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Inflammatory Bowel Disease-associated Dysbiosis and Immune Gardening of the Intestinal Microbiome

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Inflammatory Bowel Disease-associated Dysbiosis and Immune

Gardening of the Intestinal Microbiome

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Cellular and Molecular Pathology

by

Jonathan Patrick Jacobs

2015

ABSTRACT OF THE DISSERTATION

Inflammatory Bowel Disease-associated Dysbiosis and Immune

Gardening of the Intestinal Microbiome

by

Jonathan Patrick Jacobs

Doctor of Philosophy in Cellular and Molecular Pathology

University of California, Los Angeles, 2015

Professor Jonathan Braun, Chair

Inflammatory bowel disease (IBD) – comprised of Crohn's disease (CD) and ulcerative colitis (UC) – is believed to arise from a combination of genetic susceptibility and environmental factors that trigger an inappropriate mucosal immune response to constituents of the intestinal microbiome. There is now an extensive literature demonstrating that the microbiome has profound effects on immune function and, conversely, that the immune system can shape the microbiome. I hypothesized that genetic variation in mucosal immune gardening of the intestinal microbiome can result in pro-inflammatory dysbiosis, which acts as a risk factor for overt IBD. To evaluate whether individuals at risk for IBD develop dysbiosis prior to the onset of disease, a family based study was performed to characterize the microbiome and metabolome of pediatric IBD patients and their first degree relatives. These relatives are at higher risk for dysbiosis than the general population due to shared genetic and environmental factors with the IBD proband. A

subset of healthy relatives in this cohort had dysbiosis with fecal metabolomic profiles (metabotypes) shared with IBD patients. The effect of the transcription factor RORγt on the intestinal microbiome was then investigated as a model of how perturbation of immune gardening could result in dysbiosis. Mice deficient in RORγt had an altered small intestinal and colonic mucosa-associated microbiome characterized by overgrowth of segmented filamentous bacteria (SFB), a microbe previously shown to promote colitis. Further knockout and cell engraftment experiments demonstrated that small intestinal gardening of SFB was mediated by RORγt-dependent T cells in a manner independent of IL-17A. The protective rs4845604 polymorphism in the RORC gene encoding RORγt was associated with altered microbial composition in mucosal wash samples from IBD patients and healthy individuals. These findings demonstrated that RORγt-dependent T cells garden the intestinal microbiome and suggest that genetic variation in this process could influence susceptibility to IBD.

The dissertation of Jonathan Patrick Jacobs is approved.

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CHAPTER 1

THE MUCOSAL MICROBIOME: IMPRINTING THE IMMUNE SYSTEM OF THE

INTESTINAL TRACT

Abstract

The small intestine and colon house a complex ecosystem of bacteria, fungi, parasites, and

viruses that together constitute the intestinal microbiome. This environment is in part shaped by

historical exposure and diet, but is also a consequence of host genetics and immune selection.

The close interaction of host and commensals at the mucosal border poses a considerable

challenge to the intestinal immune system. The microbial environment is constantly monitored

through pattern recognition receptors (PRRs). Signals from these receptors condition the cells of

the mucosal immune system – including epithelial cells –in ways that profoundly affect host

immune responses. The intestinal microbiome promotes innate immune activity and adaptive

immune responses (e.g. Th17) that contain the intestinal microbiota and neutralize pathogens. At

the same time, gut commensals are able to promote regulatory mechanisms that prevent

inappropriate adaptive immune responses to nonpathogenic microbes.

Introduction

The human body is a complex ecosystem that harbors an incredible diversity of microorganisms

on the skin and the mucosal surfaces of the gastrointestinal, respiratory, and genitourinary

systems. The vast majority are located in the small and large intestines, comprising an estimated

10^{14} cells (10 fold greater than the number of human cells) (*1*). The intestinal microbiota is in

close proximity to the absorptive surface of the intestine, necessitating that the intestinal immune system discriminate the vast numbers of harmless or beneficial intestinal microbes from potential pathogens. This chapter will introduce the constituents of the intestinal microbiota, the factors that influence the microbiota, the various receptors that allow the immune system to sense microbial composition, and the mechanisms by which the microbiome can shape the intestinal immune system.

Composition of the intestinal microbiome

Prokaryotes and archaea

The composition of the intestinal microbiota has been difficult to characterize because only a small fraction of the microbiota can be cultured. In recent years, the intestinal microbiome has been interrogated by massive shotgun sequencing of stool samples from healthy volunteers, allowing for the identification of intestinal microorganisms by the presence of their genetic material. Pioneering work in this area was performed in the labs of David Relman and Jeff Gordon and expanded upon by two consortia: the Metagenomics of the Human Intestinal Tract (MetaHIT) and the Human Microbiome Project (HMP) (*2-5*). The non-human genes present in the microbiota collectively constitute the metagenome, which is estimated to include 100 times as many genes as the human genome. Over >99% of the metagenome is derived from prokaryotes (i.e. bacteria), while much of the remainder of the metagenome represents archaea (*4*). The archaeal component of the human microbiome is dominated by one organism, *Methanobrevibacter smithii* (*2*). Intestinal prokaryotes, on the other hand, show considerable diversity on a species level. Over 1000 bacterial species reside in the human intestine, with the most abundant falling into two bacterial phyla (Firmicutes, Bacteroidetes) (*4*). Other phyla such

as Proteobacteria (which includes *Escherichia coli*) are also represented but are found at much lower abundance. Intestinal bacteria are believed to be highly specialized for their environment, as no other ecosystem outside the digestive tract has a similar bacterial composition (*6*).

Most studies of the human microbiome have utilized stool; however, this provides an incomplete representation of the intestinal microbiome. Sequencing of microbes from mucosal biopsies has revealed that mucosa-associated microbes have a distinct composition from fecal microbiota (*2, 7*). Moreover, microbial communities differ across locations within the gastrointestinal tract (*8*). The biogeography of the human microbiome is still under investigation, but it is possible that there is diversity not just at the macroscopic level (e.g. small vs. large intestine) but also at the microscopic level with distinct microbial communities potentially existing in functional units as small as an intestinal crypt.

Sequencing studies have revealed considerable interpersonal variation in the microbiome, with each individual harboring a subset of the total diversity of the human microbiome (at least 160 species). Nevertheless, humans share a common set of commensals and variations in the abundance of common genera allow for clustering of individuals. Such studies have suggested the presence of at least 3 such clusters (referred to in some publications as enterotypes) primaily based upon the abundance of *Bacteroides*, *Prevotella*, and *Ruminococcus* (*9*). However, it is not yet clear what significance such clustering has for characterizing the entirety of an individual's microbiome.

Temporal variation in the intestinal microbiota is less than in the microbiota of external sites such as the skin, suggesting that the intestinal microbiota is a stable attribute of each individual (*10*). This has been supported by a study which used single nucleotide polymorphisms within the metagenome to track bacterial strains within individuals. The investigators found that

despite fluctuations in bacterial abundance, the bacterial strains that characterize an individual's intestinal microbiota are largely stable over time (*11*).

Sequencing of the intestinal microbiota has revealed aggregate differences in the intestinal microbiome of patients with some medical conditions compared to a healthy population, most prominently inflammatory bowel disease, obesity, and diabetes (*3, 12*). These disease associations may reflect the importance of commensal bacteria in host physiology. Their beneficial activities include harvesting energy from otherwise indigestible plant polysaccharides (transferred to the host via short chain fatty acids), triggering formation of an intestinal mucus barrier, promoting intestinal vascularization, metabolizing xenobiotics, and preventing colonization by pathogens (*13-17*). The microbiome also has profound effects on the immune system and epithelial host defense, which will be discussed later in this chapter.

Eukaroytes

Less than 0.1% of the metagenome in Westerners is derived from eukaryotes, largely fungi (the "mycobiome") and protozoans (*4*). Efforts to define the human mycobiome are in their infancy, and typically involve amplification and sequencing of 18S RNA. Sequencing studies in mice show a wide diversity of fungi in the intestine, including all 4 phyla and more than 50 genera (*18*). The human mycobiome appears to be similarly diverse. Like the prokaryotic microbiome, differences in composition have been observed between mucosa-associated fungi and fungi in stool (*19*). Intestinal protozoa fall primarily within the *Blastocystis* genus, though some individuals also harbor members of the *Entamoeba* genus (*20*). In many regions of the world, parasitic worms represent a multicellular eukaryotic component of the intestinal microbiome.

Viruses

While the microbiome typically refers to living organisms, the human intestine also includes a wide diversity of viruses which can infect human or microbial cells. Collectively, this has been referred to as the virome. Sequencing of viral particles isolated from human stool suggests that most represent bacteriophages and that there are as many distinct types of phage in each individual as phylotypes of bacteria (*21*). While in many environments phages are lytic and control bacterial populations in a predator-prey relationship, early data suggest that resident intestinal phages are predominantly lysogenic. These phages may play an important role in lateral gene transfer among bacterial populations, including antibiotic resistance genes (*22*). The human intestinal virome also contains multiple eukaryotic viruses. Chronic intestinal viruses have been poorly characterized and their significance to human health and disease is unknown; however, results in animal models suggest that chronic viral infection can evoke disease in genetically susceptible hosts (*23*).

Factors influencing the intestinal microbiome

Founder effect

The factors that give rise to an individual's microbiome are beginning to be explored. Humans are colonized initially by vaginal bacteria if they are delivered vaginally or by skin bacteria if they are delivered by C-section (*24*). It has been documented that maternal phylotypes that originally colonized an infant can be lost over time, replaced by other phylotypes of unclear provenance (*25*). These replacement microbes presumably came from the infant's environment, possibly from other individuals besides the mother. The infant's microbiota diversifies greatly after exposure to plant polysaccharides, after which an adult microbiota rapidly develops (*26*).

While the process by which an adult microbiota forms is not understood, it presumably depends upon exposure to various potential colonizers. Therefore, the adult composition of an individual's microbiota may reflect in part the history of exposure to "founder" microbes which then attain stable residence into adulthood. Data exist primarily in mice to support this concept. In syngeneic mice, it has been shown that mice within a litter have a more similar microbiota than mice of different litters that were housed in different cages (*27*). Differences in microbiota related to litter effect persist as far as four generations later (*28*). In humans, the intestinal microbiota has greater similarity within families than across unrelated individuals, but it has been difficult to separate genetic effects from shared early environment (*3*). The primary evidence for a founder effect in humans comes from studies of specific microbes. For instance, distinct subtypes of *Helicobacter pylori* in populations within southeast Asia have been correlated with their ancestral migration patterns (*29*).

Dietary influences

Studies of the gut microbiome across mammals have demonstrated a correlation between the nature of the diet (herbivore, carnivore, or omnivore) and the composition of the microbiome (*30*). This suggests that the intestinal microbiome is a reflection of dietary intake of substrates for bacterial metabolism. An extension of this insight is that fluctuations in the diet of an individual may influence the composition of the microbiota. Consistent with this hypothesis, a correlation has been made between the fecal microbiome of individuals and their dietary consumption of protein and insoluble fiber (*31*). Another study examining the microbiota of elderly patients found a correlation between microbial composition and diet as assessed by a food frequency questionnaire (*32*). A study applying the enterotype concept found that diets high in protein and animal fat favored *Bacteroides* whereas diets high in carbohydrates favored *Prevotella* (*33*). In

mice, a high fat, high sugar diet has been shown to alter the composition of a humanized microbiota (*34*). In another study, the relative abundances of 10 bacterial species that were used to colonize germ-free mice were found to predictably shift in response to dietary perturbations (*35*).

Two studies have been performed in which volunteers have been subjected to a standardized diet with longitudinal monitoring of their microbiota. One compared a high fat/low fiber diet to a low fat / high fiber diet, while the other studied a choline-deficient diet (*33, 36*). Both studies concluded that dietary modification resulted in a shift in the composition of the intestinal microbiota. In one study, this shift was seen within a single day (*33*). It is hoped that further understanding of the effects of diet on the microbiota will result in future therapeutic strategies that use dietary manipulation or prebiotics to promote microbial communities with favorable properties.

Host factors

Across mammals, the intestinal microbiome clusters primarily along taxonomic order rather than geographical location (*30*). This suggests that the host influences the composition of the microbiota through genetically encoded mechanisms that arose due to the evolutionary advantage conferred by beneficial gut microbiota. Consequently, variation in the microbiota within a species may arise from genetic polymorphisms that shape the microbiota. A genetic association with microbial composition has been demonstrated in mice in two separate quantitative trait loci analyses of interbred mouse lines (*28, 37*). Host genetics can also influence the effects of dietary change on the gut microbiota, as was shown in a genome-wide association study of mice placed on a high-fat, high-sucrose diet (*38*). In humans, monozygotic twins have

been found to have more similarity in their microbiota than dizygotic twins in one small study, though the difference did not reach statistical significance (*3*).

Much of the evidence for host selection of microbiota comes from candidate gene studies comparing the microbiota of knockout mice to littermate controls. Most of the genes that have been shown to influence the microbiota are related to the immune system, suggesting that the immune system exerts selective pressure on the microbiota to promote favorable communities. The components of the innate and adaptive immune system that have been implicated in shaping the luminal microbiota include alpha defensins (antimicrobial peptides primarily secreted by Paneth cells), the inflammasome (discussed further in the next section), regulatory T cells, CD1d restricted T cells, and immunoglobulins (*39-43*). The abnormal microbiota of inflammasome-deficient mice has been found to confer susceptibility to dextran sulphate sodium (DSS) colitis, obesity, and fatty liver disease, illustrating the importance of host immunity for maintaining a beneficial microbiota (*39, 44*). Deficiency of glycosylation enzymes has also been found to affect microbial composition, potentially by starving intestinal bacteria that use host glycans as a food source or by eliminating bacterial attachment sites in the mucus layer that derive from glycans. In mice, maternal sialyltransferase deficiency (which affects sialylation of milk oligosaccharides) gave rise to an altered microbiota that conferred resistance to DSS-induced colitis (*45*). In humans, absence of fucosyltransferase 2 (FUT2) - an enzyme that adds sugar moieties to milk and intestinal proteins - in "non-secretor" individuals is associated with shifts in microbial composition that may confer resistance to intestinal infection but risk for Crohn's disease (*46*). Finally, some genes involved in metabolism including apolipoprotein A1 and leptin have been shown to produce shifts in the microbiota through unclear mechanisms (*27, 47*).

Sensing of the microbiota by the intestinal immune system

As indicated above, the immune system plays an important role in regulating the intestinal microbiota. Under homeostatic conditions, gut microbes are restricted from the mucosal immune system by a mucus barrier that overlies the intestinal epithelium. The mucus can be divided into an outer layer which is colonized by abundant bacteria and an inner layer that excludes bacteria (*48*). In the small intestine, this inner layer is loose but due to the presence of antimicrobial peptides and IgA can remain devoid of bacteria. The colonic inner layer is much denser and is thought to physically exclude bacteria. However, even in the presence of an intact barrier, products of microbes can reach epithelial cells or immune cells residing in the lamina propria (*49*). These microbial associated molecular patterns (MAMPs) are recognized by pattern recognition receptors (PRRs), allowing the immune system to sense the microbiota and respond accordingly. In the setting of mucosal injury or invasion, immune cells come into direct contact with microbes, triggering distinct pattern recognition pathways that recognize intracellular invasion, tissue injury, and metabolic stress. These "danger" signals result in aggressive immune responses to clear enteric pathogens and restore mucosal integrity.

Toll-like receptors (TLRs)

The immune system contains multiple families of germline encoded receptors that can recognize microbial molecular patterns. The best-characterized of these is the Toll-like receptor family, which consists of transmembrane receptors that are present on the cell surface or in the endolysosomal compartment. They are characterized by N-terminal leucine-rich repeats that recognize ligands and a cytoplasmic Toll/IL-1R homology (TIR) domain that initiates signaling in response to ligand binding (*50*). The cell surface TLRs recognize external components of bacteria, mycoplasma, fungi, and viruses. TLR2 forms heterodimers with TLR1 and TLR6 to

recognize triacyl and diacyl lipoproteins, respectively, which are derived primarily from Gram positive bacteria and mycobacteria. TLR4, in conjunction with myeloid differentiation factor 2 and CD14, recognizes lipopolysaccharide (LPS) from the outer membrane of Gram negative bacteria. TLR5 binds to bacterial flagellin. Endolysosomal TLRs, in contrast, recognize microbial nucleic acids that have entered the endolysosomal compartment via ingestion of extracellular contents or capture from the cytosol within autophagosomes. TLR3 recognizes double-stranded RNA (dsRNA), which is found in the genome of reoviruses or is produced during the replication of single-stranded RNA viruses. TLR7 and TLR8 recognize viral single stranded RNA. TLR9 senses unmethylated DNA with CpG repeats, derived from bacteria and viruses.

It has increasingly been recognized that TLRs recognize endogenous ligands that are released in the setting of tissue injury. These have been referred to as damage associated molecular patterns (DAMPs). One source of DAMPs is the extracellular matrix. Degradation products of hyaluronic acid, versican, and biglycan are capable of signaling through TLR2 and TLR6 to induce inflammatory responses (*51-53*). Oxidized phospholipids generated during tissue injury (e.g. oxidized low-density lipoprotein in atherosclerotic plaques) are recognized by TLR4 and TLR6 (*54, 55*). TLRs can also recognize proteins and nucleic acids that are normally sequestered in the nucleus or other organelles but enter the extracellular space after cell death. The prototypical example is high mobility group box-1 (HMGB1), a nuclear protein that reaches the extracellular space during necrosis or by active secretion. It is recognized by TLR2, TLR4, and TLR9, possibly due to its promiscuous binding of RNA and DNA, and has been proposed to facilitate sampling of cytosolic nucleic acids by intracellular PRRs (*56, 57*). Finally, TLR9 can recognize mitochondrial double-stranded DNA – which contains unmethylated CpG similar to

that seen in bacteria – that is released from necrotic cells (*58*). Recognition of mitochrondrial

DNA released by damaged mitochondria may be a cell autonomous mechanism for detecting

cellular injury (*59*).

Signaling through TLRs can involve one of two primary TIR-adaptor molecules, MyD88

(myeloid differentiation primary response gene 88) or TRIF (TIR domain containing adapter

inducing interferon-β). MyD88 is involved in the signaling of most TLRs, except TLR3. The

critical role of TLRs in recognizing microbial pathogens is revealed by the phenotype of

MyD88-deficient humans, who develop recurrent pyogenic bacterial infections (*60*). TRIF is

utilized by TLR3 and is also involved in TLR4 signaling. The downstream signaling pathways

largely converge on NF-κB and interferon regulatory factors (*61*).

Given the importance of TLRs for sensing the intestinal microbiota, it would be expected

that they are critical for the immune system to shape the microbiome. Surprisingly, this has not

been studied systematically. Existing studies show an altered intestinal microbiome in TLR2 and

TLR5 deficient mice (*62, 63*). In the latter case, the altered bacterial community was capable of

promoting spontaneous colitis or obesity depending on the background microbiota within the

facility housing the mutant mice (*63, 64*). It is unknown whether the other TLRs also influence

the composition of the intestinal microbiota.

Nod-like receptors (NLRs)

The NLR family consists of at least 23 proteins in humans with C-terminal leucine rich repeats, a

central nucleotide-binding oligomerization (NOD) domain, and N-terminal protein binding

motifs such as a caspase activation and recruitment domain (CARD) (*65*). The NLRs are

cytoplasmic proteins that primarily recognize bacterial motifs. The best studied are NOD1,

NOD2, and the NLR components of the inflammasome. NOD2, in particular, has drawn

attention due to its strong genetic link to Crohn's disease as well as a rare familial disease, Blau syndrome. NOD1 and NOD2 recognize moieties from peptidoglycan – meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively – which are found on many bacterial and mycobacterial pathogens. NOD1 is expressed widely while NOD2 is expressed primarily in immune cells and epithelial cells. Following microbial sensing, NOD1 and NOD2 translocate to the plasma membrane and recruit RIP-like interacting caspase-like apoptosis regulatory protein kinase (RICK, also known as Rip2), resulting in a proinflammatory response through NF-κB and mitogen-activated protein kinase (MAPK) activity (*66*). NOD1 and NOD2 are also able to induce the formation of autophagosomes by recruiting Atg16L1 to the plasma membrane, resulting in engulfment of invading bacteria (*67*).

Inflammasomes are cytosolic protein complexes of a pattern recognition receptor with the adaptor protein ASC (apoptosis-associated speck-like protein containing CARD) and procaspase 1. The primary function of an inflammasome is to cleave caspase 1 into its active form, which can then activate the inflammatory cytokines IL-1β and IL-18 by proteolysis. The following NLRs have been documented to form inflammasomes: NLRP1, NLRP3, NLRP6, and NLRC4 (*68*). NLRP1 is activated by MDP, though it remains unclear whether it binds it directly. NLRC4 recognizes flagellin and PrgJ-like proteins from Gram negative bacteria in a manner that depends upon several other NLRs (Naip2, Naip5, and possibly others) and protein kinase C-theta (*69*). NLRP3 is distinct from NLRP1 and NLRC4 in that it can be activated by diverse microbial signals including pore-forming bacterial toxins, dsRNA, LPS, MDP, and lipopeptide as well as many non-microbial signals such as urea, silica, and aluminum (*70*). This broad specificity suggests that it does not sense microbes directly. There is now growing evidence that NLRP3 is activated by reactive oxygen species generated by mitochondrial stress (*71*). This is not

surprising given the critical role of mitochondria as sensors of cell injury that can initiate pathways leading to apoptosis, angiogenesis, and inflammation (*72*). NLRP6 has only recently been described to form an inflammasome (*39*). It is found predominantly in epithelial cells, but its ligand remains unknown.

An additional inflammasome has also been described that incorporates a non-NLR pattern recognition receptor, absent in melanoma 2 (AIM2), that recognizes cytoplasmic double stranded DNA (*73*). This inflammasome was capable of activating caspase-1 in response to vaccinia virus. A second member of the AIM2-like family in humans has also been described, interferon inducible protein 16 (IFI16), that recognizes intracellular DNA and is involved in the response to herpes simplex virus (*74*).

NLRs can also shape microbial composition as was seen for some TLRs. NOD2 knockout mice were found to have an altered intestinal microbiome compared to controls (*75*). Deficiency of NLRP6, or of ASC, resulted in an abnormal microbiota that conferred transmissible susceptibility to colitis and fatty liver disease (*39, 44*). As with the TLRs, it is an open area of investigation whether the many remaining NLRs affect the microbiome.

RIG-I-like receptors (RLRs)

The three RLRs - retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation−associated gene 5 (MDA5), and LGP2 - are cytoplasmic proteins that are widely expressed at low levels and are induced by viral infection. RIG-I and MDA5 recognize the double-stranded RNA replication intermediate of many viruses, triggering interferon production through binding to the mitochondrial protein interferon-beta promoter stimulator 1 (IPS-1, also known as MAVS) via a CARD domain (*76*). RIG-I recognizes short dsRNA (<1kb) that contains a 5' triphosphate end, while MDA5 recognizes longer dsRNA sequences (>2 kb) (*50*). LGP2 does not have a CARD

domain and is thought to regulate the activity of the other two RLRs. Interestingly, RLRs may also be involved in the response to DNA viruses, as cytoplasmic double-stranded DNA can be transcribed by RNA polymerase III to produce dsRNA that is recognized by RIG-I (*77*).

C-type lectin receptors (CLRs)

C-type lectins are a large group of proteins with a characteristic calcium-dependent carbohydrate recognition domain. Two families of transmembrane CLRs have been characterized that recognize microbial antigens and are expressed primarily on dendritic cells and monocytes/macrophages (*78*). The mannose receptor family includes CD205 and CD206, which mediate endocytosis/phagocytosis of diverse microbes for antigen uptake. The asialoglycoprotein receptor family includes over a dozen members. The majority induce gene expression in conjunction with signaling from Fcγ receptor or other PRRs such as the TLRs. However, several asialoglycoprotein receptors have been found that directly activate Syk through immunoreceptor tyrosine-based activation motif (ITAM) signaling upon encounter with cognate ligands (*79*). These include dectin-1, dectin-2, and macrophage-inducible C-type lectin (MINCLE). All three recognize fungal and mycobacterial ligands but have additional targets including dust mites (dectin-2), helminths (dectin-2), and the ribonucleoprotein SAP130 (MINCLE), a DAMP that is released from dying cells (*80*). Dectin-1 deficiency has recently been shown to result in an altered intestinal fungome, indicating that the immune system exerts selective pressure on fungi as well as bacteria (*18*). Moreover, the altered mycobiome in dectin-1 knockout mice resulted in increased susceptibility to colitis, providing the first evidence that fungi influence the activity of the intestinal immune system.

Galectins

Galectins are carbohydrate-binding proteins that recognize beta-galactosides. Many are divalent and can form multimeric structures with their ligands. Unlike C-type lectins, galectins are not transmembrane. They accumulate in the cytoplasm and are generally released after cell injury; some galectins can also be secreted by activated immune cells and epithelial cells. Galectins in the extracellular space are capable of binding a variety of glycans derived from the host and from pathogens. They may act as an extracellular PRR, though this is less clear than for the CLRs (*81*). A recent study has shown that cytoplasmic galectins (in particular, galectin-8) recognize host glycans on vacuoles that have been damaged by invasive *Salmonella*, triggering destruction of the vacuole by autophagy (*82*). In this manner, galectin acts as a cytoplasmic receptor for intracellular DAMPs.

Endoplasmic reticulum (ER) stress

The mucosal immune system can indirectly detect microbial infection by sensing disruptions in cell homeostasis, for instance activation of the NLRP3 inflammasome by mitochondrial injury. ER stress is another signal that activates the immune system. It is caused by the accumulation of misfolded proteins in the endoplasmic reticulum, triggering the unfolded protein response (UPR). The UPR is mediated by one of three proteins (ATF6p50, ATF4, or XBP1) that are capable of halting transcription, promoting mRNA degradation, and activating genes involved in protein folding and secretion (*83*). Triggers for the UPR include expression of mutant proteins that do not fold properly or intracellular infection by viruses, bacteria, and parasites (*84*). The exact role of the UPR in combating infection is unclear, but it is likely significant as many viruses have developed mechanisms to modulate the UPR response (*85*). Interestingly, the UPR is regulated by signaling through TLR family members. Prolonged TLR signaling can suppress some of the effects of the UPR (*86*).

The UPR has drawn great interest recently after it was linked to Crohn's disease (*87*). In mice, genetic absence of XBP1 in epithelial cells results in spontaneous ileitis and increased DSS colitis severity (*88*). The latter was abrogated by antibiotics, suggesting that the mechanism of disease involved an abnormal response to the microbiota or a shift to a pro-inflammatory microbiota. In humans, XBP1 variants have been associated with Crohn's disease. Mice lacking anterior gradient 2 (AGR2), a protein that regulates protein folding, were found to have increased ER stress and spontaneous ileitis and colitis, further supporting the association between the UPR and Crohn's (*89*). A second AGR2 knockout strain did not have inflammatory disease, suggesting that disease was related to environmental factors such as the microbiota (*90*).

Influence of the microbiota on immune development and function

As illustrated in the previous section, immune cells are equipped with an array of germline encoded receptors with overlapping specificities for bacteria, fungi, and viruses. The effects of the intestinal microbiota on the immune system have been modeled primarily using germ-free animals which are then colonized with known microbes (gnotobiotic mice) or with a standard mouse microbiota (conventionalized mice). Such studies have revealed that the presence of the intestinal microbiota shapes the development of many lineages within the immune system.

T lymphocytes

Germ-free mice have reduced numbers of CD4+ and CD8+ intestinal T cell lineages as well as intraepithelial lymphocytes, populations which can be largely restored by reintroducing a conventional mouse microbiota (*91*). Interestingly, these deficits cannot be restored by colonization with human microbiota, indicating that there are host specific microbes that induce the mouse immune system. There are now several well-characterized examples in mouse models

of T cell subsets whose development is promoted by specific microbes. One of the most striking

is the dependence of the proinflammatory Th17 subset, tied to many murine models of

autoimmune and inflammatory disease, on segmented filamentous bacteria (SFB). Germ-free

mice lack Th17 cells systemically, but this cell type can be restored by intestinal colonization

with SFB (*92*). There was also restoration, to a lesser extent, of Th1 cells (*93*). Moreover, Th17

activity in a mouse colony tracks with the level of endogenous SFB colonization (*94*). However,

SFBs are not found in humans, and an equivalent group of bacteria has not yet been identified

(*95*). Indeed, colonizing mice with a humanized microbiota does not promote Th17 development

(*93*). It is unknown by what mechanism SFB induces Th17 cells in mice, though some data

suggest that this involves an initial induction of IL-1 expression followed by signaling through

IL-1R on T cells (*96*).

The formation of regulatory T cells (Tregs) in the colon has also been found to be driven

by the microbiota. Germ-free mice have a paucity of colonic Tregs (though they have normal

numbers of Tregs in the small intestine), which can be induced to normal levels by

conventionalization or by colonization with 46 strains of *Clostridium* (*97*). These colonic Tregs

were largely IL-10-expressing and had a phenotype resembling peripheral induced Tregs. The

mechanism of Treg induction may have involved increased epithelial production of transforming

growth factor beta (TGFβ) and indoleamine 2,3-dioxygenase (IDO), but did not require MyD88.

Another group has found that a single bacterial product - polysaccharide A (PSA) - produced by

the human commensal *Bacteroides fragilis* is capable of inducing IL-10-producing colonic Tregs

(*98*). The mechanism was found to be direct recognition of PSA by TLR2 on CD4+ T cells,

triggering Treg induction (*99*). Interestingly, this immunologic manipulation by PSA was

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required for *Bacteroides fragilis* to colonize germ-free mice, highlighting the benefits to commensals of dampening host immune activity.

Invariant natural killer T (iNKT) cells are a subset of T cells expressing NK markers and a restricted T cell receptor repertoire with specificity for ligands presented by CD1d. Mouse raised under germ-free conditions have an excess of iNKT cells in the colonic lamina propria, which results in an iNKT-dependent increased susceptibility to experimental colitis and airway inflammation (*100*). This effect was not reversible by conventionalization in adulthood but was averted if a conventional microbiota was introduced at the time of birth. These findings were associated with early upregulation of CXCL16 expression by the colonic epithelium in germ-free mice. The results suggest that signals from the microbiota during the neonatal period suppress epithelial recruitment of iNKTs, representing an immunologic imprinting of early microbial exposure. Interestingly, iNKTs in the spleen and liver are decreased in number in germ-free mice and are hyporesponsive, a phenotype which can be reversed by colonization of mice with *Sphingomonas* (which expresses ligands recognized by iNKT cells) (*101*). One study using a "restricted" microbiota that is distinct from conventional mouse microbiota found further reductions in iNKT numbers in lymphoid organs of these mice compared to germ-free mice (*102*). This phenomenon was dependent upon CD8 and perforin, suggesting that the microbiota induced CD8 T cell mediated depletion of iNKT cells.

γδ T cells are abundant in the intestinal epithelium and play an important role in maintaining epithelial integrity (*103*). Their numbers are unchanged in germ-free mice, but they are functionally deficient in production of antimicrobial proteins compared to conventional mice (*104*). Antimicrobial production was inducible by exposure to a single bacterial species that invaded epithelial cells, in a manner dependent upon epithelial MyD88 expression. This suggests

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a feedback loop in which epithelial invasion by gut bacteria triggers production of antimicrobials by γδ intraepithelial lymphocytes to keep intestinal bacteria in check.

Innate lymphoid cells (ILCs)

In recent years, a group of cells have been characterized that parallel subsets of T cells but lack a rearranged antigen receptor. These innate lymphoid cells include natural killer (NK) cells, GATA3-dependent cells with a Th2-like cytokine profile, and RORγt-dependent cells (*105*). One subset of RORγt-dependent cells referred to as lymphoid tissue inducer (LTi) cells act in the fetus to organize development of lymph nodes and Peyer's patches. After birth, they organize intestinal cryptopatches which later mature into isolated lymphoid follicles (ILF). Germ-free mice have normal neonatal lymphoid development but have few ILFs. ILF formation was found to be dependent upon NOD1 recognition of peptidoglycan from the microbiota by non-hematopoietic cells (*106*). It has been proposed that the mechanism involves recognition of bacterial ligands by stromal cells in the cryptopatches (*107*).

Another subset of RORγt-dependent ILCs expresses NK receptors such as NKp46 and has been found to be the predominant intestinal source of IL-22, a cytokine which is critical for supporting epithelial production of antimicrobial peptides (*108*). These cells play a critical role in preventing systemic dissemination of intestinal bacteria (*109*). There have been conflicting reports as to whether this cell type is deficient in germ-free mice (*108, 110*). There have also been conflicting reports as to whether the presence of microbiota results in suppression or augmentation of IL-22 production (*108, 111*). The disparate results may reflect genetic or dietary variation, particularly as this ILC subset is dependent upon the aryl hydrocarbon receptor, which recognizes ligands found in food sources (*112*). Antigen-presenting cells may regulate NKp46+ RORγt-dependent ILCs by adjusting their production of IL-23, a cytokine that is critical for IL-

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22 production, in response to microbial cues. In one study, flagellin stimulated production of IL-22 by binding to TLR5 on CD103+ dendritic cells and triggering production of IL-23 (*113*).

A subset of RORγt-dependent ILCs that express T-bet and can differentiate into NKp46+ RORγt-dependent ILCs has been recently described. These cells are reduced in number in germ-free mice. A similar reduction is seen in MyD88-/-TRIF-/- mice, suggesting that TLR signaling controls this population (*114*). Tbet+ RORγt-dependent ILCs played a critical role in the intestinal response to *Salmonella* and were necessary to prevent disease in mice colonized with a colitogenic *Helicobacter* species (*114, 115*).

NK cells are also innate lymphoid cells, and like the other subtypes mentioned above are influenced by the microbiota. Germ-free mice show normal NK maturation and licensing (a process by which NK cells gain future responsiveness after recognizing host inhibitory signals) (*116*). However, NK cells in germ-free mice or mice treated with broad spectrum antibiotics showed reduced cytokine production and cytotoxicity after viral infection. This defect was dependent upon a combination of MyD88 and TRIF and could be reversed by conventionalization. Germ-free mice were found to be deficient in type 1 interferon induction by dendritic cells, with the NK defect secondary to loss of IL-15 cross-presentation by DCs to NKs (a process that requires interferon).

B cells

Germ-free mice have normal numbers and maturation of B cells, however they show reduced immunoglobulin production (*117*). This reflects the absence of exposure to pathogens that trigger antibody responses as well as reduced IgA production. As mentioned earlier, germ-free mice are deficient in ILFs, which are the primary source of IgA in the intestine. As a result, germ-free have few IgA-secreting intestinal B cells (*107*). Colonization of germ-free animals with bacteria

triggers IgA production. Interestingly, this IgA production persists even after disappearance of bacteria from the gut, suggesting that IgA-secreting B cells are imprinted by microbial exposure rather than requiring ongoing stimulation (*118*).

There is some evidence that microbiota can modulate B cell phenotype outside the intestine. Mice harboring the "restricted" microbiota alluded to earlier have been shown to be deficient in marginal zone B cells in a manner dependent upon CD8, suggesting that microbial signals can trigger CD8 T cells to target this B cell subtype (*119*).

Myeloid cells and granulocytes

Intestinal myeloid cells in mice are commonly divided into cells that express CD103 (believed to represent dendritic cells) and those that express CX3CR1 (now thought to be macrophages). CD103+ DCs sample the intestinal lumen via a mechanism involving goblet cells (*120*). After acquiring antigen, they migrate to draining lymph nodes to induce tolerogenic responses and promote IgA production (*121, 122*). Germ-free mice have normal numbers of this cell type but show reduced migration of CD103+ cells to lymph nodes (*123, 124*). CD103+ DCs produce IL-23 in response to flagellin, suggesting that their activity is controlled by signals from the microbiota (*113*).

The CX3CR1-expressing subset has been implicated in controlling invasive bacterial infection such as by *Salmonella* (*125*). These cells have transepithelial processes that can capture luminal contents in response to microbial stimulation in a manner that requires epithelial MyD88 (*126*). They can transport engulfed bacteria to mesenteric lymph nodes, a process that is inhibited by the presence of commensal bacteria in a MyD88-dependent manner (*127*). Germ-free mice have reduced numbers of the CX3CR1 subset and, moreover, this cell type does not form transepithelial cell processes (*124*). In another study, intestinal macrophages responded to the

presence of an intestinal microbiota by producing IL-1, which was critical for induction of intestinal Th17 cells (*96*).

There is evidence that microbial signals can affect systemic dendritic cell activity. As indicated earlier, the presence of a microbiota conditions dendritic cells to produce type 1 interferon in response to viral infection (*116*). In another study, the "restricted" microbiota was associated with reduced numbers of plasmacytoid dendritic cells, a population that specializes in type 1 interferon production (*128*). As with deficiency of other cell types in these mice, the phenotype was mediated by CD8 T cells.

Neutrophils have also been shown to be affected by microbial exposure. Germ-free mice or mice treated with broad spectrum antibiotics show reduced neutrophil killing of *Streptococcus pneumonia* and *Staphylococcus aureus* (*129*). This effect was dependent upon recognition of peptidoglycan through NOD1 – with NOD1 KO mice being more susceptible to streptococcal pneumonia – but it is not clear whether neutrophils directly sense peptidoglycan or receive signals from other cell types that recognize peptidoglycan.

Epithelium

Epithelial cells are on the front lines of microbial exposure, and so it does not come as a surprise that their activity is influenced by the microbiota. They express apical PRRs that undergo tonic activation due to recognition of microbial products. The importance of epithelial MyD88 is revealed by the phenotype of epithelial specific MyD88 knockout mice, which lose the normal spatial separation of the epithelium and bacteria in the distal small intestine due to reduced production of antimicrobials including RegIIIγ (*130*). Paneth cell secretion of antimicrobials including alpha-defensins and angiogenin-4 has also been found to be controlled by microbial

exposure (*131, 132*). In the case of alpha-defensins, secretion was dependent upon microbial recognition through TLRs and NOD2 (*131, 133*).

Epithelial cells perceive binding of MAMPs to cytosolic or basolateral PRRs as indicative of microbial threats. The importance of cytosolic detection of microbial products is underscored by the observation that a disease-promoting microbiota arises in mice with defective epithelial inflammasome activity (*39*). TLR5 has been the best studied example of basolateral signaling, in which flagellin recognition on the apical surface does not affect epithelial activity but basolateral recognition induces an inflammatory response (*134*).

The microbiota also promotes epithelial regeneration. MyD88 signaling has been shown to be critical for epithelial repair after injury caused by DSS, though it also promotes tumorigenesis (*135, 136*). Some data suggest that MyD88-dependent epithelial regeneration is mediated by macrophages that promote a niche for epithelial stem cells (*137*).

Outlook

The intestinal microbiota represents a complex collection of bacteria, fungi, parasites, and viruses that greatly impacts human health. Exposure to microbes imparts the mucosal immune system with the functional capacity to control enteric pathogens and avoid damaging inflammatory responses to commensals. While data only exists on the role of intestinal bacteria, it is likely that fungi and viruses also have a major role in conditioning the mucosal immune system. Interestingly, the relationship between the microbiome and the immune system appears to be bidirectional. The immune system senses the microbiota through pattern recognition receptors and is able to modulate its activity to shape the composition of the microbiota. Multiple

examples have now been described of disease-promoting microbiota that arise after perturbation of immune sensing, such as by deficiency of TLR5 and NLRP6.

Given the importance of the microbiota in immune function, inter-individual variations in microbiota are likely associated with distinct states of mucosal immune responsiveness. Defining the specific microbes, and their products, that impart immune phenotypes in humans is an active area of investigation. Advances in this field may make it possible to combine an individual's genome and metagenome to predict their immune response to pathogens as well as their susceptibility to immune-mediated disorders such as inflammatory bowel disease. The mucosal microbiome represents an attractive target for therapeutic manipulation of the mucosal immune system.

Figures
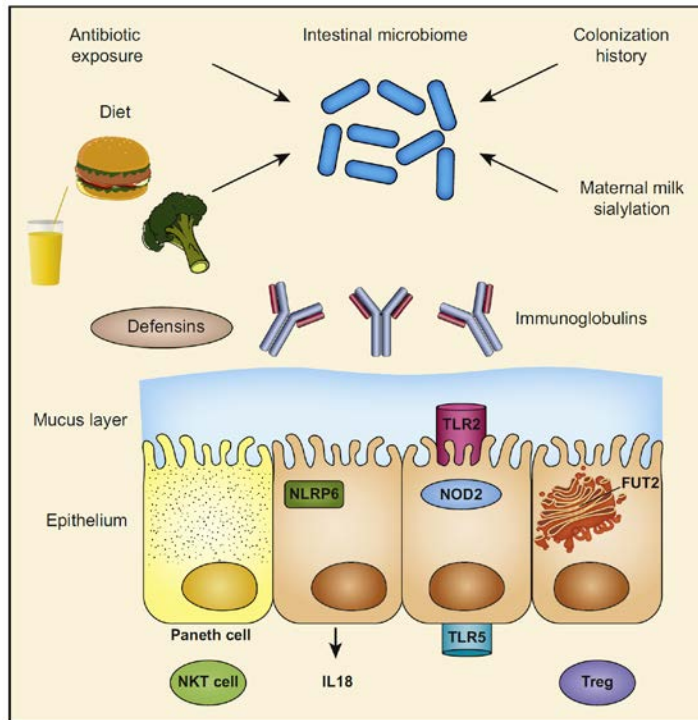
Figure 1-1. Factors that shape the intestinal microbiome. The adult microbiome is influenced by early history of exposure to potential colonizers, maternal breast milk, diet, antibiotic exposure, and host glycosylation patterns (e.g. FUT2). There is now growing evidence that the immune system shapes the microbiome through the release of antimicrobial products and the activity of immune cells in response to detection of microbial products.
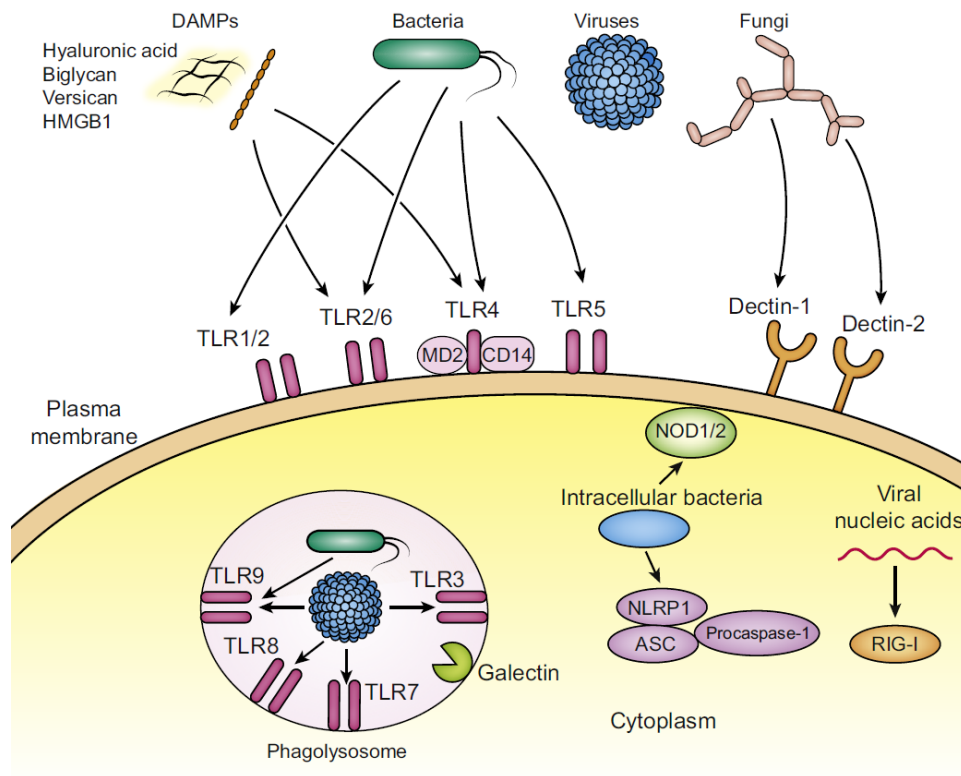
Figure 1-2. Immune pattern recognition receptors and their compartments. Sensing of microbial associated molecular patterns from bacteria, fungi, and viruses or damage associated molecular patterns occurs in three compartments: on the plasma membrane, in the cytoplasm, and in phagolysosomes.

Cell Type	Phenotype of Germ-free Mice	Changes induced by microbiota
Th17 cells	Th17 cells absent	SFB monoassociation restores Th17 cells in the intestine and systemic lymphoid organs
Regulatory T cells (Tregs)	Decreased colonic (but not small intestine) Tregs	*Clostridia* species and PSA from *B. fragilis* can induce IL-10-expressing Tregs
Invariant natural killer T (iNKT) cells	Increased iNKTs in the colonic lamina propria; decreased iNKTs in lymphoid organs	Normalization of colonic iNKT numbers only with early postnatal exposure
γδ intraepithelial lymphocytes (IELs)	IEL numbers unchanged; deficient antimicrobial production	Increased antimicrobial production
RORγt-dependent innate lymphoid cells (ILCs)	Reduced intestinal isolated lymphoid follicles; conflicting results on NKp46+ ILC number (decreased or unchanged)	Conflicting results (increased or decreased IL-22 production)
Natural killer (NK) cells	Normal NK numbers but reduced cytokine production and cytoxicity	Enhanced cytokine production and cytotoxicity
B cells	Normal B cell numbers but deficient immunoglobulin levels	Induction of IgA production
CD103+ intestinal myeloid cells	Normal numbers but reduced migration to mesenteric lymph nodes	Induction of cytokine production (e.g. IL-23)
CX3CR1+ intestinal myeloid cells	Reduced numbers and reduced transepithelial processes	Decreased trafficking to mesenteric lymph nodes
Neutrophils	Reduced bacterial killing	

Table 1-1. Effects of the intestinal microbiome on immune cell function.

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CHAPTER 2

IMMUNE AND GENETIC GARDENING OF THE INTESTINAL MICROBIOME

Abstract

The mucosal immune system – consisting of adaptive and innate immune cells as well as the epithelium – is profoundly influenced by its microbial environment. There is now growing evidence that the converse is also true, that the immune system shapes the composition of the intestinal microbiome. During conditions of health, this bidirectional interaction achieves a homeostasis in which inappropriate immune responses to nonpathogenic microbes are averted and immune activity suppresses blooms of potentially pathogenic microbes (pathobionts). Genetic alteration in immune/epithelial function can affect host gardening of the intestinal microbiome, contributing to the diversity of intestinal microbiota within a population and in some cases allowing for unfavorable microbial ecologies (dysbiosis) that confer disease susceptibility.

Introduction

The small intestine and colon house a complex bacterial ecosystem consisting of an estimated 10^{14} cells in humans, 10 fold greater than the number of human cells [1]. Many have beneficial functions such as harvesting energy from otherwise indigestible plant polysaccharides (transferred to the host via short chain fatty acids), triggering formation of an intestinal mucus barrier, promoting intestinal vascularization, metabolizing xenobiotics, and preventing colonization by pathogens [2-6]. The microbiome also has profound effects on the immune

system, the subject of other reviews in this issue. The beneficial properties of the intestinal microbiome confer an evolutionary advantage to animals that can regulate the microbial communities in their digestive tracts. Intestinal microbes also benefit from the survival and reproduction of their hosts. It has been hypothesized that in response to evolutionary pressure favoring mutual survival, hosts and intestinal microbes have co-evolved traits that foster symbiosis [7,8].

The existence of genetically encoded mechanisms for regulating microbial composition is suggested by the observation that across mammals, the intestinal microbiome clusters by taxonomic order rather than geography [9]. This could also reflect similarities in diet, which has been shown across mammals and within human populations to strongly influence the intestinal microbiome [10-12]. However, the microbiome of taxonomic groups such as bears/pandas with highly variable diets (carnivore, omnivore, herbivore) most resembles the microbiome of other members of the same taxonomic group rather than taxonomically unrelated mammals with a similar diet [9]. An alternate non-genetic explanation for taxonomic clustering of microbiota is a founder effect due to initial colonization by microbes of parental origin, allowing for stable transmission of a species-specific microbiome without active host involvement. Human neonates are known to be initially colonized by maternal vaginal or skin bacteria depending on mode of delivery and founder effects have been demonstrated for specific microbes such as H. pylori [13,14]. However, longitudinal studies of human infants and ex-germ-free mice have demonstrated wide fluctuations in microbial community structure – possibly reflecting environmental or stochastic factors – before stabilization into an adult microbiota distinct from (though still influenced by) the founding microbiota [15-17].

Since diet and founder effects, while important, do not alone explain taxonomic specificity of microbial composition, it is apparent that genetically encoded mechanisms exist to ensure that early fluctuations in intestinal microbiota result in the emergence of a stable, species-appropriate microbiota. Host factors that could potentially be utilized to regulate the microbiota include immune activity, intestinal motility, attachment surfaces, and secreted products that are metabolized by bacteria. The existence of host gardening of the intestinal microbiome has largely been probed by comparing the microbiota of mice deficient in a putative gardening gene to littermate controls using 16S ribosomal RNA sequencing. The vast majority of gardening genes identified in this manner are involved in innate (including epithelial) and adaptive mucosal immunity (Table 1).

Microbial sensing via pattern recognition receptors

The immune system is equipped with an intricate system of germline encoded pattern recognition receptors (PRRs) that recognize microbial molecular patterns. Many of the genes found to affect the microbiome are PRRs, suggesting that gardening is an active process in which mucosal cells adjust their gardening activity in response to microbial composition.

Toll-like receptors (TLRs)

TLRs are transmembrane PRRs that exist either on the cell surface - where they recognize external components of bacteria, mycoplasma, fungi, and viruses - or in the endolysosomal compartment, where they recognize microbial nucleic acids [18]. The cell surface TLRs include TLR1 (which recognizes lipoproteins), TLR2 (lipoproteins), TLR4 (lipopolysaccharide), TLR5 (flagellin), and TLR6 (lipoproteins). The endolysosomal TLRs include TLR3 (double-stranded RNA), TLR7 (single stranded RNA), TLR 8 (single stranded RNA), and TLR9 (unmethylated

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DNA with CpG repeats). TLR signaling pathways are dependent upon two adaptor molecules, MyD88 (all TLRs except TLR3) and TRIF (TLR3, TLR4). The critical role of TLRs in recognizing microbial pathogens is revealed by the phenotype of MyD88-deficient humans, who develop recurrent pyogenic bacterial infections [19].

A number of studies have suggested a role for TLRs in immune gardening of the microbiome. An early study found that MyD88-/- mice had increased Rikenellaceae and Porphyromonadaceae in cecal contents [20]. Deletion of MyD88 specifically in intestinal epithelial cells resulted in increased TM7 and decreased *Lactobacillus* in feces [21]. These mice also developed overgrowth of colonic and ileal mucosa-associated bacteria, which was associated with invasion of bacteria such as *Klebsiella pneumoniae* into mesenteric lymph nodes [21,22]. This indicates that epithelial MyD88-dependent gardening mechanisms exist that modulate microbial composition and restrain invasive pathobionts. The specific TLRs involved are largely unknown, but strong evidence exists that TLR5 has a gardening effect. TLR5-/- mice developed an altered microbiome which caused spontaneous colitis or, after rederivation into a new facility, obesity that could be transmitted to co-housed wild-type mice [23,24]. Obese TLR5-/- mice had altered abundance of 116 phylotypes and colitic TLR5-/- mice were found to have a bloom of Enterobacteriaceae, in particular *Escherichia coli*, penetrating the colonic mucus layer [25]. This suggests that colitis can be precipitated by deficient gardening of invasive pathobionts. TLR2 has also been reported to affect microbial composition, with 22 differentially abundant phylotypes in knockout (KO) mice [26].

A recent systematic study of the microbiome in TLR-deficient mice raised questions about these findings [27]. No statistically significant differences in microbial composition were observed between individually caged TLR2, TLR4, TRL5, TLR9, and MyD88 KO mice

compared to littermate controls. The reason for the discrepancy with prior studies is unknown. The earlier TLR2 data may have been misleading as the TLR2-/- and control mice were bred separately, making it possible that differences in microbial composition reflected environmental factors [26]. However, the earlier MyD88 and TLR5 studies used heterozygote breeders and the MyD88 study employed individual caging [20,23]. The lack of effect of TLR genotype in the recent study may have resulted from the mice being housed in a facility with autoclaved cages, irradiated food, and acidified water whereas other studies utilized standard SPF facilities [27]. This raises the point that gardening, while genetically encoded, acts on environmental input and so could potentially be mitigated in a clean environment.

NOD1/2

The NOD-like receptor (NLR) family consists of at least 23 cytoplasmic proteins, many with specificity for bacterial products [28]. The best studied are NOD1, NOD2, and the NLR components of the inflammasome. NOD1 and NOD2 recognize moieties from peptidoglycan – meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively – which are found on many bacterial and mycobacterial pathogens. NOD1 is expressed widely while NOD2 is expressed primarily in immune cells and epithelial cells. Following microbial sensing, NOD1 and NOD2 translocate to the plasma membrane and initiate downstream signaling pathways via RIP2 (also known as RICK) [29]. NOD1 and NOD2 are also able to induce the formation of autophagosomes by recruiting Atg16L1 to the plasma membrane, resulting in engulfment of invading bacteria [30].

NOD2 has drawn considerable attention due to the strong link between NOD2 genetic polymorphisms and Crohn's disease. NOD2-/- mice had altered microbial composition in the ileum, colon, and feces as well as an increased number of ileal mucosa-associated microbes [31-

33]. NOD2-/- mice treated with DSS (dextran sulfate sodium) and AOM (azoxymethane) to induce colitis-associated malignancy had increased colonic *Rikenella* and *Paludibacter* compared to controls [31]. The microbiome of NOD2-/- and RIP2-/- mice conferred susceptibility to DSS colitis that was transmissible to co-housed wild-type mice [31]. In humans, a study of the ileal microbiota of normal, colitis, and Crohn's disease patients demonstrated that Crohn's-associated NOD2 polymorphisms are associated with altered ileal composition independently of disease phenotype [34]. Specifically, NOD2 was found to influence the abundance of Proteobacteria, Bacillus, and Clostridium group IV by 16S sequencing and the *Clostridium Coccoides – Eubacterium Rectales* group by quantitative RT-PCR. Human patients homozygous for NOD2 polymorphisms have increased levels of both Firmicutes and Bacteroides in ileal tissue, paralleling results in mice [32].

We are unaware of any studies that have used 16S sequencing to evaluate the intestinal microbiome in NOD1-/- mice, though one paper using quantitative RT-PCR did not demonstrate any changes in abundance of a selected panel of bacterial groups [35].

Inflammasome

Inflammasomes are cytosolic protein complexes consisting of a pattern recognition receptor (generally a NLR), the adaptor protein ASC, and procaspase 1. The primary function of an inflammasome is to cleave caspase 1 into its active form, which can then activate the inflammatory cytokines IL-1β and IL-18 by proteolysis. NLRP1, NLRP3, NLRP6, and NLRC4 have been documented to form inflammasomes in response to diverse stimuli including MDP, flagellin, PrgJ-like proteins from Gram negative bacteria, and reactive oxygen species generated by mitochondrial stress [36,37]. NLRP6 is found predominantly in epithelial cells, but its ligand remains unknown [38]. Mice deficient in NLRP6, ASC, or caspase-1 developed an abnormal

microbiota that conferred transmissible susceptibility to DSS colitis and fatty liver disease [38,39]. These mice were found to have increased *Prevotellaceae* and TM7 in feces and expansion of a crypt base microbe containing electron dense intracellular material (a feature seen in some Prevotella). This demonstrates that intracellular sensing of invasive pathobionts such as Prevotella by NLRP6 can activate gardening to prevent disease-promoting dysbiosis. Interestingly, stress was recently shown to downregulate NLRP6 via corticotropin-releasing hormone, causing alteration of the small intestinal microbiome and enteritis that was transmissible to co-housed mice [40].

Epithelial gardening

IL-18

The intestinal epithelium exists in close proximity to the intestinal microbiota and is a likely site of microbial sensing and gardening. The role of the epithelium in gardening is supported by the phenotype of mice with epithelial specific MyD88 deletion and further characterization of NLRP6-mediated gardening [21]. An analysis of IL-1β, IL-1R, and IL-18 KO mice revealed that only IL-18-/- mice had an altered microbiome conferring increased DSS colitis susceptibility [38]. While the microbiome of IL-18-/- mice was not identical to that of NLRP6 and ASC KO mice, it was characterized by the same increased abundance of *Prevotellaceae* and TM7. Bone marrow chimera experiments revealed that IL-18 expression was required in non-hematopoietic cells to prevent dysbiosis, strongly suggesting that the epithelium was the source of IL-18 processed by the NLRP6 inflammasome. The mechanism of IL-18 gardening is not known but is likely multifactorial as IL-18 has diverse effects on the immune system including promoting T helper responses (Th1, Th2, and Th17 depending on the cytokine milieu), increasing cytotoxic

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CD8+ T cell activity, activating NK cells, inducing macrophage inflammatory cytokine production, and recruiting neutrophils [41].

Antimicrobial products: defensins, cathelicidin, RegIIIγ

Intestinal epithelial cells are able to produce a variety of cationic peptides with broad antimicrobial activity including beta defensins, alpha defensins, and cathelicidins [42]. Beta defensins are both constitutively expressed by epithelial cells and induced by stimuli such as TLR ligands [43]. They act as direct antimicrobial agents and as chemoattracts for immune cells (including Th17 cells) via CCR6 [42,44]. While beta defensins are attractive candidates to garden the microbiota, this has not yet been studied to our knowledge. Alpha defensins are expressed by neutrophils and Paneth cells, a specialized epithelial cell type in the small intestine that releases granules containing defensins and other antimicrobial products in response to microbial stimuli [45]. Mice lacking an enzyme required to process alpha defensins have an altered small intestinal microbiome characterized by reduced *Bacteroides* [46]. Conversely, transgenic expression of an alpha defensin in Paneth cells resulted in increased *Bacteroides* and loss of segmented filamentous bacteria (SFB) colonization in the small intestine. Transgenic mice had fewer Th17 cells in the small intestine, consistent with reports that intestinal SFB colonization is critical for induction of Th17 responses in mice [46,47]. Alpha defensin-mediated gardening may be regulated by the nutrient composition of the diet. It was recently shown that dietary tryptophan deficiency or deletion of ACE2, an enzyme required for tryptophan transport into intestinal cells, caused reduced production of antimicrobial products including alpha defensins [48]. ACE2-/- mice had an altered ileal microbiome (including increased abundance of several members of the *Limibacter* and *Paludibacter* genera) and increased DSS colitis susceptibility that was transmissible to germ-free mice. The altered microbiome was reversible

by treatment with nicotinamide (a product of tryptophan metabolism) or treatment with a dipeptide form of tryptophan which could be absorbed without ACE2.

Cathelicidin in its active peptide form, LL-37, has broad antimicrobial activity and acts as a chemoattract for neutrophils, monocytes, and T cells via the formyl peptide receptor-like 1 [42,49]. Expression of cathelicidin by epithelial cells and innate immune cells can be induced by short chain fatty acids derived from bacterial metabolism and by vitamin D [50-52]. Indeed, vitamin D deficiency resulting in inadequate cathelicidin production has been proposed as a mechanism of tuberculosis susceptibility in dark-skinned populations [52]. While we are unaware of any direct studies on microbial composition in cathelicidin deficient mice, one study has evaluated the microbiome of mice deficient in vitamin D receptor and Cyp27B1 (the enzyme that generates the active form of vitamin D, 1,25-dihydroxycholecalciferol) [53]. These mice had an altered microbiome characterized at the family level by higher abundance of Desulfovibrionaceae, Bacteroidaceae, and Erysipelotrichaceae and lower abundance of Lactobacillaceae, Lachnospiraceae, Ruminococcaceae, Streptococcaceae, and Deferribacteraceae. Intestinal expression of Cyp27B1 has recently been shown to be regulated by the microbiota, suggesting that vitamin D mediated gardening is modulated by PRR signaling [54]. Interestingly, deficiency of vitamin D receptor (VDR) or Cyp27B1 exacerbates DSS colitis [55,56]. This can be rescued by epithelial-specific transgenic expression of VDR, demonstrating the physiologic importance of vitamin D signaling for epithelial homeostasis [57]. However, vitamin D can enhance tight junctions and is involved in many immune processes including T cell homing to the intestine, maintenance of intraepithelial T lymphocytes, and development of natural killer T cells, so it is unclear if the microbial phenotype of these mice or their susceptibility to colitis is due to reduced cathelicidin expression [56,58-60].

Paneth cells and enterocytes also secrete RegIIIγ, an antimicrobial lectin produced predominantly in the ileum that has preferential activity against Gram positive bacteria [61]. It is minimally expressed in germ-free mice but rapidly induced upon conventionalization. This likely occurs through TLR-mediated microbial sensing by epithelial cells, as epithelial cell specific deletion of MyD88 greatly reduces RegIIIγ [22]. Loss of RegIIIγ expression after antibiotic treatment allowed for expansion of an antibiotic-resistant Gram positive pathogen, vancomycin-resistant *Enterococcus* [62]. RegIIIγ-/- mice have been found to have increased mucosa-associated microbiota in the ileum, characterized by overgrowth of at least two groups of Gram positive organisms - segmented filamentous bacteria (SFB) and *Eubacterium rectale* [22]. This corresponded to increased Th1 activity and IgA production in the small intestine, suggesting that the altered microbiome provoked adaptive immune responses. Increased intestinal Th17 activity was not observed in RegIIIγ-/- mice, but systemic Th17 responses were not assessed [22].

RELMβ

Intestinal goblet cells are capable of secreting not only mucus but also bioactive molecules including RELMβ, a product specific to goblet cells that is upregulated during inflammatory processes and parasite infection [63-65]. RELMβ is undetectable in germ-free mice but is induced within 48 hours of conventionalization, indicating that its expression is controlled by microbial factors [66]. It stimulates macrophages to secrete inflammatory cytokines including TNFα and IL-12, which then promotes IFNγ production by T cells [63,67]. KO mice show reduced DSS colitis severity, impaired responses to helminths, and resistance to diet induced obesity [63,65,67,68]. These mice were found to have an altered microbiome characterized by differential abundance of 15 Bacteriodetes, 15 Firmicutes, and 1 Proteobacteria lineages,

indicating that RELMβ has a gardening effect [68]. However, it is unknown whether the phenotypes of these mice were due to alteration of the intestinal microbiota.

FUT2

Epithelial gardening involves not only antimicrobial and immunomodulatory molecules but also mucus glycoproteins, which serve as attachment sites and energy sources for microbes. Considerable evidence now exists that the intestinal microbiome is influenced by fucosylation – the addition of L-fucose to the terminal β-D-galactose residue of mucus glycoproteins by α1–2 fucosyltransferase (encoded by the FUT2 gene). Fucose is utilized as a nutrient source by some bacteria, most notably *B. thetaiotaomicron*, and can be scavenged by pathogens such as *E. coli* and *Salmonella typhimurium* early during infection [69-71]. Some intestinal microbes utilize fucose to synthesize capsular polysaccharides [72]. In addition, the H antigen created by FUT2 has been shown to serve as an attachment site by pathogens such as norovirus and *Campylobacter jejuni* [73,74]. Fucosylation is a microbiota-dependent process. In germ-free mice, fucosylation occurs immediately after birth but is lost over time [75]. Fucosylation was restored by conventionalization or colonization with *B. thetaiotaomicron*, which expresses an unidentified product in response to low fucose levels that induces fucosylation [76]. Germ-free FUT2-/- mice colonized with human feces had increased abundance of *Parabacteroides*, *Eubacterium*, *Parasutterella*, *Bacteroides*, and Lachnospiraceae and decreased abundance of an unclassified Clostridiales genus in their feces [77]. Interestingly, the microbiome of FUT2-/- mice fed a polysaccharide-deficient diet was unchanged from controls, demonstrating the interplay that exists between diet and host gardening. In humans, deficiency of FUT2 – which occurs in around 20% of individuals, termed "non-secretors – was associated with shifts in microbial composition in the colonic mucosa (increased *Coprococcus* and an unclassified

Lachnospiraceae), feces (reduced *Bifidobacteria*), and bile (reduced Proteobacteria) [78-80]. The altered intestinal microbiome in non-secretors is suspected to account for their increased risk for inflammatory/autoimmune diseases including Crohn's disease, primary sclerosing cholangitis, psoriasis, and type 1 diabetes [80-83].

FUT2 is not the only reported example of non-immune genetic gardening. Sialyltransferase is an enzyme that synthesizes sialyllactose in milk, which may act as a nutrient source for some microbes and affect bacterial adhesion. In its absence, knockout and WT co-fostered mice developed an altered microbiome characterized by decreased susceptibility to DSS colitis [84]. Apolipoprotein A1 is a component of high-density lipoprotein. Knockout mice have features of metabolic syndrome, in particular impaired glucose tolerance, and alterations in microbial composition [85]. In addition, other glycosylation enzymes besides FUT2 involved in mucus formation may also affect microbial composition. For instance, deficiency of enzymes necessary for the synthesis of core 1, core 2, and core 3 O-glycans present in mucus have been reported to increase DSS colitis susceptibility or induce a microbiota-dependent spontaneous colitis [86-88]. It is not yet known if these phenotypes were related to alterations in microbial composition, but this is a plausible mechanism.

Innate lymphoid cells (ILCs)

In recent years, groups of cells have been characterized that resemble subsets of T cells but lack a rearranged antigen receptor. These innate lymphoid cells include natural killer (NK) cells, GATA3-dependent cells with a Th2-like cytokine profile, and RORγt-dependent cells [89]. RORγt-dependent ILCs are the primary intestinal source of IL-22, a cytokine induced by IL-23 which is critical for epithelial production of antimicrobial proteins including RegIIIβ, RegIIIγ,

S100A8, and S100A9 [90-92]. Mice lacking ILCs have minimal RegIIIγ expression by the

intestinal epithelium [90,93]. IL-22 production appears to be regulated by the microbiota in a

manner that depends on the route of exposure to microbial ligands. Colonization of germ-free

mice reduced IL-22 production by ILCs, possibly via induction of epithelial IL-25 by microbial

products [90]. In contrast, systemic administration of a bacterial product, flagellin, increased IL-

22 production by triggering IL-23 release from CD103+ DCs via TLR5 [94].

The ability of ILCs to regulate antimicrobial production by epithelial cells has made them

an attractive candidate gardening cell type in the mucosa. Indeed, antibody-mediated depletion of

ILCs and anti-IL-22 treatment resulted in increased SFB levels in the feces [95]. It has not yet

been reported whether ILCs influence the composition of the remainder of the microbiome in the

intestinal lumen. ILC depletion and anti-IL-22 treatment has also been found to permit systemic

dissemination of *Alcaligenes xylosoxidians*, an invasive Proteobacteria species that normally is

contained to mouse Peyer's patches and mesenteric lymph nodes [92]. This suggests that in

addition to gardening luminal SFB, possibly via regulation of IL-22, ILCs also guard against

invasive species that have penetrated the epithelial barrier.

A subset of RORγt-dependent ILCs has recently been described that expresses T-bet,

lacks CCR6 expression, and differentiates into NKp46+ RORγt-dependent ILCs [96]. These cells

are reduced in number in germ-free mice. A similar reduction is seen in MyD88-/-TRIF-/- mice,

suggesting that TLR signaling controls this population. IFNγ-producing T-bet+CCR6-RORγt-

dependent ILCs played a critical role in the intestinal response to *Salmonella* and may also be

involved in microbial gardening. This ILC subset is absent in Rag-/-Tbet-/- mice, which develop

an altered microbiota causing transmissible colitis [97,98]. These mice had differential

abundance of 69 phylotypes in feces by 16S sequencing and increased abundance of *Klebsiella*

pneumoniae and *Proteus mirabilis* (both Proteobacteria) by culture [99]. It was subsequently

discovered that the colitis phenotype was attributable to colonization with *Helicobacter*

typhlonius [98]. This invasive Proteobacteria triggered a response by an IL-17-producing CCR6+

subset of RORγt-dependent ILCs, previously shown to mediate colitis induced by *Helicobacter*

hepaticus [98,100]. Of note, the emergence of a colitogenic microbiota in Rag-/-Tbet-/- mice was

initially attributed to increased production of TNFα by dendritic cells (DCs) although it now

appears to be most likely due to a deficit in T-bet-dependent ILCs [97]. Current evidence argues

against a role for DCs in gardening, as mice deficient in the two CD103+ lamina propria DC

populations had no alteration in intestinal microbiota [98]. However, the CD103-CD11b+ DC

subset which expresses TNFα in Rag-/-Tbet-/- mice has not been studied [101].

Adaptive immune cells

B cells

Evidence for a role of the adaptive immune system in microbial gardening has come largely from

studies of B cells. Intestinal B cells are present in the lamina propria, isolated lymphoid follicles

(ILF), and Peyer's patches (PP). They develop into IgA-producing plasma cells after undergoing

class switching and affinity maturation in a T-cell-dependent manner in PP germinal centers or a

T-cell-independent manner in ILFs and the lamina propria [102,103]. This process requires

activation-induced cytidine deaminase (AID) and microbial triggers. Germ-free mice have few

IgA-secreting intestinal B cells despite normal numbers of B cells [104]. IgA is rapidly induced

upon bacterial colonization and persists even after disappearance of bacteria from the gut,

suggesting that IgA-secreting B cells are imprinted by microbial exposure rather than requiring

ongoing stimulation [105].

Mice deficient in all B cells were found to have alterations in the jejunal microbiome including increased *Paracoccus*, increased *Lactococcus*, and decreased Clostridiaceae [106]. The absence of non-IgM antibodies and somatic hypermutation due to AID deficiency caused increased culturable anaerobes and SFB in the small intestine [107,108]. This could be reversed by surgically connecting the circulatory system of AID knockouts to wild-type mice, indicating that serum factors (presumably immunoglobins) mediated the phenotype [108]. Similar microbial changes were observed in Rag-/- mice (i.e. T and B cell deficient), which could be reversed by bone marrow transplantation from WT but not AID-/- mice. The importance of somatic hypermutation in IgA gardening was recently shown using AID knock-in mice carrying a mutation that allowed for normal class switching but disrupted somatic hypermutation [109]. These mice had alterations in their small intestinal microbiome characterized by increased culturable anaerobes and increased abundance of *Lachnospiraceae*, Bacteroidales, and *Lactobacillus*. Recently, a population of small intestinal IgA-producing plasma cells was discovered that expresses inducible nitric oxide synthase (iNOS) and TNFα in a microbiota-dependent manner [110]. Absence of expression of both iNOS and TNFα in B cells resulted in deficiency of IgA-producing cells in the lamina propria. Interestingly, this was accompanied by reduced small intestinal SFB, suggesting that B cells can garden in a TNFα/iNOS manner that favors SFB whereas IgA gardens in a manner that limits SFB.

T cells

T cells, in particular T follicular helper (T$_{FH}$) cells, have been implicated in microbial gardening through their regulation of B cell class switching and affinity maturation in PP germinal centers [111]. T$_{FH}$ cells have characteristically high expression of the inhibitory receptor, programmed cell death-1 (PD1). In the absence of PD1, mice develop antibody-mediated arthritis,

glomerulonephritis, and cardiomyopathy [112,113]. PD1-/- mice also have an altered

microbiome, including increased culturable anaerobic bacteria in the small intestine and

increased Erysipelotrichaceae, Prevotellaceae, Alcaligenaceae, and TM7 in the cecum [114].

This was attributed to poor affinity maturation of the IgA repertoire due to aberrant B cell

selection in PP germinal centers [115]. Possible additional evidence for T cell regulation of B-

cell-mediated gardening comes from a study of mice lacking TGFβ-induced FoxP3-expressing

regulatory T cells due to CNS1 deficiency. These mice had increased numbers of germinal center

B cells and produced autoantibodies reacting against intestinal antigens [116]. They developed a

B-cell-mediated enteritis characterized by plasma cell expansion and Th2 cytokine expression.

These mice had increased TM7 and *Alistipes* in their feces. However, it is unclear if this was due

to altered gardening (perhaps secondary to heightened B cell activity) or was a secondary

consequence of intestinal inflammation.

CD1d-restricted T cells

Natural killer T (NKT) cells are a class of T cells that express NK markers and respond to lipid

antigens presented by CD1d as well as to cytokine stimulation by IL-12, IL-18, and type 1

interferons [117]. They have potent effector activity through their release of cytokines including

IFNγ, IL-4, IL-17, and TNFα. A subset of these cells, termed invariant NKT (iNKT) cells, has a

restricted TCR repertoire and has been shown to be strongly regulated by microbial signals. In

germ-free mice, iNKTs are increased in the colonic lamina propria in a CXCL16 dependent

manner but decreased in the liver and spleen, which could be reversed by colonization with

Sphingomonas (which expresses ligands recognized by iNKT cells) [118-120]. iNKTs respond to

a wide range of pathogens and may also be involved in gardening [121]. One study of CD1d-/-

mice observed alterations in the fecal microbiota, increased ileal SFB, and increased small

intestinal colonization by pathogens including *E. coli* and *Pseudomonas aeruginosa* [122]. NKTs may be involved in gardening downstream of NLRP6 and IL-18, but this has not yet been evaluated.

γδ intraepithelial lymphocytes (IELs)

A diverse collection of T cells reside within the intestinal epithelium, of which about 60% express TCRγδ. This IEL subset secretes growth factors that promote epithelial growth/repair and can combat enteric pathogens through IFNγ production and direct cytotoxic activity [123]. After epithelial injury, γδ IELs prevent mucosal penetration of bacteria by secreting growth factors, chemokines, and antimicrobial products in a partially MyD88-dependent manner [124]. γδ IELs may also play a role in gardening invasive pathobionts in the absence of epithelial injury. Colonization of germ-free mice induces expression of antimicrobials by γδ IELs including RegIIIγ and RegIIIβ [125]. This could be reproduced by colonization with a single endogenous mucosa-invasive *E. coli* species or wild-type *Salmonella* (but not a non-invasive mutant), suggesting that γδ T cells respond to microbes that penetrate the mucus barrier. Absence of γδ T cells resulted in an inability to control new invasive species early after exposure. WT mice co-housed with TCRγδ-/- mice showed increased RegIIIγ expression by γδ T IELs, suggesting that these mice were exposed to mucosa invasive bacteria which arose in TCRγδ-/- mice due to deficient gardening. However, it has not yet been directly demonstrated to our knowledge that loss of γδ T cells alters the composition of the microbiome.

Inter-individual variation in microbial composition due to genetic variation in gardening

The existence of multiple mechanisms for gardening the intestinal microbiome raises the possibility that variation in gardening genes can contribute to microbial diversity within populations. This has been supported by multiple mouse studies. Comparison of 8 inbred strains revealed that each had a distinct microbial composition which was not eliminated by cohabitation, though founder effects could not be excluded [126]. Two quantitative trait loci (QTL) analyses of interbred mouse lines have identified multiple genetic factors influencing the abundance of microbes at various taxonomic levels [127,128]. The first study identified thirteen QTLs affecting microbial composition, including one mapping to a region containing IL-22, IFNγ, and Irak3 (an inhibitor of TLR signaling) that affected *Lactococcus* and Coriobacteriaceae abundance [127]. The second study identified five QTLs, of which three contained candidate genes affecting immune activity (consistent with the immune system playing a major role in gardening) [128]. This included type 1 interferons, Irak4 (a signaling molecule utilized by some TLRs), and Tgfb3, which influenced the abundance of *Bacteroides*, Rikenellaceae, and Prevotellaceae, respectively. Host genetics can also interact with diet, as was shown in a study reporting wide variation among 52 strains of mice in the effect of a high-fat, high-sucrose diet on the microbiome [129]. The authors identified a single nucleotide polymorphism (SNP) near 3 amylase genes which was associated with enrichment of *Enterobacteriaceae* on a high fat, high-sucrose diet.

In humans, twin studies have been used to evaluate the global impact of genetic variation on microbial composition. The intestinal microbiome has consistently been shown to be more similar between pairs of monozygotic twins than between pairs of unrelated individuals [130-134]. However, monozygotic twins share many environmental factors including maternal colonizers, early life environment, and childhood diet. This can be controlled by comparing

monozygotic twins to dizygotic twins. Significantly increased microbial similarity between monozygotic twins compared to dizygotic twins has been reported in two studies using the Sorensen index to analyze data from 16S Sanger sequencing and temporal temperature gradient gel electrophoresis [130,133]. In contrast, two studies using high throughput 16S sequencing and a phylogenetic similarity measure, Unifrac, reported no difference in microbial similarity between monozygotic twins compared to dizygotic twins [131,132]. However, even if this is correct, the global similarity analysis used in these studies may not reveal shifts in the abundance of specific microbial groups due to genetic variation.

This review has already mentioned two genes, FUT2 and NOD2, with genetic variants that affect microbial composition [34,78-80]. Recently, a microbiome association study using 30 Crohn's associated SNPs identified a genetic variant, the rs11747270a SNP, that correlates with *Prevotella* abundance in sigmoid colon biopsies [135]. This SNP maps to the locus for immunity-related GTPase family M (IRGM) [136]. This protein regulates autophagy, a process triggered by PRRs such as NOD2 that is important for clearance of intracellular bacteria including *Salmonella*, *Mycobacterium*, and invasive *Escherichia coli* [137]. A second Crohn's associated IRGM polymorphism has been reported to reduce the effectiveness of autophagy in response to intracellular bacteria [138]. Interestingly, *Prevotella* species including *P. intermedia* and *P. bivia* have been reported to invade epithelial cell lines [139,140]. It is conceivable that the elevated abundance of *Prevotella* in colonic biopsies was due to increased survival of invasive organisms due to deficient IRGM activity. As further genome wide association studies are performed for microbial composition, it is likely that many additional examples of genetic polymorphism in gardening genes will be identified in human populations.

Acquired immune deficits can influence the microbiota

The critical role of mucosal immunity in shaping the intestinal microbiome suggests that not only genetic but also acquired deficits in immune function could affect the microbiota. A common cause of acquired immunodeficiency in humans in recent decades has been HIV. It is now appreciated that HIV transmission, replication, and persistence occur largely in mucosal tissues [141]. Rapid depletion of mucosal memory CD4+ T cells occurs during acute infection and is sustained throughout the chronic phase of disease [142]. This is accompanied by a decrease in the ratio of Th17:Tregs, increased CD8+ T cells, and decreased NK cells. As a consequence of these immune disturbances, HIV patients have increased epithelial permeability, systemic microbial translocation, and elevated serum inflammatory markers consistent with a chronic inflammatory state [143]. In most patients, these mucosal immune disturbances are not reversed completely by highly active anti-retroviral therapy [144]. In light of changes in mucosal immunity induced by HIV, it has been predicted that HIV infection would impact the intestinal microbiome. A longitudinal study of chimpanzees before and after SIV infection observed increased abundance of *Sarcina*, *Staphylococcus*, and *Selenomonas* in the feces [145]. Three recent papers evaluating the mucosal and fecal microbiome of untreated and treated HIV patients have confirmed altered microbial communities in HIV [146-148]. All three identified a reduction in Alistepes and two identified a reduction in Bacteroides. However, they each reported enrichment of different microbial groups in HIV patients. One identified enrichment of multiple members of the Proteobacteria phylum (including pathobionts such as *Pseudomonas*), another *Prevotella*, and the third *Fusobacterium* (a genus which includes pathobionts that can invade epithelial cells) [149]. The discrepancies may reflect different sampling sites in each of the studies or stochastic overgrowth of pathobionts in the face of disrupted immune gardening.

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Acquired deficits in immune function can also be seen in patients treated with immunosuppressive medication to prevent rejection of solid organ transplants. While we are not aware of published reports on the intestinal microbiome of this population, at least one study has examined the oral microbiome. Transplant patients were found to have altered microbial composition including increased abundance of pathobionts such as *Klebsiella*, *Acenitobacter*, and *Pseudomonas* [150]. More broadly, medications that affect the immune system – such as immunomodulators and biologics (not including antibiotics) used to treat inflammatory bowel disease, rheumatic diseases, and psoriasis – could also potentially affect microbial gardening and result in disease-promoting dysbiosis. There is minimal data at present on this topic, though the existence of medication-specific microbial profiles was suggested in the oral microbiome study and one of the HIV studies [148,150].

Conclusion

There is now extensive evidence that genetically encoded mechanisms exist for gardening the intestinal microbiome. These pathways pair microbial sensing by PRRs to antimicrobial effector mechanisms such as defensins, RegIIIγ, and IgA (Fig. 2-1). However, many questions remain. At present, the gardening activity of specific PRRs has been characterized in terms of shifts at the genus or higher taxonomic level in knockout mice. It is unknown what changes occur at the species or strain level, whether PRRs have overlapping activity, and which microbial features trigger gardening pathways dependent upon a single PRR (e.g. NLRP6-mediated gardening of Prevotella, which was unaffected by loss of any other inflammasome-forming NLR) [38]. The effector mechanisms that allow for targeted gardening of specific microbes are also unknown

(other than IgA), as host antimicrobial products generally have a wide spectrum of activity. While many immune cell types have been implicated in gardening, the relationships between each immune cell type and specific microbial taxa are largely uncharacterized with exceptions such as SFB gardening by B cells and NK T cells.

Despite the many unknowns, it is clear that gardening of the intestinal microbiome is critical for health. In many studies, disruption of gardening resulted in dysbiosis which conferred susceptibility to diseases including colitis, obesity, and fatty liver disease. This often corresponded to a bloom of pathobionts, especially Proteobacteria such as *Escherichia coli* in TLR5-/- mice, *Prevotella* in NLRP6-/- mice, *Helicobacter* in Rag-/-Tbet-/- mice, and *Alcaligenes* in ILC-deficient mice. The three human genetic variants associated with gardening are all also risk factors for Crohn's disease, possibly due to dysbiosis. Gardening can be affected not only by genetic variation but also by diet (as shown for tryptophan/defensins), stress (as shown for NLRP6), and acquired immune deficits (including, potentially, due to immunosuppresive medication). Disease-promoting dysbiosis arising from aberrant microbial gardening could prove to be a common theme in medicine.
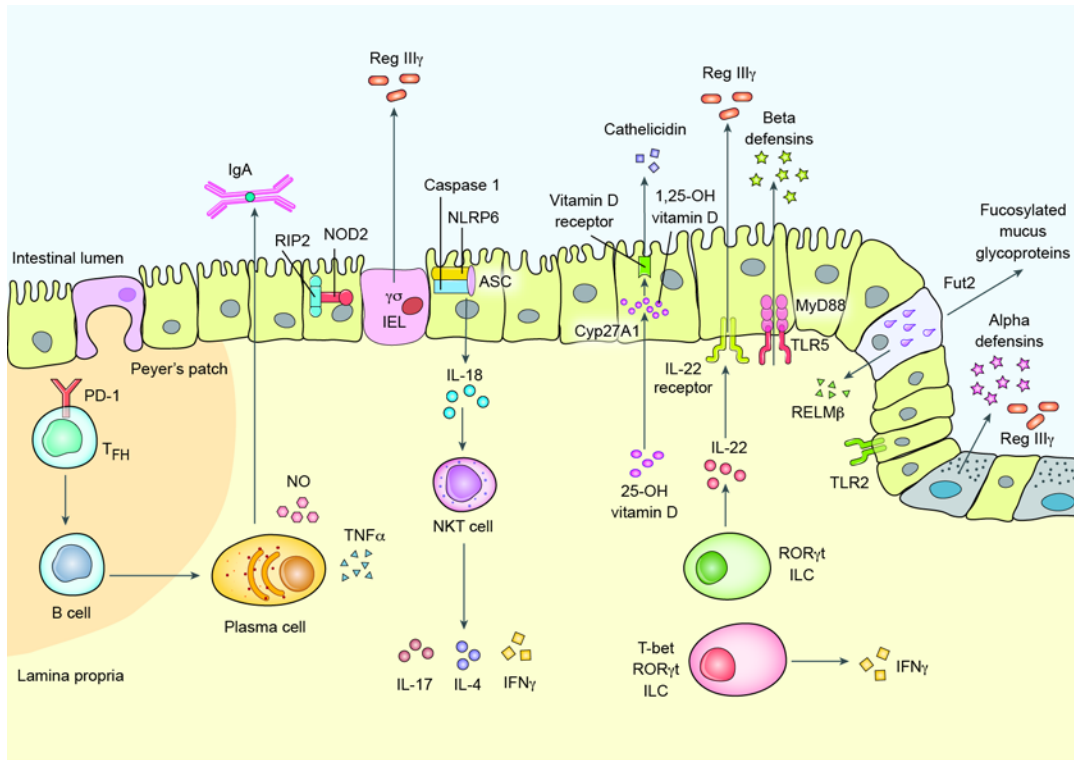
Figure 2-1. Putative mechanisms for immune gardening of the intestinal microbiome.

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CHAPTER 3

DISEASE-ASSOCIATED METABOTYPES AND ENTEROTYPES IN FAMILIES WITH

PEDIATRIC INFLAMMATORY BOWEL DISEASE

Abstract

To better understand the role of the intestinal microbiome in inflammatory bowel disease (IBD),

we investigated its effect on intestinal metabolites in IBD patients and healthy relatives in the

same household. We performed liquid chromatography / mass spectrometry (LC/MS) analysis

and 16S ribosomal RNA sequencing on fecal samples from 21 pediatric IBD patients in clinical

remission and their 69 first-degree family members, 15 of whom also had IBD. Individuals

bioinformatically grouped into three metabotypes based on their LC/MS features. The

metabotype most strongly associated with IBD was characterized by elevated bile acids while the

metabotype strongly associated with health was characterized by increased steroid hormones. By

microbial composition, individuals grouped into two enterotypes. One enterotype was associated

with IBD but irrespective of disease status was characterized by lower diversity and increased

levels of co-correlated Lachnospiraceae and Enterobacteriaceae. Metabotypes and enterotypes

were highly correlated, suggesting that they interacted as integrated ecosystems. Multivariate

analysis demonstrated that many of the differences in abundance of microbes and metabolites

between IBD patients and healthy individuals were attributable to metabotype and enterotype.

The IBD-associated enterotype and metabotypes were observed in a subset of healthy relatives

including children, arguing that they do not simply represent the effects of intestinal inflammation but rather represent a stable host-microbiome state. Healthy individuals who have an IBD-associated enterotype and metabotype may be at increased risk for developing IBD.

Introduction

Inflammatory bowel disease (IBD) – comprised of Crohn's disease (CD) and ulcerative colitis (UC) – is a chronic inflammatory disease with growing prevalence in the Western world. IBD is believed to arise from a combination of genetic susceptibility and environmental factors that trigger an inappropriate mucosal inflammatory response (*1*). A role for the microbiome in the pathogenesis of IBD has been suggested by the observation that many experimental colitis models fail to develop in germ-free animals or animals treated with broad spectrum antibiotics (*2, 3*). This is consistent with the results of genome-wide association studies, which have identified many IBD-associated genes involved in mucosal host-microbe interactions (*4*). Moreover, dysbiosis in animal models arising due to genetic defects in mucosal immunity can induce microbiota-dependent spontaneous colitis or confer enhanced susceptibility to experimental colitis that is transmissible to wild-type animals via the microbiome (*3, 5*). These findings support the hypothesis that IBD is triggered by dysbiosis arising from genetic perturbations of mucosal gardening of the microbiome (*6*). Environmental factors are likely to also be critical given that the concordance rate of IBD in monozygotic twins is 30-50% (*7*). Diet may be of particular importance in promoting or preventing dysbiosis given the strong evidence from controlled trials that diet changes the microbiome and the epidemiologic association of IBD with dietary factors such as fiber intake (*8, 9*).

A number of studies have demonstrated reduced microbial diversity and alterations in the composition and function of the intestinal microbiome in IBD patients compared to healthy controls (*10-13*). However, it remains unclear to what extent differentially abundant microbes in IBD patients are instigators of disease rather than bystanders responding to the altered environment of the inflamed intestine. These microbes may influence the inflammatory process by generating bioactive metabolites that affect immune activity and epithelial function. In animal models, microbial products can have profound effects on mucosal immune responses and epithelial homeostasis (*14*). For instance, short chain fatty acids (SCFA) such as butyrate can limit inflammatory responses in colitis models by promoting colonic regulatory T cells (*15*). *Faecalibacterium prausnitzii* is a member of the Ruminococcaceae family that produces butyrate and has been consistently reported to have reduced abundance in IBD (*12, 13, 16*). Administration of *F. prausnitzii* or its culture supernatant ameliorated experimental colitis, providing a potential mechanistic link between alterations in the IBD microbiome and disease susceptibility (*16*). It was postulated that the relevant metabolite may be butyrate; however, treatment of mice with butyrate at the same concentration as was present in the supernatant did not affect colitis severity. These findings underscore the need to characterize the IBD-associated metabolome to identify novel microbial products relevant to disease pathogenesis.

Metabolomics analysis of fecal extracts is of particular interest as this may capture microbiome-derived metabolites that influence mucosal homeostasis locally. The fecal metabolome of IBD has been reported to be enriched in amino acids and bile acid metabolites and depleted in short chain fatty acids and methylamines (*17-20*). The alterations in the fecal metabolome of IBD patients are likely influenced greatly by microbial communities in IBD based on our prior work demonstrating close correlation between the colonic mucosal

microbiome and metabolome in healthy individuals (*21*). Comparison of the microbiome and metabolome in IBD patients represents a promising strategy to reveal IBD-specific microbe-metabolite relationships that influence the inflammatory response.

If IBD develops as a consequence of a host response to dysbiosis, as has been reported in animal models, IBD patients would be predicted to harbor dysbiosis prior to the development of intestinal inflammation. Existing studies have been designed as cross-sectional cohorts of IBD patients compared to healthy patients. We performed a family based study to evaluate the microbiome and metabolome of IBD patients and their healthy relatives who would be at higher risk for dysbiosis than the general population due to shared genetic and environmental factors with the IBD proband. We found that fecal metabolomic profiles could fall into one of several states and that these states were highly correlated with disease and with dysbiosis. We propose that in families at risk for IBD, a limited number of stable intestinal microbiome/metabolome states exist including one or more that confer susceptibility to IBD.

Materials and Methods

Cohort recruitment and sample collection

21 CD and UC patients under the age of 18 were recruited at the Cedars-Sinai Medical Center. All patients were in clinical remission at the time of collection as evidenced by a Harvey-Bradshaw Index score of less than 3. Eight of the probands had a recent colonoscopy; all had a partial Mayo score of 0. A questionnaire was administered to obtain clinical metadata and peripheral blood was drawn. Participants were provided with a toilet hat, sample containers, and cold packs for home collection. Freshly defecated feces was immediately frozen at home or in

the clinic then brought to the clinic on cold packs for storage at -80°C. Frozen fecal samples were ground with mortar and pestle in the presence of liquid nitrogen then aliquoted for microbiome and metabolome analysis. This research was approved by the Cedars-Sinai Medical Center Institutional Review Board under IRB #3766.

16S rRNA gene sequencing

Genomic DNA extraction, amplification of the V4 region of 16S ribosomal RNA genes, and single-end sequencing on an Illumina HiSeq 2000 were performed as described previously (*22*). The 101 base pair reads were processed using QIIME v1.7.0 with default parameters (*23*). The number of reads per sample ranged from 17,946 (an outlier; the second lowest sequence depth was 478,168) to 848,638, with a mean of 620,720. OTUs were picked against the May 2013 version of the Greengenes database (http://greengenes.secondgenome.com), pre-filtered at 97% identity. 93.6% of the total reads were successfully mapped. Alpha diversity was assessed using phylogenetic diversity, Chao1, and Shannon index with data rarefied to 400,000 sequences. Beta diversity was calculated using unweighted UniFrac for all pairwise combinations of samples across groups. PCoA was performed with distance matrices calculated using the square root of the Jensen-Shannon divergence . Adonis with 100,000 permutations was used to assess statistical significance.

Microbial module analysis

Correlation among microbes was calculated using SparCC, a method that adjusts for compositionality effects in 16S sequence data (*24*). The SparCC correlation matrix was inputted into WCGNA, a network based approach for clustering features into modules (*25*). Network construction and selection of modules was then performed as previously described with a minimum module size of 10 (*26*). The first eigenvector of the abundance matrix for each module

was used to represent abundance of that module in interomic analyses with predicted metagenes or metabolites. Module enrichment in subsets of the cohort (e.g. IBD patients) was assessed using Gene Set Enrichment Analysis (GSEA) with modules as pre-selected sets and preranked lists of OTUs ordered by the negative logarithm of q-value from DESeq2 multivariate models (*27*). Modules were considered enriched for a particular phenotype if the enrichment statistic reached significance with q<0.05.

Predicted metagenomics analysis

The metagenomic content of samples was inferred from 16S sequence data using PICRUSt 1.0 and the KEGG database (*28*). Metagenes with differential abundance by IBD status or enterotype were identified using DESeq2. Cross-correlation of metagenes with microbial modules was performed using Spearman's correlations of the residuals of mixed effects model including gender, anti-TNF therapy, Jewish ethnicity, and mode of delivery as fixed effects and family as a random effect. Enrichment of KEGG pathways among metagenes associated with microbial modules was calculated using hypergeometric tests.

LC/MS metabolomics

50 mg of each fecal sample was dried, resuspended in 150 μL of Optima LC/MS grade water on ice, subjected to heat shock in a 37°C water bath for 90 seconds, then chilled on ice. 1 μL from each sample was removed for protein concentration measurement. 600 μL of chilled methanol containing internal standards for lipidomics and metabolomics was added to each sample and incubated on ice for 15 min as previously described (*29*). An equal volume of chloroform was then added to the mixture. The samples were then centrifuged at 16000g for 10 min. The organic and aqueous phases were separated carefully followed by addition of 600 μL of chilled acetonitrile for overnight incubation at -20°C. The samples were then centrifuged at max speed

at room temperature for 10 minutes. Supernatants were combined and transferred to new glass tubes, dried under a gentle stream of N_2, and resuspended in 100 μL of solvent A for LC/MS.

The MS analysis was performed by injecting 2 μL aliquots of each sample into a reverse-phase 50×2.1 mm H-class UPLC Acquity 1.7-μM BEH C18 column (Waters Corp, Milford, MA) coupled to a time of flight mass spectrometry (TOFMS). The mobile phase consisted of water and 0.1% formic acid (solvent A), 100% acetonitrile (solvent B), and isopropanol/acetonitrile (90:10 v:v) with 10 mM ammonium formate (solvent C). The Xevo G2-S mass spectrometer (Waters Corp, Milford, MA) was operated in the positive (ESI^+) and negative (ESI^-) electrospray ionization modes scanning a 50-1200 *m/z* range. The following 13 minute gradient was used: 95%/5% solvent A/solvent B at 0.45 ml/min for 8 minutes, 2%/98% solvent A/solvent B for 1 minute, 2%/98% solvent B/solvent C for 1.5 minutes, 50%/50% solvent A/solvent B for 1.5 minutes, and 95%/5% solvent A/solvent B for the remaining half a minute. The lock-spray consisted of leucine-enkephalin (556.2771 $[M+H]^+$ and 554.2615 $[M-H]^-$). The MS data were acquired in centroid mode and processed using MassLynx software (Waters Corp, Milford, MA) to construct a data matrix consisting of the retention time, *m/z*, and abundance value (via the peak area normalized to protein concentration) for each ion. A total of 3206 ions in the positive mode and 1420 ions in the negative mode were detected. PCoA of abundance data was performed with distance matrices calculated using the square root of the Jensen-Shannon divergence.

Our in-house statistical analysis program was used to putatively identify ions, utilizing the Human Metabolome Database (HMDB), LipidMaps, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and BioCyc. The *m/z* values were used to putatively assign IDs to the ions by neutral mass elucidation, which was accomplished by considering the possible

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adducts (H^+, Na^+, and/or NH_4^+ in the ESI^+ mode; H^- and Cl^- in the ESI^- mode). The masses were

then compared to the exact mass of small molecules in the databases, from which putative

metabolites were identified with a mass error of 20 parts per million (ppm) or less. MSMS

validation was performed by comparing the fragmentation pattern of each metabolite and lipid of

interest against that of its pure chemical form either in the METLIN database or the in-house

database (*30*). Validation was performed for 123 spectral features, of which 47 were successfully

validated (representing 37 unique metabolites).

Enterotype/metabotype analysis

Individuals were grouped into enterotypes and metabotypes by applying Dirichlet multinomial

mixture (DMM) models to 16S sequence data and normalized LC-MS metabolomic data (*31*).

This approach outperformed partitioning around medoids for clustering individuals by their

microbiome in simulation studies and using Human Microbiome Project data (*32*). Model fit for

each possible number of enterotypes or metabotypes was estimated using the Laplace

approximation. The exponent of the difference in model fit serves as an estimate of the

probability of one model over another.

Differential abundance testing

16S sequence data and LC-MS metabolomic data were filtered to remove OTUs and spectral

features present in less than 10% of samples. The sample with 17,946 16S reads was excluded.

The resulting filtered datasets were analyzed using the DESeq2 algorithm, which employs an

empirical Bayesian approach to shrink dispersion (*33*). Log fold changes for each OTU or

metagene were fitted to a general linear model under a negative binomial model. Covariates

included gender, Jewish heritage, anti-TNF therapy, mode of delivery, family, IBD group, and

enterotype/metabotype where indicated. P-values for variables in the linear models (e.g. IBD

status) were converted to q-values to correct for multiple hypothesis testing (*34*). Q-values below 0.05 were considered significant. Hierarchical clustering of metabolite abundance data and visualization with heat maps were performed in GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E).

Interomic analysis

Procrustes analysis was performed using the square root of the Jensen-Shannon divergence to compare the microbiome and metabolome (*21*). Significance of Procrustes analysis was determined using 10,000 Monte Carlo permutations in QIIME. Interomic correlation analysis was performed on the residuals of abundances of microbial modules, predicted metagenes, and spectral features from mixed effects models incorporating IBD status, enterotype (for OTUs), metabotype (for spectral features), gender, anti-TNF therapy, Jewish ethnicity, and mode of delivery as fixed effects and family as a random effect. Spearman correlation was calculated for all combinations of features in the two datasets of interest (e.g. OTUs and metabolites). P-values were computed for spearman correlation coefficients using the asymptotic *t* approximation then were converted to q-values. OTU-metabolite correlation pairs between enterotype 2 and metabotype 3 with a q-value less than 0.05 were used to construct a microbe-metabolite network in Cytoscape 3.2.1 (http://cytoscape.org).

Genetic risk score

DNA was extracted from peripheral blood and applied to Immunochip, a custom platform containing nearly 200,000 single nucleotide polymorphisms near genes related to immune function and inflammatory disease. Allele frequencies were used to calculate genetic risk score as described previously (*35*).

Logistic regression

Multivariate and univariate logistic regression was performed in R using the glm function. Multivariate models including parent/child status and Jewish ethnicity had improved fit by the likelihood ratio test relative to models without these covariates.

Results

A low-diversity enterotype is present in IBD patients and some family members without IBD

We recruited a cohort of 21 families with pediatric IBD, consisting of 36 individuals with IBD (21 probands, 6 siblings, and 9 parents) and 54 healthy first-degree relatives (Table 3-1). The 36 IBD patients included 26 with CD and 10 with UC. All were in clinical remission to avoid confounding of microbial and metabolomics analyses by the effects of active inflammation. 20 out of 22 of the probands and their siblings with CD had small intestinal involvement; of these, 14 also had colonic involvement. All but 5 of the IBD patients were on medication, including 21 on a tumor necrosis factor (TNF) inhibitor (with or without immunomodulators, i.e. methotrexate or 6-mercaptopurine), 2 exclusively on an immunomodulator, and 9 on 5-aminosalicylic acid (5-ASA) therapy. The probands and their siblings underwent an extensive characterization of their neonatal and early life medical history. No difference was seen between children with IBD and their healthy siblings in mode of delivery (vaginal vs. Cesarean), birth order, gestational age, birth weight, perinatal disease, maternal antibiotic usage pre-delivery, maternal age, and early life antibiotic treatment. The cohort largely consisted of Caucasians, the majority self-identifying as Jewish (60 individuals). Two parents were non-Caucasian (1 Asian, 1 Hispanic), accounting for four mixed race children in the cohort.

High depth 16S rRNA sequencing was performed on fecal samples (mean 620,720 reads per sample) to detect low abundance organisms that may be critical for IBD susceptibility. CD patients had lower microbial diversity than UC patients and healthy individuals as measured by phylogenetic diversity, species richness (Chao1 index), and evenness (Shannon index) (Fig. 3-1A). UC patients showed a trend towards reduced diversity compared to healthy individuals that achieved statistical significance only for phylogenetic diversity. Increased similarity of the microbiome was observed when comparing individuals within IBD group (CD, UC, healthy) versus across IBD group using a phylogenetic measure, unweighted UniFrac (Fig. 3-1B). Principal coordinate analysis (PCoA) was used to visualize microbial composition. Samples formed a gradient by microbial diversity that largely separated by IBD group (Adonis $p<10^{-5}$), with CD patients and healthy individuals occupying different ends of the PCoA plot and UC patients interspersed between the two groups (Fig. 3-1C). Family also had a statistically significant effect on microbial composition (Adonis $p < 10^{-5}$) (Fig. 3-1B). Microbial families associated with both CD and UC in multivariate models included Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, Pasteurellaceae, Streptococcaceae, Mycoplasmataceae, and Gemellaceae (Fig. 3-1D). Several families were specific to CD including Aeromonadaceae, Fusobacteriaceae, and Veillonellaceae. Our results were largely concordant with those of the RISK cohort: five out of six microbial families increased in CD patients in the RISK cohort were increased in CD patients in this cohort (*13*).

It has been reported that the human fecal microbiome can be divided into a limited number of community types, termed enterotypes (*32, 36*). To investigate whether enterotype could account for variation in microbial composition in this cohort, we classified individuals into enterotypes using Dirichlet multinomial mixture models. These models best fit the data when the

cohort was divided into two enterotypes (Fig. 3-2A, Fig. 3-2B). Enterotype 2 was associated with IBD, particularly CD, while most non-IBD individuals belonged to enterotype 1 (Fig. 3-2C). Of note, 10 non-IBD individuals had the IBD-associated enterotype 2. In PCoA plots, enterotype 1 encompassed a region with high alpha diversity whereas enterotype 2 mapped to a lower diversity region (Fig. 3-2B, Fig. 3-1C). Enterotype 2 was associated with lower alpha diversity among CD patients and among non-IBD individuals, whereas within the same enterotype smaller differences in diversity were observed between CD and non-IBD that did not achieve significance (Fig. 3-2D). Hence, enterotype 2 represents a low diversity microbial state independent of IBD status. Two families contained 5 out of 10 of the non-IBD enterotype 2 individuals, though the association between enterotype and family did not reach statistical significance (p=0.08) (Table 3-2).

The initial enterotype study reported three enterotypes that were characterized by expansion of *Bacteroides*, *Prevotella*, or *Ruminococcus* (*36*). No statistically significant difference in *Bacteroides* or *Prevotella* abundance was observed between the two enterotypes in this cohort (Fig. 3-2E). *Ruminococcus* was increased in enterotype 1but this was not the exclusive driver of enterotype as two individuals with enterotype 2 had greater *Ruminococcus* levels than all individuals with enterotype 1. In multivariate models, enterotype 2 was associated with increased abundance of a subset of IBD-associated families including Enterobacteriaceae, Enterococcaceae, Streptococcaceae, Clostridaceae, and Veillonaceae (Fig. 3-2F). Adjustment for enterotype resulted in association of fewer microbial families with IBD. When the analysis was performed at a finer taxonomic level - operational taxonomic units (OTUs) at the 97% sequence identity threshold, roughly corresponding to species - more OTUs were associated with enterotype (768) than were associated with CD (91) or UC (36) (Fig. 3-3A). The CD-specific

microbial signature included increased Enterobacteriaceae and *Fusobacterium* as well as decreased *Faecalibacterium prausnitzii* and *Roseburia*, all previously reported features of the CD microbiome (*12, 13*).

The large number of OTUs associated with enterotype suggested that whole microbial communities had shifted. To evaluate this, OTUs were grouped into modules of co-occurring microbes that were then tested for their association with enterotype or IBD status (Fig. 3-3B). Enterotype showed association with 10 modules, including the three modules with the most OTUs. This is consistent with the large ecologic change implied by the decreased microbial diversity seen in enterotype 2. The absence of large modules increased in enterotype 1 suggests that this enterotype has communities with fewer taxa or that are less consistent across individuals. The largest enterotype 2 specific module (E1) was characterized by a preponderance of OTUs in the Lachnospiracaeae family, including *Blautia* and *Dorea*. The three modules that were associated with both enterotype 2 and CD contained Lachnospiracaeae as well as Enterobacteriaceae (EC1 and EC2), *Veillonella* (EC2), *Fusobacterium* (EC3), and *Haemophilus parainfluenzae* (EC3) (Table 3-3). The three modules exclusively associated with CD or UC had few members, suggesting that disease-specific microbial changes may not involve shifts of large communities.

We analyzed the predicted metagenome to determine whether shifts enterotype-associated microbial communities perturbed the functional capabilities of the microbiome. 1783 out of 2032 metagenes associated with IBD and enterotype in multivariates models correlated with the E1 module or the EC1 and EC3 modules (Fig. 3-3C). KEGG pathway analysis demonstrated that E1 was enriched in metagenes related to translation and DNA replication,

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which may facilitate the expansion of this community at the expense of other microbes to produce a low diversity state in enterotype 2 (Fig. 3-3D). EC1/EC3 associated metagenes were enriched in signal transduction and membrane transport, attributable to increased 2 component signaling pathways and amino acid transporters that mediate nutrient uptake by the auxotrophic organisms in these modules (Fig. 3-3D, Fig. 3-4). EC1 and EC3 were also associated with increased type II secretion - used by Gram negative pathogens to secrete effectors such as cholera toxin - and increased type VI secretion, a pathway for toxin delivery into neighboring bacteria that may facilitate the expansion of these communities in enterotype 2 (Fig. 3-4) (*37, 38*).

Intestinal metabotypes are associated with IBD

LC/MS analysis was performed on fecal samples to further investigate the functional consequences of altered microbial composition in this cohort. CD patients and non-IBD patients had distinct metabolomes in both the positive and negative electrospray ionization (ESI) modes (Fig. 3-5A). As with microbial composition, non-IBD individuals and CD patients separated to opposite ends of the PCoA plots while UC patients intermingled with the two groups. The separation of metabolomes by IBD status was highly significant (Adonis $p < 10^{-5}$ in both modes) (Fig. 3-5B). Metabolomes also varied by family (Adonis $p < 10^{-5}$ in both modes), demonstrating that the shared genetic and/or environmental factors within families influenced the metabolome (Fig. 3-5B). We then employed multivariate models to identify spectral features associated with CD or UC adjusting for covariates including gender, anti-TNF therapy, Jewish ethnicity, and mode of delivery. These demonstrated that CD patients had altered abundance of 235 spectral

features whereas UC patients showed comparatively little metabolic change - 12 spectral features, of which 6 were shared with CD and two were validated as 5-ASA (a common treatment for UC in this cohort) (Fig. 3-5C). Hierarchical clustering of differential metabolites in CD demonstrated that most Crohn's patients and a subset of healthy individuals grouped together whereas other non-IBD individuals had a distinct profile (Fig. 3-5D). CD associated metabolites included amino acids (leucine, valine, proline, phenylalanine, tyrosine, and tryptophan), amino acid derivatives (serinyl tryptophan, phenylethylamine, N-acetylcadaverine), bile acids (cholic acid, 7-ketodeoxycholic acid, chenodeoxycholic acid sulfate, 3-sulfodeoxycholic acid), and an omega-6 fatty acid (7,10,13,16-docosatetraenoic acid) (Fig. 3-5E). Metabolites increased in non-IBD individuals included two heme degradation products (stercobilin, mesobilirubinogen), a glutamate derivative (acetyl-glutamic acid), and a product of steroid catabolism (1,4-androstadiene-3,17-dione) (Fig. 3-5F).

The grouping of some non-IBD individuals with CD patients by hierarchical clustering suggested the existence of metabotypes in this cohort analogous to the two enterotypes. Dirichlet multinomial mixture models supported three metabotypes in the positive ESI mode (the probability of four rather than three was estimated as 10^{-6}) and two in the negative ESI mode, which could be mapped to two of the positive mode metabotypes (Fig. 3-6A, Fig. 3-7). The positive mode metabotypes were then chosen to represent metabotype. PCoA plots showed separation of individuals in the cohort by metabotype with a notable overlap of metabotype one with non-IBD individuals (Fig. 3-6B, Fig. 3-5A). As with enterotype, metabotype was found to be highly correlated with IBD status in both modes and was correlated with family (p=0.008) (Fig. 3-6C, Table 3-2).

We then employed multivariate models that included metabotype to identify IBD-associated spectral features independent of metabotype (Fig. 3-6D). UC was found to have a limited metabolic signature with only 10 differential features (6 shared with CD), including increased 5-ASA (2) and decreased stercobilin. The number of differential features in CD was reduced from 235 to 70 with inclusion of metabotype in multivariate models. Metabotypes 2 and 3 had 115 and 387 differential spectral features, respectively, relative to metabotype 1; these largely did not overlap with CD-associated metabolites. Of the 235 features initially associated with CD status without considering metabotype, 135 partitioned to metabotype 3 and 39 to metabotype 2. This suggested that as with enterotype, metabotype accounts for much of the variation in the metabolome when comparing disease states. Of the validated metabolites that were increased in CD without adjusting for metabotype, only the two spectral features validated as N-acetylcadaverine retained significance. Hierarchical clustering demonstrated that each metabotype was characterized by a distinct set of metabolites with lower abundance in the other two metabotypes (Fig. 3-6E). Of note, non-IBD individuals in metabotypes 2 and 3 shared the expression signature of CD and UC patients in these metabotypes (i.e. they intermingled with CD patients by hierarchical clustering), consistent with metabotype representing a state that is distinct from IBD. We focused metabolite validation on metabotypes 1 and 3 as they were more clearly associated with health or CD than metabotype 2 (Table 3-4). Metabotype 3 was characterized by increased levels of multiple bile acids, taurine, tryptophan, two trytophan metabolites (serinyl tryptophan, indolelactate), an intermediate in bacterial ornithine synthesis from glutamate (N-acetylornithine), a phenylalanine derivative (phenethylamine), and a lipid A component of lipopolysaccharide (3-oxotetradecanoic acid) . Metabotype 1 had increased levels of steroid hormones (estradiol, androstenedione), androsta-1,4-diene-3,17-dione, and stercobilin.

Estradiol and androstenedione abundance was higher in females than males, but in both genders increased levels of these hormones were seen in metabotype 1 (Fig. 3-8).

Enterotype and metabotype are highly correlated

The similarity between enterotypes and metabotypes suggested a functional relationship between the microbiome and metabolome. The microbiome and metabolomics data were superimposed onto the same ordination space using Procrustes. The two datasets displayed a high degree of concordance that was statistically significant in Monte Carlo simulations with $p<0.0001$ (Fig. 3-9A). Superimposed microbiome and metabolomics data separated not only by IBD status but also by enterotype and metabotype. Contingency table analysis demonstrated a strong association between enterotypes and metabotypes, in particular of enterotype 1 with metabotype 1 and enterotype 2 with metabotype 3 (Fig. 3-9B). As enterotype-associated microbial modules affected the predicted metagenome, we evaluated whether abundance of these modules could be correlated with specific metabolites adjusting for enterotype and metabotype (Fig. 3-9C). The EC1, EC2, and EC3 modules correlated with phenethylamine and 3-oxotetradecanoic acid (Table 3-4). The modules decreased in enterotype 2 (E4, E5, EU1 EU2) as well as a module decreased in UC (UC2) were correlated with androsta-1,4-diene-3,17-dione and stercobilin.

We next sought to determine the individual microbe-metabolite associations that underpin the relationship between enterotype 2 and metabotype 3. An interomic network was constructed using all significant correlations between enterotype-associated microbes and metabotype-associated metabolites adjusting for covariates including IBD status, enterotype (for microbes), and metabotype (for metabolites) (Fig. 3-9D). Lachnospiraceae occupied a central

position in the network, with correlations to multiple bile acids, 3-oxotetradecanoic acid, 7,10,13,16-docosatetraenoic acid, and 11-oxo-androsterone glucuronide. Enterobacteriaceae and *Serratia marcescens*, representing potential pathobionts, were correlated with 3-oxotetradecanoic acid. Clostridaceae was associated with the steroid hormones estradiol and androstenedione.

We then evaluated the relative association of enterotype and metabotype in multivariate logistic regression compared to a genetic risk score derived from single nucleotide polymorphisms in IBD-associated genes. Enterotype, metabotype, and genetic score each had a statistically significant correlation with IBD in univariate regression, while enterotype dominated in multivariate regression (Fig. 3-10A). Metabotype also retained significance, though with lower magnitude of effect, whereas genetic risk score became non-significant. This raised the possibility that in the non-IBD subset, enterotype and metabotype could serve as markers of individuals at risk for developing IBD. We found that 5 out of 21 children in this cohort without IBD carried the IBD-associated enterotype and an IBD-associated metabotype (2 or 3) (Fig. 3-10B).

Discussion

We found that variation in the microbiome and metabolome among IBD patients and their first-degree relatives can be represented by a limited number of intestinal enterotypes and metabotypes. The existence of an IBD-associated enterotype and IBD-associated metabotypes in healthy relatives argues that dysbiosis with its associated metabolic products can precede disease. Healthy individuals without the disease associated enterotype and metabotypes were predominantly found in several families, indicating that genetic and/or environmental factors

shared within families such as diet influence the development of these states. Such states may have arisen due to shared genetic alterations between patients and some of their family members in immune and/or epithelial function that influence host-microbiome homeostasis. This is consistent with recent findings that human genetic variation affects microbial composition and may explain the lack of an independent association of genetic risk score with IBD in multivariate logistic regression (*39*).

The metabotypes identified in this cohort may be driven by shifts in bacterial metabolism of host-derived factors such as bile acids. Metabotype 3 showed increased levels of a conjugated bile acid (taurochenodeoxycholate) and several sulfated bile acids (chenodeoxycholic acid sulfate, 3-sulfodeoxycholic acid, 7-sulfocholic acid). This agrees with a study reporting increased conjugated and sulfated bile acids in the feces of CD patients attributed to reduced capacity of the CD microbiome to deconjugate and desulphate bile acids (*40*). We also observed increased levels of a primary bile acid, cholic acid, possibly due to decreased conversion to other bile acid metabolites. The other two bile acids associated with metabotype 3 - 7-ketodeoxycholic acid and 3-oxo-4,6-choladienoic acid - have not been previously been reported to be increased in CD or UC patients and may be products of the co-correlated *Fusobacterium*, *Blautia*, and Lachnospiraceae (*18, 20*). The bile acid shifts in metabotype 3 are likely relevant to the development of dysbiosis and IBD given the considerable evidence that bile acid signaling through farnesoid X receptor (FXR) and TGR5 is critical for maintaining the epithelial barrier, promoting antibacterial defenses, and regulating hepatic bile acid synthesis (*41-44*). There is also recent data indicating that bile acids including cholic acid can induce enterochromaffin cells to secrete serotonin, a neurotransmitter shown to promote experimental colitis (*45, 46*). Diet may be related to some of the bile acid shifts associated with metabotype 3, in particular elevated

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taurochenodeoxycholate. In mice, a high milk fat diet induces taurine conjugated bile acids that promote expansion of a pathobiont capable of exacerbating experimental colitis (*47*).

Metabotypes 1 and 3 were also differentiated by bacterial metabolism of steroid hormones. Metabotype 1 had increased levels of estradiol, a hormone known to undergo enterohepatic recirculation, and androstenedione (*48*). This could reflect increased bacterial deconjugation of these hormones, an activity that may be carried by the unidentified member of the Clostridiaceae family that correlated with these two hormones. Our findings are consistent with a published report that urinary estrogen levels correlated with abundance of fecal Clostridia and with fecal species richness (paralleling the association of metabotype 1 with the high diversity enterotype 1) (*49*). This would imply that individuals with metabotype 3 have reduced steroid deconjugation, explaining the increased levels of 11-oxo-androsterone glucuronide.

A limitation of this study is the small number of high risk families and their lack of diversity. There may be additional enterotypes and metabotypes in broader populations that include other racial or ethnic groups. Enterotypes and metabotypes may show less association with disease in cohorts of families without IBD or in cohorts that include healthy unrelated, though data from recent studies such as the RISK cohort do show non-IBD individuals in the general population with low alpha diversity and dysbiosis similar to that of CD patients (*13*). We showed association of a small number of OTUs and metabolites with IBD status after adjusting for enterotype and metabotype relative to the changes associated with enterotype/metabotype. This surprising finding may reflect the lack of disease activity in this cohort. For instance, one recent study observed that SCFAs were decreased in IBD patients with active CD or UC but were unchanged in those with inactive disease (*17*). Finally, while we obtained abundances for

nearly 5,000 spectral features in the intestinal metabolome using LC-MS, many did not have putative IDs in metabolomics databases and a validated ID is available for only a small subset of those with putative IDs.

We propose that healthy individuals with the IBD associated enterotype and metabotype have a pre-disease state placing them at heightened risk for the future development of IBD or have undiagnosed disease. Reflecting the latter possibility, one of the five children we identified with the IBD enterotype/metabotype had a history of unexplained abdominal pain associated with nonspecific duodenal inflammation. Metabolomics in particular may serve as a powerful prognostic tool as the health-associated metabotype was not observed in any of the CD patients. Validating this interpretation will require a prospective study to assess the incidence of IBD in individuals stratified by enterotype/metabotype. It will be critical that such a study include serial measurement of the fecal microbiome and metabolome to assess the stability of enterotypes and metabotypes as well as careful dietary records. Genetic data from future studies can be pooled with the data collected here to evaluate for association of genetic variants with enterotype and metabotype.

Our study provides a template for future investigations that combine microbiome and metabolomics analysis to stratify individuals based on their risk for IBD. Prospective identification of healthy individuals with a high risk microbiome/metabolome creates the opportunity to prevent disease development by targeting the dysbiosis and/or its metabolic consequences in the intestine.

Figures and Tables
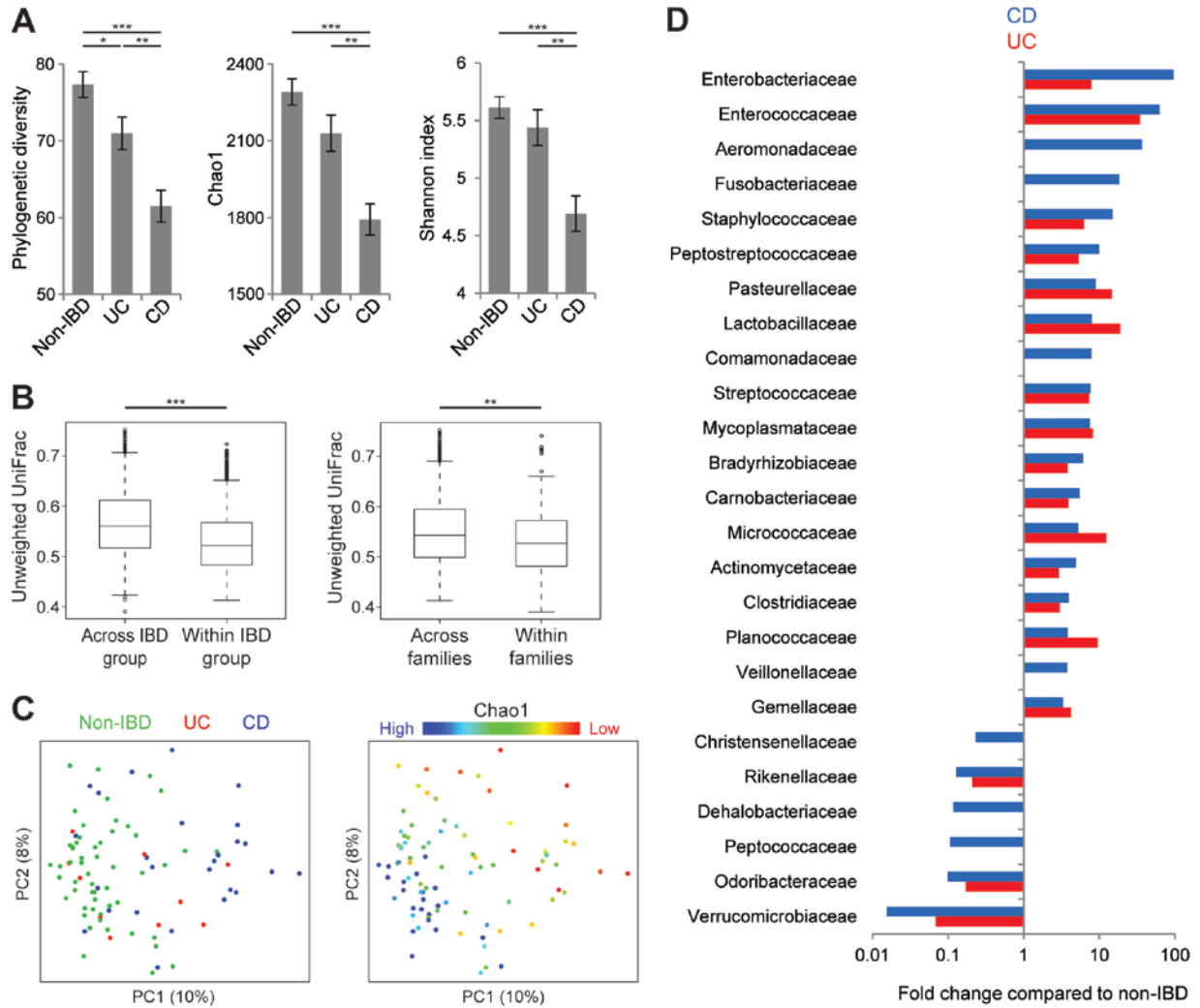
Figure 3-1. Reduced diversity and altered microbial composition in IBD patients. (**A**) Phylogenetic diversity, Chao1, and Shannon index were calculated for individuals with CD, UC, or neither (non-IBD). Mean +/- SEM. Statistical significance was calculated using a two-tailed t-test. * p<0.05, ** p<0.005, *** p<0.0001 (**B**) Boxplots showing unweighted UniFrac values for all pairwise combinations of individuals across IBD group (CD, UC, non-IBD) or within the same IBD group. The same analysis was performed for families. Statistical significance was calculated using t-tests with 10,000 Monte Carlo simulations. (**C**) PCoA plots of microbial

composition colored by IBD group or Chao1. (**D**) Relative abundance data for microbial families differentially abundant in CD or UC patients compared to non-IBD individuals with q<0.05 in multivariate models.
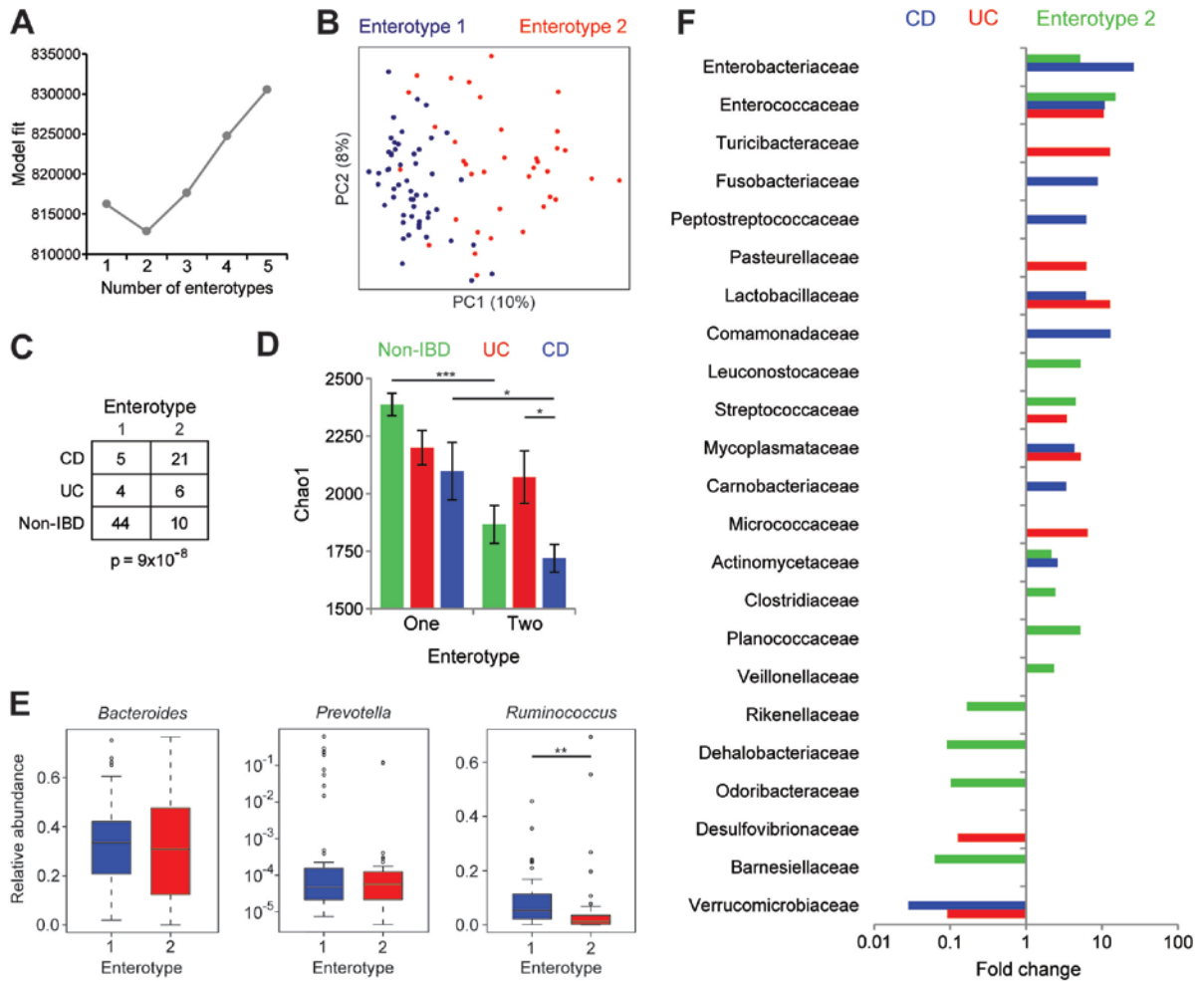
Figure 3-2. A low-diversity enterotype is associated with IBD but also is present in healthy relatives. (**A**) Dirichlet multinomial mixture models supported 2 enterotypes. (**B**) PCoA plot of microbial composition colored by enterotype. (**C**) Contingency table of IBD status by enterotype. P-value calculated using Fisher's exact test. (**D**) Chao1 by IBD status and enterotype. P-values calculated using two-tailed t-tests. * $p<0.05$, ** $p<0.005$, *** $p<0.0001$ (**E**) Abundance of three genera previously reported to distinguish human enterotypes. Statistical significance calculated using the Mann-Whitney U test. (**F**) Microbial families associated with CD (vs. non-IBD), UC (vs. non-IBD), or enterotype (2 vs. 1) with $q<0.05$ in multivariate models incorporating enterotype.
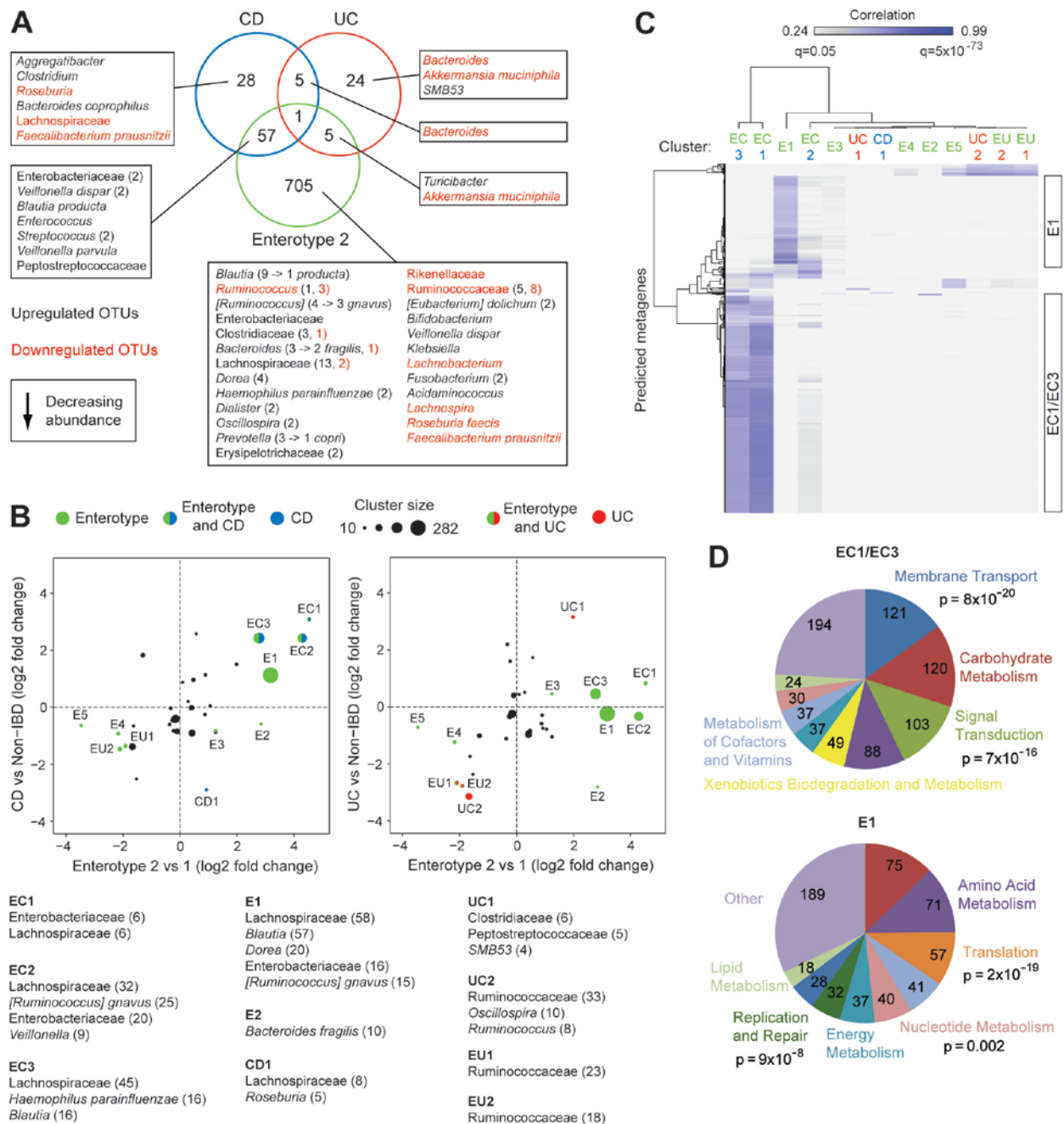
Figure 3-3. Microbial modules are associated with enterotype and influence the predicted metagenome. (A) Venn diagram indicating differential OTUs for CD (vs non-IBD), UC (vs. non-IBD) and enterotype (2 vs. 1) in multivariate models. OTUs with mean relative abundance greater than 0.1% are listed. OTUs are shown as the genus and species (if known), or family if

genus is not known. OTU counts are given in parentheses; -> is used to indicate OTUs of known species. (**B**) OTUs were grouped into modules based on co-occurrence. Median log2 fold change of the module was plotted for enterotype 2 vs. 1 compared with either CD vs. non-IBD or UC vs. non-IBD. Module association with enterotype and IBD status was determined using set enrichment analysis. Select taxa with the highest number of OTUs within a module are shown; the complete list is provided in Table S3. (**C**) Heat map showing correlation of enterotype, UC, or CD-associated modules with 2032 predicted metagenes associated with either enterotype or IBD group in multivariate models. (**D**) KEGG pathway assignments of metagenes associated with EC1/EC3 and E1. P-values are indicated for enriched pathways (p<0.005).

EC1/EC3

(Bar chart, x-axis: -Log10 Q-value for enrichment)

- Dissimilatory nitrate reduction, nitrate => ammonia
- Type II general secretion pathway
- Type VI secretion system
- Arginine transport system
- Dipeptide transport system
- Denitrification, nitrate => nitrogen
- Trans-cinnamate degradation, trans-cinnamate => acetyl-CoA
- GABA biosynthesis, prokaryotes, putrescine => GABA
- Lysine/arginine/ornithine transport system
- Histidine transport system
- Glutamate/aspartate transport system
- General L-amino acid transport system
- Putrescine transport system
- 2-Aminoethylphosphonate transport system
- Microcin C transport system
- Cytochrome o ubiquinol oxidase
- Thiamine transport system
- L-Arabinose transport system
- Vitamin B12 transport system
- Capsular polysaccharide transport system
- Heme transport system
- RcsC-RcsD-RcsB (capsule synthesis) two-component…
- C5 isoprenoid biosynthesis, mevalonate pathway
- Fumarate reductase, prokaryotes
- Octopine/nopaline transport system
- PTS system, galactosamine-specific II component
- Macrolide resistance, MacAB-TolC transporter

E1

(Bar chart, x-axis: -Log10 Q-value for enrichment)

- Ribosome, bacteria
- Aminoacyl-tRNA biosynthesis, prokaryotes
- F-type ATPase, prokaryotes and chloroplasts
- Trehalose biosynthesis, D-glucose 1P => trehalose
- Shikimate pathway, phosphoenolpyruvate + erythrose-4P =>…
- Ribose transport system
- Adenine ribonucleotide biosynthesis, IMP => ADP,ATP

Figure 3-4. KEGG modules enriched in E1 and EC1/EC3. Hypergeometric tests with correction for multiple hypothesis testing were used to generate q-values for enrichment of KEGG modules among the E1 and EC1/EC3 associated metagenes. The –log10 of the q-value is shown for enriched KEGG modules (i.e. higher values indicate greater enrichment).

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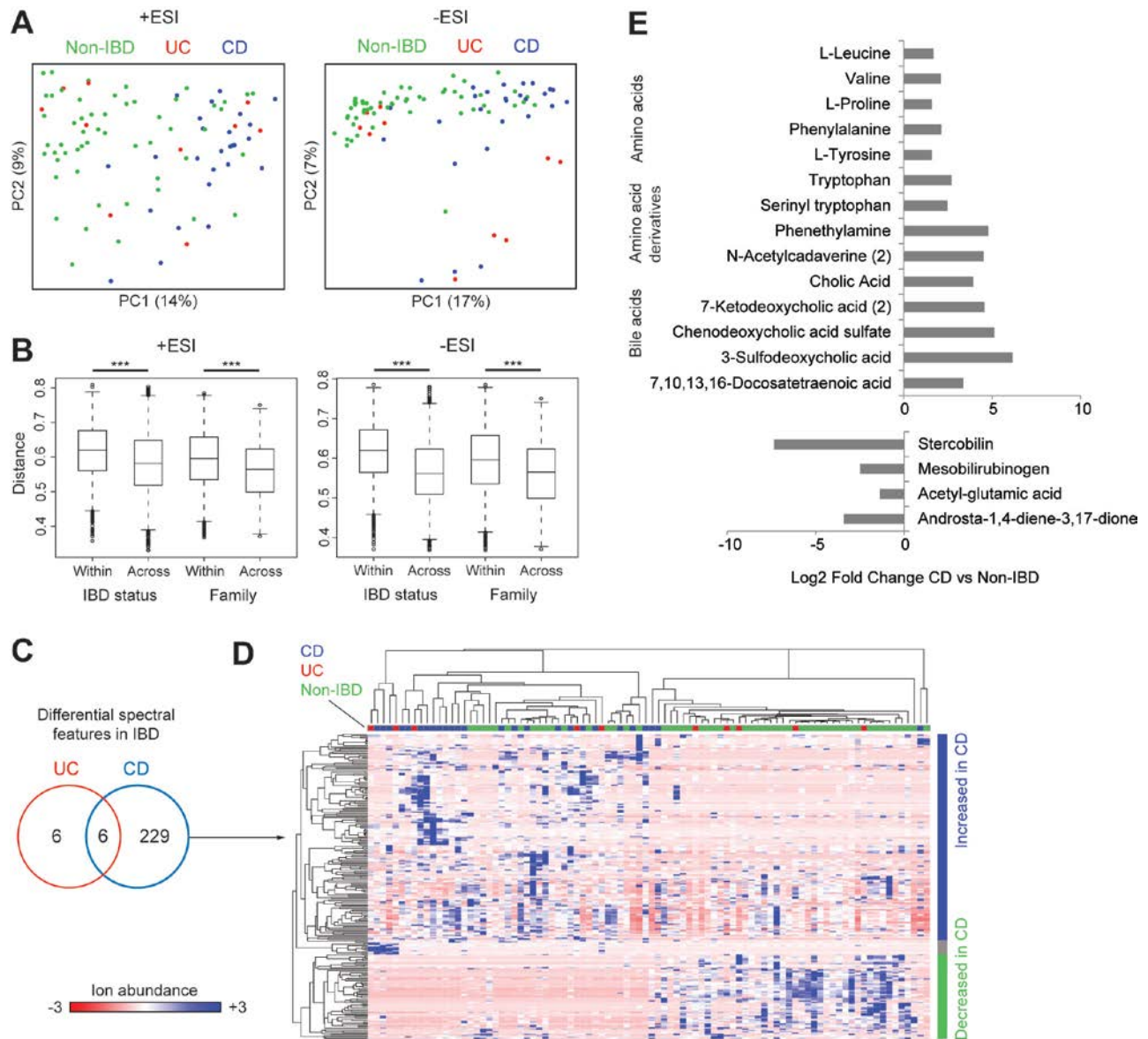
Figure 3-5. CD patients have distinct metabolomics features also seen in a subset of healthy relatives. (**A**) PCoA plots of the metabolome in the positive and negative ESI modes colored by IBD group. (**B**) Boxplots showing root square Shannon Jensen divergence ("Distance") of the fecal metabolome for all pairwise combinations of individuals across and within IBD group or family. Statistical significance was calculated using t-tests with 10,000 Monte Carlo simulations. *** p<0.0001 (**C**) Venn diagram indicating the number of differential spectral features for CD

vs. non-IBD or UC vs. non-IBD in multivariate models. (**D**) Heat map of raw abundance values for spectral features with differential abundance in CD vs non-IBD. IBD status of samples is indicated by colored boxes above the heat map. Feature abundance is represented on a color scale spanning 3 standard deviations above and below the mean. Spectral features increased or decreased in CD are highlighted to the right of the heat map; gray denotes 9 spectral features elevated in individuals on 5-ASA therapy (one validated as 5-ASA). (**E**) Log2 fold change in multivariate models is shown for validated CD-associated metabolites.
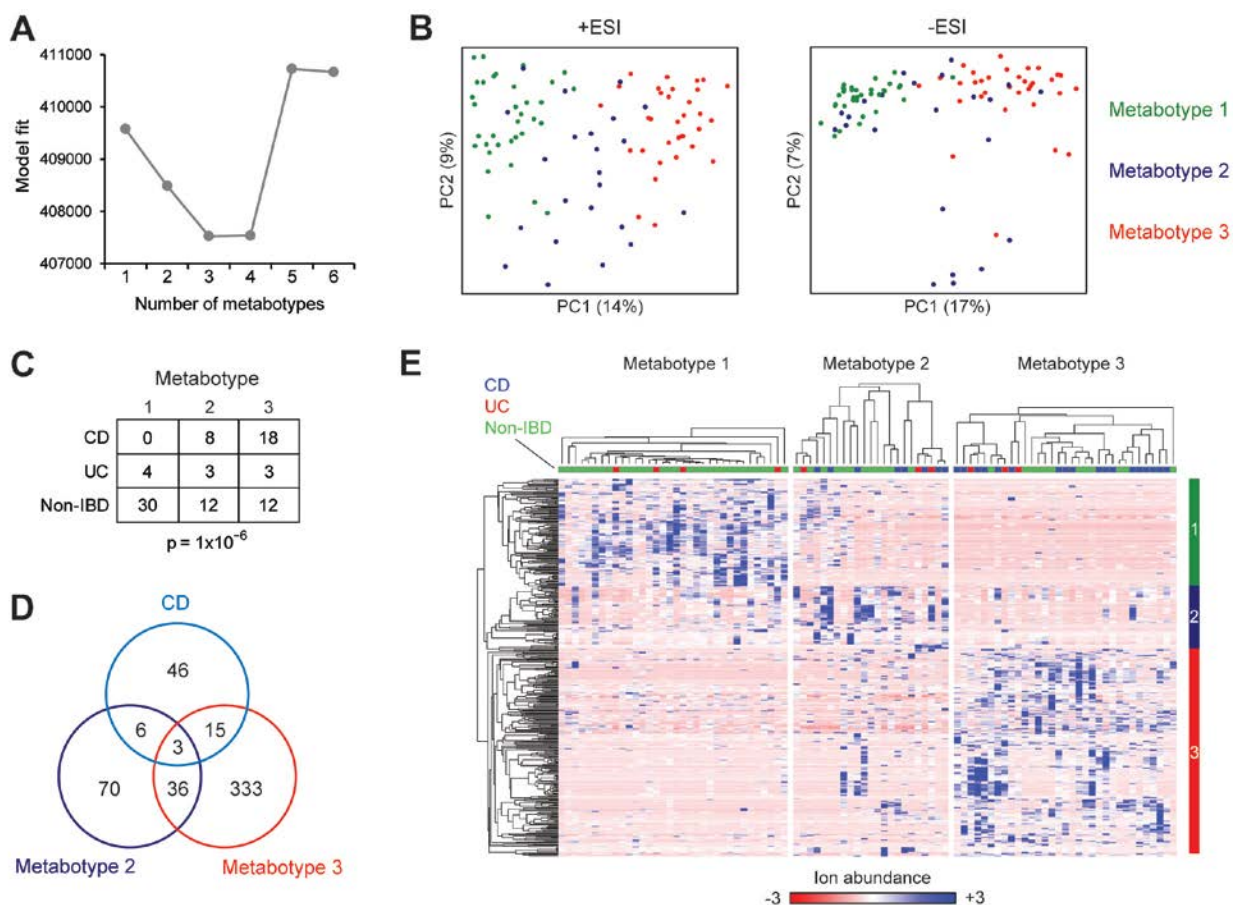
Figure 3-6. Individuals divide into metabotypes related to IBD status. (**A**) Dirichlet

multinomial mixture models were used to group samples by positive ESI mode data into

metabotypes. (**B**) PCoA plot of spectral feature abundances in the positive and negative ESI

modes colored by metabotype. (**C**) Contingency table of IBD status by metabotype. P-value

calculated using Fisher's exact test. (**D**) Venn diagram showing the number of differential

spectral features in both ESI modes for CD vs non-IBD, metabotype 2 vs. 1, and metabotype 3

vs. 1 in multivariate models. (**E**) Heat map of raw abundance values of metabotype-associated

differential spectral features in both ESI modes. IBD status of samples is indicated by colored

boxes above the heat map. Spectral features increased in each metabotype are highlighted by

colored bars to the right of the heat map.

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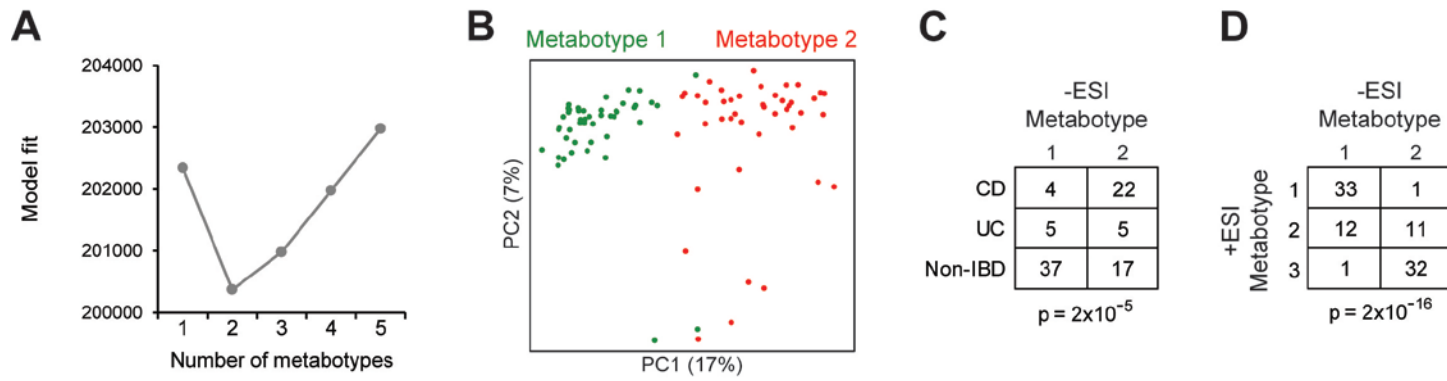
Figure 3-7. Negative ESI mode metabotypes map to positive ESI mode metabotypes. (**A**) Dirichlet multinomial mixture models were used to group samples in the negative ESI mode into metabotypes. (**B**) PCoA plot of spectral feature abundances in the negative mode colored by negative ESI mode metabotype. (**C**) Contingency table of IBD status by negative ESI mode metabotype. P-value calculated using Fisher's exact test. (**D**) Contingency table of positive and negative mode metabotypes.
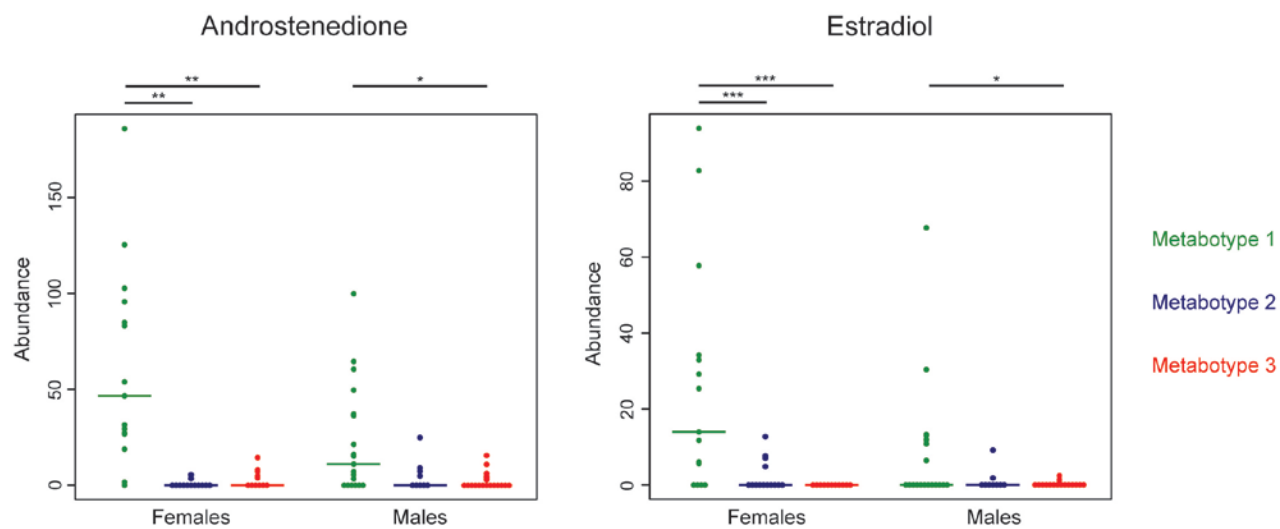
Figure 3-8. Androstenedione and estradiol are increased in metabotype 1 in both males and females. Unadjusted metabolite abundance is shown for individuals in the cohort stratified by gender and metabotype. Lines represent the median for each group. P-values were calculated using the Mann-Whitney U test. * p<0.05, ** p<0.005, *** p<0.0005
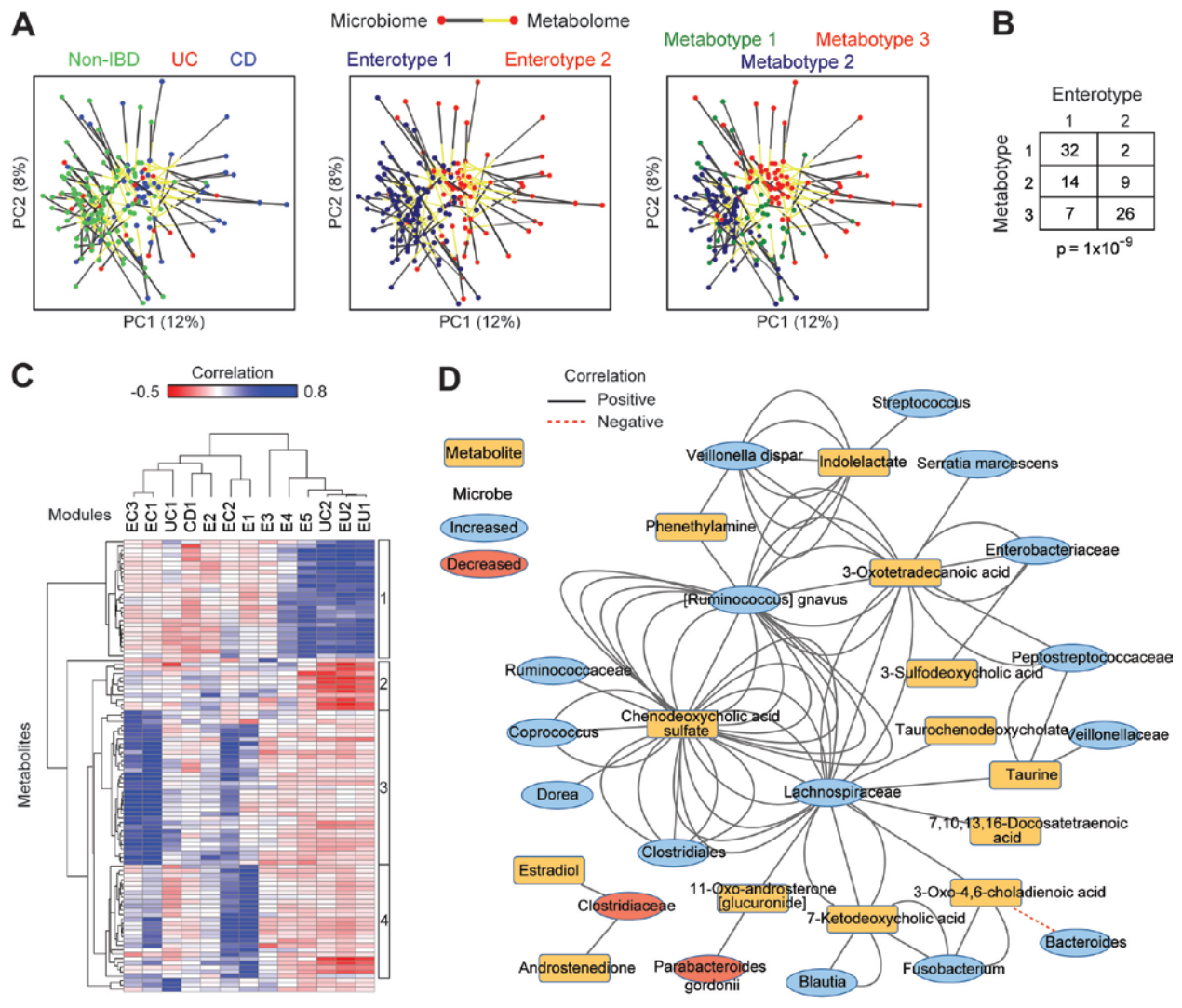
Figure 3-9. Microbe-metabolite interactions between the IBD-associated enterotype and metabotype. (A) Procrustes superimposition of the microbiome and metabolome (positive ESI mode). Points represent 16S and LC/MS data, color-coded by IBD status, enterotype, or metabotype. Each line connects the microbial and metabolomics data from one individual. (**B**) Contingency table of enterotype and metabotype. P-value calculated using Fisher's exact test. (**C**) Heat map showing Spearman correlation of microbial modules with metabolites adjusting for covariates. Only metabolites having a statistically significant association (q<0.05) with at least one module are shown. Clusters of metabolites are numbered 1-4 to the right of the heat map;

validated metabolites in these groups are indicated in Table 1. (**D**) Interomic network of

enterotype 2-associated microbes and metabotype 3-associated metabolites with a statistically

significant correlation (q<0.05) in mixed effects models adjusting for metadata.
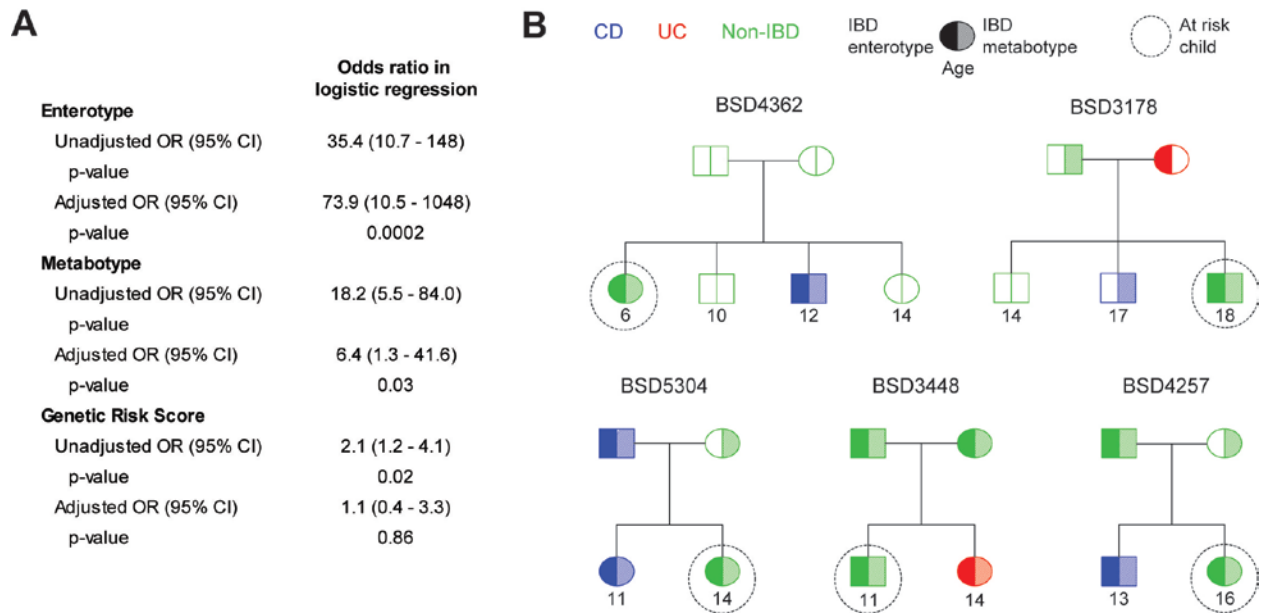
Figure 3-10. Association of enterotype, metabotype, and genetic risk score with IBD. (**A**) Unadjusted odds ratios (ORs) and 95% CI (confidence intervals) were calculated using univariate logistic regression. Adjusted ORs were obtained from multivariate logistic regression models incorporating enterotype, metabotype (2 or 3 vs. 1), parent/child status, Jewish ethnicity, and gender with or without genetic risk score. OR for genetic risk score represents the difference between the 25th and 75th percentiles. (**B**) Pedigrees are shown for 5 families with healthy children who carry an IBD-associated enterotype and metabotype.

Cohort demographics

	Probands	Siblings with IBD	Healthy Siblings	Parents
N	21	6	21	42
Crohn's disease	17	5		4
Ulcerative colitis	4	1		5
Gender				
Male	14	5	10	21
Female	7	1	11	21
Race/Ethnicity				
Caucasian	19	6	19	
Jewish	13	4	15	
Mixed	2	0	2	
Age at diagnosis	10	18.4		
Age at sampling	13.6	19.7	12.7	
Crohn's disease location				
Small intestine	15	5		
Colon involvement	10	4		
UGI involvement	6	1		
Perianal disease	7	1		
Colon only	2	0		
IBD medications at sampling				
Anti-TNF	16	3		2
*Methotrexate	10	1		0
*6-MP/azathioprine	2	0		0
5-ASA	6	1		2
None	1	1		3
Birth order	1.7	1.3	1.9	
Mode of delivery				
Vaginal	16	4	15	
Cesarian	5	2	6	
**Gestational age (weeks)	0.2	-0.3	0.0	
Birth weight (lbs)	7.4	6.8	7.0	
Perinatal disease	8	2	3	
Anemia	3	0	1	
Pre-term labor	1	2	0	
Preeclampsia	1	0	0	
Other	3	0	2	
Antibiotics pre-delivery	3	1	3	
Maternal age at delivery	33.9	32	33.4	
***Exclusive breastfeeding	20	4	17	
2+ antibiotics during 1st year	4	1	4	

Table 3-1. Cohort demographics. Immunomodulators (methotrexate, 6-MP) were combined with anti-TNF therapy in all but 2 patients. Gestational age was expressed as weeks from full-term. Exclusive breastfeeding was for at least 4 months after birth. There were no statistically significant differences in metadata between children with IBD and healthy siblings.

Family	Enterotype 1	Enterotype 2	Metabotype(+ESI) 1	2	3	Metabotype (-ESI) 1	2
BSD2205	2	0	2	0	0	2	0
BSD2780	1	0	1	0	0	1	0
BSD3178	2	1	1	0	2	1	2
BSD3205	2	0	0	1	1	0	2
BSD3448	0	3	0	1	2	0	3
BSD3486	2	0	2	0	0	2	0
BSD3590	2	0	1	1	0	1	1
BSD3724	3	0	3	0	0	3	0
BSD3803	2	0	1	1	0	1	1
BSD3888	3	0	3	0	0	3	0
BSD3905	3	0	1	1	1	2	1
BSD4031	1	1	2	0	0	2	0
BSD4241	4	0	2	0	2	2	2
BSD4257	1	2	0	1	2	1	2
BSD4287	2	0	0	2	0	1	1
BSD4343	2	1	1	1	1	3	0
BSD4346	2	0	2	0	0	2	0
BSD4352	2	0	1	1	0	1	1
BSD4362	4	1	4	1	0	5	0
BSD5304	1	1	0	1	1	1	1
BSD5502	3	0	3	0	0	3	0
Total	44	10	30	12	12	37	17
p-value	p=0.08		p=0.008			p=0.04	

Table 3-2. Metabotypes and enterotypes of non-IBD individuals are associated with family.
Contigency tables are shown for enterotype, +ESI metabotype, and –ESI metabotype of the 54
non-IBD individuals across the 21 families in this cohort. P-values were calculated using
Fisher's exact test. Two families are highlighted (BSD4257, BSD3448) that contain at least two
individuals with the IBD-associated enterotype and metabotype.

E1	
Count	**Taxa**
58	Lachnospiraceae
52	Blautia
24	Clostridiales
20	Dorea
16	Enterobacteriaceae
15	[Ruminococcus] gnavus
12	Ruminococcaceae
8	Streptococcus
6	Coprococcus
5	[Ruminococcus]
5	Blautia_producta
5	Roseburia
4	Dorea formicigenerans
3	Actinomyces
3	Oscillospira
3	Veillonella dispar
3	Veillonella parvula
3	Erysipelotrichaceae
2	Gemellaceae
2	Dialister
2	[Eubacterium] dolichum
2	Fusobacterium
2	Serratia marcescens
1	Actinomycetaceae
1	Atopobium
1	Eggerthella lenta
1	Planococcaceae
1	Lactobacillus
1	Pediococcus
1	Mogibacterium
1	Anaerococcus
1	Finegoldia
1	Clostridiaceae
1	Clostridium
1	[Ruminococcus] torques
1	Anaerostipes
1	Butyrivibrio
1	Epulopiscium
1	Oribacterium

EC1	
Count	**Taxa**
6	Enterobacteriaceae
5	Lachnospiraceae
3	Clostridiales
2	Blautia
2	Dorea
2	Peptostreptococcaceae
2	Erwinia
1	Granulicatella
1	Enterococcus
1	[Mogibacteriaceae]
1	Clostridiaceae
1	[Ruminococcus] gnavus
1	Veillonella
1	[Eubacterium] dolichum
1	Fusobacterium
1	Aeromonadaceae
1	Klebsiella

EC2	
Count	**Taxa**
32	Lachnospiraceae
25	[Ruminococcus] gnavus
20	Enterobacteriaceae
14	Clostridiales
8	Dorea
7	Veillonella dispar
6	Blautia producta
5	Ruminococcaceae
4	Peptostreptococcaceae
3	Blautia
3	Coprococcus
2	Veillonella parvula
2	Erysipelotrichaceae
2	[Eubacterium] dolichum
2	Serratia marcescens
1	Clostridiaceae
1	Butyrivibrio
1	Oscillospira
1	Veillonellaceae

EU1	
Count	**Taxa**
23	Ruminococcaceae
6	Clostridiales
5	Christensenellaceae
4	Lachnospiraceae
2	Oscillospira
1	[Mogibacteriaceae]
1	Clostridiaceae
1	Clostridium
1	Dehalobacterium
1	Ruminococcus

EU2	
Count	**Taxa**
18	Ruminococcaceae
7	Clostridiales
2	Rikenellaceae
2	Christensenellaceae
2	Blautia
2	Oscillospira
1	Adlercreutzia
1	Bacteroides
1	Clostridiaceae
1	Lachnospiraceae
1	SHA-98

CD1	
Count	**Taxa**
8	Lachnospiraceae
5	Roseburia
1	Clostridiales
1	Blautia
1	Ruminococcaceae

UC1	
Count	**Taxa**
6	Clostridiaceae
5	Peptostreptococcaceae
4	SMB53
2	Turicibacter

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Count	Taxa
1	Veillonella
1	Bradyrhizobiaceae
1	Comamonadaceae
1	Campylobacter rectus
1	Enterobacter cowanii
1	Klebsiella
1	Plesiomonas shigelloides
1	Proteus
1	Salmonella_enterica
1	Thiohalorhabdales
1	Mycoplasma

E2

Count	Taxa
10	Bacteroides fragilis
1	Bacteroides

E3

Count	Taxa
7	Lachnospiraceae
3	Clostridiales
3	Dorea
2	[Ruminococcus]
2	Blautia
1	Lachnospira

E4

Count	Taxa
11	Ruminococcaceae
8	Lachnospiraceae
3	Oscillospira
2	Odoribacter
2	Clostridiales
1	Rikenellaceae
1	Roseburia faecis

E5

Count	Taxa
7	Ruminococcaceae

Count	Taxa
1	Veillonella
1	Desulfovibrio
1	Plesiomonas
1	Serratia
1	Thiohalorhabdales

EC3

Count	Taxa
45	Lachnospiraceae
26	Clostridiales
16	Haemophilus parainfluenzae
13	Dialister
9	Blautia
8	Streptococcus
7	Blautia producta
5	Clostridiaceae
5	Ruminococcaceae
4	Coprococcus
4	Dorea
4	Fusobacterium
4	Enterobacteriaceae
3	[Ruminococcus] gnavus
2	Bacteroides
2	Bacteroides fragilis
2	Clostridium
2	Oscillospira
2	Veillonella dispar
2	Zoogloea
2	Pasteurellaceae
2	Aggregatibacter
1	Actinomyces
1	Corynebacterium durum
1	Rothia mucilaginosa
1	Bifidobacteriaceae
1	Bifidobacterium
1	Prevotella
1	Alicyclobacillus
1	Gemellaceae
1	Lactobacillales

Count	Taxa
2	Clostridiales
1	[Mogibacteriaceae]
1	Sarcina

UC2

Count	Taxa
33	Ruminococcaceae
20	Clostridiales
10	Oscillospira
8	Ruminococcus
5	Rikenellaceae
3	Christensenellaceae
2	Bacteroides
2	[Mogibacteriaceae]
2	Clostridiaceae
2	Lachnospiraceae
2	Coprococcus
1	Coriobacteriaceae
1	Clostridium
1	Dehalobacterium
1	Peptostreptococcaceae
1	Anaerotruncus
1	Ruminococcus flavefaciens

2	Clostridiales	1	Leuconostoc mesenteroides	
2	Lachnospiraceae	1	Streptococcus anginosus	
1	Rikenellaceae	1	Peptoniphilus	
1	Clostridium	1	SMB53	
1	Blautia	1	[Ruminococcus]	
1	Coprococcus	1	Anaerostipes	
		1	Oribacterium	
		1	Roseburia	
		1	Peptostreptococcaceae	
		1	Erysipelotrichaceae	
		1	[Eubacterium] dolichum	
		1	Coprobacillus	
		1	Coprobacillus cateniformis	
		1	Methylobacterium	
		1	Leptothrix	
		1	Methylophilales	
		1	Erwinia	
		1	Haemophilus	
		1	Stenotrophomonas	
		1	Mycoplasma	
		1	TM7	

Table 3-3. Taxa contained within IBD- and enterotype-associated microbial modules. OTUs included in each module (see Fig. 3B) were aggregated based upon the lowest rank of taxonomic identification (e.g. *Blautia producta*, *Blautia* for an unspeciated member of this genus, Lachnospiraceae for an OTU in this family that wasn't identified at the genus level, etc.). A count is provided of the number of OTUs in each module with the same taxonomic designation. Counts for taxa of higher rank than species do not include OTUs identified at a lower rank (e.g. E3 has 10 OTUs identified as *Bacteroides fragilis* and one OTU that was an unspeciated *Bacteroides*).

Validated metabolite	Log2 FC	Q-value	Module group	Correlated microbes
Metabotype 1				
Androsta-1,4-diene-3,17-dione	-3.4	$8x10^{-6}$	1	
Androstenedione	-5.6	0.002		Clostridiaceae
*Estradiol	-1.3, -4	0.32, 0.01		Clostridiaceae
Stercobilin	-5.4	$1x10^{-9}$	1	
Metabotype 3				
Tryptophan	2.3	0.02		
Serinyl tryptophan	1.9	0.04		
Indolelactate	3.5	0.03		*Veillonella dispar* (6), *[Ruminococcus] gnavus*, *Streptococc*
N-acetylornithine	2.7	0.02		
Phenethylamine	4.3	0.01	3	*Veillonella dispar*, *[Ruminococcus] gnavus*
Cholic acid (2)	5.5, 3.0	0.002, 0.005		
Chenodeoxycholic acid sulfate (2)	3.2, 2.9	0.02, 0.03		*[Ruminococcus] gnavus* (13), Lachnospiraceae (9), Clostrid *Coprococcus* (3), *Dorea* (1), Ruminococcaceae (1)
Taurochenodeoxycholate	6.9, 5.8	$3x10^{-4}$, 0.003		Lachnospiraceae
Taurine (2)	3.5, 2.8	$3x10^{-4}$, 0.02		Peptostreptococcaceae (2), Veillonellaceae, Lachnospiracea
7-Ketodeoxycholic acid (3)	8.9, 7.4, 6.4	$2x10^{-7}$, $4x10^{-5}$, 0.008		Lachnospiraceae (3), *Fusobacterium* (2), *Blautia* (2)
3-Oxo-4,6-choladienoic acid	9.3	$4x10^{-6}$		
3-Sulfodeoxycholic acid	6.0	$7x10^{-4}$		Enterobacteriaceae
7-Sulfocholic acid	5.3	$9x10^{-4}$		
11-Oxo-androsterone [glucuronide]	4.1	0.01		*Parabacteroides gordonii*, Lachnospiraceae
3-Oxotetradecanoic acid	6.3	0.04	3	*[Ruminococcus] gnavus* (6), Enterobacteriaceae (3), Lachno (3), Peptostreptococcaceae (2), *Serratia marcescens*, *Veillor*
7,10,13,16-Docosatetraenoic acid	6.5	$2x10^{-6}$	4	Lachnospiraceae
CD-associated				
**N-Acetylcadaverine (2)	4.9, 4.4	$3x10^{-3}$, $1x10^{-3}$	4	

Table 3-4. Validated metabolites associated with metabotypes 1 and 3. Log2 fold changes

(FC) between metabotype 3 and 1 in multivariate models are shown. Module group refers to

clusters of metabolites associated with microbial modules as indicated in Figure 6C.

*Metabotype 2 vs. 1 comparison listed second **FC refers to CD vs. non-IBD

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CHAPTER 4

RORγT-DEPENDENT CD4+ T CELLS GARDEN THE MUCOSA-ASSOCIATED

MICROBIOME OF THE SMALL INTESTINE AND COLON

Abstract

The intestinal microbiome and the mucosal immune system have reciprocal effects on

composition and function. It has previously been shown that segmented filamentous bacteria

(SFB) induce Th17 cells - which require the transcription factor RORγt - and that levels of SFB

in the feces and colonic lumen are affected by RORγt-dependent innate lymphoid cells (ILCs).

We further investigated the role of RORγt in gardening the intestinal microbiome. Mice deficient

in RORγt had an altered small intestinal and colonic mucosa-associated microbiome

characterized by overgrowth of SFB. Similar shifts were observed in the distal small intestinal

and colonic mucosa of mice lacking an adaptive immune system (RAG) or T cell receptor alpha,

implying a role for RORγt-dependent T cells. Deficiency of IL-17A or antibody neutralization

did not affect the small intestinal microbiome but increased colonic SFB, arguing that IL-17A

specifically shapes the colonic microbiome. Engraftment of lamina propria CD4+ T cells, or

fate-mapped lamina propria Th17 cells, into the small intestine of RORγt/RAG double knockout

mice reduced small intestinal SFB in a manner dependent upon RORγt but not IL-17A. In

humans, the rs4845604 polymorphism of RORC – previously found to be protective for

inflammatory bowel disease (IBD) – was associated with altered microbial composition in

mucosal wash samples from IBD patients and healthy individuals. These findings demonstrate

that RORγt-dependent T cells garden the intestinal microbiome and suggest that genetic variation in gardening could influence susceptibility to inflammatory diseases such as IBD.

Introduction

The small intestine and colon house a complex bacterial ecosystem consisting of an estimated 10^{14} cells, 10 fold greater than the number of human cells. Many have beneficial functions such as harvesting energy from otherwise indigestible plant polysaccharides (transferred to the host via short chain fatty acids), triggering formation of an intestinal mucus barrier, promoting intestinal vascularization, metabolizing xenobiotics, and preventing colonization by pathogens (*1-5*). The microbiome also exerts considerable influence on the development of many immune cell types including regulatory and IL-17-producing CD4+ T cells (Th17) (*6*). It has been hypothesized that the beneficial properties of a healthy microbiome have created evolutionary pressure for mammals to evolve mechanisms for regulating the microbial communities in their intestine (*7*).

The existence of host gardening of the intestinal microbiome has been demonstrated in mouse studies comparing the microbiome of mice deficient in a putative gardening gene to littermate controls. Nearly all the gardening genes identified in this manner are involved in innate and adaptive mucosal immunity. These include pattern recognition receptors such as Toll-like receptor 2, Toll-like receptor 5, and NOD2; antimicrobial products of epithelial cells such as defensins and RegIIIγ; and immunoglobulins (*8-13*). In several studies, disruption of immune gardening caused overgrowth of pathobionts that then induced spontaneous colitis or increased susceptibility to dextran sulfate sodium (DSS) colitis (*14-16*). For instance, loss of the NLRP6 inflammasome or its downstream target, IL-18, in epithelial cells resulted in an overgrowth of

Prevotella and increased susceptibility to DSS colitis (*14*). The potential link between altered genetic gardening and IBD was supported by early studies demonstrating an altered intestinal microbiome in individuals carrying IBD-associated genetic polymorphisms in NOD2, FUT2, and IRGM (*17-19*). Extending these results, a recent study of IBD patients reported correlations of microbial abundance with polymorphisms in 48 IBD-associated genetic variants, though specific gene-microbe interactions could not be identified other than for NOD2 (*20*).

RORγt is a transcription factor required for the differentiation of Th17 cells and a subset of innate lymphoid cells (ILCs) (*21, 22*). It drives the expression of cytokines including IL-17A, IL-17F, IL-22, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor (*23*). RORγt-dependent ILCs are reported to be the primary intestinal source of IL-22, a cytokine which induces epithelial production of antimicrobial proteins including RegIIIγ, RegIIIβ, S100A8, and S100A9 (*24-26*). RegIIIγ expression by the intestinal epithelium is greatly reduced in the absence of IL-22 (*24, 27*). There is now considerable evidence that RORγt-dependent ILCs can garden the microbiome. Antibody-mediated depletion of ILCs or functional impairment of ILCs due to aryl hydrocarbon receptor (Ahr) deficiency increases the abundance of segmented filamentous bacteria (SFB) in the colonic lumen and feces (*28, 29*). ILC gardening of SFB is believed to be mediated by IL-22, as SFB levels in ILC-depleted mice could be decreased with recombinant IL-22 and anti-IL-22 increased fecal SFB. Recent data indicate that ILC-derived IL-22 may garden the microbiome not only through antimicrobial products but also by inducing epithelial fucosyltransferase 2 (*30, 31*). The resulting mucosal fucose is metabolized by microbes, impacting their composition and function. ILCs also acts as sentinels that protect against invasive organisms. Antibody-mediated depletion of ILCs and anti-IL-22 treatment

permitted systemic dissemination of *Alcaligenes xylosoxidians*, a Proteobacteria species that normally is contained to mouse Peyer's patches and mesenteric lymph nodes (*26*).

Th17 cells play a critical role in the host defense to enteric pathogens including bacteria (*Salmonella, Citrobacter, Klebsiella*), fungi (*Candida*), and parasites (*Toxoplasma*) (*32*). However, little is known about the contribution of these RORγt-dependent T cells to gardening of the intestinal microbiome. Th17 cells are the primary intestinal source of IL-17A, a pro-inflammatory cytokine that has been linked to numerous autoimmune and inflammatory conditions but that also increases antimicrobial production by epithelial cells and may therefore have a role in gardening (*22, 33*). Th17 cells can also produce IL-22, particularly in response to Ahr ligands (*34*). The potential role of IL-22-producing T cells in gardening was suggested in a recent study of mice lacking ILCs and an adaptive immune system. Expansion of SFB in the colonic lumen of these mice could be reduced by engraftment of splenic and peripheral lymph node T cells in a partially IL-22-dependent manner (*29*).

In this study, we dissect the relative contributions of innate and adaptive immune RORγt to gardening of the mucosa-associated microbiome of the small intestine and colon. We found that RORγt-dependent T cells were critical for limiting overgrowth of SFB in the distal small intestinal microbiome and also had an effect on SFB in the colon. Gardening of the small intestinal microbiome could be mediated by fate-mapped Th17 cells but did not require IL-17A. The importance of RORγt in immune gardening of the microbiome was demonstrated in humans, in which polymorphism in RORγt was associated with alteration in the colonic mucosal microbiome.

Materials and Methods

MLI cohort

The MLI cohort consists of 85 patients with known Crohn's disease in clinical remission undergoing colonoscopy and 112 healthy controls presenting for screening colonoscopy. Mucosal washes using deionized water were obtained from the cecum and sigmoid colon of subjects as previously described (*35*). Lavaged material was then centrifuged at 5000g for 15 minutes. The pelleted material was used for 16S rRNA sequencing. DNA extracted from peripheral blood was applied to the Immunochip, a custom platform containing nearly 200,000 single nucleotide polymorphisms near genes related to immune function and inflammatory disease (*36*). This study was performed under IRB 11-002093.

Animal husbandry

C57Bl/6, RORγt gfp/gfp (referred to as RORγt-/-), RAG2-/- (referred to as RAG-/-), TCRα-/-, IL17A Cre/Cre (referred to as IL17A-/-), and R26-stop-EYFP mice were obtained from the Jackson Laboratories and bred in a specific pathogen free vivarium at the University of California, Los Angeles. All strains were on the C57Bl/6 background. Genotyping was performed as per the protocols provided by Jackson Laboratories. Initially, RORγt-/- and RAG-/- mice were crossed to generate double heterozygous mice, then double heterozygous mice were interbred to generate rare double knockout males, and finally double knockout males were bred to double heterozygous females to produce experimental mice. Other knockout strains were initially crossed to wild type C57Bl/6 mice to generate heterozygous mice which were then interbred to generate experimental mice. Pregnant females were moved to separate cages prior to delivery so that the pups would only be exposed to maternal microbiota. Upon weaning, mice were singly caged. Knockout mice and littermate heterozygote controls were sacrificed at 8-9 weeks of age for microbiome analysis.

Mucosal and luminal microbial collection

The small intestine and colon were dissected from sacrificed mice then divided into regions (cecum, proximal half of the remaining colon, distal half of the remaining colon, ileum, jejunum, and duodenum). Each region was then cut into 1 cm fragments that were lavaged repeatedly with 1 mL deionized water. Lavaged material was spun at maximum speed in a microcentrifuge for 2 minutes. The supernatant was removed and the pellet frozen for future DNA extraction. Tissue fragments after lavage were placed in DMEM + 10% fetal calf serum (D10F) then cut lengthwise to release residual luminal content. After removal of visible luminal content, the intestinal fragments were washed three times in D10F then placed in D10F with 1mM DTT for 40 minutes in an incubator shaker at 37°C. After incubation, tissue fragments were vortexed to release epithelial cells and the cell suspension was passed through a 70 micron cell strainer. The resulting cell preparation was centrifuged at 5000g for 15 minutes to pellet epithelial cells and bacteria. The supernatant was aspirated and the cells resuspended in buffer (200 mM Tris-HCl pH 8.0, 200 mM NaCl, 20 mM EDTA) then frozen at -80°C for future DNA extraction.

16S rRNA sequencing

Genomic DNA extraction, amplification of the V4 region of 16S ribosomal RNA genes, and single-end sequencing on an Illumina HiSeq 2000 were performed as described previously for microbial samples from RAG/RORγt mice and from human subjects in the MLI cohort (*37*). The 101 base pair reads were processed using QIIME v1.7.0 with default parameters (*38*). 16S rRNA sequencing of the remaining samples from experimental animals was performed on an Illumina HiSeq 2500. The 150 base pair paired end reads were joined into 254 bp amplicons in QIIME. Operational taxonomic units (OTUs) were picked against the May 2013 version of the Greengenes database (http://greengenes.secondgenome.com), pre-filtered at 97% identity. Alpha

diversity was assessed using the Shannon index with data rarefied to 100,000 sequences. Beta

diversity was calculated using unweighted UniFrac and visualized using principal coordinates

analysis (PCoA). Adonis with 100,000 permutations was used to assess statistical significance.

Differential abundance testing

Colonic and small intestinal mucosal samples were analyzed separately to identify genotype-

associated microbes in each of these two organs. 16S sequence data was filtered to remove OTUs

present in less than 10% of samples. The resulting filtered datasets were analyzed in R with

Phyloseq and the DESeq2 algorithm, which employs an empirical Bayesian approach to shrink

dispersion (*39, 40*). Log fold changes for each OTU or metagene were fitted to a general linear

model under a negative binomial model. Covariates in multivariate models for mouse data

included sample site, gender, litter, and genotype. Covariates in multivariate models for the MLI

cohort data included gender, disease status, sampling site, and rs4845604 carrier status. P-values

for variables in the linear models (e.g. genotype) were converted to q-values to correct for

multiple hypothesis testing (*41*). Q-values below 0.05 were considered significant.

IL-17A neutralization

100 µg anti-IL17A (R&D Systems) or a monoclonal isotype control antibody were administered

by intraperitoneal injection every four days over a 28 day course of treatment.

Intestinal lamina propria lymphocyte isolation

Intestinal tissue was processed as described earlier to isolate microbial samples. After vortexing,

DTT treated tissue fragments were minced with a razor, rinsed with D10F, and incubated in

D10F with 0.5 mg/mL collagenase D (Roche), DNase I (Roche), and dispase (Gibco) for 40

minutes in an incubator shaker at 37 C. The digestion media was then passed through a 70

micron cell strainer. Collected tissue fragments were mechanically disrupted with a syringe

plunger, then rinsed with D10F to release cells. The cell suspension was pelleted, resuspended with 40% Percoll, then layered carefully over 80% Percoll. Tubes containing the Percoll gradients were centrifuged at 2500 rpm in a Sorvall Legend X1 for 30 minutes. The interphase layer was then aspirated using a transfer pipette, diluted with D10F, spun down, and resuspended in D10F.

Small intestinal lamina propria cell engraftment

Lamina propria cells isolated as described above from the small intestines and colons of ROR+/gfp donors were stained for 30 minutes at room temperature with antibodies against CD3, CD19, CD8a, and CD4. Fate-mapped Th17 cells were identified by YFP fluorescence. Cell sorting was performed using the FACSAria III (BD). Flow sorted cells were collected in DMEM + 20% FCS + 1% HEPES, pelleted, then rinsed with DMEM free of phenol red. The cells were rinsed three additional times before being resuspended in DMEM. Recipients were anesthetized with isofluorane, then 100,000 lamina propria CD4+ T cells (or 10,000 fate-mapped Th17 cells) in 200 uL DMEM were transferred by retroorbital injection.

Flow cytometry

Harvested splenocytes and intestinal lamina propria cells were incubated with antibodies against CD3, CD19, CD8a, CD4, NK1.1, and NKp46 for surface staining only or with antibodies against CD3, CD19, CD8a, and CD4 for intracellular staining. RORγt expression was visualized by GFP fluorescence in cells from RORγt +/gfp mice. Fixation and permeabilization was performed using a kit (eBioscience), then intracellular staining was performed with antibodies against IL-17A and IL-22. Acquisition of multicolor flow cytometry data was performed using the LSRII (BD).

Results

RORγt regulates levels of SFB in the mucosa of the small intestine and colon

To evaluate the relative contribution of adaptive immunity and RORγt-dependent ILCs to microbial gardening, we crossed RORγt-/- mice to RAG-/- mice then bred male RAG-/-RORγt-/- mice with RAG+/-RORγt+/- female mice. The offspring consisted of four genotypes: RAG+/-RORγt+/- (controls), RAG+/-RORγt-/- (RORγt-deficient), RAG-/-RORγt+/- (RAG-deficient), and RAG-/-RORγt-/- (double knockout, DKO). These mice were individually caged at weaning so that differences in microbial composition by genotype would not be obscured by coprophagy. The mucosa-associated microbiome of the distal colon, proximal colon, cecum, ileum, mid small intestine, and proximal small intestine of these mice were evaluated by 16S sequencing, as was the microbiome of fecal pellets. RAG-deficient, RORγt-deficient, and DKO mice had reduced microbial diversity in the small intestine but not in the colon compared to controls (Fig. 4-1A). Principal coordinates analysis (PCoA) demonstrated a statistically significant effect of genotype on the microbiome, though sample site was the predominant factor affecting microbial composition (Fig. 4-1B). Analysis of the three small intestinal regions demonstrated striking overgrowth of SFB in the small intestine of knockout mice (Fig. 4-1C). RAG-/- and RORγt-/- mice had the same phenotype in the jejunum and ileum, suggesting that control of SFB in these regions depended upon an adaptive immune cell expressing RORγt. RORγt-/- and RAG-/-RORγt-/- mice had increased SFB overgrowth in the duodenum compared to RAG-/- mice, indicating that RORγt-dependent ILCs or other innate immune cells expressing RORγt are sufficient to garden SFB in the proximal small intestine.

Multivariate analysis was then performed to identify genotype-associated microbes in the small intestine and colon (Fig. 4-2A). All three knockout mice showed increased abundance of members of the Firmicutes phylum in the small intestine. SFB represented the only bacterial genus that was consistent across all the knockouts. *Ruminococcus*, *[Ruminococcus]*, and *Oscillospira* were increased in both RAG-deficient and DKO mice whereas *Acenitobacter* and *Allobaculum* were increased in both RORγt-deficient and DKO mice, suggesting distinct microbial signatures of adaptive immune and innate RORγt-dependent gardening. In the colon, SFB and *Allobaculum* were increased in RORγt-deficient and DKO mice. Across the GI tract, RORγt-deficiency was associated with elevation of SFB from the duodenum to the feces whereas RAG-deficiency predominantly affected the jejunum and ileum, with smaller increases in SFB abundance in other regions that did not reach significance (Fig. 4-2B, Fig. 4-2C).

RORγt-dependent T cells garden SFB in the distal small intestine in a IL-17A-independent manner

To investigate the potential role of RORγt-dependent T cells in gardening of SFB in the small intestine, we compared the mucosa-associated microbiome of T cell receptor alpha (TCRα) knockout mice to that of heterozygote littermate controls. TCRα–deficiency resulted in a statistically significant shift in the ileal microbiome but not the duodenal and jejunal microbiome (Fig. 4-3A). Multivariate analysis demonstrated that T cell deficiency was associated with increased SFB as well as *Ruminococcus*, *[Ruminococcus]*, and *Oscillospira* in the small intestinal and colonic mucosa (Fig. 4-3B). Analysis of SFB abundance in individual regions demonstrated that the most significant increases in SFB occurred in the ileum and distal colon (as well as feces) (Fig. 4-3C).

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Given the extensive literature on the biologic activity of Th17 cells and their known induction by SFB, we predicted that immune gardening by T cells of SFB would involve IL-17A. The mucosa-associated microbiome of IL-17A-/- mice was compared to heterozygous littermate controls. No difference in microbial composition was seen by PCoA analysis (data not shown). In the small intestine, no OTUs were associated with IL-17A genotype in multivariate models, including SFB, while in the colon the only genotype-associated microbe was SFB (Fig. 4-4A). To confirm these findings, wild-type mice were treated with IL-17A antibody or isotype control antibody for 28 days. IL-17A neutralization had no overall effect on microbial composition as visualized by PCoA (data not shown). In multivariate models, no OTUs were associated with anti-IL17 treatment in the small intestine. The only OTU associated with anti-IL17 treatment in the colon that was identified at the genus level was SFB (Fig. 4-4B). Together, these results indicate that IL-17A is dispensable for SFB gardening in the small intestine but plays a role in SFB gardening in the colon.

To demonstrate that RORγt-dependent T cells are sufficient to garden the small intestinal microbiome, a transfer model was established in which lamina propria CD4+ T cells were engrafted into the small intestinal lamina propria of DKO mice (Fig. 4-5A). Approximately 10% of transferred lamina propria T cells in the small intestine expressed RORγt and IL-17A while 4% expressed IL-22, half of which co-expressed IL-17A (Fig. 4-5A, Fig. 4-5B). No engraftment of transferred lamina propria T cells was detected in the colonic mucosa (data not shown). Mucosal microbial samples were collected 6 weeks after transfer. Recipients of wild-type or IL-17A-deficient CD4+ lamina propria T cells but not RORγt-deficient CD4+ lamina propria T cells showed a statistically significant change in the jejunal and ileal mucosa-associated microbiome (Fig. 4-5C). This corresponded to decreased SFB throughout the small intestine in recipients of

wild-type or IL-17A-deficient CD4+ T cells but not in recipients of RORγt-deficient CD4+ T cells (Fig. 4-5D). Fate-mapped Th17 cells were obtained from the lamina propria of donor mice carrying one copy of the IL17A-Cre transgene and the R26-stop-EYFP reporter. In these mice, cells with prior IL-17A expression were permanently marked with YFP expression due to removal of a stop codon from the reporter by Cre recombinase. Engraftment of Th17 cells in the small intestine suppressed SFB, indicating that this lineage mediated SFB gardening although IL-17A itself was not required (Fig. 4-5D).

Polymorphism in the RORγt gene influences the composition of the colonic mucosal bacteria in Crohn's disease patients and healthy controls

Given the evidence from animal models that RORγt shapes the mucosal microbiome, we investigated whether genetic variation in RORγt could garden the microbiome in humans. A meta-analysis of genome-wide association studies identified an IBD-protective variant (rs4845604) in the RORC gene that encodes RORγt (*36*). Microbial samples were obtained from the mucosa of the cecum and sigmoid colon of 112 healthy individuals and 85 CD patients. PCoA analysis revealed that carriers of the IBD-protective variant had altered microbial composition compared to non-carriers (Fig. 4-6A). Similar results were obtained when only health controls were analyzed, indicating that the findings did not simply reflect the negative association of the allele with IBD. Carrier status was associated with enrichment of 295 and depletion of 538 OTUs in multivariate models (Fig. 4-6B). Many of the downregulated OTUs belonged to the Proteobacteria and Fusobacteria phyla, which have been previously associated with the IBD microbiome and may be enriched in pathobionts (*42*). Subset analysis of only healthy controls yielded enrichment of 143 and depletion of 440 OTUs in carriers with a similar

pattern of decreased Proteobacteria and Fusobacteria (Fig. 4-6C). In both analyses, carrier status was also associated with increased *Akkermansia*, a mucus-resident organism depleted in IBD patients (*43*).

Discussion

In this study, we demonstrated that RORγt is critical for immune gardening of the mucosa-associated microbiome of the colon and small intestine. We confirmed earlier reports that innate RORγt-dependent cells regulate levels of SFB in the feces and additionally provided evidence that these cells control SFB levels in the colonic and duodenal mucosa (*28, 29*). We identified a role for T cells in gardening the colonic mucosal microbiome that may be mediated by IL-17A. Most significantly, RORγt-dependent CD4+ lamina propria T cells were shown to be necessary for immune gardening of SFB in the mucosa of the distal small intestine. Transfer of these cells into RAG/RORγt double knockout mice suppressed SFB levels, indicating that T cell gardening does not require B cells, non-CD4+ T cell subsets, or ILCs. Interestingly, while fate-mapped Th17 cells were capable of gardening SFB, IL-17A itself was not required. Gardening may be mediated by other RORγt-dependent cytokines produced by Th17 cells that influence epithelial antimicrobial production or mucus glycosylation (*24, 27, 30, 31*). IL-22 is an attractive candidate as the engrafted lamina propria CD4+ T cells produced IL-22 and it has been previously reported that SFB in the ileal lumen is increased with IL-22 neutralization or deficiency (*28, 29*). While this was attributed to ILCs, no experiments have been published evaluating whether ILCs or T cells are the relevant source of IL-22 in the small intestine. It will be of great interest in follow-up studies to dissect the specific cytokine requirements of RORγt-dependent CD4+ T cells and to evaluate the effects of lamina propria T cell engraftment on epithelial function.

The primary target of RORγt gardening in the small intestine was SFB, which has been shown to induce Th17 differentiation, IgA secretion, and antimicrobial production in mono-colonized mice (*44, 45*). SFB mono-colonized mice are susceptible to experimental models of arthritis and multiple sclerosis that do not develop in germ-free mice (*46, 47*). At least one study has examined SFB in gnotobiotic colitis models (*48*). SCID mice mono-colonized with SFB did not develop T-cell-transfer colitis, but SCID mice colonized with SFB in addition to microbiota from a specific pathogen free (SPF) facility developed more severe T-cell-transfer colitis than SCID mice colonized with SPF microbiota alone. These results suggested that immune activity induced by SFB – most likely Th17 responses – could augment T-cell-mediated disease elicited by other microbes. SFB has been reported in humans, raising the possibility that it or similar organisms could promote inflammatory disease mediated by Th17 cells in individuals with deficient immune gardening (*49*).

There is considerable evidence that Th17 cells contribute to IBD pathogenesis. IL-17A is upregulated in the intestinal tissue and serum of IBD patients (*50*). Polymorphism in the IL-23 receptor, which is critical for the stability and function of the Th17 phenotype, is a well characterized risk factor for IBD (*51*). IL-23R deficiency protects against colitis in mouse models of IBD including T cell transfer colitis and DSS colitis (*52, 53*). However, IL-17A itself may actually be protective against IBD. IL-17A-/- mice have exacerbated T-cell-transfer and DSS colitis and anti-IL-17A treatment was shown in a clinical trial to exacerbate Crohn's disease (*54-56*). Our data suggest a potential explanation for these puzzling results. We found that IL-17A regulates SFB in the colonic mucosal microbiome, such that anti-IL-17A treatment promotes SFB. Inhibition of IL-17A in humans may allow the overgrowth of specific IL-17A-responsive pathobionts such as SFB that can increase susceptibility to IBD. In this model, the

increased Th17 activity with SFB colonization can be viewed as an appropriate host response to regulate a pathobiont, though some of the products of Th17 cells such as IL-17F may promote IBD (*55*). In the absence of this gardening mechanism, dysbiosis could develop that confers increased risk for IBD. We speculate that other immunomodulatory therapies in clinical use or in development may also disrupt microbial gardening and will yield poor results unless paired with interventions that target the resulting dysbiosis.

A global analysis of IBD-associated genes and the fecal microbiome of IBD patients reported a statistically significant association of the rs4845604 polymorphism with the directionality of microbial abundance shifts across three cohorts (*20*). However, no associations of this RORC polymorphism with specific microbes were identified. Here, we provide evidence from Crohn's disease patients and healthy controls that this polymorphism has a profound impact on the colonic mucosal microbiome with depletion of many potential pathobionts. Our findings in animal models predict that a similar phenotype is likely to be observed in the mucosal microbiome of the small intestine. These microbial changes suggest enhanced gardening by individuals with this polymorphism, possibly due to elevated activity of RORγt-dependent CD4+ T cells and/or ILCs, and may represent a mechanism for disease resistance.

Existing literature on the effect of the immune system on microbial composition has largely focused on the fecal microbiome, potentially overlooking gardening pathways localized to specific regions such as the distal small intestine as shown in this study. We believe that the distinct gardening requirements of the distal small intestine compared to the proximal small intestine and colon may be relevant to Crohn's disease, an inflammatory disorder that can manifest anywhere in the digestive tract but predominantly affects the ileum. Further investigation into genes related to RORγt gardening may reveal genetic variants with impaired

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gardening that confer increased risk of IBD. Patients with gardening defects may require therapy

directed at specific pathobionts that bloom in a permissive immune environment.
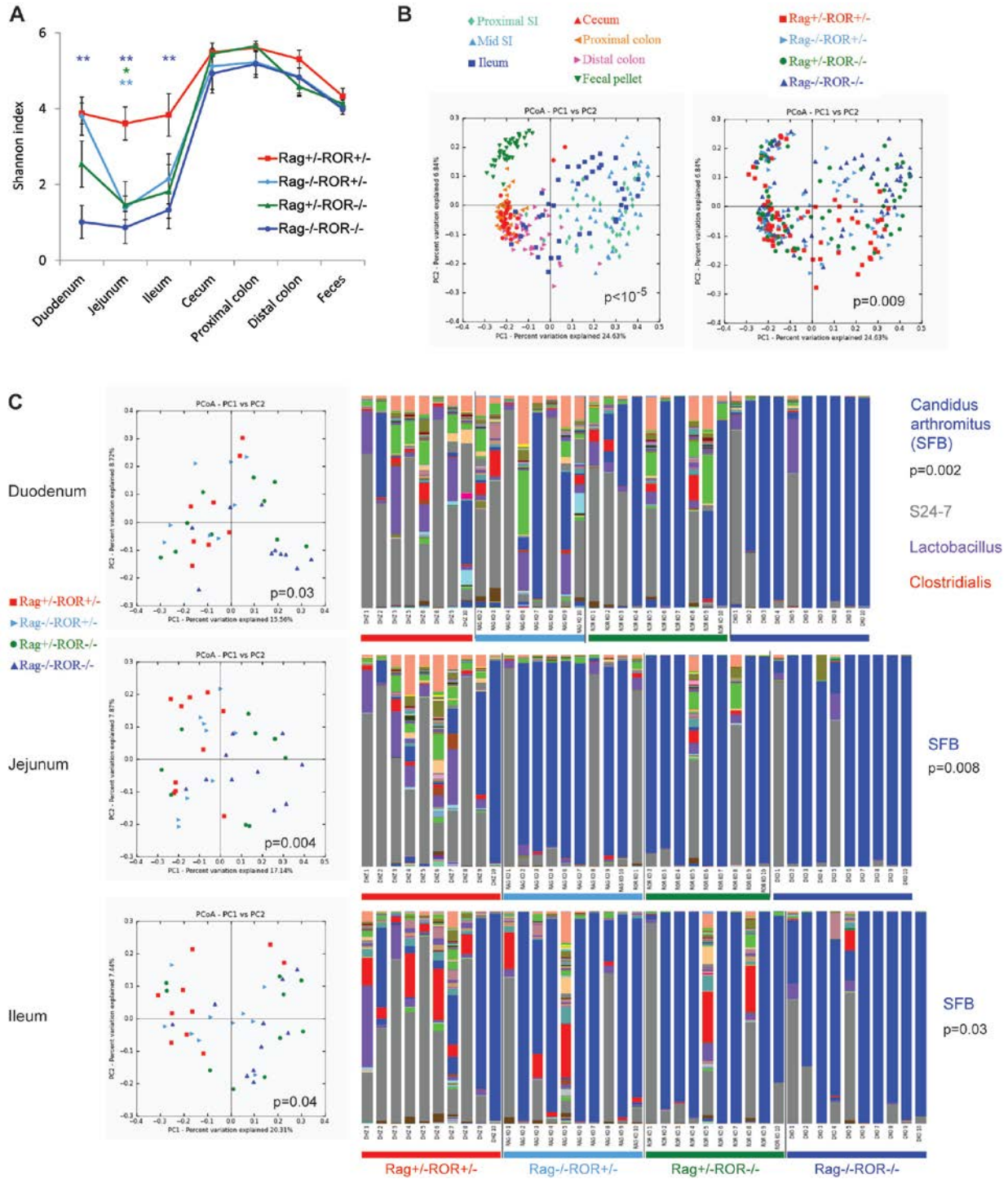
Figures

Figure 4-1. Mice deficient in RORγt or adaptive immunity have SFB overgrowth in the small intestinal mucosa. (A) Microbial diversity as measured by the Shannon index was assessed for colonic and small intestinal mucosal samples as well as fecal samples from RAG+/-RORγt+/-, RAG-/-RORγt+/-, RAG+/-RORγt-/-, and RAG-/-RORγt-/- mice. Diversity in the three knockout genotypes was compared to that of the RAG+/-RORγt+/- control mice. Statistical significance was calculated using the Mann-Whitney U test and is indicated by color-coded astericks; *p<0.05, **p<0.01. (B) Principal coordinates analysis (PCoA) plots are shown for microbial composition, colored by the sample site and mouse genotype. Statistical significance of differences among groups was calculated using Adonis and is shown in the plots. (C) PCoA plots depict microbial composition for each of the three regions of the small intestine. P-values were calculated using Adonis. Color-coded bar graphs to the right depict the relative abundance of dominant microbes in each sample. P-values for SFB enrichment (>50% abundance) were calculated using the Fisher's exact test.
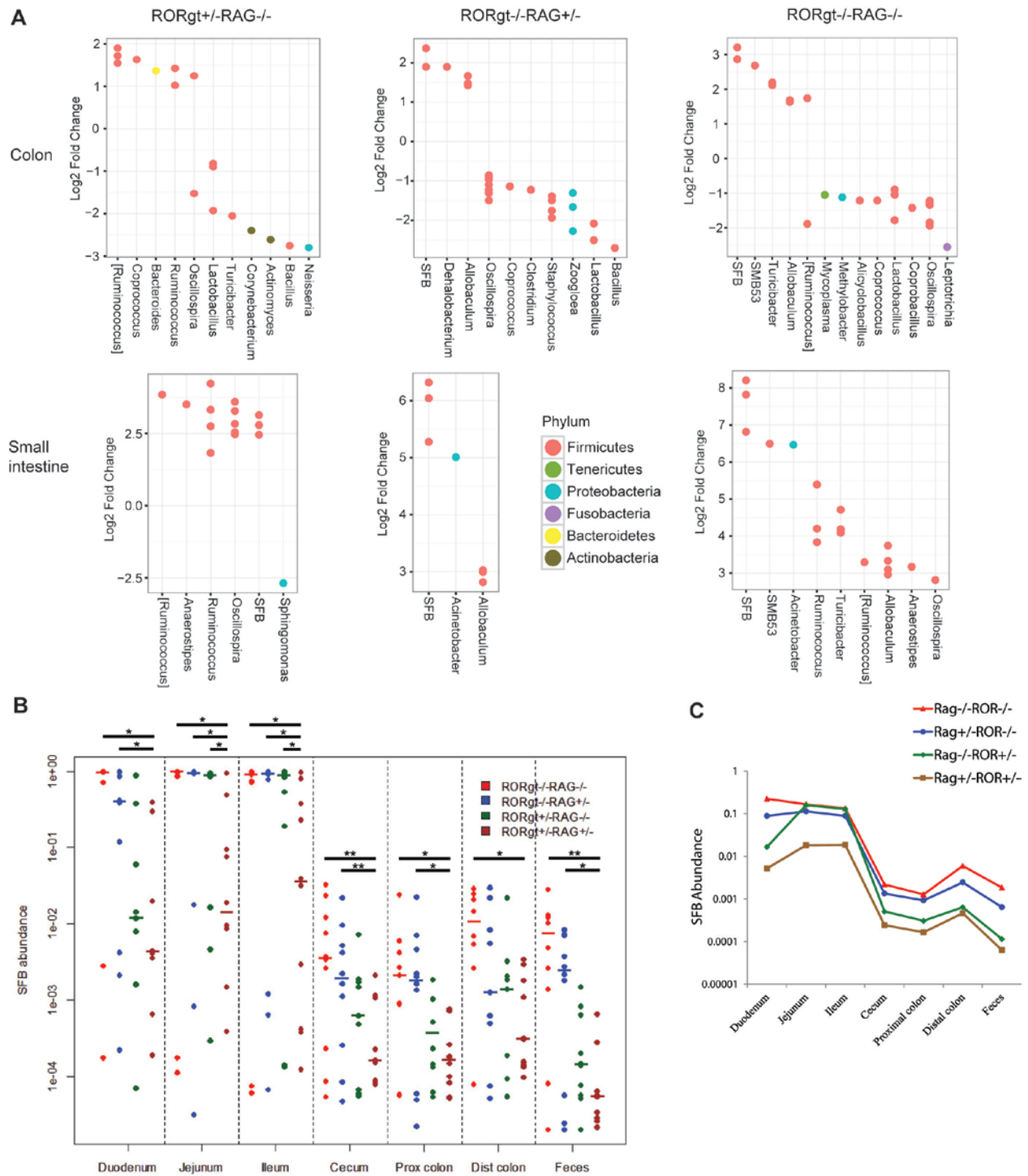
Figure 4-2. Increased SFB is seen throughout the small intestine and colon of mice deficient in RORγt. (A) OTUs in the colonic and small intestinal mucosa with differential abundance in

each of the three indicated knockout mice relative to RAG+/-RORγt+/- mice are shown, arranged

by genus and color-coded by phylum. Log2 fold change was calculated using multivariate

models incorporating sample site, gender, litter, and genotype. (B) SFB abundance is plotted for

samples from the six mucosal regions and feces, color-coded by genotype. Bars represent median

abundance. Statistical significance was calculated using the Mann-Whitney U test; *p<0.05,

**p<0.01. (C) Geometric means of SFB abundance are shown for each of the genotypes across

the six mucosal regions and feces. Error bars are not shown as the data did not follow a normal
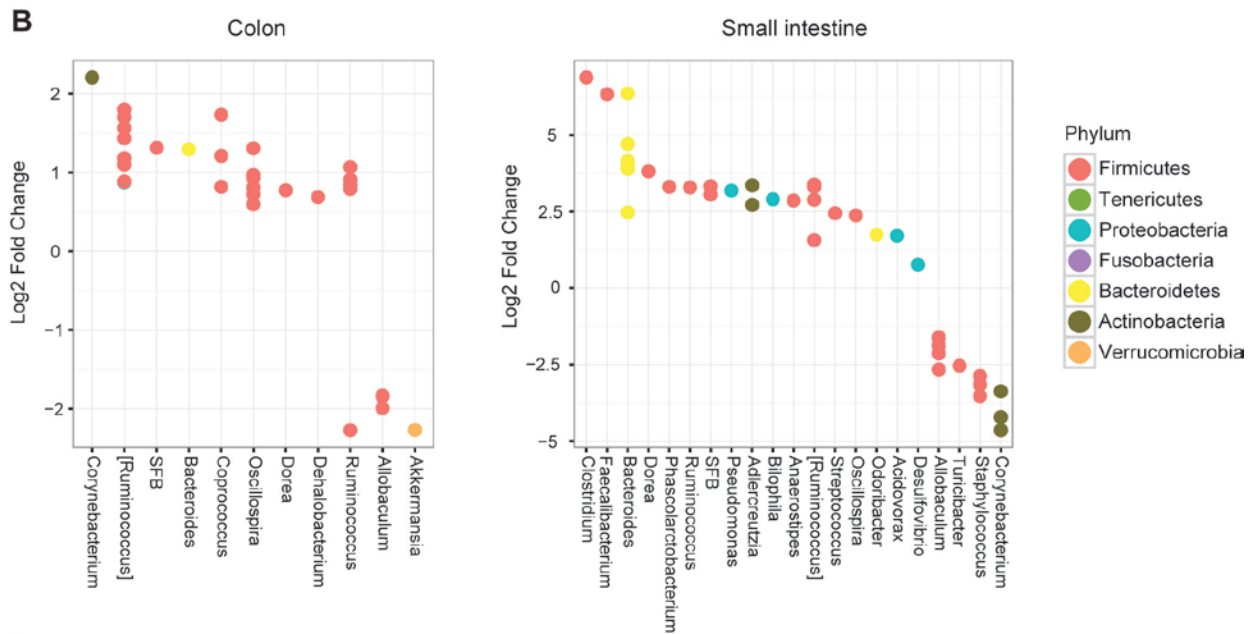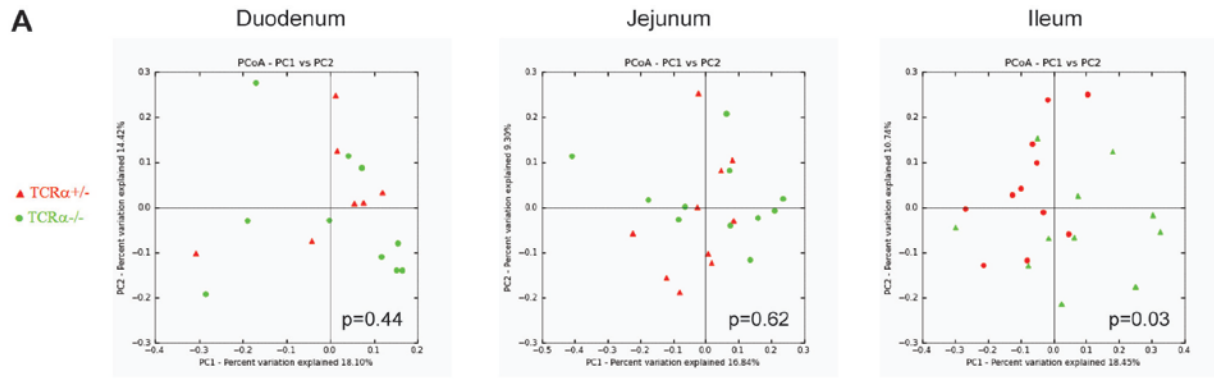
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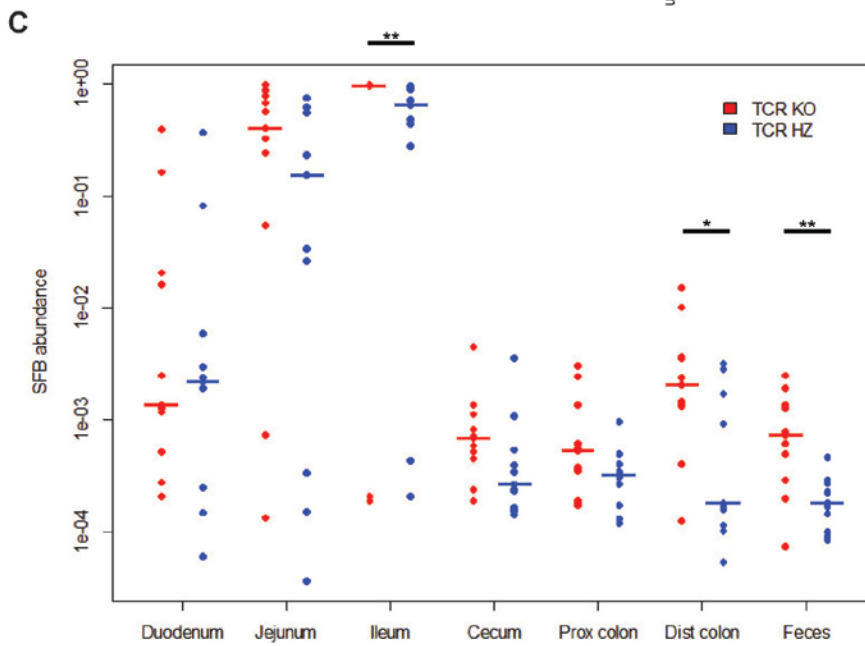
Figure 4-3. T cell deficiency promotes SFB overgrowth in the small intestine and colon. (A) PCoA plots are shown for microbial composition of TCRα+/- and TCRα-/- mice in the three regions of the small intestine. P-values were calculated by Adonis. (B) Differential OTUs in the colonic and small intestinal mucosa of TCRα-/- mice compared to TCRα+/- mice are shown. Log2 fold changes were calculated using multivariate models. (C) SFB abundance in TCRα+/- and TCRα-/- mice is plotted for samples from the six mucosal regions and feces. Statistical significance was calculated using the Mann-Whitney U test; *p<0.05, **p<0.01.
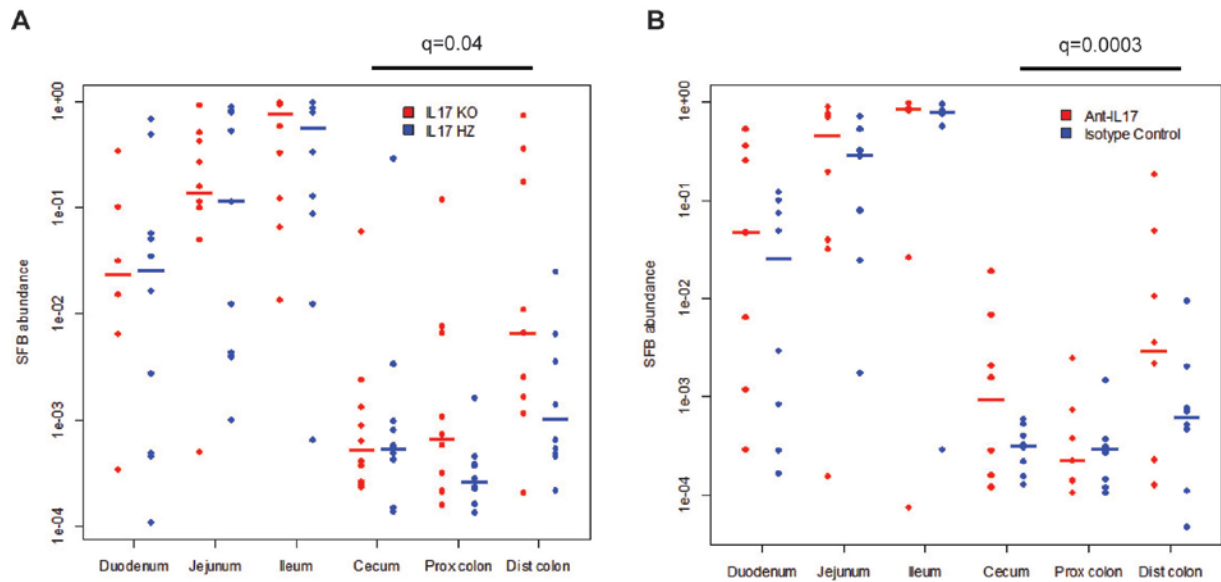
Figure 4-4. IL-17 deficiency and antibody neutralization increases colonic SFB without affecting SFB in the small intestine. (A) SFB abundance in IL-17A+/- and IL-17A-/- mice is plotted for samples from the six colonic and small intestinal mucosal regions. The q-value from multivariate models for the difference in colonic SFB abundance between the two genotypes is shown. (B) SFB abundance is shown for wild-type mice treated with anti-IL-17A or isotype control antibody for 28 days.
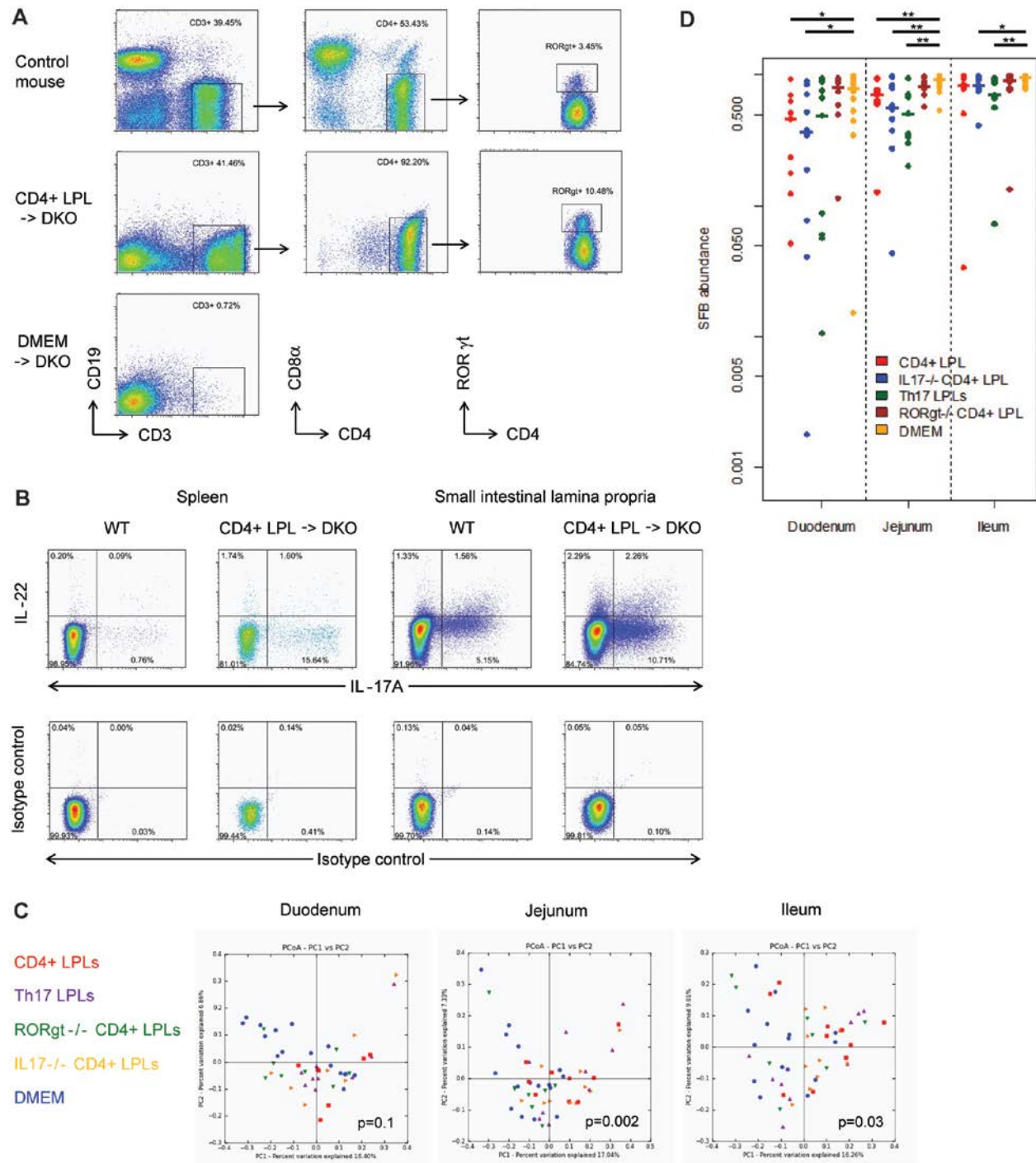
Figure 4-5. Lamina propria Th17 cells suppress SFB in the small intestine in a RORγt-dependent and IL-17-independent manner. (A) Flow cytometry of small intestinal lamina propria lymphocytes (LPLs) isolated from wild-type mice, RAG-/-RORγt-/- (DKO) mice

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receiving CD4+ LPLs, and DKO receiving DMEM. (B) IL-17A and IL-22 expression by engrafted LPLs in the small intestinal lamina propria and spleen of DKO recipients was determined by intracellular staining 8 days after transfer. Samples from wild type mice are provided for comparison. (C) PCoA plots showing microbial composition in DKO mice after engraftment of wild-type, IL-17A fate mapped, RORγt-/-, and IL17A-/- LPLs. Mice receiving DMEM serve as controls. P-values were calculated using Adonis. (D) SFB abundance is plotted for the three mucosal small intestinal regions, color-coded by donor cell type. Statistical significance was calculated using the Mann-Whitney U test; *p<0.05, **p<0.01.

Figure 4-6. The rs4845604 polymorphism in RORC is associated with shifts in the colonic mucosal microbiome of Crohn's disease patients and healthy controls. (A) PCoA plots of microbial composition in carriers and non-carriers of the rs4845604 polymorphism in the MLI cohort. The left panel shows the full cohort and the right panel shows only individuals without IBD. P-values were calculated using Adonis. (B-C) The log2 fold change between carriers and non-carriers is shown for differential OTUs identified in multivariate models using the full cohort (B) or only non-IBD individuals (C). The covariates included gender, disease status, sampling site, and carrier status.

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