EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN DIFFERENTIALLY IMPACTS KEY MEMBERS OF MICE GUT MICROBIOME

By

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ABSTRACT

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Gut microbial populations influence immune homeostasis and are associated with a number of diseases including obesity, diabetes, inflammation and inflammatory bowel disease. A review of the current literature reveals that both gut microbiota and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) influence immune responses and host diseases. Clostridia species, Bacteroides and segmented filamentous bacteria (SFB) are important members of the intestinal microbial population that participate towards initiating immune responses and maintaining immune homeostasis. Exposure to the environmental contaminant, TCDD is associated with disruption of immune balance and immunosuppression in the host. Although several studies have pinpointed the effect of both TCDD and gut microbiota separately on host health and diseases, little or no research has been done to study their interaction with each other. Knowledge of the impact of TCDD exposure on key intestinal microbial members is important to understand their role in immune regulation and metabolic disorders. The aim of this study is to investigate the changes in abundance of specific gut microbial populations including SFB, Bacteroides, *Clostridium* cluster IV, *Clostridium* cluster XIVa, *Lactobacillus* and their functional genes with exposure to TCDD. Fecal samples taken from mice exposed to varying doses of TCDD over 12, 30 and 90-day time periods were analyzed using qPCR assays targeting these key microorganisms or their functional groups. The abundance of both 16S rRNA gene and flagellin gene of intestinal SFB increases with exposure to TCDD in a dose-dependent manner, with up to 10-fold increase in SFB observed in mice exposed to 1-30 µg/kg TCDD. A relative decrease in abundance of members

of *Clostridium* IV and XIVa, and Bacteroides is found in TCDD-dosed mice groups in the short term high dose study. This work determined that TCDD exposure influences changes in abundance within key members of the intestinal microbial population, retarding healthy commensalism and thereby allowing overgrowth of pathobionts. Therefore, specific members of the gut microbial population may influence host immunity and metabolism in hosts affected by environmental toxicants such as TCDD. This work highlights that TCDD is responsible for microbial dysbiosis in the mice intestinal tract; this dysbiosis might be partially responsible for TCDD-related diseases including diabetes, inflammatory bowel disease, and autoimmune diseases observed in humans.

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KEY TO ABBREVIATIONS

TCDD - 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

SFB - segmented filamentous bacteria

qPCR – quantitative polymerase chain reaction

rRNA – ribosomal RNA

DNA – deoxyribonucleic acid

Treg – regulatory T cells

Th17 – T helper 17 cells

AhR – aryl hydrocarbon receptor

GF – germ-free

CHAPTER 1: INTRODUCTION

1.1. ROLE OF MICROBIOTA IN HOST IMMUNITY AND DISEASES

Diverse microbiota reside in the gut and play a key role in the health of their host. Dis-regulation of commensal gut microbiota is implicated in the pathogenesis of a range of diseases including obesity, diabetes, metabolic syndrome and autoimmune disorders (Kamada and Núñez 2013; Tremaroli and Bäckhed 2012). Certain bacterial species that initiate signals that impact the immune system have been identified as drivers of the gut commensal bacteria. These species include segmented filamentous bacteria (SFB), members of *Clostridium* clusters IV, XVIa and XVI and *Bacteroides fragilis*, among others.

SFB are recently cultivated bacteria, present in long filaments, either attached or floating in the terminal ilea of rodents and humans (Davis and Savage 1974; Klaasen et al. 1992; Schnupf et al. 2015). These gram-positive, filamentous, species-specific bacteria are commonly called as SFB but also known as *Candidatus Arthomitus sp.* SFB-mouse according to NCBI taxonomy database and are phylogenetically similar to *Clostridium* genus (Snel et al. 1995; Tannock et al. 1984). SFB have been identified to participate towards the immune development and maturation of the host, including B-cell and T-cell expansion and production of intestinal IgA (Chung et al. 2012; Ivanov et al. 2009; Suzuki et al. 2004). Germ-free mice have an aberrant immune system, which is partially restored back to normal after introducing SFB (Chung et al. 2012). SFB have been shown to increase several inflammatory markers including IFN-γ, TNF-α and IL-17 producing Th-17 cells (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). Increase in Th17 cells by SFB protect against some pathogenic infections including *Citrobacter rodentium* infections

(Rodrigues et al. 2012). However, SFB are known to exacerbate inflammation in colitis, experimental autoimmune encephalomyelitis, and arthritis by increasing the differentiation and expansion of auto-reactive T cells, and are therefore commonly referred to as pathobionts (Chappert et al. 2013; Lee et al. 2011). Germ-free transgenic mice when inoculated with SFB exhibited immediate reinstated arthritic conditions with higher levels of Th17 cells in the small intestine lamina propria and the spleen (Wu et al. 2010).

Although most SFB studies have been done employing rodent models, SFB has been identified in humans as well. One study was able to identify SFB like organisms in ileo-cecal valve biopsies of patients with ulcerative colitis (Caselli et al. 2013). Another recent study used stereomicroscopy to visualize SFB and clostridia in patients with Crohn's disease (Rodriguez-Palacios et al. 2015). Another human study that agrees with SFB playing an important role in initial immune development in infants, suggests that SFB are more abundant in children and decrease with age (Yin et al. 2013).

Majority of the intestinal microbiota is made up of three anaerobic groups - Bacteroides, *Clostridium* cluster IV (*Clostridium leptum* group) and *Clostridium* cluster XIVa (*Clostridium coccoides* group) and belong to the Bacteroides, Firmicutes, Actinobacteria or Proteobacteria phyla (Eckburg et al. 2006; Hold et al. 2002). The *Clostridium* cluster IV and *Clostridium* cluster XIVa (henceforth referred to as *Clostridium* IV and *Clostridium* XIVa) make up 10-40% of the intestinal microbiota and play an important role in the gut immune homeostasis (Lopetuso et al. 2013; Manson et al. 2008). Both *Clostridium* IV and *Clostridium* XIVa are non-homogeneous phylogenetically and include members from disparate phylogenetic groups. *Clostridium* IV includes *Faecalibacterium prausnitzii* and members from *Eubacterium, Roseburia Ruminococcus* and *Anaerofilum* genera whereas *Clostridium* XIVa comprises of species from

Clostridium, Eubacterium, Ruminococcus, Clostridium, Lachnospira, and *Butyrivibrio* genera (Collins et al. 1994; Lopetuso et al. 2013). Most of these Clostridia members produce short chain fatty acids (SCFAs), especially butyrate, and therefore help in regulating metabolism (Paillard et al. 2007; Vital et al. 2014).

Clostridia members are also known to maintain immune homeostasis by inducing a fraction of CD4+ T-cells in the host called regulatory T cells (Tregs) (Atarashi et al. 2011). Tregs are a subpopulation of CD4+ T-cells that have the ability to produce IL-10 cytokines, maintain tolerance to self-antigens and suppress autoimmune diseases (Romagnani 2006). A cocktail of seventeen commensal Clostridia strains that fall within the sub-class clusters IV, XIVa and XVIII when inoculated into germ-free mice are able to induce the production of Foxp3+ -expressing Tregs. They can also ameliorate symptoms of colitis in mice models with the help of these Tregs (Atarashi et al. 2011, 2013). The Tregs induced by these Clostridia produce cytokine IL-10, which has been shown to have anti-inflammatory properties and decrease inflammation associated with colitis (Atarashi et al. 2013). *Bacteroides fragilis* produces Polysaccharide A (PSA) that reduces inflammation and also protects against *Helicobacter hepaticus* induced colitis (Mazmanian et al. 2008).

There are several types of Tregs but they can be broadly classified as natural Treg (nTreg) and inducible Tregs (iTreg). Both nTreg and iTreg are responsible for maintaining immune tolerance in the host (Haribhai et al. 2011). nTregs are derived from the thymus and favor self-antigens for their development. iTreg are induced from CD4+ T-cells on contact with foreign antigens in the presence of IL-2 and TGF-β. Commensal bacteria generate iTregs that reduce inflammation and promote healthy gut microbial environment (Wang, 2015). The delicate balance between

immune tolerance promoting Tregs and activated effector cells including Th17 cells is essential for mounting effective immune responses and maintaining immune homeostasis.

1.2. TCDD ALTERS HOST IMMUNITY

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), is an environmental toxin that acts via the aryl hydrocarbon receptor (AhR) to cause several harmful health effects including embryonic and developmental toxicities, impaired immune function, weight loss and carcinogenesis in humans and animal models (Whitlock 1990). TCDD is known to target the immune system and cause extensive adaptive immune suppression, increasing the likelihood of infectious diseases in mice models (Marshall et al. 2008). T-cell populations are particularly susceptible to the effects of TCDD exposure. Several studies have shown that TCDD acts via the AhR pathway to increase the ratio of Foxp3+ Tregs (Funatake et al. 2005; Quintana et al. 2008). Studies have confirmed that TCDD reduces inflammation by showing a decrease in T-cells that cause inflammation, primarily IL-17 producing Th-17 cells and several corresponding inflammation markers including TNF-α and IL-6 (Quintana et al. 2008; Zhang et al. 2010). As a result of this altered immune response, TCDD is able to reduce inflammation associated with inflammatory bowel disease, DSS-colitis and TNBS colitis. TCDD also plays an important role in ameliorating pathogenesis of several autoimmune diseases including murine systemic lupus erythematosus (SLE), experimental autoimmune encephalomyelitis, experimental autoimmune uveoretinitis and skin transplantation by increasing Tregs via the AhR pathway (Quintana et al. 2008; Schulz et al. 2011, 2012; Zhang et al. 2010).

1.3. RELEVANCE TO HUMAN HEALTH

Both gut microbiota and TCDD influence several key processes including B-cell differentiation and the production of IgA. Production of IgA by B-cells has been observed to keep SFB populations under control (Suzuki et al. 2004). Conventional mice have higher amounts of antibodies including IgA, IgG and IgM as compared to germ-free mice (Hansson et al. 2011). Presence of SFB in intestine was also observed to positively correlate with the ability of B-cells to produce inflammation including inflammatory markers like iNOS and TNF-α and protect against the pathogen, *C. rodentium* (Fritz et al. 2012). In the absence of B-cells, altered gut microbial populations modify gene expression of gut epithelia, increasing immune responses and decreasing metabolic functions (Shulzhenko et al. 2011). B-cell differentiation and secretion of IgA in the intestinal tract was also disrupted by TCDD as observed in a few studies (De Abrew et al. 2010, 2011; Kinoshita et al. 2006; North et al. 2010). Fecal IgA levels were significantly decreased after exposing wild type mice to 0.1 and 1 μg/kg TCDD. Pups exposed to TCDD by breast-feeding their mothers also showed decreased fecal IgA, with males producing lesser IgA than females after exposure (Ishikawa 2009). From these observations, it can be surmised that TCDD reduces the production of IgA, which leads to uncontrolled outgrowth of SFB populations. It could be extrapolated that humans exposed to TCDD would have a dysbiosis in their gut bacteria, thereby compromising their immune system and increasing their risk of infection.

Gut microbiota have been implicated in the progression of metabolic endotoxemia, Type 2 diabetes, obesity and low-grade systemic inflammation (Cani et al. 2008). TCDD is known to bioaccumulate in adipose tissue and has been linked to obesity. Mice exposed to higher doses of

TCDD (100 μg/kg every 2 weeks for 8 weeks) and fed a high fat diet showed 46% increase in body weight compared to control mice fed a regular diet (Zhu et al. 2008). The ratio of the major bacterial phyla Firmicutes to Bacteroidetes was observed to be higher in genetically obese mice as compared to the corresponding lean mice group (Ley et al. 2005). Similarly, obese human patients showed increased abundance of Firmicutes and a relative reduction in Bacteroidetes relative to lean controls (Ley et al. 2006). An increased abundance of butyrate-producing bacteria including *Roseburia intestinalis, R. inulinivorans, Faecalibacterium prausnitzii* and *Eubacterium rectale* is found in controls as compared to diabetic patients (Karlsson et al. 2013; Qin et al. 2012). There is also a shift in the microbial functional genes in Type 2 diabetes patients with an increase in genes related to resistance to oxygen stress implying that the host gut becomes more formidable to bacterial colonization. Diabetic patients also displayed a decrease in genes associated with flagellar assembly and pathways for synthesis of cofactors and vitamins (Karlsson et al. 2013; Qin et al. 2012). Therefore, both TCDD and gut microbiota are able to influence metabolic disorders including Type 2 diabetes and obesity. Several incidences of increased diabetes in TCDD-exposed human populations have been documented but the exact mechanisms are still unknown. Knowledge of the impact of TCDD on the gut microbiota would pave the way toward understanding their role in the progress of metabolic syndrome including diabetes and obesity.

Both gut microbiota and TCDD are implicated in the pathogenesis of autoimmune diseases. Several intestinal bacteria including *Bacteroides*, *Eubacterium*, *Ruminococcus, Lactobacillaceae*, *Rikenellaceae* and *Porphyromonadaceae* have been associated with protection from Type 1 Diabetes (Brugman et al. 2006; Wen et al. 2008). There is a definite gender bias in the progression of this disease and some of the key bacteria that were differently abundant

included higher levels of *Roseburia, Blautia, Coprococcus 1,* and *Bilophilia* in females and *Parabacteroides* and *Peptococcus* in males (Markle et al. 2013). Protection by SFB is greater in male NOD mice along with a higher level of IFN-γ in their peripheral lymph nodes (Yurkovetskiy et al. 2013). A clear gender bias was also observed with TCDD exposure in several autoimmune diseases (Mustafa et al. 2008, 2009b). In lupus-like glomerulonephritis and Sjogren's disease, the disease progression was more rapid in females relative to males exposed to TCDD (Mustafa et al. 2008, 2009a, 2009b) (Ishimaru et al. 2009). Therefore, gender biases in autoimmune disease progression have been observed with TCDD treatment and are influenced by specific microbial populations. The involvement of gut microbial population in the progress of disease in TCDD-exposed populations has not been studied. This would contribute towards better understanding and protecting against progress of dioxin-related autoimmune diseases.

1.4. INTERACTION OF TCDD WITH GUT MICROBIOTA

Large-scale efforts are being made to find suitable treatment for inflammable bowel disease, colitis, autoimmune diseases and metabolic disorders that affect thousands of lives daily. Both TCDD and intestinal microbiota have been observed to alter immune responses and influence the outcome of these diseases, separately. Several Clostridia members are butyrate-producers that have been associated with increased Tregs and cytokines such as IL-10 that suppress self-antigens and play a beneficial role in diabetes, obesity, autoimmune disorders and other diseases (Atarashi et al. 2011). SFB are important intestinal bacteria that influence immune maturation and aid production of Th17 cells but can also be harmful under adverse conditions (Chappert et al. 2013; Chung et al. 2012; Ivanov et al. 2009). TCDD, acting via the AhR, can influence

immune responses and increase IL-10 producing Tregs that cause immunosuppression (Quintana et al. 2008; Zhang et al. 2010). However, so far, little or no research has been conducted to evaluate the direct relation of TCDD exposure to the intestinal commensal microbial population. Our goal is to study the changes in key intestinal microbial members including SFB, *Clostridium* IV and XIVa when exposed to various doses of TCDD. We will test the hypothesis that TCDD exposure disrupts two groups of specific gut microbial populations, the SFB and the *Clostridium* IV and XIVa and Bacteroides, and their populations in the mice intestine shift with time after TCDD exposure. These studies will yield important knowledge on the impact of the dioxin on key gut microbial members as well as the quantity of exposure required to alter these bacterial communities. It will indicate whether microbial populations are affected by an acute one-time exposure to TCDD or if they fluctuate only with sustained exposure to higher concentrations of the dioxin. This information will help us understand the underlying mechanisms on how they influence host health and diseases. It will also enable us to understand which microbial members are most sensitive to TCDD exposure and their role in maintaining the immune balance.

It is known that the immune system responds to both gut microbiota and TCDD exposure. Studies have shown that the gut immune maturation is strongly influenced by key microbial species including SFB and certain Clostridia species. It is important to investigate the impact of TCDD exposure on these two specific gut microbial populations in order to better understand their role in the immune system. In this study, quantitative PCR coupled with high-throughput platform along with primers targeting 16S rRNA and functional genes for some of the key intestinal members and important butyrate-producers was used. The bacterial groups targeted are SFB, *Bacteroides* and certain Clostridia members including *Roseburia* spp., *Eubacterium rectale, Clostridium* IV and XIVA, *F. prausnitzii.*

1.5. OBJECTIVES

1. To determine the role of key microbial members by analyzing changes in its population with exposure to increasing concentrations of TCDD over long (90 days) time period.
2. To determine the shift in abundance in SFB and *Clostridium* cluster IV and XIVa with exposure to repeated high concentrations of TCDD over time.
4. To determine if the observed change in abundance of SFB or Clostridia groups is reversible once TCDD dosage is terminated.
5. To determine and quantify the change in SFB, Clostridia and Bacteroides abundance with exposure to different doses of TCDD.
6. To determine and quantify the change in abundance of SFB, Clostridia groups and Bacteroides with exposure to repeated high doses of TCDD over a short time period.
7. To determine the impact of a low dose of TCDD on key microbial populations in the mice intestine over a short time period.

TCDD acts on several immune response genes and exhibits deleterious effects associated with diseases in the host. Also, commensal bacterial species including SFB and Clostridia members in the gastrointestinal tract effectively mount immune responses and maintain the immune balance in normal healthy conditions but may cause diseases during microbial dysbiosis.
We hypothesize *that exposure to TCDD causes increase in SFB abundance and relative decrease in Clostridia groups in the mice intestinal tract and the amount of change is dependent on the dosage of TCDD exposure as well as the duration of exposure.*
The outcome of this work will aid in answering the following questions:

1. Does exposure to TCDD affect commensal gut bacteria including SFB, Clostridia members and Bacteroides?
2. Do the butyrate-producers within *Clostridium* IV and *Clostridium* XIVa get impacted by exposure to TCDD?
3. At what doses of TCDD do we observe the greatest effects with regard to changes in the gut microbial population?
4. Do the microbial population revert to its original concentrations once exposure to TCDD is terminated?
5. What role does SFB play in impacting the immune system in these conditions?

This work has been divided into the following sections:

Chapter 1: Chapter 1 gives a brief introduction and states the objectives and goals of this work.

Chapter 2: This chapter presents an overview of the existing studies where both gut microbiota and TCDD influence the host immunity and several associated diseases.

Chapter 3: In Chapter 3, the results of the effect of TCDD on intestinal SFB in mice have been presented, as conducted through three separate animal studies.

Chapter 4: In this chapter, the results of the impact of TCDD on the commensal Clostridia groups and Bacteroides within the mice gut have been presented and discussed.

Chapter 5: The conclusions of this work and future directions are discussed in this chapter.

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CHAPTER 2: LITERATURE REVIEW

TCDD, GUT MICROBIOTA AND HOST IMMUNITY

Note: This chapter is to be submitted as review article to the Journal 'Current Opinion in Microbiology' with the title as "**Both TCDD And Gut Microbiota Influence Host Immunity"** by Prianca Bhaduri, James M. Tiedje and Syed A. Hashsham.

2.1. INTRODUCTION

The impact of mammalian host pathways by gut microbiota has been extensively studied. The gut microbiome is known to play a critical role in development and regulation of immune signaling pathways and functions. These host systems and their underlying metabolic, signaling and immune pathways are modified by exposure to environmental toxicants, such as dioxins. Both gut microbiota and environmental toxins have the capacity to regulate immune pathways and affect progression of related diseases. One of the most well-studied environmental contaminants is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). It is also the most environmentally persistent dioxin, making it the most potent of the polychlorinated dibenzodioxins. TCDD is known to limit the development of T-cell and B-cell immunity and adversely affect its functions. It also plays a role in the progression of several immune diseases including Type 1 diabetes (T1D) and Sjogrens's disease. However, few studies have considered the cumulative effect of both the gut microbiome as well as environmental contaminants on host immunity. A more holistic perspective is needed to discern the individual impact of each environmental stimuli, whether its bacteria or contaminants. Here we have reviewed studies that indicate gut microbiota and TCDD affect the same immune pathways and resulting immune diseases. These studies

present further undeniable evidence that exposure studies of TCDD on mammalian pathways is incomplete without considering the gut microbiome.

2.2. TCDD AND GUT MICROBIOTA INFLUENCE HOST IMMUNE RESPONSES

TCDD when introduced into the host, disrupts the normal immune system in several different ways. T-cell populations are particularly susceptible to the effects of TCDD exposure. Several studies have shown that TCDD acts via aryl hydrocarbon receptor (AhR) pathway to increase the ratio of Foxp3+ expressing regulatory T-cells (Treg). Treg cells are a subpopulation of CD4+ T-cells that have the ability to influence IL-10 and IL-22 cytokines, maintain tolerance to self-antigens and suppress autoimmune diseases. On the other hand, microbiota in the intestinal tract play a major role in the maturation of immune responses in the host and assist in the proliferation of both CD4+ T-cells and Foxp3+ Treg cells in gut associated lymphoid tissues (Chung et al. 2012; Cording et al. 2013; Ivanov et al. 2008). Therefore, both TCDD and microbiota have an impact on the immune system, specifically production of T-cells and their associated cytokines.

2.2.1. Role of T-cells in inflammatory bowel diseases

Both TCDD and microbiota modulate host immunity to fight inflammation associated with inflammatory bowel disease and colitis. TCDD acts via the AhR to upregulate Treg cells that ameliorate inflammation (Quintana et al. 2008). Studies have confirmed that dosage with TCDD reduces inflammation by showing a decrease in T-cells that cause inflammation, primarily IL-17 producing Th-17 cells and several corresponding inflammation markers including TNF-α and IL-6 (Quintana et al. 2008; Zhang et al. 2010). Mice exposed to TCDD showed a decrease in

cytotoxic T cells and T helper cells but not Foxp3+ Treg cells (Schulz et al. 2012). Certain microbial populations, specifically members of the Clostridiales order can also induce Treg cell populations by the production of short chain fatty acids (SCFA). However, other gut bacteria like segmented filamentous bacteria (SFB), induce Th17 cells that exacerbate inflammatory conditions.

Inflammatory bowel disease is studied in mice models by inducing them with dextran sodium sulfate to produce colitis. Mice dosed with TCDD showed reduced symptoms of dextran sodium sulfate-colitis along with an increase in the number of Treg cells and a decrease in Th17 cells in the lymph nodes ((Singh et al. 2011; Takamura et al. 2010). They also observed an increased production of the hormone prostaglandin E2 in TCDD-treated animals (Takamura et al. 2010). Also, introducing TNBS in mice produced another type of colitis, similar to human Crohn's disease. Dosage of TNBS-treated mice with TCDD showed a similar immune response, with reduction in Th17 cells and the inflammatory markers - IL-6, IL-12, IFN-γ, TNF-α, and IL-17, indicating occurrence of immune suppression (Benson & Shepherd 2011).

Presence of certain gut microbiota has also been implicated in altering the symptoms of colitis/inflammatory bowel disease. A cocktail of Clostridia strains including 17 Clostridial strains that fall within the clusters IV, XIVa and XVIII when inoculated into GF mice are able to induce the production of IL-10 producing Treg cells. They could also ameliorate symptoms of colitis in the mice models (Atarashi et al. 2011; Atarashi et al. 2013). They also showed increase in Foxp3 and Tgfb mRNA. IL-10 has been shown to have anti-inflammatory properties and decrease inflammation associated with colitis (Atarashi et al. 2013). For the induction of Treg cells, both commensal gut microbiota and the presence of butyrylated starch are necessary (Furusawa et al. 2013). GF mice that were fed a butyrylated starch do not show increase in Treg

cells. Also, mice colonized with Clostridial species but fed a low fiber diet were unable to replicate the Treg cell induction observed in chow fed mice (Furusawa et al. 2013). Another bacteria that protects from *Helicobacter hepaticus* induced colitis is *Bacteroides fragilis*; it produces Polysaccharide A (PSA) that reduces inflammation (Mazmanian et al. 2008). Another study showed that a single strain of clostridia – *Clostridia butyricum* is able to control colitis by induction of IL-10 by macrophages (Hayashi et al. 2013). On the other hand, SFB have been shown to exacerbate conditions of colitis by producing IL-17 and IFN-γ producing Th17 cells (Ivanov et al. 2009; Gaboriau-Routhiau et al. 2009). It has been observed that SFB proliferate in AhR-negative mice, indicating they are kept in check by AhR (Qiu et al. 2013). This increase in SFB was inversely proportional to down-regulation of IL-22 by innate lymphoid cells acting via the AhR pathway (Qiu et al. 2013).

Recently, further evidence of the mechanism used by Clostridia to modulate immune responses has come to light. Clostridial species are able to breakdown fibers in the diet to produce SCFAs that upregulate Treg cells. SCFAs are capable of differentiating CD4+ T cells to form Treg cells (Arpaia et al. 2013; Smith et al. 2013; Furusawa et al. 2013). The main SCFAs are butyrate, propionate and acetate and they exhibited induction of CD4+ T-cells to Treg cells in the peripheral lymphoid system and expansion in the number of Treg cells in the colon (Arpaia et al. 2013). SCFAs cause histone acetylation which helps increase the amount of Foxp3+ protein in the Treg cells and also increase their suppressive activity. Clostridia are able to break down high fiber in food; dosage of butyrylated starch in the absence of bacteria in GF mice failed to induce Treg cells (Furusawa et al. 2013). Therefore, Clostridia breakdown fibers to form SCFAs that then induce Treg cells or expand Treg populations in the lymphoid system. It is still not known

whether butyrate and other SCFAs also act through the AhR receptor. However, dietary lignans have been known to act as ligands to activate the AhR.

Therefore, microbial populations, specifically Clostridia groups and SFB maintain immune balance by regulating T cell populations and their cytokines. TCDD acts on this immune system primarily via the AhR to induce Treg cells that reduce inflammation but also disrupt immunity. More studies on inflammatory pathways taking into account both TCDD and gut microbiota would help elucidate the exact mechanism of inflammation and the role of each participating element.

2.2.2. Role of T-cells in autoimmune diseases

Both TCDD and intestinal microbiota play an important role in altering the pathogenesis of autoimmune diseases. SFB have been linked to several autoimmune diseases including experimental autoimmune encephalitis (EAE), arthritis, and T1D. SFB are known to exacerbate symptoms of EAE by producing Th17 cells and inflammatory conditions (Lee et al. 2011). Chappert and co-authors showed that SFB increase the differentiation and expansion of autoreactive T cells that exacerbate arthritis conditions. SFB alter the tuning of these autoreactive T-cells by lowering their activation threshold to self-antigens (Chappert et al. 2013). GF K/BxN transgenic mice when inoculated with SFB exhibited immediate reinstated arthritic conditions with higher levels of Th17 cells in the small intestine lamina propria and the spleen (Wu et al. 2010). The increased production of IL-17 cytokine acts on B cells to form germinal centers more effectively, which ultimately contribute to arthritis. IL-17 also increases the quantities of autoantibodies in the serum (Wu et al. 2010). They also found arthritis was blocked by antibiotics targeting gram positive bacteria such as ampicillin and vancomycin (Wu et al. 2010).

However, SFB were observed to protect non-obese diabetic (NOD) female mice from autoimmune T1D. Mice associated with SFB also showed increased levels of Th17 and IL-17 (Kriegel et al. 2011). SFB, present in the gut ilea, are able to both positively and negatively affect autoimmune diseases. It would be interesting to further understand the mechanisms by which SFB affect autoimmunity.

Exposure to TCDD plays an important role in predicting outcome of autoimmune diseases. TCDD has been shown to ameliorate pathogenesis of several autoimmune diseases including murine systemic lupus erythematosus (SLE), EAE, experimental autoimmune uveoretinitis (EAU) and skin transplantation by increasing Treg cells via the AhR pathway (Schulz et al. 2011; Schulz et al. 2012; Quintana et al. 2008; Zhang et al. 2010). TCDD also suppressed graft-versus-host response (Funatake et al. 2005) and reduced autoimmune T1D in NOD mice (Kerkvliet et al. 2009). Oral tolerance is the acquired suppression of immune response to a specific antigen. TCDD exposure prevented the establishment of oral tolerance against ovalbumin. Even after three booster immunization doses, anti-OVA specific antibodies remained high in TCDD treated animals while it disappeared in the controls (Chmill et al. 2010). Therefore, TCDD is able to reduce clinical signs of autoimmune diseases but it also negatively affects the acquisition of oral tolerance by the host.

Some of the possible mechanisms used by gut microbiota to initiate autoimmunity are discussed below. Different signaling pathways are used by gut microbiota to initiate autoimmunity. The retinoic acid receptor-related orphan receptor thymic isoform (RORγt) and chemokine receptor CCR7 deficient mice developed disease symptoms only in the presence of a commensal gut microbiota (Wichner et al. 2013). These deficient mice were characterized by acute mucosal inflammation. Treatment with antibiotics attenuated the acute inflammatory response indicating

the commensal microbiota were responsible for the lethal mucosal inflammation. Deficiency of RORγt resulted in an altered gut microbial population. There was a 100-fold increase in Proteobacteria/ Enterobacteriaceae in deficient mice. Therefore RORγt and CCR7 help develop immunity that in turn maintains the homeostasis of the commensal bacteria in the intestine. This prevents overgrowth of microbial populations in the intestine that cause adverse outcomes including acute inflammation (Wichner et al. 2013).

A study of the role of Treg cells and T-cell receptors (TCR) showed that mostly thymic Treg cells, but not induced Treg cells, regulate the tolerance to antigens produced by commensal microbiota. When transgenic mice with modified TCR and GFP deficient Foxp3 T-cells were treated with antibiotics, the number of colonic Treg cells were reduced and the expression of TCRs and their levels were altered. They also tested 26 hybridomas of Treg cells in pure bacterial cultures for its reactivity. Only four hybridomas responded to isolates from Clostridiales and another four responded to Bacteroides and Lactobacillus, indicating that Treg cells are specific to bacterial species (Cebula et al. 2013). The described studies point towards an interplay of gut microbial signaling after exposure to TCDD that leads to enhanced autoimmune disease progression. Further studies will need to address the role of gut microbiota and their mechanisms in modulating autoimmunity in hosts that are exposed to TCDD.

2.2.3. Role of T-cells in infectious diseases

Both commensal microbiota and TCDD affect the progression of infectious diseases. TCDD ameliorates ocular viral disease by reducing the number of Foxp3 negative T-cells via the AhR, thereby increasing the ratio of Treg cells to T-cells (Veiga-Parga et al. 2011). However, reduction of inflammatory symptoms exacerbates bacterial infection conditions: AhR-null mice

showed a greater inflammatory response and were able to clear parasital *Leishmania major* infection as compared to TCDD-treated AhR positive mice (Elizondo et al. 2011). On the other hand, gut microbiota have been noted to clear pathogenic bacterial infections including *Salmonella enteritidis* and *E. coli* in rats and rabbits, respectively (Croswell et al. 2009; Heczko et al. 2000). SFB proliferated in matrix metalloproteinase (MMP9) negative mice along with an observed increase in Th-17 cells and reduced levels of *C. rodentium* infections (Rodrigues et al. 2012). Therefore, we see that TCDD reduces numbers of Th17 cells, and affects the hosts' ability to clear pathogenic infections. However, since they increase Treg cell numbers, TCDD exposure can reduce progression of certain viral diseases. The presence of gut microbiota significantly reduces the incidence of bacterial infection and certain commensal bacterial species can assist in clearing pathogenic infections.

2.2.4. Infants are most responsive to changes in gut microbiota or TCDD exposure

Both microbiota and TCDD are seen to have the greatest effect on the immune response early in life, especially at the prenatal and natal phase. TCDD shows the greatest toxic effects when mice are exposed to it during gestation. Exposure to TCDD during the neonatal stage resulted in exacerbated autoimmune conditions in two strains of mice (Holladay et al. 2011; Mustafa et al. 2011). Another study found that low-dose of TCDD given to neonatal mice could initiate Sjogren's syndrome using NFS/sld mice models (Ishimaru et al. 2009). Therefore, we observe that when hosts are neonatally exposed to TCDD, it worsens symptoms associated with autoimmune diseases.

On the other hand, infants need to possess a complex gut microbiota in order to develop a complete immune repertoire. SFB are known to colonize prenatally and initiate immune

responses in the host. SFB induce inflammatory symptoms and leads to more than 30 fold expansion of IL-17 producing Th17 cells (Ivanov et al. 2009; Gaboriau-Routhiau et al. 2009). GF mice colonized with only SFB regained most of their T-cells as compared to wild-type mice but it was still less than the full regime of T-cells in the conventional mice. This implies that SFB have an important role but they cannot replace the entire community of commensal microbiota in the intestine (Chung et al. 2012). A complex gut microbial population is required for the maturation of the host's immune system.

One possible theory is that TCDD modifies signaling pathways that alter the commensal bacteria. This altered bacteria is unable to develop the complete immune response and fight against autoimmune diseases. More studies taking both TCDD and gut microbiota in consideration during natal condition is necessary to understand the development of the immune balance and the consequences of environmental toxin exposure in infants.

2.2.5. Role of aryl hydrocarbon receptor in T-cell immunity

TCDD acts via AhR to suppress immune responses to allergy sensitization. Mice, sensitized to peanuts after being exposed to TCDD, exhibited an increased percentage of Treg cells in the spleen and the mesenteric lymph nodes (Schulz et al. 2011). TCDD also reduced the number of precursor T-cells in the thymus and number of T-helper cells and cytotoxic T cells in the spleen and mesenteric lymph nodes. It also showed an increase in Foxp3+ Treg cells in the spleen but not in the mesenteric lymph nodes (Schulz et al. 2012). Therefore, TCDD is able to reduce the numbers of Th2 cells and ameliorate allergic reactions.

The role of AhR in modulating T-cell populations has been extensively studied and recent observations have found that TCDD affects the AhR pathway differently in vivo and in vitro

(Duarte et al. 2013). In their study, Duarte et al found that TCDD inhibits Th17 cell differentiation rather than inducing Treg cells (Duarte et al. 2013). When TCDD was introduced to mice immunized with peptide adjuvant for EAE, the disease was suppressed by 47%. However, when the same experiment was conducted in vitro conditions, TCDD activated AhR, which in turn induced Th17 differentiation leading to an increase in IL-22 and IL-17. Here, the authors claim that TCDD does not induce Treg cells in vivo but greatly inhibits Th-17 differentiation in vivo. TCDD, in vivo, also reduces the number of dendritic cells ($CD11^{high}$) that are required for producing CD4+ T-cells, but not the subset of DCs required to produce Treg cells in the spleen (Bankoti et al. 2010). During oral tolerance, TCDD induced a slight increase in the number of Th17 cells with no difference in Treg cells (Chmill et al. 2010). Similar results have been noted in humans with exposure to TCDD. One study observed that TCDD exposure increases the number of IL-22 producing cells but did not change the number of Treg cells. (Brembilla et al. 2011). Also, AhR can be induced by IL-27 and c-Maf to produce Treg cells, independent of TCDD (Apetoh et al. 2010). It is also observed that immune response can be suppressed by cells/pathways other than Treg cells using the AhR pathway. Qiu et al showed that experimental colitis is contained in AhR wild-type mice that produce IL-22 via Group 3 innate lymphoid cells. Therefore, ILCs are also capable of producing anti-inflammatory cytokines that work towards regulating the immune response. Moreover, it was seen that in $AhR^{-/-}$ mice, SFB proliferated, creating pathogenic Th17 cells that exacerbated colitis symptoms (Qiu et al. 2013). Th17 is also known to exhibit plasticity since at times Th17 may promote immune homeostasis while in other conditions it can behave either like Th1 cells or be pathogenic. Further studies will help elucidate the AhR pathway activated by TCDD under different conditions and the role of microbiota in the activation of different signaling pathways under different conditions. Since

AhR is known to induce both Treg cells and Th17 cells in humans, it is important to understand what biological conditions induce each of these effects and the role of microbiota or microbial products in triggering these pathways.

2.3. AUTOIMMUNE DISEASES IN TCDD AND MICROBIOTA

2.3.1. Role of microbiota and TCDD in type 1 diabetes (T1D)

There are several autoimmune diseases caused by self-reactive T-cells that produce autoantibodies against its own antigens. The most common autoimmune disease is T1D, which is characterized by the presence of reactive T-cells that destroy the insulin-producing beta-cells of the islets of Langerhans in the pancreas. T1D is usually preceded by inflammation in the islets (also known as insulitis), crowding of lymphocytes to the islets and presence of autoantibodies. T1D is usually studied in non-obese diabetic (NOD) mice model that are genetically susceptible to the disease. BioBreeding also generates Diabetes Prone (BB-DP) and Diabetes Resistant (BB-DR) rats for analyzing mechanisms of T1D disease progression.

Initial evidence that microbiota are involved in T1D was obtained when the host was immunized against insulin autoantibodies by introducing cow's milk (Vaarala et al. 1999). Since then, several studies have linked the presence of commensal bacteria with increased development of T1D (Wen et al. 2008). The treatment of NOD mice with antibiotics such as vancomycin reduced the progression of diabetes and lowered blood glucose levels, along with an increase in abundance of *Akkermansia mucinphilia* (Hansen et al. 2012). Antibiotics also delayed the onset of T1D and decreased severity of insulitis in BB-DP rats (Brugman et al. 2006). Gut bacteria profiles of BB-DP rats were markedly different from BB-DR rats with the latter having an increased abundance of *Bacteroides, Eubacterium*, and *Ruminococcus*.

SFB are important for protection against T1D. Gut colonization with SFB protects female mice against T1D in NOD mice (Kriegel et al. 2011). Another group also observed that SFB protection was greater in male NOD mice along with a higher level of IFN-γ in their PLNs (Yurkovetskiy et al. 2013). The protection observed in female mice could be due to the effect of other microbial strains also present in their gut community or it could be due to differences in SFB strains (Yurkovetskiy et al. 2013).

T1D was successfully ameliorated by TCDD treatment (dose of approximately 30 μg/kg) in NOD female mice. They also showed an increased proliferation of Foxp3+ Treg cells in the PLNs and did not have pancreatic insulitis, unlike the control mice. However, this protection was reversible, once TCDD treatment was ceased, the number of Treg cells decreased and they regained the ability to acquire T1D (Kerkvliet et al. 2009).

Microbiota influence the progression of T1D in the host via toll-like receptors. MyD88 is an important adaptor protein for most TLRs. T-cells were less reactive in the pancreatic lymph nodes of MyD88 Knockout (KO) NOD mice than the MyD88 controls, implying that microbiota influence disease development via the MyD88 adapter (Wen et al. 2008). Although the GF MyD88 KO mice are fully capable of developing the disease, the altered gut microbial population in the KO mice was able to protect against T1D when transferred into these GF mice. Some of the changes seen in MyD88 KO mice included a reduced Firmicutes/ Bacteroides ratio and increased *Lactobacillaceae, Rikenellaceae* and *Porphyromonadaceae* families as compared to Specific Pathogen Free NOD mice (Wen et al. 2008).

2.3.1.1. Changes in gut microbial populations with T1D in children

Several case-control studies have been done to pinpoint the shifts in bacterial populations between diabetes resistant and diabetes prone children. One study showed that four children with T1D had a decreased Firmicutes/ Bacteroidetes ratio over time compared to four healthy children. Bacteroidetes was marked by a single species *Bacteroides ovatus* that showed 20% of the increase in children with T1D (Giongo et al. 2011). Another study analyzed fecal samples from a larger cohort of children (n= 18) that were positive for two autoantibodies for T1D along with a control group (de Gaffau, 2013). Beta-cell autoimmunity was observed to be associated with a low abundance of butyrate-producing bacteria including *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, *Clostridium clostridiforme* and *Roseburia faecis*. Two species, *B. adolescentis* and *B. pseudocatenulatum* were the most abundant species in the controls but significantly decreased in the T1D group. There was an increase in Bacteroidetes phylum and Bacteroidaceae family in the T1D group. They also noted that *Clostridium perfringens* was abundant in the T1D group and *Eubacterium halli* was negatively correlated to the number of beta-cell autoantibodies in the younger children of this group. However, they did not find changes in the inflammatory marker, calproctin or IgA antibody (de Goffau et al. 2013). Another case-control study in children (n=32) also concluded that serum glucose levels are positively correlated with a decrease in Firmicutes to Bacteroidetes ratio. They also showed a decrease in *Lactobacillus* and *Bifidobacterium* and an increase in *Clostridium* species. There was also a decrease in abundance of mucin-degrading and butyrate-producing bacteria that help in maintaining intestinal barriers, in T1D groups (Murri et al. 2013).

2.3.1.2. Diet intervention prevents T1D

Diet intervention has worked well with T1D. Specific diets alter the gut microbiota thereby either protecting or increasing likelihood of the disease. The administration of *Lactobacillus johnsonii* decreased the incidence of T1D in BB-DP rats (Valladares et al. 2010; Lau et al. 2011). It decreased oxidative stress response in the host by producing decreased ROS markers such as superoxide dismutase 2 (Sod2), glutathione peroxidase (Gpx1) and inducible nitric oxide synthase (iNOS) as compared to controls not fed *L. johnsoniii*. However, they also noted a significant decrease in IFN-γ in *L. johnsonii* fed animals, which is different from other studies (Calcinaro et al. 2005; Yurkovetskiy et al. 2013). Th17 cells were also increased in the protected mice indicating Th17 cells may act against autoreactive T-cells (Lau et al. 2011; Nikoopour et al. 2010). An association between increased gut permeability and higher incidence of T1D has been observed (Neu et al. 2005). *L. johnsonii* in diet altered the microbial population to express higher amounts of tight junction proteins, occludin and claudin-1, that are required for epithelial integrity and increased mucus in the gut. Changes in intestinal permeability were also observed on a ProSobee diet that lead to protection of NOD mice against T1D progression (Alam et al. 2010). ProSobee diet fed NOD mice showed normal epithelia compared to controls that had gut inflammation and exhibited thicker crypts and thinner epithelia. This diet also decreased the amounts of CD69 and CD44 markers and increased L-selectin expression on CD4+ T-cells as compared to NOD mice on regular diet. They also displayed normal levels of IL-17, IL-23, IL-10 and TGF-β in their colons as compared to controls (Alam et al. 2010). Hydrolysed casein based diets have also delayed development of T1D in DP-BB rats and showed increased colonization by bacterial species (*Lactobacillus* and *Bacteroides*) that have been shown to be helpful for ameliorating diabetic conditions (Visser et al. 2012). This diet was also observed to reduce

reactive oxygen and nitrogen species in the distal intestines (Emani et al. 2013). Another study showed that consumption of acidic liquids alters the gut microbiota in NOD mice. It decreases the development of T1D, also increasing the Firmicutes/ Bacteroidetes ratio and increasing numbers of Foxp3 T-cells (Wolf et al. 2014).

Diet intervention may prove helpful in mitigating the harmful effects of TCDD on autoimmune diseases. Most butyrate-producing bacteria species belonging to Clostridial cluster IV and XIVa and *Akkermansia mucinphila* are associated with maintaining intestinal barriers and thereby protecting against T1D. These bacteria could be administered as probiotics in T1D populations affected with dioxin exposure.

2.3.1.3. Microbiota are responsible for gender bias for T1D; TCDD exerts toxicity in natal condition in gender biased manner – any link?

It has been observed frequently that the incidence of autoimmune diseases including T1D is higher in female rodents as compared to their male counterparts (Whitacre 2001; Markle et al. 2013). Two separate groups have elegantly studied the relation among development of T1D, sex hormones and commensal bacteria. The first group, Markle and coauthors, observed that GF NOD mice of both gender showed similar disease phenotype. However, after Altered Schaedler's Flora (ASF) was introduced to GF NOD mice, the gender bias in disease progression crept back between males and females (Markle et al. 2013). They also noted that testosterone levels for GF males were lower than ASF males, therefore implying that commensal bacteria influence production of testosterone. Moreover, when an ASF male's gut bacteria was inoculated to a GF female, the female showed reduced incidence of diabetes, and higher levels of testosterone. Transfer of male microbiome lowered serum concentrations of certain metabolites:

glycerophospholipid and sphingolipid long chain fatty acids, which were higher in control female NOD mice. The transfer of male microbiome into females also reduced insulitis, lowered number of diabetic specific autoantibodies and reduced blood glucose levels, thus protecting against autoimmune T1D. Some of the key bacteria that were differently abundant included higher levels of *Roseburia*, *Blautia*, *Coprococcus 1*, and *Bilophilia* in females and *Parabacteroides* and *Peptococcus* in males. Thus, they were able to identify the microbiome as the causative factor in androgenic changes that lead to differences in autoimmune diseases between sexes (Markle et al. 2013).

The second group lead by Leonid Yurkovitskiy also observed that gut microbiota is different between males and females in NOD mice (Yurkovetskiy et al. 2013). However, they noticed that the gut microbiome of NOD mice after puberty but not before were distinctly different. Therefore, gender differences are responsible in influencing different microbial communities in mice. The differences in specific microbial families between genders were not consistent between different studies, although, raising questions on differences in gender bias between different animal facilities. They did observe that colonization of mice with *Enterobacteriaceae* similar to *E. coli* and *Shigella* lead to a decrease in T1D incidence in GF NOD mice. They also identified signaling pathways and genes that were specifically influenced by male gut microbiota. Both the IFN-γ pathway and ILβ-1 were highly expressed in males as compared to females. Moreover, deleting the IFN-γ or its downstream genes, IFN-γ receptor-1 or signaling molecule STAT-1 removed the protection against T1D provided to male mice. Also, measurement of IFN-γ protein or mRNA revealed that it was higher in SPF males and SFB mono-colonized males as compared to females. Male PLNs and mesenteric nodes also expressed higher amounts of IFN-γ producing T-cells. They found that testosterone levels of less than 2

ng/ml are needed for diabetes protection. Overall this study was able to provide proof that both gut microbiota and sex hormones are responsible for the development of T1D. Sex hormones and gut microbiota have a feedback loop where the hormones initially influence the composition of the microbiome, which in turn influence the production of androgens that protect against T1D (Yurkovetskiy et al. 2013).

Studies have been conducted to analyze autoimmune effects of TCDD on neonatal mice, and most of the abnormalities related to exposure are also gender biased. Prenatal exposure is generally considered more harmful than adult exposure and gives rise to several abnormalities. Prenatal effects of dioxin were studied in 24-week old F1 mice by exposing pregnant mice to TCDD (Mustafa et al. 2008). A clear gender bias was observed in the effects of TCDD exposure. Thymic weights were reduced with TCDD exposure in mice but were more significant in females. This could possibly be due to the protective effects of microbiota. Cells in the thymus also decreased in females but not males with TCDD treatment. However, percentage of CD4-CD8+ T-cells in spleen of males but not females declined with 5 μg/kg TCDD. There was also a decrease in CD4+CD8- T-cells and increase in non-T cells in lymph nodes of female mice. There were signs of autoimmune glomerulonephritis in both the groups as well (Mustafa et al. 2008). F1 mice of pregnant SNF mice, that have the capacity to spontaneously develop lupus-like glomerulonephritis, exposed to TCDD also showed signs of the disease with increased autoreactive T-cells in the axillary and inguinal lymph nodes. There was a defined gender bias and the disease progression was more rapid in females. When splenocytes were treated with concalvin A, males showed increased IFN-γ whereas it decreased in females (Mustafa et al. 2009).

TCDD also exacerbates autoimmune disease conditions in the Sjogren's syndrome. Sjogren's syndrome is a T-cell mediated autoimmune disease that causes infiltration by lymphocytes and destruction of exocrine glands especially salivary glands. This disease has been reproduced in NFS/sld mice that show the disease symptoms when thymectomized 3 days after birth. It is caused by inhibition of Treg differentiation, which causes self-reactive T-cells to proliferate. Neonatal treatment of NFS/sld mice with low amounts of TCDD (0.1, 1, 10 ng/kg) produced inflammatory lesions in the salivary gland similar to those after thymectomy along with higher expression of AhR. Females had more severe lesions than males. Inflammatory lesions were also found in liver, lung and kidneys with exposure to 10 ng/kg TCDD in adult mice. However, they did not discover any changes in peripheral Foxp3+ Treg cells. There was increased IL-2 and IFN-γ production by Th1 cells (Ishimaru et al. 2009). Neonatal exposure to TCDD also increases several prostatic proteins implying a role in prostrate disease (Fujimoto et al. 2013).

TCDD alters the immune response related to viral related autoimmune diseases in a sex-dependent manner. A study using 10 ng/kg TCDD (low dose) showed decrease in survival of female mice from influenza virus (Burleson et al. 1996); also the same effect was seen with a dose of 100 ng/kg and 10 µg/kg TCDD (House et al. 1990; Warren et al. 2000). Exposure to increasing TCDD dosage as well as influenza virus decreased the total number of T-cells (CD4+ and CD8+) in the mediastinal lymph node, but did not decrease inflammation due to increase in neutrophils. There was also a suppression of IL-12 and IFN-y in the mediastinal lymph node, suppression of IgG antibodies and increase in influenza-virus specific IgA (Vorderstrasse et al. 2002). TCDD also increased the severity of Epstein Barr Virus (EBV) related Sjogren's syndrome in humans in an AhR-dependent manner (Inoue et al. 2012).

Even though several studies have been done to analyze role of gut microbiota in T1D, only one study observed that TCDD reduced progression of T1D in female NOD mice (Kerkvliet et al. 2009). The protective effect by TCDD seen in this study could well be partially due to signaling pathways that alter its gut microbial population. Although studies with TCDD have not observed gender bias in T1D, more studies may reveal this trend, since TCDD affects other autoimmune diseases in a gender-specific manner. The above studies imply that gut microbiota might be responsible for affecting some of the outcomes observed in the TCDD studies with autoimmunity. Studies in autoimmune diseases investigating changes in abundance of key bacterial species would be helpful to better understand of the underlying processes. This could also highlight ways in which to close the gender gap and protect females from autoimmune disease progression.

2.3.1.4. Gut microbiota and TCDD assist the development of autoimmune diseases

Commensal bacteria have also been associated with several other autoimmune diseases including autoimmune encephalomyelitis (EAE) and celiac disease (Berer et al. 2011; Sellitto et al. 2012). To study the effects of multiple sclerosis, the relapsing-remitting mouse model is used that spontaneously develops EAE. For EAE to develop, myelin oligodendrocyte glycoprotein (MOG) autoantibody producing B cells are required as well as transgenic CD4+ T-cells that attack the central nervous system. GF mice do not develop EAE, although re-colonization with commensal microbiota reestablished EAE in them (Berer et al. 2011). These results indicate that the intestinal microbiota is required to activate MOG-specific T-cells in the gut-associated lymphoid tissue. Treatment with antibiotics reduced the amount of T-cells required to produce anti-MOG

autoimmune B cells that aid development of demyelinating encephalomyelitis. (Berer et al. 2011).

TCDD exposure has been well studied in two autoimmune diseases, EAE and experimental autoimmune uveoretinitis (EAU). EAE was induced by immunization of mice with MOG one day after exposure to 1ug/kg TCDD (Quintana et al. 2008). TCDD-dosed mice were protected from the development of EAE and showed an expansion of Foxp3+ Treg cells. TCDD induced CD4+ T-cells to differentiate into Foxp3+ Treg cells in these mice (Quintana et al. 2008). TGF-β1 was used as a positive control since this cytokine has the ability to convert CD4+ T-cells to Foxp3+ Treg cells. AhR^d mice which have a mutated AhR gene with reduced affinity for its ligands, was not able to prevent EAE when induced with TCDD, confirming that TCDD acts via the AhR ligand. TCDD-treated mice on activation with MOG peptide produced higher amounts of TGF-β1 and lower amounts of IL-17 and IFN-γ as there were less number of CD4+ T-cells with IL-17 or IFN-γ producing capability. They also observed that EAE development was not restricted in mice with a mutant TGF- β1 indicating that TGF- β1 is essential for control of EAE by TCDD (Quintana et al. 2008). FICZ, however, has antagonist properties, and in the presence of TGF β1, IL-6 and IL-23 was able to drive the differentiation of Th-17 cells and result in increased expression of IL-17 and IFN-γ, decrease of Treg cells and an increased progression of EAE disease (Quintana et al. 2008). MicroRNA mediation maybe another mechanism through which TCDD ameliorates EAE. MiRNA-132 expression by T-cells was observed in TCDD-treated mice along with a reduced expression of reactive T-cells, IL-17 and IFN-γ (Hanieh & Alzahrani 2013). This also attenuated EAE and was anti-inflammatory as MiR-132 targets acetylcholinesterase, an enzyme that hydrolyzes acetylcholine, which has anti-inflammatory properties (Hanieh & Alzahrani 2013). Similarly, EAU was also suppressed by exposure to

TCDD. EAU was induced in mice by introducing the human IRBP peptide (hIRBP-p) by immunization. EAU destroys the photoreceptor cells of the neural retina in mice, and is induced by Th17 cells and IL-17. Clinical signs of EAU were observed in control mice but not in TCDD treated mice. Inflammatory signs in the retina and optic nerves were observed for controls but not treated mice. TCDD immunized mice did not show elevated amounts of IFN-γ, IL-17 or IL-10 in their lymph node cells or splenocytes unlike the controls. They also observed that the suppressive action of TCDD on EAU occurs by the action of Foxp3+ Treg cells (Zhang et al. 2013).

Several studies have shown that TCDD is important for immunosuppression in graft vs host (GVH) models. TCDD induces donor CD4+ T-cells to differentiate into Treg cells in an AhR dependent mechanism that are able to suppress proliferation of cytotoxic T cells in the F1 mice (Funatake et al. 2005; Funatake et al. 2008). However, characterization of these CD4+ Treg cells showed many different properties as compared to natural Treg cells. Also, surprisingly, Foxp3+ expression was found in less than 2% of the induced Treg cells (Marshall et al. 2008). TCDD also suppressed production of IL-2 and increased production of TGF-β3, BLIMP-1, granzyme B, increased response to IL-12 pathway and increased phosphorylation of STAT3. Recently, TCDD via AhR has been found to exert immunosuppression in an allogenic heart model in mice (Cai et al. 2013). Here, TCDD prolonged the allograft transplant for more than 20 days; there was an increase in CD4+CD25+Foxp3+ Treg cells, decrease in IL-17 and IFN-γ and suppression of reactive T-cells.

In summary, TCDD causes immunosuppression of autoimmune diseases by inducing Treg cells and expanding their population via the AhR receptor. Foxp3+Treg cells have the ability to inhibit autoreactive T-cells and alters levels of IFN-γ, IL-17 and other inflammatory cytokines.

However, results from different studies are often contradictory and more work needs to be done to understand the signaling pathways involved in these diseases.

2.4. INTEGRATED VIEW OF CHANGES IN GUT MICROBIOTA WITH TCDD

These findings underscore that both gut microbiota and TCDD affect the immune pathways and immune molecules, specifically by recruiting T-cell populations. However, work still needs to be conducted to tease out whether this is a direct effect, that is, gut microbiota act on immune cells or if it is an indirect effect where by TCDD acts on immune pathways that in turn alter key gut microbial populations. Future studies will need to delve into the dioxin-microbial-immune axis that exist to maintain immune balance under toxic stress conditions. Moreover, it is evident that key bacterial species and groups rather than the commensal bacteria as a whole residing in the intestine play a major role in modifying immune responses and pathogenesis of autoimmune diseases. In light of this, we have recently found that higher doses of TCDD increase the abundance of SFB whereas decreasing the abundance of *Clostridium* IV and XIVa populations in the intestine (our unpublished results, Chapter 3). Moreover, these changes in bacterial abundance are more prominent with repeated doses of TCDD over time. These results corroborate with the earlier studies reviewed here and suggests that microbial populations act to restore immune balance that has been disrupted by TCDD exposure (Figure 2.1). Since TCDD upregulates Treg cells, Clostridia species that also induce production of Treg cells, decrease in abundance to maintain immune homeostasis. On the other hand, SFB abundance increases to replenish the Th17 cells that are inhibited by TCDD (Figure 2.1). Further studies would be

needed to prove this hypothesis and elucidate the mechanisms underlying the changes observed with respect to diseases when exposed to environmental contaminants.

APPENDIX

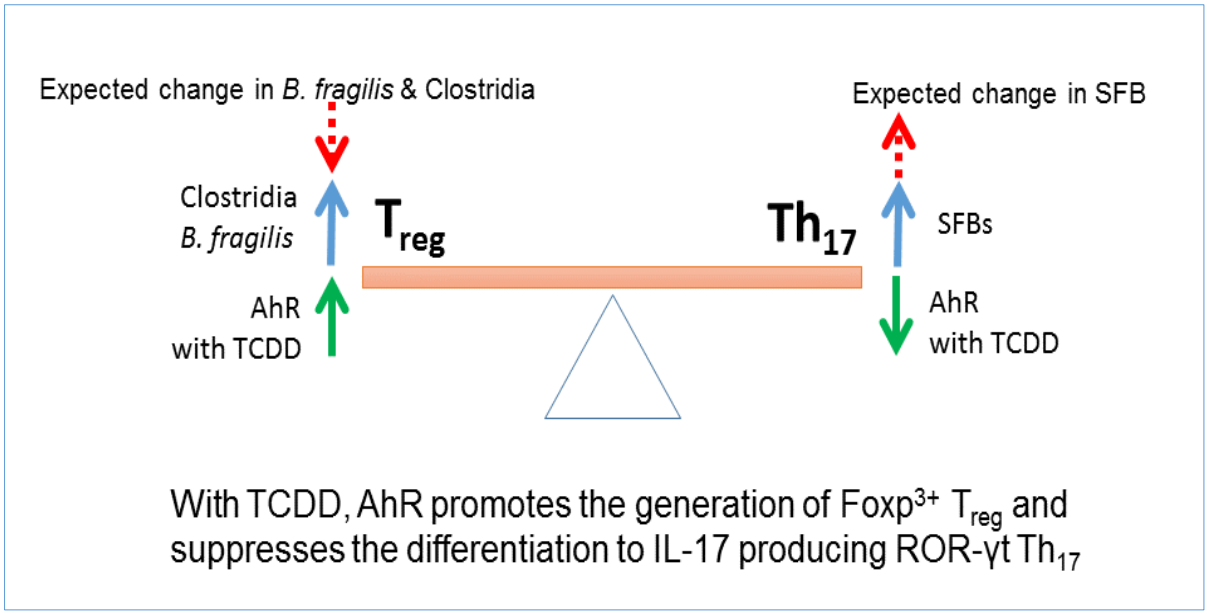

Figure 2.1. Integrated view of effect of TCDD on gut microbial populations

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CHAPTER 3: RESPONSE OF SEGMENTED FILAMENTOUS BACTERIA TO TCDD

Note: This chapter is to be submitted to the journal 'Environmental Health Perspectives' with the title "**Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin correlates with increased abundance of segmented filamentous bacteria in mouse gut microbiome**" by Prianca Bhaduri, Robert D. Stedtfeld, Tiffany Stedtfeld, Kelly A. Fader, Norbert E. Kaminski, Timothy R. Zacharewski, James M. Tiedje and Syed A. Hashsham.

3.1. ABSTRACT

Both 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) - an environmental contaminant and segmented filamentous bacteria (SFB) - a commensal gut bacteria, are implicated in modulating the host immune response via the aryl hydrocarbon receptor (AhR). In this study, abundance of SFB was examined as a function of TCDD exposure using C57BL/6 and Scd1 wild type (WT) mice. Experiments were conducted to evaluate their: i) exposure to eight different doses of TCDD over a period of 90 days followed by a 30-day recovery period (long-term with recovery), ii) exposure to repeated high doses of TCDD over a period of 30 days (short-term – high dose), and iii) exposure to low doses of TCDD over a period of 12 days (short-term - low dose). Abundance of SFB was quantified in fecal pellets over the study periods and in intestinal luminal flushing at the end of experiments. Exposure to TCDD resulted in increased abundance of SFB in all three experiments. A dose-dependent increase in abundance of SFB was also evident in the long-term experiment, with up to 10-fold change in SFB abundance (as measured by qPCR of SFB 16S rRNA gene) for TCDD doses ranging from 1-30 µg/kg of body weight administered every 4 days. When TCDD dosing stopped after 90-days, SFB abundance continued to increase

for an additional 12 days before declining to pre-exposure levels. Short-term high and low dose experiments also resulted in an increase in SFB abundance but the fold-change was lower than the highest dose in long-term experiment. These results demonstrate that SFB in mouse intestinal tract are responsive to TCDD exposure and suggest that specific gut bacterial populations play a role in addressing the challenges posed to the host by environmental toxicants such as TCDD.

3.2. INTRODUCTION

Dis-regulation of key commensal bacterial groups in the gut microbiome have been implicated in the pathogenesis of a range of diseases including obesity, diabetes, heart disease, metabolic syndrome, autoimmune disorders, and pathogen immunity (Tremaroli and Bäckhed 2012; Kamada and Núñez 2013). Segmented filamentous bacteria (SFB) participate in the development and maturation of host immunity including B-cell and T-cell differentiation and production of intestinal IgA (Suzuki et al. 2004; Ivanov et al. 2009; Chung et al. 2012). Studies have shown that ingestion of SFB partially restored the aberrant immune system of germ-free mice, (Chung et al. 2012) and protected against pathogenic infections (Heczko et al. 2000; Ivanov et al. 2009; Rodrigues et al. 2012). Dysbiosis of SFB has also been associated with patients with irritable bowel syndrome (Shukla et al. 2015). Such dysbiosis results in increased differentiation and expansion of auto-reactive T cells in mouse models exacerbating inflammation in colitis, experimental autoimmune encephalomyelitis, and arthritis (Lee et al. 2011; Chappert et al. 2013).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental toxin that acts via the aryl hydrocarbon receptor (AhR) to cause several harmful health effects including embryonic and developmental toxicities, impaired immune function, weight loss and carcinogenesis in humans

and animal models (Whitlock 1990). TCDD is known to target the immune system and cause extensive adaptive immune suppression, increasing the likelihood of infectious diseases in mice (Marshall et al. 2008). T-cell populations are particularly susceptible to the effects of TCDD exposure. Several studies have shown that TCDD acts via the AhR pathway to increase the number of Foxp3+ regulatory T-cells (Tregs) to Th17 cells (Funatake et al. 2005; Quintana et al. 2008). TCDD also reduces inflammation via decreasing T-cells, primarily IL-17 producing Th17 cells and several corresponding inflammation markers (Quintana et al. 2008; Zhang et al. 2010). As a result of altered immune response, TCDD reduces inflammation associated with inflammatory bowel disease, dextran sulfate sodium-induced and trinitrobenzene sulphonic acid colitis (Benson and Shepherd 2011; Singh et al. 2011). TCDD also plays an important role in ameliorating pathogenesis of several autoimmune diseases including murine systemic lupus erythematosus, experimental autoimmune encephalomyelitis, experimental autoimmune uveoretinitis (an autoimmune disease that targets the neural retina and related tissues) and skin transplantation by increasing Tregs via the AhR pathway (Quintana et al. 2008; Zhang et al. 2010; Schulz et al. 2011, 2012).

Previous studies have observed that SFB adapts to different host conditions by changing their abundance in the gut. Activation-induced cytidine deaminase (AID) regulates the production of certain immunoglobulins including IgA from B-cells by class switch recombination and somatic hypermutation (Durandy 2003). SFB increased from 30% to 70% of total bacteria in AID-deficient mice as quantified using qPCR of the 16S rRNA gene but subsequently returned to normal levels after the immune system in $AID^{-/-}$ mice was restored by anamastosis (Suzuki et al, 2004). Similarly, there was more than 100-fold increase in the ratio of SFB to total bacteria in the gut of MMP9-deficient mice with compromised intestinal epithelial barrier (Rodrigues et al.

2012). Also, mice immunosuppressed with cyclophosphamide displayed increase in SFB from 10% to 40% (Fuentes, 2008). Although these results indicate that SFB responds to certain changes in host pathways, studies have not explored its shift with exposure to environmental contaminants, specifically dioxins.

Since both SFB and dioxins exhibit pronounced effects on host immunity, we evaluated the influence of TCDD on the gut microbiota focusing on SFB. We hypothesized that the exposure to TCDD will cause an increase in SFB abundance, and this increase will be directly proportional to TCDD dosage. The hypothesis was tested using a number of TCDD doses over 90 days (0-30 µg/kg every 4 days) followed by a 30-day recovery period (referred to as long-term with recovery (LTR) study), a short-term high dose (SH) study (30 µg/kg every 4 days, 30 days), and a short-term low dose (SL) study (7.5 µg/kg for 4 consecutive days, 12 days). In all three experiments, TCDD-elicited significant elevation of SFB in mouse fecal pellets and intestinal luminal flushing, increasing as much as 10-fold compared to controls. A decrease in SFB was observed during the recovery period. To our knowledge, this is the first study demonstrating that TCDD exposure results in increased abundance of SFB. These findings have implications in understanding host-microbe-toxicant interactions.

3.3. MATERIALS AND METHODS

3.3.1. Animal handling and treatment

Three experiments were conducted including: i) exposure to eight different doses of TCDD over a period of 90 days followed by a 30-day recovery period (LTR study), ii) exposure to high doses of TCDD over a period of 30 days (SH study), and iii) exposure to low doses of TCDD over a period of 12 days (SL study). Additional details of these studies are provided below. All animal handling procedures were carried out with the approval of the Michigan State University All-University Committee on Animal Use and Care.

3.3.1.1. Long-term with recovery (90-day) study

Female C57BL/6 mice on postnatal day 25 (PND25) were obtained from Charles Rivers Laboratories (Portage, MI). Upon arrival, mice were housed in polycarbonate cages with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) at 30-40% humidity and a 12h light/dark cycle and acclimatized for 4 days. Mice were fed *ad libitum* with Harlan Teklad 22/5 Rodent Diet 8940 (Madison, WI) and had free access to deionized water. On PND28 and every following 4^{th} day animals (n = 10) were dosed by oral gavage with 0.1 mL sesame oil vehicle control (Sigma), 0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 30 μg/kg TCDD (Dow Chemical Company, Midland, MI) for a total of 90 days (23 doses; Figure S3.1). Additional mice (n=10) were also dosed for 90 days with either sesame oil vehicle control or 30 μg/kg of TCDD every 4 days and then allowed to recover for 30 days without dosing (henceforth referred to as recovery group).

Five mice were housed per cage and there were two cages for each TCDD dose. Mice fecal pellets were collected every 8th day starting from day 35 post initial TCDD dosing until the mice were sacrificed. Pellets were immediately stored in -80°C after collection. There were seven collections for all mice sacrificed after 90 days and 12 collections for the recovery groups (treated and control). Intestinal luminal flushings were also collected after the mice were sacrificed at the end of the dosage and recovery periods. Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS; Sigma) was flushed through the intestinal lumen of the gastrointestinal tract of each mouse and the resultant flushing was stored at -80°C until DNA was extracted.

3.3.1.2. Short-term high dose (30-day) study

Female C57BL/6 mice on PND25 were obtained from Charles Rivers Laboratories (Kingston, NY). Upon arrival, mice were housed in individual cages (Innocages, Innovive, San Diego, CA) with ALPHA-dri® bedding (Shepherd Specialty Papers, Chicago, IL). Other procedures were similar to that described in the 90-day study. The dosing of mice was staggered by day with 4 animals dosed daily. Between PND28 to PND31 and every following 4th day animals (n=8) were dosed by oral gavage with 0.1 mL sesame oil vehicle control or 30 μg/kg TCDD (Dow Chemical Company, Midland, MI) for a total of 28 days (7 doses).

Fecal pellets were collected daily beginning two days prior to the first dose of TCDD. Pellets were immediately stored at -80°C prior to DNA extraction. Luminal flushings were also collected at the end of the study period after mice were sacrificed *(*Figure S3.2).

3.3.1.3. Short-term low dose (12-day) study

B6.129-*Scd1*^{tm1Myz}/J heterozygous mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the Michigan State University Laboratory Animal Care facility. Mice were maintained on a 12-h light/dark cycle and housed in autoclaved polycarbonate cages with microisolator lids containing aspen woodchips and nesting material. Animals were allowed free access to Harlan Teklad irradiated F6 rodent diet 7964 (Madison, WI) and autoclaved deionized water throughout the study. On PND21 mice were genotyped by ear punch and weaned. Female *Scd1*$^{+/+}$ mice (n=3) on PND28 were dosed by oral gavage with sesame oil vehicle control or 7.5 μg/kg TCDD (Dow Chemical Company, Midland, MI) for 4 consecutive days. Mice were sacrificed 7 days after the first dose. Fecal pellets were collected from each cage for 6 days before TCDD treatment started and 6 days following the first TCDD dosage for a total of 12 days (Figure S3.3). Fecal pellets were stored immediately at -80°C following collection.

3.3.2. DNA extraction

One hundred milligrams of mice fecal pellets were used for DNA extractions, processed using the PowerMax Soil DNA Isolation Kit following the manufacturer's protocol (MOBIO Laboratories). DNA samples were quantified on a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Products, Wilmington, DE). DNA integrity was determined by gel electrophoresis. Extracted DNA was stored at −20°C. Luminal flushing samples were concentrated by centrifugation prior to DNA extraction which was performed using the same kit and procedure as fecal pellets.

3.3.3. Quantification of relative SFB abundance

Quantitative PCR (qPCR) was used to quantify abundance of SFB. Previously described primers were used including the 736F and 844R primers of 16S rRNA gene targeting mouse SFB and the FliC gene for mouse flagellinC3 (Table 3.1) (Bouskra et al. 2008; Prakash et al. 2011). Universal 27F was also used for validation of amplicon sequences via Sanger sequencing. Amplicons were purified and sent to the Research technology Support Facility (RTSF) at Michigan State University (MSU) to be sequenced using the 96-capillary electrophoretic ABI 3730xl platform. Quantitative PCR reactions were performed in the massively parallel Wafergen platform. Amplification was performed in 100 nL reaction wells containing a final concentration that included: 1×LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), nuclease-free PCR-grade water, 1 ng/µL DNA template, and 1 µM of each forward and reverse primer. Thermal cycling consisted of initial denaturation at 95 ^{o}C for 3 min, followed by a 40 cycles of denaturation at 95 ^{o}C for 30s, annealing at 60 ^{o}C for 1 min, and a melting curve analysis that consisted of scanning every 0.4 ^{o}C increase from 60 and 97 ^{o}C. For each primer set, amplification was conducted in triplicate. All reactions were run in triplicate and primers were also tested using a no-template control.

To confirm results and generate amplicons (used for sequencing and standard curves) some samples were also tested in the ABI Prism 7900HT using Power SYBR Green PCR Master Mix (Lifetech). For each 20 µl qPCR reaction, 1 ng of extracted DNA was added along with 18 µl Master Mix and 1 µl of 10 mM primer mix. PCR conditions were performed as previously described (Bouskra et al. 2008). Briefly, cycling consisted of an initial 95 °C for 5 min, 40 cycles of 95 °C for 55 sec, 60 °C for 55 sec and 72 °C for 1.5 min. All reactions were performed in

triplicate. The 384-well plates were processed on the ABI Prism 7900HT Sequence Detection System at the RTSF.

Concentrations of SFB in mouse fecal pellets and luminal flushings were calculated using standard curves obtained from 10-fold dilutions of purified amplicons of the 27F universal and 844R SFB primers. Amplicons were purified using the Qiagen PCR purification kit. To normalize data and calculate relative SFB abundance, the total amount of bacteria was estimated using universal primers targeting the 16S rRNA genes (Table 3.1) (Leigh et al. 2007). Standard curves of the 16S rRNA gene universal primer set was generated using 10-fold dilutions of DNA from pure culture of type strain *Shigella flexneri* ATCC 24577.

3.3.4. Validation of SFB qPCR primers and methodology

The qPCR primers were validated by amplifying DNA from fecal pellets and Sanger sequencing of purified amplicons. The 16S rDNA amplicon was obtained with fecal pellet samples and both the universal 27F forward primer and SFB-specific 844R reverse primers (Prakash et al. 2011). Both sequenced samples showed 99% identity with published mouse SFB sequences in the NCBI database (Accession Numbers: CP008713.1, AP012209.1 and AP012202.1). The mouse SFB flagellin (FliC) gene amplicon was also Sanger sequenced and showed 100% identity to *Candidatus Savagella* for rat and mouse (NCBI accession numbers: CP008713.1, AP012209.1 and AP012202.1) when queried using nucleotide BLAST.

3.3.5. Data analysis and statistics

Threshold cycle (Ct) was generated from real time amplification curves analyzed using default parameters of the SmartChip Wafergen qPCR software (V 2.7.0.1). A threshold cycle (Ct) of 30

was used as the cutoff detection limit, thus a Ct greater than 30 was considered a false positive amplification event. Only samples with amplification in two or more of the three technical replicates were considered positive. Standard curves generated for the 16S rRNA gene targeting SFB and the universal bacteria were used to estimate number of copies per reaction. Since dilution series for FliC gene was not available, copy numbers for it were estimated as described previously (Looft et al. 2012) using the following equation:

$$\text{FliC gene copy number} = 10^{\left[\frac{(30-Ct)}{3.33}\right]}$$

The relative SFB abundance was calculated by dividing SFB copy numbers over the universal bacteria copy numbers. Fold changes were calculated by dividing relative SFB abundance in TCDD treated mice over relative SFB abundance in the vehicle controls. Mean and standard error measurements (SEM) and plots were generated using Excel 2010. Box plots were generated using SigmaPlot 13.0. A paired t-test was used to test for significant differences with a significance cutoff of $P \leq 0.05$.

3.4. RESULTS

Three studies were performed including a long-term 90-day study with varying doses and a 30-day recovery period, short-term high dose study (30 days) and a short-term low dose study (12 days). Luminal flushing was obtained after sacrificing mice from the long-term study. Relative abundance of SFB was measured temporally in fecal pellets to monitor levels of SFB before and after treatments. Bacterial community DNA was extracted from the feces of vehicle (control) and treated mice, and the relative amount of SFB in total bacteria was determined by qPCR using universal primers specific to 16S rRNA genes (Lane 1991).

3.4.1. Long-term with recovery (90-day) study

Fluctuations of SFB abundance was examined in C57BL/6 mice with repeated exposure to various doses of TCDD for 90 days. For the duration of the LTR study, the vehicle group had a relative SFB abundance between 0.06% and 0.34%, while the dosed groups had increasing levels of SFB with increasing doses (Figure 3.1). A paired t-test showed that four of the TCDD treated groups, 1 μg/kg (p=0.005), 3 μg/kg (p=0.009), 10 μg/kg (p=0.002), and 30 μg/kg (p=0.005)) had significantly higher levels of 16S rRNA gene targeting SFB as compared to the vehicle control groups (Figure 3.1A). From the figure, it can also be observed that SFB abundance decreased over time within each dosed group throughout the duration of the experiment. Significant differences were also observed with the FliC gene in groups treated with 10 (P=0.002) and 30 (P=0.02) μg/kg TCDD (Figure 3.1B).

Following the last dose of 30 μg/kg TCDD, which occurred on day 87, the relative 16S rRNA gene targeting SFB and FliC gene abundance appeared to increase further, nearly doubling compared to the amount of SFB observed during the period 35 to 83 days of repeated dosing (Figure 3.1C, 3.1D). The SFB increase continued during the first 12 days of recovery, but subsequently decreased during the remaining 18 days of recovery period. Even after the 30-day recovery period, the population of SFB in the dosed mice remained higher (but not significant) compared to SFB in the control mice (0.09±0.01% in control group vs 1.45±1.15% in recovery group).

3.4.2. Luminal flushing of TCDD dosed mice in LTR

Although fecal pellets were used to measure SFB abundance over time, intestinal luminal flushing was also examined post sacrifice to evaluate correlations in relative SFB abundance between TCDD exposed and control mice. Luminal flushing samples were collected from groups of mice sacrificed immediately following 90 days of repeated 30 μg/kg TCDD dosing and from groups 30 days post cessation of dosing. There was an obvious difference in relative SFB abundance between luminal flushing of dosed and control groups (Figure 3.2), however, this difference was not significant. There was also a downward trend in relative SFB populations following 30 days of recovery, compared to the group of mice that were sacrificed prior to recovery, but this difference was also not significant. Non-significant results were partially due to a lack of replicates. Only 5 of 10 dosed mice survived the initial 90 day treatment group and 3 out of 10 dosed mice survived the entire 120 day experiment including recovery. Including both vehicle control groups, 17 out of 20 mice survived the duration of the experiments. The relative SFB abundance in luminal flushing was also higher than in fecal pellets (Table S3.1). Previously described results also observed a correlation in relative SFB abundance between fecal pellets and the distal small intestine, with intestines showing a higher abundance of SFB compared to fecal pellets (Kriegel et al. 2011).

3.4.3. Short-term high dose (30-day) study

A short-term study with repeated doses of 30 μg/kg TCDD was performed to further observe variability in individually housed mice and to examine influence during an initial dosing period. The study included analysis of fecal pellets collected for the first three days prior to dosing, followed by analysis of pellets every second day for a 30-day time period (with 30 μg/kg TCDD

dosing every 4 days). Of note, pellets were collected daily, however, only pellets from every second day were examined. Since initial populations of gut commensal bacteria can vary greatly between individual mice, this study would be able to pick up trends that were lost in the LTR study in which five mice that were cohoused per cage. The SH study showed a six-fold increase in relative SFB abundance for treated versus vehicle mice (Figure 3.3, Figure S3.4, S3.5). Differences in relative SFB abundance between TCDD and vehicle dosed mice became significant on day 7 (4 days post initial dose of TCDD, P=0.0139) and day 13 (10 days post initial dose of TCDD, P=0.0063) for the 16S rRNA gene primer set targeting SFB and the FliC gene, respectively (Figure 3.3). Differences in SFB gene copy numbers continued to be significant throughout the remaining duration of the 30 day experiment for both the 16S rRNA gene targeting SFB and the FliC gene.

3.4.4. Short-term low dose (12-day) study

Relative SFB abundance was also higher in fecal pellets of *Scd1*$^{+/+}$ mice dosed with TCDD (7.5 µg/kg). On day 12 (5 days post start of TCDD dosing), abundance of SFB was 21.1±4.03% of the total 16S rRNA gene in TCDD-dosed mice and 4.0±4.05% in the control mice (Figure 3.4). Temporally, the difference in relative SFB abundance between dosed and control mice was significant (P=0.040) only on day 12 of the experiment (i.e., 6 days after start of TCDD dosing). Error bars in Figure 3.4 reflect a variation in the initial population abundance of SFB between the *Scd1*$^{+/+}$ mice in dosed and control groups. Despite considerable variability in the initial SFB abundance between the *Scd1*$^{+/+}$ mice in the control and treated groups, the SFB abundance in all three of the TCDD-treated mice showed a significant increase by day 12 of the experiment (Table 3.2). The variation of relative SFB abundance observed between mice in all studies may

be due to inter-individual variations among mice, which has been described previously among gut microbial populations (Hildebrand et al. 2013).

3.5. DISCUSSION

SFB and TCDD exposure is known to modulate the host immune system; however, this has not been studied in conjunction. Differences in relative SFB abundance observed between control mice and TCDD-treated mice is similar to other studies that compromised mouse immunity via knockouts (Table 3.3). For example, studies by Qiu and coauthors, which also quantified relative SFB abundance of 16S rRNA gene using qPCR, observed less than 1% in control mice and 1-6% SFB in AhR deficient mice (Qiu et al. 2013). Vaishnava and coauthors observed that SFB differed from 1% in control mice to 4% in Reg3$\gamma^{-/-}$ modified mice using 16S rRNA gene group targets (Vaishnava et al. 2011). Similarly, mice deficient in lymphotoxin (which helps maintain mucosal immunity), displayed 10-fold increase in SFB abundance on both normal chow and high fat diet (Upadhyay et al. 2012). Similarly rorc $^{-/-}$ mice, deficient in lymphocytes for production of IL-22, displayed 15-fold increase in SFB when fed a high fat diet (Upadhyay et al, 2012). Likewise, studies that have utilized mice models for autoimmunity, diabetes and colitis observe that SFB exhibits a tendency to proliferate under disease conditions (Table 3.3). Recent studies have observed the production of Th17 cells by SFB is mediated by the major histocompatibility complex (MHC) Class II antigen presentation in the gut ; Geem et al. 2014; Goto et al. 2014; Zhang et al. 2014); whereas TCDD doses of 10 and 30 μg/kg were shown to suppress the expression of MHC class II antigens (Boverhof et al. 2005; Fader et al. 2014, Fader et al. 2015, manuscript submitted). The suppression of MHC class II antigens was attributed to decreased populations of intestinal lamina propria macrophages and dendritic cells, which are

both antigen presenting cells that express MHC class II markers, observed in mice exposed to repeated doses of 30 μg/kg TCDD for 28 days (Fader et al, manuscript submitted). This would influence Th17 cell induction even in the presence of SFB (Geem et al. 2014; Goto et al. 2014). This coincides with previous TCDD studies observing that the ratio of Tregs to Th17 cells increases (Bankoti et al. 2010; Duarte et al. 2013), which is likely the result of reduced levels of Th17 cells. Differences in relative SFB abundance between treated and control groups agrees with the known effect of TCDD as an agonist of the AhR pathway (Denison et al. 2011). The influence of TCDD on the MHC class II antigens causes deficiencies in CD4+ T-cells in proximal intestine in the 30-day study, thus inducing proliferation of SFB (Figure 3.5) (Fader et al, manuscript submitted). This figure illustrates the notion that TCDD exposure impacts the immune system in a manner that is counterbalanced by an increase in SFB levels. Thus, Th17-inducing SFB increase to make up for the interleukin deficiencies caused by TCDD exposure to maintain immune homeostasis.

Exposure to 2,3,7,8-tetrachlorodibenzofuran (TCDF), another persistent organic pollutant that causes inflammation associated disorders via the AhR, decreased the abundance of intestinal SFB in mice and increased Th17 inflammation markers including *Il-1β, Il-10, Tnf-α, Saa1* and *Saa3* (Zhang et al. 2015). As such, TCDF and TCDD have similar immune responses in the body but illicit opposite effects in terms of dysbiosis in SFB abundance. Decreased levels of SFB have been associated with IBD-constipation, while increased levels of SFB associated with IBD-diarrhea (Shukla et al., 2015). One possible mechanism for IBD may involve ingestion of different persistent organic pollutants. Taken together, SFB abundance in fecal matter has the potential to serve as a non-invasive biomarker for immune gut health and chronic contaminant exposure.

Overall, these results demonstrate that exposure to the environmental contaminant TCDD influences a key gut microbial population (SFB), rendering microbial dysbiosis in the host. This dysbiosis might instigate observed TCDD-related dysfunctions including immune suppression, autoimmune diseases and diabetes. More studies are needed to clarify the mechanism of TCDD-related diseases and whether key bacterial species are indirect players in disease suppression and/or progression.

3.6. ACKNOWLEDGEMENTS

The work was supported by the Superfund Research Program (2 P42 ES004911-22A1) from the National Institute for Environmental Health Sciences.

APPENDICES

APPENDIX A

TABLES AND FIGURES

Table 3.1. Primers used for qPCR to measure abundance of SFB in mouse fecal pellets and luminal flushing.

The 27F primer was combined with the SFG_844R primer to create amplicons used for Sanger sequencing and generating standard curves.

Forward primer	Sequence	Reference
27F	AGAGTTTGATYMTGGCTCAG	(Lane 1991)
SFB_736F	GACGCTGAGGCATGAGAGCAT	(Bouskra et al. 2008)
SFB_844R	GACGGCACGGATTGTTATTCA	
1132F	GGGTTGCGCTCGTTGC	(Leigh et al. 2007)
1108R	ATGGYTGTCGTCAGCTCGTG	
SFB_FliC_F	TGGGGATCGATGGTATGAAT	(Prakash et al. 2011)
SFB_FliC_R	GCTCCAAGTTTAGTTCTTGCATT	

Table 3.2. Relative SFB abundance in short-term - low dose study. Quantitative PCR of 16S rRNA gene targeting SFB on individual mice prior to dosing (days 1, 3, and 6) and post start of dosing (days 9 and 12) with vehicle or 7.5 µg/kg of TCDD for four conservative days. ND indicates no amplification, * indicates significantly different ($P \leq 0.05$) SFB population compared to mean abundance of the individual mouse during the first six days prior to treatment, shaded region represents data from mice dosed with TCDD.

Mouse	Day 1	Day 3	Day 6	Day 9	Day 12
Dosed mouse 1	ND	3e-4 ± 1e-4%	0.09 ± 0.09%	6.4 ± 1.2%*	13.1 ± 6.6%*
Dosed mouse 2	6.2 ± 2.6%	3.5 ± 1.6%	11.8 ± 5.5%	4.5 ± 0.2%	24.8 ± 1.1%*
Dosed mouse 3	20.7 ± 1.87%	16.6 ± 2.3%	8.5 ± 2.9%	6.5 ± 3.6%	25.6 ± 4.8%*
Vehicle mouse 1	0.02 ± 0.02%	2e-4 ± 3e-5%	4e-4 ± 1e-4%	3e-4 ± 1e-4%	5e-4 ± 2e-4 %
Vehicle mouse 2	3e-4 ± 1e-4%	6e-4 ± 1e-4 %	4e-4 ± 4e-5%	2e-4 ± 3e-6%	4e-4 ± 9e-5%
Vehicle mouse 3	8.4 ± 1.2%	7.4 ± 0.16%	12.1 ± 0.81%	8.9 ± 0.54%	12.1 ± 0.57%

Table 3.3. Examples of studies reporting change in abundance of SFB under various conditions.

Tissue Sample	Change in SFB level	Condition studied	Reference
Method of SFB quantification: Percentage of total bacteria			
Fecal pellets	↑	Deficiency of AhR	Qiu et al. 2013
Ileum	↑	Immunosuppression with cyclophosphamide	Fuentes et al. 2008
Ileum, jejunum	↑	Deficiency of myD88 (absence of bacterial signaling pathways)	Larsson et al. 2012
Fecal pellets	↑	Deficiency of MMP9 (MMP9 is responsible for inflammation in colitis)	Rodrigues et al. 2012
Cecum	↑	Pathogen-free mice (absence of Altered Schaedlers' Flora)	Chappert et al. 2013
Biopsy of small intestine	↑	AID deficiency (alters immune system)	Suzuki et al. 2004

Table 3.3 (cont'd)

Fecal pellets	↑	Reg3γ deficiency (Reg3γ helps maintain epithelial barrier)	Vaishnava et al. 2011
Ileum	↑	Female non-obese diabetic mice (SFB associates with protection from diabetes)	Kriegel et al. 2011
Fecal pellets	↓	Treatment with penicillin	Cox et al. 2014
Method of SFB quantification: Fold change			
Fecal pellets	↑	Germ-free mice (autoimmune model) that are prone to arthritis	Wu et al. 2010
Fecal pellets	↑	Rorc deficient mice fed high fat diet	Upadhyay et al. 2012
Fecal pellets	↑	Lymphotoxin deficient mice	Upadhyay et al. 2012
Fecal pellets	↓	Treatment with TCDF	Zhang et al. 2015

Table 3.3 (cont'd)

Method of SFB quantification: Units of SFB/ gram of tissue			
Fecal pellets	↑	In mice microbiome (compared to human-derived microbiome in mice)	Chung et al. 2012
Ileum	↑	Conventional mice (SFB correlates with increase in luminal IgA)	Ohashi et al. 2010

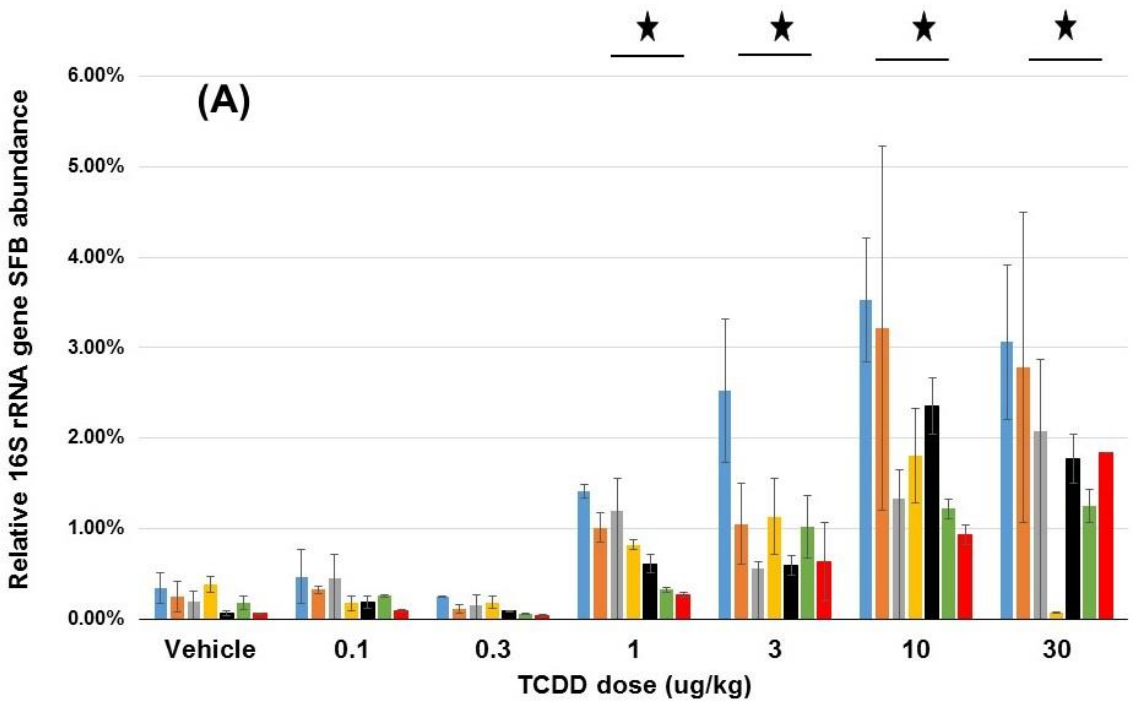
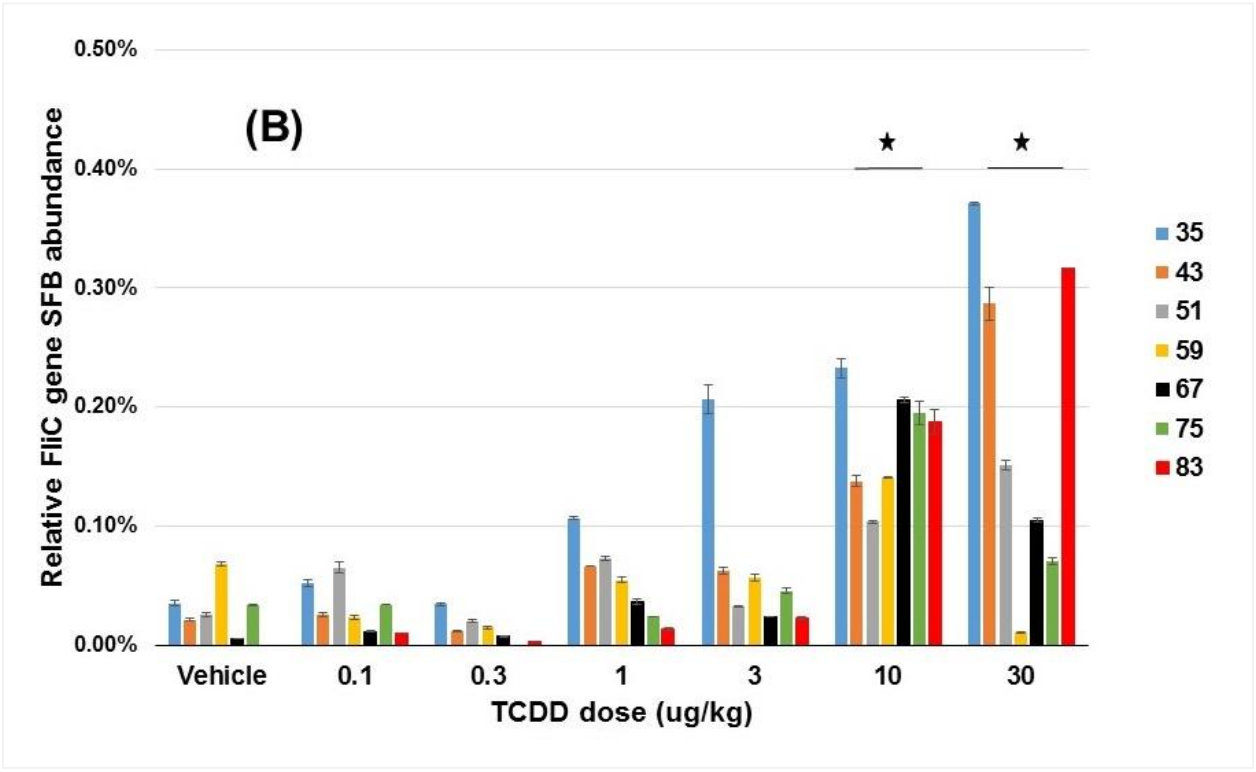


Figure 3.1. Long term dose response and recovery of TCDD-mediated influence on relative SFB abundance. (A) Relative abundance of 16S rRNA gene targeting SFB, and (B) FliC gene with vehicle and TCDD doses of 0, 0.1, 0.3, 1, 3, 10 and 30 µg/kg TCDD every 4 days. Groups dosed with 0.01, 0.03 µg/kg TCDD showed no difference in relative SFB abundance compared to vehicle group and are omitted. Fecal samples were collected on days 35, 43, 51, 59, 67, 75, and 83. (C) Relative 16S rRNA gene SFB abundance and (D) Flic gene abundance from recovery groups during TCDD dosing (days 35-83) and after dosing (days 91-120). Error bars represent the standard error of mean for two groups of five cohoused mice in each group.

Figure 3.1 (cont'd)



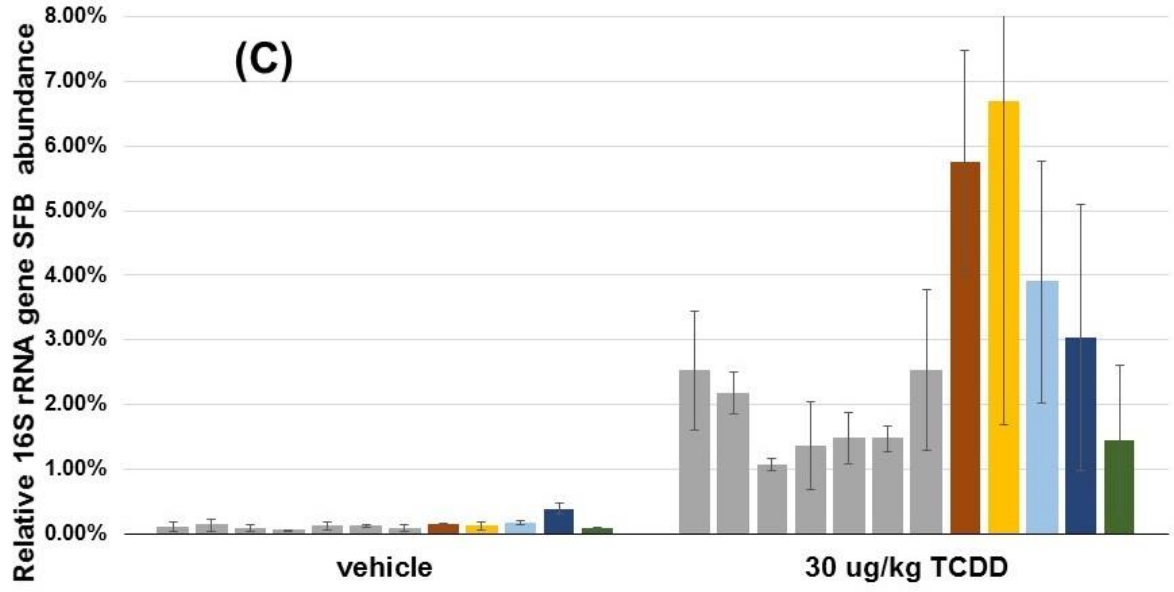
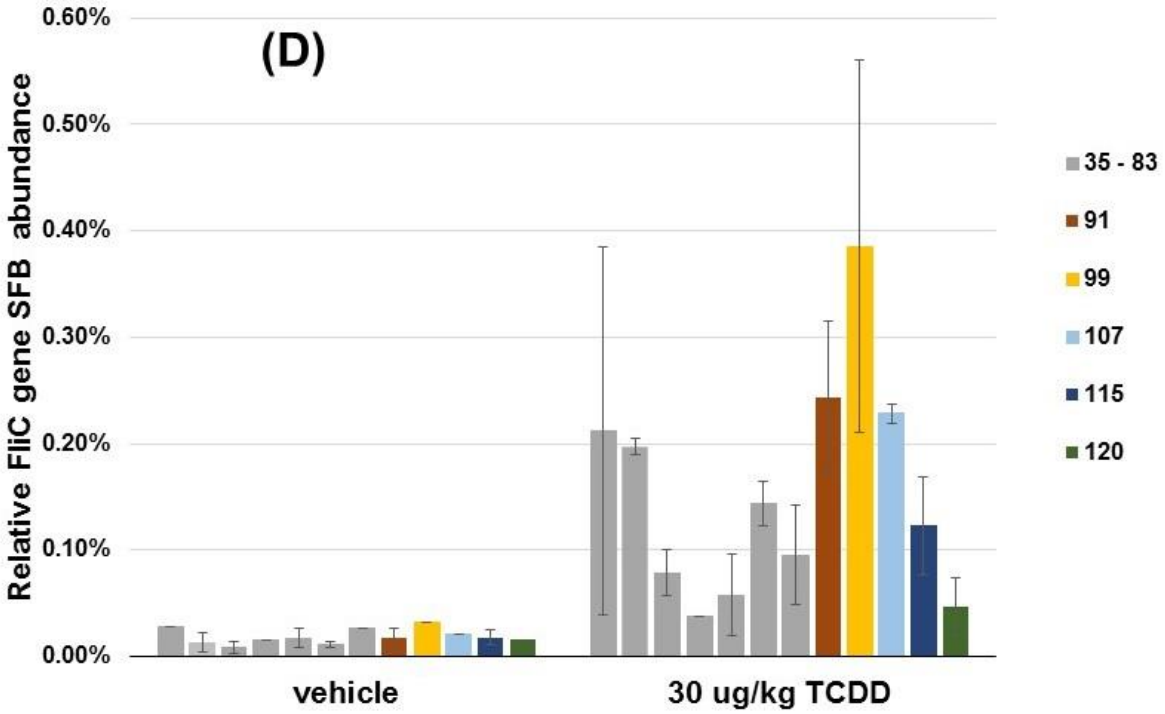


Figure 3.1 (cont'd)



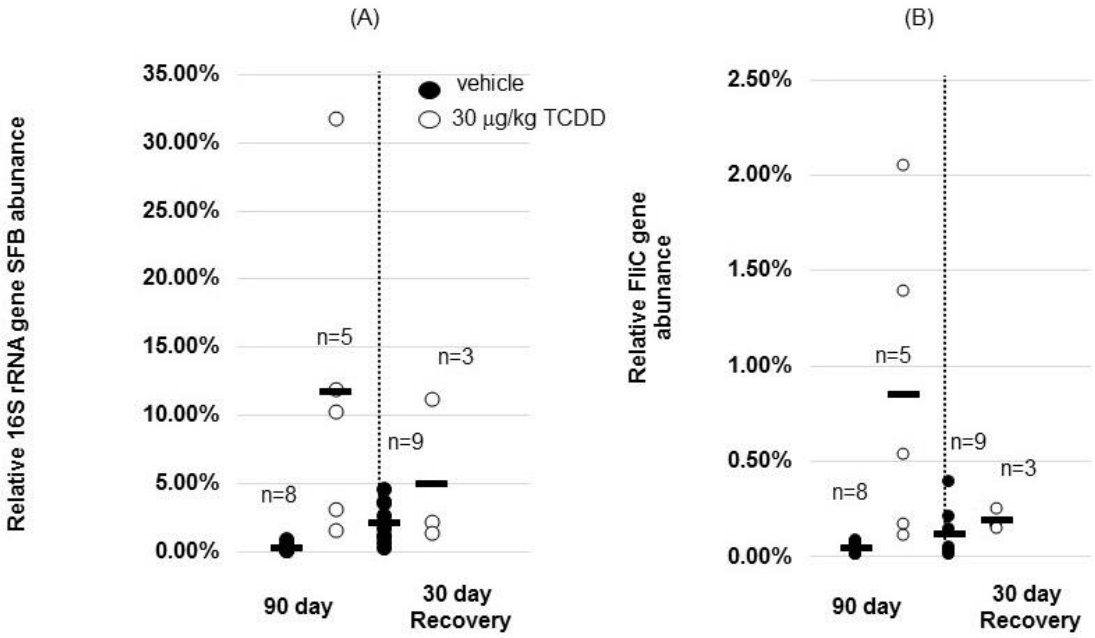


Figure 3.2. Relative SFB abundance in intestinal luminal flushing samples immediately following 90 days of repeated 30 µg/kg of TCDD dosing, and 30 day recovery post cessation of TCDD dosing. Relative SFB abundance measured using (A) 16S rRNA gene targeting SFB and (B) FliC gene. Black bars represent mean relative abundance of luminal flushing from mice that lived throughout the duration of the experiment.

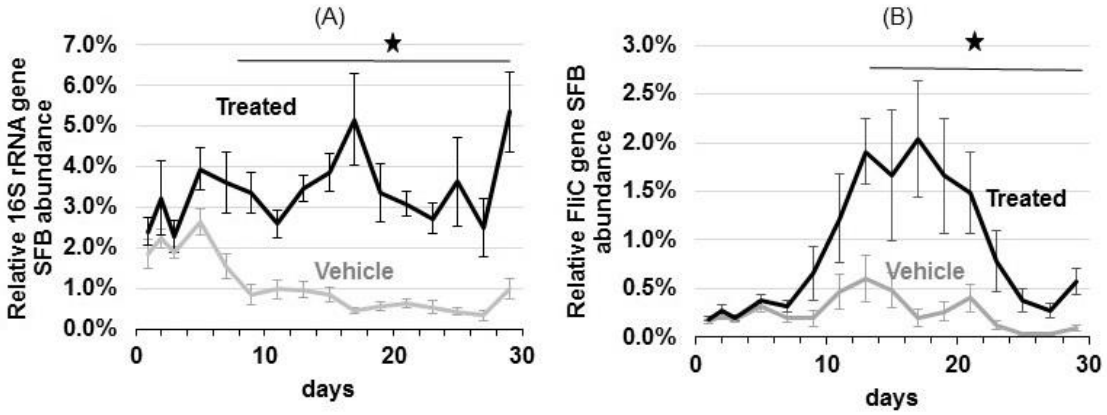


Figure 3.3. Short term relative SFB population with repeated TCDD dosing every four days. Mean of eight individually caged mice dosed with 30 µg/kg TCDD (black lines) and vehicle dosed control mice (gray lines) for the (A) 16S rRNA gene targeting SFB, and (B) FliC gene. Error bars represent the standard error of mean.

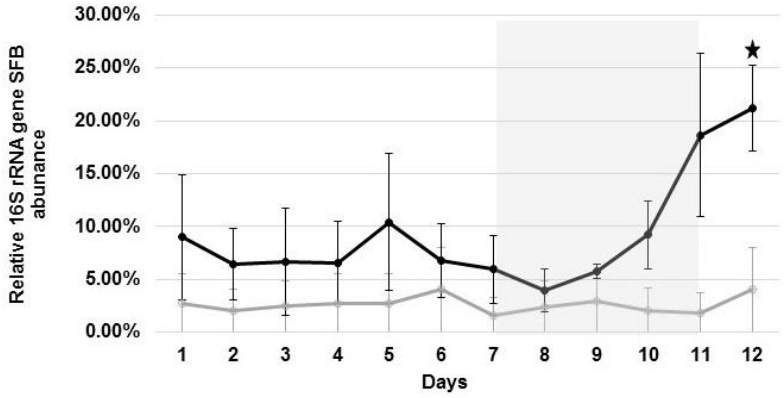


Figure 3.4. Relative SFB population during short 12 day study with 7.5 µg/kg per day for four consecutive days (gray background). Data represents mean of three mice dosed with TCDD (black lines) and vehicle dosed mice (gray lines). Error bars represent the standard error of mean.

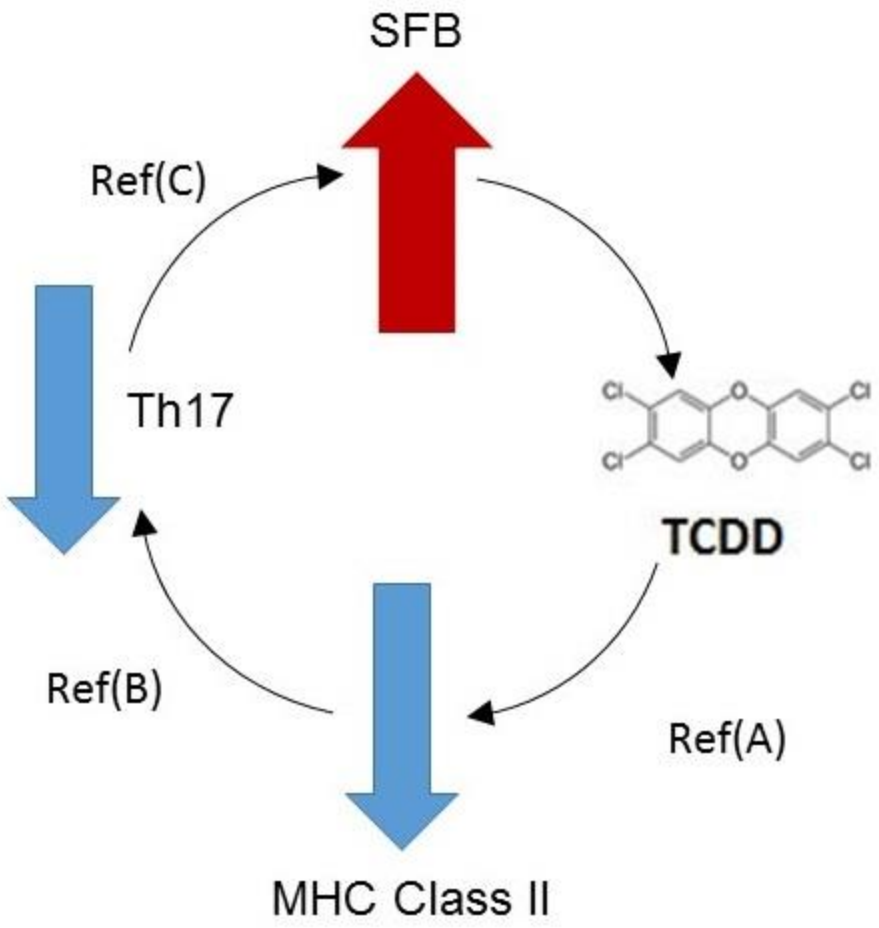


Figure 3.5. Integration of TCDD response on SFB observed in this study with immunological imbalances previously observed and TCDD studies on host expression. Key to references in figure: A –(Fader et al. 2015, maunscript submitted), B - (Boverhof et al. 2005; Geem et al. 2014; Goto et al. 2014; Zhang et al. 2014), C - this study.

APPENDIX B

SUPPLEMENTAL MATERIAL

Table S3.1. Relative SFB abundance measurements in fecal pellets and luminal flushing.

Numbers indicate mean and standard error of mean.

Assay	Sample	90 days vehicle	90 days TCDD	Recovery vehicle	Recovery TCDD
16S rRNA gene	Luminal flushing	0.31 ± 0.16%	11.77 ± 6.96%	2.15 ± 0.87%	4.99 ± 3.15%
	Fecal pellets	0.06 ± 0.01%	1.84 ± 0.03%	0.09 ± 0.01%	1.45 ± 1.15%
FliC gene	Luminal flushing	0.04 ± 0.02%	0.85 ± 0.49%	0.12 ± 0.08%	0.19 ± 0.03%
	Fecal pellets	0.03 ± 0.00%	0.1 ± 0.05%	0.02 ± 0.02%	0.05 ± 0.03%

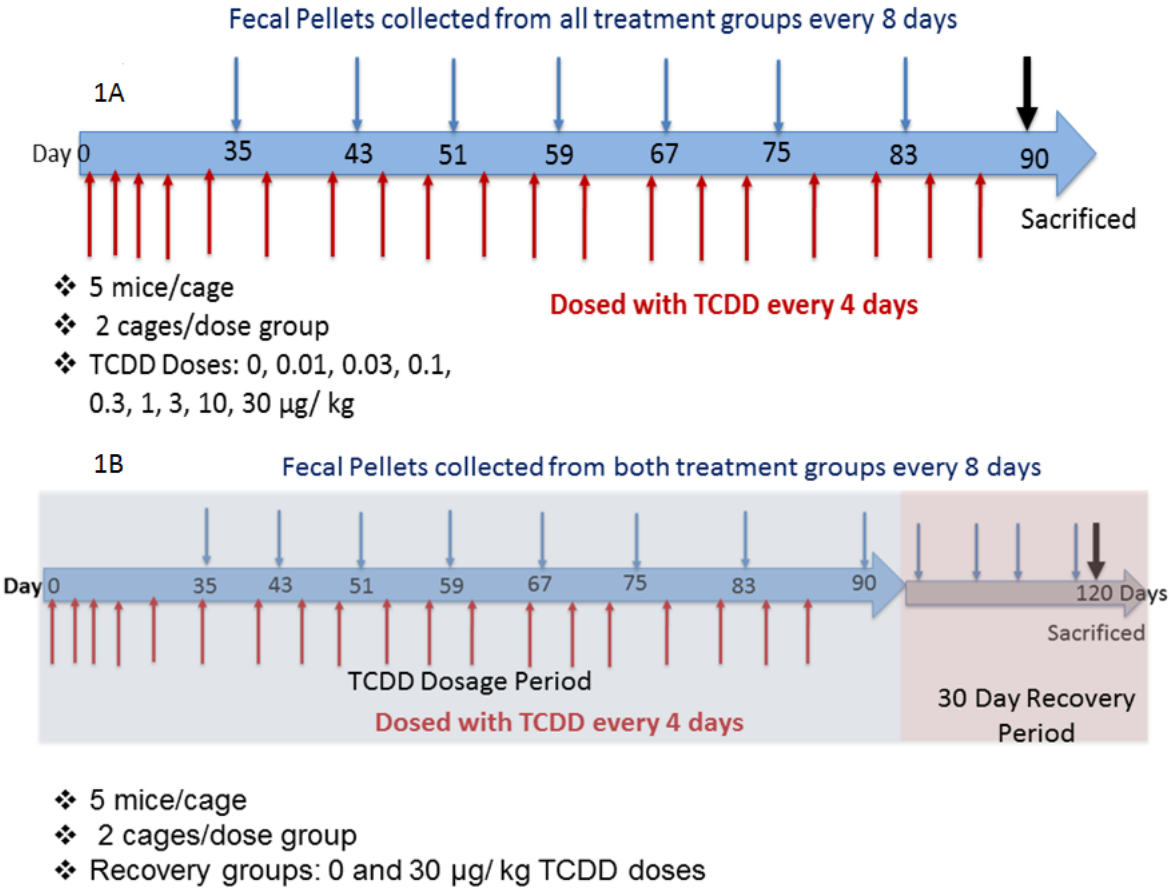


Figure S3.1. Repeated dosing study design. Female C57BL/6 mice were orally gavaged with sesame oil vehicle or 0.001 – 30 µg/kg TCDD every 4 days for 90 days (A), or with 0 or 30 µg/kg TCDD every 4 days for 90 days and allowed to recover for 30 days (B).

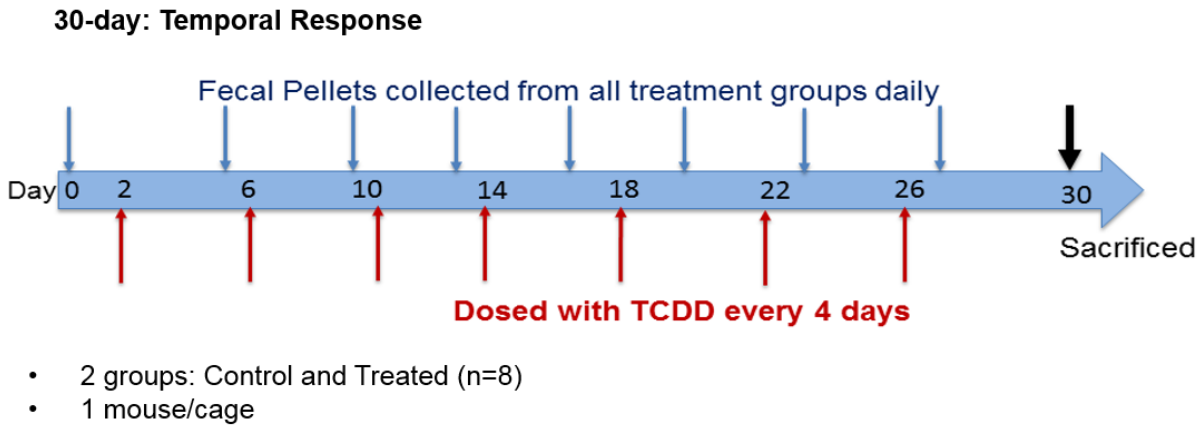


Figure S3.2. Short term design with multiple doses (1 mouse per cage). Female C57BL/6 mice were orally gavaged with sesame oil vehicle or 30 µg/kg TCDD every 4 days.

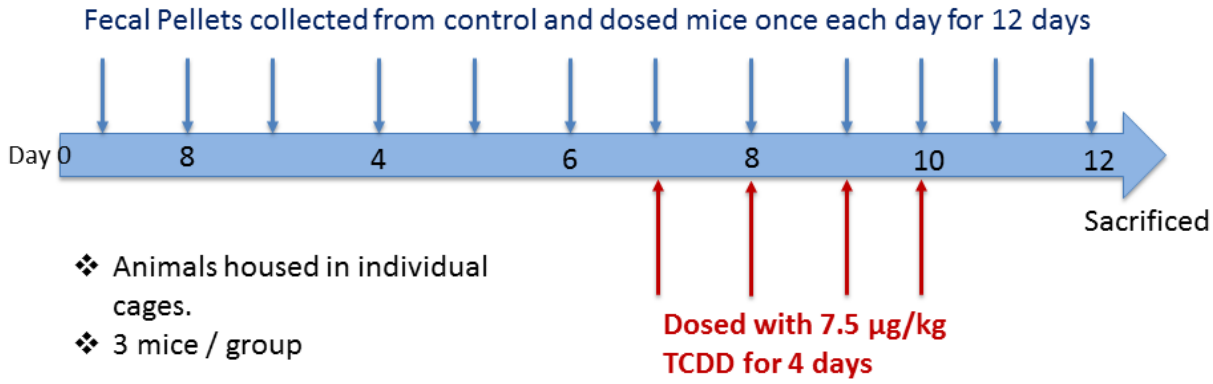


Figure S3.3. Short-term exposure to low doses study design. Female C57BL/6 mice were orally gavaged with sesame oil vehicle or 7.5 µg/kg TCDD for 4 consecutive days.

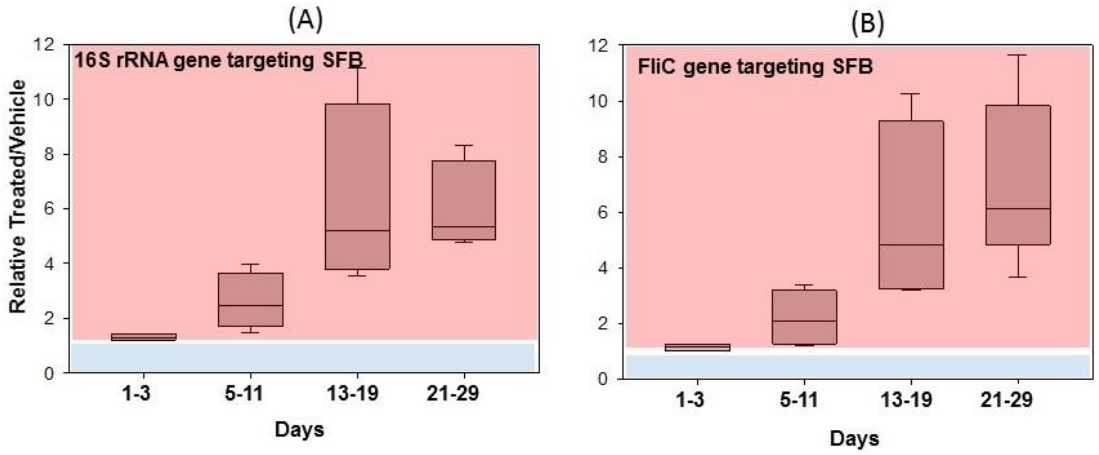


Figure S3.4. Fold change calculated as mean relative SFB abundance in TCDD treated mice divided by mean relative SFB abundance in vehicle mice during short term repeated dose study (30 µg/kg per day every 4 days). Days 1, 2, and 3 (prior to dosing) are grouped into a single box, and measurements every second day prior to dosing are grouped by week into boxes for (A) 16S rRNA gene targeting SFB, and (B) the FliC gene.

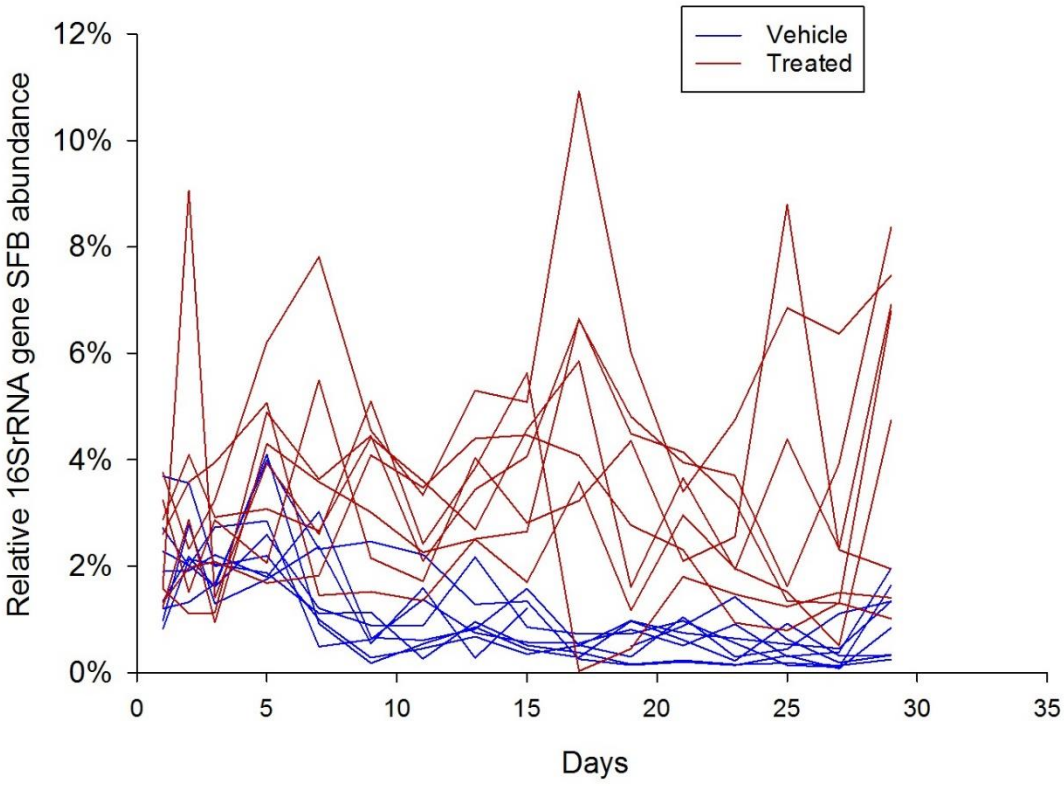


Figure S3.5. Short term high dose study. SFB abundance in individual mice across the 30 day dosage period.

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CHAPTER 4: RESPONSE OF CLOSTRIDIA CLUSTER XIV AND XIVA AND BACTEROIDES TO TCDD

Note: This chapter is to be submitted to the Journal 'Toxicologic Pathology' with the title as "**Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin correlates with decrease in abundance of Clostridia cluster XIV and XIVa and Bacteroides in Mice Gut Microbiome"** by Prianca Bhaduri, Robert D. Stedtfeld, Tiffany Stedtfeld, Kelly A. Fader, Norbert E. Kaminski, Timothy R. Zacharewski, James M. Tiedje and Syed A. Hashsham.

4.1. ABSTRACT

The most abundant commensal gut bacteria include Clostridia species and Bacteroides, which respond to changes in the micro-environment and help regulate immune response. The environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent toxin that also suppresses immune response. Using C57BL/6 mouse model, the response of Clostridia cluster IV and XIVa and *Bacteroides fragilis* abundance to exposure to TCDD was examined in fecal pellets. Experiments included monitoring their abundance over a short period (30 days) with repeated high doses of TCDD and a long-term period (90 days) with eight different dosages including a 1000-fold variation in TCDD. In the long-term study, they were also monitored over a 30-day recovery period following the 90 days of repeated TCDD dosing. Primers for each Clostridia group were redundant to maximize coverage of disparate phylogenetic species within each group. Relative abundance of *Clostridium* cluster IV and *Faecalibacterium prausnitzii* significantly decreased towards the end of the 30-day period in TCDD-dosed group. A decrease in abundance was also observed for *Clostridium* cluster XIVa including *Roseburia* and *E. rectale*

and *Bacteroides* in TCDD-dosed group as compared to controls. In the long-term study, there was a dose-dependent decrease in abundance for the *Roseburia spp and E. rectale* (subgroup of *Clostridium* cluster XIVa) group, with significant decrease in mice dosed with 10 and 30 µg/kg TCDD. This difference remained significant for the duration of the recovery period as the *Clostridium* cluster IV subgroup did not regain its original population in the 30-day recovery time. These results indicate that TCDD exposure suppresses populations within Clostridia cluster IV and XIVa, specifically the butyrate-producing bacteria. These gut commensal bacteria are responsive to TCDD and play a role in the consequent immune and metabolic responses observed in the host.

4.2. INTRODUCTION

Majority of the intestinal microbiota is made up of three anaerobic groups - Bacteroides, *Clostridium* cluster IV (*Clostridium leptum* group) and *Clostridium* cluster XIVa (*Clostridium coccoides* group) and belong to the Bacteroides, Firmicutes, Actinobacteria or Proteobacteria phyla (Eckburg et al. 2006; Hold et al. 2002). The *Clostridium* cluster IV and *Clostridium* cluster XIVa (henceforth referred to as *Clostridium* IV and *Clostridium* XIVa) make up 10-40% of the intestinal microbiota and play an important role in the gut immune homeostasis (Lopetuso et al. 2013; Manson et al. 2008). Most of these Clostridia members produce short chain fatty acids (SCFAs), specifically butyrate, and therefore help in regulating metabolism (Paillard et al. 2007; Vital et al. 2014). The most abundant butyrate-producers are *F. prausnitzii* from *Clostridium* IV and *Roseburia spp*. and *Eubacterium rectale* from *Clostridium* XIVa (Louis and Flint, 2009). Clostridia members are able to influence immunity by inducing a fraction of CD4+ T-cells in the host called regulatory T cells (Tregs) (Atarashi et al. 2011), partially through SCFA production

(Arpaia et al). Tregs maintain tolerance to self-antigens and suppress autoimmune diseases (Romagnani 2006). Clostridia strains are able to ameliorate symptoms of colitis in mice models with the help of these Tregs (Atarashi et al. 2011, 2013). The Tregs induced by these Clostridia produce cytokine IL-10, which has anti-inflammatory properties and decrease inflammation associated with colitis (Atarashi et al. 2013). Another bacterial species, *Bacteroides fragilis* produces Polysaccharide A (PSA) that reduces inflammation and also protects against *Helicobacter hepaticus* induced colitis (Mazmanian et al. 2008).

TCDD acts via the AhR pathway to increase the ratio of Foxp3+ Tregs to Th17 cells (Funatake et al. 2005; Quintana et al. 2008).TCDD exposure is correlated to decrease in T-cells that cause inflammation, primarily IL-17 producing Th-17 cells and corresponding inflammation markers including TNF-α and IL-6 (Quintana et al. 2008; Zhang et al. 2010). As a result of this altered immune response, TCDD is able to reduce inflammation associated with inflammatory bowel disease (IBD), dextran sodium sulfate-colitis and TNBS colitis. TCDD also plays an important role in ameliorating pathogenesis of several autoimmune diseases including murine systemic lupus erythematosus (SLE), experimental autoimmune encephalomyelitis, experimental autoimmune uveoretinitis and skin transplantation by increasing Tregs via the AhR pathway (Quintana et al. 2008; Schulz et al. 2011, 2012; Zhang et al. 2010).

Since both TCDD and intestinal Clostridia groups modulate immune responses by increasing Treg cell population, we hypothesized that exposure to TCDD would decrease the abundance of commensal Clostridia groups. The hypothesis was tested using TCDD doses over long term (0-30 µg/kg every 4 days for 90 days) followed by a 30-day recovery period where TCDD dosage was terminated, and a short-term high dose study (30 µg/kg every 4 days, 30 days). In the short-term study, we observed an altered gut microbial population, with the TCDD-treated groups

having a relative decrease in *Clostridium* IV and XIVa and Bacteroides with time as compared to control groups. The *Roseburia* and *E. rectale groups* (*Clostridium* IV) also showed significant decrease with repeated dosing of 10 and 30 µg/kg TCDD in the 90-day experiment. This study augments our earlier work in which we observe TCDD significantly increases SFB abundance.

4.3. MATERIALS AND METHODS

4.3.1. Animal Handling and Treatment

Two experiments were conducted including a long-term study over a 90-day period of dosing with TCDD and a short-term 30-day study. The 90-day study was conducted with various doses of TCDD (0.01-30 µg/kg dosed every 4 days), followed by a 30-day recovery period. Additional details of these studies are provided below. All animal handling procedures were carried out with the approval of the Michigan State University All-University Committee on Animal Use and Care.

4.3.1.1. Dose response, long-term (90 day) with recovery study

Female C57BL/6 mice on postnatal day 25 (PND25) were obtained from Charles Rivers Laboratories (Portage, MI). Upon arrival, mice were housed in polycarbonate cages with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) at 30-40% humidity and a 12h light/dark cycle and acclimatized for 4 days. Mice were fed *ad libitum* with Harlan Teklad 22/5 Rodent Diet 8940 (Madison, WI) and had free access to deionized water. On PND 28 and every following 4th day animals (n = 10) were dosed by oral gavage with 0.1 mL sesame oil vehicle control or 0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 30 µg/kg TCDD (Dow Chemical Company, Midland, MI) for a total of 90 days (23 doses; Figure S3.1).

Another group of mice were also dosed for 90 days with either sesame oil vehicle or 30 μg/kg of TCDD every 4 days and then allowed to recover for 30 days without dosing (henceforth referred to as recovery group).

Five mice were housed per cage, and there were two cages for each TCDD dose. Mice fecal pellets were collected every eighth day starting from day 35 post initial TCDD dosing until the mice were sacrificed. Pellets were immediately stored in -80°C after collection. There were seven collections for all mice sacrificed after 90 days and 12 collections for the recovery groups (treated and control).

4.3.1.2. Short-term high dose study (30 day)

Female C57BL/6 mice on postnatal day 25 (PND25) were obtained from Charles Rivers Laboratories (Kingston, NY). Upon arrival, mice were housed in individual cages (Innocages, Innovive, San Diego, CA) with ALPHA-dri® bedding (Shepherd Specialty Papers, Watertown, USA) at 30-40% humidity and a 12h light/dark cycle and acclimatized for 4 to 8 days. Mice were fed ad libitum with Harlan Teklad 22/5 Rodent Diet 8940 (Madison, WI) and had free access to acidified water. The dosing of mice was staggered by day with 4 animals dosed daily. Between PND 28 to PND31 and every following 4th day animals (n=8) were dosed by oral gavage with 0.1 mL sesame oil vehicle control or 30 μg/kg TCDD (Dow Chemical Company, Midland, MI) for a total of 28 days (7 doses).

Fecal pellets were collected daily including two days and the morning prior to the first exposure to TCDD (Figure S4.2). Pellets were immediately stored at -80°C prior to DNA extraction.

4.3.2. DNA Extraction

One hundred milligrams of mice fecal pellets were used for DNA extractions, processed using the PowerMax Soil DNA Isolation Kit following the manufacturer's protocol (MOBIO Laboratories). DNA samples were quantified on a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). DNA integrity was determined by gel electrophoresis. Extracted DNA was stored at −20 °C. Luminal flushing samples were concentrated by centrifugation prior to DNA extraction which was performed using the same kit and procedure as fecal pellets.).

4.3.3. Quantification of relative bacteria abundance

Quantitative PCR (qPCR) was used to quantify abundance of bacterial groups. Previously described primers were used targeting the 16SrRNA gene for *Clostridium* IV and XIVa, Bacteroides, and Lactobacillus (Table 4.1) (Figure S4.1) (Bouskra et al. 2008; Prakash et al. 2011). Functional primers for the butryl transferase emzyme (but) gene of *Faecalibacterium prausnitzii* was also included (Table 4.1). Amplicons were purified and sent to the Research technology Support Facility (RTSF) at Michigan State University (MSU) to be sequenced using the 96-capillary electrophoretic ABI 3730xl platform.

Quantitative PCR reactions were performed in the massively parallel Wafergen platform. Amplification was performed in 100 nL reaction wells containing a final concentration that included: 1×LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), nuclease-free PCR-grade water, 1 ng/μL DNA template, and 1 μM of each forward and reverse primer. Thermal cycling consisted of initial denaturation at 95ºC for 3 min, followed by a 40 cycles of denaturation at 95ºC for 30s, annealing at 60ºC for 1 min, and a melting curve analysis that

consisted of scanning every 0.4ºC increase from 60 and 97ºC. For each primer set, amplification was conducted in triplicate. All reactions were run in triplicate and primers were also tested using a no-template control.

Concentrations of bacteria in mouse fecal pellets and luminal flushing were calculated using standard curves obtained from 10-fold dilutions of anaerobic cultures of *Bacteroides fragilis, Ruminococcus obeum, Eubacterium rectale, Butyrivibrio fibrisolvens, Ruminococcus bromii* (obtained from ATCC). Amplicons were purified using the Qiagen PCR purification kit. To normalize data and calculate relative SFB abundance, the total amount of bacteria was estimated using universal primers targeting the 16S rRNA genes (Table 4.1) (Leigh et al. 2007). Standard curves of the 16S rRNA gene universal primer set was generated using 10-fold dilutions of DNA from pure culture of type strain *Shigella flexneri* ATCC 24577.

4.3.4. Data Analysis and Statistics

Threshold cycle (Ct) was generated from real time amplification curves analyzed using default parameters of the SmartChip Wafergen qPCR software (V 2.7.0.1). A threshold cycle (Ct) of 30 was used as the cutoff detection limit, thus a Ct greater than 30 was considered a false positive amplification event. Only samples with amplification in two or more of three technical replicates were considered positive. Standard curves generated for the 16S rRNA gene targeting each of the bacterial groups and the universal bacteria were used to estimate number of copies per reaction. Copy numbers of the but gene were estimated as described previously (Looft et al. 2012) using the following equation:

$$\text{but gene copy number} = 10^{\left\lceil \frac{(30-Ct)}{3.33} \right\rceil}$$

The relative bacteria abundance was calculated by dividing gene copy numbers over the universal bacteria copy numbers. Fold changes were calculated by dividing relative bacteria abundance in TCDD treated mice over relative SFB abundance in the vehicle controls. Mean and standard error measurements (SEM) and plots were generated using Excel 2010. A paired t-test was used to test for significant differences with significance cutoff of $P \leq 0.05$.

4.4. RESULTS

Two studies were performed including a long-term 90-day study with varying doses of TCDD and a 30-day recovery period and a short-term high dose study over 30 days. Relative abundance of bacterial species were measured temporally in fecal pellets to monitor population levels before and during TCDD dosing. There were two primers targeting *Roseburia* spp. and *Eubacterium rectale* (referred to as Rec-1 and Rec-2), three primers targeting *Clostridium* IV (referred to as ClosIV-1 – ClosIV-3), two primers targeting *Clostridium* XIVa (ClosXIV-1 and ClosXIV-2) and one each for *Bacteroides fragilis*, *Lactobacillus*, and the functional *but* gene of *Faecalibacterium prausnitzii*.

4.4.1. *Clostridia* cluster IV and *Clostridia* cluster XIVa

A short-term study with repeated doses of 30 µg/kg TCDD was performed to observe fluctuations in bacteria abundance in individually housed mice during the dosing period. The study included analysis of fecal pellets collected for the first three days prior to dosing, followed by analysis of pellets every second day for a 30-day time period.

The two *Roseburia* spp. and *E. rectale* primers, Rec-1 and Rec-2, showed abundance in the range of 1% to 4% in both the groups. Although the initial abundance of Rec-1 was higher in treated

group (days 1-10), their population showed a decreasing trend and from day 11 onward the population decreased below the level in the control group. From day 11 – day 29, the population of Rec-1 in treated group was 2% less than that in control group with the difference being significant on days 25 and day 27 (22 and 24 days post initial dose of TCDD, P=0.0064 and P=0.0094) (Figure 4.1). In the Rec-2 group, the abundance remained higher in the treated group for most of the study period, and only began to show a decline from day 29, near the end of this study (Figure S4.2). The vehicle group displayed a gradual increase in Rec-2 abundance through the 30 days and only at day 29 did the abundance of the treated group become less than that of the vehicle group. The treated group exhibited more fluctuation in abundance over the 30 days. All three primers used to target *Clostridium* IV (*Clostridium leptum* group) showed decreased abundance in treated groups relative to the vehicle control groups with time (Figure 4.2). The abundance of ClosIV-1 group started low for both the TCDD dosed and control groups and gradually increased with time (Figure 4.2A). The abundance in control group increased more than treated group with the difference between control and treated group becoming significant from day 23 to day 27 (20 to 24 days post initial dose of TCDD, P=0.04, P=0.005, P=0.04) (Figure 4.2A). The ClosIV-2 group also showed a similar trend with the difference between treated and vehicle being significant on day 21, day 25 and day 27 (18, 22 and 24 days post initial dose of TCDD, P=0.05, P<0.001 and P=0.0078) (Figure 4.2B). For the third primer set, ClosIV-3, the TCDD dosed group showed relatively lower abundance as compared to control group 15 days after initial TCDD dose, with the difference being significant from day 23 to day 27 (20 to 24 days post initial dose of TCDD, P=0.02, P=0.0004, and P=0.006)(Figure 4.2C). Two primer sets were used to determine the change in abundance of *Clostridium* XIVa (*Clostridium coccoides* group). For the ClosXIV-1 group, the difference between vehicle and

treated was significant only on day 25 (P=0.0078) (Figure 4.3A). For the remaining period, the vehicle group has higher abundance of ClosXIV-1, even though it was not significantly different. The ClosXIV-2 group showed more fluctuation with their abundance gradually increasing in both groups over the study period. The vehicle group had higher abundance relative to the TCDD-treated with towards the end of the experiment, and this difference was significant only on day 21 (18 days post initial dose of TCDD, P=0.02) (Figure 4.3B).

There was a relative decrease in the abundance of the butyrate transferase (but) gene of *F. prausnitzii* in the TCDD dosed group as compared to the control mice group, which stayed constantly lower from 18 days after the initial TCDD dose and became significant only on day 25 (P=0.0027) (Figure 4.4).

Fluctuations of *Roseburia* and *Eubacterium rectale* abundance (Rec-1 primer) was examined in C57BL/6 mice with repeated exposure to various doses of TCDD for 90 days. For the duration of this study, the vehicle group had a relative abundance between 4% and 7%, while the dosed groups had decreasing levels of Rec-1 with increasing doses (Figure 4.5A). A paired t-test showed that two of the TCDD treated groups, 10 μg/kg (p=0.001) and 30 μg/kg (p=0.001) had significantly lower levels of 16S rRNA gene targeting this Clostridium cluster IV sub-group (*Roseburia spp*. and *E. rectale*) as compared to the vehicle control groups (Figure 4.5A). Following the last dose of TCDD on day 87, it seems that the Rec-1 population in the gut remained significantly lowered in the treated group ($p<0.001$) compared to the control mice for the next 30 days of the recovery period (Figure 4.5B).

4.4.2. *Bacteroides fragilis*

In the short term study, *Bacteroides* increased by more than 10-fold in both controls and TCDD-dosed mice over the course of 30 days. The relative abundance of *Bacteroides* (*B. fragilis*) was less in the treated group throughout the 30 days of the study period with the difference becoming significant after day 23 (22, 24 and 26 days after initial TCDD dose, P=<0.001, P=0.0091 and P=0.0144) (Figure 4.6).

4.4.3. *Lactobacillus*

This group also displayed a difference in abundance between TCDD-treated and vehicle groups with the treated group having less abundance relative to the vehicle, though not significant, for the majority of the 30-day time period (Figure S4.3).

4.5. DISCUSSION

Reduced populations of *Clostridium* IV and XIVa have been associated with several disease conditions, including type 2 diabetes, IBD and ethesitis-related arthritis (Qin et al. 2012; Stoll et al. 2014; Wang et al. 2014). The main butyrate-producers, *Roseburia spp* and *E. rectale* from *Clostridium* XIVa and *F. prausnitzii* from *Clostridium* IV decreased significantly in abundance in TCDD-dosed mice. Here, our results agree with earlier studies that state butyrate-producing bacteria are associated with healthy conditions and decline with onset of various diseases. Butyrate-producing bacteria including *Roseburia* and *Ruminococcus* have been observed to decline in IBD along with a simultaneous increase in *Escherichia-Shigella* and *Enterococcus* pathogens (Chen et al. 2014). Patients with recurrent Crohn's disease also had a decreased level of butyrate-producing bacteria (De Cruz et al. 2015). Ulcerative colitis is marked with depletion

of two butyrate-producing species, *Roseburia hominis* and *F. prausnitzii* (Machiels et al. 2014). Children suffering from type 1 diabetes also had significantly less abundance of these bacteria than controls (de Goffau et al. 2013). Similar results were observed in patients with colorectal cancer (Zhu et al. 2014). Therefore, it follows that exposure to potent environmental toxin such as TCDD would trigger a decline in this group.

Studies have observed microbial dysbiosis observed with disease conditions characterized by decrease in Firmicutes/ Bacteroidetes ratio along with an increase in the Proteobacteria phylum (Packey and Sartor 2009). Also, an increase in opportunistic pathogens, *Prevotella*, *Escherichia coli* and *Enterobacteraceae* has been observed in disease conditions including IBD and type 2 diabetes (Chen et al. 2014; Qin et al. 2012). Inflammation causes increase in host produced nitrates that are utilized by pathogenic *E.coli* and *Shigella* but not butyrate-producing bacteria (Winter et al. 2013). Therefore, non-commensal *Enterobacteriaceae* family proliferate in the intestine inflamed condition (Winter et al. 2013). Similarly, TCDD-dosed mice also displayed an increase in *Enterobacteriaceae* family, and antibiotic resistant genes from *E.coli* and *Salmonella* in our experiments (our unpublished results). Also, TCDD, acting via the AhR, is implicated with increasing IL-22 (Vogel et al. 2013), which in turn suppresses commensal *Enterobacteraceae* bacteria and increases pathogenic bacteria including *Salmonella* enterica var typhimurium (Behnsen et al. 2014). Therefore, pathogenic members of the gut likely proliferate when the protective effect of commensal Clostridia populations is compromised in disease condition.

Recently, further evidence of the mechanism by which Clostridia gut members modulate immune responses has been reported. Certain microbially produced metabolites - SCFAs, are capable of differentiating CD4+ T cells to form Tregs (Arpaia et al. 2013; Furusawa et al. 2013;

Smith et al. 2013). The main SCFAs are butyrate, propionate and acetate and they exhibited induction of CD4+ T-cells to Tregs and expansion in the number of Tregs in the colon (Arpaia et al. 2013). SCFAs cause histone acetylation which helps increase the amount of Foxp3+ protein in the Tregs and also increases their suppressive activity. Clostridia members are able to break down the high fiber in food to form SCFAs that induce Tregs. For the induction of Tregs, the presence of these commensal bacteria is essential, as germ-free mice fed a diet high in butyrate did not show an increase in Tregs (Furusawa et al. 2013).

Bacteroides fragilis secretes PSA which is known to have anti-inflammatory properties and helps in reducing experimental colitis (Round et al, 2010). PSA also acts to protect against experimental autoimmune encephalomyelitis (animal model for human demyelinating disease) by decreasing inflammation (Ochoa-Repáraz et al. 2009). Recently, it was discovered that PSA suppresses immunity by inducing the expression of Foxp3 protein in naïve CD4+ T-cells and promotes Treg cells and IL-10 (Telesford et al. 2015). Although it is part of the commensal gut bacteria, it could become an opportunistic pathogen, and certain enterotoxigenic strains are associated with anaerobic infections and colorectal cancer (Toprak et al. 2006; Viljoen et al. 2015).

Immune homeostasis is maintained by key microbial species in the intestinal tract. The key microbial populations maintain immune balance which is disrupted by environmental contaminants, such as TCDD. In our earlier study, we reported that SFB increase in abundance with increasing TCDD dosage and over time. In this study, we observe a relative decline in abundance of *Clostridium* IV and XIVa and Bacteroides in the mice intestine. SFB is known to up-regulate Th17 cells and thereby cause an inflammatory response. On the other hand, *Clostridium* IV and XIVa and *Bacteroides fragilis* upregulate immune tolerance promoting Treg

cells that suppress autoreactive T-cells and thereby decrease inflammation. Since TCDD is known to induce Foxp3+-expressing Treg cells through the AhR, it is theorized that the immune balance is maintained by Clostridia groups and Bacteroides decreasing in abundance to keep the Treg population in check. Th17-inducing SFB proliferate to make up for the deficit in IL-17 caused by TCDD exposure.

Aryl hydrocarbon receptor is known to bind the most potently with TCDD, and cause severe immune-suppression. It also gets activated by dietary ligands including indole-3-carbinol and indirubin that are able to assist in Ahr-mediated immuno-regulation and immune suppression (Benson, 2011). It would be important to understand the role of commensal bacteria in facilitating this immune suppression, in conjunction with the AhR. Taken together, our results suggest that introduction of SCFA-producing bacteria, specifically *Eubacterium spp* and *Roseburia spp.* may be beneficial to reduce the damage caused by exposure of the host to environmental toxins. Further research may enable these bacteria to be employed as biomarkers to assess host damage caused by exposure to environmental toxins.

4.6. ACKNOWLEDGMENTS

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APPENDICES

APPENDIX A

TABLES AND FIGURES

Table 4.1. Primers used for qPCR to measure abundance of target bacteria group in mouse fecal pellets.

*Primer name 1 refers to primer notation in original reference

#Primer name 2 refers to primer notation in this publication

Target	Primer name 1*	Primer Name 2#	Sequence	Reference
Universal	27F		GGGTTGCGCTCGTTGC	(Leigh et al. 2007)
			ATGGYTGTCGTCAGCTCGTG	
Firmicutes	Firm934F	Firm	GGAGYATGTGGTTTAATTCGAAGCA	(Guo et al. 2008)
	Firm1060R		AGCTGACGACAACCATGCAC	
Faecalibacterium prausnitzii		Fpr	CCCTTCAGTGCCGCAGT	(Rinttilä et al. 2004)
			GTCGCAGGATGTCAAGAC	
Bacteroides	AllBac296F	Bac	GAGAGGAAGGTCCCCCAC	(Layton et al. 2006)
	AllBac412R		CGCTACTTGGCTGGTTCAG	
Bacteroidetes	Bact934F	Bact	GGARCATGTGGTTTAATTCGATGAT	(Guo et al. 2008)
	Bact1060R		AGCTGACGACAACCATGCAG	
Lactobacillus spp.	LactoF	Lact	TGGAAACAGRTGCTAATACCG	(Byun et al. 2004)
	LactoR		GTCCATTGTGGAAGATTCCC	

Table 4.1 (cont'd)

***Clostridium* cluster IV**	S-∗-Clos-0561-a-S-17	ClosIV-1	TTACTGGGTGTAAAGGG	(Van Dyke and McCarthy 2002)
	S-∗-Clept-1129-a-A-17		TAGAGTGCTCTTGCGTA	
***Clostridium* cluster IV (Clep)**	Clep866mF	ClosIV-2	TTAACACAATAAGTWATCCACCTGG	(Lay et al. 2005)
	Clept1240mR		ACCTTCCTCCGTTTTGTCAAC	(Sghir et al. 2000)
***Clostridium* cluster IV**	sg-Clept-F	ClosIV-3	GCACA GCAGTGGAG	(Larsen et al. 2010; Matsuki et al. 2002)
(Clostridium leptum subgroup)	sg-Clept-R3		CTTCCTCCGTTTTGTCAA	
***Clostridium* cluster XIVa–XIVb**	g-Ccoc-F	ClosXIV-1	AAATGACGGTACCTGACTAA	(Larsen et al. 2010; Matsuki et al. 2002)
(Clostridium coccoides subgroup)	g-Ccoc-R		CTTTGAGTTTCATTCTTGCGAA	
***Clostridium* cluster XIVa**	Erec482F	ClosXIV-2	CGGTACYTGACTAAGAAGC	(Rinttilä et al. 2004)
(Clostridium coccoides–Eubacterium rectale group)	Erec870R		AGTTTYATTCTTGCGAACG	

Table 4.1 (cont'd)

Roseburia spp.* and *E. rectale	Rrec630F	Rec-2	CGKACTAGAGTGTCGGAGG	(Ramirez-Farias et al. 2009)
	RrecRi630F		GTCATCTAGAGTGTCGGAGG	
	Erec870R		AGTTTYATTCTTGCGAACG	
Roseburia spp.* & *E. rectale	RrecF	Rec-1	GCGGTRCGGCAAGTCTGA	(Walker et al. 2005)
	Rrec630mR		CCTCCGACACTCTAGTMCGAC	(Ramirez-Farias et al. 2009)
		butFpr		
but genes of *Faecalibacterium prausnitzii*	G_Fprsn_F		GACAAGGGCCGTCAGGTCTA	(Vital et al. 2013)
	G_Fprsn_R		GGACAGGCAGATRAAGCTCTTGC	

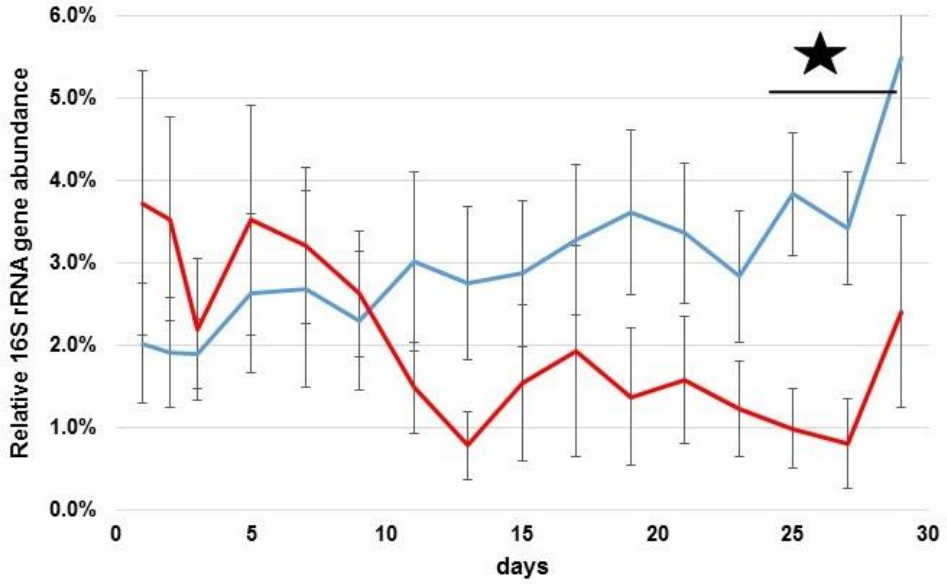

Figure 4.1. Relative *Roseburia* and *E. rectale* (Clostridia XIVa) abundance with Rec-1 (RrecF) primer during short-term 30-day study with 30 µg/kg TCDD per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

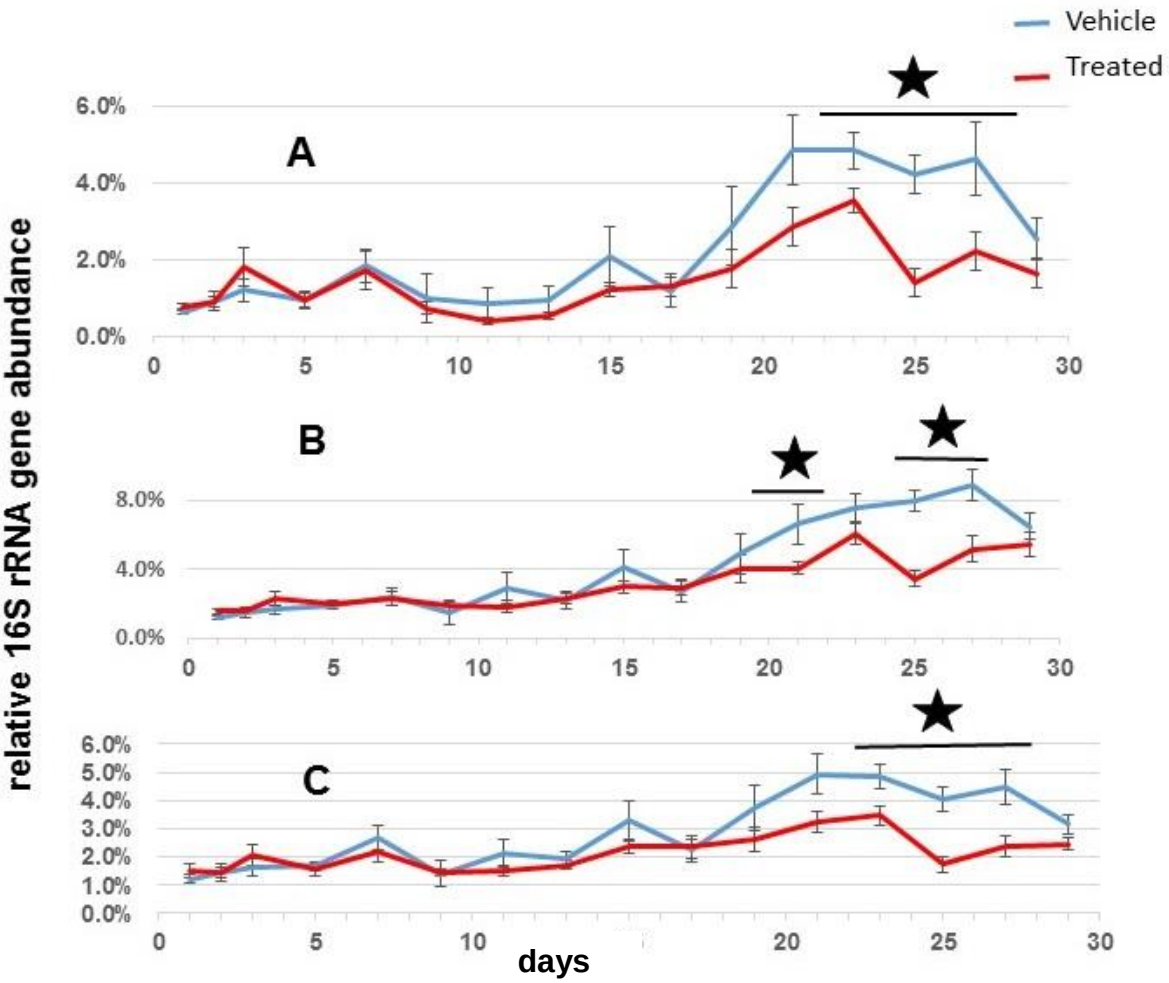

Figure 4.2. Relative Clostridia IV abundance with (A) ClosIV-1 (S*Clos-0561-a-S-1), (B) ClosIV-2 (Clep866mF) and (C) ClosIV-3 (sg-clept-F) primers during short-term 30-day study with 30 µg/kg TCDD per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

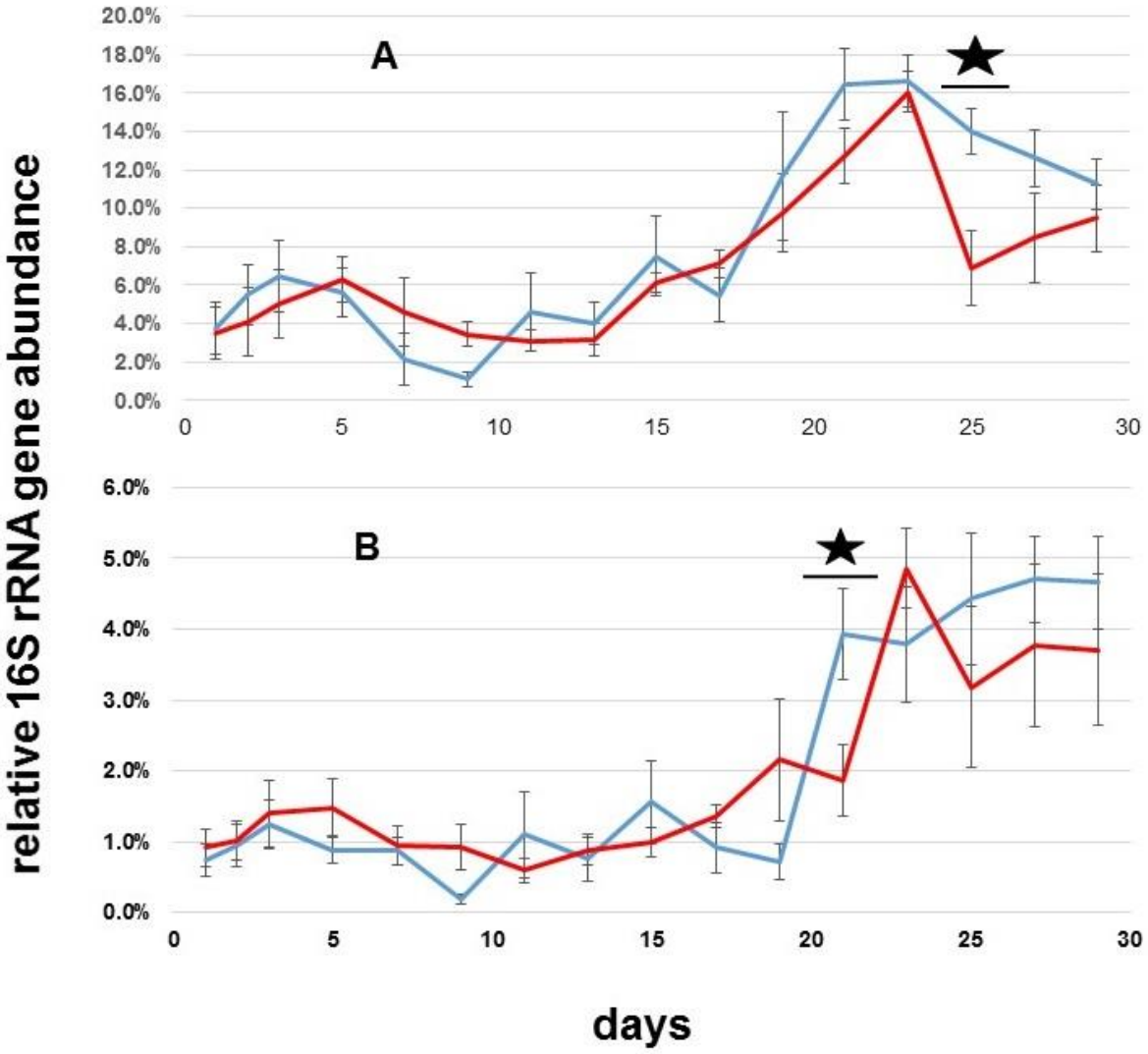

Figure 4.3. Relative Clostridia XIV abundance with (A) ClosXIV-1 (g-Ccoc-F), and (B) ClosXIV-2 (Erec482F) primers during short-term 30-day study with 30 µg/kg TCDD per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

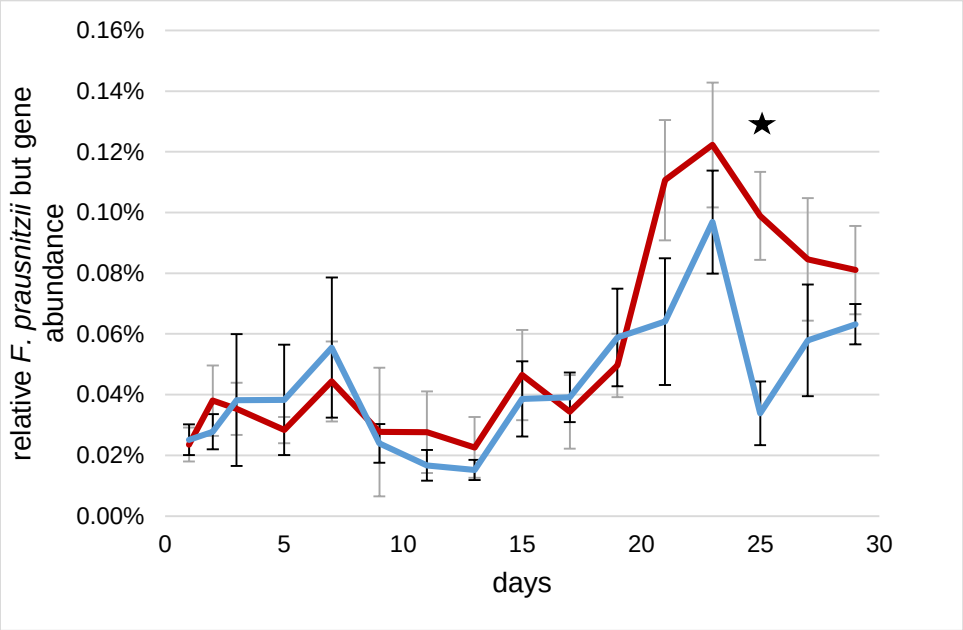

Figure 4.4. Relative *but gene of Faecalibacterium prausnitzii (Clostridium IV)* abundance during short-term 30-day study with 30 µg/kg TCDD per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

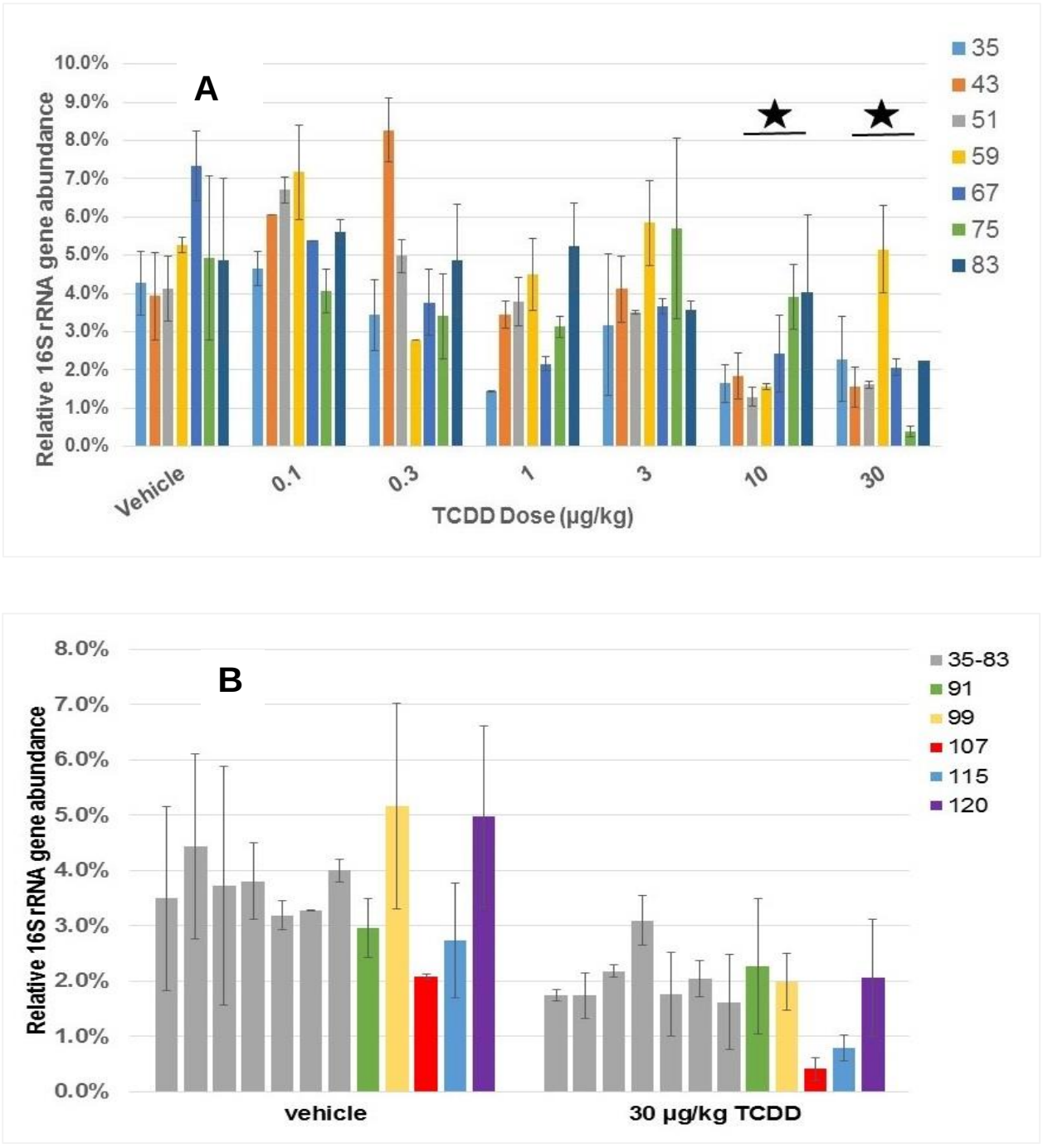

Figure 4.5. Long-term dose response and recovery of TCDD-mediated influence on relative *Roseburia* and *E. rectale* (Rec-1) abundance. (A) Relative abundance of 16S rRNA gene with vehicle and TCDD doses of 0.1, 0.3, 1, 3, 10 and 30 µg/kg TCDD every four days. Groups dosed with 0.01 and 0.03 µg/kg TCDD showed no difference in relative gene abundance

Figure 4.5 (cont'd)

compared to vehicle group and are omitted. Fecal samples were collected on days 35, 43, 51, 59, 67, 75, and 83. (B) Relative 16S rRNA gene abundance from recovery groups during 30 μg/kg TCDD dosing (days 35-83) and after dosing (days 91-120). Error bars represent the standard error of mean for two groups of five cohoused mice in each group.

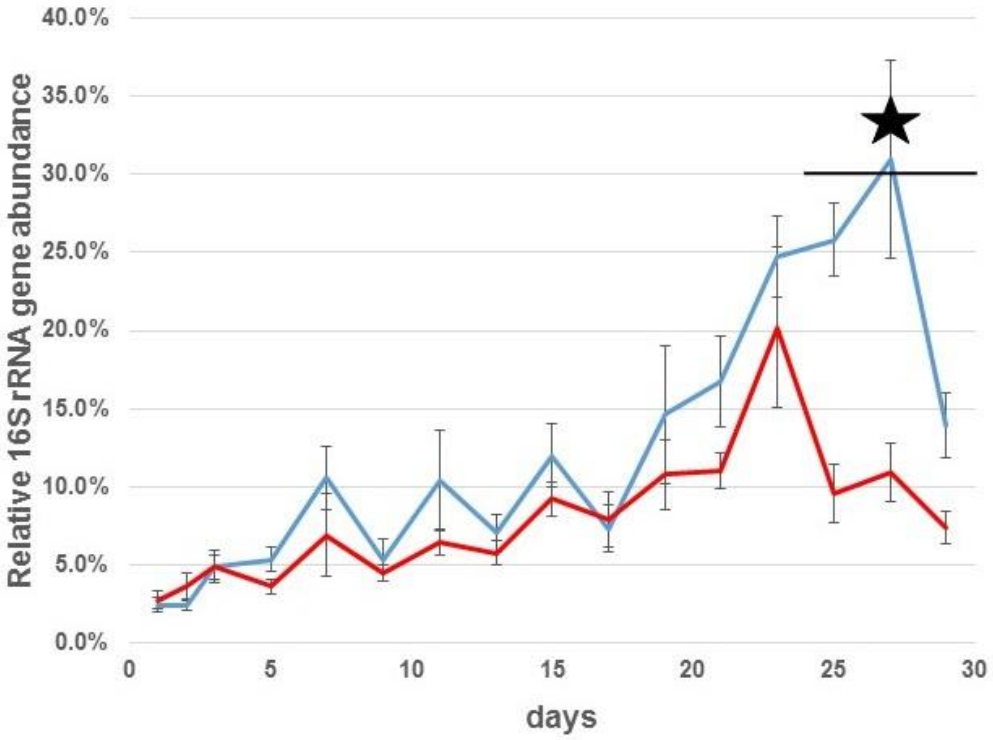

Figure 4.6. Relative *Bacteroides* abundance during the short-term 30-day study with 30 µg/kg TCDD per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

APPENDIX B

SUPPLEMENTAL MATERIAL

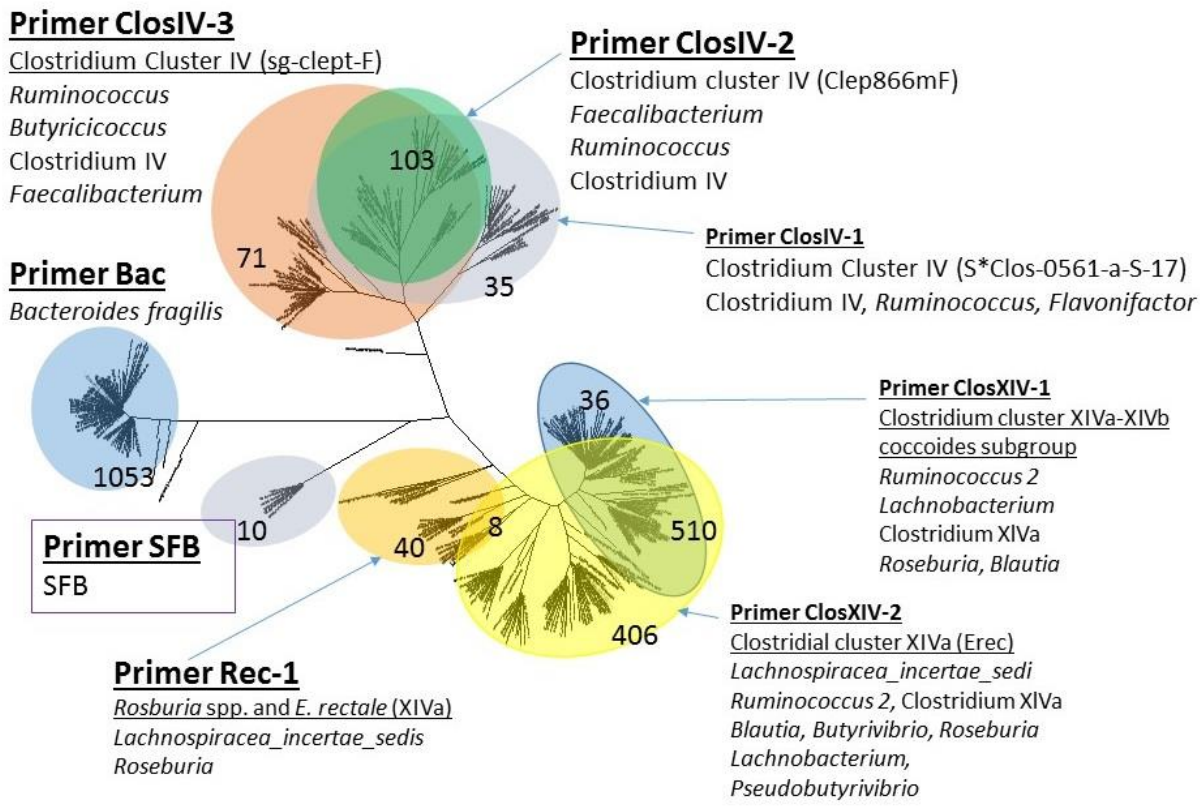

Figure S4.1. Phylogenetic distribution of primers used in this study.

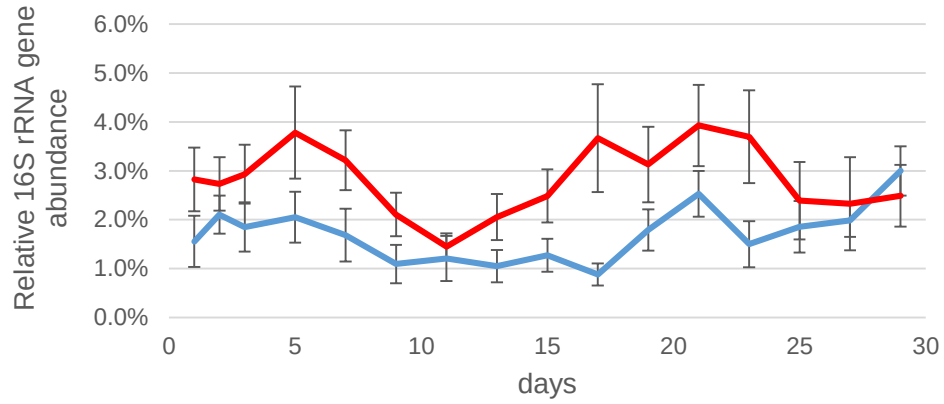

Figure S4.2. Relative *Roseburia and E. rectale* abundance with Rec-2 (Rrec630F) primers during short-term 30-day study with 30 µg/kg per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

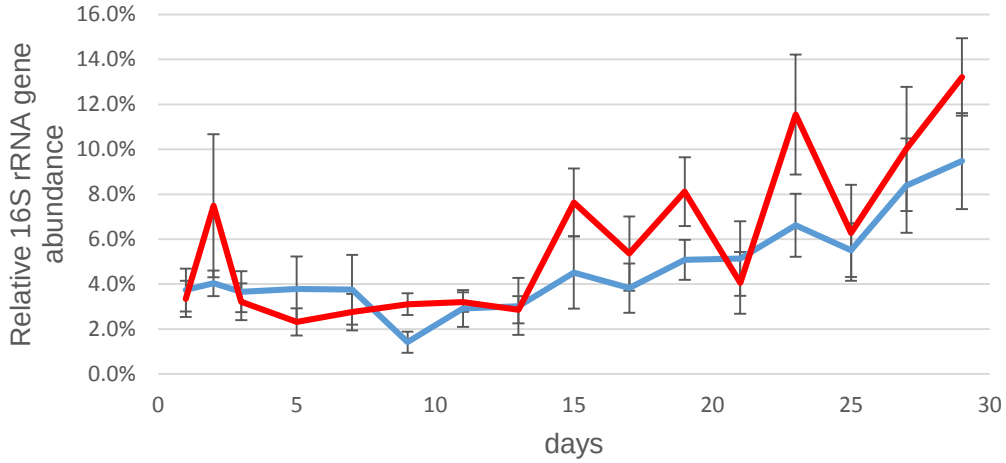

Figure S4.3. Relative *Lactobacillus* abundance during short-term 30-day study with 30 µg/kg TCDD per day every four days. Data represents mean of eight mice dosed with TCDD (red lines) and vehicle dosed mice (blue lines). Error bars represent the standard error of mean.

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The gut commensal bacteria are implicated in several essential functions for the host health including immune development, host metabolism and protection against pathogens and their dysbiosis contributes to increased risk of diseases including diabetes, inflammatory bowel diseases (IBD), ulcerative colitis and Crohn's disease (Round, 2009). The intestinal commensal bacteria are a dynamic ecosystem that responsive to perturbations in its micro-environment, whether it is external factors like diet and chemical stimulation or subjected to internal stimulation in the form of knock-out genes in animal models. Although, the adverse impact of dioxins on host systems have been studied for decades, their effect on the intestinal microbiome has not been studied. Knowledge of the effect of TCDD exposure on gut microbial members would be helpful in determining their contribution to changes in host immunity, gene expression and risk to associated diseases like autoimmunity and IBD. The goal of this study was to gain insights into the role of specific gut microbiota when exposed to TCDD in mice models. The key findings and accomplishments of this work include:

1. Gut microbiota respond to higher doses of TCDD (1-30 µg/kg body-weight of mice). Different doses of TCDD (0.01-30 µg/kg) were administered to mice every four days for 90 days. SFB displayed significant increase in abundance for 1, 3, 10 and 30 µg/kg TCDD doses whereas *Roseburia and E. rectale* was significantly less relative to vehicle abundance in the 10 and 30 µg/kg TCDD-dosed groups.
2. The duration of TCDD exposure and repeated TCDD exposure determines the amount of change observed in gut microbial populations. Higher doses of TCDD are effective in changing the abundance of key intestinal members including SFB, *Clostridium* IV and

XIVa whereas the lower doses of TCDD did not produce any visible effect on gut microbial populations. Repeated doses of TCDD over a longer time period (30 days) were more effective in significantly increasing SFB abundance. Significant change in abundance was not observed when low dose of TCDD was administered over a shorter time period (12 days).

3. Quantified the change in abundance of SFB with exposure to TCDD. The 16S rRNA gene and the Flagellin (FliC) gene increased in TCDD-treated groups as compared to control groups. The direction of SFB change was consistent throughout the three studies conducted in this work. Some of the important findings include:
    a. There is dose-dependent increase in SFB with TCDD with more than 10-fold difference in abundance observed with 10 and 30 µg/kg TCDD.
    b. SFB initially increase and then decrease in the recovery period. The SFB initially increase after 30 µg/kg TCDD dosing is terminated after 90 days, but then continue to decline to pre-exposure levels within the 30 days of recovery time.
    c. Intestinal luminal flushings from mice treated with TCDD have higher abundance of SFB after 90 days of dosage as compared to controls but their abundance declines after a 30 day recovery period and is no longer significantly different from that in control group. Also, the intestinal luminal flushing samples contain higher amounts of SFB as compared to fecal pellets collected from mice.
    d. Individual mice have different basal amounts of gut microbiota including SFB. When high repeated doses of TCDD were administered over 30 days, there was greater than 10-fold change in mean SFB levels of eight mice, as measured both

by 16S rRNA and flagellin gene. However, individually, the mice showed larger fluctuation in their gut microbial populations.

e. A low dose of TCDD given over a short period (12 days) also increases the abundance of SFB in treated group; however, there is more individual variability due to which the increase in abundance is not significant.

4. *Clostridium* IV, XIVa and *Bacteroides* show a relative decrease in abundance over 30 days with repeated 30 µg/kg TCDD.
5. Butyrate-producing bacteria within the *Clostridium* IV and XIVa, *Roseburia* and *Eubacterium rectale* and *Faecalibacterium prausnitzii* decreased in abundance relative to controls over 30 days in mice dosed with repeated 30 µg/kg TCDD.
6. Dose dependent-decrease in the butyrate producing members of *Clostridium* XIVa, *Roseburia* and *E. rectale* was observed with significant decrease significantly in abundance over 90 days when
7. The qPCR primers for SFB were validated by amplifying DNA from mice fecal pellets and Sanger sequencing the amplicons.

Immune homeostasis is maintained by key microbial species in the intestinal tract. Environmental contaminants like TCDD disturbs both the key microbial populations necessary for healthy gut and the gut immune balance. In our earlier study, we reported that SFB increase in abundance with increasing TCDD dosage and over time. In this study, we observe that abundance of butyrate-producing bacteria and *Bacteroides* declines in the mice intestine. SFB is known to up-regulate IL-17 producing Th17 cells and thereby cause an inflammatory response. On the other hand, commensal bacteria (*Clostridium* IV and XIVa and *Bacteroides fragilis*) upregulate inducible Treg cells, via MyD88 adaptor molecule and STAT3 signaling, that

decrease inflammation (Atarashi et al. 2013; Round and Mazmanian 2010; Wang et al. 2015). These inducible Tregs are antigen-specific, and are responsible for maintaining immune tolerance and for increased IgA production (Jonuleit and Schmitt 2003; Wang et al. 2015). Intestinal IgA maintains the epithelial barrier, promotes commensal bacteria and limits the growth of commensal pathobionts such as SFB (Wang et al. 2015). From our results it is evident that TCDD inhibits commensal bacteria (Clostridia and Bacteroides), thereby reducing the production of IgA, due to decrease in antigen-specific differentiated Tregs (Wang et al. 2015). Therefore, decreased IgA results in an overgrowth of SFB (Figure 5.1). TCDD has been found to directly decrease the production of intestinal IgA at the mucosal surface (Kinoshita et al. 2006). Studies in mice exposed to a less potent environmental toxin, TCDF, found that there was an increase in inflammatory markers including IL1β, TGFα, SAA3 and IL-10 along with other metabolic dysfunctions (Zhang et al. 2015).

Different types of Treg cells present in the intestine can provide a possible explanation for the increase in AhR-activated Treg observed in TCDD-related studies. Inducible Treg cells are specific Tregs that are induced by antigens at the periphery of the gut, and have antigen-specific immunosuppressive functions (Corsini et al. 2011; Jonuleit and Schmitt 2003). Thymic Tregs are natural Tregs that are derived in the thymus ((Corsini et al. 2011). TCDD activated AhR increases thymic and splenic Treg cells but not Tregs in peripheral lymph nodes (Schulz et al. 2012). Therefore the Foxp3+CD25+CD4+ Treg cells that are induced by TCDD via AhR may not have the ability to maintain healthy gut microbial commensalism.

This work can be regarded as preliminary work that established the conclusive relationship between environmental contaminants and the gut microbiota. Further studies and ongoing work will identify the cause-effect relationship between host immunity and specific

members of the gut microbiome when influenced by TCDD and other dioxins. Further studies will be necessary to understand the underlying mechanisms that are employed by gut microbiome and environmental contaminants, specifically TCDD, in influencing host disease outcomes.

Some of the possible future directions are as follows:

1. Germ-free mice are important models for isolating the effect of a single microbial group on the host. We have used germ-free mice associated with either SFB, Clostridia and *Bacteroides* species or all three groups and then exposed to high doses of TCDD at regular intervals for a month. Tissue samples from these mice after sacrifice will be analyzed for changes in quantity of immune cells including CD4+ T-cells, Treg cells and Th17 cells and cytokines including IL-17 and IL-22. The results of this study will help determine which bacterial species are play a greater role in upregulating certain immune cells (Th17, CD4+T-cells, Treg cells) and participating cytokines (IL-177, IL-22, TGFβ) in TCDD-dosed mice.
2. Metagenomic and transcriptomic studies that analyze the mice fecal samples will provide a better functional characterization of microbial genes that are up/ down-regulated due to TCDD dosage. It will also shed light on whether low doses of TCDD can stimulate certain genes or if microbes respond only to higher TCDD doses.
3. SFB has recently been cultured in cell lines and can be replicated outside of the host (Schnupf et al. 2015). SFB can be cultured and spiked with TCDD to identify if there are any direct effects of TCDD on SFB abundance. Similar studies can be done for the more abundant species in the intestine including *Clostridium* IV and XIVa, and *Bacteroides*.

4. Fecal pellets of TCDD-dosed mice can also be analyzed against the antibiotic resistance gene panel (Zhu et al. 2013) and specific qPCR of pathogenic bacteria including *Enterobacteraceae, E. coli* and *Shigella* will provide a better picture of the changes in intestinal microbial population that take place in the micro-environment.
5. It will also be useful to measure the increase in different types of Treg cells including induced Treg, natural Treg, and Foxp3+CD4+CD25+ Tregs in conventional animals dosed with TCDD along with vehicle controls. This will give valuable information to the chain of gut immune modulation occurring with TCDD exposure.

APPENDIX

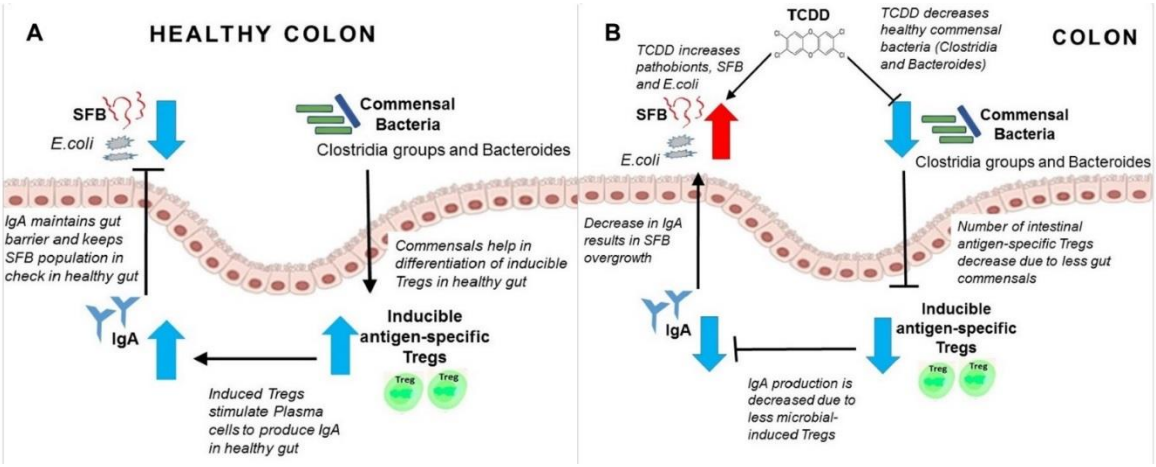

Figure 5.1. Schematic diagram illustrating potential mechanism for overgrowth of pathobionts including SFB in intestinal tract exposed to TCDD. (A) In healthy intestinal tract, commensal bacteria (Clostridia groups and Bacteroides) induce production of inducible antigen-specifc Tregs. These Tregs induce production of gut IgA via plasma cells. Gut IgA protect the epithelial barrier and keep the growth of gut microbial populations including SFB in check.

(B) TCDD causes dysbiosis of healthy commensal bacteria including Clostridia groups and Bacteroides. This results in down-regulation of antigen-specific Tregs and thereby reduces production of IgA. Reduced intestinal IgA allows SFB and *E. coli* to proliferate in the gut.

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