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Pathobionts of the Gastrointestinal Microbiota and Inflammatory Disease

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Abstract

Our immune system is charged with the vital mission of identifying invading pathogens and mounting proper inflammatory responses. During the process of clearing infections, the immune system often causes considerable tissue damage. Conversely, if the target of immunity is a member of the resident microbiota, uncontrolled inflammation may lead to host pathology in the absence of infectious agents. Recent evidence suggests that several inflammatory disorders may be caused by specific bacterial species found in most healthy hosts. Although the mechanisms that mediate pathology remain largely unclear, it appears that genetic defects and/or environmental factors may predispose mammals to immune-mediated diseases triggered by potentially pathogenic symbionts of the microbiota. We have termed this class of microbes `pathobionts', to distinguish them from acquired infectious agents. Herein, we explore burgeoning hypotheses that the combination of an immunocompromised state with colonization by pathobionts together comprise a risk factor for certain inflammatory disorders and gastrointestinal cancer.

Introduction

Microbes dominate as the most abundant life form on Earth, occupying almost every terrestrial, aquatic, and biological ecosystem on our planet. Humans are no exception. Throughout our lives, we continuously encounter microorganisms that range from those essential for health to those causing disease [1]. The human body is permanently colonized by microbial organisms on virtually all environmentally exposed surfaces. The vast majority of these microbes are harbored in the gastrointestinal (GI) tract where commensal bacteria can outnumber host cells by 10-fold (thus, we are all 90% bacteria on a cellular level). Many vital host functions are provided by the microbiota, including the synthesis of vitamins, digestion of complex polysaccharides, maintenance of the intestinal epithelial barrier, and resistance to pathogen colonization [2]. Millions of years of co-evolution have interdependently linked the health of mammals to their microbiotas [3]. The Human Microbiome Project is currently underway to sequence the microbiota of various populations of people, with a goal of identifying microbial species implicated in health and disease [4]. What is already clear is that microbes have flourished inside us since time immemorial, and have diverged to take on many functional roles that are now being uncovered at the genetic and mechanistic levels. Several descriptions of an intimate link between the microbiota and

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the immune system have recently emerged [5–8]. However, not all host-microbiota interactions promote health, and particular species of resident bacteria appear to activate the immune system resulting in inflammatory diseases. Thus, our association with the microbial world is precarious.

It is now appreciated that some symbiotic microorganisms in the GI tract induce pathology under certain conditions, usually involving environmental and/or genetic alterations. The term `pathobionts' has been suggested to describe resident microbes with pathogenic potential [9]. Organisms proposed as pathobionts are associated with chronic inflammatory conditions, unlike opportunistic pathogens which often cause acute infections and are typically acquired from the environment or other parts of the body. In addition, pathobionts are innocuous to the host under normal conditions, distinct from traditional pathogens which may cause disease even in healthy hosts. In this review, we highlight experimental evidence mostly from animal models that support the classification of specific microbes as pathobionts (see Table 1). Furthermore, we explore the role of bacterial pathobionts on intestinal health and their resulting impact on inflammatory bowel disease (IBD) and gastrointestinal cancers.

Segmented Filamentous Bacteria

Segmented filamentous bacteria (SFB) comprise a group of Gram-positive *Clostridia*-related bacteria that closely adhere to Peyer's patches in the mammalian small intestine and have been shown to potently stimulate immune responses including IgA induction and B cell activation [10]. Recently, much attention has been focused on SFB due to their ability to induce T-helper 17 (Th17) cells in the gut [7, 11]. Th17 cells, characterized by IL-17A, IL-17F, and IL-22 cytokine production, are an important contributor to adaptive immunity, conferring protection against enteric infection with extracellular pathogens. Specific-pathogen free (SPF) mice colonized with these non-culturable bacteria showed greater numbers of Th17 cells in the gut and heightened protection against *Citrobacter rodentium* infection compared to mice without SFB [7]. Germ-free (GF) mice, which have very few Th17 cells in the gut [12–14], exhibited no appreciable change in Th17 levels when reconstituted with a microbiota lacking SFB. Surprisingly, reconstitution with SFB alone was able to significantly increase the number of intestinal Th17 cells [7]. Considering the microbiota harbors a complex bacterial consortium of hundreds of species, these remarkable findings indicate the ability to induce Th17 cells in the gut may be uniquely possessed by only a small subset of the microbiota. Therefore, SFB colonization of healthy animals appears to play an important role in priming the adaptive immune system and potentially enhancing immunity against enteric pathogens.

However, the heightened immunity conferred by SFB colonization may also come at a cost to the host when inflammation is inappropriately triggered. Studies have demonstrated a pathogenic role for SFB in the gut. SCID (severe combined immunodeficiency) mice reconstituted with CD4+CD45Rb^{high} T cells and colonized with SFB developed severe colitis and intestinal inflammation [15]. In this particular animal model of colitis, SFB may synergize with the surrounding microbiota to exert its immunomodulatory effects, as mice mono-colonized with SFB did not develop intestinal pathology. Furthermore, the impact of SFB colonization on the host immune system appears to extend beyond the gut, as SFB mono-colonization in GF mice has been shown to increase the susceptibility of disease in animal models of rheumatoid arthritis and multiple sclerosis [16–17]. GF or antibiotic treated animals display reduced Th17 cells outside the gut and do not develop disease; this suggests that SFB alone can substitute for a complex microbiota in terms of driving pathology through Th17 cells induction. The observation that gut bacteria affect extra-intestinal compartments highlights the profound impact of the microbiota in modulating the

overall health of the host. Although the microbial molecules that drive immunity are unknown, these results illustrate that in the context of an autoimmune environment, SFB are pathobionts that promote diseases not observed in healthy hosts.

Helicobacter hepaticus

Helicobacter hepaticus belongs to the enterohepatic *Helicobacter* species (EHS), a diverse group of spiral bacteria that thrive on mucosal surfaces of the intestinal tract and/or the liver of humans and other animals [18]. *H. hepaticus* is a well-studied member of EHS and is prevalent in mice from commercial and academic institutions all around the world [19]. Pioneering work by Fox and co-workers led to the discovery of *H. hepaticus* and its role in hepatitis, hepatocellular carcinoma, typhlitis and colitis in several strains of immunodeficient mice [20–22]. Further studies provided evidence suggesting the involvement of *H. hepaticus* in the development of pathogenic inflammation and carcinogenesis in certain immunocompromised rodent models. Experimental infection with *H. hepaticus* induces IBD-like lesions in SCID mice reconstituted with naïve $CD4^{+}CD45RB^{high}$ T cells, as well as in C57Bl/6*IL-10*−/− mice [23–24]. Colonization with *H. hepaticus* also initiates rapid development of colitis and large bowel carcinoma in 129/SvEv *Rag2*−/− mice [25]. However, in immunocompetent wild-type (WT) mice, *H. hepaticus* fails to induce significant disease, irrespective of the mouse strains [24–25]. Therefore, *H. hepaticus* acts as a pathobiont that is able to promote colitis and in some cases colon cancer only in mouse strains with disrupted immune function.

Further questions arise from the pathobiont definition. For example, how does *H. hepaticus* interact with the host immune system to maintain a balanced relationship? Why do certain susceptible mouse strains with compromised immune systems develop inflammatory responses after *H. hepaticus* colonization, whereas WT mice do not? *H. hepaticus* infection induces Th1 and Th17 associated intestinal inflammation in *IL-10*−/− mice [26–27]. In lymphocyte-deficient *Rag*−/− mice, experimental infection with *H. hepaticus* induces colitis and colorectal cancer through proinflammatory cytokines TNF-α, IL-17, and IL-23 [25, 28–29]. *Rag*−/− mice lacking MyD88 in the hematopoietic compartment are resistant to *H. hepaticus*-induced colitis, indicating an essential role for toll-like receptor (TLR) signaling in *H. hepaticus*-induced innate inflammation [30]. Therefore, *H. hepaticus* is capable of causing both T cell-dependent and -independent inflammatory responses.

H. hepaticus induces intestinal inflammation in *IL-10*−/− mice but not WT mice, whose mesenteric lymph node (MLN) cells produce IL-10 in response to soluble *H. hepaticus* antigen (SHelAg), indicating a crucial role for IL-10 in balancing the *H. hepaticus*-induced inflammatory responses [26–27]. Furthermore, anti-IL-10R treated MLN cells derived from *H. hepaticus*-infected mice produce higher levels of IL-17 and IFN-γ compared with WT MLNs following response to SHelAg [31]. Transferring *H. hepaticus*-induced $CD4^{+}CD45RB^{low}$ regulatory T cells suppresses *H. hepaticus*-induced colitis in *Rag*−/− mice [32]. Also, $CD4^{+}CD25^{+}$ regulatory T cells isolated from *Helicobacter*-free 129SvEv mice prevent both T cell-dependent and -independent intestinal inflammation in an IL-10-dependent manner [33]. Moreover, an intact NF-κB signaling pathway is required for IL-10-mediated inhibition of *H. hepaticus*-induced colitis [34]. Therefore, it appears that *H. hepaticus* colonization induces a tolerogenic IL-10-secreting regulatory T cell response, which may be important for maintaining immunologic `balance' with the host. In addition, *H. hepaticus* was found to suppress TLR4 and TLR5-mediated immune responses in intestinal epithelial cells [35]. Our results also showed that *H. hepaticus* suppressed the expression of TLR4 in the intestinal epithelial cell line MODE-K [36], suggesting another possible regulatory strategy in epithelial cells mediated by TLR signaling. We propose that *H. hepaticus* maintains symbiotic crosstalk with the host by directing both inflammatory and tolerogenic responses in the innate and adaptive immune system during long-term

colonization. However, in genetically susceptible hosts with defects in tolerogenic immune function and/or regulatory mechanisms, *H. hepaticus* may trigger an imbalanced immune response leading to pathologic inflammation.

Most studies have focused on the host immune response to *H. hepaticus* colonization and genetic alterations in mice that lead to disease. Little is known about the bacterial components produced by *H. hepaticus* that mediate these outcomes. Our recent discovery revealed that *H. hepaticus* utilizes a type VI secretion system (T6SS) to balance host colonization and intestinal inflammation [36]. T6SS are multi-protein complexes assembled on the bacterial surface that function as a biological needle and syringe, injecting microbial molecules into eukaryotic cells. Deletion of the T6SS apparatus resulted in higher colonization levels of *H. hepaticus* during experimental colitis. Moreover, a *H. hepaticus* T6SS mutant elicited elevated inflammatory responses in the intestine of *Rag1*−/− mice reconstituted with CD4+CD45RB^{high} T cells compared to WT bacteria. Meanwhile, T6SS directed an anti-inflammatory response in an intestinal epithelial cell line, characterized by suppressed expression of TLR4, NF-κB and IL-17R. However, whether T6SS mediated suppression of innate inflammatory signaling correlates with its regulatory roles is still unknown. Identification of T6SS substrates of *H. hepaticus* may define the molecular mechanisms by which this pathobiont `communicates' with its host to establish symbiosis. Disruption of this communication, through genetic polymorphisms or mutations in the host, may form the basis for why *H. hepaticus* causes disease in compromised animals.

Helicobacter pylori

In 2005, Barry Marshall and Robin Warren won the Nobel Prize in Medicine for demonstrating that *Helicobacter pylori*, a bacterium that intimately colonizes the mucosal lining of the stomach, could directly cause peptic ulcer disease and gastritis. Classified as a class I carcinogen, *H. pylori* has been shown to lead to gastric adenocarcinoma in 1% of infected individuals. While 50% of the human population is thought to be colonized with *H. pylori*, only a small percentage actually develop gastric disorders [37]. Colonization of humans with *H. pylori* is believed to have occurred since humans migrated out of Africa 58,000 years ago. The bacteria are thought to colonize during early childhood and can thrive in the stomach for a lifetime. In countries with higher socio-economic standards (involving increased antibiotic use and hygiene), colonization appears to be less prevalent compared in developing countries.

The mechanistic details of how *H. pylori* promotes inflammation have been investigated. Using a Type IV secretion system (T4SS), *H. pylori* translocates the bacterial protein CagA into gastric epithelial cells. CagA subsequently interacts with host signal transduction pathways involved in inflammation and oncogenesis [38]. The presence of CagA, along with other virulence factors such as a pathogenicity island and additional secreted toxins, correlate well with increased virulence in strains of *H. pylori* [39]. However, even virulent strains of *H. pylori* are found in asymptomatic individuals suggesting there are other factors contributing to the induction of disease. Genetic polymorphisms in the *IL1*β gene, which encodes for a pro-inflammatory cytokine important for enhancing the inflammatory response to *H. pylori* infection, have been shown to be associated with increased risk of gastric cancer [40].

Adding another layer of complexity is the observation that colonization with *H. pylori* inversely correlates with esophageal adenocarcinoma and childhood asthma [41–42]. Furthermore, individuals colonized with CagA deficient strains of *H. pylori* are at increased risk for disease [43]. Although *H. pylori*-mediated protection against these pathologies still remains to be convincingly demonstrated, these results suggest the intriguing concept that *H. pylori* may have evolved to protect its host against disease in order to promote a healthier

environment for its long-term survival [44]. Finally, sequencing efforts have revealed highly diverse panmictic populations of *H. pylori* between geographically separated groups [45]. The extensive diversification of the *H. pylori* genome may have proven advantageous in surviving the changing immunological and environmental pressures of the stomach.

Implications for Inflammatory Bowel Disease and GI Cancers

Inflammatory bowel diseases (including Crohn's disease (CD) and Ulcerative colitis (UC)), afflict approximately 1.5 million people in the United States. Currently there is no cure for IBD, although immunosuppressive therapies and probiotics alleviate symptoms in some cases. The causes of IBD appear to be multifactorial, integrating the microbiota, host genetics, and the immune system as factors determining predisposition to disease.

Shifts in the intestinal microenvironment (due to diet, antibiotics, hygiene, etc) may lead to changes in the microbiota known as dysbiosis. Dysbiosis may increase susceptibility to intestinal inflammation [46–47]. In support of this hypothesis, *T-bet*−/−*Rag2*−/− (TRUC) mice spontaneously develop dysbiosis and colitis, which can eventually progress into colorectal cancer [48]; remarkably, microbiota transfer from these donors into wild-type mice can confer disease [5]. Subsequent studies identified two proteobacteria over-represented in TRUC mice, *Proteus mirabilis* and *Klebsiella pneumonia*, as the colitogenic microbes [49]. However, full induction of disease required the presence of a diverse microbiota, indicating that interactions with other microbes may define whether a pathobiont will display a pathogenic profile. Culture-independent 16S rDNA sequence analysis of the microbiotas of individuals with Crohn's disease revealed lower diversity and greater temporal instability compared to controls [50]. In patients with IBD, the number of commensals belonging to the phyla *Firmicutes* and *Bacteroidetes* were found to be decreased, while concomitant increases in *Actinobacteria* and *Proteobacteria* were observed [51]. These findings highlight an important link between changes to the composition of the microbiota and intestinal health in animal models and humans.

Evidence over several decades suggests that the gut microbiota is a key factor in the pathogenesis of IBD. Studies have shown increased antibody titers against gut bacteria in IBD patients compared to healthy individuals [52]. Furthermore, treatment with antibiotics can help alleviate symptoms [53]. It is well documented that in certain mouse models of experimental colitis, rederivation under germ-free conditions abolishes disease [54]. However, host genetics and their impact on the resulting immunological environment significantly determine the type of response (or lack thereof) to the microbiota. Numerous genetic variants have been identified in individuals with IBD and correlate strongly with an increased risk of disease. Many of these genes are involved in bacterial recognition (*NOD2, TLR* genes, *IRGM, ATG16L1*) and innate and adaptive immunity (*IL-23R*, *IL-10*) [55]. Balanced immune responses to the microbiota are critical for intestinal homeostasis, as the microbiota itself has been shown to coordinate intestinal immunity. Illustrating this concept, mice expressing the human defensin DEFA5 showed a reduction in SFB colonization and a corresponding decrease in lamina propria Th17 cells [56]. In addition, perturbations in the mouse NLRP6 inflammasome pathway led to overgrowth of intestinal *Prevotellaceae* and TM7 bacteria, resulting in increased susceptibility to chemically-induced colitis [57]. Thus, although certain symbionts are prominent species in the gut and typically non-pathogenic, specific host defects can trigger IBD as a result of inflammation directed to pathobionts.

Although our understanding of the role of pathobionts on human health is still in its infancy, a few studies have highlighted the dangers of disrupting the human gut microbial community. Pseudomembranous colitis which results in severe diarrhea, fever and abdominal pain, is caused by overgrowth of *Clostridium difficile* following long-term

antibiotic treatment [58]. Broad-spectrum antibiotics can also enhance vancomycin-resistant *Enterococcus* (VRE) survival and proliferation in the GI tract, which may subsequently lead to infection of the bloodstream [59–60]. As the source of *C. difficile* and *Enterococcal* infections is the microbiota, environmental factors may predispose patients to diseases caused by indigenous pathobionts. We predict that both genetic host alterations and/or environmental perturbations (such as antibiotic use) may lead to intestinal inflammation triggered by pathobionts (Figure 1).

Recent studies have suggested that chronic inflammatory conditions can contribute to the development of some cancers by promoting cell proliferation, cell survival, and/or angiogenesis [61]. Individuals with IBD (in particular ulcerative colitis) have an increased risk of developing colorectal cancer [62]. In an experimental animal model of colitis-associated cancer, *IL-10*−/− mice treated with the chemical carcinogen azoxymethane, were devoid of tumors when raised under germ-free conditions, indicating the presence of intestinal bacteria is required for carcinogenesis [63]. Similar results were found in other animal models of spontaneous colon cancer. Germ-free rederivation of *TCR*β−/−*p53*−/− mice and *TGF*β*1*−/− mice eliminated the formation of intestinal tumors [64–65]. In addition, clinical studies have identified a higher incidence of adherent and invasive *Escherichia coli* (AIEC) in biopsies from carcinoma patients compared to controls [66–67]. Colorectal cancer is the second most common cause of malignant tumors in the United States [68], and often has life-threatening consequences. Moreover, epidemiologic and clinical data show that the incidence of colon cancer is dramatically increasing in Western countries. A genetic basis for cancer is well established; however it is being increasingly appreciated that non-genetic (environmental) factors are also crucial to the disease process. Whether there is a causal relationship between the microbiota, intestinal inflammation, and colon carcinogenesis will require further investigation.

Concluding Remarks

Recent ground-breaking studies of the interactions between humans and beneficial bacteria have marked a revolution in microbiology and immunology [3]. The human gastrointestinal tract harbors astounding multitudes of symbiotic bacterial species living in homeostasis with the immune system. However, some of these permanent residents appear to take on pathogenic properties during colonization of hosts with genetic and/or environmental alterations. Based on this rationale, we have speculated a category of indigenous gut bacteria termed pathobionts which cause disease only in susceptible hosts. This designation is based on recent data from animal models, with limited but growing support from clinical studies. The combination of a compromised host with colonization by pathobionts may be a risk factor in IBD, colon cancer and perhaps for diseases outside of the intestinal compartment. Identifying the molecular interactions between pathobionts and the mammalian immune system may be critical to understanding the etiology of certain diseases with a non-infectious microbial component. Finally, the design of drugs that inhibit the processes by which pathobionts promote inflammation may represent novel therapies for chronic human diseases.

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Highlights

- Research now shows that some members of the normal gut microbiota may promote disease
- We term these microbes “Pathobionts” to distinguish them from acquired infections
- Pathobionts appear to cause chronic inflammatory diseases
- Understanding how Pathobionts induce disease may lead to anti-microbial therapies for IBD and colon cancer

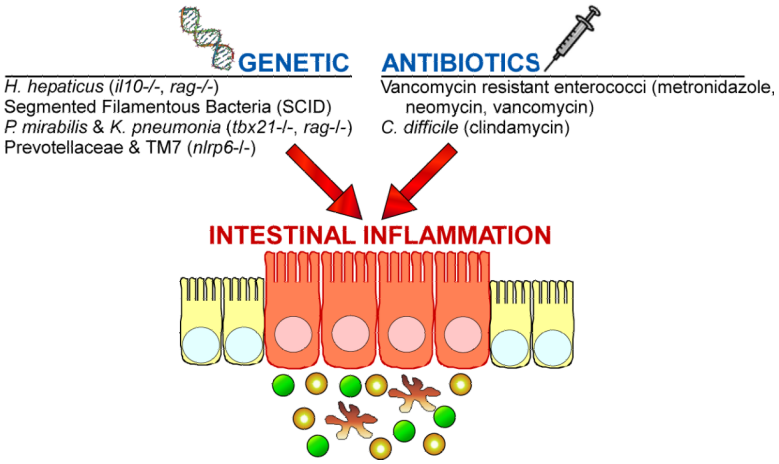

Figure 1. Genetic and environmental alterations may synergize with pathobionts to cause intestinal inflammation and disease

In addition to acquired pathogens which can cause gastroenteritis, resident gut bacteria trigger intestinal inflammation. However, unlike acute pathogens, pathobionts appear to require additional factors to cause disease. Based predominantly on animal models, certain symbionts of the microbiota can initiation gut inflammation and pathology when colonizing a genetically susceptible host (e.g., *H. hepaticus*, SFB, *P. mirabilis* & *K. pneumonia, Prevotellaceae* and TM7). In other cases, specific resident gut bacteria can expand following antibiotic use which clears competing symbionts to promote gastrointestinal disease (VRE, *C. difficile*). The associated genetic defects or antibiotics are denoted in parenthesis.

Table 1

PATHOBIONTS OF THE GASTROINTESTINAL TRACT

Bacterial strain	Conditions Promoting Pathogenesis	Refs
Segmented Filamentous Bacteria	• leads to colitis in SCID mice reconstituted with $CD4^{+}CD45Rb^{high}$ T cells	15
	• promotes disease in experimental models of rheumatoid arthritis and multiple sclerosis in mono-associated gnotobiotic mice	16,17
Helicobacter hepaticus	• induces colitis in C57BI/6 *IL-10*−/− mice	24
	• initiates colitis and large bowel carcinoma in 129/SvEv *Rag2*−/− mice	28
Helicobacter pylori	• genetic polymorphisms of *IL1B* associated with increased risk of gastric cancer	40
Proteus mirabilis Klebsiella pneumonia	• responsible for inducing colitis and colorectal cancer in *T-bet*−/−*Rag2*−/− (TRUC) mice	48
Prevotellaceae TM7	• responsible for inducing colitis in mice with mutations in the inflammasome pathway	57
Clostridium difficile	• can lead to pseudomembranous colitis in humans, following long-term antibiotic treatment	58
Vancomycin-resistant *Enterococcus*	• capable of invading the bloodstream in humans treated with broad-spectrum antibiotics	59,60