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COMMENSAL BACTERIA AND THEIR METABOLITES PREVENT SENSITIZATION TO FOOD ANTIGENS

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Abstract

The incidence of food allergy is rapidly increasing, creating a growing public health concern. The Centers for Disease Control documented an 18% increase in only 10 years (Branum and Lukacs, 2008). This rapid change in such a short time suggests that gene-by-environment interactions are involved. All barrier surfaces of the human body are colonized with symbiotic microbial communities that interface with the external environment. These microbes aid the host by providing producing nutrients and stimulating the education and maturation of the immune system. The composition of this colonizing microbiota can be strongly influenced by environmental and lifestyle factors including diet, use of antibiotics, and mode of birth (Feehley et al., 2012). We hypothesized that changes to the microbiota may alter susceptibility to sensitization with food allergens, leading to the increased incidence of allergy.

We found that treatment with broad-spectrum antibiotics (Abx) dramatically altered the composition and number of bacteria in the intestines of wild type (WT) specific-pathogen free (SPF) mice and lead to increased peanut (PN)-specific IgE and IgG1 responses after sensitization. This increased PN-specific response correlated with a decreased proportion of $Foxp3^+$ regulatory T cells (Tregs) in the colonic lamina propria (LP) and IgA in the feces. We also examined the response to sensitization in mice deficient in Toll-like receptor (TLR) 4 or TLR2 to determine if an inability to sense certain members of the microbiota could similarly increase PN-specific responses. In agreement with previously published work (Bashir et al., 2004), *Tlr4*$^{-/-}$ mice had increased levels of PN-specific IgE and IgG1 compared to *Tlr4*$^{+/-}$ littermates. *Tlr2*$^{-/-}$ mice, however, had minimal responses to sensitization.

We next used a germ free (GF) and gnotobiotic ("known biota") model to identify members of the microbiota that were protective against sensitization. Much like Abx-treated mice, GF mice, which are devoid of any colonizing bacteria, had strong responses to

sensitization as well as impaired colonic Treg differentiation and IgA production compared to SPF controls. Colonization of GF WT mice with a single species, *Bacteroides uniformis*, did not alter PN-specific responses, but colonization with a consortium of spore-forming Firmicutes from the Clostridia class was able to block the generation of a PN-specific IgE response as well as restore the Foxp3$^+$ Treg compartment of the colon and IgA production to the levels seen in SPF mice. Abx-treated mice colonized with a Clostridia-containing microbiota were also protected from sensitization to PN, but transfer of Foxp3$^+$ Tregs into Abx-treated mice was not sufficient to recapitulate this protection.

Clostridia are unique because they reside close to the host intestinal epithelium, contributing to their protective effect. A microarray on intestinal epithelial cells (IECs) from GF, *B. uniformis*-, or Clostridia-colonized mice revealed that Clostridia stimulated expression of IL-22 regulated genes. IL-22, a known barrier-protective cytokine, was both necessary and sufficient to reduce intestinal permeability to food antigens, as measured by the amount of PN protein detected in the serum after oral administration. Neutralizing IL-22 ablated the protective effect of colonization and increased PN-specific antibody responses.

Experiments to determine how Clostridia signal to the host have implicated the production of the short chain fatty acid (SCFA) butyrate in mediating protection. Treatment of GF mice with butyrate increased expression of *Il22* and *ex vivo* culture experiments have confirmed this finding. Preliminary data suggests that IL-22 production occurs through G-protein coupled receptor (GPCR) signaling after butyrate stimulation. Experiments to directly link butyrate, GPCRs, and responses to sensitization are currently ongoing. Taken together, the results presented in this thesis demonstrate that the microbiota can profoundly alter responses to food antigens. By identifying specific protective populations and their mechanisms of action, we have provided novel targets for therapeutics that we hope will promote and maintain tolerance in patients suffering from this disease.

Chapter 1: Introduction

I. The composition and functions of the microbiota

All mammals, and even many lower organisms, are colonized with microbial communities that reside at barrier sites. In humans, these colonized surfaces include the skin, intestines, lungs, and vaginal tract (Donaldson et al., 2016). Although these communities are comprised of bacteria, viruses, and fungi, bacteria are the most well-studied. Since most of what is known about host-microbiota interactions is focused on bacterial populations, the word microbiota will refer strictly to the bacterial members of this community throughout the rest of this thesis. The total number of bacteria at barrier sites is estimated to be 10^{14}, outnumbering human cells in the body 10:1 (Ley et al., 2006). These populations are also remarkably diverse; studies show there are upwards of 1,000 species represented (Qin et al., 2010). Surprisingly, although scientists have been aware of the microbiota since the mid-1800s (Smith et al., 2007), its interactions with the host have only been studied in depth for the past few decades. It was originally hypothesized that the host was ignorant of the bacterial communities residing in and on it but it is now appreciated that these bacteria are essential for host health and have a profound influence on the immune system (Eberl, 2010; Round and Mazmanian, 2009).

Most of the bacteria that comprise the human microbiota live in the intestines, increasing in density from the stomach to the rectum (Human Microbiome Project Consortium, 2012). The greatest proportion of these bacteria lives in the colon, reaching 10^{12} bacteria per gram of feces. Two phyla, Bacteroidetes and Firmicutes, dominate the intestinal community. Additional phyla, including Proteobacteria, Actinobacteria, and Verrucomicrobia, are common but represent a comparatively minor proportion of species (Human Microbiome Project Consortium, 2012). Within each phylum, certain families dominate, namely Bacteroides within Bacteroidetes and Lachnospiraceae and Ruminococcaceae within Firmicutes, but at the species levels, there can

be considerable diversity within and between individuals. These bacteria reside in a complex community with a defined structure under homeostatic conditions (Human Microbiome Project Consortium, 2012; Ley et al., 2008a). In general, Bacteroides species reside in the lumen, associated with the digesta, the material that is excreted after it has been broken down. Lachnospiraceae and Ruminococcaceae, on the other hand, live closer to the host epithelium, in rare cases, even making contact with IECs (Nava et al., 2011; Nava and Stappenbeck, 2011). Although there is cooperation within the community to execute necessary functions, bacterial taxa tend to cluster together in related groups throughout their niche, creating substructures within these broad strata (Earle et al., 2015).

Although the bacterial microbiota was originally thought to be “commensal,” implying that it derives benefits from its host while the host remains unaffected, this has been proven to be a misnomer. The microbiota is vital for the health and continued survival of the host, making this relationship generally mutualistic rather than commensal. In the absence of a colonizing microbiota, GF mice have numerous morphological and functional deficiencies (Macpherson and McCoy, 2015b; Smith et al., 2007). The microbiota increases the energy extracted from food that is available to be used by the host (Backhed et al., 2004) and is crucial for producing and metabolizing compounds that the host cannot make or breakdown on its own. One important example is the synthesis of vitamins (LeBlanc et al., 2013). Members of the microbiota can modify the chemical structure of dietary components to make vitamin K and certain B vitamins more available and biologically useful to the host (Hill, 1997; LeBlanc et al., 2011). Dietary fibers are another class of diet-derived compounds that are processed by the microbiota for the host’s benefit. Fibers such as cellulose cannot be broken down by mammals because they do not produce the necessary enzymes but many members of the microbiota can ferment these dietary fibers into SCFAs (Brestoff and Artis, 2013). The most abundant SCFAs are acetate, propionate, and butyrate (Thorburn et al., 2014). Bacteroides family members are

the dominant propionate producers, while acetate and butyrate are produced by the Firmicutes phylum (Macy et al., 1978; Maslowski and Mackay, 2011; Russell et al., 2013). Butyrate, in particular, is important to the host as a major energy source for colonocytes (Donohoe et al., 2011; Roediger, 1980).

Another essential function of the microbiota is to promote the education and maturation of the immune system. As the microbiota changes throughout the life of the host, it can influence many pathways including lymphocyte differentiation, cytokine production, and antibody generation. Several of the most well studied effects of the microbiota on the immune system and homeostasis will be discussed in detail in later sections.

II. Influence of the environment on the microbiota and dysbiosis

The host-microbiota relationship has evolved over millennia under specific selective pressures that have allowed for appropriate adaptations and functions to maintain the health of both the microbial communities and the host. Indeed, individual animal species each have their own microbiotas, adapted to their particular needs and environments (Ley et al., 2008b). A study of the microbiota of members of a variety of species living in different locations demonstrated that there are greater similarities across space within a species than there are between species in a similar geographic location, demonstrating the uniqueness and specificity of the host-microbiota relationship (Ley et al., 2008a). Transplanting the microbiota across species confirmed this idea but also emphasized that there are conserved functional characteristics of the microbiota between many host species; although the abundance of different microbial populations changed, the host response to colonization with different microbiotas demonstrated conserved patterns of gene expression (Rawls et al., 2006). This idea is similarly true within the human population; despite microbial variation at the species level between individuals, there are conserved genetic and functional capacities contained within all

microbial communities (Muegge et al., 2011; Turnbaugh et al., 2009a). More recent studies have also examined the reciprocal relationship, namely the influence exerted by host genetic variation within a species on the composition of the microbiota. This effect remains less well characterized but it seems that certain host genetic factors, such as major histocompatibility (MHC) alleles or genetic predisposition to obesity, can feed back on and alter the composition of the microbiota (Goodrich et al., 2014; Kubinak et al., 2015b; Ley et al., 2005) while others, like innate immune signaling genes, do not (Ubeda et al., 2012).

It is generally accepted that humans are first colonized at birth upon passage through the mother's vaginal canal. Bacterial DNA has been detected in the placenta, suggesting that microbial exposure may begin *in utero*, but more studies must be performed to rule out the possibility of contamination during sample processing and understand the effects of this prenatal experience (Aagaard et al., 2014). Vaginal delivery favors colonization with so-called founder species, primarily *Lactobacillus* and *Bifidobacteria* (Human Microbiome Project Consortium, 2012; Dominguez-Bello et al., 2010). These founder populations provide early protection against infection and modify the environment of the intestine to make it more hospitable for other species that come in later waves of colonization (Mueller et al., 2015; Pantoja-Feliciano et al., 2013). The founder species as well as the early colonizing anaerobes are supported by breast milk, which provides glycans and other food sources for these bacteria (Coppa et al., 2004; Marcobal et al., 2011). As the infant is weaned on to solid food, the microbiota undergoes a dramatic shift, with an increasing predominance of anaerobic bacteria and a succession toward the adult phenotype described previously, with the majority of the bacterial families falling into the Bacteroidetes and Firmicutes phyla (De Filippo et al., 2010; Yatsunenko et al., 2012). This community remains fairly stable throughout adulthood but can shift again late in life (Dominguez-Bello et al., 2011).

Changes in Western lifestyle in the past several decades, particularly the beginning of the 21st century, interfere with these evolutionarily selected host-microbiota interactions, leading to a microbial imbalance termed dysbiosis. Dysbiosis has been linked to the development and exacerbation of many diseases, including inflammatory bowel disease (IBD), obesity and diabetes, and autoimmune disease. These diseases are much more prevalent in developed, Western nations, where interventions such as Abx use and Caesarean birth as well as consumption of high fat, low fiber diets are common, suggesting a causal link (Ananthakrishnan, 2015; Bach, 2002; Cox et al., 2015).

Cesarean birth disturbs the earliest phases in the normal succession of the developing microbiota. There is evidence that infants born via C-section are not colonized with the typical founder bacteria, but are instead colonized with a microbiota that more closely resembles the skin of their mothers and caregivers (Dominguez-Bello et al., 2010). The adult skin microbiota is dominated by Firmicutes and Actinobacteria, which are clearly distinct from the standard founder populations (Human Microbiome Project Consortium, 2012). Given the large body of evidence that the commensal microbiota can educate the immune system and alter immune homeostasis, it is not difficult to imagine that this altered founder population would change the baseline or “set-point” of the host immune system, having detrimental consequences later in life (Cox et al., 2014).

Dysbiosis can also be caused at later stages of development by a variety of other factors. As described earlier, the microbiota is involved in the processing of host dietary components and derives the nutrients required for its own survival from this same food. Longitudinal studies of individuals have documented changes in the microbiota on a daily timescale in response to alterations in diet (David et al., 2014a; David et al., 2014b), indicating exactly how susceptible to influence these communities are, as well as how intertwined these bacteria are with the host food sources. In the past several generations, however, a high animal fat, low fiber, “Western”

type diet has become far more popular than the low fat, high fiber diet of our evolutionary ancestors. A direct comparison of the effect of these two diets on the microbiota demonstrated that a diet high in fat not only leads to increased obesity but also supports a bacterial community depleted of fiber fermenters that produce beneficial SCFAs and enriched for potential disease-causing members of the community called pathobionts, which are typically found in very low abundance in healthy individuals (De Filippo et al., 2010).

Multiple mouse models have confirmed that diets high in fat can drive dysbiosis and exacerbate disease states. For example, one report showed that feeding a high milk fat diet drove a bloom of the pathobiont *Bilophila wadsworthia* (Devkota et al., 2012). This species was able to grow out as a result of a diet-induced increase in luminal bile acids, which it was uniquely adapted to use as a metabolic substrate. This bloom of *B. wadsworthia* increased IFNγ production from $CD4^+$ T cells in the MLN and IL-12 from dendritic cells (DCs), leading to the development of colitis in *Il10*$^{-/-}$ mice. Different high fat diets promoted the outgrowth of other pathobionts or rare species including segmented filamentous bacteria (SFB) in lymphotoxin- or lymphotoxin signaling-deficient (*Lta*$^{-/-}$ or *Ltbr*$^{-/-}$) mice (Upadhyay et al., 2012) or an unclassified member of the Mollicutes class from the Firmicutes phylum in both mice and humans (Turnbaugh et al., 2008). Restoring a plant polysaccharide rich diet, however, reversed some of these blooms, demonstrating that the microbiota is adaptable and resilient if given the appropriate substrates (Turnbaugh et al., 2008; Turnbaugh et al., 2009b). Even non-caloric artificial sweeteners (NAS), embraced as a substitute for sugar to control obesity, caused dysbiosis that exacerbated the metabolic dysfunction they were intended to correct (Suez et al., 2014). NAS feeding caused glucose intolerance in mice and altered both the bacterial abundance and metabolic capacity encoded by the microbiota. These changes led to increased energy harvest as measured by higher levels of SCFA in the feces. The same was true when high levels of NAS were acutely introduced into a human diet (Suez et al., 2014). Metabolic

dysfuction, like pathobiont blooms, could also be reversed by consuming a high plant polysaccharide diet; a new study demonstrated that consuming such a diet reduced serum glucose and insulin levels after feeding in humans and mice. This improvement correlated with an increased abundance of *Prevotella* (Kovatcheva-Datchary et al., 2015).

Although the microbiota can fluctuate rapidly in response to changes in diet, other factors that alter the microbial community exert selective pressures that are less easily overcome. Abx use has increased dramatically over the same timeframe as the increasing incidence of allergic and autoimmune disease. It is currently estimated that the average child receives three courses of Abx in the first two years of life and 10 courses of Abx by the age of 10 (Hicks et al., 2015; Nobel et al., 2015). Although intended to treat and kill pathogenic bacteria, many of the mechanisms of action of Abx do not differentiate between pathogens and members of the microbiota. Thus, Abx use can profoundly alter the composition of the bacterial communities at barrier sites. Single, short courses reduce abundance of specific members of the microbiota, but these populations recover to a certain extent (Cox et al., 2014; Dethlefsen et al., 2008). Repeated courses, however, have lasting and marked effects on bacterial communities (Dethlefsen and Relman, 2011; Nobel et al., 2015). Many of the original taxa are never restored and the microbiota is less able to respond to or recover from secondary influences, such as a change in diet. The communities reach a new equilibrium, however the effect of this altered community on the immune system may drive a pathogenic state. Subclinical doses of Abx are even present in food sources; while the final concentrations that reach humans is very low, these levels are still enough to impact the microbial communities of the intestines and drive increased weight gain in an animal model (Cho et al., 2012). Abx-mediated alterations in the microbiota also change the metabolic profile of the community leading to impaired colonization resistance. Pathogens, such as *Salmonella* and *Clostridium*

difficile, can take advantage of the altered production of metabolites to gain a competitive advantage and establish infection (Buffie et al., 2012; Ferreyra et al., 2014; Ng et al., 2013).

These environmental factors are certainly not the only ones that can influence the composition of the microbiota, but they represent some of the most common and significant ones. Vaccinations to prevent many common childhood infections may have the unintended consequence of altering the microbiota by disrupting the community structure and allowing for colonization with other bacteria that share the same niche (Lemon et al., 2012; Mohle-Boetani et al., 1993; Takala et al., 1991). Other changes in public health, including elimination of common enteropathogens like helminths, have had a beneficial impact on the health of the general population, but may also have altered host-microbiota interactions. Indeed, recent evidence in an animal model demonstrates that infection with a helminth changes the intestinal microbiota and reduces the severity of asthma symptoms (Zaiss et al., 2015). The authors show that helminth-induced changes in the microbiota increase SCFA production, particularly propionate, which they link to the reduced severity of airway disease. While there are profound public health benefits to eliminating helminths, it may also be contributing to the rise of the so-called modern, Western diseases by altering the composition of the microbiota.

III. Specialized adaptations of the barrier to maintain homeostasis with the microbiota

In order to support colonization with the enormous population of bacteria that comprise the microbiota, the host must possess specialized adaptations to contain these communities and respond to any breaches. The first major adaptations of the host are physical ones, namely the epithelial barrier and associated effector cells. Most of the barrier sites that support a microbiota are only separated from these communities by single celled epithelial layers. In the intestine, the epithelium divides the lumen from the LP, which harbors the largest proportion of

immune cells in the body. To prevent constant activation of these underlying immune cells, the epithelium physically excludes microbes. IEC are connected by a series of junctions, with tight junctions on the apical surface, adherens junctions in the middle, and desmosomes on the basolateral side (Turner, 2009). These junctions are critical for the maintenance of barrier integrity but also regulate passage of molecules across the epithelium, creating a semipermeable barrier. Tight junctions are composed of a variety of different proteins including occludin and members of the claudin family. Different combinations of these claudins can exert selectivity on the molecules allowed to cross the epithelium. Passage through tight junctions can occur via the leak pathway, which is selective based on the size of molecules (Van Itallie et al., 2008), or through pores, which exert charge selectivity and allow for much smaller molecules to pass through (Weber et al., 2015). Some reports suggest that members of the microbiota can alter expression and stability of tight junction proteins and complexes, particularly during the neonatal period while the microbiota is maturing and the intestinal barrier is being established (Bergmann et al., 2013; Patel et al., 2012). There is additional evidence that signaling via TLRs can alter expression of occludin and certain claudin family members in different epithelial tissues (Cario, 2008; Yuki et al., 2011) and cytokines like TNFα and IFNγ can alter expression and function of tight junction-associated proteins, so it is possible that changes in the microbiota that trigger production of these cytokines in the LP may well feed back on the epithelial barrier (Wang et al., 2005). In the absence of certain tight junction proteins, there can be increased inflammation and pathology as a result of inappropriate translocation of bacterial products or even whole bacteria into the LP (Khounlotham et al., 2012).

Along with IECs, there are a variety of specialized epithelial cells that contribute to additional layers of protection and regulation. Goblet cells produce secreted and surface bound glycoproteins called mucins that form a multilayered glycocalyx to keep bacteria a safe distance from the epithelium (Earle et al., 2015; Johansson et al., 2011). The outer mucus layer, closer to

the lumen, is generally described as loose and bacteria can be found embedded in this niche (Johansson et al., 2015; Li et al., 2015). The inner mucus layer, however, is very dense and generally devoid of bacteria (Johansson et al., 2011). In this way, the mucus can help stratify the bacterial community and limit direct contact of the microbiota with the host. Bacterial penetration of the inner layer is often associated with inflammation and pathology, suggesting that the physical separation of the microbiota from the host provided by the mucus is important for the maintenance of homeostasis (Johansson et al., 2014). Certain members of the microbiota actually stimulate changes in the mucus layer to alter its integrity and thickness, making this an important homeostatic feedback loop (Derrien et al., 2004; Earle et al., 2015; Jakobsson et al., 2015). Colonization of GF mice with a conventional microbiota alters the density and organization of the mucus structure (Johansson et al., 2015) while changes in the environment can force bacteria to utilize mucus as a carbon source (Kashyap et al., 2013), thinning this protective layer and possibly activating unwanted immune responses in the LP (Earle et al., 2015).

Other specialized epithelial cells call Paneth cells produce antimicrobial peptides (AMPs), which further limit bacterial growth. These AMPs act as natural antibiotics and have a variety of bactericidal actions (Mukherjee and Hooper, 2015). One dominant family of AMPs are the C-type lectins, including RegIIIβ and RegIIIγ, which can bind to the membranes of Gram positive bacteria and form pores, thereby killing the bacteria (Lehotzky et al., 2010; Mukherjee et al., 2014). Other AMPs include cationic peptides like S100a8/a9 which heterodimerize to form calprotectin, cathelicidins, and defensins, which may exert antimicrobial activity by sequestering calcium and other ions. These AMPs are secreted into the intestinal lumen and often are found suspended within the dense mucus layer, reinforcing this barrier and preventing the encroachment of the microbiota (Sperandio et al., 2015).

The final class of specialized cells in the epithelium is intraepithelial lymphocytes (IEL). These IEL reside below and between IECs, bridging the non-hematopoietic epithelium and the underlying LP immune system. IEL come in a variety of subtypes including those with γδ TCRs and those with αβ TCRs, which can also express different coreceptors ($CD8\alpha\beta^+$ or $CD8\alpha\alpha^+$). Although it is not clear what these TCRs recognize, they are thought to be specific for non-classical MHC molecules. They survey the basolateral surface of the epithelium for damage or pathogens (Edelblum et al., 2012) and promote tissue repair and defense under a variety of conditions. Multiple reports indicate that γδ IEL can produce AMPs in response to the microbiota and can provide protection against chemically-induced colitis (Ismail et al., 2011; Klose et al., 2014). Although the mechanism of protection is not entirely clear, there is evidence that they can produce immunoregulatory cytokines and keratinocyte growth factor (Chen et al., 2002). Much of this response to injury is dependent on the presence of the microbiota (Ismail et al., 2009). Together, these findings indicate that these cells are early responders to epithelial damage and act to contain the microbiota after a potential breach of the barrier.

There are also additional immunological adaptations at barrier sites, to reinforce the physical barrier made by the IECs. One example in the gut is the production of IgA. IgA is the most abundantly produced antibody isotype in the body and the majority of this IgA production occurs in the intestine (Macpherson et al., 2008; Nagler-Anderson, 2001). IgA can sequester luminal antigen, both dietary and microbial, although the consequences of this binding are incompletely understood (Macpherson and McCoy, 2015a). Intestinal IgA is produced in a unique dimeric form where two IgA molecules are linked by their constant (Fc) regions by the J chain. This IgA is then transcytosed across the epithelium from the basolateral to apical side via the polymeric Ig receptor (pIgR). When IgA bound to pIgR reaches the apical/luminal side of the IEC, it is cleaved off and released into the lumen (Johansen and Kaetzel, 2011). In the absence

of this translocation, elevated levels of serum proteins are detected in the feces, indicating increased permeability of the epithelial barrier (Johansen et al., 1999).

IgA is produced in both T cell-dependent and T cell-independent manners in the intestine (Pabst, 2012). T cell-dependent IgA is made mostly in the organized lymphoid structures of the intestine, including Peyer's patches. The generation of these IgA molecules follows the same pathway as other antibody production in the periphery. B cells present antigen to T follicular helper (Tfh) cells in germinal centers (GCs) that then provide cytokines and costimulatory help to these B cells, driving them to class-switch and affinity mature (Hirota et al., 2013; Tsuji et al., 2009). TGFβ is the required cytokine switch-factor for IgA production, although the cellular source of this cytokine and whether $Foxp3^+$ Tregs or $ROR\gamma t^+$ Th17 cells convert to Tfh to drive IgA switching remain disputed (Hirota et al., 2013; Tsuji et al., 2009). T-independent IgA is made in isolated lymphoid follicles and possibly in the LP itself and is thought to be stimulated by cross-linking of B cell receptors by repeated epitopes, such as bacterial cell wall motifs. In the presence of cytokines like BAFF and APRIL as well as TGFβ, these B cells can class-switch and secrete IgA without T cell interaction (Pabst, 2012).

Although the exact mechanism remains controversial, there is considerable evidence that different members of the microbiota can stimulate increased production of IgA. GF mice have considerably lower levels of IgA than SPF animals, indicating that colonization with a commensal community stimulates this response (Hapfelmeier et al., 2010). Other studies demonstrated that specific members of the microbiota can promote IgA responses. Colonization with a model commensal *E. coli* stimulated T cell-independent IgA that was also reactive against other related members of the microbiota (Macpherson et al., 2000). A specific bacterial flagellin protein, CBir1, from a member of the Lachnospiraceae family can also stimulate significant IgA production (Cong et al., 2009). There is other evidence suggesting that SFB also promotes an increase in luminal IgA levels (Lecuyer et al., 2014).

The IgA produced by bacterial stimulation then feeds back on the microbiota and limits the growth of certain populations. This was first described in AID knock-out (*Aicda*$^{-/-}$) mice, which cannot class-switch to IgA. The composition of the microbiota in the absence of IgA was dramatically different than that of wild type mice, allowing for the outgrowth of potentially pathogenic members of the microbiota (Fagarasan et al., 2002). Two recent reports described a requirement for T cell-intrinsic MyD88 signaling in order to stimulate commensal-specific IgA responses (Kubinak et al., 2015a; Wang et al., 2015). In the absence of MyD88 signaling in T cells, there was reduced production of IgA from GC B cells in Peyer's patches, changes in the populations of commensal bacteria bound by IgA, and alterations to the relative abundance of different members of the microbiota. Upon challenge with chemicals that damage the epithelium (dextran sodium sulfate or trinitrobenzenesulfonic acid), these mice also had aggravated intestinal pathology and increased mortality.

Other studies have looked more specifically at which bacteria are bound by IgA. There is evidence that stimulation of IgA production is a function of localization in the intestine as opposed to a feature of particular species (Bunker et al., 2015). In a recent report, most IgA-bound bacteria were found in the small intestine and this IgA was made via T cell-independent responses in the LP. Surprisingly, many of the bacteria in the colon were not bound by IgA; those that were IgA^{+} in this site were the same taxa identified in the small intestine. Unique species, particularly those that reside in close contact with the epithelium, were found to drive T cell-dependent IgA production in the LP, but the IgA specificities were distinct from those generated by T cell-dependent mechanisms in the Peyer's patches. In the context of inflammation, however, the production of IgA may be targeted toward pathobionts in an effort to control their interaction with the host immune system (Palm et al., 2014). Certain commensals can also evade IgA responses via degradation, allowing for their own expansion and subsequent reduction in host fitness in response to challenge (Moon et al., 2015). Host genetics

also influences IgA production by dictating how broadly-reactive intestinal IgA is; greater proportions of poly-reactive IgA in BALB/c mice lead to increased diversity of the intestinal microbiota compared to C57BL/6 animals, which had less poly-reactive IgA (Fransen et al., 2015). Increased diversity of IgA also supported a more diverse colonizing microbiota and expansion of other host regulatory mechanisms such as $Foxp3^+$ Tregs (Kawamoto et al., 2014). Long term, memory B cell responses to members of the microbiota are important to facilitate long term homeostasis with the microbiota in spite of the periodic fluctuations driven by the environment as previously described (Lindner et al., 2015). Together, these studies demonstrate that, while the interaction between the microbiota and IgA is complex and involves multiple levels of control, IgA is an important host adaptation that allows for the maintenance of homeostasis with these bacterial communities.

IV. Specialized adaptations of the LP to maintain homeostasis with the microbiota

It is abundantly clear that these barrier responses work in concert with and depend on signals from the host immune system; there is constant crosstalk and tuning to maintain homeostasis. A multitude of specialized cells in the intestinal LP are essential for establishing and maintaining tolerance to the massive number of luminal antigens in the intestine. These cells include unique antigen presenting cells (APCs), innate lymphoid cells (ILCs), and T cells subsets, all of which work together to regulate and respond to commensal bacteria, dietary antigens, and pathogens.

In the LP, there are a variety of $CD11c^+$ APC subsets not found in secondary lymphoid organs. These populations are best defined by expression of 2 markers: CD103 and CX_3CR1 (Harusato et al., 2015). CD103 expression defines the DCs while CX_3CR1 defines a macrophage population that also expresses F4/80 and has functional properties of both DC and macrophages. The $CD103^+$ cells are most similar to DCs in lymphoid organs and are

considered the major APCs in the intestines (Bogunovic et al., 2009). This population can be further subdivided by the expression of CD11b to tease out very specific roles under different conditions. $CD103^+$ DCs are particularly good at inducing tolerogenic responses because they produce TGFβ and have the ability to metabolize vitamin A into retinoic acid (RA) through expression of retinaldehyde dehydrogenase (RALDH) enzymes (Coombes et al., 2007; Sun et al., 2007). These DCs are migratory and can be detected in both the LP and the mesenteric lymph node (MLN) (Schulz et al., 2009). These cells are important for the establishment of tolerance to dietary antigen, which will be discussed in detail later.

The origins and functions of CX_3CR1^+ macrophages have been more controversial and are still being defined. These cells were first identified by their ability to extend dendritic processes between IECs into the small intestinal lumen to sample contents during infection with *Salmonella* and were thought to be resident in the LP under conditions of pathogenic infection (Chieppa et al., 2006; Niess et al., 2005). Their role at steady-state was unclear. Under homeostatic conditions, they were found to be potent producers of IL-10 but generally poor at antigen processing and presentation so it was hypothesized that they were important for preventing Th17 responses (Denning et al., 2007; Hadis et al., 2011; Medina-Contreras et al., 2011). Very recently, however, it has been reported that CX_3CR1^+ cells are actually required for the differentiation of Th17 cells in response to SFB colonization (Panea et al., 2015), as well as the production of cytokines important for epithelial barrier function and protection from infection (Longman et al., 2014). These cells can also migrate under conditions of dysbiosis or stress, suggesting they also have broader functions for regulating homeostasis than previously appreciated (Diehl et al., 2013).

Another type of cell that is relatively newly identified and appreciated in the LP is the ILC (Walker et al., 2013). Much like T cells, ILCs are grouped into several subtypes that possess unique functional characteristics and cytokine producing profiles including Group 1 ILCs (ILC1s)

that mirror Th1 cells and produce IFNγ, Group 2 ILCs (ILC2s) that produce Th2 cytokines and can exacerbate allergic responses, and Group 3 ILCs (ILC3s) that are Th17-like effectors and are important in responses at the barrier and in fighting extracellular pathogens (Eberl et al., 2015). ILC3s are defined by their expression of the transcription factor RORγt and are the dominant ILC population in the intestinal LP (Mjosberg et al., 2012; Sanos et al., 2009; Sanos et al., 2011; Sawa et al., 2010; Sawa et al., 2011). ILC3s are a heterogeneous population that includes lymphoid-tissue inducer (LTi) cells, required for the development of Peyer's patches and lymph nodes (Eberl et al., 2004), as well as populations defined by expression of various NK receptors like NKp46. Each of these subpopulations has different transcriptional requirements for their developmental and functional capabilities (Rankin et al., 2015; Satoh-Takayama et al., 2011; Satoh-Takayama et al., 2008; Song et al., 2015). The ILC3s were first described as potent cytokine producers, but are also now appreciated as regulators of T cells responses. Despite their lack of antigen-specific receptors, these cells express MHC class II and can stimulate peripheral deletion of T cells specific for commensal bacteria to limit inflammatory responses and reinforce tolerance to the microbiota (Hepworth et al., 2015). ILC3s also stimulate IgA generation through lymphotoxin production, further supporting homeostasis at the barrier (Kruglov et al., 2013).

One of the most extensively studied roles for ILC3s is production of cytokines to maintain the integrity of the epithelial barrier. The most notable cytokine in this context is IL-22 (Sabat et al., 2014; Sonnenberg et al., 2011). Although predominantly produced by ILC3s under stead-state conditions (Ahlfors et al., 2014), ILC3s, γδ IEL, and Th17/Th22 cells can all make IL-22 during infection or under other stressors (Basu et al., 2012; Mielke et al., 2013; Rankin et al., 2015; Shaw et al., 2012; Zheng et al., 2008). IL-22 can be produced in response to a variety of signals, including IL-23, IL-1β, and IL-6 produced by DCs (Geremia et al., 2011; Kinnebrew et al.; Shaw et al., 2012), or directly via activation of the aryl hydrocarbon receptor (AhR) (Qiu et

al., 2013; Qiu et al., 2012; Zelante et al., 2013). These signals can also synergize to promote maximal IL-22 production (Guo et al., 2015). IL-23, IL-1β, and IL-6 are all made in response to microbial stimuli while AhR ligands are derived from dietary components and can also be made by metabolism of tryptophan (Hooper, 2011). When IL-22 is produced, it acts via the IL-22R, which is expressed only on non-hematopoietic cells, particularly IECs (Wolk et al., 2004). IL-22/IL-22R signaling drives IEC proliferation, increased mucus production and expansion of goblet cells, and increased secretion of AMPs (Sabat et al., 2014; Sonnenberg et al., 2010; Sugimoto et al., 2008). IL-22 also protects and supports the survival of epithelial stem cells, which promotes cell turnover and proper renewal of the epithelium (Aparicio-Domingo et al., 2015; Lindemans et al., 2015). Under homeostatic conditions, there is evidence that IL-22 is required to prevent outgrowth of the microbiota and block translocation of bacteria to other organs (Sonnenberg et al., 2012). IL-22 is also particularly important for the response against pathogens because it can stimulate innate antimicrobial responses like AMPs (Sonnenberg et al., 2011). The attaching and effacing pathogen *Citrobacter rodentium* is a well-studied model of infection requiring IL-22 for clearance (Guo et al., 2015; Guo et al., 2014; Tumanov et al., 2011; Zheng et al., 2008). In the absence of IL-22 or ILC3s themselves, there is increased *Citrobacter* burden, worsened pathology, and considerable mortality as a result of the infection. Thus IL-22 is critically important for maintenance of the physical barrier between the microbiota and the immune system, both at steady-state and after infectious stress.

A final unique feature of the intestine its ability to support the peripheral differentiation of lymphocyte subpopulations. Two populations for T helper cells are particularly enriched in the intestinal mucosa compared to primary or secondary lymphoid organs: $Foxp3^+$ Tregs and $ROR\gamma t^+$ Th17 cells (Littman and Rudensky, 2010). Tregs are generally considered suppressive and important to limit inflammation and prevent aberrant reactions to self and the microbiota while Th17 cells are often associated with inflammation and autoimmune disease. Multiple

recent publications, however, suggest that this stark distinction is overly simplistic and that homeostasis is maintained by a gradient of responses (Ohnmacht et al., 2015; Sefik et al., 2015).

Th17 cells were first identified about 10 years ago. Originally characterized as part of the Th1 lineage, improvements in cellular and molecular detection mechanisms demonstrated that these IL-17-producing cells were dependent on the transcription factor RORγt, making them developmentally distinct from IFNγ-producing, Tbet-dependent Th1 cells (Ivanov et al., 2006). Further insight into the origins of these cells came from the observation that mice purchased from one vendor had a sizeable portion of IL-17-producing T cells in their small intestine LP while mice obtained from a different source were lacking these cells (Ivanov et al., 2009; Ivanov et al., 2008). SFB abundance correlated with Th17 levels and transfer of SFB was sufficient to induce Th17 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). It is now understood that the CX_3CR1^+ macrophages are the APCs required for the induction of these Th17 cells (Panea et al., 2015), many of which possess T cell receptors (TCRs) specific for SFB antigens (Goto et al., 2014; Yang et al., 2014). It is also recently appreciated that epithelial responses influence Th17 cell function. Production of serum amyloid A1/2 by IECs in response to IL-22 improves IL-17 production by Th17 cells, particularly in the ileum where SFB colonizes (Sano et al., 2015).

Although SFB has been the quintessential Th17 inducing signal, it is not the only bacterial population or bacterial signal that has been able to drive this expansion. Other extracellular stimuli, including fungi (Atarashi et al., 2015), and bacterial ATP (Atarashi et al., 2008) have also been shown to promote this response. Th17 cells and their associated cytokines are important for clearance of extracellular pathogens, but since these cells were first identified, they have been implicated in the pathogenesis of many diseases. IL-17 and Th17 cells have been linked to models of autoimmune arthritis and other inflammatory joint disorders

(Langrish et al., 2005; Murphy et al., 2003; Wu et al., 2010), IBD (Geremia et al., 2011; Ivanov et al., 2006; Kullberg et al., 2006; Nishio et al., 2015), and experimental autoimmune encephalitis (EAE) (Berer et al., 2011; Haghikia et al., 2015; Lee et al., 2011).

Many of the Tregs in the intestinal lamina propria have also been shown to differentiate in response to bacterial cues. Unlike thymically-derived Tregs which differentiate during selection and leave the thymus as $Foxp3^+$ cells, these intestinal Tregs, particularly those found in the colon, arrive at this site as naïve T cells and then differentiate *in situ* in response to antigen stimulation and environmental cues (Josefowicz et al., 2012; Lathrop et al., 2011). There is evidence that the TCRs of these colonic Tregs are specific for bacterial antigens, similar to SFB-specific Th17 cells (Lathrop et al., 2011). Alterations of the microbiota, or its complete absence in the case of GF mice, have been shown to reduce the proportion of $Foxp3^+$ cells in the LP (Atarashi et al., 2011; Mazmanian et al., 2005; Round and Mazmanian, 2010; Russell et al., 2012). Much like SFB was discovered as a Th17 inducer, Clostridia are the champion Treg inducers in the colon. Consortia of both mouse and human Clostridia were shown to expand Tregs when administered to GF mice (Atarashi et al., 2013; Atarashi et al., 2011; Kashiwagi et al., 2015) however other bacteria and consortia including *Bacteroides fragilis* and Altered Schaedler flora (ASF) have been accepted as Treg inducers as well (Geuking et al., 2011; Mazmanian et al., 2005; Round et al., 2011; Round and Mazmanian, 2010). Proposed mechanisms of Treg induction include TGFβ produced by either IECs or DCs acting on naïve T cells (Atarashi et al., 2011; Kashiwagi et al., 2015), TLR signaling on naïve T cells (Round et al., 2011), and SCFA signaling in DCs and T cells (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013).

Induced Tregs have been shown to be particularly important for limiting Th2 responses in the intestine. If induced Tregs are ablated by eliminating CNS1, a intronic enhancer required for Foxp3 upregulation in the periphery, there is spontaneous Th2 inflammation in the intestine

(Josefowicz et al., 2012). Furthermore, recent reports describe specific roles for RORγt$^+$ Foxp3$^+$ Tregs in responding to the microbiota and maintaining homeostasis in the intestine, particularly the colon (Ohnmacht et al., 2015; Sefik et al., 2015). RORγt$^+$ Tregs are reduced in GF mice but can be restored upon colonization with a variety of bacterial stimuli, including Clostridia and diverse members of both the Bacteroidaceae and Lactobacillaceae families (Sefik et al., 2015). Removing vitamin A and its metabolite, RA, also had a negative effect on this population by reducing Foxp3 expression and favoring the generation of Th17 cells. Surprisingly, eliminating RORγt expression in Foxp3$^+$ cells did not increase Th17 responses. Instead it promoted the generation of intestinal and systemic Th2 responses (Ohnmacht et al., 2015). In the absence of RORγt, there was also increased coexpression of GATA3, the transcription factor required for Th2 differentiation, and Foxp3 in Tregs. Other reports have suggested that these GATA3$^+$ Tregs are also induced by IL-33, an epithelial-derived molecule that is important in Th2 responses (Schiering et al., 2014). In the colon, however, the IL-33-induced GATA3$^+$Foxp3$^+$ cells do not promote Th2 responses but are able to suppress colitis. Their function is impaired by IL-23, another microbially-induced Th17-associated cytokine, however, suggesting that there is antagonism between these different subsets. Although the picture of how these Treg subsets are generated and how their functions differ is muddy, it is clear that LP Tregs are essential for maintaining homeostasis and may also play an important role in preventing Th2 allergic responses to intestinal antigens, particularly food.

There is also emerging evidence that B cells and the pre-immune B cell receptor (BCR) and antibody repertoire are influenced by the microbiota. In many animals, lymphoid structures associated with the intestine, or even the intestine itself, are sites of B cell development and repertoire diversification (Wesemann, 2015). In mice, most B cells undergo receptor rearrangement in the bone marrow but a population of B cells in the small intestine LP also actively undergoes V(D)J recombination, as measured by RAG expression (Wesemann et al.,

2013). In agreement with a role for the microbiota in regulating these recombination events, the number of RAG-expressing B cells in the LP peaks at weaning, corresponding with a time when the microbiota is undergoing dramatic shifts. The light chain usage (Igκ segments) in the BCRs generated by these LP B cells is distinctly different compared to bone marrow-derived BCRs, possibly due to the unique types of antigens encountered in the gut. GF animals have a lack of developing B cells in the LP as well as an altered ratio of Igλ versus Igκ usage compared to conventionalized counterparts, further suggesting a microbial influence. While the functional consequences of this B cell repertoire diversification in the LP have yet to be determined, it is interesting to consider the possible importance of this population in establishing and maintaining tolerance in concert with T cell subsets.

V. Mechanisms by which the microbiota can interact with the host

How different members of the microbiota signal to the host to affect both the local and systemic immune system has been a subject of considerable research. Many of the pathways stimulated by commensal bacteria are the same ones that are triggered by pathogenic bacteria during an infection, but the outcomes are different. The reason for differential responses to these two groups of bacteria is an area of ongoing study. Pattern recognition receptors (PRRs) are both surface-bound and intracellular receptors on immune cells that can bind conserved epitopes derived from bacteria, called pathogen-associated molecular patterns (PAMPs), and drive downstream cytokine and chemokine production. They provide signals that are essential for the initiation of a productive innate immune response. TLRs are one of the major classes of PRRs and can be found both on the cell surface and in intracellular vesicles. The role of TLRs in host-microbiota interactions has been widely studied, although the consequences of activating these receptors remain disputed. IECs express a wide panel of TLRs (Otte et al., 2004), and although they are hyporesponsive to direct stimulation with TLR ligands, TLR signaling

pathways are important for maintaining homeostasis. Seminal work demonstrated that knocking out a variety of TLRs or their downstream signaling molecules, including MyD88, increased inflammatory responses, suggesting that TLR signaling is a protective signal for regulating host-microbe interactions in the intestine (Rakoff-Nahoum et al., 2004). Some TLR5 knock-out mice, particularly mice conditionally deficient in TLR5 in IECs, also display dysbiosis and increased inflammation that leads to metabolic dysfunction (Chassaing et al., 2014; Vijay-Kumar et al., 2010). This phenotype may require multiple influences, such as colonization with a pathobionts, because it is not reported in all mouse colonies, but even in the absence of basal inflammation, TLR5 deficient mice have impaired CD4 T cell responses to flagellin (Letran et al., 2011). The protective effect of TLRs and MyD88 in IECs may be related to improved epithelial repair and increased expression of AMPs as well as mucus production and IgA translocation (Brandl et al., 2007; Vaishnava et al., 2011). Regulation of NF-κB activation downstream of TLRs provides another layer of regulation and can drive differential responses. IEC-specific IKKα is necessary to produce barrier protective responses to control attaching-and-effacing pathogens while IEC IKKβ is required for mounting Th2 responses to parasites (Giacomin et al., 2015; Zaph et al., 2007). Activation of TLRs on APCs can influence gut homing of T cells (Wang et al., 2011) and production of cytokines including IL-23, IL-17, and IL-22 (Kinnebrew et al.; Van Maele et al., 2010). TLR signaling by the microbiota can also promote protective immune responses in lymphocytes such as induction of $Foxp3^{+}$ Tregs (Round et al., 2011) and IgA production (Kubinak et al., 2015a) but may contribute to the generation of autoimmune disease (Burrows et al., 2015; Wen et al., 2008). TLR2 is also expressed on human ILCs and there is evidence that these cells can respond directly to stimulation with TLR agonists (Crellin et al., 2010).

Other classes of PRRs also play a role in sensing of the microbiota. Polymorphism in NOD2, an intracellular receptor for the bacterial cell wall component peptidoglycan, is a major risk factor for IBD development in humans (Ogura et al., 2001). *Nod2*$^{-/-}$ mice may have dysbiosis

and greater pathology in response to epithelial damage from chemical disruption of the epithelium or infection with invasive pathogens due to an inability to produce AMPs, although these findings are still under investigation (Mondot et al., 2012; Robertson et al., 2013). Inflammasome activation by microbial components is also an important sensing mechanism for cytokine production. Inflammasome proteins can recognize bacterial components, including flagellin (Kupz et al., 2012), portions of Type III secretion systems (Zhang et al., 2015), and metabolites (Levy et al., 2015). Production of IL-1β and IL-18 depend on processing by inflammasome complexes, and both of these cytokines have been implicated in the maintenance of homeostasis and development of intestinal immune responses. Activation of these inflammasome-related PRRs can lead to production of IL-18 by IECs that can protect against epithelial damage (Levy et al., 2015; Nowarski et al., 2015) and synergize with TLR signaling to respond to a variety of infections, including viruses (Zhang et al., 2014). T cells in the colonic LP also express the IL-18 receptor, and IEC-derived IL-18 signaling influences the balance between regulatory and inflammatory T cell populations (Harrison et al., 2015).

In addition to PRRs, the host can respond to compounds produced by microbial metabolism. SCFAs have potent immunomodulatory effects on both hematopoietic and stromal cells. Several GPCRs can bind SCFAs with a range of affinities. GPR41 (*Ffar3*) binds propionate and butyrate with moderate affinity while GPR43 (*Ffar2*) binds acetate with high affinity and propionate and butyrate with lower affinity (Brown et al., 2003). Finally, GPR109a is a high affinity receptor for butyrate and the B vitamin niacin but does not bind either acetate or propionate (Thangaraju et al., 2009). Butyrate is also capable of altering gene expression by inhibiting histone deacetylases (HDACs) (Candido et al., 1978; Vidali et al., 1978). This activity of butyrate modulates cytokine production in intestinal macrophages (Chang et al., 2014). Butyrate and propionate also alter T cell populations in the colonic LP, promoting Treg differentiation via both HDAC inhibition (HDACi) and GPR43 signaling (Arpaia et al., 2013;

Furusawa et al., 2013; Smith et al., 2013). In the central nervous system, SCFA protect against EAE via Treg induction while long chain fatty acids increased Th1 and Th17 cells, exacerbating disease. Propionate was also shown to protect against the development of asthma by altering hematopoiesis in the bone marrow (Trompette et al., 2014). IECs and other stromal cells express GPCRs that bind SCFAs and can produce protective cytokines in response to this signaling. One example is the production of IL-18 by IECs in response to both GPR109a and GPR43 signaling by butyrate and acetate (Macia et al., 2015; Singh et al., 2014). IL-18 had a protective effect on both colon polyp formation (Singh et al., 2014) and IBD-like inflammation (Macia et al., 2015; Maslowski et al., 2009). Together, these results indicate that SCFAs can act both locally and systemically to alter host immune responses.

One newly appreciated type of host-microbe interaction is direct contact of bacteria with host cells. Although most members of the microbiota are excluded from contact with the host epithelium, a select few populations, particularly SFB, possess the ability to directly attach to IECs. A recent publication clearly demonstrated that SFB's ability to induce Th17 cells is directly related to its ability to adhere to the epithelium (Atarashi et al., 2015). In mice colonized with rat-derived SFB, there was no adhesion and significantly less Th17 induction than after colonization with mouse-derived SFB. The reciprocal was also true. Since Th17 responses are most effective at combating infection with extracellular pathogens, the requirement for cell-cell contact to stimulate this response fits logically. Infection with *Citrobacter rodentium*, one extracellular pathogen that stimulates a robust Th17-biased response, also induced generation of Th17 cells but mutant bacteria that could not attach to IECs were impaired in their ability to elicit this response. Intracellular pathogens were also incapable of driving the Th17 response. A consortium of human bacteria was able to induce Th17 cells in mice through attachment to the epithelium (Atarashi et al., 2015). Bacteria that stimulated Treg induction, on the other hand, did not contact IECs despite the fact that the two consortia shared members of the same genera

(*Ruminococcus, Clostridium,* etc.). These findings suggest that there are still aspects of host-microbiota interactions that are not fully appreciated and require future study.

VI. Increasing prevalence of allergic diseases

Food allergies are a growing public health concern (Jackson et al., 2013). Between 1997 and 2007, the Centers for Disease control have documented an 18% increase in reported food allergies (Branum and Lukacs, 2008). Similar changes in incidence have been reported for other allergic diseases such as asthma, eczema, and hay fever (Jackson et al., 2013), suggesting that there may be a common underlying cause for the increased prevalence of these diseases. Such an increase is unprecedented and cannot be explained by genetic drift through the population alone, implicating gene-by-environment interactions. Indeed, gene-by-environment interactions are essential for the health and proper development of the host at many stages throughout life (Rakoff-Nahoum et al., 2015). One hypothesis suggested in the late 1980s was the so-called "hygiene hypothesis" which proposed that children who grew up around larger numbers of older siblings had reduced incidence of hay fever because they had increased exposure to infections early in life while children who did not have such exposure had less developed immune systems and thus became susceptible to developing allergic disease (Strachan, 1989). This hypothesis gained considerable favor and has been used to explain the increase in both allergic and autoimmune disease in recent decades. It has also been expanded and modified to describe a role for general microbial exposure, particularly from the commensal microbiota, in protection (Feehley et al., 2012). The current version is more appropriately termed the "microbiota hypothesis," and this model posits that changes in the composition of the microbiota, particularly early in life when the immune system is being educated, can alter the host's susceptibility to developing allergic and inflammatory diseases (Prioult and Nagler-Anderson, 2005). A large body of evidence to support this hypothesis exists for autoimmune and inflammatory disease

but the link to allergy is less clear. This thesis aims to provide new support for a role for the microbiota in regulating allergic responses to food antigens.

VII. Oral tolerance and induction of a food allergic response

In healthy individuals under homeostatic conditions, ingested antigens, particularly food, do not provoke an immune response. The process by which the immune system learns mucosal and systemic nonresponsiveness to harmless dietary antigens is called oral tolerance (Pabst and Mowat, 2012). When food antigens are delivered orally, they reach the small intestine, particularly the ileum, where they can cross the epithelial barrier via transcytosis through specialized M cells in Peyer's patches (Ohno, 2015) or through newly described goblet cell-associated passages (McDole et al., 2012). Once in the LP, these antigens are picked up by the specialized RA-producing $CD103^+$ DCs (Coombes et al., 2007; Sun et al., 2007) that then carry the antigen from the intestine to the MLN. In the MLN, antigen is presented to naïve T cells. In the context of RA and TGFβ produced by the presenting DCs, these naïve T cells upregulate Foxp3 and become antigen-specific $Foxp3^+$ Tregs. RA also promotes expression of the homing receptors $\alpha_4\beta_7$ and CCR9, directing these Tregs back to the small intestine LP where they can prevent inflammatory responses to subsequent exposure to these oral antigens through production of IL-10 and other anti-inflammatory cytokines (Cassani et al., 2011; Coombes et al., 2007; Sun et al., 2007). There is also evidence that other cells in the LP of the small intestine such as CX_3CR1^+ macrophages promote the expansion and maintenance of the antigen-specific Tregs (Hadis et al., 2011). All together these mechanisms prevent responses to food antigens.

In contrast to tolerance, which is dominated by production of TGFβ and IL-10, allergic responses are best characterized by their Th2 bias, characterized by the production of cytokines IL-4, IL-5, and IL-13 and the generation of IgG1 and IgE antibodies (Geha et al., 2003). Such

responses are helpful to fight and expel multicellular pathogens such as helminths, but can cause life-threatening disease when produced aberrantly in response to harmless antigens such as food. The first step in an allergic response is the sensitization phase. At this time, the allergen is taken up by APCs, is processed, and the presented to antigen-specific naïve T cells, as with oral tolerance (Nagler-Anderson and Shi, 2001). During sensitization, however, naïve T cells are driven to the Th2 lineage instead of becoming Tregs. Exactly what causes this Th2 bias remains unclear; however there is evidence that signaling through Fc receptors on DCs can alter cytokine production to promote this skewing (Tjota et al., 2014; Williams et al., 2013). Another study suggests that eosinophil peroxidase produced by infiltrating eosinophils can activate $CD103^+$ DCs and drive them to a Th2 phenotype instead of the tolerogenic phenotype that promotes Treg differentiation (Chu et al., 2014). Once oral antigen-specific T cells convert to the Th2 lineage, they can produce cytokines, particularly IL-4, and provide help to GC B cells that also present allergen. IL-4 drives class-switching to the IgG1 and IgE isotypes. B cells can then produce large amounts of these allergen-specific antibodies. Allergen-specific IgE binds to FcεR1, its high affinity Fc receptor, on mast cells. In this state, there is no ongoing allergic response nor are there any acute symptoms. Upon secondary exposure to allergen, however, the system is poised to respond robustly. This subsequent allergenic exposure, or challenge, is the second phase of the allergic response. When allergen is introduced into the system again, the IgE bound to FcεR1 is cross-linked, triggering downstream signaling events that lead to the degranulation of the mast cell and release of a variety of effector molecules including histamine, leukotrienes, and vasodilators (Geha et al., 2003). In food allergy, the response is systemic, leading to manifestations throughout the body. Symptoms range from hives, diarrhea, and dizziness to an anaphylactic response characterized by constriction of the airways, gastrointestinal distress, and a drop in blood pressure (Simons et al., 2011).

There is currently no treatment for food allergies, despite the severity of responses. There is also only a poor understanding of how and why the sensitization phase is initiated. Since the prevalence of food allergy has increased so rapidly in only one generation, our lab hypothesized that certain members of the microbiota may be important for regulating the generation of an allergic response.

VIII. Influence of the microbiota on Th2 responses and oral tolerance

Many of the cell types that regulate tolerance to dietary antigen or generation of an allergic response overlap with the cell types that are responsive to the microbiota. Although it is not clear which bacteria are important for preventing allergic responses to food, there is a body of evidence supporting a role for microbial stimulation in preventing a Th2 response. One of the first descriptions for a role of the commensal microbiota in regulating allergic disease came from our lab in 2004 (Bashir et al., 2004). C3H/HeJ mice, which are unable to signal via TLR4 due to a point mutation that ablates signaling, produced high levels of PN-specific IgE in response to sensitization. TLR4-sufficient C3H/FeJ mice did not have the same elevated Th2 responses, however administration of antibiotics to C3H/FeJ mice caused them to respond similarly to the C3H/HeJ animals. Together this demonstrated that either an inability to sense certain members of the microbiota or an alteration of the microbiota could exacerbate sensitization to oral antigen.

Other evidence strongly supports a role for the microbiota in preventing Th2 responses. Multiple studies have observed that over time, GF mice spontaneously generated high levels of serum IgE (Cahenzli et al., 2013; McCoy et al., 2006). Although it is not clear what antigens this IgE is directed against, it was produced in the absence of microbial stimulation and was not generated in genetically identical SPF animals. This IgE was also not eliminated by removing solid food and placing mice on parenteral nutrition, suggesting it is not a result of sensitization to food proteins (Cahenzli et al., 2013). Subsequent studies have demonstrated that colonization

with ASF, a limited consortium of 8 bacteria developed to restore homeostasis in GF mice (Schaedler et al., 1965), blocked the generation of high levels of IgE and prevented associated pathologies. The protective effect of colonization, however, was a function of both age and bacterial diversity (Cahenzli et al., 2013). Colonization only blocked production of IgE when bacteria were introduced early in life, less than one month after birth. Colonization was also unable to prevent the generation of IgE in mice that had low diversity early after colonization (only 2 detectable species); increased microbial diversity during adulthood was unable to block the spontaneous IgE production that occurred early in development.

Other studies of GF mice indicate that Th2 effector cells like NKT cells are also dysregulated in the absence of a colonizing microbiota. Similar to the situation with IgE, GF mice had an accumulation of NKT cells in their colonic LP and lungs that could only be prevented by colonization at a young age; colonizing adult GF mice did not reduce the number of NKT cells (Olszak et al., 2012). As a result of this increased accumulation of NKT cells, GF mice also had exacerbated pathology in oxazalone-induced colitis and allergic airway responses, both Th2-biased models. Additionally, dysregulated basophil hematopoiesis has been documented in GF and Abx-treated mice, leading to an increased population of circulating basophils, which could potentiate Th2 pathology (Hill et al., 2012).

These studies do not address the initiation of allergic responses, however. Although effector cells can exacerbate responses, they are not APCs or antibody producing cells, so they cannot mediate the entirety of allergic disease. There is evidence that sensing of microbial signals by DCs is required to prevent the generation of Th2 cells. In mice lacking TRAF6, a downstream signaling component in the TLR pathway, specifically in their DCs, there was spontaneous generation of Th2 cells, cytokine production, and IgE and IgG1 production in the small intestine (Han et al., 2013). This response did not extend to the colon, but there was significant small intestinal pathology and loss of the Treg population in the LP. Abx treatment

reduced the Th2 response, demonstrating a role for commensal bacteria. In the absence of TRAF6, oral tolerance was also impaired, as measured by conversion of naïve ovalbumin (OVA)-specific OT-II T cells into Foxp3$^+$ Tregs after OVA feeding. This study did not identify which bacteria provided the protective TRAF6 signal or caused pathology in the absence of TRAF6. Interestingly, though, a follow-up study examined the phenotype of TRAF6 conditional mutant mice and found that rederiving them GF actually exacerbated the Th2 pathology in the small intestine (Han et al., 2015). This suggests that there is a balance of signals from the microbiota that must be integrated in order to maintain homeostasis. Although certain members of the microbiota can provide inflammatory signals, others may provide regulatory signals, thus completely eliminating the microbiota may not ameliorate all pathology. As discussed earlier, microbial signals also influence the differentiation of Foxp3$^+$ Tregs, which can suppress the spontaneous generation of Th2 responses in the intestinal mucosa (Josefowicz et al., 2012; Ohnmacht et al., 2015).

The establishment of oral tolerance can also be modulated and supported by the microbiota. An elegant study demonstrated that microbial signals stimulate cytokine production, particularly IL-1β, by intestinal macrophages, which then acts on ILC3s (Mortha et al., 2014). These ILCs produce Csf2 that can influence DCs and allow them to support the increased differentiation of bulk Tregs in the colonic LP as well as OT-II Treg conversion in response to oral administration of OVA. Other DC conditioning stimuli have also been shown to promote conversion of antigen-specific T cells into Foxp3$^+$ cells. In animals fed the SCFA butyrate, there was increased production of Tregs in general but also a specific expansion of Foxp3$^+$ OT-II cells after OVA feeding (Arpaia et al., 2013; Singh et al., 2014). The mechanism of this conversion remains unclear, as both inhibition of HDACs and signaling via GPR109a were shown to be required for this antigen-specific conversion. Butyrate acted, in part, to promote tolerance by driving increased expression of *Aldh1a1*, one of the RA metabolizing enzymes, on CD103$^+$ DCs

(Singh et al., 2014). There may be cooperation between these pathways or differential activation depending on the environmental cues, ultimately leading to the same outcome. Another study suggests that mucus, specifically the structural component MUC2, can condition DCs through Fc receptor signaling leading again to *Aldh1a1* upregulation and an increase in conversion of OT-II cells into $Foxp3^+$ Tregs after OVA feeding (Shan et al., 2013). MUC2 conditioning of DCs also blocked the generation of allergic delayed-type hypersensitivity responses and OVA-specific IgE and IgG1. Knocking out MUC2 or treating with Abx had a similar effect on these pathways and lead to a decrease in tolerogenic signals and a concomitant increase in allergic responses. Together, these studies demonstrate that microbial signals can direct the establishment and maintenance of tolerance to orally administered antigens; in the absence of these signals, there is increased opportunity for sensitization to food and the generation of an allergic response.

An important outstand question regarding the role of the microbiota in regulating allergic disease is whether the changes in the microbiota drive the development of allergy or whether the Th2 inflammation drives changes in the microbiota. Our laboratory favors the first model, as the data we have generated as well as evidence presented above suggests that the immune system is educated and conditioned by the members of the microbiota, which then leads to altered responses to food. There is a small literature, however, that indicates allergic disease itself changes the composition of the microbiota and may act to amplify the inflammation and exacerbate disease. Using a mouse model with overexpression of an IL-4 receptor subunit, Noval-Rivas et al. found that mice sensitized with OVA plus *Staphylococcus* enterotoxin B had decreased representation of Firmicutes and increased Proteobacteria in fecal samples compared to unsensitized counterparts (Noval Rivas et al., 2013). Transfer of OVA-specific Tregs reestablished tolerance and restored the composition of the microbiota to the unsensitized baseline, suggesting that resolution of the allergic inflammation resolved the

dysbiosis as well. Unfortunately this study did not use certain necessary controls in their comparisons, such as littermates and vehicle groups, so it is difficult to properly interpret these findings. It does present an interesting idea, however, that while there is extensive crosstalk between the microbiota and the immune system, there is also feedback of the immune system on the microbiota in allergic disease.

Based on the previous work from our laboratory and the evidence that the microbiota can extensively influence the host immune system and the development of a variety of diseases, we hypothesized that there are particular members of the microbiota that can prevent sensitization to food. We focused our studies on the effect of the microbiota during the neonatal period because the microbiota undergoes dramatic shifts and maturation at weaning and this time is a window of extensive education of the immune system, as previously described. In SPF and GF models, we used Abx-mediated alteration of the microbiota and selective colonization to identify bacterial populations that could prevent sensitization to food antigens and understand how they interact with the host to mediate protection.

Chapter 2: Methods

I. Mouse lines

All mice used in these experiments were bred and housed at the University of Chicago under a 12-hour light/dark cycle in autoclaved, positive-pressure cages with pine shaving bedding. C57BL/6, C57BL/6^{FOXP3GFP}, and *Rag*$^{-/-}$ mice were maintained under SPF *Helicobacter*-free, murine norovirus-free conditions. *Tlr4*$^{-/-}$ mice on the C57BL/6 background were maintained under SPF conditions. C57BL/6 *Tlr2*$^{-/-}$ mice were generously provided by Bana Jabri and were maintained under SPF conditions. All SPF experiments were performed with littermate controls. Knockout mice were produced by breeding a heterozygous mouse with a homozygous knockout mouse. SPF mice were fed an irradiated diet (Teklad 2918, Harlan/Envigo) and reverse osmosis water *ad libitum* except mice receiving Abx, which were given acidified water containing the Abx cocktail *ad libitum* instead. GF C57BL/6 mice were generously provided by Sarkis Mazmanian. GF *Tlr4*$^{-/-FOXP3GFP}$ mice were rederived by Kathy McCoy; GF C57BL/6^{FOXP3GFP} mice were produced from this line. All GF and gnotobiotic mice were maintained in flexible film isolators (Class Biologically Clean) and were fed an autoclaved diet (5K67, Lab Diets) and sterile water *ad libitum*. All experiments were performed in accordance with University of Chicago Animal Care and Use Protocols.

II. Purified PN extract preparation

Purified PN extract was prepared with modifications from (van Wijk et al., 2005). Dry roasted, unsalted peanuts were removed from their shells and were manually pulverized using a sterile mortar and pestle. Once peanuts reached a pasty consistency and released their oil, the mixture was transferred into a large Erlenmeyer flask and was combined with sterile 20mM Tris (pH 7.2) added at a 1:1 w:v ratio. Protein was extracted in Tris at room temperature with stirring

for 2 hr. Solution was then centrifuged at 4,000 rpm for 30 min. The aqueous fraction was collected, avoiding solids or fat, and sterile filtered through a 0.22μm filter. Protein content was assessed using NanoDrop spectrophotometry and sterile extract was frozen at -20˚C until use.

III. Sensitization with PN and assessment of PN-specific and total antibody responses

Mice were weaned at 3 weeks old and divided into cholera toxin (CT) only or PN+CT treatment groups. Within experiments, mice were littermates and across experiments, litters were related by direct maternal lineage. CT only animals received an intragastric (i.g.) gavage of 10μg CT in 150μL 20mM Tris buffer (pH 7.2) on days 0 and 2 after weaning and 15μg CT in 150μL 20mM Tris buffer on days 7, 14, 21, and 28 after weaning. PN+CT mice were given a gavage of 6mg purified PN extract plus 10μg CT in 150μL on days 0 and 2; CT was increased to 15μg for days 7, 14, 21, and 28. For GF and gnotobiotic mice, sensitization was performed following the same procedure, but CT was kept at 10μg per mouse throughout the entire sensitization. On day 35, all mice received 2 doses of 20mg purified PN extract 30 minutes apart. Core body temperature was measured before challenge and 30 minutes after the 2nd challenge using a rectal probe (Physitemp); change in temperature was calculated as T_{60}-T_0. Serum and fecal samples were collected 24 hours after challenge. Blood was obtained via heart puncture.

PN-specific IgE, IgG, and IgG1 were measured by coating ELISA plates (Immulon 2HB, ThermoScientific) with purified PN extract. Serum antibodies were detected using rat anti-mouse IgE-AP (23G3; Southern Biotech), goat anti-mouse IgG-HRP (Southern Biotech), or goat anti-mouse IgG1-HRP (Southern Biotech). Concentrations of PN-specific antibodies in experimental samples were calculated using a PN-specific standard purified from serum of sensitized mice (Bashir et al., 2004). For standard preparation, pooled serum was run over a

Protein G sepharose column (Protein G sepharose 4 Fast Flow, GE Healthcare) to deplete some IgG then flow-through was passed over a PN-coupled CNBr sepharose column (CNBr Sepharose 4B, GE Healthcare). Eluted antibodies were quantified using a total IgE, total IgG, or total IgG1 ELISA.

Total serum IgE was measured by capture ELISA. Plates were coated with purified rat anti-mouse IgE (clone R35-72, BD Pharmingen) and serum antibodies were detected with biotinylated rat anti-mouse IgE (clone R35-118, BD Pharmingen) and streptavidin-HRP (Invitrogen). Concentrations were calculated using a purified mouse IgE standard (BD Biosciences) for reference.

Samples for fecal IgA were prepared by homogenizing one fecal pellet in PBS+0.01%NaN_3 until the pellet was completely dissociated. Samples were then centrifuged at 15000 rpm for 15 minutes to remove debris and supernatants were transferred to a new tube for use in ELISA. Fecal and serum IgA were measured by capture ELISA using polyclonal goat anti-mouse IgA (Southern Biotech) to coat and polyclonal goat anti-mouse IgA-AP (Southern Biotech) to detect. Concentrations were calculated using a purified mouse IgA standard (BD Biosciences).

All ELISAs were coated and incubated overnight at 4˚C (Day 1). The next day (Day 2), plates were washed with PBS+0.05% Tween-20 and blocking was performed with 3% bovine serum albumin (BSA, Sigma) in PBS for a minimum of 2 hours at room temperature. After blocking, plates were washed again and samples were diluted in 1% BSA and added to plates. Samples were incubated on the assay plate overnight at 4˚C. On day 3, plates were washed and secondary antibody was diluted in 1% BSA in PBS and added to plates. Secondary antibody was incubated at room temperature for a minimum of 1 hour. If necessary, plates were washed again, streptavidin-HRP was diluted in 1% BSA in PBS, and incubated on plates for 1 hour minimum at room temperature. Plates were washed a final time and developed with

3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma) and stopped with 2N H_2SO_4 for HRP conjugates or *p*-Nitrophenylphosphate (pNPP, KPL Labs) for AP conjugates. Plates were read at 450nm (TMB) or 405nm (AP) on a SpectraMax plate reader (Molecular Devices) and analyzed using SoftMax Pro software (Molecular Devices).

IV. Abx treatment

The Abx cocktail was composed of five individual drugs: kanamycin (4mg/mL), gentamycin (0.35mg/mL), colistin (8500U/mL), metronidazole (2.15mg/mL), vancomycin (0.45mg/mL). All antibiotics were purchased from Sigma. This mixture was administered by i.g. gavage (100μL/mouse) beginning at 14 days old and continuing until weaning at 21 days of age (7 doses total). At weaning, this cocktail was diluted 50-fold in acidified drinking water, except for vancomycin which was maintained at 0.5g/L. Mice were allowed to drink the Abx mixture *ad libitum* and did not have access to any other source of water. Abx water was changed every 7-14 days. Mice were maintained on this Abx water for 7 to 35 days, as indicated in figure legends.

V. Isolation of bacterial populations and colonizations

For conventionalization, one fecal pellet and an equivalent amount of cecal contents were taken from an adult C57BL/6^{FOXP3GFP} mouse from our SPF facility and homogenized in 3mL PBS. The suspension was then administered by i.g. gavage to GF recipients (100μl/mouse).

Cecal contents from mice colonized with ASF were generously provided by Tatyana Golovkina (UChicago). This sample was used to create repository mice and subsequent colonizations were performed using feces from repository mice. One fecal pellet from a repository mouse was homogenized in 1mL sterile PBS. Contents were allowed to settle and recipient mice were given i.g. gavage of 100μl homogenate. Relative abundance of each strain

in ASF was measured by clone library analysis. Fecal samples were bead beaten with 0.1mm silicate zirconia beads in 1.4mL ASL buffer (Qiagen). DNA was then extracted from feces using QIAamp DNA Stool Mini Kit (Qiagen). After extraction, 16S rRNA was amplified by PCR with 8F and 1492R universal primers and Illustra PuReTaq Ready-to-go PCR beads (GE Healthcare). PCR product was purified with the MinElute PCR purification kit (Qiagen). Cloning was performed with TOPO-TA Cloning Kit for Sequencing (Invitrogen). PCR product was ligated into the TOPO vector, which was used to transform One Shot MAX Efficiency DH5α-T1 *E. coli*. Bacteria were plated on Kanamycin LB agar plates and incubated at 37˚C overnight. Plates were submitted the following day to the University of Chicago Functional Genomics core for colony picking and sequencing. The presence of all species was also measured in fecal samples by PCR using primers described in (Sarma-Rupavtarm et al., 2004).

Bacteroides uniformis was isolated by homogenizing an SPF C57BL/6^{FOXP3GFP} fecal pellet and plating homogenate on Neomycin Blood Agar plates (Remel). An individual colony was picked and replated repeatedly until only colonies of uniform morphology were present. At this time, a single colony was picked and 16S rRNA was amplified and sequenced. The results indicated >99% sequence identity to *B. uniformis* with no other contaminating species. Subsequent passages of pure *B. uniformis* were grown in Schaedler anaerobe broth (Oxoid). 10^6 CFU of *B. uniformis* in Schaedler broth was administered to a repository mouse by i.g. gavage. For subsequent colonizations, one fecal pellet from a repository mouse was homogenized in 1mL sterile PBS. Contents were allowed to settle and recipient mice were given i.g. gavage of 100µl homogenate.

The spore-forming Clostridia consortium was isolated from an SPF C57BL/6^{FOXP3GFP} fecal pellet and an equal amount of cecal material. This material was homogenized in prereduced PBS in an anaerobic chamber and chloroform was added to the suspension (3% vol/vol). The sample was further homogenized in the chloroform solution and incubated at 37˚C

for 1 hour. After this time, chloroform was removed by CO_2 percolation. The remaining solution was administered to GF mice, which acted as a repository. The resulting consortium was passaged by gavage of fecal homogenate from these repository mice to GF or antibiotic-treated SPF mice. For subsequent colonizations, one fecal pellet from a repository mouse was homogenized in 1mL sterile PBS. Contents were allowed to settle and recipient mice were given i.g. gavage of 100µl homogenate.

For heat-killed Clostridia, fecal pellets were collected from repository mice and homogenized in a sterile 50mL Falcon tube using sterile PBS in a biological safety cabinet (1 pellet/1mL PBS). The tube of homogenate was then vented, placed in an autoclave pouch, and autoclaved on a short dry cycle. Autoclaved Clostridia homogenate was administered to GF mice every other day throughout the experiment as indicated in the figure legend. These bacteria were administered repeatedly in order to mimic the amount of bacterial exposure in live colonized mice since autoclaved bacteria do not replicate. The volume of autoclaved homogenate administered was equivalent to the volume used to colonize a mouse with live Clostridia in the same experiment.

When Abx-treated mice were conventionalized or colonized with Clostridia, antibiotics were gavaged for 7 days, beginning at 14 days old. At weaning (21 days old), one day after the final Abx gavage, mice were colonized with homogenate made from fecal/cecal contents from an SPF C57BL/6^{FOXP3GFP} mouse or fecal homogenate from gnotobiotic Clostridia repositories as described. In sensitization experiments, a second colonization was performed 2 weeks post-weaning, one day before the day 14 sensitization.

For antibiotic recovery experiments, mice were treated with Abx by gavage from day 14 to day 21 after which time Abx administration ceased. Mice were placed on normal drinking water but no bacterial populations were introduced.

VI. 16S rRNA screening and sequencing

Fecal samples and ileal contents were homogenized with 0.1mm zirconia-silica beads (BioSpec) in 1.4mL ASL buffer (Qiagen) in a Mini-beadbeater (BioSpec) at top speed for 90 seconds. DNA was then extracted using the QIAamp DNA Stool Mini Kit (Qiagen), using the manufacturer's protocol. Two modifications were made to the protocol; samples were incubated with only ½ Inhibitex tablet for protease inhibition and DNA was eluted in 100µL Buffer AE.

To screen for colonization in GF or gnotobiotic mice, eluted fecal DNA was amplified by PCR using universal primers 8F and 1492R (James, 2010). PCR product was detected by gel electrophoresis. GF mice had no detectable band after PCR amplification; colonized mice had evidence of a 1.1kb product.

For sequencing of the intestinal bacterial community, the 515F/806R (V4) region of the 16S rRNA gene was then amplified from fecal or ileal DNA samples using the primers and method described in (Caporaso et al., 2011), modified for the Illumina MiSeq platform. This PCR reaction also incorporated the linker sequence, barcodes, and Illumina adaptors to the amplified V4 region of the 16S rRNA gene. PCR was performed in triplicate and pooled together for each sample. DNA concentration was measured using PicoGreen (Invitrogen) and concentrations were equalized across samples. Final products were purified using the UltraClean PCR Clean-up kit (MoBio). Sequencing was performed on the Illumina MiSeq at the Next-Generation Sequencing Core at Argonne National Laboratory. Resulting paired-end sequences were joined using fastq-join and analyses were performed using QIIME (version 1.6.0). For taxonomy-independent analyses, Uclust was used to select operational taxonomic units (OTUs; threshold was 97% sequence identity). Principal coordinates analyses (PCA) show unweighted pairwise distance for the UniFrac metric with even sampling depth. For taxonomy-dependent analyses, sequences were compared to the RDP Classifier database (Michigan State University).

VII. Bacterial load measurement

DNA was extracted from feces or ileal contents as for sequencing, using bead-beating and the QIAamp DNA Stool Mini Kit (Qiagen). After extraction, eluted DNA was diluted 10-fold and amplified using qRT-PCR with iQ Sybr Green Mix (BioRad) and universal primers 8F and 338R, described in (Buffie et al., 2012). Copies of 16S rRNA per sample were calculated from a standard curve made from the full-length 16S rRNA gene from a *Prophyromonadaceae* family member cloned into a pCR4TOPO-TA vector. Total copies 16S rRNA per sample was normalized to weight of fecal pellet or ileal contents.

VIII. IEC, LP lymphocytes, and RNA isolation

The colon and small intestine or ileum (as indicated in the figure legend) were removed, contents were collected, and tissue was flushed with PBS to clear residual debris. The small intestine was divided approximately in half, into proximal and distal sections but colon was treated as one sample. Tissue was then opened longitudinally and cut into ~1.5cm pieces. Tissue fragments were placed in 5mL of 5mM EDTA. Samples were incubated in EDTA with slow rotation (100 rpm) at 37˚C for 10 minutes then were vortexed vigorously (~15 seconds). This process was repeated a second time. The cells remaining in suspension after EDTA treatment were filtered through a 70μm filter (proximal and distal small intestine were combined at this step), washed with PBS, and further purified by centrifugation on a 40%:80% Percoll gradient (2500 rpm for 30 min, minimum acceleration and no brake). The interphase from the density gradient was collected, passed through a 40μm filter, and washed with fresh PBS. These cells were used as IECs.

For LP lymphocyte isolation, tissue was chopped into ~1mm^2 pieces after 2nd EDTA incubation/vortexing and was transferred into digestion solution containing 0.5mg/mL DNAse I (Sigma), 0.5mg/mL Collagenase D (Sigma), and 3mg/mL Dispase (Sigma) in RPMI or DMEM

plus 2% FCS. Samples were incubated for 15 minutes at 37˚C with slow rotation (100 rpm) and were then vortexed vigorously (~15 seconds). This was repeated 2-3 times until most tissue was dissociated. Samples were then filtered through a 70μm filter (proximal and distal small intestine were combined at this step) and washed with PBS. The resulting cells were further purified by centrifugation on a 40%:80% Percoll gradient (2500 rpm for 30 min, minimum acceleration and no brake). The interphase from this density gradient was collected, passed through a 40μm filter, and washed with fresh PBS. These cells were used as LP lymphocytes.

RNA was isolated from IEC or LP lymphocytes using the RNeasy Mini Kit (Qiagen) or Total RNA Isolation Kit (IBI). RNA was stored at -80˚C until use.

For extraction of RNA from whole tissue, the colon and small intestine tissue was collected in RNALater (Ambion) and left at 4˚C overnight then frozen at -80˚C until homogenization, according to manufacturer's instructions. When it was time for RNA extraction, tissue was thawed and homogenized mechanically in lysis buffer plus β-mercaptoethanol, as described in the manufacturer's protocol, using a hand-held tissue homogenizer. RNA was extracted from tissue homogenates with RNeasy Mini Kit or Total RNA Isolation Kit according to manufacturer's instructions. Extracted RNA was stored at -80˚C until use.

IX. qRT-PCR and primer tables

cDNA was made from RNA using the iScript cDNA synthesis kit (BioRad) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using the iQ SybrGreen mix (BioRad). All primers were used at 10μM (final concentration in reaction was 40nM). Primer sequences used are listed in the tables below, divided based the on housekeeping gene used for normalization (in bold):

Table 1: qRT-PCR primer sequences for genes normalized to *Hprt*

Gene	Primer Sequence	Source
Il22	F: 5'-TCC GAG GAG TCA GTG CTA AA-3' R: 5'-AGA ACG TCT TCC AGG GTG AA-3'	(Upadhyay et al., 2012)
Il17	F: 5'-CTC CAG AAG GCC CTC AGA CTA C-3' R: 5'-AGC TTT CCC TCC GCA TTG ACA CAG-3'	(Upadhyay et al., 2012)
Il23p19	F: 5'-GGT GGC TCA GGG AAA TGT-3' R: 5'-GAC AGA GCA GGC AGG TAC AG-3'	(Upadhyay et al., 2012)
Il1b	F: 5'-CAA CCA ACA AGT GAT ATT CTC CAT G-3' R: 5'-GAT CCA CAC TCT CCA GCT GCA-3'	(Iannello et al., 2013)
Il6	F: 5'-CTG CAA GAG ACT TCC ATC CAG TT-3' R: 5'-GAA GTA GGG AAG GCC GTG G-3'	(Iannello et al., 2013)
Il12p35	F: 5'-ACA GCA CCA GCT TCT TCA TCA G-3' R: 5'-TCT TCA AAG GCT TCA TCT GCA A-3'	(Kapil et al., 2009)
Il10	F: 5'-CAG GGC CCT TTG CTA TGG-3' R: 5'-GAT CTC CCT GGT TTC TCT TCC-3'	(Dube et al., 2012)
Reg3b	F: 5'-ATG GCT CCT ACT GCT ATG CC-3' R: 5'-GTG TCC TCC AGG CCT CTT T-3'	(Upadhyay et al., 2012)
Reg3g	F: 5'-ATG GCT CCT ATT GCT ATG CC-3' R: 5'-GAT GTC CTG AGG GCC TCT T-3'	(Upadhyay et al., 2012)
Hprt	F: 5'-TGA AGA GCT ACT GTA ATG ATC AGT CAA C-3' R: 5'- AGCAAGCTTGCAACCTTAACCA-3'	(Upadhyay et al., 2012)

Table 2: qRT-PCR primer sequences for genes normalized to *Gapdh*

Gene	Primer Sequence	Source
Gpr109a	F: 5'-ATG GCG AGG CAT ATC TGT GTA GCA-3' R: 5'-TCC TGC CTG AGC AGA ACA AGA TGA-3'	(Singh et al., 2014)
Gpr43	F: 5'-AAT TTC CTG GTG TGC TTT GG-3' R: 5'-ACC AGA CCA ACT TCT GGG TG-3'	(Sina et al., 2009)
Gapdh	F: 5'-AGG TCG GTG AAC GGA TTT G-3' R: 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'	(Singh et al., 2014)

Slc5a8 and *Slc16a1* expression were measured using Taqman probes/primers normalized to *Gapdh* probes/primers using Taqman Gene Expression Master Mix (Applied Biosystems).

All gene expression was measured on a Step One Plus RT-PCR system (Applied BioSystems). Samples were amplified for 40 cycles. Relative expression was analyzed using the ΔΔCt method with the gene of interest compared to expression of a housekeeping gene

(*Hprt* or *Gapdh* as indicated in primer table). Fold change in expression was normalized to the geometric mean of expression data from the negative control group in each experiment.

X. Treg and ILC flow cytometry

Spleens were extracted and mechanically dissociated through a 40μm filter, pelleted, subjected to red blood cell lysis with ACK buffer and washed, then made into a single cell suspension. Mesenteric lymph nodes were treated the same way, but did not undergo red blood cell lysis. Lymphocytes from the colonic LP were collected as described above for RNA isolation.

For analysis of Tregs, cells were stained with anti-CD4-PE (clone RM4.4, eBioscience) and intracellular Foxp3 using anti-Foxp3-FITC (clone FJK-16s, eBioscience) after fixation with the Foxp3 Fix/Perm buffer (eBioscience). In other cases, Foxp3 was detected in reporter mice expressing the Foxp3-IRES-GFP reporter.

ILC analysis was performed on lymphocytes from the colonic LP. Cells were restimulated with 50ng/mL PMA plus 500ng/mL ionomycin for 4 hours. Brefeldin A was added for the final 2 hours. After stimulation, cells were fixed and permeabilized with the Foxp3 Fix/Perm buffer (eBioscience). Samples were stained with anti-CD3, anti-CD4, anti-TCRβ, anti-NKp46, anti-RORγt, anti-IL-22, and anti-IL-17. All antibodies were purchased from eBioscience or BD Bioscience. Viability of cells was determined using the Live/Dead Violet Viability Kit (Invitrogen); only live cells were included in analysis.

All flow cytometry was performed on a FACSCanto or LSRII (BD Biosciences) and analysis was performed using FlowJo (TreeStar).

XI. T cell transfers

For isolation of Tregs or conventional T cells (Tcon) from the spleen and MLN of SPF C57BL/6^{FOXP3GFP} mice, single cell suspensions were made using mechanical homogenization

through a 40μm filter. Cells from each organ were pelleted and red blood cells were lysed with ACK buffer for spleen samples. Cells were washed again and then pooled together. CD4 was stained with anti-CD4-PE (clone RM4.4 or RM4.5, eBioscience). After staining, cells were washed and sorted sterilely (after bleach wash of instrument) based on PE and GFP expression on MoFlo XDP (Beckman Coulter) or FACSAria (BD Biosciences). Tregs were gated on $CD4^+Foxp3^+$ cells and Tcon were gated on $CD4^+Foxp3^-$. Both populations were sorted to ≥97% purity. After sorting, cells were pelleted and resuspended at $3x10^5$ cells/100μl. Recipient mice all received 100μl Abx by gavage daily for 7 days. Each mouse, at 21 days of age, received a 100μl intravenous (i.v.) injection of $3x10^5$ cells by the tail vein. Mice were then weaned onto Abx-containing water. PN+CT sensitization began one day later.

For isolation of Tregs or Tcon cells from the MLN and colonic LP of SPF $C57BL/6^{FOXP3GFP}$ mice, MLNs were treated as described above and colonic LP cells were isolated as for RNA and flow cytometric analysis. After Percoll gradient centrifugation, colonic LP cells were washed and pooled with MLN cells. CD4 staining was performed as described and cells were sorted as before. Cells were resuspended at $2.8x10^5$/100μl. Recipient mice all received 100μl Abx by gavage daily for 7 days. Each mouse received a 100μl i.v. injection of $2.8x10^5$ cells retroorbitally at weaning. Mice were weaned onto Abx-containing water. PN+CT sensitization began one day later.

For transfer of Tregs from the MLN and colonic LP of Clostridia-colonized mice, cells were collected from gnotobiotic $C57BL/6^{FOXP3GFP}$ 14 days after colonization with Clostridia following the same protocol as for SPF mice. Cells were resuspended at $2.8x10^5$/100μl. Recipient mice all received 100μl Abx by gavage daily for 7 days. Each mouse received a 100μl i.v. injection of $2.8x10^5$ cells retroorbitally at weaning. After Treg transfer, Abx treatment was ceased and mice were weaned on to normal water to ensure Tregs had appropriate antigen exposure for survival.

XII. Microarray preparation and analysis

Colonic IECs were isolated from GF, *B. uniformis*, or Clostridia colonized mice 6 days after colonization as described for RNA extraction using 5mM EDTA and Percoll gradient centrifugation. RNA was extracted with the RNeasy Mini Kit (Qiagen). IECs from three mice were pooled together to form one sample (biological replicate); each treatment was analyzed in technical duplicate or triplicate on the array (two to three independent experiments). Before submission, RNA was treated with DNA-*free* (Ambion) to remove contaminating genomic DNA. Samples were then run on an Illumina Mouse Ref-8 array at the University of Chicago Functional Genomics core. Data was normalized using Illumina software and further analyzed using dChip. Genes were considered upregulated if the *P* value of detection was >0.05 and the fold increase in *B. uniformis* or Clostridia samples was ≥1.5 compared to GF.

To verify top hits, cDNA was prepared from RNA samples used for microarray using the iScript cDNA synthesis kit (BioRad). Expression of *Reg3b* and *Reg3g* was measured by qRT-PCR using Taqman probes and master mix (Applied Biosystems) and was calculated relative to *Gapdh*. Data was normalized to GF.

XIII. Mucus staining

Tissue samples were collected from the distal colons of GF, *B. uniformis*, or Clostridia colonized mice 6 days after colonization or from PN-sensitized Abx-treated Clostridia-colonized mice treated with anti-IL-22 or isotype control 24 hours after challenge. Samples were fixed in Carnoy's fixative (60% pure EtOH, 30% chloroform, 10% glacial acetic acid) for 4 hours then transferred to 70% EtOH. For staining, tissue was embedded in paraffin and cut into 5μm sections. Samples were stained with Periodic Acid-Schiff (PAS) to visualize glycoproteins and glycolipids. Embedding, cutting, and staining were performed at the University of Chicago Human Tissue Resource Center.

Slides were visualized with the CRi Panoramic Scan Whole Slide Scanner and analyzed with Panoramic Viewer. Quantification of goblet cells was performed in an average of 11 crypts per mouse (minimum=7 crypts).

XIV. ELISA assay for detection of PN proteins in serum

Serum concentrations of the PN proteins Ara h 6 and Ara h 2 were measured 6 days after colonization in gnotobiotic mice or 6 days after weaning in SPF experiments. Mice were fasted overnight (Figures 24, 26, 28. 29, and 41) or for 3 hours plus a 200µl 0.2M $NaHCO_3$ gavage 30 minutes before PN gavage (Figures 35 and 47). At the beginning of the experiment, mice were bled from the tail vein or the superficial temporal vein ("pre" sample) and were then gavaged with 20mg of PN extract (±15µg CT, as indicated in figure legend). Subsequent bleeds occurred 15 minutes, 45 minutes, and 3 hours after gavage or 1 hour and 3 hours after gavage, as indicated. Blood was centrifuged for 10 minutes to collect serum samples for analysis.

To determine the amount of PN protein in systemic circulation, we adapted ELISA kits against Ara h 6 or Ara h 2 (Indoor Biotechnologies) for use with serum. In brief, plates were coated with a capture antibody against Ara h 6 (clone 3B8) or Ara h 2 (1C4) as per manufacturer's protocol and incubated overnight at 4˚C. The next day, plates were blocked with 1% normal mouse serum (NMS, Jackson ImmunoLabs) in PBS+0.5% Tween-20 for a minimum of 2 hours at room temperature and then washed. Experimental serum collected at the various timepoints post-gavage was diluted in 1% NMS in PBS-Tween and added to plates along with purified Ara h 6 or Ara h 2 protein as a standard (diluted according to manufacturer's protocol). Plates were incubated overnight at 4˚C. On the third day, samples were washed and probed with a secondary antibody against Ara h 6 (3E12-biotin) or Ara h 2 (polyclonal) diluted in 1% NMS in PBS-T (diluted according to manufacturer's protocol) for 1 hour minimum at room temperature. Plates were washed again then incubated with an HRP conjugate (streptavidin-

HRP for Ara h 6 [Invitrogen], goat-anti-rabbit-HRP for Ara h 2 [ThermoFisher]) diluted in 1% NMS in PBS-T for 1 hour minimum at room temperature. Plates were developed with TMB (Sigma), the reaction was stopped with 2N H_2SO_4, and plates were read at 450nm on SpectraMax plate reader (Molecular Devices) and analyzed with SoftMax Pro (Molecular Devices). Ara h 6 or Ara h 2 concentrations were calculated based on the standard curve.

XV. Neutralizing and depleting antibody treatments

Neutralizing antibody to IL-22 (clone 8E11) and isotype control (anti-GP120 clone 10E.1D2), described in (Zheng et al., 2008), were generously provided by Wenjun Ouyang (Genentech) under a Materials Transfer Agreement (MTA). 150µg of antibody was administered intraperitoneally (i.p.) 3 times per week, beginning at weaning, for one week (permeability experiment) or five weeks (sensitization experiment). For $Foxp3^+$ Treg analysis, mice were treated i.p. with one dose of 500µg anti-IL-22 (clone IL22JOP, eBioscience) after Abx treatment and Clostridia colonization.

IL-22 fusion protein (IL-22Fc) described in (Cox et al., 2012) was also provided by Wenjun Ouyang (Genentech) under MTA. 20µg IL-22Fc was given once i.p. at weaning (permeability experiment) with a boost given one week later (Treg analysis) or 20µg IL-22 was given i.p.one time per week for the first 3 weeks after weaning with a fourth dose given 3 days before challenge (sensitization experiment).

ILCs were depleted in *Rag*$^{-/-}$ mice using an antibody against CD90.2 (clone 30H12; BioXCell). 250µg anti-CD90.2 or isotype control (LFT-2; BioXCell) was administered i.p. every 3 days beginning 3 days before weaning and continuing until one week after weaning (permeability experiment).

XVI. Splenocyte culture and cytokine analysis

24 hours after challenge, spleens were harvested from PN-sensitized Abx-treated Clostridia-colonized mice treated with anti-IL-22 or isotype control. Spleens were mechanically dissociated and passed through a 40μm filter, subjected to red blood cell lysis in ACK buffer, and made into a single cell suspension. $2x10^5$ cells per well were then cultured in the presence of complete DMEM [10% FCS, 10 mM HEPES, 2 mM L-glutamine, and 1 mM sodium pyruvate (all HyClone), 100 U penicillin/mL, 100μg/mL streptomycin, 0.25μg/mL Fungizone (all purchased as 100x anti-anti, Gibco), 50μM β-mercaptoethanol, and 0.1 mM non-essential amino acids (both ThermoFisher)] media alone, 1μg/mL anti-CD3 (clone 2C11), or 200μg/mL purified peanut extract for 72 hours at 37˚C with 10% CO_2.

After 72 hours, plates were frozen at -20˚C. For cytokine analysis, plates were thawed, supernatants were harvested, and cytokine production was measured by Milliplex MAP mouse cytokine magnetic bead panel (Millipore), with modifications from the manufacturer's protocol. Magnetic beads were added at ½ of the manufacturer's recommended concentration. Samples were analyzed using a BioPlex machine (BioRad). Cytokine concentrations were calculated from standard curves prepared as described in manufacturer's protocol.

XVII. SCFA analysis

Fecal samples of ~100mg were collected from adult GF, conventionalized, or Clostridia colonized mice 14 days after colonization. Feces were homogenized in PBS and spiked with 100μL of internal standard mixture (0.5mM 4-methyl-valeric acid [Sigma], 5% metaphosphoric acid [to acidify sample and precipitate proteins], and 1.56mg/mL copper sulfate [to inhibit bacteria]). Feces were then centrifuged at 13,000 rpm for 10 min and the liquid fraction of the homogenate was used for gas chromatography. 10μL of supernatant was injected into an Agilent 6890A gas chromatograph (Agilent Technologies) and SCFAs were separated on a

fused silica capillary column (Nukol, Supelco). Identification response factors and flame ionizing detector response factors of acetate, propionate, and butyrate relative to 4-methyl valeric acid was calculated by injecting a volatile acid external standard mix (Supelco). Quantification of each SCFA was performed by measuring the peak area relative to 4-methyl valeric acid. The concentration of each SCFA was normalized to fecal weight.

XVIII. SCFA administration

Sodium butyrate (Sigma) was resuspended in sterile PBS, endotoxin-depleted using the Toxin Eraser kit (GeneScript), and then sterile filtered. It was administered at 20mg/day/mouse (182μmol/day/mouse) to GF WT or *Tlr4*$^{-/-}$ animals by i.g. gavage. Sodium acetate was prepared the same way but was administered at 44mg/day/mouse (540μmol/day/mouse), to maintain the relative ~3:1 acetate:butyrate ratio measured in human feces (Tan et al., 2014). Sodium butyrate or sodium acetate was administered once daily for 3 days (RNA isolation) or 4 days (intestinal permeability experiment).

XIX. Colon and ileum explant cultures

Protocol was adapted from (Zheng et al., 2008). Colons were removed from GF WT mice, contents were removed, then tissue was flushed with sterile PBS. After cleaning, tissue was opened longitudinally and placed in sterile HBSS containing 10μg/mL gentamycin, 100U/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL Fungizone, 10mM HEPES, and 50μM β-mercaptoethanol. Mucus was gently scraped off and tissue was sectioned into pieces ~50mg in size. These pieces were then transferred to 1.5mL eppendorf tubes containing 500μL HBSS, which had already been weighed. Tubes containing pieces of tissue were then weighed and tissue mass was calculated as (tube+HBSS+tissue)-(tube+HBSS). Ilea were treated in the same manner, but Peyer's patches were removed before the ileum was opened longitudinally.

After weighing, pieces were transferred into 1mL complete DMEM with or without an additional stimulus in a sterile 24 well tissue culture plate (Falcon or Corning). Recombinant mouse IL-23 (R&D Systems) was added at 10ng/mL, butyrate (Sigma) was added as a 5mM solution, α-cyano-4-hydroxycinnamic acid (CHC; Sigma) was added as a 1mM solution, and pertussis toxin (PTx) in glycerol (List Biologicals) was added at 100ng/mL. All solutions were resuspended in DMEM and sterile filtered prior to addition. Once in the medium, tissue was chopped into ~1mm^2 fragments. Plates were incubated for 24 hours at 37˚C with 10% CO_2. After 24 hours, supernatants were collected. IL-22 and IL-18 levels in supernatant were assayed by ELISA, following the manufacturer's instructions (IL-22: ELISA Max Deluxe, BioLegend; IL-18: Platinum ELISA, eBioscience).

XX. Control and fiber free diets

SPF C57BL/6^{FOXP3GFP} were fed a 5% cellulose diet (control; TD.10915) or a purified, low residue diet (fiber free; TD.00278) supplied by Harlan/Envigo. Diets were matched for nutritional content by a Harlan nutritionist. Beginning at weaning (21 days old), feeding of special diets was allowed *ad libitum* until the night before collection of serum for Ara h 6/2 ELISAs, when mice were fasted.

XXI. Statistical analysis

Statistical analysis was performed with GraphPad Prism 5. Student's *t*-test, one-way ANOVA with Tukey post-test, or two-way ANOVA with Bonferroni post-test was used for normally distributed data. Tests were chosen as appropriate to the number of comparisons to be made. For data that were not normally distributed, multiple comparisons were made using the Kruskall-Wallis test with Dunn's post-test was used instead of two-way ANOVA.

Chapter 3: Identification of allergy protective commensal bacteria

Work from this chapter was published in Stefka, A.T., Feehley, T., Tripathi, P., Qiu, J., McCoy, K., Mazmanian, S.K., Tjota, M.Y., Seo, G.Y., Cao, S., Theriault, B.R., *et al.* (2014). Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A *111*, 13145-13150.

I. Neonatal Abx treatment dramatically alters the fecal and ileal microbiota

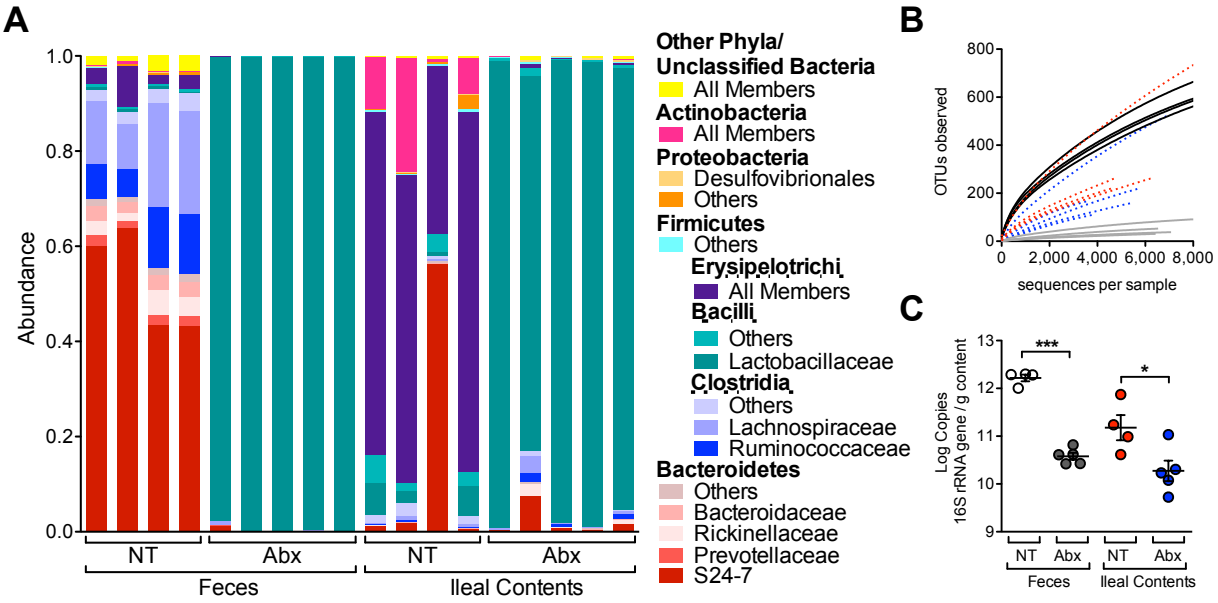


Figure 1: Neonatal Abx treatment dramatically alters the composition and load of the intestinal microbiota. Mice received no treatment (NT) or were treated with Abx beginning at 14 days of age by gavage until weaning and then in the drinking water for five weeks after weaning. After this time, fecal and ileal samples were collected for analysis. (**A**) Abundance of bacterial taxa from NT or Abx-treated mice in feces and ileal contents as determined by Illumina sequencing. (**B**) Rarefaction curves from samples in **A**. NT feces=black, Abx feces=gray, NT ileum=red, Abx ileum=blue. (**C**) Bacterial load in feces and ileal contents from mice in **A**. *n*=4-5 mice per group. Each bar/line/symbol represents an individual mouse. In **C**, bars represent mean±S.E.M. * $P<0.05$, *** $P<0.001$ determined by one-way ANOVA with Tukey post-test.

To begin to address whether the commensal microbiota regulates responses to food antigens, we treated mice with a cocktail of broad-spectrum Abx beginning in the neonatal period and analyzed the subsequent changes in the bacterial community. Abx (kanamycin, gentamicin, colistin, metronidazole, and vancomycin) were administered starting at 14 days of

age by i.g. gavage until weaning at 21 days old. At weaning, Abx were diluted in the drinking water 50-fold except for vancomycin, which remained at the gavage dose. Administration in the drinking water was maintained for 5 weeks, at which time we collected fecal samples and ileal contents from Abx-treated mice or NT controls born to mothers from the same maternal lineage to control for microbiota inheritance (littermate controls could not be used because litters cannot be split for Abx treatment pre-weaning). We extracted DNA from these samples and performed Illumina sequencing on the 16S rRNA gene. This gene is highly conserved among all bacteria but contains hypervariable regions that are unique to individual bacterial taxa. Using sequences amplified from the V4 hypervariable region, we assigned taxonomic classifications to the sequencing reads based on similarity to the RDP classifier database of known 16S sequences and determined the relative abundance of different families in NT and Abx-treated mice.

As expected, the feces of NT mice was dominated by two major phyla: Bacteroidetes and Firmicutes (Figure 1A). At the family level, there was high abundance of S24-7 (Bacteroidetes) and Lachnospiraceae (Firmicutes). This result was similar in the ileum, however there was increased abundance of Erysipelotrichi family members from the Firmicutes phylum and the appearance of a third phylum, Actinobacteria. In contrast, Abx-treated mice had minimal representation of Bacteroidetes and Firmicutes and were instead dominated in both the feces and ileum by a single family, Lactobacillaceae (Figure 1A). Lactobacilli are resistant to the Abx cocktail we used, particularly vancomycin, which lead to this over-representation. We also analyzed the Abx-induced changes in a taxonomy-independent manner by measuring OTUs observed in the feces and ileal contents of NT or Abx-treated mice as a measure of species richness. An OTU was defined as 97% sequence identity between reads. Using this strategy, we created rarefaction curves to count the number of OTUs detected among a given number of sequences. Abx-treated fecal and ileal samples had many fewer detectable OTUs than NT samples, indicating a decrease in richness (Figure 1B).

Finally, we determined if Abx-treatment reduced the number of bacteria present. Using qRT-PCR, we found that Abx-treated mice had significant lower bacterial loads in both sites than untreated mice (Figure 1C). In the feces, there was a reduction of nearly two logs. While this is a dramatic change, there were still many bacteria present, emphasizing that long-term administration of Abx does not sterilize the intestines despite many such assertions in the literature. Overall, however, we confirmed that our neonatal Abx treatment protocol had a profound effect on the number and type of bacteria present in the small intestine and colon.

II. Neonatal Abx treatment increases food allergen sensitization and reduces colonic Treg proportions and fecal IgA levels

Next, we wanted to determine if these alterations to the microbiota after Abx treatment affected responses to food allergens. We sensitized NT or Abx-treated mice by i.g. administration of PN extract plus the mucosal, Th2-skewing adjuvant CT (PN+CT) or CT alone (CT only) for 5 weeks beginning at weaning and compared the PN-specific IgE and IgG1 responses.

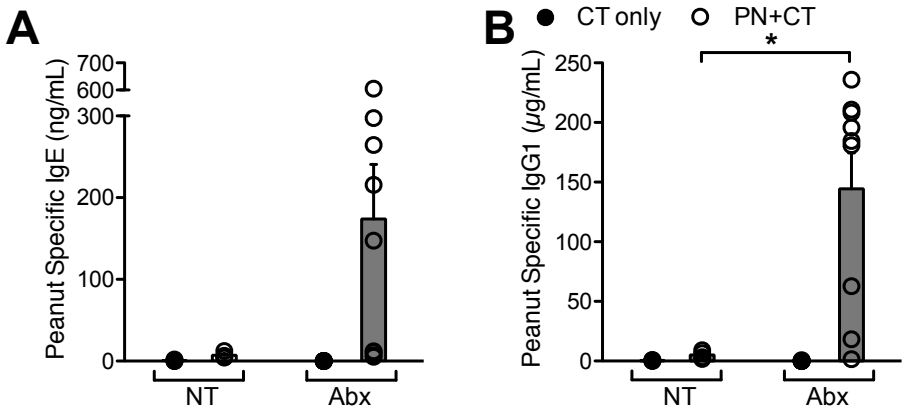


Figure 2: Neonatal Abx treatment increases PN-specific responses after sensitization. NT or Abx-treated mice were sensitized with PN+CT or CT only, beginning at weaning. After 5 weeks of sensitization, mice were challenged on day 35 with 2 doses of PN alone. Serum was collected 24 hours after challenge and (**A**) PN-specific IgE and (**B**) PN-specific IgG1 were determined by ELISA. *n*=4-9 mice per group, pooled from multiple independent experiments. Each dot represents an individual mouse; bars represent mean+S.E.M. * *P*<0.05 determined by Student's *t*-test.

NT mice had a minimal response to sensitization, as evidenced by the low concentrations of PN-specific IgE and IgG1 in their serum after challenge (Figure 2A, B). There was also no PN-specific response detected in NT or Abx-treated mice that received CT alone. In contrast, Abx-treated mice sensitized with PN+CT had high levels of both PN-specific IgE and IgG1.

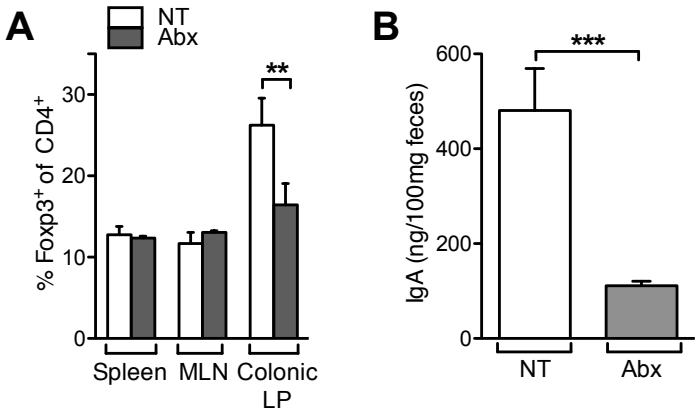


Figure 3: Abx treatment reduces colonic Foxp3$^+$ Tregs and fecal IgA production. (**A**) Proportion of Foxp3$^+$ cells among CD4$^+$ cells from spleen, MLN, and colonic LP in NT mice or mice that received Abx as in Figure 1. *n*=5 mice per group, representative of multiple experiments. (**B**) Concentration of IgA in the feces of sensitized mice from Figure 2. *n*=4-9 mice per group, representative of multiple experiments. Bars represent mean+S.E.M. ** *P*<0.01, *** *P*<0.001 by two-way ANOVA with Bonferroni post-test (**A**) or Student's *t*-test (**B**).

Since Abx treatment changed the response to sensitization and increased PN-specific antibody production, we hypothesized that there could be underlying alterations in immunoregulatory mechanisms caused by the changes in the microbiota. Because of the extensive literature about microbiota-mediated regulation of Foxp3$^+$ Tregs and IgA production, we began by looking at the effect of Abx administration on these responses. We analyzed the CD4$^+$Foxp3$^+$ Treg compartment of the spleen, MLN, and colonic LP in order to determine if Abx treatment altered Treg proportions in peripheral sites or the local mucosal environment. In mice that received no treatment, there was a baseline proportion of about 10% Foxp3$^+$ Tregs among all CD4$^+$ cells in the spleen and MLN. In the colonic LP, however, there was an enrichment of Foxp3$^+$ cells (Figure 3A), most of which are thought to be peripherally-induced and bacteria-specific (Lathrop et al., 2011). Abx-treated mice had a similar Treg profile in their lymphoid

organs as compared to untreated counterparts, in agreement with other reports that most of these Tregs are thymically-derived and not affected by the microbiota. In the colonic LP, however, there was a significant reduction in the proportion of $Foxp3^+$ cells; the average was very close to the 10% baseline seen in the other sites, suggesting that this Abx treatment eliminated most of the bacterially-induced Tregs. As with colonic Tregs, there was significantly reduced IgA in the feces of mice treated with Abx (Figure 3B). Although neither of these are food antigen-specific mechanisms of immunoregulation, these changes in intestinal immunological homeostasis may be one factor that contributed to increased PN-specific responses after Abx administration.

III. *Tlr4*$^{-/-}$ have increased PN-specific responses and reduced Treg proportions compared to *Tlr4*$^{+/-}$ littermates; *Tlr2*$^{-/-}$ mice do not have a defect

One of the first pieces of evidence to support the hypothesis that the microbiota regulates sensitization to food antigens came from the observation that C3H/HeJ mice, which have a point mutation in the gene encoding TLR4 (an innate immune sensor for lipopolysaccharide from Gram-negative bacteria) that ablates signaling, have increased allergen-specific responses to sensitization (Bashir et al., 2004). Given our results in Abx-treated C57BL/6 mice, we wanted to understand if an inability to sense certain members of the microbiota had the same effect on sensitization. We used *Tlr4*$^{-/-}$ mice on the C57BL/6 background to better understand how a genetic deficiency in sensing of the microbiota affected food allergen sensitization.

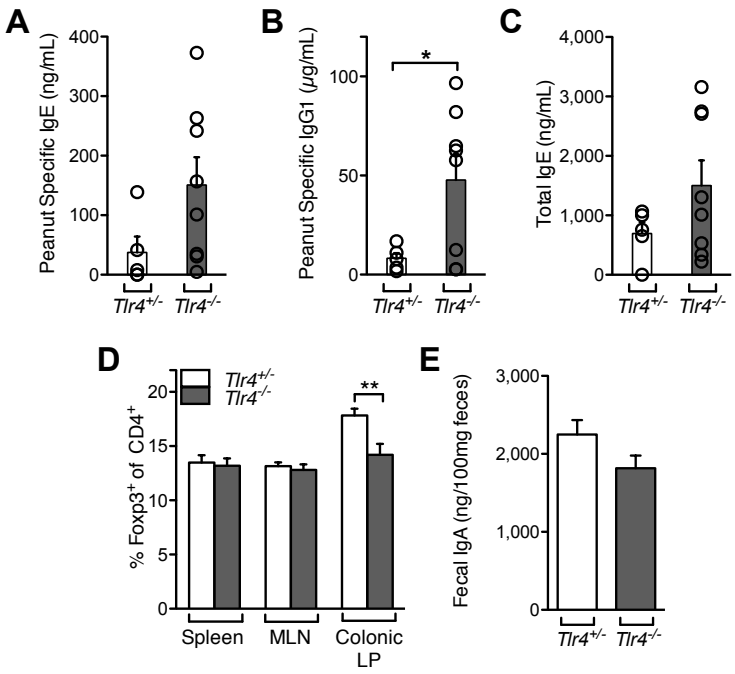


Figure 4: *Tlr4*$^{-/-}$ mice have increased PN-specific responses and reduced colonic Tregs compared to *Tlr4*$^{+/-}$ littermates. *Tlr4*$^{+/-}$ and *Tlr4*$^{-/-}$ littermates were sensitized with PN+CT for 5 weeks, beginning at weaning. Mice were challenged on day 35 with two doses of PN; serum and feces were collected 24 hours later. (**A**) PN-specific IgE (**B**) PN-specific IgG1 and (**C**) total IgE concentrations in serum as determined by ELISA. *n* =5-8 mice per group, pooled from two independent experiments. In **A-C**, each dot represents one mouse; bars represent mean+S.E.M. (**D**) Proportion of Foxp3^{+} cells among CD4^{+} cells in spleen, MLN, and colonic LP of unsensitized *Tlr4*$^{+/-}$ and *Tlr4*$^{-/-}$ littermates. *n*=3-4 mice per group, representative of multiple experiments. (**E**) Fecal IgA concentrations from mice in **A-C**. In **D** and **E**, bars represent mean+S.E.M. * $P<0.05$, ** $P<0.01$ by Student's *t*-test (**B**) or two-way ANOVA with Bonferroni post-test (**D**).

SPF *Tlr4*$^{-/-}$ mice and *Tlr4*$^{+/-}$ littermates were sensitized with PN+CT for 5 weeks and PN-specific IgE and IgG1 responses were assessed after challenge (Figure 4A, B). Although not as stark as the difference in responses between NT and Abx-treated mice, *Tlr4*$^{-/-}$ had increased concentrations of PN-specific antibodies in their serum after challenge compared to TLR4-sufficient littermates. TLR4-deficient animals also had increased levels of total IgE (Figure 4C). As with Abx-treated mice, this increased susceptibility to sensitization correlated with reduced proportions of Tregs in the colonic LP, indicating that an inability to sense a portion of the microbiota altered T cell homeostasis in the colon (Figure 4D). Fecal IgA levels were not different between *Tlr4*$^{+/-}$ and *Tlr4*$^{-/-}$ littermates (Figure 4E).

Not all commensal microbes signal through TLR4, however, so we wanted to understand whether this increased response to sensitization is due to a general inability to sense a portion of the bacteria in the microbiota or if it was due to a unique inability to sense bacteria possessing TLR4 ligands. In order to address this question, we performed the same series of experiments in SPF mice deficient in TLR2 on the C57BL/6 background. TLR2 is the receptor for many Gram-positive bacterial ligands including peptidoglycan and lipoteichoic acid, making it a functional counterpart to TLR4.

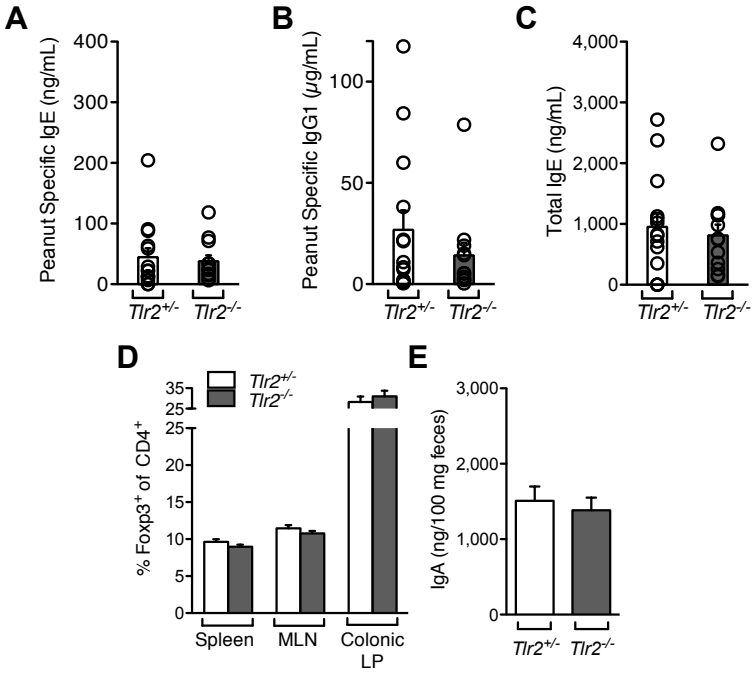


Figure 5: *Tlr2*$^{-/-}$ mice do not exhibit increased susceptibility to sensitization compared to *Tlr2*$^{+/-}$ littermates. *Tlr2*$^{+/-}$ and *Tlr2*$^{-/-}$ littermates were sensitized with PN+CT for 5 weeks, beginning at weaning. Mice were challenged on day 35 with two doses of PN; serum and feces were collected 24 hours later. (**A**) PN-specific IgE, (**B**) PN-specific IgG1, and (**C**) total IgE concentrations in serum as determined by ELISA. *n*=12-14 mice per group, pooled from three independent experiments. In **A-C**, each dot represents one mouse; bars represent mean+S.E.M. (**D**) Proportion of Foxp3^{+} cells among CD4^{+} cells in spleen, MLN, and colonic LP of unsensitized *Tlr2*$^{+/-}$ and *Tlr2*$^{-/-}$ littermates. *n*=5 mice per group, representative of multiple experiments. (**E**) Fecal IgA concentrations from mice in **A-C**. . In **D** and **E**, bars represent mean+S.E.M.

After sensitization, there was no difference in PN-specific IgE or IgG1 concentrations in the serum of *Tlr2*$^{+/-}$ and *Tlr2*$^{-/-}$ littermates; both groups had very low PN-specific antibody concentrations (Figure 5 A, B). There were also no changes in total IgE (Figure 5C). The proportions of Foxp3^{+} Tregs in spleen, MLN, and colonic LP were all normal in *Tlr2*$^{-/-}$ mice and

fecal IgA levels were equivalent between the groups (Figure 5D, E). This experiment indicated that allergic sensitization was not a consequence of an inability to sense the microbiota but was related to a specific inability to sense the microbiota via TLR4. The bacteria providing this protective signal, however, remained unclear.

IV. GF mice lacking a commensal microbiota have inherent Th2 skewing and are highly susceptible to food allergen sensitization

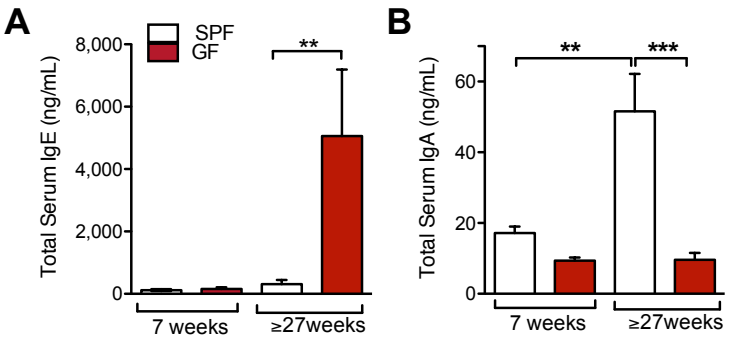


Figure 6: GF mice have dysregulated IgE and IgA production over time. (**A**) Total IgE and (**B**) total IgA concentrations in the serum of young or old SPF and GF mice as determined by ELISA. *n*=7-8 mice per group. Bars represent mean+S.E.M. ** *P*<0.01, *** *P*<0.001 by one-way ANOVA with Tukey post-test.

In order to identify which members of the microbiota may be providing protection against sensitization, we turned to a gnotobiotic ("known biota") mouse model. We began by examining baseline changes in Th2 responses in GF mice, which are born and raised in the absence of bacterial colonization and live with minimal exposure to bacterial products. In keeping with other reports in the literature (Cahenzli et al., 2013; McCoy et al., 2006), GF mice spontaneously developed high levels of IgE in their serum compared to either young GF mice or age-matched SPF mice (Figure 6A). Older GF mice also had significantly less IgA in their serum than SPF mice (Figure 6B).

Given this spontaneous Th2 bias, we predicted that these GF mice would produce very high levels of PN-specific antibodies after sensitization. GF mice were sensitized with PN+CT beginning at weaning as with SPF animals. After challenge, GF CT only-treated mice had no

increase in PN-specific antibodies, confirming that even in the absence of a microbiota, the response to sensitization was highly specific. PN+CT-treated GF mice had more PN-specific IgE and significantly more PN-specific IgG1 and total IgE in their serum than SPF counterparts (Figure 7A-C). This response was also higher than that seen in Abx-treated mice. Since the complete absence of a microbiota lead to a strong response to sensitization, these GF mice provided a model where we could test the effect of colonization with different bacterial populations in isolation in order to identify a species or group of bacteria that blocked the generation of a PN-specific response.

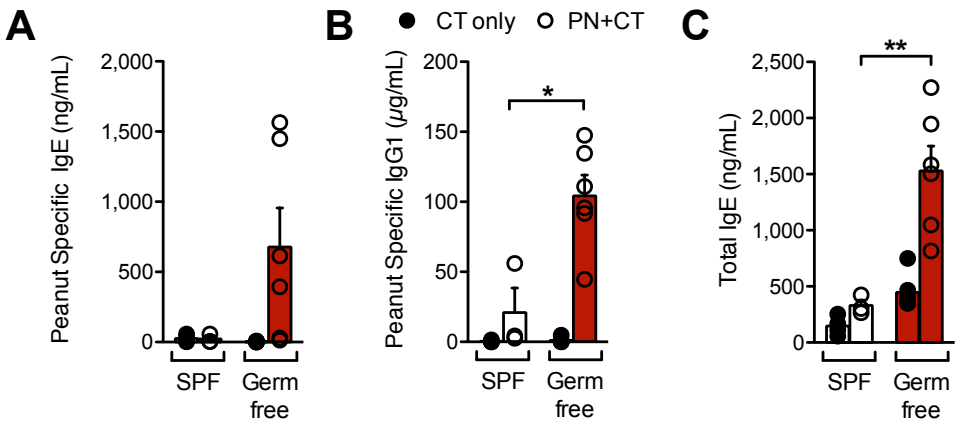


Figure 7: GF mice are much more susceptible to food allergen sensitization than SPF counterparts. SPF and GF WT mice were sensitized with PN+CT or CT only weekly beginning at weaning. After 5 weeks, mice were challenged with two doses of PN alone and serum was collected 24 hours later for antibody analysis. (**A**) PN-specific IgE, (**B**) PN-specific IgG1, and (**C**) total IgE concentrations determined by ELISA. *n*=3-6 mice per group, representative of multiple experiments. Dots represent individual mice, bars represent mean+S.E.M. * *P*<0.05, ** *P*<0.01 by Student's *t*-test.

V. Conventionalization restores GF WT mice to an SPF WT phenotype

GF mice have several documented morphological and immunological defects as a result of their microbiota-free maturation (Smith et al., 2007). One of the most obvious physical defects is enlargement of the cecum compared to SPF mice caused by increased mucus buildup and an impaired ability to digest dietary fiber. Our GF colony presented this characteristic enlargement

(Figure 8A). The immunological deficiencies are related to the bacteria-stimulated processes discussed in the introduction, including peripheral differentiation of T cells and production of secretory IgA. In our hands, GF mice had a reduced proportion of Tregs in the colonic LP (~10% baseline) and reduced IgA concentrations in the feces (Figure 8C, D), similar to the phenotype seen after Abx administration.

In order to confirm that restoring the microbiota corrects these defects, we colonized GF WT mice with cecal and fecal material from an SPF WT donor. We called mice in this proof of concept experiment "conventionalized." Conventionalization restored the gross intestinal morphology as well as the bacterial load to the level seen in SPF animals (Figure 8A, B). In the colonic LP, the proportion of $Foxp3^+$ Tregs was restored 14 days after colonization and fecal IgA levels were also the same as those seen in SPF mice raised in the barrier facility and colonized at birth (Figure 8C, D). The response to sensitization in conventionalized mice also phenocopied SPF mice. PN-specific IgE, IgG1, and total IgE levels were nearly identical to SPF controls and were significantly lower than in GF mice (Figure 8E-G). At challenge, GF mice had a drop in core body temperature indicative of an anaphylactic response. There was no drop in core body temperature in SPF or conventionalized mice, indicating that there was no systemic response to PN challenge (Figure 8H). Together, these results confirm that the microbiota of SPF mice was sufficient to reduce PN-specific responses in GF mice.

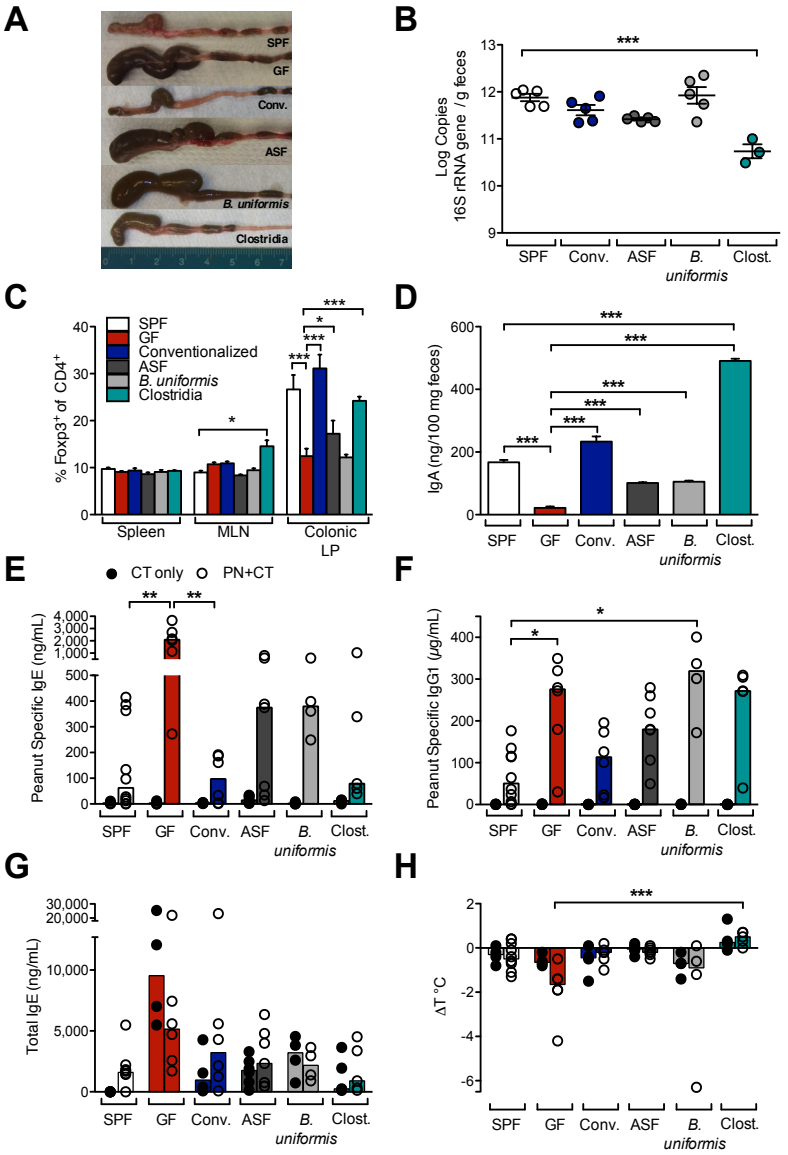


Figure 8: Colonization of GF mice with a consortium of spore-forming Clostridia prevents the production of PN-specific IgE. (**A**) Representative images of cecal morphology from SPF, GF, or gnotobiotic mice 13 days post-colonization. (**B**) Bacterial load in SPF or gnotobiotic mice 14 days post-colonization. *n*=3-5 mice per group. (**C**) Proportion of Foxp3$^+$ cells among CD4$^+$ cells in spleen, MLN, or colonic LP of SPF, GF, or gnotobiotic mice 14 days post-colonization. *n*=4-8 mice per group. (**D-H**) SPF, GF, or gnotobiotic mice were sensitized at weaning with PN+CT or CT only. After 5 weeks, mice were challenged with 2 doses of PN and serum and fecal samples were collected 24 hours later. (**D**) Concentration of IgA in the feces, (**E**) PN-specific IgE, (**F**) PN-specific IgG1, and (**G**) total IgE concentrations in the serum and (**H**) change in core body temperature, measured before and 60 minutes after challenge. *n*=4-10 mice per group, representative of multiple experiments. In **B-D**, bars represent mean+S.E.M. In **E-H**, each dot represents an individual mouse; bars represent median. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 by one way ANOVA with Tukey post-test (**B**), two-way ANOVA with Bonferroni post-test (**C, D**), or Kruskall-Wallis test with Dunn's post-test (**E-H**).

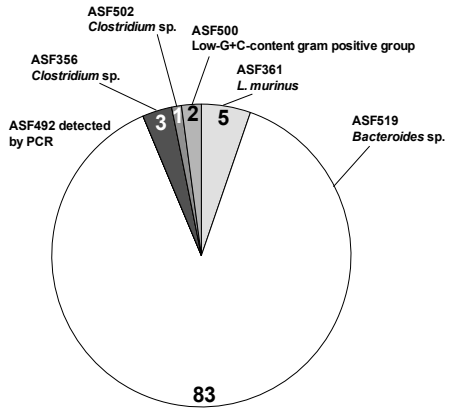
VI. Colonization with ASF is partially protective against sensitization and stimulates intermediate induction of Tregs and IgA

Although conventionalization was an important confirmation that transfer of the SPF microbiota to GF mice would reduce the response to sensitization, it did not provide any indication of which bacteria from the SPF microbiota were providing protection. To narrow down the protective populations, we colonized GF WT mice with simplified populations of bacteria to determine which, if any, were sufficient to rescue the morphological and immunological defects in the GF mice and prevent sensitization to PN. The first colonization we tried was ASF. This consortium of 8 bacteria, with members from both the Bacteroidetes and Firmicutes phyla, has been used for several decades as a minimal microbiota required to support normal immune system development and metabolic health in rodents (Dewhirst et al., 1999; Schaedler et al., 1965). Given its extensive history as a protective microbiota, including its capacity to induce $Foxp3^+$ Tregs (Geuking et al., 2011) and to block spontaneous IgE generation (Cahenzli et al., 2013), we hypothesized that it may be sufficient to reduce PN-specific responses after sensitization. We checked for the presence of all 8 bacteria in the feces of colonized mice using both clone library analysis and PCR. Only 6 of the 8 species were reliably detectable by PCR in fecal samples collected from colonized mice (Figure 9A-C).

A

Strain Identity	Species or group	Phylum
ASF356	Clostridium sp.	Firmicutes
ASF360	Lactobacillus sp.	Firmicutes
ASF361	Lactobacillus murinus	Firmicutes
ASF457	Flexistipes group	Deferribacteres
ASF492	Eubacterium plexicaudatum	Firmicutes
ASF500	Low-G+C-content gram positive group	Firmicutes
ASF502	Clostridium sp.	Firmicutes
ASF519	Bacteroides sp.	Bacteroidetes

B



C

Tag#	ASF 356	ASF 492	ASF 500	ASF 502	ASF 457	ASF 360	ASF 361	ASF 519
	Clostridium sp.	E. plexicaudatum	Clostridium sp.	Clostridium sp.	Flexistipes	Lactobacillus sp.	Lactobacillus sp.	Bacteroides sp.
1439	+	+	+	+	-	-	+(faint)	+
1440	+	+	+	+	-	-	+	+
1441	+	+	+	+	-	-	+	+
1442	+	+	+	+	-	-	+	+
1443	+	+	+	+	-	-	+	+
1444	+	+	+	+	-	-	+	+
1445	-	+	+	+	-	-	+	+
1447	+	+	+	+	-	-	+	+
1448	+(faint)	+	+	+	-	-	+	+
1449	+	+	+	+	-	-	+	+
1450	+	+	+	+	-	-	+	+
1451	+	+	+	+	-	-	+	+
1452	+	+(faint)	+	+	-	-	+	+

Figure 9: 6 members of the ASF consortium are detectable in feces after colonization. (**A**) Identity of the 8 members of ASF described in (Sarma-Rupavtarm et al., 2004) and (Dewhirst et al., 1999). (**B**) Number of clones belonging to each strain of ASF detected in feces of mice from Figure 8**C** 14 days after colonization, determined by clone library analysis. 94 clones were sequenced and are representative of 5 mice. (**C**) Identification of strains in fecal samples of ASF colonized mice by PCR using primers described in (Sarma-Rupavtarm et al., 2004).

When we examined the morphology of the cecum of ASF-colonized mice 13 days after colonization, there was still marked enlargement compared to SPF mice despite the bacterial load being comparable (Figure 8A, B). 14 days after colonization, the proportion of $Foxp3^+$ Tregs in the colonic LP was intermediate between SPF and GF mice as was fecal IgA (Figure 8C, D). This suggested that the members of ASF have some potential to stimulate an immunoregulatory response as described (Geuking et al., 2011) but that this consortium does not comprise all of the Treg/IgA-inducing capacity contained in the SPF microbiota. After sensitization with PN+CT, ASF-colonized mice had an intermediate response that correlated

with the intermediate Treg/IgA levels. PN-specific IgE and IgG1 were elevated compared to SPF or conventionalized samples but were not as high as GF (Figure 8E, F). Total IgE levels were similar to SPF mice and there was no change in core body temperature after challenge, indicating that ASF colonization did minimize some of the Th2 skewing inherent in GF mice (Figure 8G, H).

VII. Colonization with *Bacteroides uniformis* is not sufficient to reduce sensitization but colonization with a consortium of Clostridia is protective

Since both the conventionalized and ASF communities contained members from the two dominant bacterial phyla of the intestine, we also wanted to test the effects of giving back representative Bacteroidetes and Firmicutes independently. We were able to culture *Bacteroides uniformis* from the feces of our SPF mice by plating fecal homogenate on various selective media to passage and propagate a single isolate. We used a pure culture of this isolate to inoculate GF mice and study the effects. As with ASF, colonization with *B. uniformis* did not reduce cecal size despite reaching a comparable bacterial load to that of SPF mice (Figure 8A, B). There was no increase in the proportion of $Foxp3^+$ Tregs in the colonic LP and, although there was some increase in fecal IgA compared to GF mice, it was not restored to the level of SPF mice (Figure 8C, D). *B. uniformis*-colonized mice sensitized with PN+CT had PN-specific and total IgE concentrations that were similar to ASF-colonized mice, intermediate between SPF and GF mice (Figure 8E, G). PN-specific IgG1 concentrations were significantly higher than SPF mice (Figure 8F). *B. uniformis*-colonized mice also had a decrease in core body temperature after challenge, similar to GF mice, indicative of a systemic response to challenge (Figure 8H). Therefore, we concluded that monocolonization with this representative Bacteroidetes strain was not sufficient to protect against allergic sensitization.

A large proportion of species in the Firmicutes phylum are strict anaerobes and are difficult to propagate by culture-based mechanisms. Many, however, are spore formers, making them resistant to extreme stressors including chemicals. To isolate representative Firmicutes, we collected the spore-forming fraction of the microbiota of our SPF colony via chloroform extraction and used this extract to colonize GF repositories. All subsequent colonizations were performed using fecal homogenate from these repositories to ensure faithful passaging. After colonization with these spore-forming bacteria, we sequenced the 16S rRNA from the feces of repository mice to determine the identity of the chloroform-extracted population. Based on taxonomic classification, this consortium was composed of greater than 99% Firmicutes and was predominantly comprised of members of the Clostridia class from the Ruminococcaceae and Lachnospiraceae families (Figure 10A). Although we could not determine genus and species names from this sequence data because most of the members of these families remain largely unclassified, we could use phylogenetic analysis to determine that most of the bacteria in our consortium fell into *Clostridium* clusters IV, XIVa, and XIVb (Figure 10B). The consortium was very stable over time, with the most abundant OTUs remaining dominant from initial sampling until more than a year after extraction (Figure 10C).

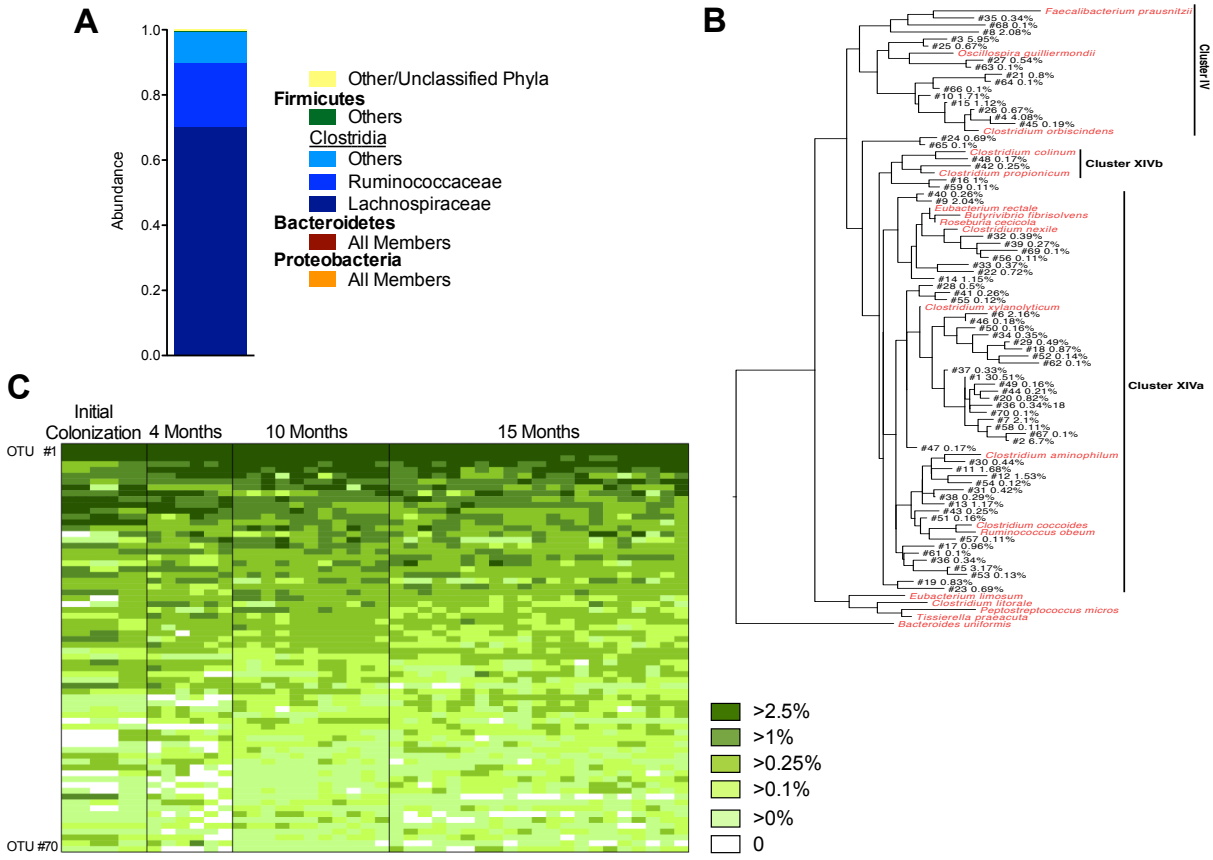


Figure 10: Chloroform-extracted bacteria are members of the Clostridia family. (**A**) Abundance of bacterial taxa based on 16S rRNA sequencing of feces from 41 repository mice colonized with chloroform-extracted consortium over 15 months. (**B**) Phylogenetic relationships between OTUs represented in **A**. OTUs were defined as 97% sequence identity; OTUs with abundance >0.1% were included in analysis. Red text signifies representative species (Pynast). (**C**) Representation of abundance of OTUs in **B** over time. Each column represents an individual mouse.

Colonization of GF mice with this consortium of Clostridia was able to reduce cecal size to that of SPF mice despite colonizing to a lower total load than the other bacterial populations tested (Figure 8A, B). Clostridia colonization also fully restored the Foxp3^{+} Treg compartment of the colonic LP after 2 weeks and even significantly increased the proportion of Tregs in the MLN (Figure 8C). There was also striking production of IgA in response to Clostridia; fecal IgA levels in Clostridia-colonized mice were significantly higher than all other groups (Figure 8D). In agreement with this restoration to an SPF-like state, both morphologically and immunologically,

Clostridia colonization reduced the production of PN-specific and total IgE after colonization to the levels seen in SPF and conventionalized mice (Figure 8E, G). There was no change in core body temperature seen at challenge; PN-sensitized Clostridia-colonized mice had a significantly lower change in body temperature than GF mice (Figure 8H). From these results, we concluded that Clostridia were sufficient to prevent food allergen sensitization, making them the major allergy-protective fraction of the microbiota.

VIII. GF *Tlr4*$^{-/-}$ mice are equally susceptible to sensitization as their GF WT counterparts but are not protected by Clostridia colonization

Given the increased allergic susceptibility seen in SPF *Tlr4*$^{-/-}$ animals, we wanted to know whether or not the lack of TLR4 further exacerbated responses in the absence of the microbiota. After being rederived GF, *Tlr4*$^{-/-}$ mice had a Foxp3^{+} Treg and fecal IgA profile that mirrored GF WT mice; both colonic LP Tregs and fecal IgA were at baseline levels (Figure 11A, B). When GF *Tlr4*$^{-/-}$ mice were sensitized with PN+CT, the PN-specific antibody profiles after challenge were also very similar to GF WT mice. Both groups had very high concentrations of PN-specific IgE and IgG1 in their serum, confirming that the increased response to sensitization in SPF *Tlr4*$^{-/-}$ mice was not due to the genetic defect itself, but was caused by an inability to sense a portion of the microbiota (Figure 11C).

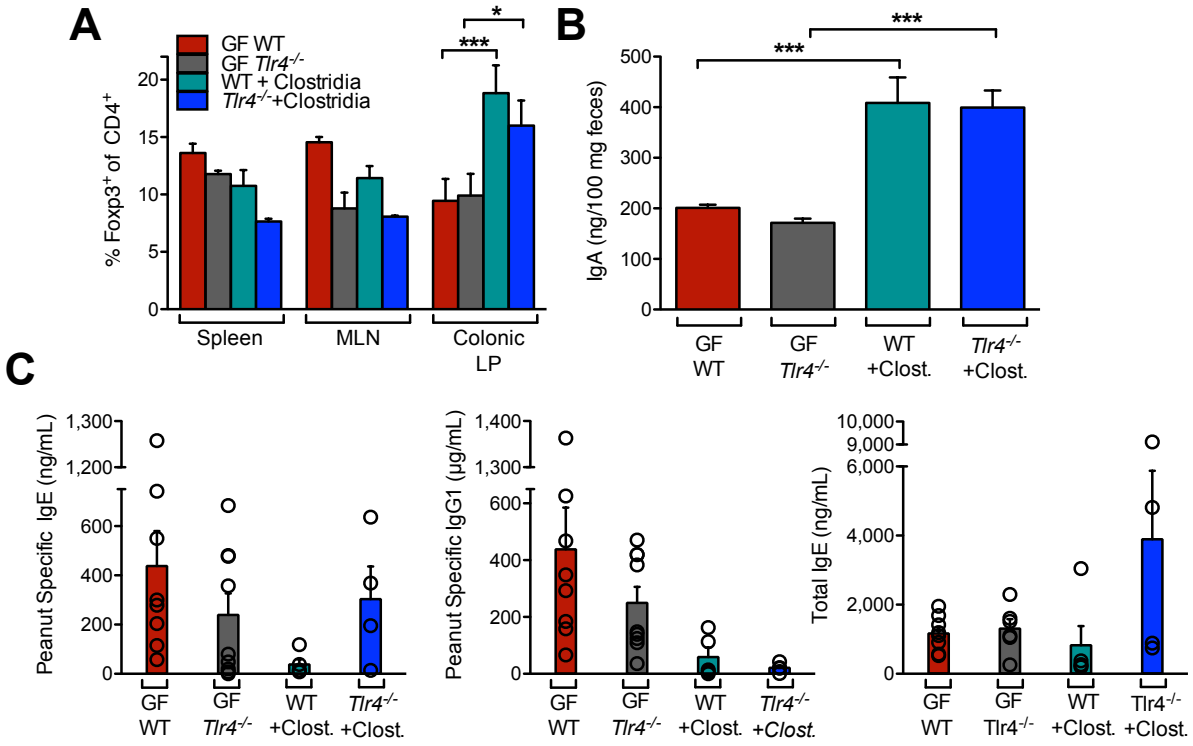


Figure 11: GF *Tlr4*$^{-/-}$ mice are equally susceptible to sensitization with PN+CT as WT counterparts but are not protected by Clostridia colonization. (**A**) Proportion of Foxp3^{+} cells among CD4^{+} cells in spleen, MLN, and colonic LP of GF WT, GF *Tlr4*$^{-/-}$, Clostridia-colonized WT mice, and Clostridia-colonized *Tlr4*$^{-/-}$ mice 14 days after colonization. (**B**) Concentration of IgA in feces from mice in **A**. Bars represent mean+S.E.M. (**C-E**) GF WT, GF *Tlr4*$^{-/-}$, and Clostridia-colonized WT and *Tlr4*$^{-/-}$ mice were sensitized at weaning with PN+CT. After 5 weeks, mice were challenged with 2 doses of PN. 24 hours later, serum was collected for antibody analysis. (**C**) PN-specific IgE, PN-specific IgG1, and total IgE concentrations in serum as determined by ELISA. Each dot represents an individual mouse; bars represent mean+S.E.M. *n*=4-9 mice per group, representative of multiple experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ determined by two-way ANOVA with Bonferroni post-test (**A**) or one-way ANOVA with Tukey post-test (**B**).

In light of the phenotype identified in *Tlr4*$^{-/-}$ mice, our discovery that Clostridia protect against sensitization was surprising. Clostridia are Gram-positive bacteria, which generally are not thought to signal via TLR4 because the outer layer of their cell wall is composed of peptidoglycan, not LPS. In order to determine whether or not there was a direct relationship between Clostridia and sensing via TLR4, we colonized GF *Tlr4*$^{-/-}$ mice with Clostridia and compared their response to that seen in GF WT mice. Clostridia colonization still induced

Foxp3^{+} Tregs in the colonic LP of GF *Tlr4*$^{-/-}$ mice, although not to the same extent as GF WT mice (Figure 11A). Interestingly, *Tlr4*$^{-/-}$ Clostridia-colonized mice had Treg proportions very similar to SPF *Tlr4*$^{-/-}$ mice, namely intermediate between GF and SPF WT mice (compare Figure 4D and Figure 11A). Fecal IgA levels were comparable between WT and *Tlr4*$^{-/-}$ mice after Clostridia colonization (Figure 11B). Interestingly, after sensitization, Clostridia-colonized *Tlr4*$^{-/-}$ animals had PN-specific and total IgE levels that were very similar to GF WT or GF *Tlr4*$^{-/-}$ mice (Figure 11C). This finding suggests that, although some of Clostridia's effect is TLR4-independent, there is a requirement for TLR4 signaling to block the generation of a PN-specific response to sensitization. It remains unclear how Clostridia signal via TLR4 and what the ligand may be.

IX. Colonization of Abx-treated mice with a Clostridia-containing microbiota protects against sensitization

After determining that Clostridia protect in the gnotobiotic model of sensitization, we wanted to test whether Clostridia colonization could also prevent the generation of a PN-specific response after Abx-treatment. The members of our Clostridia consortium are sensitive to the Abx in our cocktail, though, so we had to modify the treatment protocol to perform this experiment. Abx were administered by gavage to all animals for seven days before weaning. At weaning, mice were either weaned onto Abx-containing drinking water as before or were colonized with Clostridia from gnotobiotic fecal homogenate and placed on normal drinking water to allow the transferred Clostridia to survive. We also conventionalized a group of mice after Abx gavage following this same protocol as a positive control. Finally, we wanted to compare the effect of Clostridia administration or conventionalization to the effect of recovery from Abx treatment without intervention. This group was important to mirror the situation in

human patients who receive a course of Abx, where commensal bacteria bloom or are acquired from the environment to fill the niche created after Abx administration.

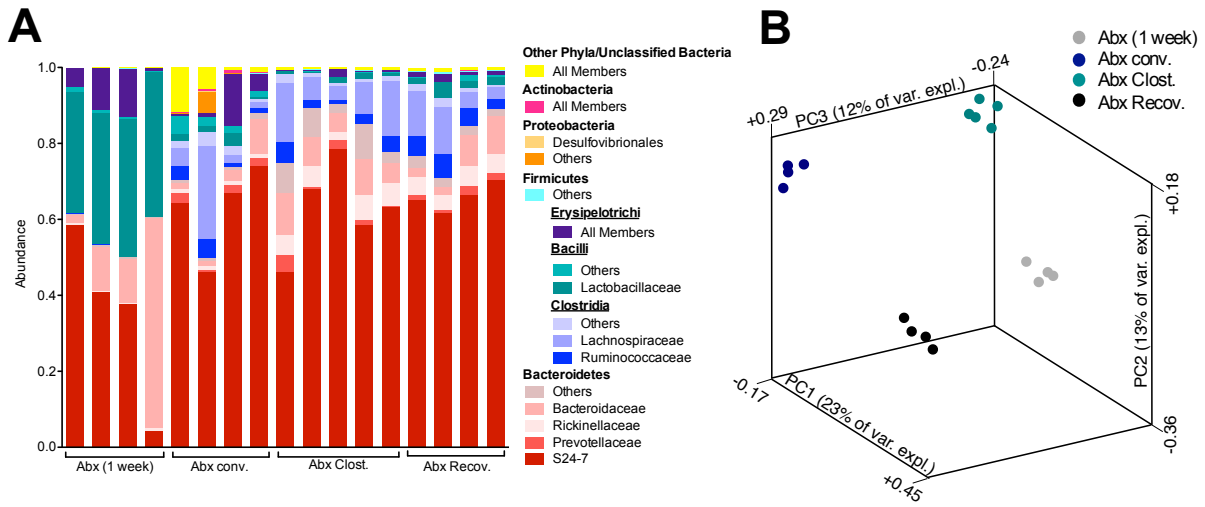


Figure 12: Colonization with Clostridia consortium after Abx treatment alters the microbiota. Mice were treated with Abx by gavage for 7 days before weaning. At weaning, mice were given a gavage of fecal/cecal material from an SPF mouse (Abx Conventionalized), feces from Clostridia-colonized repository mice (Abx Clostridia), or were left uncolonized and allowed to recover (Abx Recovery). (**A**) Abundance of various bacterial taxa in fecal samples after 1 week of Abx by gavage or 5 weeks after colonization/recovery. (**B**) PCA analysis of unweighted UniFrac for samples in **A**. Each bar/dot represents an individual mouse. *n*=4-5 mice per group.

The first question we wanted to address was whether or not colonization with our Clostridia consortium increased the abundance of Clostridia in the fecal microbiota compared to other treatments. After 1 week of Abx treatment by gavage, the time when the various consortia of bacteria were introduced, there were no detectable Clostridia family members in the fecal microbiota, although there was increased diversity as compared to the five week course of Abx treatment described in Figure 1 (Figure 12A). Five weeks after conventionalization or Clostridia colonization, there was an expansion of Clostridia compared to one week of Abx, although the expansion was equivalent between the two treatments (Figure 12A). Surprisingly, mice that were allowed to recover also had similar Clostridia abundance as the mice that received a gavage of the Clostridia consortium. Using taxonomy-independent PCA analysis, however, we

found that each treatment group segregated separately from each other, indicating that although there were no major differences in abundance of Clostridia, the community structures were distinct after each treatment (Figure 12B). This was not attributable to different starting microbiotas between groups because all mice were related by maternal lineage. Each group was also housed in multiple cages but all mice within a treatment cluster together, arguing against a cage effect.

We also examined the proportions of Foxp3$^+$ Tregs in the colonic LP after these various treatments to determine if the different bacterial communities had different Treg-inducing potential. Since Clostridia colonization maximally induced Foxp3$^+$ cells 2 weeks after colonization in the gnotobiotic system, we used the same timepoint in this model. Two weeks after being weaned on to Abx water, the proportion of Foxp3$^+$ Tregs in the colonic LP of Abx-treated mice was reduced to the baseline 10% expected from previous experiments. Both conventionalization and Clostridia colonization significantly increased the Foxp3$^+$ proportion 14 days after colonization, as predicted based on the gnotobiotic model. Recovery, however, did not expand the Foxp3$^+$ compartment at this timepoint (Figure 13).

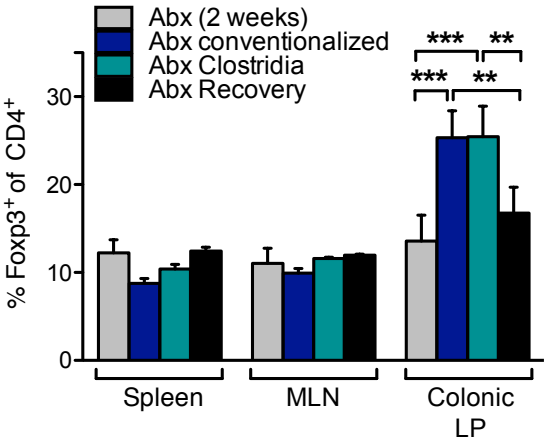


Figure 13: Conventionalization or Clostridia colonization after Abx treatment restores colonic Foxp3$^+$ Treg proportions. 14 days after weaning, the proportion of Foxp3$^+$ cells among CD4$^+$ cells was determined in spleen, MLN, and colonic LP of Abx, Abx conventionalized, Abx Clostridia, or Abx Recovery mice. Bars represent mean+S.E.M. *n*=4-6 mice per group. ** *P*<0.01, *** *P*<0.001 by two-way ANOVA with Bonferroni post-test.

Sensitization of conventionalized, Clostridia-colonized, or recovery mice with PN+CT revealed that a Clostridia-containing microbiota is sufficient to block the generation of a PN-

specific response. Compared to Abx-treated mice, all three treatments reduced PN-specific and total IgE (Figure 14A, C). PN-specific IgG1 was also significantly reduced by conventionalization or recovery (Figure 14B). Bacterial load was restored to the levels seen in NT mice in conventionalized, Clostridia-colonized, or recovery mice by the end of the experiment (Figure 14D). These results confirmed the findings from our gnotobiotic model and suggest that administration of Clostridia could have therapeutic potential as a novel type of probiotic to protect against the generation of a food antigen-specific response.

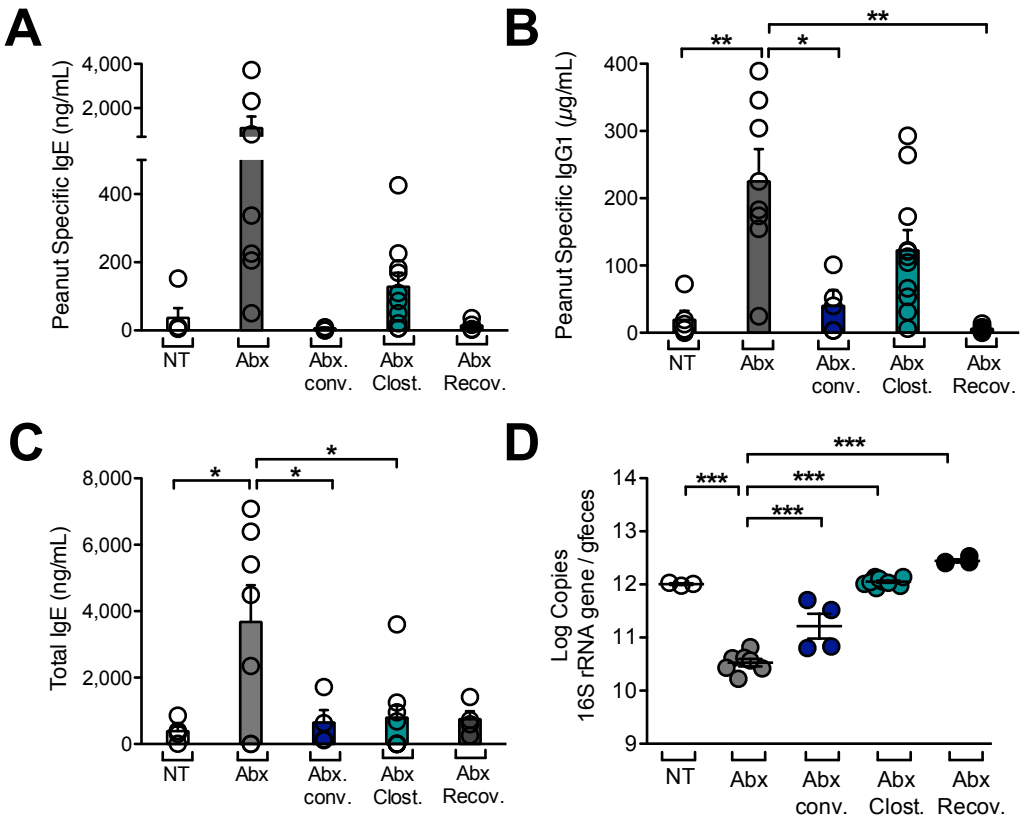


Figure 14: A Clostridia-containing microbiota protects against food allergen sensitization. Mice were treated as in Figure 12 and were sensitized with PN+CT beginning at weaning. After 5 weeks, mice were challenged with 2 does of PN. Serum and feces was collected 24 hours later for analysis. (**A**) PN-specific IgE, (**B**) PN-specific IgG1, and (**C**) total IgE concentrations in serum after challenge, determined by ELISA. (**D**) Bacterial load in feces from mice in **A-C**. In **A-C**, bars represent mean+S.E.M.; each dot represents an individual mouse. In **D**, each dot represents an individual mouse; bars represent mean±S.E.M. *n*=4-10 mice per group, pooled from two independent experiments. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 by one-way ANOVA with Tukey post-test.

X. Transfer of Foxp3$^+$ Tregs does not prevent sensitization to PN

Since induction of Tregs in the colonic LP was correlated with protection in both gnotobiotic and Abx-treated mice after Clostridia colonization, we tested if the protective effect could be phenocopied by the transfer of purified, sorted CD4$^+$Foxp3$^+$ Tregs. We began by administering Tregs from the spleen and MLN of SPF animals because they are abundant in these organs. Cells were transferred i.v. at weaning to WT mice that had received 7 days of Abx gavage. As a negative control, Abx-treated littermates received an equivalent number of CD4$^+$Foxp3$^-$ Tcon. All mice were then weaned onto Abx-containing water and sensitized with PN+CT. After challenge, the PN-specific antibody concentrations were equivalent between Abx-treated mice with or without Treg transfer and were even higher after the transfer of Tcon cells (Figure 15A, B). We considered that these cells derived from the spleen and MLN may not have had the correct homing receptors or TCRs to properly repopulate the LP, so we performed a transfer with Treg or Tcon cells from the MLN or colonic LP of SPF mice into Abx-treated recipients using the same protocol. The mice receiving mucosal Tregs in this experiment did have reduced PN-specific IgE, indicating that this transfer may have modulated responses to sensitization, however the PN-specific IgG1 remained elevated so protection was not complete (Figure 15A, B).

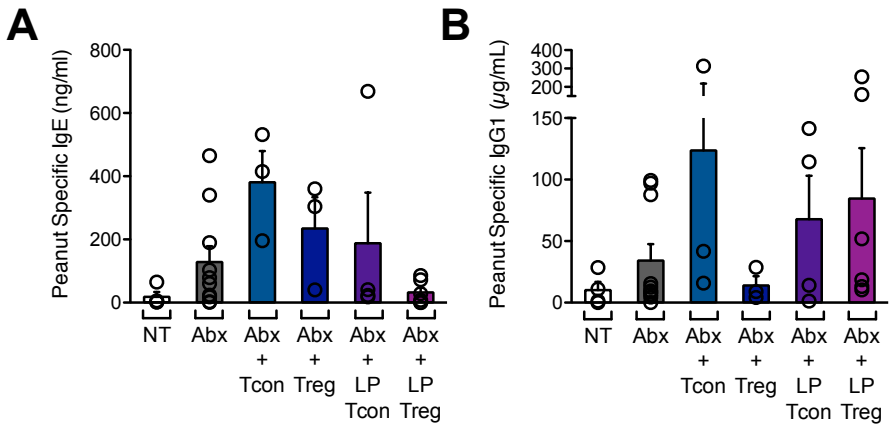


Figure 15: Transfer of Foxp3$^+$ Tregs is not sufficient to protect against sensitization to PN. Mice were treated with Abx as previously described. At weaning, mice were placed on Abx-containing water and some were given 3x10^5 CD4$^+$Foxp3$^-$ Tcon or CD4$^+$Foxp3$^+$ Tregs from spleen and MLN or 2.8x10^5 Tcon or Tregs from the MLN and colonic LP via i.v. injection. Sensitization with PN+CT began one day after transfer. After 5 weeks, mice were challenged with two doses of PN; serum was collected 24 hours after transfer. NT and Abx mice from Figure 2 were used for comparison. (**A**) PN-specific IgE and (**B**) PN-specific IgG1 concentrations in serum were determined by ELISA. Bars represent mean+S.E.M.; each dot represents an individual mouse. *n*=3-10 mice per group, pooled from two independent experiments.

Given the reduced PN-specific IgE levels after transfer of bulk colonic LP and MLN Tregs, we hypothesized that transfer of only Clostridia-induced colonic LP and MLN Tregs would be a more effective mechanism to block the PN-specific response. Following a similar procedure as in the previous experiments, Tregs from the colonic LP and MLN of gnotobiotic Clostridia-colonized mice were sorted 14 days after colonization and transferred into Abx-gavaged weanling mice. After transfer, mice were allowed to recover their microbiota in order to provide Clostridial antigens to promote the survival of transferred Tregs. Mice were then sensitized with PN+CT. Compared to a subset of mice from Figure 14, transferring Clostridia-induced Tregs had no protective effect on the PN-specific response, even after removing Abx-mediated selection of the microbiota (Figure 16A, B) but fecal IgA levels were restored by this transfer (Figure 16C). Together, these results suggest that transfer of Foxp3$^+$ Tregs was not

sufficient to restore Abx-treated mice to an NT phenotype after sensitization, although they may provide partial protection after transfer.

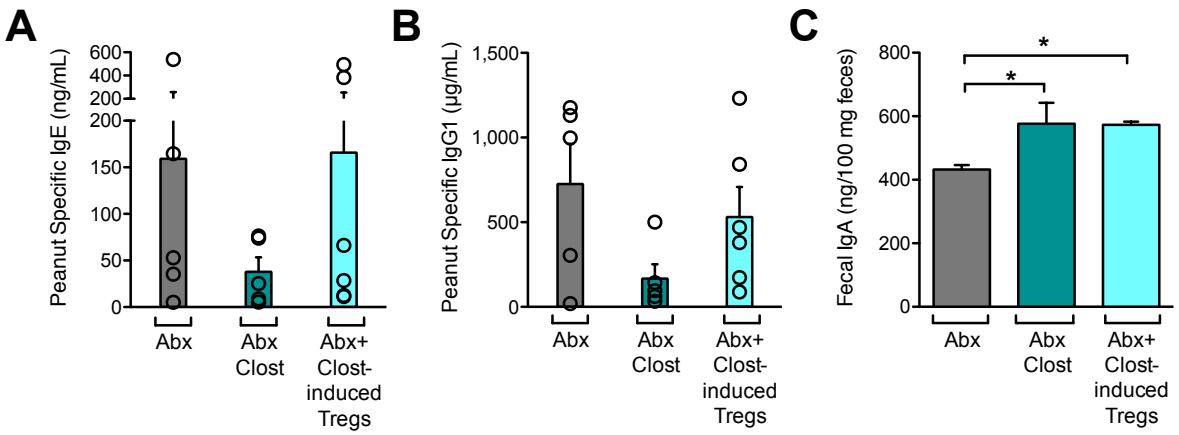


Figure 16: Transfer of Clostridia-induced Tregs is not sufficient to prevent sensitization. Mice were treated with Abx by gavage for 7 days before weaning. At weaning, mice were placed on regular water and given $2.8x10^5$ Clostridia-induced $Foxp3^+$ Tregs sorted from the MLN and colonic LP via retroorbital injection. All mice were sensitized with PN+CT. After 5 weeks, mice were challenged with 2 doses of PN; serum and feces were collected 24 hours after challenge for analysis. Abx and Abx Clostridia mice shown are a subset of mice from Figure 16. (**A**) PN-specific IgE and (**B**) PN-specific IgG1 concentrations in serum determined by ELISA. (**C**) Fecal IgA concentrations as determined by ELISA. In **A** and **B**, bars represent mean+S.E.M.; each dot represents an individual mouse. In **C**, bars represent mean+S.E.M. *n*=5-6 mice per group. * *P*<0.05 by one-way ANOVA with Tukey post-test.

Chapter 4: Clostridia colonization induces an IL-22 mediated barrier-protective response to prevent sensitization to food allergens

Work from this chapter was published in Stefka, A.T., Feehley, T., Tripathi, P., Qiu, J., McCoy, K., Mazmanian, S.K., Tjota, M.Y., Seo, G.Y., Cao, S., Theriault, B.R., *et al.* (2014). Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A *111*, 13145-13150.

I. Clostridia colonization induces a unique set of genes in the colonic epithelium

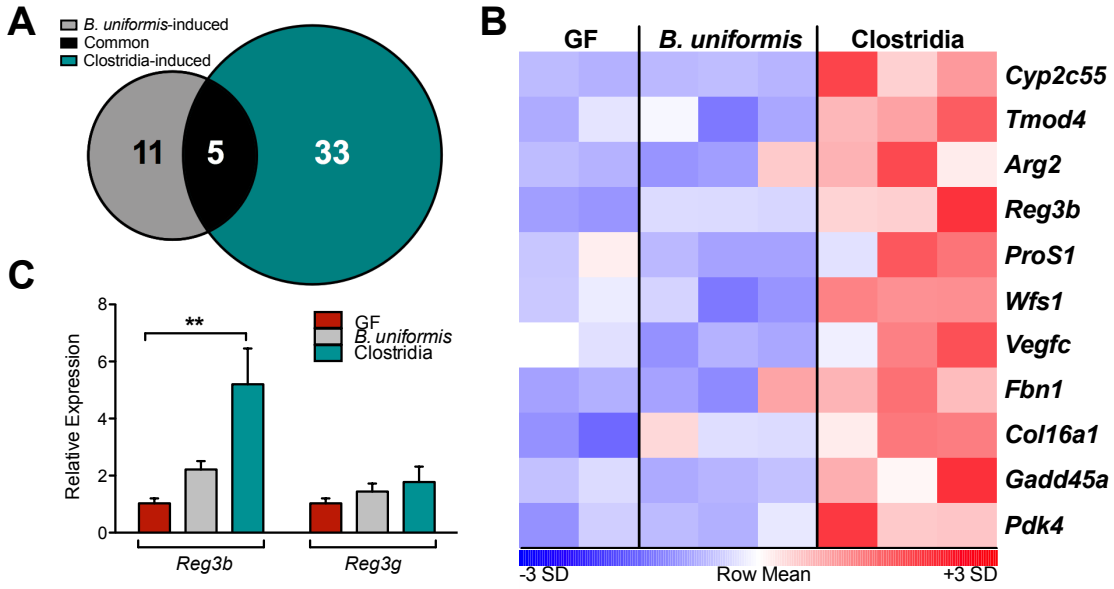


Figure 17: Colonization with Clostridia induces a unique gene signature in the colonic epithelium. Colonic IECs were collected from GF, *B. uniformis*-, or Clostridia-colonized mice 6 days after colonization. Gene expression was determined by microarray. (**A**) Number of genes upregulated by colonization with *B. uniformis* versus Clostridia, or both, relative to GF expression. (**B**) Heatmap of relative expression of 11 upregulated genes of interest. (**C**) qRT-PCR verification of *Reg3b* and *Reg3g* expression in samples used for microarray. Bars represent mean+S.E.M. *n*=2-3 samples per group; each sample was pooled from 3 individual mice. ** *P*<0.01 by two-way ANOVA with Bonferroni post-test.

Although Clostridia colonization induced Foxp3$^+$ Tregs and IgA production, transfer of Tregs was not fully protective against sensitization, so we were also interested in understanding the effect of Clostridia on the innate immune system. Clostridia are often found close to the

mucus layer of the terminal ileum and colon while most other bacteria, including *Bacteroides* species, are further away in the intestinal lumen (Nava et al., 2011). Since Clostridia occupy this specialized niche, we hypothesized that interaction with IECs may explain their unique ability to protect against sensitization. To test this hypothesis, we returned to the gnotobiotic model, which allowed us to investigate this specific interaction of Clostridia with the host. Since we were interested in early events after colonization, we performed a microarray on isolated colonic IEC from GF, *B. uniformis*-colonized, or Clostridia-colonized mice 6 days after colonization to compare the gene signature of uncolonized mice compared to mice colonized with a non-protective stimulus or a protective stimulus.

In support of our hypothesis, 33 genes were uniquely upregulated in response to Clostridia colonization. *B. uniformis* colonization only upregulated 11 genes and 5 genes were stimulated by both colonizations (Figure 17A). The top 11 upregulated genes were much more strongly expressed in mice colonized with Clostridia than either GF or *B. uniformis*-colonized mice (Figure 17B). One of most interesting hits was *Reg3b*, the gene that encodes the AMP REGIIIβ. We confirmed by qRT-PCR that *Reg3b* was indeed significantly upregulated compared to GF mice; the related AMP-encoding gene *Reg3g* was not induced to the same extent in these samples (Figure 17C).

II. Colonization with Clostridia induces expression of *Il22* and *Il17* in a TLR4-independent manner

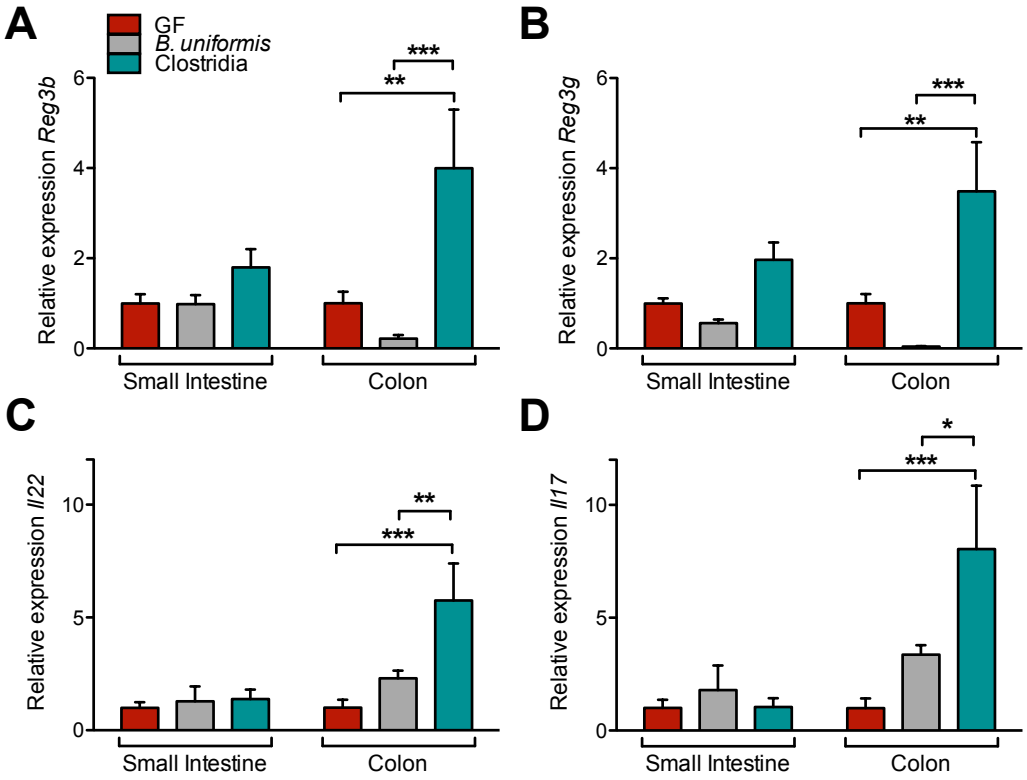


Figure 18: Colonization with Clostridia induces expression of *Il22* and related genes. Whole tissue samples and LP cells were collected from GF, *B. uniformis*-, and Clostridia-colonized mice 4 days after colonization. After RNA extraction, gene expression was determined by qRT-PCR. (**A**) Expression of *Reg3b* or (**B**) *Reg3g* in whole tissue homogenates from the small intestine or colon. (**C**) Expression of *Il22* and (**D**) *Il17* in small intestine or colonic LP cells from mice in **A** and **B**. Gene expression was measured relative to *Hprt* and normalized to GF. Bars represent mean+S.E.M. n=8-9 mice per group, pooled from two independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by two-way ANOVA with Bonferroni post-test.

Reg3b was a particularly intriguing hit because expression of this gene requires an IL-22 signal (Vaishnava et al., 2011) and IL-22 is known to be important for maintaining homeostasis at the intestinal barrier (Sonnenberg et al., 2011). To determine if Clostridia colonization stimulated the IL-22 pathway, we first validated the microarray findings in independent samples collected from GF, *B. uniformis*-, or Clostridia-colonized mice. In whole tissue homogenates

from the colon, both *Reg3b* and *Reg3g* were significantly increased after Clostridia colonization compared to GF or *B. uniformis* (Figure 18A, B). We also isolated lymphocytes from the small intestinal or colonic LP of these same animals and measured *Il22* expression. As with the AMPs, *Il22* expression was significantly higher after Clostridia colonization compared to GF or *B. uniformis* (Figure 18C). Expression of *Il17*, which is also produced by the cells that make IL-22 and is often expressed concurrently, was also increased significantly upon Clostridia colonization (Figure 18D).

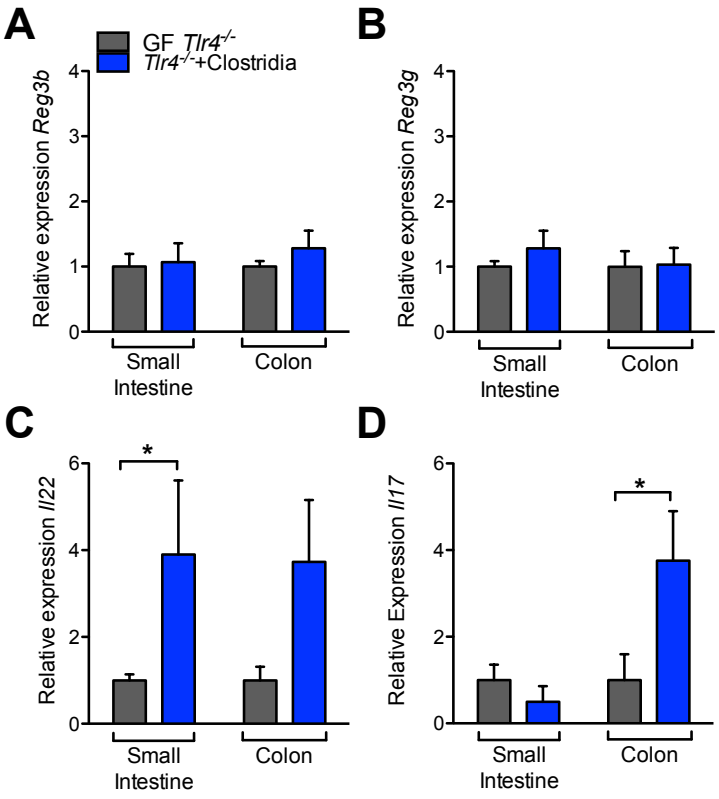


Figure 19: Induction of *Il22* and *Il17* expression by Clostridia is TLR4-independent. GF *Tlr4*$^{-/-}$ mice were colonized with Clostridia for 4 days. Gene expression in whole tissue or LP cells was determined by qRT-PCR. (**A**) Expression of *Reg3b* and (**B**) *Reg3g* in small intestine and colon tissue homogenates. (**C**) Expression of *Il22* and (**D**) *Il17* from small intestine and colonic LP cells. Expression was measured relative to *Hprt* and normalized to GF *Tlr4*$^{-/-}$. Bars represent mean+S.E.M. *n*=4-7 mice per group, pooled from 2 independent experiments. * *P*<0.05 by two-way ANOVA with Bonferroni post-test.

We performed a similar experiment in GF *Tlr4*$^{-/-}$ mice to determine if there were differences in gene expression after Clostridia colonization in the absence of TLR4 signaling that could help explain the lack of protection after sensitization. The increased expression of *Reg3b* and *Reg3g* was ablated in *Tlr4*$^{-/-}$ mice after Clostridia colonization (Figure 19A, B), in agreement with other reports in the literature demonstrating that expression of these AMPs

requires both an IL-22 signal and a TLR/MyD88 signal (Brandl et al., 2007; Vaishnava et al., 2011). *Il22* and *Il17* expression, however, was still significantly increased after colonization (Figure 19C, D), demonstrating that this portion of the Clostridia-induced response does not require TLR4.

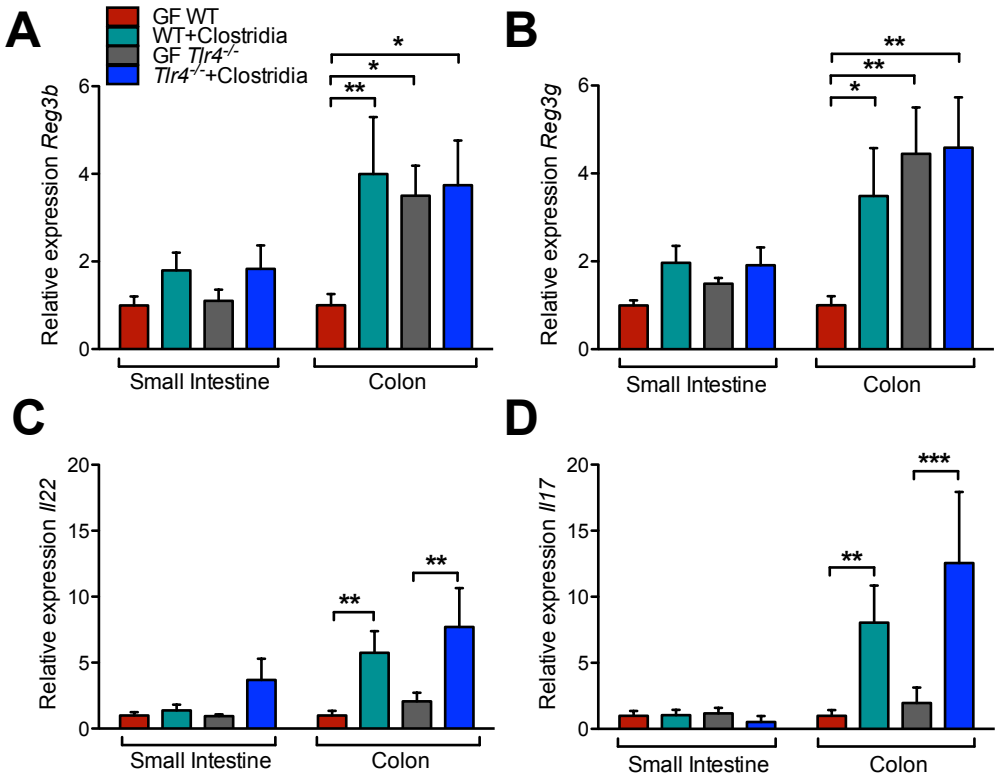


Figure 20: GF *Tlr4*$^{-/-}$ animals have dysregulated AMP expression at baseline. Samples from Figures 18 and 19 were reanalyzed and all normalized to GF WT. (**A**) Expression of *Reg3b* and (**B**) *Reg3g* in whole tissue homogenates from small intestine and colon. (**C**) Expression of *Il22* and (**D**) *Il17* in isolated LP cells from small intestine and colon. Expression was measured relative to *Hprt*. Bars represent mean+S.E.M. *n*=4-9 mice per group, pooled from 2 independent experiments. * *P*<0.05, ** *P*<0.01, ***P<0.001 by two-way ANOVA with Bonferroni post-test.

We also analyzed this data across all 4 groups. Instead of normalizing by genotype, expression was normalized to the GF WT samples used in Figure 18. This comparison presented a slightly different picture. There was still significantly increased expression of *Reg3b*

and *Reg3g* between WT GF and Clostridia-colonized mice and no marked difference in expression between the *Tlr4*$^{-/-}$ GF and Clostridia-colonized mice (Figure 20A, B). Comparing across genotypes, however, revealed that the baseline expression of both AMPs was significantly higher in the GF *Tlr4*$^{-/-}$ mice than WT counterparts. Upstream expression of *Il22* and *Il17* were not dysregulated in GF *Tlr4*$^{-/-}$ mice compared to GF WT counterparts (Figure 20C, D), so it is unclear why these AMPs are expressed at such a high level on the *Tlr4*$^{-/-}$ background. The increase in *Il22* and *Il17* after colonization was still significant between GF and Clostridia-colonized mice and also clearly independent of TLR4 when compared across all conditions (Figure 20C, D).

III. Clostridia-induced IL-22 is produced by RORγt$^+$ ILCs and CD4$^+$ T cells

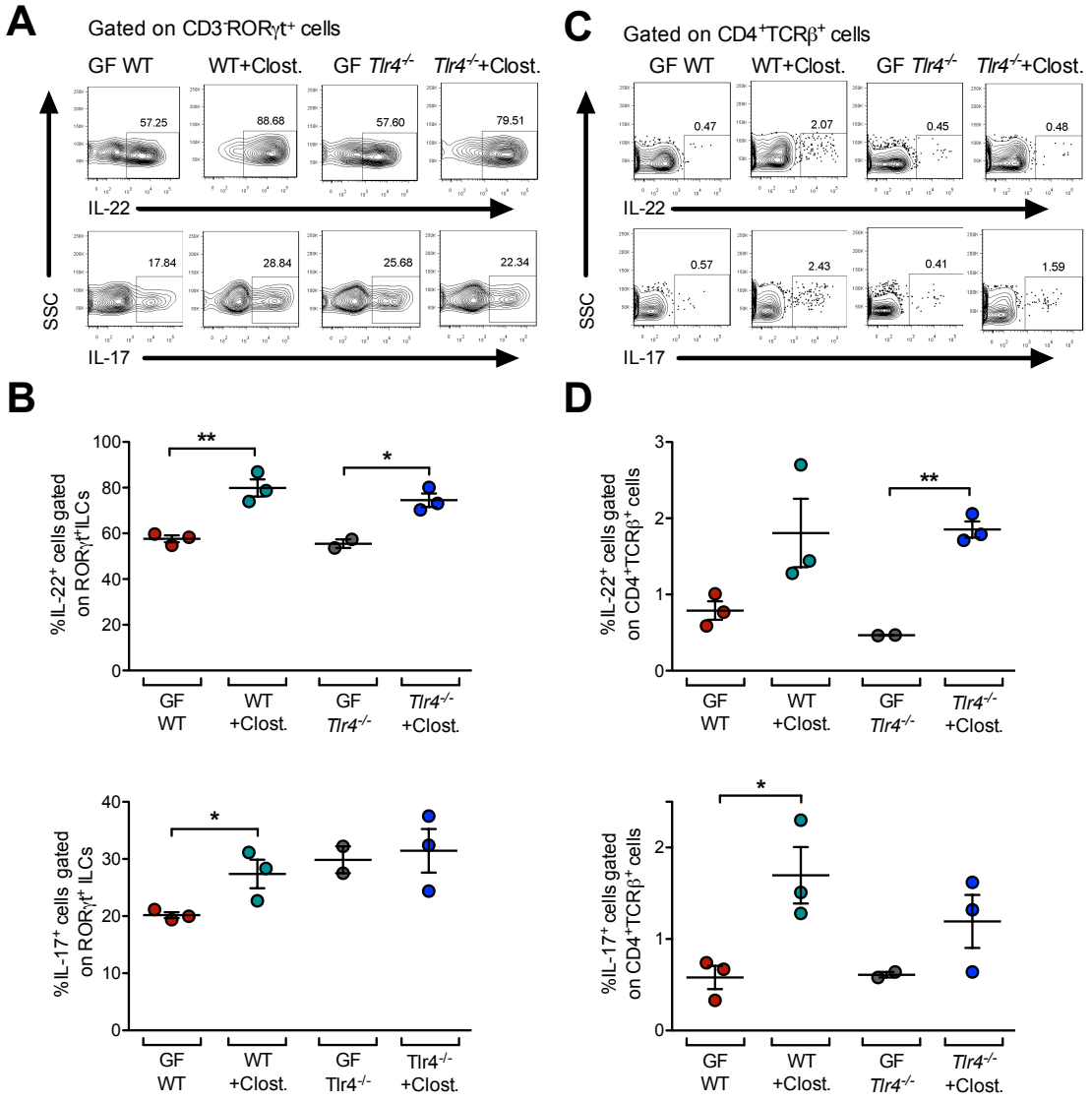


Figure 21: IL-22 and IL-17 are produced by both RORγt$^+$ ILCs and T cells in response to Clostridia colonization. IL-22 and IL-17 production were measured by flow cytometry in *ex vivo* stimulated cells from the colonic LP of GF WT and *Tlr4*$^{-/-}$ mice or Clostridia-colonized WT and *Tlr4*$^{-/-}$ mice 6 days after colonization. (**A**) Representative plots of IL-22 and IL-17 production from RORγt$^+$ ILCs. (**B**) Quantification of IL-22 and IL-17 positive populations from analysis in **A**. (**C**) Representative plots of IL-22 and IL-17 production from CD4$^+$ T cells. (**D**) Quantification of IL-22 and IL-17 positive populations from analysis in **C**. Each dot represents an individual mouse; bars show mean±S.E.M. *n*=2-3 mice per group, representative of 3 independent experiments. * *P*<0.05, ** *P*<0.01 by Student's *t*-test.

IL-22 can be produced by multiple cell types in the intestine, most notably RORγt$^+$ ILC3s and Th17 cells (Sonnenberg et al., 2011). When we examined the cellular source of IL-22 in our model by flow cytometry, we found that there were significantly increased proportions of IL-22- and IL-17-producing ILC3s 6 days after Clostridia colonization in WT mice (Figure 21B, D). *Tlr4*$^{-/-}$ mice showed a similar trend. There was also an increase in IL-22- and IL-17-producing CD4$^+$ T cells after Clostridia colonization, although the percentages of cytokine positive cells were much lower in this population, suggesting that ILCs are the major IL-22 producers at this timepoint.

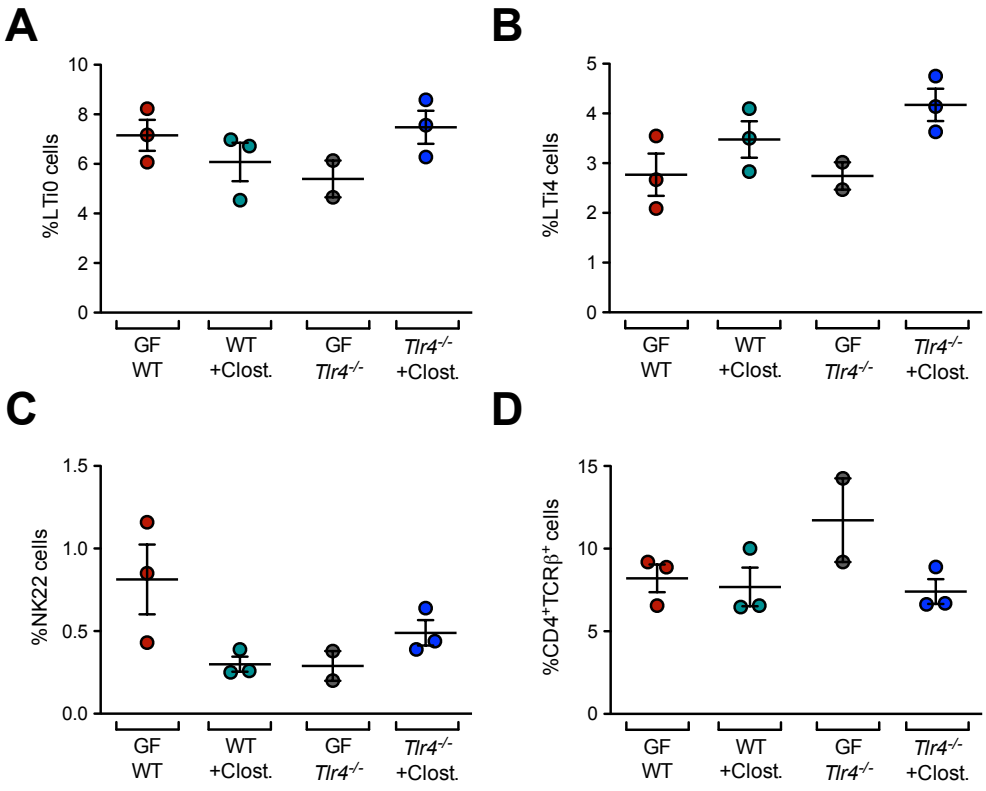


Figure 22: Clostridia colonization does not change proportions of ILC3 subsets or T cells. Proportions of different ILC3 subsets in the colonic LP from the same mice as in Figure 21. (**A**) Percent of LTi0 (CD3$^-$CD4$^-$RORγt$^+$NKp46$^-$) cells among CD3$^-$TCRβ$^-$ cells. (**B**) Percent of LTi4 (CD3$^-$CD4$^+$RORγt$^+$NKp46$^-$) cells among CD3$^-$TCRβ$^-$ cells. (**C**) Percent of NK22 (CD3$^-$ CD4$^-$ RORγt$^+$NKp46$^+$) cells among CD3$^-$TCRβ$^-$ cells. (**D**) Percent of CD4$^+$ TCRβ$^+$ cells. Each dot represents an individual mouse; bars show mean±S.E.M. *n*=2-3 mice per group, representative of 3 independent experiments.

ILCs are divided into several different subsets, including $CD4^-$ LTi (LTi0) cells, $CD4^+$ LTi (LTi4) cells, and $NKp46^+$ (or NCR^+; NK22) ILC3s (Spits et al., 2013). We compared proportions of each of these subtypes to determine if there was expansion of any particular group after colonization, but found no significant changes (Figure 22A-C). We also saw no major differences in proportions of total $CD4^+$ T cells (Figure 22D). This indicates that Clostridia increased IL-22 and IL-17 production on a per cell basis, not by changing the abundance of a certain IL-22-producing cell populations.

IV. Clostridia also induces increased mucus production and reduced intestinal permeability to PN in a gnotobiotic model

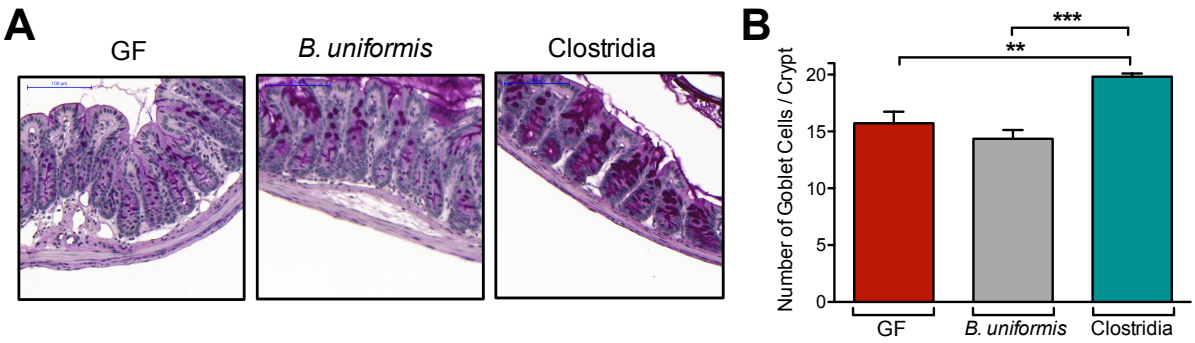


Figure 23: Clostridia colonization increases goblet cell number. Sections of distal colons from GF, *B. uniformis*-, or Clostridia-colonized mice collected 6 days after colonization were stained with PAS to identify mucus and mucus-producing cells. (**A**) Representative images and (**B**) quantification of goblet cell numbers per crypt. Scale bar=100μm. Bars represent mean+S.E.M. *n*=3-5 mice per group. ** *P*<0.01, *** *P*<0.001 by one-way ANOVA with Tukey post-test.

In addition to regulating production of the AMPs already discussed, there is also evidence that IL-22 drives mucus production and goblet cell restitution as well as increased proliferation of epithelial cells (Sonnenberg et al., 2011; Sugimoto et al., 2008). In combination, this suite of downstream effectors fortifies the epithelial barrier to increase host defenses. Since IL-22 and AMPs were both increased after Clostridia colonization, we also wanted to measure

changes in mucus production in gnotobiotic mice. Using PAS staining, we compared the number of mucus-containing goblet cells in the crypts of GF, *B. uniformis*-, and Clostridia-colonized mice. In keeping with the *Il22* expression result, there was no difference in goblet cell number between GF and *B. uniformis*-colonized mice, while Clostridia-colonized mice had significantly more than the other two groups (Figure 23A, B).

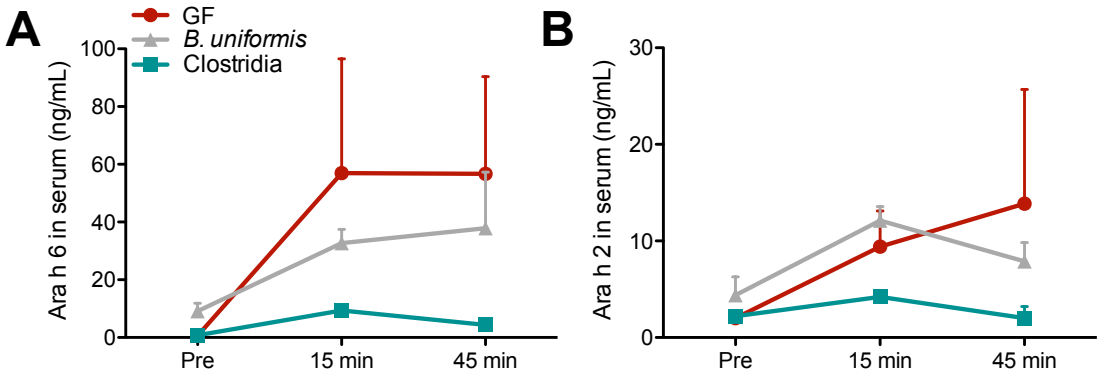


Figure 24: Clostridia colonization reduces concentration of PN proteins in serum of gnotobiotic mice. GF, *B. uniformis*-, and Clostridia-colonized mice were fasted overnight then given one gavage of 20mg PN 6 days after colonization. Serum was collected before gavage then 15 min and 45 min after gavage. Concentration of (**A**) Ara h 6 and (**B**) Ara h 2 were determined by capture ELISA. Points represent mean, bars represent S.E.M. *n*=5-12 mice per group, pooled from two independent experiments.

We next wanted to understand the functional consequences of this IL-22 program after Clostridia colonization in the context of food allergy and sensitization. We hypothesized that this fortified barrier could lead to protection by reducing permeability to luminal allergens. If less allergen crossed from the intestinal lumen into the LP and then into systemic circulation, there would be less opportunity for systemic sensitization. To test this hypothesis, we assessed intestinal permeability using a novel assay based on the standard FITC-dextran model. PN was administered via i.g. gavage and mice were bled before and after gavage at a series of timepoints. Serum was then probed for the presence of two immunodominant PN proteins, Ara h 6 and Ara h 2, over time using sensitive capture ELISAs. With this system, we determined that GF mice had detectable levels of both Ara h 6 and Ara h 2 as quickly as 15 minutes after

gavage (Figure 24A, B). *B. uniformis*-colonized mice had similar levels of these two proteins in their serum as GF mice, however Clostridia-colonized mice had much lower circulating levels of PN protein, indicating that, in agreement with our hypothesis, Clostridia colonization reduced intestinal permeability to food allergens (Figure 24A, B).

V. Colonization with Clostridia after Abx treatment induces *Il22* expression, which is necessary and sufficient to reduce intestinal permeability

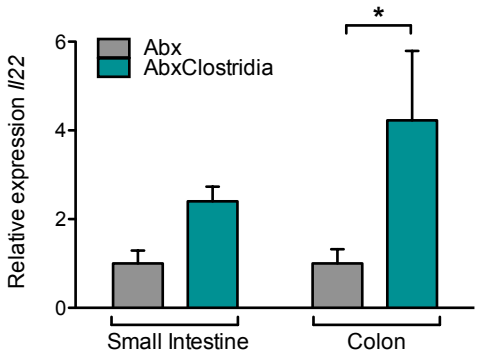


Figure 25: Clostridia colonization after Abx treatment also induces *Il22* expression. Mice were treated with Abx by gavage for 7 days prior to weaning. At weaning, mice were placed on water containing Abx or were colonized with Clostridia. RNA was collected from small intestine or colonic LP 6 days after weaning. *Il22* expression was measured by qRT-PCR, relative to *Hprt* expression, and normalized to Abx. Bars represent mean+S.E.M. n=5 mice per group. * P<0.05 by two-way ANOVA with Bonferroni post-test.

To determine if Clostridia stimulates the same pathways in the Abx-treatment model, we assessed expression of *Il22* and intestinal permeability in Abx-treated or Abx-treated Clostridia-colonized mice. As in the gnotobiotic model, Clostridia colonization after Abx-treatment significantly increased expression of *Il22* in the colonic LP compared to Abx administration alone (Figure 25). We next tested whether or not Clostridia is sufficient to reduce intestinal permeability to PN in this model. SPF mice that received no treatment had a transient rise in serum concentrations of both Ara h 6 and Ara h 2 (Figure 26A). This is in agreement with another report that OVA can be transiently detected in the serum of healthy BALB/c mice after i.g. administration, peaking 1 hour after gavage (Peng et al., 1990). Mice that received Abx had significantly more Ara h 6/2 in their serum than NT mice, indicating that changes in the microbiota alter intestinal permeability to PN (Figure 26A). Colonization with Clostridia after Abx-

treatment significantly reduced the Ara h 6/2 concentrations, corroborating our findings in the gnotobiotic model (Figure 26A). Interestingly, Clostridia-colonized mice had even lower serum allergen concentrations than NT mice. We also treated mice with Abx and administered IL-22Fc, a fusion protein of IL-22 and the constant region of mouse IgG2a to increase the stability (Ota et al., 2011). A single dose of this protein phenocopied Clostridia colonization; Ara h 6 and Ara h 2 concentrations were significantly lower than Abx-treated mice and were almost the same as the Clostridia-colonized group, demonstrating that exogenous IL-22 administration was sufficient to reduce intestinal permeability to allergens (Figure 26A).

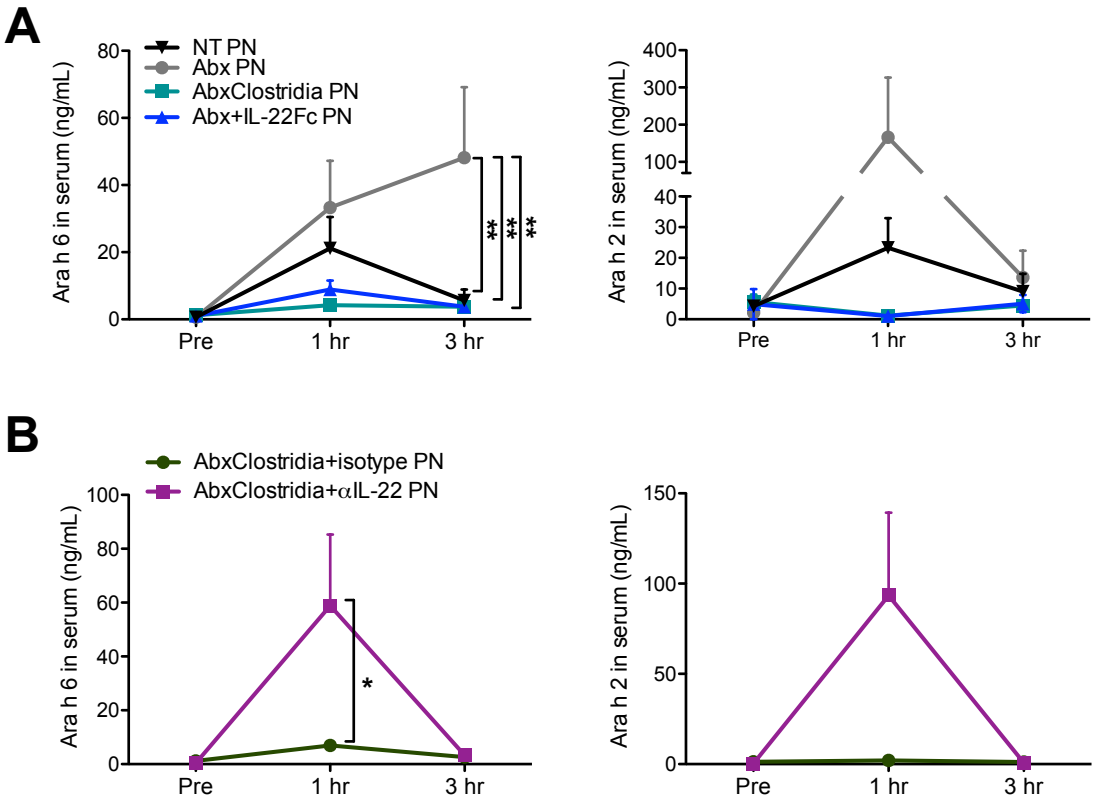


Figure 26: Clostridia-induced IL-22 is necessary and sufficient to reduce concentrations of PN proteins in systemic circulation. (**A**) SPF mice were given NT or were gavaged with Abx for 7 days prior to weaning. At weaning, mice were placed on water containing Abx with or without an i.p. injection of 20µg IL-22Fc or were placed on normal drinking water and colonized with Clostridia. 6 days after weaning, all mice were fasted and given a gavage of 20mg PN. Serum was collected before gavage and at indicated timepoints after. Serum concentrations of Ara h 6 and Ara h 2 were determined by capture ELISA. (**B**) Mice were treated with Abx as in **A**. At weaning, mice were colonized with Clostridia and given 150µg neutralizing antibody to IL-22 or an isotype control i.p. every other day. 6 days after weaning, mice were fasted and given a gavage of 20mg PN. Serum was collected before gavage and at indicated timepoints after. Serum concentrations of Ara h 6 and Ara h 2 were determined by capture ELISA. Dots represent mean, bars represent S.E.M. *n*=5-10 mice per group, pooled from two independent experiments. * *P*<0.05, ** *P*<0.01 by two-way ANOVA with Bonferroni post-test (**A**) or Student's *t*-test (**B**).

To determine if Clostridia-induced IL-22 was necessary to reduce permeability, we treated Abx-treated Clostridia-colonized mice with neutralizing antibody against IL-22 (anti-IL-22) before analyzing serum concentrations of Ara h 6/2. Mice that received an isotype control antibody had very low levels of both Ara h 6 and Ara h 2, while mice that were treated with anti-

IL-22 had significantly more PN protein in their serum (Figure 26B). Since neutralizing IL-22 ablated the protective effect of Clostridia colonization, we concluded that IL-22 was required to reduce intestinal permeability to PN.

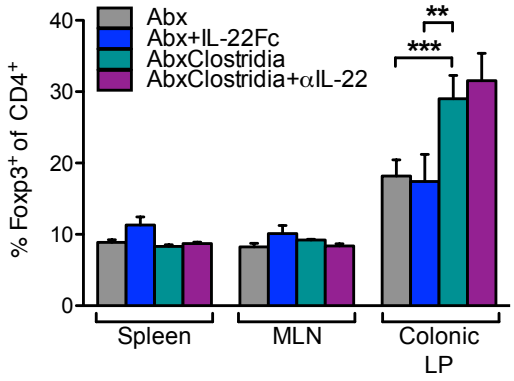


Figure 27: Treatment with IL-22Fc and anti-IL-22 do not affect Foxp3$^+$ Tregs. Analysis of proportion of Foxp3$^+$ Tregs among CD4$^+$ cells from spleen, MLN, and colonic LP of mice treated with Abx with or without two 20µg i.p. injections of IL-22Fc or treated with Abx then colonized with Clostridia with or without one i.p. injection of 500µg anti-IL-22. All mice were analyzed 2 weeks after weaning. Bars represent mean+S.E.M. *n*=3-7 mice per group. ** *P*<0.01, *** *P*<0.001 by two-way ANOVA with Bonferroni post-test.

Although the IL-22 receptor is not expressed on hematopoietic cells (Wolk et al., 2004), we wanted to confirm that neutralizing IL-22 or administering exogenous IL-22Fc did not affect colonic Treg proportions. As expected, there was no induction of Tregs in Abx-treated mice given IL-22Fc and there was no reduction of Tregs in Abx-treated Clostridia-colonized mice treated with anti-IL-22 (Figure 27). These results confirm that the changes in intestinal permeability are independent of the adaptive immune response to Clostridia represented by the induction of Tregs two weeks after colonization.

During our sensitization protocol, CT is given as a mucosal adjuvant. Other studies have documented that CT acts as an adjuvant, at least in part, by increasing intestinal permeability (Lycke et al., 1991). To extend our findings regarding Clostridia's ability to reduce permeability at steady state to our model of food allergen sensitization, we compared concentrations of Ara h 6 and Ara h 2 in the serum of Abx-treated or Abx-treated Clostridia-colonized mice after administration of PN+CT. CT administration did significantly increase the concentration of PN in the serum of Abx-treated mice compared to Abx-treated mice that received PN only (* *P* <0.05,

comparing Figure 26A to Figure 28). Even in the presence of CT, though, mice that were colonized with Clostridia had significantly lower concentrations of PN protein in their serum than Abx-treated mice that were given PN+CT (Figure 28).

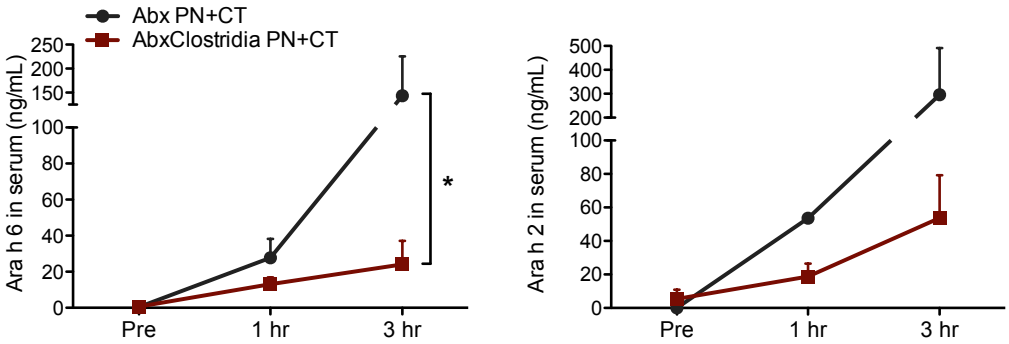


Figure 28: Clostridia colonization also reduces serum PN concentrations in the presence of CT. Mice were treated with Abx and colonized with Clostridia as in Figure 26. 6 days after weaning, mice were fasted and gavaged with 20mg PN+15µg CT. Serum was collected before gavage and at indicated timepoints after. Serum Ara h 6 and Ara h 2 concentrations were determined by capture ELISA. Dots represent mean, bars represent S.E.M. *n*=4 mice per group. * *P*<0.05 by two-way ANOVA with Bonferroni post-test.

Finally, since both ILC3s and T cells were able to produce IL-22 in response to Clostridia colonization, we wanted to understand which cell population contributed to the permeability phenotype. *Rag*$^{-/-}$ mice do not have an adaptive immune system, so they have ILC3s but no T cell compartment. In these mice, all groups of ILCs (1, 2, and 3) can be depleted by administration of antibody against CD90.2. We treated *Rag*$^{-/-}$ mice with Abx then colonized them with Clostridia while treating with anti-CD90.2 or an isotype control. In the isotype treated *Rag*$^{-/-}$ mice, there was minimal Ara h 6 or Ara h 2 detected, similar to WT mice colonized with Clostridia (Figure 29A). Depleting ILCs increased PN protein concentrations in the serum, suggesting that T cell-derived IL-22 is not necessary to mediate Clostridia's protective effect at this timepoint. The anti-CD90.2 administration also reduced *Il22* expression, verifying that IL-22-producing ILCs were effectively depleted by this treatment (Figure 29B).

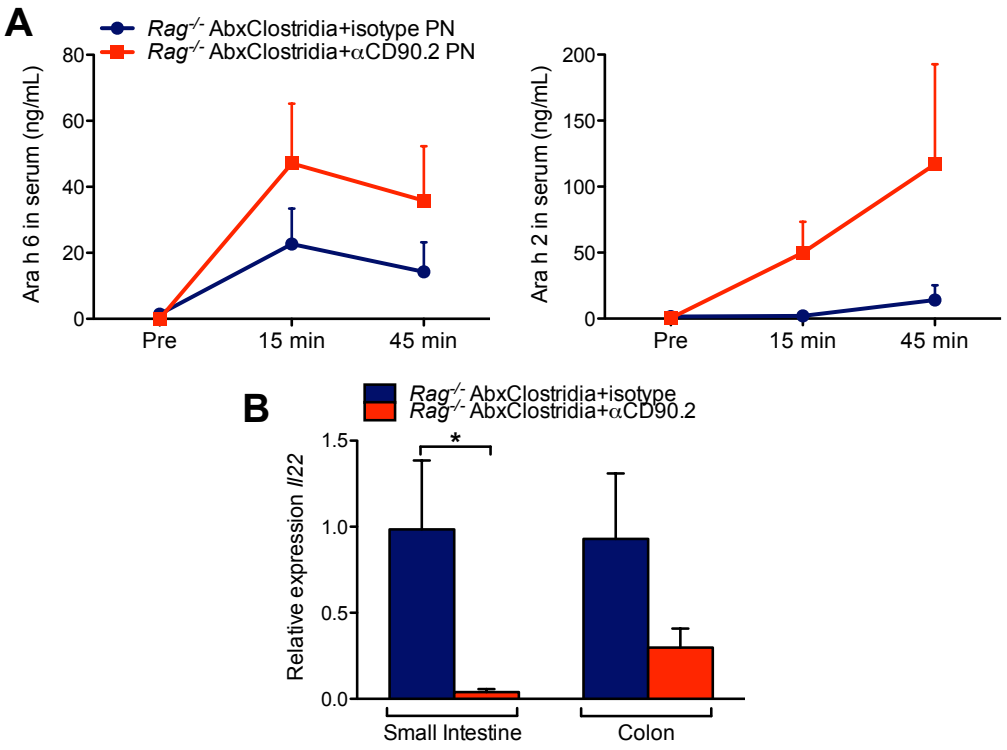


Figure 29: Depleting ILCs ablates effect of Clostridia colonization on serum PN concentrations. *Rag*$^{-/-}$ mice were treated with Abx and colonized with Clostridia at weaning as in Figure 26. Mice were also treated with 250µg anti-CD90.2 or isotype control every 3 days beginning 3 days before weaning. 6 days after weaning, mice were fasted and given a gavage of 20mg PN. Serum was collected before gavage and at indicated timepoints after. (**A**) Serum concentrations of Ara h 6 and Ara h 2, as determined by capture ELISA. (**B**) Expression of *Il22* in small intestine and colonic LP cells, measured by qRT-PCR relative to *Hprt* and normalized to isotype controls. In **A**, dots represent mean and bars represent S.E.M. In **B**, bars represent mean+S.E.M. *n*=8 mice per group, pooled from two independent experiments. * *P*<0.05 by two-way ANOVA with Bonferroni post-test.

VI. Administration of IL-22Fc to Abx-treated mice does not alter PN-specific responses

Although Clostridia-induced IL-22 was necessary and sufficient to reduce intestinal permeability, this reduced permeability only correlated with reduced PN-specific responses to sensitization. In order to demonstrate that IL-22 was able to block the generation of a PN-specific response, we administered IL-22Fc or an isotype control to Abx-treated mice throughout

sensitization with PN+CT. After challenge, there was no significant difference in responses between IL-22Fc-treated and isotype-treated animals (Figure 30A-C); both groups had PN-specific and total IgE levels similar to Abx-treated mice from Figures 2 and 14. This experiment was inconclusive, however, because the dosing protocol for this experiment was chosen empirically and may not have been optimal. We chose to modify our approach to more clearly address the requirement for IL-22 to prevent allergic sensitization.

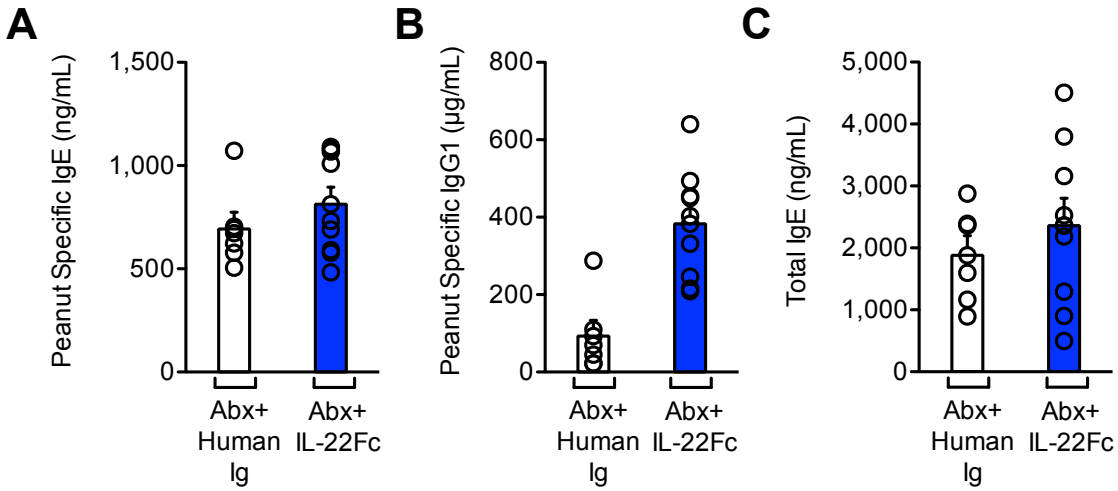


Figure 30: Treatment with IL-22Fc does not alter PN-specific responses after sensitization. Mice were treated with Abx starting at 14 days of age. Beginning at weaning, mice were given a weekly dose 20µg IL-22Fc or isotype control for 3 weeks. Mice were given a 4th dose of IL-22Fc or isotype one week prior to challenge. Mice were also sensitized with PN+CT and challenged as previously described. (**A**) Serum concentration of PN-specific IgE, (**B**) PN-specific IgG1, and (**C**) total IgE as determined by ELISA. Each dot represents an individual mouse; bars represent mean+S.E.M. *n*=6-9 mice per group.

VII. Treatment of Clostridia-colonized Abx-treated mice with anti-IL-22 increases PN-specific responses and alters the composition of the microbiota

Since neutralizing IL-22 increased intestinal permeability, we predicted that neutralizing IL-22 throughout sensitization of Abx-treated Clostridia-colonized mice would increase PN-specific responses. Using the same dosing protocol used in the permeability assay, we treated mice with Abx prior to weaning, then colonized with Clostridia and began anti-IL-22 or isotype control administration at weaning. PN+CT sensitization started one day later. After 5 weeks of anti-IL-22 treatment and sensitization, mice were challenged with two high doses of PN and samples were collected 24 hours later for analysis. We first confirmed the efficacy of the IL-22 neutralization by measuring expression of *Reg3b/g* and goblet cell numbers. Expression of *Reg3b* and *Reg3g* was significantly reduced in both the small intestine and the colon (Figure 31A) and goblet cell numbers were significantly reduced in the colonic crypts (Figure 31B) after anti-IL-22 administration, both of which confirm effective neutralization of IL-22.

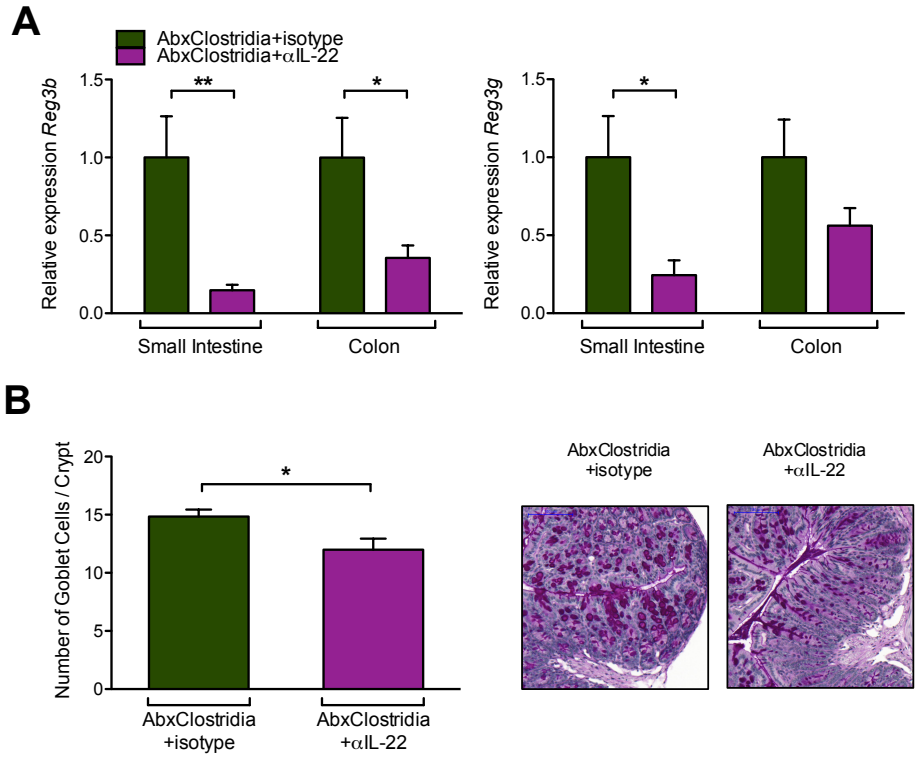


Figure 31: Neutralizing IL-22 after Clostridia colonization reduces expression of *Reg3b/g* and goblet cell numbers. Mice were treated with Abx and colonized with Clostridia as previously described. Neutralizing antibody to IL-22 or an isotype control was administered 3 times per week beginning at weaning. Tissue for RNA isolation was collected 5 weeks later for analysis. (**A**) Expression of *Reg3b* and *Reg3g* in whole tissue homogenates from small intestine and colon of isotype or anti-IL-22 treated mice. *n*=11 mice per group, pooled from 4 independent experiments. (**B**) Representative images and quantification of goblet cell numbers per crypt in the distal colon of isotype or anti-IL-22 treated mice, determined by PAS staining. Scale bar=100μm. Bars represent mean+S.E.M. *n*=5 mice per group. * *P*<0.05, ** *P*<0.01 determined by two-way ANOVA with Bonferroni post-test (**A**) or Student's *t*-test (**B**).

We assessed responses to sensitization by measuring splenocyte cytokine responses as well as serum antibody concentrations. Surprisingly, there was no difference in IL-4 production after either anti-CD3 stimulation or PN stimulation (Figure 32A). IL-13 was also only moderately increased in anti-IL-22 treated samples (Figure 32B), and there was no difference in PN-specific or total IgE concentrations between groups (Figure 32C), indicating that neutralizing IL-22 did not cause an exacerbated Th2 response. IFNγ production was also comparable between isotype and anti-IL-22 samples, so there was no change in the Th1 response (Figure 32D). IL-17 production, however, was significantly increased in splenocytes taken from anti-IL-

22 treated mice and PN-specific IgG was higher in the serum of these animals (Figure 32E, F). Thus neutralizing IL-22 did increase sensitization to food allergens, but did so by modulating a different pathway than the Th2-biased IgE response we expected. This finding is in agreement with other reports that demonstrated that IL-22 contributes to control of the adaptive arm of the Th17/Type 3 response (Qiu et al., 2013).

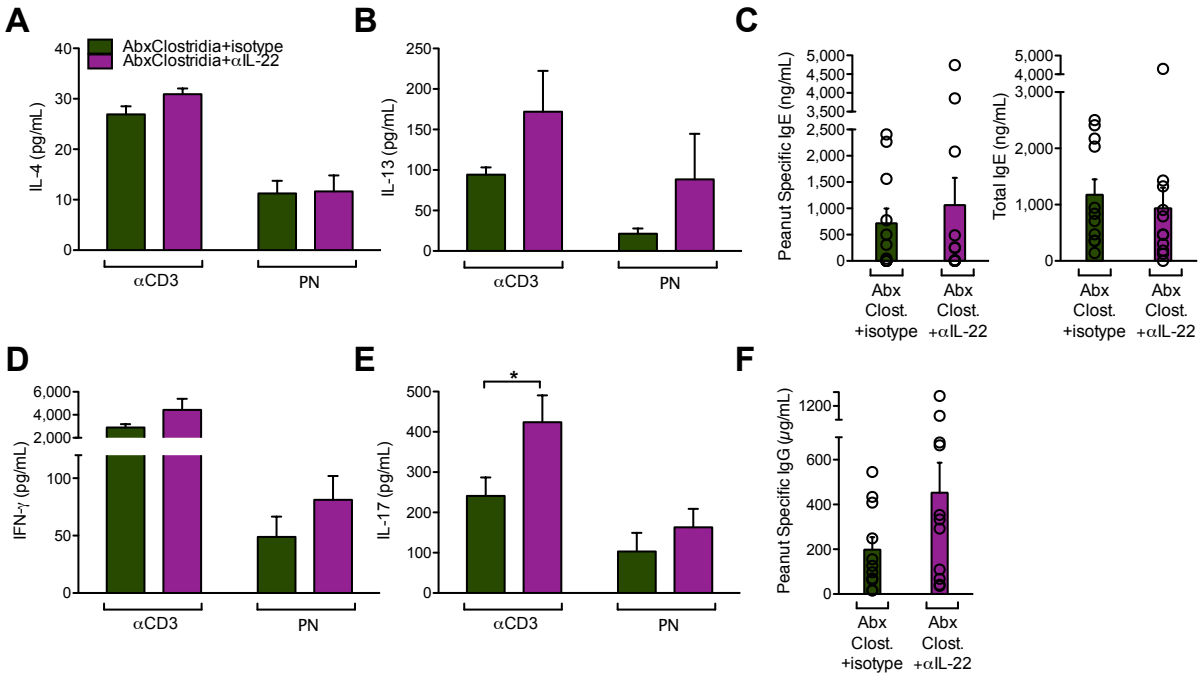


Figure 32: Neutralizing IL-22 after Clostridia colonization increases PN-specific responses after sensitization. Mice were treated with Abx and colonized with Clostridia as previously described. Neutralizing antibody to IL-22 or isotype control was administered 3 times per week beginning at weaning. Sensitization with PN+CT also began at weaning and continued for 5 weeks. After 5 weeks, mice were challenged with 2 doses of PN. Serum, feces, and splenocytes were collected 24 hours later. (**A**) IL-4 and (**B**) IL-13 concentration in splenocyte culture supernatant after stimulation with anti-CD3 or PN determined by multiplex. *n*=7 mice per group, representative of 2 independent experiments. (**C**) PN-specific and total IgE concentrations in serum determined by ELISA. *n*=11 mice per group, pooled from 4 independent experiments. (**D**) IFNγ and (**E**) IL-17 concentration in splenocyte culture supernatant after stimulation with anti-CD3 or PN determined by multiplex. *n*=7 mice per group, representative of 2 independent experiments. (**F**) PN-specific IgG concentrations in serum determined by ELISA. In **A, B, D,** and **E**, bars represent mean+S.E.M. In **C** and **F**, each dot represents an individual mouse; bars represent mean+S.E.M. *n*=11 mice per group, pooled from 4 independent experiments. * *P*<0.05 by Student's *t*-test.

We also analyzed the microbiota over the course of sensitization and treatment with the isotype or anti-IL-22 antibodies. By taxonomy-independent PCA analysis, both groups started with a very similar microbiota because all mice were littermates but as time went on, they segregated into two distinct communities based on treatment (Figure 33A). When we used taxonomy-dependent metrics to examine which bacterial populations changed, we found that mice receiving neutralizing antibody to IL-22 actually had increased abundance of the order

Clostridales in their feces compared to isotype-treated animals (Figure 33B), although Bacteroidales abundance was comparable between the two groups (Figure 33C). This suggests that, in addition to regulating intestinal permeability, IL-22 acts to titrate the abundance of Clostridia within the complex microbial community of the intestine, most likely through the production of RegIIIβ and RegIIIγ. It also reinforces how intrinsically connected these factors are; Clostridia are capable of inducing IL-22 which then acts to protect the host but also control the abundance of the Clostridia themselves.

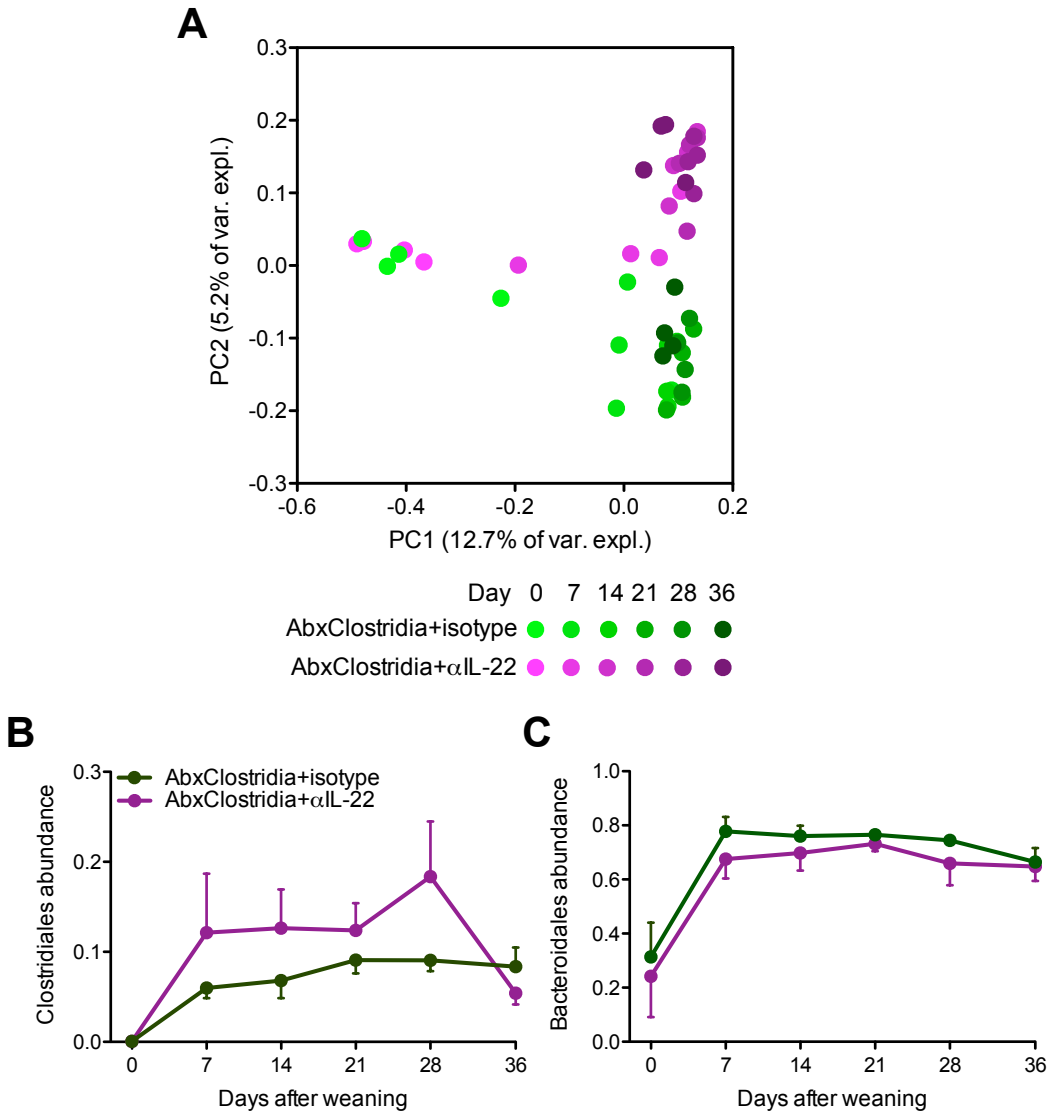


Figure 33: Neutralizing IL-22 after Clostridia colonization alters the composition of the commensal microbiota. Fecal samples collected from mice in Figure 32 were analyzed by Illumina sequencing of 16S rRNA. (**A**) PCA plot of unweighted UniFrac analysis of microbiota of isotype- or anti-IL-22-treated mice throughout sensitization. (**B**) Abundance of order Clostridiales or (**C**) Bacteroidales among total reads in fecal samples over time. In **A**, each dot represents an individual sample. In **B** and **C**, dots represent mean, bars represent S.E.M. *n*=4 mice per group, representative of mice in Figure 32.

Chapter 5: Clostridia-derived butyrate induces IL-22 production and may protect against food allergen sensitization

I. Clostridia consortium must be alive in order to induce *Il22* expression and reduce intestinal permeability

Once we identified our Clostridia consortium and determined that Clostridia-induced IL-22 was important for protection against sensitization, we were interested in determining how these bacteria were communicating with the host to induce this response. The two most simplistic hypotheses were: 1. through recognition of bacterial PAMPs via host PRRs (either extracellular or intracellular) or 2. via bacterial products/metabolites sensed by the host through a variety of receptors. To begin to dissect which, if either, of these hypotheses was true, we performed a simple experiment comparing the cytokine expression in the ileal and colonic LP of GF mice colonized with live Clostridia or treated with heat-killed Clostridia. In this system, both live and heat-killed Clostridia should signal via PRRs because both possess the same PAMPs, however only live Clostridia would be metabolically active. If hypothesis 1 was correct, then both groups should induce similar cytokine expression. If hypothesis 2 was correct, however, only the live Clostridia would stimulate cytokine expression.

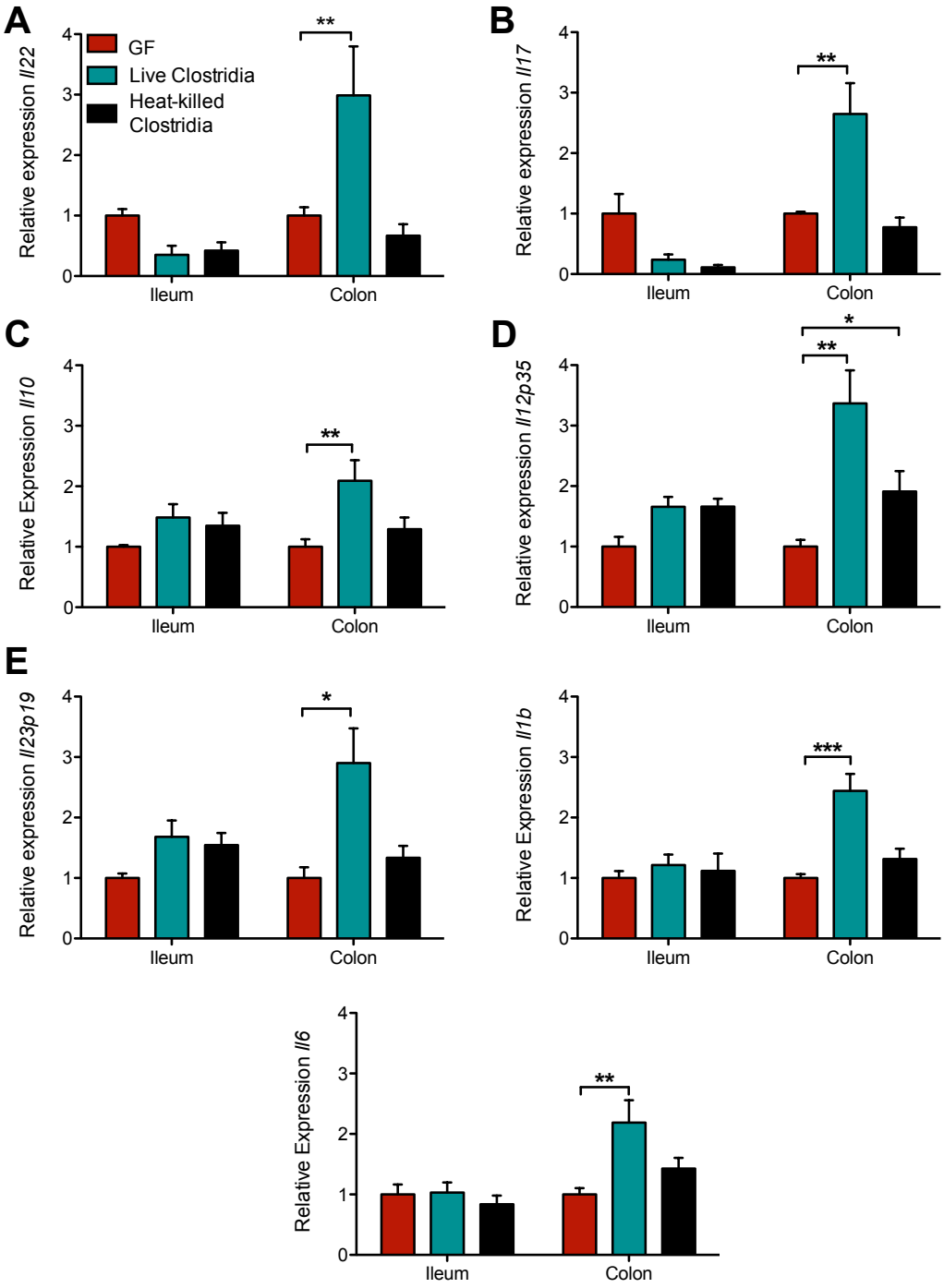


Figure 34: Clostridia must be alive in order to induce expression of *Il22* and related genes. GF mice were left untreated or were colonized with Clostridia for 4 days. Alternatively, GF mice were gavaged with autoclaved fecal homogenate from Clostridia colonized mice (heat-killed Clostridia) every other day for 4 days. RNA was collected from ileal and colonic LP. (**A**) Expression of *Il22*, (**B**) *Il17*, (**C**) *Il10*, (**D**) *Il12p35*, and (**E**) IL-22-inducing cytokines (*Il23p19*, *Il1b*, and *Il6*). Expression was measured by qRT-PCR, relative to *Hprt* and normalized to GF. Bars represent mean+S.E.M. *n*=3-4 mice per group, representative of 2 independent experiments. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 by two-way ANOVA with Bonferroni post-test.

As shown previously, colonization of GF mice with live Clostridia induced a significant increase in *Il22* expression in the colonic LP (Figure 34A). Treatment with heat-killed Clostridia, however, did not alter *Il22* expression compared to GF controls. The same was true of *Il17* expression (Figure 34B). IL-22 and IL-10 are in the same cytokine family, so we measured expression of *Il10* as well and found that only live Clostridia colonization significantly increased *Il10* expression (Figure 34C). Interestingly, both live and heat-killed Clostridia induced significantly more *Il12p35* expression than GF animals (Figure 34D), in keeping with the literature about using heat-killed bacteria as vaccine adjuvants (Yeung et al., 1998). We also examined expression of cytokines upstream of IL-22, including IL-23, IL-1β, and IL-6, because all are capable of inducing IL-22 production (Sonnenberg et al., 2011). Expression of all three of these IL-22-inducing cytokines was significantly increased in the colonic LP after colonization with live Clostridia, but there was no increase in expression after treatment with heat-killed Clostridia compared to GF mice (Figure 34E). In agreement with the *Il22* expression data, there were higher concentrations of Ara h 6 and Ara h 2 in the serum of GF mice treated with heat-killed Clostridia compared to those colonized with live Clostridia, which had very low levels of PN protein in their serum (Figure 35). Based on these results, we chose to follow up on hypothesis 2, that Clostridia's protective effect was mediated by metabolites.

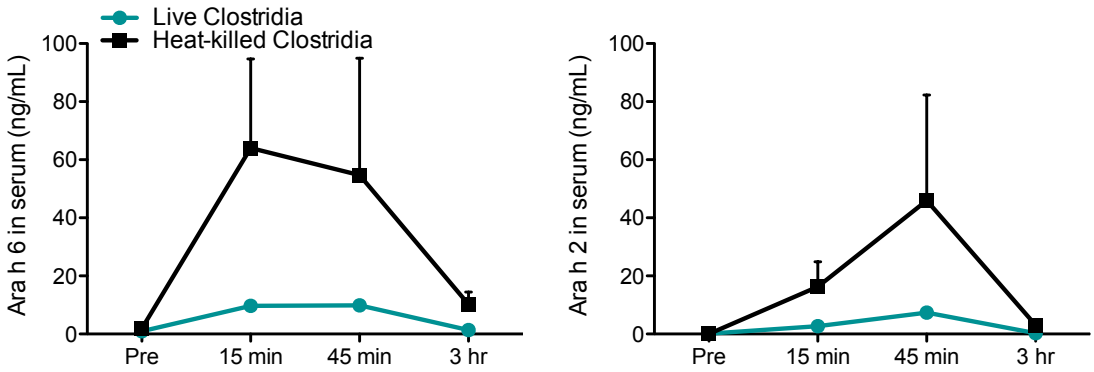


Figure 35: Clostridia must be alive in order to reduce serum concentrations of allergens. GF mice were treated as in Figure 34 for 6 days. Mice were fasted and then gavaged with 20mg PN. Mice were bled before gavage and at indicated timepoints after gavage. Ara h 6 and Ara h 2 concentrations were measured by capture ELISA. Dots represent mean, bars represent S.E.M. *n*=8-9 mice per group, pooled from two independent experiments.

II. Clostridia consortium produces high levels of acetate and butyrate

The microbiota is capable of producing an extremely wide variety of metabolites, both from components of the host diet and from products of other members of the community (Shapiro et al., 2014). To narrow down our search for potential protective metabolites produced by Clostridia, we used the sequencing data obtained during our initial characterization of the chloroform-extracted consortium. Instead of looking at bacterial abundance at the family level, we assigned a genus to as many reads as possible. Although the majority of the consortium could not be identified at the genus level, as most Clostridia remain unculturable and thus unclassified, about 25% of the total abundance could be assigned to one of five genra (Figure 36A). Within each genus, we also looked for the presence of different oligotypes, a strain-level designation defined by 100% sequence identity of the 16S rRNA gene segment. For four of the 5 genera, there were multiple oligotypes identified; at least 3 oligotypes were present in each genus (Figure 36B). This analysis indicates that there is an extensive amount of diversity contained within our consortium.

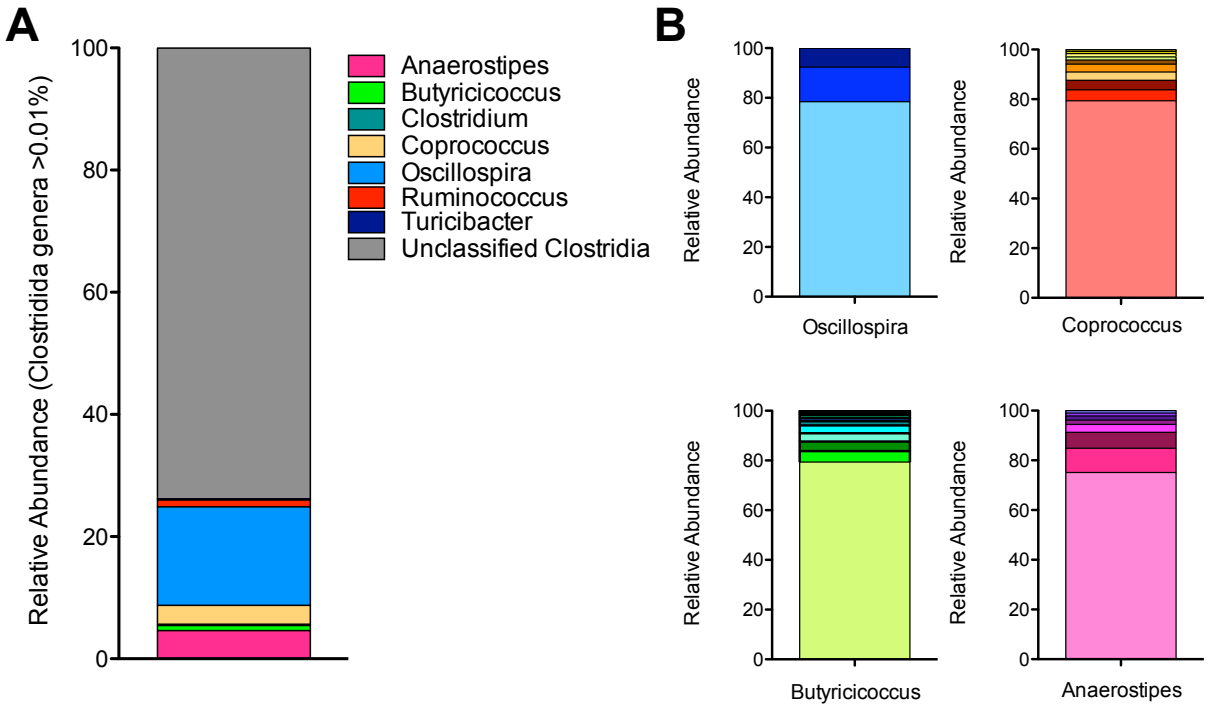


Figure 36: Clostridia consortium contains several potential butyrate producers. Illumina sequencing data of the 16S rRNA gene from a subset of 13 mice from Figure 10 was reanalyzed at different levels of classification. (**A**) Abundance of various Clostridia genera, representing >0.01% of total reads. (**B**) Abundance of unique oligotypes (100% sequence identity) within indicated genera.

It is also notable that the five genera identified share one common feature: the capacity to ferment dietary fiber (Geirnaert et al., 2014; Munoz-Tamayo et al., 2011; Pryde et al., 2002). One of the most important functions of the microbiota is to break down fibers that the host cannot metabolize on its own into SCFAs. The most abundant SCFAs in humans and mice are acetate, propionate, and butyrate, which are generally found in 60μM:25μM:15μM ratios, or ~3:1:1 (Tan et al., 2014). Because of the extensive immunomodulatory effects of SCFAs, we hypothesized that they could be some of the bacterial metabolites used by Clostridia to provide a protective signal to the host.

Using feces from GF, conventionalized, or Clostridia-colonized mice, we determined that our consortium produced high levels of both acetate and butyrate but minimal amounts of

propionate (Figure 37). This is in keeping with reports that most propionate is produced by Bacteroidetes species, while acetate and butyrate are produced predominantly by Firmicutes (Maslowski and Mackay, 2011; Russell et al., 2013). Colonization with a conventional microbiota increased concentrations of all three SCFAs, but our Clostridia consortium produced significantly more butyrate than the conventional microbiota. The molar ratio of acetate:butyrate produced by Clostridia was approximately 5:1. The concentration of acetate was higher than would be expected from other studies but this could be a function of the timepoint at which the samples were collected; the ratio of acetate:butyrate may decrease with time and reach 3:1 as estimated by other studies (Tan et al., 2014).

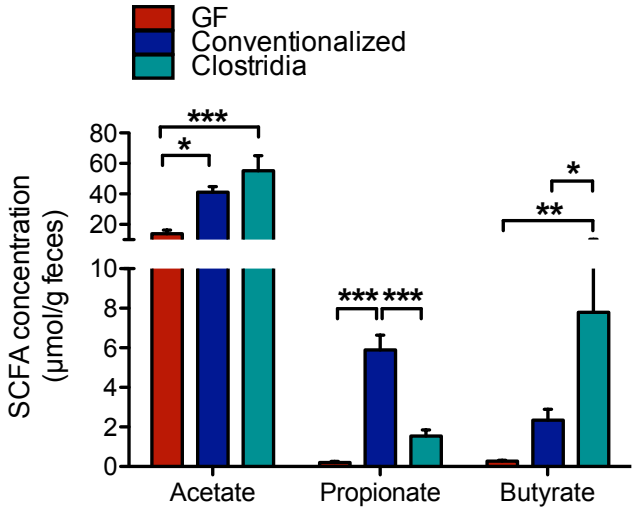


Figure 37: Clostridia consortium produces acetate and butyrate. SCFA concentrations in feces were determined by gas chromatography on samples collected from GF, conventionalized, or Clostridia-colonized mice 14 days post-colonization. Bars represent mean+S.E.M. *n*=10 mice per group, pooled from two independent experiments. **P*<0.05, ** *P*<0.01, *** *P*<0.001 by one-way ANOVA with Tukey post-test on each SCFA independently.

III. Butyrate, but not acetate, is able to induce *Il22* expression and reduce intestinal permeability

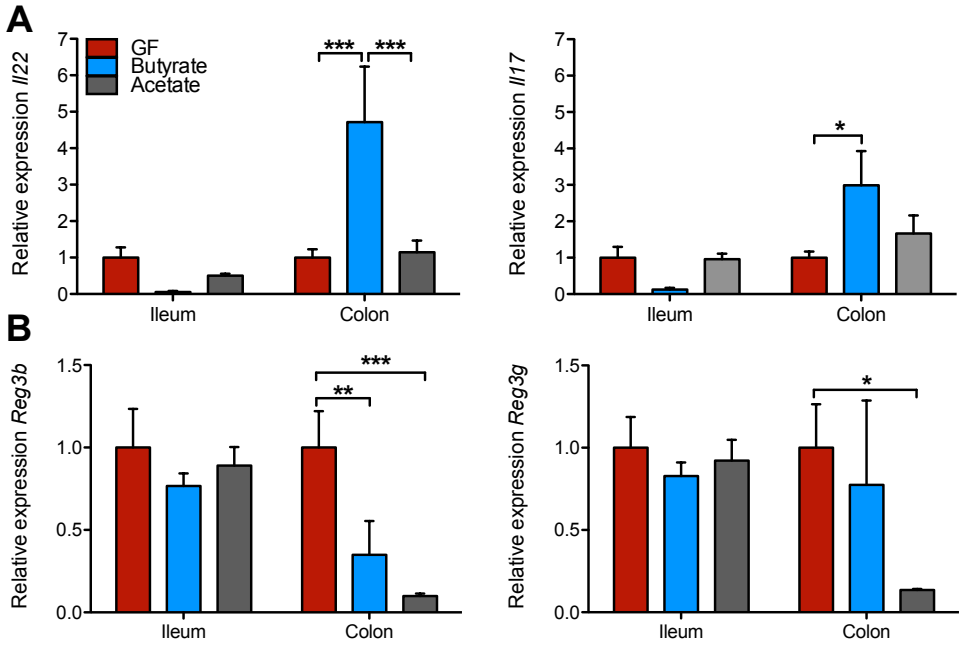


Figure 38: Butyrate, but not acetate, induces increased expression of *Il22* and *Il17* but not *Reg3b* or *Reg3g*. GF mice were left untreated or were gavaged with butyrate or acetate for 3 days. RNA was collected from ileal and colonic LP cells or IECs. (**A**) Expression of *Il22* and *Il17* in ileal and colonic LP cells. (**B**) Expression of *Reg3b* and *Reg3g* in ileal and colonic IEC. Gene expression was measured by qRT-PCR relative to *Hprt* and normalized to GF. Bars represent mean+S.E.M. *n*=4-5 mice per group, representative of 2 independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by two-way ANOVA with Bonferroni post-test.

To test whether or not either of these SCFAs was capable of inducing *Il22* expression, we administered sodium acetate or sodium butyrate to GF mice and assessed *Il22* expression in the ileal and colonic LP. Treatment with butyrate but not acetate significantly increased the expression of *Il22* and *Il17* in the colonic LP (Figure 38A), phenocopying the effect of colonization with Clostridia. Expression of *Reg3b* and *Reg3g* downstream of IL-22, however, was reduced in the presence of butyrate or acetate, suggesting that butyrate cannot recapitulate the entire Clostridia colonization phenotype (Figure 38B). Looking upstream of IL-22, butyrate did not significantly change expression of *Il23p19* (Figure 39A), however *Il1b* and *Il6* expression were significantly upregulated by butyrate (Figure 39B, C). Acetate had no effect on expression of any IL-22-inducing cytokines (Figure 39A-C). Butyrate's ability to induce *Il22* expression was not dependent on TLR4, indicating that its effect on gene expression was not due to contaminating LPS (Figure 40).

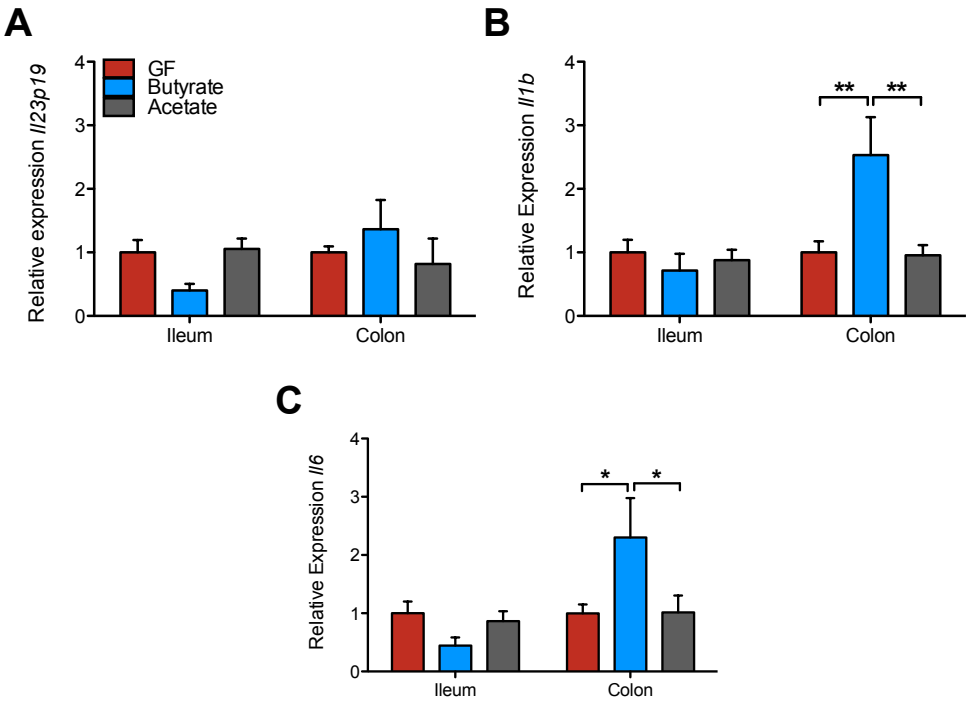


Figure 39: Butyrate induces expression of *Il1b* and *Il6*, but not *Il23p19*. Expression of cytokines upstream of IL-22 was measured in samples from Figure 38. (**A**) Expression of *Il23p19,* (**B**) *Il1b,* and (**C**) *Il6* in ileal and colonic LP cells. Expression was measured by qRT-PCR relative to *Hprt* and normalized to GF. Bars represent mean+S.E.M. *n*=4-5 mice per group. * *P*<0.05, ** *P*<0.01 by two-way ANOVA with Bonferroni post-test.

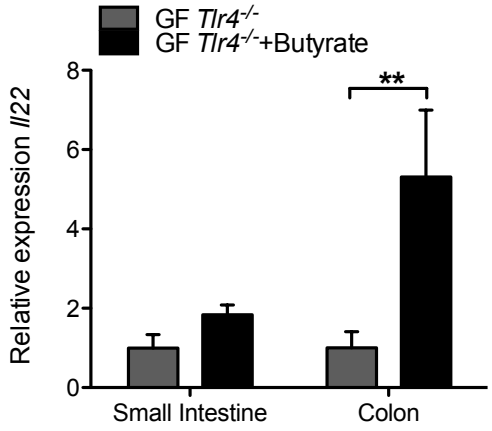


Figure 40: Butyrate induces *Il22* expression in a TLR4-independent manner. GF *Tlr4*$^{-/-}$ mice were treated with butyrate as in Figure 38. RNA was collected from the small intestine and colonic LP. *Il22* expression was measured by qRT-PCR, relative to *Hprt* and normalized to GF. Bars represent mean+S.E.M. *n*=4-5 mice per group. ** *P*<0.01 by two-way ANOVA with Bonferroni post-test.

To determine if butyrate-induced IL-22 has the same effect on intestinal permeability as Clostridia colonization, we challenged butyrate- or acetate-treated mice with PN by gavage and measured serum concentrations of Ara h 6 and Ara h 2. In agreement with the *Il22* expression data, butyrate-treated mice had reduced concentrations of both Ara h 6 and Ara h 2 in their serum compared to GF or acetate-treated animals (Figure 41). Together these results demonstrated that butyrate is a Clostridia-produced metabolite that can signal to the host to drive the key barrier protective responses we have identified as important for protection against food allergen sensitization.

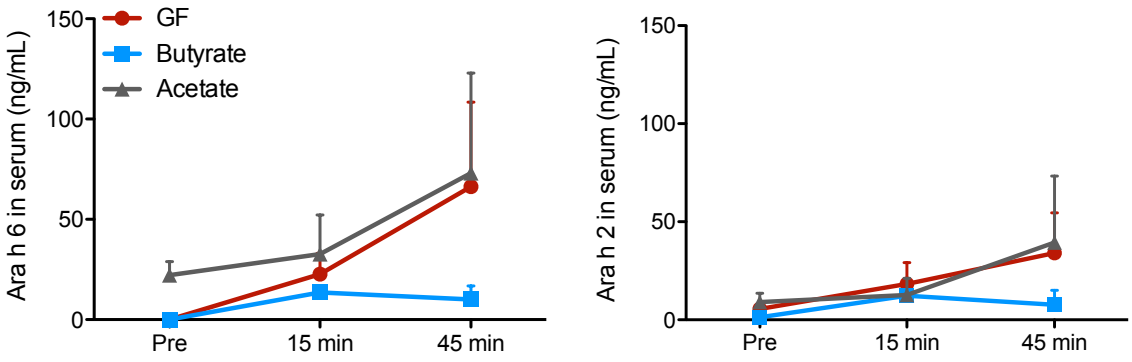


Figure 41: Butyrate reduces serum concentrations of PN proteins. GF mice were treated for 4 days with butyrate or acetate by gavage. Mice were then fasted and given a gavage of PN. Serum was collected before PN gavage and at indicated timepoints after. Ara h 6 and Ara h 2 concentrations in serum were determined by capture ELISA. *n*=4-5 mice per group.

IV. Butyrate induces IL-22 production in the ileum and colon by GPCR signaling

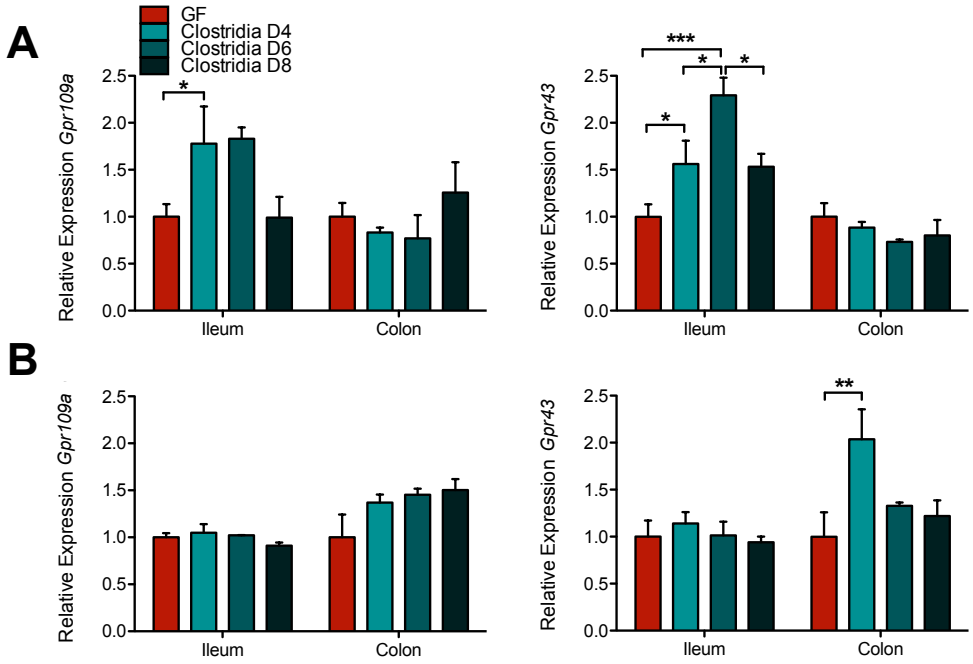


Figure 42: Clostridia colonization induces increased expression of butyrate receptors. GF mice were colonized with Clostridia for 4-8 days. RNA was collected from ileal and colonic IECs or LP cells. (**A**) Expression of *Gpr109a* and *Gpr43* in IECs or (**B**) LP cells. Expression was measured by qRT-PCR relative to *Gapdh* and normalized to GF. Bars represent mean+S.E.M. *n*=2-4 mice per group. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 by two=way ANOVA with Bonferroni post-test.

Butyrate is a very interesting molecule because it has a variety of different functions, each of which is mediated by different signaling pathways. Several reports show that butyrate can act as an HDACi in the nucleus to modify gene expression through modulation of chromatin accessibility. Some of this HDACi activity has been linked to active transport into cells via proton- or sodium-coupled monocarboxylate transporters (MCTs; (Singh et al., 2010; Stein et al., 2000)), although the necessity of these transporters for this function is not absolute (Chang et al., 2014). Butyrate is also a ligand for multiple GPCRs on the surface of a wide range of cells (Tan et al., 2014). In the immune cells of the intestine, the two most highly expressed GPCRs that are SCFA receptors are GPR43 (also called *Ffar2*) and GPR109a (also called *Niacr1*).

Butyrate binds to GPR109a with high affinity but is a lower affinity ligand for GPR43; acetate binds to GPR43 with very high affinity. A third GPCR, GPR41 (Ffar3) has been shown to mediate protective effects of propionate and is capable of binding butyrate but is not highly expressed in intestinal cells (Tan et al., 2014).

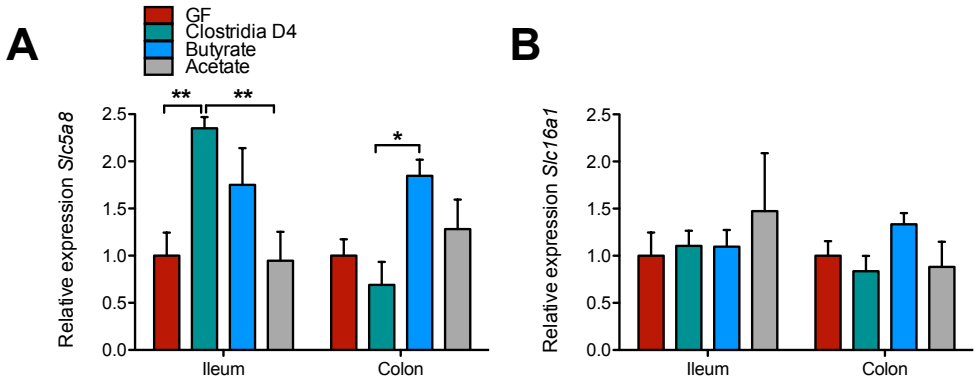


Figure 43: Clostridia and butyrate treatment increase expression of sMCT1 in IECs. GF mice were colonized with Clostridia or treated with butyrate and acetate as in Figure 37. RNA was collected from ileal and colonic IECs. Expression of (**A**) *Slc5a8* (sMCT1) and (**B**) *Slc16a1* (MCT1) was measured by qRT-PCR using Taqman primers/probes relative to *Gapdh* primers/probes and normalized to GF. Bars represent mean+S.E.M. *n*=4-6 mice per group. * *P*<0.05, ** *P*<0.01 by two-way ANOVA with Bonferroni post-test.

To understand if Clostridia colonization influences any of these butyrate signaling pathways, we assessed expression of *Gpr109a* and *Gpr43* in IECs and LP of GF versus Clostridia colonized mice. Expression of both GPCRs was increased in ileal IECs after colonization, but there was no change in expression in colonic IEC (Figure 42A). There was also no difference in expression of *GPR109a* in the LP of either the ileum or colon, but *GPR43* was significantly induced early after Clostridia colonization in the colonic LP (Figure 42B). Expression of the sodium-coupled MCT (sMCT1; *Slc5a8*) was also increased in ileal IECs after colonization, and was increased in both ileal and colonic IEC after butyrate but not acetate treatment (Figure 43A). The proton-coupled MCT (MCT1; *Slc16a1*) was unchanged by colonization or SCFA treatment (Figure 43B). Although these results do not directly link butyrate to GPCR signaling or intracellular transport, it is an interesting observation. Other reports

indicate that expression of SCFA receptors and transporters may depend on the microbiota but the data is limited (Cresci et al., 2010).

SCFAs have been implicated in immunoregulation and protection against inflammation through both HDACi activity and GPCR signaling, but much of this work has focused on induction of Foxp3^{+} Tregs or cytokine production from IECs. None of the previously described protective pathways provide a clear indication of how butyrate would induce IL-22, so we developed an *ex vivo* screening method to determine if one of the known butyrate signaling pathways could be linked to IL-22 production. As a positive control, we treated GF ileum and colon tissue with recombinant mouse IL-23 and measured IL-22 protein levels in the culture supernatant by ELISA 24 hours later. As predicted, there was significantly increased IL-22 production from both tissues in response to IL-23 stimulation (Figure 44A, B).

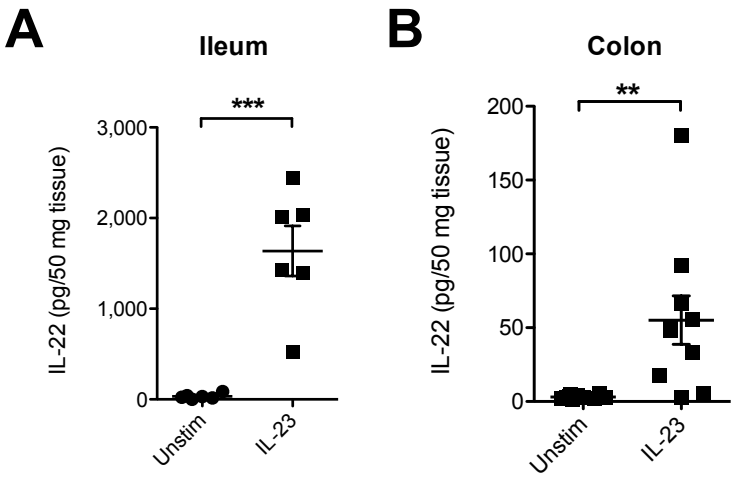


Figure 44: Treatment of GF ileum and colon tissue with IL-23 drives IL-22 production. Ileum and colon tissue from GF mice was stimulated in culture with 10ng/mL recombinant mouse IL-23 for 24 hours. (**A**) IL-22 production in ileum tissue and (**B**) colon tissue was measured in culture supernatants by ELISA. Each dot represents an individual tissue sample; bars represent mean±S.E.M. *n*=10 samples per group, pooled from two independent experiments. ** *P*<0.01, *** *P*<0.001 by Student's *t*-test.

Using this same system, we tested the effect of butyrate on IL-22 production to confirm our *in vivo* expression data. In both the ileum and the colon, there was significantly increased production of IL-22 after 24 hours of stimulation with butyrate (Figure 45A, C). The variability of the response was high, but this could be due to the crude nature of the preparation; the cultures were based on tissue weight and thus did not allow for normalization of the number of IL-22

producing cells per well. In spite of this limitation, this was a useful method as a first-pass screen to narrow down the method of signaling. Treatment with butyrate plus CHC, an MCT inhibitor, did not significantly alter IL-22 production in either the ileum or the colon, although there was some reduction in the ileum suggesting intracellular transport may contribute to production of IL-22 (Figure 45A, C). PTx inhibits the activity of all GPCRs, and was used to assess the necessity of GPR43 and/or GPR109a signaling to IL-22 production. Treatment with PTx alone did not alter production of IL-22 as compared to unstimulated tissue, however treatment with butyrate plus PTx significantly reduced IL-22 production in the ileum (Figure 45B) and brought IL-22 back down to nearly untreated levels in the colon (Figure 45D). This result indicated that GPCR signaling was required for production of IL-22 in response to butyrate. Further studies are ongoing using *Gpr109a*$^{-/-}$ mice to determine whether this receptor is involved.

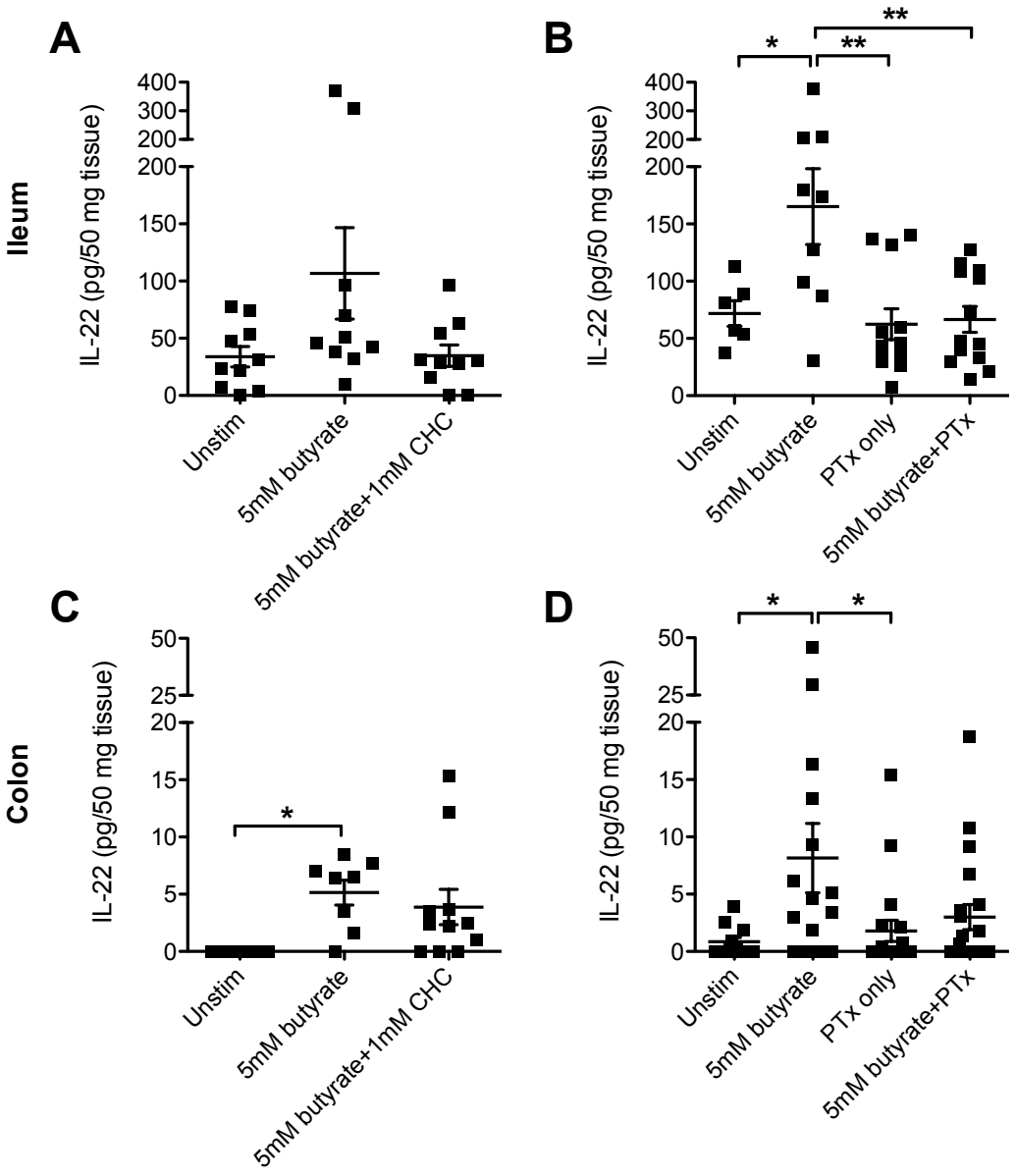


Figure 45: Treatment with butyrate stimulates IL-22 production through GPCRs. Ileum and colon tissue from GF mice was cultured in the presence of butyrate or indicated inhibitors for 24 hours. (**A**) IL-22 production from ileum after treatment with butyrate or butyrate+CHC. (**B**) IL-22 production from ileum after treatment with butyrate, PTx alone, or butyrate+PTx. (**C**) IL-22 production from colon after treatment with butyrate or butyrate+CHC. (**D**) IL-22 production from colon after treatment with butyrate, PTx alone, or butyrate+PTx. IL-22 production was measured in culture supernatants by ELISA. Each dot represents one tissue sample; bars represent mean±S.E.M. *n*=6-20 samples per group, pooled from at least two independent experiments. * $P<0.05$, ** $P<0.01$ by one-way ANOVA with Tukey post-test.

There are also reports describing a feedback loop between IL-22 and IEC-produced IL-18 (Munoz et al., 2015) as well as studies demonstrating that SCFAs exert their protective effects by promoting IL-18 production from the epithelium (Macia et al., 2015; Singh et al., 2014). We screened our cultures for IL-18 and found that, in contrast to what was seen in other reports, IL-18 production was significantly reduced in the ileum under IL-22-inducing conditions (Figure 46A). In the colon, there was no significant change in the IL-18 production in response to stimulation (Figure 46B). Consequently, we hypothesized that IL-18 does not play an important role in our model.

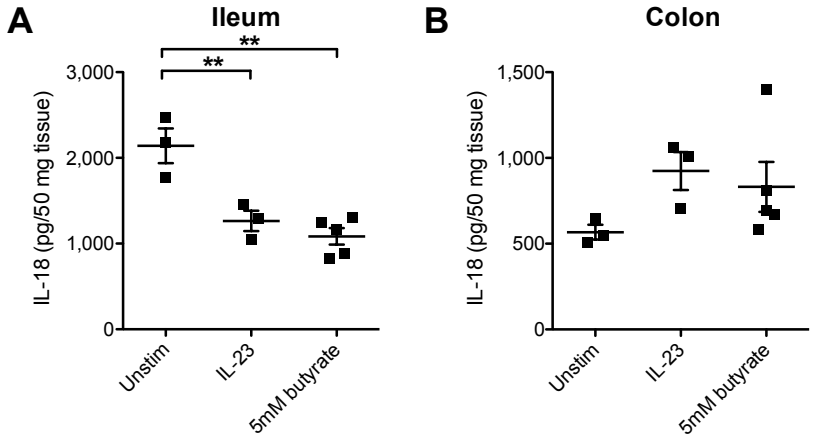


Figure 46: IL-23 and butyrate treatment do not change IL-18 production. IL-18 production was measured by ELISA in a subset of culture supernatants from Figure 45. (**A**) IL-18 production in ileum tissue cultures. (**B**) IL-18 production in colon tissue cultures. Each dot represents an individual tissue sample. *n*=3-5 samples per group; bars represent mean±S.E.M. ** *P*<0.01 by one-way ANOVA with Tukey post-test.

V. Eliminating dietary fiber increases intestinal permeability to PN

Since butyrate is derived from the metabolism of dietary fiber, we hypothesized that removing fiber in SPF mice would increase intestinal permeability to PN and subsequently increase sensitization to food antigens. To address this hypothesis, we administered a control (5% cellulose) or fiber free diet to SPF WT mice, which do not have defects in permeability or sensitization (see Figure 2 and Figure 26). After one week of fiber free diet feeding, these mice had significantly more Ara h 6 in their serum than control diet-fed littermates after PN challenge

(Figure 47). The increase in permeability after removing dietary fiber was even greater than the increase we measured after Abx treatment, indicating that dietary fiber does influence intestinal permeability. Although preliminary, this result suggests that the metabolism of dietary fiber into SCFA, particularly butyrate, could be involved in the regulation of intestinal barrier responses. Experiments to examine the effect of fiber free diet on the generation of PN-specific IgE and IgG in response to sensitization are ongoing.

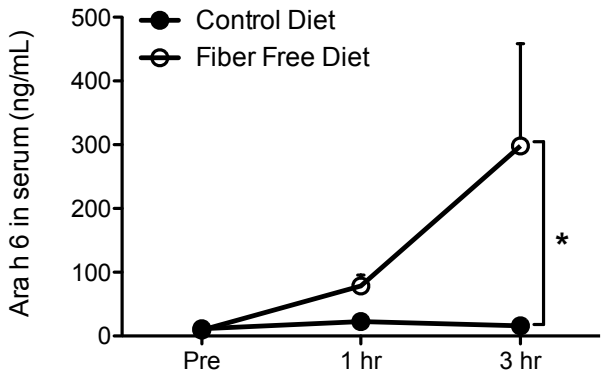


Figure 47: Feeding a fiber free diet increases intestinal permeability to PN. SPF mice were fed a control diet containing 5% cellulose or a purified, fiber free diet for 1 week beginning at weaning. After 7 days, mice were fasted overnight and challenged with 20mg PN by gavage. Serum was collected before gavage and at indicated timepoints after. Dots represent mean, bar represent S.E.M. *n*=6-11 mice, pooled from multiple experiments. * *P*<0.05 by two-way ANOVA with Bonferroni post-test.

Chapter 6: Discussion and future directions

I. Clostridia prevent sensitization to food antigens through novel barrier-protective mechanisms

To understand if environmental factors contribute to the increasing incidence of allergic disease, we investigated the role of the intestinal microbiota in regulating susceptibility to sensitization to food allergens. We found that mice treated with a cocktail of broad-spectrum Abx or GF mice that were raised in the absence of a colonizing microbiota generated much higher concentrations of PN-specific IgE in response to sensitization with PN+CT than WT SPF controls. We used selective colonization of GF mice with a series of different bacterial populations to identify protective taxa. Colonization of with a consortium of Clostridia, composed of 70 OTUs isolated from the feces of SPF mice using chloroform extraction, was particularly effective at rescuing intestinal morphology and immunological homeostasis by restoring normal, SPF proportions of Tregs in the colonic LP and increasing fecal IgA levels. After sensitization with PN+CT, mice colonized with this Clostridia consortium produced reduced levels of PN-specific IgE compared to GF animals. A Clostridia-containing microbiota also reduced PN-specific responses to sensitization after Abx treatment. Together these results confirmed our hypothesis that the microbiota is involved in regulating responses to food, as perturbations to the homeostatic host-microbe interactions allowed for the development of a PN-specific response while restoration of a specific bacterial population was sufficient to prevent sensitization.

When we examined the effect of Clostridia colonization on the host in order to understand how the microbiota provided protection against sensitization, we found that, in addition to induction of Tregs and IgA, Clostridia induced an innate IL-22-dependent response early after colonization. Colonization with *Bacteroides uniformis*, which was not protective

against sensitization and did not affect the Treg or IgA compartments, did not stimulate this pathway. The IL-22 response was characterized by increased epithelial expression of the AMPs *Reg3b* and *Reg3g* as well as increased goblet cell numbers and mucus production. Together, this suite of responses acted to reinforce the intestinal epithelial barrier and reduce the amount of orally administered antigen that entered systemic circulation; concentrations of two PN proteins were reduced in the serum of Clostridia-colonized mice compared to GF or Abx-treated mice. IL-22 was both necessary and sufficient to mediate this reduction in intestinal permeability, measured by serum allergen concentrations. We reasoned that if less allergen was available to the immune system via the blood, then there would be reduced levels of systemic sensitization. In support of this hypothesis, treatment with neutralizing antibody to IL-22 increased PN-specific responses in Abx-treated, Clostridia-colonized mice, demonstrating that Clostridia protect against sensitization to food in an IL-22 dependent manner.

Earlier work suggested that members of the Clostridia class provide protection against intestinal inflammation. The Clostridia-containing consortium ASF has long been used as a neutral or protective bacterial population and was shown to reduce the spontaneous generation of IgE and induce colonic Tregs in GF mice (Cahenzli et al., 2013; Geuking et al., 2011). Two other studies demonstrated that consortia of Clostridia, one derived from mouse feces and one derived from human feces, induce Tregs in the colonic LP in a manner similar to our consortium (Atarashi et al., 2013; Atarashi et al., 2011). Much like the bacteria we isolated and characterized from our SPF mice, both of these consortia contain a diverse set of Clostridia species that similarly fall into clusters IV, XIVa, and XIVb. These bacteria, whether obtained from humans or mice, were also shown to have a suppressive effect on a variety of intestinal inflammatory conditions, including production of OVA-specific IgE after i.p. immunization with OVA+alum. The effect of these consortia on innate immune responses, however, was not

explored. The data presented in this thesis, and the resulting publication (Stefka et al., 2014), are the first to describe a protective role for IL-22 signaling in the context of food allergy.

II. New model for maintenance of oral tolerance to dietary antigens

Based on these results above, we propose a new paradigm for oral tolerance and the prevention of aberrant responses to food (Feehley and Nagler, 2014). It is well accepted that oral tolerance requires an antigen-specific response in the small intestine, characterized by antigen-specific Tregs generated in the MLN by $CD103^+$ DCs and RA plus TGFβ, that then traffic back to and expand in the small intestine LP. We have now demonstrated that there is also a requirement for a Clostridia-induced response for the maintenance of tolerance to dietary antigen. This response is centered around innate IL-22 produced primarily by ILC3s, which drives AMP and mucus production to reinforce the epithelial barrier (Figure 48). Together, these mechanisms limit the amount of allergen that gains access to systemic circulation by reducing intestinal permeability. With less allergen crossing from the intestinal lumen into the LP and the blood stream, there is less allergen available to the immune system, thereby reducing the opportunity for systemic sensitization. In the absence of IL-22-inducing bacteria or after neutralization of IL-22, the barrier protective response is lost, and there is an increased response to dietary antigens.

This new model is particularly attractive as an explanation the growing prevalence of allergic disease over a very short time. Although a lack of antigen-specific Tregs may be the cause of food allergy in some individuals, there is no evidence suggesting that the environment affects induction of antigen-specific Tregs in the small intestine. Failure of this response alone cannot explain the major increase in food allergies in only one generation. The bacteria-induced mechanisms we have identified, however, are profoundly affected by external factors, particularly lifestyle changes adopted in the past several decades, and can also contribute to

increased sensitization and pathology when disrupted. These alterations, on a population scale, could explain the rising disease prevalence. Thus, the integration of these two models can better explain the epidemiological data and will provide novel insights into ways to reverse the allergic trends.

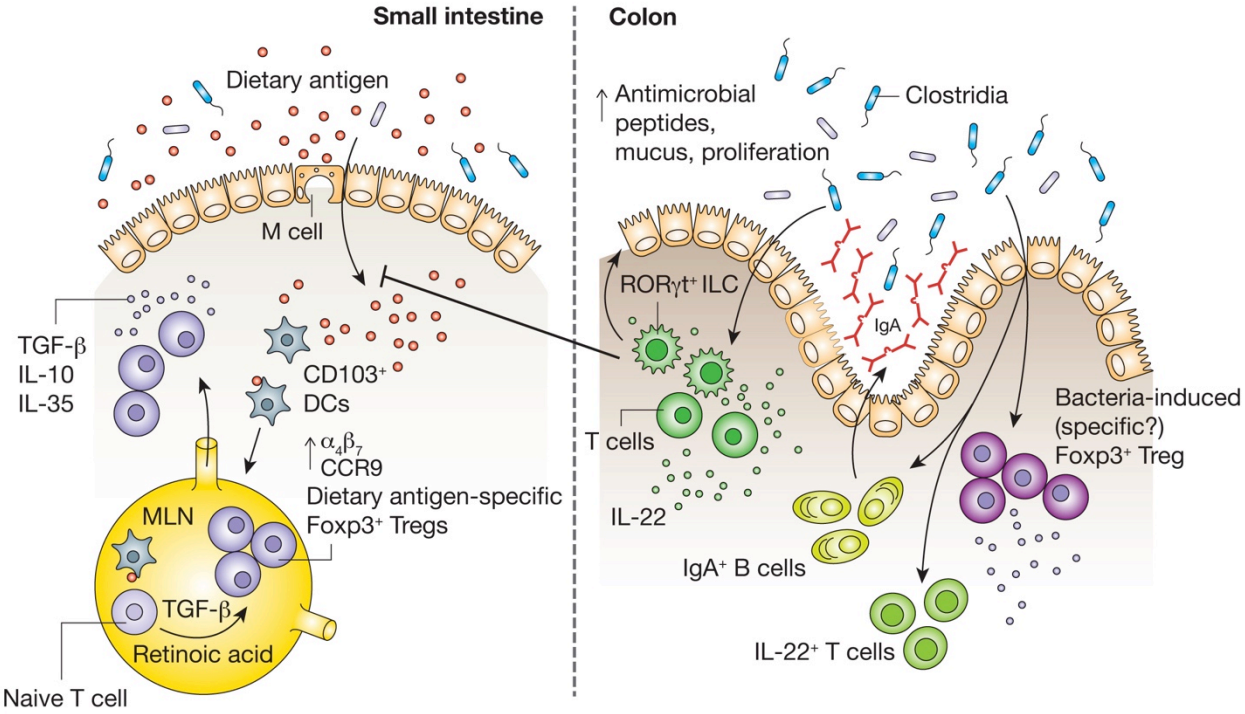


Figure 48: Tolerance to dietary antigen requires an antigen-specific response in the small intestine and a bacteria-induced, barrier-protective response in the colon. In the small intestine, dietary antigen is taken up by $CD103^+$ DCs that then traffic to the MLN where they present this antigen to naïve T cells in the presence of RA and TGFβ. Under these conditions, the naïve T cells differentiate into antigen-specific $Foxp3^+$ Tregs and upregulate the small intestine homing receptors $\alpha_4\beta_7$ and CCR9, allowing them to return to the small intestine LP where they can produce immunoregulatory cytokines and prevent inflammation upon reexposure to antigen. In the colon, colonization with bacteria, particularly Clostridia, promotes production of IL-22 by $ROR\gamma t^+$ ILC and T cells, which drives production of AMPs and mucus. This IL-22 program then reduces intestinal permeability to food antigens, preventing further uptake of antigens into the blood stream and reinforcing the tolerance maintained by antigen-specific Tregs. Clostridia colonization also drives induction of $Foxp3^+$ Tregs in the colonic LP and production of IgA at later timepoints that can reinforce colonic tolerance to promote homeostasis throughout the intestine.

Making the epithelial barrier an important checkpoint in the development of food allergies is a change in the way this disease has often been conceptualized but there are human studies that suggest the epithelium is important. Increased intestinal permeability has been documented

in both mouse models and human disease in response to allergen challenge as a result of mast cell degranulation (Groschwitz and Hogan, 2009) but increased intestinal permeability has also been reported multiple times in patients with food allergies at steady-state, in the absence of an allergic response (Jackson et al., 1981; Jarvinen et al., 2013; Ventura et al., 2006). Although these studies do not examine the causes of increased permeability, the same genetic or environmental factors that underlie the initial sensitization events, including dysbiosis, could contribute to inappropriate intestinal permeability.

Our ability to detect PN protein by ELISA in the serum also speaks to another feature of sensitization and allergy that is not often discussed. Classically, food proteins are thought to be broken down by digestive enzymes in the stomach and proximal small intestine so they can be absorbed by the time they reach the ileum. Our assay requires, however, that secondary protein structure of Ara h 6 and Ara h 2 remains intact in order to be detected by capture antibodies in the ELISA. It has been documented that common food allergens are resistant to proteolytic degradation in the stomach and this has been proposed as the cause of their allergenicity (Astwood et al., 1996). The model we present fits nicely with this hypothesis. Food proteins that can resist degradation will reach the intestinal barrier in an antigenic form. In individuals with dysbiosis or under other allergy-promoting conditions, there may be increased intestinal permeability allowing these proteins to enter circulation and elicit an immune response, leading to systemic sensitization and an allergic response.

A later, adaptive phase of the bacteria-induced response includes induction and expansion of colonic Tregs and production of IgA. These food antigen non-specific responses may help maintain and reinforce the antigen-specific tolerance mechanisms by further neutralizing luminal antigens in the distal intestine and limiting inflammatory responses. In light of new studies published after our initial characterization of the Clostridia-induced response, it would be interesting to revisit the role of these Tregs and IgA in protection. The Tregs we

identified in the colonic LP after introduction of Clostridia were defined only by their expression of CD4 and Foxp3. Recent evidence suggests that these Clostridia-induced Tregs may coexpress RORγt, making them particularly capable of suppressing Th2 responses to sensitization with PN+CT (Ohnmacht et al., 2015; Sefik et al., 2015). Since we found a central role for RORγt^{+} cells based on their production of IL-22, perhaps there is a fundamental connection between the program dictated by this transcription factor and protection against an allergic response.

We also did not characterize the specificity of the Clostridia-induced IgA in our model. Although it is increased upon colonization, it is not clear whether or not this IgA response is directed against the Clostridia consortium itself or against other luminal antigens like food. It is also not clear what causes the increased IgA production. There is evidence that ILC3s are capable of stimulating an IgA response, which would fit nicely with the central role of ILC3s in regulating intestinal permeability (Kruglov et al., 2013). It is also possible, however, that the IgA is produced in a T cell-dependent manner in response to TLR signaling (Kubinak et al., 2015a), or as a function of Clostridia localization in the intestine, close to the epithelium (Bunker et al., 2015). Further study into the signals driving IgA production after colonization may help elucidate the function of Clostridia-induced IgA in protection against sensitization.

III. Is IL-22 necessary and sufficient to prevent sensitization?

One limitation to our study is confirming the link between intestinal permeability and sensitization to food allergens. In most of the conditions we explored, reduced permeability as measured by reduced serum concentrations of PN proteins after gavage correlated with reduced production of PN-specific antibodies after sensitization with PN+CT. The two cases where the connection is not as strong are after administration of IL-22Fc to Abx-treated mice and after treatment with anti-IL-22 to neutralize IL-22 after colonization of Abx-treated mice with

Clostridia. Although both treatments significantly altered intestinal permeability, the effect on sensitization was not as dramatic. In both of these situations, we administered a biologic agent at a single dose and on an empiric schedule. When we assessed PN-specific IgE responses after PN+CT sensitization in conjunction with these treatments, there was no change compared to isotype control-treated animals. We interpreted these results to mean that these reagents had off-target effects. Both IL-22Fc and anti-IL-22 contain mouse constant regions that can bind to Fc receptors. It is know that signaling through these receptors can profoundly modulate immune responses (Bruhns and Jonsson, 2015). It seems possible that off-target effects of this binding impacted our results. Another confounding variable is the altered abundance of Clostridiales in response to manipulation of the IL-22 pathway (Figure 33). Although we only changed one experimental variable (anti-IL-22 administration) and used littermate controls, multiple biological pathways were modulated. After administration of anti-IL-22, the abundance of Clostridia increased, making it impossible to interpret the role of IL-22 in isolation, especially since Clostridia were the very bacteria we were trying to investigate. The effect of changing the microbiota throughout the course of sensitization, particularly from days 0 to 14 of sensitization, may have had unintended consequences on Th2 responses and production of PN-specific IgE.

We cannot rule out the possibility that IL-22 may regulate intestinal permeability but that this does not affect sensitization to food allergens. In order to directly address this concern and validate our findings in an independent system, we will employ some newly developed mouse models. Instead of blocking the effect of IL-22 with an antibody, we can utilize mice conditionally deficient in STAT3, a component of the signaling cascade required for the production of IL-22 as well as the response to IL-22, in RORγt^{+} cells including ILC3s and Th17 cells (*Rorc*^{Cre}*Stat3*$^{fl/fl}$ mice). These mice cannot produce IL-22 despite the presence of the cells that should be major IL-22 producers (Guo et al., 2014). Sensitization of these mice at baseline or after Abx treatment and Clostridia colonization will provide a better model to test the requirement for IL-22

to prevent a PN-specific IgE response in the absence of confounding treatments. It has also recently been published that IL-17 can regulate intestinal permeability under conditions of epithelial damage and inflammation (Lee et al., 2015; Maxwell et al., 2015). IL-17 is coregulated with IL-22 in our model, so perhaps blockade of both IL-22 and IL-17 together would have an additive effect on intestinal permeability to food and increase downstream sensitization, better establishing a role for changes in permeability in regulating food antigen-specific responses.

It also is conceivable that Tregs, which are not affected by IL-22 since they do not express the IL-22 receptor, can compensate for the lack of IL-22 over the course of sensitization (five weeks) and suppress the generation of PN-specific responses in spite of increased circulating antigen concentrations. We attempted to address the sufficiency of Tregs to prevent sensitization multiple times, with limited success. Transfer of $Foxp3^+$ cells pooled from the spleen or MLN of untreated SPF mice to Abx-treated mice had minimal effect on PN-specific IgE concentrations, possibly due to an inability of the transferred cells to home to the intestinal LP or because they expressed TCRs that were not of the appropriate specificity (ie. not bacteria specific), limiting their ability to function in this system. A similar transfer of $Foxp3^+$ Tregs from the MLN or colonic LP of SPF mice, which should have homed back to the LP easily, into Abx-treated mice did reduce PN-specific IgE concentrations but PN-specific IgG1 levels remained elevated, so protection was not fully equivalent to the response in NT mice. Finally, once we identified Clostridia as the important protective members of the microbiota, we transferred Clostridia-induced Tregs from gnotobiotic mice into Abx-treated recipients. This transfer also failed to rescue the sensitization phenotype; mice receiving these $Foxp3^+$ cells had PN-specific IgE and IgG1 levels comparable to Abx-treated mice as opposed to Abx-treated Clostridia-colonized mice.

From these experiments we concluded that $Foxp3^+$ Tregs were not sufficient to protect against food allergen sensitization, despite other reports in different models of food allergy

(Hadis et al., 2011; Noval Rivas et al., 2013; Yamashita et al., 2012). Because we wanted to only transfer the bacteria-induced, bacteria-specific Tregs, however, we had a limited number of cells to work with. It is possible that the number of cells transferred was not sufficient to affect sensitization. We also may have transferred populations of Tregs that were not as bacteria-specific as we intended. It is now clear that the nodes of the MLN are distinct and that the colon-draining nodes are populated by different cell subsets than the nodes associated with the small intestine (Houston et al., 2015). There is also evidence that the iliac lymph nodes (ILN) are important sites of induction of tolerance to colonic antigens (Veenbergen et al., 2015). Our results may have been clearer if we transferred a pool of Tregs from the colonic LP plus colon-draining MLN and ILN as these would be the most likely to represent the TCR specificities in the colon and have the capacity to repopulate this site. The efficacy of the Treg transfer might also be enhanced by enriching for and transferring a more specific subset of the colonic Treg population, particularly the RORγt^{+} Foxp3^{+} double positive population that is particularly important for blocking Th2 responses (Ohnmacht et al., 2015). After transfer, we were not able to track the transferred cells due to a lack of congenic markers, making it difficult to know whether or not the cells or their progeny survived the entire sensitization protocol. As a result, we do not rule out a protective effect of both bacteria-specific and food antigen-specific Tregs in establishing and maintaining tolerance to food but instead suggest that both an innate, IL-22 mediated response to the microbiota and a late, adaptive phase of the response cooperate to prevent aberrant reactions to harmless dietary antigens.

IV. Additional ways Clostridia may regulate intestinal permeability

Although we examined the IL-22-mediated barrier protective effects of Clostridia colonization, there are several additional facets of the intestinal epithelial barrier that may be altered in response to Clostridia that we have not yet examined. In two different colitis models,

genetic deletion or antibody-mediated blockade of IL-17 exacerbated disease. One study proposed that the absence of IL-17 altered occludin localization at the epithelial barrier leading to increased permeability and inflammation (Lee et al., 2015) while the other study demonstrated differential cytokine production and cellular infiltration in response to IL-17 neutralization (Maxwell et al., 2015). Since a change in occludin localization in the absence of IL-17 was sufficient to increase intestinal permeability, it follows that Clostridia-induced IL-22 may also affect tight junction proteins to alter intestinal permeability to food. In preliminary experiments, Clostridia colonization did not dramatically alter the expression of a panel of tight junction proteins, including a variety of claudins and occludin, but it may be that localization or organization of these proteins at the junctions in IECs is more important than changes in mRNA expression. Further studies regarding the effect of Clostridia and IL-22 on the integrity and composition of the epithelial tight junctions might help to elucidate the role of these factors in sensitization.

Another study was published very recently describing a gut-vascular barrier (GVB) similar to the blood-brain barrier (Spadoni et al., 2015). This GVB is maintained by endothelial cells rather than epithelial cells, but it functions as another structure to compartmentalize and contain the intestine and its contents to prevent excessive inflammation. Under homeostatic conditions, this GVB does not allow large molecules to permeate from the blood into the intestinal lumen or the converse, but under conditions of infection, this selectivity is lost and there is passage of large molecules into the blood stream. The proteins that maintain this barrier are related to those that form the epithelial barrier including occludin and various claudins. Although not investigated in the initial report, it would be interesting to examine whether or not the microbiota has a role in the establishment or maintenance of this GVB. It is tempting to speculate that Clostridia could have an effect on this barrier as well as the epithelial barrier in order to reduce the amount of allergen that reaches systemic circulation.

An additional consideration when measuring intestinal permeability is gut motility and transit time. It stands to reason that if luminal contents are moving faster as a result of increased peristalsis, there would be less opportunity for absorption or leakage of proteins and other contents across the intestinal barrier. Our Clostridia consortium was recently shown to stimulate production of serotonin (5-HT) in the intestine (Yano et al., 2015). A consequence of this 5-HT production was increased gut motility compared to GF mice. Indeed, GF mice are well-documented to have slower rates of transit of intestinal contents than SPF controls (Smith et al., 2007). Another study showed that the microbiota interacts with macrophage populations in the muscle layer to regulate gut motility (Muller et al., 2014). Together, these findings suggest that some of the reduced serum concentrations of Ara h 2 and 6 after colonization with Clostridia are attributable to more rapid clearance of these proteins from the lumen. Although this is not an IL-22 related mechanism, it is still a unique feature of Clostridia colonization. One way to address this influence of increased intestinal motility would be to perform the same Ara h 2/6 capture ELISAs on fecal homogenates collected over time after PN gavage instead of serum and compare if or when the proteins appear in the feces of GF versus Clostridia-colonized or Abx-treated versus Abx-treated Clostridia-colonized mice. This is a less satisfying model immunologically but it is a potential explanation for some of our findings and an important consideration for extending these studies into other contexts.

V. Role of microbial sensing by the host in Clostridia-mediated protection

After identifying Clostridia as allergy-protective members of the microbiota, we wanted to understand how this consortium was signaling to the host in order to induce protection. Previous work from our lab implicated TLR4 in protection against sensitization as TLR4-deficient C3H/HeJ mice produced increased PN-specific IgE compared to TLR4-sufficient counterparts (Bashir et al., 2004). We confirmed this finding using global TLR4 knock-outs on the C57BL/6

background. *Tlr4*$^{-/-}$ mice had increased PN-specific responses compared to *Tlr4*$^{+/-}$ littermates, demonstrating that changes in host genetics can drive an allergic phenotype that mimics the absence of certain members of the microbiota in WT animals.

Clostridia, however, are not thought to signal via TLR4 since they are Gram positive bacteria and do not express LPS on their surface. Additionally, SPF TLR4-deficient mice have a replete microbiota that contains a normal proportion of Clostridia and yet they remain susceptible to sensitization. We investigated this paradox in two different ways. First, we examined the susceptibility to sensitization in *Tlr2*$^{-/-}$ mice to understand whether the increased PN-specific responses occur in mice deficient in any individual TLR or if it is unique to animals lacking TLR4. TLR2 was the first choice for comparison because it is often considered the counterpart to TLR4 as the receptor for peptidoglycan, lipoteichoic acid, and other components of Gram positive cells walls. Surprisingly, there was no difference in the response to sensitization of *Tlr2*$^{+/-}$ and *Tlr2*$^{-/-}$ littermates, so allergic susceptibility does not increase in the context of any defect in PRR signaling. We also rederived our *Tlr4*$^{-/-}$ animals germ free to directly test the interaction with Clostridia. When GF *Tlr4*$^{-/-}$ mice were sensitized with PN+CT, they had a very similar response as GF WT mice, indicating that the increased PN-specific response was not due to the absence of TLR4 itself but rather the absence of TLR4 signaling in response to members of the microbiota. Colonization of these mice with Clostridia, however, complicated the picture. Portions of the Clostridia program appeared to be TLR4-dependent, such as induction of AMP expression and colonic Tregs. Other aspects of the protective signature of Clostridia were TLR4-independent; there was still robust production of IL-22 and IL-17 in response to Clostridia in *Tlr4*$^{-/-}$ mice. After PN+CT sensitization, however, GF *Tlr4*$^{-/-}$ mice colonized with Clostridia still had increased PN-specific IgE compared to GF WT mice colonized with Clostridia and their serum PN-specific IgE concentrations were very similar to GF *Tlr4*$^{-/-}$

mice. These findings suggest that TLR4 is involved in the sensing of Clostridia, despite the lack of a clear ligand.

We did not measure serum concentrations of Ara h 2 or Ara h 6 after challenge in GF *Tlr4*$^{-/-}$ and Clostridia-colonized *Tlr4*$^{-/-}$ animals. It is possible that, in this setting, IL-22 induction does not reduce permeability due to the incomplete execution of the IL-22 program and is thus uncoupled from protection against sensitization. This still remains to be tested, however, and, if true, would require considerable additional study to understand why. It is also interesting to consider that there is possible cross-talk between various TLRs and that the absence of TLR4 in this context is preventing appropriate responses to Clostridial ligands by other TLRs. There is some evidence that signaling through one TLR can alter the expression of others, but this has not been studied *in vivo* (van Aubel et al., 2007). Our results are not the only suggestion that Clostridia may stimulate signaling via TLR4, however. In one of the other studies that identified Clostridia as potent Treg inducers in the colon, they briefly examined the ability of Clostridia to perform this function in the absence of MyD88 (Atarashi et al., 2011). This signaling adaptor is downstream of all TLRs except TLR3, the receptor for double-stranded RNA, which signals through the adaptor TRIF. TLR4 is unique, though, in that it can also signal using the TRIF pathway, independently of MyD88. When GF *Myd88*$^{-/-}$ animals were colonized with the Clostridia consortium, there was normal induction of Tregs, suggesting that this arm of TLR signaling is not required for mediating the Clostridia Treg-inducing signal. Receptors for other PRRs, including NOD2 and the inflammasome were also dispensable for Treg induction. Although not conclusive, these results do suggest a possible TLR4-TRIF dependent signaling cascade stimulated by Clostridia. This pathway may also be important for the protection against sensitization, which would explain why our *Tlr4*$^{-/-}$ mice were sensitive to PN+CT under both SPF and gnotobiotic conditions while *Tlr2*$^{-/-}$ mice were not.

This question of how Clostridia are sensed by TLRs is certainly a subject for further investigation and we have developed tools to continue these studies. Although Clostridia do not possess an obvious TLR4 ligand, many are flagellated. Flagellin is the ligand for TLR5, and there is evidence that flagellin can stimulate IL-23-dependent IL-22 production by signaling through $CD103^+$ DCs (Kinnebrew et al.). We can sensitize *Tlr5*$^{+/-}$ and *Tlr5*$^{-/-}$ littermates to determine if there is increased susceptibility to sensitization in the absence of this signaling pathway. We can then also explore the role of Clostridia-induced IL-22 in these mice to determine if flagellin from our consortium is capable of stimulating this response. We have also developed conditional mutant mice lacking MyD88 in the epithelial (*Villin*^{Cre}*Myd88*$^{fl/fl}$) or APC (*CD11c*^{Cre}*Myd88*$^{fl/fl}$) compartments. Although we have ruled out a singular contribution of TLR2, this strategy will allow us to consider the role of signaling by TLR2 and its dimerizing partners TLRs 1 and 6, one arm of TLR4, and TLR5 simultaneously. Using these conditional mutant mice we can narrow down which cells require a TLR-mediated signal. We hypothesize that $CD11c^+$ APCs will be the population that requires this signal to prevent sensitization, but there may be an as-yet unappreciated role for IEC TLR signaling in this model. If mice lacking MyD88 in their $CD11c^+$ compartment have increased PN-specific responses after sensitization, we can dissect out which APC population is responsible for receiving the signal. Sorting of $CD11c^+CD103^+$ versus $CD11c^+CX_3CR1^+$ cells from WT mice given Abx or mice treated with Abx then colonized with Clostridia could allow us to examine differential cytokine production in these two populations. There are reports that each population of APCs is capable of stimulating IL-22 production downstream of either IL-23 (Kinnebrew et al.; Longman et al., 2014) or IL-1β (Seo et al., 2015). Clostridia colonization can stimulate increased expression of both of these cytokines, so it is difficult to speculate about which one is most important in our model; perhaps they have a synergistic effect on IL-22 production from ILCs as was described in a recent study (Seo et al., 2015). It should be noted that MyD88 is also downstream of the IL-1β receptor, however

published studies suggest that IL-1β acts directly on ILC3s (Mortha et al., 2014; Seo et al., 2015), not as a signal between APCs, so this should not confound interpretation of these results as ILC3s and all other LP lymphocytes should express MyD88 normally. We have also generated TLR4 conditional knockout animals in the same compartments (*Villin*^{Cre}*Tlr4*$^{fl/fl}$ and *CD11c*^{Cre}*Tlr4*$^{fl/fl}$), which will allow us to compare the effect of eliminating downstream signaling from many TLRs to eliminating only one TLR known to have an allergy protective effect.

VI. Butyrate as a novel IL-22 inducing stimulus and potential allergy-protective metabolite

The results presented in this thesis also demonstrate a role for protective non-TLR-mediated signaling in the regulation of IL-22 production and intestinal permeability. Treatment of GF mice with heat-killed Clostridia did not recapitulate the IL-22 induction seen with live Clostridia, which indicates that signaling stimulated by bacterial components or PAMPs is not sufficient to drive this protective response. Since Clostridia are able to metabolize dietary fiber into SCFAs, particularly acetate and butyrate, we examined whether either of these compounds could stimulate IL-22 production. Butyrate, but not acetate, was sufficient to increase *Il22* expression and treatment with butyrate reduced intestinal permeability to PN compared to GF or acetate-treated mice. The butyrate-mediated increase in *Il22* expression was TLR4-independent, much like the induction of *Il22* after colonization with Clostridia.

To study how butyrate induced IL-22, we developed an *ex vivo* culture system. Butyrate is multifunctional, acting as both an HDACi and a GPCR agonist. In *ex vivo* culture, the IL-22 production in response to butyrate was dependent on GPCR signaling in both the ileum and the colon; treatment with PTx ablated the IL-22 production in response to butyrate. Interestingly, Clostridia colonization also modulated expression of butyrate-binding GPRCs in the epithelium and LP, suggesting there may be a feedback loop between Clostridia and these SCFA

receptors. A connection between butyrate and production of IL-22 has not yet been established in the literature, making this an exciting and novel finding.

The ability of butyrate to induce IL-22 also helps to answer one of the most common questions asked about our model: since Clostridia abundance is much greater in the colon than in the small intestine, how does Clostridia-induced IL-22 regulate permeability to dietary antigens, which are absorbed in the ileum/distal small intestine? Stimulation with butyrate was even more effective at inducing IL-22 protein production in the ileum *ex vivo* than in the colon, suggesting that the immune cells at the site of food antigen uptake are highly responsive to this SCFA. Although we did not measure a difference in *Il22* message in the ileum, the *ex vivo* results suggest that this may due to technical limitations. It is possible that the limit of detection of our qRT-PCR does not allow us to distinguish the increase in *Il22* message after Clostridia colonization or butyrate administration in this tissue. Additionally, it is known that SCFAs can circulate in the blood and have effects at sites very distal to the site of their production. One study documented a protective role in asthma based on the ability of propionate to traffic to the bone marrow and modulate DC maturation (Trompette et al., 2014). Therefore, we hypothesize that Clostridia-derived butyrate may circulate in the blood or lymph in order to exert an effect on the small intestine despite the lower abundance of Clostridia in this site.

While these results are preliminary, it will be interesting to continue investigating the role of butyrate in allergic sensitization. Signaling via both GPR109a and GPR43 have recently been shown to provide protection against intestinal inflammation (Macia et al., 2015; Maslowski et al., 2009; Singh et al., 2014) and butyrate is also capable of driving the differentiation of colonic Tregs (Arpaia et al., 2013; Furusawa et al., 2013). Thus, much of the protective Clostridia-mediated program we have identified can be recapitulated by this metabolite. To determine if butyrate is sufficient to alter intestinal permeability under SPF conditions and to provide protection from sensitization, we can extend our findings into GPCR-deficient animals,

particularly *Gpr109a*$^{-/-}$ and *Gpr43*$^{-/-}$ mice. These mice and their heterozygous littermates can be sensitized with PN+CT to determine if they produce increased PN-specific IgE in the absence of butyrate signaling. We hypothesize that in the absence of one or both of these receptors, mice will generate increased PN-specific responses. It would also be of interest to study which cells are responding to butyrate and stimulating IL-22 production. CD11c^{+} APCs can be conditioned by butyrate to induce tolerogenic phenotypes in other models of inflammation (Arpaia et al., 2013; Furusawa et al., 2013) and can also produce IL-1β (Mortha et al., 2014; Seo et al., 2015), which is upregulated after butyrate administration in our model. Since IL-1β has profound effects on IL-22 production in multiple reports, we hypothesize that this may be a central pathway in protection. Indeed, as mentioned earlier, Clostridia also induce IL-1β, further supporting this pathway as an integral aspect of protection against sensitization. IL-1β must be processed from pro-IL-1β by the inflammasome. Stimulation of the NLRP3 inflammasome was demonstrated to be downstream of SCFA-driven GPCR signaling (Macia et al., 2015), suggesting that there could be a direct connection between all of these cellular and molecular elements. TLR signaling may also synergize with this butyrate-mediated signaling to drive cytokine production, integrating all of our data. A hypothetical but readily testable model can be seen in Figure 49.

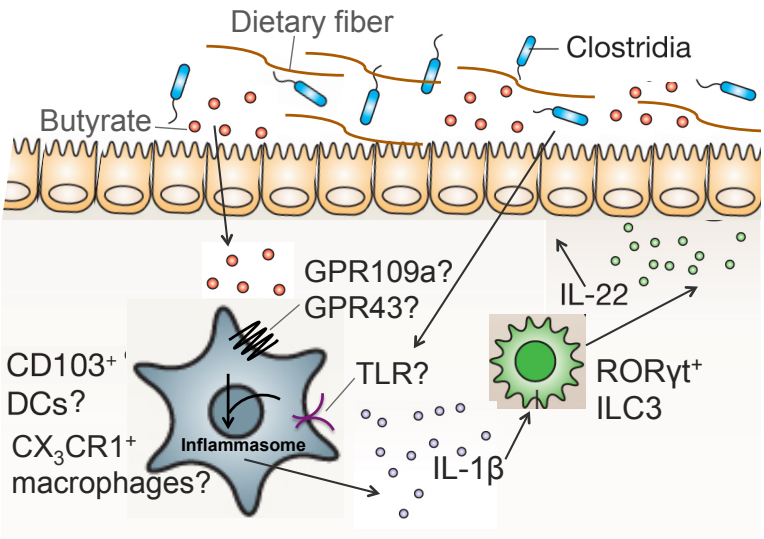


Figure 49: Clostridia-derived butyrate could promote IL-22 production via IL-1β. Clostridia ferment dietary fiber into SCFA, particularly butyrate. This butyrate can signal to APCs (either $CD103^+$ DCs or CX_3CR1^+ macrophages) via GPCR signaling, which can activate the inflammasome and promote IL-1β production. TLR signaling or direct activation of the inflammasome by other Clostridia antigens may synergize to increase cytokine production. IL-1β from these APCs promotes IL-22 production from $ROR\gamma t^+$ ILC3, reinforcing the epithelial barrier to reduce intestinal permeability and prevent sensitization to food allergens.

One of the difficulties of working with SCFAs, and butyrate in particular, is that they are volatile compounds. This makes therapeutic administration difficult, even in diseases where butyrate has been shown to be protective such as sickle cell anemia and beta thalassemia (Dover et al., 1992; Perrine et al., 1989). Patients who receive butyrate for these conditions receive continuous infusions of very high doses to ensure sufficient amounts are absorbed. In order to overcome this limitation when studying butyrate as a potential therapeutic for food allergy, we are working with engineers to encapsulate butyrate into pH sensitive nanoformulations. The encapsulant (cellulose acetate phthalate) dissolves only at pH higher than 6 (Zatz and Knowles, 1970), allowing us to deliver butyrate more directly into the distal small intestine, where food is absorbed, and the colon, where Clostridia predominantly reside. By targeting both of these sites, we expect we will see the greatest effect on sensitization. This encapsulated butyrate can be tested in Abx-treated WT mice, which we have demonstrated generate high PN-specific responses, to determine if butyrate restoration is sufficient to block

this response. *Gpr109a*$^{-/-}$ and *Gpr43*$^{-/-}$ mice can also be treated with encapsulated butyrate; if GPCR signaling is affecting sensitization due to an inability to sense butyrate, this treatment should not be protective in these animals, confirming the necessity of one or both of these receptors for butyrate-mediated protection.

Although butyrate is a difficult molecule to work with in its native form, there are some advantages to targeting it as a therapeutic. Since butyrate is derived from the breakdown of dietary fiber, it may be possible to increase butyrate concentrations, or expand populations of butyrate-producing bacteria, *in situ* by feeding a high fiber diet. Experimentally, we have demonstrated that WT animals fed a fiber free diet in order to remove the major source of butyrate have increased intestinal permeability. In addition to developing a method to deliver butyrate as a drug, we are also now working to formulate high fiber diets that will ameliorate intestinal permeability defects by expanding Clostridia and/or increasing butyrate production and, we predict, increasing *Il22* expression. Sensitization of mice on these different diets will give us further insight into both the necessity of butyrate to prevent PN-specific responses and the feasibility of dietary intervention as a "prebiotic" therapy. Several lines of evidence support a protective effect of high fiber diet feeding (Macia et al., 2015), including in multiple mouse model of asthma (Thorburn et al., 2015; Trompette et al., 2014) and metabolic dysregulation (Kovatcheva-Datchary et al., 2015), so we propose extending this strategy into models of food allergy.

VII. Role of other diet-derived compounds in regulating allergic sensitization to food

SCFA are only one type of bacterially produced immunomodulatory compound derived from dietary components. Another set of compounds that are important for responses in the intestine, particularly production of IL-22, are ligands for the AhR. Although originally described

as a receptor for xenobiotics not an immunological receptor, recent work has demonstrated that AhR can influence immunity to pathogens and maintenance of homeostasis with the microbiota (Qiu et al., 2013; Qiu et al., 2012). In addition to being a receptor for environmental toxins that promotes expression of detoxifying genes, AhR can respond to diet-derived ligands, particularly from cruciferous vegetables, or tryptophan metabolites produced by commensal bacteria (Veldhoen et al., 2008). This signaling can then drive production of IL-22 (Li et al., 2011; Martin et al., 2009; Qiu et al., 2013; Qiu et al., 2012). While the AhR is sufficient to stimulate IL-22 expression, it can also synergize with the TLR-mediated IL-23 pathway to further increase IL-22 levels (Guo et al., 2015).

In the same microarray of colonic IECs that identified *Reg3b* as a gene upregulated by Clostridia colonization, a colon-specific AhR target gene from the cytochrome P450 gene family, *Cyp2c55*, was also a top hit (Wang et al., 2004). We hypothesize that AhR signaling is also being activated by Clostridia and can contribute to the barrier protective response already described to prevent sensitization to food allergens. Indeed, preliminary studies by other labs show that treatment with an exogenous AhR ligand can block the generation of a PN-specific response by expanding $CD103^{+}$ tolerogenic DCs, altering the generation of Th2 cells, and promoting Treg conversion (Schulz et al., 2012a; Schulz et al., 2012b; Schulz et al., 2011). This work does not take the composition of commensal microbiota into consideration nor does it investigate the role of endogenous or naturally occurring AhR ligands, but it does suggest that this pathway can provide a protective signal to the host to prevent aberrant responses to food. We can investigate our finding that Clostridia can induce signaling through the AhR pathway to the suppression of sensitization through the use of global and conditional mutant mice. Much like the experimental system described for TLR4 and MyD88, we have obtained $Ahr^{+/-}$ and $Ahr^{-/-}$ mice and have also generated mice deficient in AhR in IECs ($Villin^{Cre}Ahr^{fl/fl}$) or APCs

(*CD11c*^{Cre}*Ahr*$^{fl/fl}$) to study their production of IL-22 and response to sensitization with PN+CT either at baseline or after treatment with Abx and colonization with Clostridia.

VIII. Role of Clostridia and butyrate in the establishment of antigen-specific tolerance to food

Although food allergies are an example of the breakdown of oral tolerance mechanisms, we have not yet directly examined the pathways required to induce antigen-specific tolerance in our models. As described in the introduction, there is an extensive literature implicating RA as a major driver of antigen-specific Treg conversion. RA is derived from dietary vitamin A through a series of enzymatic oxidations. Expression of RALDH enzymes, particularly RALDH1 (*Aldh1a1*), on CD103$^+$ DCs has defined these cells as a specific population that is particularly efficient at promoting conversion of naïve T cells into Foxp3$^+$ Tregs (Coombes et al., 2007; Sun et al., 2007). The role of commensal bacteria in regulating RA metabolism and subsequent induction of Tregs by these CD103$^+$ DCs has not been extensively explored. Butyrate can promote *Aldh1a1* expression via GPR109a signaling and butyrate administration increases conversion of naïve OT-II cells into Foxp3$^+$ cells after OVA feeding, but this is the only evidence linking bacteria-derived signals to the production of RA (Singh et al., 2014). We can use our system to measure changes in *Aldh1a1* expression on DCs in response to colonization with Clostridia in GF or Abx-treated mice to determine if changes in the microbiota can regulate RA production. Since butyrate has also been shown to increase *Aldh1a1* expression on CD103$^+$ DCs in the colon, we have all of the necessary tools to explore the relationship between Clostridia, dietary fiber, RA production, and antigen-specific Tregs in a model of tolerance versus sensitization to food.

Other evidence links the microbiota to ILC3 responses and antigen-specific Tregs or the inhibition of a Type 2 immune response, but no study has directly linked the microbiota-induced

ILC3 response to RA-driven antigen-specific Treg generation. One report demonstrated a connection between ILC3-derived Csf2 and $CD103^+$ DCs (Mortha et al., 2014). Production of Csf2 by ILC3s was dependent on the microbiota. In the absence of ILC-derived Csf2, there were fewer total $Foxp3^+$ Tregs in the colon and reduced proportions of $Foxp3^+$ OT-II cells in the small intestine and colon after OVA feeding. Transfer of Csf2-sufficient ILC3s was able to restore conversion of OT-II cells to Tregs after oral delivery of OVA. This paper established a relationship between the microbiota, the ILC3 program, and antigen-specific Treg responses, but it did not examine the role of RA or TGFβ in this network. Other studies have examined the effect of RA on ILC development and have demonstrated that in the absence of RA, ILC3s are reduced and functionally impaired in favor of more robust ILC2 responses (Spencer et al., 2014). Consequently there is a reduction in IL-22 production and other hallmarks of the Type 3 response. RA has also been shown to directly promote IL-22 production by γδ IEL and ILC3s, providing protection against damage in the colon (Mielke et al., 2013). Together, these studies suggest that there is a connection between the barrier protective responses we have identified as allergy protective and the tolerance-inducing mechanisms centered around RA but it remains unclear if or how they are all regulated in concert and how this affects tolerance.

Our system provides a unique opportunity to address this connection directly. We know that our Clostridia consortium stimulates induction of Tregs in the colonic LP, where Tregs are generally considered to be bacteria-specific, and prevents the generation of PN-specific IgE but we do not know if there is an effect on ileal Tregs, and more specifically food antigen-specific Tregs. We could transfer OVA-specific OT-II cells into Abx-treated or Abx-treated Clostridia-colonized hosts and feed OVA to compare the effect of Clostridia on oral antigen-specific Treg conversion. We can also use the *Rag*$^{-/-}$ model and deplete ILCs before transfer of naïve OT-II cells and OVA feeding to determine if they are required for conversion to $Foxp3^+$ OT-II cells. If Clostridia can regulate the induction of antigen-specific Tregs, we can directly address the

relationship between these cells and allergic sensitization by inhibiting RA production in the presence of Clostridia or butyrate to test whether or not this increases PN-specific responses after sensitization.

Furthermore, we can use our model to address a question that has not yet been broached in the literature, namely the role of RA in the generation of bacteria-specific colonic Tregs. Although it is accepted that RA is important in the generation of Tregs in the small intestine and MLN and it is clear that the microbiota can influence the generation of Tregs in the colon, no one has examined whether RA plays a role in this second process. We have generated mice that are unable to signal via the retinoic acid receptor α (RARα) in IECs (*Villin*^{Cre}*RARα*$^{fl/fl}$) and in APCs (*CD11c*^{Cre} *RARα*$^{fl/fl}$). The absence of RARα in global knockouts affects induction of both Tregs and effector T cells (Hall et al., 2011). With our conditional knockouts, we can examine colonic Tregs after Abx treatment or Abx treatment and Clostridia colonization in the absence of RA signaling. Feeding a vitamin A deficient diet or administering inhibitors of RALDH enzymes in WT SPF animals could also allow us to examine the necessity of RA for the induction of Tregs in the colon. Understanding how all of these processes interact will allow for the generation of more effective markers of tolerance versus disease and direct a more focused search for novel therapeutic targets.

IX. Evidence for therapeutic modulation of the microbiota in humans

Work from our laboratory in collaboration with a clinical food allergy laboratory in Naples, Italy has begun to link the role of the microbiota, particularly Clostridia, and butyrate to food allergy in humans. A recent publication comparing the microbiota of infants diagnosed with cow's milk allergy (CMA) to healthy age-matched controls demonstrated clearly that they are dramatically different (Berni Canani et al., 2015). While healthy infants have a microbiota dominated by Bifidobacteriaceae and Enterobacteriaceae as described in other studies, CMA

infants have a significantly more diverse microbiota that more closely resembles that of an adult, with large proportions of Lachnospiraceae and Ruminococcaceae. CMA infants were then treated with two different formulas, extensively hydrolyzed casein formula (EHCF) or EHCF plus the common probiotic *Lactobacillus rhamnosis GG* (EHCF+LGG). Previous reports demonstrated that feeding EHCF+LGG induced tolerance to cow's milk in significantly more infants than EHCF alone or other non-milk based formulas (Berni Canani et al., 2012; Berni Canani et al., 2013). The microbiota of CMA infants after treatment with EHCF or EHCF+LGG was altered compared to pre-treatment, but only feeding EHCF+LGG expanded the proportion of butyrate-producing Clostridia such as *Roseburia* and *Faecalibacterium*. Fecal butyrate levels were also significantly higher after this treatment compared to any of the other groups (healthy, CMA pre-treatment, or CMA+EHCF). As with the original study, treatment with EHCF+LGG induced tolerance in a significantly greater number of patients than EHCF alone, correlating with the increase in butyrate levels. Although the allergen in this study was cow's milk, it greatly strengthens our overall assertion that Clostridia are the important, allergy-protective population of the microbiota and these protective effects may be mediated, at least in part, by butyrate.

In order to understand how EHCF+LGG alters the composition of the microbiota and to interrogate the immunological effects of these changes, we have transferred the microbiota of the healthy, CMA, or CMA-tolerized infants into GF mice. We can analyze expression of *Il22* and changes in permeability in response to colonization with feces from infants fed EHCF or EHCF+LGG to understand if our findings with the PN model are applicable to the human system and if our findings can be extrapolated to other food allergens. We will also sensitize these colonized mice to determine if the microbiota is sufficient to transfer the allergic phenotype.

A second recent study in humans implicates Clostridia and SCFAs in protection against allergic disease as well. Using a cohort of infants showing signs of asthma including wheeze and positive atopic skin tests, Arrieta et al. identified four bacteria that were reduced in patients

that went on to develop active disease as toddlers compared to healthy controls (Arrieta et al., 2015). These populations included *Fecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia*, a consortium they called FLVR. Transfer of atopic infant microbiota to GF mice induced symptoms of allergic airway disease while feeding the FLVR consortium ameliorated symptoms. In combination with the results of our mouse and human studies, this finding suggests a common protective microbial signature that may prevent or treat multiple allergic diseases. Increased *Fecalibacterium* abundance also correlated with acquisition of tolerance after EHCF+LGG feeding in CMA allergic infants (Berni Canani et al., 2015), while *Rothia* and *Lachnospira* are in the same family as *Roseburia* and *Coprococcus*. Several of the species identified in both human studies are also in the same family as members of the Clostridia consortium identified in our murine model of PN sensitization.

X. Development of microbiome-modulating biotherapeutics to induce tolerance to food

We predict that our findings regarding the role of IL-22 and the protective effect of Clostridia and Clostridia-derived butyrate will extend to our new humanized mouse model and strengthen the evidence to support development of Clostridia as a microbiome-modulating biotherapeutic. Currently there is no effective treatment for food allergies and very few therapies are being tested. The best contenders are oral or sublingual desensitization, where small amounts of allergen are introduced repeatedly on or under the tongue until there is no response (Burbank et al., 2016; Sindher et al., 2016), and omalizumab, an anti-IgE antibody that acts as a sink for circulating free IgE. Neither of these is yet widely accepted nor do they have a particularly high rate of efficacy (Wood et al., 2015). The mechanism behind induction of tolerance during oral immunotherapy is hypothesized to be the induction of food-specific Tregs but this is difficult to confirm in humans. Most of the cellular assessments used to determine

efficacy of these treatments have focused on populations of Tregs in peripheral blood since this is the only readily available source of lymphocytes in humans. Recent work has demonstrated, however, that most T effector/memory cells are actually found in mucosal and lymphoid tissues (Thome et al., 2014), so the Tregs relevant to the restoration of tolerance are likely to be in the mucosa and would not be detected in blood. It is possible that finding new, more relevant immunological metrics to measure patient improvement would enhance these treatments.

A recent study also analyzed the impact of exposure versus avoidance of peanuts, during early childhood on the incidence of allergy later in life (Du Toit et al., 2015). Children exposed to peanut during infancy had a significantly reduced likelihood of developing peanut allergy by 60 moths of age compared to those who strictly avoided these allergens. This study indicates that exposure to allergens early in life may prevent sensitization better than avoidance, even in at-risk patients who present with positive skin prick tests and other diseases like severe eczema or egg allergy. This finding reinforces the importance of the neonatal period as a time where the immune system is being educated to both the microbiota and other common antigens. Allergen avoidance, however, cannot explain the entirety of the generational increase in allergic disease. It is also unclear if this finding about the beneficial effects of early introduction holds true for all allergens. Thus certain interventions during this period, such as exposure to peanut allergens, may effectively restore immune homeostasis but what can be done in patients who are past this critical phase or for whom this antigen exposure is not sufficient to prevent the development of an allergic response?

Based on the evidence in our mouse models and human study, we hypothesize that desensitization treatments would be more successful if there was also a component of the therapy aimed at altering the microbiota while reeducating the immune system. If Clostridia or butyrate was administered in conjunction with oral desensitization, our results suggest that there may be induction of a barrier protective response along with antigen-specific tolerance, better

mimicking the induction of oral tolerance in a healthy individual. This adjunctive therapeutic may also activate many protective pathways all at once, which seems like a more effective strategy than to rely only on one component of tolerance (Tregs). Since Clostridia are spore-formers, they are attractive as therapeutics because the spores are very difficult to disrupt. It may be possible to encapsulate them in this stable state and allow for easy delivery as a pill rather than repeated administration of vegetative bacteria as is done with current probiotics. As mentioned previously, we are also working to test encapsulated butyrate and dietary intervention to increase butyrate-producers as therapeutic agents that could be coupled with desensitization protocols.

We predict there would be several measurable changes in microbial and immunological homeostasis after therapeutic treatment with Clostridia. Administration of spores could increase the representation of Clostridia within the intestinal microbiota, as measured by 16S rRNA or metagenomic analysis of feces. It is possible, though, that the total abundance of Clostridia within the community will not change, due to activation of the IL-22 pathway and increased antimicrobial peptide production, which titrates the levels of these bacteria (see Figure 33). Even if Clostridia itself does not expand, however, probiotic administration should have significant effects on the community structure as we saw in the mouse model (see Figure 12). The functional capacity of the microbiota may also change, as it is well established that bacterial gene expression can be altered in the presence of other species in the niche or based on competition for resources with other members of the community (Berni Canani et al., 2015; Wu et al., 2015). Butyrate levels should also increase in response to Clostridia administration. The downstream effects of such alterations in the microbiota will be more difficult to measure due to limitations in assessing mucosal responses in humans, described above, but certain metrics can be used as proxies for improved host responses. In a therapeutic setting, intestinal permeability at baseline or in the absence of an ongoing allergic response can be measured before

treatment using sugar-based (lactulose/mannitol) readouts while the Ara h 2/6 measurements used in our murine model could be examined in humans undergoing an oral food challenge. If treatment with Clostridia is activating the same barrier-protective responses in humans, there should be a decrease in intestinal permeability over time both at steady-state and after allergen administration. Direct measurement of IL-22 in serum is also possible; other studies have measured changes in serum IL-22 levels in response to therapies for psoriasis (Caproni et al., 2009) and as a readout of Crohn's disease activity (Schmechel et al., 2008). A decrease in permeability after Clostridia administration may correlate with increased serum IL-22 concentrations. Although analysis of food-antigen specific responses in humans are limited to serum antibodies or periodic food challenges, many of the Clostridia-induced barrier protective mechanisms studied in our mouse models could be examined using minimally invasive techniques in patients.

Although there is still considerable work to be done before Clostridia-based therapeutics can be taken into the clinic, it is extremely exciting to be on the cutting edge of such translational research. Participating in these discoveries every step of the way, from establishing the mouse model to elucidating the molecular mechanisms and extending the findings into humans, has been an incredible learning experience and a very gratifying one.

References

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. Sci Transl Med *6*, 237ra265.

Ahlfors, H., Morrison, P.J., Duarte, J.H., Li, Y., Biro, J., Tolaini, M., Di Meglio, P., Potocnik, A.J., and Stockinger, B. (2014). IL-22 fate reporter reveals origin and control of IL-22 production in homeostasis and infection. J Immunol *193*, 4602-4613.

Ananthakrishnan, A.N. (2015). Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol *12*, 205-217.

Aparicio-Domingo, P., Romera-Hernandez, M., Karrich, J.J., Cornelissen, F., Papazian, N., Lindenbergh-Kortleve, D.J., Butler, J.A., Boon, L., Coles, M.C., Samsom, J.N., and Cupedo, T. (2015). Type 3 innate lymphoid cells maintain intestinal epithelial stem cells after tissue damage. J Exp Med *212*, 1783-1791.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J.R., Pfeffer, K., Coffer, P.J., and Rudensky, A.S. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T cell generation. Nature *504*, 451-455.

Arrieta, M.C., Stiemsma, L.T., Dimitriu, P.A., Thorson, L., Russell, S., Yurist-Doutsch, S., Kuzeljevic, B., Gold, M.J., Britton, H.M., Lefebvre, D.L.*, et al.* (2015). Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med *7*, 307ra152.

Astwood, J.D., Leach, J.N., and Fuchs, R.L. (1996). Stability of food allergens to digestion in vitro. Nat Biotechnol *14*, 1269-1273.

Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., Yagita, H., Ishii, N., Evans, R., Honda, K., and Takeda, K. (2008). ATP drives lamina propria T(H)17 cell differentiation. Nature *455*, 808-812.

Atarashi, K., Tanoue, T., Ando, M., Kamada, N., Nagano, Y., Narushima, S., Suda, W., Imaoka, A., Setoyama, H., Nagamori, T.*, et al.* (2015). Th17 cell induction by adhesion of microbes to intestinal epithelial cells. Cell *163*, 367-380.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K.*, et al.* (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature *500*, 232-236.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y.*, et al.* (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science *331*, 337-341.

Bach, J.-F. (2002). The effect of infections on susceptibility to autoimmune and allergic disease. N Eng J Med *347*, 911-920.

Backhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Semenkovich, C.F., and Gordon, J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A *101*, 15718-15723.

Bashir, M.E., Louie, S., Shi, H.N., and Nagler-Anderson, C. (2004). Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. J Immunol *172*, 6978-6987.

Basu, R., O'Quinn, D.B., Silberger, D.J., Schoeb, T.R., Fouser, L., Ouyang, W., Hatton, R.D., and Weaver, C.T. (2012). Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. Immunity *37*, 1061-1075.

Berer, K., Mues, M., Koutrolos, M., Rasbi, Z.A., Boziki, M., Johner, C., Wekerle, H., and Krishnamoorthy, G. (2011). Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature *479*, 538-541.

Bergmann, K.R., Liu, S.X., Tian, R., Kushnir, A., Turner, J.R., Li, H.L., Chou, P.M., Weber, C.R., and De Plaen, I.G. (2013). Bifidobacteria stabilize claudins at tight junctions and prevent intestinal barrier dysfunction in mouse necrotizing enterocolitis. Am J Pathol *182*, 1595-1606.

Berni Canani, R., Nocerino, R., Terrin, G., Coruzzo, A., Cosenza, L., Leone, L., and Troncone, R. (2012). Effect of Lactobacillus GG on tolerance acquisition in infants with cow's milk allergy: A randomized trial. J. Allergy Clin Immunol *129*, 580-582.

Berni Canani, R., Nocerino, R., Terrin, G., Frediani, T., Lucarelli, S., Cosenza, L., Passariello, A., Leone, L., Granata, V., Di Costanzo, M.*, et al.* (2013). Formula selection for managment of children with cow milk allergy influences the rate of acquisition of tolerance: a prospective multicenter study. J Pediatr *163*, 771-777.

Berni Canani, R., Sangwan, N., Stefka, A.T., Nocerino, R., Paparo, L., Aitoro, R., Calignano, A., Khan, A.A., Gilbert, J.A., and Nagler, C.R. (2015). Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J, Epub ahead of print.

Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., Liu, K., Jakubzick, C., Ingersoll, M.A., Leboeuf, M.*, et al.* (2009). Origin of the lamina propria dendritic cell network. Immunity *31*, 513-525.

Brandl, K., Plitas, G., Schnabl, B., DeMatteo, R.P., and Pamer, E.G. (2007). MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp Med *204*, 1891-1900.

Branum, A.M., and Lukacs, S.L. (2008). Food allergy among U.S. children: trends in prevalence and hospitalizations. NCHS Data Brief, 1-8.

Brestoff, J.R., and Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. Nat Immunol *14*, 676-684.

Brown, A.J., Goldsworthy, S.M., Barnes, A.A., Eilert, M.M., Tcheang, L., Daniels, D., Muir, A.I., Wigglesworth, M.J., Kinghorn, I., Fraser, N.J.*, et al.* (2003). The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem *278*, 11312-11319.

Bruhns, P., and Jonsson, F. (2015). Mouse and human FcR effector functions. Immunol Rev *268*, 25-51.

Buffie, C.G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., Ubeda, C., Xavier, J., and Pamer, E.G. (2012). Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun *80*, 62-73.

Bunker, J.J., Flynn, T.M., Koval, J.C., Shaw, D.G., Meisel, M., McDonald, B.D., Ishizuka, I.E., Dent, A.L., Wilson, P.C., Jabri, B.*, et al.* (2015). Innate and adaptive humoral responses coat distinct commensal bacteria with immunoglobulin A. Immunity *43*, 541-553.

Burbank, A.J., Sood, P., Vickery, B.P., and Wood, R.A. (2016). Oral immunotherapy for food allergy. Immunol Allergy Clin North Am *36*, 55-69.

Burrows, M.P., Volchkov, P., Kobayashi, K.S., and Chervonsky, A.V. (2015). Microbiota regulates type 1 diabetes through Toll-like receptors. Proc Natl Acad Sci U S A *112*, 9973-9977.

Cahenzli, J., Koller, Y., Wyss, M., Geuking, M.B., and McCoy, K.D. (2013). Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. Cell Host Microbe *14*, 559-570.

Candido, E.P., Reeves, R., and Davie, J.R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell *14*, 105-113.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A *108 Suppl 1*, 4516-4522.

Caproni, M., Antiga, E., Melani, L., Volpi, W., Del Bianco, E., and Fabbri, P. (2009). Serum levels of IL-17 and IL-22 are reduced by etanercept, but not by acitretin, in patients with psoriasis: a randomized-controlled trial. J Clin Immunol *29*, 210-214.

Cario, E. (2008). Barrier-protective function of intestinal epithelial Toll-like receptor 2. Mucosal Immunol *1 Suppl 1*, S62-66.

Cassani, B., Villablanca, E.J., Quintana, F.J., Love, P.E., Lacy-Hulbert, A., Blaner, W.S., Sparwasser, T., Snapper, S.B., Weiner, H.L., and Mora, J.R. (2011). Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. Gastroenterology *141*, 2109-2118.

Chang, P.V., Hao, L., Offermanns, S., and Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A *111*, 2247-2252.

Chassaing, B., Ley, R.E., and Gewirtz, A.T. (2014). Intestinal epithelial cell toll-like receptor 5 regulates the intestinal microbiota to prevent low-grade inflammation and metabolic syndrome in mice. Gastroenterology *147*, 1363-1377.

Chen, Y., Chou, K., Fuchs, E., Havran, W.L., and Boismenu, R. (2002). Protection of the intestinal mucosa by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A *99*, 14338-14343.

Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006). Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med *203*, 2841-2852.

Cho, I., Yamanishi, S., Cox, L., Methe, B.A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I.*, et al.* (2012). Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature *488*, 621-626.

Chu, D.K., Jimenez-Saiz, R., Verschoor, C.P., Walker, T.D., Goncharova, S., Llop-Guevara, A., Shen, P., Gordon, M.E., Barra, N.G., Bassett, J.D.*, et al.* (2014). Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo. J Exp Med *211*, 1657-1672.

Cong, Y., Feng, T., Fujihashi, K., Schoeb, T.R., and Elson, C.O. (2009). A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc Natl Acad Sci U S A *106*, 19256-19261.

Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med *204*, 1757-1764.

Coppa, G.V., Bruni, S., Morelli, L., Soldi, S., and Gabrielli, O. (2004). The first prebiotics in humans: human milk oligosaccharides. J Clin Gastroenterol *38*, S80-83.

Cox, A.J., West, N.P., and Cripps, A.W. (2015). Obesity, inflammation, and the gut microbiota. Lancet Diabetes Endocrinol *3*, 207-215.

Cox, J.H., Kljavin, N.M., Ota, N., Leonard, J., Roose-Girma, M., Diehl, L., Ouyang, W., and Ghilardi, N. (2012). Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. Mucosal Immunol *5*, 99-109.

Cox, L.M., Yamanishi, S., Sohn, J., Alekseyenko, A.V., Leung, J.M., Cho, I., Kim, S.G., Li, H., Gao, Z., Mahana, D.*, et al.* (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell *158*, 705-721.

Crellin, N.K., Trifari, S., Kaplan, C.D., Satoh-Takayama, N., Di Santo, J.P., and Spits, H. (2010). Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2. Immunity *33*, 752-764.

Cresci, G.A., Thangaraju, M., Mellinger, J.D., Liu, K., and Ganapathy, V. (2010). Colonic gene expression in conventional and germ-free mice with a focus on the butyrate receptor GPR109A and the butyrate transporter SLC5A8. J Gastrointest Surg *14*, 449-461.

David, L.A., Materna, A.C., Friedman, J., Campos-Baptista, M.I., Blackburn, M.C., Perrotta, A., Erdman, S.E., and Alm, E.J. (2014a). Host lifestyle affects human microbiota on daily timescales. Genome Biol *15*, R89.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A.*, et al.* (2014b). Diet rapidly and reproducibly alters the human gut microbiome. Nature *505*, 559-563.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G., and Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A *107*, 14691-14696.

Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R., and Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat Immunol *8*, 1086-1094.

Derrien, M., Vaughan, E.E., Plugge, C.M., and de Vos, W.M. (2004). Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol *54*, 1469-1476.

Dethlefsen, L., Huse, S., Sogin, M.L., and Relman, D.A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol *6*, e280.

Dethlefsen, L., and Relman, D.A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A *108 Suppl 1*, 4554-4561.

Devkota, S., Wang, Y., Musch, M.W., Leone, V., Fehlner-Peach, H., Nadimpalli, A., Antonopoulos, D.A., Jabri, B., and Chang, E.B. (2012). Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature *487*, 104-108.

Dewhirst, F.E., Chien, C.C., Paster, B.J., Ericson, R.L., Orcutt, R.P., Schauer, D.B., and Fox, J.G. (1999). Phylogeny of the defined murine microbiota: altered Schaedler flora. Appl Environ Microbiol *65*, 3287-3292.

Diehl, G.E., Longman, R.S., Zhang, J.X., Breart, B., Galan, C., Cuesta, A., Schwab, S.R., and Littman, D.R. (2013). Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX3CR1hi cells. Nature *494*, 116-120.

Dominguez-Bello, M.G., Blaser, M.J., Ley, R.E., and Knight, R. (2011). Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. Gastroenterology *140*, 1713-1719.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A *107*, 11971-11975.

Donaldson, G.P., Lee, S.M., and Mazmanian, S.K. (2016). Gut biogeography of the bacterial microbiota. Nat Rev Microbiol *14*, 20-32.

Donohoe, D.R., Garge, N., Zhang, X., Sun, W., O'Connell, T.M., Bunger, M.K., and Bultman, S.J. (2011). The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab *13*, 517-526.

Dover, G.J., Brusilow, S., and Samid, D. (1992). Increased fetal hemoglobin in patients receiving sodium 4-phenylbutyrate. N Engl J Med *327*, 569-570.

Du Toit, G., Roberts, G., Sayre, P.H., Bahnson, H.T., Radulovic, S., Santos, A.F., Brough, H.A., Phippard, D., Basting, M., Feeney, M.*, et al.* (2015). Randomized trial of peanut consumption in infants at risk for peanut allergy. N Engl J Med *372*, 803-813.

Dube, P.E., Yan, F., Punit, S., Girish, N., McElroy, S.J., Washington, M.K., and Polk, D.B. (2012). Epidermal growth factor receptor inhibits colitis-associated cancer in mice. J Clin Invest *122*, 2780-2792.

Earle, K.A., Billings, G., Sigal, M., Lichtman, J.S., Hansson, G.C., Elias, J.E., Amieva, M.R., Huang, K.C., and Sonnenburg, J.L. (2015). Quantitative imaging of gut microbiota spatial organization. Cell Host Microbe *18*, 478-488.

Eberl, G. (2010). A new vision of immunity: homeostasis of the superorganism. Mucosal Immunol *3*, 450-460.

Eberl, G., Colonna, M., Di Santo, J.P., and McKenzie, A.N. (2015). Innate lymphoid cells: a new paradigm in immunology. Science *348*, aaa6566.

Eberl, G., Marmon, S., Sunshine, M.J., Rennert, P.D., Choi, Y., and Littman, D.R. (2004). An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol *5*, 64-73.

Edelblum, K.L., Shen, L., Weber, C.R., Marchiando, A.M., Clay, B.S., Wang, Y., Prinz, I., Malissen, B., Sperling, A.I., and Turner, J.R. (2012). Dynamic migration of gammadelta intraepithelial lymphocytes requires occludin. Proc Natl Acad Sci U S A *109*, 7097-7102.

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., and Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science *298*, 1424-1427.

Feehley, T., and Nagler, C.R. (2014). Cellular and molecular pathways through which commensal bacteria modulate sensitization to dietary antigens. Curr Opin Immunol *31*, 79-86.

Feehley, T., Stefka, A.T., Cao, S., and Nagler, C.R. (2012). Microbial regulation of allergic responses to food. Semin Immunopathol *34*, 671-688.

Ferreyra, J.A., Wu, K.J., Hryckowian, A.J., Bouley, D.M., Weimer, B.C., and Sonnenburg, J.L. (2014). Gut microbiota-produced succinate promotes C. difficile infection after antibiotic treatment or motility disturbance. Cell Host Microbe *16*, 770-777.

Fransen, F., Zagato, E., Mazzini, E., Fosso, B., Manzari, C., El Aidy, S., Chiavelli, A., D'Erchia, A.M., Sethi, M.K., Pabst, O.*, et al.* (2015). BALB/c and C57BL/6 mice differ in polyreactive IgA abundance, which impacts the generation of antigen-specific IgA and microbiota diversity. Immunity *43*, 527-540.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T.*, et al.* (2013). Commensal microbe-derived butyrate induces differentiation of colonic regulatory T cells. Nature *504*, 446-450.

Gaboriau-Routhiau, V., Rakotobe, S., Lecuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G.*, et al.* (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity *31*, 677-689.

Geha, R.S., Jabara, H.H., and Brodeur, S.R. (2003). The regulation of immunoglobulin E class-switch recombination. Nat Rev Immunol *3*, 721-732.

Geirnaert, A., Steyaert, A., Eeckhaut, V., Debruyne, B., Arends, J.B., Van Immerseel, F., Boon, N., and Van de Wiele, T. (2014). Butyricicoccus pullicaecorum, a butyrate producer with probiotic potential, is intrinsically tolerant to stomach and small intestine conditions. Anaerobe *30*, 70-74.

Geremia, A., Arancibia-Carcamo, C.V., Fleming, M.P., Rust, N., Singh, B., Mortensen, N.J., Travis, S.P., and Powrie, F. (2011). IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. J Exp Med *208*, 1127-1133.

Geuking, M.B., Cahenzli, J., Lawson, M.A., Ng, D.C., Slack, E., Hapfelmeier, S., McCoy, K.D., and Macpherson, A.J. (2011). Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity *34*, 794-806.

Giacomin, P.R., Moy, R.H., Noti, M., Osborne, L.C., Siracusa, M.C., Alenghat, T., Liu, B., McCorkell, K.A., Troy, A.E., Rak, G.D.*, et al.* (2015). Epithelial-intrinsic IKKalpha expression regulates group 3 innate lymphoid cell responses and antibacterial immunity. J Exp Med *212*, 1513-1528.

Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J.T.*, et al.* (2014). Human genetics shape the gut microbiome. Cell *159*, 789-799.

Goto, Y., Panea, C., Nakato, G., Cebula, A., Lee, C., Diez, M.G., Laufer, T.M., Ignatowicz, L., and Ivanov, I.I. (2014). Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. Immunity *40*, 594-607.

Groschwitz, K.R., and Hogan, S.P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. J Allergy Clin Immunol *124*, 3-20.

Guo, X., Liang, Y., Zhang, Y., Lasorella, A., Kee, B.L., and Fu, Y.X. (2015). Innate lymphoid cells control early colonization resistance against intestinal pathogens through ID2-dependent regulation of the microbiota. Immunity *42*, 731-743.

Guo, X., Qiu, J., Tu, T., Yang, X., Deng, L., Anders, R.A., Zhou, L., and Fu, Y.X. (2014). Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. Immunity *40*, 25-39.

Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., Muller, W., Sparwasser, T., Forster, R., and Pabst, O. (2011). Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity *34*, 237-246.

Haghikia, A., Jorg, S., Duscha, A., Berg, J., Manzel, A., Waschbisch, A., Hammer, A., Lee, D.H., May, C., Wilck, N.*, et al.* (2015). Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine. Immunity *43*, 817-829.

Hall, J.A., Cannons, J.L., Grainger, J.R., Dos Santos, L.M., Hand, T.W., Naik, S., Wohlfert, E.A., Chou, D.B., Oldenhove, G., Robinson, M.*, et al.* (2011). Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity *34*, 435-447.

Han, D., Walsh, M.C., Cejas, P.J., Dang, N.N., Kim, Y.F., Kim, J., Charrier-Hisamuddin, L., Chau, L., Zhang, Q., Bittinger, K.*, et al.* (2013). Dendritic cell expression of the signaling molecule TRAF6 is critical for gut microbiota-dependent immune tolerance. Immunity *38*, 1211-1222.

Han, D., Walsh, M.C., Kim, K.S., Hong, S.W., Lee, J., Yi, J., Rivas, G., Surh, C.D., and Choi, Y. (2015). Microbiota-independent ameliorative effects of antibiotics on spontaneous th2-associated pathology of the small intestine. PLoS One *10*, e0118795.

Hapfelmeier, S., Lawson, M.A., Slack, E., Kirundi, J.K., Stoel, M., Heikenwalder, M., Cahenzli, J., Velykoredko, Y., Balmer, M.L., Endt, K.*, et al.* (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science *328*, 1705-1709.

Harrison, O.J., Srinivasan, N., Pott, J., Schiering, C., Krausgruber, T., Ilott, N.E., and Maloy, K.J. (2015). Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3(+) Treg cell function in the intestine. Mucosal Immunol *8*, 1226-1236.

Harusato, A., Flannigan, K.L., Geem, D., and Denning, T.L. (2015). Phenotypic and functional profiling of mouse intestinal antigen presenting cells. J Immunol Methods *421*, 20-26.

Hepworth, M.R., Fung, T.C., Masur, S.H., Kelsen, J.R., McConnell, F.M., Dubrot, J., Withers, D.R., Hugues, S., Farrar, M.A., Reith, W.*, et al.* (2015). Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. Science *348*, 1031-1035.

Hicks, L.A., Bartoces, M.G., Roberts, R.M., Suda, K.J., Hunkler, R.J., Taylor, T.H., Jr., and Schrag, S.J. (2015). US outpatient antibiotic prescribing variation according to geography, patient population, and provider specialty in 2011. Clin Infect Dis *60*, 1308-1316.

Hill, D.A., Siracusa, M.C., Abt, M.C., Kim, B.S., Kobuley, D., Kubo, M., Kambayashi, T., Larosa, D.F., Renner, E.D., Orange, J.S.*, et al.* (2012). Commensal bacteria-derived signals regulate basophil hematopoiesis and allergic inflammation. Nat Med *18*, 538-546.

Hill, M.J. (1997). Intestinal flora and endogenous vitamin synthesis. Eur J Cancer Prev *6 Suppl 1*, S43-45.

Hirota, K., Turner, J.E., Villa, M., Duarte, J.H., Demengeot, J., Steinmetz, O.M., and Stockinger, B. (2013). Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. Nat Immunol *14*, 372-379.

Hooper, L.V. (2011). You AhR what you eat: linking diet and immunity. Cell *147*, 489-491.

Houston, S.A., Cerovic, V., Thomson, C., Brewer, J., Mowat, A.M., and Milling, S. (2015). The lymph nodes draining the small intestine and colon are anatomically separate and immunologically distinct. Mucosal Immunol, Epub ahead of print.

Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. Nature *486*, 207-214.

Iannello, A., Thompson, T.W., Ardolino, M., Lowe, S.W., and Raulet, D.H. (2013). p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. J Exp Med *210*, 2057-2069.

Ismail, A.S., Behrendt, C.L., and Hooper, L.V. (2009). Reciprocal interactions between commensal bacteria and gamma delta intraepithelial lymphocytes during mucosal injury. J Immunol *182*, 3047-3054.

Ismail, A.S., Severson, K.M., Vaishnava, S., Behrendt, C.L., Yu, X., Benjamin, J.L., Ruhn, K.A., Hou, B., DeFranco, A.L., Yarovinsky, F., and Hooper, L.V. (2011). Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proc Natl Acad Sci U S A *108*, 8743-8748.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V*., et al.* (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell *139*, 485-498.

Ivanov, I.I., Frutos Rde, L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., and Littman, D.R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe *4*, 337-349.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

Jackson, K.D., Howie, L.D., and Akinbami, L.J. (2013). Trends in allergic conditions among children: United States, 1997-2011. NCHS Data Brief, 1-8.

Jackson, P.G., Lessof, M.H., Baker, R.W., Ferrett, J., and MacDonald, D.M. (1981). Intestinal permeability in patients with eczema and food allergy. Lancet *1*, 1285-1286.

Jakobsson, H.E., Rodriguez-Pineiro, A.M., Schutte, A., Ermund, A., Boysen, P., Bemark, M., Sommer, F., Backhed, F., Hansson, G.C., and Johansson, M.E. (2015). The composition of the gut microbiota shapes the colon mucus barrier. EMBO Rep *16*, 164-177.

James, G. (2010). Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene. In PCR for Clinical Microbiology, M. Schuller, T.P. Sloots, G.S. James, C.L. Halliday, and I.W.J. Carter, eds. (Springer Netherlands), pp. 209-214.

Jarvinen, K.M., Konstantinou, G.N., Pilapil, M., Arrieta, M.C., Noone, S., Sampson, H.A., Meddings, J., and Nowak-Wegrzyn, A. (2013). Intestinal permeability in children with food allergy on specific elimination diets. Pediatr Allergy Immunol *24*, 589-595.

Johansen, F.E., and Kaetzel, C.S. (2011). Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. Mucosal Immunol *4*, 598-602.

Johansen, F.E., Pekna, M., Norderhaug, I.N., Haneberg, B., Hietala, M.A., Krajci, P., Betsholtz, C., and Brandtzaeg, P. (1999). Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J Exp Med *190*, 915-922.

Johansson, M.E., Gustafsson, J.K., Holmen-Larsson, J., Jabbar, K.S., Xia, L., Xu, H., Ghishan, F.K., Carvalho, F.A., Gewirtz, A.T., Sjovall, H., and Hansson, G.C. (2014). Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut *63*, 281-291.

Johansson, M.E., Jakobsson, H.E., Holmen-Larsson, J., Schutte, A., Ermund, A., Rodriguez-Pineiro, A.M., Arike, L., Wising, C., Svensson, F., Backhed, F., and Hansson, G.C. (2015). Normalization of host intestinal mucus layers requires long-term microbial colonization. Cell Host Microbe *18*, 582-592.

Johansson, M.E., Larsson, J.M., and Hansson, G.C. (2011). The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A *108 Suppl 1*, 4659-4665.

Josefowicz, S.Z., Niec, R.E., Kim, H.Y., Treuting, P., Chinen, T., Zheng, Y., Umetsu, D.T., and Rudensky, A.Y. (2012). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature *482*, 395-399.

Kapil, P., Atkinson, R., Ramakrishna, C., Cua, D.J., Bergmann, C.C., and Stohlman, S.A. (2009). Interleukin-12 (IL-12), but not IL-23, deficiency ameliorates viral encephalitis without affecting viral control. J Virol *83*, 5978-5986.

Kashiwagi, I., Morita, R., Schichita, T., Komai, K., Saeki, K., Matsumoto, M., Takeda, K., Nomura, M., Hayashi, A., Kanai, T., and Yoshimura, A. (2015). Smad2 and Smad3 inversely regulate TGF-beta autoinduction in Clostridium butyricum-activated dendritic cells. Immunity *43*, 65-79.

Kashyap, P.C., Marcobal, A., Ursell, L.K., Smits, S.A., Sonnenburg, E.D., Costello, E.K., Higginbottom, S.K., Domino, S.E., Holmes, S.P., Relman, D.A.*, et al.* (2013). Genetically dictated change in host mucus carbohydrate landscape exerts a diet-dependent effect on the gut microbiota. Proc Natl Acad Sci U S A *110*, 17059-17064.

Kawamoto, S., Maruya, M., Kato, L.M., Suda, W., Atarashi, K., Doi, Y., Tsutsui, Y., Qin, H., Honda, K., Okada, T.*, et al.* (2014). Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. Immunity *41*, 152-165.

Khounlotham, M., Kim, W., Peatman, E., Nava, P., Medina-Contreras, O., Addis, C., Koch, S., Fournier, B., Nusrat, A., Denning, T.L., and Parkos, C.A. (2012). Compromised intestinal epithelial barrier induces adaptive immune compensation that protects from colitis. Immunity *37*, 563-573.

Kinnebrew, M.A., Buffie, C.G., Diehl, G.E., Zenewicz, L.A., Leiner, I., Hohl, T.M., Flavell, R.A., Littman, D.R., and Pamer, E.G. (2012). Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. Immunity *36*, 276-287.

Klose, C.S., Blatz, K., d'Hargues, Y., Hernandez, P.P., Kofoed-Nielsen, M., Ripka, J.F., Ebert, K., Arnold, S.J., Diefenbach, A., Palmer, E., and Tanriver, Y. (2014). The transcription factor T-bet is induced by IL-15 and thymic agonist selection and controls CD8alphaalpha(+) intraepithelial lymphocyte development. Immunity *41*, 230-243.

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y.S., De Vadder, F., Arora, T., Hallen, A., Martens, E., Bjorck, I., and Backhed, F. (2015). Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. Cell Metab *22*, 971-982.

Kruglov, A.A., Grivennikov, S.I., Kuprash, D.V., Winsauer, C., Prepens, S., Seleznik, G.M., Eberl, G., Littman, D.R., Heikenwalder, M., Tumanov, A.V., and Nedospasov, S.A. (2013). Nonredundant function of soluble LTalpha3 produced by innate lymphoid cells in intestinal homeostasis. Science *342*, 1243-1246.

Kubinak, J.L., Petersen, C., Stephens, W.Z., Soto, R., Bake, E., O'Connell, R.M., and Round, J.L. (2015a). MyD88 signaling in T cells directs IgA-mediated control of the microbiota to promote health. Cell Host Microbe *17*, 153-163.

Kubinak, J.L., Stephens, W.Z., Soto, R., Petersen, C., Chiaro, T., Gogokhia, L., Bell, R., Ajami, N.J., Petrosino, J.F., Morrison, L.*, et al.* (2015b). MHC variation sculpts individualized microbial communities that control susceptibility to enteric infection. Nat Commun *6*, 8642.

Kullberg, M.C., Jankovic, D., Feng, C.G., Hue, S., Gorelick, P.L., McKenzie, B.S., Cua, D.J., Powrie, F., Cheever, A.W., Maloy, K.J., and Sher, A. (2006). IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. J Exp Med *203*, 2485-2494.

Kupz, A., Guarda, G., Gebhardt, T., Sander, L.E., Short, K.R., Diavatopoulos, D.A., Wijburg, O.L., Cao, H., Waithman, J.C., Chen, W.*, et al.* (2012). NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. Nat Immunol *13*, 162-169.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., and Cua, D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med *201*, 233-240.

Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.W., Santacruz, N., Peterson, D.A., Stappenbeck, T.S., and Hsieh, C.S. (2011). Peripheral education of the immune system by colonic commensal microbiota. Nature *478*, 250-254.

LeBlanc, J.G., Laino, J.E., del Valle, M.J., Vannini, V., van Sinderen, D., Taranto, M.P., de Valdez, G.F., de Giori, G.S., and Sesma, F. (2011). B-group vitamin production by lactic acid bacteria--current knowledge and potential applications. J Appl Microbiol *111*, 1297-1309.

LeBlanc, J.G., Milani, C., de Giori, G.S., Sesma, F., van Sinderen, D., and Ventura, M. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol *24*, 160-168.

Lecuyer, E., Rakotobe, S., Lengline-Garnier, H., Lebreton, C., Picard, M., Juste, C., Fritzen, R., Eberl, G., McCoy, K.D., Macpherson, A.J.*, et al.* (2014). Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses. Immunity *40*, 608-620.

Lee, J.S., Tato, C.M., Joyce-Shaikh, B., Gulan, F., Cayatte, C., Chen, Y., Blumenschein, W.M., Judo, M., Ayanoglu, G., McClanahan, T.K.*, et al.* (2015). Interleukin-23-independent IL-17 production regulates intestinal epithelial permeability. Immunity *43*, 727-738.

Lee, Y.K., Menezes, J.S., Umesaki, Y., and Mazmanian, S.K. (2011). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A *108 Suppl 1*, 4615-4622.

Lehotzky, R.E., Partch, C.L., Mukherjee, S., Cash, H.L., Goldman, W.E., Gardner, K.H., and Hooper, L.V. (2010). Molecular basis for peptidoglycan recognition by a bactericidal lectin. Proc Natl Acad Sci U S A *107*, 7722-7727.

Lemon, K.P., Armitage, G.C., Relman, D.A., and Fischbach, M.A. (2012). Microbiota-targeted therapies: an ecological perspective. Sci Transl Med *4*, 137rv135.

Letran, S.E., Lee, S.J., Atif, S.M., Flores-Langarica, A., Uematsu, S., Akira, S., Cunningham, A.F., and McSorley, S.J. (2011). TLR5-deficient mice lack basal inflammatory and metabolic defects but exhibit impaired CD4 T cell responses to a flagellated pathogen. J Immunol *186*, 5406-5412.

Levy, M., Thaiss, C.A., Zeevi, D., Dohnalova, L., Zilberman-Schapira, G., Mahdi, J.A., David, E., Savidor, A., Korem, T., Herzig, Y.*, et al.* (2015). Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. Cell *163*, 1428-1443.

Ley, R.E., Backhed, F., Turnbaugh, P., Lozupone, C.A., Knight, R.D., and Gordon, J.I. (2005). Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A *102*, 11070-11075.

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., and Gordon, J.I. (2008a). Evolution of mammals and their gut microbes. Science *320*, 1647-1651.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., and Gordon, J.I. (2008b). Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol *6*, 776-788.

Ley, R.E., Peterson, D.A., and Gordon, J.I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell *124*, 837-848.

Li, H., Limenitakis, J.P., Fuhrer, T., Geuking, M.B., Lawson, M.A., Wyss, M., Brugiroux, S., Keller, I., Macpherson, J.A., Rupp, S.*, et al.* (2015). The outer mucus layer hosts a distinct intestinal microbial niche. Nat Commun *6*, 8292.

Li, Y., Innocentin, S., Withers, D.R., Roberts, N.A., Gallagher, A.R., Grigorieva, E.F., Wilhelm, C., and Veldhoen, M. (2011). Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. Cell *147*, 629-640.

Lindemans, C.A., Calafiore, M., Mertelsmann, A.M., O'Connor, M.H., Dudakov, J.A., Jenq, R.R., Velardi, E., Young, L.F., Smith, O.M., Lawrence, G.*, et al.* (2015). Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. Nature *528*, 560-564.

Lindner, C., Thomsen, I., Wahl, B., Ugur, M., Sethi, M.K., Friedrichsen, M., Smoczek, A., Ott, S., Baumann, U., Suerbaum, S.*, et al.* (2015). Diversification of memory B cells drives the continuous adaptation of secretory antibodies to gut microbiota. Nat Immunol *16*, 880-888.

Littman, D.R., and Rudensky, A.Y. (2010). Th17 and regulatory T cells in mediating and restraining inflammation. Cell *140*, 845-858.

Longman, R.S., Diehl, G.E., Victorio, D.A., Huh, J.R., Galan, C., Miraldi, E.R., Swaminath, A., Bonneau, R., Scherl, E.J., and Littman, D.R. (2014). CX(3)CR1(+) mononuclear phagocytes support colitis-associated innate lymphoid cell production of IL-22. J Exp Med *211*, 1571-1583.

Lycke, N., Karlsson, U., Sjolander, A., and Magnusson, K.E. (1991). The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens. Scand J Immunol *33*, 691-698.

Macia, L., Tan, J., Vieira, A.T., Leach, K., Stanley, D., Luong, S., Maruya, M., Ian McKenzie, C., Hijikata, A., Wong, C.*, et al.* (2015). Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun *6*, 6734.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222-2226.

Macpherson, A.J., and McCoy, K.D. (2015a). Independence Day for IgA. Immunity *43*, 416-418.

Macpherson, A.J., and McCoy, K.D. (2015b). Standardised animal models of host microbial mutualism. Mucosal Immunol *8*, 476-486.

Macpherson, A.J., McCoy, K.D., Johansen, F.E., and Brandtzaeg, P. (2008). The immune geography of IgA induction and function. Mucosal Immunol *1*, 11-22.

Macy, J.M., Ljungdahl, L.G., and Gottschalk, G. (1978). Pathway of succinate and propionate formation in Bacteroides fragilis. J Bacteriol *134*, 84-91.

Marcobal, A., Barboza, M., Sonnenburg, E.D., Pudlo, N., Martens, E.C., Desai, P., Lebrilla, C.B., Weimer, B.C., Mills, D.A., German, J.B., and Sonnenburg, J.L. (2011). Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe *10*, 507-514.

Martin, B., Hirota, K., Cua, D.J., Stockinger, B., and Veldhoen, M. (2009). Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity *31*, 321-330.

Maslowski, K.M., and Mackay, C.R. (2011). Diet, gut microbiota and immune responses. Nat Immunol *12*, 5-9.

Maslowski, K.M., Vieira, A.T., Ng, A., Kranich, J., Sierro, F., Yu, D., Schilter, H.C., Rolph, M.S., Mackay, F., Artis, D.*, et al.* (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature *461*, 1282-1286.

Maxwell, J.R., Zhang, Y., Brown, W.A., Smith, C.L., Byrne, F.R., Fiorino, M., Stevens, E., Bigler, J., Davis, J.A., Rottman, J.B.*, et al.* (2015). Differential roles for Interleukin-23 and Interleukin-17 in intestinal immunoregulation. Immunity *43*, 739-750.

Mazmanian, S.K., Liu, C.H., Tzianabos, A.O., and Kasper, D.L. (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell *122*, 107-118.

McCoy, K.D., Harris, N.L., Diener, P., Hatak, S., Odermatt, B., Hangartner, L., Senn, B.M., Marsland, B.J., Geuking, M.B., Hengartner, H.*, et al.* (2006). Natural IgE production in the absence of MHC Class II cognate help. Immunity *24*, 329-339.

McDole, J.R., Wheeler, L.W., McDonald, K.G., Wang, B., Konjufca, V., Knoop, K.A., Newberry, R.D., and Miller, M.J. (2012). Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature *483*, 345-349.

Medina-Contreras, O., Geem, D., Laur, O., Williams, I.R., Lira, S.A., Nusrat, A., Parkos, C.A., and Denning, T.L. (2011). CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. J Clin Invest, 4787-4795.

Mielke, L.A., Jones, S.A., Raverdeau, M., Higgs, R., Stefanska, A., Groom, J.R., Misiak, A., Dungan, L.S., Sutton, C.E., Streubel, G.*, et al.* (2013). Retinoic acid expression associates with enhanced IL-22 production by gammadelta T cells and innate lymphoid cells and attenuation of intestinal inflammation. J Exp Med *210*, 1117-1124.

Mjosberg, J., Bernink, J., Peters, C., and Spits, H. (2012). Transcriptional control of innate lymphoid cells. Eur J Immunol *42*, 1916-1923.

Mohle-Boetani, J.C., Ajello, G., Breneman, E., Deaver, K.A., Harvey, C., Plikaytis, B.D., Farley, M.M., Stephens, D.S., and Wenger, J.D. (1993). Carriage of Haemophilus influenzae type b in children after widespread vaccination with conjugate Haemophilus influenzae type b vaccines. Pediatr Infect Dis J *12*, 589-593.

Mondot, S., Barreau, F., Al Nabhani, Z., Dussaillant, M., Le Roux, K., Dore, J., Leclerc, M., Hugot, J.P., and Lepage, P. (2012). Altered gut microbiota composition in immune-impaired Nod2(-/-) mice. Gut *61*, 634-635.

Moon, C., Baldridge, M.T., Wallace, M.A., Burnham, C.A., Virgin, H.W., and Stappenbeck, T.S. (2015). Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. Nature *521*, 90-93.

Mortha, A., Chudnovskiy, A., Hashimoto, D., Bogunovic, M., Spencer, S.P., Belkaid, Y., and Merad, M. (2014). Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. Science *343*, 1249288.

Muegge, B.D., Kuczynski, J., Knights, D., Clemente, J.C., Gonzalez, A., Fontana, L., Henrissat, B., Knight, R., and Gordon, J.I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science *332*, 970-974.

Mueller, N.T., Bakacs, E., Combellick, J., Grigoryan, Z., and Dominguez-Bello, M.G. (2015). The infant microbiome development: mom matters. Trends Mol Med *21*, 109-117.

Mukherjee, S., and Hooper, L.V. (2015). Antimicrobial defense of the intestine. Immunity *42*, 28-39.

Mukherjee, S., Zheng, H., Derebe, M.G., Callenberg, K.M., Partch, C.L., Rollins, D., Propheter, D.C., Rizo, J., Grabe, M., Jiang, Q.X., and Hooper, L.V. (2014). Antibacterial membrane attack by a pore-forming intestinal C-type lectin. Nature *505*, 103-107.

Muller, P.A., Koscso, B., Rajani, G.M., Stevanovic, K., Berres, M.L., Hashimoto, D., Mortha, A., Leboeuf, M., Li, X.M., Mucida, D*., et al.* (2014). Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. Cell *158*, 300-313.

Munoz, M., Eidenschenk, C., Ota, N., Wong, K., Lohmann, U., Kuhl, A.A., Wang, X., Manzanillo, P., Li, Y., Rutz, S*., et al.* (2015). Interleukin-22 induces interleukin-18 expression from epithelial cells during intestinal infection. Immunity *42*, 321-331.

Munoz-Tamayo, R., Laroche, B., Walter, E., Dore, J., Duncan, S.H., Flint, H.J., and Leclerc, M. (2011). Kinetic modelling of lactate utilization and butyrate production by key human colonic bacterial species. FEMS Microbiol Ecol *76*, 615-624.

Murphy, C.A., Langrish, C.L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R.A., Sedgwick, J.D., and Cua, D.J. (2003). Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med *198*, 1951-1957.

Nagler-Anderson, C. (2001). Man the barrier! Strategic defenses in the intestinal mucosa. Nat Rev Immunol *1*, 59-67.

Nagler-Anderson, C., and Shi, H.N. (2001). Peripheral nonresponsiveness to orally administered soluble protein antigens. Crit Rev Immunol *21*, 121-131.

Nava, G.M., Friedrichsen, H.J., and Stappenbeck, T.S. (2011). Spatial organization of intestinal microbiota in the mouse ascending colon. ISME J *5*, 627-638.

Nava, G.M., and Stappenbeck, T.S. (2011). Diversity of the autochthonous colonic microbiota. Gut Microbes *2*, 99-104.

Ng, K.M., Ferreyra, J.A., Higginbottom, S.K., Lynch, J.B., Kashyap, P.C., Gopinath, S., Naidu, N., Choudhury, B., Weimer, B.C., Monack, D.M., and Sonnenburg, J.L. (2013). Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature *502*, 96-99.

Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G*., et al.* (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science *307*, 254-258.

Nishio, J., Baba, M., Atarashi, K., Tanoue, T., Negishi, H., Yanai, H., Habu, S., Hori, S., Honda, K., and Taniguchi, T. (2015). Requirement of full TCR repertoire for regulatory T cells to maintain intestinal homeostasis. Proc Natl Acad Sci U S A *112*, 12770-12775.

Nobel, Y.R., Cox, L.M., Kirigin, F.F., Bokulich, N.A., Yamanishi, S., Teitler, I., Chung, J., Sohn, J., Barber, C.M., Goldfarb, D.S.*, et al.* (2015). Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment. Nat Commun *6*, 7486.

Noval Rivas, M., Burton, O.T., Wise, P., Zhang, Y.Q., Hobson, S.A., Garcia Lloret, M., Chehoud, C., Kuczynski, J., DeSantis, T., Warrington, J.*, et al.* (2013). A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. J Allergy Clin Immunol *131*, 201-212.

Nowarski, R., Jackson, R., Gagliani, N., de Zoete, M.R., Palm, N.W., Bailis, W., Low, J.S., Harman, C.C., Graham, M., Elinav, E., and Flavell, R.A. (2015). Epithelial IL-18 equilibrium controls barrier function in colitis. Cell *163*, 1444-1456.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H.*, et al.* (2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature *411*, 603-606.

Ohnmacht, C., Park, J.H., Cording, S., Wing, J.B., Atarashi, K., Obata, Y., Gaboriau-Routhiau, V., Marques, R., Dulauroy, S., Fedoseeva, M.*, et al.* (2015). The microbiota regulates type 2 immunity through RORgammat(+) T cells. Science *349*, 989-993.

Ohno, H. (2015). Intestinal M cells. J Biochem, Epub ahead of print.

Olszak, T., An, D., Zeissig, S., Vera, M.P., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L., and Blumberg, R.S. (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. Science *336*, 489-493.

Ota, N., Wong, K., Valdez, P.A., Zheng, Y., Crellin, N.K., Diehl, L., and Ouyang, W. (2011). IL-22 bridges the lymphotoxin pathway with the maintenance of colonic lymphoid structures during infection with Citrobacter rodentium. Nat Immunol *12*, 941-948.

Otte, J.M., Cario, E., and Podolsky, D.K. (2004). Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology *126*, 1054-1070.

Pabst, O. (2012). New concepts in the generation and functions of IgA. Nat Rev Immunol *12*, 821-832.

Pabst, O., and Mowat, A.M. (2012). Oral tolerance to food protein. Mucosal Immunol *5*, 232-239.

Palm, N.W., de Zoete, M.R., Cullen, T.W., Barry, N.A., Stefanowski, J., Hao, L., Degnan, P.H., Hu, J., Peter, I., Zhang, W.*, et al.* (2014). Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. Cell *158*, 1000-1010.

Panea, C., Farkas, A.M., Goto, Y., Abdollahi-Roodsaz, S., Lee, C., Koscso, B., Gowda, K., Hohl, T.M., Bogunovic, M., and Ivanov, I.I. (2015). Intestinal monocyte-derived macrophages control commensal-specific Th17 responses. Cell Rep *12*, 1314-1324.

Pantoja-Feliciano, I.G., Clemente, J.C., Costello, E.K., Perez, M.E., Blaser, M.J., Knight, R., and Dominguez-Bello, M.G. (2013). Biphasic assembly of the murine intestinal microbiota during early development. ISME J *7*, 1112-1115.

Patel, R.M., Myers, L.S., Kurundkar, A.R., Maheshwari, A., Nusrat, A., and Lin, P.W. (2012). Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. Am J Pathol *180*, 626-635.

Peng, H.J., Turner, M.W., and Strobel, S. (1990). The generation of a 'tolerogen' after the ingestion of ovalbumin is time-dependent and unrelated to serum levels of immunoreactive antigen. Clin Exp Immunol *81*, 510-515.

Perrine, S.P., Miller, B.A., Faller, D.V., Cohen, R.A., Vichinsky, E.P., Hurst, D., Lubin, B.H., and Papayannopoulou, T. (1989). Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with Hb SS and beta thalassemia. Blood *74*, 454-459.

Prioult, G., and Nagler-Anderson, C. (2005). Mucosal immunity and allergic responses: lack of regulation and/or lack of microbial stimulation? Immunol Rev *206*, 204-218.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., and Flint, H.J. (2002). The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett *217*, 133-139.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T.*, et al.* (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature *464*, 59-65.

Qiu, J., Guo, X., Chen, Z.M., He, L., Sonnenberg, G.F., Artis, D., Fu, Y.X., and Zhou, L. (2013). Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. Immunity *39*, 386-399.

Qiu, J., Heller, J.J., Guo, X., Chen, Z.M., Fish, K., Fu, Y.X., and Zhou, L. (2012). The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity *36*, 92-104.

Rakoff-Nahoum, S., Kong, Y., Kleinstein, S.H., Subramanian, S., Ahern, P.P., Gordon, J.I., and Medzhitov, R. (2015). Analysis of gene-environment interactions in postnatal development of the mammalian intestine. Proc Natl Acad Sci U S A *112*, 1929-1936.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell *118*, 229-241.

Rankin, L.C., Girard-Madoux, M.J., Seillet, C., Mielke, L.A., Kerdiles, Y., Fenis, A., Wieduwild, E., Putoczki, T., Mondot, S., Lantz, O.*, et al.* (2015). Complementarity and redundancy of IL-22-producing innate lymphoid cells. Nat Immunol, Epub ahead of print.

Rawls, J.F., Mahowald, M.A., Ley, R.E., and Gordon, J.I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell *127*, 423-433.

Robertson, S.J., Zhou, J.Y., Geddes, K., Rubino, S.J., Cho, J.H., Girardin, S.E., and Philpott, D.J. (2013). Nod1 and Nod2 signaling does not alter the composition of intestinal bacterial communities at homeostasis. Gut Microbes *4*, 222-231.

Roediger, W.E. (1980). Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. Gut *21*, 793-798.

Round, J.L., Lee, S.M., Li, J., Tran, G., Jabri, B., Chatila, T.A., and Mazmanian, S.K. (2011). The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science *332*, 974-977.

Round, J.L., and Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol *9*, 313-323.

Round, J.L., and Mazmanian, S.K. (2010). Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A *107*, 12204-12209.

Russell, S.L., Gold, M.J., Hartmann, M., Willing, B.P., Thorson, L., Wlodarska, M., Gill, N., Blanchet, M.R., Mohn, W.W., McNagny, K.M., and Finlay, B.B. (2012). Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep *13*, 440-447.

Russell, W.R., Hoyles, L., Flint, H.J., and Dumas, M.E. (2013). Colonic bacterial metabolites and human health. Curr Opin Microbiol *16*, 246-254.

Sabat, R., Ouyang, W., and Wolk, K. (2014). Therapeutic opportunities of the IL-22-IL-22R1 system. Nat Rev Drug Discov *13*, 21-38.

Sano, T., Huang, W., Hall, J.A., Yang, Y., Chen, A., Gavzy, S.J., Lee, J.Y., Ziel, J.W., Miraldi, E.R., Domingos, A.I.*, et al.* (2015). An IL-23R/IL-22 circuit regulates epithelial serum amyloid A to promote local effector Th17 responses. Cell *163*, 381-393.

Sanos, S.L., Bui, V.L., Mortha, A., Oberle, K., Heners, C., Johner, C., and Diefenbach, A. (2009). RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat Immunol *10*, 83-91.

Sanos, S.L., Vonarbourg, C., Mortha, A., and Diefenbach, A. (2011). Control of epithelial cell function by interleukin-22-producing RORgammat+ innate lymphoid cells. Immunology *132*, 453-465.

Sarma-Rupavtarm, R.B., Ge, Z., Schauer, D.B., Fox, J.G., and Polz, M.F. (2004). Spatial distribution and stability of the eight microbial species of the altered schaedler flora in the mouse gastrointestinal tract. Appl Environ Microbiol *70*, 2791-2800.

Satoh-Takayama, N., Lesjean-Pottier, S., Sawa, S., Vosshenrich, C.A., Eberl, G., and Di Santo, J.P. (2011). Lymphotoxin-beta receptor-independent development of intestinal IL-22-producing NKp46+ innate lymphoid cells. Eur J Immunol *41*, 780-786.

Satoh-Takayama, N., Vosshenrich, C.A., Lesjean-Pottier, S., Sawa, S., Lochner, M., Rattis, F., Mention, J.J., Thiam, K., Cerf-Bensussan, N., Mandelboim, O.*, et al.* (2008). Microbial flora

drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity *29*, 958-970.

Sawa, S., Cherrier, M., Lochner, M., Satoh-Takayama, N., Fehling, H.J., Langa, F., Di Santo, J.P., and Eberl, G. (2010). Lineage relationship analysis of RORgammat+ innate lymphoid cells. Science *330*, 665-669.

Sawa, S., Lochner, M., Satoh-Takayama, N., Dulauroy, S., Berard, M., Kleinschek, M., Cua, D., Di Santo, J.P., and Eberl, G. (2011). RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. Nat Immunol *12*, 320-326.

Schaedler, R.W., Dubs, R., and Costello, R. (1965). Association of germfree mice with bacteria isolated from normal mice J Exp Med *122*, 77-82.

Schiering, C., Krausgruber, T., Chomka, A., Frohlich, A., Adelmann, K., Wohlfert, E.A., Pott, J., Griseri, T., Bollrath, J., Hegazy, A.N.*, et al.* (2014). The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature *513*, 564-568.

Schmechel, S., Konrad, A., Diegelmann, J., Glas, J., Wetzke, M., Paschos, E., Lohse, P., Goke, B., and Brand, S. (2008). Linking genetic susceptibility to Crohn's disease with Th17 cell function: IL-22 serum levels are increased in Crohn's disease and correlate with disease activity and IL23R genotype status. Inflamm Bowel Dis *14*, 204-212.

Schulz, O., Jaensson, E., Persson, E.K., Liu, X., Worbs, T., Agace, W.W., and Pabst, O. (2009). Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med *206*, 3101-3114.

Schulz, V.J., Smit, J.J., Bol-Schoenmakers, M., van Duursen, M.B., van den Berg, M., and Pieters, R.H. (2012a). Activation of the aryl hydrocarbon receptor reduces the number of precursor and effector T cells, but preserves thymic CD4+CD25+Foxp3+ regulatory T cells. Toxicol Lett *215*, 100-109.

Schulz, V.J., Smit, J.J., Huijgen, V., Bol-Schoenmakers, M., van Roest, M., Kruijssen, L.J., Fiechter, D., Hassing, I., Bleumink, R., Safe, S.*, et al.* (2012b). Non-dioxin-like AhR ligands in a mouse peanut allergy model. Toxicol Sci *128*, 92-102.

Schulz, V.J., Smit, J.J., Willemsen, K.J., Fiechter, D., Hassing, I., Bleumink, R., Boon, L., van den Berg, M., van Duursen, M.B., and Pieters, R.H. (2011). Activation of the aryl hydrocarbon receptor suppresses sensitization in a mouse peanut allergy model. Toxicol Sci *123*, 491-500.

Sefik, E., Geva-Zatorsky, N., Oh, S., Konnikova, L., Zemmour, D., McGuire, A.M., Burzyn, D., Ortiz-Lopez, A., Lobera, M., Yang, J.*, et al.* (2015). Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. Science *349*, 993-997.

Seo, S.U., Kuffa, P., Kitamoto, S., Nagao-Kitamoto, H., Rousseau, J., Kim, Y.G., Nunez, G., and Kamada, N. (2015). Intestinal macrophages arising from CCR2(+) monocytes control pathogen infection by activating innate lymphoid cells. Nat Commun *6*, 8010.

Shan, M., Gentile, M., Yeiser, J.R., Walland, A.C., Bornstein, V.U., Chen, K., He, B., Cassis, L., Bigas, A., Cols, M.*, et al.* (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science *342*, 447-453.

Shapiro, H., Thaiss, C.A., Levy, M., and Elinav, E. (2014). The cross talk between microbiota and the immune system: metabolites take center stage. Curr Opin Immunol *30*, 54-62.

Shaw, M.H., Kamada, N., Kim, Y.G., and Nunez, G. (2012). Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. J Exp Med *209*, 251-258.

Simons, F.E., Ardusso, L.R., Bilo, M.B., El-Gamal, Y.M., Ledford, D.K., Ring, J., Sanchez-Borges, M., Senna, G.E., Sheikh, A., and Thong, B.Y. (2011). World Allergy Organization anaphylaxis guidelines: summary. J Allergy Clin Immunol *127*, 587-593.e581-522.

Sina, C., Gavrilova, O., Forster, M., Till, A., Derer, S., Hildebrand, F., Raabe, B., Chalaris, A., Scheller, J., Rehmann, A.*, et al.* (2009). G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol *183*, 7514-7522.

Sindher, S., Fleischer, D.M., and Spergel, J.M. (2016). Advances in the treatment of food allergy: sublingual and epicutaneous immunotherapy. Immunol Allergy Clin North Am *36*, 39-54.

Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., Thangaraju, M., Prasad, P.D., Manicassamy, S., Munn, D.H.*, et al.* (2014). Activation of GPR109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity *40*, 128-139.

Singh, N., Thangaraju, M., Prasad, P.D., Martin, P.M., Lambert, N.A., Boettger, T., Offermanns, S., and Ganapathy, V. (2010). Blockade of dendritic cell development by bacterial fermentation products butyrate and propionate through a transporter (Slc5a8)-dependent inhibition of histone deacetylases. J Biol Chem *285*, 27601-27608.

Smith, K., McCoy, K.D., and Macpherson, A.J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol *19*, 59-69.

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly, Y.M., Glickman, J.N., and Garrett, W.S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science *341*, 569-573.

Song, C., Lee, J.S., Gilfillan, S., Robinette, M.L., Newberry, R.D., Stappenbeck, T.S., Mack, M., Cella, M., and Colonna, M. (2015). Unique and redundant functions of NKp46+ ILC3s in models of intestinal inflammation. J Exp Med *212*, 1869-1882.

Sonnenberg, G.F., Fouser, L.A., and Artis, D. (2010). Functional biology of the IL-22-IL-22R pathway in regulating immunity and inflammation at barrier surfaces. Adv Immunol *107*, 1-29.

Sonnenberg, G.F., Fouser, L.A., and Artis, D. (2011). Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol *12*, 383-390.

Sonnenberg, G.F., Monticelli, L.A., Alenghat, T., Fung, T.C., Hutnick, N.A., Kunisawa, J., Shibata, N., Grunberg, S., Sinha, R., Zahm, A.M.*, et al.* (2012). Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. Science *336*, 1321-1325.

Spadoni, I., Zagato, E., Bertocchi, A., Paolinelli, R., Hot, E., Di Sabatino, A., Caprioli, F., Bottiglieri, L., Oldani, A., Viale, G.*, et al.* (2015). A gut-vascular barrier controls the systemic dissemination of bacteria. Science *350*, 830-834.

Spencer, S.P., Wilhelm, C., Yang, Q., Hall, J.A., Bouladoux, N., Boyd, A., Nutman, T.B., Urban, J.F., Jr., Wang, J., Ramalingam, T.R.*, et al.* (2014). Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. Science *343*, 432-437.

Sperandio, B., Fischer, N., and Sansonetti, P.J. (2015). Mucosal physical and chemical innate barriers: Lessons from microbial evasion strategies. Semin Immunol *27*, 111-118.

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N., Mebius, R.E.*, et al.* (2013). Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol *13*, 145-149.

Stefka, A.T., Feehley, T., Tripathi, P., Qiu, J., McCoy, K., Mazmanian, S.K., Tjota, M.Y., Seo, G.Y., Cao, S., Theriault, B.R.*, et al.* (2014). Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A *111*, 13145-13150.

Stein, J., Zores, M., and Schroder, O. (2000). Short-chain fatty acid (SCFA) uptake into Caco-2 cells by a pH-dependent and carrier mediated transport mechanism. Eur J Nutr *39*, 121-125.

Strachan, D.P. (1989). Hay fever, hygiene, and household size. BMJ *299*, 1259-1260.

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C.A., Maza, O., Israeli, D., Zmora, N., Gilad, S., Weinberger, A.*, et al.* (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature *514*, 181-186.

Sugimoto, K., Ogawa, A., Mizoguchi, E., Shimomura, Y., Andoh, A., Bhan, A.K., Blumberg, R.S., Xavier, R.J., and Mizoguchi, A. (2008). IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest *118*, 534-544.

Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R., and Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med *204*, 1775-1785.

Takala, A.K., Eskola, J., Leinonen, M., Kayhty, H., Nissinen, A., Pekkanen, E., and Makela, P.H. (1991). Reduction of oropharyngeal carriage of Haemophilus influenzae type b (Hib) in children immunized with an Hib conjugate vaccine. J Infect Dis *164*, 982-986.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., and Macia, L. (2014). The role of short-chain fatty acids in health and disease. Adv Immunol *121*, 91-119.

Thangaraju, M., Cresci, G.A., Liu, K., Ananth, S., Gnanaprakasam, J.P., Browning, D.D., Mellinger, J.D., Smith, S.B., Digby, G.J., Lambert, N.A.*, et al.* (2009). GPR109A is a G-protein-

coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon. Cancer Res *69*, 2826-2832.

Thome, J.J., Yudanin, N., Ohmura, Y., Kubota, M., Grinshpun, B., Sathaliyawala, T., Kato, T., Lerner, H., Shen, Y., and Farber, D.L. (2014). Spatial map of human T cell compartmentalization and maintenance over decades of life. Cell *159*, 814-828.

Thorburn, A.N., Macia, L., and Mackay, C.R. (2014). Diet, metabolites, and "western-lifestyle" inflammatory diseases. Immunity *40*, 833-842.

Thorburn, A.N., McKenzie, C.I., Shen, S., Stanley, D., Macia, L., Mason, L.J., Roberts, L.K., Wong, C.H., Shim, R., Robert, R.*, et al.* (2015). Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. Nat Commun *6*, 7320.

Tjota, M.Y., Hrusch, C.L., Blaine, K.M., Williams, J.W., Barrett, N.A., and Sperling, A.I. (2014). Signaling through FcRgamma-associated receptors on dendritic cells drives IL-33-dependent TH2-type responses. J Allergy Clin Immunol *134*, 706-713.e708.

Trompette, A., Gollwitzer, E.S., Yadava, K., Sichelstiel, A.K., Sprenger, N., Ngom-Bru, C., Blanchard, C., Junt, T., Nicod, L.P., Harris, N.L., and Marsland, B.J. (2014). Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med *20*, 159-166.

Tsuji, M., Komatsu, N., Kawamoto, S., Suzuki, K., Kanagawa, O., Honjo, T., Hori, S., and Fagarasan, S. (2009). Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science *323*, 1488-1492.

Tumanov, A.V., Koroleva, E.P., Guo, X., Wang, Y., Kruglov, A., Nedospasov, S., and Fu, Y.X. (2011). Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. Cell Host Microbe *10*, 44-53.

Turnbaugh, P.J., Backhed, F., Fulton, L., and Gordon, J.I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe *3*, 213-223.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P.*, et al.* (2009a). A core gut microbiome in obese and lean twins. Nature *457*, 480-484.

Turnbaugh, P.J., Ridaura, V.K., Faith, J.J., Rey, F.E., Knight, R., and Gordon, J.I. (2009b). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med *1*, 6ra14.

Turner, J.R. (2009). Intestinal mucosal barrier function in health and disease. Nat Rev Immunol *9*, 799-809.

Ubeda, C., Lipuma, L., Gobourne, A., Viale, A., Leiner, I., Equinda, M., Khanin, R., and Pamer, E.G. (2012). Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J Exp Med *209*, 1445-1456.

Upadhyay, V., Poroyko, V., Kim, T.J., Devkota, S., Fu, S., Liu, D., Tumanov, A.V., Koroleva, E.P., Deng, L., Nagler, C.*, et al.* (2012). Lymphotoxin regulates commensal responses to enable diet-induced obesity. Nat Immunol *13*, 947-953.

Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland, E.K., and Hooper, L.V. (2011). The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science *334*, 255-258.

van Aubel, R.A., Keestra, A.M., Krooshoop, D.J., van Eden, W., and van Putten, J.P. (2007). Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells. Mol Immunol *44*, 3702-3714.

Van Itallie, C.M., Holmes, J., Bridges, A., Gookin, J.L., Coccaro, M.R., Proctor, W., Colegio, O.R., and Anderson, J.M. (2008). The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. J Cell Sci *121*, 298-305.

Van Maele, L., Carnoy, C., Cayet, D., Songhet, P., Dumoutier, L., Ferrero, I., Janot, L., Erard, F., Bertout, J., Leger, H.*, et al.* (2010). TLR5 signaling stimulates the innate production of IL-17 and IL-22 by CD3(neg)CD127+ immune cells in spleen and mucosa. J Immunol *185*, 1177-1185.

van Wijk, F., Hoeks, S., Nierkens, S., Koppelman, S.J., van Kooten, P., Boon, L., Knippels, L.M., and Pieters, R. (2005). CTLA-4 signaling regulates the intensity of hypersensitivity responses to food antigens, but is not decisive in the induction of sensitization. J Immunol *174*, 174-179.

Veenbergen, S., van Berkel, L.A., du Pre, M.F., He, J., Karrich, J.J., Costes, L.M., Luk, F., Simons-Oosterhuis, Y., Raatgeep, H.C., Cerovic, V.*, et al.* (2015). Colonic tolerance develops in the iliac lymph nodes and can be established independent of CD103 dendritic cells. Mucosal Immunol, Epub ahead of print.

Veldhoen, M., Hirota, K., Westendorf, A.M., Buer, J., Dumoutier, L., Renauld, J.C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature *453*, 106-109.

Ventura, M.T., Polimeno, L., Amoruso, A.C., Gatti, F., Annoscia, E., Marinaro, M., Di Leo, E., Matino, M.G., Buquicchio, R., Bonini, S.*, et al.* (2006). Intestinal permeability in patients with adverse reactions to food. Dig Liver Dis *38*, 732-736.

Vidali, G., Boffa, L.C., Bradbury, E.M., and Allfrey, V.G. (1978). Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. Proc Natl Acad Sci U S A *75*, 2239-2243.

Vijay-Kumar, M., Aitken, J.D., Carvalho, F.A., Cullender, T.C., Mwangi, S., Srinivasan, S., Sitaraman, S.V., Knight, R., Ley, R.E., and Gewirtz, A.T. (2010). Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science *328*, 228-231.

Walker, J.A., Barlow, J.L., and McKenzie, A.N. (2013). Innate lymphoid cells--how did we miss them? Nat Rev Immunol *13*, 75-87.

Wang, F., Graham, W.V., Wang, Y., Witkowski, E.D., Schwarz, B.T., and Turner, J.R. (2005). Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol *166*, 409-419.

Wang, H., Zhao, Y., Bradbury, J.A., Graves, J.P., Foley, J., Blaisdell, J.A., Goldstein, J.A., and Zeldin, D.C. (2004). Cloning, expression, and characterization of three new mouse cytochrome p450 enzymes and partial characterization of their fatty acid oxidation activities. Mol Pharmacol *65*, 1148-1158.

Wang, S., Charbonnier, L.M., Noval Rivas, M., Georgiev, P., Li, N., Gerber, G., Bry, L., and Chatila, T.A. (2015). MyD88 adaptor-dependent microbial sensing by regulatory T cells promotes mucosal tolerance and enforces commensalism. Immunity *43*, 289-303.

Wang, S., Villablanca, E.J., De Calisto, J., Gomes, D.C., Nguyen, D.D., Mizoguchi, E., Kagan, J.C., Reinecker, H.C., Hacohen, N., Nagler, C.*, et al.* (2011). MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting properties. J Immunol *187*, 141-150.

Weber, C.R., Liang, G.H., Wang, Y., Das, S., Shen, L., Yu, A.S., Nelson, D.J., and Turner, J.R. (2015). Claudin-2-dependent paracellular channels are dynamically gated. Elife *4*.

Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A.C., Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A.*, et al.* (2008). Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature *455*, 1109-1113.

Wesemann, D.R. (2015). Microbes and B cell development. Adv Immunol *125*, 155-178.

Wesemann, D.R., Portuguese, A.J., Meyers, R.M., Gallagher, M.P., Cluff-Jones, K., Magee, J.M., Panchakshari, R.A., Rodig, S.J., Kepler, T.B., and Alt, F.W. (2013). Microbial colonization influences early B-lineage development in the gut lamina propria. Nature *501*, 112-115.

Williams, J.W., Tjota, M.Y., Clay, B.S., Vander Lugt, B., Bandukwala, H.S., Hrusch, C.L., Decker, D.C., Blaine, K.M., Fixsen, B.R., Singh, H.*, et al.* (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. Nat Commun *4*, 2990.

Wolk, K., Kunz, S., Witte, E., Friedrich, M., Asadullah, K., and Sabat, R. (2004). IL-22 increases the innate immunity of tissues. Immunity *21*, 241-254.

Wood, R.A., Kim, J.S., Lindblad, R., Nadeau, K., Henning, A.K., Dawson, P., Plaut, M., and Sampson, H.A. (2015). A randomized, double-blind, placebo-controlled study of omalizumab combined with oral immunotherapy for the treatment of cow's milk allergy. J Allergy Clin Immunol, Epub ahead of print.

Wu, H.J., Ivanov, II, Darce, J., Hattori, K., Shima, T., Umesaki, Y., Littman, D.R., Benoist, C., and Mathis, D. (2010). Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity *32*, 815-827.

Wu, M., McNulty, N.P., Rodionov, D.A., Khoroshkin, M.S., Griffin, N.W., Cheng, J., Latreille, P., Kerstetter, R.A., Terrapon, N., Henrissat, B.*, et al.* (2015). Genetic determinants of in vivo fitness and diet responsiveness in multiple human gut Bacteroides. Science *350*, aac5992.

Yamashita, H., Takahashi, K., Tanaka, H., Nagai, H., and Inagaki, N. (2012). Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. Allergy *67*, 201-209.

Yang, Y., Torchinsky, M.B., Gobert, M., Xiong, H., Xu, M., Linehan, J.L., Alonzo, F., Ng, C., Chen, A., Lin, X.*, et al.* (2014). Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. Nature *510*, 152-156.

Yano, J.M., Yu, K., Donaldson, G.P., Shastri, G.G., Ann, P., Ma, L., Nagler, C.R., Ismagilov, R.F., Mazmanian, S.K., and Hsiao, E.Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell *161*, 264-276.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R.N., Anokhin, A.P.*, et al.* (2012). Human gut microbiome viewed across age and geography. Nature *486*, 222-227.

Yeung, V.P., Gieni, R.S., Umetsu, D.T., and DeKruyff, R.H. (1998). Heat-killed Listeria monocytogenes as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. J. Immunol. *161*, 4146-4152.

Yuki, T., Yoshida, H., Akazawa, Y., Komiya, A., Sugiyama, Y., and Inoue, S. (2011). Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. J Immunol *187*, 3230-3237.

Zaiss, M.M., Rapin, A., Lebon, L., Dubey, L.K., Mosconi, I., Sarter, K., Piersigilli, A., Menin, L., Walker, A.W., Rougemont, J.*, et al.* (2015). The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation. Immunity *43*, 998-1010.

Zaph, C., Troy, A.E., Taylor, B.C., Berman-Booty, L.D., Guild, K.J., Du, Y., Yost, E.A., Gruber, A.D., May, M.J., Greten, F.R.*, et al.* (2007). Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. Nature *446*, 552-556.

Zatz, J.L., and Knowles, B. (1970). Effect of pH on monolayers of cellulose acetate phthalate. J Pharm Sci *59*, 1750-1751.

Zelante, T., Iannitti, R.G., Cunha, C., De Luca, A., Giovannini, G., Pieraccini, G., Zecchi, R., D'Angelo, C., Massi-Benedetti, C., Fallarino, F.*, et al.* (2013). Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity *39*, 372-385.

Zhang, B., Chassaing, B., Shi, Z., Uchiyama, R., Zhang, Z., Denning, T.L., Crawford, S.E., Pruijssers, A.J., Iskarpatyoti, J.A., Estes, M.K.*, et al.* (2014). Prevention and cure of rotavirus infection via TLR5/NLRC4-mediated production of IL-22 and IL-18. Science *346*, 861-865.

Zhang, L., Chen, S., Ruan, J., Wu, J., Tong, A.B., Yin, Q., Li, Y., David, L., Lu, A., Wang, W.L.*, et al.* (2015). Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. Science *350*, 404-409.

Zheng, Y., Valdez, P.A., Danilenko, D.M., Hu, Y., Sa, S.M., Gong, Q., Abbas, A.R., Modrusan, Z., Ghilardi, N., de Sauvage, F.J., and Ouyang, W. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med *14*, 282-289.