The Microbiology and Immunology of Ileocecal Resection

By

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Abstract

Ileocolic resection is frequently performed for Crohn's disease; however, disease commonly recurs early in the neo-terminal ileum. The aim of this thesis was to investigate the microbial and immunologic factors inherent to ileocecal resection as they pertain to post-operative recurrence of Crohn's disease in the ileum using mouse models. The ileocolic procedure was developed in the mouse and used in models of intestinal inflammation including the IL-10$^{-/-}$ genetic knockout and the dextran sodium sulfate induced colitis models. A multitude of techniques in cell biology and biochemistry were used to characterize the local intestinal and systemic changes in the host microbiome and immune function.

Main results included local immune suppression in the intestinal tissues following ileocecal resection. This was associated with changes in macrophage and dendritic cell populations. Bacterial overgrowth in the ileum was found in both models of disease with with bacterial invasion into the lamina propria contributing to post-operative ileitis in the IL-10$^{-/-}$ mice. Significant perturbations in microbial clades defined by whole metagenome shotgun sequencing were also found.

Ileocecal resection causes significant shifts in the both the gut microbiome and mononuclear phagocyte populations. This thesis suggests immune dysfunction with impaired microbial clearance may be to blame for the early ulcerations frequently found in the ileum of Crohn's disease patients following ileocecal resection.

Preface

This thesis is an original work by Troy Perry. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Probiotic bacteria and epithelial cells and Breeding Colony.", No. AUC00000293, December 13 2013.

In accordance with standards of conducting a systematic review, chapter 2 in this thesis was co-authored by Bryan Dicken. I was responsible for study design, search strategy, data collection and writing. Bryan Dicken acted as the second reviewer for articles included in the meta-analysis.

Chapter 3 of this thesis is in press as: Perry, T., Borowiec, A., Dicken, B., Fedorak, R., Madsen, K. Murine Ileocolic Bowel Resection with Primary Anastomosis. *J. Vis. Exp*, e52106, doi:10.3791/52106 (2014). I was responsible for development of the technique and writing of the article. A. Borowiec, B. Dicken, R. Fedorak and K. Madsen assisted in development of the technique and critical reviews of the text.

Chapter 5 contains collaborative work with J. Jovel from The Applied Genomics Center at the University of Alberta. J. Jovel performed the computational analysis of the shotgun sequencing data. I was responsible for study design, experimental methods and writing of the article.

The remainder of the chapters in this thesis are original work performed by myself in consultation with my supervisory committee.

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Chapter 1. Introduction

1.1 Crohn's Disease

Crohn's disease (CD) was defined in 1932 when Crohn, Ginzburg and Oppenheimer published a paper describing "regional ileitis"(Crohn et al., 1984). As the medical community discovered the disease could affect far more than just the ileum the term "Crohn's disease" was coined to encompass the disease with all of its various manifestations.

CD is now defined as a chronic inflammatory condition with the potential to affect any part of the alimentary tract. Holding true to its original description it tends to most commonly affect the distal small bowel (terminal Ileum) and proximal colon (cecum and ascending colon). No single etiology for CD has been defined. Instead, studies to date have suggested that development of CD is multifactorial, occuring in genetically susceptible individuals under environmental and microbial influences(Abraham & Cho, 2009). The final common pathway causing symptomatic CD is the sustained immune response leading to ulceration, fistulization and stricturing of the bowel(Baumgart & Sandborn, 2012).

This section will briefly review the etiologies of CD, clinical manifestations, and current therapeutic options for active CD.

1.2 Etiology

1.2.1 Genetics of Crohn's Disease

Genetics have always appeared to play an important role in the development of CD. Observations in monozygotic twins have found concordance rates as high as 67% and there is a 15 times increase in relative risk for first degree relatives of CD patients(Bruce E Sands, 2010). The genes responsible for conferring susceptibility to CD are still the subject of investigation but new techniques mainly genome-wide association studies (GWAS) have made the identification of susceptibility genes and pathways far more efficient(Manolio, 2010). A recent meta-analysis combined all of the GWAS performed for CD. The results increased the number of known susceptibility loci to 71(Franke et al., 2010). The majority of the genes discovered to date point to a few key features of the gut immune system including the mucosal barrier, innate recognition of microbes, antigen processing through autophagy, cytokine signaling pathways and activation of lymphocytes(Franke et al., 2010).

The first gene implicated, and still the gene with the strongest association in CD is NOD2. The protein product of NOD2 is found in dendritic cells, macrophages and cells of the epithelial barrier in the gut. NOD2 functions in innate recognition of muramyl dipeptide (MDP), found in the cell wall of gram positive, and gram negative bacteria. Homozygous carriers of mutations causing a loss of function in NOD2 have a 17-fold increase in risk for CD(Economou, Trikalinos, Loizou, Tsianos, & Ioannidis, 2004). The exact role of NOD2 in the inflammation of CD is unclear, but recent studies suggest that recognition of MDP and signaling through NOD2 may have an anti-inflammatory effect in the gut(Fernandez et al., 2011; Hedl, Hedl, Abraham, & Abraham, 2011). An

alternative theory suggests that NOD2 is important in facilitating the clearance of bacteria preventing over growth and invasion by up-regulating innate immunity pathways. This is supported by the observation that NOD2 associated CD patients have decreased levels of alpha-defensins in the bowel(Wehkamp, 2004). The summation of the evidence for NOD2 suggests the interplay between the innate immune system and gut bacteria is central in the development of CD.

The discovery of ATG16L1 and IRGM mutations in CD patients turned attention to autophagy as potential pathway contributing to the inflammatory response. Autophagy is the process by which cellular components are engulfed into autophagosomes, and degraded in lysosomes in the cell cytoplasm. This process serves several purposes one of which is destruction of intracellular microbes(Fritz, Niederreiter, Adolph, Blumberg, & Kaser, 2011). Both ATG16L1 and IRGM are important in the processing of intracellular microbes through the autophagy pathway and loss of function in these renders intestinal epithelial cells susceptible to infection with invasive bacteria such as enteroadherent invasive *E. coli* (AIEC). Recently it was shown that NOD2 plays a role in autophagy specifically in bacterial handling by dendritic cells(Cooney et al., 2010) and this is dependent on an interaction with ATG16L1(Cooney et al., 2010; Homer, Richmond, Rebert, Achkar, & McDonald, 2010). This suggests that bacterial sensing by NOD2 with subsequent ATG16L1 and IRGM dependent formation of autolysosomes for bacterial killing and antigen presentation play a role in the imbalanced immune response characteristic of CD.

Down stream of the innate immune response adaptive responses are the final common pathway in the inflammation of CD. Genetic variants involving the IL-23/Th-17 adaptive

immune response pathway have been found to be protective against the development of CD(Sarra, Pallone, MacDonald, & Monteleone, 2010).

1.2.2 Bacteria and Crohn's Disease

Bacteria have long been implicated in the pathogenesis of CD. This began with the observation that mycobacterium cause a similar disease in ruminants and mycobacteria can occasionally be found in the diseased bowel of CD(Sartor, 2005). Other observations driving this theory include evidence for decreased diversity of bacteria in the bowel of CD patients(Ott et al., 2004) with increase in potentially pathogenic species such as AIEC(Darfeuille-Michaud et al., 2004; 1998), altered bacterial profiles in the bowel of discordant monzygotic twins with CD(B. Willing et al., 2009), the requirement of most IBD animals models to be colonized with bacteria(Wirtz & Neurath, 2007), and improvement in CD activity can often be seen with anti-microbial therapy(Chamberlin & Borody, 2011). No single causative organism has ever been identified for CD. Instead CD appears to develop out of a dysbiosis with a decrease in overall diversity of gut bacterial flora and a relative increase in potentially pathogenic organisms(Mac A Johnson & Winquist, 2011; Ott et al., 2004). The subject of the gut microbiome and Crohn's disease will be discussed in detail in section 1.3.

1.2.3 The Immune Response and Crohn's Disease

Deregulated immune activation is the final endpoint causing inflammation and tissue damage in CD and the cytokine environment is the defining feature of an immune response(Strober, Strober, Fuss, & Fuss, 2011). CD was classically thought to be the result of an inappropriate T helper 1 (Th1) response with production of IFN-ϒ by Th1 cells promoted by IL-12 production from innate cells mainly macrophages and DCs.

Recently the T helper 17 (Th17) response has been identified as player in CD. This was first identified in animal models of IBD and gained further attention when mutations in the Th17 pathway were found to confer a decreased susceptibility to IBD. The Th17 response is defined by IL-17 production by Th17 cells and requires TGF-β with IL-6 to cause Th17 differentiation(Bonnefoy et al., 2011). The response is further promoted by IL-23. Other cytokines, which play a role in IBD, include TNF-α, IL-1β, TL1A, and Il-13. These cytokines serve to promote and support the inflammatory response(Strober et al., 2011). See section 1.4 for a detailed discussion of the role of macrophages and DCs in mucosal immunology and CD.

1.2.4 Environment

Environmental influences on CD have been documented in large epidemiological studies. Those that appear to increase risk include smoking, NSAIDS, diet high in refined sugars, living in northern latitudes, higher socioeconomic status(Bruce E Sands, 2010), low exposure to sunlight(Nerich et al., 2011), and antibiotic use in childhood(Hviid et al., 2011). Protective factors include breast feeding, and outdoor occupations(Bruce E Sands, 2010). The relative contribution of these factors to development of disease is unclear but the single biggest modifiable risk factor for CD is smoking(Cottone, Rosselli, & Orlando, 1994).

1.3 Gut Microbiology in health and in CD

The human gut microbiome consists of and estimated 1000 to 1150 species based on large scale studies(Huttenhower et al., 2012; Qin et al., 2010) with each individual

harboring approximately 160 species although this number may increase as sequencing technology improves to provide greater depth(Qin et al., 2010).

Colonization of the gut with bacteria begins at birth(Matamoros, Gras-Leguen, Le Vacon, Potel, & La Cochetiere, 2013), and fluctuates through life, while reflecting and contributing to states of health and disease.

1.3.1 Development of the Gut Microbiome

The initial colonization of the gut is dependent on both mode of delivery and gestational age. As might be expected, vaginal delivery will lead to colonization of microbes commonly found in the vagina including *Lacotbacillus,* and *Prevotella* species(Huttenhower et al., 2012; Matamoros et al., 2013), whereas cesarian section will lead to colonization with skin flora including *Staphylococcus, Corynebacterium* and *Propionibacterium*(Matamoros et al., 2013). Pre-term infants are predisposed to colonization with microbes from the *Enterobacteriaceae* family and *Enterococcus* genus(Arboleya et al., 2012; Schwiertz et al., 2003). Before the gut can be colonized with strict anaerobes the facultative organisms create an anoxic environment(Rigottier-Gois, 2013) allowing populations of *Bifidobacterium* to dominate(Espey, 2013; Matamoros et al., 2013).

The gut microbiota in the first years of life is very volatile with large inter-individual variability and extreme shifts in response to environmental and dietary factors(Koenig et al., 2011; Palmer, Bik, DiGiulio, Relman, & Brown, 2007) however by three years a diverse community begins to form, which more closely resembles the adult microbiome(Yatsunenko et al., 2012).

1.3.2 Adult gut microbiome

In adults the human gut microbiome consists primarily of Bacteroidetes and Firmucutes with minor contributions from other phyla including Actinobacteria, Proteobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria(Koboziev, Webb, Furr, & Grisham, 2014).

Small intestine is rich in mono/disaccharides and amino acids, which support the growth of Proteobacteria and Lactobacillales. Simple sugars are depleted through the small bowel leaving polysaccharides from the mucin and diet as the primary energy source in the colon. Bacteroides and Clostridiales are capable of utilizing indigestible carbohydrates and host glycan for energy giving them an advantage in the colon(Koropatkin, Cameron, & Martens, 2012). This also follows the oxygen gradient through the gastrointestinal tract, allowing for facultative anaerobes in the small intestine and obligate anaerobes in the colon(Koboziev, Webb, Furr, & Grisham, 2013).

Controversy on the existence of a "normal" or "core" microbiome made up of essential taxa still exists. There are however several taxonomic groups that have positive associations with human health(Hollister, Gao, & Versalovic, 2014). These groups include *Lactobacillus, Bacteroides, Bifidobacterium*, and *Clostridium* clusters IV & XIV(Hollister et al., 2014). With the exception of *Lactobacillus* all of these taxa include strict anaerobes. Diversity of gut bacteria within and individual (α-diversity) appears to be a hallmark of good health. This observation has been made in studies on IBD, obesity and geriatrics(Claesson et al., 2012; Kostic, Xavier, & Gevers, 2014; Le Chatelier et al., 2014; Morgan et al., 2012; Qin et al., 2010). The absence of known pathogens and presence *Proteobacteria* also appears to be important for systemic and gut

health(Hollister et al., 2014). Despite these observations with taxonomic groups there is still wide variation in gut microbes across healthy individuals.

1.3.3 The gut metagenome

The two largest studies completed by the human microbiome and MetaHIT projects have found massive inter-individual diversity (β-diversity) (Huttenhower et al., 2012; Qin et al., 2010). These studies have led to alternative view on the gut microbiome, which may be defined as a community of microbes with a core genome and metagenome(Qin et al., 2010; Turnbaugh et al., 2008), as the genetic potential of the microbiome appears to be more consistent and more sensitive to perturbations in disease states(Morgan et al., 2012). It seems logical that the human gut would provide a specific niche, and microbes capable of specific functions may fill this niche. Genetic potential of bacteria does not necessarily segregate into taxonomic groups. Instead, many species may be capable of similar functions making species identification less important than functional determination(Shafquat, Joice, Simmons, & Huttenhower, 2014). To support this concept, the MetaHIT group released a follow up to their original publication in which they compared the metagenomes of obese and non-obese individuals(Le Chatelier et al., 2014). Instead of defining bacterial clades they instead defined “high gene count” and “low gene count” groups based on metagenomics. Obesity was strongly associated with low gene count gut metagenomes, highlighting the importance of functional diversity. Current understanding of the gut microbial genome is still in its infancy. The original landmark paper from the MetaHIT project(Qin et al., 2010) identified 3.3 million non-redundant microbial genes using WMS. They used this data to define a “minimal gut genome” to describe orthologous groups of genes carried by most bacteria necessary for

survival in the gut, and a "minimal gut metagenome" which describes genes necessary for homeostasis of the gut ecosystem. The latter of these should be found in the host gut but not necessarily in all bacteria.

The minimal genome defined by the highest abundance genes comprises "house keeping" genes present in nearly all bacteria regardless of environment (i.e. energy production, transcription and translation) and genes with functions specific for the gut. This latter group of genes are thought to function in areas such as harvesting host glycolipids for energy and adhesions to host molecules. Nearly 75% of these "gut-specific" genes are as yet undefined(Qin et al., 2010).

The minimal gut metagenome is defined by orthologous genes found in all individuals. The investigators found 6,313 functions in this category and nearly half of these are only found in <10% of known bacterial genomes, with 80% mostly uncharacterized. Of the known functions within the minimal metagenome utilization of complex sugars and glycans through degradation and fermentation was prominent. The fermentations process leads to the production of SCFAs that have multiple beneficial effects for the host as will be discussed in detail later. Other complimentary functions of the gut metagenome include production of essential amino acids and vitamins as well as degradation of xenobiotics(Qin et al., 2010). The MetaHIT and HMP studies outline the enormous complexity of the gut metagenome and highlight our significant gap in knowledge in regards to gut microbial function.

1.3.4 Gut Microbial Symbiosis

The symbiotic functions of the gut microbiome include nutritional support, immunologic development, and prevention infection with pathogenic organisms(O'Hara & Shanahan, 2006). These three categories are not mutually exclusive. Metabolic functions of the microbiota are diverse and may have a significant impact on gut health(Erickson et al., 2012; Kostic et al., 2014).

1.3.4.1 Complements to host metabolism

It has been long known that germ free mice require higher caloric input than colonized animals. Gut bacteria provide access to otherwise indigestible carbohydrates. Gene studies revealed the capacity gut microbes to metabolize carbohydrates is far greater than what is encoded in the human genome(Hollister et al., 2014). Many of the microbes associated with good health encode the ability to produce the SCFAs. Butyrate production is carried out by populations of *Clostridum*; Acetate and proprionate are produced by *Bacteroides*(Fischbach & Sonnenburg, 2011). SCFAs have been shown to have many beneficial effects for the host. They provide a source of energy for enterocytes, improve barrier function and modulate the gut immune system to maintain homeostasis(Nieuwdorp, Gilijamse, Pai, & Kaplan, 2014).

Microbial peptidases generate free amino acids in the gut lumen. Amino acids are then further processed by enzymes such as decarboxylases to produce compounds with immunomodulatory potential. Alternatively amino acids can be utilized to produce anti-microbial peptides that may prevent the growth of potential pathogens(Hollister et al., 2014).

Vitamin biosynthesis by gut microbes also benefits the host. An example of this is the production of vitamin K, which is required for hepatic synthesis of clotting factors.

1.3.4.2 Immunologic development and modulation of host responses

Much of our knowledge on the essential role of bacteria in developing and shaping the immune system is based on observations in germ free animals. Germ free animals have poorly developed gut associated lymphoid tissue including isolated lymphoid follicles, Peyer's patches, and mesenteric lymph nodes(B. P. Willing, Gill, & Finlay, 2010). Systemically they have smaller spleens. These histologic observations translate to immune dysfunction. Granulocyte killing of bacteria is diminished, as is CD8 T cell cytotoxicity. Both lamina propria and intraepithelial lymphocytes including T regulatory cells are reduced and Th1/Th17 T-helper cells are absent entirely. TLR and MHC expression is lower on the gut epithelium and secretory IgA production is nearly undetectable. When pathogens are introduced to germ free animals they are unable to responds appropriately making them fare more susceptible to infection. These defects are generally reversed when mice are conventionalized(H. Chung et al., 2012).

T-helper phenotypes Th1 and Th17 only develop in the gut after the introduction of bacteria. Studies by Ivanov et el. found that Th17 responses in mice developed in response to segmented filamentous bacteria(Ivanov & Littman, 2010; Ivanov et al., 2009; 2008). Follow up studies evaluating the effects of other populations of bacteria have also been able to induce Th17 responses(Kamada & Nùñez, 2014).

T regulatory cell induction in the gut is dependent on gut bacteria. It was first noted that *Clostridium spp.* From clusters IV and XIV were essential for development of Treg cells upon reconstitution in germ free mice. This effect was independent of signaling through

PRRs. The SCFAs produced by these clusters however demonstrate potent Treg induction by acetylation of the Foxp3 locus and activation of G-protein coupled receptors(Kamada & Nùñez, 2014). TGF-β and retinoic acid produced by lamina propria macrophages and dendritic cells induces Tregs through another mechanism involving the SCFA butyrate and niacin, in which activation of the G-protein coupled receptor Gpr109a in macrophages and dendritic cells stimulates production of TGF-β to induce Tregs(Singh et al., 2014). SCFA activation of g-protein coupled receptor-15 in Tregs will also stimulate their migration to the gut(Kamada & Nùñez, 2014). B. *fragilis* is common commensal found in the human gut which stimulates Treg induction as well; however this occurs via TLR-2 signaling in response to polysaccharide A from the bacterial cell wall(Peloquin & Nguyen, 2013).

1.3.4.3 Prevention of Pathogen Overgrowth

Metabolic products produced by commensals can prevent the pathogen colonization. Certain populations of commensal gut bacteria will secrete toxins called bacteriocins to inhibit the growth of other potential pathogens(Messaoudi et al., 2013). SCFA production by gut anaerobes alters the gut pH suppressing the growth of competitors and directly inhibits the expression of virulence factors in *Salmonella* species. Feeding children cooked green bananas led to faster recovery from shigellosis, and the likely mechanism was the increased production of SCFAs in the distal gut(Fischbach & Sonnenburg, 2011). Fischbach proposed a model of positive feedback loops in the gut microbiota to support favorable environments. By making several broad generalizations, the author simplifies Bacteroides and *Clostridium* metabolism as utilization of glycans and peptides respectively. Both produce products that feed into each others metabolic pathways

resulting in the production of SCFA's that support the gut enterocytes, which in turn release more mucin and CO_2. Mucin is then utilized by Bacteroides to produce acetate and succinate, which is then used by Clostridum to produce butyrate(Fischbach & Sonnenburg, 2011). Byproducts of metabolism from these two populations also increase the pH producing a more favorable environment for them.

Feedback loops can also be utilized to support pathogenic microbes. When a disturbance in the gut ecosystem occurs like antibiotics pathogens are given an opportunity to expand. Toxins produced by the pathogens will induce inflammation leading to tissue damage, and oxygen radicals. This in turn will further deplete the strict anaerobes and provide nutrition for auxotrophic bacteria.

Commensal microbes maintain immune tension through stimulation to prevent the colonization of pathogens. This is well recognized in the maintenance of the mucosal barrier via signaling through pathogen recognition receptors (PRRs). NOD2 is a receptor which binds muramyl dipeptide found in the bacterial cell wall and has several downstream functions(Rosenstiel, 2013). NOD2 mutations lead to defective PRR signaling which in turn results in decreased production of anti-microbial peptides from paneth cells (α-defensins, and RegIII-γ)(Rosenstiel, 2013), and bacterial translocation(Gutiérrez et al., 2013). Deficiency of α-defensins predisposes to infection with enteric pathogens. Toll-like receptors (TLRs) are another class of PRR. TLR-2 and TLR-4 which bind lipotechoic acid and LPS respectively are expressed at low levels on the apical surface of enterocytes(Abreu, 2010). MyD88 mediates downstream signaling through TLRs resulting in activation of NF-κB(Abreu, 2010). Signaling through this pathway is essential for mucosal homeostasis as demonstrated by TLR-4 and MyD88

knockout animals that spontaneously colitis. Toll signaling in the gut epithelium improves tight junctions, preventing translocation of invasive organisms and leads to increased IgA production to facilitate microbial clearance(Frantz et al., 2012; Strugnell & Wijburg, 2010). Commensal induced MyD88 signaling in macrophages stimulates the production of pro-IL-1β. This can be converted rapidly to the active form in response to pathogenic organism(Franchi et al., 2012). Together this evidence builds a strong picture whereby commensal microbes prevent infection and overgrowth of pathogens/pathobionts through direct inhibition with metabolic products, creation of microenvironments and maintenance of immune surveillance in the gut.

1.3.5 The Microbiome in IBD

Multiple studies have evaluated the changes in gut bacterial populations associated with IBD(Manichanh et al., 2006; Morgan et al., 2012; Qin et al., 2010). The most consistent finding is a loss of α-diversity due to decreases in *Bacteroides* and *Firmicutes*. Within the *Firmicutes* phylum there is often a loss of butyrate producing *Clostridum* species. *Proteobacteria* are generally increased and include adherent invasive E. coli(Kostic et al., 2014). The functional changes associated with IBD are not as well studied. The MetaHIT project included a small IBD cohort and noted 25% fewer genes present in this populations relative to the rest of the cohort(Qin et al., 2010). This is in keeping with observations of decreased diversity. Morgan et al. in 2012 evaluated functional changes in IBD by inferring expected gene abundances from reference genomes using 16s sequencing to define the populations of bacteria. Results indicated that disease activity was not associated with significant shifts in the microbiome after adjusting for other factors like age and source of DNA for sequencing (i.e. stool vs. mucosal biopsy).

However, when ileal CD was considered alone there were considerable reductions in *Ruminococcaceae* and *Feacalibacterium*, and *Roseburia*, with increases in *Escherichia*. These findings are consistent with previous observations(Darfeuille-Michaud et al., 2004; Sokol et al., 2008). A novel finding was a decrease in *Odoribacter* from the phylum Bacteroidetes. These bacteria are known producers of SCFAs. Taken together these results suggest a global reduction in SCFA synthesis and potential increased inflammatory signaling. When the inferred metagenomes were considered there were greater differences relative to bacterial clades suggesting disease associated microbial shifts may select for genotypes(Morgan et al., 2012). Functional perturbations in metabolism included increased carbohydrate metabolism and transport with higher proportion of genes involved in the pentose phosphate pathway in ileal CD. Conversely there was a global decrease in amino acid metabolism and biosynthesis, with the exception of cysteine(Morgan et al., 2012). Lipid metabolism and vitamin biosynthesis were also decreased in IBD with the exception of thiamine and riboflavin. The authors consolidated this data into a model suggesting that the metagenome in IBD and especially in iCD was adapted to an oxidizing environment likely due to the production of oxygen free radical from the immune response. Toxin secretion via type II secretory systems was also increased. Tissue damage caused by microbial products and the relatively oxygen rich environment seem to select for bacteria that can tolerate oxidizing environments through the production of glutathione coupled with riboflavin, sulfate and sulfur containing amino acids (i.e. cysteine) as well utilize nutrients released form damaged tissue by increasing carbohydrate metabolism and nutritional transport(Morgan et al., 2012).

Gevers et al. recently published a study evaluating microbial and metagenomic changes in treatment naïve CD. They included 447 CD patients and 221 controls. Utilizing the Illumina MiSeq platform to perform 16s sequencing on mucosal biopsies and stool. Mucosal specimens were superior to stool in reflecting the dysbiosis in treatment naïve disease. Consistent with previous studies, mucosal specimens demonstrated increased *Enterobacteriaciea* with decreases in Bacteroidales and Clostridales. A detailed look at the latter two demonstrated a loss of many species associated with SCFA production(Gevers et al., 2014). Due to the large sample size and significant sequencing depth several novel observations were made. Pasturellaceae, Fusobactereaceae, Neisseriaceae, Veillonellaceae and Gemellaceae were all increased in CD mucosa. Inferred microbial functional analysis was performed using PICRUSt to identify relative gene abundances in reference genomes. Genes abundances demonstrated a similar pattern to those previously described(Morgan et al., 2012), with a metagenome capable of survival in aerobic environments and utilizing auxotrophic pathways(Gevers et al., 2014). Results were compiled to build a predictive model of CD. Microbial composition of ileal and rectal biopsies both predicted CD with mean AUCs of 0.85 and 0.78 respectively. Composition found in rectal and ileal biopsies were similar regardless of disease location(Gevers et al., 2014).

The cause of the dysbiosis observed in CD is unclear. Redox potential of the gut has been recently proposed as a major contributor to the microbial shifts observed in IBD(Rigottier-Gois, 2013). The feedback loop proposed by Fischbach(Fischbach & Sonnenburg, 2011) discussed above would support this hypothesis. Based on repeat observations in IBD it is clear that obligate anaerobes (Bacteroides and Clostridium) are

depleted in favor of facultative anaerobes from the Proteobacteria phylum. As inflammation increases so to would the redox potential in the intestine. The oxidative burst produced by neutrophils and low grade blood loss from the damaged mucosa would both contribute the aerobic environment providing a competitive advantage to facultative anaerobes(Rigottier-Gois, 2013). Observations from the metagenomic data discussed above also supports this hypothesis by demonstrating increased proportion of genes to deal with oxidative environments(Gevers et al., 2014; Morgan et al., 2012).

1.4 Mononuclear phagocytes

Mononuclear phagocytes (MPs) are comprised of both dendritic cells (DCs) and macrophages. As discussed below, each serve a distinct and essential role in the gut; with DCs acting to sample antigens followed by induction of downstream immune responses, and macrophages functioning as antigen scavengers with relatively low inflammatory potential. Because the gut is constantly exposed to foreign antigens, MPs are uniquely positioned as key regulators of gut homeostasis.

1.4.1 Dendritic Cells

Dendritic cells (DCs) have been identified as an important link between innate and adaptive immunity through their ability process and present antigens to naive T cells(Bar-On, Zigmond, & Jung, 2011; MacDonald et al., 2011). DCs specific to the gut have been identified. These DC subtypes are capable of eliciting both regulatory (Treg) and inflammatory (Th1 & Th17) responses through antigen presentation on MHC II molecules with varying cytokine production/co-stimulation. DCs appear to be central in maintaining gut homeostasis(MacDonald et al., 2011). The role of DCs and the phenotypes involved in post-operative recurrence remains to be studied. This section will

discuss the DC populations present in the gut, and how DCs function in the inflammatory response.

1.4.1.2 Gut dendritic cells

DC's in the gut lamina propria (LP) are defined by the expression of the surface markers CD103+ and CD11c+. These DC's originate from pre-cDC's that replenish the DC population in the LP via migration from peripheral blood(Jaensson & Uronen-Hansson, 2008).

CD103+ DCs can be further subdivided based on the expression of CD11b. CD11b- CD103+ DCs represent a distinct population defined by their reliance on the transcription factors ID2, IRF8 and BATf3. They tend to localize in the gut associated lymphoid tissue (GALT) and LP of the distal small intestine and the colon(Denning et al., 2011). Their exact role to this point is undefined(Agace & Persson, 2011; Bar-On et al., 2011), however they are found in intestinal lymph suggesting they traffic antigens to draining lymphnodes(Cerovic et al., 2012). Functional characterization defines them as primarily tolerogenic through production of retinoic acid and TGF-β(Denning et al., 2011).

CD11b+ CD103+ DCs require GM-CSF for their differentiation. They localize to the LP and migrate to MLNs through expression of CCR-7. It is this population is also responsible for the regulatory properties attributed to CD103+ DC's(Agace & Persson, 2011; Bar-On et al., 2011), they have also been shown to accumulate in states of colitis and induce Th17 cells(Denning et al., 2011; Farache, Koren, Milo, Gurevich, Kim, et al., 2013a).

It is important to note the a similar CD103+ DC population is present in human MLNs and that this population shares functional characteristics with the murine DCs including

induction of the gut homing molecule CCR9 on T cells(Jaensson & Uronen-Hansson, 2008).

Plasmacytioid dendritic cells (pDC) are also present in the LP however their exact function is unclear. They express CCR9, and may be responsible for inducing CD103+ cells to migrate to MLN through production of TNF-alpha and type 1 IFN(C. L. Scott, Aumeunier, & Mowat, 2011).

The majority of the work on CD103+ DC's has defined them as regulators of immune tolerance in the gut by inducing FoxP3+ Treg cells. LP CD103+ DC's migrate to mesenteric lymph nodes (MLN) where they present antigens from the bowel lumen to naïve CD4+ T cells. This interaction causes differentiation to FoxP3+ Treg cells that express CCR9, a gut specific homing molecule(C. L. Scott et al., 2011). CD103+ cells are the only APC identified that has the ability to present orally administered antigens to T cells in the gut(Jaensson & Uronen-Hansson, 2008). Induction of Treg cells is dependent on both RA and TGF-beta both of which are produced by the CD103+ DCs and other cell types in the gut(Agace & Persson, 2011). Further evidence of the anti-inflammatory effect of CD103+ DCs was discovered when a mouse model of ileitis was successfully treated with Flt3-Ligand(Collins et al., 2011). Flt3-Ligand is known to preferentially induce expansion of CD103+ DCs(Varol et al., 2009) and the investigators of this study found administration of Flt3-Ligand expanded the CD103+ DC population in the gut, and this attenuated the established ileitis(Collins et al., 2011).

In contrast to the large volume of work defining gut DCs as key regulatory cells there is still very little known about the DC populations involved in the inflammatory response. A

colitis model with dextran sulfate sodium (DSS) confirmed that DC are important for gut inflammation when the investigator found ablation of DCs attenuated the colitis(Berndt et al., 2007). Unfortunately due to the nature of the model all DC populations were knocked out so there is no way of knowing which population was mediating the inflammation. Some evidence suggests that the same CD103+ DCs that induce Treg cells may also function in states of inflammation to cause Th1 and Th17 phenotypes. If they are the only APC capable of migrating to MLN and presenting luminal antigens(Jaensson & Uronen-Hansson, 2008); as the current knowledge would indicate, then a plasticity in their effect would be essential to maintain protective immunity in the gut. In a mouse T cell transfer colitis model isolated CD103+ cells displayed an inflammatory phenotype with IL-6 production and the ability to induce IFN and IL-17 producing T cells. They also found that during inflammation CD103+ cells may be recruited directly from the blood to the MLNs(Laffont, Siddiqui, & Powrie, 2010). It is difficult to say if the CD103+ cells in this model mediate the inflammation because, while the overall number of DCs increased with inflammation the number of CD103+ cells decreased. The relevance of the increase in CD103- DCs is unclear as many of them may have been macrophages in the LP(C. L. Scott et al., 2011) and resident MLN DCs in the MLNs(Jaensson & Uronen-Hansson, 2008). These populations may have increased in number purely as a result of the cytokine environment.

Another potential DC population functioning in states of gut inflammation are pDCs. Studies with both human and mouse pDCs outside of IBD have identified pDC's as mediators of Th17 commitment(Bonnefoy et al., 2011; Yu et al., 2010). In the Bonnefoy study pDCs were isolated from mouse BM(Bonnefoy et al., 2011). The pDCs exposed to

TGF-beta were found to produce IL-6 and when co-cultured with naïve T cells induced a Th17 phenotype. When the pDCs were introduced into a mouse arthritis model they significantly worsened the inflammation(Bonnefoy et al., 2011). Another study found recruitment of pDCs to colorectum in a state of immune activation in a simian model of HIV(Kwa et al., 2011). It is possible that resident CD103 DCs take a back seat during states of inflammation and pDC's instead are responsible for T cell activation in the gut.

1.4.1.2 Dendritic Cells in CD

A small number of studies have evaluated the function of DCs in human CD. In general CD patients harbor DCs with greater inflammatory potential, expressing higher levels of surface Toll like receptors (TLR)-2 and -4 as well as more markers of ma on DCs interact with peptidoglycan and lipopolysaccharide (LPS), respectively, and are known to be upregulated in active CD(Baumgart et al., 2009; Ng et al., 2010). Like murine DCs, intestinal DCs can be defined by the $CD103^+$ marker in humans(Jaensson & Uronen-Hansson, 2008) and produce retinoic acid(Sanders et al., 2014). Observations of DCs in CD support their central role in the development of pro-inflammatory T cell responses. Patients with established CD have increased numbers of myeloid and mature DCs in the gut expressing co-stimulatory molecules CD40, CD80, CD86 and the maturity marker CD83 with an increased propensity to induce Th1 cells(Baumgart et al., 2009; A. L. Hart et al., 2005; Sakuraba et al., 2009).

1.4.2 Intestinal Macrophages

In the steady state, LP macrophages have a homeostatic role. They begin as $Ly6C^+$ blood monocytes that populate the lamina propria and differentiate into resident tissue

macrophages expressing CX3CR1(Varol, Zigmond, & Jung, 2010). The half-life of these macrophages is approximately 3 weeks(Jaensson & Uronen-Hansson, 2008).

LP macrophages are intimately associated with the epithelium through the expression of CX3CR1, which drives antigen sampling through trans-epithelial dendrites 79. Once antigens are taken up by macrophages they are rapidly processed without evoking inflammatory responses(Smythies, Sellers, & Clements, 2005). In addition to antigen clearance, LP macrophages promote and maintain Treg cells in the LP through expression of IL-10 (Denning, Wang, Patel, Williams, & Pulendran, 2007). Further evidence of the homeostatic role of this population of macrophages comes from experiments where CX3CR1 expressing macrophages are depleted. These animals tend to develop a more severe DSS induced colitis with increased bacterial translocation(Medina-Contreras et al., 2011)

Continuous replenishment of the LP macrophage compartment with blood monocytes allows for plasticity in their function(Zigmond & Jung, 2013) During states of inflammation monocytes recruited to the gut take on a pro-inflammatory phenotype and have lower expression of CX3CR1. Depletion of this $CX3CR1^{int}$ cell population improves DSS colitis. When stimulated in culture these cells tend express an pro-inflammatory panel of cytokines(Farache, Zigmond, Shakhar, & Jung, 2013b)

1.4.2.1 Macrophages in CD

Macrophages in the non-inflamed human intestine are remarkably inflammation anergic while still maintaining potent phagocytic function(Smythies et al., 2005). When blood monocytes are recruited to the intestinal lamina propria they undergo maturation leading to a significant phenotypic shift from inflammation hyper-responsiveness to hypo-

responsiveness. Local tissue factors in the gut appear to be responsible for this effect. TGF-β is present in high concentrations in the LP. Signaling through TGF-β receptors on macrophages both recruits blood monocytes and down regulates NF-κB signaling. In addition, TSLP produced by epithelial cells may also promote a regulatory phenotype. Once matured in the gut, macrophages loose expression of the LPS receptor CD14, Ig receptors, as well as the potential for respiratory burst, and inflammatory cytokine expression. Despite the potential to present antigen to T cells via MHC-II expression, LP macrophages have diminished T cell priming ability due to their lack of co-stimulatory molecules(Smith et al., 2011).

In contrast to steady state LP macrophages, macrophages in the inflamed mucosa of CD are largely represented by newly recruited blood monocytes, which express the CD14 LPS receptor and maintain significant inflammatory potential. Cytokine profiles of this population of CD macrophages suggest high expression of TNF-α and IL-23 forming a positive pro-inflammatory feedback loop(Nobuhiko Kamada & Nobuhiko Kamada, 2008).

1.5 Clinical Manifestations of CD

Patients with CD typically present after a long history of intermittent abdominal pain and diarrhea resulting from bowel inflammation and stenosis. The most common regions of the bowel affected are the distal small bowel (terminal ileum) and proximal colon (cecum) with about 40% of patients having involvement of both. Disease in the bowel may manifest in several ways. The Montreal classification divides disease behavior into non-stricturing, non-penetrating (B1), stricturing (B2), and penetrating (B3)(Satsangi, 2006). B1 is pure inflammation, which will generally manifest as diarrhea. B2 disease

causes narrowing of the bowel lumen and eventually obstruction of the bowel and B3 disease causes perforation and fistulization of the bowel. A more detailed description of B3 disease is provided in the surgery section.

The next most common presentation is perianal disease. Perianal disease ranges from skin tags to fissures to fistulas and abscesses. Perianal disease frequently requires surgical intervention(Stein, 2010).

Less common manifestations in the GI tract include gastroduodenal disease, esophageal disease and isolated jejunal disease(Bruce E Sands, 2010).

1.6 Drug Therapy for CD

1.6.1 5-ASA and Sulfasalazine

Sulfasalzine (SASP) and 5-ASA are one of the oldest classes of drugs used in the treatment of IBD. Beginning in the 1930's when SASP was incidentally found to induce remission in patients with ulcerative colitis (UC) who were taking the drug for the treatment of their rheumatoid arthritis. It was found later that SASP was cleaved into two drugs in the colon: sulfapyridine and 5-ASA. Most of the clinical benefit was derived from the topical action of 5-ASA on the mucosa, whereas the sulfapyridine moiety was responsible for many of the side effects. Since then this drug has been reintroduced in various forms starting with 5-ASA alone (mesalamine) and now in several controlled release preparations(Nielsen, 2007). While their benefit in UC is well established the use of 5-ASA drugs in Crohn's disease remains controversial.

5-ASA compounds act via several pathways as immunosuppressive and anti-inflammatory agents. Although no single mechanism has been identified; the clinical efficacy is most likely multifactorial. 5-ASA inhibits proinflammatory cytokine

synthesis, prostaglandin and leukotriene synthesis, and white cell adhesion. It also acts as a free radical scavenger and prevents clonal expansion of T-cells and B-cells.

A meta-analysis of three double blind trials comparing controlled release mesalamine (Pentasa) to placebo, demonstrated a modest benefit in reduction of disease activity for patients with active Crohn's. The mean reduction in Crohn's Disease Activity Index (CDAI) for patients treated with 4g/day of mesalamine for 16 weeks was 67(A. Ford, Kane, Khan, & Achkar, 2011b). The relevance of this is questionable since a clinically significant reduction in CDAI is defined as 70-100 and the mean difference in reduction of CDAI versus placebo was only 18.

SASP may perform better than mesalamine in active Crohn's disease of the colon based on trials completed between 1974 and 1984, however direct comparison of results is difficult because of the varying methods used to assess response(Sandborn et al., 2007).

1.6.2 Azathioprine and 6-mercaptopurine

The clinical efficacy of Azathioprine (AZA) and 6-Mercaptopurine (6-MP) are based upon a balance of competing metabolic pathways, which separates responders, non-responders and those at risk of toxicity. AZA is first converted to 6-MP by glutathione-s-reductase. 6-MP is then metabolized to an inactive form by TPMT and xanthine oxidase, or it is converted to the clinically active 6-TGN, which is a collection of 6-thio-GMP/GDP/GTP. Currently the only clinical application of the enzymology is measuring patient TPMT activity prior to initiation of treatment. The assay identifies those with low TPMT activity and therefore higher risk of AZA/6-MP toxicity(Ansari et al., 2008; Compagni et al., 2008). The opposite effect has also been observed but is less consistent(Atreya & Neurath, 2008). Response and toxicity may also be predicted based

upon concentrations of the end metabolic product; 6-TGN, in patients already receiving AZA/6-MP(Osterman et al., 2006).

The mechanism of action of AZA/6-MP in Crohn's was believed to be due to random incorporation of 6-TGN into DNA causing genetic instability or the inhibition of purine synthesis leading to impaired cell division. Recently 6-TGN was found to induce apoptosis of T-lymphocytes with the 6-thio-GTP derivative as the primary mediator(Atreya & Neurath, 2008; Neurath et al., 2005).

Side effects lead to the discontinuation of AZA/6-MP in 10-25% of patients and despite strategies to identify those at risk for toxicity there are still a significant number of patients who experience side effects without any apparent predisposing factor. Major Side effects include bone marrow suppression (2-5%), pancreatitis (3.3%) and infection (7.4%). The increased risk of malignancy is still controversial but there does seem to be a slightly increased risk of lymphoma. Minor side effects include nausea, rash, fever, arthralgias, malaise and diarrhea(Arnott, Watts, & Satsangi, 2003).

Despite the known potential for adverse events, AZA/6-MP shows clear benefit in the treatment of active Crohn's disease. A recent meta-analysis evaluated eight randomized placebo controlled trials using AZA or 6-MP for induction of remission defined by various activity indices(Prefontaine et al., 2009). The overall response rate was 54% versus 33% in the study and placebo groups respectively (NNT 5). It also showed that response should only be evaluated after 17 weeks of therapy as the NNT drops from 18 before 17 weeks to 4 at 17 weeks and beyond. Other significant benefits include increased fistula healing and steroid sparing effects. Adverse events were defined as

those severe enough to cause withdrawal from the trial. The overall rates were 9.3% for AZA/6MP and 2.3% in the placebo group (NNT 14) with no mortalities.

1.6.3 Methotrexate

Methotrexate (MTX) was initially developed in 1950 as a treatment option for children with leukemia. MTX later demonstrated benefit in the treatment of rheumatoid arthritis (RA) and psoriasis. MTXs efficacy and tolerability in RA led to its evaluation as treatment for CD; however routine use is yet to be established.

MTX is a competitive folate antagonist with both anti-inflammatory and immunosuppressive properties. MTX to suppresses inflammation by inhibiting 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, thereby increasing the intracellular concentration of AICAR. The intracellular increase in AICAR is associated with increased extracellular adenosine in inflammatory exudates. The adenosine binds A2 receptors on inflammatory or connective tissue cells, which inhibits lymphocyte proliferation and the synthesis of cytokines and other inflammatory proteins(Cronstein & Naime, 1993). In high doses, MTX inhibits the dihydrofolate reductase enzyme, which impairs DNA synthesis and leads to cell death(Seitz, 1999).

A recent systematic review evaluated five randomized control trials using MTX in active CD(Alfadhli & McDonald, 2004). The paper had conflicting results. Overall, MTX exhibited no significant benefit in CD patients with active disease, however it is difficult to draw firm conclusions based on this analysis because of the variability in drug dose and administration. The negative trials used low dose oral MTX while the one positive trial used high dose intramuscular MTX and demonstrated an NNT of 5. The efficacy of

high dose intramuscular MTX was supported by another study, which demonstrated equivalence to azathioprine.

1.6.4 Corticosteroids

Corticosteroids have been mainstay of therapy for active CD since the 1950s however their use is limited because of the significant adverse events associated with long-term therapy.

Corticosteroids exert their effect by binding to a corticoid receptor located within the cytoplasm of target cells. They suppress inflammation by blocking several steps in the inflammatory process. They prevent the transcription of genes important in the inflammatory cascade, inhibit prostaglandin and cytokine production, decrease the stability of mRNA, and promote apoptosis of lymphocytes within the gut(Barnes, Adcock, & Spedding, 1993; Rhen, Rhen, Cidlowski, & Cidlowski, 2005).

Two RCTs evaluated standard corticosteroid therapy versus placebo for induction of remission in active CD. The first in 1979(Summers, Switz, & Sessions, 1979) used oral prednisone and evaluated response at 17 weeks. Overall response rate for prednisone was 47% vs. 26% for placebo. The second study in 1984 used oral methyl-prednisolone over 6 weeks(Malchow et al., 1984). This study showed an 83% response rate in the steroid group with a 38% response in the placebo control group. Taken separately both studies demonstrate significant response rates when using steroids vs placebo however when combined the ability of steroids to induce clinical remission is not significant(A. C. Ford et al., 2011a).

Budesonide was found to be effective versus placebo in two RCTs with an overall NNT of 5(A. C. Ford et al., 2011a). Budesonide is an attractive alternative to standard

corticosteroid therapy as it has fewer systemic side effects because of its rapid first pass metabolism in the liver. However, four RCTs compared budesonide to standard steroid therapy and found that it did not perform as well at inducing remission(A. C. Ford et al., 2011a).

1.6.5 Infliximab

Infliximab (IFX) is monoclonal antibody directed against tumor necrosis factor alpha (TNF); and important inflammatory cytokine. It acts by binding and neutralizing TNF. Infliximab is the most extensively studied and widely used drug in a newer generation of more directed therapies targeting various aspects of the immune system.

Risks associated with IFX continue to be debated, as there are many proposed serious side effects without much evidence to substantiate claims. Infections by common and opportunistic organisms are the most frequent event in IFX treated patients. Activation of tuberculosis is of particular concern. Other Adverse events include infusion reactions, demyelinating disease, worsening of congestive heart failure, malignancy and induction of autoimmunity. Studies completed to evaluate the risks of IFX have all concluded that the benefits out weigh the risks(Caviglia, Boskoski, & Cicala, 2008).

IFX has demonstrated efficacy for induction of remission(Akobeng & Zachos, 2004) and maintenance of remission(Behm, Behm, Bickston, & Bickston, 2008). A meta-analysis identifying studies using IFX to induce remission found that a single dose of IFX achieved a clinical in 65% of patients with nearly half of those achieving remission (NNT 4) at 4 weeks post-infusion(Akobeng & Zachos, 2004). Another meta-analysis found a significant response when IFX is continued as maintenance with remission rates in the

RCT's reviewed ranging from 28.3% to 52.9% at approximately one year. There was no significant difference in the rates of adverse events vs placebo(Behm et al., 2008).

1.7 Surgery and Crohn's Disease

The indications for surgery in CD have remained unchanged for many years; however, topics such as extent of bowel resection, type of bowel anastomosis, anastomotic technique and operative approach have been studied with the goal of optimizing outcomes and minimizing post-operative recurrence of disease. Medical prevention of post-operative recurrence has been extensively investigated. Despite the volume of work aimed at preventing recurrent disease after surgical resection the rates of recurrence remain high with little known about the etiology.

This section will review the surgical indications in CD, common procedures, varying operative approaches and techniques, risks of recurrence, treatment options, and new science exploring the post-operative recurrence of CD.

1.7.1 Indications for Surgery

Failure of medical management is generally the first indication that comes to mind when talking about surgery for CD. Traditionally this has been the most common indication accounting for up to 47% of all surgeries(Farmer, Hawk, & Turnbull, 1976; Hurst, Molinari, Chung, & Rubin, 1997). This may be changing with the wide spread use of biologic therapies. Since the introduction of infliximab the rate of surgery in those patients receiving the drug has decreased(Leombruno et al., 2011; Schnitzler et al., 2009). No recent studies have been completed to evaluate if the overall rate of surgery has declined. Failure of medical management occurs when non-surgical therapies can no

longer be used to control acute inflammatory disease either because the medications simply do not work or it may be that the patients cannot tolerate the medications.

Complete or partial intestinal obstruction is the next most common indication for surgery(Farmer et al., 1976; Hurst et al., 1997). Patients generally present after a long history of chronic obstructive symptoms secondary to stricturing disease. These patients usually require operative intervention, which could include endoscopic dilation, stricturoplasty or bowel resection. Acute obstruction is often caused by inflammatory disease and only requires operation if a trial of medical therapy fails.(Stein, 2010)

Intra-abdominal perforation and abscess occur as a consequence of penetrating disease when transmural inflammation and tissue destruction results in a hole in the bowel wall(Bruce E Sands, 2010). Free perforation is rare but it is an indication for emergency surgery as the immediate complications include progression to sepsis and death. Abscess formation is more common and can often be managed by insertion of a percutaneous drain and antibiotics. Surgical intervention is required if this fails.

Operative intervention for fistulas depends on symptomatology and location. Fistulas also arise out of penetrating disease but instead of free perforation into the peritoneum they form connections to other epithelial surfaces. Many entero-entero fistulas or entero-colonic fistulas are asymptomatic but when fistulas involve the pelvic organs or skin they often require surgery to resect the fistula tract and restore normal anatomy.(Stein, 2010)

Other less common indications for surgery include hemorrhage, cancer, toxic colitis and growth retardation in children.(Stein, 2010).

1.7.2 Surgical Technique

Intestinal manifestations of CD eventually lead to surgery in the majority of CD patients(Olle Bernell, 2000); with ileocolic resection (ICR) (removal of the terminal ileum, cecum and part of the ascending colon) being the most common procedure performed(Olle Bernell, 2000). Principles of bowel resection hold true for CD as they do for any other indication with the exception that only macroscopically abnormal bowel should be resected(V W Fazio, 1996). Otherwise, a complete exploration of the peritoneal cavity should be performed to look for other sites of disease and the anastomosis should be tension free, watertight and with good blood supply. Anastomotic configurations include end-to-end (when the transversely cut ends of colon and small bowel are put back together maintaining the configuration of a straight pipe), side-to-side (when the cut ends of small bowel and colon are closed and the two pieces of bowel are opened up longitudinally then put together), and several other less common types. They are still mostly chosen based on surgeon preference. Traditional end-to-end anastomosis were compared to all other anastomotic configurations in a meta-analysis of eight studies in CD patients(Simillis et al., 2007). The results suggested that end-end anastomosis were associated with significantly more leaks (OR, 4.37) and increased hospital stays (2.81 days); however there was no difference in peri-anastomotic recurrence rates between the two groups. The conclusions of this meta-analysis were further supported by an RCT completed in 2009, which found anastomotic configuration did not affect recurrence rates(McLeod, Wolff, & Ross, 2009).

A Cochrane review compared hand-sewn anastomosis to stapled and included studies for both benign and malignant disease as the indication for surgery. It found stapled anastomosis were associated with fewer leaks overall but when they looked specifically at CD patients there was no difference in leaks between stapled and hand-sewn techniques.

Recently a new anastomotic configuration was published termed the "S-Kono" anastomosis.(Kono et al., 2011). In this small retrospective study the authors found lower surgical recurrence rates and overall lower CD severity scores at the anastomosis 5 years post-op; despite having similar endoscopic recurrence rates 1 year post-op.

Another topic that has been debated is the role of laparoscopic surgery in CD. This question was answered in a Cochrane review that found no difference in any outcomes measured between open and laparoscopic surgery for small bowel CD. This included leak rate and disease recurrence(Dasari BVM, McKay D, Gardiner K, 2010). Some studies do suggest that post-operative recovery may be expedited in patients receiving laparoscopic procedures with lower rates of post-op ileus and fewer days in hospital(De Cruz et al., 2011).

Despite the various modifications to surgery over the years none of the advances have had any affect on long-term recurrence rates, but implementation of newer techniques may improve surgical outcomes in the short term by decreasing rates of anastomotic leaks and contributing to faster recovery.

1.7.3 Post-operative Recurrence

Unfortunately, recurrence is common after surgical resection. The neo-terminal ileum (where the ileum is hooked back up to the colon) is the most common site of recurrence

with up to 80% of patients developing endoscopic lesions one year after surgery(Rutgeerts, 2003). Repeat surgery is necessary in 44% to 65% of patients after 10 years(Olle Bernell, 2000; Rutgeerts, 2003). Repeat surgeries have a significant impact on patient health as they start to develop short bowel syndrome and other complications of surgery. So far strategies to prevent postoperative recurrence have been mostly unsuccessful and our understanding of recurrence is limited.

Risk factors for recurrence include smoking(Cottone et al., 1994), perianal fistula/penetrating phenotype(Cunningham et al., 2010; Olle Bernell, 2000), continuous ileo-colonic disease or isolated small bowel disease(Olle Bernell, 2000), previous surgical resection(De Cruz et al., 2011), and NOD2 mutation(Alvarez-Lobos et al., 2005). New insights into post-operative recurrence are being made through bacterial, and cytokine analysis.

1.7.3.1 Bacteria in Post-operative recurrence of CD

Bacteria appear to play an important role in post-operative recurrence. It is known that the neo-terminal ileum becomes heavily colonized with bacteria after an ileo-colonic anastomosis(Neut et al., 2002). This phenomenon is likely due to loss of the ileocecal valve, thus allowing colonic contents and bacteria to freely reflux into the ileum. This may also explain why patients with no small bowel CD who undergo resection of the right colon for colonic CD subsequently develop lesions in the neo-terminal ileum(Rutgeerts, 2003) and why recurrence in the neo-terminal ileum is prevented by a diverting ostomy(Rutgeerts et al., 1991). The protective effect of the diverting ostomy can be reversed be re-introducing bowel contents into the neo-terminal ileum(D'Haens et al., 1998). The effectiveness of antibiotics in CD and particular in post-op recurrence

provides another piece of evidence implicating bacteria in post-op CD(Rutgeerts, Hiele, Geboes, & Peeters, 1995; Rutgeerts et al., 2005). Animal studies lent support to these observations as well when it was shown that IL-10 deficient mice require the presence of bacteria to develop inflammation after ICR(Rigby et al., 2009).

One study evaluated mucosal associated bacteria pre and post ICR in CD patients and compared microbial profiles to cancer patients as well as patients with ileostomy(Neut et al., 2002). The study found significantly higher counts of bacteria in the neo-terminal ileum for all patients at 3 months and 1-year post-ICR, when compared to those with ileostomies. CD patients had different bacterial species present when compared to the cancer patients with increased counts of E. coli and enterococci and decreased numbers of Bifidobacterium, Eubacterium, and Ruminococci. There were also species that seemed to be specifically related to recurrence with persistently elevated levels of Bacteroides in recurrent patients. Fusobacteria were found only in CD patients after ICR and were present in 36% of patients with recurrence. E. coli counts were elevated 3 months post-operatively in patients with recurrence but the numbers decreased at 1 year, this phenomenon was observed in another study(Darfeuille-Michaud et al., 2004) that found AIEC were associated with early CD recurrence.

In summary, three lines of evidence strongly implicate bacteria as important mediators in post-operative recurrence. 1) The dysbiosis observed with relatively increased numbers of potentially pathogenic bacteria and decreased numbers of commensal bacteria, 2) Effectiveness of antibiotics in post-operative recurrence, and 3) Evidence that animal models of IBD require bacteria for inflammation to occur.

1.7.3.2 Cytokines in Post-operative CD

The cytokine evolution and milieu in post-operative recurrence is largely undefined; however, one study found levels of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in the mucosa after surgical resection correlated with disease recurrence with IL-6 having the strongest association with recurrence(Yamamoto et al., 2004). TGF-β1 has also been implicated in post-operative recurrence(Scarpa et al., 2009). TGF-β1 was elevated in normal mucosa in patients that subsequently developed post-operative recurrence. Systemic cytokines may also predict post-operative recurrence as IL-6 levels in plasma were found to correlate with CD recurrence(Ruffolo et al., 2010). Unpublished data from a group in Italy found that IL-17 expression was increased in the mucosa of patients with endoscopic recurrence (poster presentation UEGW, 2011). The opposite was true when a similar study found that levels of IL-10 expression were associated with decreased recurrence post-op(Meresse et al., 2002).

The few studies completed looking at cytokine expression, as markers of post-operative recurrence seem to have incidentally highlighted the importance of the Th17 immune response in the pathogenesis of post-operative recurrence. It is known that IL-6 and TGF-β1 are the key mediators in Th17 differentiation(Strober et al., 2011) and with the most recent observation of elevated IL-17 in recurrent lesions, this pathway stands out as a target for post-operative prevention.

1.7.4 Post-operative prevention

1.7.4.1 Probiotics

A lack of "protective" bacteria prompted several RCTs looking at the use of probiotics for prevention of post-operative recurrence(Marteau, Lémann, Seksik, & Laharie, 2006;

Prantera, Scribano, Falasco, & Andreoli, 2002; Van Gossum et al., 2007). All of the studies used oral preparations of Lactobacillus species and found no benefit in preventing post-operative recurrence. Another RCT used a combination of several bacteria VSL#3 post-operatively and this also failed to show significant benefit(Madsen et al., 2008).

1.7.4.2 Infliximab

Three studies have been published assessing IFX for prevention of post-operative recurrence. The first(Regueiro et al., 2009) included 24 patients who underwent an ileocolonic resection. The patients were randomized to receive IFX or placebo within 4 weeks post-operatively and scheduled maintenance infusions. When these patients were evaluated for endoscopic recurrence at 1 year, 9.1% of patients in the IFX group had a recurrence vs. 84.6% in the placebo group.

The next study(Sorrentino et al., 2010) followed 12 patients all of whom were started on IFX 2 weeks post-operatively. All 12 showed no evidence of clinical or endoscopic disease after 3 years at which point IFX was stopped for 16 weeks. The patients were then re-evaluated, and 10 of 12 patients had developed endoscopic recurrence. IFX was re-initiated and dose escalated until response was achieved at 3mg/kg. After re-introduction of IFX, remission was maintained over the 1-year follow up period.

Most recently, an RCT from Japan published their data from 31 CD patients randomly assigned to IFX or no therapy post ICR(Yoshida et al., 2011). They achieved significant maintenance of clinical (100%) and endoscopic (78.6%) remission in the IFX treated group at 1 year.

While the numbers are small these three studies clearly demonstrate the effectiveness of IFX for post-operative maintenance. The Sorrentino study suggests that smaller doses of

IFX may be used for post-operative maintenance. Larger studies are forthcoming to confirm these results.

1.7.4.3 Azathioprine and 6-mercaptopurine

The role of AZA/6-MP for postoperative maintenance is less clear. Two meta-analyses have evaluated the utility of AZA/6-MP in the post-operative setting. The first analysis(Doherty et al., 2009) included 5 RCT's(Ardizzone et al., 2004; D'Haens, Vermeire, Van Assche, & Noman, 2008; Hanauer et al., 2004; Herfarth et al., 2006; Nos et al., 2000) and compared AZA/6-MP to mesalamine. Two of the trials included a placebo group(D'Haens et al., 2008; Hanauer et al., 2004). When compared to mesalamine, AZA/6-MP demonstrated no significant difference in clinical recurrence at 12 or 24 months, however significance was shown in preventing endoscopic recurrence. When compared to placebo alone, AZA/6-MP was successful in preventing both clinical (NNT 7), and endoscopic (NNT 4) post-operative recurrence at 12 months. D'Haens et al(D'Haens et al., 2008) included 3 months of oral metronidazole in the immediate post-operative period for both experimental and placebo groups.

The second meta-analysis(Peyrin-Biroulet et al., 2009) included the same studies with the exception of the Spanish trial. This analysis assumed mesalamine was equivalent to placebo based on previous mesalamine studies that found no benefit in post-operative prevention. When the placebo and mesalamine arms were compared to AZA/6-MP, a significant reduction in post-operative clinical recurrence was found after 1 (NNT 13) and 2 (NNT 8) years.

Based on the two meta-analyses it is clear that AZA/6-MP is effective at preventing post-operative recurrence when compared to placebo, however given the marginal benefit

compared to mesalamine alone, and the significant side effect profile, it is unclear if it should be used routinely for prevention of post-operative recurrence.

1.7.4.4 5-ASA

Mesalamine as maintenance therapy has been extensively studied with 5 RCTs assessing the efficacy of mesalamine in preventing postoperative recurrence. The first 4 of these studies were reviewed in a meta-analysis which suggested a significant decrease in postoperative recurrence with an NNT of 8(Cammà et al., 1997). Length of treatment ranged from 12-36 months in the trials. The last RCT included 324 patients randomized to 4g/day mesalamine (Pentasa) versus Placebo. The study concluded that there was no significant reduction in postoperative recurrence(Lochs et al., 2000), and when this study is included in the analysis of the previous 4 it diminishes the benefit below significance (NNT 25)(Sutherland, 2000). Another systematic review evaluating the effectiveness of various mesalamine preparations suggested that pH 7-dependent release preparations were beneficial in the maintenance setting(Steinhart et al., 2007). It is clear that the use of mesalamine in the maintenance of Crohn's is still debatable, but the bulk of clinical data would suggest that it should not be routinely given.

1.7.4.5 Corticosteroids

Standard corticosteroids are not recommended for maintenance of disease remission in any situation because of the significant systemic side effects associated with them. Because of the improved side effect profile of budesonide it has been evaluated for use in post-operative prevention. Two studies(Ewe, Böttger, Buhr, & Ecker, 1999; Hellers, Cortot, Jewell, & Leijonmarck, 1999) found no benefit in either clinical or endoscopic recurrence at 1 year post-op after intestinal resection for CD.

1.8 Anastomotic healing

Most of our knowledge on gastrointestinal anastomotic healing comes from experiments performed on rats where colon is transected then anastomosed. Like healing in the skin, the gastrointestinal anastomosis undergoes the classic phases of wound healing. Anastomotic healing starts with the inflammatory phase. After the brief hemostatic response, Neutrophils migrate into the tissues to kill invading bacteria. Neutrophils are followed by macrophages. Macrophages act to both phagocytose debris and to release growth factors essential for wound healing such as TGF-B and IL-1B(Thompson, Chang, & Jobe, 2006; Zubaidi, Buie, Hart, & Sigalet, 2009). A unique feature of intestinal healing is the action of matrix metallo-proteases (MMP) and collagenases in the first 1-2 days of the inflammatory phase. These enzymes lead to greatly diminished wound strength. During this period, sutures provide the majority of the strength(Ågren et al., 2006).

MP subsets may be prone to significant perturbations following ICR, with intestinal anastomosis because of their role in tissue repair and wound healing. In general, peripheral blood monocytes are recruited to injured tissues during the first inflammatory phase of wound healing and persist through to the remodeling phase where phenotypes shift from IL-6, TNF-a, IL-1 producing inflammatory cells to IL-10, TGF-b producing immunosuppressive cells(Novak & Koh, 2013; Sindrilaru & Scharffetter-Kochanek, 2013).

The next phase is the proliferative phase. This is marked by the arrival of fibroblasts, which begin to form collagen rich granulation tissue. In the intestinal tract, collagen is

produced by both fibroblasts and smooth muscle cells, and consists of types 1, 3 and 5 collagen(Thompson et al., 2006). Interestingly collagen synthesis and wound strength is increased rapidly in the small intestine relative to the colon(M. F. Martens & Hendriks, 2006). The remodeling phase is not well studied in the intestine; but like skin, involves the maturation of the collagen matrix into organized bundles with increased strength.

Research aimed at improving anastomotic healing, and reducing intestinal leaks has focused on both supplementing with growth factors and inhibiting MMPs. A range of growth factors have been tested in experimental models of intestinal anastomosis. Growth factors increase extracellular matrix production, fibroblast proliferation and angiogenesis(E. R. MD, 2013b). Administration of keratinocyte growth factor (KGF) has demonstrated improved colonic healing in a rat model of colonic resection with anastomosis as determined by increased burst pressures(Egger et al., 1998). Vascular endothelial growth factor (VEGF) was used in a rabbit model of colonic anastomosis. VEGF treated animals also demonstrated improved anastomotic healing associated with increased angiogenesis, and fibroblast proliferation(Ishii et al., 2009). Rijcken et al. used local administration of insulin like growth factor-1 (IGF-1) by coating sutures used for the colonic anastomosis in a rat model of colitis. They were able to improve healing as demonstrated by increased burst pressures and hydroxyproline content(Rijcken et al., 2010).

MMPs produced largely by infiltrating macrophages lead to rapid collagenolysis. An experiment evaluating MMPs around sutures in the colonic anastomosis found elevated MMP-8 and MMP-9 associated with excessive collagenolysis(Agren, Jorgensen, &

Delaissé, 2004). Inhibition of MMPs with drugs like doxycycline has been shown to increased anastomotic strength in the newly formed anastomosis(Siemonsma et al., 2003).

No studies to date have focused on healing parameters in an ileocolonic anastomosis. Instead, the few experiments published using animal models of ICR have focused on application to human illness.

1.9 Mouse models of ICR

A mouse model of ICR was initially developed by Helmrath et al. to be used as a model of short gut syndrome. The authors experimented with various diet regiments and suture sizes to optimize animal survival following ICR. They concluded that feeding with liquid diet in the perioperative period and using 9-0 monofilament sutures resulted in optimal post-operative survival with an overall rate of 88%. The authors also performed some rudimentary investigations to identify an adaptive growth response(Helmrath, VanderKolk, Can, Erwin, & Warner, 1996). Since this initial publication, ICR in mice with 50% small bowel resection has been used in several studies to explore the dynamics of massive small bowel resection and the adaptive growth response in attempt to develop new therapies for short gut syndrome. This model was used to highlight the importance of intestinal stem cells in the adaptive growth response following massive small bowel resection. The authors of this study evaluated the growth response and expansion of intestinal stem cells from 2 days post-operatively to 16 weeks. They found an overall increase in intestinal stem cells in the crypts as defined by immunohistochemical markers and BrdU label retention. Surface area of the intestine rapidly increased in the short term via an increase in villous height. The surface area was maintained in the long term by

increasing the caliber of the bowel and number of villi after the height increase was lost(Dekaney et al., 2007). A follow up study by the same group found the presence of bacteria and inflammation enhanced the adaptive growth response, highlighting potential implications for Crohn's disease(Speck et al., 2011).

The First publication application of the ICR mouse model to Crohn's disease used the IL-10 null mouse, which spontaneously develops colitis. The authors found that after ICR these animals developed inflammation in the neo-terminal ileum similar to that seen in post-operative Crohn's disease patients, and that this inflammation was dependent on the presence of bacteria(Rigby et al., 2009). More recently this model was used to explore bacterial changes induced by ICR. In Crohn's disease there is an associated dysbiosis with relative decreases in bacteria known to have anti-inflammatory properties and increases in invasive species of bacteria(Neut et al., 2002; Sokol et al., 2008). The association holds true in cases of post-operative recurrence(Ahmed, Rieder, Fiocchi, & Achkar, 2011). Two studies sought to identify microbial changes resulting from ICR. The first used IL-10 null mice, and performed denaturing gel electrophoresis to compare bacterial similarity between the small bowel and colon after ICR. This study demonstrated that bacterial populations became similar in the small intestine and colon following ICR. A subsequent study used wild type mice and 16s pyrosequencing for phylogenetic classification of bacterial species post-operatively. This study demonstrated a marked shift in bacterial species resulting from surgery alone with clostridial species becoming dominant as well as an increase in gamma-proteobacteria. The results also confirmed the findings of the previous study with similar populations found in the small bowel and colon after ICR(Devine et al., 2013). Carrying this model forward to explore

the pathogenesis of post-operative Crohn's disease it will be important to link these microbial changes with host immune response following ICR.

1.10 Aims and hypothesis

The goal of this thesis project is to identify factors associated with ileocecal resection, that impact the high rates of post-operative recurrence, with the hypothesis that specific microbial and immunologic changes occur with ICR and these changes lead to rapid and uniform recurrence in the neo-terminal ileum.

Chapter 2. Anastomotic Leaks and Preoperative Anti-Tumor Necrosis Factor-α in IBD: A Systematic Review and Meta-analysis.

2.1 Introduction

Inflammatory bowel disease continues to be a surgical illness. 10 year surgery rates for ulcerative colitis (UC) and Crohn's disease (CD) are 16% and 47% respectively(Cosnes et al., 2011). Newer biologic therapies have been successful in treating active disease and extending surgery free survival; however, these medications are prone to failure with time, due to formation of anti-drug antibodies or refractory illness. Anti-tumor necrosis factor-α (anti-TNF) agents, more specifically infliximab (IFX), are the most commonly used biologic therapies in IBD. This class of drugs targets the TNF-α cytokine, which is an important inflammatory mediator. Because of the increasing rates of IBD(Iesalnieks et al., 2012) coupled with wide spread anti-TNF use, IBD patients are frequently presenting for surgery having recently received anti-TNF treatment.

IBD patients have higher rates of surgical complications. Anastomotic leaks are one of the most devastating of these, because of the associated morbidity(Subramanian et al., 2008; Yamamoto, Yamamoto, Shiraki, & Shiraki, 2013). This increased risk for CD patients has long been attributed to poor nutritional status and preoperative use of corticosteroids(Anstead & Anstead, 1998; Bootun, 2013) because of their known

immunosuppressive properties and detrimental effects on wound healing(Zubaidi et al., 2009). The arrival of anti-TNF drugs raised suspicion that they may further increase postoperative complication rates in IBD, because of the their potent immunosuppressive properties. Studies from the basic science literature suggest that TNF-α plays a minor role in anastomotic healing(Alam, Kim, Bonnet, Kirkpatrick, & Provido, 1996), and may itself have detrimental effects, with one study finding that neutralization of TNF-α led to improved anastomotic healing(Appau et al., 2008). However, some human studies have suggested anti-TNF medications have a negative effect on anastomotic healing(Kopylov, Ben-Horin, Zmora, Eliakim, & Katz, 2012); thus, observations from animal models may not translate into humans, making it essential to evaluate anti-TNF effects on anastomotic healing in IBD patients.

Two recent systematic reviews evaluated the overall risk of postoperative complications in patients treated with anti-TNF medications preoperatively. The paper by Kopylov *et al.* focused on CD, and evaluated articles indexed on pubmed. They found a trend toward increased postoperative complications, and an overall increased rate of infectious complications in CD(Yang, Wu, Wang, Wu, & Fan, 2012). When they looked specifically at anastomotic complications they did not demonstrate a significant increase with an OR of 1.18 [0.61-2.3]. However, the authors excluded one study which specifically evaluated anastomotic complications in CD. The study by Yang *et al.* focused on UC, and searched articles indexed in Pubmed and Embase databases(Norgard et al., 2013; 2012). Their results suggested preoperative anti-TNF medications were safe in UC patients, with no increase in total complications or infectious complications. The authors did not specifically evaluate anastomotic complications. The majority of the

studies included in these reviews have fewer than 50 patients in the exposed groups. Also, the solitary disease focus forced the authors to exclude studies including both UC and CD patients.

Because of the substantial morbidity associated with anastomotic complications, it is essential for operating surgeons to know if preoperative anti-TNF medications confer an increased risk.

This systematic review capitalizes on recent large registry studies(Fasanmade, Adedokun, Blank, Zhou, & Davis, 2011), and pools all IBD studies in a meta-analysis to determine if IBD patients receiving anti-TNF medications prior to intestinal resection are at higher risk for anastomotic leaks vs IBD patients not receiving preoperative anti-TNF therapy. Secondary outcomes assessed include 30 day re-operation rate, infectious complication rates, and total complications.

2.2 Methods

Search Strategy. MEDLINE and EMBASE data bases were searched from 1950 to February 15 2013. The search strategy was developed in consultation with a research librarian. There were no limitations placed on dates or language. The study populations were defined by the subject headings exp Crohn Disease/ or exp Inflammatory Bowel Diseases/ or exp Colitis/ or exp Colitis, Ulcerative, with keyword IBD. Titles and abstracts were searched for Crohn* and Colitis. The exposure was defined with the subject heading exp Tumor Necrosis Factor-alpha and keyword Anti-TNF. Titles and abstracts were searched for Infliximab, remicade, adalimumab, humira, certolizumab, and biologic. The outcomes were defined by subject headings exp General Surgery/ or exp Postoperative Complications/ or exp Anastomosis, Surgical. Titles and abstracts were

search for postop*, post-op*, and complication*. Results from these searches were combined to identify studies of interest (Appendix 1).

Grey literature was included by hand searching 2011-2013 conference proceedings from the largest international gastroenterology meeting - Digestive Diseases Week. An attempt was made to contact authors when abstracts did not provide enough data for inclusion in the analysis.

Study Selection. This review included published and unpublished, retrospective or prospective, observational cohort or case-control studies in IBD patients undergoing abdominal surgery for intestinal resection. Studies from IBD patient registries were also included. Patients included in the studies had a known diagnosis of IBD. Unpublished studies in abstract form were only considered if relevant data could be extracted from the abstract or the authors provided necessary data. Studies compared 30 day postoperative complication rates in anti-TNF exposed IBD patients (CD, UC or both), to unexposed IBD patients. Infliximab (IFX), adalimumab (ADA) and certolizumab (CZ) comprised the anti-TNF medications included in the studies. If the last preoperative dose was beyond 12 weeks prior to surgery in the exposure arm, studies were excluded. The 12 week exposure time point was chosen because the half life of the most frequently used anti-TNF agent; IFX, is approximately 2 weeks making it undetectable 12 weeks post-infusion(Wells, Shea, & O'connell, 2000). 30 day followup is required to capture anastomotic complications. Studies were excluded if they did not report on any of the specified outcomes with a direct comparison between anti-TNF exposed and unexposed groups.

Outcome measures. The primary outcome measure of this systematic review was anastomotic leaks within 30 days of intestinal resection and anastomosis. Studies not specifying anastomotic leaks were excluded from this portion of the review. Anastomotic leaks were defined as any breakdown of the intestinal anastomosis leading to re-operation, abscess formation, or fistula. Secondary outcomes assessed included re-operation, infectious complication, and total complication rates. Infectious complications were defined as deep or superficial surgical site infections, pneumonia, sepsis/bacteremia, urinary tract infections, and any other post-operative infections specified in the studies. Outcomes were recorded as reported by the studies because it was imposible to determine if patients were included in more than on category of complication....

Data extraction. Results of the electronic and grey literature search were screened by two independent reviewers. Relevant studies identified by one or both reviewers based on title and abstract were selected for assessment of the full text. Both reviewers completed an inclusion/exclusion form for studies under full text review. Disagreements regarding the inclusion of studies were settled by a meeting between the two reviewers to reach consensus. Studies meeting the selection criteria were subjected to a quality assessment using the Newcastle-Ottawa quality assessment scale for cohort studies or case-control studies(Appau et al., 2008; Bregnbak et al., 2012; Canedo et al., 2011; Colombel et al., 2004; El-Hussuna et al., 2012; Ferrante et al., 2009; Gainsbury et al., 2011; Indar, Young-Fadok, Heppell, & Efron, 2009; Kasparek et al., 2012; Kunitake et al., 2008; Nasir et al., 2010; Norgard et al., 2012; 2013; Regadas et al., 2011; Rizzo et al., 2011;

Schaufler et al., 2012; Selvasekar et al., 2007; Tay et al., 2003; Waterman et al., 2013). Quality scoring was performed on each study by both reviewers. Disagreements on the assignment of scores were settled by discussion. Data from the included studies was extracted using a piloted form. One reviewer collected data and the second reviewer checked the data for accuracy. Any significant discrepancies between study group and controls were noted. Variables collected in the form included the following:

1) Patient characteristics: median age, gender proportions, IBD diagnosis evaluated (CD, UC or both), proportion of elective/emergent surgeries (emergent surgeries were defined as those performed for intestinal perforation, acute obstruction, toxic mega colon and/or sepsis), corticosteroid (CS) and immunomodulator (IM) (azathioprine, 6-mercaptopurine, and methotrexate) use at the time of surgery, and median serum albumin.
2) Surgical Procedures included: intestinal resection, ostomy reversal, portion of intestine resected(colon, ileocolonic, distal small bowel or proximal small bowel), locations of anastomosis (ileocolonic, entero-entero, colo-colonic or ileal pouch-anal), diversion procedures (i.e. ostomy creation), and laparoscopic or open.
3) Anti-TNF exposure: Drug (IFX, adalimumab, or certolizumab), Median time to surgery from last dose,
4) Outcomes: anastomotic leaks, total complication rate, infectious complication rates, definitions of complications, and re-operation rate.
5) Study characteristics: retrospective or prospective, published or unpublished, country, single or multi center, years of data collection, study inclusion/exclusion criteria, number of participants and funding sources.

Data analysis. Meta-analysis was performed to assess the primary and secondary outcomes including studies where appropriate data was available. A random effects model was used to pool results. All outcomes were dichotomous and expressed as odds ratios (OR) with corresponding 95% confidence intervals. Heterogeneity was determined using the I^2 statistic.

To determine if diagnosis had an effect on postoperative complications, subgroup analysis was performed on studies evaluating CD and UC alone, and studies evaluating mixed IBD cohorts were included as a third subgroup when data for each disease could not be separated. Additional subgroup analysis was done on studies, which reported higher rates of concomitant immunomodulator use in the anti-TNF exposed group. Sensitivity analysis was performed to compare studies of high and low quality as determined by a Newcastle-Ottawa scale score of < 6.

Publication bias was explored using funnel plots for primary and secondary outcomes. Statistical analysis was performed using RevMan version 5.1 software.

2.4 Results

Search Results

The search strategy generated 2125 results (Figure 1). After duplicates were removed there was a total of 1505 references subjected to primary screening. 1462 references were excluded based on title and abstract leaving 43 references for full text review using the inclusion form. 11 of these studies were excluded because data was duplicated in two or more publications. When this was the case, the most recent publication was selected for inclusion in the study.

6 studies were excluded because they included anti-TNF use beyond 12 weeks pre-operatively in the exposed group. 4 studies presented as abstracts lacked sufficient data for inclusion; authors were contacted to provide necessary details, but no response was received. 1 study was excluded because the authors evaluated patients undergoing any abdominal surgery with or without intestinal resection and 1 study was excluded because the exposed group was compared to non-IBD patients.

After screening, 20 retrospective observational studies remained for inclusion in this review.

Study Characteristics

A summary of the study characteristics is presented in Table 1.

Of the studies included for analysis, 19 were published in peer reviewed journals(Kotze, Albuquergue, & Sobrado, 2011), and 1 was presented as an abstract at digestive diseases week(Ferrante et al., 2009). Studies were published between 2003-2013. All studies were retrospective with 18 cohort, the two largest of which were from national IBD registries, and 2 case-control studies.

In total this systematic review includes 7643 IBD patients; 1409 (18%) patients were treated with anti-TNF medications pre-operatively and 6234 (82%) were not. 6 studies included UC patients only, 10 studies evaluated CD and 4 included mixed IBD cohorts. Anti-TNF medications used included IFX only (14 studies), IFX or ADA (3 studies), IFX or CZ (1 study), and IFX, ADA or CZ (2 studies). IFX was the most common medication used In studies where other anti-TNF therapies were included. The anti-TNF preoperative treatment period was defined as 12 weeks in 12 of the studies, 8 weeks in 6 studies, and 4 weeks in 2 studies. Two of the studies also included patients if they received ant-TNFs

within 4 weeks postoperativley , but this comprised a small proportion of the patients. Procedures in the UC studies included colectomy or proctocolectomy. When reported, the majority of patients received an ileostomy in both the anti-TNF group and control group as part of the first stage of a 2 or 3 stage ileal pouch anal anastomosis (IPAA) procedure. One study reported increased rates of ileostomy creation in the anti-TNF treated group(Appau et al., 2008). The CD studies varied in procedures included; however, the majority of the studies included any intestinal procedures. The Appau study(Nasir et al., 2010; Tay et al., 2003) included only ileocolonic resections and the Nasir and Tay studies(Canedo et al., 2011; Kasparek et al., 2012) excluded patients if a diverting ostomy was created as part of the procedure. Of the 5 CD studies which reported ostomy rates, 2 had more ostomies in the anti-TNF treated group(Regadas et al., 2011). Of the 4 mixed IBD studies, 3 included any intestinal procedures for UC or CD and 1 included ileostomy reversals only(Wells et al., 2000).

Concomitant medications for the study groups of interest were reported in 16 of the studies; of those, 8 found increased IM use and 3 found increased CS use in the anti-TNF cohorts.

Quality assessment

Results of the risk of bias/quality assessment using the Newcastle-Ottawa scale(Alam et al., 1996; Zubaidi et al., 2009) is presented in Table 2. In general, most studies were of good quality. The only study with a score below 6 was the sole abstract included. The most common deficiencies noted were in the selection and comparability categories. Many studies reported significant differences in medication use and ostomy rates between groups, and failed to demonstrate the absence of preexisting outcomes prior to

surgery. This of course does not apply to anastomotic leaks, however, it is important to note if infections were present prior to surgery.

Outcomes

Definition of outcomes within studies and occurrence rates are presented in Table 3. Anastomotic leaks were reported in 15 of the studies. 10 of the studies provided a definition of anastomotic leaks. In total 6456 patients were included in this meta-analysis, of which, 1131(18%) were in the anti-TNF exposure group. Pooled results (Fig. 2a) did not demonstrate any increased risk of anastomotic leaks in the anti-TNF group (OR 1.01, 95% CI 0.65-1.58, I^2 24%). In subgroup analysis separating studies based on diagnosis (Fig. 2b) and concomitant IM use (Fig. 2c) there were no significant differences in anastomotic leaks between groups. Removal of the lone low quality study had no impact the results.

Infectious complications were reported in 13 studies. Pooled results (Fig. 3a) did not demonstrate any significant differences between anti-TNF and non anti-TNF groups (OR 1.04, 95% CI 0.68-1.57, I^2 64%). Subgroup analysis separating studies based on diagnosis (Fig. 3b) failed to demonstrate any significant differences; however a subgroup of 6 studies reporting higher rates of concomitant IM (Fig. 3c) use in the anti-TNF group showed increased odds of infectious complications in the anti-TNF treated group (OR 1.73, 95% CI 1.11-2.72, I^2 56%). Conversely, the 5 studies demonstrating equivalent IM use across groups showed anti-TNF treatment was associated with a decreased odds of infectious complications (OR 0.58, 95% CI 0.35-0.96, I^2 0%).

Total complications were reported in 14 studies. Overall, anti-TNF treatment was not associated with an increased odds of total postoperative complications (OR 1.29, 95% CI

0.91-1.84, I^2 68%) (Fig. 4a). Subgroup analysis based on diagnosis did not demonstrate any significant differences between groups (Fig. 4b); however increased complications were noted in the subgroup of studies with equivalent IM use across study groups (OR 1.49, 95% CI 1.01-2.19, I^2 69%).

Risk of bias across studies

Fig. 5 depicts funnel plots for the three outcomes explored in this meta-analysis. In all three cases a gap was noted where smaller studies reporting increased complications in the exposed group would have been plotted. Subjectively, there also appeared to be significant heterogeneity in the complications included in the studies. This was most notable in the total complications category.

2.5 Discussion

Anti-TNF medications are becoming widely used for the treatment of IBD, and while they have proven efficacy in the treatment of disease, it is important to examine their safety in the perioperative setting. This is the first systematic review and meta-analysis to evaluate anastomotic outcomes in IBD patients treated with anti-TNF medications preoperatively. This question is an important one because of the devastating consequences of anastomotic leaks, and the surgeons ability prevent them by creating a diverting ostomy or performing a three stage IPAA procedure in high risk patients. The summary of the evidence in this review suggest that preoperative treatment with anti-TNF medications does not negatively impact anastomotic healing. This is in keeping with results from the basic science literature(Kopylov et al., 2012). In the subgroup analysis it was clear that anti-TNFs had no effect in CD patients. In UC the anti-TNF group trended towards decreased anastomotic leaks. The study by Slevasekar et al. was the only UC

study to report increased anastomotic leaks and when this was removed from the analysis there was as strong trend towards fewer leaks in UC patients receiving anti-TNF therapy (OR 0.28, 95% CI 0.07-1.04, I^2 0%). When studies were evaluated based on concomitant IM use there was a trend towards increased anastomotic leaks in the anti-TNF group when IM use was higher in this group. The opposite trend was seen in studies with equivalent IM use across groups(Fig. 3c). In summary, the current evidence suggests that anti-TNF medications do not contribute to anastomotic leaks with a minimal amount of heterogeneity (I^2 24%). There are important limitations to consider when interpreting this data. Only 6 of the 15 studies included in this portion of the meta-analysis provided a clear definition of anastomotic leaks and patient characteristics between study groups did vary. The funnel plot suggests that publication bias may exist with small studies reporting increased anastomotic leak rates being omitted from the literature.

Overall infectious complications were not increased in the anti-TNF groups, however a moderate amount of heterogeneity was found when all IBD studies were pooled (I^2 64%). Subgroup analysis did not find any significant differences between anti-TNF and control groups in UC and CD, But did demonstrate a trend towards increased complication in CD and decreased complications in UC patients in the anti-TNF groups. The two previous systematic reviews evaluating preoperative anti-TNF medications in UC and CD reported similar results for infectious complications. However, the CD study reported a significantly increased odds of infectious complications (OR 1.5 95% CI 1.08-2.08)(Subramanian et al., 2008). The subgroup analysis reporting increased infectious complications in studies with increased concomitant IM use in the anti-TNF group succeeded in reducing the heterogeneity, and suggests there may be a synergistic effect of

combination IM and anti-TNF therapy placing patients at increased odds of developing an infectious complication. IM use itself does not appear to confer an increased risk of postoperative infectious complications based on a systematic review by Subramanian et al.(Kopylov et al., 2012; Yang et al., 2012).

Total complications were not significantly different between anti-TNF groups and controls. A significant increase was noted in the anti-TNF treated patients in comparison to controls with similar rates of IM use. This is a surprising finding as it contradicts the results of the infectious complication outcome. There was large heterogeneity noted in this portion of the meta-analysis and this remained in all subgroups. Sensitivity analysis to remove the single low quality study did not change the results. The previous systematic reviews also found no increase in total complications in either UC or CD patients.

Despite the large number of patients included in the review there are several key limitations. Retrospective studies are prone to measurement errors and recall bias. Definitions of complications included were not always clear and varied between studies. Furthermore, differences between anti-TNF groups and control groups were found within the studies. Concomitant medications appear to be a significant effect modifier but were not reported in all studies. Ostomy rates were reported in a minority of studies and could also act as a significant effect modifier. A major confounding factor in these studies is the possibility that anti-TNF treated patients have worse disease and may be predisposed to complications. It is impossible to control for the multitude of factors that could impact on patient outcomes in a retrospective study, however it is infeasible to evaluate this question in a randomized control trial. Perhaps the best study design to answer this

questions with more certainty would be a large prospective cohort study.

In conclusion, this is the first systematic review and meta-analysis to evaluate anastomotic leaks in IBD patients treated with anti-TNF medications preoperatively. The results suggest that the odds of anastomotic leak in anti-TNF exposed patients are no greater than unexposed patients. When planning for surgery in these patients it is essential for the surgeon to be aware of factors impacting the integrity of the anastomosis. The finding of increased infectious complications with combination IM and anti-TNF therapy warrants further investigation and should be specifically addressed in future studies.

2.6 Figures and Tables.

Figure 2.1. Study Selection.

Figure 2.2. Pooled data evaluating anastomotic leaks in anti-TNF treated groups vs. No preoperative anti-TNF treatment. (a) OR for development of anastomotic leaks in all studies reporting outcome. (b) OR for development of anastomotic leaks by diagnosis in studies. (c) OR for development of anastomotic leaks by studies reporting increased immunomodulator use in the anti-TNF groups.

Figure 2.3. Pooled data for postoperative infectious complications in anti-TNF treated groups vs. No preoperative anti-TNF treatment. (a) OR for development of infectious complications in all studies reporting outcome. (b) OR for development of infectious complications by diagnosis in studies. (c) OR for development of infectious complications by studies reporting increased immunomodulator use in the anti-TNF

groups.

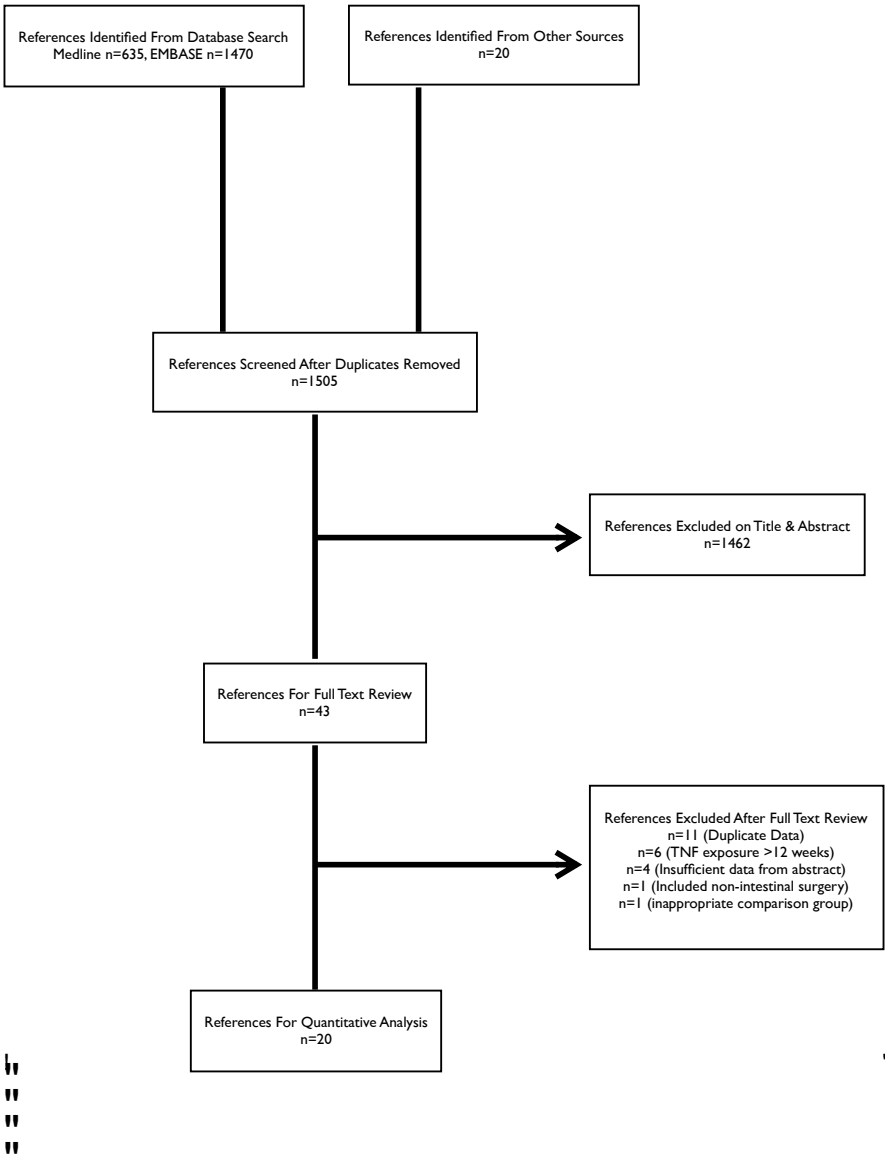
Figure 2.4. Pooled data for total postoperative complications in anti-TNF treated groups vs. No preoperative anti-TNF treatment. (a) OR for total complications in all studies reporting outcome. (b) OR for total complications by diagnosis in studies. (c) OR for total complications by studies reporting increased immunomodulator use in the anti-TNF groups.

Figure 2.5. Funnel plots for primary and secondary outcomes. (a) Anastomotic Leaks, (b) Infectious complications, (c) Total complications.

Table 2.1. Study characteristics

Table 2.2. Risk of bias and quality assessment.

Table 2.3. Study outcomes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n=6 (TNF exposure >12 weeks)
n=4 (Insufficient data from abstract)
n=1 (Included non-intestinal surgery)
n=1 (inappropriate comparison group)
References For Quantitative Analysis
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Figure 2.1.

Figure 2.2A.

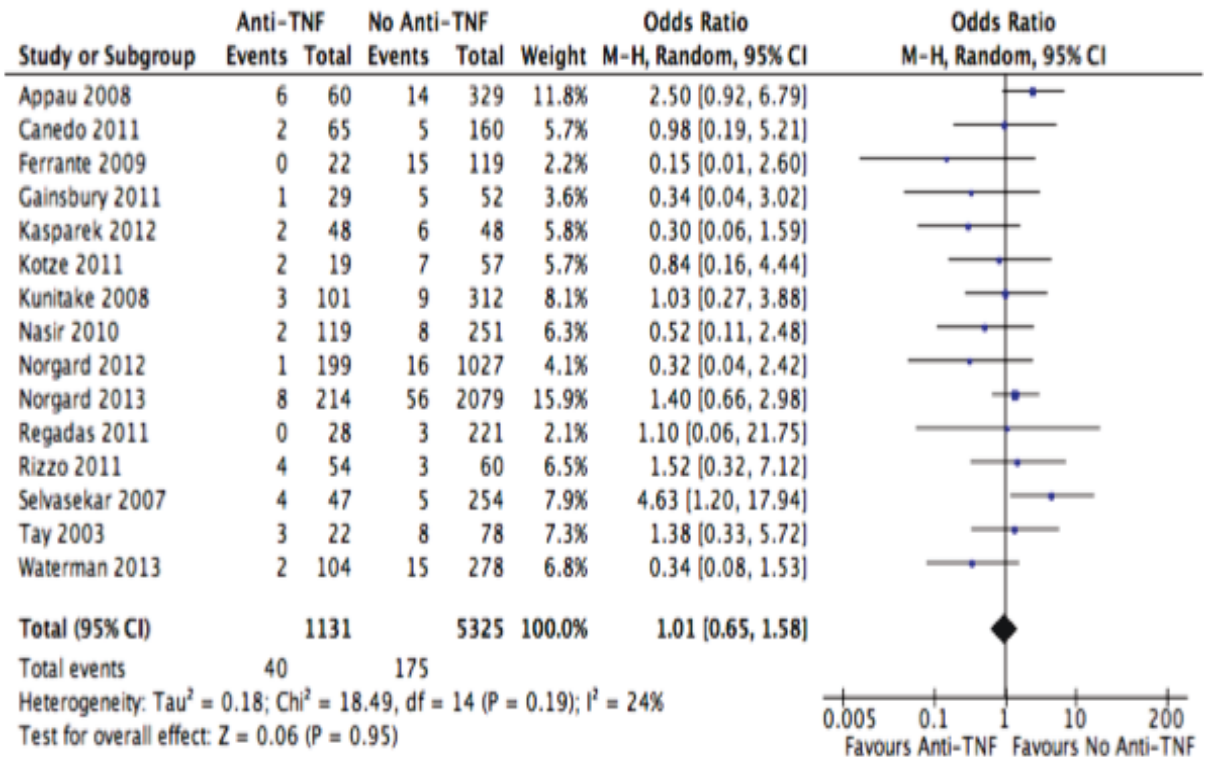

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
Appau 2008 6 60 14 329 11.8% 2.50 [0.92, 6.79]
Canedo 2011 2 65 5 160 5.7% 0.98 [0.19, 5.21]
Ferrante 2009 0 22 15 119 2.2% 0.15 [0.01, 2.60]
Gainsbury 2011 1 29 5 52 3.6% 0.34 [0.04, 3.02]
Kasparek 2012 2 48 6 48 5.8% 0.30 [0.06, 1.59]
Kotze 2011 2 19 7 57 5.7% 0.84 [0.16, 4.44]
Kunitake 2008 3 101 9 312 8.1% 1.03 [0.27, 3.88]
Nasir 2010 2 119 8 251 6.3% 0.52 [0.11, 2.48]
Norgard 2012 1 199 16 1027 4.1% 0.32 [0.04, 2.42]
Norgard 2013 8 214 56 2079 15.9% 1.40 [0.66, 2.98]
Regadas 2011 0 28 3 221 2.1% 1.10 [0.06, 21.75]
Rizzo 2011 4 54 3 60 6.5% 1.52 [0.32, 7.12]
Selvasekar 2007 4 47 5 254 7.9% 4.63 [1.20, 17.94]
Tay 2003 3 22 8 78 7.3% 1.38 [0.33, 5.72]
Waterman 2013 2 104 15 278 6.8% 0.34 [0.08, 1.53]
Total (95% CI) 1131 5325 100.0% 1.01 [0.65, 1.58]
Total events 40 175
Heterogeneity: Tau² = 0.18; Chi² = 18.49, df = 14 (P = 0.19); I² = 24%
Test for overall effect: Z = 0.06 (P = 0.95)
0.005 0.1 1 10 200
Favours Anti-TNF Favours No Anti-TNF

Figure 2.2B.

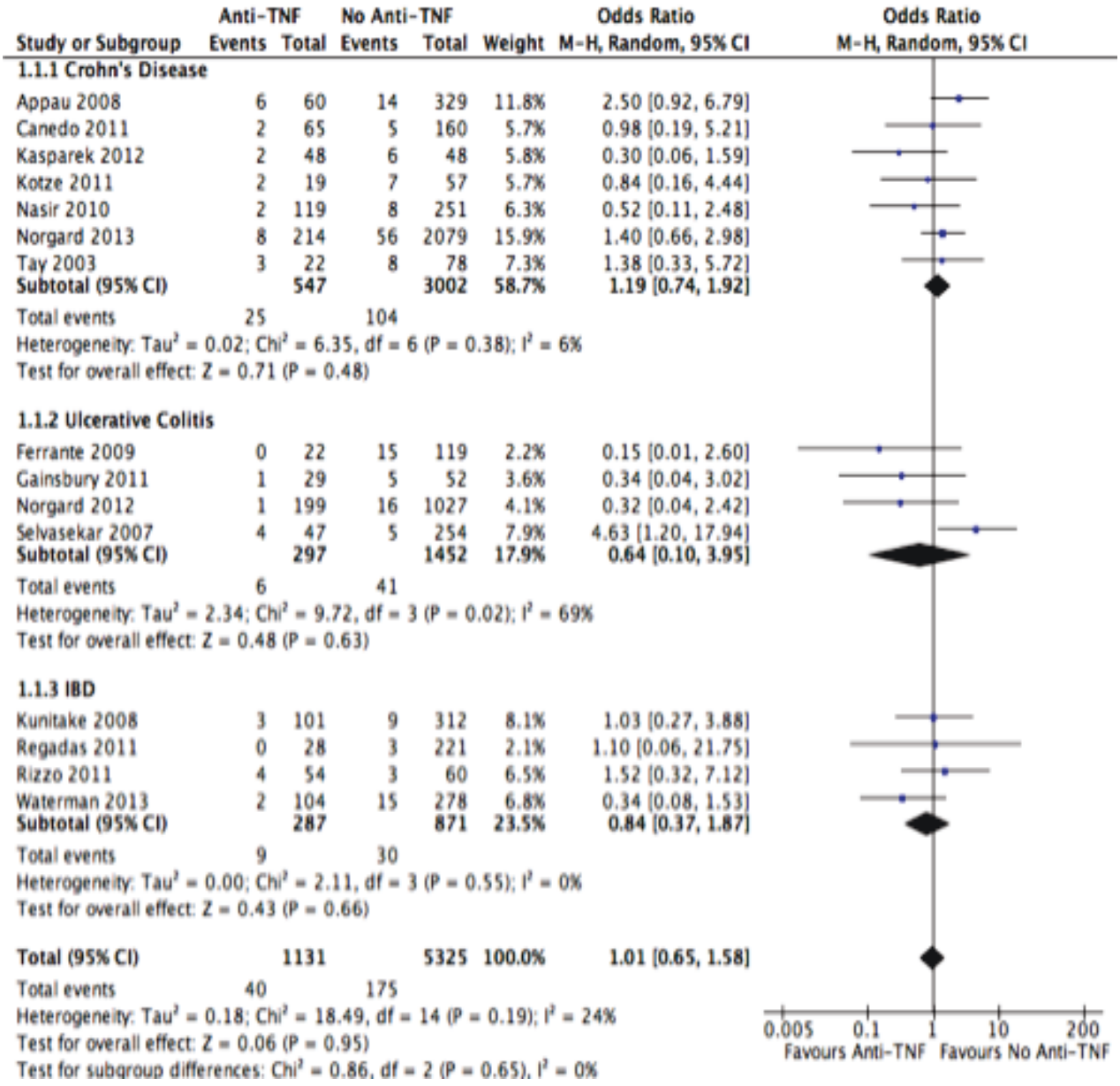

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
1.1.1 Crohn's Disease
Appau 2008 6 60 14 329 11.8% 2.50 [0.92, 6.79]
Canedo 2011 2 65 5 160 5.7% 0.98 [0.19, 5.21]
Kasparek 2012 2 48 6 48 5.8% 0.30 [0.06, 1.59]
Kotze 2011 2 19 7 57 5.7% 0.84 [0.16, 4.44]
Nasir 2010 2 119 8 251 6.3% 0.52 [0.11, 2.48]
Norgard 2013 8 214 56 2079 15.9% 1.40 [0.66, 2.98]
Tay 2003 3 22 8 78 7.3% 1.38 [0.33, 5.72]
Subtotal (95% CI) 547 3002 58.7% 1.19 [0.74, 1.92]
Total events 25 104
Heterogeneity: Tau² = 0.02; Chi² = 6.35, df = 6 (P = 0.38); I² = 6%
Test for overall effect: Z = 0.71 (P = 0.48)
1.1.2 Ulcerative Colitis
Ferrante 2009 0 22 15 119 2.2% 0.15 [0.01, 2.60]
Gainsbury 2011 1 29 5 52 3.6% 0.34 [0.04, 3.02]
Norgard 2012 1 199 16 1027 4.1% 0.32 [0.04, 2.42]
Selvasekar 2007 4 47 5 254 7.9% 4.63 [1.20, 17.94]
Subtotal (95% CI) 297 1452 17.9% 0.64 [0.10, 3.95]
Total events 6 41
Heterogeneity: Tau² = 2.34; Chi² = 9.72, df = 3 (P = 0.02); I² = 69%
Test for overall effect: Z = 0.48 (P = 0.63)
1.1.3 IBD
Kunitake 2008 3 101 9 312 8.1% 1.03 [0.27, 3.88]
Regadas 2011 0 28 3 221 2.1% 1.10 [0.06, 21.75]
Rizzo 2011 4 54 3 60 6.5% 1.52 [0.32, 7.12]
Waterman 2013 2 104 15 278 6.8% 0.34 [0.08, 1.53]
Subtotal (95% CI) 287 871 23.5% 0.84 [0.37, 1.87]
Total events 9 30
Heterogeneity: Tau² = 0.00; Chi² = 2.11, df = 3 (P = 0.55); I² = 0%
Test for overall effect: Z = 0.43 (P = 0.66)
Total (95% CI) 1131 5325 100.0% 1.01 [0.65, 1.58]
Total events 40 175
Heterogeneity: Tau² = 0.18; Chi² = 18.49, df = 14 (P = 0.19); I² = 24%
Test for overall effect: Z = 0.06 (P = 0.95)
Test for subgroup differences: Chi² = 0.86, df = 2 (P = 0.65), I² = 0%
0.005 0.1 1 10 200
Favours Anti-TNF Favours No Anti-TNF

Figure 2.2C.

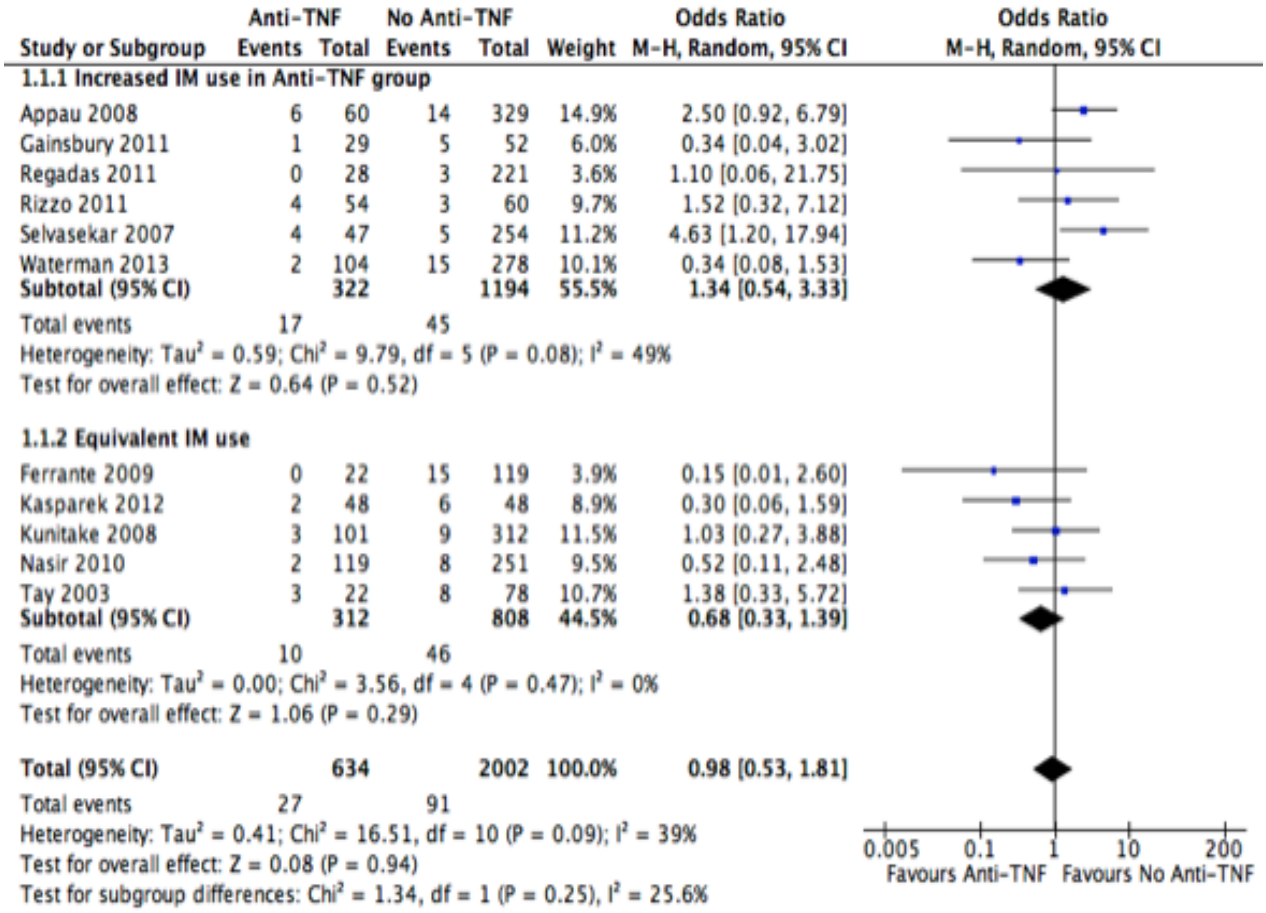
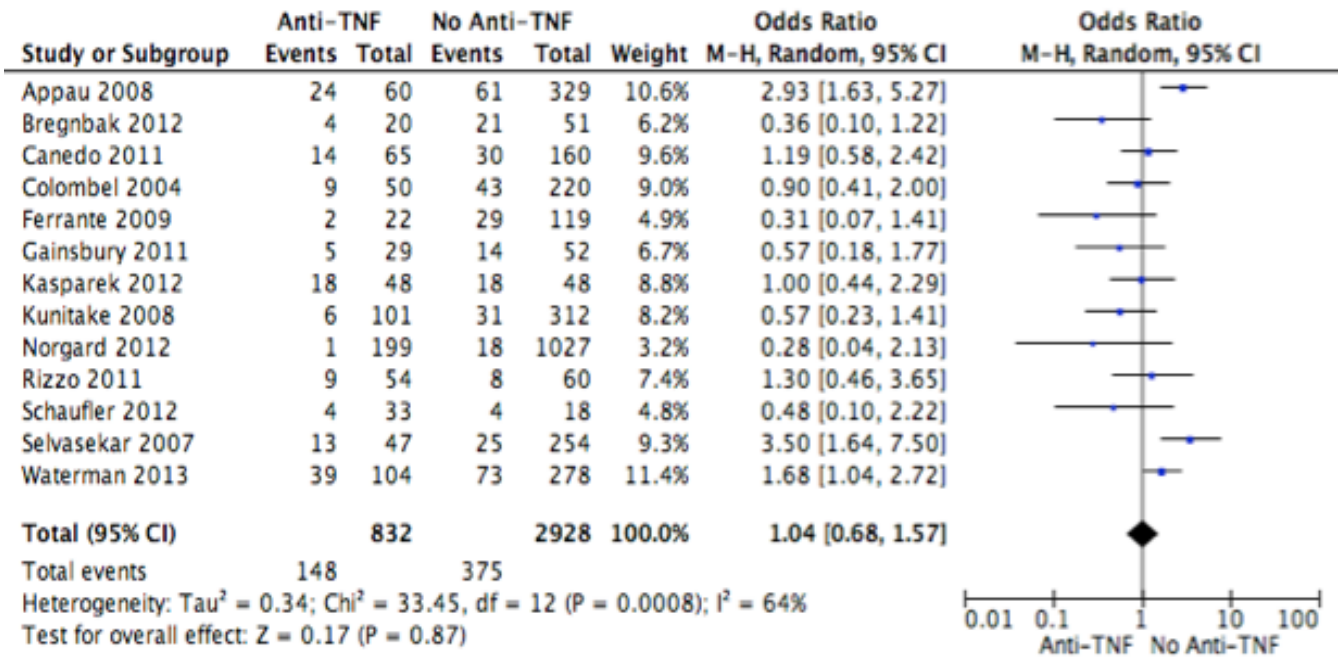

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
1.1.1 Increased IM use in Anti-TNF group
Appau 2008 6 60 14 329 14.9% 2.50 [0.92, 6.79]
Gainsbury 2011 1 29 5 52 6.0% 0.34 [0.04, 3.02]
Regadas 2011 0 28 3 221 3.6% 1.10 [0.06, 21.75]
Rizzo 2011 4 54 3 60 9.7% 1.52 [0.32, 7.12]
Selvasekar 2007 4 47 5 254 11.2% 4.63 [1.20, 17.94]
Waterman 2013 2 104 15 278 10.1% 0.34 [0.08, 1.53]
Subtotal (95% CI) 322 1194 55.5% 1.34 [0.54, 3.33]
Total events 17 45
Heterogeneity: Tau² = 0.59; Chi² = 9.79, df = 5 (P = 0.08); I² = 49%
Test for overall effect: Z = 0.64 (P = 0.52)
1.1.2 Equivalent IM use
Ferrante 2009 0 22 15 119 3.9% 0.15 [0.01, 2.60]
Kasparek 2012 2 48 6 48 8.9% 0.30 [0.06, 1.59]
Kunitake 2008 3 101 9 312 11.5% 1.03 [0.27, 3.88]
Nasir 2010 2 119 8 251 9.5% 0.52 [0.11, 2.48]
Tay 2003 3 22 8 78 10.7% 1.38 [0.33, 5.72]
Subtotal (95% CI) 312 808 44.5% 0.68 [0.33, 1.39]
Total events 10 46
Heterogeneity: Tau² = 0.00; Chi² = 3.56, df = 4 (P = 0.47); I² = 0%
Test for overall effect: Z = 1.06 (P = 0.29)
Total (95% CI) 634 2002 100.0% 0.98 [0.53, 1.81]
Total events 27 91
Heterogeneity: Tau² = 0.41; Chi² = 16.51, df = 10 (P = 0.09); I² = 39%
Test for overall effect: Z = 0.08 (P = 0.94)
Test for subgroup differences: Chi² = 1.34, df = 1 (P = 0.25), I² = 25.6%
0.005 0.1 1 10 200
Favours Anti-TNF Favours No Anti-TNF

Figure 2.3A.



Study or Subgroup	Anti-TNF Events	Anti-TNF Total	No Anti-TNF Events	No Anti-TNF Total	Weight	Odds Ratio M-H, Random, 95% CI
Appau 2008	24	60	61	329	10.6%	2.93 [1.63, 5.27]
Bregnbak 2012	4	20	21	51	6.2%	0.36 [0.10, 1.22]
Canedo 2011	14	65	30	160	9.6%	1.19 [0.58, 2.42]
Colombel 2004	9	50	43	220	9.0%	0.90 [0.41, 2.00]
Ferrante 2009	2	22	29	119	4.9%	0.31 [0.07, 1.41]
Gainsbury 2011	5	29	14	52	6.7%	0.57 [0.18, 1.77]
Kasparek 2012	18	48	18	48	8.8%	1.00 [0.44, 2.29]
Kunitake 2008	6	101	31	312	8.2%	0.57 [0.23, 1.41]
Norgard 2012	1	199	18	1027	3.2%	0.28 [0.04, 2.13]
Rizzo 2011	9	54	8	60	7.4%	1.30 [0.46, 3.65]
Schaufler 2012	4	33	4	18	4.8%	0.48 [0.10, 2.22]
Selvasekar 2007	13	47	25	254	9.3%	3.50 [1.64, 7.50]
Waterman 2013	39	104	73	278	11.4%	1.68 [1.04, 2.72]
Total (95% CI)		**832**		**2928**	**100.0%**	**1.04 [0.68, 1.57]**
Total events	148		375			

Heterogeneity: $Tau^2 = 0.34$; $Chi^2 = 33.45$, df = 12 (P = 0.0008); $I^2 = 64\%$

Test for overall effect: Z = 0.17 (P = 0.87)

Figure 2.3B.

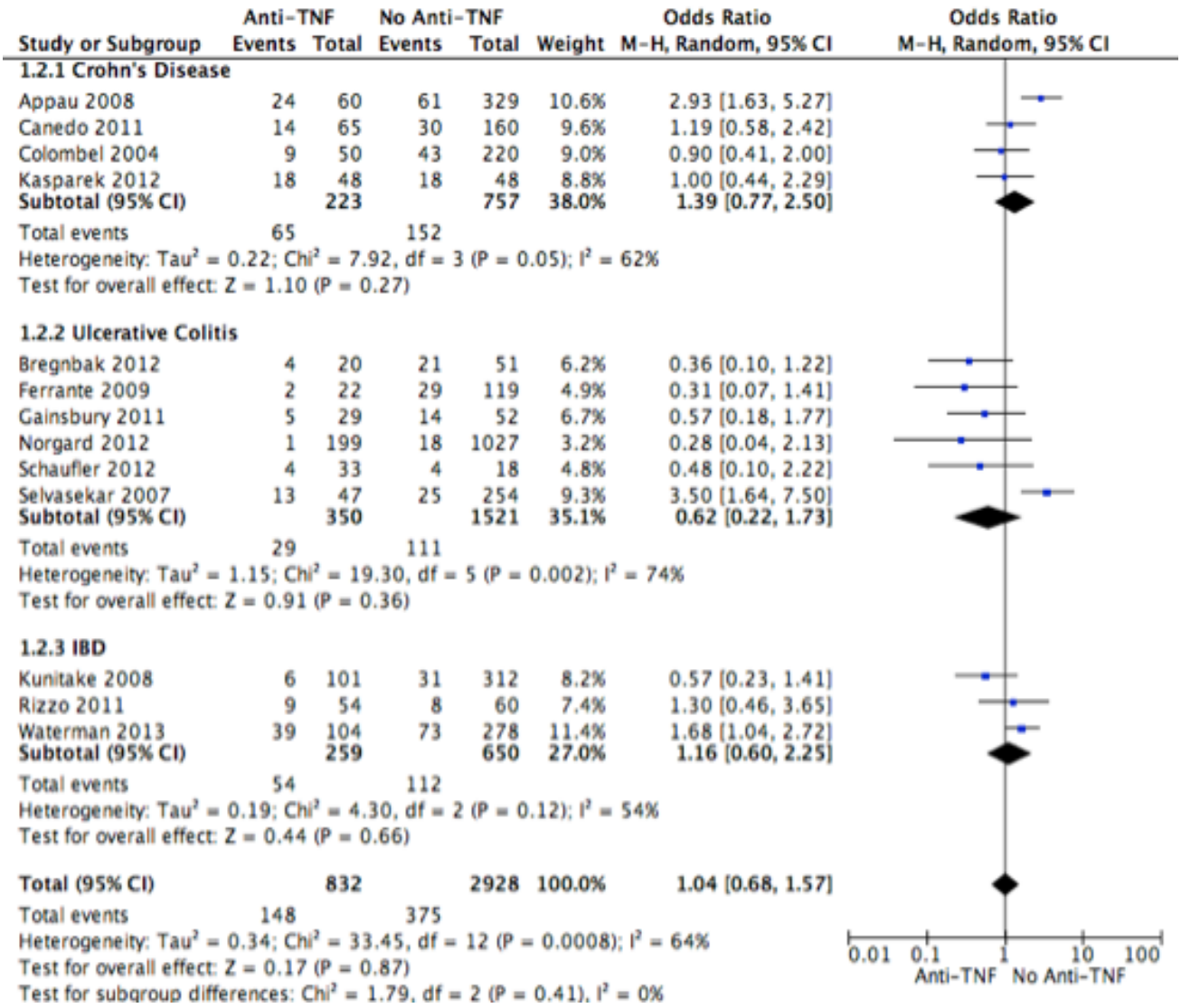

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
1.2.1 Crohn's Disease
Appau 2008 24 60 61 329 10.6% 2.93 [1.63, 5.27]
Canedo 2011 14 65 30 160 9.6% 1.19 [0.58, 2.42]
Colombel 2004 9 50 43 220 9.0% 0.90 [0.41, 2.00]
Kasparek 2012 18 48 18 48 8.8% 1.00 [0.44, 2.29]
Subtotal (95% CI) 223 757 38.0% 1.39 [0.77, 2.50]
Total events 65 152
Heterogeneity: Tau² = 0.22; Chi² = 7.92, df = 3 (P = 0.05); I² = 62%
Test for overall effect: Z = 1.10 (P = 0.27)
1.2.2 Ulcerative Colitis
Bregnbak 2012 4 20 21 51 6.2% 0.36 [0.10, 1.22]
Ferrante 2009 2 22 29 119 4.9% 0.31 [0.07, 1.41]
Gainsbury 2011 5 29 14 52 6.7% 0.57 [0.18, 1.77]
Norgard 2012 1 199 18 1027 3.2% 0.28 [0.04, 2.13]
Schaufler 2012 4 33 4 18 4.8% 0.48 [0.10, 2.22]
Selvasekar 2007 13 47 25 254 9.3% 3.50 [1.64, 7.50]
Subtotal (95% CI) 350 1521 35.1% 0.62 [0.22, 1.73]
Total events 29 111
Heterogeneity: Tau² = 1.15; Chi² = 19.30, df = 5 (P = 0.002); I² = 74%
Test for overall effect: Z = 0.91 (P = 0.36)
1.2.3 IBD
Kunitake 2008 6 101 31 312 8.2% 0.57 [0.23, 1.41]
Rizzo 2011 9 54 8 60 7.4% 1.30 [0.46, 3.65]
Waterman 2013 39 104 73 278 11.4% 1.68 [1.04, 2.72]
Subtotal (95% CI) 259 650 27.0% 1.16 [0.60, 2.25]
Total events 54 112
Heterogeneity: Tau² = 0.19; Chi² = 4.30, df = 2 (P = 0.12); I² = 54%
Test for overall effect: Z = 0.44 (P = 0.66)
Total (95% CI) 832 2928 100.0% 1.04 [0.68, 1.57]
Total events 148 375
Heterogeneity: Tau² = 0.34; Chi² = 33.45, df = 12 (P = 0.0008); I² = 64%
Test for overall effect: Z = 0.17 (P = 0.87)
Test for subgroup differences: Chi² = 1.79, df = 2 (P = 0.41), I² = 0%
0.01 0.1 1 10 100
Anti-TNF No Anti-TNF

Figure 2.3C.

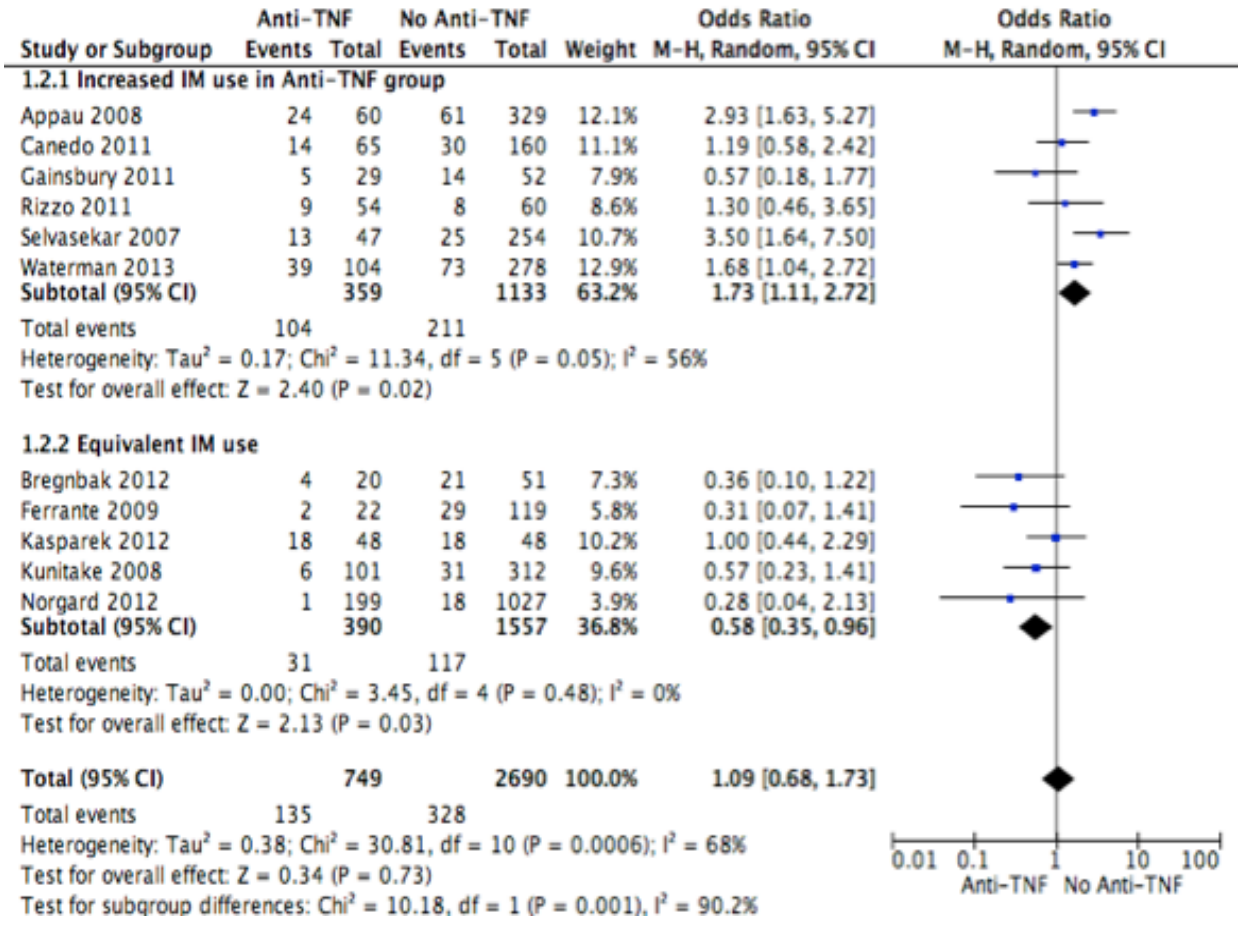

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
1.2.1 Increased IM use in Anti-TNF group
Appau 2008 24 60 61 329 12.1% 2.93 [1.63, 5.27]
Canedo 2011 14 65 30 160 11.1% 1.19 [0.58, 2.42]
Gainsbury 2011 5 29 14 52 7.9% 0.57 [0.18, 1.77]
Rizzo 2011 9 54 8 60 8.6% 1.30 [0.46, 3.65]
Selvasekar 2007 13 47 25 254 10.7% 3.50 [1.64, 7.50]
Waterman 2013 39 104 73 278 12.9% 1.68 [1.04, 2.72]
Subtotal (95% CI) 359 1133 63.2% 1.73 [1.11, 2.72]
Total events 104 211
Heterogeneity: Tau² = 0.17; Chi² = 11.34, df = 5 (P = 0.05); I² = 56%
Test for overall effect: Z = 2.40 (P = 0.02)
1.2.2 Equivalent IM use
Bregnbak 2012 4 20 21 51 7.3% 0.36 [0.10, 1.22]
Ferrante 2009 2 22 29 119 5.8% 0.31 [0.07, 1.41]
Kasparek 2012 18 48 18 48 10.2% 1.00 [0.44, 2.29]
Kunitake 2008 6 101 31 312 9.6% 0.57 [0.23, 1.41]
Norgard 2012 1 199 18 1027 3.9% 0.28 [0.04, 2.13]
Subtotal (95% CI) 390 1557 36.8% 0.58 [0.35, 0.96]
Total events 31 117
Heterogeneity: Tau² = 0.00; Chi² = 3.45, df = 4 (P = 0.48); I² = 0%
Test for overall effect: Z = 2.13 (P = 0.03)
Total (95% CI) 749 2690 100.0% 1.09 [0.68, 1.73]
Total events 135 328
Heterogeneity: Tau² = 0.38; Chi² = 30.81, df = 10 (P = 0.0006); I² = 68%
Test for overall effect: Z = 0.34 (P = 0.73)
Test for subgroup differences: Chi² = 10.18, df = 1 (P = 0.001), I² = 90.2%
0.01 0.1 1 10 100
Anti-TNF No Anti-TNF

Figure 2.4A.

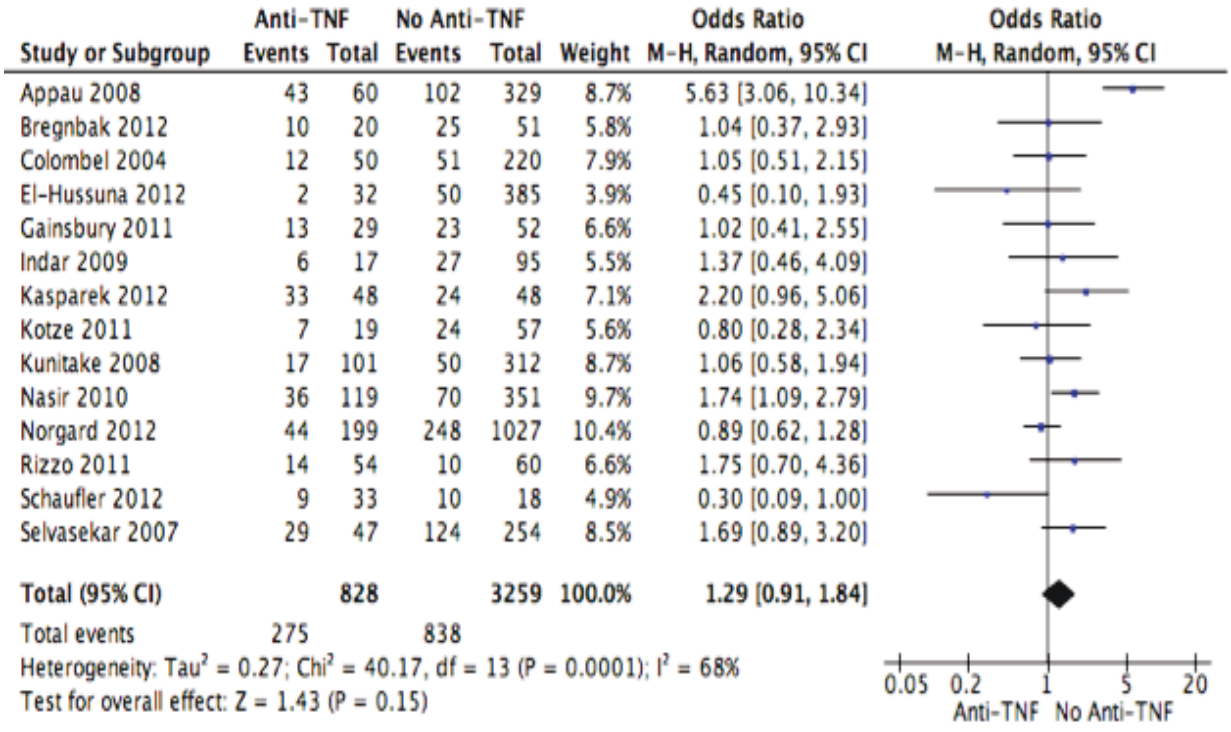

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup
Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
Appau 2008 43 60 102 329 8.7% 5.63 [3.06, 10.34]
Bregnbak 2012 10 20 25 51 5.8% 1.04 [0.37, 2.93]
Colombel 2004 12 50 51 220 7.9% 1.05 [0.51, 2.15]
El-Hussuna 2012 2 32 50 385 3.9% 0.45 [0.10, 1.93]
Gainsbury 2011 13 29 23 52 6.6% 1.02 [0.41, 2.55]
Indar 2009 6 17 27 95 5.5% 1.37 [0.46, 4.09]
Kasparek 2012 33 48 24 48 7.1% 2.20 [0.96, 5.06]
Kotze 2011 7 19 24 57 5.6% 0.80 [0.28, 2.34]
Kunitake 2008 17 101 50 312 8.7% 1.06 [0.58, 1.94]
Nasir 2010 36 119 70 351 9.7% 1.74 [1.09, 2.79]
Norgard 2012 44 199 248 1027 10.4% 0.89 [0.62, 1.28]
Rizzo 2011 14 54 10 60 6.6% 1.75 [0.70, 4.36]
Schaufler 2012 9 33 10 18 4.9% 0.30 [0.09, 1.00]
Selvasekar 2007 29 47 124 254 8.5% 1.69 [0.89, 3.20]
Total (95% CI) 828 3259 100.0% 1.29 [0.91, 1.84]
Total events 275 838
Heterogeneity: Tau² = 0.27; Chi² = 40.17, df = 13 (P = 0.0001); I² = 68%
Test for overall effect: Z = 1.43 (P = 0.15)
0.05 0.2 1 5 20
Anti-TNF No Anti-TNF

Figure 2.4B.

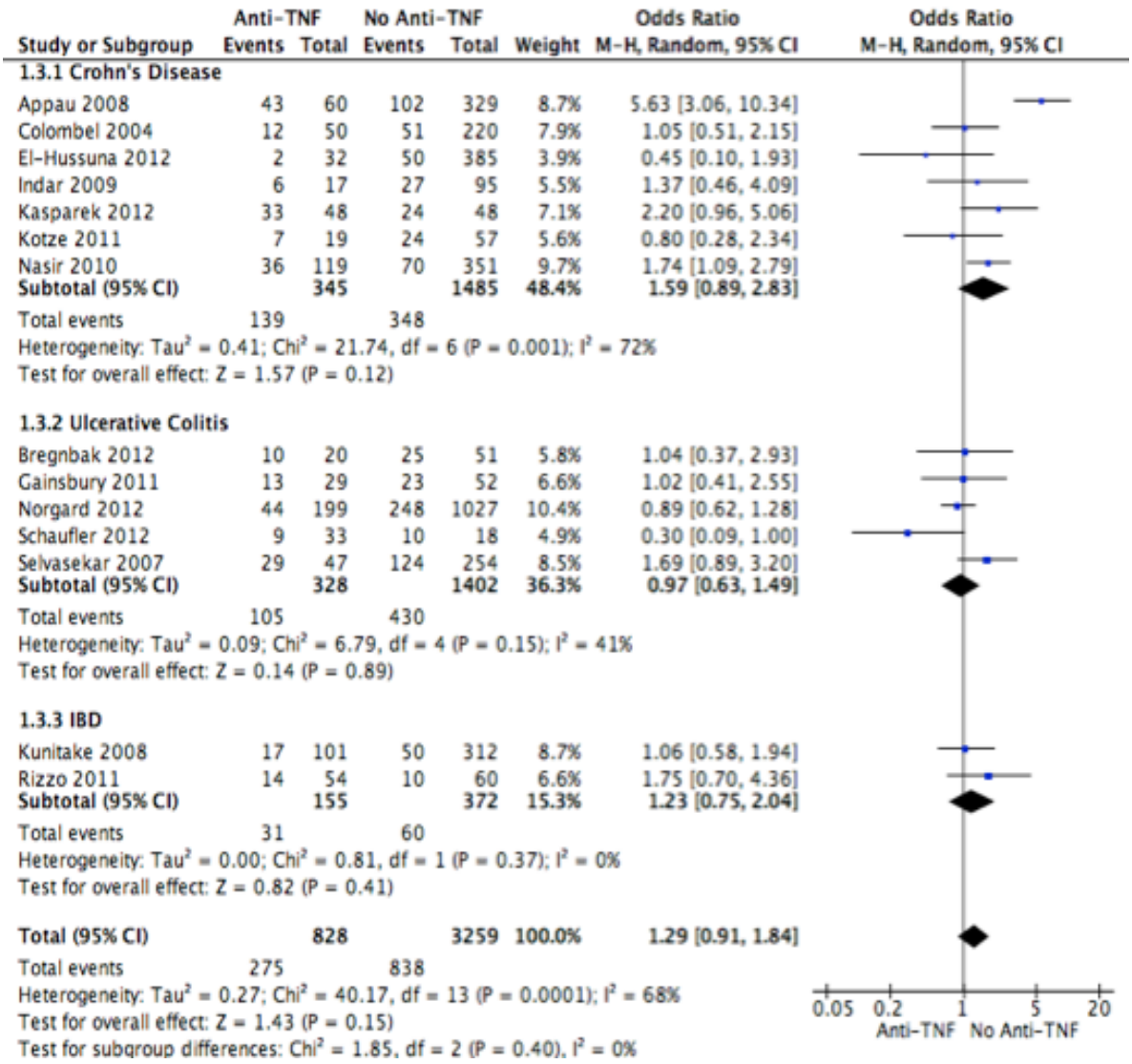

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
1.3.1 Crohn's Disease
Appau 2008 43 60 102 329 8.7% 5.63 [3.06, 10.34]
Colombel 2004 12 50 51 220 7.9% 1.05 [0.51, 2.15]
El-Hussuna 2012 2 32 50 385 3.9% 0.45 [0.10, 1.93]
Indar 2009 6 17 27 95 5.5% 1.37 [0.46, 4.09]
Kasparek 2012 33 48 24 48 7.1% 2.20 [0.96, 5.06]
Kotze 2011 7 19 24 57 5.6% 0.80 [0.28, 2.34]
Nasir 2010 36 119 70 351 9.7% 1.74 [1.09, 2.79]
Subtotal (95% CI) 345 1485 48.4% 1.59 [0.89, 2.83]
Total events 139 348
Heterogeneity: Tau² = 0.41; Chi² = 21.74, df = 6 (P = 0.001); I² = 72%
Test for overall effect: Z = 1.57 (P = 0.12)
1.3.2 Ulcerative Colitis
Bregnbak 2012 10 20 25 51 5.8% 1.04 [0.37, 2.93]
Gainsbury 2011 13 29 23 52 6.6% 1.02 [0.41, 2.55]
Norgard 2012 44 199 248 1027 10.4% 0.89 [0.62, 1.28]
Schaufler 2012 9 33 10 18 4.9% 0.30 [0.09, 1.00]
Selvasekar 2007 29 47 124 254 8.5% 1.69 [0.89, 3.20]
Subtotal (95% CI) 328 1402 36.3% 0.97 [0.63, 1.49]
Total events 105 430
Heterogeneity: Tau² = 0.09; Chi² = 6.79, df = 4 (P = 0.15); I² = 41%
Test for overall effect: Z = 0.14 (P = 0.89)
1.3.3 IBD
Kunitake 2008 17 101 50 312 8.7% 1.06 [0.58, 1.94]
Rizzo 2011 14 54 10 60 6.6% 1.75 [0.70, 4.36]
Subtotal (95% CI) 155 372 15.3% 1.23 [0.75, 2.04]
Total events 31 60
Heterogeneity: Tau² = 0.00; Chi² = 0.81, df = 1 (P = 0.37); I² = 0%
Test for overall effect: Z = 0.82 (P = 0.41)
Total (95% CI) 828 3259 100.0% 1.29 [0.91, 1.84]
Total events 275 838
Heterogeneity: Tau² = 0.27; Chi² = 40.17, df = 13 (P = 0.0001); I² = 68%
Test for overall effect: Z = 1.43 (P = 0.15)
Test for subgroup differences: Chi² = 1.85, df = 2 (P = 0.40), I² = 0%
0.05 0.2 1 5 20
Anti-TNF No Anti-TNF

Figure 2.4C.

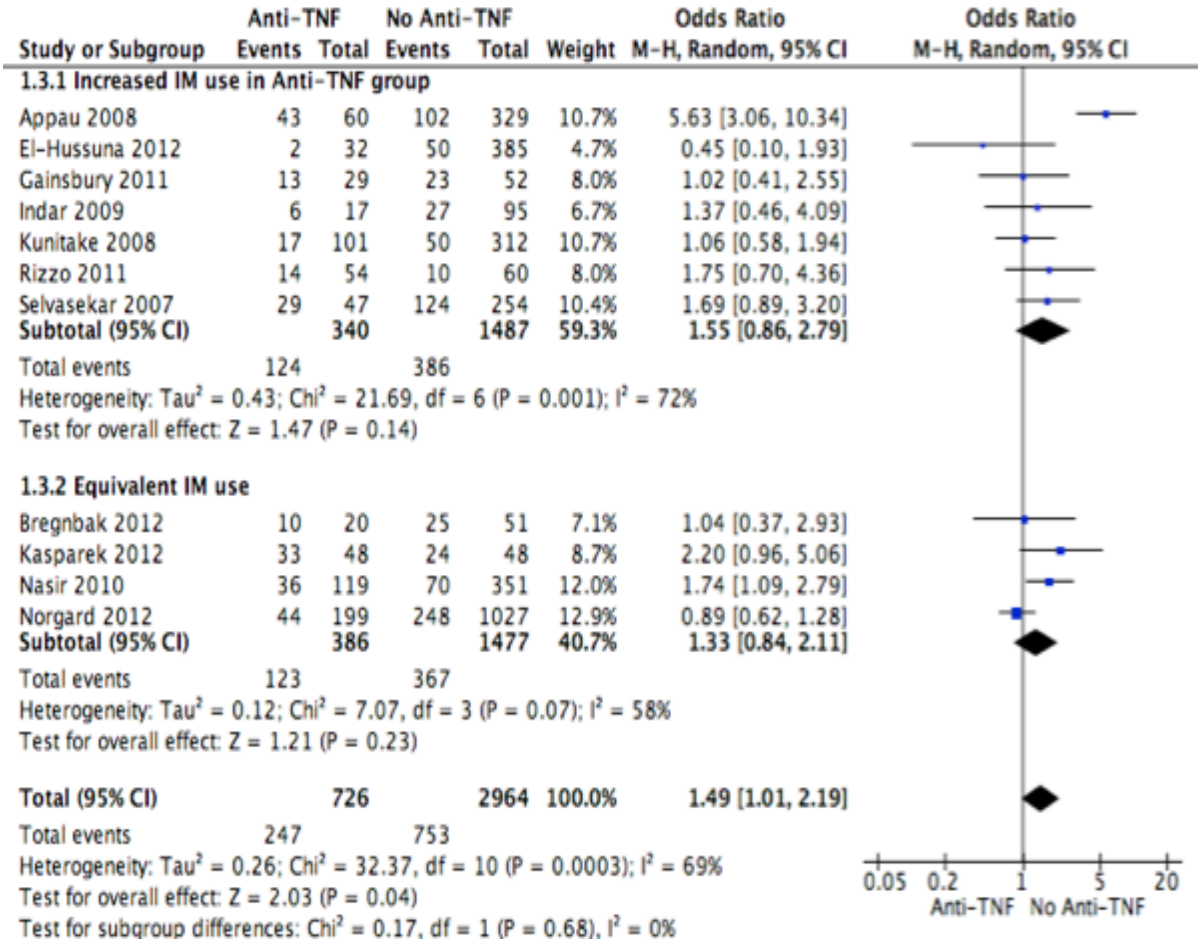
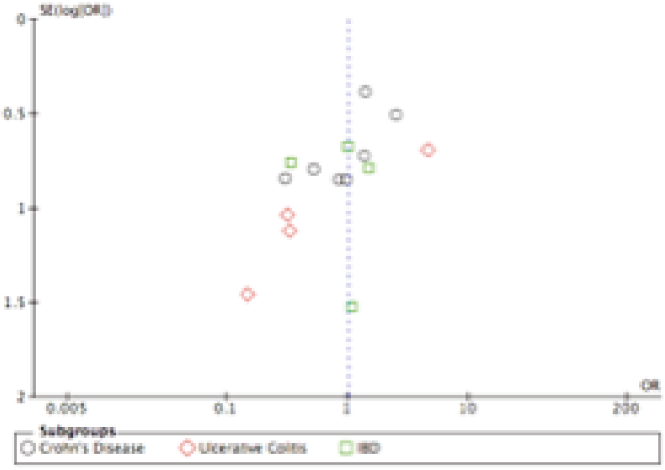
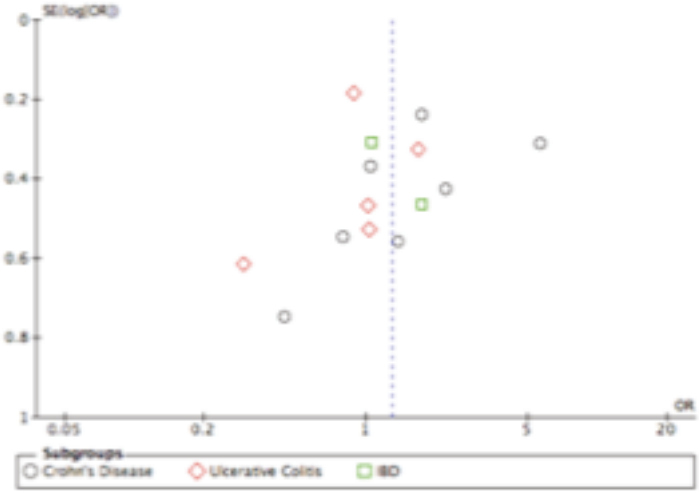

Anti-TNF
No Anti-TNF
Odds Ratio
Odds Ratio
Study or Subgroup Events Total Events Total Weight M-H, Random, 95% CI
M-H, Random, 95% CI
1.3.1 Increased IM use in Anti-TNF group
Appau 2008 43 60 102 329 10.7% 5.63 [3.06, 10.34]
El-Hussuna 2012 2 32 50 385 4.7% 0.45 [0.10, 1.93]
Gainsbury 2011 13 29 23 52 8.0% 1.02 [0.41, 2.55]
Indar 2009 6 17 27 95 6.7% 1.37 [0.46, 4.09]
Kunitake 2008 17 101 50 312 10.7% 1.06 [0.58, 1.94]
Rizzo 2011 14 54 10 60 8.0% 1.75 [0.70, 4.36]
Selvasekar 2007 29 47 124 254 10.4% 1.69 [0.89, 3.20]
Subtotal (95% CI) 340 1487 59.3% 1.55 [0.86, 2.79]
Total events 124 386
Heterogeneity: Tau² = 0.43; Chi² = 21.69, df = 6 (P = 0.001); I² = 72%
Test for overall effect: Z = 1.47 (P = 0.14)
1.3.2 Equivalent IM use
Bregnbak 2012 10 20 25 51 7.1% 1.04 [0.37, 2.93]
Kasparek 2012 33 48 24 48 8.7% 2.20 [0.96, 5.06]
Nasir 2010 36 119 70 351 12.0% 1.74 [1.09, 2.79]
Norgard 2012 44 199 248 1027 12.9% 0.89 [0.62, 1.28]
Subtotal (95% CI) 386 1477 40.7% 1.33 [0.84, 2.11]
Total events 123 367
Heterogeneity: Tau² = 0.12; Chi² = 7.07, df = 3 (P = 0.07); I² = 58%
Test for overall effect: Z = 1.21 (P = 0.23)
Total (95% CI) 726 2964 100.0% 1.49 [1.01, 2.19]
Total events 247 753
Heterogeneity: Tau² = 0.26; Chi² = 32.37, df = 10 (P = 0.0003); I² = 69%
Test for overall effect: Z = 2.03 (P = 0.04)
Test for subgroup differences: Chi² = 0.17, df = 1 (P = 0.68), I² = 0%
0.05 0.2 1 5 20
Anti-TNF No Anti-TNF

Figure 2.5.

(A)


SE(log[OR])
0
0.5
1
1.5
2
0.005
0.1
1
10
200
OR
Subgroups
Crohn's Disease
Ulcerative Colitis
IBD

(B)


SE(log[OR])
0
0.2
0.4
0.6
0.8
1
0.05
0.2
1
5
20
OR
Subgroups
Crohn's Disease
Ulcerative Colitis
IBD

(C)

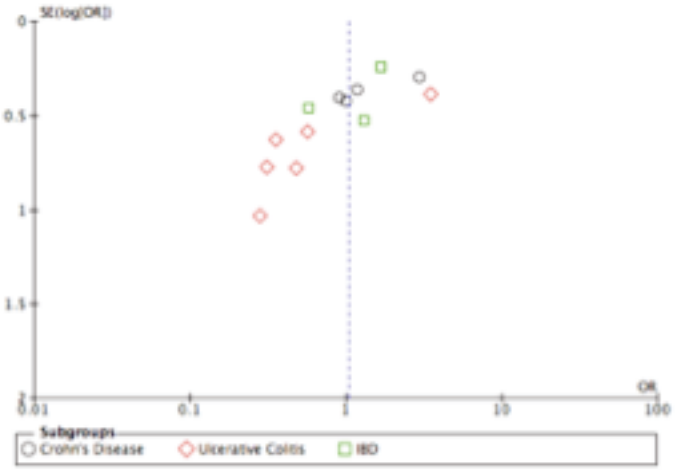

SE(log[OR])
0
0.5
1
1.5
2
0.01
0.1
1
10
100
OR
Subgroups
Crohn's Disease
Ulcerative Colitis
IBD

Table 2.1.

Study ID	Anti-TNF medications	Case-Control/ Cohort/ Registry	Country	Years of Data Collected	CD/UC/ IBD	Procedures	anti-TNF pre-op weeks	Patients E (n)	Patients U (n)	Stomas E %	Stomas U %	Medications E %	Medications U %
Appau! 2008	IFX!	Cohort	US	1998-2007	CD	Ileocolonic resection	12	60	329	28%	18%	CS 65%! IM 62%	CS 77%! IM 17%
Bregnbak 2012	IFX!	Cohort	Denmark	2005-2010	UC	Colectomy	12	20	51	N/A	N/A	CS 75%	CS 65%
Canedo 2011	IFX!	Cohort	US	2001-2008	CD	Intestinal resection	12	65	160	46%	37%	N/A	N/A
Colombel 2004	IFX!	Cohort	US	1998-2001	CD	Intestinal resection, Stricturoplasty, Intestinal bypass	8 (4)	50	220	N/A	N/A	N/A	N/A
El-Hussuna 2012	IFX! CZ	Cohort	Denmark	2000-2007	CD	Intestinal resection, Stricturoplasty	12	32	385	N/A	N/A	CS 34%! IM 88%	CS 35%! IM 36%
Ferrante 2009	IFX	Cohort	Belgium	1998-2008	UC	Proctocolectomy or Colectomy	12	22	119	91%	66%	CS 64%! IM 59%! CA 27%	CS 69%! IM 55%! CA 35%
Gainsbury 2011	IFX!	Cohort	US	2005-2009	UC	Proctocolectomy or Colectomy	12	29	52	100%	100%	CS 93%! IM 83%	CS 69%! IM 52%! CS 2%
Indar ! 2009	IFX	Cohort	US	1999-2007	CD	Intestinal Surgery	8	17	95	N/A	N/A	CS 41%! IM 29%! CS+IM 18%	CS 22%! IM 16%! CS+IM 17%
Kasparek 2012	IFX!	Case-Control	Germany	2001-2008	CD	Intestinal resection	12	48	48	19%	6%	CS 94%! IM 73%	CS 94%! IM 73%
Kotze! 2011	IFX! ADA	Cohort	Brazil	2007-2010	CD	Intestinal resection	4	19	57	N/A	N/A	N/A	N/A
Kunitake 2008	IFX!	Cohort	US	1993-2007	IBD	Intestinal resection, Stricturoplasty, IPAA	12	101	312	N/A	N/A	CS 75%! IM 37%	CS 77%! IM 26%

Table 2.1 continued.

Study ID	Anti-TNF medications	Case-Control/ Cohort/ Registry	Country	Years of Data Collected	CD/UC/ IBD	Procedures	anti-TNF pre-op weeks	Patients		Stomas		Concomitant Medications	
								E (n)	U (n)	E %	U %	E %	U %
Nasir ! 2010	IFX! ADA! CZ	Cohort	US	2005-2009	CD	Intestinal surgery for CD with anastomosis	8 (4)	119	251	0%	0%	CS 31%! IM 27%	CS 45%! IM 33%
Norgard 2012	IFX! ADA	Registry	Denmark	2003-2010	UC	Proctocolectomy or Colectomy	12	199	1027	N/A	N/A	CS 25%	CS 29%
Norgard 2013	IFX!	Registry	Denmark	2000-2010	CD	Intestinal surgery for CD	12	214	2079	N/A	N/A	CS 9%	CS 14%
Regadas 2011	IFX!	Cohort	US	2001-2008	IBD	Ileostomy reversal	8	28	221	0%	0%	CS 50%! IM 39%	CS 48%! IM 15%
Rizzo ! 2011	IFX! ADA! CZ	Cohort	US	2004-2010	IBD	CD or UC related abdominal surgery	12	54	60	N/A	N/A	CS 44%! IM 39%	CS 60%! IM 10%
Schaufler 2012	IFX!	Cohort	US	1996-2010	UC (Peds)	Colectomy	12	33	18	100%	100%	N/A	N/A
Selvasekar 2007	IFX!	Cohort	US	2003-2005	UC	Proctocolectomy or Colectomy with IPAA	8	47	254	100%	100%	CS 77%! IM 91%	CS 46%! IM 44%
Tay ! 2003	IFX!	Cohort	US	1998-2002	CD	Intestinal surgery with anastomosis or stricturoplasty	8	22	78	0%	0%	IM 59%	IM 48%
Waterman 2013	IFX! ADA	Case-Control	CAN	2000-2010	IBD	Intestinal resection, IPAA	4	104	278	N/A	N/A	CS 23%! IM 34%	CS 17%! IM 20%

E (anti-TNF exposed), U (no anti-TNF exposure), IFX (infliximab), ADA (adalimumab), CZ (certolizumab), IBD (inflammatory bowel disease), UC (ulcerative colitis), CD (Crohn's disease), IPAA (ileal pouch anal anastomosis), N/A (not available), CS (corticosteroid), IM (immunomodulator), CA (cyclosporin A).

!

Table 2.2.

	Selection				Comparability	Outcome/Exposure			
Study ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**
Appau! 2008	*	*	*	*	More IM use in anti-TNF group	*	*	*	7
Bregnbak 2012	*	*	*	Unclear	Unclear	*	*	*	6
Canedo ! 2011	*	*	*	Unclear	More Stomas in anti-TNF group	*	*	*	6
Colombel 2004	*	*	*	Unclear	Unclear	*	*	*	6
El-Hussuna 2012	*	*	*	*	More IM use in anti-TNF group	*	*	*	7
Ferrante 2009	*	*	*	*	More Stomas in anti-TNF group	*	*	*	7
Gainsbury 2011	*	*	*	Unclear	More CS use in anti-TNF group	*	*	*	6
Indar ! 2009	*	*	*	Unclear	Unclear on Stomas, more CS and IM use in anti-TNF group	*	*	*	6
Kasparek 2012	*	*	*	*	More Stomas in anti-TNF group	*	*	*	7
Kotze ! 2011	*	*	*	Unclear	Unclear	*	*	Unclear	5
Kunitake 2008	*	*	*	*	More IM use and CD in anti-TNF group	*	*	*	7

Table 2.2 continued.

	Selection				Comparability	Outcome/Exposure			
Study ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**
Nasir ! 2010	*	*	*	*	*	*	*	*	8
Norgard 2012	*	*	*	Unclear	*	*	*	*	7
Norgard 2013	*	*	*	Unclear	*	*	*	*	7
Regadas 2011	*	*	*	*	More IM use in anti-TNF group	*	*	*	7
Rizzo ! 2011	*	*	*	*	More IM use in anti-TNF group	*	*	*	7
Schaufler 2012	*	*	*	Unclear	Unclear	*	*	*	6
Selvasekar 2007	*	*	*	Unclear	More IM & CS use in anti-TNF group	*	*	*	6
Tay ! 2003	*	*	*	Unclear	*	*	*	*	7
Waterman 2013	*	*	*	*	More IM use in anti-TNF group	*	*	*	7

CS (corticosteroid), IM (immunomodulator)

Table 2.3.

		Anastomotic Leaks			Infectious Complications			Total Complications		30 Day Reoperation	
Study ID	Anastomotic Leak Definition	E n (%)	U n (%)	Infectious Complications Included	E n (%)	U n (%)	Total Complications Included	E n (%)	U n (%)	E n (%)	U n (%)
Appau! 2008	Abdominal Sepsis with surgery for Leak	6 (10%)	14 (4.3%)	Sepsis, Abscess, anastomotic leak	24 (42%)	61 (19%)	Death, re-operation, Urinary complications	43 (72%)	102 (31%)	5 (8%)	10 (3%)
Bregnbak 2012	N/A	N/A	N/A	Pneumonia, Abscess, Sepsis, Candida, UTI, Wound infection, epididymitis.	4 (20%)	21 (41%)	DVT, eczema, headache, fever, delerium.	10 (50%)	25 (50%)	N/A	N/A
Canedo 2011	N/A	2 (3%)	5 (3%)	Pneumonia, Wound infection, abscess, anastomotic leak	14 (22%)	30 (19%)	N/A	N/A	N/A	2 (3%)	9 (6%)
Colombel 2004	N/A	N/A	N/A	Wound infection, Anastomotic leak, abscess, fistula, sepsis, other	9 (17%)	43 (20%)	DVT, SBO, Bleeding.	12 (23%)	51 (23%)	N/A	N/A
El-Hussuna 2012	N/A	N/A	N/A	Abdominal Sepsis, Wound infection, pneumonia, UTI.	N/A	N/A	DVT, Wound dehiscence, death, cardiovascular complication	2 (6%)	50 (13%)	N/A	N/A
Ferrante 2009	N/A	0 (0%)	15 (13%)	Wound infection, Anastomotic leak, abscess, Pneumonia, UTI	2 (9%)	29 (24%)	N/A	N/A	N/A	N/A	N/A
Gainsbury 2011	Contrast Extravasation on Imaging	1 (3%)	5 (10%)	Wound infection, Anastomotic leak, abscess	5 (17%)	14 (27%)	DVT/PE, SBO, Ileus	13 (45%)	23 (44%)	N/A	N/A
Indar ! 2009	N/A	N/A	N/A	Anastomotic leak, Abscess, Wound Infection, UTI, Pneumonia	N/A	N/A	Flap necrosis, bleeding, MI, AKI, DVT, Ileus	6 (35%)	27 (28%)	N/A	N/A
Kasparek 2012	Leak causing abscess or fistula	2 (4%)	6 (13%)	Wound infection, Anastomotic leak, abscess, fistula,UTI	18 (38%)	18 (38%)	Ileus, Bleeding, death, stoma complications	33 (69%)	24 (50%)	11 (23%)	10 (21%)
Kotze ! 2011	Leak causing abscess	2 (11%)	7 (12%)	Abscess, Anastomotic leak, UTI, Pneumonia	N/A	N/A	N/A	7 (37%)	24 (42%)	N/A	N/A

Table 2.3 continued.

Study ID	Anastomotic Leak Definition	Anastomotic Leaks E n (%)	Anastomotic Leaks U n (%)	Infectious Complications Included	Infectious Complications E n (%)	Infectious Complications U n (%)	Total Complications Included	Total Complications E n (%)	Total Complications U n (%)	30 Day Reoperation E n (%)	30 Day Reoperation U n (%)
Kunitake 2008	N/A	3 (3%)	9 (3%)	Anastomotic leak, Abscess, Wound Infection, Pneumonia	6! (6%)	31 (10%)	SBO, Ileus, DVT/PE, Death, MI, AKI, Bleeding, Liver failure	17 (17%)	50 (16%)	N/A	N/A
Nasir! 2010	Leak causing abscess or fistula	2 (2%)	8 (3%)	Pneumonia, UTI, Wound infection	N/A	N/A	MI, DVT, AKI, Ileus	36 (30%)	70 (28%)	N/A	N/A
Norgard 2012	N/A	1 (0.5%)	16 (1.5%)	Anastomotic leak, Abscess	1 (0.5%)	18 (2%)	N/A	44 (22%)	248 (24%)	43 (21%)	230 (22%)
Norgard! 2013	N/A	8 (4%)	56 (3%)	N/A	N/A	N/A	N/A	N/A	N/A	16 (7%)	175 (8%)
Regadas 2011	N/A	0 (0%)	3 (1.5%)	Wound infection, Anastomotic leak, abscess, fistula, sepsis	N/A	N/A	SBO, Ileus	N/A	N/A	0 (0%)	8 (4%)
Rizzo ! 2011	N/A	4 (7%)	3 (5%)	Wound infection, Anastomotic leak, abscess, fistula, sepsis, other	9 (17%)	8 (13%)	DVT/PE, SBO, Ileus, AKI	14 (26%)	10 (17%)	N/A	N/A
Schaufler 2012	N/A	N/A	N/A	Sepsis, Abscess, Wound infection, other	4 (12%)	4 (22%)	SBO, readmission, stay > 10d	9 (27%)	10 (56%)	N/A	N/A
Selvasekar 2007	N/A	4 (9%)	5 (2%)	Anastomotic leak, Abscess, Wound Infection	13 (28%)	25 (10%)	Pouch complications, stricture, other	29 (62%)	124 (49%)	N/A	N/A
Tay ! 2003	Leak causing abscess or fistula	3 (14%)	8 (10%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Waterman 2013	N/A	2 (2%)	15 (5%)	Pneumonia, Abscess, Sepsis, UTI, Wound infection	39 (38%)	73 (26%)	N/A	N/A	N/A	N/A	N/A

E (anti-TNF exposed), U (no anti-TNF exposure),UTI (urinary tract infection), DVT (deep vein thrombosis), PE (pulmonary embolus), SBO (small bowel obstruction), AKI (acute kidney injury), MI (myocardial infarction).

Appendix 2.1. Medline search strategy

1. exp Crohn Disease/ or exp Inflammatory Bowel Diseases/ or exp Colitis/ or IBD.mp. or exp Colitis, Ulcerative/
2. Crohn*.ti.
3. Crohn*.ab.
4. colitis.ti.
5. colitis.ab.
6. exp General Surgery/
7. postop*.ti.
8. postop*.ab.
9. post-op*.ti.
10. exp Crohn Disease/ or exp Inflammatory Bowel Diseases/ or exp Colitis/ or IBD.mp. or exp Colitis, Ulcerative/
11. Crohn*.ti.
12. Crohn*.ab.
13. colitis.ti.
14. colitis.ab.
15. exp General Surgery/
16. postop*.ti.
17. postop*.ab.
18. post-op*.ti.
19. post-op*.ab.
20. exp Postoperative Complications/ or exp Anastomosis, Surgical/
21. exp Crohn Disease/ or exp Inflammatory Bowel Diseases/ or exp Colitis/ or IBD.mp. or exp Colitis, Ulcerative/
22. Crohn*.ti.
23. Crohn*.ab.
24. colitis.ti.
25. colitis.ab.
26. exp General Surgery/
27. postop*.ti.
28. postop*.ab.
29. post-op*.ti.
30. post-op*.ab.
31. exp Postoperative Complications/ or exp Anastomosis, Surgical/
32. exp Tumor Necrosis Factor-alpha/ or Anti-TNF.mp.
33. Infliximab.ti,ab.
34. remicade.ti,ab.
35. adalimumab.ti,ab.
36. humira.ti,ab.
37. certolizumab.ti,ab.
38. biologic.ti,ab.
39. complication*.ti,ab.
40. 21 or 22 or 23 or 24 or 25
41. 26 or 27 or 28 or 29 or 30 or 31 or 39
42. 32 or 33 or 34 or 35 or 36 or 37 or 38
43. 40 and 41 and 42

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Chapter 3. Murine ileocolic bowel resection with primary anastomosis.

3.1 Introduction

Ileocolic resection (ICR) is a common procedure performed in both emergent and elective situations for a variety of illnesses. Crohn's disease and colon cancers are the two most common indications for ICR. In both illnesses, recurrence in the bowel at the site of surgery represents a major clinical problem. Local recurrence rates for colon cancer remain an issue even with the most aggressive resections(Hallet et al., 2014). Following ICR in Crohn's disease, the disease most frequently (in up to 80%) recurs in the neo-terminal ileum at 1 year after surgery(Rutgeerts, Geboes, & Vantrappen, 1984). Given the impact of these two illnesses and their recurrence after surgery, it is important to understand local intestinal factors after ICR, which may have intrinsic influences on the natural history of these diseases. Further, it is also important to consider anastomotic healing after ICR. In the early post-operative period, anastomotic leaks can have devastating consequences for patients resulting in repeat surgeries, stoma creations, significant morbidity, and even mortality(B. D. MD et al., 2013a). Despite the importance of this topic, our current understanding of anastomotic healing remains in its infancy as a subject of research. Animal models of ICR, in particular mice, are an excellent platform for studying the intestinal and anastomotic healing following surgery.

A mouse model of ICR was initially developed by Helmrath *et al*. to be used as a model of short gut syndrome(Helmrath et al., 1996). The authors experimented with various diet

regiments and suture sizes to optimize animal survival following ICR. They concluded that feeding with liquid diet in the perioperative period and using 9-0 monofilament sutures resulted in an optimal post-operative survival of 88%. Since this initial publication, ICR in mice removing 50% of the small bowel has been used in several studies to explore the dynamics of massive small bowel resection and the adaptive growth response in attempt to develop new therapies for short gut syndrome(Dekaney et al., 2007; Speck et al., 2011).

The first application of the ICR mouse model to Crohn's disease used the IL-$10^{-/-}$ mouse model, which spontaneously develops colitis(Rigby et al., 2009). The authors found that after ICR these animals developed inflammation in the neo-terminal ileum similar to that seen in post-operative Crohn's disease patients, and that this inflammation was dependent on the presence of bacteria(Rigby et al., 2009). More recently, this model was used to explore bacterial changes induced by ICR. In Crohn's disease there is an associated dysbiosis with relative decreases in bacteria known to have anti-inflammatory properties and increases in invasive species of bacteria(Gevers et al., 2014; Kostic et al., 2014). The association holds true in cases of post-operative recurrence(Ahmed et al., 2011). Two studies sought to identify microbial changes resulting from ICR. The first used IL-10 null mice, and performed denaturing gel electrophoresis to compare bacterial similarity between the small bowel and colon after ICR(A. M. B. MD et al., 2012). This study demonstrated that bacterial populations became similar in the small intestine and colon following ICR. A subsequent study used wild type mice and 16s pyrosequencing for phylogenetic classification of bacterial species post-operatively. This study demonstrated a marked shift in bacterial species resulting from surgery alone with *Clostridium* species

becoming dominant as well as an increase in ϒ-proteobacteria. The results also confirmed the findings of the previous study with similar populations found in the small bowel and colon after ICR(Devine et al., 2013).

ICR is a common procedure for patients with colon cancer involving the cecum and ascending colon, and it is becoming increasingly recognized that the host-response to surgery likely contributes to both local and distant tumor recurrence(van der Bij et al., 2009). Despite this observation, models of ICR have not been utilized for the study of colorectal cancer and post-operative recurrence. Understanding both the systemic and local immunologic changes resulting from ICR will be important in investigating future therapies. Potential pathways involved in cancer recurrence post ICR include up regulation of growth factors, which may rescue cells from apoptosis and stimulate proliferation, mechanical tumor disruption with cell shedding, and loss of immune surveillance through post-operative immunosuppression(A. D. Scott, Uff, & Phillips, 1993; van der Bij et al., 2009).

Mouse models of ICR have the potential to be a powerful tool for the investigation of short bowel syndrome, Crohn's disease, and colon cancer. They may also provide lessons on how to prevent early post-operative anastomotic complications by further defining the cellular and biochemical pathways involved in healing the newly constructed anastomosis. A major barrier in utilizing the murine ICR model is the technical difficulty. The intestinal anastomosis requires the use of 8-0 or 9-0 suture, an operating microscope, and training in microsurgical techniques. The goal of this article is to provide clear instructions on how to perform ICR in mice with the goal of utilizing this procedure in models of disease.

3.2 Protocol

Animal use protocols were approved by the Health Science Animal Care and Use committee at the University of Alberta.

1. Preparation of instruments, animals and operative set up.

1.1) Transfer animals to a new, clean cage absent of all solid food 24 hours prior to the procedure. They may have free access to water, and liquid diet ad lib until the time of the procedure.

1.2) Autoclave all instruments required for the procedure. Clean operating surface and anesthetic nose cone with 70% ethanol.

1.3) Set up the operating surface with operating microscope, anesthetic machine and supplies in a manner which is comfortable for the operating surgeon. The elbows of the surgeon should be allowed to rest comfortably on the operating table, with hands and arms unobstructed by equipment. Instruments, sutures, cotton swabs, and a 10 ml syringe should be placed in a location that permits easy access during the procedure.

1.4) Set up overhead heat lamps to provide both warmth for the animal during the procedure and light for the operating surface.

1.5) Fill a 50 ml conical tube with 0.9% saline, and a 1.5 ml tube with petroleum jelly and place near the operating surface.

NOTE: Sterilize all instruments and surgical supplies. Because the intestine is transected, the procedure itself is not sterile. It is considered clean-contaminated. Take measures to avoid the introduction of exogenous sources of infection.

2. Ileocolic resection with anastomosis

2.1) Induce anesthesia by administering 4% isoflurane with an oxygen flow rate of 2 L/min via nose cone from the isoflurane vaporizer until the animals respiratory rate slows to approximately 30-40 breaths/min. Apply moderate pressure to the hind foot of the mouse to ensure there is no pain response prior to initiating the procedure. At this point, turn down isoflurane to 2% and oxygen flow to 0.5 L/min. Intermittently check pain response during the procedure and adjust isoflurane flow rate accordingly.

2.2) Apply petroleum jelly to the eyes to prevent drying during surgery, and immobilize the mouse in the supine position with limbs secured using transparent tape.

2.3) Clean the abdomen with povodine/iodine solution, and change into new sterile gloves.

2.4) Make a 1.5 cm skin incision in the upper midline of the abdomen using sharp point dissecting scissors to expose the fascia and peritoneum. Open the fascial/peritoneal layer in a similar fashion through the linea alba to expose the peritoneal contents.

2.5) In contrast to humans, the mouse cecum is typically found in the left upper quadrant of the abdomen. Once identified, gently grasp the cecum with forceps and deliver it through the incision. Use moistened cotton swabs to fan out approximately 3 cm of terminal ileum extending from the cecum over a sterile gauze draped on the abdominal surface (Fig. 1A). Ensure the exposed bowel is kept moist with 0.9% saline during the entirety of the procedure.

2.6) Identify the ileocecal artery branching off the superior mesenteric artery along the colon (Fig. 1A) using the operating microscope. Dissect out the avascular tissues adjacent to the ileocecal artery, encircle and ligate the artery with a 5-0 silk tie. Next, locate the regional blood supply to the terminal ileum and choose a transection point 1.5-2 cm proximal to the ileocecal junction. Ligate the branches to this section of ileum as above. Divide the arteries with micro dissecting scissors.

2.7) Divide the ischemic portions of ileum and colon ensuring there is adequate blood supply to the transected ends (Fig. 1B). It is often helpful to spatulate the ileum by dividing it at a 30 degree angle to increase the diameter of the lumen so it more closely matches the colon. Once the ileocecal portion of bowel has been removed, align the

transected ends of ileum and colon on the gauze, ensuring the mesenteric borders of each are aligned.

2.8) Construct the anastomosis by approximating the transected end of ileum to the transected end of colon using interrupted 8-0 polypropylene sutures (Fig. 1C). The first stitch is placed at the mesenteric border, with subsequent sutures placed every 0.5 mm until the Ileocolic anastomosis is watertight. When passing the suture needle through the ileum and colon, ensure that the cut edge is not rolled, and needle bites are 0.5 mm from the cut edges of the bowel. A typical anastomosis will require 14 to 16 interrupted sutures. Test the integrity and patency of the anastomosis upon completion by rolling a cotton swab proximal to distal over the ileum to force contents through the anastomosis. Small bowel contents should freely pass into the colon without anastomotic leakage.

2.9) Rinse the exposed bowel with 3-4 ml of 0.9% saline from the 10 ml syringe to wash away stool from the surface of the bowel, and deliver bowel back into the peritoneal cavity. Using 2 ml of 0.9% saline flush the peritoneal cavity, and then drain this fluid by applying gentle pressure to the abdominal wall laterally.

2.10) Close the incision with a 3-0 silk running suture, and discontinue the flow of isoflurane. Administer 0.1 mg/kg of the long acting opiate –buprenorphine- subcutaneously for post-operative pain control.

2.11) Observe the animals under the heat lamp until they are mobile then transfer them to a continually warmed cage.

3. Post-operative care and monitoring.

3.1) Monitor animals in a continually warmed cage for signs of distress for the remainder of the day. Transfer animals back to the animal care facility in a new sterile cage with access to liquid diet and water ad lib. Animals may be housed in groups of 3-4.

3.2) Perform a check on the post-operative animals the following morning, ensure the animals do not appear in distress. Feed only liquid diet. If they appear uncomfortable (i.e. hunched posture or minimal activity) administer an additional dose of subcutaneous buprenorphine. Check on the animals once more in the afternoon on post-operative day 1.

3.3) On the morning of post-operative day 2 the animals should appear fully recovered. Evidence of food consumption and stooling are positive signs of recovery. Now, resume a solid chow diet for the animals.

NOTE: Signs of distress include hunched posture, poor grooming, and minimal activity. If signs of distress are prominent the animals should be euthanized.

3.4) Euthanize the animals by inducing deep anesthesia with 4% isoflurane at an O_2 flow rate of 2 L/min until animals are unresponsive to foot pressure. Perform cervical dislocation, and observe for signs of proper euthanization.

3.5) Guidelines vary, so refer to the institutions recommendations regarding indications for and appropriate methods of euthanization in mice.

3.3 Results

Mortality rates and weight change post-op.

Mortality rates following ICR in 129S1 wild type mice are generally ~ 5%. The most common cause of morality is bowel obstruction at the anastomosis. Other causes of mortality include anastomotic leak and internal hernia leading to bowel obstruction.

Weight loss can be seen up to 14 days post-operatively, but is generally non-significant. Mice tend to completely regain pre-operative weight by post-operative day 28 (Fig. 2).

Transferability of technique

A new graduate student (BM) was taught to perform the ICR procedure to determine if an operator with no previous surgical training could learn the technique. Training began with learning how to perform the single interrupted sutures analogous to those used in the anastomosis using 8-0 nylon suture. Sutures were performed to close an incision made across a latex glove. After BM was able to efficiently place sutures through the glove without tearing, they observed three complete procedures prior to attempting one. After the first procedure was completed, they then observed another procedure and attempted

two more. Of the three animals completed, the last two went on to survive and thrive. The first animal was euthanized due to complications at the anastomosis.

3.4 Discussion

The murine ICR is a powerful model that can be used to study the effects of surgery in bowel diseases. This article describes a method of performing ICR in mice with a success rate of 95% and no issues with failure to thrive as reflected by stable weights up to 28 days post-procedure. The most significant challenges to successful ICR include avoiding bowel obstructions at the anastomosis and anastomotic leaks.

Technical elements to the surgery for preventing obstruction are aimed at maximizing lumen diameter at the anastomosis. Spatulation of the ileum at the transection point increases the lumen diameter by dividing the bowel at a 30 degree angle as depicted in Fig. 1A. When taking bites of intestinal tissue with the suture needle, it is essential to unroll the edges of the bowel and pass the needle approximately 0.5 mm from the cut edge. Excessively large bites of tissue will narrow the lumen. An option to avoid luminal narrowing when constructing the anastomosis is to employ the use of a digestible stent as described by Kiernan et al.(Kiernan, 1988). Non-technical aspects to avoid obstruction include selecting a sufficiently small suture(Helmrath et al., 1996) and avoiding bulky luminal contents by feeding animals a liquid diet one day prior to surgery and 2 days following surgery.

The second most common cause of post-operative mortality is anastomotic leak. Avoiding this complication requires strict adherence to the principles of intestinal anastomosis. The first being adequate blood supply. After the vessels supplying blood to

the region of bowel being resected have been ligated, the bowel should be visually inspected prior to transection. The ischemic segment of bowel will appear dusky; this should be completely removed leaving healthy edges. It is important to visualize a small amount of bleeding from the cut edges of the remaining intestine. A water tight anastomosis is also essential to avoid spillage of luminal contents leading to sepsis. This is achieved by avoiding large gaps between sutures, and ensuring that all layers of the intestine are included in the stitch (Fig. 3). Wound strength is greatly diminished in the first two post-operative days, thus properly placed sutures are essential to provide strength(Ågren et al., 2006; Thompson et al., 2006). A potential pitfall is inadvertently only including the mucosa as the mucosa can pucker out of the lumen of the transected colon. This can be avoided by visualizing the cut edge of serosa and gently reducing prolapsed mucosa back into the lumen with forceps.

Sham surgical controls should be considered in the experimental design of studies using the ICR model to control for the potential confounding effects of surgical stress, intestinal manipulation, and/or loss of the ileocecal valve. The choice of sham procedure will depend on the primary question being asked in the study. For example, if the research objective is to evaluate the host response after losing the ileocecal valve, then it would be prudent to include a sham surgery group in which an intestinal anastomosis is performed while leaving the ileocecal valve in place. This is typically done by transecting the small intestine without resection then performing a small bowel anastomosis in the same fashion as the ileocolic anastomosis(A. M. B. MD et al., 2012; Rigby et al., 2009). This would allow investigators to comment specifically on the effects of ileocecal valve loss by controlling for the effects of intestinal anastomosis. Alternatively, if the goal is only to

study the effects of intestinal resection and anastomosis without commenting specifically on loss of the ileocecal valve, then a sham surgery should include a laparotomy, and the bowel should be treated as it would for ICR, but without transection and anastomosis. This would control for surgical stress and intestinal manipulation. The techniques described in the protocol above can be easily adapted for performing sham procedures.

This protocol describes an ileocolic resection, which removes 1.5-2 cm of terminal ileum and the entirety of the cecum, and closely mimics an operation frequently performed in patients with ileocolic Crohn's disease. Similar procedures have been used in the IL-$10^{-/-}$ colitis model after which mice developed spontaneous inflammation and fibrosis in the small bowel analogous to Crohn's disease recurrence(A. M. B. MD et al., 2012; Rigby et al., 2009). The principles of this procedure may also apply to other models of disease. By removing 50% of the small bowel with ileocolic anastomosis, mouse models have been used to investigate the adaptive intestinal growth response in short gut syndrome(Dekaney et al., 2007; Helmrath et al., 1996; Speck et al., 2011). In the future this may also become a useful model for studying the systemic and local effects of surgery in colorectal cancer.

3.5 Figures and Tables

Figure 3.1. Stages of ileocolic resection and anastomosis. A) Ileocecal region of bowel delivered out of abdomen. Black arrows indicate vessels to be ligated, dashed lines indicate transection points for colon and terminal ileum. B) Ileocecal region removed, cut ends of bowel are aligned, remaining blood supply is demonstrated by black arrows. C) Completed ileocolic anastomosis.

Figure 3.2. Animal weights at baseline, post-operative day 14 and post-operative day 28. No significant changes in weights were observed post-procedure as determined by the Mann-Whitney U statistical test. ICR (Ileocolic resection group), Control (Non-operative controls).

Figure 3.3. Representative path of sutures through intestinal tissues. H&E stained histologic sections of ileum (right) and colon (left) are lined up to demonstrate the trajectory of the suture needle through the tissues (dashed line) starting at point (a) through to point (b). The knot should be tied at point (c). Note that less mucosa is included in the anastomosis than other tissues.

Table 3.1. Table of Materials and Equipment.

Figure 3.1.

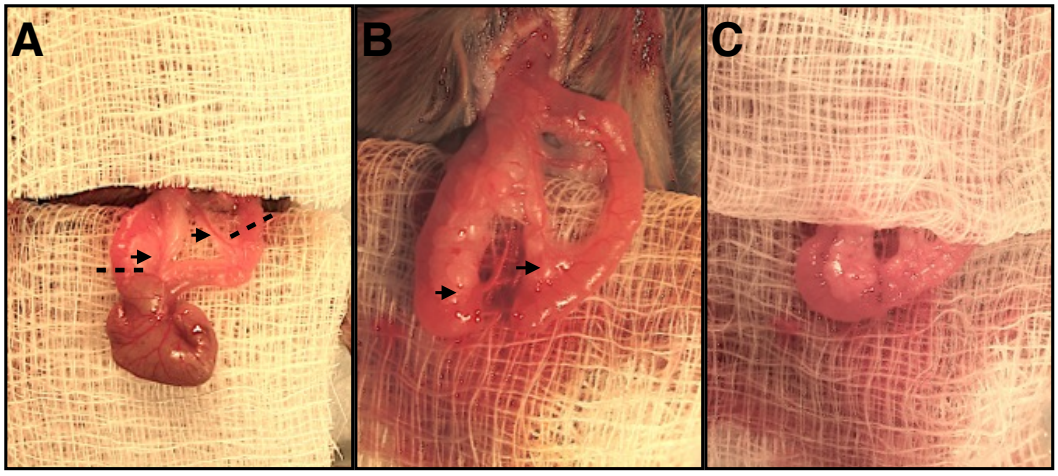

Figure 3.2.

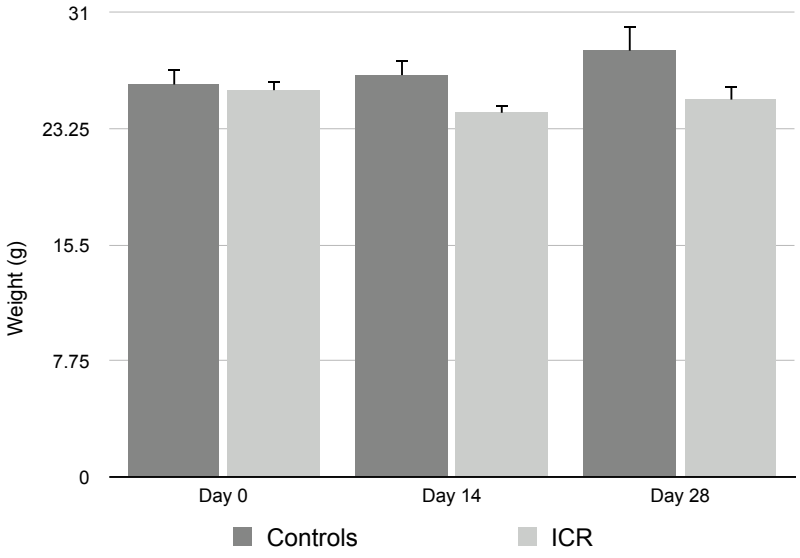

Figure 3.3.

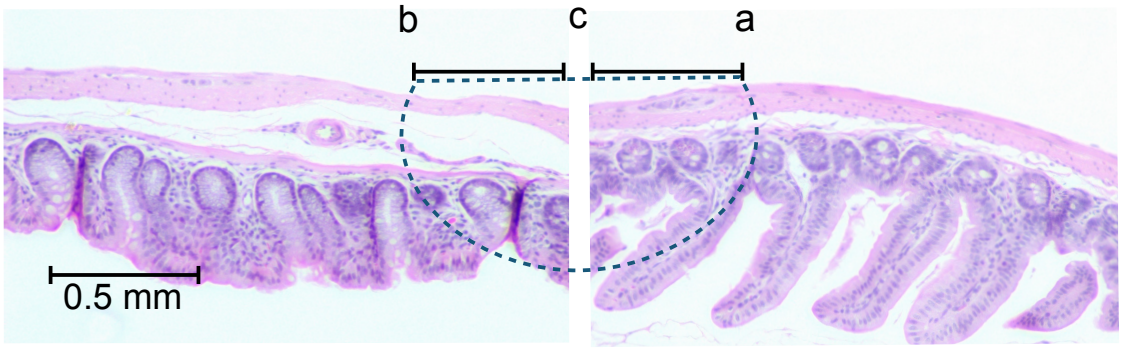

Table 3.1.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
LD101 liquid rodent diet	testdiet.com		
0.9% NaCl	Baxter	FKE1324	Injection quality saline
Operating Microscope	Ziess		
Isoflurane Anesthetic Vaporizer	Harvard Apparatus	34-0483	
Isoflurane	Abbott	05260-05	
Glass plate			For operating surface
Cotton swabs			
Micro Castroviejo Needle holder, curved	World Precision Instruments	503377	
Castroviejo straight scissors	World Precision Instruments	555530S	
Dissecting Scissors	World Precision Instruments	15922	
Dressing Forceps x 2	World Precision Instruments	500363	
5-0 silk pre-cut sutures	Ethicon	A182H	For vessel ligation
8-0 Prolene on BV130-5 needle	Ethicon	8732H	For anastomosis
3-0 Silk on FS-2 needls	Ethicon	8665G	For abdominal wall closure
Petroleum Jelly	Vaseline		
10 ml syringe	BD biosciences		
Povidone-iodine 7.5% surgical Scrub	betadine.com		
Heat lamps			
buprenorphine 0.3 mg/ml	Reckitt Benckiser Healthcare Ltd.	PL36699/0006	

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Chapter 4. Shifting immune responses following ileocecal resection: Implications for post-operative ileitis in Crohn's Disease.

4.1 Introduction

Although surgery rates may be declining with advances in medical therapy(Frolkis et al., 2013), surgical resection of the intestine is still required for many Crohn's disease patients(Cosnes et al., 2011). Because of disease location, resection of the ileocecal region (ICR) is the most common procedure performed. The natural history of recurrence following ICR is well described with rapid and uniform recurrence in the peri-anastomotic ileum(Buisson, Chevaux, Allen, Bommelaer, & Peyrin-Biroulet, 2012). Surgery for CD has been performed since its first description in 1932; however, why mucosal injury occurs in this defined section of small intestine even in patients with no previous small bowel disease is unkown.

Investigations focusing on microbes and post-operative recurrence have found that ICR leads to increased bacterial loads in the neo-terminal ileum in all patients and that CD patients with recurrence have an associated dysbiosis(Darfeuille-Michaud et al., 2004; De Cruz et al., 2014; Neut et al., 2002). These observations highlight the contributions of microbes to recurrence, but little known about the immunologic changes occurring with ICR and how these are impacting the other side of the immune-microbial interaction in the gut that is central to CD pathogenesis.

Mononuclear phagocytes (MPs), consist of macrophages (MΦ) and dendritic cells (DCs) in the gut lamina propria where they link innate and adaptive response to gut antigens. These cell populations are derived from monocyte precursors that populate the lamina propria from the circulation, which allows for plasticity in their function(Farache,

Zigmond, Shakhar, & Jung, 2013b). Homeostasis is maintained by lamina propria MΦs through phagocytosis of microbes with minimal inflammatory potential(Smythies et al., 2005) while DCs promote tolerance by inducing T regulatory cells (Tregs)(C. L. Scott et al., 2011). Both MP cells types demonstrate functional shifts with intestinal inflammation described in CD and animal models(Denning et al., 2011; Farache, Zigmond, Shakhar, & Jung, 2013b; Nobuhiko Kamada & Nobuhiko Kamada, 2008; Sakuraba et al., 2009). MP subsets may be prone to significant perturbations following ICR, with intestinal anastomosis because of their role in tissue repair and wound healing. In general, peripheral blood monocytes are recruited to injured tissues during the first inflammatory phase of wound healing and persist through to the remodeling phase where phenotypes shift from IL-6, TNF-α, IL-1 producing inflammatory cells to IL-10, TGF-b producing immunosuppressive cells(Novak & Koh, 2013; Sindrilaru & Scharffetter-Kochanek, 2013). The contribution of MPs following ICR is essentially unexplored. Owing to our poor understanding of post-operative ileitis, is a lack of animal models to investigate it. The IL-$10^{-/-}$ ICR model develops a post-operative ileitis, and supports observations in CD suggesting microbial a contribution to post-operative recurrence. Studies with this model have found shifts in the ileal flora with inflammation that is dependent on the presence of gut bacteria(A. M. B. MD et al., 2012; Rigby et al., 2009). Immunologic shifts have not been described with ICR models.

Herein we describe a new mouse model to study the immunologic changes associated with ICR and determine the impact of those changes on gut responses to antigen challenge. We performed ICR procedures in wild type 129S1/SvlmJ mice followed by a cycle of dextran sodium sulfate (DSS). DSS induces intestinal inflammation by breaking

down the mucosal barrier and allowing bacteria to penetrate into the lamina propria(Johansson et al., 2010; Laroui et al., 2012), thus mimicking the barrier defects found in CD(Baumgart & Sandborn, 2012). Results indicate that ICR induces significant pertubations in immune responses to gut microbes that are present from the intestinal mucosa to the systemic circulation, and may provide an alternative theory to the microbial overgrowth found in the post-ICR neo-terminal ileum.

4.2 Methods

Animal Model and DSS protocol.

Animal use protocols were approved by the health science animal care and use committee at the University of Alberta.

12-13 week old wild type 129S1 mice raised in conventional conditions underwent ICR with primary end-to-end anastomosis. Briefly, animals started on a liquid diet (LD101 test diet) 24 hours prior to surgery. At the time of surgery mice were anaesthetized using 2% isoflourane with O_2 flow rate of 0.5L/min. Mice were immobilized on the operating surface and the abdomen was cleansed with 7.5% povidine/iodine solution. With the aid of an operating microscope, a 1.5cm laparotomy incision was made in the midline of the abdomen. The ileocecal region was identified and delivered through the incision and draped over sterile gauze. The Ileocolic artery and regional blood supply to the distal 2cm of terminal ileum was isolated, ligated with 6-0 silk suture, and divided. Ileum was then transected 2 cm proximal to the ileocecal junction and the ascending colon was divided just distal to cecum. The Ileocolic anastomosis was constructed with interrupted 8-0 nylon sutures on a tapered needle. Upon completion, the anastomosis was inspected and confirmed to be tension free, water tight and with good blood supply. Peri-anastomotic

bowel was rinsed with 0.9% sterile saline and the bowel was placed back into the abdominal cavity. The abdominal wall was then closed with a running 5-0 silk suture. Isoflourane was discontinued and the animals were recovered under a heat lamp until alert at which point they were placed back into a clean cage and maintained on the liquid diet for 2 days. To control for the effects of surgical stress, Sham procedures were performed by making a midline incision, removing the ileocecal region from the abdomen, exposing the bowel to air for 10 min then replacing the bowel and closing the laparotomy as above. Overall survival in the operative groups was 97%.

DSS (2.5%; MW 35-45,000 kDa; MP Biomedicals) dissolved in water was given in lieu of drinking water for 5 days at two time points following ICR (see time line in figure 1A for details). Weight and fecal occult blood (FOB) were measured every two days during the protocol. FOB positivity was determined using the Hemoccult (Beckman Coulter) test and was interpreted as either positive or negative.

At sacrifice tissues were collected as in figure 1B. Weight:length ratio was determined for colon and ileum prior to division for analysis.

For MΦ depletion during DSS protocol, mice were administered 200 µl i.p. of clodronate containing liposomes as previously described(Weisser et al., 2011). See Figure 1A for time line of administration. Control PBS containing liposomes were not used because of previous results demonstrating their effect on MΦ depletion(Weisser et al., 2011).

Measurement of cytokines and myeloperoxidase.

Snap frozen peri-anastomotic colon and ileum were homogenized in PBS containing 0.05% Tween 20. Homogenates were centrifuged at 10 000 rpm for 10 min and the supernatant was used for measurement of tissue cytokines. Cytokine levels were

corrected for dry tissue weight. IFN-γ, IL-1β, IL-10, IL-12 p70, IL-2, IL-4, IL-5, IL-6, KC/GRO, TNF-α were evaluated using the Proinflammatory Panel 1 V-PLEX mouse kit (Meso Scale Discovery) as per manufacturers protocol. IL-10, IL-6, TNF-α and TGF-β were also measured using the ELISA duo sets (R&D Systems).
Myeloperoxidase (MPO) was measured using the MPO mouse ELISA kit (Hycult biotech).

Immunofluorescence.

Tissues for Immunofluorescence (IF) were placed in OCT medium and flash frozen prior to storage at -80°C. Intestinal tissues were cut using a cryostat at 6 μm then fixed and permeabilized in acetone at -20°C for 10 min. Following this tissues were blocked with 10% goat serum for 30 min rinsed in PBS three times and stained with primary antibodies in PBS with 0.5% BSA overnight at 4°C. The following morning slides were again rinsed with PBS and secondary antibodies were applied for 1 hour at room temperature, rinsed and counterstained with DAPI. Slides were mounted with FlourSave™ reagent (Calbiochem) and viewed on the Zeiss Axio fluorescence microscope and analyzed with with ZEN blue software. Cells were quantified as previously described(Weisser et al., 2011).

16s rRNA qPCR for tissue bacterial load

Sections of ileum and colon 1 cm away from the anastomosis were snap frozen. Genomic DNA was extracted from tissues using the FastDNA® Spin kit (MP Biomedicals) following manufacturers protocol, and quantified on a ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific). Samples were diluted to 50ng/ml. Diluted DNA specimens were then quantified again prior to qPCR using the PicoGreen assay

(Invitrogen). Quantitative PCR (qPCR) reactions contained 8 µl H_2O, 10 µl of Fast SYBR Green Master Mix, 1 µl each of 10µM forward and reverse primers and 2 µl target DNA. Sequence of PCR conditions was 5 min at 50 °C, 5 min at 95 °C, (15 sec at 95°C, 1 min at 60°)(40 cycles), followed by a melting curve step progressing from 60 °C to 95 °C over 12 min. qPCR was performed in MicroAmp 96 well optical plates with the 7900HT instrument, and results were analyzed with SDS 2.3 software. Non-specific amplification was determined using melting curves and visualizing products on a QIAxcel (Qiagen) instrument. All qPCR reagents and materials were obtained from Applied Biosystems unless otherwise stated.

Target DNA copy number was determined by comparison to standard curves constructed from purified PCR product obtained using the primers and quantified using the PicoGreen assay (Invitrogen) to amplify genomic DNA from stool in standard PCR reactions(Koleva, Valcheva, Sun, Gänzle, & Dieleman, 2012). Gene copy number per gram tissue was then determined.

Histology and scoring mucosal injury.

Peri-anastomotic sections of ileum and colon were taken as depicted in figure 1B, fixed in 10% buffered formalin, embedded in paraffin and cut at 5 µm. Tissue sections were stained with hematoxylin/eosin (H&E), and analyzed by a pathologist blinded to the study groups.

H&E stains were used for histologic inflammation/injury scores as described previously(Madsen et al., 2001; A. M. B. MD et al., 2012). This validated 11 point scale, scores intestinal tissues based on enterocyte injury, epithelial hyperplasia, lamina propria lymphocytes, lamina propria neutrophils and peri-intestinal inflammation.

Isolation and characterization of lamina propria cell and splenocyte populations.

Splenocytes and mesenteric lymphocytes were isolated by homogenizing spleens and mesenteric lymph nodes in PBS with 5% FBS, and passing through a 70 μm cell strainer. Red blood cells were lysed by osmotic shock. Cells were washed in PBS, passed through a 40 μm cells strainer, and aliquoted for analysis after counting. Details of isolation have been described previously(Kish et al., 2013).

Characterization of mononuclear phagocytes.

Isolated splenocytes and mesenteric lymphocytes were re-suspended in PBS and incubated for 30 min at 4 °C with LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen). Cells were washed in PBS with 5% FBS and incubated for 20 min with antibody cocktail containing PerCP-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD103, APC-conjugated anti-mouse CD11c, AlexaFluor 700-conjugated anti-mouse I-Ab, eFluor 450-conjugated anti-mouse CD11b, and PE-Cy7-conjugated anti-mouse F4/80. Following antibody incubation, cells were fixed in 2% paraformaldehyde in PBS and analyzed on a BD Biosciences FACS Canto II flow cytometer.

Stimulation of splenocytes and lamina propria cells

$1e^6$ isolated cells from the spleen and LP were pipetted into a 96 well cell culture plate and incubated in cell culture media or cell culture media containing fecal slurry (50 mg protein/ml) or cell culture media containing LPS (1 μg/ml) (Sigma-Aldrich) for 24 hours. Fecal slurries for cell stimulation were created by collecting stool from experimental animals the day prior to sacrifice and homogenizing in PBS, the homogenate was then autoclaved and protein concentration measured with a Bradford assay. Isolated cells from

each animal were stimulated with their own stool homogenate to determine response to native flora. Following incubation, culture media was preserved at -80°C for measurement of secreted cytokines.

Statistical analysis.

One-way ANOVA with log-transformation was used to determine differences across groups using Metaboanalyst 2.0 software. Individual pairwise comparisons were performed with the Mann-Whitney-U test.

4.3 Results

Defining the ICR-DSS phenotype.

Alterations in the DSS phenotype following ICR was evaluated across two time points to determine the impact of an inflammatory stimulus during the healing process. Weight change from the time of surgery to the day of DSS induction was stable in both the short-term and long-term groups (Figure 2A). In the short-term groups DSS was initiated on post-operative day (POD) #9 and resulted in significant weight loss in ICR animals ($p \leq 0.05$) relative non-operative control animals (Ct) and non-operative animals receiving DSS (Ct-D) with a mean weight loss of 1.23g during the DSS cycle. No difference was found between sham surgery animals (Sh) and sham surgery animals receiving DSS (Sh-D) when compared to ICR and control groups. For the long-term cohorts no significant weight loss was noted across all groups.

The hallmark of the DSS colitis model is the development of bloody stools(Perše & Cerar, 2012). ICR led to occult blood positive stools significantly sooner relative to shams and non-operative animals treated with DSS. Median time to occult blood stools in

both the short and long term ICR animals was 3 days versus 5 days in other DSS groups. All animals not receiving DSS remained occult blood negative (Figure 2B).

Another measure of DSS colitis is the colon weight:length ratio which increases with inflammation(Perše & Cerar, 2012). Because of anticipated effects in the neo-terminal ileum (nTI) following ICR, intestinal weight:length ratios were measured in 12 cm of distal ileum in addition to the colon. Weight:length was increased in the nTI of all ICR animals relative to controls and shams; and did not change significantly with DSS treatment (Figure 2C). This increase in the nTI may be due to the expected adaptive growth response following ICR(Dekaney et al., 2007; Speck et al., 2011). Colonic weight:length in the short term was only elevated in controls and shams treated with DSS, and none of the ICR animals developed significantly elevated weight:length. However ICR with and without DSS resulted in increased weight:length in the long term(Figure2C).

Tissue myeloperoxidase (MPO) levels were measured as a marker of neutrophil mediated inflammation. In the ileum MPO was only elevated in the nTI of ICR and ICR-D animals in the short term with a significant reduction in the long-term nTI of ICR animals (Figure 2D). In the colon, DSS treated shams and non-operative controls developed large increases in tissue MPO levels consistent with induction of DSS colitis(Chassaing et al., 2012)(Figure 2D). Both ICR and ICR-D groups in the short-term demonstrated increased MPO, but levels were significantly lower than non-ICR animals treated with DSS ($P \leq 0.005$) suggesting a blunted neutrophil response to DSS in the colonic tissues following ICR. This effect was consistent in the long-term ICR animals as well, where MPO levels fell to Ct levels and became only modestly elevated with DSS challenge.

Because ICR in humans is associated with increased bacterial loads in the nTI(Neut et al., 2002), quantitative PCR (qPCR) was performed on genomic DNA extracted from the terminal ileum to determine bacterial tissue burden by amplifying a universal portion if the 16s bacterial gene (Figure 2E). No significant increases in bacterial load were detected in ICR animals relative to non-ICR animals however when short-term ICR groups were compared to long-term ICR groups there was a significant increase in bacterial load with time ($p \leq 0.05$).

ICR recruits dendritic cells and macrophages to the intestinal lamina propria.

Shifts in mononuclear phagocyte (MP) populations in the intestine following ICR with anastomosis have not been previously studied. Murine lamina propria MP populations are defined by expression of CD11c which primarily defines monocyte derived dendritic cells (DCs), CD11b which marks macrophages (MΦ) or both CD11c and CD11b which consists of populations of both Mϕs and DCs(Denning et al., 2011; Uematsu et al., 2008). Double immunofluorescence was performed with CD11c and CD11b to detect changes in lamina propria MP populations with ICR and with DSS treatment. In non-operative controls $CD11c^+$ cells represented the dominant population in the lamina propria followed by $CD11c^+CD11b^+$ cells with relatively few single positive $CD11b^+$ cells in the ileum (Figure 3A). This is in contrast to flow cytometery studies, which found $CD11b^+$ cells to be the dominant population in the ileum. DSS did not have an affect on these ratios in controls. Likewise, no changes were noted on stains for the specific MΦ maker F4/80 and DC markers $CD11c^+CD103^+$.

With ICR, the nTI in short-term animals developed significant shifts in MP subsets with $CD11b^+$ cells representing the majority and $CD11c^+$ cells decreasing (Figure 3). As well,

there was a recruitment of $CD11c^+CD103^+$ cells, which defines a migratory population of DCs(Persson, Scott, Mowat, & Agace, 2013). The increase in $CD11b^+$ cells indicates a recruitment of MΦs to the lamina propria as supported by the increase in $F4/80^+$ cells. DSS treatment in the short-term ICR animals led to a further shift with $CD11c^+CD11b^+$ cells becoming the majority. This same pattern was seen in both long-term ICR groups with $CD11c^+CD103^+$ DCs and $F4/80^+$ MΦs remaining elevated relative to non-operative animals. Co-localization of NOS2 with F4/80, which typically labels classical macrophages was increase with ICR and subsequently decreased with DSS treatment. Colonic MP subsets were also subject to shifts with both DSS treatment and ICR (Figure 4). Non-operative DSS animals had an increase in $CD11b^+$ cells with a decrease in $CD11c^+$ cells. Previous studies have found this same increase in $CD11b^+$ cells with induction of colitis(Denning et al., 2011). ICR in the short-term group led to a similar pattern with creased $CD11b^+$ cells, and decreased $CD11c^+$ cells; while DSS treatment produced a shift toward $CD11c^+CD11b^+$ cells. In the long term ICR groups $CD11c^+CD11b^+$ and $CD11b^+$ made up the majority. It is likely that many of the $CD11c^+CD11b^+$ cells were DCs as $CD103^+$ DCs were elevated through all the ICR groups. MΦs were also increased following ICR but only in the long-term group (ICR-Lt) and demonstrated increased co-localization of NOS2.

ICR suppresses cytokine signaling in the colon with DSS.

A multiplex cytokine assay was used to evaluate changes in tissue cytokine expression with DSS treatment and ICR (Figure 5). Bar charts representing results from individual cytokines are shown in Supplementary Figure 1. CXCL1, primarily defined as a

neutrophil chemotactant; was the only cytokine different across the groups in the terminal ileum, with elevations in DSS treated controls/shams and both short-term ICR groups ($p \leq 0.05$) in keeping with MPO levels. Interestingly DSS did not evoke a CXCL1 response in the long-term ICR group.

In the colon ICR induced IL-1β and CXCL1. These two cytokines remained increased in long-term animals and levels stayed consistent with DSS treatment. DSS treatment in control and sham animals resulted in large increases in nearly all measured cytokines (Figure 5). In contrast DSS failed to increase cytokine levels in ICR groups over non-operative controls and this was consistent across both ICR time points.

Macrophage depletion induces Th1 type cytokine signaling in the neo-terminal ileum.

To determine if the intestinal immunosuppression following ICR was mediated by MΦs, liposomal clodronate (LipC) was used for MΦ depletion. This was done using the timeline for the long-term group (Figure 1).

LipC was used for macrophage depletion in a non-operative DSS treatment group (Ct-D/LipC) and an ICR DSS treated group (ICR-D/LipC). In the ileum where DSS had previously demonstrated little effect regardless of experimental group, LipC decreased expression of IL-12, IL-2, IL-5, and IL-1β in the Ct-D/LipC group relative to Ct and Ct-D groups (Figure 6). These same cytokines were decreased in the ICR-D/LipC group; however a different pattern emerged in these ICR animals with increases in CXCL1, IL-6, IFN-γ, and TNF-α (Figure 6). Suggesting that LipC had the effect of shifting the immune response in the nTI towards a Th1 pattern.

A divergent effect of LipC was found in the colon, where treatment either decreased cytokines or had no effect, failing to reverse the suppressive effects of ICR (Figure 7). Histologic injury in the peri-anastomotic ileum and colon was assessed on H&E stains using a scoring system previously derived for post-operative injury in the IL-$10^{-/-}$ model(A. M. B. MD et al., 2012). Injury was increased in the nTI of all ICR groups. The major contributing factors to the increased score were neutrophil infiltration into the lamina propria and per-intestinal inflammation in the serosal layer (Figure 8).

In the colon, injury increased with DSS administration and was unchanged with LipC treatment in non-operative animals. ICR alone also resulted in increased injury scores in the colon with no change in response to DSS or DSS with LipC.

Histologic sections of peri-anastomotic intestinal tissues were stained for F4/80 to determine if MΦs were in fact depleted with LipC (Figure9). In non-operative groups LipC actually had a paradoxical effect in the ileum, with a mean 10.2 F4/80^{+} cells/HPF in the Ct-D/LipC treatment group vs. 2.1 and 3.3 cells/HPF in the Ct and Ct-D groups respectively. In the colon F4/80 MΦs increased with DSS treatment to 20.2 cells/HPF over 13.4 in the Ct animals, and the DSS effect was unaltered with LipC treatment as the Ct-D/LipC group had 22.3 cells/HPF. This is contrast to previous work demonstrating depletion of F4/80^{+} cells in the colon with LipC treatment(Weisser et al., 2011).

ICR had the effect of increasing F4/80^{+} cells in the ileum (Figure 3) with 19.1 and 24.3 cells/HPF in the ICR-Lt and ICR-Lt/D groups respectively, and LipC had the anticipated effect of depleting MΦs in the ICR-D/LipC group to 12.4 cells/HPF. This may provide an explanation for dramatic effects of the LipC in the ileum of ICR-D mice. Like the non-

operative groups, LipC had no effect on depleting MΦs in the colons of DSS treated ICR animals.

ICR changes mononuclear phagocyte profile in the mesenteric lymph nodes and suppresses cytokine responses to microbial antigens.

Shifts in MP populations in the mesenteric lymph nodes (MLN) were evaluated by flow cytometery and functional changes were demonstrated with cell culture and quantification of cytokine secretion. To define the MP subsets cells were first gated to identify antigen presenting cells (Figure 10A). Proportions of DC subsets were determined by pre-gating for $F4/80^-$ cells to exclude MΦs, followed by quantification of cells as either $CD11c^+CD103^-$ or $CD11c^+CD103^+$. These subsets were further defined by expression of CD11b (Figure 10B). There was an increase in $CD11c^+CD103^-$ DCs in the MLNs following ICR and an increase in $CD11b^+$ expressing cells within this subset. This change persisted with DSS treatment however the addition of LipC caused a reduction in $CD11c^+CD103^-CD11b^+$ DCs. Total DCs in the MLNs were also quantified by defining cells as $CD45^+MHC\text{-}II^+CD11c^+F4/80^-$. There was no significant difference across the groups (Figure 10D).

MΦs were quantified as $CD45^+MHC\text{-}II^+CD11b^+F4/80^+$ cells and were only reduced in the LipC treatment groups (Figure 10D). An additional subset of $CD11b^+F4/80^-$ cells were increased with in the Ct-D, ICR, and ICR-D groups, and decreased with LipC treatment (Figure 10C).

Stimulation of lymph node isolates in primary cell culture was performed to determine cytokine responses in the regional lymphatic drainage of the gut resulting from ICR (Figure 10D). DSS treatment in non-operative animals led to increased IL-6, IL-10 and

TNF-α when cells were stimulated with fecal slurry or LPS. TNF-α was also elevated in the non-stimulated cultures. MΦ depletion reduced all 3 cytokines measured in non-operative animals relative to DSS treatment alone. ICR resulted in a baseline increase of IL-6 and TNF-α, which was unchanged with DSS treatment, and this effect was diminished with MΦ depletion. When stimulated with fecal slurry or LPS, the ICR group increased IL-6, IL-10 and TNF-α production. There was no significant increase in response in the ICR-D group, and neither ICR nor ICR-D groups produced IL-10 and TNF-α to the levels of Ct-D animals. The ICR-D/LipC group had significantly lower IL-6 levels in stimulated cultures, but IL-10 and TNF-α were unaffected relative to other ICR groups.

Decreased DSS induced systemic inflammatory signaling following ICR.

Systemic responses to ICR and DSS were determined by defining MP populations in the spleen with secreted cytokines from primary cell cultures and serum cytokine levels (Figure 11). Flow cytometery demonstrated no significant changes in $CD11c^+$, $CD11c^+CD11b^+$, and $CD11b^+$ subsets with ICR or DSS. LipC caused significant depletion of all 3 subsets, which was most pronounced in the $CD11c^+CDllb^+$ and $CD11b^+$ subsets. Relative proportions of MΦs within the $CD11c^+CDllb^+$ and $CD11b^+$ were determined by F4/80 expression. ICR caused a shift to more $F4/80^+$ cells in the $CD11c^+CDllb^+$ Subset. LipC caused a large depletion on all $F4/80^+$ cells (Figure 11A). When total MΦs and DCs were measured, there was no difference across all groups with the exception of LipC treated groups, which had decreased MΦs and decreased DCs in the non-operative group (Figure 11B).

Secreted cytokines from spleen isolates demonstrated some similarities to MLNs with equivalent increased levels of IL-6 in stimulated Ct-D, ICR, and ICR-D groups (Figure 11C). IL-10 increased across all groups over Ct animals with LPS stimulation with greatest elevations in the Ct-D and Ct-D/LipC groups. DSS treatment with or without LipC did not change IL-10 secretion in the ICR groups.

TNF-α secretion increased over Ct with fecal slurry and LPS stimulation in the Ct-D group. No ICR group had an increase in TNF-α secretion over Ct levels with stimulation. Serum IL-1β, CXCL-1, IL-6, TNF-α, and IFN-γ all became elevated with DSS treatment in non-operative animals. No cytokines were elevated in the serum following ICR and DSS had no effect. MΦ depletion restored IL-1β, CXCL-1, IL-6, and TNF-α signaling to reflect DSS treated non-operative controls.

4.4 Discussion

ICR with ileocolic anastomosis is a procedure frequently employed for surgical illnesses of the small bowel and colon, most notably Crohn's disease. This article describes a model, which allows the determination of physiologic responses to mucosal insult following ICR. We have demonstrated that ICR leads to significant perturbations in immunologic function from the mucosal level to the draining lymphatics and systemically. These changes contribute to a more severe DSS phenotype highlighted by shorter time to occult blood positive stools. Our data also suggests impaired microbial handling with inappropriate responses to bacteria allowing overgrowth in the ileum.

To capture responses to DSS during the evolving healing response we introduced DSS at two time points following surgery. In comparing the short term and long term ICR groups; the short term ICR group, which received DSS on POD#9 was the only group to

develop significant weight loss during the DSS cycle. Elevated MPO levels in the intestinal tissues at the short term time point suggest that there was an ongoing element of the inflammatory phase of wound healing. Serum cytokines were elevated in this group as well indicating a greater sensitivity to mucosal insult shortly after surgery. The time to occult blood positive stools did not differ across the short term and long term ICR groups with a median of 3 days vs. 5 days in sham surgery animals and non-operative controls. It is important to note that sham operative animals did not differ from controls in measurements of phenotype, and intestinal cytokine responses suggesting effects observed in our model are due to ICR and not surgical stress with laparotomy. Why ICR had this sustained effect is unclear; however blunted mucosal responses may be to blame. Blunting of immunologic responses in the intestine following ICR was perhaps the most significant finding of this study. Non-operative control animals and sham surgery animals developed large increases in colonic cytokines typical of the Th1 response in this model of colitis(Eliopoulos, 2014). With ICR the response to DSS was near absent. We hypothesized that this may be caused by shifts in mononuclear phagocyte populations through the recruitment of DCs and MΦs with suppressive properties.

In the steady state, MΦ and DC populations in the distal ileum and colon are relatively inflammation anergic; expressing low levels of inflammatory cytokines and promoting oral tolerance through Treg induction. However the MP compartment of the gut is continuously replenished with circulating precursors allowing for plasticity in reactivity and function(Farache, Zigmond, Shakhar, & Jung, 2013b). When treated with DSS, newly recruited MPs take on an inflammatory phenotype(Platt, Bain, Bordon, Sester, & Mowat, 2010). This $CD11b^{+}TLR\text{-}2^{+}CX3CR1^{int}$ monocyte subset produces high amounts

of TNF-α and is analogous to $CD14^+$ monocyte subsets that populate the inflamed CD mucosa(Nobuhiko Kamada & Nobuhiko Kamada, 2008; Thiesen et al., 2014). Consistent with these findings, DSS treatment of non-operative controls in our study led to an increase in $CD11b^+$ cells in the colonic LP and higher relative proportion of $NOS2^+F4/80^+$ cells coupled with increases in tissue inflammatory cytokines. $CD11b^+$ cells also increased with ICR; however, challenge with DSS after ICR failed to evoke an inflammatory response suggesting that the recruited MPs after ICR are highly resistive to inflammation. Based on wound healing studies in non-intestinal tissues there is an expected shift in MP phenotypes from inflammatory phase IL-6, TNF-α, and IL-1 producing MΦs to remodeling phase IL-10 and TGFβ producing immunosuppressive MΦs(Novak & Koh, 2013; Sindrilaru & Scharffetter-Kochanek, 2013). Using a model of colonic mucosal biopsy Seno et al. demonstrated efficient wound healing to dependent on M2 non-inflammatory macrophages producing IL-4 and IL-13.

To determine if the observed suppressive effects of ICR were due to recruited MΦs we combined ICR with MΦ depletion using LipC and DSS treatment. We performed this experiment using the long-term ICR-DSS protocol as this is when we observed the largest number of $CD11b^+$ and $F4/80^+$ cells. LipC did not reverse the suppressive effects of ICR in the colon and instead decreased all cytokines measured with the exception of CXCL1. This same effect was seen in LipC/DSS treated non-operative controls. These results are difficult to interpret as F4/80 staining did not demonstrate a decrease in MΦ numbers in the colonic LP.

LipC was successful with depleting MΦs in the terminal ileum and this is where the most significant effect was seen. Because DSS does not affect the ileum in non-operative

animals it is difficult to know if the lack of response to DSS in the ileum after ICR is due to the same suppressive effects seen in the colon or simply due to a lack of DSS effect in the ileum; however the LipC treatment groups suggest the latter may be true. The post-ICR terminal ileum demonstrated significant upregulation of pro-inflammatory cytokines IL-6, IFN-Y, and TNF-α typical of a Th1 response when treated with DSS combined with macrophage depletion. This suggests that LipC depleted a suppressive population of cells allowing inflammatory signaling. This effect was unique to ICR animals, and was also found in the serum.

Flow cytometery of MLN cell isolates did demonstrate changes in MP populations with DSS treatment in non-operative controls; however, stimulation of these cells in culture with fecal slurry or LPS suggested that these cells were primed for inflammatory responses leading to secretion of IL-6, TNF-α, and IL-10. A primed inflammatory response to bacterial antigens with DSS treatment was expected and DSS is known to act by increasing bacterial invasion into the lamina propria(Johansson et al., 2010). This effect of DSS was significantly diminished with MΦ depletion suggesting that DSS treatment leads to inflammatory population of MΦs in the MLNs.

With ICR there was an increase in $CD11c^{+}CD103^{-}CD11b^{+}$ DCs. This population was recently found to represent a migratory DC subset with the ability to induce Th1 and Th17 cells(Cerovic et al., 2012). Secreted cytokines from cell culture suggested a mixed picture. ICR alone increased baseline levels of IL-6 and TNF-α and primed IL-6 responses with stimulation using LPS or fecal slurry. In contrast to this TNF-α secretion did not increase from baseline with stimulation after ICR regardless of DSS treatment and was significantly lower than levels in non-operative DSS mice; indicating a

depressed TNF-α response to bacterial antigens. It is unclear which subsets are responsible for these effects. The increase in $CD11c^{+}CD103^{-}CD11b^{+}$ DCs may provide an explanation for the elevated resting levels of IL-6 and TNF-α as LipC treatment appeared to deplete this subset and ablate the baseline cytokine levels as well LipC diminished the IL-6 response to stimulation. While LipC depletion of cells helped to explain the cytokine production it did indicate which cells were responsible for depressed TNF-α responses. Results from cultured splenocytes effectively mirrored the results from the MLNs again suggesting a depressed TNF-α response to bacterial antigens following ICR.

In line with the decreased DSS response, the second major effect of ICR identified by this study is the induction of inappropriate tolerance to gut microbes. Three lines of evidence support this conclusion. The first is the measured increase in bacterial load in the terminal ileum of long-term ICR mice. This same effect has been observed in humans following ICR and was thought to result from loss of the ileocecal valve(Neut et al., 2002; Rutgeerts, 2003). Our results challenge this long held belief. Lack of intestinal cytokine response to invading microbes when DSS is used to degrade the mucosal barrier provides the second piece of evidence for impaired bacterial handling, and decreased TNF-α production in the MLNs and spleen when exposed to bacterial antigens provides the third. Prior to this work the immunologic changes in the gut associated with ICR and anastomosis were unexplored. By introducing an inflammatory stimulus after ICR, which is known to induce inflammatory bowel disease like inflammation in the intestine, we have uncovered a novel mechanism of the post-ICR immunologic contribution to

recurrence in CD. We hypothesize that the heightened DSS phenotype found in the ICR animals is due to unopposed bacterial penetration into the lamina propria. Recruited monocyte populations as part of the healing response may be partly to blame for this. This effect was most pronounced in the neo-terminal ileum where bacterial loads increased with time after ICR, and depletion of MΦs induced pro-inflammatory cytokines. If this effect is translatable to CD patients it may explain why post-operative lesions are found exclusively in the neo-terminal ileum, as blunted immune responses permit microbial overgrowth and invasion of pathobionts. Future work in this area should focus on defining the exact cells populations and signaling pathways involved in the post-ICR immune response.

4.5 Figures

Figure 4.1. Treatment time line and tissue collection protocol.

Figure 4.2. Phenotypic characterization of the ICR-DSS model across time. (A) Mean ± SEM animal weight from day of surgery to initiation of DSS and throughout the DSS cycle in short and long term groups. Mean ± SEM weight change across time was used to compare across groups. (B) Kaplan-Mier survival curves demonstrating time to occult blood positive stools across time. Significance was determined using the log-rank test for equality in survivor analysis. (C) Mean ± SEM tissue weight to length ratios in the distal 12 cm of terminal ileum and whole colon. (D) Determination of neutrophil activity with tissue MPO levels in ileum and colon expressed as mean ± SEM (E) Mean ± SEM copy number of 16s gene from tissue genomic DNA extracts. * $P \leq 0.05$ relative to Ct, # $P \leq 0.05$ across time. n=6 for Ct and Sham groups, N=9 for ICR groups.

Figure 4.3. Photomicrographs of double immunofluorescence stained tissue section from terminal ileum (40X). (A) Staining for mononuclear phagocyte markers CD11c (Alexa Flour 488-green) and CD11b (Alexa Flour 594-red). $CD11c^{+}CD11b^{-}$ cells and $CD11c^{+}CD11b^{+}$ cells were the dominant populations in all groups with the exception of short term ICR animals where $CD11c^{-}CD11b^{+}$ cells were the majority. $CD11c^{+}CD11b^{-}$ cells decreased with ICR. Cells staining positive for CD11b were primarily located in the crypts in the Ct group but were found in close association with villi epithelium following DSS and/or ICR. (B) Staining for migratory dendritic cell populations with CD11c (Alex Flour 488-green) and CD103 (Alexa Flour 594-red). $CD11c^{+}CD103^{+}$ cells were rarely detected in the villi of non-operative animals but were increased with ICR. This populations was typically visualized deep in the lamina propria. (C) Staining for macrophage populations with F4/80 (Alexa Flour 594-red) and NOS2 (Alexa Flour 488-green). $F4/80^{+}$ cells were rare within the lamina propria of non-operative groups, and increased with ICR with uniform dispersal through the lamina propria. Co-localization of F4/80 and NOS2 was higher in non-DSS treated short term and long term ICR groups. (D) Mean ± SEM cell counts from immunofloursence stained tissue sections. Counts were performed on 6 representative photomicrographs from n=3/group. * $P \leq 0.05$ relative to Ct, # $P \leq 0.05$ with DSS.

Figure 4.4. Photomicrographs of double immunofloursence stained tissue section from colon (40X). (A) Staining for mononuclear phagocyte markers CD11c (Alexa Flour 488-green) and CD11b (Alexa Flour 594-red). CD11c+CD11b- cells were the dominant

populations in controls (Ct). DSS and ICR led a a reduction in CD11c+CD11b- cells. $CD11c^{-}CD11b^{+}$ cells increased following DSS and/or long term ICR. (B) Staining for migratory dendritic cell populations with CD11c (Alex Flour 488-green) and CD103 (Alexa Flour 594-red). CD11c+CD103+ cells were rarely detected in the villi of non-operative animals but were increased with ICR. This populations was typically visualized deep in the lamina propria. (C) Staining for macrophage populations with F4/80 (Alexa Flour 594-red) and NOS2 (Alexa Flour 488-green). F4/80+ cells were dispersed through the lamina propria and increased with DSS and ICR. Co-localization of F4/80 and NOS2 was higher in DSS treated controls and long term ICR groups. (D) Mean ± SEM cell counts from immunofloursence stained tissue sections. Counts were performed on 6 representative photomicrographs from n=3/group. * $P \leq 0.05$ relative to Ct, v $P \leq 0.05$ between short and long term groups, # $P \leq 0.05$ with DSS.

Figure 4.5. Intestinal cytokine profiles do not change after ICR with DSS treatment. Tissue cytokine concentrations determined using the pro-inflammatory multiplex array (Meso Scale Discovery). Heat Maps were constructed using Metaboanalyst 2.0. *indicates significantly different across all groups $P \leq 0.005$. determined by ANOVA with log transformation. Cytokines in plots are presented in order importance for separation of the groups.

Figure 4.6. Macrophage depletion changes cytokine profiles in the terminal ileum after ICR. Tissue cytokine concentrations determined using the Pro-inflammatory

inflammatory V-plex mouse kit (Meso Scale Discovery) normalized for tissue dry weight. * $P \leq 0.05$ relative to Ct, v $P \leq 0.05$ with DSS and LipC.

Figure 4.7. Macrophage depletion does not change cytokine profiles in the colon after ICR. Tissue cytokine concentrations determined using the Pro-inflammatory inflammatory V-plex mouse kit (Meso Scale Discovery) normalized for tissue dry weight. * $P \leq 0.05$ relative to Ct, v $P \leq 0.05$ with DSS and LipC.

Figure 4.8. ICR causes injury in the terminal ileum and colon that is unchanged with DSS or macrophage depletion. (A) Photomicrographs of H&E stained tissue sections from per-anastomotic ileum and colon (20X). ICR resulted caused increased neutrophil infiltration and peri-intestinal inflammation in all groups. DSS and ICR both increased Injury scores largely due to epithelial hyperplasia. (B) Mean ± SEM injury scores. Scoring performed by pathologist blinded to groups n=6/group. * $P \leq 0.05$ relative to Ct.

Figure 4.9. Liposomal clodronate depleted macrophages in the neo-terminal ileum following ICR. Photomicrographs of double immunofloursence staining for macrophage populations with F4/80 (Alexa Flour 594-red) and NOS2 (Alexa Flour 488-green) (40X). Relative to counts presented in figure 3C, non-operative animals had a paradoxical increase in lamina propria F4/80 cells and ICR animals had a decrease in F4/80 cells with LipC treatment in the ileum. Colonic F4/80 cells were not depleted by LipC relative to stains presented in figure 4C. Bar charts represent mean ± SEM. Counts were performed on 6 representative photomicrographs from n=3/group.

Figure 4.10. ICR shifts mononuclear phagocyte populations and reduces the inflammatory priming effects of DSS in the mesenteric lymph nodes. (A) Representative gating strategy for identification of antigen presenting cells in the mesenteric lymph nodes. (B) Dendritic cell populations pre-gated as $F4/80^-$ antigen presenting cell. Dendritic cell subsets were further defined as based in CD103, and CD11b expression. The $CD11c^+CD103^-CD11b^+$ subset increased with ICR. (C) Macrophage populations defined as $CD11b^+F4/80^+$ antigen presenting cells. Macrophages decreased with LipC treatment. (D) Total mean ± SEM proportion of antigen presenting cells for gated macrophages and dendritic cells. (E) Mean ± SEM secreted cytokines from primary mesenteric lymph node cell cultures. NS (no stimulation), FecS (fecal slurry), LPS (lipopolysaccharide). Results are representative of 6 independent experiments. * $P \leq 0.05$ relative to Ct, v $P \leq 0.05$ relative to Ct-D.

Figure 4.11. ICR blunts systemic responses. (A) Macrophage and dendritic cell subsets determined by pre-gating for antigen presenting cells followed by identification of CD11c, CD11b and F4/80 expression. (B) Total mean ± SEM proportion of antigen presenting cells for gated macrophages and dendritic cells. (C) Mean ± SEM secreted cytokines from primary splenocyte cell cultures. NS (no stimulation), FecS (fecal slurry), LPS (lipopolysaccharide). (D) Mean ± SEM serum cytokine levels. Results are representative of 6 independent experiments. * $P \leq 0.05$ relative to Ct, v $P \leq 0.05$ relative to Ct-D.

Figure 4.1.

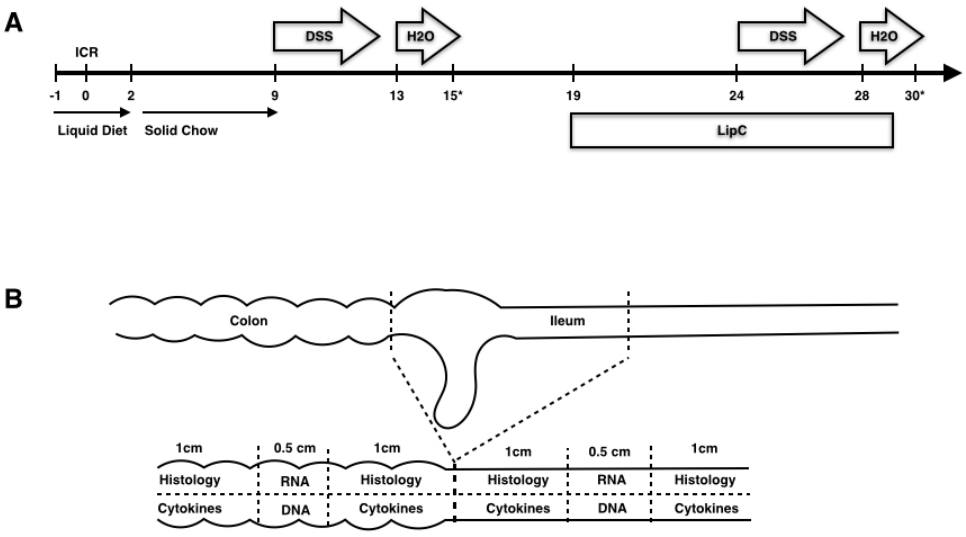
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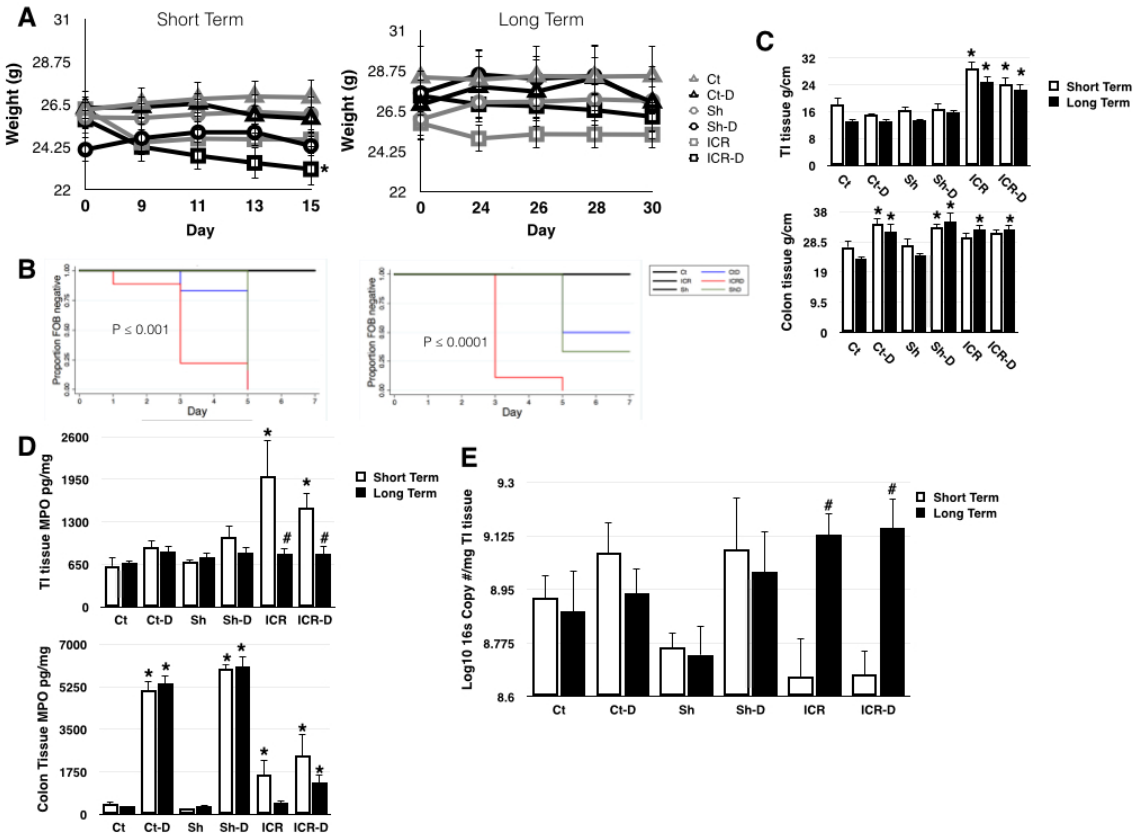
Figure 4.2.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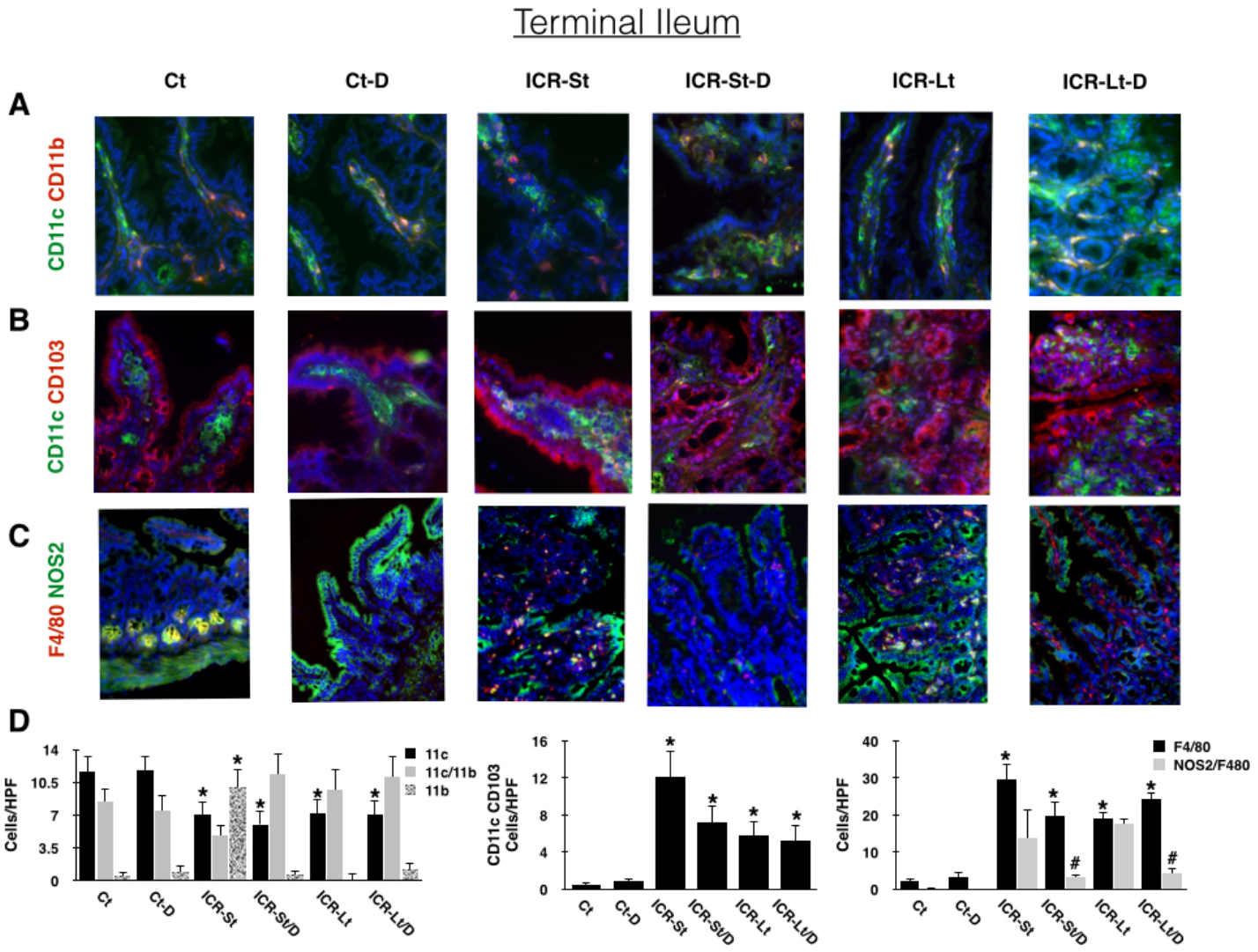
Log10 16s Copy #/mg TI tissue

Figure 4.3.


Terminal Ileum
Ct
Ct-D
ICR-St
ICR-St-D
ICR-Lt
ICR-Lt-D
A
CD11c CD11b
B
CD11c CD103
C
F4/80 NOS2
D
Cells/HPF
11c
11c/11b
11b
CD11c CD103 Cells/HPF
F4/80
NOS2/F480
Ct
Ct-D
ICR-St
ICR-St/D
ICR-Lt
ICR-Lt/D

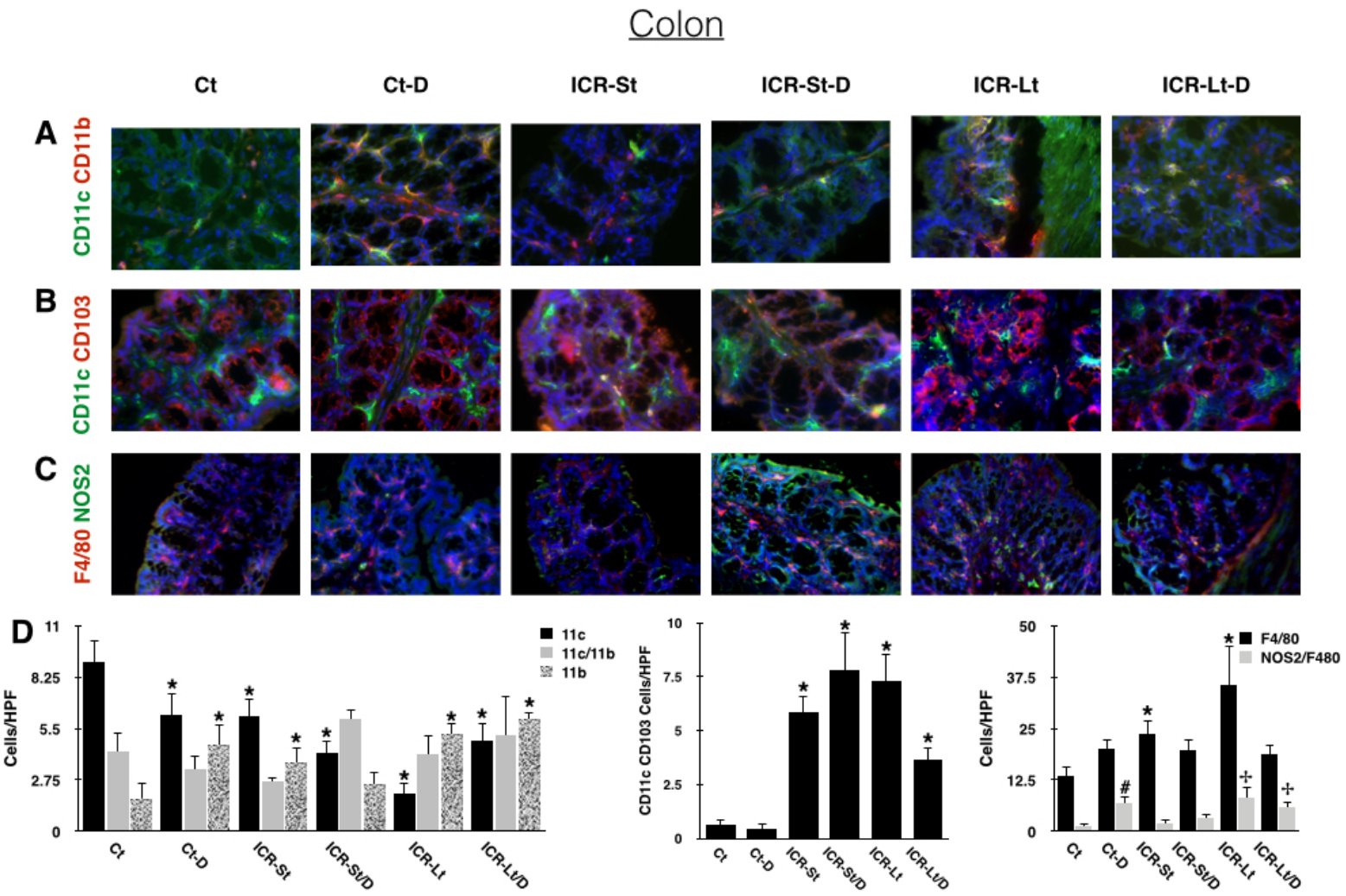

Colon
Ct
Ct-D
ICR-St
ICR-St-D
ICR-Lt
ICR-Lt-D
A
CD11c CD11b
B
CD11c CD103
C
F4/80 NOS2
D
Cells/HPF
11c
11c/11b
11b
CD11c CD103 Cells/HPF
F4/80
NOS2/F480
Ct
Ct-D
ICR-St
ICR-St/D
ICR-Lt
ICR-Lt/D

Figure. 4.5

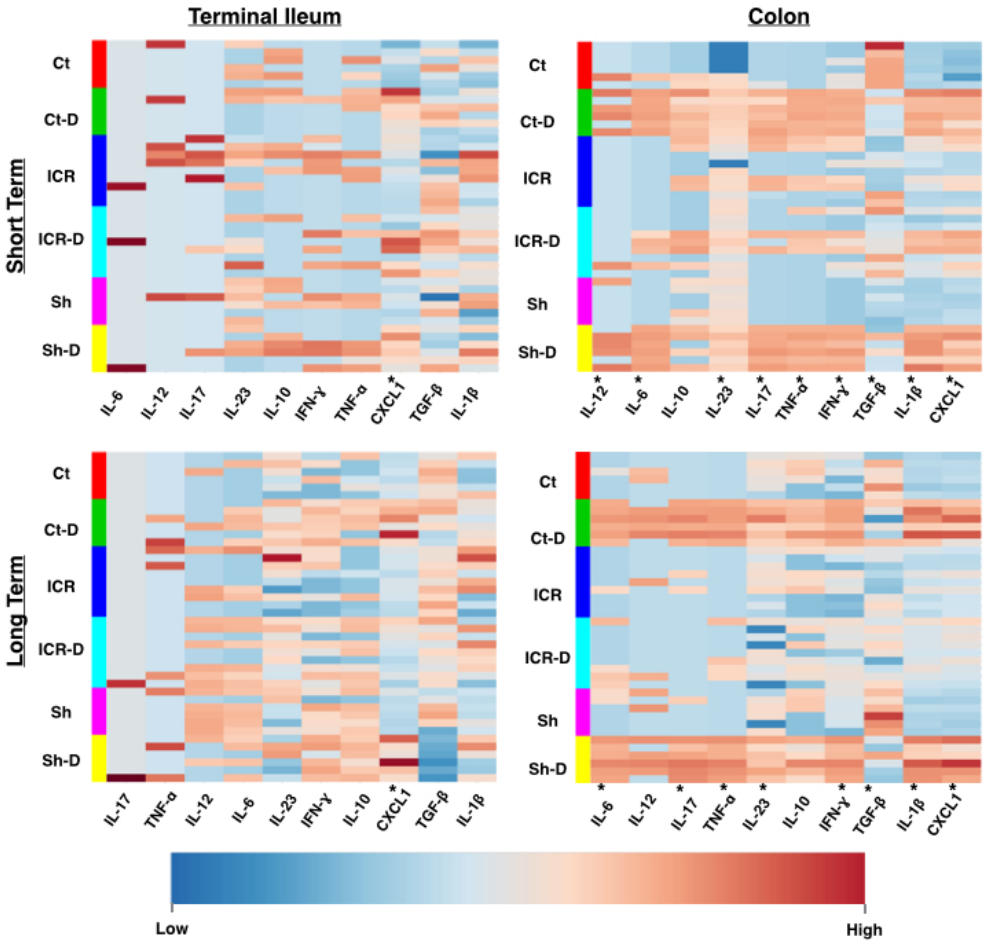

Terminal Ileum
Colon
Short Term
Long Term
Ct
Ct-D
ICR
ICR-D
Sh
Sh-D
IL-6
IL-12
IL-17
IL-23
IL-10
IFN-γ
TNF-α
CXCL1
TGF-β
IL-1β
Low
High

Figure 4.6.

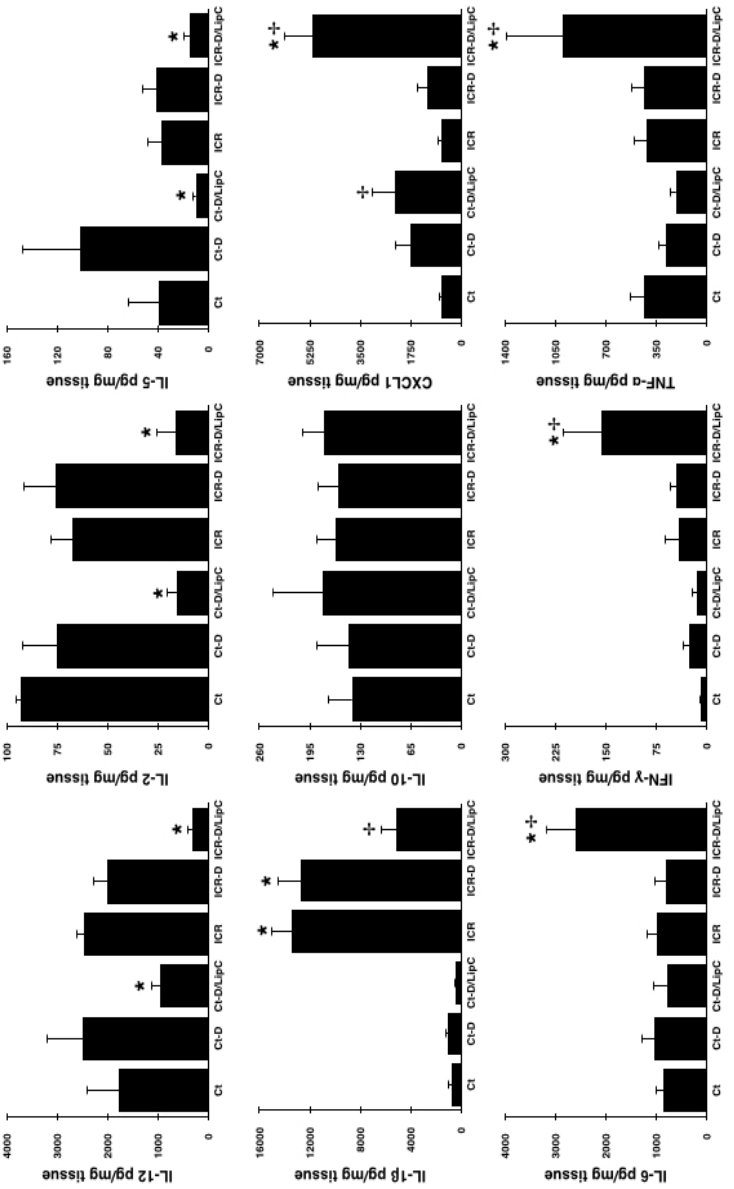

IL-12 pg/mg tissue
IL-2 pg/mg tissue
IL-10 pg/mg tissue
IL-5 pg/mg tissue
IL-1β pg/mg tissue
CXCL1 pg/mg tissue
IFN-γ pg/mg tissue
TNF-α pg/mg tissue
IL-6 pg/mg tissue
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
*
†
#

Figure 4.7.

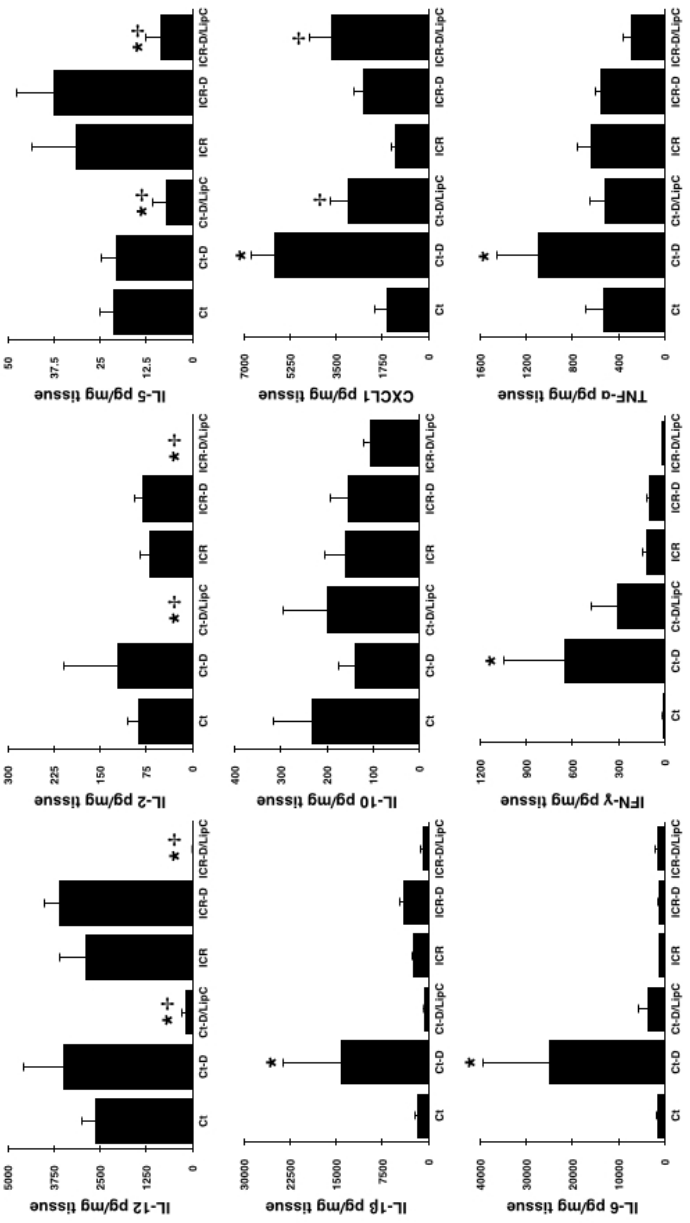

IL-12 pg/mg tissue
IL-1β pg/mg tissue
IL-6 pg/mg tissue
IL-2 pg/mg tissue
IL-10 pg/mg tissue
IFN-γ pg/mg tissue
IL-5 pg/mg tissue
CXCL1 pg/mg tissue
TNF-α pg/mg tissue
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC

Figure 4.8.

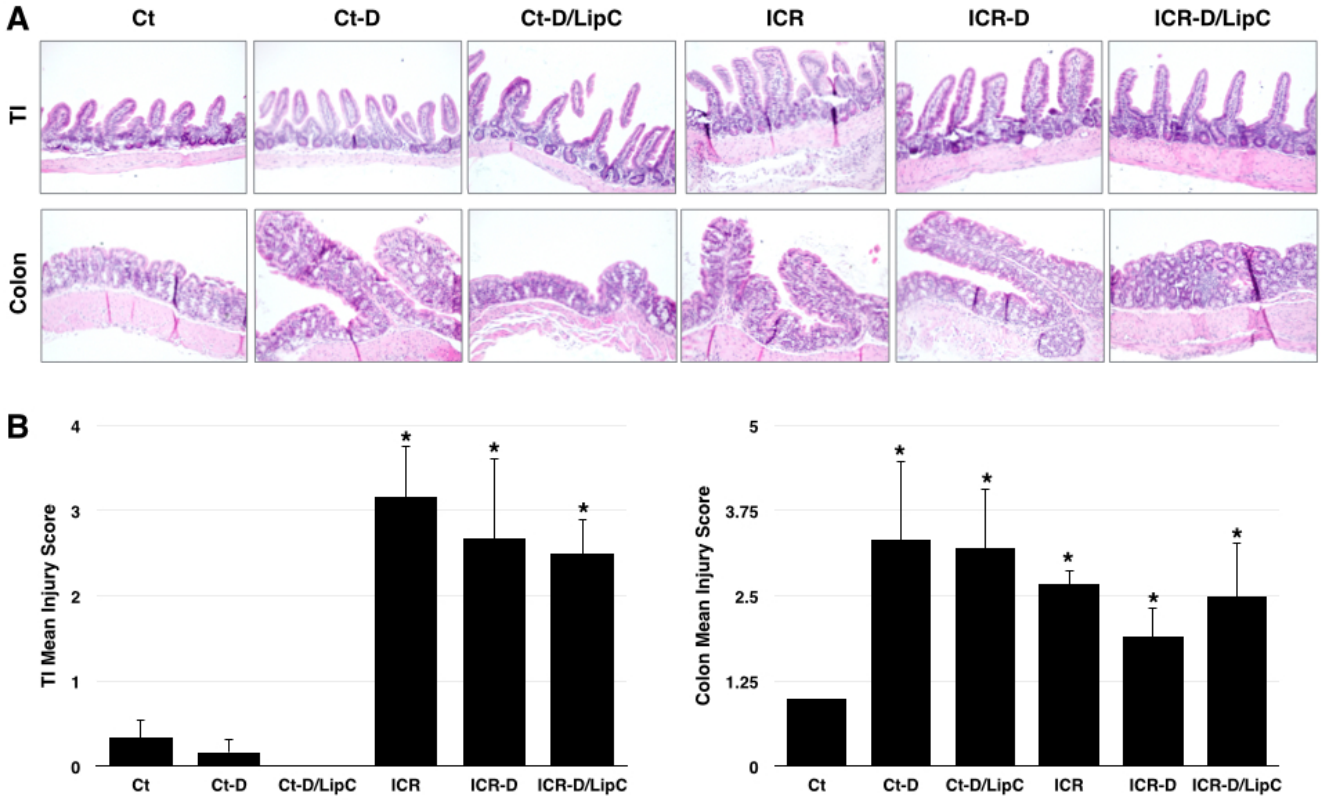

A
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
TI
Colon
B
TI Mean Injury Score
0
1
2
3
4
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
Colon Mean Injury Score
0
1.25
2.5
3.75
5
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC

Figure 4.9.

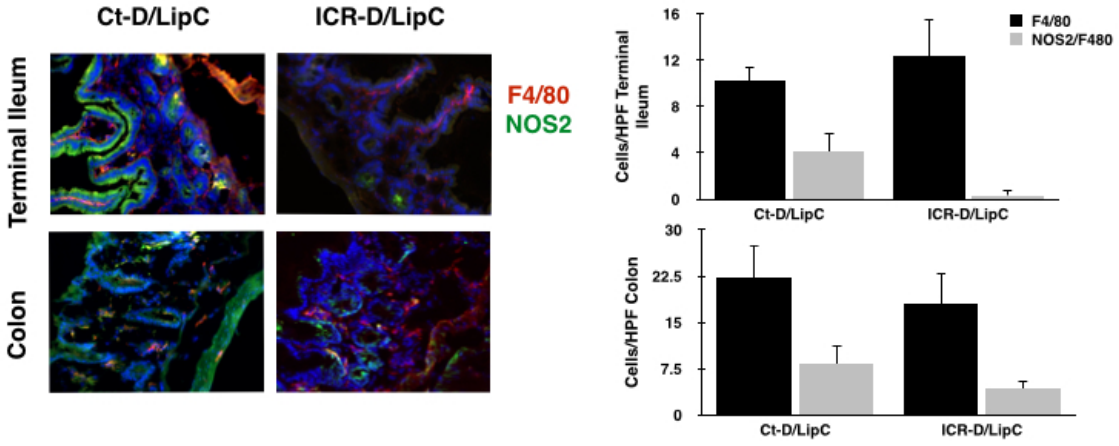

Ct-D/LipC
ICR-D/LipC
Terminal Ileum
Colon
F4/80
NOS2
F4/80
NOS2/F480
Cells/HPF Terminal Ileum
16
12
8
4
0
Ct-D/LipC
ICR-D/LipC
Cells/HPF Colon
30
22.5
15
7.5
0
Ct-D/LipC
ICR-D/LipC

Figure 4.10.

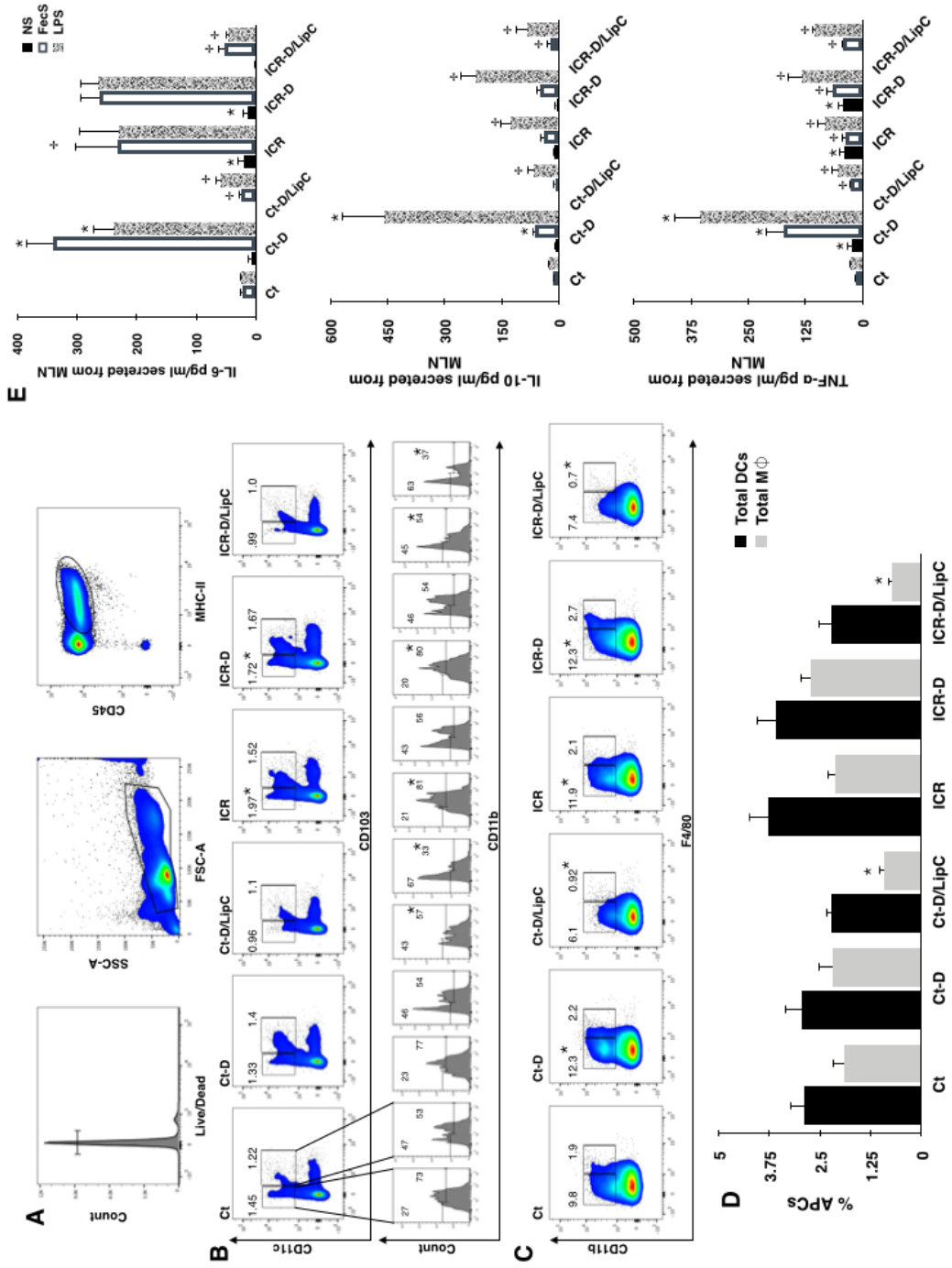
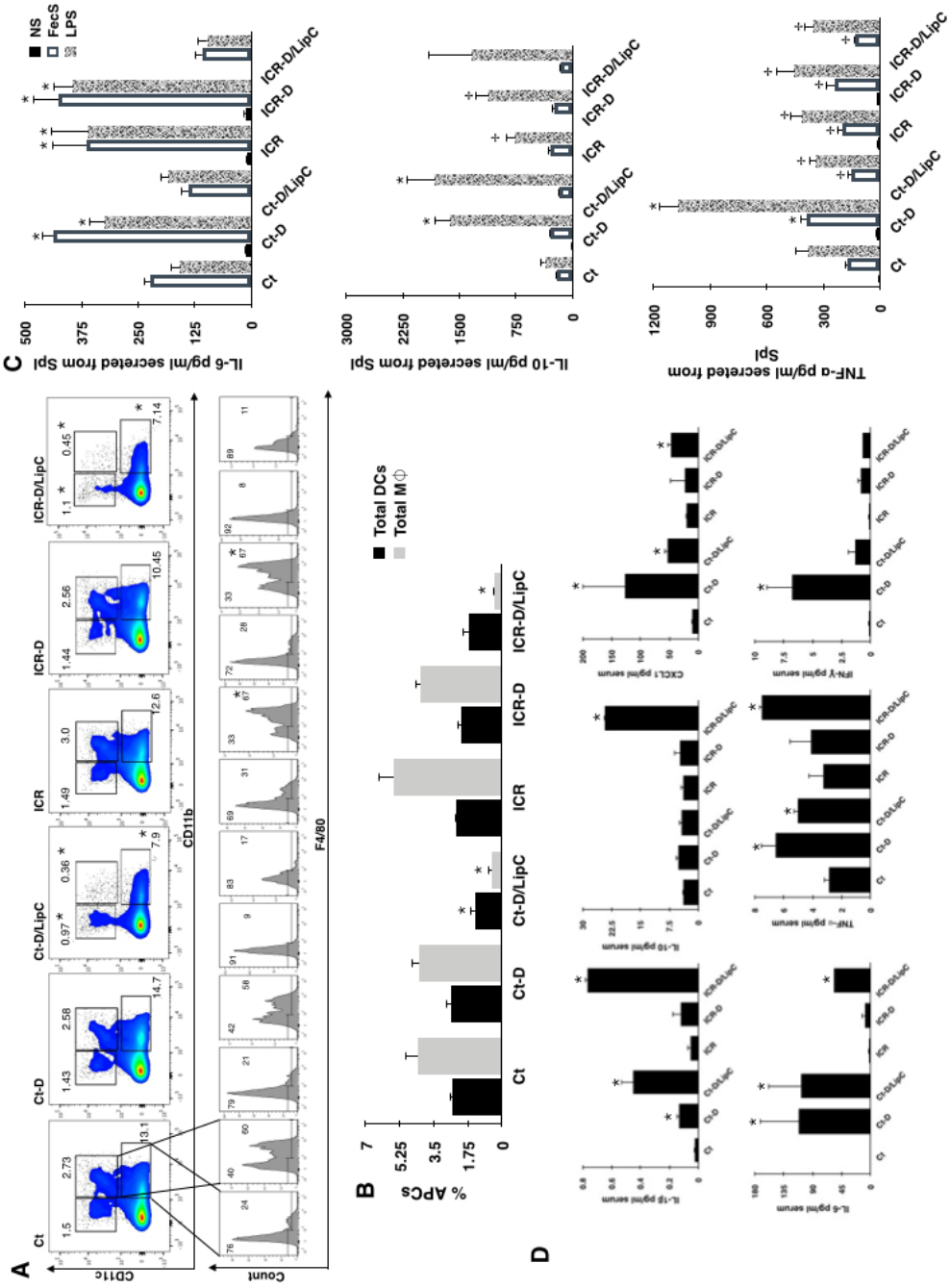

A
Count
Live/Dead
SSC-A
FSC-A
CD45
MHC-II
B
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
CD11c
CD103
Count
CD11b
C
CD11b
F4/80
D
Total DCs
Total MΦ
% APCs
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
E
NS
FecS
LPS
IL-6 pg/ml secreted from MLN
IL-10 pg/ml secreted from MLN
TNF-α pg/ml secreted from MLN

Figure 4.11.


A
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
CD11c
CD11b
Count
F4/80
B
Total DCs
Total MΦ
% APCs
Ct
Ct-D
Ct-D/LipC
ICR
ICR-D
ICR-D/LipC
C
NS
FecS
LPS
IL-6 pg/ml secreted from Spl
IL-10 pg/ml secreted from Spl
TNF-α pg/ml secreted from Spl
D

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Chapter 5. Fecal microbial transplant following ileocolic resection reduces ileitis but restores colitis in IL-10$^{-/-}$ mice.

5.1 Introduction

Surgical resection of the intestine remains a necessary treatment for most Crohn's disease (CD) patients with resection of the ileocecal region (ICR) being most common(Buisson et al., 2011). However, rapid recurrence at the anastomosis commonly occurs, and it is thought that recurrence is due to loss of the ileocecal valve thereby introducing colonic bacteria into the ileum(Rutgeerts, 2003). Studies in humans and animal models have highlighted the interaction of the gut immune system with its microbial inhabitants as central to CD pathogenesis(Koboziev et al., 2014), but little work has been done to study this relationship in post-operative disease. Mononuclear phagocytes (MP) including macrophages (MΦ) and dendritic cells (DCs) are found in large numbers in the gut lamina propria(Varol et al., 2010). Under steady state conditions, intestinal macrophages clear antigens without evoking inflammatory responses(Smythies et al., 2005) while CD103+ DCs promote tolerance by trafficking antigen to mesenteric lymph nodes and inducing T regulatory cells (Tregs)(C. L. Scott et al., 2011). Following surgery, MP subsets may be prone to significant perturbations due to their recruitment and role in tissue repair and wound healing(Novak & Koh, 2013).

A lack of animal models reflecting current surgical practices in CD has resulted in a poor understanding of post-operative ileitis. Recently, a surgical resection model was defined in IL-10 gene deficient mice (IL-10$^{-/-}$) whereby these mice developed inflammation in the peri-anastomotic small bowel following ICR(Rigby et al., 2009); moreover this

inflammation was absent both in wild-type and gnotobiotic animals, indicating that genetic susceptibility and bacteria are both required for ileitis. In this study, we developed a model of ileocecal resection in IL-10$^{-/-}$ mice analogous to the procedure commonly performed for complicated ileocecal CD in humans in order to study microbial and immune responses. As well, we performed a fecal microbial transplant (FMT) following ICR using stool from healthy wild type donor animals to restore microbial diversity and composition. In that patients undergoing surgery for CD generally have a severe microbial dysbiosis, we hypothesized that performing FMT with healthy donor stool following surgery would have an added benefit in preventing disease recurrence by restoring a healthy microbiome.

5.2 Methods

Animal model

Animal use protocols were approved by the health science animal care committee at the University of Alberta. IL-10$^{-/-}$129S1/SvlmJ mice (12-13 weeks old) underwent ileo-colonic resection (ICR) with primary end-to-end anastomosis as previously described(Perry, Borowiec, Dicken, Fedorak, & Madsen, n.d.). Ileum was transected 2 cm proximal to the ileocecal junction and the ascending colon was divided just distal to cecum (Supplementary Figure 1). The ileocolic anastomosis was constructed with 8-0 Prolene® (Ethicon). The abdominal wall was closed, and animals were maintained on liquid diet for 2 days. Overall survival in the operative groups was 97%. Non-operative control IL-10$^{-/-}$ mice (CT) received the liquid diet but did not undergo surgery.

Fecal microbial transplant

Thirteen days after surgery mice were fasted overnight and given PEG3350 17.9 mEq/L in H_20 (Golytely). On d14, stools were collected from healthy age matched wild type 129S1/SvlmJ mice (n=4) and homogenized in PBS reduced with 0.05% cysteine HCl (Sigma) at a ratio of 1 fecal pellet/ml. The fecal slurry was passed through a 100µm cell strainer and 200µl delivered to the FMT group by oral gavage (FMT). A sham transplant group was gavaged with a fecal slurry prepared from their own stools. (Sham). The non-operative control group (CT) was gavaged with PBS 0.05% cysteine HCl. Following FMT, mice were placed on chow. On d28, mice were sacrificed and tissues collected (Supplementary Figure 1).

Histological Analyses

Peri-anastomotic sections of ileum and colon were fixed in 10% buffered formalin, embedded in paraffin and cut at 5 µm. Tissue sections were stained with hematoxylin/eosin (H&E), and Masson's trichrome for subsequent analysis. H&E stains were used for histologic inflammation/injury scores(A. M. B. MD et al., 2012). This validated 11 point scale, scores intestinal tissues based on enterocyte injury, epithelial hyperplasia, lamina propria lymphocytes, lamina propria neutrophils and peri-intestinal inflammation. Masson's trichrome stains were scored from 0-2 for collagen deposition(A. M. B. MD et al., 2012).

16s rRNA qPCR

Genomic DNA was extracted using FastDNA® Spin kit (MP Biomedicals) following manufacturers protocol, and quantified on a ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific). Samples were diluted to 50ng/ml then re-quantified with PicoGreen® (Invitrogen). Quantitative PCR (qPCR) reactions contained 8 µl H_2O, 10 µl

Fast SYBR Green Master Mix, 1 µl 10µM forward and reverse primers and 2 µl target DNA. Sequence of PCR conditions was 5 min at 50 °C, 5 min at 95 °C, (15 sec at 95°C, 1 min at 60°)(40 cycles), followed by a melting curve step progressing from 60 °C to 95 °C over 12 min. qPCR was performed in MicroAmp 96 well optical plates with the 7900HT instrument. Results were analyzed with SDS 2.3 software. Non-specific amplification was determined using melting curves and visualizing products on a QIAxcel (Qiagen. All qPCR reagents and materials were obtained from Applied Biosystems unless otherwise stated. Supplementary Table 1 provides details of primers(Bartosch, Fite, Macfarlane, & McMurdo, 2004; Lee, Zo, & Kim, 1996; Matsuki et al., 2002; Rinttila, Kassinen, Malinen, Krogius, & Palva, 2004; Walter et al., 2001). Target DNA copy number was determined by comparison to standard curves constructed from purified PCR product obtained using the primers in Table 1 and quantified using the PicoGreen® assay (Invitrogen) to amplify genomic DNA from stool in standard PCR reactions. Gene copy number per nanogram of genomic DNA was then determined based on the starting concentration of genomic DNA in each reaction.

Fluorescent in situ hybridization (FISH)

Tissue sections were de-paraffinized in xylene for 15 min. Slides were washed in 100% ethanol for 15 minutes and dried at 50 °C for 25 minutes. FISH staining was performed using Cy3 labeled EUB 388 probe which specifically binds the 16s rRNA gene for the domain bacteria(Mylonaki, Rayment, Rampton, Hudspith, & Brostoff, 2005). A non-specific probe was used for negative controls. DNA probes were diluted to 5 ng/ul in hybridization buffer (0.9 M NaCl, 0.02 M Tris-HCl, 5% formamide, and 0.05% sodium dodecyl sulfate). Hybridization occurred in a humidified chamber at 50 °C for 90

minutes. Slides were rinsed in H_2O and placed in wash buffer (0.636 M NaCl, 0.02 M Tris-HCL, 0.006% sodium dodecyl sulfate) at 50 °C for 10 minutes. Slides were rinsed in H_2O, counter-stained with DAPI, and mounted with FluoroSave mounting media (Calbiochem).

Characterization of lamina propria cells.

Lamina propria cells were isolated by washing intestinal tissues in Hank's balanced salt solution with 5% fetal bovine serum (HBSS/FBS) plus 2 mM EDTA at 37° C for 20 min to remove epithelial cells. Tissues were minced and digested in HBSS/FBS plus 1.5 mg/ml collagenase type VIII (Sigma) and 40 μg/ml DNase I (Invitrogen) for 20 min. MP populations were enriched using CD11b/CD11c microbeads and the LS MACS column following manufacturers protocols (Miltenyi Biotec). Cells were stained with the LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen), washed and incubated for 20 min with antibody cocktail containing PerCP-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD103, APC-conjugated anti-mouse CD11c, AlexaFluor 700-conjugated anti-mouse I-Ab, eFluor 450-conjugated anti-mouse CD11b, and PE-Cy7-conjugated anti-mouse F4/80 in PBS with 5% fetal bovine serum(Geem, Medina-Contreras, Kim, Huang, & Denning, 2012). Cells were fixed in 2% paraformaldehyde in PBS and analyzed on a BD Biosciences FACS Canto II flow cytometer.

Tissue cytokines

Snap frozen peri-anastomotic colon and ileum were homogenized in PBS containing 0.05% Tween 20. Homogenates were centrifuged at 10 000 rpm for 10 min. Supernatant was used for measurement of cytokines after correction for dry tissue weight using the

Proinflammatory Panel 1 V-PLEX mouse kit (Meso Scale Discovery) as per manufacturers protocol. TSLP was measured using an ELISA duo set (R&D Systems).

Metagenomic Analysis

Stools were collected the day prior to ICR, post-operative d13 (prior to FMT) and post-operative d27. DNA was extracted using the FastDNA® Spin kit for feces (MP Biomedicals). Libraries were constructed using one ng of stool DNA and the Nextera XT (Illumina) protocol. The Nextera XT transposome was used to fragment DNA and to incorporate sequencing adapters into the fragments termini, followed by a 12-cycle PCR indexing reaction. Primer dimers and low-molecular-weight PCR products were removed using half a volume of Agencourt AMPure XP paramagnetic beads (Beckman Coulter). Libraries were quantified using the Agilent high sensitivity DNA kit and sequenced in a MiSeq using a paired-end 300 cycles protocol. Libraries with less than 50,000 reads were eliminated. Sequences were aligned to the NCBI non-redundant nucleotide database (nt) using the LAST algorithm(Wan, Sato, Horton, & Frith, 2011), which implements an adaptive seed approach. LAST was run with default parameters. Metagenomics classification was performed using the Meta Genome Analyzer, MEGAN5(Huson & Weber, 2012), with the following parameters: maxMatches = 100, minScore = 50.0, maxExpected = 0.01, topPercent = 10.0, minSupport = 50, minComplexity = 0.44. Paired-end analysis was enabled. For inference of the coding capacity of the bacteriome, reads classified as bacteria were mapped to the last version of the KEGG pathway database (June 2011). Principal coordinates analyses (PCoA) of taxonomic or functional data was also performed using MEGAN5 and the KEGG database.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed using Graphpad Prism version 5.04 and means compared for significant differences using Student's one-way ANOVA with Tukey–Kramer post hoc test. Results from metagenomic analysis were evaluated for significance using the LEfSe tool with p-values set at 0.05, an LDA cutoff score of 3.0 and all-against-all class comparison(Segata et al., 2011).

5.3 Results

Histological Analyses

The non-operative control group (CT) developed colitis, but not ileitis. Colitis was characterized by enterocyte injury, epithelial hyperplasia, and immune cell infiltration (Fig 1B). All mice that underwent ICR (Sham and FMT) developed fibrosis (data not shown) and histologic inflammation ($p < 0.005$) in the terminal ileum (Fig 1A). Those mice that received FMT following ICR had decreased ileitis ($p < 0.05$) as compared with the group that received a sham transplant (Sham) (Fig 1A). This was associated with reductions in enterocyte injury and neutrophilic infiltration. Colonic injury was not improved in the group receiving FMT, but was reduced in the group that received sham transplant (Fig 1B).

Mucosal-associated bacteria

To examine if the histological injury patterns were associated with changes in mucosal-associated bacteria, qPCR was used to quantify specific bacterial groups in the terminal ileum and colon. The Sham group had decreased colonic (Fig 2B) and increased ileal bacterial loads (Fig 3B), which corresponded to histologic injury. Both ICR groups had reduced colonic *Clostridium* cluster IV and *Bacteroides-Prevotella-Porphyromonas*

(BPP); Sham animals had increased colonic *Lactobacillus-Pediococcus-Leuconostoc* (LPL), while FMT animals had increased *Enterobacteriaceae* (Fig 2C). Compositional perturbations in the ileum were less dramatic as BPP organisms remained dominant across all groups (Fig 3C). *Clostridium* cluster IV was decreased in both ICR groups and LPL was elevated in Shams. Bacteria were visualized in the ileal lamina propria (LP) of the sham group (Fig 3A), which correlated with increased bacterial load and injury. LP bacteria were not seen in the FMT group or the control group. In the colon, the FMT group, but not the sham group had increased bacteria in the LP (Fig 3A).

Analyses of mononuclear phagocytes

MP subsets in the ileal lamina propria were defined as $CD45^+MHC\text{-}II^+$ cells that are $CD11b^-CD11c^+$ (R1), $CD11b^+CD11c^+$ (R2), and $CD11b^+CD11c^-$ (R3). These subsets were further defined based on expression of the dendritic cell (DC) marker CD103, and the macrophage (MΦ) marker F4/80(Denning et al., 2011; Medina-Contreras et al., 2011; Uematsu et al., 2008). Gating strategy is presented in Fig 4A. Following ICR, Sham animals had significant perturbations in ileal MP subsets (Fig 4B). The $CD11b^-CD11c^+$ population, which typically consists of $CD103^+$ DCs(Denning et al., 2011) had an increased proportion of $CD103^-$ cells. The $CD11b^+CD11c^+$ population consists of both Mϕ and DCs(Denning et al., 2011). The Sham group had decreased $F4/80^+$ MΦ and increased $F4/80^-CD103^-$ cells. MP subsets in the ileum of the FMT group were similar to non-operative controls. After gating for $CD45^+MHC\text{-}II^+$ cells, colonic MP subsets were determined by defining DCs as $CD11c^+CD103^+$ and MΦ as $CD11b^+F4/80^+$ (Fig 5A/B)(Denning et al., 2011). FMT led to an increase in $CD103^+$ DCs in the colon and a shift towards $CD11b^+CD103^+$ DCs (Fig 5A). Consistent with decreased colonic injury,

the sham group had a higher proportion of $CD11b^{-}CD103^{+}$ DCs, which are primarily defined by their pro-regulatory properties(Denning et al., 2011; C. L. Scott et al., 2011). $F4/80^{+}$ MΦs were increased in both ICR groups with a shift to the $CD11c^{-}F4/80^{+}$ subset in the FMT group (Figure 5B).

Tissue cytokine levels

All mice that underwent surgery had increased tissue levels of IL-1β, IL-5, and IFNγ in the ileum while the FMT group had a further increase in TNFα and CXCL1 (Fig 4C). The FMT group also showed a reduction in TSLP (thymic stromal lymphopoeitin). In the colon, the sham group had reduced levels of IL-1β, IL-6, TNF-α, and CXCL1 relative to non-operative controls. Donor stool transplant in the FMT group restored cytokines to the level of non-operative controls and led to further increases in TNFα, IFN-γ, and IL-2.

ICR and FMT shifted the gut microbiome.

Whole metagenome sequencing was performed on stools collected prior to surgery and transplant and at sacrifice for longitudinal analysis on taxonomic and functional genomic shifts associated with surgery and fecal transplants. At baseline, microbiota were similar across all groups (Supplementary Figure 2). Bacteroidetes and Firmicutes represented the major phyla with minor contributions from Proteobacteria and Actinobacteria (Supplementary Figure 2A). Substantial microbial diversity was seen at the genus level (Supplementary Figure 2B) and this was further supported with rarefaction analysis with 120-200 leaves detected in taxonomic analysis (Supplementary Figure 2D). Following surgery, there was a significant loss of microbial diversity in the ICR groups with predominance of Firmicutes and expansion of Proteobacteria (Fig 6A,B). At the genus level *Lactobacillus* dominated following ICR (Fig 6B/ Supplementary Figure 4). Clades

positively associated with ICR included *Streptococcus, Enterococcus, Turicibacter* and *Staphylococcus* (Supplementary Figure 3). Clades decreased with ICR largely consisted of obligate anaerobic species. PCoA of the Bray-Curtis dissimilarity index revealed differential clustering of ICR and non-operative CT animals at d13 (Fig 6C).

Fourteen days following FMT, rarefaction analysis (Figure 7D) revealed persistently decreased diversity in both ICR groups despite substantial diversity in the donor stool that had been transplanted into FMT animals (Figure 7B). The sham transplanted group continued to have increased *Lactobacillus, Enterococcus, Streptococcus and Staphylococcus* relative to control and FMT groups (Fig 7A/B). In the FMT group, the only clade elevated was *Klebsiella* (Supplementary Fig 5), which uniformly expanded in all animals following donor transplantation despite low relative abundance in donor stool (Fig 6B). PCoA at d27 demonstrated tight clustering of non-operative CT animals with wider divergence seen in the sham transplant and FMT groups (Fig 7C). An additional LEfSe analysis was performed to compare the effect of FMT in the ICR animals alone by only considering sham and FMT groups at d27 (Supplementary Fig 4). In addition to *Klebsiella,* this analysis revealed increases in *Bacteroides, Alistipes, and Parabacteroides* in FMT animals. *Klebsiella* and *Bacteroides* accounted for the majority of the relative abundance (Fig 7B).

Functional metagenomic changes

To determine the attributes of specific microbes that may have conferred a survival benefit following surgery, functional annotation was applied to the metagenomic sequences. Orthologous genes were grouped into broad functional categories and metabolic pathways based on the BRITE hierarchy. Following surgery, there was an

increased gene abundance of the glutathione metabolic pathway and thioredoxin reductase enzymes (Fig 6F), both of which are important for protection against oxidative damage(Arnér & Holmgren, 2000). There was no change in other enzymes specific to oxidative stress such as catalase, superoxide dismutase, and peroxidase (Hassett & Cohen, 1989)(data not shown). ABC transporters and the phosphotransferase system both demonstrated increased relative abundance at day 13, which may be related to bile acid resistance(Fang et al., 2009). Significant shifts in the metabolic potential of the metagenome were found following surgery suggestive of a highly auxotrophic and resistant population. Carbohydrate metabolism, lipid metabolism, membrane transport, and xenobiotic degradation were all increased following ICR (Figure 6E), but pathways for the biosynthesis of fatty acids, unsaturated fatty acids and steroid hormones were decreased (Supplementary Table 2). Moreover, despite increased butanoate metabolism, the final genes for the two butyrate synthetic pathways, acetate CoA-transferase, and butyrate kinase were undetectable following ICR (Supplementary Fig 5A) suggesting a loss of butyrate production. Loss of biosynthetic capacity in the post-ICR metagenome was also reflected by decreases in amino acid and vitamin metabolic categories (Fig 6E).

Effects of FMT: Metagenomic analysis of the sham group suggested a simple metagenome with lipid metabolism and membrane transport increased relative to the other groups (Figure 7E). Several functional pathways were increased as well, mostly within carbohydrate metabolism (Supplementary Table 2). In the FMT group numerous functional perturbations associated with the restoration of colonic injury were seen, including increased carbohydrate metabolism, xenobiotics degradation and metabolism, metabolism of other amino acids and signal transduction (Figure 7E). Mice that received

FMT showed decreased butanoate metabolism, and increased glutathione metabolism, sulfur metabolism, benzoate degradation, and two component signaling (Figure 7F). Additionally, enzymes specific to aerotolerance were increased in FMT animals (Supplementary Figure 5B). Genes for LPS biosynthesis and glycosaminoglycan degradation were elevated in FMT animals and may have contributed to the increased colonic injury (Figure 7F).

5.4 Discussion

In this study, surgical resection resulted in decreased colonic but increased ileal inflammation. Bacterial overgrowth in the neo-terminal ileum and increased bacterial invasion into the lamina propria was associated with ileal injury while decreased mucosal-associated bacteria was associated with decreased colonic injury. Impaired microbial clearance in the ileum correlated with altered antigen-presenting cell subsets. FMT following surgery failed to restore microbial diversity and allowed expansion of γ-proteobacteria and restoration of colitis. However, FMT improved ileal inflammation, reduced mucosal-associated bacterial loads, and restored MP cell subsets to similar profiles as seen in non-operative control animals.

In IL-10$^{-/-}$ mice, the ileum generally does not show any significant degree of inflammation, but ileitis can be induced following ileo-colonic resection(Rigby et al., 2009). During surgery, the bowel is exposed to atmospheric oxygen for a period of time, and it follows that microbes able to cope with oxidative stress would have a survival advantage. In this study, qPCR demonstrated that ileal mucosal associated bacteria both increased in numbers and showed compositional changes following surgery. In particular, there was an increase in the aerotolerant Lactobacilli group along with a decrease in

anaerobic Clostridium cluster IV. Clostridium cluster IV is a major butyrate producer and depletion of butyrate-producing bacteria in IBD microbiota is clearly evidenced by reduction in butyrate-producing metabolic pathways(Morgan et al., 2012) as well as levels of fecal butyrate(Marchesi et al., 2007). Butyrate plays multiple functions in the intestine as it is a major energy source for enterocytes and lack of butyrate can lead to energy deprivation, reduced barrier function, and increased bacterial translocation(B. P. Willing et al., 2010). Overall, the microbiome in CD demonstrates a loss of numerous processes important for gut health including short chain fatty acid (SCFA), vitamin, and amino acid biosynthesis, along with increased aerotolerance, nutrient import and toxin secretion(Kostic et al., 2014).

Resection in the sham group led to significant shifts in ileal lamina propria MP population. The observed reduction in MΦs was likely responsible for the increased for the increased ileal neutrophils and bacterial invasion, as these cells are essential for clearance of invading gut microbes(Medina-Contreras et al., 2011) and also function to phagocytose neutrophils as part of the healing response(Lech, Gröbmayr, Weidenbusch, & Anders, 2012). The uniform increase in cells not expressing CD103 or F4/80 in all three CD11b/CD11c subsets is intriguing, and may represent a larger relative abundance of immature monocyte populations. While further characterization is required, it is likely that a proportion of these cells are acting as myeloid derived suppressor cells (MDSCs)(Gabrilovich & Nagaraj, 2009; Ostanin & Bhattacharya, 2013). MDSCs, defined by the CD11b and Gr1 markers, are known to have potent immunosuppressive properties, which are enhanced by MHC-II expression(Ostanin & Bhattacharya, 2013). Furthermore they are known to increase following surgical or traumatic stress(Zhu,

Herrera, & Ochoa, 2010). FMT restored all MP subsets in the lamina propria to reflect controls. This was associated with a decreased bacterial load, absence of bacteria within the lamina propria, and decreased enterocyte injury and neutrophil infiltration. It is possible that the increased antigenic stimulation from the FMT was responsible for this restorative effect. Although the cytokine profiles in tissue homogenates reflected a pro-inflammatory state in both surgery groups, these profiles reflect a combination of epithelial, immune, and fibroblast cells, and are likely to represent a state of low-grade inflammation due to injury and bacterial overgrowth in the sham transplant mice, and immune activation and microbial clearance in the fecal transplant group. Indeed, the increased TNFα and IFNγ levels in the fecal transplant group suggest a Th1/M1 response and reversal of the potential immunosuppressive effects(Gabrilovich & Nagaraj, 2009). Concurrently with the induction of ileitis, surgery had the unexpected effect of suppressing colitis and inflammatory cytokines in the sham group. The underlying mechanism of this effect likely involves a combination of surgical-induced immune suppression together with a surgical-induced alteration in gut microbial composition. During healing responses it is expected that MP populations will shift towards an alternatively activated immunosuppressive phenotype(Novak & Koh, 2013). We demonstrated that $CD11c^{+}CD103^{+}CD11b^{-}$ DCs were increased in the colonic lamina propria; these cells have well established regulatory activities(C. L. Scott et al., 2011). It is possible that oxidative stress related to surgery resulted in the selection of *Lactobacillus, Enterococcus and Streptococcus* that provided a low complexity community in the colon that contributed to the suppression of inflammation. Surprisingly the decreased colitis occurred even with the loss of butyrate producers from the

Clostridium cluster IV group and butyrate producing enzymes. Relative to controls, functional characteristics with known associations to inflammation in the gut metagenome such as flagellar assembly were also reduced(Rooks et al., 2014).

Expansion of *Lactobacillus* species has been previously recognized following surgical manipulation and intestinal loss in humans(Hartman et al., 2009; Joly et al., 2010). Studies evaluating *Lactobacillus* as a probiotic demonstrate its anti-inflammatory effects, mucin stimulation, and bacteriocin production(Messaoudi et al., 2013). Some of these effects may account for the decreased bacterial load in the colonic mucosa at day 28. Once established in combination with suppressed immune responses a positive feedback loop might be formed in the colon to promote homeostasis. However, as demonstrated in our study, this balance may be finite as low complexity communities in the gut are susceptible to colonization with pathogens or pathobionts(Stecher et al., 2010).

In our study, colitis was increased following FMT. This was associated with an expansion of both *Klebsiella* and *Bacteroides,* which are known to cause colitis in $IL10^{-/-}$ mice due to the mucolytic capacity of these bacteria(Bloom et al., 2011; Högenauer et al., 2006; Rooks et al., 2014). Results from the functional metagenome analysis support this, as demonstrated by increased capacity for glycoshingolipid degradation in the fecal transplant group. Increased capacity to survive oxidative stress may form an alternative positive feedback loop selecting for pathobionts like *Klebsiella* with colitogenic properties. However, the immune response is likely also involved in the colonic injury. An antigenic load provided by the fecal transplant may have shifted the immune response from immunosuppressive to a recruitment of pro-inflammatory monocyte subsets including $CD11c^{+}CD11b^{+}CD103^{+}$ DCs, thus restoring inflammatory cytokine

signaling(Denning et al., 2011; Persson et al., 2013; Uematsu et al., 2008). The increased inflammatory cytokines in combination with mucolytic bacteria could have the additive effect of allowing for increased bacterial translocation, which we observed in the colon of FMT mice.

The increased bacterial load in the neo-terminal ileum seen in our model is similar to that seen in humans following resection(Neut et al., 2002). Results from this study may help explain the efficacy of early post-operative antibiotics in preventing post-operative recurrence(Rutgeerts et al., 2005) as the ileal segment is unable to appropriately deal with invading microbes in the post-operative period. Furthermore the induction of a gut microbiome with low complexity due to oxidative stress could facilitate the overgrowth of pathogens/pathobionts, which may eventually infect the vulnerable neo-terminal ileum providing an explanation for the occurrence of invasive E. coli species in post-operative CD lesions(Darfeuille-Michaud et al., 2004).

In conclusion, we propose a new hypothesis to describe the pathophysiology of early recurrent lesions in the ileum whereby surgery causes a loss of microbial diversity and induction of aerotolerant organisms that together with impaired microbial clearance due to immunosuppression, results in bacterial overgrowth and increased translocation in the neo-terminal ileum. If these findings are translatable to humans, ileocecal resection could be likened to a forest fire, where an ecological niche is burnt to the ground allowing a new gut ecosystem to form. The nature of this restoration may be open to manipulation to prevent the recurrence of gut inflammation with directed immunomodulation, antibiotics, probiotics and prebiotics.

5.5 Figures

Figure 5.1. Photomicrographs and histologic injury scores. Tissue sections of terminal ileum and colon at 10x magnification for non-operative control animals (CT), sham microbial transplant animals (Sham) and wild type donor microbial transplant animals (FMT). Bar graphs represent mean ± SEM cumulative injury scores and individual components of the score (n=6/group). CT animals had minimal ileal injury (A). Sham animals had increased histological injury characterized by enterocyte injury, infiltrating neutrophils, and peri-intestinal inflammation. FMT animals had increased neutrophil infiltration and per-intestinal inflammation compared with CT, but had decreased enterocyte injury and neutrophil infiltration relative to Sham. In the colon (B), CT animals had elevated injury scores with enterocyte injury and ulceration, neutrophil and lymphocyte infiltration, and epithelial hyperplasia. Lymphocyte and neutrophil infiltration were reduced in the Sham animals relative to both CT and FMT groups. Scale bar represents 100 μm. * $P \leq 0.05$ relative to CT; # $P \leq 0.05$ relative to other ICR group, (LP) lamina propria.

Figure 5.2. Quantification and localization of colonic mucosal-associated bacteria. (A) Photomicrographs of colonic tissues processed by FISH with the EUB 388 probe (orange) and DAPI (blue) at 40x magnification. CT and Sham groups demonstrate bacteria in the lumen while bacteria were found in the lamina propria of the FMT group. (B) qPCR results for total bacteria showed Sham and FMT animals to have decreased colonic bacterial loads (C) qPCR results for specific bacterial groups. Sham and FMT groups had reduced *Clostridium* cluster IV and *Bacteroides-Prevotella-Porphyromonas* (BPP). Sham animals had increased colonic *Lactobacillus-Pediococcus-Leuconostoc*

(LPL). FMT animals had increased *Enterobacteriaceae* All bar charts represents mean ± SEM 16s rRNA gene copy number per nanogram of genomic DNA (n=6/group). (*) $p \leq 0.05$ relative to CT

Figure 5.3. Quantification and localization of ileal mucosal-associated bacteria (A) Photomicrographs of ileal tissues processed by FISH with the EUB 388 probe (orange) and DAPI (blue) at 40x magnification. Sham group demonstrated bacteria in the lamina propria. Bacteria were only found in the bowel lumen in CT and FMT groups. (B) qPCR results for total bacteria showed Sham animals to have increased ileal bacterial load. (C) qPCR results for specific bacterial groups. *Clostridium* cluster IV was decreased in Sham and FMT and LPL was elevated in Sham. All bar charts represents mean ± SEM. 16s rRNA gene copy number per nanogram of genomic DNA (n=6/group). LPL (Lactobacillus-Pediococcus-Leuconostoc), BPP (Bacteroides-Prevotella-Porphyromonus), (*) $p \leq 0.05$ relative to CT.

Figure 5.4. Characterization of mononuclear phagocyte populations in the ileal lamina propria and total tissue cytokines. (A) FACS plots demonstrating gating strategy for ileal populations. Cells were positively selected for CD11b/CD11c expression using a magnetic column then pregated for live cells using a vital dye. (B) Populations R1, R2 and R3 were evaluated for expression of CD103 and F4/80. Bar charts represent mean +/- SEM proportion of cells expressing F4/80 or CD103 from the three subsets. Sham animals had an increased proportion of CD103- cells in the $CD11b^{-}$ $CD11c^{+}$ population, a decrease in $F4/80^{+}$ MΦ, and an increase in $F4/80^{-}CD103^{-}$ cells (B).

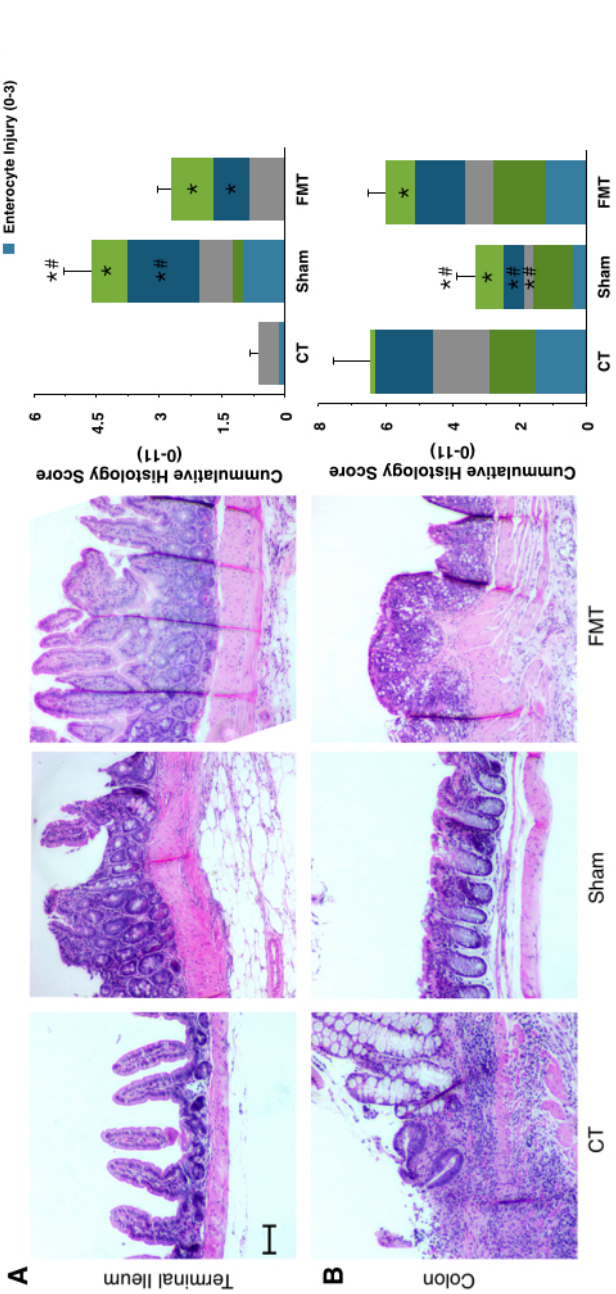
Data is representative of six independent experiments. (C) Tissue cytokines (Mean +/- SEM) from the terminal ileum corrected for tissue dry weight. Sham and FMT (n=7) animals had increased tissue levels of IL-1β, IL-5, and IFNγ. FMT animals had increased TNFα and CXCL1 and decreased TSLP. * P≤ 0.05 relative to CT, # P≤ 0.05 relative to other ICR group.

Figure 5.5. Colonic mononuclear phagocyte populations and tissue cytokines. (A) FACS plots demonstrate cells populations pre-gated for live $CD45^+$ $MHC\text{-}II^+$ cells. Results show mean +/- SEM represented by six independent experiments. (A) Proportion of antigen presenting cells expressing $CD11c^+CD103^+$ or $CD11c^+CD103^-$. $CD11c^+CD103^+$ dendritic cells were further classified by CD11b expression. (B) Proportion of antigen presenting cells expressing $CD11b^+F4/80^+$. This macrophage population was further characterized by CD11c expression. FMT animals had an increase in $CD103^+$ DCs and a shift towards $CD11b^+CD103^+$ DCs. Sham animals had a higher proportion of $CD11b^-CD103^+$ DCs. $F4/80^+$ MΦs were increased in both ICR groups with a shift to the $CD11c^-F4/80^+$ subset in the FMT group. Tissue cytokines (mean +/- SEM) from peri-anastomotic colon corrected for dry weight. Sham animals had reduced levels of IL-1β, IL-6, TNFα, and CXCL1 relative to CT. Donor stool transplant in the FMT group restored cytokines to the level of non-operative controls and led to further increases in TNFα, IFN-γ, and IL-2 (*) P≤ 0.05 relative to CT, (#) P≤ 0.05 relative to other ICR group.

Figure 5.6. **Metagenomic analysis of effects of surgery**. Relative abundance of major phyla (A) and genera (B) at d13 following surgery. Each bar represents relative abundance from one individual mouse. (C) PCoA plot of the Bray-Curtis index. (n=6/group). (D) Rarefaction analysis at d13. Curves represent mean+/-SD for each experimental group (n=6/group). Ileocolic resection caused loss of diversity with taxonomic and functional shifts in the gut microbiome. (E) Median relative abundance of functional categories in controls (n=6) vs ileocolic resection animals (n=14). (F) Median relative abundance of functional pathways and genes. Significant associations were determined using the LEfSe tool with an LDA cut off score of 3.0 (*). (PTS) Phospho-transferase system.

Figure 5.7. Metagenomic analysis of effects of FMT following surgery. Relative abundance of major phyla (A) and genera (B) at day 27. Each bar represents relative abundance from one individual mouse. (C) PCoA plot of the Bray-Curtis index. (n=6 CT group, n=7 Sham and FMT). (D) Rarefaction analysis at day 27. Curves represent mean+/-SD for each experimental group. FMT did not restore diversity but did cause taxonomic and functional shifts. (E) Median relative abundance of functional categories. (F) Median relative abundance of functional pathways. Significant associations were determined using the LEfSe tool with an LDA cut off score of 3.0 (*).

Figure 5.1.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Enterocyte Injury (0-3)
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*#

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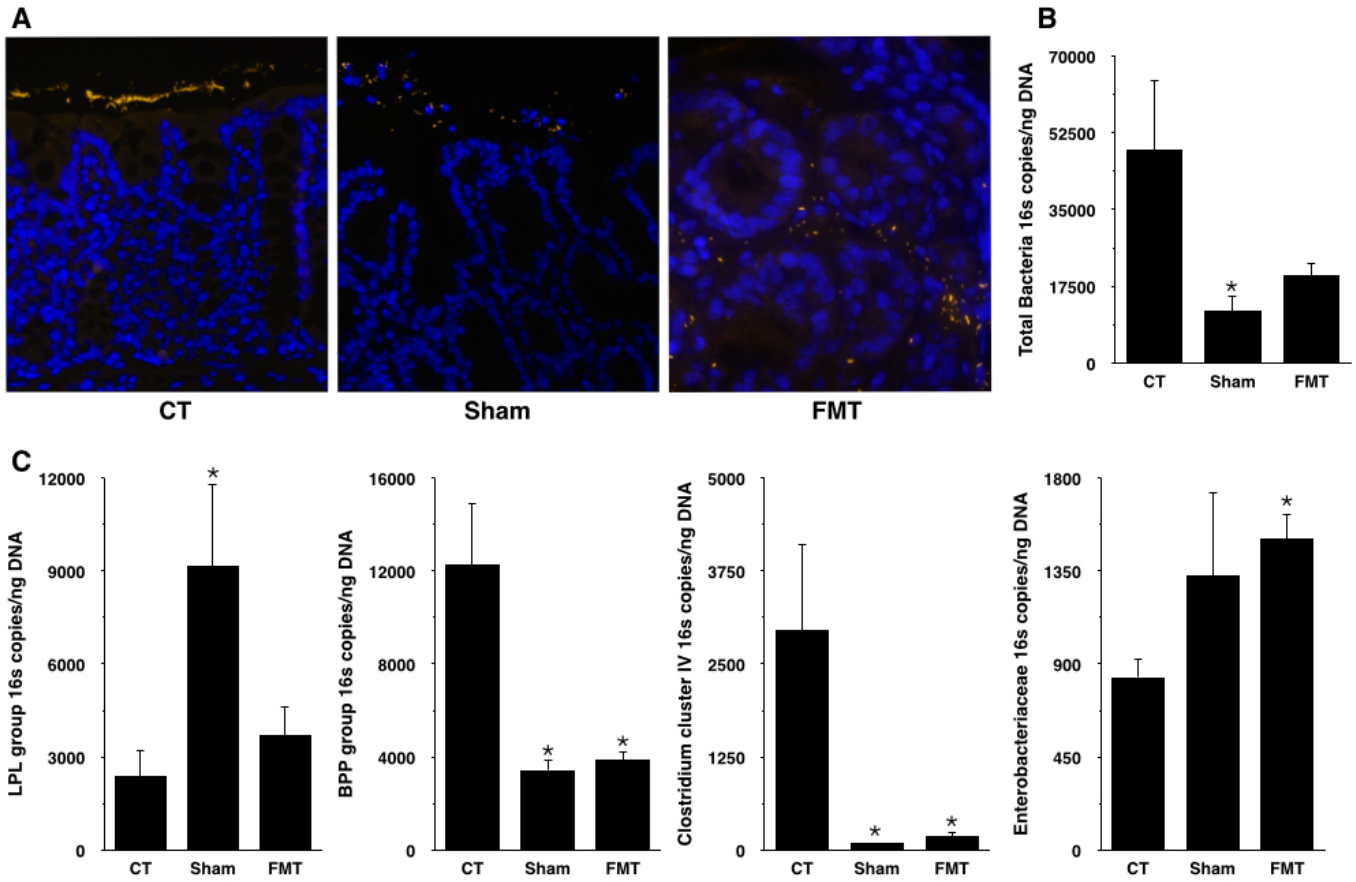
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0
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12000
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4000
0
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Figure 5.3.

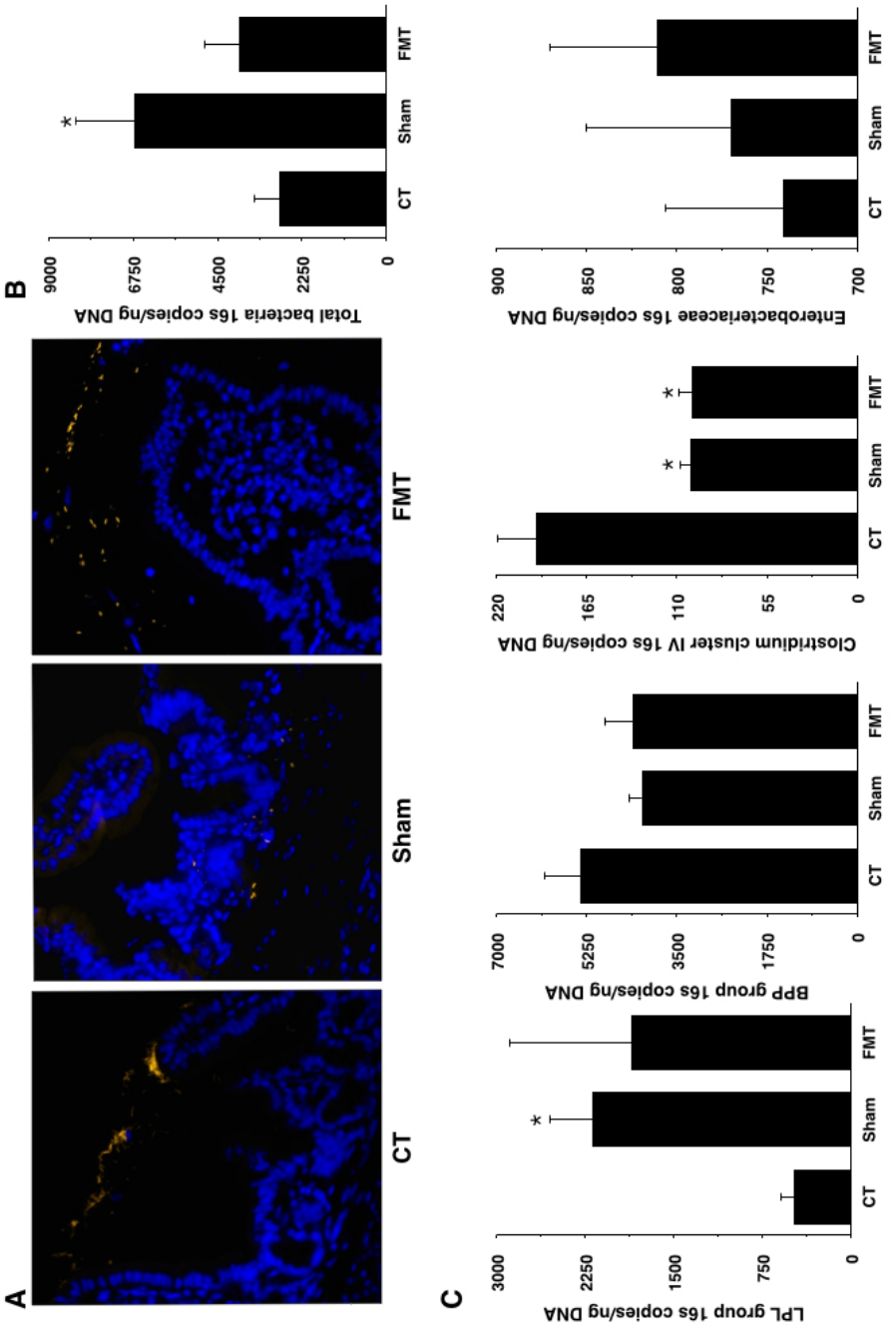
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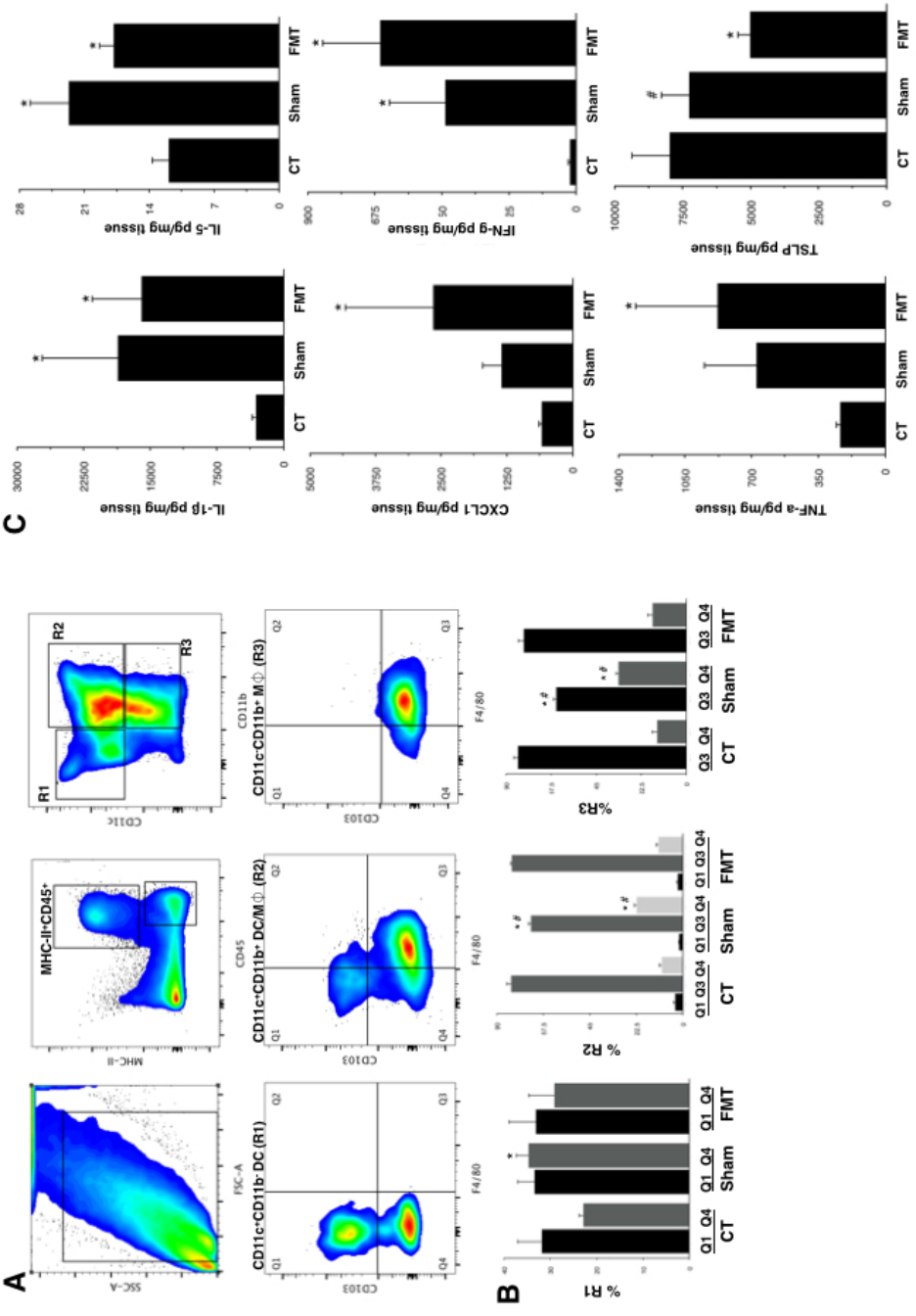
Figure 5.4.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MHC-II
CD11c
CD45
CD103
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CD11b
R1
R2
R3
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CD11c+CD11b- DC (R1)
CD11c+CD11b+ DC/MΦ (R2)
CD11c-CD11b+ MΦ (R3)
B
% R1
% R2
%R3
Q1 Q4
Q1 Q3 Q4
Q3 Q4
CT
Sham
FMT
C
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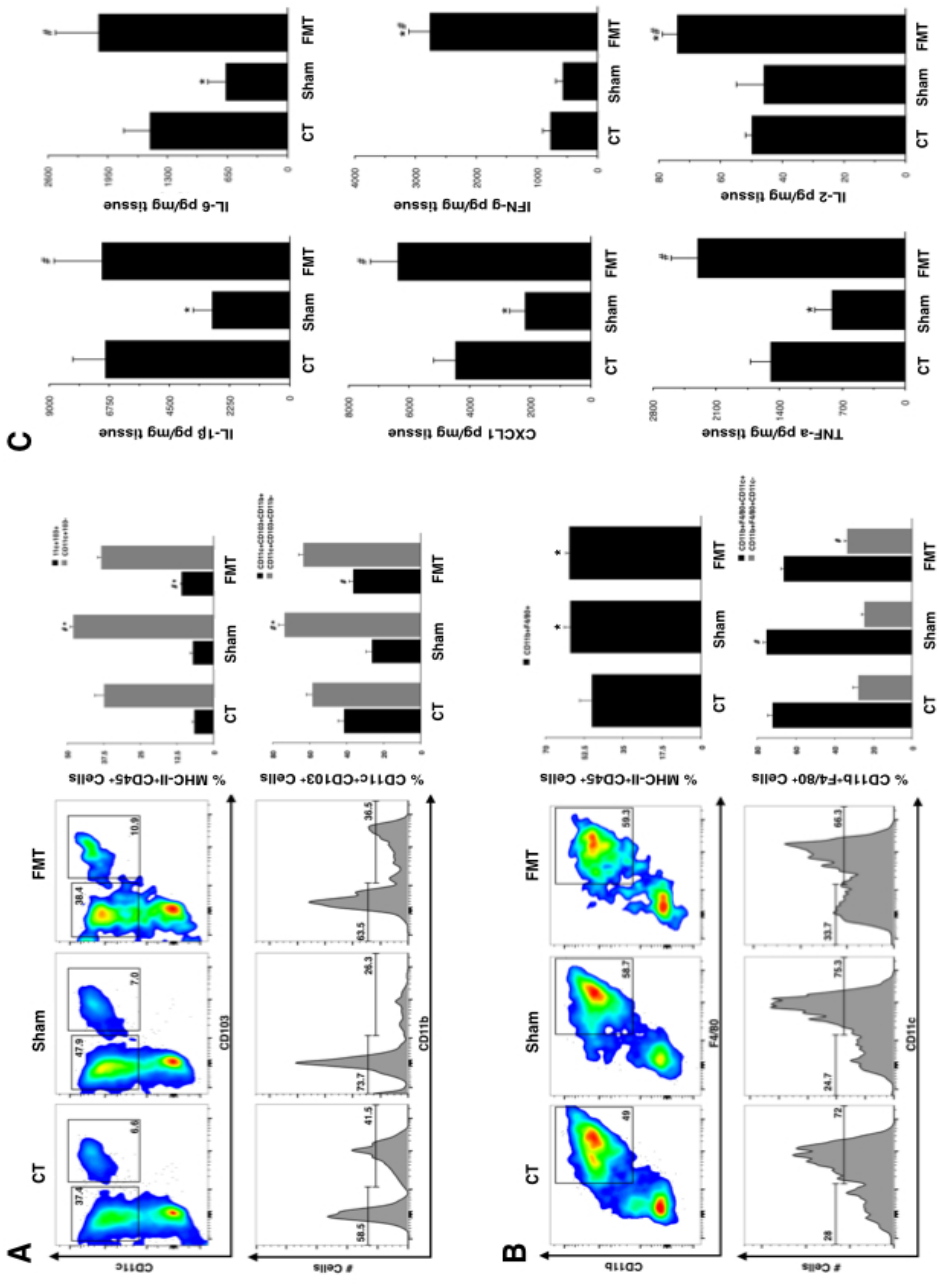
IL-5 pg/mg tissue
IFN-g pg/mg tissue
TSLP pg/mg tissue

Figure 5.5.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CT
Sham
FMT
CD11c
CD103
Cells
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% CD11c+CD103+ Cells
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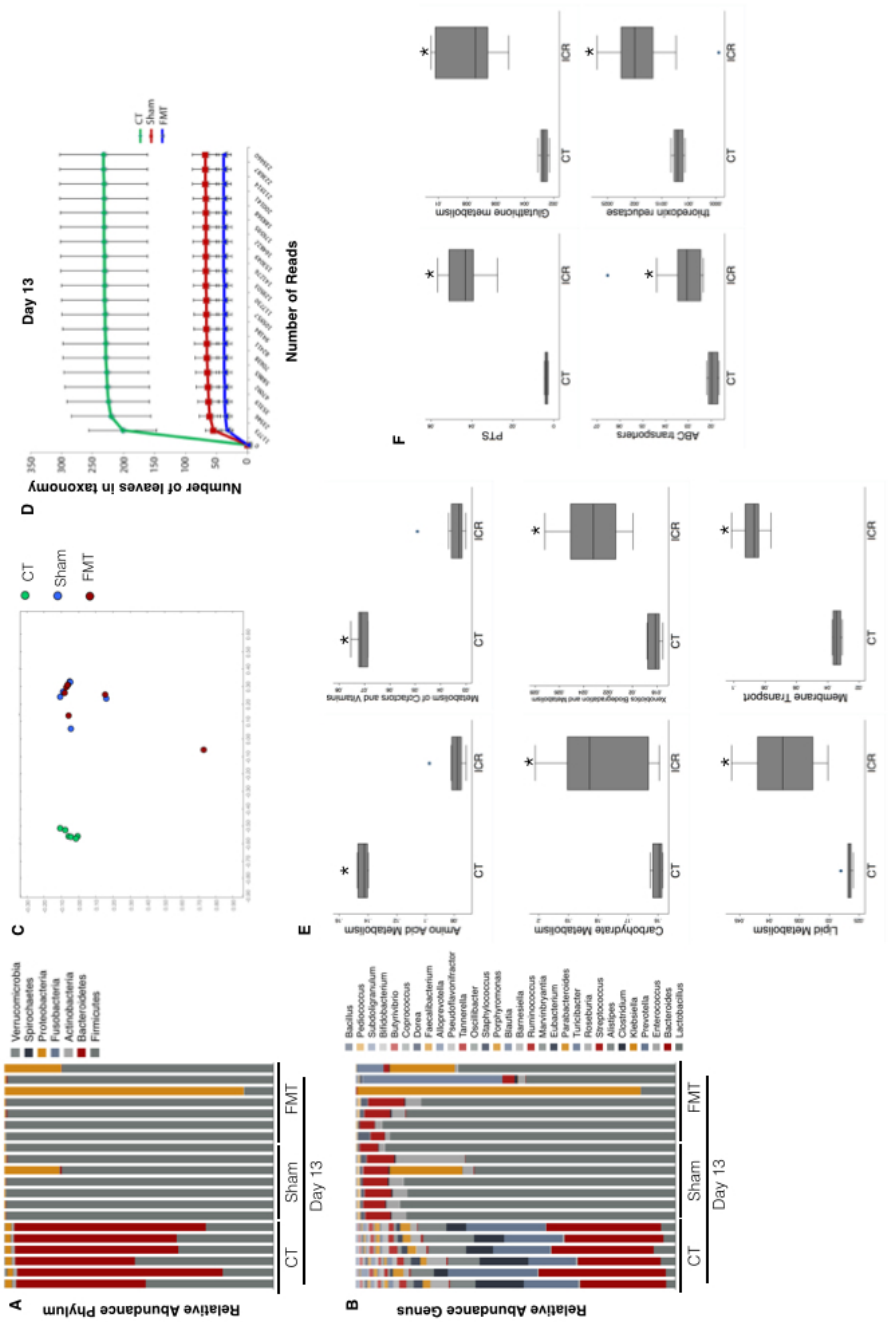
CD11c
% MHC-II+CD45+ Cells
% CD11b+F4/80+ Cells
C
IL-1β pg/mg tissue
CXCL1 pg/mg tissue
TNF-a pg/mg tissue
IL-6 pg/mg tissue
IFN-g pg/mg tissue
IL-2 pg/mg tissue

Figure 5.6.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Spirochaetes
Proteobacteria
Fusobacteria
Actinobacteria
Bacteroidetes
Firmicutes
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Sham
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Bacillus
Pediococcus
Subdoligranulum
Bifidobacterium
Butyrivibrio
Coprococcus
Dorea
Faecalibacterium
Alcaligenes
Pseudoflavonifractor
Tannerella
Oscillibacter
Staphylococcus
Porphyromonas
Blautia
Barnesiella
Ruminococcus
Marvinbryantia
Eubacterium
Parabacteroides
Turicibacter
Roseburia
Streptococcus
Alistipes
Clostridium
Klebsiella
Prevotella
Enterococcus
Bacteroides
Lactobacillus
C
CT
Sham
FMT
D
Number of Reads
Day 13
Number of leaves in taxonomy
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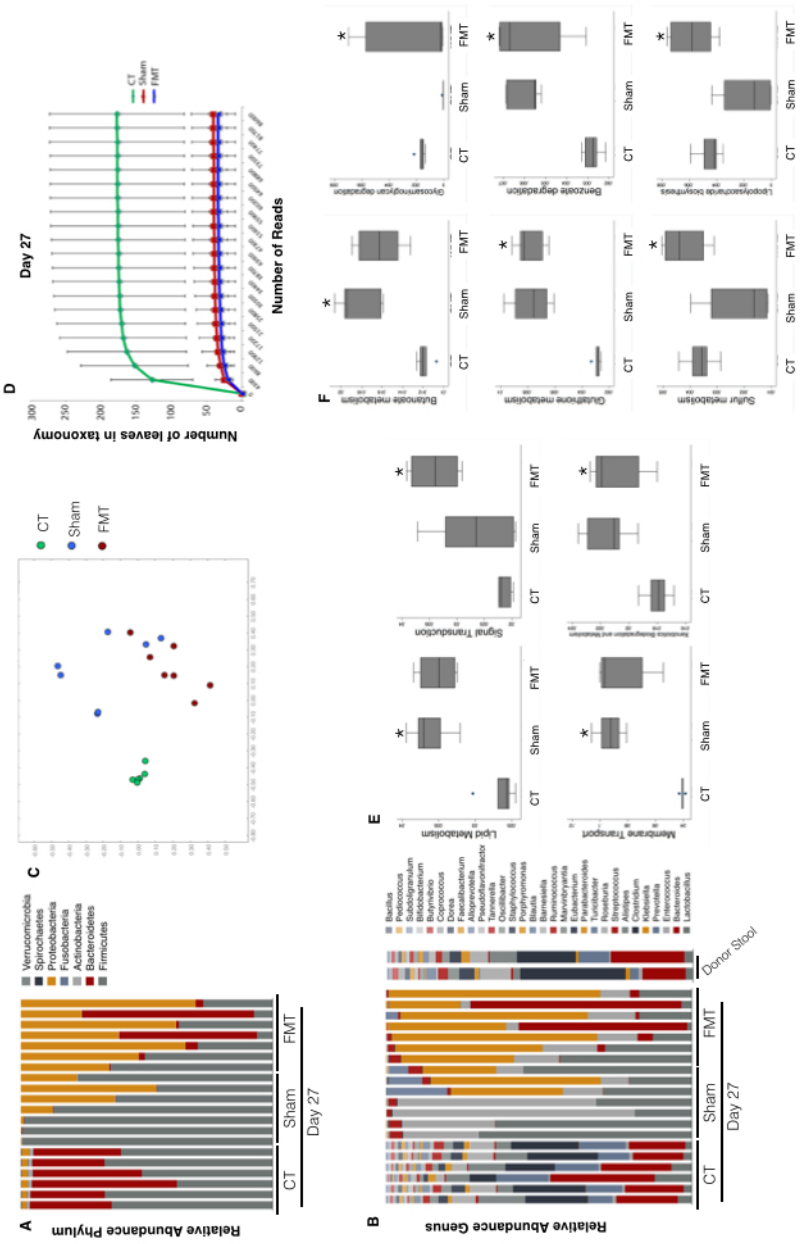
Sham
FMT
E
Amino Acid Metabolism
Carbohydrate Metabolism
Lipid Metabolism
Metabolism of Cofactors and Vitamins
Xenobiotics Biodegradation and Metabolism
Membrane Transport
CT
ICR
F
PTS
ABC transporters
Glutathione metabolism
Thioredoxin reductase
CT
ICR

Figure 5.7.


A
Relative Abundance Phylum
Verrucomicrobia
Spirochaetes
Proteobacteria
Fusobacteria
Actinobacteria
Bacteroidetes
Firmicutes
CT
Sham
FMT
Day 27
B
Relative Abundance Genus
Donor Stool
C
CT
Sham
FMT
D
Day 27
Number of Reads
Number of leaves in taxonomy
E
Lipid Metabolism
Signal Transduction
Membrane Transport
F
Glycosaminoglycan degradation
Benzoate degradation
L-proline biosynthesis
Butanoate metabolism
Carbon fixation metabolism
Sulfur metabolism

5.6 Supplementary Figures and Tables

Supplementary Figure 5.1. Outline of ileocolic resection and segments of intestine taken for analysis. Segments labeled Histology were formalin fixed and paraffin imbedded for H&E injury scoring and FISH analysis. Cytokine segments were homogenized and used for determination of tissue cytokines. Segments labelled DNA were used for DNA extraction and qPCR quantification of bacterial groups.

Supplementary Figure 5.2. Baseline stool Taxonomy. Stool samples were taken from animals prior to the experimental protocol. Metagenomic sequencing was completed. No baseline differences in diversity or bacterial clades were found between groups.

Supplementary Figure 5.3. LDA Scores of Taxonomic Shifts Between Groups. (A) Phylum level comparison between control and ICR animals at post-operative day 13. (B) Genus level comparison between control and ICR animals at post-operative day 13. (C) Genus level comparison across experimental groups on post-operative day 27 following fecal transplant. (D) Genus level comparison between sham transplanted ICR mice and donor transplanted ICR mice. Plots were generated using the LEfSe tool on the Galaxy website with and LDA cutoff score of 3.0

Supplementary Figure 5.4. Word cloud plot and coverage plots for the genus Lactobacillus. (A) Word cloud plot illustrating the taxonomic profile of each animal at day 13. Size of taxa names is proportional to their relative abundance. (B) There was increased abundance in the Sham and FMT groups compared to CT at 13 days post-

surgery. Reads were aligned to the genome of *Lactobacillus johnsonii* (NCC 533; NC_005362.1) using bowtie2 using single-end mode and default parameters. Coverage was derived from alignments.

Supplementary Figure 5.5. Word cloud plots and coverage plots for the genus Klebsiella. (A) Word cloud plot illustrating the taxonomic profile of each animal at day 27. Size of taxa names is proportional to their relative abundance. (B) There was increased abundance in the Sham and FMT groups compared to CT at 27 days post-surgery. Reads were aligned to the genome of *Klebsiella oxytoca* (KCTC 1686; NC_016612.1) using bowtie2 using single-end mode and default parameters. Coverage was derived from alignments.

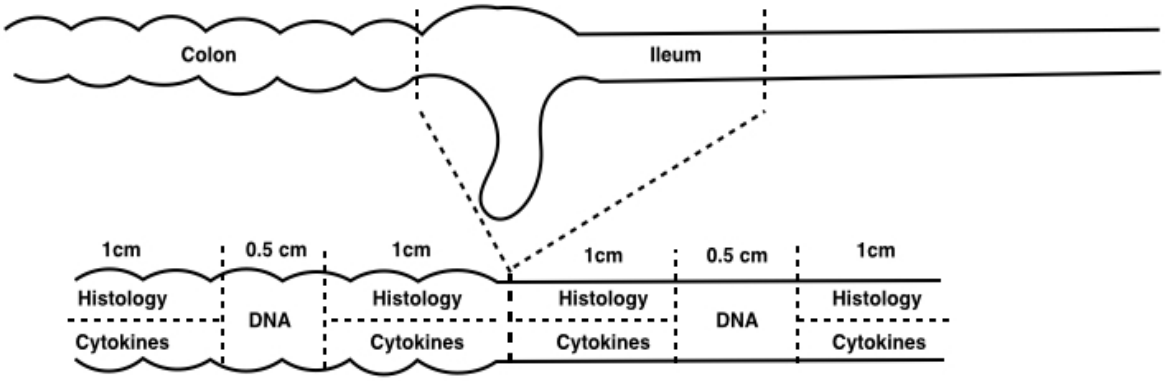
Supplementary Figure 5.6. **Relative gene abundances for butyrate production and aerotolerance determined by metagenomic analysis**. (A) Relative gene abundance for the final two enzymes in butyrate production pathways on post-operative day 13. (B) Relative gene abundances of enzymes involved in aerotolerance across groups on post-operative day 27. (*) indicates significant association with an LDA cutoff score of 3.0.

Supplementary Table 5.1. Primer sets used for quantification of mucosal associated bacterial groups.

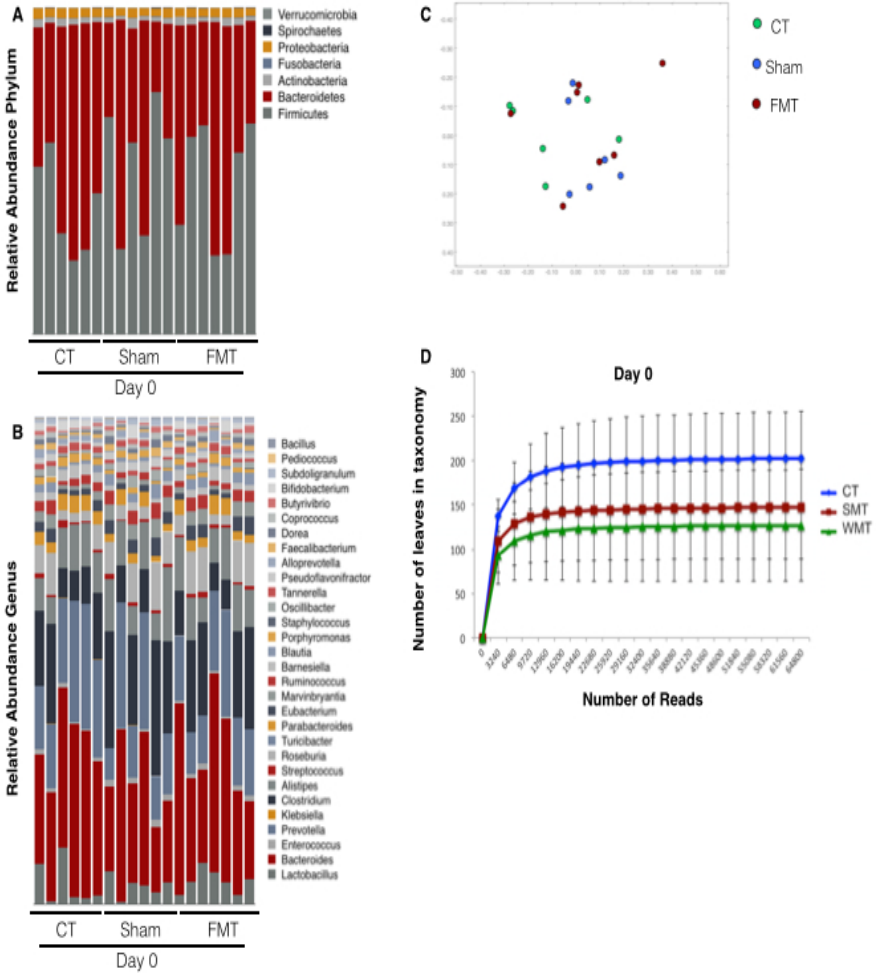
Supplementary Table 5.2. Summary results for functional annotation of metagenomic sequencing. For post-operative day 13 green color indicates increased

relative gene abundance in the ICR group, red indicates increased abundance in controls and white indicates no significant difference. For Post-operative day 27 green indicates increased abundance in the Sham group, blue indicates increased abundance in the FMT group, red indicates increased abundance in the controls and white indicates no significant difference across groups. Significant associations were determined using the LEfSe tool with an LDA cut off score of 3.0. KEGG1 represents first order cellular functions, KEGG2 represents broad functional categories, and KEGG3 represents metabolic pathways.

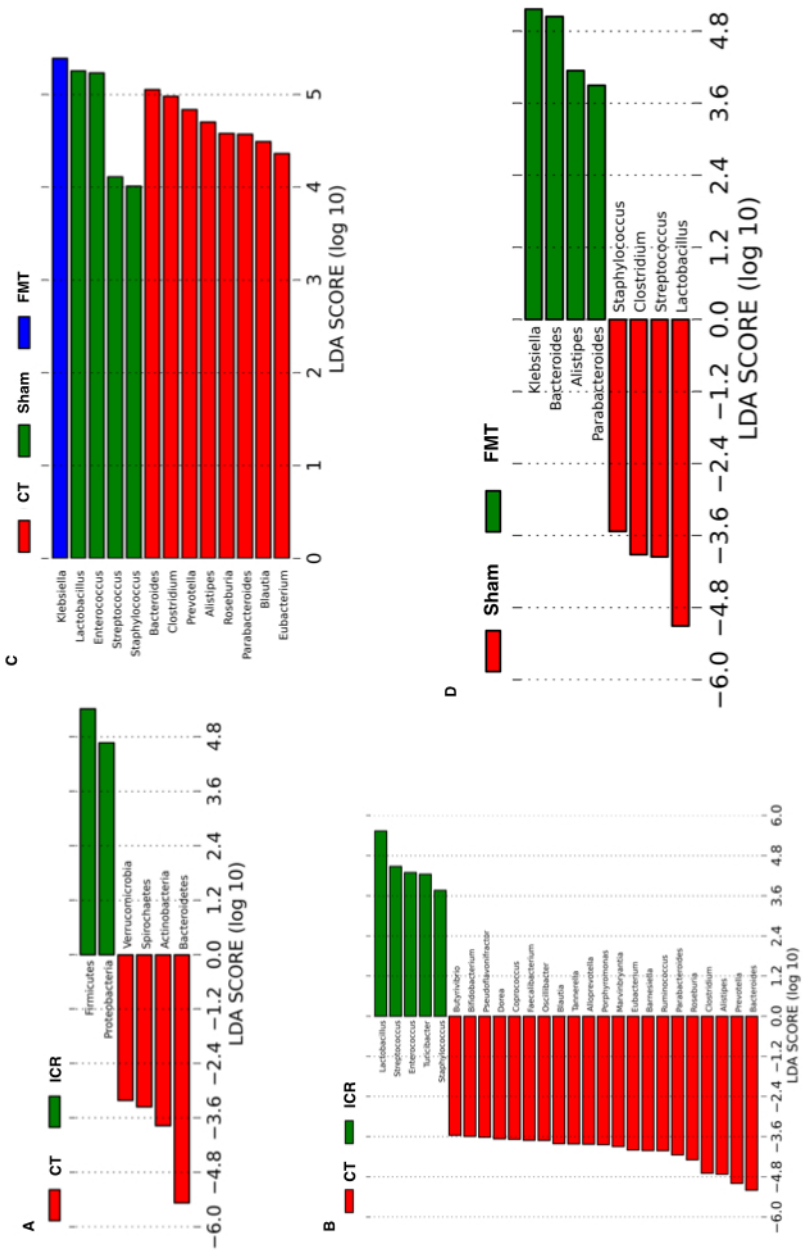
Supplementary Figure 5.1.



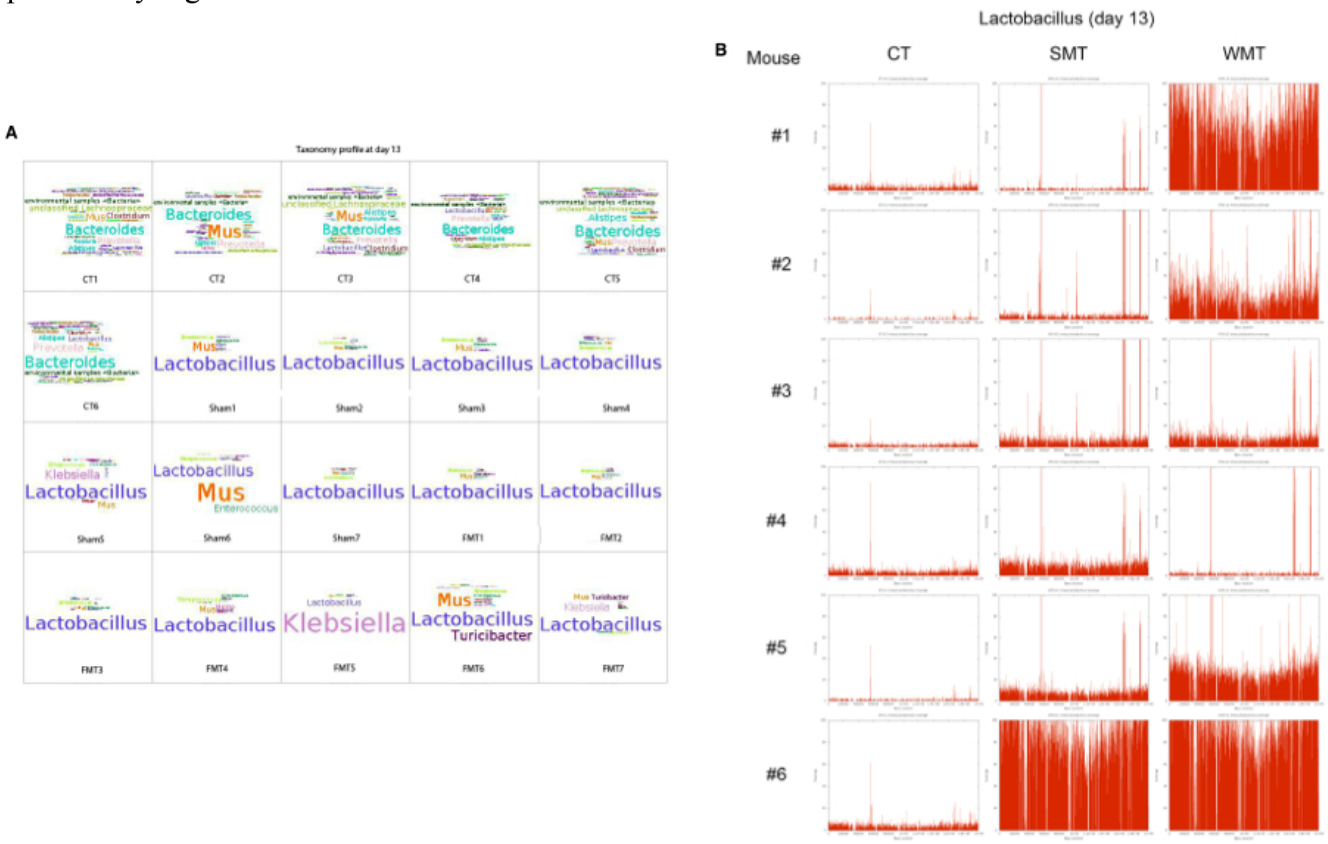
Supplementary Figure 5.2.



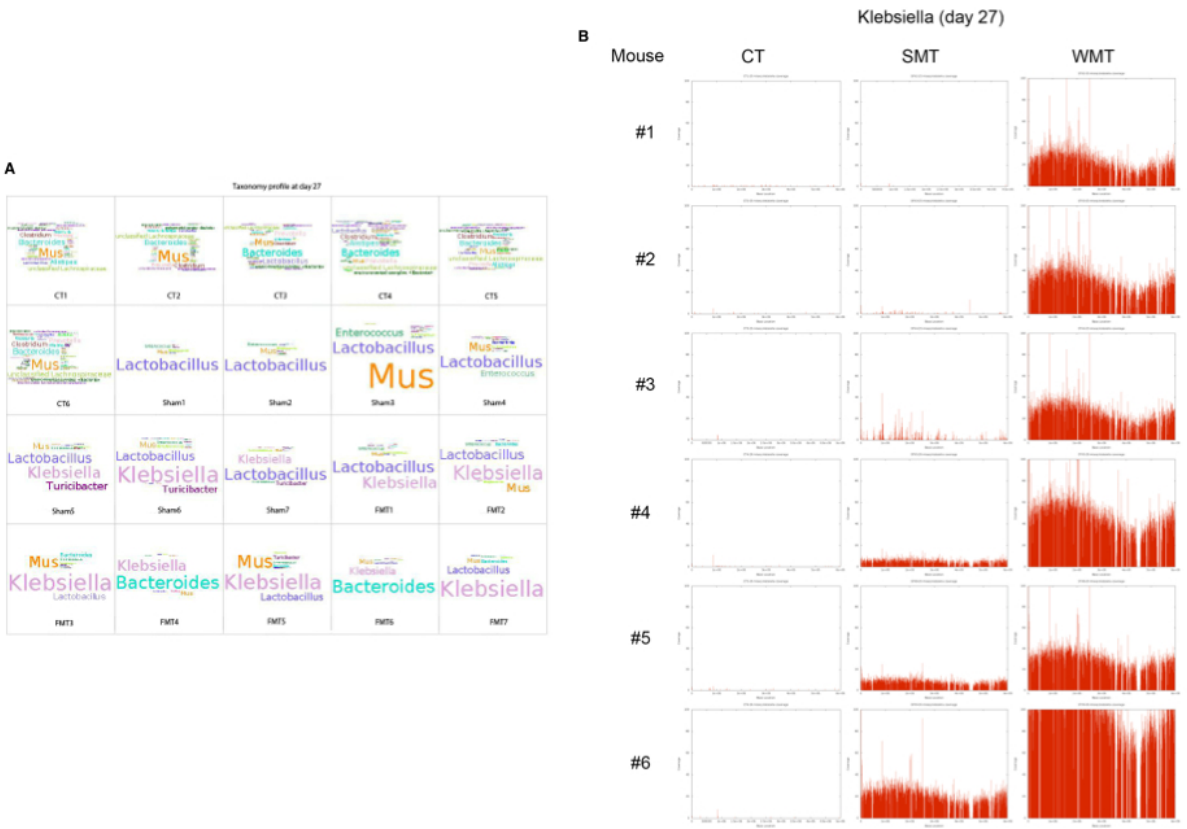
Supplementary Figure 5.3.



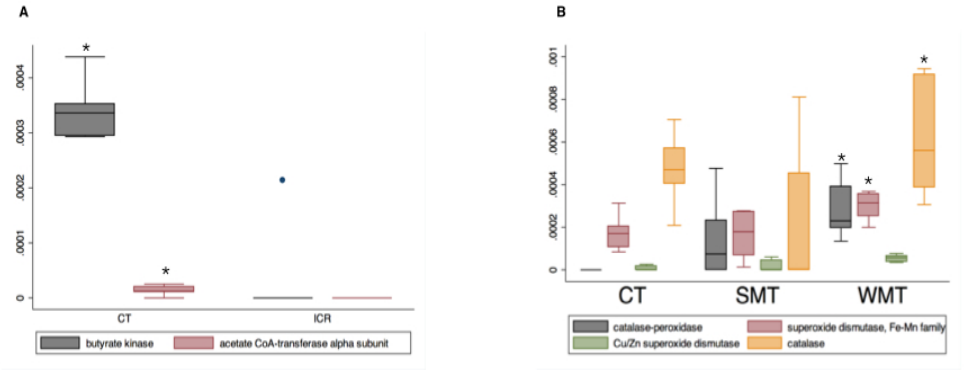
Supplementary Figure 5.4.



Supplementary Figure 5.5.



Supplementary Figure 5.6.



Supplementary Table 5.1.

Target Group	Oligonucleotide sequence (5'→3')	Tm (°C)	Product Size (bas pairs)	Reference
Domain Bacteria (total bacteria)	F:CGGYCCAGACTCCTACGGG R:TTACCGCGGCTGCTGGCAC	63	200	Lee *et al.*
Lactobacillus-Pediococcus-Leuconostoc group (LPL)	F:AGCAGTAGGGAATCTTCCA R:CACCGCTACACATGGAG *GC Clamp-CACCGCTACACATGGAG	63	341	Walter *et al.*
Bacteroides-Prevotella-Porphyromonas group (BPP)	F:GGTGTCGGCTTAAGTGCCAT R:CGGAYGTAAGGGCCGTGC *GC Clamp-CGGAYGTAAGGGCCGTGC	60	140	Rinttila *et al.*
Enterobacteriaceae Family	F:CATTGACGTTACCCGCAGAAGAAGC R:CTCTACGAGACTCAAGCTTGC	63	195	Bartosch *et al.*
Clostridium cluster IV	F:GCACAAGCAGTGGAGT R:CTTCCTCCGTTTTGTCAA GC Clamp-CTTCCTCCGTTTTGTCAA	60	239	Matsuki *et al.*

Supplementary Table 5.2.

	Day 13		Day 27			Day 13		Day 27			Day 13		Day 27	
KEGG1	KEGG2	KEGG3	KEGG2	KEGG3	KEGG1	KEGG2	KEGG3	KEGG2	KEGG3	KEGG1	KEGG2	KEGG3	KEGG2	KEGG3
Metabolism	Carbohydrate Metabolism	Glycolysis/ Gluconeogenesis	Carbohydrate Metabolism	Glycolysis/ Gluconeogenesis	Metabolism	Amino Acid Metabolism	Alanine, aspartate & glutamate metabolism	Amino Acid Metabolism	Alanine, aspartate & glutamate metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Retinol metabolism	Metabolism of Cofactors & Vitamins	Retinol metabolism
Metabolism	Carbohydrate Metabolism	Amino sugar and nucleotide sugar metabolism	Carbohydrate Metabolism	Amino sugar and nucleotide sugar metabolism	Metabolism	Amino Acid Metabolism	Glycine, serine, & threonine metabolism	Amino Acid Metabolism	Glycine, serine, & threonine metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Ubiquinone biosynthesis	Metabolism of Cofactors & Vitamins	Ubiquinone biosynthesis
Metabolism	Carbohydrate Metabolism	Butanoate metabolism	Carbohydrate Metabolism	Butanoate metabolism	Metabolism	Amino Acid Metabolism	phenylalanine, tyrosine & tryptophan biosynthesis	Amino Acid Metabolism	phenylalanine, tyrosine & tryptophan biosynthesis	Metabolism	Metabolism of Cofactors & Vitamins	One carbon pool by folate	Metabolism of Cofactors & Vitamins	One carbon pool by folate
Metabolism	Carbohydrate Metabolism	Fructose & Mannose Metabolism	Carbohydrate Metabolism	Fructose & Mannose Metabolism	Metabolism	Amino Acid Metabolism	Cysteine & methionine metabolism	Amino Acid Metabolism	Cysteine & methionine metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Porphyrin and chlorophyll metabolism	Metabolism of Cofactors & Vitamins	Porphyrin and chlorophyll metabolism
Metabolism	Carbohydrate Metabolism	Galactose Metabolism	Carbohydrate Metabolism	Galactose Metabolism	Metabolism	Amino Acid Metabolism	Valine, leucine, and isoleucine biosynthesis	Amino Acid Metabolism	Valine, leucine, and isoleucine biosynthesis	Metabolism	Metabolism of Cofactors & Vitamins	Vitamin B6 metabolism	Metabolism of Cofactors & Vitamins	Vitamin B6 metabolism
Metabolism	Carbohydrate Metabolism	Pentose phosphate pathway	Carbohydrate Metabolism	Pentose phosphate pathway	Metabolism	Amino Acid Metabolism	Histidine metabolism	Amino Acid Metabolism	Histidine metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Thiamine Metabolism	Metabolism of Cofactors & Vitamins	Thiamine Metabolism
Metabolism	Carbohydrate Metabolism	Pyruvate Metabolism	Carbohydrate Metabolism	Pyruvate Metabolism	Metabolism	Amino Acid Metabolism	Lysine biosynthesis	Amino Acid Metabolism	Lysine biosynthesis	Metabolism	Metabolism of Cofactors & Vitamins	Pantothenate and CoA biosynthesis	Metabolism of Cofactors & Vitamins	Pantothenate and CoA biosynthesis
Metabolism	Carbohydrate Metabolism	Starch and Sucrose Metabolism	Carbohydrate Metabolism	Starch and Sucrose Metabolism	Metabolism	Amino Acid Metabolism	Phenylalanine metabolism	Amino Acid Metabolism	Phenylalanine metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Riboflavin metabolism	Metabolism of Cofactors & Vitamins	Riboflavin metabolism
Metabolism	Carbohydrate Metabolism	Ascorbate and aldarate metabolism	Carbohydrate Metabolism	Ascorbate and aldarate metabolism	Metabolism	Amino Acid Metabolism	Valine, leucine, and isoleucine degradatoin	Amino Acid Metabolism	Valine, leucine, and isoleucine degradatoin	Metabolism	Metabolism of Cofactors & Vitamins	Biotin Metabolism	Metabolism of Cofactors & Vitamins	Biotin Metabolism
Metabolism	Carbohydrate Metabolism	propanoate metabolism	Carbohydrate Metabolism	propanoate metabolism	Metabolism	Amino Acid Metabolism	Lysine degradation	Amino Acid Metabolism	Lysine degradation	Metabolism	Metabolism of Cofactors & Vitamins	Nicotinate and nicotinamide metabolism	Metabolism of Cofactors & Vitamins	Nicotinate and nicotinamide metabolism
Metabolism	Carbohydrate Metabolism	Glyoxylate & dicarboxylate metabolism	Carbohydrate Metabolism	Glyoxylate & dicarboxylate metabolism	Metabolism	Amino Acid Metabolism	Tryptophan metabolism	Amino Acid Metabolism	Tryptophan metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Folate biosynthesis	Metabolism of Cofactors & Vitamins	Folate biosynthesis
Metabolism	Carbohydrate Metabolism	TCA cycle	Carbohydrate Metabolism	TCA cycle	Metabolism	Metabolism of other amino acids	Glutathione metabolism	Metabolism of other amino acids	Glutathione metabolism	Metabolism	Metabolism of Cofactors & Vitamins	Lipoic acid biosynthesis	Metabolism of Cofactors & Vitamins	Lipoic acid biosynthesis
Metabolism	Carbohydrate Metabolism	Pentose & glucuronate interconversions	Carbohydrate Metabolism	Pentose & glucuronate interconversions	Metabolism	Metabolism of other amino acids	D-Alanine metabolism	Metabolism of other amino acids	D-Alanine metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Polyketide sugar unit biosynthesis	Metabolism of Terpenoids & Polyketides	Polyketide sugar unit biosynthesis
Metabolism	Carbohydrate Metabolism	C5 branched dibasic metabolism	Carbohydrate Metabolism	C5 branched dibasic metabolism	Metabolism	Metabolism of other amino acids	Cyanoamino acid metabolism	Metabolism of other amino acids	Cyanoamino acid metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Non-Ribosomal peptide structures	Metabolism of Terpenoids & Polyketides	Non-Ribosomal peptide structures
Metabolism	Carbohydrate Metabolism	Inositol phosphate metabolism	Carbohydrate Metabolism	Inositol phosphate metabolism	Metabolism	Metabolism of other amino acids	Selenocompound metabolism	Metabolism of other amino acids	Selenocompound metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Terpenoid backbone biosynthesis	Metabolism of Terpenoids & Polyketides	Terpenoid backbone biosynthesis
Metabolism	Lipid Metabolism	Fatty acid metabolism	Lipid Metabolism	Fatty acid metabolism	Metabolism	Metabolism of other amino acids	Taurine and hypotaurine metabolism	Metabolism of other amino acids	Taurine and hypotaurine metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Geraniol degradation	Metabolism of Terpenoids & Polyketides	Geraniol degradation
Metabolism	Lipid Metabolism	Glycerolipid metabolism	Lipid Metabolism	Glycerolipid metabolism	Metabolism	Metabolism of other amino acids	D-Glutamine and D-Glutamate metabolism	Metabolism of other amino acids	D-Glutamine and D-Glutamate metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Vancomycin group Abx	Metabolism of Terpenoids & Polyketides	Vancomycin group Abx
Metabolism	Lipid Metabolism	Glycerophospholipid metabolism	Lipid Metabolism	Glycerophospholipid metabolism	Metabolism	Metabolism of other amino acids	Phosphonate & Phosphinate metabolism	Metabolism of other amino acids	Phosphonate & Phosphinate metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Limonene & pinene degradation	Metabolism of Terpenoids & Polyketides	Limonene & pinene degradation
Metabolism	Lipid Metabolism	Synthesis and Degradation of Ketone bodies	Lipid Metabolism	Synthesis and Degradation of Ketone bodies	Metabolism	Metabolism of other amino acids	beta-Alanine metabolism	Metabolism of other amino acids	beta-Alanine metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Biosynthesis of siderophore group non-ribosomal peptides	Metabolism of Terpenoids & Polyketides	Biosynthesis of siderophore group non-ribosomal peptides
Metabolism	Lipid Metabolism	Fatty acid biosynthesis	Lipid Metabolism	Fatty acid biosynthesis	Metabolism	Energy Metabolism	Carbon fixation pathways	Energy Metabolism	Carbon fixation pathways	Metabolism	Metabolism of Terpenoids & Polyketides	tetracycline biosynthesis	Metabolism of Terpenoids & Polyketides	tetracycline biosynthesis

Supplementary Table 5.2 continued.

	Day 13		Day 27			Day 13		Day 27			Day 13		Day 27	
KEGG1	KEGG2	KEGG3	KEGG2	KEGG3	KEGG1	KEGG2	KEGG3	KEGG2	KEGG3	KEGG1	KEGG2	KEGG3	KEGG2	KEGG3
Metabolism	Lipid Metabolism	Steroid hormone biosynthesis	Lipid Metabolism	Steroid hormone biosynthesis	Metabolism	Energy Metabolism	Nitrogen metabolism	Energy Metabolism	Nitrogen metabolism	Metabolism	Metabolism of Terpenoids & Polyketides	Zeatin biosynthesis	Metabolism of Terpenoids & Polyketides	Zeatin biosynthesis
Metabolism	Lipid Metabolism	biosynthesis of unsaturated fatty acids	Lipid Metabolism	biosynthesis of unsaturated fatty acids	Metabolism	Energy Metabolism	Methane metabolism	Energy Metabolism	Methane metabolism	Metabolism	Nucleotide metabolism	Pyrimidine metabolism	Nucleotide Metabolism	Pyrimidine metabolism
Metabolism	Lipid Metabolism	Arachidonic acid metabolism	Lipid Metabolism	Arachidonic acid metabolism	Metabolism	Energy Metabolism	Oxidative phosphorylation	Energy Metabolism	Oxidative phosphorylation	Metabolism	Nucleotide metabolism	Purine metabolism	Nucleotide Metabolism	Purine metabolism
Metabolism	Xenobiotics Biodegradation & Metabolism	Napthalene degradation	Xenobiotics Biodegradation & Metabolism	Napthalene degradation	Metabolism	Energy Metabolism	Sulfur metabolism	Energy Metabolism	Sulfur metabolism	Environmental Information Processing	Membrane Transport	ABC transporters	Membrane Transport	ABC transporters
Metabolism	Xenobiotics Biodegradation & Metabolism	Benzoate degradation	Xenobiotics Biodegradation & Metabolism	Benzoate degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Streptomycin biosynthesis	Biosythesis of Other Secondary Metabolites	Streptomycin biosynthesis	Environmental Information Processing	Membrane Transport	Phosphotransferase system	Membrane Transport	Phosphotransferase system
Metabolism	Xenobiotics Biodegradation & Metabolism	Dioxin degradation	Xenobiotics Biodegradation & Metabolism	Dioxin degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Phenylpropanoid biosynthesis	Biosythesis of Other Secondary Metabolites	Phenylpropanoid biosynthesis	Environmental Information Processing	Membrane Transport	Bacterial secretion system	Membrane Transport	Bacterial secretion system
Metabolism	Xenobiotics Biodegradation & Metabolism	Atrazine degradation	Xenobiotics Biodegradation & Metabolism	Atrazine degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Novobiocin biosynthesis	Biosythesis of Other Secondary Metabolites	Novobiocin biosynthesis	Environmental Information Processing	Signal Transducion	Two component system	Signal Transduction	Two component system
Metabolism	Xenobiotics Biodegradation & Metabolism	Toluene degradation	Xenobiotics Biodegradation & Metabolism	Toluene degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Tropane, piperidine and pyridine alkaloid biosynthesis	Biosythesis of Other Secondary Metabolites	Tropane, piperidine and pyridine alkaloid biosynthesis	Environmental Information Processing	Signalling Molecules and Interactions		Signalling Molecules and Interactions	
Metabolism	Xenobiotics Biodegradation & Metabolism	Steroid degradation	Xenobiotics Biodegradation & Metabolism	Steroid degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Stilbenoid, diarylheptanoid & gingerol biosynthesis	Biosythesis of Other Secondary Metabolites	Stilbenoid, diarylheptanoid & gingerol biosynthesis	Cellular Processes	Cell Motility	Bacterial chemotaxis	Cell Motility	Bacterial chemotaxis
Metabolism	Xenobiotics Biodegradation & Metabolism	Nitrotoluene degradatoin	Xenobiotics Biodegradation & Metabolism	Nitrotoluene degradatoin	Metabolism	Biosythesis of Other Secondary Metabolites	Flavanoid biosynthesis	Biosythesis of Other Secondary Metabolites	Flavanoid biosynthesis	Cellular Processes	Cell Motility	Flagellar assembly	Cell Motility	Flagellar assembly
Metabolism	Xenobiotics Biodegradation & Metabolism	Aminobenzoate degradation	Xenobiotics Biodegradation & Metabolism	Aminobenzoate degradation	Metabolism	Biosythesis of Other Secondary Metabolites	beta-lactam resisitance	Biosythesis of Other Secondary Metabolites	beta-lactam resisitance	Genetic Information & Processing	Transcription	RNA polymerase	Transcription	RNA polymerase
Metabolism	Xenobiotics Biodegradation & Metabolism	Caprolactam degradation	Xenobiotics Biodegradation & Metabolism	Caprolactam degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Flavone and flavonol biosynthesis	Biosythesis of Other Secondary Metabolites	Flavone and flavonol biosynthesis	Genetic Information & Processing	Translation	Ribosome	Translation	Ribosome
Metabolism	Xenobiotics Biodegradation & Metabolism	Cholorcyclohexane & chlorobenzene degradation	Xenobiotics Biodegradation & Metabolism	Cholorcyclohexane & chlorobenzene degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Pencillin & cephalosporin biosynthesis	Biosythesis of Other Secondary Metabolites	Pencillin & cephalosporin biosynthesis	Genetic Information & Processing	Translation	RNA transport	Translation	RNA transport
Metabolism	Xenobiotics Biodegradation & Metabolism	Ethylbenzene degradation	Xenobiotics Biodegradation & Metabolism	Ethylbenzene degradation	Metabolism	Biosythesis of Other Secondary Metabolites	Isoquinoline alkaloid biosynthesis	Biosythesis of Other Secondary Metabolites	Isoquinoline alkaloid biosynthesis	Genetic Information & Processing	Translation	Aminoacyl tRNA biosynthesis	Translation	Aminoacyl tRNA biosynthesis
Metabolism	Xenobiotics Biodegradation & Metabolism	Fluorobenzoate degradation	Xenobiotics Biodegradation & Metabolism	Fluorobenzoate degradation	Metabolism	Glycan Biosynthesis & Metabolism	Lipopolysaccharide biosynthesis	Glycan Biosynthesis & Metabolism	Lipopolysaccharide biosynthesis	Genetic Information & Processing	Folding Sorting & degradation	Protein export	Folding Sorting & degradation	Protein export
Metabolism	Xenobiotics Biodegradation & Metabolism	Styrene degradation	Xenobiotics Biodegradation & Metabolism	Styrene degradation	Metabolism	Glycan Biosynthesis & Metabolism	Glycoshpingolipid biosynthesis-globo series	Glycan Biosynthesis & Metabolism	Glycoshpingolipid biosynthesis-globo series	Genetic Information & Processing	Folding Sorting & degradation	Sulfur relay system	Folding Sorting & degradation	Sulfur relay system
Metabolism	Xenobiotics Biodegradation & Metabolism	Chloralkane & chloralkene degradation	Xenobiotics Biodegradation & Metabolism	Chloralkane & chloralkene degradation	Metabolism	Glycan Biosynthesis & Metabolism	Glycoshpingolipid biosynthesis-ganglio series	Glycan Biosynthesis & Metabolism	Glycoshpingolipid biosynthesis-ganglio series	Genetic Information & Processing	Folding Sorting & degradation	Protein processing in ER	Folding Sorting & degradation	Protein processing in ER
Metabolism	Xenobiotics Biodegradation & Metabolism	Bisphenol degradation	Xenobiotics Biodegradation & Metabolism	Bisphenol degradation	Metabolism	Glycan Biosynthesis & Metabolism	Glycosaminoglycan degredation	Glycan Biosynthesis & Metabolism	Glycosaminoglycan degredation	Genetic Information & Processing	Folding Sorting & degradation	RNA degradation	Folding Sorting & degradation	RNA degradation
Metabolism	Amino Acid Metabolism	Tyrosine metabolism	Amino Acid Metabolism	Tyrosine metabolism	Metabolism	Glycan Biosynthesis & Metabolism	N-Glycan biosynthesis	Glycan Biosynthesis & Metabolism	N-Glycan biosynthesis	Genetic Information & Processing	Replication & repair	Homologous recombination	Replication & repair	Homologous recombination
Metabolism	Amino Acid Metabolism	Arginine & Proline metabolism	Amino Acid Metabolism	Arginine & Proline metabolism	Metabolism	Glycan Biosynthesis & Metabolism	Peptidoglycan biosynthesis	Glycan Biosynthesis & Metabolism	Peptidoglycan biosynthesis	Genetic Information & Processing	Replication & repair	DNA replication	Replication & repair	DNA replication
					Genetic Information & Processing	Replication & repair	Nucleotide excision repair	Replication & repair	Nucleotide excision repair	Genetic Information & Processing	Replication & repair	Mismatch repair	Replication & repair	Mismatch repair

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Chapter 6. Conclusions

Inflammatory bowel disease (IBD) encompasses two prototypic illnesses - Crohn's disease (CD) and ulcerative colitis (UC) - that are characterized by chronic intestinal inflammation causing abdominal pain, diarrhea, intestinal bleeding, weight loss, malabsorption and nutritional deficiencies(Abraham & Cho, 2009). The onset of IBD is greatest in early adulthood, with peak incidence among those aged 18-35 at a stage of life that has significant impact on employment and psychosocial functioning(Cosnes et al., 2011). There are approximately 129,000 Canadians living with IBD, and ~6000 new cases are diagnosed every year. Canada has among the highest reported prevalence and incidence rates of IBD in the world. The prevalence of IBD in Canada is currently nearly 0.7%, equating to more than 1 in every 150 Canadians. Economic costs for IBD are estimated at $2.8 billion in Canada in 2012, with direct medical costs totalling over $1.2 billion (CCFC impact report 2012).

CD occurs in genetically-predisposed individuals who are exposed to microbial, dietary, and environmental triggers(Baumgart & Sandborn, 2012). Patients with Crohn's disease (CD) exhibit a "dysbiosis" of gut microbiota, along with decreased diversity and richness of the gut microbiome as compared with healthy individuals(Kostic et al., 2014). This has led to the hypothesis that CD occurs due to an imbalance between inflammatory-inducing and protective bacterial taxa that is accompanied by dysregulated innate and adaptive immune responses towards specific gut microbes(Morgan et al., 2012). CD most commonly involves the terminal ileum or ileocecal region. Due to disease complications or failure of medical therapy, the majority of CD patients will require surgery for ileocolonic resection (ICR) during their lifetime. Post-operatively, the disease recurs

almost always at the surgical anastomosis in the neo-terminal ileum(Buisson et al., 2012; De Cruz et al., 2011). Endoscopic lesions preclude clinical recurrence and are visualized in 54-75% of patients one year following ICR(Buisson et al., 2012; Rutgeerts, Geboes, & Vantrappen, 1990). This phenomenon in the neo-terminal ileum was identified as one of the top ten remaining mysteries in IBD by Colombel in 2008(Colombel, Watson, & Neurath, 2008) and remains that way today.

Removal of ileocecal valve allows free reflux of colonic contents into the terminal ileum after ICR, thus blending colonic and ileal flora and causing increased bacterial loads in the ileum(A. M. B. MD et al., 2012; Neut et al., 2002). This remains the predominant theory on the etiology of post-operative ileitis and is supported by observations which include; a dysbiosis in the neo-terminal ileum with recurrence, the necessity of a fecal stream for recurrence, and the efficacy of antibiotics in preventing recurrence(D'Haens et al., 1998; Neut et al., 2002; Rutgeerts et al., 2005).

Recent studies utilizing next generation sequenincg technologies have provided a detailed look at the gut microbiome. In a large study with treatment naïve CD subjects, mucosal DNA samples were submitted to high throughput sequencing. The major findings indicate that microbial profiles could predict disease, and that mucosal associated bacteria were remarkably similar between the terminal ileum and colon(Gevers et al., 2014). The application of these technologies to post-operative disease is lacking, but two small cohort studies utilized 16s based phylogenetic characterization to define microbial profiles at the time of ICR and 6 months post-operatively. The authors found microbial populations in the surgical specimen to be predictive of post-operative recurrence.

Profiles predictive of recurrence reflect those frequently found in CD with increased levels of Enterobacteriacea, enterococcus, and decreased overall diversity(De Cruz et al., 2014; Dey, Soergel, Repo, & Brenner, 2013). These results beg the question: If mucosal associated bacteria are similar between the colon and ileum, and pre-operative ileal microbes predict post-operative recurrence, then does surgical removal of the ileocecal valve actually contribute to recurrence?

What may be lacking in our understanding of post-operative recurrence is the contribution of the immune response post-operatively. The gut microbiome and mucosal immune system shape each other, and this interplay is central to gut homeostasis. Therefore, we would expect a significant contribution of the immune system to the observed dysbiosis in post-operative ileitis. The few available studies looking at immunologic responses in post-operative CD have made rudimentary observations on cytokine profiles associated with recurrence(Ahmed et al., 2011; Zorzi et al., 2013). Moreover, no studies have addressed the early changes with post-ICR immune responses and microbial populations. Thus a significant knowledge gap remains in the study of ICR associated factors impacting the recurrence of CD in the neo-terminal ileum.

The primary purpose of this thesis project was to investigate microbial and immunologic factors inherent to ICR that contribute to the uniform and rapid recurrence of CD in the ileum. The study of early changes with ICR represents a significant challenge in human subjects particularly with respect to the immunologic shifts. Based on wound healing studies in the skin, TNF-α is known to be an important part of the early healing response. If this were also the case for intestinal healing then rises in TNF-α at the anastomosis

may contribute to a persistent inflammatory response in the neo-terminal ileum. To determine is TNF-α signaling is important for anastomotic healing, we performed a systematic review and meta-analysis, which evaluated the available literature to determine if TNF-α blockade would lead to anastomotic complications. This review demonstrated that TNF-α blockade did not contribute to anastomotic leaks in IBD patients, suggesting that TNF-α is not important in anastomotic healing. This conclusion is supported by observations in the basic science literature demonstrating decreased levels of TNF-α in intestinal healing, and improved intestinal healing with TNF-α blockade(Alam et al., 1996; Seno et al., 2009; Zubaidi et al., 2009).

Animals models are typically required for controlled experiments to address specific biologic changes. A small number of mouse studies exist which demonstrate microbial changes with ICR and indicate that microbes are necessary for the induction of ileitis(Devine et al., 2013; Rigby et al., 2009). These studies utilized an ICR procedure that was initially developed for the investigation of short gut syndrome and involves a 50% small bowel resection. It is uncommon to resect this amount of bowel for CD; therefore we developed our own procedure presented in chapter 3, which resects 2 cm of ileum and is proportional to approximately 20 cm of human bowel. This new ICR procedure was then applied to mouse models of inflammatory bowel disease.

Investigation in the IL-10$^{-/-}$, and DSS models uncovered significant effects of ICR on the gut microbiome and mucosal immune system with implications for early post-operative recurrence. Immunologic queries after ICR suggested a suppressed immune response.

Increased mucosal permeability with bacterial translocation is one factor thought to underlie the pathogenesis of CD(Baumgart & Sandborn, 2012). DSS acts in similar fashion to induce intestinal inflammation by degrading the mucosal barrier(Johansson et al., 2010; Laroui et al., 2012). Taking advantage of these known effects, we introduced DSS treatment to the post-ICR immune system in wild type mice at two time points after surgery. In all cases, DSS did not evoke an inflammatory response as determined by lack of elevations in cytokines, which were significantly elevated in the colons of non-operative controls. This suppressive effect extended to the lymph nodes and spleen where stimulation of bacterial antigens did not produce an increase in TNF-α. ICR was also associated with a change in mononuclear phagocyte populations (MPs) in the intestinal tissues which was most pronounced in the neo-terminal ileum. Recruited monocytes are extremely flexible in their function and may become inflammatory effector cells(Rivollier, He, Kole, Valatas, & Kelsall, 2012) or potent immunosuppressive cells(Gabrilovich & Nagaraj, 2009; Ostanin & Bhattacharya, 2013). In the neo-terminal ileum they are most likely the latter, as macrophage depletion with liposomal clodronate induced Th1 type tissue cytokines. When ICR was performed in IL-$10^{-/-}$ mice a similar phenomenon was found with decreased cytokines in the colon after ICR relative to colitic IL-$10^{-/-}$ controls. In the ileum, post-ICR injury was associated with a decrease in F4/80^{+} macrophages and an increase in $CD11b^{+}$ cells, perhaps again representing suppressive monocytes. Fecal transplant into these animals reversed the shift in MP populations in the ileum and increased inflammatory cytokine profiles while reducing tissue injury and bacterial penetration on histology. It is also interesting to note that the post-ICR suppressive effects occurred in the absence of IL-10, which is thought to be central to the

regulatory response. In both mouse models quantitative PCR was used to measure tissue bacteria load in the ileum. Like humans, ICR lead to increased mucosal associated bacteria in the neo-terminal ileum, but results from the IL-10$^{-/-}$ model suggest this may be more of an immunologic phenomenon than an anatomical one. When normal MP populations were restored after fecal transplant bacterial load decreased as did penetration through the mucosal barrier. Longitudinal measurements of microbial changes after ICR was performed in the IL-10$^{-/-}$ model using whole metagenome shotgun sequencing to determine shifts in specific clades as well as functional changes. This demonstrated that ICR induced substantial early changes in in the gut microbiome with decreased diversity in clades and functional capacity, and that these early changes rendered the gut susceptible to colonization with pathogens and pathobionts.

While the pathogenesis of post-operative ileitis in CD still remains a mystery the results presented in this thesis provide new avenues of investigation to explore. The concept of immunosuppression leading to chronic intestinal inflammation is not new. Emerging evidence in CD suggests that innate immune dysfunction is a major contributing factor to pathogenesis(Knights, Lassen, & Xavier, 2013), and immunosuppression following solid organ transplant can both exacerbate pre-existing IBD or induce it. This is however the first description of local mucosal immunosuppression following ICR. This provides a plausible explanation for the microbial overgrowth in the neo-terminal and in the context of a leaky mucosal barrier would allow bacterial penetration and chronic infection in the ileum.

The massive loss of microbial diversity resulting from ICR also carries significant potential to result in chronic inflammation. With the decrease in diversity came a decrease in function. Metabolic products from gut microbes, specifically short-chain fatty acids, have benefits in gut health(O'Hara & Shanahan, 2006). As well, a healthy and diverse microbiome is resistant to pathogen colonization(Stecher et al., 2010). Our results demonstrate that the initial decrease in diversity made way for bacterial clades with known associations to CD and post-operative recurrence including *Enterococcus* and *Enterobacteriaceae*(De Cruz et al., 2014; Kostic et al., 2014).

This body of work has only scratched the surface of the complex immunobiology and microbiology at play in post-operative recurrence of ileal CD. Translational investigations may be able to build on this by attempting to take advantage of a seemingly hypo-responsive immune system and malleable microbiome to promote healthy re-colonization. Probiotic therapy thus far has failed to alter the natural history post-operative disease(Madsen et al., 2008), however prebiotics to promote the growth of beneficial bacteria have yet to be studied in the post-operative setting. Fecal microbial transplant was beneficial in the IL-$10^{-/-}$ model, but this appeared to be related to its activation of the immune system as it failed to restore a diverse microbiome and increased Proteobacteria.

The concept of immune activation to clear invading microbes from the vulnerable ileum is novel. However, the exact mechanisms underlying the immune suppression are unclear, and require extensive characterization in the laboratory prior to any human translation.

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