Chapter 16
Gut Microbes, Immunity, and Metabolism

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Introduction

The human gut contains a vast number of bacteria, collectively characterized as the "gut microbiome." An estimated 10^{13} individual bacteria of anywhere between 500 and 1,000 species reside in the mammalian gut, making it the most densely populated microbial communities on Earth [1]. Acting upon the assumption that there are 1,000 bacterial species, the aggregate size of all intestinal microbial genomes may exceed the size of the human genome by more than 100-fold [2]. The larger view of the mammalian physiology should take into account that, together with our microbiome, we are a biologic "supraorganism" that is dynamic and carries out functions in parallel or cooperatively.

The microbial communities associated with the gut are profoundly different from other free-living microbial communities from across the biosphere [3]. When viewed as a whole, the "supraorganism" of the gut can carry out enzymatic reactions distinct from those of the human genome and harvest energy that would otherwise be lost to the host. The consequences of these enzymatic reactions suggest that over the millennia, mammalian metabolism, physiology, and disease have shaped and been shaped by the gut microbiome. In general, we as hosts coexist in either a commensal or symbiotic relationship with our gut microbiome [4]. While we provide members of the gut microbiome a unique niche to inhabit, in turn, the gut microbiome performs critical physiologic functions that benefit the host, including education of the mucosal immune system, extraction of nutrients from undigested carbohydrates through the production of short-chain fatty acids, salvaging nitrogen through the hydrolysis of urea, production of certain vitamins, and the metabolism of bile salts, to name a few.

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Although most gut bacterial organisms are refractory to cultivation, new DNA sequencing methods and technologies now permit robust analyses of complex bacterial communities offering not only valuable information about the composition of the gut microbiome, but also a window into the upregulation and downregulation of bacterial gene representation in the face of health and disease.

Numerous diseases in both adults and children have been linked to changes in the gut microbiota, such as *Helicobacter pylori* in the development of stomach cancer [5], inflammatory bowel disease [6, 7], nonalcoholic fatty liver disease [8], irritable bowel syndrome [9], necrotizing enterocolitis in infants [10], and diet-induced obesity (DIO) [11]. The known associations with human disease, coupled with the advanced technology that is now available, make this an extremely compelling area of investigation. In this chapter, we provide a brief overview of the gut microbiome and its impact upon host metabolism with a focus on the pathogenesis of human disease.

Gut Microecology

Since the initial description of germ-free mammals over a century ago, it has become increasingly obvious that the host-associated microbiome plays a major role in a diverse set of metabolic physiologic responses [12]. With the advent of gnotobiotics, where germ-free animals are colonized with defined bacterial populations, and more sophisticated molecular techniques to characterize patterns of gene expression and metabolic function, new insights into the mechanisms by which the microbiome influences host metabolism have been revealed. Given the essential importance of the intestinal tract on nutrient absorption, it seems intuitively obvious that bacterial communities in the intestinal tract, collectively known as the gut microbiome, would likely play the most important role in this regard. These studies have revealed an important role for a single gut commensal organism, *Bacteroides thetaiotaomicron*, in regulating the expression of genes involved in multiple intestinal functions including nutrient absorption, epithelial barrier function, and xenobiotic metabolism [13]. In addition, more recent studies have revealed that the gut microbiome plays a critical role in the maturation of the mucosal immune system such as the induction of intestinal Th17 cells by the gut communal organism, *segmented filamentous bacteria* [14]. As a result, the coevolution of the mammalian host with its gut microbiome over the millennia has led to the development of complex and robust immunologic mechanisms to maintain homeostasis fostering a commensal relationship with the microbial ecosystem in the gut [15]. Alterations in these homeostatic mechanisms, imparted by host genotype, may be the pathophysiologic basis for the development of a chronic inflammatory disorder of the intestinal tract known as inflammatory bowel disease.

From the standpoint of nutrition, the gut microbiome also plays a critical role in host metabolism. Members of the *Bacteroides* genus, which are Gram-negative anaerobes, comprise a significant proportion of the bacteria in the gut [16].

B. thetaiotaomicron is a dominant member of this genus, and the first one to undergo full sequencing of its genome, revealing that this organism possesses a variety of the enzymes necessary for hydrolysis of plant polysaccharides that are otherwise indigestible by the host [17]. Humans and other mammals are able to absorb simple sugars in the proximal small intestine. Certain disaccharides are hydrolyzed to monosaccharides and then absorbed, but mammals are largely unable to digest many other complex polysaccharides, and subsequently, this underutilized dietary carbohydrate source passes into the distal gut and is lost to the host. By adopting a microbiota that has the ability to degrade these carbohydrates, mammals are able to continue harvesting energy from these molecules. *B. thetaiotaomicron* has eight identified genes that participate in starch metabolism and cleave polysaccharides to glucose and other monosaccharides in the distal intestine. The ultimate end result of this fermentative process is the production of short-chain fatty acids such as propionate, butyrate, and acetate [18]. Short-chain fatty acids account for up to 70% of the caloric requirements of ruminant animals that feed upon cellulose-rich plants [19], but in humans and rodents, they account for much less, on the order of roughly 10% of total caloric needs [20]. In the nonruminant mammals such as human and rodents, the short-chain fatty acids are used as substrate for different organs. For example, butyrate is metabolized by the colonic epithelium [21]. Propionate is transported to the liver, where it is used as a substrate for gluconeogenesis [22], and acetate is largely shunted to adipocytes for lipogenesis [19].

Short-chain fatty acids have also been implicated in the overall health of the gut. In addition to being an energy source for colonic epithelium, it is clear that butyrate is associated with the stimulation of intestinal blood flow as well as colonic epithelial proliferation.

Vitamin synthesis by the gut flora is well-known. Vitamin K is synthesized by several taxa of bacteria, including Bacteroidetes, Eubacterium, Propionibacterium, and Fusobacterium [23]. Bacterial flora has also been implicated in the formation and absorption of certain B vitamins.

Current Technology and Characterization of the Gut Microbiota/Microbiome

To fully understand the impact of the gut microbiome on host physiology, it is essential to document its composition in both health and disease. Until this decade, characterization of the gut microbiota has been limited by the methods of detection. Approximately, 10^{12} bacterial cells exist per gram of feces and anaerobic culture-based techniques have characterized roughly 400–500 different species in the intestinal tract of humans [24]. About 75% of human bacterial flora cannot be cultured by conventional microbiologic analyses that are derived by growing colonies of organisms on the polysaccharide-based agar [25]. Recent technological advances have permitted the unprecedented examination of complex microbial communities using techniques that are culture-independent. These studies have revealed the presence

of approximately 1,000 bacterial species and over 7,000 strains of bacteria in the gut microbiome of mammals.

The use of genomic fingerprinting techniques, such as T-RFLP, DGGE, and TGGE, dependent upon polymerase chain reaction (PCR) amplification of a specific gene product such as 16S rRNA, followed by separation by gel electrophoresis [26]. Clustering of band patterns can then be analyzed using statistical techniques such as principal coordinate analysis (PCoA). Although these studies can be used to determine the stability in the dominant members of a community across as large number of samples, the dynamic range is limited and no information can be obtained that relate banding patterns to changes in particular bacterial taxa.

The use of DNA microarrays to monitor the presence of previously known genes was first described in 1995 [27]. This allowed up to 20,000 genes to be monitored on a single array, expanding the approach of biological research from the study of individual genes to genome-wide study [28]. In the study of microbiota, it has been used to identify pathogens and determine host susceptibility, as well as to profile pathogen gene expression in response to antimicrobial drugs or vaccines. A lack of gold standard for microarray data analysis and a lack of consistency between laboratories or experimental conditions have remained criticisms of this technology [29].

The most robust method utilizes high throughput sequencing technology, such as pyrosequencing, which allows investigators to efficiently obtain large amounts of DNA sequence information efficiently in a cost-effective manner. Sequences of small-subunit ribosome RNA genes, in which 16S rRNA gene sequences (for archaea or bacteria) or 18S rRNA (for eukaryotes), can be used as phylogenetic markers to determine the relative abundance of bacterial taxa in a sample. Various methods can be used to isolate bacterial DNA from samples, followed by amplification of bacterial 16S rDNA using the PCR. The 16S RNA gene possesses both conserved and hypervariable segments that contain robust taxonomic information. Resulting sequences are grouped into Operational Taxonomic Units (OTUs), which are groups of sequence with identity equal to or greater than a predetermined threshold. For example, 97% identified is often used – reconstruction studies suggest this yields a number similar to the number of different bacterial genera present. OTUs are then aligned and introduced into predetermined phylogenetic trees made with full-length 16S rDNA samples using databases such as Greengenes and NAST [30]. The output trees are used for analysis in UniFrac where pairs of communities are marked on a common phylogenetic tree, and then the fraction of the branch length unique to each community determined. This provides a measure of the distance between communities in terms of their shared evolutionary history. This distance matrix can be used to generate clustering maps using dimensionality reduction by PCoA, a geometric technique that converts a matrix of distances between points in multivariate space into a projection that maximizes the amount of variation along a series of orthogonal axes. In this method, the variance in the data is used to generate axes of maximum variation, then the data are plotted in this coordinate system. In a successful analysis of this type, the axes may be attributable to specific biological phenomena [31]. Such studies have revealed that, of approximately 50 bacterial phyla on Earth, only four are associated with humans with the major bacterial phyla in the human gut being Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria [32, 33].

It must be noted, however, that 16S rRNA sequencing is limited by the fact that existing nucleotide sequence databases are biased and incomplete. Differences in detection of species could be due to differences between individuals, but also could be attributed to the biases that exist in PCR-based analysis such as DNA extraction method, PCR primer used, and preferential PCR cloning [34]. Both microarray and 16S rRNA high throughput sequencing are poor for quantification and are limited to relative comparisons unless coupled with extremely carefully controlled experimental conditions [35].

Metagonomics and Metabolomics

While 16S rDNA phylotyping can be used to characterize the composition of a microbial community, it provides little information as to the functional properties of the microbiome under investigation. To obtain this type of information, metagenomics studies where shotgun sequencing of DNA isolated from a specific sample, can be used to determine the relative abundance of genes represented in a given bacterial community. Here DNA sequences are aligned to databases such as MEGAN to identify the taxonomic origin of the sequences (e.g., bacterial vs. mammalian host) followed by alignment with preexisting known pathways such as Kyoto Encyclopedia of Genes and Genomes) (KEGG) and clusters of orthologous groups (COG) to map enzymes onto known gene ontologies [36].

As an example of this analytic approach from the standpoint of metabolic function, a metagenomic analysis of the distal gut microbiota of two human subjects showed similar patterns of enrichment for genes involved in the metabolism of starch, sucrose, glucose, galactose, fructose, arabinose, mannose, and xylose [36]. Eighty-one different glycoside hydrolases were described in the microbiome, most of which are not present in the "glycobiome" of the human genome [36]. The end products of the bacterial "glycobiome" consist of short-chain fatty acids that are then absorbed by the host. Bacterial fermentation leads to the accumulation of H_2, which can reduce the efficiency of dietary polysaccharide digestion [33], unless a methanogenic archaeon is present to shunt the H_2 towards the production of methane. *Methanobrevibacter brevii* is one of the only members of Archaea that has been cultured from the gut. Genes in the methanogenic pathway were also enriched in the same distal human colon.

A larger-scale comparative metagenomic analysis of fecal samples from 13 healthy Japanese individuals (comprised of adults, weaned children, and unweaned infants) revealed a clear structural difference between the microbiota of unweaned infants and that of adults and weaned children, with the notion that at 1 year of age, the microbiota adopted a more complex but functionally more uniform structure regardless of age or sex [37]. Metagenomic analysis showed striking enrichment of carbohydrate metabolism genes and depletion of genes for the biosynthesis of flagella and chemotaxis. There was a predominance of mobile genetic elements that implicated the distal human gut as a setting for horizontal gene transfer [37]. There were

significant differences between Japanese and American samples, suggesting a genetic or environmental influence upon the microbiota composition. In the future, analytic techniques that directly quantify gene expression through metatranscriptomics (analysis of RNA transcripts within a defined community) or metaproteomics (analysis of proteins) may lead to an additional level of information that can be used to validate the concepts derived from observations obtained through metagenomics. Although currently being used to characterize less complex bacterial communities, they have just begun to be applied to studies involving mammalian-associated bacterial communities [38].

A powerful analytic approach to examine the functional impact of the gut microbiome, inferred through metagenomics, is to correlate such data with those quantifying the presence of a large array of metabolites found in host tissues and/or fluids. The use of proton NMR or mass spectroscopy to obtain this information is known as metabolomics. In this regard, there is strong evidence demonstrating that the gut microbiome influences the metabolic phenotype of the mammalian host and participates in microbial-–host cometabolic responses [39]. One example of biochemical interactions between the host and its gut microbiome is the synthesis of bile salts in the liver, their excretion into the gut where they are modified by the gut microbiome, and their subsequent return to the host by enterohepatic circulation or reabsorption in the colon [40]. Not only are these processes important for the homeostasis of lipid metabolism in the host, but bile salt hydrolases also mediate bile tolerance in vitro and enhances survival of gut microbial symbionts in vivo [40]. Another example is the production of short-chain fatty acids by bacterial fermentation of carbohydrates in the gut and their subsequent absorption by the host where they can be used for lipogenesis and play a role in the development of DIO [41,42]. By performing broad scale untargeted profiling by either mass spectrometry [43] or ^{1}H NMR [44] on biofluids collected from conventionally housed an germ-free mice, investigators have identified large numbers of metabolites that are produced by the gut microbiome that then influence the metabolome of the mammalian host. Consistently, amino acid metabolites are among that are most greatly impacted. Interestingly, alterations in diet can have an impact on some of these same metabolites in humans suggesting that dietary alteration of the gut microbiome can alter the host metabolome [45].

As metabolic reactions in nature generally occur in the context of the communities, this approach allows the analysis of microbiota metabolism that is occurring above the organization of a single organism and instead looks at the “superorganism” that is made up of the host and the entire microbial community. As we determine the degree to which the microbiome can harvest additional energy from nutrients and supply them to the host, the value of a food becomes relative and not absolute. Further metagenomic studies, coupled with metabolomics, which characterizes metabolites generated in different physiologic conditions, can be used for further understanding of the origins of obesity and malnutrition, making specific therapeutic recommendations that are dictated by the composition of the gut microbiota and its own energy harvesting capabilities.

Further understanding of metabolomics in the context of the gut microbiome will require the construction of new modeling paradigms that incorporate the different genetic, microbial, metagenomic, and metabolomic data to fully understand the different levels of function and influence [46].

The Gut Microbiota and Obesity

The prevalence of DIO is reaching epidemic proportions in industrialized nations. In parallel, there has been a dramatic increase in type 2 diabetes mellitus (T2DM). Together, these two related disease processes are an enormous health and financial concern to the U.S. population. There are an estimated 143 million people worldwide with diabetes mellitus, 90% of which have T2DM.

The first law of thermodynamics, which states that the amount of energy stored must equal the difference between energy input and work, is highly relevant to DIO where energy is stored in fat deposits. Finely regulated mechanisms are responsible for maintaining energy balance in mammals. To maintain body mass, energy input (food intake) must match energy expenditure (a combination of physical activity, basal metabolism and adaptive thermogenesis). Thus:

> Energy input (feeding) = energy output (physical activity + basal metabolism + adaptive thermogenesis).

Physical activity includes all voluntary movement, basal metabolism is this energy required to maintain biochemical processes necessary to sustain life. By contrast, adaptive thermogenesis refers to the amount of energy expended in response to environmental factors such as cold and alterations in diet. Since triglycerides, stored as fat in white adipose tissue, is the most efficient means of energy storage, alterations in energy balance favoring "energy input" can lead to obesity.

Over the past few years, a research group at Washington University in St. Louis, led by Dr. Jeffrey Gordon, has published a series of seminal reports demonstrating the role of the gut microbiome in the development of obesity in murine systems. These investigators discovered that germ-free mice were comparatively lean with 42% less body fat in comparison to conventionally housed mice despite a 29% increase in food intake [36]. The colonization of germ-free mice with a normal colonic microbiome harvested from conventionally-housed mice led to a dramatic increase in body fat within 10–14 days. This effect of the microbiome on host adipose deposition involved an interplay between an increase in short-chain fatty acid production, intestinal absorption of monosaccharides, and enhanced hepatic lipogenesis.

Using a different model of DIO, the *ob/ob* mouse, the authors show that obesity was associated with a significant alteration in the proportion of the two major phyla in the gut microbiome, with a decrease in Bacteroidetes and a proportional increase in Firmicutes [47]. From a mechanistic standpoint, metagenomic studies demonstrate that this phylotypic alteration enhances the representation of genes involved

in the breakdown of indigestible dietary polysaccharides consistent with an increase in short-chain fatty acid concentrations in the fecal pellets of ob/ob mice. Interestingly, using microbiota transplantation, the authors showed that the obese phenotype was transmissible where germ-free mice that received an "obese microbiome" had significantly greater fat mass than those that received a "lean microbiome." Similar findings were observed in experiments where DIO was induced through a feeding of a "westernized" diet high in fat and simple sugars [48].

Several experiments have investigated the mechanism by which the microbial environment alters physiology. Short-chain fatty acids act as ligands for a G-protein coupled receptor known as Gpr41. Mice that are null for the Gpr41 gene behave similarly to germ-free mice after conventionalization, gaining less weight and adiposity in comparison to germ-free wild-type mice that have been conventionalized [49]. Another mechanism may involve fasting-induced adipose factor (*Fiaf*), a circulating lipoprotein lipase inhibitor that is inhibited by the presence of gut microbiota. [36] Germ-free mice that lack the gene for *Fiaf* are protected from DIO, demonstrating increased weight gain and intraabdominal adiposity despite similar quantities of food intake [50]. In total, these studies clearly demonstrate the ability of the gut microbiome to augment the development of DIO by enhancing the extraction of energy from the feces through the increased fermentation of indigestible carbohydrates leading to the production of short-chain fatty acids (SCFAs). The subsequent increase in short-chain fatty acid absorption in the colon auguments caloric intake favoring an increase in fat deposition in the setting of unchanged energy expenditure.

Although clearly important in murine models, is there evidence for a role of the gut microbiome in the development of DIO in humans? Some intriguing clues exist. Similar to the phylotypic alterations observed in mice, an observational study of the gut composition of 12 obese humans placed upon restricted diets over 1 year showed a relative increase in the abundance of Bacteroidetes and a relative decrease in Firmicutes [41]. In a more recent study, investigators analyzed the gut microbiome composition of obese and lean twins (31 monozygotic twin pairs, 23 dizygotic twin pairs, and 46 mothers). The twins were either concordant for obesity or leanness. The results revealed that each subject's microbiome varies significantly in composition with a comparable degree of covariation between adult monozygotic and dizygotic twin pairs. Remarkably, there was not a single abundant bacterial species that was shared among all of the subjects in the study, suggesting that there may not be a "Core" gut microbiome in humans [51]. Similar to ob/ob mice, however, obese individuals were found to have a relative decreased proportion of Bacteroidetes species and an increased proportion of Actinobacteria, although there was no significant difference in Firmicutes [51]. Interestingly, metagenomic "shotgun sequencing" and analysis revealed that obesity was associated with altered representation of bacterial genes and metabolic pathways, including those involved with nutrient extraction demonstrating that, in humans, obesity is associated with a core microbiome at a gene/functional and not an organismal level [51].

Despite these important findings, future studies may help to further define the mechanisms by which the composition of the gut microbiota is regulated and its relationship to the obese phenotype. What is the evolutionary advantage of

enhanced energy extraction by the gut microbiome in an obese host? What is the mechanism by which obesity alters the composition of the gut microbiome? What is the stability of the obese-associated gut microbiome throughout time? What is the influence of diet on gut microbiome composition? Preliminary studies with respect to this last question are described in the following section.

Gut Microbiota and Diet

Early studies examining the effect of broadly-defined diets suggested that the alterations on the gut microbiome were modest involving few genera [52]. However, more recent studies using more sophisticated technology to characterize the composition of the gut microbiome provide clear evidence that this initial impression is not correct. Studies in infants have demonstrated that dietary factors such as breast milk, formula, and solid foods have a significant impact on the composition of the gut microbiome [1, 53, 54]. Furthermore, through the use of a reductionist model system, it has been shown that a single gut commensal (*B. thetaiotaomicron*) in gnotobiotic mice adjusts its pattern of gene expression in order to adapt to alterations in host diet [55]. Importantly, a prototypic high-fat/high-sugar Western diet can reproduce some of these same alterations in C57Bl/6J mice as in the ob/ob model of obesity [41], with a significant shift of Bacteroidetes to Firmicutes [48]. This alteration was reversible, but unlike the shifts observed in *ob/ob* mice, the augmentation of Firmicutes induced by a Western diet was not division-wide, but was due to a bloom in a single class of Mollicutes [48]. Since the Western diet in this study also led to an increase in fat mass, the contribution of the host obese phenotype, much like that observed in ob/ob mice, to the diet-induced microbiome changes are unknown.

Recent studies provide more compelling evidence for the important role that diet plays in the regulation of gut microbiome composition. First, Ley et al. reported a study in which 16S rRNA sequencing was used to determine the gut microbiome composition in humans and 59 other mammalian species. Their results demonstrate that host diet has a strong influence in bacterial diversity that increases from carnivores to herbivores [56]. In this analysis, clustering by diet was highly significant, eclipsing that of order, fiber index, or gut type, supporting a strong association between gut microbiota composition and diet. Second, fasting for 24 h leads to a significant alteration in the composition of the murine gut microbiome with an increase in Bacteroidetes and a corresponding decrease in the Firmicute phylum [57]. Finally, in gnotobiotic mice colonized with a human gut microbiome, a high-fat/high-sugar "Westernized" diet leads to significant alterations in the composition of the microbiome within 18–20 h before any alteration in host phenotype, namely DIO, occurs [58]. Together with evidence from a murine KO model that reduces fat mass on a high-fat diet [30], these studies demonstrate the importance of diet in the regulation of gut microbiome composition. Although definitive data for the importance of diet in the composition of the human microbiome is currently lacking,

there is some evidence to support this notion. Analysis of major groups of fecal microbes using nine 16S rRNA FISH probes in 19 obese subjects after 4 weeks on different diets (maintenance, medium carbohydrate, and low carbohydrate) revealed significant differences in butyrate-producing bacteria (*Roseburia* and *Eubacterium rectale*) as well as *Bifidobacteria* species, although no significant difference was seen in relative counts of Bacteroidetes [59]. Total amounts of fecal short-chain fatty acids and fecal butyrate decreased as carbohydrate intake decreased [59]. These observations provide a rationale for the hypothesis that dietary interventions used to treat disease can influence the microbiome, and that the changes in the microbiome have, in turn, consequences for host metabolism.

Connecting the Gut Microbiome, Innate Immunity and Obesity

Obesity is the most important factor in the development of insulin resistance, the hallmark of T2DM. There is now compelling evidence that obesity leads to the chronic activation of inflammatory pathways leading to signaling mechanisms that directly inhibit insulin signaling [60, 61]. Indeed, adipose tissue is not only a highly active metabolic tissue, but is also a dynamic endocrine organ capable of producing a wide range of proteins that regulate both metabolism and inflammation. Collectively, these secreted factors are called adipokines. Examples include: leptin, TNF-α, IL-6, resistin, adiponectin, plasminogen activator inhibitor-1, and angiotensinogen, to name a few [62]. A number of these genes are targets of the NF-κB in the development of insulin resistance. For example, heterozygous IKKβ$^{+/-}$ mice are protected from insulin resistance when fed a high-fat diet or crossed to ob/ob mice, [63] and pharmacologic inhibition with the treatment of salicylates improves insulin resistance in several model systems [63, 64].

Activation of the innate immune system, through ubiquitously-expressed toll-like receptors (TLRs) leads to NF-κB signaling ultimately resulting in an acute inflammatory response. TLRs are a family of type I transmembrane receptors with an extracellular leucine-rich repeat domain and an intracellular Toll/IL-2 receptor (TIR) domain [65]. At least ten TLRs have now been identified, and each has a distinct role in the activation of the innate immune system. Ligands for these receptors number in the dozens, and are extremely diverse in structure and origins. For example, TLR-4 was the first characterized TLR in mammals, and is a signal-transducing receptor for bacterial lipopolysaccharide (LPS) [66] as well as saturated fatty acids [67]. Binding of TLR-4 in conjunction with coreceptors CD14 and MD-2 triggers a downstream signaling cascade that eventually leads to the transcription of proinflammatory genes that encode proinflammatory molecules in a NF-κB dependent manner [68].

Growing evidence demonstrates that the gut microbiome plays a role in the development of insulin resistance by augmenting fat mass, through its observed functional alteration in the setting of a westernized high calorie diet, and via its role in the activation of the innate immune response via TLRs and NF-κB signaling.

These effects may be either direct or indirect (Fig. 1). As described earlier, obesity-associated alterations in the composition of the gut microbiome lead to enhanced energy harvest of the luminal gut contents, resulting in the increased production of short-chain fatty acids that are utilized by the host for lipogenesis. In this manner, the gut microbiome can induce a state of insulin resistance indirectly through its ability to enhance the development of obesity. A second mechanism involves microbiome-dependent activation of TLR-5 [69]. In this recent study, Vijay-Kumar et al. showed that TLR-5$^{-/-}$ mice exhibited hyperphagia leading to the development of obesity as well as many features of metabolic syndrome including insulin resistance, hypertension, and hyperlipidemia. Although food restriction prevented the development of obesity in TLR-5$^{-/-}$ mice, there was no effect upon insulin resistance. These data suggest that the effect of TLR-5 may modulate insulin resistance by both direct and indirect pathways (Fig. 1). Remarkably, the authors also showed that the transfer of the gut microbiome from TLR-5$^{-/-}$ to wild-type germ-free mice was sufficient to establish many features of the metabolic syndrome to the recipients.

TLR-4 has also been implicated in the development of insulin resistance. One possible mechanism, independent of the gut microbiome, involves the direct activation

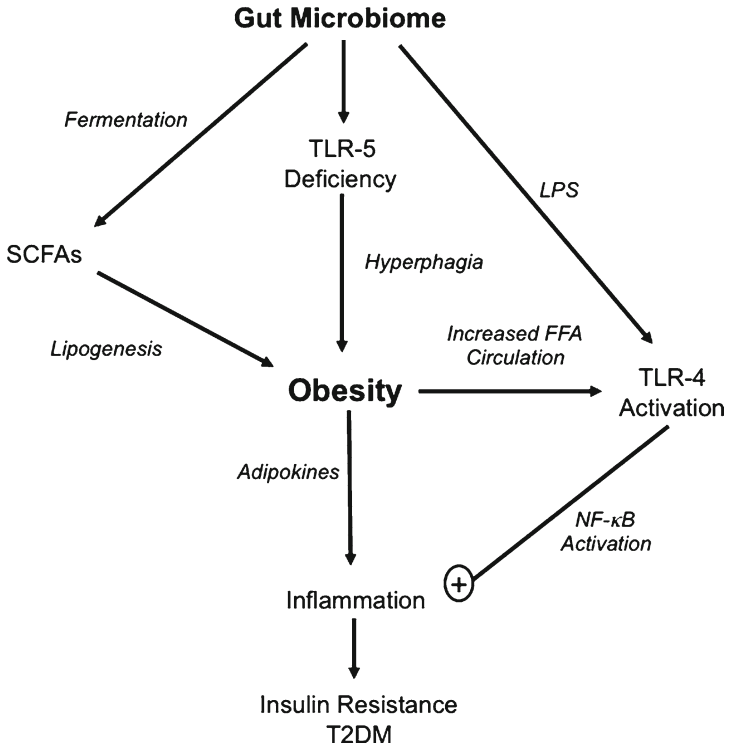

Fig. 1 Relationship between the gut microbiome and glucose homeostasis

of TLR-4 by free fatty acids (FFAs), which are often elevated in DIO and have been previously shown to mediate insulin resistance [70]. In vitro, FFAs induce TLR-4 signaling, resulting in activation of NF-κB and the production of proinflammatory cytokines such as TNF-α and IL-6, not only in macrophages, but also in adipocytes. This response occurs both in vitro and in vivo. Importantly, TLR-4 deficiency prevents lipid-induced insulin resistance. Finally, the authors show that TLR-$4^{-/-}$female C57/B6 mice are partially protected from high-fat diet-induced insulin resistance despite an increase in obesity. By contrast, interestingly, two different studies demonstrate that mice with TLR-4 deficiency are actually protected from the development of obesity as well as insulin resistance on a high saturated fat diet [71, 72]. When placed on a high-fat diet, C3H/HeJ (loss-of-function mutation in TLR-4) mice showed decrease weight gain, decreased adiposity, increased metabolic rate, improved glucose tolerance and decreased serum TNF-α, IL-6 and adiponectin levels [71]. 10ScN mice (deletion precluding expression and production of TLR 4) were protected from high-fat diet induced obesity, despite the similar caloric intake as control mice [72]. Ultimately, additional studies will be required to determine whether, like TLR-5, TLR-4 has an indirect effect on insulin resistance by either inducing or inhibiting the development of DIO. Nevertheless, through its activation by FFAs, it seems quite clear that TLR-4 has a direct effect on the development of insulin resistance.

The gut microbiome may also directly augment the development of insulin resistance through the activation of TLR-4 signaling by releasing LPS, a major membrane constituent of gram-negative bacteria, into the systemic circulation [73, 74]. High-fat diets have been reported to chronically increase serum levels of LPS by two or three-fold, possibly by increasing intestinal permeability through the reduced expression of epithelial tight junction proteins [75]. High-fat diet fed mice treated with oral antibiotics reduced body weight gain, fat mass development, expression of systemic inflammatory markers, and glucose intolerance [76]. Similar findings were observed in ob/ob mice treated with oral antibiotics. These effects may be mediated through alterations in TLR-4 signaling, since the authors also demonstrated that CD $14^{-/-}$ (a critical component of the TLR-4 complex) mice bred onto an ob/ob background mimicked the metabolic and antiinflammatory effects of oral antibiotics. Although the effects of antibiotics on glucose homeostasis may be due to an indirect effect mediated by a reduction in obesity through lower levels of short-chain fatty acid production by the gut microbiome, the results in ob/ob $CD14^{-/-}$ mice suggest that the activation of TLR-4 by LPS may have a direct impact on insulin resistance.

Gut Microbiota and Type 1 diabetes

Type I diabetes (T1D) is an autoimmune disease resulting from the destruction of insulin-producing β-cells of the pancreas. Increased incidence over the past decades suggests that environmental and possibly microbial mediated changes may influence

disease development. Wen et al., showed that the incidence of spontaneous T1D in nonobese diabetic (NOD) mice is affected by microbial environment and exposure [77]. Specifically, the effect of MyD88, an adaptor protein that facilitates the ability of ligands to stimulate the inflammatory cascade via a number of TLRs, on the development of T1D in NOD mice was determined in both germ-free and conventionally-housed conditions. NOD mice lacking MyD88 ($MyD88^{KO}$ NOD), housed under SPF conditions, fail to develop T1D. Remarkably, germ-free as well as antibiotic treated $MyD88^{KO}$ NOD mice developed T1D at higher rates than the same $MyD88^{KO}$ mice colonized with specific pathogen free bacteria, suggesting that the presence of normal gut microbiota protects against the development of diabetes [78]. Finally, examination of lymphocytes localized specifically to pancreatic lymph nodes revealed that MyD88 deficiency led to a local tolerance to pancreatic antigens. Thus, in this model system, the gut microbiome induces a state of local tolerance in the pancreas, thereby preventing the development of T1D. Together, these findings provide new insights into mechanisms by which the gut microbiome helps to shape the immunologic response in tissues distinct from those associated with mucosal surfaces. Further characterization of these mechanisms and, perhaps the components of the gut microbiome responsible for these effects, may lead to significant advances in the field of autoimmune disease processes such as T1D.

Gut Microbiota and Nitrogen Balance

Nitrogen is a crucial constituent of the diet. When body composition is constant, nitrogen intake should be equivalent to losses. In normal growth, recovery of illness, or pregnancy, a positive nitrogen balance is required. Intake of protein, amino acids, and other nitrogenous substances can be calculated from dietary intake. Between 75 and 90% of nitrogen loss from the body are through the urine and feces, primarily in the form of urea, which is produced exclusively in the liver through the urea cycle, entering as ammonia and exiting as urea, which is transported primarily to the kidney for excretion. Urea is also transported into the intestinal lumen, and stool nitrogen accounts for about 9–12% of the total loss [79].

For nearly 60 years, there has been evidence that urinary excretion does not account for all the urea that is produced by the body. Indeed, through either luminal delivery or intestinal secretion, substantial amounts of urea can be found in the colonic lumen. Once in the colonic environment, evidence from germ-free rats and animals treated with antibiotics demonstrate unequivocally that the process of urea hydrolysis, and subsequent nitrogen absorption, is exclusively a function of the gut microbiome [80, 81], with the exception of urea hydrolysis by *H. pylori* in the upper GI tract [82]. Urea hydrolysis in health is primarily a function of the colonic, and perhaps the distal ileal microflora. Indeed, using stable isotope methods, it has been estimated that approximately 15–30% of urea produced by the liver is not excreted in the urine and is hydrolyzed by bacterial urease to ammonia [83]. Previous studies have shown that the concentration of ammonia in the blood draining

the colon was 10 times than that in the inferior vena cava, and that the colonic venous concentration could be reduced by 65% through the administration of oral antibiotics [84]. The ammonia produced in the colon can either be: (1) Absorbed by the host, where it is utilized in the liver for either protein or urea synthesis; (2) Used by the gut bacteria for amino acid and protein synthesis; or (3) Excreted from the body in the feces. Based upon isotopic-labeling studies with $^{15}N15N$-urea, it has been estimated that 18% of the urea nitrogen enters the urea cycle and is excreted in the urine, approximately 74% enters the metabolic pool of the host, and only 4% is excreted in the stool [85]. Thus, it appears that the vast majority of the nitrogen obtained through urea hydrolysis in the colon is recycled where it is of benefit primarily to the host.

Interestingly, the proportion of colonic nitrogen scavenging through bacterial hydrolysis of urea appears to be proportional to the intake of dietary protein. In humans, when the intake of dietary proteins exceeds 70 grams per day, urea production is 100–120% of intake with about 70% of the urea excreted in the urine and 30% of the nitrogen being salvaged in the colon [86]. The physiologic minimal intake of dietary protein to maintain nitrogen balance in adults is approximately 35 grams. As the intake of protein falls from 70 to 35 grams per day, there is a small but insignificant decrease in urea production of approximately 10% [87]. By contrast, on a 35 grams per day protein diet, 30% of urea produced is excreted with 70% of nitrogen being salvaged. This increase in colonic nitrogen salvage approximately matches the decrease in protein intake. This adaptive metabolic response may be of particular importance in the setting of severe malnutrition [88]. On the other hand, an increase in colonic ammonia absorption may be detrimental to patients with hepatic encephalopathy, chronic renal failure, and inborn errors of the urea cycle.

Urea cycle disorders (UCD) are a group of rare inborn errors of metabolism that commonly present in childhood with episodes of vomiting, lethargy, and coma [89]. Symptoms result from the untoward accumulation of ammonia, a potentially toxic product of protein degradation, which is not adequately metabolized in the liver of affected individuals due to an enzyme deficiency present from birth. Deficiencies in each of the eight enzymes and transporters that comprise the urea cycle have been identified. All are inherited as recessive traits except for the most common disorder, ornithine transcarbamylase deficiency, which is X-linked. The mainstay of treatment is a low-protein diet in order to minimized ammonia production. In recent years, novel approaches to treatment, such as acylation therapy with benzoate and phenylbutyrate, have become commonplace [90]. Unfortunately, even with scrupulous dietary control and diligent therapeutic intervention, the risk of death or severe disability is lamentably high, probably as great as 50% [91]. A major cause of this disappointing outcome is that acute stress, usually a concurrent infection, causes the sudden release of cytokines and adrenal stress hormones that evoke catabolism of body protein and concomitant formation of ammonia in an amount that exceeds the capacity of the congenitally defective urea cycle to detoxify this potentially noxious metabolite. The result is severe hyperammonemia with resultant ataxia, seizures, and coma. Irreparable brain damage frequently ensues, as evidenced by mental retardation, epilepsy, and severe spasticity. Oral antibiotic

therapy, to reduce bacterial hydrolysis of urea and colonic ammonia absorption, has proved very useful as a therapeutic adjunct, especially during a hyperammonemic crisis. This same approach has proven to also be beneficial in patients suffering from hepatic encephalopathy [92].

Although clinically effective, the use of oral antibiotics as an adjunct to a low protein diet in the treatment of patients with UCD and hepatic encephalopathy is currently deployed in a "shotgun" manner. Indeed, given our current lack of knowledge of the gut microbiome, we are unable to "target" those organisms that most robustly hydrolyze urea to ammonia. Future studies that characterize the effect of dietary protein on the taxonomic composition of the gut microbiome as well as metagenomic studies to determine its effects on urease gene representation may provide valuable new insights that may help to more effectively modify populations of gut bacteria that will be of greater benefit in the treatment of patients that are unable to metabolize amino acids appropriately.

Conclusions and Future Directions

The coevolution between the mammalian host and its microbiome has led to the development of a largely symbiotic relationship. Nowhere is this more clearly demonstrated than the importance of the gut microbiome and its role in host immunologic and metabolic homeostasis. Nevertheless, perhaps due to recent alterations in human society such as dietary intake and other environmental conditions, this symbiotic relationship can become dysfunctional with the gut microbiome playing a role in the pathogenesis diseases such as obesity, diabetes, hepatic encephalopathy, and inflammatory bowel disease. The study of gnotobiotic mice along with recent advances in DNA sequencing technology have provided investigators with an unprecedented opportunity to explore the composition of the gut microbiome and how it may play a role in disease pathogenesis.

Despite these advances, significant questions remain to be addressed. Among these include: What is the relevance of the observations and mechanisms associated with disease pathogenesis, defined in animal models, to human biology? Current evidence suggests that there is no "Core" microbiome in humans. What, then, are the most important determinants of gut microbiome composition in humans? If a dysbiotic gut microbiome plays a role in the pathogenesis of a human disease, are there mechanisms by which its composition can be permanently altered to reduce pathogenicity? What is the effect of diet on the composition of the human gut microbiome and does this have relevance to the pathogenesis of human disease? Are the functional properties of the gut microbiome accurately reflected in the results obtained by studies examining alterations in gene abundance through metagenomics?

A key starting point in addressing some of these issues will be the expanded investigation of the microbiome in humans. This is the major focus of an international research effort known as the Human Microbiome Project [93]. Ultimately, as newer technologies in DNA sequencing technology, metatranscriptomics, metaproteomics,

and metabolomics are developed, together with advances in biocomputational techniques able to extract meaningful relationships from massive amounts of raw data, significant advances in our understanding of the gut microbiome and its role human disease pathogenesis are on the horizon.

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