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Article

Commensal *Bacteroides* Species Induce Colitis in Host-Genotype-Specific Fashion in a Mouse Model of Inflammatory Bowel Disease

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SUMMARY

The intestinal microbiota is important for induction of inflammatory bowel disease (IBD). IBD is associated with complex shifts in microbiota composition, but it is unclear whether specific bacterial subsets induce IBD and, if so, whether their proportions in the microbiota are altered during disease. Here, we fulfilled Koch's postulates in host-genotype-specific fashion using a mouse model of IBD with human-relevant disease-susceptibility mutations. From screening experiments we isolated common commensal *Bacteroides* species, introduced them into antibiotic-pretreated mice, and quantitatively reisolated them in culture. The bacteria colonized IBD-susceptible and -nonsusceptible mice equivalently, but induced disease exclusively in susceptible animals. Conversely, commensal Enterobacteriaceae were >100-fold enriched during spontaneous disease, but an Enterobacteriaceae isolate failed to induce disease in antibiotic-pretreated mice despite robust colonization. We thus demonstrate that IBD-associated microbiota alterations do not necessarily reflect underlying disease etiology. These findings establish important experimental criteria and a conceptual framework for understanding microbial contributions to IBD.

INTRODUCTION

Inflammatory bowel disease (IBD) arises from complex interactions of genetic, environmental, and microbial factors (Xavier and Podolsky, 2007). IBD is a spectrum of chronic, noncommunicable diseases primarily defined by spontaneous intestinal inflammation. The two major subtypes, ulcerative colitis and Crohn's disease, exhibit distinct and overlapping clinical and pathologic features (Stenson et al., 2009). Recent progress in IBD host genetics has provided a critical framework to evaluate microbial contributions to pathogenesis (Cadwell et al., 2010). One important challenge is to understand the role of commensal bacteria in the context of specific host genotypes.

Host genetics influence IBD susceptibility (Xavier and Podolsky, 2007). Recent meta-analyses of genome-wide association studies identified approximately 100 susceptibility loci associated with ulcerative colitis, Crohn's disease, or both IBD subtypes (Franke et al., 2010; McGovern et al., 2010). A subset of these susceptibility loci are associated with signaling pathways for IL-10 and TGF-β, two immunoregulatory cytokines with prominent roles in intestinal homeostasis (Li et al., 2006; Ouyang et al., 2011). These susceptibility alleles are common gene variants that modestly elevate disease risk. Importantly, rare recessive deficiencies in the IL-10 receptor result in fulminant, rapid-onset IBD enterocolitis within one year of age (Glocker et al., 2009).

The intestinal microbiota also plays an important role in IBD pathogenesis (Xavier and Podolsky, 2007). The microbiota includes a large and diverse community of commensal bacterial species identified by culture and culture-independent methods (Eckburg et al., 2005; Qin et al., 2010). Both clinical and laboratory data implicate the microbiota in IBD induction. Medically, antibiotics provide some therapeutic benefits to IBD patients (Ohkusa et al., 2010; Sartor, 2008). In Crohn's disease patients, surgical diversion of fecal flow produces remission in inflamed bowel segments with disease recurrence upon flow restoration (Janowitz et al., 1998). Experimental introduction of small bowel effluent into the surgically excluded bowel segments also reinduced disease, but a sterile ultrafiltrate of bowel effluent did not (Harper et al., 1985). In most spontaneous animal models of IBD, disease can be blocked by antibiotics or rederivation into a germ-free state. IBD-susceptible germ-free animals develop disease when exposed to commensal microbes from conventionally raised hosts (Sartor, 2008). Mass sequencing studies of the intestinal microbiota have revealed complex disease-associated shifts in microbiota composition, but disease is thus far not consistently associated with presence or absence of a specific microbe (Frank et al., 2007; Packey and Sartor, 2009; Qin et al., 2010).

Two related, fundamental questions about the role of the microbiota in IBD remain unresolved: in genetically susceptible hosts, do specific subsets of commensal bacteria induce IBD (Strober, 2010; Takaishi et al., 2008) and, if so, can these subsets be identified based on disease-associated alterations in levels of colonization (Tannock, 2008)? Functional tests of the ability of specific microbes to induce IBD have relied primarily on germ-free IBD-susceptible animals. These studies demonstrate that select commensal bacterial species can induce disease in some models (Sartor, 2008). However, translating these findings to conventionally raised animal models, which more closely mimic IBD patients, is challenging. Development of experimental

approaches using conventionally raised animals to functionally assess the roles of specific commensal bacteria and test hypotheses generated by culture-independent microbiota profiling is a critical priority.

Here, we address these questions using non-germ-free methods in an antibiotic-responsive mouse model with IBD-relevant deficiencies in IL-10 and TGF-β signaling. We fulfill host-genotype-specific Koch's postulates by isolating commensal bacteria, introducing them into antibiotic-pretreated mice, assessing disease development, and confirming host colonization by quantitative reisolation of the experimentally introduced bacteria. We identify distinct commensal bacterial subsets with and without disease-inducing potential in susceptible hosts and show that these subsets would not have been predicted based on disease-associated alterations in microbiota composition.

RESULTS

Establishment of a Non-Germ-free System to Screen for Disease-Inducing Microbes Using dnKO Mice

We evaluated the colitogenic potential of intestinal microbes in the dnKO mouse model of IBD (Kang et al., 2008). These mice contain a complete knockout of *Il10r2* (Spencer et al., 1998) and express a transgene-encoded dominant-negative *Tgfbr2* restricted to T cells (Gorelik and Flavell, 2000). Both mutations are closely linked to pathways implicated in human IBD: *IL10* and *IL10RB* (Franke et al., 2010; Glocker et al., 2009; McGovern et al., 2010) and *SMAD3*, a direct downstream target of TGFBR2 (Franke et al., 2010). We previously showed that dnKO mice develop spontaneous, severe, and rapid intestinal inflammation that is 100% penetrant (Kang et al., 2008). Cohoused $Il10r2^{+/-}$ littermate controls did not develop colitis, suggesting the microbial triggers of disease in dnKO mice were innocuous in nonsusceptible hosts (Figure 1A). Colitis in dnKO mice was highly responsive to treatment with metronidazole and ciprofloxacin (Figure 1A). We quantified colitis severity in several anatomically defined regions of the colon using previously validated metrics of gross and anatomic pathology (Kang et al., 2008). Intestinal whole mounts were scored for gross pathology in a blinded fashion (Figure S1). Histologic mucosal inflammation, which is a sensitive indicator of colitis severity, was quantified by measuring heights and widths of well-oriented colonic crypts (Kang et al., 2008). By these criteria, antibiotic treatment completely blocked dnKO colitis (Figures 1B–1G).

Since the combination of antibiotics we used does not sterilize the gut (Heimesaat et al., 2006), we hypothesized that disease in dnKO mice would depend on a subset of commensal bacteria that are eliminated by antibiotic treatment. The intestinal microbiota is normally dominated by members of two bacterial phyla: Firmicutes (Gram-positive) and Bacteroidetes (Gram-negative) (Eckburg et al., 2005). To assess the effects of antibiotics on the microbiota, we collected fecal samples from antibiotic-treated and untreated mice and performed qPCR using 16S rRNA gene primer sets specific for the Bacteroidetes families Bacteroidaceae, Porphyromonadaceae, and Prevotellaceae (BPP) and for the Firmicutes families Enterococcaceae, Lactobacillaceae, and Lachnospiraceae-Ruminococcaceae (Nava et al., 2011). Antibiotic-treated mice showed a decrease in BPP and Lachnospiraceae-Ruminococcaceae, whereas Enterococcaceae and Lactobacillaceae were increased (Figure S2). These data supported the hypothesis that antibiotic treatment prevented disease by selectively altering subsets of commensal bacteria within the microbiota.

To test this hypothesis, we developed a non-germ-free screen for colitogenic activity of commensal bacteria, taking advantage of the dnKO model's rapid disease onset and antibiotic responsiveness. We housed mice in a specific pathogen-free facility using stringent precautions to prevent cage-to-cage contamination. We treated dnKO and control mice at weaning with antibiotics for $\geq$3 weeks. Two days after halting antibiotic treatment, mice were orogastrically gavaged a single time with experimental inocula or sterile PBS as a control (Figure 2A). We found that antibiotic-pretreated dnKO mice gavaged with sterile PBS remained disease free (Figures 2B–2H). To demonstrate that we could induce disease in this system, we gavaged antibiotic-pretreated dnKO mice with intestinal contents harvested from untreated control animals from our colony. We used a standardized frozen stock of intestinal contents to control for microbiota variation between individual donors (Weinstein et al., 1974). Mice gavaged with freshly thawed aliquots of intestinal contents developed severe colitis by 3 weeks after inoculation (Figures 2B–2H). Gavage with freshly harvested intestinal contents or cohousing with untreated mice produced similar outcomes (data not shown). The predominant site of pathology was the colon with minor more variable effects in the lung and liver similar to the pathology that developed in postweaning, untreated, dnKO mice (Gorelik and Flavell, 2000; Kang et al., 2008). We therefore relied on colon-specific metrics of colitis to ensure results were not confounded by extraintestinal processes. These results demonstrated the feasibility of our methods of screening for colitogenic bacteria.

Mixed Cultures of Intestinal Microbes Induce Disease in Antibiotic-Pretreated Mice

We next asked whether cultivable intestinal bacteria could induce colitis in this system. Because of the rapid and widespread colitis in dnKO mice, we hypothesized that common, abundant members of the intestinal microbiota would induce disease. As an initial screen, we serially diluted stock intestinal contents from untreated mice (see above) and cultured the dilutions in parallel on nonselective and selective bacterial growth media (Figure 3A). We harvested anaerobic mixed cultures from a dilution at which ~1500 colonies grew, ensuring cultures contained only relatively abundant (approximately $\geq$0.07% abundance) intestinal bacterial cultivable on that media type (Table S1). For colonization experiments, anaerobic cultures were mixed in equal proportion with a culture grown aerobically on nonselective media and gavaged into antibiotic-pretreated dnKO mice. Mice gavaged with these cultures developed severe colitis relative to PBS-gavaged controls (Figures 3B–3E), demonstrating that colitis-inducing bacteria could be grown in culture.

To refine potential pools of colitogenic microbes, we tested cultures grown on more selective media types including LKV agar (to select for Gram-negative obligate anaerobes) or CNA agar (to inhibit Gram-negative bacilli and enrich for Gram-positive anaerobes) (Figure 3A). Gavage mixtures were prepared as above. Pathology in mice receiving the CNA culture did not significantly differ from PBS-gavaged controls (Figures 3B–3E).

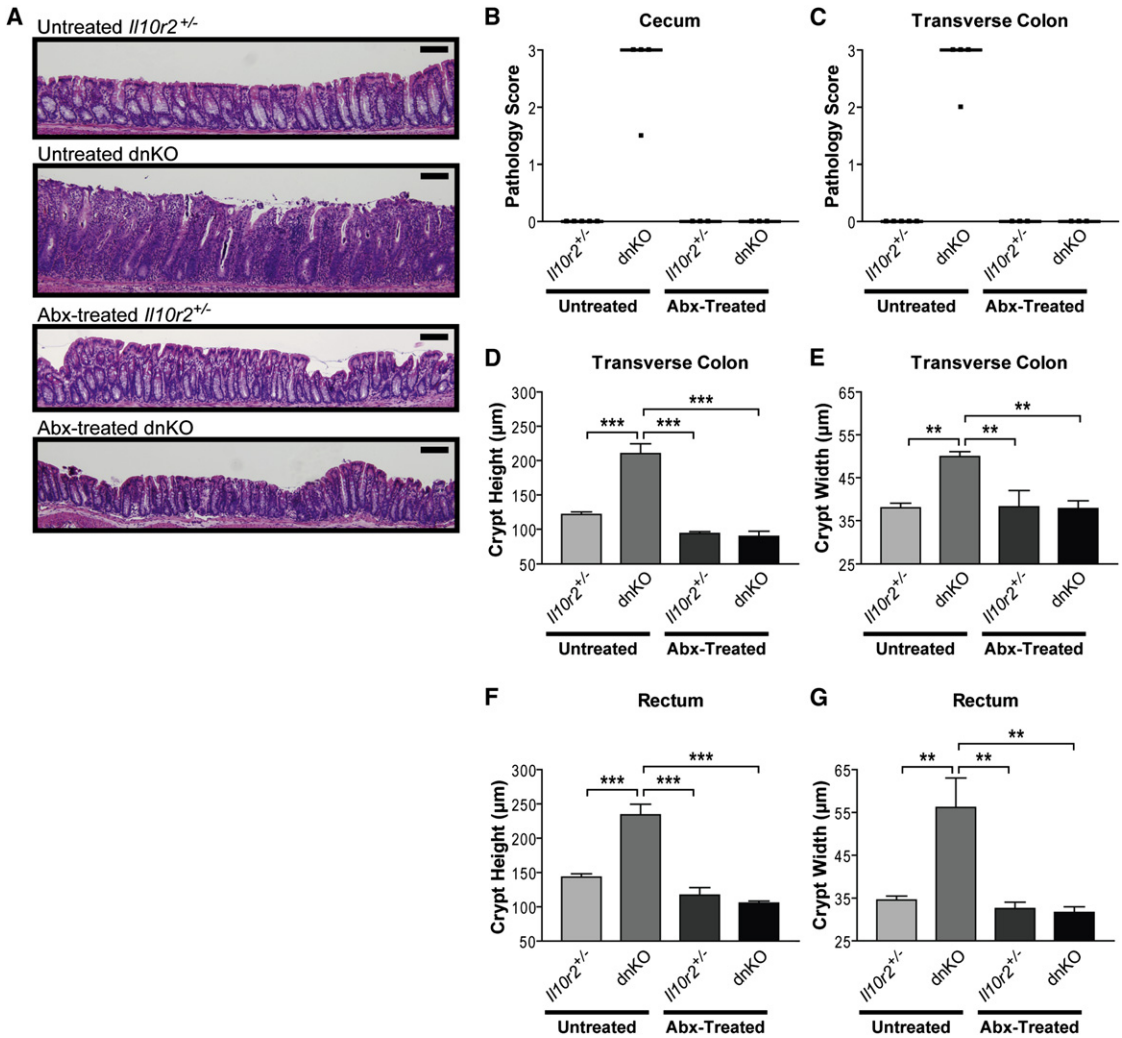

Figure 1. Antibiotic Treatment Quantitatively Prevents Colitis Development in dnKO Mice

(A) Representative images of H&E-stained rectal histology of 4-week-old untreated and antibiotic-treated *Il10r2*$^{+/-}$ and dnKO mice (distal 0.5 cm of colon). Abx = antibiotics (metronidazole + ciprofloxacin in drinking water). Scale bar = 100 μm.

(B and C) Cecum and transverse colon gross pathology scores of untreated and antibiotic-treated *Il10r2*$^{+/-}$ and dnKO mice as described in (A). Intestinal whole mounts were scored according to a validated system: ranging from 0 (no pathology) to 3 (severe pathology) (see Figure S1 for details). Individual (squares) and median (bars) pathology scores are displayed. Kruskal-Wallis test: $H_3 = 13.75$, $p = 0.0033$ (B); $H_3 = 13.75$, $p = 0.0033$ (C).

(D–G) Transverse colon and rectum crypt heights and crypt widths of mice, as described in (B) and (C), displayed as mean ± SEM, one-way ANOVA with post hoc Tukey's test: $F_{3,11} = 40.87$, $p < 0.0001$ (D); $F_{3,11} = 10.71$, $p = 0.0014$ (E); $F_{3,11} = 33.25$, $p < 0.0001$ (F); $F_{3,11} = 9.501$, $p = 0.0022$ (G). All statistically significant pairwise comparisons are displayed: **$p < 0.01$, ***$p < 0.005$. See also Figure S1.

To evaluate why the CNA culture did not induce disease, we isolated bacteria from the feces of antibiotic-treated (noncolitic) dnKO mice using nonselective ANB agar, extracted genomic DNA, and obtained partial 16S rRNA gene sequences. Sequence analysis showed the isolates belonged to a variety of families within the phylum Firmicutes, including Enterococcaceae, Lactobacillaceae, Bacillaceae, and Lachnospiraceae (Table S2). All of the isolates grew on CNA agar. Targeted 16S rRNA gene sequencing of bacterial DNA isolated from the CNA gavage inocula identified Firmicutes species (Table S2). We concluded that these species could colonize antibiotic-treated dnKO mice without inducing disease. In contrast, antibiotic-pretreated dnKO mice gavaged with the LKV culture developed robust disease similar to mice given the nonselective ANB culture (Figures 3B–3E). These results suggested that abundant Gram-negative obligate anaerobes were sufficient to induce disease in dnKO mice.

Commensal *Bacteroides* Isolates Induce Disease in IBD-Susceptible, but Not IBD-Nonsusceptible, Mice

We therefore isolated and identified abundant Gram-negative, obligate anaerobes from the stock of pooled intestinal contents described above. We isolated and propagated 17 Gram-negative isolates on LKV agar. 16S rRNA gene sequence analysis identified several redundant isolates that included six unique species from the phylum Bacteroidetes (Table S3). Isolation of

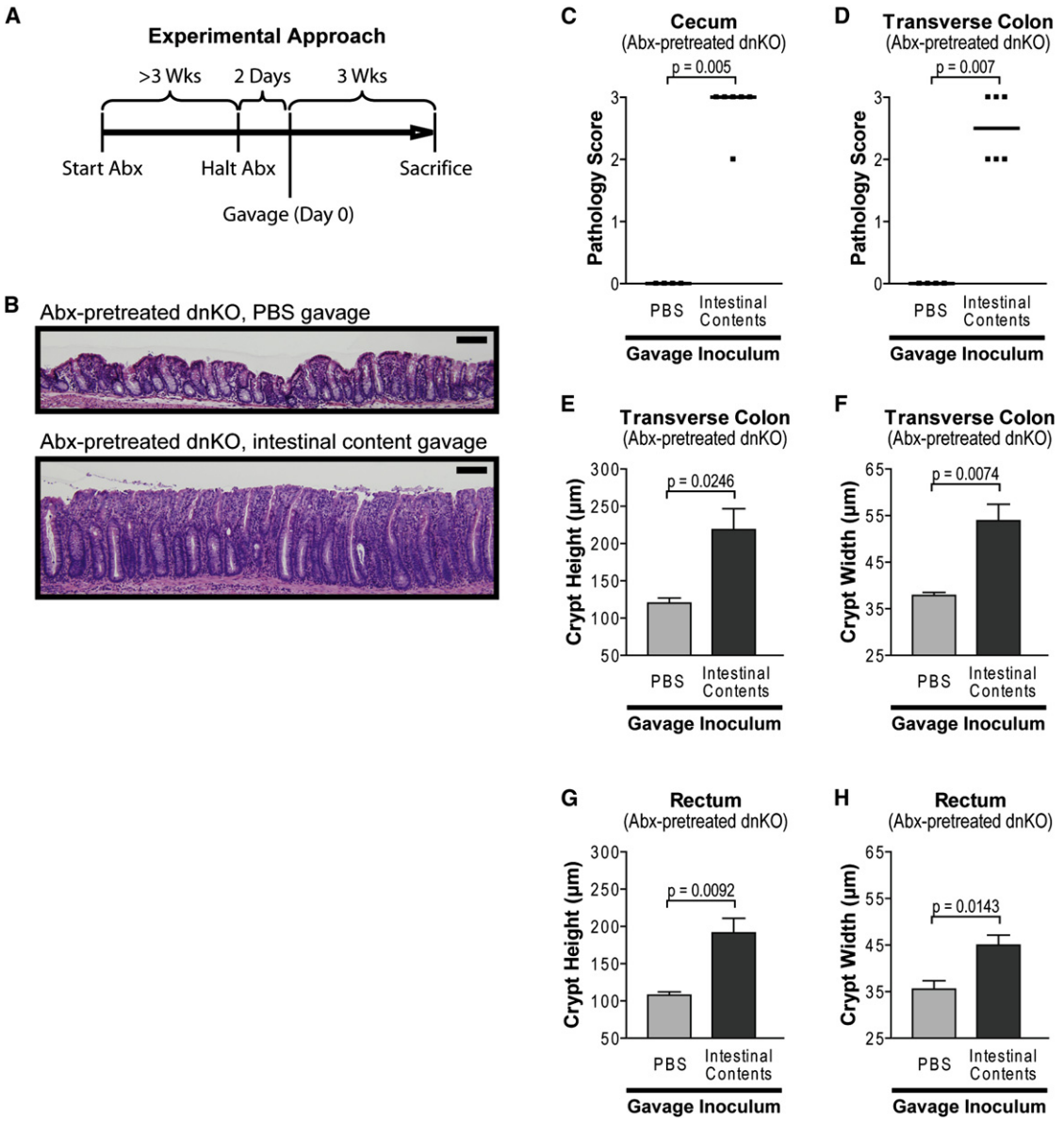

Figure 2. A Non-Germ-free System for Colitis Induction in Antibiotic-Pretreated dnKO Mice

(A) Experimental timeline. Antibiotic treatment began at weaning (age 3 weeks); mice were antibiotic treated for ≥3 weeks and gavaged 2 days after treatment cessation (Abx = antibiotics).

(B) Representative images of H&E-stained rectal histology of antibiotic-pretreated dnKO mice orogastrically gavaged with sterile PBS or with a standardized frozen stock of intestinal contents from untreated animals. Gavage dose = 2×10^7 total cfu/mouse on Anaerobic Reducible Blood (ANB) agar (nonselective anaerobic culture media; cfu = colony-forming unit). Scale bar = 100 μm.

(C and D) Cecum and transverse colon pathology scores of antibiotic-pretreated dnKO mice gavaged with sterile PBS or intestinal contents from untreated donors, as described in (B), displayed as individual (symbols) and median (bars) scores. Mann-Whitney U test.

(E–H) Transverse colon and rectum crypt heights and crypt widths of mice described in (C) and (D), displayed as mean ± SEM. Unpaired t test. See also Figure S2.

unique-appearing colonies from intestinal contents grown in parallel on *Bacteroides* bile esculin (BBE) agar produced nine additional Bacteroidetes isolates, all of which were identical to isolates obtained using LKV agar. Sequences from all isolates were ≥99% identical to 16S rRNA sequences previously detected in culture-independent studies of rodent commensal intestinal microbiota, as determined using BLAST and the Ribosomal Database Project (Cole et al., 2007, 2009). Collectively, these results confirmed the isolates were common, abundant members of the commensal microbiota.

To assess whether any of the isolates could induce disease, we screened five of them in antibiotic-pretreated dnKO mice. We found that pure cultures of each induced colitis to varying degrees. *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron*

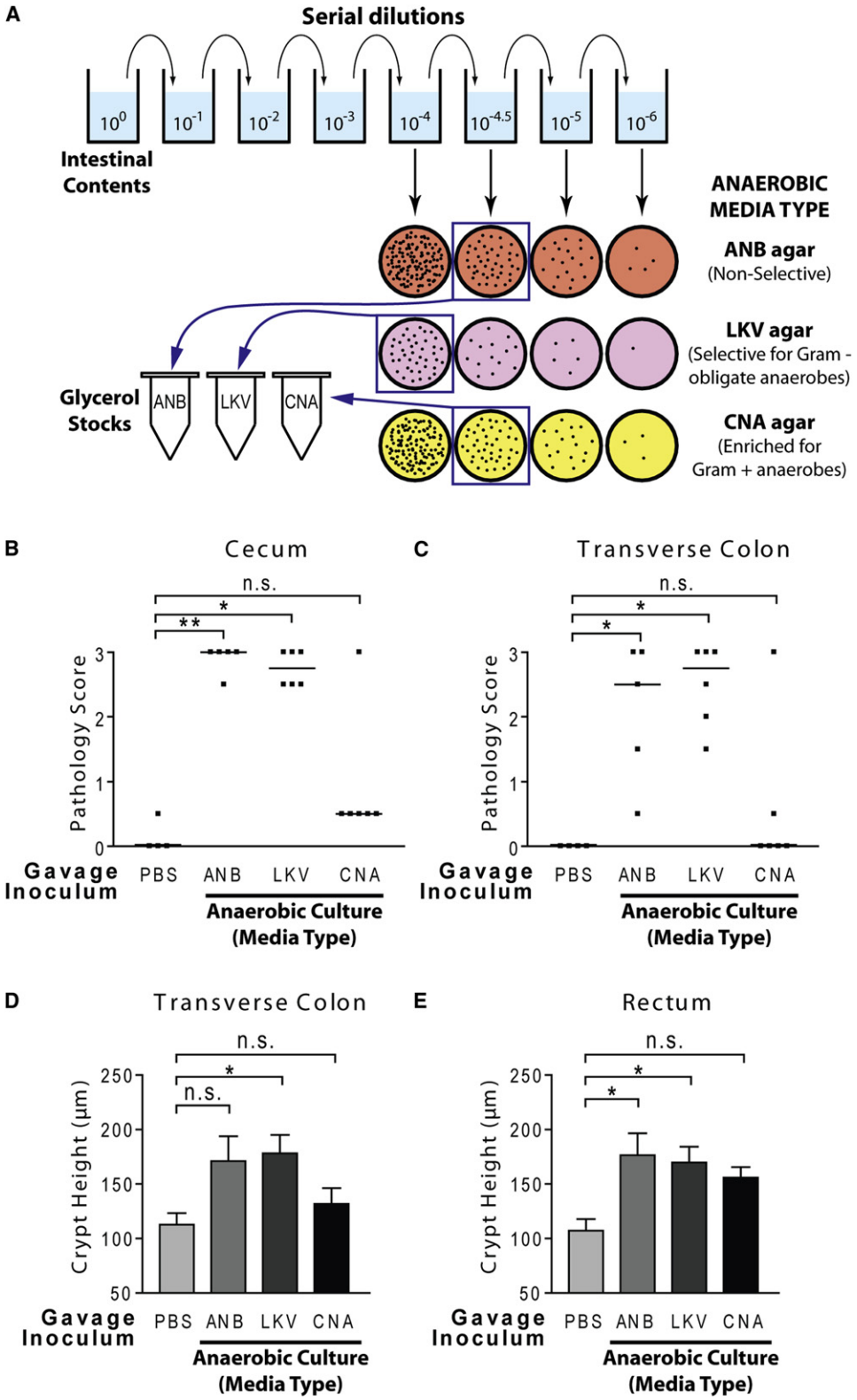

Figure 3. Mixed Cultures of Intestinal Contents Induce Disease in Antibiotic-Pretreated dnKO Mice

(A) Diagram of mixed culture preparation. Aliquots of intestinal contents (Figure 2B) were serially diluted and cultured on the indicated media types. Three day mixed cultures were harvested from the indicated culture plates (in squares) and frozen in 20% glycerol. Aliquots of each culture were subsequently thawed and adjusted to standardized doses for gavage.

induced severe ulcerative disease, while the *Bacteroides* sp. TP5 induced milder disease consisting of lymphocytic infiltrate in the mucosa (Figures 4A and 4B).

Importantly, we confirmed that *B. thetaiotaomicron,* a well-characterized symbiotic species (Goodman et al., 2009), potently induced colitis in dnKO mice, but was innocuous in *Il10r2*$^{+/-}$ controls (Figures 4C–4H and S3). The pathology in the colonized dnKO mice included a significant colonic neutrophil infiltration with transepithelial migration (Figure 4I) and a significant increase in the percentage of interferon-producing CD4+ T cells isolated from the lamina propria (Figure 4J). These findings were similar to those for untreated, spontaneously colitic dnKO mice (Kang et al., 2008).

Serum antibody responses to commensal bacterial antigens occur in IBD, but assessing cause and effect has been experimentally challenging (Brandwein et al., 1997; Lodes et al., 2004). We found that antibiotic-pretreated mice exhibited no serum IgG response to lysates of cultured Bacteroidetes species prior to gavage. In contrast, dnKO mice given either *B. thetaiotaomicron* or *B. vulgatus* developed robust IgG responses to multiple antigens of the respective isolate after gavage, whereas *Il10r2*$^{+/-}$controls given the same bacteria did not (Figures 4K and S3B). These results demonstrate that colitis induced by commensal *Bacteroides* species involves activation of both innate and adaptive immune responses in a host-genotype-specific fashion.

Antibiotic Treatment Eliminates Cultivable Intestinal *Bacteroides* from the Fecal Microbiota

As diverse intestinal *Bacteroidetes* species induced colitis in dnKO mice, we reasoned that antibiotic treatment should eradicate them from the microbiota. To test this hypothesis, we performed quantitative fecal cultures on *Bacteroides*-selective media. Fecal samples from antibiotic-treated dnKO mice exhibited no bacterial growth on BBE agar at a detection limit of 17 colony-forming units (cfu) per sample ($\sim10^3$ cfu/g of feces). In contrast, samples from untreated dnKO mice resulted in high bacterial titers ($\sim10^9$ cfu/g of feces) on BBE agar (Figure 5A). While antibiotic treatment decreased the total bacterial load as assessed by qPCR for the bacterial *rpoβ* gene (Nava et al., 2011), fecal titers on ANB media did not differ with antibiotic treatment (Figures 5A and S4A). These data confirmed that antibiotics prevented disease by selectively altering microbiota composition rather than by sterilizing the gut.

Commensal *Bacteroides* Species Fulfill Koch's Postulates in Host-Genotype-Specific Fashion

We could thus fulfill Koch's postulates for commensal *Bacteroides* species by performing serial, quantitative fecal cultures on BBE agar from single-isolate-gavaged mice. Antibiotic-pretreated mice gavaged with PBS remained free of cultivable *Bacteroides* for up to 28 weeks, confirming absence of cage-to-cage microbial contamination (Figures 5B and S4B). By contrast, mice given pure cultures of *B. thetaiotaomicron*, *B. vulgatus*, or *Bacteroides* species TP5 became stably colonized at $\sim10^{10}$ cfu/g of feces (Figures 5B, 5C, and S4B) (data not shown). Bacterial identity was determined by characteristic colony size, morphology, and pigmentation pattern as well as 16S rRNA gene sequence (Figures S4C and S4D). Colonization levels were consistent from animal to animal and experiment to experiment (Figure 5C). Antibiotic-pretreated *Il10r2*$^{+/-}$ and dnKO mice became stably colonized with gavaged bacteria at equivalent levels (Figures 5B and 5C) (data not shown). These results demonstrated that disease induction was host genotype specific.

Commensal Enterobacteriaceae, but Not *Bacteroides*, Are Strikingly Enriched in the Microbiota during Spontaneous Disease

Despite our finding that commensal *Bacteroides* induced colitis in dnKO mice, studies of *Bacteroides* dynamics in the intestinal microbiota of both IBD patients and animal IBD models have produced variable results, with several studies detecting no significant disease-associated enrichment (Dicksved et al., 2008; Frank et al., 2007; Onderdonk et al., 1998; Ott et al., 2008; Takaishi et al., 2008). We therefore examined fecal *Bacteroides* levels using quantitative titers on BBE agar and qPCR species-specific primers for the *B. thetaiotaomicron* 16S rRNA gene (Figures 6A, 6B, 6D, 6F, and S5). We found no significant difference between untreated dnKO (spontaneously colitic) and *Il10r2*$^{+/-}$ (noncolitic) mice by either of these methods. In contrast, previous studies found an IBD-associated enrichment of Enterobacteriaceae (Gram-negative, facultative anaerobes including *Escherichia coli* from the phylum Proteobacteria that can be selectively cultured on MacConkey agar) (Burke, 1997; Darfeuille-Michaud et al., 1998; Frank et al., 2007; Onderdonk et al., 1998). In agreement with these studies, we found commensal Enterobacteriaceae were strikingly enriched in spontaneous disease, accounting for ~50% of total cultivable fecal bacteria in dnKO mice, as compared to <0.5% in cohoused *Il10r2*$^{+/-}$ controls (Figures 6A, 6C, 6E, and S5).

Commensal Enterobacteriaceae Are Not Sufficient for Disease Induction

The elevation of Enterobacteriaceae in IBD led to the hypothesis that these bacteria play a pathogenic role in disease (Burke, 1997; Frank et al., 2007; Onderdonk et al., 1998). However, it is unclear whether Enterobacteriaceae enrichment is a cause or effect of disease, because enrichment also occurs during non-IBD intestinal inflammation experimentally induced by chemicals or microbial pathogens (Heimesaat et al., 2006; Lupp et al., 2007; Stecher et al., 2007). We found commensal Enterobacteriaceae were eliminated from antibiotic-treated mice (Figure 6C), allowing us to directly test their colitogenic activity. We isolated an Enterobacteriaceae species that was highly enriched in the feces

(B and C) Cecum and transverse colon pathology scores of antibiotic-pretreated 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. Individual (symbols) and median (bars) pathology scores are displayed. Gavage doses: ANB = 6.4×10^7 total cfu/mouse; LKV = 7.3×10^7 total cfu/mouse; CNA = 5.4×10^7 total cfu/mouse. Statistical significance relative to PBS determined by Dunn's multiple comparison test: not significant, $p > 0.05$; *$p < 0.05$; **$p < 0.01$.
(D and E) Transverse colon and rectum crypt heights of antibiotic-pretreated dnKO mice described in (B) and (C), displayed as mean ± SEM. Statistical significance relative to PBS determined by Dunnett's multiple comparison test. The screen was unrepeated. See also Tables S1A and S1B.

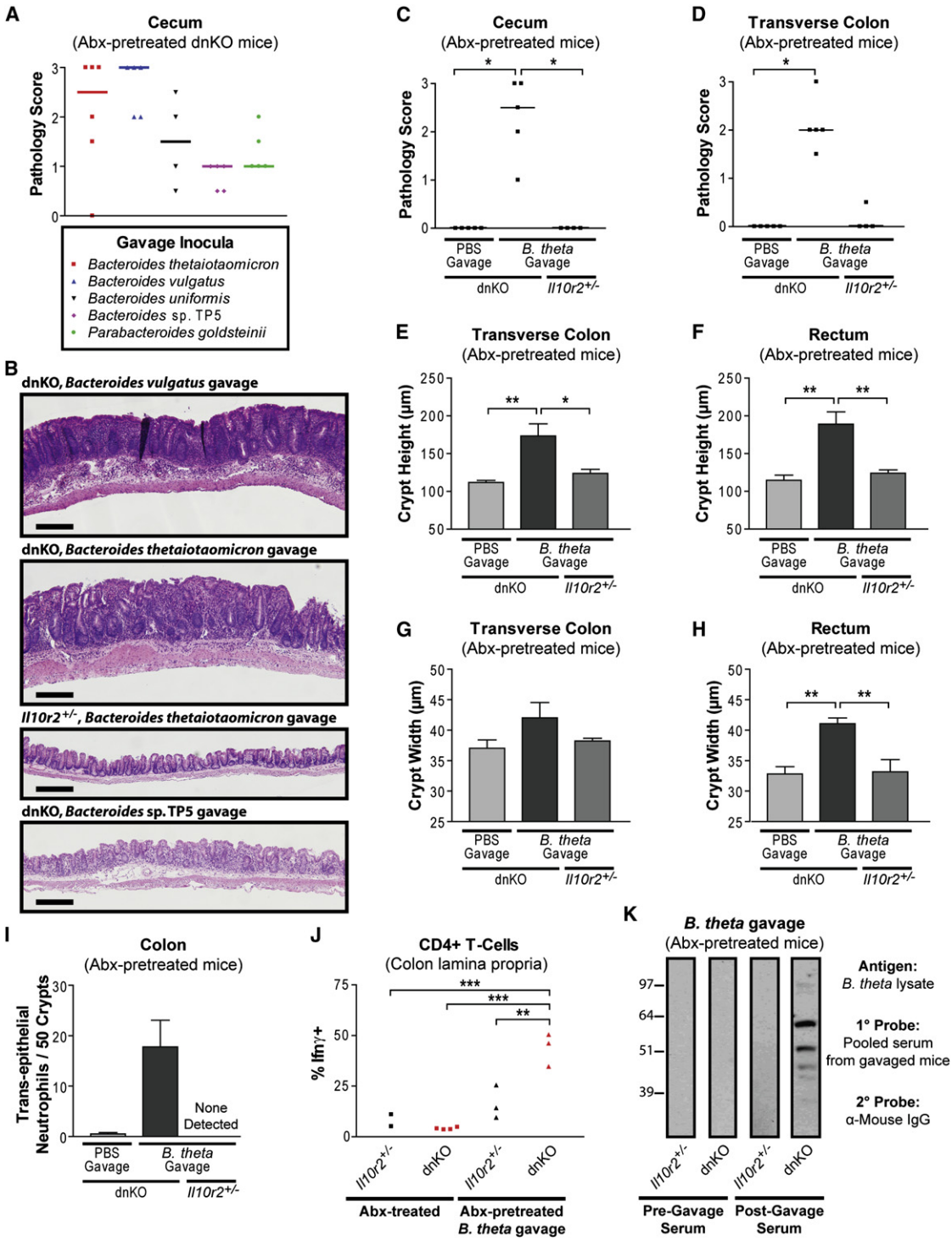

Figure 4. Commensal *Bacteroides* Induce Disease in Antibiotic-Pretreated dnKO, but Not *Il10r2*$^{+/-}$, Mice

(A) Screen for colitis induction by Bacteroidetes isolates. Cecum gross pathology scores of antibiotic-pretreated dnKO mice gavaged with 1 × 10^8 cfu/mouse of pure cultures of the indicated primary bacterial isolates (see Table S3). Not repeated for *B. uniformis* and *P. goldsteinii*.

(B) Representative H&E-stained cecal histology of mice gavaged with pure cultures of the indicated bacterial isolate as described in (A). Scale bar = 200 μm. Cecal histology of a noncolitic *Il10r2*$^{+/-}$ mouse gavaged with *B. thetaiotaomicron* is shown for comparison.

(C and D) Cecum and transverse colon pathology scores of antibiotic-pretreated mice of the indicated genotypes gavaged with PBS or a pure culture of the *Bacteroides thetaiotaomicron* isolate (7 × 10^7 cfu/mouse). Kruskal-Wallis test with post hoc Dunn's test. $H_2 = 12.26$, $p = 0.0022$ (C); $H_2 = 11.36$, $p = 0.0034$ (D). All significant pairwise comparisons are displayed: *$p < 0.05$.

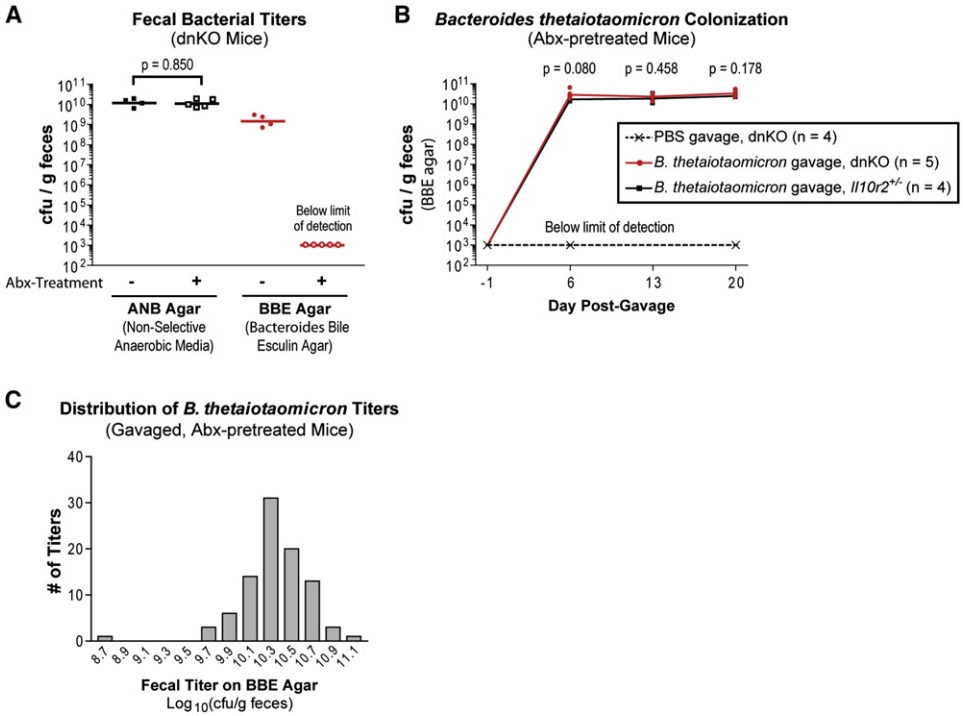

Figure 5. Intestinal *Bacteroides* Are Eliminated by Antibiotics and Stably Recolonize Gavaged, Antibiotic-Pretreated Mice Independently of Host Genotype

(A) Plot of fecal titers from untreated and antibiotic-treated dnKO mice grown in parallel anaerobically on nonselective (ANB) agar and *Bacteroides* bile esculin (BBE) agar. Titers from individual mice (symbols) and means of $\log_{10}$-transformed titers (bars) are displayed. Unpaired t test ($\log_{10}$-transformed titers).

(B) Plot of serial fecal titers on BBE agar from antibiotic-pretreated mice gavaged on Day 0 with sterile PBS or *B. thetaiotaomicron* (7×10^7 cfu/mouse). Individual titers (symbols) and means of $\log_{10}$-transformed titers (lines) are displayed. Unpaired t tests compare $\log_{10}$-transformed titers from *B. thetaiotaomicron*-gavaged dnKO and *Il10r2*$^{+/-}$ mice.

(C) Frequency histogram of all 92 *B. thetaiotaomicron* fecal titer measurements ($\log_{10}$-transformed) we performed on individual, single-isolate-gavaged, antibiotic-pretreated mice regardless of mouse genotype. Fecal titers were performed between 6 and 20 days postgavage; data are compiled from multiple independent experiments with 4–9 mice per experiment. Gavage doses ranged from 6.6×10^7 to 8.6×10^8 cfu/mouse. Excluding the single outlier value (8.78), the titers are normally distributed as assessed by the D'Agostino Pearson K^2 omnibus normality test: $p = 0.5894$, $K^2 = 1.057$, skewness = −0.153, kurtosis = 0.3345. See also Figure S4.

of an untreated, colitic dnKO mouse (~33% of total cultivable bacteria) and identified it as *E. coli* by 16S rRNA sequence analysis and phenotypic characterization (Figure S6). To test the isolate's disease-inducing potential, we gavaged it into antibiotic-pretreated dnKO mice. Other groups of dnKO mice were gavaged with *B. thetaiotaomicron* or sterile PBS, as positive and negative controls. We quantified bacterial colonization by performing serial fecal titers on BBE and MacConkey agar (Figures 7A and 7B). *E. coli* stably colonized antibiotic-pretreated animals at higher levels than those observed in untreated dnKO mice (compare Figures 6C and 7B). However, *E. coli* did not induce significant disease relative to PBS, whereas *B. thetaiotaomicron* induced significant disease relative to both *E. coli* and PBS (Figures 7C–7H).

DISCUSSION

The commensal microbiota influences pathogenesis of many diseases, including IBD, but the role of specific microbes is incompletely understood. Two central, related questions are

(E–H) Transverse colon and rectum crypt heights and crypt widths of mice described in (C) and (D), displayed as mean ± SEM, one-way ANOVA with post hoc Tukey's test: $F_{2,10} = 8.596$, $p = 0.0067$ (E); $F_{2,10} = 12.55$, $p = 0.0019$ (F); $F_{2,10} = 2.057$, $p = 0.1786$ (G); $F_{2,10} = 12.05$, $p = 0.0022$ (H). All significant pairwise comparisons are displayed if the omnibus p value by ANOVA was significant: *$p < 0.05$; **$p < 0.01$.

(I) Graph of the number of neutrophils per 50 well-oriented crypts located within the intestinal epithelium/crypt lumen in colons of the mice described in (C) and (D). Kruskal-Wallis test: $H_2 = 6.529$, $p = 0.0382$.

(J) Percent of CD4+ T cells expressing Ifnγ, determined using validated procedures (Kang et al., 2008), in the colonic lamina propria of antibiotic-treated *Il10r2*$^{+/-}$ (n = 2) and dnKO (n = 4) mice or antibiotic-pretreated *Il10r2*$^{+/-}$ (n = 3) and dnKO (n = 3) mice gavaged with 10^8 cfu/mouse of *B. thetaiotaomicron* and sacrificed 3 weeks postgavage. One-way ANOVA with post hoc Tukey's test: $F_{3,8} = 27.93$, $p = 0.0001$. All significant pairwise comparisons are displayed: **$p < 0.01$; ***$p < 0.005$.

(K) Immunoblots of *B. thetaiotaomicron* lysate probed with a 1:200 dilution of pooled serum from antibiotic-pretreated *Il10r2*$^{+/-}$ (n = 3) or dnKO (n = 8) mice collected immediately prior to gavage with 10^8 cfu/mouse of *B. thetaiotaomicron* (pregavage) or at sacrifice 3 weeks later (postgavage). Secondary antibody is α-mouse IgG. Equal amounts of bacterial lysate were separated by SDS-PAGE, transferred to nitrocellulose membranes, blotted, and exposed in parallel for all groups. See also Table S3 and Figure S3.

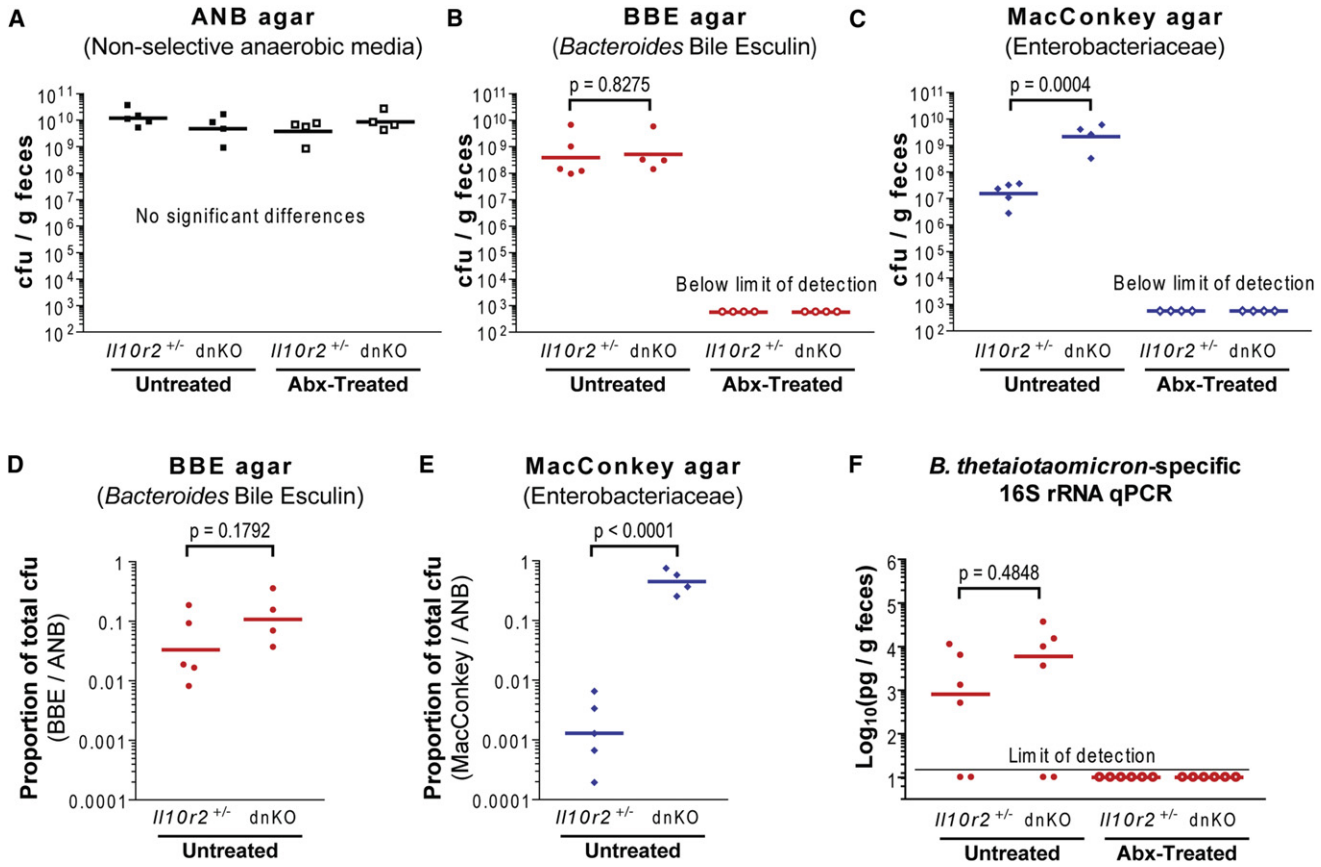

Figure 6. Commensal Enterobacteriaceae Are Enriched in Spontaneous Colitis

(A–C) Plot of fecal titers from untreated or antibiotic-treated mice of the indicated genotypes grown in parallel anaerobically on ANB and BBE agar and aerobically on MacConkey agar (selective for Enterobacteriaceae). *Il10r2*$^{+/-}$ and dnKO mice were cohoused; data compiled from ≥2 cages/group. One-way ANOVA (ANB titers: $F_{3,13} = 1.344$, $p = 0.3031$) or unpaired t test (log_{10}-transformed titers).

(D and E) Proportions of total cultivable bacteria in Figures (A)–(C) that grew on BBE agar and MacConkey agar, calculated by dividing BBE and MacConkey titers by ANB titers. Displayed as individual (symbols) and means of log_{10}-transformed proportions (bars). Unpaired t test (log_{10}-transformed proportions).

(F) Plots of fecal samples from untreated and antibiotic-treated dnKO and *Il10r2*$^{+/-}$ mice (n = 6 per group) analyzed by qPCR using primers specific for the *B. thetaiotaomicron* 16S rRNA gene. *Il10r2*$^{+/-}$ and dnKO mice within each treatment group were cohoused; data compiled from ≥3 cages/group. Results of qPCR assays were normalized to sample weight. Mann-Whitney U test (log_{10}-transformed values). See also Figure S5.

whether specific commensal bacterial subsets induce IBD and, if so, whether their proportions in the microbiota are altered during disease (Strober, 2010; Takaishi et al., 2008; Tannock, 2008). To address these questions, we utilized an antibiotic-responsive genetic mouse model with defects in IL-10 and TGF-β signaling, both of which are associated with human IBD (Franke et al., 2010; Glocker et al., 2009; McGovern et al., 2010). Using these mice, we developed a non-germ-free approach to screen for specific disease-inducing commensal bacteria. Based on the results of this screen, we fulfilled host-genotype-specific Koch's postulates for commensal *Bacteroides* species. We isolated *Bacteroides* species that were common to susceptible and non-susceptible hosts and were eliminated by disease-blocking antibiotics. We administered pure cultures of the isolates to antibiotic-pretreated mice and found that they induced colitis with features of adaptive and innate immunopathology in susceptible hosts but not in nonsusceptible hosts. Importantly, *Bacteroides* species could be quantitatively reisolated from experimentally colonized animals at equivalent levels regardless of host genotype and disease status. These results suggest the genotype-dependent disparity in disease induction was due to differences in host response rather than altered colonization susceptibility. Conversely, commensal Enterobacteriaceae were strikingly enriched in the microbiota during spontaneous disease, but a colitis-enriched Enterobacteriaceae isolate was not sufficient to induce disease, despite robust colonization. We further found that antibiotic-treated, noncolitic dnKO mice were colonized at high levels with bacteria belonging to several families of Firmicutes. We thus identified distinct subsets of commensal bacteria with and without disease-inducing potential and demonstrated that the colitogenicity of these subsets would not have been predicted based on disease-associated shifts in colonization.

Identification of specific IBD-inducing commensal microbes has been a longstanding goal (Burke, 1997; Sartor, 2008). Germ-free animals are frequently used as they provide a source of healthy yet susceptible hosts for experimental colonization with defined microbial populations. Determining colonization levels of introduced isolates in ex-germ-free animals is

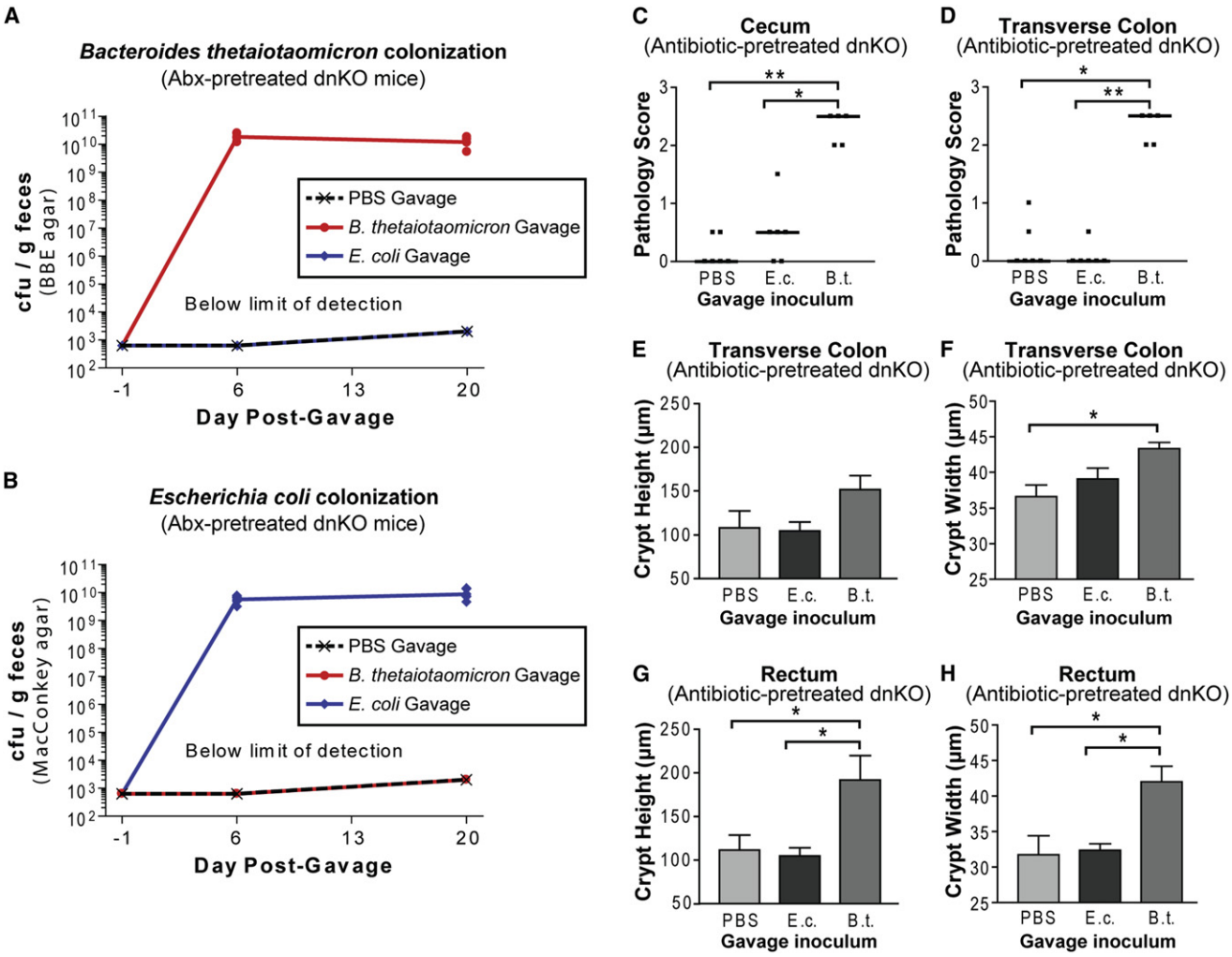

Figure 7. A Colitis-Enriched Commensal Enterobacteriaceae Isolate Robustly Colonizes Antibiotic-Pretreated dnKO Mice without Disease Induction

(A and B) Plot of serial fecal titers grown on BBE and MacConkey agar from antibiotic-pretreated dnKO mice (5–6 per group) gavaged with PBS, *B. thetaiotaomicron* (1×10^8 cfu/mouse), or *Escherichia coli* (2×10^8 cfu/mouse). Bacterial identity was determined by colony characteristics, confirmed by sequencing the 16S rRNA gene of representative colonies.

(C and D) Cecum and transverse colon pathology scores of mice described in (A) and (B) displayed as individual (symbols) and median (bars) scores. Kruskal-Wallis test with post hoc Dunn's tests: $H_2 = 11.49$, $p = 0.0032$ (C); $H_2 = 12.02$, $p = 0.0025$ (D). All significant pairwise comparisons are displayed: *$p < 0.05$; **$p < 0.01$.

(E–H) Transverse colon and rectum crypt heights and crypt widths from mice described in (A) and (B), displayed as mean ± SEM, one-way ANOVA with post hoc Tukey's test: $F_{2,14} = 2.580$, $p = 0.1112$ (E); $F_{2,14} = 5.434$ (F); $p = 0.0179$; $F_{2,14} = 6.438$, $p = 0.0104$ (G); $F_{2,14} = 7.268$, $p = 0.0068$ (H). All significant pairwise comparisons are displayed if omnibus p value by ANOVA was significant: *$p < 0.05$. See also Figure S6.

uncomplicated, and host effects can be directly attributed to the introduced bacteria (Hapfelmeier et al., 2010). While such studies show the microbiota play a role in disease induction, there are important caveats for using them to identify specific disease-inducing bacteria. Importantly, the innate and adaptive immune system of germ-free animals is immature (Ivanov et al., 2009; Lee and Mazmanian, 2010), and bacteria that induce chronic inflammation or cancer in conventionally raised animals can produce dramatically different outcomes in germ-free hosts, ranging from no disease induction to rapid death (Garrett et al., 2010; Lofgren et al., 2011; Rhee et al., 2009). These results highlight the need for new strategies to identify and characterize disease-inducing bacteria. The non-germ-free approach we developed here generates healthy, IBD-susceptible mice by pretreating conventionally raised animals with antibiotics that have been widely studied in human IBD (Elahi et al., 2009; Ohkusa et al., 2010; Rahimi et al., 2006). Antibiotic treatment eradicated certain bacterial subsets from the microbiota, enabling us to experimentally colonize antibiotic-pretreated animals with primary isolates of commensal bacteria indigenous to our mouse colony and specifically quantify colonization. Interestingly, the isolates colonized antibiotic-pretreated mice at levels higher

than those observed in untreated animals, suggesting antibiotics produce an altered microbial ecosystem permissive to expansion of individually reintroduced commensal species. This approach should be broadly applicable to a wide range of animal models and disease processes.

Multiple commensal *Bacteroides* species including *B. thetaiotaomicron* and *B. vulgatus* induced disease in antibiotic-pretreated dnKO mice. Intestinal *Bacteroides* are among the most abundant and well studied members of the commensal microbiota. They benefit the host through breakdown of complex dietary carbohydrates and modulation of mucosal glycosylation, gene expression, angiogenesis, and immune maturation (Comstock, 2009; Hooper et al., 2001; Round and Mazmanian, 2010; Stappenbeck et al., 2002; Xu et al., 2007). To our knowledge, *B. thetaiotaomicron* has not previously been implicated in IBD pathogenesis. Conflicting data exist on the role of *B. vulgatus* in IBD. Germ-free rats transgenically expressing HLA-B27, a human MHC class I allele linked to spondyloarthritis-associated IBD (Brakenhoff et al., 2010), developed T cell-dependent colitis when colonized with *B. vulgatus* but not with other bacteria, including *E. coli* (Hoentjen et al., 2007; Rath et al., 1999). Interestingly, *E. coli*, but not *Bacteroides*, species were enriched in conventionally raised, spontaneously colitic transgenic rats (Onderdonk et al., 1998). *B. vulgatus* colonization also induced disease in germ-free guinea pigs treated with carrageenan (Onderdonk et al., 1983). However, *B. vulgatus* did not induce disease in germ-free *Il10*$^{-/-}$mice and appeared to be protective in germ-free *Il2*$^{-/-}$ mice, whereas some strains of *E. coli* were colitogenic (Kim et al., 2007; Sellon et al., 1998; Waidmann et al., 2003). The *Il10*$^{-/-}$ and *Il2*$^{-/-}$ mice used in those studies were on different genetic backgrounds than the dnKO mice we used here, making it unclear whether results differed due to host genetic differences or because our studies were performed in conventionally raised rather than germ-free animals. Given the human-relevant genetic features of dnKO mice and the use of a non-germ-free approach, our findings suggest commensal *Bacteroides* species may have specific colitogenic effects in at least a subset of human IBD. Mazmanian and colleagues recently coined the term ''pathobiont'' to describe commensal or symbiotic microbes that induce disease only in certain genetic or environmental contexts (Chow and Mazmanian, 2010; Mazmanian et al., 2008). We propose that fulfillment of host-genotype-specific Koch's postulates in dnKO mice demonstrates that commensal *Bacteroides* species act as pathobionts in this genetic context.

Shifts in specific commensal intestinal bacterial subsets occur in IBD and notably include enrichment of Enterobacteriaceae and other Proteobacteria (Burke, 1997; Darfeuille-Michaud et al., 1998; Frank et al., 2007; Takaishi et al., 2008). Our finding that a colitis-enriched *E. coli* isolate did not induce disease in antibiotic-pretreated dnKO mice adds to a growing body of evidence that intestinal inflammation provides both commensal and pathogenic Enterobacteriaceae with a selective colonization advantage regardless of inflammation etiology (Heimesaat et al., 2006; Lupp et al., 2007; Stecher et al., 2007; Winter et al., 2010). Interestingly, Proteobacteria enrichment also occurs in the lung microbiota of asthma patients (Hilty et al., 2010) and the gastric microbiota of patients with *Helicobacter pylori*-induced chronic atrophic gastritis (Maldonado-Contreras et al., 2011). The pathogenic Enterobacteriaceae species *Salmonella typhimurium* directly exploits intestinal inflammation by using tetrathionate—a compound generated in the presence of host-derived reactive oxygen species—as a respiratory electron acceptor to outcompete the commensal bacteria (Winter et al., 2010). We speculate that analogous mechanisms for exploiting mucosal inflammation may account for Enterobacteriaceae enrichment in IBD and might also be related to the frequency with which nonpathogenic Enterobacteriaceae strains have independently and convergently evolved pathogenic phenotypes (Pupo et al., 1997; Wirth et al., 2006). Thus, although colitis-enriched bacteria, including some Enterobacteriaceae, may play procolitic roles in certain circumstances (Darfeuille-Michaud et al., 1998; Garrett et al., 2010), our findings underscore the importance of functionally assessing a microbe's disease-inducing potential rather than simply extrapolating from disease-associated shifts in microbiota composition.

In summary, our results provide important insights into the intestinal microbiota's role in IBD induction. This work does not support models in which a single microbial species is both necessary and sufficient for disease to occur, nor can we exclude the possibility that additional commensal species unexamined in our study may also be capable of disease induction. Nonetheless, we provide evidence that specific bacterial subsets can induce IBD, whereas other subsets cannot. We demonstrate that the identification and validation of these subsets is experimentally feasible. As additional spontaneous animal models of IBD based on other human susceptibility mutations become available, the experimental criteria and conceptual framework developed here will allow the contributions of commensal bacteria to be assessed in additional genetic contexts.

EXPERIMENTAL PROCEDURES

Mouse Care

Animal protocols were approved by Washington University's Animal Studies Committee. Breeding and antibiotic treatment (ciprofloxacin and metronidazole) of dnKO mice (*CD4-dnTgfbr2*; *Il10r2*$^{-/-}$) was previously described (Kang et al., 2008) (Supplemental Experimental Procedures). *Il10r2*$^{+/-}$ littermates served as controls. For colonization experiments, antibiotic-pretreated mice ($\geq$3 weeks) were gavaged 2 days after treatment cessation and euthanized 3 weeks post-gavage or upon loss of $\geq$30% of maximum body weight. Data are representative of at least two independent experiments per group unless otherwise indicated.

Gross Pathology Scores and Histologic Quantification of Mucosal Inflammation

Tissues were fixed and prepared for gross pathology scoring and histologic analysis as previously described (Figure S1 and Supplemental Experimental Procedures) (Kang et al., 2008).

Fecal Bacterial DNA Extraction and qPCR

Fecal pellets were collected, and bacterial DNA was extracted for quantification by qPCR assays using *rpoβ* or group-specific 16S rRNA primers (Nava et al., 2010) (Supplemental Experimental Procedures).

Intestinal Content and Mixed Culture Preparation

All anaerobic bacteriology and manipulation of uncultured intestinal contents was performed in an anaerobic glove box. Cecal contents were harvested from untreated littermates of dnKO mice, suspended in PBS/glycerol, and stored at −80°C in single-use aliquots. Mixed bacterial cultures were prepared from aliquots of intestinal contents and cultured in parallel on various media types (Supplemental Experimental Procedures).

Bacterial Isolation

Fecal bacteria were cultured on nonselective ANB agar. Pure cultures were identified by 16S rRNA gene analysis (Supplemental Experimental Procedures). Gram-negative anaerobes were isolated on LKV or BBE agar from stocks of intestinal contents (Table S1). *E. coli* for gavage experiments (Figure S5) was isolated on ANB agar from feces of an untreated dnKO mouse and identified by 16S rRNA gene analysis (Supplemental Experimental Procedures).

Gavage Experiments

Titers of frozen stock inocula were determined prior to use in colonization experiments. Aliquots of gavage inocula were volume-adjusted in sterile PBS in the anaerobic chamber and immediately transported to the mouse facility for orogastric gavage (Goodman et al., 2009). Gavage doses were confirmed by back-titering the inocula.

Titration of Fecal Bacteria

Fecal samples were titered on ANB, BBE, or MacConkey agar (Supplemental Experimental Procedures). Identity of bacteria on BBE and MacConkey titer plates from single-isolate-gavaged mice was confirmed by 16S rRNA gene sequence analysis.

Serum Immunoblots

Lysates of *B. thetaiotoaomicron* or *B. vulgatus* were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with pre- or postgavage serum collected from antibiotic-pretreated mice gavaged with pure cultures of the respective isolate (Supplemental Experimental Procedures). Goat anti-mouse IgG was used as a secondary probe.

Statistical Analysis

Statistical analysis was performed using Prism (GraphPad Software) and SPSS (SPSS Inc.) (Supplemental Experimental Procedures). Statistical significance was defined as $p < 0.05$; p values are two tailed.

ACCESSION NUMBERS

All bacterial sequences were deposited in GenBank (JF813174–JF813185).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.chom.2011.04.009.

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