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The Role of the Intestinal Microbiota in Inflammatory Bowel Disease

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by

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ABSTRACT OF THE DISSERTATION

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Inflammatory bowel disease (IBD) arises from complex interactions of genetic, environmental, and microbial factors. The intestinal microbiota is crucial for IBD induction and complex shifts in microbiota composition occur in IBD, but disease has not been consistently associated with presence or absence of a specific microbe. It is thus controversial whether fulfilling Koch's postulates for individual bacterial species is relevant to IBD and whether disease-associated alterations in microbial colonization are predictive of underlying etiology. Resolving these controversies has been challenging due to paucity of animal models with rapid disease onset, experimental reliance on gnotobiotic animals, and difficulty specifically isolating many commensal intestinal bacteria.

We fulfilled Koch's postulates in a host genotype-specific fashion, using non-gnotobiotic methods to show that common commensal *Bacteroides* species induced disease in a genetic mouse model of IBD whereas an Enterobacteriaceae species that was

significantly enriched during spontaneous disease was not itself sufficient for disease induction. We studied mice with a human-relevant IBD-susceptibility mutation which spontaneously develop intestinal inflammation resembling human ulcerative colitis. Antibiotics blocked colitis induction and mice remained disease-free after treatment cessation but developed disease if subsequently colonized with intestinal contents from untreated donors or with intestinal contents grown in mixed culture on media selective for Gram-negative obligate anaerobes. We therefore isolated common commensal *Bacteroides* species, introduced them into antibiotic-pre-treated mice, and confirmed colonization by specific, quantitative re-isolation in culture. Isolates colonized susceptible and non-susceptible mice equivalently but induced disease exclusively in susceptible animals, suggesting susceptibility was due to differences in host response rather than altered colonization susceptibility.

In contrast to commensal *Bacteroides*, Enterobacteriaceae were >100-fold enriched in the microbiota during spontaneous disease in our mice, supporting observations in other animal models and in human patients. However an Enterobacteriaceae isolate from a spontaneously colitic mouse was not sufficient to induce disease in antibiotic-pre-treated animals despite robust colonization.

We thus identified distinct subsets of commensals with and without IBD-inducing potential and showed that these subsets would not have been predicted based on disease-associated shifts in the microbiota. Our findings establish experimental criteria and a conceptual framework for understanding the intestinal microbiota's involvement in IBD.

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List of Abbreviations

16S rRNA	16S subunit of ribosomal riboxynucleic acid
Abx	Antibiotics
ACNA	Anaerobic reducible blood agar with colistin and nalidixic acid (CNA)
ANB	Anaerobic reducible blood agar
BEA	Bile esculin azide agar
BBE	*Bacteroides* bile esculin agar (*Bacteroides fragilis* isolation media)
CNA	Anaerobic reducible blood agar with colistin and nalidixic acid
CCNA	Columbia blood agar with colistin and nalidixic acid
CD4	Cluster of differentiation 4
CFU	Colony-forming unit
Choc	Chocolate agar
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
dnKO	Dominant-negative knockout (*dnTgfβrii* x *Il10r2*$^{-/-}$)
dnTgfβrii	Dominant negative transforming growth factor β receptor 2
Foxp3	Forkhead box P3
H&E	Hematoxylin and eosin
HLA-B27	Human Leukocyte Antigen B27
IBD	Inflammatory bowel disease

IL-2	Interleukin-2
IL-10	Interleukin-10
IL-10R2	Interleukin-10 receptor 2
LKV	Anaerobic reducible laked blood agar with kanamycin and vancomycin
Mac	MacConkey agar
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PSA	Polysaccharide A
RDP	Ribosomal Database Project
Rag	Recombination activating gene
Rog	Rogosa media
SEM	Standard error of measurement
SMAD3	Similar to mothers against decapentaplegic 3
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th1	T helper 1
TNFα	Tumor necrosis factor α
TSA	Trypticase Soy Agar with 5% sheep blood

CHAPTER 1

Inflammatory Bowel Disease: History and Studies of Etiology

Epigraphs

"DR. BURRILL B. CROHN, NEW YORK: In a disease of this type, in which an attempt is being made to establish the etiology of the disease, we have naturally taken great pains to exclude every known etiologic factor. Histologic sections were made of the tissues and stained with various types of stains. Cultures were made. Ground material was injected into guinea-pigs and fowl. Various types of laboratory animals were used to eliminate any possible form of tuberculosis. Löwenstein cultures were made. Dr. Klemperer, the pathologist, exhausted all the known possible scientific methods of finding an etiologic factor. I can say that no etiologic factor was found."

"Regional Ileitis: A Pathologic and Clinical Entity." (1932) Burrill B. Crohn, MD, Leon Ginzburg, MD, and Gordon D. Oppenheimer, MD. *Journal of the American Medical Association*. 99*(*6*):*1323–1329.

"*In discussing the difficult subject of the etiology of these ulcerative diseases of the terminal ileum and colon, it seems desirable in the present state of knowledge to be very conservative and careful in the expression of one's opinions. While ulcerative colitis is a fairly definite clinical and pathologic disease picture, it is doubtful if it is an etiologic entity…*

"[I]t does not seem correct to assume that all, or even a large per cent of the ulcerative colitis cases of unknown etiology are chronic bacillary dysentery… Ulcerative colitis is usually sporadic; bacillary dysentery is usually epidemic. Ulcerative colitis is almost never encountered as contact disease; nor do certain geographical localities furnish more cases than others. Ulcerative colitis usually begins insidiously; bacillary dysentery is as a rule acute and stormy in its onset….

"Our opinion in the matter of the etiology of chronic ulcerative colitis may be summarized as follows: (1) a small per cent are unrecognized amoebic colitis cases. (2) a certain per cent, approximately 20%, are instances of chronic bacillary dysentery in whom either the dysentery organisms or the secondary invaders, or both, keep up the disease. (3) the cause in the remainder is as yet unknown… Three theories seem most plausible: (1) a primary functional (neurogenic) disturbance of the bowel and then a secondary infection. (2) the mucosa is first sensitized by a transient specific or special infection and then local or distant organisms or toxins attack the susceptible tissue… (3) the specific cause, whether bacterial or viral in nature, is yet to be discovered."

"The Etiology and Therapy of Ulcerative Colitis." (1936) Asher Winkelstein, MD. *American Journal of Digestive Disease and Nutrition.* 3(11): 839-844.

Inflammatory bowel disease (IBD) consists of a spectrum of chronic, non-communicable, diarrheal diseases, including ulcerative colitis and Crohn's disease, that are characterized by inflammation of the gut wall (Xavier and Podolsky 2007). IBD affects over 1 million people in the USA alone and is rising in prevalence worldwide (Loftus 2004). Historical accounts of diseases with IBD-like symptoms date back at least two millennia to a widely cited description by a Greek physician with the oddly appropriate name Soranus of Ephesus (De Dombal 1968). The first medical report describing ulcerative colitis by its modern name is generally credited to Sir Samuel Wilks in 1859, and ulcerative colitis was formally defined as a discrete pathologic entity distinguishable from infectious colitis by Wilks and Moxon in 1875 (De Dombal 1968; Pearce 2009). Crohn, Ginzburg, and Oppenheimer proposed classifying the condition that is now called Crohn's disease as an entity distinct from ulcerative colitis in 1932 (Crohn, Ginzburg et al. 1932). By their classification, which is widely accepted today, ulcerative colitis and Crohn's disease are characterized respectively by continuous, ascending colonic inflammation that is restricted to the intestinal mucosa and by discontinuous, granulomatous inflammation that penetrates the intestinal wall and can occur in any portion of the intestinal tract (Crohn, Ginzburg et al. 1932; Xavier and Podolsky 2007). However since ulcerative colitis and Crohn's disease share many features and cannot be diagnostically distinguished in a significant fraction of cases, it remains unclear whether these two forms of IBD share a common etiology, are etiologically distinct, or represent a collection of similar diseases arising from many different etiologies (Stenson, Hanauer et al. 2009).

Beginning with many of the earliest descriptions of IBD in modern medical literature, attention has focused on the possibility that disease may have a microbial cause (see epigraphs above). Consistent with IBD's apparently non-communicable etiology, both these early investigations and many subsequent studies have failed to consistently associate most cases with infection by any known microbial pathogen (Crohn, Ginzburg et al. 1932; Winkelstein 1936; Packey and Sartor 2009). Despite the failure to identify specific etiologic agents, a wealth of clinical and laboratory data supports the hypothesis that IBD induction depends on one or more factors within the intestinal tract's indigenous microbiota (Strober 2010), which is an extraordinarily dense microbial community comprising hundreds or thousands of commensal species many of which have never been grown in culture (Eckburg, Bik et al. 2005; Qin, Li et al. 2010). Medically, antibiotics and probiotics have therapeutic benefits in several manifestations of IBD (Sartor 2008; Ohkusa, Kato et al. 2010). In patients with Crohn's disease, surgical diversion of fecal flow produces disease remission in inflamed bowel segments and disease often recurs upon flow restoration (Ginzburg, Colp et al. 1939; Harper, Lee et al. 1985; Janowitz, Croen et al. 1998). Harper and colleagues, studying IBD patients in whom surgical diversion had been performed, found that experimental introduction of small bowel effluent into the surgically excluded bowel segments re-induced disease whereas a sterile ultrafiltrate of bowel effluent did not (Harper, Lee et al. 1985). Similarly, in most spontaneous animal models of IBD, disease is blocked by antibiotics or re-derivation into germ-free (gnotobiotic) facilities and IBD-susceptible germ-free animals develop disease upon exposure to commensal microbiota from conventionally raised hosts (Sartor 2008).

However it remains a subject of active investigation whether these phenomena represent the effects of a specific microbial species or subgroup of species, constitute a non-specific response to microbial stimuli in general, or are due to the synergistic effects of multiple microbes acting together as a community (Xavier and Podolsky 2007; Sartor 2008; Takaishi, Matsuki et al. 2008; Garrett, Gordon et al. 2010; Strober 2010).

While many questions remain unresolved about the microbial factors responsible for IBD induction, advances in immunology and genetics have revolutionized understanding of disease etiology and pathogenesis. IBD is now widely understood to consist of a pathological host immune response influenced by the complex interaction of genetic and environmental, as well as microbial, factors (Xavier and Podolsky 2007; Cadwell, Patel et al. 2010). The basis of the immune response is complex and only partially understood, but components of both the innate and adaptive immune system are clearly important with substantial evidence of a critical role for T-cells and therapy targeting the inflammatory cytokine TNFα is highly efficacious in many cases (Rakoff-Nahoum, Paglino et al. 2004; Xavier and Podolsky 2007; Barnes and Powrie 2009; Stenson, Hanauer et al. 2009). Classical family-based genetic studies have shown that IBD has a strong genetic component, but disease is multigenic and genetic factors are not solely sufficient to explain its occurrence (Stenson, Hanauer et al. 2009). Recent data from multiple genome-wide association studies has led to identification of susceptibility alleles at nearly 100 different loci, some specifically associated with either ulcerative colitis or Crohn's disease and others associated with both (Barrett, Hansoul et al. 2008; Franke, Balschun et al. 2008; Franke, McGovern et al. 2010). While the functional

significance of many loci remain obscure, several are associated with genes in pathways that modulate immune and epithelial function. Interestingly, these studies have revealed susceptibility alleles associated with multiple genes in pathways related to Interleukin-10 (IL-10) and Transforming Growth Factor β (TGFβ) signaling, two inhibitory cytokines that are known from immunologic studies to play a prominent role in immunoregulation and intestinal homeostasis. In support of these findings, a recent kindred analysis identified a subset of patients with highly fulminant, rapid-onset enterocolitis due to recessive deficiencies in components of the receptor complex for IL-10 (Glocker, Kotlarz et al. 2009).

Identification of disease-inducing microbial agents in a wide variety of diseases has traditionally relied on experimental infection of laboratory animals. As expemplified by the first epigraph at the beginning of this chapter, efforts at applying this approach to IBD using standard healthy animals have largely failed to induce IBD-like disease or result in identification of causative microbial agents (Crohn, Ginzburg et al. 1932; Packey and Sartor 2009). Together with epidemiologic clues, these experiments provided some of the earliest evidence that the IBD's etiology is more complex than that of a classical infectious disease (Winkelstein 1936). Subsequent efforts to develop experimental models simulating IBD have included treating animals with colitis-inducing chemicals or drugs, transferring specific populations of immune cells into recipients lacking endogenous lymphocyte, and infecting animals with known enterocolitis-inducing microbial pathogens (Jurjus, Khoury et al. 2004; Nell, Suerbaum et al. 2010). Each of these approaches has yielded important information about the immunologic factors,

cellular pathways, potential therapies, and natural progression of intestinal inflammation. But with important exceptions (Onderdonk, Franklin et al. 1981; Onderdonk, Bronson et al. 1987; Cadwell, Patel et al. 2010), inducible models can be problematic for identifying indigenous microbial factors involved in spontaneous IBD since the proximate cause of disease in each case involves an experimental manipulation that rarely or never occurs in association with human disease.

By contrast, the relatively recent availability of technology to create genetically engineered animal models has rendered it possible to conduct experiments which would have been unimaginable in the days of Crohn, Wilks, and (especially) Soranus. In the past two decades, an ever-increasing number of genetic animal models that develop spontaneous intestinal inflammation resembling human IBD have been developed (Horwitz 2007). These animal models, primarily mice, bear mutations in genes affecting many aspects of epithelial integrity and both the adaptive and innate immune systems (Xavier and Podolsky 2007; Mizoguchi and Mizoguchi 2010). In contrast to un-manipulated healthy animals or animals in which disease has been experimentally induced, these spontaneously colitic animals have proven to be more natural candidates for efforts to identify potential IBD-inducing microbes and test hypotheses about the nature of microbial contributions to IBD etiology (Horwitz 2007; Sartor 2008). As described in greater detail in subsequent chapters, landmark studies using antibiotics and re-derivation into germ-free facilities have shown that disease in most of these models is largely dependent on the commensal microbiota and that it may be possible to identify specific commensal bacteria which play a role in disease induction (Sartor 2008).

Remarkably, there are a number of examples of in which deletion of a specific gene was observed to cause spontaneous intestinal inflammation in mice and the gene was subsequently shown to be associated with a risk allele for IBD in human patients (Mizoguchi and Mizoguchi 2010). These convergent lines of evidence raise the possibility that beyond serving as conceptually useful experimental models, discoveries in spontaneously colitic animals which bear mutations in genes linked to human IBD-susceptibility alleles may be directly relevant to the etiology and pathogenesis of human IBD.

One such animal model of particular interest was recently developed and characterized in collaboration between our laboratory and that of Dr. Paul Allen. The mice in question bear mutations affecting two different pathways that have each been identified as associated with IBD. The first genetic alteration in these mice consists of recessive deficiency in the IL-10 receptor 2 (IL10R2) protein (Spencer, Di Marco et al. 1998), deficiency in which is known to cause severe, fulminant IBD in human patients within the first year of life (Glocker, Kotlarz et al. 2009). Susceptibility alleles linked to the IL-10 locus have also been identified for both Crohn's disease and ulcerative colitis in recent genome-wide association studies (Franke, Balschun et al. 2008; Franke, McGovern et al. 2010). The second alteration consists of a transgenic dominant negative TGFβ receptor II (dnTGFβRII) expressed specifically in T-cells (Gorelik and Flavell 2000). Although we are not aware of a human IBD-susceptibility allele linked directly to this gene, a recently identified risk allele for Crohn's disease is linked to the gene encoding SMAD3 (Franke, McGovern et al. 2010), a signal-transducing protein that is

phosphorylated upon activation of the TGFβ receptor and is important for TGFβ induction of Foxp3+ regulatory T cells (Yoshimura, Wakabayashi et al. 2010). Mice bearing either of these genetic features in isolation develop somewhat variable colitis over a period of months (Spencer, Di Marco et al. 1998; Gorelik and Flavell 2000; Kang, Bloom et al. 2008). By contrast, mice that bear both genetic features, which we termed "dnKO mice", all spontaneously develop fulminant intestinal inflammation with pathologic hallmarks of severe human ulcerative colitis by 4-6 weeks of age (Kang, Bloom et al. 2008). To the extent of our knowledge, disease in this model is considerably more rapid and robust than in other spontaneous genetic models (Kang, Bloom et al. 2008). Co-housed littermate *Il10r2*$^{+/-}$ controls do not develop colitis, suggesting that any microbial triggers of colitis in dnKO mice are innocuous in exposed, non-susceptible hosts. Disease in dnKO mice is characterized by elevated Th1 serum cytokines, is partially ameliorated by neutralization of the cytokines TNFα and interferon-γ, and can be induced in *Rag*$^{-/-}$ recipient mice by adoptive transfer of CD4+ T-cells from dnKO donors (Kang, Bloom et al. 2008).

The aim of my doctoral research has been to determine whether microbial factors are important for the etiology of colitis in dnKO mice and, if so, to identify specific bacteria that play a role in disease induction. Chapter 2 of this thesis describes experiments demonstrating that colitis in dnKO mice is completely responsive to medically relevant antibiotic treatment and suggesting that disease may be due to a specific subset of anaerobic bacteria. Chapter 3 describes development of an experimental system to test the disease-inducing potential of various commensal

microbes, fulfillment of host-genotype-specific Koch's postulates for a number of commensal *Bacteroides* isolates in dnKO mice, and demonstration that although commensal Enterobacteriaceae are greatly enriched during spontaneous disease, an Enterobacteriaceae isolate is not itself sufficient for disease induction. Chapter 3 also addresses similarities and differences between our findings in dnKO mice and results from other animal models with human-relevant IBD-susceptibility mutations and proposed both experimental criteria and a conceptual framework for further elucidating commensal microbial influences on IBD etiology. Chapter 4 discusses potential future directions arising from these findings.

Chapter 2:

The dnKO mouse model of fulminant IBD is highly antibiotic responsive

INTRODUCTION

Many animal models of IBD are highly dependent on the intestinal microbiota (see Chapter 1) (Horwitz 2007). The clearest and most definitive demonstration of microbiota dependency has involved re-deriving animal models into germ-free facilities and demonstrating the absence of disease. Prominent examples in which this strategy has prevented intestinal disease include spontaneous genetic models such as HLA-B27 transgenic rats (Taurog, Richardson et al. 1994), IL-10 deficient mice (Sellon, Tonkonogy et al. 1998), T cell receptor-αβ-deficient mice (Dianda, Hanby et al. 1997), and TRUC (*Tbet*$^{-/-}$ x *Rag*$^{-/-}$) mice (Garrett, Gallini et al. 2010). Germ-free derivation also greatly mitigated colitis development in IL-2 deficient mice (Schultz, Tonkonogy et al. 1999). Germ-free re-derivation has also prevented or mitigated intestinal inflammation in a number of inducible colitis models that are sometimes used to model IBD, including carrageenan treatment of guinea pigs (Onderdonk, Hermos et al. 1977), dextran sodium sulfate (DSS) treatment of mice (Pull, Doherty et al. 2005), and transfer of $CD4^{+}CD45Rb^{high}$ T cells into *Rag*$^{-/-}$ recipient mice (Mazmanian, Round et al. 2008).

These and similar experiments have been of foundational importance, and germ-free re-derivation may be regarded as a “gold standard” in demonstrating the importance of microbial contributions to disease in a given animal model. However due to both practical reasons and a desire to more accurately simulate the situation in human IBD patients, there is also strong interest in exploring the role of intestinal microbe in animal models through use of antibiotics. Antibiotic treatment has proven efficacious to some extent in a wide variety of animal colitis models including HLA-B27 transgenic rats

(Rath, Schultz et al. 2001), IL-10 deficient mice (Hoentjen, Harmsen et al. 2003), TRUC (*Tbet*$^{-/-}$ x *Rag*$^{-/-}$) mice (Garrett, Lord et al. 2007), DSS-treated mice (Rakoff-Nahoum, Paglino et al. 2004), and DSS-treated, murine norovirus-infected, *Atg16L1* hypomorphic mice (Cadwell, Patel et al. 2010). The antibiotics used have varied from study to study, but a common theme in the majority of models has been that antibiotics with anaerobic coverage, particularly metronidazole, are partially or entirely efficacious. Some studies have distinguished between the ability of antibiotics to prevent disease before it becomes established and to therapeutically treat disease after it has already developed (Rath, Schultz et al. 2001; Hoentjen, Harmsen et al. 2003), a question with importance both for understanding disease pathogenesis and for potential relevance to medical therapies.

We assessed whether microbial factors were important in the dnKO mouse model of fulminant, rapid-onset IBD by treating mice with metronidazole and ciprofloxacin (Kang, Bloom et al. 2008). We found that disease was highly antibiotic-responsive, with antibiotics completely preventing colitis by a variety of semi-quantitative and quantitative metrics. We also found that treatment with metronidazole and ciprofloxacin could therapeutically reverse even severe colitis, reproducing classic features of quiescent IBD in treated animals. In smaller-scale experiments we further found that metronidazole monotherapy was partially efficacious in preventing disease but did not appear to be therapeutically efficacious in established disease, while ciprofloxacin monotherapy did not appear to have either preventive or therapeutic efficacy.

METHODS AND RESULTS

Generation of dnKO mice (developed by Silvia S. Kang and Paul M. Allen)

Dominant negative TGFβRII mice (supplied by Richard A. Flavell) (Gorelik and Flavell 2000) and CRF2–4-deficient (IL-10R2$^{-/-}$) mice (Genentech) (Spencer, Di Marco et al. 1998) were on the C57BL/6 background. All mice used in these experiments were bred and housed in the specific pathogen-free barrier facility on the 7th floor of the Clinical Sciences Research Building at Washington University. Animal protocols were reviewed and approved by the Washington University animal studies committee. The *Crf2–4*$^{-/-}$ mice lack the IL-10R2 receptor protein and are referred to as *Il10r2*$^{-/-}$ in this report. The dominant negative TGFβRII mice (referred to as *dnTgfβrii*) express a dominant negative TGFβRII solely in the CD4 and CD8 compartment. Mice that were unresponsive to IL-10R2 signaling in all compartments and TGFβ signaling specifically in the T cell compartment were generated by breeding *dnTgfβrii* mice (hemizygous) with *Il10r2*$^{-/-}$ mice to yield a novel strain of mouse, *dnTGFβRII* (hemizygous) × *Il10r2*$^{-/-}$, henceforth referred to as dnKO mice. The breeding scheme involved mating *dnTGFβRII* (hemizygous) × *Il10r2*$^{+/-}$, mice with *Il10r2*$^{-/-}$ mice to generate four genotypes of littermates in equal Mendelian ratios: dnKO, *Il10r2*$^{-/-}$, *dnTGFβRII* (hemizygous) × *Il10r2*$^{+/-}$, (referred to as *dnTGFβRII*), and *Il10r2*$^{+/-}$ mice (which phenotypically resemple wild-type animals).

Antibiotic treatment blocks development of fulminant disease in dnKO mice

At 2 weeks of age, the intestines of dnKO mice appeared normal both macroscopically and microscopically (Kang, Bloom et al. 2008). This observation indicated that general intestinal architecture development in these mice was not perturbed at this time. Disease induction was rapid and could be detected by 3 weeks of age, a time commensurate with known changes in the microbial ecology of the mammalian intestine that occur at the weaning–suckling transition(Palmer, Bik et al. 2007). We therefore explored the role of intestinal microbes at or after the suckling-weaning transition (3 weeks of age).

To examine whether bacteria influence disease development in the dnKO model, we treated mice with broad-spectrum antibiotics in drinking water. Beginning at 24 d of age, mice received drinking water containing high-dose ciprofloxacin and metronidazole, two commonly used antibiotics that are broadly active against aerobic and anaerobic bacterial species, respectively (Perencevich and Burakoff 2006). Mice received drinking water containing 0.66 mg/ml ciprofloxacin (Ahmed, van Vianen et al. 2003) and 2.5 mg/ml metronidazole (Sigma) (Roach, Wallis et al. 1988) beginning at 24 d of age. Previous reports (Rath, Schultz et al. 2001) have suggested occasional refusal of mice to drink water that contains antibiotics, so we included 20 mg/ml sugar-sweetened grape Kool-Aid Mix (Kraft Foods) in the water to encourage consumption. The antibiotic solution was passed through a sterilizing, 0.22-μm, nonpyrogenic cellulose acetate filter (Corning) before delivery to mice and was replaced with freshly prepared solution two to three times per week.

Antibiotic treatment blocked development of fulminant disease in treated dnKO with a high degree of efficacy as assessed by survival and weight gain. Mice were weighed every 1-2 days using a portable electronic Ohaus scale (VWR International). Weights were recorded to a tenth of a gram. Individual mice died or were humanely killed when their weight reached ≤ 70% of their maximal weight and 45-day survival was analyzed by the Kaplan-Meier method. Untreated mice rapidly developed fulminant, fatal disease (45-day survival = 10.5%; median survival = 35 days). In striking contrast, antibiotic-treated dnKO mice exhibited 100% 45-d survival (Figure 2.1), a highly significant difference ($p < 0.0001$). We were thus able to compare the growth of treated dnKO mice with treated littermate controls (*Il10r2*$^{-/-}$, *dnTGFβRII*, and *Il10r2*$^{+/-}$ combined) by measuring their weights over a 3-wk treatment period (Figure 2.2). Both groups of mice showed weight gain over the course of the experiment, and no statistically significant difference in weight gain was observed between the dnKO and control groups ($p = 0.105$). These results indicated that antibiotic treatment effectively blocked fulminant disease.

Antibiotic treatment blocks colonic gross pathology in dnKO mice

We next assessed development of intestinal pathology in antibiotic-treated dnKO mice. Disease severity was assessed using a gross pathology scoring system previously validated in this model (Kang, Bloom et al. 2008). Upon sacrifice, mice were euthanized and ceca and colons were immediately removed, flushed with PBS and Bouin's fixative (70% picric acid / 25% formaldehyde [37%] / 5% glacial acetic acid), and opened

longitudinally with scissors (ceca were opened from the ileocecal junction to the cecal tip along the greater curvature). The intestines were then pinned open mucosa-upward in square Petri dishes filled with wax (Carolina Biological), fixed in Bouin's fixative for 4-8 hours at 4°C, washed and stored in 70% ethanol. Whole mount images of fixed intestines were taken at 7X (cecum only), 20X, 40X, and 90X magnification. Images were scored for gross pathology according to a previously validated scoring system (Kang, Bloom et al. 2008) by an anatomic pathologist (T.S.S.) blinded to the identity of the samples (0 = no disease, 3 = severe disease; representative images of ceca with different degrees of disease severity are shown in Figure 2.3).

Intestinal gross pathology was assessed in a cohort of age-matched, 4 week old dnKO and *Il10r2*$^{+/-}$ mice that were either left untreated or were treated with antibiotics beginning at weaning (age 3 weeks). Consistent with previous observations (Kang, Bloom et al. 2008), we observed development of severe intestinal pathology in the cecum and transverse colon of untreated dnKO but not *Il10r2*$^{+/-}$ mice. However antibiotic treatment completely blocked pathology development in treated dnKO mice. The differences among groups were highly statistically significant ($p = 0.0033$ in both the cecum and transverse colon by Kruskal-Wallis test), although the groups were underpowered for individual pairwise comparisons. Similar results were obtained in dnKO mice treated with antibiotics for longer time periods (not shown).

Antibiotic treatment blocks colitis development in dnKO mice

We next assessed the effects of antibiotic treatment on histologic colitis in dnKO mice. Intestines were encased in 2% agar, turned on their sides (perpendicular to plane of microtome), paraffin embedded, cut in 5-μm sections, and stained with hematoxylin and eosin (H&E) at the Developmental Biology Department histology core (Washington University, St. Louis, Missouri, United States). Colonic histology of untreated dnKO mice exhibited severe mucosal inflammation with features including crypt abscessess, mucosal thickening, inflammatory infiltrates, epithelial hyperproliferation, crypt dropout, and other features characteristic of human ulcerative colitis (Figure 2.5) (Kang, Bloom et al. 2008). By contrast, colons of antibiotic-treated dnKO mice and both treated and untreated *Il10r2*$^{+/-}$ mice showed no histologic evidence of colitis (Figure 2.5).

To better quantify disease, we measured increases in crypt height and crypt width, hallmark features of the epithelial response to colonic inflammation which we have previously shown to be highly sensitive and precise metrics of disease (Kang, Bloom et al. 2008). Blinded microscopic analysis of H&E-stained histologic samples for height and width of well-oriented crypts in the transverse colon, and distal 0.5 cm of the rectum was performed at 400X magnification. By these metrics untreated dnKO mice were highly significantly more colitic than all other groups including antibiotic-pre-treated dnKO mice, which did not differ from *Il10r2*$^{+/-}$ controls (Figure 2.6). Thus, antibiotic treatment with metronidazole and ciprofloxacin completely blocked colitis development in dnKO mice.

Antibiotic treatment prevents mortality and reverses weight loss in severely colitic dnKO mice.

Given the ability of metronidazole and ciprofloxacin combination therapy to prevent colitis in dnKO mice, we next asked whether these antibiotics would also have a therapeutic role in treated established disease. To assess treatment efficacy, we allowed a group of 5 dnKO mice to age and become phenotypically sick before initiating treatment. Mice aged ≥28 days were begun on metronidazole and ciprofloxacin combination therapy if they fell below 90% of their maximum and had lost weight on 3 consecutive days or if they fell below 80% of their maximum body weight. (All mice were aged 28-32 days at beginning of treatment.) Two untreated age-, sex-, and weight-matched dnKO mice retrospectively identified from previous survival experiments were selected as controls for each antibiotic-treated mouse (total of 10 untreated control mice). Mice were weighed daily and sacrificed according to humane guidelines as described above.

We found that treatment with metronidazole and ciprofloxacin reversed even advanced disease in dnKO mice. Antibiotics resulted in significantly improved survival and weight gain (Figure 2.7). Whereas untreated matched control mice had a median survival of just 5 days, the treated mice exhibited significantly enhanced survival ($p = 0.016$, logrank test) and 3 out of 5 exhibited substantial and prolonged weight gain (Figure 2.7). Of the two treated mice that succumbed, one died within the first day of treatment (presumably before antibiotics could take effect; pathology not analyzed). The second survived longer than all untreated control mice before being sacrificed for humane reasons due to weight loss after 17 days of treatment. However its cecum and colon were

subsequently observed to be non-colitic, suggesting the weight loss occurred for extra-intestinal reasons.

Antibiotic treatment of severely colitic dnKO mice reverses mucosal thickening and ulceration and induces unusual gross mucosal characteristics

We next assessed the effects on the colonic mucosa of antibiotic treatment in severely colitic mice. Healthy colonic mucosa, such as that present in *Il10r2*$^{+/-}$ mice (Figure 2.8 A) or in dnKO mice treated with antibiotics prior to colitis development (Figure 2.8 B) has a relatively even mucosal surface with regular, densely packed crypts with circular openings. In severe colitis (Figure 2.8 C), colonic mucosa undergoes ulceration and thickening with crypt dropout (loss of many crypts), development of thickened and dysmorphic crypt openings, and occasional formation of pseudopolyps (areas of residual mucosa surrounded by ulceration). The colons of successfully treated, previously colitic dnKO mice were less thickened than colons of untreated dnKO mice (if thickened at all) and were not ulcerated, but they were grossly dysmorphic due to the residual damage from inflammation (Figure 2.8 D to F). They exhibited fewer crypts per unit area (a phenomenon known as atrophy (Riddell, Goldman et al. 1983)), less even surface, and residually dysmorphic crypt openings. Thickening of the colon in Figure 2.8 E and F is completely reversed and many areas are evident which are completely lacking in crypts although they are not ulcerated. The remaining crypts are clustered in small “islands” (usually two crypts together in a doublet), suggestive of post-colitic crypt

fission (division). All of these features are suggestive of healing mucosa still exhibiting the after-effects of severe colitis.

Antibiotic treatment of severely colitic dnKO mice reverses inflammation and induces mucosal characteristics with classic features of quiescent IBD

Remarkably, we found that antibiotic treatment in severely colitic mice produced the stereotypical histologic hallmarks of quiescent colitis (quiescent IBD). Figure 2.9 shows images of the colon of formerly colitic mice rescued with antibiotics. Colitis has largely disappeared, with reduced inflammatory infiltrates, little or no hyperplasia, and signs of healthy epithelial cells including mature, polarized surface epithelium and a relatively normal census of mature goblet cells. However the mucosa remains severely dysmorphic structurally with distorted branching and budding crypts – some showing separation from the muscularis mucosae – and widespread crypt dropout (atrophy). There are also epithelium-lined cyst-like structures that may represent the sites of former crypt abscesses. Colons from formerly colitic mice thus exhibit the classic features of quiescent colitis or quiescent IBD, which is seen in IBD patients whose colitis is in remission (Riddell, Goldman et al. 1983).

Metronidazole monotherapy provides survival benefit to dnKO mice before disease develops, but metronidazole treatment in established disease or ciprofloxacin alone does not.

We next asked whether metronidazole or ciprofloxacin played a more important role in colitis treatment in dnKO mice by treating with each of them individually. We treated mice either at weaning (21 days of age) or at ≥24 days of age. We observed that there were substantial survival benefits for mice treated with metronidazole at weaning, but not for any of the other groups ($p < 0.0001$). Although sample sizes in most groups were small and have not been repeated, the results are strongly suggestive that metronidazole treatment is of primary importance early in disease, although combination therapy may be more efficacious and appears required later in disease. Given the microbial species targeted by metronidazole (Perencevich and Burakoff 2006), these results suggested that obligate anaerobes might play a crucial role in disease induction in dnKO mice.

DISCUSSION

In many animal models of spontaneous IBD, disease is known to be microbiota dependent (Horwitz 2007). Antibiotic treatment has been one important tool for examining microbiota dependence of disease. Here we examined the effects of treatment with the antibiotics metronidazole and ciprofloxacin on colitis in the medically relevant dnKO mouse model of disease. We found that metronidazole and ciprofloxacin combination therapy were highly efficacious in completely preventing colitis in dnKO by

several metrics of disease and also efficacious in treatment of established disease. Importantly, mice successfully treated after development of severe disease showed classic colonic epithelial features seen in human patients with quiescent IBD, which may be important precursors to IBD-associated colon cancer (Greenstein, Sachar et al. 1979; Riddell, Goldman et al. 1983; Itzkowitz 2003). We further found that metronidazole monotherapy was more efficacious than ciprofloxacin monotherapy in preventing disease, although neither appeared as efficacious as combination therapy.

This is not the first study to explore the efficacy of antibiotics in murine IBD (Hoentjen, Harmsen et al. 2003; Rakoff-Nahoum, Paglino et al. 2004; Heimesaat, Bereswill et al. 2006; Garrett, Lord et al. 2007). However previous studies have either relied on experimentally administered agents to induce inflammation or on mouse strains that develop less severe forms of spontaneous IBD in which treatment efficacy is primarily assessed on the basis of histology scores without additional important metrics such as survival, weight gain, gross pathology, serum cytokine levels, and T cell activation (Kang, Bloom et al. 2008). We are aware of no other models in which antibiotic treatment has produced clinical disease remission and histologic healing as dramatic as that which we observe in antibiotic-treated dnKO mice. Our findings have similarities to those in HLA-B27 transgenic rats, where metronidazole monotherapy had preventive efficacy but not therapeutic efficacy in established disease, although that study observed some preventive efficacy of ciprofloxacin as well (Rath, Schultz et al. 2001). Metronidazole and ciprofloxacin combination therapy was not assessed.

Although antibiotics, including ciprofloxacin and metronidazole, are sometimes used to treat patients with IBD, research regarding their efficacy remains inadequate. Yamada's Textbook of Gastroenterology comments on a "surprising absence of controlled data supporting the use of antibiotics in patients with Crohn's disease", (Stenson, Hanauer et al. 2009), although a meta-analysis demonstrated beneficial effects of broad-spectrum antibiotic treatment (Rahimi, Nikfar et al. 2006). To our knowledge, the utility of metronidazole and ciprofloxacin combination therapy in ulcerative colitis has not been adequately assessed (Sartor 2003; Gionchetti, Rizzello et al. 2006; Perencevich and Burakoff 2006), and recommendations vary regarding usage in patients with fulminant ulcerative colitis (Cuffari, Present et al. 2005; Baumgart and Sandborn 2007; Ho, Lees et al. 2007). However a recent randomized, double-blind, placebo-controlled trial of a 2-week course of broad-spectrum antibiotics (amoxicillin, tetracycline, and metronidazole) in ulcerative colitis patients found significant short- and long-term benefits in terms of both clinical disease and mucosal healing, with treatment providing greater benefit in patients with more active disease (Ohkusa, Kato et al. 2010). Our results suggest that carefully controlled studies examining the benefits of combined ciprofloxacin and metronidazole in ulcerative colitis may also be appropriate.

Our findings suggest not only that disease in dnKO mice is microbiota-dependent, but that it might depend on specific microbial factors. The antibiotic regimen we used does not sterilize the gut (Heimesaat, Bereswill et al. 2006; Swidsinski, Loening-Baucke et al. 2008), suggesting that a germ-free state is not required for complete blockade of disease. Furthermore, the observation that metronidazole monotherapy had preventive

effects suggests that disease may depend in significant proportion on anaerobic microbes in the gut, given the known action and coverage of metronidazole (Rath, Herfarth et al. 1996; Perencevich and Burakoff 2006). Based on the results described here, we hypothesize that disease in dnKO mice depends on a subset of commensal bacteria, likely obligate anaerobes, which are eliminated by antibiotic treatment.

Figure 2.1: Treatment with ciprofloxacin and metronidazole promotes survival in dnKO mice.

45-day survival of untreated dnKO mice (n = 19; 45-d survival = 10.5%; median survival = 35 d) and dnKO mice receiving metronidazole and ciprofloxacin in drinking water beginning at 24 days of age (n = 8; 45-d survival = 100%). Individual mice died or were humanely killed when their weight reached ≤ 70% of maximal weight. Survival was analyzed by the Kaplan-Meier method, and statistical significance of difference between groups is $p < 0.0001$, by log-rank test. Upward arrow, antibiotic treatment begun at age 24 d. (Kang, Bloom et al. 2008). Representative of multiple independent experiments. All statistical analysis was performed using Prism v3.02 (GraphPad Software) unless otherwise indicated. (Some survival data was generated by Silvia S. Kang).

Figure 2.1

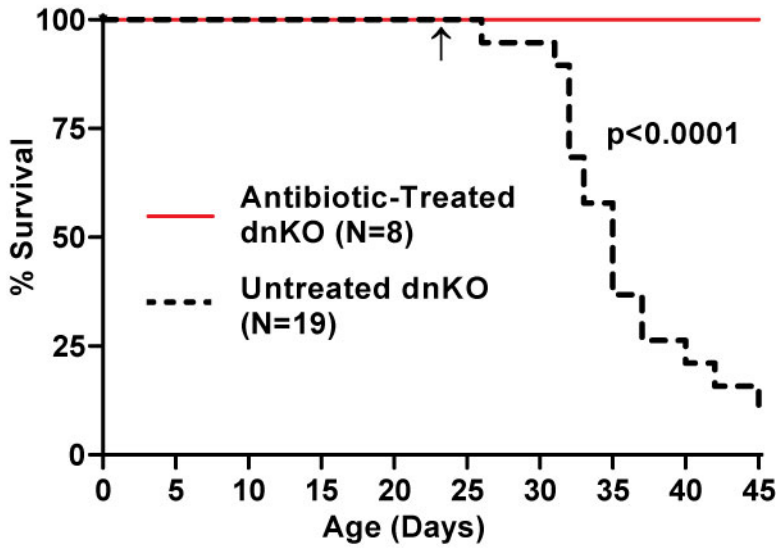
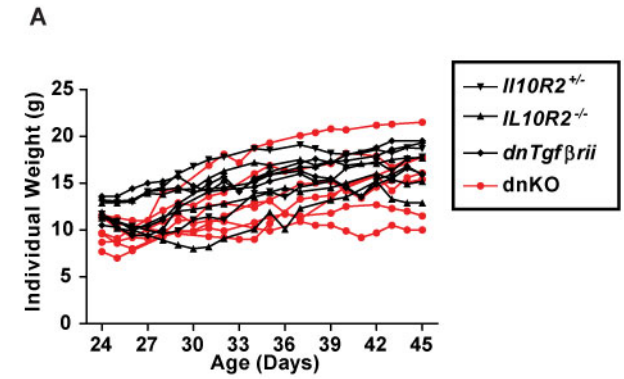
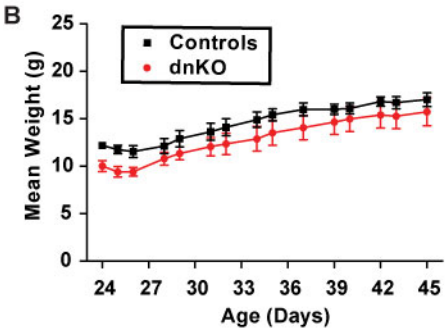

100
75
50
25
0
% Survival
0
5
10
15
20
25
30
35
40
45
Age (Days)
Antibiotic-Treated
dnKO (N=8)
Untreated dnKO
(N=19)
p<0.0001

Figure 2.2: Treatment with ciprofloxacin and metronidazole promotes weight gain in dnKO mice.

Weight gain of antibiotic-treated mice (n = 2 *Il10R2*$^{+/-}$, n = 3 *Il10R2*$^{-/-}$, n = 4 *dnTgfβrii*, and n = 7 dnKO) plotted individually (A) or as mean weights (B) of treated dnKO mice (n = 7) and treated controls (n = 9; non-dnKO genotypes combined) ± SEM. Data were pooled from three separate experiments using dnKO and littermate controls. To account for the longitudinal nature of the data, analysis of weight change over the course of treatment was performed using generalized estimating equations. The mice gained weight over the course of the experiment ($p < 0.001$), and the dnKO and control groups did not differ significantly ($p = 0.105$). Statistical analysis was performed by Michael J. Geske (Kang, Bloom et al. 2008). Representative of multiple independent experiments

Figure 2.2





Parameter	Estimate	p-value	sig.?
Age	0.295 (0.0401)	<0.0001	YES
Genotype	-1.693(1.0459)	0.105	NO

Figure 2.3: Scoring system for intestinal gross pathology.

Representative cecal whole mount samples imaged at 7X, 20X, 40X, and 90X magnification using an Olympus SZX12 dissecting microscope with an Olympus DP70 Digital Microscope Camera at 1360x1024 image size (Diagnostic Instruments). Samples were scored by an anatomic pathologist (T.S.S.) blinded to the identity of the samples according to a previously validated scoring system: 0, normal; 1, focal ulcers present; 2, ulcers and diffuse, mild mucosal thickening; and 3, ulcers and diffuse, severe mucosal thickening (Kang, Bloom et al. 2008). Samples in which different areas of the image most closely matched different scoring criteria were assigned the average of the two scores. 7X scale bar = 6 mm; 20X scale bar = 2 mm; 40X scale bar = 1 mm; 90X scale bar = 0.44 mm.

Figure 2.3

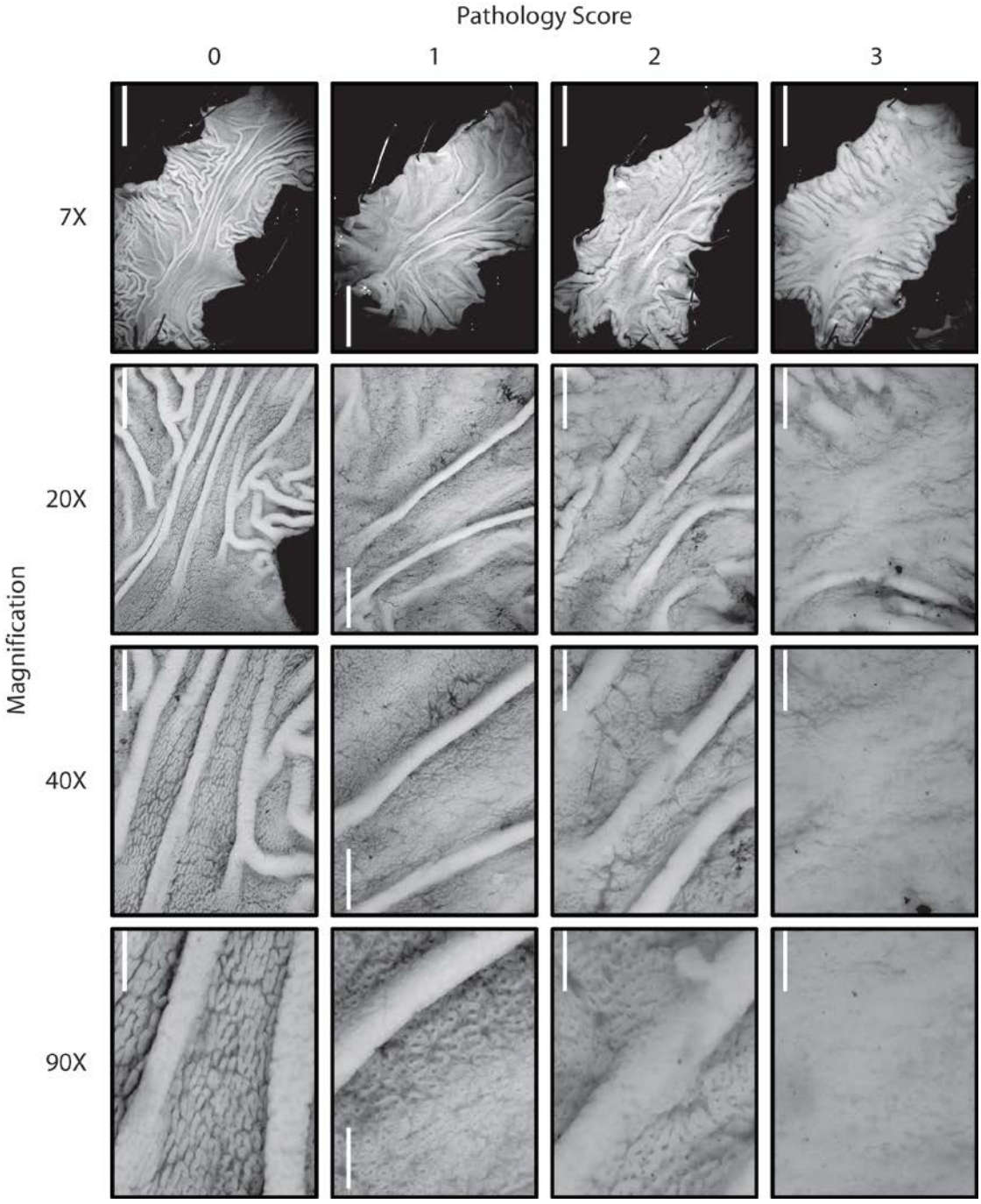


Figure 2.4: Treatment with ciprofloxacin and metronidazole blocks colonic gross pathology in dnKO mice.

Cecum and transverse colon pathology scores of 4-week-old untreated and antibiotic-treated *Il10r2*$^{+/-}$ and dnKO mice. Abx = antibiotics (metronidazole + ciprofloxacin in drinking water). Intestinal whole mounts were scored for gross pathology in a blinded fashion by an anatomic pathologist (T.S.S.) according to a validated system: 0 = no pathology, 3 = severe pathology (see Figure 2.3). Individual (squares) and median (bars) pathology scores are displayed. Kruskal-Wallis test:

(A) Cecum pathology score: $H_3 = 13.75$, $p = 0.0033$.

(B) Transverse colon pathology score: $H_3 = 13.75$, $p = 0.0033$.

Figure 2.4

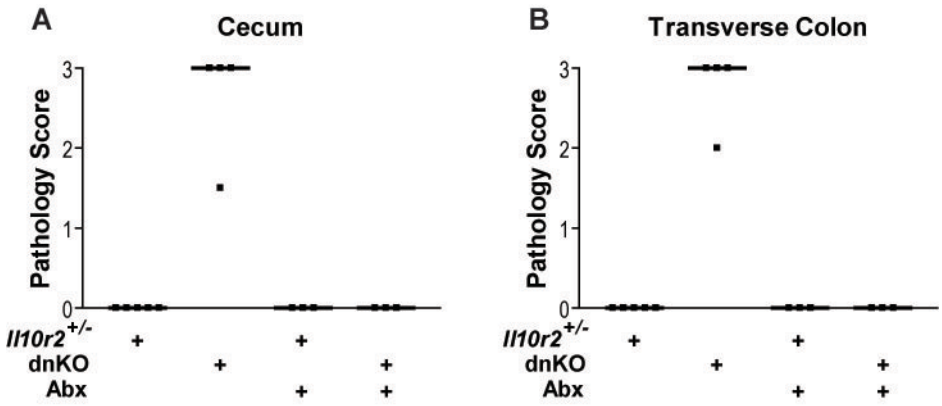

A
Cecum
Pathology Score
3
2
1
0
Il10r2+/-
dnKO
Abx
+
+
+
+
+
+
B
Transverse Colon
Pathology Score
3
2
1
0
Il10r2+/-
dnKO
Abx
+
+
+
+
+
+

Figure 2.5: Treatment with ciprofloxacin and metronidazole blocks colonic inflammation in dnKO mice.

Representative images of H&E stained rectal histology of 4-week-old untreated and antibiotic-treated *Il10r2*$^{+/-}$ and dnKO mice as described in Figure 2.4. Representative images of rectal histology (≤0.5 cm from the anorectal junction) were taken using an Olympus DP70 Digital Microscope Camera on an Olympus BX51 microscope. The rectum of the untreated dnKO mouse exhibits mucosal thickening, inflammatory infiltrates, epithelial hyperproliferation, crypt dropout, and other features characteristic of human ulcerative colitis (Kang, Bloom et al. 2008). The features of inflammation are absent from the rectum of the antibiotic-treated dnKO mouse. Scale bar = 100 μm.

Figure 2.5

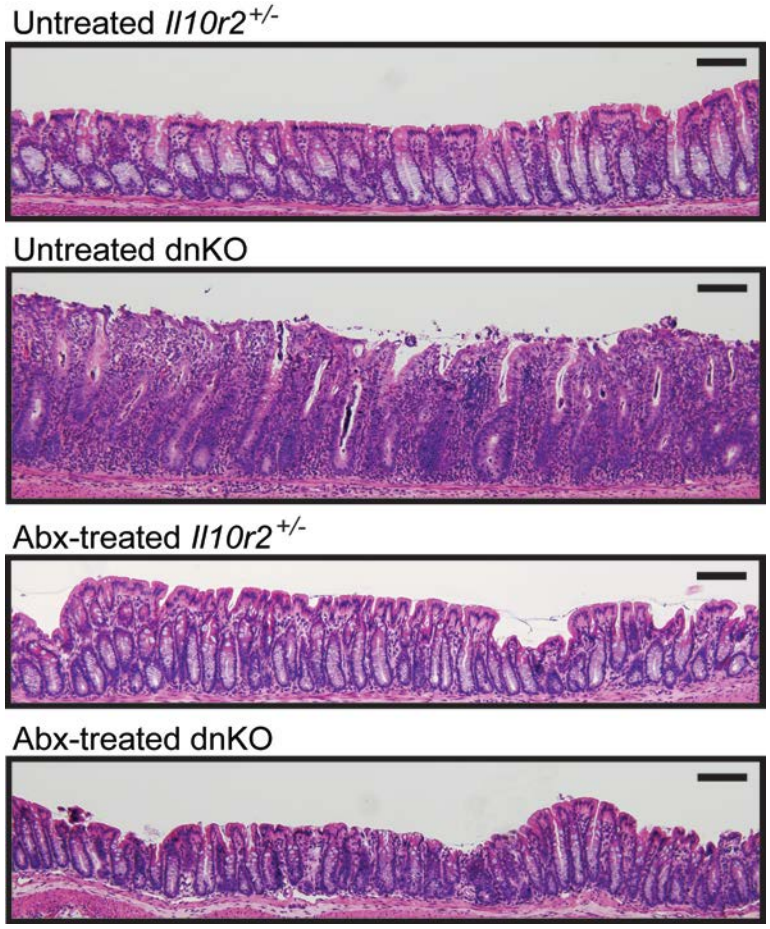

Untreated *Il10r2*$^{+/-}$
Untreated dnKO
Abx-treated *Il10r2*$^{+/-}$
Abx-treated dnKO

Figure 2.6: Treatment with ciprofloxacin and metronidazole prevents colitis in dnKO mice as assessed by quantitative metrics of the epithelial response to inflammation.

Heights and widths of well-oriented crypts from the transverse colons and rectums of 4-week-old untreated and antibiotic-treated *Il10r2*$^{+/-}$ and dnKO mice as described in Figure 2.4. Data displayed as mean +/- SEM. Statistical significance determined by 1-way ANOVA with post-hoc Tukey's test. All statistically significant pairwise comparisons are displayed: **, $p < 0.01$, ***, $p < 0.005$.

(A) Transverse colon crypt height: $F_{3,11} = 40.87$, $p < 0.0001$.

(B) Transverse colon crypt width: $F_{3,11} = 10.71$, $p = 0.0014$.

(C) Rectum crypt height: $F_{3,11} = 33.25$, $p < 0.0001$.

(D) Rectum crypt width: $F_{3,11} = 9.501$, $p = 0.0022$.

Figure 2.6

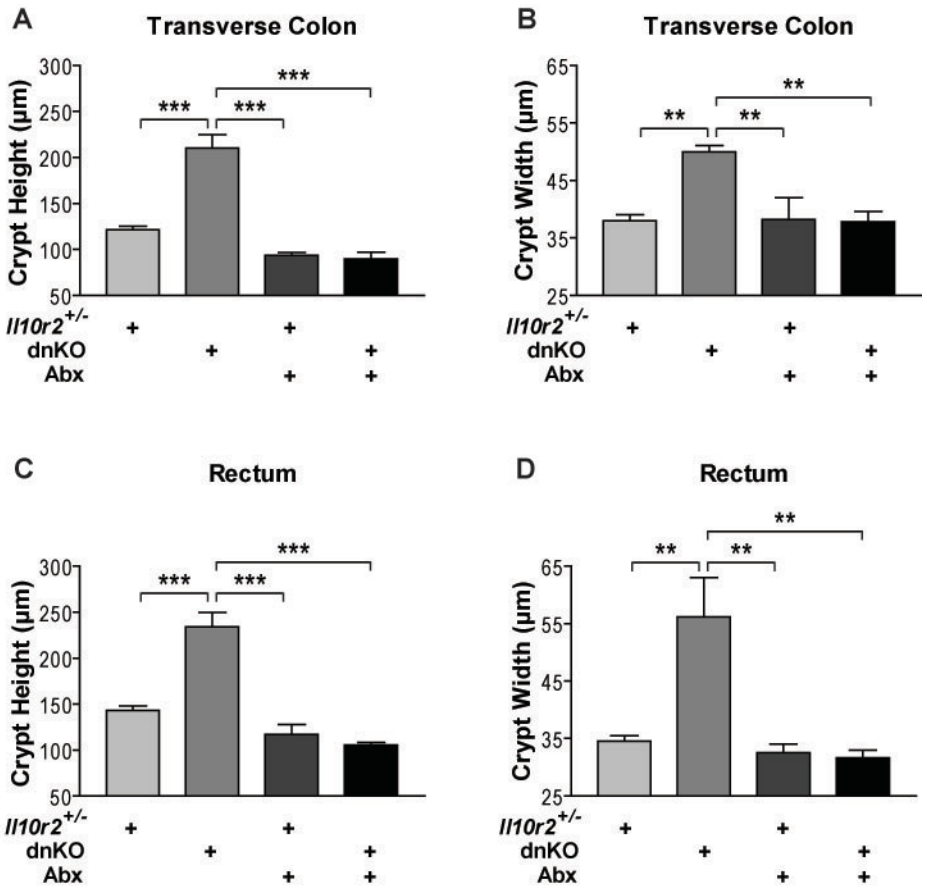

A
Transverse Colon
Crypt Height (µm)
300
250
200
150
100
50
***
***
***
Il10r2+/-
dnKO
Abx
B
Transverse Colon
Crypt Width (µm)
65
55
45
35
25
**
**
**
C
Rectum
D
Rectum

Figure 2.7: Treatment with ciprofloxacin and metronidazole prevents mortality and reverses weight loss in severely colitic dnKO mice.

dnKO mice (n = 5) treated with metronidazole + ciprofloxacin after developing severe disease (≥ 4 weeks of age) were each matched with two untreated age-, sex-, and weight-matched dnKO mice (n = 10 total untreated mice). Antibiotic treatment resulted in (A) significantly improved 25-day survival (p = 0.016, logrank test) and (B) weight gain as compared to controls. One antibiotic-treated mouse died during the first day of treatment; necropsy was not performed. A second antibiotic-treated mouse was sacrificed for humane reasons due to weight loss after 17 days of treatment and observed to be non-colitic. Data compiled from multiple independent experiments with 1-2 mice per group.

Figure 2.7

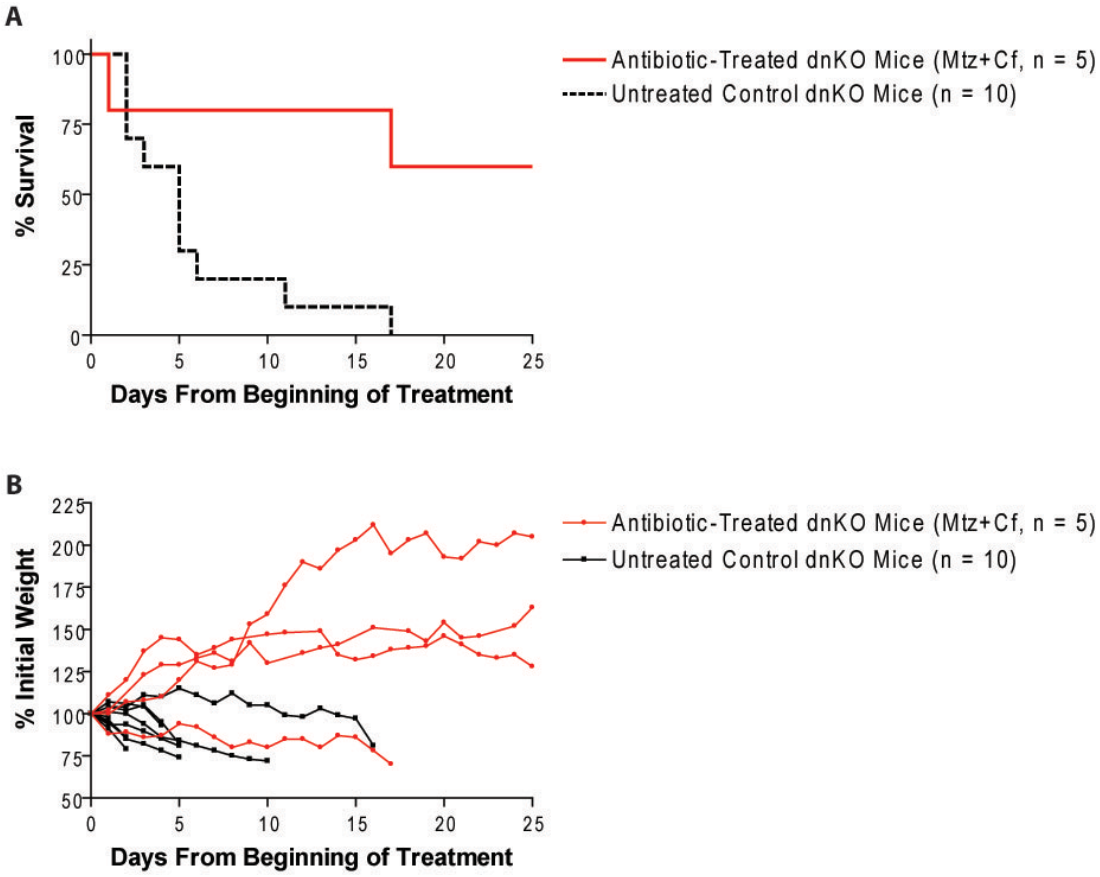

A
100
75
50
25
0
% Survival
0
5
10
15
20
25
Days From Beginning of Treatment
Antibiotic-Treated dnKO Mice (Mtz+Cf, n = 5)
Untreated Control dnKO Mice (n = 10)
B
225
200
175
150
125
100
75
50
% Initial Weight
0
5
10
15
20
25
Days From Beginning of Treatment
Antibiotic-Treated dnKO Mice (Mtz+Cf, n = 5)
Untreated Control dnKO Mice (n = 10)

Figure 2.8: Treatment with ciprofloxacin and metronidazole reverses established colitis in dnKO mice, producing grossly dysmorphic colonic mucosa with evidence of crypt dropout.

Whole mount images of descending colon mucosa.

(A) 4 week old *Il10r2*$^{+/-}$ and (B) dnKO mice treated with antibiotics before onset of severe disease (metronidazole + ciprofloxacin begun at 21 days of age). Colons exhibit even mucosal thickness with dense, regularly spaced, circular crypt openings.

(C) Descending colon from an untreated, severely colitic 4 week old dnKO mouse exhibiting areas of frank mucosal ulceration surrounding a pseudopolyp with evidence of crypt dropout (atrophy) and hyperplastic, grossly dysmorphic crypt openings.

(D to F) Descending colon mucosa from dnKO mice treated with antibiotics begun at ≥4 weeks of age (after development of severe colitis) and continued for ≥17 days.

(D) Colon exhibits no frank ulceration but has uneven mucosal thickness and residually dysmorphic crypt architecture due to prior disease.

(E) Colon is not thickened or ulcerated but exhibits severe diffuse crypt dropout as demonstrated by the reduced number of crypts per unit area, Pattern of crypts in doublets and “islands” suggests the occurrence of crypt fission.

(F) Magnified image of colon in (E), inset.

Bar = 1 mm.

Figure 2.8

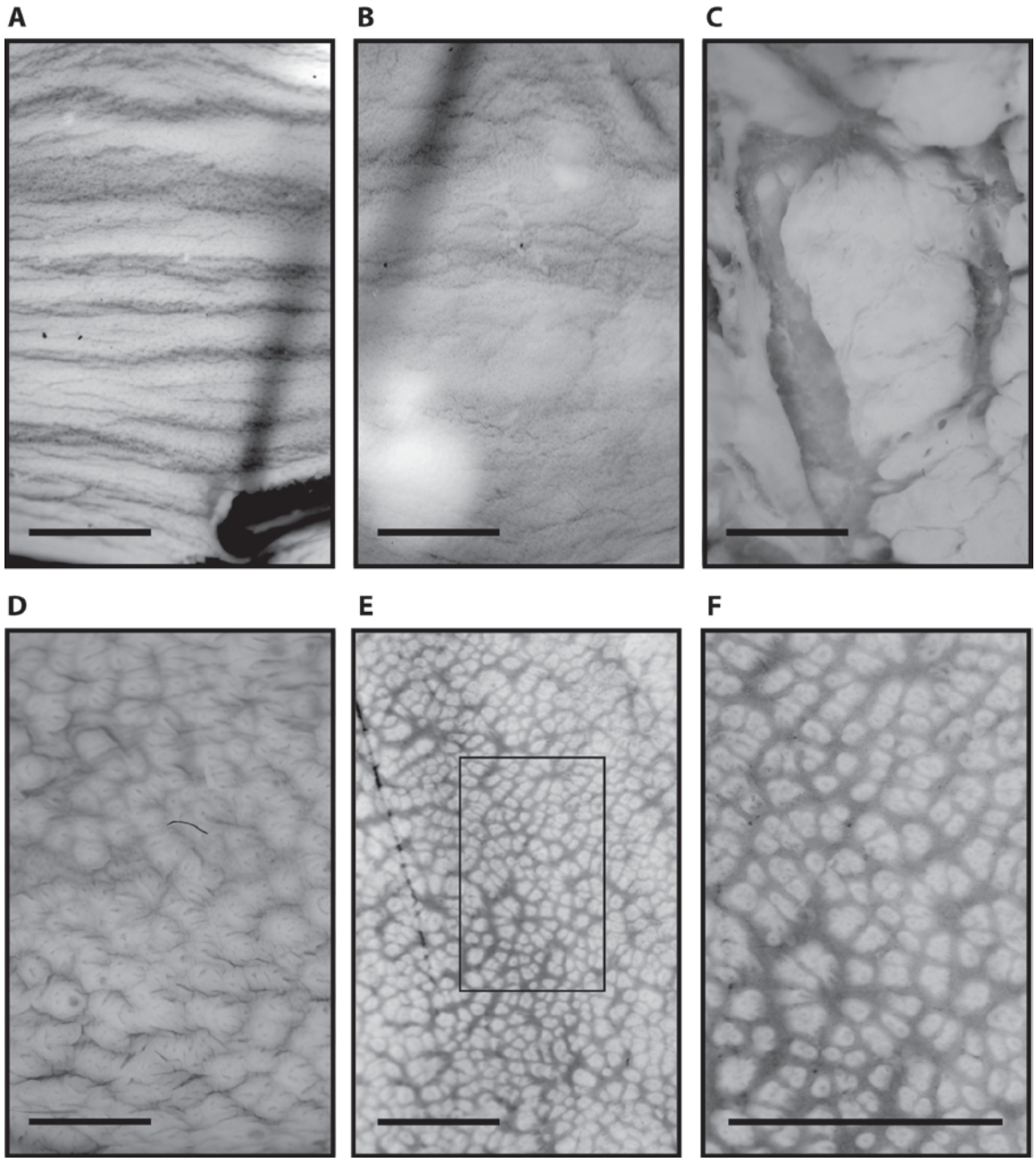


Figure 2.9: Treatment with ciprofloxacin and metronidazole reverses established colitis in dnKO mice, producing histologic hallmarks of quiescent ulcerative colitis. Images of H&E stained descending colon histology from 4-week old (A) *Il10r2*$^{+/-}$ and (B) dnKO mice treated with antibiotics before onset of severe disease (see Figure 2.8). Colons exhibit normal crypt architecture with absence of immune infiltrates and dense, regularly spaced, non-branching colonic crypts that extend to the muscularis mucosae. (C) Descending colon from an untreated, severely colitis 4-week old dnKO mouse exhibiting marked mucosal thickening, infiltration of inflammatory cells, ulceration, thinning of surface epithelium, epithelial hyperplasia in crypts, and crypt dropout. Images of (D-E) descending colon and (F) transverse colon from a dnKO mouse treated with antibiotics after developing severe colitis. Colon exhibits dramatic reductions in mucosal inflammation and crypt hyperplasia with mature surface epithelium and presence of goblet cells, especially in the transverse colon. Mucosa shows classical features of quiescent colitis including widespread crypt dropout (atrophy) and exhibit distorted, branching crypts (arrows), some of which are separated from the muscularis mucosae, and epithelium-lined cyst-like structures that may represent former crypt abscesses (Riddell, Goldman et al. 1983).

Figure 2.9

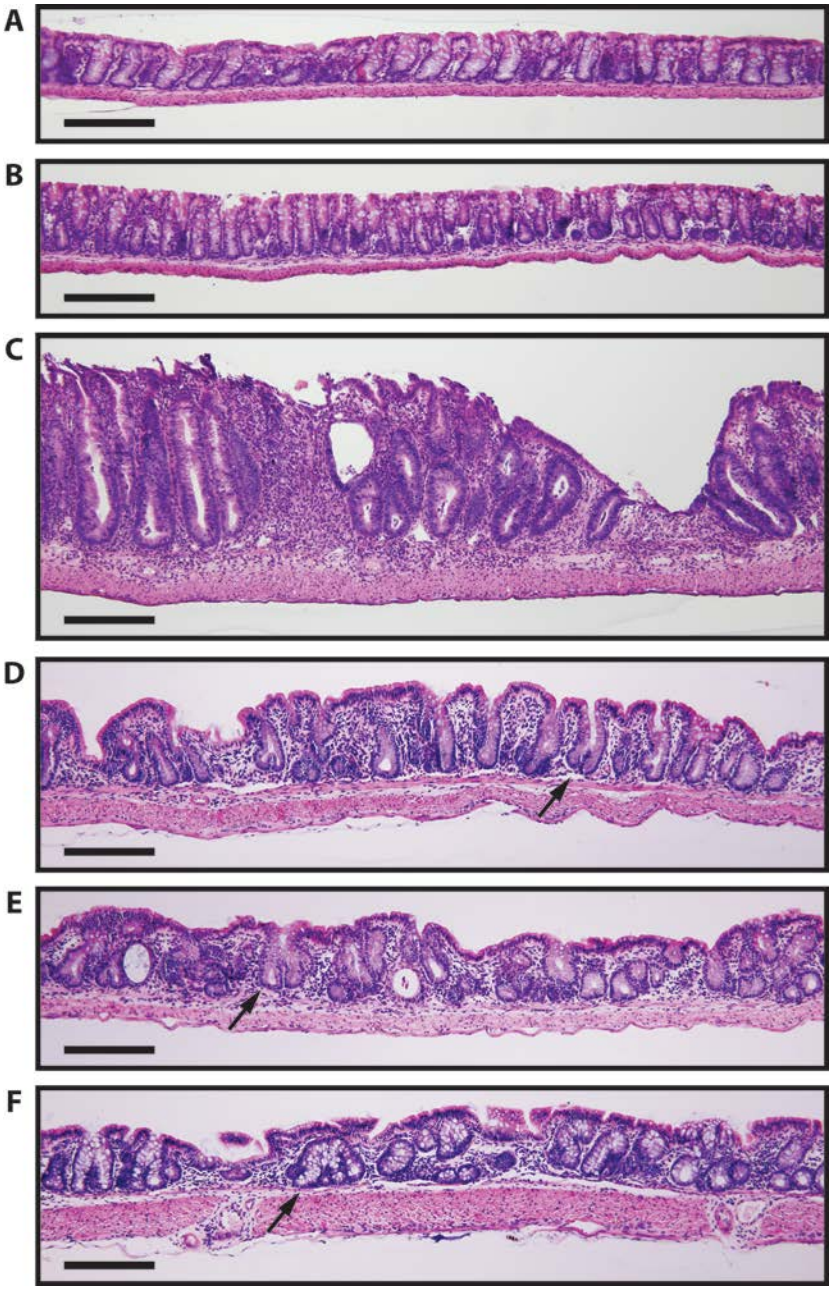
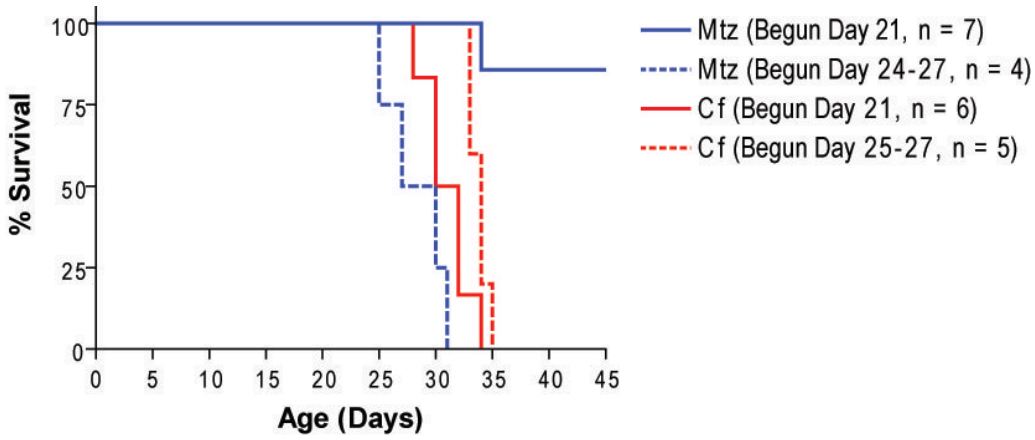


Figure 2.10: Metronidazole monotherapy begun at weaning provides survival benefit to dnKO mice, but metronidazole monotherapy begun later or ciprofloxacin monotherapy at either age does not.

45-day survival of dnKO mice treated with metronidazole or ciprofloxacin beginning at the indicated ages. Survival differences between groups are statistically significant ($p < 0.0001$, log-rank test). Survival data for metronidazole therapy begun at 21 days of age are representative of multiple independent experiments; other groups consist of compiled data from ≥2 experiments each with 1-3 mice per group.

Figure 2.10


100
75
50
25
0
% Survival
0
5
10
15
20
25
30
35
40
45
Age (Days)
Mtz (Begun Day 21, n = 7)
Mtz (Begun Day 24-27, n = 4)
Cf (Begun Day 21, n = 6)
Cf (Begun Day 25-27, n = 5)

CHAPTER 3

Commensal *Bacteroides* fulfill host-genotype-specific Koch's postulates in the dnKO mouse model of inflammatory bowel disease

INTRODUCTION

The commensal microbiota is widely recognized to play a critical role in the pathogenesis of inflammatory bowel disease (IBD) (Xavier and Podolsky 2007). But despite many decades of research (Winkelstein 1936; De Dombal 1968; Packey and Sartor 2009), there is no clear consensus on precisely how intestinal microbes induce IBD (Strober 2010). In particular, two related and fundamentally important questions remain unresolved: (1) is IBD induced by specific subsets of commensal bacteria and, if so, (2) can these subsets be identified based on disease-associated alterations in levels of colonization?

One approach to answering these questions has involved profiling the microbiota of affected individuals, a strategy recently revolutionized by the widespread availability of culture-independent mass sequencing that is an important ongoing focus of the Human Microbiome Project (Peterson, Frank et al. 2008; Peterson, Garges et al. 2009). Profiling studies have detected complex IBD-associated alterations in microbiota composition but have not found disease to be consistently associated with presence or absence of a specific microbe (Frank, St Amand et al. 2007; Takaishi, Matsuki et al. 2008; Packey and Sartor 2009; Frank, Robertson et al. 2010; Qin, Li et al. 2010). These findings have led some to theorize that community-wide alterations in the microbiota rather than specific microbiota elements may be responsible for disease (Garrett, Gordon et al. 2010), whereas others have suggested that groups of microbes which are enriched during disease likely play a pro-colitic role while microbes which are depleted may be anti-inflammatory (Sokol, Pigneur et al. 2008; Takaishi, Matsuki et al. 2008). But although

profiling studies have provided invaluable insights into the microbiota dynamics associated with disease, they have not directly addressed the question of causality (Frank, St Amand et al. 2007; Peterson, Garges et al. 2009). It thus remains an open question among many researchers whether fulfilling Koch's postulates for individual bacterial species is relevant to IBD and whether disease-associated alterations in microbial colonization are predictive of underlying disease etiology (Xavier and Podolsky 2007; Takaishi, Matsuki et al. 2008; Cerf-Bensussan and Gaboriau-Routhiau 2010; Garrett, Gordon et al. 2010).

Directly assessing disease-inducing roles of specific microbes in IBD in a reductionist fashion by applying Koch's postulates has proved challenging for several reasons (Burke 1997). Although progress has recently been made, there is a paucity of spontaneous animal models exhibiting rapid disease onset that bear human-relevant disease susceptibility mutations, with some models taking months to develop even relatively mild disease (Kang, Bloom et al. 2008). Furthermore, experiments using IBD-susceptible animal models have generally relied on gnotobiotic (germ-free) animals (Horwitz 2007; Sartor 2008). Gnotobiotic animal models have provided many foundational insights into the microbiota's influence on both homeostatic development of the immune system and on intestinal diseases (Mazmanian, Liu et al. 2005; Ivanov, Atarashi et al. 2009; Round and Mazmanian 2009). However it is not always clear how directly findings from these models translate to conventionally raised animals or to humans since many of the microbes tested have been human-derived isolates rather than rodent-adapted strains, there are well-documented examples of intestinal bacteria

inducing dramatically different disease phenotypes in gnotobiotic and conventionally raised hosts (Rhee, Wu et al. 2009; Garrett, Gallini et al. 2010), and gnotobiotic animals are immunologically underdeveloped, which could have important effects on patterns of immunopathology (Mazmanian, Liu et al. 2005; Atarashi, Nishimura et al. 2008; Ivanov, Atarashi et al. 2009; Round and Mazmanian 2009). The intestinal microbiota's vast diversity and variability (Turnbaugh, Quince et al. 2010) has posed a further challenge for identification of candidate disease-inducing bacteria, and specifically isolating and cultivating many intestinal bacteria has been prohibitively difficult (Savage 1977; Eckburg, Bik et al. 2005; Sartor 2008).

Despite these challenges, important progress has been made through screening of specific bacteria or defined bacterial mixtures for disease induction in gnotobiotic animal models. In most models, including *Il10*$^{-/-}$ mice (Kim, Tonkonogy et al. 2007), *Il2*$^{-/-}$ mice (Waidmann, Bechtold et al. 2003), TRUC (*Tbet*$^{-/-}$ x *Rag*$^{-/-}$) mice (Garrett, Gallini et al. 2010), and HLA-B27 transgenic rats (Rath, Herfarth et al. 1996; Rath, Wilson et al. 1999), certain bacteria or bacterial pools have been sufficient for disease induction whereas others have not, suggesting microbial specificity may be important in disease induction (Sartor 2008). Broader interpretation of findings is difficult since particular bacteria have produced different and sometimes apparently contradictory effects in different animal models (Rath, Wilson et al. 1999; Waidmann, Bechtold et al. 2003; Kim, Tonkonogy et al. 2007). Surprisingly, isolates of IBD-enriched bacteria from human patients have frequently failed to induce disease in gnotobiotic experiments whereas other bacteria have been colitogenic (Rath, Herfarth et al. 1996; Sellon, Tonkonogy et al.

1998) However as noted above there are several reasons why these results may not directly translate to human disease, including the fact that the isolates were not host-species-adapted. In a pioneering series of studies focusing on host-adapted bacteria, Onderdonk and colleagues isolated a panel of commensal bacteria from guinea pigs and screened them in gnotobiotic guinea pigs treated with the colitis-inducing molecule carrageenan. These studies identified *Bacteroides vulgatus* as crucial for disease pathogenesis (Onderdonk, Franklin et al. 1981; Onderdonk, Bronson et al. 1987; Onderdonk 2005). *B. vulgatus* also specifically induced colitis in gnotobiotic HLA-B27 transgenic rats (Rath, Herfarth et al. 1996; Rath, Wilson et al. 1999), but it did not induce disease in the more widely studied *Il10*$^{-/-}$ mouse model (Sellon, Tonkonogy et al. 1998) and was actually protective against colitis in *Il2*$^{-/-}$ mice (Waidmann, Bechtold et al. 2003). It is unclear which, if any, of these seemingly contradictory findings most realistically mimics the situation in human disease.

Based on our previous findings (Chapter 2), we hypothesized that disease in the antibiotic-responsive, rapid-onset, medically relevant dnKO mouse model of spontaneous IBD depends on a subset of commensal bacteria, likely obligate anaerobes, which are eliminated by antibiotic treatment. Here we use non-gnotobiotic methods to fulfill host-genotype-specific Koch's postulates for commensal *Bacteroides* species in dnKO mice. Based on screening experiments, we isolate commensal intestinal bacteria from our mouse colony, introduce them into antibiotic-pre-treated mice, assess disease development, and confirm host colonization by quantitatively re-isolating the experimentally introduced bacteria in culture. Using this approach, we show that

common commensal *Bacteroides* species colonize IBD-susceptible and non-susceptible hosts equivalently, but induce disease exclusively in susceptible animals. By contrast, we show that commensal Enterobacteriaceae are strikingly enriched during spontaneous disease, but a colitis-enriched Enterobacteriaceae isolate is not sufficient for disease induction despite robust colonization. We thus identify distinct commensal bacterial subsets with and without disease-inducing potential and show that their colitogenicity would not have been predicted based on disease-associated alterations in colonization.

METHODS AND RESULTS

Development of non-gnotobiotic methods to screen for disease-inducing microbes in antibiotic-pre-treated dnKO mice

We developed a system utilizing the dnKO model's rapid disease onset and exquisite antibiotic-responsiveness to screen commensal bacteria for colitis-inducing potential. Since antibiotic treatment was highly effective in blocking disease in dnKO mice (see Chapter 2), we hypothesized that mice pre-teated with antibiotics should remain disease-free after treatment cessation if isolated from microbial exposures but should develop disease if exposed to the microbiota of untreated mice. In initial experiments, mice were housed in our specific-pathogen free barrier facility on the 7th floor of the Clinical Sciences Research Building at Washington University. (Animal protocols for this an all subsequent experiments were approved by Washington University's animal studies committee.) In this facility, it is standard practice for individual cage components (cage bottoms, cage lids, wire racks, water bottles, and feed)

to be sterilized by autoclave or irradiation, stored in open air in the mouse facility, and assembled as necessary for use. The disinfectant Clidox®-S (Pharmacal Research Laboratories, Inc.) is used at a 1:18:1 concentration and each batch of disinfectant is used for several days before a fresh batch is made. For our experiments, we disinfected our hands and the exterior of all cages with Clidox®-S solution before handling mice. Cages containing mice are opened only in hoods with HEPA-filtered air.

In initial experiments, dnKO mice were pre-treated with antibiotics for 10 days with frequent transfer to freshly assembled cages and then removed from treatment. (All experiments in Chapter 3 were performed using combined metronidazole + ciprofloxacin treatment.) After treatment cessation, mice were either maintained in freshly assembled cages ("isolated", negative controls) or were co-housed with an untreated, non-dnKO littermate mouse in a cage containing that mouse's dirty bedding and were also smeared with 2 mL per mouse of a suspension of intestinal contents harvested from an untreated donor mouse and suspended in 10 mL of sterile PBS (Backhed, Ding et al. 2004). Mice were sacrificed after 7 days and colons were analyzed for pathology. Antibiotic-pre-treated dnKO mice (but not non-dnKO littermate controls) experimentally exposed to intestinal contents developed severe colitis with ulceration and shortening of the colon, but colons of isolated dnKO mice also showed some degree of mucosal thickening and inflammation (Figure 3.1 and not shown). These results confirmed that our approach could work in principle, but suggested that more stringent animal care practices would be required to ensure precision and reproducibility.

To enhance precision and reproducibility of mouse handling practices, we modified several experimental parameters. Instead of conventional caging, we began using "enhanced caging" that had been autoclaved after assembly and began rigorously decontaminating all surfaces with a stronger 1:8:1 solution of Clidox®-S prepared fresh daily. These techniques were previously used by the Virgin laboratory during discovery of murine norovirus (Karst, Wobus et al. 2003). We performed all animal care personally rather than allowing Division of Comparative Medicine technicians to handle the cages. We also began using enhanced contact precautions, donning an extra pair of gloves and isolation gown and changing these outer layers after handling mice or cages from each experimental group. Additionally, we began pre-treating mice with antibiotics for ≥3 weeks instead of 10 days prior to treatment cessation to ensure that no residual antibiotic-sensitive organisms remained. These enhanced animal care practices proved highly effective (see below).

Development of a standardized stock of intestinal contents

The human intestinal microbiota varies considerably from individual to individual in terms of both the bacterial species present and their relative proportions (Turnbaugh, Quince et al. 2010). We performed a qualitative experiment to examine microbiota variability between mice. Age-matched, untreated male mice (non-dnKO genotypes) were co-housed beginning at weaning (age 3 weeks). After ~3 weeks of co-housing, mice were sacrificed on each of two successive days (2 mice per day). Cecal contents were collected, pooled, serial diluted, and dilutions were cultured aerobically on chocolate

agar. The striking day to day differences in relative frequency of different colony types qualitatively illustrated that even in co-housed mice there was a high degree of microbiota variability.

To establish a reproducible source of intestinal microbes for subsequent experiments, we adapted an approach that was successfully employed in an experimental model of intra-abdominal abscesses to characterize the pathogenic contributions of various bacteria in a complex polymicrobial mixture and has also been used in experiments with “humanized” gnotobiotic mice (Onderdonk, Weinstein et al. 1974; Weinstein, Onderdonk et al. 1974; Turnbaugh, Ridaura et al. 2009). Ceca were harvested from untreated non-dnKO mice from the dnKO colony and transferred to the anaerobic chamber where contents were removed, suspended in sterile, pre-reduced PBS + 20% glycerol, and frozen at -80°C in single-use aliquots in 1.8 mL CryoTube Vials (377267, Nunc). Prior to freezing, an aliquot of the mixture was titered on non-selective anaerobic and aerobic media. Subsequently, frozen aliquots were thawed, adjusted to the same concentration of total cecal material, and titered on the same media types. We observed some loss of viability in the frozen stock, with greater loss among total anaerobic bacteria than aerobes (Table 3.1).

Sensitive, quantitative system to screen for disease-inducing microbes in antibiotic-pre-treated dnKO mice

Using the methodologic improvements described above, we validated our non-gnotobiotic screening system for disease-inducing microbes in antibiotic-pre-treated

dnKO mice. dnKO mice housed under enhanced animal care conditions (see above) were pre-treated with antibiotics for ≥3 weeks with frequent transfers to fresh sterile cages to eliminate residual antibiotic-sensitive bacteria from their environment. Two days after treatment cessation, the mice were orogastrically gavaged with sterile PBS or freshly thawed aliquots of intestinal contents from untreated animals (Table 3.1, Figure 3.3). Mice gavaged with intestinal contents but not PBS developed severe disease that was fatal in some cases (Figure 3.3). Examination of colons demonstrated that intestinal contents induced severe colonic gross and microscopic pathology resembling that seen in untreated, spontaneously colitic dnKO mice (Figures 3.4 to 3.6). Colitis induction was significant by all quantitative metrics compared to PBS-gavaged controls, which did not exhibit signs of colitis (Figure 3.4 to 3.6). Gavaging freshly harvested intestinal contents produced similar outcomes as gavaging frozen material (not shown). These results supported the hypothesis that colitis induction in dnKO mice was microbe-dependent, suggested that freezing did not eliminate all colitigenic microorganisms, and demonstrated the feasibility of our method as a sensitive and quantitative system to screen bacteria for disease-inducing potential.

Colitis-inducing microbes can be grown in mixed cultures of cecal contents

Since certain members of the colonic microbiota cannot be cultured(Savage 1977; Eckburg, Bik et al. 2005; Duncan, Louis et al. 2007), we conducted a proof-of-principle experiment to assess whether we could culture colitigenic microorganisms. Intestinal contents freshly harvested from untreated, non-dnKO donors were cultured anaerobically

on ANB agar for 48 hours or aerobically on chocolate agar for 24 hours. Cultures were removed from culture plates using sterile swabs, suspended in sterile, pre-reduced PBS to an optical density of 4 McFarland standards. Groups of antibiotic-pre-treated mice were gavaged with sterile PBS, the aerobic culture alone, or a combination of the aerobic and anaerobic cultures mixed in equal proportions. One mouse receiving the combination of aerobic and anaerobic cultures lost >30% of maximum weight and was sacrificed 5 days post-gavage; all others survived to the end of the three-week experiment. Neither survival nor weight loss differed significantly between the two groups ($p = 0.5220$ and $p = 0.4736$; Figure 3.7 A and C respectively). However we observed development of severe colitis in mice gavaged with the combination of aerobic and anaerobic cultures that was significant relative to both PBS gavage and to gavage of aerobic cultures alone ($p < 0.01$; Figures 3.7 B and 3.8). By contrast, gavage of aerobic culture alone did not induce significant disease ($p > 0.05$; Figure 3.7 B). These results, which were remniscent of findings in gnotobiotic HLA-B27 rats (Rath, Schultz et al. 2001), suggested that colitis-inducing bacteria could be grown in culture, but aerobic or facultatively anaerobic bacteria were likely not sufficient for disease induction.

This experiment also demonstrated dissociation between weight post-gavage and intestinal pathology. Although short-term antibiotic treatment blocks pro-inflammatory cytokine secretion and promotes weight gain in dnKO mice (Kang, Bloom et al. 2008), we have observed that dnKO mice treated for longer periods exhibit sporadic, variable weight loss despite remaining colitis-free (not show). Using weight as a disease metric was also problematic due to the complex breeding scheme of compound heterozygotes

used to produce dnKO mice, which ensured a constant scarcity of mice and therefore necessitated using both males and females animals from multiple litters which were not perfectly age-matched. Although neither age nor sex differences appeared to affect colitis development, they naturally produced a degree of heterogeneity in weights. In all subsequent experiments, we therefore relied on colon-specific metrics of colitis rather than weight loss or serum cytokines to ensure results were not confounded by extra-intestinal processes.

Techniques for isolation and identification of aerobic and anaerobic bacteria from cecal mixed cultures

We established a system to isolate and identify intestinal bacteria. Single colonies from mixed cultures were picked and sub-cultured on fresh media, after which a single colony from the sub-culture was passaged to ensure purity. Genomic DNA was extracted from individual isolates and the bacterial 16S rRNA gene was PCR-amplified using primers Bact-8f and Bact-1510r (Eckburg, Bik et al. 2005; Gill, Pop et al. 2006). The resulting PCR product was then sequenced using the original PCR primers. Sequences were edited and trimmed for quality using the Trev 1.9 program of the Staden Package©. Forward and reverse reads were assembled into a single contig using SeqMan from the DNAStar© Lasergene software package, Version 8.0.2. The 16S sequences were classified using the Ribosomal Database Project (RDP, Release 10, Update 15) Classifier and the closest cultured and un-cultured matches from GenBank were identified using

RDP's SeqMatch program supplemented by BLAST analysis (Altschul, Gish et al. 1990; Cole, Chai et al. 2007; Wang, Garrity et al. 2007; Cole, Wang et al. 2009).

Isolation of numerous, widely diverse bacteria from intestinal mixed cultures

We originally planned to isolate a variety of intestinal bacteria, identify them, pool them by taxonomic group, and inoculate the pooled isolates into healthy dnKO mice to test for colitigenicity. Commensal intestinal bacteria vary by several orders of magnitude in their overall abundance within the intestinal microbiota (Dethlefsen, Huse et al. 2008; Huse, Dethlefsen et al. 2008), presenting challenges for isolation of less abundant bacteria. When intestinal contents are cultured on a single non-selective media type, it is only possible to isolate relatively abundant bacteria because rarer organisms will be missed in a mixed lawn of bacteria. We therefore employed a variety of selective aerobic and anaerobic media for bacterial isolation to increase the range of bacteria we could isolate (Figure 3.9). To further increase the sensitivity of our isolation methods, we plated intestinal contents on these media types in serial, half-log dilutions, which allowed us to isolate unique-appearing colonies from the plates with the largest number of individually distinguishable colonies (Onderdonk, Weinstein et al. 1974; Sakon, Nagai et al. 2008). We allowed cultures to incubate for 2-7 days (aerobic growth) or 7-14 days (anaerobic growth), then picked unique-appearing isolates using the techniques described above.

The method of serial dilutions on selective media allowed us to sample microbes with abundances varying over an estimated 10^7-fold range (Figure 3.10 D). Despite the

likely occurrence of some redundancy of isolates from each media type and extensive redundancy between isolates from certain media types, the range of isolate titers suggests the ability to widely sample the cultivable microbiota by this approach. We calculated that sampling depth provided by culturing continues to be considerably greater than that of culture-independent broad-range bacterial 16S rRNA gene sequencing in various landmark studies (Figure 3.10 C) (Kroes, Lepp et al. 1999; Eckburg, Bik et al. 2005; Dethlefsen, Huse et al. 2008; Turnbaugh, Hamady et al. 2008). Importantly, the presence of rarer species detectable on some media types would not have been detected at the sampling depth of any of the sequencing surveys. Approaches like mass sequencing allow revolutionarily high-resolution of the taxonomy of abundant microbes within the microbiota. However mass sequencing approaches accumulate data additively with additional effort rather than exponentially, therefore our results results demonstrate that culture-based approaches remain the most sensitive and specific technique for deep sampling of cultivable bacteria within the intestinal microbiota.

We identified a subset of the isolates depicted in Figure 3.10 D by 16S rRNA gene sequencing, revealing a widely diverse range of bacteria representing the four phyla most commonly identified in culture-independent sequencing surveys of mammalian intestinal microbiota: *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria* (Figure 3.11). Interestingly, some of the isolated bacteria were apparently novel isolates that have only previously been described by culture-independent methods. All identified isolates grew on chocolate agar and/or anaerobic blood agar. Thus the pools of cultured bacteria gavaged into dnKO mice in the experiments depicted in Figures 3.7 and 3.8 were

presumably considerably more complex than the panel of bacteria depicted in Figure 3.11.

Intestinal contents cultured on media selective for Gram-negative obligate anaerobes induce disease in IBD-susceptible hosts

Due to the large numbers and broad diversity of cultivable intestinal bacteria (Figure 3.11), difficulty in growing many isolates in useful quantities, and limited availability of dnKO mice for screening experiments, we determined that testing pools of bacteria isolated from mixed cultures on general growth media would not be the most efficient approach to screen for specific colitigenic bacteria. As an alternate approach, we decided to first limit the complexity by screening mixed cultures of intestinal contents containing more restricted pools of bacteria than those used in Figures 3.7 and 3.8. The mammalian intestinal microbiota contains hundreds of bacterial species belonging primarily to the phyla Bacteroidetes (Gram-negative obligate anaerobes) and Firmicutes (Gram-positive, predominantly obligate anaerobes), with other phyla including Proteobacteria (Gram-negative, predominantly facultative anaerobes) present at lower abundance in healthy hosts (Backhed, Ley et al. 2005). However the most abundant bacteria within the microbiota belong to a relatively restricted number of highly represented taxa, primarily within the Bacteroidetes and Firmicutes phyla (Dethlefsen, Huse et al. 2008). We hypothesized that disease would likely depend on the most abundant members of the microbiota.

We adopted two approaches to restrict complexity: growing mixed cultures containing only abundant members of the microbiota and culturing on more selective media types. Dilutions of frozen intestinal contents from untreated mice (described above) were cultured in parallel on a variety of anaerobic culture media (Figure 3.12). The mixed cultures were grown on non-selective media (ANB agar), media selective for Gram-negative obligate anaerobes (LKV agar), or media inhibitory towards Gram-negative bacilli and enriched for growth of Gram-positive anaerobes (CNA agar). From each media type, we harvested mixed cultures from a dilution at which ~1500 colonies grew, ensuring the culture contained only relatively abundant (approximately ≥0.07% abundance) intestinal bacterial cultivable on that media type (Table 3.2). We also harvested a culture of concentrated intestinal contents grown anaerobically on ANB agar and a concentrated culture grown aerobically on chocolate agar (Figure 3.12 and Table 3.2). Anaerobic cultures were harvested after 3 days of incubation using sterile swabs, suspended in sterile pre-reduced PBS, and frozen at -80˚C in single-use aliquots with 20% pre-reduced glycerol. Aerobic cultures were harvested by the same method after 48 hours incubation (Table S1). The titer of each frozen stock was determined prior to use in gavage experiments.

To test whether the restricted cultures could induce disease, we gavaged groups of antibiotic-pre-treated dnKO mice with the anaerobic mixed cultures combined in 1:1 ratio with the aerobic culture. Mice receiving diluted ANB and LKV cultures developed severe intestinal pathology relative to PBS-gavaged controls while undiluted ANB culture induced a non-significant trend towards disease induction (Figure 3.13). Mice gavaged

with the CNA culture showed a trend towards development of milder disease that did not significantly differ from PBS-gavaged controls. Quantitative histologic metrics of inflammation confirmed significant disease induction by the diluted ANB and LKV cultures. These results confirmed that colitis-inducing bacteria could be grown in culture and suggested abundant Gram-negative obligate anaerobes might be sufficient for disease induction.

Isolation and classification of abundant commensal bacteria cultivable on LKV agar

To isolate Gram-negative, anaerobic bacteria, freshly thawed aliquots of intestinal contents (see above) were diluted to the concentration used in preparation of the LKV mixed culture used for gavage (Table 3.2) and cultured anaerobically on LKV agar and *Bacteroides* bile esculin (BBE) agar. Unique-appearing colonies were picked from plates, sub-cultured on fresh media, and passaged to ensure purity. Isolates were identified by sequencing the PCR-amplified 16S rRNA gene as described above; sequence identity was determined by BLAST2 analysis. Unique bacterial species isolated from LKV agar are listed in Table 3.3. For isolates <98% identical to the most similar type strain, the most similar cultured isolate in the RDP database was also identified. All isolates from BBE agar were redundant with bacteria isolated on LKV agar. All were >99% similar to sequences identified in metagenomic sequencing of rodent commensal microbiota (not shown).

As expected, the isolates were predominated by members of the phylum Bacteroidetes from the genera *Bacteroides* and *Parabacteroides* (Table 3.3), which are

Gram-negative obligate anaerobes belonging to the group known as the intestinal Bacteroidales and are highly evolved to colonize the mammalian intestine (Coyne and Comstock 2008). They include *Bacteroides vulgatus*, which has been implicated in colitis induction in the gnotobiotic HLA-B27 transgenic rat model and the carrageenan-induced colitis model in guinea pigs, but did not induce colitis when inoculated into germ-free IL-$10^{-/-}$ mice (Onderdonk, Franklin et al. 1981; Onderdonk, Bronson et al. 1987; Sellon, Tonkonogy et al. 1998; Rath, Wilson et al. 1999). *Parabacteroides distasonis*, a member of the altered Schaedler Flora, may produce milder inflammation in gnotobiotic HLA-B27 transgenic rats (Rath, Herfarth et al. 1996), but to our knowledge the other Bacteroidetes isolates have not been implicated in IBD. One *Bacteroides* isolate is >99% identical at the 16S rRNA gene level to a GenBank sequence of an unpublished isolate identified as "*Bacteroides* sp. TP-5". We are aware of no publications that have characterized *Bacteroides* sp. TP-5, but the GenBank entry is titled "The *Bacteroides* species is an important environmental risk factor for colon carcinogensis in T-cell receptor b and p53 double-knockout mice" (Kado 2009). Other than *Bacteroides* sp. TP-5, the most closely related isolate in the RDP database is only ~95% identical, suggesting this isolate may represent a novel, largely uncharacterized *Bacteroides* species.

We also initially isolated two members of the phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales* (Wang, Garrity et al. 2007). The isolates were <97% identical to each other and ~94-96% identical to the most closely related isolate in the RDP database, a recently described gram-negative strict anaerobe with the proposed name *Parasutterella excrementihominis* gen. nov., sp. nov. that is proposed to represent a

novel genus and species (Nagai, Morotomi et al. 2009). Each of these *Proteobacteria* isolates thus represented a potentially novel species, although as noted they have been previously detected in metagenomic sequencing of commensal microbiota. We are aware of no publications specifically examining either isolate's interactions with its host. However, as was reported for *P. excrementihominis*, both isolates proved extraordinarily difficult to culture in usable quantities (Nagai, Morotomi et al. 2009) and the isolates ultimately lost viability. These bacteria were therefore not characterized in subsequent experiments.

We also isolated *Lactobacillus murinus*, which is a gram-positive, microaerophilic commensal commonly found throughout the murine gastrointestinal tract and is a member of the altered Schaedler flora (Dewhirst, Chien et al. 1999; Sarma-Rupavtarm, Ge et al. 2004). A study of lactobacilli in murine colitis identified *L. murinus* in non-colitic animals but failed to detect it in IL-10 deficient colitic mice (Pena, Li et al. 2004). We did not characterize *L. murinus* in subsequent experiments because lactobacilli are known to be highly metronidazole-resistant (Hammad and Shimamoto 2010) and can be commonly isolated from the fecal microbiota of non-colitic dnKO mice treated with metronidazole and ciprofloxacin combination therapy (not shown)

Commensal *Bacteroides* isolates induce disease in IBD-susceptible but not non-susceptible hosts

We screened five of the Bacteroidetes isolates listed in Table 3.3 for colitis-inducing potential by gavaging pure cultures of each into antibiotic-pre-treated mice. For

preparation of gavage inocula, 24-hour cultures of the respective isolate were grown anaerobically in standing culture in TYG broth (Goodman, McNulty et al. 2009). Each culture was concentrated by centrifugation, mixed with sterile, pre-reduced PBS + glycerol to a final concentration of 20% glycerol, and frozen at -80°C in single-use aliquots. Titers of each frozen stock were determined prior to use in gavage. To perform colonization experiments, aliquots were thawed in a 37°C water bath, transferred to the anaerobic chamber, volume-adjusted in sterile pre-reduced PBS, and immediately transported to the mouse facility for orogastric gavage (Goodman, McNulty et al. 2009). Gavage doses were confirmed by back-titering the inocula. For sterile PBS gavage, we added sterile, pre-reduced glycerol to a concentration equivalent to that in the thawed bacterial gavage inocula.

We found that pure cultures of each of the Bacteroidetes isolates induced colitis in dnKO mice to varying degrees (Figure 3.15 and 3.16). Unexpectedly, an isolate of *Bacteroides thetaiotaomicron*, a well-characterized symbiotic species (Goodman, McNulty et al. 2009), potently induced colitis in dnKO mice. As expected, this isolate was innocuous in *Il10r2*$^{+/-}$ control animals, which did not differ from PBS-gavaged controls (Figures 3.16 to 3.19). These results demonstrated the induction of disease by commensal *Bacteroides* in a host-genotype-specific fashion.

Antibiotic treatment eliminates cultivable intestinal *Bacteroides* from the fecal microbiota

Given the ability of commensal *Bacteroides* species to induce disease in dnKO mice, we hypothesized that these bacteria must be eliminated from the microbiota by antibiotic treatment. We tested this hypothesis by culturing fecal bacteria on selective and non-selective culture media. Fecal samples from 4-week-old untreated dnKO mice or dnKO mice treated with antibiotics for ≥3 weeks were collected in sterile microcentrifuge tubes. Samples were immediately placed on ice, weighed, transferred to an anaerobic chamber within ≤1 hr of collection, suspended in 500 µl of sterile pre-reduced PBS by repeated vigorous vortexing and disruption with a sterile pipette tip, and titered by culturing 10-fold serial dilutions in parallel on ANB agar (non-selective, general anaerobic growth media) or *Bacteroides* bile esculin (BBE) agar, which is selective and differential media for the isolation of intestinal *Bacteroides* species (Livingston, Kominos et al. 1978). 10 µl of each dilution was spotted in triplicate on quadrants of titer plates. BBE titers were counted after 48 hours and ANB titers were counted at 48 hours and confirmed after 5 days. Fecal titers were calculated as cfu/g of feces.

Using these methods, we confirmed that antibiotic treatment eradicated cultivable, disease-inducing intestinal *Bacteroides* from the fecal microbiota. Fecal samples from antibiotic-treated dnKO mice exhibited no bacterial growth when quantitatively cultured on *Bacteroides* bile esculin (BBE) agar at a detection limit of 17 colony-forming units (cfu) per sample (Figure 3.20). By contrast, we observed high bacterial titers (~10^9 cfu/g of feces) on BBE agar in samples from untreated animals. Fecal titers on non-selective

media did not differ between groups, confirming that antibiotics prevented disease by selectively altering microbiota composition rather than by sterilizing the gut (Figure 3.20).

Gavaged *Bacteroides* species stably colonize antibiotic-pre-treated mice at high levels regardless of host genotype

We reasoned that BBE agar could thus be used to assess *Bacteroides* colonization of gavaged, antibiotic-pre-treated mice. Three of the *Bacteroides* isolates screened in Figure 3.15 (*B. thetaiotaomicron*, *B. vulgatus*, and *Bacteroides* sp. TP5, see Table 3.3) grew robustly by 48 hours in anaerobic culture on BBE agar (Figure 3.21 A). *B. vulgatus* forms non-pigmented, circular, convex colonies with entire edges that are 1-2 cm in diameter after 48 hrs growth. *B. thetaiotaomicron* forms colonies that are similar in size and morphology to *B. vulgatus* but strongly pigmented. *Bacteroides* sp. TP5 forms circular, convex colonies with entire edges that are 0.25-1 cm in diameter and non-pigmented or very lightly pigmented after 48 hrs.

Remarkably, we could thus monitor *Bacteroides* colonization by performing serial, quantitative, specific fecal cultures on BBE agar. Antibiotic-pre-treated mice gavaged with PBS remained free of cultivable *Bacteroides*, confirming absence of cage-to-cage microbial contamination (Figure 3.22 A). By contrast, mice given pure cultures of *B. thetaiotaomicron*, *B. vulgatus*, or *Bacteroides* species TP5 became stably colonized at ~10^{10} cfu/g of feces (Figure 3.22). Bacterial identity was determined based on characteristic colony size, morphology, and pigmentation pattern on BBE agar (Figure

3.21 B) and confirmed by 16S rRNA gene sequencing of representative colonies. Colonization levels were surprisingly high and consistent. Analysis of titers compiled from animals of various genotypes from many experiments over multiple timepoints revealed that they were normally distributed over an extremely narrow range (Figure 3.23). Antibiotic-pre-treated *Il10r2*$^{+/-}$ and dnKO mice became stably colonized with the gavaged bacteria at equivalent levels (Figure 3.22), demonstrating that genotype-dependent differences in disease induction were likely due to differences in host response rather than altered colonization susceptibility.

Commensal Enterobacteriaceae but not *Bacteroides* are strikingly enriched in the microbiota during spontaneous disease

Despite our finding that commensal *Bacteroides* induced disease in dnKO mice, studies of the intestinal microbiota in IBD have not found *Bacteroides* to be consistently enriched during disease (Onderdonk, Richardson et al. 1998; Frank, St Amand et al. 2007; Ott, Plamondon et al. 2008; Sokol, Pigneur et al. 2008; Takaishi, Matsuki et al. 2008; Frank, Robertson et al. 2010). We likewise observed that fecal titers on *Bacteroides* bile esculin agar from untreated dnKO (spontaneously colitic) and *Il10r2*$^{+/-}$ (non-colitic) mice did not significantly differ in either absolute levels or as a proportion of total cultivable bacteria (Figure 3.24). By contrast, many studies have documented IBD-associated enrichment of Enterobacteriaceae, Gram-negative, facultative anaerobes (including the species *E. coli*) from the phylum Proteobacteria that can be selectively cultured on MacConkey agar (Burke 1997; Onderdonk, Richardson et al. 1998; Frank, St

Amand et al. 2007; Lupp, Robertson et al. 2007; Sartor 2008; Frank, Robertson et al. 2010; Strober 2010). In agreement with findings from other colitis models, we observed that commensal Enterobacteriaceae were strikingly enriched in spontaneous disease, accounting for ~50% of total cultivable fecal bacteria in untreated dnKO mice compared with <0.5% of cultivable bacteria in co-housed *Il10r2*$^{+/-}$ controls (Figure 3.24).

Commensal Enterobacteriaceae are not sufficient for disease induction

The observation that Enterobacteriaceae are often elevated in IBD has led to suggestions that these bacteria may play a pathogenic role in disease (Burke 1997; Onderdonk, Richardson et al. 1998; Frank, St Amand et al. 2007; Edwards, Lucas et al. 2010). However it is unclear whether Enterobacteriaceae enrichment is a cause or effect of disease since enrichment also occurs during non-IBD intestinal inflammation experimentally induced by chemicals or microbial pathogens (Heimesaat, Bereswill et al. 2006; Heimesaat, Fischer et al. 2007; Lupp, Robertson et al. 2007; Stecher, Robbiani et al. 2007; Sartor 2008). Importantly, commensal Enterobacteriaceae were eliminated from antibiotic-treated mice (Figure 3.24 C), allowing us to directly test whether they could induce disease. We isolated an Enterobacteriaceae species that was highly enriched in the feces of an untreated, colitic dnKO mouse (~33% of total cultivable bacteria) and identified it as *Escherichia coli* based on 16S rRNA sequence similarity to the closest culture isolate (Figure 3.25). Confirmation of this species identification by phenotypic identification using the API 20E strip (biocode 5144532, bioMerieux, Inc., Durham, NC) in the Barnes-Jewish Hospital Medical Microbiology Laboratory confirmed this

classification. The isolate was maintained aerobically on MacConkey agar, ANB agar, or in Trypticase Soy Broth.

To test the isolate's disease-inducing potential, we gavaged it into antibiotic-pre-treated dnKO mice. For preparation of the gavage inoculum, 16-hour cultures of *E. coli* were grown in Trypticase Soy Broth in a 37°C shaker at 225 rpm. Each culture was concentrated by centrifugation, mixed with sterile, pre-reduced PBS + glycerol to a final concentration of 20% glycerol, and frozen at -80°C in single-use aliquots. The stock was tittered prior to use in experiments. Other groups of dnKO mice were gavaged with *B. thetaiotaomicron* or sterile PBS as positive and negative controls. We quantified bacterial colonization by performing parallel, serial fecal titers on BBE and MacConkey agar (Figure 3.26). MacConkey titers were performed as for BBE titers (see above) and were counted after 12-16 hours and confirmed for negative growth at 48 hours. *E. coli* stably colonized antibiotic-pre-treated animals at consistent levels even higher than those observed in untreated dnKO mice (compare Figure 3.24 C and 3.26 B) and comparable to *E. coli* colonization levels in mono-associated gnotobiotic mice (Hapfelmeier, Lawson et al. 2010). However *E. coli* did not induce significant disease relative to PBS, whereas *B. thetaiotaomicron* induced significant disease relative to both *E. coli* and PBS by most metrics (Figure 3.27 and 3.28).

DISCUSSION

The precise role of the intestinal microbiota in IBD induction remains an area of active investigation (Sartor 2008; Strober 2010). Multiple theories exist concerning

whether IBD can be induced by specific commensal microbes and, if so, whether the disease-inducing microbes can be identified based on enrichment in the microbiota during disease (Xavier and Podolsky 2007; Takaishi, Matsuki et al. 2008; Packey and Sartor 2009; Cerf-Bensussan and Gaboriau-Routhiau 2010; Garrett, Gordon et al. 2010). We previously hypothesized that disease in the dnKO mouse model (which bears genetic defects in pathways linked to IBD in humans and which develops spontaneous, rapid-onset, antibiotic-responsive colitis closely resembling human ulcerative colitis) depends on a subset of commensal bacteria, likely obligate anaerobes, which are eliminated by antibiotic treatment (Chapter 2). Here we fulfilled Koch's postulates for commensal *Bacteroides* species in host-genotype-specific fashion using non-gnotobiotic methods in dnKO mice. We found that *Bacteroides* species:

1) were common to susceptible and non-susceptible hosts and were eliminated by disease-blocking antibiotics,

2) could be isolated and grown in pure culture,

3) induced colitis when administered to antibiotic-pre-treated susceptible hosts but not when administered to non-susceptible hosts, and

4) could be quantitatively re-isolated from experimentally colonized animals at equivalent levels regardless of host genotype.

The disparity in *Bacteroides*-induced disease thus appears to be attributable to differences in host response rather than altered colonization susceptibility. We further showed that commensal Enterobacteriaceae were strikingly enriched during spontaneous disease but a

colitis-enriched *E. coli* isolate was not sufficient for disease induction despite robust colonization.

Intriguingly, our results in dnKO mice, which bear medically relevant deficiencies in the IL-10 receptor (Glocker, Kotlarz et al. 2009) and in TGFβ signaling (Franke, McGovern et al. 2010), independently replicate findings from the medically relevant HLA-B27 transgenic rat model (Hammer, Maika et al. 1990). Conventionally raised transgenic rats develop spontaneous colitis characterized by striking enrichment of commensal Enterobacteriaceae and unchanged levels of commensal *Bacteroides* (Onderdonk, Richardson et al. 1998) However colonization of gnotobiotic transgenic rats with the *Bacteroides* species *B. vulgatus* (and possibly *Parabacteroides distasonis*) induced disease whereas colonization with a variety of IBD-enriched human isolates including *E. coli,* a species of Enterobacteriaceae, did not (Rath, Herfarth et al. 1996; Rath, Wilson et al. 1999). The HLA-B27 allele in humans is strongly linked to spondyloarthritis (a collection of rheumatologic diseases that frequently includes IBD) but is not an independent risk factor for IBD that is not associated with spondyloarthritis (Brakenhoff, Heijde et al. 2010) It has thus been unclear whether findings in HLA-B27 transgenic rats would be more broadly relevant to IBD in general. However there are intriguing similarities between the transgenic rat model and dnKO mice. Colitis in HLA-B27 transgenic rats is T-cell-dependent (Hoentjen, Tonkonogy et al. 2006; Hoentjen, Tonkonogy et al. 2007) and there is evidence that disease may be related to impaired inhibition of T-cell activation by IL-10 and TGFβ through unknown mechanisms (Qian, Tonkonogy et al. 2008). Similarly, we previously showed that adoptive transfer of dnKO

CD4 T-cells into *Rag*$^{-/-}$ recipient mice induces fulminant colitis relative to transfer of wild-type T-cells (Kang, Bloom et al. 2008). We and others have shown that transfer of *Il10r2*$^{-/-}$ CD4 T-cells also induces enhanced disease, although to a lesser extent (Kang, Bloom et al. 2008; Murai, Turovskaya et al. 2009).

B. vulgatus colonization was also pro-colitic in the gnotobiotic guinea pig model of carrageenan-induced colitis, the model in which it was originally identified as a potential colitogen (Onderdonk, Franklin et al. 1981; Onderdonk 2005). However *B. vulgatus* mono-association did not induce disease in gnotobiotic *Il10*$^{-/-}$ mice and may actually have been protective in gnotobiotic *Il2*$^{-/-}$ mice, whereas some commensal *E. coli* species had pro-colitic effects in these models (Sellon, Tonkonogy et al. 1998; Kim, Tonkonogy et al. 2005). Alleles of the gene for IL-10 and a component of the IL-2 receptor complex were found to be associated with IBD in recent genome-wide association studies (Franke, Balschun et al. 2008; Franke, McGovern et al. 2010). Reasons for the apparent disparity between these mouse models and the dnKO mouse and HLA-B27 rat models are unclear. A recent study of intestinal biopsy material from human patients found that *Bacteroides thetaiotaomicron* induced strong IL-8 secretion *ex vivo* in tissue from IBD patients but not in tissue from healthy controls, whereas there was no difference in IL-8 response between the two groups of tissues upon exposure to an *E. coli* species (Edwards, Lucas et al. 2010). Much further work is needed, particularly from human studies, but we believe in aggregate this evidence supports the idea that commensal *Bacteroides* species may have specific colitogenic effects in at least a subset of human IBD.

Many studies have documented IBD-associated enrichment or depletion of specific commensal bacterial subsets but it remains an important unresolved question whether and how these alterations contribute to disease pathogenesis (Burke 1997; Frank, St Amand et al. 2007; Takaishi, Matsuki et al. 2008; Packey and Sartor 2009; Peterson, Garges et al. 2009; Frank, Robertson et al. 2010; Strober 2010). Our demonstration that commensal Enterobacteriaceae were enriched during spontaneous disease but were not sufficient for disease induction adds to a growing body of evidence that intestinal inflammation provides both commensal and enteropathogenic Enterobacteriaceae with a selective colonization advantage regardless of inflammation etiology (Onderdonk, Richardson et al. 1998; Heimesaat, Bereswill et al. 2006; Lupp, Robertson et al. 2007; Stecher, Robbiani et al. 2007; Ackermann, Stecher et al. 2008; Winter, Thiennimitr et al. 2010). Interestingly, disease-associated enrichment of Proteobacteria relative to Bacteroidetes has also been observed in the lung microbiota of patients with asthma, a chronic inflammatory disease (Hilty, Burke et al. 2010), and in the gastric microbiota of patients infected with *Helicobacter pylori*, a cause of chronic atrophic gastritis (Maldonado-Contreras, Goldfarb et al. 2010). In ecological terms, Enterobacteriaceae thus appear to behave as invasive species capable of exploiting new niches created by inflammation-induced ecosystem instability at mucosal surfaces. The Enterobacteriaceae pathogen *Salmonella typhimurium* directly exploits intestinal inflammation by using tetrathionate – a compound generated in the intestinal lumen in presence of host-derived reactive oxygen species – as a respiratory electron acceptor to outcompete the fermentative anaerobes that normally dominate the microbiota (Winter, Thiennimitr et al.

2010). We speculate that similar mechanisms for exploiting inflammation could account for “bystander enrichment” of commensal Enterobacteriaceae in IBD and may even be related to the frequency with which non-pathogenic Enterobacteriaceae strains have independently and convergently evolved pathogenic phenotypes (Pupo, Karaolis et al. 1997; Wirth, Falush et al. 2006). Thus, although disease-enriched bacteria may play colitogenic roles in certain circumstances and disease-depleted bacteria may have anti-colitic effects in others (Burke 1997; Sokol, Pigneur et al. 2008), our findings emphasize that IBD-associated shifts in colonization are not a sufficient basis to form conclusions about a microbe's effects on disease without additional evidence.

In this study, we have endeavored to address two key questions about IBD using dnKO mice: (1) is disease induced by specific subsets of commensal microbes and, if so, (2) can these subsets be identified based on disease-associated alterations in levels of colonization? Our work provides evidence supporting the concept of commensal specificity and disputing the concept that disease-associated enrichment necessarily implies a causative role. These findings also underscore the importance of examining microbial influences on pathogenesis in the context of host genetic predisposition (Cadwell, Patel et al. 2010). The possibility that a microbe's colonization dynamics in disease may be dissociable from its disease-inducing potential presents significant obstacles for elucidating underlying microbial etiology. Mazmanian and colleagues recently coined the term "pathobiont" to describe commensal or symbiotic microbes that induce disease only in certain genetic or environmental contexts (Mazmanian, Round et al. 2008; Chow and Mazmanian 2010), but no established criteria exist for classifying a

microbe as a pathobiont or distinguishing pathobionts from conventional pathogens. We propose refining the definition to state that unlike parasitic or opportunistic pathogens, true pathobionts colonize at equivalent levels regardless of disease induction. Formal demonstration that a microbe acts as a pathobiont would thus require fulfillment of host-genotype-specific (or environment-specific) Koch's postulates resembling those described here. Extending this concept, we note that it is even possible to envision a scenario in which pathobiotic bacteria induce a targeted immunopathologic response that leads them to become depleted within the microbiota despite playing a causative role in disease.

In summary, our results provide important insights into the intestinal microbiota's role in IBD induction and suggest a candidate group of organisms that may be relevant to disease induction in a subset of human cases. As new spontaneous animal models of IBD based on other human susceptibility mutations become available, we propose that the methods, experimental criteria and conceptual framework we develop here will allow characterization of microbial contributions to disease in additional genetic contexts.

Figure 3.1: Co-housing and exposure to intestinal contents from untreated control mice induces disease in antibiotic-pre-treated dnKO mice.

3 week old dnKO mice housed in conventional cages were pre-treated with antibiotics (metronidazole + ciprofloxacin) for 10 days, then antibiotics were halted and mice were either maintained without experimental microbial exposures or co-housed with an untreated non-dnKO mouse and smeared with intestinal contents from an untreated, non-dnKO mouse. Mice were sacrificed and colons were analyzed for gross pathology after 7 days. Colons of mice experimentally exposed to intestinal contents exhibit severe pathology with ulceration and shortening, but colons of isolated mice also show signs of mucosal thickening and inflammation. Representative of 2-3 experiments per group with 1-2 mice per experiment.

Figure 3.1

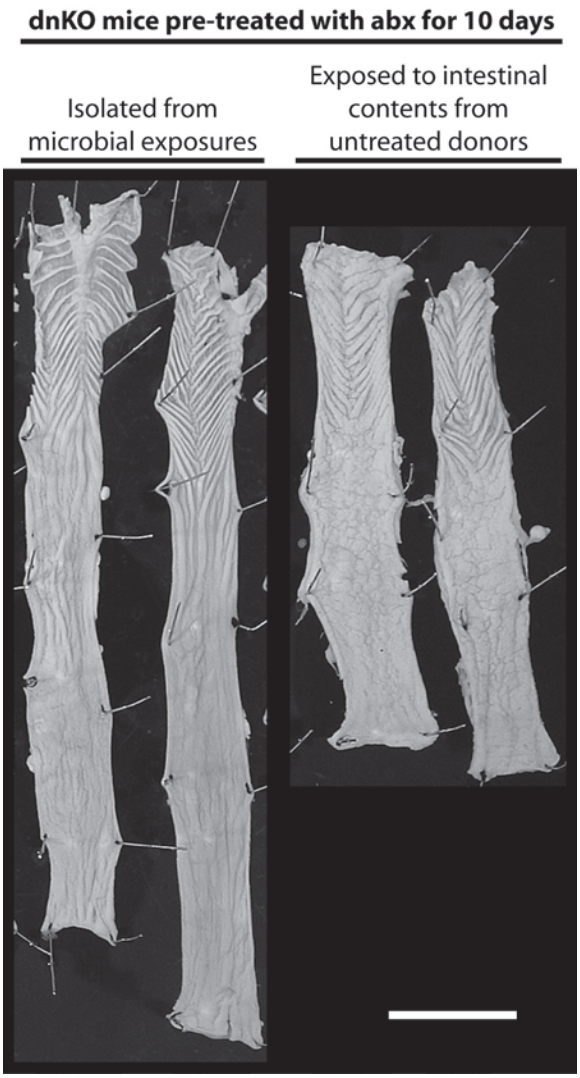

dnKO mice pre-treated with abx for 10 days
Isolated from microbial exposures
Exposed to intestinal contents from untreated donors

Figure 3.2: Bacterial composition of intestinal contents is highly variable from sample to sample.

Intestinal contents from age-matched, untreated male mice (non-dnKO genotypes) co-housed for ~3 weeks beginning at weaning were harvested on each of two successive days (2 mice per day). Serial dilutions of intestinal contents were cultured aerobically on chocolate agar. Culture plates with similar numbers of cfu (inset labels) from each culture day are shown. Note the highly variable morphology of abundant bacterial colonies between the two samples.

Figure 3.2

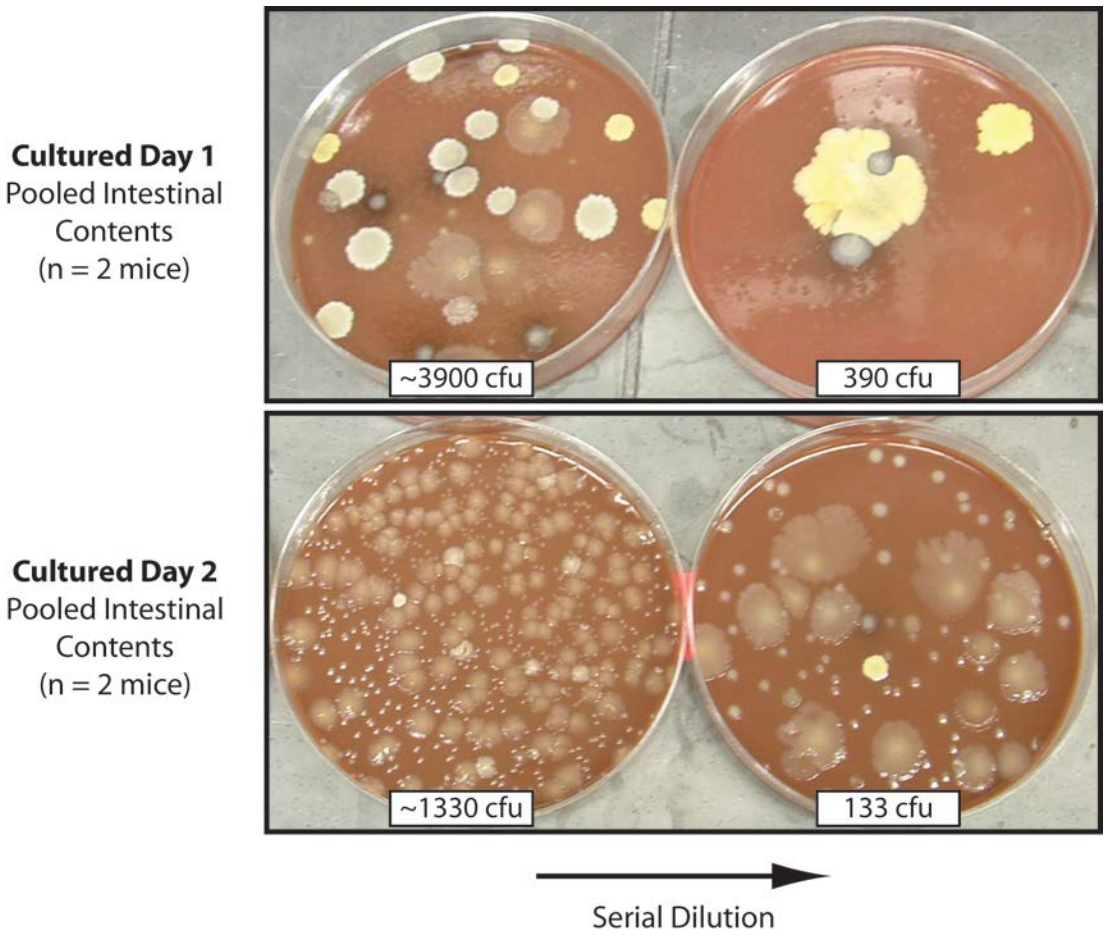

Cultured Day 1
Pooled Intestinal Contents
(n = 2 mice)
~3900 cfu
390 cfu
Cultured Day 2
Pooled Intestinal Contents
(n = 2 mice)
~1330 cfu
133 cfu
Serial Dilution

Figure 3.3: Gavaged intestinal contents from untreated donors induce fulminant disease in antibiotic-pre-treated dnKO mice.

(A) Experimental timeline. Antibiotic treatment (metronidazole + ciprofloxacin) began at weaning; mice housed were antibiotic-treated for ≥3 weeks and orogastrically gavaged with experimental inocula 2 days after treatment cessation (Abx = antibiotics). Individual mice were sacrificed after 21 days or when their weight reached ≤ 70% of maximal weight.

(B) Antibiotic-pre-treated dnKO mice housed in enhanced caging under stringent cage-care precautions were gavaged with PBS (n = 4 mice) or a freshly thawed aliquot of pooled intestinal contents from untreated non-dnKO donors that had been frozen in 20% glycerol at -80°C under anaerobic conditions (n = 6 mice). The gavage dose of intestinal contents was $2x10^7$ total cfu per mouse, titered on Anaerobic Reducible Blood (ANB) Agar. Mice were sacrificed at 3 weeks post-inoculation or upon loss of >30% of maximum body weight. Survival analyzed by Kaplan-Meier method, and statistical significance determined by the log-rank test. Results are representative of multiple independent experiments. All statistical analysis was performed using Prism v3.02 (GraphPad Software) unless otherwise indicated.

Figure 3.3

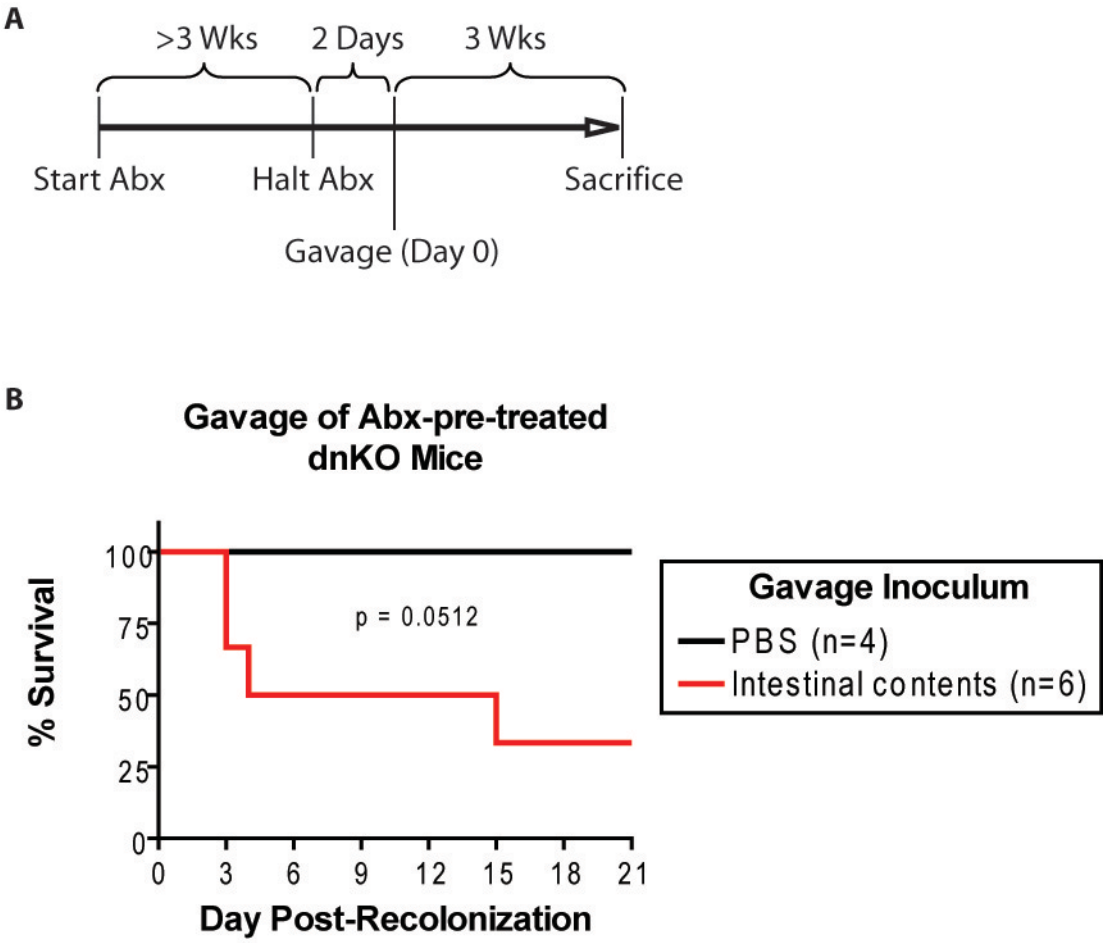

A
>3 Wks
2 Days
3 Wks
Start Abx
Halt Abx
Sacrifice
Gavage (Day 0)
B
Gavage of Abx-pre-treated dnKO Mice
% Survival
100
75
50
25
0
p = 0.0512
0
3
6
9
12
15
18
21
Day Post-Recolonization
Gavage Inoculum
PBS (n=4)
Intestinal contents (n=6)

Figure 3.4: Gavaged intestinal contents from untreated donors induce severe colonic gross pathology in antibiotic-pre-treated dnKO mice.

Pathology scores of antibiotic-pre-treated dnKO mice gavaged with sterile PBS or intestinal contents from untreated donors as described in Figure 3.3, displayed as individual (symbols) and median (bars) scores. Statistical significance determined by Mann-Whitney U-test using SPSS 16.0 (SPSS Inc.) due to a problem with Prism's algorithm.

(A) Cecum pathology scores.

(B) Transverse colon pathology scores.

Figure 3.4

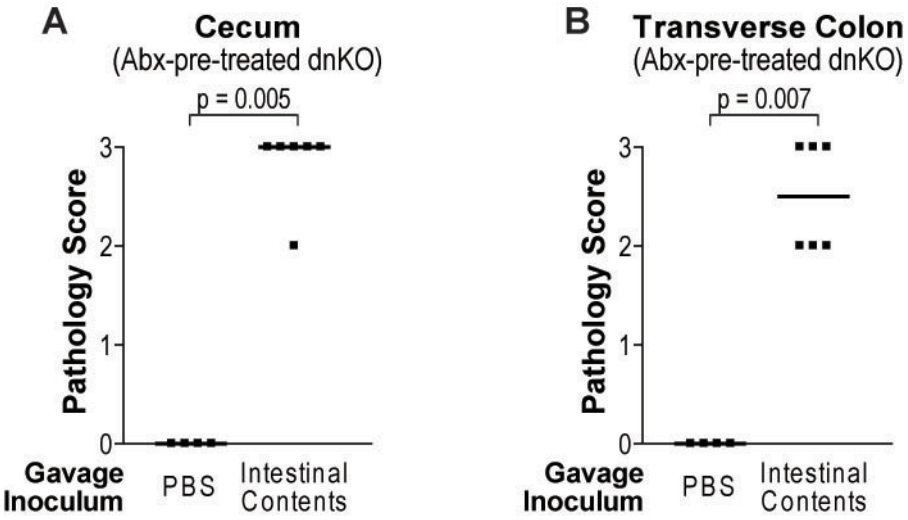

A
Cecum
(Abx-pre-treated dnKO)
p = 0.005
Pathology Score
3
2
1
0
Gavage Inoculum
PBS
Intestinal Contents
B
Transverse Colon
(Abx-pre-treated dnKO)
p = 0.007
Pathology Score
3
2
1
0
Gavage Inoculum
PBS
Intestinal Contents

Figure 3.5: Gavaged intestinal contents from untreated donors induce severe colitis in antibiotic-pre-treated dnKO mice.

Representative images of H&E stained rectal histology of antibiotic-pre-treated dnKO mice gavaged with sterile PBS or intestinal contents from untreated donors as described in Figure 3.3. Bar = 100 µm.

Figure 3.5

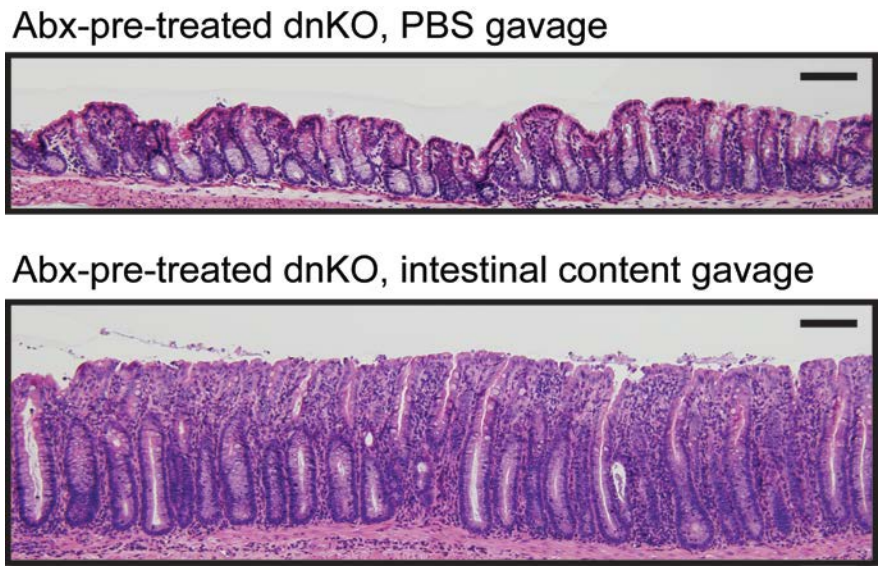

Abx-pre-treated dnKO, PBS gavage
Abx-pre-treated dnKO, intestinal content gavage

Figure 3.6: Gavaged intestinal contents from untreated donors induce severe colonic gross pathology in antibiotic-pre-treated dnKO mice.

Quantitative histologic disease metrics of antibiotic-pre-treated dnKO mice gavaged with sterile PBS or intestinal contents from untreated donors as described in Figure 3.3, displayed as mean +/- SEM. Statistical significance determined by unpaired t-test.

(A) Transverse colon crypt height.

(B) Transverse colon crypt width.

(C) Rectum crypt height.

(D) Rectum crypt width.

Figure 3.6

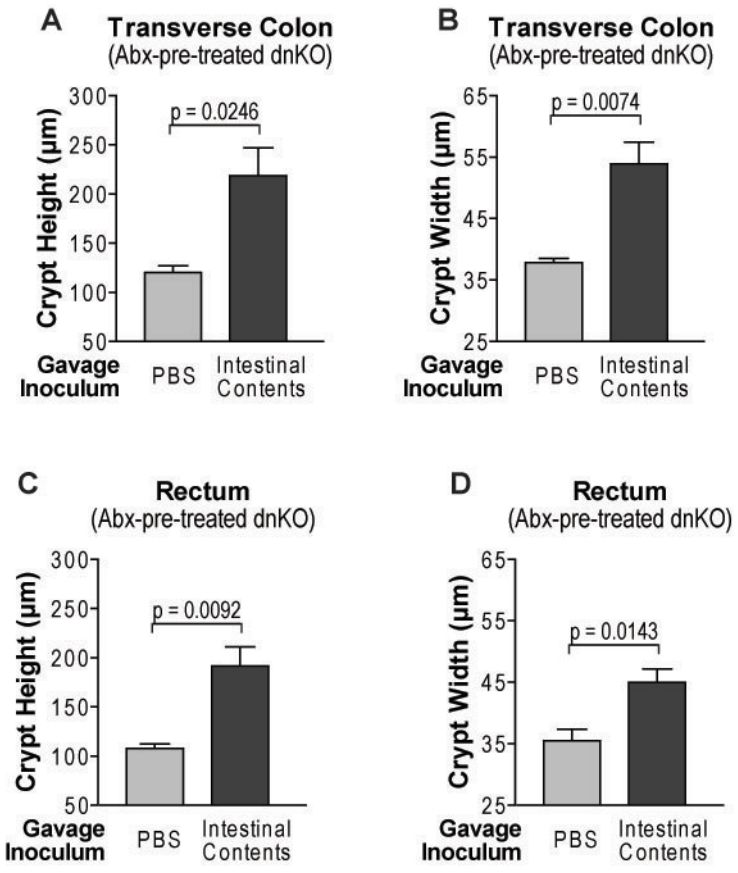

A
Transverse Colon
(Abx-pre-treated dnKO)
Crypt Height (µm)
300
250
200
150
100
50
p = 0.0246
Gavage Inoculum
PBS
Intestinal Contents
B
Transverse Colon
(Abx-pre-treated dnKO)
Crypt Width (µm)
65
55
45
35
25
p = 0.0074
Gavage Inoculum
PBS
Intestinal Contents
C
Rectum
(Abx-pre-treated dnKO)
Crypt Height (µm)
300
250
200
150
100
50
p = 0.0092
Gavage Inoculum
PBS
Intestinal Contents
D
Rectum
(Abx-pre-treated dnKO)
Crypt Width (µm)
65
55
45
35
25
p = 0.0143
Gavage Inoculum
PBS
Intestinal Contents

Figure 3.7: Combined aerobic and anaerobic bacterial cultures of intestinal contents induce non-fatal colitis in antibiotic-pre-treated dnKO mice, but mixed aerobic culture alone does not.

Intestinal contents from untreated donors were cultured anaerobically on ANB agar or aerobically on chocolate agar. Cultures were suspended in sterile, pre-reduced PBS at an optical density of 4 McFarland standards. Antibiotic-pre-treated dnKO mice were gavaged with sterile PBS, with the aerobic culture alone, or with a combination of the aerobic and anaerobic cultures mixed in equal proportions.

(A) One mouse receiving the mixed aerobic and anaerobic cultures was sacrificed 5 days post-gavage due to loss of >30% of maximum weight. All other mice were sacrificed 3 weeks post-gavage. Kaplan-Meier survival analysis; groups are not significantly different by log-rank test: $p = 0.5220$.

(B) Cecum pathology scores of mice in (A). Statistical significance determined by the Kruskal-Wallis test with post-hoc Dunn's test: $H_2 = 18.21$, $p = 0.0001$. All significant pairwise comparisons are displayed: **, $p < 0.01$.

(C) Weight change post-gavage of mice in (A). Groups are not statistically significant by 1-way ANOVA: $F_{2,20} = 0.7760$, $p = 0.4736$.

Figure 3.7

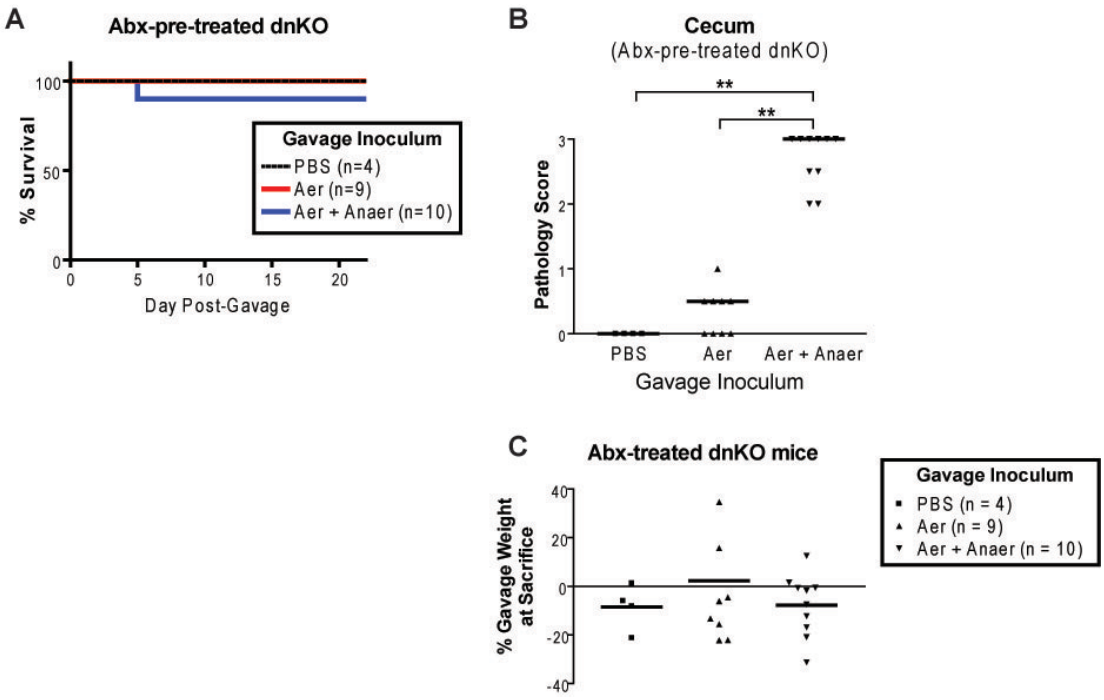

A
Abx-pre-treated dnKO
% Survival
100
50
0
0
5
10
15
20
Day Post-Gavage
Gavage Inoculum
PBS (n=4)
Aer (n=9)
Aer + Anaer (n=10)
B
Cecum
(Abx-pre-treated dnKO)
**
**
Pathology Score
3
2
1
0
PBS
Aer
Aer + Anaer
Gavage Inoculum
C
Abx-treated dnKO mice
% Gavage Weight at Sacrifice
40
20
0
-20
-40
Gavage Inoculum
PBS (n = 4)
Aer (n = 9)
Aer + Anaer (n = 10)

Figure 3.8: Representative cecal pathology of antibiotic-pre-treated dnKO mice gavaged with aerobic and anaerobic bacterial cultures of intestinal contents.

Representative cecal whole mounts of antibiotic-pre-treated dnKO mice described in figure 3.7. Righthand images are magnified views of images on left (inset areas indicated by boxes).

(A) Mouse gavaged with PBS.

(B) Mouse gavaged with aerobic culture of intestinal contents.

(C) Mouse gavaged with combined aerobic and anaerobic culture of intestinal contents. Arrows indicate ulcers.

Figure 3.8

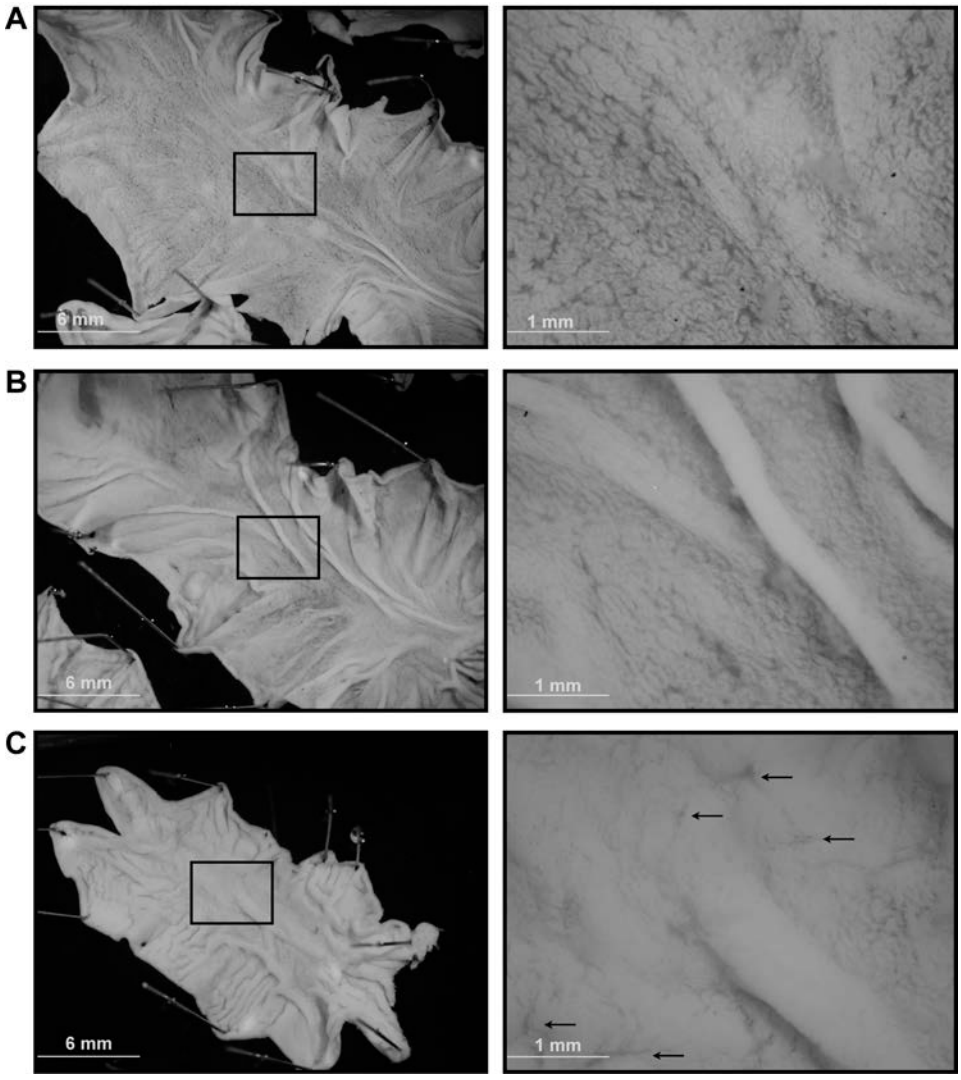


Figure 3.9: Cultivation of intestinal contents on selective culture media for bacterial isolation.

Intestinal contents from untreated, wild-type mice were harvested, suspended in sterile, pre-reduced PBS, serially diluted, and cultured on a variety of aerobic and anaerobic non-selective and selective media. The media types used for anaerobic culture were Anaerobic Reducible Blood Agar ("ANB", Remel, general anaerobic growth media), Anaerobic Reducible CNA Blood Agar ("ACNA", Remel, enriched for growth of Gram positive anaerobic organisms), Anaerobic Reducible LKV Blood Agar ("LKV", Remel, selective for growth of Gram negative obligate anaerobes), *Bacteroides fragilis* Isolation Agar ("BBE", Remel, contains gentamicin to inhibit most facultative anaerobes, bile to inhibit most anaerobic gram negative bacteria, and esculin to differentiate members of the *B. fragilis* group), and Rogosa agar ("Rog", for cultivation of lactobacilli). The media types used for aerobic culture were Tryptic Soy Agar with 5% Sheep Blood ("TSA", Remel, general aerobic growth media), Chocolate Agar ("Choc", Remel, general aerobic growth media enriched for isolation of fastidious aerobes), Columbia CNA with 5% Sheep Blood ("CCNA", Remel, for selective isolation of gram-positive cocci and inhibition of gram-negative bacilli), bile esculin azide agar ("BEA", Remel, aerobic media for isolation of enterococci), and MacConkey Agar ("Mac", selective and differential for isolation of gram negative aerobes or facultative anaerobes).

Figure 3.9

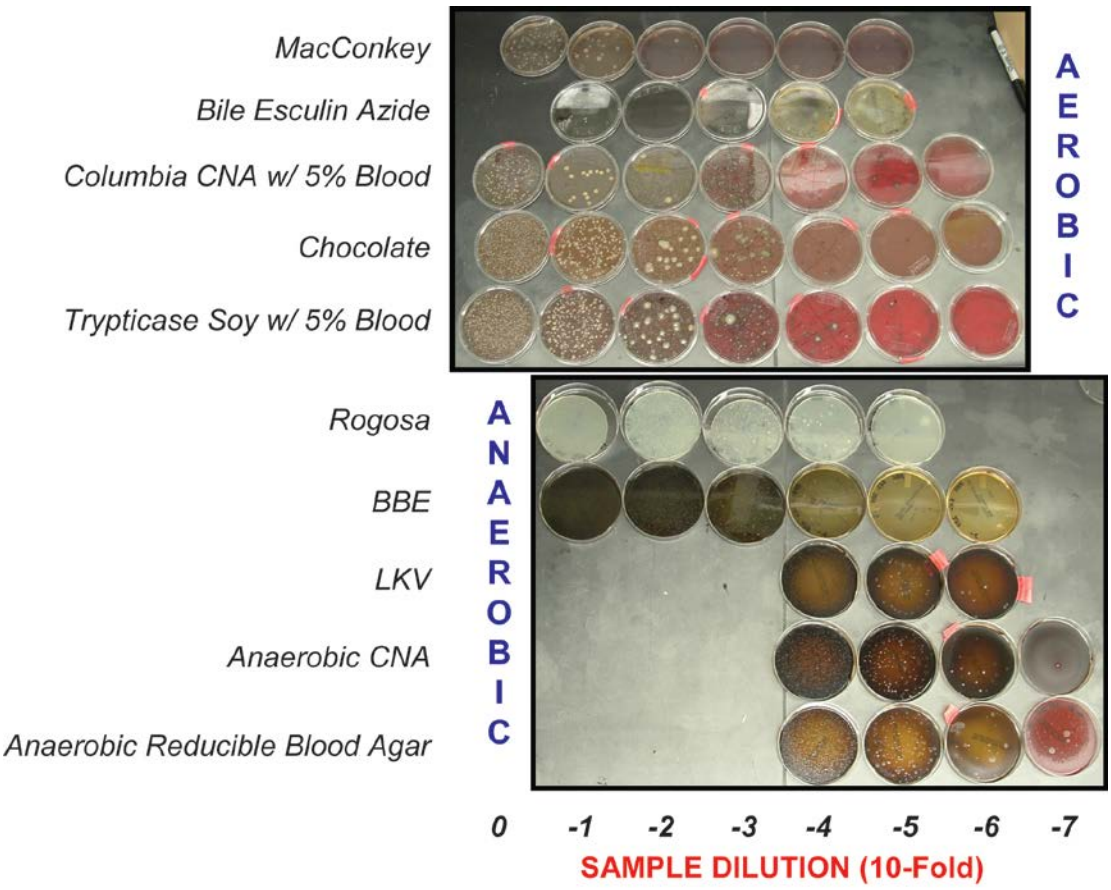

MacConkey
Bile Esculin Azide
Columbia CNA w/ 5% Blood
Chocolate
Trypticase Soy w/ 5% Blood
AEROBIC
Rogosa
BBE
LKV
Anaerobic CNA
Anaerobic Reducible Blood Agar
ANAEROBIC
0
-1
-2
-3
-4
-5
-6
-7
SAMPLE DILUTION (10-Fold)

Figure 3.10: Plating serial dilutions of intestinal contents on selective media allows deep sampling of the intestinal microbiota.

(A) Culture media from Figure 3.9 labeled to indicate the approximate limits of detection in terms of total cultivable intestinal bacterial load (cfu/g of intestinal contents) at various dilution cutoffs. Limits of detection represent the approximate titer within intestinal contents that a species would need in order to be detected at the corresponding dilution cutoff.

(B) Approximate numbers of total cfu detected on ANB agar (general, non-selective anaerobic media) at each dilution cutoff. Color-coded as in (A).

(C) Average sampling depths of various landmark microbiota surveys performed by 16S rRNA sequencing using either the Sanger methods or 454 method (Kroes, Lepp et al. 1999; Eckburg, Bik et al. 2005; Dethlefsen, Huse et al. 2008; Turnbaugh, Hamady et al. 2008). Surveys are color-coded as in (A) and (B) to indicate the corresponding dilution cutoff surpassed. Note the presence of colony types on some media that would not have been detected at the sampling depth of any of the sequencing surveys.

(D) Estimated titers of a panel of bacterial isolates picked from the plates in (A). Isolates picked based on unique-appearing colony morphology, with titers estimated by number of similar-appearing colonies on the same media type. By this method, some degree of redundancy within each media type and redundancy between certain media types should be assumed.

Figure 3.10

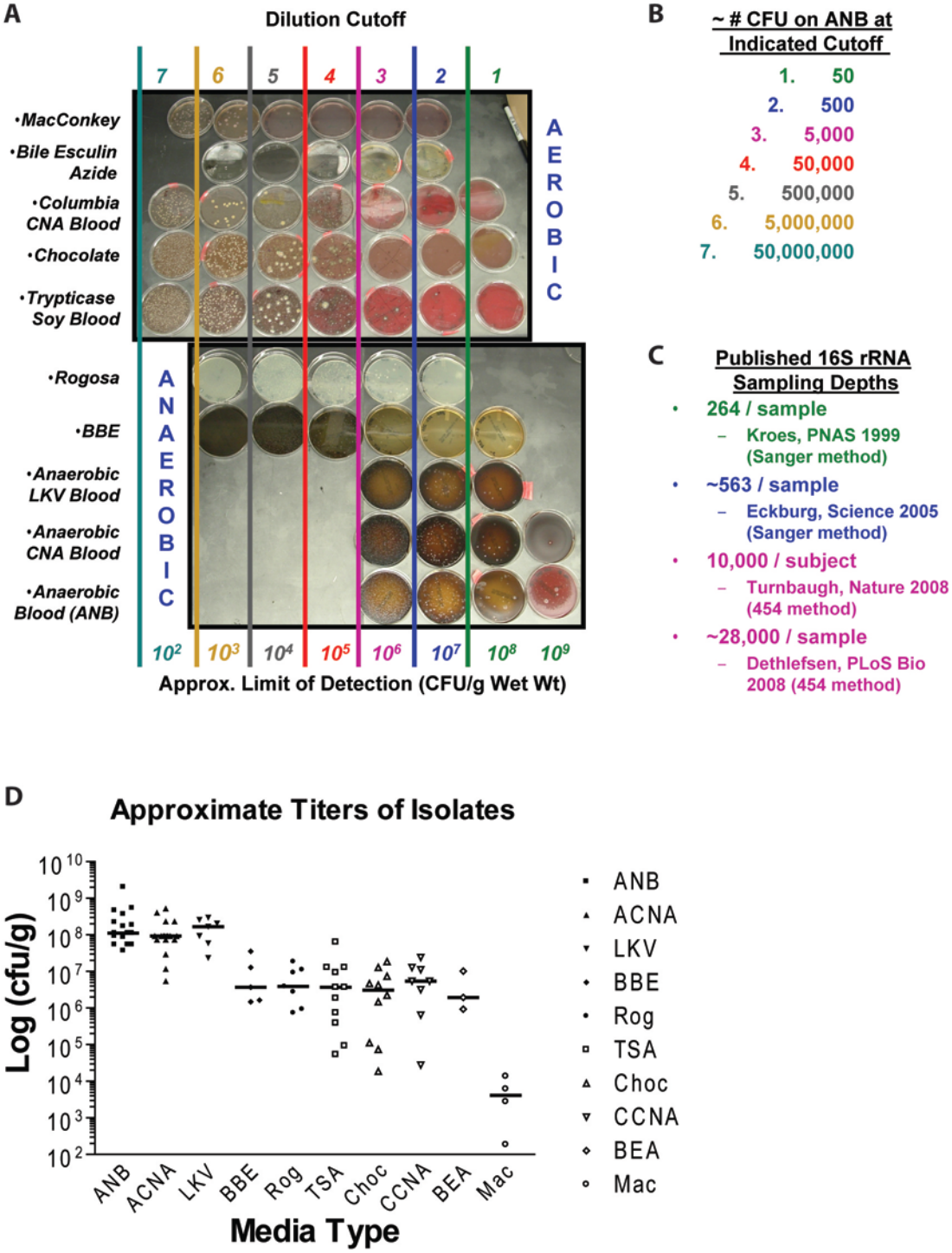
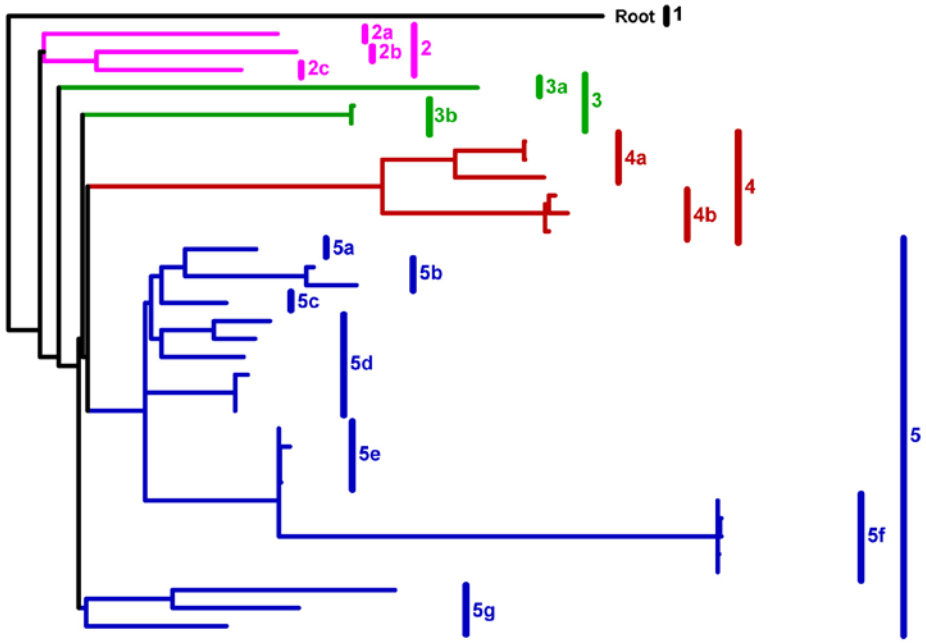

A
Dilution Cutoff
7
6
5
4
3
2
1
•MacConkey
•Bile Esculin Azide
•Columbia CNA Blood
•Chocolate
•Trypticase Soy Blood
AEROBIC
•Rogosa
•BBE
•Anaerobic LKV Blood
•Anaerobic CNA Blood
•Anaerobic Blood (ANB)
ANAEROBIC
10^2 10^3 10^4 10^5 10^6 10^7 10^8 10^9
Approx. Limit of Detection (CFU/g Wet Wt)
B
~ # CFU on ANB at Indicated Cutoff
1. 50
2. 500
3. 5,000
4. 50,000
5. 500,000
6. 5,000,000
7. 50,000,000
C
Published 16S rRNA Sampling Depths
• 264 / sample
– Kroes, PNAS 1999 (Sanger method)
• ~563 / sample
– Eckburg, Science 2005 (Sanger method)
• 10,000 / subject
– Turnbaugh, Nature 2008 (454 method)
• ~28,000 / sample
– Dethlefsen, PLoS Bio 2008 (454 method)
D
Approximate Titers of Isolates
Log (cfu/g)
10^{10} 10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2
ANB ACNA LKV BBE Rog TSA Choc CCNA BEA Mac
Media Type
ANB
ACNA
LKV
BBE
Rog
TSA
Choc
CCNA
BEA
Mac

Figure 3.11: Diversity of bacterial isolates from selective media (subset).

A subset of isolates isolated anaerobically from ANB agar, anaerobic reducible CNA blood agar, or LKV agar or aerobically from chocolate agar were classified according to 16S rRNA gene sequence using the Classifier and SeqMatch function of the Ribosomal Database Project (RDP, Release 10, Update 15). A phylogenetic tree was constructed using the RDP Treebuilder application with an archaeal 16S sequence as the outgroup. Taxonomic assignments to the genus or family level by the RDP Classifier are listed in the figure key.

Figure 3.11



KEY

1. Root: ***Methanocaldococcus jannaschii*** DSM 2661 (Archaeal sequence from RDP)
2. Phylum: *Proteobacteria*
   a. Class: *Betaproteobacteria;* Order: *Burkholderiales;* Family: *Alcaligenaceae;* Genus: *Bordetella*
   b. Class: *Gammaproteobacteria;* Order: *Enterobacteriales;* Family: *Enterobacteriaceae*
   c. Class: *Gammaproteobacteria;* Order: *Pseudomonadales;* Family: *Moraxellaceae;* Genus: *Acinetobacter*
3. Phylum: *Actinobacteria;* Class: *Actinobacteria;*
   a. Subclass: *Coriobacteridae;* Order: *Coriobacteriales;* Suborder: *Coriobacterineae;* Family: *Coriobacteriaceae;* Genus: *Eggerthella*
   b. Subclass: *Actinobacteridae;* Order: *Actinomycetales;* Suborder: *Propionibacterineae;* Family: *Propionibacteriaceae;* Genus: *Propionibacterium*
4. Phylum: *Bacteroidetes;* Class: *Bacteroidetes;* Order: *Bacteroidales*
   a. Family: *Bacteroidaceae;* Genus: *Bacteroides*
   b. Family: *Porphyromonadaceae;* Genus: *Parabacteroides*
5. Phylum: *Firmicutes*
   a. Class: *Bacilli;* Order: *Bacillales;* Family: *Bacillaceae;* Subfamily: *Bacillaceae 1;* Supergenus: *Bacillus;* Genus: *Bacillus c*
   b. Class: *Bacilli;* Order: *Bacillales;* Family: *Staphylococcaceae;* Genus: *Staphylococcus*
   c. Class: *Bacilli;* Order: *Lactobacillales;* Family: *Enterococcaceae;* Genus: *Enterococcus*
   d. Class: *Bacilli;* Order: *Lactobacillales;* Family: *Lactobacillaceae;* Genus: *Lactobacillus*
   e. Class: *Bacilli;* Order: *Lactobacillales;* Family: *Streptococcaceae;* Genus: *Lactococcus*
   f. Class: *Erysipelotrichi;* Order: *Erysipelotrichales;* Family: *Erysipelotrichaceae;* Genus: *Allobaculum*
   g. Class: *Clostridia;* Order: *Clostridiales;* Family: *Lachnospiraceae*

Figure 3.12: Preparation of selective mixed cultures of intestinal contents for gavage.

An aliquot of frozen intestinal contents (see Figure 3.3 and Table 3.1) was thawed, serially diluted, and cultured anaerobically on the indicated media types. 3-day mixed cultures were harvested from the indicated culture plates (in squares) and frozen in 20% glycerol (See Table 3.2). Titer of the frozen stocks was determined prior to use in experiments. For gavage, aliquots of each culture were subsequently thawed and adjusted to desired doses for gavage.

Figure 3.12

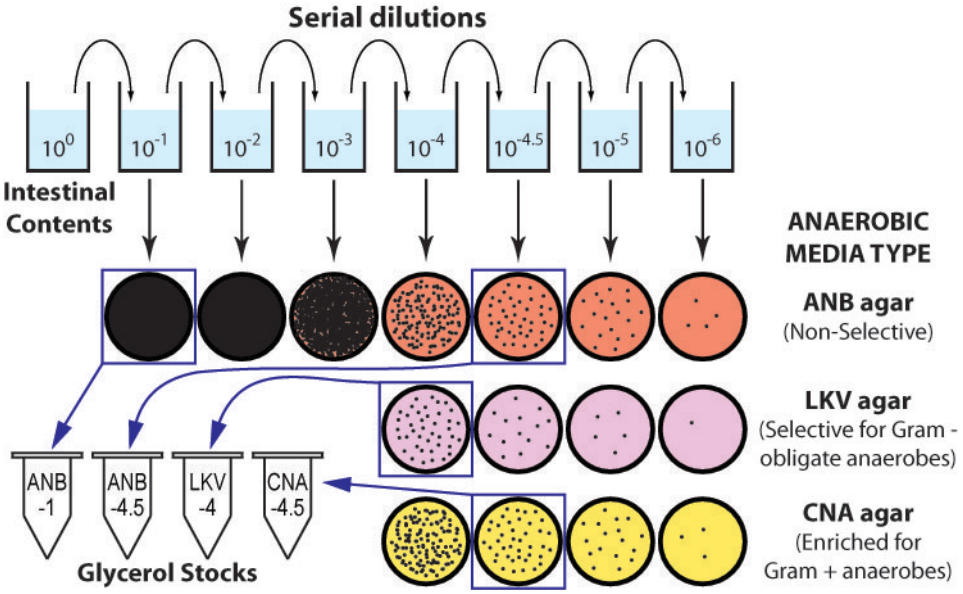

Serial dilutions
10^0
10^-1
10^-2
10^-3
10^-4
10^-4.5
10^-5
10^-6
Intestinal Contents
ANAEROBIC MEDIA TYPE
ANB agar
(Non-Selective)
LKV agar
(Selective for Gram - obligate anaerobes)
CNA agar
(Enriched for Gram + anaerobes)
ANB -1
ANB -4.5
LKV -4
CNA -4.5
Glycerol Stocks

Figure 3.13: Selective mixed cultures of intestinal contents induce intestinal gross pathology in antibiotic-pre-treated dnKO mice.

Pathology scores of intestines from antibiotic-pre-treated dnKO mice gavaged with sterile PBS or with the indicated anaerobic mixed cultures of intestinal contents mixed 1:1 with aerobic cultures grown on chocolate agar at 0 dilution (see Figure 3.12 and Table 3.2). Individual (symbols) and median (bars) pathology scores are displayed. Gavage doses: ANB -1 = $6.6x10^7$ total cfu/mouse; ANB -4.5 = $6.4x10^7$ total cfu/mouse; LKV -4 = $7.3x10^7$ total cfu/mouse; CNA -4.5 = $5.4x10^7$ total cfu/mouse. Statistical significance relative to PBS determined by Dunn's multiple comparison test: n.s., $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$. The screen was unrepeated.

(A) Cecum gross pathology scores.

(B) Transverse colon pathology scores.

Figure 3.13

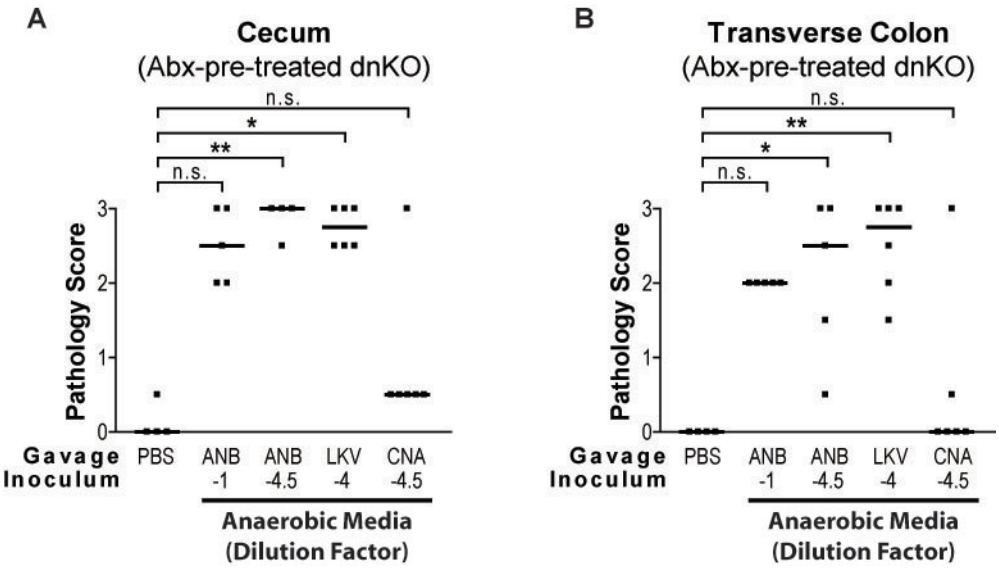

A
Cecum
(Abx-pre-treated dnKO)
n.s.
*
**
n.s.
Pathology Score
3
2
1
0
Gavage Inoculum
PBS
ANB -1
ANB -4.5
LKV -4
CNA -4.5
Anaerobic Media (Dilution Factor)
B
Transverse Colon
(Abx-pre-treated dnKO)
n.s.
**
*
n.s.
Pathology Score
3
2
1
0
Gavage Inoculum
PBS
ANB -1
ANB -4.5
LKV -4
CNA -4.5
Anaerobic Media (Dilution Factor)

Figure 3.14: Selective mixed cultures of intestinal contents induce mucosal inflammation in antibiotic-pre-treated dnKO mice.

Quantitative histologic disease metrics of intestines from antibiotic-pre-treated dnKO mice gavaged with sterile PBS or with selective mixed cultures of intestinal contents as described in Figure 3.13. displayed as mean +/- SEM. Statistical significance relative to PBS determined by Dunnett's multiple comparison test: n.s., $p > 0.05$; *, $p < 0.05$.

(A) Transverse colon crypt heights.

(B) Rectum crypt heights.

Figure 3.14

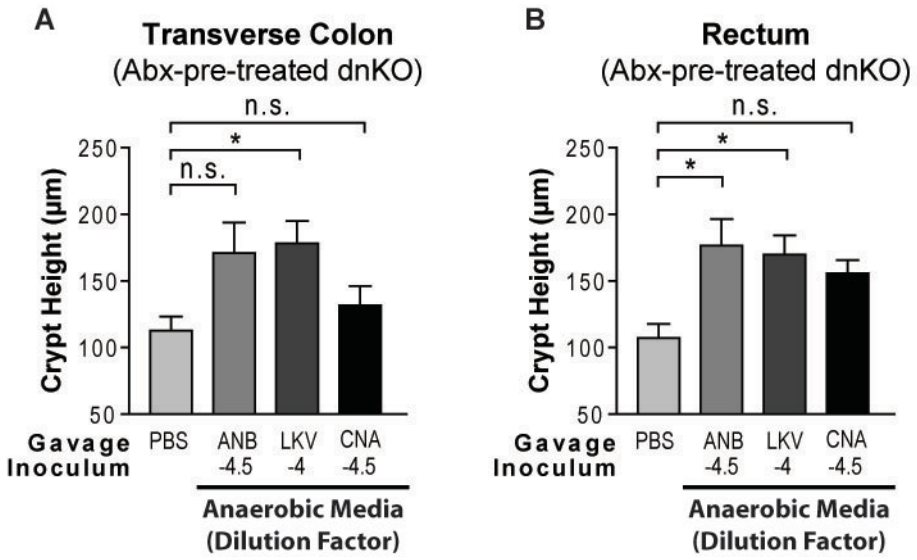

A
Transverse Colon
(Abx-pre-treated dnKO)
n.s.
*
n.s.
250
200
150
100
50
Crypt Height (µm)
Gavage Inoculum
PBS
ANB -4.5
LKV -4
CNA -4.5
Anaerobic Media (Dilution Factor)
B
Rectum
(Abx-pre-treated dnKO)
n.s.
*
*
250
200
150
100
50
Crypt Height (µm)
Gavage Inoculum
PBS
ANB -4.5
LKV -4
CNA -4.5
Anaerobic Media (Dilution Factor)

Figure 3.15: Pure cultures of diverse commensal intestinal Bacteroidetes isolates induce intestinal pathology in antibiotic-pre-treated dnKO mice.

Screen for colitis induction by Bacteroidetes isolates. Cecum gross pathology scores of antibiotic-pre-treated dnKO mice gavaged with $1x10^8$ cfu/mouse of pure cultures of the indicated primary bacterial isolates (see Table 3.3). Unrepeated for *B. uniformis* and *P. goldsteinii*.

Figure 3.15

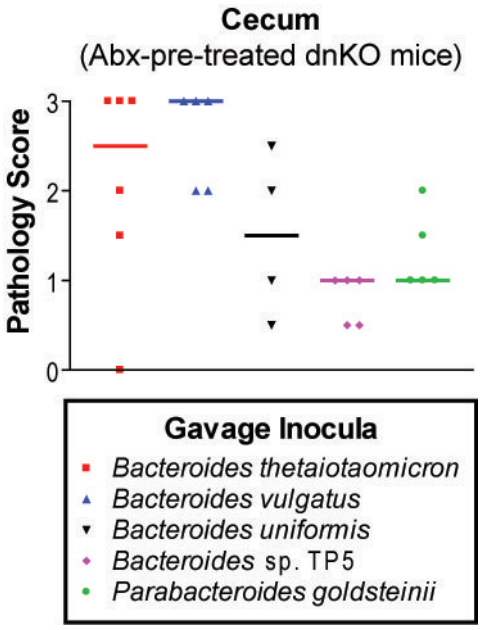

Cecum
(Abx-pre-treated dnKO mice)
Pathology Score
3
2
1
0
Gavage Inocula
Bacteroides thetaiotaomicron
Bacteroides vulgatus
Bacteroides uniformis
Bacteroides sp. TP5
Parabacteroides goldsteinii

Figure 3.16: Pure cultures of diverse commensal intestinal Bacteroidetes isolates induce colitis in antibiotic-pre-treated dnKO mice.

Representative H&E stained cecal histology of antibiotic-pre-treated dnKO mice gavaged with *Bacteroides* species as described in Figure 3.15. A non-inflamed cecum from an antibiotic-pre-treated *B. thetaiotaomicron*-gavaged *Il10r2*$^{+/-}$ control mouse is shown for comparison. Scale bar = 200 µm.

Figure 3.16

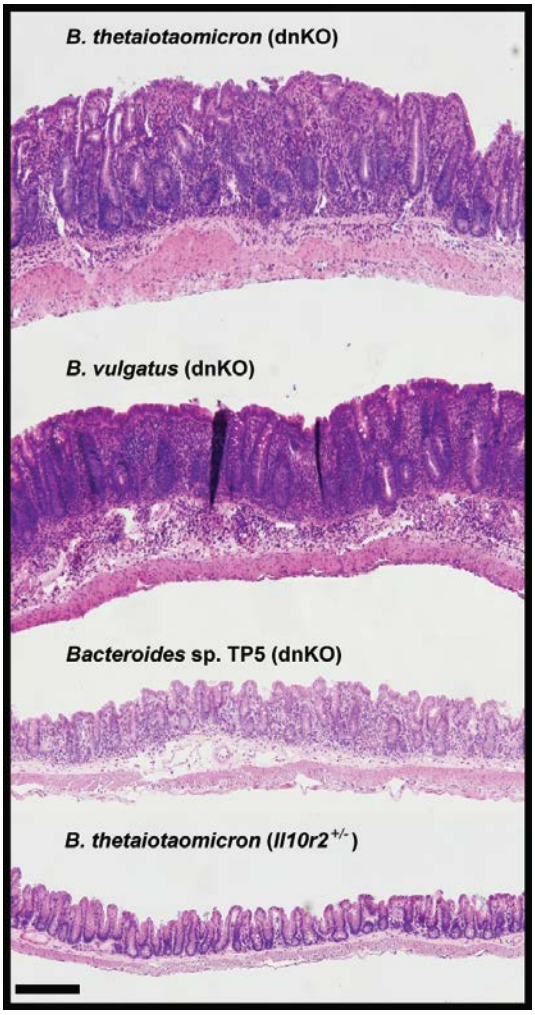

B. thetaiotaomicron (dnKO)
B. vulgatus (dnKO)
Bacteroides sp. TP5 (dnKO)
B. thetaiotaomicron (Il10r2+/-)

Figure 3.17: *Bacteroides thetaiotaomicron* induces pathology in antibiotic-pre-treated dnKO but not *Il10r2*$^{+/-}$ mice.

Pathology scores of antibiotic-pre-treated mice of the indicated genotypes gavaged with PBS or a pure culture of the *B. thetaiotaomicron* isolate described in Table 3.3 ($7x10^7$ cfu/mouse). Statistical significance determined by Kruskal-Wallis test with post-hoc Dunn's test. All significant pairwise comparisons are displayed: *, $p < 0.05$.

(A) Cecum pathology scores: $H_2 = 12.26$, $p = 0.0022$.

(B) Transverse colon pathology scores: $H_2 = 11.36$, $p = 0.0034$.

Figure 3.17

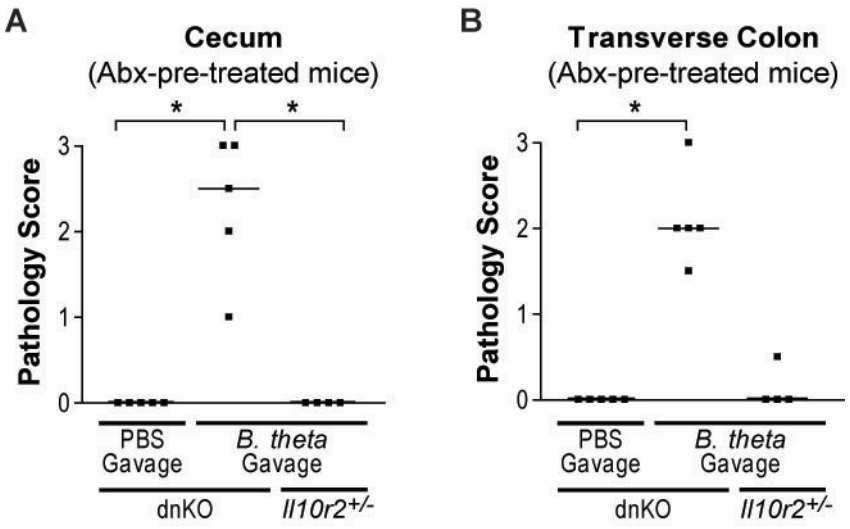

A
Cecum
(Abx-pre-treated mice)
Pathology Score
0
1
2
3
*
*
PBS Gavage
B. theta Gavage
dnKO
Il10r2+/-
B
Transverse Colon
(Abx-pre-treated mice)
Pathology Score
0
1
2
3
*
PBS Gavage
B. theta Gavage
dnKO
Il10r2+/-

Figure 3.18: *Bacteroides thetaiotaomicron* induces colitis in antibiotic-pre-treated dnKO but not *Il10r2*$^{+/-}$ mice.

Representative H&E stained rectal histology of antibiotic-pre-treated mice gavaged with PBS or a pure culture of *B. thetaiotaomicron* as described in Figure 3.17. Scale bar = 50 µm.

Figure 3.18

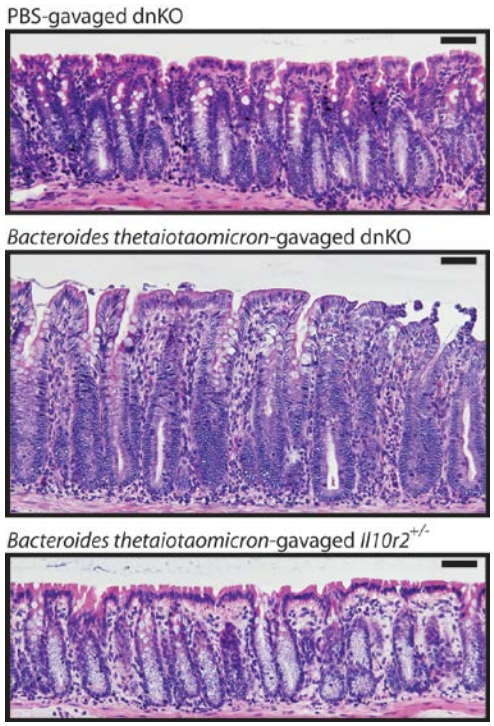

PBS-gavaged dnKO
Bacteroides thetaiotaomicron-gavaged dnKO
Bacteroides thetaiotaomicron-gavaged *Il10r2*$^{+/-}$

Figure 3.19: Quantification of *Bacteroides thetaiotaomicron*-induced colitis in antibiotic-pre-treated dnKO mice.

Quantitative histologic disease metrics of intestines from mice described in Figures 3.17 and 3.18 displayed as mean +/- SEM. Statistical significance determined by 1-way ANOVA with post-hoc Tukey's test. All significant pairwise comparisons are displayed if the omnibus p-value by ANOVA was significant: *, $p < 0.05$; **, $p < 0.01$.

(A) Transverse colon crypt height: $F_{2,10} = 8.596$, $p = 0.0067$.

(B) Rectum crypt height: $F_{2,10} = 12.55$, $p = 0.0019$.

(C) Transverse colon crypt width: $F_{2,10} = 2.057$, $p = 0.1786$.

(D) Rectum crypt width: $F_{2,10} = 12.05$, $p = 0.0022$.

Figure 3.19

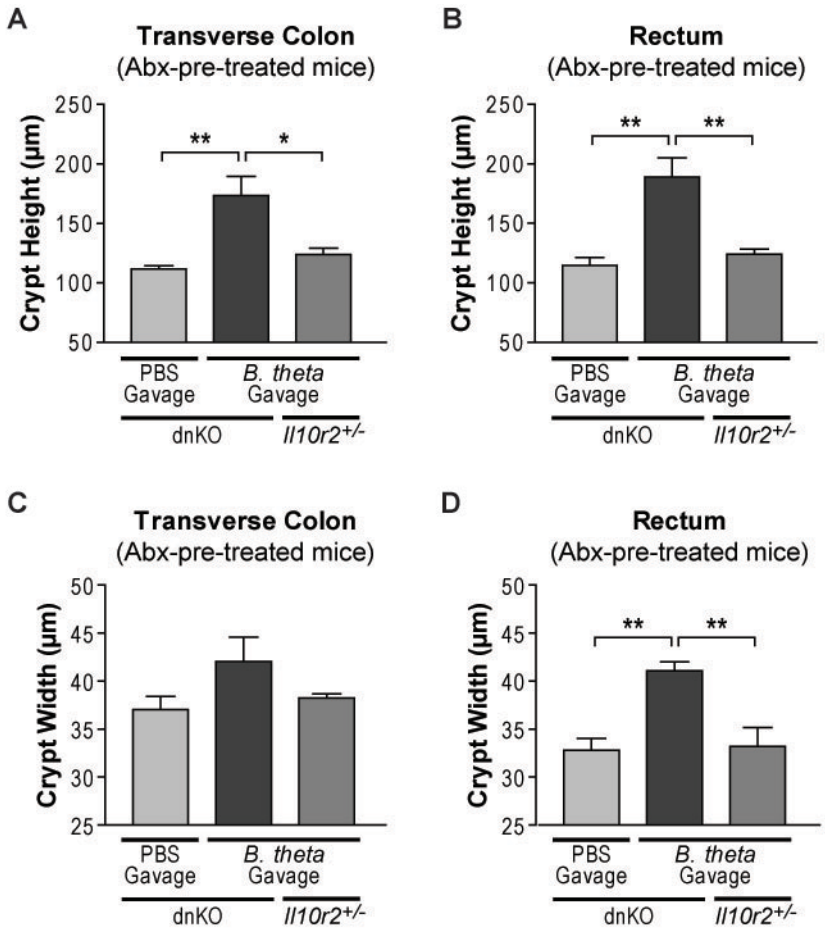

A
Transverse Colon
(Abx-pre-treated mice)
Crypt Height (µm)
250
200
150
100
50
**
*
PBS Gavage
B. theta Gavage
dnKO
Il10r2+/-
B
Rectum
(Abx-pre-treated mice)
Crypt Height (µm)
250
200
150
100
50
**
**
PBS Gavage
B. theta Gavage
dnKO
Il10r2+/-
C
Transverse Colon
(Abx-pre-treated mice)
Crypt Width (µm)
50
45
40
35
30
25
PBS Gavage
B. theta Gavage
dnKO
Il10r2+/-
D
Rectum
(Abx-pre-treated mice)
Crypt Width (µm)
50
45
40
35
30
25
**
**
PBS Gavage
B. theta Gavage
dnKO
Il10r2+/-

Figure 3.20: Antibiotic treatment eliminates cultivable intestinal *Bacteroides* from the intestinal microbiota.

Fecal samples from 4-week old untreated dnKO mice or from dnKO mice treated with antibiotics (metronidazole + ciprofloxacin) for ≥3 weeks were titered in parallel anaerobically on non-selective (ANB) agar and *Bacteroides* bile esculin (BBE) agar. Titers from individual mice (symbols) and means of $\log_{10}$-transformed titers (bars) are displayed. Limit of detection = 17 cfu per sample. Statistical significance of difference between ANB titers was determined by unpaired t-test ($\log_{10}$-transformed titers). Representative of ≥2 independent experiments per group.

Figure 3.20

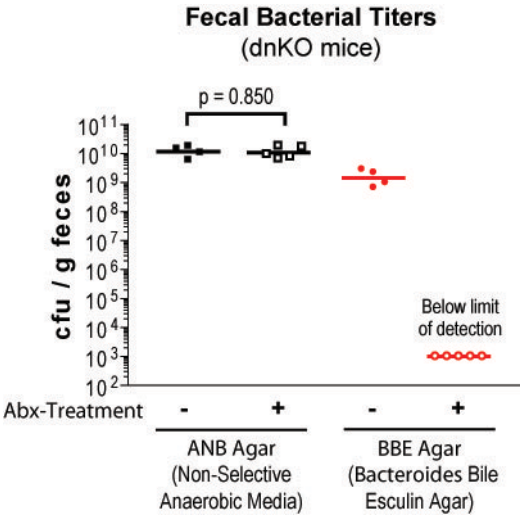

Fecal Bacterial Titers
(dnKO mice)
p = 0.850
cfu / g feces
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
Below limit of detection
Abx-Treatment
-
+
-
+
ANB Agar (Non-Selective Anaerobic Media)
BBE Agar (Bacteroides Bile Esculin Agar)

Figure 3.21: Representative *Bacteroides* colonies and fecal titer plate.

(A) Pure cultures of (1.) *B. vulgatus*, (2.) *Bacteroides* sp. TP5, and (3.) *B. thetaiotaomicron* isolates streaked on BBE media and incubated anaerobically for 48 hrs. Insets: representative colonies of each isolate exhibiting characteristic size, morphology, and pigmentation (scale bar = 1 mm). The culture plate was photographed with a digital camera, and magnified images of representative colonies of each isolate (indicated by black squares in the low-power image) were photographed on a dissecting microscope at 12.5X magnification images with both backlighting and reflected lighting at an exposure time of 1/500 s.

(B) Representative BBE fecal titer plate from a *B. thetaiotaomicron*-gavaged mouse exhibiting characteristic *B. thetaiotaomicron* colony appearance (imaged after 72-hr anaerobic incubation). Bacterial identity confirmed as *B. thetaiotaomicron* by 16S rRNA gene sequencing of representative colonies. Titer plates from mice gavaged with *B. vulgatus* and *Bacteroides* sp. TP5 also exhibited colonies with the respective characteristic morphology (not shown).

Figure 3.21

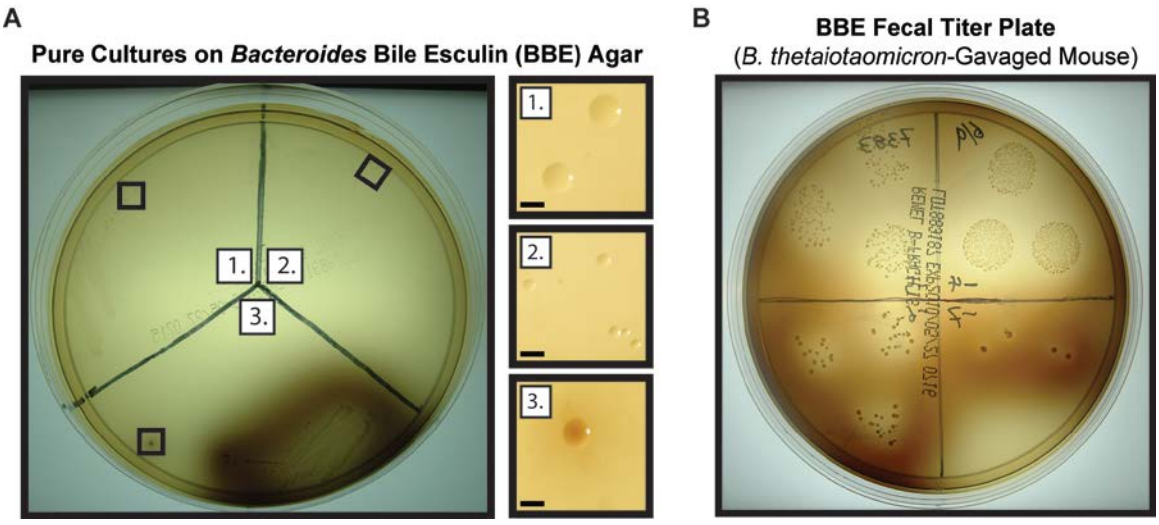

A
Pure Cultures on *Bacteroides* Bile Esculin (BBE) Agar
1.
2.
3.
1.
2.
3.
B
BBE Fecal Titer Plate
(*B. thetaiotaomicron*-Gavaged Mouse)

Figure 3.22: Gavaged *Bacteroides* stably colonize antibiotic-pre-treated mice at high levels regardless of host genotype.

Serial fecal titers on BBE agar from antibiotic-pre-treated mice gavaged on Day 0 with the indicated inoculum.

(A) Antibiotic-pre-treated mice of the indicated genotypes gavaged with sterile PBS or a pure culture of *B. thetaiotaomicron* ($6.6x10^7$ cfu/mouse). Individual titers (symbols) and means of $\log_{10}$-transformed titers (lines) are displayed. Statistical significance of differences between $\log_{10}$-transformed fecal titers from *B. thetaiotaomicron*-gavaged dnKO mice and *Il10r2*$^{+/-}$ mice determined by unpaired t-test.

(B) Antibiotic-pre-treated dnKO mice gavaged with a pure culture of *Bacteroides* sp. TP5 ($8.9x10^7$ cfu/mouse).

(C) Antibiotic-pre-treated dnKO mice gavaged with a pure culture of *Bacteroides vulgatus* ($9.1x10^7$ cfu/mouse).

(D) *Bacteroides* titers from (A-C) overlaid. Representative of ≥2 independent experiments with 2-6 mice per group for *B. thetaiotaomicron* and *Bacteroides* sp TP5; Not repeated for *B. vulgatus* at this gavage dose, but mice gavaged with a 10-fold higher dose became colonized at similar levels.

Figure 3.22

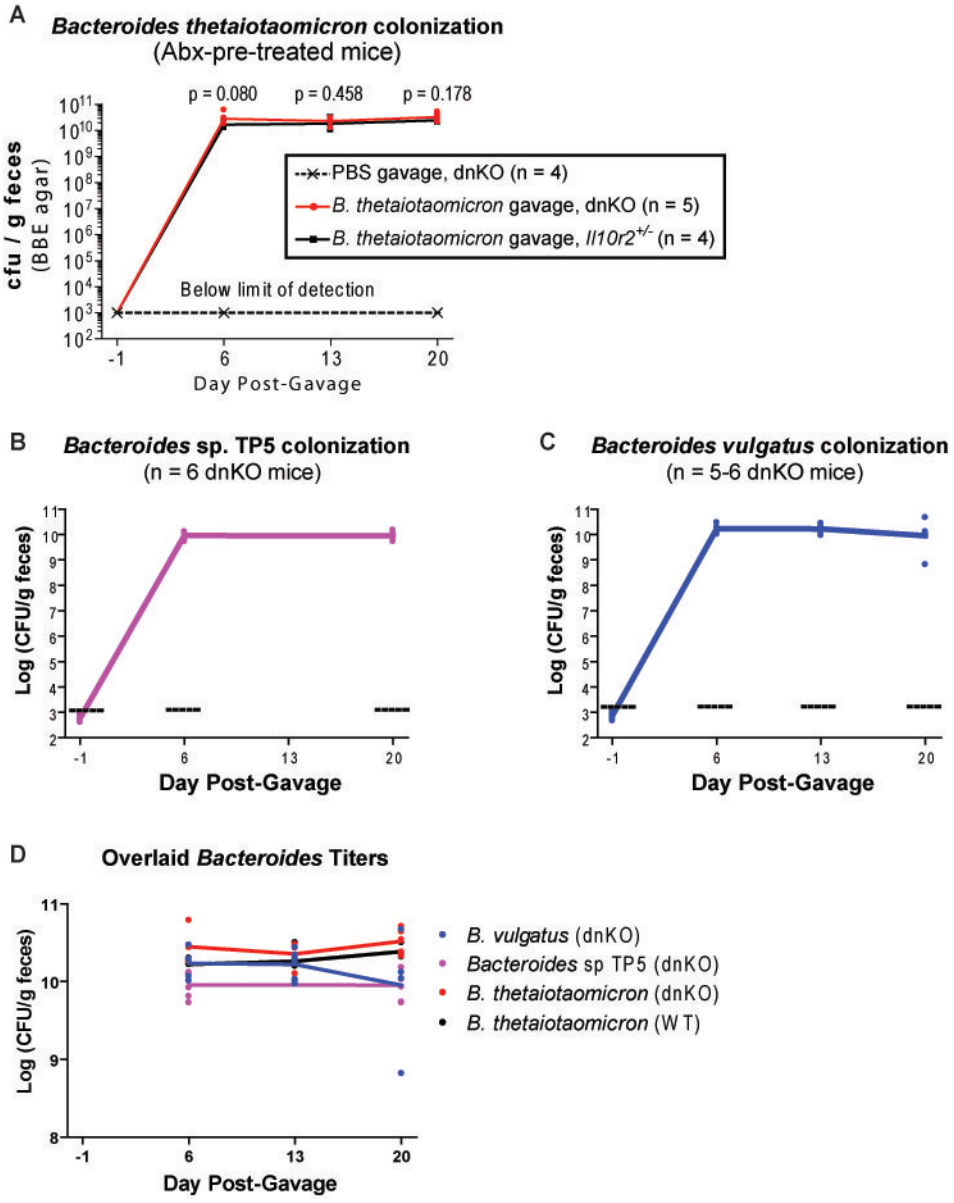

A
Bacteroides thetaiotaomicron colonization
(Abx-pre-treated mice)
p = 0.080
p = 0.458
p = 0.178
cfu / g feces
(BBE agar)
PBS gavage, dnKO (n = 4)
B. thetaiotaomicron gavage, dnKO (n = 5)
B. thetaiotaomicron gavage, Il10r2+/- (n = 4)
Below limit of detection
Day Post-Gavage
B
Bacteroides sp. TP5 colonization
(n = 6 dnKO mice)
Log (CFU/g feces)
Day Post-Gavage
C
Bacteroides vulgatus colonization
(n = 5-6 dnKO mice)
Log (CFU/g feces)
Day Post-Gavage
D
Overlaid Bacteroides Titers
Log (CFU/g feces)
Day Post-Gavage
B. vulgatus (dnKO)
Bacteroides sp TP5 (dnKO)
B. thetaiotaomicron (dnKO)
B. thetaiotaomicron (WT)

Figure 3.23: *Bacteroides* colonization of gavaged, antibiotic-pre-treated mice is high, consistent, and normally distributed.

Frequency histogram of all 92 *B. thetaiotaomicron* fecal titer measurements that we have performed on gavaged, antibiotic-pre-treated mice regardless of mouse genotype. Fecal titers were performed 6, 13, or 20 days post-gavage and are compiled from multiple independent experiments with 4-9 mice per experiment. Gavage doses ranged from $6.6x10^7$ to $8.6x10^8$ cfu/mouse. After exclusion of the outlier value of 8.78, the data are normally distributed as determined by the D'Agostino & Pearson K^2 omnibus normality test: $p = 0.5894$, $K^2 = 1.057$, skewness = -0.153, kurtosis = 0.3345. Analysis performed using GraphPad Prism version 5.01 for Windows (GraphPad Software) because the test is not available in Prism v3.02.

Figure 3.23

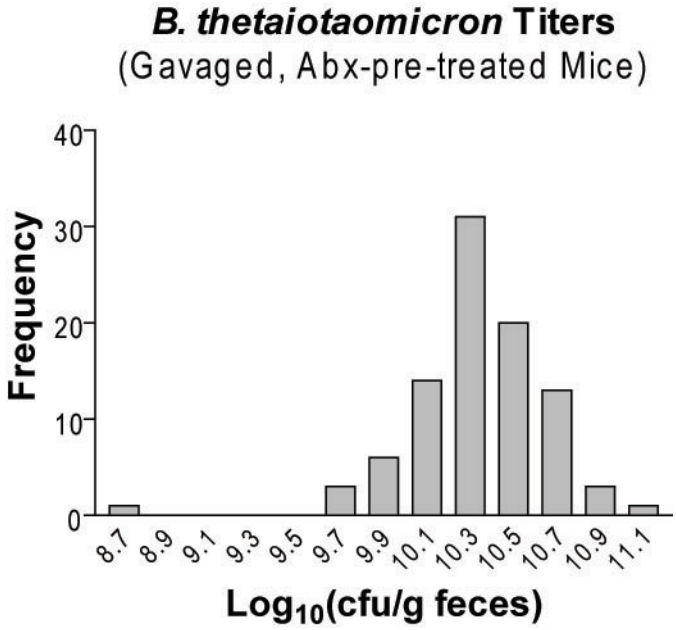

B. thetaiotaomicron Titers
(Gavaged, Abx-pre-treated Mice)
Frequency
40
30
20
10
0
8.7
8.9
9.1
9.3
9.5
9.7
9.9
10.1
10.3
10.5
10.7
10.9
11.1
Log10(cfu/g feces)

Figure 3.24: Commensal Enterobacteriaceae but not commensal *Bacteroides* are strikingly enriched in spontaneous colitis.

(A to C) Fecal samples from 4 week old untreated mice of the indicated genotypes or mice treated with antibiotics for ≥3 weeks titered in parallel anaerobically on ANB and BBE agar and aerobically on MacConkey agar (selective for Enterobacteriaceae). *Il10r2*$^{+/-}$ and dnKO mice were co-housed; data compiled from ≥2 cages/group. Statistical significance determined by 1-way ANOVA (ANB titers: $F_{3,13} = 1.344$, $p = 0.3031$) or unpaired t-test ($\log_{10}$-transformed titers).

(D) Approximate percentages of BBE- and MacConkey-cultivable fecal bacteria in untreated *Il10r2*$^{+/-}$ and dnKO mice from (A) to (C) calculated by dividing BBE titers and MacConkey titers of by the corresponding titers of total cultivable bacteria (on non-selective ANB agar). Percentages of "Other" were calculated by subtracting BBE and MacConkey percentages from 100%. Genotypes and housing arrangements of the mice are shown.

(E and F) Proportions of total cultivable bacteria from (D) displayed using a logarithmic scale. Displayed as individual (symbols) and means of $\log_{10}$-transformed proportions (bars). Unpaired t-test ($\log_{10}$-transformed proportions).

Figure 3.24

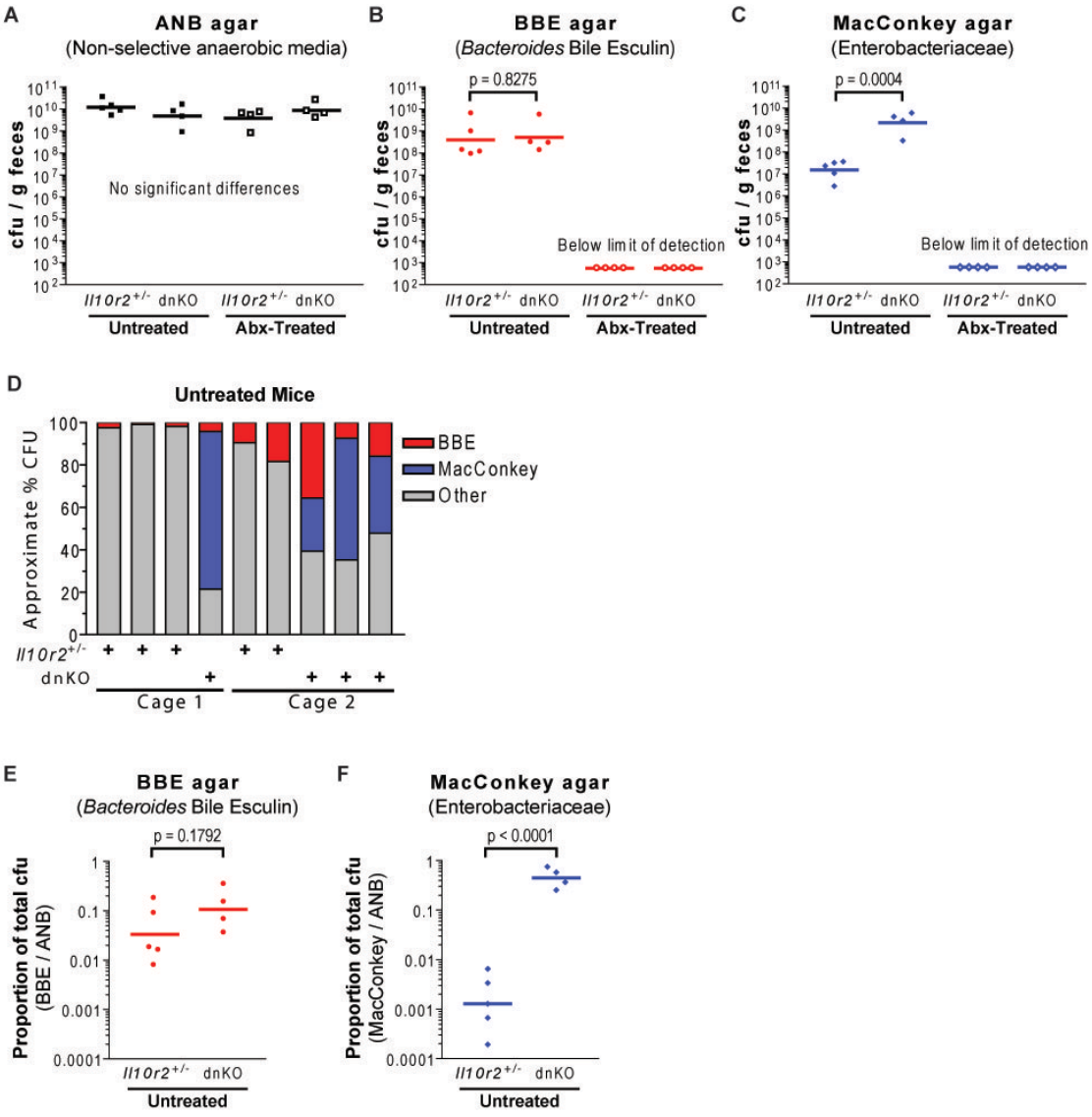

A
ANB agar
(Non-selective anaerobic media)
cfu / g feces
No significant differences
Il10r2+/- dnKO
Il10r2+/- dnKO
Untreated
Abx-Treated
B
BBE agar
(Bacteroides Bile Esculin)
p = 0.8275
Below limit of detection
C
MacConkey agar
(Enterobacteriaceae)
p = 0.0004
Below limit of detection
D
Untreated Mice
Approximate % CFU
BBE
MacConkey
Other
Il10r2+/-
dnKO
Cage 1
Cage 2
E
BBE agar
(Bacteroides Bile Esculin)
p = 0.1792
Proportion of total cfu
(BBE / ANB)
F
MacConkey agar
(Enterobacteriaceae)
p < 0.0001
Proportion of total cfu
(MacConkey / ANB)

Figure 3.25: Isolation of *Escherichia coli* from the feces of an untreated dnKO mouse.

A prominent, distinctive bacterial colony type (convex, circular, white-yellow, entire margins, strongly β-hemolytic, 2-3 mm diameter after 48 hrs incubation) was isolated from the feces of an untreated (spontaneously colitic) dnKO mouse cultured on ANB agar. The colony type comprised 33% of all colonies observed on the titer plate ($2.0x10^9$ cfu/g out of $6.0x10^9$ total cfu/g).

(A) The isolate was identified by as *Escherichia coli*, a member of the family Enterobacteriaceae, by 16S rRNA sequence identity to the closest cultured isolate, supplemented by phenotypic identification (phenotyping performed by W. M. Dunne, Jr.; in the Barnes-Jewish Hospital Medical Microbiology Laboratory. Table lists isolate ID, most similar bacterial type strain, percent sequence identity, and accession number of type strain sequence.

(B) H&E stained histology of the descending colon of the untreated dnKO mouse from which *E. coli* was isolated and of a co-housed untreated *Il10r2*$^{+/-}$ mouse. Scale bar = 200 μm.

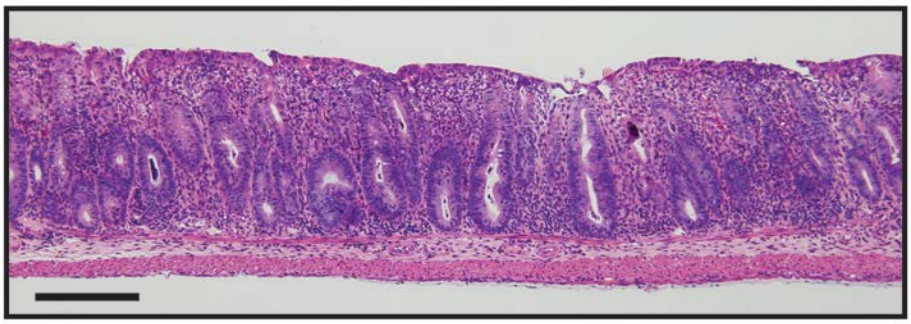
Figure 3.25

A

Isolate ID	Closest Type Strain (Closest Cultured Isolate)	% Identity
dn15.6244.1	*Escherichia fergusonii* (*Escherichia coli* ED1a)	99.8% (100.0%)

B

Untreated dnKO mouse (source of *E. coli* isolate)



Untreated *Il10r2*$^{+/-}$ mouse

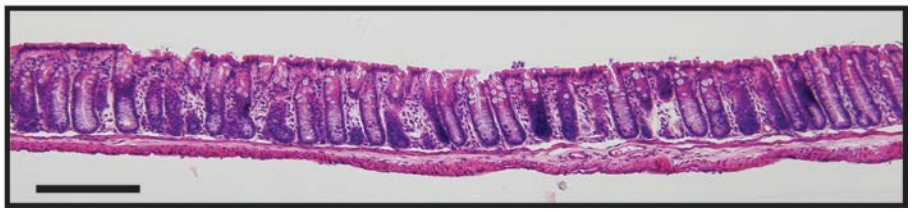


Figure 3.26: *E. coli* stably colonizes gavaged, antibiotic-pre-treated mice.

Serial fecal titers cultured in parallel on BBE and MacConkey agar from antibiotic-pre-treated dnKO mice (5-6 per group) gavaged with sterile PBS, *B. thetaiotaomicron* ($1x10^8$ cfu/mouse), or *Escherichia coli* ($2x10^8$ cfu/mouse). Bacterial identity was determined by colony characteristics, confirmed by sequencing the 16S rRNA gene of representative colonies. Representative of ≥2 experiments per group.

(A) *B. thetaiotaomicron* colonization (BBE agar).

(B) *E. coli* colonization (MacConkey agar).

Figure 3.26

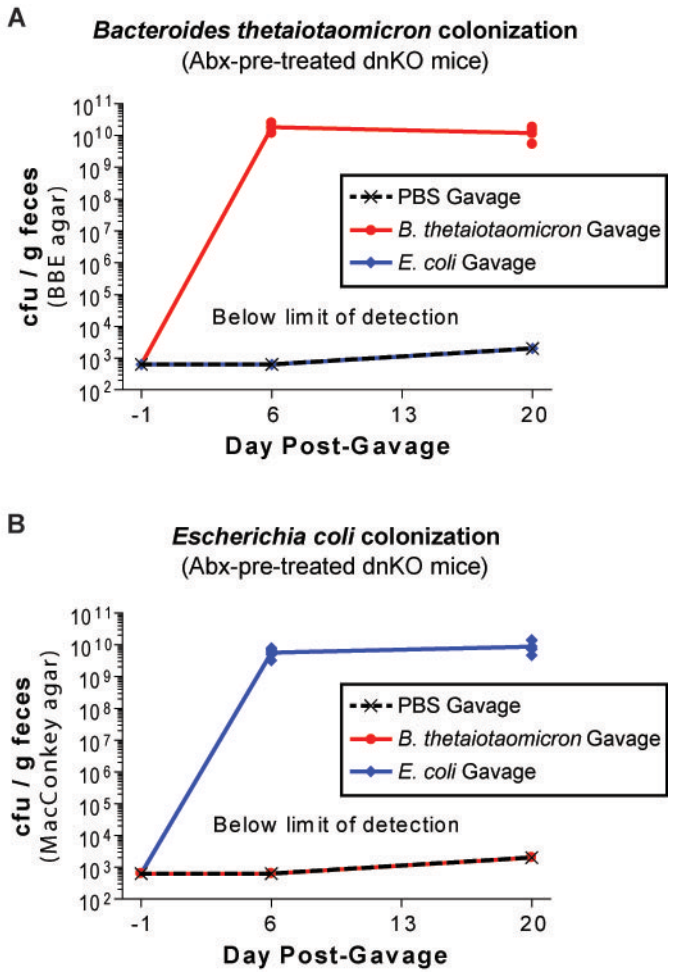

A
Bacteroides thetaiotaomicron colonization
(Abx-pre-treated dnKO mice)
cfu / g feces
(BBE agar)
PBS Gavage
B. thetaiotaomicron Gavage
E. coli Gavage
Below limit of detection
Day Post-Gavage
B
Escherichia coli colonization
(Abx-pre-treated dnKO mice)
cfu / g feces
(MacConkey agar)
PBS Gavage
B. thetaiotaomicron Gavage
E. coli Gavage
Below limit of detection
Day Post-Gavage

Figure 3.27: *E. coli* does not induce significant gross pathology in antibiotic-pre-treated dnKO mice.

Pathology scores of intestines from mice described in Figure 3.26 displayed as individual (symbols) and median (bars) scores. Statistical significance determined by Kruskall-Wallis test with post-hoc Dunn's test. All significant pairwise comparisons are displayed: *, $p < 0.05$; **, $p < 0.01$. Representative of ≥2 experiments per group.

(A) Cecum pathology scores: $H_2 = 11.49$, $p = 0.0032$.

(B) Transverse colon pathology scores: $H_2 = 12.02$, $p = 0.0025$.

Figure 3.27

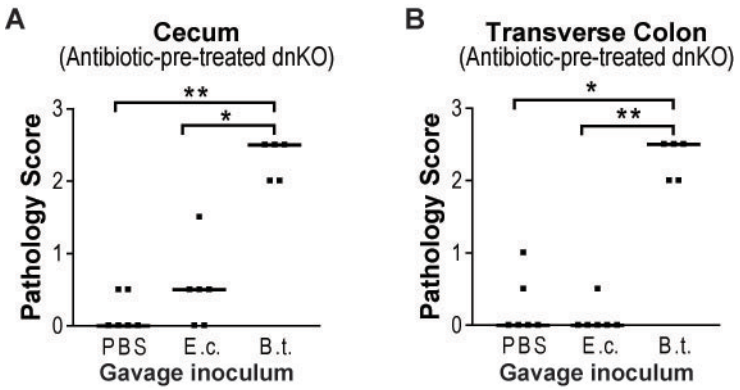

A
Cecum
(Antibiotic-pre-treated dnKO)
**
*
Pathology Score
3
2
1
0
PBS
E.c.
B.t.
Gavage inoculum
B
Transverse Colon
(Antibiotic-pre-treated dnKO)
*
**
Pathology Score
3
2
1
0
PBS
E.c.
B.t.
Gavage inoculum

Figure 3.28: *E. coli* does not induce significant histologic colitis in antibiotic-pre-treated dnKO mice.

Quantitative histologic disease metrics of intestines from mice described in Figure 3.26 displayed as mean +/- SEM. Statistical significance determined by 1-way ANOVA with post-hoc Tukey's test. All significant pairwise comparisons are displayed if omnibus p-value by ANOVA was significant: *, $p < 0.05$.

(A) Transverse colon crypt height: $F_{2,14} = 2.580$, $p = 0.1112$.

(B) Transverse colon crypt width: $F_{2,14} = 5.434$; $p = 0.0179$.

(C) Rectum crypt height: $F_{2,14} = 6.438$, $p = 0.0104$.

(D) Rectum crypt height: $F_{2,14} = 7.268$, $p = 0.0068$.

Figure 3.28

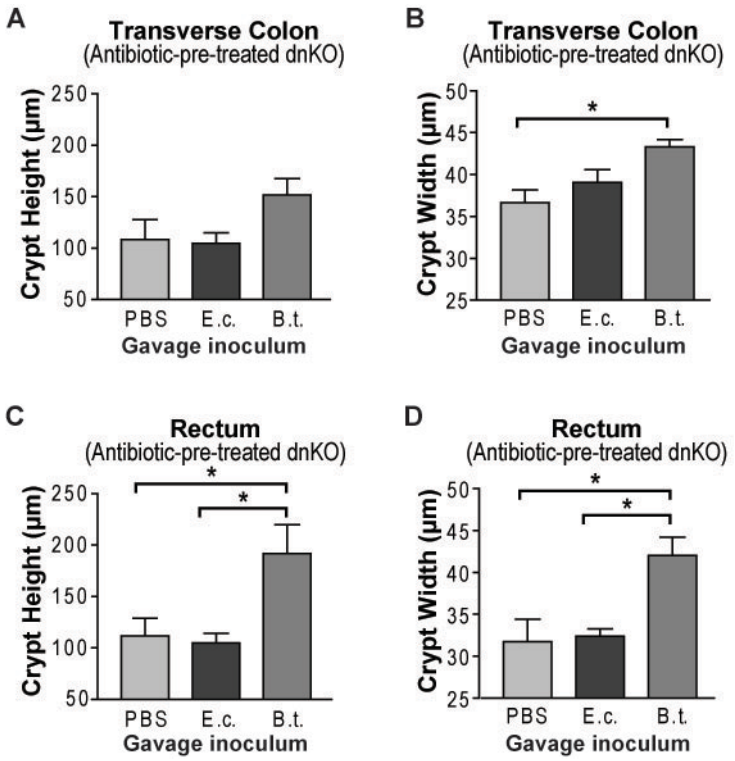

A
Transverse Colon
(Antibiotic-pre-treated dnKO)
Crypt Height (µm)
250
200
150
100
50
PBS
E.c.
B.t.
Gavage inoculum
B
Transverse Colon
(Antibiotic-pre-treated dnKO)
Crypt Width (µm)
50
45
40
35
30
25
*
PBS
E.c.
B.t.
Gavage inoculum
C
Rectum
(Antibiotic-pre-treated dnKO)
Crypt Height (µm)
*
*
250
200
150
100
50
PBS
E.c.
B.t.
Gavage inoculum
D
Rectum
(Antibiotic-pre-treated dnKO)
Crypt Width (µm)
*
*
50
45
40
35
30
25
PBS
E.c.
B.t.
Gavage inoculum

Figure 3.29: Schematic summary of findings.

dnKO mice (genetically susceptible to IBD) develop spontaneous colitis characterized by enrichment of commensal Enterobacteriaceae in the intestinal microbiota compared to *Il10r2*$^{+/-}$ (non-susceptible) mice, which do not develop disease. Antibiotic treatment prevents disease and eliminates both commensal *Bacteroides* and commensal Enterobacteriaceae from the microbiota. After cessation of treatment, mice remain healthy and free of *Bacteroides* and Enterobacteriaceae. Antibiotic-pre-treated mice experimentally colonized with individual isolates of *Bacteroides* or Enterobacteriaceae become robustly colonized independent of host genotype, but disease develops only in IBD-susceptible hosts colonized with *Bacteroides*.

Figure 3.29

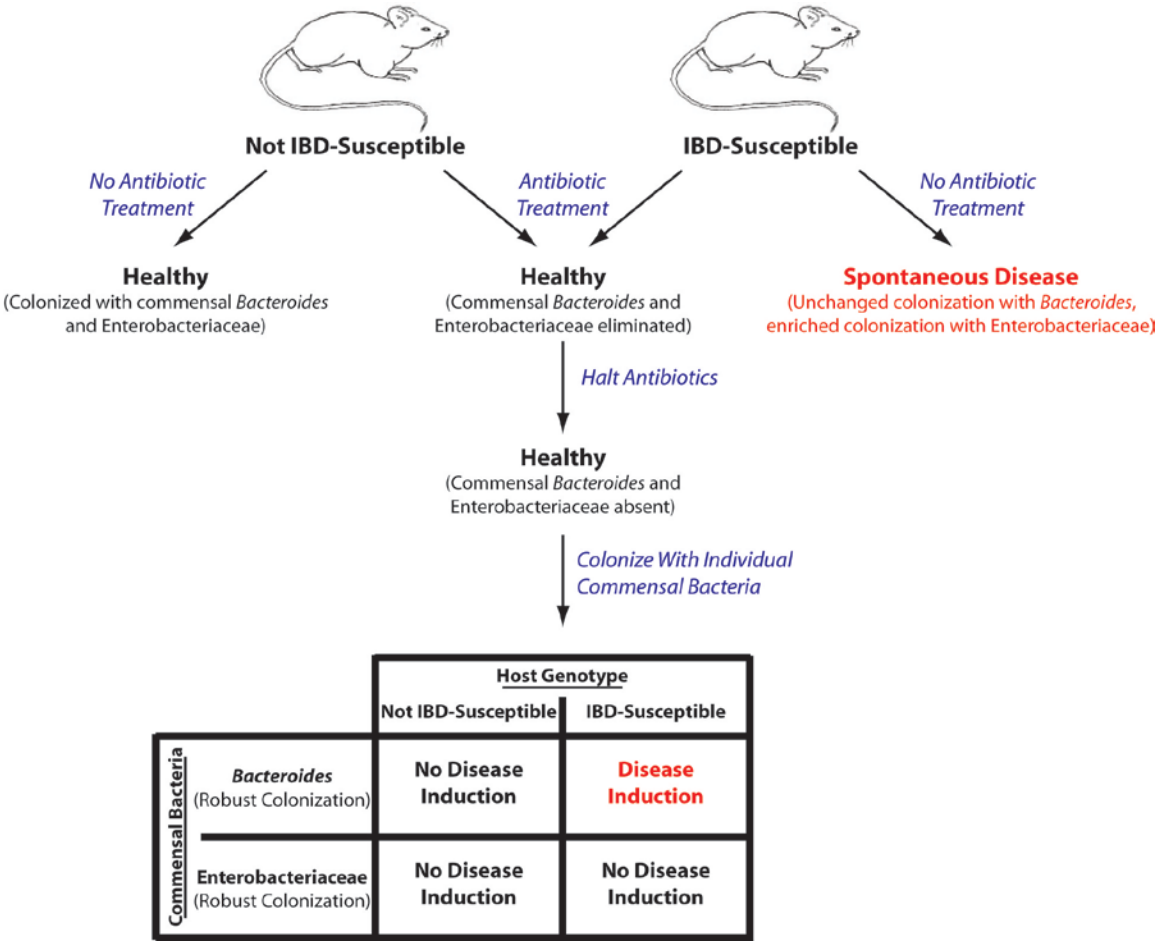

Not IBD-Susceptible
IBD-Susceptible
No Antibiotic Treatment
Antibiotic Treatment
No Antibiotic Treatment
Healthy
(Colonized with commensal Bacteroides and Enterobacteriaceae)
Healthy
(Commensal Bacteroides and Enterobacteriaceae eliminated)
Spontaneous Disease
(Unchanged colonization with Bacteroides, enriched colonization with Enterobacteriaceae)
Halt Antibiotics
Healthy
(Commensal Bacteroides and Enterobacteriaceae absent)
Colonize With Individual Commensal Bacteria
Host Genotype
Not IBD-Susceptible
IBD-Susceptible
Commensal Bacteria
Bacteroides (Robust Colonization)
No Disease Induction
Disease Induction
Enterobacteriaceae (Robust Colonization)
No Disease Induction
No Disease Induction

Table 3.1: Preparation of a standardized, frozen stock of intestinal contents.

Intestinal contents from untreated, non-dnKO mice were suspended in sterile, pre-reduced PBS in an anaerobic chamber and frozen in single-use aliquots in 20% glycerol at -80°C. Prior to freezing, an aliquot of the mixture was titered on the indicated media types. Subsequently, freshly thawed aliquots were adjusted to the same concentration of total intestinal contents and titered on the same media types.

Growth Conditions	Media Type	Colony Type	Pre-freeze mixture (no glycerol)	Post-freeze mixture adjusted to same dilution (some residual glycerol)	% Loss in viability due to freezing
			CFU/mL	CFU/mL	
Anaerobic	ANB	Total	4.20E+08	7.00E+07	16.7%
		β-hemolytic	1.41E+05	1.20E+05	85.1%
Aerobic	Chocolate	Total	4.70E+06	2.32E+06	49.4%

Table 3.2: Preparation of bacterial mixed cultures of intestinal contents to screen for colitis induction.

Serial $10^{0.5}$-fold dilutions of intestinal contents (see Methods) were plated in parallel on solid media and incubated either anaerobically or aerobically on a variety media types (see Figure 3.12). Cultures was harvested from plates at the indicated dilutions and frozen at -80˚C in single-use aliquots with 20% pre-reduced glycerol. The table lists culture conditions, media types, 10-fold dilution factors, and numbers of cfu on the plates from which cultured bacteria were harvested.

Media Type	Culture Conditions	10-Fold Dilution Factor of Intestinal Contents from Untreated Mice	# of Colonies on Source Culture Plates
ANB	Anaerobic	1	~6.3×10^6
ANB		4.5	~1.4×10^3
CNA		4.5	~1.6×10^3
LKV		4	~1.4×10^3
Chocolate	Aerobic	0	~3.9×10^5

Table 3.3: Unique bacterial isolates from LKV media.

Unique species of commensal bacteria isolated from the intestinal microbiota of untreated mice using LKV agar. Isolates were identified by 16S rRNA gene sequence analysis using the Ribosomal Database Project supplemented by BLAST. Sequence identity to bacterial type strains and closest cultured isolates was determined by BLAST2 analysis. All were >99% identical to sequences detected in metagenomic sequencing of rodent commensal microbiota (not shown).

Isolate ID	**Closest Type Strain (Closest Cultured Isolate)**	**% Identity**
dnLKV2	*Bacteroides uniformis*	99%
dnLKV3	*Bacteroides massiliensis* (*Bacteroides* sp. TP-5)	94% (99%)
dnLKV6	*Lactobacillus animalis* (*Lactobacillus murinus*, ASF 361)	99% (99%)
dnLKV7	*Bacteroides dorei* (*Bacteroides vulgatus*)	97% (99%)
dnLKV8	*Parabacteroides distasonis*	97%
dnLKV9	*Bacteroides thetaiotaomicron*	99%
dnLKV18	*Parabacteroides goldsteinii* (*Bacteroides* sp. ASF519)	99% (99%)
dnLKV27	*Parasutterella excrementihominis*, gen. nov., sp. nov.	96%
dnLKV22	*Parasutterella excrementihominis*, gen. nov., sp. nov.	93%

CHAPTER 4

Future directions

Introduction

A large number of interesting and important future directions arise from this work. I will focus primarily on those related to determining if there is molecular basis for the specificity of disease induction by *Bacteroides* species and determining whether and how this microbial specificity is related to host-genotype-specific interactions with T cells, since I believe this to be most relevant to the Stappenbeck-Allen lab collaboration. However I will first briefly mention two other potential future directions.

Brief Future Direction 1: dnKO mice as a potential model of quiescent colitis and IBD-associated colon carcinogenesis

Metronidazole and ciprofloxacin resulted in rescue of severely diseased mice within a matter of weeks (see Chapter 2), establishing a system that could serve as a straightforward and relevant model for studying the epithelial events that characterize quiescent IBD. Importantly, IBD colitis has significant association with colon cancer (Greenstein, Sachar et al. 1979), and the mechanisms by which IBD-associated cancers develop differ in important aspects from sporadic colon cancer (Itzkowitz 2003). We suggest that dnKO mice could be useful as a model for examining the progression from colitis to cancer and how it is influenced by inflammatory processes. Importantly, these experiments can be performed without relying on chemical agents to induce inflammation. At present we have only examined a small number of mice with quiescent colitis, and further work will be needed to determine whether they spontaneously show

signs of dysplasia and carcinogenesis. As a cautionary note: one obvious approach would be to cycle mice on and off of antibiotics, co-housing them with an untreated mouse after each round of treatment. Although an attractive option, care would be required to monitor and prevent the development and spread within the mouse colony of antibiotic-resistant (particularly ciprofloxacin-resistant) bacteria.

Brief Future Direction 2: Isolation of rare or novel intestinal anaerobes

The bacteriology cultivation methods described here resulted in isolation of a number of species of Firmicutes, Bacteroidetes, and Proteobacteria that appeared, based on 16S rRNA sequence analysis, to represent novel species and possibly even novel genera that have previously been detected solely in culture-independent mass sequence analysis of the intestinal microbiota. A number of these isolates did not survive during my initial, large-scale, un-targeted attempts at mass isolation. However a more focused approach would undoubtedly lead to better success in maintaining viability of even extremely fastidious and hard-to-grow bacteria. In particular, I note a number of important and promising candidates or candidate approaches.

1.) *Bacteroides* sp. TP5 (isolate dnLKV3, see Chapter 3) may be a novel species and is viable and easily grown on the culture media described here. Further phenotypic workup could result in confirming and publishing it as a novel species.
2.) We isolated two different Betaproteobacteria related to *Parasutterella excrementihominis* gen. nov., sp. nov. (Nagai, Morotomi et al. 2009). Each of

these isolates may have represented a novel species. They were extremely slow to grow and not highly abundant in the microbiota, forming very small, slow-growing colonies on BBE, LKV, or ANB agar. However they were isolatable on these selective media types and I see no reason why they or closely related Betaproteobacteria could not be isolated again by similar methods.

3.) I have succeeded in isolating a number of bacteria that appear to fall within under-studied families of Firmicutes, including Lachnospiraceae (which are Clostridiales) and Erisypelotrichaceae on anaerobic CNA agar. These organisms have been more difficult to grown in culture and some of the isolates have lost viability. However I have at least one isolate from each family that is still viable in a frozen stock and is on the verge of 16S rRNA cutoff (97% identity) for being a novel species (previously only detected by sequencing). Furthermore, our laboratory recently reported on the fact that Lachnospiraceae are preferentially enriched in the space between the transverse folds found in the mouse ascending colon (Nava, Friedrichsen et al. 2010). It would be relatively straightforward to sacrifice mice, immediately transfer their unopened colons into the anaerobic chamber, open the colons, use a small probe to scoop out the material from between the ascending colon folds, make dilutions, and plate it on anaerobic CNA agar or another even more selective medium.

4.) A variety of papers have reported that treating mice with antibiotics targeting subsets of bacteria can result in enrichment of other bacterial subsets within the microbiota that presumably expand to fill the niches left vacant by the death of the

antibiotic-sensitive organisms (Rakoff-Nahoum, Paglino et al. 2004). I have made similar observations. Treating mice with different antibiotics and then isolating bacteria that remain within the microbiota could potentially allow easier access to bacteria which are normally of too low abundance for isolation. I suggest treating for over a week before performing isolations, since the microbiota will likely take some time to normalize from the effects of antibiotics to achieve new stable microbial communities.

Major Future Direction: Mechanism of *Bacteroides* disease induction in dnKO mice.

The identification of specific disease-inducing and non-disease-inducing commensal bacteria in the dnKO mouse model presents an exciting opportunity probe the mechanisms of host-microbe interaction from both host and microbial perspectives. We have now developed a number of useful immunologic and bacteriologic tools that enable us to interrogate the underlying nature of commensal-induced immunopathology in this model. As described in Chapter 3, we have shown specificity of *Bacteroides* species as compared to at least a subset of other bacteria in inducing host-genotype-specific disease. It remains an open question why this specificity was observed: is it because of one or more specific *Bacteroides*-produced molecular factors, or is it attributable to differences in bacterial "behavior" within the intestines?

On the host side, several pieces of evidence implicate T cells in the pathogenesis of dnKO disease. The *dnTfgβrii* transgene in dnKO mice is expressed specifically in the T cell compartment (Gorelik and Flavell 2000; Kang, Bloom et al. 2008). Previous data

from our groups demonstrates that CD4 T cells from dnKO donors have substantially stronger colitis-inducing effect than wild-type T cells when adoptively transferred into *Rag*$^{-/-}$ recipients (Kang, Bloom et al. 2008). Similarly, we and others have shown that transfer of *Il10r2*$^{-/-}$ T cells also induces more severe disease than wild-type T cells (Kang, Bloom et al. 2008; Murai, Turovskaya et al. 2009). However it remains to be formally determined whether the host-genotype-specific, *Bacteroides*-induced disease is specifically attributable to T cells of the dnKO genotype or whether it also requires other cell types. Assuming that a host-genotype-specific, bacterial-species-specific T-cell-bacteria interaction is sufficient for disease, it will be important to understand what effects the bacterial stimulus is inducing in the T-cells. Do the T cells simply exhibit higher rates of activation, do they aberrantly differentiate to adopt a pathologic phenotype (e.g. Th1 vs. Th2), or is there a failure to develop or respond to regulatory T cells in the context of the colon?

Even if we confirm that disease is due a specific combination of *Bacteroides*-specific microbial stimulation and host-genotype-specific T cells, it will be important to determine whether the resulting immunopathology depends on development of T cell receptors (TCRs) directed against specific *Bacteroides*-produced antigens or whether *Bacteroides*-produced factors somehow induce more generalized host-genotype-specific T cell activation that can lead to reactivity against any potential antigen. I am personally inclined to favor the second option. I am aware of no reason why the genetic signaling defects present in dnKO mice as compared to *Il10r2*$^{+/-}$ mice would cause them to develop fundamentally different TCR repertoires in naïve T cells that could account for the host-

genotype-specific nature of the response to identical bacterial stimuli. Thus, although many dnKO T cells presumably do react against bacterial antigens during disease and some of the antigens in question may be produced by bacteria that play a direct role in disease induction, I suspect that it will be more important to determine why the T-cells have been stimulated to react pathologically rather than to identify what they are reacting against.

There is intriguing published evidence supporting the idea that *Bacteroides* species produce molecules which could cause aberrant host-genotype-specific immune responses in a non-antigen-dependent fashion. *Bacteroides fragilis* produces a zwitterionic capsular polysaccharide molecule (PSA) that in wild-type mice promotes Th1 skew but also promotes mucosal tolerance in the colon by triggering development of inducible Foxp3+ regulatory T cells (Mazmanian, Liu et al. 2005; Mazmanian, Round et al. 2008; Round and Mazmanian 2010; Strober 2010). Regulatory T cell induction appears to require stimulation of naïve T cells by IL-10 produced in a TLR2-dependent fashion, likely by antigen-presenting cells and/or the T-cells themselves (Mazmanian, Round et al. 2008; Round and Mazmanian 2010). In the absence of IL-10 production or blockade of the IL-10 receptor, the PSA molecule failed to induce tolerance and may even have been paradoxically pro-inflammatory (Mazmanian, Round et al. 2008; Strober 2010). Intriguingly, there are suggestions from genomic data that many other intestinal Bacteroidales species may make similar zwitterionic polysaccharides, with the implication that these molecules might also modulate T cell differentiation in IL-10 dependent fashion (Comstock 2009). Thus, the effects of *Bacteroides* in dnKO mice may

have a molecular cause that is independent of a particular T cell antigen or TCR specificity.

Hypothesis: A molecular factor specifically present in commensal intestinal *Bacteroides* species[(Aim 1)] induces host-genotype-specific immunopathologic T-cell activation and/or altered differentiation[(Aim 2)] in a process that is not dependent on a specific bacterial antigen.[(Aim 3)]

Specific Aims:

1.) Assay for the presence of molecules that are present specifically in *Bacteroides* species but not in non-colitogens (e.g. *E. coli* and the residual bacteria in antibiotic-treated mice) which have pro-colitic or pro-inflammatory activity in dnKO but not in *Il10r2*$^{+/-}$ mice.

- **a.** Treat dnKO and *Il10r2*$^{+/-}$ mice currently receiving antibiotic therapy with lysates of colitis-inducing and non-colitis-inducing bacteria in drinking water to assess whether host-genotype-specific, *Bacteroides*-specific disease-induction occurs.
  - **i.** If so, consider biochemically fractionating the lysate.
- **b.** Perform intraperitoneal and/or foot-pad injections of dnKO and *Il10r2*$^{+/-}$ mice currently receiving antibiotic therapy using lysates of colitis-inducing and non-colitis-inducing bacteria and assess whether a host-genotype-specific, *Bacteroides*-specific hyperactive immune response

occurs in the injected region, draining lymph node, or spleen (as appropriate to the site of injection). Characterize the response.

c. Harvest naïve T-cells, preferably from antibiotic-treated, non-colitic dnKO and *Il10r2*$^{+/-}$ mice, culture them with antigen-presenting cells, pulse with bacterial lysates, and assess whether a host-genotype-specific, *Bacteroides*-specific response occurs.

2.) Assess whether adoptively transferred T-cells from dnKO donors but not from *Il10r2*$^{+/-}$ donors preferentially induce disease in *Rag*$^{-/-}$ mice colonized with *Bacteroides*, but not in mice colonized with *E. coli* or mock-colonized.

a. Assess the phenotypes of the transferred cells in each case to determine if there are differences in activation, polarization, or regulatory T cell development.

b. Alternate approach: T cell deplete dnKO mice to determine whether disease course is affected.

3.) Assess whether the adaptive immunes response in *Bacteroides*-colonized, colitic dnKO mice is specifically targeted against particular *Bacteroides* molecules or whether it is a more general phenomenon wherein *Bacteroides*-colonized dnKO mice also develop pathological immune responses against normally non-colitogenic bacteria or antigens.

a. Assess whether pre-immunization with killed *Bacteroides* induces a more strongly immunopathologic response upon colonization or re-challenge (Onderdonk, Cisneros et al. 1983).

b. Inject antigens that normally lack intrinsic colitis-inducing activity into dnKO mice along with the killed *Bacteroides* and assess whether an immune response targeting them develops.

i. Assess whether immune responses develop against bacteria that were present at high levels in the intestinal tract of dnKO mice prior to *Bacteroides* colonization (when the mice were non-colitic).

c. Perform *in vitro* assays to determine the percent of T cells reactive to the introduced *Bacteroides* species as compared to other antigens from the gut milieu.

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