Individual Microbes Shape Various Parts of the Immune System

Citation

Sefik, Esen. 2015. Individual Microbes Shape Various Parts of the Immune System. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

Individual microbes shape various parts of the immune system

A dissertation presented

by

Esen Sefik

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

Harvard University

Cambridge, Massachusetts

September 2015

Dissertation Advisor: Drs. Christophe Benoist and Diane Mathis Esen Sefik

Individual microbes shape various parts of the immune system

Abstract

The gastrointestinal tract, home to a vast number of bacteria, requires finely-tuned regulatory and effector immune mechanisms to maintain homeostasis and tolerance. In a large-scale screen, we studied the impacts of single microbes on major immune populations, whole intestinal tissue homeostasis and metabolism. Bacteria interacted with the host at multiple levels including cytokine responses, accumulation of various T cells, alterations in composition of mononuclear phagocytes and induction of epithelial cell genes as measured by transcriptome analysis of whole intestinal tissue. Interestingly, taxonomically unrelated bacteria elicited similar immune phenotypes and metabolic effects. A more focused analysis of the induction of regulatory mechanisms revealed a microbiota-dependent, context-specific transcriptional control of Foxp3+ regulatory T cells and of IL17 producing T cells. These facets were both regulated by Rorγ, a transcription factor known for its antagonistic effects on Foxp3. Paradoxically, Rorγ expression induced by bacteria in colonic Foxp3+ regulatory T cells was necessary for function of these cells especially in the context of IL17 and IFNγ-mediated colitis. Overall, this large-scale screen provides a comprehensive study of how individual bacterial species shape many aspects of the host immunity and metabolism, and exemplifies a microbiota-dependent, context-specific mechanism that potentiates function in Foxp3+ regulatory T cells.

Acknowledgements

Throughout my graduate studies, I was very lucky to have the support of many amazing people. I would like to express my sincerest gratitude to all for their endless support, patience and love.

I would like to sincerely thank my advisors, Dr. Christophe Benoist and Dr. Diane Mathis. Their invaluable guidance, mentorship and the example they set allowed me to grow as a scientist and as a person. Thank you for having confidence in me and teaching to reach for higher with patience. I could not have asked for a better research environment.

I would also like to express my gratitude to all of the CBDM lab members, past and present. You've provided a very friendly environment for me. I was very happy to share all the excitements of science with all of you.

Adriana Ortiz-Lopez, Kimie Hattori and Natasha Asinovski deserve a very special thank you. You have helped me with long gut preps, fed me on busy days and greeted me with a smiling face. Thank you for all your support and love.

I would like to also thank David Zemmour, Wilson Kuswanto, Tze Tan, Lindsay Kua, Marisella Panduro-Sicheva, Evgeny Kiner, Dmitriy Kolodin and Jon Sitrin for all the tiramisu, Saturday morning chats, gut preps, baked goodies and insightful discussions by the coffee machine. I would also like to acknowledge all of the close friendships that I've gained during graduate school.

Thank you Angela Morton, Ho-keun Kwon and Francesca Gazzaniga for many interesting and fun collaborations.

Thank you Catherine Laplace for all your help in figure preparation and more importantly for inspirational discussions.

Thank you Andrew Rhoads, Katie Rothamel, Henry Paik and Liang Yang for all outstanding technical help.

Thank you to the Joslin Diabetes Research Center Flow Cytometry Core and especially Joyce LaVecchio and Girijesh Buruzula. I have spent long days in challenging sorts, but your expertise and friendship made it all possible.

I would like to thank all of my collaborators, past and present across various labs. It was a pleasure to have worked with such wonderful people. We had a long-standing collaboration with Dennis Kasper's Lab and I would like to especially thank Naama Geva Zatorsky, Tsering Sherpa, Lesley Pasman and Neil Surana.

I would like to acknowledge and thank all the past and present members of my thesis committee, Dr. Mike Carroll, Dr. Scott Snapper, Dr. Dennis Kasper, Dr. Rodrigo Mora and Dr. Shannon Turley for all of your helpful guidance with this project. Also, I would like to thank Boehringer Ingelheim Fonds for funding and many educational opportunities.

On a personal note, I would like to especially thank my parents, Nurcihan and Bedri Sefik and my little sister Esra for being there every step of the way and for making me who I am. I would also like to thank my husbands' parents, Aysegul and Kudret Karatepe for all their guidance. Finally, I would like to thank my beloved husband, Kutay Karatepe. You have provided me with an unbelievable amount of love and support in the tough times and shared my happiness in my successes.

TABLE OF CONTENTS

Chapter 1: Introduction

1.1: Gut homeostasis and the microbiota

The gastrointestinal (GI) tract, in close proximity to a vast number of luminal microbes requires finely-tuned regulatory mechanisms to prevent excessive immune responses against commensals and allow healthy colonization by commensals, but not pathogens. In this section, we will introduce the basic components of the mammalian (more specifically human) microbiota and highlight the reciprocal relationship between the microbiota and the host immune system. In the next section (Chapter 1.2), we will detail how regulatory T cells mediate tolerance to various components of the microbiota and ensure healthy colonization by these microbes.

1.1.1: Characterization of the human gut microbiota

The human gastrointestinal tract (GI) is estimated to harbor approximately 100 trillion microorganisms, most of which are bacteria. Approaches based on 16SrRNA sequencing of bacteria allowed characterization of more than 1000 bacterial species, which account for 100 times more bacterial genes vs. human genes in the mammalian host (Qin et al., 2010; Backhed et al., 2005). More recently, Human Microbiome Project (HMP) Consortium set out to characterize microbial species that colonize various sites on human body and develop a repository that would serve as a reference tool. HMP has improved our understanding of the impacts of microbiome on human health and may have implications in inflammatory or autoimmune diseases. Various body parts were screened from healthy individual. Microbial communities were characterized by 16S rRNA and shotgun metagenomics sequencing. Major body parts included the skin, oral cavity, vagina, nasal cavity and lower GI tract represented by stool. Since the HMP has been the most comprehensive study with defined standards in terms of sample collection, data generation and analysis, I will summarize general patterns observed in these cohorts, but also acknowledge earlier work. The HMP identified *Bacteroidetes* and *Firmicutes* as the dominant phyla, but also reported contribution from *Verrucomicrobia*, *Tenericutes, Proteobacteria, Fusobacteria, Actinobacteria, Spirochaetes, Cyanobacteria* (Consortium, T.H.M.P., 2012; Costello et al., 2009; Turnbaugh et al., 2008; Qin et al., 2010; Backhed et al., 2005; Ley et al., 2006). The diversity observed in many taxonomical orders but also in phylum level in HMP studies was defined at two levels.

1. Alpha diversity considers the abundance of different types of organism within a sample (e.g how many different operational taxonomic units (OTUs) or roughly species are there in a sample?).
2. Beta diversity looks into community differences at a given body site among members of a population and compares differences and determines whether there are shared genera, OTUs across individuals (e.g how do OTUs from vagina relate to other vaginal samples from different subjects? Do members of a population share similar organisms at certain body sites?).

The human microbiota has different degrees of alpha and beta diversity in a site-specific manner (Consortium, T.H.M.P., 2012; Costello et al., 2009; Grice et al., 2009). For instance, the vagina has a low beta diversity meaning organisms are shared among individuals. The vagina also has a low alpha diversity compared to stool with one of the highest alpha diversities, meaning vagina has fewer OTUs compared to the stool (Consortium, T.H.M.P., 2012).

Interestingly, microorganisms identified from different body parts cluster in a site-specific manner. *Staphyloccocus aureus* is abundant in nostrils but absent in the vagina and the opposite is true for *Bifidobacterium dentium*. *Bacteroides* are more abundant in the stool compared to skin, nostrils, vagina and mouth (Consortium, T.H.M.P., 2012). *Bacteroides thetaiotaomicron,* with 46% prevalence in stool samples, is a common bacterium found across many individuals in the lower GI tract (Consortium, T.H.M.P., 2012). These observations also agree with niche-specific colonization described earlier in lower scale studies of humans, mice and other mammals (Costello et al., 2009; Grice et al., 2009; Backhed et al., 2005). Although not distinguished in the HMP studies, the small intestinal lumen, cecum and colon also differ significantly in their alpha and beta diversity and bacterial load (Eckburg et al., 2005). Small intestine not only has fewer OTUs described, but is also colonized with at least 1000 (10^5 counts/ml in the small intestine vs. 10^9 counts/ml in the colon) times fewer bacteria than the colon (reviewed in (Mowat et al., 2014)).

This niche-specific effect can also be extended to the host level (man vs mouse or male vs female) where genes, diet, prior infections, habitat, body temperature etc. define host-specific properties and the composition of the microbiota (Ley et al., 2008; Turnbaugh et al., 2008; HMCNtaure2012, Grice et al., 2009). The host-specific colonization was highlighted in a set of studies where the zebrafish, mouse or human microbiota were transplanted into non-native hosts and the microbial composition when stabilized did not resemble the donor host (Chung et al., 2012; Rawls et al., 2006).

Although much higher diversity is observed among individuals and different host species, diversity over time within the same individual is also present (Consortium, T.H.M.P., 2012; Turnbaugh et al., 2008; Faith et al., 2013; Grice et al., 2009). However, beyond transient fluctuations many bacterial strains have been shown to be stable for decades (Faith et al., 2013; Costello et al., 2009; Grice et al., 2009; Ley et al., 2008). The GI tract is colonized during early stages of development, with first bacterial exposure during birth (Koenig et al., 2011). This reaches the highest rate of phylogenetic and functional diversification with transition to solid food or table food and stabilizes around weaning (2.5 years of age in humans) (Koenig et al., 2011).

The mouse microbiome shares most of the key concepts described for the human microbiome -distribution of main phyla, niche-specific colonization, alpha and beta level diversity which varies across the body (Ley et al., 2008; Backhed et al., 2005). Genetically and environmentally more controlled host, easy manipulation of microbial communities, availability of germ free models (germ free mice do not have any bacteria in their GI tract) and the studies in gnotobiotic mice (germ free mice colonized with various bacteria in combination or alone) have advanced our understanding of how bacteria benefit the host or vice versa.

What constitutes a symbiont vs. pathogen has been discussed in many studies. A pathogen in very broad terms is described as a microorganism that can trigger disease. However, the pathogenicity of a microbe involves many factors highly dependent on the host's genetic makeup, immune state, as well localization of the microbe. Although *E. faecalis* colonizes healthy neonates, some *E. faecalis* strains, which display different capacity for producing reactive oxygen species, can be classified as pathogens (Nicholson et al., 2012). When these *E. faecalis* strains colonize IL10-/- germ free mice, they induce DNA damage on epithelial cells and promote tumorigenesis and inflammation (reviewed in (Sears et al., 2014)). Symbionts are described as microorganisms that live together with host. Mutualistic symbionts benefit the host, but also benefit from the host. Commensalistic symbionts benefit from the host, but the host is not harmed or helped in any significant way. Many aspects of the host environment such as competition with other microbes, availability of nutrients or immune state of the host influence symbiosis. A study on *B. thetaiotaomicron* mutants in gnotobiotic mice colonizing in presence or absence of competition highlighted the importance of community composition (presence of bacteria with ability to make Vitamin B12) and competition for nutrients (availability of Vitamin B12; Goodman et al., 2009). *B. thetaiotaomicron mutants* which poorly competed for Vitamin B12 had a fitness disadvantage only when other members of the community could not produce Vitamin B12 (Goodman et al., 2009).

It was known that the bacteria benefit from a nutrient-rich, protected environment while the host benefits from bacterial metabolites such as vitamins and more efficiently digested food. In the last decade, this nutrient-based simplistic view of symbiosis has been elevated by studies focusing on how bacteria facilitate development of the host immune system, control metabolism, protect against infections and influence the brain function (reviewed in (Belkaid et al., 2014; Nicholson et al., 2012; Cryan et al., 2012)). In the intestinal lumen, bacteria contribute to direct or indirect synthesis of various metabolites (Nicholson et al., 2012). These include short-chain fatty acids, bile acids, choline products, vitamins, lipids and phenols (Nicholson et al., 2012). Perturbations of the host-microbiota interaction may have pathogenic outcomes and disrupt biological fitness of the host. In the next sub-sections, we will focus on immune pathways, summarize some of these studies and report novel findings from our studies to provide a more comprehensive, but immunology-centered, view of how microbiota benefit the mammalian host.

1.1.2: An overview of the immune and non-immune cells in the intestinal mucosa

The intestines, primarily important for digestion and absorption of nutrients, are an active site of immunological responses. Diverse sets of immune and non-immune cells populate the intestines. The distribution and function of these cells vary significantly along the intestines. Here, we will mostly focus on how microbial burden impacts the distribution and function of these cells, but also mention the influence of intestinal physiology when necessary.

Briefly, the small intestine, colon and cecum have remarkable differences in length and thickness. The longest part, the small intestine has small, specialized protrusions called villi, essential for absorption of nutrients. The size of these structures varies along the length of the intestine and correlates with absorption of nutrients. Villi are shorter in the ileum (most distal part of the small intestine) and flatten in the cecum and colon where there is very little nutrient absorption but mostly reabsorption of water. The intestines could be divided into multiple parts: the lumen where bacteria live, the mucosa where the majority of immune responses take place, the submucosa, a dense layer of connective tissue, the muscle layer which separates the intestines from peritoneal cavity and finally the gut associated lymphoid tissue (GALT) responsible for induction of immune responses (reviewed in (Mowat et al., 2014)). GALTs are organized structures, which include Peyer's Patches of the small intestine, colonic patches and mesenteric lymph nodes (reviewed in (Mowat et al., 2014)). Columnar epithelial cells separate the lymphoid areas of the Peyer's patches from the intestinal lumen. Peyer's Patches like lymph nodes have B cell follicles and T cell areas (reviewed in (Mowat et al., 2014)). The mucosa has three immunologically and anatomically distinct parts: the epithelial layer, the lamina propria and a thin layer of muscle.

Epithelial cells along the intestines are mostly absorptive and are adapted for metabolic and digestive functions (Peterson et al., 2014). On the other hand, secretory epithelial cells, which include enteroendocrine cells, paneth cells and goblet cells, are specialized for digestive and barrier functions (Peterson et al., 2014). Paneth cells and goblet cells sustain a chemical and physical antimicrobial barrier that sequesters bacteria in the lumen (Salzman et al., 2013; Vaishnava et al., 2011; Jakobsson et al., 2014). Their distribution and significance vary along different parts of the intestines. Although immune functions of epithelial cells have been studied extensively, the immunological contribution of other stromal cells is still largely unknown. There are several types of epithelial cells but we will focus on paneth cells and goblet cells.

Paneth cells, epithelial cells found in the crypts, secrete antimicrobial peptides such as RegIIIγ (Vaishnava et al., 2011) and defensins (Salzman et al., 2013), which help establish a chemical barrier against microbes. Secretion of these antimicrobial molecules in part is regulated by the microbiota as antibiotic-treated mice have lower levels of RegIIIγ (Brandl et al., 2008). Paneth cells can increase production of RegIIIγ in response to IL22 (Vaishnava et al., 2011), a cytokine that will be detailed in discussion of innate lymphocytes and T cells. IL22 could be induced both by microbial colonization and tissue injury (Wolk et al., 2004).

Goblet cells secrete mucins -glycoproteins that form a gel-like barrier (mucus)- and concentrate in the distal colon. Abundance of bacteria correlates with the structural differences in the mucus layers of the small intestine and colon. Bacterial concentration in the colon requires a thicker, stratified mucus layer while a less dense mucus layer supported by antimicrobial peptides suffices in the small intestine (Jakobsson et al., 2014). Cytokines such as IL9, IFNγ and IL13 promote mucus production (Peterson et al., 2014). More recently, tolerogenic effects of mucins have been highlighted in a study where *Muc2* induces β-catenin (a transcription factor induced in tolerogenic DCs) in DCs and mediates IL10 production by DCs (Shan et al., 2013).

Epithelial cells also express pattern recognition receptors (PRR), which are required to initiate immune responses and recruit cells. TLR2, TLR3, TLR4, TLR5, TLR9, NOD1, NOD2, NLRP3, NLRP6 and NLRC4 are expressed by the intestinal epithelial cells (reviewed in (Peterson et al., 2014)). PRR signaling is hyporesponsive in the epithelial cells, but anatomic segregation of these PRRs allows distinction of pathogen vs commensal-derived signals. For instance, stimulation of epithelial cells by TLR9 ligands from basolateral but not the apical side induces NFκB activation (Lee et al., 2006).

The immune cells of the intestines can be grouped into two categories: **innate cells**, which comprise mononuclear phagocytes, innate lymphocytes, neutrophils, eosinophils and mast cells, and **adaptive cells**, which include T and B lymphocytes. Immune cells are mostly found in the lamina propria, but also in the epithelial layer. The immune cell composition markedly differs between the epithelial layer and the lamina propria.

Both the lamina propria and the epithelial layer are enriched for a variety of innate **mononuclear phagocytes**, characterized based on expression of CD11b, CD11c, CX3CR1, F4/80, CD103 and PDCA. These include major tissue-resident macrophages, dentritic cells (conventional dendritic cell (DCs) and plasmacytoid dendritic cells pDCs) and monocyte populations. Classification of these cells as dendritic cells or macrophages has been confusing as macrophages in the intestines also express high levels of CD11c (ImmunologicalGenomeProject (ImmGen); Miller et al., 2012), a molecule that has been long considered DC specific. More recently, the following nomenclature was suggested: (1) CD11c+CD11b-CD103+ cells as cDCs that show similarities to the ones in lymph nodes, (2) CD11c-CD11b+F4/80+ cells as tissue-resident macrophages, (3) CD11b+CD11c+CD103+CX3CR1- cells as CD103+CD11b+DCs and (4) CD11b+CD11c+F4/80+CX3CR1+ cells as CX3CR1+ mononuclear phagocytes (MP) with DC and macrophage properties (Varol et al., 2010). These mononuclear phagocytes, predominantly involved in immune surveillance, uptake and transport of antigen (Farache et al., 2013; Varol et al., 2009; Bogunovic et al., 2009), prime T cells (Sun et al., 2007; Jaensson et al., 2008; Johansson et al., 2005; Denning et al., 2007; Uematsu et al., 2008), facilitate tissue repair (Pull et al., 2005; Seno et al., 2009) and promote IgA class switch (Tezuka et al., 2007; Macpherson et al., 2000).

The two cells types that express CD11b are primarily involved in antigen sampling in the small intestine (and possibly in the colon): **CD103+CD11b+ DCs** and **CX3CR1+ MPs** (Farache et al., 2013; Varol et al., 2009; Bogunovic et al., 2009). CD103+CD11b+ DCs, mainly residing in the lamina propria, may capture soluble antigen or crawl into the epithelial layer to uptake antigen. The actual mechanisms of antigen capture are not very clear in CD103+CD11b+ DCs (Farache et al., 2013). On the other hand, CX3CR1+ MPs, located in both the epithelial layer and the lamina propria, use transepithelial dendrites to sample luminal bacterial antigens (Niess et al., 2005; Farache et al., 2013). Formation of these dendrites depends on CX3CR1 (Niess et al., 2005).

Which cells carry antigens to the draining lymph nodes has been a subject of controversy. Initially, CD103+ DCs cells were proposed to carry commensal derived antigens to mesenteric lymph nodes (Farache et al., 2013) and promote gut homing molecules such as CCR9 on the surface of T cells (Sun et al., 2007; Jaensson et al., 2008; Johansson et al., 2005). However, more recent findings suggest that CX3CR1+ MPs are not stationary and carry the commensal derived antigens to MLNs via CCR7-mediated trafficking (Diehl et al., 2013). Conversely, in this study, CD103+ DCs were only mobile after TLR stimulation by pathogens (Diehl et al., 2013). CD103+ DCs have been proposed to induce regulatory T cells by production of TGF-β and retinoic acid (Sun et al., 2007), but more recently this role has been attributed to macrophages (Denning et al., 2007). CD103+ DCs can contribute to differentiation of IL17 producing T cells in a TLR5 dependent manner (Denning et al., 2007; Uematsu et al., 2008). Intestinal macrophages also facilitate regulatory T cell differentiation by producing both TGF-β and IL10, acting in synergy (Denning et al., 2007; Murai et al., 2009). Macrophages are also important for tissue repair and replenishment of epithelial cells by inhibition of TNF (Pull et al., 2005; Seno et al., 2009).

Although less abundant Ly6Chi **monocytes** and Ly6C+PDCA+CD11b- **pDCs** are also found in the intestines. pDCs can contribute to oral tolerance by cross presentation of antigens to T cells (Goubier et al., 2008; Mouries et al., 2008) and may regulate T cell-mediated protection from colitis (Dasgupta et al., 2014).

Innate lymphocytes are a heterogeneous group of cells implicated in tissue repair and immunity against bacteria and helminths. Innate lymphoid cells (ILCs) mimic many features of T cells (detailed later) and are characterized in a similar manner. T-bet expressing ILCs which rely on IL15 and secrete IFNγ are known as **ILC1s** and include NK cells (Hesslein et al., 2011; Klose et al., 2013). These cells have important cytotoxic roles in cancer and viral infections.

ILC2s express GATA3 and secrete cytokines IL5 and IL13, associated with TH2 immunity. These cells are particularly important in immunity against helminth infections, promote IgE and IgA production and regulating eosinophils (Saenz et al., 2010; Monticelli et al., 2011). Recently, ILC2s have been shown to control lipid metabolism (Nussbaum et al., 2015; Lee et al., 2015). ILC2 can expand in response to alarmin IL33, IL25 and thymic stromal lymphopoietin (TSLP) secreted by epithelial cells (Saenz et al., 2010; Kim et al., 2013a; Brestoff et al., 2014). ILC2s can limit adiposity in white adipose tissue, and control beige fat biogenesis in response to IL33 by eosinophil and IL4 dependent and independent mechanisms (Lee et al., 2015; Brestoff et al., 2014).

Finally, **ILC3s** express RORγ and like their T cell counterparts secrete IL17 along with high levels of IL22 (Sanos et al., 2008; SawaNatImm2008). Lymphoid tissue inducer cells (LTi) found in fetus and neonatal mice and humans (and in lower levels in adults) are a type of IL17+IL22+ ILC3s. LTis, the first ILC3s described (Mebius et al., 1997; Eberl et al., 2004; Cupedo et al., 2008), are necessary for formation of secondary lymphoid tissues (Cupedo et al., 2008). Although ILC2s and ILC1s have been described in the intestinal mucosa, ILC3s are found at higher proportions. Single or double producers of IL17 and IL22 populate the intestines. Some IL22-producing ILC3s express NKp46 (Cella et al., 2009; Sanos et al., 2008 and Satoh-Takayama et al., 2008) and regulate tissue repair and intestinal epithelial cell function (Cella et al., 2009; Sanos et al., 2008; Goto et al., 2014). IL22 is important for intestinal barrier function at steady state and during enteric intestinal infections (Cella et al., 2009; Sanos et al., 2008). In response to IL22, epithelial cells proliferate and secrete defensins and antimicrobial peptides such as RegIIIγ. During infections, IL23 secreted by DCs promotes IL22 production by ILC3s (Cella et al., 2009; Sanos et al., 2008). ILC3s also regulate macrophage function by secreting GM-CSF (Mortha et al., 2014). Finally, major histocompatibility class II (MHCII)-dependent antigen presentation by ILC3s has been shown to regulate CD4 T cell responses against commensals (Hepworth et al., 2013).

Intestines contain a large number of **plasma cells** that predominantly secrete IgA in the lamina propria. For easier transport to lumen, a dimeric form of IgA is secreted in the intestines. The dimeric IgA secretion follows a similar gradient to the mucus production and correlates with bacterial burden. There are two types of IgA, adapted for the demands of the small intestine and colon (Kett et al., 1986; Lin et al., 2013; Kett et al., 1995). IgA1 is concentrated in the small intestine while IgA2, a more protease-resistant form, is localized to the colon (Kett et al., 1986; Lin et al., 2013). Interestingly, increasing bacterial burden of the small intestine can switch IgA1 dominance to IgA2, which is favored by bacteria-rich colon (He et al., 2007; Kett et al., 1995). Bacteria in the lumen can promote IgA class-switch by epithelial-cell or DC-mediated cytokine APRIL or iNOS (He et al., 2007; Tezuka et al., 2007). IgA class switch can also occur with the help of T cells in the germinal centers of lymphoid associated tissues such as Peyer's Patches of the small intestine (Shikina et al., 2004; Kawamato et al., 2014; Tsuji et al., 2009; Linterman et al., 2011; Victora et al., 2000). Bcl6+ T follicular helper cells and Foxp3+ regulatory T cells have been shown to mediate IgA class switch in the Peyer's

Patches (Kawamato et al., 2014; Tsuji et al., 2009). Plasma cells secreting IgM and IgG, although not as abundant as IgA, are also detected along the intestines.

T cells that bear αβ T cell receptors (Tαβ) or γδ T cell receptors (Tγδ) have been found in both the small intestine and colon. Tγδ cells located primarily in the intraepithelial layer regulate the replenishment of epithelial cells (Komano et al., 1995) and tight junction formations (Dalton et al., 2000). Tαβ cells on the other hand are involved in antigen specific immune responses, which include effector and regulatory T cell responses. All major types of Tαβ cells -MHC Class I (CD8+), Class II (CD4+) restricted or Natural Killer T cells (NKT)- have been described. These cells produce a variety of cytokines including IFNγ, IL17, IL22, IL4, IL5, IL13 and IL10.

NKT cells recognize self and foreign lipids presented on MHC-like CD1d molecule (reviewed in (Cohen et al., 2009)). Based on their TCR rearrangement, NKT cells are grouped as variant or invariant. Invariant NKT (iNKT) cell have a restricted TCR repertoire and use a limited set of TCRα (Vα24 in human, Vα14 in mouse rearranged to Jα18) and TCRβ chains (Vβ11 in human, Vβ8.2, Vβ7, and Vβ2 in mice) (reviewed in (Cohen et al., 2009)). The best marker for distinguishing these cells is their specificity to CD1d-bound α-galactosylceramide, a potent lipid antigen from marine sponges. iNKT cells can be activated by microbial lipids in antigen-specific manner or by cytokines produced by other innate cells (Brigl et al., 2011). iNKT cells are more concentrated in the small intestinal lamina propria and are almost absent in the epithelial layer or in the colon (Olszak et al., 2012; An et al., 2014).

The intestinal mucosa contains **CD8+ T cells and CD4+ T cells**, but the distribution of these cells vary in the epithelial layer and the lamina propria. Epithelial layer is enriched with CD8 T cells, but most of these cells express unconventional CD8aa co-receptor and display an oligoclonal TCR repertoire. These cells colonize the gut early in development (reviewed in (Mowat et al., 2014))

Conventional T cells that express conventional co-receptors **CD4αβ or CD8αβ** are more abundant in the lamina propria. The CD4:CD8 ratio in the small intestinal lamina propria (2:1) is higher than the colonic lamina propria (almost 1:1). CD8ab cytotoxic T cells display an effector-memory phenotype in the lamina propria (few naïve CD8 T cells have also been reported) (reviewed in (Mowat et al., 2014)). These cells express high levels integrin α4β7, necessary for homing to the intestinal mucosa, and rely on IL15 and IL7 for survival (reviewed in (Mowat et al., 2014)). Their main role is to provide antigen-specific protection by killing pathogen-infected cells. CD4+ T cell subsets will be described later.

Regulatory T cells are essential in restraining inflammatory responses mediated by effector T cells. In very broad terms, regulatory T cells can be divided into 2 classes based on their Foxp3 expression. The distribution, differentiation and dependence of Foxp3-TR1cells and Foxp3+Tregs show marked differences, but importance of IL10 produced by both cells has been highlighted in colitis-prone IL10-deficient mice (Asseman et al., 1999). GI Tregs will be described in detail later.

1.1.3: The effects of microbial colonization on some immune populations

The relationship between the components of the microbiota and the immune system has been the focus of numerous studies. Here, I will attempt to summarize some of these exciting observations. Some of these responses involve direct interaction with bacteria or bacterial products, while some rely on cues delivered from intestinal epithelial cells or other immune cells.

- **ILCs**: Although germ free mice or mice treated with antibiotics have normal ontogeny of ILC1s, ILC2s and ILC3s (Ganal et al., 2012; Monticelli et al., 2011, Sawa et al., 2011), the function of ILC1 and ILC3s is compromised (Ganal et al., 2012; Sawa et al., 2011). ILC1s, and in particular NK cells, isolated form germ free mice have defects in priming when stimulated with various adjuvants and display decreased cytotoxicity (Ganal et al., 2012).

The effect of commensals on IL22 production by ILC3 is controversial (Sawa et al., 2011; Takayama et al., 2008). IL22 production in some IL22+Nkp46+ ILC3s is reduced in germ free mice and requires colonization with commensals (Takayama et al., 2008). Conversely, in another study, IL22 production by Nkp46+ILC3s is higher in germ free mice and repressed in specific pathogen free mice (Sawa et al., 2011). This repression upon bacterial colonization is regulated by IL25 secreted by epithelial cells. Which bacterial species mediate these microbiota-dependent ILC functions is not known.

- **Conventional DCs and pDCs**: The microbiota also regulates the function of various mononuclear phagocytes. Polysaccharide-A (PSA) produced by B. fragilis is important for DC (Shen et al., 2012) and pDC (Dasgupta et al., 2014) response in stimulation of IL10-producing T cells.

- **Macrophages:** The crosstalk between the microbiota, ILC3s and macrophages is important for tolerogenic effects mediated by macrophages (Mortha et al., 2014). Tissue-resident macrophages respond to microbial stimuli by production of IL1β, which promotes CSF2 production by ILC3s, which then regulates retinoic acid and IL10 production by CD103+ DCs and macrophages (Mortha et al., 2014).

- **IgA+ B cells**: IgA production by plasma cells relies on microbial colonization (Macpherson et al., 2000). Germ free mice have few IgA-secreting plasma cells, but colonization with mouse fecal flora restores the IgA phenotype (Macpherson et al., 2000). Interestingly, this responsiveness is mutual as lack of IgA results in overgrowth of specific anaerobes such as the Segmented Filamentous Bacteria in the lower small intestine (Suzuki et al., 2004). Mice with a specific mutation, which does not affect IgA numbers but alters affinity to antigens, also had a similar dysbiosis, highlighting IgA's importance in calibrating the microbial composition (Wei et al., 2011). IgA-mediated responses against commensals are required for compartmentalizing bacteria mainly to the lumen and controlling the composition of the bacteria. Although this response may be considered an immune response, commensal-specific IgA does not show classic features of systemic immune memory. Sequential oral immunization leads to additive but not synergistic effects (Hapfelmeier et al., 2010). IgA repertoire adapts to the most recent dominant changes in the microbiota with no specificity retained against former colonizers (Hapfelmeier et al., 2010).

- **Invariant NKT cells:** iNKT cells, which contribute to the intestinal pool of cytokines by producing IL4 and IFNγ, are more numerous in germ free mice (Olszak et al., 2012). The scarcity of iNKT cells in colons of specific pathogen free mice was attributed to the inhibitory effects of sphingolipids produced by bacteria (such as *B. fragilis*) (An et al., 2014).

Although not a complete list, interactions highlighted here provide a view of how microbiota educates the immune system. These interactions have consequences in protecting against colitis or healthy colonization by the microbiota. IgA production mediated by the microbiota controls the composition of the microbiota (Rogier et al., 2014). Mice that lack ILC3s succumb to DSS mediated-colitis, but the role of microbiota in inducing or repressing IL22 may be irrelevant in this model of colitis as IL22 can be induced in response to injury (Sawa et al., 2011). The inhibition by sphingolipids produced by bacteria provides protection against NKT cell-mediated, oxazalone-induced colitis (An et al., 2014).

1.1.4: The microbiota-dependent induction of CD4+ T cells

CD4+ T cells differentiate into different types of effector T cells to facilitate protection against different classes of pathogens. Naive CD4+ T cells leave the thymus with a diverse set of T cell receptors (TCR) that can be activated in an antigen specific manner. Costimulation by CD28 in presence of the correct cocktail of cytokines (IL-12, IL4, IL-6, TGF-B, IL21) commits an activated T cell to a particular effector lineage. T helper (TH) subsets acquire transcription factors and produce characteristic cytokines controlled by different STAT signaling. TH cells facilitate a wide range of pathogen-specific immune responses by distinct, but overlapping functions. These subsets include:

- **T helper type 1 (TH1)** cells express transcription factor T-bet and produce cytokines IFNγ, lymphotoxin. T-bet expression in these cells is induced by cytokine IL12. IFNγ production mediated by STAT4 can activate macrophages and other innate cells to promote their ability to eliminate intracellular pathogens (Mosmann et al., 1986). TH1 cells also enhance function of cytotoxic CD8 T cells. Many autoimmune diseases (Type 1 diabetes, Multiple sclerosis (MS)) manifest TH1 driven pathologies.

- **T Helper type 2 (TH2)** cells express transcription factor GATA3 and produce cytokines IL4, IL13, IL5 to expel extracellular parasites (Mosmann et al., 1986). TH2 cells require IL4 presence during differentiation and upregulate STAT6. They also promote Ig class switch, particularly IgE. On the flip side, pathologies associated with TH2 cells drive allergy and asthma.
- **IL-17 producing T helper cells (TH17),** which express RORγ secrete IL17A, IL17F and IL22 (Ivanov et al., 2006; Xang et al., 2008). Th17 cells -mostly found in the skin, lung, and intestines- protect against fungi and extracellular bacteria (Denning et al., 2007; Uematsu et al., 2008; Happel et al., 2005 and Robinson et al., 2009). They are also necessary for maintaining epithelial barrier and are involved in tissue repair via IL22 (Pickert et al. 2009). TH17-driven pathologies (mainly by IL17) are described in rheumatoid arthritis and MS (EAE in mice) (Wu et al., 2009; Jager et al., 2009 and Peters et al., 2011). TH17 differentiation with respect to peripheral Treg differentiation will be detailed later.
- **T follicular helper cells (TFH)** that facilitate T cell-dependent Ig class switch in the germinal centers express BCL6 and BLIMP1. BCL6, required for programming of TFH cells, is induced by IL6 (and IL21) and utilizes STAT3 in downstream effector functions (Yu et al., 2009; Johnston et al., 2009; Nurieva et al., 2009).

- **IL22-producing T helper cells (TH22)** express both AHR and RORγ and produce IL22 during tissue repair. In humans IL22 is produced by a T cell subset distinct from TH17 cells. In mice TH17 cells can produce both IL17 and IL22 (Trifari et al., 2009; Zenewicz et al., 2008 and Wolk et al., 2004).
- **IL-9 producing T helper cells (TH9)** differentiate in response to TGFβ and IL4. Th9 cell express a variety of overlapping transcription factors described in the context of other TH cells (GATA3, STAT6, STAT3) (reviewed in (Kaplan et al., 2015)). TH9 cells, involved in immunity against helminths and tumors, is a non-classical TH subset that has also been described in atopic disease, inflammatory bowel disease and EAE (reviewed in (Kaplan et al., 2015)).

Although these lineages have been considered by some as fixed or terminal, recent work has highlighted the plasticity and the functional overlap (mostly by cytokine production and transcription factor utilization) of these cells under various inflammatory, infectious and autoimmune settings (Bending et al. 2009; Antebi et al., 2013; Wang et al., 2014, Duhen et al., 2013; Yang JEM2009).

All classic CD4+ helper subsets (TH1, TH2, TH17, TFH) have been described in the intestines, particularly in the lamina propria, where they provide immunity against various pathogens. On the flip side, accumulation, proliferation or effector functions of these cells have been linked to inflammatory bowel disease (reviewed in (Mathis and Benoist, 2011)). On many accounts microbiota has been portrayed as drivers or inducers of these T cells. Mice raised with conventional microbiota have higher numbers of cytokine producing effector T cells at steady state compared with germ free mice (Niess et al., 2008). The evidence from transfer experiments also argues that the microbiota induce generation of memory T cells that are better at inducing colitis when transferred into lymphopenic recipients (Asseman et al., 2003).

Although many of these early studies failed to identify direct, microbe-specific evidence, more recently a well-characterized, microbe-driven effect was presented for generation of TH17 cells. These cells are abundant in the intestinal lamina propria (LP), particularly in the small intestine (Ivanov et al., 2006). TH17 cells have been implicated in many autoimmune and inflammatory diseases either associated with protection or pathogenesis depending on the model or the disease (reviewed in (Mathis and Benoist, 2011)). The transcription factor RORγ, is the key regulator of TH17 cells and controls differentiation and functions of these cells (Ivanov et al., 2006). FOXP3, a key regulator of Tregs, is thought to antagonize RORγ (Zhou et al., 2008; Bettelli et al., 2006; Yang et al., 2008; Mucida et al., 2007) during TH differentiation. TGFβ induces FOXP3 in-vitro and skews T cell-differentiation into the Treg lineage (Chen et al., 2003). However, supplementation of these cultures with the inflammatory cytokine IL6 inhibits FOXP3I and induces TH17 cells by upregulation of RORγ (Mangan et al., 2006; Bettelli et al., 2006). Similarly, abrogation of the FOXP3II-RORγ molecular interaction (Zhang et al., 2008) or inhibition of the retionic acid pathway (Mucida et al., 2007) (another molecule important for peripheral Treg generation, especially in the intestines) leads to an increase in RORγ expression and IL17. Many cytokines regulate TH17 cell differentiation at various stages. In addition to TGFβ and IL6 (Zhou et al., 007), IL21 also (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007) acts on early stages of TH17 cell differentiation. IL23 plays a role in stabilization and expansion of TH17 cells (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007; Mangan et al., 2006), which then produce cytokines IL17A, IL17F, IL22.

Germ free mice or mice treated with antibiotics have very few TH17 cells in the small intestinal lamina propria. Notably, mice housed in different animal facilities had varying levels of TH17 cells (Taconic Farms had increased TH17 numbers compared with Jackson Laboratory) (Ivanov et al., 2008). These observations led to the discovery of Segmented Filamentous Bacteria (SFB) as the major driver of TH17 cells at steady state (Ivanov et al., 2008; Ivanov et al., 2009). Colonization of mice with SFB was sufficient to induce generation and accumulation of TH17 cells (Ivanov et al., 2008; Ivanov et al., 2009). This finding has been the most compelling evidence linking a very specific component of the microbiota to a specific immune cell type.

1.1.5: Dysbiosis of the microbiota and implications in autoimmune and inflammatory diseases

Dysbiosis, an imbalance in the composition of the healthy microbiota, has been associated with many gut proximal and distal autoimmune and inflammatory diseases. Correlative studies in humans or experimental work in mice have identified bacterial alterations that affect various immune cell types.

In a spontaneous model of rheumatoid arthritis (RA), colonization with SFB triggers disease via induction of TH17 cells in the small intestine (Wu et al., 2010). Similarly, in a mouse model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), disease severity was reduced in germ free mice, and SFB-mediated TH17 induction exacerbated the disease (Lee et al., 2010). Treatment with antibiotics also affected the severity of EAE and conferred protection in mice (Ochoa-Reparaz et al., 2009). On the other hand, colonization of NOD mice with SFB conferred protection against type 1 diabetes (T1D) in females (Kriegel et al., 2011).

Although the clear link between the microbiota and mouse autoimmune diseases has not been fully translated to human diseases, microbial dysbiosis have been observed in individuals with T1D, MS and RA. Autoantibody positive individuals display decreased diversity of the microbiota (reviewed in (Vaarala et al., 2013)). Children with

T1D have lower abundance of butyrate-producing bacteria and increased representation of *Bacteroidetes* in feces (particularly *B. ovatus*) (reviewed in (Vaarala et al., 2013)). Variety of factors, which adversely affect the healthy establishment of the microbiota such as use of antibiotics early in life (Ochoa-Reparaz et al., 2009), formula feeding instead of breast feeding (Norgaard et al., 2011), cesarean-section birth (Conradi et al., 2013), have been linked to increased risk of MS in humans. These studies do not directly analyze the effect of microbial communities on MS, but rather focus on factors that are known to shape the microbiota. The microbial composition of the RA patients compared to healthy individuals differs in the gut, mouth and saliva (Zhang et al., 2015). A variety of microbial species, such as gram-positive *Lactobaccilus salivarius* or acetate producer *Clostridium* spp, are enriched in RA patients, while others such as gram-negative *Haemophilus*, *Aggregatibacter* and *Cardiobacterium* are depleted in RA patients (Zhang et al., 2015). The oral or gut dysbiosis observed in RA patients correlate with disease indices such as RA serum markers.

Crohn's disease (CD) and ulcerative colitis (UC) are two complex diseases. Discordance of incidence of these diseases in twin studies and rise in incidence over the past few decades indicate environmental factors. Recently, the microbiota has been implicated to have a role in the onset or prognosis of UC and CD (reviewed in (Huttenhower et al., 2014)). Early cohorts comparing the microbiota of diseased and healthy individuals identified multiple but inconsistent changes in the microbiota during the disease onset or progression. Some of these studies suggested an increase in microbial diversity (Seksik et al., 2003) while others suggested a decreased diversity (Ott et al., 2004; Bibiloni et al., 2006) in diseased individuals. CD patients compared to healthy individuals showed decreased community diversity and enrichment of *Enterobacteriaceae* and depletion of *Clostridiales* (Baumgart et al., 2007; Frank et al., 2011). In a more recent study of new onset CD, dysbiosis of microbiota involved more bacterial families than outlined earlier (Gevers et al., 2014). The enrichment of *Enterobacteriaceae* was accompanied by an increase in some other taxanomic orders (Gevers et al., 2014). *Bacteroidales and Clostridiales were both* depleted (Gevers et al., 2014).

Clearly, more work is needed to have a comprehensive view of the role of the microbiota in the onset and prognosis of inflammatory bowel disease and autoimmune disease in humans, but the discoveries so far are very promising.

1.2: Foxp3+ regulatory T cell (Treg) biology

Foxp3+ regulatory T cells (Tregs) are critical for the maintenance of immunological tolerance and immune homoeostasis (Brunkow et al., 2001; Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001; Hori et al., 2003; Williams and Rudensky, 2007). The absence or loss of function of Foxp3 results in multi-organ autoimmunity as demonstrated in clinical findings (lymphoproliferation and multi-organ autoimmunity) from patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) and from *scurfy* and Foxp3-deficient mice (Brunkow et al., 2001; Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001; Hori et al., 2003; Williams and Rudensky, 2007). Several lines of evidence including adoptive transfer of Tregs to Foxp3-deficient mice, germ line, T cell-specific or other immune cell specific deficiencies in Foxp3 (Gavin et al., 2007; Fontenot et al., 2003; Liston et al., 2007; Hsieh et al., 2006) supported that Foxp3 in T cells have a non-redundant, critical role in mediating suppression and preventing autoimmunity. Here, we will highlight the heterogeneity of Tregs and discuss their differentiation, activities and function in various tissues, and more specifically in the intestines.

1.1.1: Origin of Tregs

Tregs can differentiate in thymus (tTregs) from CD4+ single positive T cells based on their expression of TCR that recognize self-peptide MHC complexes. The affinity for antigen and efficiency of MHC-peptide-TCR interaction are higher in Foxp3+ Tregs than those that mediate positive selection of conventional CD4+ T cells (Tconv) (Hsieh et al., 2004). On the other hand, Tregs can be generated in periphery (pTregs) as a result of conversion from Tconv cells under various inflammatory or non-inflammatory conditions (reviewed in (Bilate and Lafaille, 2012)). These conditions include: adoptive transfer to lymphopenic hosts (Curotto de Lafaille et al., 2004), foreign antigen presentation by immature APCs (Kretschmer et al., 2005), oral tolerance in OVA-specific TCR transgenic mice (Mucida et al., 2005) and tolerogenic microenvironment of the intestinal mucosa (Sun et al., 2007). The intestinal mucosa favors generation of pTregs. Foxp3+ T cells can also be generated in vitro by TGFβ supplementation and are referred to as iTregs (Chen et al., 2003).

Both tTregs and pTregs have similar amounts of FoxP3 protein and transcript, which is critical for differentiation and function of both. Although FoxP3 regulates an important part of the Treg transcriptome, some features of Tregs are FoxP3-independent (Hill et al., 2007; Fu et al., 2012). Consistent with FoxP3-independent features of Tregs, transcriptomes of tTregs and pTregs markedly differ in lymphopenia-induced model of colitis (Haribhai et al., 2009). Profiles of Tregs from spleen or lymph nodes are enriched for tTregs. When splenic tTregs are compared to Tregs isolated from the small intestinal lamina propria, there are marked differences (Feuerer et al., 2010). This striking difference is attributed to enrichment of pTregs within the small intestinal lamina propria, and the phenotypic differences between tTregs and pTregs (Feuerer et al., 2010). Surprisingly, small intestinal Tregs are also strikingly different from in-vitro differentiated iTregs (Feuerer et al., 2010), but similar to Tconv cells isolated from the same mucosal microenviroment (induction of *Ccr9* and *Gzmb*, repression of *Eomes* in both Tconvs and Tregs in the small intestine). These differences include activation-induced genes, chemokine receptors, transcription factors and effector molecules. Overall, these findings suggest that although absolutely necessary, FoxP3 alone is not sufficient for all Treg features and function.

The specific contribution of pTregs is still under debate. Evidence suggests that pTregs are especially involved in oral tolerance, response to the microbiota, fetal maternal tolerance and protection against colitis (reviewed in ((Josefowicz et al., 2012a)).

In various transfer models of colitis a non-redundant role of pTregs has been demonstrated (Haribhai et al., 2009). In rescue experiments of Foxp3-deficient mice, pTregs are necessary for restraining inflammation in the lungs (Haribhai et al., 2011). Specific depletion of pTregs, when tTreg numbers remain similar, results in aggregation of inflammatory infiltrates in the lung, liver and colon (Haribhai et al., 2011). Direct evidence of the functional importance and specification of pTregs is provided in mice with the conserved non-coding DNA sequence 1 (CNS1) of *Foxp3* deleted. CNS1 is one of the three intronic enhancer regions of *Foxp3* gene. CNS3 is involved in *Foxp3* induction in thymocytes while specifically unmethylated CNS2 is involved in the maintenance of the mature Treg phenotype (Zheng et al., 2010). Finally, CNS1, which has no impact on tTregs, is required for generation of pTregs, especially at mucosal sites (Zheng et al., 2010; Josefowicz et al., 2012b). CNS1-deficient mice display dysbiosis of the microbiota and increased allergic-type inflammation in the intestines and the lung mucosa (Josefowicz et al., 2012b).

There have been attempts to provide a set of markers that differentiates tTregs from pTregs. Expression of the transcription factor Helios and surface marker Nrp1 has been associated with thymic Tregs while the lack of expression marks pTregs (Thornton et al., 2010; Yadav et al., 2012; Weiss et al., 2012). However, the significance of these markers is still under debate as Helios expression has been detected in both tTreg and pTreg populations in mice and humans under various settings of in-vitro differentiation, lymphopenic proliferation and activation (Himmel et al., 2013; Gottschalk et al., 2012; Akimova et al., 2011).

The discussion of antigen specificity of tTregs and pTregs has led to complicated interpretations. Non-overlapping TCR repertoires of pTregs and tTregs have been suggested to confer non-redundant functional roles to these cells (Haribhai et al., 2011). Although not universally accepted, tTregs, educated in the thymus, are thought to have specificity against self-antigens (Hsieh et al., 2004), while pTregs more likely to be generated in response to environmental or peripheral stimuli can recognize non-self (Sun et al., 2007; Haribhai et al., 2011). However, this notion has been challenged in a study, which described similar frequency of non-self specific tTreg TCR clones and naïve non-Treg clones in TCRmini mice, which have fixed TCRβ but diverse TCRα (Pacholczyk et al., 2007). These Tregs only had a small proportion of autoreactive TCRs, but were specific to alloantigens (Pacholczyk et al., 2007). Multiple lines of somewhat contradictory work on TCR repertoires and function of Tregs especially in the intestinal mucosa suggest that both tTregs and pTregs can recognize non-self antigens and mediate tolerance to commensal bacteria (and/or food antigens) (Cebula et al., 2013; Lathrop et al., 2011).

Multiple effector mechanisms of suppression are used by both tTregs and pTregs in different inflammatory contexts. Tregs can secrete anti-inflammatory cytokines, mediate suppression via expression of cell surface effectors, which inhibit the activation or proliferation of effector T cells or induce apoptosis of target cells. Tregs have been shown to produce high levels of suppressive cytokines such as interleukin 10 (Rubtsov et al., 2008), TGF-β (Li et al., 2007) and interleukin 35 (Morse, 1978) to maintain homeostasis especially in the lung and intestines. CTLA-4, expressed on the cell surface of Tregs, inhibits dentritic cells and effector T cells and is critical for Treg function (Wing et al., 2008; Friedline et al., 2009). CTLA-4 mediated downregulation of CD80 and CD86 on DCs interfere with DC's ability to activate T cells (Wing et al., 2008; Friedline et al., 2009). Similarly, ectoenzymes CD39 and CD73 start adenosine signaling which inhibits antigen presenting cells and effector T cells (Deaglio et al., 2007; Borsellino et al., 2007; Kobie et al., 2006). CD25, a molecule important for Treg survival, inhibits T or NK cell activation by depleting IL2 (Sitrin et al., 2013; Thornton et al., 2004). The co-receptor LAG3 inhibits co-stimulatory capacity of DCs (Huang et al., 2004), while TIGIT inhibits

DC’s ability to produce to immunosuppressive cytokines such as IL10 (Yu et al., 2009). Finally, contact-mediated Granzyme-B secretion by Tregs facilitates apoptosis of target effector T cells (Gondek et al., 2005).

More recently, heterogeneity beyond pTregs and tTregs was highlighted in elegant sets of studies where different, but somewhat overlapping, subsets control resolution of various types of infections and non-sterile inflammation (Wohlfert et al., 2011; Duhen et al., 2012; Cretney et al., 2011; Koch et al., 2009; Chaudhry et al., 2009). These subsets draw similarities to T helper effector lineages (TH1, TH2, TH17 and TFH). The acquisition of various transcription factors and effector molecules specialize Tregs to better handle different sources of immunopathology. Expression of GATA3 and IRF4 endows Tregs with the ability to suppress TH2 and TH17 responses (Wohlfert et al., 2011; Duhen et al., 2012; Cretney et al., 2011). T-bet+ Tregs regulate the migration, proliferation and survival of Treg cells during TH1-mediated immune responses (Koch et al., 2009), while mice with Stat3-deficient Tregs develop spontaneous colitis and cannot restrain TH17-mediated inflammation (Chaudhry et al., 2009). Finally, Tregs that acquire BCL6 can suppress germinal center reactions, including differentiation of plasma cells and antibody affinity maturation (Chung et al., 2011).

Overall, tTregs and pTregs originate from different precursors with different stimulatory requirements but share a variety of mechanisms, which they use to mediate immune suppression. In the next section, we will detail tissue-specific effects that provide additional heterogeneity within Tregs.

1.2.1: Tregs in non-lymphoid tissues

Transcriptional profiles of Tregs from various sources have highlighted the heterogeneity within Tregs and encouraged distinction of Treg sub-phenotypes based on the tissue of residency. Distinct populations of Tregs have been described in many nonlymphoid tissues including skin, lung, liver, placenta, intestines, adipose tissue and injured muscle (unpublished work from our lab, (Feuerer et al., 2009; Cipolletta et al., 2012; Burzyn et al., 2013b; Samstein et al., 2012; Sather et al., 2007; Aluvihare et al., 2007). These "tissue" Tregs respond to chemokine-mediated signals and express a variety of chemokine receptors such as CCR4, CXCR5, CXCR3 and CCR2 (unpublished work from our lab). Loss of CCR4 impairs Treg homing to the skin and lung and manifests as severe inflammation in these specific tissues (Sather et al., 2007). The requirement of specific chemokine receptors for other tissue Tregs is not well-defined, but an overabundance of chemokine receptors in various tissue Tregs is striking (data from transcriptional profiles in our lab, but also reviewed in (Campbell and Koch, 2011)).

In these tissues, beyond their well-described role in regulating the activity of other immune cells, Tregs located can also control other non-immunological processes. The mechanisms of action, gene expression profiles and TCR repertoires of these tissue Tregs are distinct from their lymphoid counterparts. The best characterized of these Tregs include those that reside in chronically inflamed visceral adipose tissue (VAT) where they regulate metabolic parameters (Feuerer et al., 2009; Cipolletta et al., 2012) or those that accumulate in acutely injured muscle where they mediate muscle repair post-injury (Burzyn et al., 2013b). These "tissue" Tregs acquire tissue-specific adaptations or features that allow functional advantage, but also enhance their survival or accumulation in that tissue. VAT Tregs express the transcription factor PPARγ, which drives the phenotype, accumulation and function of these cells (Cipolletta et al., 2012). Similarly, muscle Tregs express high levels of the growth factor amphiregulin, which improves muscle repair by acting on muscle satellite cells (Burzyn et al., 2013b). In both VAT and injured muscle, specific Treg clones expand, and the population is enriched for certain TCR sequences which make up to 80% of total Tregs (Burzyn et al., 2013b;

Kolodin et al., 2015). TCR specificity of these tissue Tregs may facilitate not only their function but also tissue specific localization (reviewed in (Burzyn et al., 2013a)).

1.2.2: The intestinal Tregs

Microbially challenged gastrointestinal (GI) tract is populated with particular Tregs. These intestinal Tregs, more abundant in the colonic lamina propria, but also found in the small intestinal lamina propria, modulate responses to commensal microbes and dietary antigens (reviewed in (Ai et al., 2014)).

The intestinal mucosa is a favorable site for generation of pTregs. Several subsets of intestinal DCs (Coombes et al., 2007; Sun et al., 2007), macrophages and MPs (Diehl et al., 2013) with tolerogenic properties (mostly mediated by IL10 secretion) have been described. The intestinal mucosa has large reservoir of TGFβ (reviewed in (Podolsky et al., 1993)) and retinoic acid (Sun et al., 2007). All these factors contribute to the generation of pTregs in the intestines (Coombes et al., 2007).

Intestinal Tregs are an unusual a mix of tTregs and pTregs, which display heterogeneity based on expression of a variety of transcription factors and surface receptors. These Tregs acquire expression of transcription factors *Tbx21, Irf4, Gata3, Stat3* and various effector molecules described in TH1, TH2 and TH17-like Tregs and are equipped to restrain various types of inflammation (Koch et al., 2009; Chaudhry et al., 2009; Cretney et al., 2011; Duhen et al., 2012; Wohlfert et al., 2011).

Colonic Tregs are also unique in terms of their diverse TCRs (unpublished work from our lab) that show marked reactivity against microbial antigens, which seem important drivers of their differentiation and/or expansion (Lathrop et al., 2011; Cebula et al., 2013). Both tTregs (Cebula et al., 2013) and pTregs (Lathrop et al., 2011) from the colonic lamina propria were reported to have specificity against different luminal antigens. The TCRα repertoire of intestinal (small intestinal and colonic) Tregs showed marked resemblance to the repertoire of thymic Tregs in TCRmini mice. These colonic thymic TCRs when cloned to hybridomas showed reactivity against caecal lysates from conventionally raised mice but not to the lysates from antibiotic treated mice (Cebula et al., 2013). The bacterial strains tested in the caecal content included a number of *Bacteroidetes and Clostridia* species.

In a contrasting report, TCRs from colonic Tregs were unique and different from any other Tregs including thymic Tregs in their TCRα chain diversity. These colonic Treg TCRs when expressed in a hybridoma cell line also showed reactivity to a variety of bacterial antigens and again these bacteria were enriched for *Clostridia* and *Bacteroidetes* species (Lathrop et al., 2011). Studies focusing on the TCRα diversity did not provide a conclusive decision whether the antigen-specificity of colonic Tregs is governed by tTregs or pTregs.

1.2.3: Tregs as mediators of gut homeostasis

Regulation of immune responses to prevent excessive inflammation and to allow healthy colonization by the components of the microbiota is crucial for intestinal tissue integrity and effective host-bacteria coexistence. Evidence from multiple studies outlined here support that functionally specialized intestinal Tregs are crucial for this regulation. The GI tract harbors numerous diverse bacteria and requires Tregs that can recognize intestinal antigens and prevent pathogenic immune responses against commensal bacteria.

In T cell transfer models of colitis, transfer of naive CD4+ T cells to SCID mice results in microbiota-dependent intestinal inflammation (Powrie et al., 1993; Aranda et al., 1997). The importance of Tregs in intestinal homeostasis was demonstrated by prevention of colitis when Tregs were transferred along with Tconvs (Coombes et al., 2007; Read et al., 2000). The need for Tregs to have experienced bacteria is still debated as Tregs from germ free mice provided the same protection in some studies, but not in others (Singh et al., 2001; Strauch et al., 2005). As previously mentioned, the GI tract and particularly the colon are enriched with pTregs. Most early transfer studies did not distinguish contribution of tTreg and pTreg populations in mediating protection against colitis. Since Tconvs when transferred to Rag-deficient recipients gave rise to very few colonic pTregs, the contribution of pTregs could be demonstrated only in mice deficient in various cytokine-signaling pathways such as the cytokine IL23. IL23 deficient mice developed higher levels of pTregs following transfer of T convs (Izcue et al., 2008). In these mice, colitis developed when Foxp3-deficient mice were used as donor cells so that transferred Tconv cells could not differentiate into pTregs (Izcue et al., 2008).

A different study highlighted the synergy between in-situ generated pTregs and tTregs in mediating protection against lymphopenia-induced model of colitis (Haribhai et al., 2009). In this study, the division of labor between pTregs and tTregs was driven by differences in their transcriptome.

The severe lymphoproliferative disease seen in *Foxp3*-deficient, Balb/c mice could be rescued by adoptive transfer of tTregs and pTregs. Acute depletion of pTregs (when tTreg numbers remained similar) from these rescued mice led to the accumulation of infiltrates and loss of tolerance in particular tissues such as the colon (Haribhai et al., 2011). In these studies the non-redundant function of pTregs was mediated by diversification of the Treg TCR repertoire (Haribhai et al., 2011).

The most definitive evidence proving the role of pTregs in controlling intestinal inflammation came from studies in CNS1-deficient mice (described earlier), where loss of pTregs manifested as alterations in the composition of the microbiota as well as TH2 type pathology in the small intestine (Josefowicz et al., 2012b).

1.2.4: Microbiota-dependent induction of colonic Tregs

The previous section, on VAT, muscle and particularly intestinal Tregs have highlighted the importance of specialized Tregs in maintaining homeostasis at various tissues that have different types and degrees of inflammation. The importance of Tregs in mediating tolerance against commensal bacteria to ensure gut homeostasis was outlined earlier, but the nature of this relationship is reciprocal where the microbiota also mediate Treg phenotype in the intestines (with some systemic presentation). Specific components of the microbiota have been reported to induce Tregs with specific phenotypes. Colonization with *Bacteroides fragilis* modified intestinal Treg phenotype by microbial PSA and induced IL10 production (Mazmanian et al., 2008; Round and Mazmanian, 2010). Some other microbes, mostly probiotics (a mix *of Bifidobacteria, Lactobacilli and Streptococci*), have also been suggested to modify the intestinal Tregs and provide protection against airway inflammation or TNBS-induced colitis (Di Giacinto et al., 2005; Karimi et al., 2009; Kwon et al., 2010). More recently, colonic Tregs were reported to be significantly underrepresented in germ free mice or in mice treated with antibiotics (Atarashi et al., 2011; Geuking et al., 2011), while small intestinal or lymphoid tissue Tregs remained unaffected. Colonization of germ free mice with different combinations of bacteria (Schadler's flora (Geuking et al., 2011) or Clostridia combinations (Atarashi et al., 2011; Atarashi et al., 2013)) elicited the differentiation or expansion of colonic Tregs and more specifically colonic pTregs that lack expression of Helios and NRP1. In these studies, the induction of colonic Tregs conferred protection in various models of colitis (Atarashi et al., 2011; Atarashi et al., 2013) but also had systemic manifestations as measured by decreased systemic IgE (Atarashi et al., 2011). Another study examined the ability of single microbes to induce colonic Tregs (Faith et al., 2014) and challenged the need for complex combinations.

Microbe-derived short chain fatty acids (SCFA), more specifically acetate, butyrate and propionate, were shown to mediate colonic Treg cell phenotype and accumulation (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013; Faith et al., 2014), but there were noteworthy contradictions among these studies in the impact, route or concentration of administration. In some studies butyrate was effective when added to the drinking water or mixed in the food while others claimed the need for intrarectal administration. In addition there were some discrepancies in the ability of these SCFA, more specifically butyrate and propionate, to induce tTregs or pTregs. Thymic stromal lymphopoietin (TSLP), produced by epithelial cells in response to the elements of the microbiota, was also shown to induce expansion of colonic pTregs and inhibit TH17 cells in mice colonized with Shaedler's flora (Mosconi et al., 2013), although effects of single microbes in mediating TSLP is still unclear.

Although the function, heterogeneity and specificity of colonic Tregs have been vastly studied in many inflammatory and steady state settings, little is known about their transcriptional profile and how this profile capacitates Treg function.

So far, the reciprocal and dynamic interactions between the microbiota and the host immune system and the need for fine-tuning of immune responses in the intestines have been established in numerous studies. These studies focused on limited immune populations or a few bacterial species. Here, we set out to have a comprehensive unbiased study asking how a diverse set of single bacterial species shapes all major immune populations, affects whole tissue homeostasis and influences metabolism. Finally, we took our findings one step further by focusing on how single microbes potentiate specific colonic Treg function and phenotype.

Chapter 2: Individual microbes influence various immune and non-immune cell populations

Microbial species, in combination or alone have been reported to influence lymphocyte and myeloid populations and cytokines (reviewed in chapter 1). Yet, little is known about the ability of single microbes to drive these phenotypes and the redundancy in this ability as well as the molecular controls of these phenotypes. To study the impacts of various microbial species alone on various components of the immune system, we generated a large-scale screen in which germ free mice were colonized with single species from a panel of 64 bacterial strains selected from the microbiota of the human gastrointestinal tract and obtained from various clinical human isolates. These bacteria have been selected on three criteria: (1) reflect the diversity seen in the human microbiota and maintain the prevalence of the dominant phyla; (2) have genome sequences available for phylogenetic comparisons (which may help identification of genes that are responsible for the outcomes we observe); (3) allow strain-specific effects or effects of closely related species to be explored. Major immune phenotypes, whole tissue intestinal transcriptomes and metabolite composition in the blood plasma were analyzed as read-outs of this screen. The data acquisition step of this large screen is completed, but the analysis presented here is still preliminary. We asked 5 main questions to better analyze and understand the large set of data generated:

1. Do all these 64 strains of microbes show symbiosis (mutualism or commensalism), especially in absence of community pressures or competition? Can all microbes colonize all parts of the GI tract or other tissues with similar efficiency?

2. Which single microbes induce gut proximal phenotypes? Is there a systemic manifestation of these phenotypes?

3. Is there any redundancy in microbes' ability to induce specific immunological or non-immunological phenotypes? Which phenotypes does one microbe uniquely induce? Are there any phenotypes that are induced by all microbes?

4. Is there any correlation between the phenotypes observed? Are there any phenotypes that show similar or opposite patterns upon colonization? Is this specific to intestinal phenotypes or could this be extended to systemic effects?

5. Is there a correlation between microbes that induce similar phenotypes? Can this be explained by phylogeny?

The work presented in this chapter was performed in distinct collaboration with Naama Geva-Zatorsky and Lesley Pasman from Dennis Kasper's Lab and Lindsay Kua, Tze Tan and Adriana-Ortiz Lopez on the complete screen of germ free mice colonized with single bacterial species. Henry Paik and Liang Yang helped with database management and whole tissue gene expression analysis. Naama Geva-Zatorsky and Lesley Pasman were responsible for microbiology and colonization aspects of the study and also involved in data analysis. Lindsay Kua, Tze Tan and Adriana-Ortiz Lopez partook in the tissue processing and cytometry analysis.

2.1: Profile of microbes studies in this screen

In our large screen, we wanted to maintain the bacterial diversity at the phylum level, but also parse out how species level diversity and the genetic distance affect immune phenotypes. The microbes we chose displayed this diversity, but also allowed focused assessment of strain-specific effects on a given phenotype.

Since early age marks the most dramatic changes in the microbiota and the corresponding immune phenotypes (Koenig et al., 2011), we chose to colonize 4-week old germ free mice with a total of 64 different strains, one microbe at a time (schematized in Fig1). Overall the large screen covered 5 phyla (*Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria*), 29 genera, 52 species (21 of which belonged to *Bacteroidetes),* 64 strains (6 identical species with at least 2 strains) (Fig. 2A, Table 1). This enabled comparison of taxonomic effects of bacteria on various immune or non-immune phenotypes and assessment of redundancy in induction capabilities of related or unrelated microbes.

Over 90 percent of bacteria successfully colonized the intestines of germ free mice and could be quantified in fecal material. Neither of these strains manifested gross inflammation assessed by histopathology in H&E sections or by whole tissue assessment of inflammatory genes (not shown), supporting the true commensal nature of the bacteria analyzed.

Niche-specific colonization has been the highlight of many studies where community pressure, competition and physiological features of a given location define an ecological niche. Here, even in absence of competition, some niche-specific colonization was observed, in line with these earlier studies (Fig.1B) (Consortium, T.H.M.P., 2012, Costello et al., 2009; Grice et al., 2009; Backhed et al., 2005). It was noteworthy that *Porphyromonas gingivalis*, *Prevotellae intermedia*, *Prevotellae melaninogenica,*

Table 1: Phylogeny and origin of microbes

	Microbe_Name	Key_Microbe_Name	Bacterial_Species	Origin	An/aerobic	Phyla	Family	Genus	Strain
1	Bifidobacterium adolescentis_L2-32	Badol.L2-32	Bifidobacterium adolescentis	BEI	Anaerobic	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	L2-32
2	Bifidobacterium breve_SK134	Bbrev.SK134	Bifidobacterium breve	BWH, Onderdonk lab	Anaerobic	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	SK134
3	Bifidobacterium longum_AO44	Blong.AO44	Bifidobacterium longum	Clinical isolate, BWH	Anaerobic	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	AO44
4	Collinsella aerofaciens_VPI1003	Caero.VPI1003	Collinsella aerofaciens	ATCC	Anaerobic	Actinobacteria	Coriobacteriaceae	Collinsella	VPI1003
5	Eubacterium lentum_AO28	Elent.AO28	Eggerthella lenta	Clinical isolate, BWH	Anaerobic	Actinobacteria	Coriobacteriaceae	Eggerthella	AO28
6	Propionibacterium granulosum_AO42	Pgran.AO42	Propionibacterium granulosum	BWH, Onderdonk	Anaerobic	Actinobacteria	Propionibacteriaceae	Propionibacterium	AO42
7	Bacteroides caccae_AO1	Bcacc.AO1	Bacteroides caccae	Clinical isolate, BWH	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	AO1
8	Bacteroides dorei_CL03T12C01	Bdore.CL03T12C01	Bacteroides dorei	BEI	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	CL03T12C01
9	Bacteroides dorei_DSM17855	Bdore.DSM17855	Bacteroides dorei	DSMZ (Germany)	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	DSM17855
10	Bacteroides eggerthii_DSM20697	Begge.DSM20697	Bacteroides eggerthii	DSMZ (Germany)	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	DSM20697
11	Bacteroides finegoldii_DSM17565	Bfine.DSM17565	Bacteroides finegoldii	DSMZ (Germany)	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	DSM17565
12	Bacteroides fragilis_3_1_12	Bfrag.3.1.12	Bacteroides fragilis	BEI	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	3_1_12
13	Bacteroides fragilis_CL03T00C08	Bfrag.CL03T00C08	Bacteroides fragilis	BEI	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	CL03T00C08
14	Bacteroides fragilis_NCTC9343	Bfrag.NCTC9343	Bacteroides fragilis	Dennis Kasper	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	NCTC9343
15	Bacteroides massiliensis_DSM17679	Bmass.DSM17679	Bacteroides massiliensis	DSMZ (Germany)	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	DSM17679
16	Bacteroides oleiciplenus_DSM22535	Bolei.DSM22535	Bacteroides oleiciplenus	DSMZ (Germany)	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	DSM22535
17	Bacteroides ovatus_ATCC8483	Bovat.ATCC8483	Bacteroides ovatus	BWH, Onderdonk	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC8483
18	Bacteroides ovatus_CL02T12C04	Bovat.CL02T12C04	Bacteroides ovatus	Comstock lab	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	CL02T12C04
19	Bacteroides salanitronis_DSM18170	Bsala.DSM18170	Bacteroides salanitronis	DSMZ (Germany)	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	DSM18170
20	Bacteroides thetaiotaomicron_ATCC29148	Bthet.ATCC29148	Bacteroides thetaiotaomicron	ATCC	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC29148
21	Bacteroides thetaiotaomicron_ATCC29741	Bthet.ATCC29741	Bacteroides thetaiotaomicron	BWH, Onderdonk	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC29741
22	Bacteroides uniformis_ATCC8492	Bunif.ATCC8492	Bacteroides uniformis	ATCC	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC8492
23	Bacteroides vulgatus_ATCC8482	Bvulg.ATCC8482	Bacteroides vulgatus	BWH, Onderdonk	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC8482
24	DeltaPSABacteroides fragilis_NCTC9343	Dfrag.NCTC9343.deltaPSA	Bacteroides fragilis	Kasper lab	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	NCTC9343_dPSA
25	DeltaSPTBacteroides fragilis_NCTC9343	Dfrag.NCTC9343	Bacteroides fragilis	Kasper lab	Anaerobic	Bacteroidetes	Bacteroidaceae	Bacteroides	NCTC9343
26	Parabacteroides distasonis_ATCC8503	Pdist.ATCC8503	Parabacteroides distasonis	BWH, Onderdonk	Anaerobic	Bacteroidetes	Porphyromonadaceae	Parabacteroides	ATCC8503
27	Parabacteroides johnsonii_CL02T12C29	Pjohn.CL02T12C29	Parabacteroides johnsonii	BEI	Anaerobic	Bacteroidetes	Porphyromonadaceae	Parabacteroides	CL02T12C29
28	Parabacteroides merdae_CL03T12C32	Pmerd.CL03T12C32	Parabacteroides merdae	Comstock lab	Anaerobic	Bacteroidetes	Porphyromonadaceae	Bacteroides	CL03T12C32
29	Parabacteroides merdae_CL09T00C40	Pmerd.CL09T00C40	Parabacteroides merdae	BEI	Anaerobic	Bacteroidetes	Porphyromonadaceae	Bacteroides	CL09T00C40
30	Porphyromonas gingivalis_W83	Pging.W83	Porphyromonas gingivalis	Forsyth	Anaerobic	Bacteroidetes	Porphyromonadaceae	Porphyromonas	W83
31	Porphyromonas uenonis_BAA906	Pueno.BAA906	Porphyromonas uenonis	ATCC	Anaerobic	Bacteroidetes	Porphyromonadaceae	Porphyromonas	BAA906
32	Porphyromonas uenonis_UPII60-3	Pueno.UPII60-3	Porphyromonas uenonis	BEI	Anaerobic	Bacteroidetes	Porphyromonadaceae	Porphyromonas	UPII60-3
33	Prevotellae intermedia_AO10	Pinte.AO10	Prevotellae intermedia	Clinical isolate, BWH	Anaerobic	Bacteroidetes	Prevotellaceae	Prevotella	AO10
34	Prevotellae melaninogenica_ATCC25845	Pmela.ATCC25845	Prevotellae melaninogenica	BWH, Onderdonk	Anaerobic	Bacteroidetes	Prevotellaceae	Prevotella	ATCC25845
35	Clostridium histolyticum_AO25	Chist.AO25	Clostridium histolyticum	BWH, Onderdonk	Anaerobic	Firmicutes	Clostridiaceae	Clostridium	AO25
36	Clostridium perfringens_ATCC13124	Cperf.ATCC13124	Clostridium perfringens	BWH, Onderdonk	Anaerobic	Firmicutes	Clostridiaceae	Clostridium	ATCC13124
37	Clostridium ramosum_AO31	Cramo.AO31	Clostridia ramosum	BWH, BWH, Onderdor	Anaerobic	Firmicutes	Clostridiaceae	Clostridium	AO31
38	Clostridium sordellii_AO32	Csord.AO32	Clostridium sordellii	Clinical isolate, BWH	Anaerobic	Firmicutes	Clostridiaceae	Clostridium	AO32
39	Coprobacillus_8_2_54BFAA	Copr.8.2.54BFAA	Coprobacillus	BEI	Anaerobic	Firmicutes	Erysipelotrichaceae	Coprobacillus	8_2_54BFAA
40	Enterococcus faecalis_HH22	Efaec.HH22	Enterococcus faecalis	BEI	Anaerobic	Firmicutes	Enterococcaceae	Enterococcus	HH22
41	Enterococcus faecalis_OG1RF	Efaec.OG1RF+GFP	Enterococcus faecalis	Gilmore Lab	Anaerobic	Firmicutes	Enterococcaceae	Enterococcus	OG1RF+GFP
42	Enterococcus faecalis_TX0104	Efaec.TX0104	Enterococcus faecalis	BEI	Anaerobic	Firmicutes	Enterococcaceae	Enterococcus	TX0104
43	Enterococcus faecium_TX1330	Efaec.TX1330	Enterococcus faecium	BEI	Anaerobic	Firmicutes	Enterococcaceae	Enterococcus	TX1330
44	Eubacterium rectale_ATCC33656	Erect.ATCC33656	Eubacterium rectale	ATCC	Anaerobic	Firmicutes	Eubacteriaceae	Eubacterium	ATCC33656
45	Lachnospiraceae_sp_2_1_58FAA	Lach.2.1.58FAA	Lachnospiraceae sp.	BEI	Anaerobic	Firmicutes	Lachnospiraceae	Lachnospira	2_1_58FAA

Table 1 continued…

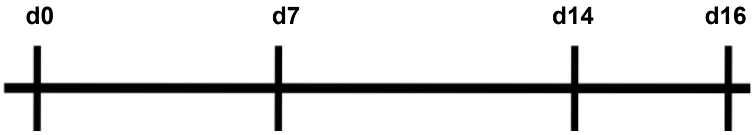
Table 1: Phylogeny and origin of microbes

	Microbe_Name	Key_Microbe_Name	Bacterial_Species	Origin	An/aerobic	Phyla	Family	Genus	Strain
46	Lactobacillus casei_AO47	Lcase.AO47	Lactobacillus casei	Clinical isolate, BWH	Anaerobic	Firmicutes	Lactobacillaceae	Lactobacillus	AO47
47	Lactobacillus johnsonii_AO12	Ljohn.AO12	Lactobacillus	Clinical isolate, BWH	Anaerobic	Firmicutes	Lactobacillaceae	Lactobacillus	AO12
48	Lactobacillus rhamnosus_LMS2-1	Lrham.LMS2-1	Lactobacillus rhamnosus	BEI	Anaerobic	Firmicutes	Lactobacillaceae	Lactobacillus	LMS2-1
49	Peptostreptococcus magnus_AO29	Pmagn.AO29	Peptostreptococcus magnus	Clinical labs BWH	Anaerobic	Firmicutes	Clostridiaceae	Peptostreptococcu	AO29
50	Peptostreptococus asaccharolyticus_AO33	Pasac.AO33	Peptostreptococus asaccharolyt	Clinical isolate, BWH	Anaerobic	Firmicutes	Peptoniphilaceae	Peptoniphilus	AO33
51	Ruminococcus gnavus_ATCC29149	Rgnav.ATCC29149	Ruminococcus gnavus	ATCC	Anaerobic	Firmicutes	Lachnospiraceae	Blautia	ATCC29149
52	Staphylococcus saprophyticus_ATCC15305	Ssapr.ATCC15305	Staphylococcus saprophyticus	ATCC	Aerobic	Firmicutes	Staphylococcaceae	Staphylococcus	ATCC 15305
53	Staphylococcus saprophyticus_DLK1	Ssapr.DLK1	Staphylococcus saprophyticus	Clinical isolate, HMS	Aerobic	Firmicutes	Staphylococcaceae	Staphylococcus	DLK1
54	Streptococcus mitis_F0392	Smiti.F0392	Streptococcus mitis	BEI	Anaerobic	Firmicutes	Streptococcaceae	Streptococcus	F0392
55	Veillonella_6_1_27	Veil.6.1.27	Veillonella	BEI	Anaerobic	Firmicutes	Veillonellaceae	Veillonella	6_1_27
56	Fusobacterium mortiferum_AO16	Fmort.AO16	Fusobacterium mortiferum	Clinical isolate, BWH	Anaerobic	Fusobacteria	Fusobacteriaceae	Fusobacterium	AO16
57	Fusobacterium nucleatum_F0419	Fnucl.F0419	Fusobacterium nucleatum	Bei	Anaerobic	Fusobacteria	Fusobacteriaceae	Fusobacterium	F0419
58	Acinetobacter baumannii_ATCC17978	Abaum.ATCC17978	Acinetobacter baumannii	Mekelanos lab	Aerobic	Proteobacteria	Moraxellaceae	Acinetobacter	ATCC17978
59	Acinetobacter lwoffii_F78	Alwof.F78	Acinetobacter lwoffii	Harald Renz	Aerobic	Proteobacteria	Moraxellaceae	Acinetobacter	F78
60	Campylobacter jejuni_AS-84-79	Cjeju.AS-84-79	Campylobacter jejuni	BEI	Anaerobic	Proteobacteria	Campylobacteraceae	Campylobacter	AS-84-79
61	Escherichia coli_Nissle1917	Ecoli.Nissle1917	Escherichia coli	Mekelanos lab	Aerobic	Proteobacteria	Enterobacteriaceae	Escherichia	Nissle1917
62	Helicobacter pylori_ATCC700392	Hpylo.ATCC700392	Helicobacter pylori	ATCC	Anaerobic	Proteobacteria	Helicobacteraceae	Helicobacter	ATCC700392
63	Klebsiella_sp_4_1_44FAA	Kleb.sp.4.1.44FAA	Klebsiella	BEI	Anaerobic	Proteobacteria	Enterobacteriaceae	Klebsiella	sp. 4_1_44FAA
64	Neisseria flavescens_SK114	Nflav.SK114	Neisseria flavescens	BEI	Aerobic	Proteobacteria	Neisseriaceae	Neisseria	SK114

Helicobacter pylori and *Eubacterium lentum* did not populate the intestinal lumen, but maintained niche-specificity. *P. gingivalis,* an anaerobe mostly described as part of the oral microbiome in healthy individuals*,* colonized only the oral cavity (Yilmaz et al., 2008). Similarly, colonization with *P*. *melaninogenica, a* clinical isolate from the sputum*,* was restricted to the oral cavity. As expected, *Helicobacter pylori* colonization was localized to the stomach. Colonization even in absence of competition may suggest that these bacterial niches are defined by unique physical features of the location such as the high acidity provided by the stomach, where *H. pylori* is better fitted. Alternatively, limited niche occupation may suggest that resources provided by other bacteria are necessary for colonization or diversification of niches. This was supported by detection of live *P. melaninogenica* (anaerobic) colonies in the fecal material when co-colonized with *Staphylococcus saprophyticus* (aerobic).

Interestingly, a significant proportion of the bacteria could be detected live in the caudal and mesenteric lymph nodes (MLNs) (Fig. 1B-second and third circles from inside), suggesting that these microbes may be transported live to the MLNs (Macpherson et al., 2004; Dielh et. al. 2013). Both CX3CR1+ MPs and CD103+ DCs have been described to carry live bacteria to the MLNs. While CX3CR1+ MP responses involve commensals, CD103+ DCs are mobilized as part of an immune response involving TLR activation by pathogens (Diehl et al., 2013). A total of 43 microbes were detected live in the MLNs and colonization with half of these bacteria induced higher levels of either CD103+ DCs or CX3CR1+ MPs (compared to germ free) in the small intestinal or colonic lamina propria. However, in the MLNs there was no clear correlation between the detection of live bacteria and proportions of CD103+ DCs or CX3CR1+ MPs (Pearson coefficient of correlation=-0.08 for CD103+ DCs; Pearson coefficient of correlation= -0.3 for CX3CR1+ MPs; p value>0.05).

Figure 1



Germ-free CB6 mice gavaged with bacteria in the isolator

Collect feces : CFU check

Sacrifice mice: Analysis of immune populations

Sacrifice mice: Analysis of colonic transcriptome CD4+ Tcells and CD45+ T cells sorted from small intestinal lamina propria (not discussed here)

Tissues of interest:
Spleen+ peripheral lymph nodes (SP)
Mesenteric lymph node (MLN)
Peyer's Patches (PP)
Colonic Lamina propria (Co-LP)
Small intestinal lamina propria (SI-LP)

Immune Populations recorded:

1. Myeloid populations:

CD45+CD19- cells
- CD11b+CD11c- cells
    - macrophages
- CD11b+CD11c+ cells
    - CD103+CD11b+ DCs
    - CX3CR1+ MPs
- CD11b-CD11c cells
    - CD103+DCs
- CD11b- cells
    - pDCs
- Monocytes

2. Lymphoid populations

CD45+ cells
- B cells
- Tαβ cells
    - CD4+ T cells
        - Treg
        - Helios- Tregs
        - Helios+ Tregs
        - Rorγ+Helios- Tregs
        - Rorγ-Helios- Tregs
        - Rorγ-Helios+ Tregs
    - CD8+ T cells
        - Helios+ CD8+ T cells
    - DN(CD8-CD4-TCR+)
- Tγδ cells
- ILC3

3. Cytokines

CD4+ T cells
- IL17+ CD4+ T cells
- IL10+ CD4+ T cells
- IL22+ CD4+ T cells
- IFNγ+CD4+ T cells

Figure 1. Experimental set up for each microbial colonization
Germ free mice were gavaged with single bacterial species at 4 weeks of age. The innocula was allowed to grow for 14 days. Mice were sacrificed for analysis at 6 weeks of age.

Figure 2

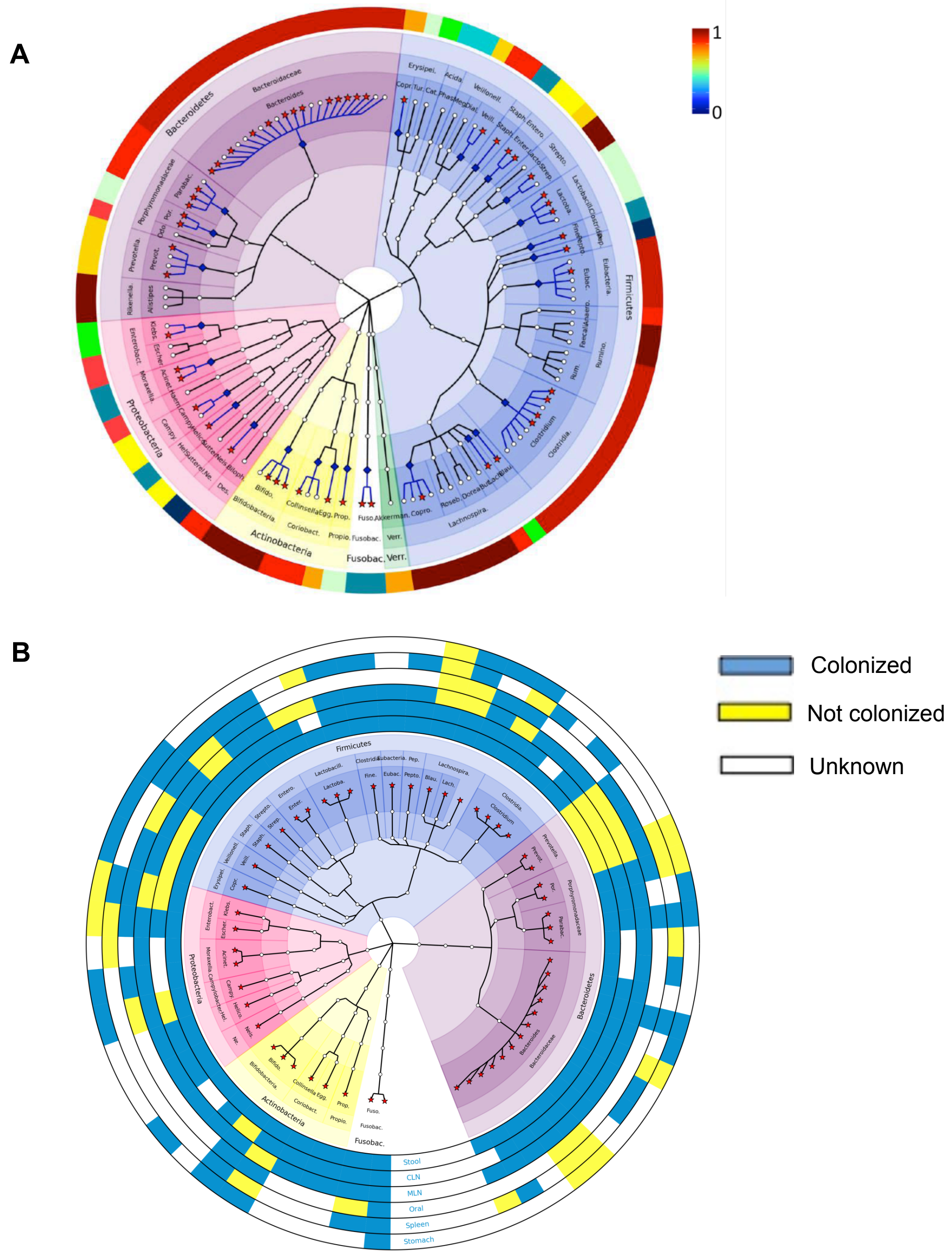


Figure 12. Phylogeny of bacterial species used in the large-scale screen

A. Phylogeny of bacterial species used. Phyla, family and genus names provided in outermost circles. Branching indicates phylogenetic distance. Mice were colonized with bacterial species marked with red stars. Prevalence of bacteria in the human microbiota are color-coded in the outermost circle where red marks highest abundance.

B. Different tissues where bacteria colonized. Circles on the outside mark different tissues which were plated to measure bacterial colonization (Inside out order: Stool, caudal lymph node(CLN), mesenteric lymph node (MLN), oral cavity, spleen pooled with inguinal lymph nodes and stomach) Stool represents the lower GI tract. Blue indicates that bacteria colonized; yellow means no live bacteria was detected; white is unknown.

2.2: Profiles of the immune populations that are altered by microbial colonization

In this screen, proportions of 140 different populations of lymphocytes (Fig. 3) or myeloid cells (Fig. 4) were analyzed from various intestinal and lymphoid tissues, covering either local or systemic responses. These cells covered B and T lymphocytes (Tαβ and Tγδ), T cell subsets (CD4+, CD8+, CD4-CD8-), Tregs and their corresponding pTregs and tTreg subsets, RORγ+ ILC3s, CD103+ DCs, CD103+CD11b+ DCs, macrophages, CX3CR1+ MPs, pDCs and monocytes (gating strategy outline in Fig. 3-4). These cells were reported from the colonic lamina propria, small intestinal lamina propria, caecal lamina propria (epithelial layer was stripped off prior to isolation of lamina propria cells), Peyer's patches, MLNs, spleen and inguinal lymph nodes. In addition, we measured cytokine production by CD4 T cells (IL17, IL10, IFNγ and IL22 - Fig. 5).

This comprehensive analysis revealed numerous interesting phenotypes that are shaped by colonization with single microbes. To better evaluate these, we asked specific questions:

I. Does colonization with single bacterial species elicit local responses? Are there any systemic extensions of these responses? What are the bacteria and the host immune populations they have an effect on?

II. Is there any redundancy in bacteria's ability to induce specific immune cells?

III. Is there a correlation between microbes that induce similar phenotypes? Can this be explained by phylogeny?

IV. Do the systemic or local immune populations presented here correlate with one another? Can we use this analysis to parse out microbe driven cellular interactions not described before?

Figure 3

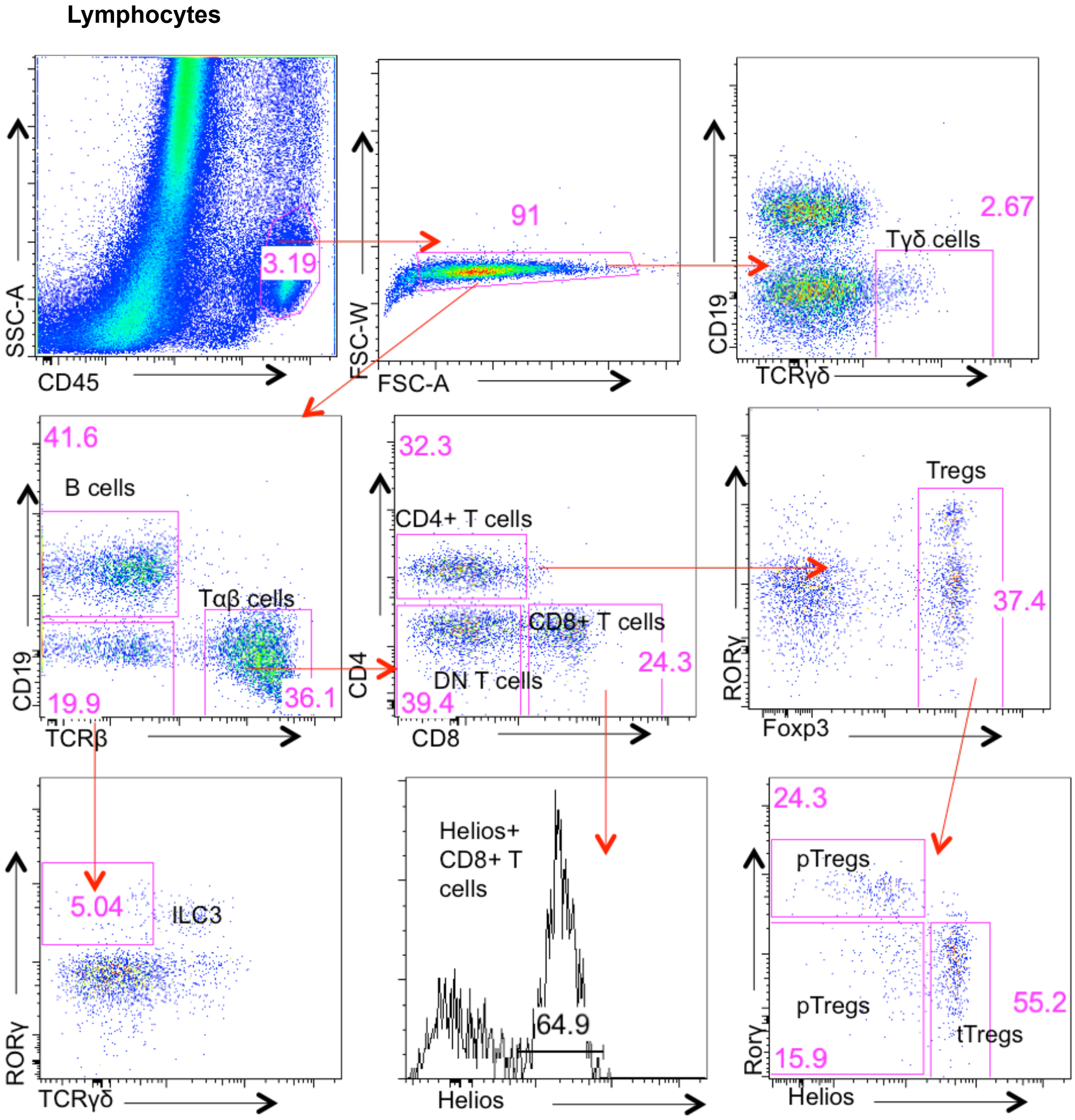


Figure 3: Representative gating scheme for lymphocytes
Cells were isolated from colonic lamina propria and stained with antibodies against CD45, CD19, TCRβ, TCRγδ, CD4, CD8, Foxp3, Rorγ, Helios

Figure 4

Mononuclear phagocytes

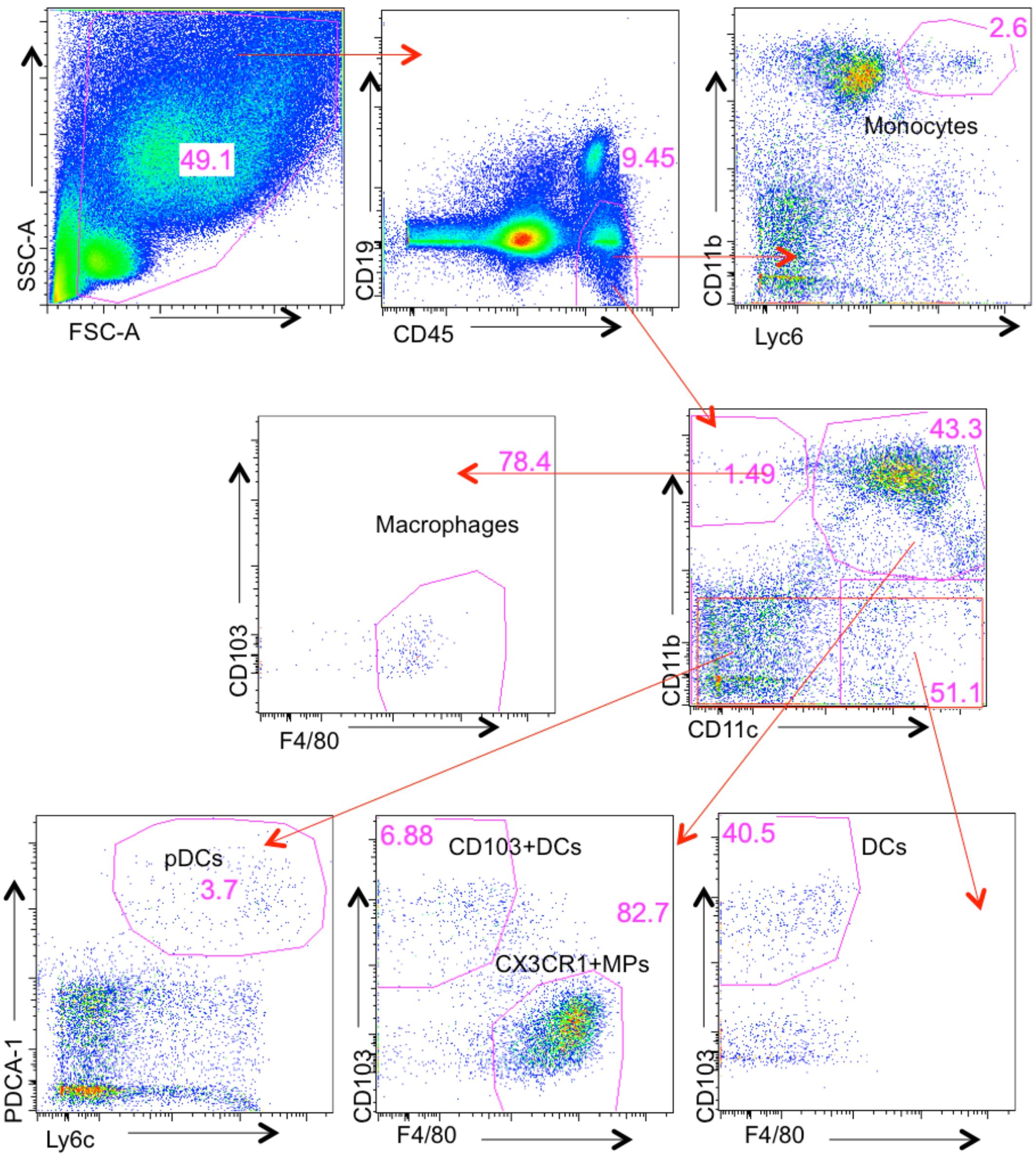


Figure 3: Representative gating scheme for mononuclear phagocytes
Cells were isolated from colonic lamina propria and stained with antibodies against CD45, CD19, CD11b,CD11c, Ly6c, F/480, CD103 and PDCA-1.

Figure 5

Cytokines

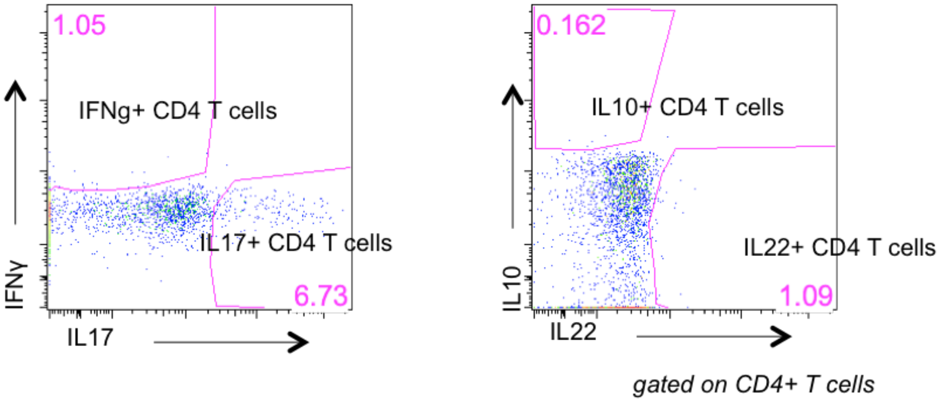


Figure 3: Representative gating scheme for cytokines
Cells were isolated from colonic lamina propria, treated with PMA and ionomycin for 3h and stained with antibodies against CD45, CD19, TCRβ, TCRγδ, CD4, CD8, IL17α, IFNγ, IL10 and IL22.

Although majority of the immunological phenotypes remained unaffected in the systemic lymphoid tissues represented by spleen and lymph nodes, many bacterial species elicited unique or shared effects on local cell populations such as effector T cells, regulatory T cells, CD4-CD8- T cells, TCRgd T cells and various myeloid populations including macrophages, CXC3R1+ MPs, CD103+DCs, pDCs and monocytes in the intestinal mucosa (colon, small intestine or cecum) and the Peyer's Patches. I will describe several of these populations (that have been confirmed in multiple repeats) and give examples of bacteria, which induce these populations upon colonization.

1. Different pools of microbes have been shown to induce generation or accumulation of **Tregs** in the colonic lamina propria (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013; Faith et al., 2014). To our surprise, 43% of microbes induced total regulatory T cells in the colonic lamina propria comparable to SPF levels. Upon colonization with these microbes, Treg proportions in the colons of colonized mice were higher than the median of Treg proportions in SPF mice (Fig.6A). This induction did not correlate with SCFA levels measured in caecal content or with bacterial phylogeny (discussed in detail in the next chapter).

2. An increase in **Tαβ cell that do not express co-receptor CD4 or CD8** (DN- double negative T cells) was observed upon colonization with many bacteria, but specifically with *Fusobacterium mortiferum* (Fig6B). The composition of this T cell population is not entirely characterized, but includes NKT

Figure 6

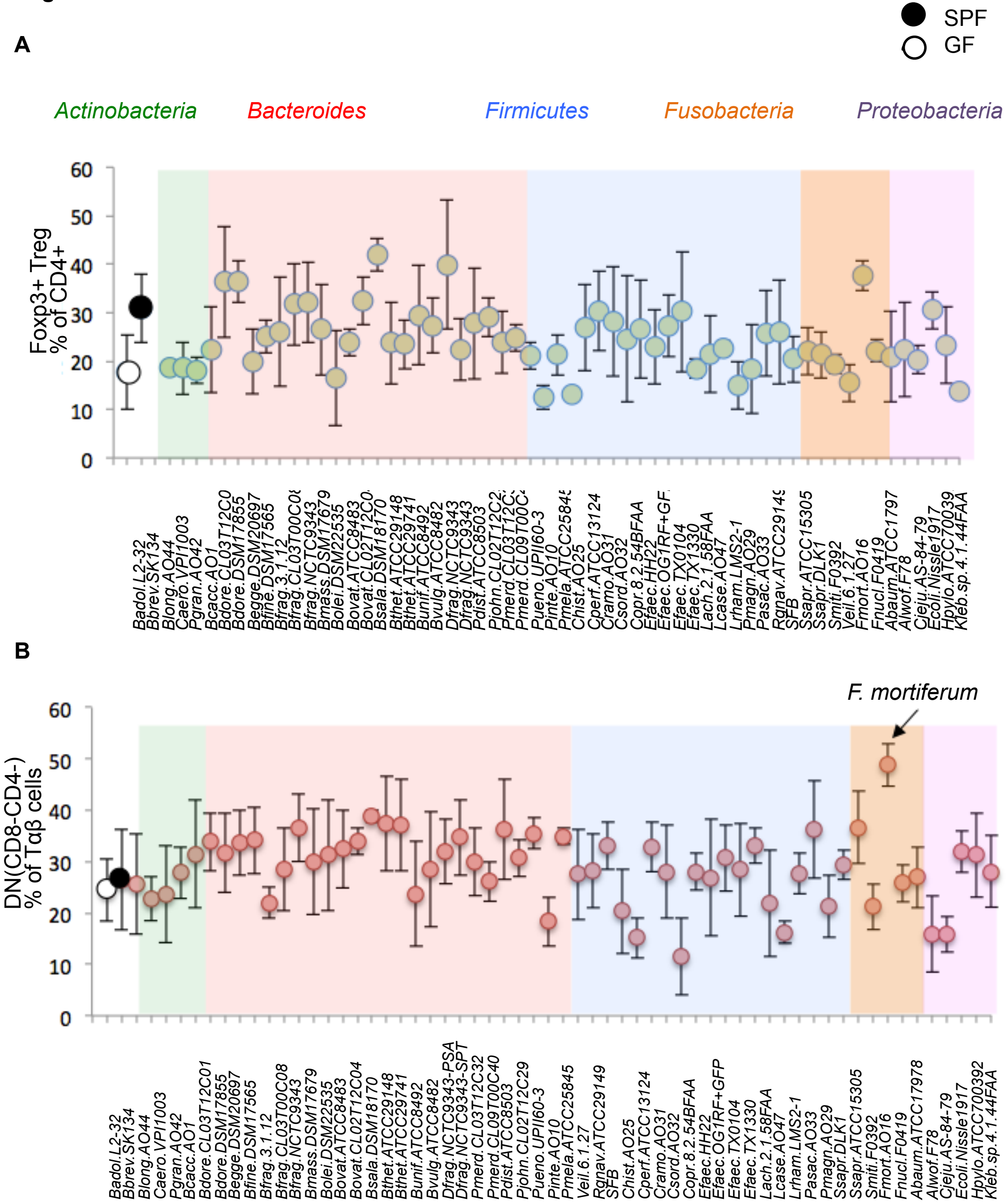


Figure 6. Microbial colonization induces a distribution of Tregs and CD4-CD8- T cells in the colonic lamina propria.

A. Frequencies of colonic Tregs within the CD4+TCR+ population. Each dot is the mean of at least 3 mice with SD. Color coded per phyla.

B. Frequencies of CD4-CD8- T cells within the TCR+CD45+ population. Each dot is the mean of at least 3 mice with SD. Color coded per phyla.

cells. The increase in DN T cells did not correlate with an increase in iNKT assessed by iNKT-specific α-galactosylceramice tetramer staining (not shown).

3. Many bacteria alone influenced a variety of mononuclear phagocytes. Colonization with *Bacteroides vulgatus* increased proportions of **pDCs** specifically in the small intestinal lamina propria (Fig 7A), while colonization with *Neisseria flavescens* induced pDCs in the colonic lamina propria (Fig7B). Earlier work has described that *B. fragilis* mediates its immunomodulatory effects via pDCs, but this effect was localized to the MLNs (Dasgupta et al., 2014). pDCs treated with PSA (isolated from *B. fragilis*) mediated protection against colitis in an IL10-dependent manner (Dasgupta et al., 2014). Here, multiple bacterial species induced pDCs in the small intestinal and the colonic lamina propria. Phenotypic and functional differences mediated by bacteria on pDCs (and T cells) in our context remain unknown. The induction of pDCs did not increase IL10 production by intestinal or MLN CD4+ T cells in mice colonized with *B. vulgatus*. Other polysaccharides (or bacterial products) produced by *Bacteroides* or other bacteria may be involved in this regulation given the diversity of these polysaccharides and other products in bacteria, and more in specifically Bacteroides, from clinical isolates (reviewed in (Wexler et al., 2007)).

Figure 7

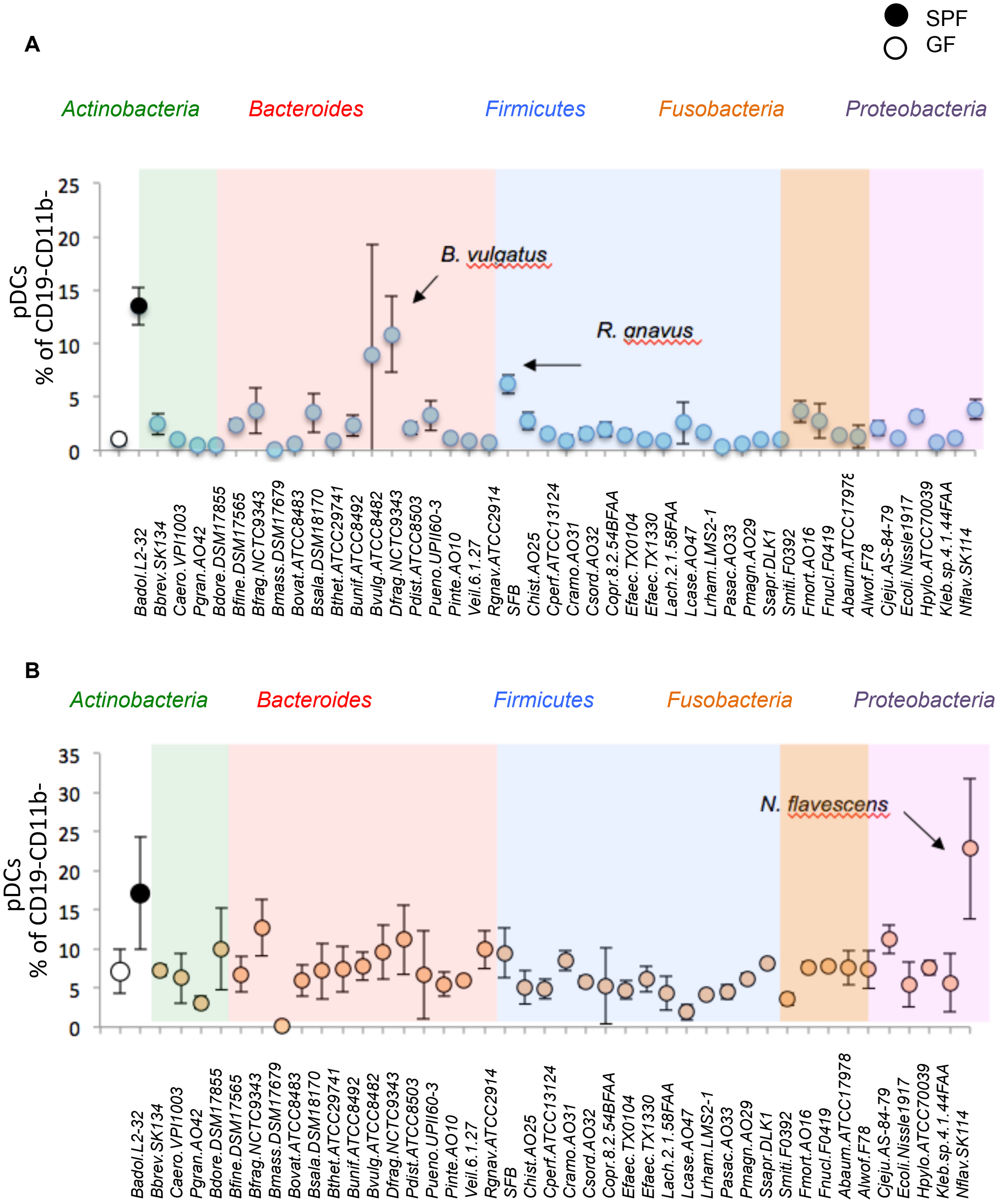


Figure 7. Microbial colonization induces pDCs in the colon and the small intestine. Frequencies of small intestinal (A) and colonic (B) pDCs within the CD45+CD19-CD11b-population. Each dot is the mean of at least 3 mice with SD. Color coded per phyla.

4. Proportions of small intestinal **monocytes** were increased in mice colonized with *Clostridium sordellii* (Fig. 8). An increase in monocytes can be associated with inflammation, but induction of monocytes in *C. sordellii* colonized mice did not correlate with any intestinal inflammation assessed by histopathology (not shown). At the mucosal sites, Ly6chi monocytes are precursors to CX3CR1+ MPs (Varol et al., 2007). This role of monocytes may be a more likely explanation since there is a corresponding increase in small intestinal CX3CR1+ MPs in *C. sordellii* colonized mice.

5. Colonization with *Bifidobacterium adolescentis, Enterococcus faecalis, Staphylococcus saprophyticus* induced **TH17 cells** in the small intestinal lamina propria (Fig. 9A). Although the major driver of small intestinal TH17 cells in mice is SFB, the bacteria that are responsible for TH17 cells in healthy mucosa of humans are not well described. SFB (or bacteria with sequences that resemble the sequence of SFB) have been identified in many vertebrates including humans (Sczesnak et al., 2011; Prakash et al., 2011; Yin et al., 2013). In humans, SFB probably colonize infants and disappear by 3 years of age (Sczesnak et al., 2011; Prakash et al., 2011; Yin et al., 2013). At this point it has been confirmed whether the SFB are the main drivers of mucosal TH17 cells in humans (Yin et al., 2013). Findings here, identify multiple species from the human microbiota aside from SFB that

Figure 8

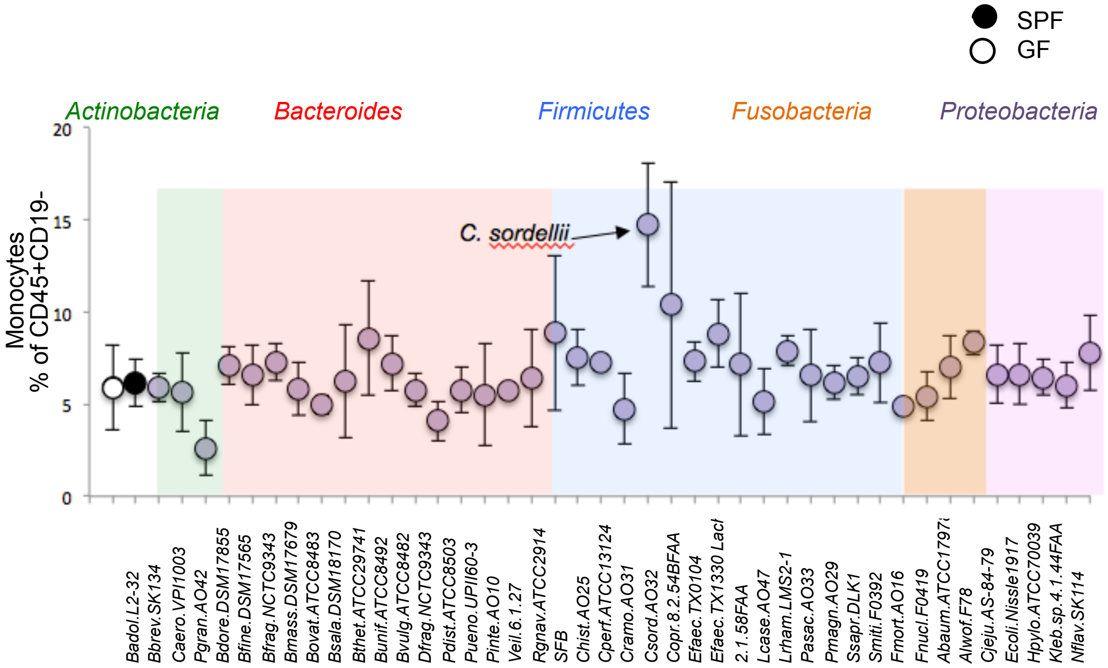


Figure 8. Colonization with *C. sordellii* colonization induces monocytes in the small intestine.
Frequencies of small intestinal monocytes within the CD45+CD19- population. Each dot is the mean of at least 3 mice with SD. Color coded per phyla.

are capable of inducing TH17 cells at least in the setting of the mouse gut.

6. Colonization with *Lachnospiraceae sp.* or *Campylobacter jejuni* elicited **TH17** responses in the Peyer's Patches (Fig 9B), but not in the small intestinal (Fig. 9A) or colonic lamina propria. Th17 cells have not been described in the Peyer's patches.

There was some redundancy in bacteria's ability to induce (or repress) similar immune populations (Fig. 6-9). Many unrelated bacteria from different phyla have been noted to induce regulatory T cells in the colon (Fig. 6A). Similarly unrelated SFB (isolated from mouse flora), *B. adolescentis* (a member of *Actinobacteria*)*, E. faecalis* (a member of *Firmicutes)* induced TH17 cells in the small intestine (Fig. 9A). However, this ability did not correlate with any phylogenetic distribution (Fig. 6-9, phyla color coded) or the genetic distance between bacteria. Induction of these cell types by different bacteria may involve different pathways, which raise the possibility of synergy when bacteria of interest are colonized together. To test this possibility we colonized mice with SFB and *E. faecalis* at the same time. Co-colonization of *E. faecalis* with SFB did not induce a higher frequency of small intestinal TH17 cells than did the colonization with either SFB or *E. faecalis* alone. This may suggest the mouse SFB and the human *E. faecalis* may use similar pathways to induce TH17 cells. Similarly, *C. ramosum* and *B. thetaiotaomicron,* bacteria with equal abilities to induce colonic Tregs, did not have synergistic effects when co-colonized. Colonic Treg levels were comparable in mice colonized with *C. ramosum* and *B. thetaiotaomicron* together or alone. Alternatively, the lack of synergy observed may be due to reaching a saturation point in TH17 and Treg proportions (with no room for synergistic effects to be observed) upon colonization with a single bacterial

Figure 9

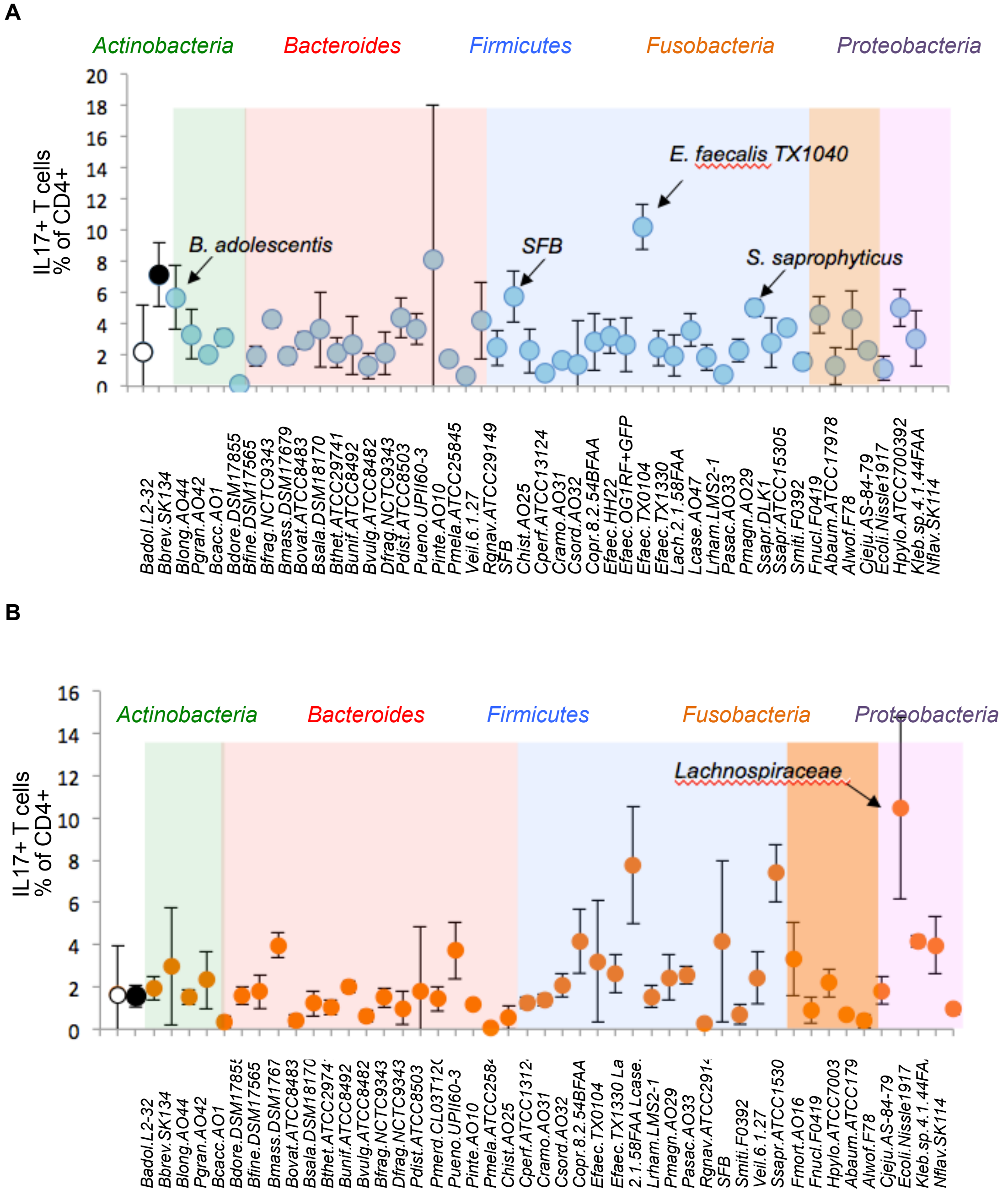


Figure 9. Microbial colonization induces TH17 cells in the small intestinal lamina propria and the Peyer's Patches.

A. Frequencies of small intestinal IL7+ cells within the CD4+TCR+ population. Each dot is the mean of at least 3 mice with SD.

B. Frequencies of IL17+ cells within the TCR+CD45+ population in the Peyer's patches. Each dot is the mean of at least 3 mice with SD. Color coded per phyla.

species. Co-colonization experiments with two bacteria that induce medium levels of Tregs may better address the question of synergy and provide evidence for independent induction mechanisms.

To have a more inclusive view of this redundancy, we used hierarchical-clustering analysis to cluster the bacteria and the corresponding immune population they induce or repress upon colonization (Fig. 10). We compared proportions of all immune cells types (rows) across different microbial colonizations (columns) and clustered these colonizations based on the phenotypes of immune cells elicited. This analysis, grouped by tissue, revealed that there were many bacteria, which had a similar patterns of induction (red) or repression (blue) compared to the rest of bacteria studied in this screen. Proportions of total Tregs and pTregs in the colonic lamina propria were increased after mono-colonization (colonization with one bacterial species at a time) with multiple bacterial species. Similarly, colonization with multiple microbes increased proportions of TH17 cells in the small intestinal lamina propria. Although there were few changes in the systemic lymphoid tissues, the phenotypes observed in the local intestinal tissues were similar after colonization with various single microbes, highlighting the redundant abilities of microbes (Fig. 10, presented as columns here).

Next, we asked whether there is any correlation between microbes, based on their ability to induce or repress immune populations upon colonization. For this analysis, we took two approaches: all-inclusive and strain-specific.

- **All-inclusive**: First, we calculated correlation coefficients (Spearman) for each microbe against one another using all the proportions of immune populations described before. Then we clustered these results to identify any relationships between bacteria (Fig. 11). Groups of bacteria that elicit similar immune responses were clustered in

Figure 10

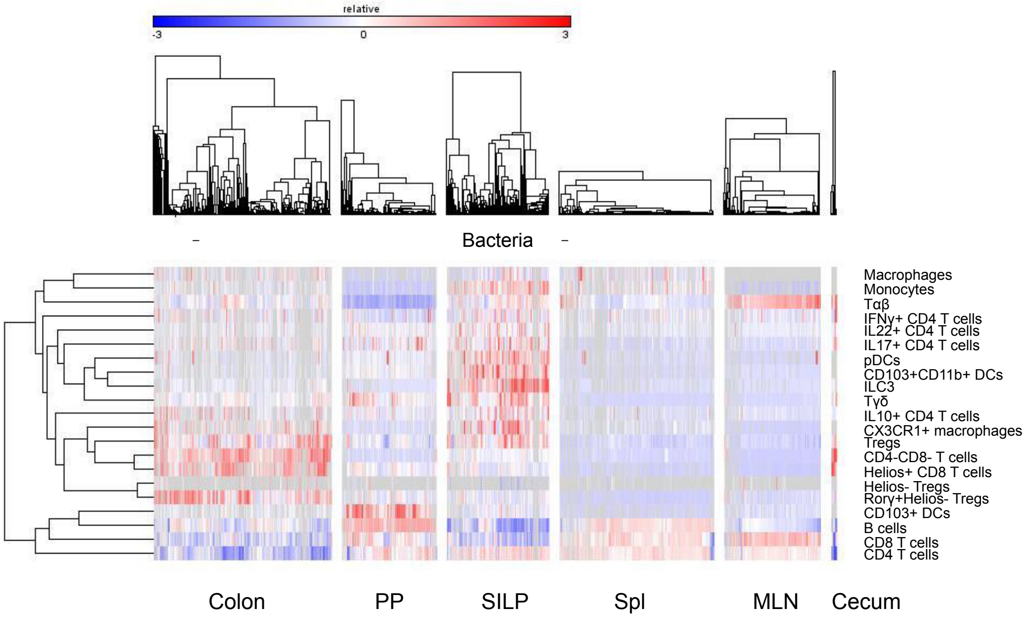


Figure 10: Redundancy in bacteria's ability to induce immune populations. Hierarchical clustering analysis of proportions of immune populations induced by microbial colonization(rows: Immune populations, columns= microbes that induce the corresponding immune populations). Means of at least 3 mice grouped by tissue. Row normalized by subtracting row means and dividing by the standard deviation.

Figure 11

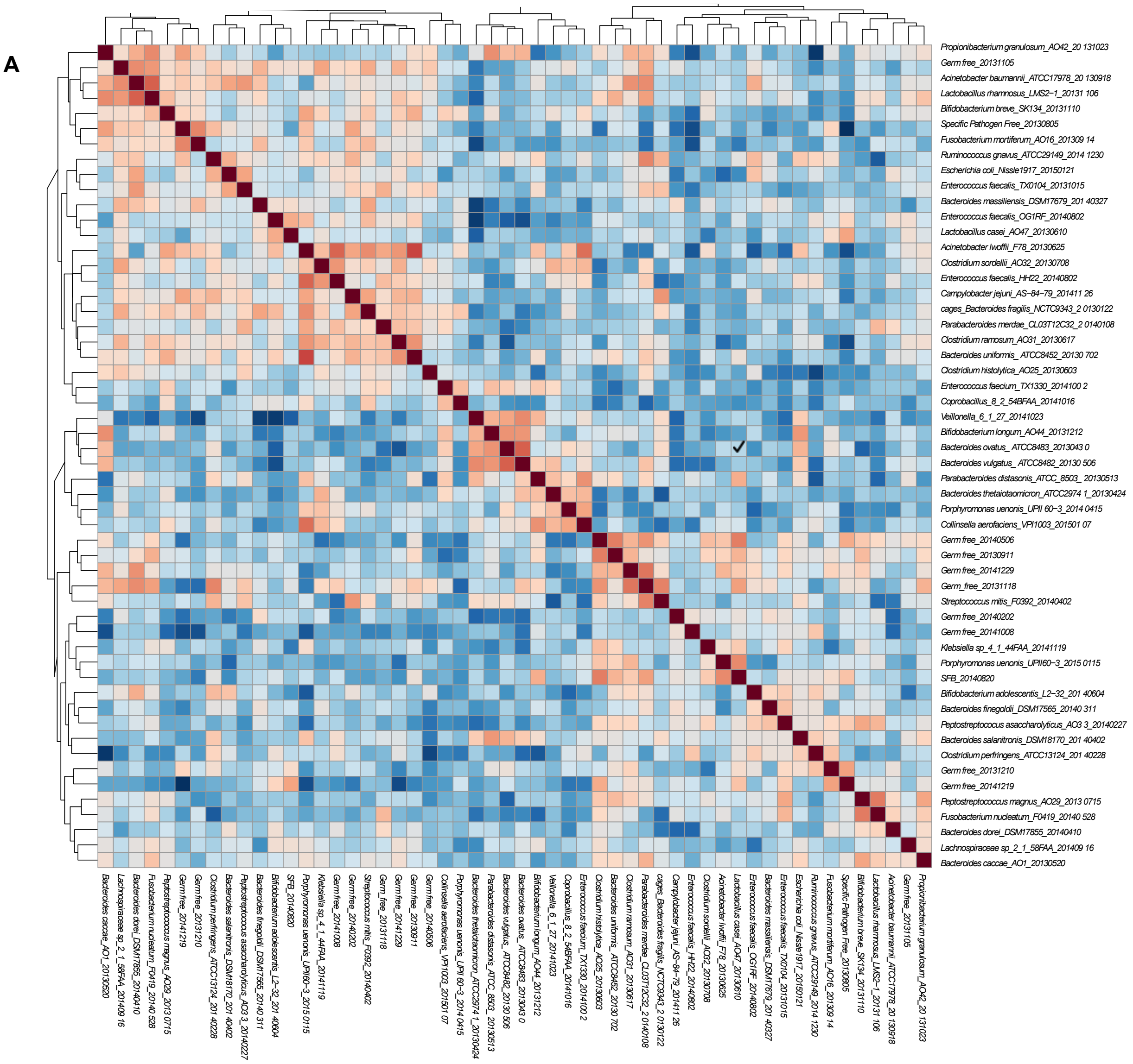


Figure 11: Overlapping abilities of bacteria to induce similar immune populations does not correlate with phylogeny

Spearman correlation and clustering of microbes based on their effect on immune populations. This analysis includes all the species that have the data for majority of the immune population in all tissues. *(figure prepared by NGZ)* close proximity along the diagonal, represented by different shades of red. Interestingly, we did not observe overrepresentation of any bacterial phylogeny that correlates with a microbe's ability to induce or repress local or systemic immune populations in the host. Mice colonized with bacteria from *Bacteroidetes* had comparable proportions of immune cells compared with mice colonized with bacteria from *Firmicutes* or *Fusobacteria.*

- **Strain-specific**: Next, we wanted to see whether mice colonized with the same species, but different strains of bacteria, have similar composition or proportions of intestinal immune cells. For this analysis, we only focused on a small set of microbes and the immune populations localized to the colonic lamina propria. The small set of microbes included 6 bacterial species each with at least two different strains. We asked whether there are strain-specific effects of colonization with these microbes on the host immune system. Interestingly, when bacteria were clustered based on how they shape the composition and the phenotype of the colonic immune cells, the clustering pattern did not mirror the phylogenetic distance (Fig. 12). In other words, different strains of the same species did not cluster together. Surprisingly, mice colonized with closely related *B. fragilis CL03T00C08* and B fragilis *NCT9343* did not elicit similar Treg responses in the colon (*Bacteroides* phylogeny is discussed in detail in Chapter 3). On the other hand, distant *S. saprophyticus DLK1* and *E. faecalis HH22* had similar immune profiles of the colon (measured by proportions of colonic lymphocytes, mononuclear phagocytes and cytokine producing T cells). The microbial effects of colonization with *S. saprophyticus DLK1* on the colonic immune cells

Figure 12

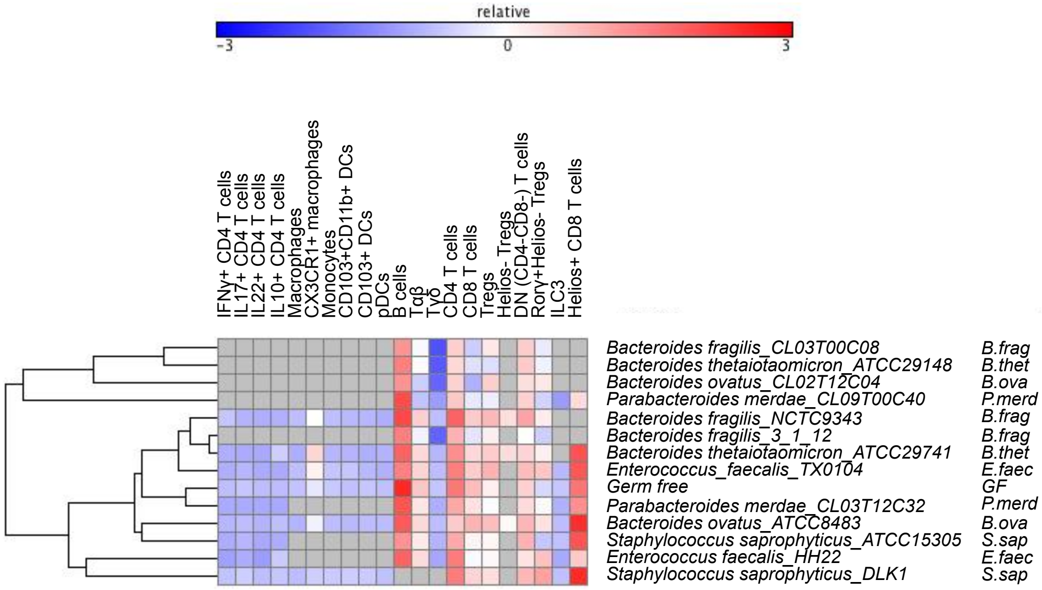


Figure 12: Overlapping abilities of bacteria to induce similar immune populations does not correlate with phylogeny

Hierarchical clustering of microbes and their effects on immune populations (microbes vs. proportion of immune populations) This analysis includes only the species that have at least two different strains and is focused on how colonization impacts on the colonic immune populations.

differed significantly from its close relative *S. saprophyticus ATCC15305.* Similarly, colonization with *E. faecalis HH22* and *E. faecalis TX0104* had different immune outcomes on the host (Fig. 12).

Overall, overlapping abilities of bacteria to induce similar immune populations in mice suggest some redundant pathways or metabolites shared by these bacteria (Consortium, T.H.M.P., 2012;). The phylogenetic diversity observed in the human microbiome does not necessarily translate functional diversity. This is evident at two levels: (1) similar functions shared by taxonomically unrelated bacteria and (2) different functions carried out by taxonomically related bacteria. Bacteria from different phylogeny express similar genes that are important in vitamin biosynthesis (Consortium, T.H.M.P., 2012). Conversely, strains from the same species such as *S. mitis* may have different expression of choline-binding proteins (Consortium, T.H.M.P., 2012). All together these may account for overlapping abilities of bacteria to induce similar immune cell types such as TH17 cells, Tregs or pDCs.

Finally, we asked whether the immune cells induced or repressed by bacterial colonization correlate with one another. To address this, we took two slightly different approaches that drew similar conclusions (Fig. 13A-B):

- First, we analyzed all the cells types in each tissue and calculated the correlation among these populations with respect to one another(Fig 13A). These correlation coefficients were then clustered to reveal how all the immune populations we analyzed relate to one another. This analysis considered colonic Tαβ cells and splenic Tαβ cells as different immune populations and allowed characterization of how tissue-specific responses relate to one another and how immune populations in the

Figure 13

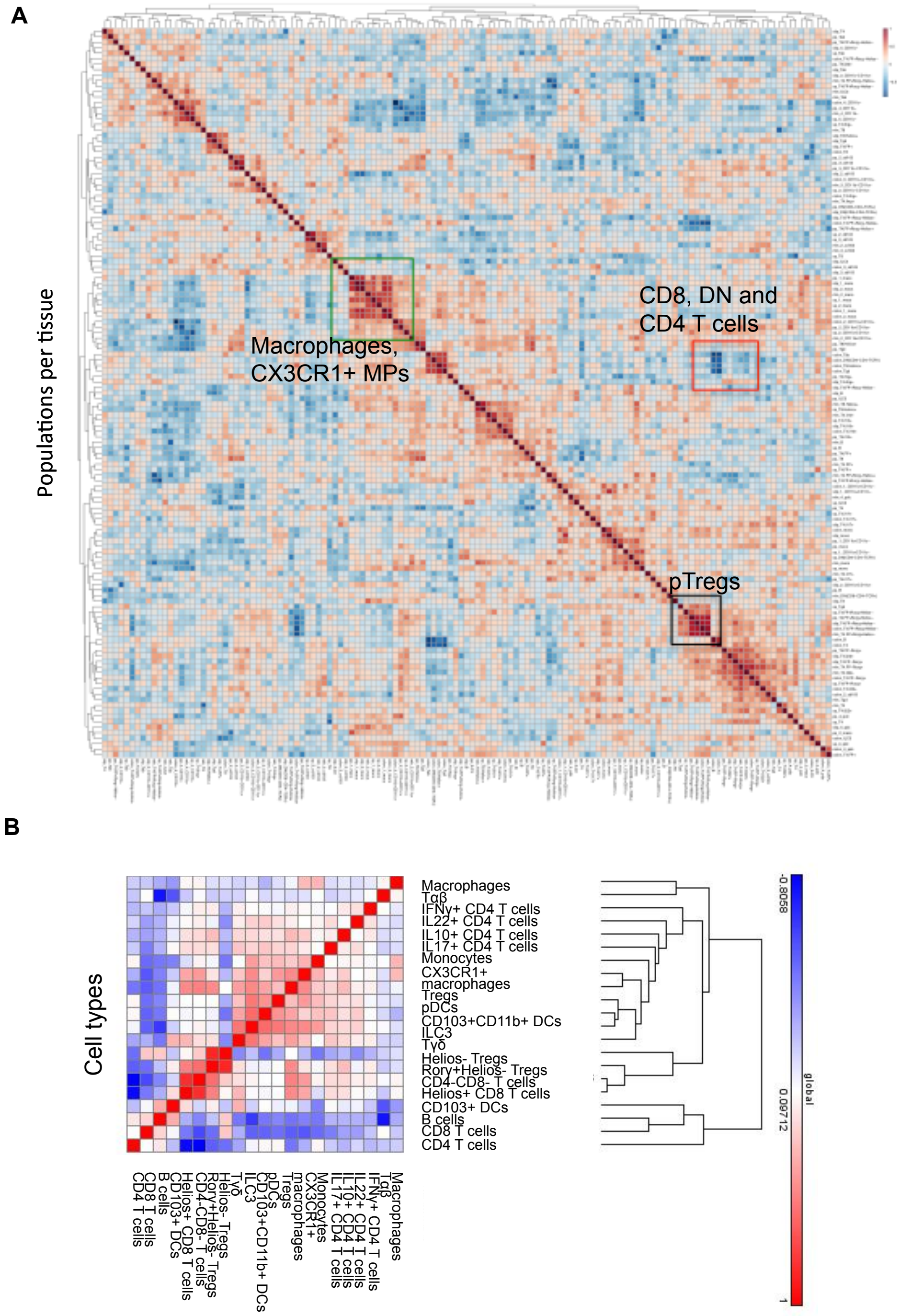


Figure 13: Relationship between different immune populations
A. Spearman correlation and clustering of immune populations. This analysis considers all the immune populations in different tissues separately. Correlation coefficient for each immune population for each tissue was calculated separately *(figure prepared by NGZ).*
B. Pearson correlation and clustering of immune populations. This analysis is simplified and correlation coefficient was calculated for each immune cell type (not per tissue).

same tissue relate to one another. This analysis revealed that some population changes in the systemic lymphoid tissues mirrored the changes of that same population in the intestinal tissues (Fig 13A).

- In a second approach (B), we grouped immune populations by cell types and combined all cell types across all tissues. In this analysis there was only one correlation coefficient value calculated for comparison of Tαβ and B cells pooled from all tissues. We asked if there were any general changes Tαβ cells elicited by microbial colonization irrespective of the tissue. Tαβ and B cell proportions from colon, small intestine, spleen, MLN and PPs were used together in determining how similar Tαβ and B cell phenotypes are upon colonization. Although the first approach is more inclusive, the second approach displays very similar patterns, suggesting local changes in the intestines have similar systemic outcomes.

These two approaches identified various mostly overlapping patterns. Macrophages and CX3CR1+ MPs from all tissues behaved similarly upon colonization (Fig13A-green square). Induction of total Tregs and their corresponding pTreg proportions (measured as Helios- or RORγ+Helios-), were strikingly similar among all tissues including the Peyer's Patches and MLNs (Fig13A-black square). The degree of Treg induction in the MLNs was at a lower scale and was disregarded earlier. The same was true for macrophages where few tissue-resident macrophages are found in the spleens and lymph nodes. Activated Helios+CD8+ T cells and DN T cells correlated well in all tissues of monocolonized mice and this pattern was also present in the colon-only analysis performed earlier). Conversely, CD4+ T cells anti-correlated with both Helios+CD8+ T cells and DN T cells (Fig13A-red square and 13B). Another strong and intriguing correlation was among CD103+ DCs (Fig13B) and B cells especially given the role of DCs in IgA class switch (Tezuka et al., 2007). These parallels or patterns among immune cells need to be further explored and may provide insights into fine-tuning of immune system. The effects of microbial colonization may involve multiple cell types and the clusters we observe here may provide insight to how cells interact with the components of the microbiota and with one another

Overall, the assessment of various immune phenotypes serves as a repository for the immunomodulatory aspects of each bacterium and highlights the redundancy in microbe's ability to drive these outcomes.

2.3: Colonic gene expression profiles of mice colonized with microbes

We asked how colonization with single bacterial species affects the whole tissue transcriptome of the colon. Colons of mice colonized with various single bacterial species were collected in Trizol and homogenized. For consistency, the same 5mm-piece was cut from the distal colon (3cm from rectum). We looked for microbe-specific or shared effects of microbial colonization on the activity or differentiation of immune and non-immune populations in the colon. Specifically, we asked:

1. Are there general effects elicited by all microbes upon colonization of the intestines?
2. Does microbial colonization promote microbe-specific effects that are uniquely modulated by one microbe but not any others?
3. Are there any shared microbial effects on the colonic transcriptome of colonized mice? Can this be explained by phylogeny?
4. Is there any relationship between the genes that are induced or repressed and the immune cell populations affected by microbial colonization?

First, we assessed the variability within the sample replicates of colons from mice colonized with single bacterial species or from non-colonized germ free mice. To our surprise, there were more genes with high coefficients of variation (CV) within the colons of germ free mice (Fig. 14A, x-axis) compared with the colons colonized mice (Fig. 14A, y-axis). This analysis did not look into microbe-specific effects, but highlighted the general trends that microbial colonization elicits as a whole when all effects are pooled. This analysis also allowed identification of 184 colonic genes that change during microbial colonization compared to the germ free state (will be discussed later).

Figure 14

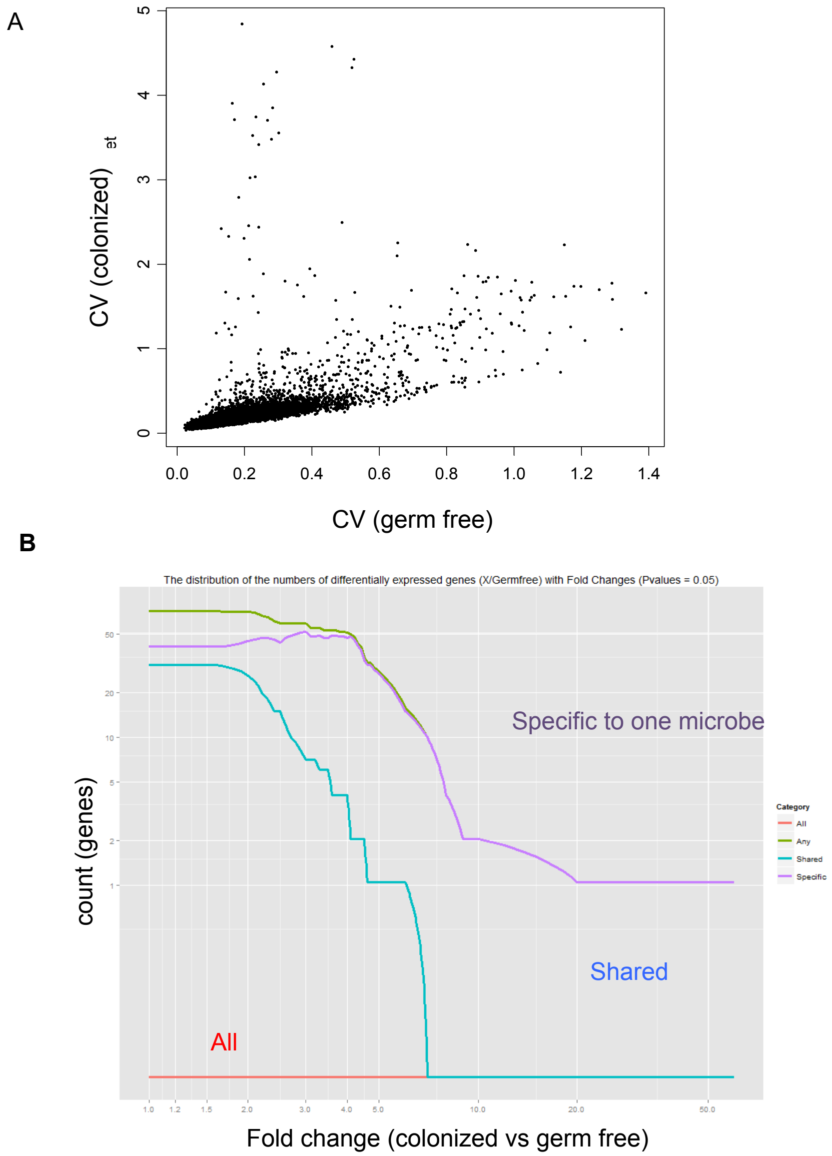


Figure 14. microbe-specific and shared effects of microbial colonization on the colonic transcriptome.

A. Coefficient of variation (CV) analysis of colonic transcriptome from mice colonized with single bacteria (all colonized pooled- y axis) and germfree mice (x-axis).

B. Number of genes (y-axis) that are induced by all(red), some(blue) and one bacteria(purple) following microbial colonization across a distribution of fold changes (x-axis). All: genes that are induced or repressed by all microbial colonizations compared to germ free. Some microbes: genes that are induced by at least two microbial colonizations compared with germ free. One-microbe: genes that are uniquely induced by one microbe upon colonization but not by others.

Next, we wanted to determine whether the stabilization of the gene expression we observe upon colonization is a result of a set of genes that are induced or repressed in the colon by all bacteria. Since we were interested in significant gene hits, we only counted the genes that were differentially expressed either in colonized mice or germ free mice with p-values lower than 0.05. We counted the number of genes that are induced or repressed by all microbes (1), by some microbes (2) or by one microbe (3) across all possible fold change values.

1. **All microbes**: In this analysis we calculated the fold change value for each gene between the mice colonized with single microbe and the germ free mice. Then, we counted the number of colonic genes with a fold change value significantly higher or lower (p value<0.05) in all of the microbial colonizations compared with the germ free state. For instance, we counted the number of genes that are induced by all microbes at a fold change of at least 1.5 or higher compared to germ free. This analysis included all possible distribution of fold change values. Finally, we graphed these gene counts (y-axis) across their corresponding of fold change values (x-axis). As the flat red line indicates, there were no genes that all microbes affected in the similar direction in the colonic whole tissue (Fig14B, red).
2. **Some microbes**: This analysis followed similar steps as described in “1. All microbes,” but instead of looking for gene expression changes that are promoted by all the microbes, this analysis looked for changes that are elicited by at least two

microbes or more. Interestingly, there were a number of shared effects of microbes on the colonic transcriptome (Fig14B, blue).

3. **One-microbe**: This analysis looked for unique microbe-specific effects on the colonic transcriptome that are elicited by only one microbe compared to the rest. One difference here compared to the earlier analysis (all or some microbes) was including another criteria to the calculation of gene counts. For a given microbial colonization, this analysis was not restricted to identifying genes that change with respect to germ free but also with respect to other microbes. For instance, this analysis set out to identify significant changes that colonization with *Ruminococcus gnavus* induced compared with colonization with all other bacteria as well as the germ free state state. Indeed, we identified numerous unique-microbe specific effects of colonization. Bacteria, which induce these microbe specific effects, will be described later.

We looked deeper in the microbe-specific and shared effects of microbial colonization. For this analysis we used 184 genes that were differentially regulated by at least one microbe. The criteria for selection of these genes were a fold change of at least 1.5 (colonized vs. germ free) and a low CV. We used hierarchical clustering to determine how expression of these genes changes upon colonization with different single bacterial species (Fig. 15). Some of these genes were shared in a patch-work manner in colons of colonized mice. Colonization with *C. jejuni* and *Lachnospiracea* induced a large set of shared genes in the colon, but a subset of these genes were also induced upon colonization with *Acinetobacter lwoffii, Coprobacillus* and

Peptostreptococus asaccharolyticus. A different set of genes was induced in *Lachnospiracea, A*. *lwolfii*, *Coprobaccilus, B. vulgatus, Parabacteroides distasonis,* but not in *C. jejuni or P. asaccharolyticus.* The microbes that elicited these shared responses did not show any taxonomical enrichment.

We used another clustering module that groups genes that have similar induction or repression patterns upon microbial colonization (Fig. 16). This analysis gives more control on setting an optimum number of clusters. This analysis allowed identifications of 10 gene clusters each with 5-30 genes that show similar expression patterns that microbe-specific (Fig 16- cluster 4, 6) or shared effects of bacteria (Fig. 16- cluster 1, 2, 7, 10).

The latter analysis showed a clearer set of microbe-specific effects (Fig 16),that were also seen in the heatmap (Fig. 15). Colonization with *R. gnavus* had the most microbe-specific effects seen in clusters 4 and 6. This finding was surprising as colonization with *R. gnavus* induced very few immune populations in the colonic lamina propria (induction of pDCs and CX3CR1+ MPs) with respect to colons of germ free mice or mice colonized with other bacteria (Fig10A-B).

We asked whether the activity or differentiation of some immune and non-immune populations could be explained by induction of these differential genes. We looked for representation of these genes (as small groups in clusters or as a whole list) in the transcriptomes of various immune and non-immune cell types reported in the ImmGen consortium. These bacteria-induced genes were enriched in mostly stromal cells but also at low levels in some myeloid and B cells (Fig. 17A). I will summarize some of these intriguing microbe-specific or shared effects:

Figure 15

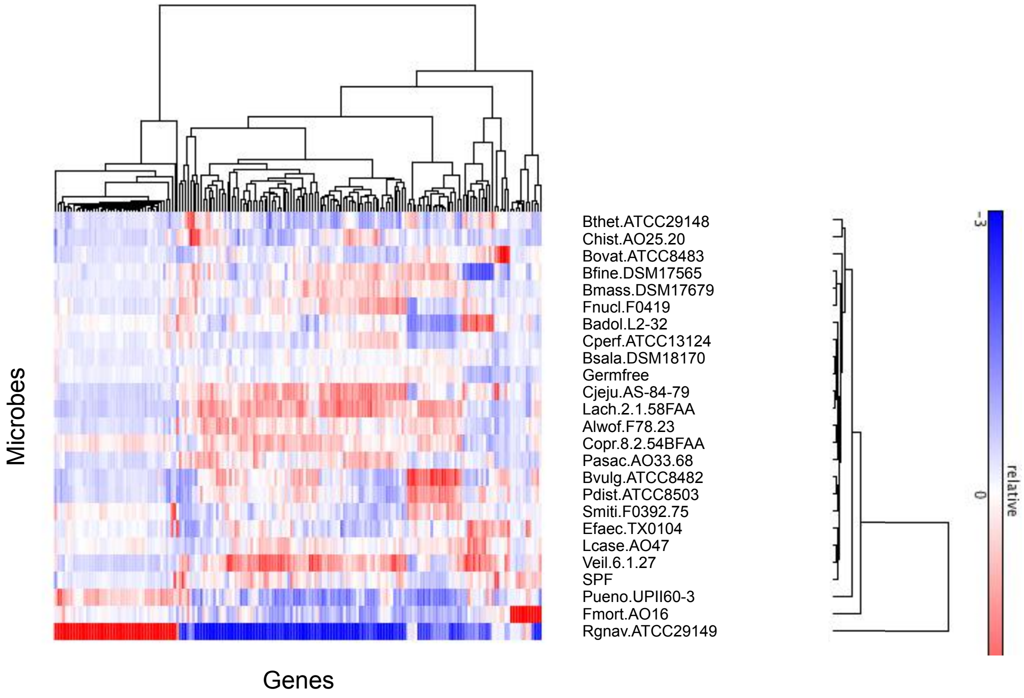


Figure 15: Microbe-specific or shared effects of colonization on the colonic transcriptome.
Hierarchical clustering analysis of genes enriched in microbially-colonized mice compared to germ free mice. Genes shown here are 184 colonic genes that change after colonization with microbes compared to the germ free state. Class means of at least duplicates.

Figure 16

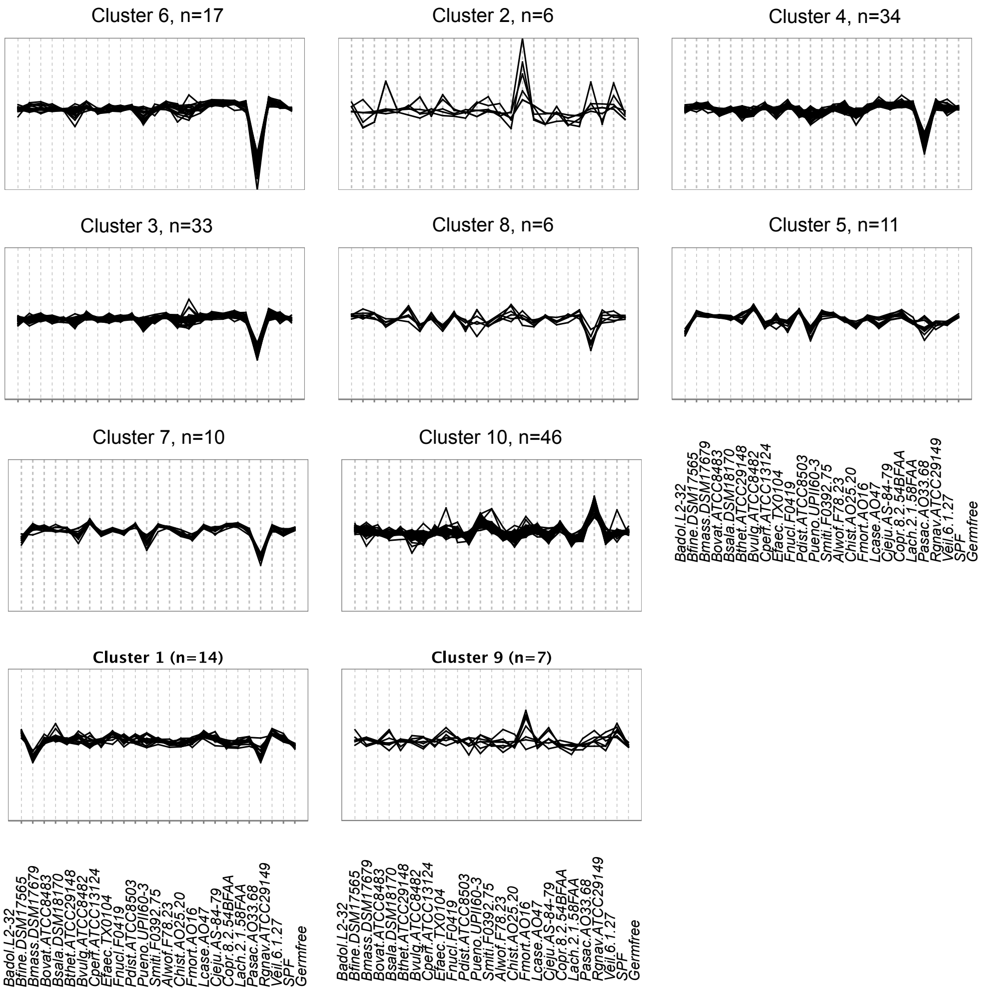


Figure 16: Microbe-specific or shared effects of colonization on the colonic transcriptome.
K-means clustering analysis of genes enriched in microbially-colonized mice compared to germ free mice. Genes shown here are 184 colonic genes that change after colonization with microbes compared to the germ free state. Class means of at least duplicates.

Figure 17

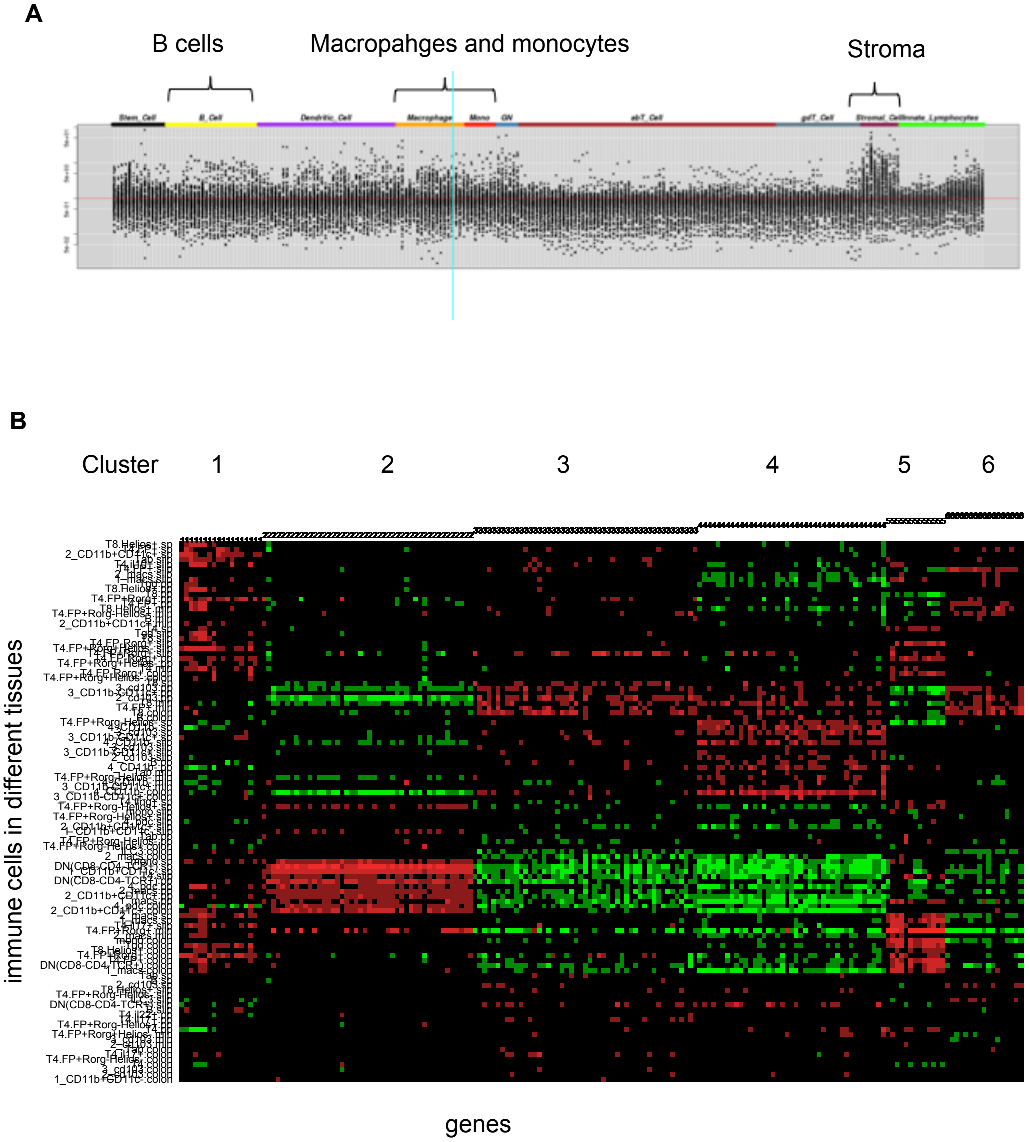


Figure 17: Genes that are induced or repressed by microbial colonization are enriched in stromal cells.

A. W-plot from ImmGen Consrotium for 184 genes that were identified to be differentially regulated in the colonic tissue by microbial colonization. This is a scatter plot of the means-normalized expression value of each of 184 genes in all of the populations in the ImmGen data set.

B. Clustering analysis of correlation between genes and the immune populations induced by microbial colonization. Red: positive correlation; green- negative correlation.

- The repression *Muc1* (Fig. 16-induced cluster 10) in mice colonized with *R. gnavus, P. uenonis, F. mortiferum* suggests effects on epithelial cells, especially in mucin-producing goblet cells. Bacterial induction of antimicrobial peptides such as RegIIIγ in the small intestine and mucins in the colon are well established in earlier studies (Vaishnava et al., 2011; Jakobsson et al., 2014). However, these studies did not address ability of microbes to suppress mucin production.
- Overrepresentation of *Ighg* and *Igk-v28* were part of the shared effects of bacteria and marked changes in B cells. These effects of microbes on B cells were shared by many and complementary to the wide range of colonic B cell proportions observed in colonized mice.
- A decrease in *Tgfb3* transcript was noted in mice colonized with *R. gnavus*, *B. finegoldii,* while a slight increase in *Tgfb3* transcript was present in mice colonized with *Veillonella* and *L. casei* (cluster 1). Specifically, *Tgfb3* but not other isoforms, confers pathogenic potential to TH17 cells during differentiation (Lee et al., 2012). However, there was no difference in IL17 levels produced by CD4 T cells in colons of mice colonized with *R. gnavus*, *B finegoldii, Veillonella* or *L. casei*.
- *Maf,* a transcript which has very high expression in macrophages and monocytes (ImmGen), was induced in the colons of mice colonized with in *R. gnavus* (highest induction with *R. gnavus* colonization), *P. uenonis* and *Coprobaccilus* (Fig. 16-cluster 10). This finding agrees with high proportions of CX3CR1+ MPs observed in *R. gnavus* colonized mice but not in others.

- Additionally microbe-specific repression of *Ly6g*, a transcript specifically expressed in neutrophils (ImmGen), in *R. gnavus* colonized mice (Fig. 16-cluster 6) was puzzling. Histological sections showed no neutrophilic accumulation in mice colonized with other bacteria. Since the analysis of immune cells did not include neutrophils, it is not clear if there were slightly lower proportions of neutrophils (that can be detected in colonic transcriptional analysis but not in histopathological analysis) in the colons of *R. gnavus* colonized mice.

The characterization of shared or microbe-specific effects suggested many links to immune populations described earlier. We systematically compared how gene expression data relates to the immune populations discussed earlier. These genes and cell types clustered in 6 well-defined groups (Fig. 17B). These correspond to large changes in DN (CD8-CD4-) T cells, CD103+ DCs and macrophages and may help explain how microbes affect the function or activity of these populations.

Overall, microbial colonization, which stabilized expression of many genes that fluctuate in the colons of germ free mice, had microbe-specific or shared effects. These shared effects conferred by bacteria could not be explained by phylogenetic relationships.

2.4: Discussion

The human gastrointestinal tract harbors a vast number of microorganisms with enormous diversity (Consortium, T.H.M.P., 2012). Understanding the impact of the microbiota on human health has been the motivation for many studies. There have been many advances in characterization of the components of the human microbiota from healthy and diseased individuals (Consortium, T.H.M.P., 2012; Gevers et al., 2014). Dysbiosis in the human microbiota has been linked to many autoimmune and inflammatory diseases (reviewed in (Huttenhower et al., 2014; Mathis and Benoist, 2011)). Gnotobiotic and germ free studies in mice have highlighted the impact of commensals, in combination or alone, on the host immune system (Atarashi et al., 2011; Geuking et al., 2011; Ivanov et al., 2009; Mazmanian et al., 2008; Faith et al., 2014; Chung et al., 2012). Although the particulars of the murine microbiota have been studied extensively, the specific effects of single microbes from the human microbiota are not well described (Atarashi et al. 2013; Chung et al., 2012).

We were inspired by these studies and set out to characterize the impact of single microbes from the human gastrointestinal tract in gnotobiotic mice. Our large-scale screen targeted to reflect the microbial diversity seen in the human microbiota covered 64 different strains, 52 species, 29 genera and 5 phyla. None of the mice colonized with one of the 64 strains of microbes chosen showed any signs of inflammation. Although not all microbes had similar success in colonizing the mouse GI tract, the ones that colonized could be referred as symbionts as they all affected the host immunity at some level.

Some bacteria did not colonize the lower GI tract, but maintained their niche even in absence of competition. This opens up a discussion for how niche-specificity is maintained and how microbial interactions affect the host. The pressures applied from the host and the microbial communities balance each other during co-colonization and may have synergistic, inhibitory or harmonious effects on the host. The few attempts we made to address the effects of co-colonization in microbes' ability to colonize and affect the host led to interesting observations. Colonization of an anaerobic bacterium with an aerobic bacterium diversified the niche of the anaerobe. We did not observe any synergistic or inhibitory immune effects mediated by co-colonization. Although beyond the scope of this study, understanding microbe-to-microbe interactions and their outcome in shaping the host immune system could be the motivation for other comprehensive and systematic studies.

Here, we identified multiple lymphoid, myeloid and stromal populations that are altered by microbial colonization with single species. Although these microbe-driven effects were mostly enriched in the intestinal tissues, systemic extensions were also present. Correlation analysis between the immune populations analyzed from different tissues suggested multiple effects of colonization in the lymph nodes and the spleen. These may have implications in autoimmune diseases such as T1D, MS and RA for which a concordance between the dysbiosis of the microbiota and the disease risk has been evaluated in humans (Vaarala et al., 2013; Zhang et al., 2015). . A closer look at the systemic outcomes driven by bacteria may help predict in which autoimmune disease these bacteria from the human microbiota may play a role.

Our analysis was focused on primarily screening for the effects of microbial colonization at steady state, but we addressed the functional importance of microbe-driven immunological phenotypes at various colitis models. Treg response elicited by multiple single bacteria in the colon correlated with protection in TNBS induced-colitis. Similarly, in a DSS induced colitis model, supplementation of the normal flora with a high Treg inducer bacteria (*C. ramosum*) conferred protection. These findings highlight the functional importance and capacity of the cells that are promoted by microbial colonization.

To our surprise there were many shared effects of microbial colonization on the proportion of immune populations and colonic transcriptome. Many bacteria promoted similar immunological and non-immunological effects on the host. The redundancy in bacteria's ability to induce TH17 cells was remarkable. It is tempting to speculate that there may be more bacteria like the SFB even from the mouse microbiota that can generate TH17 cells. The striking redundancy seen in the microbes' ability to have similar outcomes was independent of phylogeny as many taxonomically unrelated bacterial species elicited similar responses at either gene expression or cellular level on the host. Two different strains of *S. saprophyticus* had different abilities to induce TH17 cells in the small intestinal lamina propria and Tregs in the colonic lamina propria. This observation supports the functional redundancy in the phylogenetically diverse microbiota, revealed by shotgun metagenomics (Consortium, T.H.M.P., 2012). Focusing on functional networks and pathways may help explain how different bacteria mediate similar pathways. For instance, choline-binding proteins show remarkable differences in various strains of *S. mitis* (Consortium, T.H.M.P., 2012*).* Similar observations may help us predict why colonization with two different strains of S. *saprophyticus* has different immune outcomes. This approach may still be insufficient and call for identification of bacterial transcripts by RNA sequencing or proteins to fully understand how not even remotely related bacterial species have similar outcomes in the host.

Although there were remarkable shared effects, bacteria still elicited microbe-specific effects, which require a more detailed analysis. The colonic transcriptome of mice colonized with *R. gnavus* was the most differential compared to other microbial colonizations. These *R. gnavus* specific effects mostly involved stromal responses. Although response of the epithelial cells to microbial colonization has been somewhat described in studies comparing goblet cells and paneth cells from SPF or germ free mice, not much is known about microbe-specific changes in the intestinal epithelium

(Jakobsson et al., 2014; Vaishnava et al., 2011). Focusing on the function and activity of stromal genes from mice colonized with several single microbes may help resolve this.

Overall, this large-scale screen provides a comprehensive study on effects of individual bacteria on many aspects of host immunity and colonic transcriptome and may have implications at the diseased state.

Chapter 3: Microbe-dependent induction of a distinct population of RORγ$^+$ regulatory T cells in the colonic lamina propria

We have summarized various microbe-specific or shared effects of microbial colonization on the host immune and stromal populations that cover, either local or systemic responses. This chapter, which is a copy of our manuscript published in *Science* on August 13th 2015, focuses on a very specific regulatory response that can be mediated by various single microbes. As previously mentioned, in 43% of the microbial colonizations, there was an increase in colonic Tregs comparable to SPF levels. Here, we dissected this microbe-induced effect to test whether microbes can shape activities of these colonic Tregs. In this analysis, we asked how colonic Tregs relate to other "tissue" Tregs and described how multiple microbes can have shared effects on shaping the phenotype and function of colonic Tregs. We discovered a subset of colonic Tregs that uniquely express transcription factor RORγ. Expression of RORγ could be induced by multiple microbes and was crucial for constraining intestinal inflammation.

The work presented in this chapter especially benefited form collaborative studies (in order of appearance) with, Dr. Liza Konnikova and Dr. Scott Snapper on human colonic biopsies, Dr. Dennis Kasper, Dr. Naama Geva-Zatorsky, Dr. Adriana Ortiz-Lopez on the large scale screen of germ free mice colonized with single bacterial species, Dr. Abby Mcguire on bacterial genome comparison, Dr. Sungwhan Oh on SCFA analysis of caecal material, David Zemmour on RNA sequencing analysis of colonic Tregs, Drs. Mercedes Lobera, Jianfei Yang and Shomir Ghosh from Tempero Pharmaceuticals on Rorγ antagonist treatments.

3.1: Individual intestinal symbionts induce a distinct population of RORγ$^+$ regulatory T cells

Esen Sefik,[1] Naama Geva-Zatorsky,[1] Sungwhan Oh,[1] Liza Konnikova,[3] David Zemmour,[1] Abigail Manson McGuire,[4] Dalia Burzyn,[1] Adriana Ortiz-Lopez,[1] Mercedes Lobera,[5] Jianfei Yang,[5] Shomir Ghosh,[5] Ashlee Earl,[4] Scott B. Snapper,[3] Ray Jupp,[6] Dennis Kasper,[1] Diane Mathis,[1,2] Christophe Benoist[1,2†]*

[1]Division of Immunology, Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA.

[2]Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA USA.

[3]Division of Gastroenterology and Hepatology, Brigham and Women's Hospital, Boston, MA, and Department of Medicine, Harvard Medical School, Boston, MA, USA.

[4]Broad Institute of MIT and Harvard, Cambridge, MA, USA.

[5]Tempero Pharmaceuticals, a GSK Company, Cambridge, MA, USA.

[6]UCB Pharma, Slough, Berkshire, UK

*Present address: Jounce Therapeutics, Inc., Cambridge, MA 02138, USA.

†Corresponding author. E-mail: cbdm@hms.harvard.edu.

T regulatory cells that express the transcription factor Foxp3 (Foxp3$^+$ T_{reg}) promote tissue homeostasis in several settings. We now report that symbiotic members of the human gut microbiota induce a distinct T_{reg} population in the mouse colon, which constrains immuno-inflammatory responses. This induction—which we find to map to a broad, but specific, array of individual bacterial species—requires the transcription factor Rorγ, paradoxically, in that Rorγ is thought to antagonize FoxP3 and to promote T helper 17 (T_H17) cell differentiation. Rorγ's transcriptional footprint differs in colonic T_{regs} and T_H17 cells and controls important effector molecules. Rorγ, and the T_{regs} that express it, contribute substantially to regulating colonic T_H1/T_H17 inflammation. Thus, the marked context-specificity of Rorγ results in very different outcomes even in closely related cell-types.

FoxP3 regulatory T (Treg) cells are essential regulators of immunologic homeostasis and responses (Josefowicz et al., 2012). Beyond their well-described role in regulating the activity of other immunocytes, Tregs located in parenchymal tissues control other, non-immunological, processes. These "tissue Tregs" include those that reside in visceral adipose tissue and regulate metabolic parameters (Feuerer et al., 2009; Cipolletta et al., 2012) and those that help channel inflammatory and regenerative events in injured muscle (Burzyn et al., 2013b). The activities, transcriptomes, and T cell receptor (TCR) repertoires of these tissue Tregs are distinct from their counterparts in secondary lymphoid organs.

Another essential and quite specific population of tissue Tregs resides in the lamina propria (LP) of the digestive tract, in particular in the colon, where these cells modulate responses to commensal microbes (reviewed in (Ai et al., 2014)). Colonic Tregs are an unusual population that has provoked some contradictory observations.

TCRs expressed by colonic Tregs show marked reactivity against microbial antigens that seem to be important drivers of their differentiation and/or expansion (Lathrop et al., 2011; Cebula et al., 2013). Many of them appear to arise by conversion from FoxP3– conventional CD4+ T cells (Tconv) (Lathrop et al., 2011), although arguments for a thymic origin have been made (Cebula et al., 2013). Many colonic Tregs express marker profiles (Helios– and Nrp1–) that differ from Tregs of thymic origin (reviewed in (Bilate and Lafaille, 2012)), although the significance of these markers has been questioned (Bilate and Lafaille, 2012). Accordingly, most studies have found a decreased abundance of colonic Tregs in germ-free (GF) mice (reviewed in (Ai et al., 2014)), and colonization of GF mice by pools of microbes (Schadler's flora (Geuking et al., 2011) or Clostridia combinations (Atarashi et al., 2011; Atarashi et al., 2013) elicited the differentiation or expansion of Helios–Nrp1– colonic Tregs. The ability of single microbes to induce colonic Tregs has been more controversial, and the need for complex combinations (Atarashi et al., 2011; Atarashi et al., 2013) has been questioned (Faith et al., 2014).

The transcriptomes of tissue-resident Tregs adapt to their location, most strikingly in terms of transcription factors (TFs) (Burzyn et al., 2013a), and we searched for such elements in colonic Tregs. Comparison of transcriptomes of highly purified CD4+FoxP3+ Tregs (from Foxp3$^{ires–gfp}$ reporter mice (Bettelli et al., 2006)) from colon or spleen uncovered 933 differential transcripts (at a fold change > 2 and false discovery rate (FDR) < 0.1) (Fig. 18A (top), Fig. 19A). These encompassed important signaling and effector pathways (*Icos, Gzmb, Lag3, Areg,* and *Il1rl1*) (Fig. 18A (top)), shared in a patchwork manner by other tissue Tregs. Yet ~39% (at a colon-specific bias of >1.5-fold) had preferential expression in colonic Tregs (including *Il10, Ctla4, Havcr2, Ccl20, Jak2,* and *Fosl2*) (Fig. 18A (bottom)).

Figure 18

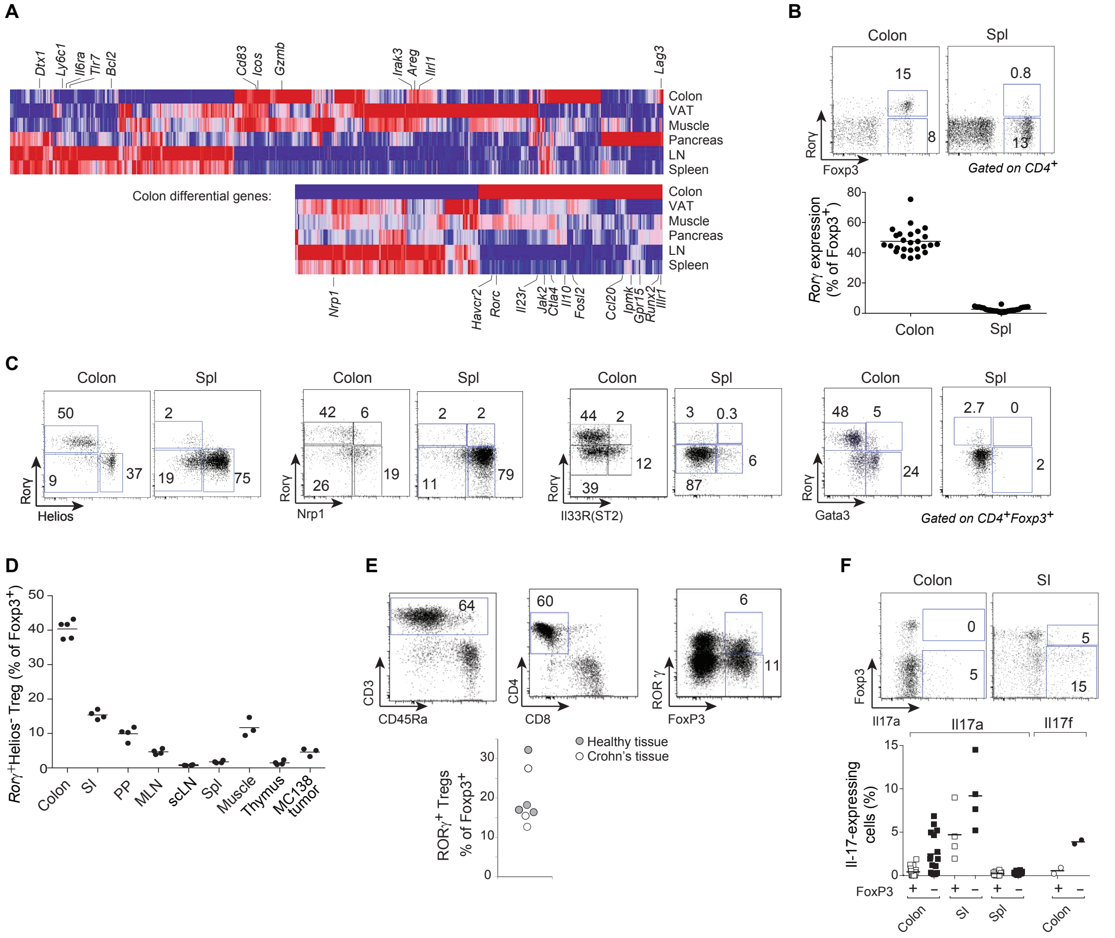


Figure 18: Rorγ, encoded by *Rorc*, is preferentially expressed in colonic T_{regs}.
Gene expression profiles from purified T_{reg} cells of various origins. (**A**) Transcripts that are enriched in tissue and colonic T_{regs}. (Top) Transcripts differentially represented in tissue versus splenic T_{regs} (at a fold change > 2). VAT, visceral adipose tissue. (Bottom) Transcripts that are most biased in colonic T_{regs} (fold change > 1.5 versus any other tissue T_{reg}). Means of at least two duplicates. (**B**) Representative flow cytometry plots of $CD4^+$ T cells and a compilation of frequencies (bottom) of Rorγ $^+$Helios$^-$ T_{regs} within the $FoxP3^+CD4^+TCR\beta^+$ population. Each point is an individual mouse. Data are representative of more than three independent experiments. **(C)** Representative Rorγ vs Helios, Nrp1, Il33R or Gata3 plots for colon or spleen $Foxp3^+CD4^+TCR\beta^+$ Tregs (see Fig. S2 for quantification). (**D**) Frequencies of Rorγ$^+$Helios$^-$ T_{regs} among $FoxP3^+CD4^+TCR\beta^+$ cells of different tissues (SI, small intestinal lamina propria; PP, Peyer's patches; MLN, mesenteric LNs; scLN, subcutaneous LNs; spleen, Spl). Each point is an individual mouse. Data pooled from at least two independent experiments. (**E**) Flow cytometry analysis of human colon biopsies and frequencies of human RORγ$^+$ T_{regs} within the $FOXP3^+CD4^+CD8^-CD3^+CD45^+$ population. Healthy tissue samples were endoscopically determined normal areas from chronic constipation or irritable bowel syndrome patients; inflamed tissue was from Crohn's lesions. Each point is an individual patient. Data pooled from five independent experiments. (**F**) IL-17a (after phorbol 12-myristate 13-acetate + ionomycin activation and intracellular staining) or IL-17f (reporter in *Il17f*^{rfp} mice) expression among Foxp3$^+$ T_{reg} or FoxP3$^-$ T_{conv} mice. Each point is an individual mouse. Data are representative of three independent experiments.

Figure 19

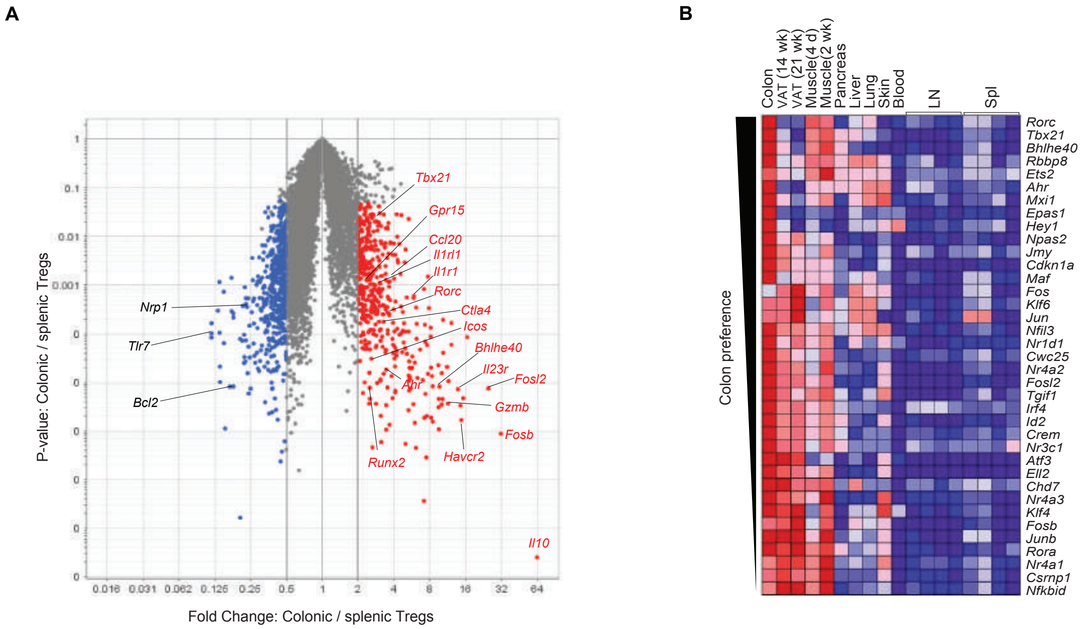


Figure 19: Comparison of gene expression profiles of Tregs from colon and spleen uncovered 933 differential transcripts.
A. Gene expression profiles from purified Treg cells of colon and spleen, sorted from Foxp3ires-gfp mice. X-axis FoldChange between colonic (mean of quadruplicate) and splenic (mean of triplicate) Tregs; Y-axis: corresponding p.values. Colonic signature is highlighted in red (induced) and blue (repressed) at a FoldChange>2 and p.value <0.05.
B. Transcription factors overrepresented in colonic Tregs vs other tissue and lymphoid organ Tregs.

GeneOntology analysis revealed no enriched function or pathway, except for a high proportion of TFs, including *Ahr, Epas1, Hey1, Bcl6, Npas2, Nr1d1,* and *Maf*. To our surprise, the most differential of these TFs proved to be *Rorc* (encodes Rorγ) (Fig. 19B). Rorγ controls many aspects of immunocyte differentiation (Cua and Tato, 2010) but is perhaps best known as the key regulator of interleukin-17 (IL-17)–producing CD4+ T cells (TH17), and as a reciprocal antagonist of FoxP3 during in vitro differentiation in which CD4+FoxP3+ Treg and TH17 represent alternative cell fates (reviewed in (Korn et al., 2009)).

Cytometry confirmed that many colonic CD4+FoxP3+ Tregs express Rorγ (40 to 60% in C57BL/6J or other inbred mouse strains) (Fig. 18B and Fig. 20A), a phenotype largely absent in spleen or lymph node (LN) and, among FoxP3+ cells, induced in vitro. Helios and Nrp1, described as markers of thymus-derived Tregs (reviewed in (Bilate and Lafaille, 2012)), were absent on colonic Rorγ+ Tregs (Fig. 18C); this absence demarcated three distinct subsets of colonic Tregs, with Rorγ+ representing the majority of Helios– cells (Fig. 18C and Fig. 20, B and C). Consistent with the RNA data, Rorγ+ Tregs were also detected in low proportions in the small intestine (SI) and regenerating muscle (Fig. 18D and Fig. 20D). In keeping with a recent report (Schiering et al., 2014), Rorγ+ Tregs were distinct from those expressing the IL-33 receptor, most of which were Helios+ (Fig. 18D and Fig. 20, B, C, E, and F), and from Gata3hi Tregs (Wohlfert et al., 2011), which also belong to the Helios+ Treg subset

We asked whether RORγ is also expressed by colonic Tregs in humans, by staining cells from healthy or inflamed (Crohn's) colon biopsies. Rorγ+ Tregs were indeed detected at comparable levels in both contexts (Fig. 18E).

Rare Tregs expressing IL-17 and Rorγ have been observed during chronic inflammation or cancer, usually being Helios^{hi} (reviewed in (Du et al., 2014)). We tested IL-17

Figure 20: Rorγ$^+$ Tregs express low levels of Nrp1, Helios and Il33R.

A: Representative Rorγ vs Helios plots (right) and frequencies (left) for colon FoxP3$^+$CD4$^+$TCRβ$^+$ Tregs in different inbred strains of mice (C57BL/6 and NOD from Jackson).
Each point is an individual mouse. Data representative of ≥ 3 independent experiments.

B: Representative Rorγ vs Helios, Il33R (ST2) or Gata3 plots for colon Foxp3$^+$CD4$^+$TCRβ$^+$ Tregs. Rorγ$^+$Helios$^-$ Tregs in red; Gata3$^+$ Tregs in blue.

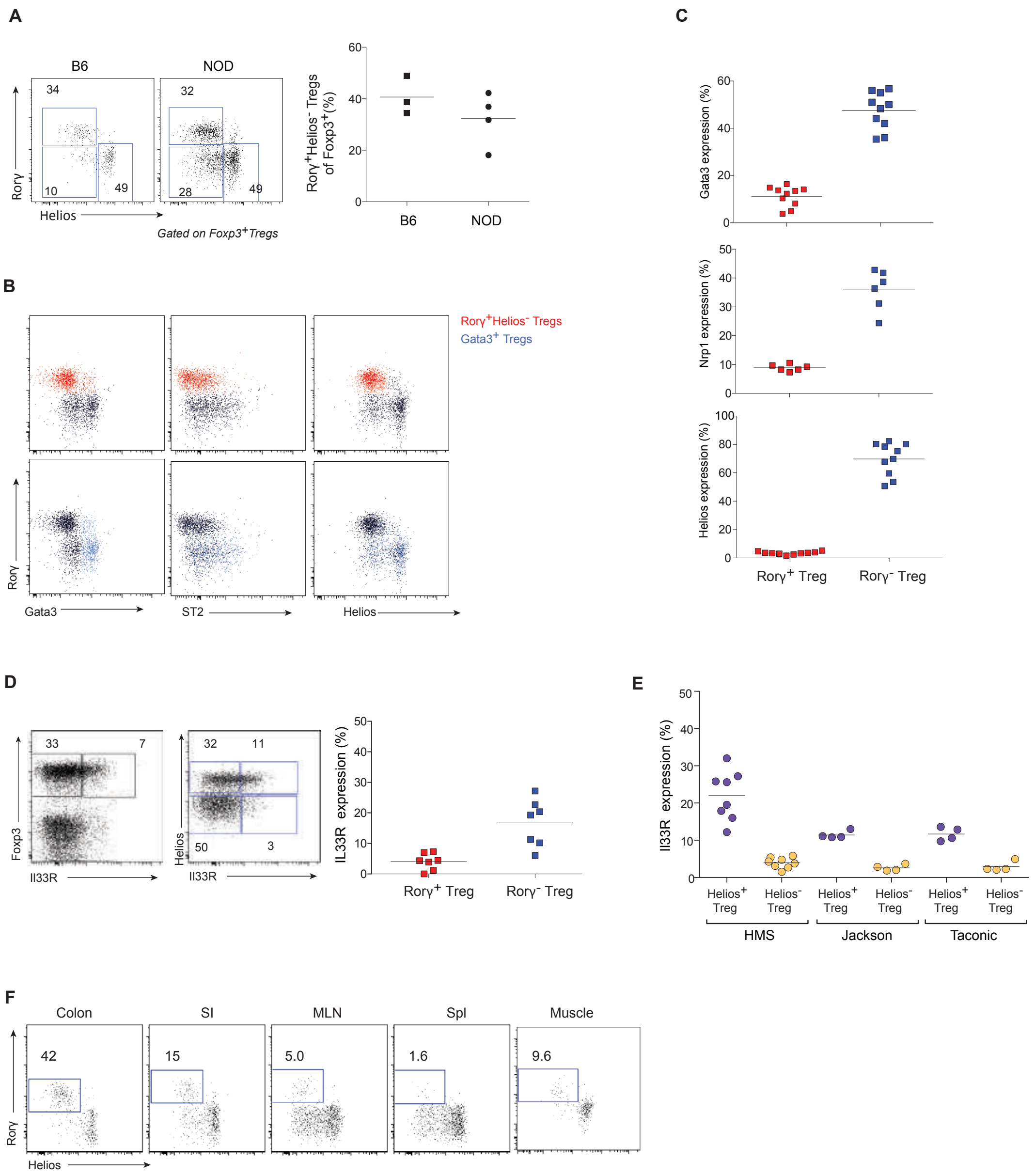
C: Colonic lymphocytes were stained with FoxP3, Helios, Nrp1, Gata3 and Rorγ. Frequencies of Gata3$^+$, Helios$^+$ or Nrp1$^+$ cells in Rorγ$^+$ or Rorγ$^-$ Tregs. (Quantification of flow cytometry plots shown in Fig. 2D). Each point is an individual mouse. Data representative of ≥ 3 independent experiments.

D: Representative Il33R vs FoxP3 plots for colon CD4$^+$TCRβ$^+$ cells (left) and Il33R vs Helios plots for colon FoxP3$^+$CD4$^+$TCRβ$^+$ Tregs (right). Frequencies of Il33R$^+$ Tregs among colonic Rorγ$^+$ or Rorγ$^-$ Tregs. Each point is an individual mouse. Data representative of ≥ 3 independent experiments.

E: Frequencies of Il33R$^+$Helios$^+$ or Il33R$^+$Helios$^-$ Tregs from several colonies (HMS, Taconic, Jackson). Each point is an individual mouse. Data representative of ≥ 3 independent experiments.

F: Representative Rorγ vs Helios plots of FoxP3$^+$CD4$^+$TCRβ$^+$ cells of Rorγ$^+$Helios$^-$ among Tregs of different tissues (SI: small intestinal lamina propria; PP: Peyer's patches; MLN: mesenteric lymph nodes; scLN: subcutaneous lymph nodes).Quantified in Fig.1D.

Figure 20 continued



production in colonic Rorγ+ Tregs. Although IL-17–expressing Tregs could be detected in the SI LP, colonic Rorγ+ Tregs did not secrete detectable IL-17a or f (Fig. 18F).

The properties of this dominant colonic Rorγ+Helios– Treg population suggested a link to the gut microbiota. Indeed, GF mice had a lower proportion of Rorγ+ Tregs than their conventionally raised specific pathogen–free (SPF) counterparts (Fig. 21A). During normal maturation in the mouse, Rorγ+ Tregs appeared between 15 and 25 days of age (Fig. 21B), coincident with the changes in the gut microbiota that accompany the transition to solid food. Note that Rorγ+ Tregs appeared a few days after Rorγ–Helios– Tregs. Antibiotic treatment strongly affected Rorγ+ Tregs (Fig. 21C), a large reduction followed a broad-spectrum antibiotic combination, whereas individual antibiotics had less or no effect, which suggested the contribution of several microbes. As the reported impacts of various microbial species on total colonic Tregs have differed ((Atarashi et al., 2011; Faith et al., 2014), we took advantage of a panel of mice generated in a large-scale screen in which GF mice were colonized with a single species from a panel of 22 bacterial species from the human gastrointestinal tract (Table S1). A number of microbes elicited colonic Rorγ+ Tregs, with a gradient of responses and, for some, at frequencies comparable with those of SPF mice (Fig. 21D). This restoration of Rorγ+ Tregs was independent of bacterial load and not accompanied by inflammation (Fig. 22). Bacteria able to induce Rorγ+ Treg (and colonic FoxP3+ Tregs more generally) belonged to several phyla and genera and were not restricted to *Clostridiae* (Atarashi et al., 2013; Atarashi et al., 2011). Segmented filamentous bacteria (SFB)—which are classic inducers of Rorγ-dependent TH17 cells (Ivanov et al., 2009) and which elicit IL-17–producing Tregs in the SI (Lochner et al., 2011)—were only mediocre inducers of colonic Rorγ+ Tregs, which reinforced the distinction between the cell populations. We noticed diversity within the *Bacteroides* genus and assessed a wider Bacteroides panel (Fig. 23A and Table S1). Here again, a range of colonic Rorγ+ Tregs was observed.

Figure 21

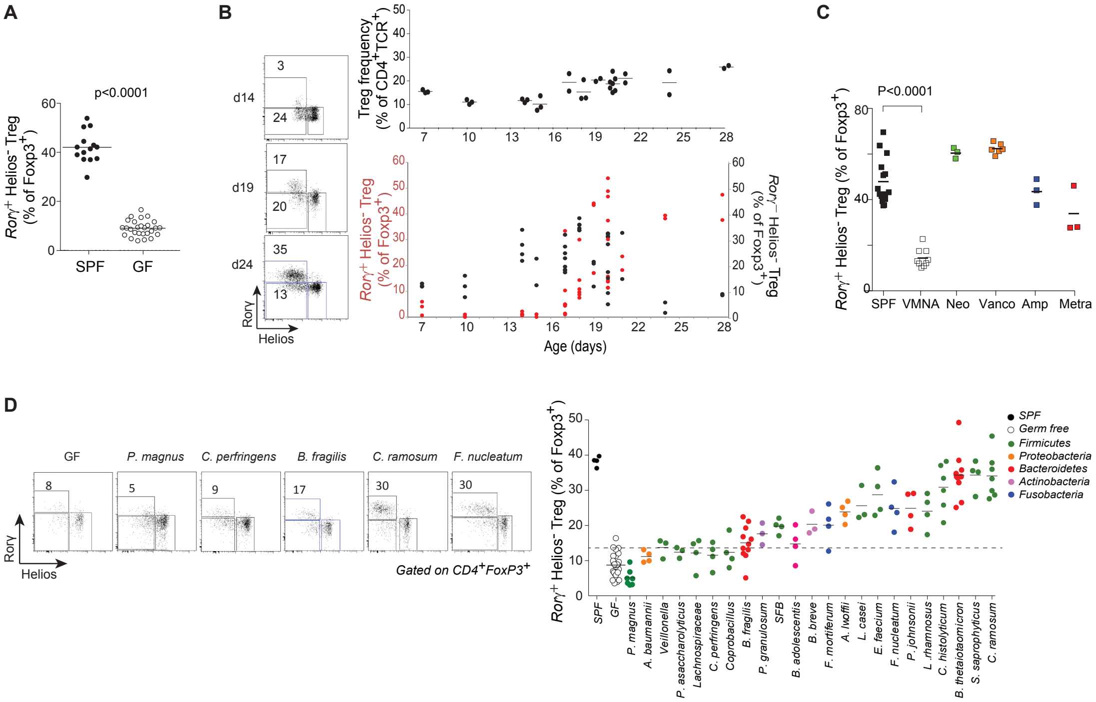


Figure 21: Rorγ^{+}Helios^{-} T$_{\text{regs}}$ can be induced by several bacterial species.
(**A**) Frequency of Rorγ^{+}Helios^{-} within colon FoxP3^{+}CD4^{+}TCRβ^{+} T$_{\text{regs}}$ of SPF and GF mice, $P < 0.0001$ as determined by Student's *t* test. Each point is an individual mouse. Data pooled from more than three experiments. (**B**) Induction of Rorγ in colonic T$_{\text{regs}}$ during postnatal development in SPF mice (left). Representative FACS plots (right); frequencies across ages of FoxP3^{+} T$_{\text{regs}}$ within CD4^{+}TCRβ^{+} cells, as well as Rorγ^{+}Helios^{-} (red) and Rorγ^{-}Helios^{-} (black) cells within T$_{\text{regs}}$. Each point is an individual mouse. Data pooled from four or more experiments. (**C**) SPF mice were treated with single antibiotics (abbreviations for neomycin, vancomycin, ampicillin, metronidazole) or all four (VMNA) antibiotics for 4 weeks. Frequency of colonic Rorγ^{+}Helios^{-} T$_{\text{regs}}$ within the FoxP3^{+}CD4^{+}TCRβ^{+} population. $P = 0.0004$, Bonferroni-corrected Student's *t* test. Each point is an individual mouse. Data pooled from two experiments. (**D**) GF mice were colonized with single bacterial species, and colonic T$_{\text{regs}}$ were analyzed after 2 weeks (top). Representative plots and frequencies of Rorγ^{+}Helios^{-} within FoxP3^{+}CD4^{+}TCRβ^{+} T$_{\text{regs}}$, color-coded per phyla (bottom). Each point is an individual mouse. Data are representative one to three experiments for each microbe. *Different from GF at an FDR of <0.05.

Figure 22

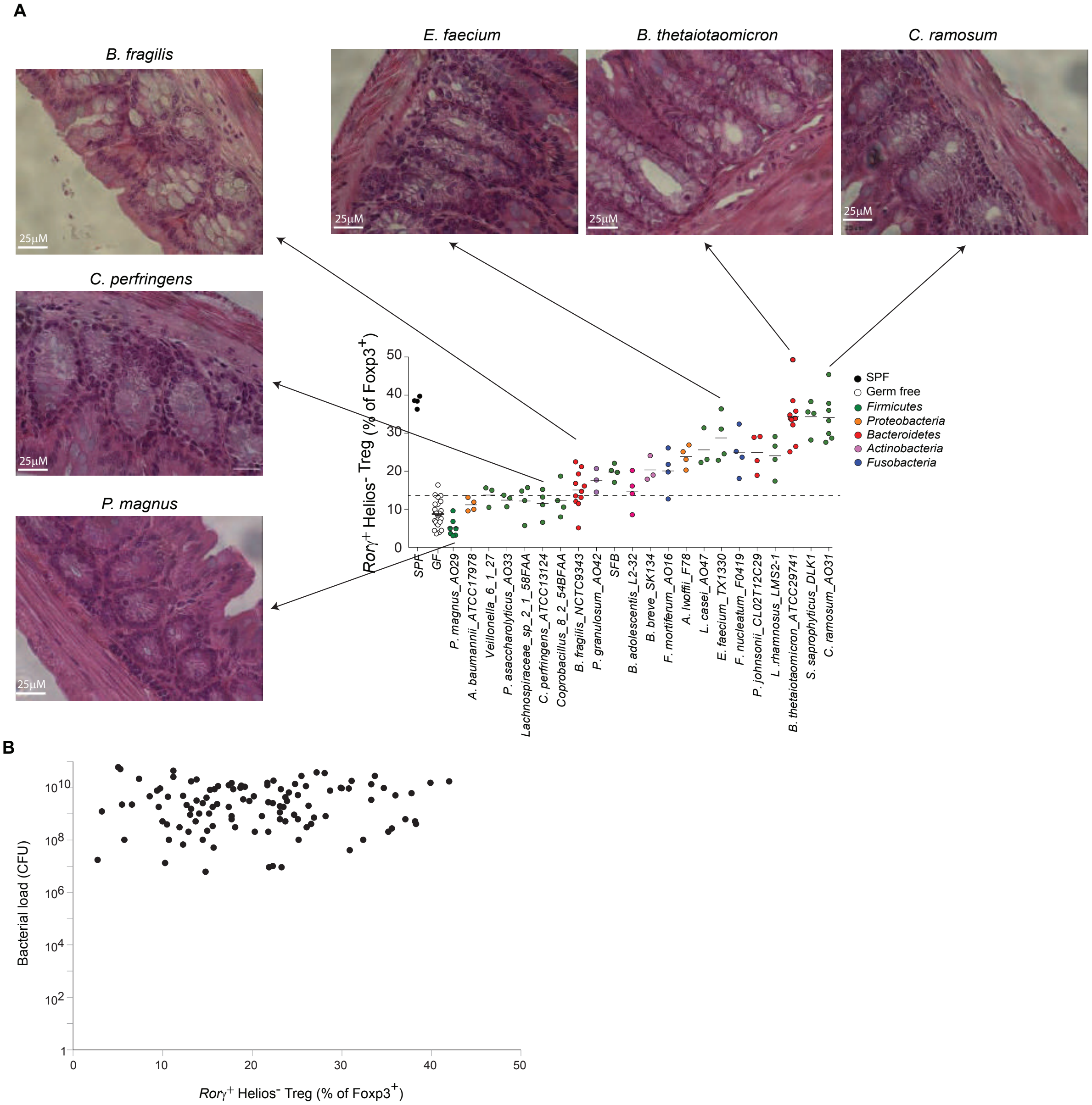


Fig. 22: Induction of Rorγ$^+$Helios$^-$ Tregs is not accompanied by inflammation and is independent of bacterial load.
A: Haematoxylin and eosin staining of the distal colon from mono-colonized mice.
B: Correlation between bacterial load (measured as CFU) and frequency of colonic Rorγ$^+$Helios$^-$ Tregs in mono-colonized mice. Pearson r=-0.046. Each point is an individual mouse. Data pooled from ≥ 3 independent experiments.

This distribution did not relate to the *Bacteroides* phylogeny for these strains with no unique correlation between Treg-inducing ability and gene content (Fig. 23B). Colonic Rorγ+ Tregs did not appear immediately after GF colonization but only after a few days, again after Rorγ–Helios– cells (Fig. 23C).

Several reports have suggested that short-chain fatty acids (SCFAs) promote increased colonic Tregs (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). To test their relevance to Rorγ+ Tregs, SCFAs were quantified by liquid chromatography–mass spectrometry (LC-MS) in cecal content of monocolonized mice. No significant correlation between any SCFA and Rorγ+ Treg frequency or to other Treg parameters, was observed (Fig. 24, A and B, and Table S2). In addition, we could not reproduce previously reported effects of oral or rectal SCFA administration (Fig. 24, C and D). Although SCFA combinatorial effects or inter-colony variation cannot be ruled out, SCFAs cannot alone explain the microbial impact on colonic Tregs observed here.

To integrate our observations with intercellular pathways that influence intestinal T cells, we measured the relative abundance of Rorγ+ Tregs in mice lacking receptors for key cytokines and alarmins. Signaling through IL-23, IL-1, or IL-33 receptors was not required to sustain Rorγ+ Tregs, nor was IL-10 (Fig. 25, A to D). In fact, only the Helios+ population expanded after IL-33 administration (Fig. 25E).

We then asked what transcripts Rorγ controls in Rorγ+ Tregs and whether Rorγ is necessary to specify this particular Treg lineage. We compared transcriptomes of Rorγ+ and Rorγ– colonic Tregs (sorted from *Foxp3Thy1.1* × *Rorcgfp* intercrossed mice). Rorγ+ cells were enriched in some, but not all, transcripts of the colonic Treg signature, notably *Il23r, Cxcr3, Tbx21,* and *Havcr2* (Fig. 26A), as validated at the protein level, including the unexpected CXCR3 (Fig. 26B). Conversely, *Il1rl1* (encodes IL-33R), *Nrp1*, and Ikzf2 were underrepresented in Rorγ+ Tregs.

Figure 23

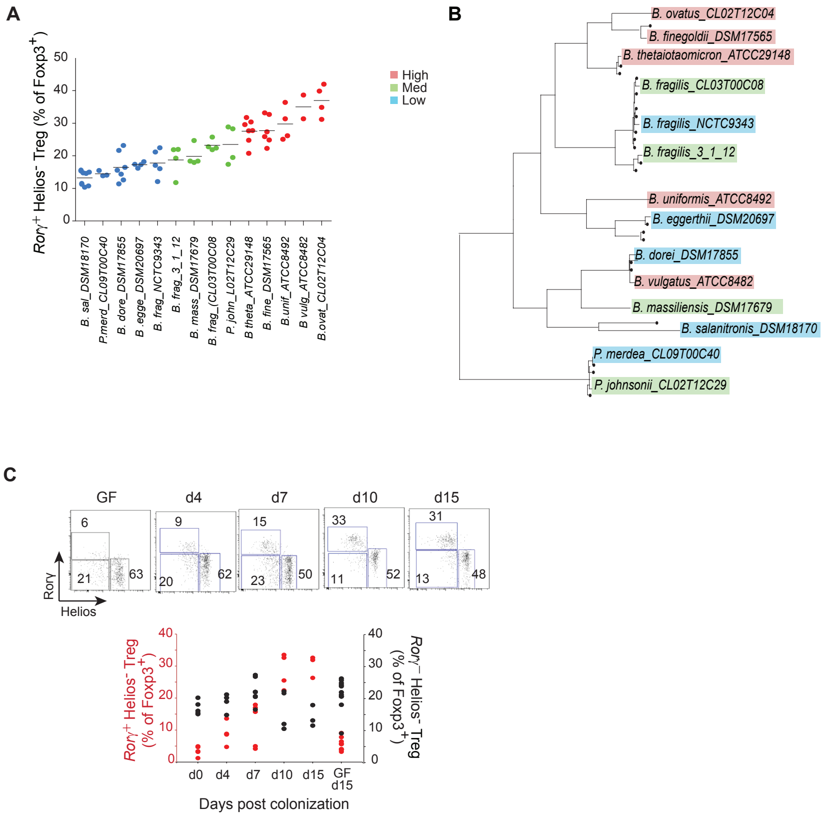


Figure 23: The distribution Rorγ$^+$Helios$^-$ Tregs induced by *Bacteroides* did not relate to the phylogeny for these strains.

A: GF mice were monocolonized with a set of species within the *Bacteroides* genus, and the frequency of Rorγ$^+$Helios$^-$ within colonic Tregs determined. Color-coded by their Treg-inductive ability. Each point is an individual mouse. Data representative of 1-3 independent experiments per microbe.

B: Dendrogram of genetic distances of the *Bacteroides* species analyzed in E, color-coded by their Treg-inductive ability. *See text for Bacteroides genomics by Abby Manson Mcguire.*

C: Representative plots and frequencies of Rorγ$^+$Helios$^-$ (red) and Rorγ$^-$Helios$^-$ (black) Tregs at different times after colonization with the inductive *B. thetaiotaomicron.* GF d15 mice are maintained as germ free for another 15 days, an important control needed to ensure no age-related differences. Each point is an individual mouse. Data pooled from 3 independent experiments.

Figure 24. SCFA do not induce Rorγ$^+$Helios$^-$ Tregs.
A: SCFA concentration in cecal material of monocolonized mice vs frequency of Rorγ$^+$Helios$^-$ Tregs; Each point is an individual mouse. Data pooled from ≥ 3 independent experiments.
p=0.06 (acetate), p=0.14 (butyrate), p=0.04 (propionate) as determined by Student's t test.
B: SCFA concentration in cecal material of monocolonized mice vs frequency of total, Helios$^-$ Helios$^+$Tregs. Each point is an individual mouse. Data pooled from > 3 independent experiments. Total Tregs: p=0.6 (acetate), p=0.9 (butyrate), p=0.15 (propionate); Helios$^-$ Tregs: p=0.45 (acetate), p=0.36 (butyrate), p=0.39 (propionate); Helios$^+$Tregs: p=0.43 (acetate), p=0.36(butyrate), p=0.39 (propionate) as determined by Student's t. test.
C: Frequencies of total, Rorγ$^+$Helios$^-$ and Helios$^-$ colonic Tregs of mice orally treated with SCFA, from three independent experiments. In first and second experiment (orange and green), mice treated with 36mM of individual or combined SCFA in drinking water containing antibiotics. In the third experiment (blue) mice were treated with 150mM acetate, 150mM propionate or 100mM butyrate in drinking water containing antibiotics. Each point is an individual mouse. Data pooled from 3 independent experiments.
D: Frequencies of total, Rorγ$^+$Helios$^-$ and Helios$^-$ colonic Tregs from mice treated intrarectally with butyric acid for 7 days. Each point is an individual mouse. Data representative of ≥ 2 independent experiments.

Figure 24 continued

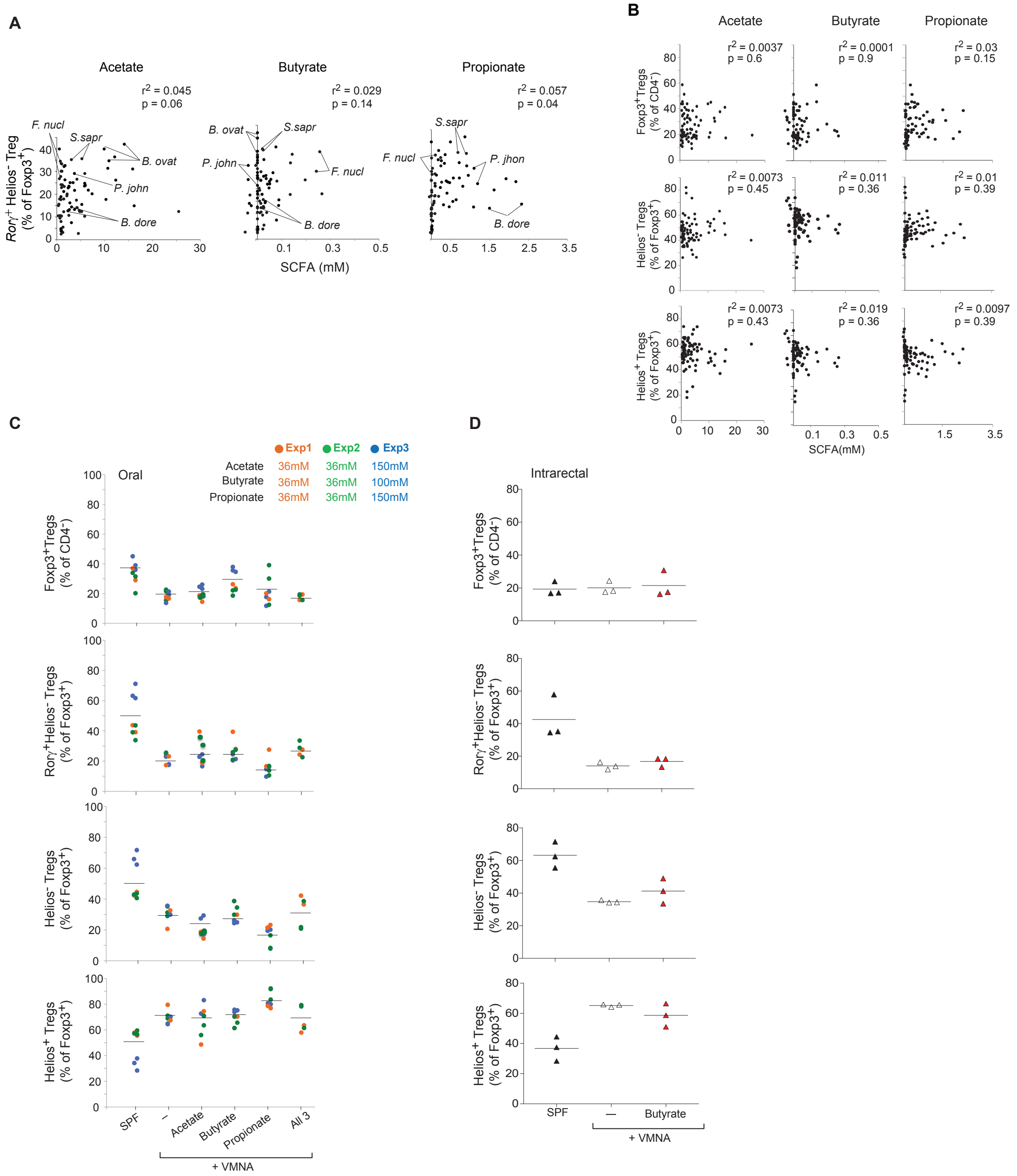


Figure 25

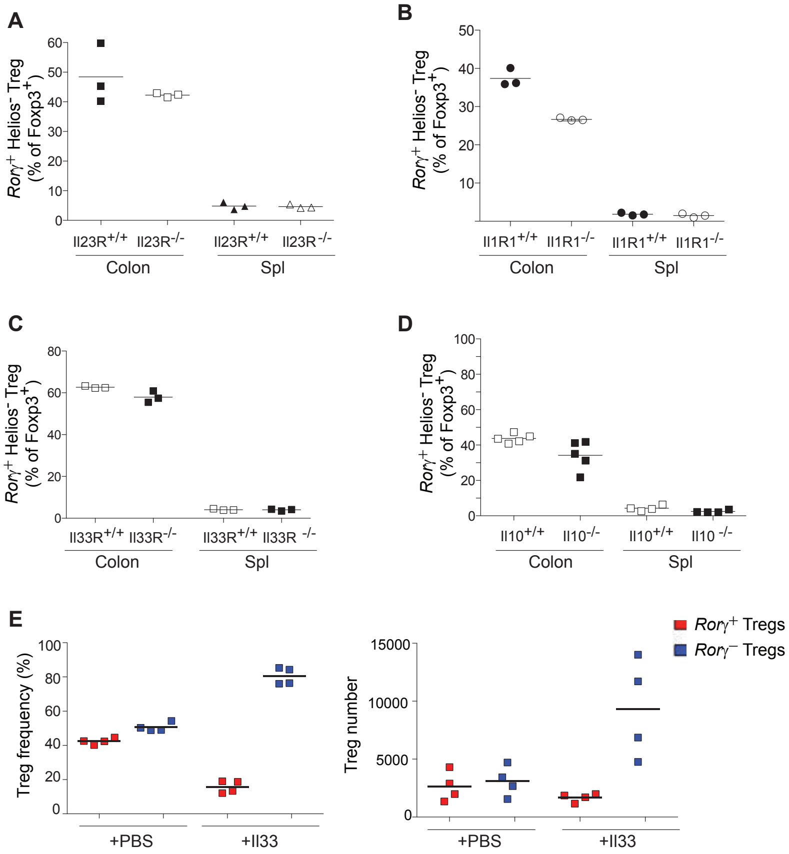


Figure 25: Signaling through Il23R, Il1R, Il33R and Il10 was dispensable for induction of Rorγ$^+$Helios$^-$ Tregs.

Frequency of Rorγ$^+$Helios$^-$ Tregs within the FoxP3$^+$CD4$^+$TCRβ$^+$ population in
A: *Il23R$^{-/-}$*; **B:** *Il1r1$^{-/-}$;* **C:** *Il1rl1$^{-/-}$*(*IL33R$^{-/-}$*); and **D:** *Il10$^{-/-}$* mice and controls. Each point is an individual mouse. Data representative of ≥ 2 independent experiments.
E: Mice were treated with mouse recombinant Il33 for 7 days. Frequencies (left) and numbers (right) of Rorγ$^+$ or Rorγ$^-$ Tregs within the FoxP3$^+$CD4$^+$TCRβ$^+$ population. Each point is an individual mouse. Data representative of ≥ 2 independent experiments.

To further delineate the transcriptional signature of Rorγ in Treg cells, RNA sequencing profiles were generated from Nrp1– cells of *Foxp3-cre.Rorcfl/fl* mice, which have a Treg-selective deletion of Rorc (Fig. 27A), or paired wild-type (WT) littermates. Differentially expressed genes were related to the Rorγ-dependent signature in conventional TH17 cells (defined from a comparison of SI CD4+ T cells of mice colonized, or not, with SFB) (Fig. 26C and Table S3). Part of the classic TH17 signature was unrelated to Rorγ in colonic Tregs (blue in Fig. 26C) or *Il1r1* or the canonical TH17 cytokines *Il17a/f* and *Il22*; some were shared (*Rorc* itself, *Il23r*); and a third segment was controlled by Rorγ in Nrp1– colonic Tregs but not in TH17 cells (*Havrc2*, *Irak3*, and *Il1rn*). Thus, the transcriptional footprint of Rorγ is context-dependent in different T cells.

Next, we explored whether Rorγ contributes to colonic Treg homeostasis. First, mice were treated for 3 weeks with a pharmacologic Rorγ antagonist (Skepner et al., 2014), which reduces SI TH17 levels. This treatment partially decreased both the total frequency of colonic FoxP3+ cells and their Rorγ+ component (Fig. 26D). Second, *Foxp3-cre.Rorcfl/fl mice*—which have no systemic Treg deficiency or *scurfy*-like pathology nor any change in FoxP3 intensity—showed a reduced frequency of colonic Tregs, and, more specifically, of Helios– Tregs; the proportion of Helios+Gata3+ Tregs was correspondingly increased (Fig. 26E and Fig. 27B).

We noted that the loss of Rorγ+ Tregs in *Foxp3-cre.Rorcfl/fl* mice led to increased production of Il17 and IFNγ, but not Th2 cytokines like IL5 or IL13, by Tconv cells in colons of otherwise unchallenged mice (Fig. 28A), suggesting a decreased ability of colonic Tregs lacking Rorγ to regulate inflammatory responses. We thus assessed *Foxp3-cre.Rorcfl/fl* mice in the trinitrobenzenesulfonic acid (TNBS)–induced colitis model and found an exacerbation of disease severity, in colitis score and histopathology (Fig. 28, B and C). Second, after TNBS challenge of GF mice monocolonized with different microbes, the frequency of Rorγ+ Tregs correlated with the colitis score (Fig.

Figure 26

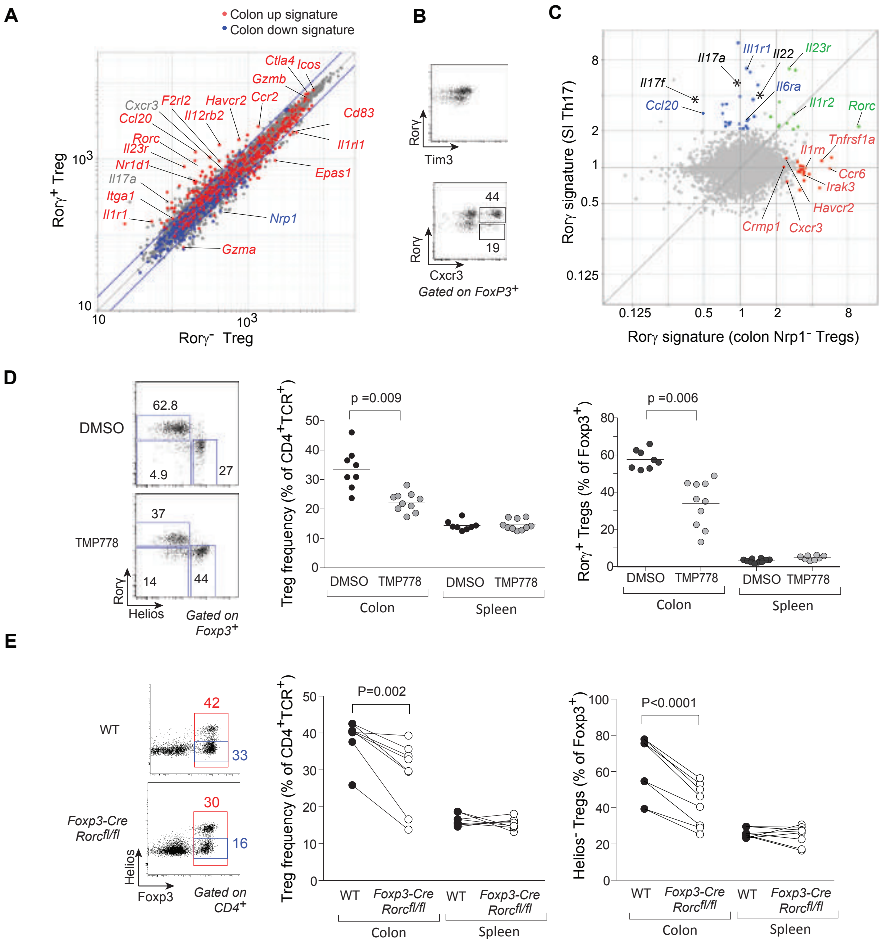


Figure 26: Rorγ determines a specific signature and function in colonic T_{regs}.

(**A**) Rorγ$^+$ or Rorγ$^-$ T_{regs} were sorted from the colon of *Foxp3*$^{thy1.1}$x *Rorc*^{gfp} intercross male mice, and gene expression profiles were determined. Expression values (triplicate averaged) are compared and highlighted according to the colon T_{reg} signature of fig. S1. (**B**) Flow cytometric validation of some of the Rorγ$^+$/Rorγ$^-$ T_{regs} differential genes (*Havcr2,* which encodes Tim3, and *Cxcr3*) that represent two experiments. (**C**) Comparison by gene expression profiling of the Rorγ signature in different contexts (all mean of triplicates). Fold change between colonic Nrp1$^-$ T_{regs} from WT or *Foxp3-cre* × *Rorc*$^{fl/fl}$ mice is shown on the *x* axis; fold change between SI CD4$^+$ T cells sorted from GF mice monocolonized with T_H17-inducing SFB or from unmanipulated GF is shown on the *y* axis. Shared or specific signature genes are color-coded. (**D**) SPF mice were treated with Rorγ antagonist TMP778 or control dimethyl sulfoxide (DMSO) for 3 weeks. Representative cytometry plots of colonic T_{regs} (left) or compiled frequencies of FoxP3$^+$ T_{regs} (middle) and of Rorγ$^+$Helios$^-$ (right) within FoxP3$^+$CD4$^+$TCRβ$^+$ T_{regs} (right); *P* = 0.009 as determined by Student's *t* test. Each point is an individual mouse. Data are representative of two or more independent experiments. (**E**) Analysis of Rorγ–deficient T_{regs} from *Foxp3-cre* × *Rorc*$^{fl/fl}$ mice or control (*Foxp3-creRorc*$^{+/+}$*)* littermates. Cytometry plots of colonic T_{regs} (left) or compiled frequencies of FoxP3$^+$ T_{regs} (middle) and of Rorγ$^+$Helios$^-$ (right) within FoxP3$^+$CD4$^+$TCRβ$^+$ T_{regs} (right); *P* values were determined by paired Student's *t* test. Each point is an individual mouse. Data are representative of more than three independent experiments.

Figure 27

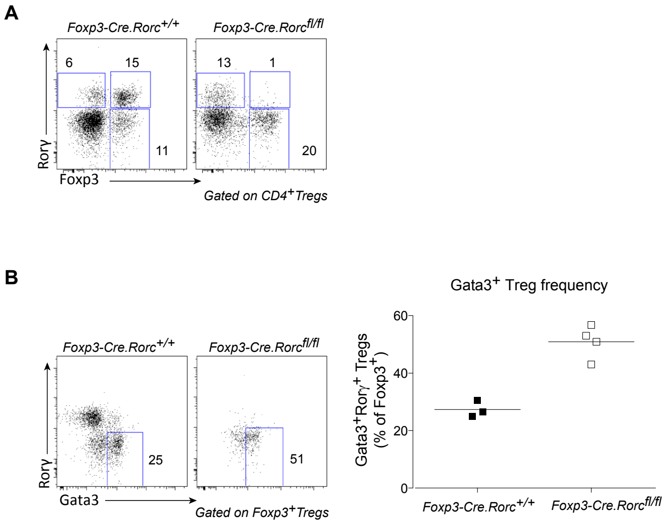


Figure 27. Rorγ is deleted specifically in Tregs of Foxp3-cre.Rorcfl/fl mice.
A: Representative Foxp3 and Rorγ plots for colon $CD4^+TCR\beta^+$ cells from Foxp3-cre.Rorcfl/fl and Foxp3-cre.Rorc+/+ littermates.
B: Representative Gata3 and Rorγ plots and frequencies of $Ror\gamma^-Gata3^+$ Tregs among $FoxP3^+CD4^+TCR\beta^+$ Tregs from Foxp3-cre.Rorcfl/fl and Foxp3-cre.Rorc+/+ littermates. Each point is an individual mouse. Data representative of ≥ 2 independent experiments.

Figure 28

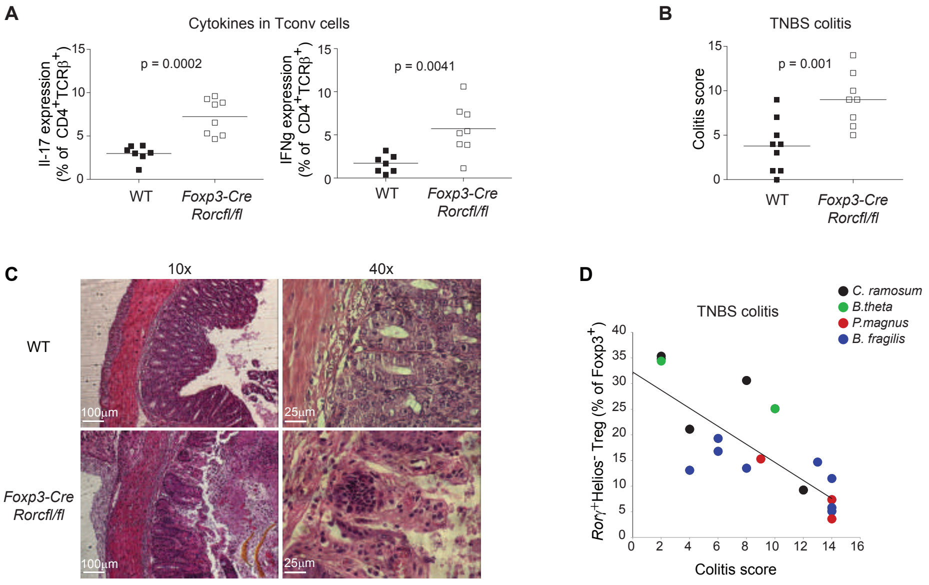


Figure 28: Rorγ$^+$ T$_{regs}$ control gut inflammation. (**A**) Frequency of IL-17a and IFN-γ expression in Foxp3$^-$ CD4$^+$ T$_{conv}$ cells from *Foxp3-cre* × *Rorc*$^{fl/fl}$ mice and control *Foxp3-cre* × *Rorc*$^{+/+}$ littermates at steady state; *P* values determined by paired Student's *t* test. Each point is an individual mouse. Data are representative of three or more independent experiments. (**B** and **C**) Colitis score (B) and histology (C) of *Foxp3-cre* × *Rorc*$^{fl/fl}$ mice and control *Foxp3-cre* × *Rorc*$^{+/+}$ littermates challenged with TNBS, calculated on the basis of weight loss, histologic score, and other physical parameters; *P* value as determined by paired Student's *t* test. Each point is an individual mouse. Data representative of more than three independent experiments. (**D**) Correlation between TNBS-colitis score (*x* axis) with frequency of Rorγ$^+$Helios$^-$ within colonic T$_{regs}$ in GF mice monocolonized for 2 weeks with bacteria that elicit different levels of Rorγ$^+$Helios$^-$ T$_{regs}$ before TNBS colitis induction. *B. theta*, *B. thetaiotaomicron*. Pearson's correlation coefficient $r = 0.82$, $P < 0.0001$. Each point is an individual mouse. Data pooled from four experiments.

28D). These results imply a non-redundant role for Rorγ and Rorγ+ Tregs in colonic homeostasis.

Thus, Rorγ contributes unexpectedly but in an important way to the Treg response to commensal microbes. This role contrasts with the accepted dichotomy between FoxP3 and Rorγ, a notion stemming mainly from their antagonism in vitro (Bettelli et al., 2006; Zhou et al., 2008; Bettelli et al., 2006; Yang et al., 2008; Mucida et al., 2007),); perhaps this relation has been overinterpreted. There had been indications that the two TFs are not incompatible (Du et al., 2014), but these data suggest a collaborative transcriptional impact, consistent with the overlap between their chromatin-binding sites (Xiao et al., 2014). The context-specificity of Rorγ's transcriptional footprint is in line with its broad involvement in many immunological and non-immunological processes (organogenesis, circadian rhythm, and lipid metabolism) (Cua and Tato, 2010; Jetten, 2009). Rorγ-dependent *Il23r* expression in Tregs also raises the intriguing speculation that human IL23R genetic variants associated with inflammatory bowel disease (Abraham and Cho, 2009) might involve balancing effects in effector and regulatory T cells.

Rorγ+ Tregs form the majority of the Helios– Tregs that differentiate locally in response to antigens of commensal microbes in the gut (Lathrop et al., 2011) and do not respond to the alarmin IL-33, in contrast to Gata3+Helios+ cells that expand during tissue damage (Schiering et al., 2014; Wohlfert et al., 2011). Mutually exclusive expression of Gata3 and Rorγ in colonic Tregs suggests that they may distinguish Treg responses to symbiotic (Rorγ) versus aggressive (Gata3) microbes. Contrary to expectations, many individual *microbes* proved able to elicit Rorγ+ and Helios– Tregs, a property not restricted to *Clostridiae* (Atarashi et al., 2011). The graded range suggests that several mechanisms may be involved. The molecular mediator of Rorγ+ Treg induction remains elusive but is unlikely to be SCFAs alone. Rorγ+ induction must follow different routes inTH17 versus colonic Tregs, because the best Rorγ+ Treg inducers do not affect SI TH17 and vice-versa

In conclusion, these studies show Rorγ as a uniquely microbe-responsive factor induced in two different cellular contexts, in response to different microbes, with distinct transcriptional consequences, and with diametrically opposite functional outcomes

ACKNOWLEDGMENTS

We thank A. Onderdonk, C. Dong, A. Rudensky, R. Lee, V. Kuchroo, and L. Bry for microbial and mouse strains and S. Edwards, A. T. Sherpa, K. Hattori, K. Rothamel, and R. Cruse for help with mice or profiling. TMP778 is available to academic investigators from G.S.K. under a material transfer agreement. The data are tabulated in supplementary materials and deposited at the National Center for Biotechnology Information, NIH, Gene Expression Omnibus (GSE68009). E.S., N.G.Z., D.K., D.M., C.B., and H.M.S. have filed a provisional patent application related to work presented in this paper. This work was supported by NIH R01-AI51530 and R56-AI110630 and the J.P.B. Foundation (D.M. and C/B); a Sponsored Research Agreement from UCB Pharma (D.M., C.B., D.K., and A.E.); the Helmsley Charitable Trust and the Wolpow Family Chair in the Center for Inflammatory Bowel Disease Treatment and Research (S.P.S.). E.S. and D.Z. were supported by fellowships from the Boehringer Ingelheim Fonds, N.G.Z. by the Human Frontier Science Program and European Molecular Biology Organization (ALTF 251-2011) fellowships and the Weizmann - National Postdoctoral Award for Advancing Women in Science.

3.2: Bacteroides genomics by Abby Manson Mcguire

Here, we attempted to identify specific elements in the genomes of the *Bacteroides* genus, which would correlate with the ability to induce colonic Rorγ+ Treg cells after colonization of GF mice.

We first constructed a table of genetic orthogroups present in the genomes of the *Bacteroides* species analyzed. Orthogroups are sets of genes predicted to be descended from a single common ancestral gene, containing both paralogs and orthologs, For better resolution, in addition to the genomic sequences for the 14 phenotyped Bacteroides and *Parabacteroides* strains, we included in the comparative analysis genomes from 78 Bacteroides and *Parabacteroides* strains with genomic sequences available in GenBank. These 92 strains cover the breadth of these two genera, representing at least 28 species (23 Bacteroides and 5 *Parabacteroides*), as well as 22 unclassified Bacteroides strains and 4 unclassified *Parabacteroides* strains.

To assure consistency and to reduce artifacts among the genomes analyzed, all genomes were re-annotated in a uniform manner. Protein-coding genes were predicted with Prodigal (Hyatt et al., 2010) and filtered to remove genes with ≥70% overlap to tRNAs or rRNAs. The tRNAs were identified by tRNAscan-SE (Lowe et al., 1997). The rRNA genes were predicted using RNAmmer (Lagesen et al., 2007). The gene product names were assigned based on top blast hits against SwissProt protein database (≥70% identity and ≥70% query coverage), and protein family profile search against the TIGRfam hmmer equivalogs.

Additional annotation analyses performed included PFAM (Finn et al., 2008), TIGRfam (Haft et al., 2001), KEGG (Ogata et al., 1999), COG (Tatusov et al., 1997), GO (Conesa et al., 2005), EC (Tian2004), SignalP (Petersen et al., 2011), and TMHMM (Krogh et al., 2001). Finally, SYNERGY2 (Wapinski et al., 2007, Wapinski et al., 2011), available at http://sourceforge.net/projects/synergytwo/, was used to identify orthogroups across our set of 92 genomes. Phylogenetic trees were generated by applying RAxML (Stamatakis et al., 2006) to a concatenated alignment of 253 single-copy core orthogroups (excluding orthogroups with paralogs) across all 92 organisms. Bootstrapping was performed using RAxML's rapid bootstrapping algorithm.

We first attempted a categorical approach to the identification of orthogroups related to Treg phenotypes. Of the 14 phenotyped strains, we selected the five with mean value for Rory+Helios- (% of FoxP3+) greater than 25 as having "high" values (*B. ovatus CL02T12C04, B. vulgatus ATCC 8482, B. uniformis ATCC 8492, B. finegoldii DSM 17565,* and *B. thetaiotaomicron ATCC 29148*). We selected the five with mean Rory+Helios- (% of FoxP3+) values lower than 18 as having "low" values (*B. fragilis NCTC 9343, B. eggerthii DSM 20697, B. dorei DSM 17855, P. merdae CL09T00C40, B. salanitronis DSM 18170*). We searched for orthogroups found in all members of the "high T-reg" or "low T-reg" subset, but present in no members of the opposite subset Three orthogroups met this criterion (encoding two hypothetical proteins and one glycoside hydrolase). However, a permutation test (randomly assigning the 14 phenotyped strains, into 5 "high-Treg" and 5 "low-Treg" groups, 1000 permutations) resulted in 3.5 orthogroups on average, indicating that the three orthogroups observed in the real data are not significantly different from the noise in this analysis.

We also tested a linear fit between all Rory+Helios- Treg values to orthogroup distribution. Here again, the distribution of coefficients and p.values observed with the real data was not significantly different from that generated with random permutations of the phenotypes.

We concluded that there was no correlation between the phylogenetic distribution of 14 phenotypes Bacteroides strains and their ability to drive Rory+Helios- Tregs. We could not identify any sets of bacterial genes that could explain Treg inductive ability.

3.3: Discussion

We observed a gradient of responses in bacteria's ability to induce colonic Tregs. This was a particularly interesting observation that challenged some of the early work on colonic Tregs (Geuking et al., 2011; Atarashi et al., 2011; Atarashi et al., 2013). When focused on this phenotype we discovered that bacteria also shape the phenotype and the function of colonic Tregs in a specific somewhat controversial manner. Microbe-dependent expression of RORγ, a transcription factor known best for its role in inflammatory TH17 cells, potentiates Treg function in colonic Tregs. Mice with a Treg-specific deletion of *Rorγ* or gnotobiotic mice that have few RORγ+ Tregs had more severe TNBS-induced colitis.

In polyp-prone mice or human colon cancer, FoxP3+Rorγ+Tregs, which resemble colonic Rorγ+ Tregs, but have relatively low anti-inflammatory activity, have been described (Blatner et al., 2012). The gut microbiota may contribute to carcinogenesis by inducing different Tregs through different pathways.

The induction mechanism of Rorγ is still not known in the context of colonic Tregs. The molecule(s) that induces Rorγ may be microbe-derived or host-derived (microbe-induced). Although we could not rule out the combinatorial effects of SCFA, butyrate, acetate and propionate did not induce Rorγ expression in colonic Tregs.

Primary bile acids are synthesized in the liver and converted to secondary bile acids by bacteria in the colon. Anti-inflammatory roles of bile acids have been described in DCs where treatment with bile acids lowers production of inflammatory cytokines, such as IL12 (Ichikawa et al., 2012). Treatment of mice with cholestyramine, a sequestrant that blocks secondary bile acid conversion, had no effect on Rorγ expression in colonic Tregs.

A variety of sterol lipids (oxysterols, chosterol biosynthetis intermediates) have been described to induce Rorγ in the context of TH17 cells (Santori et al., 2015; Soroosh et al., 2014; Wang et al., 2010). The effect of these agonists has not been tested in colonic Tregs, but opens up an important discussion for agonists and antagonists. These ligands may be used to control Rorγ- dependent responses in different contexts. Based on bacteria's abilities to induce different levels of TH17 cells and Rorγ + Tregs, one may speculate that the induction of Rorγ follows different routes in TH17 cells and Tregs. The specificity of microbe- derived Rorγ ligands in the context of TH17 cells, colonic Rorγ+ Tregs and other Rorγ+ Tregs is not known, but may serve useful in choosing therapeutic targets.

Chapter 4: Materials and Methods

The experimental procedures described here cover the materials and methods for all chapters.

4.1: Mice

C57BL/6, NOD, B6.129P2(Cg)-*Rorc*$^{tm2Litt/J}$ (*Rorc*^{gfp}, (Eberl et al., 2004)), B6.129S7-*Il1r1*$^{tm1Imx/J}$ *(Il1R1*$^{-/-}$*,* (Glaccum et al., 1997)) and *NOD.Il10*$^{-/-}$ (Gonzalez et al., 2001) mice were obtained from Jackson. *Il17f*^{rfp} (Yang et al., 2008) mice were obtained from C. Dong, *Il1rl1*$^{-/-}$ (Townsend et al., 2000) from R. Lee, *Foxp3*$^{thy1.1}$ (Liston et al., 2008) and *Foxp3*$^{cre-yfp}$ (Rubtsov et al., 2008) from A. Rudensky, *CD4-Cre.Rorc*$^{fl/fl}$ (Eberl et al., 2004; Sawada et al., 1994), *IL23r*$^{-/-}$ (Awasthi et al., 2009) and *Foxp3*$^{ires-gfp}$ *(Bettelli et al., 2006)* mice from V. Kuchroo, and bred in HMS specific-pathogen-free facilities.

A germfree C57BL/6J breeding nucleus was obtained from L. Bry, and maintained in germ-free isolators. All experimentation was performed following animal protocol guidelines of HMS and GSK (reviewed and approved HMS IACUC protocols 02954 and 8604781-R98).

Four week-old mice were injected sc with 20mg/kg of TMP778 (Tempero Pharmaceuticals, Inc) in DMSO twice a day for 21 days. Recombinant mouse Il33 (BioLegend) was administered i.p. (2µg/ injection) every other day for 7 days. Mice were treated with 0.5mg/ml vancomycin (Acros Organics), 1mg/ml metronidazole (Sigma-Aldrich), 1mg/ml neomycin (Fisher Scientific), 1mg/ml ampicillin (Sigma-Aldrich) (VMNA) dissolved in drinking water.

For SCFA treatment, mice were pretreated with VMNA for 2 weeks, and butyrate, acetate and propionate (Sigma-Aldrich) were then added to drinking water containing antibiotics at a final concentration of 36mM (per (Arpaia et al., 2013)) or at 100mM butyrate, 150mM propionate, 150mM acetate (per (Smith et al., 2013)) for 2-3 weeks.

For intrarectal administration, anesthetized mice were injected intrarectally with 200mL of 50mM butyric acid (Sigma-Aldrich) or pH-matched water (pH=4) once a day for 7 days.

4.2: Bacteria

Bacteria were obtained from the ATCC, BEI or DSMZ, or from laboratory collections (Kasper, Onderdonk), and grown in media listed in Table S1. Anaerobic bacteria were cultured under strictly anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37^oC in an anaerobic chamber.

4.3: Generation of mono-colonized mice

Germ free C57BL/6 mice were inoculated with single bacterial species at 4wk of age, then housed in gnobiotic isolators for 2 weeks. Fecal material was collected and plated at 1wk and at 2wk to verify colonization by a single bacterial species.

4.4: SCFA quantitation

Cecal contents of monocolonized mice were resuspended in 75% acetonitrile containing deuterated internal standards (d3-acetate, d5-propionate and d7-butyrate). Samples were sonicated for 10 minutes, vortexed and centrifuged at 8000xg for 5 minutes. Supernatant was collected, treated with activated charcoal and re-centrifuged to remove nonpolar lipids. Supernatant was collected again and dried under nitrogen stream, then resuspended in 95% acetonitrile for injection. An HP-HILIC-MS/MS system, equipped with Agilent HP1100 HPLC system (quad pump with autosampler), Waters BEH amide HILIC column (2.1 mm x 100 mm x 2.5 μm) and LTQ XL (Thermo scientific) tandem mass spectrometer was used for SCFA quantitation. A linear gradient of acetonitrile:water=95:5 to 60:40 with 2mM ammonium formate at pH 9.0 was applied for separation of SCFAs. Formate adduct ($(M+HCOO^-)^-$) of each SCFA was quantitated in negative ion mode and peak areas of SCFAs calculated for each sample (Thermo Scientific Xcalibur 2.0), and normalized relative to the recovery of each paired internal standard.

4.5: Preparation of mouse lymphocytes and flow cytometry

Intestinal tissues were treated with RPMI containing 1 mM DTT, 20 mM EDTA and 2% FBS at 37^{o}C for 15 min to remove epithelial cells, minced and dissociated in collagenase solution (1.5mg/ml collagenase II (Gibco), 0.5mg/ml dispase and 1%FBS in RPMI) with constantly stirring at 37^{o}C for 45min. Single cell suspensions were then filtered and washed with 4% RPMI solution. The Peyer's Patches were treated in a similar fashion except for the first step of removal of epithelial cells. Lymph nodes and spleens were mechanically disrupted. Single-cell suspensions were stained with antibodies against CD4, CD8, TCR-β, CD45, Il17A, IFNγ, Helios (Biolegend), Rorγ, FoxP3, Il33R/ST2 (eBioscience), Nrp1 (R&D Systems), anti-Il33R/ST2 conjugated to biotin (mdBioproducts).

For cytokine analysis, cells were treated with RMPI containing 10% FBS, 10ng/ml phorbol 12-myristate 13-acetate (Sigma), 1μM Ionomycin (Sigma) in presence of GolgiStop (BD Biosciences) for 3.5 hours. For intracellular staining of cytokines and transcription factors, cells were stained for surface markers and fixed in eBioscience Fix/Perm buffer overnight, followed by permeabilization in eBioscience permeabilization buffer for 45 min in the presence of antibodies. Cells were acquired with a BD LSRII and analysis was performed with FlowJo (Tree Star) software.

4.6: Preparation of human lamina propria lymphocytes and flow cytometry:

All human studies were approved by the Boston Children's Hospital Institutional review Board (IRB-P00000529, "Pediatric Inflammatory Bowel Disease Biospecimen

Repository). Biopsies from a combination of ascending, descending and transverse colon (at least 2 from each site and 6-10 in total per patient) were obtained. Healthy tissue was obtained from individuals being tested for chronic constipation or irritable bowel syndrome, with no gross inflammation observed by endoscopy. Crohn's tissue was obtained from areas with gross colonic inflammation, as determined by the endoscopist. Biopsies for the same patient were pooled together. The epithelial layer was stripped with agitation in 10mM EDTA (CA^-/Mg^- HBSS) at 37^oC for 40 min, remaining tissue digested with 200 Units/ml of Collagenase VIII (25% FBS, 2mM CaCl HBSS) for 60-90 min with agitation at 37^oC.

Single-cell suspensions were stained with antibodies against CD4, CD8, CD3, CD45, CD4RA, RORγ and FOXP3 (eBioscience). For intracellular staining of transcription factors, cells were stained for surface markers and fixed in eBioscience Fix/Perm buffer overnight, followed by permeabilization in eBioscience permeabilization buffer for 45 minutes in the presence of antibodies.

4.7: TNBS colitis

Mice were sensitized via administration of 1% TNBS (Sigma, in 4:1 acetone: olive oil solution) on shaven skin between the shoulders. A week later, colitis was induced by intrarectal administration of 150-200µg TNBS per gram of mouse in 50% ethanol into anaesthetized mice via a thin round-tip needle. The TNBS concentration was optimized for each batch of TNBS. For TNBS induction in gnobiotic mice, mice were colonized with single bacterial species for 2 weeks prior to TNBS administration. The tip of the needle was inserted 4 cm proximal to the anal verge, and mice were held in a vertical position for at least 1 min after the injection. Care was taken to ensure stool was not ejected during the process, as this would cause loss of material. Mice were observed and weighed daily and were sacrificed on day 4 after intrarectal TNBS administration at the peak of the disease.

A combined colitis score was calculated based on weight loss, histology, diameter of the colon, and the appearance of the stool. Weight loss was scored as follows: 0, 0-4% weight loss or weight gain; 1, 4-10% weight loss; 2, 10-15% weight loss; 3, 15-20% weight loss; 4, more than 20% weight loss. The appearance of the stool was assessed as follows: 0, hard; 1, softened stool; 2, diarrhea; 3, bloody diarrhea. The thickness of the colon was assessed based on differential in diameter (Δdiameter=diameter of TNBS treated colon- diameter of untreated colon): 0, no change; 1, Δdiameter< 1mm; 2, Δdiameter= 1-2mm; 3, Δdiameter= 2-3mm; 4, Δdiameter> 3mm. For histology, colons were fixed with Bouin's fixative, sectioned, and stained with haematoxylin and eosin. The degree of inflammation in the distal part of colon was graded from 0 to 4 as follows: 0, normal intact structure; 1, mild inflammation with intact structure; 2, infiltration of leukocytes and some damage to structure; 3, severe inflammation accompanied by complete loss of structure; 4, necrosis of the tissue.

4.8: Gene expression profiling

For microarray analysis, 15,000 to 30,000 cells were double-sorted into Trizol (Invitrogen) using a MoFlo sorter, and RNA prepared (pooling from different mice). For whole tissue colon analysis, 5 mm long piece was cut from the distal colon (3 cm away from rectum) of monocolonized, germ free or SPF mice. The tissue was collected in TRIZOL and homogenized.

Profiling was performed on Affymetrix Mouse Genome M1.0 ST arrays as previously described (Cipolletta et al., 2012), in biological triplicate (duplicate in rare instances). Datasets from other tissue Tregs have been described (Feuerer et al., 2009; Cipolletta et al., 2012; Burzyn et al., 2013b)

For low-input RNAseq, 1,000 cells were double-sorted into Trizol, RNA extracted and reverse-transcribed using ArrayScript (Ambion) using a specific primer containing T7 promoter, the 5' TruSeq Illumina adapter, a 8-positions with random nucleotide assignment as a unique molecular identifier (UMI), and a oligodT sequence. Second-strand synthesis was performed using the mRNA Second strand synthesis module (NEBNext #E6111L). After cDNA size selection using AMPure XP beads (0.8x and 1x, BeckmanCoulter- A63987), the product was amplified via *in vitro* transcription (MEGAshortscript, Invitrogen) for 10 hours and then fragmented (Magnesium RNA Fragmentation Module, New England Biolabs). 3' indexing adaptor was ligated (truncated T4 RNA ligase 2 -Enzymatics), reverse-transcribed (Superscript II, Invitrogen), and amplified by PCR for 15 cycles (HiFi hotstart PCR kit, Kapa). cDNA cleanup and size selection were performed on AMPure XP beads. Libraries were quantitated by BioAnalyzer using the Agilent High Sensitivity DNA Kit (Agilent 5067-4626) and qPCR using Kapa library quantification kits, and sequenced on a MiSeq (nano kit) and HiSeq 2500 (rapid mode).

Raw sequencing reads were processed using custom scripts. Read 1 contains the transcript sequence, Read 2 the UMIs. Raw reads were first trimmed using the FASTX-Tollkit v0.0.13 (fastx_trimmer –Q 33). Read 2 was trimmed in order to extract the UMI (5-12bp), and Read 1 was trimmed to 30bp eliminate a potential oligo-dT sequence. Reads were filtered for quality (more than 80% of the sequence having a Sanger Phred+33 quality score > 33) using fastq_quality_filter -v -Q 33 -q 20 -p 80. Mapping was performed with Tophat2 to the mm10 mouse transcriptome (Kim et al., 2013b) keeping the strand information with the following options: tophat -p 2 --library-type fr-firststrand --read- mismatches 5 --read-gap-length 5 --read-edit-dist 5 --no-coverage-search --segment- length 15 --transcriptome-index. Duplicated mapping reads were filtered out using the UMIs as follows. First duplicated mapped reads were marked using picard-tools- 1.79/MarkDuplicates.jar. Then the genomic position of the duplicated reads were extracted and for each of these positions, only reads having different UMIs were kept. Reads that mapped to multiple positions were filtered out using samtools 0.1.19 flag 256 (Li et al., 2009). Finally, reads were assigned to genes using a modified version of htseq-count with the mm10 gene annotation from UCSC Transcript database. Samples were normalized with DESeq using the estimateSizeFactors function (Anders and Huber, 2010; Dillies et al., 2013).

4.9: Bioinformatic and statistical analysis

Significance was assessed by Student's t-Test (paired t test when comparing littermates) and a p value of < 0.05 was deemed statistically significant. Microarray data were background-corrected and normalized using the robust multi-array average (RMA) algorithm implemented in the GenePattern software package (Reich et al., 2006) and replicates were averaged. Analysis focused on genes with a mean expression value > 120 in at least one sample with a coefficient of variation < 0.25. Scatter analysis (MultiplotStudio) and hierarchical clustering were performed in GenePattern.

Chapter 5: General discussion

To our knowledge, this large-scale screen is the first of its kind and presents a detailed view of how various microbial species alone shape various components of the host immune system. Findings here identify multiple species from the human microbiota that are capable of inducing various immune and non-immune populations, at least in the setting of the mouse gut.

The ability of different components of the human gut microbiota to colonize a non-native host challenges some of the early work (Rawls et al., 2006; Ley et al., 2008; Chung et al., 2012). It has been suggested that gut immune maturation is dependent on the host-specific microbiota and the host genetics determined the colonization success of bacteria. We propose that the ability of bacteria to colonize and interact with the host does not solely depend on the host-genetics.

When the zebrafish microbiota were transplanted into a non-native host such as the mouse, the stabilized microbial composition did not resemble the donor host (Rawls et al., 2006). Here, all microbes from the human microbiota colonized some part of the mouse with some niche-specificity. This finding may suggest that bacterial community factors and competition may play a bigger role than the host genetics alone in determining the microbial composition. A study on colonization success of *B. thetaiotaomicron* mutants highlighted some aspects of these bacterial interactions within a community. The ability of *B. thetaiotaomicron* mutants to colonize germ free mice was largely influenced by the other members of the community and the competition for nutrients (Goodman et al., 2009). Our findings here do not dispute the importance of host-derived factors such as diet, specialized niches, availability of nutrients, but prompt us to reconsider how much the microbial diversity is shaped by the genetics of the host.

Colonization of the mouse GI tract with the complete human microbiota was not sufficient to drive the “full” maturation of the immune system (Chung et al., 2012). Here in our study, multiple bacterial species alone were sufficient to restore many of the immune-defects associated with germ free mice. When germ free mice were reconstituted with the human or the mouse flora, the maturation of the immune system was assessed by the restoration of immunological differences between the germ free and SPF mice. These included increased cytokine production and T cell numbers. We have not observed any difference in total T cells (or CD4, CD8 subsets) between the germ free mice, SPF mice or mice colonized with one bacterial species.

In line with the earlier work, germ free mice in our study had lower levels of IL17 production by CD4 T cells in the small intestine (Chung et al., 2012, Ivanov et al., 2009). The induction of small intestinal TH17 cells relies on the SFB and colonization of germ free mice with the mouse flora that lacks SFB can’t restore TH17 levels. We confirmed that the higher levels of TH17 cells in SPF mice were driven by the SFB. SPF mice that lacked SFB and germ free mice had similar proportions of small intestinal TH17 cells. The “full” maturation of the immune system, which encompassed generation of TH17 cells (Chung et al., 2012), in this case was not driven by the host-specific mouse flora, but SFB alone. We also identified multiple bacterial species from the human microbiota that can drive TH17 cell differentiation in the setting of a mouse gut. Finally, there are other microbe-driven effects in the colonic transcriptome and immune populations such as the colonic Tregs that can be restored by several members of the human microbiota. In the earlier study, these were not included in the evaluation of the gut immune maturation. Overall, multiple microbes isolated from the human microbiota could drive maturation of the different components of the gut immune system.

We cannot rule out the inhibitory or combinatorial effects of colonizing germ free mice with a complex community of bacteria. Colonization with many microbes, instead of a single microbe, may have affected bacteria's ability to promote maturation of the immune system. In the few co-colonization experiments we performed, we have not observed any synergistic or inhibitory effects of bacteria co-colonized. Yet, the scale of interactions among the bacterial species of the human microbiota or the mouse microbiota as a whole is more complex than those addressed in our bi-colonization experiments. How synergistic, harmonious or inhibitory interactions of microbes drive different immune outcomes is an intriguing question that could motivate many other large-scale studies, for which our findings here may provide a starting point.

The redundancy and strain specificity observed in the outcome of microbial colonization was independent of phylogeny and may be explained by overlapping bacterial products or antigens. Alternatively, the overlapping host responses to multiple microbial colonizations may suggest different routes of activation and induction. The comparison of genome sequences of the bacteria did not reveal any correlation between the phenotypes and the bacteria's ability to drive these phenotypes in mice. The profiling of bacterial transcripts by RNA sequencing may improve our understanding of immunological and non-immunological phenotypes generated here. It is known that PSA, which confers immunomodulatory effects on IL10 producing T cells (Mazmanian et al., 2008), is not uniformly expressed by different *B. fragilis* (Pantosti et al., 1993). *B. fragilis NCTC9343* strain has variable expression of PSA and PSB (Pantosti et al., 1993). Along these lines, transcriptome, metabolome or proteome characterization of bacterial strains during colonization may be the next step in deciphering the source of redundant or strain-specific effects observed in the outcome of colonizations. This may also introduce another level of complexity where the bacterial metabolism or transcriptome may be influenced by the host factors, highlighting the complex two-way communication between the microbiota and the host.

The human microbiota and the host together are a dynamic organism and our results support that the host and the bacteria interact in many unique, specialized, complementary or redundant ways. Findings outlined here challenge the need for pools of host-specific bacteria to promote immune populations (Chung et al., 2012, Atarashi et al., 2011, Atarashi et al., 2013), as single bacterial species are sufficient to drive functional immune cells. Yet, these findings also highlight the specialized functions of bacteria as many bacteria promote tissue-specific effects. These leave us wondering what more bacteria do given the complexity of responses that colonization with a single bacterium drives. If strong inducers of TH17 and Treg cells are co-colonized, will bacteria complement or inhibit one another? Based on the host phenotypes we observe, can we engineer cocktails of bacteria that would deliver the desired outcome? If bacterial characterization proposed earlier identifies bacterial products, is there a need for live bacteria to affect the host immune system? Can single bacteria promote non-immunological processes such as the metabolism, tissue regeneration and behavior?

More than 1000 species have been described in the human GI tract, and our study focused on 5% of these species. Once the strain-specific effects, the impact of the diseased states or different host genetics are considered, our findings may only be the tip of an iceberg. Deciphering how the human microbiota shapes the host responses still remains to be a complex problem, but our work has made significant contributions to understanding this complexity and will hopefully inspire many future studies.

References

Abraham, C. and Cho, J.H. (2009). IL-23 and autoimmunity: new insights into the pathogenesis of inflammatory bowel disease. Annu. Rev. Med. 60, 97-110.

Ai, T.L., Solomon, B.D., and Hsieh, C.S. (2014). T-cell selection and intestinal homeostasis. Immunol. Rev. 259, 60-74.

Aluvihare, V.R., Kallikourdis, M. and Betz, A.G. (2004). Regulatory T cells mediate maternal tolerance to the fetus. Nature Immunology. 5, 266–271.

An, D. et al. (2014). Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. Cell. 156, 123–133.

Anders, S. and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biol. 11, R106.

Antebi, Y.E. et al. (2013). Mapping Differentiation under Mixed Culture Conditions Reveals a Tunable Continuum of T Cell Fates. PLoS. 11, e1001616.

Aranda, R. et al. (1997). Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. Journal of Immunology. 158, 3464–3473

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van, d., V, Deroos, P., Liu, H., Cross, J.R., Pfeffer, K., Coffer, P.J. et al. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 504, 451-455.

Asseman, C. et al. (1999). An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. Journal of Experimental Medicine. 190, 995–1004.

Asseman, C. et al. (2003). Colitogenic Th1 Cells Are Present in the Antigen-Experienced T Cell Pool in Normal Mice: Control by CD4+ Regulatory T Cells and IL-10. Journal of Immunology. 171, 971–978.

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.

Awasthi, A., Riol-Blanco, L., Jager, A., Korn, T., Pot, C., Galileos, G., Bettelli, E., Kuchroo, V.K., and Oukka, M. (2009). Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. J. Immunol. 182, 5904-5908.

Backhed, F. et. al. (2005). Host-Bacterial Mutualism in the Human Intestine. Science. 307, 1915–1920.

Baumgart, M. et al. (2007). Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. The ISME Journal. 1, 403–418.

Belkaid, Y. & Hand, T.W. (2014). Role of the microbiota in immunity and inflammation. Cell. 157(1), pp.121–141.

Bending, H.D. et al. (2009). Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. The Journal of Clinical Investigation. 119, 565.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 441, 235-238.

Bibiloni, R. (2006). The bacteriology of biopsies differs between newly diagnosed, untreated, Crohn's disease and ulcerative colitis patients. Journal of Medical Microbiology. 55(8), pp.1141–1149.

Bilate, A.M. and Lafaille, J.J. (2012). Induced CD4+Foxp3+ regulatory T cells in immune tolerance. Annu. Rev. Immunol. 30, 733-758.

Blatner, N.R., Mulcahy, M.F., Dennis, K.L., Scholtens, D., Bentrem, D.J., Phillips, J.D., Ham, S., Sandall, B.P., Khan, M.W., Mahvi, D.M. et al. (2012). Expression of RORgammat marks a pathogenic regulatory T cell subset in human colon cancer. Sci Transl. Med. 4, 164ra159.

Bogunovic, M. et al. (2009). Origin of the lamina propria dendritic cell network. Immunity. 31, 513–525.

Brandl, K. et al. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature. 455(7214), pp. 804–807.

Brestoff, J.R. et al. (2014). Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. Nature. 519, 242–246.

Brigl, M. et al., Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. Journal of Experimental Medicine. 208, 1163–1177.

Burzyn, D., Benoist, C., and Mathis, D. (2013a). Regulatory T cells in nonlymphoid tissues. Nat Immunol. 14, 1007-1013.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J.L., Cerletti, M., Jang, Y., Sefik, E., Tan, T.G., Wagers, A.J., Benoist, C. et al. (2013b). A special population of regulatory T cells potentiates muscle repair. Cell. 155, 1282-1295.

Cebula, A., Seweryn, M., Rempala, G.A., Pabla, S.S., McIndoe, R.A., Denning, T.L., Bry, L., Kraj, P., Kisielow, P., and Ignatowicz, L. (2013). Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. Nature. 497, 258-262.

Cella, M. et al. (2008). A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature. 457, 722–725.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. 198, 1875-1886.

Chung, Y. et al. (2011). Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nature Medicine. 17(8), pp.983–988.

Chung, H. et al. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 149, 1578–1593.

Cipolletta, D., Feuerer, M., Li, A., Kamei, N., Lee, J., Shoelson, S.E., Benoist, C., and Mathis, D. (2012). PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. Nature. 486, 549-553.

Cohen, N.R., Garg, S. and Brenner, M.B. (2009). Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. Advances in immunology, 102, 1–94.

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 21, 3674.

Conradi, S. et al. (2013). Breastfeeding is associated with lower risk for multiple sclerosis. Multiple Sclerosis Journal. 19(5), pp.553–558.

Consortium, T.H.M.P. (2012). Structure, function and diversity of the healthy human microbiome. Nature. 486, 207–214.

Coombes, J.L. et al. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. The Journal of experimental medicine. 204, 1757–1764.

Costello, E.K. et al. (2009). Bacterial Community Variation in Human Body Habitats Across Space and Time. Science. 326, 1694–1697.

Cryan, J.F. & Dinan, T.G. (2012b). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nature Reviews Neuroscience. 13, 701–712.

Cua, D.J. and Tato, C.M. (2010). Innate IL-17-producing cells: the sentinels of the immune system. Nat. Rev. Immunol. 10, 479-489.

Cupedo, T. et al. (2008). Human fetal lymphoid tissue–inducer cells are interleukin 17–producing precursors to RORC+ CD127+ natural killer–like cells. Nature Immunology. 10, 66–74.

Dalton, J.E. et al. (2006). Intraepithelial gammadelta+ lymphocytes maintain the integrity of intestinal epithelial tight junctions in response to infection. Gastroenterology. 131, 818–829.

Dasgupta, S. et al. (2014). Plasmacytoid Dendritic Cells Mediate Anti-inflammatory Responses to a Gut Commensal Molecule via Both Innate and Adaptive Mechanisms. Cell Host and Microbe. 15(4), pp.413–423.

Denning, T.L. et al. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17–producing T cell responses. Nature Immunology. 8, 1086–1094.

Di Giacinto, C. et al. (2005). Probiotics Ameliorate Recurrent Th1-Mediated Murine Colitis by Inducing IL-10 and IL-10-Dependent TGF- -Bearing Regulatory Cells. The Journal of Immunology. 174(6), pp.3237–3246.

Diehl, G.E. et al. (2013). Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX3CR1hi cells. Nature. 494, 116–120

Dillies, M.A., Rau, A., Aubert, J., Hennequet-Antier, C., Jeanmougin, M., Servant, N., Keime, C., Marot, G., Castel, D., Estelle, J. et al. (2013). A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief. Bioinform. 14, 671-683.

Du, R., Zhao, H., Yan, F., and Li, H. (2014). IL-17+Foxp3+ T cells: an intermediate differentiation stage between Th17 cells and regulatory T cells. J. Leukoc. Biol. 96, 39-48.

Duhen, T. et al. (2012). Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. Blood. 119, 4430–4440.

Eberl, G. et al. (2003). An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nature Immunology. 5, 64–73

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.

Eckburg, P.B. et. al. (2005). Diversity of the Human Intestinal Microbial Flora. Science. 308, 1635–1638.

Faith, J.J. et al. (2013). The Long-Term Stability of the Human Gut Microbiota. Science. 341, 1237439–1237439.

Faith, J.J., Ahern, P.P., Ridaura, V.K., Cheng, J., and Gordon, J.I. (2014). Identifying gut microbe-host phenotype relationships using combinatorial communities in gnotobiotic mice. Sci Transl. Med. 6, 220ra11.

Farache, J. et al. (2013). Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 38, 581–595.

Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S. et al. (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med. 15, 930-939.

Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, Hotz HR, Ceric G, Forslund K, Eddy SR, Sonnhammer EL, Bateman A. (2008). The Pfam protein families database. Nucleic Acids Research. 36, 281.

Frank, D.N. et al. (2011). Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. Inflammatory bowel diseases. 17, 179–184.

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 the differentiation of colonic regulatory T cells. Nature. 504, 446-450.

Ganal, S.C. et al. (2012). Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. Immunity. 37, 171–186.

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.

Gevers, D. et al. (2014). The treatment-naive microbiome in new-onset Crohn's disease. Cell Host and Microbe. 15, 382–392.

Glaccum, M.B., Stocking, K.L., Charrier, K., Smith, J.L., Willis, C.R., Maliszewski, C., Livingston, D.J., Peschon, J.J., and Morrissey, P.J. (1997). Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. J. Immunol. 159, 3364-3371.

Gonzalez, A., Andre-Schmutz, I., Carnaud, C., Mathis, D., and Benoist, C. (2001). Damage control, rather than unresponsiveness, effected by protective DX5+ T cells in autoimmune diabetes. Nat. Immunol. 2, 1117-1125.

Goodman, A.L. et al. (2009). Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host and Microbe. 6, 279–289.

Goto, Y. et al. (2014). Innate lymphoid cells regulate intestinal epithelial cell glycosylation. Science. 345, 1254009–1254009.

Goubier, A. et al. (2008). Plasmacytoid dendritic cells mediate oral tolerance. Immunity. 29, 464–475.

Grice, E.A. et al. (2009). Topographical and Temporal Diversity of the Human Skin Microbiome. Science. 324, 1190–1192.

Haft DH, Loftus BJ, Richardson DL, Yang F, Eisen JA, Paulsen IT, White O. (2001). TIGRFAMs: a protein family resource for the functional identification of proteins. Nucleic Acids Research. 29, 41.

Hapfelmeier, S. et al. (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science. 328, 1705–1709.

Happel, K.I. et al. (2005). Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. Journal of Experimental Medicine. 202, 761–769.

Haribhai, D. et al. (2009). A Central Role for Induced Regulatory T Cells in Tolerance Induction in Experimental Colitis. The Journal of Immunology. 182(6), pp.3461–3468.

Haribhai, D. et al. (2011). A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. Immunity. 35, 109–122.

He, B. et al. (2007). Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity. 26, 812–826.

Hepworth, M.R. et al. (2013). Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature. 498, 113–117.

Hesslein, D.G.T. & Lanier, L.L. (2011). Transcriptional Control of Natural Killer Cell Development and Function 1st ed., Elsevier Inc.

Himmel, M.E. et al. (2013). Helios+ and Helios- Cells Coexist within the Natural FOXP3+ T Regulatory Cell Subset in Humans. The Journal of Immunology. 190(5), pp.2001–2008.

Huang, C.T. et al. (2004). Role of LAG-3 in regulatory T cells. Immunity. 21(4), pp.503–513.

Huttenhower, C. et al. (2014). Inflammatory Bowel Disease as a Model for Translating the Microbiome. Immunity. 40, 843–854.

Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 11, 119.

Ichikawa, R. et al., (2012). Bile acids induce monocyte differentiation toward interleukin-12 hypo-producing dendritic cells via a TGR5-dependent pathway. Immunology, 136, 153–162.

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, R.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.

Izcue, A. et al. (2008). Interleukin-23 Restrains Regulatory T Cell Activity to Drive T Cell-Dependent Colitis. Immunity. 28, 559–570.

Jaensson, E. et al. (2008). Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. Journal of Experimental Medicine. 205, 2139–2149.

Jäger, A. et al. (2009). Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. The Journal of Immunology. 183, 7169–7177.

Jakobsson, H.E. et al. (2015). The composition of the gut microbiota shapes the colon mucus barrier. EMBO reports. 16, 164–177.

Jeremy E Koenig et. al. (2011). Colloquium Paper: Succession of microbial consortia in the developing infant gut microbiome. Proceedings of the National Academy of Sciences. 108, 4578–4585.

Jetten, A.M. (2009). Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. Nucl. Recept. Signal. 7, e003.

Johansson, M.E.V., Larsson, J.M.H. & 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. Proceedings of the National Academy of Sciences. 108, 4659–4665.

Johnston, R.J. et al. (2009). Bcl6 and Blimp-1 Are Reciprocal and Antagonistic Regulators of T Follicular Helper Cell Differentiation. Science. 325, 1006–1010.

Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. Annu. Rev. Immunol. 30, 531-564.

Kaplan, M.H., Hufford, M.M. & Olson, M.R. (2015). The development and in vivo function of T helper 9 cells. Nature Reviews Immunology. 15, 295–307.

Karimi, K. et al. (2009). Lactobacillus reuteri–induced Regulatory T cells Protect against an Allergic Airway Response in Mice. American Journal of Respiratory and Critical Care Medicine. 179, 186-193.

Kawamoto, S. et al. (2014). Foxp3+ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis. Immunity. 41, 152–165.

Kolodin, D. et al. (2015). Antigen- and Cytokine-Driven Accumulation of Regulatory T Cells in Visceral Adipose Tissue of Lean Mice. Cell Metabolism. 21(4), pp.543–557.

Komano, H. et al. (1995). Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. Proceedings of the National Academy of Sciences of the United States of America. 92, 6147–6151.

Kett, K. et al. (1986). Different subclass distribution of IgA-producing cells in human lymphoid organs and various secretory tissues. Journal of Immunology. 136, 3631–3635.

Kett, K. et al. (1995). Intestinal B-cell isotype response in relation to local bacterial load: Evidence for immunoglobulin A subclass adaptation. Gastroenterology. 109, 819–825.

Kim, B.S. et al. (2013a). TSLP Elicits IL-33-Independent Innate Lymphoid Cell Responses to Promote Skin Inflammation. Science Translational Medicine, 5, 170ra16–170ra16.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013b). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Klose, C.S.N., Kiss, E.A., Schwierzeck, V., Ebert, K., Hoyler, T., d'Hargues, Y., Göppert, N., Croxford, A.L., Waisman, A., Tanriver, Y. & Diefenbach, A. (2013). A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. Nature. 494, 261–265.

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. (2009). IL-17 and Th17 Cells. Annu. Rev. Immunol. 27, 485-517.

Kriegel, M.A., Sefik, E., Hill, J.A., Wu, H.J., Benoist, C., and Mathis, D. (2011). Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. Proc Natl Acad Sci U S A. 108, 11548-11553.

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 305, 567.

Kwon, H.K. et al. (2010). Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proceedings of the National Academy of Sciences. 107(5), pp.2159–2164.

Lagesen K, Hallin P, Rodland EA, Staerfeldt HH, Rognes T, Ussery DW. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Research. 35, 3100.

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.

Lee, J. et al. (2006). Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nature Cell Biology. 8, 1327–1336.

Lee, M.-W., Odegaard, J.I., Mukundan, L., Qiu, Y., Molofsky, A.B., Nussbaum, J.C., Yun, K., Locksley, R.M. & Chawla, A. (2015). Activated Type 2 Innate Lymphoid Cells Regulate Beige Fat Biogenesis. Cell. 160, 74–87.

Lee, Y.K. et al. (2011). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proceedings of the National Academy of Sciences. 108, 4615–4622.

Ley, R.E. et al. (2008). Evolution of Mammals and Their Gut Microbes. Science. 320, 1647–1651.

Ley, Ruth E, Peter J Turnbaugh, Samuel Klein, and Jeffrey I Gordon. (2006). Microbial Ecology: Human Gut Microbes Associated with Obesity. Nature. 444, 1022–1023.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics. 25, 2078-2079

Lin, M. et al. (2013). IgA subclass switch recombination in human mucosal and systemic immune compartments. Mucosal Immunology. 7, 511–520.

Linterman, M.A. et al. (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nature Medicine, 17, 975–982.

Liston, A. et al., 2007. Lack of Foxp3 function and expression in the thymic epithelium. Journal of Experimental Medicine. 204, 475–480.

Liston, A., Nutsch, K.M., Farr, A.G., Lund, J.M., Rasmussen, J.P., Koni, P.A., and Rudensky, A.Y. (2008). Differentiation of regulatory Foxp3+ T cells in the thymic cortex. Proc Natl. Acad Sci U S. A. 105, 11903-11908.

Lochner, M., Berard, M., Sawa, S., Hauer, S., Gaboriau-Routhiau, V., Fernandez, T.D., Snel, J., Bousso, P., Cerf-Bensussan, N., and Eberl, G. (2011). Restricted microbiota and absence of cognate TCR antigen leads to an unbalanced generation of Th17 cells. J Immunol. 186, 1531-1537.

Lowe TM, Eddy SR. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic acids. 25, 955.

Macpherson, A.J. et al. (2004). Compartmentalization of the Mucosal Immune Responses to Commensal Intestinal Bacteria. Annals of the New York Academy of Sciences. 1029, 36–43.

Macpherson, A.J. et al. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science. 288, 2222–2226.

Mangan, P.R., Harrington, L.E., O'Quinn, D.B., Helms, W.S., Bullard, D.C., Elson, C.O., Hatton, R.D., Wahl, S.M., Schoeb, T.R., and Weaver, C.T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 441, 231-234.

Mathis, D. and Benoist, C. (2011). Microbiota and autoimmune disease: the hosted self. Cell Host. Microbe. 10, 297-301.

Mebius, R.E., Rennert, P. & Weissman, I.L. (1997). Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity. 7(4), pp.493–504.

Miller, J.C. et al. (2012). Deciphering the transcriptional network of the dendritic cell lineage. Nature Immunology. 13, 888–899.

Monticelli, L. et al. (2011). Innate lymphoid cells promote lung tissue homeostasis following acute influenza virus infection. Nature Immunology. 12, 1045.

Morse,H. (1978). Origins of inbred mice: proceedings of a workshop, Bethesda, Maryland, February 14-16, 1978. (New York: Academic Press).

Mortha, A. et al. (2014). Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis. Science. 343, 1249288–1249288.

Mosconi, I. et al. (2013). Intestinal bacteria induce TSLP to promote mutualistic T-cell responses. Mucosal Immunology. 6, 1157-1167.

Mosmann, T.R. et al. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of immunology (Baltimore, Md. : 1950). 136, 2348–2357.

Mouries, J. et al. (2008). Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. Blood. 112, 3713–3722.

Mowat, A.M. & Agace, W.W. (2014). Regional specialization within the intestinal immune system. Nature Reviews Immunology. 14, 667–685.

Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., and Cheroutre, H. (2007). Reciprocal Th-17 and Regulatory T Cell Differentiation Mediated by Retinoic Acid. Science. 317, 256-260.

Murai, M. et al. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature Immunology*. 10(11), pp.1178–1184.

Nicholson, J.K. et al. (2012). Host-Gut Microbiota Metabolic Interactions. Science. 336, 1262-1267.

Niess, J.H. (2005). CX3CR1-Mediated Dendritic Cell Access to the Intestinal Lumen and Bacterial Clearance. Science. 307, 254–258.

Norgaard, M. et al. (2011). Use of Penicillin and Other Antibiotics and Risk of Multiple Sclerosis: A Population-based Case-Control Study. American Journal of Epidemiology. 174(8), pp.945–948.

Nurieva, R. et al. (2007). Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 448, 480–483.

Nurieva, R.I. et al. (2009). Bcl6 Mediates the Development of T Follicular Helper Cells. Science. 325, 1001–1005.

Nussbaum, J.C., Yun, K., Locksley, R.M. and Chawla, A. (2015). Activated Type 2 Innate Lymphoid Cells Regulate Beige Fat Biogenesis. Cell. 160, 74–87.

Ochoa-Reparaz, J. et al. (2009). Role of Gut Commensal Microflora in the Development of Experimental Autoimmune Encephalomyelitis. The Journal of Immunology. 183(10), pp.6041–6050.

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. (1999). KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Research. 27, 29.

Olszak, T. et al. (2012). Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. Science. 336, 489–493.

Ott, S.J. et al. 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut. 53, 685–693.

Pantosti, A. et al. (1993). Bacteroides fragilis strains express multiple capsular polysaccharides. Journal of clinical microbiology. 31(7), pp.1850–1855.

Peters, A., Pitcher, L.A., et al. (2011). Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. Immunity. 35, 986–996.

Petersen TN, Brunak S, von Heijne G, Nielsen H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 8, 785.

Peterson, L.W. and Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature Reviews Immunology. 14, 141–153.

Pickert, G. et al. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nature Medicine. 206, 1465–1472.

Podolsky, D.K. (1993). Regulation of intestinal epithelial proliferation: a few answers, many questions. The American journal of physiology. 264, G179–186.

Powrie, Flona et al. (1993). Phenotypically distinct subsets of CD4 +T cells induce or protect from chronic intestinal inflammation in C. B-17 scidmice. International Immunology. 5, 1461–1471.

Prakash, T., Oshima, K., Morita, H., Fukuda, S., Imaoka, A., Kumar, N., Sharma, V.K., Kim, S.W., Takahashi, M., Saitou, N. et al. (2011). Complete genome sequences of rat and mouse segmented filamentous bacteria, a potent inducer of th17 cell differentiation. Cell Host. Microbe. 10, 273-284.

Pull, S. et. al. (2005). Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. Proceedings of the National Academy of Sciences. 102, 99–104.

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

Rawls, J.F. et al. (2006). Reciprocal Gut Microbiota Transplants from Zebrafish and Mice to Germ-free Recipients Reveal Host Habitat Selection. Cell, 127, 423–433.

Reich, M., Liefeld, T., Gould, J., Lerner, J., Tamayo, P., and Mesirov, J.P. (2006). GenePattern 2.0. Nat. Genet. 38, 500-501.

Robinson, M.J. et al. (2009). Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. Journal of Experimental Medicine. 206, 2037–2051.

Rogier, E.W. et al. (2014). Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. Proceedings of the National Academy of Sciences. 111, 3074–3079.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., Jr. et al. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 28, 546-558.

Samstein R. M. et al. (2012). Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. Cell. 150(1), 29-38.

Sawada, S., Scarborough, J.D., Killeen, N., and Littman, D.R. (1994). A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell. 77, 917-929.

Saenz, S.A. et al. (2010). IL25 elicits a multipotent progenitor cell population that promotes T. Nature. 464, 1362–1366.

Salzman, N.H. et al. (2003). Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature. 422, 522–526.

Sanos, S.L. et al. (2008). RORγt and commensal microflora are required for the differentiation of mucosal interleukin 22–producing NKp46+ cells. Nature Immunology. 10, 83–91.

Santori, F.R. et al. (2015). Identification of Natural RORγ Ligands that Regulate the Development of Lymphoid Cells. Cell Metabolism. 21, 286–297.

Satoh-Takayama, N. et al. (2008). Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46. Immunity. 29, 958–970.

Sawa, S. et al. (2011). RORγt+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. Nature Immunology. 12, 320–326.

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.

Sczesnak, A., Segata, N., Qin, X., Gevers, D., Petrosino, J.F., Huttenhower, C., Littman, D.R., and Ivanov, I.I. (2011). The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. Cell Host. Microbe. 10, 260-272.

Sears, C.L. & Garrett, W.S. (2014). Microbes, Microbiota, and Colon Cancer. Cell Host and Microbe. 15, 317–328.

Seksik, P. et al. (2003). Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut. 52, 237.

Seno, H. et al. (2009). Efficient colonic mucosal wound repair requires Trem2 signaling. Proceedings of the National Academy of Sciences. 106, 256–261.

Shan, M. et al., 2013. Mucus Enhances Gut Homeostasis and Oral Tolerance by Delivering Immunoregulatory Signals. Science. 342, 447–453.

Shen, Y. et al. (2012). Outer Membrane Vesicles of a Human Commensal Mediate Immune Regulation and Disease Protection. Cell Host and Microbe. 12, 509–520.

Shikina, T. et al. (2004). IgA Class Switch Occurs in the Organized Nasopharynx- and Gut-Associated Lymphoid Tissue, but Not in the Diffuse Lamina Propria of Airways and Gut. The Journal of Immunology. 172, 6259–6264.

Skepner, J., Ramesh, R., Trocha, M., Schmidt, D., Baloglu, E., Lobera, M., Carlson, T., Hill, J., Orband-Miller, L.A., Barnes, A. et al. (2014). Pharmacologic inhibition of RORgammat regulates Th17 signature gene expression and suppresses cutaneous inflammation in vivo. J. Immunol. 192, 2564-2575.

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

Soroosh, P. et al. (2014). Oxysterols are agonist ligands of ROR t and drive Th17 cell differentiation. Proceedings of the National Academy of Sciences. 111, 12163–12168.

Stamatakis A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 22, 2688.

Strauch, U.G. (2005). Influence of intestinal bacteria on induction of regulatory T cells: lessons from a transfer model of colitis. Gut. 54(11), pp.1546–1552.

Suzuki, K. et al. (2004). Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proceedings of the National Academy of Sciences. 101, 1981–1986.

Tatusov RL, Koonin EV, Lipman DJ. (1997). A genomic perspective on protein families. Science. 278, 631.

Tezuka, H. et al. (2007). Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature. 448, 929–933.

Tian W, Arakaki AK, Skolnick J. (2004). EFICAz: a comprehensive approach for accurate genome-scale enzyme function inference. Nucleic Acids Research. 32, 6226.

Townsend, M.J., Fallon, P.G., Matthews, D.J., Jolin, H.E., and McKenzie, A.N. (2000). T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. J Exp Med. 191, 1069-1076.

Trifari, S. et al. (2009). Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from TH-17, TH1 and TH2 cells. Nature Immunology. 10, 864–871.

Tsuji, M. et al. (2009). Preferential Generation of Follicular B Helper T Cells from Foxp3+ T Cells in Gut Peyer's Patches. Science. 323, 1488–1492.

Turnbaugh, Peter J, Micah Hamady, Tanya Yatsunenko, Brandi L Cantarel, Alexis Duncan, and others. (2008). A core gut microbiome in obese and lean twins. Nature. 457, 480–84.

Uematsu, S. et al. (2008). Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nature Immunology. 9, 769–776.

Vaarala, O. (2013). Human Intestinal Microbiota and Type 1 Diabetes. Current Diabetes Reports. 13(5), pp.601–607.

Vaishnava, S. et al. (2011). The Antibacterial Lectin RegIII Promotes the Spatial Segregation of Microbiota and Host in the Intestine. Science. 334, 255–258.

Varol, C. et al. (2007). Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. Journal of Experimental Medicine. 204, 171–180.

Varol, C. et al. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity. 31, 502–512.

Varol, C., Zigmond, E. & Jung, S. (2010). Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. Nature reviews. Immunology. 10, 415–426.

Victora, G.D. et al. (2010). Germinal Center Dynamics Revealed by Multiphoton Microscopy with a Photoactivatable Fluorescent Reporter. Cell. 143, 592–605.

Wang, Y., Kumar, N., et al. (2010). Modulation of Retinoic Acid Receptor-related Orphan Receptor and Activity by 7-Oxygenated Sterol Ligands. Journal of Biological Chemistry. 285, 5013–5025.

Wapinski I, Pfeffer A, Friedman N, Regev A. (2007). Automatic genome-wide reconstruction of phylogenetic gene trees. Bioinformatics. 23, 549.

Wapinski I, Pfeffer A, Friedman N, Regev A. Natural history and evolutionary principles of gene duplication in fungi. (2011). Nature. 449, 54.

Wei, M. et al. Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. Nature Immunology. 12, 264–270.

Wexler, H.M. (2007). Bacteroides: the Good, the Bad, and the Nitty-Gritty. Clinical Microbiology Reviews. 20, 593–621.

Wohlfert, E.A., Grainger, J.R., Bouladoux, N., Konkel, J.E., Oldenhove, G., Ribeiro, C.H., Hall, J.A., Yagi, R., Naik, S., Bhairavabhotla, R. et al. (2011). GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. J. Clin. Invest. 121, 4503-4515.

Wolk, K. et al. (2004). IL-22 increases the innate immunity of tissues. Immunity. 21, 241–254.

Wu, H.J., Ivanov, I.I., 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.

Xiao, S., Yosef, N., Yang, J., Wang, Y., Zhou, L., Zhu, C., Wu, C., Baloglu, E., Schmidt, D., Ramesh, R. et al. (2014). Small-molecule RORgammat antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. Immunity. 40, 477-489.

Yang, X.O., Nurieva, R., Martinez, G.J., Kang, H.S., Chung, Y., Pappu, B.P., Shah, B., Chang, S.H., Schluns, K.S., Watowich, S.S. et al. (2008). Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 29, 44-56.

Yilmaz, O. (2008). The chronicles of Porphyromonas gingivalis: the microbium, the human oral epithelium and their interplay. Microbiology, 154, 2897–2903.

Yin, Y., Wang, Y., Zhu, L., Liu, W., Liao, N., Jiang, M., Zhu, B., Yu, H.D., Xiang, C., and Wang, X. (2013). Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens. ISME. J. 7, 615-621.

Yu, Di et al. (2009). The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment. Immunity. 31, 457–468.

Yu, X., Harden, K., Gonzalez, L.C., Francesco, M., Chiang, E., Irving, B., Tom, I., Ivelja, S., Refino, C.J., Clark, H. et al. (2009). The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol. 10, 48-57.

Zenewicz, L.A. et al. (2008). Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity. 29, 947–957.

Zhang, X. et al. (2015). The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nature Medicine. 21(8), pp.895–905.

Zhou, L. et al. (2007). IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nature Immunology. 8, 967–974.

Zhou, L., Lopes, J.E., Chong, M.M., Ivanov, I.I., Min, R., Victora, G.D., Shen, Y., Du, J., Rubtsov, Y.P., Rudensky, A.Y. et al. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature. 453, 236-240.

Appendix

Supplementary tables

Table S1: Bacteria able to induce Rorγ$^+$ Tregs belong to several phyla and genera. Phylogeny, origin and growth conditions of bacteria used in mono-colonizing GF mice. (shown in Fig. 2D/E).

Table S2: Footprint of Rorγ is different in colonic Tregs and classic TH17 cells. Expression values and FoldChange differences of transcripts enriched in Rorγ signature of colonic Tregs (comparing Nrp1$^-$ Tregs from WT or *Foxp-cre.Rorcfl/fl*) or SI TH17 cells (SFB-CD4$^+$T cells/GF-CD4$^+$T cells). t.test p.value calculated for Rorγ$^+$ and Rorγ$^-$ Tregs.

Table S3: SCFA concentration in caecal content of monocolonized mice does not correlate with Treg phenotype.

SCFA concentrations and corresponding frequencies of Rorγ+Helios-, Helios-, Helios+ and total Tregs per individual mice.

Table S1: Bacteria able to induce Rorγ$^+$ Tregs belong to several phyla and genera.

Microbe Name	Strain Number	Phylum	Family	Genus	Origin	Growth Conditions
Peptostreptococcus magnus	AO29	Firmicutes	Peptoniphilacea	Finegoldia	Onderdonk lab	YPG
Acenitobacter baumannii	ATCC17978	Proteobacteria	Moraxellaceae	Acinetobacter	Mekelanos lab	SB
Veillonella	6_1_27	Firmicutes	Veillonellaceae	Veillonella	BEI	YPG
Peptostreptococcus asaccharolyticus	AO33	Firmicutes	Peptostreptococcaceae	Peptostreptococcus	Onderdonk lab	YPG
Lachnospiraceae	2_1_58FAA	Firmicutes	Lachnospiraceae	unclassified Lachnospiraceae	BEI	Chopped meat + Glucose
Clostridium perfringens	ATCC13124	Firmicutes	Clostridiaceae	Clostridium	BEI	YPG
Coprobacillus	8_2_54_BFAA	Firmicutes	Erysipelotrichaceae	Coprobacillus	BEI	Chopped meat
Bacteroides fragilis	NCTC9343	Bacteroidetes	Bacteroidaceae	Bacteroides	Kasper lab	YPG
Propionibacterium granulosum	AO42	Actinobacteria	aceae	um	Onderdonk lab	YPG
SFB (Candidatus Arthromitus)		Firmicutes	Clostridiaceae	Candidatus	Umesaki lab	Fecal transfer from monocolonized mice
Bifidobacterium adolescentis	L2-32	Actinobacteria	Bifidobacteriace	Bifidobacterium	BEI	YPG
Bifidobacterium breve	SK134	Actinobacteria	Bifidobacteriace	Bifidobacterium	BEI	YPG
Fusobacterium mortiferum	AO16	Fusobacteria	Fusobacteriacea	Fusobacterium	Onderdonk lab	YPG
Acenitobacter lwofii	F78	Proteobacteria	Moraxellaceae	Acinetobacter	Renz lab	SB
Lactobacillus casei	AO47	Firmicutes	Lactobacillaceae	Lactobacillus	Onderdonk lab	YPG
Enterococcus faecium	TX1330	Firmicutes	Enterococcacea	Enterococcus	BEI	YPG
Fusobacterium nucleatum	FO419	Fusobacteria	Fusobacteriacea	Fusobacterium	BEI	YPG
Parabacteroides johnsonii	CLO2T12C29	Bacteroidetes	Porphyromonad	Parabacteroides	BEI	YPG
Lactobacillus rhamnosus	LMS2-1	Firmicutes	Lactobacillaceae	Lactobacillus	BEI	YPG
Bacteroides thetaiotaomicron	ATCC29741	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC	YPG
Staphylococcus saprophyticus	DLK1	Firmicutes	Staphylococcace	Staphylococcus	Kasper lab	LB
Clostridium ramosum	AO31	Firmicutes	Clostridiaceae	Clostridium	Onderdonk lab	YPG
Bacteroides salanitronis	DSM18170	Bacteroidetes	Bacteroidaceae	Bacteroides	DSMZ	YPG
Parabacteroides merdae	CLO9T00C40	Bacteroidetes	Porphyromonad	Parabacteroides	BEI	YPG
Bacteroides dorei	DSM17855	Bacteroidetes	Bacteroidaceae	Bacteroides	DSMZ	YPG
Bacteroides eggerthii	DSM20697	Bacteroidetes	Bacteroidaceae	Bacteroides	DSMZ	YPG
Bacteroides fragilis	3_1_12	Bacteroidetes	Bacteroidaceae	Bacteroides	BEI	YPG
Bacteroides massiliensis	DSM17679	Bacteroidetes	Bacteroidaceae	Bacteroides	DSMZ	YPG
Bacteroides fragilis	CLO3T00C08	Bacteroidetes	Bacteroidaceae	Bacteroides	BEI	YPG
Bacteroides thetaiotaomicron	ATCC29148	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC	YPG
Bacteroides finegoldii	DSM17565	Bacteroidetes	Bacteroidaceae	Bacteroides	DSMZ	YPG
Bacteroides uniformis	ATCC8492	Bacteroidetes	Bacteroidaceae	Bacteroides	ATCC	YPG
Bacteroides vulgatus	ATCC8482	Bacteroidetes	Bacteroidaceae	Bacteroides	Onderdonk lab	YPG
Bacteroides ovatus	CLO2T12C04	Bacteroidetes	Bacteroidaceae	Bacteroides	BEI	YPG
Clostridium histolyticum	AO25	Firmicutes	Clostridiaceae	Clostridium	Onderdonk lab	YPG

Table S2: SCFA concentration in ceacal content of monocolonized mice does not correlate with Treg phenotype.
SCFA concentrations and corresponding frequencies of Rorg+Helios-, Helios-, Helios+ and total Tregs per individual mice.

Mouse ID	Phylum	Species	Strain	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Total Tregs (% of CD4+)	Rorg+Helios- Treg (% of Foxp3+)	Helios+ Treg (% of Foxp3+)	Helios- Treg (% of Foxp3+)
I_20140609_631	Bacteroidetes	Bacteroides dorei	CL03T12C01	3.097186	2.322967	0.03157	38.7	14.4	58.3	41.7
I_20140609_629	Bacteroidetes	Bacteroides dorei	CL03T12C01	2.332531	1.497426	0	39.2	12.7	62.5	37.5
I_20140630_236	Bacteroidetes	Bacteroides eggerthii	DSM20697	2.728873	0.639479	0	20.5	16.2	54.6	45.4
I_20140530_727	Bacteroidetes	Bacteroides fragilis	3_1_12	2.551179	0.470868	0.01428	43.8	23.2	53.9	46.1
I_20140530_200	Bacteroidetes	Bacteroides fragilis	3_1_12	3.718705	0.809479	0.04119	24.2	21.7	53.8	46.2
I_20140530_251	Bacteroidetes	Bacteroides fragilis	3_1_12	2.628409	1.086942	-0.0254	29.4	13.2	65.0	35.0
I_20140527_653	Bacteroidetes	Bacteroides fragilis	CL03T00C08	5.966071	0.383238	0.03605	41.8	23.9	54.8	45.2
I_20140527_612	Bacteroidetes	Bacteroides fragilis	CL03T00C08	2.545086	0.422471	-0.0381	33.1	23.5	51.7	48.3
I_20140527_672	Bacteroidetes	Bacteroides fragilis	CL03T00C08	3.399444	0.566678	0.03467	32.3	25.1	48.6	51.4
I_20130707_890	Bacteroidetes	Bacteroides fragilis	NCTC9343	1.441237	0.233112	0	21.6	21.2	55.0	45.0
I_20140623_749	Bacteroidetes	Bacteroides ovatus	CL02T12C04	0.542945	0	0	43.8	39.9	45.3	54.7
I_20140623_792	Bacteroidetes	Bacteroides ovatus	CL02T12C04	15.85583	0.336317	0	41.1	31.1	46.5	53.5
I_20140623_757	Bacteroidetes	Bacteroides ovatus	CL02T12C04	10.89742	0.354975	0	45.0	34.7	46.5	53.5
I_20140623_749	Bacteroidetes	Bacteroides ovatus	CL02T12C04	9.89629	0.491782	0	43.8	39.9	45.3	54.7
I_20140623_782	Bacteroidetes	Bacteroides ovatus	CL02T12C04	14.1124	0.865142	0	37.6	42.0	36.9	63.1
I_20140407_740	Bacteroidetes	Bacteroides salanitronis	DSM18170	3.058143	0.034858	0.07818	14.4	7.5	60.8	39.2
I_20140407_719	Bacteroidetes	Bacteroides salanitronis	DSM18170	16.16694	0.067003	0.03314	17.3	14.9	55.7	44.3
I_20140407_722	Bacteroidetes	Bacteroides salanitronis	DSM18170	10.26742	0.084994	0.01923	17.8	17.7	54.0	46.0
I_20140407_738	Bacteroidetes	Bacteroides salanitronis	DSM18170	6.585995	0.10056	0.05037	26.9	21.8	59.8	40.2
I_20140526_677	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29148	7.375217	1.614291	0.04765	30.9	20.2	56.2	43.8
I_20140526_620	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29148	5.837587	1.922424	0.02543	16.8	24.8	54.5	45.5
I_20140526_658	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29148	5.799698	2.17525	0.0553	22.1	23.6	47.5	52.5
O_20140729_M1	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29148	1.06349	0.910679	0	34.7	30.1	47.7	52.3
I_20140323_742	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29741	1.702813	0.130797	0	32.1	26.3	50.5	49.5
I_20140323_793	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29741	1.731335	0.176616	0	41.0	32.6	40.1	59.9
I_20140323_785	Bacteroidetes	Bacteroides thetaiotaomicron	ATCC29741	1.517641	0.257375	0	51.2	32.2	52.5	47.5
I_20140616_232	Bacteroidetes	Bacteroides uniformis	ATCC8492	2.776476	0.722068	0	34.9	25.1	55.1	44.9
I_20140616_789	Bacteroidetes	Bacteroides uniformis	ATCC8492	12.09774	0.188072	0.07597	32.7	36.4	43.2	56.8
I_20140616_226	Bacteroidetes	Bacteroides uniformis	ATCC8492	10.6534	0.717915	0.02658	21.4	31.4	48.9	51.1
I_20140616_778	Bacteroidetes	Bacteroides uniformis	ATCC8492	12.30871	0.951012	0.06402	30.2	26.2	52.3	47.7
I_20131013_573	Actinobacteria	Bifidobacterium breve	SK134	1.449314	0	0	18.8	17.9	65.1	34.9
I_20131013_572	Actinobacteria	Bifidobacterium breve	SK134	1.177819	0	0	18.7	24.1	56.7	43.3
I_20131013_550	Actinobacteria	Bifidobacterium breve	SK134	0.889016	0	0	18.3	24.2	58.9	41.1
I_20150212_1945	Firmicutes	Clostridium histolyticum	AO25	0.919775	0.242371	0.14298	23.6	33.5	49.5	50.5
I_20150212_1946	Firmicutes	Clostridium histolyticum	AO25	0.913029	0.245545	0.13584	45.5	30.1	56.6	43.4
I_20140206_713	Firmicutes	Clostridium perfringens	ATCC13124	0.669289	0	0.02135	38.7	9.5	61.7	38.3
I_20140206_711	Firmicutes	Clostridium perfringens	ATCC13124	1.294703	0.0176	0.02694	20.8	5.5	64.1	35.9
I_20140907_293	Firmicutes	Clostridium ramosum	AO31	0.822102	0.006549	0	21.4	27.8	45.3	54.7
I_20140907_294	Firmicutes	Clostridium ramosum	AO31	1.045083	0.006847	0	20.8	33.3	38.7	61.3
I_20140916_198	Firmicutes	Coprobacillus	8_2_54BFAA	0.887188	0	0	34.3	8.0	53.2	46.8
I_20140916_197	Firmicutes	Coprobacillus	8_2_54BFAA	0.705904	0	0	17.8	10.6	51.5	48.5
I_20140916_178	Firmicutes	Coprobacillus	8_2_54BFAA	1.445443	0	0	36.2	12.3	58.6	41.4
I_20150125_1958	Fusobacteria	Fusobacterium mortiferum	AO16	0.999676	0.093707	0.08019	43.7	22.9	55.1	44.9
I_20150125_1959	Fusobacteria	Fusobacterium mortiferum	AO16	0.846089	0.187741	0.13468	58.6	17.6	56.7	43.3
I_20150125_1966	Fusobacteria	Fusobacterium mortiferum	AO16	1.5539	0.264526	0.1965	28.1	23.4	59.3	40.7
I_20140501_726	Fusobacteria	Fusobacterium nucleatum	F0419	0.555267	0.094375	0.24215	23.2	26.8	48.4	51.6
I_20140501_752	Fusobacteria	Fusobacterium nucleatum	F0419	0.601699	0.096543	0.26513	18.9	17.9	52.6	47.4
I_20140501_704	Fusobacteria	Fusobacterium nucleatum	F0419	0.746081	0.10609	0.25603	22.3	34.5	45.6	54.4
I_20131025_415		Germ free		0.873455	0	0	15.0	5.0	65.1	34.9
I_20131025_435		Germ free		0.875038	0	0	10.6	0.9	60.6	39.4
I_20131009_486	Firmicutes	Lactobacillus rhamnosus	LMS2-1	4.10718	-0.049807	-0.0309	12.8	17.4	67.8	32.2
I_20131009_487	Firmicutes	Lactobacillus rhamnosus	LMS2-1	4.209345	0.019444	0.01452	9.1	26.6	58.3	41.7
I_20131009_481	Firmicutes	Lactobacillus rhamnosus	LMS2-1	5.858404	0.030744	-0.0068	18.9	29.1	57.5	42.5
I_20140616_255	Bacteroidetes	Parabacteroides johnsonii	CL02T12C29	3.688913	1.100601	-0.0378	31.3	29.1	52.0	48.0
I_20140616_229	Bacteroidetes	Parabacteroides johnsonii	CL02T12C29	2.366541	1.18949	0.04944	16.3	22.8	46.3	53.7
I_20140616_799	Bacteroidetes	Parabacteroides johnsonii	CL02T12C29	0.872533	0.402572	0	26.5	18.9	55.1	44.9
I_20140609_659	Bacteroidetes	Parabacteroides merdae	CL09T00C40	4.236698	0.313153	-0.0333	23.8	14.1	47.0	53.0
I_20140609_618	Bacteroidetes	Parabacteroides merdae	CL09T00C40	3.703395	0.461199	0.08701	21.0	13.3	59.2	40.8
I_20140609_622	Bacteroidetes	Parabacteroides merdae	CL09T00C40	5.218288	0.553494	0.08831	18.2	15.5	39.1	60.9
I_20140630_238	Firmicutes	Peptostreptococcus magnus	AO29	4.510515	-0.023165	-0.0503	38.5	2.8	71.4	28.6
I_20140630_241	Firmicutes	Peptostreptococcus magnus	AO29	5.825996	-0.007109	-0.0402	33.6	10.3	73.6	26.4
I_20140907_288	Firmicutes	Peptostreptococcus magnus	AO29	0.904501	0	0	17.9	2.5	73.8	26.2
I_20140907_287	Firmicutes	Peptostreptococcus magnus	AO29	1.275876	0.014832	0	20.0	3.2	65.2	34.8
I_20140907_291	Firmicutes	Peptostreptococcus magnus	AO29	0.976538	0.025182	0.02224	13.3	4.0	56.1	43.9
I_20140130_709	Firmicutes	Peptostreptococus asaccharoly	AO33	0.991269	0.010683	0.01051	35.2	10.4	64.9	35.1
I_20140130_712	Firmicutes	Peptostreptococus asaccharoly	AO33	25.46501	0	0.02626	19.4	12.4	60.0	40.0
I_20140130_760	Firmicutes	Peptostreptococus asaccharoly	AO33	5.196418	0.003231	0.01529	19.4	12.4	60.0	40.0
I_20140130_741	Firmicutes	Peptostreptococus asaccharoly	AO33	2.529292	0.00339	0.01507	23.8	10.7	63.9	36.1
I_20140130_750	Firmicutes	Peptostreptococus asaccharoly	AO33	4.791512	0.00534	0.02532	19.4	12.4	60.0	40.0
I_20140130_756	Firmicutes	Peptostreptococus asaccharoly	AO33	4.689477	0.006242	0.02438	18.0	12.9	55.9	44.1
I_20140130_758	Firmicutes	Peptostreptococus asaccharoly	AO33	5.13821	0.009245	0.02429	19.4	12.4	60.0	40.0
I_20140723_225	Firmicutes	SFB(Candidatus Arthromitus)		2.164468	0.008266	0.00692	19.5	20.1	22.5	77.5
I_20140723_216	Firmicutes	SFB(Candidatus Arthromitus)		2.108153	0.010821	0.00927	18.7	19.3	18.0	82.0
I_20140723_223	Firmicutes	SFB(Candidatus Arthromitus)		4.203449	0.038868	0.01408	17.1	14.1	26.5	73.5
I_20131205_516	Firmicutes	Staphylococcus saprophyticus	DLK1	5.247058	0.684766	0.02051	27.7	35.6	43.2	56.8
I_20131205_514	Firmicutes	Staphylococcus saprophyticus	DLK1	3.047405	0.903492	0.02357	17.8	35.2	42.2	57.8
I_20140926_191	Firmicutes	Veillonella	6_1_127	0.359954	0.064311	0.00899	19.2	15.0	51.7	48.3
I_20140926_181	Firmicutes	Veillonella	6_1_127	0.443647	0.07021	0.02397	15.5	15.6	49.8	50.2
I_20140926_182	Firmicutes	Veillonella	6_1_127	0.344145	0.093449	0.038	11.6	10.5	57.6	42.4

Table S3: Footprint of Rorγ is different in colonic Tregs and classic TH17 cells.

		Mean expression values				Fold change		
ProbeSetID	**GeneSymbol**	**SFB-CD4+ T cells**	**GF-CD4+ T cells**	**WT Nrp1-Treg (RNA seq)**	**KO Nrp1-Treg (RNA seq)**	**FoldChange WT/KO Nrp1- Treg**	**FoldChange SFB/GF-CD4+T cells**	**t.test p.value WT/KO Nrp1-Treg (-log10)**
10494023.0	Rorc	244.0	111.0	67.1	6.8	9.9	2.2	3.0
10441633.0	Ccr6	247.1	252.7	45.2	8.0	5.7	1.0	2.8
10524034.0	Idua	371.2	368.8	27.7	8.6	3.2	1.0	2.2
10469816.0	Il1rn	99.3	100.8	24.3	6.8	3.6	1.0	2.1
10586614.0	C2cd4b	234.4	350.7	48.8	10.5	4.7	0.7	1.8
10476889.0	Nxt1	263.4	259.4	42.6	12.6	3.4	1.0	1.6
10544596.0	Tmem176b	680.1	423.3	57.3	16.4	3.5	1.6	1.6
10606058.0	Cxcr3	835.2	1108.0	119.8	48.3	0.8	2.5	1.5
10541895.0	Tnfrsf1a	499.7	443.4	84.5	17.5	4.8	1.1	1.5
10587748.0	Adamts7	148.2	162.9	20.5	6.0	3.4	0.9	1.5
10373740.0	Pik3ip1	665.5	284.7	31.2	12.6	2.5	2.3	1.4
10581151.0	Rrad	558.9	870.5	115.6	36.3	3.2	0.6	1.4
10521498.0	Crmp1	213.4	209.8	129.6	52.3	1.0	2.5	1.4
10372781.0	Irak3	343.8	395.4	69.1	20.2	3.4	0.9	1.2
10471535.0	Fam129b	123.8	120.4	67.1	20.9	3.2	1.0	1.2
10515920.0	Ppcs	196.6	206.8	51.4	16.0	3.2	1.0	1.2
10365098.0	Tbxa2r	491.9	235.3	21.1	7.6	2.8	2.1	1.2
10467739.0	Avpi1	175.6	159.5	18.6	6.1	3.0	1.1	1.1
10408557.0	Serpinb1a	413.1	188.5	20.8	6.8	3.1	2.2	1.0
10446214.0	Alkbh7	153.9	127.6	66.6	11.4	5.8	1.2	1.0
10589113.0	Nckipsd	133.9	120.2	16.4	5.4	3.0	1.1	1.0
10375443.0	Havcr2	364.0	307.0	189.4	77.7	1.2	2.4	1.0
10545154.0	Il23r	1096.2	163.3	71.1	27.4	2.6	6.7	1.0
10494428.0	Txnip	2604.1	1189.5	166.9	79.5	2.1	2.2	1.0
10531126.0	Igj	435.7	66.5	64.1	22.0	2.9	6.6	0.9
10376899.0	Trim16	84.2	91.4	24.0	7.8	3.1	0.9	0.9
10441055.0	Pigp	204.7	224.6	52.4	16.3	3.2	0.9	0.9
10552824.0	Rras	440.1	460.0	51.9	15.4	3.4	1.0	0.9
10472235.0	Dapl1	4032.8	1201.1	15.0	21.1	0.7	3.4	0.9
10490724.0	Samd10	240.1	274.4	23.9	6.3	3.8	0.9	0.9
10345752.0	Il1r2	279.0	99.9	147.1	52.1	2.8	2.8	0.8
10347888.0	Ccl20	347.2	121.1	9.3	19.1	0.5	2.9	0.8
10411226.0	F2rl1	476.5	211.1	23.7	11.2	2.1	2.3	0.8
10425283.0	Maff	227.8	295.8	176.4	51.3	3.4	0.8	0.7
10429568.0	Ly6c1	3013.5	1066.9	11.5	7.1	1.6	2.8	0.5
10589654.0	Als2cl	517.6	219.9	44.5	55.5	0.8	2.4	0.4
10593123.0	Tagln	428.8	87.3	5.5	3.9	1.4	4.9	0.4
10386058.0	Sparc	412.8	116.6	48.9	22.9	2.1	3.5	0.3
10369688.0	Tet1	233.1	82.4	38.9	54.6	0.7	2.8	0.3
10481574.0	Fam78a	1357.4	365.3	43.3	33.6	1.3	3.7	0.3
10351197.0	Sell	5171.4	1946.3	19.1	10.5	1.8	2.7	0.3
10445767.0	Treml2	1254.9	369.6	18.6	24.3	0.8	3.4	0.3
10545707.0	Actg2	893.7	125.6	14.1	9.0	1.6	7.1	0.3
10404132.0	Cmah	1788.8	780.3	149.1	185.1	0.8	2.3	0.3
10480238.0	St8sia6	1325.0	554.4	24.2	32.1	0.8	2.4	0.2
10499655.0	Il6ra	1629.6	664.4	73.8	64.7	1.1	2.5	0.2
10589994.0	Eomes	169.8	76.7	16.6	21.9	0.8	2.2	0.2
10345762.0	Il1r1	331.6	49.0	42.3	37.3	1.1	6.8	0.1
10344750.0	Sgk3	362.8	152.1	26.1	31.5	0.8	2.4	0.1
10415662.0	Rcbtb1	773.8	364.4	31.8	29.9	1.1	2.1	0.1
10406852.0	Cnn3	384.3	144.1	7.7	5.9	1.3	2.7	0.1
10588495.0	Dusp7	1490.6	587.2	35.7	30.6	1.2	2.5	0.1
10462587.0	Stambpl1	782.0	353.3	36.3	32.3	1.1	2.2	0.1
10375019.0	Nsg2	504.0	237.6	34.7	30.1	1.2	2.1	0.1
10568165.0	Zg16	550.5	87.1	8.3	6.8	1.2	6.3	0.1
10467124.0	Acta2	1616.4	145.0	9.5	9.9	1.0	11.2	0.1
10499378.0	Sema4a	449.9	194.5	6.7	6.8	1.0	2.3	0.0