CellPress

Immunity
Perspective

Finding the Missing Links among Metabolites, Microbes, and the Host

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http://dx.doi.org/10.1016/j.immuni.2014.05.015

The unexpected diversity of the human microbiome and metabolome far exceeds the complexity of the human genome. Although we now understand microbial taxonomic and genetic repertoires in some populations, we are just beginning to assemble the necessary computational and experimental tools to understand the metabolome in comparable detail. However, even with the limited current state of knowledge, individual connections between microbes and metabolites, between microbes and immune function, and between metabolites and immune function are being established. Here, we provide our perspective on these connections and outline a systematic research program that could turn these individual links into a broader network that allows us to understand how these components interact. This program will enable us to exploit connections among the microbiome, metabolome, and host immune system to maintain health and perhaps help us understand how to reverse the processes that lead to a wide range of immune and other diseases.

Introduction

The human microbiota (the collection of microbes that inhabit our bodies) and human microbiome (the collection of DNA from microbes) are remarkably, and unexpectedly, diverse. Although the Human Microbiome Project (HMP) (Peterson et al., 2009; Turnbaugh et al., 2007) was predicated on the assumption that we would share a large core of microbial lineages, sprinkled with a diversity of "peripheral" lineages that make each of us unique (Turnbaugh et al., 2007), this hypothesis was not validated by empirical evidence after the completion of the HMP. Indeed, the first deep sequencing of multiple fecal samples from each of three individuals revealed that the differences between individuals are large. The differences between individuals are substantially greater than the differences within an individual at different sampling sites along the distal large intestine (Eckburg et al., 2005). This pattern of diversity has subsequently been reinforced in different body habitats (Costello et al., 2009; Findley et al., 2013; Grice et al., 2009; Human Microbiome Project Consortium, 2012) and with larger subject populations, especially in the gut (Qin et al., 2010; Turnbaugh et al., 2009; Yatsunenko et al., 2012). Even within healthy Western adults, studies routinely show that different people can be >90% different in terms of the populations of microbes in their gut; in other words, a microbial cell chosen from person A and a microbial cell from person B will be different at the species level more than 90% of the time. Additionally, the dynamic range of the most common microbes is hugely variable. Within the microbes identified by the European MetaHIT Project as "core," meaning that they were found in at least 90% of the healthy European cohort studied, the dynamic range was several orders of magnitude different for each species—in other words, any microbial species found with at least 10% abundance in one person was at least as rare as one cell in 1,000 in another person in the cohort (Qin et al., 2010). We are at the leading edge of understanding the implications of this tremendous diversity at the metabolic level and of the interplay among the gut microbiota, metabolic function, and the immune system. To address the molecular connections between gut bacteria and immune function, there now exists a collection of synergistic technologies that will allow rapid progress in untangling these complex interactions in ways that can be harnessed to promote health.

Grappling with a Diverse Microbiota

Although the microbiota is incredibly diverse, and much of this diversity still consists of uncharacterized species and genes, defining this diversity for some populations appears within reach given the multitudes of microbiome profiling projects to date. Recent large-scale studies, such as the HMP and MetaHIT efforts, are beginning to saturate the gene catalog for healthy Western cohorts (Human Microbiome Project Consortium, 2012; Qin et al., 2010). This diversity is usually assessed by a technique called rarefaction: as additional subjects are examined, a curve is plotted with the number of subjects on the x axis and the number of unique taxa or genes on the y axis. As discovery of this microbial "parts list" becomes more complete, finding a new unique part requires additional people and subsets of populations, so the curve levels off until, at last, it reaches an asymptote when all the parts have been discovered. Similar saturation of rarefaction curves is now being observed for several clinical conditions in which the microbiome is involved, such as obesity (Le Chatelier et al., 2013) and diabetes (Qin et al., 2012). This finding has several important implications: first,

that the depth of microbial diversity is addressable via currently available technologies; second, that a reference database can be constructed, allowing interpretations of new sequences on the basis of matching findings to what is known already rather than computationally driven de novo assembly and annotation procedures; and third, that markers can be discovered within this known universe and then prioritized for further laboratory characterization and experimental work, including studies in animal models, such as those using gnotobiotic mice. However, a cautionary note appears needed: studies of children and of non-Western individuals reveal completely different configurations of the microbiome and the microbiota (Yatsunenko et al., 2012). Consequently, substantial additional work will be required for extending these techniques to cover the diverse complexity found in humanity, a notion that can be extended to various conditions in relevant animal models.

Another previously made key assumption that was not validated by subsequent studies is that diversity in the microbiota correlates directly to diversity in the microbiome in terms of overall metabolic functions. This property, that different assemblages of species converge on very similar functional profiles, was first observed in the human gut (Turnbaugh et al., 2009) and then more recently extended to other human body sites (Human Microbiome Project Consortium, 2012). Conceptually, this makes sense in retrospect and by analogy to larger-scale ecosystems: two grasslands might look relatively similar to each other, especially in comparison to two forests, even in situations where essentially none of the species in either grassland is shared (Hamady and Knight, 2009). Although particular species and functions seem to be highly individualized (Fierer et al., 2010; Schloissnig et al., 2013) and even stable over time (Caporaso et al., 2011; Faith et al., 2013) and although associations between genetic lineages of microbes and functional capacities are so highly conserved that functional profiles can be predicted accurately from species distributions (Langille et al., 2013), the overall functional profiles appear remarkably static in healthy subjects overall. Although a few studies have correlated the microbiota to the microbiome (Muegge et al., 2011) and to the metabolome (Ridaura et al., 2013; Smith et al., 2013a), the relationships among the various "omics" levels and with the host immune system remain largely unexplored.

Metabolite Diversity Might Exceed Even Microbial Diversity

In contrast to the microbiota and the microbiome, which we are now starting to saturate for some populations, the metabolome is extremely diverse and remains largely undescribed and undefined. For example, tissue-culture cells grown in pure culture remain poorly characterized in terms of their metabolic profiles, underscoring the dearth of knowledge about more complex biological systems. The human genome contains approximately 20,000 protein-coding genes, but our metabolomic profile is much more complex—it numbers at least 500,000, and individual classes of compounds, such as lipids and oligosaccharides, potentially harbor very high levels of diversity simply on combinatorial grounds (Quehenberger et al., 2010; Shevchenko and Simons, 2010). For example, the molecular family of six common triacylglycerides that use at least 20 different fatty acids provides the combinatorial capacity to make more than 40,000 different lipids (and in reality, there are many more fatty acids, leading to further combinatorial explosion). This calculation does not take in account the many modifications that fatty acids undergo or the many modifications triacylglycerides undergo themselves. This is just one description of one subfamily of one molecular family. Furthermore, many specialized metabolites, including secondary metabolites, natural products, quorum sensors, and small-molecule virulence factors, are produced by nearly all microbes. Some organisms have the metabolic capacity to make as many as 50 of these. These molecules are optimized to regulate the surrounding environment and to control and alter biology at the host-microbe interface by driving microbial community composition and host immune regulation (Figure 1). In turn, these molecules drive complex physiological feedback loops (Nicholson et al., 2005). These microbial molecules have been exploited in the clinic as cholesterol-lowering drugs, immune regulators, or antibiotics. On the basis of the genome sequences now available, there are strong indications that thousands more of these microbial effector molecules remain to be discovered in the human microbiota. Finally, given that the diversity of diet-derived cells and microbial cells present in the gut dwarf human cell diversity, as well as outnumber them, it seems appropriate to assume that the vast majority of metabolites in our bodies are not of human origin and that microbes significantly alter the human microbiome (Figure 1).

The number of potential gut bacterial metabolites is thus currently unknown and includes molecules of dietary, host, and microbial origin. Furthermore, there is complex crosstalk among hosts, microbes, and the transformations that the metabolites undergo. These complex transformations often define the ultimate function of the molecules that are present (Martin et al., 2007). The tools currently available for harvesting and visualizing the diversity of microbial metabolites, including both primary and specialized metabolites, do not presently capture this complexity. Consequently, the state of knowledge for metabolites resembles the situation for microbes a decade ago: we know that there are myriad metabolites of potential microbial origin or of microbial transformation, and we know that some are shared among people and some are unique. In a handful of examples that are clinically interesting, metabolites and their association with clinical phenotypes have been described via targeted techniques (Martin et al., 2007). For example, people are similar in some of the common metabolites they contain (such as amino acids or short-chain fatty acids in the gut), and some of this variation correlates with disease. However, we lack a clear understanding of how many (and which) microbial metabolites influence host biology, as well as the overall nature of variation in the general population. This is due to the current limitations in technical approaches capable of building a comprehensive metabolomic "parts list" on a population-level scale. If we perform the analysis at the level of pathways rather than individual metabolites, they might appear to be more similar among individuals, but this apparent similarity might arise from limitations in the bioinformatics tools used. Just as we are starting to appreciate that our microbiomes augment our genome's capabilities and indeed can be more predictive than our human genome for classifying people as healthy or diseased (Knights et al., 2011; Le Chatelier et al., 2013), we need to expand the concept of our metabolome to include the microbially produced and microbially modulated metabolites as factors contributing to pathogenesis.

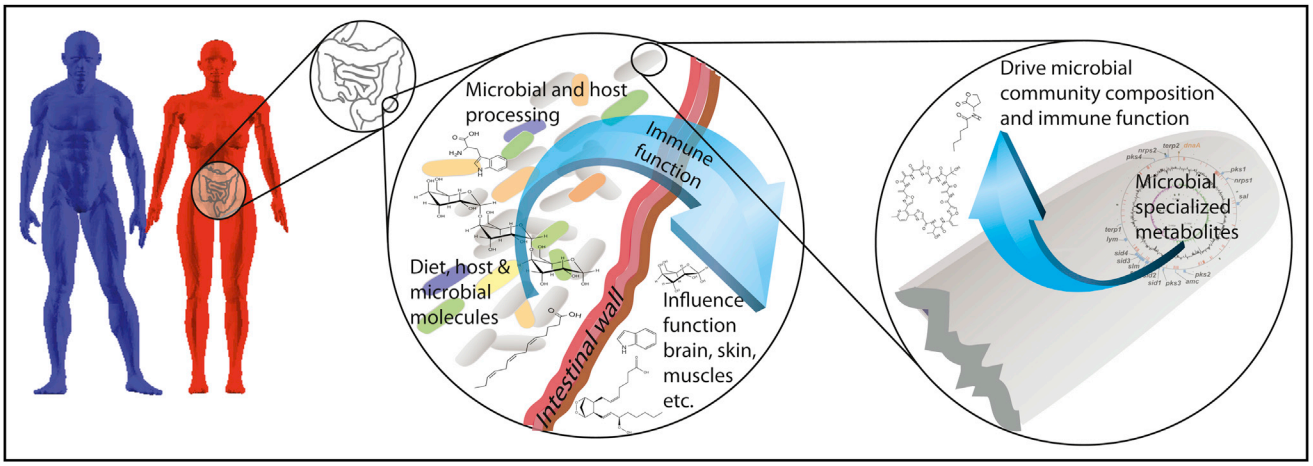

Figure 1. The Microbe-Host Interface
Individual differences, including sex-specific differences between men and women (blue and red, respectively), might control the microbiota or interact with the microbiota to influence immune function in several ways, including the release of metabolites and direct microbial interactions. Much of this interface occurs in the gut (shown in the inset in the female figure), where the intestinal wall provides an interface between metabolites and immunological processes in the gut and the influence of these metabolites and immunological processes on the host systemically. In addition to having normal metabolic function, specialized microbially produced metabolic products (second inset), such as signaling molecules and antibiotics, might play a key and specific role in driving microbial community composition and subsequently the microbiome.

Integrating Immunology with Metabolomics Studies: A New Frontier

Immunological responses by the host are also diverse, although our view of this diversity has been limited by the application of targeted approaches. Traditional research in immunology has focused on diverse immune responses to pathogenic infection. Viewed through this perspective, conventional wisdom in the field has guided studies to determine how the immune system attempts to control infectious agents, how pathogens subvert the arsenal of innate and adaptive immune mechanisms, and how individual mutations in the host might affect these processes. We now appreciate that host-microbial interactions at various body surfaces cannot be fully captured in this simple framework but will most likely need to involve complex and dynamic processes. These processes include not only immunity to pathogens but also immune ignorance and/or tolerance mechanisms for symbiotic bacteria and opportunistic pathogens that are routinely found in microbiome studies. The functions of the immune system might even include promoting the growth of beneficial microbes, as well as limiting the growth of harmful microbes, given that the same microbe could be harmful or beneficial in the context of different body sites, host physiological status, and so forth. Conceivably, individual members of the gut microbiota can have diverse effects on the immune system such that some indigenous organisms promote proinflammatory responses while others promote anti-inflammatory reactions. A handful of microbes have been experimentally shown to directly affect infection (by other species, including bacteria, eukaryotes, and viruses), autoimmunity, and inflammation in animal models. These examples together with the genomic information indicate that a vast constellation of microbes with bioactive properties is waiting to be discovered. Consequently, the interplay between the gut microbiota and the immune response is likely to be far more diverse and dynamic than we currently appreciate, and the role that metabolites play in the evolutionary connection between symbiotic microbes and their hosts is a frontier of research in the field.

To date, observations connecting the microbiome, the metabolome, and immunological responses have been sporadic and incomplete. Investigators have made observations, like flashbulbs going off in the dark, but a systematic approach has not yet been applied in any complex biological system. Therefore, although we know that connections exist (as detailed below), only a small fraction of these connections are known at present. In part, this lack of a unified approach is due to "language barriers" among the practitioners of the different disciplines and the lack of a unified research community making a concerted effort to bring their collective expertise to bear on these problems. Going forward, better ways of representing and visualizing the data—especially methods that translate across the various highly multivariate data sets collected by each discipline, as well as research practices and communities that allow translation of results among disciplines—will be critical for uncovering the diverse molecular interplay between gut microbial metabolites and the immune system. This perspective describes the current state of the art in our understanding of microbial-metabolite-immune connections and provides a framework for the vast and exciting potential of developing an integrated program to decode the molecular conversation between mammals and their microbiomes. In doing so, we describe a microbial-metabolite-based roadmap for explaining current observations and discovering new interactions in ways that will be useful for basic research, predictive modeling, patient stratification, and potentially the development of transformative treatments for various diseases.

Microbial Metabolism and Inflammation

The microbiome produces a wide range of metabolites and has a pervasive effect on various host processes, although many of these effects are just starting to be explored in detail. It has

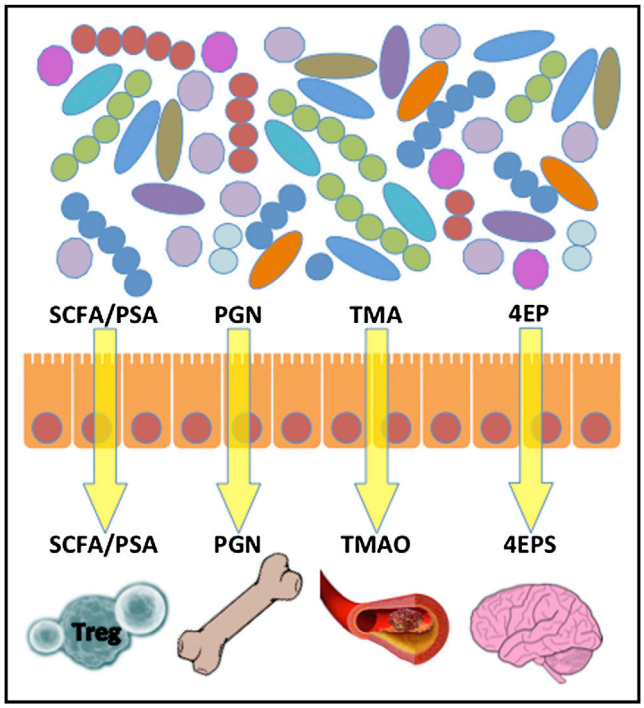

Figure 2. A Simplified Depiction of the Effects of Specific Gut Microbial or Microbial-Mammalian Cometabolites on Different Organ Systems, Including the Immune System

Short-chain fatty acids (SCFAs) and polysaccharide A (PSA) affect Treg cell development. Peptidoglycan (PGN) affects bone resorption. Trimethylamine N-oxide (TMAO) affects the vascular system and influences the risk of heart disease. 4-ethylphenol sulfate (4EPS) affects brain function and leads to anxiety-like behavioral defects similar to those observed in autism. Although some of these examples illustrate harmful effects, it is likely that many microbially produced metabolites in addition to SCFAs and PSA produce beneficial effects on the host, although these tend to be less well studied. Additional abbreviations are as follows: 4EP, 4-ethylphenol; and TMA, trimethylamine.

long been known that bacteria in the gut are involved in metabolizing otherwise indigestible food components, such as dietary fiber, but additional functions are being discovered at a rapid pace. For example, microbial production of vitamins and amino acids is important during human infant development (Yatsunenko et al., 2012) and has been implicated in malnutrition (Smith et al., 2013a), which in turn affects susceptibility to a variety of infectious diseases. Similarly, different bacteria in different people have variable effects on the metabolism of drugs, ranging from the painkiller acetaminophen (Clayton et al., 2009) to the cardiac glycoside digoxin (Haiser et al., 2013) to the cancer drug cyclophosphamide (Viaud et al., 2013), and most likely also have an effect on immunomodulatory drugs.

Microbial metabolites have long been implicated in metabolic functions both inside and outside the gut (Figure 2). Of these, short-chain fatty acids (SCFAs), breakdown products of dietary fiber (such as butyrate), are especially important as an energy source for intestinal epithelial cells. Recent studies have expanded the role of SCFAs from gut bacteria to reveal impacts on the immune system (for more detail, see the accompanying review by Thorburn et al., 2014 in this issue of *Immunity*). In brief, in ulcerative colitis, an inflammatory bowel disease (IBD), SCFAs are reduced, and treatment with dietary fiber provides clinical benefits. Germ-free mice without any gut bacteria have lower SCFA levels than do animals with a complex microbiota (Maslowski et al., 2009). This phenotype is attributed to increased intestinal inflammation in a mouse model of IBD, suggesting that SCFAs provide beneficial or immunosuppressive functions. Accordingly, deletion of GPR43, a sensor of SCFAs, leads to exacerbated intestinal disease in mice (Figure 2).

SCFAs directly affect the development and function of anti-inflammatory regulatory T (Treg) cells, which restrain uncontrolled inflammation. SCFAs, and in particular propionate, increase both the proportion and the absolute count of Treg cells in germ-free mice and augment Treg cell function to promote suppression in colitis models (Smith et al., 2013b). Bacteria such as spore-forming group XIVa Clostridia appear to produce butyrate that also promotes Treg cell differentiation both in vivo and in vitro (Furusawa et al., 2013). The effects of SCFAs extend beyond Treg cells and the gut. For example, mice fed a high-fiber diet have both increased SCFA levels and protection from allergic inflammation in the lungs, attributed to effects on hematopoiesis of innate immune cells by propionate (Trompette et al., 2014). Collectively, these studies highlight how gut microbial metabolites, specifically SCFAs, provide benefits to the host via enhancing the innate and adaptive immune systems in various animal models and contexts. Future work will be needed for determining whether this mechanism translates to humans. Dietary and microbial interventions for allergic, inflammatory, and autoimmune diseases would be relatively safe and feasible approaches that could be rapidly validated in many populations and societies around the world.

Specific Immune Signals from Microbially Produced Metabolites

Many of the diseases that have been linked to the microbiome also have an immunological component. These conditions include IBD (Frank et al., 2011; Frank et al., 2007; Gevers et al., 2014), obesity (Cotillard et al., 2013; Le Chatelier et al., 2013; Ley et al., 2006; Ridaura et al., 2013; Turnbaugh et al., 2009), rheumatoid arthritis (Scher et al., 2013), food allergies (Ling et al., 2014), asthma (Fuchs and von Mutius, 2013), and animal models of multiple sclerosis (Berer et al., 2011; Lee et al., 2011), autism (Hsiao et al., 2013), resistance to infection (Abt et al., 2012; Ichinohe et al., 2011; Khosravi et al., 2014), and a wide range of other conditions. Examples of specific microbial triggers, including lipopolysaccharide (LPS), double-stranded RNA (dsRNA), and quorum-sensing molecules (homoserine lactones and their derivatives), are also well known. We will summarize some of this literature, but these topics have been extensively reviewed elsewhere. Microbe-brain connections are also increasingly emerging as interesting and important (Cryan and Dinan, 2012). For example, neurotransmitters, such as dopamine and serotonin, are either produced by bacteria or stimulated in host cells by the presence of bacteria; viewing these neurotransmitters as metabolites that affect the immune system is an important emerging perspective (Baganz and Blakely, 2013).

Metabolites and Pattern Recognition by the Immune System

Of particular interest is how molecules produced by bacteria, including metabolites, can act in pattern recognition by the

immune system. The innate immune system has evolved pattern-recognition receptors (PRRs) that recognize conserved microbial ligands. Binding to these ligands alerts the host to the presence of the microbes that produce them. This class of molecules, commonly termed pathogen-associated microbial patterns (PAMPs), has primarily been studied in the context of pathogenesis. Remarkably, some PAMPs produced by the gut microbiota can also positively affect the immune system and health. For example, in a model of intestinal injury and inflammation, more severe disease has been shown to occur in the absence of commensal microbes, but this effect can be ameliorated by the addition of LPS or lipotiechioc acid, two highly conserved products produced by microbes (Rakoff-Nahoum et al., 2004). The beneficial effects of microbial molecules extend beyond the gut. For example, peptidoglycan (a PAMP) from gut bacteria augments the function of innate immune cells that originate from bone marrow, such as neutrophils, to help fight off systemic bacterial infection (Clarke et al., 2010). Depletion of gut bacteria also renders mice susceptible to influenza virus infection in the lungs and to systemic lymphocytic choriomeningitis virus infection (Abt et al., 2012; Ichinohe et al., 2011). The administration of PAMPs restores the host's ability to control infection, demonstrating that microbial molecules previously studied in the context of infection can actually have host-protective functions when produced at physiological amounts by gut bacteria. Finally, germ-free animals are defective in the differentiation of specific innate immune cell subsets that are critical for resistance to systemic bacterial infection (Khosravi et al., 2014). Oral administration of microbial ligands restores these defects, illustrating that microbial molecules regulate immune system development at its core—during hematopoiesis.

Another example of a PAMP that can act as a beneficial signal, and not just as a pathogenic trigger, is polysaccharide A (PSA) from the human commensal *Bacteroides fragilis* (Mazmanian et al., 2005). PSA signals to the immune system through a specific PRR to induce development and function of Foxp3$^+$ Treg cells, which prevent inflammation in the gut and CNS through the effects of interleukin-10 (IL-10) (Ochoa-Repáraz et al., 2010; Round and Mazmanian, 2010). Therefore, not all interactions between microbial molecules and PRRs lead to inflammation and disease (Chu and Mazmanian, 2013). One can therefore view PRRs as sensors not of pathogens but of microbes in general. Thus, PRRs can be considered the immune system's "eyes," which observe the microbial world and can be potentially beneficial or harmful to the host depending on the context of the interaction. This perspective suggests a reconsideration of the term PAMP to a more broad terminology proposed by many advocating the use of microbial-associated molecular patterns, or MAMPs (Mackey and McFall, 2006). The situation is somewhat analogous to antibiotics, which are often used in lower concentrations as signaling molecules in natural microbial ecosystems (Linares et al., 2006).

Microbially Derived Metabolites Affect the Immune System

The human immune system is significantly affected by many common microbially derived molecules. LPS is perhaps the best-studied microbially produced molecule affecting the inflammatory status of humans or mice; as of this publication, there are over 85,000 articles on this topic in PubMed. However, other bacterial molecules are also important. Tryptophan (which is an essential amino acid in humans and which we obtain both from our diet and from tryptophan biosynthesis by our microbial symbionts), as well as its microbially produced breakdown products, plays an important role in the immune system. For example, in mice fed unrestricted tryptophan diets, lactobacilli (typically thought of as anti-inflammatory, although the genus *Lactobacillus* contains considerable genetic and phenotypic diversity) produce indole-3-aldehyde, which upregulates IL-22 in the host and induces a mucosal response limiting colonization of the gut by the fungal pathogen *Candida albicans* (Zelante et al., 2013). Another interesting case is the common quorum-sensing molecule N-(3-oxo-dodecanoyl) homoserine lactone, which disrupts NF-κB signaling (Kravchenko et al., 2008). Although no homoserine lactones have yet been detected in the gut, the gut microbiome harbors many genes capable of producing this class of signaling molecules (Swearingen et al., 2013). Part of the challenge in detecting homoserine lactones is that they are rapidly degraded by microbes (Moroboshi et al., 2005; Tinh et al., 2007) and that they are only produced under specific conditions, even by bacteria that contain the relevant genes (Wang et al., 2006). Better methods for detecting transiently produced and rapidly degraded molecules might be required in order to pinpoint the role of such molecules in shaping the gut immune system.

Microbes also contribute to the alteration of arachidonic-acid-derived lipids. Although mostly studied in the context of pathogenesis (Eberhard et al., 2002), many organisms that can metabolize these lipids are found in the normal human microbiota and alter the amount of arachidonic acid and its downstream metabolites, such as prostaglandins and leukotrienes. For example, colonizing germ-free mice with *Bacteroides thetaiotaomicron* (*B. theta*) or colonizing them with *B. theta* and *Bifidobacterium longum* together increases prostaglandin E2 production (Rath et al., 2012). If insufficient arachidonic acid is present in the diet, it can also be obtained from hydrolysis of membrane lipids, which store arachidonic acid. Arachidonic acid release can be mediated by several mechanisms. Although arachidonic acid release has not yet been correlated with or attributed to the gut microbiota, many microbes, including many members of the normal gut microbiota, have the necessary hydrolases to produce arachidonic acid and most likely perform roles similar to that of phospholipase A2.

Unknown Functions of Specialized Gene Clusters in the Microbiome: New Natural Products?

Finally, many specialized metabolite-producing gene clusters (e.g., natural products, including polyketides, sterols, isoprenoids, and nonribosomally synthesized peptides) are found within the gut microbiome. The functions of these metabolites are almost completely all unknown. Given that many specialized metabolites isolated from other microbes, such as rapamycin, are now in clinical use to control immune-mediated diseases, it is likely that the microbiome has coevolved numerous gene clusters whose products interact with the immune system directly or indirectly, perhaps in concert with other microbial inhabitants of the gut. Many microbes produce the classes of molecules we have described, although understanding the full phylogenetic

spectrum of microbes capable of their production, and the conditions under which they are produced, remains largely uncharacterized especially in the context of the community.

Defining a Research Program to Integrate Microbiota, Metabolism, and Immunity

As noted above, many connections have been made between microbes and metabolism, between microbes and immunity, and between metabolites and immunity. However, connections that link all three are scarce at present. An integrated, comprehensive discovery- and hypothesis-driven research program could yield immense dividends in terms of insights into all three fields (microbes, metabolism, and immunity) and their fascinating connections. We therefore define approaches that can systematically identify these connections. In particular, these approaches will identify small-molecule phenocopies of what are currently thought to be host immune issues, which might include known bacterial components. We should aim to design a pipeline that transcends descriptive cataloging studies (microbiomes, metabolomes, etc.) and instead gets to the underlying mechanisms that represent the molecular foundations of host-microbial symbiosis.

A Paradigm for Studies Linking Metabolites, Microbes, and the Immune System

An example of what this research program might look like is provided by a recent study examining the links among the immune system, the microbiota, and metabolism in a mouse model of autism (Hsiao et al., 2013). In brief, mice born to mothers subject to maternal immune activation, which simulates viral infection, developed symptoms resembling autism spectrum disorders (ASD), including lack of social interaction, repetitive behavior, deficits in communication, gut barrier dysfunction, immunological changes, and dysbiosis of the microbiome. This combination of symptoms has been reported in ASD.

Importantly, the authors observed systematic changes in the serum metabolome, including overproduction of a metabolite (4-ethyl phenyl sulfate [4-EPS]) that when individually administered to normal mice recapitulates some of the same phenotypes. Introduction of a probiotic strain of *B. fragilis* resulted in lowered 4-EPS production and also ameliorated intestinal and behavioral abnormalities. This combination of immunological manipulation, generation of lead microbes and metabolites through untargeted microbiome and metabolite profiling, and tests in gnotobiotic mice (initially germ-free mice colonized with defined communities of microbes) provides a paradigm for identifying connections, although we emphasize that such studies that have been performed to date only scratch the surface of the full range of microbial and metabolic components of the altered response.

Toward an Integrative Pipeline

A pipeline for identifying novel connections in higher throughput might include a program to screen small-molecule compound libraries for effects on immune cells in tissue culture to both identify metabolites produced by bacteria or by bacterial communities that have been linked to diseases and test candidate microbes and metabolites, alone or in combination, in gnotobiotic mice. Additionally, screening bacteria in high throughput in organoid systems with reporter gene assays to identify immunomodulatory effects might be more facile than using live animals. Essentially, the pipeline needs to include (1) case-control studies to establish that there are microbial or metabolic differences to be explained in the first place, (2) spatial mapping and multivalent characterization (microbiome, metabolome, immune repertoire) to inform hypotheses about possible connections, and (3) prospective longitudinal studies in humans and preclinical experimental manipulation studies in mice or other model systems to establish causality. This approach might ultimately lead to drug candidates for clinical trials in humans.

This pipeline will be complicated by the bidirectional connections between the microbiome and the immune system. For example, genetic deletion of TLR5, an innate immune system component that recognizes bacterial flagellin, results in a substantially altered bacterial community, which in turn (depending on the microbial background) can lead to phenotypes ranging from metabolic syndrome (Vijay-Kumar et al., 2010) to colitis (Carvalho et al., 2012a; Carvalho et al., 2012b), the latter of which stems from an inability to regulate proinflammatory proteobacteria. Additional research has shown that, instead of producing a single altered microbiome, TLR5-deficient mice produce different microbiota coupled to different phenotypes that can be transmitted vertically within a family (this is facilitated by the fact that mice are coprophagous) (Ubeda et al., 2012). Microbiome profiling and specific cytokine assays have been performed in these animals, and some of the phenotypes have been transferred to previously germ-free mice by transmission of the altered microbiota. Fascinatingly, such phenotypes include the behavioral phenotype that causes the TLR5-deficient mice to overeat and thereby develop metabolic syndrome. Unfortunately, however, the combination of microbiome, metabolite, and immunological profiling in a longitudinal study design that would be ideal for identifying lead microbes and metabolites has not yet been performed.

Moving Beyond the Gut

In our discussions thus far, we have mainly focused on the gut and on the systemic effects of gut microbes at distal sites because these have been the best studied to date. However, there is intriguing but preliminary evidence that there might be microbes at sites previously thought to be sterile in healthy adults, including the lungs, adipose tissue, the pancreas, the liver, amniotic fluid, and even the brain. Studying whether microbes inhabit these sites in gnotobiotic mice and the immunological and host responses at distal sites, together with the metabolites produced by the host, the bacteria, or the combination of the two, has substantial potential for uncovering fundamentally new mechanisms of disease. Microbial metabolites are dramatically understudied in the context of the human nervous system, immune system, and metabolism. We propose that future studies should focus on interactions between microbial metabolites and the host in various contexts, conditions, and diseases.

Finally, most studies to date have treated a given body compartment, such as the skin or the gut, as a homogeneous assemblage of microbes, yet the spatial and dynamic associations of particular microbes, their metabolites, and components of the immune response are also critical for understanding

function at these sites. For example, knowing which bacteria colonize which crypts in the gut (Lee et al., 2013; Pédron et al., 2012), and how they stimulate stem cell proliferation and other factors linked to IBD or cancer, might provide important information not accessible in readouts of the microbiome and/or metabolome through stool samples. In the context of IBD, for example, biomarkers in treatment-naive patients can be more clearly assessed in mucosal biopsies than in stool (Gevers et al., 2014), the latter of which is frequently used for microbiome assessments because of its accessibility via noninvasive sampling. Especially given the success of imaging mass spectrometry in understanding how bacteria interact with one another to produce metabolites that they would not produce in isolation in pure culture (Traxler et al., 2013; Yang et al., 2009), as well as interkingdom interactions between bacteria and fungi (Moree et al., 2012), the potential for extending these spatially defined studies into the human body is immense. Furthermore, understanding how microbes and metabolites change over time in various contexts might lead to predictive models for disease diagnosis and patient stratification. The pipeline we propose should reveal how microbes are located spatially: correlating specific metabolite features with the microbiome distribution and then testing whether the molecules that colocalize affect the immune system will provide an especially powerful mechanism for generating lead compounds for the downstream studies that might extend into the clinic. Unfortunately, this sampling is destructive by current methods; the discovery of nondestructive readouts of the microbiome and metabolome would permit longitudinal within-subjects study designs, which, given high variability among individuals, might be important for clinical translation of these discoveries to improve human health.

Conclusion

A number of specific interactions among microbes, metabolites, and the immune system have now been discovered; although many of these examples were identified in the context of specific diseases, the broader network of these connections is most likely critical for a wide range of normal biological processes in the host. Development of a pipeline that allows for larger-scale discovery of these connections in both health and disease in humans and animal models is most likely within our reach via technologies that exist today or are in current development. In particular, in the same way that microbe and gene catalogs are now saturating in many populations, saturating the metabolite repertoire and building reference databases that allow matching to known standards will accelerate metabolomic discoveries considerably. Similarly, "multiomics" approaches in which the microbiome, the metabolome, and the immune repertoire are assessed simultaneously in the same biological specimens will provide considerable advances over current approaches. The prospect of a fundamental advance in our understanding of biology, not only of the parts list but also of the interactions that lead to a healthy supraorganism, and an engineering-level basis for developing technologies to arrest or reverse processes that lead to disease is exciting and feasible. Overcoming the barriers in communication and philosophies among diverse disciplines and among different experimental and computational technologies might catalyze a revolution in understanding the microbe-metabolite-immune connection in numerous ways that will benefit mankind.

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