Chem. *281*, 26041–26050.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. PNAS *72*, 3666–3670.

CDER, C. for D.E. and R. (2009). Therapeutic Biologic Applications (BLA) - Etanercept Product Approval Information - Licensing Action 12/2/98.

Cesaro, A., Abakar-Mahamat, A., Brest, P., Lassalle, S., Selva, E., Filippi, J., Hébuterne, X., Hugot, J.-P., Doglio, A., Galland, F., et al. (2009). Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia. Am. J. Physiol. Gastrointest. Liver Physiol. *296*, G1332–G1343.

Chalaris, A., Adam, N., Sina, C., Rosenstiel, P., Lehmann-Koch, J., Schirmacher, P., Hartmann, D., Cichy, J., Gavrilova, O., Schreiber, S., et al. (2010). Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice. J. Exp. Med. *207*, 1617–1624.

Chan, F.K., Chun, H.J., Zheng, L., Siegel, R.M., Bui, K.L., and Lenardo, M.J. (2000). A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science *288*, 2351–2354.

Cohen, R.D., Woseth, D.M., Thisted, R.A., and Hanauer, S.B. (2000). A meta-analysis and overview of the literature on treatment options for left-sided ulcerative colitis and ulcerative proctitis. Am. J. Gastroenterol. *95*, 1263–1276.

Collart, M.A., Baeuerle, P., and Vassalli, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. Mol. Cell. Biol. *10*, 1498–1506.

Coope, H.J., Atkinson, P.G.P., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G.G.B., Johnston, L.H., and Ley, S.C. (2002). CD40 regulates the processing of NF-kappaB2 p100 to p52. EMBO J. *21*, 5375–5385.

Cooper, H.S., Murthy, S.N., Shah, R.S., and Sedergran, D.J. (1993). Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab. Invest. *69*, 238–249.

Davies, A.M. (2003). Regulation of neuronal survival and death by extracellular signals during development. EMBO J. *22*, 2537–2545.

Dempsey, P.W., Doyle, S.E., He, J.Q., and Cheng, G. (2003). The signaling adaptors and pathways activated by TNF superfamily. Cytokine and Growth Factor Reviews *14*, 193–209.

Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., and Liu, Z. (2000). The Distinct Roles of TRAF2 and RIP in IKK Activation by TNF-R1: TRAF2 Recruits IKK to TNF-R1 while RIP Mediates IKK Activation. Immunity *12*, 419–429.

D'Haens, G., Geboes, K., Peeters, M., Baert, F., Ectors, N., and Rutgeerts, P. (1997). Patchy cecal inflammation associated with distal ulcerative colitis: a prospective endoscopic study. Am. J. Gastroenterol. *92*, 1275–1279.

Dick, A.P., Grayson, M.J., Carpenter, R.G., and Petrie, A. (1964). Controlled trial of sulphasalazine in the treatment of ulcerative colitis. Gut *5*, 437–442.

Dieleman, L.A., Ridwan, B.U., Tennyson, G.S., Beagley, K.W., Bucy, R.P., and Elson, C.O. (1994). Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology *107*, 1643–1652.

Ea, C.-K., Deng, L., Xia, Z.-P., Pineda, G., and Chen, Z.J. (2006). Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell *22*, 245–257.

Edwards, D.R., Handsley, M.M., and Pennington, C.J. (2008). The ADAM metalloproteinases. Mol. Aspects Med. *29*, 258–289.

Etchevers, M.J., Aceituno, M., García-Bosch, O., Ordás, I., Sans, M., Ricart, E., and Panés, J. (2009). Risk factors and characteristics of extent progression in ulcerative colitis. Inflamm. Bowel Dis. *15*, 1320–1325.

Faveeuw, C., Preece, G., and Ager, A. (2001). Transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is affected by metalloproteinases. Blood *98*, 688–695.

Gearing, A.J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A.H., Drummond, A.H., Galloway, W.A., Gilbert, R., and Gordon, J.L. (1994). Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature *370*, 555–557.

Glocker, E.-O., Kotlarz, D., Boztug, K., Gertz, E.M., Schäffer, A.A., Noyan, F., Perro, M., Diestelhorst, J., Allroth, A., Murugan, D., et al. (2009). Inflammatory Bowel Disease and Mutations Affecting the Interleukin-10 Receptor. New England Journal of Medicine *361*, 2033–2045.

Gómez, M.I., Sokol, S.H., Muir, A.B., Soong, G., Bastien, J., and Prince, A.S. (2005). Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling. J. Immunol. *175*, 1930–1936.

Gore, R.M., Balthazar, E.J., Ghahremani, G.G., and Miller, F.H. (1996). CT features of ulcerative colitis and Crohn's disease. AJR Am J Roentgenol *167*, 3–15.

Gout, T., Östör, A.J.K., and Nisar, M.K. (2011). Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: a systematic literature review. Clin Rheumatol *30*, 1471–1474.

Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., et al. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell *83*, 793–802.

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.-Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L., et al. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell *15*, 103–113.

Grossman, R.M., Chevret, S., Abi-Rached, J., Blanchet, F., and Dubertret, L. (1996). Long-term safety of cyclosporine in the treatment of psoriasis. Arch Dermatol *132*, 623–629.

Gutiérrez, J.M., Rucavado, A., Escalante, T., and Díaz, C. (2005). Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. Toxicon *45*, 997–1011.

Gyrd-Hansen, M., and Meier, P. (2010). IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat. Rev. Cancer *10*, 561–574.

Haas, T.L., Emmerich, C.H., Gerlach, B., Schmukle, A.C., Cordier, S.M., Rieser, E., Feltham, R., Vince, J., Warnken, U., Wenger, T., et al. (2009). Recruitment of the Linear Ubiquitin Chain Assembly Complex Stabilizes the TNF-R1 Signaling Complex and Is Required for TNF-Mediated Gene Induction. Molecular Cell *36*, 831–844.

Hamamoto, N., Maemura, K., Hirata, I., Murano, M., Sasaki, S., and Katsu, K. (1999). Inhibition of dextran sulphate sodium (DSS)-induced colitis in mice by intracolonically administered antibodies against adhesion molecules (endothelial leucocyte adhesion molecule-1 (ELAM-1) or intercellular adhesion molecule-1 (ICAM-1)). Clin Exp Immunol *117*, 462–468.

Heinecke, J.W. (1999). Mechanisms of oxidative damage by myeloperoxidase in atherosclerosis and other inflammatory disorders. J. Lab. Clin. Med. *133*, 321–325.

Hiscott, J., Marois, J., Garoufalis, J., D'Addario, M., Roulston, A., Kwan, I., Pepin, N., Lacoste, J., Nguyen, H., and Bensi, G. (1993). Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. Mol. Cell. Biol. *13*, 6231–6240.

Hooper, N.M. (1994). Families of zinc metalloproteases. FEBS Letters *354*, 1–6.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495–504.

Huang, T.F., Holt, J.C., Lukasiewicz, H., and Niewiarowski, S. (1987). Trigramin. A low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb-IIIa complex. J. Biol. Chem. *262*, 16157–16163.

Ito, H., Takazoe, M., Fukuda, Y., Hibi, T., Kusugami, K., Andoh, A., Matsumoto, T., Yamamura, T., Azuma, J., Nishimoto, N., et al. (2004). A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. Gastroenterology *126*, 989–996; discussion 947.

Kanarek, N., London, N., Schueler-Furman, O., and Ben-Neriah, Y. (2010). Ubiquitination and Degradation of the Inhibitors of NF-κB. Cold Spring Harb Perspect Biol *2*.

Kappelman, M.D., Rifas-Shiman, S.L., Kleinman, K., Ollendorf, D., Bousvaros, A., Grand, R.J., and Finkelstein, J.A. (2007). The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States. Clin. Gastroenterol. Hepatol. *5*, 1424–1429.

Kappelman, M.D., Rifas-Shiman, S.L., Porter, C., Ollendorf, D.A., Sandler, R.S., Galanko, J.A., and Finkelstein, J.A. (2008). Direct Health Care Costs of Crohn's Disease and Ulcerative Colitis in United States Children and Adults. Gastroenterology *135*, 1907–1913.

Kawaguchi, M., Mitsuhashi, Y., and Kondo, S. (2005). Overexpression of tumour necrosis factor-alpha-converting enzyme in psoriasis. Br. J. Dermatol. *152*, 915–919.

Kedia, S., Ahuja, V., and Tandon, R. (2014). Management of acute severe ulcerative colitis. World J Gastrointest Pathophysiol *5*, 579–588.

Kim, M.L., Zhang, B., Mills, I.P., Milla, M.E., Brunden, K.R., and Lee, V.M.-Y. (2008). Effects of TNFalpha-converting enzyme inhibition on amyloid beta production and APP processing in vitro and in vivo. J. Neurosci. *28*, 12052–12061.

Kitajima, S., Takuma, S., and Morimoto, M. (2000). Histological analysis of murine colitis induced by dextran sulfate sodium of different molecular weights. Exp. Anim. *49*, 9–15.

Kornbluth, A., and Sachar, D.B., Practice Parameters Committee of the American College of Gastroenterology (2010). Ulcerative colitis practice guidelines in adults: American College Of Gastroenterology, Practice Parameters Committee. Am. J. Gastroenterol. *105*, 501–523; quiz 524.

Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S.D. (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell *53*, 45–53.

Kruglov, A.A., Lampropoulou, V., Fillatreau, S., and Nedospasov, S.A. (2011). Pathogenic and protective functions of TNF in neuroinflammation are defined by its expression in T lymphocytes and myeloid cells. J. Immunol. *187*, 5660–5670.

Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., and Müller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263–274.

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol *1*, a001651.

Leighton, J.A., Shen, B., Baron, T.H., Adler, D.G., Davila, R., Egan, J.V., Faigel, D.O., Gan, S.-I., Hirota, W.K., Lichtenstein, D., et al., Standards of Practice Committee,

American Society for Gastrointestinal Endoscopy (2006). ASGE guideline: endoscopy in the diagnosis and treatment of inflammatory bowel disease. Gastrointest. Endosc. *63*, 558–565.

Li, Y., Brazzell, J., Herrera, A., and Walcheck, B. (2006). ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding. Blood *108*, 2275–2279.

Libermann, T.A., and Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol. Cell. Biol. *10*, 2327–2334.

Lichtenstein, G.R., Abreu, M.T., Cohen, R., and Tremaine, W. (2006). American Gastroenterological Association Institute Technical Review on Corticosteroids, Immunomodulators, and Infliximab in Inflammatory Bowel Disease. Gastroenterology *130*, 940–987.

Lichtiger, S., Present, D.H., Kornbluth, A., Gelernt, I., Bauer, J., Galler, G., Michelassi, F., and Hanauer, S. (1994). Cyclosporine in Severe Ulcerative Colitis Refractory to Steroid Therapy. New England Journal of Medicine *330*, 1841–1845.

Lindsay, J., Van Montfrans, C., Brennan, F., Van Deventer, S., Drillenburg, P., Hodgson, H., Te Velde, A., and Sol Rodriguez Pena, M. (2002). IL-10 gene therapy prevents TNBS-induced colitis. Gene Ther. *9*, 1715–1721.

Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology. Cell *104*, 487–501.

Ludwiczek, O., Vannier, E., Borggraefe, I., Kaser, A., Siegmund, B., Dinarello, C.A., and Tilg, H. (2004). Imbalance between interleukin-1 agonists and antagonists: relationship to severity of inflammatory bowel disease. Clin. Exp. Immunol. *138*, 323–329.

Lum, L., Reid, M.S., and Blobel, C.P. (1998). Intracellular Maturation of the Mouse Metalloprotease Disintegrin MDC15. J. Biol. Chem. *273*, 26236–26247.

Luminex (2011). The New MAGPIX® Multiplexing System. BioProbes *66*, 14–15.

Marchetti, L., Klein, M., Schlett, K., Pfizenmaier, K., and Eisel, U.L.M. (2004). Tumor Necrosis Factor (TNF)-mediated Neuroprotection against Glutamate-induced Excitotoxicity Is Enhanced by N-Methyl-D-aspartate Receptor Activation ESSENTIAL ROLE OF A TNF RECEPTOR 2-MEDIATED PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT NF-κB PATHWAY. J. Biol. Chem. *279*, 32869–32881.

Marlow, G.J., van Gent, D., and Ferguson, L.R. (2013). Why interleukin-10 supplementation does not work in Crohn's disease patients. World J Gastroenterol *19*, 3931–3941.

Maskos, K., Fernandez-Catalan, C., Huber, R., Bourenkov, G.P., Bartunik, H., Ellestad, G.A., Reddy, P., Wolfson, M.F., Rauch, C.T., Castner, B.J., et al. (1998). Crystal structure of the catalytic domain of human tumor necrosis factor-α-converting enzyme. Proc Natl Acad Sci U S A *95*, 3408–3412.

McDermott, M.F., Aksentijevich, I., Galon, J., McDermott, E.M., Ogunkolade, B.W., Centola, M., Mansfield, E., Gadina, M., Karenko, L., Pettersson, T., et al. (1999). Germline Mutations in the Extracellular Domains of the 55 kDa TNF Receptor, TNFR1, Define a Family of Dominantly Inherited Autoinflammatory Syndromes. Cell *97*, 133–144.

McGeehan, G.M., Becherer, J.D., Bast, R.C., Boyer, C.M., Champion, B., Connolly, K.M., Conway, J.G., Furdon, P., Karp, S., and Kidao, S. (1994). Regulation of tumour necrosis factor-alpha processing by a metalloproteinase inhibitor. Nature *370*, 558–561.

Melgar, S., Karlsson, A., and Michaëlsson, E. (2005). Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. American Journal of Physiology - Gastrointestinal and Liver Physiology *288*, G1328–G1338.

Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J. wu, Young, D.B., Barbosa, M., Mann, M., Manning, A., et al. (1997). IKK-1 and IKK-2: Cytokine-Activated IκB Kinases Essential for NF-κB Activation. Science *278*, 860–866.

Micheau, O., and Tschopp, J. (2003). Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes. Cell *114*, 181–190.

Mohler, K.M., Sleath, P.R., Fitzner, J.N., Cerretti, D.P., Alderson, M., Kerwar, S.S., Torranee, D.S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., et al. (1994). Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. Nature *370*, 218–220.

Moss, M.L., Jin, S.-L.C., Milla, M.E., Burkhart, W., Carter, H.L., Chen, W.-J., Clay, W.C., Didsbury, J.R., Hassler, D., Hoffman, C.R., et al. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. Nature *385*, 733–736.

Moss, M.L., White, J.M., Lambert, M.H., and Andrews, R.C. (2001). TACE and other ADAM proteases as targets for drug discovery. Drug Discov. Today *6*, 417–426.

Moss, M.L., Sklair-Tavron, L., and Nudelman, R. (2008). Drug Insight: tumor necrosis factor-converting enzyme as a pharmaceutical target for rheumatoid arthritis. Nature Clinical Practice Rheumatology *4*, 300–309.

Mózes, T., Baráth, I., Gornicsar, K., Grosz, A., Mózes, T., Göndöcs, C., Széphalmi, P., Gaál, K., and Madarász, E. (2011). Deviations in circulating TNFα levels and TNFα production by mononuclear cells in healthy human populations. Mediators Inflamm. *2011*, 972609.

Mudter, J., and Neurath, M.F. (2007). Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. Inflamm. Bowel Dis. *13*, 1016–1023.

Munkholm, P., Langholz, E., Davidsen, M., and Binder, V. (1994). Frequency of glucocorticoid resistance and dependency in Crohn's disease. Gut *35*, 360–362.

Munoz, C., Carlet, J., Fitting, C., Misset, B., Blériot, J.P., and Cavaillon, J.M. (1991). Dysregulation of in vitro cytokine production by monocytes during sepsis. J Clin Invest *88*, 1747–1754.

Murray, P.J. (2005). The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. PNAS *102*, 8686–8691.

Nath, D., Slocombe, P.M., Webster, A., Stephens, P.E., Docherty, A.J., and Murphy, G. (2000). Meltrin gamma(ADAM-9) mediates cellular adhesion through alpha(6)beta(1)integrin, leading to a marked induction of fibroblast cell motility. J. Cell. Sci. *113 (Pt 12)*, 2319–2328.

Nelson, R., Liao, C., Fichera, A., Rubin, D.T., and Pekow, J. (2014). Rescue therapy with cyclosporine or infliximab is not associated with an increased risk for postoperative complications in patients hospitalized for severe steroid-refractory ulcerative colitis. Inflamm. Bowel Dis. *20*, 14–20.

Oh, J., Arkfeld, D.G., and Horwitz, D.A. (2005). Development of Crohn's disease in a patient taking etanercept. J Rheumatol *32*, 752–753.

Okayasu, I., Hatakeyama, S., Yamada, M., Ohkusa, T., Inagaki, Y., and Nakaya, R. (1990). A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology *98*, 694–702.

Patel, I.R., Attur, M.G., Patel, R.N., Stuchin, S.A., Abagyan, R.A., Abramson, S.B., and Amin, A.R. (1998). TNF-alpha convertase enzyme from human arthritis-affected cartilage: isolation of cDNA by differential display, expression of the active enzyme, and regulation of TNF-alpha. J. Immunol. *160*, 4570–4579.

Peppel, K., Crawford, D., and Beutler, B. (1991). A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. J Exp Med *174*, 1483–1489.

Peppercorn, M.A., and Goldman, P. (1972). The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J. Pharmacol. Exp. Ther. *181*, 555–562.

Peschon, J.J., Slack, J.L., Reddy, P., Stocking, K.L., Sunnarborg, S.W., Lee, D.C., Russell, W.E., Castner, B.J., Johnson, R.S., Fitzner, J.N., et al. (1998). An Essential Role for Ectodomain Shedding in Mammalian Development. Science *282*, 1281–1284.

Present, D.H., Korelitz, B.I., Wisch, N., Glass, J.L., Sachar, D.B., and Pasternack, B.S. (1980). Treatment of Crohn's Disease with 6-Mercaptopurine. New England Journal of Medicine *302*, 981–987.

Pruessmeyer, J., and Ludwig, A. (2009). The good, the bad and the ugly substrates for ADAM10 and ADAM17 in brain pathology, inflammation and cancer. Semin. Cell Dev. Biol. *20*, 164–174.

Pulli, B., Ali, M., Forghani, R., Schob, S., Hsieh, K.L.C., Wojtkiewicz, G., Linnoila, J.J., and Chen, J.W. (2013). Measuring Myeloperoxidase Activity in Biological Samples. PLoS One *8*.

Qian, M., Bai, S.A., Brogdon, B., Wu, J.-T., Liu, R.-Q., Covington, M.B., Vaddi, K., Newton, R.C., Fossler, M.J., Garner, C.E., et al. (2007). Pharmacokinetics and Pharmacodynamics of DPC 333 ((2R)-2-((3R)-3-Amino-3{4-[2-methyl-4-quinolinyl) methoxy] phenyl}-2-oxopyrrolidinyl)-N-hydroxy-4-methylpentanamide)), a Potent and Selective Inhibitor of Tumor Necrosis Factor α-Converting Enzyme in Rodents, Dogs, Chimpanzees, and Humans. Drug Metab Dispos *35*, 1916–1925.

Qiu, W., Wu, B., Wang, X., Buchanan, M.E., Regueiro, M.D., Hartman, D.J., Schoen, R.E., Yu, J., and Zhang, L. (2011). PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. J Clin Invest *121*, 1722–1732.

Rauert, H., Wicovsky, A., Müller, N., Siegmund, D., Spindler, V., Waschke, J., Kneitz, C., and Wajant, H. (2010). Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). J. Biol. Chem. *285*, 7394–7404.

Reinecker, H.C., Steffen, M., Witthoeft, T., Pflueger, I., Schreiber, S., MacDermott, R.P., and Raedler, A. (1993). Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol *94*, 174–181.

Rothe, M., Pan, M.-G., Henzel, W.J., Ayres, T.M., and V. Goeddel, D. (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell *83*, 1243–1252.

Rutgeerts, P., Sandborn, W.J., Feagan, B.G., Reinisch, W., Olson, A., Johanns, J., Travers, S., Rachmilewitz, D., Hanauer, S.B., Lichtenstein, G.R., et al. (2005). Infliximab for Induction and Maintenance Therapy for Ulcerative Colitis. New England Journal of Medicine *353*, 2462–2476.

Rutter, M., Saunders, B., Wilkinson, K., Rumbles, S., Schofield, G., Kamm, M., Williams, C., Price, A., Talbot, I., and Forbes, A. (2004). Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. Gastroenterology *126*, 451–459.

Ruuls, S.R., Hoek, R.M., Ngo, V.N., McNeil, T., Lucian, L.A., Janatpour, M.J., Körner, H., Scheerens, H., Hessel, E.M., Cyster, J.G., et al. (2001). Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. Immunity *15*, 533–543.

Sandborn, W.J., Hanauer, S.B., Katz, S., Safdi, M., Wolf, D.G., Baerg, R.D., Tremaine, W.J., Johnson, T., Diehl, N.N., and Zinsmeister, A.R. (2001). Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. Gastroenterology *121*, 1088–1094.

Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nat Rev Immunol *10*, 170–181.

Scheinin, T., Butler, D.M., Salway, F., Scallon, B., and Feldmann, M. (2003). Validation of the interleukin-10 knockout mouse model of colitis: antitumour necrosis factor-antibodies suppress the progression of colitis. Clin Exp Immunol *133*, 38–43.

Schoepfer, A.M., Beglinger, C., Straumann, A., Safroneeva, E., Romero, Y., Armstrong, D., Schmidt, C., Trummler, M., Pittet, V., and Vavricka, S.R. (2013). Fecal calprotectin more accurately reflects endoscopic activity of ulcerative colitis than the Lichtiger Index, C-reactive protein, platelets, hemoglobin, and blood leukocytes. Inflamm. Bowel Dis. *19*, 332–341.

Schultz, J., and Kaminker, K. (1962). Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. Arch. Biochem. Biophys. *96*, 465–467.

Seals, D.F., and Courtneidge, S.A. (2003). The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev. *17*, 7–30.

Sharma, M., Mohapatra, J., Wagh, A., Patel, H.M., Pandey, D., Kadam, S., Argade, A., Deshpande, S.S., Shah, G.B., Chatterjee, A., et al. (2014). Involvement of TACE

in colon inflammation: a novel mechanism of regulation via SIRT-1 activation. Cytokine *66*, 30–39.

Sibilia, M., and Wagner, E.F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. Science *269*, 234–238.

Simpkins, K.C. (1977). Aphthoid ulcers in Crohn's colitis. Clin Radiol *28*, 601–608.

Smith, C.W. (1993). Endothelial adhesion molecules and their role in inflammation. Can. J. Physiol. Pharmacol. *71*, 76–87.

Smolen, J.S., Beaulieu, A., Rubbert-Roth, A., Ramos-Remus, C., Rovensky, J., Alecock, E., Woodworth, T., and Alten, R. (2008). Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. The Lancet *371*, 987–997.

Solomon, K.A., Pesti, N., Wu, G., and Newton, R.C. (1999). Cutting edge: a dominant negative form of TNF-alpha converting enzyme inhibits proTNF and TNFRII secretion. J. Immunol. *163*, 4105–4108.

Song, H.Y., Rothe, M., and Goeddel, D.V. (1996). The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation. Proc Natl Acad Sci U S A *93*, 6721–6725.

Stöcker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Rüth, F.X., McKay, D.B., and Bode, W. (1995). The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci *4*, 823–840.

Strober, W., Fuss, I.J., and Blumberg, R.S. (2002). The immunology of mucosal models of inflammation. Annu. Rev. Immunol. *20*, 495–549.

Targownik, L., Singh, H., Nugent, Z., and Bernstein, C.N. (2012). The epidemiology of colectomy in ulcerative colitis. AJG *107*, 1228–1235.

Tartaglia, L.A., Ayres, T.M., Wong, G.H.W., and Goeddel, D.V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. Cell *74*, 845–853.

Toruner, M., Loftus, E.V., Harmsen, W.S., Zinsmeister, A.R., Orenstein, R., Sandborn, W.J., Colombel, J.-F., and Egan, L.J. (2008). Risk factors for opportunistic infections in patients with inflammatory bowel disease. Gastroenterology *134*, 929–936.

Truelove, S.C., and Witts, L.J. (1954). Cortisone in Ulcerative Colitis. Br Med J *2*, 375–378.

Tulchinsky, H., Hawley, P.R., and Nicholls, J. (2003). Long-term failure after restorative proctocolectomy for ulcerative colitis. Ann. Surg. *238*, 229–234.

Varfolomeev, E., Goncharov, T., Fedorova, A.V., Dynek, J.N., Zobel, K., Deshayes, K., Fairbrother, W.J., and Vucic, D. (2008). c-IAP1 and c-IAP2 Are Critical Mediators of Tumor Necrosis Factor α (TNFα)-induced NF-κB Activation. J Biol Chem *283*, 24295–24299.

Wajant, H., Henkler, F., and Scheurich, P. (2001). The TNF-receptor-associated factor family: Scaffold molecules for cytokine receptors, kinases and their regulators. Cellular Signalling *13*, 389–400.

Wallach, D., Engelmann, H., Nophar, Y., Aderka, D., Kemper, O., Hornik, V., Holtmann, H., and Brakebusch, C. (1991). Soluble and cell surface receptors for tumor necrosis factor. Agents Actions Suppl. *35*, 51–57.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., and Baldwin, A.S. (1998). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680–1683.

Van Wart, H.E., and Birkedal-Hansen, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci U S A *87*, 5578–5582.

Wiens, G.D., and Glenney, G.W. (2011). Origin and evolution of TNF and TNF receptor superfamilies. Developmental & Comparative Immunology *35*, 1324–1335.

Wirtz, S., Neufert, C., Weigmann, B., and Neurath, M.F. (2007). Chemically induced mouse models of intestinal inflammation. Nat. Protocols *2*, 541–546.

Yan, Y., Kolachala, V., Dalmasso, G., Nguyen, H., Laroui, H., Sitaraman, S.V., and Merlin, D. (2009). Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. PLoS ONE *4*, e6073.

Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., and Su, B. (2001). The essential role of MEKK3 in TNF-induced NF-κB activation. Nat Immunol *2*, 620–624.

Zhang, X.P., Kamata, T., Yokoyama, K., Puzon-McLaughlin, W., and Takada, Y. (1998). Specific interaction of the recombinant disintegrin-like domain of MDC-15 (metargidin, ADAM-15) with integrin alphavbeta3. J. Biol. Chem. *273*, 7345–7350.