Regulation of Host Immunity by the Gut Microbiota

Hannah Partney and Nissan Yissachar

Abstract Constant exposure to diverse microorganisms has accompanied human evolution and continues to shape immunological development throughout life. In mucosal tissues, both innate and adaptive arms of the immune system are required to support healthy mutualistic interactions with the resident microbiota, while aggressively fighting pathogenic infections. Technological breakthroughs over the past decade facilitated groundbreaking discoveries that transformed our understanding of intestinal immunology and established the gut microbiota as a critical factor that shapes immunological development and function. Indeed, alterations to microbiota composition (dysbiosis) are associated with a wide array of human diseases, including autoimmune diseases, chronic inflammation, the metabolic syndrome, and cancer. In this chapter, we discuss fundamental concepts that underlie microbiota-immune system crosstalks, and their subsequent impact on host immunity, in health and disease. Given the widespread scope of this growing research field, we focus primarily on adaptive immune responses induced by the gut microbiota and mediated by intestinal effector and regulatory T cells. We further highlight non-immune cellular components that facilitate homeostatic host-microbiota communications in the gut. Finally, we discuss how disruptions to microbiota-immune system interactions provoke inflammatory responses in the gut and beyond and propose that rationally designed microbiota-based therapy may serve as an innovative avenue for future therapeutics.

Keywords Gut microbiota · Immune system · Effector and regulatory T cells · Autoimmunity · Inflammation

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1 Introductory Remark: Overview of Intestinal Tissue Structure

From the esophagus to the stomach and the intestine, the gastrointestinal tract functions to digest and absorb nutrients and water and also serves as a one-way exit for waste products formed. More recently, the gut has been discovered as an invaluable environment for immunoregulation and housing specialized intestinal immune cells. For the purposes of this chapter, we will shortly summarize the anatomical makeup of the intestines as their particularities are what offer insight in understanding homeostatic and pathogenic inflammatory responses (for a detailed review, see [1]).

The small intestine is distinguished by its finger-like projections called villi while the large intestine is reflected by a flat epithelial surface more suitable for water absorption. There are three compartments making up the small intestine that, in total, reach 6–7 m in length: the duodenum, the jejunum, and the ileum. The large intestine, while much wider, measures approximately 1.5 m. The mucosa is the innermost layer of the intestines (i.e., adjacent to the lumen of the intestines) and plays an extremely important part in the immune reactions as it is exposed to the luminal environment consisting of symbionts and pathogens. Moving outwards lie the submucosa, muscularis, and serosa layers [1]. Taking a closer look at the mucosa, mucus, made up of glycoproteins called mucins (the most abundant being MUC2), acts as the buffer between the epithelium and lumen; a single, unattached mucus layer is secreted in the small intestine, and a combination of a loose mucus layer and epithelial-attached dense mucus inner layer is formed in the colon. Bacteria, which penetrate the single mucus layer of the small intestine and the outer mucus layer of the large intestine, reside in the mucus, feed on the glycoproteins, and interact with a number of immune mediators that affect the microbiota such as IgA, defensin, and lysozyme [2, 3]. Antibacterial mediators in the small intestine keep the bacteria away from the epithelium, and in the large intestine the inner mucus layer remains mostly impenetrable [3].

Specialized intestinal epithelial cells (IECs) that make up the epithelial layer serve as unconventional immune cells that sense luminal content and maintain homeostasis among the harsh conditions and constant exposure to antigens. Invaginations of the epithelium called crypts of Lieberkühn penetrate the underlying connective tissue (termed lamina propria (LP)) within the mucosa [4]. Transit-amplifying cells, generated by intestinal stem cells (ISCs), residing at the base of these intestinal crypts differentiate into mature cell types known as IECs; the entire process takes only 3–5 days and characterizes the intestines through rapid-cell proliferation. Enterocytes are absorptive columnar epithelial cells and are the most prolific out of all the IECs. Enteroendocrine and goblet cells are also found among the cell types migrating upwards to the tips of the villi that secrete hormones for digestive regulation and mucins with antimicrobial properties, respectively. Paneth cells are another specialized IEC with antimicrobial properties as well as MUC2-producing capabilities [3] that remain beside ISCs at the base of intestinal crypts. Further, tuft

cells are responsible for sensing luminal contents [4, 5]. Identified in the ileum by decreased microvilli, Peyer's patches are lymphoid follicles with a barrier made up of microfold (M) cells that also sense luminal content and transport antigens as well as bacteria into the LP [5]. M cells-mediated sampling of luminal antigens facilitates antigen presentation by cells of the innate immune system (i.e., intestinal dendritic cells) to cells of the adaptive immune system [1].

Throughout this chapter, we will review the main cellular components that underlie microbial regulation of the enteric immune system and promote local and systemic immunological homeostasis.

2 Regulatory T Cells and Gut Microbiota

Regulatory T cells (Tregs) are critical regulators of host immunity. Although they constitute about 10% of total CD4+ T cell populations, they control the activity of most immunological cell types (both innate and adaptive arms) and can be found in every organ in the body. Thus, Tregs are involved in autoimmunity, allergy, inflammation, anti-tumor responses, and more. Tregs express the lineage-specific transcription factor (TF) FoxP3, and co-expression of additional TFs differentiates between major Treg subsets. Along the gastrointestinal (GI) tract, Tregs that co-express Helios and Gata3 are abundant in the small intestine, while Tregs co-expressing the RAR-related orphan receptor RORγ are most abundant in the colon [6].

2.1 Molecular Mechanisms of Microbiota-Mediated Immunoregulation: The Case of Bacteroides fragilis

The importance of the gut microbiota to homeostatic immunological maturation and function has long been proposed, most notably by Elie Metchnikoff by the end of the nineteenth century [7]. In more recent years, fundamental mechanistic studies have pinpointed Myd88-dependent pathways and toll-like receptors (TLRs) as central factors for recognition of gut microbiota by the intestinal epithelium. These findings contribute to our understanding of gut homeostasis and protection from intestinal injury [8].

Until recently, bacterial molecules that facilitate host-microbiota mutualism remained enigmatic. Groundbreaking studies by Dennis Kasper and colleagues revealed that a bacterial polysaccharide A (PSA) expressed by the gut commensal *Bacteroides fragilis* [9, 10] directs proper development and maturation of the intestinal immune system [11]. Monocolonization of germ-free (GF) mice with *B. fragilis* alone (but not PSA-mutant *B. fragilis*) corrected systemic T cell deficiencies, T-helper 1/2 (Th1/Th2) imbalances, and abnormal lymphoid organogenesis

observed in GF mice. Supplementing in vitro co-cultures of dendritic cells (DCs) and T cells with purified PSA molecule resulted in PSA presentation by DCs and triggered dose-dependent expression of the Th1 cytokine interferon gamma (IFNγ). Similarly, in vivo colonization of GF mice with PSA-deficient *B. fragilis* resulted in Th2-mediated pathologies of the thymus, including outgrowth of B cell-like follicles in the thymic medulla. These disorders were probably caused by inability to maintain microbiota-mediated Th1/Th2 balance in GF mice, and it has been proposed that *B. fragilis*-derived PSA protects against immune-mediated pathologies [11].

In a follow-up study [12], Mazmanian and Kasper demonstrated that treating mice with purified PSA protects them from experimentally induced colitis. Purified PSA induced anti-inflammatory interleukin (IL)-10 expression and suppressed pro-inflammatory IL-17 expression in intestinal immune cells. Collectively, these findings illustrate that microbiota-derived molecules possess immunomodulatory capacity that may be harnessed for therapeutic purposes in chronic inflammatory diseases [12].

Deeper mechanistic investigations enhanced our understanding of the immunomodulatory capabilities of *B. fragilis*. This bacterium was shown to induce Treg development and to increase their suppressive capacity (including IL-10 production), an effect mediated by PSA not only when administered prior to the induction of inflammation, but also after the induction of experimental colitis in mice [13]. Furthermore, *B. fragilis* was shown to activate the TLR pathway in order to establish host-microbial symbiosis and to actively suppress immunity, rather than to induce pathogen elimination [14]. PSA was found to signal via TLR2 expressed on intestinal Tregs to promote immunological tolerance; in the absence of PSA, *B. fragilis* was unable to restrict inflammatory T helper 17 (Th17) cell responses. This study suggests that colonization of symbiotic bacteria is accompanied by immunological recognition of the microbiota via specific recognition molecules that discriminate between mutualistic commensals and pathogens [14].

In a different inflammatory model, TLR2-dependent sensing of *B. fragilis*-derived PSA was shown to protect against demyelination and inflammation of the central nervous system (CNS) in animal models for autoimmune multiple sclerosis (MS) (experimental autoimmune encephalomyelitis (EAE) in mice) thus suggesting a systemic anti-inflammatory effect for *B. fragilis* [15].

2.2 PSA Molecule Transportation to Host Cells

How are PSA molecules delivered to host cells? One mechanism of such interkingdom communication is the release of PSA by *B. fragilis* via outer membrane vesicles (OMVs), which are then sensed by TLR2-expressing DCs, resulting in induction of Tregs and production of anti-inflammatory cytokines [16]. *B. fragilis*-derived OMVs were shown to protect the host from experimentally induced colitis [16], yet this OMV-mediated protection requires *Atg16l1* and *Nod2* genes, which are

associated with an increased risk for developing inflammatory bowel disease (IBD) in humans [17]. The expression of *ATG16L1* in CD11c + DCs is required to trigger a non-canonical autophagy pathway in a NOD2-dependent manner, required for Treg priming and protection from colitis [17]. Accordingly, immunocytes derived from human Crohn's disease (CD) patients containing the *ATG16L1* T300A risk variant failed to promote Treg development in response to *B. fragilis* OMVs. Thus, genetic polymorphisms in CD susceptibility genes result in failure to sense microbiota-derived protective signals, collectively suggesting that gene-environment interactions underlie the etiology of IBD [17].

2.3 B. fragilis *Modulates* iNKT *Cells Activity*

In addition to PSA production, *B. fragilis* shapes the host immune system by another thought-provoking mechanism: controlling the activity of invariant natural killer T (iNKT) cells by bacterially derived inhibitory sphingolipids [18]. iNKT cells are critical regulators of both innate and adaptive immunity, due to their ability to quickly release large quantities of cytokines upon activation by lipid antigens presented by non-polymorphic major histocompatibility complex (MHC) class I-like, CD1d protein [19–21]. *B. fragilis*-derived inhibitory sphingolipids were shown to significantly inhibit iNKT cell proliferation in the colon, a process that takes place early in life, during neonatal development. As a result, colonic iNKT cell numbers are constrained throughout development and into adulthood, which subsequently protect the host from experimental iNKT cell-mediated, oxazolone-induced colitis. This novel mechanism may support future development of bacterially derived sphingolipids as a potential therapy for autoimmune and allergic disorders that are mediated by damaging activity of iNKT cells [18].

2.4 Tissue Tregs

In addition to the established function of the immune system in protection from microbial challenges, recent studies highlight another critical function in maintaining local and systemic homeostasis. For instance, distinct populations of Tregs that reside in non-lymphoid tissues, including the visceral adipose tissue, skeletal muscle, and colonic LP, promote tissue functions, repair, and homeostasis in response to local signals [22].

2.5 Colonic Tregs and the Microbiota

Over the past decade, a collection of ground-breaking studies identified specific microbial species within the human microbiome that, when transferred into mice, can induce Treg development in the intestine as well as systemically. Similarly, microbe-derived metabolites (such as short-chain fatty acids (SCFA)) were found to mediate some of these effects and emerge as a new therapeutic approach to immunological diseases, including autoimmunity and allergy.

Seminal work by Kenya Honda and his colleagues revealed that spore-forming members of the gut microbiome, specifically the *Clostridium* genus (clusters IV and XIVa), induce the development and colonic accumulation of Tregs [23]. Moreover, oral supplementation of these microbes into mice triggered resistance to experimentally induced colitis and allergic immunoglobulin E (IgE) responses [23]. In follow-up work, a rationally selected mixture of 17 bacterial strains (clusters IV, XIVa, and XVIII of *Clostridia*, which lack virulence factors) isolated from a healthy human gut induced colonic Treg development when transferred into mice [24]. In addition to increasing Treg abundance, these microbes also induced the expression of anti-inflammatory mediators such as IL-10 and inducible T cell costimulator (ICOS) in Tregs and ameliorated inflammatory and allergic responses in mouse models of colitis and allergic diarrhea [24].

A unique subset of colonic Tregs was recently identified and thoroughly characterized [25, 26]. Surprisingly, these colonic Tregs co-express the RAR-related orphan receptor RORγ (a lineage marker of pro-inflammatory Th17 cells) in addition to the expression of FoxP3. Yet in the context of colonic Tregs, RORγ controlled the expression of major effector molecules (including *Il23r*, *Cxcr3*, *Tbx21*, and *Havcr2*), and promoted a transcriptional signature that differs from its activity in Th17 cells. Interestingly, the majority of RORγ+Helios-colonic Tregs are induced by the microbiota, and specific bacterial species of the human gut microbiota induced the accumulation of colonic Tregs when transferred into GF mice [25, 26]. The functional role of microbiota-induced colonic Tregs was demonstrated by monocolonization of GF mice with selected bacterial strains that induced different levels of colonic Tregs. In these mice, the colitis score was improved in association with increased levels of colonic Tregs [25]. Another factor that favors the development of FoxP3 + Tregs and of FoxP3 + RORγ+ colonic Tregs over Th17 cells is the vitamin A metabolite, retinoic acid (RA) [27, 28]. In mice, vitamin A deficiency specifically blocked the development of RORγt+ Tregs but not of Helios+ Tregs or Th17 cells [25, 26]. Mice lacking RORγ in Tregs were generated in two independent studies [25, 26], resulting in the absence of colonic Tregs, and subsequently increased frequencies of GATA3+ Helios+ Tregs, and increased production of the type 2 cytokines IL-4 and IL-5 in T cells. Compared with their wild-type littermates, these mice were more sensitive to experimental colitis, as indicated by trinitrobenzenesulfonic acid (TNBS)–induced [25] and oxazolone-induced colitis models [26]. The latter colitis model is dependent on the type 2 cytokines IL-4 and IL-13, suggesting that RORγ+ colonic Tregs regulate type 2 immune responses.

Furthermore, mice lacking RORγt in Tregs were more resistant to helminth infection (*Heligmosomoides polygyrus*) due to the increased production of IL-4, IL-5, and IL-13 throughout the infection [26]. Collectively, these studies identify *RoR*γ as a bacterially modulated factor that directs distinct cellular and transcriptional immunological responses and regulates opposite functional outcomes.

2.6 Commensal Gut Bacteria with Immunomodulatory Abilities

The quest for microbes that naturally inhabit the human gut, and possess unique immunomodulatory abilities, was significantly advanced by a recent systematic in vivo screen [29]. Cohorts of GF mice were monocolonized at 4 weeks of age with 53 individual bacterial species (selected based on the Human Microbiome Project [30]) representing the variety of phyla in the human gut microbiota (phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria*). A comprehensive analysis (microbiologic, immunologic, and transcriptomic) was performed at 2 weeks post-colonization, spanning the small and large intestines, as well as lymphoid organs including draining lymph nodes and spleen. This study generated a broad atlas that systematically dissects immune system-microbiota interactions in the gut. It yielded both anticipated and unanticipated insights, such as the identification of microbial strains capable of driving Th17 cell development in the SI (reaching a similar level as driven by segmented filamentous bacteria (SFB) [31, 32]; the identification of an array of bacterial strains, encompassing a diversity of species, that could induce RORγ+ Tregs in the colon (further investigated at: [25]; and the identification of bacterial strains that modulate the enteric immune system immune in unexpected ways (i.e., expansion of colonic, IL-10-producing CD4+ T cells by *Veillonella* 6.1.27 species, and reduction of plasmacytoid dendritic cell (pDC) numbers by *L. rhamnosus*) [29]. Furthermore, these findings suggest that bacterially induced changes, both immunologic and transcriptional changes, are not related to a particular microbial phylogeny. The experimental approach selected in this study – examination of the immunological effects induced by individual bacterial strains, one by one, in a gnotobiotic environment – simplifies the immense complexity of the intestinal microbiota. Clearly, these important findings (and their underlying mechanisms) should be further evaluated in a "real-world" environment, where complex inter-species interactions (between different microorganisms and the host) take place in the gut.

2.7 Colonic Treg Abundance Is Determined by Maternal Transmission of IgA in Breast Milk

The intestinal immune system maintains a homeostatic balance that tolerates the heavy burden of mutualistic microbiota, while retaining the capacity to eliminate gastrointestinal pathogens. The abundance of intestinal Tregs in the tissue greatly influences this balance, as Tregs limit tissue inflammation but delay pathogen clearance. Diverse inbred mouse strains differ in their colonic RORγ+ Treg proportions, which are stable for life and transmitted through multiple generations. But how is the intestinal setpoint for RORγ+ colonic Tregs determined? A recent study approached this question by a series of cross-fostering experiments between two lines of inbred mice that differ in their colonic proportions of RORγ+ Tregs: the B6 background (in which RORγ+ Treg abundances were ~ 40%–60% of total colonic Tregs) and the BALB/c background in which much lower frequencies were observed (~20%) [33, 34]. In a sequence of elegant experiments, the authors showed that the RORγ+ Treg setpoint is not driven by genetics or maternal microbiota transfer. Instead, colonic RORγ+ Tregs form a double-negative regulatory loop with immunoglobulin A (IgA), in which variable amounts of IgA are transferred by mothers to their offspring. This results in differences in neonatal IgA coating of gut microbes, which in turn condition RORγ+ Treg proportions in adults and regulate levels of intestinal IgA+ plasma cells. This phenotype is transferred through multiple generations because in female offspring, IgA+ plasma cells expand and migrate in late gestation via the entero-mammary axis, resulting in the IgA differences that are reflected in their breast milk. Thus, the abundance of RORγt+ Tregs in mouse offspring and in subsequent generations is dictated by a non-genetic, non-epigenetic mode of maternal inheritance during a critical time window after birth through IgA presence in breast milk [34].

2.8 Regulation of Intestinal Tregs by Cytokines

The cytokine IL-2 plays a major role in the development, maintenance, and function of effector and regulatory T cells [35–37]. A recent study identified IL-2 as a central factor that maintains intestinal Tregs, and thus immunological homeostasis [38]. Interestingly, cellular and transcriptional analysis of intestinal IL-2-expressing immunocytes identified type-3 innate lymphoid cells (ILC3s) as the major source of intestinal IL-2. IL-2 expression by ILC3s was induced by interleukin (IL)-1β produced by macrophages in response to microbiota-derived signals, and in a MYD88- and NOD2-dependent manner. Loss of IL-2 production by intestinal ILC3s led to a significant reduction in peripherally induced small intestinal (but not colonic) Tregs (detected by low neuropilin-1 expression) and an increase in Th1 effector T cells. Induction of small intestinal Tregs specific to the dietary antigen ovalbumin (OVA) was also impaired in mice deficient in IL-2-producing ILC3s, and

consequently OVA feeding did not induce oral tolerance in these mice. Intestinal biopsies collected from Crohn's disease (CD) patients contained reduced frequencies of both Tregs and ILC3s, compared with biopsies collected from healthy individuals. Remarkably ILC3s isolated from inflamed regions of resected small intestinal tissues of CD patients expressed low levels of *IL2* transcript compared with non-inflamed regions, and similarly the number of IL-2+ ILC3s was reduced in the terminal ileum of CD patients compared with healthy controls. Thus, microbiota-derived signals drive IL-1β production in macrophages, which triggers IL-2 secretion by ILC3s, required to maintain intestinal Tregs, oral tolerance to dietary antigens, and small intestinal homeostasis [38].

Another cytokine that regulates colonic Treg functions is the IL-1 family member, IL-33. In response to tissue damage, epithelial IL-33 functions as an intestinal danger signal (alarmin). Indeed, high levels of IL-33 were found in inflamed intestinal lesions of human IBD patients. Interestingly, ST2 (the IL-33 receptor) is highly expressed on colonic Tregs, and ST2 signaling promotes Treg function in the inflammatory environment, by supporting Treg accumulation and maintenance, and by inducing transforming growth factor (TGF)-β1-mediated Treg differentiation [39]. Intriguingly, IL-23, a pro-inflammatory cytokine involved in IBD pathogenesis, was found to confine Treg function by inhibition of IL-33 signaling in Tregs. Thus, the balance between IL-33 and IL-23 controls Treg responses and immunological tolerance in the intestine, in health and in disease [39].

2.9 Microbial Metabolites and Treg Induction: From Bench to Bedside

Despite significant advances in the identification of specific microbial strains within the human microbiota that drive anti-inflammatory (Tregs) or pro-inflammatory (Th17 cells) responses, the underlying molecular signals remain mostly elusive. Three groundbreaking studies revealed that the microbial metabolites, SCFAs, control Treg development and function to maintain homeostasis in the gut [40–42].

Undigestible dietary components, such as complex dietary fibers, are fermented by the microbiota in the colon. The metabolic by-products of this microbial fermentation are the SCFAs propionate, acetate, and butyrate, for which the luminal concentrations range from 50 to 100 mM under physiological conditions. However, in GF mice, SCFA concentrations are significantly reduced to ~10% of their concentrations in mice reared under specific pathogen-free (SPF) conditions [40]. Similarly, Treg abundance within the GF colon is similarly impaired. Strikingly, administering SCFAs to GF mice successfully restored Treg numbers and function (i.e., IL-10 production) in the colon. SCFAs could also augment Treg quantities and suppressive capacity in SPF mice. Moreover, administration of propionate [40] or butyrate [41, 42] alone could induce Treg differentiation and function, in vitro and in vivo. Functionally, SCFA administration to mice

ameliorated experimentally induced T cell–dependent colitis in a Treg-intrinsic, *Ffar2*-dependent manner. How do SCFAs exert their anti-inflammatory effects? Mechanistically, SCFAs signal directly on Tregs via the GPCR43 receptor, encoded by the *Ffar2* gene which is highly expressed by Tregs in response to microbiota-derived signals. SCFA signaling (most notably butyrate and propionate [41, 42]) inhibits histone deacetylase (HDAC) activity and thus triggers epigenetic chromatin modification (acetylation) at the *Foxp3* locus (CNS1, CNS2, and CNS3 enhancers) which promotes *FoxP3* transcription and Treg development. These findings mechanistically link colonic production of SCFAs (primarily butyrate and propionate) by microbiota-mediated fermentation of dietary fibers, with colonic Treg accumulation and the maintenance of anti-inflammatory immune homeostasis in the gut and systemically.

The substantial clinical potential of the use of SCFAs to treat autoimmune and chronic inflammatory diseases was evaluated in several follow-up studies. An impressive example of the effects of dietary fatty acids (FAs) on systemic autoimmunity was demonstrated for multiple sclerosis (MS), a T cell-mediated autoimmune disease of the CNS [43, 44]. Administration of propionic acid (PA) to mice, using the EAE model for MS, resulted in the accumulation of small intestinal Tregs, increased production of anti-inflammatory cytokines (i.e., TGF-β and IL-10), and an improvement in clinical outcome, as reflected by reduced inflammatory cell infiltration in the spinal cord, as well as reduced demyelination, and increased axonal preservation [44]. In sharp contrast, long-chain FAs (the most abundant component of the Western diet) exacerbated disease, by enhancing Th1 and Th17 differentiation in the small intestine via the p38-MAPK pathway in T cells [44]. Collectively, these findings show that dietary-induced changes in the gut modulate pro- and anti-inflammatory T-helper cell responses, leading to exacerbation or amelioration of CNS autoimmunity.

These findings were leveraged for a translational study that examined the effects of PA administration in human MS patients [43]. Metabolite analysis in serum and stool samples revealed that PA levels are reduced in MS patients, compared with healthy individuals. These reduced PA levels were accompanied by a dysbiotic gut microbiome composition, and altered Treg/Th17 cell balance, where MS patients exhibit increased levels of Th17 and a reduced level of Tregs in their serum.

MS patients (both therapy-naïve and as an add-on to already administered therapy) were then supplemented with PA pills (orally, on a daily basis). Remarkably, only 2 weeks following PA therapy, Th1/Th17 proportions were decreased, while Tregs proportions were significantly increased, and their suppressive capacity was augmented. In agreement, analysis of clinical data (including MRI scans) revealed a significant reduction in annual relapse rate (ARR), reduced disease progression, and an increase in volume of subcortical gray matter in MS patients under PA supplementation. These effects lasted at least 3 years after PA intake. Examination of the functional effects of post-PA microbiome was performed by transfer of microbiota samples collected from MS patients before and after PA therapy, into a 3D gut organ culture system. Surprisingly, post-PA microbiota elicited rapid transcriptional responses that correlate with Treg induction

(as determined by [45]), suggesting that PA therapy transforms gut microbiome composition into a Treg-inducing configuration. Collectively, these findings suggest that the anti-inflammatory effects of PA could serve as a potent immunomodulatory treatment that supplements available MS drugs, and may potentially benefit other autoimmune diseases [43].

2.10 Bile Acid Metabolites

In addition to SCFAs, two recent studies reveal another group of microbe-derived bioactive molecules that interact with the host and modulate colonic Treg development and function: these are microbiota bile acid (BA) metabolites [46, 47]. In the liver, hepatocytes synthesize primary BAs that are released into the small intestine (duodenum) to enable the absorption of lipids and fat-soluble vitamins. Although most BAs are utilized in the small intestine, a small portion (~5%) reach the colon, where the gut microbiota converts them into a complex collection of steroid hormones that regulate host metabolism (including energy balance and cholesterol metabolism) via nuclear- and G-protein-coupled receptors. Two independent studies revealed that enteric BA composition is determined by both dietary and microbial factors, which also modulate tissue accumulation and function of colonic RORγ+ Tregs.

In the first study, SPF and GF mice were fed either a nutrient-rich diet or minimal diet, in order to identify microbially modified metabolites that possess Treg-inducing capabilities [47]. Analysis of colonic RORγ+ Treg abundance detected lower numbers of colonic Tregs in both minimal-diet SPF mice and rich-diet GF mice, compared with rich-diet SPF mice. This effect was specific to the colon, as no difference in RORγ+ Treg abundance was detected in other regions of the GI tract or in other lymphoid organs (including mesenteric lymph nodes, Peyer's patches, thymus, or spleen). Colonic RORγ+ Treg frequency was recovered by switching from a minimal to nutrient-rich diet (in a microbiota-dependent manner), suggesting that dietary components modified by bacterial symbionts are probably responsible for colonic Treg induction. Colonizing mice with specific gut microbes that were genetically modified and could not support bacterial BAs metabolism resulted in a significant decrease of this colonic Treg population. Vice versa, restoration of the enteric BAs triggered colonic RORγ+ Treg expansion and ameliorated intestinal inflammation in experimentally induced colitis via BA nuclear receptors [47].

In the second study, major types of deconjugated BAs were screened for their ability to induce colonic Treg differentiation [46]. The secondary BA, 3-β-hydroxydeoxycholic acid (isoDCA), was found to increase Treg development by direct impact on dendritic cells (DCs), resulting in reduced immunostimulatory activity of DCs. In addition, genetic depletion of the farnesoid X receptor in DCs mimicked the effects of isoDCA on cellular transcription and Treg induction. Likewise, colonization of mice with engineered consortia of gut *Bacteroides* strains

that produce isoDCA induced colonic RORγ+ Treg differentiation and tissue accumulation [46].

Collectively, these studies identify a novel class of bioactive molecules that mediate the immunological effects of the gut microbiota, control immunological homeostasis in the host, and may be utilized as future therapeutic interventions (Fig. 1).

3 Effector T Cells and Gut Microbiota

3.1 T Helper 17 Cells: Development and Function

CD4+ T helper 17 (Th17) cells are characterized by the expression of the RAR-related orphan receptor RORγt and by the production of hallmark cytokines: IL-17, IL-22, and IL-21 [48–50]. Th17 cells are most abundant in the intestinal LP, where they control immunological responses to various extracellular bacterial and fungal infections (e.g., by promoting high-affinity secretory IgA humoral responses and antimicrobial proteins secretion by IECs). The cytokines IL-6 and TGF-β are required to induce Th17 differentiation from naïve T cell precursors, while IL-23 promotes the expansion of differentiated Th17 cells [48, 51]. TGF-β is required for differentiation of both Th17 and Treg cells, although development of these two cell types depends on distinct transcription factors (RORγt and FoxP3, respectively). Both TFs are co-expressed in naive CD4+ T cells, and the decision of antigen-stimulated T cells to differentiate into Th17 or Tregs is regulated by TGF-β concentrations, as well as the balance between TGF-β concentrations and the presence of pro-inflammatory cytokines such as IL-6 and IL-21 [52].

Besides their role in mediating immunity against infection, Th17 cells were shown to promote autoimmunity and inflammatory diseases in mouse models and in humans, including MS, IBD, rheumatoid arthritis, psoriasis, and more [53]. In addition to infiltration of pathogenic Th17 cells into the affected tissues during pathology, Th17 cells facilitate tissue accumulation of other immunocytes including other T-helper cells, macrophages, and neutrophils, which foster pathologic inflammation and tissue damage.

Thus, Th17 cells can promote both homeostatic, non-inflammatory roles (i.e., by supporting intestinal barrier integrity) and pathogenic, pro-inflammatory roles in many inflammatory diseases [54]. Interestingly, the gut microbiota regulates differentiation and function of homeostatic and pathogenic Th17 cells, as we will discuss below [55].

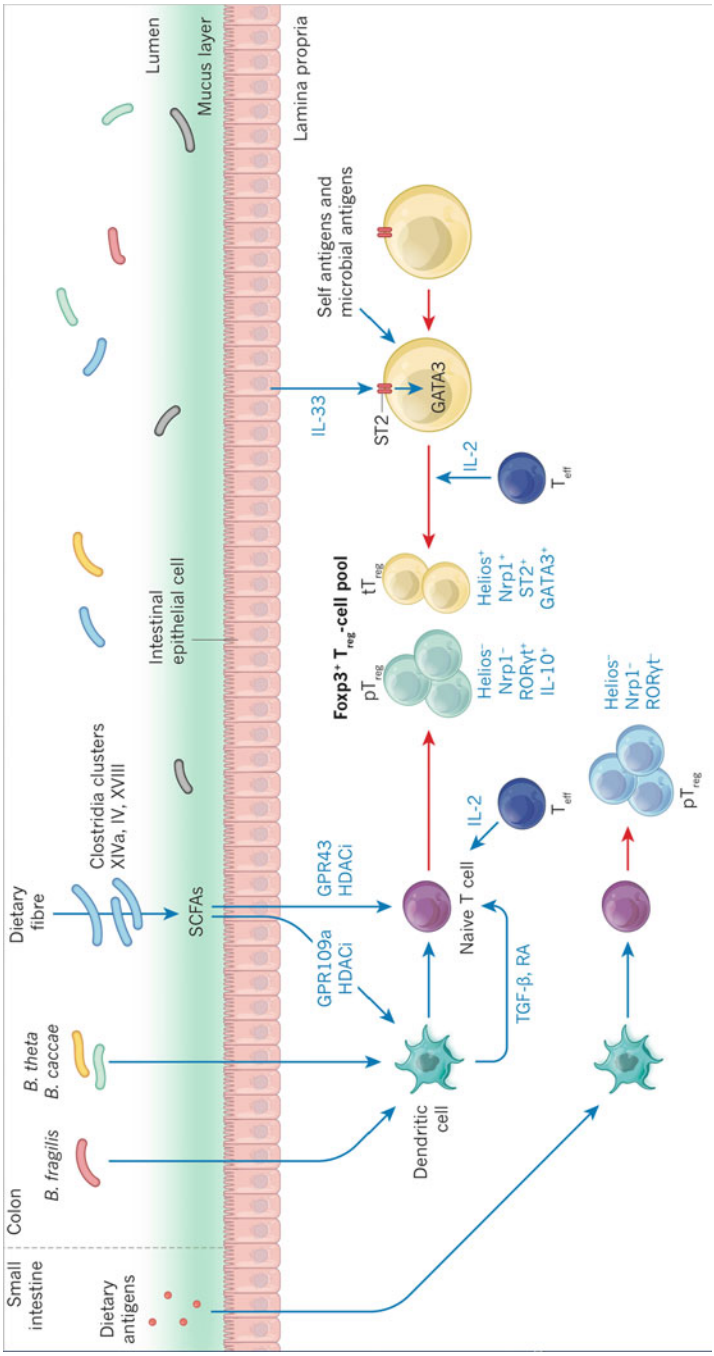

Fig. 1 Influence of the microbiota and diet on subsets of regulatory T cells in the intestine. Foxp3-expressing T_{reg} cells in the intestine can be subdivided into at least three subsets on the basis of their expression of RORγt, GATA3, Helios, and Nrp1. T_{reg} cells that express RORγt but not Nrp1 are induced at peripheral sites by antigens derived from the microbiota. Known as pT_{reg} cells, they are the main producers of IL-10, which suppresses the aberrant activation of myeloid cells, γδ T cells, and T_H17 cells. Dendritic cells produce mediators of pT_{reg}-cell differentiation, including TGF-β and retinoic acid (RA). Short-chain fatty acids (SCFAs), which are produced from dietary fiber by certain members of the microbiota, particularly species of Clostridia, also contribute to the induction of pT_{reg} cells. On binding to the G-protein-coupled receptor (GPR)109a on dendritic cells, SCFAs induce the expression of aldehyde dehydrogenase (ALDH), which

3.2 *Induction of Th17 Cells by Segmented Filamentous Bacteria (SFB)*

The notion that the gut microbiota controls the development and accumulation of intestinal Th17 cells was established by a series of seminal studies by Dan Littman and Ivaylo Ivanov [31, 56], a discovery that was followed up by intensive research efforts that deeply characterized how microbial signals regulate effector T cell development and function.

Littman and colleagues noticed that inbred mice purchased from different vendors markedly differ in their intestinal Th17 numbers, despite an identical genetic background [56]. Moreover, they showed that Th17 cells (which are particularly enriched in the small intestinal LP) do not develop in mice lacking microbiota. However, Th17 development could be induced by co-housing Th17 cell-sufficient mice with Th17-deficient mice through horizontal microbiota transfer.

Strikingly, transfer of a single bacterial species, segmented filamentous bacterium (SFB), is sufficient to induce Th17 development in the small intestinal LP [31, 57]. SFB was found to colonize mainly the small intestinal terminal ileum, and to tightly adhere to the intestinal epithelium. SFB colonization activated inflammatory gene expression as well as antibacterial defenses including antimicrobial peptides and mucosal IgA responses, collectively resulting in increased resistance to pathogenic infection by the murine pathogen *Citrobacter rodentium* [31, 57]. Moreover, SFB colonization induces the production of serum amyloid A (SAA) in the terminal ileum, which acts on DCs to promote Th17 cell differentiation. *Saa* genes (*Saa1/Saa2/Saa3*) encode acute-phase response proteins that function as cytokines to induce pro-inflammatory cytokine secretion (including IL-8, TNFα, IL-1β, and IL-23) during infection or tissue damage.

Of note, SFB-induced Th17 cells produce the Th17 hallmark cytokines IL-17 and IL-22 and are fully capable of promoting pro-inflammatory responses, not only during infection but also in an autoimmune context. This was demonstrated using the K/BxN mouse model for autoimmune arthritis [58]. Inflammatory arthritis in the

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Fig. 1 (continued) metabolizes vitamin A into RA. SCFAs entering dendritic cells act as inhibitors of histone deacetylase (HDACi) to suppress the expression of pro-inflammatory cytokines. They also directly act on naive T cells through GPR43 or the upregulation of Foxp3 expression through HDAC inhibition. IL-2 derived from T_{eff} cells probably helps to stabilize the differentiation of T_{reg} cells. Several species of *Bacteroides* contribute to the induction of pT_{reg} cells that express RORγt but not Nrp1 through dendritic cells. A second pool of pT_{reg} cells expresses neither RORγt nor Nrp1; these T_{reg} cells are induced by, and maintain immune tolerance to, dietary antigens. It should be noted that induction of pT_{reg} cells through dietary antigens occurs largely in the small intestine, whereas the induction of pT_{reg} cells by the microbiota occurs largely in the colon. T_{reg} cells that express both GATA3 and Nrp1 are thought to be generated in the thymus and are known as tT_{reg} cells. $GATA3^+$ T_{reg} cells express ST2 (a component of the IL-33 receptor that is also known as IL1RL1). IL-33, which is probably released from the epithelial cells of the intestine at steady-state, is markedly upregulated under conditions of inflammation. IL-33 acts with IL-2 (from T_{eff} cells) to induce the expression of GATA3 in T_{reg} cells. (Adapted from [55])

K/BxN T cell receptor (TCR) transgenic mouse model is provoked by an initiation phase and an effector phase [59, 60]. During the initiation phase, which relies on the adaptive immune system, a self-peptide derived from glucose-6-phosphate isomerase (GPI) is presented by the major histocompatibility complex class II (MHCII) molecule, Ag7, and recognized by T lymphocytes that display a transgenic TCR. These autoreactive T cells effectively help GPI-specific B cells to produce GPI autoantibodies that drive the effector stage, which is executed primarily by cells of the innate immune system. Here, GPI/anti-GPI immune complexes initiate an inflammatory response that recruits mast cells, neutrophils, the complement pathway, etc. Shortly after (beginning at 4 weeks of age), arthritis develops with almost 100% penetrance. Intriguingly, the effector phase can be mimicked by transfer of serum from K/BxN mice into wild-type healthy mice.

In an elegant study by the Mathis/Benoist lab [58], the authors demonstrated that autoimmune arthritis in the K/BxN mouse model was significantly attenuated under GF conditions, establishing a role for the gut microbiota in disease initiation and progression. Under GF conditions, the anti-GPI serum autoantibody titer was significantly reduced, and similar reduction was observed in splenic autoantibody-secreting B cells, splenic and small intestinal Th17 cells, and in overall ankle thickening. The pathological link to Th17 cells was further demonstrated by treating SPF K/BxN mice with neutralizing anti-IL-17 antibodies which prevented B-cell responses and arthritis development. Remarkably, monocolonizing GF mice with SFB alone restored the intestinal Th17 cell compartment, and autoantibody production, and rapid arthritis developed. These findings highlight the role of microbiota-induced Th17 cells in driving extra-intestinal autoimmune disease [58].

3.3 How Does SFB Trigger Intestinal Th17 Development?

Whole-genome sequencing and annotation analysis reveals that the 1.57 Mb SFB genome contains no virulence genes, despite its resemblance to pathogenic Clostridia [61, 62]. SFB was found to be an auxotroph for most essential cofactors and amino acids, and the SFB genome contains TLR5 agonists that are flagellin proteins, which stimulate Th17 cell production.

Intestinal induction of Th17 cells was shown to be mediated by MHCII molecules expressed by CD11c + intestinal dendritic cells (DCs) that present SFB antigens [63]. Comprehensive analysis of the TCR repertoire demonstrated that intestinal Th17 cells are specific for SFB- and other microbiota-derived antigens [64]. T cells having a TCR specific for SFB-derived peptides were shown to differentiate into Th17 cells. Moreover, T cell differentiation toward Th1 or Th17 lineages was found to depend on bacterial context, or which type of luminal bacteria delivered the antigen [64].

Further studies identified a role for the intestinal epithelium, as well as for type 3 innate lymphoid cells (ILC3), in mediating Th17 induction in the small intestinal ileum [65, 66]. Adherence of SFB to the ileal IECs was found to be a critical signal

for Th17 induction. Indeed, colonizing mice with a non-adherent SFB strain isolated from rats failed to induce Th17 cells. Epithelial adherence of SFB facilitates IL-22 secretion by ILC3 cells, which then trigger the production of serum amyloid A proteins 1 and 2 (SAA1/2) by IEC (in a STAT3-dependent manner). SAA1/2 proteins act directly on intestinal Th17 cells and amplify the production of IL-17A as well as other hallmark effector cytokines. Recently, SAA1/2/3 proteins were shown to function as pro-inflammatory factors that induce pathogenic Th17 cells and promote intestinal inflammation independently of TGF-β [67].

Interestingly, bacterial adhesion to the IEC was required for Th17 induction not only by SFB, but also by a wide array of Th17-inducing microbes, including the murine pathogen *Citrobacter rodentium*, *Escherichia coli O157*, and a mixture of 20 bacterial strains isolated from feces of a human colitis patient, which induced Th17 development in an IEC-adherence dependent manner [66]. The importance of epithelial adhesion was clarified in a recent study by using electron tomography, illustrating that SFB proteins are transferred into IECs by adhesion-directed endocytosis [68]. In this process, which is different from the endocytosis induced by invasive pathogens, proteins associated with bacterial cell wall are delivered into the cytoplasm of the IECs, eventually leading to the induction of Th17 cells. This mechanism illustrates how the host and the microbiota communicate under physiological conditions, and how T cell responses specific to commensal antigens are triggered by IECs [68].

3.4 Th17-Inducing Microbes in Humans

The identification of SFB as a potent inducer of intestinal Th17 cells in rodents motivated a search for microbial symbionts that naturally inhabit the human gut and possess Th17-inducing properties. A systemic approach for in vivo monocolonization of GF mice with a large collection of human symbionts led to the identification of several bacterial species that possess the ability to induce the development and tissue accumulation of Th17 cells in the small intestinal LP [32]. These phylogenetically diverse species provoked Th17 development as efficiently as SFB. The most robust Th17-inducer identified in this screen was *Bifidobacterium adolescentis*, a human symbiont that (together with other *Bifidobacterium* strains) can be found in the healthy human gut microbiota across age and geography. When introduced into GF mice, *B. adolescentis* was closely associated with the gut epithelium – similar to SFB. Moreover, as with SFB, *B. adolescentis* inoculation exacerbated autoimmune arthritis in the K/BxN mouse model. Yet the transcriptional responses induced by *B. adolescentis* in the small intestine were different than SFB-induced gene programs, suggesting that these two microbes elicit Th17 development using distinct molecular mechanisms. Overall, this study proposes that Th17-inducing bacteria exist in the healthy human microbiota, in numbers and diversity yet to be determined [32] (Fig. 2).

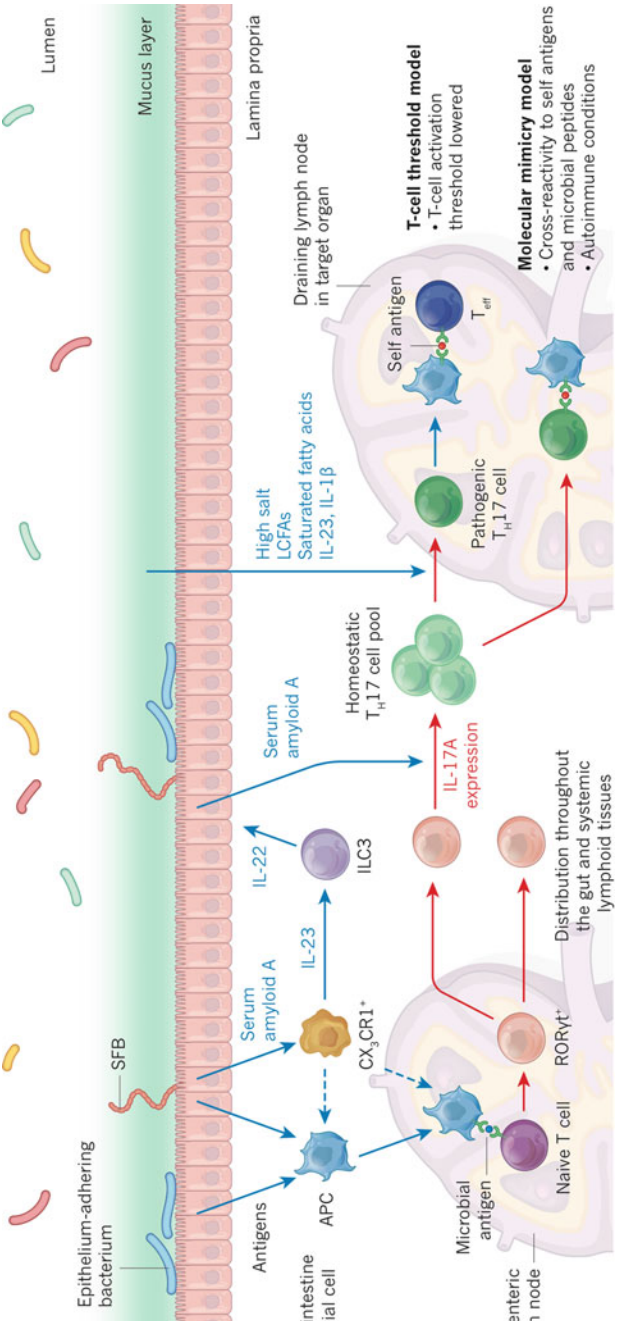

Fig. 2 Microbiota-mediated induction of T_H17 cells and autoimmunity. Epithelium-adhering bacteria initiate the differentiation of naive $CD4^+$ T cells into RORγt-expressing T cells (T_H17 polarized cells) (red) in the mesenteric lymph node through as-yet-undefined antigen-presenting cells (APCs). T_H17 polarized cells then accumulate and further differentiate into IL-17-expressing homeostatic T_H17 cells (green) in the lamina propria of the small intestine. These homeostatic T_H17 cells then stimulate epithelial cells to enhance the integrity of the intestinal mucosal barrier. The adhesion of segmented filamentous bacteria (SFB) elicits a unique program of gene expression in the epithelial cells, including the upregulation of serum amyloid A. Serum amyloid A from epithelial cells of the small intestine seems to function as a cytokine, and it modulates CX_3CR1-expressing cells (that are derived from monocytes) to produce IL-23, which stimulates the production of IL-22 by ILC3s. As well as its effects on CX_3CR1-expressing cells, serum amyloid A can stimulate RORγt-expressing T cells directly to upregulate the expression of IL-17A. Dendritic cells that express the antigens CD11b and CD103 have also been implicated in the expansion and maintenance of T_H17 cells (not shown). T_H17 cells become pathogenic when they are stimulated with IL-23, IL-1β, higher concentrations of salt, long-chain

3.5 Induction of Intestinal Th1 Cells by the Oral Microbiota

The oral cavity contains a large number of oral-resident bacteria which, in heathy individuals, hardly colonize the healthy intestine when ingested [69, 70]. However, transmission of oral bacteria to the gut can be detected under pathological conditions, such as IBD [71], liver cirrhosis [72], and colon cancer [73]. A recent study by Kenya Honda and collogues isolated a group of antibiotic-resistant *Klebsiella* strains from human saliva [74]. These oral bacteria were found to colonize the gut under dysbiotic conditions, where they act as potent inducers of pro-inflammatory, effector T-helper 1 (Th1) cells. Oral administration of isolated *Klebsiella* species to *Il10−/−* GF mice elicited potent induction of colonic Th1 cells, accompanied with severe gut inflammation. In agreement, the relative abundance of these *Klebsiella* species was significantly higher in human IBD patients compared with healthy individuals. These finding propose that the microbiota of the oral cavity may serve as a pool of pathobionts that exacerbate intestinal inflammation in genetically susceptible individuals [74].

Interestingly, periodontal inflammation within the oral cavity (periodontitis) facilitates the expansion of oral pathobionts, such as *Klebsiella* and *Enterobacter* species [75]. When ingested, these oral microbes trigger colonic inflammation by activating the inflammasome in colonic mononuclear phagocytes. Separately, Th17 cells that possess oral pathobiont specificity are generated in the oral cavity during periodontitis and migrate to the inflamed gut, where they are activated by translocated oral pathobionts (but not gut-resident microbiota) and elicit colitis development. Thus, parallel pathways of oral microbiota translocation and migration of orally generated effector T cells mediate and exacerbate gut inflammation [75].

3.6 Effector T Cells, Microbiota, and Disease

The concept that regulation of effector T cell activity by the gut microbiota may control both intestinal and systemic pathological processes was demonstrated in numerous studies over the past decade.

In the gut, a large-scale in vivo analysis of GF mice colonized with gut microbiotas from healthy and IBD individuals followed enteric T cell responses following colonization [76]. Interestingly, GF mice colonization with IBD-related

Fig. 2 (continued) fatty acids (LCFAs), and saturated fatty acids. Pathogenic T_H17 cells can migrate to the draining lymph nodes of target organs, where they contribute to autoimmune disease through cross-reactivity between peptides from microbes and self antigens (the molecular mimicry model). Alternatively, microbiota-specific T_H17 cells migrate to the lymph nodes and lower the threshold of activation of auto-reactive T cells such as T_{eff} cells (the T cell threshold model). (Adapted from [55])

microbiota promoted differentiation and enteric accumulation of Th17 and Th2 cells and decreased RORγt+ Tregs levels, thus altering the balance between Th17 and colonic Treg cells. Moreover, IBD microbiota transfer exacerbated disease in a mouse model of experimentally induced colitis. Strikingly, Th17/Treg proportions induced by each microbiota were predictive of disease status in the human donors, and of disease severity induced in the colitis mouse model. This study illustrates the impact of the gut microbiota in controlling the balance between inflammation and immunological tolerance in the gut, and emphasizes that the gut microbiota is a major factor determining IBD pathogenesis [77].

In the spinal cord, synergistic effects induced by two strains of gut microorganisms, OTU0002 (a newly isolated strain of the *Erysipelotrichaceae* family) and *Lactobacillus reuteri*, were shown to exacerbate spinal cord inflammation in mice by enhancing Th17 responses [78].

In the kidney, intestinal Th17 cells were shown to participate in an aggressive autoimmune kidney disease called crescentic glomerulonephritis (cGN) [79]. cGN is associated with high morbidity and mortality as it destroys the kidneys and leads to end-stage renal failure within weeks, or as little as several days. Th17 immune responses play a major role in cGN pathogenesis. Labeling and tracking intestinal Th17 cells using photoconversion of intestinal cells in Kaede mice upon glomerulonephritis induction led to the identification of Th17 cell migration from the intestine to the kidney. A functional role for intestinal Th17 cells in renal disease pathogenesis was demonstrated by depletion of Th17 cells (in GF mice and in mice treated with broad-spectrum antibiotics) which ameliorated disease, and by expanding Th17 cells (during *Citrobacter rodentium* infection) that exacerbated pathology. Thus, microbiota-induced Th17 cells migrate from the intestine to distal organs, where they participate in pathological immune responses [79].

In the eye, activation of retina-specific T cells was shown to be dependent on the gut microbiota [80]. These cells break through the blood-retinal barrier (which normally sequester retinal antigens to prevent pathogenic T cell activation) and promote the development of autoimmune uveitis, a major cause of blindness in humans. In this study, Caspi and colleagues used mice that express a TCR specific to interphotoreceptor retinoid binding protein (IRBP), a major uveitogenic epitope. These mice spontaneously develop uveitis at weaning age and reach 100% incidence by 2 months. The authors showed that retina-specific Th17 cells are activated by microbiota-derived signals in the intestinal lamina propria, leading to disease onset in the eyes. Interestingly, activation of retina-specific T cells was independent of the endogenous retinal autoantigen, but involved TCR signaling in response to non-cognate antigen in the gut. Thus, commensal microorganisms signal to autoreactive T cells that drive spontaneous uveitis in the eyes – an immune-privileged site. The notion that autoreactive T cells are activated by the gut microbiota might represent an underappreciated mechanism for autoimmunity in general [80].

In the heart, myocarditis is a chronic inflammation of the heart muscle associated with heart failure. It can be developed by chronic stimulation of Th1 and Th17 cells that specifically recognize myosin heavy chain 6 (MYH6)-derived peptides. Using a

mouse model of spontaneous autoimmune myocarditis, a recent study discovered that the progression of autoimmune myocarditis to dilated cardiomyopathy is microbiome-dependent [81]. Peptide mimics from commensal *Bacteroides* species were shown to imprint cardiac myosin-specific Th17 cells in the intestine, a process which could be prevented by antibiotics. In agreement, human myocarditis patients display immune reactivity against *Bacteroides* and cardiac myosin antigens, characterized by significantly elevated *Bacteroides*-specific CD4+ T cell and B cell responses. This study suggests that microbiome manipulation may restrain cardiotoxic T cell responses, and thus turn inflammatory cardiomyopathy into a targetable disease.

3.7 Diet, Microbiome, and Th17 Cells

Diet is one of the most significant factors that shapes human microbiome composition and function [82, 83]. In the past decades, several groundbreaking studies began to unravel the effects of diet on host immunity, and to identify mechanistic links that associate a diet-modified microbiome to immunological development and function.

One example is salt (sodium chloride), a nutritional component that is highly abundant in "western" diets (in which processed foods high in salt, sugar, and fat are abundant, and fresh fruits and vegetables are scarce). In addition to sodium in the plasma (in concentrations of approximately 140 mM), immunocytes are also exposed to sodium in the interstitial fluid and in lymphoid tissues, where sodium concentrations are significantly higher (between 160 mM and up to 250 mM). Two independent studies found that increased salt concentrations enhance Th17 induction in mice and humans [84, 85]. Mechanistically, high-salt conditions during cytokine-induced Th17 polarization activate the TF nuclear factor of activated T cells 5 (NFAT5) as well as serum/glucocorticoid-regulated kinase 1 (SGK1) via the p38/MAPK pathway. In response to increased salt concentrations, SGK1 promotes IL-23R expression by deactivation of mouse Foxo1, resulting in stabilization of the Th17 phenotype. These high-salt-induced Th17 cells present a pathogenic, pro-inflammatory phenotype, characterized by the production of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and IL-2. Moreover, these Th17 cells amplified autoimmunity in a mouse model for MS (murine EAE). Thus, increased dietary salt intake induces pathogenic Th17 cell development, and serves as a risk factor that promotes autoimmune inflammation [84, 85].

Another more recent study investigated the effect of high-salt intake on gut microbiome composition and Th17 development [86]. Interestingly, a high-salt diet broadly alters gut microbiome composition in mice, and particularly depletes the symbiont *Lactobacillus murinus*. In two disease models, EAE and salt-sensitive hypertension, supplementing mice with *L. murinus* cultures ameliorated pathological phenotypes by reducing Th17 levels. Moreover, a proof-of-principle study in healthy humans showed that a moderate high-salt challenge increased blood pressure

and Th17 cell abundance and reduced *Lactobacillus* species levels in the gut. Thus, Th17 induction via the diet-microbiome axis may account for the striking increase in the incidence of autoimmune diseases in developed countries, within the past half-century [87].

Another example is the effect of a ketogenic diet on microbiome composition and intestinal Th17 cell abundance. Ketogenic diets are very low in carbohydrate and very high in fat. This triggers a metabolic shift that increases the levels of circulating ketone bodies, and then uses them for energy production. A recent study illustrates that transition to a ketogenic diet modifies gut microbiome composition, including alteration in the main microbial phyla (*Bacteroidetes*, *Firmicutes*, and *Actinobacteria*) in a representative cohort of healthy humans [88]. Shotgun metagenomic analysis revealed that these changes were distinct from changes observed in response to a high-fat diet, and partly resulted from direct inhibition of gut bacteria, especially bifidobacteria species, by ketone bodies. Some of these microbes, such as *B. adolescentis*, were shown to induce Th17 development in the small intestinal LP [32]. Indeed, using monocolonizations and human microbiome transplantations into germ-free mice, the authors revealed that reduced levels of intestinal *B. adolescentis* during a ketogenic diet resulted in deficiency of intestinal Th17 induction, in part due to the production of ketone bodies. This study further demonstrates the importance of diet-microbiota interactions on pro-inflammatory Th17 cell development [88]. Collectively, understanding how dietary changes modulate host immunity via alterations to gut microbiome composition represents a novel and promising frontier that may lead to the development of personalized approaches to prevent and treat human diseases by dietary interventions.

4 Enteric Neuro-Immune-Microbiota Interactions

4.1 The Enteric Nervous System

The enteric nervous system (ENS) is the largest component of the autonomic nervous system with over 100 million neurons (comparable to the amount in the spinal cord) making up the ENS in humans. Intrinsic ENS sensory neurons (whose cell bodies inhabit the gut wall), interneurons, and motor neurons are spatially organized in two layers of interconnected ganglia: the myenteric (or Auerbach) plexus (MP) and the inner, submucosal (or Meissner) plexus (SP). In addition to enteric neurons, the ENS contains enteric glial cells (EGCs) that can be found in the enteric ganglia, the smooth muscle layer, and the LP. These complex neuronal networks synchronize smooth muscle contractions (MP) and control gut secretions and nutrient absorption (SP). Remarkably, the ENS can control gastrointestinal functions independently of CNS input (although normally, bi-directional communication between the CNS and ENS mutually regulate these two systems).

Many anatomical features, neurotransmitters, and signaling pathways are shared between the ENS and CNS. The use of cutting-edge approaches for single-cell RNA

sequencing allowed, only recently, deep characterization of the mouse and human ENS [89, 90]. Besides the generation of detailed cellular atlases of the ENS at single-cell resolution, and the identification of dozens of neuronal subsets, these studies revealed novel intercellular communication modules that connect the ENS to the intestinal epithelium, immune system, and stromal cells, as well as to extraintestinal organs such as the brain.

These studies are in line with emerging research, collectively suggesting that the close interactions of the ENS with cells of the enteric immune system and the microbiota profoundly affect host immunity and homeostasis [91, 92].

4.2 Neuron-Macrophage Interactions with the Microbiota Control Gut Homeostasis

Tissue-residing macrophages are highly abundant in all layers of the small and large intestine [1]. They preserve mucosal integrity by scavenging dead cells, promoting epithelial cell renewal and tissue remodeling, and producing the anti-inflammatory cytokine IL-10, in addition to their phagocytic activity towards pathogenic and commensal bacteria. After decades of simplistic partition of macrophage into "M1-like" and "M2-like" subtypes, they are now perceived as a highly heterogeneous and diverse population that retain specialized, tissue-specific properties [93].

In a seminal study, Bogunovic and colleagues identified a unique subset of muscularis macrophages (MMs) residing along nerve fibers in the colonic muscularis externa, forming close interactions with enteric neurons [94]. Intriguingly, these cells were found to regulate intestinal peristaltic contractions, as specific depletion of MMs resulted in intestinal dysmotility. Mechanistically, MMs were shown to produce high quantities of bone morphogenetic protein 2 (BMP2)—a secreted protein of the TGF- ß superfamily—and perturbations to BMP signaling altered gut peristalsis and accelerated colonic transit time. Remarkably, MM-derived BMP2 stimulated enteric neurons via BMP receptor II (BMPRII), to secrete the growth factor colony-stimulating factor 1 (CSF1), a crucial factor required for MM development and homeostasis. Strikingly, manipulating the gut microbiota (by antibiotic treatment) altered MM-ENS crosstalks and impaired GI motility, however this phenotype could be restored by microbial reconstitution.

A follow-up study by Mucida and colleagues further characterized the interactions between enteric macrophages and neurons using a combination of state-of-the-art transcriptional, genetic, and imaging techniques [95]. Substantial differences in transcriptional profiles were identified by comparing lamina propria (LpM) and muscularis (MM) macrophages, especially in genes related to immunologic and metabolic processes. Gene expression in MMs resembled alternatively activated (M2) macrophages, expressing tissue-protective genes which were upregulated following *Salmonella* infection, via neuron-derived adrenergic signaling that induced tissue-protective gene expression in MMs expressing the adrenergic

receptor ß$_2$AR (*Adrb2*). Taken together, both gut motility and innate responses to bacterial infection are controlled by reciprocal neuro-immune crosstalks. In both cases, microbiota-derived signals influence these interactions and thus control GI motility and tissue-protective responses to luminal perturbations [94, 95].

4.3 Neuronal Interactions with Enteric Innate Lymphoid Cells

The discovery and characterization of ILCs over the past decade greatly expanded our understanding of immunological regulation of tissue homeostasis [96]. ILCs are lymphocytes that do not express the classic, genetically rearranged, adaptive antigen receptors as T and B cells do. These cells sense, and quickly respond to diverse stimuli, and promote immunity by serving as the innate counterparts of T lymphocytes. Several ILC subtypes were identified, which mainly include type-1 ILCs (ILC1) that respond to intracellular pathogens with Th1 cells; ILC2s that respond to extracellular parasites and allergens with Th2 cells; and ILC3s that react to extracellular microbes along with Th17 cells. ILCs are especially abundant at barrier surfaces. In the gut, the activity of ILC2 and ILC3 cells was recently found to be regulated by enteric neurons and glial cells, in response to microbiota-derived signals.

4.4 Modulation of ILC2 Activity by Enteric Neurons

The neuropeptide neuromedin U (NMU) was recently identified as a potent stimulator of ILC2 activity and type 2 immunity in the gut and the lungs [97–99]. Interestingly, NMU is expressed in cholinergic neurons of the myenteric and submucosal plexus, and the receptor for NMU (Nmur1) is specifically expressed on ILC2 cells, which are close in proximity to these neurons. Functionally, neuronally derived NMU triggered potent ILC2 activation and proliferation, as well as extensive expression of the pro-inflammatory type-2 cytokines IL5, IL13, Amphiregulin (Areg), and Colony-stimulating factor 2 (Csf2). Mechanistically, stimulation of Nmur1 on ILC2 cells activated the calcineurin-NFAT signaling pathway and ERK1/2 phosphorylation, which mediated ILC2 responses to NMU. Using multiple in vivo models, neuronally derived NMU was shown to boost ILC2 responses to infection by the gastrointestinal helminth, *Nippostrongylus brasiliensis*, as well as to allergic lung inflammation, where NMU acts together with IL-25 to expand inflammatory ILC2. These findings reveal a novel neuro-immune circuit, in which cholinergic neuron-derived NMU rapidly stimulates ILC2 responses to promote type 2 immunity in mucosal tissues.

In addition to neuronally mediated ILC2 activation, two recent studies identified neuron-derived molecules that suppress intestinal ILC2 activity [100, 101]. In the first study [101], both murine and human ILC2 cells were shown to express high levels of the adrenergic receptor ß$_2$AR gene (*Adrb2*). Additionally, ILC2 cells were located near TH+ adrenergic neurons in the small intestine, suggesting that ILC2 cells may respond to the ß$_2$AR ligand norepinephrine (NE). Indeed, ß$_2$AR-deficiency enhanced ILC2 responses to *N. brasiliensis* infection, including excessive eosinophilia, goblet cells hyperplasia, and reduced parasite burdens. These results and others reveal that the adrenergic nervous system negatively regulates type 2 inflammation at mucosal tissues through ß$_2$AR signaling on ILC2 cells. Another study [100] identified a subset of ILC2 cells in the lungs and in the intestine that express high levels of the neuropeptide calcitonin gene-related peptide (CGRP; encoded by the gene *Calca*) and its receptor components. Here again, CGRP was found to act as a negative regulator of ILC2 activity and type 2 inflammation following parasitic infection.

4.5 Enteric Glial Cells Control ILC3 Responses to Luminal Microbes

Enteric glial cells (EGCs) are a fundamental component of the ENS and, similar to enteric neurons, form extensive cellular networks in the gut mucosa that were shown to be controlled by the microbiota [92, 102, 103]. EGCs are the main producers of neurotrophic factors including the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) neurturin (NRTN), artemin (ARTN), and persephin (PSPN), which modulate enteric neuronal function.

In a study by Veiga-Fernandes and colleagues, ILC3 were found to express high levels of RET, a tyrosine kinase receptor activated by GFLs [104]. Gain of function of RET increased the abundance of IL-22-producing ILC3, which acts on the intestinal epithelium, to express reactivity and repair genes, including antimicrobials and mucins. *Ret*-deficient mice were highly sensitive to experimentally induced colitis and to *Citrobacter rodentium* infection. In contrast, *Ret*-gain of function protected mice from developing colonic inflammation. Mechanistically, short-term activation of ILC3 by GFLs triggered ERK, AKT, p38, and STAT3 phosphorylation and increased *Il22* transcription, demonstrating a direct effect of RET signaling on ILC3 activity. Interestingly, both microbiota-derived products and host-derived alarmins (IL-1ß and IL-33) regulated GFL expression in glial cells, in a MYD88-dependent manner. Collectively, these findings reveal that enteric glial cells sense microbiota-derived products and host alarmins and control ILC3 activity and barrier defense in the gut.

4.6 *ENS-Derived Neuropeptides Control Intestinal T Cell Development*

The notion that the gut microbiota induces differentiation of naïve T cells into Tregs or Th17 lineages was well established over the past decade. Yet, despite significant progress, a comprehensive understanding of the cellular and molecular mechanisms by which complex microbial communities promote Tregs and Th17 development is still missing.

Microbiota-induced T cell differentiation occurs in a matter of days upon microbial exposure. Thus, most studies dissect immune responses in animal models, days or weeks after microbial colonization. The recent development of a microfluidic-based, 3D gut organ culture system allowed a zoomed in look at the early events that initiate Tregs and Th17 induction [45]. Monocolonization of intact gut fragments ex vivo with an array of human commensals revealed that Treg- and Th17-inducing microbes elicit rapid and diametrically opposite transcriptional responses from the enteric immune and nervous systems. Remarkably, these findings suggest that Treg/Th17-inducing microbes modulate the same intestinal pathways, but in an opposite direction than during initial encounter, resulting in opposite Treg/Th17 outcomes in the longer term. For example, the gene *Tac1* which encodes a precursor protein to four neuropeptides of the tachykinin family, including neurokinin A and substance P, was rapidly (2 h) downregulated by Treg-inducing microbes and upregulated by the Th17-inducing microbe SFB. In agreement, the frequency of colonic Tregs in *Tac1*−/− mice was higher than in their *Tac1*-proficient littermates. These findings suggest that immediate-early neuronal responses to the microbial colonization promote long-term Treg/Th17 development and balance immunological tolerance and inflammation [45].

A molecular mechanism by which microbial stimulation of enteric neurons controls colonic Treg development was recently discovered [105]. RORγ+ colonic Tregs were found to reside close to nitrergic and peptidergic nerve fibers in the colonic LP. In vitro, enteric neuron and Treg co-culture experiments indicated that enteric neurons prevented Treg induction, independently of cell contact. A search for ENS-secreted factors led to the identification of neuronally derived IL-6 as a major modulator of RORγ+ Treg formation. Conditional ablation of IL-6 in neurons increased total FoxP3 Treg cells but decreased the colonic RORγ+ subset. These findings suggest that microbial signals regulate enteric neuronal density and activation, thus controlling Treg cell generation and immunological tolerance in the gut (Fig. 3).

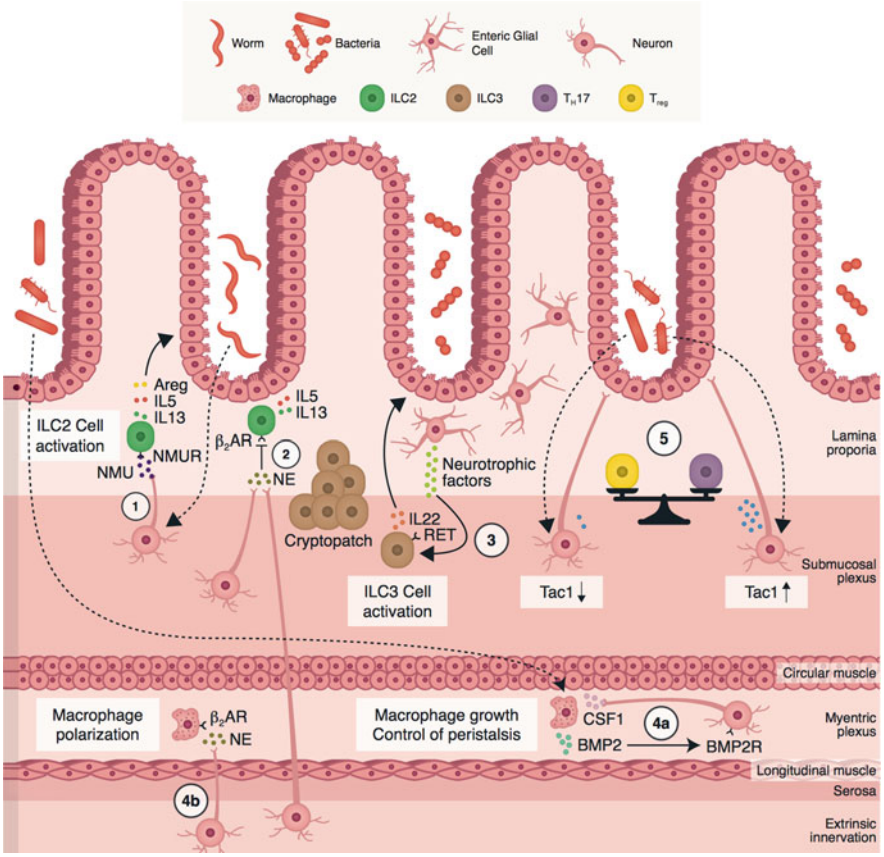

Fig. 3 Summary of enteric neuro-immune interactions, and their regulation by the gut microbiota. (1) Parasitic worm infection triggers neuropeptide neuromedin U (NMU) production by cholinergic neurons which activates NMU receptor (NMUR)-expressing innate lymphoid cells (ILC2s) and leads to type 2 cytokine production and host defense. (2) Adrenergic neuron-derived norepinephrine (NE) inhibits ILC2 proliferation and effector functions via ß2-adrenergic receptor ($ß_2$AR) signaling. (3) Neurotrophic factors secreted by enteric glial cells in response to bacterial infection and tissue damage activate RET-expressing ILC3s and trigger the release of tissue-protective interleukin (IL)-22. (4a) Microbiota-derived signals induce bone morphogenetic protein 2 (BMP2) release from myenteric macrophages. Neuronal signaling via the BMP2 receptor (BMP2R) stimulate enteric neurons to secrete colony stimulating factor 1 (CSF1) that supports macrophage growth and control of gut peristalsis. (4b) Adrenergic neuron-derived NE promotes myenteric macrophage polarization upon bacterial infection, via $ß_2$AR signaling. (5) Differential activation of enteric neurons by immunomodulating microbes rapidly control neuronal gene expression and neuropeptides production, ultimately affecting regulatory and effector T cell balance. (Adapted from [128])

5 Humoral Immunity and the Microbiota

Antibody-mediated immunity shapes gut microbiota composition and tissue localization and thus serves as a critical component in the establishment of life-long host-microbiota mutualism [106]. In the gut, large quantities of immunoglobulin A (IgA) antibodies are produced and secreted from intestinal plasma cells (PCs) in a T cell-dependent or independent manner. Dimerization of IgA antibodies is facilitated by the J chain polypeptide and polymeric immunoglobulin receptors, which facilitate transcytosis-mediated IgA secretion into the gut lumen. IgA antibodies protect from pathogenic infection and regulate symbiotic microbiota growth and diversity by binding to specific microorganisms, subsequently leading to bacterial neutralization and exclusion.

Experiments in GF mice illustrated that the production and secretion of bacteria-specific IgA antibodies is largely regulated by commensal microbiota [107, 108]. However, microbiota-induced IgA is considered to be of relatively low affinity and specificity, compared with pathogen-induced IgA. Thus, a dysbiotic microbiota developed in pathological conditions may drive high-affinity antigen-specific IgA responses, resulting in increased IgA "coating" of the dysbiotic microbiota compared with homeostatic microbiota. A recent study tested this hypothesis by the development of a unique flow cytometry-based method to isolate IgA-coated and uncoated bacteria from human and mouse fecal samples, and to analyze their taxonomic composition (IgA-SEQ) [109]. Remarkably, high IgA coating identified colitogenic bacteria in a colitis mouse model and in human IBD patients. These highly coated gut bacteria conferred significant susceptibility to colitis when transferred to GF mice. These findings propose that targeted elimination of highly IgA-coated gut bacteria may ameliorate or even prevent disease development [109].

In addition to their role in protection from pathogenic infection, antibodies play an important role in facilitating the establishment and stability of the gut microbiota. For example, the common human commensal *Bacteroides fragilis* was found to change its surface capsule architecture in order to induce specific IgA responses [110]. This IgA response enhances its epithelial adherence and allows *B. fragilis* to stably colonize a defined mucosal niche while gaining a competitive edge over exogenous bacterial competitors.

High affinity antibodies to various antigens are developed in B cells undergoing B cell antigen receptor (BCR) selection and affinity maturation in germinal centers, formed in response to infection or immunization. In the gut, germinal centers are chronically present due to constant exposure to antigens derived from diet and the microbiota. Interaction of antigens with cells of the immune system takes place in gut-associated lymphoid tissues (GALT), located in the mucosa and submucosa. Hypermutation of immunoglobulin genes takes place in gut-associated germinal centers (gaGCs) in the gut-draining mesenteric lymph nodes (mLNs) and Peyer's patches. A recent study followed the kinetics of clonal selection in steady-state gaGCs using a combination of cell-fate mapping techniques and single cell

sequencing of immunoglobulin genes [110]. Commensal-binding B cell clones are selected in gaGCs in steady-state, so that 5–10% of gaGCs from mice contain dominant B cell clones at steady-state. These B cell clones produce antibodies with increased binding properties to commensal bacteria. Overall, positive selection of B cells takes place in gaGCs under homeostatic conditions, at a rate that is tunable by microbiota presence and composition. Thus, targeted control of individual bacterial species shapes overall microbiota composition by the generation of specific antibody-mediated responses to the gut microbiota.

The multifaceted composition of the gut microbiota drives constant B cell stimulation and the development of complex and highly individualized immunoglobulin repertoires. In a recent study, Macpherson and colleagues used a unique mouse model that enables transient exposures of GF mice to different microbial taxa, followed by analysis of the B cell pool and immunoglobulin repertoires by deep sequencing [111]. Interestingly, exposing mice to the same microorganism (*Escherichia coli* strain HA107) but in different body sites (intestine or bloodstream) resulted in the generation of distinct immunoglobulin repertoires, characterized by distinct IgA or IgG diversity. Compared with mucosal exposure, the thresholds of systemic exposure are lower and result in responses that diversify the B cell and IgA repertoire. In addition to the effect of differing body sites, the order of exposures to different microbial antigens determines functional B cell immune responses and immunoglobulin repertoire generation. Collectively, these findings highlight the differences in humoral responses to mutualistic microorganisms at mucosal sites (restricted response) and to systemic exposure that elicits a flexible response required to avoid lethal sepsis.

Taken together, antibody-mediated humoral immunity is a crucial component in the establishment of healthy host-microbiota homeostasis, in controlling overall microbial load and bacterial tissue localization, and in shaping global microbiota composition, which in turn, regulate immunological development and function.

6 Perspective

Since the early 2000s, next-generation sequencing technologies have enabled deep characterization of microbiome composition in a culture-independent manner and revolutionized our understanding of host-microbiota interactions [112]. Pioneering studies introduced technical and computational methods to study the microbiome, initiating an explosion of research that analyzed microbiome composition in healthy individuals, throughout development, or in response to environmental and physiological changes associated with changes in diet or disease [30, 113–116].

Further studies established the realization that the gut microbiota is crucial for proper development and function of the immune system [55, 117], and promoted mechanistic dissections of the cells and molecules that mediate immunological

modulation by the gut microbiota. Translational and clinical studies supported the feasibility of microbiota-based therapies in treatment of recurrent *Clostridium difficile* infection using fecal microbiota transplantation (FMT) [118], and in boosting the efficacy of anti-cancer immunotherapy [119–125].

Although significant progress has been made over the past decade, many fundamental questions remain mostly unanswered. In the upcoming years, research efforts will likely address these questions in order to promote translational studies and clinical applications of microbiome-based therapeutics.

First, host-microbiota research will have to advance beyond a general description of changes to microbiome composition, to the identification of functional connections between specific microbial taxa and their physiologic impact on the host. Moreover, the relevance of an individual taxon to a specific host phenotype will have to be validated within the physiological context of the whole microbiota community. This may require the development of new experimental tools for the identification of functional host-microbe communication modules.

Another major issue is the relevance of current immunological research, mostly based on inbred lab mice, to real-life microbiota-immune system interactions in humans. One interesting approach to address this issue is the use of wildling mice—a combination of laboratory inbred mice colonized with natural "wild" microbiota—which stimulates physiological immune maturation, increases bench-to-bedside safety, and promotes more realistic pre-clinical immunological studies [126].

Additional challenges and questions stem from the substantial variability in immunological and microbial configurations between individuals. What is the definition of a "healthy" microbiome in an individual? Should microbiome-based therapeutics (such as FMT or probiotics) be tailored for each patient? Will universal immunomodulatory pathways be identified? Moreover, the available toolbox required for microbiome manipulation (including probiotics, prebiotics, bacterial-derived metabolites, and more) will have to be improved and expanded to support long-term colonization of desired microbial species, or to diminish an undesired microbial colonizer. For example, the use of bacteriophages is expected to enable precise depletion of specific bacterial strains from the microbial milieu [127].

Lastly, there is a critical need to set up an experimentally validated framework that defines guidelines for microbiome "editing" and facilitates rational intervention with microbiome configuration in order to safely shift a particular microbiome from an undesired ecological steady-state (dysbiosis) to a stable heathy state (eubiosis).

Collectively, based on the substantial technological and intellectual advancement over the past two decades, we anticipate that the near future will provide fascinating insights into microbe-immune crosstalks that will significantly expand our scientific knowledge and will revolutionize medicine as we know it.

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Compliance with Ethical Standards
The article does not contain any studies with human participants performed by the authors.

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