Mediators of Inflammation

The Microbiota and Immune System Crosstalk in Health and Disease 2019

Lead Guest Editor: Rossella Cianci
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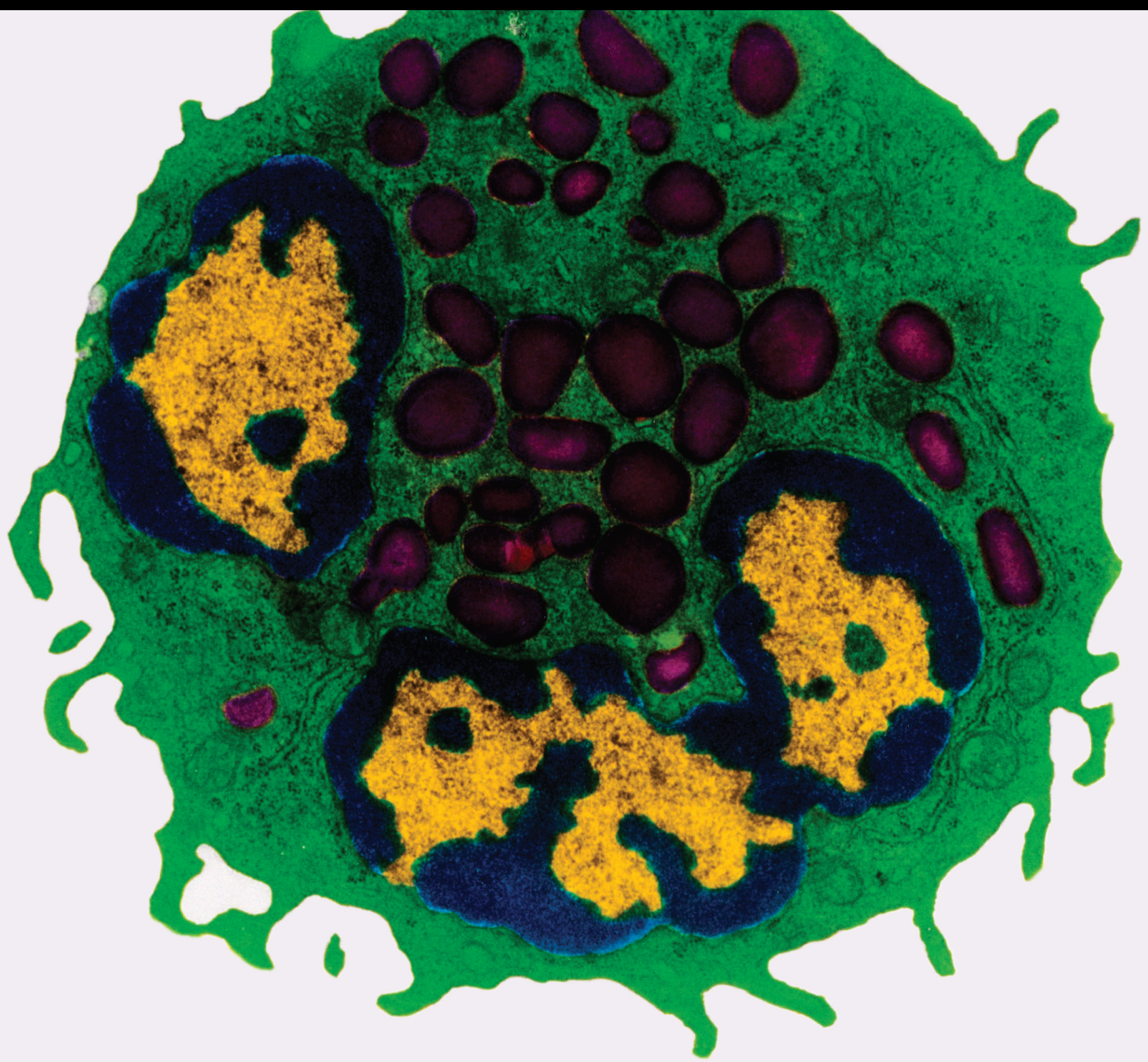

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Review Article

The Interplay between Immune System and Microbiota in Osteoporosis

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Osteoporosis is a disease characterized by low bone mass and alterations of bone microarchitecture, with an increased risk of fractures. It is a multifactorial disorder that is more frequent in postmenopausal women but can be associated to other diseases (inflammatory and metabolic diseases). At present, several options are available to treat osteoporosis trying to block bone reabsorption and reduce the risk of fracture. Anyway, these drugs have safety and tolerance problems in long-term treatment. Recently, gut microbiota has been highlighted to have strong influence on bone metabolism, becoming a potential new target to modify bone mineral density. Such evidences are mainly based on mouse models, showing an involvement in modulating the interaction between the immune system and bone cells. Germ-free mice represent a basic model to understand the interaction between microbiota, immune system, and bone cells, even though data are controversial. Anyway, such models have unequivocally demonstrated a connection between such systems, even if the mechanism is unclear. Gut microbiota is a complex system that influences calcium and vitamin D absorption and modulates gut permeability, hormonal secretion, and immune response. A key role is played by the T helper 17 lymphocytes, TNF, interleukin 17, and RANK ligand system. Other important pathways include NOD1, NOD2, and Toll-like receptor 5. Prebiotics and probiotics are a wide range of substances and germs that can influence and modify microbiota. Several studies demonstrated actions by different prebiotics and probiotics in different animals, differing according to sex, age, and hormonal status. Data on the effects on humans are poor and controversial. Gut microbiota manipulation appears a possible strategy to prevent and treat osteopenia and/or osteoporosis as well as other possible bone alterations, even though further clinical studies are necessary to identify correct procedures in humans.

1. Introduction

Osteoporosis is the most common bone disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, with increased risk of fractures.

Fractures severely affect patients' quality of life and mortality, especially in case of major fractures (femur and vertebrae) and represent a serious public health problem due to population aging, with high impact on the health care costs. In fact, the incidence of osteoporotic fractures is rapidly increasing in both sexes because of longer life expectancy [1].

Osteoporosis is classically distinguished in primary and secondary. Primary form includes postmenopausal osteoporosis, due to the fall of estrogen levels. Secondary form is due to endocrine diseases (i.e., hypercortisolism, hyperthyroidism), kidney diseases, hematologic diseases (i.e., multiple myeloma and malignant neoplasms infiltrating the bone), autoimmune or rheumatic diseases (i.e., inflammatory bowel disease, rheumatoid arthritis), drugs (i.e., steroids), malnutrition, malabsorption (i.e., celiac disease), or prolonged immobilization [2].

Bone loss is an asymptomatic process, so that the diagnosis of osteoporosis may often be made only after a fracture has occurred. Fractures can be prevented by reducing the risk of falling, changing lifestyle and nutrition, smoking, and alcohol abstention [3]. In case of vitamin D deficiency, osteoporosis is more frequent. Therefore, the first line treatment is characterized by calcium and vitamin D supplementation,

which is essential for a good bone activity [4]. Calcium may be taken with food and tablet. In addition, several drugs are available to treat osteoporosis and reduce the risk of fracture blocking bone reabsorption (such as bisphosphonates and denosumab), by stimulating bone formation or both (such as teriparatide or abaloparatide).

However, such drugs have safety and tolerance problems in long-term treatment. Concerns about rare side effects of antiresorptive drugs (osteonecrosis of the jaw, gastritis, and atypical fractures) lead many patients to discontinue such therapy [5]. Therefore, new tools are necessary to develop new treatments. These new options should have low side effects, improved efficacy, and adherence to treatment as well as overall patient outcome.

Recently, gut microbiota (GM) has been highlighted to have strong influence on bone metabolism, attracting the attention of endocrinologist and gastroenterologist as a potential new target to modify bone mineral density. The basis of these evidences are mainly focused on an involvement in modulating the interaction between immune system and bone cells [6–9].

GM is composed by all commensal, symbiont, and pathogenic microorganism consisting of bacteria, fungi, and viruses that colonize human intestine. GM is acquired at birth, mainly from the mother, and it is influenced by several factors such as genetic background, diet, age, eventual treatments, and antibiotics [10–12]. GM differs among people, and it is important to have a coexistence of different phyla in the intestine. Scientific community is increasing interest in studying such new "organ" to deeply understand its role and potentiality to treat diseases. It is strongly involved in human development, especially of the immune system; in fact, GM is necessary for an appropriate education and evolution of the innate and adaptive immune response [13].

GM plays an important role in maintaining gut barrier function, protecting the host against pathogens, food digestion, and modulating systemic immune responses by interacting with dendritic cells, macrophages, granulocytes, T- and B-cells, and intestinal epithelial cells [13].

The relationship between host and GM is complex and is based on variety of interactions, which are mainly controlled by the immune system. In case of alteration of the GM, such homeostatic balance may be interrupted, and the host may develop some pathologic conditions. A lack of variety among germs is a risk factor for the development of diseases (mainly immune mediated disorders) such as obesity [14, 15], insulin resistance [16, 17], inflammatory bowel diseases [18, 19], neurodegenerative disorders [20], and other metabolic diseases [21].

The intent of this review is to expose the mechanism underlying the interaction between GM and osteoporosis.

2. Studies on Germ-Free Models

The role of GM has been investigated looking at germ-free mouse models. These mice are raised in sterile cages, so that they cannot acquire any germ in the gut. They grow up weak, with a deficient formation of immune system and lymphoid organs. In this model, data on bone density are controversial, as in some studies, germ-free mice showed a low bone mass, while in other papers, they presented an increased bone mass density compared to normal mice.

Schwarzer et al. [22] observed that male germ-free mice presented a very weak bone development, including femur length, cortical thickness, and cortical/trabecular bone fraction. This condition has been supposed to be linked to low IGF1 levels that have been documented in such models [23].

Instead, Sjögren et al. [24] demonstrated that female germ-free mice presented an increased bone mineral density and a lower number of osteoclasts compared with conventionally raised mice. Moreover, these models are protected from developing osteoporosis in steroid deprivation settings. In fact, ovariectomy does not induce bone loss on germ-free mice [25].

The same controversial data have been reported in mice treated with oral antibiotics, in which GM is severely affected [26]. Several unclear pathways have been found in these opposite results; for that, such differences may be due to the lack of standardization among studies, differing for mouse breed, age, sex, antibiotics used, and technic of checking bone mineral density.

Male mice treated with antibiotics presented a decreased bone density, while female mice had an increased bone density [27]. On the basis of this, it is possible that GM composition and antibiotics response may be influenced by sexual hormones or other sex-related factors.

Anyway, Yan et al. [23] demonstrated that subsequent colonization of germ-free mice, with a normal GM composition, caused a reduction of bone mass in short period. In fact, analysis of bone density one month after colonization showed a reduction of bone mass, while 8 months after colonization, mice showed a bone density which was comparable with mice raised in conventional condition.

3. Role of Gut Microbiota in Vitamin D and Calcium Absorption

Vitamin D sufficiency and normal calcium phosphate metabolism play a key role in developing and holding an appropriate bone mass. A correct absorption of such trace elements is crucial.

The active form of vitamin D is 1,25-dihydroxyvitamin D (1,25(OH)2D3). It is produced in the skin after sun exposition or absorbed from diet and activates the vitamin D receptor (VDR). VDR is a nuclear receptor and transcription factor expressed in a wide variety of tissues, including the intestine, and it modulates metabolic and immune system processes.

Classically, vitamin D is known to regulate bone development and calcium homeostasis through its actions in the intestine, kidney, and bone. Vitamin D regulates calcium absorption in the intestine and kidney by activating transcellular calcium channel (TRVPV and CalbindingD9K) which mediates intracellular calcium diffusion [28].

Low levels of vitamin D or inactivating polymorphisms in VDR have been associated with inflammatory and metabolic disorders. In particular, the variation of human VDR gene shapes the gut microbiome at the genetic level. In fact,

it has been observed that mouse models carrying VDR deletion in gut epithelia cells present dysbiosis and an increased susceptibility to inflammatory bowel diseases [29]. Furthermore, vitamin D induces the expression of cathelicidin antimicrobial peptide (CAMP) gene, which is expressed by immune and epithelial cells and enhances barrier function, suggesting that vitamin D has antibacterial effects [30]. Moreover, vitamin D reduces the permeability of intestinal cells in animal models of colitis [31].

However, data on biological function of vitamin D and VDR in GM are limited. The GM has effects on the host immune system as well as vitamin D, and it also plays a critical role in the synthesis of vitamins and trace elements. For that, there is a strong direct interaction between vitamin D levels and GM composition, also influencing calcium absorption. A reduction in the production of 1,25(OH)2D3 may lead to gut inflammation and a shift in the balance of the GM composition [32].

4. Role of Gut Microbiota in Bone Homeostasis

Bone is a dynamic tissue whose homeostasis is based on several different mechanisms. A wide variety of factors influences bone strength such as hormones, physical activity, diet, weight, and lifestyle. Moreover, bone metabolism seems to be influenced by several gastrointestinal peptides, such as ghrelin, peptide tyrosine-tyrosine (peptide YY), incretins, glucose-dependent insulinotropic polypeptide or gastric inhibitor polypeptide (GIP), and glucagon-like peptide (GLP) 1 and 2 [33].

The main factor involved on bone density is the balance between the osteoblastic (that replace bone) and osteoclastic (that reabsorb bone) activity. This process, called remodelling, is necessary to build the skeleton during growth, regulate calcium homeostasis, and repair microdamages. Remodelling involves multiple molecular events, such as cooperation between osteoblasts, osteoclasts and other cell populations (i.e., immune cells), and several hormones such as parathyroid hormone (PTH), vitamin D, calcitonin, growth hormone (GH and IGF1), sexual hormones, growth factors and cytokines. An increased osteoclast activity or reduced osteoblast activity can cause reduction in the architecture of bone mass, causing osteoporosis. In this setting, risk of fracture is increased.

Remodelling is triggered by signalling from osteoblasts and osteocytes that produce receptor activator of nuclear factor kB ligand (RANKL), a member of the tumor necrosis factor (TNF) receptor family, which binds to the RANK receptor on osteoclast precursors. This binding is essential for activation of osteoclast precursors to mature form which can destroy bone matrix.

Osteoclasts attach firmly to the bone surface and secrete hydrochloric acid and cathepsin K to dissolve bone mineral. After resorption is complete, osteoblasts lay down bone collagen matrix, which is then mineralized.

GM has supposed to influence bone homeostasis through the effect on the systemic immunity. In fact, microbiota is involved in production of circulating cytokines and development of lymphoid cells, particularly of T helper 17 (Th17) lymphocytes. This correlation between GM and immune system is important, because the latter plays an essential role in regulating bone density. In fact, RANKL is expressed not only by mesenchymal cells, osteoblasts and osteocytes, but also by activated T CD4+ lymphocytes, indicating that this is a molecule that bridges the skeletal and immune systems [34]. Moreover, lymphocytes produce tumor necrosis factor α (TNFα) and interleukin (IL)-17, both involved in osteoclastogenesis [35, 36]. However, T-cells also produce interferon gamma (IFN-γ), which counterbalances the action of RANKL, determining an inhibitory effect on osteoclastogenesis. The only osteoclastogenic Th subset is represented by Th17 cells through the production of IL-17 that induces the expression of RANKL. Th17 cells are a subset of proinflammatory T helper cells which play an important role in maintaining mucosal barrier and preventing intestinal colonization by pathogenic germs. They have also been implicated in autoimmune and inflammatory disorders. Th17 cells activated by intestinal inflammation migrate into the bone matrix, where IL-17 enhances local inflammation leading to an increase of inflammatory cytokines, such as TNFα and IL-1, raising RANKL expression and activating osteoclast precursor cells [37]. In vivo, Th17 have been associated to increased osteoclast differentiation both in mouse models and in humans affected by inflammatory diseases. In fact, Th17 lymphocytes were also detected in peripheral blood of patients suffering from Crohn's disease, and for that, Th17 may be involved in decreased bone density frequently detected in these patients [38]. Th17 lymphocytes may be a promising therapeutic target for the bone reabsorption associated with T-cell activation. It is also remarkable to point out that germ-free mice do not present Th17 cells in their bowel tissue, but their development and maturation may be induced by germ colonization.

Moreover, the relationship between GM and bone is also mediated by innate immunity through several receptors such as nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) receptors and Toll-like receptor 5 (TLR5). NOD1 and NOD2 are ubiquitary intracellular sensors of pathogen-associated molecular patterns (PAMPs), mainly expressed on epithelial and immune cells, that bind bacterial peptidoglycans and activate the NFkB pathway playing a key role in the effects of microbiota on bone. In fact, neither the expression of TNFα and RANKL nor the bone density is affected by the microbiota variations in mice which are knocked out for these two genes [39].

TLR5 is the innate immune receptor known to recognize flagellin, one of the main bacterial proteins. It is expressed on both immune and not-immune cells, such as enterocytes. A recent study has identified TLR5 as a new mediator in the process of inflammation-induced bone loss and osteoclastogenesis, through the activation of RANKL pathway [40]. Mice that are knocked out for this receptor develop deficiencies in the immune system leading to change in the GM composition, with the prevalence of *Proteobacteria*, *Firmicutes*, and flagellated bacteria that invade bowel mucosal barrier [41]. Therefore, these mice exhibit hyperphagia, obesity, insulin resistance, hypertension, hyperlipidemia, and increased inflammation, due to GM imbalance and show a

decreased bone strength, while mice lacking TLR5 that are raised in germ-free condition do not present the metabolic phenotype [42]. The use of antibiotics leads to a greater reduction of the whole-bone femur bending strength in mice knocked out for this receptor with respect to wild type [26].

Another important molecule implicated in immunomodulation is Lipopolysaccharides (LPS), which is the main component of bacterial cell wall in gram-negative bacteria. Such molecule stimulates inflammation by activating transformed growth factor (TGF) and Toll-like receptors 4 [43]. LPS has been documented to be involved in bone metabolism. A mouse model, in fact, was implanted with LPS to induce inflammation [44]. These mice showed femoral bone loss, suggesting a potential role of LPS in reducing bone mineral density. In mice treated with high dose LPS, trabecular bone volume of the proximal tibial metaphysis tended to be decreased, while an upregulation of the inflammatory mediators, interleukin-1, cyclooxygenase-2, and TNF was found.

Anyway, the major cause of osteoporosis is sexual hormone deficiency, mainly estrogen lack in postmenopausal women. During menopause, there is a progressive bone loss, due to the upregulation of osteoclast maturation and activity mediated by several cytokines. In fact, estrogen deficiency leads to an alteration of the immune response and enhances the production of TNFα, which directly induces osteoclast differentiation and indirectly increases the expression of RANKL and macrophage colony-stimulating factor (M-CSF) by monocytes and T-cells. Moreover, estrogen deficiency increases bowel permeability and promote inflammation. Furthermore, the lack of estrogens increases the expression of Class II TransActivator (CIITA), a transcriptional factor involved in the upregulation of major histocompatibility complex class II on macrophages, improving the antigen presentation [45]. Mouse models of osteoporosis caused by ovariectomy suggest that osteoclast activity is mainly stimulated by activated T-cells, which promote macrophage differentiation and the expression of M-CSF and RANKL by stromal cells, through the activation of the CD40/CD40L system [46]. Ovariectomy increases T-cell activation through the upregulation of several intracellular pathways, such as STAT3, ROR-ct, and ROR-a, and the downregulation of Foxp3 [47]. Several studies suggest that T CD4+ lymphocytes are the most relevant source of TNFα in condition of estrogen deficiency. Moreover, the lack of T-cell in nude mice seems to preserve against postovariectomy bone loss. However, the transfer of wild-type T-cells restores the capacity of ovariectomy to induce bone loss. Such alteration of the immune system has also been demonstrated in humans and seems to be more relevant in osteoporotic women [48]. Furthermore, in postmenopausal women, hormone replacement therapy decreases the production of osteoclastogenic cytokines [49].

In this contest, GM is central in controlling lymphocytic activation on the basis of sexual hormonal change. The same data have been confirmed in mouse models treated with leuprolide and gonadotropin realising hormone agonist inducing menopause. Moreover, a pilot study [50] conducted on a small number of patients has shown GM modification among osteopenic patients, osteoporotic patients, and control. Such preliminary data suggest a connection between GM and osteoporosis, but further investigations are needed to confirm this hypothesis. However, in our opinion, it is reasonable to speculate that dysbiosis may exacerbate the bone loss in postmenopausal women.

Recent studies have confirmed a close connection between GM and bone diseases. This linkage is not only limited to disorders connected to hormonal changes but may include a huge number of different pathways all associated by inflammation. In fact, GM modification has been linked to several rheumatic and autoimmune diseases, and the inflammation is one of the factors that contributes to osteoporosis in patients with inflammatory bowel diseases. In fact, cytokines produced during intestinal inflammation may alter osteoblast action and bone density. Furthermore, it has been observed that osteoporotic patients with inflammatory bowel diseases have higher circulating proinflammatory cytokines levels. In this contest, TNF antagonists (infliximab and adalimumab), used as conventional treatment for inflammatory bowel diseases, appear to have beneficial effects on bone metabolism, increasing bone formation. It has also been observed that TNF blockade leads to an important increase of bone formation markers, such as osteocalcin and procollagen type 1 N-terminal propeptide, and to a stabilization of bone mass density [51]. Moreover, TNF inhibitors seem to influence the GM composition. Indeed, mice treated with TNF inhibitors have shown alterations of the GM, with differences between genders and age. Thus, you can speculate that the effect of TNF blockade on bone may also be mediated by the modulation of GM. However, available data are still limited.

Another important model to support the connection between GM and bone is characterized by patients affected by small intestinal bacterial overgrowth syndrome. This syndrome is characterized by malabsorption due to destruction of nutrients by bacteria, causing alteration in intestinal calcium and vitamin D absorption. These patients present a chronic bowel inflammatory status and develop bone alteration such as osteomalacia.

5. Prebiotics

Prebiotics are fermentable food ingredients that cannot be digested by humans while stimulate the growth and activity of GM as substrate of their metabolism. After this process, GM produces specific metabolic products that can be subsequently used by the host. Prebiotics include a large group of nondigestible oligosaccharides composed by short-chain sugar, the most common of which are galactooligosaccharides (GOS), fructooligosaccharides (FOS), inuline, xylooligosaccharides (XOS), polydextrose, and lactulose. In the group of prebiotics, a list of metabolizable food ingredients can also be included, as compounds of human milk, onions, garlic, and other vegetables.

Prebiotics are safe and can be given to any age after the fifth month of life. The only side effect may be bloating, gas, and increased bowel movements.

Fermentation of fibres in the large intestine causes the production of short-chain fatty acid (SCFA), such as acetate,

propionate, valerate, isovalerate, butyrate, and isobutyrate [52]. These molecules increase calcium intestinal absorption reducing bowel pH and promote the gut villi development, causing GM modifications. They also improve the deconjugation of phytoestrogens and may modulate immune system [53].

In female mice, the use of prebiotics reduces bone loss due to estrogens deficiency after ovariectomy [54]. Estrogens lack, in fact, causes reduction in calcium absorption. The administration of inuline and FOS have been demonstrated to increase calcium absorption in ovariectomized rats [55].

Administration of GOS to male rats induces a pH decrease in gross intestine, an increase of bifidobacteria, and calcium and magnesium absorption leading to improvement of bone density [56]. Anyway, it is not clear if such increased calcium absorption also causes an increase in BMD in all animals (such effect has been demonstrated in mice and rats but not in pigs).

In humans, the effect of FOS administration is controversial. In fact, administration of one-year treatment did not modify bone density in adolescent girls [57], while it reduces bone loss in postmenopausal women [58].

In male mice, ingestion of FOS increases cortical and trabecular bone [59]. Ingestion of food with a high amount of fibres improves cortical thickness, cortical bone mineral content, bone strength, and trabecular BMD in rats. The same evidence has been found in case of GOS supplementation [56].

Lactitol is a nonabsorbed sugar that can increase calcium absorption in rats reducing intestinal pH, which raises calcium bioavailability [60].

Milk of mothers with new born affected by malnutrition are poorer of human milk sialylated oligosaccharides, an important energy source for gut bacteria, and this condition may affect bone health. In fact, germ-free mice colonized with stools from malnourished Malawian infants showed a delayed growth that improved after administration of sialylated milk oligosaccharides [61].

Effects of prebiotics on bone homeostasis are controversial and seem to be related among others to the type of prebiotic. A recent study has been demonstrated that administration of agave fructans and inulin increases serum osteocalcin levels in female mice [55], while a GOS/FOS and calcium combination increased bone mineralization [62]. In contrast, the use of inulin and FOS documented an increased bone resorption in ovariectomized rats [63]. In postmenopausal women treated with FOS for 2 years, a decrease of serum and urine bone turnover markers was recorded [58]. Another important tool to improve bone density is represented by the use of FOS in combination with soy isoflavone treatment in ovariectomized rats, which has shown to decrease bone resorption improving BMD [64].

Prebiotics can also alter the GM composition. In fact, FOS and GOS increase the proportion of bifidobacteria in GM, affecting the rate of production of SCFA [52]. The direct connection between prebiotics and bone is not fully clear, but it is definitively a promising research field due to the effect on local GM and its metabolites.

6. Probiotics

Probiotics are live microorganism that, if administrated in appropriate amount, can provide health benefits to the host. Several species of microbes can be defined as probiotics, such as *Lactobacilli*, *Bifidobacteria*, *Escherichia*, *Enterococcus* and *Bacillus subtilis*, and *Saccharomyces*.

Probiotics are usually provided in dairy products, such as yoghurt as concentrated cultures or as inoculants in milk-based food or dietary supplements in form of capsules, bags, or tablets. Recently, they have been added to new products, such as ice cream, beer, and toothpaste.

Their effect on bone status has been extensively studied, both in mice and in human. In particular, *Lactobacillus reuteri* was able to improve bone mineral density in ovariectomized murine models, even in the absence of milk [65], but it does not have effects on female intact mice [66]. However, it is interesting to note that *Lactobacillus reuteri* has been shown to increase bone mineral content in male mice [66], confirming the role of sex hormones in the probiotics effect on bone. Moreover, this bacterium has been supposed to have an anti-TNFα activity, modulating bone metabolism through the immune system [67].

Similar effects have been observed in case of oral supplementation of *Lactobacillus paracasei* and *helveticus* that affect osteoclastogenesis decreasing the production of IL1β and TNFα in ovariectomized murine models [68].

Another study on sex hormone-deficient mice has demonstrated that twice-weekly treatment with *Lactobacillus rhamnosus* inhibits bowel inflammation and decreases bone loss through a reduced expression of RANKL and TNFα, unlike what happens using of nonprobiotic breed of *Escherichia coli*. Moreover, this study has confirmed that estrogen deficiency affects gut barrier integrity leading to immune system activation. In fact, hypogonadal germ-free mice did not exhibit the same bone damage of wild-type hypogonadal mice [69].

Decreased bone loss due to sex-steroid lack seems to be partially restrained also with the use of *Bifidobacterium longum* in ovariectomized rats [70].

As to the effect on humans, it has been recently demonstrated that the oral administration of *Lactobacillus reuteri* in postmenopausal women increases tibial bone density [71] and circulating vitamin D levels [72]. *Lactobacillus rhamnosus* and *Lactobacillus plantarum* also increase serum vitamin D, through a higher expression of VDR in both mouse and human enterocytes [73]. VDR knockout animals exhibit a decreased presence of lactobacilli compared to clostridium and bacteroides [74]. Furthermore, these models do not benefit of the protective effect of probiotics on Salmonella infections.

Bifidobacteria have also demonstrated healthy effects in yoghurt consumers. However, not all dairy products have the same effect on bone metabolism. In fact, the Framingham Offspring Study has highlighted that yoghurt and milk absorption improves hip but not spine bone density [75]. The positive effect of yoghurt was also confirmed in elderly people. Indeed, high yoghurt intake led to better physical performances and higher bone mineral density [76].

Furthermore, the use of probiotics may be important also in oral pathologies. In fact, in rats affected by periodontitis, oral *Saccharomyces cerevisiae* administration as monotherapy or in combination with standard therapy improves local inflammation and decreases alveolar bone loss [77].

It has also been suggested that combined use of probiotics and prebiotics, called symbiotics, may increase effects on bone homeostasis. For that, Michaëlsson et al. [78] have studied the effect of high intake of fruits and vegetables in combination with fermented dairy products (i.e., yoghurt) in postmenopausal women. They observed that high absorption of symbiotics reduces the risk of hip fracture more than low intake of vegetables, fruit, and fermented milk. However, it is important to note that the beneficial effect of prebiotics is considerably increased by concomitant assumption of probiotics, whereas the use of probiotics alone has already a notable effect on bone mineral content.

7. Conclusions

Osteoporosis is a multifactorial disorder associated with reduced bone density and high risk of fracture. Such condition is more frequent in postmenopausal woman but can be associated to other disease (inflammatory bowel disease, celiac disease, etc.).

Several studies have defined a central role of GM in the modulations of immune response in regulating bone activity, mainly in mouse models. GM manipulation appears to be a possible strategy to prevent and treat osteopenia and/or osteoporosis as well as other possible bone alterations, even though further clinical studies are necessary to identify correct procedures in humans. GM modification may play a role together with diet, lifestyle, and drugs.

The chance of using appropriate prebiotics and probiotics to increase bone density in different ages is also a possible new path that may be followed in the next few years, and the role of dairy products is still central. Another possible option is the development of functional food to improve prebiotic effects.

GM transplant is another option that may be considered in severe diseases. At present, its role is clear in treating antibiotic resistant colitis infections, but the chance of using GM transplant for treating bone disease needs further investigations.

Conflicts of Interest

None of the authors has anything to declare.

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Research Article

Psychobiotics Regulate the Anxiety Symptoms in Carriers of Allele A of IL-1β Gene: A Randomized, Placebo-Controlled Clinical Trial

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Background. Probiotic oral intake, via modulation of the microbiota-gut-brain axis, can impact brain activity, mood, and behavior; therefore, it may be beneficial against psychological distress and anxiety disorders. Inflammatory cytokines can influence the onset and progression of several neurodegenerative mood disorders, and the IL-1β rs16944 SNP is related to high cytokine levels and potentially affects mood disorders. The aim of this study was to examine the combined effect of IL-1β polymorphism and probiotic administration in mood disorder phenotypes in the Italian population. *Methods*. 150 subjects were randomized into two different groups, probiotic oral suspension group (POSG) and placebo control group (PCG), and received the relative treatment for 12 weeks. Psychological profile assessment by Hamilton Anxiety Rating Scale (HAM-A), Body Uneasiness Test (BUT), and Symptom Checklist 90-Revised (SCL90R) was administered to all volunteers. Genotyping was performed on DNA extracted from salivary samples. *Results*. After 12 weeks of intervention, a significant reduction of HAM-A total score was detected in the POSG ($p < 0.01$), compared to the PCG. Furthermore, IL-1β carriers have moderate risk to develop anxiety (OR = 5.90), and in POSG IL-1β carriers, we observed a reduction of HAM-A score ($p = 0.02$). *Conclusions*. Consumption of probiotics mitigates anxiety symptoms, especially in healthy adults with the minor A allele of rs16944 as a risk factor. Our results encourage the use of probiotics in anxiety disorders and suggest genetic association studies for psychobiotic-personalized therapy.

1. Introduction

In the last years, the increased scientific interest about microbiota and its relationship with health maintenance and disease onset underlined the importance of bacterial composition in the gastrointestinal tract. In the neuroscience field, the recognized complex bidirectional communication between host microbiota and brain-gut axis opened to new discoveries on the neurological disorders and disease onset and tailored treatments for affected patients.

Two neuroanatomical pathways are involved in the brain-gut interaction. The central nervous system (CNS) shares information with the lumen and the enteric nervous system (ENS) [1], through the sympathetic and parasympathetic branches of the autonomic nervous system (ANS), and they mutually modulate gut functions and environment [2, 3].

Secondly, psychophysical stress can set off adaptive processes by the neuroendocrine system, which in turn regulates the hypothalamic-pituitary-adrenal (HPA) axis, increasing

the levels of inflammatory cytokines and prostaglandins in the gut. These events lead to changes in the microbiota composition and increased gastrointestinal permeability [4].

HPA axis activity influences physiological and behavioral states, including anxiety and depressive disorders [5]. A balanced microbiota-gut-brain (MGB) axis improves CNS and ENS functions [4–6]. Conversely, MGB axis impairment damages gut microbiota, destroys intestinal epithelium integrity, and affects permeability, increasing circulating levels of endotoxin, bacterial lipopolysaccharide (LPS), and inflammatory mediators [7]. Currently, it is well known that intestinal inflammation and gut microbiota imbalance are related to chronic abdominal pain syndromes and eating disorders, and increasing evidences highlighted a link between gut microbiota and neurological and psychiatric disorders, such as anxiety and depression [8, 9].

Anxiety is a feeling characterized by agitation, anguish, fear, and disproportionate worry, usually without triggers, accompanied by various somatic signs [10]. This disturbance is related to different adverse health outcomes, especially in the elderly [11].

In vivo studies observed the development of anxiety signs and symptoms after fecal microbiota transplants, highlighting the ability to affect neuropsychiatric conditions through the changes of the microbial composition [12]. Although these effects were observed also in germ-free mice [13], this suggests to use probiotics as a treatment in neuropsychiatric disorders.

For this purpose, *Lactobacillus*, *Bifidobacterium*, and others species were used in animal and human studies as probiotic supplements to enhance the biodiversity and health of the gut microbiota [14] and to treat anxiety disorders, through the improvement of the MGB axis balance [15], obtaining the title "psychobiotics."

Interleukin-1 beta (IL-1β) was clearly identified as an important player in the onset and progression of several neurodegenerative diseases [16], and numerous studies have already proved the link between mood disorder symptoms and proinflammatory cytokine expression and circulating levels [17].

Psychophysical stress increases the proinflammatory cytokines through the HPA axis, impairing the gut barrier integrity and causing dysbiosis related to anxiety disorder [18–21]. In particular, IL-1β, after a psychophysiological stress stimulus, can affect the gut microbiota balance and mood status [22]. The IL-1β expression is strongly influenced by some polymorphisms in the IL-1β gene, which increase the related cytokine levels, thus affecting the magnitude of inflammatory disorders, making them a determining cofactor in several chronic diseases and potentially in the onset mood disorders [23].

In particular, increased levels of IL-1β were observed in the presence of the rs16944 polymorphism (NM_0000576.2:c.-598T>C), which is found in the promoter region of IL-1β [24]. The rs16944 is located in the functional promoter region (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). The presence of the allele A of rs16944 increases the IL-1β production, and it was associated with elevated risk of depression in schizophrenic spectrum disorders [25], depressive symptoms in Alzheimer disease [26, 27], and depressed state in breast cancer patients [28]. The relationship between IL-1β polymorphism and anxiety disorder was observed by Kovacs et al. [29], but no other study has investigated the combined effect of IL-1β polymorphism and probiotic administration in mood disorder phenotypes.

Therefore, in the present study, we investigated if the administration of psychobiotic suspension could represent a novel, safe, and long-term solution to treat or prevent anxiety disorders, to reduce associated symptoms, and to ameliorate their psychological state, in carriers of IL-1β rs16944 gene polymorphism. To this end, a randomized, placebo-controlled clinical trial was conducted on female and male volunteers.

In this study, the primary objective was to investigate the effects of the SNP rs16944 within the IL-1β gene on anxiety development in a sample of the Italian population. The secondary outcome was to evaluate the possible beneficial effect of a new probiotic formulation on anxiety and related symptoms according to the IL-1β SNP rs16944. The third objective was to assess a change in body shape perception before and after probiotic intervention. To this end, a randomized, placebo-controlled clinical trial was conducted on volunteers.

2. Materials and Methods

2.1. Study Design and Outcomes. The study protocol was conducted between January 2017 and July 2017, using an interventional randomized placebo-controlled clinical trial. At the time of recruitment, the patients were submitted to nutritional status and psychometric test evaluation. A medical history was performed and saliva samples were collected. The subjects received the psychobiotic mixture or the placebo at the beginning of the trial with consumption instructions. Volunteers consumed the relative treatments at home, once daily (1 sachet/day), two hours before lunch, in order to ensure adequate gastrointestinal transit and absorption. Eligible patients were randomly divided into two groups: (1) psychobiotic oral suspension group (POSG) and (2) placebo control group (PCG).

Both groups followed the assigned treatment for a 12-week period. The subjects were asked to maintain their usual lifestyle and dietary habits and to report any illness or adverse reaction emerging during study conduction. The subjects were asked to report any missed consumption of the products during the intervention. The POSG and PCG arms were double-blinded. The subjects repeated nutritional visit 12 weeks after intervention initiation of each arm (±3 days).

Nutritional status evaluation, psychometric tests, and buccal mucosa sample extraction were carried out at the time of enrollment (T0) and after the 12-week period intervention (T1). All participants recruited into the study authorized their participation by reading and signing the informed consent form, drafted in accordance to the provisions of the Ethics Committee of Medicine, University of Rome "Tor Vergata," and with the Helsinki Declaration of 1975, as

revised in 1983. Trial registration: this protocol has been registered with ClinicalTrials.gov Id: NCT01890070.

2.2. Subjects. 150 volunteers were initially recruited during routine medical check-up visits at the Section of Biomedicine and Prevention, Division of Clinical Nutrition and Nutrigenomics of the University of Rome "Tor Vergata." Exclusion criteria were age < 18 and >65, pregnant and lactating women, type 1 diabetes, established altered intestinal bacterial flora (intestinal bacterial overgrowth), history of psychiatric or psychological disturbance, absence of depression evaluated with Symptom Checklist-90 (SCL90) Global Severity Index (GSI) (score < 1), acute disease, endocrine, metabolic, liver, and gastrointestinal disease, cardiovascular or kidney dysfunction, cancer, and HIV infection. Subjects that were recently under antibiotic treatments, chronic pharmacological therapy with anti-inflammatory drugs or oral contraceptives, other probiotics or dietary supplements, subjects who are following dietary treatments, smokers, and alcohol and drug abusers were also excluded from the protocol. No subjects with known alterations of intestinal transit following organic pathologies (abdominal surgery, diabetes mellitus, scleroderma, hypothyroidism, etc.) were included in the study. The subjects enrolled into the study were asked to not consume any other probiotics or food supplements for the whole duration of the study.

2.3. Interleukin 1 Beta Genotyping. The DNA extraction from salivary samples collected with swabs was performed according to Hochmeister et al. [30]. gDNA was quantified with NanoDrop. Master Mix Taq DNA Polymerase and dNTPs (TaqPath ProAmp Master Mix, Life Technologies, CA, USA) and a two allele-specific fluorescent probes (TaqMan SNP Genotyping Assays, Life Technologies, CA, USA) were used to prepare the gDNA for the genotyping. The IL-1β gene rs16944 (NM_0000576.2:c.-598T>C) context sequence was as follows: TACCTTGGGTGCTGTTCTCTGCCT C(G/A)GGAGCTCTCTGTCAATTGCAGGAGC.

Genotyping was carried out using the StepOnePlus™ Real-Time PCR System (Applied Biosystems StepOnePlus Real-Time PCR, Life Technologies, CA, USA), according to the manufacturer's instructions.

2.4. Psychodiagnostic Instruments

2.4.1. Hamilton Anxiety Rating Scale (HAM-A). The Hamilton Anxiety Rating Scale (HAM-A) revised version questionnaire consists of 14 items used to define several anxiety-related symptoms, including both psychological and somatic symptomatology. The 14 items included are as follows: anxious mood; tension (startles, restlessness, and crying); fears (dark/strangers/crowds/animals); insomnia; "intellectual" (poor memory/difficulty concentrating); depressed mood (including anhedonia); somatic symptoms (aches, stiffness, and bruxism); sensory (tinnitus, blurred vision); cardiovascular (e.g., tachycardia and palpitations); respiratory (chest tightness, choking); gastrointestinal (irritable bowel syndrome-type symptoms); genitourinary (urinary frequency, impotence); autonomic (dry mouth, tension headache), and observed behavior at interview (restless, fidgety, etc.) [27].

In this study, HAM-A was administered by instructed physicians pre- and posttreatment. To each item, a score between 0 and 4 was attributed, considering 0 the absence and 4 the presence of severe symptoms. The total score ranges from 0 to 56 and was interpreted as follows: <17 mild anxiety, 17-24 mild-moderate anxiety, and 25-30 moderate-high anxiety.

Anxious individuals were considered the ones that had a score equal to or higher than 18 (≥18).

2.4.2. Body Uneasiness Test (BUT). The Body Uneasiness Test (BUT) is a self-assessment scale used for body image studies and related pathologies. BUT allows to calculate the Global Severity Index (GSI) or total average score, which is obtained from the sum of clinical scores (BUT-A), divided by their number (34). Item number with score ≥ 1 corresponds to Positive Symptom Total (PST). The sum of item scores ≥ 1 divided by PST produces the Positive Symptom Distress Index (PSDI) [31].

Five factors were defined: WP (Weight Phobia), BIC (Body Image Concerns), A (Avoidance), CSM (Compulsive Self-Monitoring), and D (Depersonalization). In our study, we considered as positive for altered perception of body image a GSI score ≥ 1.2.

2.4.3. Symptom Checklist-Revised (SCL90R). Symptom Checklist-Revised (SCL90R) is a general psychopathology self-assessment scale composed of 90 items, which investigates the presence of symptoms in the week before the test check. These 90 items, which have 5-level Likert answers, have 10 reference factors: (1) somatization (Som); (2) obsessive/compulsive (Obs); (3) interpersonal sensitivity (Interp Sens); (4) Depression (Dep); (5) anxious (Anx); (6) anger/hostility (Anger Host); (7) phobia (Phob); (8) psychoticism (Psych); (9) paranoia (Paran); and (10) sleep disorders. The score goes from 0 to 4, and a score above 1 is an index of pathology [32].

2.5. Composition of Probiotic Oral Suspension (POS). The POSG received 3 g/day of probiotic oral suspension (POS) containing *Streptococcus thermophiles* (1.5×10^{10} colony-forming unit (CFU), CNCM strain number I-1630), *Bifidobacterium animalis subsp. Lactis* (1.5×10^{10} colony-forming unit (CFU)), *Bifidobacterium bifidum* (1.5×10^{10} colony-forming unit (CFU)), *Streptococcus thermophiles* (1.5×10^{10} colony-forming unit (CFU)), *Lactobacillus bulgaricus* (1.5×10^{10} colony-forming unit (CFU), CNCM strain numbers I-1632 and I-1519), *Lactococcus lactis subsp. Lactis* (1.5×10^{10} colony-forming unit (CFU), CNCM strain number I-1631), *Lactobacillus acidophilus* (1.5×10^{10} colony-forming unit (CFU)), *Lactobacillus plantarum* (1.5×10^{10} colony-forming unit (CFU)), *Lactobacillus reuteri* (1.5×10^{10} colony-forming unit (CFU), DSM 17938), corn maltodextrin, anticaking agent (silica), casein, lactose, and gluten < 3 ppm LLOQ (lower limit of quantitation) (Biocult Strong, HOMEOSYN, Rome, Italy).

The placebo was 3 g/day of inert material (flour type 00), maltodextrin from corn, anticaking agent (silica), casein, lactose, and gluten < 3 ppm LLOQ (lower limit of quantitation) (HOMEOSYN, Rome, Italy). The appearance of the placebo was indistinguishable in color, shape, size, packaging, smell, and taste from that of the probiotic supplement.

2.6. Statistical Analysis. Hardy-Weinberg equilibrium (HWE) was assessed using SNP-HWE program and tested using the χ^2 analysis (Wigginton et al. 2005). To analyze the sample, subjects were divided into carriers (IL-1β rs16944, -598C) and noncarriers (IL-1β rs16944, -598T). The power of the study was calculated with the Quanto Program (USC Biostats, California, US). Shapiro-Wilk test was performed to determine parametric and nonparametric data. For comparisons between averages and medians, nonparametric tests for asymmetrically distributed data were conducted in all analyses and presented as mean (±standard deviation). In order to determine the presence of statistically significant differences among treatments and IL-1β carriers/-noncarriers, *t*-test or Mann–Whitney test was performed. Percent frequency variation was analyzed using the McNemar and Pearson chi-square test. The association of IL-1β and the categorical Hamilton score was assessed by binary logistic regression (LBM) represented as odds ratio (OR) and 95% confidence intervals (CI). In all the statistical tests performed, the null hypothesis was rejected at the probability level greater than or equal to 0.05 ($p \geq 0.05$). General Estimated Equations (GEE) were used to model the effects of risk and protective factor correlation between treatment, A carriers and noncarriers, time, and HAM-A results [33]. Statistical analyses were carried out using the IBM SPSS21.0 software for Windows (Armonk, NY: IBM Corp. USA).

3. Results

3.1. Population Characteristics. Out of the 150 patients recruited, 8 were excluded as they did not meet the inclusion criteria. The remaining 142 subjects were randomized equally into two groups. The first group (POSG) consumed the probiotic mixture formulation, and the second group (PCG) consumed the placebo formulation. During this clinical trial, 6 subjects from POSG and 34 subjects from PCG abandoned the study for the poor performances of treatments (Figure 1).

The final sample consisted of 97 patients, with ages ranging from 18 to 62 years old (POSG: mean 43.81 (±14.88), PCG: mean 32.92 (±11.75)). These patients successfully participated and completed the study protocol. At baseline, the total sample was divided according to A carrier and noncarrier for SNP rs16944. The tested SNPs of the IL-1β gene was in Hardy-Weinberg equilibrium ($p > 0.05$). The power of the study was 0.95, with fixed $\alpha = 0.05$ and 2-sided. Genotype frequencies shown in TSI population (GG: 0.38, AA: 0.14, AG: 0.48) [34] are similar to the ones of our subjects (GG: 0.47, AA: 0.14, AG: 0.39), as well as the allele frequencies for TSI (A: 0.38; G: 0.62) and for our sample (A: 0.34; G: 0.66) (Table 1).

3.2. Influence of IL-1β Polymorphism on HAM-A, BUT, and SCL-90R Tests. The overall description of the total sample population at baseline can be seen in Table 2. Of the 65 subjects in POSG, 31 subjects were noncarrier (47.69%) and 34 (52.31%) A carrier. Of the 34 subjects in PCG, 15 (44.12%) subjects were noncarrier and 19 (55.88%) A carrier. At baseline, among treatment groups, no statistically significant difference ($p \geq 0.05$) for total BUT score, BUT GSI score, total SCL-90R score, and SCL90R GSI score (Table 3) was highlighted. However, at baseline, there is a difference between frequencies of A carrier and non carrier for HAM-A within the two groups ($p < 0.01$) (Table 3). Moreover, A carriers, according to HAM-A, had significantly higher risk to be anxious compared to noncarriers ($p < 0.01$; OR = 5.90 (1.73; 20.16)) (Table 4), showing an interaction between IL-1β polymorphism and anxiety state. Frequencies of A carriers and noncarriers, according to psychometric results, before and after treatment, were reported in Table 5.

3.3. Effect of POS Treatment on HAM-A, BUT, and SCL-90R Questionnaires according to IL-1β SNP. After 12 weeks of intervention, we noticed an improvement in the psychometric parameters according to HAM-A test. POS treatment reduced score significantly (Table 3) and the frequency of anxious patients ($\Delta\% = -10.64\%$), more than in PCG ($\Delta\% = -5.10\%$) (Table 5).

Furthermore, GEE analysis highlighted a significant reduction of the HAM-A total score after POS treatment compared to the PC ($\beta = -0.33$; $p < 0.01$; OR = 0.68 (0.40; 1.15)). POS treatment determined a significant reduction of anxiety risk in A carriers ($\beta = -0.32$; $p = 0.02$; OR = 0.73 (0.56; 0.94)), but not in noncarriers ($p \geq 0.05$) (Table 6). These results highlighted the beneficial effect of POS treatment on anxiety state and the increased sensitivity of IL-1β A carriers to probiotic administration on anxiety reduction. Conversely, BUT and SCL-90 questionnaire results did not show significant changes after POS treatment compared to placebo (Table 3 and Figure 2).

4. Discussion

Among the most prevalent psychiatric disorders, anxiety is a condition that occurs worldwide, affecting the normal functioning of millions of people and burdening national health system economies. Despite the worldwide high prevalence, such disorders are often neglected and misdiagnosed. It is common for anxiety-affected individuals to be also suffering from other physical symptoms or concomitant mood disorders, like depressive conditions, drugs abuse, and even suicide (National Institute of Mental Health 2010).

Many studies focused on the role of inflammation on the CNS functions and relative diseases. In particular, IL-1β has pleiotropic effects on the CNS, where the proinflammatory cytokine, released by neurons and glial cells, acts in an autocrine and/or paracrine fashion, participates in the onset and progression of different neurodegenerative diseases and stroke [16]. Furthermore, proinflammatory cytokine expression and circulating levels, like interferon gamma (INFγ),

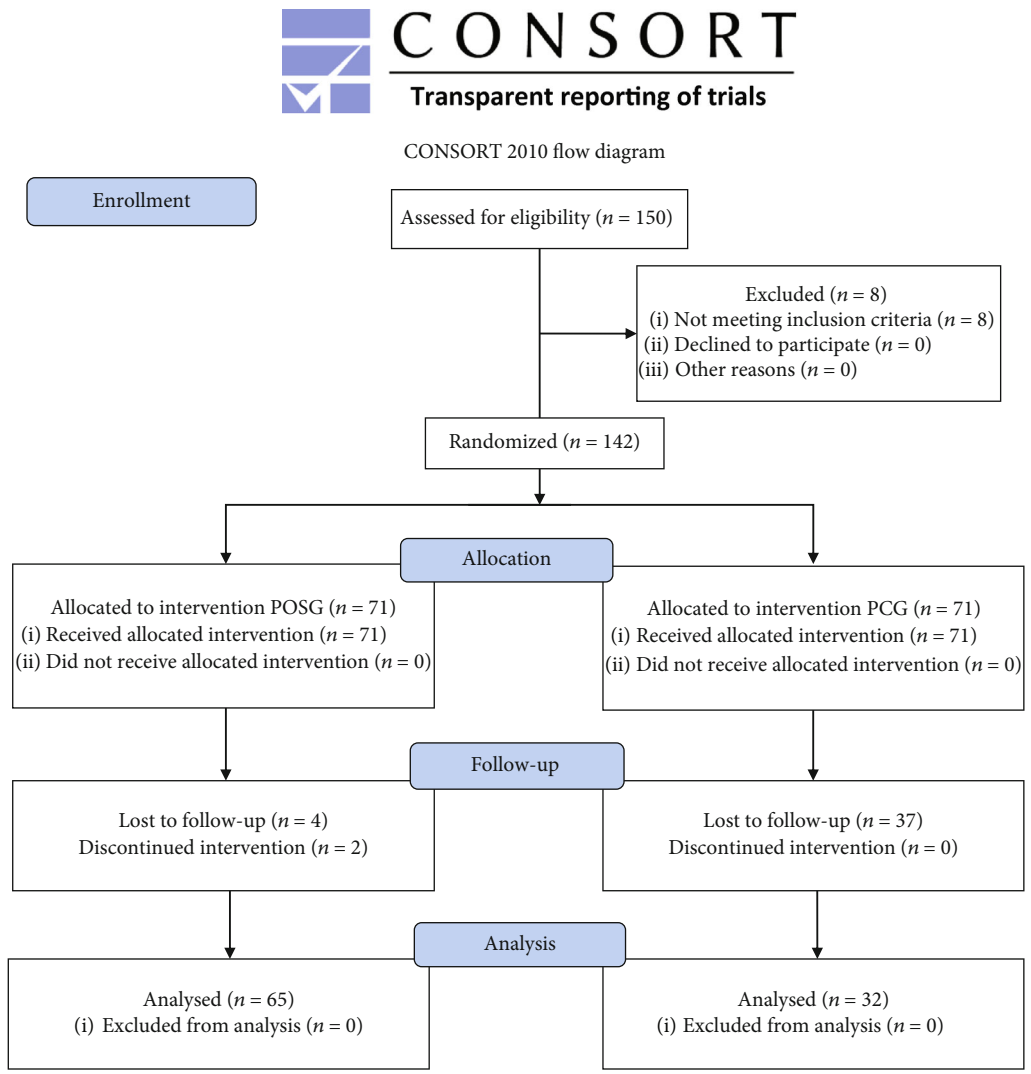

Figure 1: Study design. Consort flow diagram of the study. Probiotic oral suspension group (POSG) and placebo control group (PCG).

Table 1: Study population allele and genotype frequencies for IL-1β rs16944 compared to Tuscan Italians from Southern Europe (TSI).

IL-1β rs16944		
Allele frequency	A	G
TSI	0.38	0.62
Study population	0.34	0.66
Genotype frequency	AA	AG
TSI	0.14	0.48
Study population	0.14	0.39

Table 2: Descriptive characteristics of recruited study population.

Parameter ($n = 97$)	Mean (±SD)
Gender (%)	Female = 61.9% Male = 38.1%
Age	41.29 (±14.90)
Total BUT score	37.17 (±33.36)
BUT GSI score	1.09 (±0.98)
Total SCL-90R score	61.83 (±47.33)
SCL-90R GSI score	0.69 (±0.53)
Hamilton score	10.91 (±7.31)

Descriptive table. Results are expressed in mean ± SD. BUT: Body Uneasiness Test (BUT); SCL90R : Symptom Checklist-Revised; HAM-A: Hamilton Anxiety Rating Scale.

TNFα, IL-6, and IL-1β, are associated with mood disorder symptoms [17].

In mouse models, IL-1β concentrations have been linked not only to neurodegenerative diseases but also to memory impairment [35] and anxiety disorders [36, 37].

In humans, IL-1β polymorphisms are linked to the levels of related cytokine expression and consequently to elevated risk of depression in different populations [25, 26, 28].

Nevertheless, the emerging knowledge of the MGB axis highlights the role of the gut microbiota as an important modulator of neuroinflammation, stress response, mood, and behavior and increases its importance in psychiatric

Table 3: Descriptive characteristics of study population ($n = 97$) according to treatments and IL-1β.

(a)

	POSG	PCG		POSG			PCG			A carrier POSG vs. PCG	Noncarrier POSG vs. PCG
	Baseline	Baseline	p	A carrier Baseline	Noncarrier Baseline	p	A carrier Baseline	Noncarrier Baseline	p	p	p
Total BUT score	40.42 ± 13.81	37.67 ± 10.67	0.133	42.16 ± 41.46	40.65 ± 22.89	0.871	41.67 ± 37.64	49.67 ± 25.86	0.721	0.605	0.231
BUT GSI score	1.19 ± 1.02	0.52 ± 0.31	0.123	1.45 ± 1.22	0.90 ± 0.67	0.091	0.76 ± 1.22	0.78 ± 0.17	0.925	0.128	0.085
Total SCL-90R score	64.17 ± 50.19	47.83 ± 21.54	0.441	78.53 ± 58.81	68.12 ± 43.18	0.325	62.67 ± 40.60	63.30 ± 38.72	0.374	0.325	0.525
SCL-90R GSI score	0.71 ± 0.56	0.53 ± 0.24	0.146	0.87 ± 0.65	0.53 ± 0.37	0.565	0.70 ± 0.23	0.67 ± 0.10	0.567	0.095	0.525
Hamilton score	10.77 ± 7.63	11.50 ± 5.91	0.738	13.03 ± 8.00	8.50 ± 6.63	0.025	14.38 ± 4.37	7.67 ± 5.75	0.012	0.401	0.217
Age	43.81 ± 14.88	37.92 ± 11.75	0.091								

(b)

	POSG						PCG					
	A carrier			Noncarrier			A carrier			Noncarrier		
	Baseline	T1	p	Baseline	T1	p	Baseline	T1	p	Baseline	T1	p
Total BUT score	42.16 ± 41.46	47.42 ± 43.11	0.787	40.65 ± 22.89	35.83 ± 40.40	0.565	41.67 ± 37.64	45.09 ± 45.89	0.521	49.67 ± 25.86	43.33 ± 3.51	0.258
BUT GSI score	1.45 ± 1.22	1.39 ± 1.27	0.341	0.90 ± 0.67	0.91 ± 1.19	0.566	0.76 ± 1.22	1.33 ± 1.35	0.128	0.78 ± 0.17	0.83 ± 0.10	0.162
Total SCL-90R score	78.53 ± 58.81	74.36 ± 58.22	0.140	68.12 ± 43.18	59.60 ± 62.56	0.374	62.67 ± 40.60	63.54 ± 58.76	0.296	63.30 ± 38.72	60.33 ± 42.52	0.624
SCL-90R GSI score	0.87 ± 0.65	0.83 ± 0.65	0.302	0.53 ± 0.37	0.64 ± 0.69	0.064	0.70 ± 0.23	0.82 ± 0.65	0.652	0.67 ± 0.10	0.90 ± 0.33	0.136
Hamilton score	13.03 ± 8.00	9.33 ± 7.42	0.001	8.50 ± 6.63	6.07 ± 6.61	0.152	14.38 ± 4.37	14.13 ± 4.26	0.351	7.67 ± 5.75	7.33 ± 5.28	0.363

Results are expressed in mean ± standard deviation. t-test was used for all parameters. Significant values are for $p < 0.05$. POSG: probiotic oral suspension group; PCG: placebo control group; BUT: Body Uneasiness Test; SCL90R: Symptom Checklist-Revised; HAM-A: Hamilton Anxiety Rating Scale; BUT GSI: Body Uneasiness Test Global Severity Index; SCL90R GSI: Symptom Checklist-Revised Global Severity Index.

Table 4: IL-1β A carrier risk for depression, dysmorphic, and anxiety symptoms.

	χ^2 value	$\chi^2 p$	β	SE	p	OR (minimum-maximum)	R^2
BUT	1.91	0.17	0.92	0.67	0.17	2.50 (0.67; 9.31)	0.06
SCL-90R	1.63	0.20	0.97	0.78	0.21	2.64 (0.58 12.09)	0.06
HAM-A	9.08	<0.01	1.78	0.63	<0.01	5.90 (1.73; 20.16)	0.18

IL-1β A carrier risk for depression, dysmorphic, and anxiety symptoms evaluated with Body Uneasiness Test (BUT), Symptom Checklist-Revised (SCL90R), and Hamilton Anxiety Rating Scale (HAM-A).

Table 5: BUT, SLC-90R, and HAM-A frequencies.

			BUT		SCL-90		HAM-A	
			Healthy	Dysmorphic symptoms	Healthy	Depressive symptoms	Healthy	Anxiety symptoms
		T0	54.55%	45.45%	68.18%	31.82%	56.76%	43.24%
	Total population	T1	61.54%	38.46%	66.67%	33.33%	70.97%	29.03%
		Δ%		-6.99%		1.52%		-14.21%
		T0	100.00%	0.00%	100.00%	0.00%	37.50%	62.50%
A carrier	PCG	T1	100.00%	0.00%	100.00%	0.00%	50.00%	50.00%
		Δ%		0.00%		0.00%		-12.50%
		T0	47.37%	52.63%	63.16%	36.84%	62.07%	37.93%
	POSG	T1	58.33%	41.67%	63.64%	36.36%	78.26%	21.74%
		Δ%		-10.96%		-0.48%		-16.19%
		T0	75.00%	25.00%	85.00%	15.00%	88.57%	11.43%
	Total population	T1	88.89%	11.11%	87.50%	12.50%	93.10%	6.90%
		Δ%		-13.89%		-2.50%		-4.53%
		T0	100.00%	0.00%	100.00%	0.00%	100.00%	0.00%
Noncarrier	PCG	T1	100.00%	0.00%	100.00%	0.00%	100.00%	0.00%
		Δ%		0.00%		0.00%		0.00%
		T0	70.59%	29.41%	82.35%	17.65%	86.21%	13.79%
	POSG	T1	83.33%	16.67%	80.00%	20.00%	91.30%	8.70%
		Δ%		-12.75%		2.35%		-5.10%
		T0	64.29%	35.71%	76.19%	23.81%	72.22%	27.78%
	Total population	T1	64.29%	35.71%	76.19%	23.81%	81.67%	18.33%
		Δ%		0.00%		0.00%		-9.44%
		T0	100.00%	0.00%	100.00%	0.00%	64.29%	35.71%
Total	PCG	T1	100.00%	0.00%	100.00%	0.00%	71.43%	28.57%
		Δ%		0.00%		0.00%		-7.14%
		T0	58.33%	41.67%	72.22%	27.78%	74.14%	25.86%
	POSG	T1	66.67%	33.33%	68.75%	31.25%	84.78%	15.22%
		Δ%		-8.33%		3.47%		-10.64%

Frequencies for positive/negative classification on depression, dysmorphic, and anxiety symptoms evaluated with Body Uneasiness Test (BUT), Symptom Checklist-Revised (SCL90R), and Hamilton Anxiety Rating Scale (HAM-A). Results are expressed as percentage. POSG: psychobiotic oral suspension group; PCG: placebo control group.

Table 6: Association of IL-1β and POSG treatment with HAM-A results.

	β	Error SD	p	OR (minimum-maximum)
POSG vs. PCG	-0.33	0.11	<0.01*	0.68 (0.40; 1.15)
POSG vs. PCG in IL-1β rs16944 noncarriers	-0.29	0.19	0.12	0.75 (0.52; 1.08)
POSG vs. PCG in IL-1β rs16944 A carriers	-0.32	0.13	0.02*	0.73 (0.56; 0.94)

HAM-A results associated with polymorphism rs16944 within the IL-1β gene with 12 weeks in the psychobiotic oral suspension group (POSG). GEE analysis for HAM-A results, significant values (*$p \leq 0.05$) are expressed for POSG vs. placebo control group (PCG), POSG vs. PCG in IL-1β rs16944 noncarrier group, and POSG vs. PCG in IL-1β rs16944 carrier group.

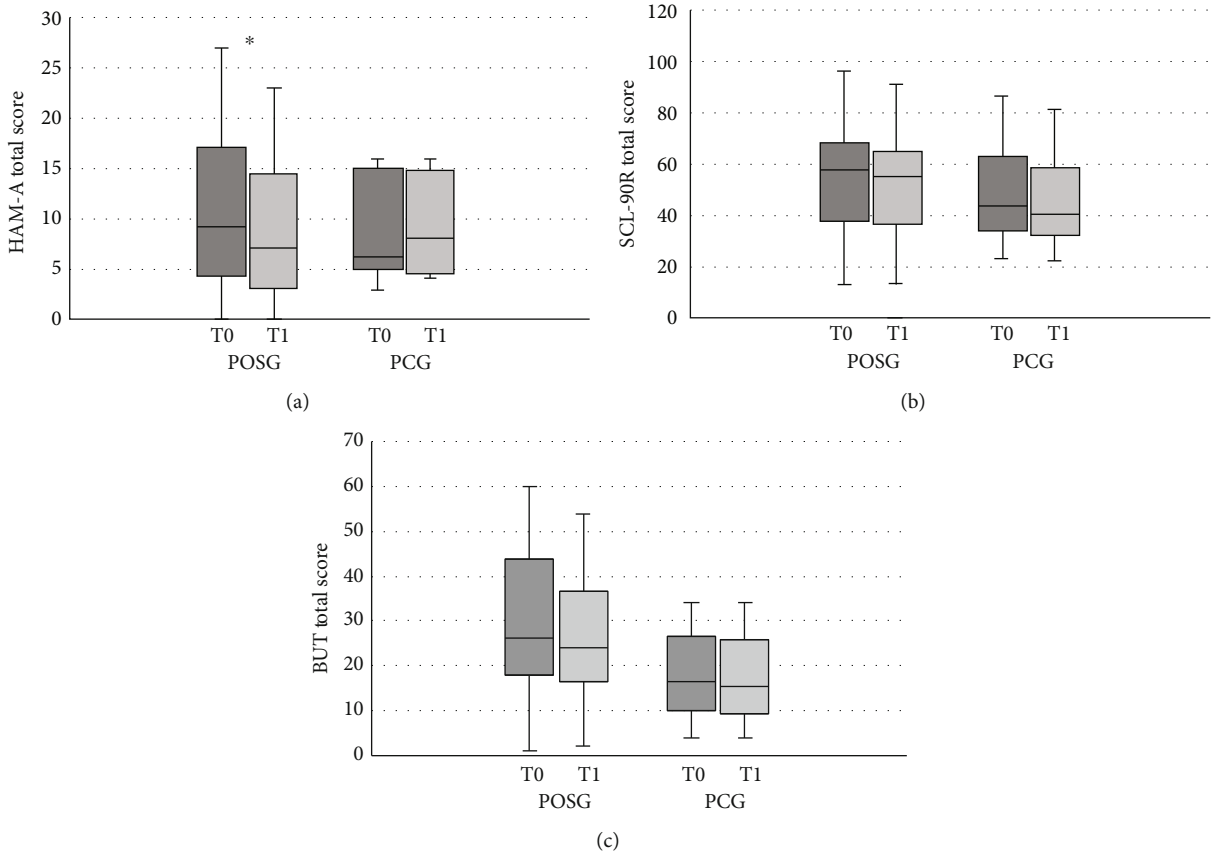

Figure 2: Comparison of psychometric test results at baseline and after treatment in POSG and PCG. Comparison of POSG and PCG before and after treatment. HAM-A: Hamilton Anxiety Rating Scale; SCL-90R: Symptom Checklist-90 Revised; BUT: Body Uneasiness Test; POSG: probiotic oral suspension group; PCG: placebo control group. Values are presented as median with min and max. Statistical significance attributed to results with $^{*}p < 0.05$.

disorder onset and progression, including anxiety [14, 15, 19, 21]. The gut microbiota is able to regulate systemic IL-1β concentrations [38], potentially modulating anxiety disorders [39–41].

Despite the numerous studies, the mechanism that links systemic inflammation and neurological disorders is still poorly understood, and nowadays, there is a gap in the scientific literature about the role of IL-1β in human anxiety.

In this study, we genotyped the IL-1β gene (rs16944) to observe the relationship of the polymorphism and anxiety state in an Italian population sample. At baseline, we found frequency differences between IL-1β A carriers and noncarriers, according to HAM-A scores ($p < 0.01$). In fact, anxious subjects were 43.24% A carriers and 11.43% noncarriers. At baseline, A carriers had moderate but significative increased risk to be anxious compared to noncarriers (5.90 (1.73; 20.16)).

Our observations implicate a bidirectional relationship between anxiety disorders and rs16944 polymorphism in an Italian population. The present results are in line with previous data shown by Kovacs et al. [29], which found a relationship between high life stress and anxiety symptoms, measured by the Brief Symptom Inventory, and the minor A allele of rs16944 polymorphism in Hungarian population. In that study, however, the increase in anxiety symptoms was related to childhood adversity, suggesting that both early life stress and the presence of the minor allele A are synergic contributing factors in disorder development. The number of studies investigating the role of IL-1β SNPs in anxiety disorders is few; hence, the present results should stimulate scientific interest on the influence of genetic asset and anxiety disorders.

In the light of this association, we investigated the combined effect of IL-1β polymorphism and probiotic administration in mood disorder phenotypes. Logan and Katzman assumed for the first time that the use of probiotics as adjuvant treatment in patients with major depressive disorder, a condition with complex pathophysiology associated with neurotransmitter and neuromodulator deficiencies, increased proinflammatory cytokine levels, gastrointestinal disturbances, and HPA axis dysfunction [42]. More recently, literature has shown that selective modulation of gut microbiota by exogenous agents, such as probiotic administration, could represent a novel therapeutic approach for mood and anxiety disorders [43, 44]. The beneficial effects of anxiety- and depression-related behavior are mainly obtained through the administration of the genera *Bifidobacterium* and *Lactobacillus*, but only some specific strains have

ensured positive results [15]. Therefore, the secondary outcome of the present study was to evaluate the potential anxiolytic effect of the novel psychobiotic formulation to treat or prevent anxiety disorders, assessed with HAM-A scale, according to the IL-1β SNP rs16944.

After the 12-week intervention, we observed an improvement in the psychometric parameters. As determined by GEE analysis, HAM-A total score was significantly reduced in subjects who consumed the probiotic formulation ($p < 0.01$) compared to PCG results. Furthermore, the probiotic mixture lowered the percentage of anxious patients ($\Delta\% = -10.64\%$), more than in PCG ($\Delta\% = -5.10\%$) (Table 5).

These data suggest that probiotic intake has an impact on anxiety and confirmed our previous results of the concomitant administration of probiotics and hypocaloric diet in obese subjects [45] that suggested a greater improvement of anxiety symptoms.

For the evaluation of body image perception, BUT-A was performed both at baseline and on follow-up visit. As can be observed in Table 3, at baseline, BUT did not highlight a difference between POSG and PCG GSI score results (1.19 ± 1.02 and 0.52 ± 0.31, respectively), regardless of being A carrier or noncarrier. At the end of the 12-week intervention, we did not notice a significant reduction in BUT-A GSI in both POS and PC groups. Thus, we cannot conclude that the administered probiotic formulation is able to modify body image disorders. Moreover, our results are not in line with a previous study performed by De Lorenzo et al. [46], probably because the groups selected in that study included only women, making it difficult to compare the results, knowing the test's limitation according to Cuzzolaro et al. [31] in the male population. Therefore, in contrast to Messaoudi et al. [47], our GSI results of psychological distress measured by SCL-90R after 12-week intervention were not significant ($p \geq 0.05$) in this study. In our opinion, those results can be explained by the low GSI score (GSI < 1) since the baseline time point (Table 3).

Family environment and genetics are established risk factors in the etiology of psychiatric disorders as well as anxiety development [48]. Multiple genes of small effect contribute to the disorder vulnerability, and the interaction between genetic and distressing environmental factors may lead to the onset of anxiety disorders [49]. There is a number of convincing studies that have recognized a direct transmission of anxiety within families, mainly observed in first-degree relatives, with an overall four- to sixfold increased risk [50]. The genetic contribution to the pathophysiology of psychiatric disorders is highly complex. Previous studies found higher risk of depression in IL-1β gene rs16944 carriers of the higher synthesizing A allele, in schizophrenia [25] and Alzheimer disease patients [26].

Interestingly, in this study, after the 12-week probiotic intake, IL-1β A carriers, but not noncarriers, had a significant reduction of HAM-A score ($p = 0.02$), and the frequency percentage of anxious carriers has been cut from 37.93% to 21.74% (Table 5). Although we cannot exclude an independent impact of the minor A allele of rs16944 on microbiota composition and modulation, our results suggest that the psychobiotic administration determined a reduction of anxiety and related symptoms and restored psychological equilibrium in the treated sample.

In conclusion, despite the limitations related to the lack of IL-1β blood level measurement in this clinical study, our results suggest that the consumption of probiotics mitigates anxiety symptoms, especially in healthy adults with the minor A allele of rs16944 as a risk factor. This study provides further evidences that gut microbiota is involved in the psychological state and that its modulation may improve the overall quality of life. Furthermore, the 12-week intervention was sufficient to afford significant results without manifestation of adverse events, and so, the psychobiotic intake represents a good approach to attenuate anxiety-related feelings. Thus, probiotics might serve as a new therapeutic approach for neuropsychiatric disorder treatment and/or prevention. Although preclinical data suggest the benefits of probiotic use in anxiety-related disorders, clinical evidence is somewhat lacking as well as the establishment of which probiotic strains clearly have psychobiotic properties. In the light of these observations, clinical studies on the role of psychobiotics in anxiety are at the very least necessary in order to establish more accurately the probiotic therapeutic efficiency.

The research field related to gut microbiota manipulation and mood disorders is far from exhausted. Hence, our results are aimed at further contributing to the scientific evidences on psychobiotic ability to manage anxiety disorders and improve related symptomatology and identifying the potential mechanisms implicated. The next step would be the assessment of the minor A allele of rs16944 on microbiota composition and modulation and then the "psychobiotics" effect of probiotics compared to anxiolytic drugs on anxiety-diagnosed subjects, to further confirm their psychotropic properties.

Abbreviations

ANS: Autonomic nervous system
BUT: Body Uneasiness Test
CFU: Colony-forming unit
CNS: Central nervous system
DNA: Deoxyribonucleic acid
ENS: Enteric nervous system
GAD: Generalized anxiety disorder
GI: Gastrointestinal
HAM-A: Hamilton Anxiety Rating Scale
HPA: Hypothalamic-pituitary-adrenal
IL: Interleukin
LPS: Lipopolysaccharide
MGB: Microbiota-gut-brain
POS: Probiotic oral suspension
SCL: Symptom Checklist
SNP: Single-nucleotide polymorphism.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

PG, GC, and LDR conceived, designed the experiments, and drafted the manuscript; GC performed the experiments; GC and LR collected and analyzed the data; MM and ADL drafted the manuscript; AC, CC, and RC collected the data; LDR had primary responsibility for the final content. All the authors read and approved the final manuscript. All the authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. PG and GC contributed equally to this work.

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Review Article

The Interplay between Immune System and Microbiota in Diabetes

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Diabetes is not a single and homogeneous disease, but a cluster of metabolic diseases characterized by the common feature of hyperglycemia. The pathogenesis of type 1 diabetes (T1D) and type 2 diabetes (T2D) (and all other intermediate forms of diabetes) involves the immune system, in terms of inflammation and autoimmunity. The past decades have seen an increase in all types of diabetes, accompanied by changes in eating habits and consequently a structural evolution of gut microbiota. It is likely that all these events could be related and that gut microbiota alterations might be involved in the immunomodulation of diabetes. Thus, gut microbiota seems to have a direct, even causative role in mediating connections between the environment, food intake, and chronic disease. As many conditions that increase the risk of diabetes modulate gut microbiota composition, it is likely that immune-mediated reactions, induced by alterations in the composition of the microbiota, can act as facilitators for the onset of diabetes in predisposed subjects. In this review, we summarize recent evidence in the field of gut microbiota and the role of the latter in modulating the immune reactions involved in the pathogenesis of diabetes.

1. Introduction

Diabetes can be described as a cluster of metabolic diseases characterized by the common feature of hyperglycemia. However, it is not a single and homogeneous disease and is therefore difficult to classify.

In the past, it was categorized on the basis of age at diagnosis and the need for insulin therapy. The latest pathogenetic [1] classification identifies four forms of diabetes; in particular, the subdivision into type 1 (T1D) and type 2 (T2D) diabetes was introduced to replace insulin-dependent and noninsulin-dependent diabetes.

T1D is the most common metabolic disorder in children and young adults, and is due to a progressive autoimmune or idiopathic β-cell destruction, with the end result of absolute insulin deficiency. It is a multifactorial disease, in which a genetic predisposition, combined with a triggering event, initiates the activation of self-reactive lymphocytes. Although in the early stages the disease is clinically silent, it is already possible to detect autoantibodies directed against β-cell antigens. Several factors have been hypothesized to contribute to T1D onset, including chemicals, viruses, commensal bacteria, and diet. T2D, on the other hand, most commonly occurs in adulthood, against a background of obesity and insulin resistance. It is characterized by an initial phase of compensatory hyperinsulinemia, creating an overload for pancreatic β-cells, leading to a progressive loss of insulin secretive function, and consequently to hyperglycemia.

However, several studies have shown that this subdivision does not accurately describe some intermediate forms of diabetes, with overlapping features [2]. It is increasingly common to see obese young people with metabolic characteristics of T2D with autoantibodies for β-cells typical of T1D now defined as "double diabetes" or type 1.5 diabetes [3]. Moreover, there is another well-known type of diabetes, called Latent Autoimmune Diabetes in Adults (LADA),

which shares mechanisms belonging to the two abovementioned diseases: a progressive reduction in insulin secretion due to autoimmune destruction of β-cells and, although to a lesser extent than in T2D, insulin resistance.

Over the past decades, there has been an increase in all forms of diabetes, accompanied by changes in eating habits and consequently a structural evolution of gut microbiota [4]. It is likely that all these events could be related and that gut microbiota alterations might be involved in the immunomodulation of diabetes.

Immunomediated pathogenesis is a common feature of almost all forms of diabetes, in terms of inflammation and/or autoimmunity. On the other hand, alterations of gut microbiota seem to be linked to several immune-driven inflammatory diseases [5–7]. These observations have led researchers to hypothesize a possible link between diabetes onset and gut microbiota alterations.

Against this complex background, recent studies have shown that the development of diabetes is closely related to alterations of gut microbiota, an important "organ" consisting of bacteria, viruses, protozoa, and fungi living in the gastroenteric tube [8]. The microbiota provides protection against pathogenic microbes by maintaining local intestinal integrity and regulating intestinal barrier permeability.

All this is possible thanks to a symbiotic relationship favored by the balance between gut microbiota, intestinal epithelial cells, and the mucosal immune system [9]. The disruption of this equilibrium, called dysbiosis, seems to be involved not only in the pathogenesis of several intestinal diseases, such as inflammatory bowel disease (IBD) [10], celiac disease, irritable bowel syndrome (IBS), and colorectal cancer [11], but also in metabolic diseases including obesity, metabolic syndrome, and diabetes [12, 13]. Recently, intestinal microbiota composition has been shown to play a role in obesity [14] and diabetes [15], but the exact molecular mechanisms through which a given intestinal microbiota induces metabolic diseases still need to be clarified. In the case of T2D, increased energy harvesting and the triggering of a low-grade inflammatory status in insulin resistance and obesity [16] are two of the possible mechanisms involved. Therefore, recent studies also suggest that gut microbiota contributes to the risk of developing T1D in genetically predisposed individuals; indeed, environmental factors that may affect the risk of developing T1D, including birth delivery mode [17], diet in early life [18], and possibly the use of antibiotics [19], are all related to the intestine and its microbiota.

Thus, gut microbiota seems to have a direct, even causative role in mediating connections between the environment, food intake, and chronic disease. Whereas many conditions that increase the risk of diabetes modulate gut microbiota composition, it is likely that immune-mediated reactions induced by alterations in the composition of the microbiota can act as facilitators of the onset of diabetes in predisposed subjects.

In this review, we will summarize the recent evidence in the field of gut microbiota and the role of the latter in modulating immune reactions involved in the pathogenesis of diabetes.

2. Gut Microbiota Composition in Diabetes

Intestinal microbiota is made up of five dominant bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* [20]. In particular, *Bacteroidetes* and *Firmicutes* are the main bacterial phyla known to be correlated with obesity and T2D. The *Firmicutes* phylum is composed of *Ruminococcus*, *Clostridium*, *Lactobacillus*, and butyrate-producing bacteria, while the *Bacteroidetes* phylum consists of *Bacteroides*, *Prevotella*, and *Xylanibacter* [20].

Human and animal studies have been used to demonstrate that gut microbiota composition is altered in diabetes. Comparing the gut microbiota of lean mice and mice with diet-induced obesity, some authors found an increase in the abundance of *Firmicutes* associated with diet-induced obesity [21]. These observations were supported by the identification of an increase in the *Firmicutes*/*Bacteroidetes* ratio in ob/ob mice and in mice fed a high-fat diet compared with lean mice. Furthermore, this increase was more significant in the high-fat diet-fed mice than in the ob/ob mice [22].

Other studies have also demonstrated a strong connection between T2D and changes in the composition of gut microbiota. A study conducted on diabetic patients compared to nondiabetic controls showed that the proportions of phylum *Firmicutes* and class *Clostridia* were significantly reduced in the diabetic group compared to the control group, while there was a greater quantity of *Bacteroidetes* and *Proteobacteria*. Consequently, the ratios of *Bacteroidetes* to *Firmicutes* were found to be significantly and positively correlated with reduced glucose tolerance [15].

In humans, however, there are still doubts as to whether the state of intestinal microbiota is the consequence or the cause of the altered metabolic condition. To clarify this, studies using germ-free mice have demonstrated the central role of intestinal microbiota in triggering metabolic impairments, even though it remains to be demonstrated whether genetic background can influence the development of a specific microbiota.

Diet is one of the main determinants of intestinal microbiota composition and an extremely important causal factor in the development of T2D. Turnbaugh et al., for example, have shown that microbiome structure is rapidly altered in response to a switch from a low-fat, plant polysaccharide-rich diet to a high-fat, high-sugar "Western" diet [23].

In the last decades, human food habits have changed, with fats being preferred over fibers; thus, gut microbiota has changed in response to the new feeding habits. It has therefore been hypothesized that the diabetes epidemic could be related to the structural change of gut microbiota.

Studies have found that in T1D there is an imbalance in intestinal microbiota; thus, children with T1D showed higher levels of *Bacteriodetes* than controls, who instead had higher levels of *Prevotellla* [24]. Other studies have found a reduction in beneficial anaerobic bacteria in children with T1D and an increase in *Enterobacteriaceae*, and proposed this as a possible immune trigger for T1D onset [25]. A Finnish study that evaluated children at high genetic risk for T1D, following them from birth to 2.2 years of age, showed that the species *Bacteroides dorei* and *Bacteroides vulgatus* were

found in greater numbers in T1D cases compared to controls prior to seroconversion, suggesting that early changes in microbiota composition could be useful in predicting T1D autoimmunity in genetically susceptible infants [26].

Diabetes-related alterations in gut microbiota composition have also been associated with exposure to xenobiotics, such as heavy metals, persistent organic pollutants (POPs), and organophosphate. In the last decades, there has been a massive production and release of toxic chemicals affecting the entire globe. Many of these chemicals interfere with the endocrine system altering hormone production, release, transport, and activities and are known as endocrine-disrupting chemicals (EDCs) [27]. EDCs enter the human body mainly through the mouth, and gut microbiota plays a central role in their metabolism, thus contributing to obesity and diabetes [28]. Among heavy metals, arsenic exposure in mice seems to affect gut microbiota composition, not altering *Bacteriodetes* but decreasing several species of *Firmicutes* (*Eubacterium*, *Faecalibacterium*, and *Roseburia*), leading to metabolomic changes in gluconeogenesis, adipogenesis, lipogenesis, and inflammation [29]. Regarding POPs, exposure to 2,3,7,8-tetrachlorodibenzofuran (TCDF) affected gut microbial structure, with a decrease in the *Firmicutes*/-*Bacteriodetes* ratio. TCDF also increased levels of Flavobacteriia and *Butyrivibrio* spp. and decreased Clostridia and *Oscillobacter* spp. resulting in an increase in bile acid metabolites. Increased levels of SCFAs were also observed in fecal and cecal contents of TCDF-exposed mice and may be responsible for altered hepatic lipogenesis, gluconeogenesis, and glycogenolysis [30]. Organophosphates (OPs) are chemical substances used in insecticides, herbicides, etc., which inhibit acetylcholine esterase; animal studies have shown that prolonged intake of the OP insecticide monocrotophos induces hyperglycemia, dyslipidemia, cardiac oxidative stress, and myocardial infarction in rats [31] An altered hepatic gluconeogenesis mediated by OP-degrading gut microbiota has been demonstrated to be the key mechanism underlying OP-induced hyperglycemia. [32]

Hence, structural alterations of gut microbiota seem to characterize all forms of diabetes. In fact, these compositional alterations may play a role in both inducing the onset of autoimmune diabetes in young predisposed subjects and in speeding up the process of β-cell failure in obese/insulin-resistant subjects.

3. Gut Microbiota in Immunopathogenesis of Diabetes

The microbes making up the gut microbiome utilize nutrients and produce metabolites which are able to influence metabolism, leading to obesity, insulin resistance, and diabetes. For example, short-chain fatty acids (SCFAs) are produced by fermentation of dietary fibers; these molecules act both as energy substrates used by colonocytes and the host, and as ligands for G-protein-coupled receptors (GPCRs) [33]. These GPCRs, under SCFA stimulation, induce peptide YY (PYY) production, which can modulate intestinal motility and nutrient absorption. A study of murine germ-free and cocolonized Gpr41-/- and +/+ littermates showed that Gpr41-deficiency is associated with reduced expression of PYY, increased intestinal transit rate, and reduced harvest of energy from the diet. These results reveal that Gpr41 is a regulator of host energy balance through effects that are dependent upon the gut microbiota [34].

The obese microbiome has an increased capacity to harvest energy from the diet, and this feature is genetically transmissible: some researchers have shown that colonization of germ-free mice with an obese microbiota led to a greater increase in total body fat than colonization with lean microbiota [21]. Furthermore, germ-free (GF) animals are protected against obesity after consuming a Western-style, high-fat, sugar-rich diet. Two complementary but independent mechanisms that result in increased fatty acid metabolism are involved here: elevated levels of fasting-induced adipose factor (Fiaf), a circulating lipoprotein lipase inhibitor which induces peroxisomal proliferator-activated receptor coactivator (Pgc-1α), and increased phosphorylated AMP-activated protein kinase (AMPK) activity [35]. These findings suggest that manipulation of the gut microbiota may impact on muscle activity, regulating fatty acid oxidation. Thus, the host energy metabolism may be protected against a high-calorie westernized diet. Furthermore, exercise intervention has been shown to directly affect gut microbiota composition; germ-free mice exhibited worse exercise performance compared to mice colonized by a single bacterial species, while mice colonized by multiple nonharmful bacteria displayed the best exercise performance [36]. Moreover, another study showed that in obese mice, even under high-fat diet conditions, exercise protects gut microbiota by reducing inflammatory markers such as cyclooxygenase 2 (Cox-2) in both the proximal and distal gut [37]. Human studies have also revealed the importance of physical exercise in modulating gut microbiota in order to prevent/ameliorate metabolic diseases. Estaki et al., after normalizing BMI, diet, and age, analyzed fecal microbiota and fecal SCFAs in 39 healthy subjects and found that a higher fitness level correlated with gut microbiome diversity. Furthermore, increased production of butyrate, a marker of gut health, and increased abundance of butyrate-producing species were found in individuals with greater levels of aerobic fitness [38]. Another study showed that in T2D patients, a six-month endurance, resistance, and flexibility training program decreased intestinal mycetes overgrowth, gut permeability, and systemic inflammation, resulting in improved glycemia and functional and anthropometric variables [39].

Obesity and insulin resistance, leading to T2D, are characterized by low-grade inflammation, consequent to a morbid activation of the immune system. Lipopolysaccharides (LPS), which are components of the outer membrane of Gram-negative bacteria, with their high inflammatogenic properties, were thought to be the precipitators of the inflammatory processes leading to obesity and insulin resistance [40]. LPS are able to cross the intestinal epithelial barrier either via leaky intestinal tight junctions or carried by chylomicrons [41]. Once they reach systemic circulation, LPS bind the plasma LPS-binding protein (LBP), which activates the receptor protein CD14 located in the plasma membrane of macrophages. This complex is able to bind Toll-like receptor

4 (TLR4) on the membrane of macrophages, triggering the synthesis of several inflammatory effectors, such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1) [42]. This has been confirmed in mice with deletions of the LPS receptor TLR4, or part of the TLR4 machinery such as CD14, that showed attenuated inflammatory response and increased glucose transport; in addition, TLR4 inactivation blunted insulin resistance induced by LPS in differentiated adipocytes [43]. The involvement of gut microbiota has been further demonstrated since, in a study by Cani et al., chronic antibiotic treatment reduced metabolic endotoxemia and the cecal content of LPS in both high-fat-fed and ob/ob mice. This effect was correlated with reduced glucose tolerance and body weight gain. Furthermore, a high-fat diet was shown to greatly increase intestinal permeability and reduce the expression of genes coding for proteins of the tight junctions [44].

Thus, gut microbiota can affect intestinal mucosal permeability, leading to an increased absorption of exogenous antigens [45]. Furthermore, some microbial toxins have been reported to directly impair pancreatic β-cell function [46], which could be one of the mechanisms underlying autoimmune diabetes. In fact, in mice models, the injection of *Streptomyces* toxin and bafilomycin A1 resulted in smaller islets and reduced the entire pancreatic β-cell mass, concurrently impairing glucose tolerance [46]. Moreover, Lee et al. have also demonstrated that gut barrier disruption induced by *C. rodentium* infection accelerated insulitis in NOD mice [47]. Therefore, innate immune cells are directly involved in linking gut microbiota and diabetes pathogenesis. Any alteration in the communication between innate immunity components and gut microbiota may lead to diabetes onset, as explained above.

Several studies have investigated the role of the innate immune system at the intestinal level and also in T1D pathogenesis. Deletion of the innate immune adaptor myeloid differentiation primary response gene 88 (MyD88) in a NOD mouse model of T1D provided microbiota-dependent protection from the disease: MyD88-negative mice in germ-free (GF) but not in specific pathogen-free conditions develop the disease. The same authors also found that colonization of GF mice with a variety of intestinal bacteria reduced the occurrence of T1D in MyD88-negative but not wild-type NOD mice, favoring the balanced signal hypothesis: i.e., that both inflammatory and regulatory responses are induced by the microbiota and that TLR4-mediated Trif signaling causes a tolerizing immune response, which protects against T1D development [48].

Going back to talk about T2D, another mechanism involved in dysbiosis-induced immunopathogenesis of obesity and T2D is that of alterations in T helper 17/regulatory T cell (Th17/Treg) balance. Th17 and Treg cells are two $CD4^+$ T helper cells; Treg cells regulate and control immune tolerance in healthy individuals, while Th17 cells mainly produce IL-17. Th17 cells have been implicated in the control of adipogenesis and glucose homeostasis in obesity [49]. In a recent study, intestinal $ROR\gamma t^+$ IL-17^+CD4^+ T-cells were shown to participate in energy metabolism in mice, and specifically, a reduction of $ROR\gamma t^+$ and IL-17-producing $CD4^+$ T-cells contributed to the development of insulin resistance [50]. This observation supports the role of the intestinal Th17 lineage in the regulation of insulin sensitivity.

Several studies have shown that gut microbiota alterations are associated with abnormalities of the mucosal immune system, likely involved in the autoimmunity underlying T1D. In a murine study, the transfer of intestinal *Lactobacillus johnsonii* N 6.2 from diabetes-resistant biobreeding rats to diabetes-prone biobreeding (BBDP) rats resulted in a delay in disease pathogenesis through a mechanism that might involve the upregulation of Th17 cells [51]. These findings concur with those of another study, in which protection from the disease, probably mediated by the upregulation of intestinal T helper 17 cells, was observed after segmented filamentous bacteria were naturally transmitted to NOD mice [52]. These data seem to prove that bacteria provide protection against disease in both BBDP and NOD murine models.

Th17 cells are involved in different ways in T1D pathogenesis. In a spontaneous autoimmune diabetes model, IL-17A and IL-17F expressions in islets are related to insulitis in NOD mice. However, islet antigen-specific Th17 cells need to be transformed into Th1-like cells to induce diabetes [53]. Furthermore, a recent study has revealed that the exposure of nonobese diabetic NOD mice to acidified water was able to delay T1D onset: NOD mice exposed to neutral water, in fact, were more predisposed to the development of diabetes, while exhibiting a decrease in *Firmicutes* and an increase in *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. They also had lower levels of Foxp3 expression in CD4(+)Foxp3(+) cells, as well as decreased CD4(+)IL-17(+) cells, and a lower ratio of IL-17/IFN-γ $CD4^+$ T-cells, indicating that a change in liquid acidity dramatically alters the intestinal microbiome, the presence of protective Th17 and Treg cells, and the incidence of diabetes [54]. Moreover, in NOD mice, a Th17/Treg imbalance compromises the ability of Treg cells to suppress self-reactive effector T-cells and to impede the destruction of pancreatic islets, which may potentially induce or aggravate T1D [55].

The balance of Th1/Th2 lymphoid cells also seems to play a role in diabetes onset mediated by gut microbiota. Th1/Th2 lymphoid cells are $CD4^+$ T-cells, whose differentiation into Th1 or Th2 depends on stimulation by IFN-γ and IL-4, respectively. Several studies have shown that gut microbiota and its metabolites can modulate the equilibrium of Th1/Th2 cells in the intestinal tract. For example, the polysaccharide A produced by an anaerobic gram-negative bacterium *Bacteroides fragilis* could promote the expression of proinflammatory cytokines, such as IL-12 and p40, leading to Th1 activation [56]. Furthermore, Pam3 of gram-positive bacteria can activate IFN-γ production, in turn inducing the differentiation of Th1 cells [57]. Commensal A4 bacteria belonging to the Lachnospiraceae family produce an immunodominant microbiota CBir1 antigen by inducing TGF-β production by dendritic cells [58].

IL-12 is also the primary immunoregulatory factor secreted by Th1 cells and plays a key role in the pathogenesis of diabetes. IL-12 is able to bind to IL-12 receptors on pancreatic β-cells and activate proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ), inducing their apoptosis via

the STAT4 signaling pathway [59]. In addition, IL-12 is involved in complications of T2D; Ali et al. recently determined that the disruption of IL-12 promotes angiogenesis and increases blood flow in obese type 2 diabetic mice by an endothelial nitric oxide synthase/Akt/vascular endothelial growth factor receptor 2/oxidative stress-inflammation-dependent mechanism [60].

The nucleotide-binding oligomerization domain-containing protein 2 (Nod2) has been identified as a key factor for T1D susceptibility; $Nod2^{-/-}$NOD mice had different gut microbiota compared to $Nod2^{+/+}$NOD mice and were protected from diabetes, but only when kept separate from $Nod2^{+/+}$NOD mice, suggesting that T1D susceptibility in $Nod2^{-/-}$NOD mice is dependent on the alteration of gut microbiota. In fact, colonizing germ-free NOD mice with $Nod2^{-/-}$NOD microbiota significantly reduced the number of cells secreting proinflammatory cytokines but increased T-regulatory cells [61].

A recent study also evaluated the ability of human gut microbiota to delay the onset of T1D when transferred into germ-free NOD mice; diabetes onset was significantly delayed in all bacteriome humanized colonies vs. germ-free NOD mice, but the pace of beta cell loss was not transferable to the mouse model [62].

Physical exercise also seems to play a role in the immunomodulation of gut microbiota involved in T1D pathogenesis. A recent study revealed that NOD mice subjected to moderate intensity exercise benefited from glucose-lowering effects in the late stages of diabetes, while control sedentary NOD mice showed larger infiltrates at the end of the 12-week study. These findings suggest that exercise could promote a beneficial immune-modulation in T1D [63].

Thus, gut microbiota can modulate both innate and adaptive immunity, resulting in conditions which facilitate diabetes onset. This is possible due to gut microbiota's ability to affect glucose metabolism under predisposing conditions such as obesity and metabolic syndrome, and in conditions at risk of autoimmunity.

4. Gut Microbiota as a Novel Therapeutic Target for Prevention and Treatment of Diabetes

4.1. Probiotics. The aforementioned evidence has raised interest in targeting gut microbiota as an effective strategy to prevent and manage diabetes.

Probiotics are living microorganisms that can be ingested either alone or with food, conferring benefits to their host [64]. Yadav et al. showed that a probiotic dahi-supplemented diet, containing *Lactobacillus acidophilus* and *Lactobacillus casei*, significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress in high fructose-fed diabetic rats, thus lowering the risk of diabetes and its complications [65]. Other authors have investigated the effects of probiotic yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 and conventional yogurt on blood glucose and antioxidant status in type 2 diabetic patients; the probiotic yogurt improved fasting plasma glucose values and HbA1c and antioxidant status in diabetic subjects [66]. Moreover, daily consumption of 200 ml of a shake containing 4×108 CFU/100 ml of *Lactobacillus acidophilus*, 4×108 CFU/100 ml of *Bifidobacterium bifidum*, and 1 g/100 ml of fructooligosaccharides decreased blood glucose in T2D individuals. Recently, a growing interest for *Akkermansia muciniphila* developed since it seems to ameliorate gut permeability, obesity, and glucose tolerance [67, 68]. A recent randomized, double-blind, placebo-controlled study first evaluated metabolic effects of *Akkermansia muciniphila* administration in overweight/obese insulin-resistant humans. It showed a positive effect on insulin sensitivity and total cholesterol, and it turned out to be safe and well tolerated [69].

Several studies have also investigated fecal transplants as a therapeutic strategy. An animal study showed that the bacterial transfer from MyD88-deficient NOD mice, which are protected from T1D development, reduced insulitis and significantly delayed the onset of diabetes. Moreover, after the oral transfer of fecal bacteria over 3 weeks, the composition of the gut microbiota was stably altered, as it showed an increase in *Lachnospiraceae* and *Clostridiaceae* and a decrease in *Lactobacillaceae* [70]. Furthermore, the TEDDY study, a prospective cohort study that followed children at high risk for autoimmune diabetes, observed a reduction in the risk of islet autoimmunity in children who had received probiotics before or at the age of 27 days compared with those who had first received probiotics after 27 days or not at all [71].

A number of studies in humans have explored the effects of infusing intestinal microbiota from lean donors to male recipients with metabolic syndrome. Vrieze et al. demonstrated that six weeks after infusion of microbiota from lean donors, insulin sensitivity of recipients and levels of butyrate-producing intestinal microbiota increased [72], and they concluded that butyrate-producing bacteria prevent translocation of endotoxic compounds derived from gut microbiota, one of the factors driving insulin resistance. Similarly, another study has suggested that the butyrate synthesizing microbiota could improve insulin sensitivity through signaling pathways and direct effects on glucose metabolism [73]. Thus, intestinal microbiota transplantation, especially *F. prausnitzii*, from a normal individual to a diabetic one, seems to be able to synthesize abundant quantities of butyrate, which stabilizes the leaky gut and inhibits downstream proinflammatory mechanisms.

Probiotic supplementation or microbiota transplantation are two promising novel therapeutic strategies that could be used to prevent or treat diabetes by modulating the host's preexisting microbiota.

4.2. Prebiotics. Prebiotics are defined as food able to induce a selective growth and/or activity of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host [74]. Prebiotics are mainly inulin, fructooligosaccharides, galactooligosaccharides, and lactulose [75]. Kim et al. conducted a trial to evaluate the effect on glucose, lipid metabolism, and fecal microbiota composition of a one-month strict vegetarian diet in six

Table 1: Mechanisms of immunomodulation of gut microbiota in diabetes.

Animal model/study group	Main finding	Mechanisms involved	Reference
GF mice Gpr41-/- and +/+	Gpr41 is a regulator of host energy balance through modulation of gut microbiota	Reduced expression of PYY, increased intestinal transit rate, and reduced harvest of energy from the diet	B.S. Samuel et al. [34]
GF mice	Protected against obesity after consuming a Western-style, high-fat, sugar-rich diet	Elevated levels of Fiaf Increased AMPK activity	F. Backhed et al. [35]
GF mice Specific GF mice *Bacteroides fragilis* gnotobiotic mice	GF mice had a worse exercise performance compared to mice colonized by a single bacterial species and to mice colonized by multiple nonharmful bacteria	Higher serum levels of glutathione peroxidase (GPx) in SPF than GF mice. Lower serum superoxide dismutase activity in BF than SPF and GF mice	Y.J. Hsu et al. [36]
Healthy subjects	Higher fitness level is correlated to gut microbiome diversity	Increased production of butyrate	M. Estaki et al. [38]
T2D subjects	Improved glycemia, functional and anthropometric variables	Reduction of intestinal mycetes overgrowth, gut permeability, and systemic inflammation	E. Pasini et al. [39]
ob/ob mice High-fat diet-fed mice	Chronic antibiotic treatment reduced metabolic endotoxemia and the cecal content of LPS	Increased intestinal permeability Reduced expression of genes coding for proteins of tight junctions	P.D. Cani [44]
Mice injected with *Streptomyces* toxin and bafilomycin A1	Impaired glucose tolerance	Smaller islet pancreatic β-cell mass	M.A. Myers [46]
MyD88-negative mice NOD mice	Colonization of GF mice with intestinal bacteria reduced T1D in MyD88-negative but not in wild-type NOD mice	TLR4-mediated Trif signaling causes a tolerizing immune response	M.P. Burrows [48]
Diabetes-resistant biobreeding rats Diabetes-prone biobreeding (BBDO) rats NOD mice	Bacteria provide protection against diabetes	Transfer of intestinal *Lactobacillus johnsonii* N 6.2 from diabetes-resistant biobreeding rats to diabetes-prone biobreeding rats. Transmission of segmented filamentous bacteria to NOD mice	K. Lau et al. [51] M.A. Kriegel [52]
NOD mice placed on neutral or acidified water	Acidified water delays T1D onset	Increase in *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* and decrease in *Firmicutes* in NOD mice exposed to neutral water. Lower levels of Foxp3 expression in CD4(+)Foxp3(+) cells, decreased CD4(+)IL-17(+) cells, and a lower ratio of IL-17/IFN-γ $CD4^+$ T-cells in NOD mice exposed to neutral water.	K.J. Wolf et al.[54]
Obese diabetic mice (wt, $p40^{-/-}$and $p35^{-/-}$)	Disruption of IL-12 promotes angiogenesis and increases blood flow recovery	Increase in capillary/arteriole density, endothelial nitric oxide synthase/Akt/ vascular endothelial growth factor receptor 2 signaling, and a reduction in oxidative stress and inflammation	M. Ali et al. [60]
$Nod2^{-/-}$ NOD mice $Nod2^{+/+}$NOD mice	$Nod2^{-/-}$ NOD mice are protected from T1D	Colonization of germ-free NOD mice with $Nod2^{-/-}$NOD microbiota reduced the number of inflammatory cells and their cytokines, but increased T-regulatory cells	Y. Y. Li et al. [61]
Trained NOD mice Untrained NOD mice	Exercise enhances a beneficial immune-modulation in T1D	Reduced pancreatic infiltrates. Reduced levels of IL-6 and MIP-1β	R. Codella et. al [63]

obese subjects with T2D and/or hypertension. A strict vegetarian diet reduced body weight and the concentration of triglycerides, total cholesterol, low-density lipoprotein cholesterol, and HbA1c and improved fasting glucose and postprandial glucose levels. In addition, it determined compositional changes in gut microbiota, such as a reduced ratio of *Firmicutes* to *Bacteroidetes* and an increase in the species of *Bacteroides fragilis* and *Clostridium*, which decreased intestinal

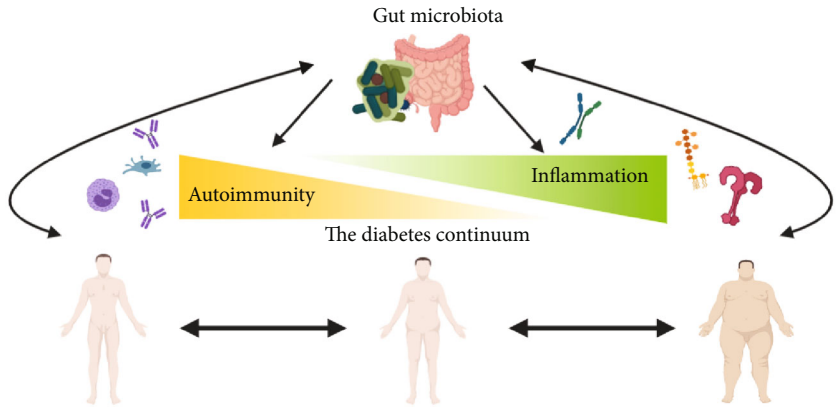

Figure 1: Gut microbiota alterations predisposing both autoimmunity and inflammation facilitate the appearance of all forms of diabetes: from T1D to T2D passing through LADA and other intermediate forms of diabetes. Likewise, diabetes itself can modulate gut microbiota, inducing structural and functional alterations that contribute to the disease.

inflammation and SCFA levels [76]. Another randomized, placebo-controlled trial assessed the effects of the administration of high performance inulin on glycemic status and lipid profile in women with T2D. Forty-nine subjects were randomized to receive 10 g/d inulin or 10 g/d maltodextrin for 8 weeks. The inulin-treated group showed a significant reduction in fasting plasma glucose, HbA1c, total cholesterol, and triglycerides and an increase in HDL-C [77]. Furthermore, a cross-sectional study by Beretta et al. revealed that a higher fiber intake in T1D is also associated with lower systolic and diastolic blood pressure [78].

Several antidiabetic drugs act as prebiotics, inducing changes in gut microbiota composition. Metformin, which represents the first-line therapy in T2D management, is able to positively modulate gut microbiota composition, both in humans and animals [79], contrasting the effects of a high-fat diet [80]. The exact mechanism by which metformin acts on gut microbiota composition is still unknown, but it seems to decrease the abundance of *Intestinibacter* [81] while increasing butyrate production [82]. Some studies have shown that it is able to inhibit bacterial complex I and also has an antimalarial function [83]. The degree to which gut microbiota is altered by metformin depends on host factors, such as the dosage used, the oral availability of the drug, and personal variability in absorption.

Glucagon-Like Peptide-1 Receptor Agonists (GLP-1RAs), another class of widely used antidiabetic drugs, also seem to have a role in modulating gut microbiota. Wang et al. showed that in mice treated with liraglutide, compared to saxagliptin, a lean-related gut microbiota profile was consistent with the loss of body weight [84]. Other studies have shown that liraglutide prevents diabetes onset in male rats and that this effect seems to be correlated with structural changes in gut microbiota, specifically an increase in SCFA-producing bacteria (*Bacteroides*, *Lachnospiraceae*, and *Bifidobacterium*) [85].

5. Conclusions

It is well known that innumerable pathogenic mechanisms are involved in all forms of diabetes.

Considering the data presented above, it appears evident that structural and functional alterations of intestinal microbiota are present not only in overt diabetes but also in conditions which predispose towards diabetes, such as obesity, metabolic syndrome, and the presence of antibodies associated with immune-mediated diabetes. Moreover, many studies (summarized in Table 1) have shown that these alterations trigger an innate and adaptive immune response which finally leads to overt diabetes. Although not conclusive, the evidence points towards the microbiota inflammation/autoimmunity diabetes hypothesis. It is likely that microbiota alterations facilitate the appearance of diabetes in already predisposed subjects, as explained in Figure 1.

If this is confirmed, attempts to stem the progression of diabetes could begin with preventive nutritional strategies, not only to decrease calorie intake but also to modulate gut microbiota with prebiotic and probiotic supplements or even through fecal transplants.

Treatment for patients who already have diabetes would also change, in that they would receive supplements to modulate the gut microbiota and improve glucose metabolism. In this regard, it would seem that some drugs already used for the treatment of diabetes, such as metformin and GLP-1RAs, are effective in lowering glycemia thanks to their action on intestinal microbiota.

To conclude, we can say that in a not too distant future, prevention and treatment for both T1D and T2D should encompass the modulation of gut microbiota and its immune responses.

Conflicts of Interest

The authors declare that they have no competing interests.

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Review Article

Lung and Gut Microbiota as Potential Hidden Driver of Immunotherapy Efficacy in Lung Cancer

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Lung cancer is one of the deadliest and most common malignancies in the world, representing one of the greatest challenges in cancer treatment. Immunotherapy is rapidly changing standard treatment schedule and outcomes for patients with advanced malignancies. However, several ongoing studies are still attempting to elucidate the biomarkers that could predict treatment response as well as the new strategies to improve antitumor immune system response ameliorating immunotherapy efficacy. The complex of bacteria, fungi, and other microorganisms, termed microbiota, that live on the epithelial barriers of the host, are involved in the initiation, progression, and dissemination of cancer. The functional role of microbiota has attracted an accumulating attention recently. Indeed, it has been demonstrated that commensal microorganisms are required for the maturation, education, and function of the immune system regulating the efficacy of immunotherapy in the anticancer response. In this review, we discuss some of the major findings depicting bacteria as crucial gatekeeper for the immune response against tumor and their role as driver of immunotherapy efficacy in lung cancer with a special focus on the distinctive role of gut and lung microbiota in the efficacy of immunotherapy treatment.

1. Introduction

The small (SCLC) and non-small-cell lung cancer (NSCLC) (referred as lung cancer "LC" hereafter) is one of the deadliest malignancies in the world. For 2019, the American Cancer Society estimates 116,440 and 111,710 new LC cases with 24% and 23% of new deaths per year for men and women, respectively [1]. Over the past few decades, the research on genetics of LC improved the opportunity to select patients that could benefit from the most recent immune-based therapeutic strategies [2–8].

Several clinical trials established the efficacy of immunotherapy on different tumors bringing to the approval of this new therapeutic regimen. The clinical trials CheckMate 017, CheckMate 057, and Keynote 010 demonstrated that the monoclonal antibodies (mAbs) against programmed cell death-1 (PD-1) nivolumab [9] and pembrolizumab [10] significantly improved the overall survival (OS) over docetaxel in NSCLC patients after the failure of prior platinum-based chemotherapy. Similarly, the OAK trial showed that atezolizumab [11], an anti-PD-ligand 1 (PD-L1) mAb, produced a survival benefit compared with docetaxel in the same NSCLC population. In details, the anti PD-(L)1 therapy blocks the binding of PD-1 to its ligand (PDL-1) restoring the functions of "exhausted" T cells and resulting in tumor shrinkage [12]. The immunoblocking between PD-1 and activated cytotoxic

T lymphocytes (CTLs), and between PD-L1 and tumor cells, has exhibited significant clinical efficacy in different types of cancer and was currently approved for treating tumors, including advanced stage of NSCLC [13]. Consistently, nivolumab and pembrolizumab showed impressive efficacy also in SCLC [14].

Actually, five monoclonal antibodies targeting immune checkpoints have been approved by the U.S. Food and Drug Administration (FDA) for cancer treatment alone or in combination with platinum-based chemotherapy [9], although ongoing study attempts to discover new predictive biomarker of treatment response as well as new strategies to improve immunotherapy efficacy, including the combination of anti-PD-(L)1 and anti-Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) agents [15, 16].

Several studies demonstrated that the gut microbiome regulates the power by which immunotherapy may stimulate the anticancer immune response (reviewed in [17]).

Commensal microorganisms are required for the maturation, education, and function of the immune system. A tight and continuous interaction of immune cells with microorganisms allows learning the difference between commensal and pathogenic bacteria. Indeed, the haematopoietic and nonhaematopoietic cells of the innate immune system are strategically located at the host-microbiome interface and are rich of pattern recognition receptors (PRRs) that sense microorganism presence [18]. This relationship leads to the concept of humans as mammalian holobionts resulting from parallel coevolution of host-eukaryotic and microbe-prokaryotic elements.

The gastrointestinal tract hosts are the most abundant and diversified microbial population. The gut microbiota is composed of 10^{13} to 10^{14} microorganisms whose genome is collectively at least 100 times the human genome [19]. Moreover, behind gut epithelia, bacteria colonize other specialized epidermal surfaces like the ductal system of exocrine organs and respiratory tract.

The human respiratory tract is the main portal of entry for numerous microorganisms. Interestingly, gut and lung microbiota are connected by a complex bidirectional axis via lymphatic [20] and blood circulation, and modification of one mucosal compartment can directly impact distant mucosal site [21].

Recent high-depth metagenomic sequencing techniques have changed our understanding of the complex microbiome ecosystem enabling the identification and quantification of individual bacterial strains and the correlation between specific microbiome asset and disease status. More interesting, wide efforts are now focused on how variations in these populations may influence response to immunotherapy.

In this review, we discuss some of the major findings depicting bacteria as crucial gatekeeper for the immune response against tumor and their role as driver of immunotherapy efficacy in lung cancer.

2. Role of Commensal Bacteria in Cancer Response to Immunotherapy

During early life, the immune system is broadly stimulated with the first contact to microorganisms at gastrointestinal and lung barriers [22]. This primary wave of microbial exposure exerts a long-lasting effect on immune cell function [23].

Increasing evidence supports the idea of a dynamic interaction between immune cells, microbiota, and tumor microenvironment. Gene expression analysis of tumors from antibiotic-treated mice showed a downregulation of genes related to inflammation, phagocytosis, antigen presentation, and adaptive immune response. Moreover, microbiota disruption impairs the efficacy of CpG-oligonucleotide immunotherapy affecting myeloid-derived cell functions in the tumor microenvironment [24].

Furthermore, it has been demonstrated that oral administration of *Bifidobacterium* improves response to anti-PD-L1 antibody in mouse models of cancer by inducing dendritic cell function and increasing $CD8^+$ T cell accumulation in the tumor microenvironment [25]. Microbiota composition has also a key role in the immunostimulatory effects of Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) blockade. In details, Bacteroides species affect interleukin- (IL-) 12-dependent Th1 immune response facilitating tumor control in mice and patients [26].

A recent study analyzed baseline stool samples from 42 metastatic melanoma patients before immunotherapy treatment demonstrating an abundance of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* in responding patients. Fecal transplantation of germ-free mice with stool from responding patients improved efficacy of anti-PD-L1 therapy increasing immune-mediated tumor control through the induction of T cell response [27].

The different microbiota composition between cancer patients and healthy individuals not only demonstrated diagnostic and prognostic potentials of special microbial pathogens in cancer but also suggested the idea that the manipulation of the microbiota could be a valid approach for a better therapeutic response, acting on drug efficacy or enhancing the immune system (discussed below).

The fecal microbiota transplantation (FMT) (i.e., the transfer of fecal bacteria from a donor into a recipient) that has been applied to clinical practice for the treatment of Clostridium difficile infection [28], ulcerative colitis [29–31], and irritable bowel syndrome [32] demonstrated an effect also on the systemic immune response and particularly on the mechanisms of immune surveillance against LC (Routy et al.). Routy and colleagues demonstrated that a specific host gut microbiota might contribute to patient immunotherapy response. Antibiotic-induced alterations of gut microbiota during immunotherapy treatment dampens patient response to the therapy. Interestingly, the FMT from patients sensitive to immunotherapy is able to revert the immunotherapy response in treatment-resistant patients. These findings lead to intriguing hypothesis that the modification of gut microbiota through FMT could enhance the response also in tumors resistant to immunotherapy.

The overall results of these studies open the avenue to propose a multiparameter prediction model integrating conventional parameters, such as tumor genetic alterations, with microbiota assessment to select patients most likely to respond to immunotherapies.

3. Effects of Gut Microbiota on LC

The role of the human gut microbiome is being increasingly accepted. From 2015 to present, more than 158 papers on high-impact journals were published and several research groups indicated the role of the gut microbiome in different diseases with a particular emphasis on cancers (https://www.ncbi.nlm.nih.gov/pubmed?term=(LUNG%20CANCER%20MICROBIOME)%20AND%20(%222015%2F01%2F01%22%5BDate%20-%20Publication%5D%20%3A%20%223000%22%5BDate%20-%20Publication%5D).

More than 100 trillion bacteria colonize the human intestines [33]. The crosstalk between the gut microbiota and the immune system contributes to the health status of the host. The application of this concept in oncology field is particularly important, and several recent papers highlighted the role of gut microbiota as one of the regulatory factors affecting both the tumor proliferation and the immunological environment of cancer, determining thus the efficacy of the treatment with the immune checkpoint inhibitors. The specific role of gut microbiota in supporting cancer development and growth is yet unclear. However, there are compelling evidences of the gut microbiota role in modulating both innate and adaptive immune response and how this influences tumor growth and immune escape [34]. Moreover, the gut microbiota is able to regulate host immunity both locally and at distal sites [35] modulating the expansion and differentiation of T cell populations. Briefly, the pathogen-associated molecular patterns (PAMPs) of the microorganisms in the intestines are recognized by the Toll-like-receptors (TLRs) on the membrane of intestinal epithelial cells. The activation of TLRs leads to the activation of signal cascade that finally results into the stimulation of immunological cells in the lamina propria. Dendritic cells and macrophages, activated in mesenteric lymph nodes (MLN), prime the naïve B and T cells to mature and differentiate, producing, thus, IgA. Differentiated T cells assume both profile of Th1 and/or Th17 proinflammatory cells activating additional effector cells as neutrophils or anti-inflammatory cells to control immune response [36–43]. Moreover, high diversity of gut microbiome supports M1 macrophage and Th1 lymphocyte differentiation, activation of helper/cytotoxic T cell, and upregulation of PD-1 expression on lymphocytes [44].

All these studies highlighted the potential of gut microbiota manipulation in cancer treatment, especially in tumors where the immunotherapy is currently adopted in clinical practice such as the LC and melanoma.

In melanoma, PD-1 inhibitors produce long-lasting responses in 30-40 percent of patients. However, these drugs do not work in the other 60-70 percent of melanoma patients for a multitude of reasons, including not having the right microbes in the gut—a condition termed "intestinal dysbiosis." Likewise, several phase III LC clinical trials revealed that immunoblockade treatment leads to only approximately 20% of patients' overall objective response (OOR) and that median duration of response is significantly heterogeneous [45–47]. Recent studies demonstrated that gut microbiota could modulate immunotherapy response. Indeed, gut commensals such as *B. thetaiotaomicron* or *B. fragilis* are predictive factors for anti-CTLA-4 treatment in a mouse melanoma model [26].

It is therefore desirable to identify patients who would benefit more from immunotherapy and to understand what drives resistance in the patients who do not respond.

A study by Routy et al. proved that the gut microbiota plays a critical role in the response to PD-1 blockade and may have a prognostic value in LC. Moreover, the gut microbiota of patients who respond to immunotherapy with checkpoint inhibitors was different from those who do not. In particular, the authors identified an increased level of *Akkermansia muciniphila* (*A. muciniphila*) in patients who experienced longer survival. They demonstrated that gut microbiota not only was a predictor of response but also regulated the efficacy of anti-PD1 in murine models. In fact, the fecal microbiota transplantation from responder mice restored PD-1 blockade sensibility in the same models. Interestingly, the authors demonstrated that gut microbiome, and in particular *A. muciniphila*, influences efficacy of PD-1-based immunotherapy against epithelial tumors increasing the presence of tumor-infiltrated $CCR9^{+}CXCR3^{+}CD4^{+}$ T cells through a IL-12-dependent signaling pathway [48].

A recent paper using data from 37 advanced NSCLC patients receiving nivolumab enrolled in the study from the clinical trials CheckMate 078 (NCT02613507) and CheckMate 870 (NCT03195491) demonstrated a strong correlation between the level of gut microbiome diversity and anti-PD-1 efficacy in advanced NSCLC Chinese patients. The patients with high gut microbiome diversity (reported as favorable gut microbiome) exhibited an increase of memory T and NK cell signatures in the peripheral blood samples. These findings provide important implications for the prediction of anti-PD-1 immunotherapy response in Chinese population with NSCLC [49].

To date, a single study examined the association among antibiotics and efficacy of immune checkpoint inhibitors. In this retrospective analysis of the data from 90 NSCLC patients treated (13 patients) or untreated (77 patients) with antibiotics prior to nivolumab therapy as second or later line of therapy, the authors demonstrated that antibiotic treatment reduced significantly both Progression-Free Survival (PFS) and OS. Although, in multivariate analysis, no statistically significant association was found between survival and prior antibiotic use, a trend concerning the negative influence of antibiotic use was conveyed. These data, although need further validations, confirmed that gut microbiota could have an important role in shaping systemic immune responses [50].

Botticelli and colleagues demonstrated that a specific gut microbiome may influence the response to immunotherapy. In particular, by using the NGS technique, the authors showed that there are higher levels of Rikenellaceae, Prevotella, Streptococcus, Lactobacillus, Bacteroides plebeius, Oscillospira, and Enterobacteriaceae in the stool of NSCLC patients than in healthy controls. Moreover, patients who respond to nivolumab treatment had less abundance of *Ruminococcus bromii*, *Dialister*, and *Sutterella* spp. than not responders [51].

The concept of immunomodulatory ability is also applicable to the chemotherapy regimen able to regulate the immune system. Cyclophosphamide is well known for its antineoplastic and immunomodulating ability and was registered for early and advanced breast cancer. In a transgenic tumor mouse model of autochthonous lung carcinogenesis, this alkylating agent alters the composition of microbiota in the small intestine inducing translocation of specific Gram-positive bacteria, including *Lactobacillus johnsonii* (growing in >40% cases), *Lactobacillus murinus*, and *Enterococcus hirae*, into secondary lymphoid organs [52]. Here, the Gram-positive bacteria stimulate the generation of a specific subset of "pathogenic" T helper 17 (pTh17) cells and memory Th1 immune response. In germ-free or antibiotic-treated animal models, the absence of these bacteria leads to a reduction in pTh17 response and cyclophosphamide tumor resistance. Adoptive transfer of pTh17 cells partially restored the antitumor efficacy of cyclophosphamide. These results suggest that the gut microbiota helps shape the anticancer immune response in LC patients [53].

4. Effects of Lung Microbiota on LC

The lung is constantly exposed to microorganisms from the air and the upper respiratory tract; therefore, it is not a "sterile place" as previously believed. Acquisition of lung microbiome is a crucial event in newborn to protect the lung from injuries [54]. Lung tissue hosts a unique microbiome asset with less diversity, compared to the intestinal one, but equally affected by drugs, disease, and eating habits, which can create a selective pressure on reproducing communities. The specific composition of the lung microbiome results from the balance of three phenomena: microbial immigration, microbial elimination, and the relative reproduction rates of its members [55].

Dysbiosis of lung microbiome ecosystem and the epithelial integrity loss in heavy smokers could be the initial cause of inflammation in chronic obstructive pulmonary disease and LC [56]. A comparative analysis of 142 LC patients and 33 healthy controls reveals a distinct lung microbiome profile associated with tumor tissue [57]. Moreover, epidemiological evidence indicates a significant association between prolonged antibiotic exposure and incidence of LC [58].

Exacerbations of chronic lung disease have shown correlation with microbiota disorder of the respiratory tract. Respiratory dysbiosis is closely linked to a dysregulated host immune system, which in turn further affects lung microenvironment promoting inflammation [59].

On the other hand, a recent study claims that depletion of local commensal microbiota or blockade of the downstream cellular/molecular immune mediators suppresses the development of lung adenocarcinoma. By using the conditionally genetically engineered mouse model (GEMM) of lung adenocarcinoma, the authors demonstrated that commensal bacteria stimulate production of IL-1β and IL-23 from myeloid cells via a Myd88-dependent pathway. This event leads to proliferation and activation of tissue resident $\gamma\delta$ T cells with a consequent increased production of effector molecules, such as IL-17, to promote inflammation and tumor cell proliferation [60]. However, this study does not deep investigate the specific strain composition of the lung microbiota responsible for lung tumor development.

Many efforts have been focused on the discovery of bacterial diagnostic biomarkers for LC [61, 62].

These biomarker discovery studies often used saliva, sputum, bronchoscopic samples, or bronchoalveolar lavage fluid instead of direct lung biopsy, which is not performed on healthy subjects. However, lung tissue remains the most accurate sample to study lung microbiome alternations [63]. A study evaluating saliva microbiota revealed that bacterial profiles are significantly altered in LC patients compared to those from control subjects. In particular, *Capnocytophaga*, *Selenomonas*, and *Veillonella* were found to be more abundant in both lung squamous cell carcinoma and adenocarcinoma patients whereas *Neisseria* was less abundant than in the controls [64].

Another study compared bronchial brushing samples from cancerous site and contralateral noncancerous site of 24 LC patients and 18 healthy controls. The authors demonstrated that LC-associated microbiota profile is extremely divergent from that found in healthy subjects with a significant decrease in microbial diversity. More interestingly, the alterations of microbiota composition in unilateral lobe LC patients are extended to the contralateral noncancerous site suggesting a deep change of the whole lung microenvironment, which is linked to the development of LC [65].

Although increasing evidence has highlighted the key role of commensal microbiota in tumor-immune system interaction and treatment response, the main efforts have been focused on gut microbiota. Less is known on how lung microbiota could affect antitumor immunity and immunotherapy response.

Evidence suggests that manipulation of the composition of local flora may influence the ability of the host to generate an immune response that could mount both local and distal antitumor protective responses ameliorating the efficacy of immunotherapy treatment.

To date, several interesting clinical trials are attempted to study the role of lung microbiota on the efficacy of immunotherapy-based treatment in LC (Table 1).

An ongoing observational clinical trial (NCT03688347) at Iowa Institute of Human Genetics (Iowa, US) is currently recruiting patients with advanced or recurrent LC (and other solid tumors) that initiate a new line of immunotherapy, either alone or in combination with chemotherapy, targeted therapy, or other immunotherapy agents.

Recently, Stevenson et al. isolated and identified *Enterococcus gallinarum* MRx0518, a commensal Gram-positive species, demonstrating the antitumor efficacy of this bacteria strain in mouse models of different solid tumors, including LC. MRx0518, and more specifically its flagellin, acts on both the innate and the adaptive immune system showing strong immunostimulating properties. Its inactivation resulted in complete abrogation of the TLR5-mediated activation of NF-κB [66, 67].

Based on these exciting results, the NCT03934827, a single center, open label clinical trial, is aimed at studying

Table 1: Clinical trials investigating the role of microbiota in lung cancer patients receiving immunotherapy.

ClinicalTrial.gov identifier	Title	Conditions	Study type	Intervention/treatment	Estimated enrollment (patients)	Primary outcome	Secondary outcome
NCT03688347	Microbiome in lung cancer and other malignancies	Lung cancer and other solid tumors	*Observational*	Nasal, skin, and oral swab, stool collection, and microbiota analysis	40	Identify and compare bacteria within given samples through a standard protocol and 16S rRNA amplicon; correlate data from samples with patient clinical information regarding overall response rates	Correlate data from samples with patient clinical information regarding overall response rates
NCT03934827	MRx0518 in patients with solid tumours waiting surgical removal of the tumour	Lung cancer and other solid tumors	*Phase 1*	MRx0518 *vs.* placebo capsules	120	Safety and tolerability of MRx0518 as determined through the collection of the number and severity of AEs, SAEs, changes in biochemistry, haematology, urinalysis laboratory results, and vital signs	Response of MRx0518 determined by the measurement of tumor markers; OS of patients who receive MRx0518 compared to placebo
NCT03168464	Radiation and immune checkpoints blockade in metastatic NSCLC (BMS # CA209-632)	Metastatic NSCLC	*Phase 1, 2*	Nivolumab, ipilimumab, and radiation therapy	45	Enhance ORR to the combination of nivolumab/ipilimumab in chemorefractory NSCLC and double the ORR of ipilimumab/RT, from 18% based on intent to treat to 36%	Changes in TCR repertoire in peripheral blood are associated with response to treatment; serum markers IFN-b, CXCL11, sMICA, sMICB levels/changes associated with patients' response to the treatment; PFS; OS; associations of ORR with changes in the microbiome

vs.: versus; AEs: adverse events; SAEs: serious adverse events; OS: overall survival; NSCLC: non-small-cell lung cancer; ORR: overall response rate; RT: radiotherapy; TCR: T cell receptor; PFS: progression-free survival.

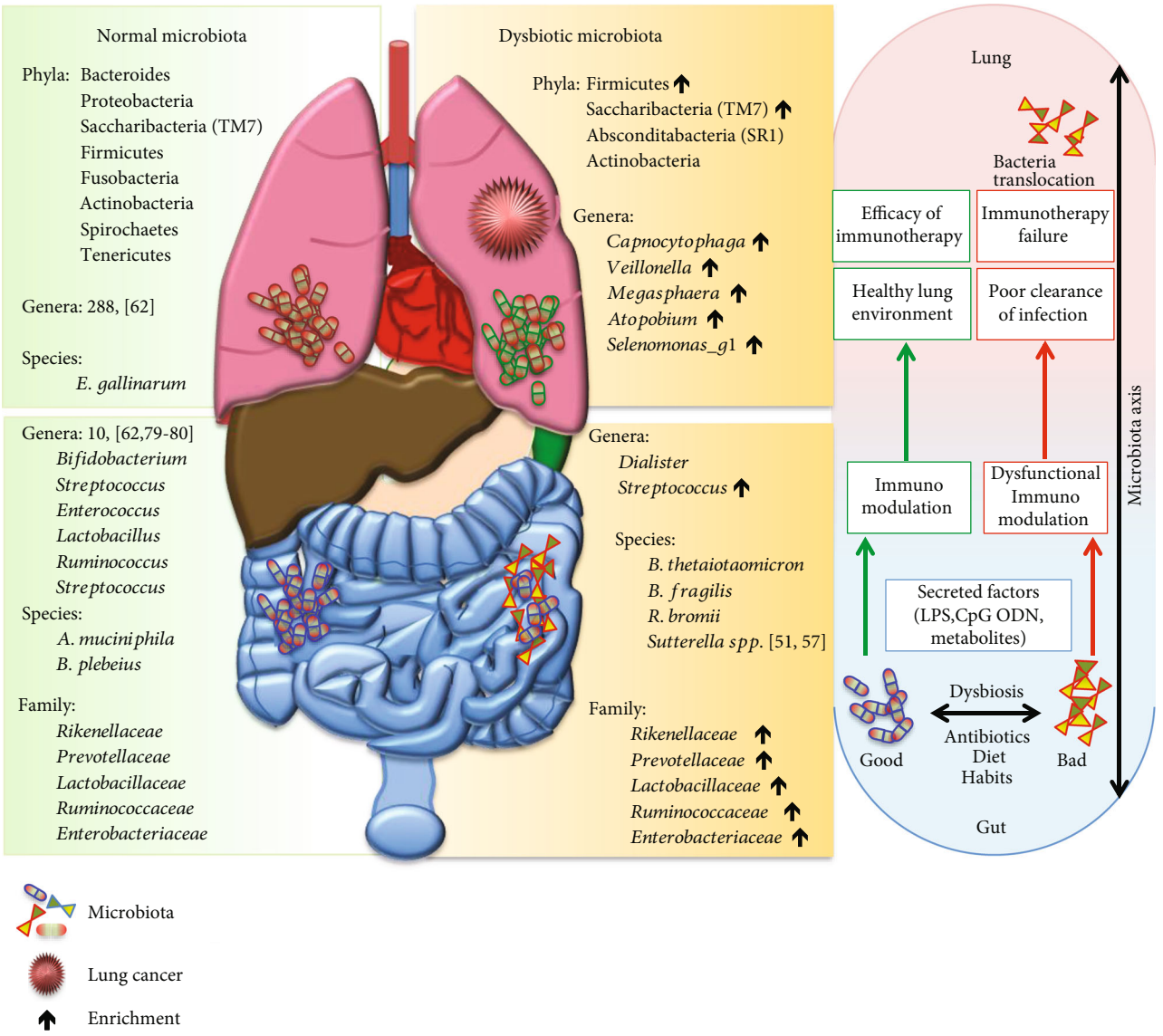

FIGURE 1: Increasing evidence supports the idea of a dynamic influence between host and microbiota. The fine line between human health and disease can be driven by friend (green) or foe (red) microbiota. We reported the main bacteria that could be responsible for the transition from a health to a pathological status. Commensal microorganisms are required for the maturation, education, and function of the immune system. A tight and continuous interaction of immune cells with microorganisms allows learning the difference between commensal and pathogenic bacteria that could influence immunotherapeutic treatment.

MRx0518 in combination with pembrolizumab in patients with LC and other solid tumors (at MD Anderson Cancer Center Houston, Texas, US). This study will assess the safety and tolerability and clinical benefit of MRx0518 in combination with pembrolizumab through the collection of adverse events.

Moreover, the NCT03168464 Interventional Clinical Trial at Weill Medical College of Cornell University (New York, US) is aimed at evaluating the association of ORR with changes in the microbiome in NSCLC patients with metastatic disease who have failed at least one prior treatment.

Although these studies are still in their infancy, they will provide a valid contribution in the exact determination of the role of the local microbiota in the response to immunotherapeutic agents and, on the other hand, will provide both new prognostic biomarkers and a powerful alternative tool to modulate the patient outcome.

5. Gut-Lung Microbiota Axis

The interaction between gut microbiota and host cells in the intestinal mucosa occurs in several ways. The pathogen-associated molecular patterns (PAMPs), provided by gut microbiota, serve as ligands for different Toll-like receptors (TLRs) on the surface of the intestinal epithelial cells (IECs). PAMPs from different microbiomal origin, such as lipopolysaccharide (LPS) or CpG ODN from bacteria, or viral double-stranded RNA, or toxin from parasites and fungi could activate TLR innate-adaptive immunity [68, 69] (Figure 1). In a similar way, also lipoteichoic acid (LTA),

the main component of the Gram-positive cellular wall seems to function as potent immune activator with a signaling similar to the LPS activation pathway.

Indeed, the immune system through plasma cells and IgA secretion into the lumen of the gut could regulate in turn microbiota population [70]. Moreover, commensal bacteria and their metabolites (i.e., short-chain fatty acids (SCFAs) like butyrate, propionate, and acetate) directly stimulate IECs regulating immune cells. SCFAs might regulate the immune system through regulation of G-protein-coupled receptors (GPRs) and histone deacetylase [71], modulating epithelial and immune cell functions. Other cell types have also emerged as targets of SCFAs, including monocytes, dendritic cells, T cells, and intestinal epithelial cells [72].

In dendritic cells, treatment with SCFA butyrate is associated with decreased expression of the proinflammatory cytokines IL-12 and IFN-γ and increased expression of Th2 cytokines [72]. Some evidence suggests that butyrate may regulate the ability of dendritic cells to present antigen and to prime T cells [73].

The gastrointestinal and respiratory tracts, although physically distant organs, are part of a shared mucosal immune ecosystem named the gut-lung axis [74]. Gut microbiota dysbiosis has been implicated in several lung diseases. Indeed, restoring microbiota in the gut of mice resulted in reduced severity of pneumonia [75].

It has been hypothesized a bidirectional crosstalk between the two microbiota entities which means that alteration of one compartment could impact on the other one.

This concept opens the possibility to indirectly modify lung bacterial composition, which represents the population physically close to lung tumor microenvironment, through gut microbiota modification strategies, such as fecal transplantation.

The dynamic crosstalk between the two compartments occurs through a direct translocation of bacteria from one to the other site or through the release into the bloodstream and the lymphatic system of bacteria-derived immunomodulatory molecules, which affect systemic immunity [75–80].

The massive crosstalk between the microbiota of gut-lung axis and its decisive role in inflammation and against lung infections could open to new therapeutic and immunization strategies.

6. Conclusions

The straight interaction between microbiota and host epithelial barrier is required for the maturation, education, and function of the immune system impacting the host's health but also the power of immunotherapy to boost anticancer response. The molecular crosstalk between the gut and lung microbiota and anticancer immune regulation represents a novel area of research. Potentially, the microbiota could modulate and eventually potentiate an immune response by the release of proinflammatory cytokines, metabolites, or nucleic acids, allowing a microbiota-based selection of patients who could benefit from specific immunotherapy treatment.

However, microbiota composition differs widely according to host genetics and racial characteristic as well as diet and eating habits. These variables are closely related to geographical location, suggesting therefore the need of more in-depth clinical research studies, looking at ethnic diversity as well as eating habits and environment-related factors.

These substantial divergences in the basal microbiome components of different study populations question the universality of the microbiome-based findings and recommend taking into consideration more geographically tailored approaches [81]. Because this research area is still in its infancy, new efforts are necessary to determine the role of the microbiota in the response to immunotherapeutic agents and also to comprehensively illustrate the gut-lung axis and its implications.

Conflicts of Interest

E.B. received honoraria or speakers' fee from MSD, AstraZeneca, Celgene, Pfizer, Helsinn, Eli-Lilly, BMS, Novartis, and Roche; E.B. also received research grants from I.A.S.L.C. (International Association for the Study of Lung Cancer), L.I.L.T. (Lega Italiana per la Lotta contro i Tumori), Fondazione Cariverona, Astra-Zeneca, Roche, and Open Innovation, not related to the submitted work. S.P. reports personal fees from Astra-Zeneca, Eli-Lilly, BMS, Boehringer Ingelheim, Roche, MSD, and Istituto Gentili, outside the submitted work. G.T. reports grants and others from Celgene, Novartis, Roche, Incyte, and Merck Serono and grants from Fondazione Cariverona, outside the submitted work. The remaining authors have nothing to disclose.

Authors' Contributions

Giampaolo Tortora and Emilio Bria share the last coauthorship.

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Research Article

IL-17 Inversely Correlated with IL-10 via the STAT3 Gene in *Pneumocystis*-Infected Mice

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Background. *Pneumocystis* pneumonia (PCP) remains a common opportunistic infection in immunosuppressed individuals. Current studies showed that multiple immune cells and cytokines took part in the host defense against *Pneumocystis* (PC). However, the roles of IL-17 and IL-10 in the development of PCP have not been elucidated. *Methods*. IL-10 and IL-17 levels in serum from PCP mice were detected via ELISA. The percentages of B10 cells, IL-10^+ macrophages, and IL-10^+ T cells in the lung from IL-$17^{-/-}$ PCP mice and Th17 cells and IL-17^+ $\gamma\delta$T cells in IL-$10^{-/-}$ PCP mice were examined via flow cytometry. Also, antibody neutralization examination was also performed to elucidate the relationship of IL-17 and IL-10 in the PCP model. *Results*. We noted the increase of IL-17 and IL-10 levels in serum from mice infected with *Pneumocystis*. Furthermore, deficiency of IL-17 or IL-10 could lead to the delayed clearance of *Pneumocystis* and more severed lung damage. Our data also demonstrated that IL-17 deficiency enhanced the serum IL-10 level and the percentages of B10 cells, IL-10^+ macrophages, and IL-10^+ T cells in the lung from PCP mice. Interestingly, we also noted an increase of the IL-17 level in serum and Th17 cell and IL-17^+ $\gamma\delta$T cell percentages in the lung from IL-$10^{-/-}$ PCP mice. Using antibody neutralization experiments, we found that the STAT3 gene might play a critical role in the interplay of IL-17 and IL-10 in PCP. *Conclusion*. Taken together, our results demonstrated that IL-17 and IL-10 could play the protective roles in the progression of PCP and the inverse correlation of them might be mediated by STAT3.

1. Introduction

Pneumocystis pneumonia (PCP) is the leading cause of lung infections in HIV-positive individuals worldwide [1, 2]. Recently, newer use of immunosuppressive agents and chemotherapeutics on patients with autoimmune conditions, transplantation, and hematologic malignancies lends to the development of PCP. In addition, HIV-negative PCP hosts tend to have a higher mortality rate and have a more fulminant presentation with substantial dyspnea, fever, and chills. Furthermore, HIV-negative patients are more likely to require mechanical ventilation [3–6]. The immune system can mount a pathologic response against *Pneumocystis* and result in severe damage to the host lung. Recent studies have demonstrated that multiple immune cells and cytokines participate in the development of PCP. These include macrophages, Th1 cells, Th2 cells, Th17 cells, B cells, and the other immune cells. However, the pathogenesis of PCP has not been elucidated.

The alveolar macrophages (AMs) are the first line of host defense to *Pneumocystis*. The critical role of AMs lies in their capability to directly kill both trophozoites and cysts, leading to adaptive immune responses [7, 8]. $CD4^+$ T cells are demonstrated to play a critical role in memory cell functions via recruiting and activating the effector cells [9]. Several studies suggested that Th1, Th2, and Th17 cells could play the protective roles in host inflammatory responses. Mounting IFN-γ could attenuate the lung damage of the

Pneumocystis-infected rat [10]. Th2 cell deficiency leads to the persistent eosinophilic infiltration in PCP mice [11]. An increase of Th17 cells was noted in PCP hosts; however, Ripamonti et al. found that IL-17 could not help to eliminate the *Pneumocystis* cysts [12, 13]. Nowadays, accumulating evidence indicates that B cells might play a vital role of promoting the proliferation and activation of $CD4^+$ T cells during *Pneumocystis* infection [14]. Our previous study also demonstrated that B10 cells regulated the Th1/Th17 cell immune responses in the PCP model [15].

IL-17 is a tissue-signaling cytokine that favors protection of barrier organs such as the skin, lung, and gastrointestinal system [16]. It is one of the critical proinflammatory cytokines and related to multiple diseases [17, 18]. IL-17 was secreted by Th17 cells, $\gamma\delta$T cells, iNKT cells, and group 3 ILCs [19]. IL-10 is one of the most significant anti-inflammation cytokines produced during infectious diseases and cancer [20]. During *Pneumocystis* infection, IL-10 was demonstrated to play a protective role in reducing the immune response to pathogen, alleviating lung damage, and mediating B cell protection-demand hematopoiesis in PCP hosts [21, 22]. Several studies have demonstrated that IL-10 could inhibit immune responses in multiple diseases [23–25]. However, the roles of IL-17 and IL-10 in PCP hosts have not been clearly elucidated.

In this study, we focused on the functions of IL-17 and IL-10 and their interactions in *Pneumocystis*-infected individuals.

2. Materials and Methods

2.1. Mice. Wild-type (WT) C57BL/6 mice and severe combined immunodeficient (SCID) mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). IL-$10^{-/-}$ mice (stock no. 002251) with the C57/BL6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). IL-$17^{-/-}$ mice on a C57BL/6 background were provided by Dr. Iwakura (University of Tokyo, Tokyo, Japan). Mice used for experiments were 6-8 wk females. They were bred on a chow diet in ventilated cages in the Animal Care Facility of Beijing Chaoyang Hospital. All of the animal studies were approved by the Capital Medical University Animal Care and Use Committee.

2.2. PCP Models and Sample Processing. Pneumocystis murina was maintained in CB17 SCID mice, and lung homogenates were used to get *Pneumocystis* cysts as previously described [15, 26]. After the lung homogenates were stained using Diff-Quick (Baxter, McGaw Park, IL), the number of *Pneumocystis* cysts was determined microscopically. PCP models were prepared by intratracheally inoculating with 1×10^6 cysts in 100 μl of PBS. Mice were sacrificed at serial time postinfection. Periodic acid silver methenamine staining of the lung was used to confirm *Pneumocystis* infection (Supplementary Fig. 1). *Pneumocystis* burden in the lung was detected by real-time PCR as previously described. Primers and probes for the *P. murina* RNA were described in the online supplement.

2.3. Flow Cytometry. Cells from tissue and blood were stained with innate cell-specific, B cell-specific, and T cell-specific panels, as described previously [15], and analyzed using FACSCanto II (BD Biosciences, San Jose, CA, USA). The antibody panel is described in the online supplement.

2.4. Real-Time PCR. mRNA expression of STAT3, STAT5, RORγT, IFN-γ, STAT1, GATA3, and Irf4 in the lung from infected mice was determined by real-time PCR (RT-PCR). Primers and probes were described in the online supplement.

2.5. Enzyme-Linked Immunosorbent Assay. Blood from PCP patients and mice was centrifuged at 1,000 g to obtain sera. IL-10 and IL-17A in serum samples were detected using ELISA Kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.6. IL-17 and IL-10 Neutralization In Vivo. C57BL/6 mice were inoculated intraperitoneally twice weekly with 200 μg of anti-mouse IL-17A clone 17F3 (Bio X cell, West Lebanon, NH) [27] or 200 μg of anti-mouse IL-10 clone 1B1.3A (Bio X cell, West Lebanon, NH) [28]. The control group received an equal volume of PBS. Mice were sacrificed at 2 wk postinfection.

2.7. Statistical Analysis. Statistical analysis was performed using Prism 5.0 (GraphPad Software, San Diego, CA). Data were described as mean ± SEM. We performed statistical analysis by Student's t-test for two-group comparison. All hypothesis tests were conducted at the 0.05 level of statistical significance.

3. Results

3.1. IL-17A/IL-10 Levels Increased in Pneumocystis-Infected Mice. ELISA data demonstrated a significant increase of the IL-17A level in PCP mice compared with that in the corresponding serum from WT mice ($1.25 \pm 0.18 \times 10^2$ vs $0.45 \pm 0.05 \times 10^2$ pg/ml, $P < 0.01$, Figure 1(a)). Also, the percentages of Th17 cells increased in the lung from *Pneumocystis*-infected mice than those from uninfected mice (7.50 ± 0.15 vs $3.07 \pm 0.36\%$, $P < 0.01$, Figure 1(b)). In addition, $\gamma\delta$T cells from PCP mice were expressing more IL-17A than those from WT mice (14.2 ± 0.18 vs 8.5 ± 0.12, $P < 0.01$, Figure 1(c)).

We also noted that IL-10 concentrations in the serum from PCP mice were higher than those from WT mice (5.9 ± 0.2 vs 3.0 ± 0.3 pg/ml, $P < 0.01$, Figure 2(a)). FACS data showed the significant increase of IL-10-producing B cell (5.7 ± 0.4 vs $2.9 \pm 0.6\%$, $P < 0.05$, Figure 2(b)), macrophage (43.5 ± 2.5 vs $29.3 \pm 1.8\%$, $P < 0.05$, Figure 2(c)), and T cell (5.8 ± 0.9 vs $2.9 \pm 1.2\%$, $P < 0.05$, Figure 2(d)) percentages in the lung from *Pneumocystis*-infected mice than those from uninfected mice. Furthermore, we detected the percentages of IL-17- and IL-10-expressing mononuclear cells in blood from mice. The results demonstrated that there were few IL-17-producing cells and IL-10-producing cells in blood from mice. Also, we did not note significant differences of the percentages of these cells in blood from PCP mice and WT mice (Supplementary Fig. 2). Meanwhile, the PCP model was built by intratracheally inoculating with cysts and severe infection was observed in the lung of mice in our previous

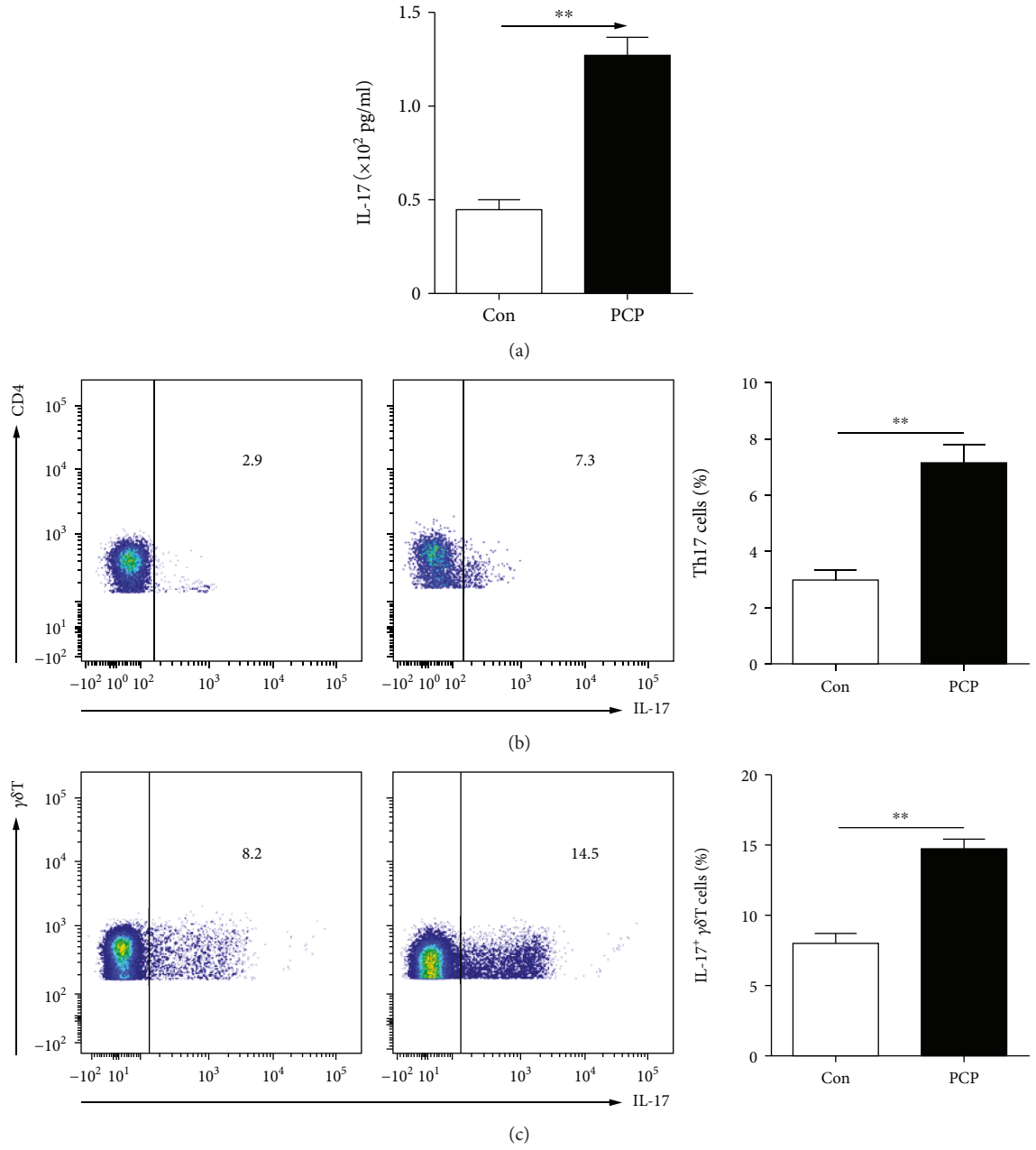

Figure 1: IL-17 levels increased in *Pneumocystis*-infected mice. IL-17 levels in the sera of PCP mice and WT mice were examined via ELISA (a). Representative flow cytometric dot plots and comparisons of Th17 ($CD4^+IL\text{-}17^+$) cells (b) and $IL\text{-}17^+$ $\gamma\delta$T ($\gamma\delta T^+IL\text{-}17^+$) cells (c) in the lungs from PCP mice and WT mice. Comparisons were evaluated by Student's *t*-tests for two-group comparisons. $^{**}P < 0.01$ and $^{***}P < 0.001$. Con: control; PCP: *Pneumocystis* pneumonia.

study [15]. According to these results, we focused on the immune cells in the lung from mice after *Pneumocystis* infection in the next experiments.

3.2. IL-17 and IL-10 Were Associated with the Clearance of Pneumocystis Cysts. $IL\text{-}17^{-/-}$ mice and $IL\text{-}10^{-/-}$ mice were used to investigate the roles of IL-17 and IL-10 in the clearance of *Pneumocystis*. *Pneumocystis*-infected $IL\text{-}17^{-/-}$ and $IL\text{-}10^{-/-}$ mice were sacrificed at 1-5 wk postinfection. Using RT-PCR, we found that after 3 wk postinfection, *Pneumocystis* burden in WT mice started to decrease. However, $IL\text{-}17^{-/-}$ mice and $IL\text{-}10^{-/-}$ mice showed delayed clearance of *Pneumocystis* in the lung (Figures 3(a) and 3(b)). We performed hematoxylin and eosin (H&E) staining of the lung homogenates from $IL\text{-}17^{-/-}$ PCP mice and $IL\text{-}10^{-/-}$ PCP mice at 2 wk postinfection. Compared with WT PCP mice, $IL\text{-}17^{-/-}$ PCP mice and $IL\text{-}10^{-/-}$ PCP mice showed more severe alveolar hemorrhage and inflammation cell infiltration in the lung (Figures 3(c) and 3(d)).

3.3. IL-17 and IL-10 Inversely Correlated with Each Other in Pneumocystis-Infected Mice. To further explore the role of

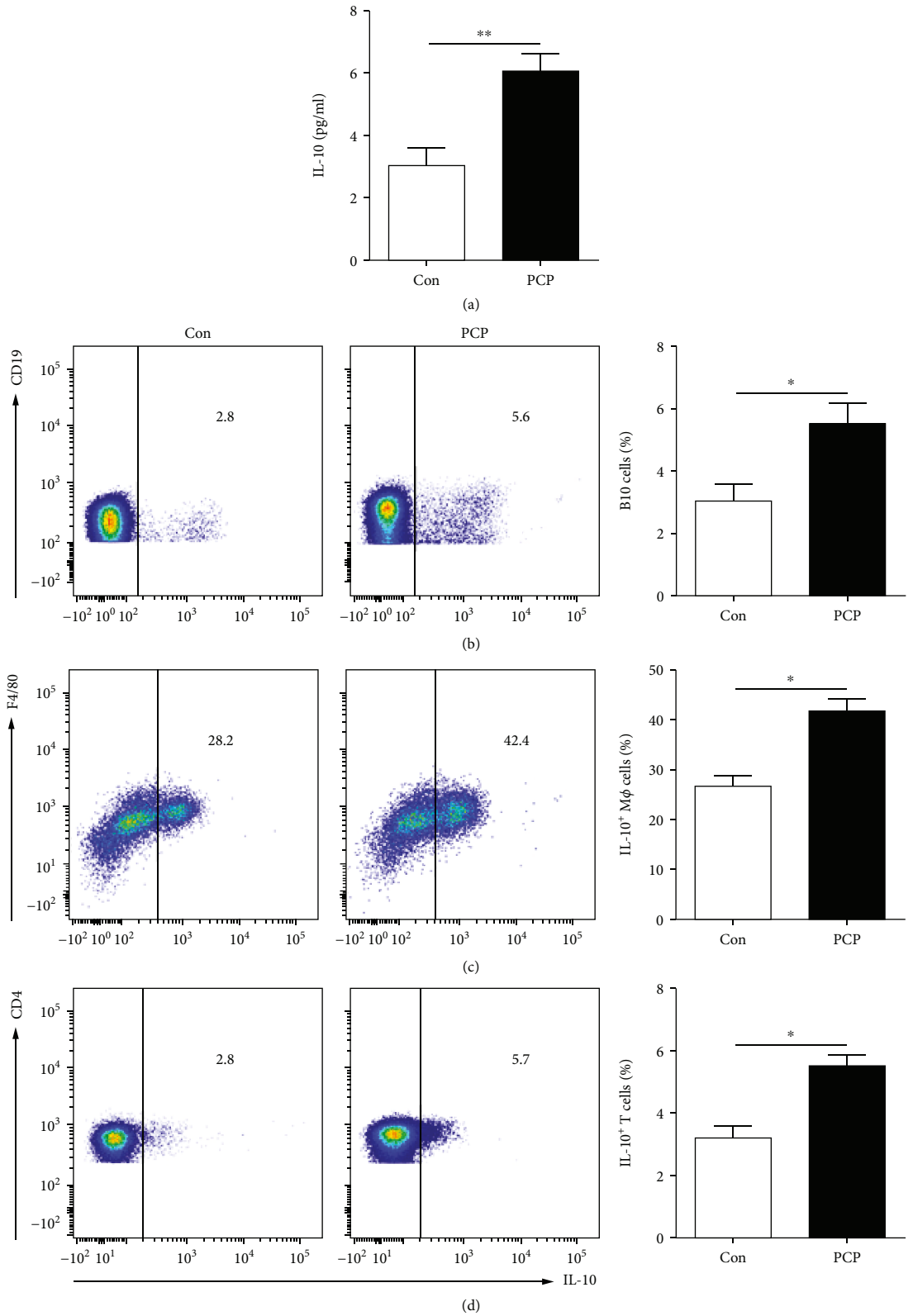

Figure 2: IL-10 levels increased in *Pneumocystis*-infected mice. The levels of IL-10 in the serum of PCP mice (a) were examined by ELISA. Representative flow cytometric dot plots and comparisons of B10 cells ($CD19^+IL\text{-}10^+$) (b), $IL\text{-}10^+$ macrophages ($F4/80^+IL\text{-}10^+$) (c), and $IL\text{-}10^+CD4^+$ T cells ($CD4^+IL\text{-}10^+$) (d) in the lung from PCP mice and WT mice. Comparisons were evaluated by Student's *t*-tests for two-group comparisons. $^*P < 0.05$. Con: control; PCP: *Pneumocystis* pneumonia.

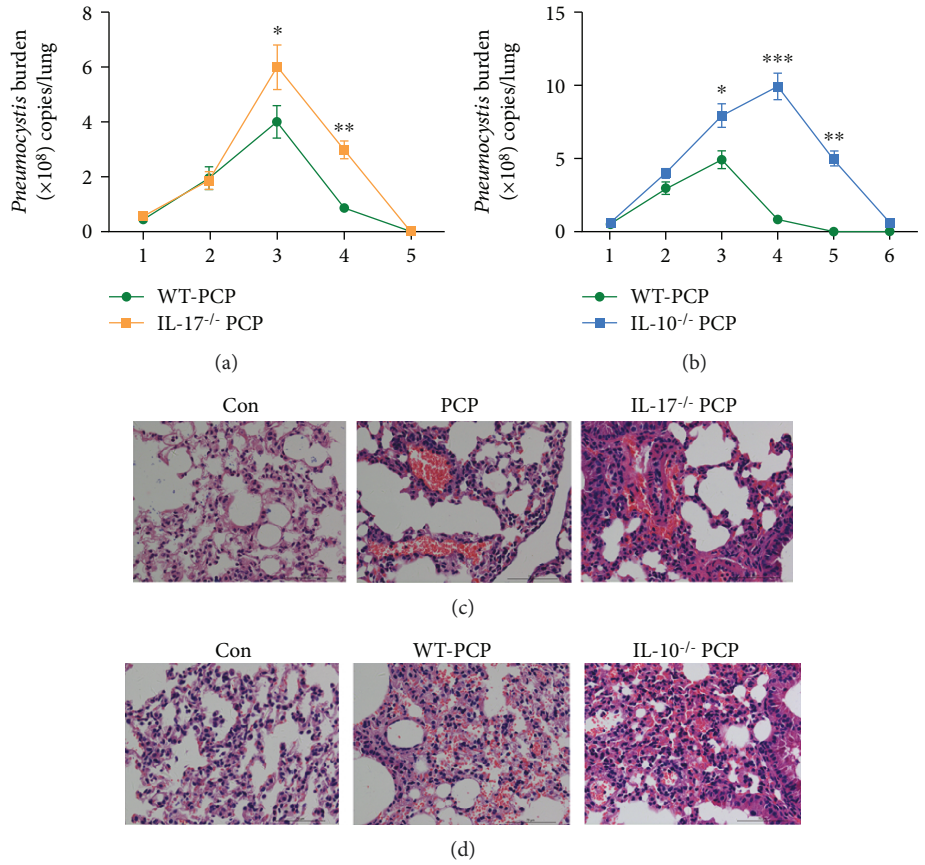

FIGURE 3: IL-17 and IL-10 were associated with the clearance of *Pneumocystis* cysts. Comparisons of *Pneumocystis* lung burden in the lungs from WT PCP mice ($n = 5$) and IL-17$^{-/-}$ mice ($n = 5$) (a). Comparisons of *Pneumocystis* lung burden in the lungs from WT PCP mice ($n = 5$) and IL-10$^{-/-}$ mice ($n = 5$) (b). H&E-stained histological features of the lungs in WT mice and IL-17$^{-/-}$ PCP mice (c). H&E-stained histological features of the lungs in WT mice and IL-10$^{-/-}$ PCP mice (d). In (a, b), the results are presented as means ± SE of 5 mice per group in each experiment, performed in triplicate at different time points. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Comparisons were evaluated by Student's *t*-test for two-group comparisons. Con: control; H&E: hematoxylin-eosin; PCP: *Pneumocystis* pneumonia.

IL-17 in *Pneumocystis* infection, we detected the percentages of B cells, T cells, and macrophages in the lungs from IL-17$^{-/-}$ PCP mice and WT PCP mice at 2 wk postinfection. The results did not show significant differences of the percentages of these cells between WT PCP mice and IL-17$^{-/-}$ PCP mice (Supplementary Fig. 3). However, flow cytometry data showed the significant increase of IL-17-producing B cells (16.9 ± 1.5 vs $6.7 \pm 1.2\%$, $P < 0.01$, Figure 4(a)), macrophages (58.5 ± 2.4 vs $39.5 \pm 1.9\%$, $P < 0.01$, Figure 4(b)), and T cells (8.5 ± 0.2 vs $4.4 \pm 0.1\%$, $P < 0.01$, Figure 4(c)) in the lung from IL-17$^{-/-}$ PCP mice than those from WT PCP mice. Also, the percentages of T cells, B cells, and $\gamma\delta$T cells were detected in WT-PCP mice and IL-10$^{-/-}$ PCP mice and we noted the decreased B cells and increased $\gamma\delta$T cells in IL-10$^{-/-}$ PCP mice (Supplementary Fig. 4). Next, we performed experiments to investigate if IL-10 influences the production of IL-17 in the PCP model. Similar to what we found in IL-17$^{-/-}$ PCP mice, we noted that CD4^{+} T cells (10.0 ± 1.5 vs $6.5 \pm 0.9\%$, $P < 0.01$, Figure 4(d)) and $\gamma\delta$T cells (35.2 ± 2.1 vs $16.5 \pm 1.6\%$, $P < 0.01$, Figure 4(e)) were expressing more IL-17 in the lung from IL-10$^{-/-}$ PCP mice than WT PCP mice.

3.4. IL-17-Related Gene Expression in PCP Mice. Since IL-17-expressing B cells, macrophages, and T cells were significantly increased in the lung from IL-10$^{-/-}$ PCP mice, we explored whether IL-10 would make an impact on IL-17-related genes. RT-PCR data demonstrated that IL-17 and STAT3 gene expression was significantly increased in the lung from IL-10$^{-/-}$ PCP mice than that from WT PCP mice at 2 wk after *Pneumocystis* infection. The expression of RORγT was downregulated in IL-10$^{-/-}$ PCP mice. There were no significant differences of the other related genes such as STAT5, STAT1, GATA3, IFN-γ, IRF4, NFκB, and IL-6 between IL-10$^{-/-}$ PCP mice and WT PCP mice (Figures 5(a) and 5(b)). The above data indicated that IL-10 deficiency might promote IL-17 expression via the STAT3 gene.

Next, we elucidated the change of IL-10 expression and IL-17-related genes in the lung of IL-17$^{-/-}$ PCP mice. Our data demonstrated that IL-10 and the STAT3 gene were upregulated in the lung from IL-17$^{-/-}$ PCP mice compared with WT-PCP mice after 2 wk of infection with *Pneumocystis* (Figure 5(c)). However, RORγT, STAT1, IRF4, IL-6, and IFN-γ genes were downregulated in IL-17$^{-/-}$ PCP mice.

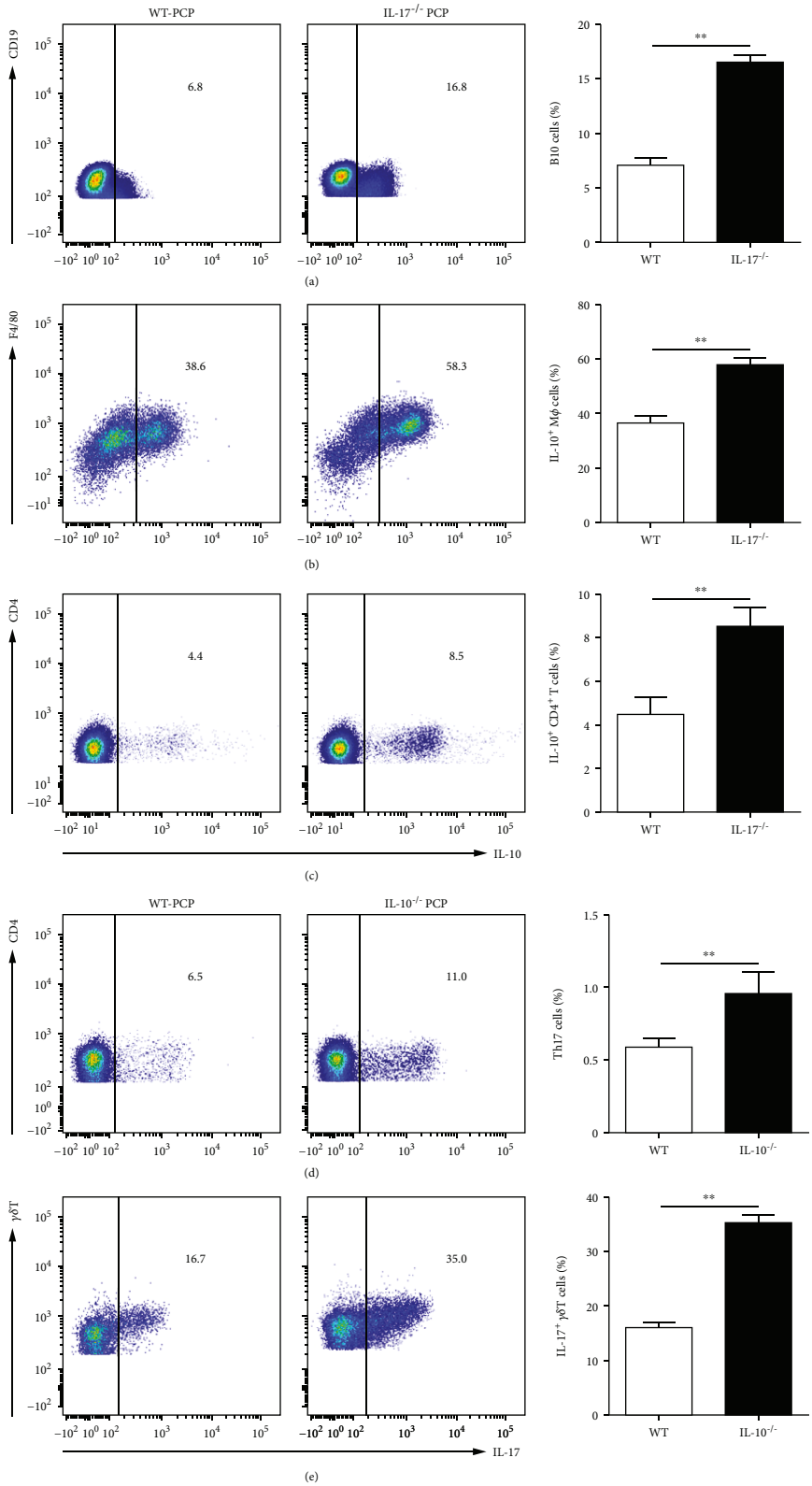

Figure 4: IL-17 inversely interplayed with IL-10 in the PCP model. Representative flow cytometric dot plots and comparisons of B10 cells ($CD19^+IL\text{-}10^+$) (a), $IL\text{-}10^+$ macrophages ($F4/80^+IL\text{-}10^+$) (b), and $IL\text{-}10^+$ T cells ($CD4^+IL\text{-}10^+$) (c) in the lungs from WT PCP mice and $IL\text{-}17^{-/-}$ PCP mice. Representative flow cytometric dot plots and comparisons of Th17 cells ($CD4^+IL\text{-}17^+$) (d) and $IL\text{-}17^+$ $\gamma\delta$T cells ($\gamma\delta T^+IL\text{-}17^+$) (e) in the lungs from WT PCP mice and $IL\text{-}10^{-/-}$ PCP mice. $^{**}P < 0.01$. Comparisons were evaluated by Student's t-test for two-group comparisons. WT: wild type; PCP: *Pneumocystis* pneumonia.

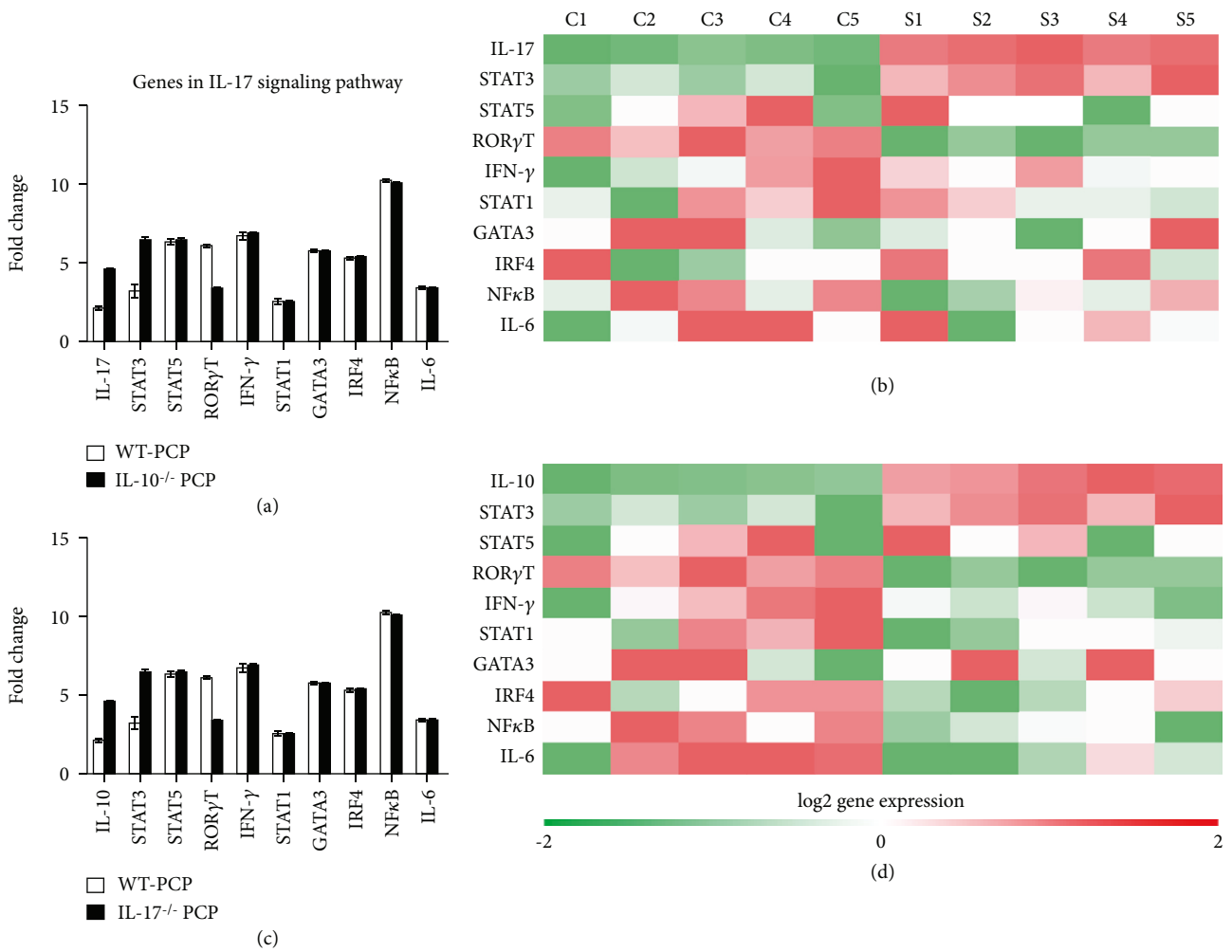

Figure 5: IL-17-related gene expression in IL-10$^{-/-}$ PCP mice and IL-17$^{-/-}$ PCP mice. Genes in the IL-17 signaling pathway from the lungs of WT PCP mice and IL-10$^{-/-}$ PCP mice were examined by RT-PCR (a), and the expression of genes were shown in the heat map (b). IL-10 and genes in the IL-17 signaling pathway from the lungs of WT PCP mice and IL-17$^{-/-}$ PCP mice were examined by RT-PCR (c), and the expression of genes was demonstrated in the heat map (d). Comparisons were evaluated by Student's t-test for two-group comparisons. WT: wild type; PCP: *Pneumocystis* pneumonia.

Furthermore, there was no significant difference of the other genes between IL-17$^{-/-}$ PCP mice and WT PCP mice. Thus, STAT3 may play an important role in the interplay of IL-10 and IL-17 in the *Pneumocystis*-infected mouse model.

3.5. STAT3 Played a Role in the Interplay of IL-17 and IL-10 in the PCP Model. As STAT3 may play a role in the interplay of IL-17 and IL-10 in the PCP model, we performed IL-17 and IL-10 antibody neutralization experiments in *Pneumocystis*-infected mice (Figure 6(a)). We depleted IL-17 and IL-10 in WT PCP mice. The results showed that after injection of anti-IL-17 mAb, IL-10-expressing B cells (Figure 6(b)), macrophages (Figure 6(c)), and T cells (Figure 6(d)) were induced significantly in PCP mice. Also, after injection of anti-IL-10 mAb, the expression of STAT3 increased and Th17 cell (Figure 6(e)) and IL-17^{+} $\gamma\delta$T cell (Figure 6(f)) percentages were higher in the lung from infected mice. Meanwhile, RT-PCR data demonstrated that depletion of IL-17 and IL-10 both promoted the expression of STAT3 (Figure 6(g)).

Thus, STAT3 may play an important role in the interactions of IL-17 and IL-10 in *Pneumocystis*-infected mice.

4. Discussion

Accumulating evidence indicates that PCP remains to be one of the most devastating diseases among non-HIV individuals receiving immunosuppressive therapy [1]. Multiple immune cells and cytokines have been studied in PCP hosts; however, it has been difficult to determine conclusively the cellular and molecular pathogenesis of PCP. Our present study focused on the immune regulatory roles of IL-17 and IL-10 in *Pneumocystis* pneumonia.

IL-17 is one of the founding members of the family of inflammatory cytokines, and IL-17 signaling is related to immunopathology and autoimmune diseases [17]. The

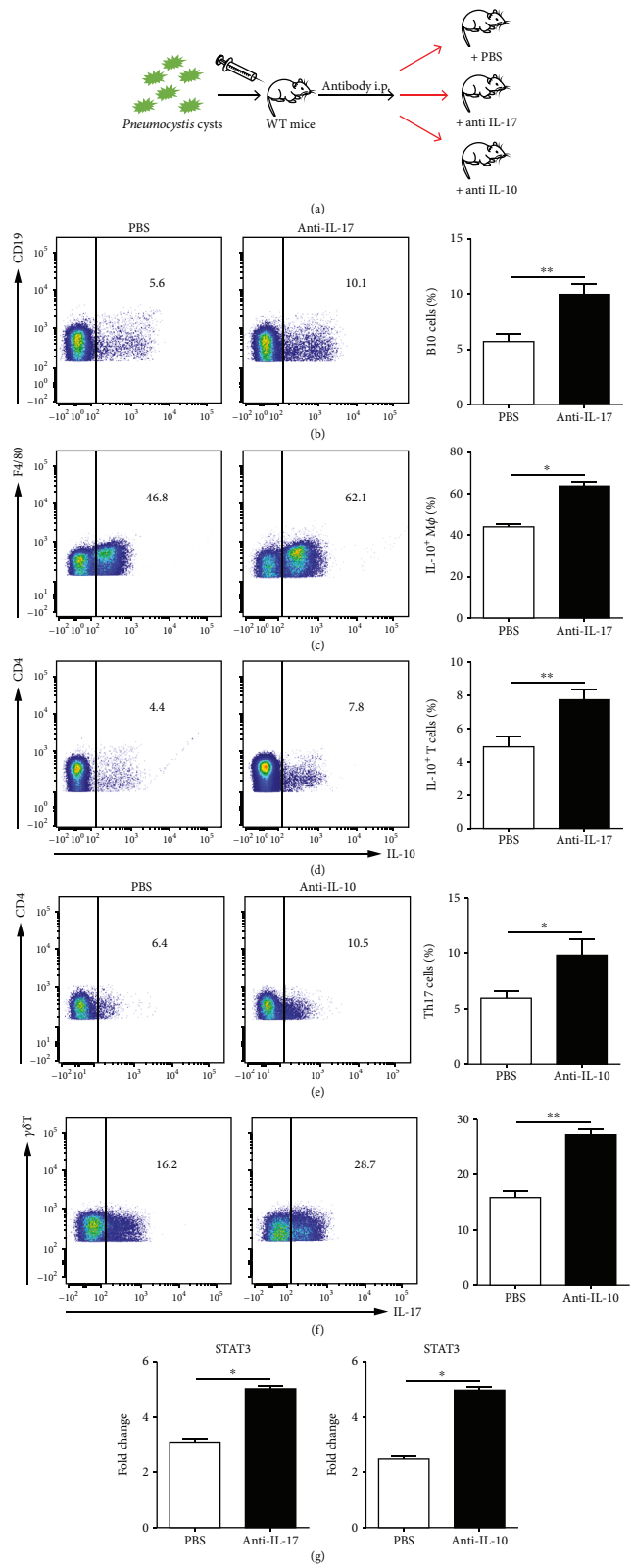

Figure 6: STAT3 was in association with the interactions of IL-17 and IL-10. The experimental design for the antibody neutralization is shown (a). Representative flow dot plots and comparisons of B10 cells ($CD19^+IL\text{-}10^+$) (b), $IL\text{-}10^+$ macrophages ($F4/80^+IL\text{-}10^+$) (c), and $IL\text{-}10^+$ T cells ($CD4^+IL\text{-}10^+$) (d) in the lung from WT PCP mice that received PBS or anti-IL-17 antibody. Representative flow dot plots and comparisons of Th17 cells ($CD4^+IL\text{-}17^+$) (e) and $IL\text{-}17^+$ $\gamma\delta$T cells ($\gamma\delta T^+IL\text{-}17^+$) (f) in the lung from WT PCP mice that received PBS or anti-IL-10 antibody. STAT3 gene expression in the lung from WT PCP mice the received anti-IL-17, anti-IL-10, or PBS was examined by RT-PCR (g). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Comparisons were evaluated by Student's t-test for two-group comparisons.

proinflammatory role of IL-17 was demonstrated in host defense against pathogen in a number of chronic inflammatory diseases [18]. IL-17A and IL-17F act on various immune cells and increase the production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and the granulocyte-macrophage colony-stimulating factor (GM-CSF) [29]. According to the related previous studies, IL-17 has two opposite contributions: its deficiency results in the loss of control of infections, while its overproduction could cause some chronic inflammatory diseases [18]. The study of Yen et al. demonstrated that IL-17$^{-/-}$ mice are more susceptible to Staphylococcus aureus [30]. Awasthi and Kuchroo found that IL-17$^{-/-}$ Candida albicans-infected mice show a higher fungal burden in skin lesion [31]. However, high levels of Th17 cells and CD8$^+$ IL-17$^+$ T cells were found in blood from patients with rheumatic diseases [32]. Meanwhile, the role of IL-17 in Crohn's disease remains unclear; IL-17 production leads to intestinal inflammation in several studies but could also be protective in others' researches [33, 34]. Thus, these data suggest that IL-17 could play a dual role in hosts defense against pathogens in chronic inflammatory diseases.

There are several studies focused on the immune function of IL-17 in the PCP model, but these results did not clarify the exact immune modulatory role of IL-17. Our present study showed that at 2 wk postinfection, IL-17 concentration was increased in serum and immune cells expressed more IL-17 in the lung from PCP mice. Our data is consistent with some studies from other investigators: Carmona et al. found that β-glucan surface components of *Pneumocystis* drive the activation of the IL-23/IL-17 axis, thus stimulating Th17 cell immunity in infected mice [13]; using a nude mouse model, Hu et al. verified that deficiency in IFN-γ promoted the differentiation of Th17 cells and IL-17 is essential for inflammatory responses in PCP [35]; Ripamonti et al. noted that IL-17$^+$ $\gamma\delta$ T cells and CD4$^+$ T cells in the lungs were increased during *Pneumocystis* infection in immunocompetent mice. However, the data of this study also demonstrated that IL-17A is not required for control of *Pneumocystis* infection [12], which is inconsistent with our present study. We found that the clearance of *Pneumocystis* was delayed in IL-17$^{-/-}$ mice compared with WT mice. Likewise, depletion of IL-17 could not provide an experimental model for the formation of fungal-driven inducible bronchus-associated lymphoid tissue (iBALT), which is responsible for the *Pneumocystis* burden in the lung of infected mice [36]. The present study focused on the immune regulatory role of IL-17 in PCP hosts, and the results indicated that IL-17 levels elevated in infected individuals and it was essential in the clearance of *Pneumocystis*. Meanwhile, we found that depletion of IL-17 leads to the induction of IL-10 in the PCP model.

Interleukin-10 (IL-10) has long been recognized to be one of the vital anti-inflammatory cytokines, which has been unequivocally established in various models of infection, inflammation, and even cancer [20, 37]. IL-10$^{-/-}$ mice could develop chronic inflammatory bowel disease [23]. In transgenic models, IL-10 reduced the ability of mice to mount significant T- or B-cell responses to ovalbumin, Listeria monocytogenes, and Leishmania [24]. Also, IL-10 expression constitutes a crucial element in the impairment of antiviral immunity [25]. According to these results, it is increasingly apparent that IL-10 might have a key role in inflammatory diseases. During *Pneumocystis* infection, IL-10 downregulates the immune response to pathogen in WT mice and plays an important role in controlling lung damage [38]. Furthermore, IL-10 was demonstrated to play a role in mediating B cell protection-demand hematopoiesis in PCP hosts [22]. In our previous study, we noted that B10 cells could play the immune regulatory role of Th1 and Th17 cell responses in infected mice [15]. In the current study, we further studied the immune modulatory role of IL-10. We noticed that IL-10 deficiency increased the proportion of the IL-17 level. These results suggested that during *Pneumocystis* infection, IL-17 inversely correlated with IL-10. In consistency with our data, some investigators also noted the interplay of IL-17 and IL-10 in inflammatory immunity. Mice lacking B10 cells were found to develop exacerbated disease and present with increased Th17 cell percentages [39]. Inhibiting IL-13 may inhibit Th17 production in an IL-10-dependent manner [40]. Mavropoulos et al. found that IL-10-producing B cells were impaired in psoriatic arthritis and inversely correlate with IL-17 and IFN-γ production [41]. Hansen et al. noted that IL-10 regulated an arthritic IL-17 response following infection with Borrelia burgdorferi [42]. These results indicated that IL-10 could play a significant role in the immune control and regulate the immune responses of the other cytokines during *Pneumocystis* infection.

Next, we detected the expression of IL-17-related genes in IL-17$^{-/-}$ and IL-10$^{-/-}$ PCP mice. Our data revealed the upregulation of STAT3 expression in IL-10$^{-/-}$ PCP mice. Interestingly, IL-17-related genes were all downregulated in IL-17$^{-/-}$ PCP mice except for the STAT3 gene. Thus, we suggested that the inverse correlation of IL-17 and IL-10 might be regulated via the STAT3 gene. STAT3 is one of the important transcription factors responsible for transmitting cytokine signals from the cellular membrane to the nucleus thus to alter gene expression, such as IL-6, type I and II interferon receptors, the IL-10 family receptors, and the IL-12 and IL-23 family receptors [43]. STAT3 activation is the downstream of a large number of cytokines via multiple receptor types [44, 45]. Recent evidences suggested a significant role of STAT3 in selectively maintaining a procarcinogenic inflammatory microenvironment [46]. In addition, STAT3 was reported to play a protective role in regulating virus-mediated proinflammation [47] and be associated with multiple immunodeficiency autoimmunity diseases [48]. Holland et al. found that mutations in the gene encoding STAT3 were identified in patients with autosomal dominant hyper-IgE syndrome (AD-HIES). Furthermore, the regulatory T cell and Th17 cell counts were reduced in these patients [49, 50]. Tangye et al. also suggested that STAT3 could play a critical part in the development of Th17 cells via affecting transcription of the genes encoding IL-17A, IL-17F, RORγT, and RORα [51]. Meanwhile, activation of STAT3 is critical for IL-10 production [52]. These data and

our results all indicated that STAT3 might play a key role in the inverse correlation of IL-17 and IL-10 in the PCP model.

In summary, all of the above demonstrated the pivotal roles of IL-17 and IL-10 in PCP hosts. IL-17 and IL-10 could both play protective roles in *Pneumocystis* infection via attenuating lung damage and assisting the clearance of pathogen. In addition, IL-17 and IL-10 inversely correlated with each other in the PCP model. We also noted that STAT3 might play a central role in the interplay of IL-17 and IL-10 during infection and it may be a new target for the therapy of PCP in the future.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared no conflict of interest.

Authors' Contributions

Heng-Mo Rong and Xiao-Jun Qian contributed equally to this work.

Acknowledgments

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Supplementary Materials

1. Supplementary Fig. 1: comparisons of periodic acid silver methenamine-stained histological features of the lungs of WT mice (A) and WT-PCP mice (B) at 2 wk postinfection. 2. Supplementary Fig. 2: IL-17 and IL-10 levels in serum from Pneumocystis-infected mice. Representative flow cytometric dot plots and comparisons of Th17 ($CD4^+IL\text{-}17^+$) cells (A) and IL-17^+ $\gamma\delta$T ($\gamma\delta T^+IL\text{-}17^+$) cells (B) in the blood from PCP mice and WT mice. Representative flow cytometric dot plots and comparisons of B10 cells ($CD19^+IL\text{-}10^+$) (C) and IL-17^+CD4^+ T cells ($CD4^+IL\text{-}10^+$) (D) in blood from PCP mice and WT mice. Comparisons were evaluated by Student's t-tests for two-group comparisons. $^*P < 0.05$. Con: control; PCP: Pneumocystis pneumonia. 3. Supplementary Fig. 3: IL-17 deficiency did not influence the percentages of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages in the lung from PCP mice. Representative flow dot plots and comparisons of $CD4^+$ T cells ($CD3^+CD8^-$), $CD8^+$ T cells ($CD3^+CD8^+$) (A), B cells ($CD3^-CD19^+$) (B), and macrophages ($CD45^+F4/80^+$) (C) in the lungs from WT PCP mice and IL-$17^{-/-}$ PCP mice. Comparisons were evaluated by Student's t-test for two-group comparisons. 4. Supplementary Fig. 4: IL-10 deficiency influenced the percentages of B cells and $\gamma\delta$T cells in the lung from PCP mice. Representative flow dot plots and comparisons of $CD4^+$ T cells ($CD3^+CD8^-$), $CD8^+$ T cells ($CD3^+CD8^+$) (A), B cells ($CD3^-CD19^+$) (B), and $\gamma\delta$T cells ($CD3^+$ $\gamma\delta T^+$) (C) in the lungs from WT-PCP mice and IL-$10^{-/-}$ PCP mice. Comparisons were evaluated by Student's t-test for two-group comparisons. *(Supplementary Materials)*

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Review Article

Bacteriophages: Uncharacterized and Dynamic Regulators of the Immune System

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The human gut is an extremely active immunological site interfacing with the densest microbial community known to colonize the human body, the gut microbiota. Despite tremendous advances in our comprehension of how the gut microbiota is involved in human health and interacts with the mammalian immune system, most studies are incomplete as they typically do not consider bacteriophages. These bacterial viruses are estimated to be as numerous as their bacterial hosts, with tremendous and mostly uncharacterized genetic diversity. In addition, bacteriophages are not passive members of the gut microbiota, as highlighted by the recent evidence for their active involvement in human health. Yet, how bacteriophages interact with their bacterial hosts and the immune system in the human gut remains poorly described. Here, we aim to fill this gap by providing an overview of bacteriophage communities in the gut during human development, detailing recent findings for their bacterial-mediated effects on the immune response and summarizing the latest evidence for direct interactions between them and the immune system. The dramatic increase in antibiotic-resistant bacterial pathogens has spurred a renewed interest in using bacteriophages for therapy, despite the many unknowns about bacteriophages in the human body. Going forward, more studies encompassing the communities of bacteria, bacteriophages, and the immune system in diverse health and disease settings will provide invaluable insight into this dynamic trio essential for human health.

1. Introduction

The human gut is a dense and diverse ecosystem containing a collection of trillions of bacteria, archaea, viruses, and eukaryotic microorganisms, collectively termed the gut microbiota. Advances in single-cell techniques, animal models, and "omics" approaches to study the human gut microbiota have unveiled the role of these commensal microorganisms as an active component of human physiology and health. Indeed, the gut bacterial community expands human metabolism by providing its host with metabolic pathways involved in breaking down otherwise indigestible nutrients and xenobiotics, compounds foreign to a living organism [1, 2]. The gut microbiota also protects against the invasion of pathogens by occupying all available niches in the gut and producing inhibitory compounds preventing the colonization of the gut by these and other microorganisms [3, 4]. Furthermore, the development of a mature immune system has been tied to bacterial colonization of the infant gut [5, 6].

Several genetic and environmental factors shape the composition of the gut microbiota. As such, a number of human diseases, including inflammatory bowel diseases (IBD), obesity, allergies, and diabetes, have all been associated with disease-specific shifts in gut microbial communities [7–12]. Despite the tremendous recent advances in this field, most studies on the gut microbiome remain incomplete, as they do not consider one of the main agents of bacterial death and horizontal gene transfer in nature, namely, bacteriophages (phages) [13]. For example, it is estimated that up to 50% of bacterial mortality in the oceans worldwide is due to daily phage infection and a selection of human bacterial pathogens, such as *Vibrio cholerae*, acquires their pathogenicity through phage-encoded toxins [14–16]. In the gut, these bacteria-specific viruses are estimated to be as

abundant as their bacterial hosts and constitute a source of polysaccharide and carbohydrate metabolism genes and antibiotic resistance, as well as cofactors that increase bacterial growth and fitness [13, 17–19]. Yet, their interactions with their bacterial hosts and the human immune system remain poorly described.

Phages were first discovered in 1915 by Twort and independently rediscovered and named in 1917 by d'Herelle, who named them after their lethal mode of action on bacteria (bacteriophage means "bacteria eater") [20, 21]. Both researchers studied phages in attempts to use them to cure bubonic plague or cholera, but their unsuccessful attempts and the concomitant discovery of antibiotics in the 1940s led to the widespread abandon of phages for therapy, except in Russia, Georgia, and Poland [22]. Despite this, phages remained studied in the laboratory context, where they have been instrumental for the development of molecular biology [23]; in aquatic systems, where they have been shown to play major roles in biogeochemical cycles [24, 25]; and in the food industry to control food-borne pathogens [26]. With the recent and dramatic increase in antibiotic resistance, phages have returned to the spotlight as a promising therapeutic tool, despite the many unknowns about their roles in the human body. After an overview of phage communities in the human gut during human development, we then detail their effects on the immune response through their actions on their bacterial targets and summarize the recent evidence for direct interactions between them and the immune system. Finally, we conclude with opportunities and challenges these interactions can represent in the context of phage therapy.

2. Bacteriophages in the Human Gut: Diversified, Numerous, and Uncharacterized

Despite advancements in high-throughput sequencing technologies, the characterization of phages in the human gut remains limited, mostly due to difficulties in phage isolation and genome annotation [27]. The inherent mosaic nature of phage genomes, their small size (approx. 30 kb in the gut [28]), and absence of universal genetic markers make annotation of phages challenging. Regardless, recent characterizations of the collection of phage genes (i.e., the phageome) have led to better identification of phages in the mammalian gut in health and disease, shedding some light on the compositional and functional diversity of these entities [29].

2.1. Phage Communities in the Healthy Human Gut. Phage sequences dominate the viral sequences detected in the human gut (the gut virome), despite most of the phage sequences corresponding to "dark matter" remaining to be characterized [27]. Within the characterized phages in the gut, the tailed dsDNA phages of the *Caudovirales* order are the most abundant, composed of the *Myoviridae*, *Podoviridae*, and *Siphoviridae* families, followed by the ssDNA *Microviridae* phage family [19, 30]. As RNA phages are currently considered to be transient members of the gut originating from our diet [31], most of our discussion here will focus on DNA phages. Phage diversity typically follows that of the main bacterial hosts in the gut, namely, the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria [32, 33], even during the transitions from childhood to adulthood.

Phages have been detected at low levels in newborns shortly after birth and are suggested to be from maternal and environmental origins [34, 35]. Within 2 weeks of life, phage communities go through drastic changes in their diversity and abundances in the infant gut [35]. Characterization of the viromes from mother-infant pairs suggests that breast milk may be an important initial source of phages in the infant gut [35–38]. Until approximately 2 years of age, the bacterial communities in the gut follow rapid expansions in their numbers and diversity (Figure 1) [39, 40]. Initially, this is also the case for the phage communities, but they rapidly contract and decrease in diversity with age (Figure 1) [34]. The rich collection of different *Caudovirales* phages found in the first few months of life decreases and seems to be replaced by the *Microviridae* species (Figure 1) [34]. The mechanisms underlying this dichotomy between bacterial and phage communities remain unclear, as not all shifts in phage diversity reflect the bacterial shifts. However, as we further detail, this could be driven in part by changes in phage replication cycles. Interestingly, one year after birth, phage communities were still different between children born vaginally and through C-section, despite their gut bacterial communities being similar, highlighting the importance of vertical transmission for some phage taxa [41].

From early childhood into adulthood, phage communities in the gut are unique to each individual, as demonstrated by the study of monozygotic and dizygotic twin pairs [32]. Similar to gut bacterial communities, relatives and unrelated household members share more phages than unrelated individuals [32], but each individual harbours a unique phage signature. There is increasing evidence for clusters of phage species that are shared among many healthy individuals, which include the ubiquitous *crAssphage* [19, 42, 43]. Approximately 40% of phages in these clusters are not found in adults with IBD, suggesting that these phages could be important biomarkers of health [19], yet these phages represent only a fraction (<5%) of the estimated phage diversity in the gut [42, 44]. More studies characterizing gut phage communities in adults from a variety of locations and diet are thus warranted to better understand the roles of these phages as markers of health. In the gut of healthy adults, phage communities remain relatively stable over time, with 80% of the same phage sequences detected in a given individual for 2.5 years [32, 42]. Unlike other ecosystems, the abundance of phages relative to their bacterial hosts, determined with the virus-to-bacteria ratio (VBR), is low and between 0.1 : 1 and 1 : 1. This suggests a dominance of the lysogenic replication cycle over the lytic cycle in the healthy adult gut, and as detailed below, there is increasing evidence linking disease with modifications of phage replication cycles.

2.2. Phage Replication Strategies and Implications for Development and Health. Phages replicate mostly through the lytic or lysogenic replication cycles, which have been extensively described elsewhere [24, 44]. In brief, the lytic cycle is characterized by the direct production of new phages

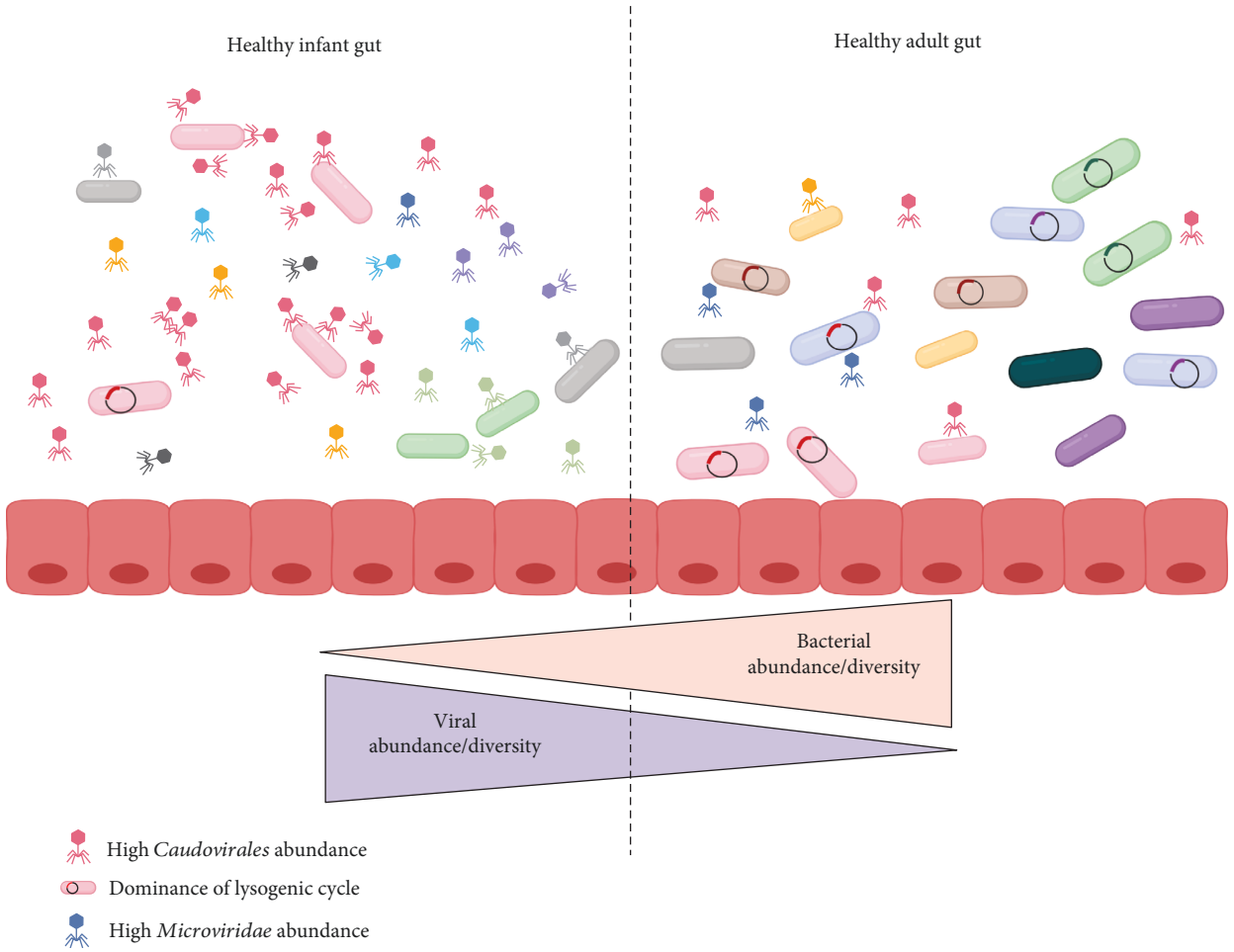

Figure 1: Characteristics of phage-host dynamics in the healthy infant and adult gut. During the first 2-3 years of life, there are drastic changes in the bacterial and phage communities in the healthy gut. Kill the Winner dynamics dominate during childhood, resulting in lytic replication and high phage abundance and diversity, particularly within the phage order *Caudovirales* (red). Piggyback the Winner dynamics are hypothesized to be prevalent in the healthy adult gut, where an increase in lysogenic replication coincides with a decrease in overall phage abundance and diversity. The abundance of *Microviridae* (blue) increases, and the phage community remains relatively stable over time. An absence of phage predation may lead to the expansion of bacterial abundance and diversity observed in the adult gut. Image created using BioRender.

after infection of a bacterial cell, causing bacterial cell death. Lysogeny is characterized by the integration of the phage genome into the bacterial genome or maintained as a plasmid. The integrated phage, or prophage, remains in its bacterial host until induction occurs, triggering a return to the lytic production of new phages [44]. It is currently considered that phages in the gut of infants up to 24 months old replicate through the lytic cycle, as both bacterial and phage communities are highly dynamic and go through drastic changes in abundances and composition [34, 44]. During this developmental period, phages are suggested to alter bacterial populations and maintain high levels of bacterial diversity through "Kill the Winner (KtW)" dynamics [34, 45, 46]. In these predator-prey interactions, phage infection controls the abundance of the dominant members of the bacterial community.

In contrast, phages in the gut of healthy adults seem to be integrated prophages, leading to the dominance of the lysogenic cycle (Figure 1). This is supported by the low VBRs, stability of phage abundance and diversity, absence of KtW dynamics, and the abundance of phages classified as temperate based on sequence homology and the presence of the *integrase* gene necessary for genome integration into the bacterial host [32, 33, 44]. The lysogenic cycle is typically found in low-nutrient and low bacterial abundance settings, which are not prevailing conditions in the gut. The prevalence of lysogeny despite the high abundance of actively replicating bacteria in the gut has led to the "Piggyback the Winner (PtW)" model, whereby phages may undergo lysogenic replication in such conditions to take advantage of the high fitness of their bacterial hosts [47]. In extension of this idea, it is hypothesized that there is a gradient of lysogenic to lytic replication across the gut mucus layer. In the lumen and the top mucus layer, where the bacterial load is higher, lysogenic replication dominates in agreement with PtW dynamics; while in the inner mucus layer, with lower bacterial load, lytic replication dominates [47]. Diseases where the mucosal layer is disrupted could thus lead to more lytic replication, further

enhancing the changes in bacterial communities and associated pathologies.

Interestingly, metagenomic studies report that most detected prophage sequences in the human and murine gut are integrated within bacteria from the Firmicutes phylum [32, 33, 42, 48]. This could have strong implications for human health, as the diversity and abundance of bacterial taxa within the Firmicutes are typically altered and possibly implicated in a variety of diseases [49]. The ubiquity of phages in the gut and their ability to modulate bacterial communities in other ecosystems suggest that they could be active players in human health and interact with the host immune system. Several immunological diseases, including inflammatory bowel diseases (IBD), Parkinson's disease, and Type 1 and Type 2 diabetes, have been associated with alterations of the gut phage community [50–54]. Understanding the direct and indirect ways by which phages interact with the immune system, as summarized in Figure 2, will help us gain insight into the functional role that these viruses play in human health and disease.

3. Bacterial-Mediated Interactions between Phages and the Immune System

As previously detailed, phage communities are specific to their bacterial hosts and can alter bacterial diversity and metabolism in a number of ways: by undergoing different replication cycles, infecting different bacterial hosts, carrying unique suites of genes augmenting host fitness, and having distinct binding properties. Given the many intricate interactions between the immune system and our resident bacterial communities, phages could be indirectly influencing these interactions by manipulating their hosts.

3.1. The Intestinal Bacterial Community and the Immune System. In order to understand how phage-mediated changes in the gut microbiota can influence immunity, it is important to consider the interactions between bacteria and the immune system. The bacterial component of the microbiota has been heavily implicated in the development of immune cells and the regulation of immune responses [55]. Initial exposure to microbial products is important in developing tolerance to commensals [56, 57]. In addition, the development of isolated lymphoid follicles, secretion of IgA, and maturation and homeostasis of CD4+ T cells and invariant natural killer T cells have all been tied to early exposure to microbes or microbial products [58–61]. The commensal bacterial community also plays an important role in the regulation of immune responses. For instance, various *Clostridia* species from the clusters IV and XIVa have been shown to induce mucosal regulatory T cell (Treg) accumulation and IL10 production, central to dampening proinflammatory immune responses [62, 63]. Many of these regulatory interactions can be linked to the production of short chain fatty acids (SCFAs), often produced by microbial fermentation of diet-derived fibres [64].

The intestinal bacterial communities also play an important role in preventing the colonization and systemic dissemination of potentially pathogenic enteric microbes [65–68]. The outgrowth of these pathogens, often belonging to the Proteobacteria phyla, has been associated with inflammatory diseases, with evidence indicating that some of these microorganisms can thrive in an inflamed environment [69–72]. It has been suggested that the increase in abundance of pathogens with increased inflammatory capabilities could trigger a feedback loop, whereby the proliferation of pathogenic organisms leads to increased inflammation and an environment that further selects for pathogen dissemination [55]. Consequently, a number of immunological disorders have been associated with shifts in microbial community composition [10, 73, 74]. We are now beginning to gain some insight into how phages might be driving these changes.

3.2. Phage-Mediated Alterations in the Intestinal Bacterial Communities: Implications for Immune Disorders. Despite the prevalence of lysogeny in the gut, there is growing evidence that phage predation can shape microbial communities in this environment [75–79]. Reyes et al. staged a "phage attack" of isolated virus-like particles (VLPs) from the feces of 5 unrelated volunteers to germ-free mice colonized with a collection of 15 bacterial isolates. Following phage administration, changes in the relative abundance of members of the bacterial community could be detected, suggesting that gut-derived phages were still infectious [75]. Using a similar approach, Hsu et al. colonized germ-free mice with a mock community of 10 known bacterial isolates before administering phages specific to a subset of these bacteria. They concluded that phage predation had cascading effects on the microbiota due to knockdown of susceptible species and subsequent disturbances to networks of interbacterial interactions. Further, these phage-induced changes of the microbiota were sufficient to alter the concentrations of a number of bacterial-derived metabolites, including neurotransmitters, amino acids, and bile salts [77].

These phage-mediated changes of gut bacterial communities could have downstream effects on immune signaling by allowing for the proliferation of proinflammatory or pathogenic microorganisms or altering the production of immunomodulatory bacterial-derived products (Figure 2(a)). The detection of bacterial DNA systemically following oral phage administration supports the idea that phage-mediated cell lysis could be responsible for the release of immunostimulatory pathogen-associated molecular patterns (PAMPs) [80]. With increased gut permeability, these PAMPs could translocate the epithelial layer and cause immune activation (Figure 2(a)) [80].

Both phage and bacterial communities have been shown to be altered in the context of intestinal inflammation [10, 50, 51, 81, 82]. Norman et al. concluded that the increase in *Caudovirales* and the expansion of overall phage richness observed in IBD patients were not driven by increases in bacterial richness [50]. The authors also found significant associations between the expansion of *Caudovirales* and specific members of the bacterial community [50]. These findings suggest that changes in the bacterial community associated with IBD could be driven by an imbalance of phages infecting these bacteria. In line with this hypothesis,

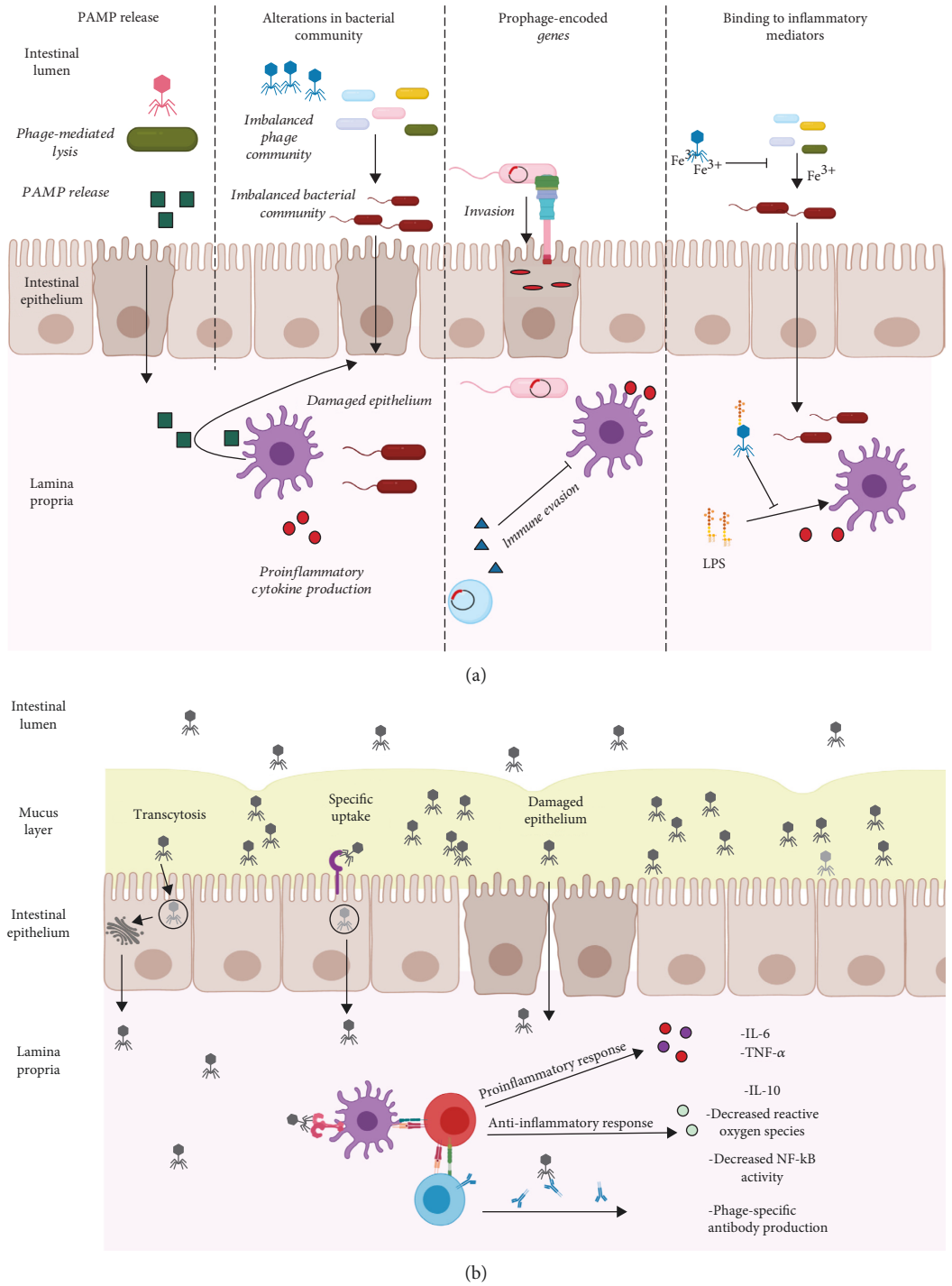

Figure 2: Crosstalk between phages and the immune system. (a) Indirect influences on immune responses. Phage infection may lead to the release of PAMPs, which can translocate the gut epithelium and induce proinflammatory responses. In the case of imbalanced phage communities, infection of certain bacterial species may lead to an altered microbiota, overgrowth of pathogens, and chronic inflammation. Prophage-encoded genes can aid pathogens in their abilities to damage and invade the epithelium and evade the immune system by directly inhibiting phagocytic cells. Sequestration of iron by phage tail domains could prevent pathogen overgrowth in the intestines. Binding of LPS by phage head proteins may dampen LPS-induced inflammation. (b) Direct stimulation of immune responses. Phages may cross the intestinal epithelium in 3 ways: nonspecific transcytosis, specific recognition of eukaryotic cells via structures that resemble bacterial receptors, and passage through damaged epithelial cells with defects in permeability. Once in the lamina propria, phages can interact with the intestinal immune system to generate pro- or anti-inflammatory responses and generate specific antiphage-neutralizing antibodies. The image was created using BioRender.

Cournault et al. found that phages which infect the bacterium *Faecalibacterium prausnitzii* were elevated in the feces of IBD patients [83]. Since levels of *F. prausnitzii*, a producer of the SCFA butyrate, are depleted in the gut of IBD patients, the expansion of phages infecting these bacterial taxa could contribute to its loss and increased inflammation during the course of disease [84]. Similar associations have been made in Parkinson's disease (PD), where the gut microbiota has been implicated in disease progression through the regulation of inflammatory responses and subsequent interactions with the enteric nervous system [85–88]. In PD patients, there is an increase in lytic *Lactococcus* phages and a corresponding decrease in *Lactococcus* bacteria, which have been shown to be potent inducers of anti-inflammatory responses and involved in the production of neurotransmitters [52]. Most recently, Tetz et al. found that children who presented seroconversion or developed Type 1 diabetes (T1D) had a high abundance of lysogenic *E. coli* phages compared to their bacterial hosts [54]. Interestingly, these data could suggest that prophage induction could cause release of DNA-amyloid complexes and trigger autoimmune cascades leading to T1D development [54].

The findings mentioned above show clear associations between altered phage and bacterial communities, and inflammatory diseases. Additional studies will need to identify factors that influence the changes in phage communities during disease. Different diets and specific dietary components have now been shown to shape the intestinal phage communities and the phageome [33, 89, 90]. Xenobiotics have also been shown to increase the expression of prophage induction genes, which could have widespread effects on bacterial and phage community composition [91]. Given that KtW or predator-prey interactions between phages and their hosts are most prevalent in early childhood, the infant phageome may be key in driving the appropriate maturation of the gut microbiota. Understanding the factors that shape the initial phage community during early childhood will provide insight into how microbial imbalances and their associated inflammatory diseases develop.

3.3. Phage-Encoded Genes Involved in Crosstalk with the Immune System. Beyond regulating the diversity, abundance, and metabolism of bacterial communities, phages are also powerful agents of horizontal gene transfer between bacteria. Prophages integrated into bacterial chromosomes or maintained as plasmids within bacterial cells account for important genetic differences between strains of the same species [92, 93]. In a process known as lysogenic conversion, genes within these integrated prophages can confer a fitness advantage to their bacterial host [94]. Many of these phage-encoded genes are involved in "superinfection exclusion," where integrated prophages are involved in preventing their bacterial host from further infection by closely related phages [95, 96]. Importantly, several genes carried by prophages have been found to increase the pathogenic potential of their host, either through the expression of phage-encoded virulence factors or other proteins that assist in immune evasion (Figure 2(a)). Thus, the genetic material that prophages provide to their lysogens has strong implications for how the immune system responds to, or can control, certain members of a microbial community.

Prophage-encoded toxins can be found in several unrelated bacterial species. Enterohemorrhagic *E. coli* (EHEC), *Clostridium botulinum*, *C. difficile*, *Vibrio cholerae*, and *Streptococcus pyogenes*, among others, rely on genetic material provided by prophages to produce toxins or proteins that regulate toxin production [97–100]. In *C. difficile* infections specifically, toxin B causes increased IL-8 production and immune-mediated damage of the intestinal epithelium [101]. *C. difficile* prophages do not encode this toxin [99]; however, lysogeny of several strains can increase its levels, suggesting a mechanism where phage integration could drive toxin B production and downstream proinflammatory responses [99]. Other phage-encoded genes, which are not toxins, may assist the invasive properties of enteric pathogens. *Salmonella typhimurium* expresses the rho GTPase, *sopE*, which is derived from the SopEφ temperate phage [102]. SopE is secreted into host cells via a type 2 secretion system and aids the entry of the bacterium by inducing membrane ruffling (Figure 2(a)) [103]. Delivery of SopE into stromal cells has also been shown to elicit mucosal inflammatory responses via caspase-1 activation and contribute to murine colitis [104, 105]. In turn, gut inflammation can accelerate the transfer of *sopE* between *Salmonella* strains through activation of the SOS stress response and subsequent prophage induction [106]. Some bacteria use prophage-encoded genes to evade the immune system to aid in their dissemination. For instance, *Staphylococci* prophages contain several genes involved in immune evasion, which integrate within the β-haemolysin gene [107]. The prophage-encoded chemotaxis inhibitory protein (CHIPS) and the Staphylococcal complement inhibitor (SCIN) block complement activation and neutrophil-mediated killing [108]. The Panton-Valentine leukocidin, which has been associated with methicillin-resistant *Staphylococcus aureus* (MRSA), can directly inhibit phagocytes by forming pores in the membranes of these cells [109, 110]. Collectively, these studies demonstrate that phage-encoded genes can have a diverse and profound influence on the interactions between bacteria and the immune system.

3.4. Phage Binding to Inflammatory Mediators. The exposed phage protein coat and tail fibres provide opportunities for unique binding sites between phages and their direct environment. Most studied interactions focus on phage binding to receptors on the surface of bacterial cells and subsequent infection [111, 112]. However, there is increasing evidence that the binding properties of phages and their associated functions are more complex. Structural analysis of the tail fibre region in T4 phages revealed that the needle domain contains 7 iron ions coordinated by histidine residues [113]. Iron binding has now been associated with several phages (Figure 2(a)) [114, 115]. Interestingly, Penner et al. found that the Pf4 phage could sequester Fe^{3+} and subsequently inhibit the formation of *Aspergillus fumigatus*-associated biofilms [115]. Increases in the amount of free iron have similarly been associated with increased risk of infection, virulence, and the outgrowth of pathogens including *V.*

vulnificus, *S. typhimurium*, and *Yersinia* species [116–119]. Phages can also alter immune responses by directly binding to inducers of inflammation: for example, the tail adhesin gp12 has been shown to mediate adsorption of T4 phages to *E. coli* cells [120]. More recently, Miernikiewicz et al. built on these findings to show that recombinant gp12 could not only bind to LPS but could also prevent LPS-induced production of proinflammatory cytokines in mice (Figure 2(a)) [121].

The ubiquity of phage-mediated binding of LPS and iron sequestration in the gut remains unclear, and other mechanisms could also be taking place. As we better characterize and annotate the phages in the human gut, we will gain a greater appreciation for how phage-mediated binding interactions might modulate inflammatory responses. Studying the immune response to both bacterial and phage communities in the gut will unveil many underlying interactions between these three parties, with some studies already demonstrating direct crosstalk between phages and the immune system.

4. Direct Crosstalk between Bacteriophages and the Immune System in the Gut

Phages are unable to infect eukaryotic cells, mostly due to differences between prokaryotic and eukaryotic replication and transcriptional machinery. Still, the human body is under constant exposure to diverse and abundant phage communities. Phages have been found in the gut, skin, lung, and bloodstream and have even been detected in cerebrospinal fluid and *in utero* following systemic dissemination. Understanding how phages access these disparate sites and how they interact with the mammalian immune system has important implications for human health and disease.

4.1. Crossing the Epithelial Barrier. In the mucosal layer above the epithelium, phage abundance has been shown to be over four times higher than the adjacent luminal area in a number of metazoan species [122]. The presence of phages systemically in several mammalian species suggests that the phages found in the mucosal layer can cross the epithelial cell layer and interact with underlying immune cells. Tight junctions between epithelial cell layers prevent passage of molecules greater than 0.4 nm, which includes phages [123]. It was thus suggested that the most probable mode of transportation of phages through this layer would be when the epithelium is compromised. In this case, a loss in tight junction functionality, responsible for tight cell-cell adhesion, may cause points of entry for phages (Figure 2(b)). Yet, phages have been detected in humans and rodents without any deficiencies in intestinal permeability, suggesting alternative pathways by which phages cross the epithelium [124–128].

In one example of phages interacting with mammalian cells, Lehti et al. described that phages could be internalized by eukaryotic cells by binding to moieties that resemble bacterial phage receptors (Figure 2(b)) [129]. Here, the *Escherichia coli* phage PK1A2 was shown to be internalized by neuroblastoma cells, which contain surface polysialic acid that are identical in structure to the bacterial K1 polysialic acid capsule [129, 130]. While phage DNA was shown to be degraded in the lysosome, this suggests that molecular mimicry could allow for direct interactions between phages and eukaryotic cells. Similarly, several groups have expressed eukaryotic surface structures on phage capsids to enter various eukaryotic cells for gene delivery [131]. Namdee et al. demonstrated this in the gut using a filamentous phage expressing an integrin binding motif [132]. Another and more nonspecific mechanism of phage uptake was described by Nguyen et al. (Figure 2(b)) [133]. The authors used an *in vitro* transwell system to measure transcytosis of various phage families through colonic (T84 and Caco2), lung (A549), and liver (Huh7) epithelial cell lines. While the percentage of transcytosed phages varied between families, transcytosis was preferred in the apical to basal direction in all cases [133]. Microscopy and cellular fractionation revealed that phages were internalized by endocytosis and were trafficked through the Golgi apparatus before being released basally [133]. Inhibitors of endocytosis block the uptake of natural and engineered phages, suggesting that this could be a prominent mode of access to eukaryotic epithelial cells [134–136]. Current estimates suggest that approximately 2×10^{12} phages inhabit the human colon [133, 137, 138]. Based on these numbers, Nguyen et al. speculated that over 30 billion daily transcytosis events occur through the epithelium. This nonspecific mode of uptake is likely a powerful mechanism that accounts for the presence of phages systemically in healthy individuals [133]. Another possible mechanism for phages crossing the epithelium barrier includes the Trojan horse theory, whereby a phage-infected bacterium is taken up by an epithelial cell, although there currently is no evidence of this [139, 140].

4.2. Immune Recognition and Responses to Phages. After crossing the epithelium, it is hypothesized that phages drain into the lymphatic system where they interact with circulating dendritic cells (DCs) and macrophages to stimulate cytokine production and generate humoral immune responses (Figure 2(b)). The vast genetic diversity of phages in the human gut reflects wide differences in phage morphologies, replication cycles, and structural proteins. Consequently, the direct interactions between phages and the immune system are complex and specific between the phage and the immune cell of interest. Still, most data suggest that phages have either weak proinflammatory or immunomodulatory effects. In a study where 5×10^8 pfu $\cdot$ ml^{-1} T4 phages were individually administered to bone marrow-derived dendritic cells, human plasma, or healthy mice, no increase in cytokine production or production of reactive oxygen species (ROS) was detected [141].

In another study, Miedzybrodzki et al. found that the T4 phage was immunomodulatory by reducing ROS production [142]. Indeed, a preparation of T4 phages inhibited ROS production from peripheral blood polymorphonuclear leukocytes (PMNs) stimulated by LPS or several *E. coli* strains [142]. These findings are all in agreement with the observations that T4 phages reduce immune cell infiltration of an allogeneic skin transplant and reduce T cell proliferation and NF-κB activation in mouse models [143]. Similarly, it

has been shown that NF-κB activity can been be modulated by the *Staphylococcus aureus* phage, vB_SauM_JS25. In LPS-stimulated MAC-T bovine mammary epithelial cells, vB_SauM_JS25 inhibited production of several proinflammatory cytokines and inhibited NF-κB signaling [144]. The abilities of T4 and *S. aureus* phages to inhibit the NF-κB pathway could represent a common mechanism for phages to elicit anti-inflammatory responses. The systemic presence of phages in the human body and their anti-inflammatory properties could be important in modulating immune responses and limiting autoimmune or inflammatory disorders [145]. Indeed, when phages infect their bacterial hosts in the bloodstream, dampening the immune response would be important because of the massive release of PAMPs resulting from bacterial lysis.

This perspective on phage-immune system interactions is likely oversimplistic, as there is substantial evidence that certain phages or phage communities can elicit proinflammatory immune responses. For example, *S. aureus* phage A20/R was shown to mediate costimulatory activity in splenocyte proliferation and induce production of the proinflammatory cytokine, IL-6 [146]. There are also examples of phage nucleic acids stimulating antiviral immune responses by activating Toll-like receptors (TLRs) [139]. The archetype filamentous phage M13 was shown to stimulate interferon production and protect mice against tail lesions caused by the vaccinia virus [147]. Eriksson et al. found that the use of tumor-specific phages led to a B16 tumor regression resulting from neutrophil infiltration [148]. Using MyD88-deficient mice, the authors found that this immune activation was dependent on phage induction of TLRs, which causes polarization of tumor-associated macrophages (TAM) to a proinflammatory M1 state [148].

Importantly, there is now increasing evidence that these proinflammatory interactions between immune cells and phages could be relevant in immunological disorders. A recent study showed that a cocktail of 3 *E. coli* phages isolated from IBD patients increased the proportion of CD4+ T cells, CD8+ T cells, and IFN-γ-producing T cells in Peyer's patches of germ-free mice [136]. The authors found that this T cell-mediated IFN-γ production was dependent on interactions with DCs [136]. Using an *in vitro* approach, they found that these phages were endocytosed by DCs and interacted with TLR9 within endosomes, important sensors implicated in immunity against eukaryotic viruses [136]. The authors then went on to demonstrate that specific pathogen-free mice given this phage cocktail had exacerbation of dextran sodium sulfate- (DSS-) induced colitis and increased levels of TLR9-mediated production of IFN-γ [136]. They further assessed that DCs cultured with VLPs isolated from UC patients stimulated higher IFN-γ production in comparison to healthy controls *in vitro*, suggesting that certain phage communities might generate more proinflammatory responses [136]. Dysbiosis of phage communities has been correlated with several inflammatory diseases [50–53]. In humans and in a T cell mouse model of colitis, increased abundance of *Caudovirales* has been observed relative to household controls. While it is unclear whether this dysbiosis could drive the development of these disorders, the proinflammatory potential of phage-immune cell interactions should be considered when studying these diseases and developing therapeutics.

Adding to the complexity of the phage-host immune crosstalk, there are several examples of phages which simultaneously elicit pro- and anti-inflammatory responses. Van Belleghem et al. analyzed the expression profiles of 12 immune-related genes in blood monocytes after individual exposure to a *S. aureus* phage and several *Pseudomonas aeruginosa* phages [149]. After exposure to each of these phages, genes involved in both pro- and anti-inflammatory immunological pathways were activated in the peripheral blood monocytes. For instance, the induction of the proinflammatory cytokines IL1α and IL1β coincided with induction of the IL1 receptor antagonist, which reduces proinflammatory responses [149]. These findings are in agreement with the discovery that filamentous *Pseudomonas* prophages (Pf4) are recognized by TLR3, resulting in transcription of type-1 interferons (IFN), often responsible for clearance of eukaryotic viral infections [150, 151]. This increase in type-1 IFN inhibited TNF, allowing for *P. aeruginosa* to persist and cause infection [150]. In support of their findings, a majority of *P. aeruginosa*-infected wounds contain detectable Pf4 [150].

4.3. Antibody Response to Phages. Once across the epithelial layer, neutralizing antibodies could limit further body-wide phage dissemination (Figure 2(b)). Immunization studies have indeed shown that humoral immune responses to phages can be generated. Some early investigations showed that various phages administered to animals or humans can generate specific neutralizing antibody responses [152–154]. It has long been thought that only antibodies that bind to the tail fibre region and inhibit phage-host interactions could abrogate phage infectivity. However, several studies demonstrate that phage capsid proteins, including the T4 highly antigenic outer capsid protein (Hoc), can generate antibody responses [155]. Dąbrowska et al. found that antibodies generated against T4 phages specific to the phage surface proteins, gp23 and Hoc, decreased phage activity [156]. The authors suggested that the antibodies generated against head proteins could prevent phage activity by causing aggregation of phage particles or interaction with the immune complement system to destabilize phage capsids or sterically inhibit phage-bacterial interactions [156].

The production of antiphage antibodies is not exclusive to individuals immunized with phages. The detection of antibodies specific to the T4 phage in the serum of animals with no history of immunization was discovered by Jerne in 1956 [152]. More recently in a group of 50 healthy human volunteers with no prior exposure to phage therapy or immunization, 81% had antibodies in their serum specific to the T4 phage [156]. These data support the idea that natural phage communities could indeed transcytose the epithelium and elicit a humoral immune response.

5. Considerations for Phage Therapy

Given the alterations in phage and microbial communities that are observed in a number of inflammatory diseases, there is a potential to use phages to manipulate the

microbiota towards a less proinflammatory composition. The long-term stability of phages in the gut and their capacity to alter bacterial hosts offer promise for the design of narrow or whole community phage cocktails that target members of the microbial communities implicated in disease. Before these therapeutic cocktails become a reality, we need to understand phage-host interactions that occur in the context of health and how they differ in inflammation. The contributions of prophage induction to changes in bacterial and phage communities, the host range of phages in the gut, phage-phage interactions, and whether predator-prey dynamics shift during inflammation are questions that still remain unanswered.

Nevertheless, we are beginning to characterize the diversity of phages in the human gut and understand how they might interact in various ways with the immune system. The ability for phages to cross the epithelium barrier and stimulate immune responses has strong implications for the effectiveness of phage therapy. The production of antibodies against phages and their proinflammatory potential raise questions for the efficacy and safety of such approaches. Understanding which phage taxa elicit pro- or anti-inflammatory responses will go a long way in determining which phages might be appropriate for a given condition. Much of the data summarized here on the direct interactions between phages and the immune system focus on a narrow group of phages, often in isolated settings. Elucidating these interactions at a whole community level will help us appreciate the degree to which phages influence immune responses in the human body. Either through their abilities to regulate bacterial populations or through their potential to directly stimulate immune responses, it is clear that phages are active and dynamic players in human health and cannot remain unconsidered in gut microbiome studies.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Role of Microbiome in Modulating Immune Responses in Cancer

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The complex interactions between genes and the environment play important roles in disease susceptibility and progression. One of the chronic diseases that is affected by this gene-environment interplay is cancer. However, our knowledge about these environmental factors remains limited. The microorganisms that inhabit our bodies have recently been acknowledged to play a crucial role as an environmental factor, to which we are constantly exposed. Studies have revealed significant differences in the relative abundance of certain microbes in cancer cases compared with controls. It has been reported that changes in the composition of normal gut microbiota can increase/decrease cancer susceptibility and progression by diverse mechanisms including, but not limited to, inflammation—a well-known hallmark of carcinogenesis. The microbiota can also affect the response to various treatments including immunotherapy. The microbiome-immune-cancer axis will continue to provide insight into the basic mechanisms of carcinogenesis. In this review, we provide a brief understanding of the mechanisms by which microbiota affects cancer development, progression, and treatment.

1. Introduction

The number of microbial cells in the human body was initially thought to be approximately 10-fold more than the sum of our own cells [1], suggesting the importance of their abundance in the human body. A recent study has shown that the estimation of these numbers is not true and that the ratio between the number of human and microbial cells in a human body is 1 : 1 [2]. However, this finding in no way undermines the active roles our microbiome plays in the body; on the contrary, it signifies that regardless of the ratio of microbial cells to human cells, the microbiome is capable of contributing to the physiological processes. Based on next-generation sequencing platforms [3, 4], it is known that the composition of microbial communities varies across different anatomical sites [5, 6]. Most microbes are bacteria, viruses, and fungi residing within our gastrointestinal (GI) tract. These together make up the human microbiome (bacteriome, virome, and fungome). However, there are differences in the microbiome composition between species and within the same species [6, 7], mainly attributed to host genetics and environmental factors, and their interactions with each other. Human disease susceptibility is primarily influenced by gene-environment interactions, and the microbiome is now believed to be a critical factor. Differences in the microbiome are evident between cases and controls for a growing list of human diseases including Crohn's disease, type-2 diabetes, autism, and chronic allergies [5, 8, 9]. In the past decade, studies have indicated that disturbance in the composition of normal microbiota influences cancer development and progression, as well as response to therapy.

2. Role of Microbiota in Cancer

Microbiota composition varies with tissues, indicating that their effects on inflammation and carcinogenesis are tissue-specific. The interindividual variability of microbiomes [10] determines key differences in disease development and progression. There are evidences of tumor-promoting effects of certain microbes in spontaneous, genetically driven and carcinogen-induced cancers in different organs of germ-free animals, for example, the skin, colon, liver, breast, and lungs [11–23]. In mice, depletion of intestinal microbiota using antibiotics reduces the development of cancer in the liver and colon [11, 23–30]. Although most of the studies show tumor-promoting effects of the microbiota, antitumor effects

of exogenous bacterial infections have also been observed. Towards the end of the nineteenth century, antitumor effects were observed in patients with sarcomas, after bacterial infections which was later developed as Coley's toxin (heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens*). Similarly, for over 40 years, one of the standard treatments for bladder cancer is BCG (mixture of bacterial extracts from *Bacillus Calmette–Guérin*) [31]. Later studies showed that specific bacterial components, such as Toll-like receptor (TLR) and NOD-like receptor (NLR) agonists, were responsible for many antitumor effects. This led to the concept that activation of innate immunity may convert tumor tolerance into antitumor immune responses [30, 32–34]. Microbes are recognized by multiple pattern recognition receptors (PRRs), which monitor the microbial status and barrier integrity, and initiate regulatory responses. These PRRs not only may control the microbiota through antibacterial mediators and thereby suppress cancer but also may promote resistance to cell death and trigger cancer-promoting inflammation. Moreover, the microbes release carcinogenic molecules, such as genotoxins and tumor-promoting metabolites [35]. The recognition of microbial patterns by TLRs is a powerful proinflammatory stimulus and a major effector of innate immunity [36]. It is well established that microbe-associated molecular patterns (MAMPs) and TLRs promote carcinogenesis. TLR4, the receptor for Gram-negative bacterial cell wall component LPS, promotes carcinogenesis in the liver, pancreas, colon, and skin, as shown by reduction in tumor development in *Tlr4*-deficient mice [37–40], and increases tumor load in mice that express constitutively activated components like peptidoglycan and lipoteichoic acid, promoting gastric cancer [41]. A key cancer-promoting downstream action of TLR signalling involves induction of survival pathways by activation of nuclear factor-κB (NF-κB) and STAT3 [17, 34, 39].

The composition and role of the human virome in health are understudied. A completely new avenue of research involving the viruses inhabiting the human body has changed the way viruses were looked upon. A phage is a virus that is known to infect only prokaryotic cells and not interact with eukaryotic cells. The human body has an abundance of these bacteriophages, mainly populating the areas of the blood, lymph, and organs. However, the mechanisms employed by the phages to cross epithelial barriers and access the body's organs have not yet been identified. A recent study reported that there was apical-to-basal transcytosis with every type of phage investigated across different cell lines. However, paracellular transport across an intact epithelial barrier was not found to be a likely mechanism of transcytosis [42]. This study also revealed that phages have access to membrane-bound vesicles and the cytosol. Further investigation showed that bacteriophages were found in all subcellular fractions of the eukaryotic cell with intracellular transport probably trafficking through the Golgi apparatus [42].

The main reservoir of phages in the human body is the GI tract. These phages have coevolved with the gut bacteria over the course of our life, and they have the potential to prevent pathogenic attack to their host. The presence of phages throughout the human body is very well documented. Unfortunately, articles on the issue of the microbiome in health and disease, as well as the role of microbial interactions with the immune system and with the intestinal mucosa, hardly explain the role of phages [43]. Phages, however, have been found to have antitumor effects in mouse models of melanoma [44].

As mentioned earlier, the microbiome also consists of a huge number of fungi which has been collectively named as mycobiome or fungome [45]. Despite the potential significance of the mycobiome, only few studies have analysed its composition. Great interindividual variation in mycobiome and predisposition to opportunistic infections owing to this variation has been proposed by many studies [46]. Many fungal species including *Candida*, *Aspergillus*, and *Cryptococcus* have been found to inhabit and influence infections in the human body [46]. There are studies suggesting an antagonistic relationship between *Pichia* and *Candida* species by different mechanisms [47]. Moreover, a negative correlation between *Candida* and *Campylobacter* in HIV-infected patients was also reported in this study, whereas in healthy subjects, no correlation between *Candida* and bacterial species was found [47]. Candida species is a well-known oral fungal pathogen, and studies have shown that infection with this species can significantly increase overall and some individual cancer risks, for example, head and neck, pancreatic, skin, and thyroid cancers [48]. A study in colorectal cancer patients has revealed dysbiosis in mycobiome characterised by change in fungal composition and ecology, which suggests the important role of gut mycobiome also in CRC [49].

Several reports with mouse models provide data on the fact that the composition of the gut microbiota is modulated by diet [50]. The composition of the microbiota differs among individuals living in different geographic regions and on the long-term diet [50]. A balanced microbial composition could be achieved through symbiosis that occurs through the consumption of balanced diets [50]. Dysbiosis, caused by an imbalanced diet, disturbs the microbe-immune interaction making the host susceptible to inflammation and diseases [50]. However, there is still a lack of understanding of how microbiome composition is modulated by diet [50].

3. Host-Microbiota Interaction

A key factor to develop symbiosis between host and microbes is the anatomical separation of microbial entities from the host compartments by layers of well-maintained physical barriers. Disturbance in these barriers leads to inflammation and diseases, including cancer [37]. The barriers include an intact epithelial lining that acts as a sensing system to detect and eliminate invading bacteria, the mucous layer surrounding the gut, and the low pH in the skin and stomach. Moreover, bacterial numbers and location are monitored by specific cell types: such as in the gut, Paneth cells defend the immune system by secreting antimicrobial molecules into the lumen, goblet cells secrete mucin to lubricate the intestinal contents and protect the epithelium, and in the skin, keratinocytes regulate the microbes by secreting antibacterial peptides [51, 52]. In the gut, secreted

immunoglobulin A (IgA) provides additional protection against microbes and limits the access of intestinal antigens to the circulation and invasion of potentially dangerous bacterial species [53]. The gastrointestinal (GI) tract is considered the largest immunological organ in the body playing a significant role in regulating immune homeostasis. The interplay between epithelial cells, immune cells, and microbiome influences immune system mediators and thus affects the intestinal barrier [54]. The lining of the lower intestine contains finger-like projections that form structures called villi which increase the mucosal surface. Underlying the epithelium, the lamina propria contains the important antigen-presenting dendritic cells, which regulate humoral and cellular immunity [54]. Tight junctions, or the zonula occludens, interact with different proteins with their intracellular domains and regulate vesicular import and export [55]. They facilitate the passage of small ions and water-soluble molecules through the paracellular space and prevent the passage of antigens, microorganisms, and their toxins [55]. Apart from the host control mechanisms, the natural host microbiome nurtures a functional luminal barrier [56] by maintaining epithelial cell turnover and producing mucins, as well as by competing for resources, which suppresses the growth of pathogenic microbes. A classic example of the protective role of commensal microbiota is opportunistic infection with *Clostridium difficile,* which only causes disease when the normal resident gut microbiota is suppressed by antibiotics. This infection can be cured by transplantation of microbiota from healthy individuals [57]. Similarly, germ-free mice have an increased susceptibility to infection with pathogens [58]. Production of bacteriocins is another way by which the natural microbiota restricts the growth of pathogenic microbes [59]. Failures of these control mechanisms—that is, defective barrier, immune suppression, and dysbiosis—have been associated with microbe-driven carcinogenesis. These regulatory mechanisms are inextricably linked, and failure of one typically disturbs the overall equilibrium. For instance, infection with *H. pylori* not only injures host cells but also alters the gastric environment and barrier, which increases inflammation and disturbs the microbiota [60].

4. Microbiome in Immunoregulation

Microbiota shapes the innate and adaptive immunity significantly, although the intricate details are still unknown [61]. The development of the microbial flora at birth influences the maturation of the immune system and development of tolerance and containment of microbial infections [62, 63]. It continues throughout life via signalling through receptors of the innate immune cells, through sampling of the microbiota by adaptive immune response, and by generating metabolic products [64, 65]. For example, data from germ-free and antibiotic-treated mice show a markedly reduced response to CpG stimulation in the setting of cancer immunotherapy [66]. Upregulation of TLRs by LPS and other microbial products can activate the NF-κB, c-Jun/JNK, and JAK/STAT3 pathways, which play roles in cell proliferation and immunosuppression [67, 68]. Overall, antibiotics, particularly during immunosuppression, may interfere with effective anticancer immune responses [69].

Apart from bacteria, the presence of bacteriophages in huge numbers in the human body naturally triggers the question of whether these are mere spectators of the whole interaction between the bacterial species and the immune system. The potential role of phages present in the GI tract is of special interest. Studies have reported that these intestinal phages may have immunosuppressive properties when administered *in vivo*, inhibiting both humoral and cell-mediated immunities [70, 71]. Therefore, intestinal phages not only may help eliminate harmful bacteria and reduce the number of commensal bacterial species, thus reducing the heavy bacterial load on local mucus membrane, but also may suppress local immune reactions [43], for example, inhibition of dendritic cells and NF-κB [43]. This suppression plays a crucial role in maintaining immune homeostasis. Therefore, phages appear to have a protective role in the development of gut inflammation in healthy people, and any disturbance in the phage composition breaks the phage-mediated tolerance [43]. This breakdown may promote the development of inflammatory bowel diseases and other opportunistic infections [43].

5. Microbiota in Modulating Immunotherapy

Cyclophosphamide (an immunostimulatory alkylating agent used to treat solid sarcomas) alters natural microbiota in the small intestine of mice and causes the translocation of certain gram-positive bacteria, mainly *Lactobacillus johnsonii* and *Enterococcus hirae*, into secondary lymphoid organs [72]. These bacteria stimulate the generation of a specific subset of Th17 and Th1 cells (which produce IL-17 and IFN-γ), underscoring how particular microbial components present in the gut lumen can regulate the polarity of Th responses to cyclophosphamide treatment. Furthermore, alteration of the gut microbiota influences the efficacy of immune checkpoint blockers (ICB). Immunotherapy has been among the most recent developments in cancer care, especially with the advent of ICBs. These inhibitors function by reactivating T cells that have been rendered ineffective by the tumor microenvironment, thus making them respond again to tumor antigens [37]. As of now, blockade of two checkpoints by monoclonal antibodies has been successful: cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed-cell-death protein 1 (PD-1)/programmed-cell-death ligand 1 (PD-L1) [8]. Recent research shows that the immunostimulatory and antitumor effects of the CTLA-4 antibody depend on distinct bacterial species of the gut [73]. The anti-CTLA-4 monoclonal antibody has been found to lose its therapeutic efficacy against established sarcomas, melanomas, and colon cancers in germ-free or antibiotic-treated mice (Figure 1).

A seminal study reported that response to an ICB could be improved by changing the gut microbiome of a mouse [74]. Data for many patients with different types of cancer were examined. Some of these patients were on antibiotic therapy for routine causes like dental pain or a urinary tract infection before or shortly after starting a PD-1 drug. It was

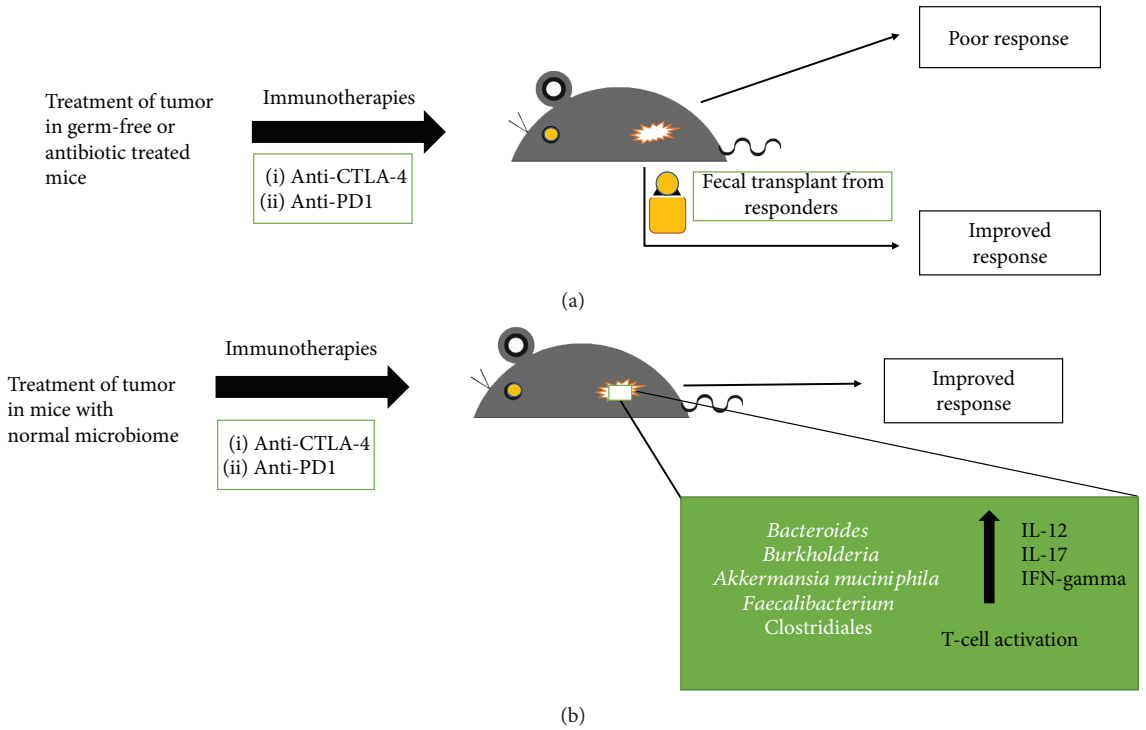

Figure 1: (a) Treatment of tumor in germ-free or antibiotic-treated mice shows poor response to immune checkpoint blockers. When fecal transplant is made to these germ-free or antibiotic-treated mice from responders, the mice show improved response to the same immune checkpoint blockers. (b) Treatment of tumor in mice with normal microbiome shows improved response to immune checkpoint blockers, and the prevalent species of microbiota include *Bacteroides* [74], *Burkholderia* [74], *Akkermansia muciniphila* [75], *Faecalibacterium* [77], and Clostridiales [77].

found that certain bacteria of the genera *Bacteroides* and *Burkholderia* were responsible for the antitumor effect of the microbiome [74] (Figure 1). Interleukin-12 is released in response to these *bacterial species*, which may aid in triggering immune responses by stimulating the T cells [74]. To confirm the results, microbes were transferred into mice that had no intestinal bacteria, either by feeding them with the microorganisms or by giving them the *Bacteroides*-rich feces of some ipilimumab-treated patients. In both cases, growth of these bacterial species improved the response to a checkpoint inhibitor [74]. Later, studies on the differences in the gut bacteria of responders and nonresponders revealed the presence of *Akkermansia muciniphila*, a bacterial species associated with mucus lining of the gut that may provide protection against obesity and diabetes. Germ-free mice devoid of gut bacteria responded better to PD-1 blockers on receiving fecal transplants from responders, compared to mice receiving feces from nonresponders. On feeding them *A. muciniphila*, poorly responding mice could be turned into responders [75].

Studies have also found that differences in composition of gut microbiota could explain why mice purchased from different vendors showed different responses to PD-1 blockers [76] (Figure 1). In a recent study, it was reported that the gut microbiome significantly affects melanoma patients receiving PD-1 blockers [77]. Like other studies, mice that received fecal transplants from responders showed better response to drugs compared to the mice that received fecal transplants from nonresponders. In this report, the bacterial species found were mainly *Faecalibacterium* and Clostridiales [77] (Figure 1).

6. Concluding Remarks

The crosstalk between the natural host microbiome and immune system clearly modulates local and systemic inflammatory responses, oncogenic signalling, and tumor progression. The microbiome-induced innate and adaptive immune responses have an impact on the efficacy of immunotherapy. It is therefore imperative to uncover the underlying immune mechanisms and find targetable molecules associated with the host's personal microbiota that influence immune responses. It has been shown that transplants of certain microbes restore eubiosis in chronic disease states, which reduces inflammation induced by microbial dysbiosis. Narrow-spectrum and nonabsorbable antibiotics may be used to target genotoxic or translocating bacteria. Since host diet affects normal microbiota, natural restoration of commensals through foods that help them thrive could reduce the harmful effects of chronic diseases. Genetically manipulated species of microbiota expressing or lacking specific enzymes [73] along with matched diets might be used to achieve higher levels of tumor-suppressive effects or lower levels of tumor-promoting effects or suppress the growth of tumor-promoting bacterial species [37]. Targeting the inflammatory pathways that are activated by the translocated

bacterial species may reduce inflammation and slow down tumor growth and/or enhance the efficacy of certain immunotherapy strategies.

Targeting bacterial genotoxins and enzymes that promote cancer could be useful. Understanding the multifarious mechanisms by which microbiota promotes carcinogenesis will open new avenues to identifying diagnostic, preventative, and therapeutic approaches. Continued unravelling of natural microbiota and its alteration during infections, antibiotic therapy, and varied diets could lead to identification of biomarkers that determine the escape phase of an abnormal cell from immunological pressures. Intratumor heterogeneity and response to therapy can also be explained based on differences in microbial composition. Therefore, it is possible that combining anticancer therapy with certain microbes known to provide protection from cancer may be considered in the future. Certain microbial peptides have anticancer effects. For instance, azurin, which is secreted by *Pseudomonas aeruginosa*, has been found to work well against tumors [78]. Therefore, biochemical analysis of microbial peptides with potential anticancer activities could be helpful. Further insight into the microbiome-immune interplay may aid in the development of preventative vaccines against cancer. Culture conditions supporting growth of most microbes inhabiting the human body, especially anaerobic bacteria residing deep within our GI tract, need to be established. These studies should be combined with epidemiological data, genome-wide association studies, and metabolomics. It is necessary to culture specific bacteria to analyse their functional role in gnotobiotic mouse models in which either the microorganisms are excluded or their composition is known. Improved probiotic/prebiotic strategies to prevent diseases may be developed. Immunotherapy might be improved based on the knowledge of microorganisms that influence their efficacy. Since microbiota varies in different tissues, it could provide information about factors that cause certain cancers to be more aggressive. Microbiome signatures in different cancers could be developed for research on personalized medicine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

The Role of the Microbiota in the Diabetic Peripheral Artery Disease

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Vascular complications of diabetes mellitus represent a major public health problem. Although many steps forward have been made to define the causes and to find the best possible therapies, the problem remains crucial. In recent years, more and more evidences have defined a link between microbiota and the initiation, promotion, and evolution of atherosclerotic disease, even in the diabetic scenario. There is an urgency to develop the knowledge of modern medicine about the link between gut microbiota and its host's metabolic pathways, and it would be useful to understand and justify the interindividual diversity of clinical disease presentation of diabetic vascular complication even if an optimization of pharmacological treatment has been made or in the case of young patients where hypertension, dyslipidemia, and diabetes are not able to justify a very quick progress of atherosclerotic process. The aim of the present review is to gather all the best available evidence in this regard and to define a new role of the microbiota in this field, from biomarker to possible therapeutic target.

1. Type 2 Diabetes Mellitus: A Chronic Low-Grade Inflammatory Disease

Type 2 diabetes mellitus (T2DM) represents a chronic metabolic disease characterized by a relative insulin deficiency due to pancreatic β-cell dysfunction and insulin resistance in target organs, with consequent hyperglycemia. It has become a global public health problem because of an endemic progression worldwide, also resulting from an increasing prevalence of obesity and sedentary lifestyle [1]. Indeed, T2DM is considered as a chronic, low-grade inflammatory disease determined by long-term immune system imbalance, metabolic syndrome, and/or nutrient excess [2].

Emergent evidences support the implication of inflammatory processes with an abnormal production of cytokines and activation of inflammatory signaling pathways in the development of this metabolic disease [3–6]. In the early 1990s, Hotamisligil et al. described an increase of tumor necrosis factor- (TNF-) α in adipose tissue and, conversely, an improved peripheral glucose uptake with the neutralization of TNF-α in animal models of obesity and diabetes [7, 8]. This finding marked a new era in understanding that a subclinical inflammatory process triggers both insulin resistance and metabolic dysfunction, which precede T2DM. Advances in this field have recognized components of both innate and adaptive immune responses in regulating the inflammatory process [9]. Even, Tsai et al. have hypothesized that T2DM could be considered as an autoimmune disease [10]. In addition, T2DM is clearly associated with macro- and microvascular complications that are considered as the expression of the inflammatory process [11]. In particular, atherosclerosis is a complex process resulting from an inflammatory response to injury with the interaction of numerous cell types and formation of fatty streaks that could progress to atheromatous plaques, plaque destabilization, and plaque rupture [12]. Endothelial dysfunction is an early

event of this process that determines the alteration of vascular homeostasis, and it stimulates the production of proinflammatory cytokines [12]. Chronic hyperglycemia condition accelerates the progression of atherosclerosis because of the overproduction of reactive oxygen species (ROS) by the mitochondrial electron transport chain, the formation of intracellular advanced glycation end products, the activation of protein kinase C, and the increase of polyol pathway flux [13]. Excess of ROS also increases the expression of inflammatory and adhesion factors, the formation of oxidized low-density lipoprotein, and insulin resistance by activating the ubiquitin pathway, inhibiting the activation of AMP-protein kinase and adiponectin, and decreasing endothelial nitric oxide synthase activity [12].

1.1. Lower Extremity Arterial Disease in Diabetic Patients. Diabetes is associated with accelerated atherosclerotic disease that affects arteries of the brain, heart, and lower extremities [14]. Therefore, diabetic patients have a higher risk of stroke, myocardial infarction, and limb amputation [15]. In particular, peripheral artery disease (PAD), defined as the atherosclerotic occlusive disease of the lower extremities, is one of the most severe conditions in patients with T2DM. Nowadays, PAD represents a public health problem with a significant impact on healthcare and high economic burden [12]. Over 200 million people are affected with lower extremity artery disease worldwide [13], and its prevalence increases with the prevalence of T2DM, one of the major risk factors [16]. Furthermore, PAD has special features and poorer prognosis in diabetic than in nondiabetic patients [17]. Clinical onset is frequently characterized by critical limb ischemia and gangrene, typical manifestations of advanced disease stages, due to a poorly symptomatic progression of these patients during the earlier stage of disease and to their reduced pain perception related to the concomitant presence of peripheral neuropathy [18]. As a consequence, patients with diabetes are at higher risk of lower extremity amputation than those without diabetes [6, 19–21]. In addition, diabetic patients with PAD, compared with diabetic patients without PAD, have also a higher risk of cardiovascular disease [22–25]. Despite its severity, PAD is still the least studied compared to other diabetic vascular complications [26].

2. The Microbiota: The Oldest Guest

The human organism owns several metabolic pathways to counter the inflammatory process determined by the continuous exposition to the external environment and pathogens and to endogenous oxidative factors [27]. The infection results as one of both local and systemic principal inflammation-promoting factors [28]. In the latter case, the role of a cross-mimicry process [28–31] and a systemic bloodstream translocation from a local origin [30, 32–34] has been demonstrated as initiating and promoting events of the systemic inflammation burden far from the site of the original colonization or infection [35].

Specifically, several studies have enhanced a clear correlation between *Helicobacter pylori* and atherosclerosis, known as the plaque inflammation process [28, 30, 31, 36]. In addition, genetic fragments of human-colonizing microbes (*Helicobacter pylori* and periodontal microbes) have been found in carotid artery samples [28, 37, 38] of patients affected by PAD, demonstrating an atherosclerotic plaque colonization. Moreover, other bacteria or viruses have been discovered colonizing the atherosclerotic plaques (e.g., *Chryseomonas*, *Veillonella*, *Streptococcus*, *Cytomegalovirus*, human immunodeficiency virus, *Mycobacteria*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Fusobacterium nucleatum*, and *Streptococcus mutans*) [28, 39–49]. The examples of microorganisms implied in the indirect [30, 40, 41, 50–63] and direct activation of the immunological system determining the plaque atherosclerotic burden are continuously increasing [30, 64–67], confirming the real necessity to deeply understand this topic and so compensate for the actual lack of knowledge about the basic mechanism of the microbiota's role in atherosclerosis [68–71].

Recent studies support the predominant role of the infection at the base of the inflammation load focusing on the outcomes of the actual available therapeutic solutions in lower-limb PAD, such as endovascular revascularization procedures and major vascular surgery. The influence of bacterial activity has been demonstrated in several unfavorable outcomes, such as the restenosis after arterial angioplasty [41, 50, 71–77] or any major adverse cardiovascular event (MACE) [30, 59, 78], which represent the first cause of exitus of patients affected by lower-limb PAD [79]. An interesting possible explanation has been proposed, defining the role of the bacterial atherosclerotic plaque colonization as an additional promoting factor of inflammation burden, after the angioplasty trauma-induced local inflammation [80–83]. The sum of the two stimuli determines the increased production of cytokines, the endothelial dysfunction, the induction of the foam cells, the proliferation and migration of the vascular smooth muscle cells (VSMCs), the powered tendency of platelets to aggregate, and the proinflammatory behavior of the perivascular adipose tissue (PVAT) [30, 40, 41, 51–58, 60, 62, 63, 84–86].

2.1. The Microbiome. Recent hypothesis supports a complementary role of microbiota as a constitutive component of the human organism rather than an inevitable and casual colonization from the environment around us. This complementarity has just been introduced and partially understood by the increasing studies that try to highlight the microbiome, the collection of microbial genomes, and the plasticity that completes our genomic feature [64]. The microbiome is the genetic characterization of the entire microbiota in a specific tissue [87]. Its crosstalk with the immune system modulates and regulates the immune response against the host [88]. In particular, the gut microbiome plays a fundamental role in this modulation for its location and microbiota. The gut microbial community is composed mainly of phyla *Bacteroides*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* [89], in different proportions. Interindividual variation is determined by a difference in the microbiome and also by environmental factors, such as lifestyle, diet, antibiotics, and drug use [90, 91]. This amount of genetic data

has been playing an unexplored role in the modulation of our metabolism pathways and in our pathologies, such as obesity [64, 92, 93] and diabetes [64, 94–99]. In the latter case, a different geographic origin influences the gut microbiome showing as similar metagenomes that could encode similar functions presenting a differently marked microbe species composition [64, 99]. The microbiome plasticity is directly influenced by influencing factors of the host itself, such as the intrapartum neonatal colonization through the vaginal canal transit, or totally host-independent factors, such as the change of diet from maternal milk to the introduction of solid food, the level of hygiene to which everyone differently has been exposed since birth, and the use of antibiotic therapy during lifetime [64, 100].

Although the microbiota becomes definitive and adult-like in the host at around three years of age [64, 101], the microbiome still changes through the epigenetic mechanisms that are induced by endogenous and exogenous factors [64].

3. The Microbiota and Microbiome in Type 2 Diabetes Mellitus

Evidence in animal and human models supports the hypothesis that obesity and T2DM are associated with a deep gut dysbiosis. Overnutrition could represent one of the main starting points to alter gut microbiota locally and to initiate systemic inflammatory processes through the mucosal barrier [102, 103]. Qin et al. performed the first metagenome-wide association study in T2DM using stool samples from Chinese patients with T2DM [98]. They found that T2DM patients had only a moderate degree gut bacterial dysbiosis. Functional annotation analyses, however, indicated a decline in butyrate-producing *Roseburia intestinalis* and *Faecalibacterium prausnitzii*, which may be metabolically beneficial, and an increase in several opportunistic pathogen levels. Another metagenome-wide association study was performed on T2DM and conducted in Europe on postmenopausal female patients with normal, impaired, or diabetic glucose regulation [99]. In this study, Karlsson et al. found that *Roseburia intestinalis* and *Faecalibacterium prausnitzii* were highly discriminant for T2DM, in contrast to the Chinese cohort. The authors suggest that the two studies were considerably different, not only for the different sequencing techniques used but also for ethnic and dietetic influences. Moreover, a previous smaller study found that T2DM patients showed higher levels of *Lactobacillus* species in comparison to nondiabetics [94], as showed by both Chinese and European studies. In addition, Zhang et al. found that normal subjects differed from patients with prediabetes with higher levels of *Faecalibacterium prausnitzii* and *Haemophilus parainfluenzae* T3T1, whereas *Verrucomicrobiaceae*, *Akkermansia muciniphila*, and *Clostridiales* sp. SS3/4 were less abundant [104]. The last result differs from the findings of Qin et al., which described a reduction of *Akkermansia muciniphila* in Chinese patients with diabetes. These results, however, suggest that patients with T2DM have evidence of gut dysbiosis. The reasons for the discrepancies may be determined by various confounding factors, such as different study populations, different sequencing techniques used, and different diets and drugs used [105].

Recent studies suggest that short-chain fatty acids (SCFAs), such as acetate, butyrate, and propionate, as well as the end products of fermentation of dietary fibers by the anaerobic intestinal microbiota, might constitute a link between the microbiota and systemic inflammatory diseases. In particular, butyrate seems to have a direct role in the development of extrathymic anti-inflammatory regulatory T cells [106]. Trompette et al. demonstrated that mice fed a high-fiber diet have an altered microbiota and are protected from allergic airway inflammation [107]. They showed that propionate regulated allergic inflammation, bone marrow hematopoiesis, and dendritic cell function. These findings suggest that metabolites produced by the gut microbiota influence hematopoiesis and immune responses in the lung. Thus, these microbiota-derived products might be important players in the generation of local and systemic immunity/inflammation. According to the studies mentioned before, the alteration on the production of SCFAs, especially butyrate, observed in T2DM patients, might have a key role in the development of low-grade inflammation [105].

Another important role in the development of a metabolic syndrome has been demonstrated for the pattern recognition receptor such as the toll-like receptor 5 (TLR5), a component of the innate immune system expressed in the gut mucosa and one that helps defend against infection [108]. TLR5-deficient mice exhibited hyperphagia and developed hyperlipidemia, hypertension, insulin resistance, and obesity, as well as an altered microbiota. Interestingly, the transfer of intestinal microbiota from TLR5-deficient mice to germ-free mice led to metabolic syndrome. These data support the crosstalk of gut microbiota with the innate immune system and suggest that the alteration of this link is critical in the development of the metabolic syndrome. In addition, studies show that gut-derived endotoxin—lipopolysaccharide (LPS)—might be involved in the chronic inflammation observed in T2DM. Cani et al. described that a high-fat diet (HFD) increased the LPS content of the gut microbiota and resulted in metabolic endotoxemia [95]. They observed that subcutaneous infusions of LPS into mice determined insulin resistance and obesity similar to that after feeding an HFD. Gut dysbiosis might increase LPS production by gram-negative bacteria and lead to metabolic endotoxemia and low-level inflammation that could contribute to the development of insulin resistance and T2DM [12].

3.1. The Microbiota in Atherosclerosis. An evident promoting role of microbes in a nonspecific inflammatory mechanism has been observed supporting an active participation of microbiota in systemic metabolic processes of the human body. At the base of this "inflammasome," there are several processes, such as an overproduction of proatherogenic mediators (C-reactive protein (CRP); interleukin 18 (IL18), IL1β, and IL6; and TNF-α), a hyperstimulated expression of adhesive molecules (vascular cell adhesion molecule 1 and intercellular adhesion molecule 1) [28, 30, 40, 41, 50, 85, 109], synthesis and release of growth factors and PVAT-derived adipokines, production of ROS, hormones

(corticosteroids and sex hormones), and free fatty acids, and a cytokine-related direct influence on the autonomic nervous system [28, 60, 62, 63]. The latter phenomenon is known as the neuroendocrine-immunitary crosstalk, which finally causes an homeostatic unbalance that initiates and promotes hypertension, insulin resistance, diabetes, altered levels of low-density lipoprotein- (LDL-) cholesterol, plasma triglycerides, high-density lipoprotein- (HDL-) cholesterol [28, 110–112], and a rise of oxidative molecules that determine the LDL-cholesterol oxidation, with a worsening of the atherosclerotic plaque instability and progression [28, 113, 114].

Bacterial colonization/infection of the vascular wall may contribute to the pathogenesis of atherosclerosis by the activation of a local, and eventually systemic, immunological response [115]. This process may involve each of the vascular wall layers (the intima, media, and adventitia) [28]. The main effect of a possible infection on the intima layer is the induction of endothelial dysfunction with a resulting dysregulation in vasomotor function, thrombotic complications, and initiation and progression of atherosclerosis [28]. There are several lines of evidence to suggest that bacterial infection activates platelets by a stimulatory effect on von Willebrand factor binding and factor VIII associated with a hyperfibrinogenemia state [116, 117].

The infection of the media layer may affect VSMC function and connective tissues that participate in the regulation of blood pressure, the vascular lumen, and the modulation of shear stress [84]. The adventitia layer is composed of adventitial compacta and adventitial fat, the aforementioned PVAT [118]. PVAT has recently been defined as the widest endocrine tissue that humans own [62]. It produces adipokines, hormones (corticosteroids and sex hormones), cytokines (TNF-α, IL6, and IL8), growth factors (visfatin, platelet-derived growth factor-BB, and transforming growth factor-β), and other substances such as ROS, nitric oxide (NO), hydrogen sulfide (H_2S), free fatty acids, and plasminogen activator inhibitor type 1 [28]. These substances regulate inflammation, vasoreactivity, and vascular VSMC growth, proliferation, and migration in the adjacent layers of the vasculature [84]. Bacterial infection may modify the PVAT functions [28].

An increasing amount of study evidences the association of periodontal bacteria, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Eikenella corrodens*, *Parvimonas micra*, and *Campylobacter rectu*s, and cardiovascular disease [33, 34, 119]. The study conducted by Tapashetti et al. shows higher CRP plasma level and a greater mean carotid intima-media thickness (c-IMT) value in patients with chronic periodontitis than in patients with healthy gums [119]. Kosaka et al. found that higher levels of salivary inflammatory cytokines were associated with periodontal disease. Among these, higher salivary IL6 and TNF-α were positively associated with both periodontal disease and intensity of carotid atherosclerosis [120]. In the case control study conducted by Chen et al., periodontal bacteria was in 13 of the 25 (52%) atherosclerotic samples obtained from patients with aortoiliac and/or femoropopliteal occlusive disease [121]. These results confirm that periodontitis increased fivefold the risk of having PAD and was associated with increased serum IL6 and TNF-α concentrations.

New evidences of the importance of microbiota are continuously found in the multifaceted human metabolic network as the proven reduction of the prevalence of *Eubacterium* and *Roseburia* in gut microbiota of patients who have already had an atherosclerotic symptomatic event, with an opposite pattern of prevalence for *Colinsella* [28, 64]. Moreover, a different gut microbiota composition was found in patients affected by diabetes [98] and atherosclerosis, giving the basis to hypothesize an atherosclerotic process induced by a possible gut microbiota dysbiosis [28, 122]. The discovery of the increased dimension and lipid content of the atherosclerotic plaques observed in mice fed with a hyperlipid diet [111, 123] is an interesting example of lipid trim imbalance mediated by the action of colonizing microbes. Chen et al. have demonstrated that levels of *Helicobacter pylori* immunoglobulin G (IgG) and serum IL18 were significantly higher in subjects with increased c-IMT [85]. This evidence suggests a positive association between *Helicobacter pylori* infection and subclinical carotid artery atherosclerosis mediated by IL18. In addition, the coronary atherosclerosis in patients affected by chronic heart disease with an infection of Cag A-positive *Helicobacter pylori* was explained by an infection related to the imbalance between lipid metabolism and LDL-cholesterol oxidation burden, the aggravation of which proved the progression and instability of the atherosclerotic plaque [28, 124]. The importance of this elegant crosstalk between microbiota and the host metabolism is clearly enhanced by a more aggressive disease phenotype observed in a specific group of patients, according to their microbial composition. Indeed, the presence of *Chlamydia pneumoniae* in blood and plaques of these patients has been defined as a promoter of hypercholesterolemia-induced atherosclerosis [111] that could be a possible cause of restenosis after an angioplasty procedure [28, 125, 126]. Another example of microbiotal influence on prognosis could be the evidence of a raised severity of stroke in *Chlamydia pneumoniae* seropositive patients with an increased c-IMT [124, 127, 128].

Several data suggest that infections resulting from periodontal or gut microbes have a direct influence on our endocrine system, on PVAT, and on pituitary-suprarenal action, with a possible derived imbalance of the autonomic sympathetic nervous system and metabolism homeostasis that could induce hypertension, insulin peripheral resistance, T2DM, increase of LDL-cholesterol and triglycerides, decrease of HDL cholesterol associated to an even more oxidative burden by ROS overproduction, and restenosis phenomenon [28, 84]. Moreover, a leaky gut phenomenon that allows a bloodstream translocation of bacterial fragments and a direct atherosclerotic plaque colonization [28, 64] could facilitate several processes, including neuroimmune crosstalk [28, 40, 41, 51–56, 58], macrophage-specific reverse cholesterol transport process modulation [28, 129–131], and the development of many diseases, such as obesity and T2DM [28, 64].

In addition, it was observed that diabetic patients have higher baseline plasma levels of LPS than the healthy control group and a low prevalence of butyrate-producing bacteria (e.g., *Roseburia* and *Faecalibacterium* spp.) known for anti-inflammatory abilities [64, 98, 99].

The atherosclerotic plaque peroxidation is essential for promoting LDL-cholesterol accumulation inside macrophage cells, which become foam cells. These cells promote the upregulation of inflammasomes created by an overproduction of cytokines [111, 132] and an overexpression of adhesive molecules [111, 133]. Specifically, it has been documented that *Porphyromonas gingivalis* plays a main role in the promotion of LDL oxidation and plaque instability and rupture caused by metalloproteinase, as an initiating and promoting factor of peroxidation [111]. *Porphyromonas* is also involved in the progression of abdominal aortic aneurism [28, 134] and in inducing endothelial activation or dysfunction through a state of systemic inflammation with cytokines and metalloproteinase [111, 135–140]. In support of this evidence, there is a suggestive experiment demonstrating the effect of *Porphyromonas gingivalis* injection in mice fed with a hyperlipidic diet, where an increase of the atherosclerotic plaque thickness and of its lipid content has been observed [111, 123]. Similarly, a hypercholesterolemia-induced atherosclerotic process has been found following the injection of *Chlamydia pneumophila* [111, 141].

Finally, obesity, a growing problem in modern society, shares part of its pathogenesis and natural history with the host-colonizing microbiota, finding several meeting points with microbial metabolic influence. A characteristic *Firmicutes*/*Bacteroides* ratio has been discovered in obese patients with a surprising restoration of the normal proportion or lean-like proportion, once patients experienced a loss of weight [64, 92].

Several evidences are enlarging our knowledge and beliefs about the unavoidable influence of microbiota and its metabolome on our metabolic system, and the comprehension of this complex network is an absolute priority to introduce new therapeutic means and preventive solutions to slow or even stop the progression of atherosclerosis and its clinical manifestations, such as the strongly disabling diseases like the lower-limb PAD.

3.2. Restenosis after Percutaneous Angioplasty: The Possible Role of Microbiota. Angioplasty proves to be one of the most effective nonmedical treatments in diabetic PAD of the lower limbs, with the erroneous belief of gaining the vessel lumen enlargement in stented arteries rather than a simple balloon-angioplasty procedure. A recent study has demonstrated a loss in the lumen enlargement of the arteries treated by endovascular revascularization stenting caused by a neointimal hyperplasia that progressively reduces the vessel lumen and determines a restenosis of the treated vascular segment [80].

The effect of this hyperplastic phenomenon is the in-stent restenosis, a local process caused by hypercellularity and a low apoptosis rate [142].

This evidence deserves a notable scientific resonance because, according to collected data, the gut-related systemic inflammatory burden could be implicated in the neointimal hyperplasia, with a possible involvement in the in-stent microbe colonization as a further promoting factor [142].

In addition, different innate anatomic-functional characteristics of the arterial samples obtained from different body districts (e.g., the coronary artery and internal iliac artery) have been observed, suggesting an emergent necessity for new target-specific endovascular revascularization procedures and major vascular surgery for the PAD-affected population, rather than a translation of nonmedical treatments from the better-known coronary district to a totally different scenario as PAD [72, 143, 144].

Finally, more and more bacteria, correlated to the inflammation in the atherosclerotic process at the base of the restenosis mechanism, are being found (e.g., *Helicobacter pylori*, *Chlamydia pneumophila*). This evidence could introduce new therapeutic solutions against the in-stent restenosis, such as the addition of a microbe-specific antibiotic to the already used antiproliferation factors added to stent devices (e.g., Rapamycin) [28, 76] or the adjacent extravascular tissue antibiotic injection therapy with an expected prevention of neointimal hyperplasia and consequently in-stent restenosis [28, 71].

4. The Metabolome: From Waste to Biomarker

Since medical researchers have focused on the metabolic products of human multisliced colonizing microbiota film, a new interesting scenario has been proposed. The role of metabolites in host inflammatory process modulation and, consequentially, in atherosclerotic clinical manifestations as PAD has been defined to be much more essential and incisive than the producing microbe itself [145, 146].

An increasing number of studies enrich the knowledge about the gut metabolome by studying tryptophan (trp), kynurenine/tryptophan (kyn/trp) ratio, indole sulfonate, p-cresyl sulfonate (PCS), hippuric acid (HA), indole-3-carboxaldehyde (i3a), indole 3-proprionic (i3p), H_2S, and phenylacetylglutamine and their influence on atherosclerotic phenomenon [147], like PAD in patients affected by a high grade of severe atherosclerosis, an end-stage disease characterized by an hemodynamic stenosis of carotid arteries aimed at an endarterectomy, disabling claudication, or critical limb ischemia undergoing an endovascular revascularization procedure or "demolitive" surgery with amputation in nonsolvable PAD [145]. Specifically, the tryptophan depletion determines an overactivation of the transduction signal of a stress pathway [145, 148] and an elevated value of the kyn/trp ratio is found in inflammatory statements, including infections with a proven positive relation to MACE. In addition, a low value of the kyn/trp ratio has been observed in germ-free mice with an interesting opposite tendency of this relation in case used for the first colonization of the same mice [145, 149, 150]. Data about the gut microbes' metabolic products are continuously developing with several examples of their effect on host homeostasis; for example, indole sulfonate has been observed to have an active role in VSMC dysfunction, vessel calcification, and thickening of arteries [145, 151]. It has been proven that PCS has a positive relation

with cardiovascular death [145], while HA has an influencing role on postvascular surgery cardiac events and also a partially demonstrated positive correlation with ankle-brachial index (ABI), an accepted approximation of the high grade of atherosclerosis in PAD-affected patients [145].

In support of the demonstrative data showing the growing role of gut microbe metabolites in initiating and promoting the PAD process, a negative relation between indole, trp, i3p, and i3a and a high grade of carotid stenosis, disabling claudication, and critical limb ischemia (CLI) has been defined [145]. Meanwhile, higher baseline plasma concentrations of 3-hydroxyanthranilic acid and higher kyn/trp ratio have been traced in the advanced atherosclerosis group, mostly accepted in populations affected by CLI undergoing amputation of the lower limbs, clearly suggesting how a plasmatic concentration of trp greatly reduces the predisposition and risk of progression to an advanced phase of disease [145]. Similarly, increased levels of indole, i3p, i3a, and HA are detectable in patients with a higher ABI index, while on the other side, a negative correlation has been observed between the ABI index and high levels of 3-hydroxyanthranilic acid and high kyn/trp ratio [145].

Trimethylamine N-oxide (TMAO) deserves a particular description and focus. Recently, it has been defined as an independent risk factor for MACE [129, 152–157]. Trimethylamine (TMA) is a gut microbiota metabolite originating from the microbial metabolism of choline and found in many kinds of food as free choline or as a part of several compounds, such as betaine, L-carnitine derived from food [152, 153], and ergothioneine found in mushrooms, beans, and the liver and kidney of animals [152]. After the absorption from the gut lumen and once circulating in the bloodstream, TMA reaches the host liver where hepatic flavin monoxygenase produces TMAO [152]. The interest on this metabolite is derived from the observed positive relation between high levels of TMAO and markedly increased risk of atherosclerosis [129, 152–154, 158, 159]. The plasmatic levels of TMAO are influenced by diet with a higher plasma concentration in the case of elevated-fat-content diet, western diet, and red meat consumption [152, 153, 160–168]. On the other hand, a lower determination has been detected in patients affected by chronic kidney disease who respect a low-protein diet [152, 169]. The glomerular filtration rate acts like a determining factor of TMAO plasma concentration with an inverse proportion; therefore, there is an increase of TMAO levels in the case of a reduction of renal filtration ability and a restoration of healthy patient-like levels after kidney transplant [152].

It has been demonstrated that many human gut-colonizing bacteria are able to produce TMA increasing the TMAO plasma concentration (*Streptococcus sanguinis*, *Desulfovibrio alaskensis*, *Desulfovibrio desulfuricans*, *Acinetobacter*, *Serratia*, *Escherichia coli*, *Citrobacter*, *Klebsiella pneumoniae*, *Providencia*, *Shigella*, *Achiomobacter*, *Sporosorcine* that belongs to *Firmicutes phylum*, *Actinobacteria* [152, 170]). In contrast, bacteria belonging to *Bacteroidetes* are not capable of producing TMA [152, 166].

The characteristics of TMAO justify the importance of improving our knowledge about this metabolite. In fact, several responsibilities on creating an imbalance of host homeostasis have been described, such as endothelial dysfunction, oxidative-stress status promotion, overexpression of proinflammatory cytokines, and a positive relation with elevated inflammation biomarkers, incidence of T2DM, and chronic kidney disease [152].

In addition, TMAO appears essential for explaining part of the lipid balance and the increase of scavenger receptors (CD36 and scavenger receptor class A type 1 (SR-A1)), contributing to the rise of fat accumulation inside foam cells, a fundamental event of atherosclerotic plaque progress [129, 152, 171]. Flavin-containing monooxygenase 3 (FMO3) has been declared the most active in converting the liver enzyme of TMA in TMAO, and its activity is positively related to higher plasmatic levels of TMAO. FMO3 activation and the derived high levels of TMAO are strictly linked to an alteration of reverse cholesterol transport [152, 172], to facilitated hyperglycemia and hyperlipidemia (defined as increased levels of very-low-density lipoprotein- (VLDL-) and LDL-cholesterol) [152, 159, 172], to overexpression of TNF-α, IL6, CRP [162, 167], and insulin resistance [152, 159], and finally to the promotion of atherosclerosis [129, 152–154, 158, 159]. Therefore, TMAO has been demonstrated to be an independent influencing factor of the imbalance between the host metabolism and the inflammatory process. Moreover, it has been defined as transmissible atherosclerosis susceptibility factor [160].

Further studies have been conducted to understand clinical implications of TMAO. In fact, elevated plasmatic levels of this metabolite have a predictive role for the of 5-year all-cause mortality in stable patients affected by PAD [161]. This evidence could result essentially in the establishment of a new prognostic measurable blood marker to improve the stratification assessment of patients by detecting who deserves specific dietary supplementation or pharmacologic therapy [152, 153, 173–175].

Moreover, TMAO has been seen to be a quite precise predictive factor of the future risk of MACE and increased incidence of stroke, myocardial infarction, and death [129, 152–156, 160, 176–178]. This metabolite also has a positive correlation with Syntax scores I and II (angiographic grading tools to determine the complexity of coronary artery disease, the high values of which are related to cardiac mortality and MACE in patients undergoing multivessel and, specifically, unprotected left main percutaneous coronary intervention) even after adjustments for traditional risk factors [160].

5. Therapeutic Intervention

One of the first therapeutic proposals that have been suggested for application in the clinical practice is oral tolerance induction with self-antigens capable of reducing the inflammatory burden caused by the cross-mimicry phenomenon [72, 179, 180]. Another interesting proposal, which is even more successful, consists in dietary supplementation of immune-modulator, anti-inflammatory, and antiangiogenetic molecules containing food, such as catechin and epigallocatechine-3-gallate found in green tea. Surprisingly,

a decrease of *Porphyromonas gingivalis*-related cytokine production in patients affected by periodontitis has been unveiled, giving discrete hopes in new therapeutic options to prevent and reduce the atherosclerotic process [181–185].

An additional possible new treatment could be fecal transplantation or the fragmented intestinal microbiota transplantation from lean healthy people that is described to be a hopeful treatment that reduces the insulin resistance and increases the butyrate producing microbiota [64, 186].

Furthermore, given the data regarding TMAO, the new therapeutic solutions could include a multifactorial reduction of TMAO plasmatic levels, for example, through targeting the gut microbiota TMAO producer [152] or the FMO3 enzymatic activity that reduces the conversion of TMA to TMAO [152]. An alternative option could be a change of dietary habits [152, 187], but it is not possible to reduce the carnitine and choline intake according to their nutritional importance [152]; therefore, it could be useful to encourage the consumption of marine fish which is rich in cardioprotective molecules such as ω3-polyunsaturated fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) that are implicated in the amelioration of impaired glycemic tolerance, in the reduction of adipose tissue-induced inflammation, in the reduction of monocyte chemoattractant protein-1 (MCP-1/CCL2), and in the increase of IL10 [152, 165].

Other possible solutions are the introduction of new effective prebiotics (all nondigestible food that stimulates the growth of beneficial bacteria) [152, 188] and probiotics (administrating specific bacterial strains such as *Lactobacillus paracasei*) [152, 189]. The use of antibiotics aimed at eliminating the TMAO producer microbes has also been proposed [152, 155]. Further therapeutic options are represented by the administration of an oral nonabsorbent binder to remove TMAO or its precursors [35, 170]; the inhibition of TMA precursors, for example, through 3,3-dimethyl-1-butanol (DMB) (contained in balsamic vinegar, red wine, extra virgin olive oil, and grape seed oil), that is an analogue of choline that competes and inhibits choline-TMA-lyase [152, 190]; and the inhibition of enzymes involved in TMA biosynthesis [152] using dietary supplements such as *Gynostemma pentaphyllum* [168] (an herbal product used in China to treat hyperlipidemia and obesity that is associated to a reduction of TMAO levels) or Gancao (the root of *Glycyrrhiza uralensis*) coadministered with a derivative of the *Aconitum carnichoelii* root [191]. Finally, it has been shown that enalapril is able to promote the renal excretion of TMAO [152, 175].

5.1. The Role of Antibiotics. The strong connection between the hosting organism and the colonizing microbiota has already been demonstrated and the scientific community continuously tries to collect new evidences about this crosstalk to find out new therapeutic ways and to manage the outcomes of the natural history of the disease.

The urgent necessity to fight a life-limiting disease such as PAD presents new challenges such as the achievement of cardioprotective therapeutic solutions through available sources. Nowadays, the use of available local modulators of gut microbiota, such as antibiotics and probiotics, has been demonstrated to be an effective protective factor for biologically different organs such as the cardiovascular system [192]. Mass spectrometry allows studying this revolutionary administration of exogenous influencing factors of microbes and the derived metabolome [192, 193] and permits comparing the metabolic paradigm/pattern between the examined case and the germ-free control [192, 194].

The oral administration of antibiotics and probiotics becomes the key to fully understand the role of human organism-colonizing microbes on our metabolic pathways. In fact, a direct modulation of the gut microbiome composition could indirectly determine an evident cardiovascular protective effect; on the other hand, the local injection of the same antibiotics in the coronary arterial circulation is associated to an ineffective cardioprotective outcome. In support of this evidence, during the trial of Lam et al., a group of mice premedicated with vancomycin alone or a combination of antibiotics (streptomycin, neomycin, bacitracin, and polymyxin B) showed a reduction of the necrotic myocardium after the induced coronary ischemia, compared to the control group treated with the same medication, directly injected in the coronary arterial circulation. Surprisingly, the administration of metabolites derived from phenylalanine, tryptophan, and tyrosine, at a sufficient concentration to restore the pretreatment serum levels, provokes the loss of the cardioprotective effect defined by the reduction of the size of the necrotized tissue area [192]. Notably, the used antibiotics are not absorbed and cannot reach the bloodstream, confirming the totally indirect cardioprotective mechanism. The direct effect on the gut microbe composition results in a reduction of *Clostridia* and a rise of *Bacilli* and *Proteobacteria* in the vancomycin-treated group, while it presents a reduction of *Bacilli* and no effect on *Clostridia* and *Proteobacteria* in the group treated with the mixture of antibiotics [192]. This evidence generates a new hypothesis: the cardioprotection mainly originates from the modification of the metabolome derived from the complex bacterial composition and interrelationship, rather than a specific *phylum*. It is possible that the interaction between circulating metabolites and cell surface receptors mutates the transduction signals of cellular survival pathways, leading to a worse cardiovascular outcome than the examined treated group, or they can be implied in the mitochondrial dysfunction worsening the evolution of the adverse event [192, 195].

Encouraging data have been derived from the comprehension of possible implicated cell signaling pathways, such as the Jak2 activity [192, 196]; the role of pyrazolopyrimidine on the Src family protein kinases [192, 197, 198]; the TGFβ-mediated response [192, 199]; the effect of the fungal metabolite Wortmannin on mammalian target of rapamycin (m-TOR), a member of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI-3) kinase superfamily; and other cellular transduction trails/paths worthy of further studies.

The established protective effect of orally administered antibiotics on remote organs such as the cardiovascular system demonstrated an effective reduction of the risk of restenosis or narrowing of the vessel treated with angioplasty,

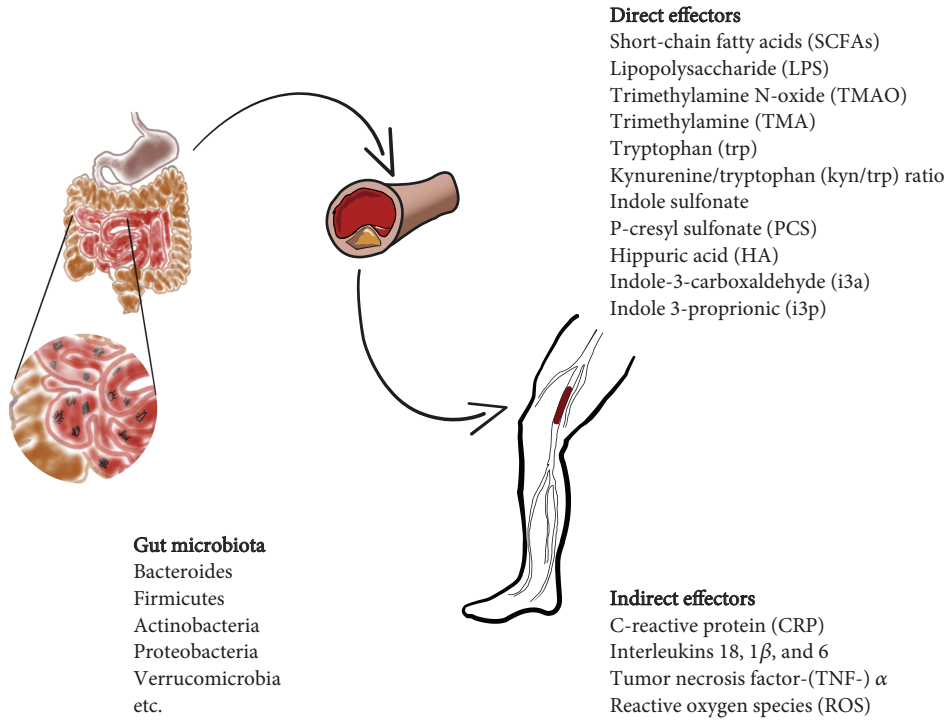

Figure 1: The different components of the intestinal microbiota (left) are able to worsen atherosclerosis at the base of the diabetic PAD by direct (top) and indirect (bottom) effectors.

stenting, or bypass with graft. The production of short-chain fatty acids by the colonic bacteria fermentation of fibers taken with diet influences the function of VSMCs, the principle responsible for the vascular restenosis phenomenon [142]. The future will rely on a standardized antibiotic or probiotic administration as a treatment and a protective factor against the failure of the endovascular revascularization procedure that actually remains the main therapeutic option in PAD of the lower limbs.

Furthermore, the oral administration of vancomycin affects the gut microbiota composition of the host, at the cost of a minimal systemic absorbance, showing a relative decrease of Gram-positive bacteria belonging to *Firmicutes phylum* and the reduction of the plasmatic level of sodium butyrate [142, 200, 201] and presenting an increase of the *Bacteroidetes*/*Firmicutes* ratio and a rise of the Gram-negative *Proteobacteria* [142]. The result is a relative decrease of sodium butyrate Gram-positive producers, with a proof of an expected major neointimal hyperplasia observed in the vancomycin-treated group and an abolition of this same effect if the plasmatic concentration of sodium butyrate would have been restored by dietary supplementation, although with the concomitant administration of vancomycin. This confirms the antiproliferative and antimigratory properties of sodium butyrate on VSMC [142].

5.2. Probiotics in Diabetic PAD. Probiotics could gain an important role in the medical treatment of diabetic PAD. The rationale behind their use is based on the effect of an oral supplementation of microbes that shows a direct modification of the gut microbiota composition, which could be a further intervention against the dysbiosis found in this kind of patients [68].

Probiotics are revealing a complemental action with the antibiotic therapy in the selection of a protective combination of colonizing microbes. In fact, their systemic influence appears effective in ameliorating the lipid profile imbalance by reducing cholesterol plasmatic levels, increasing LDL-lipoprotein resistance against the oxidation, and inducing a decrease of the onset of insulin resistance in diabetic controls [202–204].

The contemporary administration of ω3 fatty acids has further empowered the effect of probiotics on host metabolism with an excellent result on lipid control, insulin resistance, and inflammatory response [202, 205–207].

Many alternatives have already been suggested as a possible probiotic therapy, in particular the probiotic VSL#3 (VSL Pharmaceuticals Inc., Fort Lauderdale, FL) which is notably interesting. It contains three strains of *Bifidobacteria* (*B. longum*, *B. infantis*, and *B. breve*), four strains of *Lactobacilli* (*L. acidophilus*, *L. paracasei*, *L. delbrueckii* subsp. *bulgarius*, and *L. plantarum*), and one strain of *Streptococcus salivaris* subsp. *termophilus*. The results observed during the use of VSL#3 confirm the usefulness of its introduction in the pharmacological therapy of PAD, with a further empowerment of the beneficial effect obtained by the addition of ω3 fatty acids as dietary supplementation. By the administration of probiotic VSL#3, interesting modulations on host metabolism have been described, such as the modest reduction of IL1, TNF-α, and IL6, the main responsible cytokines of the

inflammatory atherosclerosis. Moreover, an increase of HDL cholesterol levels; a decrease of triglycerides, LDL, and VLDL lipoprotein levels; a decrease in fasting glycaemia and atherosclerotic index; and a marked modification of microbes in stool samples have been also demonstrated [202].

An effective risk factor control is at the base of the medical management of diabetic PAD, and the dietary supplementation of probiotics could appear as a new means to modify the natural history of this chronic, disabling, and progressive disease. It is necessary, however, to discover new combinations of supplementing microbes, focusing on their beneficial properties on systemic metabolism. It also important to respect the selection of the species contained in the probiotics, because there are strains of microbes that do not present an effective ability in the metabolic profile modulation, such as *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus acidophilus*, all of which have been observed ineffective in the improvement of serum lipid control [202, 208–210].

Moreover, the addition of prebiotics to the previously described probiotic treatment presented further interesting results, such as the reduction of plasmatic insulin with a consequential amelioration of the insulin resistance, a reduction of total and LDL cholesterol levels, and a reduction of triglycerides, accompanied by an elevation of HDL serum levels. In addition, an improvement of the inflammatory state has been described thanks to the decrease of CRP, IL1β, and TNF-α plasmatic concentrations. Moreover, in the symbiotic group, the one treated with probiotic and prebiotic supplementation, the count of *Lactobacilli* was higher, and the count of *Escherichia coli* and fecal coliform was lower [211].

A summary of the direct and indirect effectors involved in the connection between intestinal microbiota and PAD is reported in Figure 1.

6. Conclusions

In recent years, more and more evidences have documented the relationship between intestinal microbiota and diabetic PAD. The use of antibiotics is very frequent in patients affected by T2DM and PAD, since they often suffer from infected ulcers of the lower limbs. This could represent the first type of intervention: the choice of antibiotic therapies able to modulate, in one way or another, the intestinal microbiota represents an important objective. Furthermore, also the use of prebiotics and probiotics, useful for modifying the composition of the microbiota and the production of harmful metabolites, represents a further field of study. Finally, considering precision medicine, the study of a personalized therapy based on antibiotics, prebiotics, probiotics, and targeted diet could provide a new therapeutic instrument for the treatment of diabetic PAD.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors have read the paper and agree that it can be published.

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