The Pennsylvania State University

The Graduate School

College of Medicine

INTEGRATIVE ANALYSIS OF THE MICROBIOME AND HUMAN INTESTINAL TRANSCRIPTOME: IMPLICATIONS FOR THE PATHOGENESIS OF DIVERTICULITIS

A Dissertation in

Biomedical Sciences and Clinical and Translational Sciences

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2017

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ABSTRACT

The formation of diverticula, or herniations of the colonic mucosa and submucosa through the bowel wall, is prevalent in the sigmoid colon of aged individuals in Western countries. Individuals with diverticula have diverticulosis, a condition which is usually asymptomatic. However, in 4-25% of individuals with diverticulosis, inflammation of the diverticula results in diverticulitis. Acute diverticulitis is commonly treated with empiric antibiotics and increased dietary fiber. Despite the high prevalence of disease in the United States, the pathophysiology of diverticulitis is largely unknown. Previous data suggests that the immune system may play a role in diverticulitis pathogenesis. A key function of the intestinal immune system is to discriminate pathogens from commensal microorganisms. Microbial dysbiosis contributes to the pathogenesis of inflammatory bowel disease; however, whether the microbiome is also involved in diverticulitis pathogenesis is unknown. Therefore, we hypothesized that deregulation of the innate immune system contributes to diverticulitis pathogenesis by promoting a dysbiotic microbial ecosystem.

To test this hypothesis, we used RNA-seq, an unbiased and genome-wide approach, to globally survey the intestinal transcriptome of full-thickness sigmoid colon tissue collected adjacent to areas chronically affected by diverticulitis compared to controls. Gene Ontology pathway analysis found increased expression of genes associated with the innate immune response. Expanding on these findings, we identified an increased number of macrophages expressing the scavenger receptor CD163L1 in diverticulitis tissues. In diverticulitis patients, $CD163L1^{+}$ macrophages localized at the sub-epithelium and co-expressed the chemokine CXCL10. Furthermore, heightened serum CXCL10 was found in diverticulitis patients relative to controls. As the intestinal microbiome influences immune homeostasis, bacterial 16S rRNA and fungal ITS sequencing was performed from chronically diseased tissue and adjacent tissue in diverticulitis patients. We found distinct microbial interactions segregated chronically diseased tissue from adjacent tissue within the same colonic segments.

The findings from this dissertation propose that deregulation of the innate immune response to an altered microbial ecosystem results in chronic inflammation and the development of diverticulitis. There are currently no prognostic or diagnostic biomarkers or targeted therapeutic options for diverticulitis; thus this work may help drive development of such targets within molecular pathways of disease relevance.

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LIST OF ABBREVIATIONS

3' UTR	3’ untranslated region
5-ASA	5-aminosalicylic acid
AIDS	Acquired immunodeficiency syndrome
APC	Adenomatous polyposis coli
ARGHAP15	Rho-GTPase-activating protein 15
AT	Adjacent sigmoid colon tissue
AXIN2	Axis inhibition protein 2
BLK	B lymphoid tyrosine kinase
BMI	Body mass index
CARD9	Caspase domain-containing protein 9
CASP	Caspase
CCR7	C-C motif chemokine receptor 7
CD	Crohn's disease
CD163L1	CD163 molecule-like 1
CI	Confidence interval
CLR	C-type lectin receptors
COLQ	Collagen-like tail subunit of asymmetric acetylcholinesterase
CR3	Complement receptor 3
CT	Computed tomography
CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C motif chemokine receptor 3
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
DSS	Dextran sodium sulfate
DT	Chronically diseased sigmoid colon tissue
ELISA	Enzyme linked immunosorbent assay
eQTL	Expression quantitative trait loci
FAM155A	Family with sequence similarity 155A
GAGE	Generally applicable gene set enrichment
GO	Gene ontology
GWAS	Genome-wide association study
HHV-6	Human Herpesvirus 6
HTF-Microbi.Array	High taxonomic fingerprint-Microbi.Array
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICC	Interstitial cells of Cajal
ICD-9	International Classification of Disease, Ninth Edition
IFIT	Interferon-induced proteins with tetratricopeptide repeats

IFN	Interferon
IFNAR	Interferon alpha and beta receptor subunit
IL	Interleukin
IL10R	IL-10 receptor
INPP5D	Inositol polyphosphate-5-phosphatase D
IP-10	Interferon-γ induced protein 10
IRB	Institutional Review Board
IRF	Interferon regulatory factor
ISG15	Interferon-stimulated gene 15
ITS	Internal transcribed spacer
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JUN	Jun proto-oncogene AP-1 transcription factor subunit
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LPS	Lipopolysaccharide
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage-colony stimulating factor
ME	Module eigengene
MMP	Matrix metalloproteinase
MUC2	Mucin 2
MyD88	Myeloid differentiation primary response gene 88
NCATS	National Center for Advancing Translational Sciences
NFKBIA	NF-κB inhibitor alpha
NF-κB	Nuclear factor kappa B
NLR	Nucleotide binding domain and leucine-rich repeat containing receptors
NLRP3	NOD-, LRR-, and pyrin domain containing 3
NSTI	Nearest sequenced taxon index
OAS	2’-5’-oligoadenylate synthetase
OR	Odds ratio
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular patterns
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PICRUSt	Phylogenetic investigation of communities by reconstructed states

PRR	Pattern recognition receptors
PtdIns(3,4)P_2	Phosphatidylinositol-3,4-biphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-triphosphate
PTPRC	Protein tyrosine phosphatase, receptor type C
RAF	Rapidly accelerated fibrosarcoma
RASAL3	RAS protein activator-like 3
RELA	RELA proto-oncogene, NF-KB subunit
RIG I	Retinoic acid inducible gene I
RLR	RIG I-like receptors
RPKM	Reads per kilobase of transcript per million mapped reads
RT-qPCR	Reverse transcription and quantitative PCR
SASH3	SAM and SH3 domain-containing 3
SD	Standard deviation
sIgA	Secretory IgA
SNP	Single nucleotide polymorphism
SUDD	Symptomatic uncomplicated diverticular disease
SxAge	Age at surgery
SYK	Spleen tyrosine kinase
TAB1	TGF-beta activated kinase 1 binding protein 1
TBK1	TANK binding kinase 1
TBX21	T-box 21
TCL1A	T-cell leukemia/lymphoma 1A
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNFSF15	Tumor necrosis factor superfamily 15
TOM	Topological overlap matrix
TRAF6	Tumor necrosis factor-receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
TUBB3	Beta-tubulin 3
U1102	Uganda-1102
UC	Ulcerative colitis
WGCNA	Weighted gene co-expression network analysis

PREFACE

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ACKNOWLEDGEMENTS

First and foremost, my sincerest gratitude is bestowed upon my thesis advisors, Dr. Walter Koltun and Dr. Greg Yochum. Their unwavering support, work ethic, and intellectual guidance has provided me with amazing opportunities for professional growth over the past two years, allowing me to become the scientist I have always dreamed I could be. Dr. Koltun, your knowledge and clinical expertise is unsurpassed and the heart of our lab. My clinical rotation was a highlight of my graduate school career - meeting the patients afflicted with the diseases you are studying really puts the research into a new perspective. Dr. Yochum, thank you for constantly challenging me as a scientist and pushing me past my boundaries. Your dedication to my scientific career helped me accomplish a wealth of success over the past year and even revive a few projects. I would also like to acknowledge my previous thesis advisor, Dr. Carla Gallagher. Thank you for your guidance and introducing me to public health and epidemiology. Through the work in your lab, I realized my career goals.

I would also like to thank my thesis committees, past and present, for their commitment to my education and future as a scientist. Drs. Shannon Kelleher, Vijay Kumar, and Gina Lamendella, thank you for providing your knowledge and support over these past two years. Thank you, Drs. Rob Bonneau, Laura Carrel, Doug Leslie, and John Richie for your advice during my first years in graduate school. The diverse expertise and invaluable intellectual input from all of these individuals helped me become a well-rounded scientist. My warmest regards are also extended to Drs. Jim Pawelczyk and Gail Thomas in the CTS program. Thank you for opening the doors for many opportunities and your encouragement over the years.

Thank you to the members of the Koltun and Yochum labs for the friendships and intellectual contribution to this work. To Melanie Eshelman and Stephen Matthews, I am so glad that the Yochum lab joined up with the Koltun lab. Thank you for being great friends and always

lending an open ear or suggestion. I look forward to what the future has in store for you both. To Leonard Harris, Sue Deiling, and John Hegarty, thank you for welcoming me into the lab with open arms and introducing me to our Biobank and translational research. Thank you to Andrea Angstadt, Jen Modesto, and Abid Kazi for all of the guidance and advice during my first years of graduate school.

Importantly, my sincerest gratitude is bestowed upon my family for always believing in me. To my parents, Ken and Dianne, thank you for providing me with strength, curiosity, and empowering me to pursue my dreams even when things were difficult. I dedicate my dissertation to you and cannot put into words how thankful I am for both of you. To my sisters, Kelley and Erin, thank you for keeping me grounded and keeping life enjoyable. There is never a dull moment when you are around. Thank you to all my friends for their support and the fun. I have truly enjoyed my time in Hershey.

Finally, thank you to the patients who donated their tissue to our Biobank. We are indebted to their generosity and without them this work would not have been possible.

Chapter 1

INTRODUCTION

Diverticular Disease Classification

The lower gastrointestinal tract is comprised of the small intestine and colon. Distal from the small intestine, the colon is segmented into the cecum, ascending colon, transverse colon, descending colon, and sigmoid colon, followed by the rectum. Pseudodiverticula (hereafter referred to as diverticula), or herniations of the mucosa and submucosa through the circular muscle of the colon [1], primarily develop in the sigmoid colon of individuals in Western countries [2]. Diverticulosis is an asymptomatic condition arising from the formation of diverticula. Some individuals develop symptomatic diverticulosis, which has also been termed diverticular disease. This clinical condition may arise from inflammation or other mechanisms such as visceral hypersensitivity [3]. A subtype of diverticular disease, symptomatic uncomplicated diverticular disease (SUDD), is defined by persistent abdominal symptoms despite the lack of overt inflammation [3]. Finally, acute or chronic diverticular inflammation is termed diverticulitis [3]. Uncomplicated diverticulitis is defined by localized inflammation while complicated diverticulitis is associated with complications such as fistula, phlegmon, perforation, bleeding, or abscess [4]. A small percentage of patients develop recurring episodes of diverticulitis, defined as chronic, recurrent diverticulitis [3]. These classifications will be used throughout the remainder of this literature review (Table 1-1). Diverticular disease may also be defined as the spectrum of changes occurring to the colon, encompassing both diverticulosis and diverticulitis [5]. This definition will be used throughout Chapters 2-5.

Table 1-1. Disease definitions.

Condition	Definition
Diverticulosis	Asymptomatic diverticula
Diverticular disease	Symptomatic diverticulosis or the spectrum of changes to the colon including diverticulosis and diverticulitis
Symptomatic uncomplicated diverticular disease (SUDD)	Persistent abdominal pain despite the absence of overt diverticular inflammation
Diverticulitis	Inflammation of the diverticula
Uncomplicated diverticulitis	Localized diverticular inflammation
Complicated diverticulitis	Diverticulitis associated with complications such as fistula, bowel obstruction, phlegmon, perforation, bleeding, and/or abscesses
Chronic, recurrent diverticulitis	Two or more episodes of diverticulitis

Clinical Characteristics and Treatment

Most patients (80-85%) with diverticulosis remain asymptomatic [6]. A diagnosis of diverticulitis is based primarily on clinical symptoms, a physical examination, and radiographic tests, such as computed tomography (CT) [6]. Symptoms associated with diverticulitis usually include lower left abdominal pain, constipation, leukocytosis, fever, and nausea/vomiting [2]. In patients with diverticulitis, pericolic wall thickening and fat stranding are common while complications such as fistulas, bowel obstruction, and abscesses are found in patients with complicated diverticulitis [7]. These abnormalities are readily assessed by CT [7]. Uncomplicated diverticulitis is commonly treated with a course of empiric antibiotics and increased dietary fiber intake [6]. The prevalence of chronic, recurrent disease is 13-23% in patients with uncomplicated diverticulitis and increases to 40% in those with complicated diverticulitis [8]. Current guidelines from The American Society for Colon and Rectal Surgeons recommends elective colonic resection on a case-by-case basis, taking into consideration the patient's overall medical condition, age, whether symptoms persist after medical treatment, and the frequency and severity of episodes [9]. Emergent surgery is required if the patient presents with diffuse peritonitis or those that fail non-operative treatment of acute disease [9].

Epidemiology

Diverticulosis is commonly associated with aging, as it affects over 50% of individuals at age 60 and the incidence increases to 65% for individuals over 70 years old [10]. However, a small subset of individuals (~20%) develop diverticulosis at a youthful age (<40 years old) [10]. Diverticulosis is the most common incidental finding during routine colonoscopies in individuals ≥60 years [3]. In 10-25% of individuals, inflammation of the diverticula results in diverticulitis

[5]. However, the lifetime risk of developing diverticulitis was recently proposed to be lower (~4%) [11]. With the introduction of surveillance colonoscopy, more individuals have been identified with incidental diverticulosis [11]. Therefore, these data suggest that the previous prevalence rate of 10-25% likely under-estimated the true prevalence of diverticulosis, resulting in an over-estimation of diverticulitis [11]. Nevertheless, the incidence rate of diverticulitis is increasing in the United States [12,13]. A Minnesota-based temporal study found that the age- and sex-adjusted incidence rate of diverticulitis has been steadily increasing since 1993, from 100 per 100,000 person-years to 250 per 100,000 person-years in 2007 [12].

Similarly, the healthcare burden of diverticulitis is significant and increasing. Analysis of the 2009 National Ambulatory Medical Care Survey of physician principal diagnoses for outpatient clinic visits found that diverticular disease was a leading cause of gastrointestinal visits [14]. Diagnoses associated with diverticular disease (International Classification of Disease, Ninth Edition (ICD-9): 562.10, 562.11) contributed to an estimated 2,682,168 visits in 2009, which is higher than the estimated 1,893,799 visits for inflammatory bowel diseases (IBD) (ICD-9: 555.9, 556.9) [14]. A similar trend was described for the 2009 Nationwide Inpatient Survey of principal discharges [14]. Diverticulitis with hemorrhage (ICD-9: 562.11) ranked third amongst gastrointestinal principal diagnoses, with 219,133 discharges and 1,235 hospital deaths, resulting in an aggregate cost of $2,115,989,000 per year [14]. For comparison, IBD as a principal diagnosis contributed to 100,687 total discharges and 44 hospital deaths, totaling an aggregate cost of $1,007,990,000 in 2009 [14]. Moreover, a study of the National Emergency Department Sample found that emergency department visits for acute diverticulitis increased 26.8% between 2006 and 2013, leading to an aggregated national cost increase of about 105%, from $822,000,000 to over $1,600,000,000, after adjusting for inflation [15].

Geographic location plays a key role in spatial location of diverticula within the colon. In Western countries, such as Europe and the Americas, the formation of diverticula is primarily

limited to the left colon, with a majority of cases found within the sigmoid colon [2]. The incidence of right-sided diverticula is rare (1-2%) [16]. In contrast, right-sided disease is more prevalent (43-70%) in Asian countries [16,17]. For comparison, of Japanese diverticular disease cases, 70-76% had right-sided disease, 13-16% had left-sided disease, and 11-14% had both left- and right-sided disease [17,18]. Additionally, demographic differences were described between those with right-sided and left-sided disease. Those with right-sided disease tended to be younger men while left-sided disease was associated with older age and both sexes [17]. A potential mechanism to explain the higher incidence of right-sided disease amongst Asians is genetics [2,16]; although to date, no genetic associations have been established. As native Asians have adjusted to a Westernized lifestyle, the incidence of both right-sided and left-sided disease increased, with similar demographic trends as previously described [17]. Furthermore, migratory studies found that that the risk for diverticular disease was lower in individuals immigrating to Westernized countries from non-Western countries relative to the native population [19–22]. As time from settlement increased, the risk of diverticular disease increased in non-Western immigrants [19]. Moreover, a study reported that diverticular disease remained right-sided in Japanese settling in Hawaii and consuming a Westernized diet [22], suggesting that although increased risk for diverticular disease may be attributed to diet, the spatial location of diverticula is likely highly associated with genetics.

Pathophysiology

Despite the prevalence and healthcare burden of diverticular disease and diverticulitis, its pathogenesis is understudied. Since diverticulosis is associated with advanced age [23], factors associated with weakening of the bowel wall and the aging process are considered as potential etiological mechanisms for diverticulosis [24]. Dietary factors and genetic susceptibility have

been implicated as potential etiological causes of diverticulosis, as well as diverticular disease and diverticulitis [24] (Figure 1-1). However, the most commonly considered mechanism for diverticulitis onset is the obstruction of a diverticulum by a fecalith, resulting in bacterial overgrowth and mucosal inflammation [24]. This notion provides the basis for broad spectrum antibiotic therapy. Alterations in the microbial community have been implicated in the pathogenesis of diverticular disease [3,25] and will be discussed in a later section of this literature review.

Colonic wall structural abnormality

The muscularis propria of the colon encompasses a single inner circular muscle and an outer longitudinal layer, comprised of three bands of taenia coli at the mesenteric and antimesenteric poles [1]. The vasa recta supplies blood to the mucosa and submucosa of the colon, penetrating the bowel wall between the mesenteric and antimesenteric taenia coli [1]. This architecture results in an area of structural weakness that is susceptible to the formation of diverticula [1]. This anatomy is common to all individuals, so additional factors must contribute to the pathogenesis of diverticulosis. Abnormalities of the colonic muscle were demonstrated in the sigmoid colon of diverticular disease patients. An accordion-like appearance is characteristic of the taenia coli in diverticular disease patients, caused by a thickening and shortening of the muscle associated with increased elastin deposition [26]. Similarly, smaller collagen fibrils with tighter crosslinking were described in individuals with diverticulosis [27] and those requiring surgery for complications associated with diverticular disease [28]. Abnormal collagen crosslinking likely results in reduced submucosal compliance, increasing the risk for small tears in the bowel wall under enhanced pressure and potentially resulting in herniation [5].

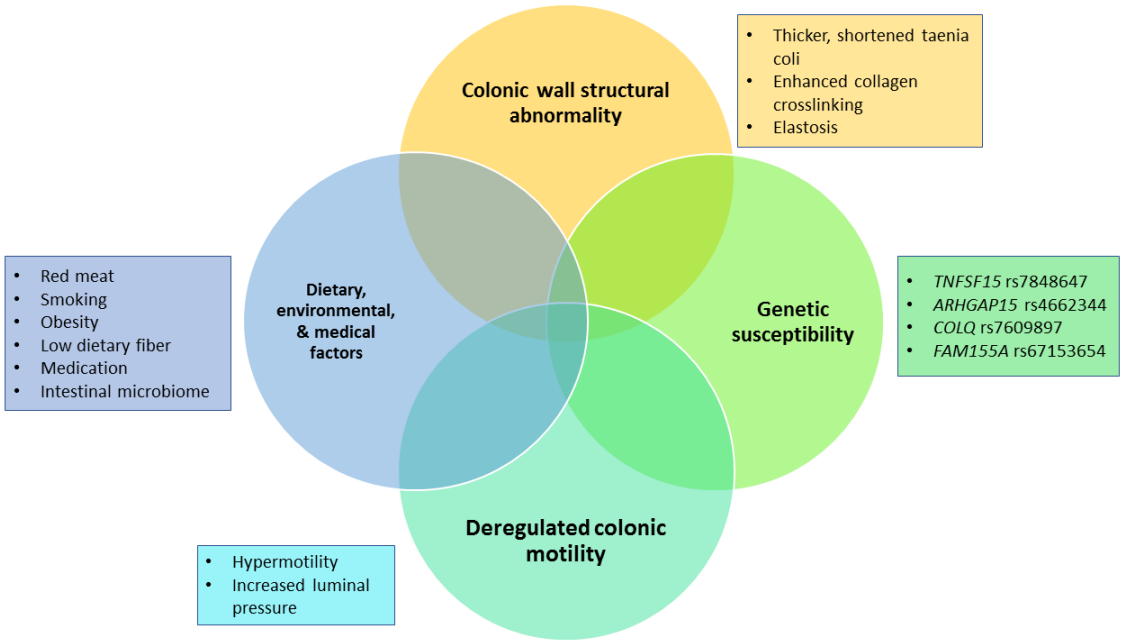

Figure 1-1. Risk factors associated with diverticulitis pathogenesis.

Current data suggests that four potential mechanisms are involved in the pathogenesis of diverticulitis. These mechanisms include: colonic wall structure abnormality, genetic susceptibility, deregulated colonic motility, and dietary, environmental, and medical factors. Potential causal factors or genes associated with increased risk of disease are boxed.

Deregulated colonic motility

Deregulated colonic motility and increased pressure within the lumen may strain the abnormal and weakened bowel wall, making it susceptible to herniation and formation of diverticula [2]. During resting conditions, the sigmoid colon of patients with diverticular disease [29,30] and SUDD [31] displayed an increased motility index (percent duration of activity x median maximal amplitude) and/or contractile strength compared to healthy controls. Similarly, this trend was described in individuals with right-sided diverticulosis, diverticular disease, and diverticulitis [32]. Another study found that asymptomatic diverticular disease patients also demonstrated enhanced motility within the sigmoid colon relative to patients with irritable bowel syndrome (IBS) [30]. However, reports of hypermotility in diverticular disease patients have been inconsistent [33].

Defects of the enteric nervous system may also affect gastrointestinal motility. The interstitial cells of Cajal (ICC) are the pacemaker cells of the gastrointestinal tract, mediating input from the enteric motor nerves to the smooth muscle [34]. Reduced numbers of S100 positive glial cells and three subtypes of ICCs were found in patients with diverticulosis relative to controls [35]. Thus, a defect within the enteric nervous system, specifically of ICC pacemaker cells, could contribute to gastrointestinal motor abnormality. If deregulated colonic motility and thus increased intraluminal pressure is involved in the development of diverticula, dietary supplementation to restore normal motility would be beneficial. In fact, studies evaluating treatment with fiber supplements, including unprocessed bran [36], methylcellulose tablets [37], and bran tablets [38] demonstrated beneficial effects in patients with diverticular disease, including symptom relief [36–38] and mitigation of intracolonic pressure [37,38].

Dietary, environmental, and medical factors

In the 1970s, Painter and Burkitt published a seminal paper describing the causal role of reduced dietary fiber in the development of diverticular disease [39]. They found that individuals from Westernized countries with deficiency in dietary fiber had a higher incidence of diverticular disease compared to those from low-risk African countries where consumption of high levels of dietary fiber is common [39]. A second study confirmed this association, demonstrating that insoluble fiber intake decreased the risk for diverticular disease [40,41]. Similarly, a British group found that a high fiber diet reduced the risk of hospital admissions and mortality from diverticular disease [42,43]. However, these findings have been challenged recently as an association between a low dietary fiber diet and diverticulosis was not found [23,44]. Although the evidence for dietary fiber and diverticular disease pathogenesis is inconsistent, these data suggest there may be some benefit to dietary fiber.

Apart from fiber, other dietary factors were shown to influence the risk for diverticulitis. Increased risk of diverticulitis was demonstrated in individuals with low levels of vitamin D [45] and in men who eat a high quantity of red meat [46]. Moreover, a vegetarian lifestyle [42] and consumption of nuts and popcorn [47] reduced the risk for diverticulitis.

Environmental factors also contribute to diverticular disease risk. The geographic locale of individuals may have a role in disease risk as those living in areas of low ultraviolet light exposure [48] and those living in a rural area had a higher risk of diverticulitis-associated hospitalizations [48,49]. Smoking was found to be a risk factor for complicated diverticular disease and diverticulitis-associated hospitalizations [49–52]. Similarly, obesity was associated with diverticulitis [53], diverticular bleeding [53], and hospitalization [49].

Finally, medical therapy can also be attributed to disease risk. Use of oral corticosteroids [52], opiate analgesics [52], and non-steroidal anti-inflammatory drugs [54,55] have been

associated with complicated diverticulitis. Statin use [52] and calcium channel blockers [56] may reduce the risk for diverticular bleeding. Apart from dietary fiber which likely influences colonic motility and intraluminal pressure [57], the molecular mechanisms behind these associations still remain unknown.

Genetic predisposition

The genetic contribution to diverticular disease was evaluated through two large European monozygotic and dizygotic twin studies [58,59]. Both studies included patients with a primary or secondary diagnosis of diverticulosis or diverticular disease [58,59]. These patients were combined and referred to as diverticular disease [58,59]. The first study used the Danish twin registry and evaluated 923 twins with diverticular disease and 29,399 control twins [58]. The estimated heritability, or percentage of the disease phenotype attributed to genetics, of diverticular disease was found to be 53% [58]. Comparable results were obtained from a cohort of twins with diverticular disease identified from the Swedish twin registry (n=2,296) compared to non-diverticular disease control twins (n=102,156) [59]. The heritability of diverticular disease in the Swedish cohort was 40% [59]. For reference, the heritability of siblings to IBD probands has been reported at a lower percentage. Crohn's disease (CD) heritability was estimated to be 25-42%, while ulcerative colitis (UC) heritability was lower at 4-15% [60].

Despite the higher predicted heritability for diverticular disease, research studying the genetic contribution to disease risk has been sparse. Our group reported an association of individuals with the single nucleotide polymorphism (SNP) rs7848647, a genetic variant mapping upstream of tumor necrosis factor superfamily 15 (*TNFSF15*), and individuals requiring surgical treatment for diverticulitis [61]. To date, one genome wide association study (GWAS) has been reported for diverticular disease and diverticulitis [62]. Intronic variants (rs4662344 and

rs7609897) found within a DNase hypersensitivity site located in the Rho-GTPase-activating protein 15 gene (*ARGHAP15*) and collagen-like tail subunit of asymmetric acetylcholinesterase gene (*COLQ*), respectively were associated with diverticular disease in the Icelandic and Dutch populations [62]. A SNP (rs67153654) within the family with sequence similarity 155A gene (*FAM155A*) was associated with diverticulitis and interestingly, this SNP seemed to be associated with disease progression rather than development [62]. *FAM155A* rs67153654 was not associated with diverticular disease but showed a protective association for diverticulitis [62]. To evaluate the impact of the SNPs on RNA expression, the target gene and nearby gene expression data was evaluated in blood and adipocytes, with no significant differences in expression reported [62]. Importantly, none of the IBD-associated loci [63] were associated with diverticular disease or diverticulitis [62]. Therefore, although three SNPs were identified by GWAS, the functional impact of these genetic variants remains unknown.

Interplay of the host immune response and microbial ecosystem in the pathogenesis of diverticulitis

Based on the previously identified mechanisms of diverticular disease pathogenesis, our lab developed a potential model describing the step-wise defects leading to diverticulitis [61] (Figure 1-2). We proposed that genetic variation in immune-associated genes, such as the previously identified *TNFSF15* variant [61], predisposes individuals to diverticulitis. Deregulation of the host immune response, resulting in chronic low-grade inflammation, contributes to microbial dysbiosis and influences the development of diverticulitis. This interplay between the host innate immune system and gut microbes has been understudied in diverticulitis.

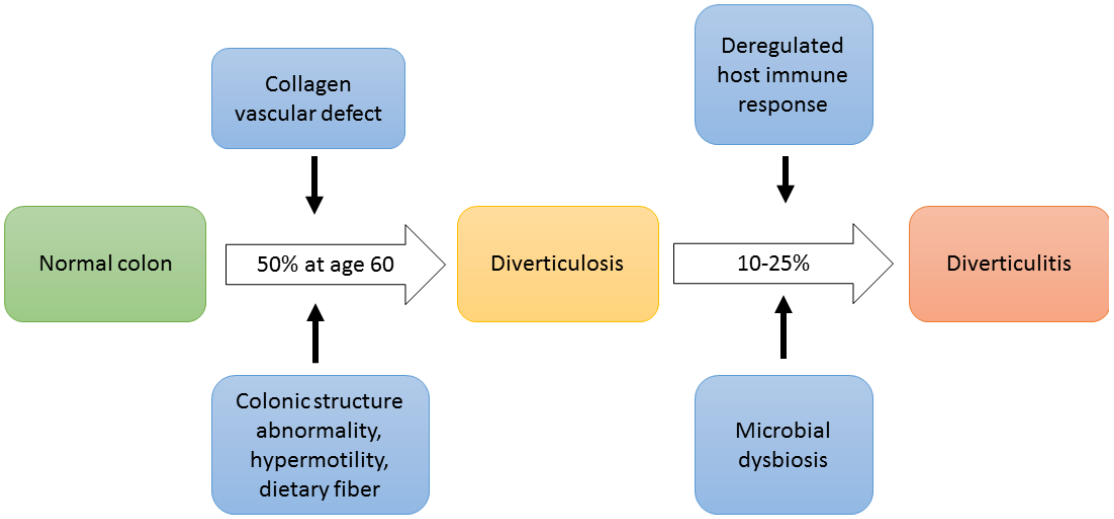

Figure 1-2. Previously proposed model of diverticulitis pathogenesis.

Previous work from our lab identified a potential model for diverticulitis pathogenesis. Genetic predisposition in genes associated with the collagen vascular system and physiological alterations within the bowel may alter susceptibility to diverticulosis. Genetic variation in immune-associated genes may be associated with a deregulated host immune response that contributes to microbial dysbiosis. The inability of the host to maintain microbial homeostasis increases the risk for diverticulitis onset. Figure adapted from Connelly *et al.* [61].

Innate Immune Response

The intestines are colonized by an abundance of diverse bacteria, fungi, and other microorganisms that live symbiotically with the host [64] to educate and promote immune tolerance [65]. The innate immune response is the critical first line of defense against pathogenic insults and early activation of the inflammatory response in the intestinal tract [66]. Thus, a primary function of the innate immune system is to discriminate between pathogenic and non-pathogenic foreign antigens and maintain intestinal homeostasis [67]. Phagocytic cells, such as macrophages and dendritic cells, are key mediators of the innate immune response [67]. Deregulation of the innate immune response, in particular through toll-like receptor (TLR) signaling, has been shown to potentiate intestinal inflammation [68]. In particular, TLR4 is stimulated by bacterial and fungal antigens to promote a pro-inflammatory response [69] and disrupt intestinal homeostasis [70]. While the role of macrophages and the innate immune response has been studied in IBD, very little is known about the innate immune response in diverticulitis.

Macrophage polarization: M1 and M2 phenotypes

Intestinal macrophages localize in the lamina propria throughout the gastrointestinal tract [71,72]. Macrophages are also found in the colonic smooth muscle [73] where they have been shown to regulate colonic motility [74]. An oversimplified approach to classifying macrophages is by activation phenotype [75]. M1 macrophages are considered “classically activated”, demonstrating a pro-inflammatory phenotype while M2 macrophages are “alternatively activated” with anti-inflammatory functions [75]. These cell populations can be identified by their upstream activation signals, including cell surface markers and cytokine and chemokine secretion

[76–78] (Table 1-2). *In vitro*, mature macrophages are polarized into M1 and M2 phenotypes by culturing with pro- or anti-inflammatory mediators [79]. Supplementing media with interferon (IFN)-γ or lipopolysaccharide (LPS) polarizes mature macrophages to the M1 phenotype and interleukin (IL)-4, IL-10, or IL-13 polarizes macrophages towards that M2 phenotype [79]. M1 macrophages are defined by their role in stimulating T_H1 immune responses [75,80], pathogen resistance, bactericidal properties, and production of reactive oxygen species [81]. On the contrary, M2 macrophages are phagocytic cells involved in T_H2 responses [80] by promoting tissue remodeling, angiogenesis, and the response to parasites [81]. M2 macrophages can also be subdivided into M2a, M2b, M2c, and M2d based on the response to upstream immune mediators and cytokines [76] (Table 1-2). Resident intestinal macrophages are considered to have an M2-like phenotype [82]. Macrophage polarization is dynamic as cells can repolarize in the presence of extrinsic factors such as cytokines [83]. This process requires both changes at the transcriptional and translational levels as well as changes in metabolite profiles [83].

Toll-like receptor signaling initiates the innate immune response

Microbial recognition by macrophages occurs through pattern recognition receptors (PRRs) [67]. PRRs recognize pathogen-associated molecular patterns (PAMPs), or microbial components that are essential to the function of the microorganism, such as cell wall proteins or microbial DNA and RNA [67]. In total, ten human toll-like receptor (TLRs) exist with varying specificity for PAMPs [66] (Table 1-3). Binding of TLR4 by LPS, a component of the Gram-negative bacterial cell wall [84], is of particular interest as IBD patients demonstrate strong up-regulation of TLR4 [85,86]. This receptor is expressed on myeloid cells and intestinal epithelial cells [87], with higher expression found in inflamed intestinal tissue [86]. Interestingly, overexpression of *Tlr4* in the mouse intestine influenced the mucosa-associated bacterial

Table 1-2. M1 and M2 macrophage differentiation profile.

	M1	Tissue resident M2	M2a	M2b	M2c	M2d
Upstream signals	IFN-γ LPS GM-CSF	IL-4 IL-13 IL-10 C1q C3b Microbiota	Fungal and helminth infections IL-4 IL-13	Immune complexes LPS	IL-10 TGF-β GCs	IL-6 adenosine
Marker expression	CD16 CD68 CD86 CD80 MHC-II IL-1R TLR2 TLR4 SOCS3	CD163 CD206 CD208 MGL-1	CD163 CD206 MHC-II TGM2	CD86 MHC-II	CD163 CD206 MERTK	VEGF-A
Cytokine secretion	TNFα IL-1β IL-6 IL-12 IL-23	IL-10	IL-10 TGF-β IL-1RA	IL-1 IL-6 IL-10 TNF-α	IL-10 TGF-β	IL-10 IL-12 TNF-α TGF-β
Chemokine secretion	CCL2 CCL3 CCL4 CCL5 CCL8 CCL9 CCL10 CCL11 CXCL8 CXCL9 CXCL10 CXCL11		CCL17 CCL22 CCL24	CCL1	CCR2	CCL5 CXCL10 CXCL16

Adapted from Röszer *et al.* 2015 [76], Mantovani *et al.* 2002 [77], and Duluc *et al.* 2007 [78]
IFN-γ, interferon-γ; LPS, lipopolysaccharide; GM-CSF, granulocyte macrophage colony-stimulating factor; TGF-β, transforming growth factor-β; GCs, glucocorticoids; MHC-II, major histocompatibility factor class II; TLR, toll-like receptor; SOCS3, suppressor of cytokine signaling 3; MGL-1, macrophage galactose-type lectin-1; TGM2, transglutaminase 2; MERTK, MER proto-oncogene, tyrosine kinase; VEGF-A, vascular endothelial growth factor-A; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; CCR, C-C chemokine receptor.

Table 1-3. Pathogen-associated molecular patterns sensed by pattern recognition receptors.

Microbial Component	Pattern Recognition Receptor
Lipopolysaccharide (LPS)	TLR4
Diacyl lipopeptides	TLR2 TLR6
Triacyl lipopeptides	TLR1 TLR2
Lipoteichoic acid	TLR2 TLR6
Peptidoglycan	TLR2
Porins	TLR2
Lipoarabinomannan	TLR2
Flagellin	TLR5
CpG-DNA	TLR9
Zymosan	TLR2 TLR6
Phospholipomannan	TLR2
Mannan	TLR4 DC-SIGN
Glucuronoxylomannan	TLR2 TLR4
β-glucans	Dectin-1
High mannose structures	Dectin-2
Viral DNA	TLR9
Viral dsRNA	TLR3
Viral ssRNA	TLR7 TLR8
Viral envelope proteins	TLR4
Hemagglutinin protein	TLR2

Adapted from Akira *et al.* 2006 [67].

composition and microbial translocation into the lamina propria [88]. Conversely, *Tlr4*$^{-/-}$ mice also demonstrated enhanced bacterial translocation and reduced neutrophil chemotaxis and infiltration following dextran sodium sulfate (DSS)-induced colitis [70], indicating that TLR4 signaling is critical to maintain intestinal homeostasis.

TLR4 signaling leads to the induction of pro-inflammatory genes through myeloid differentiation primary response gene 88 (MyD88)-dependent and –independent pathways [89]. MyD88-dependent or –independent signaling may be differentially determined by pathogen, as *Escherichia coli 55*:B5 and *Vibrio cholerae* LPS activated the MyD88-dependent pathway while *Salmonella* LPS induced the MyD88-independent signaling pathway [90]. The MyD88-dependent pathway results in the transcription of pro-inflammatory cytokines by activation of the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling cascades (Figure 1-3) [67].

The MyD88-independent, TIR-domain-containing adapter-inducing IFN-β (TRIF)-dependent pathway can also activate NF-κB signaling through TRIF interaction with tumor necrosis factor-receptor associated factor 6 (TRAF6) [91] (Figure 1-3). Subsequently, the TRIF-dependent pathway is activated following internalization of the receptor to mediate transcription of the type I interferon, IFN-β [92]. IFN-β signaling activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway and promotes expression of IFN-inducible genes [93], such as C-X-C motif chemokine ligand 10 (CXCL10) [94,95]. CXCL10 is a pro-inflammatory chemokine secreted by many cells types, including monocytes and macrophages [96]. CXCL10 recruits cells expressing its cognate receptor C-X-C motif chemokine receptor 3 (CXCR3), including those involved in potentiating a T_H1 immune response, such as T and B cells, natural killer cells, and monocytes/macrophages [97].

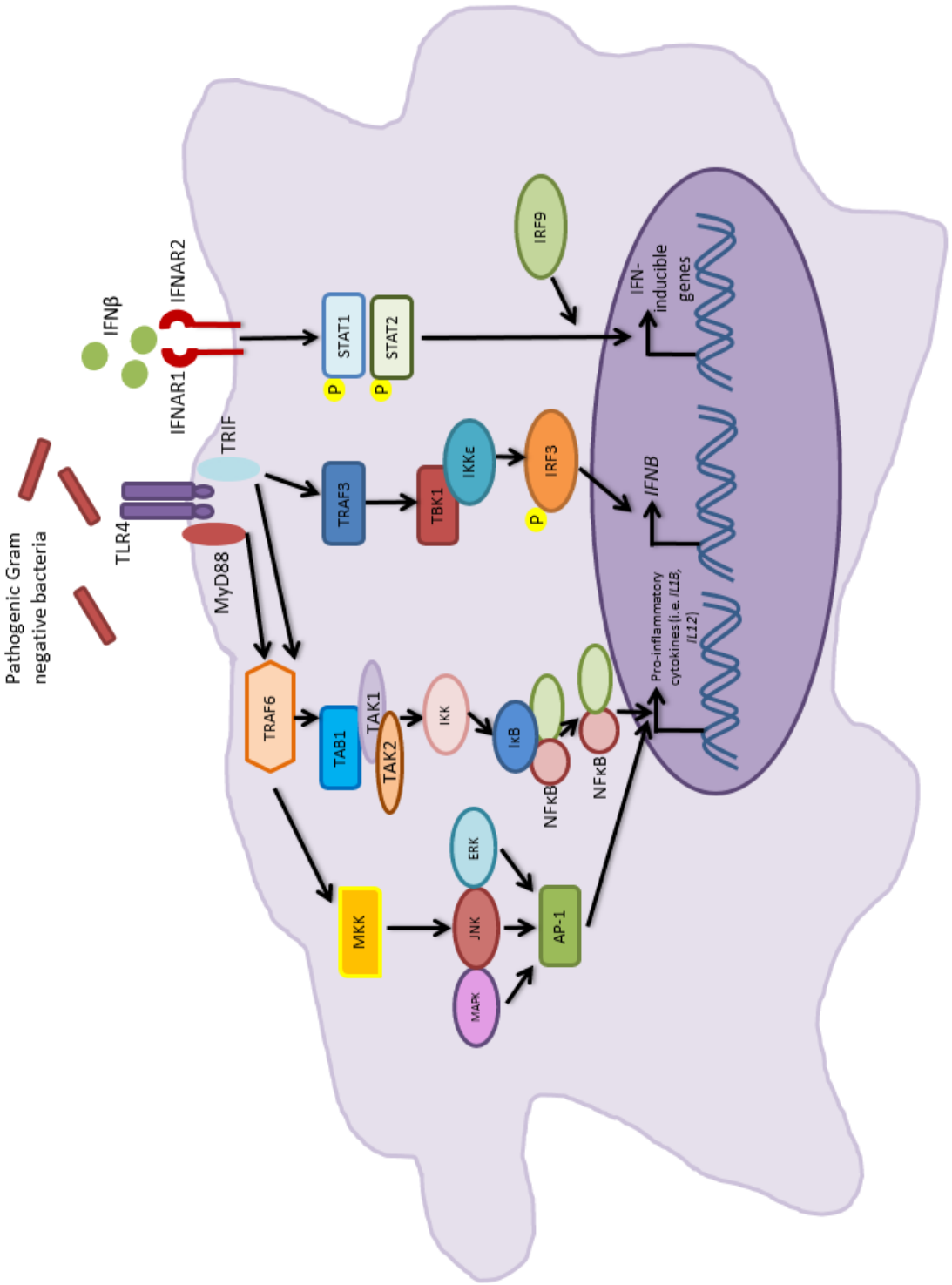
IFNβ
IFNAR2
IFNAR1
STAT1
STAT2
P
P
IRF9
IFN-inducible genes
TRIF
TRAF3
TBK1
IKKε
IRF3
P
IFNB
TLR4
MyD88
Pathogenic Gram negative bacteria
TRAF6
TAB1
TAK1
TAK2
IKK
IκB
NFκB
NFκB
Pro-inflammatory cytokines (i.e. IL1β, IL12)
MKK
ERK
JNK
MAPK
AP-1

Figure 1-3. TLR4 signaling promotes expression of pro-inflammatory mediators.

Pro-inflammatory macrophages express TLR4 in response to lipopolysaccharide present in the cell wall of Gram negative bacteria. Ligand binding to TLR4 promotes activation of the MyD88-dependent pathway and activation of NF-κB and MAPK signaling. Following receptor internalization, the MyD88-independent, TRIF-dependent pathway is activated to induce IFN-β expression. See text for details. Figure adapted from Mandraju *et al.* [98].

Whether the TLR signaling pathway is affected in diverticulitis has not been well-studied. Patients without intestinal inflammation lack expression of TLR2 or TLR4 protein in the lamina propria [85]. However, a limited report of two diverticulitis patients found that macrophages expressing the pan-macrophage marker CD68 also co-expressed TLR2 and TLR4 protein, although to a lesser degree than IBD patients [85]. Therefore, further investigation is required to better understand the role of the TLR signaling pathway in patients with diverticulitis.

Macrophage conditioning to toll-like receptor ligands

Maintaining inflammatory anergy through macrophage conditioning is critical as resident intestinal macrophages are constantly exposed to antigens, through invading commensal bacteria [99] or sampling of microbial antigens [100]. Under homeostasis, macrophages residing in the lamina propria are bactericidal [101], phagocytize apoptotic cells [102], and promote cellular proliferation through crosstalk with the epithelium [103]. In an inflammatory state, macrophages stimulate a pro-inflammatory response through activation of TLRs by foreign antigens or cellular products [67]. However, in the homeostatic intestinal environment, stimulation of a pro-inflammatory response would be detrimental. Therefore, intestinal factors are involved in macrophage conditioning, or the process of rendering intestinal resident M2 macrophages unresponsive to TLR stimuli [104,105].

Following homing of peripheral monocytes to the intestinal lamina propria [106], differentiation and conditioning occurs, by which IL-10 and transforming growth factor-β (TGF-β) are important regulators [105,107,108]. IL-10 is constitutively expressed by macrophages and a subset of T cells [109]. The critical role of IL-10 in maintaining intestinal homeostasis is epitomized by the *Il10*$^{-/-}$ mouse model which spontaneously develops severe enterocolitis [110]. Similarly, increased risk for very early onset IBD is found in individuals with genetic variants in

IL10 and IL-10 receptor (*IL10R*) [111,112]. In a mouse model, TLR hyporesponsiveness positively correlated with IL-10 levels [113]. Similarly, inhibiting IL-10 in intestinal macrophages *in vitro* reversed TLR hyporesponsiveness [107]. These findings confirm the critical role of IL-10 in maintaining intestinal homeostasis by dampening TLR signaling in macrophages.

Intestinal macrophages also express high levels of TGF-β [105]. Similar to IL-10, monocyte-derived macrophages treated with conditioned media were hyporesponsive to LPS [108]. One mechanism by which TLR stimulation mediates a pro-inflammatory response is through activation of the NF-κB pathway [67]. Stromal-derived TGF-β down-regulated NF-κB signaling in blood monocytes by inhibiting phosphorylation of IκBα and promoting its dissociation from NF-κB [105].

CD163L1$^+$ macrophages display an anti-inflammatory M2 phenotype

CD163L1 was identified as a novel Group B scavenger receptor cysteine-rich type 1 transmembrane protein with a high degree of homology to CD163 [114]. CD163 is a scavenger receptor of the hemoglobin-haptoglobin complex found specifically on monocytes and macrophages [115,116]. Despite the high homology, CD163L1$^+$ cells do not display affinity towards the hemoglobin-haptoglobin complex or various bacterial species [117]. Thus, its ligand is still unknown. However, the receptor does demonstrate clathrin-mediated endocytosis [117]. As expected, CD163L1-expressing macrophages were localized to the colonic lamina propria [117]. *In vitro* analysis of monocyte-derived CD163L1$^+$ macrophages demonstrated that these cells possess an M2 macrophage phenotype, displaying up-regulation of CD163L1 only after polarization with the anti-inflammatory mediators macrophage-colony stimulating factor (M-CSF) and IL-10 [117,118]. Despite the identification of the CD163L1 receptor almost two

decades ago, very minimal progress has been made to identify the role of $CD163L1^+$ cells in disease.

Macrophages in diverticular disease

Our understanding of the role for macrophages in the pathogenesis of diverticular disease is limited. A recent report found that patients with diverticulosis and SUDD had higher numbers of $CD68^+$ macrophages relative to controls [119]. No differences were seen between areas affected by diverticula and distant tissue [119], suggesting that low-grade inflammation may affect the entire sigmoid colon rather than remain localized to diverticula. A second study found that increased expression of $CD68^+CD163^+$ macrophages in the sigmoid colon tissue was associated with complicated diverticulitis and steroid use [120]. $CD163L1^+$ macrophages have not been evaluated in diverticular disease patients. However, these cells were analyzed in patients with active IBD [118]. Cells expressing CD163L1 were the most abundant cell population in these patients and contrary to their proposed role as M2 resident macrophages, they did not secrete IL-10 [118]. Based on the critical role of IL-10 in mediating macrophage conditioning and intestinal homeostasis, determining whether the $CD163L1^+$ cellular phenotype is potentiating inflammation in diverticulitis patients is of interest.

The Human Gut Microbiome and Mycobiome

The human gut microbiome is a dynamic ecosystem of bacteria, fungi, viruses, and other microorganisms that live in symbiosis with the host [64]. As previously mentioned, diverticulitis is commonly treated with empiric antibiotics [6], suggesting a role for the gut microbiome in disease pathogenesis. However, the role of the microbiome, as a cause or consequence of

diverticulitis has yet to be unveiled. Translocation of bacteria through the epithelium evokes an inflammatory response [121], disrupting the host-microbiome homeostasis and promoting bacterial dysbiosis [122]. Therefore, classifying bacterial populations in normal and diseased intestinal epithelium is required to fully understand their role in pathogenesis.

The structure of the human intestinal tract

A single layer of epithelial cells lines the intestines and protects the underlying tissues from commensal bacteria. The epithelium is coated by a mucus layer composed of large, highly glycosylated proteins called mucins, of which mucin 2 (MUC2) is the primary mucin secreted by intestinal goblet cells [123]. The importance of the mucus barrier was shown in *Muc2*$^{-/-}$ mice, where bacteria directly contacted the intestinal epithelium [124]. The small intestine is lined by a single, unattached layer of mucus that is penetrable to bacteria [124], but limits bacterial interaction with the epithelium through the secretion of antimicrobial peptides by Paneth cells and enterocytes [125,126] (Figure 1-4). On the contrary, the colon has a dual layer of mucus, which is comprised of a densely packed inner layer that is impenetrable to bacteria and an outer layer that is colonized by mucosa-associated bacteria [124]. In addition, a steep oxygen gradient encompasses the colonic crypt which proves an additional barrier to microbial habitation [127]. Hypoxic conditions are found at the apex of the crypt [127], which is conducive to the anaerobic bacteria that inhabit the colon [128] (Figure 1-4). Unlike the small intestine, the colon does not contain Paneth cells [128], although the colonic epithelium may express a unique subset of antimicrobial peptides that help protect against microbial invasion [129].

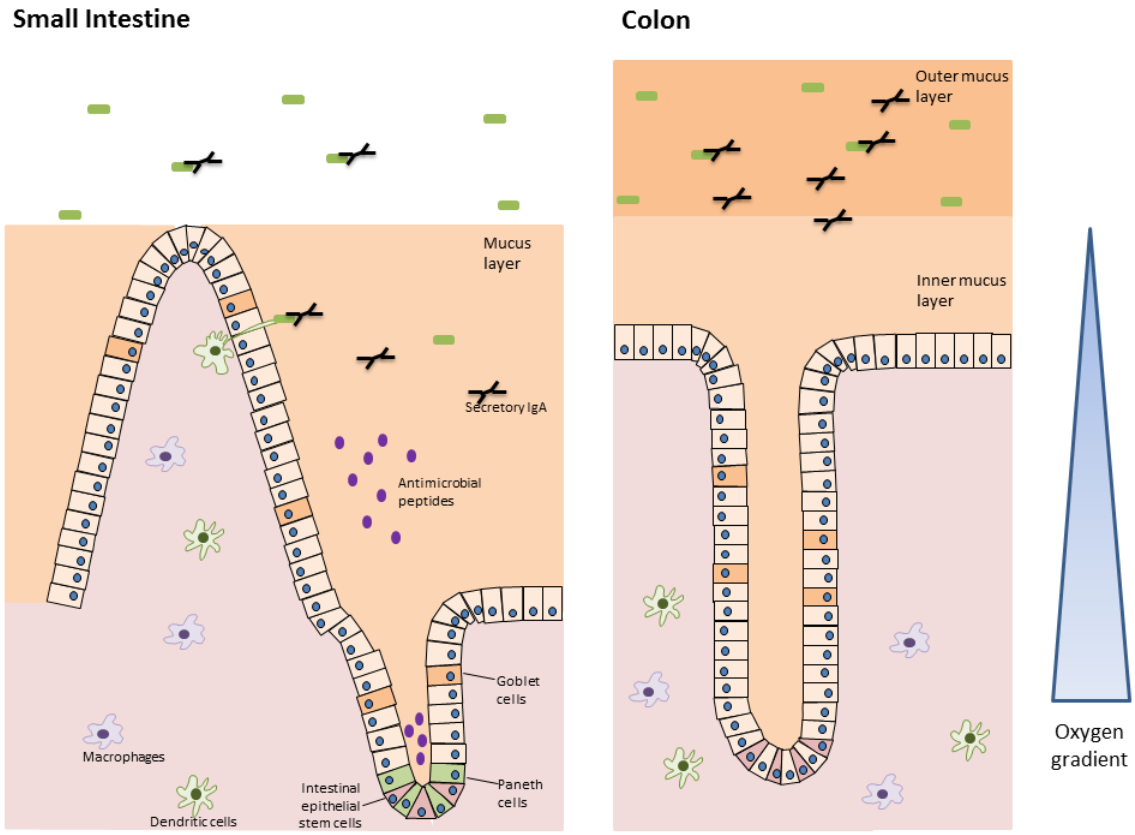

Figure 1-4. Structure of the human intestinal tract.

The protective barriers of the small intestine and colon differ. The villi of the small intestine are covered by a single mucus layer while the colon contains two layers of mucus, with the inner layer being impenetrable to bacteria. The Paneth cells of the small intestine secrete antimicrobial peptides to prevent bacterial contact with the epithelium. Secretory IgA (sIgA) binds to both commensal and pathogenic bacteria to maintain mucosal homeostasis in both the small intestine and colon. Within the crypt, a steep oxygen gradient helps to limit bacterial colonization.

Human intestinal bacterial composition at homeostasis

The human gut microbiome is a complex ecosystem comprised almost entirely of two distinct phyla, Bacteroidetes and Firmicutes, of which 95% of Firmicutes are classified as *Clostridia* [130,131]. Although complex, the gut microbiome is relatively stable over time in adults. A fecal core microbiome of about 40 operational taxonomic units (OTUs) persisted over a one year time period [132]. A study of over 500 fecal isolates found that about 60% of bacterial strains within each individual were consistent over a five year period [133]. This stability may be partially due to factors that influence the biogeography of the lower gastrointestinal tract. The oxygen and pH gradient found throughout the lower gastrointestinal tract influences microbial habitats. Relative to the small intestine, the colonic environment is more anaerobic [127] and at a higher pH [128]. Thus, significant intra-individual heterogeneity is found when sampling the mucosal microbiota from terminal ileum to rectum [130,134,135].

Bacterial metabolism also contributes to spatial localization. The microbial community requires fermentation of complex polysaccharides, including resistant starches and non-starch polysaccharides, as principal carbon and energy sources within the colon which promote colonization of specific bacterial niches [136]. As these complex polysaccharides are obtained from dietary sources, dietary variation has a major influence on the overall microbial composition [137–141]. Another subset of symbiotic bacteria colonize the outer mucus layer of the colon, such as Bifidobacteriaceae and *Akkermansia muciniphila* [128]. They utilize the host glycan, mucin as a primary carbon and energy source [128]. These symbiotic processes results in the production of metabolic by-products called short chain fatty acids (i.e. butyrate, propionate, and acetate) [142]. Butyrate is an anti-inflammatory metabolite and the primary energy substrate of the colonocytes [142,143]. Due to its role in intestinal homeostasis, the potential therapeutic use of butyrate in IBD is under investigation [144].

The large quantity of non-pathogenic commensal bacteria must also be tolerated by the host to promote intestinal homeostasis. Between 20-70% of the commensal bacteria, pathogenic bacteria, and toxins are coated by secretory IgA (sIgA) [145] (Figure 1-5A). Coating of commensal bacteria in sIgA helps identify these microorganisms as "non-pathogenic" by the host [146]. In a process known as immune exclusion, agglutination and entrapment of these bacteria within the outer mucus layer results in removal of these microorganisms by peristalsis [147]. The large sIgA-bacterial complex prevents direct interaction with the epithelial cell surface [147]. sIgA-mediated formation of microbial biofilms [148] protect the epithelium by preventing invasion by pathogens [147] (Figure 1-5B).

Occasionally, commensal bacteria come in contact with the epithelial layer. To mediate induction of a pro-inflammatory response, activation of basolateral TLR9 by microbial DNA on intestinal epithelial cells elicits an NF-κB-mediated inflammatory response [149]. However, activation of apical TLR9 results in phosphorylation and ubiquitination of IκBα and defective NF-κB activation; thus, inhibiting the inflammatory cascade [149] (Figure 1-5C). These data suggest that the intestinal epithelial cells have a mechanism to protect against unwarranted inflammatory responses to bacteria. Finally, studies in mice found that dendritic cells can extend transepithelial dendrites to sample and internalize sIgA-coated luminal bacteria to mediate bacterial translocation to Peyer's patches [150,151]. Additionally, Peyer's patch M cells are able to directly sample the luminal bacteria through interaction with either dectin-1 or an unclassified IgA receptor [152]. Peyer's patch dendritic cells promote the production of gut-homing commensal-specific IgA-producing B cells [153], secretion of commensal-specific IgA from naïve B cells through secretion of IL-6 [154], and a reduction in pro-inflammatory cytokines

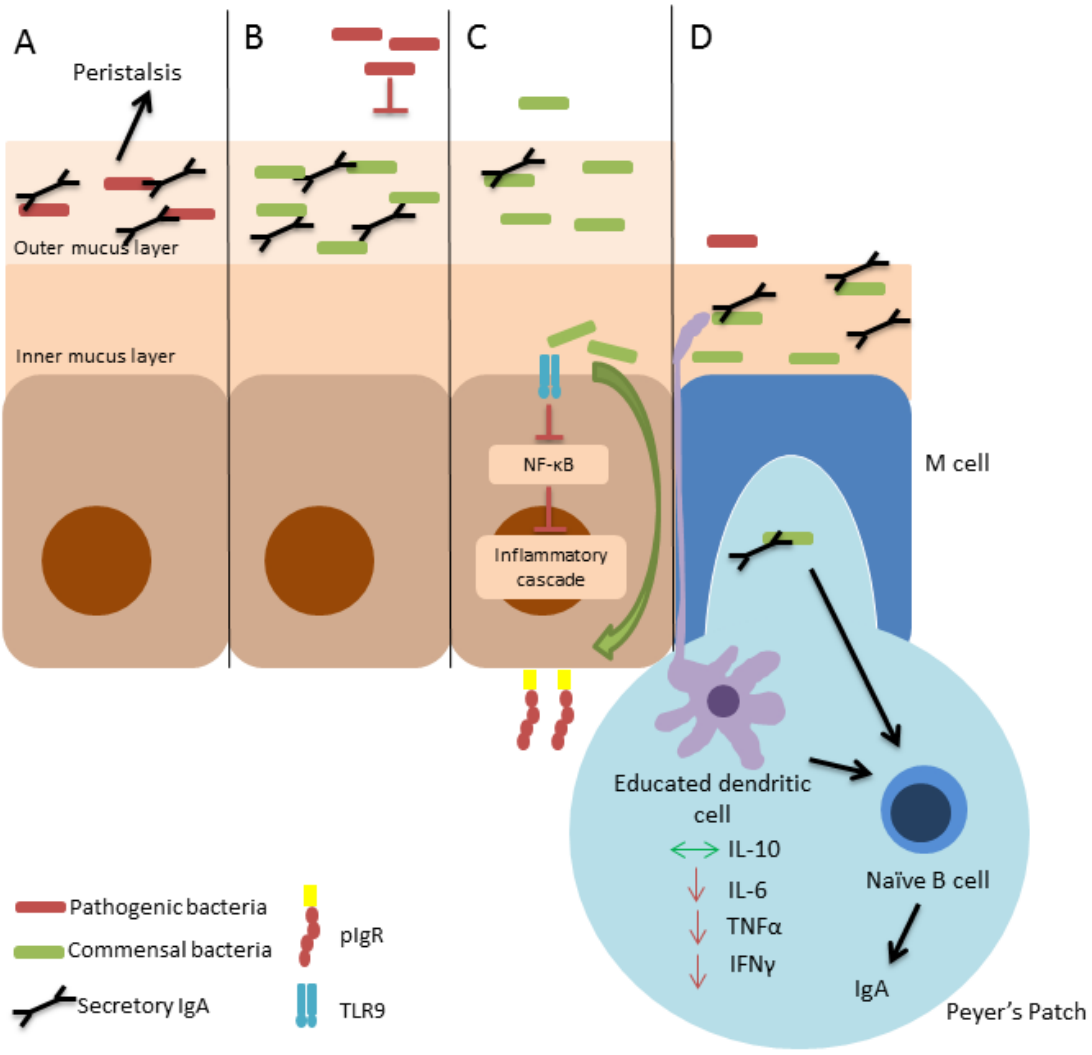

Figure 1-5. Mechanisms of intestinal homeostasis to commensal bacteria.

(A) Secretory IgA binds to pathogenic bacteria, trapping it in the mucus barrier for clearance during peristalsis. (B) Secretory IgA promotes the formation of microbial biofilms, excluding pathogens from entering the mucus barrier. (C) Binding of non-pathogenic bacteria to apical TLR9 inhibits NF-κB signaling and the pro-inflammatory cascade. Association of the commensal bacteria with the epithelium promotes expression of polymeric immunoglobulin receptor (pIgR) to transport IgA across the epithelium. (D) Peyer's patch M cells and dendritic cells directly sample luminal IgA-bound commensals. Both mechanisms result in direct IgA secretion from naïve B cells or activation of naïve B cells to commensal specific-IgA-producing B cells which enter circulation and home to the lamina propria. Following sampling of non-pathogenic microorganisms, downregulation of pro-inflammatory cytokines are found within the Peyer's patch, while IL-10 remains stable. Figure adapted from Mantis *et al.* 2011 [147].

within the Peyer's patch [155] (Figure 1-5D). Increased production of IgA within the mucosa to target commensal bacteria will aid in further promoting intestinal homeostasis [156–158].

Microbial dysbiosis in diverticulosis, SUDD, and diverticulitis

A disruption of the homeostatic microbial balance is known as dysbiosis. Dysbiosis is associated with many gastrointestinal diseases, including IBD [131], IBS [159], and colorectal cancer [160]. As the result of non-specific colonic inflammation in mice, the composition of the microbiome becomes altered, with an increase in Enterobacteriaceae [161]. One potential mechanism promoting Enterobacteriaceae growth is through bacterial utilization of nitrate produced by the host in response to the inflammatory environment for anaerobic respiration [162]. Additionally, sIgA-mediated formation of biofilms that associate with *E. coli* may promote colonization [148,163]. Alterations in the gut microbiota has been recognized as a potential etiological factor involved in the pathogenesis of diverticular disease and diverticulitis [3,25]. However, whether bacterial dysbiosis causes or promotes diverticular disease remains an open question.

To evaluate the global bacterial composition, one of the most common culture-independent methods is 16S rRNA gene sequencing. By taking advantage of the variable regions of the 16S rRNA gene (Figure 1-6) and high throughput metagenomic sequencing methods, many applications are capable of categorizing bacterial OTUs to the genus level [164]. The 16S rRNA gene is highly conserved and sequencing across variable regions provides a taxonomic bacterial fingerprint which can be identified by various databases, such as Greengenes [165]. Four studies have evaluated the microbial composition of patients with diverticulosis, SUDD, or diverticulitis (Table 1-4). However, there is no consistency amongst the results, likely due to differences in sequencing methods, sample collection, and utilization of control groups that potentially harbor a

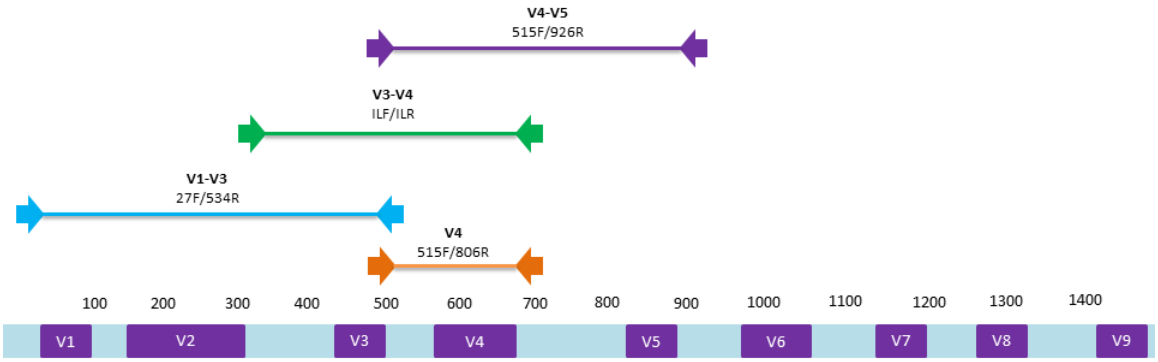

Figure 1-6. Bacterial 16S rRNA gene and sequencing primers.

The bacterial 16S rRNA gene is comprised of nine variable regions (purple) and ten conserved regions (blue). 16S rRNA sequencing utilizes primers that sequence across multiple variable regions as designated by the above primer sets.

Table 1-4. Microbiome alterations in diverticulosis, SUDD, and diverticulitis.

Disease	Comparison Group	Source	Increased Taxa	Reduced Taxa	Method	Reference
Diverticulosis	Healthy controls	Fecal	None	*Clostridium* cluster IV	HTF-Microbi.Array	[119]
Diverticulosis	Healthy controls	Fecal	*Akkermansia muciniphila*	None	Real time PCR	[166]
Diverticulosis	Healthy controls	Mucosa	*Bacteroides/ Prevotella*	Enterobacteriaceae	HTF-Microbi.Array	[119]
SUDD	Healthy controls	Fecal	*Akkermansia muciniphila*	None	Real time PCR	[166]
SUDD	Healthy controls	Mucosa	*Bacteroides/ Prevotella*	Enterobacteriaceae	HTF-Microbi.Array	[119]
SUDD	Diverticulosis	Fecal	None	*Clostridium* cluster IX, *Fusobacterium*, Lactobacillaceae	HTF-Microbi.Array	[119]
Diverticulitis	Colorectal cancer, IBD	Mucosa	*Bifidobacterium longum, Bifidobacterium animalis*	None	Quantitative PCR	[167]
Diverticulitis	Control cohort[1]	Fecal	Proteobacteria	None	16S-23S Interspace profiling	[168]

[1]Control cohort includes the following indications: follow-up after polypectomy, anemia, benign neoplasm, malignant neoplasm, Crohn's disease, ulcerative colitis, indeterminate colitis, irritable bowel syndrome, abdominal pain, surveillance for familial cancer susceptibility, and diverticulosis

HTF-Microbi.Array, High taxonomic fingerprint-Microbi.Array; SUDD, symptomatic uncomplicated diverticular disease; PCR, polymerase chain reaction; IBD, inflammatory bowel disease

confounding dysbiotic microbial composition. Interestingly, only Daniels *et al.* provided a global view of the microbial composition [168]. The remaining studies used methods that provided a bias towards particular subsets of bacteria through either PCR-based identification [166,167] or a bacterial array of 30 phylogenetically-related groups [119]. The studies also differ among mucosal [119,167] and fecal [119,166,168] sampling of the bacterial population (Table 1-4). As the anatomy of the diverticulum demonstrates limited communication with the lumen, evaluating only the fecal microbiota may lack important mucosa-associated microbes that are potentiating or protecting against disease. As many of these studies were performed with small sample sizes, determining if adequate statistical power was obtained is critical to data interpretation.

An increase in *Akkermansia muciniphila* was demonstrated in patients with diverticulosis and SUDD [166]. A reduced level of *A. muciniphila* colonized the mucosa of the diverticular region relative to a distant site of the colon unaffected by diverticula in patients with SUDD [119]. Despite its role in mucin degradation, *A. muciniphila* is considered beneficial or anti-inflammatory, due to its production of short chain fatty acids and involvement with maintaining intestinal homeostasis [169,170]. The protective role of *A. muciniphila* may explain the preferential colonization of distant healthy mucosa compared to that affected by diverticula.

Both studies evaluating the microbiome of diverticulitis patients utilized a control group that harbored a dysbiotic microbial composition [167,168]. Therefore, it is difficult to distinguish cause from consequence. Gueimonde *et al.* characterized the differences in *Bifidobacteria* in patients with diverticulitis, IBD, and colorectal cancer [167]. An increase in *Bifidobacterium longum* and *Bifidobacterium animalis* were seen in diverticulitis patients relative to both IBD and colorectal cancer patients [167]. *Bifidobacteria* are commonly utilized as a probiotic and the study [167] did not report if diet or use of dietary supplements were obtained at the time of surgery. A second study found that at the first episode of acute diverticulitis, the fecal microbiome differs significantly from controls, with the largest difference defined by a higher

diversity of the phylum Proteobacteria [168]. It is important to note that the controls for this study included a wide variety of diseases and conditions [168] (Table 1-4). Although some work has been done to evaluate microbial dysbiosis in diverticulosis, SUDD, and diverticulitis there are many limitations to these studies that warrant: (1) more comprehensive analysis of the entire microbiome, (2) identification and utilization of appropriate controls, and (3) analysis of mucosal-associated bacteria to address the those microbes mechanistically involved in disease pathogenesis.

Human intestinal fungal composition at homeostasis and disease

In comparison to the microbiome, the human gut mycobiome, comprising the fungal communities, is understudied [171]. As such, the methodology required for comprehensive analysis of the fungal composition still requires optimization. However, fundamental studies of the mycobiome in both health and disease can provide critical insights into intestinal disease processes.

Although evidence of a core microbiome has been demonstrated throughout the lower gastrointestinal tract [132], a core mycobiome has not been established nor is it stable over time [172]. Rather, a study in mice detected longitudinal variation in fungal composition while the bacterial population remained stable, indicating that the mycobiome may be more highly influenced by the environment [172]. Additionally, evidence that the fungal composition differs throughout the lower gastrointestinal tract has not been reported. This is likely because studies from mucosal samples are lacking as most studies have isolated fungal DNA from fecal samples [173,174].

Common metagenomic sequencing methods target the 18S rRNA gene, 28S rRNA gene, or the internal transcribed spacer (ITS) regions [171] (Figure 1-7). The ITS regions lie between

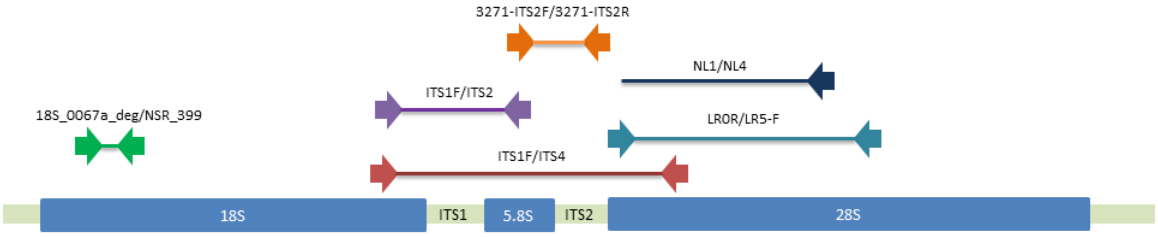

Figure 1-7. Fungal ITS region and sequencing primers.

The fungal internal transcribed spacer (ITS) regions (green) lie between the 18S rRNA, 28S rRNA, and the intercalary 5.8S rRNA gene (blue). Various primer sets have been designed to amplify locations within this genomic segment. Figure adapted from Cui *et al.* 2013 [171].

the 18S and 5.8S rRNA genes and the 5.8S and 28S rRNA genes. Although a variety of primers have been established for this region, there is inherent bias for each primer set as these methods are still undergoing optimization [171] (Figure 1-7). Additionally, unlike the bacterial 16S rRNA databases for profiling taxonomic identification, ITS-based databases are limited for fungi, resulting in misclassification or unidentified taxa [171]. However, as these databases have improved, more uniform identification methodologies have allowed for a well-characterized mycobiome [175,176].

In approximately 70% of adults, fungi are found within the gastrointestinal tract [177]. This niche is dominated by *Candida* species, with *Candida albicans* and *Candida tropicalis* identified most frequently by meta-analysis [173,174]. *Malassezia* is another frequently reported yeast in the gastrointestinal tract, although they are commonly considered skin commensals [173,174]. Therefore, whether these fungi are identified as skin contaminants in fecal samples or are indigenous to the human gut requires investigation. Other commonly reported gastrointestinal fungi include yeast and filamentous fungi [173,174]. The physiology of many filamentous fungi are not suitable to the human gastrointestinal tract [173]; therefore, if these culture-independent methods are identifying the true indigenous mycobiome or fungi ingested from the environment or food sources remains to be answered.

Innate immunity to fungi occurs primarily through recognition of the cell wall components chitin, β-glucans, and mannans [178,179] (Table 1-3). Fungal elements can be indirectly recognized through opsonization, such as the complement system which coats β-glucans in C3b and C3d for recognition by complement receptor 3 (CR3) on neutrophils and monocytes [180,181] (Figure 1-8A). Similar to bacteria, direct recognition of cell wall components and fungal DNA can be sensed by membrane-bound PRRs [182] (Figure 1-8B, C). The C-type lectin receptors (CLRs) DC-specific ICAM3-grabbing non-integrin (DC-SIGN), dectin-1, dectin-2, and mincle recognize *N*-linked mannans [183], β-glucans [184,185], high

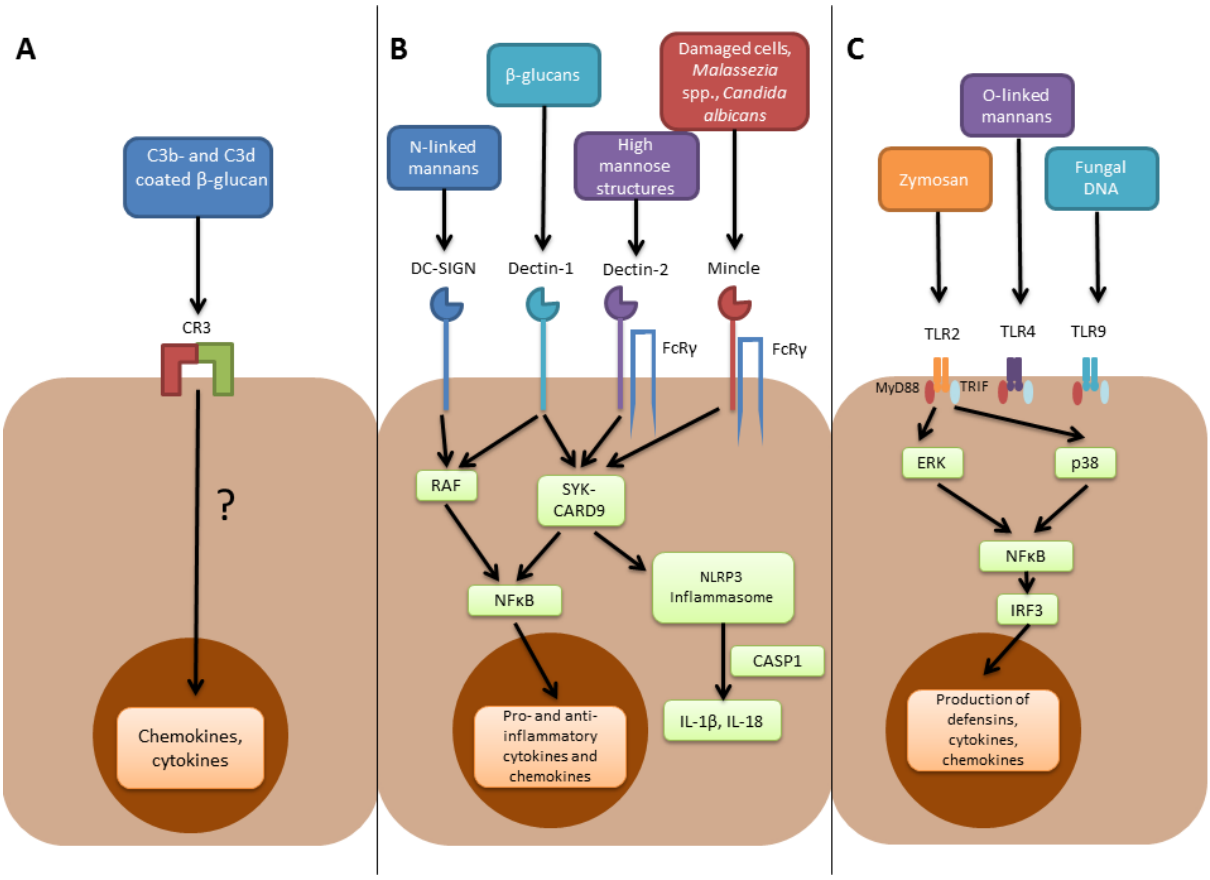

Figure 1-8. Mechanisms of innate fungal immunity.

(A) CR3 is a heterodimer of CD11b and CD18 that recognizes C3b- and C3d-coated β-glucans and through a currently unestablished mechanism, promotes an immune response through induction of chemokines and cytokines. (B) The CLRs DC-SIGN, dectin-1, dectin-2, and mincle recognize their respective PAMPs, activating either RAF or SYK-CARD9 pathways resulting in downstream activation of NF-κB. The SYK-CARD9 pathway can also activate the NLRP3 inflammasome, resulting in cleavage of pro-IL1β and pro-IL18. (C) TLR2, TLR4, and TLR9 activated by fungal ligands stimulate downstream activation the NF-κB pathway and IRF3 to promote an inflammatory response. Figure adapted from Romani *et al.* 2011 [182].

mannose structures [186], and specific fungal species (i.e. *Candida* spp. and *Malassezia* spp.) and damaged cells [187–189], respectively. Dectin-1 recognition of β-glucans is the primary mechanism of fungal phagocytosis in macrophages [184]. Dectin-1 induces downstream activation of canonical and non-canonical NF-κB signaling through a rapidly accelerated fibrosarcoma (RAF)-dependent mechanism or through synergistic activity of spleen tyrosine kinase (SYK) and caspase domain-containing protein 9 (CARD9) pathways [190]. In addition to activating NF-κB, the SYK-CARD9 pathway can also activate the NOD-, LRR-, and pyrin domain containing 3 (NLRP3) inflammasome which proteolytically cleaves pro-IL1β and pro-IL18 to the active forms [191] (Figure 1-8B). TLR2, TLR4, and TLR9 also recognize fungal antigens [192–194] (Table 1-3). Upon binding of the antigen to the receptor, downstream activation of canonical and non-canonical NF-κB occurs through a MyD88-dependent mechanism to induce transcription of pro-inflammatory mediators [93,195] (Figure 1-8C).

Bacterial-fungal interactions shape the microbial ecosystem

The diverse composition of the intestinal microbial ecosystem results in interactions between bacteria and fungi. In fact, examination of the murine gut found that commensal fungi are found in contact with bacteria in the lumen of the colon [196]. These microorganisms co-exist in the environment through symbiotic, synergistic, antagonistic, cooperative, or commensal interactions [197] (Table 1-5). Such interactions may be physical in nature, with bacteria and fungi co-existing as a biofilm, as a planktonic relationship, or through intrahyphal colonization [197]. Molecular communication of bacteria and fungi define the type of interaction. Antibiosis, or diffusion of typically antimicrobial metabolites from one organism to another, is one type of communication [197]. Other interactions may alter signaling mechanisms, physiochemical

Table 1-5. Bacterial-fungal interactions.

Interaction	Definition
Symbiotic	Close association of two or more groups which do not necessarily benefit from each other
Synergistic	Activity of one group benefits another
Antagonistic	Activity of one group harms another
Cooperative	Activity of one group benefits the recipient more than itself
Commensal	One group derives benefits from another without hurting or helping it

environmental conditions, chemotaxis, cellular contacts, nutrition, cooperative metabolism, protein secretion, and genetic transfer [197].

Under intestinal homeostasis in healthy individuals, bacteria and fungi exist commensally, providing additional benefit to the host [197]. Disruption of this ecosystem, through antibiotic and antifungal therapy or dysbiosis associated with disease states, can positively influence the growth of pathogenic microorganisms [198–200]. Diet may also play a role in defining bacterial-fungal relationships. A positive syntrophic relationship was demonstrated between *Candida*, *Prevotella*, *Ruminococcus*, and the archaea *Methanobrevibacter* [201]. This relationship relies on the degradation of starches by *Candida* to simple sugars which are fermentable by *Prevotella* and *Ruminococcus* [201]. The fermentation byproducts are a fuel source for *Methanobrevibacter* [201]. Therefore, studies that examine this microbial ecosystem in diverticulitis patients are warranted to gain a comprehensive understanding of the dysbiotic community. Understanding the role of the microbial ecosystem in diverticulitis patients and the interplay with the host innate immune response are imperative to gain insight into this complex disease.

Dissertation Overview

Diverticulosis is a common condition in Western countries that manifests in the sigmoid colon of affected individuals [1]. This condition results from the formation of diverticula, or the herniation of the mucosa and submucosa through the circular muscle of the colon [1]. Inflammation of the diverticula, or diverticulitis, occurs in 10-25% of individuals with diverticulosis [5]. Complications associated with diverticular disease account for a significant healthcare burden in the United States [14,15]. Despite the high prevalence of disease, its pathophysiology is largely unknown. Current data suggests that diverticula formation occurs through genetic predisposition, abnormal colonic wall structure, deregulation of colonic motility, and potentially low dietary fiber [2]. Diverticulitis was thought to develop due to obstruction of a diverticulum by a fecalith, resulting in inflammation and bacterial dysbiosis [3,25]. However, a previous study from our lab found that a genetic variant upstream of *TNFSF15* was associated with increased risk for diverticulitis [61], thus suggesting that defects in the immune response may be involved in diverticulitis pathogenesis.

The innate immune response is the first line of defense against foreign antigens. Literature aimed at understanding the influence of the immune system on the development of diverticular disease is limited. An increased number of macrophages have been described in patients with varying disease severity (diverticulosis, SUDD, and acute diverticulitis) [119,120]. Of interest to our group are M2 macrophages expressing CD163L1, a scavenger receptor with unknown ligand specificity [114,117,118]. In patients with IBD, these cells demonstrate a phenotype unlike other M2 resident macrophages as they are unable to secrete the anti-inflammatory cytokine IL-10 [118]. IL-10 is critical for macrophage conditioning to TLR ligands and promoting intestinal homeostasis [107].

Several investigators have proposed a role for bacterial dysbiosis in the pathogenesis of diverticulitis [3,25]; however, a comprehensive analysis of the microbial contribution to disease has been lacking. Few studies have reported on the bacterial composition of patients with diverticulosis, SUDD, and acute diverticulitis [119,166–168] but the data is difficult to interpret due to lack of consistency in results, bias in methodology, and use of control populations that harbor dysbiotic communities. Additionally, the microbial ecosystem is comprised of a dynamic population of bacteria, fungi, viruses, and other microorganisms. Thus, a comprehensive analysis of other microbial populations and identifying their contribution to the ecosystem as a whole through positive or negative interactions is imperative to determine whether microbial dysbiosis contributes to the pathogenesis of diverticulitis.

Based on the data suggesting that the immune system may be involved in diverticulitis pathogenesis, the work presented in this dissertation sought to evaluate the interplay of the innate immune system and microbial dysbiosis. Therefore, we hypothesized that **deregulation of the innate immune system contributes to diverticulitis pathogenesis by promoting a dysbiotic microbial ecosystem.** The following aims were developed and completed to test this hypothesis:

Aim 1: Identify the molecular pathways associated with diverticulitis compared to non-diverticulosis controls.

Although various etiological pathways have been described for diverticular disease, the molecular mechanism of disease pathogenesis remains to be elucidated. RNA-seq, an unbiased and genome-wide approach to evaluate the transcriptome of full-thickness sigmoid colon tissue was performed on patients with chronic, recurrent diverticulitis and non-diverticulosis controls. Differentially expressed genes were identified and subjected to Gene Ontology pathway analysis. Additionally, to gain an understanding of the co-expression network of commonly expressed genes, weighted gene co-expression network analysis (WGCNA) was performed to identify hub genes, or those that were highly interactive within the network.

Aim 2: Elucidate the molecular phenotype of CD163L1$^+$ macrophages in diverticulitis patients.

A previous report demonstrated a higher number of CD68$^+$ macrophages in the sigmoid colon of patients with diverticulosis [119]. Using the previously reported RNA-seq data set from Aim 1, we performed Spearman correlation of transcripts associated with macrophage signaling to identify molecular signatures. Using indirect immunofluorescence, we evaluated expression of the scavenger receptor, CD163L1 and the pro-inflammatory chemokine CXCL10 in the lamina propria of diverticulitis patients relative to controls. Furthermore, serum CXCL10 levels were measured relative to diverticulosis patients and non-diverticulosis controls.

Aim 3: Determine how the bacterial and fungal composition, as well as transkingdom interactions differs amongst chronically diseased tissue and non-affected adjacent tissue in diverticulitis patients.

Diverticulitis is commonly treated with empiric antibiotics, thereby suggesting a role for bacterial dysbiosis in disease pathogenesis [6]. However, studies evaluating the microbial contribution to disease have been limited or subject to bias. Therefore, by utilizing each patient as their own control and studying full-thickness sigmoid colon tissue obtained from areas of chronic disease and non-affected adjacent tissue, we can control for differences due to diet, genetics, or other confounders. 16S rRNA sequencing and ITS sequencing was performed to identify OTUs that were significantly different between chronically diseased tissue and adjacent tissue. Phylogenetic Investigation of Communities by Reconstructed States (PICRUSt) was performed from virtual metagenomic data to infer the functional mechanistic pathways associated with each tissue type. Additionally, since the human intestines function as a dynamic ecosystem, transkingdom interactions were evaluated and compared for each tissue type.

Although mortality associated with diverticular disease and diverticulitis contributes to a higher healthcare burden than IBD [14], research into the pathogenesis of disease has been

limited. Many of the currently identified mechanisms (i.e. colonic wall structure abnormality, deregulated colonic motility, dietary factors) are non-specific and likely contribute to diverticulum formation. Thus, identifying factors involved in diverticulitis pathogenesis are of immediate need. Previous work found a genetic variant upstream of *TNFSF15* associated with surgical diverticulitis [61], suggesting that deregulation of the immune response may be involved in the risk for developing diverticulitis. The aims presented above address this need by examining the interaction of the microbial ecosystem with the innate immune response in patients with chronic, recurrent diverticulitis. Future directions based on this work will further elucidate the molecular and genetic mechanisms of disease pathogenesis, identify serum biomarkers of disease severity, and evaluate objective biological criteria in medical and surgical decision making.

Chapter 2

RNA-SEQ IMPLICATES DEREGULATION OF THE IMMUNE SYSTEM IN THE PATHOGENESIS OF DIVERTICULITIS

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Author Contributions

Conception and design of experiments: GSY and WAK; Identified patients and procured clinical data: KMS, CSC, LH, and SD; Performed experiments: KMS, DMK, SE, AS, and YIK; Performed histology: DMK; Analyzed and interpreted results: KMS, DMK, AS, YIK, GSY, and WAK; Drafted the manuscript: KMS and GSY; Edited and approved the final version of the manuscript: KMS, CSC, SE, LH, SD, AS, YIK, GSY, WAK.

Abstract

Individuals with diverticula or outpouchings of the colonic mucosa and submucosa through the colonic wall have diverticulosis, which is usually asymptomatic. In 10-25% of individuals, the diverticula become inflamed, resulting in diverticulitis. Very little is known about the pathophysiology or gene regulatory pathways involved in the development of diverticulitis. To identify these pathways, we deep sequenced RNAs isolated from full-thickness sections of sigmoid colon from diverticulitis patients and control individuals. Specifically for diverticulitis cases, we analyzed tissue adjacent to areas affected by chronic disease. Since the tissue was collected during elective sigmoid resection, the disease was in a quiescent state. A comparison of differentially expressed genes found that Gene Ontology (GO) pathways associated with the immune response were up-regulated in diverticulitis patients compared to non-diverticulosis controls. Next, weighted gene co-expression network analysis was performed to identify the interaction between co-expressed genes. This analysis revealed *RASAL3*, *SASH3*, *PTPRC*, and *INPP5D* as hub genes within the brown module eigengene, which highly correlated (r=0.67, P=0.0004) with diverticulitis. Additionally, we identified elevated expression of downstream interacting genes. In summary, transcripts associated with the immune response were up-regulated in adjacent tissue from the sigmoid colons of chronic, recurrent diverticulitis patients. Further elucidating the genetic or epigenetic mechanisms associated with these alterations can help identify those at-risk for chronic disease and may assist in clinical decision management.

Introduction

Diverticular disease, encompassing both diverticulosis and diverticulitis, describes a spectrum of changes occurring most frequently within the sigmoid colon of adults in Western countries [5]. This condition initiates as diverticula or outpouchings of the colonic mucosa and submucosa through the colonic wall where blood vessels penetrate the muscle layer [5,202]. This asymptomatic condition is known as diverticulosis and its incidence increases with age. In approximately 10-25% of individuals with diverticulosis, inflammation of the diverticula leads to the development of diverticulitis [5]. Complications associated with diverticular disease, including surgery, account for a significant healthcare burden in the United States and overall, contributed to more healthcare visits than inflammatory bowel disease (IBD) in 2009 [14]. Yet the pathobiology of this disease is very poorly understood. Which patients will follow a benign course versus those who will develop diverticulitis is poorly predicted by clinical criteria. A better understanding of the pathogenesis of diverticulitis is required to uncover more effective disease management strategies.

Historically, a low-fiber diet was considered a causal factor in the development of diverticulosis [39], but this notion has since been refuted [203]. Obesity is a well-established risk factor for diverticulitis [53,204] and smokers have an increased risk of diverticular perforation/abscess relative to non-smokers [51]. Although both environmental factors and genetic susceptibility have been implicated in the risk for diverticulitis, little is known about the biological mechanisms associated with development of diverticulosis and subsequently, diverticulitis. Genetics may play a major role in the disease phenotype with the estimated heritability of diverticular disease found to be 40-53% by two twin studies [58,59]. Although genome-wide association studies (GWAS) have the advantage of identifying at-risk alleles for complex diseases, the ability of GWAS to study the mechanism of disease pathogenesis is limited

since many of the identified single nucleotide polymorphisms (SNPs) map to non-coding regions of the genome [205]. More recently, examining the overlap of GWAS with expression quantitative trait loci (eQTL) have helped understand how SNPs located within a regulatory element affects gene expression [206]. Unfortunately, studies examining the genetic contribution to diverticular disease are sparse and GWAS has yet to be reported.

We conducted RNA-seq analysis on transcripts isolated from full-thickness adjacent sigmoid colon tissues from individuals with chronic, recurrent diverticulitis during disease quiescence (n=20) and non-diverticulosis controls (n=5) to identify pathways associated with disease. We found 1,381 differentially expressed genes, of which 314 had a |fold change| >1.60. Gene ontology (GO) pathway analysis identified that a majority of these genes were associated with up-regulation of the immune response in diverticulitis patients relative to controls. Network analysis found that highly connected hub genes included the immune regulators: RAS Protein Activator-Like 3 (*RASAL3*), SAM and SH3 Domain-Containing 3 (*SASH3*), Protein Tyrosine Phosphatase, Receptor Type C (*PTPRC*), and Inositol Polyphosphate-5-Phosphatase D (*INPP5D*). Overall, these data suggest that immunoregulatory defects may be involved in the pathogenesis of diverticulitis.

Materials and Methods

Study design and specimen collection

This was a retrospective cohort study performed at the Pennsylvania State University College of Medicine with Institutional Review Board (IRB) approval. Chronic, recurrent diverticulitis patients were consented between April 2010 and August 2014. At the time of elective sigmoid resection, colonic tissue was collected into the Penn State Hershey Colon and

Rectal Diseases Biobank. Diverticulitis was confirmed by pre-operative CT scans and histopathologic evaluation of surgical specimens. Immediately after resection, surgical tissues were transported from the operating room to the surgical pathology lab where several full-thickness segments of tissue were obtained and stored in RNAlater (Invitrogen, Carlsbad, CA). Adjacent tissue was obtained 5-10 cm away from areas of chronically-diseased tissue. Diverticulitis patients with a concurrent diagnosis of IBD or other inflammatory conditions, recent use of immunosuppressives, cancer, dysplasia, or dysmotility disorders were excluded. Controls were identified from patients undergoing sigmoid resection without evidence of diverticulosis for the following conditions: endometriosis, rectal prolapse, slow transit, and sigmoid volvulus.

RNA isolation

Approximately 250 mg of tissue stored in RNAlater was pulverized in a MultiSample BioPulverizer (Biospecs Products, Bartlesville, OK) atop a bath of liquid nitrogen. RNA was first isolated using TRIzol (Ambion, Waltham, MA) and chloroform. Following centrifugation, the aqueous phase was subsequently purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was analyzed using the Agilent Bioanalyzer 2100 and samples had an RNA integrity number ≥7.

RNA-seq

The cDNA libraries were prepared using the SureSelect Strand Specific RNA Library Preparation Kit (Agilent Technologies, Santa Clara, CA) as per the manufacturer's instructions. RNA-sequencing was performed as previously described [207]. The Illumina CASAVA pipeline v1.8

was used to extract the de-multiplexed sequencing reads. FastQC (v0.11.2) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to validate the quality of the raw sequence data. Additional quality filtering used FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) set at a quality score cutoff of 20. Next, the filtered reads were aligned to the human reference genome (build hg38) using Tophat (v2.0.9) [208] allowing 2 mismatches. Read counts were calculated using HTSeq [209] as provided with the Ensembl gene annotation package (release 78). RPKM (Reads per Kilobase per Million mapped reads) values were calculated after applying GC-content and quantile normalizations using an R package "*cqn*" (The R Project for Statistical Computing, Vienna, Austria). Volcano plots were constructed using R. Gene Ontology (GO) pathway analysis was performed using the Generally Applicable Gene Set Enrichment (*GAGE*) pathway analysis package for R [210].

Reverse transcription and quantitative PCR (RT-qPCR)

cDNA synthesis was performed from 100 ng of RNA using the SuperScript III First-Strand Synthesis kit (ThermoFisher Scientific, Leesport, PA) according to the manufacturer's instructions. *RASAL3*, *PTPRC*, *INPP5D*, and *SASH3* gene expression levels were assessed by RT-qPCR. The following primers were used to detect *RASAL3* (forward: TGGCTATCTCTCTGCTCCCAGACC; reverse: CCTCAAAGCACCCTGCCTCAAT), *PTPRC* (forward: GCCAGCACCTACCCTGCTCAGAAT; reverse: GGGGCACCAAGTGGATTAACACAA), *INPP5D* (forward: CTCCTCCTGCCCAGCTTCCTATG; reverse: TTTCTTCCAGCCTCAGCACTTGGT), and *SASH3* (forward: TACTCAATGGCAAGGTGGGCTCTT; reverse: AGCTCATGCAGGGTCTTAGGCTTG). cDNA was diluted to 10 ng/µl for qPCR in reactions containing SensiFAST SYBR® & Fluorescein master mix (Bioline, Taunton, MA) as previously

described [207]. Thermocycling conditions were 94°C for 3 min, 45 cycles of 94°C for 10 s and 68°C for 40 s. *TUBB3* (beta-tubulin 3) served as an internal reference gene and its expression was measured using the primers (forward: ACAACGAGGCGCTCTACGACATCT; reverse: AAGGAGGTGGTGACTCCGCTCA) under the same thermocycling conditions. Values were normalized to *TUBB3* and fold change was calculated using the formula $2^{-\Delta Ct}$. The data were subsequently log-transformed and two-tailed *t*-test performed.

Network analysis

Network analysis was performed from log2 RPKM values using the weighted gene co-expression network analysis (*WGCNA*) package in R [211]. Student's *t*-test was performed on log2 RPKM values to identify a set of genes with $P<0.05$ to be subjected to WGCNA (1,381 genes). The dataset was evaluated for outliers and one diverticulitis patient was subsequently removed from the analysis. Following the standard WGCNA protocol, using a soft thresholding power of 14, clustering was first performed using topological overlap matrix (TOM)-based dissimilarity followed by a clustering of consensus module eigengenes (MEs). This analysis resulted in multiple MEs comprised of genes that are highly co-expressed. The grey ME consisted of genes that were unassigned within an ME and were not further evaluated. Pearson correlation was performed to identify the relationship between MEs and clinical traits. The final data was exported into Cytoscape v3.4.0 [212] to visualize the network between the top weighted gene interactions for each module. GO pathway analysis was performed for each module using the "*GAGE*" package for R [210].

Statistical analysis

Statistical analyses were performed using R software v3.3.3. Two-tailed statistical tests were determined to be significant at $P \leq 0.05$. For demographics and clinical indices, two-tailed chi-squared tests were used to compare categorical variables while two-tailed *t*-tests were used to compare continuous variables. For the RNA-seq analysis, *q*-values were obtained by the false discovery rate method [213] and were considered significant if $q \leq 0.05$.

Results

Immune-associated differentially expressed genes distinguish diverticulitis patients and controls

Transcriptomic profiling is an unbiased approach that has been used effectively to identify deregulated signaling pathways in intestinal diseases, such as colorectal cancer [214]. We performed RNA-seq analysis on full-thickness sigmoid colon tissue obtained 5-10 cm away from areas of diseased tissue to identify pathways that could be involved in the development of disease while avoiding the inflammation associated with diverticulitis. We analyzed the transcriptome of diverticulitis patients (n=20) and non-diverticulosis controls (n=5) (Table 2-1). In total, 1,381 genes were differentially expressed ($P<0.05$), of which 314 genes displayed a |fold change| >1.60 (Figure 2-1A). Several genes displaying the greatest difference in expression were involved in immune system processes, including T-box 21 (*TBX21*), B Lymphoid Tyrosine Kinase (*BLK*), C-C Motif Chemokine Receptor 7 (*CCR7*), and T-Cell Leukemia/Lymphoma 1A (*TCL1A*).

To uncover pathways represented amongst the 314 differentially expressed genes, we subjected the list to GO pathway analysis. Of the significantly up-regulated pathways ($q \leq 0.05$), we highlighted three categories of pathways that were most prominently featured in our analysis,

Table 2-1. Demographics and clinical information.

	Diverticulitis n (%)	Control n (%)	*P*-value
Total n	20	5	
Sex			
Male	10 (50.0%)	2 (40.0%)	0.6889
Female	10 (50.0%)	3 (60.0%)	
Race			
Caucasian	20 (100%)	7 (100%)	1.0000
Age at surgery (years) mean ± SD (range)	57.3 ± 14.2 (36.5-78.0)	57.1 ± 10.6 (43.9-71.0)	0.9769
Body mass index (kg/m^2) mean ± SD (range)	26.8 ± 4.8 (20.0-42.0)	28.2 ± 7.6 (19.0-40.0)	0.6085
Smoking status			
Positive history	13 (65.0%)	1 (20.0%)	0.1904
Former smoker[a]	9 (40.0%)	1 (20.0%)	
Current smoker	5 (25.0%)	0 (0%)	
Negative history	7 (35.0%)	4 (80.0%)	

[a]Smoking cessation of ≥ 1 year prior to surgery, mean time between smoking cessation and surgery 18.1±14.6 years (range 1-43 years)

SD, standard deviation. Continuous variables analyzed by two-tailed *t*-test and categorical variables analyzed by two-tailed chi-squared test.

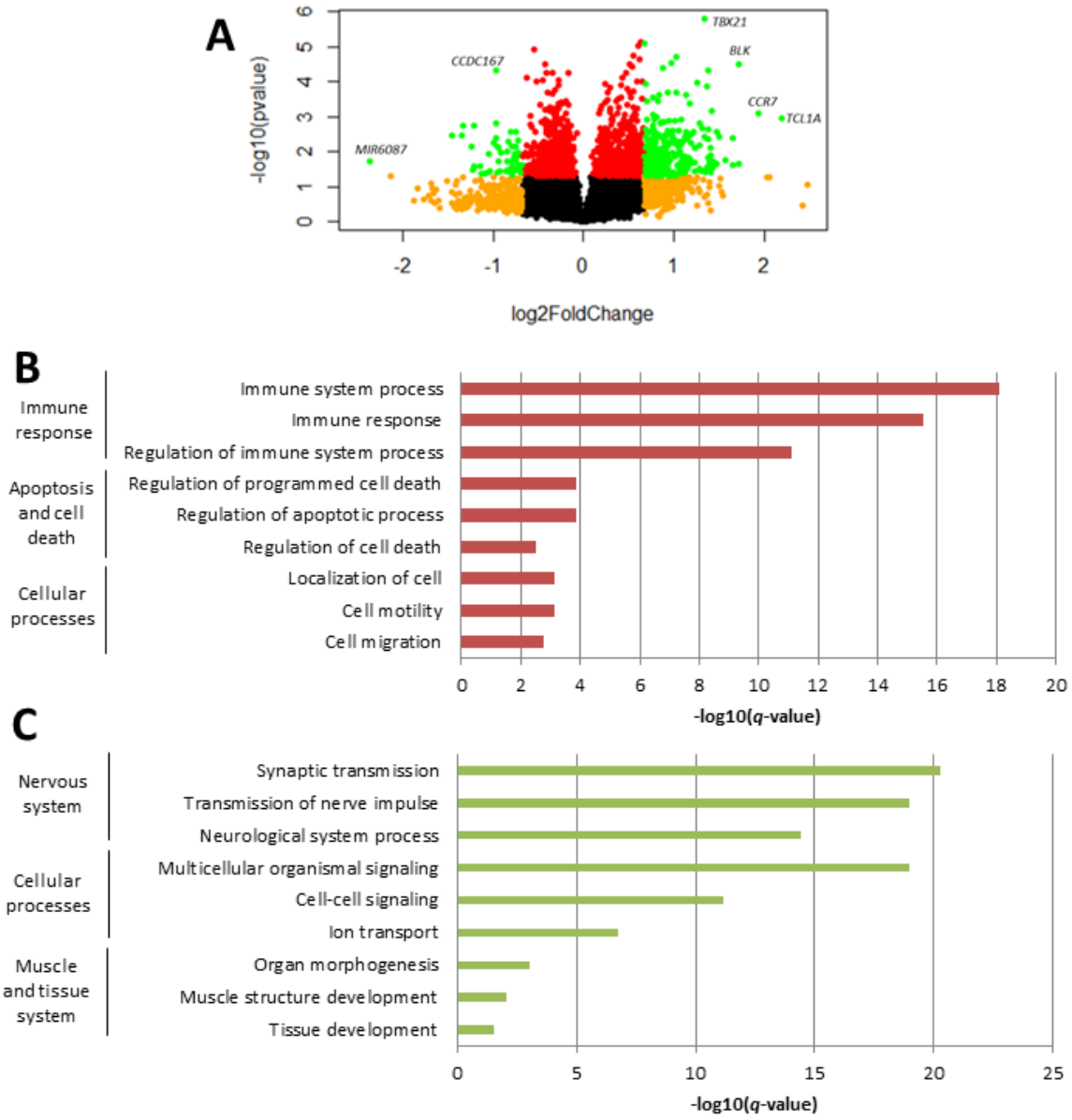

Figure 2-1. Expression of immune-associated transcripts is up-regulated in diverticulitis patients.

*(*A) Volcano plot of differentially expressed genes in diverticulitis (n=20) and control (n=5) sigmoid colon tissues with each gene represented by a point. Red represents genes with $P < 0.05$. Orange represents genes with |fold change| >1.60. Green represents genes with $P < 0.05$ and |fold change| >1.60. (B) Gene ontology categories of up-regulated genes. (C) Gene ontology categories of down-regulated genes.

including those involved in the immune response, apoptosis and cell death, and cellular processes (Figure 2-1B). Over 50% of the identified pathways were classified under the immune response with both the innate and adaptive immune systems represented. In addition, we identified significantly down-regulated pathways in diverticulitis patients compared to controls (Figure 2-1C). These data were also categorized into three groups: nervous system, cellular processes, and muscle and tissue system, with similar representation in each category.

To determine whether differentially expressed genes could segregate diverticulitis patients from controls, we subjected the immune response pathway, consisting of 55 genes, to hierarchical clustering analysis (Figure 2-2). Overall, the diverticulitis patients segregated from the controls, with the exception of one patient. Thus, differences in the immune-associated transcriptome delineate diverticulitis and from controls.

Immune-associated module eigengenes correlate with the presence of diverticulitis

To define the interactive network of co-expressed genes, weighted gene co-expression network analysis (WGCNA) was performed on the 1,381 genes that were differentially expressed between diverticulitis and controls ($P<0.05$). These genes were designated into five module eigengenes (MEs) based on co-expression (Figure 2-3A). The grey ME includes genes that were not assigned to another ME and thus, were not analyzed further. GO pathway analysis was performed to identify the pathway contribution for each ME. Similar to our previous GO analysis, immune-associated genes comprised the brown, yellow, and turquoise MEs. The blue ME included genes involved in the transcription process. Pearson correlation (r) was performed for each ME and clinical traits of interest (Figure 2-3A). The brown ME was positively correlated with diverticulitis ($r=0.67$, $P=0.0004$) and smoking status ($P=0.01$). Additionally, the turquoise ME was trending towards significance with age at surgery ($P=0.08$).

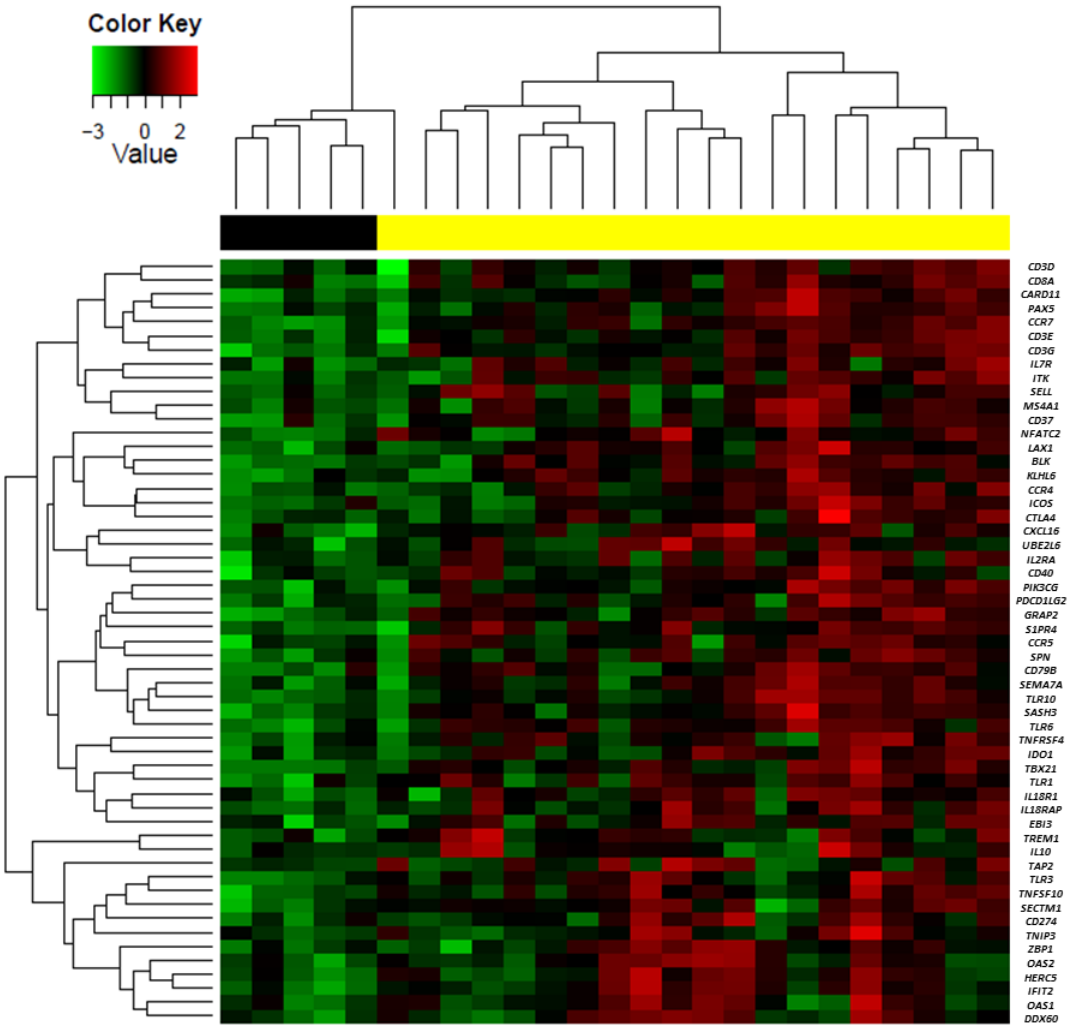

Figure 2-2. Hierarchical clustering analysis of genes comprising the immune system pathway segregates diverticulitis patients and controls.

Heat map depicting 55 genes clustered by diverticulitis patients (yellow bar) and controls (black bar). The color key indicates log2 RPKM values.

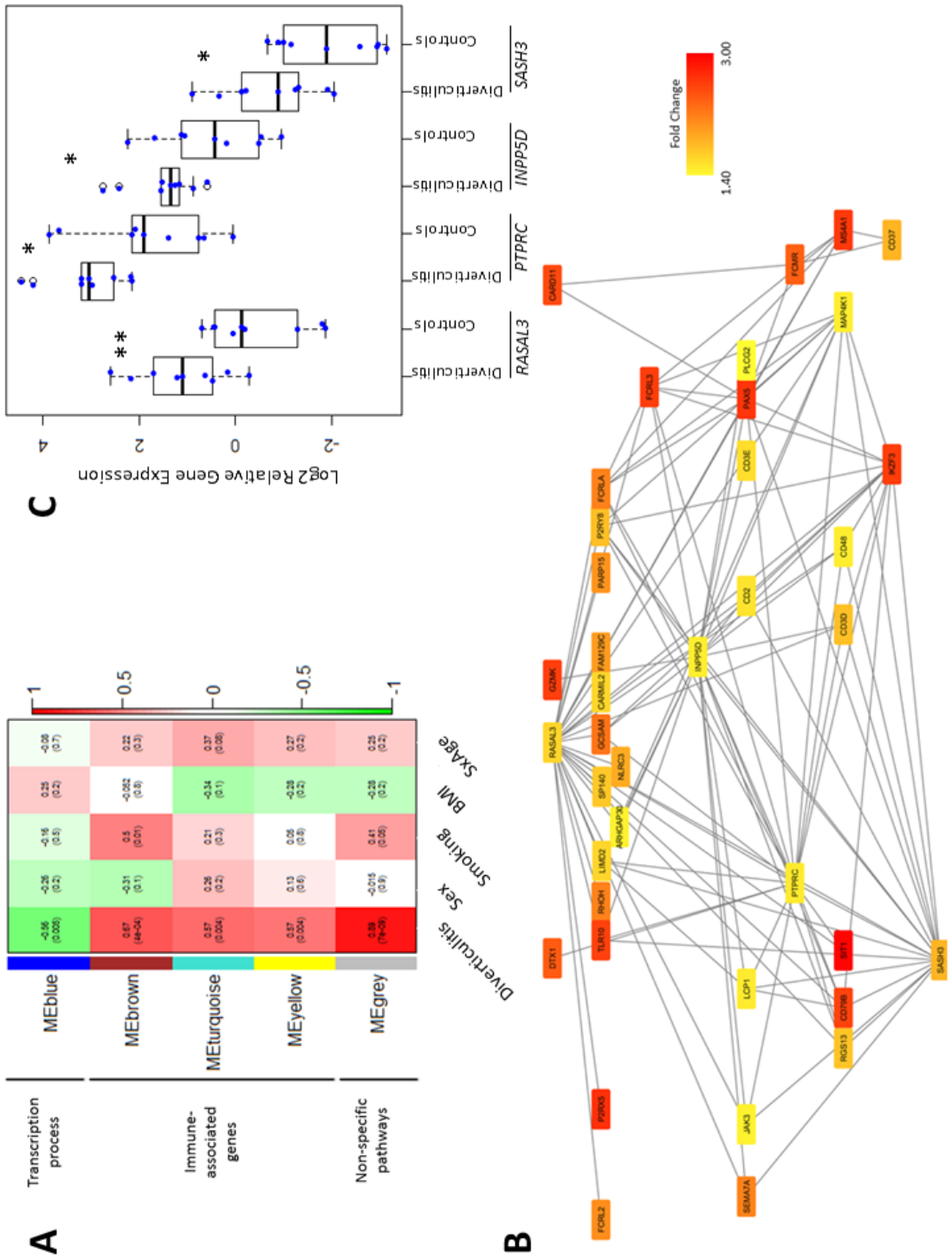
A
Transcription process
Immune-associated genes
Non-specific pathways
MEblue
MEbrown
MEturquoise
MEyellow
MEgrey
Diverticulitis
Sex
Smoking
BMI
xAge
1
0.5
0
-0.5
-1
B
Fold Change
3.00
1.40
C
Log2 Relative Gene Expression
4
2
0
-2
RASAL3
PTPRC
INPP5D
SASH3
Diverticulitis
Controls

Figure 2-3. Immune-associated module eigengenes (MEs) correlate with the presence of diverticulitis.

*(*A) Pearson correlation was performed on MEs to evaluate the association between expression and clinical traits: diagnosis of diverticulitis, sex, smoking history (positive or negative history), body mass index (BMI), and age at surgery (SxAge). The heatmap is color-coded by correlation coefficient identified by the color key with the *P*-value listed in parenthesis. (B) Weighted gene co-expression network analysis identified gene co-expression networks for each module eigengene (ME), with the top interactions (weight ≥0.20) illustrated from the brown ME. Fold change gene expression of diverticulitis patients (n=20) relative to non-diverticulosis controls (n=5) was overlaid on the pathway as designated by the color key. (C) RT-qPCR from an independent cohort of diverticulitis patients (n=9) and controls (n=9) demonstrating log2 relative expression of *RASAL3, PTPRC, INPP5D,* and *SASH3*. Boxplots display median and interquartile range. **P*<0.05, ***P*<0.01.

Network interaction analysis identifies up-regulation of hub genes and downstream genes in patients with diverticulitis

An undirected network was constructed from the top weighted interactions for each module to identify hub genes and potential downstream effects. The brown ME is presented as it was highly correlated with diverticulitis (Figure 2-3B). We identified four hub genes, or genes with high connectivity, in the brown ME: *RASAL3*, *SASH3*, *PTPRC*, and *INPP5D* (more commonly known as *SHIP1*). For all four hub genes, gene expression in the diverticulitis cohort was higher relative to non-diverticulosis controls. Additionally, higher expression of genes downstream of the hub genes was also seen in the diverticulitis patients compared to controls. Biological replication of hub genes was performed in an independent cohort of diverticulitis patients (n=9) and controls (n=9) which confirmed up-regulated expression of these targets (Figure 2-3C). Overall, these data suggest that these hub genes may be involved in regulating the immune response and that this signaling network is deregulated in diverticulitis patients.

Discussion

Previously, our group uncovered a genetic association between tumor necrosis factor superfamily member 15 (*TNFSF15*) and surgical diverticulitis [61], suggesting that intrinsic defects in the host immune response may be involved in diverticulitis pathogenesis. To evaluate in a non-biased fashion the potential immunologic involvement and the global transcriptome contribution to diverticulitis, we performed RNA-seq on colonic tissues obtained from diverticulitis and control individuals. We identified an enrichment of pathways involved in the immune response, including both the innate and adaptive immune systems in patients with diverticulitis. These data were unexpected since the tissue used for this study was collected from patients during disease quiescence, away from an area of disease involvement. Identification of

deregulated highly interactive hub genes associated with immunoregulation provides evidence to suggest that defects in the host immune response may be involved in the pathogenesis of diverticulitis.

Association of MEs with clinical traits identified various potential associations with smoking status and patient age at surgery. We found that the brown ME also correlated with smoking status. This result is not surprising since smoking is an environmental risk factor for diverticulitis [50], with one predicted mechanism being deregulation of the immune system [215,216]. There are currently no data indicating how smoking cessation influences the colonic immune system. Therefore, we included both former and current smokers in our analysis, which might be contributing to the significance of this ME. However, we also found a potential association with age at surgery and the turquoise ME. This ME includes immune-associated genes and those involved in the protein kinase cascade. Although diverticulitis is commonly associated with the older population, a small percentage (<5%) develop this disease at a younger age (<40 years old) [217]. More robust studies are needed that compare diverticulitis patients diagnosed prior to 40 years old to age-matched controls. This analysis would be imperative to confirm this association and to identify the transcriptomic contribution to age-related diagnosis of diverticulitis.

Network interaction of the brown ME identified four immune-associated hub genes: *RASAL3, SASH3, PTPRC,* and *INPP5D*. Neither *RASAL3* nor *SASH3* have been studied in inflammatory diseases, although *RASAL3* regulates natural killer T-cell expansion and function in mice [218]. *INPP5D,* also known as *SHIP1*, is a leukocyte-specific molecule that negatively regulates phosphatidylinositol 3-kinase (PI3K) activation. SHIP1 dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) to phosphatidylinositol-3,4-biphosphate (PtdIns(3,4)P_2) [219,220]. *SHIP*-deficient mice are prone to a Crohn's disease (CD)-like mucosal inflammation [221]. Similarly, reduced levels of INPP5D/SHIP1 were found from intestinal

biopsies from CD patients [222]. This is contrary to the results seen in our study, which demonstrates increased *INPP5D/SHIP1* gene expression in diverticulitis patients (fold change: 1.50), and thus warrants further investigation to understand the potential differences in disease processes. Increased expression of *INPP5D/SHIP1* may result in enhanced dephosphorylation of PtdIns(3,4,5)P_3, disrupting various signaling pathways, including the Akt pathway, which is involved in apoptosis [223]. In fact, overexpression of *INPP5D/SHIP1* induced caspase (CASP)-3 and -9 activity *in vitro* [224]. Although we did not detect significant up-regulation of *CASP3* (fold change: 1.11) or *CASP9* (fold change: 1.18), we identified up-regulation of apoptosis pathways in diverticulitis patients relative to controls (Figure 2-1B). Overall, we describe an up-regulation of *INPP5D/SHIP1* and the precise role for this gene in diverticulitis requires further investigation.

PTPRC, also known as CD45, is an abundant cell surface glycoprotein involved in protein tyrosine phosphorylation [225]. The primary substrates for PTPRC are Src-family tyrosine kinases (SFKs), involved in initiation of leukocyte-specific immune responses [225]. A previous report suggested that up-regulation of PTPRC results in high levels of inflammatory cytokines [226]. A similar mechanism was described in our study. Diverticulitis patients demonstrated higher levels of *PTPRC* transcripts relative to controls (fold change: 1.52). Up-regulation of the PTPRC pathway may contribute to the chronic nature of disease in these individuals, increasing the risk for recurrent diverticulitis.

In summary, we used RNA-seq analysis on full thickness sigmoid colon tissues to identify molecular pathway that differed between diverticulitis patients and controls. Previously, the pathophysiology of diverticulitis was thought to involve diet, age, and obesity [2]. The findings from the current study provide additional, non-biased evidence that implicates a deregulation of the immune system in the pathogenesis of diverticulitis. Further characterizing

these specific immunologic differences may allow the development of objective biologic criteria that could eventually help in clinical decision making.

Acknowledgements

Thank you to Drs. K. McKenna, E. Messaris, D. Stewart, and F. Puleo for contributing patient samples to the Biobank, the patients for their participation in the study, and the members of the Koltun lab for helpful discussion throughout the course of this work. This publication was supported by the Carlino Fund for IBD Research and in part by the National Center for Advancing Translational Sciences (NCATS), grants UL1 TR000127 and TL1 TR000125 (Schieffer).

Chapter 3

ACCUMULATION OF CXCL10-EXPRESSING CD163L1^{+} MACROPHAGES IN THE SIGMOID COLON OF SURGICALLY MANAGED DIVERTICULITIS PATIENTS

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This work was submitted to *Annals of Surgery.*

Author Contributions

Conception and design of experiments: KMS, WAK, and GSY; Performed experiments: KMS and SE; Analyzed and interpreted results: KMS, SE, WAK, and GSY; Drafted the manuscript: KMS and GSY; Edited and approved the final version of the manuscript: KMS, SE, WAK, GSY

Abstract

Objective: We sought to investigate whether CD163L1^{+}CD68^{+} macrophages are potentially involved in the deregulated innate immune pathway associated with surgical diverticulitis.

Background: While the exact clinical indications for surgery for diverticulitis patients are variable, most cases undergo multiple episodes of acute disease. Understanding the molecular basis of surgical diverticulitis may allow for a more objective assessment of disease status. By RNA-seq analysis of intestinal tissue, we previously found that the innate immune response was deregulated in surgical diverticulitis patients.

Methods: We assessed tissue from an uninvolved area adjacent to a region of the sigmoid colon chronically affected by diverticulitis. We performed Spearman's correlation on transcripts associated with macrophage signaling identified from our prior RNA-seq analysis. We confirmed altered *CD163L1* and *CXCL10* gene expression levels via RT-qPCR from an independent cohort of diverticulitis patients and controls. We used immunofluorescence microscopy to localize CD163L1^{+}CD68^{+} macrophages and CXCL10 levels in intestinal tissue and ELISA to measure CXCL10 levels in patient serum.

Results: We identified a positive correlation between *CD163L1* and *CXCL10* gene expression using our previous RNA-seq data. We found an increased number of CD163L1^{+}CD68^{+} macrophages in the sigmoid colons of diverticulitis patients relative to controls (P=0.036). Those at the apex of the crypt expressed the chemokine CXCL10. These diverticulitis patients also displayed heightened CXCL10 levels in their serum (P=0.007).

Conclusions: Our results suggest that CD163L1^{+}CD68^{+}CXCL10^{+} cells contribute to the pathogenesis of diverticulitis. CXCL10 may serve as a prognostic biomarker to aid in clinical decision making for diverticulitis patients.

Introduction

Diverticulosis is a benign condition characterized by the formation of diverticula, which are herniations of the colonic mucosa and submucosa through the colonic wall [202]. These outpouches usually form in weakened areas where blood vessels penetrate the bowel wall [5,202]. In the United States, approximately 40-50% of individuals aged 60 years will have diverticulosis with the incidence increasing with age [5,10]. In 10-25% of individuals with diverticulosis, inflammation of the diverticula results in diverticulitis [5]. The standard medical therapy for patients with uncomplicated diverticulitis is the administration of antibiotics [227]. However, some patients undergo multiple episodes of diverticulitis within a relatively short time period and are therefore viewed as suffering from chronic, recurrent diverticulitis. In severe cases, these patients require surgical resection of the affected tissue to manage their disease [228]. The exact clinical indication for surgery is variable but often requires multiple bouts of disease episodes. Typically, when these individuals undergo elective surgery, the disease is in a quiescent phase, several weeks after their most recent period of active inflammation. Treatment of individuals with diverticular disease is a substantial healthcare burden in the United States, costing about $2.6 billion dollars for inpatient care in 2009 [14]. A biomarker that would prognosticate the course of disease would aid in clinical decision making and possibly avoid multiple bouts of disease by identifying surgical candidates earlier.

The innate immune system functions as an intrinsic host defense mechanism against infection from enteric pathogens [229]. Macrophages are critical mediators of the innate immune response in the gut as they reside in the lamina propria, engulf foreign pathogens, and secrete immunostimulatory factors, such as cytokines and chemokines [230,231]. CD163 molecule-like 1 (CD163L1) is a scavenger receptor found on macrophages which demonstrates endocytic properties against a currently unknown ligand [117]. Based on its *in vitro* cytokine differentiation

profile, CD163L1-expressing macrophages display an anti-inflammatory M2 phenotype [118]. Little is known about the phenotype of these cells in disease states or during an acute inflammatory process; however, patients with inflammatory bowel disease (IBD) were found to harbor $CD163L1^+$ macrophages with impaired secretion of interleukin 10 (IL-10) [118].

Macrophages are recruited to sites of inflammation by sensing immunostimulatory cytokines and chemokines [96]. C-X-C motif chemokine 10 (CXCL10), also known as interferon-gamma induced protein 10 (IP-10), is a pro-inflammatory chemokine whose expression is induced by interferon-gamma (IFNγ) and type 1 interferon [232]. This chemokine is secreted by many cell types, including monocytes, macrophages, activated neutrophils, and intestinal epithelial cells [96,233]. CXCL10 mediates the inflammatory response by recruiting pro-inflammatory cells expressing its cognate receptor C-X-C motif chemokine receptor 3 (CXCR3) (i.e. activated T lymphocytes and macrophages) to the site of pathogenic insult [233,234].

Few reports have investigated the role of the innate immune system in diverticulitis. Enrichment of $CD163^+CD68^+$ macrophages in the sigmoid colon was associated with complicated diverticulitis [120]. Similarly, an increase in $CD68^+$ macrophages was seen in the sigmoid colon of both diverticula-laden and distant areas of the bowel in patients with diverticulosis and symptomatic uncomplicated diverticular disease [119]. We have previously identified an up-regulation of innate immune response signaling pathways in surgical diverticulitis patients relative to non-diverticulosis controls by profiling transcripts isolated from colonic tissues using RNA-seq [235]. Therefore, these reports suggest that deregulation of the innate immune system could contribute to diverticulitis. Here, we sought to determine whether $CD163L1^+$ macrophages are potentially involved in the deregulated innate immune pathways we identified previously in our RNA-seq profiling of diverticulitis patients requiring surgery [235].

Materials and Methods

Specimen collection

This study was approved by the Pennsylvania State University College of Medicine Institutional Review Board (IRB). Patients with chronic, recurrent diverticulitis (n=37) were identified and consented to have their tissue collected into the Penn State Hershey Colon and Rectal Diseases Biobank following elective sigmoid resection. Previous episodes of diverticulitis were confirmed by computed tomography (CT) scans and surgical pathology. Surgical specimens were obtained as previously described [236]. Full-thickness sections of sigmoid colon were obtained and stored in either RNAlater (Invitrogen, Carlsbad, CA) or fixed in formalin. The tissue was collected from an uninvolved area of sigmoid colon 5-10 cm away from tissue displaying the consequences of chronic diverticulitis, specifically increased bowel wall thickness and rigidity, as previously described [236]. The tissue examined had normal bowel wall thickness and an absence of macroscopic inflammation. Diverticulitis patients were excluded if they had a history of IBD, cancer, or dysplasia. Our control population consisted of patients requiring sigmoid resection for diseases other than diverticular disease and without a history of diverticulosis and included sigmoid cancer (n=1), anal cancer (n=1), rectal prolapse (n=1), slow transit (n=1), sigmoid volvulus (n=1), and endometriosis (n=2). Tissue was obtained at least 5 cm away from the diseased portion of bowel to ensure that tissue reflected grossly normal adjacent bowel. None of the patients used immune modifying medications.

Reverse transcription and quantitative PCR (RT-qPCR)

RNA was isolated from tissue stored in RNAlater using TRIzol reagent (Ambion, Waltham, MA) as previously described [235]. In total, 100 ng of RNA was used for cDNA

synthesis using the SuperScript III First-Strand Synthesis kit (ThermoFisher Scientific, Leesport, PA) according to the manufacturer's instructions. *CXCL10* and *CD163L1* gene expression levels were assessed in cDNA using real-time qPCR. The following primers were used to detect *CXCL10* (forward: TCCATGTAGGGAAGTGATGGGAGAG; reverse: TCCTTAAAACCAGAGGGGAGCAAA) and *CD163L1* (forward: TCCTGTGGTTGAGAGAGCAGTCC; reverse: CAGTGTGACGGGCAAGTGGAGAT). cDNA was diluted to 10 ng/µl for quantitative PCR analysis (qPCR) in reactions containing SensiFAST SYBR® & Fluorescein master mix (Bioline, Taunton, MA) as previously described [237]. Thermocycling conditions were 94°C for 3 min, 45 cycles of 94°C for 20 s and 58°C for 30 s for *CXCL10* and 95°C for 3 min, 45 cycles of 95°C for 10 s and 55°C for 30 s for *CD163L1*. *TUBB3* (beta 3 tubulin) served as an internal reference gene and its expression was measured using the primers (forward: ACAACGAGGCGCTCTACGACATCT; reverse: AAGGAGGTGGTGACTCCGCTCA) and the following thermocycling conditions: 94°C for 3 min, 45 cycles of 94°C for 10 s and 68°C for 40 s. Values were normalized to *TUBB3* and fold change was calculated using the formula $2^{-\Delta Ct}$. The data were subsequently log-transformed.

Indirect immunofluorescence

Formalin-fixed tissue sections were paraffin-embedded and sectioned with 5 µm thickness. Slides were deparaffinized by two 5 min xylene washes followed by an alcohol gradation (100% ethanol for 3 min, thrice; 95% ethanol for 3 min, twice; 70% ethanol, once; water for 5 min). Antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a steamer for 20 min then cooled to room temperature for one hour. Slides were incubated with primary antibody overnight. The following antibodies were used at a 1:500 dilution: rabbit polyclonal anti-CD163L1 (HPA015663, Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-CD68 (sc-

70761, Santa Cruz Biotechnology, Dallas, TX), and mouse monoclonal anti-interferon-γ induced protein (IP-10) (also known as CXCL10) (ab8098, abcam, Cambridge, MA). The appropriate secondary antibody (1:800) was added (Alexa Fluor 647 goat anti-mouse IgG (H+L) and Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen, Carlsbad, CA) and slides were incubated for one hour prior to setting with the VECTASHIELD HardSet Mounting Media with DAPI (Vector Laboratories, Burlingame, CA). Slides were visualized using the 20x/ 0.75 Plan S Apo objective and under oil immersion using the 60x/ 1.42 Oil Plan Apo N objective on the DeltaVision Elite deconvolution microscope (GE Healthcare Life Sciences, Pittsburgh, PA) by Z sectioning. Using 3D images obtained from 60x magnification, cells were visualized from 5-6 random fields per patient using the same image acquisition settings (DAPI 10% transmission, 0.039 s exposure; FITC/Alexa Fluor 488 10% transmission, 0.0497 s exposure, CY-5/Alexa Fluor 647 32% transmission, 0.5903 s exposure). A program was developed in Volocity software version 6.3 (PerkinElmer, Akron, OH) to identify cells that were positive for CD163L1 and CD68. The program separated touching objects to ensure that only individual cells were counted.

CXCL10 measurement in serum

Blood samples from diverticulitis patients (n=13) were collected on the day of surgery and prior to sigmoid resection. Blood samples from non-diverticulosis controls (n=9) were collected either prior to surgical procedures for sigmoid cancer (n=1) or benign neoplasm (n=1) and from healthy controls (n=7) recruited as part of the Penn State Hershey Colon and Rectal Diseases Biobank. The serum was then stored in aliquots at -80°C until analysis. CXCL10 levels were quantified using the IP-10 (also known as CXCL10) enzyme linked immunosorbent assay (ELISA) kit (KAC2361, ThermoFisher, Leesport, PA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using R software version 3.3.1 (The R Project for Statistical Computing, Vienna, Austria). Two-tailed statistical tests were determined to be significant at $P \leq 0.05$. Spearman's correlation coefficient (ρ) was calculated from log2 reads per kilobase per million mapped reads (RPKM) values from previously reported RNA-seq data [235]. A correlation heatmap was generated using the *ggplot2* package in R [238]. For RT-qPCR, fold change values were log-transformed and two-tailed *t*-test performed. The non-parametric Mann Whitney U-test was used to compare the difference in cell counts between diverticulitis and controls.

Results

Expression of *CD163L1* in diverticulitis tissue correlates with a pro-inflammatory macrophage signature

The clinical demographics of surgical diverticulitis patients are presented in Table 3-1. Previously, we reported on RNA-seq analysis of full-thickness sigmoid colon tissue from diverticulitis patients requiring surgery relative to non-diverticulosis controls and found an up-regulation of pathways associated with the innate immune response [235]. It is important to note that in these individuals (hereafter referred to as diverticulitis), the disease was in a state of relative quiescence and the tissue we assessed was adjacent to areas of chronically diseased tissue [236]. To identify a potential mechanism contributing to these pathways, we selected a panel of fourteen transcripts involved in macrophage signaling and performed Spearman's correlation of log2 RPKM values from the diverticulitis patients (n=20) (Figure 3-1A). These transcripts included markers characteristic of pro-inflammatory M1 macrophages (*CD14*, *CD80*, *CD86*,

Table 3-1. Surgical patient demographics and clinical information

	Diverticulitis n (%)	Controls n (%)	*P*-value
Total n	37	7	
Sex			0.7785
Male	19 (51.4%)	4 (57.1%)	
Female	18 (48.6%)	3 (42.9%)	
Race			
Caucasian	37 (100%)	7 (100%)	1.000
Age at surgery, years mean ± SD (range)	58.9 ± 13.4 (36.5 – 78.4)	57.8 ± 8.7 (43.9 – 71.0)	0.8363
Body mass index (kg/m^2) mean ± SD (range)	26.3 ± 5.1 (20 – 42)	27.4 ± 6.6 (19 – 40)	0.6199
Smoking status			0.0992
Positive history	21 (56.8%)	1 (14.3%)	
Negative history	16 (43.2%)	6 (85.7%)	

SD, standard deviation. Continuous variables analyzed by two-tailed Student's *t*-test and categorical variables analyzed by two-tailed chi-squared test.

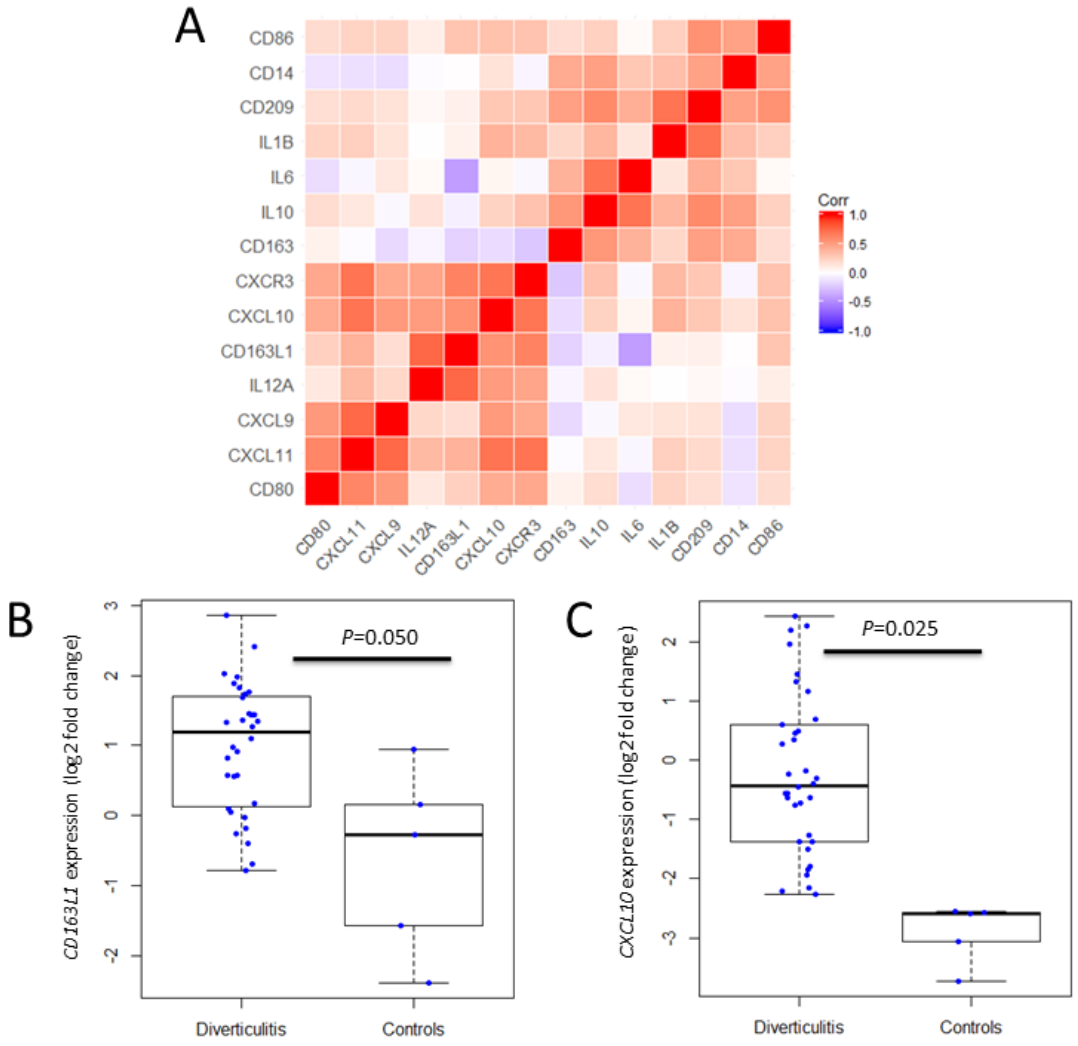

Figure 3-1. Expression of *CD163L1* in diverticulitis tissue correlates with a pro-inflammatory macrophage signature.

(A) Heatmap of Spearman correlation values of transcripts associated with macrophage signaling from log2 RPKM values obtained from RNA-seq of diverticulitis patients (n=20). Positive (red) and negative (blue) correlations are defined by the scale. (B) RT-qPCR analysis of *CD163L1* transcripts in RNA isolated from the sigmoid colon of diverticulitis patients (n=32) and controls (n=5). (C) RT-qPCR analysis of *CXCL10* transcripts in RNA isolated from the sigmoid colon of diverticulitis patients (n=34) and controls (n=5). In (B) and (C), boxplots display the median and interquartile ranges. Each point on the boxplot is the average of three PCR replicates from a single patient sample. The data is normalized to beta tubulin (*TUBB3*) levels.

CXCL9, CXCL10, CXCL11, CXCR3, IL1B, IL6, and *IL12A*) and anti-inflammatory M2 macrophages (*CD163, CD163L1, CD209,* and *IL10*). From this panel, we identified two distinct phenotypes associated with *CD163* and *CD163L1* when transcripts were clustered hierarchically. The cytokines *IL10* (ρ=0.53, *P*=0.015) and *IL6* (ρ=0.40, *P*=0.019), and the M2 marker *CD209* (ρ=0.49, *P*=0.014) were positively correlated with *CD163,* encoding the gene for a scavenger receptor commonly associated with the anti-inflammatory M2 macrophage phenotype. *CD163L1*, encoding the gene for an M2 macrophage scavenger receptor of unknown ligand, was positively correlated with transcripts associated with a pro-inflammatory phenotype, including *IL12A* (ρ=0.75, *P*<0.0001), the pro-inflammatory chemokine *CXCL10* (ρ=0.55, *P*=0.027), and the cognate receptor for CXCL10, *CXCR3* (ρ=0.63, *P*=0.0002).

As our previous study found an up-regulated immune response was present in these tissue sections [235] and CXCL10 has been associated with IBD [239,240], we focused on the correlation between *CD163L1* and *CXCL10.* To confirm these findings, RNA was isolated from full-thickness sigmoid colon tissue from an independent cohort of diverticulitis patients (n=18) and RT-qPCR analysis of *CD163L1* and *CXCL10* performed. We found that both *CD163L1* (Figure 3-1B) and *CXCL10* (Figure 3-1C) were up-regulated in these patients relative to controls (n=5). Thus, these data suggest that CD163L1$^+$ macrophages may localize in the sigmoid colon lamina propria of individuals with diverticulitis.

Increased levels of CD163L1$^+$CD68$^+$ macrophages localize within the lamina propria of surgical diverticulitis patients

To evaluate CD163L1$^+$ macrophages, we co-stained populations in sigmoid colon tissue sections from non-diverticulosis control (n=3) and diverticulitis patients (n=6) with the pan-macrophage marker CD68. Cells co-expressing CD163L1 and CD68 localized at the apex of the

colonic crypt within the lamina propria in both diverticulitis patients (Figure 3-2A) and controls (Figure 3-2B). We next quantified macrophages in multiple fields per tissue section and found an increased number of $CD163L1^{+}CD68^{+}$ macrophages in diverticulitis tissues relative to non-diverticulosis controls (14.4 ± 8.9 vs. 8.9 ± 6.0 cells, respectively; Figure 3-2C). Thus, these data indicate that diverticulitis patients requiring surgery harbor an increased level of $CD163L1^{+}CD68^{+}$ macrophages in the sigmoid colon, as compared to non-diverticulosis controls.

$CD163L1^{+}$ macrophages express CXCL10 in the sigmoid colon of surgical diverticulitis patients

As *CD163L1* expression was positively correlated with *CXCL10* expression in diverticulitis tissues (Figure 3-1A), we sought to determine how $CD163L1^{+}$ macrophages and CXCL10 may be associated. We utilized indirect immunofluorescence to evaluate CXCL10 protein localization in the sigmoid colon. CXCL10 localized within at the apex of the crypt in diverticulitis patients (Figure 3-3A). Upon co-staining sections with anti-CD163L1 antibodies, we noticed that $CD163L1^{+}$ macrophages at the crypt apex co-localized with CXCL10 (Figure 3-3B-C). Notably, this expression only involved those $CD163L1^{+}$ macrophages located at the apex of the crypt of the lamina propria, while those located deeper in the tissue towards the bottom of the crypt did not express the chemokine. $CXCL10^{+}$ cells that did not co-express CD163L1 were also detected (Figure 3-3B). Control patients lacked expression of CXCL10 (Figure 3-3D-F). These data indicate that a subset of $CD163L1^{+}$ macrophages express the pro-inflammatory chemokine CXCL10 and appears to be localized at the most luminally exposed portions of the intestinal epithelium.

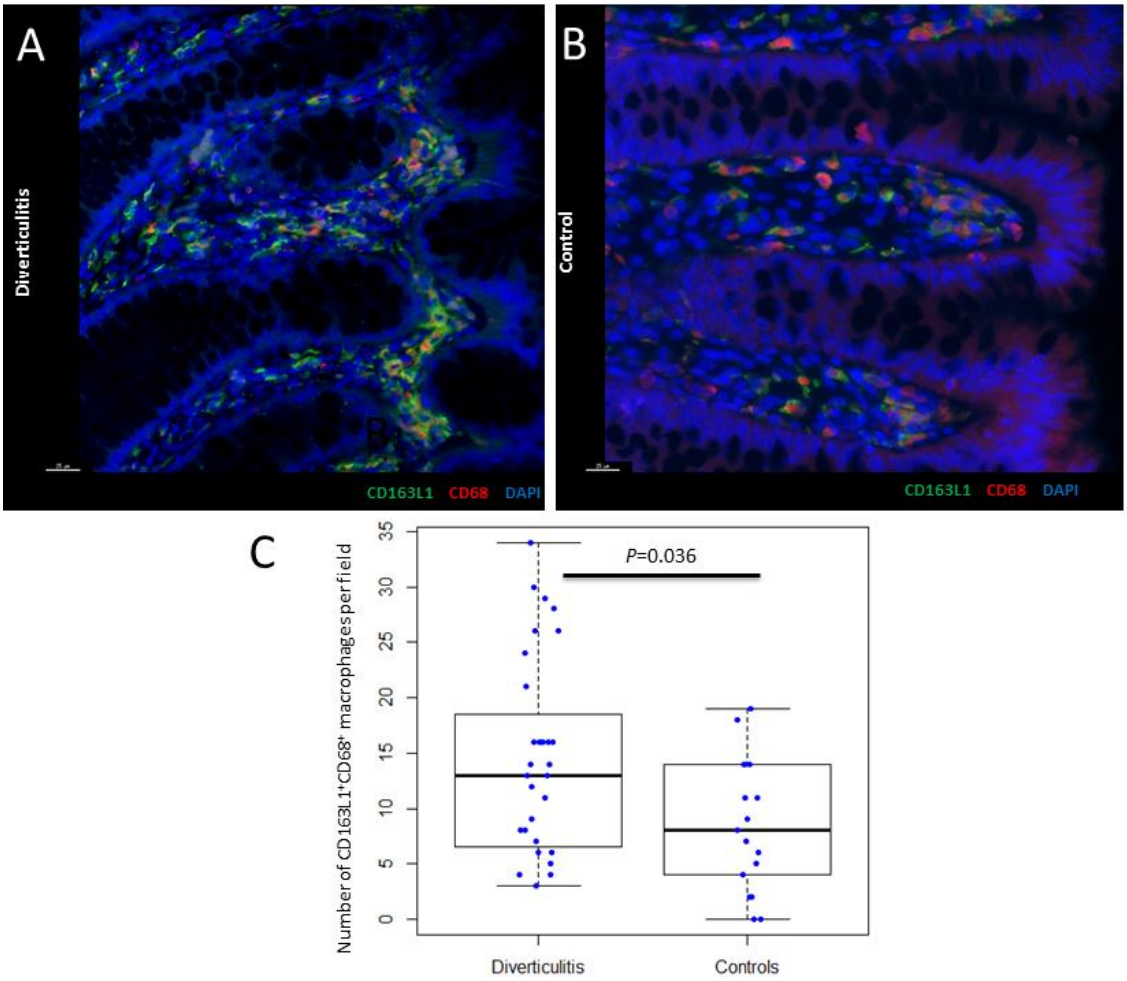

Figure 3-2. Increased levels of $CD163L1^{+}CD68^{+}$ macrophages are found within the lamina propria of diverticulitis patients.

(A) Representative image of a section of sigmoid colon from a diverticulitis patient stained with antibodies directed against CD163L1 (green) and CD68 (red). The slides were stained with nuclear stain DAPI as a reference. (B) As in (A) except tissue was obtained from a control patient. (C) Quantification of $CD163L1^{+}CD68^{+}$ cells in tissue sections. A total of 9 independent sections (from n=6 diverticulitis and n=3 controls) were evaluated with positive cells counted in 5-6 fields per sample. Boxplots display median and interquartile ranges.

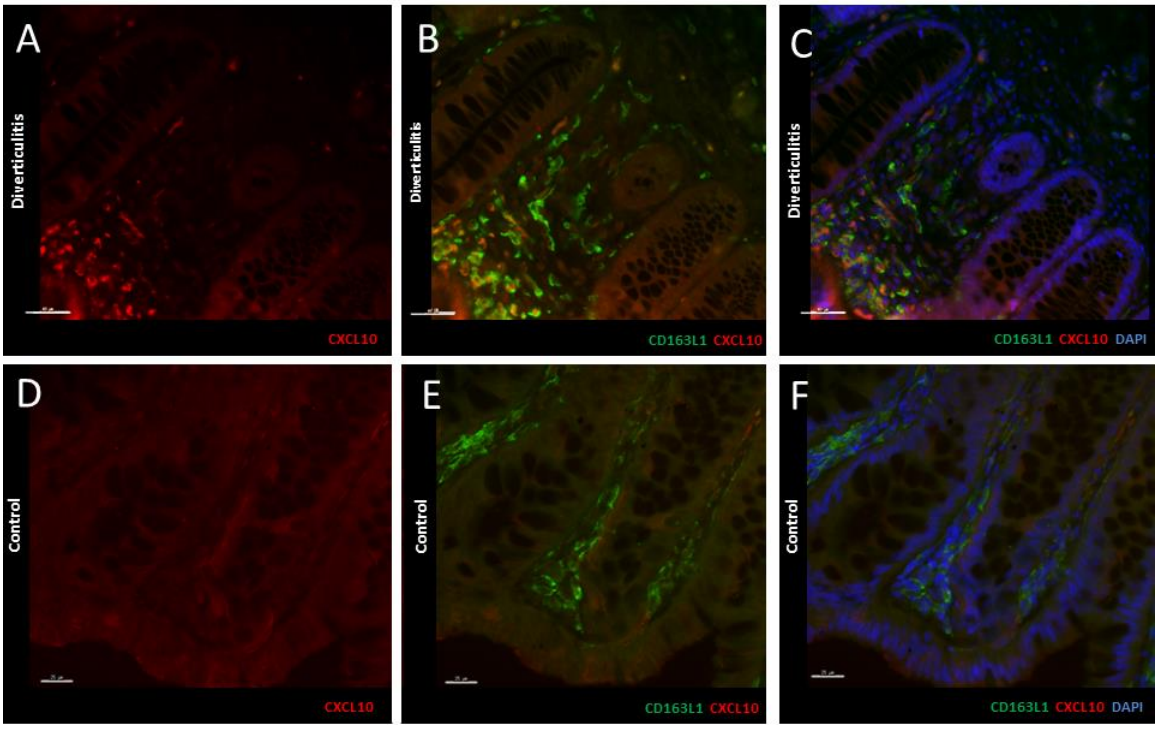

Figure 3-3. CD163L1^{+} macrophages express CXCL10 in the sigmoid colon of diverticulitis patients.

(A) Representative image of the sigmoid colon of a diverticulitis patient stained with an antibody against CXCL10 (red). (B) Representative image of the sigmoid colon of a diverticulitis patient stained with antibodies against CXCL10 (red) and CD163L1 (green). (C) Merged staining with CXCL10, CD163L1, and nuclear stain DAPI. (D) Representative image of the sigmoid colon of a control stained with an antibody against CXCL10 (red). (E) Representative image of the sigmoid colon of a control patient stained with an antibody against CD163L1 (green). (F) Merged staining with CXCL10, CD163L1, and DAPI.

Serum CXCL10 is increased in surgical diverticulitis patients

Because we found increased levels of *CXCL10* transcripts and CXCL10 protein within the diverticulitis intestine, we reasoned that elevated CXCL10 levels may be found in the serum of these patients. At the time of elective sigmoid resection, blood was collected from diverticulitis patients (n=13) and serum isolated. We used ELISA to quantitatively analyze serum CXCL10 compared to non-diverticulosis controls (n=9). The clinical demographics for these patients are described in Table 3-2. Overall, mean serum CXCL10 was significantly higher in diverticulitis patients (173.7 ± 101.2 pg/mL) compared with levels found in controls (74.7 ± 51.1 pg/mL, P = 0.007, Figure 3-4). These data indicate that diverticulitis patients undergoing surgery demonstrate increased levels of serum CXCL10 relative to non-diverticulosis controls.

Discussion

Studies aimed at understanding the immunologic mechanisms of diverticulitis have been limited [241]. For many years, it was widely believed that constipation and a low-fiber diet were the major causal factors for diverticulitis [241]. However, a recent report provided compelling evidence that refutes this assertion [23]. Empiric antibiotic usage and increased fiber intake are used in the treatment of uncomplicated diverticulitis; however, these treatments have recently been brought into question as well [242,243]. Thus, the pathophysiology of diverticular disease is very poorly understood with long standing caveats being brought into question. Our previous data suggested that an alteration in the innate immune system is associated with diverticulitis [235]. In this report, we provide evidence of an increased number of $CD163L1^{+}CD68^{+}$ macrophages in the lamina propria of surgical diverticulitis patients. $CD163L1^{+}$ macrophages localizing at the luminally exposed surface expressed the pro-inflammatory chemokine CXCL10. We also found

Table 3-2. Patient demographics and clinical information for serum CXCL10

	Diverticulitis n (%)	Non-diverticulosis Controls n (%)	*P*-value
Total n	13	9	
Sex			0.3858
Male	8 (61.5%)	3 (33.3%)	
Female	5 (38.5%)	6 (66.7%)	
Race			
Caucasian	13 (100%)	10 (100%)	1.000
Body mass index (kg/m^2) mean ± SD (range)	28.8 ± 5.2 (23 – 41)	27.9 ± 9.2 (19 – 46)	0.7723
Smoking status			0.8782
Positive history	3 (23.1%)	1 (11.1%)	
Negative history	10 (76.9%)	8 (88.9%)	

SD, standard deviation. Continuous variables analyzed by two-tailed Student's *t*-test and categorical variables analyzed by two-tailed chi-squared test.

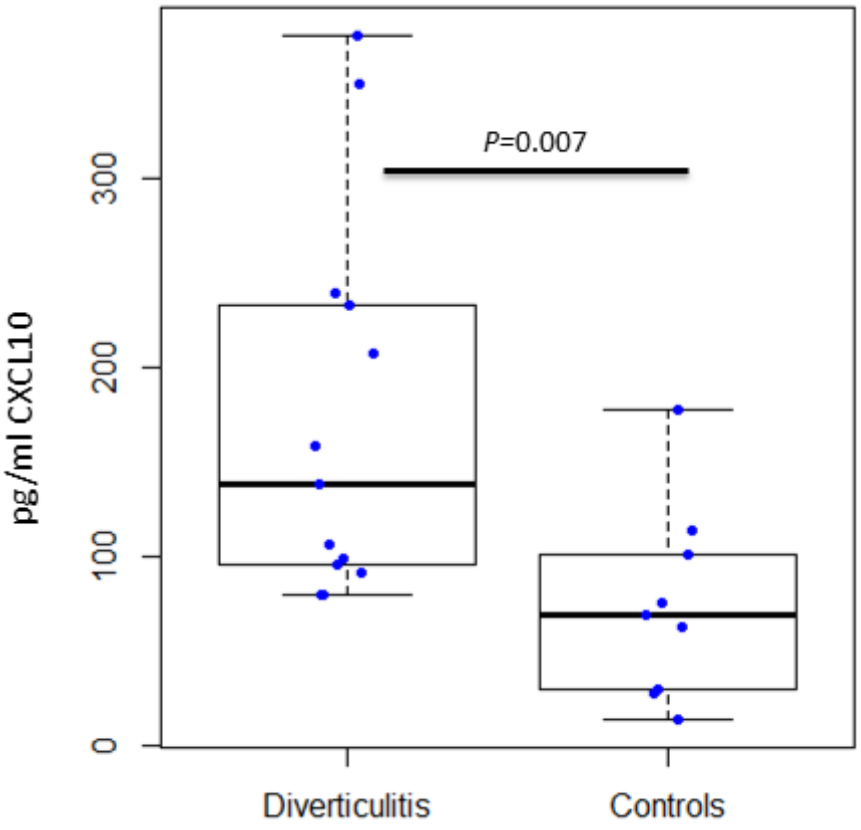

Figure 3-4. Serum CXCL10 is increased in diverticulitis patients.

Serum CXCL10 levels from chronic, recurrent diverticulitis patients (n=13) and non-diverticulitis controls (n=9) as detected by ELISA. Boxplots display the median and interquartile ranges. Each point on the boxplot is the average of two replicates from a single patient sample.

increased serum levels of CXCL10 in surgical diverticulitis patients even during relative disease quiescence. This implies a relatively chronic inflammatory state which may contribute or reflect recurrent diverticulitis leading to surgery in these individuals.

The *CD163L1* gene encodes a scavenger receptor expressed on macrophages and may have arisen by gene duplication of *CD163* [114]. $CD163L1^+$ cells contain clathrin-mediated endocytic properties [117]. Although the ligand for CD163L1 is currently unknown, cells expressing this receptor may be involved in resolving the inflammatory response [117]. $CD163L1^+$ macrophages localize primarily in the lamina propria of the intestines [117,118] and we confirmed this localization in the sigmoid colon of our specimens (Figures 3-2 and 3-3). We found an increased number of $CD163L1^+$ macrophages in colonic tissue sections from diverticulitis patients that were resected during a quiescent state of the disease (Figure 3-2). *In vitro*, cells expressing CD163L1 are designated as anti-inflammatory M2-like macrophages, as expression of the receptor was only associated with anti-inflammatory mediators, such as macrophage-colony stimulating factor (M-CSF) and IL-10 [117,118]. $CD163L1^+$ cells are a predominant macrophage population in the healthy colon [118]. In IBD patients, the proportion of $CD163L1^+$ macrophages was reduced as the local microenvironment likely stimulated differentiation of newly recruited monocytes to pro-inflammatory macrophages. Furthermore, those cells retaining the CD163L1 receptor demonstrated impaired IL-10 secretion [118]. Despite the classical anti-inflammatory phenotype of these cells, our RNA-seq data suggested that in the adjacent tissue of diverticulitis patients requiring surgery, these cells display a pro-inflammatory phenotype, as *CD163L1* positively correlated with *CXCL10* (Figure 3-1A).

Although none of the patients in our study were undergoing an active inflammatory response at the time of tissue collection, we found that the pro-inflammatory chemokine CXCL10 was expressed in the sigmoid colon of diverticulitis patients (Figure 3-3). CXCL10 is induced by

IFNγ and type I interferon [232]. This chemokine induces chemotaxis of $CXCR3^+$ cells, including macrophages, natural killer cells, and activated T cells, to activate a T_H1 immune response [234]. Our data suggest that only a subset of the $CD163L1^+$ macrophages express CXCL10, as those $CD163L1^+$ cells residing at the base of the crypt did not co-localize with CXCL10 (Figure 3-3). However, numerous other cell types, including monocytes, activated neutrophils, and intestinal epithelial cells can also secrete CXCL10 in the presence of an inflammatory response [233]. Indeed, we also identified $CXCL10^+$ cells that did not co-localize with $CD163L1^+$ macrophages. Future studies using patient-derived $CD163L1^+$ macrophages would address if these cells are secreting CXCL10 or merely responding to secretion by other cell types.

Patients with diverticulitis requiring surgery also demonstrated increased levels of serum CXCL10 relative to non-diverticulosis controls (Figure 3-4). These data are similar to a previous report of elevated serum CXCL10 in IBD patients during both the acute disease phase and during remission [239]. An increased level of CXCL10 during disease quiescence defines a potential mechanism for chronic inflammation, as elevated levels of CXCL10 could mediate prolonged recruitment of T_H1 immune cells to potentiate inflammation. Further studies should be performed to determine if serum CXCL10 can be used to objectively identify patients who may undergo a more severe disease course and are thus more likely to require surgery. Early identification of diverticulitis patients who will require surgical intervention may improve quality of life by reducing the risk for multiple episodes of diverticulitis. Furthermore, pharmacologic mitigation of the CXCR3/CXCL10 axis is under evaluation as a potential treatment for individuals with IBD [244]. As we provide evidence that CXCL10 is also elevated in diverticulitis, assessing the use of CXCR3/CXCL10 antagonists in diverticulitis patients may lead to a more targeted medical therapy.

This study has several limitations. First, patients were enrolled from a single institution which may present bias based on geographic locale. Future studies should confirm these findings in other larger populations. Second, tissue was obtained from diverticulitis patients during disease quiescence. Ideally, we would analyze tissue samples prior to diverticulitis onset; however, obtaining tissue from diverticulosis patients is difficult. Therefore, we chose to assess the tissue adjacent to chronically diseased tissue with a normal bowel wall appearance and no overt inflammation to minimize the effects of the adjacent chronic disease.

In summary, we identified $CD163L1^{+}CD68^{+}$ macrophages as potential pro-inflammatory cells in surgically managed diverticulitis patients using RNA-seq data. Increased levels of $CD163L1^{+}CD68^{+}$ macrophages were found in the sigmoid colon of diverticulitis patients requiring surgery. Some of the macrophages localizing at the crypt apex also expressed CXCL10. Moreover, surgical diverticulitis patients demonstrated elevated levels of serum CXCL10, which may potentiate a chronic inflammatory response in patients with recurrent diverticulitis by recruiting pro-inflammatory immune cells. Alternatively, it may represent a biochemical marker of ongoing disease progression in these patients. Future studies evaluating serum CXCL10 as a biomarker of diverticulitis onset or severity would be beneficial as there are currently no objective biologic criteria for medical or surgical decision making for diverticulitis.

Acknowledgements

This work was supported by the Carlino Fund for IBD Research and in part by the National Center for Advancing Translational Sciences UL1 TR000127 and TL1 TR000125. We are indebted to the patients who graciously provide their tissues for our research. We thank the colorectal surgeons and all members of the colorectal disease biobank, including Leonard Harris and Sue Deiling for their help with recruiting patients and obtaining tissues. We thank Dr. Thomas Abraham in the microscope core facility at the Pennsylvania State University College of Medicine for his assistance with imaging and quantification of stained cells.

Chapter 4

THE MICROBIAL ECOSYSTEM DISTINGUISHES CHRONICALLY DISEASED TISSUE FROM ADJACENT TISSUE IN THE SIGMOID COLON OF CHRONIC, RECURRENT DIVERTICULITIS PATIENTS

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doi: 10.1038/s41598-017-06787-8.

Author Contributions

Designed the study: KMS, WAK, RL, and DBS; Processed patient samples and obtained clinical data: KMS, SD, and LRH; Performed 16S rRNA and ITS sequencing and bioinformatics analysis: KS, JRW, DRT, RD, VT, and RL; Analyzed and interpreted the data: KMS, KS, MAE, JPH, GSY, WAK, RL, and DBS; Wrote the manuscript: KMS and KS; Read and revised the manuscript: KMS, KS, JRW, DRT, RD, VT, LRH, SD, MAE, JPH, GSY, WAK, RL, DBS.

Abstract

Diverticular disease is commonly associated with the older population in the United States. As individual's age, diverticulae, or herniation of the mucosa through the colonic wall, develop. In 10-25% of individuals, the diverticulae become inflamed, resulting in diverticulitis. The gut ecosystem relies on the interaction of bacteria and fungi to maintain homeostasis. Although bacterial dysbiosis has been implicated in the pathogenesis of diverticulitis, associations between the microbial ecosystem and diverticulitis remain largely unstudied. This study investigated how the cooperative network of bacteria and fungi differ between a diseased area of the sigmoid colon chronically affected by diverticulitis and adjacent non-affected tissue. To identify mucosa-associated microbes, bacterial 16S rRNA and fungal ITS sequencing were performed on chronically diseased sigmoid colon tissue (DT) and adjacent tissue (AT) from the same colonic segment. We found that *Pseudomonas* and Basidiomycota OTUs were associated with AT while Microbacteriaceae and Ascomycota were enriched in DT. Bipartite co-occurrence networks were constructed for each tissue type. The DT and AT networks were distinct for each tissue type, with no microbial relationships maintained after intersection merge of the groups. Our findings indicate that the microbial ecosystem distinguishes chronically diseased tissue from adjacent tissue.

Introduction

The gut comprises an ecological network of microbes, which together are a major determinant of the colonic microenvironment. As a part of the colonic ecosystem, bacteria (microbiome) and fungi (mycobiome) cohabitate, potentially influencing one another's pathogenicity, nutritional states, symbiosis, and development of community structures [197]. In states of good health, gut microbes synergistically coexist with the host to the benefit of each, providing host nutrition, maintaining homeostasis, and preventing disease [197]. Disruption of the gut ecosystem can result in pathogenic consequences. In particular, bacterial dysbiosis has been associated with various systemic disease states, including obesity [245], inflammatory bowel disease (IBD) [246], and *Clostridium difficile* infection [247]. More recently, the involvement of fungal communities associated with disease, such as in IBD [248,249], has gained attention. Comprehensive analysis of the microbial ecosystem may help explain the development of these various disease states.

Diverticular disease affects about 2.5 million people in the United States each year [250]. It is commonly a disease associated with age, being found in 40% of people over the age of 60 with an increasing incidence with advancing age [5]. Diverticular disease includes a spectrum of changes to the gut that begin with diverticulosis, or the herniation of mucosa and muscularis mucosa through the wall of the colon, usually in areas where mural blood vessels pierce the muscle layer of the bowel wall [5,202]. In Western countries, 75-90% of diverticulosis occurs in the sigmoid colon [251–253]. In approximately 10-25% of diverticulosis patients, the diverticulae becomes inflamed, leading to diverticulitis [5]. Diverticulitis is empirically treated with broad spectrum antibiotics, suggesting that bacteria may contribute to its pathogenesis. In some cases, individuals with chronic, recurrent episodes of diverticulitis may require surgical resection of the sigmoid colon.

There is limited data suggesting that bacterial dysbiosis may be an important determinant in the pathogenesis of diverticulitis [167,168]. However, research on this topic is challenged by the difficulty in identifying an appropriate control group since, for reasons that are still unclear, many persist with asymptomatic diverticulosis. Additionally, the microbial ecosystem relies on relationships between bacteria and fungi, so the exclusion of fungal organisms in prior studies on this subject omits a potential key causal factor in this disease. In the present study, both bacteria and fungi were investigated separately as well as through transkingdom interactions to determine whether microbial differences in tissue chronically affected by diverticulitis (DT) versus adjacent non-affected tissue (AT) can distinguish these two tissue types.

Using 16S rRNA and internal transcribed spacer (ITS) gene sequencing, we assigned bacterial and fungal sequencing reads to operational taxonomic units (OTUs) and analyzed how the microbial ecosystem differed between patient-matched DT from the sigmoid colon and AT from the same colonic segment. Underlying the high similarity of commensal organisms, we hypothesized that a distinctive subset of mucosa-associated pathogenic microbes would be associated with DT relative to AT, and that the microbial network of bacteria and fungi would differ between the two tissue types. Inferred metagenomic analyses were performed to evaluate the predictive metagenomes of the microbial communities associated with DT and AT as further explanation of an association between the microbiome and diverticulitis. Our study describes distinct microbial ecological networks which distinguish DT and AT, and these data suggest that a dysbiotic network of microbes is associated with the mucosa of chronically diseased sigmoid colon.

Materials and Methods

Study design and specimen collection

This retrospective cohort study was performed at the Penn State Hershey Medical Center with Institutional Review Board (IRB) approval. Informed consent was obtained from all subjects and all methods were performed in accordance with these guidelines. Between April 2010 and August 2014, chronic, recurrent diverticulitis patients were consented to collect colonic tissue into the Penn State Hershey Colon and Rectal Diseases Biobank at the time of elective sigmoid resection. Surgical specimens were immediately transported from the operating room to the surgical pathology laboratory where Biobank staff obtained several full-thickness sections of tissue. Chronically diseased tissue (DT) was an area of chronic inflammation that demonstrated a thickened bowel wall. Adjacent tissue (AT) sections were taken from an area of sigmoid colon with normal appearing bowel wall thickness, and as far away from DT as possible. This section comprised our patient-matched control that was not influenced by chronic, recurrent diverticulitis. Tissue was flash-frozen at -80°C until processing for analysis. Confirmation of diverticulitis was based on preoperative CT scans and surgical pathology. Patients with IBD, cancer, or dysplasia were excluded.

Analysis of the microbiome

To analyze populations of bacteria associated with the mucosa as opposed to those present in the stool, DNA was extracted from approximately 250 mg of colonic tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Frederick, MD). DNA was quantified using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA), aliquoted, and shipped on dry ice to Dr. Lamendella at Juniata College. DNA concentrations were quantified with the Qubit 2.0

Fluorometer High Sensitivity dsDNA kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. PCR was performed using the Illumina-barcoded 806R reverse primer and 515F forward primer as previously described [247]. Pooled PCR products were gel purified using the Qiagen Gel Purification Kit (Qiagen, Frederick, MD), quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and samples were combined in equimolar amounts. Prior to submission for sequencing, libraries were quality checked using the 2100 Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA). Pooled libraries were stored at -20 ˚C until they were shipped on dry ice to the California State University (North Ridge, CA) for sequencing.

Library pools were size verified using the Fragment Analyzer CE (Advanced Analytical Technologies Inc., Ames, IA) and quantified using the Qubit High Sensitivity dsDNA kit (Life Technologies, Carlsbad, CA). After dilution to a final concentration of 1 nM and addition of a 10% spike of PhiX V3 library as an internal control (Illumina, San Diego CA), pools were denatured for 5 minutes in an equal volume of 0.1 N NaOH then further diluted to 12 pM in Illumina's HT1 buffer. The denatured and PhiX-spiked 12 pM pool was loaded on an Illumina MiSeq V2 300 cycle kit cassette with 16S rRNA library sequencing primers and set for 150 base, paired-end reads.

Forward and reverse reads were merged using VSEARCH version 1.9.10 with a minimum overlap set to 40 bp [254]. Using USEARCHv7, paired sequences were quality filtered at a maximum expected error of 0.5% and truncated at a length of 253 bp. Filtered reads maintained an average Phred Q score of 37.9. Chimeric sequences were identified and removed using the UCHIME algorithm with default settings [255]. A total of 172 out of 4,308 OTUs were removed after chimera checking and a total of 391,649 paired sequences were used in downstream analyses. OTUs were picked *de novo* using the UPARSE pipeline [256] within USEARCHv7 using a 97% ID setting. Taxonomy was assigned using the assign_taxonomy.py

script in QIIME 1.9.0 [257] with default parameters using the Greengenes 16S rRNA gene database (13-5 release, 97%) [165]. Results were compiled into a biological observation matrix (biom) format OTU table in which singleton sequences were removed.

Analysis of the mycobiome

The ITS region between the 18S and 5.8S rRNA genes was amplified in 25 µL PCR reactions using the same concentrations as previously mentioned, with the exception of adding 5 µL of undiluted template DNA, and the use of ITS1 forward (5' - AATGATACGGCGACCACCGAGATCTACACGGCTTGGTCATTTAGAGGAAGTAA - 3') and Illumina-barcoded ITS2 reverse primers (5' - CAAGCAGAAGACGGCATACGAGAT TACCGCTTCTTC CG GCTGCGTTCTTCATCGATGC - 3') designed to avoid common PCR-related biases in generating fungal amplicons of the variable target region [258]. The protocol described by Smith and Peay is one of the only one-step PCR assays compatible with Illumina sequencing and ITS1F is highly fungal specific and other primers for ITS2 are often less specific and can co-amplify host tissue [259]. The thermocycling conditions performed using an MJ Research PTC-200 thermocycler (Bio-Rad, Hercules, CA) were: 94°C for 1 min, 30 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s; 68°C for 7 min and a 4°C hold. PCR products were visualized on a 1% SYBRsafe E-gel (Life Technologies, Carlsbad, CA). Purified libraries were pooled and sequenced on the Illumina NextSeq platform using the 150 bp paired end chemistry at University of California Davis Genome Center.

A total of 6,116,765 forward reads and 419,207 reverse reads were retrieved from ITS sequencing. Due to the higher read counts and the fact that we could retain more samples in our fungal dataset, only the forward reads were analyzed. This approach was used by Nguyen *et al*. who found that analyzing forward reads for ITS region 1 yielded a more robust analysis in a

mock community analysis [260]. Forward ITS sequences were quality filtered using VSEARCH 1.11.1 with a maximum expected error of 0.5% and truncated at a length of 150 bp to retain an average Phred Q score of 35.6 throughout the entire read length. The sequences were trimmed using Trimmomatic to remove low-quality regions [259]. OTUs were picked using the open-reference UCLUST algorithm in QIIME 1.9.0 [257] at the default OTU threshold of 0.97 and singleton sequences were discarded. Taxonomy was assigned using the BLAST option in the assign_taxonomy.py script against version 7 of the UNITE fungal ITS database [261,262] with the maximum e-value set to the default of 0.001. Taxa with no BLAST hits were removed from the OTU table for downstream analysis.

Diversity and statistical analyses

Alpha diversity rarefaction curves were created within the QIIME 1.9.1 package [257] using the untransformed OTU table. Multiple rarefactions were performed on the 16S rRNA OTU table from all samples using a minimum depth of 0 sequences to a maximum depth of 7000 sequences, with a step size of 700 for 20 iterations. Multiple rarefactions were performed on the ITS OTU table from all samples using a minimum depth of 0 sequences to a maximum depth of 6000 sequences, with a step size of 600 for 20 iterations. Rarefactions were then collated and plotted using observed species, Chao1, PD Whole Tree, and Heip's evenness diversity metrics. Alpha diversity was compared between disease states, as well as age, sex, BMI, smoking history, and antibiotic administration. Richness plots were made within Phyloseq using the untransformed OTU table against the Observed OTUs, Chao1, and ACE metrics with lines connecting each patient's diverticular and normal samples.

Both 16S and ITS OTU tables were normalized using metagenomeSeq's Cumulative Sum Scaling (CSS) algorithm [263]. Beta diversity analyses were performed using weighted

UniFrac distance matrices and visualized using a 3-dimensional principal coordinate analysis (PCoA) plots in EMPeror. To assess within-group variation in DT and AT samples, average weighted UniFrac distances within each tissue grouping were calculated and compared using a two-sample *t*-test. Core microbiome analyses were also completed within QIIME 1.9.1 [257]. The ANOSIM method within QIIME was employed on the weighted UniFrac distance matrices to test if there was a difference in beta diversity between DT and AT groups. LEfSe analysis was used to identify bacterial taxa whose sequences are differentially abundant between DT and AT groups [264]. LEfSe uses a Kruskal-Wallis test followed by pairwise Wilcoxon rank sum test correction, with both alpha values set to 0.05. The effect size cutoffs to determine significantly differentiating taxa between DT and AT groups were set at a genus-level LDA score >1.5 for both 16S rRNA gene and ITS data.

Co-occurrence networks were built and visualized in Cytoscape 3.3.0 using the CoNet plugin [265]. As created in QIIME 1.9.1, untransformed OTU tables consisting of exclusively chronically diseased tissue and adjacent tissue were uploaded. In a preprocessing step, any taxa appearing in <50% of samples were discarded and taxa had to appear as non-zero values in at least two samples to be considered for correlations to account for read count sparsity. Two correlational measures, Pearson and Spearman, and two dissimilarity measures, Bray-Curtis and Kullback-Leibler, were used to calculate correlations between the remaining taxa. The use of all four measures reduces the chance of spurious correlations due to outliers, matching zeroes, or data compositionality. The Benjamini-Hochberg-Yekutieli multiple testing correction was used to adjust *P*-values in the final step of CoNet processing.

Unassigned taxa were removed from networks. Remaining taxa were then labeled to the furthest identified taxonomic rank. Green lines were used to connect positive correlations, while red lines showed negative correlations. Chronically diseased tissue and adjacent tissue networks were additionally merged using both difference and intersection parameters. There were no

shared correlations between DT and AT networks, suggesting differential correlations between bacteria and fungi in the different tissue types.

Predicted metagenomes were calculated with 16S rRNA gene data for the DT and AT sample groups using PICRUSt software [266]. A closed reference 16S rRNA gene OTU table was imported into PICRUSt version 1.1.0 mapped against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and summarized at the Level 3 functional annotation.

Power analysis

The *micropower* R-package was used to calculate the PERMANOVA power of our study design [267]. The input for this analysis was the distance matrix made using weighted UniFrac distances from the 16S rRNA OTU table. PERMANOVA power was calculated with five, nine, and 15 subjects per treatment group specified. Each sample size was observed at alpha cutoffs of 0.01, 0.05, and 0.1 performed over 100 bootstrap iterations. The PERMANOVA power calculated from our study design of nine subjects per treatment group at an alpha cutoff of 0.05 was found to be 0.89 (Supplemental Table 4-1). Varying the alpha cutoffs to 0.01 and 0.1 changed our power calculations as described in Supplemental Table 4-1.

Supplemental Table 4-1. PERMANOVA power analysis using the weighted UniFrac distances from our 16S rRNA OTU data table

Subjects/Group	Alpha	Average Effect Size (omega2)	Power
5	0.01	0.059	0.1
9	0.01	0.055	0.4
15	0.01	0.057	1
5	0.05	0.056	0.24
9	0.05	0.059	0.89
15	0.05	0.058	1
5	0.1	0.058	0.5
9	0.1	0.061	0.95
15	0.1	0.059	1

Results

Unique bacterial taxa colonize chronically diseased and adjacent tissue

Prior to assessing the mucosa-associated bacterial community structure, we evaluated the cellular architecture and inflammatory cell infiltrate present in DT and AT. Hematoxylin and eosin stained tissue sections were examined from nine patients whose clinical demographics are described in Table 4-1. DT is an area chronically affected by diverticulitis that demonstrated a thickened bowel wall, while AT is obtained from an area adjacent to DT and is unaffected by disease (Figure 4-1A, B). The cellular architecture for both DT and AT was normal-appearing, with an intact epithelium and crypts. AT showed negligible mucosal neutrophilic inflammation (Figure 4-1C, D), whereas DT also showed minimal inflammation but demonstrated increased numbers of neutrophils within the lamina propria (Figure 4-1E, F).

We then performed 16S rRNA gene sequencing to analyze the microbiome of patient-matched DT and AT and found that bacterial communities in both tissue types were dominated by Proteobacterial taxa (Figure 4-2A). Alpha diversity measures did not reveal differing trends between DT and AT, likely due to the use of patient-matched samples resulting in high similarity of commensal organisms (Supplemental Figure 4-1A). Overall clustering based on weighted UniFrac distance was not significant when comparing tissue type (ANOSIM test $P = 0.521$) (Supplemental Figure 4-1B) or patient identification (ANOSIM test $P = 0.110$) (Supplemental Figure 4-1C).

We next analyzed the core microbiome, which typically refers to the population of bacteria comprising two or more habitats [268]. In our study, the core microbiome was defined as common bacteria with OTUs present in ≥80% of all samples. This analysis revealed that subsets of bacterial taxa were distinct between DT and AT (Figure 4-2B). Although a majority of OTUs

Table 4-1. Disease definitions.

	Diverticulitis cohort (n = 9)
Sex (male/female)	5/4
Age (years) at surgery, mean ± SD (range)	60.9 ± 14.1 (43-77)
Body mass index (kg/m^2), mean ± SD (range)	25.0 ± 2.8 (20-28)
Episodes of diverticulitis prior to surgery	
1-2 episodes	4
3-4 episodes	2
≥5 episodes	3
Antibiotics usage within 3 months prior to surgery	
Metronidazole and Neomycin[1]	9
Metronidazole and Ciprofloxacin	2
Amoxicillin/Clavulanic Acid	1
Doxycycline and Cephalexin	1

SD, standard deviation
[1]Received day prior to surgery as part of mechanical bowel prep

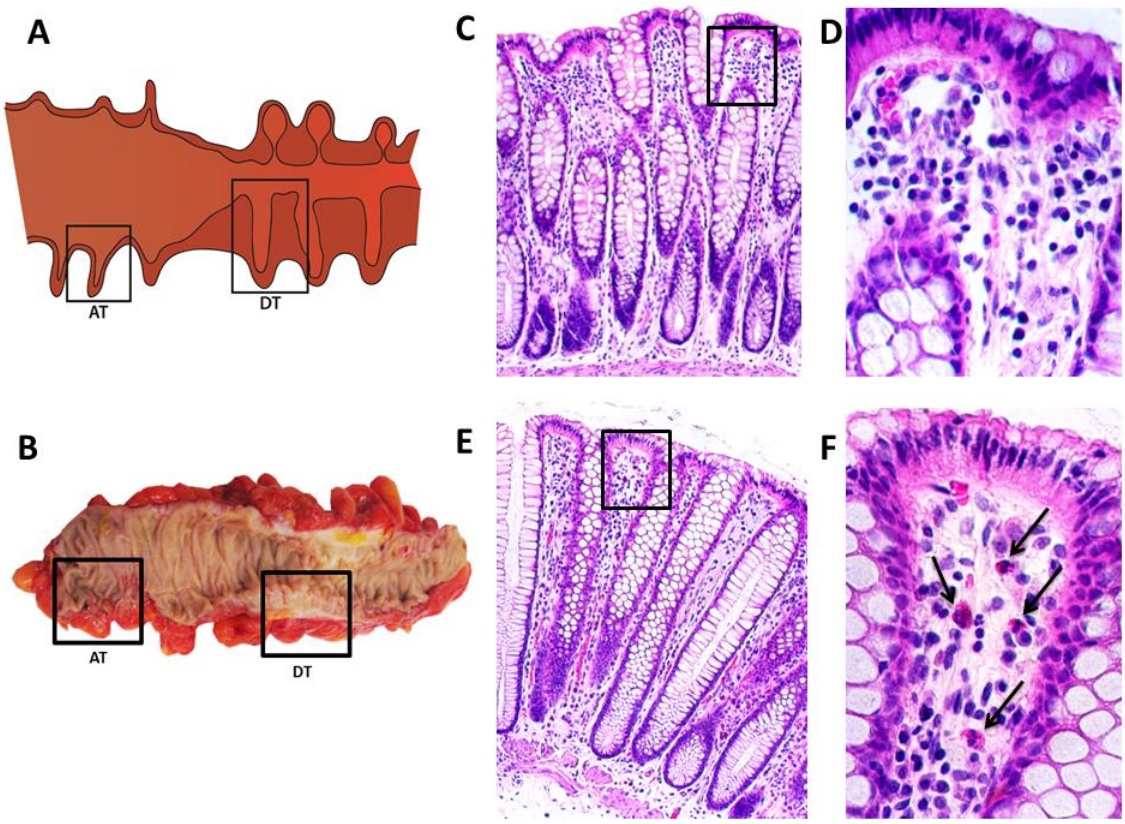


Figure 4-1. Evaluation of chronically diseased tissue and adjacent tissue obtained from the sigmoid colon of chronic, recurrent diverticulitis patients.

(A) Illustrated representation of the sigmoid colon with areas obtained for analysis shown in boxes. Chronically diseased tissue (DT) is an area of thickened bowel wall. Adjacent tissue (AT) is an area of normal bowel wall thickness. Diverticulae may be present in either tissue section. (B) Sigmoid colon taken from a patient immediately after resection and representative tissue sections shown in boxes. (C) Representative H&E stained section from AT shows minimal mucosal neutrophilic inflammation. (D) Higher magnification of the boxed area of (C) showing negligible mucosal inflammation. (E) Representative H&E stained section from DT with minimal mucosal inflammation demonstrated by the presence of few mucosal neutrophils within the lamina propria. (F) Higher magnification of the boxed area of (E) with neutrophils marked by an arrow.

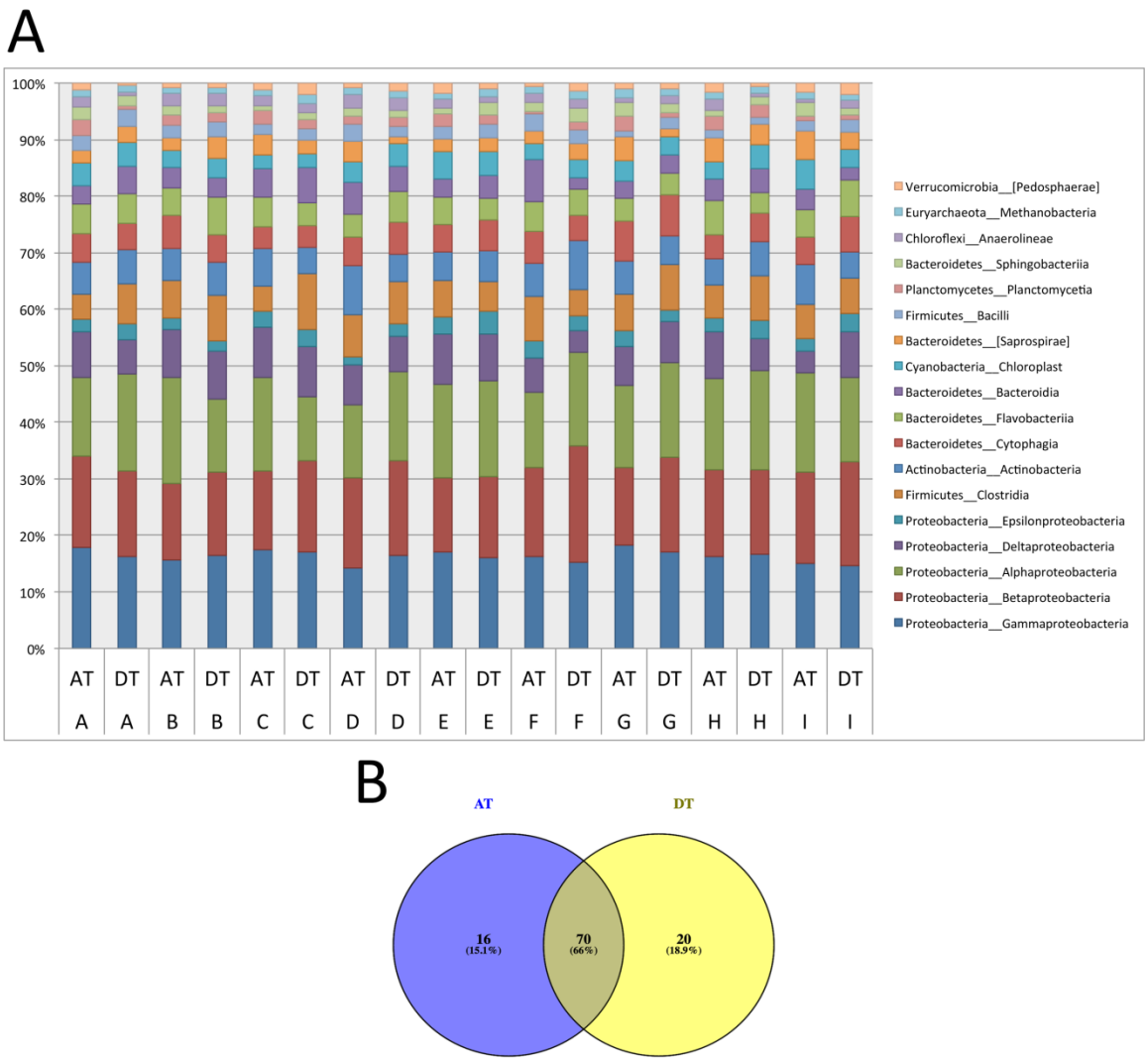
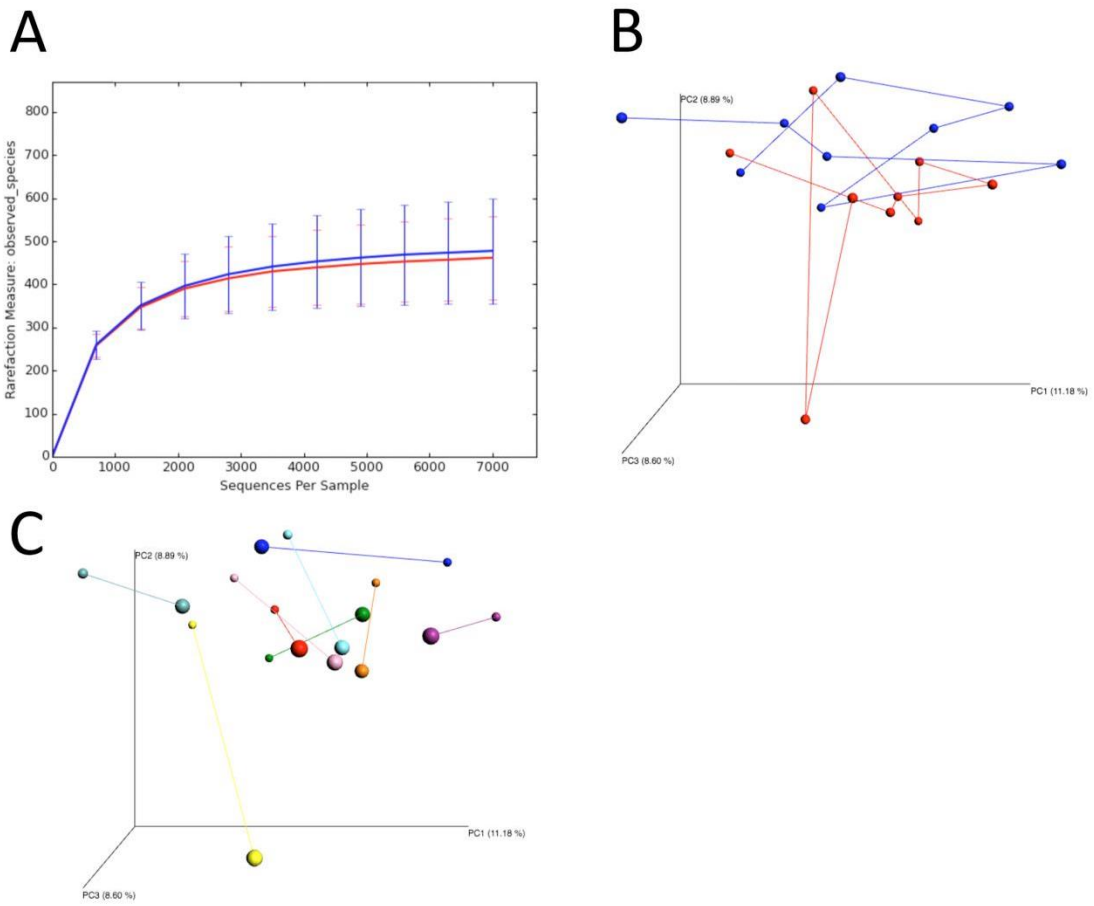


Figure 4-2. Bacterial alpha and beta diversity of chronically diseased tissue and adjacent tissue.

(A) Bacterial community composition profiles illustrate abundances of prevalent taxonomic classes. All taxa unassigned at the kingdom level were removed. Patients are arbitrarily labeled A through I with chronically diseased tissue (DT) and adjacent tissue (AT) indicated. (B) Venn diagram of core microbiome (including OTUs found in ≥80% of the samples) presents the number of OTUs that are shared (66.0%) and unique between chronically diseased tissue (DT) (18.9%) and adjacent tissue (AT) (15.1%).



Supplemental Figure 4-1. Microbial alpha and beta diversity analysis.

(A) Alpha diversity rarefaction plots of observed taxa within diverticulitis (red) tissue and adjacent (blue) tissue were generated within QIIME 1.9.0. Differences in alpha diversity were not found to be statistically significant. (B) Principle coordinate analysis plot calculated from weighted UniFrac distances comparing bacterial community structures between diverticulitis (red) tissue and adjacent (blue) tissue. (C) Principle coordinate analysis plot calculated from weighted UniFrac distances comparing bacterial community structures between individual patients. Each color is indicative of an individual patient, with large points representing diverticular tissue, and small points representing adjacent tissue. Clustering was not found to be statistically significant based on neither tissue type nor patient.

(66.0%) were shared among the two tissue types, 18.9% and 15.1% of OTUs were specific to DT and AT, respectively (Supplemental Table 4-2).

To further define which bacterial taxa were enriched in each tissue type, Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was used (Figure 4-3A). A total of 33 taxa (25 being taxonomically identified) were enriched in both groups, with an LDA score >1.5 (Supplemental Table 4-3). In general, bacteria within the phyla Proteobacteria and Actinobacteria were most represented in the LEfSe plot for DT and AT. The OTU *Pseudomonas* (P=0.038) was most predictive of AT while Microbacteriaceae (P=0.019) were enriched in DT. We then used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to infer the potential mechanistic function from virtual metagenomics data collected from 16S rRNA sequencing (Figure 4-3B). Nearest sequenced taxon index (NSTI) scores were calculated on all 23 samples included in PICRUSt metagenome prediction analysis and yielded an average score of 0.108 ± 0.013. Five pathways were identified with an LDA score >1.5. AT was associated with enrichment of glycosyltransferase (P=0.002) and carbohydrate metabolism (P=0.024) pathways while DT was correlated with methane metabolism (P = 0.047); valine, leucine, and isoleucine biosynthesis (P=0.031); and the C5-branched dibasic acid metabolism pathway (P=0.009) (Supplemental Table 4-4). In accordance with the observed association between methane metabolism and DT, methanogenic archaea, such as *Thermoplasmata*, were found to be predictive of DT (Figure 4-3A).

Supplemental Table 4-2. Differentially expressed bacterial taxa unique between diverticulitis tissue (DT) and adjacent tissue (AT).

Phylum	Class	Order	Family	Genus	Species
DT Core (80%) Bacteria					
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*Desulfosporosinus*	*meridiei*
Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	*Syntrophomonas*	
Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	*Shewanella*	
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae		
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae		
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae		
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*veronii*
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		
Synergistetes	Synergistia	Synergistales	Synergistaceae	*vadinCA02*	
Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae		
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae		
Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae		
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae		
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae		
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*foraminis*
AT Core (80%) Bacteria					
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Hymenobacter*	
GN04					
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	
Bacteroidetes	Sphingobacteriia	Sphingobacteriales			
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae		
Bacteroidetes	Bacteroidia	Bacteroidales			
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	*Methylotenera*	*mobilis*
Cyanobacteria	Chloroplast	Stramenopiles			
Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	*Geobacter*	
Chloroflexi	Anaerolineae	H39			
Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	*Fluviicola*	
Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	*Fluviicola*	
Acidobacteria	[Chloracidobacteria]	Ellin7246			
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Rhodoferax*	

Only taxonomically assigned OTUs at the kingdom are listed.

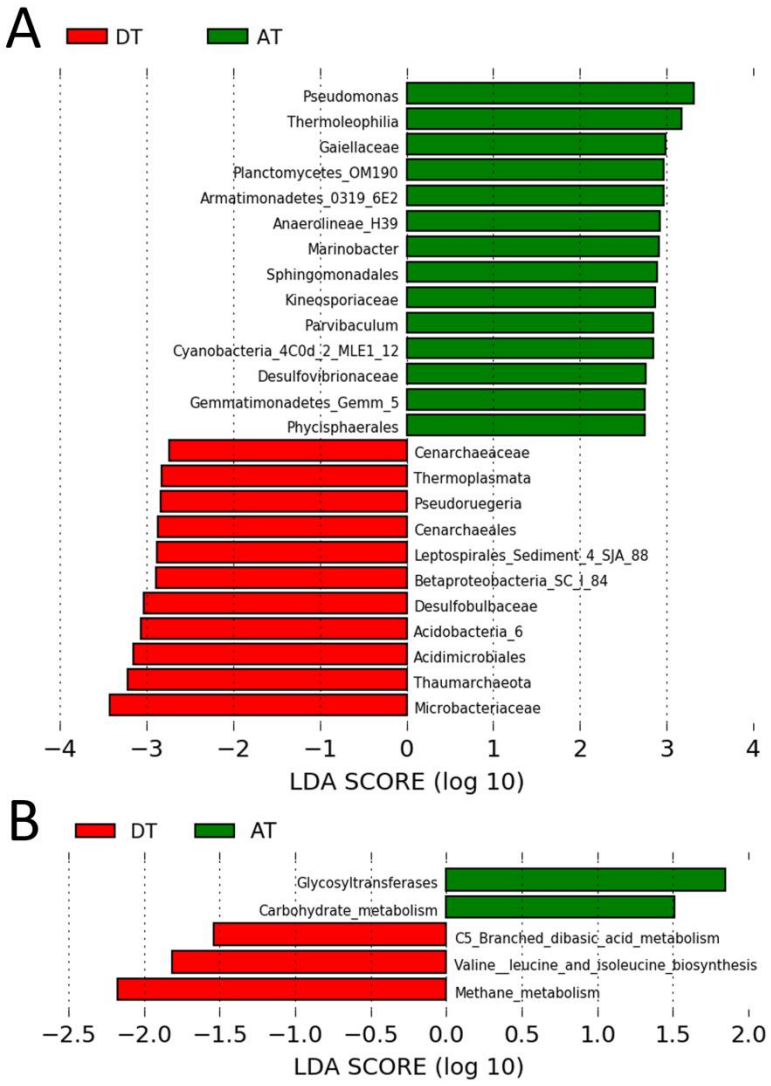


Figure 4-3. Differential bacterial and archaeal compositions comprise chronically diseased tissue and adjacent tissue.

(A) LDA Effect Size (LEfSe) plot of taxonomic biomarkers identified within chronically diseased tissue (DT) and adjacent tissue (AT) samples. Red bars are indicative of enrichment within DT samples, whereas green bars are indicative of enrichment within AT samples. A Kruskal-Wallis test was employed at an alpha=0.05 to identify significantly enriched taxa, whereas a pairwise Wilcoxon rank sum test was utilized to test biological consistency across all subgroups (alpha=0.05). A linear discriminant analysis (LDA) was calculated to determine effect size and we present all taxa that yielded an LDA score >1.5. A total of 33 taxa (25 being taxonomically identified) were found to be enriched in both sample groupings. (B) PICRUSt-inferred metagenomics and LEfSe predicted-function enrichment plots. Functions with an LDA score >1.5 are shown. Inferred molecular functions of the bacterial populations identified by 16S rRNA gene sequencing are stratified by tissue type (chronically diseased tissue (DT) and adjacent tissue (AT)).

Supplemental Table 4-3. Enriched bacterial and archaeal taxa identified by LEfSe analysis.

	Enriched Bacterial and Archaeal Taxonomy	LDA Score	LEfSe *P*- value
AT	Archaea.Crenarchaeota.Thaumarchaeota	3.22	0.007
	Archaea.Crenarchaeota.Thaumarchaeota.Cenarchaeales	2.87	0.031
	Archaea.Crenarchaeota.Thaumarchaeota.Cenarchaeales.Cenarchaeaceae	2.73	0.039
	Archaea.Euryarchaeota.Thermoplasmata	2.82	0.029
	Bacteria.Acidobacteria.Acidobacteria_6	3.07	0.038
	Bacteria.Actinobacteria.Acidimicrobiia.Acidimicrobiales	3.15	0.020
	Bacteria.Actinobacteria.Actinobacteria.Actinomycetales.Microbacteriaceae	3.42	0.019
	Bacteria.Proteobacteria.Alphaproteobacteria.Rhodeobacterales.Rhodobacteraceae.*Pseudoruegeria*	2.84	0.031
	Bacteria.Proteobacteria.Betaproteobacteria_SC_I_84	2.89	0.029
	Bacteria.Proteobacteria.Deltaproteobacteria.Desulfobacterales.Desulfobulbaceae	3.03	0.024
	Bacteria.Spirochaetes.Leptospirae.Leptospirales_Sediment_4_SJA_88	2.88	0.029
DT	Bacteria.Actinobacteria.Actinobacteria.Actinomycetales.Kineosporiaceae	2.87	0.031
	Bacteria.Actinobacteria.Thermoleophilia	3.18	0.038
	Bacteria.Actinobacteria.Thermoleophilia.Gaiellales.Gaiellaceae	2.99	0.007
	Bacteria.Armatimonadetes_0319_6E2	2.97	0.012
	Bacteria.Chloroflexi.Anaerolineae_H39	2.92	0.047
	Bacteria.Cyanobacteria_4C0d_2_MLE1_12	2.85	0.029
	Bacteria.Gemmatimonadetes_Gemm_5	2.75	0.029
	Bacteria.Planctomycetes_OM190	2.97	0.024
	Bacteria.Planctomycetes.Phycisphaerae.Phycisphaerales	2.75	0.029
	Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.Hyphomicrobiaceae.*Parvibaculum*	2.85	0.029
	Bacteria.Proteobacteria.Alphaproteobacteria.Sphingomonadales	2.89	0.018
	Bacteria.Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae	2.75	0.012
	Bacteria.Proteobacteria.Gammaproteobacteria.Alteromonadales.Alteromonadaceae.*Marinobacter*	2.91	0.008
	Bacteria.Proteobacteria.Gammaproteobacteria.Pseudomonadales.Pseudomonadaceae.*Pseudomonas*	3.31	0.038

Supplemental Table 4-4. Enriched predicted function from PICRUSt analysis.

	Enriched Function	LDA Score	LEfSe *P* value
DT	C5 Branched dibasic acid metabolism	1.60	0.009
	Methane metabolism	2.30	0.047
	Valine, leucine, and isoleucine biosynthesis	1.85	0.031
AT	Carbohydrate metabolism	1.58	0.024
	Glycosyltransferases	1.85	0.002

Pathogenic fungal species are found in chronically diseased tissue

Fungal organisms also constitute a large part of the intestinal ecosystem and interact with bacteria to influence their community structure, so we next analyzed the mycobiome of DT and AT. At the fungal class level, we detected large abundance profile differences for within-subject variation and between-subject variation among tissue types (Figure 4-4A). Similar to the bacterial analysis, alpha diversity measures for fungal communities did not reveal significant differences between DT and AT (Supplemental Figure 4-2A). While weighted UniFrac distance analysis showed no significant clustering of overall fungal community structure based on tissue type (ANOSIM test $P = 0.635$) (Supplemental Figure 4-2B), analysis by patient identification did reveal significant clustering (ANOSIM test $P = 0.010$) (Supplemental Figure 4-2C). Core mycobiome analysis (OTUs present in ≥80% of all samples) revealed that a majority of OTUs (75.0%) were shared between DT and AT (Figure 4-4B). Only 8.3% and 16.7% of OTUs were specific to DT and AT, respectively (Supplemental Table 4-5). Using LEfSe to identify fungal taxonomic biomarkers for DT and AT with an LDA score >1.5, the OTU *Exophiala* found in the division Ascomycota was enriched in DT ($P = 0.037$) while three Basidiomycota OTUs identified to Plutaceae ($P = 0.039$), *Pluteus* ($P = 0.039$), and Agaricales ($P = 0.039$) were correlated with AT (Figure 4-4C, Supplemental Table 4-6). Because examining the mycobiome did not identify sufficient evidence to suggest that fungal organisms alone may be involved in diverticular disease, we analyzed the interactive network of bacterial-fungal relationships.

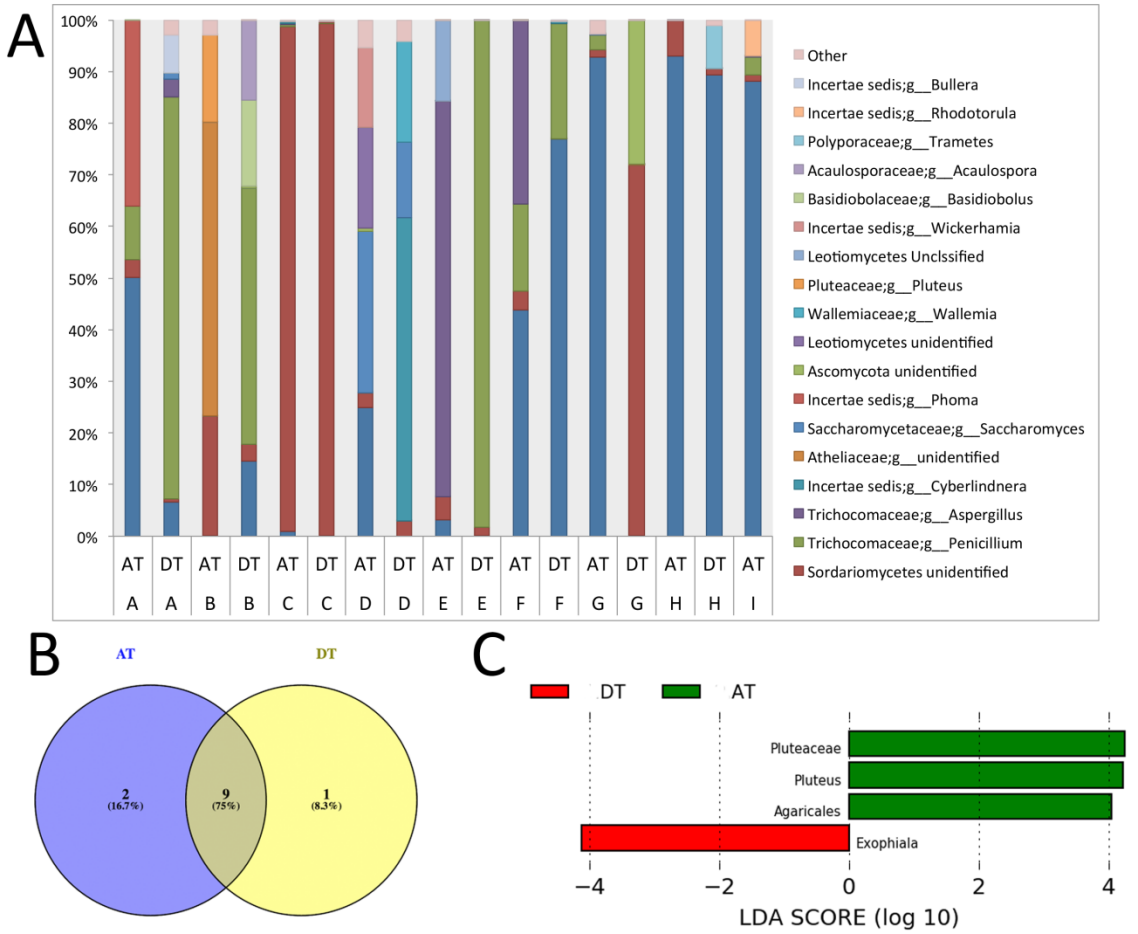
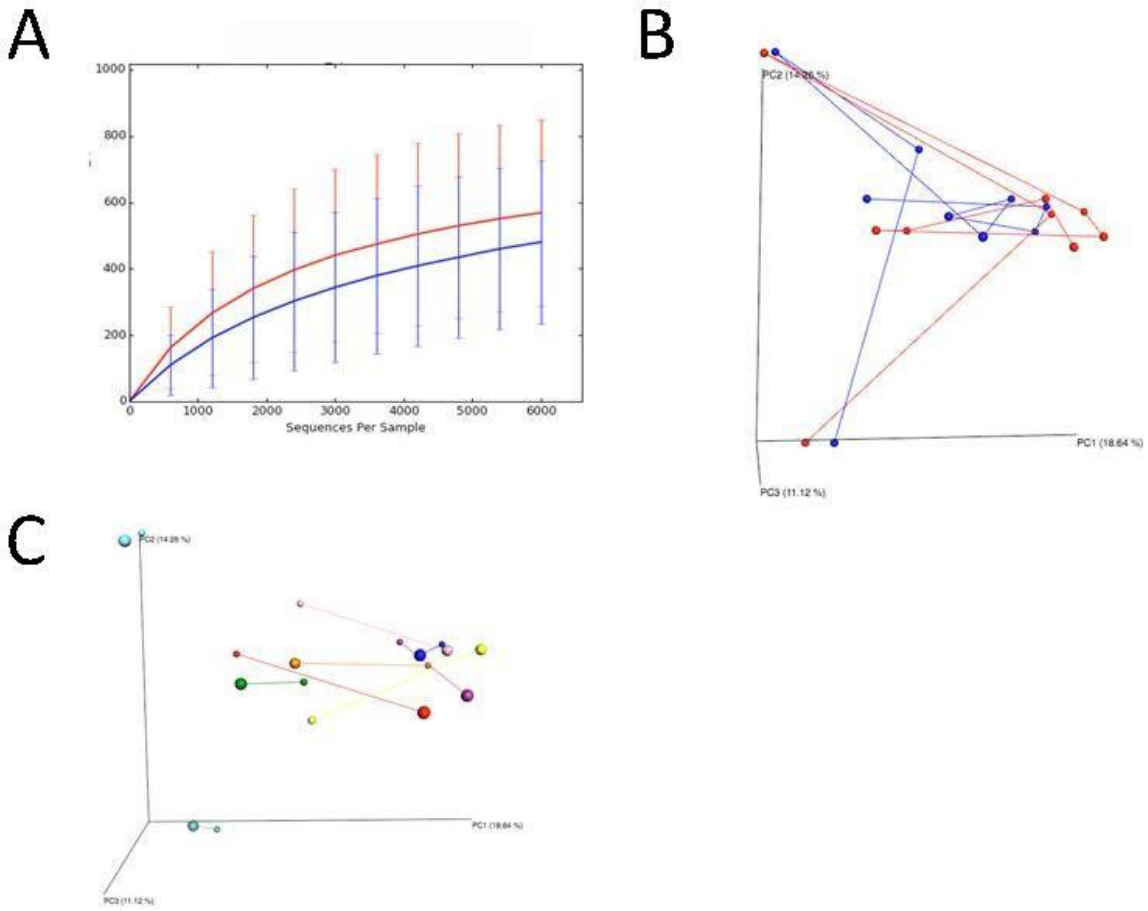


Figure 4-4. Alpha and beta diversity analysis of fungal communities associated with chronically diseased tissue and adjacent tissue

(A) Fungal community composition profiles illustrate abundances of prevalent taxonomic orders and families. All taxa unassigned at the kingdom level were removed. Patients are arbitrarily labelled A-I with chronically diseased tissue (DT) and adjacent tissue (AT) indicated. (B) Venn diagram of core mycobiome (including OTUs found in ≥80% of the samples) displays the number of OTUs that are similar (75.0%) and unique between chronically diseased tissue (DT) (8.3%) and adjacent tissue (AT) (16.7%). (C) LDA Effect Size (LEfSe) enrichment plots reveal significantly enriched fungi identified within each tissue type (chronically diseased tissue (DT) and adjacent tissue (AT)). Taxa with an LDA score >1.5 are presented.



Supplemental Figure 4-2. Fungal alpha and beta diversity analysis.

(A) Alpha diversity rarefaction plots of observed taxa within diverticulitis (red) tissue and adjacent (blue) tissue were generated within QIIME 1.9.0. Differences in alpha diversity were not found to be statistically significant. (B) Principle coordinate analysis plot calculated from weighted UniFrac distances comparing bacterial community structures between diverticulitis (red) tissue and adjacent (blue) tissue. (C) Principle coordinate analysis plot calculated from weighted UniFrac distances comparing bacterial community structures between individual patients. Each color is indicative of an individual patient, with large points representing diverticular tissue, and small points representing adjacent tissue. Clustering was not found to be statistically significant based on tissue type, but was found to be statistically significant based on patient ID (ANOSIM P=0.01).

Supplemental Table 4-5. Differentially expressed fungal taxa unique between diverticulitis tissue (DT) and adjacent tissue (AT).

Phylum	Class	Order	Family	Genus	Species
DT Core (80%) Fungi					
Ascomycota	Leotiomycetes				
AT Core (80%) Fungi					
Ascomycota	Saccharomycetes	Saccharomycetales	Incertae sedis	*Cyberlindnera*	*jadinii*
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	*subversicolor*

Only taxonomically assigned OTUs at the kingdom are listed.

Supplemental Table 4-6. Enriched Fungal Taxonomy from LEfSe Analysis.

	Enriched Function	LDA Score	LEfSe *P* value
DT	Fungi.Ascomycota.Chaetothyriomycetes.Chaetothyriales.Herpotrichiellaceae.*Exophiala*	4.11	0.037
AT	Fungi.Basidiomycota.Agaricomycetes. Agaricomycetidae.Agaricales	4.04	0.039
	Fungi.Basidiomycota.Agaricomycetes.Agaricomycetidae.Agaricales.Plutaceae	4.24	0.039
	Fungi.Basidiomycota.Agaricomycetes.Agaricomycetidae.Agaricales.Plutaceae.*Pluteus*	4.21	0.039

Distinct bipartite co-occurrence networks describe chronically diseased tissue and adjacent tissue

The gut ecosystem thrives on relationships between microbes to promote a healthy environment and physiological homeostasis, and in turn, disruption of this homeostatic ecosystem may promote disease states [245–249]. To examine how bacteria and fungi co-exist to potentially maintain the ecosystem of DT and AT, bipartite co-occurrence networks were constructed and analyzed. Positive and negative bacterial-fungal relationships were determined for each tissue type identifying co-existence or competitive exclusion, respectively [269]. The bipartite co-occurrence network plot for DT is presented (Figure 4-5). As predicted, both positive and negative correlations (Spearman's rho > 0.80) were observed, confirming the presence of an interactive network of microorganisms. Using the intersection merge method to evaluate the bacterial-fungal relationships that are similar and different between DT and AT bipartite networks, we found that each tissue type exhibited differential microbial interactions, suggesting distinct microbial ecologies. For example, enrichment of the OTU *Pseudomonas* was identified as a taxonomic biomarker of AT (Figure 4-3A) and this OTU had a positive relationship with the fungal OTU *Aspergillus* in AT; however, in DT, this relationship was not observed. Thus, this analysis identified differences in the microbial ecosystem and bacterial-fungal relationships between DT and AT. Such transkingdom interactions allows for a broad overview of the ecological niche that may play a role in the inflammatory processes seen in areas of chronic disease.

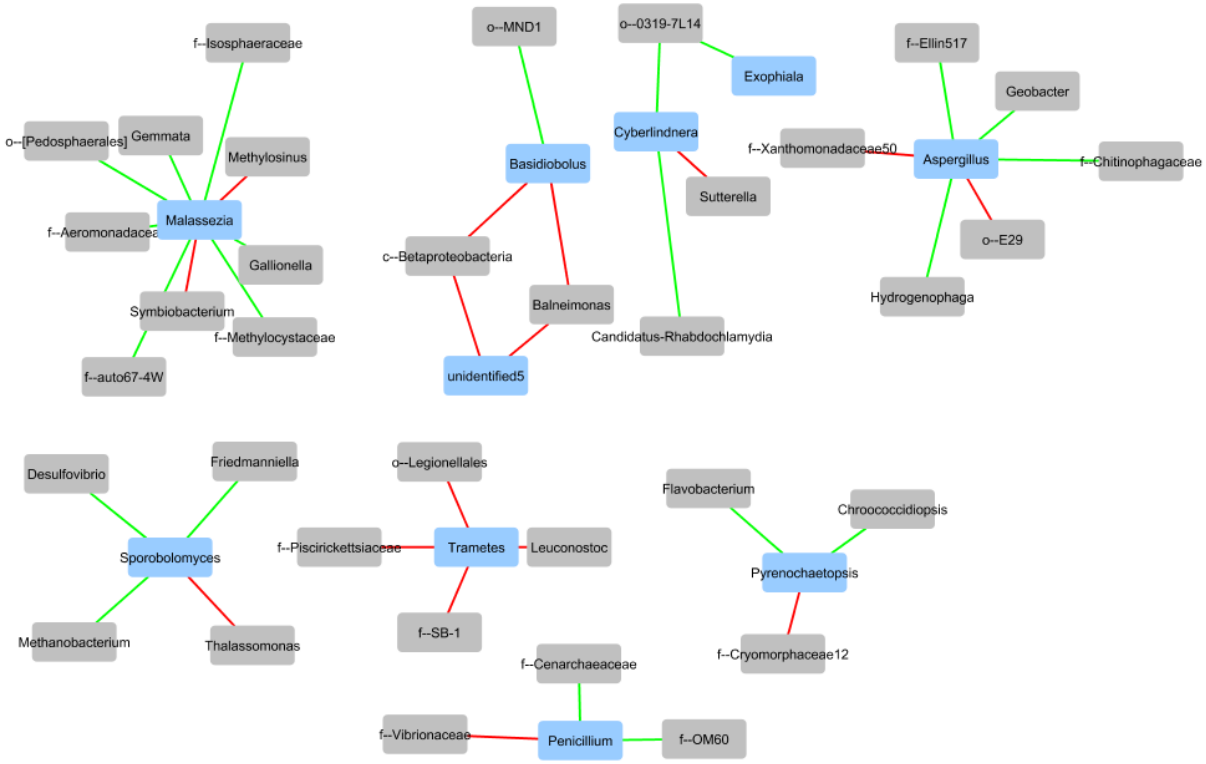


Figure 4-5. Bipartite co-occurrence network of chronically diseased tissue.

Bipartite co-occurrence network of chronically diseased tissue showing positive (green) and negative (red) correlations between fungal (blue) and bacterial (gray) organisms (Spearman's rho > 0.80). Nodes are labeled to the furthest identified taxonomic level. When merged with the adjacent tissue bipartite co-occurrence network plot, none of the described relationships were maintained.

Discussion

Our multi-faceted approach of analyzing the microbiome, the mycobiome, and the ecological relationship between bacteria and fungi allowed us to glean several potentially important insights into the differences between chronically diseased diverticular tissue and adjacent non-inflamed sigmoid colon. LEfSe analysis found that AT was associated with enrichment of *Pseudomonas* and Basidiomycota OTUs while Microbacteriaceae and Ascomycota were enriched in DT. Unique microbial ecological networks distinguished the two tissue types, with no relationships maintained upon merge of the two bipartite co-occurrence network plots. These data suggest that distinct microbial ecosystems may have a role in the inflammatory process associated with diverticular disease.

In line with our results, a previous study found higher diversity of Proteobacteria with *Pseudomonas* as one of the predictive OTUs for diverticulitis patients compared to IBD and colorectal cancer patients [168]. However, whether this enrichment is associated with non-specific intestinal inflammation [161] or the diverticular disease process will require further research. *Pseudomonas* is an organism which in healthcare settings is known to have a broad range of behavior, but with a potential for antibiotic resistance [270]. Animal models of intestinal anastomoses have shown that *Pseudomonas* can release collagenases in response to tissue injury [271]. This may have a correlation with diverticular disease, where an initial mechanical insult from biochemical stimuli or stool within the diverticulae may promote the release of degradative enzymes by this opportunistic pathogen.

PICRUSt allowed the inference of mechanistic pathways potentially associated with different bacterial communities. One of the enriched pathways predicted by PICRUSt was methane metabolism, which is consistent with LEfSe findings that *Thermoplasmata* are predictive of DT, as archaea within this class have been identified as methylamine-degrading, gut

methanogens [272–274]. Previous studies similarly found an enrichment of methanogenic bacteria, such as *Methanobrevibacter smithii,* in patients with diverticulosis [275] and with constipation [276]. A predominance of methanogenic microorganisms may, therefore, be associated with gut motility patterns which lend to the development of diverticulae. It should be emphasized that PICRUSt is a computational method used to analyze virtual metagenomic data, and due to its inferred approach, physiological interpretations of these results should be treated with caution as they require experimental confirmation. Future work should include shotgun metagenomics and meta-transcriptomic sequencing to elucidate the genetic potential and activities within these gut microbial communities.

Beta diversity analyses revealed that overall fungal community structure was more strongly driven by inter-individual variation than tissue type, suggesting that diverticulitis is perhaps not associated with fundamental differences in mycobiome composition, but shifts in a subset of taxa and their accompanying interactivity with the rest of the community. Fungal sequencing data found an enrichment of OTUs associated with Ascomycota in DT, while AT was associated with Basidiomycota OTUs. A prior study that correlated diet with gut ecology noted an inverse relationship in enrichment between these two taxa, and a correlation between these fungi and certain bacterial OTUs such as *Prevotella* and *Bacteroides* [201]. How these fungal organisms might potentially contribute to diverticulitis is unknown at this time, although in IBD, a gut disease known to be associated with a chronic intestinal dysbiosis and immune dysregulation, at least one previous study reported an increased Basidiomycota/Ascomycota ratio from fecal samples [248]. The variable size of the ITS region along with relatively poor sequencing of reverse reads in our study prevented analysis of paired-end sequence data. However, a previous report indicates that the analysis of high quality single reads provides robust representation of present communities [254]. Nevertheless, how different fungal organisms may

contribute to varying bacterial ecologies and host immune responses represents a currently unstudied aspect of diverticulitis as well as most other gut diseases.

Using tissue samples to assess the mucosa-associated microbiome rather than stool limits our study to only patients who require surgery for their disease. Additionally, these patients demonstrate a more severe phenotype by virtue of their need for surgery. All such patients required antibiotics pre-operatively; however, only four patients were on antibiotics in the three months prior to surgery. Antibiotic usage (Table 4-1) did not influence bacterial species richness (cephalexin $P = 0.192$, neomycin $P = 0.175$, amoxicillin and clavulanic acid $P = 0.151$, and doxycycline $P = 0.192$) or fungal species richness (cephalexin $P = 0.080$, neomycin $P = 0.162$, amoxicillin and clavulanic acid $P = 0.085$, and doxycycline $P = 0.101$). By utilizing tissue, we are able to examine the mucosa-associated population of microbes rather than cataloguing those excreted in the stool. This obtains a more complete analysis of mechanistically-relevant taxa for a disease whose hallmark is changes to the colon wall. Given the shape of diverticulae, we believe that using mucosa-associated organisms is of particular importance. Diverticulae can have narrow necks, limiting communication with the lumen of the colon and helping to create a physically partitioned, distinct microbial environment.

While perhaps at the cost of a larger sample size, another priority in experimental design was to limit the study population to well-matched and carefully selected cohorts of diverticulitis patients. To overcome inter-individual differences, each subject was used as their own control, by assaying AT collected from a macroscopically non-diseased section of sigmoid colon at the time of surgical resection. The resulting small sample size also hindered the ability to incorporate clinical data as a correlate to microbial findings, and larger studies will be necessary in this regard. Consequently, this is the first diverticulitis study to examine the microbiome using a control group that does not harbor a confounding bacterial community structure, such as in IBD patients who harbor an intestinal dysbiosis at baseline [168].

In summary, we provide a further description of the microbial communities associated with diverticulitis. The inclusion of fungal organisms in microbial analyses of gut diseases has been lacking, and this study represents the first investigation of the mycobiome in diverticulitis. The finding of unique communities of both bacteria and fungi indicate the need to incorporate both kingdoms in future microbiome analyses. Our study additionally demonstrates potential differences between DT and AT in terms of microbial functionality. Future directions in this aim could incorporate matched metagenomics and meta-transcriptomics to glean possible roles of gut microbes in shaping diverticular etiology and progression, as well as host cell transcriptomics to define host-pathogen interactions. Development of a currently unavailable animal model for diverticulitis would also aid in characterizing pertinent microorganisms and potential treatment therapies. In total, examining the interactions between bacterial and fungal species in diverticular disease, evaluating the role anti-fungal agents may have in the treatment of diverticulitis, and exploring microbial metabolic activity, would help to further understand the impact of these organisms on disease pathophysiology.

Acknowledgements

This publication was funded by the Carlino Fund for Inflammatory Bowel Disease Research and in part by Grants UL1 TR000127 and TL1 TR000125 from the National Center for Advancing Translational Sciences (NCATS).

Chapter 5

DISCUSSION

Overview

The development of diverticulitis is most commonly attributed to obstruction of a diverticulum by a fecalith, resulting in inflammation and bacterial dysbiosis [24]. However, as only a small percentage of individuals with diverticulosis develop diverticulitis [5], other factors likely contribute to disease risk and pathogenesis. We hypothesized that deregulation of the innate immune system contributes to diverticulitis by promoting a dysbiotic microbial ecosystem. In Chapter two, RNA-seq of unaffected full-thickness sigmoid colon tissue identified up-regulation of immune response pathways, and in particular expression of genes associated with the innate immune response in diverticulitis patients relative to non-diverticulosis controls. In Chapter three, we found that a particular subset of macrophages expressing CD163L1 localized to the sub-epithelial lamina propria within the colons of diverticulitis patients and displayed a pro-inflammatory phenotype, notably secretion of CXCL10. In Chapter four, by analyzing bacteria and fungi in patient-matched sigmoid colon mucosa from chronically diseased and adjacent tissues, we found that the chronically diseased tissue harbored a unique microbial ecosystem, importantly distinctive bacterial-fungal interactions, compared to adjacent tissue. In this final Chapter, these data will be evaluated as a whole, identifying a potential mechanism defining the interplay between a deregulated immune response and the microbial ecosystem. In addition, we present preliminary data that implicates the potential involvement of a virus in the pathogenesis of diverticulitis. Finally, we will discuss how the findings in this thesis may provide distinct biological criteria to inform medical and surgical decision making to treat individuals afflicted by diverticulitis.

Using RNA-seq as a Tool to Study Diverticulitis Pathogenesis

Recent work in the IBD field has primarily focused on increasing the power of GWAS to detect genetic variants associated with disease, and to date, over 200 IBD-associated SNPs have been identified [63,277]. However, as many of these are tag SNPs that lie in non-coding regions of the genome [205], the causal variants and the functional contribution of this genetic variation is unknown. As the heritability of diverticular disease is significantly higher than IBD [58,59], studies to identify SNPs associated with disease and clinical outcomes are warranted. However, the first GWAS study was recently reported and the results were largely inconclusive as only three SNPs were associated with either diverticular disease or diverticulitis [62]. As diverticulitis is a complex disease, identifying SNPs associated with disease through haplotype analysis or those that segregate within a molecular pathway would be more powerful than the single SNP associations performed by GWAS. In addition, it is established that environmental factors, both extrinsic and the intestinal microenvironment are critical contributors to diverticulitis pathogenesis. Genetic association studies do not take the environmental contribution to disease into account. A method to study both the genetic and environmental contribution to disease is through surveying the transcriptome. RNA-seq is an unbiased and global approach which allows for the analysis of deregulated genes and molecular pathways. Furthermore, bioinformatic methods are available to identify SNPs located within mRNA transcripts that may have a functional impact [278]. To evaluate the overall genetic and environmental contribution to diverticulitis pathogenesis, we used RNA-seq to gain insight into the molecular mechanisms involved in diverticulitis pathogenesis.

Integrative Analysis of the Intestinal Immune Response and Microbiome

Identifying the intrinsic causes of diverticulitis is met with difficulty as the ideal study design relies on sigmoid colon tissue obtained prior to the development of diverticulosis and following these individuals longitudinally until diverticulitis onset. However, this approach is not feasible as healthy tissue is often difficult to obtain and diverticulitis may take many decades to manifest. As an alternative approach, we obtained sigmoid colon tissue collected at surgery that is adjacent (5-10 cm) to areas of chronic diverticulitis (Figure 4-1A, B). Gross examination of these tissue sections did not reveal overt inflammation and a normal bowel wall thickness was noted. Comparatively, areas of chronic disease displayed a thickened bowel wall. Histological examination of tissue sections revealed a lack of neutrophilic inflammation in adjacent tissue (Figure 4-1C, D) and chronically diseased tissue (Figure 4-1E, F). As all surgeries were performed electively, none of the patients were undergoing acute diverticulitis at the time of tissue collection. By utilizing the adjacent tissue in our analyses, we aimed to identify potential intrinsic molecular factors that increase the risk for diverticulitis relative to a non-diverticulosis control cohort.

Based on the data presented in this dissertation, we developed a general model of disease pathogenesis (Figure 5-1). As individuals age, the connective tissue of the bowel wall begins to weaken, increasing its susceptibility to the formation of diverticula and the onset of diverticulosis [279]. We hypothesize that the presence of diverticula alters the microbial ecosystem, including the bacteria, fungi, and viruses. This notion is supported by previous reports of differences in bacterial composition between diverticulosis patients and healthy controls [119,166]. Although aging is associated with diminished immune function [280], we hypothesize that the innate immune system maintains microbial homeostasis in diverticulosis patients. Supporting this

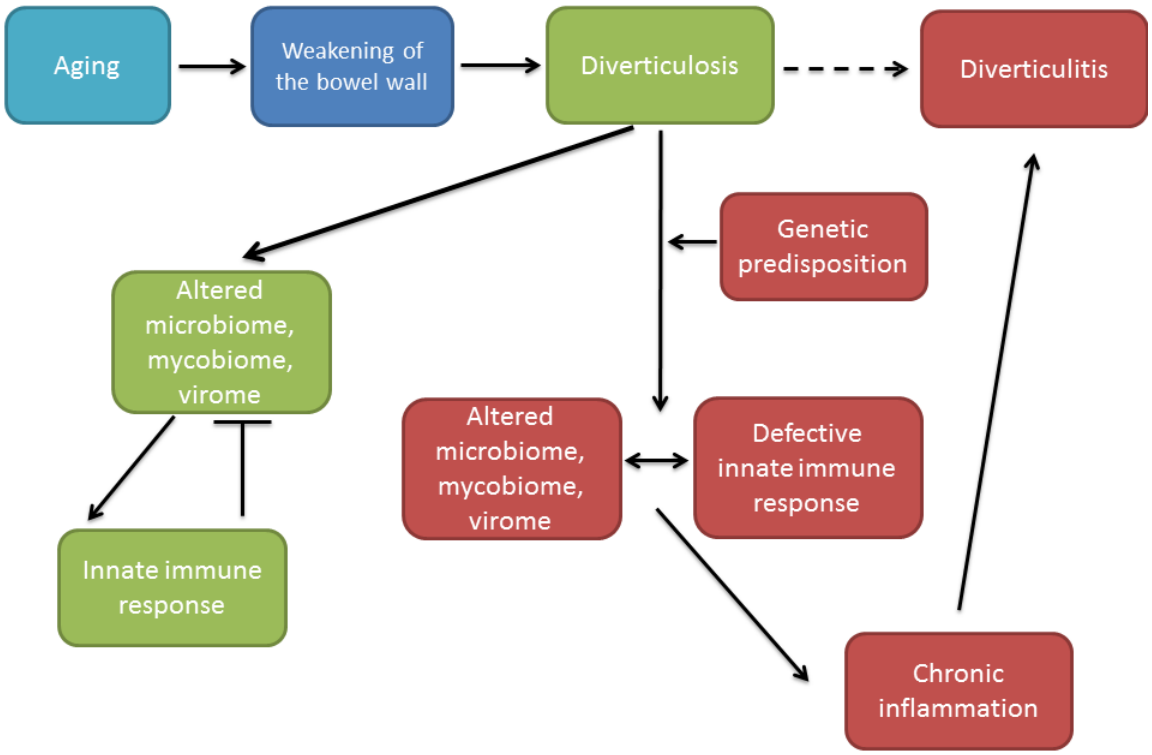


Figure 5-1. Proposed mechanism of diverticulitis pathogenesis.

As part of the aging process, the bowel wall and immune system begin to weaken. This process contributes to the development of diverticula. A majority of individuals will remain asymptomatic throughout their lifetime (diverticulosis). We predict that the presence of diverticula alters the microbial ecosystem and activates the innate immune system to maintain homeostasis. In a subset of individuals, this dysbiosis is unable to be controlled, potentially due to a genetic predisposition, resulting in chronic inflammation. Prolonged chronic inflammation may result in diverticulitis.

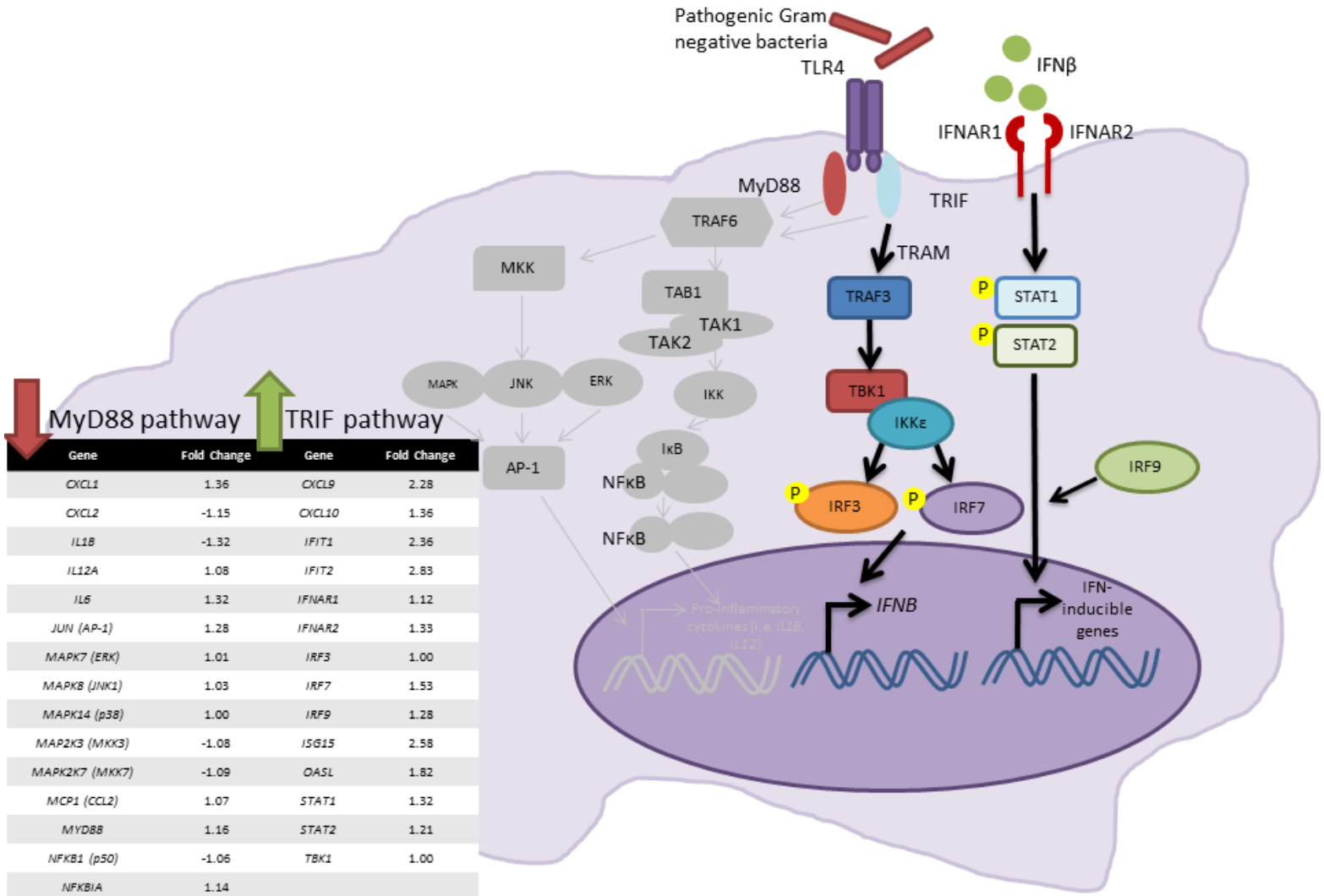
hypothesis, increased abundance of $CD68^+$ macrophages were found in sections of the sigmoid colon harboring diverticula and non-diverticular areas of diverticulosis patients [119]. We propose that in a small proportion of diverticulosis patients, a defect in the innate immune response results in chronic inflammation. In our cohort of chronic, recurrent diverticulitis patients, we found an increased number of $CD163L1^+CD68^+$ macrophages (Figure 3-2), of which a subset of these cells co-expressed the pro-inflammatory chemokine CXCL10 in regions adjacent to chronically diseased tissue (Figure 3-3). As the incidence of diverticulitis is relatively low, we hypothesize that this immune defect may be associated with a genetic predisposition. Consequently, deregulation of the innate immune response disrupts microbial homeostasis, as was demonstrated by our microbiome analysis of adjacent and chronically diseased tissue. We found that the core microbiome specific to the adjacent tissue of diverticulitis patients was comprised of Bacteroidetes and Proteobacteria while the chronically diseased section of sigmoid colon harbored primarily Proteobacteria (Supplemental Table 4-2). Supporting these findings, a previous report found that OTUs within Proteobacteria were predictive of diverticulitis [168].

The molecular mechanism of diverticulitis pathogenesis is largely unstudied. We sought to address this by using RNA-seq to identify candidate pathways associated with disease. One differentially expressed gene we detected encoded CD163L1, a scavenger receptor on resident macrophages. Within the innate immune response, we showed that a subset of $CD163L1^+$ macrophages co-expressed with CXCL10 (Figure 3-3). One pathway promoting CXCL10 secretion is through TLR4 signaling upon binding of LPS. Our microbiome data suggested that TLR4 signaling may be one mechanism of CXCL10 secretion as a *Pseudomonas* OTU was highly associated with the adjacent tissue (Figure 4-3). Moreover, CXCL10 was only co-localized with CD163L1 at the sub-epithelium, indicating that expression may be mediated by infiltrating bacteria. There are two arms driving LPS-mediated TLR4 signaling, including: (1) MyD88-independent, TRIF-dependent and (2) MyD88-dependent pathways. We evaluated components of

these pathways in our RNA-seq data set to determine whether defects in TLR4 signaling correlated with diverticulitis.

Binding of LPS to TLR4 stimulates the MyD88-independent, TRIF-dependent pathway to promote IFN-β production through binding of the transcription factor interferon regulatory factor (IRF) 3 to their consensus sequence 5'-GAAANNGAAANN-3' within the *IFNB* promoter [281]. IFN-β interacts with its cognate receptor, a heterodimer of interferon alpha and beta receptor subunit (IFNAR) 1 and IFNAR2, to promote expression of IFN-inducible pro-inflammatory genes, including CXCL10, through the JAK/STAT pathway [93]. We found that *IFNAR2* and the transcription factors *STAT1* and *STAT2* were also overexpressed in diverticulitis patients relative to controls. This may mediate the production of the MyD88-independent, TRIF-dependent signaling pathway downstream target genes *CXCL10,* interferon-induced proteins with tetratricopeptide repeats (*IFIT) 1, IFIT2,* interferon-stimulated gene 15 (*ISG15*), and 2'-5'-oligoadenylate synthetase like (*OASL*) [282] (Figure 5-2).

On the contrary, the MyD88-dependent pathway results in the transcription of pro-inflammatory cytokines by NF-κB and mitogen-activated protein kinase (MAPK) signaling [67]. We found that transcription of NF-κB signaling pathway genes, including *NFKB1,* RELA Proto-Oncogene (*RELA*), and NFKB inhibitor alpha (*NFKBIA*) were unchanged in diverticulitis patients relative to non-diverticulosis controls (Figure 5-2). Similarly, the MAP kinase signaling pathway also demonstrated minimal change in transcript levels for Jun proto-oncogene AP-1 transcription factor subunit (*JUN*), *MAPK7, MAPK14, MAPK8*, mitogen-activated protein kinase kinase (*MAP2K*) *3*, and *MAP2K7*. These findings correlated with minimal change in downstream target genes of the MyD88-dependent pathway, including *CXCL1, CXCL2, IL1B, IL12A, IL6,* monocyte chemoattractant protein 1 (*MCP1*), and suppressor of cytokine signaling 3 (*SOCS3*) [282].



MyD88 pathway TRIF pathway

Gene	Fold Change	Gene	Fold Change
CXCL1	1.36	*CXCL9*	2.28
CXCL2	-1.15	*CXCL10*	1.36
IL1B	-1.32	*IFIT1*	2.36
IL12A	1.08	*IFIT2*	2.83
IL6	1.32	*IFNAR1*	1.12
JUN (AP-1)	1.28	*IFNAR2*	1.33
MAPK7 (ERK)	1.01	*IRF3*	1.00
MAPK8 (JNK1)	1.03	*IRF7*	1.53
MAPK14 (p38)	1.00	*IRF9*	1.28
MAP2K3 (MKK3)	-1.08	*ISG15*	2.58
MAPK2K7 (MKK7)	-1.09	*OASL*	1.82
MCP1 (CCL2)	1.07	*STAT1*	1.32
MYD88	1.16	*STAT2*	1.21
NFKB1 (p50)	-1.06	*TBK1*	1.00
NFKBIA	1.14		
RELA (p65)	1.05		
SOCS3	1.17		
TAB1	1.01		

Figure 5-2. Proposed mechanism of TLR4 signaling in diverticulitis.

We propose that patients with diverticulitis have defective TLR4 signaling. RNA-seq was performed from full-thickness sigmoid colon tissue obtained from diverticulitis patients (n=20) and non-diverticulosis controls (n=5), with fold change values listed in the table for selected genes in this pathway. Lipopolysaccharide derived from pathogenic Gram negative bacteria, detected by 16S rRNA sequencing, activate TLR4. The RNA-seq data suggests that the MyD88-dependent signaling is down-regulated in diverticulitis patients. Up-regulation of TRIF-dependent signaling results in transcription of type I interferon which binds IFNAR1/2 and stimulates secretion of IFN-inducible genes, such as *CXCL10* via the JAK/STAT pathway. Figure adapted from Mandraju *et al.* [98].

Overall, these data suggest that the MyD88-dependent pathway is not engaged and the MyD88-independent, TRIF-dependent arm of TLR4 signaling is up-regulated in diverticulitis patients.

A previous report described a similar mechanism in UC patients, whereby monocyte-derived macrophages demonstrated a TLR4-specific defect, correlating with enhanced IFN-β and CXCL10 secretion [283]. CXCL10 functions as a chemokine, recruiting cells expressing CXCR3, including activated T and B cells, natural killer cells, and monocytes/macrophages [97,284,285]. As these immune cells are involved in potentiating a T_H1-mediated immune response [97], aberrant recruitment of $CXCR3^+$ cells to colonic tissues may result in chronic inflammation. Interestingly, blocking CXCL10 expression protects against colitis in mice [286,287] by impairing T_H1 immune cell recruitment into the colon [288], suggesting that therapeutic mitigation of this pathway may be beneficial for diverticulitis patients.

However, future studies are required to determine if defective MyD88-dependent signaling through the MAPK and NF-κB arms downstream of TLR4 is associated with diverticulitis, as the current data only evaluated gene expression. One limitation of our RNA-seq approach is that we utilized full-thickness tissue for analysis and thus is representative of a heterogeneous cell population. Purification of macrophages derived from the intestinal mucosa would allow for a more directed test of our hypothesis. Western blot analysis of MAPK and NF-κB activation from patient-derived intestinal macrophages will help determine if this arm of the TLR4 signaling pathway is, in fact, deregulated in diverticulitis patients under basal conditions and following stimulation with LPS. Western blot should also be performed for targets within the TRIF-dependent pathway to confirm up-regulation of this arm of the signaling pathway.

RNA-seq from patient-derived intestinal macrophages would delineate specific pathways associated with macrophage signaling that are deregulated in diverticulitis patients. Based on the data presented in this dissertation, we would ideally analyze $CD163L1^+$ macrophages; however, the available antibodies directed against CD163L1 have not been validated for flow cytometry.

Thus, we may have to expand this analysis to conventional M1 and M2 markers (Table 1-2). Additionally, our Biobank has whole blood collected for RNA studies. Evaluating the peripheral blood transcriptomic profile may allow greater insight into the intrinsic immune-mediated mechanisms of diverticulitis pathogenesis.

Although our data suggests that diverticulitis pathogenesis may result from a deregulated interaction between the host immune system and intestinal microbiota, many questions still remain unanswered and must be addressed to advance the field. One lingering question pertains to whether the diverticulum serve as a niche for a unique microbial ecosystem [289]. We attempted to address this question through our microbiome and mycobiome analysis by studying tissue adjacent to and affected by chronic disease, thereby determining whether differing ecosystems populated the same colonic segment (Chapter 4). The diverticulum potentially harbors fecal material and thus a unique bacterial composition. A similar model has been described in mice whereby transverse folds in the ascending colon that anatomically display some similarities to a diverticulum [289] harbor a different bacterial composition relative to the luminal contents [290]. To our knowledge, similar studies have not been performed from the diverticulum of humans but would indeed be feasible using similar methods and tissues in our Biobank. This study would be of particular interest as more microbiome studies are performed from tissue samples to gain a better understanding if the full-thickness or mucosal tissue sections are truly representative of the diverticular ecosystem.

We hypothesize that patients who develop diverticulitis have a dysbiotic microbiome which increases their susceptibility for disease. We could address this by evaluating the mucosal microbiome assessed from biopsies or brushings of the mucosa obtained near multiple diverticula at the time of colonoscopy in individuals with asymptomatic diverticulosis. As patients with asymptomatic diverticulosis do not undergo surgery, samples would need to be obtained during routine surveillance colonoscopy. Since we found that the microbial ecosystem differs between

adjacent and chronically diseased tissue, we would compare the microbiome of an individual with diverticulosis to an unaffected area near diverticula from diverticulitis patients. Additionally, prospective studies should be performed to identify if predictive microbial markers of disease can be delineated similar to Daniels *et al.* [168]. Biopsy or brushing samples would be collected during asymptomatic diverticulosis and patients segregated by future diverticulitis onset. Although this prospective analysis will be the most powerful to identify predictive microbial targets, this study would be difficult to complete as the subjects analyzed would be required to develop disease.

Barbara *et al.* found that patients with diverticulosis and SUDD had an increased quantity of macrophages within the intestinal lamina propria [119]. Our data supports this finding, by demonstrating an increased number of $CD163L1^{+}$ macrophages within the adjacent tissue of our chronic, recurrent diverticulitis patients (Figure 3-1). These findings beg the question: Is the presence of diverticula alone sufficient to stimulate an enhanced immune response? We show that pathways associated with the innate and adaptive immune systems are up-regulated in adjacent tissue (Figure 2-1). However, the next step is to compare adjacent tissue from patients with diverticulitis to those with diverticulosis. We hypothesize that the deregulated immune response is involved in the pathogenesis of diverticulitis. To test this, RNA-seq and GO pathway analysis could be performed from biopsies to compare adjacent tissue from diverticulitis patients to diverticulosis. We would predict that although the presence of the diverticula may slightly alter the immune profile of diverticulosis patients, this will be minimal compared to the adjacent tissue from patients with diverticulitis. Thus, these findings could suggest that an inherent defect contributes to diverticulitis pathogenesis. A prospective analysis should be performed to identify if this deregulation is, in fact, intrinsic and occurs prior to the onset of diverticulitis.

A powerful approach to identify disease mechanisms is to integrate multiple “omics” data sets derived from the same patient cohort. Barbara *et al.* found a negative correlation between

both *Clostridium* cluster IV and *A. muciniphila* and macrophage counts in patients with diverticulosis and SUDD [119]. As our lab is interested in the molecular mechanism of disease pathogenesis, bioinformatic methods have been developed to correlate 16S rRNA microbiome data with host RNA-seq reads [246,291,292]. By applying these methodologies to our data sets, we may elucidate how the host-microbial relationship contributes to the development of diverticulitis. One limitation of these studies is that they require large sample sizes to provide adequate statistical power to interpret conclusions. Thus, increasing the number of subjects in our Biobank is required before embarking on such an endeavor.

In summary, using RNA-seq, we found that the adjacent sigmoid colon tissue from diverticulitis patients has an altered TLR4 signaling gene expression profile. Notably, these patients demonstrate an increase in TRIF-mediated type I interferon signaling and reduced MyD88-dependent signaling through NF-κB and MAPK pathways. Future directions to confirm these findings at the protein level are needed. Additional studies using patient-derived intestinal macrophages will allow us to directly test our hypothesis and identify relevant mechanisms within the innate immune system that are deregulated in diverticulitis.

Viral Etiology to Diverticulitis

Standard medical therapy for acute sigmoid diverticulitis is empiric antibiotics [6]. However, the use of antibiotics for uncomplicated diverticulitis is under scrutiny as two randomized clinical trials of antibiotic versus no antibiotic treatment in uncomplicated diverticulitis cases have demonstrated no differences in recovery, presence of complications, or recurrence [242,243]. Thus, the American Gastroenterological Association proposed a change in the clinical guidelines to prescribe antibiotics selectively rather than routinely for acute diverticulitis [293]. In Chapter 4, we evaluated the role of the microbiome and mycobiome in

patient-matched tissue from diverticulitis patients. Although we found unique microbial ecosystems segregated adjacent tissue from chronically diseased tissue, whether these changes are a cause or consequence of diverticulitis is still undetermined. The virome, or viral composition, of the intestines is also a component of the intestinal ecosystem that interacts symbiotically with the host and other microbes [294]. The majority (~90%) of the intestinal virome is comprised of bacteriophages [295], which are viruses that infect bacteria to promote lateral exchange of genetic material and virulence factors of pathogenic bacteria [296]. Viruses are also present in healthy individuals, although they contribute to a much smaller percentage of the virome [296]. Nonetheless, viruses also interact with the host immune system through TLRs [294,297]. TLR3, TLR7/8, and TLR9 are localized in the endosome [297]. All TLRs, except TLR3, recruit the adapter molecule MyD88 [298]. TLR3 signals through a TRIF-dependent mechanism to activate both the NF-κB signaling pathway and IRF-mediated transcription of pro-inflammatory genes and type I interferon, respectively [297] (Figure 5-3). TLR7/8 and TLR9 recruit MyD88 and induces expression of pro-inflammatory cytokines and type I interferons [297].

In Chapter 2, we used GO pathway analysis of the RNA-seq data to identify the molecular pathways associated with diverticulitis. Further evaluation of our RNA-seq dataset identified multiple pathways associated with viral infection and type I interferon-mediated signaling that were up-regulated in diverticulitis patients (Figure 5-4A). The type I interferon signaling pathway is the first line of defense against viral pathogens, mediating production of antiviral proteins [299]. Twenty-seven genes from the type I interferon-mediated signaling pathway segregated most diverticulitis patients from controls (Figure 5-4B). The IFIT family of proteins are induced following infection by DNA and RNA viruses [300]. In particular, cytomegalovirus and adenovirus type 12 induced IFIT proteins late in infection [301,302]. Stimulation of TLR3, TLR7/8, and TLR9 by viral ligands (Table 1-3) activates the JAK/STAT pathway and induces transcription of the *IFIT* family of genes [300]. The OAS family of proteins

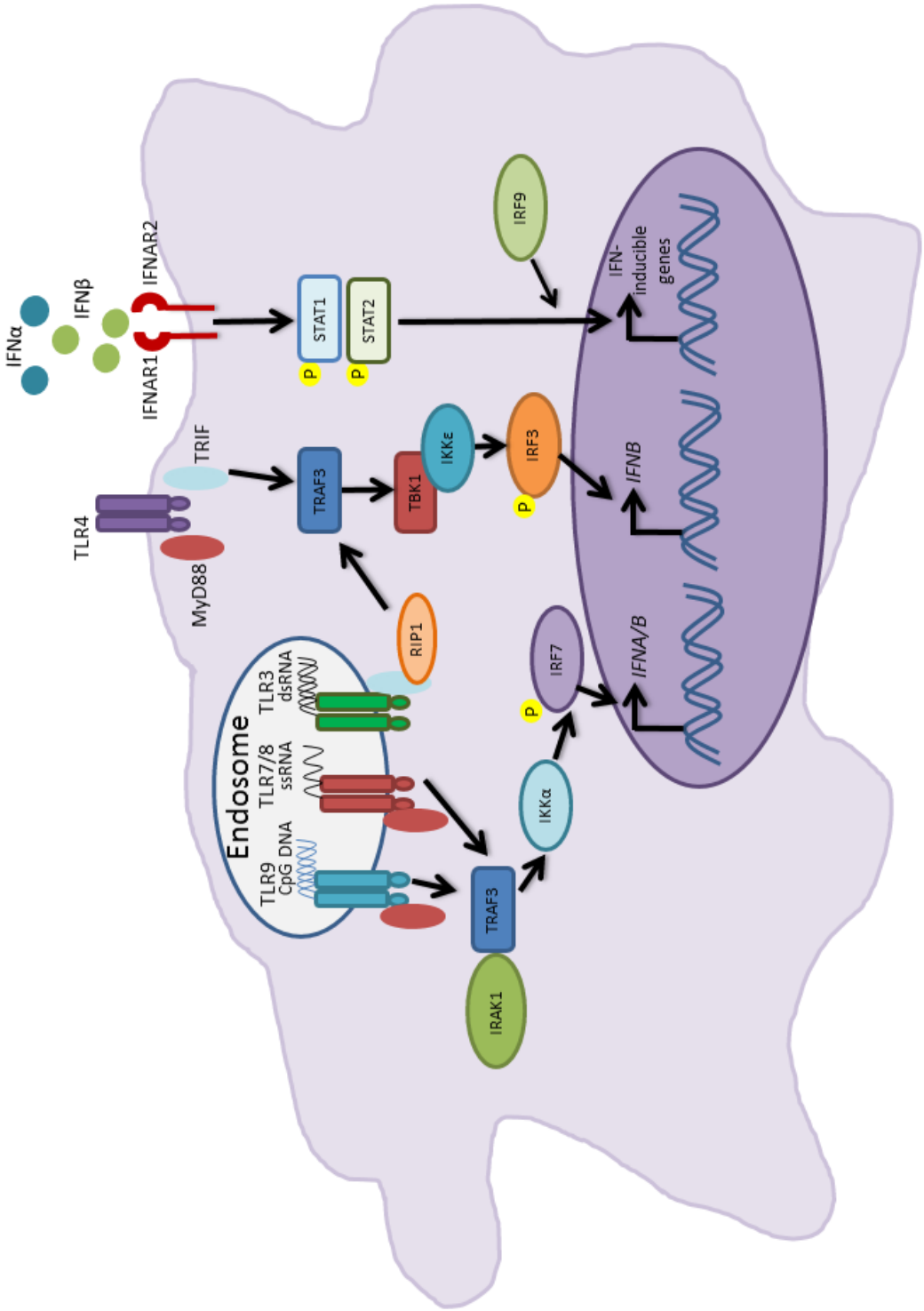

IFNα
IFNβ
IFNAR2
IFNAR1
STAT1
STAT2
P
P
IRF9
IFN-inducible genes
TRIF
TLR4
MyD88
TRAF3
TBK1
IKKε
IRF3
P
IFNB
RIP1
Endosome
TLR3 dsRNA
TLR7/8 ssRNA
TLR9 CpG DNA
IRF7
P
IFNA/B
IKKα
TRAF3
IRAK1

Figure 5-3. TLR signaling pathway in innate viral immunity.

Macrophages express TLR3, TLR7/8, and TLR9 within endosomes. Recognition of viral products activate the MyD88-independent, TRIF-dependent pathway and type I interferon expression. All TLRs shown also signal through TRAF6 to activate the NF-κB signaling pathway (not shown for brevity). See text for details. Figure adapted from Mandraju *et al.* [98].

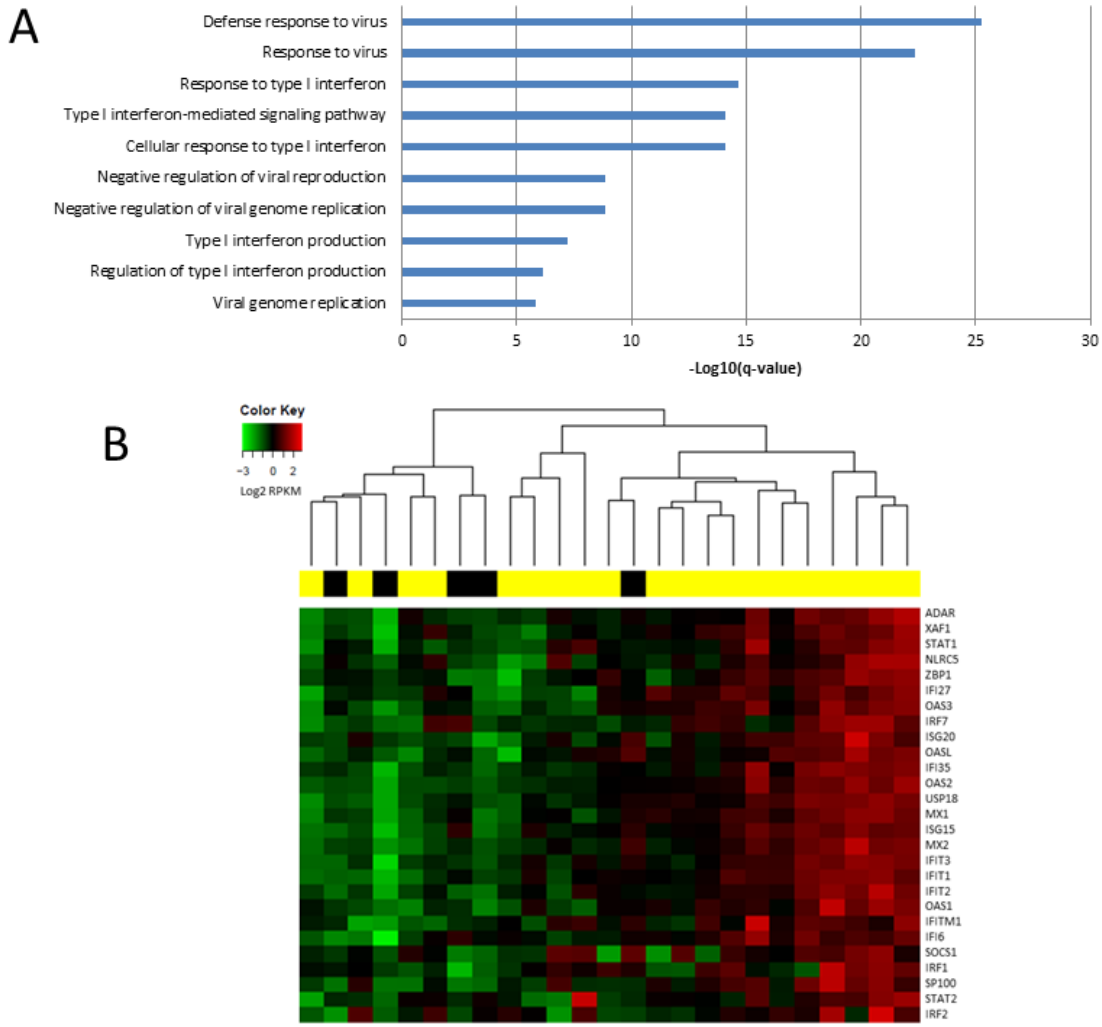


Figure 5-4. Up-regulation of viral signaling pathways in diverticulitis patients by RNA-seq.

(A) Gene ontology categories of virally-mediated and type I interferon signaling pathways obtained from RNA-seq of diverticulitis patients (n=20) and controls (n=5). (B) Hierarchical clustering of log2 RPKM values from RNA-seq demonstrates segregation of diverticulitis patients and controls for 27 genes involved in type I interferon-mediated signaling.

degrade viral RNA in combination with 2'-5'-linked oligoadenylate, activating endoribonuclease to inhibit early viral infection [303]. Based on this evidence, we hypothesized that viral infection may be involved in the pathogenesis of diverticulitis.

To identify viral transcripts in our RNA-seq data set, we subjected raw RNA-seq reads to analysis using Taxonomer [304] (Appendix A). The most abundantly expressed human virus identified in our patient cohort was Human Herpesvirus 6A (HHV-6A) (strain Uganda-1102 (U1102)) (Table 5-1). HHV-6 is a double stranded DNA virus and a member of the *Betaherpesviridae* family and *Roseolovirus* genus [305]. Two distinct variants are found, HHV-6A and B, with 90% homology [306]. Additionally, HHV-6A U1102 is a strain variant first isolated from acquired immunodeficiency syndrome (AIDS) patients in Uganda [307] and is a prototype strain for HHV-6A [308]. HHV-6 has a tropism for mature $CD4^+$ T cells [309,310] and monocytes/macrophages [311]. In $CD4^+$ T cells, HHV-6 signals through TLR9 and activates the MAPK pathway to induce a pro-inflammatory response by AP-1-mediated transcription [312]. However, HHV-6 infection of dendritic cells increased the expression of TLR3, TLR4, and TLR7 [313]. Interestingly, dendritic cells infected with HHV-6 demonstrated impaired NF-κB signaling following LPS stimulation relative to those mock infected [313]. These data correlate with our proposed model of TLR4 signaling and RNA-seq expression data of the MyD88-dependent and –independent signaling pathways (Figure 5-2). Therefore, we chose to pursue HHV-6 as a top candidate in diverticulitis pathogenesis. However, the median number of viral transcripts identified by Taxonomer was not significantly different between diverticulitis and control cohorts (Table 5-1). Unfortunately, Taxonomer does not provide the genes associated with transcripts and those identified may be associated with both latency and active replication.

We sought to identify actively replicating viral proteins in our diverticulitis patients. Indirect immunofluorescence against the gp60/110 envelope glycoprotein of HHV-6A and B was

Table 5-1. Top viruses identified by Taxonomer.

	Diverticulitis median reads (range)	Controls median reads (range)	*P*-value
Human Herpesvirus-6 (strain Uganda-1102)	46 (8-72)	22 (14-74)	0.8550
Hepatitis E virus	3.5 (0-38)	3 (1-13)	0.8913
Measles virus	2.5 (0-8)	2 (1-3)	0.6790

Data analyzed by two-tailed Mann-Whitney *U*-test.

performed. Structural proteins, such as those used to envelop the virus, are produced during the late lytic phase of infection [314]. We found that the tissue adjacent to chronic disease in diverticulitis patients demonstrated increased levels of actively replicating virus in the lamina propria of the sigmoid colon (Figure 5-5A) relative to non-diverticulosis controls (Figure 5-5B). Viral envelope proteins co-localized with $CD4^+$ cells in the lamina propria of diverticulitis patients (Figure 5-5C). Future analyses will also evaluate if HHV-6 gp60/110 co-localizes with the monocyte/macrophage marker CD68. Studies are in progress to quantify *U22*, a highly conserved late gene specific to HHV-6 [315], in intestinal tissue sections to confirm active replication [316].

HHV-6A has yet to be linked to disease as the causal variant; however, acute infection has been associated with the immune-mediated diseases, Hashimoto's thyroiditis [317] and multiple sclerosis [318]. Primary infection with HHV-6B causes the common childhood disease exanthem subitum (sixth disease or roseola infantum) [319]. Following primary infection, the virus actively replicates in the salivary glands while remaining latent in mononuclear immune cells [320]. Reactivation of the virus is rare in immunocompetent individuals [320]. HHV-6 has also been isolated from the intestinal mucosa in immunocompromised patients. HHV-6B was found in patients with IBD, correlating with endoscopic activity [321]. Our Taxonomer data suggests that HHV-6A is present in our intestinal tissues. Although HHV-6A and B variants are 90% homologous [306], restriction enzyme digestion with *Hin*dIII can distinguish the two variants [316]. Viral subtyping is in progress to confirm that HHV-6A was identified by RNA-seq.

To confirm an active infection is present, patient serum will be analyzed for viral antibodies. HHV-6 IgM is found in the serum only during an active infection and during the 2-3

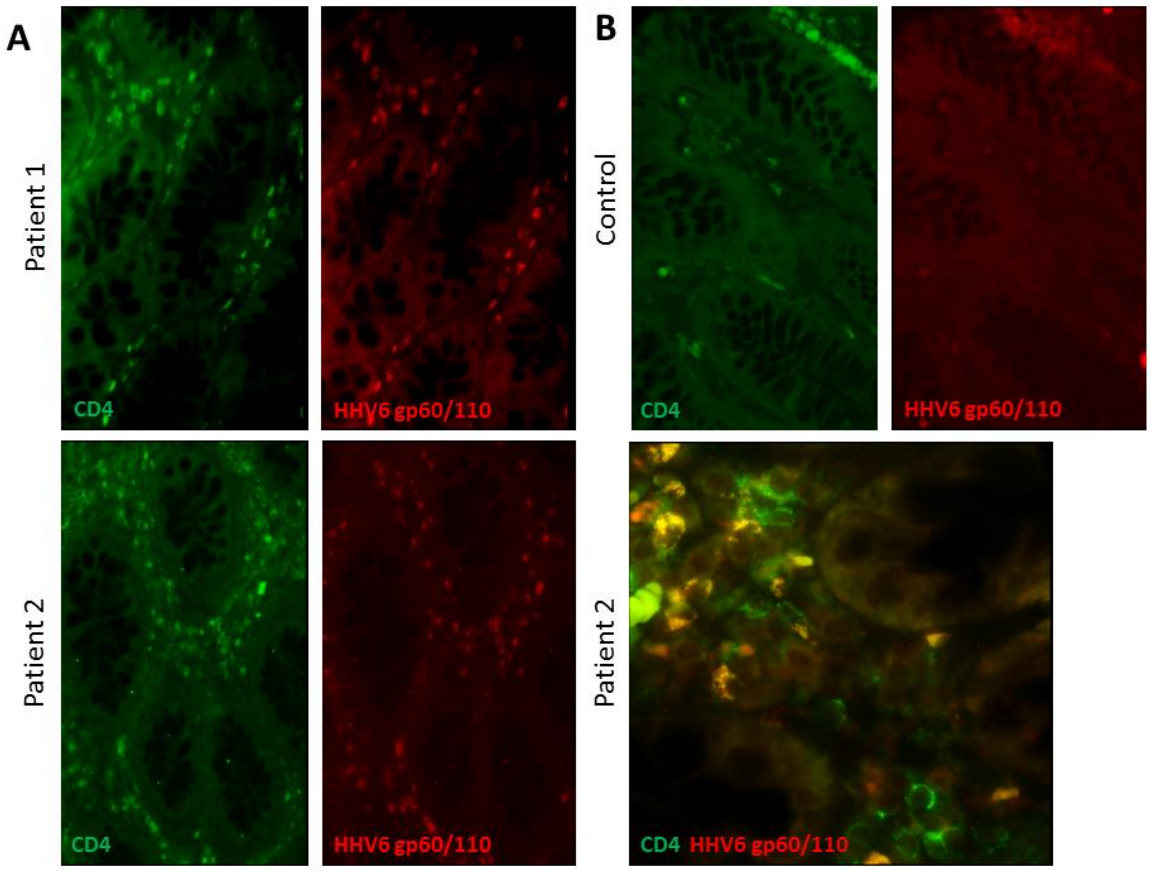


Figure 5-5. A subset of diverticulitis patients demonstrates actively replicating human herpesvirus 6 (HHV-6) within $CD4^+$ T cells in the lamina propria.

(A) Sigmoid colon tissue section from two independent diverticulitis patients stained with an antibody directed against CD4 T cell marker (green) and HHV-6 gp60/110 envelope glycoprotein (red). (B) Sigmoid colon tissue section from a non-diverticulosis control individual. (C) Magnified image of a section of the sigmoid colon from patient 2 depicting co-localization of CD4 and HHV-6 (yellow).

months following an infection [322]. Many diagnostic methods rely on indirect fluorescent antibody assays rather than ELISA, as reduced specificity and difficulty interpreting results is a concern for commercially available ELISA kits [323]. HHV-6 DNA is only found in the serum during acute infection [324]. qPCR of the *U22* gene, similar to that performed in intestinal tissue, can also be performed in serum to quantify viral loads in diverticulitis patients.

Using RNA-seq, we were able to interrogate a previously uncharacterized facet of diverticulitis pathogenesis, the virome. Limitations of this analysis are to be considered as our identification method was restricted to transcripts and thus may have resulted in bias against DNA viruses. However, our top candidate, HHV-6 is a dsDNA virus and we found actively replicating virus to be present in higher abundance in diverticulitis patients through indirect immunofluorescence. Future directions to confirm the unresolved questions related to HHV-6 and diverticulitis and expanding the analysis on a global scale would enhance our understanding of the viral contribution to diverticulitis. By identifying a viral component to diverticulitis, we address the clinical problem associated with lack of efficacy of antibiotics [242,243] and offer alternative therapeutic options for clinical trials, including anti-viral therapy.

Serum CXCL10 as a Clinical Biomarker

There are currently no prognostic or diagnostic biomarkers for diverticular disease. Early mechanistic studies, such as those using RNA-seq, will help identify new potential biomarkers within a molecular pathway of relevance to the disease. Our RNA-seq analysis identified deregulation of innate immunity, in particular macrophage signaling and CXCL10 secretion. To expand on our finding of increased expression of CXCL10 in the sub-epithelial lamina propria of diverticulitis patients (Figure 3-4), we evaluated serum CXCL10 from non-diverticulosis controls and quiescent diverticulitis patients (Figure 3-3). The CXCR3/CXCL10 axis is involved in T_H1

immune cell recruitment, and in particular $CD4^+$ T cells, to areas of inflammation [325]. Activation of this axis has been demonstrated locally in colonic tissue sections from patients with IBD [210,326,327] and systemically in the serum of pediatric and adult patients with CD [239]. Interestingly, serum CXCL10 levels were heightened during both active disease and remission relative to controls [239]. The authors propose that elevation of CXCL10 during both stages of disease may be attributed to its function as a mediator of T_H1 lymphocyte recruitment to propagate chronic intestinal inflammation [239].

Similar to CD patients [239], significantly elevated serum CXCL10 was seen in quiescent diverticulitis compared to controls (Figure 3-3). The clinical characteristics of these patients would be attributable to IBD patients in remission. To be considered as a biomarker for diverticulitis onset, prospective trials that evaluate serum CXCL10 prior to diverticulitis diagnosis are required. Ideally, blood samples would be collected at a similar time point for diverticulosis patients to identify a homogenous cohort (i.e. time from diverticulosis diagnosis, time of day, absence of comorbidities or medications). Moreover, studies that evaluate CXCL10 as a biomarker of surgical outcomes would be of interest. By identifying patients at the time of acute disease and following serum CXCL10 levels through recovery may help to identify which patients will require future surgical intervention. We do not currently have serum banked from patients with active diverticulitis recruiting these individuals would be ideal as we begin to assess the role of CXCL10 as a biomarker.

There is greater sensitivity and specificity in a panel of biomarkers rather than a single measurement [328]; therefore, identifying a panel of serum cytokines and chemokines to determine risk for diverticulitis outcomes would be imperative. Finally, cutoff values for multiple cytokines and chemokines will need to be established to determine high positive and negative predictive values for patients at high risk and low risk for diverticulitis, respectively. Thus,

further studies are required to determining whether serum CXCL10 serves as a biomarker for diverticulitis onset or outcomes.

Conclusions

There is currently limited data regarding the molecular mechanisms of diverticulitis pathogenesis. To overcome this gap in knowledge, we utilized a global transcriptomic approach, allowing us to gain insight into the genetic and environmental contribution of diverticulitis onset. We identified a new role for the innate immune system in diverticulitis, possibly influencing pathogenesis through a deregulated TLR4-mediated mechanism. These data correlate with our microbiome studies, suggesting an increased abundance of Proteobacteria, in particular *Pseudomonas* OTUs, colonizing tissue adjacent to areas of chronic disease.

Empiric antibiotic therapy is used selectively to treat diverticulitis [6,293]. However, as clinical trials have not demonstrated a benefit to antibiotics [242,243], identifying other treatment options is needed. By identifying common molecular pathways that are deregulated in diverticulitis patients, new drug targets can be evaluated. Additionally, through our own work, we show that both the fungal and viral compositions differ in patients with diverticulitis. Thus, including both anti-fungals and anti-virals in clinical trials may be beneficial, especially for patients with chronic, recurrent diverticulitis.

Clinically, being able to identify patients who are likely to develop chronic diverticulitis from those that will undergo an asymptomatic disease course would provide long-term benefit to the population. We provide evidence implicating molecular and genetic biomarkers in prognostic evaluation of diverticulitis patients. Serum CXCL10 was significantly higher in diverticulitis patients and expanding this marker to a panel of serum cytokines and chemokines could lead to a robust biomarker of disease outcomes.

In summary, by utilizing an unbiased global approach to survey the transcriptome, we identified a previously unconsidered role for the interaction between the innate immune response and microbial ecosystem in the pathogenesis of diverticulitis. These findings identified a potential biomarker of diverticulitis, CXCL10, which following further investigation may be beneficial in clinical decision making. Studies in progress evaluating the virome of diverticulitis patients identified HHV-6 as a possible viral contributor to diverticulitis. Evaluating anti-viral therapy in clinical trials will address the lack of efficacy of current therapeutic options in patients with diverticulitis.

Appendix

SUPPLEMENTAL METHODS

Identification of Viral Sequences from RNA-seq Reads

To identify viral genomic sequences within the RNA reads generated by RNA-seq, a bioinformatic pipeline called Taxonomer [304] was utilized. Briefly, reads are binned as human, bacterial, fungal, viral/phage, unknown, ambiguous, or user-specific and taxonomically classified using the appropriate databases. Total number of reads for each taxonomically identified virus was obtained and only human viruses were evaluated. Median values are reported for diverticulitis and control cohorts and significance determined by Mann-Whitney *U*-test performed in R version 3.3.3 (The R Project for Statistical Computing, Vienna, Austria).

Indirect Immunofluorescence for HHV-6 gp60/110

Formalin-fixed tissue sections were paraffin-embedded and sectioned with 5 μm thickness. Slides were deparaffinized by two 5 min xylene washes followed by an alcohol gradation (100% ethanol for 3 min, thrice; 95% ethanol for 3 min, twice; 70% ethanol, once; water for 5 min). Antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a steamer for 20 min then cooled to room temperature for one hour. Slides were incubated with primary antibody overnight. The mouse monoclonal anti-HHV-6 gp60/110 envelope glycoprotein (MAB8537, EMB-Millipore, Billerica, MA) was used at a 1:50 dilution to visualize actively replicating virus. The mouse monoclonal antibody anti-CD4 (ab133616, abcam, Cambridge, MA) was used at 1:500 dilution to visualize T cells. The appropriate secondary antibody (1:800) was added (Alexa Fluor 647 goat anti-mouse IgG (H+L), Invitrogen, Carlsbad, CA) and slides were incubated for one hour prior to setting with the VECTASHIELD HardSet Mounting Media with DAPI (Vector Laboratories, Burlingame, CA). Slides were visualized using the 20x/ 0.75 Plan S Apo objective (GE Healthcare Life Sciences, Pittsburgh, PA).

HHV6 *U22* Cloning

HHV-6A HEK293 (clone D2) in U1102 cells (HHV6 Foundation, Santa Barbara, CA) were cultured in Dulbecco's Modified Eagle Medium (ThermoFisher, Waltham, MA), 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. DNA was isolated from pelleted cells using the DNeasy kit (Qiagen, Germantown, MD). The *U22* late gene, a highly conserved region specific for HHV-6 encoding for a glycoprotein, was amplified using the following primers [316] (forward: TCGAAATAAGCATTAATAGGCACACT; reverse: CGGAGTTAAGGCATTGGTTGA). Thermocycling conditions were 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s followed by 72°C for 5 min. The PCR product was electrophoresed on a 1% agarose gel for 1 hour at 100V and the 99 bp product was gel purified using the QIAquick Gel Extraction kit (Qiagen, Germantown, MD). TOPO-TA cloning with TOP10 competent *Escherichia coli* cells (Life Technologies, Carlsbad, CA) was used to clone the full length cDNA into bacteria following the manufacturer's instructions. Bacterial colonies were picked and cultured for 18 hours in Luria-Bertani (LB) broth with ampicillin then subjected to the Nucleospin Plasmid Miniprep Kit (Macherey-Nagel, Bethlehem, PA). Plasmid DNA was sent to the Genomics Core Facility (University Park, PA) for Sanger sequencing using the same primers as previously described to identify potential mutations.

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SELECTED PEER-REVIEWED PUBLICATIONS

1. **Schieffer KM**, Wright JR, Harris LR, Deiling S, Yang Z, Lamendella R, Yochum GS, Koltun WA. (2017) NOD2 signaling pathway variants and microbiome differences contribute to the occurrence of pouchitis in one of two FAP siblings following ileal pouch-anal anastomosis. *J Crohn's Colitis* in press.

2. **Schieffer KM**, Choi CS, Emrich S, Harris LR, Deiling S, Karamchandani DM, Salzberg A, Kawasawa YI, Yochum GS, Koltun WA. (2017) RNA-sequencing implicates deregulation of the immune system in the pathogenesis of diverticulitis. *Am J Physiol Gastrointest Liver Physiol* **313**(3):G277-84.

3. **Schieffer KM***, Sabey K*, Wright JR, Toole DR, Drucker R, Tokarev V, Harris LR, Deiling S, Eshelman MA, Hegarty JP, Yochum GS, Koltun WA, Lamendella R, Stewart Sr. DB. (2017) The Microbial Ecosystem Distinguishes Chronically Diseased Tissue from Adjacent Tissue in the Sigmoid Colon of Chronic, Recurrent Diverticulitis Patients. *Sci Rep* **7**(1):8467. *co-first authors

SELECTED SCIENTIFIC MEETING PRESENTATIONS

1. **Schieffer KM**, Poritz L, Yochum GS, Koltun WA. Using Endoscopy to Longitudinally Study the Development of Colonic Metaplasia in a Rat Ileorectal Model of Pouchitis. Advances in Inflammatory Bowel Disease. December 8-10, 2016. Orlando, FL. (poster presentation)

2. **Schieffer KM**, Wright JR, Harris LR, Deiling S, Yochum GS, Lamendella R, Koltun WA. *NOD2* allele variant and microbiome differences contribute to the occurrence of pouchitis in one of two FAP siblings following ileal pouch-anal anastomosis. Digestive Diseases Week. May 6-9, 2017. Chicago, IL. (poster presentation)