THE UNIVERSITY OF CHICAGO

THE ROLE OF LYMPHOTOXIN SIGNALING ON COMMENSAL BACTERIA-INDUCED INFLAMMATION

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PATHOLOGY

BY

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CHICAGO, ILLINOIS

AUGUST 2016

DEDICATION

I dedicate this to my father, Zuopei Zhang, MS,

who had to stop pursuing his doctorate

in order to provide for our family.

Thank you for giving me the luxury to pursue mine, Dad.

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List Of Abbreviations

ASF: Altered Schaedler's flora

CD11c: Integrin, alpha X (complement component 3 receptor 4 subunit), a marker for DCs

CD3ε: Cluster of Differentiation 3 epsilon subunit, a marker for T cells

CD19: Cluster of Differentiation 19, a marker for B cells

CD45: Cluster of Differentiation 45, a marker for leukocytes

CD90: Cluster of Differentiation 90, a marker for peripheral T cells

CFU: Colony forming units

Cre: Cre recombinase, catalyzes LoxP sites

DC: Dendritic Cell

DIO: Diet Induced Obesity

F/F: Flanking/Flanking by LoxP

GF: Germ Free

HFD: High Fat Diet

IACUC: Institutional Animal Care and Use Committee

IL: Interleukin

IL-22R: Interleukin 22 Receptor

ILC: Innate Lymphoid cell

ILC3: Group 3 Innate Lymphoid cell

IP: Intraperitoneal injection

IV: intravenous injection

LPL: Lamina propria lymphocytes

LPS: Lipopolysaccharide

LT: Lymphotoxin

LTβR: Lymphotoxin β Receptor

LTi: Lymphoid Tissue Inducer

MLN: Mesenteric Lymph Node

NCD: Normal Chow Diet

NK: Natural killer cells

PCR: Polymerase Chain Reaction

RAG1: Recombination Activating Gene 1

RNA: Ribonucleic acid

RORγt: Retinoic acid receptor-related orphan receptor gamma

SEM: Standard Error of the Mean

SFB: Segmented Filamentous Bacteria

SPF: Specific Pathogen Free

T_H17: $CD4^+$ effector T cells that produce IL-17 and IL-22

TNF: Tumor Necrosis Factor

T_{regs}: Regulatory T cells

WT: Wild type

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Acknowledgements

First, I have to acknowledge the role of my advisor on my graduate career, Dr. Yang-Xin Fu. When deciding between graduate schools, my interview with Dr. Fu convinced me to come to the University of Chicago to pursue my doctorate. Dr. Fu's personality and optimism truly is the reason I am here today. During the periods when my project was not going well (and there were a lot of those periods), Dr. Fu kept on pushing and nudging me forward. In my moments of greatest self-doubt, Dr. Fu's approachability, understanding and commitment to see me finish the project, drove me to tackle it every day. I honestly can say that if Dr. Fu were not my advisor, I would not be finishing my Ph.D. program. So thank you, Dr. Fu, thank you for seeing me complete this project before I could see it myself.

Secondly, I am forever indebted to Dr. Cathryn Nagler, the chair of my committee; though, after everything, Dr. Nagler is more like a co-advisor. Following the move of Dr. Fu, Dr. Nagler stepped in, and went above and beyond, to look over Dr. Joanna Wroblewska, Kevin Muite and me, the remaining Fu graduate students. Dr. Nagler incorporated us into her lab meetings and made herself available to us in a way that felt like we had joined her lab. She answered all of our emails and made time to sit-down with us individually to update her on our progress. Without Dr. Nagler advocating for us, I am certain the remaining Fu graduate students would not be where we are today. Thank you Dr. Nagler, you pushed me to become the best scientist I could be and you did it all because you fundamentally cared about my growth and development, and for that, I am forever grateful.

In addition, I have to extend my warmest gratitude to Dr. Julie Bubeck-Wardenburg, who was not only generous with her time during my committee meetings, but who also let me pester her lab about all things bacteria-related and use her anaerobic chamber whenever I needed it. I especially have to thank Drs. Vivian Choi and Aaron Hecht for their patience and time guiding me through their protocols. I also have to thank Dr. Eugene Chang, not only for his time spent on my committee, but also for letting me borrow his lab's expertise to help tackle a lot of the gut pathology and bacterial culture problems. I specifically have to thank Drs. Daina Ringus, Anuradha Nadimpalli, Mark Musch, and Kristina Martinez for taking the time to show me how to do various techniques. I also have to thank Dr. Maria-Luisa Alegre, who always asked really insightful questions that propelled this thesis forward. Last but not least, I have to thank Dr. Stephen Meredith since he fundamentally cares about the MPMM students and our complete development as a scientist.

I have to thank the Fu lab members who were all incredibly generous with their time. It goes without saying that my project would not have existed without Dr. Vaibhav Upadhyay. I hope this thesis makes you proud and thanks for sharing the project with me! This work could not have been done without the time and training provided by Drs. Xiaohuan Guo (who even walked me through the ILC transfer from Beijing!), Haidong Tang (who taught me how to do retro-orbital injections!), Tae-Jin Kim (who helped me on the splenomegaly project!), Byron Burnette, Xuanming Yang, Tony Tu, Christine Pu, and Michelle Xu. Last but not least, Yang Wang who made sure everything in the lab ran as smooth as possible, so we could just focus on our projects; we definitely noticed her absence when she moved.

I have to also thank Drs. Elizabeth McNally (for letting me join her lab during my college and post-college life) and Megan Roy-Puckelwartz for convincing me to go to graduate school. I also have to thank everyone in the McNally lab that cheered me on, especially Drs. Kay-Marie Lamar, Quan Gao, Jeffrey Goldstein and Brandon Gardner! Thanks also to Drs. Maxwell Rovner and Paige Marnell, you guys are amazing! I have to acknowledge the friends, some new, some old, I have made during my time here that really guided me and supported me throughout, especially Drs. Melissa Tjota, Noel Pauli, Emily Davenport and Susan Stanley, and Ai-Lin Sui.

I have to thank my dad, Zuopei Zhang, whom this dissertation is dedicated to. He came from a farming village in China, where his family struggled to put food on the table, to the States because he wanted a better life for my sister and me. He taught me the meaning of sacrifice, the ability to “吃苦 chi ku” and the power of education. I am forever grateful to my sister, Tina Zhang; my greatest accomplishment in life is becoming best friends with you. Thank you so much for the support of the Schram family, Peggy, Jeff and Bud. I also have to acknowledge my cats, the Meowingtons, who always made sure to lend a helping paw; thank you for rescuing me.

Finally, there is Dr. Andrew Schram. You, amazingly, started this journey with me and have been with me every step of the way, through every failed experiment and every seemingly small lab victory. Thank you for making me a better person, for making me amazing meals, for making me feel loved and taken care of every single day and for starting/ending everyday with “I love you.” Thank you so much for always cheering me on and never letting me give up and quit. I cannot wait to see what life has in store for us next, and I could not have asked for a better life partner. I love you, buns.

Abstract

The microbiota plays a critical role in the development of diet-induced-obesity (DIO). Previous work has demonstrated the importance of lymphotoxin (LT) signaling in regulating the microbiota to promote weight gain. It demonstrated that LT-mediated IL-22 production promoted obesity on a high fat diet. However, the mechanism of how a defect in LT signaling leads to resistance to DIO is unclear. Specifically, which cells need to express $LT\alpha_1\beta_2$ and how it regulates the microbiota community is unknown. The enclosed work demonstrates that active LT signaling from Type 3 Innate Lymphoid cell (ILC3s) is needed to prevent bacterial-induced inflammation. The observed splenomegaly phenotype is characterized by increased splenocytes and increased splenic neutrophils. Mice deficient in $LT\alpha_1\beta_2$ or LTβR develop splenomegaly in a microbiota-dependent manner. This was observed following the treatment of antibiotics and using germfree (GF) mice. The derivation of GF *Ltbr*$^{-/-}$ mice resulted in the absence of splenomegaly. Furthermore, this work also suggests the presence of specific gut bacteria that causes splenomegaly in an LT-dependent manner. In addition, previous work has demonstrated the importance of ILC3 in mediating mucosal immunity and maintaining mucosal barrier homeostasis in the intestinal tissue. Through the use of various LTβ and LTβR conditional knockout mice, we were able to demonstrate that retinoic acid-related orphan receptor γT (RORγt) cells expressing $LT\alpha_1\beta_2$, and providing active LT signaling, prevents the development of splenomegaly. Specifically, ILC3s expressing $LT\alpha_1\beta_2$ is both necessary and sufficient to prevent microbial-induced inflammation. Going further, previous work had characterized the importance of the gut microbiota. In processing carbohydrates for nutrient absorption and demonstrated that

in the absence of the gut microbiota, GF mice fed a high fat diet (HFD) were resistant to weight gain. This work demonstrates that the complexity of the carbohydrates used in HFD experiments in GF mice alter weight gain, not necessarily the presence or absence of the microbiota. Specifically, we characterized that GF mice fed a diet rich in maltodextrin were resistant to DIO compared to GF mice fed a diet rich in sucrose. Thus, we have contributed to the current understanding of how the gut microbiota promotes DIO. Finally, we sought to understand whether the increased basal inflammation in specific pathogen-free (SPF) *Ltbr*$^{-/-}$ mice prevented the mice from gaining weight on HFD. We determined that a HFD alters the colonic tissue and decreases colonic tissue density in a diet-dependent manner. We demonstrated that in addition to GF *Ltbr*$^{-/-}$ mice not developing splenomegaly, but GF *Ltbr*$^{-/-}$ mice also were able to gain weight and developing DIO. This implies the microbiota-induced inflammation has a role in the previously observed leanness phenotype. Interestingly, RORγt cells expressing $LT\alpha_1\beta_2$ were not involved in DIO but active LT-signaling from ILC3s has a partial role in increased adiposity. The following work demonstrates the importance of LT-mediated immune responses at the colonic mucosal barrier for the prevention of splenomegaly and systemic inflammation. In addition, we have demonstrated that this particular inflammation can lead to resistance to DIO in an LT-dependent manner due to HFD decreasing colonic density. Finally, we have highlighted the importance of microbiota-dependent dietary carbohydrates for weight gain. In summary, LT-signaling, the gut microbiota, and diet together affects host health and obesity.

Chapter 1: Introduction

The prevalence of obesity

The existing paradigm attributes obesity simply to caloric abundance. A "western diet," characterized by excess calories and fat, has been associated with the rampant obesity epidemic in the US and in many parts of the world. Unfortunately, obesity has become a well-established disease, so problematic that a new field of surgery, called bariatric surgery, has been developed to help address the most severe cases of obesity, usually categorized as patients with a body mass index (BMI) of >40 (Azagury and Lautz 2011). In United States, if the current rates of developing obesity remain unchanged, by 2022, 80% of American adults will be considered at least overweight (Youfa Wang et al. 2008a).

Diet-induced obesity (DIO) is a pandemic health problem linked to increased death from a wide variety of co-morbidities. An analysis of 900,000 adults has described increased BMI associated with a 30% higher overall increase in mortality (Prospective Studies Collaboration 2009). Beyond increased mortality, obese patients also have higher incidences of preventable comorbidities like heart disease, stroke and diabetes. It is estimated that by 2030, in United States alone, $860 to $956 billion will be spent on health-care associated with managing obesity and its complications (Youfa Wang et al. 2008a).

Obesity and inflammation

Obesity evokes a chronic inflammatory state in the host (Youfa Wang et al. 2008b; Kau et al. 2011a). When examining the contribution of inflammation to DIO, most studies have focused on inflammation in adipose tissues, where inflammatory cytokines can antagonize insulin signaling and contribute to type II diabetes (Hotamisligil 2006; Spencer et al. 2010; Yang et al. 2010; Xu et al. 2003). The chronic inflammation found in visceral adipose tissue, associated with obesity, is thought to play an important role in the development of insulin resistance in patients (Bouloumié, Casteilla, and Lafontan 2008; Fain 2010; Hotamisligil 2006; Iyer et al. 2010; Yang et al. 2010).

Specifically, tissue resident macrophages in adipose tissue have been implicated in increasing the prevalence of metabolic disease (Bouloumié, Casteilla, and Lafontan 2008; Xu et al. 2003; Weisberg et al. 2003). In addition, activation of the adipose tissue macrophages has been shown to alter the adaptive immune response and decrease the prevalence of T_{regs} within the adipose tissue (Ilan et al. 2010). The adaptive immune system components, like B and T cells, have also been shown to promote obesity's low-grade inflammation (S. Winer et al. 2009; D. A. Winer et al. 2011). Taking advantage of this relationship, immunotherapy, has been shown to impact weight gain and decrease the incidence of metabolic disease (S. Winer et al. 2009; Ilan et al. 2010).

Interestingly, the low-grade inflammatory response associated with obesity is also similar to the inflammatory response typically seen during pathogenic infections (Spencer et al. 2010; Hotamisligil 2006). During pathogenic infections, pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns

(PAMPs), like lipopolysaccharide, flagellin and bacterial and/or viral DNA and RNA (Takeuchi and Akira 2010). Activation of PRRs is crucial for the development of the innate immune response and activation of the adaptive immune response for protection against pathogen infections. Recently, It has been appreciated that the gut-resident bacteria, called the commensal microbiota, could be a constant source of PAMPs that can also be recognized by host PRRs for immune activation (Bergstrom et al. 2012; Neish 2009; Nobuhiko Kamada et al. 2013).

One of the immune signaling pathways associated with this response is myeloid differentiation primary response gene 88 (MYD88) (Hosoi et al. 2010). MYD88 activation is downstream of various PAMPs that can also be activated by food metabolites (Fessler, Rudel, and Brown 2009; Vijay-Kumar et al. 2010). Mice deficient in MYD88, Toll-Like receptor (TLR) 4, or TLR5 have been shown to have an altered sensitivity to metabolic disease when compared to wildtype (WT) mice (Hosoi et al. 2010; Fessler, Rudel, and Brown 2009; Vijay-Kumar et al. 2010). The involvement of TLR4/TLR5 in metabolic disease and DIO has led to the theory that the chronic, low-grade inflammation may be attributed to the gut microbiota. However, it is still unclear how this relationship occurs (Musso, Gambino, and Cassader 2010).

Obesity and the microbiota

The human microbiota is home to tens of trillions of microbes that are primarily categorized as Bacteria, Archaea, and Eukarya (Consortium 2012). Human co-evolution with the microbiota has lead to the development of a symbiotic relationship where the microbiota contributes to host physiological processes, like food metabolism, and the

host provides a special niche for the microbiota to inhabit (Hooper and Macpherson 2010). Emerging evidence suggests the gut microbiota has an active role in food metabolism (Kau et al. 2011b; Bäckhed et al. 2007a). Firstly, the difference in diet and environments experienced by children from rural Africa and children from urban Europe can be seen in their respective microbial communities (De Filippo et al. 2010). Impressively, due to differences in diet, the African cohort contains a more diverse gut microbial community and contains two bacterial species unseen in the European cohort (De Filippo et al. 2010). This supports the idea that humans rely on the microbiota to breakdown particular plant polysaccharides since only the microbial communities have the enzymes necessary for processing cellulose and xylan (De Filippo et al. 2010; Maslowski and Mackay 2011). Therefore, diet has the ability to affect the microbial community, which in turn, can affect nutrient availability.

This idea is further supported by the observation that dietary components can alter the gut microbiota composition, as quickly as within 24 hours of dietary changes. This was demonstrated using mice colonized with a human microbiota. When mice were switched from a low-fat, high-plant polysaccharides diet to a high-fat, high-sugar diet for one day, the group noticed changes in the mice's microbiota communities (Turnbaugh, Ridaura, et al. 2009). Specifically, high fat, high sucrose diet resulted in an increase in the Firmicutes phylum and a decrease in the Bacteroidetes phylum (Turnbaugh, Ridaura, et al. 2009). Furthermore, this microbial change even predates the development of obesity.

This observation has been validated in various animal models of obesity as well. Obese animals have been noted to possess a different gut microbiota from their

genetically similar lean counterparts. (Ley et al. 2005; Ley et al. 2006; Turnbaugh et al. 2008). One proposed mechanism for how the microbiota can lead to obesity suggests that the obesity-associated microbiota processes dietary components differently, and more efficiently, thus leading to the development of DIO (Ussar et al. 2015; Turnbaugh et al. 2006). It has been suggested that the primary influences on gut microbiota composition are dietary components like fat and carbohydrates (Muegge et al. 2011a; Faith et al. 2011a; Ley et al. 2005; Turnbaugh, Ridaura, et al. 2009; Kashyap et al. 2013), but the mechanism behind these changes are unclear.

In particular, obesity is described to be minimized in germfree (GF) rodents fed a high calorie diet (Bäckhed et al. 2007b). This represents a paradigm shift from the model wherein obesity was linked to caloric intake alone. Although early twin studies suggested a critical role of host genetics in obesity (Stunkard et al. 1986), recent evidence demonstrates that obesity is also associated with differences in the intestinal microbiota found in lean and obese twins (Turnbaugh, Hamady, et al. 2009).

Microbiota and malnutrition

On the other hand, childhood malnutrition and stunting occurs at a frequency that cannot be explained by food scarcity alone (J. I. Gordon et al. 2012). A variety of factors have been shown to correlate with stunting, like the environment a child is exposed to *in utero* and post-natally (Ahmed et al. 2014). Stunted growth in childhood can lead to diminished survival, developmental delay, and poor health. (Dewey and Begum 2011). Just as the gut microbiota has been linked to obesity, it has also been linked to malnutrition. Specifically, the gut microbiota has been shown to drive kwashiorkor (M. I.

Smith et al. 2013; J. I. Gordon et al. 2012). In particular, the group noted that temporary meal supplementation resulted in transient microbial community changes and increased host growth (M. I. Smith et al. 2013). This observation was further explored in mouse models. Germ free mice conventionalized with the microbial communities from malnourished donors were leaner and had diminished growth compared to the germ free mice conventionalized with the microbial communities from well-nourished children. This allowed for the conclusion that the microbial community was partially responsible for the stunted growth phenotype (M. I. Smith et al. 2013).

Another group observed that although malnourished children could be partially rescued by nutrient intervention, their microbiota never fully normalized and still contained members associated with severe malnutrition (Subramanian et al. 2014). Exactly how this “malnourished” microbiota affects the mucosal immune system is not well understood. Recently, it has been proposed the functional defects in IgA production, found in malnourished Malawian children, could contribute to the disease (Kau et al. 2015).

The commensal microbiota and colonic pathology

The dense population of the microbiota is maintained and separated from host tissue by a mucosal epithelial layer that is approximately 10μm thick (Hooper, Littman, and Macpherson 2012). Thus, a physical immune barrier helps to prevent opportunistic infections by commensal microbes (McFall-Ngai 2007; Hooper, Littman, and Macpherson 2012). Thus, the intestinal morphology of GF mice is also very different from that of colonized mice. For instance, the total mass of the intestine and total

surface area of the small intestine is decreased in GF mice (Meslin, Sacquet, and Delpal 1984; K. Smith, McCoy, and Macpherson 2007). The intestinal epithelium is altered by the presence of the microbiota as evidence, for example, by the slower rate of cell turnover and increased numbers of goblet cells present in the cecum of GF rodents (Khoury, Floch, and Hersh 1969; Johansson et al. 2015; K. Smith, McCoy, and Macpherson 2007). One mechanism that could explain the pathological difference is the idea that paneth cells can sense the microbiota and thus can upregulate intestinal angiogenesis to maintain intestinal homeostasis along the host-microbe barrier (Stappenbeck, Hooper, and Gordon 2002; Vaishnava et al. 2008).

When comparing mice reared in microbiota-free environment to normal microbiota colonized mice, it was quickly apparent that the presence of the microbiota is critical to the development of the normal immune system; GF mice possess fewer lymphoid structures in the intestinal tissue, and less IgA production compared to normal mice (K. Smith, McCoy, and Macpherson 2007). In addition to an impaired immune system, GF animals have an altered physiology (K. Smith, McCoy, and Macpherson 2007). For example, the basal metabolic rate of a GF mouse is lower than colonized mice, as measured by O_2 consumption. GF mice also have increased rates of absorption of certain vitamins and minerals (Bernard S. Wostmann, Bruckner-Kardoss, and Knight 1968).

The presence of bacterial ligands like LPS and flagellin has been shown to up-regulate intestinal epithelial proliferation, paneth cell proliferation and expression of antimicrobial peptides (AMPs) like RegIIIγ through the IL-22 pathway (Peterson et al. 2015; Deplancke and Gaskins 2001; Loonen et al. 2014; Specian and Oliver 1991).

AMPs are a diverse group of peptides produced by invertebrates, plants and animals that provide natural resistance to microorganisms (Skarnes and Watson 1957; Loonen et al. 2014; Brogden 2005). Generally speaking, AMPs are capable of killing bacterial pathogens by forming pores in the membranes of pathogens and/or inhibit bacterial replication by preventing cell-wall synthesis, nucleic-acid synthesis and protein synthesis (Y. J. Gordon, Romanowski, and McDermott 2005; Brogden 2005). These immune and physical responses play an important role in compartmentalizing the microbiota (Deplancke and Gaskins 2001). Mice deficient in IgA antibody production have been shown to have increased systemic antibodies directed against the microbiota, thus implying that bacterial components can be exposed to the systemic immune system (Macpherson et al. 2000; Peterson et al. 2015).

Therefore, it is possible that the compartmentalization of the commensals within the gut lumen is compromised in immune-deficient mice. For example, mice deficient in the phosphatase SHP-1 have a type of systemic autoimmune disease that is dependent on the presence of the microbiota (Croker et al. 2008). In addition, various genetic mutations have been found to be associated with severity of Crohn's disease and colitis. Genome-wide association studies has demonstrated that certain genetic variants within genes typically associated with mucosal immune responses also correlate with disease severity (Franke et al. 2010; Glocker et al. 2009; McGovern et al. 2010). Furthermore, treatments with antibiotics and/or probiotics have been shown to dampen inflammation within the colon, thus demonstrating a microbial component to inflammatory bowel disease (Cummings and Kong 2004).

Emerging evidence has also linked various autoimmune diseases like autoimmune encephalomyelitis (EAE) to the microbiota; GF mice experience less incidences of EAE, arthritis and colitis when compared to mice colonized with bacteria (Wu et al. 2010; Lee et al. 2011; Hooper, Littman, and Macpherson 2012). It has also been shown that mice lacking the transcription factor T-bet, which affects both the innate and adaptive immune cells, develop colitis in a microbiota-dependent manner, so much so, that the transfer of this particular microbiota can also increase colitis incidence in WT mice (Garrett et al. 2007).

Microbiota and immune development

The microbiota plays a pivotal role in promoting proper gut immunity for host mucosal defense (Costello et al. 2012). For instance, studies have implicated the commensal microbiota in directing T cell development to regulate immune responses to pathogens (Kau et al. 2011a; Littman and Pamer 2011). Specifically, colonization of mice with segmented filamentous bacteria (SFB) results in increased T_H17 cells, that produce IL-17 and IL-22, within the colonic tissue (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). Although the exact mechanism for T_H17 cell induction has not been fully elucidated, it is believed that SFB's direct interaction with the colonic epithelium promotes T_H17 cell polarization and activation (Ivanov et al. 2009). The microbiota also appears to have similar effects on B cells and can change the repertoire of antibodies to become specific for particular bacterial components (Peterson et al. 2015).

Similarly, another microbial member has been shown to modulate the host's immune system. Polysaccharide A (PSA) of *Bacteroides fragilis* (Round and

Mazmanian 2010), promotes the up regulation of IL-10 and T_{regs} within the intestine by activating TLR2 on T cells (Round et al. 2011). However, *B. fragilis* ΔPSA promotes a more pro-inflammatory response that tends to polarize intestinal immune cells towards T_H17-type responses (Round et al. 2011). A consortium of Clostridial strains has also been shown to up regulate the presence of inducible T_{regs} not only within the colonic tissue but also within peripheral tissue (Atarashi et al. 2011). Furthermore, the presence Clostridia can regulate intestinal barrier permeability and function to prevent the development of food allergies (Stefka et al. 2014). However, exactly how the microbiota can influence the peripheral immune system has not been well understood.

Type 3 innate lymphoid cells (ILC3s)

Recently, a group of innate cells called innate lymphoid cells (ILCs) has been found to be important in regulating inflammation at barrier sites, like at the lung and the colon. ILCs are innate lymphocytes that have the capability to respond quickly to a variety of stimuli to influence adaptive immune cells (Sonnenberg and Artis 2015). ILCs are derived from common lymphoid progenitors, but lack somatic recombination capabilities, so ILCs are unable to rearrange their cell surface receptors that bind antigens. They develop in the fetal liver and adult bone marrow (Eberl et al. 2004; Sonnenberg and Artis 2015; Sonnenberg 2014). ILCs are mainly broken into 3 groups, based on the distinct transcription factors that are critical for their development to maturity. ILC1s express T-bet and are mainly found to produce interferon (IFN) and TNF (Klose et al. 2014). ILC2s express GATA3 and are mainly found to produce IL-4, IL-5, IL-13 and IL-9 (Monticelli et al. 2011). ILC2s can mainly be found in the lungs, skin and

adipose tissue (Sonnenberg and Artis 2015). ILC3s express RORγt and mainly produce IL-22, IL-17 and IFNgamma (Sawa et al. 2011). ILC3s can only be found in the skin, intestinal lamina propria and mucosal immune sites (Hepworth et al. 2015).

Furthermore, ILC3s play an important role in modulating colonic inflammation (Sonnenberg, Fouser, and Artis 2011; Hepworth et al. 2015). ILC3s can regulate intestinal myeloid cells by producing granulocyte macrophage colony-stimulating factor (GM-CSF) (Mortha et al. 2014). Since cryptopatches, comprised mainly of ILC3s, only differentiate into isolated lymphoid follicles in the presence of the commensal microbiota, it is believed that microbial ligands can activate ILC3s (Lorenz et al. 2003; Giacomin et al. 2015; Hepworth et al. 2015). Specifically, the activation of MHCII on ILC3s is required for proper tissue homeostasis, since the absence of MHCII results in microbiota-mediated, T-cell-dependent colonic inflammation (Fung et al. 2016; Hepworth et al. 2015; Hepworth et al. 2013).

Lymphotoxin signaling and mucosal immunity

The lymphotoxin (LT) pathway is traditionally thought to be important for the development and function of the gut-associated-lymphoid-tissue (GALT). LT is also known to promote the production of antimicrobial peptides, IgA and IL-22 which are involved in mucosal defense (Upadhyay and Fu 2013). LTα, also known as TNFSF1, is part of the tumor necrosis factor (TNF) superfamily (Ware et al. 1995). LTα forms a soluble homotrimer that can bind to herpes virus entry mediator (HVEM) and TNF receptor 1/2 (Ware et al. 1995). Only the lymphotoxin-signaling pathway results in the formation of secondary lymphoid structures and organization of lymphoid organs

(Alimzhanov et al. 1997; De Togni et al. 1994; Fütterer et al. 1998; Mariathasan et al. 1995; Tumanov et al. 2003). Although TNF can also bind TNF-receptor, TNF-mediated signaling alone has a limited role in organogenesis (Matsumoto et al. 1997; Roach et al. 2005). The primary signaling pathway for the generation of lymphoid organs comes from LTα forming a heterotrimer with two LTβ to make a membrane-bound ligand that binds to LTβR (De Togni et al. 1994; Fütterer et al. 1998; Mariathasan et al. 1995; Tumanov et al. 2003).

Thus, mice deficient in LTα, LTβ, or LTβR lack all GALT and have compromised mucosal defenses (Alimzhanov et al. 1997; De Togni et al. 1994; Fütterer et al. 1998; Onder et al. 2013; Tumanov et al. 2003; Fu and Chaplin 1999). $LT\alpha_1\beta_2$ is expressed on the surface of T, B, LTi (lymphoid tissue inducer cells) and ILC3s (van de Pavert and Mebius 2010). ILC3s expressing $LT\alpha_1\beta_2$ or soluble $LT\alpha_3$ regulate IgA production in both T-cell-dependent and T-cell-independent pathways (Tsuji et al. 2008; Kruglov et al. 2013). LTβR is expressed on stromal cells, myeloid cells, hepatocytes, epithelial cells and endothelial cells (Ware et al. 1995; van de Pavert and Mebius 2010; Upadhyay and Fu 2013). One of the primary roles of active lymphotoxin signaling is to organize immune cells within follicular structures to promote specific immune responses (Berger et al. 1999; GeurtsvanKessel et al. 2009; Kumar et al. 2010; Macpherson et al. 2000; Ota et al. 2011; Tumanov et al. 2002).

We and others have demonstrated that the LTβR pathway controls IL-22 and antimicrobial peptide expression in a bacterial colitis model, like the *Citrobacter rodentium* infection model (Ota et al. 2011; Satoh-Takayama et al. 2011; Tumanov et al. 2011). The LT-IL-23/IL-22 pathway, controlling the expression of antimicrobial peptides

and IgA, can shape the microbiota to protect the host against bacterial infections (Macho-Fernandez et al. 2015). In addition, the IL-22RA pathway has been implicated in the establishment of the microbiota and mucosal homeostasis that prevents dissemination of commensal bacteria during colonic inflammation (Pham et al. 2014).

Lymphotoxin signaling and obesity

Several epidemiological studies have linked polymorphisms in TNF and LTα to obesity and type II diabetes (Mahajan et al. 2010). While most studies have focused on TNF, several polymorphisms linking TNF to obesity lie within the coding exons of LTα (Mahajan et al. 2010). Another group observed that *Lta*$^{-/-}$ mice were resistant to DIO (Pamir et al. 2012). They noticed that *Lta*$^{-/-}$ mice had increased basal systemic inflammation on normal chow. The overabundance of macrophages was seen at baseline in only normal chow *Lta*$^{-/-}$ mice compared to WT controls.

Interestingly, *Lta*$^{-/-}$ mice had increased macrophage and T cell accumulation in the adipose and systemic tissues, without significant increases in insulin resistance following high fat diet (HFD) challenge (Pamir et al. 2012). Following HFD, more M2-like macrophages were found in the adipose tissue of *Lta*$^{-/-}$ mice compared to WT controls. In addition, the authors noticed an increase in T cell concentration within the adipose tissue of WT mice, as determined by relative CD3 mRNA expression in adipose tissue. However, opposite to WT mice, *Lta*$^{-/-}$ mice had more T_{regs} and T_H1, instead of T_H17 cells, in adipose tissue (Pamir et al. 2012).

Furthermore, the group noticed increased serum inflammatory cytokines, like TNFα and IL-6, that were elevated in *Lta*$^{-/-}$ mice at baseline and further exacerbated

during HFD compared to WT mice (Pamir et al. 2012). The authors also noted that the high levels of inflammatory cytokines in HFD fed *Lta*$^{-/-}$ mice were even more elevated than in other mouse models of obesity, like *ob/ob* mice (Pamir et al. 2012).

Previous work also revealed an essential role for the microbiota and LT in DIO since *Lta*$^{-/-}$ and *Ltbr*$^{-/-}$ mice resist weight gain (Upadhyay et al. 2012; Upadhyay and Fu 2013). The cecal microbiota community of *Ltbr*$^{-/-}$ mice also differed from *Ltbr*$^{+/-}$ mice after HFD. Housing lean *Ltbr*$^{-/-}$ mice with their obese *Ltbr*$^{+/-}$ littermates rescues weight gain in *Ltbr*$^{-/-}$ mice, demonstrating the communicable nature of the obese phenotype. In addition, microbiota transfer of *Ltbr*$^{-/-}$ cecal contents into WT GF recipients confers the leanness phenotype. Specifically, HFD fed *Ltbr*$^{-/-}$ mice maintains higher levels of SFB within their feces compared to littermate controls. Taken together these observations raise the possibility that the LT pathway might be important for immune-mediated selection of the gut microbiota. In addition, we noticed a decrease in colonic tissue IL-22 expression in HFD fed *Ltbr*$^{-/-}$ mice that is not seen in obese littermate controls (Upadhyay et al. 2012). We hypothesize that LT-dependent gut immune responses can select the commensal microbiota to facilitate weight gain (body growth) in response to HFD.

How does lymphotoxin signaling prevent weight gain on HFD?

The laboratory's animal models (*Ltbr*$^{-/-}$ mice and all the conditional knockouts along the LT pathway) give us a unique opportunity to dissect out the molecular and cellular elements of gut immunity that contribute to weight gain, as well as which commensal bacteria might promote weight gain after HFD. We hypothesize that the

dynamic interactions between the microbiota and LT-mediated gut immunity is essential for the establishment of a microbiota that enables obesity following HFD. Alternatively, the gut immune system may prevent alterations in the microbiota that can lead to leanness.

Some host responses like IgA, goblet cells, antimicrobial peptides, and IL-22 control the gut microbiota and immune response to promote host growth and obesity. The absence of these host responses could result in an inability to regulate the gut microbiota, thus leading to an inability to gain weight in the presence of excess calories. Using gnotobiotic mouse models and conditional LTβR and LTβ mice, we explored the mechanistic role of how the LT pathway interacts with the gut microbiota to result in obesity.

Chapter 2: Methods

Mice

C57BL/6, *Rag1*$^{-/-}$ and *Ragγc*$^{-/-}$ (DiSanto et al. 1995) mice were originally obtained from Harlan, Jackson, and Taconic Labs, respectively, and were bred and maintained in-house for multiple generations. *Lta*$^{-/-}$, *Ltb*$^{-/-}$, *Ltbr*$^{-/-}$, *Rorc*$^{-/-}$, *Cd4*$^{cre+}$*Ltb*$^{f/f}$ (LTβ$^{\Delta T}$), *Cd19*$^{cre+}$*Ltb*$^{f/f}$ (LTβ$^{\Delta B}$), *Cd4*$^{cre+}$*Cd19*$^{cre+}$*Ltb*$^{f/f}$ (LTβ$^{\Delta T, B}$), *Rorc*$^{cre+}$*Ltb*$^{f/f}$ (LTβ$^{\Delta ILC, T}$), *Cd11c*$^{Cre+}$*Ltbr*$^{f/f}$ (LTβR$^{\Delta DC}$), and *LysM*$^{Cre+}$*Ltbr*$^{f/f}$ (LTβR$^{\Delta M\phi}$) mice were bred as littermates in a vivarium at the University of Chicago and were initially characterized elsewhere (Mariathasan et al. 1995; Alimzhanov et al. 1997; Fütterer et al. 1998; Eberl et al. 2004; Tumanov et al. 2003; Tumanov et al. 2002; Tumanov et al. 2011; Yugang Wang et al. 2010). *Ltb*$^{-/-}$ mice were crossed onto the *Rag1*$^{-/-}$ background for this study. LTβ$^{\Delta ILC, T}$ mice were crossed onto the *Rag1*$^{-/-}$ background for this study. Littermates were generated by breeding of a null parent (usually the dam) to a heterozygous mate. In the case of conditionally deficient mice, littermate *Ltb*$^{f/f}$ or *Ltbr*$^{f/f}$ mice were used as WT controls. Mice were genotyped before weaning between 21-28 days after birth. All mice were bred and kept under specific pathogen-free conditions. All experimental procedures were approved and carried out in accordance to University of Chicago's IACUC (Protocol #71866).

Germ free derivation of LTβR littermates

Derivation of *Ltbr*$^{-/-}$ mice was contracted through Taconic Biosciences via the standard GF derivation protocol using GF isolator reared, GF surrogate mother. Briefly,

Taconic Biosciences received male *Ltbr*$^{-/-}$ mice from us to be bred to C57BL/6 female mice. At day 19-20 of gestation, the 'uterine package' was surgically removed from the female donor. Under sterile conditions, the 'uterine package' was opened and the *Ltbr*$^{+/-}$ pups were transferred to a GF isolator housing a surrogate mother. The microbiological status of the isolator was monitored following transfer. Once the *Ltbr*$^{+/-}$ mice reached adulthood, they were shipped to the University of Chicago's Gnotobiotic Core Facility using Taconic GF Shipper. Specialized technicians at the Gnotobiotic Core transferred the animals from the Taconic GF Shipper to the designated flexible isolator. GF heterogynous LTβR mice were bred together to generate GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ were maintained by homozygous breeding in the gnotobiotic facility. Fecal samples were collected weekly to monitor for microbiological status on the experimental isolators by culture and PCR of bacterial 16s ribosomal DNA. All experimental procedures were approved and carried out in accordance to University of Chicago's IACUC (Protocol #71866).

Specific pathogen-free and germfree high fat diet experiments

All mice used in diet experiments were age and sex matched. All specific pathogen-free (SPF) mice were maintained on normal chow (Harlan Teklad 2918) until 9 weeks of age. At 9 weeks of age, animals received autoclaved and irradiated high fat, high sucrose diet (Harlan Teklad 88137) or were maintained on normal chow for the duration of the 9-week long high fat diet experiments. Mice were monitored and weighed weekly until the end of 9 weeks. Mice were sacrificed by IACUC standards and weighed again before serum and tissues were collected for further analysis. Similarly,

for GF obesity experiments, all GF mice were maintained on standard autoclaved and irradiated chow (2016S) until 9 weeks of age. At 9 weeks of age, GF mice were switched to either autoclaved and irradiated 88137 or 97222 or maintained on 2016S for the duration of the 9-week long experiments. Gnotobiotic technicians monitored and weighed the mice weekly. Fecal samples were collected weekly to monitor for microbiological status on the experimental isolators by culture and 16s PCR. Table 1 shows the macronutrient contents of the diets used.

Table 1: Diets used in study.

Diet	Normal Chow Diet	GF Normal Chow Diet	High Fat, High Sucrose Diet	High Fat, Maltodextrin-rich Diet
Make Catalog #	Tekland 2018/2918	Tekland 2016S	Tekland 88137	Tekland 97222
Sucrose, g/kg	N/A	N/A	341.46	254.924
Maltodextrin	N/A	N/A	N/A	140
Protein, % kcal	18.6	16.4	15.2	15.8
Carbohydrate, % kcal	44.2	48.5	42.7	46.9
Fat, % kcal	6.2	4	42.0	37.4
kcal/g	3.1	3	4.54	4.38

Stool DNA extraction

Stool samples were freshly collected and frozen at <-20°C. Stool DNA extraction was performed with Mini-beadbeater (BioSpec Products) homogenization with beads followed by extraction with QIAamp ® DNA Stool Mini Kit's (Qiagen) protocol. The DNA was eluted and saved at -20°C until further analysis.

Gnotobiotic experiments

GF mice were weaned and transferred to a gnotobiotic isolator. Cecal and fecal contents containing SFB (generously provided by Alexander Chervonsky's lab) and/or Altered Schaedler's Flora (ASF) (originally obtained from purchased ASF-colonized Taconic mice) were vortexed in sterile 1x PBS (Invitrogen). A sterile 18-gauge gavage needle was used to administer 100 µl/mouse before 4 weeks of age. Fecal samples were collected weekly to monitor colonization status of the gnotobiotic colony using 16s qRT-PCR (Table 2). At 9 weeks of age, mice were used for HFD experiments following standard procedures. Fecal samples were collected to measure levels of bacterial colonization between the experimental groups, before the start of HFD, during the HFD and before sacrifice. Conventionalization of GF mice was performed by gavage of cecal content of an *Ltbr*$^{+/-}$ donor. Conventionalized mice were aged for 4-6 weeks before spleens and systemic tissue was dissected and analyzed for cellularity.

Table 2: 16s Primers used.

Target	Forward	Reverse	Reference
EUA	AGAGAGTTTGATCCTGGCTCAG	ATTACCGCGGCTGCTGG	(Turner et al. 1999; Muyzer, de Waal, and Uitterlinden 1993)
SFB	GACGCTGAGGCATGAGAGCAT	GACGGCACGGATTGTTATTCA	(Bouskra et al. 2008)
Universal 16s	AGAGTTTGATCMTGGCTCAG	TACGGYTACCTTGTTACGACTT	(Weisburg et al. 1991)
ASF360	CTTCGGTGATGACGCTGG	GCAATAGCCATGCAGCTATTGTTG	(Sarma-Rupavtarm et al. 2004)
ASF361	GCAATGATGCGTAGCCGAAC	GCACTTTCTTCTCTAACAACAGGG	(Sarma-Rupavtarm et al. 2004)
ASF356	CGGTGACTAATACCGCATACGG	CCTTGCCGCCTACTCTCCC	(Sarma-Rupavtarm et al. 2004)

Table 2, continued.

ASF457	CCGAAAGGTGAG CTAATGCCGG	GGGACGCGAGTC CATCTTTC	(Sarma-Rupavtarm et al. 2004)
ASF492	CTGCGGAATTCCT TCGGGG	CCCATACCACCG GAGTTTTC	(Sarma-Rupavtarm et al. 2004)
ASF500	GTCGCATGGCAC TGGACATC	CCTCAGGTACCG TCACTTGCTTC	(Sarma-Rupavtarm et al. 2004)
ASF502	CGGTACCGCATG GTACAGAGG	CAATGCAATTCCG GGGTTGG	(Sarma-Rupavtarm et al. 2004)
ASF519	CACAGTAAGCGG CACAGCG	CCGCTCACACGG TAGCTG	(Sarma-Rupavtarm et al. 2004)

Culture conditions for bacteria in peripheral tissues

Using autoclaved instruments, spleens and lungs were sterilely harvested under flame and placed in sterile 1x PBS (Invitrogen). Tissues were mechanically disrupted either in 1x PBS or 1x PBS + 0.5% TritonX100 (ThermoFisher). Tissue homogenates were plated on bacterial plates and/or used to inoculate a liquid culture in both anaerobic and aerobic incubator conditions (Table 3) either in lab or by the University of Chicago Clinical Microbiology Core lab (generously assisted by Vera Tesic). Spleens

and livers were sterilely homogenized with a tissue homogenizer before plating serial dilutions on bacterial culture plates. Plates were monitored for bacterial growth at 24 hours, 48 hours and 72 hours in a 37°C incubator. Fecal samples were collected, processed, and used as positive controls. For the GF mice colonization experiment, a spleen from a specific pathogen-free (SPF) *Ltbr*$^{-/-}$ mouse was homogenized and gavaged into GF mice. Fecal samples were collected weekly for 4 weeks following gavage and monitored for the presence of bacterial colonization via 16s RT-PCR.

Table 3: Culture conditions used to identify bacteria in systemic tissues.

Culture media type	Anaerobic	Aerobic	Lab or Clinical Microbiology Core
LB plate and broth	Yes	Yes	Lab
Nutrient plate and broth	Yes	Yes	Lab
5% sheep's blood agar (BD)	Yes	Yes	Lab
BHI+hemin+VitK supplement plate	Yes	Yes	Lab
MaConkey plate	Yes	Yes	Lab
Blood plate	Yes	Yes	Microbiology Core
Chocolate plate	Yes	Yes	Microbiology Core
Brucella plate	Yes	Yes	Microbiology Core
BBL plate	Yes	Yes	Microbiology Core

Flow cytometry and antibodies

Viable cells from lungs, spleens and LPL were counted on a microscope (Zeiss) using a hemocytometer. All samples were stained with ZombieAqua® in 1x PBS following Biolegend's instructions. Cell samples were then washed with FACs wash buffer (1 x PBS + 2% Fetal bovine serum), before incubating the samples in anti-CD16/CD32 FC block (clone 2.4g2), in the dark, for at least 15 minutes, at room temperature. Samples were stained with a master mix of antibodies purchased from Biolegend for 30 minutes in the dark at 4°C. Samples were then washed again with FACs wash buffer before fixation with eBioscience IC fixation buffer overnight, in the dark at 4°C. Data was collected on an LSR-Fortessa (BD) and analyzed using FlowJo.

Sorting and transfer of ILC3s

ILC3s from lamina propria lymphocytes (LPL) of *Rag1*$^{-/-}$ mice were sorted based on 7AAD, CD45 and CD90 expression using a FacsAria (BD) as was initially described elsewhere (Xiaohuan Guo et al. 2015). Briefly, the small intestine and colonic tissue, including the cecum, was removed and cleaned using sterile 1x PBS and mechanical scraping. The mucosal tissue was washed using 1x HBSS + 0.5% dithiothreitol before enzymatic digestion using DNase and Liberase (Roche). The intestines were further disrupted using gentleMACs™ C tubes (Miltenyl Biotec). Lymphocytes were further purified by gradient centrifugation using a 40%/80% Percoll (GE) gradient. LPL were collected at the interphase, washed and counted. The cells were stained at 1x10^{7} cells/ml before sorting. Purification checks were performed after each sort. The cells

were kept on ice until ≥2.5x10^5 cells were transferred via retro-orbital, IV injection into 3-4 week old mice. The mice were aged for another 4-6 weeks before analysis.

Blocking lymphotoxin and IL-22 signaling in vivo

The LTβR:hIg used in this study has been previously described (Anders et al. 2005). Briefly, 100 μg per mouse of LTβR:hIg was administered by intraperitoneal (i.p.) injections weekly from weaning for the duration of the experiment. Anti-IL-22 antibody (generously provided by Wenjun Ouyang at Genentech) was administered i.p. 150 μg/mouse 3x/week from weaning for the duration of the experiment. Mouse weights were monitored before each injection to ensure normal health status.

Histology

Tissues were dissected from mice and fixed in 10% neutral buffered formalin. The University of Chicago's Pathology Core facility paraffin-embedded, cut and strained with H&E. Histological images were taken at the University of Chicago's Microscope Core facility using a Zeiss light-microscope and camera.

Statistical methods

Data was analyzed using Student's *t*-test or ANOVA with multiple comparisons correction, when appropriate, using GraphPad Prism 6.0 program. Data from such experiments are presented as mean values ± S.E.M. $P<0.05$ was considered statistically significant.

Chapter 3: Active LT Signaling From ILC3s Prevents The Development Of Splenomegaly In The Presence Of The Commensal Microbiota.

Lymphotoxin beta-receptor signaling prevents gut microbiota-induced splenomegaly and systemic inflammation.

It was previously noticed that *Ltbr*$^{-/-}$ mice have enlarged spleens, which were presumed to be due to the absence of peripheral lymph nodes (Zhu et al. 2011; Browning et al. 2005; Chyou et al. 2008; Liao and Ruddle 2006; Scheu et al. 2002). We also observed significant differences in spleen size (Figure 1**A**) and splenocyte number (Figure 1**B**) in SPF *Ltbr*$^{-/-}$ mice when compared to *Ltbr*$^{+/+}$ mice or littermate *Ltbr*$^{+/-}$ mice. To determine whether the increased basal inflammation in lymphotoxin deficient mice was microbiota-dependent, we rederived the *Ltbr*$^{-/-}$ mice GF. Initially, we observed an alteration of colonic pathology (Figure 1**E-F**), which included a decrease in the colonic weight-to-length ratio when compared to SPF colons (Figure 1**F**). We did not see a difference in colon density between SPF littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice, nor between GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice (Figure 1**F**). In other words, the difference in colonic density between GF and SPF mice appears to be microbiota-dependent, not lymphotoxin-dependent. This difference in colonic density could be attributed to the lamina propria being thinner and less cellular as previously reported in GF mice (Glaister 1973; K. Smith, McCoy, and Macpherson 2007). Intriguingly, the splenomegaly phenotype was no longer observed in the GF mouse colony. Specifically, GF *Ltbr*$^{-/-}$ mice had spleen sizes (Figure 1**C**) and splenocyte numbers (Figure 1**D**) comparable to GF *Ltbr*$^{+/+}$ mice.

Figure 1: The commensal microbiota influences splenomegaly phenotype seen in Lymphotoxin-deficient mice and colonic pathology.

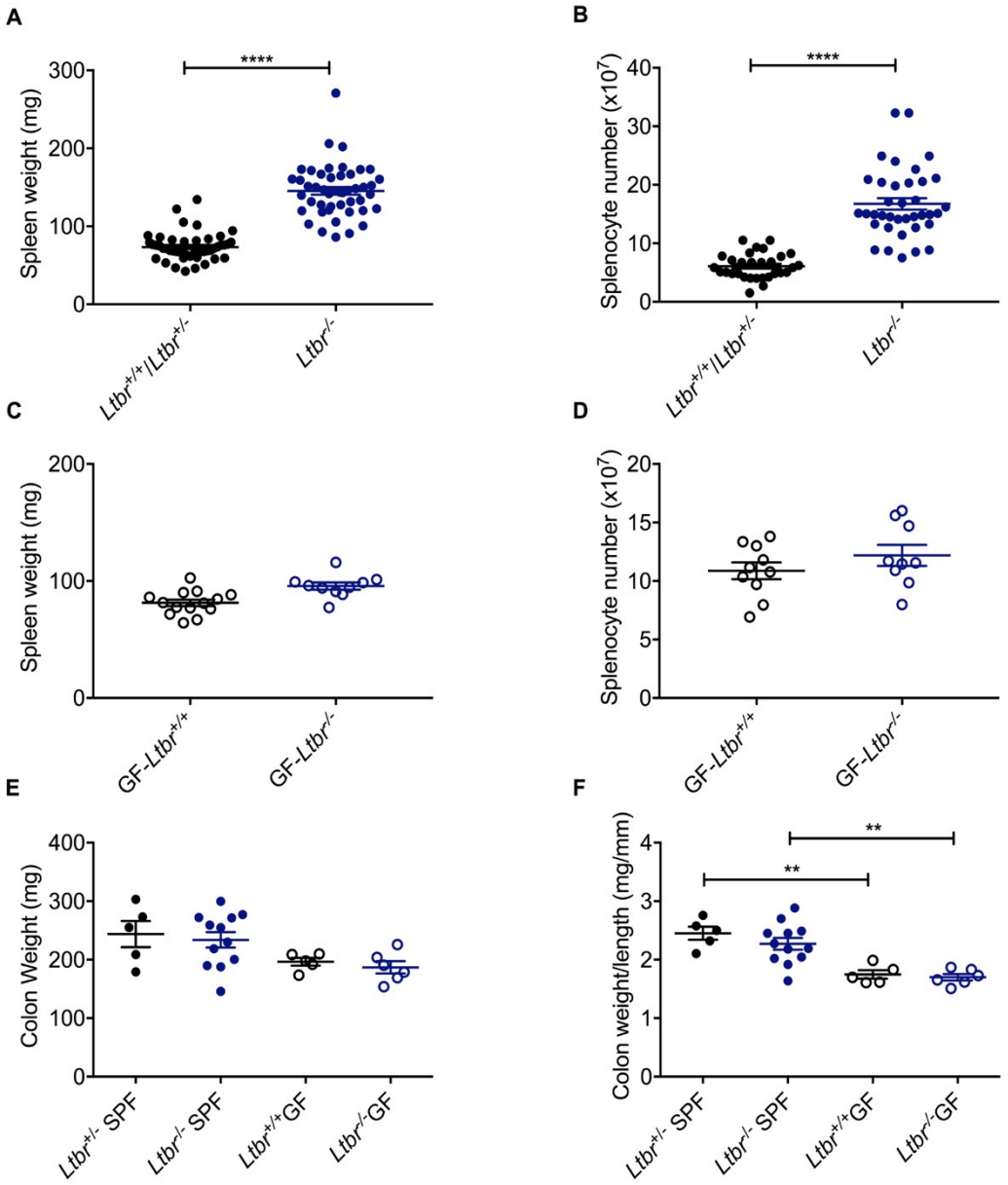


Figure 1: The commensal microbiota influences splenomegaly phenotype seen in Lymphotoxin-deficient mice and colonic pathology. **A)** Spleens from SPF *Ltbr*$^{+/+}$, littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice were harvested and weighed. *n* = 47, pooled from 6 repeats. **B)** Trypan blue negative splenocytes counted with a hemocytomer from spleens in **A**. **C)** Spleens from germfree (GF) *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ spleen weights were harvested and weighed. *n* = 10-14, pooled from 6 repeats. **D)** Trypan blue negative splenocytes counted with a hemocytomer from spleens in **C**. **E)** Colon weights were measured and **F)** colon weight/length ratio was calculated. Error bars represents mean and SEM. **A-D)** Unpaired t-test was performed, **E-F)** One-way ANOVA was performed. **, *P*<.01, ****, *P* < .0001.

The presence of systemic immune infiltrates in peripheral tissues, especially in the lung, in SPF *Ltbr*$^{-/-}$ mice were thought to be associated with either lack of lymph nodes and/or increased autoimmunity (Kang et al. 2003). Interestingly, systemic infiltrates were also absent in GF *Ltbr*$^{-/-}$ mice when compared to SPF *Ltbr*$^{-/-}$ mice (Figure 2). The presence of immune infiltrates was visualized by histology. Together, these data suggest that the splenomegaly and increased systemic immune infiltrates phenotype originally seen in SPF *Ltbr*$^{-/-}$ mice might be microbiota-dependent.

Figure 2: The presence of systemic immune infiltrates in Lymphotoxin-deficient mice is dependent on the presence of the commensal microbiota.

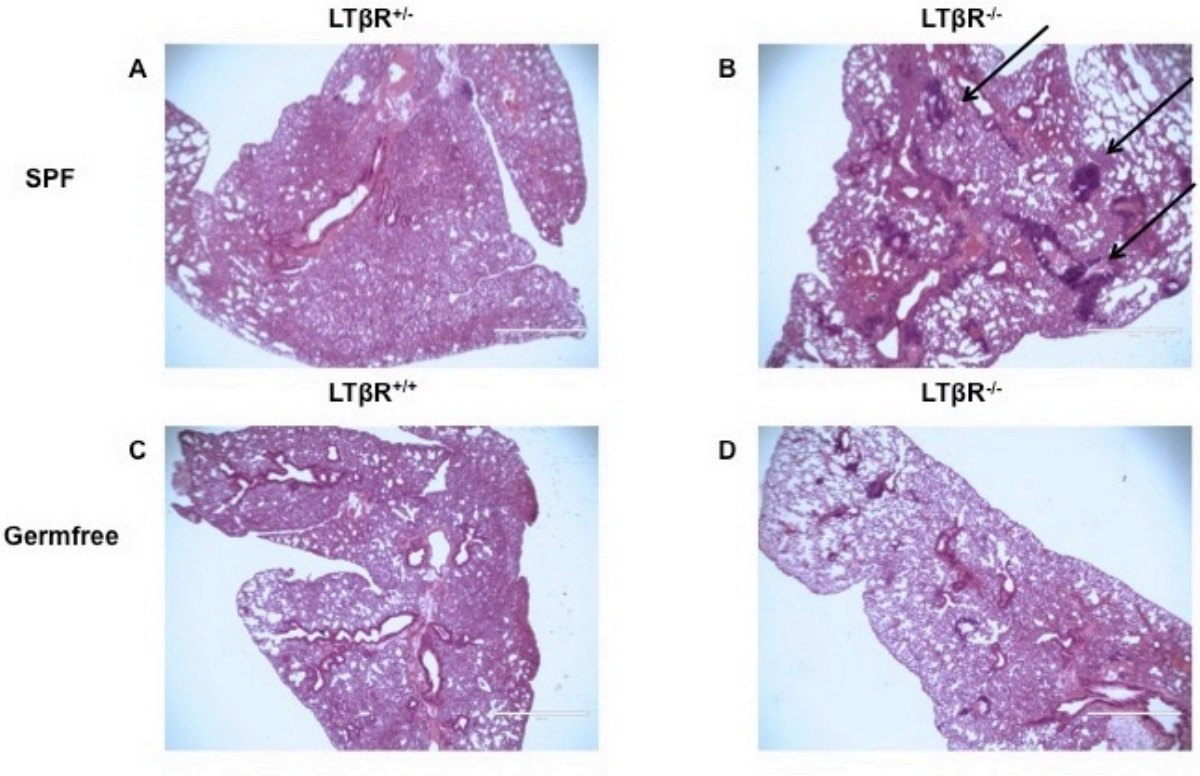


Figure 2: The presence of immune infiltrates in Lymphotoxin-deficient mice is dependent on the presence of the commensal microbiota. H&E sections from the lungs of SPF littermate **A)** *Ltbr* $^{+/-}$ and **B)** *Ltbr* $^{-/-}$ mice. H&E sections from the lungs of GF **C)** *Ltbr* $^{+/+}$ and **B)** *Ltbr* $^{-/-}$ mice. Black arrows denotes immune infiltrates. The same lobe was sectioned across the 4 groups. Images were taken at 4x.

To understand whether the development of splenomegaly observed in *Ltbr*$^{-/-}$ mice was microbiota-dependent, we wanted to understand whether the development of splenomegaly occurred concurrently with the acquisition of the microbiota (Costello et al. 2012). To determine whether splenomegaly corresponded with the acquisition of a mature microbiota, we performed a time-course experiment to see when splenomegaly first appears. Splenomegaly was measured in SPF littermates *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ before weaning until 12 weeks after birth. We observed that *Ltbr*$^{-/-}$ and *Ltbr*$^{+/-}$ littermate mice had similar spleen sizes before and after weaning. The splenomegaly phenotype appeared when *Ltbr*$^{-/-}$ mice were greater than 6 weeks of age, presumably only after the adult commensal microbiota has established in the mice (Figure 3) (Johansson et al. 2015; Costello et al. 2012).

Figure 3: The splenomegaly phenotype develops during development of the adult commensal microbiota.

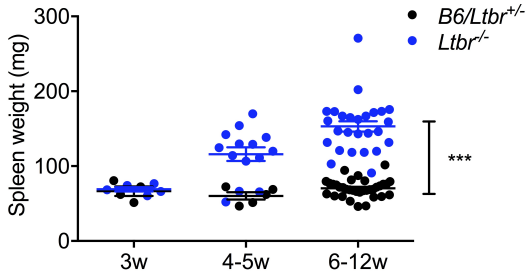


Figure 3: The splenomegaly phenotype develops during development of the adult commensal microbiota. SPF *Ltbr*$^{+/+}$ and littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice were sacrificed before weaning (3 weeks of age), post-weaning (4-5 weeks) and at adulthood (6-12 weeks) and the spleens were harvested and weighed. Error bars represents mean and SEM. One-way Anova with Tukey correction was performed. ***, $P < .001$.

To determine whether the splenomegaly phenotype was comprised of one particular immune cell type or the expansion of all immune cells, we analyzed the splenocytes via flow cytometry. When we examined the splenocyte cell population, we noticed only a significant increase in the percentage of $Gr1^{+}CD11b^{+}$ cells (Figure 4**A**), most likely neutrophils, in the SPF *Ltbr*$^{-/-}$ splenocytes when compared to *Ltbr*$^{+/-}$ littermates. Since neutrophils typically respond to bacterial infections and because splenomegaly in *Ltbr*$^{-/-}$ mice is not observed under GF conditions, it suggests that bacteria might contribute to this phenotype. In other words, it implies that the presence of colonic bacteria promotes the expansion of neutrophils within the enlarged spleen.

Figure 4: Oral antibiotics prevent splenomegaly in Lymphotoxin-deficient mice.

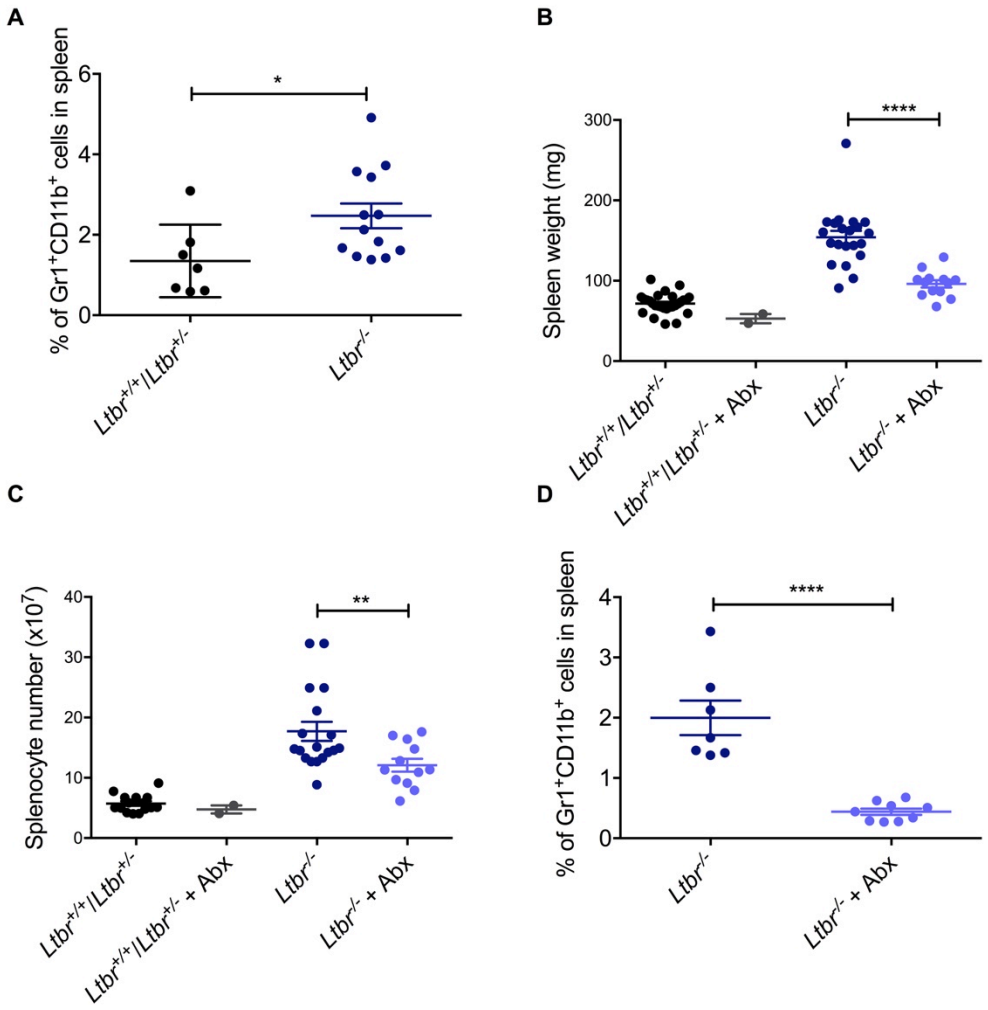


Figure 4: Oral broad-spectrum antibiotics prevents splenomegaly in Lymphotoxin-deficient mice. A) SPF spleens were examine for percentage of neutrophils (CD45+, CD3-, B220-, Gr1+ and CD11b+) out of total splenocytes. **B-D)** SPF *Ltbr*$^{+/+}$ and littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice were placed on ABX water or water alone at 4 weeks of age. Mice were sacrificed after an additional 4 weeks. The spleens were harvested and **B)** weighed, **C)** counted and percentage of neutrophils determined.. Error bars represents mean and SEM. Student's *t*-test was performed on **A** and **D.** One-way Anova with Tukey correction was performed on **B** and **C**. *, $P < .05$; **, $P < .01$; ****, $P < .0001$.

In order to determine whether the bacteria within the microbiota led to the expansion of neutrophils in the spleen of *Ltbr*$^{-/-}$ mice, we utilized antibiotics to deplete the commensals in the SPF colony (Rakoff-Nahoum et al. 2004). In order to test this hypothesis, broad-spectrum antibiotics (ampicillin, vancomycin, neomycin, and metronidazole) were administered to SPF *Ltbr*$^{-/-}$ and *Ltbr*$^{+/-}$ mice, starting at weaning for 4 weeks, *ad libitum* in the drinking water. Compared to water alone, spleen size of ABX-treated *Ltbr*$^{-/-}$ mice (Figure 4**B**) and splenocyte numbers (Figure 4**C**) were significantly reduced. ABX treatment had no effect on spleen sizes (Figure 4**B**) or splenocyte numbers (Figure 4**C**) of *Ltbr*$^{+/+}$ or littermate *Ltbr*$^{+/-}$ mice. Consistent with the idea that increased neutrophils were dependent on bacteria, we also saw a reduction in neutrophils in the spleen of ABX-treated *Ltbr*$^{-/-}$ mice (Figure 4**D**). Together, these data suggest that the presence of bacteria in *Ltbr*$^{-/-}$ mice allows for the development of neutrophilic splenomegaly. In other words, lymphotoxin signaling prevents peripheral inflammation in the presence of the microbiota.

Development of splenomegaly depends on the lack of lymphotoxin and the presence of selected microbiota.

To determine if bacteria was sufficient to promote splenomegaly in *Ltbr*$^{-/-}$ mice, we utilized a gnotobiotic approach. GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were colonized at weaning via oral gavage with a modified altered Schaedler's flora (ASF) with the addition of segmented filamentous bacteria (SFB) that we called ASFB. We decided to utilize ASF since it is a simplified mouse microbiota consisting of 8 cultivatable members (Dewhirst et al. 1999). We decided to also colonize the gnotobiotic (known bacteria) colony with

SFB since a previous work from our laboratory correlated SFB levels with lymphotoxin signaling (Upadhyay et al. 2012). After colonization with ASFB for 4 weeks, *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice did not develop splenomegaly (Figure 5**A**) nor increase splenocyte numbers (Figure 5**B**). These data suggested that the ASFB is not sufficient to promote these phenotypes.

Figure 5: The splenomegaly phenotype develops only in the presence of selected microbiota members.

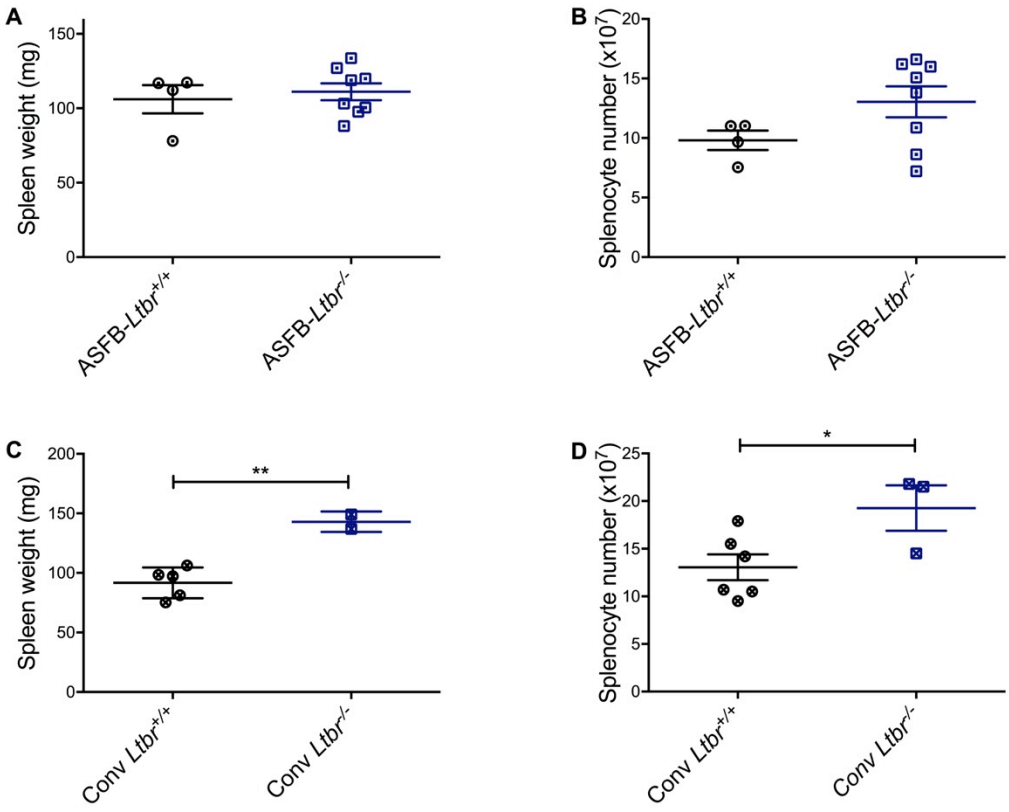


Figure 5: The splenomegaly phenotype develops only in the presence of selected microbiota members. ASFB-colonized *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were sacrificed and the spleens were harvested and **A)** weighed and **B)** splenocytes counted. n = 4-8, repeated 2 times. GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were conventionalized with *Ltbr*$^{+/-}$ cecal contents and the spleens harvested and **C)** weighed and **D)** splenocytes counted. n = 4-8, repeated 2 times with **C)** a representative graph with error bars representing mean and SD. **A,B,D)** Error bars represents mean and SEM. Unpaired t-tests was performed. *, $P < .05$; **, $P < .01$.

To determine whether a complete commensal microbiota is sufficient to promote splenomegaly in GF mice deficient in lymphotoxin β receptor, we conventionalized GF *Ltbr*$^{+/+}$and *Ltbr*$^{-/-}$ mice with SPF littermate *Ltbr*$^{+/-}$ cecal contents at 3-4 weeks of age. 4 weeks post-conventionalization, we observed significant spleen size differences (Figure 5**C**) and splenocyte number differences (Figure 5**D**) between conventionalized *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. These data demonstrate that the commensal microbiota is sufficient to promote splenomegaly in lymphotoxin-deficient mice.

Splenomegaly occurs in mice without detectable disseminating commensals.

Previous work has demonstrated the possibility that disseminating microbiota members can promote splenomegaly (Sonnenberg et al. 2012). To determine whether the presence of disseminating microbiota members could be promoting splenomegaly in lymphotoxin deficient mice, we utilized various cultures techniques in both aerobic and anaerobic conditions to try to culture bacteria from spleen and liver tissues. We used colonic tissue as the positive control for bacterial growth. No bacteria were consistently detected from systemic tissue using the culture conditions we attempted, while colonic tissue grew positive cultures at all the tested conditions (Table 4).

Table 4: Conditions attempted to detect bacteria.

Undetectable systemic bacteria from spleen and liver

Bacterial growth media	Aerobic Conditions	Anaerobic Conditions	Bacteria recovered consistently?
LB (broth and plate)	✔	✔	No
BHI+Hemin+VitK (broth and plate)	✔	✔	No
Blood Plates	✔	✔	No
Nutrient plate (broth and plate)	✔	✔	No

UofC Microbiology Core	Bacteria recovered consistently?
Blood Plate	No
Chocolate Plate	No
Brucella plate from chopped meat broth	No
BBL plate from chopped meat broth	No

Culture independent	Bacteria recovered?
16s PCR from tissue	Not detectable, problem with sensitivity
Gavage spleen into Germfree mouse	No

As a secondary method, we utilized the University of Chicago Pathology Microbiology Core to see if other culture conditions would allow for the detection of systemic bacteria. The Clinical Microbiology Core homogenized the tissue and plated the tissue homogenates on various bacterial plates and bacterial broth in both aerobic and anaerobic conditions at 37°C and 42°C. Again, colonic tissue was used as a positive control for bacterial growth. Again, no consistent bacterial growth was detected under these culture conditions from the spleen or liver homogenates, while the colonic tissue had positive cultures in all tested conditions (Table 4).

Previous work has implicated the important roles of mucosal lymphoid tissues in containing and regulating the colonic commensal microbiota to prevent inflammation, specifically Th17 immunity (Sonnenberg et al. 2012; Fung et al. 2016; Withers et al. 2016; Sonnenberg et al. 2011; Sonnenberg, Fouser, and Artis 2010). One particular microbiota member, *Alcaligenes faecalis,* was found to promote splenomegaly in the absence of IL-22 secreting ILC3s (Sonnenberg et al. 2012). Due to the lack of lymph nodes in lymphotoxin deficient mice, we wondered whether the splenomegaly observed in the SPF colony could be replicated by *A. faecalis*. Thus, we asked whether using known microbial inducers of Th17 immune response and/or known microbiota members that can be found systemically could be lymphotoxin-signaling dependent.

We utilized a gnotobiotic approach to promote Th17 immune responses in mucosal tissue with a previously identified pathobiont. We colonized GF *Ltbr*$^{+/+}$and *Ltbr*$^{-/-}$ mice at weaning with *A. faecalis*, SFB, and *B. fragilis*ΔPSA for a few days before gavaging ASF cecal contents, which we called ASF+3. Successful colonization was determined by PCR. Interestingly, we did not observe splenomegaly in either ASF+3 colonized *Ltbr*$^{+/+}$and *Ltbr*$^{-/-}$ mice. It is possible that this gnotobiotic community does not contain the proper microbial ligand that requires lymphotoxin signaling to promote inflammation.

To rule out the possibility that uncultivable bacteria might be promoting splenomegaly in *Ltbr*$^{-/-}$ mice, we utilized two different culture-independent techniques. One technique was the use of PCR to detect 16S ribosomal DNA. We used a phenol-chloroform DNA extraction method on the spleens collected from littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice. We verified the presence of DNA via Nanodrop. We used a fecal sample as

a positive control. We did not detect a positive PCR signal via standard PCR and qRT-PCR in DNA from systemic tissues, but did detect a positive signal in the positive control (Table 4).

To ensure the PCR techniques did not produce a false negative, we gavaged a homogenized spleen from SPF *Ltbr*$^{-/-}$ mice into GF *Ltbr*$^{+/+}$and *Ltbr*$^{-/-}$ mice. Fecal samples were collected from the gavaged GF mice 2 days post gavage and 7 days post gavage. Fecal samples were processed using the Stool DNA Extraction kit from Qiagen. We used an SPF fecal sample as a positive control. Again, we did not detect a positive 16S r DNA PCR signal via standard PCR and qRT-PCR in the fecal samples retrieved from GF mice fed the SPF spleen (Table 4).

Lymphotoxin on Group 3 innate lymphoid cells prevents splenomegaly.

To better understand how the host immune system responds to bacteria in an LT-dependent manner, we decided to determine which immune cell was involved. To determine which immune cells expressing lymphotoxin ligand $\alpha_1\beta_2$ and/or LTβR were important in regulating spleen size, we generated LTβ and LTβR conditional knockout mice. We measured the spleen sizes in *LysM*$^{Cre+}$*Ltbr*$^{f/l}$ mice (myeloid lineage), *Tie2*$^{Cre+}$*Ltbr*$^{l/l}$ mice (stromal cells) (Figure 6**A**) and *Cd19*$^{Cre+}$*Ltb*$^{f/f}$ mice (B cells), *Cd4*$^{Cre+}$ *Ltb*$^{f/f}$ mice (T cells), *Cd19*$^{Cre+}$*Cd4*$^{Cre+}$*Ltb*$^{f/f}$ mice (B/T cells) (Figure 6**B**) and *Rorc*$^{Cre+}$*Ltb*$^{f/f}$ mice (ILC3 and T cells). We examined the lymphotoxin conditional mouse models and observed that *Rorc*$^{Cre+}$*Ltb*$^{f/f}$ mice had significantly larger spleens when compared to littermate controls (Figure 6**C**).

Figure 6: The absence of Lymphotoxin on RORγt expressing cells results in splenomegaly.

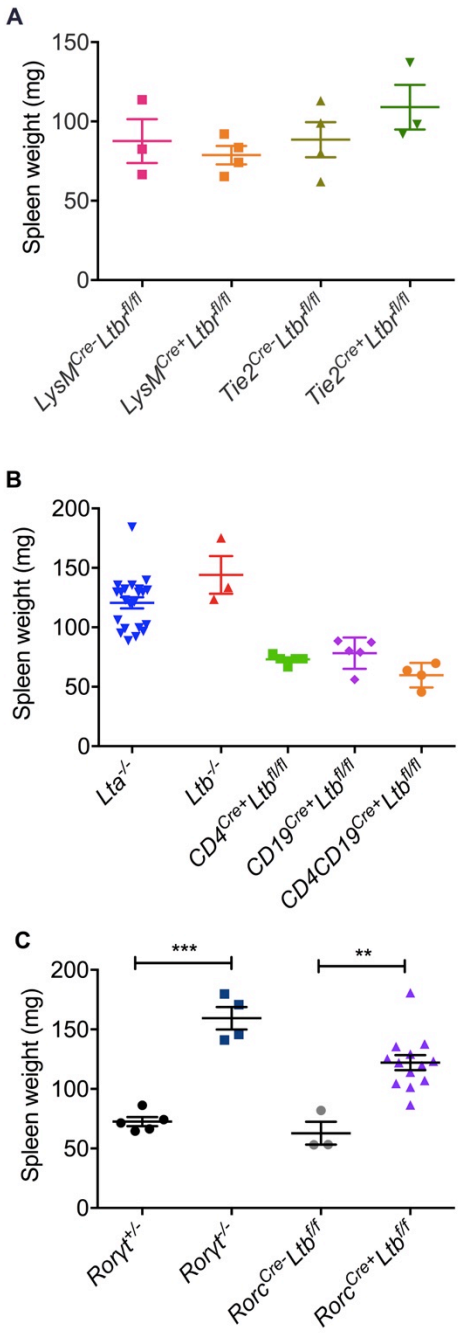


Figure 6: The absence of Lymphotoxin on Rorc expressing cells results in splenomegaly. Conditional **A)** *Ltbr*$^{fl/fl}$ (*n* = 3-4, repeated 2 times) and **B-C)** Lymphotoxin ligand deficient and *Ltb*$^{fl/fl}$ mice (*n* = 3-13, repeated 5 times) were bred and the spleens were harvested and weighed. Error bars represents mean and SEM. One-way ANOVA was performed. **, *P* <.01; ***, *P* < .001.

We wondered whether ILC3s expressing LTβ regulated spleen size since T cells deficient in lymphotoxin did not have significantly larger spleens compared to littermate controls (Figure 6**B**). Since T cells and ILC3s both express lymphotoxin, we crossed *Rorc*$^{Cre+}$*Ltb*$^{f/f}$ mouse onto the *Rag1*$^{-/-}$ background to determine whether the lack of $LT\alpha_1\beta_2$ on ILC3 was sufficient for responding to the microbiota in the absence of adaptive immunity. Interestingly, *Rag1*$^{-/-}$*Rorc*$^{Cre+}$*Ltb*$^{f/f}$ mice had significantly larger spleens (Figure 7**A**) and increased splenocyte numbers (Figure 7**B**) when compared to SPF *Rag1*$^{-/-}$ mice. In order to verify that ILC3s expressing lymphotoxin were needed following exposure to the same microbiota, we generated littermate *Rag1*$^{-/-}$*Ltb*$^{+/-}$ and *Rag1*$^{-/-}$*Ltb*$^{-/-}$ mice and observed that mice that lack lymphotoxin on only ILC3s had significantly larger spleens (Figure 7**C**) and slightly more splenocyte numbers (Figure 7**D**) compared to littermate controls. Together, these data demonstrate that lymphotoxin expressing ILC3s are needed in the presence of the commensal bacteria to prevent the development of splenomegaly.

Figure 7: The absence of Lymphotoxin on ILC3s results in the development of splenomegaly.

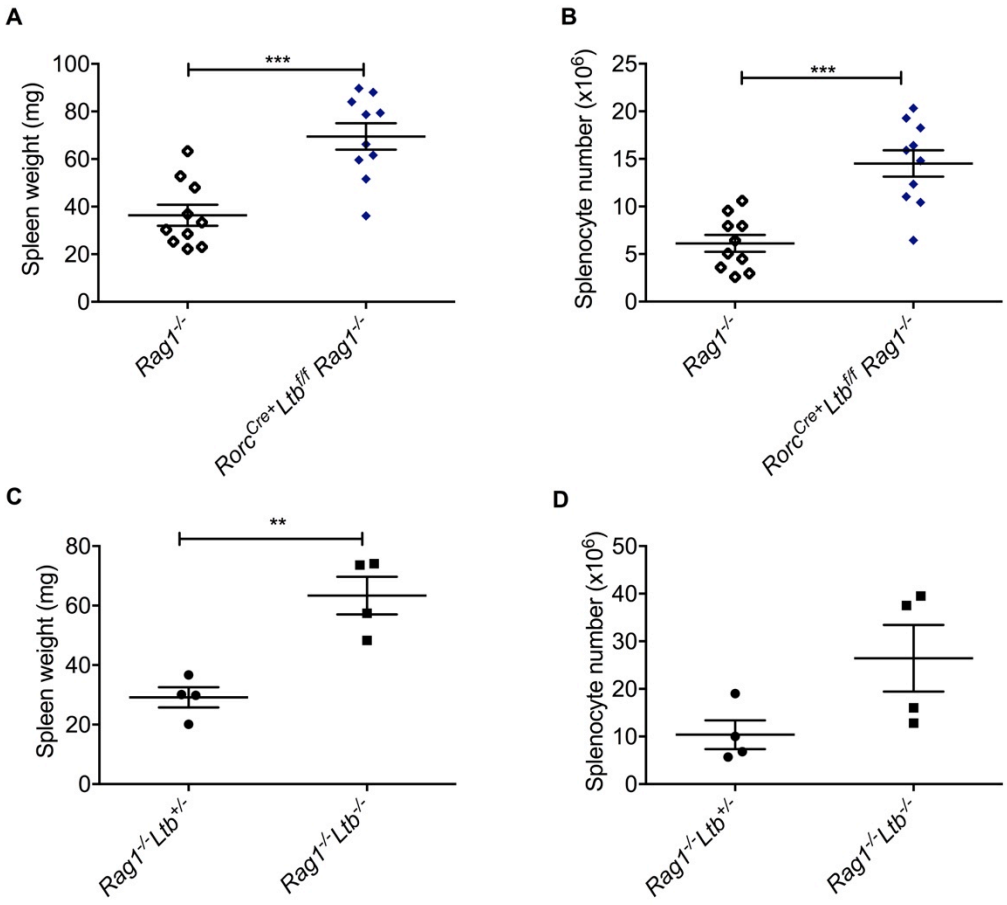


Figure 7: The absence of Lymphotoxin on ILC3s results in the development of splenomegaly. *Rorc*$^{Cre+}$*Ltb*$^{fl/fl}$ mice were crossed onto B6 *Rag1*$^{-/-}$ background. The mice were sacrificed and the spleens were harvested and **A)** weighed and **B)** splenocytes counted in comparison to spleens from B6 *Rag1*$^{-/-}$ mice. *n* = 8, repeated 2 times. Littermate *Rag1*$^{-/-}$*Ltb*$^{+/-}$ and *Rag1*$^{-/-}$*Ltb*$^{-/-}$ mice were generated. The mice were sacrificed and the spleens were harvested and **C)** weighed and **D)** splenocytes counted. *n* = 4, repeated 2. Error bars represents mean and SEM. Unpaired t-tests was performed. **, *P* <.01; ***, *P* < .001.

Active lymphotoxin signaling from group 3 innate lymphoid cells prevents splenomegaly.

Since expression of LTβ on ILC3s can dampen splenomegaly, we wondered whether active lymphotoxin signaling was required. To determine whether ILC3s needed to constitutively provide lymphotoxin to prevent the development of systemic inflammation, we examined whether blocking lymphotoxin signaling would have an effect on spleen size. We decided to treat *Rag1*$^{-/-}$ mice with LTβR:hIg fusion protein, since in *Rag1*$^{-/-}$ mice ILC3s were the only immune cells expressing $LT\alpha_1\beta_2$. Littermate *Rag1*$^{-/-}$ mice were treated with LTβR:hIg fusion protein or IgG control for 4 weeks, starting at weaning. In LTβR:hIg treated *Rag1*$^{-/-}$ mice, we saw an increase in spleen size (Figure 8**A**) and splenocyte numbers (Figure 8**B**) compared to control *Rag1*$^{-/-}$ mice. When we examined the splenocyte cell populations, we again saw an increase in both the percentage (Figure 8**C**) and absolute number (Figure 8**D**) of neutrophils. We concluded that active lymphotoxin signaling from ILC3s is needed to prevent the development of bacteria-induced neutrophilic splenomegaly in SPF *Rag1*$^{-/-}$ mice.

Figure 8: The absence of active signaling from ILC3s results in the development of neutrophilic splenomegaly.

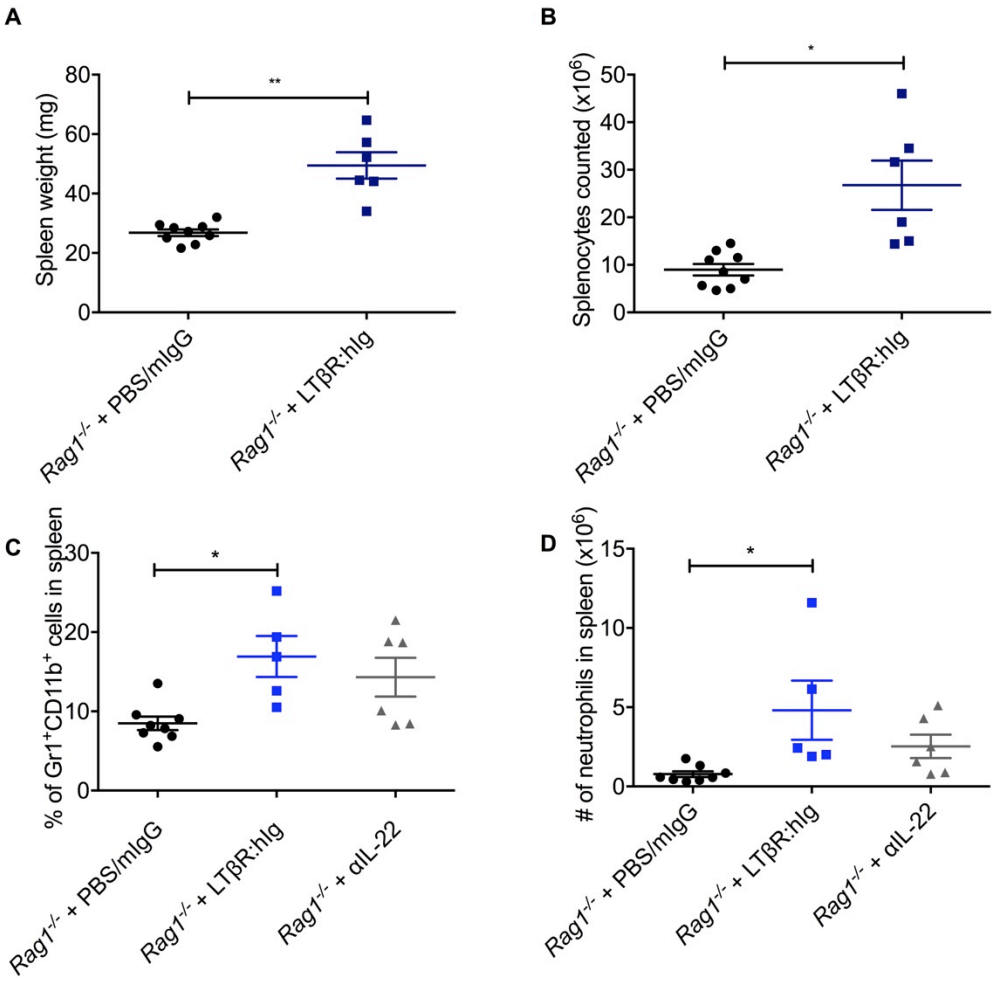


Figure 8: The absence of active signaling from ILC3s results in the development of neutrophilic splenomegaly. SPF littermate *Rag1*$^{-/-}$ were weaned and given LTβR:hIg, IgG control or αIL-22 for 4 weeks. The mice were sacrificed and the spleens were harvested and **A)** weighed and **B)** splenocytes counted. **C)** Percentage of neutrophils in the spleen and **D)** absolute number of neutrophils were counted. *n* = 5-8, repeated 3 times. Error bars represents mean and SEM. Unpaired t-tests was performed on **A-B**, One-way ANOVA was performed on **C-D**. *, *P* <.05; **, *P* < .01.

Blocking innate IL-22 signaling did not affect splenomegaly.

It is known that *Rorγt*$^{-/-}$ mice, which also lack ILC3s, have a splenomegaly defect (Garidou et al. 2015; Zhang, Guo, and He 2003). Research has shown that IL-22 plays

an important role in preventing splenomegaly in an ILC3 and microbiota-dependent manner (Garidou et al. 2015; Sonnenberg et al. 2012; Sawa et al. 2011). Since the lymphotoxin pathway regulates IL-22 signaling in the context of bacterial pathogen infections (Ota et al. 2011; Tumanov et al. 2011), we wondered whether IL-22 signaling was also downstream of lymphotoxin signaling in the splenomegaly phenotype.

To determine whether the IL-22 pathway was directly involved in regulating microbiota-induced inflammation, we blocked IL-22 signaling in *Rag1*$^{-/-}$ mice alongside the LTβR:hIg treated *Rag1*$^{-/-}$ mice. Littermate *Rag1*$^{-/-}$ mice were weaned and given αIL-22 or IgG control i.p. 3x weekly for 4 weeks. However, we did not observe a significant difference in spleen size between αIL-22-treated *Rag1*$^{-/-}$ mice and control *Rag1*$^{-/-}$ mice (Figure 9**A**) nor in splenocyte count (Figure 9**B**). Furthermore, we did not see a corresponding increase in percentage (Figure 8**C**) or absolute number (Figure 8**D**) of neutrophils. These data show that only inhibiting IL-22 signaling, and not lymphotoxin signaling, is insufficient to promote the development of splenomegaly in *Rag1*$^{-/-}$ mice. One caveat for this experiment is that we cannot rule out the possibility that we did not administer enough αIL-22 blocking antibodies to cause the development of splenomegaly, as that has previously been described (Sonnenberg et al. 2012). These data allowed for the conclusion that, in this particular SPF colony, ILC3 expressing $LT\alpha_1\beta_2$, and actively providing lymphotoxin signaling, are needed to prevent the development of splenomegaly in the presence of bacteria.

Figure 9: The absence of IL-22 signaling does not result in splenomegaly in Rag1 mice.

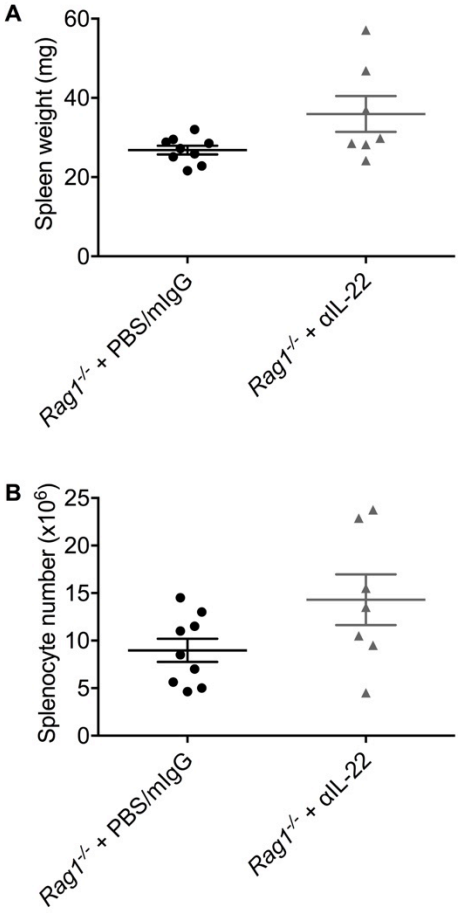


Figure 9: Blocking IL-22 signaling in B6 *Rag1*$^{-/-}$ mice does not result in splenomegaly. B6 *Rag1*$^{-/-}$ mice were given IL-22 blocking antibody for 4 weeks or IgG/PBS as control. The mice were sacrificed and the spleens were harvested and **A)** weighed and **B)** splenocytes counted. *n* = 7-9, repeated 3 times. Error bars represent mean and SEM. Unpaired t-tests was performed and determined to be insignificant.

Lymphotoxin-expressing ILC3s is necessary to promote splenomegaly.

Since we have determined that active lymphotoxin signaling from ILC3s was needed to prevent the development of splenomegaly and systemic inflammation, we wondered whether ILC3s expressing $LT\alpha_1\beta_2$ by themselves were capable of preventing

splenomegaly. In order to determine whether a lack of LT signaling from ILC3 was sufficient to promote splenomegaly, we sorted out ILC3s based on CD90 and CD45 expression (Figure 10) and transferred them into *Ragγc*$^{-/-}$ hosts. *Ragγc*$^{-/-}$ mice lack T cells, B cells, natural killer cells, but most importantly, ILCs, due to lack of functional receptors for many cytokines and recombinase activating gene-2 (DiSanto et al. 1995; Garcia, DiSanto, and Stockinger 1999; Greenberg and Riddell 1999). *Ragγc*$^{-/-}$ hosts do have a small number (1-3 million) of cells with low CD45 expression that are assumed to be part of the macrophage/monocyte lineage (Biosciences 2016). To test whether ILC3s expressing $LT\alpha_1\beta_2$ were necessary to prevent splenomegaly, we isolated lamina propria lymphocytes (LPLs) from littermate *Rag1*$^{-/-}$*Ltb*$^{+/-}$ and *Rag1*$^{-/-}$*Ltb*$^{-/-}$ mice (Figure 10).

Figure 10: Example sort for collecting ILC3s.

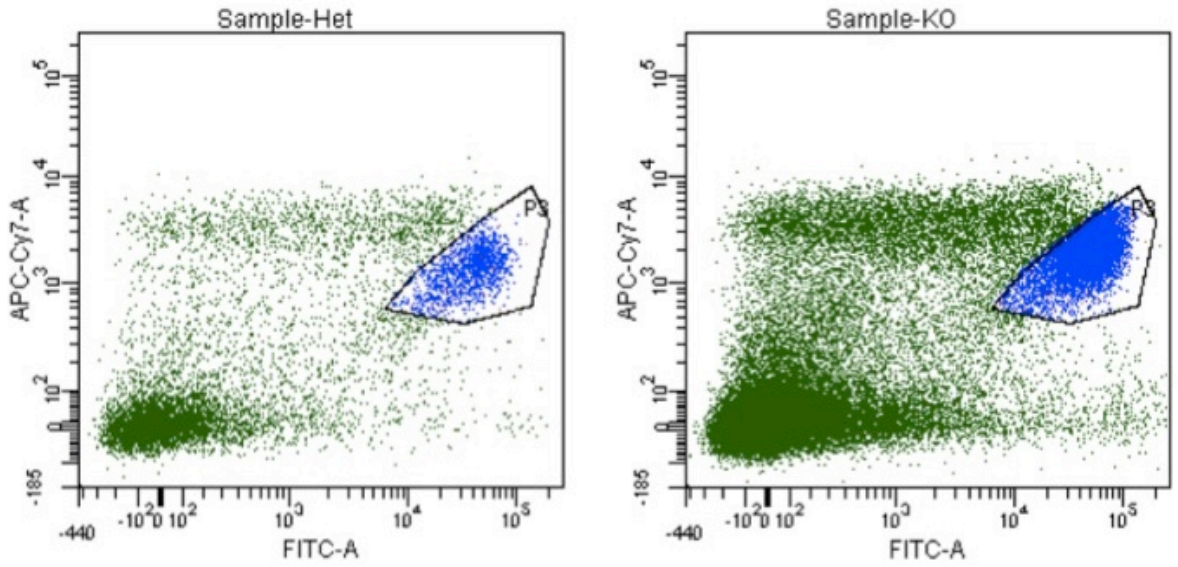


Figure 10: Example sort flow graph where CD45 is on the Y-axis and CD90 is on the X-axis. The blue group of cells are ILC3s that were sorted and collected for transfer immediately following collection.

ILC3s were sorted from the corresponding LPLs based on CD45+ and CD90+ expression and transferred via retro-orbital IV injection into littermate *Ragγc*$^{-/-}$ mice before weaning. The *Ragγc*$^{-/-}$ mice who received ILC3s sorted from *Rag1*$^{-/-}$*Ltb*$^{+/-}$ LPLs had significantly smaller spleens than the *Ragγc*$^{-/-}$ mice who received ILC3s sorted from *Rag1*$^{-/-}$*Ltb*$^{-/-}$ LPLs (Figure 11**A**). In addition, the *Ragγc*$^{-/-}$ mice who received ILC3s sorted from *Rag1*$^{-/-}$*Ltb*$^{+/-}$ LPLs had significantly fewer splenocytes compared to the *Ragγc*$^{-/-}$ mice who received ILC3s sorted from *Rag1*$^{-/-}$*Ltb*$^{-/-}$ LPLs (Figure 11**B**). Furthermore, when we examine the splenocyte populations, we see a corresponding increase in absolute numbers of neutrophils (Figure 11**C-D**) in the *Ragγc*$^{-/-}$ mice who received ILC3s sorted from *Rag1*$^{-/-}$*Ltb*$^{-/-}$ LPLs. Given these data, we determined that $LT\alpha_1\beta_2$ expressed on the membrane of ILC3s was necessary and sufficient to prevent the development of neutrophilic splenomegaly in the presence of the microbiota.

Figure 11: The absence of Lymphotoxin on ILC3s is sufficient to promote splenomegaly.

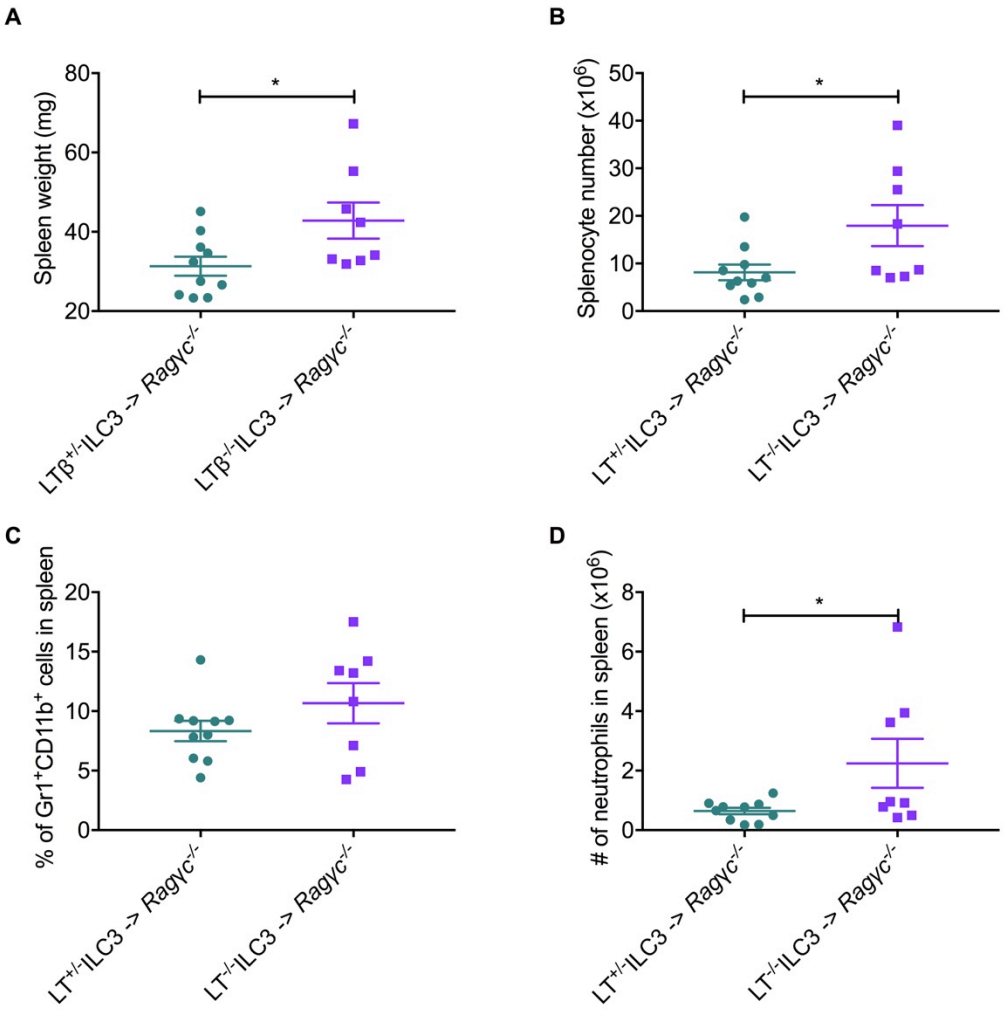


Figure 11: The absence of Lymphotoxin signaling from ILC3s results in the development of neutrophilic splenomegaly. SPF littermate *Rag*γ*c*$^{-/-}$ were weaned and given ILC3s from *Rag1.Ltb*$^{+/-}$ or *Rag1.Ltb*$^{-/-}$ transfer via retro-orbital injections. The mice were sacrificed after 5 weeks and the spleens were harvested and **A)** weighed and **B)** splenocytes counted. **C)** Percentage of neutrophils in the spleen and **D)** absolute number of neutrophils were counted. *n* = 8-10, repeated 3 times. Error bars represents mean and SEM. Unpaired t-tests was performed. *, *P* <.05.

Chapter 4: A High Fat, High Sucrose Diet Allows For Development Of DIO In Different Gnotobiotic Conditions.

GF mice are susceptible to DIO, independent of lymphotoxin signaling.

We had previously shown that mice deficient in lymphotoxin signaling had a different microbiota community on TD.88137 compared to littermate *Ltbr*$^{+/-}$ TD.88137-fed mice and, thus, were resistant to weight gain (Upadhyay et al. 2012). Previous work has demonstrated that the deregulated microbiota was able to transfer resistance to weight gain to GF *Ltbr*$^{+/+}$ mice (Upadhyay et al. 2012). Since SPF *Ltbr*$^{-/-}$ mice have increased systemic inflammation that is dependent on the presence of bacteria, we wondered whether the resistance to weight gain was also a result of the increased in basal inflammation. In other words, we wondered whether lymphotoxin signaling would affect weight gain, on TD.88137, in the absence of the microbiota.

To determine if lymphotoxin signaling regulated weight gain, independent of the commensal microbiota, we utilized the GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mouse colony. GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were maintained on normal standard chow diet (NCD) until 9 weeks of age. At 9 weeks of age, *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were placed on TD.88137 for an additional 9 weeks. Other GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were maintained on NCD to control for normal host growth in GF conditions. Following 9 weeks of weekly weight monitoring, the TD.88137-fed mice, and their corresponding littermate NCD controls, were examined for adiposity.

Surprisingly, GF mice fed TD.88137 were capable of developing DIO, independent of lymphotoxin signaling (Figure 12). Regardless of genotype, GF mice

gained significantly more weight, as measured by absolute weight (Figure 12**A**) and percent of original weight gained (Figure 12**B**). Similarly, perigonadal fat pad mass also increased in TD.88137-fed GF mice compared to NCD fed mice (Figure 12**C**). These data demonstrated that lymphotoxin signaling did not regulate DIO independent of the microbiota. Going further, these data suggest that the increased basal inflammation of *Ltbr*$^{-/-}$ mice colonized with bacteria could contribute to the resistance to weight gain.

Figure 12: GF mice are susceptible to DIO.

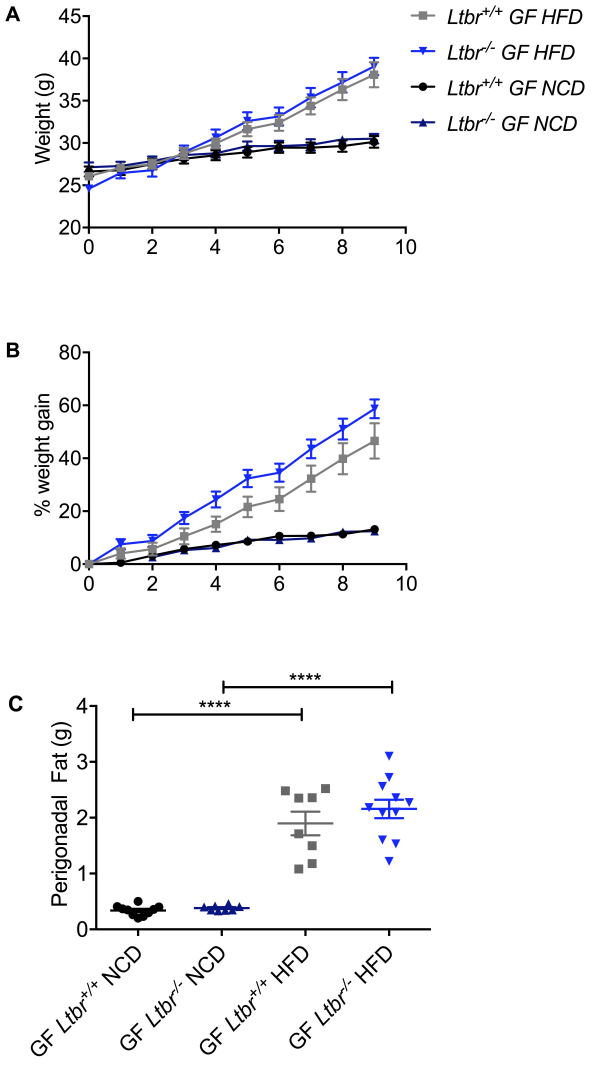


Figure 12: GF mice are susceptible to diet-induced-obesity, in a Lymphotoxin-independent manner. GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were placed on HFHSD at 9 weeks of age for an additional 9 weeks. Weekly weights were recorded. **A)** Absolute weight and **B)** Percent weight gain are plotted. **C)** Diet-fed mice and age matched NCD controls were sacrificed and perigondal fat pad was weighed. n = 20, repeated 4 times. Error bars represent mean and SEM. 2-way ANOVA was performed on **A)** and **B).** One-way ANOVA was performed on **C)**. ****, $P < .0001$. Representative graphs with male mice.

SFB monocolonized mice were resistant to DIO, independent of lymphotoxin signaling.

As a result, we asked whether the presence of bacteria was sufficient to promote resistance to DIO in *Ltbr*$^{-/-}$ mice. Given that GF mice gain weight on TD.88137, we wondered whether the presence of particular members of the gut microbiota suppressed weight gain in a lymphotoxin-dependent manner. Previous work by the lab showed that, in SPF conditions, Segmented Filamentous Bacteria (SFB) levels decreased in TD.88137 obese *Ltbr*$^{+/-}$ mice compared to lean *Ltbr*$^{-/-}$ mice (Upadhyay et al. 2012).. However, it was not known whether SFB directly contributed to resistance to DIO in *Ltbr*$^{-/-}$ mice or not.

To determine if SFB regulated weight gain in a lymphotoxin-dependent manner, or was a biomarker for leanness in SPF mice, we orally gavaged cecal SFB-monocolony contents into GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice at weaning. At 9 weeks of age, SFB-monocolonized mice were placed on TD.88137 for an additional 9 weeks. Surprisingly, SFB monocolonized mice were susceptible to TD.88137-induced-obesity, but not in a lymphotoxin-dependent manner (Figure 13**A-B**). In other words, both *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were relatively obese, in both absolute weights (Figure 13**A**) and in percent weight gained (Figure 13**B**). These data allowed us to determine that SFB-alone may not directly promote leanness in a lymphotoxin-dependent manner.

Figure 13: SFB and ASFB gnotobiotic conditions resulted in mice having DIO.

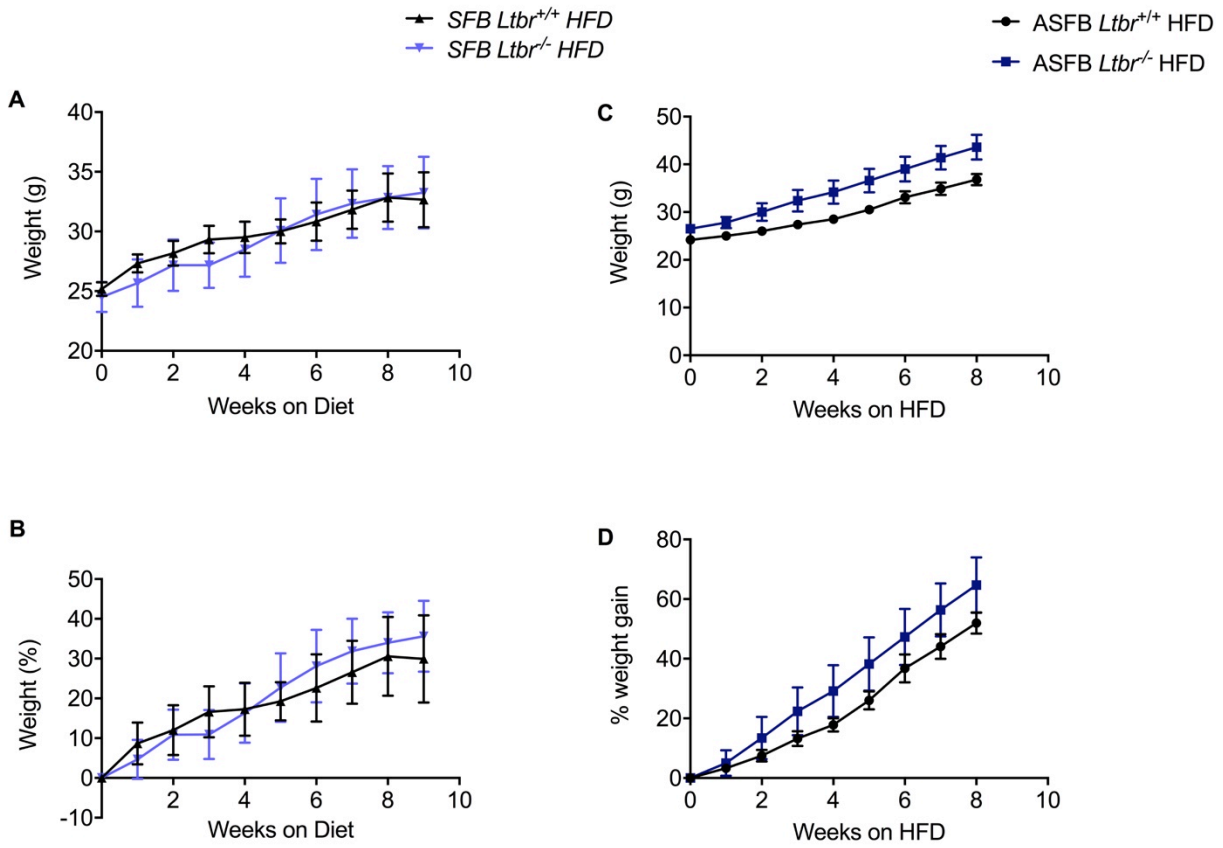


Figure 13: SFB and ASFB gnotobiotic conditions resulted in DIO, independent of Lymphotoxin. SFB colonized *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were placed on HFHSD at 9 weeks of age for an additional 9 weeks. Absolute weight gained **A)** percent weight gained **B).** *n* = 6-8, repeated 2 times. ASFB colonized SFB colonized *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were placed on HFHSD at 9 weeks of age for an additional 9 weeks. Absolute weight gained **C)** percent weight gained **D).** *n* = 8-10, repeated 2 times. Error bars represent mean and SEM. Graphs are representative data with male mice.

ASFB colonized mice were susceptible to DIO, independent of lymphotoxin signaling.

One possible explanation for why a monocolony of SFB does not recapitulate the observed SPF phenotype, could be that SFB need to interact with other members of the microbiota in order to influence weight gain in a lymphotoxin-dependent manner. In order to ensure sufficient SFB colonization in the presence of a more complex

microbiota, we colonized 3-4 week old GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice with SFB cecal contents for 2 days before orally gavaging cecal ASF into the same mice. This method allowed us to generate an ASFB gnotobiotic colony as verified by 16S rRNA qPCR (Figure 14).

Figure 14: ASFB gnotobiotic colony.

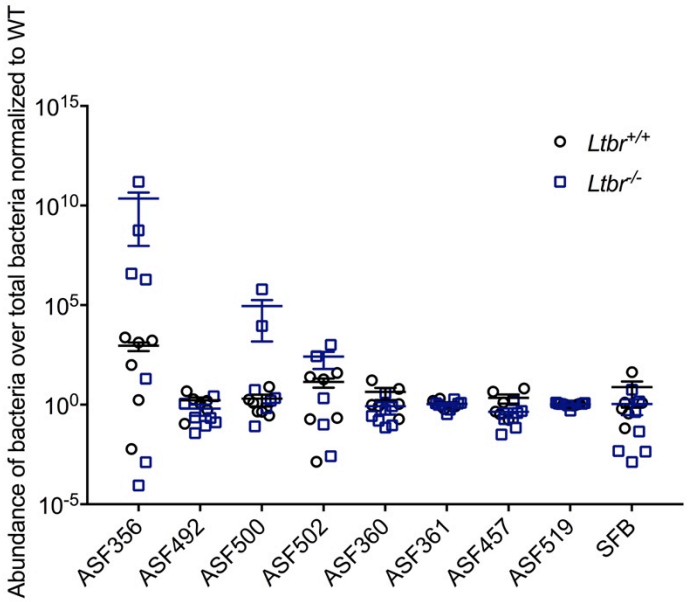


Figure 14: Levels of individual members of ASFB in *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. *n* = 6-7, with technical triplicates for each mouse. Error bars represent mean and SEM.

At the start of 9 weeks of age, the mice were placed on TD.88137 or maintained on NCD. Weekly weights were measured for an additional 9 weeks. Interestingly, gnotobiotic mice colonized with ASFB were susceptible to TD.88137-induced obesity (Figure 13**C-D**). ASFB colonized *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice had similar absolute weight gain (Figure 13**C**) and similar percent weight gained (Figure 13**D**). These data allowed us to conclude that SFB levels might be a biomarker of weight gain in SPF. Going further,

these data suggested that the resistance to weight gain phenotype, in SPF *Ltbr*$^{-/-}$ mice, could not be attributed simply to the presence of microbes. Rather, these data suggest that particular bacteria may influence not only splenomegaly but also leanness in a lymphotoxin-dependent manner.

A high sucrose diet enables the development of DIO in germfree conditions.

Based on the importance of complex carbohydrate processing, we wondered whether TD.88137 contained different carbohydrate components from previously used high fat diets. To address this hypothesis, we examined and compared (Table 5) the ingredients of TD.88137 against TD.96132, the diet utilized by the Gordon Lab, with macronutrients shaded in blue (Bäckhed et al. 2004; Bäckhed et al. 2007a; Turnbaugh et al. 2006). We noticed the absence of maltodextrin and the presence of more sucrose in TD.88137 when compared to TD.96132 (denoted by red font in Table 5).

Table 5: High-fat-diets used in germfree conditions.

Ingredient, g/kg	TD.88137 Adjusted Calories Diet (42% from fat)	TD.96132 Adjusted Fat Diet	TD.97222 18% Milkfat Diet	TD.97223 18% Safflower Diet
Casein	195	236	195	195
DL-Methionine	3	3.54	3	3
Sucrose	341.46	182.62	254.924	254.924
Corn Starch	150	160	130	130
Maltodextrin		120	140	140
Anhydrous Milkfat	210		180	
Beef Tallow		100		
Vegetable Shortening, hydrogenated (Primex)		100		
Safflower Oil, linoleic				180

Table 5, continued.

Cellulose	50	40	34	44.54
Cholesterol	1.5		2.04	2.5
Mineral Mix, AIN-76 (170915)	35		35	35
Mineral Mix, AIN-93G-MX (94046)		41.3		
Calcium Carbonate	4		4	4
Calcium Phosphate, dibasic		4.72		
Vitamin Mix, Tekland (40060)	10	11.8	10	10
Choline Bitartrate			1	1
Ethoxyquin, antioxidant	.04	.02	.036	.036

Table 5, continued.

Kcal/g	4.54	4.48	4.38	4.38
Protein (% by weight)	17.3	20.9	17.3	17.3
Carbohydrate (% by weight)	48.5	45.5	51.3	51.3
Fat (% by weight)	21.2	20.2	18.2	18.2
Protein (% kcal)	15.2	18.7	15.8	15.8
Carbohydrate (% kcal)	42.7	40.6	46.9	46.9
Fat (% kcal)	42	40.7	37.4	37.4

To determine whether the absence of maltodextrin and the presence of only sucrose in TD.88137 conferred DIO to GF mice, we challenged GF mice with TD.97222 and TD.88137. We chose to compare TD.88137 to TD.97222 since it matched TD.88137 closely in macronutrient sources while only differing in carbohydrate sources (Table 5). Interestingly, GF mice fed TD.97222, which had maltodextrin, were significantly leaner compared to GF mice fed TD.88137, which lacked maltodextrin (Figure 15). A maltodextrin-rich diet resulted in less absolute weight gain (Figure 15**A**) and less percent weight gained of original weight (Figure 15**B**) when compared to a non-maltodextrin diet in GF conditions. The maltodextrin-rich high fat diet-fed mice were further found to have less perigonadal fat pad mass when compared to non-

maltodextrin, sucrose-rich diet fed mice (Figure 15**C**). These data allowed for the conclusion that a high sucrose, high fat diet confers obesity to germfree mice due to the host's ability to process sucrose. In other words, only the presence of complex carbohydrates that depends on microbial metabolism will result in resistance to obesity in GF mice.

Figure 15: GF mice are susceptible to DIO only on simplified carbohydrates.

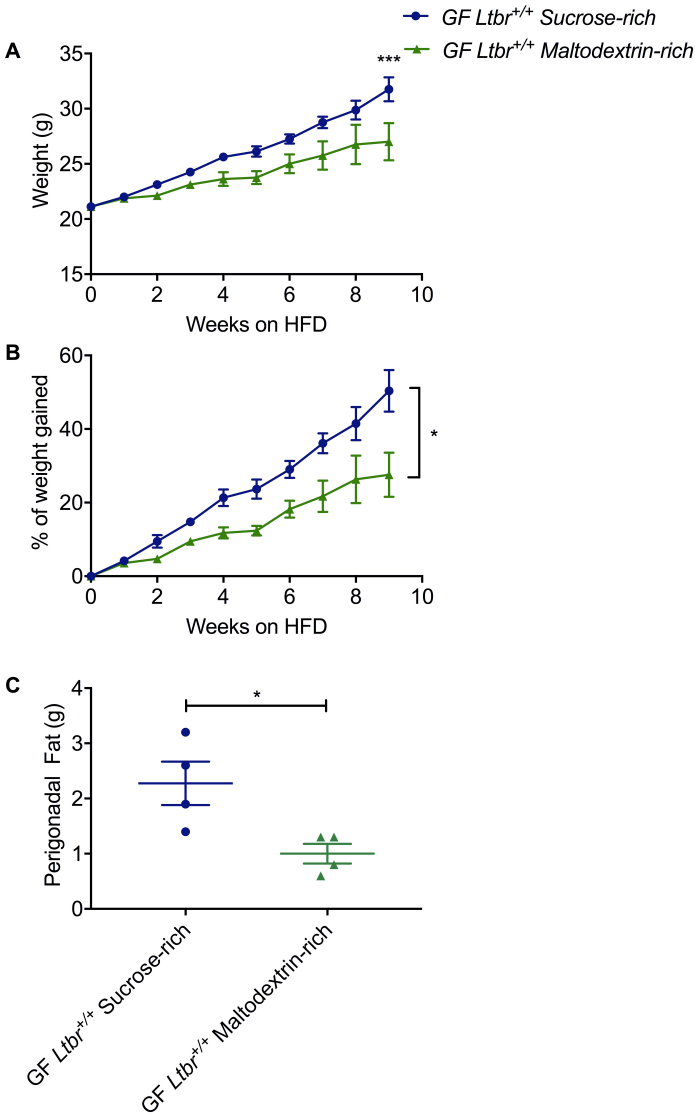


Figure 15: GF mice are susceptible to diet-induced-obesity only on simplified carbohydrates, in a Lymphotoxin-independent manner. Controlling for fat contents in the diet, GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were placed on either a sucrose-rich diet or a maltodextrin-rich diet at 9 weeks of age for an additional 9 weeks. Weekly weights were recorded. **A)** Absolute weight and **B)** Percent weight gain are plotted. **C)** Diet-fed mice and were sacrificed and perigondal fat pad was weighed. *n* = 6-8, repeated 2 times. Error bars represent mean and SEM. 2-way ANOVA was performed on **A)** and **B).** Unpaired Student's t-test was performed on **C)**. *, *P*<. 05; ***, *P*<.001. Representative graphs with female mice.

Carbohydrate content of diet affects colonic gross pathology.

Since we noticed that a difference in carbohydrates could affect weight gain in GF mice, we wondered whether the different carbohydrates also altered the intestinal tissue because GF mice have altered metabolism and tissue homeostasis, especially of the intestines (K. Smith, McCoy, and Macpherson 2007). This altered physiology could potentially impact absorption of nutrients, and thus alter weight gain potential. To determine whether the presence of different carbohydrates alters the colonic pathology, in the absence of the microbiota, we challenged GF *Ltbr*$^{+/+}$ mice to either a sucrose-rich or a maltodextrin-rich diet, while controlling for the fat content. Following the standard DIO protocol, we examined the colonic tissue after 9 weeks of HFD feeding. Interestingly, we noticed that the mice fed a maltodextrin-rich diet were not only leaner (Figure 15) but also had a reduction in colonic tissue mass when compared to the mice fed a sucrose-rich diet (Figure 16**C-E**). We observed a reduction in overall colon length (Figure 16**C**), colon weight (Figure 16**D**) and a corresponding decrease in the density of the colonic tissue (Figure 16**E**). These data demonstrated that the different carbohydrate sources seem to alter colonic tissue pathology and the density of the colons could potentially correlate with weight gain.

Figure 16: Different carbohydrates affect gross colonic pathology.

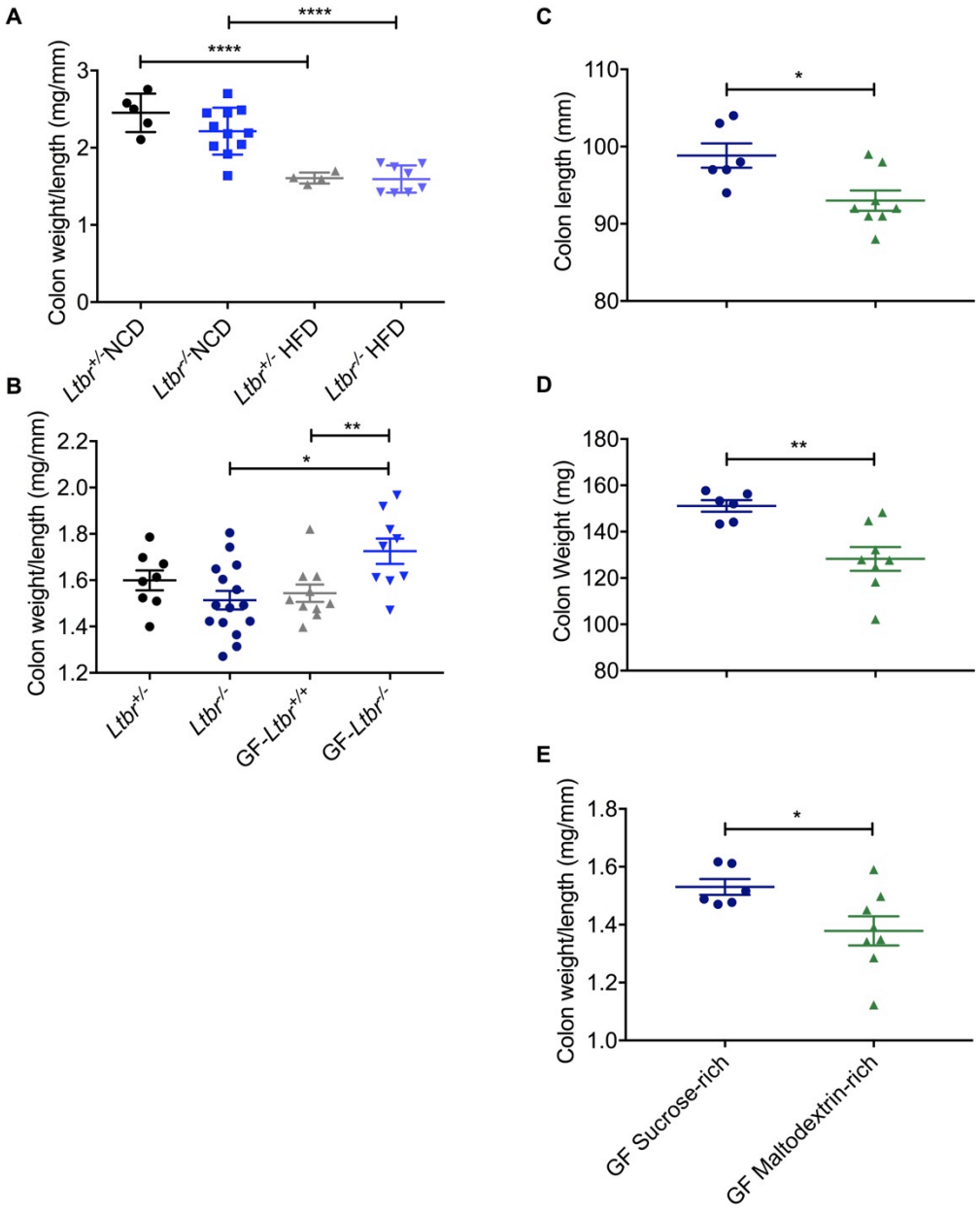


Figure 16: Different microbiota conditions and different carbohydrate components of diet changes the colonic gross pathology. SPF littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice were placed on NCD or HFD following our standard diet protocol. **A)** Colon weight over length ratio for SPF mice on NCD vs HFD. **B)** Colon weight over length ratio of littermate *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice on HFD compared to age-matched GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice on HFD. Controlling for fat contents in the diet, GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice were placed on either a sucrose-rich diet or a maltodextrin-rich diet at 9 weeks of age for an additional 9 weeks. **C)** Colon length and **D)** Colon weight are plotted. **E)** The colonic weight over length ratio was plotted. n = 6-8, repeated 2 times. Error bars represent mean and SEM. **A-B)** One-way ANOVA was used and **C-E)** Student's t-test was performed. *, P<.05; **, P<.01, ****, P<.0001.

To determine whether colonic density correlated with weight gain, we utilized SPF *Ltbr*$^{-/-}$ and *Ltbr*$^{+/-}$ fed TD.88137. Following high fat diet challenge, the colons from the lean *Ltbr*$^{-/-}$ mice and the obese *Ltbr*$^{+/-}$ mice were analyzed and compared to NCD littermate controls. We noticed that when SPF mice are placed on TD.88137 compared to NCD, the colon weight-to-length ratio was reduced (Figure 16**A**). To determine if this pathological change is a result of the microbiota, we challenged SPF and GF mice to TD.88137 and measured their colonic tissue. Interestingly, although we do not see a difference in colonic density in SPF lean *Ltbr*$^{-/-}$ mice and SPF obese *Ltbr*$^{+/-}$ mice (Figure 16**A-B**), we observed that the colons of GF *Ltbr*$^{-/-}$ fed TD.88137 were heavier when compared to SPF *Ltbr*$^{-/-}$ fed TD.88137 (Figure 16**B**). Furthermore, although we do not see a difference in weight gain between GF *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice, unlike the SPF colony, we observed that GF *Ltbr*$^{-/-}$ fed TD.88137 also have heavier colons compared to GF *Ltbr*$^{+/+}$ fed TD.88137. These data allowed us to conclude that the presence of the microbiota and the different types of carbohydrates both alter colonic pathology. However, these pathological changes do not appear to correlate with weight gain on TD.88137.

Chapter 5: Active Lymphotoxin Signaling From RORγt^{+} Cells Has A Partial Effect On The Development Of DIO With A Sucrose-Rich Diet.

Lymphotoxin on RORγt^{+} cells is not required for DIO.

Previous work in the lab has shown that lymphotoxin signaling from DCs and myeloid cells did not control weight gain (Table 6) (Upadhyay 2013) and T/LTi/B cells, expressing membrane bound $LT_{\alpha1\beta2}$, did not affect weight gain (Table 6). However, mice deficient in RORγt were resistant to weight gain when compared to mice with RORγt^{+} cells (Upadhyay et al. 2012). Given the role of membrane bound $LT_{\alpha1\beta2}$ on ILC3s in regulating microbiota-induced splenomegaly, we asked whether membrane bound $LT_{\alpha1\beta2}$ on ILC3s may also influence weight gain on TD.88137. To determine whether RORγt^{+} expressing cells need to express membrane bound $LT_{\alpha1\beta2}$ to promote weight gain on TD.88137, we generated littermate *Rorc*$^{Cre+}$*Ltb*$^{f/f}$ and *Rorc*$^{Cre-}$*Ltb*$^{f/f}$ mice.

Table 6: Conditional lymphotoxin signaling mice and DIO.

Mouse Genotype	Phenotype	Weight gain on TD.88137?
Cd11c^{Cre+}Ltbr$^{f/f}$	DCs deficient in signaling through LTβR	Yes
LysM^{Cre+}Ltbr$^{f/f}$	Myeloid cells deficient in signaling through LTβR	Yes
Cd4^{Cre+}Ltb$^{f/f}$	T/LTi cells deficient in membrane bound $LT_{\alpha1\beta2}$	Yes
Cd19^{Cre+}Ltb$^{f/f}$	B cells deficient in membrane bound $LT_{\alpha1\beta2}$	Yes, partial
Cd4/Cd19^{Cre+}Ltb$^{f/f}$	T/LTi/B cells deficient in membrane bound $LT_{\alpha1\beta2}$	Yes, partial

At 9 weeks of age, *Rorc^{Cre+}Ltb$^{f/f}$* and *Rorc^{Cre-}Ltb$^{f/f}$* mice were placed on TD.88137. Weekly weights were monitored for an additional 9 weeks. Surprisingly, TD.88137-fed *Rorc^{Cre+}Ltb$^{f/f}$* were as susceptible to weight gain as littermate TD.88137-fed *Rorc^{Cre-}Ltb$^{f/f}$* mice (Figure 17). Mice deficient in T cells and ILC3s expressing $LT_{\alpha1\beta2}$ had the same absolute weight (Figure 17**A**), percent weight gain (Figure 17**B**), and perigonadal fat pad mass (Figure 17**C**) when compared to littermate mice sufficient in T cells and ILC3s expressing $LT_{\alpha1\beta2}$. These data demonstrate that membrane lymphotoxin on RORγt^{+} cells may not be essential for DIO on TD.88137.

Figure 17: Mice deficient in Lymphotoxin on RORγt+ cells are susceptible to DIO.

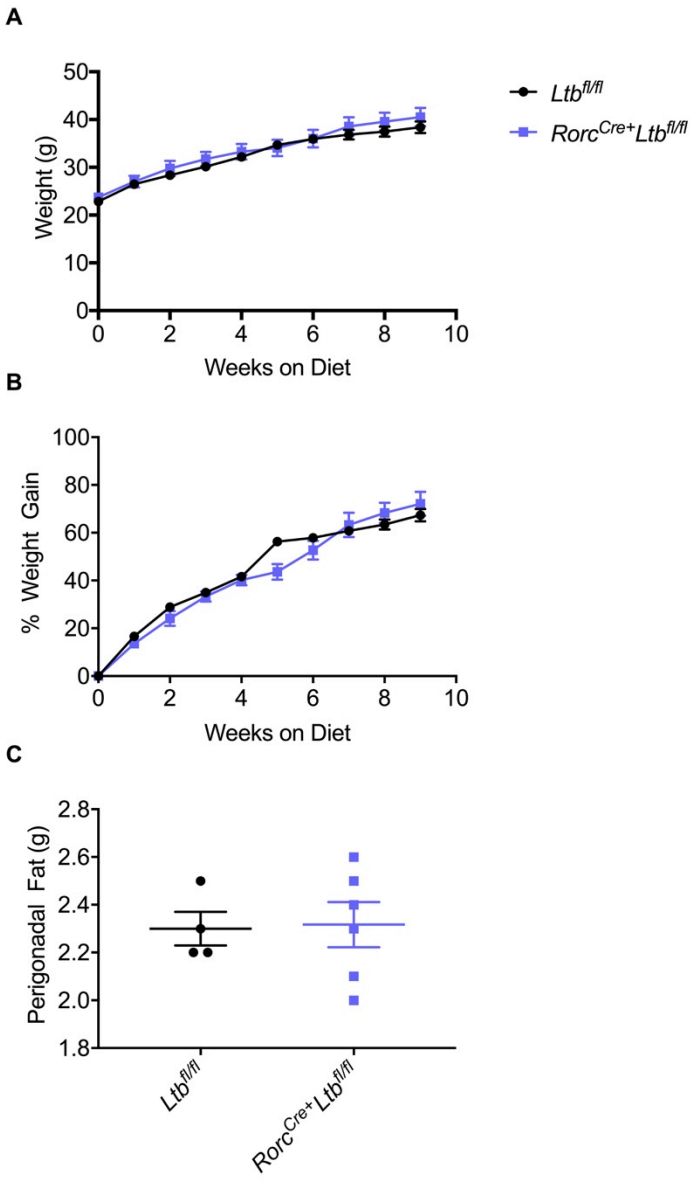


Figure 17: Mice deficient in Lymphotoxin on RORγ+ cells are susceptible to DIO. SPF littermate *Rorc*$^{Cre+}$*Ltb*$^{fl/fl}$ and *Ltb*$^{fl/fl}$ mice were placed on HFD following our standard diet protocol. **A)** Absolute weight gain **B)** Percent weight gained. **C)** Perigondal fat pad mass. *n* = 8-12, repeated 4 times. Error bars represent mean and SEM. 2-way ANOVA was used on **A-B**. Student's *t*-test was used on **C**. Representative graphs depicting male mice.

Mice defective in T/B cells are partially susceptible to DIO.

Since B cells have been shown to regulate DIO in the presence of the microbiota, we asked whether B cells expressing $LT_{\alpha1\beta2}$ could be promoting DIO in *Rorc*$^{Cre+}$*Ltb*$^{f/f}$ mice (Shulzhenko et al. 2011). Thus, we determined whether *Rorc*$^{Cre+}$*Ltb*$^{f/f}$ mice having other immune cells, like B cells that can express $LT_{\alpha1\beta2}$ could compensate for the lack of ILC3/T cells expressing $LT_{\alpha1\beta2}$. To understand whether lymphotoxin signaling from the adaptive immune cells or innate immune cells were more important for the development of obesity we fed B6 *Rag1*$^{-/-}$ mice TD.88137. After feeding mice TD.88137, we were able to determine that mice deficient in T and B cells had a partial weight phenotype compared to in-house bred *Rag1*$^{+/+}$ mice (Figure 18). In particular, when compared against *Rag1*$^{+/+}$ mice, *Rag1*$^{-/-}$ mice were leaner on TD.88137 based on absolute weight (Figure 18**A**) and percent of weight gained (Figure 18**B**). *Rag1*$^{-/-}$ mice also had reduced adiposity, as determined by less perigonadal fat pad weight when compared to *Rag1*$^{+/+}$ mice fed TD.88137 (Figure 18**C**). These data allowed us to conclude that T/B cells expressing LTβ may play a role in DIO, and that the innate immune cell compartment providing lymphotoxin signaling may be able to partially compensate for the lack of T/B cells to promote for host growth.

Figure 18: Rag mice are partially susceptible to DIO.

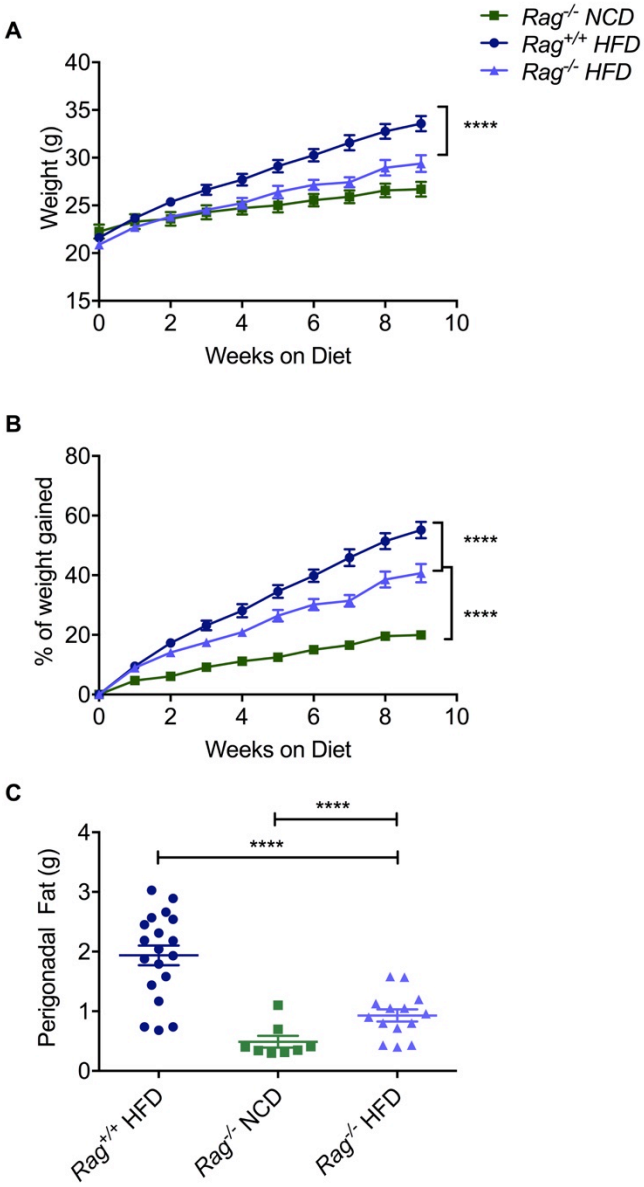


Figure 18: Rag deficient mice are partially susceptible to diet-induced obesity. SPF littermate *Rag1*$^{-/-}$ mice were placed on NCD or HFD following our standard diet protocol. **A)** Absolute weight gain **B)** Percent weight gained. **C)** Perigondal fat pad mass. *n* = 15-20, repeated 4 times. Error bars represent mean and SEM. 2-way ANOVA was used. ****, *P*<.0001. Representative graphs depicting male mice.

Preliminary data suggests active lymphotoxin signaling from ILC3s may play a partial role in DIO.

Since we determined that active lymphotoxin signaling is needed for the prevention of neutrophilic splenomegaly, we wondered whether leanness on TD.88137 might also be a result of a defect in active lymphotoxin signaling, perhaps from ILC3s. To determine whether active signaling from ILC3s regulated weight gain on TD.88137, we used LTβR:hIg fusion protein to block active lymphotoxin signaling. Littermate *Rag1*$^{-/-}$ mice were weaned and given LTβR:hIg or IgG control i.p. weekly. At 9 weeks of age, these *Rag1*$^{-/-}$ mice were placed on TD.88137 for an additional 9 weeks. Weekly LTβR:hIg fusion protein was given i.p. and the mice were monitored for weight gain. Interestingly, TD.88137-fed LTβR:hIg-treated *Rag1*$^{-/-}$ mice had similar body growth when compared to TD.88137-fed IgG control *Rag1*$^{-/-}$ mice (Figure 19**A**). Interestingly, *Rag1*$^{-/-}$ mice that lacked consecutive LTβR signaling had less percent weight gain compared to littermate controls (Figure 19**B**). However, we do not observe a significant perigonadal fat pad mass difference in the *Rag1*$^{-/-}$ mice without lymphotoxin signaling when compared to control mice (Figure 19**C**). These data was inconclusive so we were not able to determine whether mice lacking lymphotoxin signaling from ILC3s can also promote resistance to weight gain in the presence of excess calories. Due to the inability to control for LTβR:hIg treatment variance, further experiments will need to be done in LTβ-deficient ILC3 mice to determine conclusively the role of LTβ on ILC3 in mediating weight gain in the presence of the microbiota.

Figure 19: Active Lymphotoxin signaling may play a partial role in weight gain in Rag deficient mice.

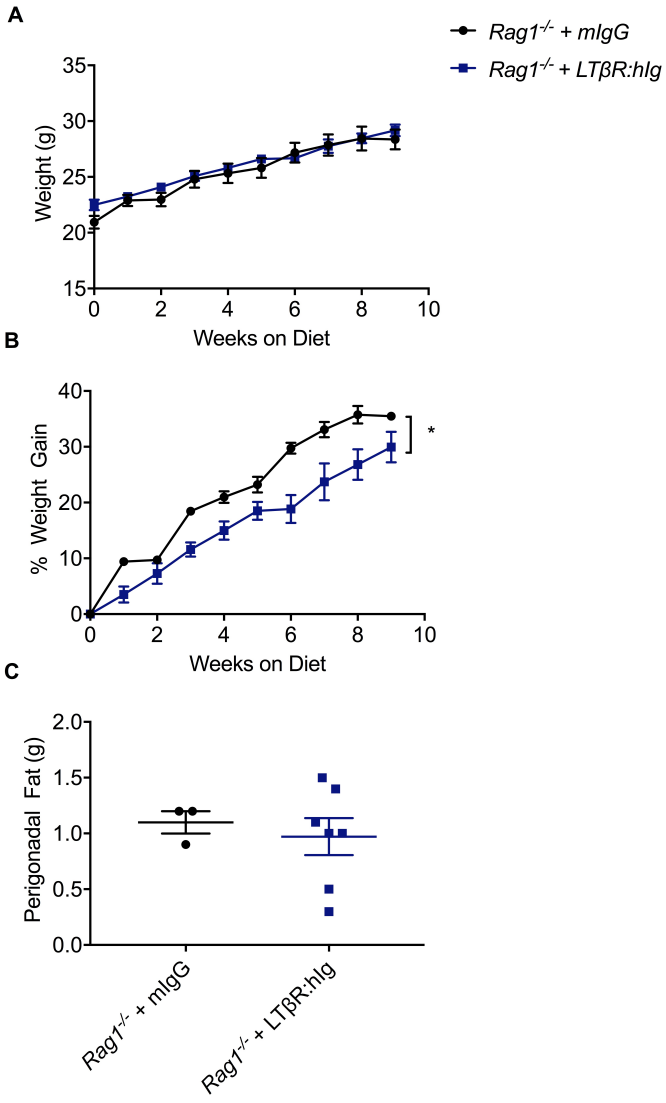


Figure 19: Active Lymphotoxin signaling may play a partial role in weight gain in Rag deficient mice. SPF littermate *Rag1*$^{-/-}$ mice were placed on HFD following our standard diet protocol and either treated with IgG control or LTβR:hIg. **A)** Absolute weight gain **B)** Percent weight gained. **C)** Perigondal fat pad mass. n = 3-8, repeated 2 times. Error bars represent mean and SEM. 2-way ANOVA was used. *, P<.0005. Representative graphs depicting male mice.

Chapter 6: Conclusions And Future Directions

Summary

As mentioned in the introduction, obesity is traditionally thought to cause, and be the result of, systemic inflammation (Fain 2010; Hotamisligil 2006). The lab's previous paper revealed an essential role for LTα in DIO due to *Lta*$^{-/-}$ and *Ltbr*$^{-/-}$ mice being resistant to weight gain on TD.88137 (Upadhyay et al. 2012). The observation that GF mice fed the high fat diet TD.88137 were obese was initially surprising. Previous reports have demonstrated the importance of the microbiota in processing carbohydrates (Faith et al. 2011b; Muegge et al. 2011b). We wondered if the diet composition could be responsible for this discrepancy. After examining and comparing TD.88137 to other diets that have been used during GF obesity experiments, we came upon the realization that TD.88137 contains minimal complex carbohydrates, specifically maltodextrin. In order to determine whether the absence of maltodextrin was sufficient to alter weight gain in GF conditions, we compared another diet that contained the same diet composition except for differences in carbohydrate source. We were able to determine that the current dogma of leanness in the GF mice was mainly dependent on certain carbohydrates that needed microbial enzymes to efficiently breakdown the nutrients for host absorption.

In order to understand mechanistically how the absence of LTα signaling regulates weight gain only in the context of the microbiota, we examined *Ltbr*$^{-/-}$ mice at baseline to determine if any of the defects in gut immunity predisposes the mice to becoming resistant to weight gain. Surprisingly, the characteristic increased systemic

inflammation seen in LT deficient animals could partially be attributed to the presence of the microbiota. GF *Ltbr*$^{-/-}$ did not exhibit the same level of systemic inflammation, as determined by the neutrophilic splenomegaly and increased immune infiltrates in the lungs, when compared to age-matched SPF *Ltbr*$^{-/-}$ mice. Furthermore, we were able to determine that the mere presence of bacteria is insufficient to promote systemic inflammation in LT-deficient mice since only conventionalization with SPF cecal material resulted in neutrophilic splenomegaly in ex-GF *Ltbr*$^{-/-}$ mice.

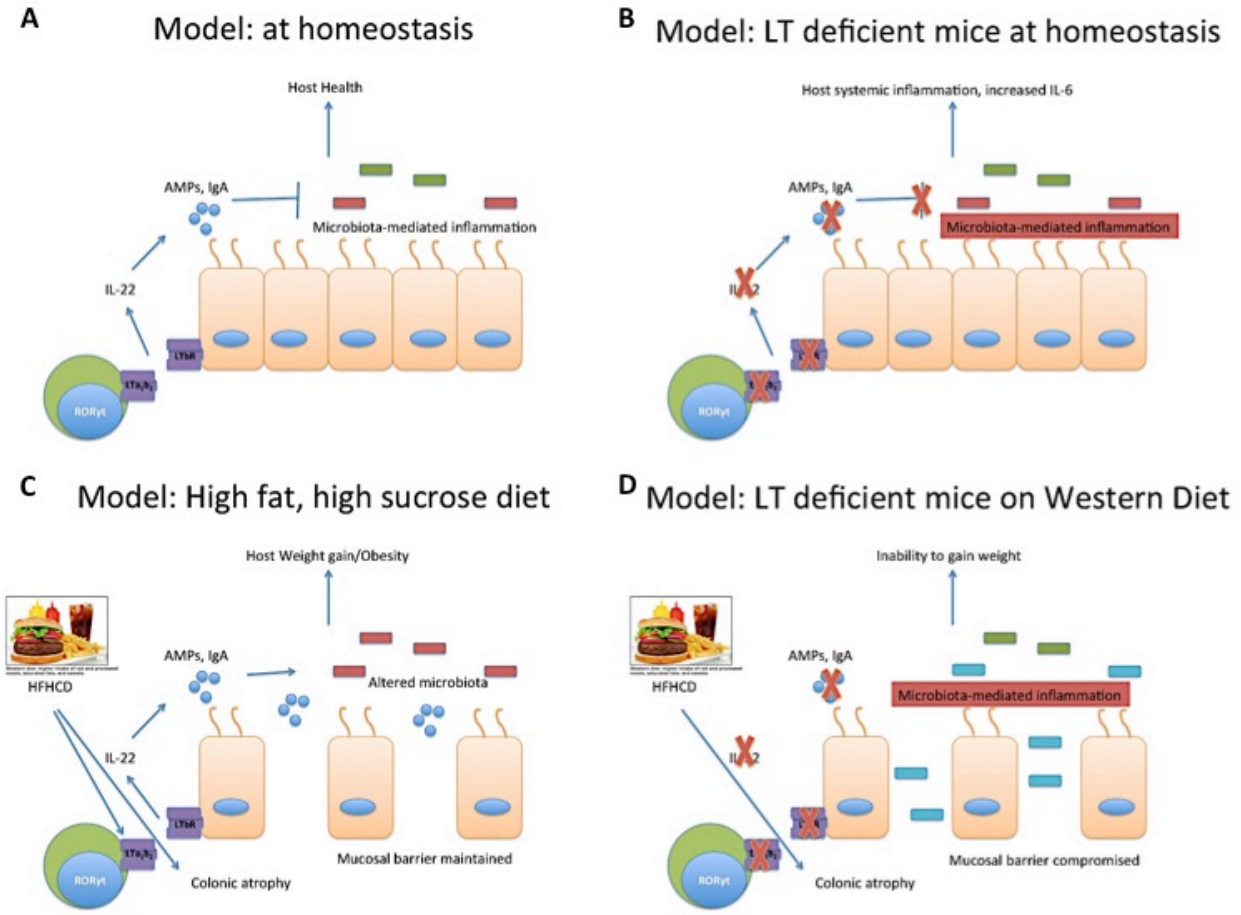
Through the use of different conditional mouse models that deleted *Ltb* and *Ltbr* on various immune cells, we were able to determine that cells expressing RORγt transcription factor and LTβ were important for mediating the host's immune response to the microbiota. Specifically, LTβ expression and active lymphotoxin signaling on ILC3s was necessary and sufficient to prevent the development of neutrophilic splenomegaly. Furthermore, we were able to demonstrate that active lymphotoxin signaling from ILC3s plays a partial role in the resistance to weight gain on TD.88137.

A new model to take into accounts the role of mucosal immunity on DIO.

Therefore, this thesis gives rise to the following model (Figure 20): Lymphotoxin signaling is needed at baseline to promote IL-22 signaling pathway at mucosal sites. Active LTβ expression on ILC3s binds and activates LTβR, on an unidentified cell type, to modulate IL-22 signaling (Figure 20**A**). In the absence of LT, ILC3s no longer express LTβ to activate LTβR to promote proper mucosal barrier defense (Figure 20**B**). Thus, with a compromised barrier, LT-deficient mice have increased systemic inflammation due to its inability to regulate and compartmentalize microbial members.

In the context of HFD, this inability to compartmentalize the microbiota becomes more exacerbated, since TD.88137 decreases the density of the colonic tissue. Since this decrease in colonic density occurs independently of lymphotoxin-signaling, the up regulation of LTα expression during HFD becomes critical since HFD tends to exacerbate inflammation in LTα deficient mice (Upadhyay et al. 2012; Pamir et al. 2012). Therefore the model suggests that LTα gets up regulated following TD.88137 because lymphotoxin signaling is needed to maintain the immunological mucosal barrier in the presence of decrease colonic tissue density (Figure 20**C**). In the absence of active lymphotoxin signaling, the colon tissue still becomes less dense and the mucosal barrier is further compromised. Therefore, LT-deficient animals are unable to gain weight on HFD since the excess nutrients are probably diverted to try to dampen the increase in systemic inflammation (Figure 20**D**).

Figure 20: Proposed lymphotoxin and weight gain model.



Implications of the new model.

We have demonstrated that GF mice are not necessarily resistant to DIO. Instead, we have shown that GF animals fed an excess caloric diet with increased fat components and no complex carbohydrates are able to become obese. This work supports the notation that microbiota members are important for degrading complex carbohydrates (Bäckhed et al. 2007a; Muegge et al. 2011b; Ley et al. 2006; Turnbaugh et al. 2006; Ley et al. 2005; Musso, Gambino, and Cassader 2010). In addition, we have demonstrated that the increase in systemic inflammation markers typically seen in LT-

deficient animals is at least partially dependent on the presence of specific microbiota members, rather than microbial components. Furthermore, we, and others, have shown that increased inflammation does not always lead to increased susceptibility to DIO and insulin resistance (Pamir et al. 2012). Thus, we have described a difference in microbial-induced inflammation from host cell stress-induced inflammation, like from excess calories, and how these two types of inflammation promote weight gain and obesity.

In the absence of lymphotoxin expression and signaling, we noticed a decrease in IL-22 expression after HFD, which could explain the increased microbiota-mediated systemic inflammation (Upadhyay et al. 2012). This implies that IL-22 maintenance during TD.88137 could play an important role in maintaining mucosal barrier integrity (Ouyang et al. 2011; Colonna 2009). Therefore, in the presence of the commensal bacteria, the lack of lymphotoxin-induced IL-22 signaling could lead to a compromised mucosal barrier that prevents weight gain during excess caloric feedings. This is why, in the absence of the microbiota, *Ltbr*$^{-/-}$ mice are able to gain weight and become as obese as *Ltbr*$^{+/+}$ mice. GF *Ltbr*$^{-/-}$ mice lack the microbiota-mediated inflammation in the context of HFD so the mice can use the excess calories to promote host growth and weight gain. Furthermore, one explanation for why the colons of HFD-fed GF *Ltbr*$^{-/-}$ mice are denser compared to the colons of HFD-fed GF *Ltbr*$^{+/+}$ mice and SPF-fed mice could be that the combination of lymphotoxin signaling and the microbiota promotes epithelial cell turnover. To verify this hypothesis, further research will need to be done to determine whether GF and SPF *Ltbr*$^{-/-}$ mice have different rates of epithelial proliferation, possibly by BrdU staining.

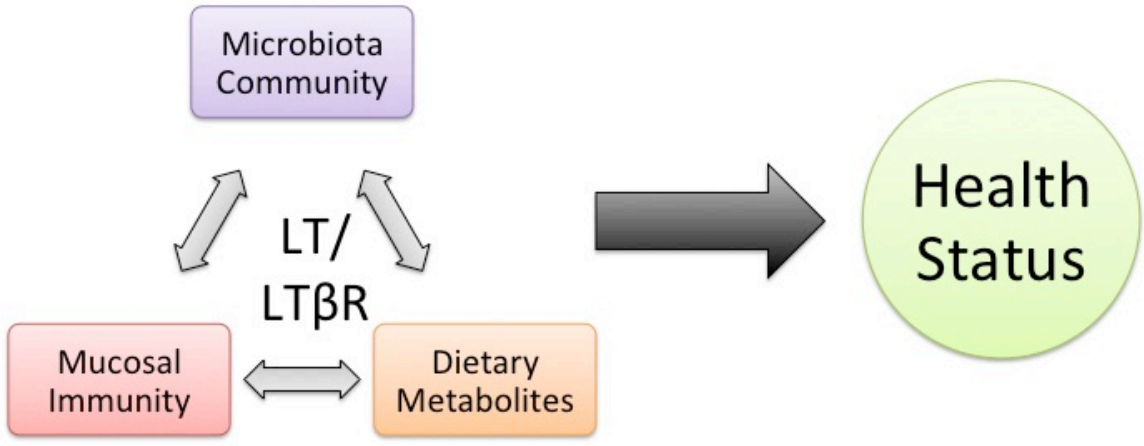
This new model (Figure 20) also implies that dietary components and metabolism of said dietary components can alter mucosal immunity. Different dietary components can directly alter colonic pathology, as seen in the GF mice (Figure 16). Specifically, mice deficient in fatty acid synthase (*Fas*) along the gut epithelium have a leaky gut barrier, as measured by both *in vivo* detection of traceable 10 kDa dextran and circulating traceable dextran (Wei et al. 2012). *Fas,* downstream of insulin receptor signaling, is an enzyme that is needed for de novo lipogenesis for the conversion of simple precursors into saturated fatty acids for cell membranes and energy-storage (Wakil 1989; Lodhi, Wei, and Semenkovich 2011). *Fas*-deficient mice have increased gut permeability, increased proinflammatory cytokines and high serum endotoxin (Wei et al. 2012). Furthermore, another group demonstrated that leptin, traditionally thought to be important for the satiety response after food consumption, also regulates mucosal immunity (X. Guo et al. 2011; Myers, Cowley, and Münzberg 2008; Chehab, Lim, and Lu 1996). Specifically, the group discovered that mice deficient in leptin receptor along the colonic epithelium have increased susceptibility to *Entamoeba histolytica* infections, which causes diarrheal diseases (X. Guo et al. 2011). This result demonstrates that host metabolism along the gut has consequences that can change the mucosal barrier and affect systemic inflammation.

Dietary impact on mucosal immunity has recently been characterized in germfree mice fed an elemental diet devoid of dietary antigens (Kim et al. 2016; B. S. Wostmann et al. 1970). The group demonstrated that majority of the peripheral T_{regs} (pT_{regs}) seen in GF animals is the result of the presence dietary antigens (Kim et al. 2016). GF mice fed a diet minimizing said dietary antigens have limited pT_{regs} when compared to GF mice

fed the standard chow (Kim et al. 2016). The group further differentiates pT_{regs} by noticing that dietary antigens promote absolute number increases of pT_{regs} in the small intestine while the microbiota promotes absolute number increases pT_{regs} in the colon (Kim et al. 2016). This implies that pT_{regs} found in the intestine might have different immune functions.

Thus, a more general model arises (Figure 21). This thesis supports the idea that changes to either one of the following microbiota community, mucosal immunity or dietary composition will affect the host's health status (Ussar et al. 2015). Furthermore, this thesis also suggests that changes to the microbiota community will also affect mucosal immunity and dietary absorption, which will alter health status and *vice versa.* This model also synthesizes and explains how the microbiota can influence obesity, leanness and malnutrition. For example, particular microbiota members in the context of HFD and a healthy mucosal barrier will result in the development of DIO. Conversely, particular microbiota members in the context of undernutrition with a compromised mucosal barrier will result in malnutrition.

Figure 21: Proposed general model.



A recent study has demonstrated a strong correlation between host genetics, microbiota composition and dietary challenges that converges to affect development of metabolic syndrome, like obesity and insulin resistance (Ussar et al. 2015). Ussar et al helped to elucidate why C57Bl/B6J (Jackson Laboratories), 129S1/SvImJ (Jackson Laboratories), and 129S6/SvEvTac (Taconic Farms) mice differ in weight gained and inflammation while on the same high fat diet compared to standard normal chow (Almind and Kahn 2004; Ussar et al. 2015). The group characterized and determined specific metabolic phenotypes that were correlated with different microbiota communities, which were also mouse strain specific and mouse strain independent. The group was able to identify operational taxonomic units (OTUs) that correlated with metabolic phenotype using 16s rRNA sequencing and Spearman's rank correlations. For example, they determined that HFD increased the relative abundance of Firmicutes, and *Mucispirillum schaedleri* positively correlated with weight while *Clostridium scindens*

negatively correlated with weight gain, independent of host genetics (Ussar et al. 2015). Together, this thesis and this paper put forth the idea that host genetics (like mucosal immunity-defects), the gut microbiota and dietary changes all act together to modulate the severity of the associated metabolic syndrome.

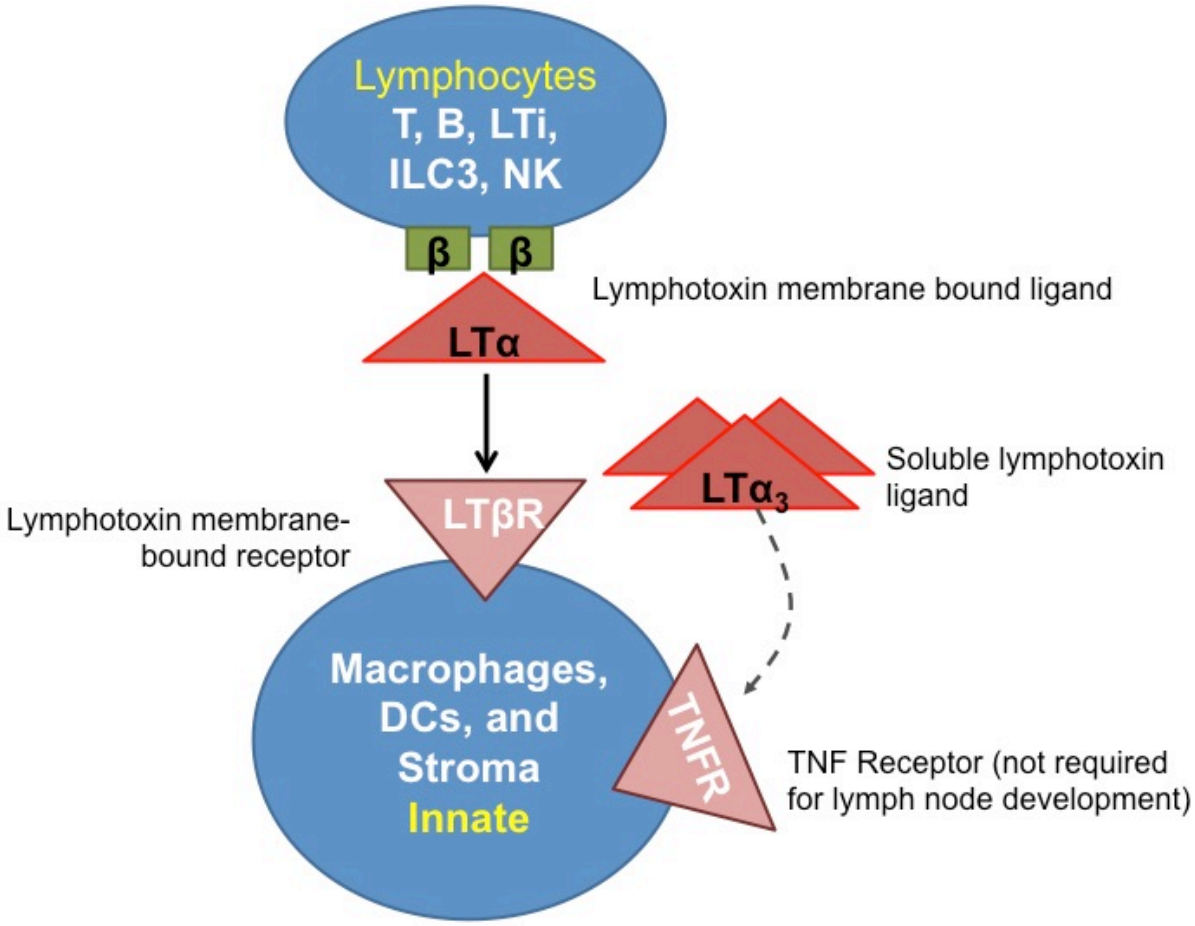
Limitations of this study.

Although that we have found the cell type that needs to express the LTβ ligand for mucosal barrier maintenance, we have yet to determine which cell type is receiving the signal and expresses LTβR. In addition, since LTβR is expressed on stromal, endothelial and epithelial cells, it is very possible that these cell types are also needed to regulate colonic inflammation and promote tissue homeostasis (Browning et al. 2005; Chyou et al. 2008; Ehlers et al. 2003; Fütterer et al. 1998; Hu et al. 2015; Schneider et al. 2008; Sun et al. 2015; Tumanov et al. 2003). In addition, as shown in (Figure 22), there are multiple immune cells that express the ligand $LT\alpha_1\beta_2$ or $LT\alpha_3$. Thus, the lymphotoxin pathway has inherent signaling redundancies that can compensate in lymphotoxin-deficient conditional mice (Ware et al. 1992; Ware et al. 1995; Banks, Rickert, and Ware 2006). Unfortunately, due to lack of a proper Cre that can selectively and specifically delete LTβR on these cell types individually, it might be difficult to determine precisely which cell type is required to express LTβR to prevent splenomegaly and promote weight gain (Chai et al. 2013; Yugang Wang et al. 2010).

Another limitation of this thesis is the fact that we were unable to definitively prove the absence of systemic bacteria. Furthermore, we have not identified a particular bacteria or consortium that is both responsible for the splenomegaly phenotype as well

as the leanness phenotype in *Ltbr*$^{-/-}$ mice. However, we have tried multiple different culture and culture-independent techniques without being able to *consistently* retrieve systemic bacteria, thus, it is still possible that viable systemic bacteria cannot be found in *Ltbr*$^{-/-}$ mice.

Figure 22: Possible compensation signaling with lymphotoxin pathway.



Furthermore, we have not characterized the exact differences caused by lymphotoxin-sufficient and lymphotoxin-deficient signaling on mucosal immunity. The previous paper noticed a reduction in AMPs, like RegIIIγ expression in the colonic tissue (Upadhyay et al. 2012). In addition, administration of IL-22 not only rescues weight gain

but also AMP expression (Upadhyay et al. 2012). However, IL-22 signaling does not fully address how and why the pathological changes undergone by colonic tissue occurs during TD.88137 and/or in the presence/absence of the commensal bacteria. Further research is needed to determine whether these colonic tissue changes occurs during all high fat diet experiments or is specific to TD.88137. We would propose that the changes in colonic pathology could explain why obesity is associated with increased endotoxaemia (Gregor and Hotamisligil 2011; Kanneganti and Dixit 2012; Vijay-Kumar et al. 2010). This also implies that endotoxaemia does not directly promote obesity-associated inflammation because this thesis suggests a difference between DIO inflammation and microbial-induced inflammation.

Confusingly, a recent paper determined that mice deficient in IL-22 receptor are more susceptible to obesity and metabolic syndrome on an adjusted calories diet (containing 60% fat, Harlan) (X. Wang et al. 2014). The group further demonstrates that IL-22 administration in obese mice (both adjusted calories diet and leptin-deficient mice) can reverse hyperglycaemia and insulin resistance (X. Wang et al. 2014). Interestingly, although naïve obese mice have sufficient IL-22 production from ILC3s, the obese mice, when challenged with *C. rodentium* infections, have a 50% lethality and lower colonic IL-22 expression when compared to lean infected WT mice (X. Wang et al. 2014). This research in the context of this thesis suggests that IL-22-mediated mucosal immunity might not be capable of controlling chronic inflammation, from high fat diet, and severe, acute inflammation, like from *C. rodentium* infections. One caveat of Wang et al study is the use of leptin deficient mice, which has been previously shown to have an impaired mucosal immune response to colonic pathogens (X. Guo et al. 2011).

It is also possible that various HFD promotes IL-22 in a lymphotoxin-dependent manner to preserve mucosal barrier functions to compensate for colonic tissue atrophy. However, HFD induction of IL-22 is not sufficient to overcome bacterial infections, which explains why the obese mice die at an increased frequency compared to lean mice during *C. rodentium* infections. Furthermore, it is possible that high fat diet causes colonic tissue atrophy that makes the host more susceptible to colonic infections. Further research will need to be done to understand if/how IL-22-mediated barrier function following high fat diet differs from IL-22-mediated barrier function in the presence of the microbiota. One way to determine this would be to see if obese GF WT mice also succumb to *C. rodentium* infections at the same rate as SPF obese WT mice. Given that GF WT mice do not have colonic pathology following *C. rodentium* infections and the model suggested by this thesis, we would predict that obese GF WT mice, with high fat diet induced colonic barrier dysfunction, will now be susceptible to *C. rodentium* infections (N. Kamada et al. 2012).

Germfree animals are not inherently resistant to DIO.

The DIO phenotype observed in the GF mice was unexpected. Previous work had demonstrated that GF mice were resistant to weight gain due to their inability to process complex carbohydrates without a microbiota (Bäckhed et al. 2004; Bäckhed et al. 2007a; Turnbaugh et al. 2006). Supporting this idea, we were able to determine that the presence or absence of a complex carbohydrate, maltodextrin, can affect weight gain and obesity.

Maltodextrin is considered to be a polysaccharide (Baer et al. 2014; “21 CFR 184.1444 - Maltodextrin.” 2016) that is not well digested by humans due to the presence of α-1 -> 6 glucosidic linkages which cannot be easily processed by disaccharidases typically found on the epithelial surface of human intestines (Olano-Martin et al. 2000). In addition, maltodextrin is known to enable growth of variety of commensal microbiota members due to the presence of multiple maltodextrin enzymes found in bacteria (Baer et al. 2014; Boos and Shuman 1998; Olano-Martin et al. 2000; Rowan and Anderson 1997; Seibold, Wurst, and Eikmanns 2009). Thus, the absence of complex carbohydrates, like maltodextrin, in TD.88137 was able to confer weight gain and obesity in GF mice. We have yet to determine whether this phenomenon only occurs in diets that manipulate maltodextrin, or whether this phenomenon could also be observed in diets composed of other types of complex carbohydrates.

With this thesis, we have demonstrated that there is nothing inherently wrong in GF mice that make them incapable of weight gain. This suggests that the current understanding that GF mice are resistant to weight gain could partially be contributed by the dietary components used in previous GF high fat diet experiments. Therefore, without the microbiota to break down the complex carbohydrates in those particular diets, there was a limited amount of nutrients available to the host for absorption. This idea implies that the previous high fat diets resulted in resistance to obesity in GF mice from the lack of excess calories. Examining how the different diets used in GF mice differ in caloric intake and availability for the host could test this hypothesis.

In the absence of bacterial-induced inflammation, lymphotoxin is not needed for DIO.

Interestingly, GF *Ltbr*$^{-/-}$ mice develop DIO at similar rates compared to GF *Ltbr*$^{+/+}$ mice following TD.88137. This result was initially unexpected. Upon further examination, this data supports the idea that LTα prevents bacterial-induced inflammation. Therefore, the presence of the bacterial-induced inflammation actively prevents weight gain on high caloric diets. One could speculate that *Ltbr*$^{-/-}$ mice fed TD.88137 utilize the excess nutrients to bolster the mucosal barrier in a non-lymphotoxin-dependent manner. This explanation could explain a previously inexplicable result that *Ltbr*$^{-/-}$ mice fed TD.88137 have a higher core body temperature compared to littermate *Ltbr*$^{+/-}$ mice fed TD.88137 and *Ltbr*$^{-/-}$ mice fed standard normal chow (Upadhyay 2013). This data also implies that LT signaling does not directly affect weight gain and obesity in the absence of the microbiota-induced inflammation.

However, this study only examined the consequences of changing carbohydrates to determine its affects on weight gain in GF mice. It is known that the presence of saturated fatty acids could contribute to colonic inflammation and alter weight gain (Devkota et al. 2012). It has also been demonstrated that different saturated fats affect weight gain and metabolic syndrome differently in SPF mice (de Wit et al. 2012). Therefore the role of fat metabolism on DIO and mucosal immunity has yet to be examined. In addition it was previously determined that LTβR can regulate lipoprotein homeostasis and lipidemia (Lo et al. 2007). Given that lipid homeostasis and obesity both impact liver function, and LT signaling is known to contribute to liver regeneration and pathogenesis, the effects of different fats on weight gain in *Ltbr*$^{-/-}$ mice have yet to be explored (de Wit et al. 2012; Anders et al. 2005; Ruddell et al. 2009).

Could lymphotoxin from Type 3 ILCs have a role in the development of DIO?

Blocking active lymphotoxin signaling in *Rag1*$^{-/-}$ mice, thus blocking lymphotoxin signaling from ILC3s, only partially recapitulated the lean weight phenotype. One possibility is that the fusion protein was not dosed appropriately throughout the course of the 18-week long experiment. Temporary insufficient dosing could result in some sufficient lymphotoxin signaling for weight gain. It should be noted that WT mice treated with LTβR:hIg and placed on TD.88137 were not more resistant to weight gain compared to control IgG treated mice. One possible explanation is that due to the mouse's immune system capability to generate an anti-fusion protein immune response, it limits the length of time we can give LTβR:hIg to mice. These data demonstrates that the length of LTβR:hIg treatment is critical for the weight phenotype.

Given that we saw only a partial weight phenotype in LTβR:hIg treated *Rag1*$^{-/-}$ mice and we did not see a significant difference in weight in mice with LTβ-deficient ILC3s, it is possible that the presence of any immune cell expressing LTβ can compensate for the lack of LTβ from ILC3s. In order to fully test this hypothesis, we would need to generate *Rorc*$^{Cre+}$*Ltb*$^{f/f}$*Rag1*$^{-/-}$ mice and it's littermates and put them on TD.88137 for 9 weeks. In the meantime, we could also use the newly generated *Ltb*$^{-/-}$ *Rag1*$^{-/-}$ and *Ltb*$^{+/-}$*Rag1*$^{-/-}$ mice. The predication would be that *Rorc*$^{Cre+}$*Ltb*$^{f/f}$*Rag1*$^{-/-}$ mice will not only have splenomegaly but also a resistance to weight gain on TD.88137 when compared to littermate controls. In other words, we predict that when $LT\alpha_1\beta_2$ on ILC3s are the only cell type that can signal through LTβR, mice with sufficient LT signaling will gain more weight than mice lacking LT signaling on TD.88137.

Final remarks…

In summary, I have worked towards furthering our understanding of not only how mucosal immunity changes in the presence of the microbiota, but also how these changes relate to overall host health before and after high fat diet. Throughout my time working on this project, I have become incredibly humbled and amazed by how our selves are more of an ecosystem rather than just a host. Given the progress and interests in treating *Clostridium difficile* with fecal microbiota transplant (a recently FDA-approved treatment), and understanding the role of antibiotics on colonic health, I truly believe we, as a society, have changed the way we look at microbes and how they impact disease. We are just at the very beginning of understanding how to harness the gut microbiota to manipulate disease and I am incredibly grateful to have been able to experiment and experience it firsthand. I will end it here: be good to your gut, and your gut will be good to you.

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