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Review

Rapidly expanding knowledge on the role of the gut microbiome in health and disease☆

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ABSTRACT

The human gut is colonized by a wide diversity of micro-organisms, which are now known to play a key role in the human host by regulating metabolic functions and immune homeostasis. Many studies have indicated that the genomes of our gut microbiota, known as the gut microbiome or our "other genome" could play an important role in immune-related, complex diseases, and growing evidence supports a causal role for gut microbiota in regulating predisposition to diseases. A comprehensive analysis of the human gut microbiome is thus important to unravel the exact mechanisms by which the gut microbiota are involved in health and disease. Recent advances in next-generation sequencing technology, along with the development of metagenomics and bioinformatics tools, have provided opportunities to characterize the microbial communities. Furthermore, studies using germ-free animals have shed light on how the gut microbiota are involved in autoimmunity. In this review we describe the different approaches used to characterize the human microbiome, review current knowledge about the gut microbiome, and discuss the role of gut microbiota in immune homeostasis and autoimmunity. Finally, we indicate how this knowledge could be used to improve human health by manipulating the gut microbiota. This article is part of a Special Issue entitled: From Genome to Function.

1. Introduction

We are not alone. Immediately after birth, various body habitats, such as our skin surface, oral cavity and gut, are colonized by an extensive range of microbes: these are mainly bacteria, but also archaea, fungi, viruses and protozoa. These microbial communities, also called the human microbiota, outnumber human cells by a factor of 10. In addition, these microbes carry 150 times as many genes as those contained in our human genome [1,2] which constitute the microbiome. These micro-organisms essentially inhabit all the mucosal surfaces, with the gastrointestinal tract (GI) and mainly the large intestine being colonized by high densities of micro-organisms, which are collectively known as gut microbiota. Under normal physiological conditions, these microbes are commensal, mediating in digesting our food, strengthening our immune system, and preventing pathogens from invading our tissues and organs. The involvement of bacteria in digestive functions not only benefits the human host, but microbes can also steadily provide themselves or others with nutrient sources, thereby producing a great variety of metabolites with a potential impact on human health [3]. It has been reported that other factors are also involved in the microbiota composition. For example, diet influences the activity and composition of the microbiome. A recent study showed that increased protein intake in mice can lead to altered transcription activation in *Eggerthella lenta* and thereby cause changes in metabolism of the drug digoxin [4]. Several other studies report on the role of dietary factors on microbiota composition, such as a lower abundance of the genera *Bacteroides*, *Bifidobacterium* and *Enterobacteriaceae* in people on a vegan diet compared to controls on an omnivorous diet [5–9]. It has recently been suggested that the human gut microbiota can be divided into different enterotypes based on the abundance of specific bacterial groups, dominated by *Bacteroides*, *Prevotella* or *Ruminococcus* [10] and these enterotypes seem to be strongly associated with long-term diets, particularly the levels of proteins and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*) [11]. Furthermore, it has been demonstrated that the mode of delivery (vaginally or by cesarean section) has a strong influence on shaping the initial gut microbiota composition. The analysis of the meconium of newborn infants revealed a strong correlation between the first microbial communities of the digestive tract and the microbial communities found in either the mother's vagina (*Lactobacillus*, *Prevotella* or *Sneathia*) in the case of vaginal delivery or the mother's skin (*Staphylococcus*, *Corynebacterium* and *Propionibacterium*) in the case of delivery by cesarean section [12]. In addition, it has been recently reported that CS seems to be correlated with decreased gut microbiota diversity, delayed Bacteroidetes

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colonisation and reduced Th1 responses [13]. The fact that metabolic functions performed by gut bacteria resemble those of an organ but cannot be executed by humans themselves has led to the human microbiome being called a "forgotten" organ [14]. This mutually beneficial relationship is a result of millions of years of co-evolution between the human host and its microbes. This shared evolutionary history has, unavoidably, led to additional inter-relationships between microbial communities and their human host that extend beyond metabolic functions. In this context, the human immune system and gut microbiota interact with each other in such a way that the microbiota shape the immune system and, vice versa, the immune system shapes the composition of the microbiota. In particular, the human immune system has evolved in such a way as to avoid the opportunistic invasion of the commensal bacteria into the host tissues leading to detrimental effects on human health [15]. More than a century ago the Russian scientist Ilya Metchnikov, who is regarded as "the father of modern probiotics," suggested that the microbial communities within the GI tract had a profound influence on general human health and claimed that some of the bacterial organisms present in the large intestine were a source of toxic substances that contributed to illness and aging. Metchnikov observed that the regular consumption of lactic acid bacteria in fermented dairy products was associated with enhanced health and longevity in Bulgarian peasant populations and he suggested that supplementing the diet with lactic acid bacteria, an early probiotic intervention, would have health benefits, including promoting longevity [16]. He foresaw that any change in the composition, function and dynamics of human microbiota could increase susceptibility to certain diseases. However, although the impact of the human microbiome in health and disease has long been recognized [1], a comprehensive analysis of human gut microbiota in terms of their genetic and metabolic potential is still needed to unravel all the mechanisms by which the microbiome is involved in human health and disease.

Traditional microbiology was almost entirely culture-dependent and involved microbial species being grown in specific media under laboratory conditions. Despite the great benefits derived from cultivating specific, easily-grown microbial species in a vast number of studies, other studies involving, for instance, the culture of anaerobic bacteria, are still limited. The culturing of most gut microbes has been unsuccessful, as "normal" gut microbiota are dominated by anaerobic bacteria, such as members of the genus *Bacteroides* and of the phyla Actinobacteria and Firmicutes. In addition, poor knowledge about the carbon sources needed for microbial growth means the cultivation of most of the microbes colonizing the human gut is inefficient. Historically (Fig. 1), the first important approach related to studying microbiota was the establishment of germ-free mice [17]. These axenic animals are raised in completely sterile environments and can be colonized with specific human intestinal micro-organisms; they have been widely used as models for human microbiome research, providing information on how the gut microbiota are involved in health and disease. Later on, fluorescent in situ hybridization (FISH) was the first DNA-based technique to be used without prior DNA extraction or DNA amplification using polymerase chain reaction (PCR). In this technique, fluorescently-labeled oligonucleotide probes are hybridized to marker genes, such as 16S rRNA, that characterize a microbial community [18]. The main

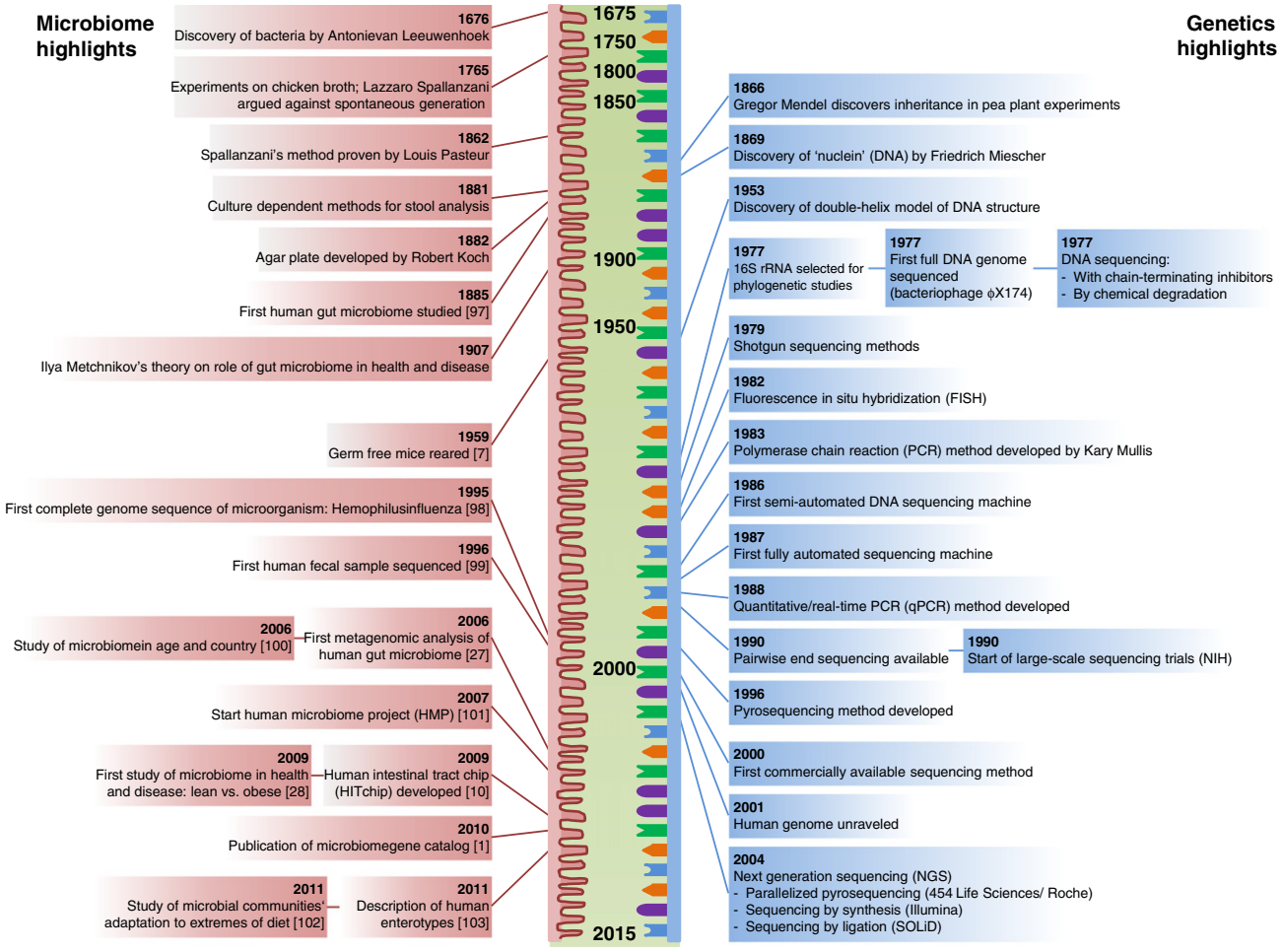

Fig. 1. Timeline of important events in the development of microbiome studies and related advances in genetics. The left-hand column shows the historical highlights in microbiome research. The right-hand column shows the historical highlights in genetic research, with an emphasis on the analytical techniques. References are indicated as mentioned in the text, except for references [97–103].

advantage of FISH is that it allows the quantitative analysis of microbial species, although only known species can be detected simultaneously. Fortunately, after the discovery of PCR in 1983 [19], the introduction of DNA-based methods that were culture-independent provided new opportunities for studying and characterizing gut microbiomes [18]. The 16S ribosomal RNA (rRNA) gene is highly conserved among all the bacteria, but it also contains regions that are variable among bacterial taxa. Studies based on PCR using universal and group-specific 16S rRNA gene primers followed by electrophoreses were widely performed for analyzing microbiomes before the introduction of sequencing. In particular, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rRNA sequences was used to obtain the banding patterns of the identified species, allowing the semi-quantitative analysis of the bacterial species. However, only the most abundant species could be detected by this method. Another method, using terminal restriction fragment length polymorphisms (T-RFLP), was based on the digestion of a mixture of PCR-amplified variants of a single gene; it is a cost-effective method, but not a quantitative one. Among PCR-based methods, real-time quantitative PCR (qPCR) is one of the most commonly used techniques, with a high sensitivity and specificity that provides a quantitative estimation of the amount of the PCR products; however, it can only be used to detect known species. In 2009, a rapid, high-resolution phylogenetic microarray-based method was developed, known as the human intestinal tract chip (HITChip). In this method, a microarray platform was developed on which oligonucleotides probes could be hybridized, allowing the analysis of a many 16 small subunit ribosomal RNA (SS rRNA) microbial genes. HITChip allows the simultaneous comparison of the relative amount of over 1000 genus-like groups of gut bacteria, but only known species can be detected [20]. Importantly, this method can be used as a complementary method to the 16S rRNA sequencing-based methods for gaining a better insight into human gut microbiota.

Although DNA-based, culture-independent methods contributed to the identification of uncultured species (whose number is much greater than the number of cultured species identified in the last decade), the characterization of the microbial communities in a high-throughput manner has benefited greatly from the introduction of next-generation DNA sequencing techniques. These provide a better quantitative picture of the microbial communities [21], even allowing determination of species of low abundance as well as previously unknown species.

In 2005, the International Human Microbiome Consortium (IHMC) was founded with the aim of establishing a cooperative effort to analyze the microbiome in human health and disease, with the ultimate goal of using this knowledge to prevent and/or treat these diseases. In addition, recent coordinated efforts, such as the Human Microbiome Project (HMP) of the US National Institutes of Health (NIH), and the MetaHIT project funded by the European Union, have contributed to this goal. In 2010, Qin et al. published the first extensive catalogue of non-redundant human intestinal microbial genes based on cohort studies on 124 individuals of European origin and using Illumina-based metagenomic sequencing [1]. By 2011, the Human Microbiome Project had published the sequences of 178 bacterial species [22], but there is still much to be done. In this review we focus on the most recent strategies using next-generation DNA sequencing technology and our current knowledge on the role of the gut microbiome in human health. In particular, we describe how the gut microbiome seems to be involved in immune homeostasis and in autoimmune diseases, both in gastrointestinal-associated diseases as well as extra-intestinal diseases.

2. Next-generation sequencing technologies and the meta-omics era

Although DNA sequencing has been used since the 1970s [23,24], it was long unaffordable, time-consuming and too laborious for scientists to apply it for high-throughput studies. However, the development of next-generation sequencing (NGS) has greatly facilitated metagenomic studies, making them increasingly common and affordable to the scientific community [25]. The term metagenomics is used to describe genetic studies of uncultured microbial communities from environmental samples, using sequence-based studies and bioinformatics tools [26–28]. These studies aim to identify the taxonomic diversity of the microbiota (how many and which microbes are present in a community) and/or to characterize the biological tasks of the members of such a community by performing functional metagenomics.

Nowadays, the different NGS-based methods used for metagenomic studies [29–31] are mostly performed as one of two types (Fig. 2): (1) using specific primers for amplifying 16S rRNA genes, and (2) using random primers for amplifying all microbial genes (whole-genome shotgun sequencing). In typical 16S rRNA next-generation sequencing-based studies, DNA is extracted from a sample without the need to culture microbes. The amplification of 16S rRNA genes is followed using specific PCR primers, with further sequencing using NGS technologies. Subsequently, the 16S sequences identified are clustered into Operational Taxonomic Units (OTUs) according to sequence similarity. Using published databases of previously annotated sequences, a taxonomic label is attached [32,33]. The community can be described in terms of which OTUs are present, their relative abundance, and/or their phylogenetic relationships. Furthermore, the relative abundances of genes and pathways can be determined by comparing the sequences to functional databases such as KEGG [34] or SEED [35]. Recently, computational approaches, such as implemented in PICRUSt, allow us to predict the gene composition of a metagenome using 16S rRNA gene data and a database of reference genomes [36]. It is important to note that this 16S rRNA NGS-based method is widely used for microbiome studies, but it is limited to bacteria, as parasites and viruses do not have 16S rRNA genes. The first 16S rRNA metagenomic study of healthy, human, distal gut microbiota dates back to 2006, when Gill et al. analyzed the DNA sequences obtained from fecal samples of two healthy adults using 16S rRNA sequence-based methods [37]. They reported that the human microbiome plays a significant role in metabolic pathways, such as the processing of glycans, amino acids and xenobiotics, methanogenesis, and the 2-methyl-D-erythritol 4-phosphate pathway-mediated biosynthesis of vitamins and isoprenoids. These results imply that humans are super-organisms, whose metabolism depends on the presence of a large number of micro-organisms that are found in the human host [37]. The first microbiome study that showed an association of the human gut microbiome to a disease was done on obese and lean adults – monozygotic and dizygotic twin pairs – using 16S rRNA-based methods. This showed that obesity was linked with changes in the microbiota: specifically that obese individuals were seen to have reduced bacterial diversity and a differential bacterial gene expression profile compared with lean individuals [38]. The microbiota of intestinal biopsies and stool samples from inflammatory bowel disease (IBD) patients was analyzed by 16S gene sequencing and followed up in a subset of samples using shotgun metagenomics. This revealed major shifts in oxidative stress pathways, as well as decreased carbohydrate metabolism and amino acid biosynthesis in favor of nutrient transport and uptake [39]. Another study also combined 16S gene sequencing and shotgun metagenomics to analyze the microbiome in patients with arthritis and it was observed that the expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis [40].

As an alternative to 16S sequencing, in whole-genome shotgun sequencing the DNA is first extracted, amplified using random primers, subcloned, and then sequenced to produce a representative DNA library of the microbial and viral populations being studied. After sequencing, the enormous numbers of reads obtained can be analyzed to identify the species represented by the sequences and the community's functional capabilities, using databases such as KEGG [34] or SEED [35]. For example, a metagenome-wide association study (MGWAS) based on deep shotgun sequencing of gut microbial DNA showed that patients with type 2 diabetes were characterized by a moderate degree of gut microbial dysbiosis, a decrease in the abundance of some universal butyrate-producing bacteria, an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring

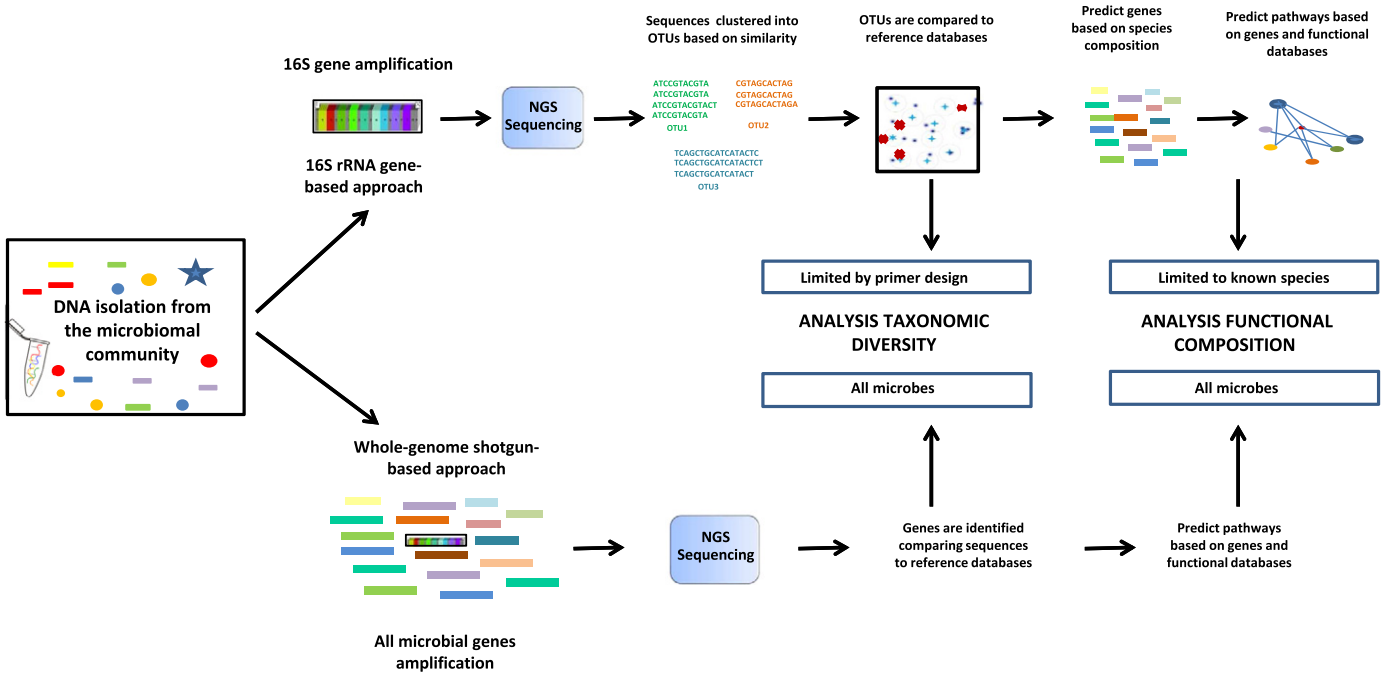

Fig. 2. Different NGS methods for metagenomic studies. First, DNA is extracted from a sample without prior culturing. The 16S rRNA gene-based approach is performed by amplification of 16S rRNA gene using specific PCR primers. The whole-genome shotgun sequencing is based on the amplification of all microbial genes using random primers. Both these high-throughput methods are used to characterize the taxonomic diversity in detail, including describing known and unknown taxa, and the functional composition of the microbial community. The 16S rRNA NGS-based method is extensively used for microbiome studies, but is widely limited by primer design, while the shotgun sequencing method is used to describe all the microbes present within the community.

sulphate reduction and oxidative stress resistance [41]. Recently, a new model has been developed, based on shotgun metagenomic sequencing, to distinguish between patients with type 2 diabetes and healthy women. This model has better predictive value than the classical predictive markers used at the moment, such as body mass index and waist–hip ratio [42]. Another study has showed that individuals with a low bacterial richness are characterized by more marked overall adiposity, insulin resistance and dyslipidemia, and a more pronounced inflammatory phenotype compared with individuals with a highly rich bacteria [43]. Furthermore, it has been suggested that alteration in the gut metagenome is associated with the inflammatory status of the host and that patients with symptomatic atherosclerosis harbor characteristic changes in their gut metagenome [44].

As well as investigation of bacteria, the metagenomic approach allows the virome and mycobiome composition of the human gut to be identified. The first results of virome and mycobiome studies have indicated a wide range of mycobacteria and viruses in the mouse and human gut, that compositional changes are diet-dependent, and that the mycobacteria and viruses play a role in predisposition to diseases [45–49].

It is worth mentioning that other meta-omic approaches along with (functional) metagenomics need to be used as complementary ways to obtain a comprehensive picture of the role of the human microbiome in health and disease and to investigate whether changes in the microbiome are the cause or the consequence of a disease [50]. Metatranscriptomics aims to sequence microbial mRNAs to investigate which genes (and to what extent) are expressed in a microbial community, revealing fluctuating expression profiles depending on microenvironmental conditions. Remarkably, meta-transcriptomic analysis of microbiomes using fecal samples from healthy individuals showed that highly expressed genes were involved in the metabolism of carbohydrates, energy production, and the synthesis of cellular components [51]. Meta-proteomics aims to identify, quantify and define the potential role of the microbial proteins. A meta-proteomic study of two healthy individuals showed that microbial proteins that are involved in post-translational modifications, protein folding and turnover were overproduced in contrast to proteins involved in inorganic ion metabolism, cell wall biogenesis and membrane biosynthesis [52]. Last but not least, the study of metabolomics aims to identify and quantify all the small-molecule, microbial-produced metabolites under specific physiological and micro-environmental conditions, in order to unravel the dynamic nature of the metabolic function of a microbial community and understand how it influences its human host. For instance, such studies have looked at fecal samples from healthy individuals, and patients with IBD (i.e. Crohn's disease (CD) or ulcerative colitis (UC)) and found reduced levels of butyrate, acetate, but also of methylamine and trimethylamine, and an elevated level of amino acids in IBD patients compared to healthy people [53]. Although all these approaches are still in their infancy, their potential usefulness in investigating the gut microbiome in a wider context and its relationship with the human host is clear.

3. The role of human gut microbiota in immune homeostasis and autoimmunity

Throughout evolution, the human host and its microbes have developed further complex inter-relationships, in which microbes regulate the normal development and function of the mucosal immune system [54,55]. This inter-relationship ensures that the host's immune system does not attack the microbes found in and on the human host, resulting in the establishment of an immune homeostasis. However, the microbiota protect the host from infection by direct and indirect mechanisms [56]: they mainly increase the epithelial barrier function through the production of different metabolites, such as short-chain fatty acids (SCFAs) [57] and the presence of mucus [58], or by promoting the production of antimicrobial molecules such as REGIIIγ and REGIIIβ by epithelial cells in the small and large intestines [59]. The immune system plays a vital role in protecting humans from invading pathogens and in maintaining their self-tolerance. However, in the case of autoimmune diseases, the immune system fails to properly distinguish self-

tissue and non-self tissue, and it attacks part of its own cells [60,61]. Given that humans can be considered as super-organisms colonized by many micro-organisms, the theory of the recognition of self-tissue by the human immune system should also include the recognition of human microbiota [62]. Thus, it could be speculated that the host's mucosal immune system has developed a tolerance or non-responsiveness to bacteria colonizing the human mucosal surfaces, leading to a beneficial symbiosis between the human host and the micro-organisms. The human gut microbiota regulate the immune homeostasis but their regulative role is not limited to just the local intestinal immune system but also influences the host's systemic immune responses.

Advances in NGS technologies have facilitated the characterization of gut microbiota and provided evidence that alterations of these microbial communities can cause immune dysregulation, leading to autoimmune diseases. Along with these technologies, the use of germ-free animals seems to offer a powerful approach for investigating the role of the gut microbiota in regulating the immune system [63]. These animals have been raised in completely sterile environments and have thus never been exposed to any micro-organisms, so they can be colonized with human intestinal micro-organisms and be used as models for studying the human microbiome.

3.1. Gut microbiota and innate immune homeostasis

An interesting mechanism has been proposed to contribute to the innate immune homeostasis: when the intestinal macrophages derived from blood monocytes are recruited to intestinal mucosa, they acquire a unique phenotype, which is called "inflammation anergy" [64,65]. Surprisingly, previous studies showed that intestinal macrophages lack the key receptor CD14 involved in the recognition of bacterial lipopolysaccharides (LPS) and LPS-binding proteins [66,67], with macrophages found in the colon expressing low levels of CD14 [68,69]. In contrast to blood monocytes, intestinal macrophages were found to express reduced levels of other innate response receptors, including Fcα (CD89), Fcγ (CD64, CD32, CD16),CR3 (CD11b/CD18), and CR4 (CD11c/CD18); the growth factor receptors IL-2 (CD25) and IL-3 (CD123); the integrin LFA-1(CD11a/CD18), and, lastly, pro-inflammatory cytokines were found to be downregulated, such as IL-1, IL-6, IL-12, RANTES, TNF-β and TNF-α, in response to inflammatory triggers. Hence, it is evident that intestinal macrophages are significantly distinct in phenotype and function from blood monocytes, promoting an absence of inflammatory response despite the presence of immunostimulatory bacteria. In addition, toll-like receptors (TLRs) induce an inflammatory response that must be strongly regulated to avoid tissue damage and many negative regulators of TLRs have been identified. Once TLR and ligand interaction has occurred, TLR signaling can be further controlled by intracellular regulators, which can inhibit TLR signaling pathways. Some of the intracellular regulators are even present constitutively to control TLR activation at a physiological level [70]. Even when the immune response is activated, despite of all the aforementioned forms involved in its regulation, production of anti-inflammatory cytokines could further control the inflammatory response.

Further evidence on the regulation of APCs by the gut microbiota was provided by studies using germ-free animals. In these studies, intestinal DCs were found in lower levels, while an increase in their number was observed when the animals were colonized with *Escherichia coli* [71,72]. Additional studies found that macrophages of germ-free mice lacked major macrophage functions, such as phagocytosis, chemotactic and microbicidal activities [73,74], and also the cell surface molecules that mediate interactions with leucocytes, such as major histocompatibility complex class II [75].

Another essential part of the innate immune system, the neutrophils with phagocytic activity, has been shown to be influenced by gut microbiota. For instance, experiments using germ-free rats revealed that these rats are neutropenic [76]. In addition, circulating peripheral blood neutrophils in germ-free rats have been found with decreased phagocytic activity and subsequently they could not release superoxide anions as a result of a phagocytic event. The release of superoxide anions could not even be restored when the germ-free rats were transferred back to the initial environment [77]. In addition, innate lymphoid cells (ILCs) are a group of innate immune cells that belong to the lymphoid lineage; they produce many Th cell-associated cytokines, but do not respond in an antigen-specific manner. This group of cells has an important role in regulating homeostasis and inflammation [78]. There is increasing evidence for the important role played by group 3 (RORγt) ILCs in intestinal immunity. These ILCs resemble Th17 cells in their cytokine profile (production of IL-22 and/or IL-17 upon IL-23 and IL-1b stimulation), inducing increased anti-apoptotic signals as well as the production of antibacterial peptides by epithelial cells [79,80] important in maintaining mucosal homeostasis. Within the ILCs group 3 there is a second type of NK cells in the gut mucosa that differ from the classical NK cells with respect to two features: [81]: (1) they showed a limited production of INFγ and no production of perforin, whereas they did produce interleukin-22, which promoted the production of antimicrobial molecules; and (2) they expressed the NK-cell natural cytotoxicity receptor NKp46 as the classical NK cells, but they also express the nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t (RORγt). It is interesting to note that germ-free mice lacked this second type of IL-22+NKp46+ cells, suggesting that the gut microbiota play a critical role in differentiating this type of NK cell in the gut mucosa [82]. Recently, it has been described that microbiota-driven IL-1β production by macrophages stimulated the release of Csf2 by ILC3, which in turn promote DCs and macrophages to maintain colonic Treg homeostasis [83].

Last but not least, the intestinal epithelial cells (IECs) constitute the first physical barrier preventing commensals and potential pathogens inhabiting the intestinal lumen from invading the underlying tissue. These cells also produce some antimicrobial peptides, cytokines and chemokines to protect tissue from the invasion of microbes by modulating the immune system. Importantly, it has been suggested that the proliferation of IECs and the production of antimicrobial peptides are influenced by the gut microbiota. Specifically, in germ-free mice treated with broad-spectrum antimicrobials, the IECs showed a reduced proliferation and also a lower production of antimicrobial peptides [84,85].

Taking into account all the above data, we can support that the gut microbiota influence several essential parts of the innate immune system and, specifically, the intestinal immune system.

3.2. Gut microbiota and adaptive immune homeostasis

CD4+ T cells are the key components of the adaptive immune system that play critical roles in determining an individual's health status. They are classified into four major subpopulations depending on the production of cytokines: T helper 1 (Th1), Th2, Th17, or regulatory T cells (Treg). The Treg cells are further classified into two subpopulations: CD4+FOXP3+ Treg cells or CD4+FOXP3−IL−10+ Treg cells [86]. The pro- and anti-inflammatory T cell-mediated responses must be balanced to ensure the individual's immune homeostasis and subsequent health.

Certain microbial species have been associated with the initiation of specific T cell responses. For instance, members of the Gram-negative bacterium *Bacteroides fragilis* induce the differentiation of naive CD4+ T cells into Treg cells through interactions with the polysaccharide A (PSA) molecules located on the outer membrane of the bacteria and resulting in an anti-inflammatory immune response, not only locally in the intestinal mucosa, but also in the systemic circulation [87]. Furthermore, 46 *Clostridia spp*. belonging to clusters IV and XIVa, were associated with the differentiation of CD4+ T cells into IL-10 producing Treg cells in the intestinal mucosa when germ-free mice were colonized with this bacterial mixture [88]. Segmented filamentous bacteria (SFB), which inhabit the small intestine of mice, have been associated with a

pro-inflammatory response by inducing the differentiation of naive CD4+ T cells into Th17 cells and, to a lesser extent, into Th1 cells [88,89]. Remarkably, when germ-free mice were colonized with SFB, they became susceptible to Th17 cell-mediated arthritis and experimental autoimmune encephalomyelitis [90,91], suggesting that changes in specific bacterial species in the gut microbiota can enhance susceptibility to certain autoimmune diseases.

Although there is growing evidence that the gut microbiota regulates the innate and adaptive immune responses and that certain species elicit specific responses, the precise underlying mechanisms are still unclear. Nonetheless, the investigation into how gut microbiota are involved in immune homeostasis and how a perturbation of this homeostasis can lead to autoimmune diseases is of great importance in developing alternative strategies to treat or prevent many chronic diseases.

4. Gut microbiota and autoimmune and inflammatory diseases

Given that the gut microbiota modulate the innate and adaptive immune systems locally in the intestinal mucosa and also systemically outside the gut, it is not surprising that microbial species have been associated with certain autoimmune and inflammatory diseases and that changes in the gut microbiome can contribute to an increased susceptibility to diseases both within and outside the gut. In this section, we discuss the role of gut microbiota in gastrointestinal (GI) and extra-intestinal autoimmune and inflammatory diseases.

4.1. Gut microbiota and GI-associated autoimmune and inflammatory diseases

Inflammatory bowel disease (IBD) consists of several inflammatory conditions of the colon and small intestine, with Crohn's disease (CD) and ulcerative colitis (UC) being the major types. The main difference between these two types is that the inflammation in CD can affect any part of the gastrointestinal tract, whereas in the UC it is limited to the colon. There is genetic evidence showing that the impaired recognition and killing of commensal bacteria contributes to IBD development: many of the IBD-susceptibility genes that have been identified regulate host–microbial interactions (Fig. 3), [92,93]. For instance, *NOD2*, which is an intracellular sensor of bacterial peptidoglycan, was identified as a susceptibility gene for Crohn's disease, and Crohn's disease-associated *NOD2* mutations are associated with a loss-of-function of the protein [94–96]. NOD2 is highly expressed in Paneth cells and regulates their function, which is to release granules containing antimicrobial peptides in response to bacteria [97]. Individuals carrying the rs41450053 (SNP13) variant in *NOD2* have shown lower levels of α-defensins, which are normally expressed by Paneth cells, compared to wild-type *NOD2* or other *NOD2*-mutant genotypes [98]. Specifically, this mutation has been associated with CD of the ileum, where Paneth cells are abundant [99]. Nod2-deficient mice displayed a diminished ability to kill bacteria as well as increased loads of commensal bacteria, demonstrating that NOD2 is essential for developing intestinal microbial communites [100]. It has also been reported that dysbiosis caused by Nod2 deficiency in mice seems to be a risk factor for colitis [101]. Later, it was demonstrated that NOD2 genotypes also affect the microbial composition in humans [102]. Furthermore, individuals homozygous for loss-of-function mutations for the *FUT2* gene, which encodes an α-1,2-fucosyltransferase that regulates expression of ABO histo-blood group antigens on the GI mucosa and in body fluids, showed a "nonsecretor" phenotype and, interestingly, an increased susceptibility to CD [103]. A recent study described how the *FUT2* genotype seems to explain substantial differences in microbiota composition and how nonsecretor individuals exhibit an altered mucosa-associated microbiota. These likely lead to an increased risk to CD, indicating that host genetic factors may influence the composition of gut microbiota [104]. It has also been recently reported that the rare G allele of the rs11747270 polymorphism, which is located within the *IRGM* gene (involved in autophagy and with a potential role in microbiota homeostasis), shows a significant correlation with a *Prevotella*-predominant enterotype [105]. With all the evidence described above, it is now clear that different genetic factors seem to influence host–microbial diversity [92] and it is therefore important to take interactions between genetics and microbiota into account when studying the role of the microbiome in diseases.

Typically, IBD patients show an overall decreased diversity of gut bacteria and, specifically, a reduction in the members of the dominant Firmicutes and Bacteroidetes compared to healthy people [106,107]. In colonic CD patients, increased levels of Firmicutes, Actinobacteria and Terenicutes were found compared to healthy individuals, whereas in ileal CD patients fewer Firmicutes and more Proteobacteria and Fusobacteria were found [107]. Additional studies have shown an increase in the *Lachnospiraceae* family in ileal CD patients [108,104]. Interestingly, ileal and colonic CD patients showed contrasting microbial profiles and some bacterial commensals were absent in ileal CD patients. Moreover, a greater relative abundance of proteobacteria, which include common enteric pathogens such as the family of Enterobacteriaceae [109] and a reduction in beneficial microbes, such as those producing butyrate, with *Faecalibacterium prausnitzii* being the main producer, have been observed in IBD patients [110,111]. In a recent study, Gevers et al. studied samples from new-onset cases of IBD collected prior to treatment from multiple gastrointestinal locations. They reported that an increased abundance in certain bacteria (including *Enterobacteriaceae*, *Pasteurellacaea*, *Veillonellaceae* and *Fusobacteriaceae*) and a decrease in *Erysipelotrichales*, *Bacteroidales* and *Clostridiales* correlated strongly with disease status, suggesting that the rectal mucosal-associated microbiome analysis offers a unique opportunity for the early diagnosis of CD [112]. However, it should be noted that the mucosal tissue samples in which increased levels of aerobic and facultative anaerobes (e.g. *Proteobacteria*) were dominant, did not reflect the gut microbial dysbiosis in the same way as stool samples, in which obligate anaerobes (e.g. *Bacteroides* and *Clostridiales*) were found to be prevalent [112].Last but not least, an overall increase in fungal diversity in UC and CD has been reported, but more studies are needed to shed light on the relationship between these organisms and IBD [108].

4.2. Gut microbiota and extra-intestinal diseases

4.2.1. Gut microbiota and rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic systemic inflammation, which may affect many tissues and organs but principally affects the joints. Previous studies have suggested that both genetic and environmental factors influence the etiopathogenesis of RA. However, studies on monozygotic twins have shown that environmental factors strongly influence the development of RA, as the concordance rate of RA has been estimated to be 15%, compared with a higher rate of 50% in type 1 diabetes [113,114]. Thus, it has been suggested that the gut microbiota play a significant role in the pathogenesis of RA, among other environmental factors [115,116]. Recent studies using 16S rRNA-based methods described a different composition of fecal bacteria in patients with recent onset RA compared to control individuals with fibromyalgia [117]. A reduction in the *Bifidobacterium*, *Bacteriodes–Porphyromonas–Prevotella*, *Bacteroides fragilis* subgroups and *Eubacterium rectal–Clostridium coccoides* group was observed in the guts of RA patients compared to the control group. Recently, a marked over-representation of Prevotella species, in particular *P. copri*, has been observed in patients with new-onset rheumatoid arthritis and a potential role for *P. copri* in the susceptibility to arthritis has been suggested [40]. Wu et al. used K/BxN mice as a model for arthritis and proposed a mechanistic model to explain how gut microbiota could be associated with the extra-intestinal disease of RA by alteration of the systemic immune system [91]. According to their proposed model (Fig. 3), the colonization of mouse gut with SFB

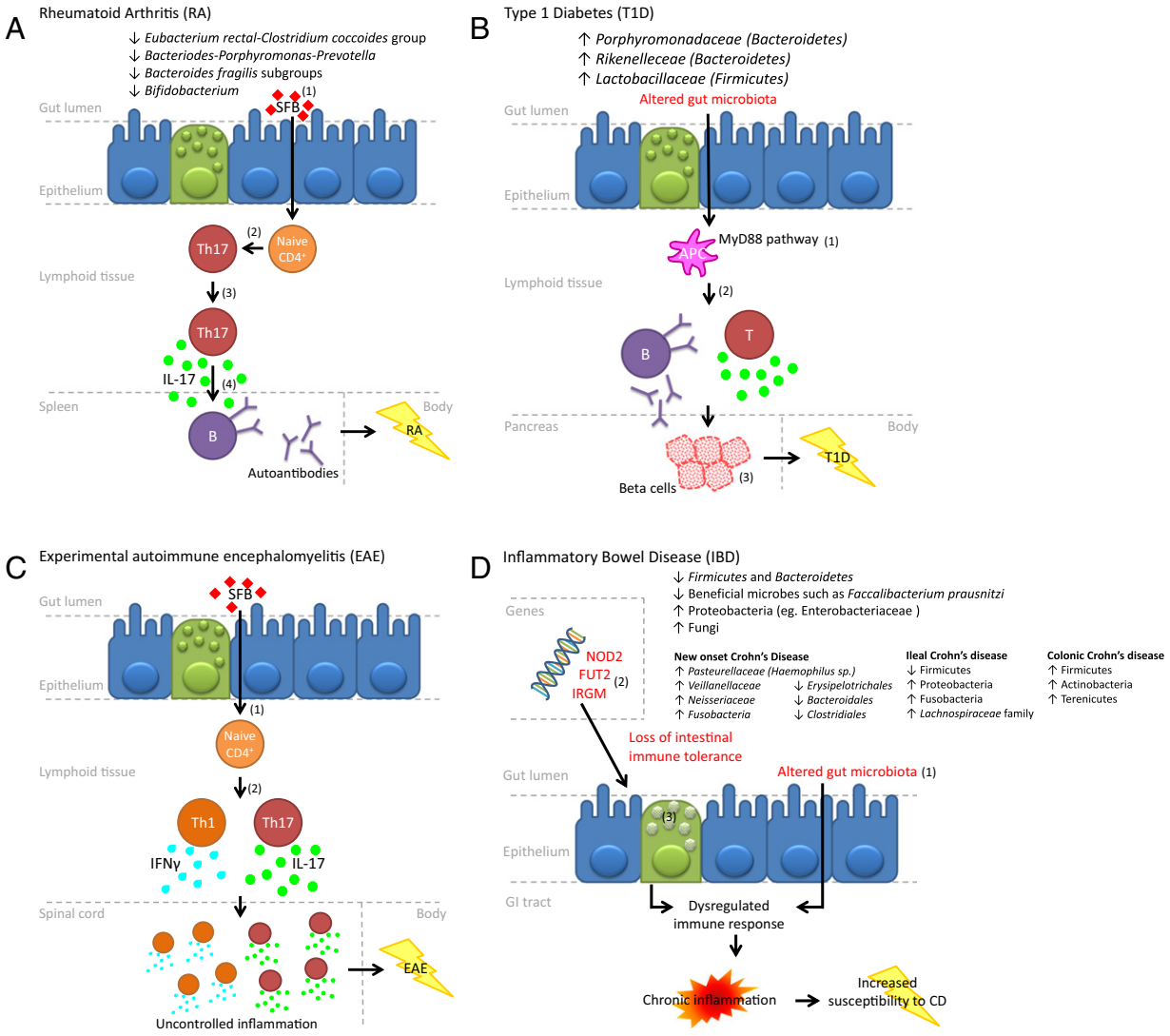

Fig. 3. (A) Rheumatoid arthritis (RA) model. (1) The presence of some bacterial species, for example, segmented filamentous bacteria (SFB) in the gut (2) induces the differentiation of naïve CD4$^+$ T cells into Th17 cells. (3) The activated Th17 cells produced in the gut migrate into the peripheral lymphoid tissue. (4) In the spleen, IL-17 acts on germinal center B cells leading to the differentiation of B cells and the production of autoantibodies which, in turn, attack the joints leading to RA. A reduction of certain bacterial species has been observed in patients with early onset of RA. (B) Type 1 diabetes (T1D) model. (1) Interactions of gut microbiota with the innate immune system via the MyD88-signaling pathway lead to (2) activation of the innate and adaptive immune system and as a consequence is produced (3) destruction of pancreatic beta cells. In mice an increase of three bacterial families has been observed as shown above. (C) Experimental autoimmune encephalomyelitis (EAE) model. (1) Naïve CD4 + T cells circulating in the periphery sense signals derived from the presence of SFB in the gut. (2) These SFB induce CD4 + T cell differentiation into IL-17A producing Th17 cells and CD4+IFNγ+ T cells indicating that certain microbial species control extra intestinal immune responses that extend to the central nervous system. Interestingly, germ-free mice colonized with segmented filamentous bacteria showed increased Th17 cell and CD4+IFNγ+ T cell responses in both colon and small intestine as well as within the spinal cords. (D) Inflammatory bowel disease (IBD) model. (1) An unbalanced microbiota is associated with a dysregulated immune response leading to chronic inflammation in the gastrointestinal (GI) tract, such as that seen in IBD. (2) A number of genes related to IBD development have been associated with alterations in the gut microbiota composition by different mechanisms involved in the loss of the intestinal immune tolerance.

induces the differentiation of CD4 + cells into Th17 cells. Subsequently, Th17 cells migrate into the peripheral lymphoid tissue and produce IL-17. IL-17 acts directly on the germinal center B cells in spleen leading to differentiation of the B cells, which subsequently produce auto-antibodies. The auto-antibodies could then be directed against target joints, ultimately leading to the development of RA.

4.2.2. Gut microbiota and type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune disease that results from the destruction of insulin-producing beta cells of the pancreas from T-cells. Studies using a non-obese, diabetic (NOD) mouse model for human T1D could not observe any amelioration of the disease phenotype when mice were raised in pathogen-free conditions, which was observed for the mouse models used to study the autoimmune and inflammatory diseases mentioned above. NOD mice showed a higher incidence of T1D in germ-free conditions compared with specific-pathogen free (SPF) mice housed in the same conditions [118]. This observation was in agreement with the fact that T1D has a higher incidence in countries with stringent hygiene practices [119]. However, inconsistent with the previous studies, germ-free NOD mice deficient for MyD88 protein (an adaptor protein for multiple innate immune receptors that recognize microbial antigens) showed an amelioration of the disease phenotype when hosted in an SPF environment, implying that the gut microbiota might protect against the disease [118]. This protective effect in MyD88-deficient mice can be explained by the growth of beneficial bacteria, which would otherwise have been under the control of MyD88. These findings indicate that the interaction of the gut microbiota with the innate immune system via the MyD88-signalling pathway has a critical role in the progression of diabetes (Fig. 3). Moreover, germ-free NOD mice, when colonized with the gut microbiota of SPF NOD mice deficient for MyD88, showed an amelioration of diabetes. In addition, a significant increase of

three bacterial families was observed in MyD88-deficient NOD mice compared to SPF NOD animals using 16S rRNA-based methods [118]. These families include the *Lactobacillaceae* (Firmicutes), *Rikenelleceae* and *Porphyromonadaceae* (both Bacteroidetes), suggesting that these members of the gut microbiota have an immuno-regulative role in diabetes.

4.2.3. Gut microbiota and experimental autoimmune encephalomyelitis (EAE)

Multiple sclerosis (MS) is a demyelinating neurodegenerative autoimmune disease that affects the central nervous system (CNS). Several studies have provided evidence that the gut microbiota plays a role in experimental autoimmune encephalomyelitis (EAE), which is the murine model for human multiple sclerosis. One interesting observation was that antibiotic treatment may alleviate the disease severity, implying that alterations in the gut microbiota can influence susceptibility to MS [120]. Furthermore, germ-free mice induced for EAE showed an amelioration of the disease phenotype, which is in agreement with the reduced secretion of pro-inflammatory cytokines observed, such as IL-17. In contrast, when these mice were colonized with SFB, they showed an enhanced disease phenotype, which was consistent with an increase of Th17 cells in the CNS (Fig. 3) [90]. Remarkably, it has been suggested that a specific regulatory B cell population induced by altering the gut microbiota could be involved in regulating autoimmunity, by shifting the immune esponses from Th1/Th17 towards anti-inflammatory Th2-type responses, and thereby leading to a reduced susceptibility to EAE [121].

4.2.4. Gut microbiota and obesity

Different changes in the gut microbiota composition as well as a reduced bacterial diversity have been associated with obesity by human and mice studies. An enrichment of *Firmicutes* and a decrease in *Bacteroidetes* levels has been reported in obese individuals compared to lean individuals. This was found both in humans [122] and in mice genetically predisposed to obesity [123]. Interestingly, the *Firmicutes*/*Bacteroidetes* ratio was normalized to that observed in lean individuals after weight loss [122]. More recently, Kalliomäki et al. have showed that early differences in gut microbiota composition in children, higher levels of *Staphylococcus aureus*, and lower levels of *Bifidobacteria* may predict overweight [124]. So far, different studies have been performed to investigate whether the altered gut microbiota contribute to obesity or whether obesity alters the gut microbiota. These studies have suggested a causal relationship between changes in microbiota composition and obesity development, showing that the obese phenotype can be transferred by the microbiota and also that the obese microbiome has an increased capacity to harvest energy from the diet [125,126]. Analysis of the metagenome of twins concordant for obesity showed altered representation of bacterial genes and metabolic pathways in obese individuals, who harbored more genes for phosphotransferase systems involved in carbohydrate processing [38]. It has been demonstrated that prevention of obesity by the microbiota in mice was diet-dependent, pointing to the connection between gut microbes and diet as a key factor in the path to obesity [127]. It is remarkable to note that the intestinal microbiota metabolism may also contribute directly to other phenotypes associated with obesity, such as CVD risk [128], while inflammasome-mediated microbiota dysbiosis also seems to exacerbate more processes associated with the metabolic syndrome, such as obesity and non-alcoholic fatty liver disease (NAFLD) [129].

4.2.5. Gut microbiota and immunodeficiencies

Last but not least, it is important to highlight that although only a few studies on the microbiome in patients with immunodeficiencies have been performed, this field will likely develop strongly in the future. Patients with chronic mucocutaneous candidiasis (CMC) and hyper IgE syndrome (HIES) have an increased risk for skin and mucosal infections with fungal pathogens, mainly *Candida albicans* and *Staphylococcus aureus*. Recently, it has been reported that the microbiota composition in these patients shows dysbiosis, with an increase of gram negative bacteria, especially *Acinetobacter spp*, and a decrease of *Corynebacterium spp* compared with healthy individuals [130]. The increased bacteria (*Acinetobacter spp*) showed suppression of the cytokine response to *C. albicans* and *S. aureus*, both of which are involved in the most prevalent infections developed by patients with the above primary immunodeficiencies. Furthermore, HIV infection is associated with highly characteristic changes in the gut community, including increased diversity, which are not restored to an HIV-negative state during antiretroviral therapy [131]. It has been suggested that fecal transplantation could be used to treat *Clostridium difficile* infection in patients with HIV [132].

5. Cause or consequence of disease development?

Despite all the studies performed so far, it is still unclear whether the changes in the microbiome are a cause or a consequence of disease development. However, there is growing evidence, provided by both human fecal microbiota transplantation and germ-free mice studies, to support a causal role for gut microbiota in regulating diseases. The use of animal models, in which the intestinal flora can be widely manipulated, provides a powerful tool for such mechanistic studies. For example, a recent study in a non-obese, diabetic (NOD) mouse model of type 1 diabetes indicated that the commensal microbial community affects the course of autoimmune disease by altering the levels of sex hormones [133]. NOD female mice are significantly more susceptible to disease than males, although this difference is not apparent under germ-free conditions. This study showed that fecal transplantation from male NOD mice to females was associated with increased testosterone in the female mice and protection against the development of diabetes [133]. Another study showed that obesity-related metabolic abnormalities observed in mice genetically deficient in Toll-like receptor 5 (TLR5) correlated with changes in the composition of the gut microbiota. Transfer of gut microbiota from TLR5-deficient mice to wild-type germ-free mice conferred many of those features associated with obesity to the recipients [134]. Similarly, TLR2 genetically deficient mice are protected from high-fat-induced insulin resistance and this feature can be reproduced in wild-type mice by microbiota transplantation from TLR2-deficient mice and reversed by antibiotics [135]. Another remarkable observation in EAE was that treatment with a broad-spectrum antibiotic may alleviate the disease severity, implying that alterations in gut microbiota induced by antibiotics can influence the susceptibility to autoimmune demyelinating processes of the central nervous system (CNS) [120]. Another study in an animal model of ulcerative colitis, the T-bet −/− x Rag2 −/− ulcerative colitis (TRUC) knockout mice, showed that the transfer of the microbiota from TRUC mice to wild-type mice resulted in colitis in them [136]. Similar to TRUC mice, colitis could be also induced in wild-type mice when the gut microbiota of NLRP −/− mice was transferred to them [137]. Importantly, both animal studies have shown that there is an overgrowth of certain colitis-associated bacterial species in mice with colitis, and that these can cause disease in wild-type mice that do not have a predisposition to the disease. The fact that certain antibiotics (i.e. sulfazalazine and minocycline) seem to alleviate the symptoms of some RA patients has provided clues that bacteria may play an important role in RA [138]. Animal studies using the IL-1 receptor antagonist deficient (IL-1Rn −/−) mouse model for arthritis have indicated a pathological role for the gut microbiota in RA as germ-free IL-1Rn −/− mice did not develop disease, whereas when germ-free IL-1Rn −/− mice were colonized with *Lactobacillus bifidus* they did develop RA [139]. In addition, fecal microbiota transplantation in humans has shown successful results in treating several conditions [140,141].Thus, taking all these considerations into account, it is possible that certain disease-associated gut microbial species could be the cause of a disease rather than a result of it.

6. Conclusions and future perspectives

The role of the microbiome in its entirety in health and disease has emerged as an area of major scientific and clinical importance in the past 10 years. It is now evident that changes in gut microbiota have a profound effect on the human immune system, which can affect the development of autoimmune and inflammatory diseases both within and outside the gut. Despite the incredible complexity of the microbial communities in the gut, advancements in next-generation sequencing, in parallel with improved bioinformatic tools, have provided new opportunities to characterize human gut microbiota without needing to perform cultures. In addition, the use of animal models for human autoimmune diseases, in which the intestinal microbiota can be manipulated (such as in the germ-free animals), as well as human studies of fecal microbiota transplantation, have provided further insights into how the microbiota are involved in the development of certain diseases. It is important to understand the exact relationship between the gut microbiota and the human immune system in order to find new opportunities to prevent and treat certain diseases. Recent evidence strongly suggests that, in the near future, we will be able to use individual microbiota profiles in clinical practice as a biomarker for patients' gut health, in order to predict who is at risk of developing certain diseases [142]. The recent success story of fecal transplantation in a patient suffering from *Clostridium difficile*-associated diarrhea has opened up a promising new future for treating such patients by restoring their intestinal microbial balance [143] and the potential of fecal microbiota transplantation therapy is now being explored in other conditions. It is therefore important to understand how the gut microbiota shape the human immune system, as well as how the immune system shapes the gut microbiota – this will be possible by combining functional metagenomic studies with others omics approaches. Ultimately, this knowledge should provide exciting new opportunities for improving human health: specifically for preventing and treating autoimmune diseases by manipulating the human gut microbiota to restore a healthy balance in the gut's microbial communities.

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