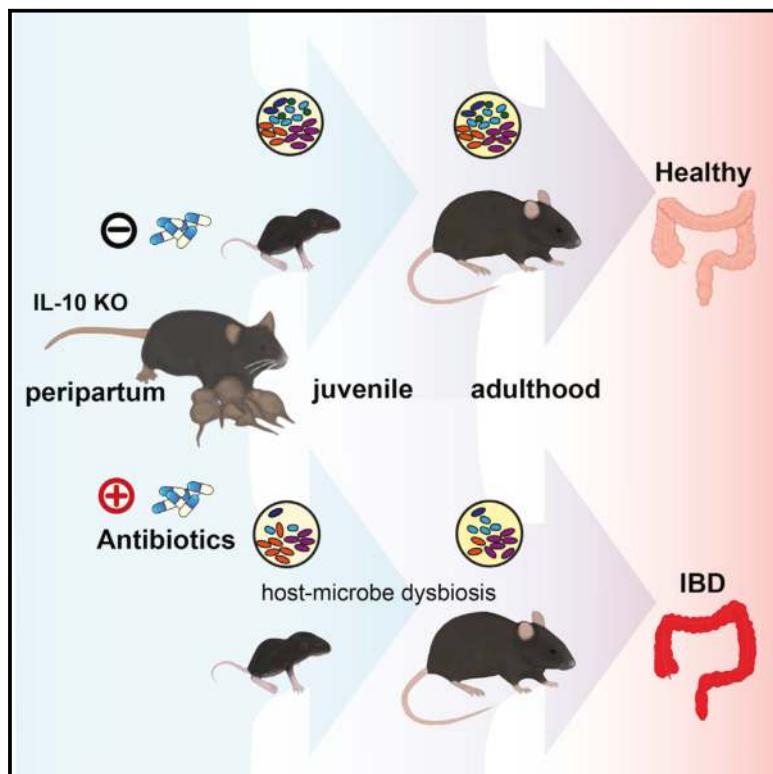


Peripartum Antibiotics Promote Gut Dysbiosis, Loss of Immune Tolerance, and Inflammatory Bowel Disease in Genetically Prone Offspring

Graphical Abstract



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In Brief

Miyoshi et al. show that maternal and neonatal exposure to antibiotics can cause enduring changes in the gut microbiome that adversely affect the development of the immune system. In genetically susceptible individuals, the persistence of intestinal dysbiosis and immune imbalance may increase risk for immune disorders like inflammatory bowel diseases.

Highlights

- Peripartum antibiotics promote offspring gut dysbiosis, immune dysfunction, and IBD
- Antibiotics given after the developmental period do not increase IBD
- Antibiotic-perturbed maternal microbiota likely contribute to neonatal gut dysbiosis
- Gut dysbiosis from peripartum antibiotics and genotype may be useful markers for IBD



Peripartum Antibiotics Promote Gut Dysbiosis, Loss of Immune Tolerance, and Inflammatory Bowel Disease in Genetically Prone Offspring

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SUMMARY

Factors affecting the developing neonatal gut microbiome and immune networks may increase the risk of developing complex immune disorders such as inflammatory bowel diseases (IBD). In particular, peripartum antibiotics have been suggested as risk factors for human IBD, although direct evidence is lacking. Therefore, we examined the temporal impact of the commonly used antibiotic cefoperazone on both maternal and offspring microbiota when administered to dams during the peripartum period in the IL-10-deficient murine colitis model. By rigorously controlling for cage, gender, generational, and murine pathobiont confounders, we observed that offspring from cefoperazone-exposed dams develop a persistent gut dysbiosis into adulthood associated with skewing of the host immune system and increased susceptibility to spontaneous and chemically dextran sodium sulfate (DSS)-induced colitis. Thus, early life exposure to antibiotic-induced maternal dysbiosis during a critical developmental window for gut microbial assemblage and immune programming elicits a lasting impact of increased IBD risk on genetically susceptible offspring.

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic disorders that include two main clinical phenotypes, ulcerative colitis (UC) and Crohn's disease (CD). Diseases like IBD are often referred to as "new-age" disorders because of the alarming increase in their incidence and prevalence over the past century, particularly in populations that have undergone rapid changes in industrialization, hygiene, and diet (Bager et al., 2012; Benchimol et al., 2015; Devkota et al., 2012; Pugazhendhi et al., 2011). While there is a genetic basis (Anderson et al., 2011; Franke et al., 2010; Jostins et al., 2012; Liu et al., 2015; Liu and Stappenbeck, 2016; Van Limbergen et al., 2014), it is unlikely that genetic drift alone over

this short period of time accounts for these diseases, raising the more likely role of environmental risk factors in triggering disease in genetically predisposed individuals. These factors can affect individuals in many ways, but their impact on the gut microbiome, resulting in disturbances in host-microbe interactions, may be of relevance to the development and pathogenesis of IBD (Frank et al., 2007; Gophna et al., 2006; Manichanh et al., 2006; Ott et al., 2004; Seksik et al., 2003). This may be particularly relevant during the early stages of life when critical events in the development of the gut microbiome and immune system are taking place. The identification and avoidance of tipping factors in early life, therefore, represents a logical and important strategy for lowering risk of disease. In this regard, the promiscuous use of antibiotics during the preterm and post-natal (peripartum) periods that affect both maternal and neonatal microbiota has been suggested as a risk factor for human IBD, although compelling scientific evidence is lacking.

In the United States, it is estimated that antibiotics are prescribed with unclear indications at ~21% of pediatric ambulatory visits, where half of the prescriptions are broad spectrum (Hersh et al., 2011). Approximately 40% of pregnant women at term and greater than 30% of neonates are exposed to antibiotics (Broe et al., 2014; Stokholm et al., 2013). In developed countries, broad-spectrum antibiotics, such as cephalosporins, are prescribed more frequently during pregnancy (Petersen et al., 2010). Furthermore, several studies have shown an association between early life exposure to antibiotics and increased risk of IBD development, especially treatment-naïve pediatric CD (Gevers et al., 2014; Hviid et al., 2011; Kronman et al., 2012; Ungaro et al., 2014; Virta et al., 2012). However, these studies were limited in their ability to establish a causal link due to large differences in inter-individual gut microbiomes, challenges in controlling retrospectively for confounding variables, and constraints of observational clinical design. To address this issue, we took an alternate approach, using the well-accepted interleukin (IL)-10 knockout (KO) mouse model, where the immunomodulatory cytokine IL-10 is genetically deleted (Kühn et al., 1993). Genetic risk in IL-10 KO mice is not sufficient to cause disease, because these animals rarely develop disease if they are raised germ-free (GF) or housed in a *Helicobacter hepaticus*-free environment (Keubler et al., 2015; Sellon et al., 1998). *H. hepaticus* rarely causes colitis in wild-type mice but can cause a nearly 100%



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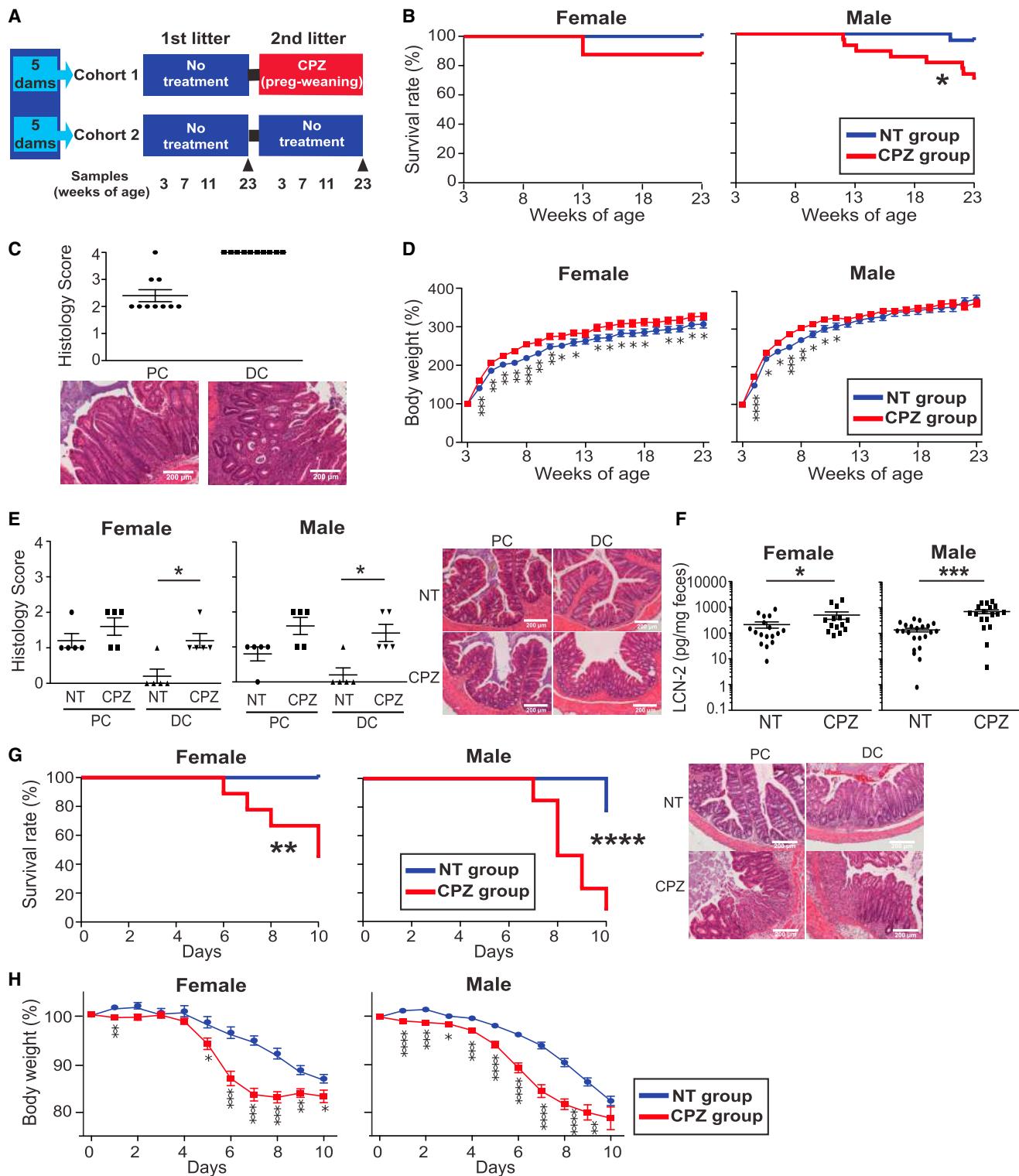


Figure 1. Maternal Peripartum Exposure to CPZ Increases the Risk for Colitis in Offspring

(A) Study design using IL-10 knockout (KO) mouse model. All mice (cohorts 1 and 2) were obtained from ten breeding pairs subjected to mixed-bedding protocol to normalize microbiota among parent cages. Cohort 1 (first row) included two sequential litters from five breeders divided into the non-treatment (NT) tracking group (litter 1) and the cefoperazone (CPZ) tracking group (litter 2). Cohort 2 (second row) included two sequential litters from five breeders that were used as additional NT controls to assess/control for generational drift in gut microbiota across litters 1 and 2.

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disease penetrance in IL-10 KO mice (Kullberg et al., 1998). We examined the temporal impact of the broad-spectrum antibiotic cefoperazone (CPZ) on both the maternal and offspring microbiota when administered to dams during the peripartum period. Here, we reasoned that vertical transmission of maternal microbiota is the major source of microbes for the development of the infant gut microbiome (Adlerberth and Wold, 2009; Asnicar et al., 2017; Caufield et al., 1993, 2007; Funkhouser and Bordenstein, 2013; Jiménez et al., 2008; Nayfach et al., 2016; Pantoja-Feliciano et al., 2013). Under experimental conditions that controlled for cage, gender, and age effects, as well as the confounding presence of common murine pathobionts, we observed that offspring from CPZ-exposed dams develop gut dysbiosis that persists into adulthood. This effect is associated with a skewing of the host immune system and increased susceptibility to the development of spontaneous and chemically (dextran sodium sulfate; DSS)-induced colitis in IL-10 KO mice. Fecal microbiota transplantation (FMT) of CPZ-exposed dams' microbiota into GF IL-10 KO mice resulted in a similar skewing of the host immune response in the offspring of FMT-CPZ recipients to that observed in the offspring of donor animals. Together, our findings support the notion that early life exposure to antibiotic-induced maternal dysbiosis during a critical developmental window for gut microbial assemblage and immune programming can have a lasting impact in genetically susceptible offspring, increasing the risk for complex immune-related disorders such as IBD.

RESULTS

Antibiotic Treatment in Early Life Increases the Risk for Colitis in Offspring

The mice used for this study were derived from GF IL-10 KO mice on a C57BL/6 background conventionalized with *H. hepaticus*-free donor gut microbiota and subsequently housed in an *H. hepaticus*-free room under continuous monitoring. To control for genetics and maternal contribution, the ten breeding pairs used for two sequential parturitions underwent a vetted normalization protocol involving mixed-bedding transfers between cages of the same generation before pairs were set up for breeding (Figure 1A). In cohort 1, the first litters from each dam were used as the non-treatment (NT) group. For their second pregnancy, dams were treated with CPZ from day 14 of gestation and throughout the pre-weaning period (Figure S1A). The rationale for this protocol was to expose pups to maternal microbiota that had been conditioned with CPZ at

the time of the pup's birth, analogous to common practice scenarios in human populations. Cohort 2 served as additional controls, with dams untreated throughout both sequential parturitions to control for the potential impact of sequential litters on colitis development. Pups were tracked from 3 weeks of age through 23 weeks of age. Both genders were studied, with 17 females and 23 males used for the NT tracking group and 16 females and 26 males used for the CPZ tracking group. No differences in the incidence of colitis (0%) were observed between the first and second litters of cohort 2 NT dams (Figure S1B). In contrast, peripartum CPZ exposure led to a significantly decreased survival rate in IL-10 KO pups, particularly in males ($p = 0.018$) (Figure 1B). This included 12.5% of female (2/16) and 30.8% of male (8/26) mice that were sacrificed because of severe weight loss (a euthanasia criteria). Histological examination of their intestines confirmed the presence of severe colonic inflammation (Figure 1C). As noted by others, both female and male mice exposed to peripartum CPZ treatment gained more weight initially (Figure 1D), which can be attributed to the obesogenic effects of antibiotic-induced mucosal inflammation and gut dysbiosis (Cho et al., 2012). Females from CPZ-exposed dams continued to exhibit heavier body weights throughout the 23 weeks relative to NT controls; however, 8 out of 26 males exposed to CPZ began to lose weight as early as 11 weeks of age due to spontaneous colitis development. Despite this, no significant differences were observed in histopathology scores for colitis at 3 and 7 weeks of age between NT and CPZ treatment groups (Figure S1C) prior to decreases in body weight. Thus, peripartum CPZ exposure in the absence of *H. hepaticus*, a known pathobiont, appears to promote increased risk for spontaneous colitis in genetically susceptible offspring later in life.

We next examined the mice that remained grossly healthy and never developed overt signs of spontaneous colitis (NT group: 17 females, 22 males; CPZ group: 14 females, 18 males). At 23 weeks of age, histologic grading of colons revealed higher inflammatory scores, particularly in the distal colons of the CPZ group compared to NT ($n = 5$ mice per gender; $p = 0.021$ in females, and $p = 0.019$ in males) (Figure 1E). Fecal lipocalin-2 (LCN-2), a marker for colitis (Chassaing et al., 2012), showed that both females and males from CPZ-exposed dams had significantly higher LCN-2 levels as compared to the NT group ($p = 0.037$ in females, and $p < 0.001$ in males) (Figure 1F). Thus, even in IL-10 KO mice that did not develop frank disease, peripartum exposure to CPZ is associated with subclinical histologic evidence of colonic inflammation.

(B) Survival rates of NT and CPZ offspring during the observation period (3 to 23 weeks of age) (NT group: $n = 17$ females and 23 males; CPZ group: $n = 16$ females and 26 males).

(C) Histology of proximal and distal colons (PC and DC, respectively) for CPZ mice euthanized due to colitis onset ($n = 2$ females and 8 males). Representative H&E histological sections of PC and DC for NT and CPZ are shown.

(D) Percent weight change (expressed as percent starting weight) of tracked pups beginning at 3 weeks of age (weaning).

(E) Histology of PC and DC in mice that did not develop colitis at 23 weeks of age ($n = 5$ per gender per treatment).

(F) Fecal lipocalin-2 (LCN-2) levels were measured in mice that did not develop colitis at 23 weeks of age (NT group: $n = 17$ females, 22 males; CPZ group: $n = 14$ females and 18 males).

(G) Survival curves for NT and CPZ mice without frank colitis treated with 2.5% dextran sulfate sodium (DSS) at 23 weeks of age. Representative H&E histology of PC and DC are presented for NT and CPZ, respectively, following DSS.

(H) Weight change following onset of DSS treatment. Mice showing more than 20% body weight loss were euthanized.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. Data are indicated as mean \pm SEM for (C)–(F) and (H). See also Figure S1.

To examine how CPZ-exposed IL-10 KO mice that did not develop frank colitis would respond to a colitogenic challenge, mice were administered 2.5% DSS in drinking water at 23 weeks of age. We observed that both female and male mice from CPZ-exposed dams had significantly lower survival rates, lost significantly more weight, and exhibited more severe histologic injury from the DSS challenge compared to their 23-week-old NT counterparts ($p = 0.003$ in females, and $p < 0.0001$ in males) (Figures 1G and 1H). Moreover, as expected, pups from wild-type C57BL/6 (WT) dams that underwent an identical CPZ exposure protocol did not develop colitis. Furthermore, at 23 weeks of age, there were no differences in LCN-2 levels or DSS-induced colitis susceptibility, regardless of gender (Figures S1D and S1E), highlighting the importance of genetic susceptibility for disease development in the case of maternal antibiotic-induced dysbiosis. Together, these data revealed that maternal CPZ exposure during the peripartum period in IL-10 KO mice leads to an increased susceptibility to developing both spontaneous and chemically induced colitis later in life.

Peripartum Antibiotic Exposure Leads to Aberrant Development of the Offspring Immune System

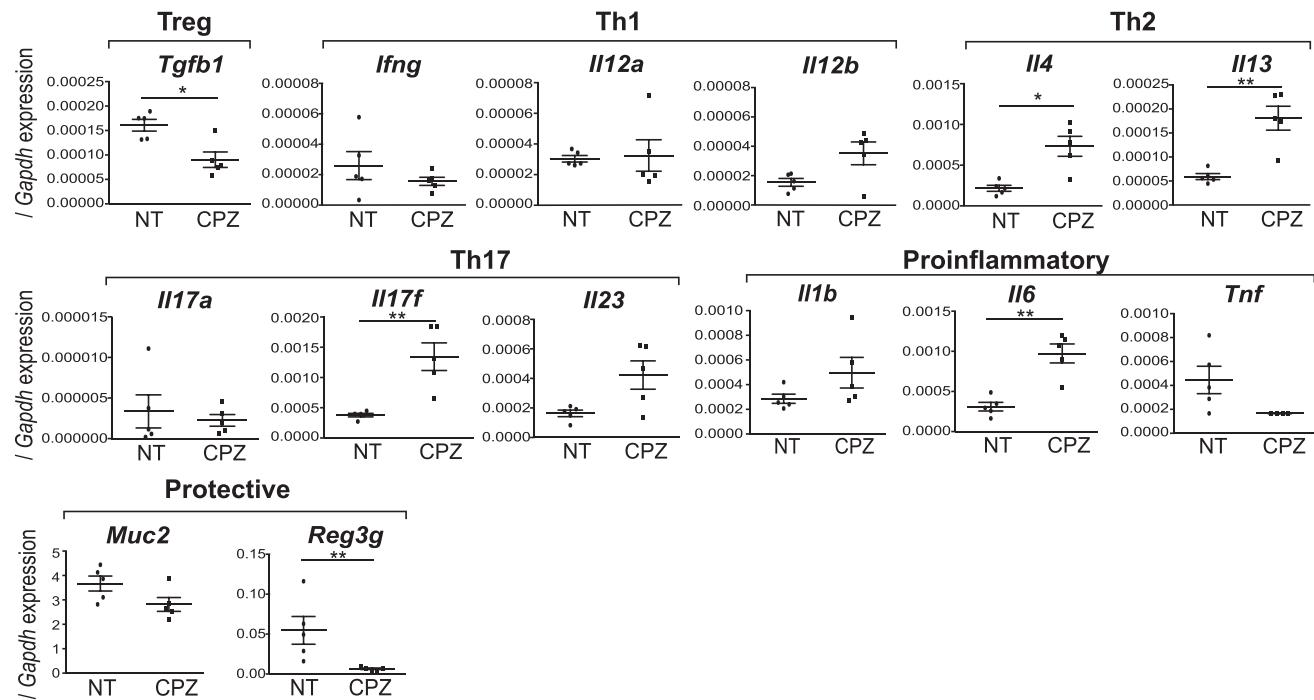
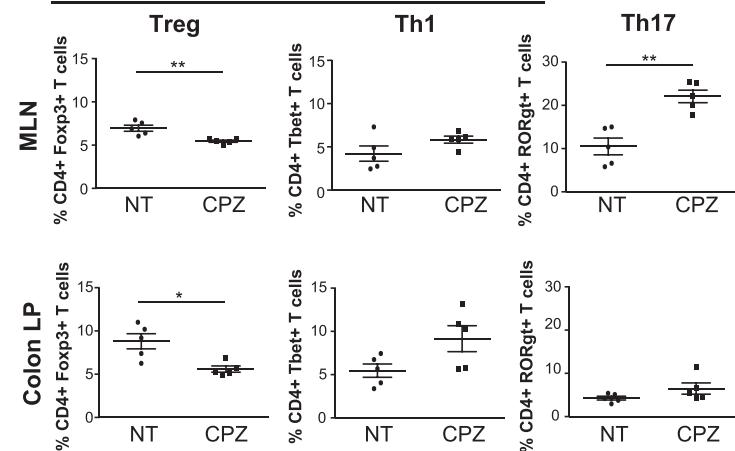
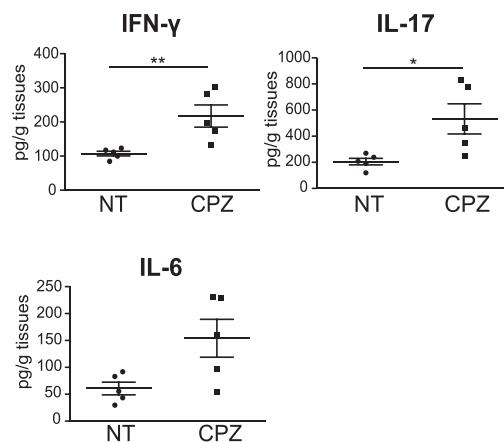
To determine whether and how maternal CPZ exposure during early development alters immune profiles, we first compared mRNA levels of cytokine genes indicative of T cell subtypes (Treg, Th1, Th2, and Th17 cells), pro-inflammatory cytokine genes, and mucosal protective factors relevant to IBD in colons of 3-week-old pups from CPZ-exposed dams versus NT controls. Male data are shown in Figure 2, and female data are shown in Figure S2. Exposure to maternal CPZ-induced dysbiosis elevated mRNA levels of key immune mediators such as *Il17f* ($p < 0.01$ in males), *Il4* ($p < 0.05$ in males), *Il13* ($p < 0.01$ in males), *Il1b* ($p < 0.05$ in females), and *Il6* ($p < 0.01$ in males). Conversely, anti-inflammatory and trophic mediators, including *Tgfb1* ($p < 0.05$ in males), *Muc2* ($p < 0.05$ in females) and *Reg3g* ($p < 0.01$ in females and males), were significantly decreased (Figures 2A and S2A). In spite of the increased mRNA expression of *Il4* and *Il13*, there were no significant differences in plasma immunoglobulin E (IgE) level in CPZ pups compared to NT controls (Figure S2D). Next, we examined markers of several T cell populations prior to the onset of colitis in NT and CPZ 3-week-old IL-10 KO pups using flow cytometry. We determined the proportion of T cells expressing transcription factors indicative of both regulatory and inflammatory T cell populations, including Forkhead box P3 (Foxp3), T-box transcription factor (T-bet), and RAR-related orphan receptor gamma t (ROR γ t). We harvested cells from the mesenteric lymph nodes (MLNs) and colonic lamina propria (LP) of 3-week-old NT and CPZ pups. Here, we focused on CD4 $^{+}$ T cell populations, as shown in the gating strategies used for flow cytometry in Figure S2F. Specifically, we examined the percentage of live CD45 $^{+}$ TCRb $^{+}$ CD4 $^{+}$ cells (CD4 $^{+}$ T cells) that expressed Foxp3 $^{+}$ (regarded as regulatory T cells; Tregs), T-bet $^{+}$ (Th1 cells), or ROR γ t $^{+}$ (Th17 cells). We observed that male CPZ IL-10 KO pups exhibited a significant reduction in Tregs in both the MLN ($p < 0.01$) and colonic LP ($p < 0.05$), with significant increases in Th17 cells in the MLN ($p < 0.01$) as compared to NT counterparts (Figure 2B). Simi-

larly, CPZ IL-10 KO female pups showed significant decreases in MLN Tregs ($p < 0.01$) and significant increases in LP Th17 cells ($p < 0.05$) as compared to NT pups (Figure S2B). These immune changes were not observed in 3-week-old NT versus CPZ WT mice, with the exception for significant differences detected in MLN Th1 cells from WT females (Figure S2E). As shown in Figure 2C, 23-week-old male CPZ IL-10 KO mice that survived and did not develop spontaneous colitis still showed significantly higher levels of interferon (IFN)- γ ($p < 0.01$) and IL-17 ($p < 0.05$), compared to their NT counterparts. In contrast, females at 23 weeks of age did not show such drastic differences (Figure S2C). These findings suggest that an early immune skewing induced by maternal peripartum CPZ exposure in IL-10 KO mice contributes to a proinflammatory milieu that lasts into adulthood.

Peripartum Antibiotic Exposure Induces a Persistent Maternal and Neonatal Gut Dysbiosis

Examination of 16S rRNA gene copy number, as determined by qPCR using universal 16S primers, showed a significant decrease in dams following CPZ exposure as compared to NT, which recovered by 4 and 8 weeks following CPZ cessation (Figure 3A). Despite the recovery of 16S gene copy number, the microbial community structure of dams based on 16S rRNA gene amplicon sequencing revealed that peripartum administration of CPZ caused persistent shifts in the microbiota (Figures 3B and 3C). The Shannon diversity index was decreased (Figure 3B), and microbial β -diversity (Figure 3C) did not recover, even after 8 weeks of CPZ cessation. At the taxonomic level of phyla shown in Figure S3A, CPZ dramatically reduced the relative abundance of Bacteroidetes and increased the relative abundance of Firmicutes and Verrucomicrobia. These changes, particularly in Bacteroidetes and Verrucomicrobia, were also observed 4 and 8 weeks following the cessation of CPZ exposure (Figure S3A). Interestingly, despite the persistent changes in the maternal gut microbiota, none of the dams exposed to CPZ developed colitis. This observation suggested that disruption of the microbial community due to CPZ exposure in adult IL-10 KO mice with already matured gut microbiota may not predispose these animals to develop colitis. To test this, we treated adult female and male IL-10 KO mice with CPZ for 4 weeks (identical to IL-10 KO dams) and tracked their microbial community structure as well as body weight. Here, we observed no significant differences in survival rate between NT and CPZ-treated IL-10 KO adults, and both females and males exhibited a significant increase in body weight following CPZ exposure (Figure S3B). Contrary to the 16S rRNA changes observed in IL-10 KO dams, no appreciable differences were observed with regard to the relative abundance of Bacteroidetes in adult IL-10 KO mice of either gender following the cessation of CPZ; however, Verrucomicrobia appeared to be elevated (Figure S3C). Together, these data suggest that exposure to CPZ following microbial maturation may be beyond the window of immune maturation and may not significantly impact disease prognosis, even in genetically susceptible mice.

We next examined the microbial community composition of pups from NT and CPZ dams at 3, 7, and 11 weeks of age.

A qPCR Cytokine Gene Expression**B Flow Cytometry T cell Populations****C ELISA Cytokine Expression****Figure 2. Maternal Peripartum Exposure to Cefoperazone Leads to Aberrant Development of the Host Immune System in IL-10 KO Mice**

(A) Real-time qPCR mRNA levels of cytokine and pro- versus anti-inflammatory genes involved in colonic inflammation from colonic mucosal scrapings in non-treated (NT) versus cefoperazone (CPZ) male IL-10 KO mice at 3 weeks of age. mRNA levels are expressed as $\Delta\Delta CT$ relative to the housekeeper gene *Gapdh*.

(B) Flow-cytometric analyses of live CD45 $^+$ TCR β $^+$ CD4 $^+$ T cells expressing Foxp3 $^+$ (Treg), T-bet $^+$ (Th1), or ROR γ t $^+$ (Th17) in MLNs and colonic LPs of NT versus CPZ males at 3 weeks of age. Data indicate percentage of live CD4 $^+$ cells.

(C) Protein levels of inflammatory cytokines in MLNs of NT males (black circles) versus CPZ-exposed males (black squares) at 23 weeks of age determined via ELISA.

n = 4–5 per group. *p < 0.05; **p < 0.01, via Mann-Whitney U test. Female data are shown in Figure S2. Data are indicated as mean \pm SEM.

See also Figure S2.

Analysis of 16S rRNA gene copy number revealed significant reduction at 3 weeks of age in both male and female CPZ pups ($p < 0.0001$), with a significant compensatory increase

at 7 and 11 weeks of age relative to NT pups (Figure 3D). The pups from CPZ-exposed dams showed a significantly lower Shannon diversity index compared to that of pups in

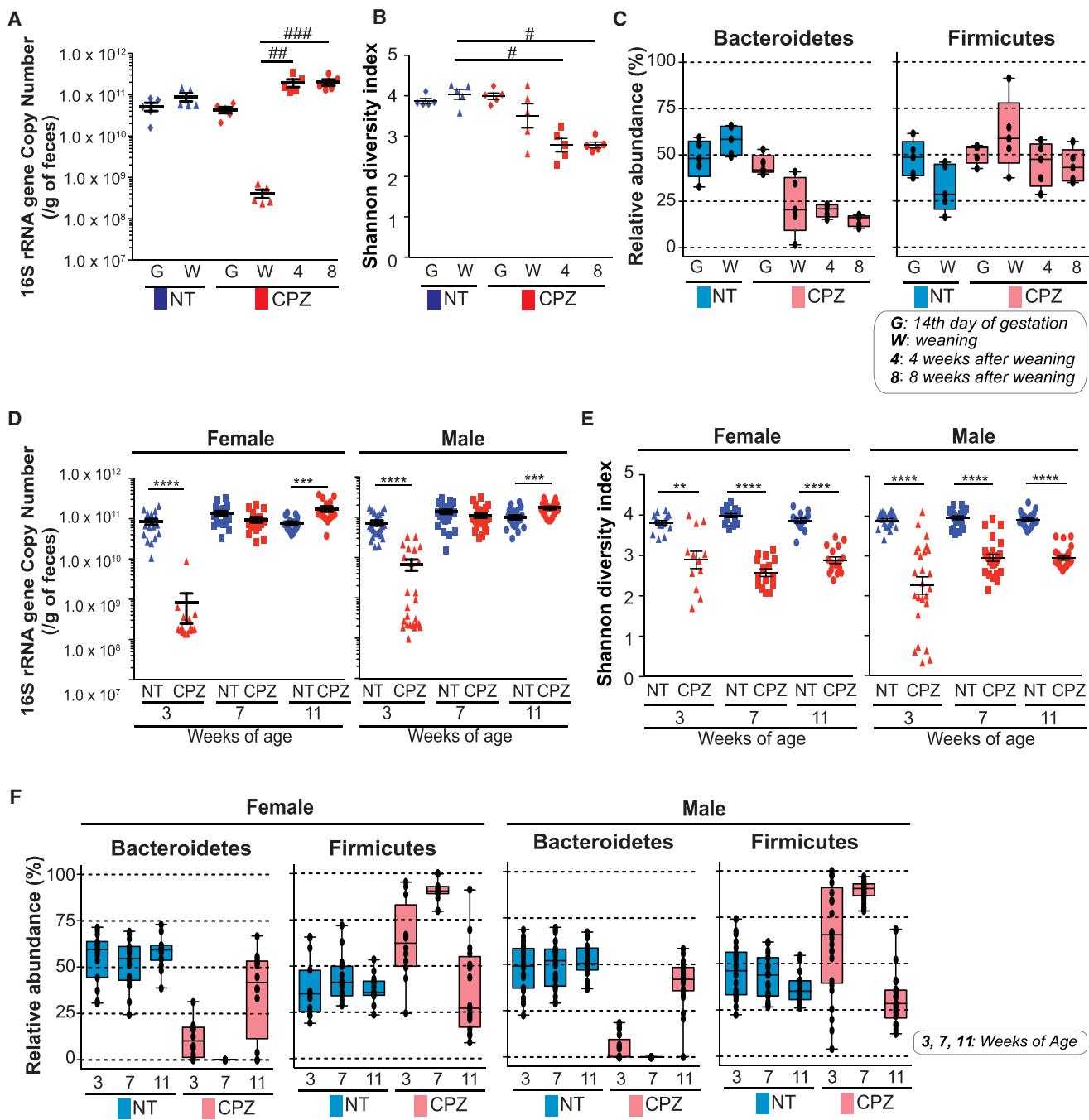


Figure 3. Maternal Peripartum Exposure to Cefoperazone Induces a Persistent Gut Dysbiosis in Dams and Pups

(A) Fecal 16S rRNA gene copy number in non-treated (NT) versus cefoperazone (CPZ)-exposed dams at the 14th day of gestation (start of CPZ treatment), at weaning (end of CPZ treatment), and at 4 weeks and 8 weeks after CPZ cessation.

(B) Shannon diversity index in dams at the 14th day of gestation (G), at weaning (W), and at 4 weeks and 8 weeks after CPZ cessation.

(C) Bacterial community composition in NT and CPZ-exposed dams tracked until 8 weeks after CPZ cessation.

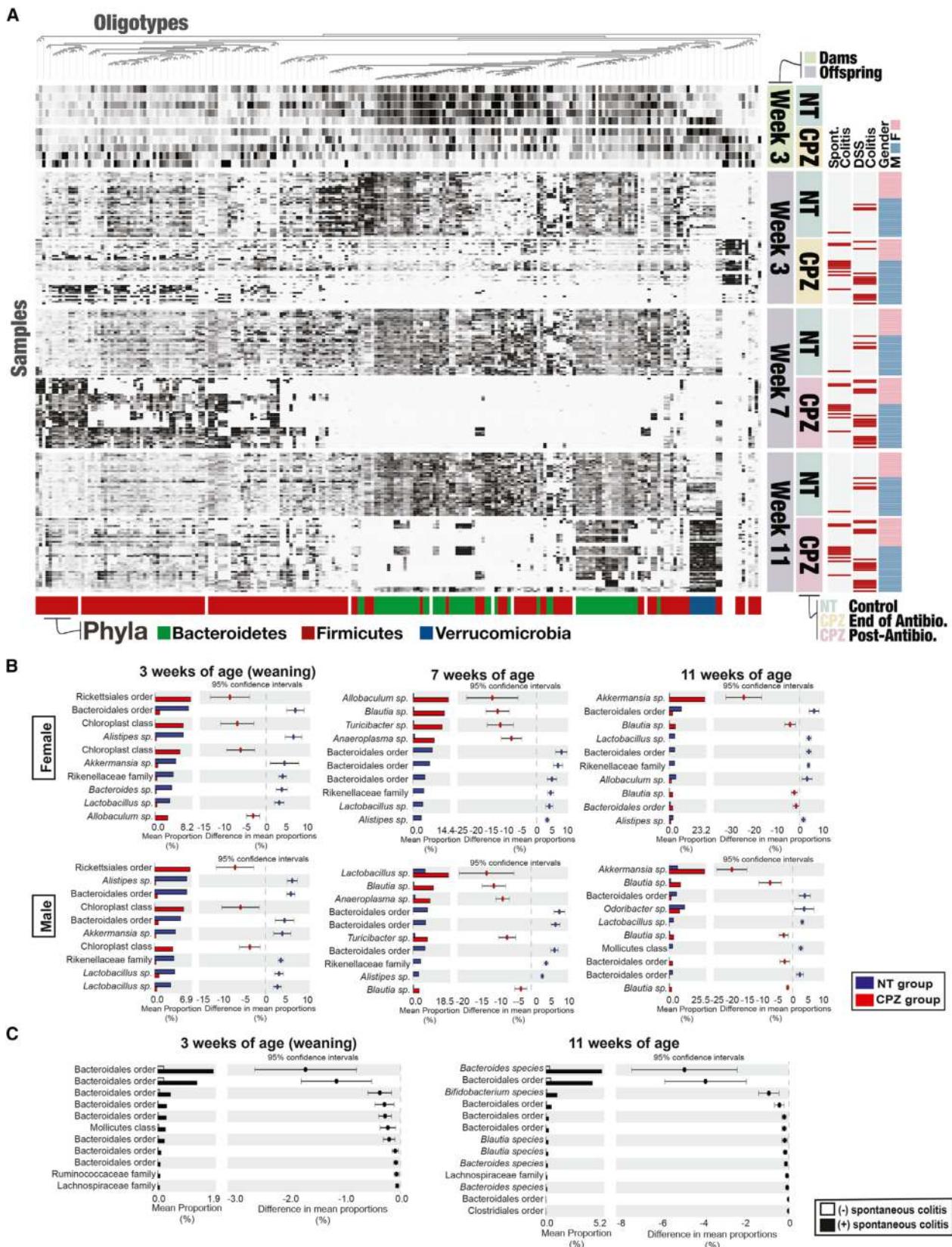
(D) Fecal 16S rRNA gene copy number in NT and CPZ IL-10 KO offspring at 3, 7, and 11 weeks of age.

(E) Shannon diversity index in NT versus CPZ IL-10 KO offspring at 3, 7, and 11 weeks of age.

(F) Bacterial community composition was assessed at 3, 7, and 11 weeks of age in female and male offspring.

Two dominant phyla, Bacteroidetes and Firmicutes, are presented for (C) and (F). Additional phyla are shown in Figure S3. # $p < 0.05$; ## $p < 0.01$; and ### $p < 0.001$, via Dunn's test. * $p < 0.01$; ** $p < 0.001$; and *** $p < 0.0001$, via Mann-Whitney U test. Data are represented as mean \pm SEM for (A), (B), (D), and (E). Oligotype phyla taxa are presented as relative abundance and represented with boxplots. Dots represent individual samples.

See also Figure S3.



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the NT group ($p < 0.01$ at 3 weeks of age, and $p < 0.0001$ at 7 and 11 weeks of age in females; $p < 0.0001$ at 3, 7, and 11 weeks of age in males) (Figure 3E). A persistently low α -diversity was observed as late as 11 weeks of age in CPZ pups. At 3 weeks of age, the microbial community composition differed dramatically between the NT and CPZ groups. However, each group exhibited community profiles similar to that of their respective dams. The alterations to the microbiota in CPZ pups were persistent, showing different patterns of maturation through adulthood (11 weeks of age), 8 weeks post-exposure to maternal CPZ-induced dysbiosis, and at a time point prior to the onset of inflammation in either group (Figures 3F and S3D).

Exposure to Antibiotics in the Peripartum Period Induces Persistent and Significant Changes in Gut Microbiota in IL-10 KO Dams and Offspring

A high-resolution analysis of 16S rRNA gene amplicons using Minimum Entropy Decomposition (MED) revealed that CPZ exposure of dams resulted in significant changes in bacterial oligotypes as compared to NT dams at the time of weaning (Figure 4A, top heatmap). Interestingly, the samples from dams suggest that CPZ exposure leads to persistent changes in specific oligotypes, even following CPZ cessation, in that little to no recovery of oligotypes was observed at 4 or 8 weeks post-treatment (Figure S4A). We observed specific alterations in oligotype abundance in maternal microbiota of NT versus CPZ-exposed dams at weaning (3 weeks post-birth; approximately 4 weeks after the start of CPZ treatment) that were consistent with changes in the microbiota of the offspring at weaning (3 weeks of age) (Figure 4A, top and bottom heatmaps). As described, nearly 100% of the mice that developed either spontaneous colitis or severe DSS-induced colitis were from CPZ-exposed dams, where dysbiosis was clearly evident, suggesting that maternal vertical transmission of specific oligotypes, and not vertical transmission of CPZ itself, may play a role in colitis development. To rule out a significant contribution of direct exposure of CPZ to the pups' microbiota via maternal transmission, we performed antimicrobial assays using cecal contents from NT versus CPZ-exposed dams and their respective offspring at weaning. We observed that only cecal contents from CPZ-exposed dams elicited antimicrobial effects against *E. coli*, while no appreciable killing effect was observed in NT or CPZ pups, suggesting that maternal transmission of CPZ was minimal (Figure S4B). Together, these data provide evidence that maternal CPZ-induced gut microbiota is

vertically transferred to the offspring and does not fully recover, despite cessation of antibiotic intake.

Further analysis of gut microbes of the offspring from NT or CPZ-exposed dams at 3, 7, and 11 weeks of age showed persistent dysbiosis over time in CPZ pups (Figure 4A, bottom). The changes in oligotypes in pups from CPZ-exposed dams persisted well beyond maternal influence in that CPZ pups do not exhibit recovery at 7 or 11 weeks of age, while oligotypes in NT pups appear to retain some stability over time (Figure 4A, bottom heatmap). Pups from CPZ-exposed dams showed significant alterations at 3, 7, and 11 weeks of age in oligotypes belonging to the phyla Bacteroidetes, Firmicutes, and Verrucomicrobia relative to age-matched NT controls. Overall, offspring from CPZ-exposed dams exhibited a decrease in a large portion of oligotypes assigned to Bacteroidetes across all time points, despite some evidence for a reemergence of several of these oligotypes, along with several others belonging to both Firmicutes and Verrucomicrobia at 11 weeks of age. Oligotypes across all samples visualized in the heatmap can be further explored in depth at https://anvi-server.org/merenlab/il10ko_peripartum_cpz.

At each time point, significant differences were observed in the relative abundance of particular oligotypes between offspring from NT versus CPZ-exposed dams. To determine which oligotypes were the most significant contributors to the overall differences between NT and CPZ microbial communities, we utilized the Statistical Analysis of Metagenomic Profiles (STAMP) to identify significantly enriched oligotypes. The top ten oligotypes exhibiting the greatest differences in mean relative abundance among females and males and across all three time points are shown in Figure 4B. Specifically, at 3 weeks of age, CPZ males and females exhibited a larger proportion of oligotypes belonging to the Rickettsiales order from the phyla Proteobacteria, as compared to NT controls. At 7 weeks of age both male and female offspring from CPZ-exposed dams exhibited a significant increase in specific *Allobaculum* sp., *Blautia* sp., *Turicibacter* sp., *Lactobacillus* sp., and *Anaeroplasma* sp. oligotypes. By 11 weeks of age, *Akkermansia* sp. and *Blautia* sp. were significantly elevated in female and male CPZ offspring as compared to NT. Furthermore, analysis of the male offspring from CPZ-exposed dams at 3 and 11 weeks of age showed unique oligotype signatures from those mice that eventually developed spontaneous colitis as compared to mice that remained healthy. These oligotypes that are significantly different are presented in Figure 4C and include bacteria belonging to the order Bacteroidales at both 3 and 11 weeks of age.

Figure 4. Maternal Peripartum Exposure to Cefoperazone Induces Persistent and Significant Changes in Specific Microbial Oligotypes in IL-10 KO Dams and Offspring

(A) Comprehensive comparison of gut microbes in fecal samples collected from non-treatment (NT) and cefoperazone (CPZ)-treated IL-10 KO dams at weaning (week 3) and their offspring at 3, 7, and 11 weeks. Oligotype abundance was determined in samples from dams and offspring that were tracked for spontaneous colitis or DSS treated. Sixteen samples were excluded due to low number of sequences. Columns represent an individual oligotype, and rows represent individual samples. Samples with red horizontal bars represent mice that developed spontaneous colitis (Spont. Colitis) or DSS colitis following exposure at 23 weeks of age and were euthanized due to frank colitis. Colored bars at the bottom of the figure represent dominant phyla.

(B) Significant changes in mean proportions of oligotypes between NT and CPZ offspring at 3, 7, and 11 weeks of age as determined by STAMP. The top ten oligotypes with the greatest changes in relative abundance are represented with extended error bar plots (error bars represent 95% confidence intervals). All oligotypes significantly different between groups are presented in Table S1.

(C) Oligotypes with significant differences in relative abundance were identified at 3 and 11 weeks of age between males from CPZ-exposed dams that did or did not develop spontaneous colitis. All oligotypes identified as significantly altered between groups are sorted by changes in relative abundance. See also Figure S4 and Table S1.

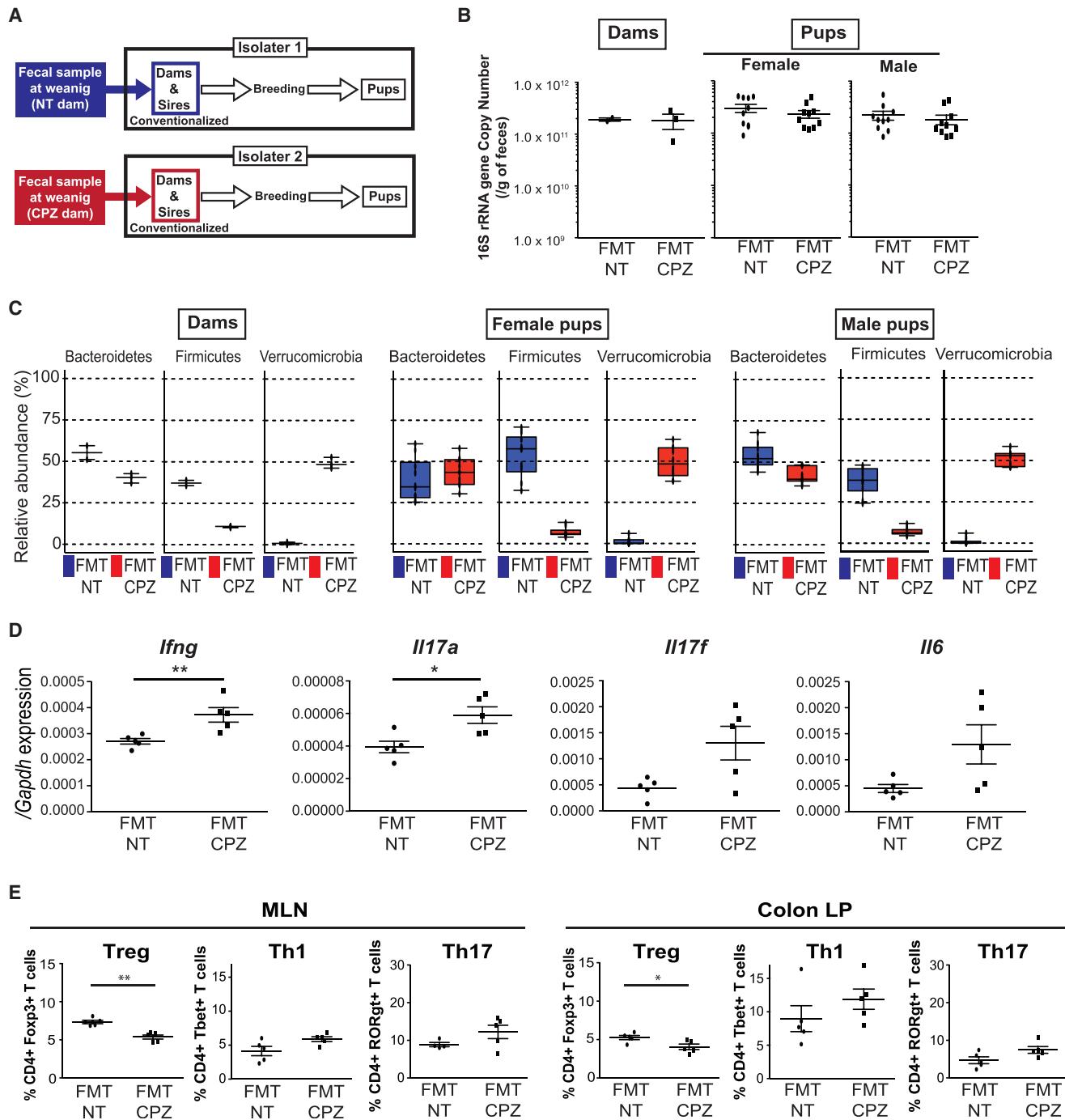


Figure 5. Conventionalization of GF IL-10 KO Mice with CPZ-Induced Dysbiosis Skews Host Immune Status in the Offspring

(A) Fecal microbiota transplant (FMT) study design. GF IL-10 KO mice were gavaged with fecal slurries prepared from non-treated (NT) or cefoperazone (CPZ)-treated dams at weaning from cohort 1 in separate flexible film isolators. Breeding pairs were then set up (isolator 1: two FMT-NT dams; isolator 2: three FMT-CPZ dams). Offspring from FMT dams in each isolator were analyzed at weaning ($n = 10$ per gender per group).

(B) Fecal 16S rRNA gene copy number in dams and offspring at weaning.

(C) Bacterial community composition in dams and offspring. Three dominant phyla—Bacteroidetes, Firmicutes, and Verrucomicrobia—are presented. Additional phyla are shown in Figure S5.

(D) Real-time qPCR mRNA levels of inflammatory cytokines from colonic mucosal scrapings in FMT-NT (black circles) versus FMT-CPZ (black squares) male IL-10 KO offspring at 3 weeks of age. mRNA levels are expressed as $\Delta\Delta CT$ relative to housekeeper gene *Gapdh*.

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FMT of Maternal CPZ-Induced Dysbiosis into GF IL-10 KO Dams Elicits a Pro-inflammatory Milieu in Offspring

We next examined whether CPZ-induced dysbiosis observed in dams would transfer an inflammatory phenotype in the absence of CPZ exposure. Here, we performed FMT using stool obtained from NT or CPZ-exposed dams at weaning into GF IL-10 KO females maintained in separate flexible film isolators (Figure 5A). Following FMT, these mice were set up into breeding pairs. Examination of dams and pups at the time of weaning revealed identical 16S rRNA gene copy numbers as measured by qPCR in recipients regardless of donor treatment (Figure 5B). Analysis of 16S rRNA amplicons via MED showed that dams, female pups, and male pups within the FMT-CPZ group exhibited significant decreases in Firmicutes, with concomitant increases in Verrucomicrobia relative to FMT-NT (Figure 5C). Slight but insignificant changes in Bacteroidetes were observed only in dams and male FMT-CPZ pups (Figure 5C). Significant changes were not observed in other phyla between FMT-NT and FMT-CPZ dams and pups (Figures S5A and S5B). At 3 weeks of age, we examined similar immune parameters in FMT pups as shown in specific-pathogen-free (SPF) IL-10 pups from NT or CPZ-exposed dams in Figure 2. Male FMT-CPZ pups exhibited significantly increased expression of *Ifng* and *I17a* in MLNs relative to FMT-NT and increased but not significant trends of *I17f* and *I6* (Figure 5D). Similar observations were also seen in female FMT-CPZ 3-week-old pups (Figure S5C). We next examined markers of T cell populations in FMT-NT and FMT-CPZ 3-week-old pups via flow cytometry. Again, we determined the proportion of T cells expressing transcription factors, including Foxp3, T-bet, and ROR γ t in the MLN and colonic LP, focusing on CD4 $^+$ T cell populations (Figure S2F). We observed that FMT-CPZ male IL-10 KO pups exhibited a significant reduction in Tregs in both the MLN ($p < 0.01$) and colonic LP ($p < 0.05$), with increases, although not significant, in Th1 and Th17 cells in the MLN and LP, as compared to FMT-NT counterparts (Figure 5E). Interestingly, FMT-CPZ female IL-10 KO pups exhibited no significant differences in MLN or LP proportions of Tregs; however, both Th1 and Th17 cells were significantly increased in the MLN ($p < 0.05$) (Figure S5D). To rule out the direct impact of CPZ on these immune parameters, we analyzed 3-week-old pups from GF IL-10 dams that had or had not received peripartum CPZ in a manner identical to that presented in Figure 1A. Analysis of the proportion of T cells expressing Foxp3, T-bet, or ROR γ t showed that they were not significantly altered by maternal exposure to CPZ under GF conditions (Figure S5E), suggesting that exposure to maternal dysbiosis induced by CPZ under SPF conditions in early life served as the main driver of changes.

DISCUSSION

The findings of this study support a potential role of broad-spectrum antibiotics as a risk factor for IBD when used during the

peripartum period. While this possibility has been entertained by several retrospective human studies, a clear causal link between peripartum antibiotics and IBD risk has never been proven (Gevers et al., 2014; Hviid et al., 2011; Kronman et al., 2012; Ungaro et al., 2014; Virta et al., 2012). Using a genetic risk model, the IL-10 KO mouse, and using a prospective study design that rigorously controls for starting gut microbiomes and generational drift, we were able to observe the impact of CPZ, a broad-spectrum antibiotic, on maternal and offspring gut microbiota during and after antibiotic exposure. Our data indicate that peripartum CPZ exposure of dams causes gut microbial dysbiosis in dams, which is vertically transmitted to the offspring and is still evident following cessation of CPZ exposure. These changes were associated with a skewing of the immune profile in IL-10 KO pups from dams exposed to peripartum CPZ, rendering them at higher risk for the development of spontaneous and DSS-induced colitis later in life.

We designed our study to follow a common practice scenario in humans where antibiotics are used widely among late-term pregnancies and during the neonatal period. By using a carefully designed bedding transfer protocol, we ensured that the various treatment groups had similar starting microbiomes and, by using a model of sequential parturitions, we controlled for generational drift in microbiota. Finally, we used MED to characterize the microbial community structure at high resolution. The impact of CPZ-induced perturbations in maternal microbiota on the development of offspring gut dysbiosis is particularly notable, as the changes in respective taxa over time were similar, except more pronounced in the offspring. We speculate that the severe gut dysbiosis observed in the offspring of CPZ-exposed dams stems from the antibiotic-induced selective pressure on maternal microbiota that reduces the abundance, diversity, and likelihood of susceptible organisms to establish in the neonate. The CPZ-induced changes in maternal gut microbiota show that Bacteroidetes are significantly reduced at the time of CPZ cessation, which persists in the dams up to 8 weeks after cessation of the antibiotic (Figure S3A). However, these changes did not occur to the same extreme as to those observed in offspring, perhaps indicative of a greater resilience of a mature adult gut microbiota to broad-spectrum antibiotics. Consistent with this notion, dams were only exposed to CPZ during adulthood, and none developed colitis. We further showed that exposure of both male and female IL-10 KO mice in adulthood failed to produce the same degree of dysbiosis and also did not result in colitis. In contrast, neonates that acquired microbes from maternal antibiotic-induced gut dysbiosis during this critical window of microbial assemblage displayed a dysbiotic gut microbiome from the very start, which was associated with immediate and long-term skewing of their immune system.

As shown in Figure 4, it is striking to see the near absence of most members of the Bacteroidetes phylum and the elevated abundance of members of the Firmicutes phylum

(E) Flow-cytometric analyses of live CD45 $^+$ TCR β $^+$ CD4 $^+$ T cells expressing Foxp3 $^+$ (Treg), T-bet $^+$ (Th1), or ROR γ t $^+$ (Th17) in MLNs and colonic LPs of 3-week-old FMT-NT versus FMT-CPZ male offspring ($n = 4\text{--}5$ per group).

Data indicate percentage of live CD4 $^+$ cells. * $p < 0.05$; ** $p < 0.01$, via Mann-Whitney U test. Data are represented as mean \pm SEM for (B), (D), and (E). See also Figure S5.

at 3 (weaning) and 7 weeks of age (4 weeks post-exposure to maternal CPZ-induced dysbiosis). We attribute the nadir of Bacteroidetes at 7 weeks of age to the possibility that other more fit groups (Firmicutes and Verrucomicrobia) developed stable networks that limited the ability of Bacteroidetes to establish (Figure S3D). By 11 weeks of age, we observe some appearance of Bacteroidetes taxa, but their diversity remained limited. This gut dysbiosis persisted to adulthood (11 weeks of age), even before frank colitis was observed in any of the groups. Additionally, we identified oligotypes that appeared to be significant contributors to the overall differences observed between NT and CPZ groups, as well as among CPZ male pups that did or did not develop colitis (Figure 4). Furthermore, FMT of maternal CPZ-induced communities into GF IL-10 KO mice resulted in immune skewing in these offspring that was consistent with that observed in SPF IL-10 KO pups exposed to maternal CPZ-induced dysbiosis. This reveals that maternal CPZ-induced dysbiosis alone in the absence of direct exposure to CPZ can elicit a negative impact on immunological outcomes and possibly further predispose genetically susceptible offspring to develop colitis. Future studies involving shotgun metagenomics should provide additional insights into maternal-offspring influences, host disease development, and potential predictive markers of risk.

The maternal CPZ-induced gut dysbiosis transmitted to the offspring is associated with disruptions in immune development and protective processes that would normally occur at the time of weaning to adulthood (Figure 2). The increased mRNA expression of *Il4* and *Il13* implied the possibility that Th2-mediated mechanisms, including invariant natural killer T (iNKT) cells, may play a role in the development of colitis, as previously reported (Olszak et al., 2012). However, in our model, plasma IgE was not significantly altered by maternal CPZ exposure (Figure S2D); hence, we focused on Th1 and Th17 cells, which have been shown to be relevant in the IL-10 KO mouse (Keubler et al., 2015). While we observed significant increases specifically in Th17 ROR γ t cells, previous work in WT C57BL/6 mice from dams experiencing repeat exposure to subtherapeutic doses of penicillin exhibited decreases in IL-17 and IFN- γ T cell populations. However, in our model, genetically susceptible hosts such as the IL-10 KO mouse respond in a different manner relative to WT mice, resulting in a relatively increased risk for “spontaneous” colitis, as was observed (Figure 1). It is notable that this risk continues to exist for mice that do not develop frank spontaneous colitis, as these mice exhibited increased histological and subclinical mucosal inflammation and were highly susceptible to the colitogenic effects of DSS compared to NT controls. These findings are likely relevant to human IBD where genetic risk, while necessary, is, by itself, insufficient to cause frank disease. It is also possible that a significant number of these at-risk subjects have subclinical disease that would be detectable histologically or through examination of immune/inflammatory markers, which has been previously suggested (Howarth et al., 2002; Sakata et al., 2001). Moreover, these individuals are likely to be more sensitive to risk factors that set the stage for events that trigger the immune response to cause frank clinical disease. We speculate that this may involve improper imprinting of

immune networks of genetically susceptible individuals created by the gut dysbiosis caused through peripartum antibiotic exposure.

In summary, our studies provide compelling evidence that peripartum maternal antibiotic exposure skews maternal gut microbiota, and that of the subsequent offspring, and increases IBD risk in genetically susceptible offspring by affecting a critical stage of their microbial and immune development. These effects can be persistent into adulthood and promote the risk of complex immune disorders such as IBD in genetically susceptible hosts. Identification of potential risk factors and a better understanding of the underlying mechanisms that lead to increased IBD risk is paramount for developing strategies for the prevention and/or treatment of human IBD. In this case, we may have to rethink common practices of indiscriminate and empirical use of antibiotics during pregnancy and infancy and perhaps give thought to the development of microbial and host metrics that can assess states of host-microbe interactions so that course corrections can be made to promote good health.

EXPERIMENTAL PROCEDURES

Animals

GF IL-10 KO mice on a C57BL/6J genetic background were bred in the University of Chicago Gnotobiotic Research Animal Facility (GRAF) and FMT (conventionalized) with *Helicobacter hepaticus*-free microbiota (kindly provided by Dr. Cathryn Nagler, University of Chicago), as previously described (Gilliland et al., 2012). Following conventionalization, these SPF IL-10 KO breeding pairs were bred in house under *H. hepaticus*-free conditions (University of Chicago Institutional Animal Care and Use Committee [IACUC] protocol 71084). Five initial breeding pairs were prepared, and their progeny were transferred to bedding to normalize gut microbes. Bedding was mixed at 4 days and 9 days following cage changes every 2 weeks until breeding was initiated. Following this normalization procedure, ten breeding pairs were prepared to obtain two parturitions for NT and CPZ-treatment groups (Figure 1A). In cohort 1, the first and second litters were used as NT controls or the CPZ group, respectively. In cohort 2, the first and second litters were used as NT controls. Wild-type C57BL/6J mice underwent the same breeding, microbial normalization, and CPZ treatment protocol for comparison.

Antibiotic Treatment

CPZ sodium salt was purchased from Sigma-Aldrich. CPZ (0.5 mg/mL) was administered in drinking water beginning at the third week of gestation until weaning of pups (Figure S1A). Mice had free access to water throughout the treatment period (University of Chicago IACUC protocol 72101). The same dose of CPZ was administered to adult IL-10 KO mice between 12 and 20 (18.9 ± 2.9) weeks of age for 4 weeks.

Clinical Evaluation of Spontaneous Colitis

Body weights of mice in cohorts 1 and 2 were measured weekly beginning at weaning. The euthanasia criteria for spontaneous colitis included rectal prolapse; more than 15% body weight loss; or signs of pain/distress, including poor grooming, decreased activity, and hunched posture (Hale and Greer, 2012).

DSS-Induced Colitis

Mice were given 2.5% DSS (36–50 kDa) (MP Biomedicals) in drinking water for 10 days (IACUC protocol 72101). All mice were weighed and monitored daily. Mice exhibiting more than 20% body weight loss were euthanized.

FMT in GF IL-10 KO Mice

Fecal samples collected from NT and CPZ-exposed dams at weaning were used to gavage FMT of 200 μ L fecal supernatant (100 mg feces per 1 mL sterile

PBS) into GF IL-10 KO female recipients maintained in separate flexible film isolators (see model in *Figure 5A*). NT and CPZ-FMT females were paired with GF IL-10 KO males for breeding. Pups were utilized for flow cytometry and qPCR analysis at 3 weeks of age (weaning). Fecal pellets were collected from dams and offspring for microbial analysis.

Histological Analysis

Colon samples were fixed in 4% formaldehyde and embedded in paraffin followed by H&E staining. Colitis histological score for colitis was used as previously described (Erben et al., 2014).

Fecal DNA Extraction and 16S rRNA Gene Amplicon Analysis

Fecal samples of tracked offspring in cohort 1 were harvested and rapidly frozen at -80°C at 3, 7, 11, and 23 weeks of age. DNA was extracted as previously described (Wang et al., 2009), and the V4-V5 region of the 16S rRNA gene was amplified following Earth Microbiome Project (EMP) protocols (<http://www.earthmicrobiome.org/emp-standard-protocols/16s/>). Sequencing was performed on an Illumina MiSeq sequencer at the High-Throughput Genome Analysis Core, Argonne National Laboratory. Raw sequencing data were de-multiplexed, and partially overlapping paired-end reads were merged using illumina-utils (Eren et al., 2013b). Mismatches at the overlapping regions of pairs were resolved using the base with the higher Q score, and the merged sequences were kept for downstream analyses only (1) if they contained up to three mismatches at the overlapping region, and (2) if 66% of the bases in the first half of each read had an average Q score of 30. The quality filtered reads were partitioned into ecologically relevant units using MED (Eren et al., 2015b) with default parameters. Using Shannon entropy, MED resolves a given amplicon dataset iteratively into high-resolution oligotypes (Eren et al., 2013a). Taxonomy was assigned to oligotypes using GAST (Huse et al., 2008), and Anvi'o v2.3.1 (Eren et al., 2015a) was used to visualize the relative abundance of each oligotype across samples in the context of metadata. Oligotype community data were normalized, and taxonomic relative abundances in NT and CPZ groups were compared for females and males separately at each time point. Oligotypes with significant differences between NT and CPZ communities were filtered for oligotypes with greater than 10-fold changes in relative abundance, and then further filtered for the top ten oligotypes exhibiting the greatest difference in mean relative abundance. Extended error plots were created using a statistical analysis of taxonomic and functional profiles (STAMP, v2.1.3) (Parks et al., 2014).

Statistical Analysis

Spontaneous and DSS-induced colitis incidence by NT or CPZ treatment was estimated by "survival analysis," performed using log-rank test followed by Kaplan-Meyer plot. The Mann-Whitney U test was used to compare body weights, histological scores, T cell populations, mRNA expression levels, protein levels, or 16S rRNA gene copy number between the NT and CPZ groups. The Kruskal-Wallis test and Dunn's test were also used for 16S rRNA gene copy number. These tests were performed with GraphPad Prism (GraphPad Software). Statistical significance was assumed when $p \leq 0.05$. Raw counts of oligotypes were normalized using the "decostand" function implemented in the R/CRAN package "vegan." Alpha diversity via the Shannon diversity index was computed using the "diversity" function implemented in the "vegan" package. Student's t test was used to compare oligotype enrichment at 3, 7, and 11 weeks of age between IL-10 KO pups from NT and CPZ-exposed dams and between male pups from CPZ-exposed dams that eventually developed spontaneous colitis and male pups that remained healthy. The p values were adjusted for multiple test using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). The criterion of significance was set at a false discovery rate (FDR) <0.05.

ACCESSION NUMBERS

The accession numbers for the mouse sample information and the microbial dataset reported in this paper are BioProject: PRJNA376026 and SRA: SRP108147, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.06.060>.

AUTHOR CONTRIBUTIONS

J.M., A.M.B., V.L., and E.B.C. conceived the study, designed experiments, and prepared the manuscript. J.M., A.M.B., and S.M. performed experiments and analyzed data. J.M., Y.H., N.H., T.O.D., and A.M.E. analyzed microbial data sets. E.B.C. oversaw the entire project.

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