BACTERIAL MODULATION OF THE EPITHELIAL BARRIER IN CROHN'S DISEASE

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by *Dillon Duhon*, titled *Bacterial Modulation of the Epithelial Barrier in Crohn's Disease* and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.

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List of Abbreviations

AMP – Antimicrobial Peptide

ASCA – Anti-*Saccharomyces cerevisiae* Antibodies

BA – Bile Acid

CD – Crohn's Disease

CLDN1 – Claudin-1

CREB – cAMP Response Element Binding Protein

CRP – C-reactive Protein

DAMP – Damage-associated Molecular Pattern

DCA – Deoxycholic Acid

DSS – Dextran Sodium Sulfate

EGFR – Epidermal Growth Factor Receptor

ER – Endoplasmic Reticulum

FXR – Farnesoid X Receptor

GF – Germ-free

HPLC – High-performance Liquid Chromatography

IBD – Irritable Bowel Disease

IBS – Irritable Bowel Syndrome

IEC – Intestinal Epithelial Cell

IRAK – IL-1R-associated Kinase

LCA – Lithocholic Acid

MAMP – Microbial-associated Molecular Pattern

MYD88 – Myeloid Differentiation Primary Response Protein 88

NF-κB – Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

NMR – Nuclear Magnetic Resonance

PPARγ – Proliferator-activated Receptor Gamma

PRR – Pattern Recognition Receptor

SCFA – Short-chain Fatty Acid

SPF – Specific Pathogen-free

TIR – Toll/IL-1 Receptor

TLR – Toll-Like Receptor

Tollip – Toll-interacting Protein

TNF – Tumor Necrosis Factor

TRAF6 – Tumor Necrosis Factor Receptor-associated Factor 6

TRIF – TIR-domain-containing Adapter-inducing IFNβ

UC – Ulcerative Colitis

UDCA – Ursodeoxycholic Acid

UPR – Unfolded Protein Response

XBP1 – X-box-binding Protein 1

ZO1 – Zonula occludins 1

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Abstract

Crohn's Disease is a complex, multifactorial disorder induced by chronic, disproportionate inflammatory responses to the bacteria that reside in the digestive tract. One mechanism by which this inflammatory response is induced is a dysfunctional epithelial barrier, in which bacteria are exposed to the immune system in an unregulated fashion that leads to inflammatory-mediated bowel damage. As discussed in this thesis, the epithelial barrier is regulated by a number of different cell types, receptors, as well as the products of microbial metabolism. Microbial metabolites, such as short-chain fatty acids, indole derivatives, and secondary bile acids, have recently been elucidated to have significant physiological effects on the epithelial barrier. Thus, microbial metabolites may have useful implications as Crohn's Disease biomarkers or as therapeutic agents in disease treatment and remediation.

Overview of Crohn's Disease

Introduction

Since the turn of the 21st century, the developed world has incurred a marked increase in the occurrence of chronic inflammatory diseases. Specifically, in Westernized nations, the incidence of inflammatory bowel disease has increased to a point in which 1 in 200 individuals are affected [46]. Inflammatory bowel disease may be stratified into two disease categories, ulcerative colitis (UC) and Crohn's disease (CD). While these diseases are categorized under the broader umbrella of inflammatory bowel disease, they differ significantly in their location within the gastrointestinal (GI) tract, symptomatology, pathophysiology, disease complications, and treatment options. The substantial increase in the occurrence of inflammatory bowel diseases in the 21st century across the developed nations presents a significant burden to healthcare. Patients diagnosed with Crohn's Disease experience serious detriments to quality of

life, including complications that can lead to irreversible tissue damage, surgical resection, and hospitalization.

UC is a disease restricted to the colon, characterized by continuous lesions and superficial inflammation, which can progress to symptoms such as epithelial erosion, ulcers, and bloody diarrhea [46]. CD is a chronic, progressive inflammatory disease characterized by intestinal skip lesions that can occur anywhere along the length of the GI tract. In addition to the more systemic presentation of CD, the complications associated with the disease present serious quality of life consequences for the patients, resulting in a significant disease burden to society. Fifty percent of patients diagnosed with CD will develop serious intestinal complications such as intestinal fistulae and strictures within 10 years of diagnosis, which often require hospitalization and surgery [46]. Furthermore, nearly 30% of patients with CD already have bowel damage at diagnosis, with half of these requiring surgery within 20 years of the diagnosis [46]. Given that colon damage may already be present at diagnosis, in addition to the observation that CD is most often diagnosed in people under the age of 30, there is an urgent need for more effective screening technologies in the identification of at-risk populations.

Risk Factors for Crohn's Disease

The risk factors for the development and progression of Crohn's Disease include both genetics and the environment. From a genetic perspective, more than 200 disease loci have been identified as CD risk factors, however the majority of these variants only modestly increase disease risk [46]. The most significant genetic risk variants identified thus far have been mutations in loci for the pattern-recognition receptor *NOD2*, the IL-23 receptor gene *ILR23R*, and the autophagy gene *ATG16L1* [46]. While discovery and investigation of polymorphisms in these genes has been valuable in identifying potential disease mechanisms and pathogenesis, genetic variance alone has been ineffective thus far in addressing the wide range of disease phenotypes, differences in disease location, or differences in the age of patients at diagnosis. This observation strongly suggests that there are environmental and epigenetic influences on the pathogenesis and development of CD.

Despite the identification of new environmental risk factors in the last decade, smoking remains to be the largest modifiable risk factor for Crohn's Disease [46]. Not only does smoking double the risk of developing CD, it also increases the risk of surgical intervention, disease recurrence after surgery, and can be associated with earlier disease onset. In addition to smoking, the use of antibiotics during childhood, regular use of non-steroidal antiinflammatory

drugs (NSAIDs), and oral contraceptives have been noted to increase the risk of developing CD [46].

Another risk factor that has gained significant traction in the past decade is diet, which can significantly alter the composition of the gut microbiome. The term "gut microbiome" refers to the trillions of microorganisms that colonize the entire length of the digestive tract. It is estimated that nearly $4\ x\ 10^{13}$ microorganisms, including viruses, bacteria, fungi, and archaea, live within the digestive tract [9]. The large majority of these microorganisms are considered to be mutualistic or commensal, and indeed many of these microorganisms are instrumental in digestion, absorption of nutrients, host immunity, endocrine signaling, and disease prevention [21]. The contents of the human diet can dramatically influence the microbial communities within the gut, which consequently impact the function and composition of metabolic, endocrine, neurological, and immunological systems that propagate along the length of the digestive tract. More specifically, the advent of the high-fat, low-fiber "Westernized Diet" has been shown to decrease microbial diversity and lead to a condition called gut dysbiosis [21].

Gut dysbiosis, which is defined as a microbial imbalance, is not only a symptom of Crohn's Disease but has recently been implicated as a significant risk factor for disease pathogenesis. The term "dysbiosis," however, is not particularly valuable in elucidating a role in disease pathogenesis, as a microbial imbalance is a non-specific terminology. For example, an imbalance of microbes could refer to a proliferation or depletion of a specific species of bacteria, but it may also refer to a general depletion of bacterial diversity within the gut. Therefore, it is important and relevant to characterize the specific forms of dysbiosis that could contribute to disease pathogenesis, disease progression, or even disease resolution.

While significant progress has been made in identifying genetic risk variants for CD, it is estimated that the known risk loci only explain about 13% of disease liability [32]. This finding exemplifies the potential for epigenetic and environmental influences in the context of disease etiology and progression. Perhaps the most promising environmental factor currently under study is the interface of the gut microbiome with the intestinal epithelial cells (IECs) that form a protective barrier within the lining of the digestive tract. Recent advances in metabolomic, metagenomic, and biomarker sequencing technologies have revealed that the bacteria present in the gut produce an impressive number of microbial metabolites, some of which have already been identified to have significant physiological mechanisms, including regulation of intestinal barrier function, inflammation, and bacterial recognition. Given that a compromised epithelial barrier and chronic inflammation are hallmarks of CD, investigation into the potential roles of microbial metabolites in the context of Crohn's Disease is warranted. The intent of this thesis is

to summarize the interface between the gut microbiome and IECs, highlight microbial metabolites relevant to the function of the epithelial barrier, and discuss the implications of microbial metabolites in improving screening technologies and treatment opportunities for Crohn's Disease.

Anatomy of the Gut

The Gut Microbiome & Biogeography of the Gut

Critical to the study of Crohn's Disease, or any disease state, is a thorough understanding of the underlying anatomy and the conditions that exist under homeostasis, or what may be considered "normal function." For this reason, an introduction into the biogeography of the digestive tract is warranted to establish a baseline understanding of the physiology that drives the function of the multi-organ system that constitutes the "gut." Additionally, because this thesis will discuss the relationship of the gut microbiome and CD, an understanding of how the conditions within the digestive tract drive microbial colonization is also relevant. While the digestive tract is often described as one long, continuous tube, it is important to note that the environment within the gut changes drastically from the mouth to the distal colon. These environmental parameters, both chemical and physical, include abundance of oxygen, pH, type and density of mucus, rates of peristalsis, nutrient availability, and levels of antimicrobial peptides [33]. The physical and chemical conditions within distinct regions of the digestive tract are critical to their function in digestion and nutrient absorption, as well as influencing the microbial communities that reside within.

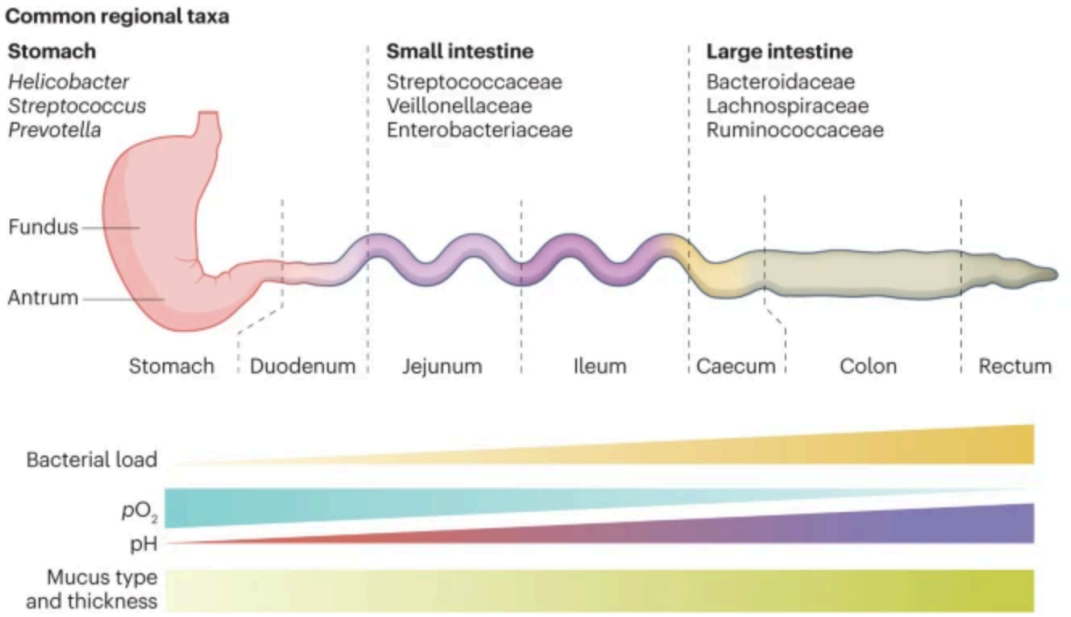

Figure 1: Regional biogeographical features of the gastrointestinal tract at the scale of the whole gut (McCallum et al) The chemical and physical characteristics of the digestive tract change signficantly from the beginning of the stomach to the distal colon. Bacterial colonization along the length of the digestive tube is dependent on the physical and chemical properties that exist within these physiologically distinct sections.

Figure 1 above depicts the biogeographical organization of the gut along the longitudinal axis from the stomach to the colon. Importantly, bacterial load and diversity increase significantly towards the distal end of the digestive tract. The proximal portion, namely the oral cavity and the stomach, are highly acidic and oxygenated compared to the more distal portions. As such, facultative anaerobes and bacteria that can evade the acidic environment tend to proliferate in these cavities [33]. The presence of a thick mucus layer within the stomach is key to both normal physiological function and bacterial colonization. The pH of the stomach lumen is between 2-3.5, however the pH of the mucus layer is dynamic, reaching a neutral pH (~7.4) within the glands of the mucosa.

The biogeography of the small intestine is unique due to the length of the organ and the gradual transformation of contents passing through the lumen. The proximal end of the small intestine (duodenum) is similar to the stomach in terms of bacterial diversity, owing to the high rate of flow, prominent secretion of antimicrobial peptides (AMPs), and presence of oxygen. However, as the small intestine transitions into the jejunum and ileum there is a neutralization of

pH (~7.5) and a significant reduction in oxygen pressure. Highly acidic conditions are inhibitory to the growth of most bacteria, so this neutralization is favorable for bacterial colonization [33]. The transit time of luminal contents in the jejunum and ileum is still relatively fast (2-6 hours), so fast-growing bacteria that have tools to adhere to the mucus layer are more likely to proliferate. In addition, simple carbohydrates are the most abundant nutrient available during this stage of digestion, so bacteria which can ferment simple carbohydrates are more successful [33]. Interestingly, microbial diversity in the distal small intestine is lower than in the upper small intestine, despite the increased bacterial load that is present.

As the ileum transitions into the caecum and distal colon, the physiological environment transitions to conditions that allow for the highest bacterial burden and greatest bacterial diversity. The lumen of the colon is more acidic than the small intestine due to the fermentation of complex polysaccharides and dietary fiber, which are critical for microbial metabolism [33]. Many of the microbes within this region of the gut are critical for human metabolism as well, as the fermentation of dietary fibers liberates short-chain fatty acids (SCFAs) that are an important energy source for intestinal epithelial cells [21]. The most well-characterized SCFAs, propionate, butyrate, and acetate, have been implicated in numerous biological processes that can have both local and systemic effects. It is estimated that fermented microbial products produced in the colon account for 5-10% of daily energy requirements in humans [21]. In addition to the lower pH, the environment of the colon is also highly anaerobic and has the slowest transit time compared to the other regions of the digestive tract (10-59 hours) [33]. As discussed previously, it is these physiological parameters that drive the proliferation of specific microbes. As such, the highly anaerobic environment of the colon facilitates the growth of bacteria that rely on anaerobic metabolism. The phylums that dominate the colon include Bacteroidota, Pseudomonadota, and Bacillota [33]. The slow transit time in the colon creates a larger window for bacteria to form colonies, which results in the highest density of microbial growth compared to any other region of the body. Given this immense microbial burden, the physiology of the colon is highly specialized to regulate the interactions between the bacteria present and the underlying immune system, which is poised to detect and destroy foreign entities. Critical to the regulation of these interactions are intestinal epithelial cells, which not only form a protective physical barrier against the bacteria, but also possess a host of receptors that can modulate how the immune system reacts to them. The cross-talk between the microbiome and IECs have important implications for both the development and amelioration of CD.

Anatomy of the Small and Large Intestines

The human digestive system has a unique occupation in the body; not only is it the site of digestion and absorption of nutrients critical for nearly all bodily processes, it is also a battleground between the outside world and the immune system. The environment of the gut presents the difficult challenge of facilitating absorption while also acting as a barrier to prevent invasion of dangerous and opportunistic pathogens. As such, the regulation of the barrier that lines the digestive tract is paramount to create a homeostatic environment that benefits both host and symbiotic microbes.

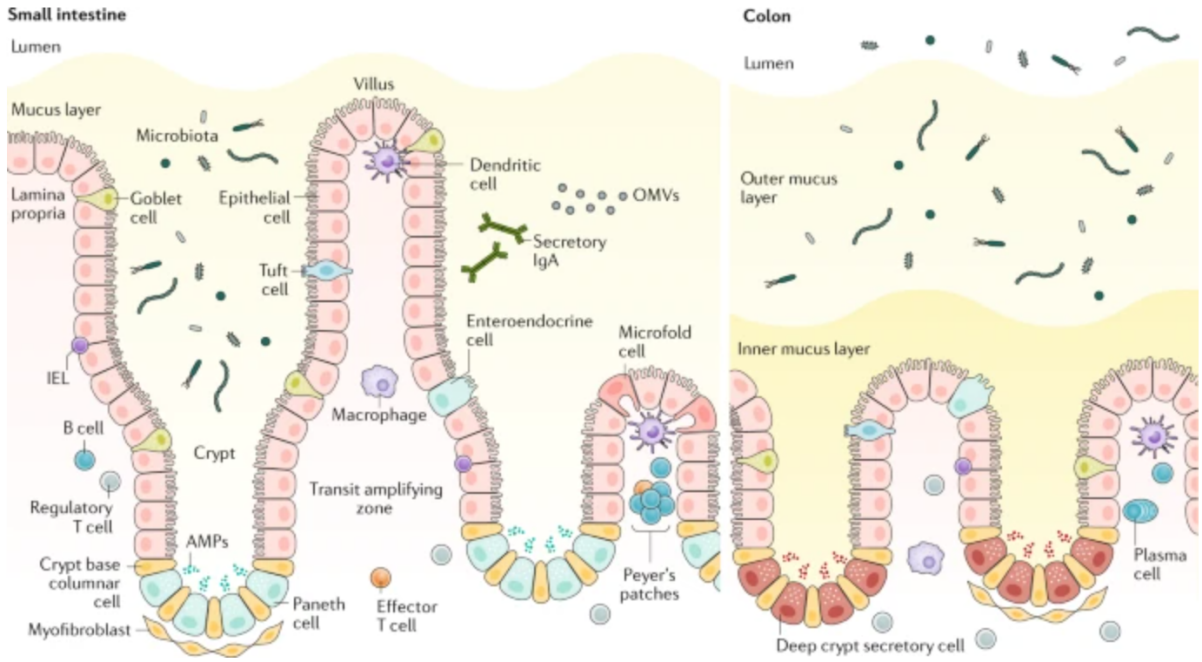

Figure 2: Anatomy of the Intestinal Immune System (Burgueño et al) The architecture and cell types of the small and large intestines differ significantly. The small intestine lacks Paneth cells, which secrete the majority of antimicrobial products present in the mucus of the small intestine. Because of this, the epithelial layer of the large intestine is covered by two distinct mucus layers (inner and outer), which decreases the potential for microbial invasion.

Figure 2 from Burgueño et al depicts the complex cellular and architectural landscape of the small intestine and colon. The physical barrier that separates the lumen of the intestines from the largest immune compartment in the body is composed of a 10μm layer of epithelial cells (enterocytes) that form invaginations called crypts [9]. Interspersed among the sea of epithelial cells are a host of specialized cells that hold critical functions in digestion, immune defense,

mucus production, hormone secretion, and barrier regeneration. Intestinal crypts are composed of columnar stem cells that can rapidly replenish the epithelium in response to damage. Impressively, the process of division at the base of the crypt to apoptosis and cell shedding at the tip of the villus takes only 3-5 days [19], resulting in regular regeneration of the intestinal epithelium. Paneth cells, also located on the basement membrane at the bottom of small intestinal crypts, produce antimicrobial peptides like lysozyme C and α-defensin which protect the crypt cells from bacterial invasion [9]. Paneth cells also secrete growth factors that support stemness of intestinal stem cells. Located proximally to the gut-associated lymphoid tissues (including Peyer's Patches and diffuse lymphoid tissue) are microfold cells. Microfold cells sample the environment of the lumen, capturing luminal antigens before transporting them across the membrane to be exposed to dendritic cells [33]. Dendritic cells that are exposed to luminal antigens can then migrate to lymph nodes where they may drive T and B cell maturation to respond to potentially harmful pathogens within the digestive tract. Another important cell type in the intestinal epithelial layer is the enteroendocrine cell. These secretory cells produce a variety of hormones and peptides such as serotonin, somatostatin, and cholecystokinin, which can contribute to intestinal peristalsis, blood flow, and fluid secretion [1]. While these specialized cell types play vital roles in homeostasis, immunity, and nutrient absorption, the enterocytes of the small and large intestines are of particular importance to this thesis because of their relevance to the pathophysiology of Crohn's Disease.

The Intestinal Barriers in Health and Disease

Intestinal Epithelial Cells and the Glycocalyx

Intestinal epithelial cells form the physical barrier between the luminal compartment of the intestines and the sensitive, vascularized stroma below. Increased translocation of bacteria beyond this epithelial lining is directly associated with development of inflammatory bowel disease [41], so maintenance of the physical and chemical properties of the barrier are of utmost importance. In addition, the interactions of microbes at this barrier have been demonstrated to influence extra-intestinal inflammatory and autoimmune diseases, such as multiple sclerosis, Type I Diabetes, and rheumatoid arthritis [41]. The enterocytes of the intestinal tract are polarized, columnar epithelial cells that have microvilli on their apical surface

that increase the surface area of the tissue, critical for digestion. IECs are adjacently linked together by tight junction proteins, which allow selective transport of solutes while preventing free translocation of larger molecules [3]. Although Paneth cells are the primary producer of antimicrobial peptides (AMPs), enterocytes are also capable of secreting AMPs such as C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ) [41]. Secretion of REGIIIγ by IECs and Paneth cells is thought to be an important mediator of segregation between the epithelium and commensal microbes. Another mediator of this segregation is secretion of IgA by enterocytes. IgA, the most abundant immunoglobulin in mucosal surfaces, is produced by mature plasma cells in the lamina propria below the epithelial barrier. IECs capture dimeric IgA complexes on their basolateral surface via the polymeric immunoglobulin receptor (polyIgR), where they are then transcytosed to the apical surface and secreted into the intestinal lumen [41]. The secretion of REGIIIγ and IgA by IECs is an example of how these cells can generate a chemical barrier in addition to their function as a physical barrier.

Integrated with the apical membrane of IECs is a unique structure called the glycocalyx, which is composed of a range of transmembrane glycoproteins and glycolipids. The glycocalyx within the intestinal tract is densely loaded with glycosylated mucins such as MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC17 and MUC20, each containing an extracellular O-glycosylated filament that sometimes extends even beyond the microvilli of IECs [37]. These glycoproteins are continuously replenished, with an average turnover rate of 6-12 hours [37].The length of these transmembrane filaments, in addition to their high turnover rate, are thought to contribute to the barrier function in IECs, as pathogens that manage to bind them are released back into the mucus layer above the glycocalyx. Glycocalyx dysfunction has been suggested as a possible risk factor in the development of Crohn's Disease, specifically in the ileal small intestine. A recent study from Layunta et al investigated the effects of MUC17 deficiency in disease pathogenesis. In this study, *Muc17*$^{\Delta IEC}$ mice (which lack production of this mucin in IECs) exhibited a compromised glycocalyx barrier, which resulted in increased extra-intestinal translocation of commensal bacteria and consequent loss of epithelial homeostasis. In addition to this mouse model, MUC17 expression in histological specimens of patients with ileal CD were compared to non-IBD controls. This experiment indicated MUC17 levels in the enterocyte brush border of ileal CD sections were markedly decreased compared to the control group [29]. To date, there are no known disease associations between *MUC17* genetic mutations and CD [29], however the findings of this study suggest that this should be further investigated in future studies. Additionally, these data indicate that mucins could play an important role in the development of CD, but also as mediators of cell-microbe interactions.

The Importance and Function of Intestinal Mucus Layers

While the physical barrier established by epithelial cells is of undeniable importance in preventing bacterial translocation, there is another barrier present within the small and large intestines that has recently gained significant attention in the context of inflammatory bowel disease. As shown in Figure 2 above, a mucus layer (or layers) covers the surface of the intestinal epithelium from the stomach to the distal colon. The mucus layer within the gut is a dynamic barrier, changing in thickness and function depending on its location within the digestive tract. Under normal physiological conditions, intestinal mucus functions as a lubricant to prevent damage to the epithelium, while also acting as a semi-permeable barrier allowing small molecules to permeate through to be absorbed by IECs. The mucus lining of the intestines is continuously replenished by goblet cells, which are interspersed throughout the intestinal epithelium, including within the intestinal crypts. Goblet cells are mucus-producing cells that secrete gel-forming glycoproteins, such as mucin 2 (MUC2), which polymerize and become hydrated to form a gel-like structure [26]. The continuous production of mucus by goblet cells is critical to maintain an outward flow of mucus towards the lumen, which aids in preventing bacterial colonization within the epithelial crypts that could endanger the stem cells that proliferate within [33]. The importance of this mucus barrier is highlighted by the observation that $MUC2^{-/-}$ mice develop spontaneous colitis due to the lack of protection for the underlying epithelium [37].

As indicated in Figure 2, the mucus layer in the small intestine is thin and penetrable by some bacteria, so additional protective mechanisms are required to deter microbial contact with IECs. Mucus-secreting goblet cells secrete regulatory factors that influence intestinal barrier function. For example, goblet cells secrete trefoil factor 3 (TFF3) which can increase the structural integrity of the mucus, as well as signal for epithelial repair and apoptosis resistance [16]. Additionally, resistin-like molecule-β (RELMβ) secreted by goblet cells increases MUC2 secretion and helps regulate macrophage and adaptive T cell function in the context of inflammation [16]. Mucus secreted into the lumen of the small intestine mixes with IgA synthesized by B cells in the lamina propria, in addition to antimicrobial peptides secreted by the Paneth cells [9]. These defensive molecules within the mucus layer are produced to modulate the level of bacterial colonization within the mucus, which is critical to mitigating inflammatory responses to commensal microbes.

The proximal and distal colon possess two distinct mucus layers, an outer (luminal) thin layer and a dense inner layer. Much like its function in the small intestine, mucus layers within the colon act as a lubricant to protect epithelial cells from physical damage. Given that the colon harbors the greatest density of microbial communities anywhere in the body, the additional, dense mucus layer is paramount to maintain a buffer between the gut microbiota and the underlying IECs. The dense mucus layer functions similar to the glycoclayx, in which the innermost layer is attached to the epithelium [26]. Under normal physiological conditions, the goblet cells of the large intestine continuously replenish the dense mucus layer, which moves outwards towards the lumen. The density of the mucus, coupled with the outward flow create a bacteria-restricted zone. As the mucus moves luminally, endogenous proteases separate the mucins from the epithelium, softening the mucus [26]. The softer, luminal mucus is more penetrable to bacteria, which creates an environment in which they may proliferate. The luminal mucus is also an important energy source for many commensal microorganisms that possess enzymes to break down the glycans in colonic mucus [26]. Therefore, the variation of mucus thickness in colon accomplishes two important outcomes: the creation of a habitat in which microbes can adhere and aid in digestion, and maintenance of a bacteria-restricted zone that prevents unrestricted contact with the immune system.

Intestinal Mucus in Inflammatory Bowel Disease

As highlighted by the previous section, the protective functions of mucus layers in the small and large intestines are critical to a homeostatic environment in which the microbiome is effectively held “at arm's length.” As one might expect, compromisation of the thickness or contents of these mucus layers have physiological consequences. In the context of CD, mucus barrier dysfunction is most relevant to the small intestine due to defects in the secretion of antimicrobial factors. Because the mucus of the small intestine acts more as a diffusion barrier rather than an exclusion barrier [26], deficiency in antimicrobial protein secretion increases the bacterial burden at the epithelial barrier, resulting in an inflammatory response. Genetic defects in the unfolded protein response (UPR) in secretory cells of the small intestine have recently been implied as a mechanism for this compromised mucus function.

The implications for *XBP1* mutations in CD come from a mouse study published by Kaser et al, in which it was demonstrated that deletion of *XBP1* in intestinal epithelial cells led to ER stress and spontaneous enteritis. X-box-binding protein 1 (XBP1) is a transcription factor that is critical to regulation of the unfolded protein response in all cell types (Kaser et al, 2006).

The UPR is a method of removing misfolded proteins from the endoplasmic reticulum (ER), which can occur as a result of ER stress. XBP1 directs transcription of a host of genes that are necessary for ER function, in addition to being necessary for the development of secretory cells in the small intestine, such as goblet cells and Paneth cells (Kaser et al, 2006). Another key observation from this study is that the small intestine of *XBP1*$^{-/-}$ mice was devoid of Paneth cells, which are the most potent secretors of antimicrobial compounds. Together, these results suggest that XBP1 polymorphisms could compromise the protective function of the mucus layer, leading to increased inflammation at the epithelial barrier.

While the dynamics and regulation of the colonic mucus barrier have been under rigorous investigation in the context of inflammatory bowel disease, the current data indicate that this mucus barrier is not dysfunctional in Crohn's Disease. Recent studies have indicated that mucus thickness is slightly higher in cases of active CD [48], however the thickness of the adherent (or dense) mucus layer is unchanged. The implications of the increased mucus thickness is unclear in the context of CD, however it may imply that mucus secretion is upregulated. Because crypts in the large intestine lack secretory Paneth cells, specialized "sentinel" goblet cells are present at the edges of colonic crypts. These goblet cells are equipped with toll-like receptors (TLRs) that can sense the components of the cell wall of bacteria (lipopolysaccharide or LPS) and respond by increasing the outward flow of mucus [33]. Toll-like receptors, therefore, have important regulatory roles in the function of both mucosal and epithelial barriers within the intestines. Thus, a discussion of TLRs and their relevance to CD is warranted.

Toll-Like Receptors and Barrier Function

TLR Signaling and Function

Given the dual-function of the gut epithelium in facilitating both absorption and protection, it is clear that the cells composing the epithelial barrier must possess a host of regulatory mechanisms to allow the cells to respond to the physiological conditions within the gut. Key to the ability to respond to the physiological environment is the ability to detect changes and elicit responses to adapt to the current challenge. Toll-like receptors, found on nearly all cell types within the gut epithelium, have been identified as important modulators of the gut barrier

in both health and disease [1]. TLRs are transmembrane pattern-recognition receptors (PRRs) that can recognize microbial-associated molecular patterns (MAMPs), which are molecules present within and produced by microbes. Some TLRs also recognize damage-associated molecular patterns (DAMPs), which are molecules released by damaged or dying cells that can have proinflammatory effects. TLRs are most commonly associated with the innate immune system, as phagocytic cells (macrophages, neutrophils, and dendritic cells) possess a large range of TLRs that drive specific downstream signaling mechanisms. TLRs are differentially expressed throughout the digestive tract depending on location, cell type, and physiological conditions. Variable expression, cell polarization, and chemical regulation of toll-like receptors create context-driven responses that can maintain homeostasis or lead to undesirable outcomes within the lining of the gut epithelium.

Currently there are 10 TLRs described in humans (and 13 in mice), some possessing unique ligands while others can be stimulated by the same ligand. While there is diversity in what ligands can stimulate TLRs, there is considerable overlap in the downstream signaling molecules. Figure 3 below outlines some of the ligands for TLRs and the adaptor proteins that are recruited as a result of receptor-ligand interactions.

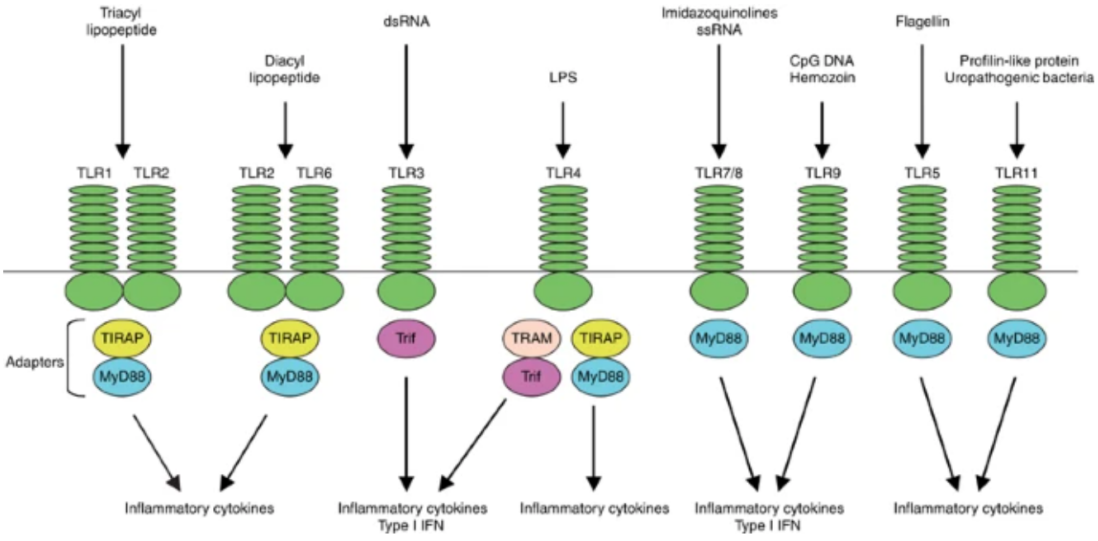

Figure 3: TLR-Mediated Immune Responses (Kawai et al) Downstream toll-like receptor signaling is mediated by a host of adapter proteins (TIRAP, MyD88, Trif, TRAM) that can regulate the effects of TLR stimulation. The variety in the downstream signaling effects of TLRs is dependent on what adapter proteins are engaged as a result of ligand binding.

Upon recognition of a specific ligand in the extracellular domain, TLRs dimerize which allows the intracellular Toll/IL-1 receptor (TIR) to interact with different TIR-containing adapter proteins [54]. Adapter proteins, such as myeloid differentiation primary response protein 88 (MYD88), IL-1R-associated kinase (IRAK), tumor necrosis factor receptor-associated factor 6 (TRAF6), and TIR-domain-containing adapter-inducing IFNβ (TRIF), induce downstream signaling pathways that cause activation of transcription factors like AP-1, NF-κB, and IRF3 [54]. Not only can these transcription factors stimulate production of proinflammatory cytokines like tumor necrosis factor (TNF), IL-6 and Type I interferons, they can also activate proliferative and anti-apoptotic pathways [28]. In this way, TLR signaling is a double-edged sword that is critical to proinflammatory processes, but also important for tissue repair in response to injury.

Spatial Organization and Expression of Toll-Like Receptors

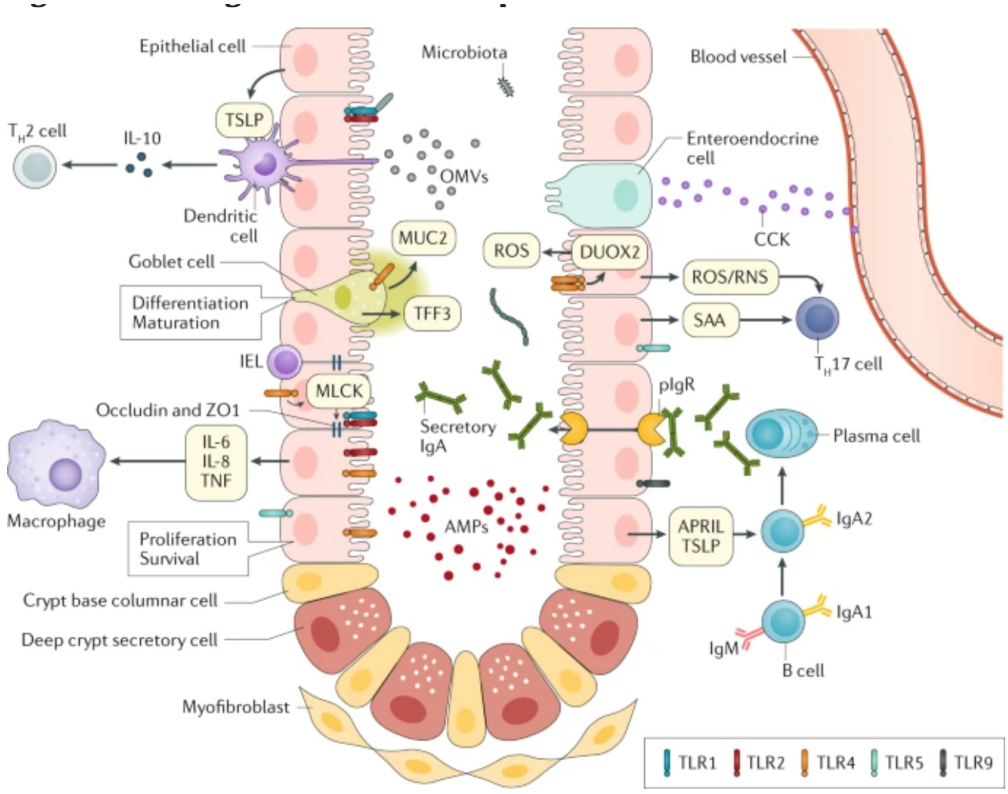

Figure 4: Toll-like Receptor Expression in the Intestinal Epithelial Cells (Burgueño et al)
Toll-like receptors are differentially expressed in different cell types of the large intestine. This differential expression translates to a wider range of functionality in TLR signaling, as stimulation of TLRs in a goblet cell can induce a different outcome than TLR stimulation in an

IEC. Furthermore, apical vs. basolateral expression of TLRs contribute to context-dependent responses as a result of ligand binding.

Figure 4 above depicts the differential expression of TLRs in various cell types of the intestinal epithelium of the colon. Notably, there are many types of TLRs present within the epithelium, with some cells possessing multiple TLRs on their plasma membrane. The expression of TLRs also changes depending on the location within the digestive tract. For example, TLR3 is expressed in both the small intestine and the colon, whereas TLR5 is primarily expressed in the colon [1]. Almost all TLRs have been implied to be expressed at the mRNA level within the colon, however this does not infer their level of protein expression or how they are spatially localized [1]. The expression of TLRs is also largely dependent on the physiological environment within the mucosa. For example, under normal conditions it has been observed that TLR expression is generally low on IECs [8]. However, in conditions where inflammation is aberrant, such as in Crohn's Disease, TLR4 expression is markedly higher on the apical side of the epithelium [11].

Additionally, Figure 4 highlights the polarization of TLRs within the epithelium. Some TLRs are preferentially expressed on the apical surface, whereas others are normally only located on the basolateral surface within the lamina propria. This is an example of how TLRs may be spatially regulated, ensuring that signaling will occur in a context-dependent manner. TLR5 is a PRR that recognizes bacterial flagellin as a ligand and is primarily localized to the basolateral surface of epithelial cells [23]. Stimulation of basolateral TLR5 by this ligand causes activation of the proinflammatory NF-κB pathway, which is a key mediator of the innate immune response [23]. In this way, TLR5 regulates activation of an innate immune response to occur in the context of a barrier breach or an invasive-flagellated bacteria (i.e. *Salmonella*). Another example of spatial regulation in TLRs is evident in the interactions of TLR9 with its ligand CpG ODN, which is a motif present in bacterial DNA. TLR9 is expressed on both the apical and basolateral surfaces of IECs, however the location of ligand binding infer different downstream effects. Apical activation of TLR9 results in the inhibition of the proinflammatory NF-κB pathway and expression of the WNT protein receptor Frizzled 5, which is critical in differentiation of Paneth cells [30]. Conversely, basolateral stimulation of TLR9 by CpG ODN leads to activation of the NF-κB transcription factor and eventual secretion of IL-8, a chemoattractant for neutrophils [30]. These contradictory signaling pathways highlight the notion that TLR signaling

is intended to be context-dependent, where stimulation of the immune system is most likely to be employed in the context of a barrier breach or after detection of invasive pathogens.

Regulation of Toll-like Receptors

Because TLRs are non-specific in their recognition of bacterial ligands (i.e. commensal vs. pathogenic), it is implied that there must also be regulatory elements in place to prevent disproportionate reactions towards commensal microbes. Indeed, it has been demonstrated that enterocytes can modulate TLR signaling by inactivating downstream signaling molecules or by limiting ligand-receptor interactions. Toll-interacting protein (Tollip) is an adapter protein, similar to MYD88, that associates with the TIR domain of TLR2 and TLR4 to block phosphorylation of IRAK [56]. Inhibition of IRAK phosphorylation halts the downstream signaling cascade that results in the production of the pro-inflammatory NF-κB transcription factor. Because of this antiinflammatory mechanism, overexpression of Tollip in IECs has been described as a mechanism to prevent disproportionate inflammatory reactions towards commensal bacteria [56], and also may have therapeutic opportunities in the pathology of inflammatory bowel disease. Another regulatory protein involved in TLR signaling is proliferator-activated receptor gamma (PPARγ), which is an inhibitor of the NF-κB pathway by direct binding to NF-κB subunits [20]. Interestingly, PPARγ mRNA expression is increased when TLR4 is activated by its ligand, LPS [20]. Because TLR4 activation is well-established as a pro-inflammatory signal, it would appear that upregulation of PPARγ expression as a result of TLR4 activation is an example of a negative feedback loop by which the pro-inflammatory signal can be quenched. Notably, PPARγ is abundantly expressed in the colonic epithelium of healthy individuals, but has been noted to be downregulated in the tissues of individuals diagnosed with CD [20]. This observation may have therapeutic implications for the use of PPARγ ligands in reducing production of inflammatory mediators.

Models of TLR Dysfunction in Disease

Given the range of expression and the importance of spatial regulation in toll-like receptors, they have significant implications in both health and disease. The majority of studies investigating the mechanisms of TLR interactions are performed in mouse models, where genetic ablation of TLR genes can elucidate potential functions. In particular, TLRs have been observed to be critical to regulation of the epithelial barrier of the intestines. This is highlighted

by the observation that MYD88-deficient mice experience increased microbial burden and impaired mucosal permeability when subjected to *Citrobacter rodentium*-induced colitis [25]. Additionally, an experiment where *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$ mice were subjected to DSS (dextran-sodium sulfate)-induced colitis revealed that the tight junctions of the knockout mice were compromised compared to the control group [10]. This study identified the role of TLR2 signaling in the translocation of tight junction proteins zonula occludins 1 (ZO1) and occludin to the epithelial tight junctions, where they can reinforce the barrier integrity of epithelial cells. A similar mechanism has been identified in TLR1 signaling, where *Tlr1*$^{-/-}$ mice experience increased bacterial translocation to other organ systems (i.e. liver, blood, and spleen) in addition to increased intestinal permeability to small molecules [27]. This observation in *Tlr1*$^{-/-}$ mice may also be due to the function of TLR1 signaling in MUC2 production in goblet cells. TLR1-deficient mice exhibit a compromised mucus layer in the colon, leading to increased exposure of the epithelium to the microbial community [27]. These models of TLR1/2 function imply that stimulation of these receptors has a protective outcome for the epithelial barrier, specifically in maintenance of the tight junctions of the epithelium.

Toll-like Receptor Polymorphisms and Inflammatory Bowel Disease

While mouse models have been invaluable in mechanistic study of TLR function, the effects of mutations in TLR genes in humans are more difficult to interpret. One of the first susceptibility loci identified as a risk factor for Crohn's Disease was the PRR NOD2, which is similar to some TLRs in function and form. NOD2 is essential for intracellular processing of bacteria, specifically in formation of the phagosome and initiation of autophagy in epithelial cells [9]. NOD2 recruits ATG16L1 to the membrane where it initiates formation of the phagosome to capture absorbed bacteria. The process of autophagy ensures that the contents of the phagosome are destroyed and its contents can be transferred to antigen-presenting dendritic cells. Mutations in either NOD2 or ATG16L1 can interrupt this process, resulting in impaired bacterial clearance and antigen presentation [50]. Impaired antigen presentation of engulfed bacteria leads to defective priming of regulatory T cells and a consequent overactive effector T cell response. The overactive T cell response manifests as the destructive, chronic intestinal inflammation that is a hallmark of CD.

TLR9 polymorphisms have also been genetically associated with IL23 and NOD2 mutations [49], in which some gene-gene interactions have been observed. As such, TLR9 mutations may play a role in the development of IBD, however the mechanism has yet to be

elucidated. Interestingly, polymorphisms in other TLRs like TLR1, TLR2, TLR4, and TLR6 are not currently linked to the pathogenesis of IBD, but they are more frequent in patients diagnosed with IBD [9]. These findings justify additional research into how TLR polymorphisms may contribute to disease pathogenesis or disease prognosis, in both CD and UC. The apparent barrier dysfunction and disproportionate inflammatory responses in the tissues of Crohn's Disease patients are likely linked to TLR dysregulation, however genetic mutations do not explain the heterogeneity of the disease that is observed in patients. In addition, TLR signaling and expression can be modulated epigenetically and chemically through environmental influences, such as bacteria. Thus, further exploration into another regulatory factor of the epithelial barrier, the gut microbiome, is justified.

Microbial Metabolites Regulate Barrier Functions in the Gut

The Microbiome and its Metabolites

The human gut microbiome represents a complex, dynamic environment of microbes that have a long-standing evolutionary relationship with the host tissues in which they reside. As mentioned previously, the gut microbiome formally refers to the organisms (bacteria, fungi, viruses, and bacteriophages) that proliferate within the intestinal tract, but also to the genomes of those organisms that can drive the production of compounds that can be both beneficial and pathogenic in humans. The study of these organisms in the past relied on bacterial culture from stool samples for identification of microbes. This proved to be an extremely limited tool for microbiome research, as many colonic residents are difficult to culture *ex-vivo* given their preference for anaerobic conditions. Additionally, bacterial culture of stool samples does not strongly represent microbial communities present in other parts of the digestive tract (i.e. mouth, stomach, or small intestine) and cannot detect the presence of phages or viruses. As such, the decreased cost of culture-independent sequencing technologies have become instrumental in progressing the field of microbiome research. Specifically, metabolomic, metagenomic, metatranscriptomic, metaproteomic, and biomarker sequencing techniques have begun to unveil a completely novel landscape of microbiome influence in health and disease [24]. It is currently estimated that the microbes in the human gut produce over 50,000 metabolites, 22,500 of which are believed to have antibiotic properties [5]. As discussed previously, the

biogeography of the gut has significant influence on the bacterial communities that can successfully colonize it. Similarly, bacterial diversity is heavily influenced by the availability of nutrient substrates that come from the human diet. Some metabolites are derived from the dietary compounds themselves, such as Compound K, while other metabolites like SCFAs are produced as a result of bacterial metabolism [24]. An additional category of metabolites is secondary bile acids, which are produced by the host secreted into the lumen of the intestines where they can incur biochemical modifications by the bacteria [14]. Of relevance to this review, analysis of the microbial metabolites produced in the digestive tract has demonstrated non-trivial roles in the regulation of the epithelial barrier and inflammatory signaling pathways that have implications for disease prevention, prognosis, and treatment of Crohn's Disease.

Short Chain Fatty Acids

Dietary fiber and indigestible carbohydrates are a critical nutrient resource for bacteria that ferment these compounds into SCFAs, such as butyrate, acetate, and propionate. Not only have these compounds been identified as an energy source for IECs, they have also been demonstrated to play important roles in cell differentiation, mucus production, and epithelial barrier function. For example, administration of 1-10nM of sodium butyrate to E12 human colon cells improved barrier function by increasing secretion of MUC2 mucin [35]. This effect of low-dose sodium butyrate was also reproduced in Caco-2 cell lines [40]. Interestingly, high concentrations of sodium butyrate (50-100nM) had a negative effect on barrier function, as many cells underwent an apoptotic response. This highlights the importance of dosage in the function of sodium butyrate in IECs.

Butyrate may also influence epithelial barrier integrity by modulation of tight junction proteins. IECs stimulated with butyrate upregulate the transcription factor SP1, which induces upregulation of the tight junction protein claudin-1 (CLDN1) [53]. Additionally, an increase in paracellular transport of tight junction proteins such as occludin and ZO1 have been observed as a result of SCFA treatment [47]. SCFAs have also been shown to play a role in modulating inflammatory responses. Administration of butyrate has been shown to downregulate IL-1β, which is a potent mediator of inflammation produced by macrophages and dendritic cells [15].

Indole Derivatives

Another group of microbial metabolites relevant to both epithelial barrier functions and inflammatory signaling are indole derivatives. Indole is produced as a result of catabolization of the dietary amino acid, tryptophan, by the enzyme tryptophanase, which is employed by several species of bacteria [24]. Bacteria, such as *Clostridium sporogenes, Escherichia coli*, and *Bacteroides ovatus* produce indole and indole derivatives for functions such as intracellular signaling and quorum sensing [24]. Indole has been detected in human feces at significant concentrations (250-1100μM), which implies that the colonic epithelium may be regularly exposed to indole [4]. A study performed by Bansal et al on HCT-8 cell lines elucidated a number of physiological functions for indole, including epithelial barrier properties, modulation of proinflammatory and anti-inflammatory signaling, as well as decreased pathogen colonization. In this study, HCT-8 cells were exposed to 1mM of indole for 4-24H, and gene expression was measured via whole-transcriptome sequencing. The results showed significant upregulation of genes involved in epithelial barrier function, specifically genes responsible for tight junction organization, actin cytoskeleton, mucin production, and adherens junctions [4].

Indole exposure in HCT-8 cells also showed notable effects in toll-like receptor expression. Expression of TLR3 and TLR9 were increased as result of indole treatment, in addition to upregulation of anti-inflammatory TLR regulatory proteins such as TOLLIP and SIGIRR. This important finding suggests that indole shapes IEC immune function to a more tolerant state, in which commensal microbes will not trigger disproportionate immune responses from IECs. This is highlighted by the figure from Bansal et al below, which shows a reduction in IL-8 levels in cells treated with indole.

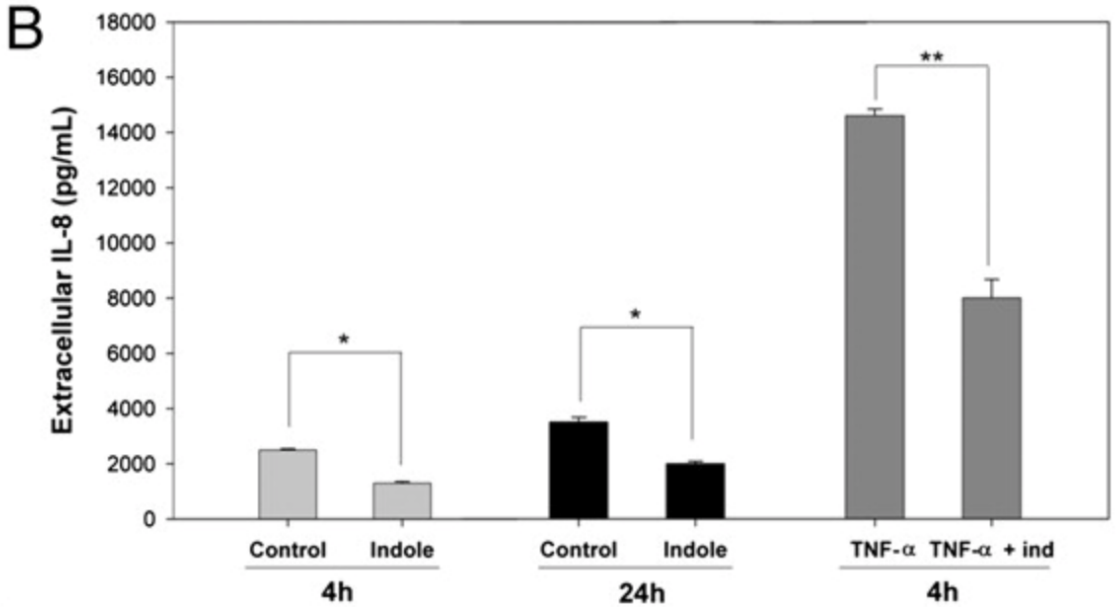

Figure 5: Changes in TLR, IL-8 Signaling (Bansal et al) HCT-8 cell lines were treated with 1mM indole and extracellular IL-8 levels were measured over a period of 4-24 hours. Cells pre-treated with indole exhibited decreased secretion of IL-8 at both the 4h and 24h time points. Additionally, cells pretreated with TNFα also exhibited decreased IL-8 secretion.

Importantly, IL-8 levels were significantly lower even in cells pre-treated with TNFα, an inducer of IL-8 secretion which is highly expressed in patients with Crohn's Disease. In addition to modulating TLR expression, indole exposure also had effects on gene expression of anti-inflammatory cytokines. Of note, receptors for anti-inflammatory cytokines IL-10 and IL-11 were upregulated, and expression of pro-inflammatory cytokines IL-8 and CXCL5 were decreased. Although this study was conducted on cell lines, the results indicate a promising role for indole in the mitigation of damage from chronic inflammatory disease.

Further evidence for the application of indole as a mediator of barrier functions has been described in a germ-free (GF) mouse model. In a study performed by Shimada et al, the expression of tight junction proteins was compared between GF mice and a control group of pathogen-free mice. The results indicated that GF mice had significantly lower expression of tight-junction and adherens-junction proteins such as *claudin-7, occludin, tjp1, Cdh1*, and *Ctnnb1*. When the two groups of mice were challenged with oral DSS, the GF mice had significantly lower survival rates than the control group as a result of more severe DSS-induced colitis. When indole levels were compared between the test and control groups, the GF mice had 27-fold lower levels of indole, which is expected as indole is a microbial metabolite. Once this initial finding of decreased barrier function was established, indole supplementation was employed as a method of restoring expression of barrier function. Figure 6A below shows indole levels in the feces of GF mice after two week supplementation of indole capsules. While indole levels did not reach concentrations equivalent to the control group (SPF), indole supplementation was sufficient to induce expression of the tight junction and adherens junction proteins that were previously depleted (Figure 6B).

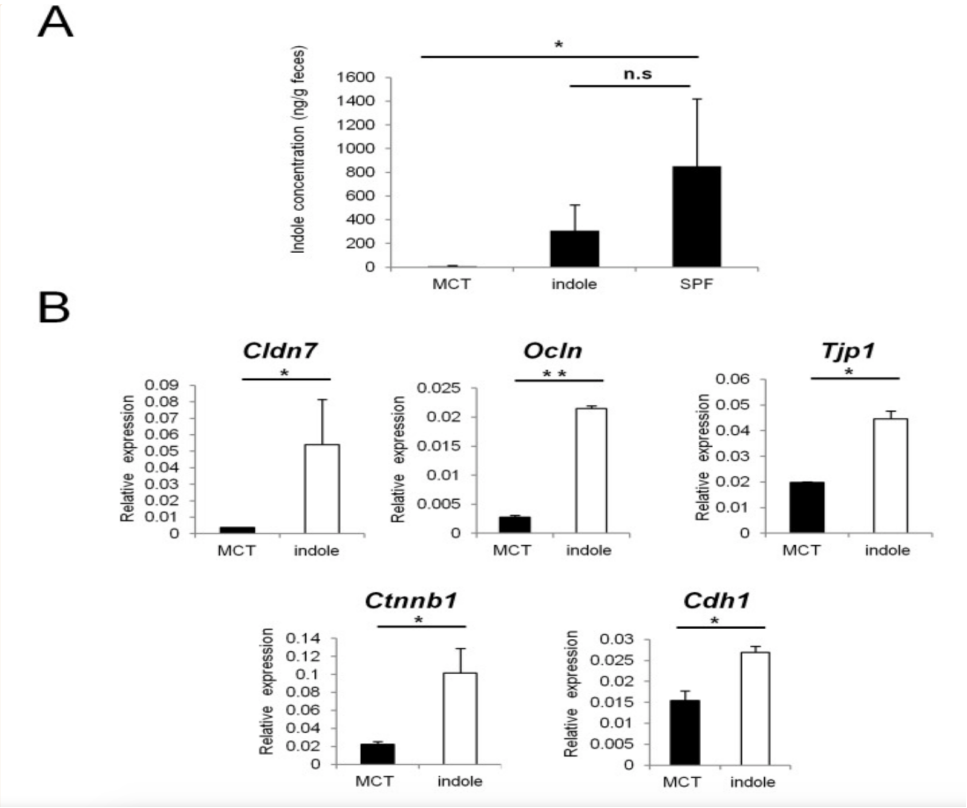

Figure 6: Indole-containing Capsules Promote Epithelial Barrier Function in GF Mice (Shimada et al) (A) Concentration of indole in the feces of either germ-free (GF) or specific pathogen-free (SPF) mice treated with medium-chain triglycerides (MCT), or indole. Concentration of Indole was measured using HPLC-FL. (B) Real-time quantitative PCR analysis of mRNA expression of *Cldn7, Ocln, Tjp1, Ctnnb1*, and *Cdh1* in colonic epithelial cells of GF mice treated with MCT or indole containing capsules.

These indole-supplemented GF mice were then subjected to DSS treatment, in which 90% survived after 5 days. The findings from this study strongly suggest that indole is a critical microbial metabolite for modulation of barrier function, and further suggests that indole supplementation could be an avenue for restoring barrier function in Crohn's Disease.

Secondary Bile Acids

Bile acids (BA) represent a relatively novel signaling molecule in the context of microbial metabolites. While bile acids have been recognized for decades as critical components of dietary fat absorption, the discovery that bile acid receptors are encoded in the human genome

sparked considerable interest in additional functionalities. These cholesterol-derived surfactants are produced in the liver before being transported to intestines via the hepatobilliary system where they are secreted into the lumen and further modified by microbes into secondary bile acids, which have recently been demonstrated to have a range of physiological activity, specifically in metabolic and inflammatory pathways [12].

The most common secondary bile acids found in the human gut include deoxycholic acid (DCA), ursocholic acid, ursodeoxycholic acid (UDCA), and lithocholic acid (LCA) [24]. In the intestines, secondary bile acids agonize the farnesoid X receptor (FXR) in the nuclear membrane, as well as the G protein-coupled receptor TGR5/M-BAR. Stimulation of these receptors by their respective BA ligands have important regulatory roles in inflammatory signaling, which may have implications for reducing inflammation in Crohn's Disease.

In DSS-induced mouse models of colitis, FXR-deficient mice experience increased levels of mucosal inflammation whereas pre-treatment of mice with FXR-agonists resulted in decreased levels of inflammation [22]. This protective response is likely due to decreased expression of inflammatory cytokines, as another study showed that colonic mouse tissues treated with FXR-agonist INT-747 display reduced expression of IL-8, IL-1β, and chemokine CCL2 [22]. FXR signaling has also been demonstrated to influence TLR signaling. This is exemplified by the observation that INT-747-specific FXR activation causes a reduction in TLR4-mediated proinflammatory gene expression in IECs [51]. Additionally, proinflammatory chemokine and cytokine expression in cultured human CD14+ monocytes and dendritic cells was markedly decreased as a result of FXR stimulation [22], implying a regulatory role in innate immune cell function as well. Importantly, FXR is primarily expressed in hepatocytes and ileal IECs, which must be considered in the context of therapeutic avenues for FXR-dependent mechanisms.

Conversely, the bile acid receptor TGR5 is expressed in a broader range of cell types, including hepatocytes, IECs, monocytes, and macrophages [18]. Stimulation of TGR5 by endogenous BAs activates adenylate cyclase, which leads to expression of the cyclic AMP-responsive transcription factor, cAMP response element binding protein (CREB) [43]. TGR5-dependent CREB activation has been shown to suppress TLR4-mediated NF-κB transcriptional activation of several pro-inflammatory cytokine genes, such as IL-1β, IL6, IL8, and TNF [43]. TGR5 signaling has also been implied in the context of both innate and adaptive immune cells. In the context of chemically-induced colitis, TGR5 activation recruited regulatory T cells to the inflamed tissue by promoting epidermal growth factor receptor (EGFR) and SRC kinase (SRC)

signaling [6]. Additionally, TGR5-dependent cAMP production can suppress activity of the NLRP3 inflammasome, which is critical in the processing of IL-18 and IL-1β.

Stimulation TGR5 by another agonist, BAR501, induced transformation of proinflammatory mucosa-associated M1 macrophages to the M2 phenotype, which are critical for tissue repair after injury [6]. In the context of CD, M1 macrophages are the predominant macrophage isotype and are potent producers of cytokines that drive T cell differentiation towards a proinflammatory Th1 response [31]. A study by Yonano et al demonstrated that mucosa-associated macrophages isolated from the tissues of CD patient biopsies highly express the TGR5 receptor. Moreover, when these macrophages were treated with a TGR5 agonist *ex-vivo* their expression of TNFα was markedly reduced [55]. Importantly, the TGR5 receptor has a higher affinity for secondary bile acids compared to FXR, which has higher affinity for primary bile acids [12]. This observation, in addition to spatial expression of FXR and TGR5, will likely dictate how bile acids may be used as therapeutic substrates in inflammatory bowel disease.

The experiments around microbial metabolites presented in these sections highlight significant roles for microbial metabolites in both health and disease. Relevant to CD, it would appear that SCFA, indole derivatives, and secondary bile acids have the potential to regulate barrier function of tight junctions in IECs, the production of proinflammatory cytokines that are relevant to chronic inflammation, and modulate TLR signaling pathways to mitigate overstimulation of the immune system. While the results of these experiments in mice are compelling, studies of the effects of these metabolites in humans will be paramount in gauging their therapeutic value.

Discussion - Microbial Metabolites in Detection and Treatment of Crohn's Disease

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Gaps in CD Screening Technology

The diagnosis and treatment of CD represent difficult challenges to overcome, largely due to the heterogeneity of symptoms and sometimes invasive procedures involved in creating the diagnosis. Because a diagnosis cannot be created based on a single finding or by pathognomic features, diagnosis relies on physical examination, clinical history, complementary diagnostic tests, endoscopic imaging, and often a confirmatory histological biopsy [45]. To complicate things, CD has unique phenotypic characteristics that can change over time in the

same individual, with recurring flare-ups being a hallmark of the disease. Endoscopic imaging is the current gold-standard for CD diagnosis, and is also useful for biopsy collection. Additionally, cross-sectional imaging modalities such as bowel ultrasonography (BUS), CT Enterography and MRI enterography (MRE) are also useful in assessing disease extent or inflammatory manifestations such as stenosis, abscesses, and fistulas [45]. Lastly, histological examination of biopsy or resection samples are often employed for a confirmatory diagnosis, despite a lack of CD-specific histological features. In this case, pathologists identify focal (discontinuous) chronic inflammation, focal crypt irregularity (discontinuous crypt distortion), granulomas, and irregular villous architecture to create a diagnosis of CD [45].

The inherent issue with these diagnostic tools is that they require symptoms to already be present and problematic in the patient to justify their application. Cross-sectional imaging modalities are not useful in identifying at-risk patients, and they often require ingestion or injection of contrast agents followed by radiation exposure. Endoscopy can be performed non-surgically, however there are risks for complications and sedation is often required to perform the procedure. Moreover, endoscopy and colonoscopy is only recommended for people over the age of 45 by the American Cancer Society, or for people with alarm symptoms (weight loss, bleeding, recurrent vomiting). As previously mentioned, CD is most often diagnosed in people under the age of 30, and more than 30% of patients diagnosed with CD have already incurred significant bowel damage [45]. These observations highlight a critical need for screening modalities in the identification of at-risk individuals. Additionally, the diagnosis of younger individuals implies that recommendations for screening modalities should likely be applied earlier in life to detect early signs of disease. A potential solution to the lack of non-invasive screening technologies is the use of biomarkers as a window into the metabolome of the gut.

Current Biomarker Screening Modalities in CD

There are currently several biomarkers employed in disease management and the diagnosis of CD, however the majority of them rely on the pre-existing presence of inflammation. For example, serological autoantibodies and antimicrobial antibodies are useful for CD diagnosis. Autoantibodies like antineutrophil cytoplasmic antibodies (pANCAs) and antimicrobial antibodies such as anti-*Saccharomyces cerevisiae* antibodies (ASCAs) are currently in use [45]. ASCAs are currently the most-used serological marker in CD diagnosis, with an ASCA-positive rate of 60-70% in patients with CD [45]. pANCAs are less valuable in CD diagnosis (10-15% positivity rate in CD), but they are often identified in patients with ulcerative

colitis (60-70% positivity) [45]. Additionally, serological markers of inflammation such as C-reactive protein (CRP) are used to monitor CD activity in patients who have already been diagnosed. This is often an unreliable serological marker however, as a third of CD patients have normal CRP levels despite having active disease [45].

Faecal biomarkers represent a relatively new non-invasive approach to disease diagnosis and management. Faecal calprotectin is the only faecal biomarker currently used in practice today, but has proven to be useful in distinguishing irritable bowel syndrome (IBS) from IBD, in addition to diagnosis of CD [39]. Calprotectin is calcium/zinc-binding protein that is abundantly expressed in the cytosol of neutrophils. The presence of calprotectin in the stool is indicative of acute or chronic inflammation within the GI tract, as neutrophil infiltration is a hallmark of inflammation. Analysis of faecal calprotectin is accomplished through enzyme linked immunosorbent assays (ELISA), of which there are already several on-market options available [39]. Although there are no clinically-validated cut-off values for faecal calprotectin yet in place, there have been studies linking calprotectin levels in the stool to histological scores. This was demonstrated in a study by Pous-Serrano et al, in which pre-operative faecal calprotectin levels were found to be significantly associated with the degree of histological inflammation measured in small bowel resections from CD patients [44]. Faecal calprotectin is also useful in managing disease treatment, as repeated measurements of calprotectin along the course of treatment can be an indicator of decreasing inflammation in the GI tract [39]. Importantly, the use of this faecal biomarker as a screening tool has reduced the requirement for endoscopy in adults by 67% [45].

Microbial Metabolites as a Screening Tool for CD

The use of faecal calprotectin in CD disease management is a promising tool for reducing the necessity of invasive procedures, but also as a non-invasive screening method. Given that microbial metabolites are present in detectable levels in the stool, especially by high-performance liquid chromatography (HPLC), the application of microbial metabolites as biomarkers should be investigated in the context of Crohn's Disease. To date, the investigation of the levels of microbial metabolites in human stool are significantly lacking, likely due to the fact that the identification of these compounds as physiologically-relevant is novel in itself. One metabolite that has been moderately explored in the context of CD is the SCFA, butyrate. In a study performed by Bjerrum et al, they explored the levels of different metabolites present in the stool samples of both healthy patients and IDB patients by nuclear magnetic resonance (NMR)

spectroscopy. The study showed that SCFAs (namely butyrate and propionate) were significantly lower in the samples collected from patients with active CD.

As discussed in this thesis, other microbial metabolites such as indole and bile acids have been implicated as physiologically-relevant players in epithelial barrier function, inflammatory cytokine signaling, and TLR regulation. This warrants further investigation into the levels of these metabolites present in both healthy individuals and patients with active inflammatory disease, such as CD. A recent study performed by Desmons et al effectively utilized HPLC to measure a wide variety of tryptophan-dependent metabolites present in the stool of either healthy participants or patients with IBD. Indeed, this study showed that indole-3-lactic acid was a key discriminator between the two test groups (Desmons et al, 2022). If additional data can be generated around the levels of these microbial metabolites present in faecal samples, it may be possible to link them to histological relevance as well, as was demonstrated in the calprotectin study mentioned above. Moreover, perhaps regular screening for microbial metabolites present in stool samples of patients with familial history of CD, or in patients with identified risk-factors for CD could be useful in predicting the onset of disease.

A different approach to screening methodologies may be found in analysis of the bacteria present in the microbiome of patients. As the microbiome is largely responsible for production of the metabolites discussed thus far, this approach would entail identifying the bacteria that are critical to the metabolism of these compounds and further verification of their presence or absence in health or disease states. The idea of sequencing the microbiome of patients to diagnose CD has been explored, however it has been less explored in the context of the microbial metabolites that these bacteria produce. For example, a decreased presence of *Fusobacterium prausnitzii* and an increased presence of *Fusobacterium nucleatum,* has been effective in distinguishing patients with CD from healthy individuals [45]. Additionally, the microbiome of patients with active CD have been shown to have an enrichment of *Escherichia* spp. and a decreased abundance of *Firmicutes* present in the stool [45]. While these observations are certainly clinically relevant, evaluation of bacteria that are known producers of metabolites such as SFCA, indole and indole derivatives, and secondary bile acids could prove to be useful in determining the drivers of the metabolic profile of the microbiome. For example, Zhou et al identified *Clostridium* AP sp000509125, *Bacteroides ovatus*, and *Eubacterium limosum* as active modifiers of primary bile acids (taurochenodeoxycholic acid and glycochenodeoxycholic acid) into secondary BAs (UDCA and LCA) [57], which are known agonists for the TGR5 receptor. On a similar note, many *Bacteroides* species are known producers of SCFAs as a product of dietary fiber fermentation [52].

The immense diversity of microbes and their products within the digestive tract poses challenge for the use of sequencing in screening technologies. To make matters more challenging, the microbiome is highly dynamic in response to the diet of the patient, inflammatory status of the intestines, as well as other factors like antibiotic or antiinflammatory treatments. However, targeted, longitudinal research of the microbiotic profiles of CD patients, paired with the metabolomic profile of the stool could be useful in predicting disease onset or recurrence. Additionally, the mechanisms of action of many of these microbial metabolites have only been demonstrated in mouse models or on human cell lines. Clinical validation of these metabolites as physiologically-relevant will increase their value as a diagnostic tool.

Microbial Metabolites as a Therapeutic Opportunity in CD

As evidenced in the prior discussions of SCFAs, indole derivatives, and secondary bile acids, these products of bacterial metabolism have been demonstrated to have physiologically relevant activity in the strengthening of the epithelial barrier, signaling of inflammatory cytokines, as well as the modulation of TLR signaling pathways. The effects of these compounds may have clinical relevance in the treatment and management of Crohn's Disease because the primary treatment strategy for the majority of patients is mucosal healing [45]. This treatment strategy is rooted in the mitigation of the inflammation present in the mucosa (i.e. epithelium and stroma) of the intestines. As tissue healing cannot occur in the presence of aberrant inflammation, specific targeting of pro-inflammatory cytokines like TNFα has had significant success in disease treatment and management of CD. In a phase 3 clinical trial (CALM trial) that assessed the efficacy of a monoclonal antibody anti-TNFα therapy (adalimumab) in patients with CD, 46% of patients exhibited complete mucosal healing at the week 48 time point [13]. Notably, this trial also documented that 86% of patients reported treatment-induced adverse events as a result of anti-TNF therapy, including nasopharyngitis, headache, nausea, and worsening of CD [13]. The results of this study indicate two potential therapeutic gaps in the standard of treatment for CD: anti-TNF therapy is not effective in mucosal healing for many patients, and side effects of treatment are highly likely. As such, novel therapeutic options are an urgent need in the field of CD treatment and management.

Perhaps the most promising microbial metabolites discussed in this review that have implications for mucosal healing in patients with CD are indole derivatives and secondary bile acids. In the case of indole, the studies discussed in the previous section indicate that indole may have significant effects on the upregulation of tight junction proteins and the

downregulation of pro-inflammatory cytokines such as IL-8 and CXCL5. Moreover, indole treatment of HCT-8 cell lines led to upregulation of TLR regulatory proteins TOLLIP and SIGIRR, which have been shown to mitigate the pro-inflammatory potential of TLRs [4]. Together, these results indicate that indole treatment may reduce the burden of inflammation while also upregulating proteins that strengthen the epithelial barrier by tight junction repair.

Secondary bile acids represent another potential therapeutic agent, especially in the mitigation of the inflammatory damage associated with CD. As discussed, secondary bile acids can antagonize the FXR and TGR5 receptors, which are differentially expressed in intestinal epithelial cells. Importantly, TGR5 is expressed in both IECs and macrophages which may increase its value as a therapeutic target since both cell types are present at the site of inflammation in CD. The study by Pols et al demonstrated that TGR5-dependent CREB activation suppressed TLR4-mediated NF-κB transcriptional activation, resulting in reduced production of several inflammatory cytokines (IL-1β, IL6, IL8, and TNF). This is highly relevant to CD pathology, as the damaged epithelium in CD patients is more chronically exposed to commensal bacteria resulting in chronic stimulation of TLR4. Additionally, M1 macrophages are the most common macrophage subtype present in the inflammatory environment of the intestines and responsible for production of pro-inflammatory cytokines. The study by Biagioli et al demonstrated that stimulation of TGR5 induced a transformation of M1 macrophages to the M2 phenotype, which are critical for tissue healing. Coupled with the observation that TGR5 receptors are highly expressed in macrophages from tissue biopsies of patients with CD [55], the hypothesis that secondary bile acids could have therapeutic benefit to patients with CD is plausible.

As is the case with any novel therapeutic agent, clinical validation of the effects of indole and secondary bile acids in humans is of utmost importance. Not only should the mechanism of action be validated, but proper dosing and timing of administration must be thoroughly investigated, as repeat administration of these agents may be necessary to generate a physiological benefit. In the context of dosing, the route of administration should also be considered. The majority of anti-TNF antibody therapies are administered intravenously, however this may not be beneficial for administration of a metabolite that is typically produced locally by the microbiome. With this in mind, perhaps oral or suppository administration of the metabolites are a more viable option, as the likelihood that they will reach the site of damage is increased. The use of HPLC for the detection may also be useful to measure active concentrations of the compound during the course of treatment.

While indole derivatives and secondary bile acids both represent promising opportunities in the field of CD therapeutics, it is important to note that the anti-inflammatory effects of these compounds may have similar drawbacks to anti-TNF therapy. In most cases, broad suppression of TNFα increases susceptibility to infection due to its importance in many inflammatory pathways. Moreover, the physiologically relevant dosage has yet to be determined in humans, so the risks associated with delivering too much of these microbial metabolites have yet to be elucidated. This being said, the plausibility of these compounds as a treatment option for patients who do not respond to anti-TNF treatment is an exciting development, in which further investigation into their applicability is certainly warranted.

The increasing incidence and financial burden to healthcare of Crohn's Disease in recent history implicate a need to improve treatment opportunities for patients. The current screening methodologies available for CD are not effective in identifying patients that are at risk for disease development, and many methods are invasive by nature. Furthermore, current treatment strategies for CD have been effective for many patients, however the data indicate that nearly half of patients may not benefit from this treatment, and side effects are highly probable. Microbial metabolites produced by the resident microbes of the digestive tract have the potential to address both of these issues in CD screening and treatment. Specifically, short-chain fatty acids, indole derivatives, and secondary bile acids represent promising compounds for CD-specific biomarkers, as well as therapeutic agents to induce repair of the epithelial barrier. Longitudinal studies, as well as clinical data in humans will be paramount in elucidating how these compounds may be applied in the context of Crohn's Disease management.

Appendix 1 – Permissions for Figures

Figure 1

McCallum, G., Tropini, C. The gut microbiota and its biogeography. *Nat Rev Microbiol* 22, 105–118 (2024).

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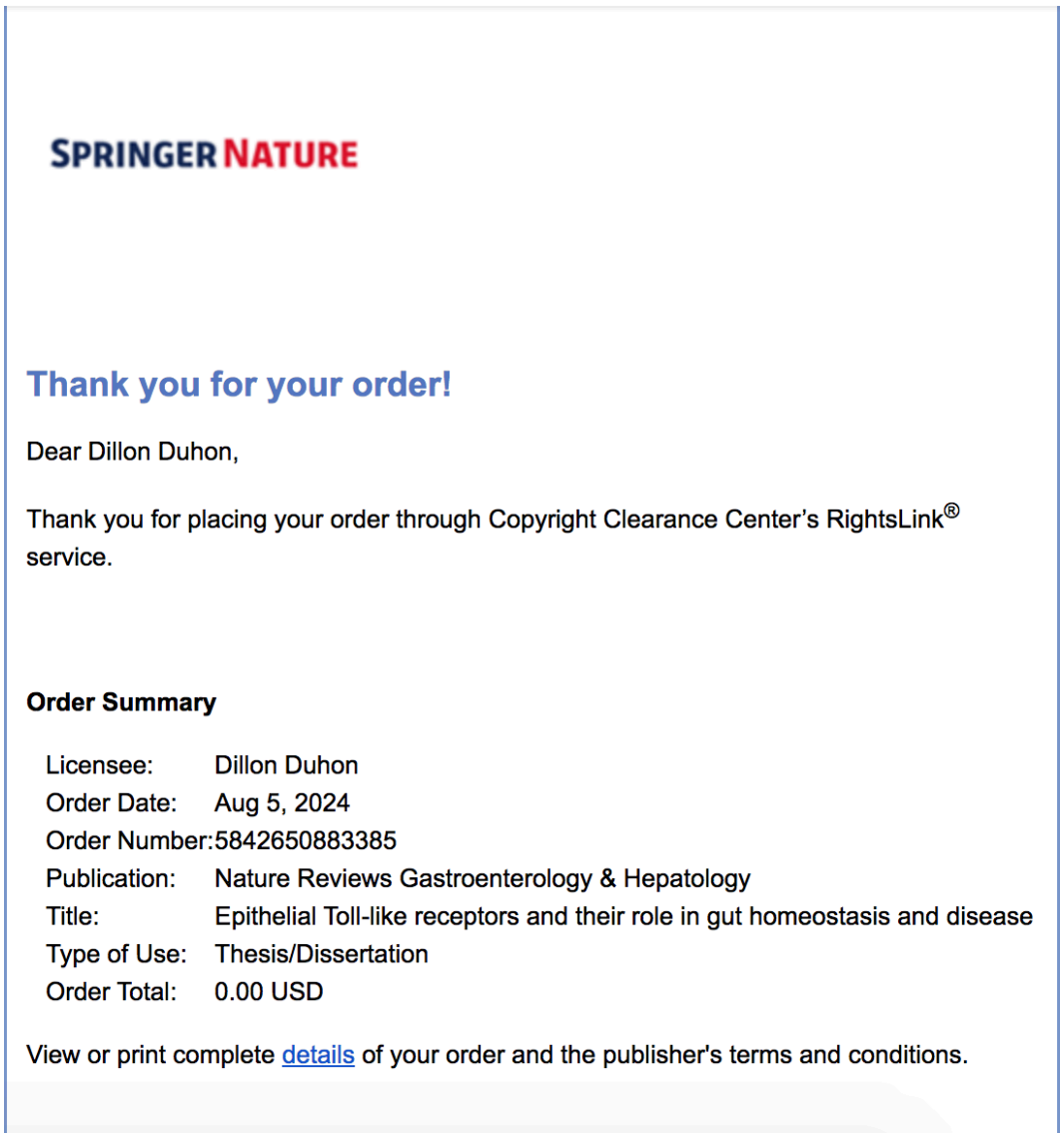

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Figure 3

Kawai, T., Akira, S. TLR signaling. *Cell Death Differ* 13, 816–825 (2006).

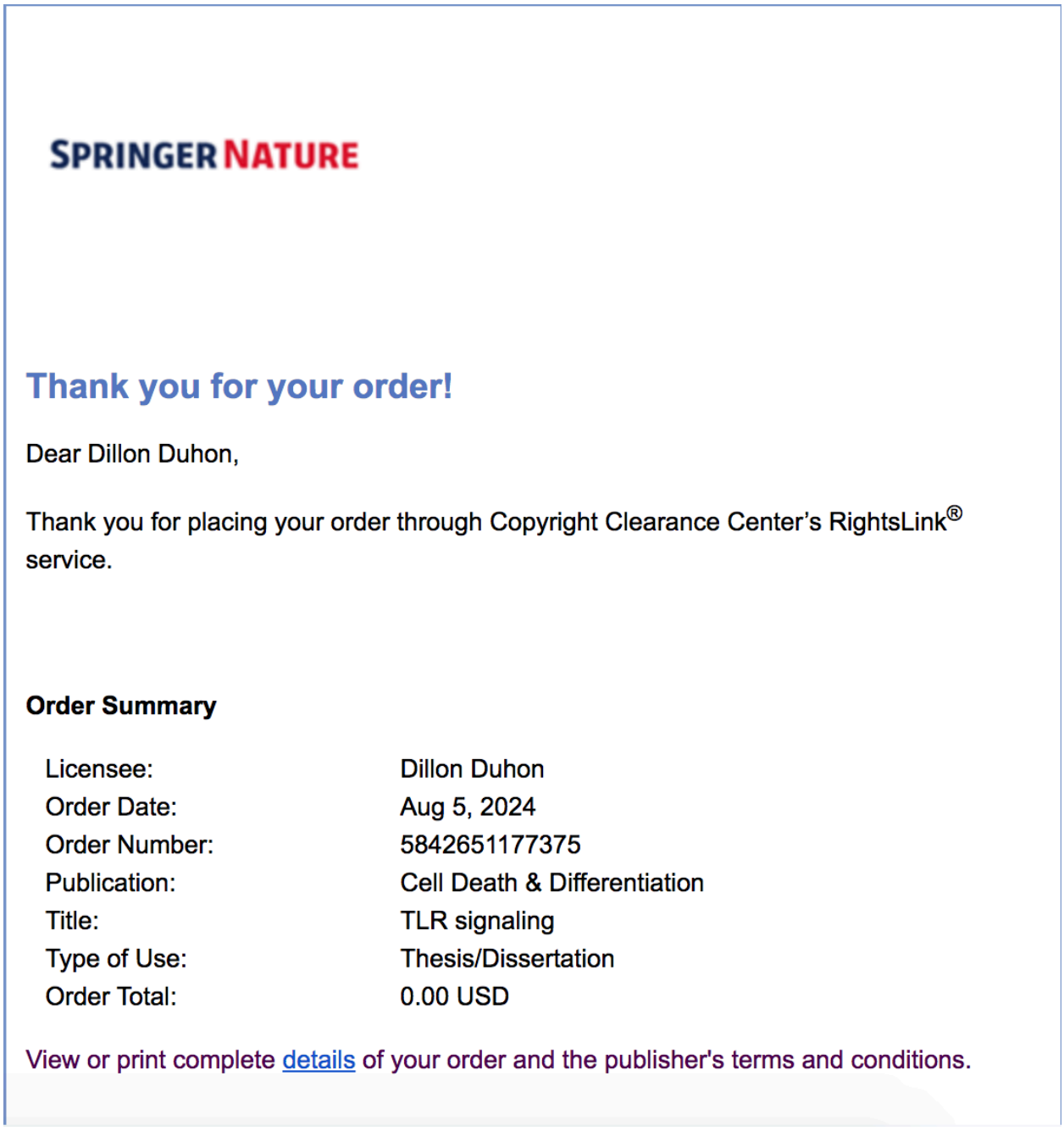

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Figure 5:

Bansal, Tarun, et al. “The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation.” *Proceedings of the National Academy of Sciences*, vol. 107, no. 1, 29 Dec. 2009.

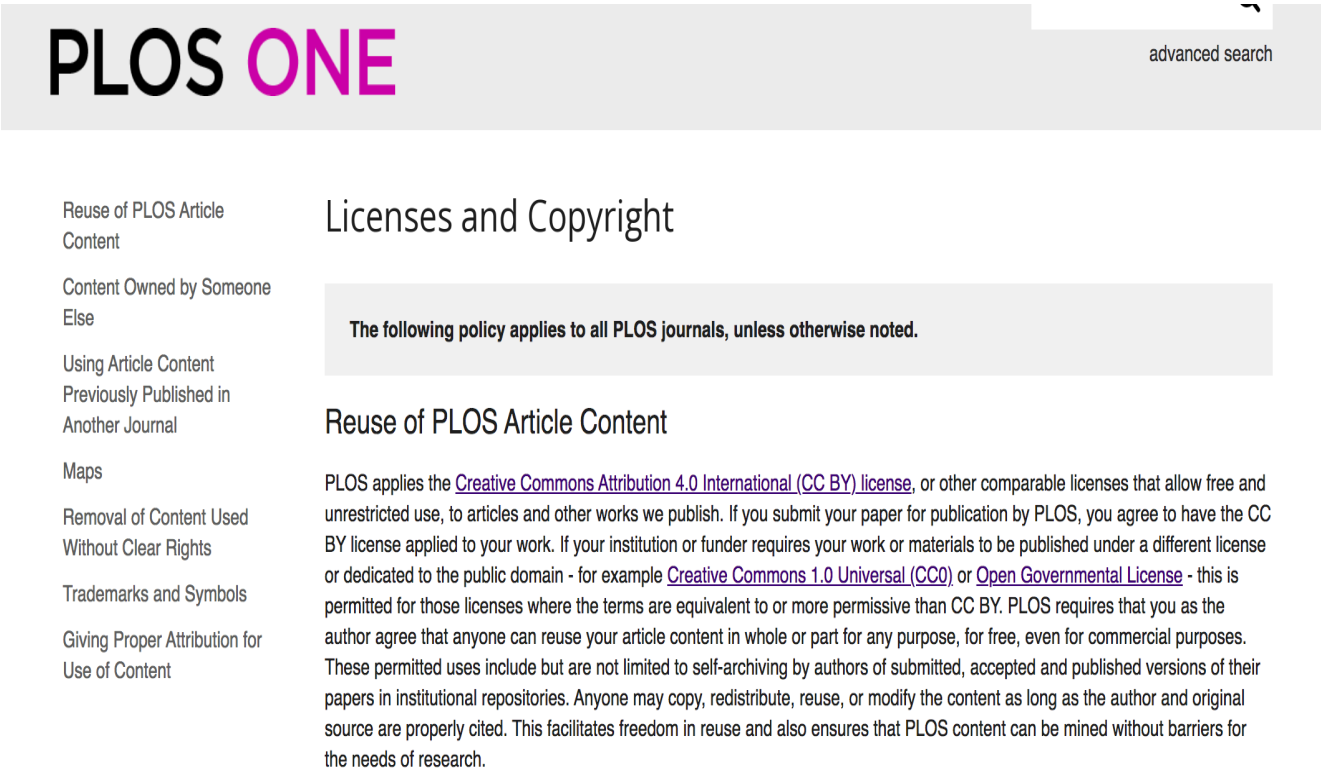

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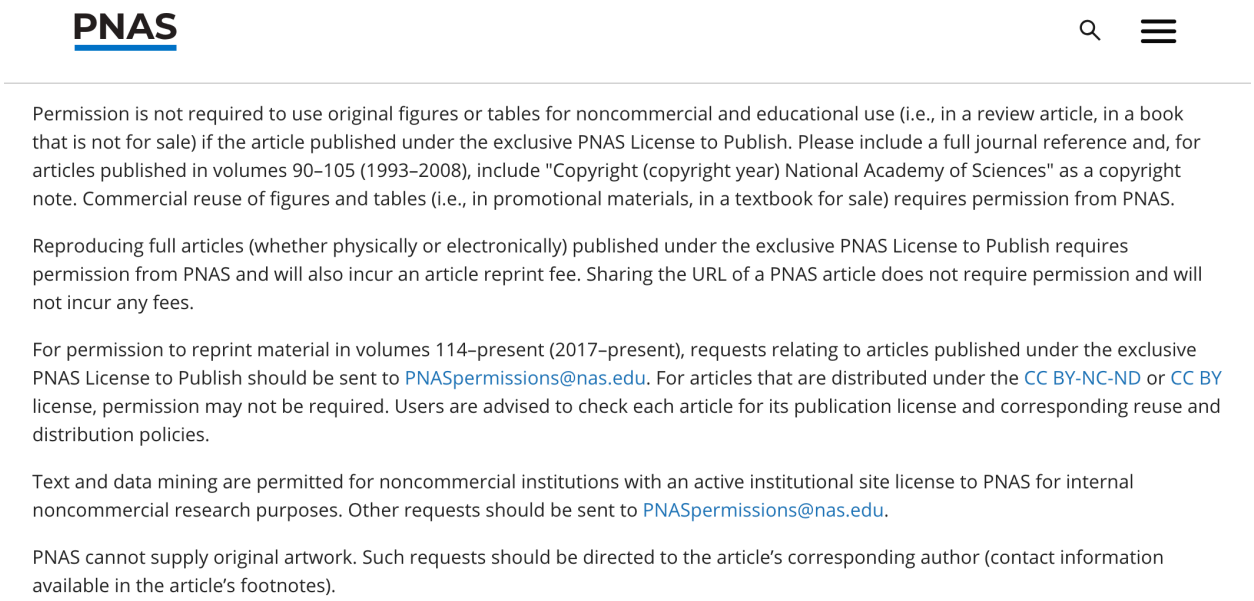
Figure 6:

Shimada Y, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, Takeda K. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. PLoS One. 2013 Nov 20



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