



Review

# The Enteric Nervous System and the Microenvironment of the Gut: The Translational Aspects of the Microbiome-Gut-Brain Axis

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**Abstract:** The proper functioning of the gastrointestinal tract is essential for digestion, absorption and the elimination of waste products. It protects us against pathogens, allergens and toxins, continuously monitoring and regulating the internal environment. The vast majority of these tasks are carried out by the nervous and immune systems of the gut in close cooperation by constantly adapting to internal and external stimuli, maintaining its homeostasis. In this review, we would like to summarize the most recent findings about the cytoarchitecture and functional microanatomy of the enteric nervous system and the immune microenvironment of the gut highlighting the essential role and inevitable molecular crosstalk between these two highly organized networks. Gut neuroimmunology is a rapidly evolving field and might help us to understand the etiology of inflammatory bowel disease and the systemic consequences of chronic intestinal inflammation. Finally, we also included a brief outlook to present the most recent research depicting the multifaceted role of the gut microbiome, its contribution to the gut-brain axis and human disease.

**Keywords:** enteric nervous system; muscularis macrophage; intraganglionic macrophage; barrier; gut neuroimmunology; microbiome; gut-brain axis



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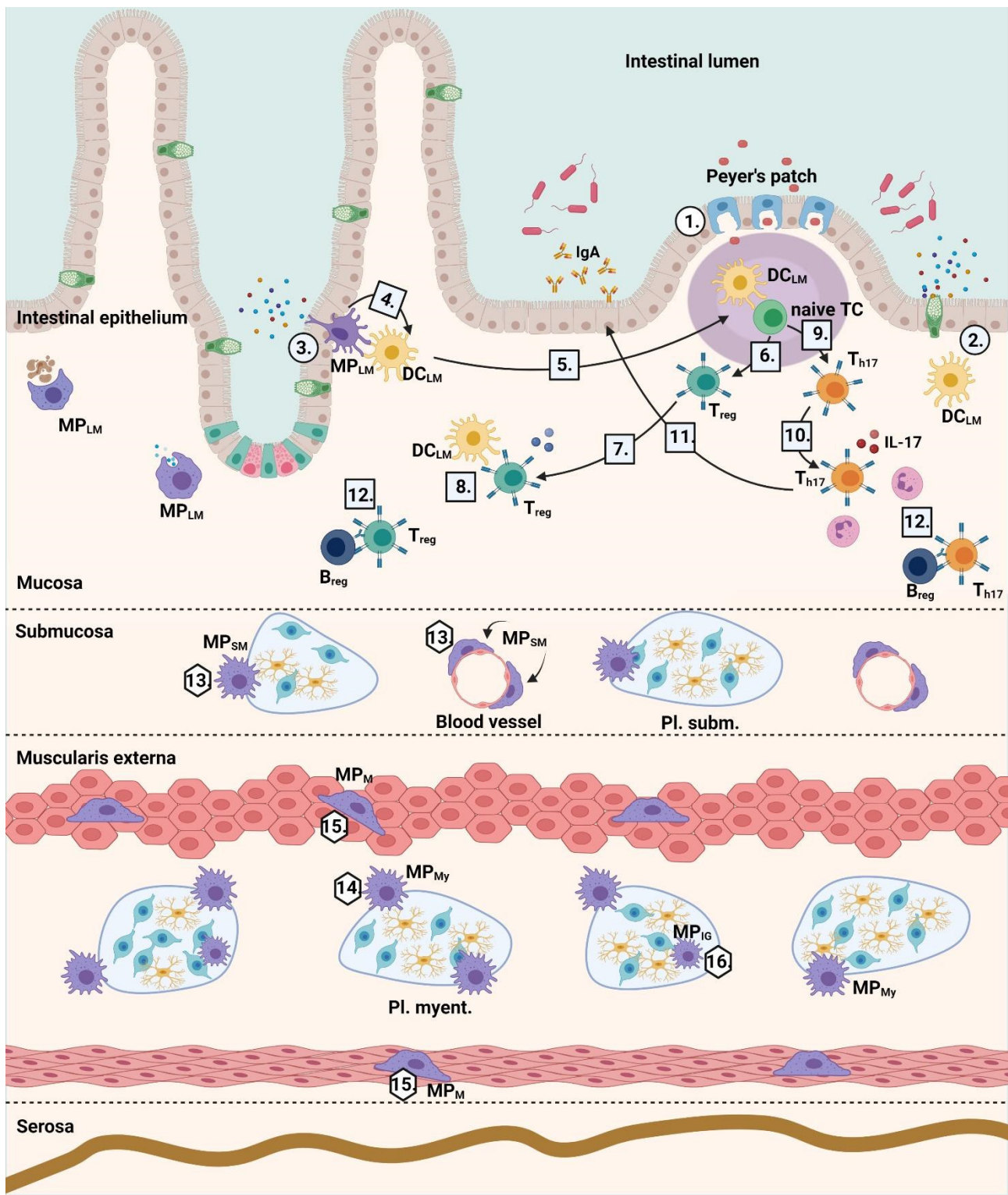
## 1. The Immune Microenvironment of the Gut

A properly functioning gastrointestinal tract digests food, absorbs nutrients and water and excretes the waste. To help digestion, it invites a microbiota of commensalists (symbionts), but inevitably faces hostile pathogens as well. The complex task of telling friend and foe apart is delegated to the innate and adaptive immune systems, the former is met with 'oral tolerance', while the latter is struggled by a controlled inflammation.

Antigens from the gut lumen make their way through the epithelium—via intestinal epithelial cells (IEC), macrophages (MP), or directly—into dendritic cells (DC; here considered as a subset of MP) of the lamina propria [1]. DCs then migrate to nearby lymph nodes to present those antigens to naive T cells (TC). TCs are familiar with 'friendly' antigens, which they encounter during their maturation in the thymus, thus they can respond selectively to luminal antigens. These 'friendly' antigens can induce a phenotype-shift to regulatory TCs ( $T_{reg}$ ) with anti-inflammatory effects, whereas foreign antigens promote their differentiation to IL-17 producing helper TCs ( $T_{H17}$ ), initiating a pro-inflammatory cascade with the recruitment of neutrophil granulocytes and natural killer cells (a group of innate lymphoid cells (ILCs)) [2,3]. The onset of IBD (Inflammatory Bowel Disease) is associated with the upset of this immunological equilibrium leading to uncontrolled inflammation [1].

The lumen of the gut is lined by intestinal epithelial cells (IECs) acting as a physical barrier for microbes: interconnected with tight junctions and coated with secreted mucus. Some IECs, goblet- and M cells (in the vicinity of Peyer's patches) help to deliver antigens to the subepithelial space via the transcytosis of microbes [4] (Figure 1 (1),(2)). In addition,

IECs have a role in the pro-inflammatory pathway, providing chemical protection by producing antimicrobials, cytokines, and IgA [5] (Figure 1).



**Figure 1.** (1) M-cells in the vicinity of Peyer's patches sample the luminal microbes via transcytosis. (2) Goblet cells deliver antigens to the subepithelial space. (3) Lamina propria mucosae macrophages (MP<sub>LM</sub>-s) grow transepithelial dendrites (TED) to phagocytose the antigens of the luminal microbial flora directly. (4) MP<sub>LM</sub>-s pass on their antigens to CD103<sup>+</sup> lamina propria mucosae dendritic cells (DC<sub>LM</sub>) through connexin-43-dependent gap junctions. (5) DC<sub>LM</sub>-s migrate to the

nearest lymph node. (6) Naive TCs differentiate to  $T_{reg}$  cells. (7)  $T_{reg}$  cells migrate to the mucosa where they produce anti-inflammatory interleukin 10 (IL-10). (8)  $T_{reg}$  cells induce the IL-10 production of  $MP_{LM}$  cell. (9) Naive TCs differentiate into  $T_{h17}$  cells. (10)  $T_{h17}$  cells migrate to the mucosa and produce IL-17 (IL-17A, IL-17F) which recruit further neutrophil granulocytes and ILC3 cells. (11)  $T_{h17}$  cells also produce IL-22 which induces IECs to produce antimicrobial peptides (RegIII- $\beta$  and RegIII- $\gamma$ ) and IgA. (12)  $B_{reg}$  cells fine-tune the quantities of  $T_{reg}$  and  $T_{h17}$  cells. (13)  $MP_{SM}$ : submucosal macrophage. (14)  $MP_{My}$ : myenteric macrophage. (15)  $MP_M$ : muscular macrophage. (16)  $MP_{IG}$ : intraganglionic macrophage. Created with BioRender.

Subepithelially, in the *lamina propria mucosae* (LM), reside the mononuclear population of lamina propria macrophages ( $MP_{LM}$ -s) and lamina propria dendritic cells ( $DC_{LM}$ -s), ordained to phagocytose antigens from the subepithelial space by growing transepithelial dendrites (TEDs) to sample the antigens of the luminal microbial flora [6]. TEDs do not compromise the integrity of the epithelial barrier, as they keep the adhesive structures of IECs intact (Figure 1, (3)).  $MP_{LM}$ -s pass on their antigens to  $CD103^+$   $DC_{LM}$ -s through connexin-43-dependent gap junctions [7,8] (Figure 1, (4)). Once equipped with antigens,  $CD103^+$   $DC_{LM}$ -s migrate to the nearest lymph node, where they activate naive TCs with TGF- $\beta$  and retinoic acid [9] (Figure 1, (5)). By presenting their antigens to TCs, handing over the response to the adaptive immune system,  $DC_{LM}$ -s delimit their role to a continuous 'surveillance' [10].

When friendly antigens (food or commensal microbiota) prime naive TCs, those differentiate to  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells [9] (Figure 1, (6)).  $T_{reg}$  cells migrate to the LM where they produce anti-inflammatory interleukin 10 (IL-10) and induce the IL-10 production of  $MP_{LM}$  cells as well (Figure 1, (7),(8)). IL-10 leads to the expansion of  $T_{reg}$  cells, as a self-reinforcing effect, while  $T_{reg}$  cells also inhibit effector TCs [5]. The latter phenomenon is called 'oral tolerance', an active immunosuppressive process. The presence of commensal flora in the gut is essential in developing oral tolerance. This is underpinned by the fact that, in germ-free animals, the host becomes susceptible to food allergies [11]. Moreover, certain kinds of intestinal bacteria (e.g., *Clostridia* spp.) increase oral tolerance by priming TCs towards  $T_{reg}$  phenotype [12,13].

Pathogen-activated naive TCs become  $T_{h17}$  cells [13].  $T_{h17}$  cells produce the pro-inflammatory interleukin, IL-17 (IL-17A, IL-17F) which recruit further neutrophil granulocytes and ILC3 cells [14,15]. ILC3 cells are a group of ILCs that act as part of the innate immune response to extracellular bacteria and intestinal commensalists that produces IL-22 [3] (Figure 1, (9)).  $T_{h17}$  cells also produce IL-22, which induces IECs to produce antimicrobial peptides (RegIII- $\beta$  and RegIII- $\gamma$ ) and IgA [16] (Figure 1, (10),(11)) supporting the local immune defense of the intestine. In healthy organisms, immunoprotection and tolerance are in a regulated balance. Regulatory B-cells ( $B_{reg}$ ) can maintain this balance through IL-10 production and the fine-tuning of  $T_{reg}$ - and  $T_{h17}$  cellular differentiation [17,18] (Figure 1, (12)).

Chronic inflammation occurs in the upset of this equilibrium. In IBDs, such as Crohn's disease or ulcerative colitis, large amounts of imperfectly differentiated macrophages (with monocyte-like phenotypes) migrate into the mucosa and produce inflammatory cytokines, which may then feed a self-reinforcing process of increasingly severe inflammation with long-term effects [19].

In addition to mucosal inflammation, the muscularis externa layer is also involved in the pathophysiology of IBDs. As recently shown, long-term motility decline and increased transit time are caused by macrophages associated with the smooth muscle cells and ganglia of the deeper layers of the intestine [20,21]. These are the submucosal macrophages ( $MP_{SM}$ -s), located in the highly vascularized surroundings of the submucosal plexus, and the muscular macrophages ( $MP_M$ -s) of the muscularis externa layer, situated in the vicinity of the myenteric plexus or in the circular and longitudinal smooth muscle layers [19] (Figure 1, (13)). Until recently, we had a sparse knowledge about the role of these macrophages.

While the embryonic cells of  $MP_{LM}$  macrophages are rapidly replaced after birth by monocytes of hematopoietic origin, migrating through the bloodstream,  $MP_{SM}$  and  $MP_M$

macrophages form a long-lived and self-sustaining population (resident macrophages) [22,23].  $MP_{SM}$  cells play an important role in supporting blood vessels, enteral neurons regulating intestinal secretion, as well as in tissue homeostasis [20] (Figure 1, (13)).

In their absence, a decrease of submucosal neural density and an increase of vascular permeability and disintegration occurs [24]. The primary task of  $MP_M$  cells is to regulate gastrointestinal motility [20,25,26], which we elaborate on in the following paragraphs.

## 2. The Heterogeneity of Muscularis Macrophages

$MP_M$  cells differ considerably from  $MP_{LM}$  cells both in terms of molecular expression profile and morphology. They are described as  $CD45^+$  and  $MHCII^+$  cells, with their high  $CX3CR1$  expression separating them from  $MP_{LM}$ -s and their low  $CD11c$  expression from  $DC_{LM}$ -s. In addition, they show high tissue protective *Retnla*, *Mrc1*, and *CD163* gene expression [24]. Morphologically, two types of  $MP_M$ -s are distinguished: bipolar with two long parallel protrusions, located between smooth muscle cells, or star-shaped ones, with microglia-like dendritic arborization, situated in close proximity to the myenteric plexus [27] (Figure 1, (14),(15)). Some  $MP_M$  cells were shown to be in direct contact with the smooth muscle cells and interstitial cells of Cajal (ICCs). It has been shown, that  $MP_M$ -specific expression of TRPV4 receptor directly controls GI motility by interacting with smooth muscle cells [27,28].  $MP_M$ -derived TRPV4 mediates the production of prostaglandin E2, which induces muscle contraction, a process independent of enteric neurons [28] (Figure 1, (15)). Furthermore,  $MP_M$ -s express BMP2 in the intestinal smooth muscle layer, which acts on enteric neurons. This is underlined by the fact that the blockade of BMP2 signaling or  $MP_M$  depletion, causes dysmotility and prolonged intestinal transit time in mice [25].

Myenteric macrophages ( $MP_{My}$ ) are described as  $MP_M$  cells closely related to the myenteric plexus and ENS ganglia [26] (Figure 1, (14)). Their maturation is largely influenced by factors of neural origin, as they express various neurotransmitter receptors, such as glycine-, nicotinic acetylcholine-, adrenergic-, and purine receptors [20]. Interestingly, these resident macrophages in mice already appear on the 8.5th embryonic day, before the formation of the myenteric plexus. Hence,  $MP_{My}$  may play an important role in the development of the myenteric plexus, and the survival of enteric neurons, since depletion of a group of  $MP_{My}$  causes a 50% decrease in the number of enteric neurons with consequent intestinal dysmotility in mice [20]. Characteristics and phenotypic distinction of intestinal macrophages are elucidated in Table 1.

Enteric neural cells play a pivotal role in gastrointestinal motility, secretion and—as discovered recently—in the regulation of the gut immune microenvironment. For comprehension of how the neuroimmunological crosstalk happens in the gut, first we summarize the latest knowledge about the diversity and function of this cell population.



**Table 1.** The heterogeneity of intestinal macrophages according to surface markers, morphology, localization and function.

Cell Type	DC <sub>LM</sub>	MP <sub>LM</sub>	MP <sub>SM</sub>	MP <sub>M</sub>	MP <sub>My</sub>	MP <sub>IG</sub>
Markers	CD45 MHCII CD11b F4/80 CX3CR1 <sup>+/−</sup> CD11c CD103 <sup>+/−</sup> connexin-43	CD45 MHCII CD11b F4/80 CX3CR1 <sup>low/+</sup> connexin-43	CD45 MHCII CD11b F4/80 CX3CR1 <sup>low/+</sup> CD11c <sup>low/+</sup>	CD45 MHCII CD11b F4/80 CX3CR1 Retn1a Mrc1 CD163 TRPV4 BMP2	MP <sub>M</sub> markers glycine-, nicotinic acetylcholine-, adrenergic-, purine receptors	MP <sub>M</sub> markers Iba1, CSF1R
Morphology	amoeboid	amoeboid	amoeboid	bipolar star shaped	star shaped	star shaped
Presence	Lamina propria mucosae	Lamina propria mucosae	Submucosa	Muscularis	Myenteric plexus	Within the ECM barrier of myenteric ganglions
Role	antigen presentation, TC activation	phagocytosis of antigens	supporting of blood vessels and enteral neurons regulating intestinal secretion tissue homeostasis	regulation of gastrointestinal motility	development of the myenteric plexus, survival of enteric neurons	phagocytosis of apoptotic ganglionic cells

### 3. Neurons and Neurochemistry of the Enteric Nervous System

The oldest classification of enteric neurons was based on morphology [29]; this usually distinguished two morphological subpopulations: ‘Dogiel type II’ neurons with several long processes and ‘Dogiel type I’ neurons with numerous short processes. However, the restrictive dichotomy of the morphological data does not reflect the well-known chemical and functional diversity of enteric neurons. Electrophysiological classification of enteric neurons is also useful in defined circumstances. The first experiments to capture their electrical properties (which later provided the basis for classification) were recorded in the 1970s [30–32], where S (synaptic)- and AH (after-hyperpolarizing)-type neurons were identified. Despite the practicality of this classification, this kind of nomenclature has become obsolete now. Other functional subsets of enteric neurons can be inferred based on their axonal projection patterns, their synthesizing enzymes, calcium-binding proteins, cytoskeletal proteins and expression of primary transmitters [33,34]. Nowadays, the enteric neurons are mostly divided into four main classes: motoneurons, intrinsic primary afferent neurons (IPANs), interneurons and intestinofugal neurons. Motoneurons directly innervate the smooth muscle, blood vessels or glands, while intrinsic primary afferent neurons process signals from the intestinal microenvironment and transmit them to interneurons. The intestinofugal neurons are a special group of neurons that synapse directly with neurons of the prevertebral sympathetic ganglia. Altogether, 14 different subtypes had been described previously (summarized in Table 2), but in a recent study, a new intrinsic afferent neuron subtype was also identified [35]. The substances that act as neurotransmitters in the central nervous system are also found in the enteric nervous system, so that in addition to the classical neurotransmitters, several neuropeptides, and other neurotransmitters (adenosine triphosphate (ATP) and nitric oxide (NO)) also play an important role. To date, 20 different enteric neuron types and projections have been isolated, each using a different neurotransmitter combination (chemical coding).

**Table 2.** Types of neurons in the enteric nervous system with their chemical coding and proportion.

# ID	Neuron Type	Incidence	Neurotransmitters
Myenteric neurons			
6	Excitatory circular smooth muscle motor neurons	12%	Acetylcholine/GABA (enkephalin, tachykinins)
4	Excitatory longitudinal smooth muscle motor neurons	25%	Acetylcholine/Calretinin (tachykinins)
7	Inhibitory circular smooth muscle motor neurons	16%	NOS/VIP (PACAP)
5	Inhibitory longitudinal smooth muscle motor neurons	~3%	NOS/VIP/GABA
1	Ascending interneurons (local reflex circuit)	5%	Acetylcholine/Calretinin (tachykinins)
8	Descending interneurons (local reflex circuit)	5%	Acetylcholine/NOS/VIP
9	Descending interneurons (secretomotor reflex circuit)	3%	Acetylcholine/Serotonin
10	Descending interneurons (migrating myoelectric complex)	4%	Acetylcholine/Somatostatin
2	Myenteric intrinsic primary afferent neurons (IPAN)	27%	Acetylcholine/Calbindin (tachykinins)
3	Intestinofugal neurons	1%	Acetylcholine/VIP (CCK, enkephalin)
Submucosal neurons			
12	Non-cholinergic secretomotor/vasodilator neurons	45%	VIP/Galanin
13	Cholinergic secretomotor/vasodilator neurons	15%	Acetylcholine/Calretinin
14	Cholinergic secretomotor non-vasodilator neurons	29%	Acetylcholine/NPY/CCK (somatostatin, CGRP)
11	Submucosal intrinsic primary afferent neurons	11%	Acetylcholine/Calbindin (tachykinins)

The neurons of the mammalian enteric nervous system can be divided into three major groups based on their transmitter molecules: cholinergic, catecholaminergic and the so-called non-adrenergic cholinergic (NANC) groups of neurons. The main neurotransmitter of the latter is nitric oxide (NO). In nitrergic neurons, the enzyme nitric oxide synthase (NOS) is responsible for the production of NO- which is formed by the metabolism of arginine. There are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Because of its short-lived free radical nature, NO is fundamentally different from classical neurotransmitters and synaptic vesicles but is delivered to the target cell as a diffusible gas, where it has no receptor in the usual sense. In the postsynaptic cell, NO directly activates the guanylate cyclase that catalyzes the synthesis of cyclic guanosine monophosphate (cGMP), which leads to an increase in the cell's cGMP levels. The proportion of nitrergic neurons varies in the different stages of the intestinal tract. The nitrergic neurons are essentially inhibitory motoneurons of the longitudinal muscle layer and are therefore also responsible for the inhibition of descending peristalsis via interneurons, resulting in aboral relaxation of the intestinal tract.

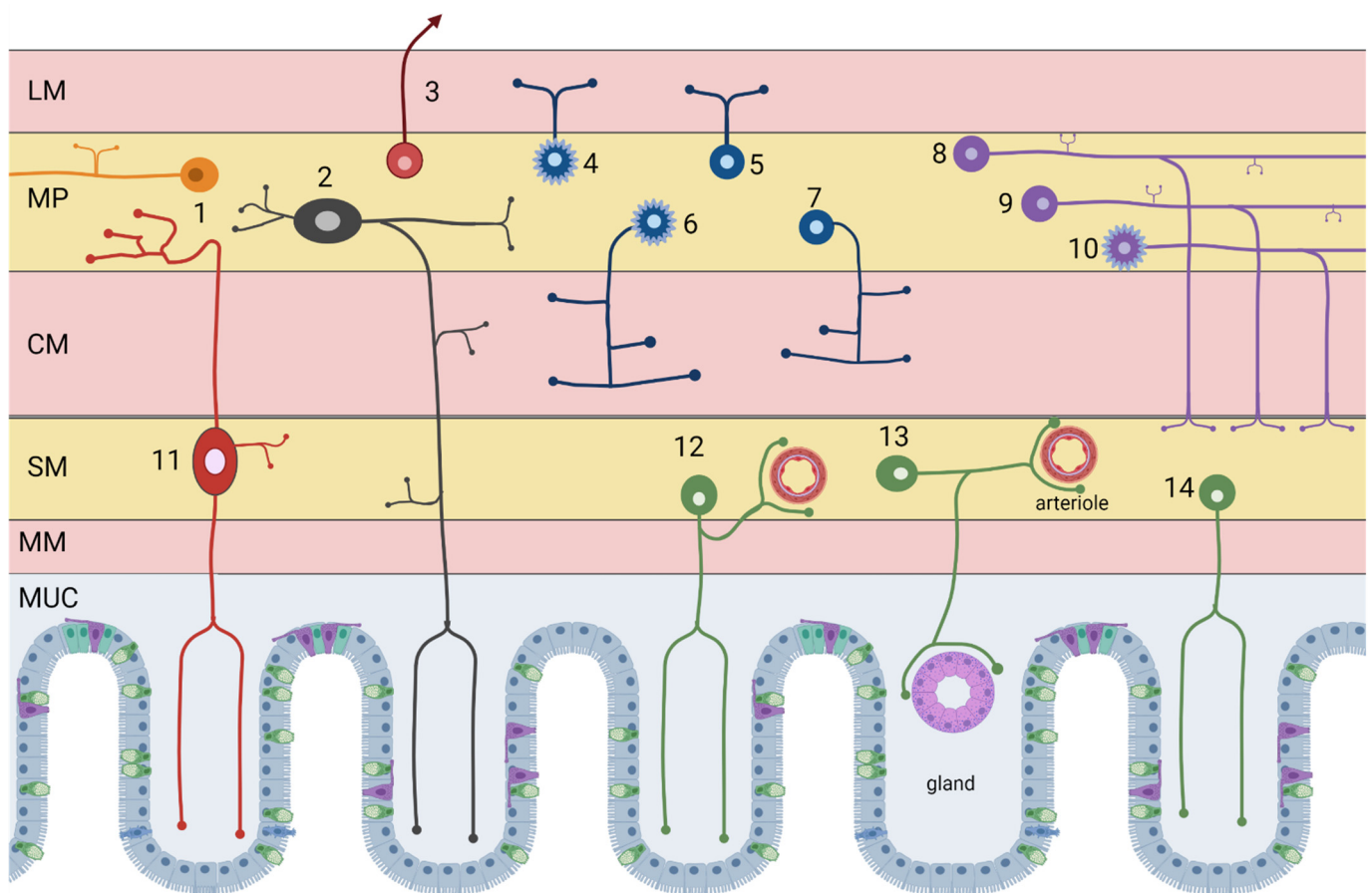
In the enteric nervous system, neuropeptides constitute a major group of signaling molecules: neuropeptide Y (NPY) is found in both the peripheral and central nervous systems. NPY-ergic neurons are found in all layers of the intestine; some of them are extrin-

sis, located mainly around blood vessels and synaptic in myenteric ganglia. The intrinsic NPY-ergic system includes motoneurons that inhibit the circular muscle layer, descending projections that modulate other neurons, and secretomotor neurons. It often co-exists as a co-transmitter with other neurotransmitters, such as NO or vasoactive intestinal peptide (VIP). Otherwise, the three NANC neurotransmitters have similar roles in the enteric nervous system: they can simultaneously affect motility, microcirculation, and secretion. Their inhibitory effects differ molecularly in that, while NO acts directly, NPY exerts its inhibitory effect on the intestinal wall smooth muscle via intrinsic cholinergic motor fibers. While VIP essentially executes the relaxation phase of the descending component of peristaltic movement, its homologue, substance P (SP), is responsible for the ascending, contractile component. Neurons expressing VIP are found in all four enteric neuron types. While NO modulates cGMP levels, VIP can activate the enzyme adenylate cyclase, thereby increasing cellular cAMP levels. In a subset of enteric neurons, VIP and NO act as co-transmitters in synergy to promote smooth muscle cell relaxation.

Glutamate acts in concert with other neurotransmitters. It is co-expressed with acetylcholine, SP, in rodent enteric neurons and its stimulatory effects on NOS have also been described. Glutamic acid produced in the intrinsic motor neurons of the myenteric plexus acts on two types of ionotropic receptors: the normally fast-response  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and the slowly activating *N*-methyl-D-aspartate (NMDA) receptors. Glutamate's special role is reflected in the fact that it was the first neurotransmitter to be detected in ascending intestinofugal projections that reach up to the brain. The role of this neurotransmitter in the functioning of the gut-brain axis has since become unquestioned.

Interestingly, one of the most common inhibitory neurotransmitters of the central nervous system, gamma-aminobutyrate (GABA), can depolarize postsynaptic cells in the alimentary canal. Smooth muscle contraction and relaxation can occur as a result of the GABAergic effect. GABAergic neurons appear to function as interneurons and can exert their effects on two types of receptors: excitatory GABA<sub>A</sub> and inhibitory GABA<sub>B</sub> receptors. Through the GABA<sub>A</sub> receptor, it exerts a motility stimulating effect on excitatory cholinergic and noradrenergic enteric projections, while it generates smooth muscle relaxation by activating nitrergic neurons. There is more evidence that GABA is also expressed in neurons of the submucosal plexus and is secreted paracrinally in mucosal enteroendocrine cells, thereby affecting absorption, secretion, and the intestinal immune system [36,37].

However, it is hard to build a universal classification of enteric neurons. Our conceptual understanding of enteric neurons and circuits comes from guinea pig studies: intrinsic afferent neurons, different types of ascending and descending interneurons, and muscle or mucosal motor neurons were identified in this species. Detailed knowledge on neuronal substances occurs from variety of gut segments from guinea pig, such as the small intestine [38,39], the colon [40] and the stomach [41]. Regional differences in the connectivity of the enteric nervous system (ENS) were observed in mice between the proximal versus distal colon, as well [42]. Our knowledge of human enteric neuron classes [43,44] and circuits [45] is much more limited due to the restricted access to human tissues. It is not possible to get a general picture—among other reasons—because of the differences between species. Furthermore, the 'one neuron—one function' concept is not supported anymore: intrinsic primary afferent neurons (IPANs) have been shown to be interneurons [46] and non-IPANs can also be regarded as sensory neurons [47,48]. Under experimental and pathological conditions the chemical coding of neurons may change [44]. The difficulty of combining different groupings, the difficulty of resolving discrepancies and technological advances have given rise to recent groupings based on scRNA sequencing data [49–52], which must be also integrated into the framework of ENS. The different cell types are shown in Figure 2 corresponding to their number.



**Figure 2.** Neuron types in the ENS that have been defined by their functions, cell body morphologies, chemistries, key transmitters and projections to targets. LM longitudinal muscle, MP myenteric plexus, CM circular muscle, SM submucosal plexus, MM muscularis mucosae, MUC mucosa. The numbers neuron types corresponds to the numbers in ascending interneurons; 1: Ascending interneurons; 2: Myenteric intrinsic primary afferent neurons (IPANs); 3: Intestinofugal neurons; 4: Excitatory longitudinal muscle motor neurons; 5: Inhibitory longitudinal muscle motor neurons; 6: Excitatory circular muscle motor neurons; 7: Inhibitory circular muscle motor neurons; 8: Descending interneurons (local reflex); 9: Descending interneurons (secretomotor and motility reflex); 10: Descending interneurons (migrating myoelectric complex); 11: Submucosal IPANs; 12: Non-cholinergic secretomotor/vasodilator neurons; 13: Cholinergic secretomotor/vasodilator neurons; 14: Cholinergic secretomotor non-vasodilator neurons. Created with BioRender, modified from Lomax, 2000 [40].

#### 4. Intraganglionic Macrophages and the Myenteric Plexus Barrier

Early histological studies identified macrophages closely juxtaposed to nerve fibers [53] and enteric neurons [27,54], but according to recent findings, some  $MP_M$ -s are entirely located within the extracellular matrix (ECM) barrier surrounding the myenteric plexus. These macrophages were identified as a unique cell population, called intraganglionic macrophages ( $MP_{IG}$ ) (Figure 1, (16)), characterized by the expression of multiple microglial markers and a distinct ultrastructure from their extraganglionic counterparts [21,55]. Although  $MP_{MY}$ -s include cells whose cell bodies occur outside the ganglion, with their extensions reaching in the intraganglionic space (periganglionic macrophages), haemopoietic cells that are entirely located within the neuronal microenvironment of the ENS and express an immunological signature similar to central nervous system's (CNS), microglia (CD45, CD11b, Iba1, CSF1R) is a novel finding that further stratifies the already heterogeneous population of  $MP_M$ -s [55].

It is hypothesized that MHC-II+ macrophages in the vicinity of ganglion cells phagocytose apoptotic ganglion cells [56]. This feature is reminiscent of the so-called “scavenger” function of microglia. Based on gene expression studies, intestinal macrophages are closest



to the microglia of the CNS [57]. These include genes related to the expression profile of the two cell populations: *Hnmt*, *Mtus1*, *C3a1A*, *Dagla*, *Wrb*, *Fkbp9* or *Gab1* [57]. The fractalkine receptor (*CX3CR1*) is one of the few molecular markers that are uniquely specific to intestinal macrophages and microglia, but not expressed by other tissue macrophages [8]. Signs of cellular degeneration, extensive cytoplasmic vacuolization and accumulation of swollen mitochondria but no membrane-blebbing or other morphological sign of apoptosis make  $MP_{IG}$ -s unique in terms of cellular morphology. Moreover,  $MP_{IG}$ -s show distinct ultrastructure from extraganglionic  $MP_{My}$ -s and have an active translational machinery and Golgi apparatus [21]. This might mean that  $MP_{My}$ -s and  $MP_{IG}$ -s have distinct roles in pathological conditions. A phenomena in the sciatic nerve underpins these observations, where transcriptionally unique perineurial and endoneurial macrophages are activated differentially in response to a crush injury [58].

Unlike the blood-brain-barrier (BBB) of the CNS we do not have much information about the blood-nerve barrier (BNB) protecting the ENS. While sensory dorsal root ganglia and autonomic parasympathetic and sympathetic ganglia include blood vessels, where monocytes can enter directly into the nervous tissue [59], there are no blood or lymphatic vessels inside enteric ganglia [60,61]. The extracellular matrix (ECM) capsule covering the myenteric plexus has a structure similar to the BBB, made of agrin and collagen matrix proteins and covered by glial endfeet, surrounded by a collagen-rich periganglionic space. Due to its similar barrier function, it was described as the intestinal myenteric plexus barrier (MPB) [21]. In a mouse model of DSS-induced intestinal inflammation, the MPB disintegrates and the intravenously injected 4 kDa FITC-dextran protein, which does not cross this barrier under physiological conditions, leaks inside the ganglia. The phenomenon is accompanied by an increase in  $MP_{IG}$  cell number and MPB degradation does not occur in the absence of  $M_M$ -s [21]. Neuronal or glial alteration and dysfunction in the ENS as a consequence of colitis were observed in multiple studies [62–64], with the direct causes underlying remaining unidentified. Disruption of the MPB might be responsible for long term dysmotility in IBD and other GI pathologies with acquired enteric neuronal dysfunction.

## 5. The Neuroimmunological Crosstalk in the Gut: The Central Role of the ENS

Alternating, peristaltic contractions of the smooth muscle layer of the intestinal tract (muscularis externa) are responsible for the gastrointestinal motility essential for nutrient transport and waste excretion, coordinated by the intrinsic system of the ENS. Although the neurons of the enteric ganglia receive extrinsic innervation from the sympathetic and parasympathetic fibers of the autonomic nervous system, they are able to function as a single autonomic unit, which is why the enteric nervous system is often referred to as the 'second brain' [65].

While there is extensive literature on the role of mucosal macrophages in gut immunity and secondary mucosal lymphatic tissue, little has been reported for a long time on macrophages of the muscularis externa. The two essential survival factors of the mononuclear cell line are *FLT3* and *CSF1*. *FLT3* is required for the survival of dendritic cells, whereas *CSF1R* is required for the survival of  $MP_M$ -s, as evidenced by the disappearance of macrophages from the muscularis externa in *CSF1R*—KO mice and after intraperitoneal administration of a *CSF1R*-blocking antibody [25]. While  $MP_M$  cells show an 80% depletion in response to the *CSF1R*-blocking antibody, the  $LpM$  population is not drastically affected by depletion treatments [25,66].

BMP-like growth factors also play a role in the interaction of  $MP_M$ -s and the ENS. *BMP2* and its receptor are well-known factors in the development and organization of embryonic smooth muscle and ENS [67,68]; *BMP2* expression is specific for  $MP_M$ -s in the intestinal smooth muscle layer, and blocking BMP signaling with dosomorphin, similar to the  $MP_M$ -depletion that is achieved with a *CSF1R* blockade, results in dysmotility and a prolonged intestinal transit time in mice [25]. In ex vivo colon cultures of mice treated with

anti-CSF1R antibodies, the addition of BMP2 can reduce the motility impairment caused by MP<sub>M</sub>-depletion [25].

BMP2 produced by MP<sub>M</sub>-s in the smooth muscle layer acts on the BMPRII receptor (BMPRII) of enteric neurons. Upon ligand binding of the receptor, a SMAD phosphorylation cascade is initiated [69], suggesting constitutive activation of MP<sub>M</sub>-s on enteric neurons mediated by BMP2 [25]. This study suggests that the ENS produces CSF1 as part of a macrophage-neuron crosstalk in 'exchange' for ensuring the survival of the MP<sub>M</sub> population. However, subsequent studies have suggested that MP<sub>M</sub>-s colonize the gut even in the absence of ENS, and that CSF1 is predominantly produced by mucosal cells rather than ENS neurons [54].

In addition to the chemical crosstalk between intestinal macrophages and the ENS, the presence of a luminal microbial flora is required for proper motility of the intestinal tract, as supported by the development of severe intestinal dysmotility in homozygous mice reared in a germ-free environment [70] and expressing the TLR4 (toll-like receptor 4) gene [71]. In germ-free and antibiotic-treated TLR4-KO mutant mice, alterations in the neuronal phenotype of the ganglion, in particular a reduction in the number of nitrergic neurons, are thought to be responsible for reduced intestinal motility [71]. Antibiotic treatment of mice *in vivo* clearly reduces BMP2 expression levels, which is due to the depletion of the MP<sub>M</sub> population. As a consequence of BMP2 deficiency, the number of pSMAD1/5/8 positive enteric neurons in the intestinal tract is also reduced. A direct link between the commensal flora, macrophages and ENS is also suggested by the finding that adding LPS to primary enteric neuron cell culture (LPS, a systemic inflammatory mediator released from bacteria that dissociate during infection) increases neuronal CSF1 secretion *in vitro* [25]. Supplementation of antibiotic-treated mice with LPS may prevent downregulation of ENS CSF1 expression and a reduction in the number of MP<sub>M</sub>-s [25]. These results suggest that constant immunological stimulation of MP<sub>M</sub>-s and enteric neurons by commensal flora is required to maintain healthy gut motility.

Both MP<sub>LM</sub>-s and MP<sub>M</sub>-s possess neurotransmitter receptors, such as  $\beta$ 2 adrenergic receptor ( $\beta$ 2AR), however, MP<sub>M</sub>-s show a much higher expression rate for the sympathetic neurotransmitter receptor. MP<sub>My</sub>-s, a population of MP<sub>M</sub>-s closely associated with the myenteric plexus of the ENS, show particularly high  $\beta$ 2AR expression levels. The receptor ligand, the major source of noradrenaline, tyrosine hydroxylase (TH), was shown to be positive extrinsic sympathetic neurons, whose central extensions come into proximity with enteric neurons and MP<sub>My</sub>-s from outside the muscular layer, through the serosa. TH+ neuronal extensions do not reach the lamina propria [26]. Catecholaminergic activation of the intestinal wall is predominantly mediated by postganglionic sympathetic fibers originating from the coeliac ganglion and the superior mesenteric ganglion, as confirmed by RiboTag cell labeling studies [26]. Noradrenergic sympathetic activation of the intestinal wall contributes primarily to the smooth muscle relaxation required for normal motility [72]. Oral administration of an enterocolitis-inducing bacterial strain (e.g., *Salmonella Typhimurium*) results in strong noradrenergic activation in the muscularis externa, with the source of this activation being the coeliac and the superior mesenteric ganglion. The released noradrenaline can indirectly regulate intestinal motility by acting on the  $\beta$ 2AR of MP<sub>M</sub>-s [26]. Intestinal infection or  $\beta$ 2AR activation by the adrenergic agonist salbutamol also induces changes in the gene expression profile of MP<sub>M</sub>-s, triggering an alternative macrophage activation pathway, whereby cells release anti-inflammatory cytoprotective mediators and proinflammatory factors are downregulated [26]. Other studies have also shown that nociceptors innervated by the intestinal wall, extrinsic and intrinsic afferent fibers, can induce gene expression changes in Treg (regulatory T cells) cells upon microbial stimulation [73]. Based on the infection of an intestinal *ex vivo* experimental system with *Clostridium ramosum* bacteria, researchers hypothesize that certain microbes are able to directly activate intestinal nociceptive sensory fibers, triggering an early inflammatory and pain response in gastrointestinal infections [73].

## 6. The Microbiome-Gut-Brain Axis

The human body has more bacterial cells than eukaryotic. The skin, the respiratory tract, the urogenital tract and the intestine provide the largest and most diverse habitat for these microorganisms that live in commensal relationships with us, and which in turn influence the developmental and maturational processes of the alimentary tract together with its function. In many cases, the intestinal tract and its microbial flora perform digestion together and the molecular milieu that they produce provides a common input for higher immune, neuroendocrine and neurotransmitter capabilities. We call this unified structure the gut-brain axis, or, extendedly, the microbiome-gut-brain axis, which is based on the neuroimmunological interactions between the enteric nervous system and immune microenvironment of the gut. In a broader sense, the neuroendocrine and neuroimmune systems, such as the hypothalamic-pituitary-adrenal axis (HPA axis); the sympathetic and parasympathetic branches of the autonomic nervous system also contribute to this vast network. The first experiments suggesting the existence of a gut-brain axis connection were carried out by Pavlov, who found that certain sensory signals, such as the smell and sight of food, triggers the secretory function of the stomach and pancreas even before the food enters the oral cavity [74].

Interest in this research field was sparked by a 2004 study, which showed that mice kept in a germ-free environment showed an increased HPA response to stress [23]. The role of the microbiome in the gut-brain axis has been demonstrated in various experimental animals and a number of neuroactive substances produced by gut flora have been described. The role of gut flora composition in patients with various psychiatric and neurological conditions and the effects of probiotics began to be generally accepted [75]. However, it is still not fully understood whether changes in gut flora are a consequence, a cause, or both, of disease, through certain feedbacks mediated by the gut-brain axis [76,77].

Multiple bacterial species are identified as exogenic neurotransmitter producers. *Lactobacillus* and *Bifidobacterium* species secrete GABA; they can influence the social behavior of the host and thus contribute to alleviating symptoms of anxiety, stress and depression in humans by restoring the disrupted oxytocin balance [78].

The gut-brain axis not only plays an important role in providing local functions, but we are also starting to understand its wider implications. The late prenatal and early postnatal development of the human brain, mental development and changes in the microbiome coincide in time [79]. However, the correlation is not only apparent: in germ-free mice, not only brain structure abnormalities, but also abnormal mental development have been described [80], which may even be due to alterations in fetal microglia populations [81]. Environmental influences that disrupt the normal gut flora (e.g., maternal stress, prenatal infections, antibiotic use) increase the risk of neurodevelopmental and mental disorders [82]. Thus, as surprising and neglected as it may seem, gut flora is a critical player in the development of normal behavioral patterns [83]. Germ-free mice were shown to be more sensitive to visceral pain, but after fecal transplantation with wild-type mice, this sensitization was abolished. A similar situation was observed in response to antibiotic treatment or infection, a condition that was also restored by probiotic treatment [84]. Not only the neuroendocrine, immune and nervous systems are responsible for the stress response, but the composition of the commensal flora may also be affected. Some research suggests that a normal bacterial composition helps our resilience to stress [83,85]

The microbiome, which acts through the gut-brain axis, is becoming an increasingly important therapeutic target because of its presumptive link with mental and neurological disorders. Fecal microbiota transplantation (FMT), pre- and probiotic treatments have shown positive results in smaller cohorts of patients with depressive symptoms [86], and in mouse models of obsessive-compulsive disorder (OCD) [87]. In addition, abnormal alterations in gut flora have been reported in a number of psychiatric disorders and neurodevelopmental disorders, such as bipolar disorder, schizophrenia, autism (ASD) and attention deficit hyperactivity disorder (ADHD) [88].

According to recent studies, the microbiome influences the efficacy of cancer immunotherapy. Antigens of commensals can pass the intestinal barrier and result in T-cell priming, along with the stimulation of cytokine and interferon production, promoting an anti-tumor immune response on the basis of molecular mimicry [89]. Intestinal fungal species may interact with bacterial growth or can direct immune cell trafficking, especially inflammatory or tolerant immune responses that can emerge or evolve [90–92]. A beneficial effect of Bacteroidales in the immunostimulation associated with immune-checkpoint blockade was shown in recent studies [93,94], whereas Li and colleagues showed that *Bifidobacterium*'s oral administration had a similar effect as PD-L1-specific antibody therapy in experimental mouse models [95]. Another group described an association between the composition of the gut microbiome and immunotherapy efficacy on a sizable patient cohort of malignant melanoma [96]. Nonetheless, the prospect of the gut microbiome as a prognostic, and in the future, a potential therapeutic factor is immense and not negligible anymore. Despite the fact that FMT still has many limitations and risks, altering the human microbiome for therapeutic purposes cannot be rendered as an 'unscientific' or inceptive approach to fight diseases in the long term.

Several hypotheses have been proposed to describe the relationship between the aforementioned conditions and the microbiome, the most important of which are the 'old friend' hypothesis and the 'leaky gut theory'. The former, also known as the 'early immune challenge' hypothesis, is a key element in the tens of thousands of years of coevolution between our gut and elements of the commensal flora [97]. Substantial changes in our modern society (health, lifestyle, nutrition) have reduced our contact with our 'old friends' and thus altered immunological maturation processes. Indirectly, this leads to an increased incidence of allergic and autoimmune diseases and chronic inflammatory processes [98]. The leaky gut theory is associated with a reduction in the function of the barriers (BBB, epithelial barrier of the gut), as the altered microbiome causes the tight junctions to loosen and changes in intestinal permeability. Thus, the potential for bacterial translocation increases, circulating lipopolysaccharide and immunoglobulin levels arise, where cyclic macromolecules cross the BBB, generating neuroinflammatory processes [99]. Although each theory has a slightly different focus, they all recognize immunological dysfunction and the underlying changes in the gut-brain axis as a central background.

The gut microbiome plays an exceptional role in gut physiology. Being the largest contact area with the outer world lined by living cells it is continuously bombarded by immunological stimuli. We have already a plethora of circumstantial evidence of the fact that commensal microbiota affect the central nervous system and consequently, mental health. However, the identification of a direct link between the two networks supported by rigorous evidence has not been presented yet.

## 7. Further Strategies on Microbiome Research

The ENS and macrophages of the muscularis externa are perfect candidates to carry the role of these 'middlemen'. To find new directions and perspectives in the research field, there is an unmet need for novel and innovative research approaches. In addition to correlative studies between human disease and microbial communities, providing *in vivo* validation on the influence of microbiota on the immune and the nervous system is essential for the initiation of phase I clinical trials. Experiments involving FMT in germ-free murine models are resource-intensive and still have some limitations. Nonetheless, more and more of these studies succeed in proving not just the correlation but also the causation *in vivo* between microbiota and clinical parameters, including immunotherapy efficacy, systematic inflammation, IBD and neuropsychiatric conditions [94,100–103]. Microbial 'cocktails' may serve as future therapies for these burdening health conditions. Apart from preclinical studies, randomized clinical trials have already been started for a selected population of UC patients [104,105], where FMT was associated with a higher rate of combined clinical and endoscopic remission compared with placebo. Moreover, there was no increased risk of adverse events in FMT-treated patients compared to controls [106]. With the help of



publicly available metagenome and transcriptomic databases, researchers can now find their target microbial taxa cost-effectively and prove their disease-modulator role on widely accessible animal models to create a basis for clinical trials in the future.

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