THE UNIVERSITY OF CHICAGO

EXPLORING THE ROLE OF GUT MICROBIOTA AS MODIFIERS OF AUTOIMMUNE PATHOGENESIS IN INTERLEUKIN-10 DEFICIENT MICE

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BY

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Dedication

I dedicate this to my parents and loved ones,

for your unconditional support, kindness, courage, and wisdom.

I dedicate this to my teachers and mentors,

for your guidance, inspiration, and support.

I dedicate this to The Multicultural Graduate Community and SACNAS,

for your community of support, innovation, and motivation.

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Abstract

Complex immune disorders such as Type 1 diabetes (T1D) and inflammatory bowel diseases (IBD) have increased dramatically over the past century. These “new age” disorders have risen particularly in populations that have undergone rapid changes in industrialization (Thia *et al.*, 2008; Bager *et al.*, 2012; Molodecky *et al.*, 2012; Ungaro *et al.*, 2014). While a genetic basis for these diseases exist, environmental risk factors play a role in triggering disease in genetically susceptible individuals (Anderson *et al.*, 2011; Noble and Erlich, 2012; J. Z. Liu *et al.*, 2015; Mejía-León and Barca, 2015; Liu and Stappenbeck, 2016). Increasing evidence supports the role of the gut microbiome, including disturbances in microbial communities (dysbiosis) and host-microbe interactions, in the pathogenesis of these diseases. This may be particularly relevant during infancy when critical developmental events in microbial assemblage and immunity are occurring. We utilized the interleukin-10 deficient (IL-10 KO) murine model of IBD under several environmental conditions to understand the role of gut microbiota as modifiers of disease development. We examined this model under three main scenarios: 1) raised germ-free (GF) in the absence of bacteria; 2) conventionally-raised specific-pathogen-free (SPF) in the presence of bacteria; and 3) conventionally-raised in the absence of *Helicobacter hepaticus* but exposed to antibiotic-induced maternal dysbiosis at birth. Under the 1st and 2nd scenarios, we made the surprising observation that the absence of gut microbiota leads to the manifestation of immune infiltration in the pancreas of GF IL-10 KO mice, which resembles the pathology of T1D. This was absent in SPF IL-10 KO, which instead manifest with spontaneous colitis resembling IBD. Under the 3rd scenario, we discovered that disruptions to microbial communities early in life, via acquisition of an antibiotic-induced maternal dysbiosis at birth, perturbs the immune system and increases the risk for IBD development. Thus, the IL-10 KO model represents two different facets of complex immune disorders, T1D and IBD, in which gut microbiota modify disease pathogenesis.

Chapter 1: Introduction

1.1 The gut microbial "organ" and host immunity

The gastrointestinal tract (GI), also referred to as the gut, and its trillions of microbial communities, or microbiota, play an important role in maintaining host metabolic and immunological homeostasis. Hundreds of studies highlight the importance of gut microbiota in orchestrating health and disease (Round and Mazmanian, 2009; Wu and Wu, 2012; Arrieta *et al.*, 2014). Although many studies primarily focus on bacteria, our microbial "organ" comprises of bacteria, as well as smaller populations of fungi and archaea (Eckburg *et al.*, 2005). Given that the greatest amounts of bacteria reside in the colon, which provides the largest surface area for environmental interactions, the colon is a likely site for autoimmune initiation (Solly, Honeyman and Harrison, 2001; Turley *et al.*, 2005; Vaarala, 2012; Paun, Yau and Danska, 2016a). The active, symbiotic relationship between the gut microbial "organ" and the host is crucial for host development, metabolism, and immunological homeostasis, as disruptions in the gut microbiota lead to an array of diseases (Arrieta *et al.*, 2014). In particular, disruptions in gut microbial communities, or dysbiosis, are associated with complex immune disorders in genetically susceptible hosts, such as inflammatory bowel diseases (IBD) and Type 1 diabetes (T1D). A number of large observational studies in genetically susceptible individuals have taken on the challenge of deciphering environmental contributors to disease development (Bager *et al.*, 2012; Jostins *et al.*, 2012; Schokker *et al.*, 2014; Ungaro *et al.*, 2014; Benchimol *et al.*, 2015) however, identifying and translating mechanisms of action remain a work in progress.

Several recent publications highlight the role of environmental factors and gut microbiota on host immunity and disease development (Chehoud *et al.*, 2015; Ojeda *et al.*, 2016; Coretti *et al.*, 2017; Igartua *et al.*, 2017; Lin and Zhang, 2017; Plaza-Díaz *et al.*, 2017). Microbes are intimately connected with our immune systems via mucosal immunity, which is a significant generator of peripheral tolerance (Sudo *et al.*, 1997; Fink *et al.*, 2012; Gensollen *et al.*, 2016).

Hence, gut microbes can have potentiating effects on the host immune system. Innate and adaptive immunity work concomitantly to activate appropriate immune responses and combat non-self-threats. As so, autoimmunity, a consequence of aberrant immune responses or loss of tolerance, can initiate in the gut and impact multiple organs of the body via the generation and activation of pathogenic lymphocytes (Th1, Th17 T cell subtypes) and loss of regulatory T cells (Treg) (Wu and Wu, 2012; Dunne *et al.*, 2014). Imbalances in Th1, Th17, and Treg responses contribute to the pathogenesis of inflammatory diseases in part by the production of proinflammatory cytokines. Th1-subtype T cell responses are involved in cell-mediated immunity and mainly produce interferon gamma (INF-γ), while Th17-subtype responses induce inflammation and produce cytokines such as tumor necrosis factor alpha (TNFα), IL-17, and IL-6 (Annunziato *et al.*, 2007; Wen *et al.*, 2008; Feng *et al.*, 2011; H. P. Liu *et al.*, 2015). Moreover, Treg suppression via anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β), are key to maintaining tolerance and are implicated in the pathogenesis of autoimmunity (Kuhn *et al.*, 1993; Cortés *et al.*, 2014; Shouval *et al.*, 2014).

1.2 The IL-10 KO murine model

The establishment of several murine models has increased our understanding of disease pathogenesis and promoted the development of current therapeutic strategies (King, 2012; Mizoguchi *et al.*, 2016). We focused on the interleukin-10 deficient (IL-10 KO) mouse model to dissect the impact of gut microbiota as an environmental factor modulating disease development. The IL-10 KO model is a well-established transgenic mouse model of colonic inflammation, or colitis, manifested in IBD (Kuhn *et al.*, 1993; Rennick, Davidson and Berg, 1995; Keubler *et al.*, 2015). The histopathology of colitis is characterized by the severity of immune infiltration of the lamina propria, epithelial hyperplasia, loss of goblet cells, and crypt abscess (Erben *et al.*, 2014). IL-10 is a key immunomodulatory cytokine produced by immune cells to regulate anti-inflammatory responses and maintain immune balance with a key role in

intestinal homeostasis (Saraiva and O'Garra, 2010; Shouval *et al.*, 2014). The loss of IL-10 promotes Th1 and Th17-mediated colonic inflammation, which can be modified by environmental factors such as antibiotics, diet and changes in microbial communities (Chaudhry *et al.*, 2011; Devkota *et al.*, 2012; Candon *et al.*, 2015; Keubler *et al.*, 2015). The multifactorial nature of disease in this model provides an opportunity to refine the role of genetic susceptibility, environmental factors, and microbial communities in disease.

1.3 IBD and T1D are complex immune disorders

The striking increase in complex immune disorders, such as IBD and T1D, over the last few decades has focused attention on the role of environmental factors and the microbiome in disease pathogenesis. IBD is generally classified into two clinical manifestations of GI inflammation, Crohn's disease and ulcerative colitis. In Crohn's disease, immune-mediated lesions and inflammation can manifest throughout the GI tract, while manifestations of ulcerative colitis impact the colon (Hendrickson, Gokhale and Cho, 2002; Tontini *et al.*, 2015). Several reviews have highlighted mechanisms by which alterations in gut microbiota can impact IBD (Manichanh *et al.*, 2012; Hold *et al.*, 2014; Huttenhower, Kostic and Xavier, 2014; Coretti *et al.*, 2017). T1D is characterized by slow and progressive pathogenic stages, with mononuclear islet infiltrates destroying insulin-secreting β-cells, leading to insulin deficiency and hyperglycemia. Since the earliest phases of T1D immunopathology manifests in the absence of chronic hyperglycemia, clinical diagnosis is typically made once significant tissue damage has occurred. Thus, millions of type 1 diabetics depend on insulin treatment to live (Flier, Underhill and Eisenbarth, 1986). Unfortunately for both IBD and T1D, the heterogeneity of disease can make disease diagnosis and treatment challenging. However, the discovery of microbial signatures and microbe-mediated mechanisms associated with each disease has led to increased interest in modulating the gut microbiome as a potential therapy (Dunne *et al.*, 2014; Burrows *et al.*, 2015; Knip and Siljander, 2016; Plaza-Díaz *et al.*, 2017).

Although significant advances have been made in understanding disease development and treatment options for patients, there is currently no cure or effective prophylactic treatment for IBD or T1D. Thus, both diseases can present life-threatening challenges and increase lifestyle burden (Rubin, 2000; Devlen *et al.*, 2014; Gonder-Frederick, 2014). One difficulty for the development of cures for patients is that both diseases are heterogeneous, with complex gene-environment interactions and manifestations across a diversity of individuals (Gjymishka *et al.*, 2013). Both IBD and T1D share susceptibility loci, including polymorphisms in IL-10, and aberrant immune responses that manifest either in the gut or the pancreas, two organs that share anatomic proximity, an integrated lymphatic system, and endodermal origin (Turley *et al.*, 2005; Paun, Yau and Danska, 2016b). Interestingly, many patients with T1D also manifest with symptoms of IBD and disrupted gut integrity (Leeds *et al.*, 2011; Gulden, Wong and Wen, 2015; Pellegrini *et al.*, 2017). Moreover, deficiencies in IL-10 expression levels are associated with both IBD and T1D (Rennick, Davidson and Berg, 1995; Maul *et al.*, 2005; Holmén *et al.*, 2006). Given the important homeostatic functions of IL-10 and Treg cells, immune therapies aimed at targeting effector T cells or impacting cytokine expression, including IL-10, in IBD and T1D have been explored, but without significant success (Sellon *et al.*, 1998; Ko *et al.*, 2001; Sasaki *et al.*, 2005; Baker *et al.*, 2007; Tamagawa *et al.*, 2007; Manichanh *et al.*, 2012). Thus, the IL-10 KO model presents a suitable model of genetic susceptibility to investigate the impact of environmental factors and gut microbiota on host immunity and disease outcome.

1.4 Gut microbiota modify disease pathology in IL-10 KO mice

The role of gut microbiota as modifiers of disease pathophysiology is a rapidly growing field of research. Comprehensive population-based studies in parallel with technological advancements of the study of gut microbial ecosystems have strengthened our current understanding of the gut microbiota's contribution to disease (Gevers *et al.*, 2014; Knights *et al.*, 2014; van den Elsen *et al.*, 2017). The development of colitis in IL-10 KO mice is dependent on

the presence of gut microbiota (Rennick, Davidson and Berg, 1995; Sellon *et al.*, 1998). In particular, the presence of certain bacteria, specifically pathobionts such as *Helicobacter hepaticus* (*H. hepaticus*), within a defined microbial community impact the risk of colitis development ((Matharu *et al.*, 2009; Nagalingam *et al.*, 2013; Yang *et al.*, 2013). The specific-pathogen-free (SPF) IL-10 KO mice in our colony phenotypically mimic ulcerative colitis, with approximately 20-30% of IL-10 KO mice developing spontaneous colitis. These mice are protected against colitis under germ-free (GF) conditions, highlighting the role of microbiota for disease development. The absence of the microbiome in murine models has also been associated with impaired T cell function, including decreased number and function of regulatory T cells (Treg), defined by their expression of CD4 and CD25 surface markers and the transcription factor Forkhead box P3 (FoxP3) (Östman *et al.*, 2006; Chinen *et al.*, 2010). Moreover, lack of functional FoxP3 expressing T cells induces severe colitis and widespread autoimmunity in GF mice (Kim, Rasmussen and Rudensky, 2007; Chinen *et al.*, 2010). The reintroduction of bacteria to GF IL-10 KO mice via fecal microbiota transplantation (FMT) leads to the development of colitis (Sellon *et al.*, 1998). Thus, microbe-mediate modulation of the immune system in genetically susceptible host influences T cell mediated responses to initiate inflammation in the gut (Kullberg *et al.*, 2002; Mazmanian, Round and Kasper, 2008).

1.5 Dissertation overview

The main goal of my thesis is to examine the role of gut microbiota in two facets of complex immune disorders, T1D and IBD, in the IL-10 KO mouse model. Previous assessment of GF IL-10 KO mice has primarily focused on immunity in the GI tract. Our surprising discovery that pancreatic islets displayed lymphocytic infiltrates resembling the histopathology of T1D in GF IL-10 KO, but not SPF IL-10 KO mice, lead us to further investigate this model. In Chapter 2 I present the results of cross-sectional studies focused on the characterization of pancreatic infiltration and diabetes development in the presence and absence of microbiota in IL-10 KO

mice. Our characterization of this previously unobserved phenotype in the pancreas represents an opportunity for future studies to expand our understanding of the role of gut microbiota on autoimmune responses in T1D development. Since gut microbiota modulates disease manifestation under GF versus conventionally-raised SPF conditions, we investigated the impact of microbial dysbiosis acquired from birth on immune skewing and colitis development. In Chapter 3, I present the results of a prospective study focused on the role of gut microbiota during a critical developmental window for immunity, microbial assemblage, and later IBD development in IL-10 KO mice. These data were published in Cell Reports and demonstrate that acquisition of a dysbiotic maternal microbiota, following peripartum maternal antibiotic exposure, perturbs the immune system of offspring early in life and increases the risk for colitis development in offspring. Both chapters highlight two examples of how the presence and absence of gut microbiota, including microbial dysbiosis, impact the clinical manifestation of disease in genetically susceptible hosts. In Chapter 4, I discuss the overall conclusions, significance, and future directions for the work presented in this dissertation.

Chapter 2: Characterization of type 1 diabetes in germ-free interleukin-10 deficient mice

2.1 Abstract

Type 1 diabetes (T1D) and inflammatory bowel diseases (IBD) are chronic immune disorders that manifest in the pancreas or gastrointestinal tract of individuals respectively. These debilitating diseases lack cures and are expected to increase in prevalence across all ethnic groups, impacting millions of people worldwide. The association between disturbances in gut bacterial communities, or microbial dysbiosis, and IBD is well established. However, far less is known about the role of gut microbiota in T1D onset and progression. In particular, the initial autoimmune response is difficult to study in humans due to lack of accessibility to the pancreas. Thus, we used the interleukin-10 deficient (IL-10 KO) mouse model as a tool to understand mechanisms of disease and uncover potential prophylactic approaches. In IBD-prone IL-10 KO mice, the absence of gut microbiota under germ-free (GF) conditions prevents IBD development. Interestingly, we noticed GF IL-10 KO mice exhibited polyuria, a classic symptom of T1D. Upon examining the pancreas, we discovered pancreatic lymphocytic infiltration resembling T1D lesions. These findings prompted the hypothesis that the presence of gut microbiota play a protective role in the pancreas. Our first objective was to determine whether C57BL6/J GF IL-10 KO mice exhibit an increased incidence of pancreatic infiltration associated with T1D and identify the immune mediators compared to conventionally-raised counterparts. We monitored IL-10 KO mice across various ages and collected pancreatic tissue for histological assessment. This assessment revealed that an astonishing 50% of male and female mice above 6-months of age develop pancreatic infiltration. Immunofluorescent staining of islet infiltrates are positive for adaptive and innate immunological markers, including lymphoid and myeloid cells markers, which are typically used to characterize T1D lesions. A subset of GF IL-10 KO mice are also positive for insulin autoantibodies (IAA), a hallmark of human T1D;

however, the majority of mice do not become diabetic. Overall, the incidence of pancreatic infiltration in the GF IL-10 KO cohort is approximately 30%, as compared to 0% in conventionally-raised and fecal microbiota transplanted (FMT) IL-10 KO counterparts. The GF IL-10 KO mouse potentially represents a new model for T1D independent of the major MHC risk haplotypes found in other T1D mouse models such as the non-obese diabetic (NOD) mouse model. Our findings of early stage lymphocytic infiltrates in the pancreas and IAA in the absence of overt diabetes in GF IL-10 KO mice embody the first of three progressive stages of T1D pathogenesis in humans. Thus, our model provides the unique opportunity to understand the role of the microbiome in immune pathogenesis and target early stages of disease progression to eventually develop microbe-mediated prophylactic strategies.

2.2 Introduction

The incidence of T1D worldwide is rising dramatically with an estimated increase of 23% in the USA within the next 40 years. This complex autoimmune disease affects all ethnic groups, but particularly individuals of European ancestry, followed by Hispanics and African Americans (Menke *et al.*, 2013). This poses a serious health concern that motivates the need for biomedical research to understand disease pathophysiology and develop effective preventative approaches that can translate to human disease. Millions of T1D patients depend on insulin treatment to live and struggle to maintain appropriate fluctuations in glycaemia, especially post-prandial. Immune cell infiltration of the pancreas reveals a prominent role for auto-reactive T cells in disease pathogenesis, along with antigen presenting cells and ineffective regulatory T cells (Tregs) (Tritt *et al.*, 2008; Phillips *et al.*, 2009; Tai, Wong and Wen, 2016); however, the precise etiology in humans still remains relatively elusive.

The pathophysiology of T1D is multifactorial, with disease risk influenced by a combination of genetic predisposition and environmental factors that trigger the autoimmune

disease process. Genetic predisposition is a major determinant of T1D risk, with a higher incidence of disease among relatives of T1D patients. Genetic studies have discovered over 40 loci associated with disease. One of the strongest associations to T1D first identified in individuals of European ancestry was the Human Leukocyte Antigen (HLA) region, or Major Histocompatibility Complex (MHC). The HLA-DQ and -DR alleles are major determinants of autoimmune susceptibility because the region contains many vital immune genes required for proper T cell responses. Several non-MHC loci also contribute to disease risk, including the insulin gene, which contains the 2nd strongest genetic association with T1D. Interestingly, HLA and non-HLA haplotypes, as well as specific genotypic combinations, confer susceptibility or protection against T1D. Despite significant genetic associations to disease risk, exact mechanistic insights have yet to emerge (Noble and Erlich, 2012).

The rapid rise in T1D incidence over the last half century reflects the significance of environmental factors to disease pathogenesis. Because the heritability of T1D is estimated to be 72-88%, environmental factors are thought to contribute significantly to the phenotypic variability noted in patients. The contribution of these environmental triggers is best supported by the discordance rate of disease observed in identical twins (Hyttinen *et al.*, 2003). Several studies propose triggers, including viral infections, dietary components, antibiotic use, and more recently, gut microbial dysbiosis, contribute to disease onset and progression (Mejía-León and Barca, 2015; Hu, Wong and Wen, 2017). Over time, these environmental triggers may fuel metabolic and immune dysfunction that trigger β-cell suicide or homicide. Localized physiological changes may also perpetuate autoimmune responses directed toward β-cells. While our understanding of these complex interactions continues to expand; further collaboration and research is needed to optimized effective treatment strategies for patients at various stages in disease progression (Atkinson *et al.*, 2011). Thus, a new model of disease course has been established to capture the earliest presymptomatic stages of disease development (Insel *et al.*, 2015).

Studies from our lab and others have demonstrated the pathogenic effects microbial dysbiosis can have on host metabolism and inflammation. Studies in susceptible animal models also indicate the incidence of disease can be altered by the colonization of particular gut microbiota, with age and mode of microbial colonization playing a key role in later disease development (Miyagawa *et al.*, 2010; Collado *et al.*, 2012; Morgan *et al.*, 2012; Kump *et al.*, 2013; Hansen *et al.*, 2014; Mizoguchi *et al.*, 2016). Distinct gut microbial profiles have been associated with T1D patients as compared to healthy controls (Dunne *et al.*, 2014; Asnicar *et al.*, 2017; Pellegrini *et al.*, 2017). For example, T1D patients have been shown to display an "autoimmune microbiota" defined by a higher proportion of Bacteroidetes phylum and decreased intestinal microbial diversity. These bacterial communities also exhibited a low abundance of short chain fatty acid (SCFA)-producing bacteria, particularly butyrate-producing and mucin-degrading bacteria (Giongo *et al.*, 2011). However, exact causation could not be determined from these human studies and the gut microbial dysbiosis noted in these patients may be secondary to an already altered metabolic state in patients. Thus, mouse models provide a valuable opportunity to test causation and dissect underlying mechanisms of disease pathogenesis.

Several hypothetical models implicating gut microbial contributors in autoimmune development have been proposed (Castiglione *et al.*, 2012; Chervonsky, 2013). The 'hygiene hypothesis' predicts the absence of early childhood exposure to diverse microbiota contributes to host autoimmune development (Wills-Karp, Santeliz and Karp, 2001; Castiglione *et al.*, 2012). For instance, the increased incidence of insulitis in the non-obese diabetic (NOD) mouse model of spontaneous T1D raised under GF conditions indicates gut bacteria predominantly protect against T1D pathogenesis (Alam *et al.*, 2011). However, the gut microbiota can stimulate pro- and anti-diabetogenic signals, with the development of disease mediated via innate immune sensing in the gut (Burrows *et al.*, 2015). Another model of growing interest, the 'perfect storm hypothesis', attributes the combination of genetic predisposition, altered gut microbial

composition, and decreased gut barrier integrity to autoimmune disease pathogenesis (Alam *et al.*, 2011; Paun, Yau and Danska, 2016a). While many studies shed light on the significance of host-microbe interactions, further investigation is needed to uncover the role of the gut microbiota in immune pathogenesis and disease prevention. An understanding of how the gut microbiota protect against the onset of pancreatic inflammation may allow for new preventative strategies to decrease the incidence of T1D.

The anti-inflammatory cytokine IL-10 plays a vital role in the regulation of host immune responses. IL-10 is important for stabilization of Tregs and inactivation of self-reactive T cells, which affects peripheral lymphocyte tolerance. Sarvetnick and other groups have focused on the role of IL-10 in autoimmune diabetes development for a long time; however, results from these studies indicate paradoxical effects of IL-10 in T1D development (Balasa *et al.*, 2000; Goudy *et al.*, 2003; Baker *et al.*, 2007; Thompson *et al.*, 2014). It is likely that age, dose, and location of IL-10 expression dictate variation in the phenotypic outcomes of these studies. Thus, the effects of IL-10 compel further elucidation of mechanisms underlying pancreatic autoimmunity in the IL-10 KO model. The novel discovery of pancreatic inflammation in GF IL-10 KO mice provides a new model for gaining insights into the role of IL-10 and gut microbiota in autoimmune initiation, which may contribute to future preventive therapies via immune and gut microbial modulation.

2.3 Results

2.3.1 GF IL-10 KO mice exhibit pancreatic infiltration characteristic of T1D histopathology.

Our initial observation of polyuria in GF IL-10 KO mice prompted us to investigate whether these mice develop T1D lesions via histological assessment of pancreata. To our surprise, H&E stained pancreata slides revealed GF IL-10 KO mice exhibited islet and ductal lymphocytic infiltrates resembling the histopathology of T1D islet infiltrates (representative slide,

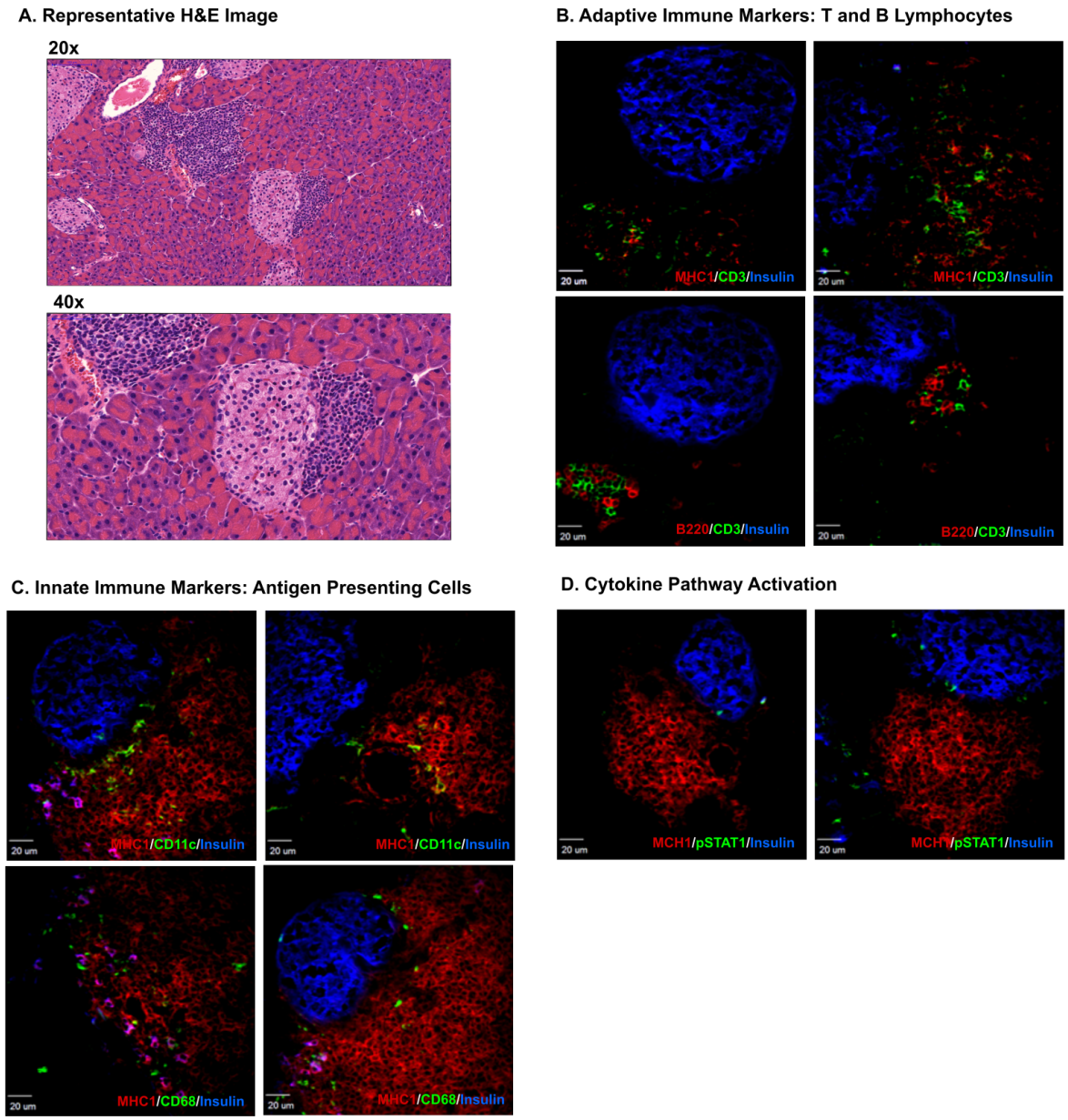
Figure 2.1A). We investigated the identity of these immune infiltrates via immunofluorescence staining performed in collaboration by Patrick Moore at The University of Chicago. Pancreatic infiltrates were positive for adaptive and innate immune markers. Infiltrates located adjacent to insulin positive β-cells were positive for $CD3^+$ T lymphocytes, $B220^+$ B lymphocytes, as well as $CD11c^+$ and $CD68^+$ macrophage and dendritic cell markers (Figure 2.1B-C). Interestingly, we visualized co-localization of insulin with antigen presenting cell infiltrates (Figure 2.1C). We also found preliminary evidence of cytokine pathway activation with $pSTAT1^+$ cells at the periphery of islets near infiltrates, suggestive of islet stress (Figure 2.1D). To further investigate the role of these infiltrates in mediating immunopathology, we utilized adoptive transfer models in our GF system.

2.3.2 Adoptive transfer of immune cells from GF IL-10 KO donors does not transfer pancreatic immunopathology into immunocompromised GF RAG1 KO recipients.

We utilized a GF adoptive transfer model to test the functional potential of immune cells from GF IL-10 KO mice to elicit pancreatic infiltration into GF RAG1 KO recipients, which are immunocompromised because they lack mature adaptive T and B lymphocytes. Younger GF RAG1 KO female and male recipients were intraperitoneal (IP) injected with isolated splenocytes from older GF IL-10 KO female and male donors respectively (mouse information in Table 1). Since 50% of GF IL-10 KO mice above 6-months of age developed pancreatic infiltration, we selected older GF IL-10 KO donors, approximately 29 weeks of age, to increase the probability of pancreatic immunopathology in the donors. To determine the success of transferred immune cells, we analyzed the ratio of T and B lymphocytes in donor versus recipients. T and B lymphocytes were successfully detected via flow cytometry in recipients, but were in significantly lower frequencies as compared to donors (Figure 2.2A). Both female and male GF RAG1 KO recipients of IL-10 KO donors gained weight and appeared healthy

throughout the 10-week observation period (Figure 2.2B). Likewise, wild-type (WT) donor RAG1 KO recipients appeared healthy and gained weight over the course of 9 weeks with the

Figure 2.1: GF IL-10 KO mice develop pancreatic islet and ductal immune cell infiltrates.
A. Representative H&E stained infiltrated pancreatic sections at 20x and 40x magnification from a GF IL-10 KO female 33 weeks of age (FBG=205mg/dL). IF staining performed on sections from two GF IL-10 KO males ~20 weeks of age reveal infiltrates are MHC class I positive (B-D). **B.** Infiltrates are positive for $B220^{+}$ B cell and $CD3^{+}$ T cell markers. **C.** Several insulin-positive cells co-stain with cells positive for CD11C or CD68, dendritic and pan-macrophage markers. **D.** Evidence of $pSTAT1^{+}$ cells suggest activation of interferon or stress-induced responses.



exception of females because they were older than WT-RAG1 KO recipient males (Figure 2.2C, Table 1). Although 4/4 female donors and 1/4 male GF IL-10 KO donors were positive for pancreatic infiltration, GF RAG1 KO recipients did not exhibit pancreatic infiltration (Figure 2.2D). Furthermore, no differences were found in non-fasted blood glucose levels between female and male recipients from either GF IL-10 KO or GF WT donors (Figures 2.2E and 2.2F).

Figure 2.2: Adoptive transfer of immune cells from GF IL-10 KO donors into GF RAG1 KO recipients does not recapitulate pancreatic immunopathology.
A. Percent body weight of GF IL-10 KO – GF RAG1 KO female and male recipients (n=9-12/sex). **B.** Percent body weight of GF WT – GF RAG1 KO female and male recipients (n=2-3/sex). **C.** Representative H&E stained pancreas slide from a GF IL-10 KO female donor and a GF RAG1 KO female recipient at 40x magnification. **D.** Non-fasted A.M. blood glucose levels in female versus male GF IL-10 KO – GF RAG1 KO recipients. **E.** Non-fasted A.M. blood glucose levels in female versus male GF WT – GF RAG1 KO recipients. ****p=0.0001, **p=0.006, *p=0.03 via Sidak's Multiple Comparison.

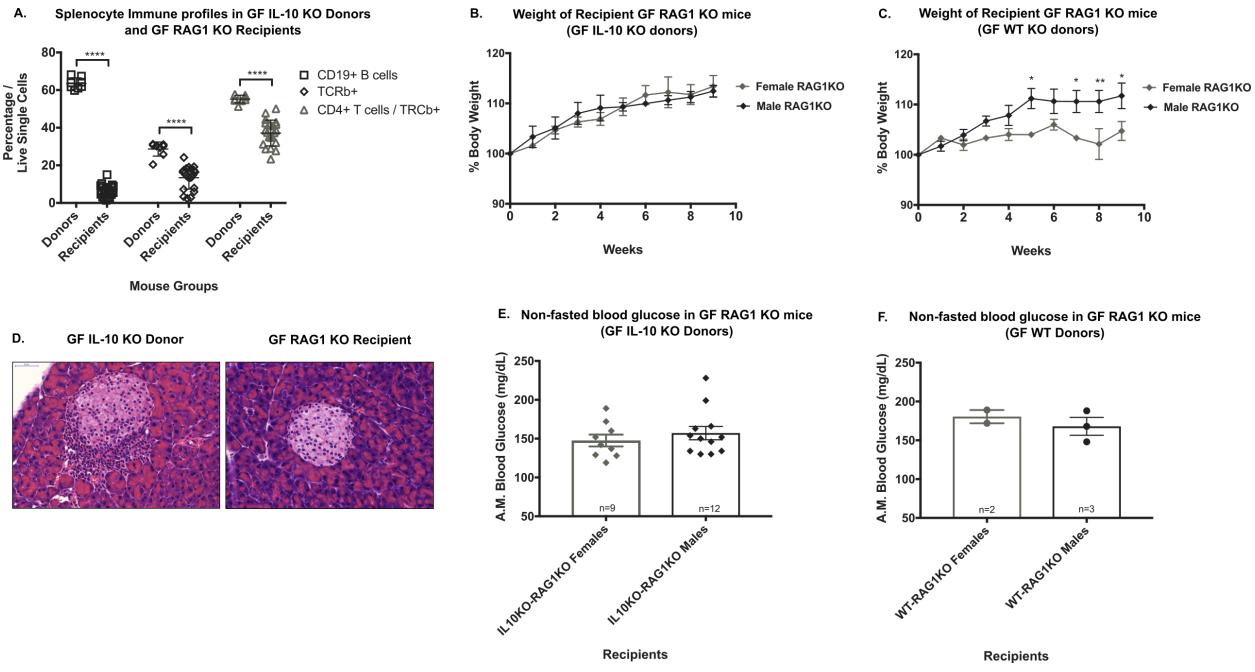


Table 1: Adoptive Transfer Donor and Recipient Mouse Information.

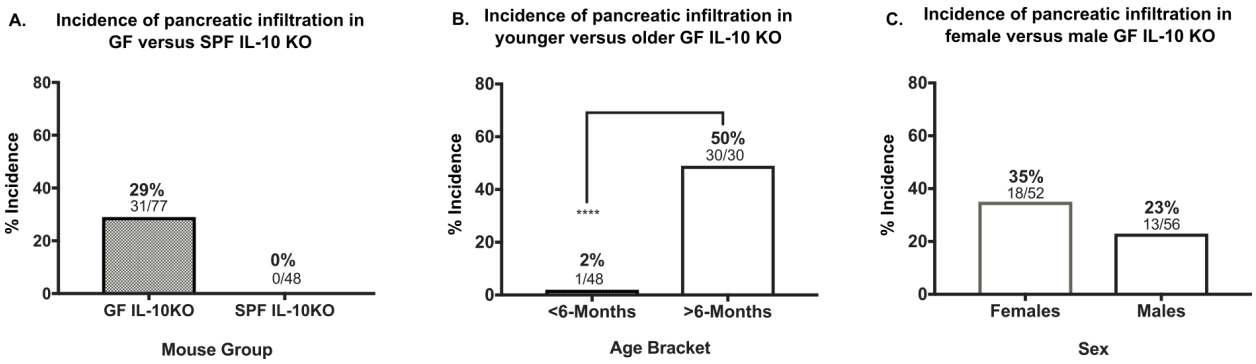
GF Donors	Number of GF RAG1 KO Recipients per Donor	Age of Donor at start (weeks)	Age of Recipient at start (weeks)	Age of Recipient at end (weeks)
5 Male Donors	15 Male Recipients			
M1 IL-10	3	35	12	22
M2 IL-10	3	31	14	25
M3 IL-10	3	27	9	19
M4 IL-10	3	27	8	18
M1 WT	3	18	16	26
5 Female Donors	12 Female Recipients			
F1 IL-10	3	31	12	22
F2 IL-10	2	31	16	26
F3 IL-10	2	31	9	19
F4 IL-10	2	31	13	23
F1 WT	3	24	21, 23	32, 34

2.3.3 The presence of gut microbiota protects against pancreatic infiltration and GF IL-10 KO mice exhibit increased incidence of pancreatic infiltration.

The presence of infiltrates resembling T1D histopathology under GF conditions led us to hypothesize (1) whether gut microbiota is protective against pancreatic infiltration and (2) whether GF IL-10 KO mice exhibit an increased incidence of pancreatic infiltration associated with T1D. We found IL-10 KO mice raised in the presence of bacteria are 100% protected against pancreatic infiltration. Conversely, GF IL-10 KO mice raised in the absence of bacteria exhibited significant pancreatic infiltration with approximately 30% of the mice across all ages positive for pancreatic immunopathology (Figure 2.3A). Interestingly, histological scoring for the presence of infiltrates in GF IL-10 KO mice revealed 50% of mice above 6-months of age were positive for pancreatic infiltration, demonstrating the slow development of immunopathology in these mice (Figure 2.3B). Female and male mice displayed similar incidences, with 35% of females and 23% of males positive for pancreatic infiltration (Figure 2.3C). Moreover, GF WT

mice displayed some signs of pancreatic infiltration in older mice (3/10 mice), but this was not significant compared to the incidence found in older GF IL-10 KO mice. This finding suggests a role for gut microbiota and IL-10 in the development of pancreatic infiltration.

Figure 2.3: The presence of gut microbiota protects against pancreatic infiltration in IL-10 KO mice.
A. Incidence of pancreatic infiltration in GF versus SPF IL-10 KO mice across all ages (GF n=77, SPF n=48). **B.** Incidence of pancreatic infiltration in GF IL-10 KO mice below or above 6-months of age (<6-months n=48, >6-months n=30). **C.** Incidence of pancreatic infiltration in GF IL-10KO females versus males (female n=52, male n=56). ****p>0.0001 Unpaired T test.



2.3.4 A subset of GF IL-10 KO mice develop overt diabetes, but the majority of mice maintain euglycemic control.

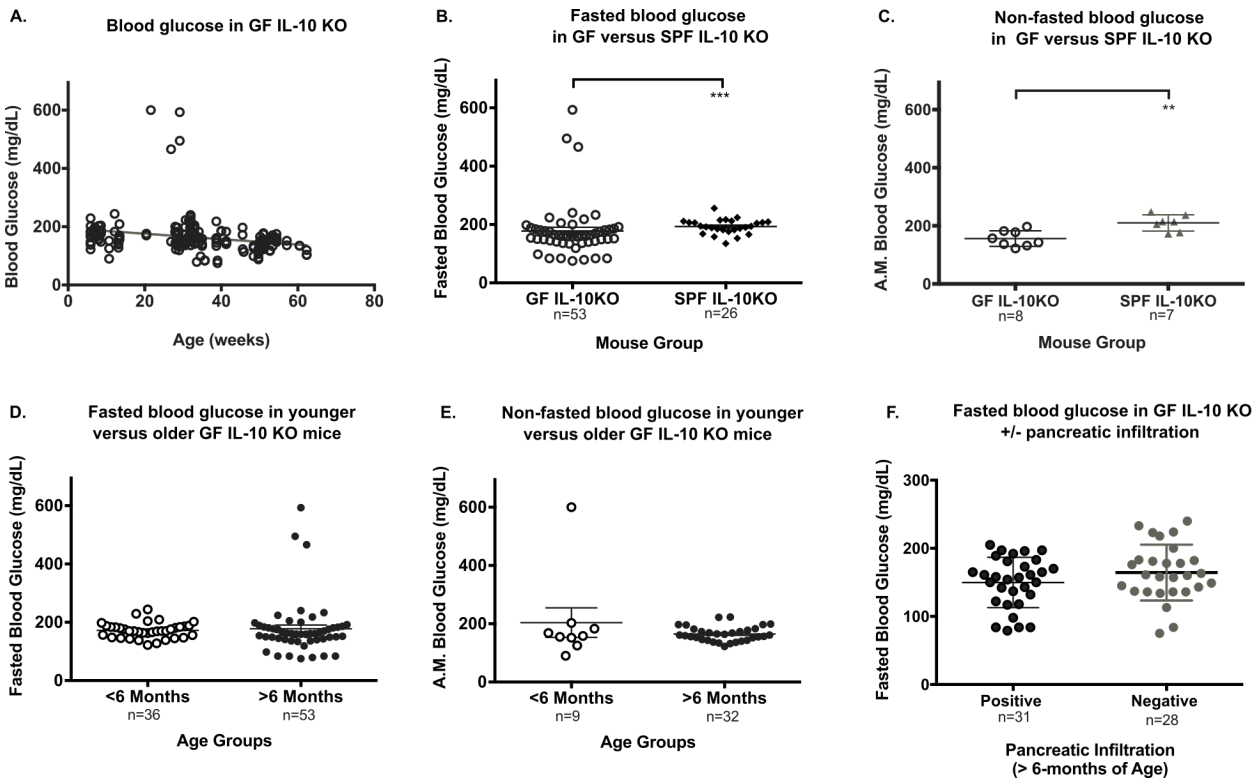
Since GF IL-10 KO mice exhibit an increased incidence for pancreatic infiltration, we tested whether this finding was associated with diabetes development, defined by blood glucose levels above 250 mg/dL. We found 4 mice, only 7% of mice above 6-months of age, developed overt diabetes. It is possible a small number of additional mice, found extremely ill or dead and for which we did not have recorded blood glucose readings, could also have been attributed to hyperglycemia but were not included in our calculation. Overall, the majority of GF IL-10 KO mice did not develop overt diabetes (Figure 2.4A). We measured fasted blood glucose levels, a standard measure for diabetes assessment, in age-matched GF IL-10 KO and SPF IL-10 KO

mice. Despite SPF IL-10 KO mice being protected from pancreatic infiltration, they displayed slightly higher levels of fasted blood glucose levels compared to GF IL-10 KO mice, but did not develop diabetes (Figure 2.4B). This finding was consistent with non-fasted A.M. blood glucose readings between age-matched GF IL-10 KO mice (Figure 2.4C).

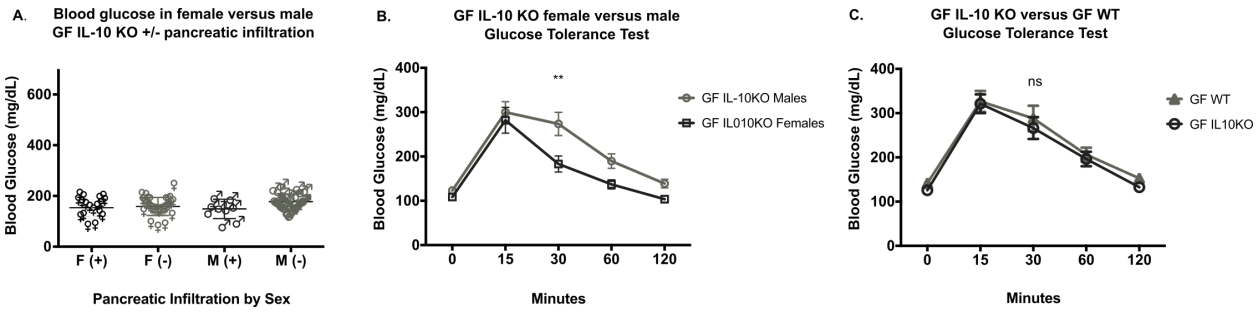
Since pancreatic infiltration appeared in GF IL-10 KO mice at an older age, we compared fasted blood glucose levels in GF IL-10 KO mice above and below 6-months of age. No significant differences in fasted and non-fasted A.M. blood glucose levels were found in GF IL-10 KO mice based on these age brackets (Figure 2.4D-E). We next tested the hypothesis that GF IL-10KO mice with pancreatic lymphocytic infiltrates resembling the pathology of T1D would have higher fasted blood glucose levels compared to mice without infiltration. Despite differences in the presence or absence of pancreatic immunopathology, age-matched GF IL-10 KO mice positive for pancreatic infiltration did not exhibit significant differences in fasted blood glucose levels (Figure 2.4F). Additionally, no gender dimorphism was observed in blood glucose levels between female and male GF IL-10 KO mice (Figure 2.S1A). Although no significant differences in blood glucose levels were observed between sexes, we found slight differences in glucose tolerance between older GF IL-10 KO females and males. GF IL-10KO females appear to be slightly more glucose tolerant compared to males (Figure 2.S1B). No differences in glucose tolerance were found in GF WT females versus males (Figure 2.S1C).

Figure 2.4: Blood glucose diabetes assessment in IL-10 KO mice.
A. Cross-sectional blood glucose levels in GF IL-10 KO males and females (n=184). **B.** Fasted blood glucose in GF versus SPF IL-10 KO mice (GF n=53, SPF n=26). **C.** Non-fasted A.M. blood glucose levels in GF versus SPF IL-10 KO mice (GF n=8, SPF n=7). **D.** Fasted blood glucose levels in GF IL-10 KO mice less than or greater than 6-months of age (>6-months n=36, >6-months n=53). **E.** Non-fasted A.M. blood glucose levels in GF IL-10 KO mice less than or greater than 6-months of age (>6-months n=9, >6-months n=32). **F.** Fasted blood glucose in older GF IL-10 KO mice positive or negative for pancreatic infiltration pathology (Positive n=31, Negative n=28). ***p=0.0003, **p=0.0024 via Mann-Whitney Test.

Figure 2.4, continued.



Supplementary Figure 2.S1, related to Figure 2.4.
A. Blood glucose levels in female and male GF IL-10 KO mice positive or negative for pancreatic infiltration. **B.** Glucose tolerance blood glucose levels in GF IL-10 KO female versus male (n=12-13/sex). **C.** Glucose tolerance blood glucose levels in GF IL-10 KO versus GF WT mice (n=12-13/group). ***p*=0.002 via 2way ANOVA.



2.3.5 A subset of GF IL-10 KO mice are positive for insulin autoantibodies.

Since the majority of mice maintain glycaemia and IF staining revealed co-localization of infiltrates with insulin, we investigated if this model was analogous to stage 1 of T1D in humans, in which patients are euglycemic but are positive for the presence of autoantibodies (Insel *et al.*, 2015). We hypothesized that GF IL-10 KO mice would be positive for insulin autoantibodies (IAA), one of the first autoantibodies to appear in pre-diabetic patients (Ziegler *et al.*, 1993). We collaborated with Dr. Mark Atkinson and Clive Wasserfall at the University of Florida Diabetes Center to test for the presence of IAA in the sera of GF IL-10 KO mice originally suspected of diabetes (Cohort 1). These mice exhibited varying degrees of pancreatic infiltration and had elevated fasting blood glucose (FBG) levels but were not diabetic. In Cohort 1, 61% (11/18 mice) were positive (pos) for IAA (Table 2). We also tested a second cohort of mice (Cohort 2) at the Barbara Davis Center. These mice included 11 GF IL-10 KO mice above 6-months of age that were positive (pos) or negative (neg) for pancreatic infiltration (Panc_Infil). However, none of the mice in Cohort 2 were positive for IAA (Table 3).

Table 2: Insulin Autoantibody Testing in GF IL-10 KO Cohort 1.

Sample_ID	Bacterial_Status	Sex	Age_wks	FBG	IAA
120905-125	GF	F	n/a	141	pos
120905-129	GF	M	n/a	111	pos
121220-5	GF	M	n/a	124	pos
100512-12	GF	M	49	154	pos
100512-8	GF	F	49	130	neg
100512-9	GF	F	49	148	pos
121220-6	GF	M	n/a	132	pos
121220-7	GF	M	n/a	135	pos
121220-1	GF	M	n/a	144	pos
121220-4	GF	M	n/a	151	pos
122012-3	GF	M	n/a	137	neg
100512-10	GF	F	43	130	pos
121220-2	GF	M	n/a	167	pos
120905-126	GF	F	n/a	179	neg
100512-1	GF	F	78	199	neg
100512-3	GF	M	34	155	neg
100512-11	GF	M	43	138	neg
100512-14	GF	M	43	151	neg

Table 3: Insulin Autoantibody Testing IL-10 KO Cohort 2.

Sample_ID	Bacterial_Status	Sex	Age_wks	BG*	Panc_Infil	IAA
812AT.F1	GF	F	28	122	Pos	Neg
812AT.F2	GF	F	29	143	Pos	Neg
812AT.F3	GF	F	29	132	Pos	Neg
118.F1	GF	F	31	199	Pos	Neg
118.F4	GF	F	31	173	Pos	Neg
812AT.M1	GF	M	28	137	Neg	Neg
812AT.M2	GF	M	28	178	Neg	Neg
812AT.M3	GF	M	28	157	Neg	Neg
509AT.M4	GF	M	29	197	Pos	Neg
118.M3	GF	M	31	152	Pos	Neg
118.M4	GF	M	34	159	Pos	Neg
911.45	SPF	F	8	N/A	Neg	Neg
94.1	SPF	M	32	**195**	Neg	Neg
911.48	SPF	M	8	N/A	Neg	Neg
1392	SPF	M	20	**165**	Neg	Neg
*BG readings are non-fasted. Bold numbers indicate 4-6 hour fasted reading.						

2.3.6 The reintroduction of gut microbiota via fecal microbiota transplantation protects against pancreatic infiltration in GF IL-10 KO recipients.

Fecal microbiota transplantation (FMT) has become a useful tool for patients clinically and in research as a means to study the role of gut microbiota in disease pathogenesis in the lab. Previous studies and work in our lab demonstrate that the presence of pathobionts, such as *H. hepaticus*, and age of FMT GF IL-10 KO recipients impacts colitis onset (Kullberg *et al.*, 1998). Previous work also demonstrates the importance of microbial differences due to gender in the protection of T1D in the NOD mouse model of T1D (Yurkovetskiy *et al.*, 2013). Thus, we sought to test the role of gut microbiota in the protection against pancreatic infiltration in younger and older GF IL-10 KO recipients in the absence of a confounding pathobiont, *H. hepaticus*. FMT donor gut microbiota were introduced into GF IL-10 KO females and males at 5

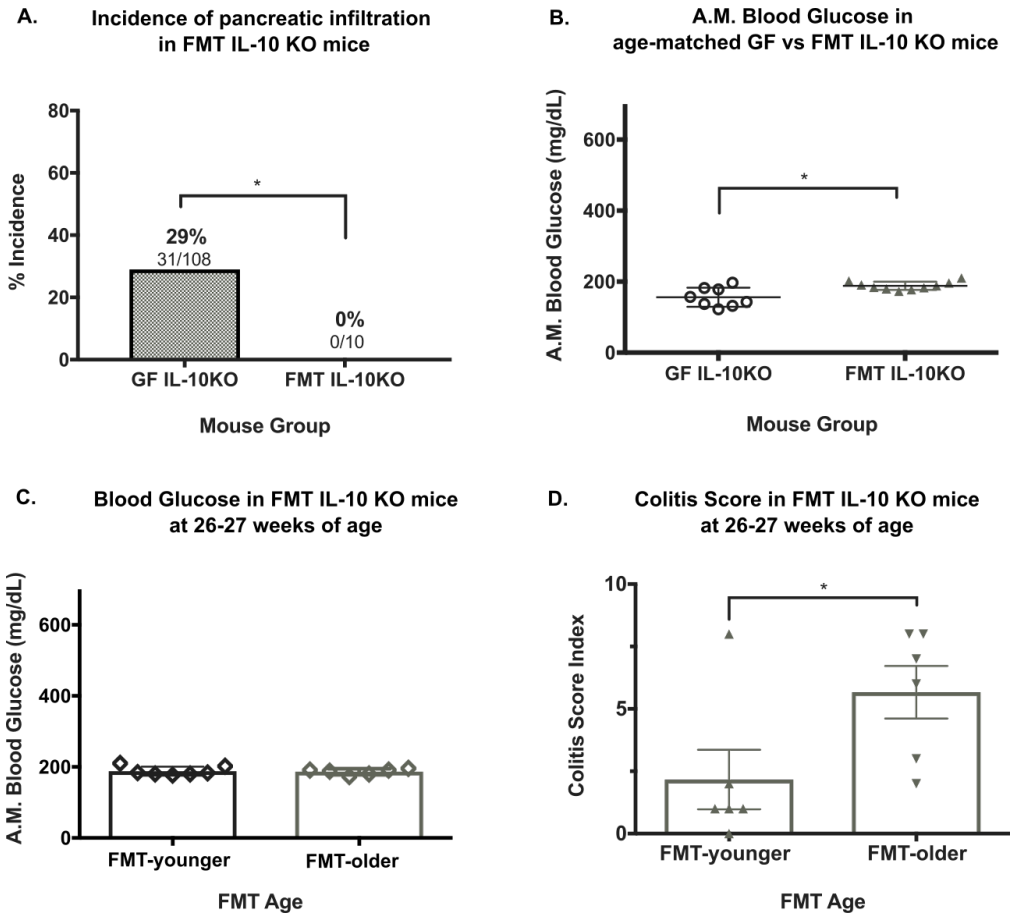
weeks (FMT-younger) or 17 weeks of age (FMT-older) from respective female or male helicobacter-free SPF 13-week-old WT donors. Interestingly, we found mice in both FMT age groups appeared to be protected from pancreatic infiltration (Figure 2.5A).

2.3.7 FMT does not impact blood glucose levels in FMT IL-10 KO recipients.

We next investigated the impact of FMT on blood glucose levels. Similar to our finding in SPF IL-10 KO mice, FMT IL-10 KO mice had slightly higher non-fasted A.M. blood glucose levels compared to age-matched GF IL-10 KO mice (Figure 2.5B). Moreover, age of bacterial introduction into younger versus older recipients did not impact overall blood glucose levels in age-matched FMT recipients at time of harvest, 26-27 weeks of age (duration of FMT observation period 22 weeks in younger-FMT versus 9 weeks in older-FMT recipients) (Figure 2.5C). Likewise, no differences were found in glucose tolerance in FMT-younger versus FMT-older IL-10 KO and WT mice 8-11 weeks post-FMT (Figure 2.S2A and 2.S2B). FMT-younger IL-10 KO females and males at 15-16 weeks of age displayed similar glucose tolerances (Figure 2.S2C). However, slight differences were noted in glucose tolerance of FMT-older females versus males at 26 weeks of age, which was similar to results observed in older GF IL-10 KO mice. FMT-older IL-10 KO females appeared to be slightly more glucose tolerant compared to males (Figure 2.S2D). Lastly, FMT-older IL-10 KO mice developed more severe colitis scores compared to FMT-younger mice (Figure 2.5D). Pan-colitis was observed with a higher degree of inflammation in both the proximal and distal colon of FMT-older IL-10 KO mice (Figure 2.S2E). As expected, FMT WT mice did not develop colitis (Figure 2.S2F).

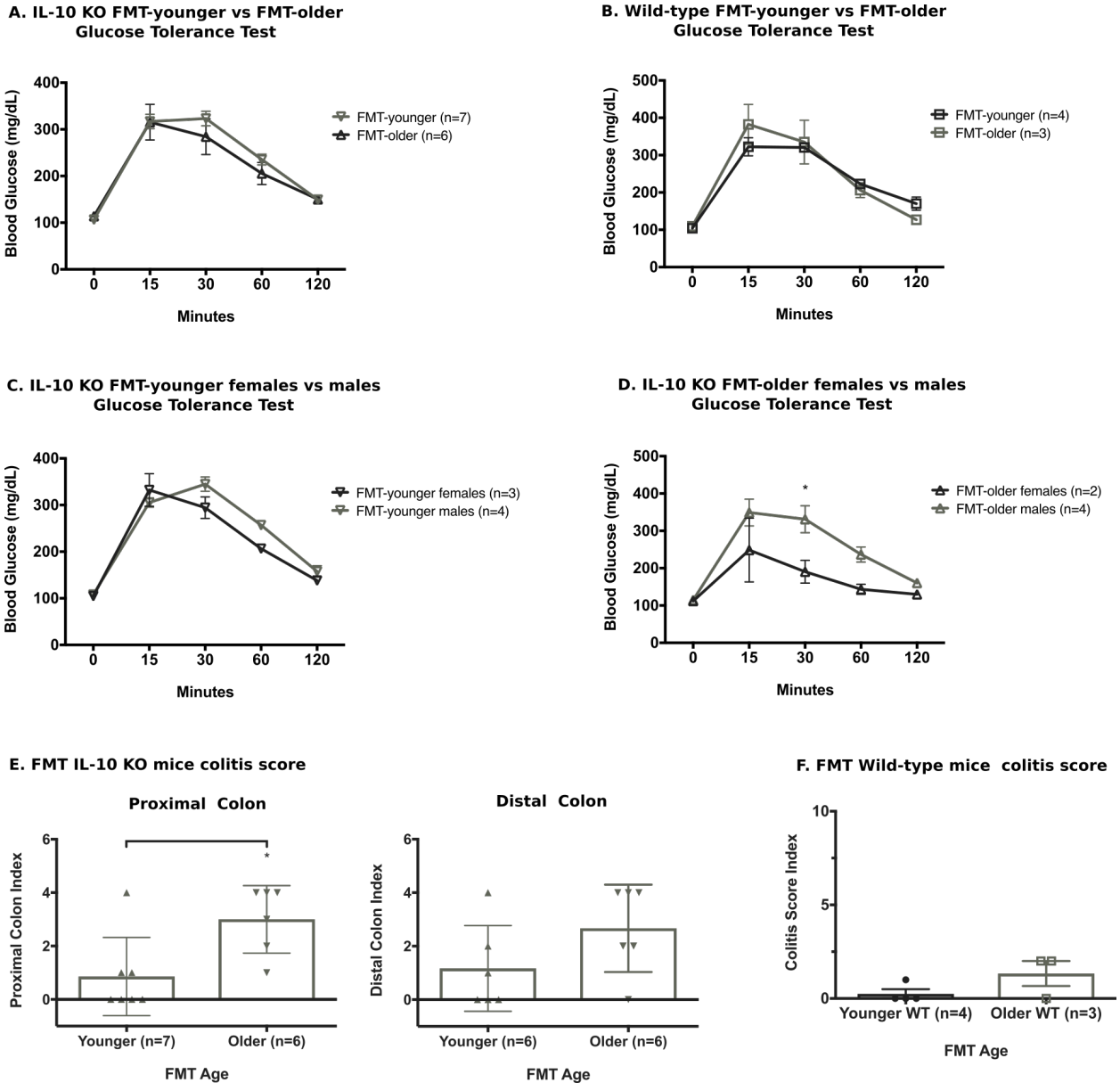
Figure 2.5: Fecal microbiota transplantation (FMT) of GF IL-10 KO recipients protects against pancreatic infiltration.
A. Incidence of pancreatic infiltration in GF versus FMT IL-10 KO mice (GF n=108, FMT n=10). **B.** Non-fasted A.M. blood glucose levels in age-matched GF versus FMT IL-10 KO mice (n=8-10/group). **C.** Non-fasted A.M. blood glucose levels in FMT-younger (5 weeks) versus FMT-older (17 weeks) IL-10 KO mice (n=6-7/group). **D.** Combined proximal and distal colitis score in FMT-younger versus FMT-older IL-10 KO mice at the time of sacrifice (26-27 weeks of age) (n=6/group). *p=0.02 Mann-Whitney Test.

Figure 2.5, continued.



Supplementary Figure 2.S2, related to Figure 2.5

A. Glucose tolerance blood glucose levels in FMT-younger versus FMT-older IL-10 KO mice (n=6-7/group). **B.** Glucose tolerance blood glucose levels in FMT-younger versus FMT-older wild-type mice (n=3-4/group). **C.** Glucose tolerance blood glucose levels in FMT-younger female versus male IL-10 KO mice (n=3-4/sex). **D.** Glucose tolerance blood glucose levels in FMT-older female versus male IL-10 KO mice (n=2-4/sex). **E.** Proximal and distal colitis score in FMT IL-10 KO mice. **F.** Combined colitis score in FMT WT mice. **p*=0.03 via Sidak's Multiple Comparison Test in D and Mann-Whitney in E.



2.4 Discussion and Future Directions

Deciphering the course of T1D pathogenesis in humans has been relatively difficult due to the variability and inaccessibility of human diabetic pancreatic samples. This inhibits longitudinal sampling of the pancreas over time and presents a substantial limitation to studying immunoregulatory mechanisms before the onset of clinical diabetes. However, significant progress has been made in identifying key players involved in islet invasion and β-cells destruction (S. Kim *et al.*, 2007; Coppieters *et al.*, 2012; Magnuson *et al.*, 2014; Lundberg *et al.*, 2017). Current knowledge and outstanding questions regarding the immune mechanisms involved in the development of autoimmune T1D have been previously reviewed (Wå and Cooke, 2013; Christoffersson and von Herrath, 2016; Pugliese *et al.*, 2016; Tai, Wong and Wen, 2016; Walker and von Herrath, 2016). Given the barriers to studying T1D in humans, rodent models provide important clues to environmental factors and underlying mechanisms impacting disease incidence. Additionally, the advent of GF animal systems has allowed for the expansion of more mechanistic studies focused on the role of gut microbiota in disease incidence and development.

The use of GF IL-10 KO mice to initially study the role of gut microbiota in inflammatory bowel disease led us to the observation of polyuria and the surprising discovery of pancreatic infiltration and diabetes in a subset of older GF IL-10 KO mice. We hypothesized that the loss of microbial signals and IL-10 play a protective role in the development of pancreatic immunopathology. Several strategies were considered to test this hypothesis. To first determine whether GF IL-10 KO exhibit an increased incidence of pancreatic infiltration and an association with T1D, we focused on immune-histopathology, blood glucose assessment, and an adoptive transfer model. To determine whether gut microbiota are protective, we utilized fecal microbiota transplantation models and assessed the pancreas of SPF counterparts. While further characterization is required, the GF IL-10 KO model exhibits early phases of pancreatic infiltration similar to the pathology of T1D, which could address current limitations to the field by

allowing us to study early stages of disease initiation and increase our understanding of mechanisms at play.

As part of our initial characterization, we examined the pancreas of GF IL-10 KO males and females and discovered pancreatic infiltration resembling T1D infiltrates or lesions. Pancreatic data from T1D donors and murine models support the role of B and T lymphocytes and antigen-presenting cells in disease pathology (In't Veld, 2011; Magnuson *et al.*, 2014; Walker and von Herrath, 2016). Our immunofluorescent staining confirms the presence of T and B lymphoid cells and myeloid cells, such as macrophages and dendritic cells, in pancreatic infiltrates (Figure 2.1). We also observed positive pSTAT1 staining at the periphery of islets near infiltrates, which has been implicated in T1D pathogenesis and β-cells death (Sunshin Kim *et al.*, 2007). Our immunostaining results are compatible with previous human and mouse staining of islet infiltrates (Willcox *et al.*, 2009; Coppieters *et al.*, 2012; Thompson *et al.*, 2014; Carrero, Ferris and Unanue, 2016; Lundberg *et al.*, 2017). Obstacles and opportunities for targeting effector T cells in T1D have been recently described (Buckner and Nepom, 2016). Further characterization of immunity in these mice would require the use transgenic mouse lines of interest. For example, the dynamic nature of pancreatic islet infiltrates was investigated via traceable photoconvertible fluorescent reporters in immunocytes via the NOD x Kaede transgenic mouse model. Cells in the Kaede transgenic mouse line express the green fluorescent form of the Kaede protein, which can be photoconverted into a red fluorescent form at one site in the body in order to monitor cell trafficking in vivo. The use of this model revealed the constant cell influx and turnover of invading immune cells in the pancreas, which were predominantly comprised of T and B lymphocytes but also $CD11b^+$ and $CD11c^+$ myeloid cells (Magnuson *et al.*, 2014).

Following immune histological assessment of the pancreas, we focused on a secondary strategy to characterize the immunogenicity of GF IL-10 KO mice via adoptive transfer of immune cells into immunodeficient GF RAG1 KO recipients lacking mature T and B

lymphocytes (Mombaerts *et al.*, 1992). Adoptive transfer of immune cells from diseased GF IL-10 KO donors did not transfer pancreatic immunopathology into immunocompromised GF RAG1 KO recipients. Further investigation is required to decipher the role of the adaptive immune system in this model. There were several caveats to this adoptive transfer experiment that may have contributed to the failure to propagate the disease phenotype in recipients. Although donor splenic adaptive immune cells were successfully transferred, donors exhibited only mild pancreatic infiltration and were not overtly diabetic. The frequency of transferred T and B cells in recipients was also significantly lower in spleens of recipients (Figure 2.2). Thus, transferred donor cells may not have lived long enough or may not have migrated to the pancreatic lymph nodes and pancreas to propagate disease pathology. Additionally, the interaction between donor innate and adaptive immune cells with recipient innate cells, which are capable of expressing IL-10, may have prevented disease transfer. While future transfer of purified T and B cells into younger GF RAG1 KO mice would better test the role of specific adaptive immune cells in disease pathology, further baseline characterization of the immune system is required. For example, several reports highlight signaling pathways that contribute to changes in T cell subtypes (Alonso *et al.*, 2015) and T cell migration into the pancreas via CXCR3 (Roep *et al.*, 2010; Hu *et al.*, 2011). Thus, further characterization of these cell populations in GF IL-10 KO mice would establish a baseline for further analysis of immune responses.

To confirm whether the presence of pancreatic infiltration was increased in GF IL-10 KO mice, we performed a cross-sectional study to examine the pancreas for the presence of immunopathology before and after 6-months of age. Interestingly, we observed significantly higher pancreatic infiltration in both female and male GF IL-10 KO mice at an older age, above 6-months of age (Figure 2.3). This finding demonstrates the slow and progressive nature of disease, which may require additional triggers in order to progress to overt diabetes. This finding also suggests age plays a role in disease development in the absence of microbial

signals and potentially IL-10 mediated immunoregulatory mechanisms (Thompson *et al.*, 2014). One possible explanation for the pancreatic phenotype in our mice is immune dysregulation due to the lack of IL-10 signals and microbial-mediated signals. IL-10 signaling is important for Treg cell function in restraining T cell mediated inflammation (Chaudhry *et al.*, 2011; Keubler *et al.*, 2015). When Foxp3-expressing Treg cells are ablated, conventionally-raised mice develop widespread immune-mediated lesions (Kim, Rasmussen and Rudensky, 2007; Chinen *et al.*, 2010). Treg cells also regulate diabetogenesis in NOD mice, with a temporal decline in functional potency (Tritt *et al.*, 2008). Furthermore, GF conditions may potentiate immunogenicity because mice raised GF contain fewer Treg cells compared to conventionally-raised mice (Östman *et al.*, 2006). Although the absence of microbial signals in GF IL-10 KO prevents the development of spontaneous colitis (Sellon *et al.*, 1998), the loss of potentially protective microbial-mediated signals may contribute to the immunopathology we observe in the pancreas. Since immune imbalance may also impact other organs, we collected salivary glands, thymus, liver, spleen, and kidney for future histological assessment of organ-specific immunopathology in GF IL-10 KO mice.

Interestingly, only a small subset of mice became overtly diabetic, while the majority of GF IL-10 KO mice maintained euglycemia (Figure 2.4). The delayed onset of pancreatic immunopathology and diabetes may in part be due to the C57Bl/6 genetic background and MHC b haplotype, which is thought to be more protective against diabetes compared to the g7 haplotype found in the NOD mouse model (Leiter, Coleman and Hummel, 1981). While genetic analyses of GF IL-10 KO mice were beyond the scope of this study, the model also offers an intriguing opportunity to refine genetic and environmental factors that dictate the natural history of disease development. A modified disease classification system has recently been proposed to capture the sequential nature of disease starting from presymptomatic to symptomatic stages (Insel *et al.*, 2015). Our model reflects the first of three progressive stages in T1D development in which patients maintain euglycemia but are positive for autoantibodies. The presence of

autoantibodies specific to islets before disease diagnosis has been a hallmark of human T1D, with insulin as one of the major targets of humoral autoimmunity in humans and the diabetes-prone NOD model (Bonifacio *et al.*, 2001; Ziegler *et al.*, 2011). Previous reports indicate autoantibodies in NOD and C57Bl/6 mice demonstrated differences in IgM and IgD autoantibody reactivity, which may reflect disease susceptibility mechanisms (Bonifacio *et al.*, 2001; Quintana and Cohen, 2001). We observed a subset of GF IL-10 KO positive for insulin autoantibodies in our first cohort analyzed via radioimmunoassay at University of Florida Diabetes Center (Table 2). Our second cohort of GF IL-10 KO mice did not test positive and were analyzed via a non-radioactive autoantibody assay method with electrochemiluminescence (ECL) technology at the Barbara Davis Center for Diabetes (Table 3). Further investigation on whether autoantibodies play a role in pathology should be considered via longitudinal testing of sera starting in younger GF IL-10 KO mice. H2-b MHC I and II tetramer staining provides one quantitative method for assessing antigen-specific T cell responses in these mice and quantifying CD4 and CD8 T cells that bind to suspected antigens of interest (Kurtulus and Hildeman, 2013). Greater knowledge on autoreactive cells in this model would allow for more sophisticated techniques to uncover immune signatures. For example, a recent study in human T1D patients identified T1D-associated immune receptor signatures at the nucleotide and amino acid level via high-throughput immunosequencing of T cell receptor ß-chain and B cell receptor immunoglobulin heavy chain from peripheral blood mononuclear cells, splenocytes, and pancreatic lymph nodes (Seay *et al.*, 2016).

Two major areas of focus within the last decade pertain to the contribution of the gut microbiome, in particular microbial maturation and dysbiosis, to disease pathogenesis, as well as underlying mechanisms that lead to autoimmunity (Dunne *et al.*, 2014). A large body of literature supports microbial dysbiosis as a major contributor to disease pathogenesis (Vaarala, 2012; Gulden, Wong and Wen, 2015; Knip and Siljander, 2016; Paun, Yau and Danska, 2016a; Hu, Wong and Wen, 2017), highlighting microbial modulation as an exciting avenue for

prevention or treatment. Our final characterization strategy focused on the protective capacity of gut microbiota against pancreatic immunopathology via a cross-sectional study of SPF IL-10 KO mice and a fecal transplantation study in younger and older GF IL-10 KO recipients. We hypothesized that exposure to microbial signals at an early age during immune development would be more protective against pancreatic infiltration compared to exposure in adulthood. Interestingly, both younger (5 weeks of age) and older (17 weeks of age) FMT IL-10 KO recipients were protected from pancreatic infiltration (Figure 5). These preliminary results support a role for microbial signals in immune regulatory mechanisms presumably via connections between the gut and the pancreas (Turley *et al.*, 2005; Paun, Yau and Danska, 2016a). The nature of the IL-10 KO model leads to the development of spontaneous colitis in the presence of specific gut microbiota. Thus, FMT may stimulate different microbe-mediated immune signaling pathways which direct inflammation in the gut rather than the pancreas. We identified colitis development in both younger and older FMT IL-10 KO recipients, however older mice developed more severe colitis and at a faster rate (Figure 2.5), which differs from a previous report indicating age of microbial colonization did not impact colitis severity (Sydora *et al.*, 2003). Further assessment of gut integrity, microbial assemblage, and microbe-mediated immune signaling pathways would contribute to the growing understanding of potential microbial-based therapies for complex immune disorders such as IBD and T1D (Wu and Wu, 2012; Gulden, Wong and Wen, 2015).

To dissect the specific role of gut microbial communities and age-related immunological mechanisms at play, future FMT experiments would require a larger cohort of GF IL-10 KO recipients at birth, via FMT of dams, and late adulthood. Initially, utilizing a defined bacterial community such as Altered Schaedler Flora (ASF), which represents a simplified community of bacteria normally present in the mouse gut, for FMT, would aid in deciphering the contribution of specific microbial communities to microbe-mediated immune responses in disease development. Work in the NOD model by the Chervonksy lab at The University of Chicago

demonstrate the importance of gut microbiota-mediated innate immune signaling pathways for the development of T1D (Wen *et al.*, 2008; Burrows *et al.*, 2015). The deletion of the innate immune adaptor myeloid differentiation primary response gene 88 (MyD88) or specific innate immune toll-like receptors in NOD mice results in T1D under GF conditions. However, these mice are more protected against T1D if they are raised SPF in the presence of microbiota or if GF MyD88-negative mice are given FMT with specific bacterial communities (Burrows *et al.*, 2015). These findings highlight the involvement of innate immune sensing receptors in pro- and anti-diabetic regulatory mechanisms underlying T1D and incite future immunological investigations in our IL-10 KO model in the absence and presence of gut microbiota.

Since diabetes is halted at an early stage in T1D pathogenesis in our GF IL-10 KO mouse model, further investigation into immunological and metabolic pathways at play would yield more mechanistic insights and contribute to potential prophylactic treatment strategies. More sophisticated dissection of molecular signals at play would require the use of knockout models of single molecular factors or cross-breeding with other transgenic models to examine immunological and metabolic responses involved in disease development. To provide an initial landscape of immune expression profiles and populations at immunologically relevant sites, experiments could initially investigate immune responses via microarray and multi-parametric flow cytometry of immune cells in colonic lamina propria, mesenteric lymph nodes, and pancreatic lymph nodes from FMT IL-10 KO recipients, as compared to GF IL-10 KO and GF WT counterparts.

Moreover, recent studies focused on metabolic mechanisms underlying T1D pathogenesis suggest metabolomic disturbances precede the onset of T1D (Mejía-León and Barca, 2015; Overgaard *et al.*, 2016). Many environmental triggers are capable of fueling underlying metabolic and immune dysfunction, particularly through cellular mechanisms that originate in the gut and impact host immune responses and metabolism. For example, postprandial nutrients and microbial signals impact enteroendocrine cells, such as L cells which

secrete hormones like glucagon-like peptide-1 (GLP-1) (Greiner and Bäckhed, 2016). GLP-1 and GLP-1 receptors regulate several physiological functions in the pancreas and the gut. As an incretin, GLP-1 stimulates insulin release from β-cells and inhibits glucagon release from α-cells in the pancreas to help regulate glucose control. However, GLP-1 signaling also modulates innate immune responses in the gut via enteroendocrine-immune interactions (Yusta *et al.*, 2015). Interestingly, GF mice exhibit elevated GLP-1 levels, as well as altered metabolism (Wichmann *et al.*, 2013; Greiner and Bäckhed, 2016). Thus, elevated GLP-1 and other enteroendocrine hormones may participate in driving immune responsiveness and immunopathology in the pancreas of GF IL-10 KO mice. Future investigation of both systemic and more localized physiologically changes in the gut and in the islets of the pancreas may reveal novel mechanisms perpetuating autoimmune responses directed toward β-cells in the pancreas. Characterization of metabolic profiles in our model would shed light on the cross talk between the enteroendocrine system, immunity, and metabolism.

In summary, the IL-10 KO mouse model allows for the investigation of gut-mediated signaling in disease pathogenesis of the intestine, as well as the pancreas in the presence and absence of microbiota. While further characterization if required to dissect specific immune compartments and role of microbial-mediated signaling pathways, our initial characterization provides the foundation to understanding the role of gut microbiota in early stages of pancreatic immunopathology in IL-10 KO mice. The amalgamation of knowledge from distinctive but associated research fields—from islet biology, to peripheral immune tolerance, to gut microbial assemblage—in combination with human clinical trials is necessary for the progression toward prophylactic treatment strategies (Gulden, Wong and Wen, 2015; Garyu, Meffre and Cotsapas, 2016).

2.5 Experimental Procedures

2.5.1 Animals

Murine experimental procedures were performed at The University of Chicago and approved by The Institutional Animal Care and Use Committee (IACUC). Male and female C57Bl/6 Interleukin-10 deficient (IL-10 KO), wild-type (WT), and recombination-activating gene 1 deficient (RAG1 KO) mice were bred in-house, fed ad libitum, and maintained under a normal 12-hour light/dark cycle. Germ-free (GF) cohorts were group-housed and maintained on irradiated chow (Harlan, 2018 diet) in plastic isolators in The University of Chicago Gnotobiotic Research Animal Facility (GRAF). Specific pathogen free (SPF) cohorts were group-housed and maintained on standard chow (Harlan, 2016 diet) in the same SPF vivarium room.

2.5.2 Histological assessment

Harvested pancreata were OCT embedded and frozen in a methyl-butane bath, or paraformaldehyde (4%) fixed at 4°C for 4-6 hours, processed, and paraffin embedded. Serial sections, 5-µm thick, were stained with immunofluorescence or Hematoxylin/Eosin (H&E). Colon, salivary gland, kidney, and thymus were formaldehyde (4%) fixed, paraffin embedded, serial sectioned, and H&E stained for assessment. Samples were processed and sectioned at The University's Human Tissue Resource Center (HTRC) or in-lab following HTRC's protocol guidelines. Histological scoring for colitis was employed as previously described (Erben *et al.*, 2014).

2.5.3 Pancreatic infiltration incidence

To determine pancreatic infiltration incidence, whole H&E stained pancreas sections were scanned and scored for the presence or absence of lymphocytic infiltrates on a blinded basis in Panoramic Viewer. This scoring was used to determine the incidence of pancreatic infiltration by calculating the number of mice positive for pancreatic immune cell infiltrates divided by the total number of mice scored in each mouse group.

2.5.4 Immunohistochemistry

Immunostaining was performed by Patrick Moore from the lab of Dr. Christopher Rhodes at The University of Chicago. To identify immune cell types within pancreatic infiltrates, frozen sections of pancreas were stained with primary antibodies: insulin (Sigma-Aldrich, MO); CD3 (Sigma); MHC-1 (Abcam, CA); CD4 (Abcam); CD68 (Abcam); B220 (BD Pharm, CA); CD8 (Thermo Fisher, MA); CD11c (StemCell Tech, Canada); and pSTAT1 (BD Bioscience, CA). Donkey derived secondary antibodies (Jackson ImmunoResearch) were used for fluorescence visualization on a DSU confocal microscope.

2.5.5 Diabetes assessment

To assess diabetes development, several procedures were performed to tracked blood glucose levels in gnotobiotic isolators and the animal vivarium. To initially track blood glucose measurements of mice housed in isolators, submandibular cheek bleeds were performed by gnotobiotic special technicians and serum was stored at -80C. Mice were monitored for increased frequency of urination and urinalysis was performed to test for glucose in the urine (Bayer, Keto-diastix Reagent Strips). Blood glucose levels were recorded with a glucometer (Accu-Chek Compact Plus) from small tail snips of mice (1) fasted in the early morning for 4-6 hours and/or (2) non-fasted in the morning (A.M.). To assess glucose tolerance, mice fasted overnight for no more than 15-hours received intraperitoneal injections (IP) of 20% dextrose solution. Blood glucose readings were measured at 0, 15, 30, 60, and 120 minutes.

2.5.6 Flow cytometry

Spleens were mashed through a sterile 40 or 70μm filter, washed with sterile filtered CRPMI (RPMI + 10% FCS) and spun down at 4C 600G for 5 minutes. Red blood cells were lysed (RBL Buffer: H_2O 100ml, NH_4Cl 0.824g, $KHCO_3$ 0.1g, 0.5M EDTA 20uL), cells were spun down again and resuspended in sterile FACs PBS (PBS, 2% FCS) for cell counting with a hemocytometer. For cell population analysis, 1-2 million cells from cell suspensions isolated were stained 1:1000

for Aqua Live/Dead (Life Technologies), and 1:100 or 1:200 for anti-CD4 (eBioscience, CA), anti-CD8α (eBioscience), anti-CD19 (eBioscience), anti-TCRβ (eBioscience), or anti-CD45 (Biolegend, CA). Samples were analyzed with a FACSCanto (BD Biosciences) and FlowJo v10.1 (FLOWJO, OR, Ashland).

2.5.7 Adoptive transfer

GF RAG1 KO mice recipients (15 males, 12 females ranging from 7-14 weeks of age) were IP injected with splenic immune cells (splenocytes) isolated from older GF IL-10 KO donors (4 males, 4 females ranging from 27-35 weeks of age) under sterile conditions. One older GF WT male and female were used as control GF WT donors. Each adoptive transfer was performed with a Donor to Recipient ratio of 1:2 or 1:3 (Table 1). Fecal aerobic and anaerobic cultures were collected throughout the study to ensure the absence of microbial contamination in the isolator. GF RAG1 KO recipients were weighed weekly and removed from the isolator after 10-weeks for metabolic and histological assessment.

2.5.8 Insulin autoantibody analyses

Frozen serum samples were analyzed for the presence of insulin autoantibodies in collaboration with Dr. Mark Atkinson and Clive Wasserfall at the University of Florida Diabetes Center (Cohort 1) and Dr. Liping Yu at the Barbara Davis Diabetes Center (Cohort 2). Cohort 1 was analyzed via radioimmunoassay previously described with an IAA cutoff based on NOD versus C57Bl/6 positive Index above 10.2 (Yu *et al.*, 2003). Cohort 2 was analyzed via an electrochemiluminescence assay (Miao *et al.*, 2015).

2.5.9 Fecal microbiota transplantation

GF IL-10 KO mice at 4-5 weeks and 17 weeks of age were transferred from the Gnotobiotic Research Animal Facility into a *H. hepaticus*-free vivarium. Donor *H. hepaticus*-free mice were kindly provided by Dr. Cathryn Nagler at the University of Chicago. Fresh fecal pellets from donor WT female and male mice were diluted respectively in sterile PBS (2 pellets per 1 mL

PBS). 150μL was processed by oral gavage into each of the recipient mice. Recipient mice were monitored until 27 weeks of age.

2.5.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism v7.0b via nonparametric ANOVA or unpaired t tests. Data are represented as mean ± SEM.

Chapter 3: Peripartum exposure to antibiotics promotes gut dysbiosis, loss of immune tolerance, and inflammatory bowel disease risk in genetically prone offspring

3.1 Abstract[1]

Factors impacting the developing neonatal gut microbiome and immune networks may increase risk for developing complex immune disorders such as inflammatory bowel diseases (IBD). In particular, antibiotics commonly used during the peripartum period have been suggested as risk factors for human IBD, although direct evidence are lacking. We therefore 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 the offspring from cefoperazone-treated dams develop a persistent gut dysbiosis into adulthood associated with skewing of the host immune system and increased susceptibility to spontaneous and chemically (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 in genetically-susceptible offspring to increase IBD risk.

[1] Citation for chapter: Miyoshi J, Bobe AM et al. 2017. Peripartum exposure to antibiotics promotes gut dysbiosis, loss of immune tolerance, and inflammatory bowel disease in genetically prone offspring. Cell Reports *20,* 491-504.

3.2 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 (Pugazhendhi *et al.*, 2011; Bager *et al.*, 2012; Devkota *et al.*, 2012; Benchimol *et al.*, 2015). While there is a genetic basis (Franke *et al.*, 2010; Anderson *et al.*, 2011; Jostins *et al.*, 2012; Van Limbergen, Radford-Smith and Satsangi, 2014; J. Z. Liu *et al.*, 2015; Liu and Stappenbeck, 2016), 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 ((Seksik *et al.*, 2003; Ott *et al.*, 2004; Gophna *et al.*, 2006; Manichanh *et al.*, 2006; Frank *et al.*, 2007). 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 (Stokholm *et al.*, 2013; Broe *et al.*, 2014). 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 with increased risk IBD development, especially treatment-naïve pediatric CD (Hviid, Svanstrom and Frisch, 2011; Kronman *et al.*, 2012; Virta *et al.*, 2012; Gevers *et al.*, 2014; Ungaro *et al.*, 2014). 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, employing the well-accepted IL-10 knock-out (KO) mouse model, where the immunomodulatory cytokine IL-10 was genetically deleted (Kuhn *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 (Sellon *et al.*, 1998; Keubler *et al.*, 2015). *H. hepaticus* rarely causes colitis in wild-type mice, but can cause nearly a 100% 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 (Caufield, Cutter and Dasanayake, 1993; Caufield *et al.*, 2007; Jimenez *et al.*, 2008; Adlerberth and Wold, 2009; Funkhouser and Bordenstein, 2013; Pantoja-Feliciano *et al.*, 2013; Nayfach *et al.*, 2016; Asnicar *et al.*, 2017). 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 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 risk for complex immune-related disorders such as IBD.

3.3 Results

3.3.1 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 a *H. hepaticus*-free room under continuous monitoring. To control for genetics and maternal contribution, the 10 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 3.1A). 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 3.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 3.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 3.1B). 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 3.1C). As noted by others, both female and male mice exposed to peripartum CPZ treatment gained more weight initially (Figure 3.1D), 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 at 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 3.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.

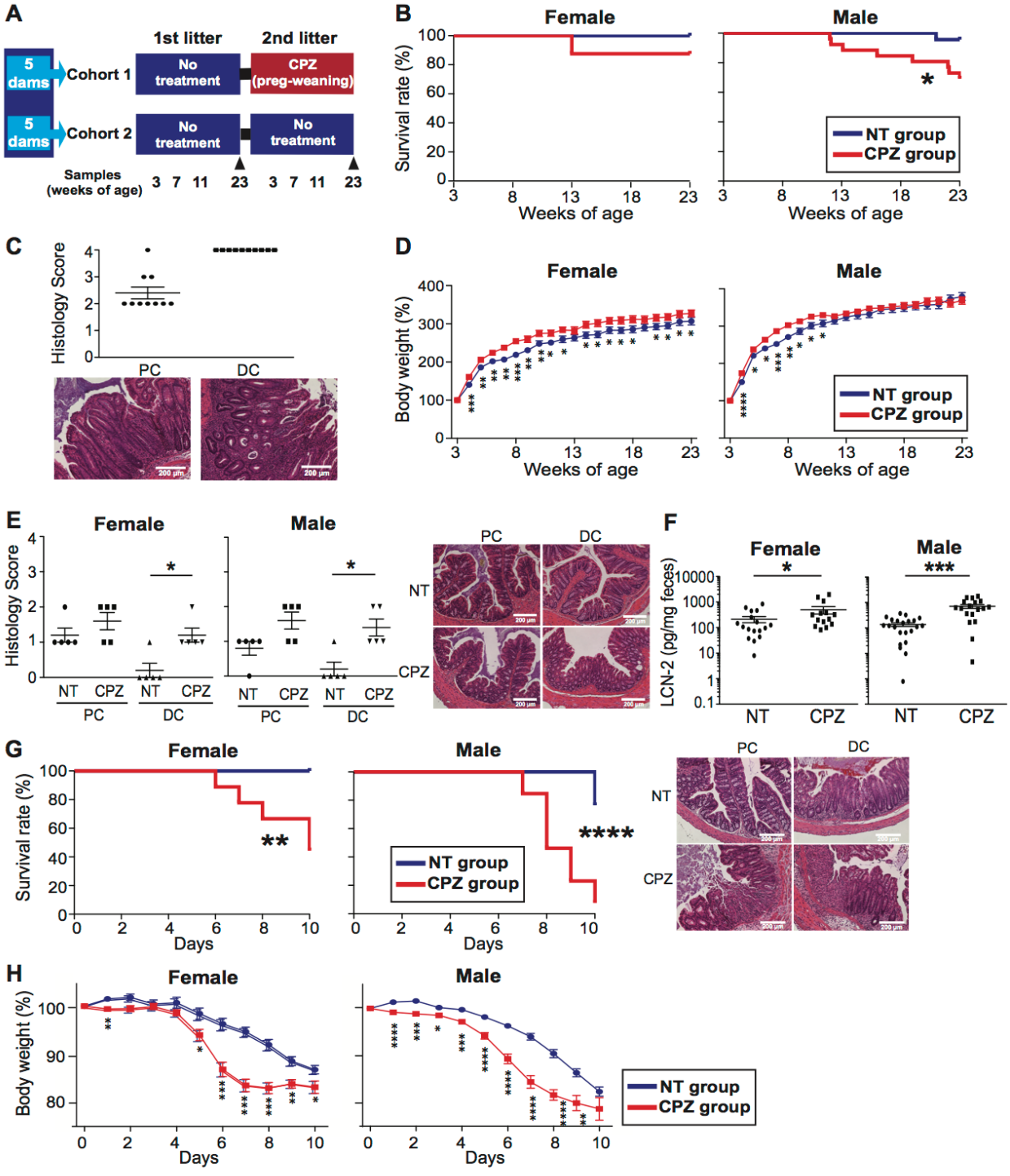
We next examined the mice that remained grossly healthy and never developed overt signs of spontaneous colitis (NT group: 17 females, 22 males; CPZ group: 14 females, 18 males). At 23 weeks of age, histologic grading of colons revealed higher inflammatory scores, particularly in the distal colons of the CPZ group compared to NT (n=5 mice/gender, p=0.021 in females and p=0.019 in males) (Figure 3.1E). 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 3.1F). 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.

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) (Figure 3.1G and 3.1H). 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 (Figure 3.S1D and 3.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.

Figure 3.1: Maternal peripartum exposure to cefoperazone increases the risk for colitis in offspring.
(A) Study design using IL-10 knock-out (KO) mouse model. All mice (cohort 1 and 2) were obtained from 10 breeding pairs subjected to mixed bedding protocol to normalize microbiota among parent cages. Cohort 1 (1st row) included two sequential litters from 5 breeders divided into non-treatment (NT) tracking group (litter 1) and cefoperazone (CPZ) tracking group (litter 2). Cohort 2 (2nd row) included two sequential litters from 5 breeders that were used as additional NT controls to assess/control for generational drift in gut microbiota across litters 1 and 2. (B) Survival rates of NT and CPZ offspring during the observation period (3 to 23 weeks of age) (NT group n=17 females; 23 males. CPZ group n=16 females, 26 males). (C) Histology of proximal (PC) and distal colon (DC) for CPZ mice euthanized due to colitis onset (n=2 females, 8 males). Representative H&E histological sections of PC and DC for NT and CPZ. (D) Percent weight change (expressed as % of 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/gender/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, 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 represented as mean ± SEM for (C)-(F) and (H). See also Figure 3.S1.

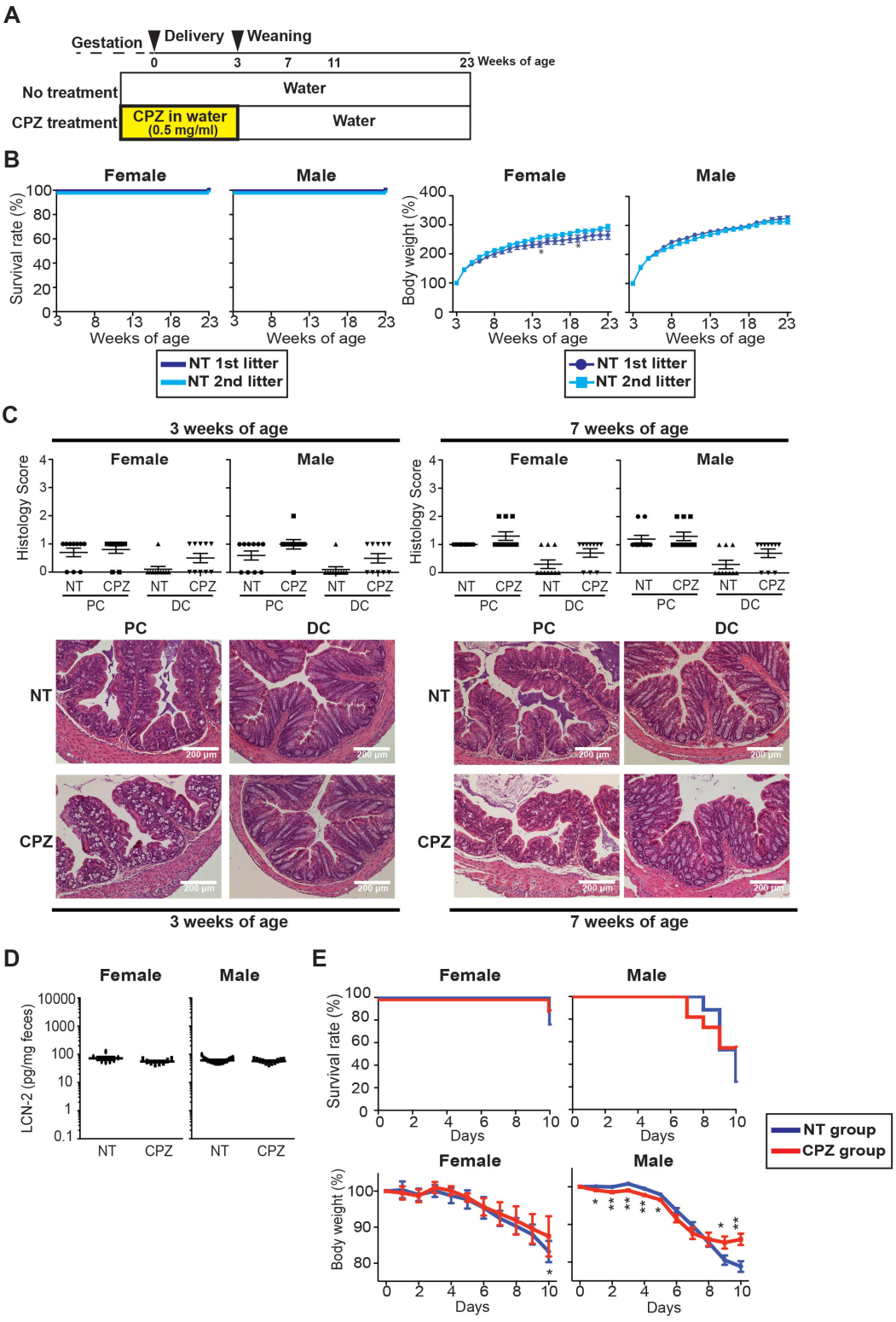
Figure 3.1, continued.



Supplementary Figure 3.S1: No significant differences were observed in colitis outcome between litters of cohort 2 NT IL-10 KO mice and between NT versus CPZ WT mice following DSS-challenge.

(A) Cefoperazone (CPZ) treatment protocol. Cohort 1 dams were treated with CPZ in drinking water during the 2nd pregnancy from day 14 of gestation until pup weaning. (B) Survival curves and weight changes for 1st and 2nd litters (NT) in cohort 2 (n=7 females in 1st litter, n=14 in 2nd litter; n=15 males in 1st litter, n=7 in 2nd litter). (C) Histological assessment of proximal colon (PC) and distal colon (DC) at 3 and 7 weeks of age. No significant differences in histological scores in PC and DC were observed between groups at 3 and 7 weeks of age in both genders. Representative pictures are presented for PC and DC at each age. (D) Fecal lipocalin-2 (LCN-2) levels were measured in all wild type (WT) subjects at 23 weeks of age (NT n=8 females; 17 males. CPZ n=8 females, 11 males). (E) Survival curves and weight changes for NT and CPZ WT mice that were treated with 2.5% dextran sulfate sodium (DSS) at 23 weeks of age. Mice showing more than 20% body weight loss were euthanized. *p < 0.05, **p < 0.01. Data are represented as mean ± SEM for (B)-(E).

Supplementary Figure 3.S1, continued.



3.3.2 Peripartum antibiotic exposure leads to aberrant development of the offspring immune system

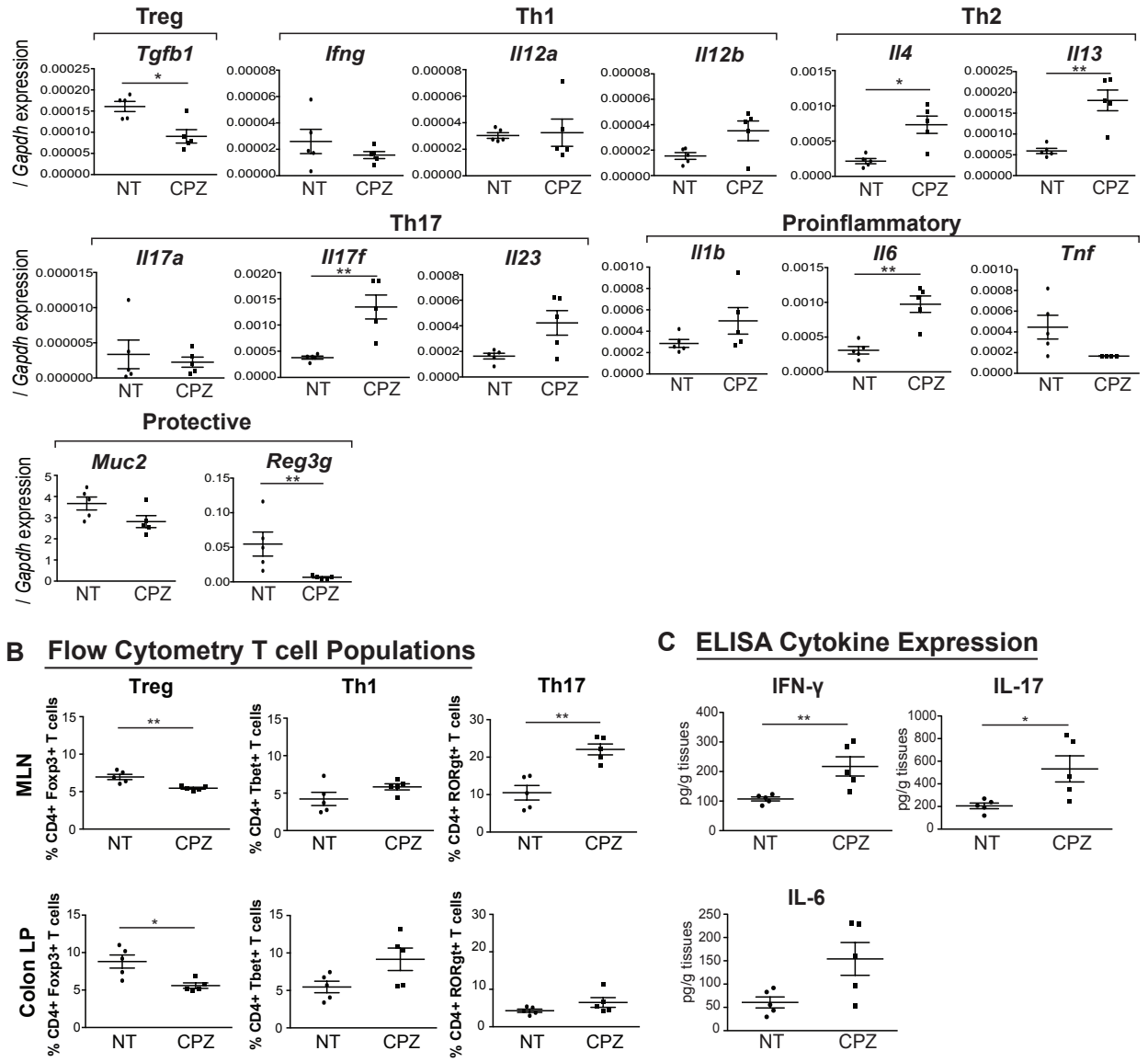
To determine if and how maternal CPZ exposure during early development alters immune profiles, we first compared mRNA levels of cytokine genes indicative of T cell subtypes (Treg, Th1, Th2 and Th17 cells), pro-inflammatory cytokine genes, and mucosal protective factors relevant to IBD in colons of 3-week-old pups from CPZ-exposed dams versus NT controls. Male data are shown in Figure 3.2 and female data in Figure 3.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 (Figure 3.2A and Figure 3.S2A). In spite of the increased mRNA expression of *Il4* and *Il13*, there were no significant differences in plasma IgE level in CPZ pups compared to NT controls (Figure 3.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 (RORγt). We harvested cells from the mesenteric lymph nodes (MLN) 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 3.S2F. Specifically, we examined the percentage of live $CD45^+TCRb^+CD4^+$ cells ($CD4^+$ T cells) that expressed $Foxp3^+$ (regarded as regulatory T cells; Treg), $T\text{-}bet^+$ (Th1 cells) or $ROR\gamma t^+$ (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 3.2B). Similarly, CPZ IL-10 KO female pups showed significant decreases in MLN Tregs (p<0.01) and significant increases in LP Th17 cells as compared to NT (p<0.05) (Figure 3.S2B). These immune changes were not observed in 3-week-old NT versus CPZ group WT mice with the exception for significant differences detected in MLN Th1 cells from WT females (Figure 3.S2E). As shown in Figure 3.2C, 23-week-old male CPZ IL-10 KO mice that survived and did not develop spontaneous colitis still showed significantly higher levels of 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 3.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.

Figure 3.2: 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- vs. anti-inflammatory genes involved in colonic inflammation from colonic mucosal scrapings in non-treated (NT) vs. cefoperazone (CPZ) male IL-10 KO mice at 3 weeks of age. mRNA levels expressed as ΔΔCT relative to housekeeper gene *Gapdh*. (B) Flow cytometric analyses of live $CD45^+TCR\beta^+CD4^+$ T cells expressing $Foxp3^+$ (Treg), $T\text{-}bet^+$ (Th1) or $ROR\gamma t^+$ (Th17) in MLNs and colonic LPs of NT vs. CPZ males at 3 weeks of age. Data represent percentage of live $CD4^+$ cells. (C) Protein levels of inflammatory cytokines in MLNs of NT vs. CPZ-exposed males at 23 weeks of age determined via ELISA. *N=4-5/group.* NT (black circles), CPZ (black squares). *p<0.05, **p<0.01 via Mann-Whitney *U*-test. Data are represented as mean±SEM. Female data shown in Figure 3.S2. See also Figure 3.S2.

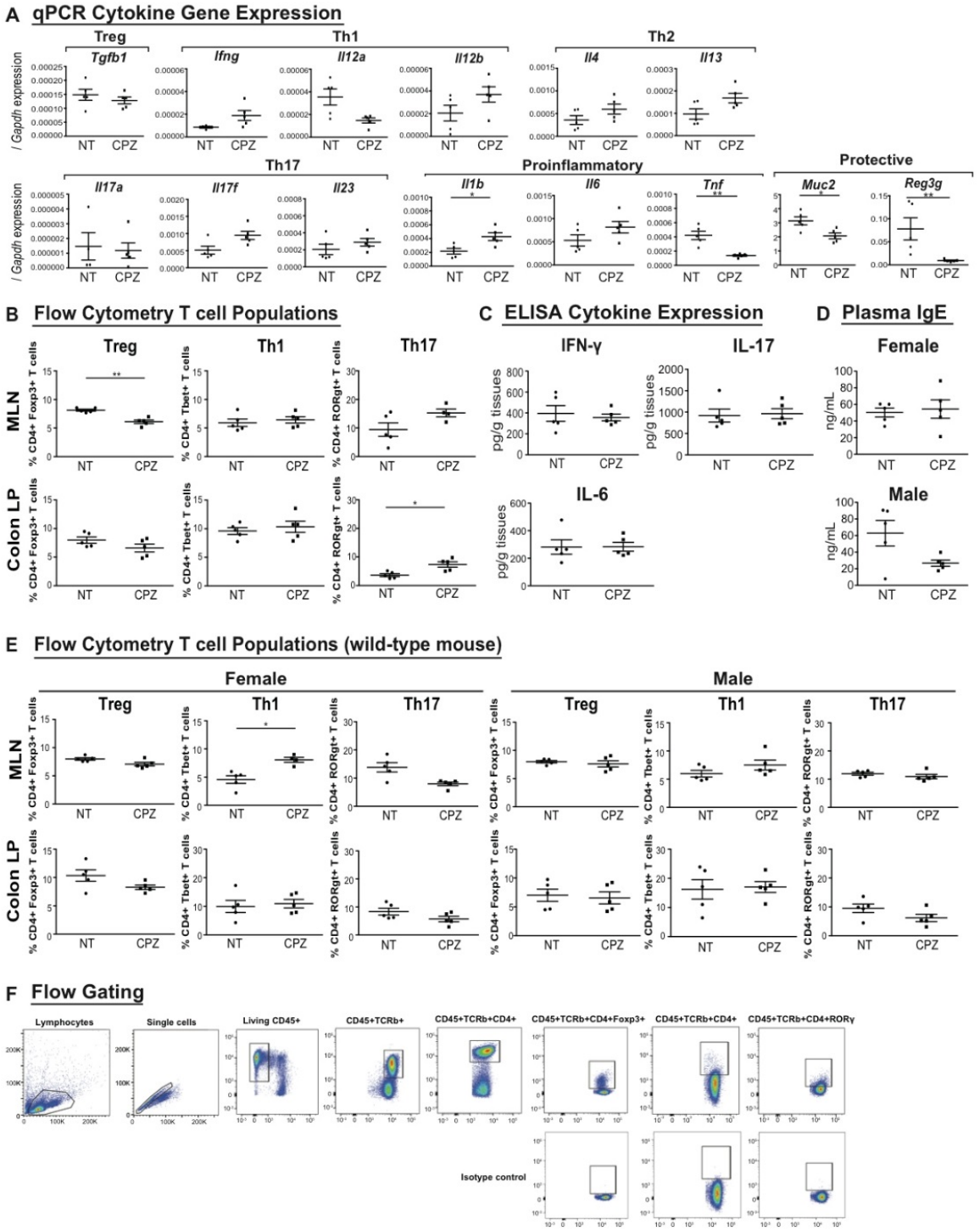
Figure 3.2, continued.



Supplementary Figure 3.S2: Female IL-10 KO offspring from CPZ-exposed dams exhibit aberrant immunity similar to male offspring, but WT offspring from WT CPZ-exposed dams do not regardless of gender.

(A) Real-Time qPCR mRNA levels of pro- vs. anti-inflammatory cytokine genes involved in colonic inflammation from colonic mucosal scrapings in female IL-10 KO mice of non-treated (NT; black circles) versus cefoperazone (CPZ; black squares) exposed dams at 3 weeks of age. mRNA levels are expressed as ΔΔCT relative to the housekeeper gene *Gapdh*. (B) Flow cytometric analyses of live $CD45^+TCR\beta^+CD4^+$ T cells expressing $Foxp3^+$ (Treg), $T\text{-}bet^+$ (Th1) or $ROR\gamma t^+$ (Th17) in MLNs and colonic LPs of NT versus CPZ females at 3 weeks of age. Data represents percentage of live $CD4^+$ cells. (C) Protein levels of inflammatory cytokines in MLNs of NT versus CPZ IL-10 KO females at 23 weeks of age determined via ELISA. (D) Flow cytometric analyses in MLNs and colonic LPs of NT versus CPZ WT female and male offspring at 3 weeks of age. (E) Representative images of flow cytometry gating strategy for analyzing MLN and colonic LP T cell populations with representative isotype controls. n=4-5/group. $^*p < 0.05$, $^{**}p < 0.01$ via Mann-Whitney U-test. Data are represented as mean ± SEM.

Supplementary Figure 3.S2, continued.



3.3.3 Maternal peripartum antibiotic exposure induces a persistent maternal and neonatal gut dysbiosis

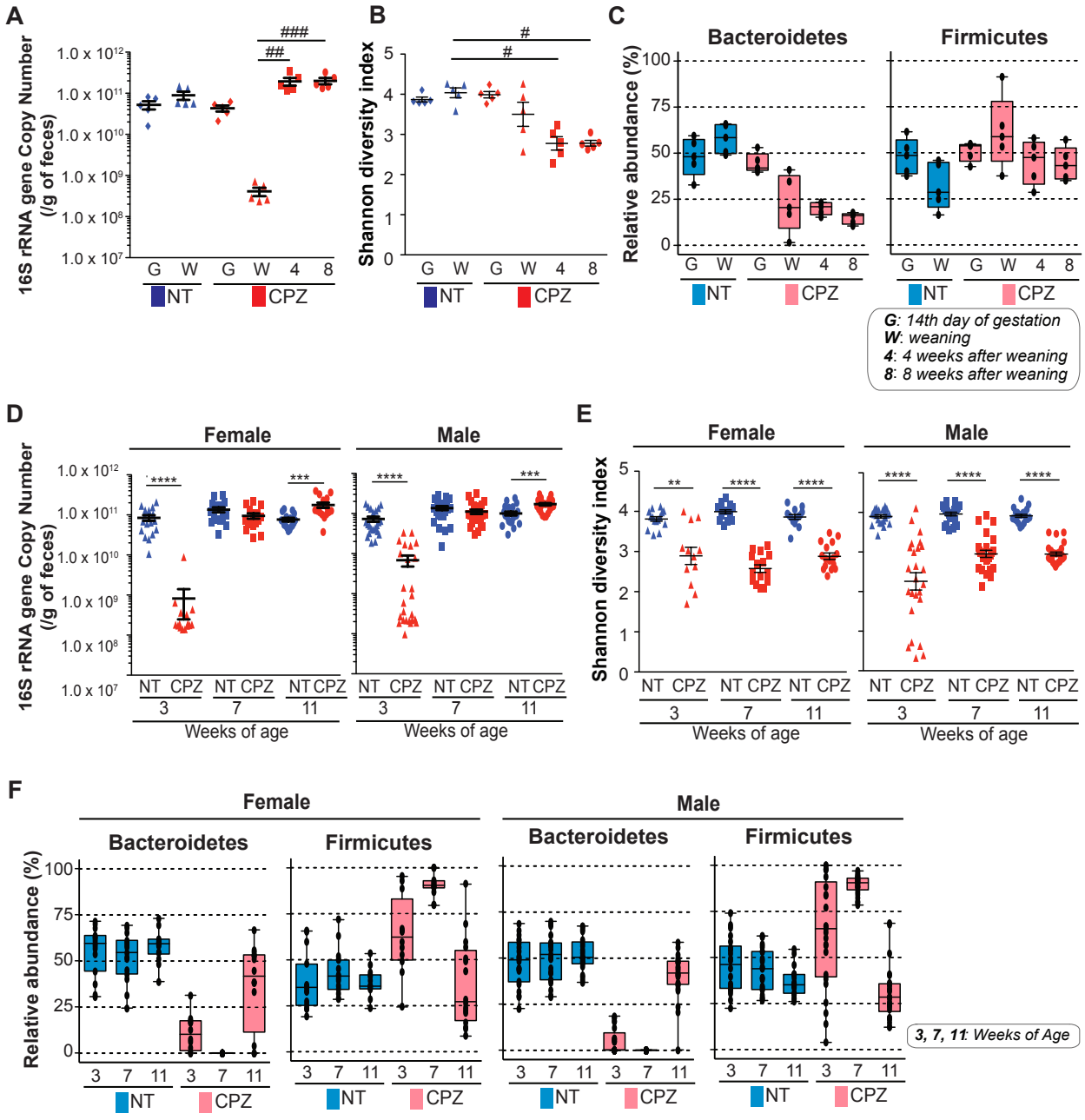
Examination of 16S ribosomal RNA (rRNA) gene copy number, as determined by qPCR using universal 16S primers showed a significant decrease in dams following CPZ exposure as compared to NT, which recovered by 4 and 8 weeks following CPZ cessation (Figure 3.3B). Despite recovery of 16S gene copy number, the microbial community structure of dams based on 16S rRNA gene amplicon sequencing revealed peripartum administration of CPZ caused persistent shifts in the microbiota (Figure 3.3B and Figure 3.3C). Shannon diversity index was decreased (Figure 3.3B) and microbial β-diversity (Figure 3.3C) did not recover, even after 8 weeks of CPZ cessation. At the taxonomic level of Phyla shown in Figure 3.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 cessation of CPZ exposure (Figure 3.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 with already maturated 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 3.S3B). Contrary to the 16S rRNA changes observed in IL-10 KO dams, no appreciable differences were observed in regards to relative abundance of Bacteroidetes in adult IL-10 KO of either gender following the cessation of CPZ, however, both Firmicutes and Verrucomicrobia appeared to be elevated (Figure 3.S3C). Together, this data suggests 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. 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 those in the NT group (p<0.01 at 3 weeks of age and p<0.0001 at 7 and 11 weeks in females; p<0.0001 at 3, 7 and 11 weeks of age in males) (Figure 3.3E). 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 NT and CPZ groups. However, each group exhibited community profiles similar to 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 (Figure 3.3F and Figure 3.S3D).

Figure 3.3: 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 14th day of gestation (start of CPZ treatment), at weaning (end of CPZ treatment), 4 weeks and 8 weeks after CPZ cessation. (B) Shannon diversity index in dams at 14th day of gestation (G), at weaning (W), 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 vs. 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 shown in Supplemental Figure 3.3. $^{\#}p$<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. Oligotype phyla taxa presented as relative abundance and represented as box plots. Dots represent individual samples. See also Figure 3.S3.

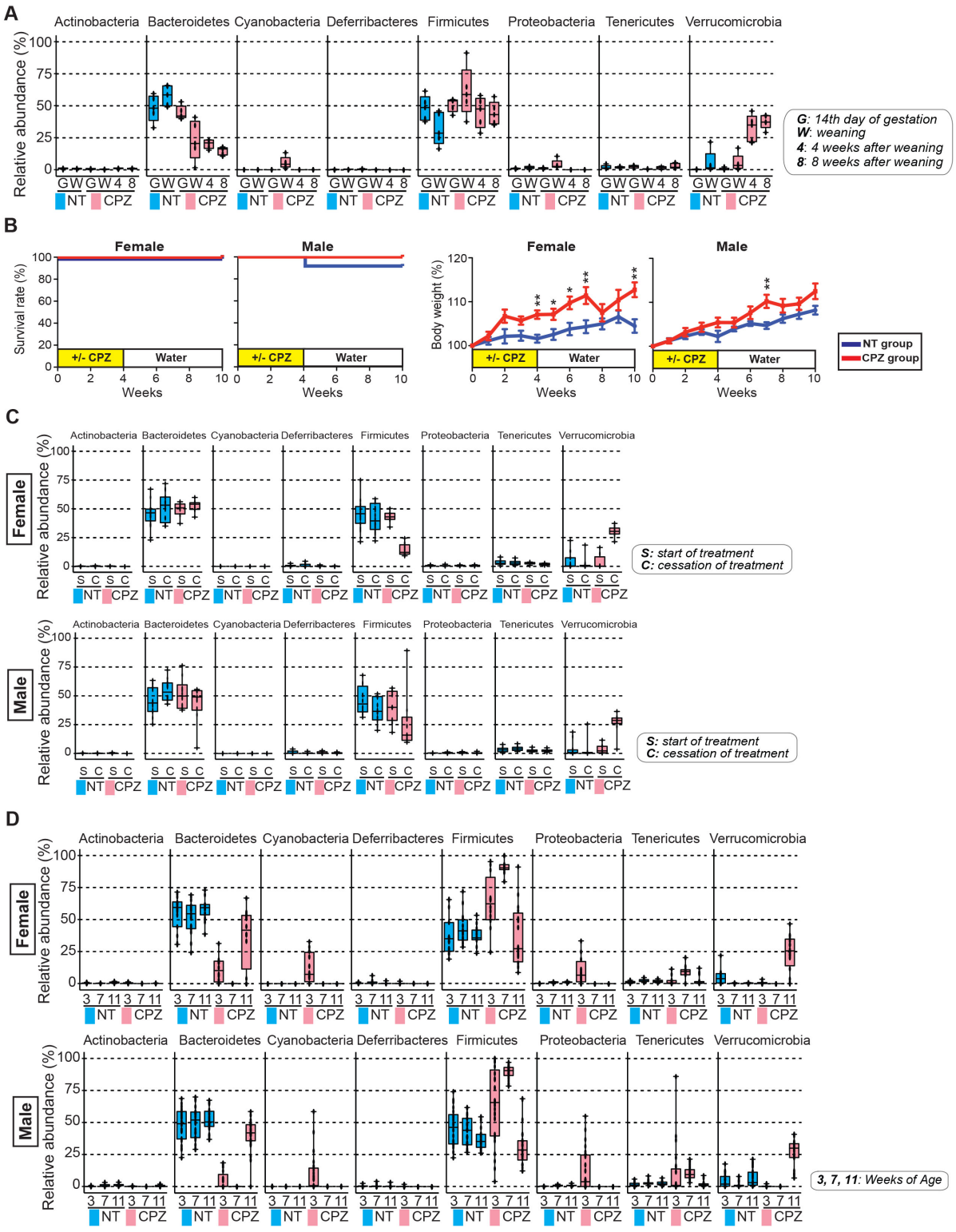
Figure 3.3, continued.



Supplementary Figure 3.S3: Maternal peripartum exposure to CPZ induces a persistent gut dysbiosis in dams and offspring, but CPZ-induced dysbiosis in adulthood does not lead to colitis outcome.

(A) The gut microbiota of IL-10 KO dams before and after CPZ treatment. Relative abundance (%) of oligotype phyla taxa in non-treated (NT; light blue bars) versus CPZ-exposed (CPZ; pink bars) dams at the 14th day of gestation (G; before treatment), weaning (W; at the end of treatment), 4 weeks and 8 weeks after CPZ cessation (4 and 8; 4 and 8 weeks after weaning). Dots represent individual samples. (B) Survival curves and weight changes in NT and CPZ-treated adult IL-10 KO mice (NT *n*=15 females, 12 males. CPZ *n*=8 females, 10 males). (C) Adult NT versus CPZ IL-10 KO female (top panel) and male (bottom panel) microbiota at the start (S, week 0) and the end (C, week 4) of treatment. Relative abundance (%) of oligotype phyla taxa in NT versus CPZ adult mice. (D) IL-10 KO female pup (top panel) and male pup (bottom panel) microbiota over time. Relative abundance (%) of oligotype phyla taxa of NT versus CPZ pups 3, 7, and 11 weeks of age. Dots represent individual samples.

Supplementary Figure 3.S3, continued.



3.3.4 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 3.4A, 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 oligoypes was observed at 4 or 8 weeks post-treatment (Figure 3.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 3.4A, top and bottom heatmap). 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, is playing 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 3.S4B). Together, this data provides 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 3.4A, bottom).

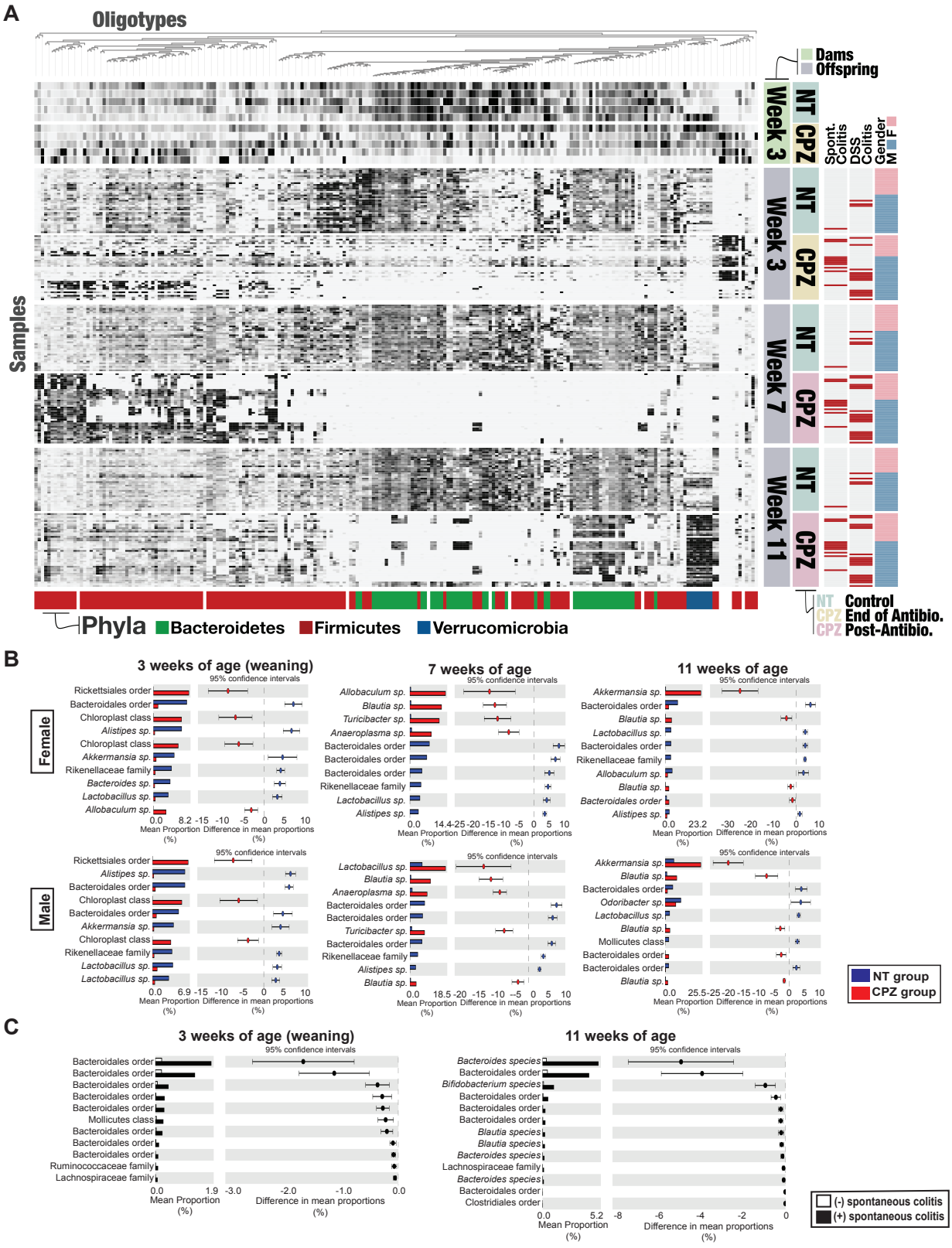
The changes in oligotypes in pups from CPZ-exposed dams persisted well beyond maternal influence, in that CPZ pups do not exhibit recovery at 7 or 11 weeks of age, while oligotypes in NT pups appear to retain some stability over time (Figure 3.4A, bottom heatmap). Pups from CPZ-exposed dams showed significant 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 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 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 3 time points are shown in Figure 3.4B. Specifically, at 3 weeks of age, CPZ males and females exhibited a larger proportion of oligotypes belonging to Rickettsiales order from the phyla Proteobacteria, as compared to NT controls. At 7 weeks of age both male and females from CPZ-exposed dams exhibited a significant increase in specific *Allobaculum* sp., *Blautia* sp., *Turicibacter* sp., *Lactobacillus* sp., and *Anaeroplasma* sp. oligotypes, respectively. 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 3.4C and include bacteria belonging to the order Bacteroidales at both 3 and 11 weeks of age.

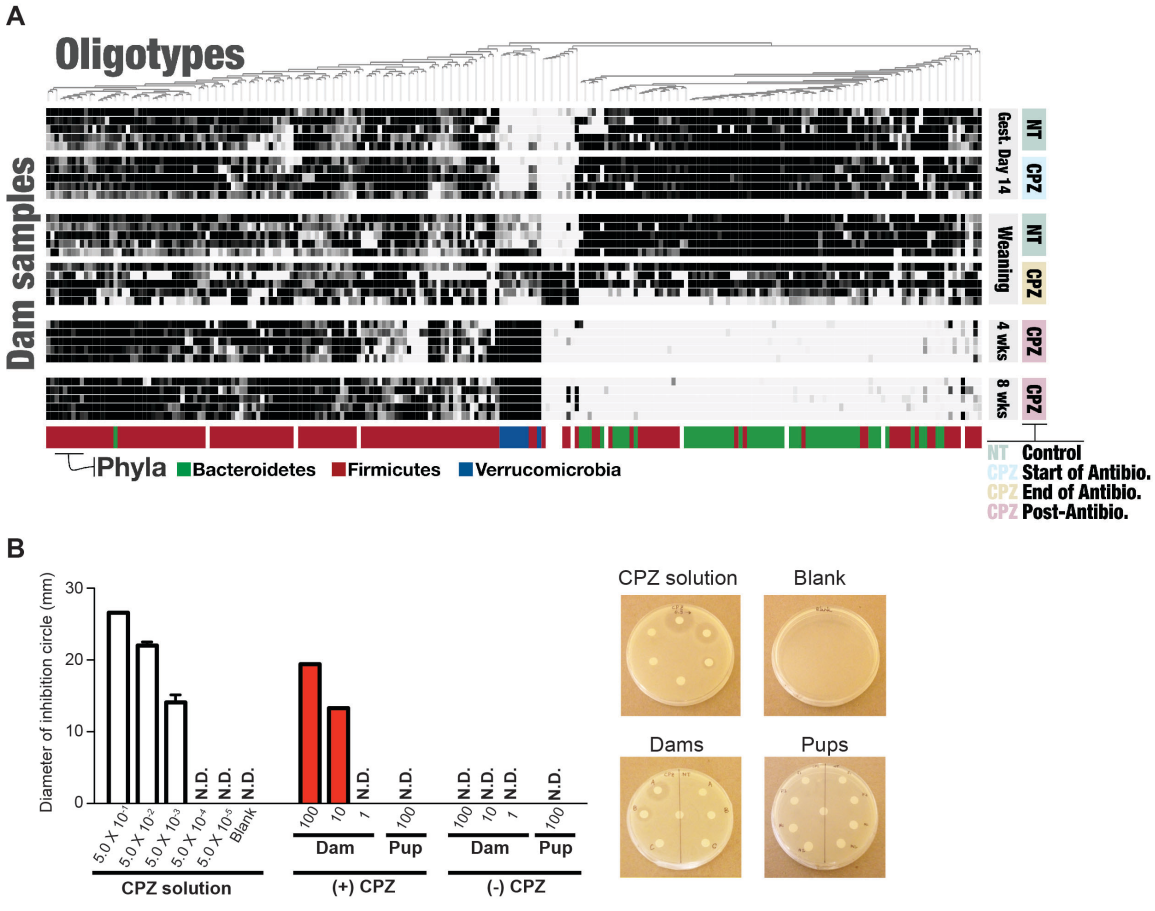
Figure 3.4: Maternal peripartum exposure to cefoperazone induces persistent and significant changes in specific microbial oligotypes in IL-10 KO dams and offspring.
(A) Comprehensive comparison of gut microbes in fecal samples collected from non-treatment (NT) and cefoperazone (CPZ)-treated IL-10 KO 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 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 determined by STAMP. The top 10 oligotypes with the greatest changes in relative abundance are presented as extended error bar plots (bars represent 95% confidence intervals). (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 3.S4.

Figure 3.4, continued.



Supplementary Figure 3.S4: Dams exposed to peripartum CPZ exhibit persistent changes in specific microbial oligotypes with minimal to no CPZ transfer between dams and pups during the peripartum CPZ exposure period.

(A) Comprehensive comparison of gut microbes in fecal samples of IL-10 KO dams collected over time. Oligotypes were determined in samples obtained from non-treated (NT) and cefoperazone (CPZ)-exposed dams at the 14th day of gestation (Gest. D. 14; just before the start of antibiotic exposure) and weaning (Weaning; end of antibiotic exposure), as well as 4 and 8 weeks (4, 8 weeks; post-antibiotic exposure) after CPZ cessation in CPZ-exposed dams. Each column presents an individual oligotype and each row represents an individual sample. Dominant phyla are represented by the colored bars at the bottom. (B) Antibiotic sensitivity patterns of E. *coli* K12 to CPZ solution, cecal contents from NT and CPZ-exposed dams, and cecal contents from their respective pups at weaning [(-) CPZ: NT dams and their pups, (+) CPZ: CPZ dams and their pups]. For examining E. *coli* sensitivity to CPZ water solution, six paper discs were impregnated with 5.0 x 10^{-1} (concentration in dams' drinking water) 5.0 x 10^{-2}, 5.0 x 10^{-3}, 5.0 x 10^{-4}, 5.0 x 10^{-5} mg/ml of CPZ and water (blank), respectively. For examining E. *coli* sensitivity to dam intestinal contents, paper discs were impregnated with sterile-filtered cecal supernatant (100 mg/1 ml of water) obtained and diluted 10-fold sequentially (100, 10 and 1) from NT and CPZ dams. For examining E. *coli* sensitivity to pup intestinal contents, testing pup samples, paper discs were impregnated with sterile-filtered cecal supernatant (100mg/1 ml of water) from NT and CPZ pups. Representative pictures of plates are shown. Plates with blank LB broth were prepared to rule out the possibility of contamination (plate labeled Blank). Data are represented as mean ± SEM for CPZ solution plates.



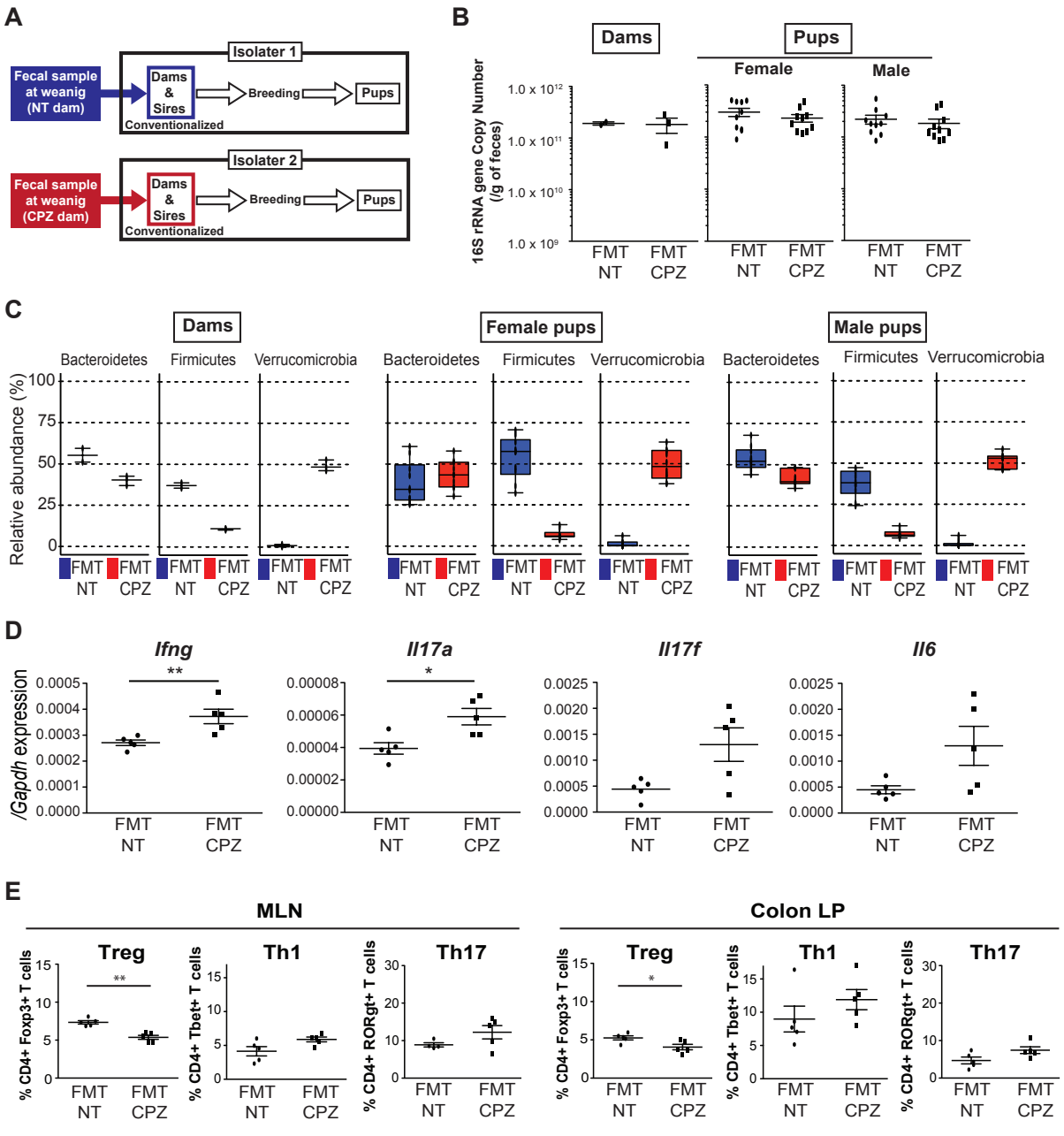
3.3.5 Fecal microbiota transplant of maternal CPZ-induced dysbiosis into germ-free IL-10 KO dams elicits a pro-inflammatory milieu in offspring

We next wanted to examine whether CPZ-induced dysbiosis observed in dams would transfer an inflammatory phenotype in the absence of CPZ exposure. Here, we performed fecal microbiota transplantation (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 3.5A). 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 number as measured by qPCR in recipients regardless of donor treatment (Figure 3.5B). 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 3.5C). Slight but insignificant changes in Bacteroidetes were observed only in dams and male FMT-CPZ pups (Figure 3.5C). Significant changes were not observed in other Phyla between FMT-NT and FMT-CPZ dams and pups (Figures 3.S5A and 3.S5B). At 3 weeks of age, we examined similar immune parameters in FMT pups as shown in SPF IL-10 pups from NT or CPZ-exposed dams in Figure 3.2. Male FMT-CPZ pups exhibited significantly increased expression of *Ifng* and *Il17a* in MLNs relative to FMT-NT, and increased but not significant trends of *Il17f* and *Il6* (Figure 3.5D). Similar observations were also seen in female FMT-CPZ 3-week-old pups (Figure 3.S5C). We next examined markers of T cell populations in FMT-NT or 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 RORγt in the MLN and colonic LP, focusing on $CD4^+$ T cell populations (Figure 3.S2E). 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 3.5B). 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 3.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 3.1A. Analysis of the proportion of T cells expressing Foxp3, T-bet, or RORγt showed that they were not significantly altered by maternal exposure to CPZ under germ-free conditions (Figure 3.S5E), suggesting that exposure to maternal dysbiosis induced by CPZ under SPF conditions in early life served as the main driver of changes.

Figure 3.5: Conventionalization of germ-free IL-10 KO mice with cefoperazone-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: 2 FMT-NT dams, isolator 2: 3 FMT-CPZ dams). Offspring from FMT dams in each isolator were analyzed at weaning (n=10/gender/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 supplemental Figure 3.5. (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 expressed as ΔΔCT relative to housekeeper gene *Gapdh*. (E) Flow cytometric analyses of live $CD45^+TCR\beta^+CD4^+$ T cells expressing $Foxp3^+$ (Treg), $T\text{-}bet^+$ (Th1) or $ROR\gamma t^+$ (Th17) in MLNs and colonic LPs of 3-week-old FMT-NT versus FMT-CPZ male offspring (n=4-5/group). Data represent percentage of live $CD4^+$ cells. *$p<0.05$, **$p<0.01$ via Mann-Whitney U-test. See also Figure 3.S5.

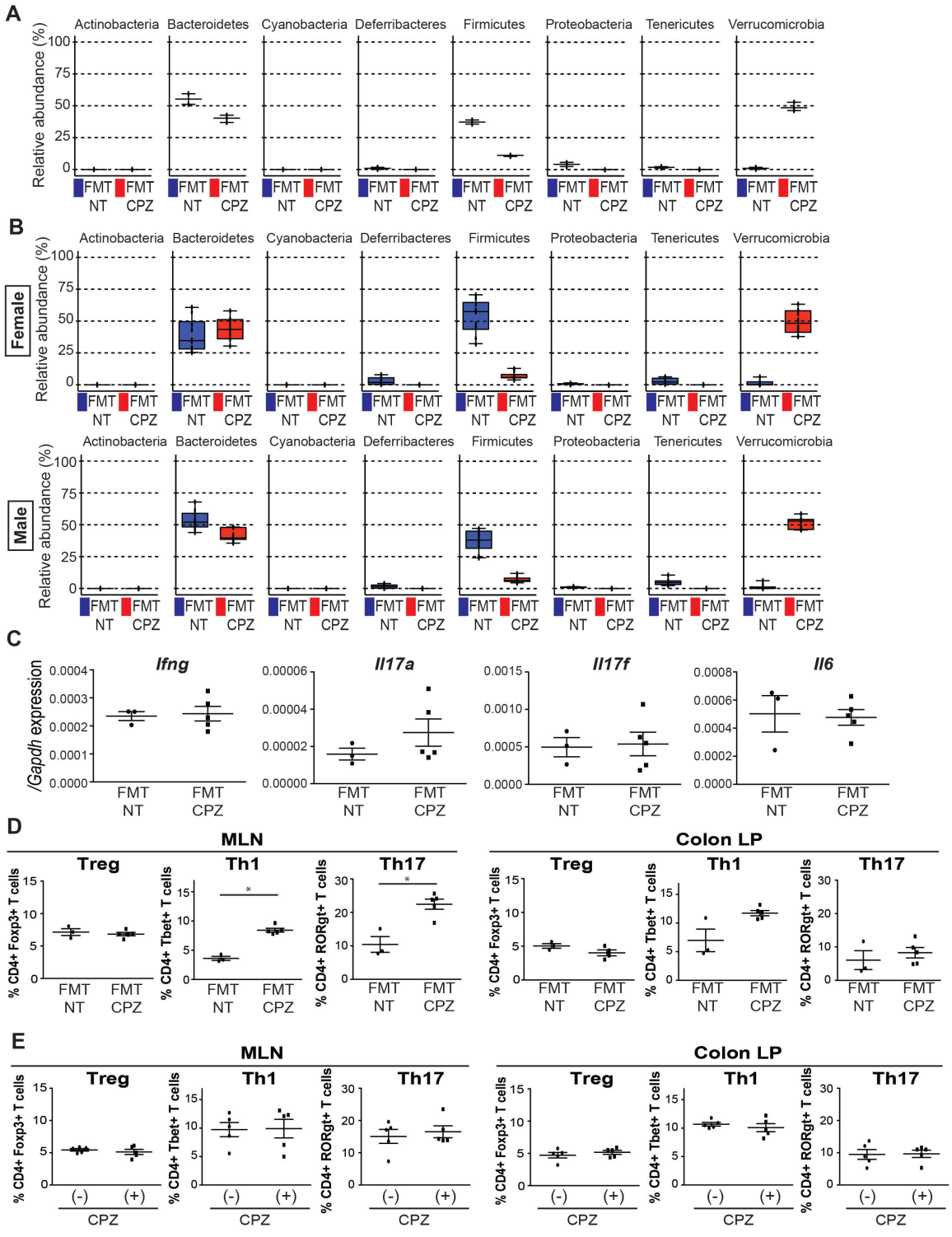
Figure 3.5, continued.



Supplementary Figure 3.S5: Fecal transplantation of CPZ-induced maternal dysbiosis recapitulates changes in the host immune system of offspring from FMT-dams.

(A) Bacterial community composition of fecal-microbiota-transplanted (FMT) IL-10 KO dams. (B) IL-10 KO female pups (top panel) and male pups (bottom panel) microbiota at 3 weeks of age from FMT-NT (blue bars) versus FMT-CPZ (red bars) dams. Oligotype phyla taxa are presented as relative abundance and represented as box plots. Dots represent individual samples. (C) Real-Time qPCR mRNA levels of cytokines from colonic mucosal scrapings of FMT-NT (black circles) versus FMT-CPZ (black squares) female IL-10 KO mice at 3 weeks of age (*n*=3-5/group). mRNA levels are expressed as ΔΔCT relative to the housekeeper gene *Gapdh*. (D) Flow cytometric analyses of live $CD45^+TCR\beta^+CD4^+$ T cells expressing $Foxp3^+$ (Treg), $T\text{-}bet^+$ (Th1) or $ROR\gamma t^+$ (Th17) in MLNs and colonic LPs of FMT-NT versus FMT-CPZ female offspring. (E) Flow cytometric analyses of live $CD45^+TCR\beta^+CD4^+$ T cells expressing $Foxp3^+$ (Treg), $T\text{-}bet^+$ (Th1) or $ROR\gamma t^+$ (Th17) in MLNs and colonic LPs of germ-free male pups with/without maternal peripartum exposure to CPZ (*n*=5/group). Data represents percentage of live $CD4^+$ cells.

Figure 3.S5, continued.



3.4 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 (Hviid, Svanstrom and Frisch, 2011; Kronman *et al.*, 2012; Virta *et al.*, 2012; Gevers *et al.*, 2014; Ungaro *et al.*, 2014). Using a genetic risk model, the IL-10 KO mouse, and employing a prospective study design that rigorously controls for starting gut microbiomes and generational drift, we were able to observe the impact of cefoperozone (CPZ), a broad-spectrum antibiotic, on maternal and offspring gut microbiota during and after antibiotic exposure. Our data indicates 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 pregnancy 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 (Figure 3.3A) 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 3.S3B). However, these changes did not occur to the same extreme as 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 3.4, 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 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, Verrucomicrobia) developed stable networks that limited the ability of Bacteroidetes to establish (Figure 3.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 3.4). Furthermore, FMT of maternal CPZ-induced microbial communities into GF IL-10 KO mice resulted in immune skewing in their offspring that was consistent with immune profiles observed in SPF IL-10 KO pups exposed to maternal CPZ-induced dysbiosis. These findings show that maternal CPZ-induced dysbiosis alone in the absence of direct exposure to CPZ can have a negative impact on immunological outcomes and possibly further predispose genetically-susceptible offspring to developing 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 3.2). 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 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 3.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 RORyt 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 relative increased risk for “spontaneous” colitis, as was observed (Figure 3.1). 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 as 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 (Sakata *et al.*, 2001; Howarth *et al.*, 2002). 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 involves 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 persist 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.

3.5 Experimental Procedures

3.5.1 Animals

Germ-free IL-10 KO mice on a C57Bl/6J genetic background were bred in the University of Chicago Gnotobiotic Research Animal Facility (GRAF) and fecal microbiota transplanted (conventionalized) with *Helicobacter hepaticus*-free microbiota (kindly provided by Dr. Cathryn Nagler, University of Chicago) as previously described (Gillilland 3rd *et al.*, 2012). Following conventionalization, these SPF IL-10 KO breeding pairs were bred in-house under *H. hepaticus*-free conditions (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, 10 breeding pairs were prepared to obtain two parturitions for no treatment (NT) and CPZ treatment groups (Figure 3.1A). In cohort 1, the 1st and 2nd litters were used as NT controls or the CPZ group,

respectively. In cohort 2, the 1st and 2nd litters were used as NT controls. Wild-type C57Bl/6J mice underwent the same breeding, microbial normalization, and CPZ treatment protocol for comparison.

3.5.2 Antibiotic treatment

CPZ sodium salt was purchased from Sigma-Aldrich (St. Louis, MO). CPZ (0.5 mg/ml) was administered in drinking water beginning at the 3rd week of gestation until weaning of pups (Figure 3.S1A). Mice had free access to water throughout the treatment period (IACUC protocol 72101). The same dose of CPZ was administered to adult IL-10 KO mice between 12 to 20 (18.9 ±2.9) weeks of age for 4 weeks.

3.5.3 Clinical evaluation of spontaneous colitis

Body weights of mice in cohort 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).

3.5.4 DSS-induced colitis

Mice were given 2.5% DSS (36-50 kDa) (MP Biomedicals, Santa Ana, CA) 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.

3.5.5 Fecal microbiota transplantation

Fecal samples collected from NT and CPZ-exposed dams at weaning were used for fecal transplantation (FMT) via gavage of 200uL of fecal supernatant (100mg feces per 1mL sterile PBS) into GF IL-10 KO female recipients maintained in separate flexible film isolators (See model, Figure 3.5A). 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.

3.5.6 Histological analysis

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

3.5.6 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 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, Illinois. Raw sequencing data were de-multiplexed, and partially overlapping paired-end reads were merged using illumina-utils (Eren, Vineis, *et al.*, 2013). 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 three or less mismatches at the overlapping region, and (2) 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 Minimum Entropy Decomposition (MED) (Eren, Morrison, *et al.*, 2015) with default parameters. Using Shannon entropy, MED resolves a given amplicon dataset iteratively into high-resolution oligotypes (Eren, Maignien, *et al.*, 2013). Taxonomy was assigned to oligotypes using GAST (Huse *et al.*, 2008), and Anvi'o v2.3.1 (Eren, Esen, *et al.*, 2015) 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 female and male 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, then further filtered for the

top ten oligotypes exhibiting the greatest difference in mean relative abundance. Extended error plots were created using Statistical analysis of taxonomic and functional profiles (STAMP) v2.1.3 (Parks *et al.*, 2014).

3.5.7 Statistical analyses

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. 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 NT and CPZ groups. Kruskal-Wallis test and Dunn's test were also employed for 16s rRNA gene copy number. These tests were performed with GraphPad Prism (GraphPad Software, CA, USA). Statistical significance was assumed when $p \leq 0.05$. Raw counts of oligotypes were normalized using the "decostand" function implemented in R/CRAN package "vegan". Alpha diversity via Shannon diversity index was computed using "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 versus CPZ-exposed dams and between male pups from CPZ-exposed dams that eventually developed spontaneous colitis versus male pups that remained healthy. The *p*-values were adjusted for multiple-test using Benjamini-Hochberg method (Benjamini and Hochberg, 1995). The criterion of significance was set at false discovery rate (FDR)<0.05.

3.5.8 Antibiotic sensitivity assay

Kirby Bauer disk diffusion assays were performed with E. *coli* K12 lawns grown on LB agar plates with slight modifications (Bauer *et al.*, 1966; Chait *et al.*, 2010). To test whether the presence of CPZ in intestinal contents would inhibit *E. coli* growth, lawns were incubated overnight with circular discs impregnated with either: (1) CPZ solution starting at the treatment concentration given to pregnant dams (0.5 mg/ml; diluted 10-fold 4x); (2) cecal contents from

NT or CPZ-exposed dams (sterile-filtered (0.22μM) supernatant of 100 mg of cecal content/1 ml of water; diluted 1, 10, 100-fold), and (3) cecal contents of offspring from NT or CPZ-exposed dams immediately prior to weaning (sterile-filtered (0.22μM) supernatant of 100 mg of cecal content/1 ml of water). The diameter of the zone of inhibition circle (mm) was measured as an indicator of the level sensitivity of E. coli to the presence of CPZ.

3.5.9 Flow cytometry

MLN and colon LP were harvested for analyzing T cell populations with/without CPZ treatment using previously described methods (Davies and Parrott, 1981; Little *et al.*, 2005). FcR blocking was performed for all samples with anti-mouse CD16/CD32 antibody (BD Biosciences, CA, San Jose). Cells were stained using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, MA, Waltham) to assess viability. For surface stains, anti-mouse CD45 (Biolegend, San Diego, CA), anti-mouse TCRβ (eBioscience, San Diego, CA), and anti-mouse CD4 antibodies (Biolegend) were used. For intranuclear stains, the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used followed by staining with the anti-mouse Foxp3 (eBioscience), anti-mouse T-bet (eBioscience) and anti-RORγt antibodies (eBioscience). The Rat IgG2a Kappa Isotype, Mouse IgG1 Kappa Isotype, and Rat IgG1 Kappa Isotype controls (eBioscience) were used. Samples were analyzed with a FACSCanto (BD Biosciences) and FlowJo v10.1 (FLOWJO, OR, Ashland).

3.5.10 Real-Time qPCR

Messenger RNA was extracted from total colonic mucosal scrapings with TRIzol Reagent (Thermo Fischer Scientific). Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Corporation, IN, Indianapolis) was used to obtain cDNA samples. Real-time qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, CA, Hercules) with LightCycler 480II (Roche Diagnostics Corporation). The primers were designed based on the Primer Bank reference base (https://pga.mgh.harvard.edu/primerbank/). Forward (F) and

reverse (R) primer sequences are as follows: Tgfb1, F-CTCCCGTGGCTTCTAGTGC; R-GCCTTAGTTTGGACAGGATCTG; Ifng, F-ATGAACGCTACACACTGCATC; R-CCATCCTTTTGCCAGTTCCTC; Il12p35, F-CTGTGCCTTGGTAGCATCTATG; R-GCAGAGTCTCGCCATTATGATTC; Il12p40, F-TGGTTTGCCATCGTTTTGCTG; R-ACAGGTGAGGTTCACTGTTTCT; Il4, F-GGTCTCAACCCCCAGCTAGT; R-GCCGATGATCTCTCTCAAGTGAT; Il13, F-CCTGGCTCTTGCTTGCCTT; R-GGTCTTGTGTGATGTTGCTCA; Il17a, F-GGCCCTCAGACTACCTCAAC; R-TCTCGACCCTGAAAGTGAAGG; Il17f, F-TGCTACTGTTGATGTTGGGAC; R-AATGCCCTGGTTTTGGTTGAA; Il23, F-ATGCTGGATTGCAGAGCAGTA; R-ACGGGGCACATTATTTTTAGTCT; Il1b, F-GCAACTGTTCCTGAACTCAACT; R-ATCTTTTGGGGTCCGTCAACT; Il6, F-TAGTCCTTCCTACCCCAATTTCC; R-TTGGTCCTTAGCCACTCCTTC; Tnf, F-CCCTCACACTCAGATCATCTTCT; R-GCTACGACGTGGGCTACAG; Muc2, F-ATGCCCACCTCCTCAAAGAC; R-GTAGTTTCCGTTGGAACAGTGAA; Reg3g, F-ATGCTTCCCCGTATAACCATCA; R-GGCCATATCTGCATCATACCAG; Gapdh, F-AGGTCGGTGTGAACGGATTTG; R-TGTAGACCATGTAGTTGAGGTCA.

3.5.11 ELISA

Frozen samples were resuspended in 1 ml of PBS per 100mg of feces or MLN with cOmplete, Mini Protease Inhibitor Tablets (Sigma-Aldrich). Mouse Lipocalin-2/NGAL DuoSet ELISA (R&D Systems, MN, Minneapolis) was used for fecal samples. Mouse ELISA Ready-SET-Go! for IFN-γ, IL-17 and IL-6 (eBioscience) was used for MLN samples. To analyze plasma IgE level, we performed ELISA using goat anti-mouse IgE antibody (capture antibody) and goat anti-mouse IgE antibody labeled with HRP (secondary antibody) (SouthernBiotech, Birmingham, AL).

3.6 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.

3.7 Database

Mouse sample information and microbial dataset can be found via BioProject ID PRJNA376026 and Sequence Read Archive accession **SRP108147.**

3.8 Acknowledgements

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3.9 Conflict of Interests

The authors declare no conflict of interests.

Chapter 4: Conclusions

Within the last half-century, the scientific community has propelled our understanding of host-microbe interactions underlying health and disease. In particular, the ability to sequence and identify complex microbial communities, particularly those living in the gut, and determine host-microbe signaling mechanisms, has excited research focused on the role of microbial ecosystems for human health. These microbial communities essentially form a pseudo organ within the gut, which plays a key role in educating the host immune system and maintaining health (Lathrop *et al.*, 2011; Littman and Pamer, 2011; Huttenhower *et al.*, 2012; Lee *et al.*, 2014). Disruptions to gut microbial communities, or dysbiosis, can have adverse effects on host metabolism and immune function, contributing to the development of complex immune disorders such as Type 1 diabetes (T1D) and inflammatory bowel diseases (IBD), particularly in genetically susceptible individuals (Manichanh *et al.*, 2012; Gulden, Wong and Wen, 2015). Within this thesis, I demonstrate the role of gut microbiota as modifiers of disease pathogenesis by utilizing the interleukin-10 deficient (IL-10 KO) mouse model. This model reflects two facets of complex immune disorders, T1D and IBD, under specific microbial conditions. I present three scenarios in which gut microbiota modify disease pathology in the pancreas or colon of IL-10 KO mice (Figure 4.1).

The first scenario presents the role of gut microbiota in one facet of complex immune disorders, T1D. Here, the absence of gut microbiota is associated with increased pancreatic infiltration in older germ-free (GF) IL-10 KO mice that resembles the pathology of T1D (Figures 2.1 and 2.3). Metabolic and immunological characterization of the model reveals a subset of these mice develop insulin autoantibodies, a hallmark of human T1D, and the majority of mice maintain blood glucose regulation (Table 2 and 3). The recent restaging of T1D pathogenesis into three progressive stages of disease positions our model at the first presymptomatic stage of T1D in which patients with underlying islet autoimmunity are positive for autoantibodies but

maintain normal glycemic levels (Insel *et al.*, 2015). The absence of gut microbial signals and IL-10 may set the stage for the slow progression of disease in these mice; however as with the complexity of human disease, additional external triggers are likely needed for disease to progress to the symptomatic stage of disease.

The second scenario presents the role of gut microbiota in a different facet of complex immune disorders, IBD (Figure 4.1). Here, the presence of specific gut microbial communities protects against pancreatic infiltration (Figure 2.3); but leads to the development of spontaneous colitis in approximately 20-30% of our colony. The diversity and variation in gut microbiota, along with epigenetic factors and other environmental triggers introduced over time may account for differences in IBD incidence observed within our colony. In particular, the presence of specific gut bacteria considered pathobionts, such as *Helicobacter hepaticus* (*H. hepaticus*), drastically increases the incidence of colitis up to 100% (Kullberg *et al.*, 1998; Yang *et al.*, 2013). Interestingly, fecal microbiota transplantation (FMT) of GF IL-10 KO mice with *H. hepaticus*-free microbiota protected mice from the development of pancreatic infiltration; however, IL-10 KO mice given FMT at an older age, developed more rapid and more severe colitis (Figure 2.5). Thus, older FMT IL-10 KO mice may not be as able to cope with microbial stimuli introduced later in life because they lack proper immune education from an early age, which is important for establishing immunological tolerance (Gensollen *et al.*, 2016; Tamburini *et al.*, 2016).

We tested the importance of early life microbial exposure in the absence of *H. hepaticus* for immunological development and colitis risk in IL-10 KO mice in the third scenario (Figure 4.1). By treating pregnant IL-10 KO dams with a broad-spectrum antibiotic, cefoperazone (CPZ), and tracking their offspring for the development of IBD, we determined vertical transmission of an antibiotic-induced dysbiotic maternal microbiota early in life perturbs the immune system and increases colitis risk in IL-10 KO offspring (Figure 4.2). IL-10 KO offspring from CPZ-exposed dams were more susceptible to developing spontaneous colitis compared to

offspring from non-treated (NT) dams. Adult IL-10 KO offspring from CPZ-exposed dams that did not develop frank colitis were also more susceptible to chemically-induced colitis challenge (Figure 3.1), which suggests the absence of specific microbial-mediated signaling early in life has lasting effects on the immune system of offspring. IL-10 KO mice from CPZ-exposed dams exhibited perturbed immunological profiles, with an increase in proinflammatory markers at 3 weeks of age (Figure 3.2), and persistent decreases in the diversity of gut bacterial communities well into adulthood (Figure 3.3). Interestingly, adult IL-10 KO mice treated with CPZ did not develop colitis and did not exhibit significant changes to immunological and microbial profiles in adulthood (Figure 3.S3). This suggests the adult microbiome and immune system are more resilient to antibiotics. Moreover, we determined minimal to no CPZ itself was transferred from dams to offspring (Figure 3.S4) and the bacterial oligotypes present in offspring primarily derived from their mother's microbiota (Figure 3.4). Thus, the acquisition of a dysbiotic maternal microbiota early in life has long lasting consequences on the immune system and gut microbial communities of genetically susceptible offspring, which contribute to loss of tolerance and colitis development later in life.

Further investigations of microbial-mediated host responses in the IL-10 KO mice are required to understand the mechanisms underlying the role of gut microbiota as modifiers of disease pathogenesis. In particular, determining the specific cross-talk between host cells in the gut, such as epithelia, enteroendocrine, and specialized immune cells, with gut microbiota would improve our understanding of how gut microbial modulation could positively impact human health. In the first scenario, further investigations focused on metabolic and immunological pathways in GF IL-10 KO mice are needed to determine how the absence of specific gut microbial signals, loss of IL-10, and immune-mediated pathways result in presymptomatic T1D. The halt of disease at a presymptomatic stage suggest protective signaling pathways may be at play and additional environmental factors are required to trigger further immunological and metabolic dysfunction. Viral infections, particularly in the gut, are one plausible trigger

underlying human T1D and IBD (Ohashi *et al.*, 1991; Cadwell *et al.*, 2010; Yeung, Rawlinson and Craig, 2011; Perez-Brocal *et al.*, 2013; Krogvold *et al.*, 2015; Rodriguez-Calvo and von Herrath, 2015). It would be interesting to first confirm whether the stool and organs, particularly the pancreas, of GF IL-10 KO mice are positive for traces of viruses. For example, detection methods, including Real-Time Polymerase Chain Reaction (RT-PCR) and immunostaining, could be used to determine the presence of viruses, such as enteroviral capsid protein vp1, in stored tissue samples from IL-10 KO mice (Richardson *et al.*, 2009). GF mice could also be challenged with enterovirus infection to determine whether infection renders GF IL-10 KO more susceptible or protected against T1D or IBD development.

Moreover, a significant portion of scientific literature, including the work presented in this thesis, primarily addresses bacterial communities, although it is acknowledged that other microbes, such as viruses and fungi, play a key role in the pathogenesis of disease as well. While we've developed a deeper understanding of gut bacterial communities, far less is known about the host's relationship with the mycobiome, virome, and bacteriophagome. By taking a well-controlled, targeted approach to rigorously investigate the role of gut microbiota on host immunological development and IBD risk, we were able to perform a comprehensive analysis of bacterial oligotypes over time in IL-10 KO mice from CPZ-exposed or NT dams. This study demonstrates the importance of early life microbial assemblage and immune education for disease risk in genetically susceptible hosts. Further microbial analyses, including metagenomics, transcriptomic, and metabolomic analyses, would provide greater insight on the functional potential of bacteria that may confer susceptibility or protection against disease. Additionally, antibiotic-induced perturbations to bacterial communities may trigger opportunistic fungi to respond to changes in their surrounding environment and induce immune responses involved in IBD pathogenesis (Ott *et al.*, 2008; Iliev *et al.*, 2012; Li *et al.*, 2014; Chehoud *et al.*, 2015). Future microbial analyses should expand to include the role of the mycobiome in disease risk.

Concomitant investigations of host immune-signaling pathways would complement our understanding of microbe-mediated immune signaling pathways underlying IBD risk or protection. While the role of early life exposure to dysbiotic microbiota on T1D risk was not examined in this study, it is possible peripartum CPZ exposure and maternal gut dysbiosis impacts pancreatic histology and host metabolism. Thus, pancreata were collected at various ages from CPZ and NT IL-10 KO offspring and could be examined to assess islet architecture and the presence/absence of pancreatic inflammation. Although further mechanistic information is needed to contribute to the development of effective treatments or prevention strategies that are translatable to humans, our findings in the third scenario prompt clinicians to reconsider the indiscriminate use of antibiotics during infancy, especially in genetically susceptible individuals.

In summary, I demonstrate the role of gut microbiota as modifiers of disease pathogenesis by utilizing the genetically susceptible interleukin-10 deficient (IL-10 KO) mouse model. This model reflects two facets of complex immune disorders, T1D and IBD, under specific microbial conditions. I present three scenarios in which gut microbiota modify disease manifestation in the pancreas or colon of IL-10 KO mice (Figure 4.1). In the first scenario, the absence of microbes is associated with increase pancreatic infiltration in older germ-free (GF) IL-10 KO mice. In the second scenario, the presence of gut microbiota protects SPF IL-10 KO mice from developing pancreatic infiltration but instead these mice develop spontaneous colitis. In the third scenario, vertical transmission of a dysbiotic maternal microbiota early in life contributes to loss of tolerance and increases the incidence of disease in the colon of SPF IL-10 KO offspring. Further investigations are needed to dissect the nature of host-microbe, as well as microbe-microbe interactions, in modifying disease processes. These are exciting times for host-microbe research and complex immune disorders such as T1D and IBD. As our understanding increases with more mechanistic studies and advancement of microbial analyses in animal models and human clinical trials, the advent of effective microbial-mediated prophylactic therapies for T1D and IBD may be in site.

Figure 4.1: Three scenarios in which the (1) absence, (2) presence, or (3) disruption of gut microbiota modify disease risk in the IL-10 KO mouse model.

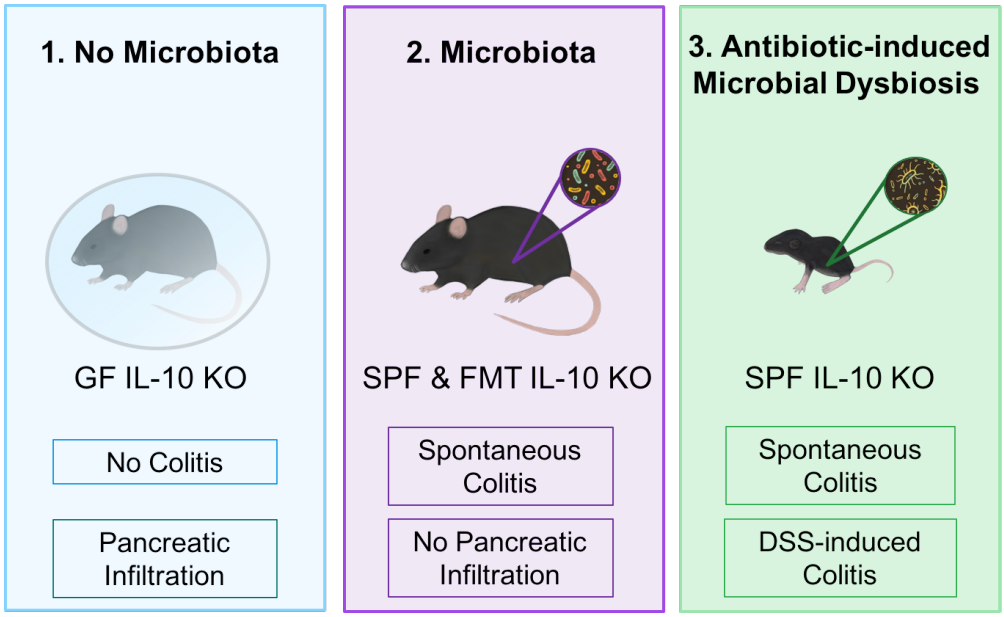
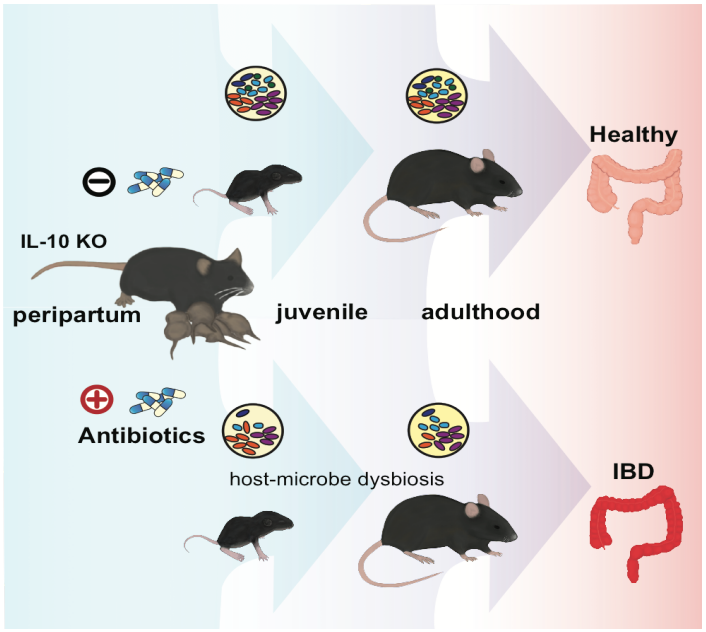


Figure 4.2: Peripartum antibiotic-induced maternal dysbiosis has long lasting effects on microbial assemblage and host immunity and increases IBD risk in IL-10 KO offspring.



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