3-2017

# The Host Microbiome Regulates and Maintains Human Health: A Primer and Perspective for Non-Microbiologists

Sunil Thomas
*Lankenau Institute for Medical Research, Wynnewood, Pennsylvania*

Jacques Izard
*University of Nebraska–Lincoln*, jizard@unl.edu

Emily Walsh
*Harvard School of Dental Medicine*, emily_walsh@hsdm.harvard.edu

Kristen Batich
*Duke University Medical Center*

Pakawat Chongsathidkiet
*Duke University Medical Center*

*See next page for additional authors*

Published in *Cancer Research,* 2017. doi: 10.1158/0008-5472.CAN-16-2929

Submitted October 26, 2016; revised December 19, 2016; accepted December 21, 2016; published online March 14, 2017.



# The Host Microbiome Regulates and Maintains Human Health: A Primer and Perspective for Non-Microbiologists

Sunil Thomas,[1] Jacques Izard,[2] Emily Walsh,[3] Kristen Batich,[4,5,6] Pakawat Chongsathidkiet,[4,5,6] Gerard Clarke,[7] David A. Sela,[8,9,10] Alexander J. Muller,[1] James M. Mullin,[1] Korin Albert,[11,12] John P. Gilligan,[1] Katherine DiGuilio,[1] Rima Dilbarova,[1] Walker Alexander,[1] and George C. Prendergast[1]

1 Lankenau Institute for Medical Research, Wynnewood, Pennsylvania
2 Department of Food Science and Technology, University of Nebraska–Lincoln, Lincoln, Nebraska
3 Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, Massachusetts
4 Department of Neurosurgery, Duke Brain Tumor Immunotherapy Program, Duke University Medical Center, Durham, North Carolina
5 Department of Surgery, Duke Brain Tumor Immunotherapy Program, Duke University Medical Center, Durham, North Carolina
6 Department of Pathology, Duke University Medical Center, Durham, North Carolina
7 Department of Psychiatry and Neurobehavioural Science, APC Microbiome Institute University College Cork, Cork, Ireland
8 Department of Food Science, University of Massachusetts, Amherst, Massachusetts
9 Department of Microbiology, University of Massachusetts, Amherst, Massachusetts
10 Center for Microbiome Research, University of Massachusetts Medical School, Worcester, Massachusetts
11 Molecular and Cellular Biology Graduate Program, University of Massachusetts, Amherst, Massachusetts
12 Department of Food Science, University of Massachusetts, Amherst, Massachusetts

*Corresponding author* — Sunil Thomas, Lankenau Institute for Medical Research, 100 E. Lancaster Ave., Wynnewood, PA 19096; tel 484 476-3507, fax 484 476-2205, email thomass-2@mlhs.org ; suntom2@gmail.com

## Abstract

Humans consider themselves discrete autonomous organisms, but recent research is rapidly strengthening the appreciation that associated microorganisms make essential contributions to human health and well being. Each person is inhabited and also surrounded by his/her own signature microbial cloud. A low diversity of microorganisms is associated with a plethora of diseases, including allergy, diabetes, obesity, arthritis, inflammatory bowel diseases, and even neuropsychiatric disorders. Thus, an interaction of microorganisms with the host immune system is required for a healthy body. Exposure to microorganisms from the moment we are born and appropriate microbiome assembly during childhood are essential for establishing an active immune system necessary to prevent disease later in life. Exposure to microorganisms educates the immune system, induces adaptive immunity, and initiates memory B and T cells that are essential to combat various pathogens. The correct microbial-based education of immune cells may be critical in preventing the development of autoimmune diseases and cancer. This review provides a broad overview of the importance of the host microbiome and accumulating knowledge of how it regulates and maintains a healthy human system.

## Role of the Microbiome in Maintaining Host Health

At all stages of life, humans are associated with microorganisms and their products. Humans coevolved with microbes in the environment, and each body habitat has a unique set of microorganisms in its microbiota (1). The microbiome (term coined by Joshua Lederberg) consists of the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body (2). The host organism together with its microbiome constitutes the "holobiont" (Greek, *holos,* whole/entire), and the totality of the genome is the "hologenome" (3, 4). Changes in the holobiont may impact the complex signaling network, thereby influencing the hologenome leading to health or disease.

The human body is estimated to be composed of $3 \times 10^{13}$ eukaryotic cells and $3.9 \times 10^{13}$ colonizing microorganisms, such that host cells and microbiota are of nearly the same number in an individual (5). The largest concentrations of microbes occupy the gut, skin, and oral cavity. The microbiota of our system is well tolerated by our immune system due to the coevolution of these microorganisms over time. The overwhelming majority of gut microbiota are *Eubacteria*. The collective genome, or metagenome, of the enteric microbiota contains over 100 times the number of genes in the human genome, and there are approximately 10-fold more genes in each of our microbiomes than in each of us, encoding the greatest source of potential antigens with which the immune system must cope (6). The microbiome in humans significantly enriches the metabolism of glycans, amino acids, and xenobiotics. It is also responsible for the synthesis of vitamins, isoprenoids, and other nutrients, making humans "superorganisms" whose metabolism represents an amalgamation of microbial and human attributes (7).

Each individual emits a distinct and personalized cloud of microorganisms into his or her surroundings (8). The microbiome in humans is also not constant during lifespan, but rather changes with age. Culture and location also have a profound impact on

the microbiome (9). Health status is yet another factor influencing the microbiome compositional status. In one study, the growth dynamics of gut microbiota in health and disease have been inferred from a single metagenomic sample (10). These authors copy the number and ratio at origin and terminus to detect the actively growing species in a microbiome. In this way, they showed differences between virulent and avirulent strains, population diurnal oscillations, and bacterial species predominant during disease and diet changes.

Diet is a particularly important factor in determining the microbiota composition of the gut (11). Thus, vegans, vegetarians, and omnivores have distinct microbiomes. Total counts of *Bacteroides* spp., *Bifidobacterium* spp., *Escherichia coli* (*E. coli*), and *Enterobacteriaceae* spp. were significantly lower in vegan samples than in controls. In contrast, total counts of *Klebsiella* spp., *Enterobacter* spp., other *Enterobacteriaceae, Enterococcus* spp., *Lactobacillus* spp., *Citrobacter* spp., and *Clostridium* spp. were similar in people with different diets. Subjects on a vegetarian diet ranked between vegans and omnivores. The total microbial count did not differ between the dietary groups (12). Notably, the microbiome of a person can be altered rapidly by changes in dietary patterns. It has been demonstrated by David and colleagues (13) that short-term consumption of diets composed entirely of animal or plant products can alter the microbial community structure. An animal-based diet increased the abundance of bile-tolerant microorganisms, including Alistipes, Bilophila, and *Bacteroides* and decreased the levels of *Firmicutes* that metabolize dietary plant polysaccharides (*Roseburia spp, Eubacterium rectale*, and *Ruminococcus bromii*). Thus, the gut microbiome rapidly responds to diet.

## Microbiome Taxonomy and Its Future

The taxonomy of microbiomes reflects their complexity and the challenges encountered in their understanding. Microbiomes include species across all major kingdoms, including viruses as well as Archaea, bacteria, and microbial eukaryotes. Our current depth of knowledge is associated with different methods of investigation, targeted surveys, and scope of studies conducted. To date, the most comprehensively investigated phylogenic group in health and disease has been bacteria.

### *Prokaryotes*

List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net) includes two prokaryotic domains (or empires), subdivided into 30 phyla in the domain Bacteria and 5 phyla in the domain Archaea. Together, these 35 phyla encompass about 2,400 genera and 12,400 species (14). This list is based on strict requirements, including the availability of reference strains, and does not include all available reference strains deposited in culture collections, including the ones for which genome sequences are already available (15–17). The addition of whole-genome phylogenetic analysis allows a refined positioning in the phylogenetic hierarchy as new tools are being developed (18–21). This approach brings some conflicts with the current classification, as has happened when the 16S rRNA gene phylogenetic classification competed with the phenotypic classification (22–24). In addition, the ability to target and obtain the sequence of the genes used for phylogenetic classification (16S rRNA, recA, rpoB, gyrB, etc.) using culture-independent methods also adds to the known diversity. Specialized curated databases that allow the propagation of this knowledge include SILVA, Ribosomal Database Project, and the Human Oral Microbiome Database (23, 25, 26). This culture-independent approach raised to 46 the number of phyla (23). How much of this diversity is in the human microbiome is unclear. However, it is already clear that organisms known to be environmental are also associated with health and that at least 30 prokaryotic phyla and 950 genera are associated with the human microbiome (27, 28).

### *Microbial eukaryotes*

The microbial eukaryotes are extremely diverse and do not fit under a single keyword. Although accurate, the eukaryotic supergroups defined by phylogenomics [Opisthokonta, Amoebozoa, Excavata, Archaeplastida, and SAR (Stramenopiles + Alveolates + Rhizaria)] are unfortunately uninformative compared with previous classification methods used in the literature. From the clinician to the lay person, terms such as fungi, protists, parasites, protozoa, and amoebae are much more familiar. In this area, current knowledge is based mostly on their roles as causative agents of disease; few studies have focused on healthy individuals or within a defined illness in a restricted number of individuals (29–33). Until recently, the focus on single disease agents also meant neglecting the remainder of the eukaryotic microbiome (34, 35).

The human eukaryotic microbiome includes pathogens, commensals, and beneficial organisms. The fungi (Opisthokonta) harbor a wide diversity of organisms, with an overlap for the skin with the local environment (35). The fungi include the Ascomycota (*Candida albicans*), Basidiomycota (*Cryptococcus neoformans*), Microsporida (*Encephalitozoon intestinalis*), and Zygomycota (*Rhizopus microsporus*). As fungi are part of the environment and human alimentation, it may be difficult to differentiate between transient and commensal organisms without a longitudinal study, unless a disease or an opportunistic infection occurs (36, 37). The Acanthocephala, most closely related to the rotifers, include *Macracanthorhynchus hirudinaceus*. The helminths (Opisthokonta), which are classified as part of the animals, include the cestodes (tapeworms, *Taenia saginata*), trematodes (flukes, *Schistosoma mansoni*), and nematodes (roundworms, *Enterobius vermicularis*). Although the majority of the helminths cause illness in millions of people worldwide, a few helminth species have been used in therapy (38, 39). The protozoa include the Amoebozoa (Amoebozoa; amoeba: *Entamoeba histolytica*), Metamonada (Excavata; flagellates: *Giardia intestinalis*), Parabasilia (Excavata; *Dientamoeba fragilis*), Ciliophora (SAR; ciliates: *Ballentidium coli*), Apicomplexa (SAR; *Cryptosporidium parvum*), and Stramenopile (SAR; *Blastocystis hominis*). These protozoa are all medically important even though not all carriers are symptomatic (30, 35). The Archaeplastida (including green and red algae) can be present in the microbiome of the skin and digestive tract. Additional sequences available through the sequencing of targeted genes, including via metagenomics, have expanded this knowledge and are maintained and curated in databases such as SILVA (23). A current view of the tree of life, encompassing the total diversity represented by sequenced genomes, was published recently by Hug and colleagues (40).

### *Viruses*

The gut microbiome includes bacteriophages that influence the bacterial hosts. The bacteriophage in the human gut are of three classes: a set of core bacteriophages shared among more than half of the human population, a common set of bacteriophages found in 20% to 50% of individuals, and a set of bacteriophages that are

unique to a person. Healthy gut phageome (aggregate of bacteriophage in the gut) is significantly decreased in individuals with gastrointestinal diseases (41). The International Committee on Taxonomy of Viruses in its 2014 release listed 104 families, 505 genera, and 3,186 species of all known viruses (42). The human virome overlaps with other animal viromes. These dsDNA, ssDNA, dsRNA, $ssRNA^-$ $ssRNA^+$ viruses, and dsDNA and ssRNA retroviruses can affect any of the tissues within the body. Human protists (nonfungal microbial eukaryotes) have their own viral challenges, which are being uncovered within the human virome (43). Much more is known about mycoviruses and their intracellular transmission during cell division and sporogenesis, and it is recognized that their life cycles generally lack an extracellular phase. Most known mycoviruses have dsRNA genome, but an increasing number of positive- or negative-strand ssRNA and ssDNA viruses have been isolated and characterized (44). Most of the archaeal viruses have been isolated from members of the Euryarchaeota and Crenarchaeota with broader morphologic characteristics than their bacterial counterpart (45). Little is known of the impact of archaeal viruses on the human virome. The dsDNA, ssDNA, dsRNA, and ssRNA bacteriophages have a great impact on prokaryotic ecology through their ability to modify population structure. A list of viral pathogens is maintained by the ViPR resource, whereas prophages are available at PHAST (46, 47).

***Future of taxonomy***

An open challenge in taxonomy is to refine the classifications to be more compatible with the emerging methods of molecular biosurveillance and detection, requiring targets associated to an outcome or being able to identify strains at multiple body-sites across the domains of life (21, 48). This work is dependent on a greater understanding of the true diversity in the population with the direct sequencing of large sample sets and/or large cohorts, such as the Human Microbiome Project, MetaHIT, BioMarks, and future large-scale projects (49–51). New resources are now attempting to bridge the different approaches and topics of detection across domains, such as the Human Pan-Microbe Community database (34). The ability to isolate and sequence single cells offers the opportunity to deepen both our understanding of the genomic composition of taxonomic diversity, as well to put this diversity in context of its environment, microbial partners, biogeography, and host physiologic status both at the local and systemic levels (52–54).

Beyond this time, taxonomy and the species level classification focused on vertical transmission of conserved information to descendant cells. The strain definition is associated with gene composition and gene modification, including mutations and antigenic variation following homologous DNA recombination, CRISPR system (clustered regularly interspaced short palindromic repeats), gene transfer, mobile elements, epigenetics, etc. Moving from targeted gene phylogeny to whole-genome comparison has its own limitations that can be complemented by the inclusion of other omics as once metabolic panels, protein, and DNA fingerprinting profiles were used. This polyphasic analysis allows understanding genetic relatedness and phylogenic relationship in the context of disease, reservoir, niche transmission within a single individual, propagation within a population, and dissemination among environments. An increasing number of gene transfer events among domains is being documented as well as across ecological niches (55–57). The genetic modification can be due to increases in genetic content, but genetic loss also has critical consequences in competitiveness or niche settlement (58, 59). As no clear general phylogenetic definition of strain has emerged in this era of genomics, efforts are to differentiate the different isolates with markers not yet found in other genomes and/or SNP (20, 21, 60, 61).

## The Infant Microbiome and Transgeneration Effects

Until recently, the placenta was considered a "sterile" intrauterine environment. Aagaard and colleagues (62) reported that the placental microbiome is consistently different from other parts of the body, including the skin and urogenital tract. Interestingly, the placental microbiome is most similar to that of the oral cavity. Thus, these authors suggested that microbes travel to the placenta from the mouth via blood. The results reinforce data that have suggested a link between periodontal disease in the mother and the risk of preterm birth.

Infant-associated microbial communities initially possess high concentrations of facultative anaerobes, such as *E. coli* and *Streptococcus* spp., but these populations are replaced by strict anaerobes coinciding with a reduction in oxygen tension (63–65). In addition to environmental routes of inoculation, the specific mode by which the infant is delivered is now known to influence the early gut microbiome structure and trajectory.

Although there is emerging evidence that the fetus encounters placental and amniotic bacteria *in utero*, it is clear that parturition contributes to the infant's first major inoculation of colonizing microbes (63, 66, 67). Microbial communities colonize external surfaces of the infant immediately following birth. This includes various microbial populations that are established and maintained along the gastrointestinal tract. The percentage of babies delivered through cesarean section (C-section) has risen in many countries. Although a number of C-section deliveries are performed for obstetrical indications, a large proportion is not medically indicated and may be due to maternal request and may incur several risks for the child (68). Obstetricians in many medical settings are paid more for cesarean delivery, and it is well known that private hospitals and practitioners encourage cesarean delivery (69). However, recent studies demonstrated that babies born vaginally are healthier compared with babies born by cesarean delivery. As such, infants delivered vaginally tend to harbor microbiotas that are typically encountered in the female reproductive tract, such as *Lactobacillus*. In contrast, cesarean delivery is typically associated with *Staphylococcus* spp. and other bacteria that are associated with the mother's skin and hospital environment (65, 70, 71). Children delivered by C-section have significantly increased risk of asthma, systemic connective tissue disorders, juvenile arthritis, inflammatory bowel disease, immune deficiencies, leukemia (72), and Crohn disease (73). Although there are some indications that infants born via C-section may be more susceptible to colonization by *Clostridium difficile* (*C. difficile*) or methicillin-resistant *Staphylococcus aureus* (*S. aureus*), and may be at an increased risk for pathologies later in life (71, 72), additional mechanistic studies are required to conclude causal relationships in this regard.

The infant microbiome exhibits several shared attributes regardless of birth method. In general, the infant microbiome is often dominated by the genera *Bifidobacterium, Bacteroidetes*, and members of clostridial taxa (74). In the seminal 2012 study

conducted by Yatsunenko and colleagues (9), gut microbiomes were characterized from individuals located in three distinct geographic locations (i.e., United States, rural Malawi, and rural Venezuela). Regardless of the host's location, microbial populations converge toward an adult community by 3 years of age (9). Furthermore, microbial community diversity increased as the host aged across all populations (9). Infant microbiomes exhibit hallmarks of functional redundancy, in that interindividual taxonomic variation is common despite sharing a stable and uniform metabolic potential (75). This functional redundancy during neonatal development may contribute to metabolic, digestive, and immune system homeostasis (74, 76). During infancy, the impact of alterations in community assembly on function has been linked to outcomes such as malnourishment, *C. difficile*–associated diarrhea, and necrotizing enterocolitis (74, 76, 77). Early-life microbiome disruption may potentially increase risk for developing celiac disease, asthma, type I diabetes, and obesity (70, 72, 78–81). These conditions could have long-term medical implications that interact reciprocally with the gut microbiome.

Perturbations or durable disruptions of the infant microbiome may proceed via several paths, with hospitalization and antibiotic use considered to be primary causes. Preterm and term infants who are hospitalized early in life are at a greater risk for nosocomial *C. difficile* infection (74, 82). Thus, the hospital environment is a reservoir for infectious agents that may be deleterious for at-risk populations, such as preterm infants with underdeveloped immunologic function. As antibiotics select for resistant and resilient strains, indiscriminant usage of antimicrobials may drive gut dysbiosis in certain instances. Several studies have characterized the influence of antibiotic usage on restricting gut microbiota diversity, potentially increasing susceptibility to aggressive bacterial infections like *C. difficile* and vancomycin-resistant *Enterococcus* bacteremia (82–84).

As it does in adults, diet exerts a strong influence on the structural composition of infant-hosted microbiomes. Culture-dependent and independent studies indicate that breastfed infants often possess a significantly different and less diverse gut microbiome relative to formula-fed infants (9, 64, 85–87). Accordingly, human milk incorporates several bioactive compounds important for infant nutrition, including lipids, proteins, and lactose. In addition, several milk molecules enhance immunologic and neurologic development (88–90). Escaping digestion by host glycosyl hydrolases, soluble milk glycans are transferred to the distal colon, where they are exposed to the gut microbiota of the infant. Thus, these human milk oligosaccharides (HMO) are available to guide the establishment and function of the infant microbiome. HMOs are heterogeneous carbohydrate polymers that are the third most abundant milk component at several grams per liter (91). HMO structures incorporate the monosaccharides D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and N-acetylneuraminic acid, with more than 200 unique HMO isomers composed of these components identified to date (91, 92).

Breastfeeding infants often display a microbiome enriched for commensal *Bifidobacteria* that can utilize HMOs. *Bifidobacteria* are Gram-positive anaerobes of the phylum *Actinobacteria*, which typically colonize infants and adults to a lesser degree (93–95). Accordingly, *Bifidobacterium longum* (*B. longum*) subsp. *infantis* is a major commensal of breastfed infants, with this lineage possessing a large genomic cluster that enables HMO utilization (95). That unique gene assemblage permits the catabolism of specific small mass HMOs that other *Bifidobacteria* cannot process. For example, in comparison with other *Bifidobacteria, B. longum* subsp. *infantis* flourishes in the presence of milk that contains α1,2-fucosylated HMOs (96). An individual's complement of HMO structures is somewhat dependent on the mother's genotype and may vary by gestational age and stage of lactation. The relative concentrations of α1,2-linked fucosyl moieties depend on the fucosyltransferase 2 allele (96). Women with a functional copy of this gene, termed secretors, may confer certain health benefits to their infant such as a decreased risk for diarrheal diseases (96–98). HMOs can decrease the presence of gastrointestinal pathogens using two primary mechanisms. HMOs themselves mimic pathogen-binding sites of receptors that decorate the surface of host cells (99–101). Studies have documented this effect using *Vibrio cholerae, Streptococcus pnemoniae*, and *E. coli* (102–104). In addition, high levels of *Bifidobacteria* are correlated with lower incidence of potentially dangerous neonatal infections, potentially due to competitive exclusion (105, 106). Gut microbiota development during infancy can have long-lasting effects on the individual's future health. Colonization of fucosyllactose-utilizing *Bifidobacteria* is due to an ABC transporter that acts as a key genetic factor for fucosyllactose utilization (107).

Human milk is generally accepted as the best nutrition for newborns and has been shown to support the optimal growth and development of infants (108). Human milk also provides bioactive components that are important to optimize gut microbial colonization, immune maturation, metabolic development, and even cognitive development. Breast milk has a low buffering capacity, which would make the gut more susceptible to a lowering of pH due to acid production from bacterial fermentation in the colon. The fecal pH of the breastfed infant is between 5 and 6 dominated by *Bifidobacteria,* whereas formula-fed infants have a fecal pH in the range of 8 to 9. The acetic acid in the gut of breastfed infants is frequently present as an acetate buffer. This effect was not observed in formula-fed infants. The lower pH in the gut is an important factor in restricting the growth of Enterobacteria, Clostridia, and the Bacteroides and favors the proliferation of the acid-tolerant *Bifidobacteria* and *Lactobacilli* (109). Human milk also contains many antimicrobial factors, such as partially digested or fermented peptides, milk-borne fatty acids, human lactoferrin, lysozyme, and secretory IgA. These factors may decrease the prevalence of pathogens in the gut's ecosystem in infants. The broad range of nondigestible oligosaccharides specifically found in human milk but not in other mammals' milk (108) is a major factor in the prevention of pathogen growth in the gastrointestinal tract. Stunted infants fed poorly have low amounts of sialylated HMOs in the gut. These oligosaccharides are not used by the body, but rather used by the gut microbes. Charbonneau and colleagues (110) colonized germ-free mice with a consortium of bacterial strains cultured from the fecal microbiota of a 6-month-old stunted infant and fed recipient animals with normal diet with or without purified sialylated bovine milk oligosaccharides (S-BMO). S-BMO produced a microbiota-dependent body weight gain, indicating growth promotion in the presence of gut microbiota. However, control animals that were germ free did not increase body weight, demonstrating some bacteria in the gut are involved in weight gain.

Infant formula is often based on bovine milk unless it is plant derived. Fluid dairy milk contains oligosaccharides with a similar structure to HMOs, which may suggest similar functionality

despite being incorporated at relatively low concentrations (108, 111–113). At the moment, there are efforts to supplement infant formula with oligosaccharides, although HMO structures are difficult to synthesize and may not be commercially viable (114). However, oligosaccharides from other sources may increase bifidobacterial concentration as a preferred endpoint, including galacto-oligosaccharides and fructo-oligosaccharides.

Use of oral probiotics by the mother during pregnancy is thought to help the developing baby. Microbes in the placenta or amniotic fluid affect fetal innate immune gene expression during late pregnancy. Maternal probiotic supplementation significantly modulated the expression of Toll-like receptor (TLR)- related genes both in the placenta and in the fetal gut. Thus, fetal and placental immune physiology may be modulated by maternal dietary interventions, including using specific probiotics (115, 116). It has also been shown that maternal probiotic supplementation during pregnancy and breastfeeding reduces the risk of eczema in the infant (116). Probiotic supplements continue to impact infants in their early years. It has been shown that infant formula supplemented with the probiotics *Lactobacillus rhamnosus* (*L. rhamnosus*) GG and *Bifidobacterium lactis* Bb-12 offers a safe means of reducing the risk of early acute otitis media and antibiotic use and the risk of recurrent respiratory infections during the first year of life (117). Probiotics enhance gut-specific IgA responses, which are frequently defective in children with food allergy (118). Kainonen and colleagues (119) have demonstrated that exclusive breastfeeding promotes an anti-inflammatory cytokine milieu, which is maintained throughout infancy. Such an immunologic environment limits hyperresponsiveness and promotes tolerization, thereby prohibiting the onset of allergic disease.

Infantile colic (excessive crying) is a common problem in about 20% of healthy thriving infants in the first 3 months of life (120). The risk factors associated with the development of infantile colic include maternal smoking, increased maternal age, and firstborn status. Infantile colic could also be related to cow's-milk protein allergy and atopy (121). Several studies have demonstrated that administration of probiotics containing *Lactobacillus reuteri* (*L. reuteri*) DSM 17938 significantly improved colic symptoms by reducing crying and fussing times in breastfed infants with colic (122, 123). Treatment of colic with *L. reuteri* did not affect the global composition of the microbiota. The decrease in colicky symptoms was linked to changes in the microbiota, with a relative increased abundance of the phyla *Bacteroidetes* and genus *Bacteroides* after treatment with *L. reuteri* (124).

## Microbiome and Aging

As humans age, the composition of the microbiome also changes (9). Aging is accompanied by the onset of a myriad of clinical changes, including a basal proinflammatory state ("inflamm-aging") that directly interfaces with the microbiota of older adults and enhances their susceptibility to diseases that accompany aging. Studies in older adults demonstrate that the gut microbiota correlates with diet, basal level of inflammation, and location of residence (e.g., community dwelling, long-term care settings; refs. 125–127). Links between the microbiota and a variety of clinical problems plaguing older adults have been made, including physical frailty, *C. difficile,* colitis, vulvovaginal atrophy, colorectal carcinoma, and atherosclerotic disease (128).

The most drastic change associated with the aging gut is a change in the relative proportion of organisms, for example, *Firmicutes* dominate in the young and *Bacteroidetes* in the elderly. Reduction in the diversity of bacteria comprising subpopulations is seen in individuals with high frailty, although living in a community undermines this alteration (125, 129).

Aging-associated oxidative stress induces aggressive potential and virulence factors of anaerobic bacteria, thereby causing morphologic alterations of bacterial cells that could impact the host. The microbiota may also influence host gene expression by regulating miRNAs (130). Analysis of the network functions revealed that differentially regulated miRNAs between infants and adults and miRNAs that decreased during aging shared two network functions: inflammatory disease and inflammatory response. miRNAs promote aging by modulating their targets to drive cell senescence and aging in different tissues or organs. There is significant variation in the expression of miRNA during aging. Genome-wide assessment of miRNA expression revealed that the majority of miRNAs decreased with age (131, 132). Interestingly, host-derived miRNAs may also influence the composition of the gut microbiome (133).

It has been documented that calorie restriction can increase the life span of model organisms (134). Notably, Zhang and colleagues (135) demonstrated that calorie restriction enriches bacterial phylotypes positively correlated with lifespan. Bacteria of the genus *Lactobacillus* have been shown to increase in animals on low-fat diet, and this environment reduces phylotypes that are negatively correlated with lifespan. Caloric restriction– induced changes in the gut microbiota occur concomitantly with a significant reduction in serum levels of lipopolysaccharide (LPS)-binding protein, suggesting that animals undergoing calorie restriction establish a structurally balanced architecture of gut microbiota that exerts a health benefit through the reduction of antigen load from the gut. Strikingly, dietary changes can detectibly influence host environment in as little as 24 hours, with longer term changes correlating with novel enterotype clustering in the host (136).

Multiple studies in centenarians indicate extreme aging is characterized by microbial changes deemed unique from other age groups, with emphasis placed on organismal composition and increased inflammatory effects (137, 138). Fecal sampling by Rampelli and colleagues (138) revealed a distinctive functional profile with a decrease in short-chain fatty acid (SCFA) production and saccharolytic potential but an increase in proteolytic potential. A total of 116 microbial genes were found to be correlated with aging, including those essential to the metabolism of tryptophan, phenylalanine, tyrosine, valine, and lysine. Implications of such variability include changes in well being, aging, and disease susceptibility. This was accompanied by an increase in the occurrence of pathobionts, bacteria usually present in low numbers in adults. Proinflammatory effects of the pathobionts are exaggerated by a decrease in *Faecalibacterium prausnitzii* (*F. prausnitzii*), a species associated with anti-inflammatory influences (137).

The relationship between aging and the microbiome is not strictly one-sided; it has been demonstrated that host aging can actually be impacted by interspecies communication. Animal fecundity, development time, and lifespan were all dependent on the amount and type of bacteria they were fed. There are multiple lines of evidence demonstrating the ability of microbes to substantially

change host physiology, as it pertains to these parameters (139). Accordingly, manipulating the microbiome of older adults holds promise as an innovative strategy to positively influence the development of comorbidities associated with aging (128).

Rozsa and colleagues (140) recently proposed the "microbiome mutiny hypothesis," whereby some microorganisms of the microbiome could switch to higher virulence (microbiome mutiny) in old or seriously ill people, to optimize their transmission under the conditions of increased background mortality. This proposed virulence shift might contribute to the death of old or seriously ill people even in the absence of apparent disease.

In the central nervous system (CNS), polyphenols present in many edible plants exert anti-inflammatory effects (141) and act on the brain in several ways. Like antioxidant vitamins, dietary polyphenols contribute to the regulation of oxidative stress and improve vascular health. Notably, intestinal microbiotas convert dietary polyphenols to phenolic acids, stimulating the proliferation of *Bifidobacteria* and decreasing the ratio of *Firmicutes* to *Bacteroidetes*, relative to controls. Polyphenols also stimulate SCFA production by bacteria (142). Wang and colleagues (143) recently reported that the microbiome can convert grape-derived polyphenol to the phenolic acids, 3-hydroxybenzoic acid and 3-(3′-hydroxyphenyl)propionic acid, which interfere with the assembly of β-amyloid peptides into neurotoxic β-amyloid aggregates that play key roles in the pathogenesis of Alzheimer disease. Thus, in the brain and other tissues, many healthful effects of polyphenols may relate to their conversion to various metabolic derivatives by the gut microbiome while aging.

## Microorganisms and Immune Function

Through coevolution with their hosts, microbes exert a major influence in shaping the development of the immune system, putting it under selective pressure to develop the capability to discern between invasive pathogens that it is imperative to control and commensal resident microbes that are beneficial to tolerate (144, 145). Many developmental aspects of the adaptive immune system are influenced by the composition of bacterial colonization of the gut. Thus, the mammalian immune system, which is tasked with the duty of controlling microorganisms, is in turn fundamentally shaped by microorganisms (146). For example, it has been demonstrated that changes to the symbiotic microbiota early in life, or the absence of it, can lead to exacerbated type II immunity and allergies due to aberrant immune functionality. The microbiota is a strong inducer of proinflammatory Th17 cells and regulatory T cells (Treg) in the intestine. The microbiota-induced Tregs express the nuclear hormone receptor RORγt and differentiate along a pathway that also leads to Th17 cells. In the absence of RORγt$^+$ Tregs, Th2-driven defense against helminths is more efficient, whereas Th2-associated pathology is exacerbated. Thus, the microbiota regulate type II responses through the induction of type III RORγt$^+$ Tregs and Th17 cells, thereby balancing immune responses at mucosal surfaces (147). Exercise can also influence the immune system and how they modulate microorganisms (148, 149). Intense exercise causes immunosuppression, whereas moderate-intensity exercise improves immune function and potentially reduces risk and severity of respiratory viral infection by increasing stress hormones, reduce excessive local inflammation, and skew the immune response to a Th2 phenotype (148). Similarly, exercise can also influence bacterial infections. Pape and colleagues (150) demonstrated a reduction of bacterial infection in people with physical activity compared with those that maintain a sedentary lifestyle.

Similar to adaptive immunity, the innate immunity is also influenced by the microbiome. One example of this is neutrophil aging. Aged neutrophils exhibit impaired migration and reduced proinflammatory properties. Microbiota influence neutrophil aging via TLR- and MyD88-mediated signaling pathways. Depletion of the microbiota significantly reduces circulating numbers of aged neutrophils and improves pathogenesis and inflammation-related organ damage in models of sickle cell disease or endotoxin- induced septic shock. Thus, host microbiota may play a role in regulating a disease-promoting neutrophil subset that promotes tissue injury in various inflammatory diseases (151).

Although active immunity is essential to combat infection, inadequate control over immune responsiveness due to the inability to establish immune tolerance can also have dire consequences, regardless of whether the response is directed against a foreign pathogen or self. Meanwhile, one of the major benefits of immune tolerance is the ability to maintain a commensal microbiome consisting of a multitude of foreign microorganisms. Thus, the mechanisms for establishing tolerance are a vital aspect of the immunoregulatory framework. One crucial element in instructing the immune system to be self-tolerant is the education of thymus T cells during development. In the thymus, self-reactive cells are either eliminated or differentiated into tolerogenic Foxp3$^+$ Tregs (152). Apart from the thymus, the immune system is also educated in the gut, where it has been shown that the interaction of T cells with commensal microbiota results in the peripheral generation of Tregs rather than pathogenic effector cells. Failure of this tolerogenic process can lead to the development of autoimmune diseases, including colitis (152).

## Microorganisms Encountered Early in Life Prevent Autoimmunity and Allergy

The human microbiome is important for human health, behavior, and disease, yet its function and dynamics during healthy and disease states are not fully understood (153). It is also not fully understood how the microbiome interacts with the host immunity thereby preventing autoimmunity. The hygiene hypothesis first put forward by Strachan (154) postulates that the lack of early exposure to microorganisms (either beneficial or pathogenic) would lead to poor development of the immune system. The leading idea is that some microorganisms that coevolved with us are able to protect against a large spectrum of immune-related disorders (155). Although the hygiene hypothesis is not universally applicable, it offers some explanative power to interpret the effects of microorganism exposure in early life on preventing autoimmunity and allergy.

Children growing up on dairy farms are protected from allergy, hay fever, and asthma (156, 157). Asthma is a chronic inflammatory disease triggered by acute inflammation of the bronchial tube, leading to production of extra mucus. This can make breathing difficult and trigger coughing, wheezing, and shortness of breath. The number of asthma cases is increasing all over the world, but the causes remain obscure. It has been hypothesized that increased cleanliness, reduced family size, and subsequent

decreased microbial exposure could explain the increases in global asthma prevalence (158). Evidence from bronchial brushings implicates phyla present in healthy individuals with variation present in disease conditions such as cystic fibrosis, chronic obstructive airways disease, and asthma. The microbiome can exacerbate the phenotypes seen in the condition, as well as explain the variability in phenotypes observed (159). Many cytokines and chemokines are involved in the pathophysiology of asthma. Th2 cytokines may play an important role in the pathophysiology of asthma. The Th1 cells secrete IL2 and IFNγ, whereas the cytokines, IL4, IL5, IL9, and IL13 are derived from Th2 cells, although they may also be derived from other cell types. The distinction between Th1 and Th2 cells is not as distinct in humans as in mice (160, 161). According to the hygiene hypothesis, the lack of infection and exposure to environmental endotoxins may alter the balance between Th1 and Th2 cells.

Although children on farms are much less likely to get asthma, the underpinnings of protection are not clearly understood. Early-life contact with livestock and their fodder and consumption of unprocessed cow's milk have been identified as the most effective protective exposures. Studies of the immunobiology of farm living point to activation and modulation of innate and adaptive immune responses by intense microbial exposures (162). Schuijs and colleagues (157) demonstrated that chronic exposure to low-dose endotoxin or farm dust protects mice from developing house dust mite (HDM)-induced asthma. Endotoxin reduced epithelial cell cytokines that activate dendritic cells (DC), thus suppressing type II immunity to HDM. Loss of the ubiquitin-modifying enzyme A20 in lung epithelium abolished the protective effect. An SNP in the gene encoding A20 has been associated with allergy and asthma risk in children growing up on farms. Thus, the farming environment protects from allergy by modifying the communication between barrier epithelial cells and DCs through A20 induction.

From delivery, the microbiome assembly might influence asthma. Babies born via C-section, who experience an altered trajectory of microbiome assembly, are more prone to asthma than those born vaginally. Similarly, children treated with antibiotics are also more prone to asthma attack (163). Lif Holgerson and colleagues (164) analyzed the oral biofilm in healthy 3-month-old infants born by C-section or delivered vaginally. Among more than 300 bacterial taxa analyzed, *Slackia exigua* was detected only in infants delivered by C-section. Furthermore, significantly more bacterial taxa were detected in the infants delivered vaginally (79 species/species clusters) compared with infants delivered by C-section (54 species/species clusters). Overall, the vaginally delivered infants had a higher number of bacterial taxa. A higher prevalence of salivary *Streptococcus salivarius, Lactobacillus curvata, Lactobacillus salivarius (L. salivarius*), and *Lactobacillus casei* (*L. casei*) was detected in infants delivered vaginally (165).

A longitudinal human study by Arrieta and colleagues (166) reported that infants at risk of asthma have transient gut microbial dysbiosis during the first 100 days of life. The authors collected stool and urine samples from more than 300 babies at 3 months and 1 year, as well as information on their health at 1, 3, and 5 years. Then, they analyzed levels of gut microbes in each stool sample. Babies that had low or undetectable levels of four bacteria, *Lachnospira, Veillonella, Faecalibacterium*, and *Rothia*, at 3 months all went on to show early signs of asthma, wheezing and skin allergies, at 1 year. The babies that did not develop these symptoms invariably had high levels of the four microbes in their 3-month stool samples. The authors also used the stool samples from the asthma-prone 3 month olds to colonize the guts of mice that had been raised in a bacteria-free environment. The animals developed inflamed lungs indicative of asthma. However, upon inoculating the four missing microbes to the digestive tracts of these mice along with the feces, they no longer had a heightened risk of developing asthma. The studies demonstrated that certain bacterial species that are encountered early in life could train the immune system to prevent asthma.

Microbial dysbiosis in early life can alter the trajectory of immune development and provide the setting for allergic disorders in later life (167). Dysbiosis may trigger autoimmune diseases via inappropriate posttranslational modification of host proteins (168). Endogenous and microbial enzymes have the capacity of intestinal enzymatic neoantigen generation by posttranslational modification of host proteins. The hygiene hypothesis stipulates that microbial exposure during early life induces immunologic tolerance via immune stimulation and hence reduces the risk of allergy development. Several common lifestyle factors and household practices, such as dishwashing methods, may increase microbial exposure. Hesselmar and colleagues (169) investigated whether lifestyle factors are associated with allergy prevalence. The authors demonstrated that in families that used hand dishwashing, allergic diseases in children are less common than in children from families who use machine dishwashing. The authors were of the opinion that a less efficient dishwashing method may induce immune tolerance via increased microbial exposure.

Autoimmunity is more prevalent in the population of some northern European countries, such as Finland and Estonia, when compared with Russia. It was found that *Bacteroides* species are less abundant in Russians but dominate in Finnish and Estonian infants. Their LPS exposures arose primarily from *Bacteroides* rather than from *E. coli*, which is a potent innate immune activator. The *Bacteroides* LPS was found to be structurally distinct from *E. coli* LPS and inhibits innate immune signaling and endotoxin tolerance. It was observed that unlike LPS from *E. coli*, *Bacteroides dorei* LPS does not decrease incidence of autoimmune diabetes in nonobese diabetic mice. Early colonization by immunologically silencing microbiota may thus preclude aspects of immune education (170).

Rheumatoid arthritis (RA) is an autoimmune disease in which the immune system attacks the joints, leading to swollen and painful joints. The mucosal surfaces are sites of rheumatoid arthritis initiation. The common occurrence of periodontal dysbiosis in rheumatoid arthritis suggests that oral pathogens may trigger the production of disease-specific autoantibodies and arthritis in susceptible individuals. Periodontitis is characterized by the presence of citrullinated autoantigens that are primary immune targets in rheumatoid arthritis. The citrullinome in periodontitis is similar to the hypercitrullination observed in the rheumatoid joint, implicating this mucosal site in rheumatoid arthritis pathogenesis. Recent studies identified the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) as a bacterial trigger of autoimmunity in rheumatoid arthritis by inducing hypercitrullination in host neutrophils. The pore-forming toxin leukotoxin A secreted by *A. actinomycetemcomitans* triggers autoantigen citrullination in the rheumatoid arthritis joint (171). Zhang

and colleagues (172) reported alterations in the gut, dental, or saliva microbiome that distinguished patients with rheumatoid arthritis from healthy controls. Individuals with rheumatoid arthritis had low numbers of *Haemophilus* spp., whereas *L. salivarius* was very high in these patients. It has been reported in experiments in mice that inoculation with *Bifidobacterium adolescentis* (*B. adolescentis*) exacerbated autoimmune arthritis. *B. adolescentis* is known to induce Th17 cells in the intestine (173). Interestingly, the frequencies of Th17 cells and levels of IL17 strongly correlated with systemic disease activity at both the onset and the progression of rheumatoid arthritis (174).

## Role of Microbiome in Obesity

Obesity results from an imbalance of food intake, basal metabolism, digestive tract microbial composition, and energy expenditure (175, 176). According to Turnbaugh and colleagues (177), the gut microbiome should be considered as a set of genetic factors that together with host genotype and lifestyle contribute to the pathophysiology of obesity. It is observed that the intestinal bacteria in obese humans and mice differ from those in lean individuals. Obese mice microbiota was found to be rich in *Firmicutes* compared with the lean mice microbiota, which was abundant in *Bacteroidetes* (177). Strikingly, colonization of germ-free mice with microbiota from obese mice was sufficient to cause a significant increase in total body fat, as compared with colonization with microbiota from lean mice (177). The obese microbiome has an increased capacity to harvest energy from the diet, thereby increasing weight gain in the host (177, 178). Colonization of adult germ-free mice with a gut microbial community harvested from conventionally raised mice increased body fat within 10 to 14 days, despite an associated decrease in food consumption. This change involves several linked mechanisms: microbial fermentation of dietary polysaccharides that cannot be digested by the host; subsequent intestinal absorption of monosaccharides and SCFA; their conversion to more complex lipids in the liver; and microbial regulation of host genes that promote deposition of the lipids in adipocytes (179).

Transfer of human microbiota to mice can phenocopy such effects, as shown by Ridaura and colleagues (180). Cohabitation of mice harboring an obese microbiota with mice containing the lean microbiota prevented the development of increased body mass and obesity-associated metabolic phenotypes in obese cage mates. Rescue correlated with invasion of specific members of *Bacteroidetes* from the lean microbiota into obese microbiota and was diet dependent. The study confirmed that specific bacteria along with diet could induce obesity.

Childhood obesity is considered one of the most serious global health issues in our society. Obese children are more likely to be obese in adulthood and are at greater risk of premature death and adverse health outcomes in later life (181). Administration of three or more courses of antibiotics before children reach age 2 years is associated with an increased risk of early childhood obesity (182). When given early in life, antibiotics that disrupt microbiota composition, and consequently the metabolic activity of the microbiota, can affect the body mass of the host by either promoting weight gain or stunting growth (183). The correlation of antibiotics to obesity has been earlier shown in animal models (184).

Food is broken up into components that tend beneficial microorganisms. Bacterial fermentation of a diet rich in fibers leads to the production of SCFA, which as noted above includes acetate, propionate, and butyrate in the gut (185, 186). Interestingly, butyrate promotes colonic health and helps prevent cancer (185, 187, 188). The consumption of high fat and high calorie foods negatively impacts the beneficial microbes, which are believed in turn to promote obesity. Notably, obese people have lower *Bacteroidetes* and more *Firmicutes* in their distal gut than lean subjects, and the introduction of low fat and carbohydrate diets increased the proportion of *Bacteroidetes* (175, 189).

Obesity is also known to impair cognition and produces atrophy of brain regions associated with learning and memory. In animal studies, it has been shown that, even before the onset of diabetes or metabolic syndrome, early-stage obesity produced deficits on cognitive tasks that require the prefrontal cortex. Impaired cognition was associated with synapse loss, including reduced numbers of dendritic spines and expression of synaptic proteins, as well as structural alterations in the microglia. Thus, obesity must be considered as a contributing factor to brain dysfunction mediated through the gut–brain axis (190, 191).

It has been demonstrated recently that some bacterial species are beneficial to the host by preventing obesity. In animal models and humans, the abundance of *Akkermansia muciniphila* (*A. muciniphila*) is decreased in obese and type II diabetic mice (192), and use of the bacterium as a probiotic is beneficial to the host. Interestingly, whole bacterium is not essential to prevent obesity. Intake of the membrane protein of the bacterium *per se* could be beneficial to the host. Amuc_1100, a specific protein isolated from the outer membrane of *A. muciniphila*, interacts with TLR2, is stable at temperatures used for pasteurization, improves the gut barrier, and recapitulates the beneficial effects of the bacterium (193).

Metformin is a well-established drug in the management of type II diabetes and obesity. Recent studies suggest that the microorganisms are involved in mediating the beneficial effects of metformin on glucose metabolism. Metformin shifts gut microbiota composition through the enrichment of mucin-degrading *A. muciniphila* as well as several SCFA-producing microbiota, including *Butyrivibrio, Bifidobacterium bifidum, Megasphaera*, and *Prevotella* (194). Overall, there is substantial evidence of the key role of microbiota in obesity and its adverse effects.

## Microbiome and Cardiovascular Diseases

The gut microbes produce a large range of metabolites that act not only in the gut, but also systemically, and this large pool of known and unknown metabolites is not fully understood (195). The metabolite trimethylamine *N*-oxide (TMAO) is the first potentially direct link between the gut microbiota and atherosclerotic heart disease. Trimethylamine is produced by the gut microbiota from nutrients containing L-carnitine, choline, and phosphatidylcholine and is subsequently oxidized by hepatic flavin-containing monooxygenases to TMAO. TMAO has been proposed to interfere with cholesterol transportation, and TMAO precursors promote foam cell formation and atherosclerosis in animal models, but not in the presence of antibiotics to the drinking water, suggesting a microbiota-dependent mechanism (195).

Hypertension is a risk factor for coronary heart disease, yet whether gut microbiota dysbiosis is involved in the development of hypertension remains largely unknown. In a recent study, it was observed that the bacterial genus *Prevotella* and *Klebsiella* were

overrepresented in individuals with hypertension. Fecal transplantation from hypertensive human donors to germ-free mice increased blood pressure in animals, thereby demonstrating the direct influence of gut microbiota on high blood pressure (196).

Beneficial microorganisms are known to protect against atherosclerosis. The lack of gut microbiota in germ-free apolipoprotein E (ApoE)-null mice, an experimental model of human atherosclerosis, was found to induce the development of atherosclerotic plaques even when animals were fed a standard low-cholesterol diet. Colonization with normal human microbiota prevented atherogenesis in germ-free ApoE-null mice fed a standard low-cholesterol diet but not a diet with high-cholesterol content (197). The bacterial genera *Eubacteria, Anaeroplasma, Roseburia, Oscillospira*, and *Dehalobacteria* appeared to be protective against atherosclerosis and showed significant negative correlation with atherosclerotic plaque size and plasma adipocyte fatty acid binding protein and cholesterol (198). *A. muciniphila* is also beneficial to the heart. The bacteria attenuate atherosclerotic lesions by ameliorating metabolic endotoxemia-induced inflammation through restoration of the gut barrier (199).

## Microbiome and Behavior

The exponential growth in our collective knowledge of the human microbiome has seen the study of gut microorganisms move beyond the traditional preserve of strictly microbiological disciplines. As our appreciation of the structure and dynamics of the gut microbiome has flourished, so too has our grasp of the implications for host physiology in health and disease. Perhaps one of the more surprising aspects of this host–microbe dialogue is the complex interactions that manifest as alterations in brain and behavior. Moreover, the bidirectional nature of this conversation needs to be considered in the context that disruptions to CNS function can be expressed distally as alterations in microbiome composition and function in the gastrointestinal tract. These aspects of host–microbe dialogue are generally important to medicine, due to the impact of behavioral states that widely impact and/or reflect the operation and progression of pathogenic processes and their treatment (e.g., the negative impact of depression on general therapeutic compliance).

These complex reciprocal interactions are facilitated by the microbiome–gut–brain axis, which incorporates the gut microbiome as a critical node of the communication network encompassing the CNS, the neuroendocrine and neuroimmune systems, the sympathetic and parasympathetic arms of the autonomic nervous system, and the enteric nervous system (200). The focus on the gut microbiome has proved to be a surprisingly fertile ground, and the evidence garnered from a variety of preclinical approaches has converged to illuminate how the gut microbiome regulates multiple behaviors, physiologic readouts, and indeed many fundamental aspects of brain function.

In this regard, surveys of microbiota-deficient germ-free animals have proved particularly informative. The use of these animals in general is not new, but their application to CNS-directed queries has been a notable feature of recent research efforts (201, 202). From a behavioral perspective, these animals display a less anxious phenotype (203–206), and this atypical performance can be normalized if the animals are colonized postweaning (205). Remarkably, it has also been demonstrated using both the germ-free paradigm and an antibiotic-induced microbiota deficiency that anxiety-like behaviors can be transferred via the gut microbiota by means of a fecal transplant (207, 208). Germ-free animals also display alterations in social behaviors (209, 210) and, insofar as it has been logistically possible to address in detail in this paradigm, aspects of cognitive function (211). Gut microbiota depletion using a cocktail of antibiotics from early adolescence in mice replicates many of the behavioral characteristics of germ-free mice, including reduced anxiety-like behaviors and impaired cognitive performance (212).

Other approaches have both largely supported and extended the behavioral picture painted by microbiota-deficient animals. For example, administration of a probiotic *L. rhamnosus* strain reduced anxiety and depression-related behaviors (213), while alternative candidate probiotics, including a *B. longum* strain, exerted a beneficial impact on cognitive processes (214). Prebiotics (e.g., fiber-rich foods that can influence the microbiome) can also exert anxiolytic effects (215), while bacterial infection with an enteric pathogen can impact both learning and memory (211) and modulate pain behaviors (216).

Physiologically, germ-free animals also exhibit profound differences with conventionally colonized controls. These differences include a defective immune system and exaggerated corticosterone outputs to acute stressors (205, 217, 218). The microbiome is also required for the development of microglia, cells that defend the CNS. Microglia from germ-free mice had altered gene expression that influenced its development (219).

An increased availability of tryptophan, the amino acid precursor to neuroactives such as serotonin and kynurenine pathway catabolites, as generated respectively by indoleamine 2, 3-dioxygenase (IDO1) or TPH, is one feature of the germ-free state (205, 220, 221). Many aberrant physiologic features can be rescued if the animals are colonized with a normal microbiota, especially if this intervention takes place during specific time windows postweaning (205, 217). As is the case for behavior, other microbiota manipulations, such as rendering mice microbiota deficient or probiotic ingestion by rodents, can also impact parameters such as corticosterone outputs or tryptophan availability (212, 213, 222).

The reciprocal interaction between stress and the microbiome is a particularly interesting facet of this bidirectional relationship. As indicated above, the gut microbiota exert an influence on the hypothalamic–pituitary adrenal axis, the main host stress response system, and this can be captured by measuring cortisol in humans or corticosterone in rodents (223). There are now studies showing that the opposite is also true and that a variety of stressful insults that are linked to psychopathology in adulthood can alter the composition of the gut microbiome in animal models. This is reflected in studies that have examined early-life stress (224, 225), prenatal stress (226, 227), and psychosocial stress (228–230). Interestingly, the gut microbiota seems necessary for the expression of some of the pathologic behavioral features induced by maternal separation (231), a well-validated early-life stress-based model of gut–brain axis dysfunction (232). In the clinical setting, maternal prenatal stress is also associated with alterations in the infant gut microbiome (233). Another example of such feedback loop is stress-related microbiome–gut–brain axis dysfunction in irritable bowel syndrome (234).

Growing up, germ-free influence biological function such as blood–brain barrier integrity (235), transcriptional regulation (203, 236), neurogenesis (237) microglial function (238), and myelination

(239). Recently, it has been demonstrated that a germ-free mouse model of Alzheimer disease displayed a marked reduction of cerebral amyloid pathology and that colonization of these mice with the gut microbiota of their conventionally colonized counterparts reinstated the amyloid pathology (240). Although the behavioral implications of these altered amyloid phenotypes requires elaboration, this intriguing study does provide support for the hypothesized role of the gut microbiota in neurodegenerative disorders (241).

The mechanisms underpinning influence of the gut microbiome on brain and behavior are under investigation. The gut microbiota is required for motor deficits, microglia activation, and α-synuclein pathology. Colonization of microbiota from Parkinson disease–affected patients enhances physical impairments compared with microbiota transplants from healthy human donors. Thus, the gut bacteria are involved in movement disorders, and alterations in the human microbiome represent a risk factor for Parkinson disease (242). Recently, the role of the vagus nerve (the main neural communication highway between the gut and the brain) has attracted much attention (243). It has been demonstrated, for example, that the beneficial CNS impact of a probiotic was abolished in vagotomized mice (213), while the anxiety-like behaviors that emerge in a colitis model were absent in previously vagotomized mice (207). The vagus nerve is not the sole conduit linking the gut and the brain (244), and a variety of alternatives have been considered. These include microbial regulation of tryptophan metabolism (245), microbial metabolites, such as SCFAs (246) or indoles derived from tryptophan (247), neuropeptide production (248) as well as immunomodulation (249). The important role of the gut microbiota in maintaining intestinal barrier integrity also needs to be taken into account (250, 251).

The landscape for manipulating the microbiome is broad and increasingly financed (252). Considerations are being given to priming interventions that promote assembly of the infant microbiome (65, 253, 254), sustain the gut microbiota in healthy aging (125, 126), more radical options such as fecal microbiota transplantation (255) as well as less controversial options such as psychobiotics (256), exercise (257, 258), and diet-based manipulations (259, 260). Indeed, a number of small studies using healthy volunteers have now demonstrated that ingestion of certain cocktails of probiotics, a fermented milk product with probiotic or prebiotics, can impact on the CNS (261–264). Autism spectrum disorders are complex neurobiological disorders characterized by impairment in social interaction and communication and restricted, repetitive, and stereotyped patterns of behavior, interests, and activities. Autistic children suffer from gastrointestinal disorders. Autistic children have less diverse microbial population in the gut and significantly lower abundances of the genera *Prevotella, Coprococcus*, and *Veillonellaceae* involved in carbohydrate metabolism (265).

Future directions will likely see further elaboration of the role of the gut microbiome in sleep (266) and circadian rhythms (267–269). Our awareness of the interface between natural and built environments, the gut microbiota, and human behavior is also growing (270–272). Of course, a key caveat is to what degree this promising but largely preclinical body of research will effectively impact the clinical setting. Moving from mouse to man, be it in the context of CNS-directed or gastrointestinal-focused research, is of course complicated for stress-related neuropsychiatric and other heterogeneous disorders associated with the gut microbiome (273, 274). Of equal importance is the necessity to address the issue of whether the correlations that have been noted thus far between multiple disorders and the gut microbiota alterations are in fact causal relationships. Such obstacles are not insurmountable with due diligence and the necessary multidisciplinary expertise required to exploit the considerable opportunities presented by host–microbe interactions.

## Beneficial Microorganisms Restrict the Outgrowth of Pathogens in the Gut

The human microbiota encompasses all the microorganisms that reside on the skin and in all other tissues and organs, including the gastrointestinal tracts. Of these body sites, the gastrointestinal tract is the most densely colonized organ. The microbiome includes bacteria, fungi, and archaea (275). There are approximately 1,000 species of microbes colonizing the gut, with densities of $10^4$ to $10^5$ bacteria per millimeter of digestive effluent in the proximal small intestine and $10^{11}$ bacteria per gram of luminal content in the colon (276). The physicochemical conditions in the gut influence the composition of the intestinal microbiota (277). The gastrointestinal tract harbors many distinct niches, each containing a different microbial ecosystem that varies according to the location within the gastrointestinal tract. The microbial density increases along the gastrointestinal tract with $10^1$ to $10^4$ microbial cells in the stomach and duodenum, $10^4$ to $10^8$ cells in the jejunum and ileum, to $10^{10}$ to $10^{12}$ cells per gram in the colon and feces (277–279).

The majority of all microorganisms in the human digestive tract are bacteria and belong to two phyla, the *Bacteroidetes* and the *Firmicutes* (280). In addition, the other significant phyla occupying the digestive tract include *Proteobacteria, Actinobacteria, Fusobacteria, Spirochaetes, Verrucomicrobia*, and *Lentisphaerae* (281, 282). The methanogens *Methanobrevibacter* and *Methanosphaera* are the most dominant archaeal groups (7, 283). The two common fungal phyla in the gut include *Ascomycota* (which includes the genera *Candida* and *Saccharomyces*) and *Basidiomycota* (30, 284).

Intestinal microbiota play a central role in the metabolic, nutritional, physiologic, and immunological processes of the human body, processing indigestible dietary polysaccharides, including resistant starch and dietary fibers, thereby leading to the production of important nutrients, such as SCFAs, vitamins (vitamin K, vitamin B12, folic acid), and certain amino acids that humans are unable to synthesize themselves (279, 285, 286). The plant polysaccharides in our diet are rich in xylan-, pectin-, and arabinose-containing carbohydrate structures. The human genome lacks most of the enzymes required for degrading these glycans. Nevertheless, the distal gut microbiome provides us with this capacity to process these polysaccharides. The human gut microbiome is enriched for genes involved in glucose, galactose, fructose, arabinose, mannose, and xylose, starch, and sucrose metabolism. Our microbiome also has significantly enriched metabolism of glycans, amino acids, and xenobiotics, methanogenesis, and 2-methyl-D-erythritol 4-phosphate pathway–mediated biosynthesis of vitamins and isoprenoids (7). The intestinal microbiota also participates in the defense against pathogens by mechanisms such as colonization resistance and production of antimicrobial compounds. Furthermore, the intestinal microbiota is involved in the development, maturation, and maintenance of the gastrointestinal sensory and motoric functions, the intestinal barrier, and the mucosal immune

system (279). The microbiota of the intestine is also involved in promoting bone formation as well as resorption, leading to skeletal growth. Microbiota induces the hormone insulin-like growth factor 1 (IGF-1), promoting bone growth and remodeling. When the microbiota ferment fiber, SCFAs are produced, leading to induction of IGF-1 that promote bone growth (287).

The very high microbial content of the large intestine poses a major challenge to the mucosal immune system, as it needs to tolerate commensal microbiota and dietary antigens while maintaining the ability to eliminate pathogens. Induction of colonic Treg is crucial in fostering this immune homeostasis (288). $CD4^+CD25^+FOXP3^+$ Tregs are of two types: thymus-derived Tregs (tTreg) and peripherally derived Tregs (pTreg). Although it is difficult to distinguish these types phenotypically, both are thought to have an essential role in immune regulation (288). Although tTregs develop in the thymus, the major site for pTreg development is the colon, resulting in a large population of regulatory cells that have a distinct T-cell receptor (TCR) repertoire and are critical for intestinal homeostasis (152). Notably, the development of pTregs requires microbiota to be present in the colon. Although the mechanism of induction of colonic pTregs is not understood, several microbial components have been found to enhance their expansion and function, including SCFAs and polysaccharide A of *Bacteroides fragilis* (*B. fragilis*; ref. 288). Acetate, propionate, and butyrate are the three main SCFAs, and butyrate has been found to be the most potent inducer of colonic Tregs.

The newborn infant is colonized at birth with microbes from the mother's vaginal and fecal microbiota as well as with other environmental microbes encountered in the first days of life (289). The mode of delivery influences the microbial composition in man. An article by Penders and colleagues (93) demonstrated that the important determinants of the gut microbiome composition in infants were the mode of delivery, type of infant feeding, gestational age, infant hospitalization and antibiotic use in the infant. Term infants born vaginally and exclusively breastfed had the most "beneficial" gut microbiota (had the highest numbers of *Bifidobacteria* and lowest numbers of *C. difficile* and *E. coli*). In contrast, infants born through C-section had lower numbers of *Bifidobacteria* and *Bacteroides*, and they were more often colonized with *C. difficile*, as compared with vaginally born infants. Exclusively formula-fed infants were more often colonized with *E. coli, C. difficile, Bacteroides*, and *Lactobacilli*, as compared with breastfed infants. Hospitalization and prematurity were associated with higher prevalence and counts of *C. difficile*. Administration of antibiotics to infants was associated with decreased numbers of *Bifidobacteria* and *Bacteroides*. Infants with older siblings had slightly higher numbers of *Bifidobacteria*, compared with infants without siblings (93, 290).

*C. difficile* is an opportunistic, anaerobic Gram-positive, spore-forming, toxin-producing bacillus that is transmitted among humans through the fecal–oral route. Notably, the pathogen is generally present in the human gut, but it does not cause any disease under normal conditions. Abuse/misuse of antibiotics destroys beneficial microbiota that enables the proliferation of *C. difficile*, leading to pathogenic conditions. Ampicillin, amoxicillin, cephalosporins, clindamycin, and fluoroquinolones are the antibiotics most frequently associated with disease, but almost all antibiotics have been associated with increased rates of opportunistic infection. *C. difficile* colonizes the large intestine and releases the exotoxins TcdA and TcdB that cause colitis in susceptible persons (291, 292). *C. difficile* has emerged as a major enteric pathogen with worldwide distribution. In the United States, *C. difficile* is the most frequently reported nosocomial (i.e., hospital-acquired) pathogen. A recent surveillance study identified 453,000 cases of *C. difficile* infection and 29,000 deaths associated with *C. difficile* infection; approximately, a quarter of those infections were community acquired (293). The antibiotics prescribed to control *C. difficile* include metronidazole, vancomycin, fidaxomicin, or surgery in extreme cases of infection (294). Fecal transplant is emerging as an alternative strategy for treating recurrent *C. difficile* infections (295). Use of probiotics (such as beneficial bacteria and yeast), which help restore a healthy balance to the intestinal tract, is safe and effective for preventing *C. difficile*–associated diarrhea (296). Thus, beneficial microorganisms are essential to maintain the human gut immune homeostasis, thereby preventing pathogenic infections. Further contributing to such defenses, beneficial microorganisms also modulate epithelial cell proliferation, villus architecture, and angiogenesis within the intestine, along with xenobiotic metabolism, bone mineral density, behavior, and key metabolic functions (297, 298). Tipping the balance favoring the expansion of enterobacteria is one of the causes of several inflammatory bowel diseases. However, it is not known how the favorable bacteria prevent dysbiosis. A recent study demonstrated that microcins, the small proteins secreted by several favorable bacteria could limit the expansion of competing *Enterobacteriaceae* (299).

## Microbiome Effects on Intestinal Barrier Function and Inflammatory Bowel Disease

Mammals, including humans, support one of the most complex microbial ecosystems. Although the immune system is classically thought to have evolved to provide protection from infection by microbial pathogens, animals peacefully coexist with a vast and complex microbiota, which extensively interacts with the immune system. It has recently been proposed that the total information encoded by the mammalian genome is not sufficient to carry out all functions that are required to maintain health and that products of our microbiome are crucial for protection from various diseases (300). It is possible that alterations in the development or composition of the microbiota (dysbiosis) disturb the partnership between the microbiota and the human immune system, ultimately leading to altered immune responses that may underlie various human inflammatory disorders (146).

In inflammatory bowel disease (IBD), the role and interplay of the microbiome (301–303) with a gastrointestinal barrier compromise (304–306) has been the subject of extensive review (307–311). Gastrointestinal barrier function is not governed solely by the tight junctional complex, although this focus has certainly attracted the greatest basic research interest. Tight junctions form the continuous intercellular barrier around epithelial cells, which are required to separate tissue spaces and regulate selective movement of solutes across the epithelium (312). From a wider perspective, gastrointestinal barrier function also may be compromised by an impaired mucus layer over the epithelium [a topic reviewed nicely by others recently (Chen and colleagues; ref. 313)], as well as by cell death in the epithelium or an epithelial–mesenchymal transition leading to impaired cell adhesion/attachment (to other cells and substratum). Leak at the sites of compromised tight junctions may be quite distinct in nature from the leak at the sites of cell death (314). Likewise, the remediation of leak is very different in these cases; repair of leak from impaired tight junctions may be a purely transcription/ translation/ phosphorylation–based process, whereas remediation of leak due

to cell death/dedifferentiation/detachment could also require a careful orchestration of cell motility and cell replication. In these different cases, the microbiome may exert control over very different processes. It is worth considering that any given specific case of IBD likely involves gastrointestinal leak from all of these causes, and therefore alleviation of such leak, and the inflammatory cascades it gives rise to, is a quite complex task. Further research is required to determine whether microbiome may be better at repair of one type of leak than the other.

There is no doubt that IBD is in part driven by a breakdown or compromise of the gastrointestinal epithelial barrier, and many reviews on IBD have dealt with this feature as mentioned above. There is some controversy concerning whether a compromise of barrier function is the initial causation of the disease. The findings that asymptomatic, first-degree relatives of IBD patients in fact harbor molecular-level leak in their gastrointestinal tract mucosa have traditionally been powerful evidence tilting the argument toward causality (315, 316). The question of course that leaps to the fore then is what induces the leak in the first place? The very fact that a genetic element exists in IBD (e.g., in first-degree relatives) indicates a role for genetics in the disease, but equally obvious is that genetics is probably a necessary but insufficient condition.

Studies focusing on this involvement of gastrointestinal microbiome in IBD take two forms: (i) whether the microbiome is abnormal in IBD and possibly playing a role in etiology; (ii) whether a microbiome modification can be designed as a therapeutic option in the disease. The second possibility appears achievable: Some of the best clinical evidence, on the basis of its very applicability, is the success of "fecal transplant" procedures in achieving therapeutic efficacy in IBD (317, 318). Prior to the recent advent of these protocols, there was the use of butyrate enemas to achieve therapeutic relief (286, 319, 320). The therapeutic efficacy of luminal administration of butyrate is cogent testimony to the positive role played by the normal microbiome in maintaining a functional epithelial barrier in the gastrointestinal tract, as well as to the utility of targeting the microbiome as a viable clinical approach to IBD. Butyrate is a significant metabolite of dietary fiber by the normal gastrointestinal microbiota (321), with butyrate levels in the gastrointestinal lumen being the highest in the body. Butyrate has been found in many recent *in vitro* studies to be highly effective in inducing structural changes to the epithelial tight junction complex, resulting in improved epithelial barrier function (322, 323). A similar literature also exists for the gastrointestinal microbiota metabolite, indole, a product of tryptophan metabolism by commensal bacteria (324, 325). In combination, the fecal transplant and butyrate enema clinical studies, along with the *in vitro* studies of tight junction modification and enhancement by butyrate and indole, provide a very powerful argument of not only maintenance and modification of the gastrointestinal barrier by the microbiota, but also for targeting the microbiota as a viable, effective therapeutic strategy.

Better delineated proof of the ability of the microbiota to both positively and negatively affect the gastrointestinal barrier comes out of animal model and epithelial cell culture studies. For example, the probiotic bacterium, *L. casei,* both strengthened barrier function and decreased proinflammatory cytokine content in BALB/c mice (326). In addition, such treatment also modified the gastrointestinal microbiota overall. The probiotic and commensal bacteria *L. rhamnosus* and *F. prausnitzii* have also improved barrier function, as demonstrated in studies with CACO-2 cell culture models and experimentally induced colitis in C57BL/6 mice (327, 328). The microbiome is also known to be involved in the wound healing of the mucosa of the gut (329). In mice, it has been demonstrated that mucosal injury leads to increase in the expression of formyl peptide receptor 1 (FPR1) and neutrophilic NADPH oxidase (NOX2) that causes depletion of oxygen resulting in the enrichment of anaerobic bacteria. The anaerobic, mucinophilic gut symbiont, *A. muciniphila,* stimulated proliferation and migration of enterocytes adjacent to the colonic wounds mediated through FPR1 and intestinal epithelial cell–specific, NOX1-dependent redox signaling, thereby leading to wound healing of the mucosa. These findings highlight a very important consideration in studies of the microbiota, barrier function, and IBD, namely that not only can microbiota affect barrier function, but modification of barrier function (good and bad) may well affect microbiota composition, a "research road less traveled." This less investigated area is well illustrated by the finding that anti-TNF immunologic medications, which reduce proinflammatory cytokines and allow for barrier repair, also result in changes in microbiota composition (330).

It is worth noting too that a factor as omnipresent as diet can affect both barrier function and microbiota while also simultaneously affecting cytokine production. Administering a high-fat "Western" diet to CEABAC10 mice induced deleterious changes in gastrointestinal microbiota (e.g., increased content of adherent-invasive *E. coli*), decreased mucus layer protection, and led to gastrointestinal barrier compromise (331). Pathogenic *E. coli* have been implicated in Crohn disease, in part due to an ability to not produce cell death while inducing synthesis of copious amounts of TNF (332). The dietary connection to an altered microbiota in terms of pathogenic bacteria is also apparent in the finding that vitamin D deficiency enables the barrier-disruptive effects of pathogenic *E. coli* to be manifested (333). Even more fascinating and less intuitive is an effect of the environment at large on barrier function, microbiota, and inflammatory status, as Kish and colleagues (334) show for particulate air pollutants. It will be instructive to discover in future research whether the principal actions of diet/nutrition/ environment are on microbiota directly and barrier function indirectly, or vice versa.

As more and more studies reveal the intricate interplay between gastrointestinal barrier function, gastrointestinal microbiota, and degree of inflammation, it is worth considering that the effects of microbiota on barrier function, and barrier function on microbiota, will in large degree derive from actions of protein kinases in signaling pathway transduction systems. This has been recently very well reviewed by Yang and Yan (335). One would caution however against taking an aggressively reductionist approach in dealing with the interplay of microbiota, barrier function, and inflammation: The complexity of the gut microbiome in the gastrointestinal tract, the complexity of the signaling pathways known to regulate barrier function, and the complexity of cytokine interactions all suggest strongly that one needs to tread carefully in what may well be overly ambitious undertakings to find and utilize specific molecular mechanisms involved in this intricate relationship. A properly functioning gastrointestinal mucosa is in essence a symphony scored by microbiota "strings," barrier function "woodwinds," and immune regulatory "brass." To search for an all-pivotal kinase or phosphatase responsible for gastrointestinal mucosal homeostasis may be analogous to trying to claim a single

flute or viola as the fulcrum of Beethoven's Ninth, a fool's errand that ignores the extreme complexity and subtlety of the gastrointestinal environment.

Because of the still singular importance of the studies showing epithelial barrier compromise in asymptomatic first-degree relatives of IBD patients to the field of IBD research, it will be very interesting to observe the outcome of studies yet to be performed on the microbiome of first-degree relatives of IBD patients. It is likely that those results could be just as pivotal to the future understanding of IBD as were the now-long-ago studies of May and Hollander on barrier function and IBD (315, 316).

Increased risk of developing IBD may be due to improved hygiene practices. Ramanan and colleagues (336) showed that intestinal helminth infection, caused by parasitic worms, protects IBD-susceptible mice from developing the disease. The infection of parasitic worms increased specific protective species and limited other inflammatory members of the microbiota. People from helminth-endemic regions harbored a similar protective microbiota, and their deworming led to an increase in inflammatory *Bacteroidales*, as observed in the mice. Thus, a changing microbial environment may shape susceptibility to inflammatory disease.

Although we currently know at least a partial membership of the human microbiome, we are yet to fully understand how these microorganisms are contained in the intestine. Anatomy of the colon by light microscope reveals a mucus layer, mucosa, submucosa, gut-associated lymphoid follicle, and muscularis. The intestinal/colonic mucus is an efficient system for protecting the epithelium from bacteria by promoting their clearance and separating them from the epithelial cells, thereby inhibiting inflammation and infection (337). Colonic mucus is produced by the goblet cell. The main mucus component in the intestine is MUC2 mucin, a large and heavily *O*-glycosylated gel-forming mucin that forms enormous polymeric nets by C-terminal dimerization and N-terminal trimerization. Upon secretion from the goblet cells, the mucus expands rapidly and builds a stratified dense layer that is attached to the epithelium. Normal human sigmoid colon has an inner mucus layer that is impenetrable to bacteria. At a distance far from the epithelial surface, the inner mucus is transformed into a soluble and less organized outer mucus layer that by proteolytic expansion generates the preferred habitat for the commensal microbes (338).

IBDs are characterized by aberrant innate and adaptive immune responses to commensal luminal bacteria (339). Ulcerative colitis is thought to be caused by some strains of *E. coli* (340). In cell culture models, it has been shown that ulcerative colitis-associated *E. coli* producing α-hemolysin can cause rapid loss of tight junction integrity (341). The human intestinal epithelium is formed by a single layer of epithelial cells that separates the intestinal lumen from the underlying lamina propria and the space between these cells is sealed by the tight junction, which regulates the permeability of the intestinal barrier (342). Tight junction complexes allow passive absorption of small hydrophilic molecules (nutrients and ions), but they restrict passage of large molecules and infectious microbes. Ulcerative colitis is characterized by a leaky intestinal barrier due in part to defective tight junction. Our group had reported that attenuation of the *Bin1* gene in a mouse model would protect against experimental colitis (343). On the basis of the study, we recently demonstrated that treatment of experimental colitis with Bin1 mAb would support mucosal barrier function by inducing the expression of tight junction proteins, thereby protecting the integrity of the lymphoid follicle. The therapy may be a novel strategy to treat ulcerative colitis and possibly limit risks of colorectal cancer (344). Thus, lowering Bin1 levels may be a strategy that would lead to enhanced tight junction proteins that, in turn, protects against pathogenic microorganisms crossing the epithelial cells.

Other strategies may be used by the epithelial cells to protect against pathogenic microbes from entering the tissues. Recently, while working on subconfluent CACO-2 cells (derived from the intestine), we observed that EEA1 endosomes (early endosome marker) were confined more toward the periphery of CACO-2 cell monolayers. To confirm whether these endosomes are present in the colon tissues, we stained for EEA1 in mouse colonic tissues. We observed EEA1 endosomes in the peripheral mucosa as well as in the muscularis. Insofar, as endosomes are traditionally thought to protect against foreign bodies, including microorganisms, one might speculate that early endosomes help protect against bacteria crossing the mucus. Microbes that overcome this barrier to cross the mucus layer might be destroyed within endosomes, with further protection afforded by the endosomes lining the muscularis (Fig. 1).

Additional endosomal strategies may be used to protect colonic tissues against the entry of pathogenic microbes. Endosomes are traditionally thought to protect colonic epithelial cell layers against microorganisms, possibly helping eliminate bacteria that

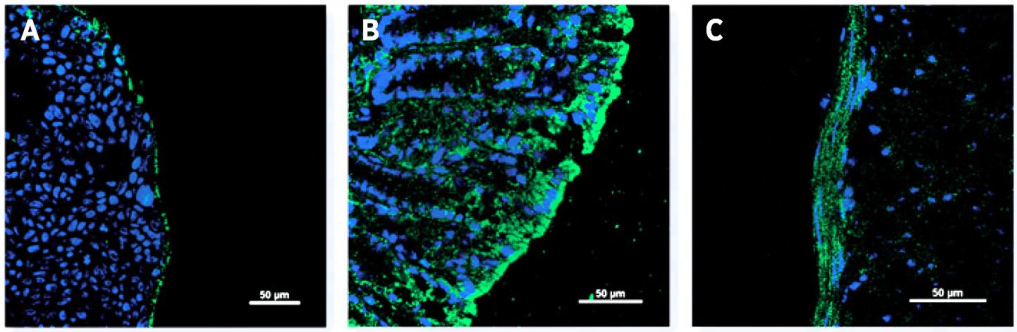




**Figure 1.** Early endosomes localize mainly to the periphery of colon mucosa and muscularis. **A,** Peripheral localization of EEA1 in a colony of human colonic Caco-2 cells. Cells were stained with the early endosomal marker EEA1 (green) and DAPI to visualize cell nuclei (blue) and processed for immunofluorescence microscopy. **B,** Peripheral localization of EEA1 in murine colon mucosa processed as above. **C**, Peripheral localization of EEA1 in murine colon muscularis processed as above.

cross the mucus layer, a strategy that also may be extended into the subordinate muscularis layer (Fig. 1). In subconfluent human colonic cells, which form island-like colonies in monolayer culture, the early endosome marker EEA1 can be seen to preferentially localize to the colony periphery. Similarly, in colon tissues, early endosomes display the same localization with EEA1 staining in the periphery of the mucosa and muscularis. Beclin1-dependent autophagy associated with the endosome pathway also has been implicated in the bacterial and viral pathogen elimination (345), which downregulates Beclin1 to promote virulence and infection. Beclin1 associates with endosomes and regulates EEA1/early endosome localization and late endosome formation (346). Upon TLR signaling, Beclin1 rapidly translocates to the phagosome and mediates efficient phagosome–lysosome fusion to ensure rapid acidification and efficient destruction of the pathogen (345). In human colonic cells, Beclin1 staining occurs throughout the colon tissue at endosomes (Fig. 2), possibly helping direct pathogenic cargo to lysosomes and thereby restricting microbiome ecology to the gut lumen.

## IDO1 and the Microbiome–Host Interaction

As noted above, establishing and maintaining the symbiotic mutualism that exists between the microbiome and its mammalian host necessitates the engagement of mechanisms of acquired immune tolerance, as these microorganisms represent the epitome of non-self. IDO1 is a metabolic enzyme that has gained recognition as an important mediator of acquired immune tolerance. IDO1 catalyzes the rate-limiting first step in the degradation of the essential amino acid tryptophan, but is not involved in maintaining tryptophan homeostasis, which instead is the role of the distinct liver enzyme TDO2 (tryptophan dioxygenase; ref. 347). The concept of IDO1 as an immune regulator emerged from findings that tryptophan catabolism could suppress cytotoxic T-cell activation (348, 349). The demonstration that the IDO pathway inhibitor 1-methyl-tryptophan (1MT) could elicit T-cell– dependent rejection of allogeneic mouse concepti (350, 351) established the physiologic relevance of tryptophan catabolism as a mediator of acquired immune tolerance. Subsequent findings linking attenuation of the tumor suppressor gene *Bin1* to IDO1 dysregulation and tumoral immune escape (352) provided experimental substantiation for the corollary proposition that tumor cells might, by inducing IDO1, appropriate this mechanism of protection for the "foreign" fetus to overcome immunosurveillance. Within the complex inflammatory milieu of the tumor microenvironment, IDO1 induction is not necessarily restricted to tumor cells, and nonmalignant stromal cells expressing IDO1 can promote tumoral immune escape as well (353). In particular, IDO1 induction in antigen-presenting cells (APC), such as DCs and macrophages, has been implicated in promoting immune tolerance by suppressing effector cytotoxic T lymphocytes, converting naïve T lymphocytes to FoxP3$^+$ Treg cells and elevating the suppressive activity of "natural" Tregs (354).

A great deal of attention is now focused on the therapeutic potential of small-molecule inhibitors of IDO1 for treating cancer patients (355), particularly in combination with cancer chemotherapy or "immune checkpoint" antibodies (352, 356). It is not yet clear how IDO1 may influence host interactions with the microbiome, but there has been much attention to the related topic of its role in the host response to infection by various pathogens, which has been a topic of interest for a number of years. Indeed, well prior to findings of its involvement in immune modulation, it was noted that intraperitoneal administration of bacterial LPS could induce IDO1 activity in the lungs of mice by 30- to 50-fold (357). This initial indication that IDO1 induction might be associated with the inflammatory response to microbial infection was followed by reports of pulmonary IDO1 induction in response to virus infection (358) and IFNγ (359).

As IFNγ plays a major role in controlling a variety of infections, the finding that IDO1 is highly responsive to IFNγ spurred investigations addressing whether IDO1 might have a downstream antimicrobial effector role. In 1984, Pfefferkorn and colleagues reported that tryptophan degradation was responsible for the IFNγ-mediated restriction of the growth of the obligate intracellular protozoan *Toxoplasma gondii* (*T. gondii*) in human fibroblasts (359). IFNγ-mediated restriction of the growth of the obligate, intracellular, Gram-negative bacterium *Chlamydophila psittaci* was likewise linked to tryptophan deprivation (360). These studies focused attention on IFNγ-elicited tryptophan deprivation resulting from the induction of IDO1 as mediating the antiproliferative effect on these intracellular pathogens. This assessment, that IDO1 provides a beneficial effect in combating infections, was complicated by additional studies demonstrating the activation of genes for *Chlamydia* persistence triggered by IDO1-mediated

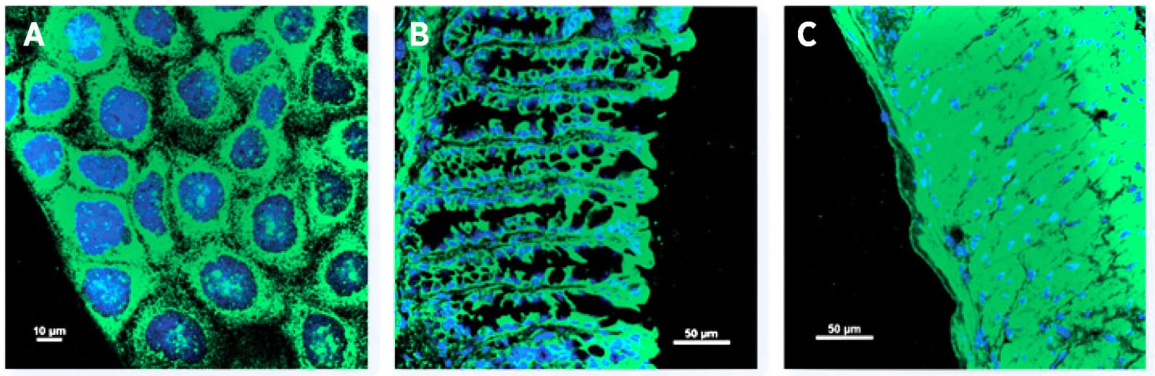




**Figure 2.** Late endosomes locate throughout colon mucosa and muscularis. **A,** Punctate cytosolic localization in a colony of human colonic Caco-2 cells. Cells were stained with Beclin1, an autophagic regulator associated with late endosomes (green), and DAPI to visualize cell nuclei (blue) and processed for immunofluorescence microscopy. **B,** Cytosolic localization of Beclin1 in murine colon mucosa processed as above. **C,** Cytosolic localization of Beclin1 in murine colon muscularis processed as above.

tryptophan depletion. Because the bacterium is sensitive to the antibiotic treatment only when it is metabolically active, IDO1 activity in this context was detrimental to clearing infections, leading to the suggestion that tryptophan supplementation might help overcome antibiotic resistance (361). Furthermore, although the effects of IDO1 on microorganisms were initially attributed to depletion of tryptophan, evidence of microbial effects produced by downstream tryptophan metabolites, from what is collectively referred to as the kynurenine pathway, were also reported (362, 363). In particular, 3-hydroxykynurenine was shown to suppress the proliferation of *S. aureus* in vascular allografts (364), and both 3-hydroxykynurenine and 3-hydroxyanthranilic acid were found to contribute to controlling the replication of *Trypanosoma cruzi* in mice (365). In a single report, treatment with 1MT produced three different outcomes depending on the nature of the infection, exacerbating *T. gondii* toxoplasmosis, restraining *L. major* leishmaniasis, and having no apparent effect on HSV-1 replication or latency (366). In aggregate, the implication from these studies of infectious pathogens is that the overall impact of IDO1 activity, both in terms of tryptophan depletion and the production of various metabolites, on the diverse ecology of the commensal microbiome is likely to be complex and contextual.

Perhaps even more consequential than the direct effects of IDO1 activity on particular microbes are the effects that IDO1 can exert on the overall inflammatory environment. In accord with its ability to elicit T-cell suppression, the general assumption has been that IDO1 should act in an immunosuppressive manner to limit the severity of inflammation. Data supporting this interpretation have been reported in a mouse model of chronic granulomatous disease in which defective IDO1 function in mice lacking an essential component of NADPH oxidase, $p47^{phox}$, was implicated in the exaggerated inflammatory response to infection with *Aspergillus fumigatus* (367). The more severe illness was associated with higher numbers of IL17-producing γδT cells and fewer IL10-producing αβTregs, which could be reversed by the provision of exogenous kynurenine. However, as with the effects of tryptophan catabolism on microorganisms, the categorization of IDO1 as strictly immunosuppressive may be an oversimplification (368). In a mouse model of chemical carcinogenesis, genetic loss of IDO1 did not exacerbate inflammation in response to phorbol ester–elicited tumor promotion, as would be expected if it were broadly immunosuppressive, but did result in a dramatically reduced incidence of premalignant lesions (369). Perhaps even more strikingly, in a mouse model of rheumatoid arthritis, 1MT treatment suppressed rather than exacerbated joint inflammation (370), whereas in a contact hypersensitivity model, genetic loss of IDO1 resulted in diminished ear swelling (371). Why IDO1 has such varied effects on the inflammatory response remains to be fully elucidated, but suggests that the outcome of the interactions with the complex microbiome may be contextual and difficult to predict.

The study in the rheumatoid arthritis model noted above also highlights a particular complication with interpreting results of the many studies that have relied on the use of the compound 1MT to inhibit IDO1 activity. Biochemical and pharmacologic evaluation of this compound clearly indicates that it is not directly inhibiting the enzyme at the dose ranges administered *in vivo*, and it is able to signal as a mimetic for tryptophan sufficiency and thereby interfere with activation of downstream response pathways (372). However, a tryptophan deficiency signal can be provided by any of the enzymes that catalyze tryptophan degradation (IDO1, IDO2, TDO2, as well as TPH, the latter of which initiates an alternate pathway of tryptophan catabolism to serotonin). Therefore, 1MT is not a valid tool to discriminate which of these particular enzymes is involved. Indeed, in the rheumatoid arthritis model, genetic analysis revealed that the recently identified paralog IDO2, and not IDO1, is likely to be responsible for the effect of 1MT on joint inflammation, as the effect of 1MT administration was phenocopied in mice lacking IDO2 but not IDO1 (370, 373). Studies using later generation, direct enzyme inhibitors (355), coupled with studies in genetically modified animals (371, 374, 375) can overcome ambiguities in data interpretation associated with 1MT treatment. Indeed, a recent study of LPS responses utilizing the *IDO1, IDO2*, and *Tdo2* gene deletion mouse strains provides confirmatory evidence that these three genes have distinct, nonoverlapping roles in the host immune response to this microbial signal (376).

Although the regional microbiome present at all barrier surfaces is likely to influence immunity locally, the microbiome of the gastrointestinal tract is of particular interest because of the broader role it has been found to play in shaping systemic immune homeostasis (377). Correspondingly, current microbiome research is largely focused on the gastrointestinal tract, where commensal microorganisms have been found to contribute to host defense by limiting the growth of enteric pathogens and producing symbiosis factors that control intestinal inflammation and pathology (377). Evidence implicating IDO1 in this process has come from a study of the protective capacity of *L. salivarius*, which is abolished in mice lacking the gene encoding NOD2, an intracellular pattern recognition molecule that regulates inflammatory pathways in response to detection of bacterial peptidoglycans. In this model, IDO1 upregulation was found to correlate with NOD2-dependent protection (378). The increased regulatory complexity imposed by the gut microbiome may help also explain counterintuitive findings associated with IDO1 in this tissue. In a dextran sodium sulfate/1,2-dimethylhydrazine–elicited model of colon carcinogenesis, genetic loss of IDO1 resulted in increased tumor frequency (379), unlike other organ systems in which IDO1 loss has been associated with resistance to carcinoma development (380, 381). This outcome is similar to the atypical impact on tumor formation ascribed to Tregs in the gut, where their presence appears to be protective against carcinogenesis (382), despite evidence that Tregs are generally associated in other organs with tumor promotion. It has been proposed that the effect of immunosuppressive mechanisms on inflammatory pathology of the gut may be quite different depending on whether there is any initial involvement of tissue damage, as the resulting microbial translocation produces a severe tumor-promoting inflammation (383). Under these circumstances, dampening the inflammatory response via immunosuppressive mechanisms may provide a more consequential benefit that overshadows any detrimental role in promoting immune escape. This interpretation is consistent with the findings of two otherwise apparently contradictory studies in colitis models, where IDO1 blockade resulted in augmented colitis induced by trinitrobenzene sulfonic acid (384), but diminished colitis induced by *Citrobacter rodentium* (385).

In conjunction with the complex immunoregulatory effects attributed to IDO1 activity in the gut, tryptophan metabolites produced by the microbiota affect mucosal reactivity. When

switching from sugar to tryptophan as an energy source, the highly adaptive Lactobacilli in the gut expand and produce an aryl hydrocarbon receptor (AhR) ligand, indole-3-aldehyde, that contributes to AhR-dependent IL22 transcription. The resulting IL22- dependent mucosal response promotes the survival of mixed microbial communities, while providing colonization resistance to the fungus *Candida albicans* and mucosal protection from inflammation. This example of coevolutionary commensalism through the microbiota–AhR axis represents yet another way in which tryptophan catabolism appears to be involved in fine tuning host mucosal reactivity (386). As further investigations into the physiologic and pathophysiologic interactions between IDO1, the commensal microbiome and host immunity are conducted, the indications from these early studies are that IDO1 is likely to play an integral but contextual role at the interface between homeostasis and dysbiosis.

## Microbiome and Cancer

The interplay between microbes, cancer, and the immune system is in no manner fully defined. However, accumulating evidence argues provocatively that microbes exert a variety of functions on host oncogenesis, tumor progression, and response to immunotherapy. Thus, selectively manipulating the gut microbiome is a critical parameter to consider in the ongoing battle against established cancers.

The metabolic potential of the gut microbiota is now regarded as vital to the process of malignant transformation. Disruption of the intimate relationship between the host and intestinal bacteria, known as dysbiosis, can affect oncogenesis, tumor progression, and response to cancer therapy. Dysbiosis can occur for several reasons: (i) direct occupancy of unwanted, foreign microbes (as discussed above) that outcompete friendly gut flora; (ii) a response to immunosenescence with aging; and (iii) direct environmental insults such as antibiotics and smoking (387). In the setting of chronic autoimmune processes, such as Crohn's disease and ulcerative colitis, the integrity of gut epithelial, myeloid, and lymphoid components is disrupted (Fig. 3). These chronic insults ultimately increase host's risk for neoplastic transformation (388). Indeed, several factors that favor carcinogenesis similarly recapitulate dysbiosis.

One well-studied model of the dysbiosis/cancer connection is that of repeated intra-abdominal infections, the use of antibiotics, or both leading to an increased incidence of colorectal cancer (389). In several preclinical studies, interventions that abrogate or directly alter gut microbiome composition increase the incidence and progression of colorectal carcinoma in both genetic and carcinogen-induced models of tumorigenesis (390–392). Moreover, several byproducts of the gut microbiota directly target intestinal epithelial cells and either facilitate oncogenesis (as reported for hydrogen sulfide and the *B. fragilis* toxin) or suppress tumorigenesis (in the case of SCFAs; ref. 393). Intestinal microbes have been characterized to participate in more than just colorectal carcinogenesis. Experimental models of gut flora also elucidate the development of other extraintestinal cancers, such as hepatocellular carcinoma (394, 395), presumably through systemically disseminated metabolic networks. *Helicobacter pylori* (*H. pylori*) is a Gram-negative bacterial pathogen that selectively colonizes the gastric epithelium. It is postulated that half of the world's population is infected with *H. pylori*, although colonization of the pathogenic bacteria does not cause any symptoms in a majority of the population. Nevertheless, long-term carriage of *H. pylori* significantly increases the risk of developing diseases. Among infected individuals, approximately 10% develop peptic ulcer disease, 1% to 3% develop gastric adenocarcinoma, and <0.1% develop mucosa-associated lymphoid tissue (MALT) lymphoma. However, at initial stages, gastric MALT lymphoma can be cured completely by the eradication of *H. pylori* with antibiotics (396).

Apart from antibiotics, probiotics may also inhibit tumorigenesis and cancer progression. Konishi and colleagues (397) reported that the culture supernatant of *L. casei* has tumor-suppressive effect on colon cancer cells. The authors reported that ferrichrome produced by *L. casei* is the molecule that provides tumor protection and is exerted via the JNK signaling pathway.

The etiology of breast cancer is still not understood, although it is believed the disease is due to a combination of both genetic and environmental factors. It is posited that environmental factors influence breast cancer, as there is an increased incidence of breast cancer among migrants and their descendants after they move from a region of low breast cancer risk to a region of high risk. Bacterial communities within the host could be one such environmental factor that may influence breast cancer development. Different bacterial profiles in breast tissue exist between healthy women and those with breast cancer. Breast cancer patients had higher levels of *Bacillus, Enterobacteriaceae*, and *Staphylococcus*. *E.coli* and *Staphylococcus epidermidis* isolated from breast cancer patients induced DNA double-stranded breaks in HeLa cells. There was also a decrease in some lactic acid bacteria, known for their beneficial health effects, including anticarcinogenic properties (398). It has been demonstrated that women who drink fermented milk products have a reduced risk of breast cancer development (399). Oral administration of *Lactobacillus* species has been shown to be protective in animal models of breast cancer (400).

Case studies back to the 1700s have recounted the development of bacterial infections in cancer patients that led to remissions of their malignant disease. One of the pioneers in this field, the U.S. surgeon William B. Coley, engaged in a lifelong study of this phenomenon after the loss of his very first patient in the late 1800s to a rapidly invasive sarcoma (401). Searching the literature available, Coley discovered records of another sarcoma patient with relentless sarcomatous recurrences following surgical resection and an ultimate wound infection (erysipelas) with *Streptococcus pyogenes* (*S. pyogenes*) and high fever. To his surprise, after each attack of fever, the ulcer improved, the sarcoma shrank, and the lesion ultimately regressed completely. Coley suspected that in some manner, the infection had induced tumor regression and began a series of trials to "cure" his cancer patients with pathogen inoculation. He infected his next 10 patients, but observed intrinsic variability in efficacy using this method (402). Because of this unpredictability, he elected to create a formulation containing two killed bacteria: *S. pyogenes* and *Serratia marcescens*. Under the form of an inactivated vaccine, he could simulate an infection (inflammation, chills, fever) without the actual risks of a life-threatening disease. This vaccine became known as ``Coley toxins." The relative success with Coley's vaccine was by no means limited to sarcomas. For decades, this vaccine form had been used by other contemporaries for carcinomas, lymphomas, melanomas, and myelomas (403, 404).

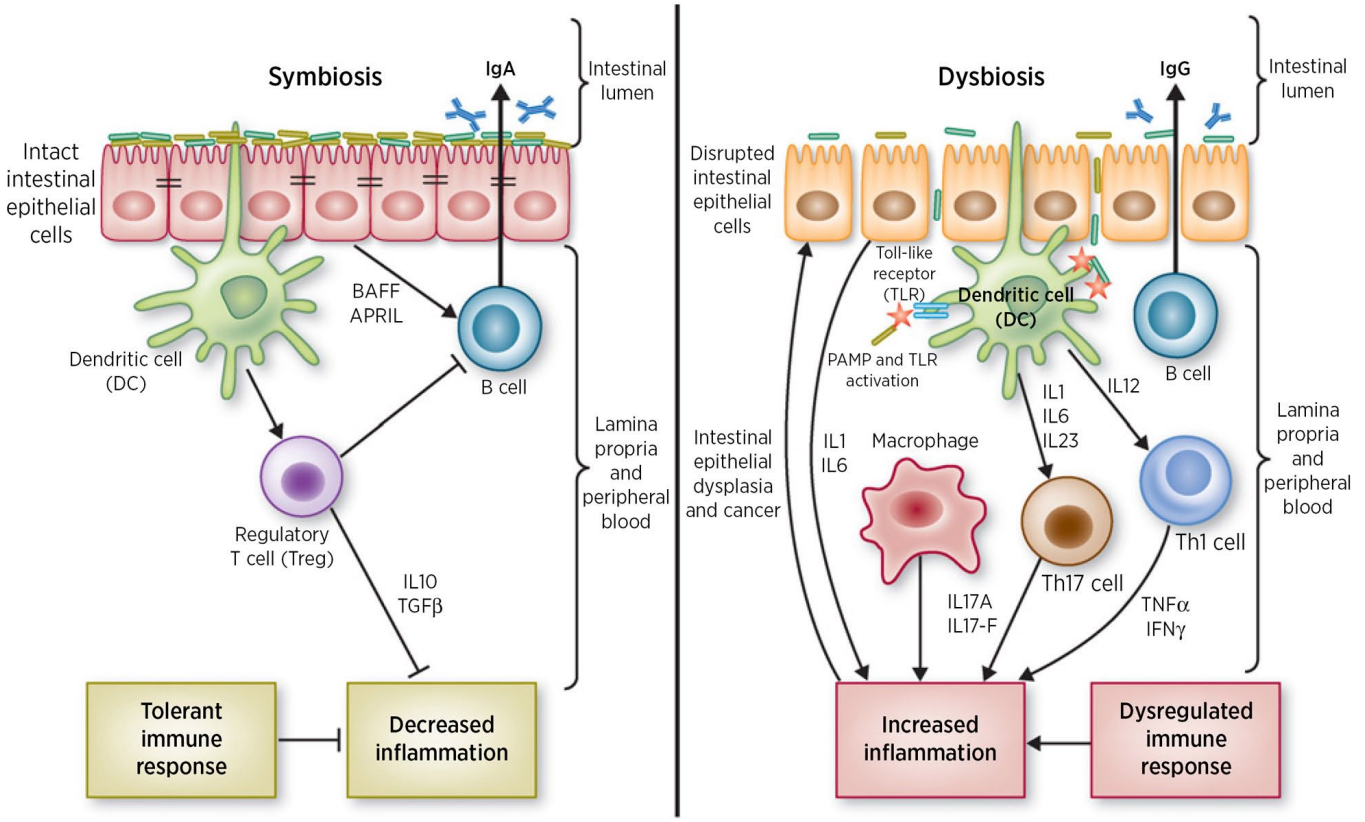




**Figure 3.** Dysbiosis: an immunocompromised state characterized by pathobiont colonization that leads to hyperinflammation, dysplasia, and tumorigenesis. Symbiosis (left): a symbiotic gut microbiota operates under a functional intestinal epithelial cell barrier, with steady-state proportions of mucus, pattern recognition receptors, antimicrobial peptides, and secretory IgA, which in turn contain the microbiota in the intestinal lumen. Under tight control by intestinal epithelial cells, the intestinal immune system within the gut lamina propria becomes largely tolerant to the resident commensals. Signaling cascades that occur downstream of TLRs are used by intestinal epithelial cells to detect microbes through pattern recognition receptors. Upon LPS stimulation of TLRs, the MYD88 protein is recruited, activating the NF-kB pathway, leading to production of antimicrobial proteins and proinflammatory cytokines. In a symbiotic gut, intestinal epithelial cells are desensitized by repeated exposure to LPS or are attenuated by LPS-mediated downregulation of the IL1 receptor–associated kinase 1 (IRAK1), an activator of the NF-kB cascade. Exposure to LPS induces epithelial cells to secrete TGFβ, B-cell–activating factor of the TNF family (BAFF), and a proliferation-inducing ligand (APRIL), all of which promote the development of tolerogenic responses to the microbiota. CD103+ DCs support the development of Tregs to secrete IL10 and TGFβ, and together, they stimulate the production of commensal-specific IgA. Dysbiosis (right): increased intestinal exposure of diverse PAMPs, proinflammatory cytokines, apoptotic debris, and toxins leads to microbial dysbiosis and overgrowth of "pathobionts," transformed symbiotic bacteria now under pathologic conditions. Pathobiont overgrowth leads to the loss of barrier integrity and a breach in the intestinal epithelial cell barrier. Translocation of bacteria and bacterial components triggers the intestinal immune system through TLR activation, resulting in potentially harmful effector T-cell responses set to clear invading bacteria. Ultimately, the secretion of IL1 and IL6 from intestinal epithelial cells fuels a Th1 and Th17 response by DCs and macrophages and leads to higher levels of commensal-specific IgG by B cells.

However, with the advent of radiotherapy and chemotherapy, and the empowerment of the FDA in 1964 that restricted clinical use of "Coley toxins," the use of microbial toxins in oncology fell out of use. There were, however, rare instances in which this line of thinking endured and eventually received FDA approval. Perhaps the most prominent example is the use of Bacillus Calmette–Guerin (BCG) for the treatment for superficial bladder cancer (405). BCG is currently the only conventional bacterial vaccine in use for direct tumor killing. Unlike Coley toxins, BCG is not administered with the ultimate goal of induced fever. But similar to Coley's methods, the vaccine is applied directly to the tumor site with repeated courses following initial resection to prevent recurrence (406). After intravesicular administration of this vaccine, a wide range of cytokines become detectable in the urine, including IL1, IL2, IL6, IL8, IL10, IL12, IL18, IFNγ, IFNγ-inducible protein-10, macrophage colony-stimulating factor, and TNFα (407–409). This inflammatory host response illustrates the point that individual immunomodulating cytokines are partial components of a much more complex immunologic response to infection, and correspondingly, tumor regression. Some insights were gleaned from Coley toxins and other historical reports on live or attenuated bacterial inoculations. One was that a local inoculation produces only a local response. Thus, BCG use is limited to superficial bladder cancer. The heat and immune activation associated with local

inflammation are perceived to be a minimized febrile response, and correspondingly, this local response is only effective in the immediate region where it occurs (401). Since Coley's passing, the field of tumor immunology has developed into a better founded and more sophisticated specialty, with investigators not only employing a variety of basic immunologic principles (e.g., antitumor cytokines, cytotoxic T cells, immunostimulatory antibodies, cell-based vaccines) but pivotal insights into the dominance of tumoral immune suppression in blunting the effectiveness of any immunotherapy. Overcoming this historical source of failure in the efficacy of cancer immunotherapy is empowering this field anew today.

There are more than a hundred chemotherapy drugs to treat many types of cancers. However, it is not fully understood the mechanism of some of these drugs. Cyclophosphamide is a clinically important chemotherapeutic cancer drug that stimulates antitumor immune responses. Viaud and colleagues (410) demonstrated that cyclophosphamide alters the composition of microbiota in the gut and induces the translocation of several Gram-positive bacteria into mesenteric lymph nodes and spleen. In the lymphoid organs, the Gram-positive bacteria stimulated the generation of pathogenic Th17 (pTh17) cells and memory Th1 immune responses. Germ-free tumor-bearing mice or treated with antibiotics to kill Gram-positive bacteria showed a reduction in T-cell responses, and their tumors were resistant to cyclophosphamide. Adoptive transfer of pTh17 cells restored the antitumor efficacy of cyclophosphamide. Overall, the study suggested that the gut microbiota help shape the anticancer immune response.

## Turning the Tables: Using Engineered Microbes to Attack Cancer

Over the past century, knowledge gained on how selective microbes either facilitate the growth of cancer or alternatively act as tumoricidal agents permits us open access to utilize this double-edged sword to our advantage. Such interventions have already begun and span several modalities, including but not limited to the use of helper peptide sequences from bacterial subunits, bacterial toxin–fusion proteins, oncolytic viral vaccines, and, as recent studies elucidate, leveraging the metabolism of host flora to potentiate immune modulators.

Broadly defined, an immunotherapeutic is any modality that manipulates the immune system for enhanced therapeutic outcome. These include nonspecific activation of the immune system with microbial components or cytokines, antigen-specific adoptive immunotherapy with antibodies or lymphocyte transfers, and active immunotherapy by direct vaccination against tumor-specific proteins, or antigens. We traditionally regard vaccines as educators for the naïve immune system that are administered prophylactically in anticipation of any infection. However, in the setting of aggressive cancers, the use of cellular vaccines to mount reactive immune responses is now being employed with promising results.

A variety of cancer vaccines are currently under investigation, but perhaps the most widely investigated to date are (i) cellular vaccines composed of APC loaded with tumor antigen (411–414) and (ii) peptide vaccines (415, 416). Peptide vaccines are comprised of 8–25 amino acids that encompass an epitope, a recognizable sequence coding for an antigen. The transient nature and low magnitude of responses in many cancer patients has elucidated that tumors themselves are inherently proficient at downplaying immune responses as well as escaping antigen recognition altogether. Thus, there is an urgent need for improving vaccine immunogenicity and for ensuring that cancer antigens are sufficiently immunogenic. To enhance peptide vaccine immunogenicity, these small peptides are often conjugated to a carrier protein, such as keyhole limpet hemocyanin (417, 418) and tetanus toxoid (419, 420). These helper proteins enable recognition by and activation of the immune system with great potency and generate complimentary bystander activation with cytokine release and maturation of effector cell phenotype. Peptide vaccines are appealing in cancer therapy because they are relatively easy to manufacture and store, and they do not require laborious preparations. Because of their "off-the-shelf" feature, repeated boosting for enhanced immune activation also distinguishes peptide cancer vaccines as an expandable modality (421).

Adoptive immunotherapy has also been utilized to eradicate established tumors (422). This process involves *ex vivo* activation of autologous immune cells, isolated from either peripheral blood or intratumoral lymphocytes (423, 424), into lymphocyte- activated killer cells. Lymphocyte-activated killer cells are generated by culturing autologous peripheral lymphocytes with IL2, a vital growth cytokine for generating T cells and natural killer (NK) cells. These killer cells are then returned to the patient intratumorally or intravenously, where they become activated by host APCs and exert their tumoricidal effects. Using the knowledge we have gained from microbial components, NK cells have been potentiated by leveraging the cytolytic capacity of microbial diphtheria toxin. One study demonstrated that haploidentical NK cells for relapsed and refractory acute myeloid leukemia could be augmented and improved with a lymphodepletive platform using diphtheria toxin conjugated to IL2. Using the immunotoxin IL2DT to deplete immunosuppressive Tregs, investigators appreciably improved rates of *in vivo* NK-cell expansion (10% vs. 27%) and AML 28-day remission (53% vs. 21%; $P = 0.02$) compared with the cohort without IL2DT (425).

Oncolytic viruses represent another immunotherapy modality that has gained recent traction in the field of tumor immunology. Use of these microbes was first based on early reports of spontaneous cancer remissions coincident with natural infection or upon the use of live attenuated vaccines (426). Since then, an improved understanding of the molecular basis for viral host cell tropism, cytotoxicity, and cell type specificity has opened up avenues for the very selective design of virally based anticancer strategies. To be efficacious and safe, an oncolytic virus must possess (i) an inherently low human pathogenic potential (i.e., the orphan reovirus; ref. 427); (ii) a veterinary pathogen with unknown human pathogenicity (i.e., vesicular stomatitis virus; ref. 428); or (iii) a human pathogen genetically engineered to selectively kill cancerous cells without collateral cytotoxicity in normal cells (i.e., herpes simplex virus-1; ref. 429). One such group is utilizing a prototype nonpathogenic poliovirus recombinant, known as PVSRIPO. Poliovirus naturally targets the vast majority of ectodermal/ neuroectodermal cancers expressing its cellular receptor, CD155. Evidence from glioblastoma patients suggests that the CD155 receptor is ectopically upregulated on tumor cells. Preclinical studies have shown that treatment of glioma xenografts with intratumoral inoculation of PVS-RIPO produced rampant tumor cell death, potent host-mediated inflammatory reactions against infected tumors, and rapid tumor decline (430, 431). The use of PVS-RIPO is now being evaluated in the phase I setting for recurrent glioblastoma (432).

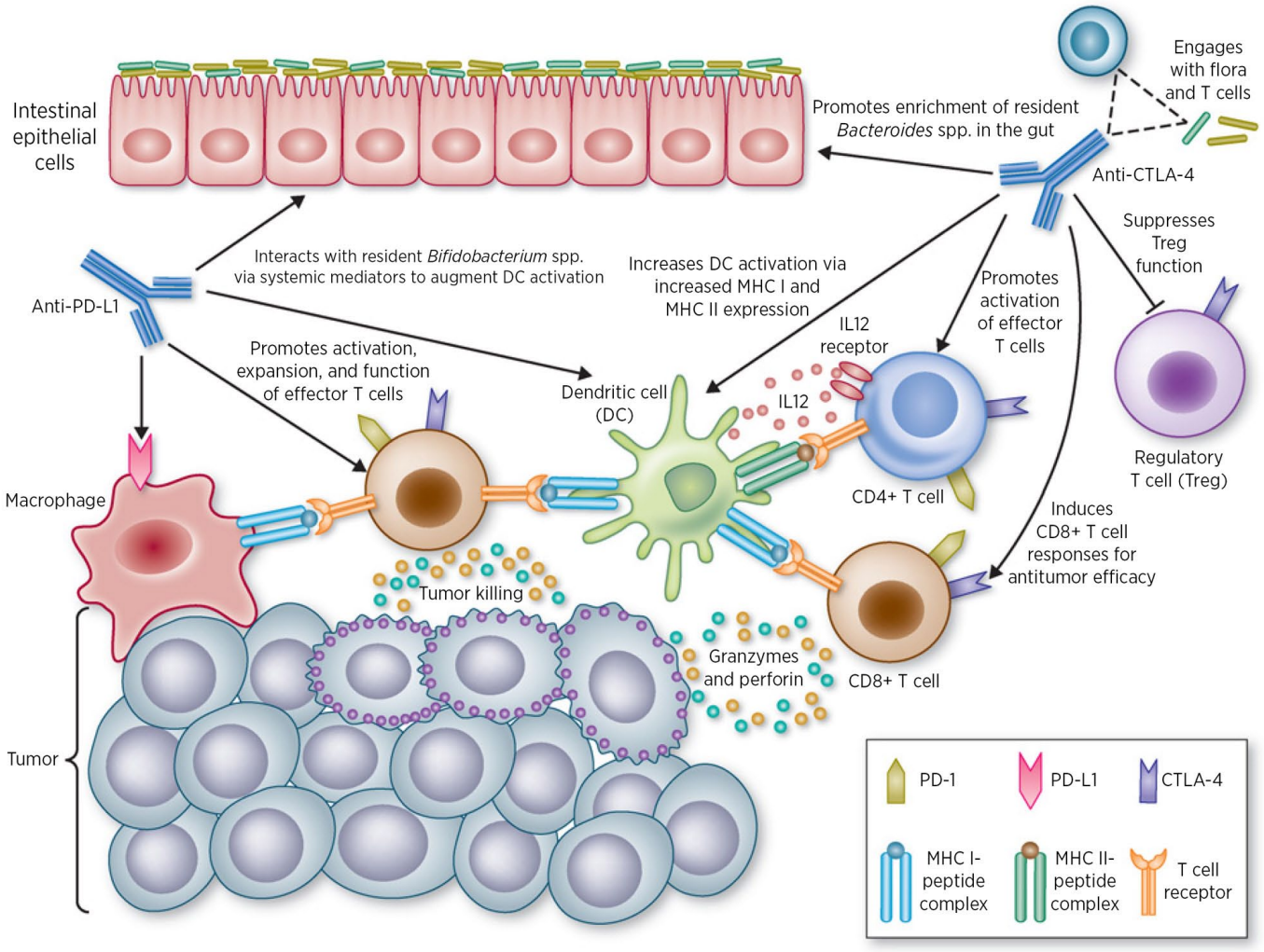




**Figure 4.** Gut microbiome directs the efficacy of immune checkpoint therapy. Both anti-CTLA-4 and anti-PD-L1 therapies rely on gut microbiota for efficacy in immune activation. Anti-PD-L1 therapy has been shown to rely on the preexistence of sufficient Bifidobacterium species, which are also thought to augment responses via PD-L1 binding on APCs, such as DCs and macrophages. Subsequent ligation results in the prevention of suppressive signals to PD-1–expressing T cells. Similarly, anti-CTLA-4 indirectly alters the intestinal flora and enriches the Bacteroides species, possibly by promoting deterioration of the intestinal epithelial cell barrier via activation of local lymphocytes. These bacteria then promote the activation of DCs, which present tumor antigens to prime and maintain antitumor T-cell responses. Anti-CTLA-4 holds additional activation functions, including (i) preventing CTLA-4 from blocking activation of the costimulatory molecule CD28 on T cells and (ii) blocking the immunosuppressive function of Tregs, which are required in the deactivation of immune responses against tumors.

As multiple studies are being carried out yearly, investigators continue to uncover key barriers to efficacy that further clarify our strategies. As with any antigen-specific immune response, several homeostatic mechanisms remain at play to prevent rampant damage to the host or autoimmune toxicity. One of the most exciting regulatory axes to be studied recently is that of programmed death (PD)-1/PD ligand-1 (PD-L1). PD-1 is a coinhibitory receptor that is inducibly expressed by T and B cells upon activation. Antigen-specific T cells expressing the PD- 1 receptor will engage with either of its ligands, PD-L1 or PD-L2 expressed on APC, which elicits an inhibitory cascade and subsequent inhibition of TCR-induced cytokine production and proliferation (433). PDL-1 is expressed in several other cell types, including tumor cells and some epithelial cells, lymphoid cells, and myeloid cells (434). Another axis involved in regulating self-recognition is that of cytotoxic T lymphocyte antigen-4 (CTLA-4). Studies have demonstrated that tumor cells stimulate CTLA-4, promoting a cascade of inhibitory immune processes and ultimate T-cell inactivity against tumors themselves (435, 436).

In cancer immunotherapy, mAbs against the immune checkpoints CTLA-4, PD-1, and its ligand PD-L1 have demonstrated high activity in melanoma and other tumors (437). Ipilimumab,

an anti-CTLA-4 antibody, was the first approved "immune checkpoint inhibitor." Although the response rate with ipilimumab is low (less than 20% of patients have objective responses), many of those positive responses were associated with long-term survival (438), with similar results in the first- and second-line settings. Nivolumab and pembrolizumab, both anti-PD-1 inhibitors, have now also been approved for the treatment of melanoma, with response rates of up to 40% and a demonstrated survival advantage in phase III trials (434).

Strikingly, recent findings in preclinical models of cancer stress the importance of intact gut microbiota for effective immune checkpoint blockade. One recent study found that antitumor effects of CTLA-4 blockade depended on the presence of distinct *Bacteroides* species. In both mice and patients, T-cell responses specific for *B. thetaiotaomicron* or *B. fragilis* were associated with the efficacy of CTLA-4 blockade. Using antibiotic- treated as well as germ-free mice, tumors lacking these strains did not respond to CTLA blockade. This deficiency was rescued by *B. fragilis* gavage, by immunization with *B. fragilis* polysaccharides, or by adoptive transfer of *B. fragilis*–specific T cells. Ultimately, fecal microbial transplantation from humans to mice confirmed that treatment of melanoma patients with antibodies against CTLA-4 favored the outgrowth of *B. fragilis* with anticancer properties (439). A different preclinical study similarly elucidated that mice treated with gut commensals of *Bifidobacterium* displayed significantly improved suppression of melanoma growth in comparison with non- *Bifidobacterium*–treated counterparts. These observed differences in spontaneous antitumor immunity were eliminated upon cohousing or after fecal transfer, eluding to the importance of shared bacterial colonization. Furthermore, administration of the bacteria to *Bifidobacterium*-naïve mice with established melanoma significantly enhanced tumor-specific immunity and response to anti-PD-L1 mAb therapy (440). Although the mechanisms from both these studies are not fully understood, they laid the foundation behind the requirement for an intact microbiome to enact antitumor responses. One key observation from both these studies was that they employed subcutaneous tumor models, meaning that intestinal microbiota exerted antitumor immunity in a systemic fashion. Both studies relied on the presence of $CD8^+$ T cells. Second, both demonstrate that altered DC activation was a responsible intermediate event between the presence of gut microbiota and provision of checkpoint inhibitors (Fig. 4).

## Conclusions

It is humbling to consider how much biomedical research has been conducted since the molecular biology revolution of the past century without appreciation of the importance of microbiomes in health and disease. Like all realms of biomedical investigation, the field of cancer research can no longer ignore the "other half" of the organism; it must become as familiar with the genetics, biology, physiology, and immunologic effects of host microorganisms as with the hosts themselves. In considering sources of experimental irreproducibility in biomedical publications that have been suggested recently to be disturbingly high, it seems likely that natural variations in microbiome infections present in experimental models and vivariums at different sites will provide one more challenge to the exquisitely difficult problem of how one defines a "molecular mechanism" in disease. More focus on practical applications (sought by most funding organizations) along with empirical explorations once traditional to biology may offer two paths forward, as, to paraphrase the pragmatic American philosopher Charles S. Peirce, "You know something if you can do something." How the current obsession with molecular mechanism will change in the face of the challenge the microbiome poses to preclinical research is unclear. Nevertheless, as the molecular biology revolution continues to wash up on the shores of reductionism this century, it will be impossible not to reembrace the roots of traditional biological thought, where ecology, evolution, and a focus on emergent principles in complex organisms can help recenter the pursuit of new knowledge and its applications to improve disease management and healthy lifespans.

### Disclosure of Potential Conflicts of Interest

The Editor-in-Chief of *Cancer Research,* George C. Prendergast, is a co-author of this article. E. Walsh is a scientist at Seres Therapeutics. D.A. Sela has received speakers bureau honoraria Artugen and Premier Medical Group and has ownership interest (including patents) in a patent (2010/0113383 A1). G.C. Prendergast is the Editor-in-Chief (*Cancer Research*) at AACR, reports receiving commercial research grants from Janssen Pharmaceuticals, has ownership interest (including patents) in Dynamis Pharmaceuticals, Man's Best Friend Therapeutics, Meditope Biosciences Inc., and New Link Genetics Inc., and is a consultant/ advisory board member for Biogen Inc., Guidepoint Global LLC, Kyn Therapeutics Inc., New Link Genetics Inc., OrbiMed Advisors LLC, Ribonova Inc., and Vitae Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

### Grant Support

The laboratories of Jacques Izard are supported by the NIH grants R01CA202704 and Nebraska Tobacco Settlement Biomedical Research Development Funds. The APC Microbiome Institute is supported by Science Foundation Ireland (SFI; grant number SFI/12/RC/2273) and has conducted studies in collaboration with several companies including GSK, Pfizer, Wyeth, and Mead Johnson. G. Clarke's contribution to this review was specifically supported by the Irish Health Service Executive (grant number HaPAI/2015/GC). S. Thomas, A.J. Muller, J.M. Mullin, and G.C. Prendergast acknowledge support from the Lankenau Medical Center Foundation, the Women's Board of Lankenau Medical Center, and Main Line Health.

### Disclaimer

The content of this review was neither influenced nor constrained by the organizations that funded this work.

## References

1. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial community variation in human body habitats across space and time. Science 2009;326:1694–7.
2. Lederberg J, McCray AT. 'Ome Sweet 'Omics—a genealogical treasury of words. Scientist 2001;15:8.
3. Margulis L. Symbiogenesis and symbionticism. Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. In: Margulis L, Fester R, editors. Cambridge, MA: MIT Press; 1991. p. 1–14.
4. Bordenstein SR, Theis KR. Host biology in light of the microbiome: ten principles of holobionts and hologenomes. PLoS Biol 2015;13: e1002226.
5. Sender R, Fuchs S, Milo R. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. Cell 2016;164:337–40.
6. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature 2012;489: 231–41.
7. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science 2006;312:1355–9.
8. Meadow JF, Altrichter AE, Bateman AC, Stenson J, Brown GZ, Green JL, et al. Humans differ in their personal microbial cloud. PeerJ 2015;3: e1258.

9. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–7.
10. Korem T, Zeevi D, Suez J, Weinberger A, Avnit-Sagi T, Pompan-Lotan M, et al. Growth dynamics of gut microbiota in health and disease inferred from single metagenomic samples. Science 2015; 349:1101–6.
11. Graf D, Di Cagno R, Fak F, Flint HJ, Nyman M, Saarela M, et al. Contribution of diet to the composition of the human gut microbiota. Microbial Ecol Health Dis 2015;26:26164.
12. Zimmer J, Lange B, Frick JS, Sauer H, Zimmermann K, Schwiertz A, et al. A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. Eur J Clin Nutr 2012;66:53–60.
13. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559–63.
14. Parte AC. LPSN–list of prokaryotic names with standing in nomenclature. Nucleic Acids Res 2014;42:D613–6.
15. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. J Bacteriol 2010;192:5002–17.
16. Nelson KE, Weinstock GM, Highlander SK, Worley KC, Creasy HH, Wortman JR, et al. A catalog of reference genomes from the human microbiome. Science 2010;328:994–9.
17. Human Microbiome Project Consortium. A framework for human microbiome research. Nature 2012;486:215–21.
18. Bouckaert R, Heled J, Kuhnert D, Vaughan T, Wu CH, Xie D, et al. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput Biol 2014;10:e1003537.
19. Huang K, Brady A, Mahurkar A, White O, Gevers D, Huttenhower C, et al. MetaRef: a pan-genomic database for comparative and community microbial genomics. Nucleic Acids Res 2014;42:D617–24.
20. Kerepesi C, Banky D, Grolmusz V. AmphoraNet: the webserver implementation of the AMPHORA2 metagenomic workflow suite. Gene 2014;533:538–40.
21. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, et al. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat Methods 2015;12:902–3.
22. Stackebrandt E. Forces shaping bacterial systematics. Microbe 2007; 2:283–8.
23. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, et al. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Res 2014;42:D643–8.
24. Zuo G, Xu Z, Hao B. Phylogeny and taxonomy of archaea: a comparison of the whole-genome-based CVTree approach with 16S rRNA sequence analysis. Life 2015;5:949–68.
25. Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database 2010;2010:baq013.
26. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res 2014;42:D633–42.
27. Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. Genome Biol 2012;13:R42.
28. Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T, et al. Biogeography of the ecosystems of the healthy human body. Genome Biol 2013;14:R1.
29. Agirbasli H, Ozcan SA, Gedikoglu G. Fecal fungal flora of pediatric healthy volunteers and immunosuppressed patients. Mycopathologia 2005;159: 515–20.
30. Scanlan PD, Marchesi JR. Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture- dependent and -independent analysis of faeces. ISME J 2008; 2:1183–93.
31. Chen Y, Chen Z, Guo R, Chen N, Lu H, Huang S, et al. Correlation between gastrointestinal fungi and varying degrees of chronic hepatitis B virus infection. Diagn Microbiol Infect Dis 2011;70:492–8.
32. Hamad I, Sokhna C, Raoult D, Bittar F. Molecular detection of eukaryotes in a single human stool sample from Senegal. PLoS One 2012;7:e40888.
33. Gouba N, Raoult D, Drancourt M. Plant and fungal diversity in gut microbiota as revealed by molecular and culture investigations. PLoS One 2013;8:e59474.
34. Hamad I, Raoult D, Bittar F. Repertory of eukaryotes (eukaryome) in the human gastrointestinal tract: taxonomy and detection methods. Parasite Immunol 2016;38:12–36.
35. Parfrey LW, Walters WA, Lauber CL, Clemente JC, Berg-Lyons D, Teiling C, et al. Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. Front Microbiol 2014;5:298.
36. Irlinger F, Layec S, Helinck S, Dugat-Bony E. Cheese rind microbial communities: diversity, composition and origin. FEMS Microbiol Lett 2015;362:1–11.
37. Miceli MH, Diaz JA, Lee SA. Emerging opportunistic yeast infections. Lancet Infect Dis 2011;11:142–51.
38. Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology 2005;128:825–32.
39. Wammes LJ, Mpairwe H, Elliott AM, Yazdanbakhsh M. Helminth therapy or elimination: epidemiological, immunological, and clinical considerations. Lancet Infect Dis 2014;14:1150–62.
40. Hug LA, Baker BJ, Anantharaman K, Brown CT, Probst AJ, Castelle CJ, et al. A new view of the tree of life. Nat Microbiol 2016;1:16048.
41. Manrique P, Bolduc B, Walk ST, van der Oost J, de Vos WM, Young MJ. Healthy human gut phageome. Proc Natl Acad Sci U S A 2016;113: 10400–5.
42. International Committee on Taxonomy of Viruses. The International Committee on Taxonomy of Viruses; 2015. Available from: http://www.ictvonline.org.
43. Reteno DG, Benamar S, Khalil JB, Andreani J, Armstrong N, Klose T, et al. Faustovirus, an asfarvirus-related new lineage of giant viruses infecting amoebae. J Virol 2015;89:6585–94.
44. Ghabrial SA, Caston JR, Jiang D, Nibert ML, Suzuki N. 50-plus years of fungal viruses. Virology 2015;479–80:356–68.
45. Snyder JC, Bolduc B, Young MJ. 40 years of archaeal virology: expanding viral diversity. Virology 2015;479–80:369–78.
46. Pickett BE, Sadat EL, Zhang Y, Noronha JM, Squires RB, Hunt V, et al. ViPR: an open bioinformatics database and analysis resource for virology research. Nucleic Acids Res 2012;40:D593–8.
47. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: a fast phage search tool. Nucleic Acids Res 2011;39:W347–52.
48. Gardy J, Loman NJ, Rambaut A. Real-time digital pathogen surveillance - the time is now. Genome Biol 2015;16:155.
49. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174–80.
50. Consortium HMP. Structure, function and diversity of the healthy human microbiome. Nature 2012;486:207–14.
51. Massana R, Gobet A, Audic S, Bass D, Bittner L, Boutte C, et al. Marine protist diversity in European coastal waters and sediments as revealed by high-throughput sequencing. Environ Microbiol 2015; 17:4035–49.
52. Beall CJ, Campbell AG, Dayeh DM, Griffen AL, Podar M, Leys EJ. Single cell genomics of uncultured, health-associated *Tannerella* BU063 (Oral Taxon 286) and comparison to the closely related pathogen *Tannerella forsythia*. PLoS One 2014;9:e89398.
53. Mason OU, Hazen TC, Borglin S, Chain PS, Dubinsky EA, Fortney JL, et al. Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. ISME J 2012;6: 1715–27.
54. Yoon HS, Price DC, Stepanauskas R, Rajah VD, Sieracki ME, Wilson WH, et al. Single-cell genomics reveals organismal interactions in uncultivated marine protists. Science 2011;332:714–7.

55. Hehemann JH, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature 2010;464:908–12.
56. Manna S, Harman A. Horizontal gene transfer of a Chlamydial tRNA guanine transglycosylase gene to eukaryotic microbes. Mol Phyl Evol 2016;94(Pt A):392–6.
57. Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ. Ecology drives a global network of gene exchange connecting the human microbiome. Nature 2011;480:241–4.
58. Cisse OH, Pagni M, Hauser PM. Comparative genomics suggests that the human pathogenic fungus *Pneumocystis jirovecii* acquired obligate biotrophy through gene loss. Genome Biol Evol 2014;6:1938–48.
59. Elias AF, Stewart PE, Grimm D, Caimano MJ, Eggers CH, Tilly K, et al. Clonal polymorphism of *Borrelia burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain background. Infect Immun 2002;70:2139–50.
60. Rawat A, Engelthaler DM, Driebe EM, Keim P, Foster JT. MetaGeniE: characterizing human clinical samples using deep metagenomic sequencing. PLoS One 2014;9:e110915.
61. Schloissnig S, Arumugam M, Sunagawa S, Mitreva M, Tap J, Zhu A, et al. Genomic variation landscape of the human gut microbiome. Nature 2013;493:45–50.
62. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Sci Transl Med 2014;6:237ra65.
63. Scholtens PA, Oozeer R, Martin R, Amor KB, Knol J. The early settlers: intestinal microbiology in early life. Annu Rev Food Sci Technol 2012; 3:425–47.
64. Underwood MA, German JB, Lebrilla CB, Mills DA. *Bifidobacterium longum* subspecies infantis: champion colonizer of the infant gut. Pediatr Res 2015;77:229–35.
65. Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Dominguez-Bello MG. The infant microbiome development: mom matters. Trends Mol Med 2015;21:109–17.
66. Braundmeier AG, Lenz KM, Inman KS, Chia N, Jeraldo P, Walther- Antonio MR, et al. Individualized medicine and the microbiome in reproductive tract. Front Physiol 2015;6:97.
67. Ardissone AN, de la Cruz DM, Davis-Richardson AG, Rechcigl KT, Li N, Drew JC, et al. Meconium microbiome analysis identifies bacteria correlated with premature birth. PLoS One 2014;9:e90784.
68. Neu J, Rushing J. Cesarean versus vaginal delivery: long-term infant outcomes and the hygiene hypothesis. Clin Perinatol 2011;38:321–31.
69. Johnson EM, Rehavi MM. Physicians treating physicians: information and incentives in childbirth. Am Econ J Econ Policy 2016;8:115–41.
70. Mueller NT, Whyatt R, Hoepner L, Oberfield S, Dominguez-Bello MG, Widen EM, et al. Prenatal exposure to antibiotics, cesarean section and risk of childhood obesity. Int J Obes 2015;39:665–70.
71. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 2010;107:11971–5.
72. Sevelsted A, Stokholm J, Bonnelykke K, Bisgaard H. Cesarean section and chronic immune disorders. Pediatrics 2015;135:e92–8.
73. Malmborg P, Bahmanyar S, Grahnquist L, Hildebrand H, Montgomery S. Cesarean section and the risk of pediatric Crohn's disease. Inflamm Bowel Dis 2012;18:703–8.
74. Johnson CL, Versalovic J. The human microbiome and its potential importance to pediatrics. Pediatrics 2012;129:950–60.
75. Kostic AD, Gevers D, Siljander H, Vatanen T, Hyotylainen T, Hamalainen AM, et al. The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. Cell Host Microbe 2015;17:260–73.
76. Murgas Torrazza R, Neu J. The developing intestinal microbiome and its relationship to health and disease in the neonate. J Perinatol 2011; 31Suppl 1:S29–34.
77. Rousseau C, Levenez F, Fouqueray C, Dore J, Collignon A, Lepage P. Clostridium difficile colonization in early infancy is accompanied by changes in intestinal microbiota composition. J Clin Microbiol 2011; 49:858–65.
78. McLoughlin RM, Mills KH. Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. J Allergy Clin Immunol 2011;127:1097–107.
79. Ajslev TA, Andersen CS, Gamborg M, Sorensen TI, Jess T. Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. Int J Obes 2011;35:522–9.
80. Cheng J, Palva AM, de Vos WM, Satokari R. Contribution of the intestinal microbiota to human health: from birth to 100 years of age. Curr Topics Microbiol Immunol 2013;358:323–46.
81. Decker E, Hornef M, Stockinger S. Cesarean delivery is associated with celiac disease but not inflammatory bowel disease in children. Gut Microbes 2011;2:91–8.
82. Hunter PA, Dawson S, French GL, Goossens H, Hawkey PM, Kuijper EJ, et al. Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies. J Antimicrob Chemother 2010;65Suppl 1:i3–17.
83. Stewardson AJ, Huttner B, Harbarth S. At least it won't hurt: the personal risks of antibiotic exposure. Curr Opin Pharmacol 2011;11:446–52.
84. Blaser MJ. Who are we? Indigenous microbes and the ecology of human diseases. EMBO Rep 2006;7:956–60.
85. Schwartz S, Friedberg I, Ivanov IV, Davidson LA, Goldsby JS, Dahl DB, et al. A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in immune response. Genome Biol 2012;13:r32.
86. Stark PL, Lee A. The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. J Med Microbiol 1982; 15:189–203.
87. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, et al. Analysis of intestinal flora development in breastfed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 2000;30:61–7.
88. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. Pediatr Clin North Am 2013;60:49–74.
89. Eidelman AI. Breastfeeding and the use of human milk: an analysis of the American Academy of Pediatrics 2012 Breastfeeding Policy Statement. Breastfeeding Med 2012;7:323–4.
90. Isaacs EB, Fischl BR, Quinn BT, Chong WK, Gadian DG, Lucas A. Impact of breast milk on intelligence quotient, brain size, and white matter development. Pediatric research 2010;67:357–62.
91. Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. Proc Natl Acad Sci U S A 2011;108Suppl 1:4653–8.
92. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr 2000; 20:699–722.
93. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006;118:511–21.
94. Sela DA. Bifidobacterial utilization of human milk oligosaccharides. Int J Food Microbiol 2011;149:58–64.
95. Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, et al. The genome sequence of *Bifidobacterium longum* subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A 2008;105:18964–9.
96. Lewis ZT, Totten SM, Smilowitz JT, Popovic M, Parker E, Lemay DG, et al. Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. Microbiome 2015;3:13.
97. Davidson B, Meinzen-Derr JK, Wagner CL, Newburg DS, Morrow AL. Fucosylated oligosaccharides in human milk in relation to gestational age and stage of lactation. Adv Exp Med Biol 2004;554:427–30.
98. Newburg DS, Ruiz-Palacios GM, Altaye M, Chaturvedi P, Meinzen-Derr J, Guerrero M de L, et al. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants.

Glycobiology 2004;14:253–63.
99. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr 2005;25:37–58.
100. Newburg DS. Oligosaccharides in human milk and bacterial colonization. J Pediatr Gastroenterol Nutr 2000;30Suppl 2:S8–17.
101. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. J Nutr 2005;135:1304–7.
102. Idota T, Kawakami H, Murakami Y, Sugawara M. Inhibition of cholera toxin by human milk fractions and sialyllactose. Biosci Biotechnol Biochem 1995;59:417–9.
103. Andersson B, Porras O, Hanson LA, Lagergard T, Svanborg-Eden C. Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. J Infect Dis 1986;153:232–7.
104. Newburg DS, Pickering LK, McCluer RH, Cleary TG. Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of *Escherichia coli*. J Infect Dis 1990;162:1075–80.
105. Duijts L, Jaddoe VW, Hofman A, Moll HA. Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy. Pediatrics 2010;126:e18–25.
106. Abt MC, Pamer EG. Commensal bacteria mediated defenses against pathogens. Curr Opin Immunol 2014;29:16–22.
107. Matsuki T, Yahagi K, Mori H, Matsumoto H, Hara T, Tajima S, et al. A key genetic factor for fucosyllactose utilization affects infant gut microbiota development. Nat Commun 2016;7:11939.
108. Oozeer R, van Limpt K, Ludwig T, Ben Amor K, Martin R, Wind RD, et al. Intestinal microbiology in early life: specific prebiotics can have similar functionalities as human-milk oligosaccharides. Am J Clin Nutr 2013;98: 561s–71s.
109. Heavey PM RI. The gut microflora of the developing infant: microbiology and metabolism. Microbial Ecol Health Dis 1999;11:75–83.
110. Charbonneau MR, O'Donnell D, Blanton LV, Totten SM, Davis JC, Barratt MJ, et al. Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. Cell 2016;164:859–71.
111. Zivkovic AM, Barile D. Bovine milk as a source of functional oligosaccharides for improving human health. Adv Nutr 2011;2:284–9.
112. Tao N, DePeters EJ, Freeman S, German JB, Grimm R, Lebrilla CB. Bovine milk glycome. J Dairy Sci 2008;91:3768–78.
113. Tao N, DePeters EJ, German JB, Grimm R, Lebrilla CB. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. J Dairy Sci 2009;92:2991–3001.
114. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 2012;22:1147–62.
115. Rautava S, Kalliomaki M, Isolauri E. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. J Allergy Clin Immunol 2002;109:119–21.
116. Rautava S, Kainonen E, Salminen S, Isolauri E. Maternal probiotic supplementation during pregnancy and breast-feeding reduces the risk of eczema in the infant. J Allergy Clin Immunol 2012;130:1355–60.
117. Rautava S, Salminen S, Isolauri E. Specific probiotics in reducing the risk of acute infections in infancy–a randomised, double-blind, placebo-controlled study. Br J Nutr 2009;101:1722–6.
118. Isolauri E, Rautava S, Salminen S. Probiotics in the development and treatment of allergic disease. Gastroenterol Clin North Am 2012;41:747– 62.
119. Kainonen E, Rautava S, Isolauri E. Immunological programming by breast milk creates an anti-inflammatory cytokine milieu in breast-fed infants compared to formula-fed infants. Br J Nutr 2013;109:1962–70.
120. Xu M, Wang J, Wang N, Sun F, Wang L, Liu XH. The efficacy and safety of the probiotic bacterium *Lactobacillus reuteri* DSM 17938 for infantile colic: a meta-analysis of randomized controlled trials. PLoS One 2015;10:e0141445.
121. Savino F. Focus on infantile colic. Acta Paediatr 2007;96:1259–64.
122. Sung V, Collett S, de Gooyer T, Hiscock H, Tang M, Wake M. Probiotics to prevent or treat excessive infant crying: systematic review and metaanalysis. JAMA Pediatr 2013;167:1150–7.
123. Chau K, Lau E, Greenberg S, Jacobson S, Yazdani-Brojeni P, Verma N, et al. Probiotics for infantile colic: a randomized, double-blind, placebo-controlled trial investigating Lactobacillus reuteri DSM 17938. J Pediatr 2015;166:74–8.
124. Roos S, Dicksved J, Tarasco V, Locatelli E, Ricceri F, Grandin U, et al. 454 pyrosequencing analysis on faecal samples from a randomized DBPC trial of colicky infants treated with *Lactobacillus reuteri* DSM17938. PLoS One 2013;8:e56710.
125. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 2011; 108Suppl 1:4586–91.
126. O'Toole PW, Jeffery IB. Gut microbiota and aging. Science 2015;350: 1214–5.
127. Jackson MA, Jeffery IB, Beaumont M, Bell JT, Clark AG, Ley RE, et al. Signatures of early frailty in the gut microbiota. Genome Med 2016;8:8.
128. Zapata HJ, Quagliarello VJ. The microbiota and microbiome in aging: potential implications in health and age-related diseases. J Am Geriatr Soc 2015;63:776–81.
129. Saraswati S, Sitaraman R. Aging and the human gut microbiota-from correlation to causality. Front Microbiol 2014;5:764.
130. Patrignani P, Tacconelli S, Bruno A. Gut microbiota, host gene expression, and aging. J Clin Gastroenterol 2014;48Suppl 1:S28–31.
131. Noren Hooten N, Abdelmohsen K, Gorospe M, Ejiogu N, Zonderman AB, Evans MK. microRNA expression patterns reveal differential expression of target genes with age. PLoS One 2010;5:e10724.
132. Lai CY, Wu YT, Yu SL, Yu YH, Lee SY, Liu CM, et al. Modulated expression of human peripheral blood microRNAs from infancy to adulthood and its role in aging. Aging Cell 2014;13:679–89.
133. Liu S, da Cunha AP, Rezende RM, Cialic R, Wei Z, Bry L, et al. The host shapes the gut microbiota via fecal microRNA. Cell Host Microbe 2016;19:32–43.
134. Heilbronn LK, Ravussin E. Calorie restriction and aging: review of the literature and implications for studies in humans. Am J Clin Nutr 2003;78:361–9.
135. Zhang C, Li S, Yang L, Huang P, Li W, Wang S, et al. Structural modulation of gut microbiota in life-long calorie-restricted mice. Nat Commun 2013;4:2163.
136. Wu Z, Song L, Liu SQ, Huang D. A high throughput screening assay for determination of chronological lifespan of yeast. Exp Gerontol 2011;46:915–22.
137. Biagi E, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P. Aging of the human metaorganism: the microbial counterpart. Age 2012;34: 247–67.
138. Rampelli S, Candela M, Turroni S, Biagi E, Collino S, Franceschi C, et al. Functional metagenomic profiling of intestinal microbiome in extreme ageing. Aging 2013;5:902–12.
139. Heintz C, Mair W. You are what you host: microbiome modulation of the aging process. Cell 2014;156:408–11.
140. Rozsa L, Apari P, Muller V. The microbiome mutiny hypothesis: can our microbiome turn against us when we are old or seriously ill? Biol Direct 2015;10:3.
141. Caracciolo B, Xu W, Collins S, Fratiglioni L. Cognitive decline, dietary factors and gut-brain interactions. Mech Ageing Dev 2014;136–7:59–69.
142. Parkar SG, Trower TM, Stevenson DE. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. Anaerobe 2013; 23:12–9.
143. Wang D, Ho L, Faith J, Ono K, Janle EM, Lachcik PJ, et al. Role of intestinal microbiota in the generation of polyphenol-derived phenolic acid mediated attenuation of Alzheimer's disease beta-amyloid oligomerization. Mol Nutr Food Res 2015;59:1025–40.

144. Raberg L, Sim D, Read AF. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science 2007; 318:812–4.
145. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity 2013;39:372–85.
146. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 2009;9:313–23.
147. Ohnmacht C, Park JH, Cording S, Wing JB, Atarashi K, Obata Y, et al. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat(+) T cells. Science 2015;349:989–93.
148. Martin SA, Pence BD, Woods JA. Exercise and respiratory tract viral infections. Exerc Sport Sci Rev 2009;37:157–64.
149. Simpson RJ, Kunz H, Agha N, Graff R. Exercise and the regulation of immune functions. Prog Mol Biol Transl Sci 2015;135:355–80.
150. Pape K, Ryttergaard L, Rotevatn TA, Nielsen BJ, Torp-Pedersen C, Overgaard C, et al. Leisure-time physical activity and the risk of suspected bacterial infections. Med Sci Sports Exerc 2016;48:1737–44.
151. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. Nature 2015;525:528–32.
152. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. Nature 2011;478:250–4.
153. Integrative HMP (iHMP) Research Network Consortium. The Integrative Human Microbiome Project: dynamic analysis of microbiome-host omics profiles during periods of human health and disease. Cell Host Microbe 2014;16:276–89.
154. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989; 299:1259–60.
155. Okada H, Kuhn C, Feillet H, Bach JF. The `hygiene hypothesis' for autoimmune and allergic diseases: an update. Clin Exp Immunol 2010; 160:1–9.
156. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C, et al. Exposure to environmental microorganisms and childhood asthma. N Engl J Med 2011;364:701–9.
157. Schuijs MJ, Willart MA, Vergote K, Gras D, Deswarte K, Ege MJ, et al. Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. Science 2015;349:1106–10.
158. Brooks C, Pearce N, Douwes J. The hygiene hypothesis in allergy and asthma: an update. Curr Opin Allergy Clin Immunol 2013; 13:70–7.
159. Chotirmall SH, Burke CM. Aging and the microbiome: implications for asthma in the elderly? Expert Rev Respir Med 2015;9:125–8.
160. Barnes PJ. Cytokine-directed therapies for asthma. J Allergy Clin Immunol 2001;108(2 Suppl):S72–S76.
161. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2008;8:183–92.
162. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. Nat Rev Immunol 2010;10:861–8.
163. Couzin-Frankel J. Bacteria and asthma: untangling the links. Science 2010;330:1168–9.
164. Lif Holgerson P, Harnevik L, Hernell O, Tanner AC, Johansson I. Mode of birth delivery affects oral microbiota in infants. J Dent Res 2011;90: 1183–8.
165. Nelun Barfod M, Magnusson K, Lexner MO, Blomqvist S, Dahlen G, Twetman S. Oral microflora in infants delivered vaginally and by caesarean section. Int J Paediatr Dent 2011;21:401–6.
166. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med 2015;7:307ra152.
167. Marsland BJ, Salami O. Microbiome influences on allergy in mice and humans. Curr Opin Immunol 2015;36:94–100.
168. Lerner A, Aminov R, Matthias T. Dysbiosis may trigger autoimmune diseases via inappropriate post-translational modification of host proteins. Front Microbiol 2016;7:84.
169. Hesselmar B, Hicke-Roberts A, Wennergren G. Allergy in children in hand versus machine dishwashing. Pediatrics 2015;135:e590–7.
170. Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, et al. Variation in microbiome LPS immunogenicity contributes to autoimmunity in humans. Cell 2016;165:1551.
171. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. Aggregatibacter actinomycetemcomitans–induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. Sci Transl Med 2016;8:369ra176.
172. Zhang X, Zhang D, Jia H. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med 2015;21:895–905.
173. Tan TG, Sefik E, Geva-Zatorsky N, Kua L, Naskar D, Teng F, et al. Identifying species of symbiont bacteria from the human gut that, alone, can induce intestinal Th17 cells in mice. Proc Natl Acad Sci U S A 2016;113:E8141–50.
174. Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, et al. Role of Th17 cells in human autoimmune arthritis. Arthritis Rheum 2010;62:2876–85.
175. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006;444:1022–3.
176. Wang CY, Liao JK. A mouse model of diet-induced obesity and insulin resistance. Methods Mol Biol 2012;821:421–33.
177. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.
178. Kallus SJ, Brandt LJ. The intestinal microbiota and obesity. J Clin Gastroenterol 2012;46:16–24.
179. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718–23.
180. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 2013;341:1241214.
181. Koleva PT, Bridgman SL, Kozyrskyj AL. The infant gut microbiome: evidence for obesity risk and dietary intervention. Nutrients 2015;7: 2237–60.
182. Scott FI, Horton DB, Mamtani R, Haynes K, Goldberg DS, Lee DY, et al. Administration of antibiotics to children before age 2 years increases risk for childhood obesity. Gastroenterology 2016;151:120–9.
183. Cox LM, Blaser MJ. Antibiotics in early life and obesity. Nat Rev Endocrinol 2015;11:182–90.
184. Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, et al. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell 2014;158:705–21.
185. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, Topping DL, et al. Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. J Nutr 2011;141:883–9.
186. De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 2015 Sep 28; doi: 10.1136/gutjnl-2015-309957.
187. Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 2002;217: 133–9.
188. McIntyre A, Gibson PR, Young GP. Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. Gut 1993;34: 386–91.
189. Musso G, Gambino R, Cassader M. Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded? Diabetes Care 2010;33:2277–84.
190. Bocarsly ME, Fasolino M, Kane GA, LaMarca EA, Kirschen GW, Karatsoreos IN, et al. Obesity diminishes synaptic markers, alters microglial morphology, and impairs cognitive function. Proc Natl Acad Sci U S A 2015;112:15731–6.
191. Burokas A, Moloney RD, Dinan TG, Cryan JF. Microbiota regulation of the mammalian gut-brain axis. Adv Appl Microbiol 2015;91:1–62.
192. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et

al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A 2013;110: 9066–71.
193. Plovier H, Everard A, Druart C, Depommier C, Van Hul M, Geurts L, et al. A purified membrane protein from *Akkermansia muciniphila* or the pasteurized bacterium improves metabolism in obese and diabetic mice. Nat Med 2017;23:107–13.
194. de la Cuesta-Zuluaga J, Mueller NT, Corrales-Agudelo V, Velasquez-Mejia EP, Carmona JA, Abad JM, et al. Metformin is associated with higher relative abundance of mucin-degrading *Akkermansia muciniphila* and several short-chain fatty acid-producing microbiota in the gut. Diabetes Care 2017;40:54–62.
195. Troseid M. Gut microbiota and acute coronary syndromes: ready for use in the emergency room? Eur Heart J 2017 Feb 4. [Epub ahead of print].
196. Li J, Zhao F, Wang Y, Chen J, Tao J, Tian G, et al. Gut microbiota dysbiosis contributes to the development of hypertension. Microbiome 2017;5:14.
197. Stepankova R, Tonar Z, Bartova J, Nedorost L, Rossman P, Poledne R, et al. Absence of microbiota (germ-free conditions) accelerates the atherosclerosis in ApoE-deficient mice fed standard low cholesterol diet. J Atheroscler Thromb 2010;17:796–804.
198. Chan YK, Brar MS, Kirjavainen PV, Chen Y, Peng J, Li D, et al. High fat diet induced atherosclerosis is accompanied with low colonic bacterial diversity and altered abundances that correlates with plaque size, plasma AFABP and cholesterol: a pilot study of high fat diet and its intervention with Lactobacillus rhamnosus GG (LGG) or telmisartan in ApoE-/- mice. BMC Microbiol 2016;16:264.
199. Li J, Lin S, Vanhoutte PM, Woo CW, Xu A. Akkermansia muciniphila protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in ApoE-/- mice. Circulation 2016;133:2434–46.
200. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. Front Physiol 2011;2:94.
201. Kirk RG. "Life in a germ-free world": isolating life from the laboratory animal to the bubble boy. Bull Hist Med 2012;86:237–75.
202. Williams SC. Gnotobiotics. Proc Natl Acad Sci U S A 2014;111:1661.
203. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, et al. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 2011;108:3047–52.
204. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 2011;23:255–64.
205. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 2013;18:666–73.
206. Crumeyrolle-Arias M, Jaglin M, Bruneau A, Vancassel S, Cardona A, Dauge V, et al. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. Psychoneuroendocrinology 2014;42:207–17.
207. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 2011;141:599–609.
208. Bruce-Keller AJ, Salbaum JM, Luo M, Blanchard Et, Taylor CM, Welsh DA, et al. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. Biol Psychiatry 2015;77:607–15.
209. Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. Mol Psychiatry 2014;19:146–8.
210. Arentsen T, Raith H, Qian Y, Forssberg H, Diaz Heijtz R. Host microbiota modulates development of social preference in mice. Microbial Ecol Health Dis 2015;26:29719.
211. Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, et al. Bacterial infection causes stress-induced memory dysfunction in mice. Gut 2011;60:307–17.
212. Desbonnet L, Clarke G, Traplin A, O'Sullivan O, Crispie F, Moloney RD, et al. Gut microbiota depletion from early adolescence in mice: implications for brain and behaviour. Brain Behav Immun 2015;48:165–73.
213. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 2011;108:16050–5.
214. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive processes in an anxious-mouse strain. Behav Brain Res 2015;287:59–72.
215. Savignac HM, Couch Y, Stratford M, Bannerman DM, Tzortzis G, Anthony DC, et al. Prebiotic administration normalizes lipopolysaccharide (LPS)- induced anxiety and cortical 5-HT2A receptor and IL1-beta levels in male mice. Brain Behav Immun 2016;52:120–31.
216. Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, et al. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut 2006;55:182–90.
217. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary- adrenal system for stress response in mice. J Physiol 2004;558 (Pt 1):263–75.
218. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep 2006;7:688–93.
219. Matcovitch-Natan O, Winter DR, Giladi A, Vargas Aguilar S, Spinrad A, Sarrazin S, et al. Microglia development follows a stepwise program to regulate brain homeostasis. Science 2016;353:aad8670.
220. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 2009;106: 3698–703.
221. Mardinoglu A, Shoaie S, Bergentall M, Ghaffari P, Zhang C, Larsson E, et al. The gut microbiota modulates host amino acid and glutathione metabolism in mice. Mol Syst Biol 2015;11:834.
222. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 2008;43:164–74.
223. Dinan TG, Cryan JF. Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 2012;37:1369–78.
224. Bailey MT, Coe CL. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 1999;35: 146–55.
225. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 2009;65:263–7.
226. Golubeva AV, Crampton S, Desbonnet L, Edge D, O'Sullivan O, Lomasney KW, et al. Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. Psychoneuroendocrinology 2015;60:58–74.
227. Jasarevic E, Howerton CL, Howard CD, Bale TL. Alterations in the vaginal microbiome by maternal stress are associated with metabolic reprogramming of the offspring gut and brain. Endocrinology 2015; 156:3265–76.
228. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 2011;25:397–407.
229. Galley JD, Nelson MC, Yu Z, Dowd SE, Walter J, Kumar PS, et al. Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota. BMC Microbiol 2014;14:189.
230. Bharwani A, Mian MF, Foster JA, Surette MG, Bienenstock J, Forsythe P. Structural & functional consequences of chronic psychosocial stress on the microbiome & host. Psychoneuroendocrinology 2016;63:217–27.
231. De Palma G, Blennerhassett P, Lu J, Deng Y, Park AJ, Green W, et al. Microbiota and host determinants of behavioural phenotype in maternally separated mice. Nat Commun 2015;6:7735.
232. O'Mahony SM, Hyland NP, Dinan TG, Cryan JF. Maternal separation

as a model of brain-gut axis dysfunction. Psychopharmacology 2011;214: 71–88.
233. ZijImans MA, Korpela K, Riksen-Walraven JM, de Vos WM, de Weerth C. Maternal prenatal stress is associated with the infant intestinal microbiota. Psychoneuroendocrinology 2015;53:233–45.
234. Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Irritable bowel syndrome: a microbiome-gut-brain axis disorder? World J Gastroenterol 2014;20: 14105–25.
235. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Toth M, et al. The gut microbiota influences blood-brain barrier permeability in mice. Sci Transl Med 2014;6:263ra158.
236. Stilling RM, Ryan FJ, Hoban AE, Shanahan F, Clarke G, Claesson MJ, et al. Microbes & neurodevelopment–Absence of microbiota during early life increases activity-related transcriptional pathways in the amygdala. Brain Behav Immun 2015;50:209–20.
237. Ogbonnaya ES, Clarke G, Shanahan F, Dinan TG, Cryan JF, O'Leary OF. Adult hippocampal neurogenesis is regulated by the microbiome. Biol Psychiatry 2015;78:e7–9.
238. Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci 2015;18:965–77.
239. Hoban AE, Stilling RM, Ryan FJ, Shanahan F, Dinan TG, Claesson MJ, et al. Regulation of prefrontal cortex myelination by the microbiota. Transl Psychiatry 2016;6:e774.
240. Harach T, Marungruang N, Dutilleul N, Cheatham V, Mc Coy KD, Neher JJ, et al. Reduction of Alzheimer's disease beta-amyloid pathology in the absence of gut microbiota. Ithaca, NY: Cornell University;2015. Available from: https://arxiv.org/abs/1509.02273.
241. Friedland RP. Mechanisms of molecular mimicry involving the microbiota in neurodegeneration. J Alzheimers Dis 2015;45:349–62.
242. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. Cell 2016;167:1469–80.e12.
243. Forsythe P, Bienenstock J, Kunze WA. Vagal pathways for microbiome-brain-gut axis communication. Adv Exp Med Biol 2014;817:115–33.
244. Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology 2010; 139:2102–12.
245. O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res 2015;277:32–48.
246. De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, et al. Microbiota-generated metabolites promote-metabolic benefits via gut-brain neural circuits. Cell 2014;156:84–96.
247. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 2015;161:264–76.
248. Holzer P, Farzi A. Neuropeptides and the microbiota-gut-brain axis. Adv Exp Med Biol 2014;817:195–219.
249. El Aidy S, Dinan TG, Cryan JF. Gut microbiota: the conductor in the orchestra of immune-neuroendocrine communication. Clin Ther 2015;37:954–67.
250. Kelly JR, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. Front Cell Neurosci 2015;9:392.
251. Quigley EM. Leaky gut - concept or clinical entity? Curr Opin Gastroenterol 2016;32:74–9.
252. Garber K. Drugging the gut microbiome. Nat Biotechnol 2015;33: 228–31.
253. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. Trends Mol Med 2014;20:509–18.
254. Clarke G, O'Mahony SM, Dinan TG, Cryan JF. Priming for health: gut microbiota acquired in early life regulates physiology, brain and behaviour. Acta Paediatr 2014;103:812–9.
255. Kelly CR, Kahn S, Kashyap P, Laine L, Rubin D, Atreja A, et al. Update on fecal microbiota transplantation 2015: indications, methodologies, mechanisms, and outlook. Gastroenterology 2015;149:223–37.
256. Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. Biol Psychiatry 2013;74:720–6.
257. Clarke SF, Murphy EF, O'Sullivan O, Lucey AJ, Humphreys M, Hogan A, et al. Exercise and associated dietary extremes impact on gut microbial diversity. Gut 2014;63:1913–20.
258. O'Sullivan O, Cronin O, Clarke SF, Murphy EF, Molloy MG, Shanahan F, et al. Exercise and the microbiota. Gut Microbes 2015;6:131–6.
259. Dash S, Clarke G, Berk M, Jacka FN. The gut microbiome and diet in psychiatry: focus on depression. Curr Opin Psychiatry 2015;28:1–6.
260. Luna RA, Foster JA. Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression. Curr Opin Biotechnol 2015;32:35–41.
261. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, et al. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 2011;105:755–64.
262. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 2013;144:1394–401.
263. Schmidt K, Cowen PJ, Harmer CJ, Tzortzis G, Errington S, Burnet PW. Prebiotic intake reduces the waking cortisol response and alters emotional bias in healthy volunteers. Psychopharmacology 2015;232: 1793–801.
264. Steenbergen L, Sellaro R, van Hemert S, Bosch JA, Colzato LS. A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. Brain Behavior Immun 2015;48: 258–64.
265. Kang DW, Park JG, Ilhan ZE, Wallstrom G, Labaer J, Adams JB, et al. Reduced incidence of *Prevotella* and other fermenters in intestinal microflora of autistic children. PLoS One 2013;8:e68322.
266. Opp MR, Krueger JM. Sleep and immunity: a growing field with clinical impact. Brain Behav Immun 2015;47:1–3.
267. Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, et al. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 2014;159:514–29.
268. Voigt RM, Forsyth CB, Green SJ, Mutlu E, Engen P, Vitaterna MH, et al. Circadian disorganization alters intestinal microbiota. PLoS One 2014;9: e97500.
269. Forsyth CB, Voigt RM, Burgess HJ, Swanson GR, Keshavarzian A. Circadian rhythms, alcohol and gut interactions. Alcohol 2015;49:389–98.
270. Rook GA. Regulation of the immune system by biodiversity from the natural environment: an ecosystem service essential to health. Proc Natl Acad Sci U S A 2013;110:18360–7.
271. Hoisington AJ, Brenner LA, Kinney KA, Postolache TT, Lowry CA. The microbiome of the built environment and mental health. Microbiome 2015;3:60.
272. Logan AC. Dysbiotic drift: mental health, environmental grey space, and microbiota. J Physiol Anthropol 2015;34:23.
273. Slattery DA, Cryan JF. The ups and downs of modelling mood disorders in rodents. ILAR J 2014;55:297–309.
274. Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? Dis Models Mech 2015;8:1–16.
275. Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol 1977;31:107–33.
276. Walter J, Ley R. The human gut microbiome: ecology and recent evolutionary changes. Annu Rev Microbiol 2011;65:411–29.
277. Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM. Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2007;2:285–95.
278. Dethlefsen L, Eckburg PB, Bik EM, Relman DA. Assembly of the human intestinal microbiota. Trends Ecol Evol 2006;21:517–23.

279. Gerritsen J, Smidt H, Rijkers GT, de Vos WM. Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr 2011;6:209–40.
280. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Dore J, et al. The *Firmicutes/Bacteroidetes* ratio of the human microbiota changes with age. BMC Microbiol 2009;9:123.
281. Rajilic-Stojanovic M, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. Environ Microbiol 2007;9: 2125–36.
282. Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut 2008;57:1605–15.
283. Mihajlovski A, Alric M, Brugere JF. A putative new order of methanogenic Archaea inhabiting the human gut, as revealed by molecular analyses of the mcrA gene. Res Microbiol 2008;159:516–21.
284. Ott SJ, Kuhbacher T, Musfeldt M, Rosenstiel P, Hellmig S, Rehman A, et al. Fungi and inflammatory bowel diseases: alterations of composition and diversity. Scand J Gastroenterol 2008;43:831–41.
285. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005;307:1915–20.
286. Hamer HM, Jonkers DM, Bast A, Vanhoutvin SA, Fischer MA, Kodde A, et al. Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. Clin Nutr 2009;28:88–93.
287. Yan J, Herzog JW, Tsang K, Brennan CA, Bower MA, Garrett WS, et al. Gut microbiota induce IGF-1 and promote bone formation and growth. Proc Natl Acad Sci U S A 2016;113:E7554–E63.
288. Hoeppli RE, Wu D, Cook L, Levings MK. The environment of regulatory T cell biology: cytokines, metabolites, and the microbiome. Front Immunol 2015;6:61.
289. Karlsson F, Tremaroli V, Nielsen J, Backhed F. Assessing the human gut microbiota in metabolic diseases. Diabetes 2013;62:3341–9.
290. Ly NP, Litonjua A, Gold DR, Celedon JC. Gut microbiota, probiotics, and vitamin D: interrelated exposures influencing allergy, asthma, and obesity? J Allergy Clin Immunol 2011;127:1087–94.
291. Voth DE, Ballard JD. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev 2005;18:247–63.
292. Leffler DA, Lamont JT. *Clostridium difficile* Infection. N Engl J Med 2015;373:287–8.
293. Lessa FC, Winston LG, McDonald LC. Burden of *Clostridium difficile* infection in the United States. N Engl J Med 2015;372:2369–70.
294. Cornely OA, Nathwani D, Ivanescu C, Odufowora-Sita O, Retsa P, Odeyemi IA. Clinical efficacy of fidaxomicin compared with vancomycin and metronidazole in *Clostridium difficile* infections: a meta-analysis and indirect treatment comparison. J Antimicrobial Chemother 2014;69: 2892–900.
295. Burke KE, Lamont JT. Fecal transplantation for recurrent Clostridium difficile infection in older adults: a review. J Am Geriatr Soc 2013;61: 1394–8.
296. Goldenberg JZ, Ma SS, Saxton JD, Martzen MR, Vandvik PO, Thorlund K, et al. Probiotics for the prevention of *Clostridium difficile*-associated diarrhea in adults and children. Cochrane Database Syst Rev 2013;5: Cd006095.
297. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature 2012;489:242–9.
298. Sommer F, Backhed F. The gut microbiota–masters of host development and physiology. Nat Rev Microbiol 2013;11:227–38.
299. Sassone-Corsi M, Nuccio SP, Liu H, Hernandez D, Vu CT, Takahashi AA, et al. Microcins mediate competition among *Enterobacteriaceae* in the inflamed gut. Nature 2016;540:280–3.
300. Zaneveld J, Turnbaugh PJ, Lozupone C, Ley RE, Hamady M, Gordon JI, et al. Host-bacterial coevolution and the search for new drug targets. Curr Opin Chem Biol 2008;12:109–14.
301. Bejaoui M, Sokol H, Marteau P. Targeting the microbiome in inflammatory bowel disease: critical evaluation of current concepts and moving to new horizons. Dig Dis 2015;33Suppl 1:105–12.
302. Ohkusa T, Koido S. Intestinal microbiota and ulcerative colitis. J Infect Chemother 2015;21:761–8.
303. Serban DE. Microbiota in inflammatory bowel disease pathogenesis and therapy: is it all about diet? Nutr Clin Practice 2015;30:760–79.
304. Klag T, Stange EF, Wehkamp J. Defective antibacterial barrier in inflammatory bowel disease. Dig Dis 2013;31:310–6.
305. Atreya R, Neurath MF. IBD pathogenesis in 2014: molecular pathways controlling barrier function in IBD. Nat Rev Gastroenterol Hepatol 2015;12:67–8.
306. Lee SH. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intestinal Res 2015;13:11–8.
307. Chichlowski M, Hale LP. Bacterial-mucosal interactions in inflammatory bowel disease: an alliance gone bad. Am J Physiol Gastrointestinal Liver Physiol 2008;295:G1139–49.
308. Michielan A, D'Inca R. Intestinal permeability in inflammatory bowel disease: pathogenesis, clinical evaluation, and therapy of leaky gut. Mediators Inflamm 2015;2015:628157.
309. Tang Y, Forsyth CB, Keshavarzian A. New molecular insights into inflammatory bowel disease-induced diarrhea. Expert Rev Gastroenterol Hepatol 2011;5:615–25.
310. Coskun M. Intestinal epithelium in inflammatory bowel disease. Front Med 2014;1:24.
311. Merga Y, Campbell BJ, Rhodes JM. Mucosal barrier, bacteria and inflammatory bowel disease: possibilities for therapy. Dig Dis 2014;32:475–83.
312. Anderson JM, Van Itallie CM. Physiology and function of the tight junction. Cold Spring Harb Perspect Biol 2009;1:a002584.
313. Chen SJ, Liu XW, Liu JP, Yang XY, Lu FG. Ulcerative colitis as a polymicrobial infection characterized by sustained broken mucus barrier. World J Gastroenterol 2014;20:9468–75.
314. DiGuilio KM, Mercogliano CM, Born J, Ferraro B, To J, Mixson B, et al. Sieving characteristics of cytokine- and peroxide-induced epithelial barrier leak: Inhibition by berberine. World J Gastrointest Pathophysiol 2016;7:223–34.
315. Hollander D, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. Ann Intern Med 1986; 105:883–5.
316. May GR, Sutherland LR, Meddings JB. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? Gastroenterology 1993;104:1627–32.
317. Wei Y, Zhu W, Gong J, Guo D, Gu L, Li N, et al. Fecal microbiota transplantation improves the quality of life in patients with inflammatory bowel disease. Gastroenterol Res Pract 2015;2015:517597.
318. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. Gastroenterology 2015;149:102–9.
319. Luhrs H, Gerke T, Muller JG, Melcher R, Schauber J, Boxberge F, et al. Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. Scand J Gastroenterol 2002;37:458–66.
320. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology 1992;103:51–6.
321. Cummings JH. Short chain fatty acids in the human colon. Gut 1981;22:763–79.
322. Valenzano MC, DiGuilio K, Mercado J, Teter M, To J, Ferraro B, et al. Remodeling of tight junctions and enhancement of barrier integrity of the CACO-2 intestinal epithelial cell layer by micronutrients. PLoS One 2015;10:e0133926.
323. Peng L, Li ZR, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr 2009; 139:1619–25.
324. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proc Natl Acad Sci U S A 2010;107:228–33.

325. Shimada Y, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, et al. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. PLoS One 2013;8:e80604.
326. Zakostelska Z, Kverka M, Klimesova K, Rossmann P, Mrazek J, Kopecny J, et al. Lysate of probiotic *Lactobacillus casei* DN-114 001 ameliorates colitis by strengthening the gut barrier function and changing the gut microenvironment. PLoS One 2011;6:e27961.
327. Laval L, Martin R, Natividad JN, Chain F, Miquel S, Desclee de Maredsous C, et al. *Lactobacillus rhamnosus* CNCM I-3690 and the commensal bacterium *Faecalibacterium prausnitzii* A2–165 exhibit similar protective effects to induced barrier hyper-permeability in mice. Gut Microbes 2015;6:1–9.
328. Carlsson AH, Yakymenko O, Olivier I, Hakansson F, Postma E, Keita AV, et al. *Faecalibacterium prausnitzii* supernatant improves intestinal barrier function in mice DSS colitis. Scand J Gastroenterol 2013;48:1136–44.
329. Alam A, Leoni G, Quiros M, Wu H, Desai C, Nishio H. The microenvironment of injured murine gut elicits a local pro-restitutive microbiota. Nat Microbiol 2016;1:15021.
330. Busquets D, Mas-de-Xaxars T, Lopez-Siles M, Martinez-Medina M, Bahi A, Sabat M, et al. Anti-tumour necrosis factor treatment with adalimumab induces changes in the microbiota of Crohn's Disease. J Crohn's Colitis 2015;9:899–906.
331. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 2014;63:116–24.
332. Darfeuille-Michaud A. Adherent-invasive *Escherichia coli*: a putative new *E. coli* pathotype associated with Crohn's disease. Int J Med Microbiol 2002;292:185–93.
333. Assa A, Vong L, Pinnell LJ, Rautava J, Avitzur N, Johnson-Henry KC, et al. Vitamin D deficiency predisposes to adherent-invasive Escherichia coli-induced barrier dysfunction and experimental colonic injury. Inflamm Bowel Dis 2015;21:297–306.
334. Kish L, Hotte N, Kaplan GG, Vincent R, Tso R, Ganzle M, et al. Environmental particulate matter induces murine intestinal inflammatory responses and alters the gut microbiome. PLoS One 2013;8:e62220.
335. Yang L, Yan Y. Protein kinases are potential targets to treat inflammatory bowel disease. World J Gastrointest Pharmacol Ther 2014;5:209–17.
336. Ramanan D, Bowcutt R, Lee SC, Tang MS, Kurtz ZD, Ding Y, et al. Helminth infection promotes colonization resistance via type 2 immunity. Science 2016;352:608–12.
337. Hansson GC. Role of mucus layers in gut infection and inflammation. Curr Opin Microbiol 2012;15:57–62.
338. Johansson ME, Sjovall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol 2013;10: 352–61.
339. Ciorba MA, Bettonville EE, McDonald KG, Metz R, Prendergast GC, Newberry RD, et al. Induction of IDO-1 by immunostimulatory DNA limits severity of experimental colitis. J Immunol 2010;184:3907–16.
340. Campieri M, Gionchetti P. Bacteria as the cause of ulcerative colitis. Gut 2001;48:132–5.
341. Mirsepasi-Lauridsen HC, Du Z, Struve C, Charbon G, Karczewski J, Krogfelt KA, et al. Secretion of alpha-hemolysin by Escherichia coli disrupts tight junctions in ulcerative colitis patients. Clin Transl Gastroenterol 2016;7:e149.
342. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr 2011;141:769–76.
343. Chang MY, Boulden J, Valenzano MC, Soler AP, Muller AJ, Mullin JM, et al. Bin1 attenuation suppresses experimental colitis by enforcing intestinal barrier function. Dig Dis Sci 2012;57:1813–21.
344. Thomas S, Mercado JM, DuHadaway J, DiGuilio K, Mullin JM, Prendergast GC. Novel colitis immunotherapy targets Bin1 and improves colon cell barrier function. Dig Dis Sci 2016;61:423–32.
345. Wirawan E, Lippens S, Van den Berghe T, Romagnoli A, Fimia GM, Piacentini M, et al. Beclin1: a role in membrane dynamics and beyond. Autophagy 2012;8:6–17.
346. McKnight NC, Zhong Y, Wold MS, Gong S, Phillips GR, Dou Z, et al. Beclin 1 is required for neuron viability and regulates endosome pathways via the UVRAG-VPS34 complex. PLoS Genet 2014;10: e1004626.
347. Hayaishi O, Ryotaro Y, Takikawa O, Yasui H. Indoleamine-dioxygenase— a possible biological function. In: Progress in Tryptophan and Serotonin Research. Berlin: Walter De Gruyter and Co.; 1984. p. 33–42.
348. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol Today 1999;20:469–73.
349. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 1999;189:1363–72.
350. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281:1191–3.
351. Mellor AL, Sivakumar J, Chandler P, Smith K, Molina H, Mao D, et al. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. Nat Immunol 2001; 2:64–8.
352. Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11:312–9.
353. Munn DH, Sharma MD, Hou D, Baban B, Lee JR, Antonia SJ, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest 2004;114:280–90.
354. Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends Immunol 2013;34:137–43.
355. Prendergast GC, Smith C, Thomas S, Mandik-Nayak L, Laury-Kleintop L, Metz R, et al. Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. Cancer Immunol Immunother 2014;63:721–35.
356. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med 2013; 210:1389–402.
357. Yoshida R, Hayaishi O. Induction of pulmonary indoleamine 2,3-dioxygenase by intraperitoneal injection of bacterial lipopolysaccharide. Proc Natl Acad Sci U S A 1978;75:3998–4000.
358. Yoshida R, Urade Y, Tokuda M, Hayaishi O. Induction of indoleamine 2,3-dioxygenase in mouse lung during virus infection. Proc Natl Acad Sci U S A 1979;76:4084–6.
359. Pfefferkorn ER. Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. Proc Natl Acad Sci U S A 1984;81:908–12.
360. Byrne GI, Lehmann LK, Landry GJ. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. Infect Immun 1986;53:347–51.
361. Beatty WL, Belanger TA, Desai AA, Morrison RP, Byrne GI. Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. Infect Immun 1994;62:3705–11.
362. Bozza S, Fallarino F, Pitzurra L, Zelante T, Montagnoli C, Bellocchio S, et al. A crucial role for tryptophan catabolism at the host/Candida albicans interface. J Immunol 2005;174:2910–8.
363. Silva NM, Rodrigues CV, Santoro MM, Reis LF, Alvarez-Leite JI, Gazzinelli RT. Expression of indoleamine 2,3-dioxygenase, tryptophan degradation, and kynurenine formation during in vivo infection with *Toxoplasma gondii*: induction by endogenous gamma interferon and requirement of interferon regulatory factor 1. Infect Immun 2002;70: 859–68.
364. Saito A, Motomura N, Kakimi K, Narui K, Noguchi N, Sasatsu M, et al. Vascular allografts are resistant to methicillin-resistant *Staphylococcus aureus* through indoleamine 2,3-dioxygenase in a murine

model. J Thorac Cardiovasc Surg 2008;136:159–67.
365. Knubel CP, Martinez FF, Fretes RE, Diaz Lujan C, Theumer MG, Cervi L, et al. Indoleamine 2,3-dioxigenase (IDO) is critical for host resistance against *Trypanosoma cruzi*. FASEB J 2010;24:2689–701.
366. Divanovic S, Sawtell NM, Trompette A, Warning JI, Dias A, Cooper AM, et al. Opposing biological functions of tryptophan catabolizing enzymes during intracellular infection. J Infect Dis 2012;205:152–61.
367. Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, et al. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. Nature 2008;451:211–5.
368. Muller AJ, Mandik-Nayak L, Prendergast GC. Beyond immunosuppression: reconsidering indoleamine 2,3-dioxygenase as a pathogenic element of chronic inflammation. Immunotherapy 2010;2:293–7.
369. Muller AJ, Sharma MD, Chandler PR, Duhadaway JB, Everhart ME, Johnson BAIII, et al. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. Proc Natl Acad Sci U S A 2008;105:17073–8.
370. Scott GN, DuHadaway J, Pigott E, Ridge N, Prendergast GC, Muller AJ, et al. The immunoregulatory enzyme IDO paradoxically drives B cell-mediated autoimmunity. J Immunol 2009;182:7509–17.
371. Metz R, Smith C, DuHadaway JB, Chandler P, Baban B, Merlo LM, et al. IDO2 is critical for IDO1-mediated T-cell regulation and exerts a non-redundant function in inflammation. Int Immunol 2014;26:357–67.
372. Metz R, Rust S, Duhadaway JB, Mautino MR, Munn DH, Vahanian NN, et al. IDO inhibits a tryptophan sufficiency signal that stimulates mTOR: A novel IDO effector pathway targeted by D-1-methyl-tryptophan. Oncoimmunology 2012;1:1460–8.
373. Merlo LM, Pigott E, DuHadaway JB, Grabler S, Metz R, Prendergast GC, et al. IDO2 is a critical mediator of autoantibody production and inflammatory pathogenesis in a mouse model of autoimmune arthritis. J Immunol 2014;192:2082–90.
374. Baban B, Chandler P, McCool D, Marshall B, Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase expression is restricted to fetal trophoblast giant cells during murine gestation and is maternal genome specific. J Reprod Immunol 2004;61:67–77.
375. Kanai M, Funakoshi H, Takahashi H, Hayakawa T, Mizuno S, Matsumoto K, et al. Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. Mol Brain 2009;2:8.
376. Bessede A, Gargaro M, Pallotta MT, Matino D, Servillo G, Brunacci C, et al. Aryl hydrocarbon receptor control of a disease tolerance defence pathway. Nature 2014;511:184–90.
377. Dzutsev A, Goldszmid RS, Viaud S, Zitvogel L, Trinchieri G. The role of the microbiota in inflammation, carcinogenesis, and cancer therapy. Eur J Immunol 2015;45:17–31.
378. Macho Fernandez E, Valenti V, Rockel C, Hermann C, Pot B, Boneca IG, et al. Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan- derived muropeptide. Gut 2011;60:1050–9.
379. Chang MY, Smith C, DuHadaway JB, Pyle JR, Boulden J, Soler AP, et al. Cardiac and gastrointestinal liabilities caused by deficiency in the immune modulatory enzyme indoleamine 2,3-dioxygenase. Cancer Biol Ther 2011;12:1050–8.
380. Muller AJ, DuHadaway JB, Chang MY, Ramalingam A, Sutanto-Ward E, Boulden J, et al. Non-hematopoietic expression of IDO is integrally required for inflammatory tumor promotion. Cancer Immunol Immunother 2010;59:1655–63.
381. Smith C, Chang MY, Parker KH, Beury DW, Du Hadaway JB, Flick HE, et al. IDO is a nodal pathogenic driver of lung cancer and metastasis development. Cancer Discov 2012;2:722–35.
382. Erdman SE, Rao VP, Poutahidis T, Ihrig MM, Ge Z, Feng Y, et al. CD4(+) CD25(+) regulatory lymphocytes require interleukin 10 to interrupt colon carcinogenesis in mice. Cancer Res 2003;63:6042–50.
383. Saleh M, Trinchieri G. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. Nat Rev Immunol 2011;11:9–20.
384. Gurtner GJ, Newberry RD, Schloemann SR, McDonald KG, Stenson WF. Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. Gastroenterology 2003;125:1762–73.
385. Harrington L, Srikanth CV, Antony R, Rhee SJ, Mellor AL, Shi HN, et al. Deficiency of indoleamine 2,3-dioxygenase enhances commensal-induced antibody responses and protects against *Citrobacter rodentium*-induced colitis. Infect Immun 2008;76:3045–53.
386. Zelante T, Iannitti RG, Fallarino F, Gargaro M, De Luca A, Moretti S, et al. Tryptophan feeding of the IDO1-AhR axis in host-microbial symbiosis. Frontiers Immunol 2014;5:640.
387. Zitvogel L, Galluzzi L, Viaud S, Vetizou M, Daillere R, Merad M, et al. Cancer and the gut microbiota: an unexpected link. Sci Transl Med 2015;7:271ps1.
388. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol 2013;13: 321–35.
389. Wang JL, Chang CH, Lin JW, Wu LC, Chuang LM, Lai MS. Infection, antibiotic therapy and risk of colorectal cancer: a nationwide nested case-control study in patients with Type 2 diabetes mellitus. Int J Cancer 2014;135:956–67.
390. Bonnet M, Buc E, Sauvanet P, Darcha C, Dubois D, Pereira B, et al. Colonization of the human gut by E. coli and colorectal cancer risk. Clin Cancer Res 2014;20:859–67.
391. Arthur JC, Perez-Chanona E, Muhlbauer M, Tomkovich S, Uronis JM, Fan TJ, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 2012;338:120–3.
392. Zhan Y, Chen PJ, Sadler WD, Wang F, Poe S, Nunez G, et al. Gut microbiota protects against gastrointestinal tumorigenesis caused by epithelial injury. Cancer Res 2013;73:7199–210.
393. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol 2014;12:661–72.
394. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature 2013;499:97–101.
395. Dapito DH, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell 2012;21:504–16.
396. Wroblewski LE, Peek RM Jr, Wilson KT. Helicobacter pylori and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev 2010;23: 713–39.
397. Konishi H, Fujiya M, Tanaka H, Ueno N, Moriichi K, Sasajima J, et al. Probiotic-derived ferrichrome inhibits colon cancer progression via JNK-mediated apoptosis. Nat Commun 2016;7:12365.
398. Urbaniak C, Gloor GB, Brackstone M, Scott L, Tangney M, Reid G. The microbiota of breast tissue and its association with breast cancer. Appl Environ Microbiol 2016;82:5039–48.
399. van't Veer P, Dekker JM, Lamers JW, Kok FJ, Schouten EG, Brants HA, et al. Consumption of fermented milk products and breast cancer: a case-control study in The Netherlands. Cancer Res 1989; 49:4020–3.
400. de Moreno de LeBlanc A, Matar C, Theriault C, Perdigon G. Effects of milk fermented by Lactobacillus helveticus R389 on immune cells associated to mammary glands in normal and a breast cancer model. Immunobiology 2005;210:349–58.
401. Hoption Cann SA, van Netten JP, van Netten C. Dr William Coley and tumour regression: a place in history or in the future. Postgraduate Med J 2003;79:672–80.
402. Coley W. The treatment of malignant tumors by repeated innoculations of erysipelas: with a report of ten original cases. Am J Med Sci 1893;10:487–511.
403. Nauts HC, Fowler GA, Bogatko FH. A review of the influence of bacterial infection and of bacterial products (Coley's toxins) on malignant tumors in man; a critical analysis of 30 inoperable cases treated by Coley's mixed toxins, in which diagnosis was confirmed by microscopic examination selected for special study. Acta Med Scand Suppl 1953;276:1–103.

404. Hoption Cann SA, van Netten JP, van Netten C, Glover DW. Spontaneous regression: a hidden treasure buried in time. Med Hypotheses 2002; 58:115–9.
405. Bassi P. BCG (Bacillus of Calmette Guerin) therapy of high-risk superficial bladder cancer. Surg Oncol 2002;11:77–83.
406. Bohle A, Jocham D, Bock PR. Intravesical bacillus Calmette-Guerin versus mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity. J Urol 2003;169:90–5.
407. Bohle A, Nowc C, Ulmer AJ, Musehold J, Gerdes J, Hofstetter AG, et al. Elevations of cytokines interleukin-1, interleukin-2 and tumor necrosis factor in the urine of patients after intravesical bacillus Calmette-Guerin immunotherapy. J Urol 1990;144:59–64.
408. Fleischmann JD, Toossi Z, Ellner JJ, Wentworth DB, Ratliff TL, Imbembo AL. Urinary interleukins in patients receiving intravesical Bacillus Calmette-Guerin therapy for superficial bladder cancer. Cancer 1989;64:1447–54.
409. Taniguchi K, Koga S, Nishikido M, Yamashita S, Sakuragi T, Kanetake H, et al. Systemic immune response after intravesical instillation of bacille Calmette-Guerin (BCG) for superficial bladder cancer. Clin Exp Immunol 1999;115:131–5.
410. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science 2013;342:971–6.
411. Liau LM, Black KL, Prins RM, Sykes SN, DiPatre PL, Cloughesy TF, et al. Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. J Neurosurg 1999;90:1115–24.
412. Liau LM, Black KL, Martin NA, Sykes SN, Bronstein JM, Jouben-Steele L, et al. Treatment of a patient by vaccination with autologous dendritic cells pulsed with allogeneic major histocompatibility complex class I-matched tumor peptides. Case Report. Neurosurg Focus 2000;9:e8.
413. Brossart P. Dendritic cells in vaccination therapies of malignant diseases. Transfus Apher Sci 2002;27:183–6.
414. So-Rosillo R, Small EJ. Sipuleucel-T (APC8015) for prostate cancer. Expert Rev Anticancer Ther 2006;6:1163–7.
415. Speiser DE, Lienard D, Pittet MJ, Batard P, Rimoldi D, Guillaume P, et al. In vivo activation of melanoma-specific CD8(+) T cells by endogenous tumor antigen and peptide vaccines. A comparison to virus-specific T cells. Eur J Immunol 2002;32:731–41.
416. Sampson JH, Archer GE, Mitchell DA, Heimberger AB, Herndon JEII, Lally-Goss D, et al. An epidermal growth factor receptor variant III-targeted vaccine is safe and immunogenic in patients with glioblastoma multiforme. Mol Cancer Ther 2009;8:2773–9.
417. Heimberger AB, Sampson JH. The PEPvIII-KLH (CDX-110) vaccine in glioblastoma multiforme patients. Expert Opin Biol Ther 2009;9: 1087–98.
418. Ragupathi G, Cappello S, Yi SS, Canter D, Spassova M, Bornmann WG, et al. Comparison of antibody titers after immunization with monovalent or tetravalent KLH conjugate vaccines. Vaccine 2002;20:1030–8.
419. Slingluff CL Jr, Yamshchikov G, Neese P, Galavotti H, Eastham S, Engelhard VH, et al. Phase I trial of a melanoma vaccine with gp100(280–288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. Clin Cancer Res 2001;7:3012–24.
420. La Rosa C, Wang Z, Brewer JC, Lacey SF, Villacres MC, Sharan R, et al. Preclinical development of an adjuvant-free peptide vaccine with activity against CMV pp65 in HLA transgenic mice. Blood 2002;100:3681–9.
421. Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. Nat Rev Drug Discov 2007;6:404–14.
422. Alexander RB, Rosenberg SA. Adoptively transferred tumor-infiltrating lymphocytes can cure established metastatic tumor in mice and persist long-term in vivo as functional memory T lymphocytes. J Immunother 1991;10:389–97.
423. Bartels CJ, Rosenberg SA, Yang JC. Adoptive cellular immunotherapy of cancer in mice using allogeneic T-cells. Ann Surg Oncol 1996;3:67–73.
424. Barth RJ Jr, Bock SN, Mule JJ, Rosenberg SA. Unique murine tumor-associated antigens identified by tumor infiltrating lymphocytes. J Immunol 1990;144:1531–7.
425. Bachanova V, Cooley S, Defor TE, Verneris MR, Zhang B, McKenna DH, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. Blood 2014;123:3855–63.
426. Bluming AZ, Ziegler JL. Regression of Burkitt's lymphoma in association with measles infection. Lancet 1971;2:105–6.
427. Coffey MC, Strong JE, Forsyth PA, Lee PW. Reovirus therapy of tumors with activated Ras pathway. Science 1998;282:1332–4.
428. Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N, et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat Med 2000;6:821–5.
429. Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM. Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science 1991;252:854–6.
430. Gromeier M, Lachmann S, Rosenfeld MR, Gutin PH, Wimmer E. Intergeneric poliovirus recombinants for the treatment of malignant glioma. Proc Natl Acad Sci U S A 2000;97:6803–8.
431. Dobrikova EY, Broadt T, Poiley-Nelson J, Yang X, Soman G, Giardina S, et al. Recombinant oncolytic poliovirus eliminates glioma in vivo without genetic adaptation to a pathogenic phenotype. Mol Ther 2008;16:1865–72.
432. Brown MC, Dobrikova EY, Dobrikov MI, Walton RW, Gemberling SL, Nair SK, et al. Oncolytic polio virotherapy of cancer. Cancer 2014;120: 3277–86.
433. Hobo W, Maas F, Adisty N, de Witte T, Schaap N, van der Voort R, et al. siRNA silencing of PD-L1 and PD-L2 on dendritic cells augments expansion and function of minor histocompatibility antigen-specific CD8+ T cells. Blood 2010;116:4501–11.
434. Marquez-Rodas I, Cerezuela P, Soria A, Berrocal A, Riso A, Gonzalez-Cao M, et al. Immune checkpoint inhibitors: therapeutic advances in melanoma. Ann Transl Med 2015;3:267.
435. Agarwala SS. Novel immunotherapies as potential therapeutic partners for traditional or targeted agents: cytotoxic T-lymphocyte antigen-4 blockade in advanced melanoma. Melanoma Res 2010;20:1–10.
436. Ansell SM, Hurvitz SA, Koenig PA, LaPlant BR, Kabat BF, Fernando D, et al. Phase I study of ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients with relapsed and refractory B-cell non-Hodgkin lymphoma. Clin Cancer Res 2009;15:6446–53.
437. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;369:122–33.
438. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 2011;364:2517–26.
439. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 2015;350:1079–84.
440. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science 2015;350: 1084–9.