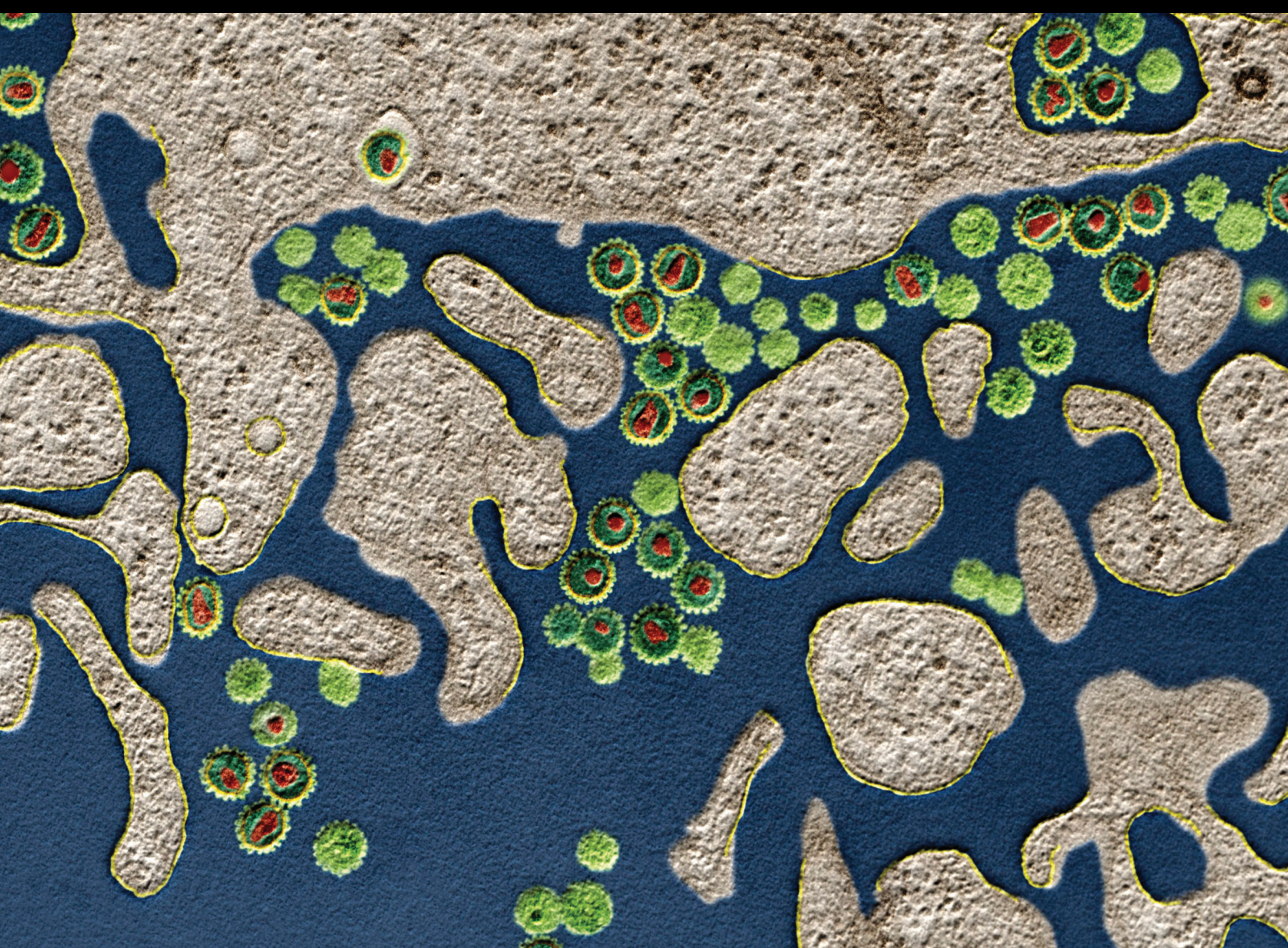
Journal of Immunology Research

Alteration of Immune-Mechanisms by Human Microbiota and Development and Prevention of Human Diseases

Lead Guest Editor: Mitesh Dwivedi
Guest Editors: Ansarullah, Ilian Radichev, and Elizabeth H. Kemp



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Editorial Board

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 6985256, 2 pages
https://doi.org/10.1155/2017/6985256

Editorial

Alteration of Immune-Mechanisms by Human Microbiota and Development and Prevention of Human Diseases

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Received 1 October 2017; Accepted 8 October 2017; Published 28 December 2017

The role of microbiota in regulating the immune system has gained much importance in recent years, since it plays a crucial role in disease development and prevention. The human body harbors 100 trillion commensal microbes, exceeding the number of host cells by more than 10-fold, with a huge amount of genetic information, that is, 100-fold more genes than the human genome. With this enormous pool of genetic information, the microbiota may influence human life at many levels including host immunity and its regulation. The immune mechanisms elucidating the molecular interactions between the microbiota and host immune system components have come up with new and interesting therapeutic approaches. Recent studies focusing on the interactions between microbiota and the host immune system have revealed the fundamental importance of the microbiome in shaping host immune responses, affecting susceptibility against immune-mediated and infectious diseases.

A total of 15 manuscripts were received for this special issue, and 9 manuscripts have been accepted for publication after rigorous rounds of review process. This issue provides a glimpse of some of the ongoing efforts in the area of immune regulation by microbiota and management of the human health. For example, some featured papers provide useful information regarding the role of microbiota in altered immune mechanisms in human diseases such as rheumatoid arthritis, multiple sclerosis, erythema nodosum, connective tissue diseases and vasculitides, and allergic asthma.

One of the interesting research articles presented by A. Fusco et al. explores the role of β-defensins in protecting the intestinal epithelium against *S. typhimurium* and their interaction with the gut microbiota. In particular, the experiments of coinfection with *S. typhimurium* and probiotic *E. faecium* showed that, in the presence of *E. faecium*, *Salmonella* infection caused a much less intense inflammatory response. Overall, the findings of this study propose that antimicrobial peptides (AMPs) could be considered, in the future, a new class of therapeutics since they are able to induce lesser resistance and have a selective antimicrobial activity to protect the host without the need for the immune system memory.

The research article presented by L. M. Rocha-Ramírez et al. reveals the innate immune mechanisms generated due to probiotic *Lactobacillus* strains. In particular, the study demonstrates that strains of *Lactobacillus* exert early immunostimulatory effects that may be directly linked to the initial inflammation of the response of human macrophages. The study further proposes that the effect of these probiotics as potential immunomodulators in immunocompromised hosts could be evaluated.

The research article presented by C. F. Michael et al. provides interesting insights into repair of epithelial damage in asthmatic airways by using prebiotic mannan (SC-MN) derived from *S. cerevisiae*. Unlike the mannan derived from bacteria, SC-MN does not stimulate Th17 inflammatory cytokines and the study reports that SC-MN showed an increased expression and activation of Krüppel-like factors (KLFs) 4 and 5, key transcription factors for epithelial cell differentiation, survival, and proliferation. Since anti-

inflammatory therapy alone has not been shown to halt disease progression in asthma, the study proposes a therapy that could promote epithelial repair and provide adjunctive therapeutic benefit for asthma. In this line, another research article by D. B. Lew et al. demonstrated the beneficial effects of SC-MN on allergic asthma mouse models. In particular, the study showed that the antismooth muscle (ASM) hyperplasia/hypertrophy in the mouse model due to allergen challenge can be preventable with SC-MN treatment. The study further concludes that the prebiotic mannose receptor blocker SC-MN is a promising agent that can render dual benefits in asthma, that is, anti-inflammatory and ASM remodeling at the level of both large and small airways. Another research article by O. Ozbagcivan et al. presents a retrospective descriptive study which examines the frequency and type of infections in the etiology of erythema nodosum (EN). The study identified, besides *Streptococci*, many other microbes including the ones living on and inside the human body to be of importance in the etiology of EN.

The current issue also involves interesting review articles. G. Horta-Baas et al. present a review article which provides some evidence linking intestinal dysbiosis with the autoimmune mechanisms involved in the development of rheumatoid arthritis (RA). The article reviewed various studies that have evaluated the influence of gut microflora on the etiopathogenesis of RA as well as RA severity. The article proposes that a specific or systematic manipulation of certain intestinal microbiota associated with host diseases could change therapeutic strategies in subjects with RA.

Further, G. Ranucci et al. present a review article on the role of early life intestine microbiota in the lungs' health in children. In particular, the review article discusses the recent development in gut-lung axis research, with emphasis on the effects of targeting microbiota of infants and children at a risk of or with progressive lung diseases. The article proposes that such knowledge could open new possibilities for therapeutic interventions in modifying the progression of chronic lung diseases as well as preventing its onset in population at risk.

The recent advances in sequencing technologies allowed for an unprecedented growth in the information about the diversity of bacterial species providing a valuable information how this diversity is associated with human health and disease. In this special issue, R. Talotta et al. review the latest information about the role of human microbiota in the pathogenesis of connective tissue diseases (CTDs) and vasculitides. This is a highly understudied field, and the authors discuss the potential links between dysbiosis and pathogenesis of inflammatory arthritides, such as systemic lupus erythematosus, systemic sclerosis, Sjögren's syndrome, and Behçet's disease. A more targeted evaluation of the impact of gut microflora on the pathophysiology of multiple sclerosis (MS) has been discussed by M. Adamczyk-Sowa et al. The results showed that the MS patients' intestinal microflora is characterized by moderate dysbiosis in combination with the protective effect of some *Bacteroides* metabolites against demyelinating in animal models, which suggests the existence of a very close interaction between the intestinal mucosa and the brain.

Thus, many previous and current reports suggest that there is a captivating relationship between microbiota and immune system. Although a few mechanisms of immune regulation by microbiota have been described, the exact role of microbiota in various diseases is still unknown. However, interventional studies are needed to elucidate such functional aspects of microbiota or their product's role in immune regulatory mechanisms. This knowledge will be of importance in order to optimize clinical studies and gain deeper mechanistic insights.

In summary, this special issue covers many important aspects of microbiota role in immune regulation and human health. We hope that this special issue can provide valuable information to investigators in the field of microbiota and immune regulation and also give the readers a sense of the advancements made in this field.

Acknowledgments

We would like to express our great appreciation to all the authors, who have quality submissions and provided useful insights into the current state of the field. We would also like to thank the special issue reviewers and editors, whose efforts substantially contributed to the improvement of the overall quality of this thematic issue.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 8450496, 5 pages
https://doi.org/10.1155/2017/8450496

Review Article

Early-Life Intestine Microbiota and Lung Health in Children

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Received 5 March 2017; Revised 3 July 2017; Accepted 20 July 2017; Published 21 November 2017

Academic Editor: Mitesh Dwivedi

The gastrointestinal microbiota plays a critical role in nutritional, metabolic, and immune functions in infants and young children and has implications for future lung health status. Understanding the role of intestinal dysbiosis in chronic lung disease progression will provide opportunities to design early interventions to improve the course of the disease. Gut microbiota is established within the first 1 to 3 years of life and remains relatively stable throughout the life span. In this review, we report the recent development in research in gut-lung axis, with focus on the effects of targeting microbiota of infants and children at risk of or with progressive lung diseases. The basic concept is to exploit this approach in critical window to achieve the best results in the control of future health.

1. Introduction

Clinical research in the last decades focused on host-microbe crosstalk especially at intestinal level raising the hypothesis that the gut microbiota is one of the key factors that determine host health. The immune and metabolic functions are influenced by the colonization of intestine by friendly bacteria [1] and a growing number of diseases derive from the gut microbiota composition. The change in microbiota structure often starts in the early life in children with or at risk of chronic diseases, such as cystic fibrosis [2, 3].

The microbiome is established within the first 1 to 3 years of life and remains relatively stable throughout the life span [4]. The neonatal period is a critical window for immune programming that affects the global health status for a lifetime, and the development of the gut microbiota may help predicting specific disease risk and disease progression or elimination of disease altogether [5].

In this review, we analyze the main factors and phases of early microbial imprinting, gut-lung axis in the progression of chronic lung diseases in children, and the manipulation of early-life gut microbiota for modifying history of lung diseases in children.

2. Factors Influencing Gut Microbiota Composition in Early Infancy

Environmental factors together with genetics and immune system activity influence microbial colonization pattern in newborns Table 1. The concept that the fetus is sterile has been reconsidered after detection of microbial traces in fetal and maternal compartments [6, 7]. It is now believed that there is a prenatal colonization with vertically transferred microbial material.

The type of delivery influences the pattern of colonization in infant gut. Vaginally delivered infants are colonized with bacterial communities from the mother's vaginal and intestinal tract. Infants born to cesarean section lack such exposure, leading to the first establishment of bacteria similar to the human skin microbiota [8]. In addition, the intestinal colonization by *Lactobacillus*, *Bifidobacteria*, and *Bacteroides* in infants born by cesarean section is delayed [9].

Table 1: Main factors and phases of early microbial imprinting.

(1) Prenatal (colonization with vertically transferred microbial material)
(2) Delivery (cesarean section versus natural)
(3) Feeding (human milk versus formula milk)
(4) Infections
(5) Antibiotics
(6) Intestinal environment (cystic fibrosis, short gut, celiac disease)

Infant feeding influences the gut microbial composition with long-lasting effects. Breastfeeding has been associated with a variety of long-term beneficial effects including lower incidence of obesity [10], diabetes [11], and allergies [12, 13]. Breast milk is the gold standard in infant nutrition, because it is species specifically adapted to the infant nutritional needs and functional to drive the development of the immune system. In addition to nutritional support, breast milk provides bioactive constituents that promote the growth of a wide and dynamic array of microorganisms [14]. Colostrum has higher microbial diversity than transitional and mature milk, and factors influencing the microbial community in breast milk also depend on the mother's nutritional status and the type of delivery.

An infant gut microbiota dominated by *Bifidobacteria* has consistently been associated with host health. The predominance of *Bifidobacteria* in breastfed infant stools was firstly found over 100 years ago, suggesting that breast milk contains specific molecules that stimulate the growth of these bacteria, defined as bifidus factors [15]. Today, compelling evidence show that prebiotics in breastmilk promote the growth of *Bifidobacteria*. The latter dominate the microbiota of breastfed infants, whereas formula-fed infants had higher proportions of *Bacteroides* and members of the *Clostridium coccoides* and *Lactobacillus* groups [16]. In addition, breastfeeding modulates innate immune response [17].

Antibiotics have a major impact on gut microbiome composition particularly in young infants. In the latter, the gut bacteria structure is very unstable, and the continuous and repeated use of antibiotics, especially if the antibiotic used is a broad-spectrum one, profoundly alters gut microbial structure with major late clinical consequences. Recent papers show that prenatal use of antibiotics is associated with an increased risk of subsequent asthma [18].

Of course, antibiotics are often necessary for certain conditions, but their use should be considered in the light of the new data summarized in Table 2. Russell and colleagues found that perinatal antibiotic use exerts highly selective modifications on resident gut flora, which in turn lead to very specific alterations in susceptibility to TH2- or TH1-/TH17-driven lung inflammatory disease [19]. A recent study found that children lacking four bacteria types were more likely to develop asthma, but the only significant environmental factor among these children was receiving more antibiotics in their first year of life than children with lower asthma risk [18]. Early infantile infections including common intestinal infections do change intestinal microbial structure with long-lasting effects [20].

3. Gut-Lung Axis in the Progression of Chronic Lung Diseases in Children

The gut and respiratory epithelia provide a physical barrier against microbial penetration, and colonization with the normal microbiota generates resistance to pathogens. The role of microbiota in lung homeostasis and immunity is supported by the poor outcomes of germ-free mice that were exposed to acute infections [21] and their susceptibility to allergic airway disease [22]. Dysbiosis in the gut has recently been linked to alterations in immune responses and to disease development in the lungs. In the last few years, chronic lung diseases, such as asthma and cystic fibrosis, have been investigated to evaluate the potential role of intestinal dysbiosis in their development. Primarily, exacerbations of chronic gut and lung diseases share key conceptual features with the dysregulation of intestinal microbial ecosystem.

Cystic fibrosis (CF) is a disease in which recurrent and chronic infection leads to a progressive decline of lung function and ultimately to death. In observational studies in humans, CF has been associated with aberrant microbial colonization of the intestinal and the respiratory tracts. These findings are the likely consequences of the loss of CF transmembrane conductance regulator (CFTR) function and the resulting altered microenvironment [23]. Mutations in CFTR fundamentally affect the airway and intestinal microenvironment and result in abnormal colonization pattern of microorganisms in patients with CF even in the absence of antibiotic exposure [24]. More severe CFTR allelic variants such as homozygous DF508 correlate with more significant alterations in gut microbiome pattern. Chronic gut inflammation is seen in CF even in the absence of overt gastrointestinal symptoms and is thought to be a driver of systemic inflammation, a hallmark of the disease [25]. The association between gut microbial colonization in early life and respiratory outcomes in patients with CF has been investigated, and breastfeeding was associated with delayed exacerbations, gut diversity, and prolonged periods of well-being and specific bacterial communities in the gut prior to respiratory complications [26]. Bacteria in the respiratory tract in CF originate from intestinal microbiota and are thought to contribute to the dynamic interactions between the host and microbial communities in CF [27].

Studies have demonstrated also a relationship between poor nutrition and the development of bronchopulmonary dysplasia in preterm infants who require prolonged supplemental oxygen therapy [28]. No data are available regarding gut dysbiosis in this setting.

Children developing asthma have a reduced gut microbial diversity in the first year of life as compared to healthy children [29]. In particular, delivery mode has been associated with wheezing and asthma until school age, mediated by specific gut bacterial groups [30]. The presence of beneficial bacteria, such as *Bifidobacterium longum*, and a reduction in *Bacteroides fragilis* [31] in the gut have been associated with a lower incidence of asthma. The relative abundance of the bacterial genera *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* seems significantly decreased in children at risk of asthma, with consequent reduced

Table 2: Gut microbiota alterations induced by antibiotic use.

Antibiotics	Gut microbiota alterations	Study	References
Ciprofloxacin	Decrease of the taxonomic richness, diversity, and evenness of the community	Healthy adult humans	[41, 42]
Amoxicillin Cefoperazone	Long-lasting alterations in the gut microbial community including a decrease in overall diversity	Young mice	[43]
Ampicillin Vancomycin Metronidazole Neomycin	Decrease of microbial diversity and colonization with antibiotic-resistant microbes	Young mice	[44]
Ceftriaxone	Gut microbiota dysbiosis	Adult mice	[45]

levels of fecal acetate and dysregulation of enterohepatic metabolites [31].

4. Manipulation of Early-Life Gut Microbiota in Lung Diseases in Children

Oral administration of probiotics and prebiotics or a combination of both (the so-called "synbiotic approach") could indirectly influence the composition of airway microbiota through the release of bacterial products or metabolites that reach the lung and promote the outgrowth of beneficial bacteria or directly, via microaspiration of the probiotic strain from the intestinal tract to the airways [32]. These mechanisms may restore a health-promoting microbiota and have a beneficial effect on the course of the disease. Moreover, few probiotic strains have a role in modifying the course of lung diseases. In animal studies, *Lactobacilli* have profound immunoregulatory effects on the lung, but the results of clinical trials in humans have been highly variable. Strain differences may in part explain the observed variability.

In humans, administration of *Lactobacillus* GG (LGG) negatively influenced the incidence of ventilator-associated pneumonia [33] and reduced respiratory infections in healthy as well as hospitalized children [34].

Asthma is a major potential target for probiotics, due to its frequency, related pathogenesis, and the lack of consistently effective preventive strategies. However, data on early intestine microbial manipulation are missing, because asthma develops progressively until school age, and the long-term follow-up of probiotic and prebiotic study is limited.

Manipulation of gut microbiota in CF by changing dietary content of indigestible carbohydrate and short-chain fatty acids, namely, butyrate, may improve undernutrition and play an anti-inflammatory effect on animals [35]. Restoration of gut microbiota by probiotics improves nutritional status, energy intake, and respiratory function in cystic fibrosis [35]. Three RCT concluded that probiotic administration in particular LGG and *Lactobacillus reuteri* has been related with a reduction of episodes of pulmonary exacerbations in children with CF [36–38]. The first evidence of the potential benefits of probiotic administration in CF came from a prospective randomized placebo-controlled crossover trial performed in two groups of patients with CF chronically colonized by *Pseudomonas aeruginosa*. Nineteen children were given LGG for 6 months followed by placebo (oral rehydration solution) for the subsequent 6 months. In parallel, 19 children were given the placebo during 6 months then the probiotic for the same period of time. The patients on LGG had a significant reduction of intestinal inflammation and of episodes of pulmonary exacerbations and hospitalization rates, with a decrease in IgG, suggesting that there is a relationship between intestinal and pulmonary inflammation. The intake of this probiotic was associated with a significant increase of the maximal forced expiratory volume in 1 second (FEV1) compared to the placebo as well as to a significant increase of body weight [36]. In another prospective randomized, double-blind, placebo-controlled study, 61 patients with CF were randomly assigned to receive 10^{10} colony-forming units *Lactobacillus reuteri* per day or placebo for 6 months. Pulmonary exacerbations and the number of upper respiratory tract infections were significantly reduced in the treatment group compared with the placebo group [39].

As for bronchopulmonary dysplasia, no studies on humans have been conducted with pre- or probiotics. Moreover, in intermittent hypoxia induced in animal models, the supplementation with probiotics may improve pulmonary status, by acting on the specific matrix metalloproteinases suggesting a beneficial effect on lung inflammation. In addition, prebiotic uses have been associated with arrested lung vascular endothelial growth factor signaling highly involved in lung microvascular development, suggesting preservation of angiogenesis [40].

However, the effects of probiotics are likely to be time-dependent, indicating the need for comparative clinical trials evaluating early-life microbiota modulation impact on lung health status. In particular, future studies are required in order to analyze how gut microbial composition in early life influence lung disease occurrence and history, investigating also the role of functional foods in preventing and/or modifying lung health status in children.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This paper has been produced with a grant support by the North American Cystic Fibrosis Foundation (Grant no. GUARIN10A0).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 6976935, 9 pages
https://doi.org/10.1155/2017/6976935

Research Article

Beta-Defensin-2 and Beta-Defensin-3 Reduce Intestinal Damage Caused by *Salmonella typhimurium* Modulating the Expression of Cytokines and Enhancing the Probiotic Activity of *Enterococcus faecium*

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Received 29 May 2017; Revised 9 August 2017; Accepted 5 September 2017; Published 9 November 2017

Academic Editor: Mitesh Dwivedi

The intestinal microbiota is a major factor in human health and disease. This microbial community includes autochthonous (permanent inhabitants) and allochthonous (transient inhabitants) microorganisms that contribute to maintaining the integrity of the intestinal wall, modulating responses to pathogenic noxae and representing a key factor in the maturation of the immune system. If this healthy microbiota is disrupted by antibiotics, chemotherapy, or a change in diet, intestinal colonization by pathogenic bacteria or viruses may occur, leading to disease. To manage substantial microbial exposure, epithelial surfaces of the intestinal tract produce a diverse arsenal of antimicrobial peptides (AMPs), including, of considerable importance, the β-defensins, which directly kill or inhibit the growth of microorganisms. Based on the literature data, the purpose of this work was to create a line of intestinal epithelial cells able to stably express gene encoding human β-defensin-2 (hBD-2) and human β-defensin-3 (hBD-3), in order to test their role in *S. typhimurium* infections and their interaction with the bacteria of the gut microbiota.

1. Introduction

The gastrointestinal tract is the most important immune organ of the human body. The intestinal surface has a strategic position at the interface between the antigenic luminal environment and the internal milieu of the host and is constantly exposed to various antigens from food or from different pathogens.

The human intestine hosts a large and diverse microbial community and contains approximatively 400–1000 different species of bacteria, virus, and fungi. These microbes are collectively referred to as the commensal microbiota.

The importance of the homeostatic maintenance of human health by the intestinal microbiota has become a topic of great interest [1–4]. Commensal bacteria modulate the expression of genes involved in several major intestinal and extraintestinal functions, including the xenobiotic metabolism, postnatal intestinal maturation, nutrient absorption, and fortification of the mucosal barrier, and inhibit the growth of pathogenic species through the production of antimicrobial substances. In addition, the human microbiota is involved in the synthesis of essential amino acids and vitamins (K, B2, B1, B6, B12, folic acid, biotin, and pantothenic acid) in the absorption of calcium, magnesium, and iron, in the extraction of energy from components in the diet, and in the regulation of fat storage [5, 6].

The genus *Enterococcus* is a group of lactic acid bacteria (LAB) whose use as probiotic microorganisms is controversial [7] as they are sometimes associated with infections in humans [8–11]. However, it has been shown that several

enterococcal strains, which may rebalance the intestinal bacterial flora in antibiotic-induced dysbiosis [12], can intervene in the antitumoral protective response [13] and can have antiviral activity [14].

Of great interest is *Enterococcus faecium*, for which the European Food Safety Authority (EFSA) has recently established new guidelines for distinguishing between beneficial or potentially pathogenic strains based on their susceptibility to ampicillin and on the presence of specific genetic markers of virulence (esp, hylEfm, IS16).

It has been demonstrated that the culture supernatant of the *E. faecium* strain in the human intestinal epithelial cells has a strong bactericidal effect on enteroaggregative *Escherichia coli*, including the induction of membrane damage and cell lysis [15]. The ability of these bacteria to produce enterocins is remarkable, and these instead can be applied as food biopreservatives [16, 17]. In fact, *E. faecium* RZS C5, a natural cheese isolate, has a strong activity against *Listeria monocytogene* adhesion and invasion of Caco-2 cells [18].

E. faecium SF68® (NCIMB 10415) is present in pharmaceutical preparations as a feed additive for different animals [19, 20], since it is capable of lowering the bacterial concentration of *E. coli* and stimulates an anti-inflammatory response [21].

Therefore, the human intestinal microbiota contributes to maintaining the integrity and impermeability of the intestinal wall, which represents the first line of defense against pathogens. Among these, *Salmonella enterica* serovar t*yphimurium* (*S. typhimurium*) is one of the most common nontyphoidal *Salmonella* (NTS) considered a major cause of acute food infection [22]. This Gram-negative bacillus can cause severe diarrhoea, vomiting, fever, and death in severe cases, especially in children, the elderly, and immunocompromised patients.

S. typhimurium can survive and replicate within host macrophages and induces the activation of NF-kB and the secretion of proinflammatory cytokines, such as interleukin- (IL-) 8 [23] and tumor necrosis factor alpha (TNF-α) [24]. This inflammation also helps it to compete with the microorganisms of the host microbiota [25].

Probiotics attenuate NF-kB activation and inflammatory cytokine production in the intestinal epithelial cells *in vitro* [26, 27] and *in vivo* [28–30].

In addition to serving as a protective barrier, the intestinal epithelium plays an active role in the intestinal immune response through the secretion of inflammatory cytokines, chemokines, and antimicrobial peptides such as β-defensins [31].

The family of β-defensins is composed of small cationic peptides produced by epithelial cells, Paneth cells, neutrophils, and macrophages, constitutive or induced by microorganisms or cytokines that contribute to the broad spectrum innate immunity.

Human β-defensin-2 (hBD-2) is an inducible antimicrobial peptide with a molecular mass of 4–6 kD and acts as an endogenous antibiotic in the defense against Gram-negative bacteria, among which the potential pathogenic microbes of the gut [32, 33], and can be induced by endogenous stimuli, infections, or wounds.

Human β-defensin-3 (hBD-3) is identified in psoriatic scales [34] and is expressed in the skin, placenta, and oral tissue [34, 35] and shows antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. Being insensitive to high salt concentrations, its antimicrobial activity results to be greater than that of hBD-2 [36].

Both hBD-2 and hBD-3 are chemoattractants for neutrophils [37] and memory T-cells, induce histamine release from mast cells and prostaglandin synthesis, and play a role also in allergic responses.

In the light of the growing interest of the use of antimicrobial peptides as natural defense molecules against pathogens and due to the increased antibiotic resistance by a number of pathogenic bacteria, this study aims to create a line of intestinal epithelial cells expressing high concentrations of the antimicrobial peptides hBD-2 and hBD-3 and to assess their role in the host inflammatory response resulting from bacterial infections.

2. Materials and Methods

2.1. Cloning. Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics) from primary cultures of human keratinocytes stimulated with the LPS of *Pseudomonas aeruginosa* and TNF-α in order to obtain a high production of antimicrobial peptides. It was subsequently transcribed into complementary cDNA using random hexamer primers (Random hexamers, Roche) at 42°C for 45 minutes, according to the manufacturer's instructions. Two pairs of degenerate primers, designed on their specific amino acid sequence (hBD-2 for 5′-CCAGCCATCAGCCATGAGGGT-3′, hBD-2 rev-5′-GGAGCCCTTTCTGAATCCGCA-3′ 254 bp; and hBD-3 for 5′-CGGCAGCATTTTGCGCCA-3′, hBD-3 rev 5′-CTAGCAGCTATGAGGATC-3′), were used to amplify, by RT-PCR, gene coding hBD-2 and hBD-3 with FastStart High Fidelity (Roche Diagnostics). The amplification programs were the following: 35 cycles at 94°C for 1′, 63°C (for hBD-2) or 58°C (for hBD-3) for 1′, and 72°C for 1′; the PCR products were 254 and 206 base pairs.

The amplified DNA fragments were subjected to restriction and sequencing analysis and cloned into the pEF/V5-HIS TOPO (Invitrogen) vector using the T4 DNA ligase (Invitrogen), in accordance with the manufacturer's protocol, and then transformed into *E. coli* TOP 10 (Invitrogen).

The cloning vectors, pEF/V5-HIS TOPO-hBD-2 and pEF/V5-HIS TOPO-hBD-3, were extracted from the bacterial culture and amplified using a QIAprep Spin Midiprep Kit (QIAGEN).

2.2. Transfection. Caco-2 cells were transfected using the IBAfect reagent (IBA), according to the manufacturer's manuals. Briefly, 3×10^5 cells were seeded in 6-well plates, and immediately after seeding, plasmids conjugated with the transfection reagent were added. The mixture was incubated for 24 and 48 hours. After incubation, the success of the experiment was verified by the extraction of mRNA from

Table 1: Primer sequences and amplification programs.

Gene	Primer sequence	Conditions	Product size (bp)
IL-6	5′-ATGAACTCCTTCTCCACAAGCGC-3′ 5′-GAAGAGCCCTCAGGCTGGACTG-3′	5″at 95°C, 13″ at 56°C, and 25″at 72°C for 40 cycles	628
IL-8	5′-ATGACTTCCAAGCTGGCCGTG-3′ 5′-TGAATTCTCAGCCCTCTTCAAAAACTTCTC-3′	5″at 94°C, 6″ at 55°C, and 12″at 72°C for 40 cycles	297
IL-1β	5′-GCATCCAGCTACGAATCTCC-3′ 5′-CCACATTCAGCACAGGACTC-3′	5″at 95°C, 14″ at 58°C, and 28″at 72°C for 40 cycles	708
TGF-β	5′-CCGACTACTACGCCAAGGAGGTCAC-3′ 5′-AGGCCGGTTCATGCCATGAATGGTG-3′	5″at 94°C, 9″ at 60°C, and 18″at 72°C for 40 cycles	439
IL-1α	5′-CATGTCAAATTTCACTGCTTCATCC-3′ 5′-GTCTCTGAATCAGAAATCCTTCTATC-3′	5″at 95°C, 8″at 55°C, and 17″at 72°C for 45 cycles	421
TNF-α	5′-CAGAGGGAAGAGTTCCCCAG-3′ 5′-CCTTGGTCTGGTAGGAGACG-3′	5″at 95°C, 6″ at 57°C, and 13″at 72°C for 40 cycles	324

treated cells and by the amplification of hBD-2 and hBD-3 genes by PCR.

Cell-free supernatants of the transfected cells were recovered by centrifugation and assayed for the hBD-2 and hBD-3 concentration by an enzyme-linked immunosorbent assay (Phoenix Pharmaceuticals Inc.).

For blasticidin selection, untransfected and transfected cells were cultured at 37°C and 5% CO_2 for 14 days in the presence of the following increasing concentrations of blasticidin S (Sigma-Aldrich): 5, 10, 20, 50, 100, and 200 μg/ml. Then, MTT-labelling reagent was added at a final concentration of 0.5 mg/ml. After 4 hours, a solubilization solution was added to each well and the plates were incubated overnight. Spectrophotometric absorbance was measured using a microplate (ELISA) reader at a wavelength of 570 nm.

2.3. Bacterial Strains. S. enterica subsp. *enterica* serovar *typhimurium* (ATCC® 14028GFP™) was cultured on Luria-Bertani agar (Oxoid, Unipath, Basingstoke, UK). *E. faecium* (ATCC 27270™) was cultured on Bacto Tryptic Soy agar (TSA, Difco Laboratories). These strains were grown at 37°C for 18 h.

2.4. Cell Culture and Infection. Caco-2 cells (human Caucasian colon adenocarcinoma cells) were routinely cultured in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 1% Penstrep, 1% glutamine, and 10% fetal calf serum (Invitrogen) at 37°C at 5% CO_2. After transfection, the cells were grown in a sterile 75 cm^2 flask at a concentration of 3×10^5 to confluence for 21 days to reach full differentiation and polarization. The culture medium was changed every two days.

Subsequently, fully differentiated cells were seeded into six-well plates and then infected with exponentially growing bacteria at a multiplicity of infection (MOI) of 100 for 6 hours (for gene expression analysis) and 24 h (for ELISA assay) at 37°C in 5% CO_2 in DMEM without antibiotics. In the case of coinfection, preincubation of one hour with *E. faecium* was followed by the addition of *S. typhimurium* without the removal of the probiotic bacterium.

At the end of the experiment, bacteria present in the supernatants of infected and coinfected cells were counted (CFUs) by spreading serial dilutions on selective medium HiCrome™ *E. faecium* Agar Base (Sigma-Aldrich) and Brilliance *Salmonella* agar (OXOID) and were incubated at 37°C overnight.

2.5. Real-Time PCR. In order to evaluate the expression of pro- and anti-inflammatory cytokines, the cells at the end of treatments were washed three times with sterile PBS, and the total RNA was extracted using High Pure RNA Isolation Kit (Roche Diagnostics).

Two hundred nanograms of total cellular RNA were reverse transcribed (Expand Reverse Transcriptase, Roche) into complementary DNA (cDNA) using random hexamer primers (Random hexamers, Roche) at 42°C for 45 minutes, according to the manufacturer's instructions [38]. Real-time PCR for IL-6, IL-8, TNF-α, IL-1α, IL-1β, and TGF-β was carried out with the LC FastStart DNA Master SYBR Green kit using 2 μl of cDNA, corresponding to 10 ng of total RNA in a 20 ml final volume, 3 mM MgCl2, and 0.5 mM sense and antisense primers (Table 1). After amplification, melting curve analysis was performed by heating to 95°C for 15 s with a temperature transition rate of 20°C/s, cooling to 60°C for 15 s with a temperature transition rate of 20°C/s, and then heating the sample at 0.1°C/s to 95°C. The results were then analyzed using LightCycler software (Roche Diagnostics). The standard curve of each primer pair was established with serial dilutions of cDNA. All PCR reactions were run in triplicate. The specificity of the amplification products was verified by electrophoresis on a 2% agarose gel and visualization by ethidium bromide staining.

2.6. ELISA Assay for Pro- and Anti-Inflammatory Cytokines. Caco-2 cell monolayers were infected with *S. typhimurium* and/or *E. faecium* for 24 h at 37°C, as described above. At the end of the experiment, supernatants were harvested and the presence of cytokines IL-6, IL-8, IL-1β, TNF-α, and TGF-β was analyzed by enzyme-linked immunosorbent assay (ELISA, ThermoFischer Scientific Inc.).

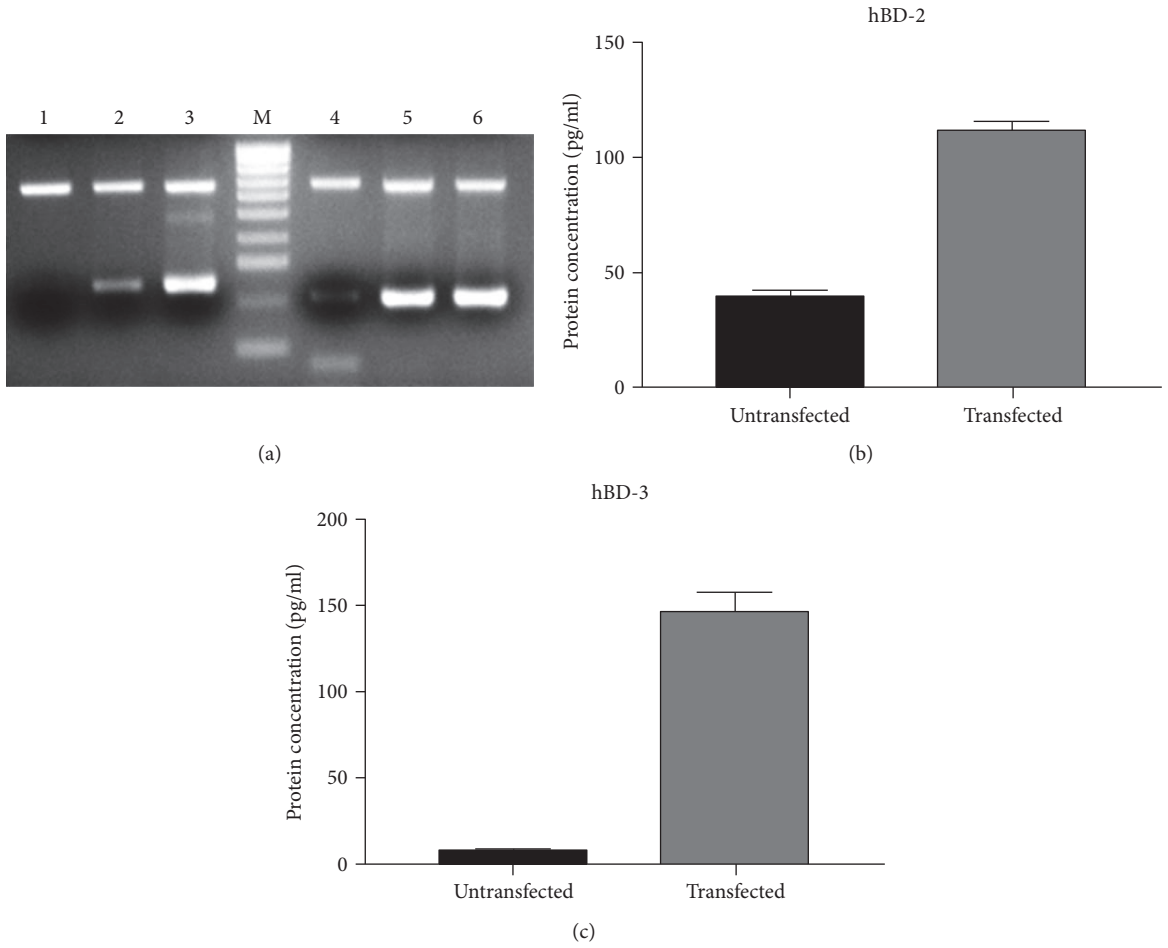

Figure 1: (a) hBD-2 mRNA expression in untransfected cells (lane 1), 24 hours (lane 2), and 48 hours (lane 3) after transfection; hBD-3 mRNA expression in untransfected cells (lane 4), 24 hours (lane 5), and 48 hours (lane 6) after transfection. (b) hBD-2 concentration in cell supernatants 48 hours after transfection. (c) hBD-3 concentration in cell supernatants 48 hours after transfection.

2.7. Bacterial Internalization Assay. Untransfected Caco-2 cell cultures were infected with *S. typhimurium* alone or coinfected with *S. typhimurium* and *E. faecium* as previously described. In another set of experiments, *E. faecium* was heat killed by incubating at 60°C for 45 min and subcultured on TSA plates (Difco Laboratories) overnight at 37°C to prove that no viable organisms remained. Killed bacterial preparation was resuspended in DMEM without antibiotics and added to cell monolayer an hour before the addition of *S. typhimurium*. After 2 h of incubation at 37°C, infected monolayers were extensively washed with sterile PBS and further incubated for another two hours in the DMEM medium, and supplemented with gentamicin sulphate (250 μg ml-1) (Sigma-Aldrich) in order to kill the extracellular bacteria. At the end of the experiments, infected monolayers were extensively washed in PBS then lysed with a solution of 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 minutes at room temperature to count internalized bacteria. Aliquots of cell lysates were serially diluted and plated on Brilliance Salmonella agar (OXOID) and incubated at 37°C overnight to quantify viable intracellular bacteria (CFUs/ml). The efficiency was calculated as the ratio of the number of cell-internalized bacteria with the number of bacteria used to infect the cell monolayers.

2.8. Statistical Analysis. Significant differences among groups were assessed through two-way ANOVA by using GraphPad Prism 6.0. The data are expressed as means ± standard deviation (SD) of three independent experiments.

3. Results

3.1. Cloning and Transfection. The hBD-2 and hBD-3 genes were successfully amplified by RT-PCR from a total cellular RNA. As expected, the PCR products were 254 and 206 bp in length. These products were inserted with high efficiency in the pEF/V5-HIS TOPO vector.

The success of transfection of the cloning products in colorectal adenocarcinoma Caco-2 cells was verified after 24 and 48 hours by RT-PCR and after 48 hours by ELISA assay on cell supernatants (Figure 1).

3.2. Blasticidin Selection and Cellular Viability. The toxicity curve performed on transfected and untransfected cells

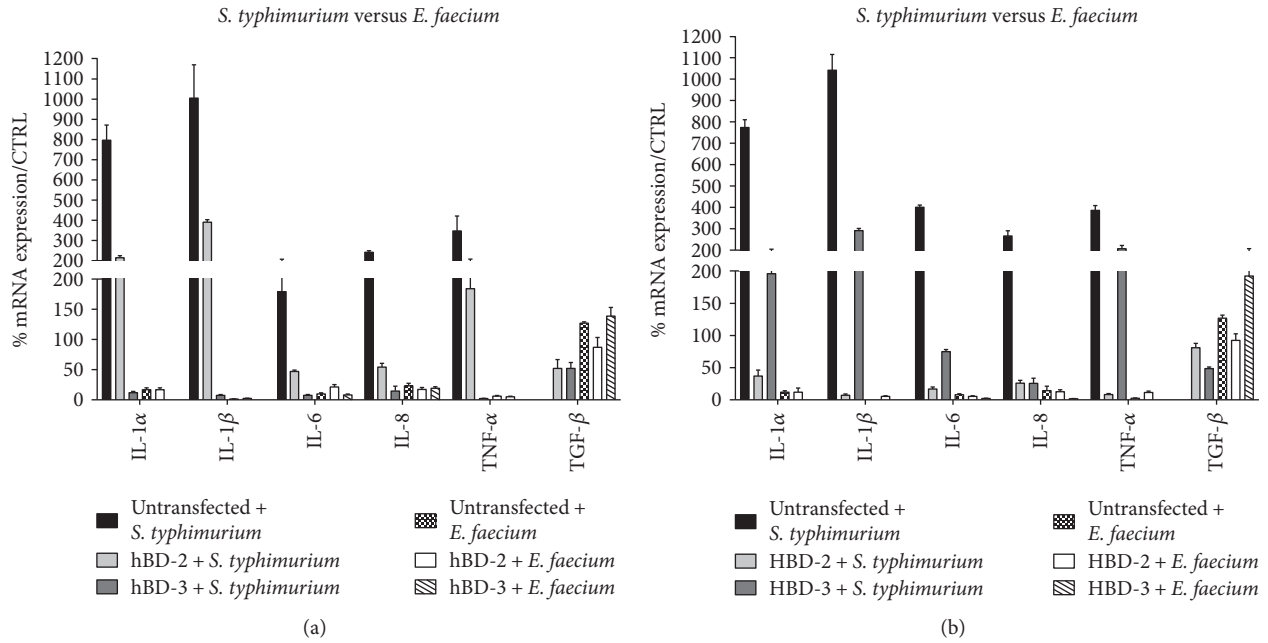

Figure 2: Comparison between relative gene expression (a) and protein concentration (b) in Caco-2 cells infected with *S. typhimurium* and Caco-2 cells infected with *E. faecium*. Data are mean ± SD and are expressed as the percentage of increment compared to uninfected controls.

showed that the optimal antibiotic concentration for the selection of stable clones was 200 μg/ml. These data were also supported by the results of the cellular viability assay (see Supplementary Material available online at https://doi.org/10.1155/2017/6976935). The selected clones were then cultured for an additional 21 days to obtain their differentiation, which was characterized by polarization and the formation of microvilli.

3.3. Evaluation of the Host Inflammatory Response. After the infection of untransfected and hBD-2- or hBD-3-transfected Caco-2 cells with *E. faecium* and/or *S. typhimurium*, we examined the host response by evaluating the expression of proinflammatory cytokines IL-6, IL-8 Il-1α, and IL-1β and anti-inflammatory cytokine TGF-β by real-time PCR.

The data obtained showed that the cells transfected with the hBD-2 and hBD-3 genes and infected with *S. typhimurium* showed a lesser expression of proinflammatory cytokines compared to the untransfected control. Instead, an infection of Caco-2 cells with *E. faecium* resulted only in a slight increase of expression of proinflammatory cytokines and an increase in anti-inflammatory cytokine TGF-β, which was more apparent in the presence of antimicrobial peptides; these data confirm that *E. faecium* did not act as a pathogen and did not induce an increase in the inflammatory response (Figure 2).

In addition, during the coinfection with *S. typhimurium* and *E. faecium*, the already significant decrease in expression levels of proinflammatory cytokines revealed in the transfected cells during infection with *S. typhimurium* alone is even more pronounced, indicating that the antimicrobial peptides have enhanced probiotic antibacterial activity (Figure 3).

These data were also confirmed by ELISA protein assay.

3.4. Evaluation of Bacteria Viability. In order to test the toxicity of antimicrobial peptides against *S. typhimurium* and *E. faecium*, the supernatants of the coinfected cells were subjected to serial dilutions and plated on selective media.

Our results indicate that both hBD-2 and hBD-3 possess selective toxicity towards *S. typhimurium* and did not interfere with the growth of *E. faecium* (Table 2).

3.5. Effect of E. faecium and AMPs on S. typhimurium Invasiveness. Preincubation of untransfected Caco-2 cells with live *E. faecium* significantly affected *S. typhimurium* internalization, reducing it by 45.8%. Conversely, pretreatment with heat-killed *E. faecium* does not interfere with the invasive capacity of the pathogen (Figure 4).

4. Discussion

Innate immunity, in particular through antimicrobial peptides (AMPs), plays a key role in maintaining the balance between protection against pathogens and normal microbial tolerance; AMPs are structurally heterogeneous peptides of amphipathic nature isolated from a wide variety of organisms, plants, insects, amphibians, and mammals that are able to kill bacteria, fungi, and viruses quickly. Among these, the human β-defensins have received considerable interest. These peptides are produced by epithelial cells, constitutively, or as a result of certain stimuli such as microorganisms or cytokines. Defensins are able to attract inflammatory cells such as neutrophils, T cells, macrophages, and epithelial cells capable of releasing inflammatory mediators such as IL-6, IL-8, and IL-1β, as well as destabilizing microbial membranes; moreover, they have the ability to remodel the tissues and bind LPS.

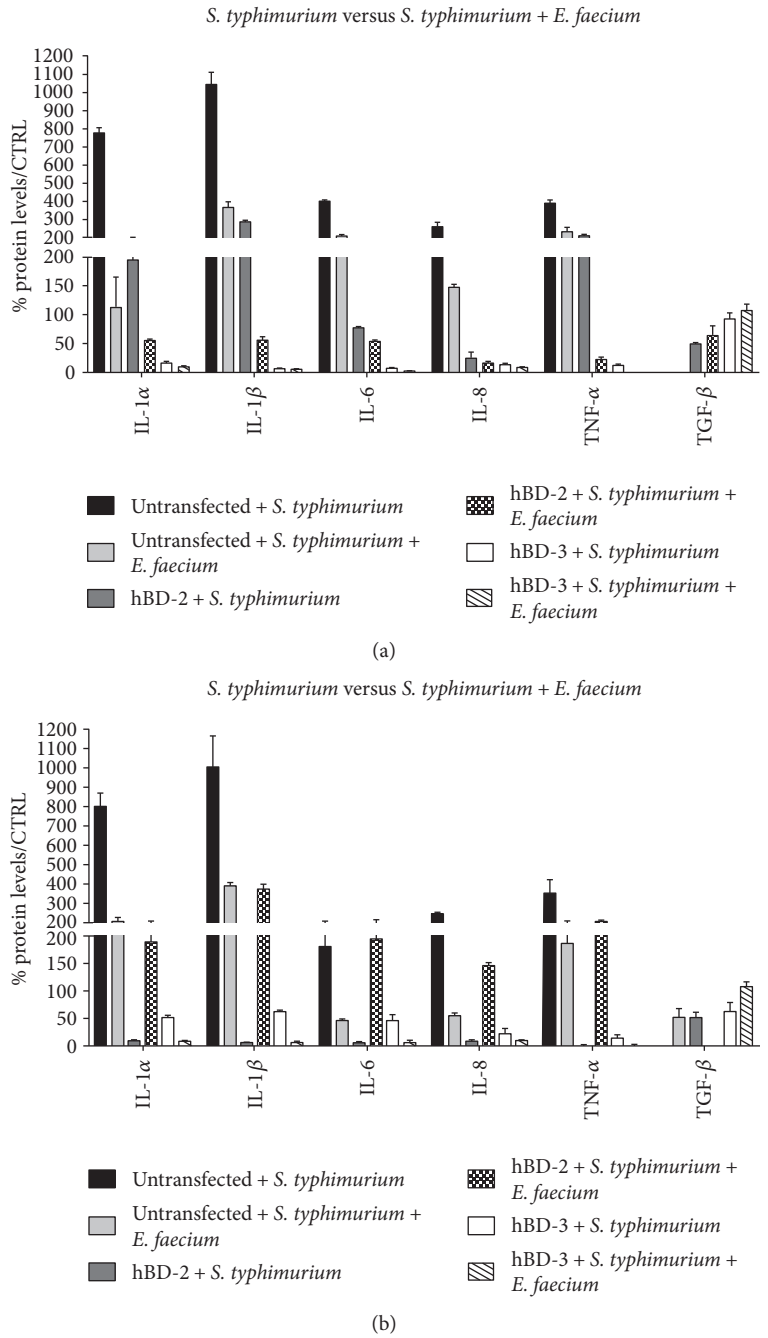

Figure 3: Comparison between relative gene expression (a) and protein concentration (b) in Caco-2 cells infected with *S. typhimurium* alone and Caco-2 cells coinfected with *S. typhimurium* and *E. faecium*. Data are mean ± SD and are expressed as the percentage of increment compared to uninfected controls.

In particular, β-defensin-2 (hBD-2) and β-defensin-3 (hBD-3) are present in various epithelia, such as skin [39], oral cavity [40], paranasal sinuses, gingival [41], corneal [42], intestinal, respiratory, and urogenital epithelium [31], and show antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi.

It has been estimated that the number of microbes present throughout the human body amounts to approximately 100 trillion cells, tenfold the number of human cells, and suggested that they encode 100-fold more unique genes than our own genome [43]. Most of them are components of the gut microbiota, which contains between 1000 and 1150 prevalent bacterial species that play a central role in human health [43, 44].

This community is defined as a "metabolic organ," as it plays a primary role in maintaining homeostasis by intervening in the regulation of metabolism and nutritional, physiological, and immunological functions.

In the first phase of this work, we worked on creating, by cloning and gene transfection techniques, a line of

Table 2: CFUs/ml of *S. typhimurium* and *E. faecium* in supernatants of coinfected cells.

	Inoculum	Untransfected	hBD-2-transfected	hBD-3-transfected
S. typhimurium	1×10^7	2×10^6	5×10^4	$4{,}3 \times 10^3$
E. faecium	3×10^8	3×10^8	3×10^8	2×10^8

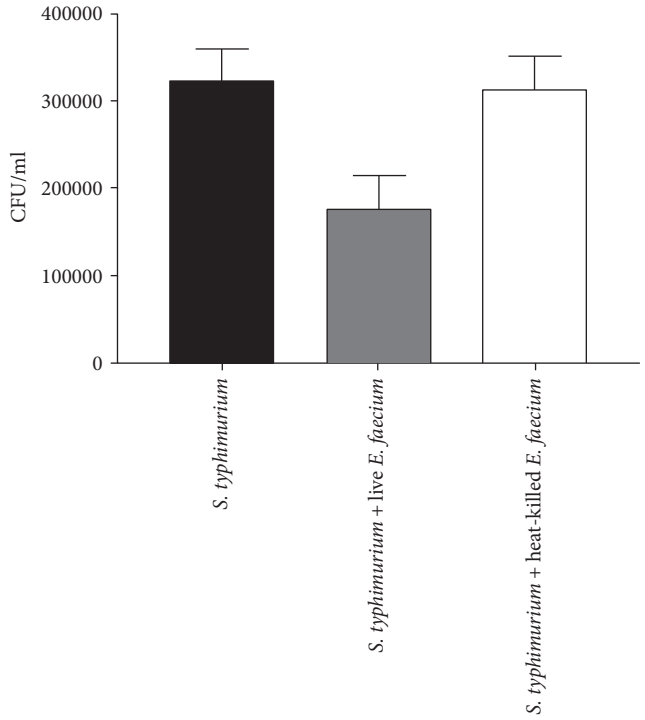

Figure 4: *S. typhimurium* internalization assay. Untransfected Caco-2 cells were infected with *S. typhimurium* alone or coinfected with live or heat-killed *E. faecium* for 4 hours. The number of internalized bacteria was determined by host cell lysis, plating, and counting CFU/well. The data shown are representative of three different experiments ± SD. Error bars represent standard deviations.

intestinal epithelial cells (Caco-2 cells) that expresses hBD-2 and hBD-3 genes. This allowed us to evaluate the role of these peptides in protecting the intestinal epithelium against *S. typhimurium*, alone or in cooperation with *E. faecium*, which is one of the major components of the human gut microbiota [45, 46].

The first data obtained from the CFUs/ml counts following infection and coinfection showed that there was a marked reduction in the number of colonies of *S. typhimurium* compared to untransfected cells in transfected cells, while the number of colonies of *E. faecium* remained unchanged, which shows that the antimicrobial peptides selectively carried out their microbicidal activity against the pathogen.

Mucosal surfaces are lined with epithelial cells that form a barrier between potentially pathogenic microorganisms and the host tissues. Penetration of this layer by invasive bacteria initially leads to an acute inflammatory response, a hallmark of which is the local accumulation of polymorphonuclear leukocytes. After the infection with pathogenic bacteria, epithelial cells at mucosal surfaces can secrete chemical mediators, such as proinflammatory and chemoattractant cytokines constituting surveillance and warning system for the immune and inflammatory cells present in the underlying mucosa [47]. However, in sites where there is a physiologically high bacterial concentration due to the resident microbial flora, that is, the colon, cytokine production is closely dependent on bacterial invasiveness, as only invasive bacteria induce cytokine secretion [48–50].

Among these, IL-6, IL-1, and TNF-α are highly expressed in most inflammatory states so as to often be considered a target of therapeutic intervention.

IL-8 chemokine is also thought to be an early signal of acute inflammation, as it is secreted by the intestinal epithelial cells following bacterial invasion, and accumulates in the mucosa underlying the epithelial cell layer where the IL-8 responsive effector cells reside. In addition, it has been shown that the presence of the IL-8 in serum is a diagnostic marker for neonatal bacterial infection [51, 52].

The results obtained show that the inflammatory response in hBD-2- and hBD-3-transfected cells is modified with respect to untransfected cells, since the expression of proinflammatory cytokines IL-6, IL-8, TNF-α, IL-1α, and IL-1β is greatly reduced, while the expression of anti-inflammatory cytokine TGF-β is increased. These data indicate that the invasive and inflammatory potential of *S. typhimurium* is significantly reduced in the presence of antimicrobial peptides.

Experiments of coinfection of untransfected cells with *S. typhimurium* and probiotic *E. faecium* showed that in the presence of *E. faecium*, *Salmonella* infection caused a much less intense inflammatory response, and this data is confirmed by invasive assays in which the presence of *E. faecium* results in a reduction in the internalization of *S. typhimurium* by 45.8%. However, the more interesting result is that the decrease in the level of inflammatory response due to the presence *of E. faecium* is further reduced in the transfected cells, that is, in the presence of high concentrations of antimicrobial peptides, suggesting that antimicrobial peptides may enhance the beneficial probiotic activity.

In our experimental system, the ability of AMPs to significantly reduce the inflammatory response in infected and coinfected cells is also due to their killing activity against *Salmonella*, as also demonstrated by the count of CFUs/ml following coinfection, in which the concentration of pathogen is considerably reduced in the presence of AMPs with respect to untransfected cells. AMPs could be considered, in the future, as a new class of therapeutics since they are able to induce lesser resistance and have a selective antimicrobial activity to protect the host without

the need for the immune system memory [53]. Having an *in vitro* system that will produce these proteins will allow us to better clarify the mechanisms underlying these different behaviors.

Conflicts of Interest

The authors declare that they have no conflict of interest regarding the publication of this article.

Acknowledgments

This study was supported by the MIUR, Project PON03PE_00060_3.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 4835189, 13 pages
https://doi.org/10.1155/2017/4835189

Review Article

Intestinal Dysbiosis and Rheumatoid Arthritis: A Link between Gut Microbiota and the Pathogenesis of Rheumatoid Arthritis

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Received 23 April 2017; Revised 17 June 2017; Accepted 12 July 2017; Published 30 August 2017

Academic Editor: Mitesh Dwivedi

Characterization and understanding of gut microbiota has recently increased representing a wide research field, especially in autoimmune diseases. Gut microbiota is the major source of microbes which might exert beneficial as well as pathogenic effects on human health. Intestinal microbiome's role as mediator of inflammation has only recently emerged. Microbiota has been observed to differ in subjects with early rheumatoid arthritis compared to controls, and this finding has commanded this study as a possible autoimmune process. Studies with intestinal microbiota have shown that rheumatoid arthritis is characterized by an expansion and/or decrease of bacterial groups as compared to controls. In this review, we present evidence linking intestinal dysbiosis with the autoimmune mechanisms involved in the development of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a systemic, inflammatory, and chronic disease characterized by a persistent immune response that leads to inflammation and destruction of joints. The etiopathogenic mechanisms involved are complex and include an interaction between the innate and acquired immune response, involving antigen-presenting cells (APCs), autoreactive T cell formation, and production of autoantibodies directed against their own cellular structures, like rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPAs). These antibodies are often present in the blood, long before any sign of joints' inflammation, suggesting that the triggering of autoimmunity might occur at different sites to the joints, for example, the gastrointestinal tract or airway [1]. Epidemiological studies suggest rheumatoid arthritis as the result of complex interactions between genes, environmental and hormonal factors, and the immune system [2, 3].

There is a genetic susceptibility to rheumatoid arthritis by a tendency to familial aggregation, a concordance between monozygotic twins and an association with some histocompatibility antigens [4, 5]. Heritability estimates have suggested a 60–70% genetic risk factors, responsible for developing rheumatoid arthritis [6]. Candidate gene or genome-wide association studies have identified different

risk loci associated with rheumatoid arthritis etiology. Currently, in this disease, about 100 described genes are associated with susceptibility, protection, severity, activity, and treatment response [6]. Human leukocyte antigen (HLA) polymorphisms are the most important genetic risk factors. HLA are an important part of the immune system triggering T cells of the immune system to produce antibodies. Associations of RA with HLA-DRB1 alleles have been observed in all racial and ethnic populations [7, 8]. The shared epitope (SE), a 5-amino acid sequence motif in positions 70–74 of the HLA–DRβ chain, is the most significant genetic risk factor for rheumatoid arthritis [9]. Some SE alleles, such as HLA-DRB1*0401, appear to confer a higher risk than others; moreover, the presence of two SE alleles and in particular HLA-DRB1*0401/*0404 confers a high risk to develop the disease and has also an influence on disease severity [10]. SE alleles are associated with ACPA-positive rheumatoid arthritis, but only relatively weakly with ACPA-negative rheumatoid arthritis [8]. SE alleles might contribute to the genetic predisposition to rheumatoid arthritis causing an immune dysregulation (controlling both specificity and amount of ACPA production) or a premature immunosenescence [10].

In genetically disease-susceptible individuals, subsequent environmental triggers might induce rheumatoid arthritis development. The bacterial and viral components are an attractive source of antigens capable of inducing rheumatoid arthritis and, therefore, have been the most investigated as potential causal agents [3]. However, there is no conclusive evidence to date of a causal relationship of a microorganism with rheumatoid arthritis.

In recent years, characterization and understanding of this gut microbiota has increased and constitutes a wide research field, especially in autoimmune diseases. The gut microbiota is the major source of microbes that may exert beneficial as well as pathogenic effects on human health [11]. Encouraged by studies that show alterations in intestinal microbiota composition in autoimmune diseases, such as rheumatoid arthritis, the interest of studying microorganisms as potential candidates in the development of autoimmunity has been renewed [11–14].

Findings supporting the idea that the onset of autoimmunity may be related to gastrointestinal tract are as follows: (1) microbial composition in subjects with early rheumatoid arthritis differs to controls, with a reduction of certain bacteria belonging to the family *Bifidobacterium* and *Bacteroides* [15, 16], and a marked increase of species belonging to the genus *Prevotella* [17]. (2) In murine models, the parenteral injection of cell wall fragments from various intestinal bacteria is arthritogenic [17] and in this model, arthritis is not developed when bred in germ-free conditions; otherwise, it is presented when intestinal bacterial species are introduced [18]. (3) Diet has been shown to influence inflammatory activity levels [19]. (4) Some drugs used to treat rheumatoid arthritis have antimicrobial effects (chloroquine, sulfasalazine, minocycline, and roxithromycin) [20–23]. (5) Altered microbiome was partially restored to normality in patients showing clinical improvement after prescribing disease-modifying antirheumatic drugs [5, 18]. So, differences in composition of intestinal microbiota and in the immune system function could determine which patients develop the disease.

A great effort is currently being made to study subjects with rheumatoid arthritis in preclinical phase. Awareness of the mechanisms that initiate the autoimmune process, as well as the ones involved in the transition from pre- to clinical phase, might guide to intervention strategies which allow its prevention or treatment in very early stages of the disease. Some publications suggest that rheumatoid arthritis early treatment leads to better long-term outcomes and perhaps to increased rates of drug-free remission [24]. Prevention concept is an emerging research field in rheumatology area, in which modifications of the microbiota could be a new way of modulating the disease.

2. Immunopathogenesis of Rheumatoid Arthritis

Understanding the complex molecular processes that play a role in the pathogenesis of rheumatoid arthritis is still a challenge. Susceptible individuals under genetic and environmental factors with loss of immunological tolerance to self-antigens trigger the autoimmune phenomena and the autoantibody formation [25]. This lack of immunological tolerance represents the first step towards autoimmunity. Immune system dysregulation is characterized by the presence of autoantibodies and autoreactive T cells. The inappropriate generation of autoreactive B cells is the most obvious alteration of the immune system in these patients; they are detected long before the disease appears. The most important are RF and ACPAs that recognize different proteins in the citrullinated form [26]. Together with increased autoantibody production, proinflammatory cytokines' level is elevated in the joint synovium of patients with rheumatoid arthritis. The joints of patients with RA are complicated tissues where innate and adaptive immune cells along with joint resident cells, like synoviocytes and chondrocytes, are involved [27].

Multiple cell types have been identified to contribute to the pathogenic context in rheumatoid arthritis. In rheumatoid synovium, dendritic cells are found mainly in lymphocytic aggregates and peripheral vessels, suggesting that they come from peripheral blood. MHC alleles are expressed by APCs that processed extracellular peptides to $CD4^+$ T cells, driving the secretion of proinflammatory cytokines that stimulate B cells to produce antibodies [25]. Patients with the disease present a defective function of circulating regulatory T cells (Treg) and an increase in T helper 17 (Th17) cells in plasma and synovial fluid [28]. Macrophage-derived and dendritic cell-derived transforming growth factor β and interleukin-1β, 6, 21, and 23 provide a milieu that supports Th17 differentiation and suppresses differentiation of regulatory T cells, thus shifting T cell homeostasis towards inflammation [29].

Posttranslational modifications (PTMs) are critical for the function and antigenicity of proteins. The three PTMs primarily involved in rheumatoid arthritis are glycosylation, carbamylation, and citrullination [25]. Citrullination results

Table 1: Natural history of rheumatoid arthritis.

Phase of initiation of the disease (interaction between genetic-hormonal-environmental factors)			Preclinical RA	Clinical RA
Genetic and epigenetic factors	Hormonal factors	Environmental factors	*Immunological changes*	*Immunological changes*
Shared epitope, PTPN22, STAT4, CTLA4, TRAF1, PADI4, FCRL3, TNFIP3 DNA methylation Dysregulated histone marks	Relationship man : woman 4 : 1 Arthritis is improved in the pregnancy but relapse in the postpartum	Microbiota oral, pulmonary and intestinal Smoking Silica dust Obesity Diet	Inadequate response to peptides Expansion of autoreactive T cells and B cells Expansion of antibody isotype usage and class switching Changes in soluble cytokine and chemokine networks Altered Th17 cells and Th17/regulatory T cell ratios	Upregulation of signalling molecules Immune-mediated tissue inflammation Alterations of autoantibodies, such as glycosylation Cellular expansion
			Clinical manifestations	*Clinical manifestations*
			Presence of autoantibodies (RF, ACPAs) Nonspecific symptoms	Arthritis Bone erosions Systemic symptoms
Forms of intervention				
Suspension of smoking Avoid exposure to silica Healthy diet Maintaining an adequate weight Modifications of the microbiota?			In research, the early use of rituximab or abatacept Modifications of the microbiota?	Anti-inflammatory Biological and nonbiological disease-modifying drugs Glucocorticoids

from the conversion of arginine onto citrulline by the enzyme peptidyl arginine deiminases (PAD), and this is the major crucial posttranscriptional modification associated to self-antigen recognition in rheumatoid arthritis [25]. Citrulline may alter protein structure and generate new epitopes associated with the production of ACPAs. ACPAs present in rheumatoid arthritis patients show differing fine specificities and cross-reactivity degrees with various citrullinated and/or posttranslationally modified peptides/proteins, including fibrinogen, fibronectin, α-enolase, collagen type II, and histones [30].

Table 1 summarizes the relevant aspects in the evolution of this disease. Preclinical rheumatoid arthritis comprises the period in which the autoimmunity is detectable until the beginning of the inflammation and/or injury of clinically apparent tissue, genetic and environmental risk factors interact, probably sequentially, to initiate and propagate the development of autoimmunity, finally resulting in detectable tissue inflammation and lesion [24, 31]. If any other risk factor is involved in the onset and/or spread of the disease, it is still unknown.

ACPAs' response might be important to transition from preclinical phase to clinical expression of rheumatoid arthritis. ACPAs' repertoire analysis prior to diagnosis in patients with rheumatoid arthritis revealed that this immune response starts in a very restricted manner and expands to several months or even years (epitope spreading, from one initially recognized epitope towards reactivity to many different epitopes) before the diagnosis of rheumatoid arthritis [10, 24, 32, 33]. Epitope spreading towards more citrullinated ones is compatible with the possibility that a single antigen (but not always the same) is responsible for starting up the immune response [16, 32]. Sokolove et al. [33] reported that earlier identified autoantibodies were targeted against various ligands of the innate immune response including citrullinated histones, fibrinogen, and biglycan. Over time, the ACPAs' titers and epitope diversity of ACPAs increase, especially before arthritis onset. ACPAs could be isotypes IgG, IgA, or IgM with an altered glycosylation status that confers enhanced Fc-receptor and citrullinated antigen binding [34]. ACPAs themselves can be pathogenic by activating either macrophages or osteoclasts via immune complex formation and Fc-receptor engagement or probably by binding membrane citrullinated vimentin, thus promoting bone loss [34].

Since the original description of antibodies as citrullinated antigens in a subpopulation of rheumatoid arthritis patients, it has become clear that citrullinated epitopes of a large number of autoantigens as well as antigens derived from microorganisms can be recognized by highly specific antibodies for rheumatoid arthritis [30]. Alterations at specific mucosal sites suggest that microbial factors might affect mucosal immune response, also playing an important role in early pathogenesis of rheumatoid arthritis [35]. Alterations in compositional diversity and abundance levels of microbiota, that is, dysbiosis, can trigger several types of autoimmune and inflammatory diseases through the imbalance of T cell subpopulations, such as Th1, Th2, Th17, and Treg cells [27].

Dysbiosis in one or more mucosal sites leads to immune alterations and breaks in self-tolerance to citrullinated autoantigens [35]. Mucosal body surfaces such as respiratory and gastrointestinal tract carry out complex tasks as they must (1) remain tolerant against innocuous environmental, nutritional, and microbial antigens to ensure organ function and (2) set efficient immune responses against invading

pathogens [36]. Lung and gut tissues contain immunological cells able to initiate an immune response; an attractive possibility is that the triggering of T cell-mediated immunity to citrullinated autoantigens can occur in the mucosae after presentation of neoantigens by APCs [1, 31]. In mucosal site, possible roles for citrullinated microbial antigens and molecular mimicry, Toll-like receptor (TLR) signals, and other innate immune activators and danger signals might exist [35]. Mucosa-associated bacterial flora and smoking or environmental particles (silica dust) act on immune cells (neutrophils, dendritic cells, and macrophages) as pathogen-associated molecular patterns and damage them, leading to inflammation onset, circulating cytokines, and chemokine increase along with autoantigen production. The citrullinated antigens are processed and presented by the APCs to the T cells, which are activated and in turn activate the B cell, leading to the production of autoantibodies [31]. Autoantigens in rheumatoid arthritis are not tissue- and organ-specific but comprise a large collection of posttranslational modified proteins [31]. Smoking and other stimuli might initiate citrullination by PAD activation, formation of lymphoid structures that could enhance antigen presentation, and T and B cell production [31]. Potential mechanisms by which cigarette smoke (CS) promotes rheumatoid arthritis include release of intracellular proteins from reactive oxidative substances activated or injured cells, augmentation of autoreactive B cell function, and alteration of (a) many cell signaling pathways involved in cellular activation, (b) cigarette smoke-impaired antigen-presenting cells, (c) regulatory T cell functions, and (d) T cell activation by antigens found in cigarette smoke [37].

3. Microbiota and the Immune System

Microbial exposures in gastrointestinal and respiratory tracts are key determinants of the overall immune tone at these mucosal barriers and represent a leading target for future intervention strategies [36]. Gut is an entryway for various environmental antigens in the form of food or infectious agents. Intestinal microbiota is a factor influencing metabolic homeostasis and the immune system [5]; it is a site of remarkable interaction between microorganisms and human body. Microorganisms establish a symbiotic relationship with epithelial and lymphoid tissue [12, 25]. Intestinal bacteria synthesize and change a variety of compounds that affect physiology and immunity. However, not all host-microbiota interactions promote health, particularly species of resident bacteria seem to activate the immune system resulting in inflammatory diseases [38, 39]. A diverse and balanced microbiota is necessary to develop an appropriate immune response [40].

Benefits provided by gut microbiota to the host rely on intricate interactions with host cells [41]. Studies using germ-free and gnotobiotic animals, colonized with defined bacteria, provided direct evidence about microbiota's crucial role in development and maintenance of the host immune system [41] and preservation of its functions such as maturation of intestinal lymphoid tissue, secretion of immunoglobulin A, and the production of important antimicrobial peptides [42]. In axenic mice, scarce growth of lymphoid tissue and alterations in T cells and subpopulations of B lymphocyte development were observed; in some cases, these mice did not develop diseases presented in ordinary subjects, probably due to defects in the adaptive immune system in the absence of the microbiota, rather than to the absence of microorganisms per se. Conserved molecular patterns, either expressed on the surface of symbiotic bacteria or secreted in the intestine, may interact with pattern recognition receptors (PRRs), which are expressed on or within epithelial and lymphoid cells to initiate transduction and transcription of signals from a set of molecules that mediate host defense or metabolic activities within the gut [40].

Intestinal commensal microbiota have been shown to modulate T cell and Treg responses that are required for effective host defense against pathogens while circumventing autoimmune responses and other immunopathologic consequences [43]. As the first line defense of host against pathogens, innate immune responses rely on a family of receptors known as PRRs including TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs). TLRs are key innate immune receptors to perceive pathogen-associated molecular patterns (PAMPs), which are specific pathogenic "molecular signature." Subsequent to sensing microbial PAMPs, TLRs enable the initiation of inflammatory responses and eventually eliminate the pathogenic invaders [43]. Components of gram-positive and gram-negative bacteria interact with TLRs to mediate both innate and adaptive immunity, as well as other cellular functions of the mucosal barrier [40]. Epithelial cells have TLRs on their cell membrane, which allow the recognition of PAMPs and the activation of MyD88 coupling protein-mediated signaling that ends with the induction of an inflammatory response and the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6, or interleukin-1β. The innate immune response cells of the lamina propria constantly examine the contents of the intestinal lumen for foreign antigens and constitute another defense mechanism [28]. Because commensal bacteria differ in their ability to stimulate receptors for innate immunity (TLRs and NLRs), the pattern of released chemical mediators varies significantly by determining proinflammatory or anti-inflammatory responses. Lipopolysaccharide of gram-negative bacteria binds to TLR-4, whereas peptidoglycan and other components of the cell wall of gram-positive bacteria signal via the TLR-2 pathway generating an immune response [11]. Gram-positive anaerobic bacteria contain a greater amount of polysaccharides and peptidoglycans that may act as antigenic stimuli.

After interaction with an antigen, dendritic cells play an important role in the differentiation of immature $CD4^+$ lymphocytes into Th1, Th17, or Th2 cells. The differentiation of the T helper cells seems to be deeply influenced by the intestinal microbiota [11, 16, 42]. Dendritic cells act as APCs by displaying charged peptides in their MHC class II molecules. Presentation of these molecules to B cells or to T cell receptors sensitizes these cells to initiate an adaptive immune response [28]. In rheumatoid arthritis, dendritic cells could participate in maintenance of inflammatory process by

regulating antigenic presentation, and the presentation of the antigen or the arthritogenic antigens would then be abnormally prolonged, which might favor the perpetuation of inflammation. Macrophages and dendritic cells continuously detect intestinal lumen antigens and evaluate the presence of deleterious antigens. Antigens are presented by MHC II molecules and interact with B cells or T cell receptors to induce adaptive immune responses. Depending on the microbial antigen, a specific cytosine medium is then generated to influence a specific type of T helper cell differentiation. While type 1 T helper (Th1) cells develop in response to intracellular pathogens and produce interferon, both type 2 T helper (Th2) cells and Th17 cells are stimulated by extracellular microorganisms. Th17 cells contribute to the defense against extracellular pathogens by the production of IL-17 and IL-22, which induce the change of immunoglobulin class in B lymphocytes and the production of Reg3g by epithelial cells, respectively [14]. The immune response generated by effector T cells is regulated by the subpopulation known as regulatory Treg cells. Intestinal Treg cells play an important role in maintaining immune tolerance to dietary antigens and gut microbiota [44]. Treg cells retain tolerance to self-antigens and eliminate autoimmunity. $CD4^+CD25^+$ Treg cells are suppressive cells, which express the transcription factor Foxp3, and are indispensable for maintenance of immune self-tolerance and homeostasis by suppressing aberrant or excessive immune response. *Lactobacillus* and *Bifidobacterium infantis* exert an anti-inflammatory effect through the induction of $CD4^+CD25^+FoxP3^+$ Treg cells [11]. *Bacteroides fragilis* polysaccharide A acts as an immunomodulator and stimulates $CD4^+$ Treg cells through an interleukin-2-dependent mechanism to produce IL-10 [11].

Taking into account the fact that dendritic cells are fundamental in generating immune response, it has been hypothesized that commensal bacteria influence the function and differentiation of dendritic cells, thus modulating immune response [11, 23]. Thus, intestinal dysbiosis can induce arthritis by influencing the differentiation of T cell subgroups. It also influences the expression degree of Toll-like receptors of antigen-presenting cells and may contribute to an unbalance in the Th17/Treg cell ratio. With the development of Th17 cells, activating local inflammatory cascades with tissue damage and in predisposed individuals, this local immune response can lead to systemic autoimmunity with self-reactive Th17 cells.

The microbiota are the most prominent influence from the environment on the differentiation of Th17 cells. Recently, much attention has been paid to segmented filamentous bacteria (SFB) because of its ability to induce the production and activation of Th17 cells in the intestine, with the secretion of interleukin-17 [23, 27]. SFB comprise a group of Clostridia-related gram-positive bacteria that adhere closely to Peyer's plaques in the small intestine and can stimulate the immune response by inducing IgA secretion and activating B cells. These bacteria are necessary for the development of autoimmunity in the murine K/BxN arthritis model [45], and the use of antibiotics prevents the advance of arthritis [13, 30, 31]. Studies in murine models revealed that induction of T_{FH} and Th17 cells precedes the onset of arthritis, indicating a role for both types of cells. Monocolonization with SFB enhances the production of autoantibodies and accelerates the progression of disease through the generation of Th17 cells although a microbiota-induced T_{FH} cell-dependent process can also precipitate disease [44]. Teng et al. [46] demonstrated that SFB trigger autoimmune arthritis by inducing differentiation and migration of gut T follicular helper cells (T_{FH}) to systemic lymphoid sites, leading to increased autoantibody production and arthritis exacerbation. In contrast, Block et al. [45, 47] confirmed a role for gut microbiota in the differentiation of T_{FH} cells and germinal center formation. Depletion of gut microbiota in mice by antibiotics reduced the number of T_{FH} cells and antibody production levels. They concluded that intestinal microbiota regulates the development of arthritis through T_{FH} independently of Th17 cells.

4. Dysbiosis in Rheumatoid Arthritis

Importance of gut microbiome in autoimmunity related to rheumatoid arthritis has been implicated in both mouse models and human disease. Alterations of microbiota are related to risk and severity of the disease. Three sites have been associated particularly with it, mainly the lungs, oral mucosa, and gastrointestinal tract. However, the site(s) of initial immune response triggering remains to be verified. Whereas precise mechanisms that enhance risk are not fully understood for each, it is likely that local tissue stress leads to posttranslational modification of peptides with subsequent antibody formation serving as a common mechanism [48].

Airway abnormalities and lung tissue citrullination are found in both rheumatoid arthritis patients and individuals at risk. This suggests the lung as a possible site of autoimmunity generation [49]. Evidence of an early role of the adaptive immunity and immune activation in the lungs of these patients comes from a proteomic study where two shared citrullinated vimentin peptides have been described in bronchial tissue form early rheumatoid arthritis patients and synovial tissue from patients with established disease, offering some clues on an immune process initiated in the lungs [25]. The pulmonary mucosa as an autoimmune process origin is based on the following observations: ACPAs are presented in the sputum of ACPA-positive patients without arthritis; there are microscopic and macroscopic changes in the lungs with early rheumatoid arthritis and untreated ACPA-positive patients; pulmonary alterations have been demonstrated in high-resolution computed tomography in subjects without the disease but with ACPA-positive; pulmonary biopsy samples from patients with ACPA positive with established AR suggest that ACPAs are produced locally. However, the precise molecular mechanisms that could be responsible for the triggering of immunity in the lung mucosa are relatively unexplored. It is known that lung exposure to harmful agents, including smoke, may induce increased expression and activation of PAD [1]. It has been hypothesized that the citrullinated proteins may become an autoantigen and thereby trigger an immune system response in people with genetic predisposition for rheumatoid arthritis.

Smoking and periodontitis promote protein citrullination and ACPA production [50]. Attention in the gums is based on periodontal disease, more frequent in subjects with rheumatoid arthritis or correlated with its activity. It has been speculated that periodontal pathogens drive systemic inflammation or disseminate to affected tissue. Indeed, increased specific IgG to periodontal pathogens, including *Prevotella intermedia* and *Porphyromonas gingivalis*, has been reported in RA [51]. The presence of *P. intermedia* and *P. gingivalis* in the subgingival dental plaque, as well as synovial fluid, supports a role of microbiota in initiating or maintaining chronic inflammation [52]. Cross-sectional studies have revealed correlations between RA and higher titers of serum antibodies against proposed periodontal pathogens such as *P. gingivalis*, *Prevotella melaninogenica*, and *Tannerella forsythia* [53]. Oral inoculation with *P. gingivalis* and *Prevotella nigrescens* aggravated the severity of arthritis in an experimental mouse model by directing the immune pathway towards production of IL-17 and generation of a Th17 response [54]. The effects of the two bacteria diverged in that *P. nigrescens*, in contrast to *P. gingivalis*, suppressed the joint-protective type 2 cytokines, including IL-4. *P. gingivalis* expresses an enzyme with amino deiminase activity that converts C-terminal arginine to citrulline, similar to the one involved in the etiology of rheumatoid arthritis. Citrullination of bacterial and human proteins by PAD can expose hidden epitopes leading to tolerance loss in genetically susceptible individuals. It is believed that the resulting immune response together with endogenous citrullination produces ACPAs [4]. The fact that smoking is strongly linked to the presence of periodontitis and *P. gingivalis* infection provides circumstantial evidence in favor of this hypothesis. However, recent epidemiological data have not demonstrated a clear relationship between periodontitis and rheumatoid arthritis [1].

Recently, gut microbiota has been proposed as an indispensable environmental factor in the progression of rheumatoid arthritis [5, 18, 27, 55–57]. Bennike et al. [43] identified 21 citrullinated peptides in the colonic tissue from both rheumatoid arthritis patients and controls, which have been previously found in lung tissue and synovial fluid from RA patients. Three citrullinated proteins (citrullinated vimentin, fibrinogen-alpha, and actin) are known targets for ACPAs, supporting that colon mucosa could be a potential break site for immune tolerance towards citrullinated epitopes. Citrullinated vimentin was found with increased abundance in the colonic tissue of these patients compared to the controls, which could indicate that initial rheumatoid arthritis triggering is not limited just to a specific location in the body but can take place in many other locations [43]. Therefore, this study supports the hypothesis that colon mucosa could serve as a break site for immune tolerance to citrullinated proteins, triggering ACPA production in patients with impaired immune system.

5. Gut Microbiota in Rheumatoid Arthritis

Potential role of intestinal microbiota in etiopathogenesis of rheumatoid arthritis is supported by studies in animal models, by research on intestinal microbioma, and indirectly, by the effect of diet and probiotics in the degree of inflammatory activity. Pathophysiologic mechanisms by which gut microbiota is associated with arthritis is probably multifactorial; proposed mechanisms include activation of antigen-presenting cells through an effect on TLRs or NLRs, ability to produce citrullinization of peptides by enzymatic action, antigenic mimicry, alterations in permeability of intestinal mucosal, control of host immune system (triggering T cell differentiation), and increase of T helper type 17-mediated mucosal inflammation.

Despite the existence of animal models, it is well known that there is no animal model that represents rheumatoid arthritis entirety [58]. However, murine arthritis models have shown to induce erosive polyarthritis by intraperitoneal injection of cell wall fragments of *Streptococcus pyogenes*, *Lactobacillus casei*, and *Eubacterium aerofaciens* [59–61]. The arthritogenicity of bacterial structures depends on bacterial species, and remarkably, even bacteria from the normal intestinal microbiota cause experimental arthritis in animals [17]. Gnotobiotic mice do not develop arthritis, and introduction of SFB is enough to reintegrate Th17 cells from the lamina propria, a greater production of autoantibodies and a rapid development of destructive arthritis [62]. Antibiotic treatment prevents and suppresses a phenotype similar to rheumatoid arthritis in several murine models [4, 18, 28], and in genetically susceptible mice, dysbiosis increases sensitivity to arthritis through activation of autoreactive T cells in the gut [63].

Intestinal microbiota's role in pathogenesis of arthritis was demonstrated by the induction/exacerbation of arthritis in experimental murine models [55, 64–66]. Studies with gnotobiotic mice have shown that disruptions in the intestinal microbiota could induce production of proinflammatory cytokines, interleukin-17, and increased levels of Th17 cells, even in extraintestinal tissues [11]. Th17 cells then migrate into the peripheral lymphoid tissue and secrete IL-17, which in turn, acts directly on B cells and induces systemic B cell differentiation and antibody production [67]. This ultimately can lead to development of autoimmune disease via molecular pattern recognition from gut microbiota [67]. The IL-1 receptor antagonist (IL-1Ra) knockout mice, which spontaneously develop autoimmune T cell-mediated arthritis, do not develop disease when raised in a germ-free environment. However, colonization with commuter *Lactobacillus bifidus* produces a rapid onset of the disease, severity, and incidence comparable to the arthritis observed in mice. *L. bifidus* triggers arthritis in this model by promoting an imbalance in Treg–Th17 cell homeostasis and mediated through Toll-like receptor signaling (TLR2-TLR4) [28, 65]. Liu et al. [64] found that the genus *Lactobacillus* was significantly more abundant in collagen-induced arthritis- (CIA-) susceptible mice prior to arthritis onset than in CIA-resistant mice. Notably, germ-free mice conventionalized with the microbiota from CIA-susceptible mice showed a higher frequency of arthritis induction than those conventionalized with the microbiota from CIA-resistant mice. Monocolonization of germ-free K/BxN mice with SFB was sufficient to drive production of autoantibodies and pathogenic Th17 cells as well as to trigger arthritis [66]. Systemic deficiencies of germ-

free animals reflect a loss of Th17 cells from the intestinal lamina propria. Introduction of a single species of resident intestinal filamentous bacteria restored the Th17 cell compartment into the lamina propria; autoantibodies production and arthritis occurred rapidly. Thus, a single commensal microbe, through its ability to promote a specific subpopulation of T helper cells, can lead to an autoimmune disease [66]. These results provide the evidence that commensal bacteria can drive autoimmune arthritis by inducing a Th17 response in the intestine. Therefore, composition of gut microbiota plays a pivotal role in the balance between inflammatory Th cells and suppressive Treg cells to maintain immune tolerance under healthy conditions [27].

Interaction between host genetic factors such as MHC and intestinal microbiota and its impact on development of rheumatoid arthritis is difficult to study in humans because of the high variability of genetic factors and diet [11]. Research conducted by Gomez et al. [68] provided the first demonstration that HLA genes and intestinal environment interact to affect the susceptibility of arthritis. This study showed differences in fecal microbiome of rheumatoid arthritis-susceptible HLA-DRB1*0401 transgenic mice compared to DRB1*0402 mice resistant to development of rheumatoid arthritis. Specifically, DRB1*0401 female mice had significantly different microbial to DRB1*0402 females, and this resulted in an increase in intestinal permeability and transcripts of Th17 type cytokines in DRB1*0401 mice. The analysis showed that the intestinal flora of arthritis-susceptible mice had a greater amount of *Clostridium* sp. bacteria, while those of DRB1*0402 resistant to arthritis were enriched by the families *Porphyromonadaceae* and *Bifidobacteriaceae* [68]. The latter organism has been associated with the anti-inflammatory response in the intestinal mucosal immune systems through the suppression of T cell proliferation and the production of proinflammatory cytokines and by inhibition of nuclear factor kB. The results show a difference in intestinal microbial composition between the two strains, suggesting that MHC genes may be directly or indirectly involved in determining the intestinal microbial composition and that interactions between bowel commensals [13, 18]. In addition, they demonstrated that dysbiosis is not enough since it requires a genetic susceptibility of the host, due to an induction inability in inflammatory response in wild animals, even with proarthritogenic intestinal flora [13, 28].

Several studies attempting to link gut mucosal and joint inflammation have been followed during the past decades. Eerola et al. [69] reported that fecal profile of bacterial cell fatty acids was significantly different in subjects with rheumatoid arthritis, mainly by anaerobic bacteria compared to controls. They support the idea that rheumatoid arthritis represents a state of chronic inflammation that could be motivated or aggravated by pathogenic bacteria overgrowth or by a lack of common immunomodulating bacteria [5, 16, 18, 57, 70]. Vaahtovuo et al. [16] also described differences in fecal diversity in individuals with rheumatoid arthritis compared to those with fibromyalgia, characterized by lower bundi-bacteria, *Bacteroides-Porphyromonas-Prevotella*, subgroup *Bacteroides fragilis*, and the group *Eubacterium rectale-Clostridium coccoides* in subjects with rheumatoid arthritis [6]. On the other hand, Newkirk et al. [71] identified differences in the types of *E. coli* pathogen colonization among subjects with rheumatoid arthritis, RF-positive patients were more commonly colonized with *E. coli*, phylogenetic group D, whereas RF-negative patients were more commonly colonized with *E. coli*, phylogenetic group B2, and these individuals also had lower joint scores and inflammatory markers yet higher IgA anti-*E. coli* antibody responses.

The observation that gut microbiota differs in subjects with early rheumatoid arthritis compared to controls has renewed the interest in studying intestinal microbiota as a possible site of origin of the autoimmune proces; studies that evaluate the intestinal microbiota show that rheumatoid arthritis is characterized by an expansion and/or decrease of bacterial groups as compared to controls [5, 18, 55–57]. High-throughput sequencing of stool samples in 5 studies of RA patients showed gut dysbiosis [5, 18, 55, 57, 63], and 2 study reported overexpansion of *Prevotella* sp. in patients with early RA, particularly *P. copri* (Table 2). Differences between the bacteria reported in the studies may be influenced by the time course of the disease (i.e., early versus established), subjects included in the control groups (healthy or first degree relatives), the treatment received, and the geographical location, since the studies do not show the same pattern.

Toivanen et al. [57] compared fecal microbiota from 25 patients with early RA to the microbiota of patients with noninflammatory pain using oligonucleotide probe against 16S RNA. Patients with early RA had significantly fewer bacteria belonging to the gender *Bacteroides* sp., *Prevotella* sp., and *Porphyromonas* sp. In other study, Scher et al. [18] reported that individuals with early RA were more likely to harbor *Prevotella copri* compared to controls. In addition, they demonstrated that oral administration of *P. copri* increased local inflammatory response in a colitis murine model. Therefore, *P. copri* would alter intestinal permeability. Such increase might lead to bacteria penetration and/or its components throughout the body; this is one of the proposed mechanisms that link dysbiosis with the pathogenesis of arthritis. Interestingly, the relative abundance of *P. copri* showed a negative correlation with the presence of shared epitope, suggesting that the composition of the human intestinal microbiome could also be partially dependent on the host genome and suggesting a dysbiosis before the appearance of the clinical phenotype.

In line with these results, Maeda et al. [63] observed that *P. copri* was in abundance within gut microbiota in Japanese patients with early RA who had not received drug treatment. They identified that *P. copri* per se had a high capacity to induce Th17 cell-related cytokines, such as IL-6 and IL-23. Increased *Prevotella* sp. abundance is associated with augmented T helper type 17-mediated mucosal inflammation, which is in accordance with the marked capacity of *Prevotella* sp. in driving Th17 immune responses in vitro [51]. In other study, Pianta et al. [72] identified that subgroups of rheumatoid arthritis patients have differential IgG or IgA immune reactivity with *P. copri*. In both new onset rheumatoid

Table 2: Summary of studies that have evaluated the influence of gut flora on the etiopathogenesis of RA.

Author (year of publication)	Design	Subjects included	Method employed	Results in the RA group versus the controls
Shinebaum et al. [70]	Case-control	25 patients with RA compared with controls	Estimation of bacterial counts in fecal culture	Significantly higher carriage rate of *Clostridium perfringens* in the RA population than controls (88% versus 48%, $p < 0.01$). Coliform counts also tended to be higher
Eerola et al. [69]	Case-control	74 treatment-naive early RA and 91 non-RA controls	Gas-liquid chromatography of bacterial CFAs	Variation in CFA profile of RA as compared to controls likely caused by anaerobic bacteria
Vaahtovuo et al. [16]	Case-control	50 individuals with RA and 50 individuals with fibromyalgia	Flow cytometry, 16S rRNA hybridization, and DNA-staining	The RA patients had significantly less bifidobacteria and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *Bacteroides fragilis* subgroup, and *Eubacterium rectale-Clostridium coccoides* group
Toivanen et al. [57]	Case-control	25 treatment-naive individuals with early RA patients and 23 control patients suffering from noninflammatory pain	16S ribosomal DNA	Patients with early RA had significantly less bacteria belonging to the *Bacteroides*, *Prevotella*, and *Porphyromonas* genera than the controls (4.7% versus 9.5%, $p < 0.01$). The number of bacteria belonging to the *Bacteroides-Prevotella-Porphyromonas* group was, on average, in RA patients only half that of the controls
Scher et al. [18]	Cross-sectional	44 treatment-naive individuals with RA, 26 treated RA, 16 patients with psoriatic arthritis, and 28 healthy controls	16S ribosomal DNA	Increases in *Prevotella copri* (75% versus 21.4%) abundance and decrease in *Bacteroides*
Liu et al. [56]	Case-control	15 individuals with early RA and 15 healthy controls	Quantitative real-time PCR	Fecal microbiota of RA patients contained significantly more *Lactobacillus* (10.62 ± 1.72 copies/g) than the control group (8.93 ± 1.60 copies/g)
Zhang et al. [5]	Cohort	77 treatment-naive individuals with RA and 80 unrelated healthy controls; 17 treatment-naive individuals with RA paired with 17 healthy relatives; and 21 samples from DMARD-treated individuals with RA	Metagenomic shotgun sequencing and a metagenome-wide association study	The RA gut was enriched in gram-positive bacteria and depleted of gram-negative bacteria, including some *Proteobacteria* and *gram-negative Firmicutes* of the Veillonellaceae family. The RA-enriched MLGs formed a large cluster including *Clostridium asparagiforme*, *Gordonibacter pamelaeae*, *Eggerthella lenta*, and *Lachnospiraceae bacterium*. There was a trend towards increased abundance of *P. copri* as a function of RA duration in the first year
Maeda et al. [63]	Cross-sectional	25 treatment-naive individuals with early RA patients and 23 healthy controls	16S rRNA-based deep sequencing	A subpopulation of early RA patients harbored intestinal microbiota dominated by *Prevotella copri*
Chen et al. [55]	Case-control	40 Subjects with RA with treatment 32 controls (15 relatives of 1 degree with AR and 17 healthy subjects)	16S ribosomal DNA	Increased number of reads from the phylum Actinobacteria in the RA group (0.45 versus 0.04%) Decrease in Faecalibacterium and expansion of Collinsella aerofaciens and Eggerthella lenta

MLGs: metagenomic linkage groups.

arthritis patients and chronic ones, a subgroup had IgA antibody responses to either Pc-p27 or the whole organism, which correlated with Th17 cytokine responses and frequent ACPAs. The second subgroup had IgG *P. copri* antibodies, which were associated with *Prevotella* DNA in synovial fluid, *P. copri*-specific Th1 responses, and less frequent ACPAs. Patients with RA had IgA, IgG, or no specific antibodies, indicating that different immune responses to *P. copri* can develop within the individual patient, which in turn may have implications for disease risk and outcomes.

Molecular mimicry, due to its sequence similarities between foreign and self-peptide, is one such mechanism known to result in cross-activation of pathogen-derived autoreactive T or B cells. These T and B cells can cross-

react with host epitopes, thus leading to autoimmunity [67]. In a recently work, Pianta et al. [73] described two proteins derived from common types of gut bacteria that could evoke the immune responses in RA patients. *N*-Acetyl-glucosamine-6-sulfatase (GNS) and filamin A (FLNA) were identified as autoantigens that produce responses from both T and B cells, in over 50% of RA patients, but not in healthy controls or patients with other rheumatic diseases. The HLA-DR-presented GNS peptide has marked sequence homology with epitopes from sulfatase proteins of the *Prevotella* sp. and *Parabacteroides* sp., whereas the HLA-DR-presented FLNA peptide has homology with epitopes from proteins of the *Prevotella* sp. and *Butyricimonas* sp., another gut commensal. T cell responses to the corresponding microbial and self-peptides were strongly correlated. These study provides evidence that T cell epitopes of a related order of gut microbes, particularly *Prevotella* sp., may cross-react with self-epitopes of highly expressed proteins in joints, especially in patients with SE alleles.

Moreover, *Prevotella* sp. may not be the only genus participating in inflammatory disease. Zhang et al. [5] used metagenomic shotgun sequencing technology to analyze fecal, dental, and salivary samples from a large cohort of RA patients as well as from healthy controls. Analysis of differentially represented metagenomic linkage groups revealed significant microbiome differences between RA patients and healthy controls, not only in fecal but also in salivary and dental samples. They demonstrated that gram-positive bacteria enriched microbiota versus few gram-negative bacteria and, furthermore, that dysbiosis was associated with inflammation markers and clinical rheumatoid arthritis activity. Besides, they also detected alterations in redox environment and transport and metabolism of iron, sulfur, and zinc in the microbiota of RA patients, indicating that the altered microbiome could play an important role in the pathogenesis of RA. Noteworthy, altered microbiome was partially restored to normal (microbiome of healthy controls) in patients who showed clinical improvement after prescribing disease-modifying antirheumatic drugs. Remarkably, *P. copri* in RA-affected individuals showed a trend of increasing relative abundance in the first year, consistent with its reported expansion in early RA. Many *Prevotella* species were enriched in the saliva of RA subjects compared with controls. One notable exception was *Prevotella intermedia*, which was enriched in the control group. However, in dental plaque samples, there was a very different picture and most *Prevotella* sp. were present at higher proportions in healthy subjects.

Chen et al. [55] reported that bacteria belonging to the phylum *Actinobacteria* play a significant role in the pathogenesis of rheumatoid arthritis, *Collinsella* sp. and *Eggerthella* sp. predicted its presence, and dysbiosis in the intestinal microbiome is partially restored after treatment with disease-modifying antirheumatic drugs, similar to results reported in the previous study. The potential association of *P. copri* as previously reported with new onset untreated RA and DR4 was not observed in this cohort of RA patients (in contrast to previous studies, all the patients in the present study were currently on a treatment regimen). They showed that the decreased gut microbial diversity of RA patients is associated with disease duration after adjusting for various drugs used for treatment. *Collinsella* sp. may contribute to the development of rheumatoid arthritis through molecular mimicry as it presents sequences shared with DRB1*0401. The role of the RA-associated bacteria *Collinsella* sp. was confirmed using a human epithelial cell line and a humanized mouse model of arthritis. *Collinsella* sp. enhanced disease severity in a humanized mouse model. One mechanism by which Collinsella contributes to disease pathogenesis is by increasing gut permeability as observed by the lower expression of tight junction proteins. Additionally, Collinsella influences the epithelial production of IL-17A [55].

Although studies in subjects with rheumatoid arthritis show a correlation of *Prevotella* sp. in its pathogenesis, other studies suggest that it may be considered a beneficial bacterial species rather than pathogenic [74, 75]. Culture collections now include approximately 40 different *Prevotella* species, most of them oral isolates, and three of which are found in the gut (*P. copri* is generally the more abundant). The vastly different genome gene repertoires of strains within and between *Prevotella* sp. and across hosts probably underlie some of the differences observed in responses at the genus level to diet and health conditions across individuals [75]. A recent comprehensive study comparing several bacterial species suggests that membership of a specific phylum does not predict immunological properties, underlining the importance of characterizing properties at species level. Marietta et al. [76] evaluated the ability of 2 species of *Prevotella* sp. for arthritis prevention and treatment in HLA-DQ8 mice. It was shown that the ability to modulate the immune response differed between the *Prevotella* strains. Treatment with *P. histicola* suppressed arthritis development by modulating the immune response (regulation of dendritic cells and generation of Treg cells), resulting in suppression of Th17 responses and reduction of inflammatory cytokines (IL-2, IL-17, and tumor necrosis factor). In contrast, administration of *P. melaninogenica* showed no significant change in cytokine levels, either preventing arthritis development. The ability of *P. histicola* to modulate was validated using a DBA/1 mouse model, demonstrating that mice treated with *P. histicola* developed milder arthritis compared to controls. It is clear that a single strain of *Prevotella* sp. can act in what has been interpreted as a beneficial or detrimental manner, depending on the context. This may explain why *Prevotella* sp. is abundant in healthy microbiota and suggests that only certain strains may exhibit pathogenic properties.

In new onset RA patients, *Prevotella* abundance in the gut was at the expense of *Bacteroides fragilis*, an organism that is important for Treg function [18, 72]. In fact, high levels of *P. copri* and similar species are related to low levels of beneficial microbes, which are believed to suppress the immune system and metabolize vitamins in forms absorbed into the bloodstream [17]. When discussing possible mechanisms by which diet could influence rheumatoid arthritis, effects of intestinal flora should be considered [77–79]. A diet rich in protein and animal fat is associated with the presence of *Bacteroides* sp. while a diet rich in carbohydrates is

Table 3: Summary of studies evaluating the effect of probiotics on the level of rheumatoid arthritis activity.

Author (year of publication)	Design	Population and intervention	Outcomes
Hatakka et al. [89]	Clinical trial	*Lactobacillus* group ($n = 8$). Placebo group ($n = 13$)	There were no statistically significant differences in: CRP, ESR, IL-6, IL-10, IL-12, and HAQ
Pineda et al. [86]	Clinical trial	15 subjects Lactobacillus rhamnosus GR-1 and *Lactobacillus reuteri* RC-14 administered for 3 months versus 14 subjects in the control group	Probiotics did not statistically improve the percentage of ACR 20 response (20% versus 7%, $p = 0.33$)
Alipour et al. [84]	Clinical trial	22 subjects with *Lactobacillus casei* versus 24 subjects with placebo	Statistically significant decrease in CRP level, count of inflamed and painful joints and in DAS-28
Vaghef-Mehrabany et al. [85]	Clinical trial	22 subjects with *Lactobacillus casei* versus 24 subjects with placebo (maltodextrin)	Statistically significant decrease in level of TNF-α, IL-6, and IL-12 count of inflamed and painful joints and in DAS-28

CRP: C-reactive protein; DAS28: disease activity score-28; IL: interleukin; ESR: erythrocyte sedimentation rate; ACR: American College of Rheumatology.

associated with the presence of *Prevotella* sp. [80]. Studies have shown that Mediterranean or vegan diet reduces inflammatory activity, increases physical function, and improves vitality [19]. However, some other studies find benefits without achieving a significant improvement in composite indices to measure disease activity [81, 82]. Nevertheless, in these studies, it has not been determined whether a rheumatoid arthritis improvement was actually due to a change in intestinal flora composition.

Probiotics are living organisms that can confer a health benefit to the host. Probiotics mainly exert their beneficial effects through the following three methods: antimicrobial effects, enhancement of mucosal barrier integrity, and immune modulation [83]. Studies [84–86] assessing the relationship between probiotic supplementation with the level of RA activity have not shown conclusive results (Table 3). Improvements in probiotic interventions have not been evident enough to demonstrate the effectiveness of treating RA patients, due to the limited studies. Better-designed research needs to be conducted in order to identify the best species that relieve RA and optimize the intake method and doses of the probiotics [83].

Finally, evidence that intestinal microbiota motivates arthritis development comes from murine experimental models. In humans, this association is based in differences between microbiome within comparison groups, which do not necessarily represent its causality. Results in humans suggest dysbiosis as a significative etiologic agent promoting rheumatoid arthritis progression or, even more, that inflammation caused by some microorganisms like *P. copri*, may probably contribute to arthritis maintenance. In order to predict intestinal microbiota's role in pathogenesis of rheumatoid arthritis, an accurate comprehension related to this species potential, its ecology and interaction with other microbes and/or hosts would still be necessary. Furthermore, although gut mucosal got probably the highest potential for host-microbe interaction, all mucosae have resident microbiota and react dynamically to its presence with an immune modulation. Gram-negative bacteria could cause infections at any part of the body; among the most common types are oral, dental, pleuropulmonar, intra-abdominal, genital mucosal, skin, and soft tissues. In other words, it does not mean that all subjects with rheumatoid arthritis must have dysbiosis in a single site.

6. Microbiota as a Possible Mechanism for Rheumatoid Arthritis Prevention

Current research projects are focused on prevention with biological drugs that inhibit antibody formation or activate T cells [87]. Recent findings showed intestinal dysbiosis as a major advance in our understanding of rheumatoid arthritis. Nevertheless, there is no study demonstrating the antigen or antigens that trigger the autoimmune process. Logical indications point mucosal dysbiosis as an attractive site for elucidating autoimmunity pathways, particularly the mechanisms that induce loss of immune tolerance and specific mechanisms by which a person evolves from a preclinical to a clinical disease.

Gut microbiota has been shown to play role in rheumatoid arthritis although the mechanism of this association remains obscure. Understanding these mechanisms is crucial for a better treatment efficacy and personalized patient management [67]. Plasticity of microbiome may allow a specific or systematic manipulation of a certain intestinal microbiota associated with host diseases [88], speculating that, in the future, this manipulation could change therapeutic strategies in subjects with rheumatoid arthritis.

Conflicts of Interest

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

Acknowledgments

The authors thank Gloria Mercado for her helpful comments during the preparation of this manuscript.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 6836498, 11 pages
https://doi.org/10.1155/2017/6836498

Review Article

The Microbiome in Connective Tissue Diseases and Vasculitides: An Updated Narrative Review

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Received 27 April 2017; Revised 4 July 2017; Accepted 12 July 2017; Published 1 August 2017

Academic Editor: Ilian Radichev

Objective. To provide a narrative review of the most recent data concerning the involvement of the microbiome in the pathogenesis of connective tissue diseases (CTDs) and vasculitides. *Methods*. The PubMed database was searched for articles using combinations of words or terms that included systemic lupus erythematosus, systemic sclerosis, autoimmune myositis, Sjögren's syndrome, undifferentiated and mixed CTD, vasculitis, microbiota, microbiome, and dysbiosis. Papers from the reference lists of the articles and book chapters were reviewed, and relevant publications were identified. Abstracts and articles written in languages other than English were excluded. *Results.* We found some evidence that dysbiosis participates in the pathogenesis of systemic lupus erythematosus, systemic sclerosis, Sjögren's syndrome, and Behçet's disease, but there are still few data concerning the role of dysbiosis in other CTDs or vasculitides. *Conclusions.* Numerous studies suggest that alterations in human microbiota may be involved in the pathogenesis of inflammatory arthritides as a result of the aberrant activation of the innate and adaptive immune responses. Only a few studies have explored the involvement of dysbiosis in other CTDs or vasculitides, and further research is needed.

1. Introduction

The human microbiota harboured by each person consists of 10–100 trillion symbiotic microbial cells, mainly bacteria in the gut, but also viruses, yeasts, protozoa, and even helminths. The sum of human microbes and their genes existing within and on the human body (collectively known as the microbiome) has been found to be a principal factor in human health and disease [1]. Humans and microbes have coevolved to establish a symbiotic relationship over time, but perturbations, known also as dysbiosis, may occur and drive several diseases, including autoimmune disorders. Over the last few decades, new insights provided by DNA sequence-based analyses of human microbial communities have renewed interest in mucosal immunology and suggest that alterations in the human microbiome can also affect the development of rheumatic diseases.

The concept that human microbiota may modulate systemic autoimmunity is not new, but the underlying mechanisms of autoimmune regulation by the microbiome are just beginning to emerge [2]. Studies of animal models published 30 years ago demonstrated a relationship between the development of inflammatory arthritis and the presence/absence of some intestinal bacteria [3, 4], and, more recently, many studies have drawn attention to the potential role of the oral microrganism *Porphyromonas gingivalis* in the development of rheumatoid arthritis (RA) [5]. A recent study of the lung microbiome in a cohort of patients with early RA has found distal airway dysbiosis similar to that detected in sarcoidol lung inflammation [6], but, although various studies have investigated the different composition of gut microbiota in patients affected by RA and spondyloarthritis (SpA), the complex mechanisms by which microbes influence the pathogenesis of autoimmune diseases are still unknown.

Connective tissue diseases (CTDs) encompass a wide group of immune-mediated diseases, characterized by the inflammation of the connective tissues of the body sustained

by the activation of the immune system against self-epitopes expressed on cells and matrix. Vasculitides share similar pathogenetic mechanisms and are characterized by an immune-mediated response against components of the vascular tree. Genetic predisposition is necessary but not sufficient to give raise to these diseases, and an environmental trigger, including infections or changes in microbiome composition, is thought to be required for the onset. Given the spread of connective tissues all over the body, clinical manifestations of CTDs are polyhedral and may involve skin, joints, and visceral organs. Similarly, vasculitides have a wide range of clinical manifestations, according to the vascular district involved by the inflammatory process.

Like in inflammatory arthritides, it is presumable that, in genetic predisposed individuals, dysbiosis activates several immune pathways favouring in turn the development of CTDs or vasculitides.

As there are currently only limited animal and human data available concerning the potential link between the microbiome and systemic autoimmune diseases other than SpA and RA, the aim of this narrative review is to provide an updated view of the involvement of microbiome in the pathogenesis of CTDs and vasculitides.

2. Materials and Methods

2.1. Sources and Selection Criteria. The PubMed database was searched for articles using combinations of words or terms that included connective tissue diseases (systemic lupus erythematosus, systemic sclerosis, autoimmune myositis, Sjögren's syndrome, undifferentiated and mixed connective tissue disease, and vasculitis), microbiota, microbiome, and dysbiosis. Papers from the reference lists of the articles and book chapters were reviewed, and relevant publications were identified. Abstracts and articles written in languages other than English were excluded.

3. Results

We selected 55 articles concerning the role of the microbiome in CTDs and vasculitides; the findings are described below, divided by the type of rheumatic disease.

3.1. Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE), the most emblematic CTD, mainly affects women of childbearing age. As in the case of other autoimmune diseases, it has been hypothesized that an infectious stimulus triggers the onset of SLE by inducing chronic immune system activation [7]. No specific infective agent has yet been isolated, but various candidates have been proposed (including *Epstein-Barr virus*, *cytomegalovirus*, retroviruses, and *human parvovirus B19*), and there is some concern about the possible role of unbalanced microbial microbiota and the prevalence of some pathogens in the gut and mucosa of SLE patients [8].

Up to 50–80% of SLE patients can suffer from skin and mucosal manifestations, in which changes in mucocutaneous commensal flora composition can lead to the proliferation of pathogen species driving chronic inflammation [9]. The innate immunity is activated as the first step during infections sustained by intracellular pathogens, and the secretion of cytokines like type I interferons, which include IFN-α and IFN-β, is required to modulate the immune response leading to cell apoptosis and pathogen clearance. In SLE, type I interferons are typically overexpressed and associated to disease activity and can induce the hyperactivation of myeloid dendritic cells activating in turn autoreactive T lymphocytes that play a role in SLE pathogenesis [10].

In a study of 84 SLE patients, Conti et al. detected *Staphylococcus aureus* colonising the nasal mucosa and, although the rate of occurrence was similar to that observed in healthy controls, this may be associated with a distinct SLE phenotype characterized by renal and skin involvement and a higher likelihood of anti-dsDNA, anti-Sm, anti-SSA, anti-SSB, and anti-RNP antibody positivity [11]. The activation of cells belonging to the innate immune system via the interactions of pathogen-associated molecular patterns (PAMPs) with Toll-like receptors (TLRs) or the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) cascade, along with the subsequent production of IFNs, plays a key role in the clearance of infections sustained by *Staphylococci,* although it may contribute at the same time to the virulence of some strains of these microorganisms, like strain 502A [12]. However, the authors did not distinguish among different strains of *Staphylococcus aureus* colonising nasal mucosa of SLE patients, which may account for different burdens of cytokine production and different pathogenetic pathways, but only described the association between bacteria and clinical and laboratory alterations.

Periodontal inflammation has also been associated with SLE. In a study of 52 SLE patients and 52 controls, Corrêa et al. found that the risk of periodontitis sustained by *Fretibacterium*, *Prevotella nigrescens*, and *Selenomonas* spp. was higher in patients than in controls and was related to the local release of interleukin- (IL-) 6, IL-17, and IL-33 [13]. The hyperexpression of C3 has been associated with the maintenance of periodontitis sustained by *Porphyromonas gingivalis* in experimental animal models. According to Maekawa et al., neutralising C3 by adding the compstatin analogue Cp40 may prevent local inflammation and the subsequent release of IL-17 or receptor activator of nuclear factor kappa-B ligand (RANKL), perhaps by restoring dysbiosis [14]. Immune complexes formed by ribonucleoproteins, autologous nucleic acids, and immunoglobulin G (IgG) bind to fragment cristallizable receptors (FcgRIIa) and trigger intracellular responses by binding endosomal TLRs, thus inducing complement activation and IFN production. C3 is a crucial molecule in generating IFN signature in SLE, and C3-deficient mutant mice have less type I IFN-related apoptosis and cytokine production [15]. Although the role of complement fractions in favouring or counteracting the growth of pathogenic species in the gingival mucosa of this population of patients is still unknown, the findings suggest that gingival inflammation may represent a chronic stimulus for the local activation of innate immune cells and induce complement consumption and IFN signature generation.

A number of SLE studies have investigated the composition of gut bacteria in animal models and humans and have

been recently addressed in a review by Neuman and Koren [16]. In rare cases, SLE patients may suffer from noninfectious SLE enteritis, which is usually sustained by a visceral or serosal vasculitis of the bowel. Gut dysbiosis could be at the basis of local or systemic inflammation, by modulating the immune response [17]. Engagement of TLR9 by commensal flora may modulate the T-effector/regulator ratio in nonautoimmune animal models and lead IFN signature in SLE. However, SLE enteritis and the unbalance in gut flora composition are still unclear.

Zhang et al. found a gender-dependent alteration in the composition of gut microbiota in stool and colon samples taken from lupus-prone MRL/Mp-Faslpr (MRL/lpr) mice: female mice had higher levels of Lachnospiraceae and Bacteroidetes S24-7 and lower levels of *Bifidobacterium* and Erysiopelotrichaceae, with no significant difference in Lactobacillaceae, although the levels of the latter family (and Firmicutes phylum) were significantly lower in the MRL/lpr mice than in controls and were restored after treatment with oral retinoic acid [18]. Hevia et al. evaluated the intestinal microbial composition of 20 SLE patients and 20 matched controls by analysing fecal samples using Torrent 16S rRNA gene-based sequencing [19] and found a significant reduction in the Firmicutes/Bacteroidetes phyla ratio in the patients, which may account for the glycan degradation, lipopolysaccharide biosynthesis, and oxidative phosphorylation described in SLE patients. Similar results were also obtained in a Chinese SLE study cohort, in which the authors also isolated nine genera (*Rhodococcus, Eggerthella, Klebsiella, Prevotella, Eubacterium, Flavonifractor*, and Incertae sedis) that were significantly increased in comparison with controls [20]. According to Rodríguez-Carrio et al., the altered Firmicutes/Bacteroidetes ratio in the gut of SLE patients may account for the hyperproduction of free fatty acids (FFAs) that are associated with impaired endothelial dysfunction characterized by increased levels of leptin, interferon gamma-inducible protein-10, epidermal growth factor, IL-8, and monocyte chemoattractant protein-1 [21]. Lopez et al. have shown that altered gut microbial composition may affect the final differentiation of T helper (Th) subsets in SLE patients in an *in vitro* study of 37 SLE patients and 36 controls: they showed that an imbalance of Firmicutes and Bacteroidetes in fecal samples was associated with Th17 responses whereas enrichment with *Bifidobacterium bifidum LMG13195* or a mixture of two clostridium strains, *Ruminococcus obeum DSM25238* and *Blautia coccoides DSM935*, restored the number of T regulatory cells, thus providing a rationale for the use of probiotic therapy [22]. In another study, Rojo et al. found significant differences in the metabolite landscape but not in the microbial composition in SLE patients versus healthy controls. SLE patients had reduced levels of homoserine lactone and N-acetylmuramic acid and increased level of ribose-1,5-bisphosphate, which, in contrast to healthy subjects, were not influenced by body mass index (BMI) [23]. Furthermore, studies on gut dysbiosis in SLE demonstrated a reduced ratio of Firmicutes/Bacteroidetes that may be at the basis of an altered differentiation of Th effectors, selecting Th17 clones to the detriment of T regulator ones, with the subsequent production of proinflammatory cytokines. The use of prebiotics or probiotics in these patients seems promising; however, randomised controlled trials (RCTs) are still unavailable.

Some experiments in animal models have shown that caloric restriction, as well as the assumption of polyunsaturated fatty acids, vitamins A, D, and E, and phytoestrogens may ameliorate SLE disease activity [24]. Dietary changes may also affect intestinal virome thus preventing cytotoxic and proapoptotic responses aiming to eradicate intracellular microorganisms. Cuervo et al. reported a significant association between the intake of flavone, flavanones, dihydrochalcones, and polyphenols and the growth of *Blautia*, *Lactobacillus*, and *Bifidobacterium* in a cohort of 20 SLE female patients [25]. In another experiment on lupus-prone SNF_1 mice, the administration of acidic water restrained the course of glomerulonephritis and the production of cytokines and autoantibodies, inducing meantime a restore in the gut microbial flora (higher *Lactobacillus reuteri* and *Turicibacter* spp. colonisation in acidic water-drinking mice) [26]. Similar results have been reported with the use of *Lactobacilli* GMNL-32, GMNL-89, and GMNL-263 on the course of SLE hepatitis in lupus-prone mice [27].

To summarise, there are emerging evidences that dysbiosis represents one rawplug inside the complex pathogenesis of SLE; however, given the modest knowledge on the beneficial effects of probiotics and prebiotics and the lack of RCTs, no clear therapeutic strategies can be currently drawn. Additionally, the use of immunomodulators and immunosuppressive drugs may alter the composition of commensal flora, selecting pathogens able to chronically stimulate the immune system.

3.2. Systemic Sclerosis. Systemic sclerosis (SSc) is a connective tissue disease that may affect the entire gastrointestinal (GI) tract in up to 90% patients [28], who often have oral telangiectasias, esophageal dysmotility, small intestinal bacterial overgrowth, or delayed intestinal transit. The most representative characteristic of GI involvement is fibrosis of the muscular tunic, which is associated with reduced peristalsis [29, 30]. However, there is some evidence that there are mucosal alterations associated with altered intestinal permeability and possibly dysbiosis. Increased intestinal permeability would favour the translocation of bacterial products from the lumen to the submucosal layer, thus leading to aberrant activation of the local innate and adaptive immune system [31–33].

Several studies have demonstrated that vascular damage occurs throughout the intestinal tract and that mucosal abnormalities (including watermelon stomach, intestinal telangiectasias, and angiodysplasia) may be present in more than 50% of SSc patients and are usually associated with a more pronounced vasculopathic phenotype (i.e., digital ulcers and pulmonary artery hypertension) [34]. Together with endothelial dysfunction, defective control of microvascular tone may be responsible for tissue hypooxygenation and the production of reactive oxygen species (ROS), thus leading to chronically defective enterocytes, which may

lose their ability to counteract the proliferation of pathogenic microorganisms.

Only a few published studies have so far investigated the role of dysbiosis in SSc.

Andréasson et al. used a genome-based microbiota test of stool samples to study the gut flora composition of 98 SSc patients [35] and found that 75.5% had dysbiosis (low levels of *Faecalibacterium prausnitzii* and/or Clostridiaceae) and that this was significantly associated with esophageal dysmotility, micronutrient deficiency, skin telangiectasias, pitting scars, pulmonary fibrosis, and high serum levels of inflammation markers. Volkmann et al. examined by means of Illumina HiSeq 2000 16S sequencing of cecum and sigmoid mucosal lavage samples taken from 17 SSc patients and a healthy control group and found decreased levels of commensal bacteria such as *Faecalibacterium* and *Clostridium* and increased levels of pathogenic bacteria such as *Fusobacterium* and *g-Proteobacteria* in the patients, who also had increased levels of *Bifidobacterium* and *Lactobacillus* and a reduced *Bacteroides fragilis*/*Fusobacterium* ratio, especially in the case of the most severe GI symptoms [36].

Measurement of fecal calprotectin is a noninvasive tool for monitoring bowel inflammation and may also give indirect information on the innate immune cells' activation following a microbial insult. The molecule, formed by the dimerization of the proteins S100A8 and S100A9 and mainly expressed in the cytosol of polymorphonuclear cells, prevents the infections sustained by virulent strains of bacteria or fungi, but its concentration is also arisen in autoimmune inflammatory bowel diseases (IBDs). Marie et al. detected increased levels of fecal calprotectin (>50 μg/g) in 93 of their 125 SSc patients and found that fecal calprotectin concentration significantly correlated with intestinal motor dysfunction and small intestinal bacterial overgrowth as assessed by means of glucose H_2/CH_4 breath testing [37].

Changes in the lung and skin microbiome of SSc patients may also occur. Polyhedral cutaneous and pulmonary manifestations, including puffy fingers, skin and lung fibrosis, and alveolitis, led to the discovery of four genotypes that may lead to a more pronounced fibrotic or inflammatory phenotype [38]. The inflammatory phenotype is characterized by the upregulation of genes involved in the autoimmune and autoinflammatory cascades triggered by the stimulation of TLRs, converging to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and then activating a complex cascade involving Th2 lymphocytes, M2 macrophages, Th17 cells, and fibroblasts [39].

Many microbial products have been postulated as inducing the activation of lung and skin macrophages and dendritic cells through TLRs. Fungi are commensal components of the microbiome of the skin and lung, and the lung mycobiome of healthy subjects is mainly represented by *Aspergillus* spp., whereas *Candida* spp. become prevalent under pathological conditions (including idiopathic pulmonary fibrosis and interstitial lung disease) [40]. However, no study has yet evaluated microbiome or mycobiome composition in the lungs of SSc patients. One study used ribosomal RNA sequencing of forearm skin biopsies taken from patients with early (<6 months) diffused and limited SSc and healthy controls and found the increased expression of *Rhodotorula glutinis* sequences in the patient samples, but no significant differences in bacterial or viral characterisation [41]. It can be argued that *Rhodotorula* can cause SSc skin fibrosis as it may activate the innate immune system and induce lung granulomatous diseases and peritoneal fibrosis, but there is a lack of studies characterising the mycobiome of unaffected skin sites and those affected by disease.

In brief, similarly to SLE, there is some evidence that SSc patients may also suffer from a multidistrict imbalance of commensal and pathogenic microorganisms and antibiotic or probiotic treatments may have some beneficial effects [42, 43]. However, it is still unknown where the primitive dysbiotic insult occurs (skin or visceral organs), which organ may be firstly affected, and whether dysbiosis could be the consequence rather than the cause of the anatomic modifications affecting connective tissues.

3.3. Sjögren's Syndrome. Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease involving the exocrine glands (mainly the lacrimal and salivary glands) that has extraglandular systemic repercussions in 71% of cases [44]. Glandular tissue inflammation characterized by the infiltration of dendritic cells, CD4+ T lymphocytes, and B cells leads to the final destruction of the acini and reduces the secretion of fluids containing antimicrobial factors, and the subsequent disruption of the skin and mucosal barrier may lead to dysbiosis and a higher risk of colonisation by pathogenic species.

However, in a study from Lugonja et al., the authors did not find any increase in the prevalence of periodontitis in 39 SS patients in comparison with 36 RA and 23 osteoarthritis controls on the basis of a search for specific serum antibodies against ten oral/periodontal bacteria [45].

It has been hypothesized that dysbiosis may condition the activation of the immune system and influence the severity of SS. For example, it has been demonstrated that postnatal gut colonisation by different *Bifidobacterium species* may influence the response of the immune system to microbial stimulation by modulating the production of salivary secretory IgA and that *Bacteroides fragilis* colonisation is associated with a less intense production of cytokines and chemokines by immune cells following stimulation with lipopolysaccharide (LPS) [46]. de Paiva et al. used animal models and humans to demonstrate that, in comparison with controls, there was significant dysbiosis in the stools of SS model mice (with a prevalence of *Enterobacter*, *Escherichia*/*Shigella*, and *Pseudomonas*) and that histological changes in their mucosae (monocyte infiltration, a reduction in the number of goblet cells, and barrier disruption) were more pronounced after 10 days of exposure to antibiotics [47]. However, they did not find any significant difference in the overall microbial composition of the conjunctiva and tongue between SS patients and healthy controls, although there was a prevalence of *Streptococcus* and a decrease in *Leptotrichia* and *Fusobacterium* levels in the SS tongue samples. The fecal microbiota of the SS patients contained a higher proportion of *Pseudobutyrivibrio*, *Escherichia*/*Shigella*, *Blautia*,

and *Streptococcus* and fewer *Bacteroides*, *Parabacteroides*, *Faecalibacterium*, and *Prevotella*. Interestingly, the severity of ocular (but not systemic) symptoms assessed using the unweighted 12-domain European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index (ESSDAI) was significantly related to gut flora diversity.

Szymula et al. investigated whether the production of autoantibodies against RoSSA, which represent a distinctive trait and classification criterion for SS [48], may be triggered by the recognition of a cross-reactive bacterial peptide by the adaptive immune system [49]. Using Ro60 reactive T cell hybridomas from HLA-DR3 transgenic mice, they demonstrated that a cross-reactive peptide from the von Willebrand factor type A domain protein (vWFA) produced by *Capnocytphaga ochracea* was a potent activator of T cells. *C. ochracea* is a gram-negative, anaerobic bacterium that is usually isolated in gingival sites and dental plaques, and so the disrupted mucosal barrier in the oral cavity or gut of SS patients may underlie the proliferation of pathogens capable of chronically activating the immune system, possibly by means of a mechanism of molecular mimicry.

In conclusion, the altered functioning of the mucosal and cutaneous barrier observed in SS may favour the colonisation of microbial pathogen species, which may further activate the immune response by molecular mimicry or by a direct interaction with TLRs on local dendritic cells. However, given the paucity of scientific evidence, the exact role of the microbiome in SS is still unclear.

3.4. Idiopathic Inflammatory Myopathies. No data are currently available concerning the microbial composition or pathogenic role of the microbiome in autoimmune myositides. Idiopathic autoimmune myopathies include dermatomyositis, polymyositis, necrotising autoimmune myositis, and sporadic inclusion-body myositis. Each form affects skeletal muscle and may overlap with other CTDs, but inflammatory myopathies differ in terms of their clinical aspects, histological findings, and myositis-specific antibodies [50].

There is very little evidence supporting a pathogenic role of dysbiosis in the onset of inflammatory myopathies. A review by Bleau et al. discussed the effect of a high-fat diet on intestinal microbial composition and its further repercussions on adipose tissue and skeletal muscles [51]. A high intake of saturated fatty acids may select the survival of LPS-bearing bacterial species, including Enterobacteriaceae, at the expense of Bacteroidetes, thus inducing the TLR activation of resident macrophages in the gut, adipose tissue, and skeletal muscles. Saturated fatty acids per se may also trigger TLR2 and TLR4 signalling, which may pave the way for chronic proinflammation and the consequent release of tumour necrosis factor-alpha (TNF-α), IL-6, and chemokines that may contribute to decreasing protein synthesis, oxidative stress, and insulin-resistance in skeletal muscle tissue. However, despite this favourable pathogenic pathway and some evidence indicating the potential development of inflammatory myopathies after infections or vaccinations [52], no study has yet investigated the differences in microbial taxonomic composition in autoimmune myopathies.

3.5. Other Connective Tissue Diseases. There are no available data concerning the role of the microbiome in undifferentiated CTDs (UCTDs), mixed CDT (MCTD), relapsing polychondritis, or other overlap syndromes.

3.6. Vasculitides. The vasculitides are a wide spectrum of diseases characterized by chronic inflammation of small, medium-sized, and large vessels. Healthy vessels may harbour their own commensal microbiome, but colonisation of the vascular tree by pathogenic microbial agents, including *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Staphylococcus*, and *Stenotrophomonas* spp., has recently been described in a number of vascular diseases, including atherosclerosis and aneurysms [53]. Although little evidence is currently available, it can be presumed that vascular dysbiosis may account for the chronic inflammation occurring in systemic vasculitides.

Giant cell arteritis (GCA) is a systemic granulomatous vasculitis affecting large vessels that mainly occurs in the elderly and has a typical cyclic pattern every 5–7 years. It has been postulated that an infectious stimulus may underlie its onset, but the etiological agent is still unknown. In a DNA sequencing study of the temporal artery biopsies of 17 GCA patients and five controls, Bhatt et al. did not find any distinctive microbiome signature in the patients and the isolation of *Propionibacterium acnes*, *Escherichia coli*, and *Moraxella catarrhalis* in both GCA and healthy samples mainly reflected external contamination [54].

Kinumaki et al. made a longitudinal metagenomic analysis to assess intestinal microbial variations in a cohort of 28 patients with Kawasaki disease (KD) [55], a form of vasculitis affecting the medium-sized vessels of mainly Asiatic children aged 6–11 months. It is usually associated with febrile spikes, lymphadenopathy, and mucosal and skin manifestations, which suggest an infectious etiopathogenesis although its cause is unknown. The authors found that five streptococcal species (*S. pneumonia*, *pseudopneumoniae*, *oralis*, *gordonii*, and *sanguinis*) and, in some cases, the genera *Rothia* and *Staphylococcus* were the most abundantly represented microorganisms in the intestinal tract of KD patients, and their prevalence increased during the phases of disease reactivation. On the contrary, the presence *Ruminococcus*, *Blautia*, *Faecalibacterium*, and *Roseburia bacteria* increased during disease remission.

There are no published data concerning dysbiosis in small vessel vasculitides, although it has been demonstrated that gut dysbiosis in glomerulonephritis mice models (with a prevalence of *Escherichia coli* or *Citrobacter rodentium*) may locally expand Th17 lymphocytes, which could then migrate to the kidney through a chemokine pathway involving C-C motif chemokine ligand 20 (CCL20) and C-C motif chemokine receptor 6 (CCR6) [56]. Accordingly, patients with antineutrophil cytoplasmic antibody- (ANCA-) associated vasculitis develop a form of necrotising glomerulonephritis sustained by the infiltration of Th17 cells that may be preactivated in the gut by a change in microbial composition. Disrupted gut tolerance of microbial agents may also be reflected by the detection of ANCAs in patients with inflammatory bowel diseases such as Crohn's disease, in whom

(together with anti-glycoprotein 2, anti-granulocyte macrophage colony-stimulating factor, anti-Saccharomyces cerevisiae antibodies, and other antibody subsets) they represent a valid means of diagnosing and monitoring the course of the disease [57].

A number of studies have investigated the role of dysbiosis in the pathogenesis of Behçet's disease (BD), a variable-vessel vasculitis characterized by recurrent mouth and genital ulcers, ocular inflammation, and skin rashes. Its etiopathogenesis is still unknown, but it shares some of the traits of autoinflammatory diseases. Coit et al. investigated the oral microbiome of 31 BD patients and 15 controls by analysing salivary samples using high-throughput sequencing of the 16S rRNA V4 region [58] and found a reduced prevalence of *Alloprevotella rava* and increased colonisation by *Haemophilus parainfluenzae*, neither of which was significantly affected by concomitant immunosuppressive treatments (cyclosporine A, azathioprine, and prednisone) or genetic predisposition (HLA-B/MICA locus variants), but they were slightly restored by periodontitis treatment. The same authors reported a significant reduction in species diversity in BD patients, as measured by the inverse Simpson index.

Seoudi et al. found differences in the microbial composition of the saliva of 54 BD patients, eight subjects affected by recurrent aphthous stomatitis, and 25 controls [59]. Nonulcerous BD oral cavities were mainly colonised by *Rothia denticariosa*, whereas ulcerous BD oral cavities had higher prevalence of *Streptococcus salivarius* and *Streptococcus sanguinis* than those of the subjects in the two control groups.

Using pyrosequencing of the V3-V4 hypervariable regions of the 16rDNA gene, Consolandi et al. found a significant reduction in the genera *Roseburia* and *Subdoligranulum* in fecal samples of 22 BD patients in comparison with 16 controls [60]. These microorganisms belonging to the Clostridiales order are responsible for the production of short-chain fatty acids (SCFAs) that have anti-inflammatory properties, and, accordingly, biochemical stool analysis revealed lower SCFA levels in the BD patients that positively correlated with the reduced *Roseburia* (but not *Subdoligranulum*) colonisation. Similarly, Shimizu et al. made a metagenomic analysis of stool samples from 12 BD patients and 12 healthy subjects [61] and found a reduced prevalence of *Clostridia* and an increased prevalence of *Actinobacteria* and *Lactobacillus* spp. in the patients, with only minimal effects of concomitant immunosuppressive treatments.

In summary, vasculitides represent a heterogeneous group of immune-mediated diseases sustained by different immunologic pathways and involving different-sized vessels. The role of microbiome in vessel inflammation may be based on microbiome unbalance in genetically predisposed patients. However, because data concerning vascular dysbiosis are still unvailable, it can be hypothesized that inflammation may arise from the attempt to counteract dysbiotic changes occurring in other districts, such as the gut or oral mucosa. Among vasculitides, only BD showed altered microbiome in saliva and stool, but the link between alterated microbiome and vascular inflammation is still unclear. Table 1 resumes the results of the principal studies evaluating dysbiosis in CTDs and vasculitides.

4. Discussion

The role of the microbiome in inducing and worsening autoimmune diseases has been widely investigated over the last years. The commensal and pathogenic microorganisms resident in the skin or mucosa are in mutual balance, protected by the integrity of the skin/mucosal barrier and the active surveillance of the innate and adaptive immune systems. The broad spectrum of CTDs is characterized by profound changes in the anatomical and physiological characteristics of mucosa and skin and therefore provides a predisposing environment for the onset of dysbiosis. Once it has occurred, dysbiosis leads to the release of microbial peptides and other molecules that amplify a state of chronic inflammation as a result of the activation of effector cells belonging to both innate and adaptive immune systems. There is considerable evidence that dysbiosis can trigger autoimmune diseases, including RA and SpA, but less is known about microbial composition in CTDs or vasculitides.

Nevertheless, studies of SLE and SSc have demonstrated an imbalance in the composition of the gut and mucocutaneous flora. A reduced Firmicutes/Bacteroidetes ratio has been found in the gut of SLE patients and reduced levels of Clostridiaceae in fecal samples of SSc patients. These changes in the microbial composition are at the basis of an increased production of proinflammatory FFAs and a reduction in the amount of anti-inflammatory SCFAs, with a subsequent modulation in the cytokine pattern and in the release of leptin. Moreover, reduced gut colonisation by Firmicutes has been related to the inbalance between Th17 and T regulator lymphocytes.

Chronic infections sustained by pathogen strains, including *Staphylococcus aureus*, may activate the innate immune system via the recognition of PAMPs by TLRs or NOD2, or following opsonization with C3 and immunoglobulins, thus fomenting the production of type I IFNs that represent a peculiar signature in SLE pathogenesis. So far, two studies have associated nasal colonisation by *Staphylococcus aureus* and periodontitis sustained by *Fretibacterium*, *Prevotella nigrescens,* and *Selenomonas* spp. with clinical manifestations and the production of some proinflammatory cytokines in SLE patients.

Alterations in the mycobiome may underlie fibrotic skin and lung diseases as the fibrotic skin areas of SSc patients may be colonised by *Rhodotorula glutinis*, which is known to be involved in fibrosing diseases.

Intestinal dysbiosis has also been demonstrated in SS patients, and, interestingly, gingival colonisation by *Capnocytphaga ochracea* in genetically predisposed subjects may lead to the activation of the adaptive immune system through the cross-reactivity against a von Willebrand factor type A domain protein (vWFA) that is produced by the microorganism and Ro-SSA self-antigens.

The evidence that dysbiosis may be involved in the pathogenesis of myositides and vasculitides is weaker, but some studies of BD patients have found taxonomic variations in the microbial composition of the gut that were not affected by concurrent immunosuppressive therapies. Similarly to SLE and SSc, a dysbiosis consisting in a reduction in

Table 1: Principal studies aiming to evaluate the alterations of microbiota in connective tissue diseases and vasculitides.

Author, year	Country	Models	Disease	Sample type	Technology employed	Implicated microbiota	Reference
Hevia et al., 2014	Spain	Human	SLE	Stool	16S rRNA (Ion Torrent PGM Sequencing, PCR analysis)	↓ Firmicutes/Bacteroidetes ratio in SLE pts than HC	[19]
He et al., 2016	China	Human	SLE	Stool	16S rRNA (Illumina Miseq)	↓ Firmicutes genera *Dialister* and *Pseudobutyrivibrio* ↑ Bacteroidetes *Rhodococcus*, *Eggerthella*, *Klebsiella*, *Prevotella*, *Eubacterium*, *Flavonifractor*, and Incertae sedis	[20]
Corrêa et al., 2017	Brazil and USA	Human	SLE	Subgingival dental plaque samples	V4 region of 16S rRNA (Illumina MiSeq)	Higher bacterial loads and decreased microbial diversity ↑ *Fretibacterium*, *Prevotella nigrescens*, and *Selenomonas*	[13]
Arron et al., 2014	USA	Human	SSc	Skin	Integrated Metagenomic Sequence Analysis, DNA microarrays, and 16S rRNA sequencing (Illumina HiSeq 2000)	No difference in bacterial microbiome between SSc and HC ↑ *Rhodotorula glutinis* in SSc	[41]
Andréasson et al., 2016	Sweden	Human	SSc	Stool	The GA-map™ Dysbiosis Test	↓ *Faecalibacterium* and *Clostridium* in SSc than HC More severe dysbiosis in pts with esophageal dysmotility, skin telangiectasias, pitting scars, pulmonary fibrosis, and elevated serum markers of inflammation	[35]
Volkmann et al., 2016	USA	Human	SSc	Cecum and sigmoid mucosal lavage samples	16S rRNA sequencing (Illumina HiSeq 2000)	↓ *Faecalibacterium* and *Clostridium* ↑ *Fusobacterium*, g-Proteobacteria, *Bifidobacterium* and *Lactobacillus* ↓ *Bacteroides fragilis* ↑ *Fusobacterium* in SSc patients with moderate/severe GI tract symptoms	[36]
de Paiva et al., 2016	USA	Human and mice	SS	Conjunctival samples, tongue samples, stool	Human: Ocular conjunctiva: V1-V3 region of 16S rRNA Tongue mucosa & stool: V4 region of 16S rRNA (454 Sequencing/Illumina Sequencing) Mice: Ready-To-Go™ You-Prime First-Strand kit	Mice stool: ↓ *Blautia*, *Alistipes*, *Lactobacillus*, *Allobaculum*, *Bacteroides*, *Desulfovibrio*, *Intestinimonas*, and *Clostridium* ↑ *Enterobacter*, *Parasutterella*, *Escherichia/Shigella*, *Pseudomonas*, and *Staphylococcus* Human stool: ↑ *Pseudobutyrivibrio*, *Escherichia/Shigella*, *Blautia*, and *Streptococcus* ↓ *Bacteroides*, *Parabacteroides*, *Faecalibacterium*, *Prevotella* versus HC Tongue samples: ↑ *Streptococcus*, ↓ *Leptotrichia* and *Fusobacterium*, ↓ *Bergeyella*, *Peptococcus*, and *Butyrivibrio* genera	[47]
Seoudi et al., 2015	UK	Human	BD	Saliva	Human oral microbe identification microarray (HOMIM) analysis	↑ *Rothia denticariosa* in BD and RAS ulcer sites ↑ *Streptococcus salivarius* in ulcer sites in BD versus RAS ↑ *Streptococcus sanguinis* in BD ulcer sites versus HC	[59]
Consolandi et al., 2015	Italy	Human	BD	Stool	Pyrosequencing of the V3-V4 hypervariable regions of the 16 rDNA gene and biochemical analysis	↓ *Roseburia* and *Subdoligranulum* than HC	[60]

Table 1: Continued.

Author, year	Country	Models	Disease	Sample type	Technology employed	Implicated microbiota	Reference
Coit et al., 2016	Turkey, USA, and Sweden	Human	BD	Saliva	V4 region of 16S rRNA (Illumina Sequencing)	↑ *Haemophilus parainfluenzae* ↓ *Alloprevotella rava* and species in the genus *Leptotrichia*	[58]
Shimizu et al., 2016	Japan	Human	BD	Stool	16S rRNA sequencing (Ion Torrent PGM)	↑ *Bifidobacterium* and *Eggerthella* ↓ *Megamonas* and *Prevotella* genera in BD pts versus HC	[61]
Bhatt et al., 2014	USA	Human	GCA	Temporal artery biopsy specimens	Illumina HiSeq V3 sequencing	↑ *Propionibacterium acnes* and *Escherichia coli* in GCA and HC	[54]
Kinumaki et al., 2015	Japan	Human	KD	Stool	Metagenomic Shotgun Sequencing (Illumina Sequencing)	↑ *Rothia*, *Staphylococcus*, and *Streptococcus* in the acute phase; ↑*Ruminococcus*, *Blautia*, *Faecalibacterium*, and *Roseburia* in the nonacute phase	[55]

SLE: systemic lupus erythematosus; SSc: systemic sclerosis, GI: gastrointestinal; SS: Sjögren's syndrome; BD: Behçet disease; RAS: recurrent aphthous stomatitis; GCA: giant cell arteritis; KD: Kawasaki disease; HC: healthy controls; pts: patients; PGM: personal genome machine; PCR: polymerase chain reaction; rRNA: ribosomal ribonucleic acid.

Clostridiales has been reported in fecal samples of BD patients, with a subsequent hypoproduction of SCFAs having anti-inflammatory properties.

An isolated study assessing the intestinal microbiome in patients affected by KD found an increased expression of *Streptococci*, *Staphylococci*, and *Rothia* during disease's flares. No studies addressed the role of dysbiosis in the onset of small vessel vasculitides, although some experiments on animal models could support the role of intestinal microbiome in the activation of autoreactive Th17 cells in ANCA-associated vasculitides.

Overall, the contribution of dysbiosis in tuning the immune response has a rationale in CTDs and vasculitides, although there are currently less evidences than in chronic autoimmune arthritides. Moreover, the multifaceted expression of these diseases raises the questions about the primitive district in which dysbiosis would occur. In chronic autoimmune arthritides, changes in gut microbiome have been considered the main source of immune system activation. Since joints are sterile sites, the most accredited hypothesis is that effector cells could be primed and activated following the presentation of microbial peptides in the gut and then migrate into joints where they could give raise to the inflammatory cascade via a mechanism of *molecular mimicry*. Alterations in the composition of the commensal flora harboured in the intestinal tract have been detected in SLE, SSc, and SS; however, dysbiosis occurring at mucosal sites, including the mouth, nose, and lungs as well as in the skin, may represent another trigger in these diseases characterized by a high burden of skin and mucosal inflammation. The impairment of the muco-cutaneous barrier, partly related to chronic inflammation, may further favour the chronicity of this mechanism by which pathogen species could survive and maintain inflammation in already inflammed sites. Several studies on BD, characterized by recurrent mouth aphtosis, have demonstrated an unbalanced flora of the mouth, which was associated also to disease activity. Similarly, the onset of other vasculitides may be related to alterations in vessel microbiome, but studies are lacking.

According to above described data, counteracting dysbiosis by means of prebiotics or probiotics may represent a useful tool in preventing or limiting inflammation in CTDs and vasculitides. Implementation with *Lactobacilli* or their substrates has shown some benefits in experimental studies on SLE human and animal cohorts. Unfortunately, no RCTs on the effectiveness of such strategies are available, and currently published studies are not conclusive due to heterogenicity in methodology. It is likely that a normocaloric diet with a high fiber and vitamin content together with a weight control could help patients in reducing the inflammatory burden and could be considered beside the pharmacologic intervention in the future algorithm for the treatment of these diseases.

5. Conclusions

In conclusion, several studies suggest that alterations in human microbiota may be involved in the pathogenesis of CTDs or vasculitides; however, due to the lack in a methodologic standardization, the absence of RCTs, the polyhedral manifestations of the diseases, and concomitant treatments as well as the still scarce amount of research in this field, further studies are needed to better understand the real impact of dysbiosis on the course of these diseases and to conceive preventive or therapeutic strategies to counteract microbiome-driven inflammation.

Conflicts of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 3432701, 10 pages
https://doi.org/10.1155/2017/3432701

Research Article

Beneficial Effects of Prebiotic *Saccharomyces cerevisiae* Mannan on Allergic Asthma Mouse Models

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Received 10 February 2017; Revised 31 May 2017; Accepted 4 June 2017; Published 1 August 2017

Academic Editor: Ansarullah

One of the unmet needs for asthma management is a new therapeutic agent with both anti-inflammatory and anti-smooth muscle (ASM) remodeling effects. The mannose receptor (MR) family plays an important role in allergen uptake and processing of major allergens Der p 1 and Fel d 1. We have previously reported that ASM cells express a mannose receptor (ASM-MR) and that mannan derived from *Saccharomyces cerevisiae* (SC-MN) inhibits mannosyl-rich lysosomal hydrolase-induced bovine ASM cell proliferation. Using a humanized transgenic mouse strain (huASM-MRC2) expressing the human MRC2 receptor in a SM tissue-specific manner, we have demonstrated that ASM hyperplasia/hypertrophy can occur as early as 15 days after allergen challenge in this mouse model and this phenomenon is preventable with SC-MN treatment. This proof-of-concept study would facilitate future development of a potential asthma therapeutic agent with dual function of anti-inflammatory and anti-smooth muscle remodeling effects.

1. Introduction

The currently available asthma therapeutics are effective in controlling inflammation but ineffective in controlling pathological changes of airway smooth muscle (ASM) remodeling that can occur at a young age [1, 2]. Therefore, an anti-inflammatory agent that can successfully inhibit ASM remodeling is extremely desirable to reduce asthma-related morbidity and mortality.

There has been a growing interest in the therapeutic use of living microorganisms (probiotics) for many human diseases, including asthma [3–5]. Similarly, prebiotics that are nonliving and indigestible polysaccharides can have beneficial effects on the host. Multiple mannose-rich oligosaccharides are capable of blocking antigen-driven T cell proliferation and antigen uptake and presentation [6, 7]. Acemannan from the Aloe plant induces maturation of dendritic cells [8] and inhibits the proliferative responses of tumor cells by affecting the expression of T lymphocytes [9]. Since the discovery of beer by a Mesopotamian farmer, *Saccharomyces cerevisiae* (Brewer's or Baker's yeast) is quantitatively and economically one of the most important groups of microorganisms exploited by man for food and alcoholic beverages. Mannan derived from *S. cerevisiae* (SC-MN), a prebiotic polymer of mannose (Man9 and a Gln residue, connected by α linkages), composes 45% of the cell wall of *S. cerevisiae* [10].

The mannose receptor (MR) family is an intricate part of innate immunity and the homeostatic clearance system [11] and plays an important role in uptake and processing of major allergens Der p 1 and Fel d 1, although the binding sites of these antigens differ [12, 13]. We have previously reported that airway smooth muscle cells express a mannose receptor (ASM-MR) [14] and SC-MN can inhibit bovine

ASM cell proliferation induced by endogenous mannosyl-rich MR ligands such as lysosomal hydrolases (β-hexosaminidases Hex A and Hex B and β-glucuronidase) [15].

In a murine allergic asthma model, mycobacterial acyl chains and mannose groups of lipoglycans have been shown to suppress allergic disease and increase IL-10 secretion from cervical lymph nodes and splenocytes [16]. We have chosen to investigate the effects of SC-MN because mannan from pathogenic microorganisms is capable of eliciting IL-17 production [17]. However, this is not true of SC-MN [17], making it an appealing therapeutic agent as its beneficial effects could be achieved without IL-17-driven inflammation. We have confirmed this finding in our study as well as documented its lack of ability to induce IL-13 production in the lungs of mice despite a potential concern of its ability to regulate dendritic cell function in favor of T_H2 polarization [18]. Furthermore, SC-MN had no effect on IL-33 that can stimulate group 2 innate lymphoid cells important in fungal allergy [19].

Here, we present evidence that by cloning of the huASM-MRC2 and development of a humanized transgenic mouse model, overexpression of huASM-MRC2 results in accelerated ASM remodeling and SC-MN can offer dual anti-inflammatory and anti-smooth muscle remodeling benefits.

2. Materials and Methods

2.1. Animals, Cloning huASM-MRC2, and Transgene Construct. All protocols were approved by the University of Tennessee Health Science Center (UTHSC) Institutional Biosafety Committee and Institutional Animal Care and Usage Committee. Wild-type (WT) FVB/NJ and BALB/c were purchased from the Charles River Laboratories or the Jackson Laboratory and housed in a pathogen-free vivarium at the UTHSC. The human ASM-MRC2 was cloned by RT-PCR using primers designed from cDNA of MRC2 and mRNA isolated from human bronchial ASM cells. The human ASM-MR cDNA (5658 bp) is 99% identical to the EST clone KIAA0709 (5641 bp; adult human brain), and the coding region is identical to that of MRC2 cDNA (Endo180, 4639 bp). Full-length cDNA of the huASM-MRC2 coding region was reconstructed, and a transgenic mouse model overexpressing huASM-MRC2 from the smooth muscle-specific SM22α gene promotor was developed for the purpose of testing their susceptibility to atopic asthma phenotype. Sense and antisense oligonucleotide primers were designed based on the deposited sequence of SM22α gene (5′ region and exon 1; 3892 bp; accession U36589). Hind III sites were added to the 5′ end of both primers for cloning purposes. The primers were used to generate a 502 bp fragment containing the 445 bp SM22 alpha promoter including exon 1 [20] by PCR on genomic mouse DNA. The promoter fragment was ligated to the 5′ end of the full-length coding region of ASM-MR cDNA (4437 bp) in the plasmid pCR 3.1-Uni, and a fragment containing the β-globin intron and polyadenylation signal (1.2 kb) was ligated to the 3′ end of ASM-MR cDNA. The 6.2 kb transgene fragment was excised with the restriction endonuclease Pme I and used to generate transgenic mice in the FVB/NJ strain.

The transgenic mice (FVB/NJ) were then backcrossed onto an allergy-prone BALB/c strain, and N10 generation mice of ages 6–8 wk were used for the study. Transgene expression was confirmed using qPCR by Transnetyx Inc. (Memphis, TN, USA): target sequence 3′-cagcgaggacctatgtgct ctgccctacacgaggtctacaccatccagggaaactcccacggaaagccg-5′.

For smooth muscle remodeling studies, mice with qPCR signal values between 7.5 and 13.0 were used.

2.2. Allergen Sensitization and Challenge. WT BALB/c or humanized transgenic mice were OVA-immunized on days 0 and 14 with 20 μg OVA (Sigma, St. Louis, MO, USA) in Imject® Alum (Pierce, Rockford, IL, USA) [21], i.p.; nonimmunized (sensitized) mice received alum only. Increased serum OVA-specific IgE levels on day 1 versus day 21 were confirmed using ELISA (AbD Serotec, Oxford, UK). Responsive mice (sIgE $\geq$ 500 ng/ml) were pretreated with control saline or SC-MN (Sigma, prepared for patented use for asthma therapeutic, endotoxin level < 2 EU/ml) 30 min before OVA challenge on days 28, 29, and 30 (100 μg OVA i.n. in 25 μl phosphate-buffered saline (PBS).

2.3. Airway Physiology Measurements. To assess isolated thoracic flow, compartmentalized double chambers (Buxco) were used to exclude nasal airway resistance. Conscious spontaneously breathing animals were retrained in the chambers to analyze thoracic-specific airway resistance (sRaw) in response to aerosolized saline or escalating doses of Mch (2.5–25 mg/ml, 1 ml) for 3 min [22]. Readings were taken averaged for 5 min following each nebulization.

2.4. Liposome Construction. Liposomes were prepared by the evaporation method using different molar ratios of HSPC : Chol : DSPE-PEG2000 (e.g., 50 : 45 : 5) (Dr. George C. Wood, Department of Pharmaceutical Sciences, UTHSC). Appropriate quantities of lipids and near-infrared fluorescent dye (DiIC18 = DiR) were dissolved in 1 : 9 solvent mixtures of chloroform and methanol. The mixture was evaporated in a Rota-evaporator at 40°C under vacuum at 100 rpm overnight. The lipid film was hydrated at 65°C with HEPES buffer containing 0.15 M NaCl and 10 mM EDTA on the Rota-evaporator at 100 rpm for 2 hr. The resulting fluorescent liposomes were large multilamellar vesicles of approximately 0.8–1.0 μm. Depending on the vesicle size required, liposomes were extruded through stacked nucleopore filters for a total of 6–10 passes, producing a narrow particle size distribution of unilamellar liposomes. Targeting of PEG-maleimide liposomes with the cRGD peptide was performed via covalent coupling through thioester bond formation between the maleimide group of the liposomes and cyclic RGD [23].

2.5. Fluorescent Reflectance Imaging (FRI). On day 32, 48 hr after OVA challenge, mice were anesthetized using isofluorane. Fluorescent liposome (-DiR) (800 nm, 90 μg/ml, 25 μl, each nostril, i.n.) or fluorescent cRGD-liposome (-DiR) (80 nm, 20 μg/ml, 200 μl, i.v.) was administered to the mice. The imaging procedures were performed at 2, 6, and 24 hr postadministration of fluorescent liposome with fixed fiducial markers for optical imaging with different dye

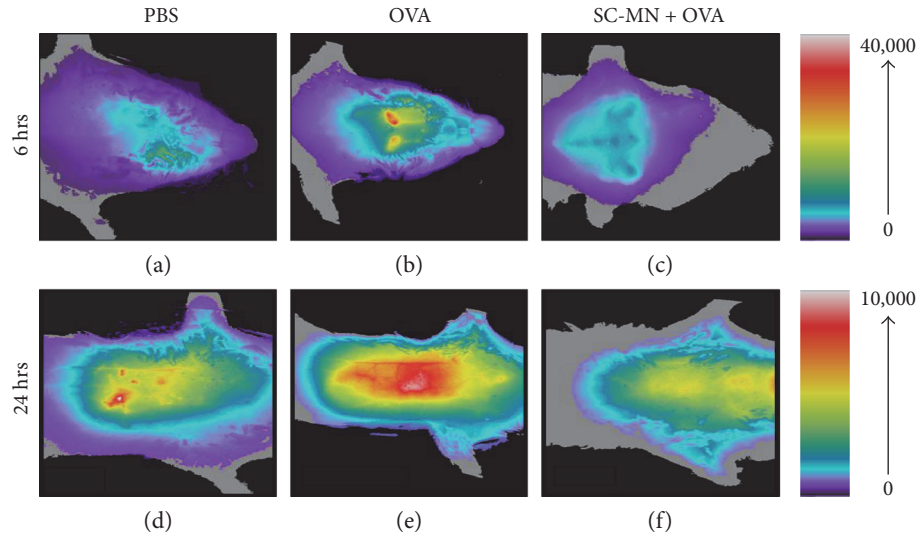

Figure 1: Homing of cyclic RGD-liposomes to cervical lymph nodes and lungs in allergic asthmatic mice. Cyclic RGD- and DiR-labeled liposomes were injected intravenously into OVA-allergic BALB/c mice. (a, d) PBS-challenged; (b, e) OVA-challenged; (c, f) mannan pretreatment (1 mg, i.n., 30 min before each of the three OVA challenges). FRI images were acquired 2, 6, and 24 hr postinjection. The intensity of the signals in the cervical lymph nodes (c) and lungs (f) in SC-MN-pretreated mouse was markedly diminished compared to that in the OVA-challenged mouse (b, e). Signals in the lungs were detected at the 24 hr time point (d–f). The results are representative of 2 separate experiments with similar results.

concentrations as a standard. The FRI system includes a liquid nitrogen CCD camera (Photometric Chemipro, Roper Scientific, Trenton, NJ) mounted onto a darkbox and a shutter mounted with a filter wheel (Sutter Instruments Co., Lambda 10-2 Optical Filter Changer, Novato, CA) that allows for multiple excitation filters to be controlled through software (Metamorph, Universal Imaging, Downingtown, PA). An excitation filter centered at 710 nm (Chroma Technology Corp., Rockingham, VT) and a 3-cavity narrow bandpass filter (10 nm spectral width) (780 nm at peak wave) (Andover, Salem, NH) were used to visualize the near-infrared light. The biologic autofluorescence region is approximately 400–600 nm wavelength, and therefore, DiR signals were able to be detected without interference by autofluorescence.

2.6. Bronchoalveolar Lavage (BAL) and Lung Tissue Collection. Bronchoalveolar lavage was performed with 0.5 ml of warm PBS (37°C) under ketamine/xylazine sedation (100 mg/kg each). Cell count was performed by cytospin, and cell-free BAL fluid (BALF) was stored at −80°C. Total lung tissue was homogenized in 1 ml of PBS containing protease inhibitor cocktail (Sigma) on ice. Protein was quantified by using a Bradford Protein Assay kit (Thermo Fisher). Both BAL fluids and lung homogenates were analyzed for cytokines by a Quantikine ELISA kit from R&D systems according to the manufacturer's instructions. Muc5ac in BAL fluid was analyzed by an ELISA kit (USCN Life Sci. Inc., Houston, TX) according to the manufacturer's instructions.

2.7. IL-10 ELISPOT Analysis. Naïve mice were fed once with SC-MN (80 mg/kg) or saline, via gavage. Eighteen hours later, BAL cells and unselected splenocytes (1×10^5 cells/well of each cell type) were plated onto a Millipore microtiter well plate for IL-10 ELISPOT analysis. Cells were stimulated with or without SC-MN (1 mg/ml) and cultured for 72 hrs at 37°C in CO_2 incubator (5% CO_2/95% air). The IL-10-producing cells were identified following the manufacturer's instructions (BD Pharmingen) and sent out to BD Biosciences analysis service.

2.8. Histology, Immunohistochemistry, Histochemistry, and Analysis. Mice were sacrificed (cervical dislocation under anesthesia) and perfused with 4% paraformaldehyde. Paraffin-embedded trachea and lung tissue sections were cut at 10 μm for H&E staining, immunohistochemistry was performed for smooth muscle isoactin, and Alcian blue-periodic acid Schiff (PAS) stains were used for mucin-containing goblet cells. Smooth muscle α-isoactin was probed with the α-smooth muscle actin antibody clone 1A4 according to the manufacturer's instruction using a kit (Sigma). All slides were scanned using the ScanScope® XT at 0.25 μm/pixel resolution (~400x magnification). Trachea cross sections were analyzed for the trachealis muscle area at day 38, 45, or 52. The small airway smooth muscle area was analyzed by selecting 3–5 small airway branches with cross-sectional cut (120–300 μm calibers) at day 45. Data collection and analyses were performed blinded ($n = 5$-6 mice) using the fiduciary marker 200 μm average (i.d.) airway caliber and 31,416 μm^2 luminal area. Each data point represents an averaged value from one mouse.

2.9. Statistical Analysis. All animal experiments were repeated three times. Data are expressed as mean or median ± 95% CI (for PC200R, provocative methylcholine concentration effecting a 200% increase in airway resistance) or mean ± SD (or SEM) and analyzed by the Kruskal-Wallis test followed by post hoc tests for unpaired data or one-way

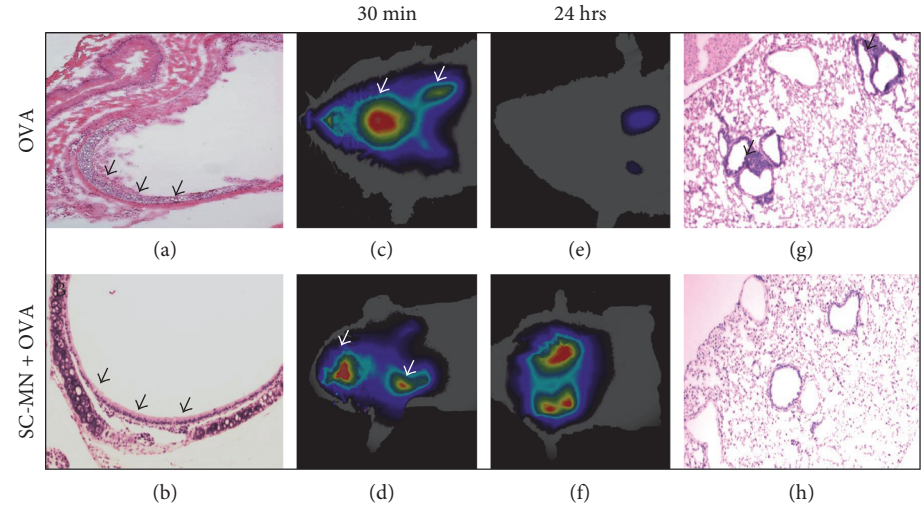

Figure 2: Effect of SC-MN on OVA-induced airway pathology in BALB/c mice. Upper panels: OVA-allergic BALB/c mouse. Lower panels: SC-MN-treated allergic BALB/c mouse (1 mg, i.n., 30 min prior to each OVA challenge). (a) H&E staining of the trachea shows epithelial denudation (arrow) compared to that of the intact epithelium in SC-MN-treated mouse (b). (c–f) FRI of mice 30 min (c, d) and 24 hrs (e, f) post i.n. administration of liposome. Notice the increase in central airway signals (arrow head) with less lung deposition (c) and faster clearance (arrow) (e) in untreated allergic mouse compared to the SC-MN-treated mouse (d, f). Images of the abdomen showed no signals in the stomach. Color intensity: red > yellow > green > blue. The FRI images are representative of 2 separate experiments with similar results. (g) Peribronchial cellular infiltration in H&E lung sections compared to the paucity of peribronchial cellular infiltration in SC-MN-treated mouse (h), representative of 3 separate experiments. Final magnification: 100x.

Table 1: Effect of intranasally administered SC-MN on airway inflammation in WT BALB/c allergic mice.

Aerosol challenge	Total cell number/ml	Macrophages	Lymphocytes	Eosinophils	Neutrophils
PBS	326,000 ± 6000	245,000	55,000	9780	6520
OVA	530,000 ± 30,000*	260,000	196,000	27,000	11,000
SC-MN + OVA	242,000 ± 18,000	138,980	96,960	4040	2020

Mice pretreated with SC-MN (i.n.) showed marked reduction in total BAL cell numbers compared to the OVA group. The decrease is also reflected in all four cell types analyzed. Results are mean ± SEM for total cell numbers in BALF and mean differential cell counts ($n = 3$). $^*P < 0.05$, OVA group versus PBS or SC-MN + OVA group.

analysis of variance followed by Dunnett's post hoc test using Prism 6 software (GraphPad, San Diego, CA). For FRI data, software (Metamorph, Universal Imaging, Downingtown, PA) was used to quantify the intensity of fluorescence. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Anti-Inflammatory Effects of SC-MN on OVA-Allergic WT BALB/c Mice. For a global view of anti-inflammatory effects of SC-MN, we employed a whole-body imaging system to identify sites of inflammation; fluorescent cyclic arginine-glycine-aspartic acid- (cRGD-) labeled liposomes were used to detect integrin upregulation as a marker of inflammation. Intravenously (i.v.) administered cRGD first homed to the cervical lymph nodes and then to the lungs in the OVA-allergic mice, which was partially mitigated by intranasally (i.n.) administered SC-MN treatment. The upper limit of signals from lymph nodes was 4 times higher than that of the signals in the lungs. Cyclic RGD homing to the cervical lymph nodes and lungs was prominent compared to the control or SC-MN treatment in mice (Figures 1(a), 1(b), 1(c), 1(d), 1(e), and 1(f)), suggesting that SC-MN treatment suppressed inflammation in the draining lymph nodes and lungs. Intranasal pretreatment with SC-MN (1 mg, 30 min before each of three OVA challenges) inhibited allergic airway inflammation allowing i.n. administered liposome to reach the lungs of SC-MN-pretreated allergic mice, compared to the little deposition of liposome in the lungs of control allergic mice (Figures 2(a), 2(b), 2(c), 2(d), 2(e), 2(f), 2(g), and 2(h)). This finding is presumably due to

Table 2: A single oral administration of SC-MN increases IL-10 producing cell numbers in naïve mice.

Specimen	Gavage feeding	Immunospot	Mean spot sizes
BAL	Saline	163 ± 161	0.0052 ± 0.0022
BAL	SC-MN	586 ± 211*	0.0060 ± 0.006
Splenocytes	Saline	35 ± 13	0.0133 ± 0.0064
Splenocytes	SC-MN	427 ± 221	0.0077 ± 0.0009

Results are mean ± SEM ($n = 3$). We observed that administering SC-MN to mice increased the number of IL-10-producing cells in the BAL ($^*P < 0.05$). A similar trend was seen for splenocytes from SC-MN-treated mice.

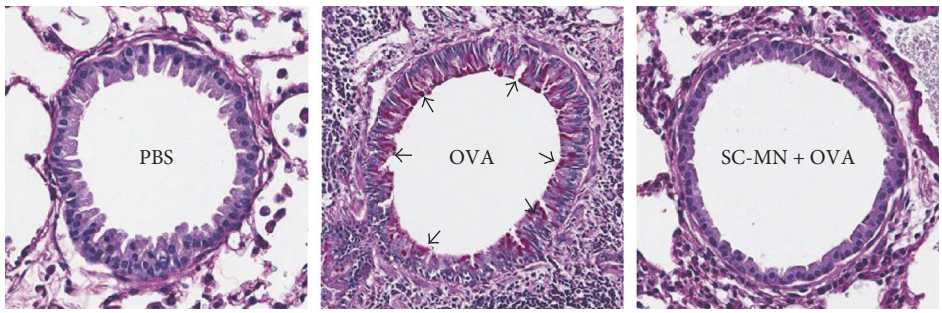

Figure 3: Effect of SC-MN on airway mucin in allergic mice. PAS staining visualizes neutral mucins (magenta material indicated by arrows) from tissue morphology at day 31. Intranasally administered SC-MN effectively blocked the ovalbumin- (OVA-) induced increase in mucin in small airway tissue sections. Similar results were obtained at day 45.

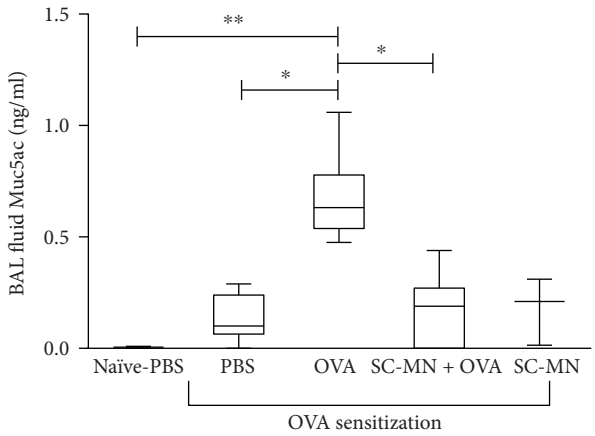

Figure 4: SC-MN effectively inhibits Muc5ac protein levels in BAL fluid of allergic mice. BAL fluid was obtained at day 45. Results are mean ± SEM ($n = 3$ for naïve control with PBS challenge); $n = 7$ for OVA-sensitized groups). $^{**}P < 0.01$ versus PBS-challenged naïve mice; $^{*}P < 0.05$ versus PBS-challenged allergic mice or SC-MN-treated allergic mice.

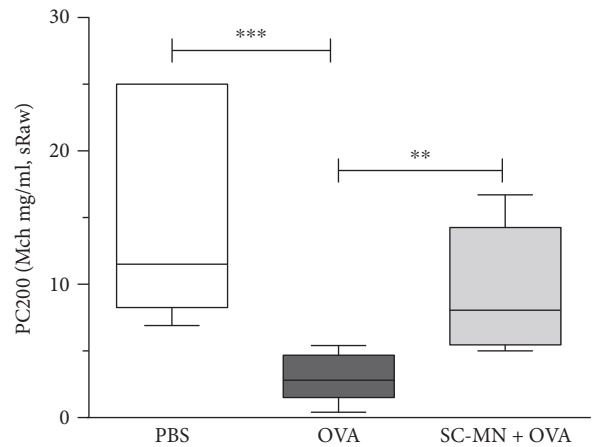

Figure 5: Inhibition of AHR by intranasal SC-MN in WT BALB/c OVA-allergic mice. All mice were OVA-sensitized and OVA-challenged, and some mice were pretreated with SC-MN 30 min before each challenge with OVA. Thoracic PC200R (sRaw), median ± 95% CI. $^{**}P < 0.05$ versus OVA challenge with saline pretreatment. $^{***}P < 0.001$ versus PBS challenge with saline pretreatment.

mucociliary clearance of the liposome particles in the inflamed airways of untreated allergic mice. Allergic mice pretreated with SC-MN showed marked inhibition of cellular infiltration (Table 1).

Similar to the report on lipoglycan [16], SC-MN can stimulate IL-10 regulatory cytokine as shown in Table 2.

To confirm that SC-MN does not cause potentially harmful effects of IL-17 production [17], T_H2 polarization [18], or IL-33 production that can stimulate ILC2 cells [19], we have measured IL-13, IL-17, IL-22, and IL-33 in BALF and supernatants of lung homogenates in naïve BALB/c mice after daily SC-MN (or saline) for 3 consecutive days. There was no increase in any of the abovementioned cytokines in SC-MN- treated naïve BALB/c mouse lung homogenate supernatants: IL-13, 115 ± 82 versus 51 ± 11; IL-17, 125 ± 31 versus 123 ± 28; IL-22, 8 ± 7 versus 8 ± 9; and IL-33, 5792 ± 2012 versus 5553 ± 1257 pg/mg protein (saline versus SC-MN group, resp., $n = 7$ mice). Similarly, there was no discernable difference in any of these cytokines measured in the BALF of these two groups.

3.2. Effect of SC-MN on Airway Mucin. Mucous hypersecretion from goblet cells is a hallmark of asthma, which can lead to airway obstruction and increased morbidity [24]. The effect of SC-MN on airway neutral mucin was examined by PAS staining in the midlung sections (Figure 3); stored mucin within the epithelium was inhibited by SC-MN in allergic mice (59–81% inhibition, $n = 6$). Muc5ac protein levels in BAL fluid were significantly elevated in allergic mice that were effectively inhibited by SC-MN treatment (Figure 4).

3.3. Inhibition of AHR by SC-MN (Intranasal) in WT Allergic BALB/c Mice. Based on preliminary dose response studies (6–60 mg/kg), we have selected 45 mg/kg as an optimum efficacious SC-MN dose targeting ASM cells and thoracic sRaw parameter. Mice pretreated with SC-MN (45 mg/kg, i.n., 30 min before OVA challenges) showed significantly blunted AHR, measured by PC200R (provocative Mch concentration effecting a 200% increase in airway resistance), compared to untreated allergic mice (OVA group) (Figure 5).

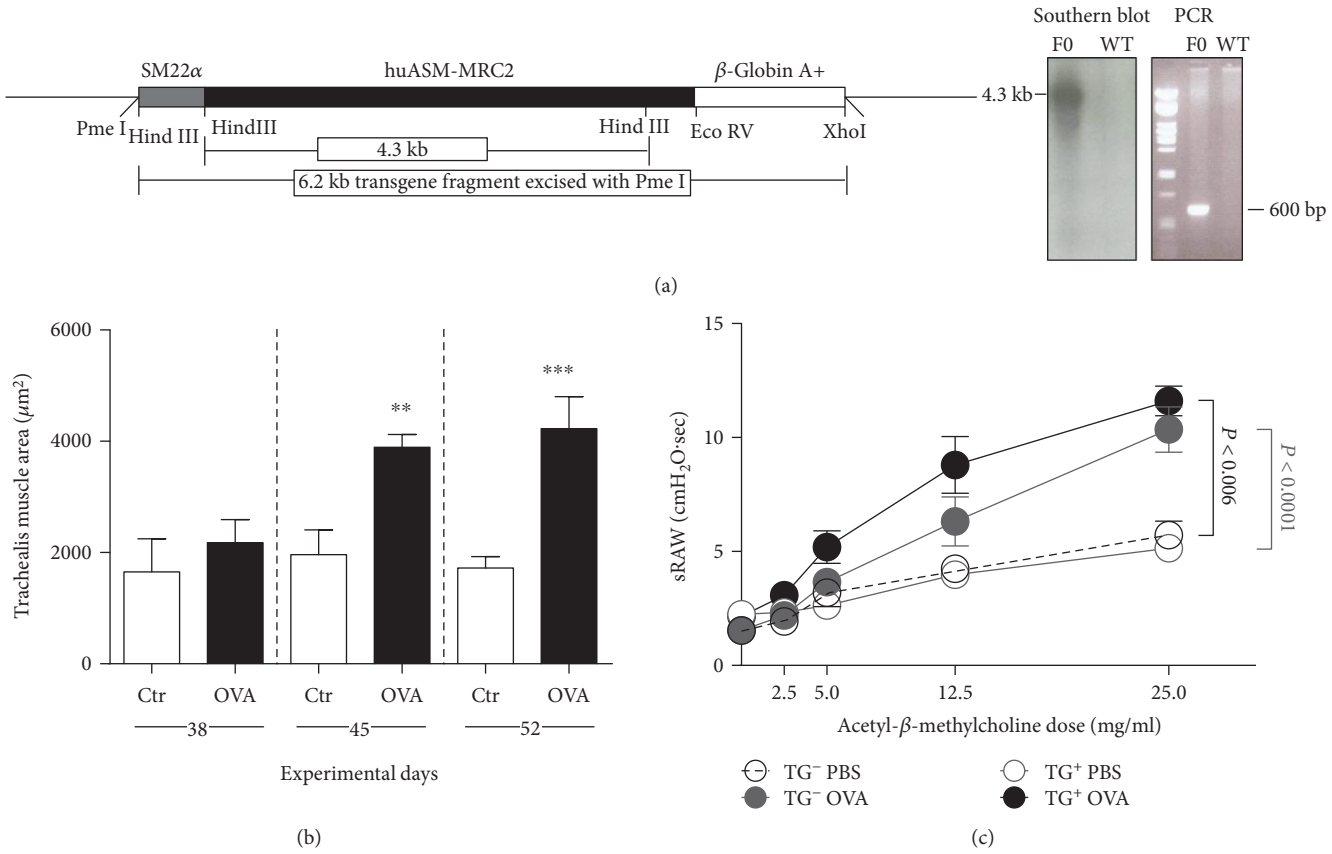

Figure 6: Accelerated large airway smooth muscle remodeling in a huASM-MRC2 transgenic allergic asthma mouse model. (a) Schematic of the construct used to generate huASM-MRC2 transgenic (Tg+) mice. The resulting transgenic founders were confirmed by Southern blot, probing Hind III-digested DNA with a 1 kb fragment of the huASM-MRC2 gene (hybridizing to a ~4.3 kb DNA fragment), and PCR analysis using huASM-MRC2 oligonucleotide primers (600 bp product). (b) Mice were OVA-sensitized and OVA-challenged. Control mice received OVA sensitization and PBS challenge. Histology was performed on day 38, 45, or 52. Results are mean ± SD ($n = 6$–11). $^{**}P < 0.01$. $^{***}P < 0.001$. (c) Thoracic sRaw in allergic huASM-MRC2 transgenic mice (N10, backcrossed onto BALB/c). A shift of the curve to the left indicates Mch sensitivity (AHR). Numerous breaths were rejected from the automated data acquisition due to shallow labored breathing at Mch 25 mg/ml. Data are mean ± SD ($n = 6$).

3.4. Accelerated ASM Remodeling in a huASM-MRC2 Mouse Model. Groneberg et al. have demonstrated airway smooth muscle remodeling in OVA-allergic BALB/c mice using a long-term, labor-intense protocol [24]. We have developed a mouse model to substantially shorten the process from a total of 163 days to 45 days (Figures 6(a) and 6(b)). Trachealis muscle mass in OVA-sensitized and OVA-challenged mice remained unchanged in WT (data not shown) and Tg-negative littermate control groups at day 52 (Figure 6(b)). The transgenic allergic mice showed AHR by a shift to the left of the thoracic airway resistance curve (Mch sensitivity) compared to Tg-negative littermate control allergic mice (Figure 6(c)). At the maximum dose of Mch (25 mg/ml), numerous breaths of Tg+ allergic mice were rejected from data acquisition due to shallow labored breathing.

3.5. Inhibition of Large and Small Airway Smooth Muscle Remodeling and AHR by SC-MN in Smooth Muscle-Specific huASM-MRC2 Transgenic Allergic Mice. The level of β-Hex in the BAL fluid of allergic mice is 3 times higher than that of naïve counterparts (data not shown). To examine the impact of blocking ASM-MR/β-Hex-mediated remodeling using SC-MN, the universal allergen OVA was used to induce general allergic inflammatory states in transgenic mice. SC-MN not only significantly inhibited large and small airway smooth muscle mass (Figures 7 and 8) but also inhibited the increase in the number of small airway smooth muscle nuclei (Figure 8(d)). SC-MN treatment (45 mg/kg) significantly restored the decrease in PC200R seen in transgenic allergic mice (Figure 9).

4. Discussion

In this study, we have demonstrated the efficacy of SC-MN in suppressing inflammation including stored and secreted mucin and airway hyperreactivity in WT BALB/c allergic mice. In addition, our newly developed transgenic huASM-MRC2 mouse model enabled us to demonstrate ASM remodeling in a reasonable experimental time period, compared to other models [25], that was successfully modified by SC-MN. Airway hyperreactivity was inhibited in this model as well. Airway remodeling in this model showed two elements:

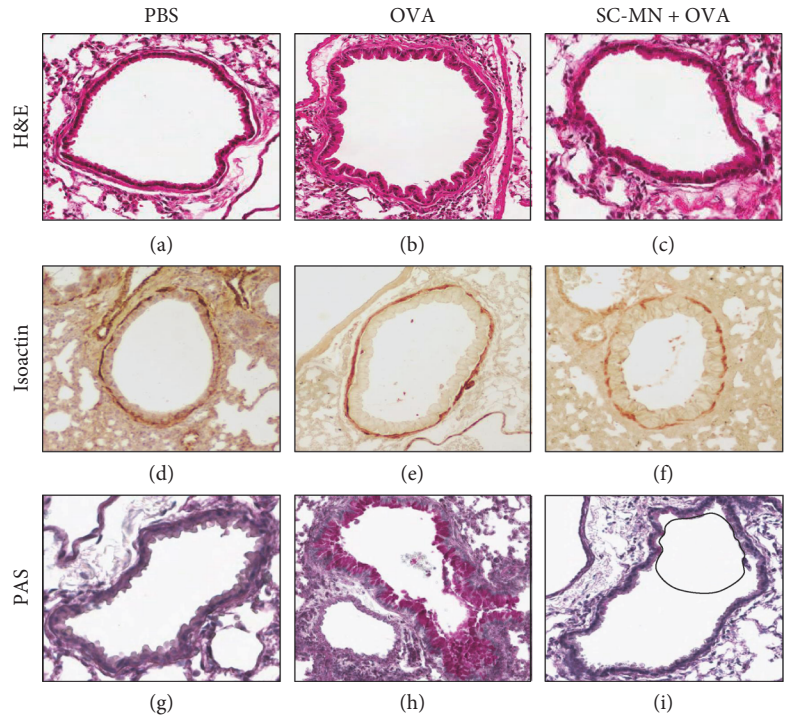

Figure 7: Intranasal mannan blocked the OVA-induced increase in small airway smooth muscle as well as in cellular infiltration, in allergic transgenic mice. OVA-sensitized mice treated with SC-MN prior to OVA or PBS challenge. Representative sections show tissue morphology at day 45, visualized by H&E staining (midlevel left lung). (a) Saline + PBS (control); (b) saline + OVA; (c) SC-MN 45 mg/kg + OVA. Isoactin staining shows organized and thickened ASM in the OVA group (e) compared to the control (d) and SC-MN-treated mice (f). PAS staining shows magenta neutral mucin staining in OVA-sensitized and OVA-challenged mice (h).

(1) an accelerated increase in ASM mass and (2) proliferation of ASM, indicated by ASM nucleus count. Both of these elements were effectively inhibited by SC-MN, consistent with antimitogenic/antiproliferative benefits seen in bovine ASM cells [15].

The mannose receptor blocker, mannan, elicits structure- and source-dependent differential responses in dendritic cells [26]. For instance, the response to monomeric engagement of mannan-MR stimulates IFN-γ and IL-12, aiding host immunity against pathogens and regulating T_H2 microenvironment. On the other hand, mycobacterial lipoglycans have lipid appendages tending to aggregate MRs and suppress IFN-γ and IL-12 production, causing immunosuppression in host. The SC-MN preparation used in this study lacks acyl moieties (confirmed by Avanti Polar Lipids Inc. analytical service). Therefore, a higher dose of SC-MN compared to that of lipoglycans [16] was required to exert an anti-inflammatory effect. Regarding the mechanism of anti-inflammation, a single dose of oral SC-MN to naïve FVB/NJ mice, followed by *in vitro* stimulation with SC-MN for 72 hr, resulted in IL-10 production by BAL cells and splenocytes. Sayers et al. also reported a robust induction of IL-10 by lipoglycan from splenocytes and mediastinal lymph nodes from allergic mice using anti-CD3/CD28 stimulation *in vitro* [16]. However, transgenic overexpression of IL-10 can cause lung fibrosis, suggesting potential harmful effects of excess IL-10 beyond its beneficial immunoregulatory role [27]. There are multiple advantages of SC-MN over mannan from other sources: SC-MN is water-soluble and the molecular mass is relatively small (36 kD) compared to that of MN from *Aloe barbadensis* (1000 kD) [8]. Additionally, acemannan and MN from many other sources have beta linkages that are likely to be antigenic [28].

Our findings highlight an important role of huASM-MRC2 (a phylogenetically conserved pattern recognition receptor) that is encoded within chromosome17q23.2 near ORMDL3 17q12-21 of which pathogenic variants increase the risk of asthma [29]. Therefore, members of the MR family, endogenous ligands, and receptor blockers can be exploited for the treatment of human asthma and other diseases. Glycoprotein ligands for the MR family members may be distinct with predilection to bind to different domains [30], and specific function of each member may be diverse depending on the type of ligand, cell, and tissue. For instance, complex protease-resistant endogenous glycoproteins bind to MR at the high-affinity binding domains (CTLD4-5) while SC-MN binds through CTLD4–8 [14]. Single-nucleotide polymorphisms (SNPs) of MRC1 have been associated with asthma in Japanese and African-American populations [31]. Investigation to determine possible association between MRC2 SNPs and asthma is underway. It is reasonable to assume that a family of receptors with such an intricate role in innate and adoptive immunity as well as homeostatic clearance could play an important role in immunomodulation. Recently, gene variants of glycoproteins, affecting serum levels of respective glycoproteins, have

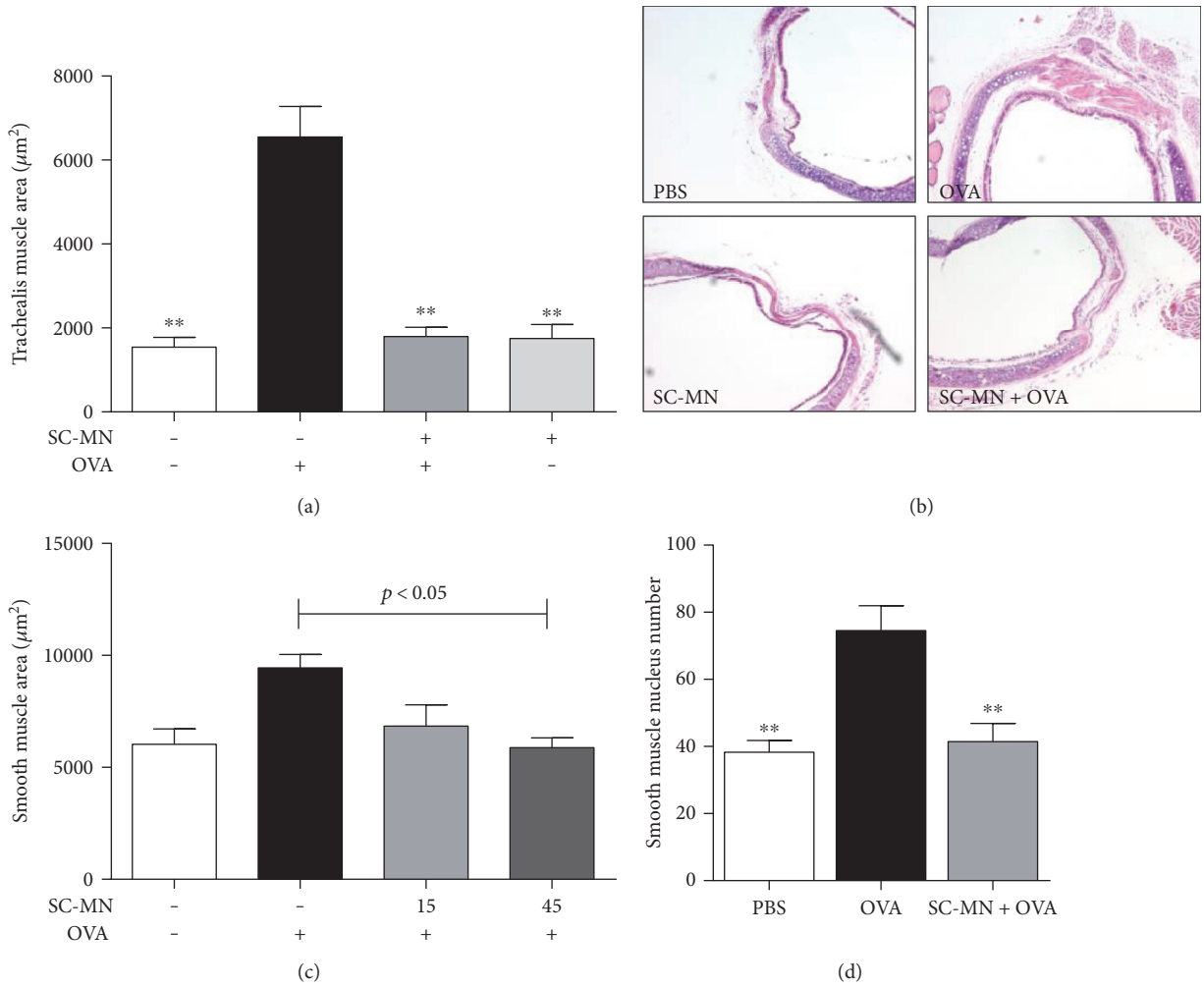

Figure 8: Intranasal SC-MN pretreatment inhibited the OVA-induced increase in small airways ASM, trachealis muscle mass, and number of ASM nuclei of small airways in huASM-MRC2 mice. (a) SC-MN inhibited the trachealis muscle remodeling induced by OVA (day 52) ($n = 6$–11); (b) H&E staining of trachea cross section; (c) small airway ASM area (day 45) ($n = 5$-6); (d) SC-MN inhibition of the increased small airway ASM nuclei in allergic transgenic mice ($n = 4$–7). $^{**}P < 0.01$ versus OVA-challenged group with saline pretreatment.

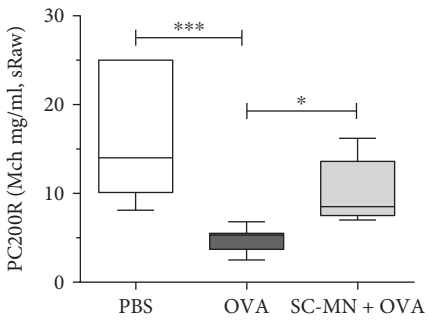

Figure 9: Inhibition of AHR by intranasal SC-MN in huASM-MRC2 transgenic OVA-allergic mice. All mice were OVA-sensitized and OVA-challenged, and some mice were pretreated with SC-MN 30 min before each challenge with OVA. Thoracic PC200R (sRaw), median ± 95% CI. $^{*}P < 0.05$ versus OVA challenge with saline pretreatment. $^{***}P < 0.001$ versus PBS challenge with saline pretreatment ($n = 7$ in each group).

been reported as important factors in asthma [32]. Regarding glycoprotein endogenous ligands for MR, β-hexosaminidase levels are significantly elevated in serum of severe asthmatic patients [33]. There is also a growing interest in immunoregulatory capacity of glycans and glycan-binding proteins such as galactins, selectins (C-type lectins, MR is a ligand for L-selectin) [34], and siglecs [35].

In conclusion, the prebiotic mannose receptor blocker SC-MN is a promising agent that can render dual benefits in asthma: anti-inflammatory and anti-smooth muscle remodeling at the level of both large and small airways.

Abbreviations

AHR: Airway hyperreactivity
ASM: Airway smooth muscle
BAL: Bronchoalveolar lavage
β-Hex A and B: β-Hexosaminidase A and B (aka NAGA (N-acetyl-β,D-glucosaminidase), NAG, and NAGase)

CCD: Charge-coupled device
CTLD: C-type (calcium-dependent binding) lectin-like domain
cRGD: Cyclic arginine-glycine-aspartic acid (D)
DiR: DiIC18-indotricarbocyanine iodide-phycoerythrin
DSPE-PEG: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol
FRI: Fluorescent reflectance imaging
huASM-MRC2: Mannose receptor C-type 2 cloned from human airway smooth muscle cells
MRC1: C-type mannose receptor 1 (CD206, macrophage mannose receptor)
MRC2: C-type mannose receptor 2 (CD280, Endo180, urokinase plasminogen activator receptor-associated protein)
OVA: Ovalbumin
PC200R: Provocative methacholine (methylcholine) concentration effecting a 200% increase in airway resistance
PC: Phosphatidylcholine
SC-MN: Mannan derived from *Saccharomyces cerevisiae*.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors wish to acknowledge Dr. Erik Bonten, Dr. Alessandra d'Azzo, and St. Jude Children's Research Hospital Transgenic Core Facility for huASM-MRC2 transgenic mouse development; Dr. George C. Wood (UTHSC, Department of Pharmaceutical Sciences) for the liposome construction; Dr. Elizabeth A. Tolley (UTHSC, Department of Biostatistics and Epidemiology) for the statistical consultation; Histology Digitizing Service Core at UTHSC; and Yuling Zhao, Ethan Stranch, Gregory McGraw, Ashley Roberson, and Julie Chang for the technical assistance. This work was supported in part by the American Academy of Allergy, Asthma, and Immunology/Aventis (D. Betty Lew), NIH R01HL56812 and R41HL090108 (D. Betty Lew), University of Tennessee Research Foundation-Cumberland Pharmaceuticals & Emerging Technologies (D. Betty Lew), and University of Tennessee Clinical Translational Science Institute (D. Betty Lew).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 8903982, 7 pages
https://doi.org/10.1155/2017/8903982

Research Article

Airway Epithelial Repair by a Prebiotic Mannan Derived from *Saccharomyces cerevisiae*

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Received 10 February 2017; Revised 5 May 2017; Accepted 30 May 2017; Published 9 July 2017

Academic Editor: Elizabeth H. Kemp

In asthmatic airways, repeated epithelial damage and repair occur. No current therapy directly targets this process. We aimed to determine the effects of mannan derived from *S. cerevisiae* (SC-MN) on airway epithelial wound repair, in vitro. The presence of functional mannose receptors in bronchial epithelial cells was shown by endocytosis of colloidal gold-Man BSA via clathrin-coated pits in 16HBE cells. In primary normal human bronchial epithelial cells (NHBEC), SC-MN significantly facilitated wound closure. Treatment with SC-MN stimulated cell spreading as indicated by a significant increase in the average lamellipodial width of wound edge 16HBE cells. In addition, NHBEC treated with SC-MN showed increased expression and activation of Krüppel-like factors (KLFs) 4 and 5, transcription factors important in epithelial cell survival and regulation of epithelial-mesenchymal transition. We conclude that SC-MN facilitates wound repair in human bronchial epithelium, involving mannose receptors.

1. Introduction

The hallmarks of asthma exacerbation include inflammation, denudation of the bronchial epithelial barrier, and focal airway damage that is evident on bronchial biopsy even in mild asthmatics [1, 2]. The consequences of repeated epithelial damage and repair are not completely understood, but the disrupted epithelial barrier and epithelial dysfunction are crucial in the development and maintenance of asthma symptoms [3]. This process, triggered by a variety of factors such as ozone [4], radical-containing particles [5], viral infections [6], cigarette smoke [7], and allergens, can ultimately contribute to airway remodeling [3, 7]. Inhaled corticosteroids have been shown to prevent epithelial damage in asthmatics [8]. However, this is likely an indirect result of anti-inflammatory effects rather than a direct protective or repair function. A report by Dorscheid et al. demonstrated that steroid treatment prior to wounding airway epithelial cells in vitro resulted in impairment of both epithelial cell migration and wound closure and induced apoptosis of inflammatory cells and epithelial cells [9]. Indeed, none of the current acute or control therapies for asthma specifically target epithelial integrity or repair. Resolution of airway hyperreactivity (AHR) has been reported to correlate with restoration of the epithelium [1, 10], whereas AHR may persist after resolution of cellular inflammation in human asthma [11].

Moreover, anti-inflammatory therapy alone has not been shown to halt disease progression in asthma [12]. Thus, a therapy that could promote epithelial repair should provide adjunctive therapeutic benefit for asthma.

Mannan, polymannose glycoprotein in the cell wall of various bacterial and fungal species, may have species-specific structure and lipid modifications that regulate the

degree of dendritic cell mannose receptor clustering, differential cytokine profile, and subsequent effector functions [13]. Mannan derived from mycobacteria has been shown to suppress allergic airway disease in a murine-allergic asthma model [14]. On the other hand, mannan derived from pathogenic fungi is likely to induce Th17 inflammatory cytokines [15]. This is in contrast to mannan derived from the outer wall of the commensal yeast *Saccharomyces cerevisiae* (SC-MN), which does not stimulate Th17 cytokines [15]. However, direct effects of mannan, from any source, on airway epithelium, are unknown. In this study, we show that human bronchial epithelial cells (HBEC) express a functional mannose receptor. We also present evidence that SC-MN facilitates wound healing of bronchial epithelium in vitro, complementary to its antiproliferative effect in airway smooth-muscle cells that we have previously described [16, 17]. At a transcriptional level, we present evidence that SC-MN stimulates expression and activation of Krüppel-like factors (KLFs) 4 and 5, key transcription factors for epithelial cell differentiation, survival, and proliferation [18].

2. Methods

2.1. Cell Cultures. Either primary normal human bronchial epithelial cells (NHBEC, Lonza, Walkersville, MD) or an immortalized line of human bronchial epithelial cells, 16HBE (kindly provided by Dr. Gruenert, California Pacific Institute) was cultured either in 6-well plates, in 24-well plates on Transwell inserts with 0.45 μm pores at a liquid/liquid interface, or on 18 mm glass coverslips in 12-well plates to confluence at 37°C and 5% CO_2. The NHBEC were cultured in basal epithelial growth media (BEM) supplemented with BulletKit™ (Lonza) at 37°C and 5% CO_2.

2.2. Mannose Uptake Studies. 16HBE cells were grown in on a nylon sheath in Transwell culture plates and were incubated with gold-labeled mannose-bovine serum albumin (10 nm) (ManBSA) or gold-BSA (control) (EY Labs Inc., San Mateo, CA) for 30 min and processed for electron microscopy as we previously described [17].

2.3. Wound Repair Assay. Using a pipette tip (5–200 μl capacity), mechanical wounds were inflicted across the diameter of the wells. Wounded cells were cultured with medium containing 10% fetal bovine serum (HyClone, Logan, UT) and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO). Cells were incubated with vehicle control (saline, final concentration: 0.005%), SC-MN (0.5–1.0 mg/ml, approximately 14–28 μM) (Sigma, prepared for patented use for asthma therapeutic), budesonide (10^{-7} – 10^{-6} M), heat-inactivated (50°C, 3 hr) beta-hexosaminidase A (HI-Hex A, endogenous ligand for mannose receptor, 50 nM, microdialyzed to remove ammonium sulfate and stored in 5% glycerin-containing phosphate buffer, Sigma), or a combination of SC-MN and HI-Hex A. Endotoxin levels of these agents were less than 2 EU/ml. Primary NHBEC were fixed and stained with crystal violet for overall assessment of wound closure under phase microscopy. Analysis of the perimeter area of the remaining wound in each image was performed using *ImageJ* software (National Institutes of Health, Bethesda, MD). For lamellipodial analysis, cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X, followed by staining with rhodamine phalloidin (Molecular Probes, Eugene, OR) according to the manufacturer's protocol and then images were acquired using fluorescence microscopy [19]. A number of total wound edge cells expressing lamellipodia were counted, and measurements were made using MetaMorph imaging software (version 4.6; Universal Imaging, West Chester, PA).

2.4. Western Blot Analysis. NHBE cell lysates were analyzed for Krüppel-like factors 4 and 5 (KLF4 and KLF5) and phospho-KLF4 and phosphor-KLF5 by Western blot. Protein concentration was determined by Bradford method (Thermo Scientific, Rockford, IL) and 10 μg was loaded per lane for SDS-polyacrylamide gel electrophoresis (8%). Blots of protein bands were probed with primary (KLF4 (rb, 1 : 500 dilution, EMD Millipore, Billerica, MA); p-KLF4 (Ser245) (rb, 1 : 500, Abgent, San Diego, CA); KLF5 (rb, 1 : 500, AVIVA System Biology, San Diego, CA); and p-KLF5 (Ser311) (rb, 1 : 500, Bioss Inc., Woburn, MA; beta-actin) (mo, 1 : 1000, Santa Cruz, Dallas, TX)) and appropriate secondary antibodies and intensity of the bands was measured with *ImageJ* 1.42 software.

2.5. Statistical Analysis. Data are presented as median +/− SD. Data were analyzed using the nonparametric Kruskal-Wallis one-way analysis of variance test (Graphpad Prism 6, La Jolla, CA). p values less than 0.05 were considered significant.

3. Results

3.1. Presence of Functional Mannose Receptors in Human Epithelial Cells. To assess whether bronchial epithelial cells express mannose receptors, 16HBE cells grown on 24-well Transwell plate inserts were exposed to colloidal gold-Man BSA (10 nm particle size) for 30 min at 37°C. In control experiments, the cells were treated with gold-BSA (Figure 1(a)). Electron microscopic examination of the cells showed gold particles being endocytosed via clathrin-coated pits in 16HBE cells, indicating the presence of functional mannose receptors (Figure 1(b)) [17].

3.2. Facilitation of Repair by SC-MN on Mechanically Inflicted Wounds in NHBEC. Mannan from *S. cerevisiae* enhanced wound closure in primary NHBEC. SC-MN-treated cells (1 mg/ml) showed accelerated wound closure compared to the control cultures and budesonide-treated cells (10^{-7} M) (Figure 2, images acquired at the 32 hr time point). Budesonide was used for comparison because this inhaled corticosteroid is frequently used to treat asthma in young children. The average number of wound edge cells expressing lamellipodial extensions analyzed at the 24 hr time point under high-power magnification was 5/14 (36%) for untreated controls, 11/11 (100%) for mannan-treated cultures, and 8/14 (57%) for budesonide-treated cultures. Measurements of the remaining wound area at the 32 hr time point are shown in Figure 3. SC-MN (0.5 and 1.0 mg/ml)

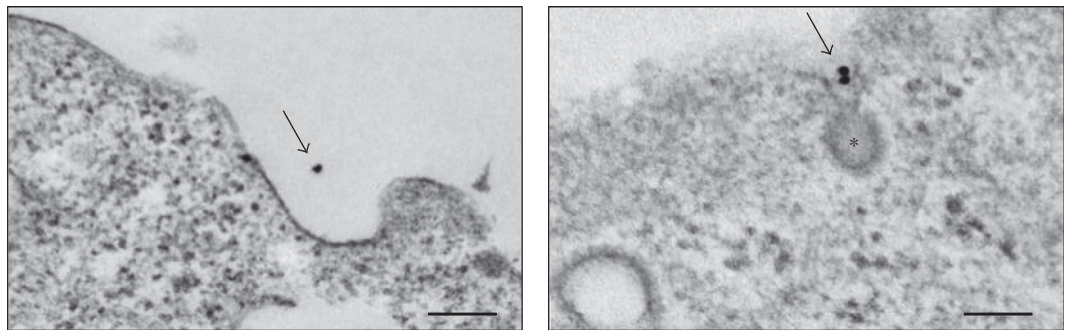


Figure 1: Endocytosis of colloidal gold-mannose bovine serum albumin (ManBSA) via clathrin-coated pits in 16HBEC. (a) Gold-BSA; (b) gold-ManBSA. *Clathrin-coated pit. Arrows: gold particles. Electron microscopy scale bar: 100 nm.

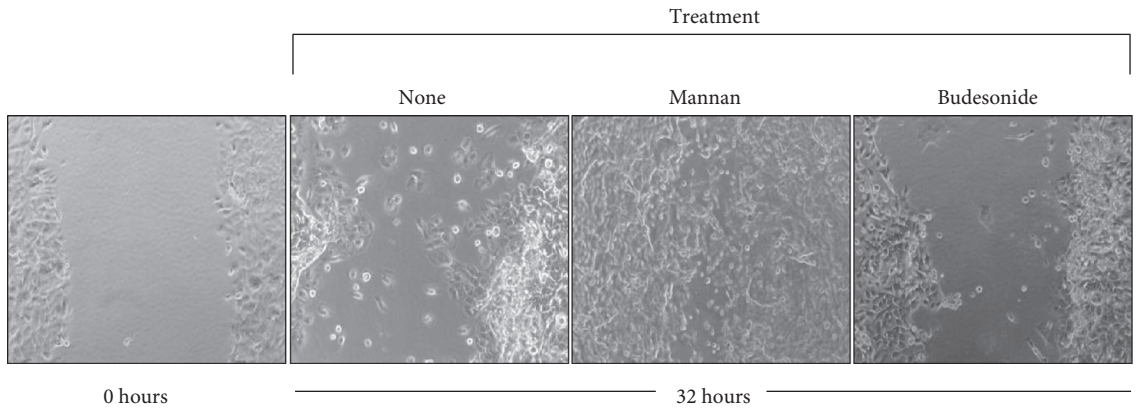


Figure 2: SC-MN facilitates wound closure in NHBEC. Mechanically injured NHBEC were treated with saline (none), SC-MN (1 mg/ml), or budesonide (10^{-7} M). Final magnification: 50x.

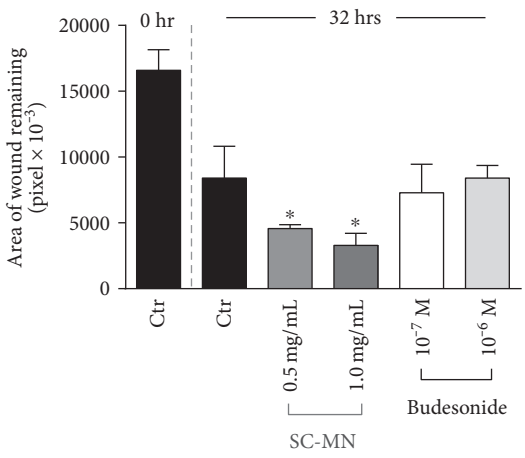


Figure 3: SC-MN's beneficial effect on mechanical wound closure in NHBEC. SC-MN at 0.5 and 1.0 mg/ml concentration significantly facilitated wound closure compared to the control-(saline-) treated cultures. Budesonide had no such effect. $^{*}p < 0.05$.

significantly facilitated wound closure compared to the control cultures in contrast to the lack of beneficial effect by budesonide treatment.

3.3. Facilitation of Repair by SC-MN on Mechanically Inflicted Wounds in 16HBEC. Similar results were obtained in a faster growing 16HBE cells (Figure 3) when wound closure was examined over time. There was no significant difference in closure at 8 hrs, but by 13 hrs, differences became evident. At 17 hours, the SC-MN- or HI-Hex A-treated wounds had 30% or less wound area remaining, versus the 50% or greater remaining for control cultures, respectively (data not shown). Combining SC-MN (1 mg/ml) and HI-Hex A (50 nM) appeared to maximize wound closure (Figure 4). Accelerated 16HBE cell spreading and migration were apparent at the cellular level as early as 8 hrs in all three conditions (mannan treated, HI-Hex A treated, and a combination of these agents), compared to those of control cells. Figure 5 shows images of rhodamine-phalloidin-stained actin. Average lamellipodial width of wound edge cells at 13 hours after wounding was significantly increased in SC-MN-treated (4.47+/− 0.90 microns, $n = 10$) versus control 16HBE cells (1.57+/− 0.59 microns, $n = 6$) ($p < 0.05$), indicating increased spreading and enhanced migration. The overall appearance of the wound edge cells in the HI-Hex A or SC-MN-treated groups was consistent with an active healing process not only by lamellipodial extensions but also by directionality toward the opposing wound edge (Figure 5).

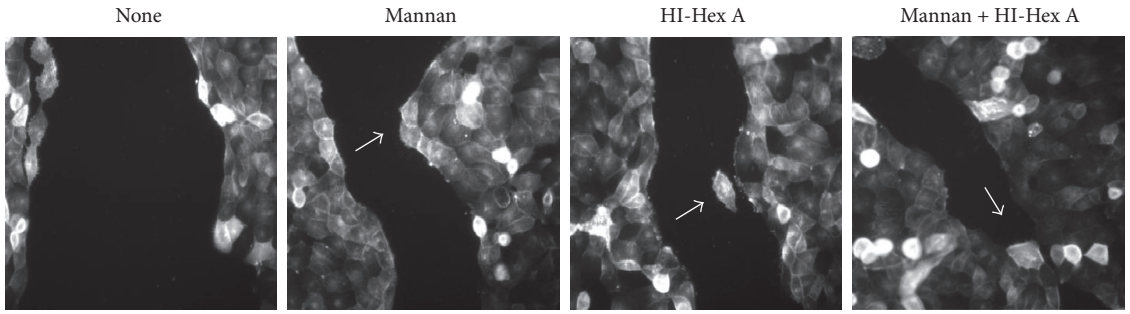
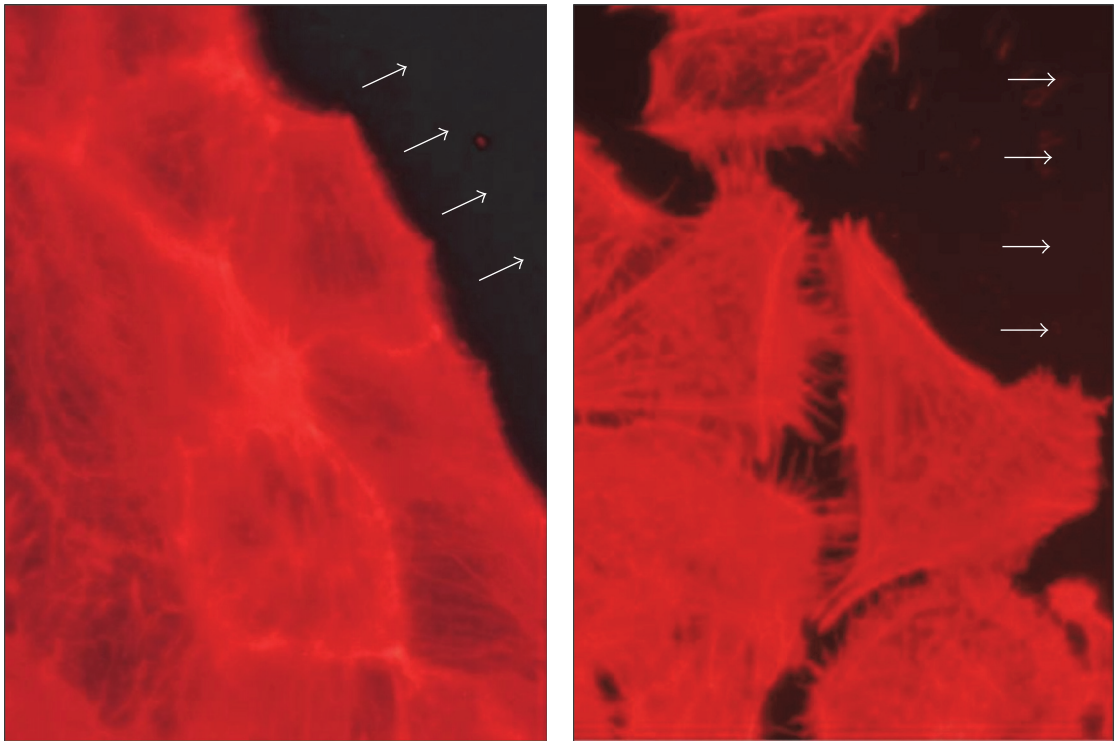


Figure 4: SC-MN and heat-inactivated (HI) Hex A facilitate wound closure in 16HBE cells. Digital images of cells after 17 hrs incubation (final magnification: 300x). Arrows: evagination of migrating wound edges.



(a) (b)

Figure 5: Effect of SC-MN on 16HBEC migration. Mechanically injured cells on glass coverslips were treated with saline (a) or SC-MN (1 mg/ml) (b) for 13 hrs. Arrows: the direction of cell migration (final magnification: 500x).

3.4. Effect of SC-MN on KLF4 and KLF5 Expression and Activation in NHBE Cells. Although mannose receptor (MR) activation in airway smooth muscle cells is known to involve signal transduction pathways including transient elevation of cAMP [20], PKC activation [21], and ERK1/2 activation [22], the mechanism by which SC-MN affects epithelial repair is unknown. To gain insight into postreceptor mechanisms at the transcriptional level, we assessed whether the reparative effects of SC-MN on airway epithelial cells involved transcriptional factors KLF4 or KLF5, which are known to be associated with cell differentiation, survival, and proliferation [18]. Mannan from *S. cerevisiae* (1 mg/ml) stimulated the expression and phosphorylation of KLF4 and KLF5 (Figure 6) at 2–18 hr time points in NHBEC.

4. Discussion

We present evidence that a functional MR family member is expressed in human bronchial epithelial cells; this is supported by endocytosis of mannosylated neoglycoprotein in clathrin-coated pits. This adds to the list of MR in the epithelium: retinal pigment epithelial cells [23] and nasal polyposis [24]. In search of a therapeutic that can directly impact the epithelial damage occurring in asthma, we have discovered that SC-MN facilitates healing of human bronchial epithelial wounds in vitro. The healing effect of SC-MN on primary NHBEC was significant compared to that of the control cells and contrasted to budesonide (glucocorticosteroids) treatment which did not improve wound healing. Asthma

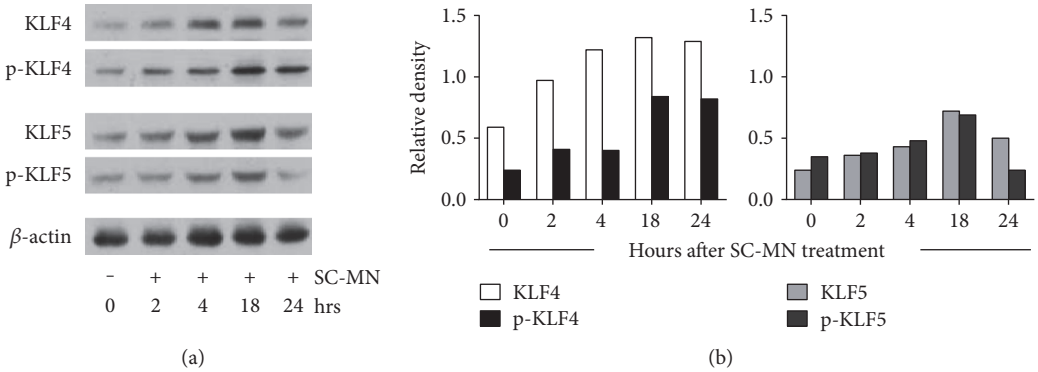


Figure 6: Expression and activation of KLF4 and KLF5 by SC-MN in NHBEC. (a) Phosphorylated forms indicate protein activation. (b) Density of each protein relative to β-actin. Data are representative of two independent experiments.

research on beta-hexosaminidases (Hex A and Hex B), endogenous enzymes, and known ligands for mannose receptors, has been hampered by the lack of highly purified enzyme (the preparation used for our previous work was 150–170 times more pure compared to the commercially available beta-hexosaminidases) [16]. Nevertheless, heat-inactivated Hex A, produced a similar healing effect on airway epithelium, further supporting the presence of a mannose receptor in human bronchial epithelium.

Epithelial repair mechanisms involve cell migration and proliferation, promoted by multiple growth factors, chemokines (MCP-1), cytokines (IL-1β, IL-2, IL-4, and IL-13), and prostaglandins (PGE2) that work in coordination with integrins and other matrix materials (fibronectin, collagen, and laminin) [25]. Treatment of bronchial epithelial cells with SC-MN does not produce the abovementioned cytokines (preliminary unpublished data). Many cellular molecular factors, such as Sonic hedgehog, Rho GTPases, MAP kinase pathways, STAT3, and Wnt [25], contribute to epithelial cell migration and proliferation. In addition to promoting wound closure, SC-MN appears capable of initiating directional migration of human bronchial epithelial cells. However, the specific mechanisms of how SC-MN directs epithelial migration and proliferation downstream of MR are unknown.

In order to determine the underlying mechanisms of airway epithelial wound healing by SC-MN, KLF4 and KLF5 expression were determined. These transcription factors are known to be associated with differentiation, survival, and cell proliferation. Within 2 hrs of cell exposure to SC-MN, KLF4 expression and activation (by phosphorylation) were apparent. This association of KLF4 expression and activation is likely a contributing factor to directional migration of epithelial cells during wound closure. SC-MN also induced KLF5 expression and activation in NHBEC at 2–18 hrs (peak at 18 hrs), and KLF5 is known to inhibit epithelial mesenchymal transition by microRNA 200 transcription [26].

There are limitations of this study in respect to cell types and culture system. Primary human bronchial cells from asthmatic patients would have provided further insight to the beneficial effect of mannan in asthma; however, they are extremely difficult to acquire. In addition, the culture of NHBEC in an air-liquid interface model could elucidate mannan's effect on differentiated cells. Regarding the mechanisms of SC-MN's action, it is possible that mannan may work through alternative mechanisms in epithelial cells other than a receptor-mediated phenomenon. For example, it may act as a barrier protection as it does in yeast. Another possibility is that endotoxin of SC-MN, even though the concentration is minimal, might have had some influence in epithelial cells.

The mannose receptor and other endogenous glycan-binding proteins, implicated in both innate and adaptive immunity, represent a largely unexplored opportunity to modulate immune responses. Glycoprotein endogenous ligands for MR (β-Hex A and B, aka NAGA (N-Acetyl-β, D-Glucosaminidase), NAG, and NAGase) are elevated in bronchoalveolar lavage fluids of guinea pigs exposed to ozone [4] and in serum of asthmatic patients, more so in severe asthmatics [27]. Moreover, gene variants of glycoprotein and resulting abnormal serum levels of the respective glycoproteins are associated with asthma [28]. Although the specific subtype of mannose receptor expressed in airway bronchial epithelial cells is yet to be determined, sufficient evidence exists to indicate the presence of a functional receptor in the mannose family. SC-MN is an appealing agent with therapeutic potential for repairing wounded bronchial epithelium in addition to its already known effect of antimitogenic effect in airway smooth muscle cells [16]. We conclude that SC-MN mediates a direct healing effect on human bronchial epithelial cell wounds, in part, by engagement of a mannose receptor. Intricate regulation by SC-MN at the transcriptional level involving KLF4 and KLF5 and their posttranslational modifications (phosphorylation, acetylation, and methylation) needs further investigation.

Abbreviations

EMT: Epithelial-mesenchymal transition
NHBEC: Normal human bronchial epithelial cells
KLF: Krüppel-like factor
ManBSA: Mannosylated bovine serum albumin
OVA: Ovalbumin
SC-MN: *Saccharomyces cerevisiae* mannan
Hex A: β-Hexosaminidase A
HI: Heat inactivated.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors wish to thank Dr. Kenneth Chapman for his scientific contribution and Julie Chang and Carolyn Stewart for their technical assistance. This work was supported in part by Le Bonheur Foundation (Christopher M. Waters), NIH-HL56812 and NIH-HL090108 (Dr. Betty Lew), University Tennessee Research Foundation-Cumberland Pharmaceuticals & Emerging Technology (Dr. Betty Lew), University of Tennessee Clinical Translational Science Institute (Dr. Betty Lew), NIH-HL123540 and NIH-HL094366 (Christopher M. Waters), and NIH-HL079109 (Kafait U. Malik).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 4607491, 14 pages
https://doi.org/10.1155/2017/4607491

Research Article

Probiotic *Lactobacillus* Strains Stimulate the Inflammatory Response and Activate Human Macrophages

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Received 9 February 2017; Revised 16 May 2017; Accepted 23 May 2017; Published 5 July 2017

Academic Editor: Ansarullah

Lactobacilli have been shown to promote health functions. In this study, we analyzed the mechanism by which four different strains of probiotics affected innate immunity, such as regulation of ROS, cytokines, phagocytosis, bactericidal activity, signaling by NF-κB pp65, and TLR2 activation. The production of ROS was dependent on the concentration and species of *Lactobacillus*. The results obtained from the tested strains (*Lactobacillus rhamnosus* GG, *L. rhamnosus* KLSD, *L. helveticus* IMAU70129, and *L. casei* IMAU60214) showed that strains induced early proinflammatory cytokines such as IL-8,TNF-α, IL-12p70, and IL-6. However, IL-1β expression was induced only by *L. helveticus* and *L. casei* strains (after 24 h stimulation). Phagocytosis and bactericidal activity of macrophages against various pathogens, such as *S. aureus*, *S. typhimurium*, and *E. coli*, were increased by pretreatment with *Lactobacillus*. The nuclear translocation NF-κB pp65 and TLR2-dependent signaling were also increased by treatment with the probiotics. Taken together, the experiments demonstrate that probiotic strains of *Lactobacillus* exert early immunostimulatory effects that may be directly linked to the initial inflammation of the response of human macrophages.

1. Introduction

The FAO and WHO (Food and Agriculture Organization of the United Nations and World Health Organization) define probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. In particular, *Lactobacillus* is an important member of the probiotic bacteria that plays essential roles of immunomodulation in the intestinal mucosa [2]. Clinical and experimental studies of probiotic *Lactobacillus strains* have reported that these bacteria efficiently prevent and treat antibiotic-associated diarrhea, traveler's diarrhea, and infections caused by intestinal pathogens [3, 4]. In addition, some studies have shown that they provide a positive effect by promoting the secretion of immunoglobulin IgA and the production of antimicrobial molecules (i.e., bacteriocins), which are capable of inhibiting some intestinal pathogens [5]. In this context, the immunomodulatory effects of probiotic *Lactobacillus* strains have also been shown to decrease the inflammatory response under some pathological conditions such as necrotizing

enterocolitis and allergies in children [6, 7]. In other in vitro studies, some strains produced an increase in the production of proinflammatory cytokines such as TNF-α, IL-12, and IL-8 whereas others produced increases in the secretion of anti-inflammatory cytokines such as IL-10 [8, 9]. A study by Lopez et al. [10] showed that UV-inactivated and live *Lactobacillus rhamnosus* GG (LGG) are equally effective in decreasing IL-8 in the intestinal epithelium. In addition, Li et al. [11] showed that live and heat-killed LGG are able to exert similar effects on the secretion of pro- and anti-inflammatory cytokines and chemokines when included in the diet of infant rats. However, these beneficial effects have been associated with only a limited number of strains, and other strains and species cannot be presumed to exert the same effects [12, 13]. The immune effects of probiotic bacteria have therefore been shown to be extremely diverse and strain-dependent in addition to cell type-specific.

Monocytes and macrophages are cellular components of the innate immune system that prevent the invasion of pathogens by releasing cytotoxic molecules such as reactive oxygen species (ROS) and by secreting proinflammatory cytokines such as TNF-α and IL-8 [14, 15]. Macrophages sense bacteria because bacteria express conserved pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). Macrophages also mediate responses when they recognize microbe-associated molecular patterns (MAMPs), which are expressed on the cell surface of probiotic bacteria [16]. Some studies have shown that TLR2 and TLR4 are constitutively expressed on macrophages [17]. The activation of TLR results in the induction of a signaling cascade that modulates the expression of various response genes such as cytokines and may activate signaling pathways, such as the NF-*k*B signaling pathway [18]. Miettinen et al. [19] showed that both viable and dead *L. rhamnosus* GG increased macrophage functions by activating NF-*k*B, STAT1, and STAT3 DNA-binding activity. Although immunological activities have been attributed to some probiotics, the molecular mechanisms underlying these effects have not been established. Determining the probiotic effects of *Lactobacillus* on the innate immune response and host health could support applications aimed at preventing and treating different diseases. A better understanding of how probiotic bacteria interact with host cells is therefore needed to optimize such applications.

2. Materials and Methods

2.1. Bacteria Strains and Growth Conditions. Four strains of lactic acid bacteria were used in this study: *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* KLDS, *Lactobacillus helveticus* IMAU70129, and *Lactobacillus casei* IMAU60214 previously isolated from commercial products by Cruz-Guerrero et al. [20, 21]. In the stimulation experiments, the *Lactobacillus* was cultured prior to use in MRS broth (Difco) overnight at 37°C. The bacterial cells were harvested at stationary phase using centrifugation (4000 ×g, 10 min) and washed twice with sterile physiological saline solution. The concentrated bacterial cells were inactivated by heating the cells to 85°C for 15 min. Frozen stocks of bacterial cells were stored in SSF at −80°C until use. Additionally, *Staphylococcus aureus* (ATCC 2913), *Escherichia coli* (ATCC 35218), and *Salmonella* enteric serovar *Typhimurium* (ATCC 14028) were grown overnight in tryptic soy broth (Difco Laboratories) at 37°C. After the cells were centrifuged (4000 ×g 5 min) and washed three times in sterile physiological saline, the bacterial strains were normalized to a density of ~10^8 CFU/ml. In some experiments, the bacteria were inactivated using heat and the loss viability was confirmed by plating the inactivated bacteria on agar plates (at 37°C for 48 h). FITC-labeled bacteria (*S. aureus*, *S. typhimurim*, and *E. coli*) were killed by heating the cells at 80°C for 60 min and then resuspended at a concentration of 5 mg/ml in 50 mM carbonate buffer (pH 9.6). FITC isomer I (Sigma) was then added to the cells, which were incubated at 37°C for 30 min, and then washed with sterile PBS and stored at −80°C until use.

2.2. Macrophages Derived from Human Monocytes. Leukocyte-rich buffy coat was obtained from volunteer donors (Blood Bank, Children's Hospital of Mexico Federico Gomez), with approval from the local Ethics Committee. Mononuclear cells were separated using gradient centrifugation, as previously described [22]. Briefly, the interface mononuclear cells were washed twice with sterile physiological saline solution and then collected in Falcon tubes. The cells were immediately counted in a Neubauer chamber and cell viability was evaluated by staining the cells with trypan blue. To isolate the monocytes, we performed magnetic labeling with negative selection using a Monocyte Isolation Kit II (Miltenyi Biotec), according to the manufacturer's instructions. Purified monocytes were then differentiated into macrophages by culturing the cells in RPMI-1640 medium supplemented with 10% FBS (Gibco, Invitrogen USA) for 7 days.

2.3. ROS Production. ROS secretion was quantitated using chemiluminescence in a luminol-amplified system in an LKB-1251 luminometer, which coverts photons in a photomultitiplier tube into an electric current in the presence of a peroxidase such as HRP, according to the method described in Rellstab et al. [23]. Briefly, the macrophages were adhered to round glass coverslips (9 mm) at concentration of 1×10^5 cells per coverslip and then placed in cuvettes containing 1 ml of Hank's solution (luminol 0.8×10^{-4} M and HRP at 4 U). After the cuvettes were warmed at 37°C for 10 min, baseline chemiluminescence was recorded. Subsequently, different concentrations of heat-inactivated *Lactobacillus* were added to the reaction mixture (e.g., MOI, 1 : 10, 1 : 100, 1 : 250, and 1 : 500). Opsonized zymosan (ZAS) and unstimulated macrophages (Hank's solution) were used as the positive and negative controls, respectively.

2.4. Cytokines. Macrophages were cultured and unstimulated or stimulated with heat-inactivated lactobacillus at a MOI of 500 : 1 (bacteria : macrophage), and the supernatants were collected after 6 and 24 h. Cytokine levels (IL-8, TNF-α, IL-6, IL-12p70, IL-1β, and IL-10) were quantified using an ELISA detection kit (BD OptEIA Pharmingen, San Diego,

CA, USA) according to the manufacturer's instructions. A standard curve was used to calculate the concentrations of the different cytokines in pg/ml.

2.5. Macrophage Phagocytosis Assay. For the phagocytosis assay, 1×10^4 macrophages in RPMI-1640 were plated onto 12 mm glass coverslips (Corning) and placed in sterile 24-well tissue culture plates (Costar). The cells were allowed to adhere in an atmosphere containing 5% CO_2. Opsonized zymosan was added to the macrophages at (a MOI of 10 : 1). Simultaneously, macrophages in separate wells were pretreated with lactobacillus at a MOI (of 500 : 1 for 1 h at 37°C) and subsequently challenged with zymosan and opsonized bacteria such as *Staphylococcus aureus*, *E. coli*, and FITC-labeled *Salmonella typhimurium* a MOI of 10 : 1. To terminate phagocytosis, the cells were vigorously washed with PBS (pH 7.4) and then fixed in 4% paraformaldehyde (pH 7.4). Subsequently, the nuclei of the cells were stained with DAPI (1 mg/ml). Glass coverslips were air-dried and mounted onto glass microscope slides using Vectashield (Vysis). The phagocytic bacteria were visualized using an epifluorescence Zeiss Axioskop 2 microscope equipped with a Zeiss Axiocam (Zeiss AG, Oberkochen, DE). The percentage of ingesting bacteria was determined by evaluating 100 cells per field across four separate experiments. The phagocytic index was calculated as the percentage of cells performing phagocytosis using the mean number of particles per cells.

2.6. Macrophage Bactericidal Activity. Bacterial uptake and survival was measured using a gentamicin protection assay [24]. Before the cells were infected, 1×10^4 macrophages in RPMI-1640 supplemented with 10% SFB were placed in sterile 12-well tissue culture plates (Costar). The cells were allowed to adhere in an atmosphere containing 5% CO_2. Simultaneously, macrophages in separate wells were pretreated with lactobacillus at a MOI (of 500 : 1 for 1 h at 37°C) and subsequently challenged with *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* serovar *Typhimurium* at a MOI of 1 : 10. The cells were then incubated for 60 min at 37°C in atmosphere containing 5% CO_2. The end of this incubation period was considered time 0. Each well was then washed and treated with media containing 200 μg/ml gentamicin for 30 min at 37°C in an atmosphere containing 5% CO_2 to kill any extracellular bacteria. The media was then replaced with media containing 20 μg/ml gentamicin for the duration of the experiments. At time point 1 (120 min), the wells were washed 4 times with 1 ml of PBS each time. They were then incubated for 10 min at room temperature in sterile water containing 1% Triton-X-100 to lyse the macrophages. Dilutions of the resulting lysates were plated on LB agar plates and the number of viable intracellular bacteria was counted as colony-forming units (CFU).

2.7. NF-κB (p65) Detection Using Indirect Immunofluorescence and Transnuclear Activity Assays. Human macrophages were attached to round glass coverslips (9 mm) and then either unstimulated or stimulated using zymosan (10 μg/ml) and probiotic heat-inactivated lactobacillus at a MOI of 1 : 500. NF-*k*B activity was measured in the macrophages using indirect immunofluorescence with a Cellomics NF-*k*B kit (Thermo Scientific) according to the manufacturer's protocols. Fluorescence emitted within 30 min of the addition of different stimuli was photographed using an epifluorescence Zeiss Axioskop 2 microscope equipped with a Zeiss Axiocam (Zeiss AG, Oberkochen, DE). The percentage of cells that were positive for pp65 was determined in 100 cells per field across four separated experiments.

2.8. Transient Expression of TLR2 in HEK293 Cells (293-hTLR2). Human embryonic kidney (HEK293-hTLR2) cells were obtained from InvivoGen (SanDiego, CA) and grown in 24-well culture plates in DMEM supplemented with 10% FCS and 10 μg/ml blasticidin (InvivoGen) at 37°C in a 5% CO_2 atmosphere. Cultures at 70–80% confluence were challenged with the probiotic bacteria lactobacillus at a MOI of 1 : 500. The plates were then incubated at 37°C for 24 h in an atmosphere containing 5% CO_2. The supernatants were collected, and TLR activation was analyzed using ELISA for the quantity production of IL-8, according to the manufacturer's protocols. The control, cultures were stimulated with the TLR2 agonist zymosan 10 μg/ml or LTA (10 μg/ml). Blocking assays were performed using 10 μg/ml of anti-hTLR2-IgA. The levels of IL-8 were then determined as described above for cytokines.

2.9. Statistical Analysis. The results are reported as the mean ± SD, for each experiment. A total of four donors were included in the duplicate samples. Differences between the conditions assays were analyzed using one-way analysis of variance (ANOVA) in Graph Pad Prism 5 Software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. ROS Production. The effect of heat-killed lactobacillus on the generation of ROS species was measured using chemiluminescence (Figure 1). The results showed that adding heat-killed lactobacillus induced active respiratory bursts in human macrophages. ROS production was dependent on the bacteria: macrophage MOI and the species of *Lactobacillus*. The ROS-induced immunostimulatory effects were maximized at a MOI of 500 : 1 for *L. rhamnosus* GG, *L. rhamnosus* KLSD, *L. helveticus* IMAU70129, and *L. casei* IMAU60214, which produced peak chemiluminescence (190 ± 15, 196 ± 24, 175 ± 12, and 200 ± 14 Mv, resp.), (Figure 1(a)). However, the oxidative response induced by lactobacillus was lower than that induced by zymosan (200 ± 14 Mv versus 600 ± 20 Mv).

In addition, as shown in Figure 1(b), the zymosan-induced ROS production kinetic was fast and short (500 sec), whereas the heat-killed lactobacillus-induced ROS production kinetic was delayed (2000 sec) and took longer to return to baseline. These results indicate that heat-killed lactobacillus activates ROS production in human macrophages.

3.2. Cytokine Production in Macrophages. Macrophages treated with *Lactobacillus* were induced to produce cytokines

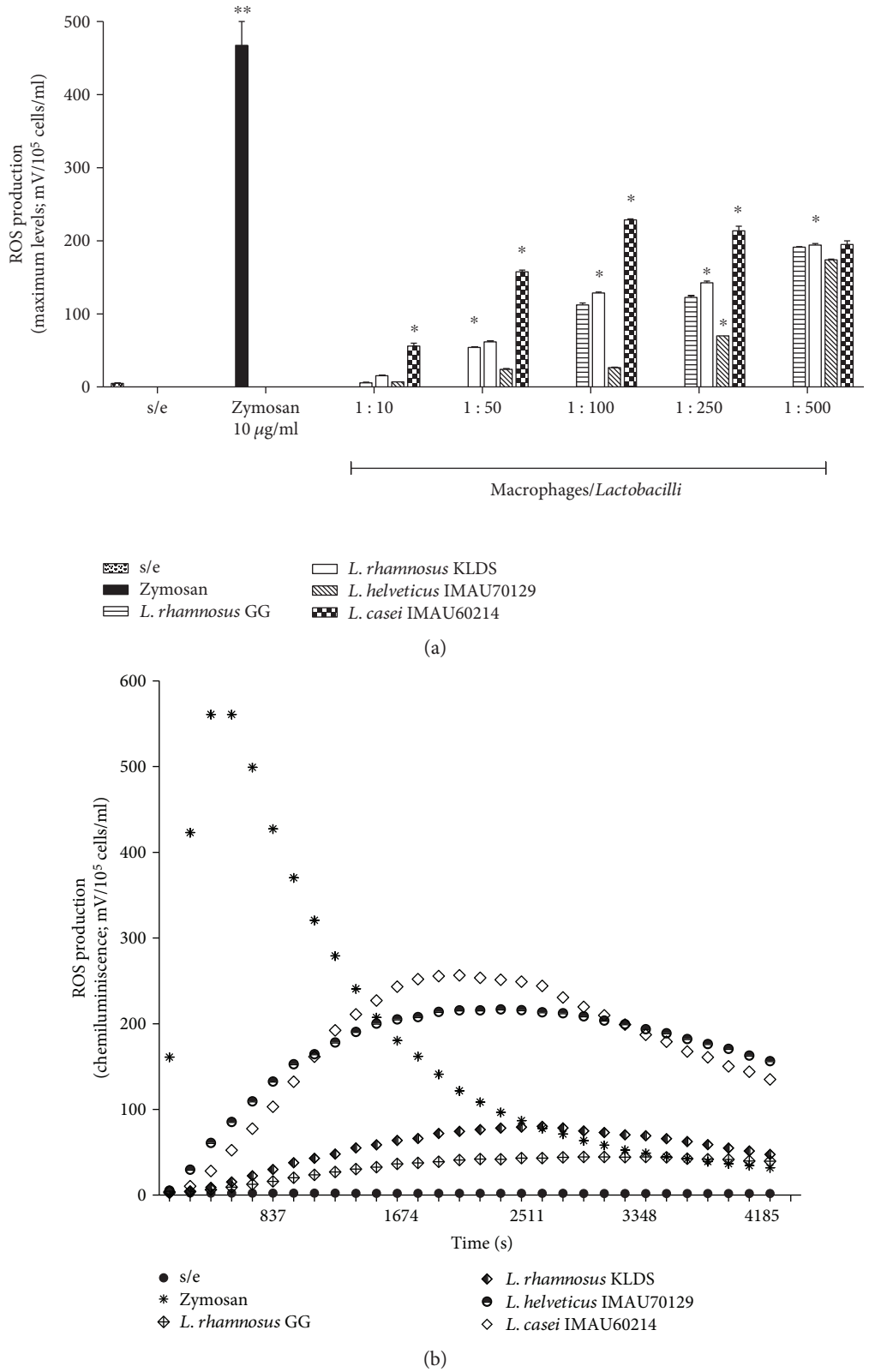


Figure 1: ROS production by human macrophages in response to probiotic heat-killed lactobacillus was measured by luminol-enhanced chemiluminescence. (a) Mixtures were prepared using 1 ml of Hank's solution, containing luminol 0.8×10^{-4} M, 4 U HRP, and lactobacillus bacteria at different MOI (1 : 1 and 1 : 500; bacteria : macrophages). The kinetics of the oxidative response induced in human macrophages by lactobacillus was monitored (b). The values shown represent four experiments that were performed in duplicate. Asterisks indicate the level of statistical significance between conditions. Significant difference: $p < 0.05$.

between 6 and 24 h after the treatment began. In macrophages challenged with a MOI 1 : 500 of heat-inactivated lactobacillus, the maximum concentration of IL-8 was observed at 6 h (Figure 2). All strains of lactobacillus induced a significant level of IL-8. The results showed that in the treated macrophages, there was one logarithmic increase more than what was observed in the untreated macrophages, but similar values to those in the zymosan-stimulated macrophages (Figure 2). The level of IL-8 produced after 24 h was not different across the groups and remained high in the cells treated with all probiotic strains of lactobacillus. The level obtained of TNF-α was similar across treatments (Figure 2). In contrast, the maximum concentration of IL-6 was obtained after of 24 h and the level of this cytokine was different across the treatments (Figure 2). *L. casei* IMAU60214 and *L. helveticus* IMA70129 induced the highest levels of IL-6 (e.g., higher than levels produced by *L. rhamnosus* KLSD and *L. rhamnosus* GG). In addition, the synthesis of the cytokine IL-12p70 was significantly higher in these strains. IL-1β expression was increased after 24 only following stimulation with *L. helveticus* IMA70129 and *L. casei* IMAU60214. In contrast, neither *L. rhamnosus* GG nor *L. rhamnosus* KLSD induced a significant increase in the cytokine IL-1β (Figure 2). In this experiment, we found that all strains of probiotic lactobacillus induced the production of IL-10 later, after 24 h, and the levels produced were different across the strains (Figure 2). These data collectively indicate that each one of the strains of lactobacillus induced a strong inflammatory response in macrophages.

3.3. Lactobacillus Modulates Phagocytosis in Macrophages. The phagocytosis of bacteria is an effective mechanism employed by the innate immune response. We therefore preincubated macrophages with probiotic strains of lactobacillus to determine their ability to increase phagocytosis in response to challenge with different pathogens (Figure 3). In this study, we applied four previously selected stimuli and zymosan (prepared from the yeast *Saccharomyces cerevisiae*) to two species of Gram-negative (*S. typhimurium* and *E. coli*) and one species of Gram-positive (*S. aureus*) microorganism. In cells challenged with FITC-labeled phagocytic stimuli, the increased rate of ingestion of phagocytized particles was higher in macrophages that were pretreated for 1 h than in untreated cell (Figure 3). In addition, pretreatment with lactobacillus resulted in significantly higher efficiency of macrophage phagocytosis than was observed in the untreated cells (Figure 3(b)). *Lactobacillus helveticus* IMAU70129 and *L. casei* IMAU60214 most strongly activated the phagocytic functions of the macrophages. However, in summary, all of lactobacillus strains exerted a positive effect on the ingestion of FITC-labeled bacteria, including *S. aureus*, *S. typhimurium*, *E. coli*, and yeast (zymosan) by human macrophages (Figure 3(c)).

3.4. Bactericidal Activity. Macrophages were infected as described in the Materials and Methods. As shown in Figure 4, preincubating human macrophages for 120 min with heat-killed lactobacillus induced a decrease in the number of viable intracellular *S. typhimurium* (Figure 4(a)). The effects of this treatment on bactericidal activity were similar to those observed in cells treated with *E. coli* (Figure 4(b)) and *S. aureus* (Figure 4(c)). Furthermore, these results showed that was no difference in these effects across different species of *Lactobacillus*. Taken together, these data confirm that *Lactobacillus* bacteria restrict the survival of these pathogens.

3.5. Lactobacillus Bacteria Induce the Translocation of NF-κB. Various proinflammatory and anti-inflammatory cytokines are known to be regulated at least in part by the transcription activator NF-*k*B. As shown in Figure 5, the activation and translocation NF-*k*B pp65 were induced in macrophages challenged with lactobacillus (Figure 5(a)). The expression of the NF-*k*B translocation factor was observed in more than 60% of the treated cells after 30 min of incubation (Figure 5(b)). In macrophages stimulated with zymosan, more than 90% of the cells expressed this factor. However, in untreated macrophages, approximately 20% of the cells expressed it (Figure 5). Taken together, these indicate that heat-killed lactobacillus activates the expression of the NF-*k*B factor pp65.

3.6. TLR2 Mediates the Recognition of Lactobacillus. We next sought to determine whether TLR2 was stimulated by lactobacillus in HEK293 cells. The results showed that the concentration of IL-8 induced in response to challenge with heat-killed *Lactobacillus* was similar to that produced by an agonist of TLR2, zymosan, and LTA (Figure 6). In addition, to verify that *Lactobacillus* plays a role in TLR2 recognition in inflammatory responses in human macrophages, we performed a blocking assay by applying anti-hTLR2-IgA antibodies at a concentration of 10 μg/ml for 30 min and then adding the stimuli. As shown in Figure 6, after 24 h, the production of IL-8 was nearly completely inhibited by all strains of *Lactobacillus*. This inhibitory effect on IL-8 production was also induced by the TLR2 agonist ligands zymosan and LTA. These results suggest that TLR2 participates in cytokine-mediated proinflammatory responses.

4. Discussion

In intestinal homeostasis, macrophages play an important role by acting as immunological sentinels in the gastrointestinal tract [25]. Most of these cells infiltrate the lamina propria, which produces a variety of molecules (cytokines) that regulate and activate the innate immune response. This infiltration of macrophages, which are derived from monocytes, into the intestine may initiate an inflammatory response, in response to infection, and disruptions in the homeostasis of these cells may contribute to the immunological damage observed in chronic inflammatory diseases [26]. Several groups have proposed that intestinal homeostasis can be restored by administering supplements containing fermented dairy products and beneficial microorganisms (probiotics), such as *Lactobacillus*. In this study, we evaluated the immunological impact of four probiotic bacteria in the genus

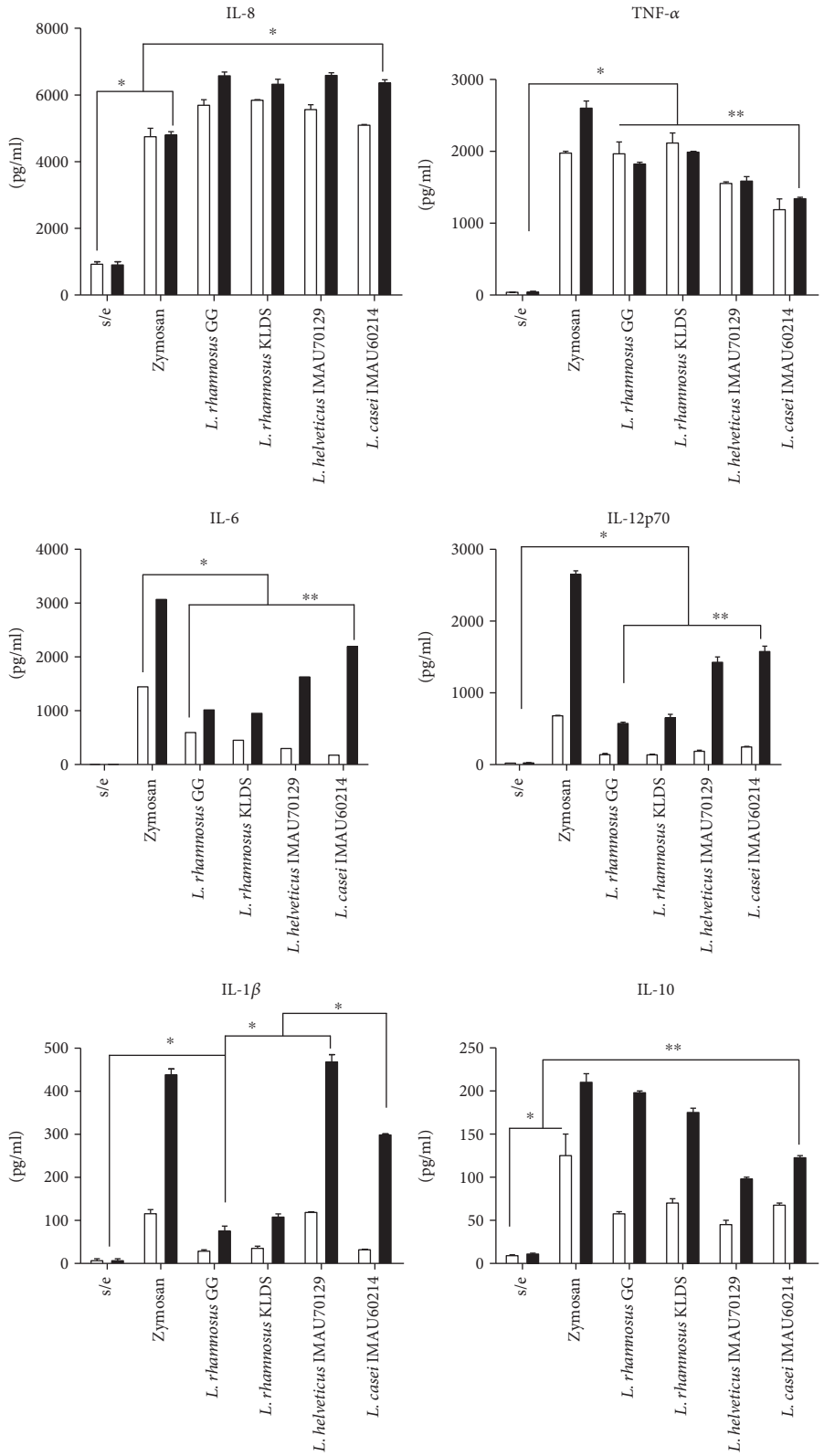


Figure 2: Cytokine production in human macrophages that were stimulated using heat-killed lactobacillus. The supernatants of stimulated macrophages were analyzed using ELISA to determine the level of expression of the cytokines IL-8, TNF-α, IL-6, IL-12, IL-1β, and IL-10. The macrophages were treated with zymosan (10 μg/ml) or heat-killed strains of lactobacillus at a MOI of 1 : 500 or untreated (s/e). Cytokine concentrations were determined at 6 h (□) and 24 h (■) after stimulation. The results are shown as the means ± of the standard deviations and are representative of four independent experiments. Significance was set at $p < 0.05$ ($*$) in the comparison between 6 and 24 h of stimulation. $p > 0.05$ ($**$).

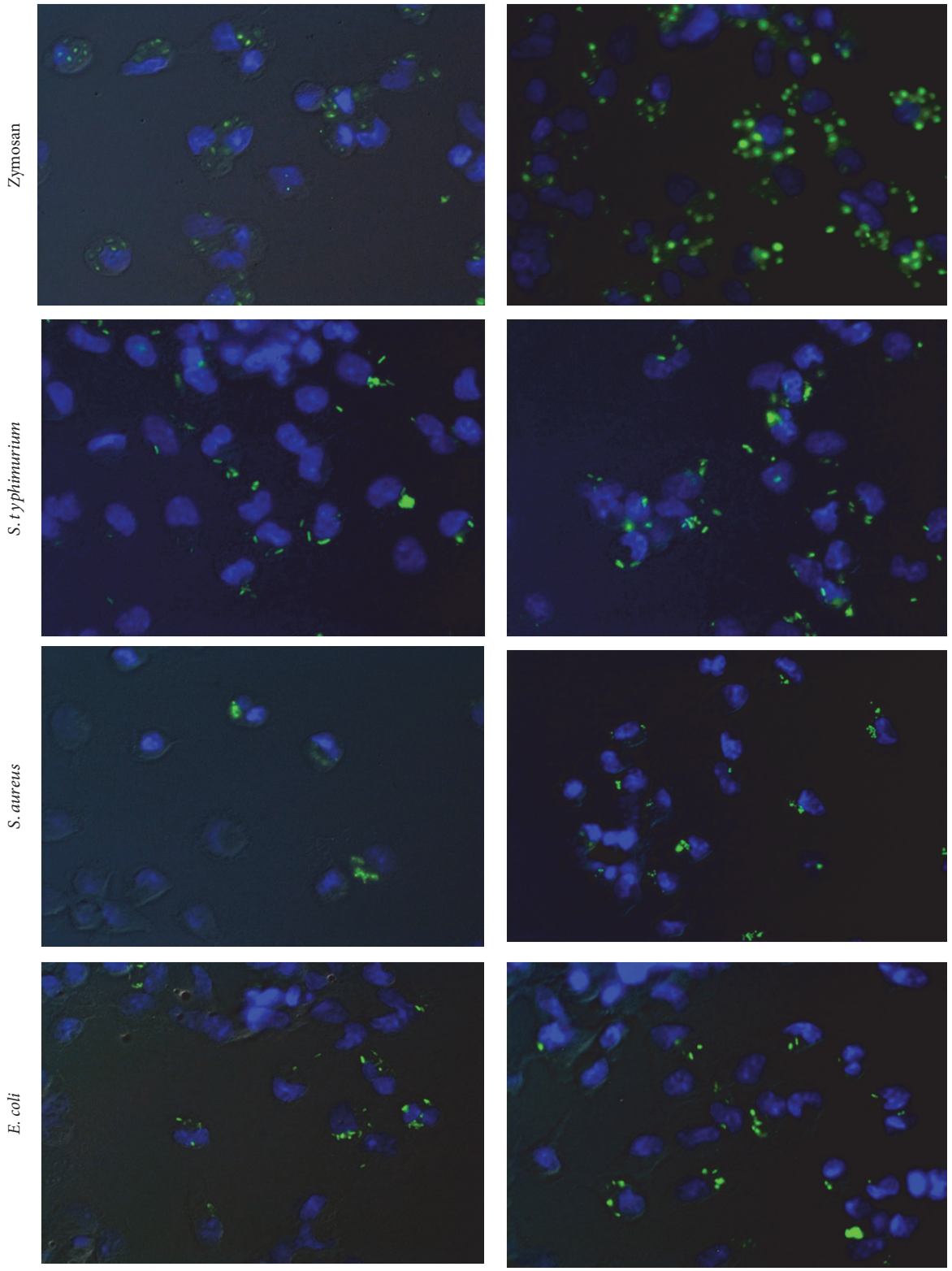
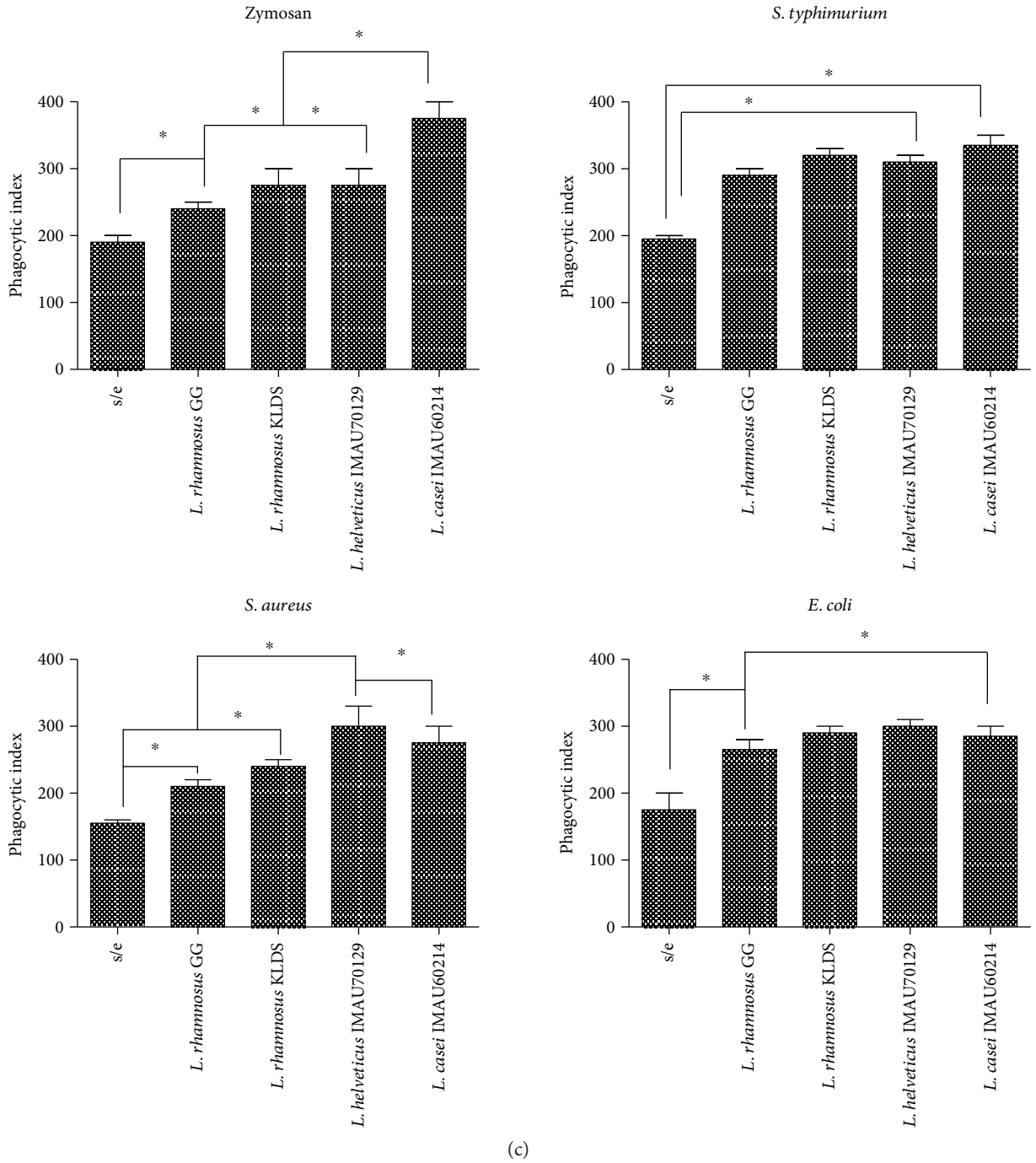


Figure 3: Continued.



(c)

Figure 3: Effect of heat-killed lactobacillus on phagocytosis in human macrophages. Cells (1×10^4) were treated with heat-killed strains of lactobacillus at a MOI of 1 : 500 for 1 h before zymosan particles, *S. aureus*, *S. typhimurium*, or *E. coli* (each at a MOI of 1 : 10) were added. Phagocytosis was allowed to proceed for 1 h at 37°C in an atmosphere containing 5% $C0_2$, before phagocytosis was terminated. The cells were then fixed, and the nuclei were stained with DAPI. Macrophages, untreated (a) and treated (b) cells. Fluorescence photomicroscopy in macrophages treated with different stimuli was performed using an epifluorescence Zeiss Axioskop2 microscope equipped with a Zeiss Axiocam (Zeiss AG, Oberkochen, DE). This figure shows the representative results with the strain (*L. casei* IMAU60214). (c) Heat-killed lactobacillus enhanced the phagocytic index of human macrophages. The ingestion of cells was determined in macrophages that were incubated in medium alone or preincubated with lactobacillus strains. The following formula was used: the percentage of cells undergoing phagocytosis × the number of particles per cells. The cells were incubated with zymosan, *S. typhimurium*, *S. aureus*, and *E. coli*, as indicated. Asterisks indicate a significant difference between conditions ($p > 0.05$).

Lactobacillus (i.e., *L. rhamnosus* GG, *L. rhamnosus* KLDS, *L. helveticus* IMAU70129, and *L. casei* IMAU60214) on the induction of the synthesis of inflammatory mediators, including ROS. The immunological role of ROS has been highlighted in individuals with defects in the biochemical machinery required for protein synthesis. ROS entities are

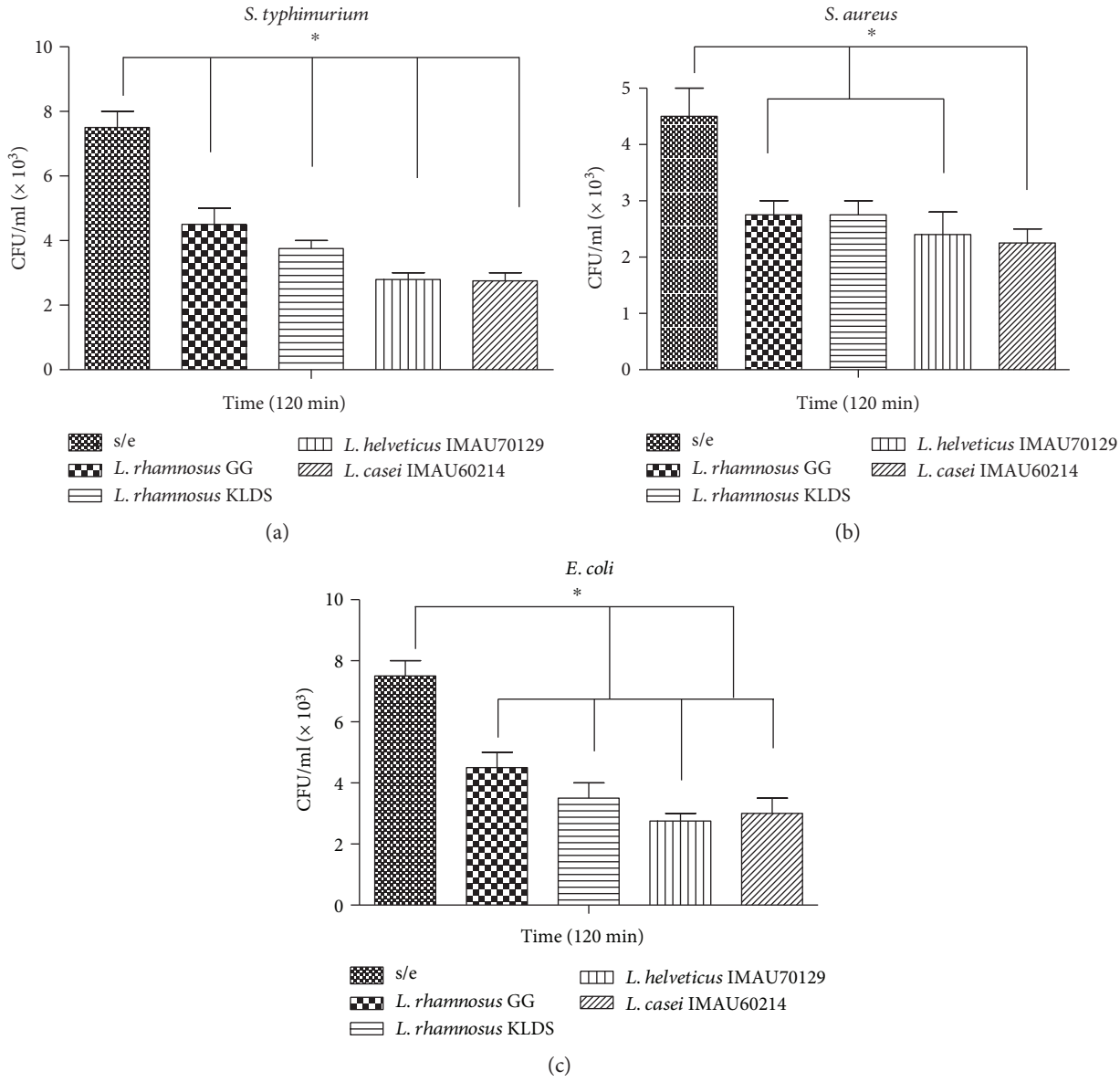


Figure 4: Effect of lactobacillus on the bacterial activity of macrophages against the pathogens *S. typhimurium, S. aureus*, and *E. coli*. Human macrophages were infected with (a) *S. typhimurium*, (b) *S. aureus*, or (c) *E. coli* and then incubated for 90 min at 37°C to allow the bacteria to be internalized. External bacteria were then killed by applying gentamicin for 30 min at 37°C. Samples were taken immediately before gentamicin treatment (0 h) and every 120 min thereafter to determine viable counts following Triton X-100 lysis. The results are expressed as CFU per milliliter with means ± SD from no fewer than four separate experiments that were performed in duplicate using macrophages obtained from different human donors. Asterisks indicate a significant difference between conditions ($p < 0.05$).

extremely toxic and have deleterious effects on a variety of pathogens, such as fungi, parasites, and bacteria [27]. Activated mononuclear phagocytes (i.e., monocytes and macrophages) are the main source of microbicidal ROS. We demonstrated that in macrophages, the production of ROS in response to challenge with lactic bacteria was dependent on the concentration and species of bacteria. Both of the probiotic bacteria used in this study (*L. casei* IMAU60214 and *L. helveticus* IMAU70129) induced the highest levels of ROS in human macrophages obtained from healthy hosts. In support of these results, Marcinkiewicz et al. [28] demonstrated that ROS production was also induced in murine macrophage stimulated with lactobacillus. Likewise, the production of new ROS molecules in response to lactobacillus was associated with the mechanisms underlying IL-12 synthesis [29].

In contrast, other investigations have demonstrated that free extracts of LGG and *L. paracasei* had antioxidant activities in cells grown in the presence of superoxide dismutase [30]. The variability in the biological effects exerted by different probiotics may be associated with a number of factors, such as secreted factors, including bacteriocins, lactic acid, short chain fatty acids, and the presence of SOD activity. Different studies have reported that the probiotic effects of different lactobacillus species affect the immunomodulatory

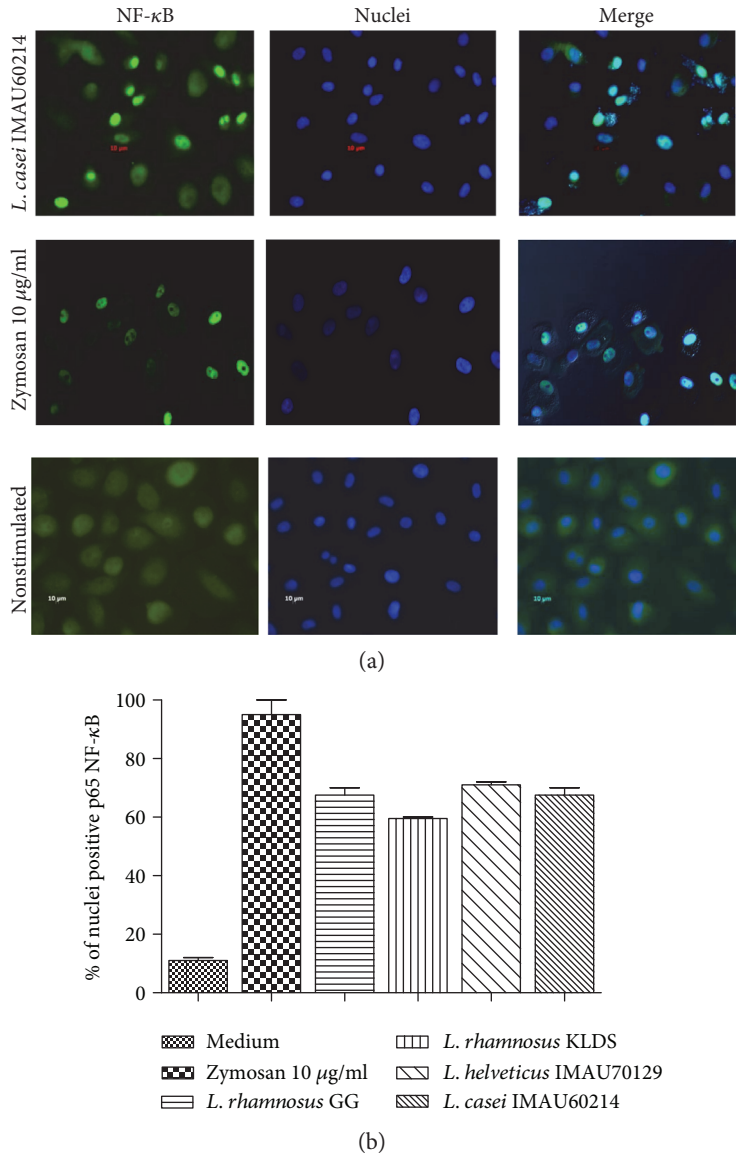


Figure 5: Immunofluorescence analysis of NF-*k*B activation. Human macrophages were stimulated for 30 min with heat-killed lactobacillus at a MOI of 1 : 500, zymosan (positive control) or left unstimulated. (a) Nuclear translocation was defined as blue fluorescence (Hoechst staining), and the NF-*k*B pp65 subunit was identified using green fluorescence (Dylight 488). (b) The percentage of nuclei that were positive for pp65. The results represent three independent experiments.

capacity of various cellular components in the innate and mucosal immune systems, such as T, B, and NK lymphocytes [31]. We demonstrated that the heat-killed lactobacillus *L. rhamnosus* GG, *L. rhamnosus* KLSD, *L. helveticus* IMAU70129, and *L. caseí* IMAU60214 induce the production of the cytokine IL-8 in human macrophages. This chemokine plays a very important role in the recruitment of others immune cells during an inflammatory response [32]. The chemokine IL-8 is produced during the early stages of the interaction lactobacillus and macrophages (i.e., within 6 h of stimulation), and this response was sustained for 24 h at much higher levels (> 2000 pg/ml) than that of the production of other cytokines analyzed in this study. In similar studies, other authors have demonstrated that the TH1 lymphocytes display chemotaxis in response to the expression of the IL-8 mRNA and other soluble factors derived from LGG [33]. In contrast, Jiang et al. [34] showed that IL-8 synthesis was not induced when lines of epithelial intestinal (i.e., CaCo2, T84, and HT-29) and THP-1 monocyte cells were stimulated with *L. reuteri*. These discrepancies were attributed to differences in the study models and the types of cells used in them. In the current study, we also demonstrated that TNF-α and IL-8 were similarly induced and that they were maintained at high levels for 24 h. Both TNF-α and IL-8 are proteins that play very important biological roles in the regulation of the inflammatory responses of macrophages [35]. These results collectively suggest that the probiotic strains analyzed in this study have immunostimulatory effects on the synthesis of high level cytokines, including IL-6, IL-

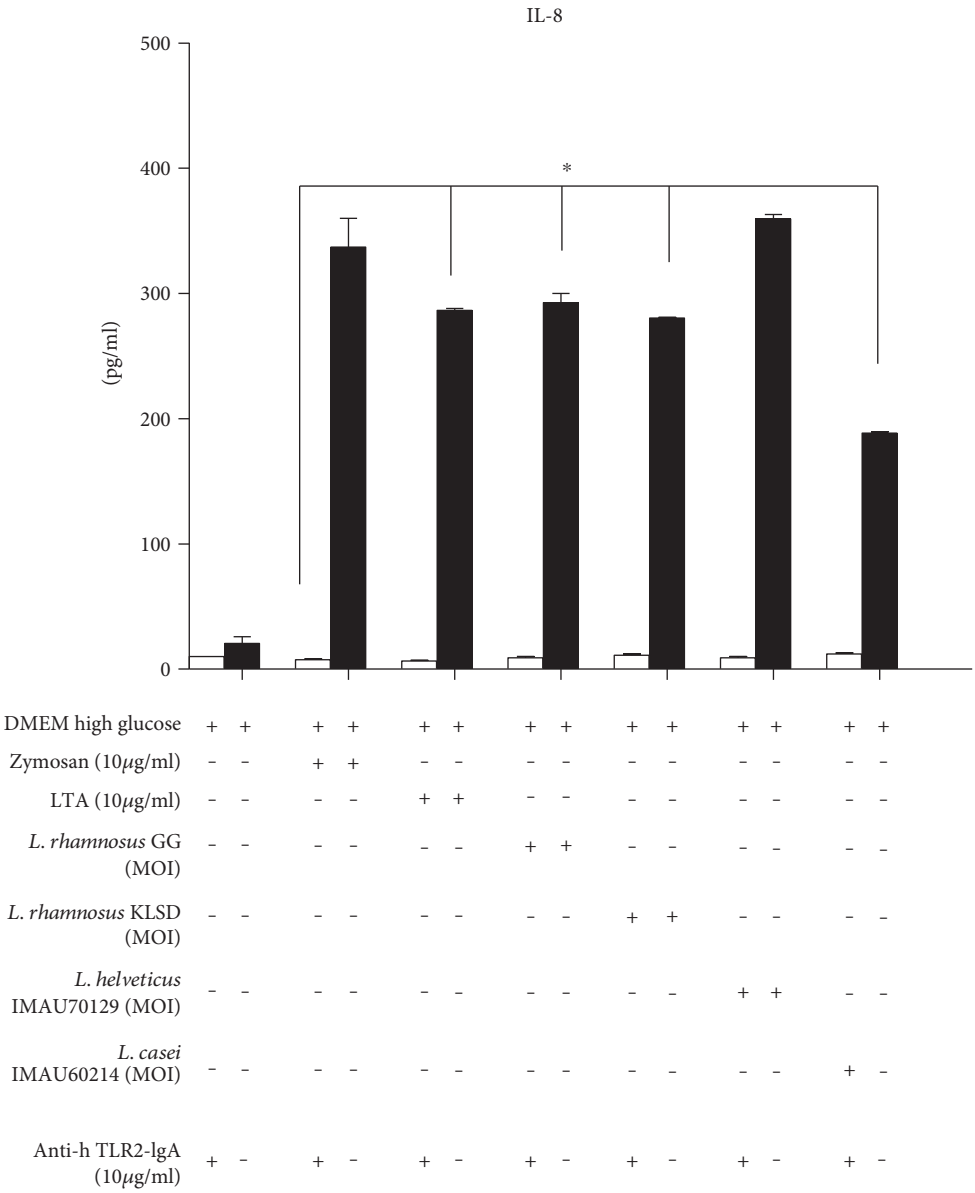


Figure 6: Transfection assay of HEK293-hTLR2 cells showing IL-8 was secreted in response to strains of lactobacillus. The recognition of lactobacillus strains was evaluated using a model in which HEK293-hTLR2 cells were transfected, and then blocking assays were performed using anti-hTLR2-IgA antibodies at a concentration of 10 μg/ml. $p < 0.05$ ($*$).

12, and IL-1β, for at least 24 h in human macrophages. One interesting result of this study was that the production of IL-1β, which plays a very important role in the costimulation of T lymphocyte functions, was not induced in response to all strains. Part of the impact of lactobacillus on the regulation of the immune response was to induce the synthesis of IL-10, which was observed at high levels at 24 h after stimulation. IL-10 has also been shown to play a role in chronic gastrointestinal problems, and its modulation by probiotic bacteria has been observed in patients with ulcerative colitis and inflammatory bowel disease [36]. There is no doubt that IL-10 downregulates proinflammatory cascades. We also demonstrated that these lactic bacteria had an impact on the phagocytic activity of macrophages against extracellular pathogens such as *S. aureus* and *E. coli* and intracellular pathogens such as *S. typhimurium*. In support of these results, a previous study showed that in a murine model supplementing the diet with *L. plantarum* CGMC 1.557 for 20 days enhanced phagocytic activity in macrophages [37]. In addition, Kausahal et al. [38] demonstrated that *Lactobacillus acidophilus* and *Bifidobacterium bifidum* improved the phagocytic potential of macrophages in aged mice. However, the signaling induced by the production of several mediators, such as cytokines, involves the activation of downstream transcription factors, such as NF-*k*B [39]. In the present work, we demonstrated that NF-*k*B activated an inflammatory response in response to heat-inactivated lactobacillus. Similar reports have shown that

stimulation with *L. casei* induced the MAPK and NF-*k*B signaling pathways, which was associated with the secretion of the cytokines TNF-α and IL-12 from murine spleen cells [40]. *L. rhamnosus* GG also initiated signaling cascades including the NF-*k*B and STAT signaling pathways in human macrophages. In this study, we also found that in combination with the activation of NF-*k*B, TLR2 also plays an important role by activating the synthesis of IL-8 in HEK-hTLR2 cells in response to stimulation with lactobacillus (*Lactobacillus rhamnosus* GG, *L. rhamnosus* KLSD, *L. helveticus* IMAU70129, and *L. casei* IMAU60214). In contrast, Shida et al. [41] reported that PGN derived from *L. jonhsonii* and *L. plantarum* exerted inhibitory effects via both TLR2-dependent and independent mechanisms. In other studies, using murine macrophages, stimulation with LTA reversed the ability of some lactobacillus strains to induce the synthesis of IL-12 by increasing the production of IL-10 via a mechanism involving the activation of ERK family dependent effects on the activation of TLR2 [42]. Other investigations have indicated that viable and lyophilized lactobacillus exerts different effects on immunomodulation and that these differences are partially attributable to the involvement of the TLR receptor but not TLR4 and TLR9 [43].

Finally, investigations into the mechanisms underlying the activation of macrophages by lactobacillus probiotics are important, and the results of studies such as this one suggest that this group of lactic bacteria, including *Lactobacillus rhamnosus* GG, *L. rhamnosus* KLSD, *L. helveticus* IMAU70129, and *L. casei* IMAU60214, has potential adjuvant effects on the immune response of host organisms.

5. Conclusion

Four heat-killed lactobacillus probiotic strains exerted immunostimulatory properties by activating the in vitro inflammatory response of macrophages via mechanism involving the synthesis of proinflammatory mediators, including cytokines, ROS, and participation in signaling cascades, such as the NF-*k*B and TLR2 pathways. These observations suggest that the properties of heat-killed probiotics may improve the innate immune response. However, in vitro tests not necessarily represent which may occur in vivo, so it is important to evaluate the effect of probiotics in models with immunosuppressed animals and clinical studies in humans. Depending on the results obtained, the effect of these probiotics as potential immunomodulators in immunocompromised hosts could be evaluated.

Abbreviations

PRRs: Pattern recognition receptors
TLRs: Toll-like receptors
MAMPs: Microbe-associated molecular patterns.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Rocha-Ramírez LM and Perez-Solano RA conceived the study, and Eslava C contributed to its experimental design. Rocha-Ramírez LM and Eslava C wrote the manuscript. Eslava C and García Garibay M participated in revising the manuscript. The phagocytosis and bactericidal activity experiments were performed by Castañón-Alonso SL. Cell culture and human macrophages were prepared by Ramírez Pacheco A. Cytokine levels were quantified (ELISA) by Moreno Guerrero SS. The immunofluorescence assays used to evaluate NF-*k*B expression and chemilumiscence were performed by Rocha-Ramírez LM and Pérez Solano RA. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by grants from the Fondos Federales México (HIM/2011/007 SSA.929 and HIM/2014/013 SSA.1120).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 8139591, 8 pages
https://doi.org/10.1155/2017/8139591

Research Article

Examination of the Microbial Spectrum in the Etiology of Erythema Nodosum: A Retrospective Descriptive Study

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Received 9 February 2017; Revised 14 April 2017; Accepted 4 May 2017; Published 28 May 2017

Academic Editor: Mitesh Dwivedi

Even though infections are the most common cause of erythema nodosum (EN), only certain microorganisms take the great interest such as streptococci in knowledge. Our aim was to examine the frequency and type of infections in EN, to determine the characteristics of patients with an infectious etiology, and to discuss the role of these microbes in EN pathology in the context of their interactions with humans. Charts of 81 patients with EN who were seen between 2003 and 2017 were retrospectively reviewed. Identified etiological factors were classified into three groups: infectious, noninfectious, and idiopathic. While there were no significant demographic and clinical differences between the infectious and idiopathic groups, systemic symptoms ($p = 0.034$) and the number of EN lesions ($p = 0.016$) were significantly lower; the mean erythrocyte sedimentation rate was significantly higher ($p = 0.049$), but the mean aspartate aminotransferase value was significantly lower in the infectious group compared to the noninfectious group ($p = 0.019$). Besides streptococci, many other microbes, including the ones living on and inside us, were identified in the etiology of EN. There is a need for large-scale prospective studies involving control groups for a better understanding of the microbial immunopathology of EN.

1. Introduction

Erythema nodosum (EN) is the most common clinical form of panniculitis defined with hypodermal septal inflammation. It usually appears on the anterior surfaces of the lower extremities as erythematous, warm, and painful nodules ranging from 1 to 20 cm in diameter [1]. Lesions do not have a tendency to necrosis and scars but may leave residual hyperpigmentation. Cutaneous symptoms reach their maximum in 1-2 weeks and then spontaneously resolve in 1–6 weeks, sometimes taking up to 12 weeks to fully resolve [2, 3]. In addition to the cutaneous symptoms, a prodrome commonly occurs a few weeks before the onset of EN including weight loss, malaise, low-grade fever, cough, and arthralgia with or without arthritis [1, 2, 4].

The exact prevalence of EN in Turkey is unknown, but the estimated worldwide prevalence of the disease is reported as one to five per 100,000 persons [2, 5]. Although EN can occur at any age, it is most common in the third and fourth decades of the life, and there is a female predominance with the prevalence being three to five times higher in women than men [3].

EN is generally considered a reactive process that may be triggered by a wide variety of stimuli [1, 2]. Most direct and indirect evidence support the involvement of a type IV delayed hypersensitivity response to numerous antigens. Although no specific cause can be documented in most of the patients, it is imperative to investigate the possible triggers [3]. Etiological factors may vary according to many conditions including age, gender, race, and geographic location of the patient, but infections are the most frequently related factors [1, 2, 6]. Because of the well-known association of EN with streptococcal infections, they take the great part of interest in most studies; however, other infections are not sufficiently focused in this regard. Therefore, there is a need to think about the possible roles and locations of other microorganisms in the etiology of EN.

The purpose of this study was to examine the frequency and type of infections in EN etiology, to determine the characteristics of patients with an infectious etiology and to discuss the role of these microbes in EN pathology in the context of their interactions with humans.

2. Materials and Methods

2.1. Study Design. This is a retrospective chart review of all patients with EN who were seen at the Department of Dermatology of Dokuz Eylul University Faculty of Medicine in Izmir, Turkey, between January 2003 and 2017. The study protocol was approved by the Local Ethical Committee which follows the guidelines set by the Declaration of Helsinki.

2.2. Collection of the Data. A total of the 159 patients' file records with the diagnosis of EN were screened. The diagnosis was made on clinical grounds based on the presence of tender erythematous nodules and plaques without ulceration mainly on the legs. Skin biopsies were carried out in 64 patients with unilateral involvement and in those with involvement at sites other than the pretibial areas. Histopathological verification was based on the presence of septal inflammation, with an inflammation of the septal vessels, but without signs of true vasculitis. 18 patients were excluded because of the absence of typical histopathological findings, and 60 patients were excluded because of the missing data, lack of ≥1-year follow-up period in their file records, and having more than single etiological factor. As a result, a total of 81 patients were included in this study, and 46 of them were verified by histopathologic examination due to the clinical suspicion. Demographic and clinical data were collected from patients' file records. Clinical features including systemic manifestations, location and number of EN lesions, past history of EN, season in the EN attack, associated disorders, and used medications were noted. The following investigations were done in all included patients: complete blood count; erythrocyte sedimentation rate; C-reactive protein; routine blood chemistry including fasting blood glucose, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyl transferase, lactate dehydrogenase, total bilirubin, direct bilirubin, and total protein; serum electrolytes including sodium, potassium, chloride, and calcium; viral serologies including human immunodeficiency virus, hepatitis B virus, and hepatitis C virus; serologies for hydatid cyst, brucella, and syphilis; antistreptolysin O titer (two consecutive determinations were performed in a 2–4 week interval if it was >200 U/mL); antinuclear antibody; routine urine analysis; urine culture; throat culture; direct fecal smear microscopy and culture; tuberculin skin test; chest X-ray; and abdominopelvic ultrasonography. In each case, the results of routinely requested dental, otorhinolaryngological, and gynecological consultations were also reviewed in order to investigate focal infections. Vaginal smears and cultures were obtained from all patients who underwent vaginal examination. Specific investigations such as angiotensin converting enzyme, pathergy test, computed tomography scan, and special microbial smears, cultures, and/or serologies were also performed in individual patients if there were associated signs and symptoms.

Table 1: Demographic and clinical characteristics of the study subjects ($n = 81$).

Characteristics	EN patients
Gender, *n* (%)	
Female	64 (79%)
Male	17 (21%)
Age, mean ± SD (min–max)	38.54 ± 19.17 (7–83)
Location of EN	
Pretibial areas	59 (72.8%)
Pretibial areas + other areas**	14 (17.3%)
Other areas**	8 (9.9%)

EN: erythema nodosum; **femoral areas, gluteal areas, arms, ankles, and/or knees.

2.3. Etiological Classification of EN. EN etiology was classified into three groups: infectious (associated with any infection), noninfectious (associated with rheumatological diseases, sarcoidosis, inflammatory bowel diseases, malignancies, and/or medications), and idiopathic (when no underlying disease or precipitating event was found). Criteria for including the patients in the infectious group were the absence of any other identifiable factors and detection of no recurrences of EN within 1 year after the appropriate treatment of the identified infection. In order to provide a clear distinction in the classification, only the patients who had only single well-established etiological cause were included in this study. In other words, patients who had both infectious and noninfectious etiologies were excluded.

2.4. Statistical Analysis. The statistical analyses were performed with the SPSS/PC software (Version 23.0 for Windows; SPSS Inc., Chicago, Ill). The Mann Whitney *U* test was used to compare the mean values of quantitative variables as the two samples were obtained independently. Qualitative variables were analyzed with chi-squared test and Fisher's exact test. $p < 0.05$ was considered significant in all analyses.

3. Results

Demographic and clinical characteristics of the patients with EN are shown in Table 1. Overall, the most common etiological factor was infections which were found in 32 (39.5%) of the patients (Table 2). Among all patients with EN, 19 (23.4%) had recent upper respiratory tract infections and 13 (16%) of them had serological evidence for streptococcal pharyngitis (a positive throat swab culture for *Streptococcus pyogenes* in one patient and high antistreptolysin O titers in the others). All infections and identified microbes in EN patients are demonstrated in Table 3.

In the comparison of three groups (infectious, noninfectious, and idiopathic), no significant differences were

Table 2: Etiological factors in patients with erythema nodosum ($n = 81$).

Etiological factors	n (%)
Infectious	32 (39.5%)
Noninfectious	24 (29.6%)
Rheumatological diseases	11 (13.5%)
Sarcoidosis	6 (7.4%)
Inflammatory bowel diseases	(3.7%)
Malignancies	2 (2.5%)
Medications	2 (2.5%)
Idiopathic	25 (30.9%)

Table 3: Infections and identified microbes in patients with erythema nodosum ($n = 81$).

Infections	Identified microbes	Patients with EN
URTI		19 (23.4%)
Streptococcal	Streptococci ($n = 13$)	13 (16%)
Nonstreptococcal	—	3 (3.7%)
Viral	—	3 (3.7%)
GIT infection		3 (3.7%)
Intestinal infection	*Blastocystis hominis* ($n = 2$)	2 (2.5%)
Stomach infection	*Helicobacter pylori* ($n = 1$)	1 (1.2%)
UTI	*Escherichia coli* ($n = 3$)	3 (3.7%)
Pneumonia	*Mycoplasma pneumoniae* ($n = 1$)	2 (2.5%)
Vaginitis	*Gardnerella vaginalis* ($n = 1$) *Candida albicans* ($n = 1$)	2 (2.5)
Breast abscess	*Staphylococcus aureus* ($n = 1$)	1 (1.2%)
Rectal abscess	—	1 (1.2%)
Tuberculosis	*Mycobacterium tuberculosis* ($n = 1$)	1 (1.2%)

EN: erythema nodosum; URTI: upper respiratory tract infection; GIT: gastrointestinal tract; UTI: urinary tract infection.

observed between the infectious and idiopathic groups in means of all demographic and clinical data. But, while infectious and noninfectious groups did not show significant differences in terms of age, gender, past EN history, location of EN lesions, and season in the EN attack, the presence of systemic symptoms and number of EN lesions were significantly lower in the infectious group ($p = 0.034$ and $p = 0.016$, resp.). In a detailed examination of the systemic symptoms, even though they did not reach statistical significance, malaise and arthralgia were the markedly lower symptoms in the infectious group (Table 4). In the infectious group, a majority of patients had the EN attack in autumn (34.4%) while the number of patients were the lowest in autumn for the other two groups. However, these differences did not show statistical significance.

In laboratory evaluations, higher white blood cell count, neutrophil percentage, C-reactive protein value, and an erythrocyte sedimentation rate were found in the infectious group compared to the other groups (Figure 1). Among these, only the differences in the erythrocyte sedimentation rate (31.5 mm/h versus 24.1 mm/h) and in the proportion of patients with the high erythrocyte sedimentation rate (65.6% versus 33.3%) showed statistical significance between the infectious and noninfectious groups ($p = 0.049$ and, resp.). By contrast, many of the biochemical markers were found to be higher in the noninfectious group compared to the infectious group. However, only the difference in the proportion of patients with high aspartate aminotransferase (0% versus 16.7%) reached the statistical significance ($p = 0.029$) (Table 5).

4. Discussion

In this report, we present data based on the spectrum of the microbial etiology of EN in a series of patients seen during a 14-year period in a university hospital for a defined population of Western Turkey. Not as a surprising result, the etiology of EN was associated with infectious causes in a high proportion of patients, and streptococci were the most frequently identified etiological agents. But the main outcome of this study is that, besides the well-known associations of certain microbes with EN, even some others that we consider to be harmless or microorganisms which do not constitute the criteria for an active infection, may also involve in EN etiology.

In addition, to examine the microbial diversity in EN etiology, we also aimed to determine the specific characteristics of patients with an infectious etiology. We found that systemic symptoms and the number of EN lesions were lower in these patients when compared to the patients with a noninfectious etiology. Since most of the patients with an infectious etiology had no current active infection or their infections were in the regressive phase at the time when EN lesions were detected, systemic symptoms may also be reduced in this period. Another reasonable explanation of these results may be the detection of some silent infections in these patients such as *Blastocystis hominis* (*B. hominis*), *Helicobacter pylori* (*H. pylori*), *Gardnerella vaginalis* (*G. vaginalis*), or *Candida albicans* (*C. albicans*). Additionally, the higher detection of the long standing symptoms such as weight loss, malaise, arthralgia, and night sweats in the other group is already an expected finding as a result of the course of the chronic systemic diseases. In laboratory findings, a significantly higher erythrocyte sedimentation rate in patients with an infectious etiology may be reflecting the slow regression of this acute phase reactant which might be increased in the active phase of the infections. On the other hand, significantly higher aspartate aminotransferase values in the noninfectious group may be related with the accompanying systemic disorders in these patients. Although this enzyme is basically known as a diagnostic liver enzyme, various conditions may increase the value since it is located in many

Table 4: Demographic and clinical data of erythema nodosum patients according to the etiological classification ($n = 81$).

Variables	Etiological classification			p value	
	Infectious (A)	Noninfectious (B)	Idiopathic (C)	A versus B	A versus C
Number of patients, n (%)	32 (39.5)	24 (29.6)	25 (30.9)	—	—
Age, years, mean ± SD	36.16 ± 18.17	43.88 ± 17.39	36.48 ± 21.65	0.110	0.987
Female gender, n (%)	26 (81.3)	18 (75)	20 (80)	0.573	1.00
Systemic symptoms, n (%)	15 (46.9)	18 (75)	6 (24)	**0.034***	0.076
Fever	6 (18.8)	5 (20.8)	2 (8)	1.00	0.444
Weight loss	2 (6.3)	4 (16.7)	1 (4)	0.385	1.00
Malaise	2 (6.3)	6 (25)	0	0.063	0.499
Arthralgia	10 (31.3)	13 (54.2)	5 (20)	0.085	0.339
Night sweats	1 (3.1)	2 (8.3)	0	0.571	1.00
Number of EN lesions, mean ± SD	1.90 ± 0.92	2.95 ± 1.82	2.24 ± 1.47	**0.016***	0.603
Past history of EN, n (%)	3 (9.4)	5 (20.8)	5 (20)	0.268	0.280
Location of EN, n (%)					
Pretibial areas	25 (78.1)	17 (70.8)	17 (68)	0.533	0.389
Pretibial areas + other areas**	4 (12.5)	5 (20.8)	5 (20)	0.475	0.485
Other areas**	3 (9.4)	2 (8.3)	3 (12)	1.00	1.00
Season in the EN attack, n (%)					
Spring	5 (15.6)	7 (29.2)	6 (24)	0.222	0.508
Summer	8 (25)	7 (29.2)	8 (32)	0.728	0.559
Autumn	11 (34.4)	3 (12.5)	5 (20)	0.061	0.231
Winter	8 (25)	7 (29.2)	6 (24)	0.728	0.931

EN: erythema nodosum. *Significant values. **Femoral areas, gluteal areas, arms, ankles, and/or knees.

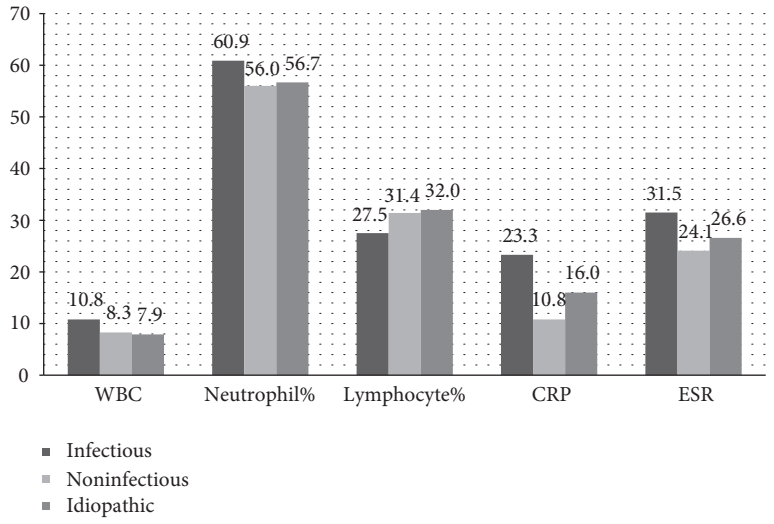

Figure 1: Laboratory parameters associated with infectious processes in erythema nodosum patients according to the etiological classification ($n = 81$). WBC: white blood cell; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

tissues of the human body. Two of the patients with high aspartate aminotransferase values in the noninfectious group had malignancies (pancreas cancer in one patient and myelodysplastic syndrome in the other), one of the patients had systemic sarcoidosis, and the other one had long-term oral contraceptive use, which may be the factors responsible for the high values in patients in this study.

The spectrum of the microbial etiology of EN may vary depending on a range of factors such as the population studied, the location, reflecting the geographical distribution of microbes, and even the department in the same geographical area in which patients were admitted [1, 2, 5]. Although all the infective agents including aerobic and anaerobic bacteria, viruses, fungi, parasites, and mycobacteria can induce

Table 5: Laboratory characteristics of erythema nodosum patients according to the etiological classification ($n = 81$).

Variables	Etiological classification			p value	
	Infectious (A) ($n = 32$)	Noninfectious (B) ($n = 24$)	Idiopathic (C) ($n = 25$)	A versus B	A versus C
Complete blood count					
WBC > 10,000/mm^3, n (%)	11 (34.4)	6 (25)	5 (20)	0.450	0.231
Neutrophil > 73%, n (%)	5 (15.6)	1 (4.2)	1 (4)	0.085	0.215
Lymphocyte > 45%, n (%)	2 (6.3)	4 (16.7)	3 (12)	0.385	0.645
Acute phase reactants					
CRP > 5 mg/L, n (%)	18 (56.3)	11 (45.8)	10 (40)	0.440	0.223
ESR > 20 mm/h, n (%)	21 (65.6)	8 (33.3)	12 (48)	**0.017***	0.181
Biochemical markers					
FBG > 100 mg/dL, n (%)	8 (25)	6 (25)	5 (20)	1.00	0.655
Urea > 20 mg/dL, n (%)	0	1 (4.2)	0	0.429	NA
Creatinine > 0.95 mg/dL, n (%)	1 (3.1)	3 (12.5)	1 (4)	0.303	0.439
AST > 35 U/L, n (%)	0	4 (16.7)	1 (4)	**0.029***	0.439
ALT > 35 U/L, n (%)	1 (3.1)	5 (20.8)	1 (4)	0.074	1.00
LDH > 220 U/L, n (%)	10 (31.3)	8 (33.3)	8 (32)	1.00	1.00
GGT > 38 U/L, n (%)	3 (9.4)	5 (20.8)	2 (8)	0.268	1.00
ALP > 120 U/L, n (%)	3 (9.4)	4 (16.7)	3 (12)	0.447	1.00
Total bilirubin > 1.2 mg/dL, n (%)	0	1 (4.2)	0	0.429	NA
Direct bilirubin > 0.2 mg/dL, n (%)	3 (9.4)	5 (20.8)	4 (16)	0.268	0.687
Total protein < 8.3 g/dL, n (%)	4 (12.5)	5 (20.8)	3 (12)	0.475	1.00

WBC: white blood count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; FBG: fasting blood glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; GGT: gamma glutamyl transferase; ALP: alkaline phosphatase; NA: not applicable. *Significant values. (The upper or lower limits in laboratory values were based on the reference intervals given in Central Laboratory of Dokuz Eylul University Hospital.)

eruption of EN, a specific cause can be documented in only half of the patients in clinical practice. Streptococcal pharyngitis is still the most frequent etiological factor in all over the world both in developed and developing countries [7–10]. They have been reported to be detected up to 44% of patients in adults and 48% of patients in children with EN [4]. The increased incidence of EN patients in the first half of the calendar year and the detection of familial EN cases may also be associated with this infectious etiology [1]. EN eruptions typically appear two to three weeks after the episode of a streptococcal pharyngitis so that the pharyngeal swabs are usually negative at the time when EN lesions are detected, as in our cases. Therefore, patients should have repeated streptococcal antistreptolysin O titers or polymerase chain reaction assays for group A streptococci to demonstrate the previous infection [4].

Apart from upper respiratory tract infections, some gastrointestinal system infections were also identified as the etiological factor in this study. Intestinal *B. hominis* infection was determined in stool in two of the patients with intermittent abdominal pain and diarrhea. *B. hominis* is an intestinal protozoan regarded by some as a mere commensal and by others as a parasite that can cause diarrhea and intestinal disease [11]. Although there has been no documentation in literature to specify the association of *B. hominis* with EN, it has been known that some intestinal parasites such as *Giardia* species have been reported to be associated with EN in literature [12, 13]. Furthermore, *B. hominis* has already been known to cause cutaneous hypersensitivity reactions by alternating the immune system via activation of specific Th2 immune cells producing interleukins including IL-3, IL-4, IL-5, and IL-13, and some immunological disorders such as urticaria, Hashimoto's thyroiditis, and Henoch-Schönlein purpura have been mentioned to be associated with *B. hominis* infection [11, 14, 15]. While all human intestinal parasitic infections have been shown to induce both humoral and cellular immune responses and can cause the hypersensitivity reactions in their hosts, we suggest a possible role of *B. hominis* infection in the pathogenesis of EN.

The occurrence of EN lesions was associated with *H. pylori* infection of the stomach in another patient in this study. Both stool antigen assay and endoscopic biopsy were performed as the diagnostic methods for this infection. *H. pylori* is the dominant member of the gastric microbiota in over half of the human population, and it can persist in a human stomach for a lifetime. Approximately 20% of *H. pylori* organisms adhere to the surface of gastric epithelial cells, and this physical contact causes cellular damage and activates the immune system [16]. Since this immunological response caused by the bacterium is oriented both locally and systemically, several immune-mediated extraintestinal disorders including hematological, cardiovascular, neurological, metabolic, and autoimmune have been considered to be potentially induced by the infection. Even though the presence of reports in literature on the association of *H. pylori* with various cutaneous diseases such as chronic urticaria,

rosacea, psoriasis, Henoch-Schönlein purpura, Behçet's disease, alopecia areata, and Sweet syndrome, there exists only one report to demonstrate the association with EN [17]. In this short report of Capella GL. consisting of two patients, the author discussed the possible role of *H. pylori* infection in the etiology of EN by providing some scientific evidence. These were its biological similarity with *Campylobacter* species which are the recognized causes of EN, the presence of EN-like eruptions in immunosuppressed patients caused by *Helicobacter cinaedi* bacteremia, the disappearance of EN-like lesions of Behçet's disease after *H. pylori* eradication, and the improvement of EN lesions after *H. pylori* eradication [18].

Urinary tract infection due to *Escherichia coli* (*E. coli*) was the associated factor with EN lesions in three of the patients in our study. *E. coli* is one of the most versatile bacterial species which is either harmless as a commensal in gut microbiata or pathogenic with the ability to cause intestinal or extraintestinal infections [19]. It is becoming increasingly clear that various microbes have been postulated to trigger a cascade of immunological events in patients, and urinary tract infections are among the most common infectious diseases of humans caused by a range of pathogens, with uropathogenic *E. coli* being the most common etiological agent [20]. Even with this high frequency, the association of *E. coli* infections with EN has not been adequately addressed in literature. In only one study, investigating the associated diseases with EN, *E. coli* infection was mentioned among the well-established associations. However, the authors gave no detailed information about the type or anatomical location of the infection in this study [7].

Two of the study patients had EN lesions associated with pneumonia in our study. An empiric antibiotic therapy was started without the ability to determine the infectious agent in one patient, and *Mycoplasma pneumoniae* (*M. pneumoniae*) was the identified agent in the other. There are many publications in literature reporting the association between *M. pneumoniae* and EN [21, 22]. Unfortunately, the link between EN and mycoplasma infection has not been fully understood yet. In some instances, *M. pneumoniae* has been isolated directly from skin lesions, suggesting the direct effect of the microorganism to elicit the development of cutaneous lesions [23]. It has been speculated that *M. pneumoniae* can be transferred hematogenously to the dermis, thus causing a hypodermal inflammation. However, it is also impossible to exclude the role of immunologic mechanisms and autoimmunity [21] because most cutaneous lesions associated with microorganisms are thought to be caused not by the organisms themselves but by the host response to antigens on these microbes. Many different mechanisms such as immune-complex-mediated damage, cytotoxic T cell-mediated immune responses, cytokines and chemokines released against mycoplasmal lipopeptides, and autoimmune reactions may play a role in some phases of the immune responses, and the innate immune response to mycoplasmas may also enhance the role of the adaptive immunity [23].

Genital infections were detected in two of the EN patients in this study. The first one was bacterial vaginosis caused by *G. vaginalis*. Bacterial vaginosis is the worldwide leading vaginal disorder among women of reproductive age caused by polymicrobial agents in which *G. vaginalis* is the founder organism [24]. However, *G. vaginalis* is also considered to be a part of the normal vaginal microbiota in a significant proportion of both girls and women by some researchers while others think of this entity as the asymptomatic infection [25]. When we consider the association of this microbe with some immunological conditions such as erythema multiforme and reactive arthritis, it may not be irrational to consider it among the microbial etiological causes of EN [26, 27]. The other detected genital infection was vulvovaginal candidiasis caused by *C. albicans* in the second patient. Vulvovaginal candidiasis is estimated to be the second most common cause of vaginitis after bacterial vaginosis, and *C. albicans* accounts for 85% to 90% of cases [28]. *C. albicans* is a commensal yeast species that is found in the human gastrointestinal and vaginal microbiota. When tolerance mechanisms become defective, the commensal form changes into the pathogenic form and expresses its virulence traits [29]. In our literature search, we found no specific documentation on the association of *C. albicans* with EN, although the delayed-type hypersensitivity reaction to *C. albicans* is already well known and has been historically used to evaluate the immune competence experimentally in humans [30]. In fact, the essential subject that might be considered in this aspect is whether these microorganisms are among the overlooked etiological factors in EN cases in which there exists a female predominance, especially in patients who fall into the "idiopathic" category.

Breast abscesses are among the relatively rare reported causes of EN in literature. In a patient series involving two EN patients associated with breast abscesses, *Staphylococcus aureus* (*S. aureus*) was the causative agent in one of the patients as compatible with our results [31]. In spite of the pathogenesis associated with *S. aureus*, it is also acknowledged to be a typical member of the complex community of microbiota living on and within our skin. But little is known about the transition from the asymptomatic colonization to an invasive infection, despite the fact that persistently colonized individuals are most often infected by their own colonizing strains [32]. Although *S. aureus* is the most frequently detected microorganism in breast abscesses, the relationship with EN is still unclear due to the low level of evidence [31].

Tuberculosis has long been linked with EN and is the second most common infectious disease associated with EN after streptococcal infections in most reports from Turkey [6, 33]. EN may occur in the course of primary tuberculosis and may even manifest before the development of a skin reaction to the tuberculin skin test. Furthermore, EN may be found in patients with highly positive reactions to the tuberculin skin test with no detectable focus of active tuberculosis infection. It is believed to reflect the strong immunologic response to *Mycobacterium tuberculosis* (*M. tuberculosis*) antigens in immunocompetent individuals [4, 34]. Consistent with this, an excess cytokine response to *M. tuberculosis* antigens was demonstrated in EN patients in a recent study, and blood from EN patients was shown to exhibit an enhanced ability to restrict the

mycobacterial growth in vitro. On the other hand, in some studies, it was suggested that EN should be considered a strong predictor of tuberculosis or an early symptom of tuberculosis rather than just a response to newly acquired *M. tuberculosis* antigens. It has been suggested to screen all patients with EN for *M. tuberculosis* infection and even to initiate the tuberculosis treatment in certain patients who are in hard-to-reach populations with limited possibility of continuous monitoring for the symptoms [34]. In our study, contrary to the expected for our country, tuberculosis did not constitute a major portion of the EN etiology. The reason for this may be the lower incidence of tuberculosis in our region compared to the other regions of the country [35].

The major limitations of this study are the retrospective nature, lack of a control group to compare the results obtained, and limited number of patients due to our intention to take the patients only who had complete data and single well-established etiological factor for inclusion. Additionally, the follow-up periods were not uniform in each case; thus, it may have caused some missing follow-up data for the detection of recurrences of EN in patients. However, as far as we know, this is the first detailed study in literature that examines and discusses the possible roles of microbes in EN occurrence, in the context of their interactions with humans.

5. Conclusion

In the present study, a significant proportion of cases with EN was associated with the infectious etiology, and streptococcal pharyngitis was the most commonly detected cause in accordance with previous reports. Besides this well-known association, *B. hominis*, *H. pylori*, *E. coli*, *M. pneumoniae*, *G. vaginalis*, *C. albicans*, *S. aureus*, and *M. tuberculosis* were the other identified microbes in EN development in our patients. Since all microbes, either pathogenic or commensal, are the critical regulators of the host immune system and have the potential to induce the activation of immunological events in their hosts through complex mechanisms, the microbial spectrum of EN may be much greater than we know. There is a need for prospective large-scale studies involving control groups in the future for a better understanding of the microbial immunopathology of EN.

Conflicts of Interest

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

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 7904821, 14 pages
https://doi.org/10.1155/2017/7904821

Research Article

Does the Gut Microbiota Influence Immunity and Inflammation in Multiple Sclerosis Pathophysiology?

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Received 20 September 2016; Revised 31 December 2016; Accepted 2 February 2017; Published 20 February 2017

Academic Editor: Ilian Radichev

Aim. Evaluation of the impact of gut microflora on the pathophysiology of MS. *Results*. The etiopathogenesis of MS is not fully known. Gut microbiota may be of a great importance in the pathogenesis of MS, since recent findings suggest that substitutions of certain microbial population in the gut can lead to proinflammatory state, which can lead to MS in humans. In contrast, other commensal bacteria and their antigenic products may protect against inflammation within the central nervous system. The type of intestinal flora is affected by antibiotics, stress, or diet. The effects on MS through the intestinal microflora can also be achieved by antibiotic therapy and *Lactobacillus*. EAE, as an animal model of MS, indicates a strong influence of the gut microbiota on the immune system and shows that disturbances in gut physiology may contribute to the development of MS. *Conclusions.* The relationship between the central nervous system, the immune system, and the gut microbiota relates to the influence of microorganisms in the development of MS. A possible interaction between gut microbiota and the immune system can be perceived through regulation by the endocannabinoid system. It may offer an opportunity to understand the interaction comprised in the gut-immune-brain axis.

1. Introduction

1.1. Definition of Multiple Sclerosis. Multiple sclerosis (MS) is a progressive disease of the central nervous system (CNS) characterized by the presence of lesions in the brain and the spinal cord [1]. Nylander and Hafler [2] demonstrated that the inflammatory factor in MS contains CD4 and CD8 T cells, B cells, and activated monocytes that result in the degradation of the myelin sheath surrounding nerves. Traditionally, inflammatory demyelination has been considered the primary form of the pathogenesis in MS.

Although the etiology of MS remains unclear, several hypotheses suggest that autoimmunity plays a major role in the development of the disease. The most widely supported view is that MS is a CD4+ T cell-driven autoimmune disorder [3]. In MS lesions, astrocytes play a paradoxical role during disease development [4]. Experimental data show that astrocytes not only mediate inflammation but also diminish the detrimental effects of proinflammatory factors. Activated astrocytes secrete compounds including reactive oxygen and nitrogen species [5], which have toxic effects on neurons. Oxidative stress is a key factor in the pathogenesis of MS. Activated macrophages and microglia in the CNS produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) and secrete cytokines (tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6)) and chemokines (macrophage inflammatory protein (MIP-1), monocyte chemoattractant protein (MCP-1), and interferon-gamma-(IFN-γ-) induced protein (IP-10)). Neurodegeneration during MS may result from chronic oxidative stress and excitotoxicity [6].

Recent clinical and experimental studies indicate that MS arises from a negative interaction between genetics and environmental factors [7, 8]. Multiple sclerosis has a complex genetic component including the haplotype HLA-DR15 and over 100 of other types of minor risk alleles involved in the

immune response. Additionally, several environmental and lifestyle factors contribute to the onset of MS, including latitude, deficiency of vitamin D3, obesity early in life, cigarette smoking, and the Epstein-Barr virus (EBV). Hedström et al. [9] and Alotaibi attempted to find a relationship between the active form of vitamin D3, 1,25-OH D3, and the major histocompatibility complex (MHC) HLA-DRB1∗15 allele, which is linked to an increased risk of developing MS [10]. The study results of Hedström and Alotaibi [11] suggest that HLA-DRB1∗15 only contributes to the risk of disease when susceptible individuals are deficient in vitamin D.

In the 1970s, a relationship between the 6p21 region of the *HLA* gene and MS risk was discovered. In the following three decades, this region was only considered a genetic risk factor that increased susceptibility to MS. It was not until the introduction of genome-wide association studies (GWAS) that new genetic risk factors were found (the International Multiple Sclerosis Genetics Consortium). There is considerable variability in *HLA* genes in patients with MS. Research in Europe and the United States has revealed the existence of three HLAs that occur more frequently in MS compared to the general population. Further research in the US has shown that MS is more likely to occur in individuals who possess more than one of these HLAs. It can be assumed that the HLA system correlates with the course of the disease [11]. There are at least two genes, apart from HLA-DRA, on which single-nucleotide polymorphisms (SNPs) can occur, which may increase susceptibility to MS: the IL2R gene (an encoding subunit of the IL-2) and IL7RA gene (a subunit of IL-7) (the International Multiple Sclerosis Genetics Consortium). Furthermore, the risk of MS may be altered by polymorphic variants of ALCAM, CD6, CD80, CD86, and CD40 genes. Lastly, there may be sex-related genetic factors that contribute to the risk of MS. Reduced levels of CD6 mRNA in men and a reduced expression of the CD40L gene in women may increase the risk of developing MS (Pender).

Multiple human epidemiological studies have revealed the effects of environmental factors on the prevalence of MS [11] and demonstrated that viral infections, lack of sun exposure, vitamin D deficiency, active or passive smoking, season of birth, obesity, vitamin A deficiency, dietary habits (especially high levels of salt and fat), stress, and the intestinal microflora play a significant role in the initiation of the disease. EBV is a widely recognized risk factor. A seroepidemiological study of MS found that nearly 100% of the patients were infected with EBV [13]. If EBV infection occurs in late childhood, it is considered the largest risk factor for the development of MS. There is a strong EBV-specific CD8+ response in the blood during the onset of MS; the intensity of the response decreases during the course of the illness [14, 15].

Neuropathological studies support roles for the human herpes virus (HHV-6) and human endogenous retroviruses (HTLV-1/2, HIV-1/2) as risk factors for the development of MS. Herpes viruses can reactivate or cause de novo infections, leading to neurological disabilities [16]. Other risk factors include lack of sun exposure and deficiency of vitamin D [17]. Spelman et al. [18] demonstrated latitude-dependent lag between seasonal ultraviolet radiation (UVR) and relapse probability, which showed that the probability of a relapse increases in MS patients living at latitudes further from the equator. Additionally, MS patients living at further latitudes have significantly lower levels of vitamin D in all seasons of the year. These patients reach a low threshold level of 25(OH)D, long immunomodulatory effect sooner after the winter solstice compared to people residing at more equatorial latitudes. Low levels of vitamin D have been observed in other neurological diseases, including Alzheimer's disease, Parkinson's disease, and stroke [19]. A meta-analysis showed that individuals born in autumn have a high risk of MS, based on their exposure to UV light and vitamin D levels [20]. Studies show that the risk of the disease decreases with diets low in salt and fat and with the use of essential oils from *Pterodon emarginatus*, a Brazilian legume [21].

Symptoms of MS include cognitive deficits, impaired information processing, and long-term memory deficits [22, 23]. Clinical symptoms are muscle weakness, blurred vision, dizziness, and fatigue [2].

2. Main Text

2.1. The Definition of Gut Flora. Gut microflora is the population of microorganisms living in the human gut. Microflora can be loosely divided into harmless, beneficial, or pathogenic forms. Intestinal microflora develops within the first few years of life and then remains largely stable. There are an estimated 1000+ different types of intestinal flora (Table 1). The main factors that influence the composition of the intestinal flora after birth are the duration of pregnancy and the method of delivery (natural childbirth or caesarean section) [24]. Premature birth is associated with the presence of *Staphylococcus*. Children born vaginally have a higher incidence of *Lactobacillus* and *Prevotella,* while children born by caesarean section have a higher incidence of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* [25]. Facultative anaerobic bacteria such as *Escherichia coli* and other coliform bacteria are the first colonizers of the intestine in infants. In the first year of life, the intestine is colonized by *Bacteroides*, *Clostridium*, *Ruminococcus*, and *Bifidobacteria* [26]. Breastfeeding promotes *Bifidobacteria* and *Lactobacillus* proliferation. The intestinal microflora of a 3-year-old child is identical to that of an adult [27, 28]. Mariat et al. [29] suggested that the changes in the composition of the intestinal flora might result in the dysfunction of the immune system in elderly adults. Collins et al. [30] reported that the adult intestinal flora is mainly composed of four main clusters: *Firmicutes*, *Bacteroidetes*, Actinobacteria, and *Proteobacteria*. Tuohy et al. [31] reported that the intestinal flora can be divided into three groups, including health benefits, bacteria of the genera *Bifidobacterium* and *Lactobacillus*, and opportunistic microorganisms [32, 33].

2.2. GALT System. The gut-associated lymphoid tissue (GALT) is the largest immune system in the body. Intestinal bacteria act through the lymphatic system, which is associated with the digestive tract, that is, the GALT system. The GALT consists of organized cellular complexes (Peyer's patches and solitary lymphoid follicles) and dispersed cells

Table 1: The composition of human intestinal flora.

The name of the bacteria	The amount of bacteria	Age of occurrence	The origin of the bacteria	Influence	Role
Staphylococcus	$<10^5$ CFU ml^{-1}	Acute prematurity	Caesarean section	Pathogenic	Unfolding mucin whose parts are used as food for the body. One of the first pathogens inhabiting the intestinal flora [Palmer et al., 2007]
Actinobacteria	10^4 CFU ml^{-1}	From the first years of life	With food	Beneficial	Coordination of the immune system Stimulation of gastrointestinal motilityProduction of bioactive compounds, including antibacterial, antifungal, and anthelmintic drugs as well as antitumor and antiviral drugs, for example, streptomycin and kanamycin [Kumar et al., 2010]
Bacteroides	10^{10} to 10^{11} per 1 g of the content	From the age of 3	With a decreasing amount of oxygen in the intestine There are obligatory anaerobes	Endogenous bacteria due to infection	Production of vitamins B7, B2, and C Inhibition of the growth of pathogens and harmful bacteria [Round et al., 2011] They stimulate the formation of iTreg [Round et al., 2011]
Clostridium	$<10^5$ CFU ml^{-1}	The largest number in the adulthood	With a decreasing amount of oxygen in the intestine There are obligatory anaerobes	Pathogenic	They stimulate the formation of Treg relevant in reducing the pathology dependent on Th2 cells in the mucous membranes of the respiratory system [Atarashi et al., 2011] [Josefowicz et al., 2012]
Ruminococcus	10^5–10^6 CFU ml^{-1}	From the age of 3	Natural childbirth	Beneficial	Unfolding mucin, which helps the intestine absorb the fragments used as food for the body [Præsteng et al., 2013]
Bifidobacteria	10^{10} to 10^{11} CFU ml^{-1}	From the first years of life if the child is breastfed	Mother's milk	Beneficial	They form a natural protective barrier against pathogens by producing bacteriocin and organic acids. Stimulation of gastrointestinal motility [Bottacini et al., 2014]
Veillonella	10^5 CFU ml^{-1}	From the first years of life	With food	Pathogenic	Coordination of the immune system Stimulation of gastrointestinal motility This increases the concentrations of IL-8, IL-6, IL-10, and TNF-α [van den Bogert et al., 2014]

Table 1: Continued.

The name of the bacteria	The amount of bacteria	Age of occurrence	The origin of the bacteria	Influence	Role
Prevotella	10^6-10^7 CFU ml^{-1}	From birth	Natural childbirth	Pathogenic	Production of vitamin B1 and folic acid Inhibiting the growth of pathogens and harmful bacteria [Koskey et al., 2014]
Streptococcus	$<10^5$ CFU ml^{-1}	From birth	The first organisms that colonize the intestine	Pathogenic	Stimulation of the immune system by the ability of adhesion to the bowel mucosa [Wang et al., 2014]
Lactobacillus	10^8 to 10^{10} per 1 g of the content	From the first years of life if the child is breastfed	Natural childbirth	Beneficial	Create natural barriers to the growth of pathogenic bacteria by production of bacteriocin and organic acids [Bottacini et al., 2014]
Escherichia coli	$>10^6$ CFU ml^{-1}	The first few hours of life	With a decreasing amount of oxygen in the intestine There are obligatory anaerobes	Pathogenic	Production of vitamins B and K It produces fecal enzymes and synthesized carcinogens [Grudniak et al., 2015]
Propionibacterium	10^6 CFU ml^{-1}	From birth	Caesarean section	Pathogenic in immunocompromised individuals	Coordination of the immune system Stimulation of gastrointestinal motility Induced expression of proteins iNOS and COX-2 by ROS-dependent NF-kB and AP-1 activation of macrophages [Jahns et al., 2015]

iTreg: regulatory T cells; CFU: colony-forming unit; Th2: lymphocytes; IL: interleukin; TNF-α: tumor necrosis factor-alpha; iNOS: nitric oxide synthase; COX-2: induced cyclooxygenase; ROS: reactive oxygen species; NF-kB: transcription factor; AP-1: activator protein 1.

(T and B cells, macrophages, and dendritic cells) which are located in the intestinal lamina propria, near the epithelium. In the intestinal epithelium, covering Peyer's patches, are specialized cells, known as M cells, which capture antigens and transmit them to T and B cells. Unlike pathogens, commensal bacteria are unable to penetrate other internal organs or the bloodstream. Weng and Walker reported that antigen stimulation of lymphocytes causes proliferation of naive T and B cells, which are activated in the digestive tract, migrate to the lymph vessels and mesenteric lymph nodes, and enter the bloodstream. In the blood, T and B cells are transported back into the lymphatic structures of the digestive tract and the mucous membranes of other systems (respiratory, urogenital, and endocrine glands), where they remain as effector cells. Germ-free animals had only free B cell. Intestinal microflora colonization by commensal bacteria in GF animals stimulates the immune system, resulting in the formation of active Peyer's patches, proliferation of lymphocytes in the lamina propria, and increased production and circulation of secretory antibodies, mainly immunoglobulin A (IgA) and immunoglobulin M (IgM). Nonpathogenic intestinal bacteria stimulate the formation of primarily natural antibodies, which are essential components of the nonspecific immune mechanisms and constitute the first line of defense in the immune response [34]. Research in the Institute of Mother and Child in Prague on probiotic strains of *E. coli* (O86 and Nissle 1917) showed that neonatal *E. coli* leads to long-term stimulation and production of secretory antibodies. The most commonly used probiotics are strains of *E. coli* Nissle 1917, *Saccharomyces boulardii*, and a probiotic mixture containing four strains of *Lactobacillus* and *Bifidobacterium* and one of three strains of *Streptococcus salivarius* [35]. Probiotics may also directly influence the permeability of the intestinal barrier. In the recent studies on epithelial cell lines derived from the colon tissue, it was confirmed that the probiotic *Lactobacillus acidophilus* restores proinflammatory cytokines such as TNF-α and interferon-gamma (IFN-γ) [36]. Probiotics contribute to the balance of cytokines and can favorably affect the course of those allergic and inflammatory diseases. The clinical effects of probiotics are beneficial and activities of the specific strain cannot be transferred to another strain of the same species. Clinical observations indicate that the probiotic strains may be potentially useful in the treatment of inflammatory diseases [37].

2.3. The Impact of Drugs on the Flora. An increased amount of *Bifidobacterium* was detected in stool samples of people who consume caffeine [38]. The researchers found that smoking and drinking coffee can alter the composition of the intestinal flora. Caffeine in coffee increases the level of granulocyte colony-stimulating (G-CSF) levels, which leads to significant improvement in memory in mice [39].

2.4. Interaction of Intestinal Flora with Other Systems. Organisms perform a number of metabolic processes, including the synthesis of vitamins B2, B7, and C, which can affect the bioavailability and metabolism of drugs. Some species of bacteria activate the immune system and can cause the development of inflammatory bowel disease (IBD) and other diseases including myasthenia gravis and diabetes [40]. Turnbaugh et al. [41] demonstrated that the intestinal microflora is related to obesity. In the experiment, human intestinal microflora was transferred to GF mouse and was monitored during manipulation of the diet of mice. The introduction of diet resulted in changes after one day [42]. Increasing the energy production by methanogenic bacteria may contribute to the development of obesity. After surgical treatment of obesity, the number of *F. prausnitzii* in patients with type 2 diabetes (T2D) increased but was lower than that in the controls. After surgery, reduced blood glucose, insulin, and glycosylated hemoglobin were noted in patients and there was also decreased resistance to insulin, based on the ELISA results of HOMA-IR (Homeostasis Model Assessment of Insulin Resistance). Some bacteria, such as Firmicutes, contribute to an increase in the absorption of short-chain fatty acids [43]. The effect of lipopolysaccharides and peptidoglycans on the circulatory system by the permeability of the intestinal epithelial barrier stimulates the production of cytokines. These substances have an impact on the synthesis of low-density lipoproteins and can cause damage to the endothelial cells, foam formation, and proliferation of smooth muscle cells [44], the factors that are closely related to the development of atherosclerosis. In patients with heart failure, colorectal microvascular changes may induce the production of cytokines, which contribute to the impaired myocardial function. The bacteria will also be found in the blood circulation, so they may also play a role in the development of heart failure [45]. Sun et al. [46] demonstrated that cathelicidin antimicrobial peptide that is produced in the beta cells of the pancreas in mice with diabetes is also present in normal mice. In another study, intestinal bacteria were transferred from normal mice into the intestine of mice with type 1 diabetes. The bacteria are involved in the initiation of fatty acid production, which leads to the formation of cathelicidin and inhibiting the development of type 1 diabetes [47]. The immune system protects against the invasion of pathogens on the mucosal surface through the production of IgA. [48]. Germ-free animals reduced the number of intestinal intraepithelial T lymphocytes. T cells are important for the detection of pathogens and antigen presentation. Intestinal colonization by microorganisms is required for optimal growth, development, and function of the intestinal immune system [49, 50]. Microbial action on the central nervous system is expressed by modulating cytokine levels. This action also includes changes in the expression of receptors in the brain which affect the interaction of the intestines and the autonomic nervous system, including regulation of the hypothalamic-pituitary-adrenal axis [51].

2.5. The Influence of Gut Flora on the Development and Maturation of the CNS. Changes in the microbiome intestine can lead to abnormal immune responses in both the distal regions of the gut and immune system, that is, the CNS. The microflora modulates the immune system through by-products (Table 2) [52]. Human intestinal flora can play very important role in the pathogenesis of MS, and recent studies suggest that replacing some of the bacterial population in the gut can lead to a state of the proinflammatory potential mechanism of pathogenesis of MS. Bacteria and their antigenic products may protect against inflammation within the central nervous system of man. Specific bacterial antigens, such as polysaccharides (PSA) from *B. fragilis*, mediate the migration of microflora, which indicates a significant interaction between the intestinal mucosa and human brain. These observations suggest that populations of effector cells and regulatory T (Treg) cells are involved in the pathogenesis of MS [53].

Pantazou et al. [54] described the gut-brain axis and demonstrated that intestinal bacteria affect neuroendocrine function and the maturation of the brain by stimulating migration of CD4+ T cells that express CD39 in the brain [55]. Experimental autoimmune encephalomyelitis (EAE) has been used as a model to explore MS and other demyelinating autoimmune diseases of the CNS. Predominantly Th1 and Th17 characterize the immune response to EAE [56]. Lee et al. have shown that oligodendrocyte glycoprotein sensitized mice are susceptible to EAE, but the authors concluded that the segmented bacterial colonization caused her susceptibility [57]. EAE resistant mice were also infected with *Lactobacillus casei* Shirota [58] and *Bifidobacterium animalis,* which were found to reduce symptoms [59]. A potential therapeutic strategy for MS is oral administration of probiotic *Lactobacillus* species, which has been shown to result in IL-10-dependent activation of Tregs in the CNS followed by reduction of IFN-γ, TNF-α, and IL-17. Treatment targeting the gut of EAE mice can suppress chronic inflammation. Effect of intestinal microflora may act to both increase and decrease the occurrence of autoimmune diseases by affecting the permeability of the intestinal immune tolerance to the loss of constituents of the microflora and the formation of antibodies to the antigens of intestinal bacteria [60].

2.6. Latitude and Specific Gut Flora. According to Miyake et al. [61], the composition of intestinal microflora in Japanese patients with MS is not significantly different from that of healthy people. However, the analysis of this study by UniFrac showed significant differences ($p < 0.05$) in the overall structure of the intestinal microflora in MS patients and healthy controls. The intestinal microflora in MS patients has greater interindividual variability than that of healthy controls [62–64]. This study also showed a decrease in the percentage of several *Bacteroides*, including *B. stercoris*, *B. coprocola,* and

Table 2: Effect of gut microflora on the immune system in MS.

Authors	Materials	Models of diseases	Conclusions
Sriram et al. (1999)	17 patients with relapsing-remitting MS, 20 patients with progressive MS, and 27 patients with other neurological diseases (OND)	MS	CNS infections; *Chlamydia pneumoniae* is a common occurrence in patients with MS. Although *Chlamydia pneumoniae* may be a pathogenic factor of MS, it may simply be a secondary infection of damaged tissues of the CNS.
Becher et al. (2001)	Mice	EAE	CD40-CD154 interactions in the CNS are key determinants for the development and progression of the disease. No CD40 expression in cells of the CNS reduces the intensity and duration of myelin oligodendrocyte and glycoprotein-induced EAE and reduces the degree of infiltration of inflammatory cells into the CNS.
Oksenberg et al. (2008)	Mice	EAE	EAE is considered a model of autoimmune diseases, including MS.
Ezendam and van Loveren (2008); Ezendam et al. (2008)	Mice	EAE	Reduced symptoms in mice infected with *Lactobacillus casei* Shirota and *Bifidobacterium animalis*.
Yokote et al. (2008)	Mice	MS	Low-calorie diet alleviates the symptoms of MS.
Hawker et al. (2009)	Adults with primary progressive MS	MS	Rituximab monoclonal antibody, selective cell killing CD20, proved effective in reducing disease activity in relapsing-remitting MS.
Lavasani et al. (2009)	Mice with the strains of *Lactobacillus*	EAE	The administration of probiotic lactic acid has a positive effect on the autoimmune disease by the production of IL-10 and stimulation of Treg cells.
Barnett et al. (2009)	Patients with MS and other neurological diseases	MS	IgG disrupted myelin in MS.
Ochoa et al. (2010)	Mice after treatment with antibiotics and infected with *B. fragilis*	EAE	Antibiotic therapy can protect against EAE; a similar effect is observed for *B. fragilis* (commensal bacteria).
Farooqi et al. (2010)	Mice	EAE	EAE has similar features to inflammation, demyelination, axonal loss, and gliosis.
Lee et al. (2011)	Mice	EAE	SFB induce Th17 immune response.
Farrokhi et al. (2013)	Patients with and without MS concentration of lipid 624	MS	Lipid 624 (TLR2 ligand) occurs in lower concentrations in patients with MS [12].
Rumah et al. (2013)	Patients with MS and healthy individuals	MS	CSF obtained from two tissues; immunity to ETX is 10 times more frequent in individuals with MS compared to healthy subjects, indicating prior exposure to ETX in MS population.
Chiurchiù et al. (2013)	Healthy people and MS patients	MS	pDC from patients with MS and production of higher levels of interleukin-12 and interleukin-6, whereas pDC had lower levels of interferon-α compared to healthy subjects.

Table 2: Continued.

Authors	Materials	Models of diseases	Conclusions
Tauschmann et al. (2013)	Healthy young people	Autoimmune disease	The imbalance between the bacteria and the intestinal immune system leading to overstimulation of the immune system.Treg cells have inhibitory effects on the cells in autoimmune diseases.
Reichelt et al. (2014)	Patients with MS	MS	The increase of IgA may be secondary to an increase in the intestinal absorption.
Miyake et al. (2015)	Patients with MS	MS	Patients with MS are characterized by moderate dysbiosis. The decrease in the percentage of several *Bacteroides*, including *B. stercoris*, *B. coprophilus*, and *B. coprocola*.
Nicol et al. (2015)	Mice	EAE	Reduction of the severity of symptoms after antibiotic treatment. Decrease in the amount of IFN-γ, MIP-1a, MIP-1 p, MCP-1, IL-17, and IL-6 and increase in the amount of IL-10 and IL 13.

EAE: experimental autoimmune encephalomyelitis; *B. fragilis*: *Bacteroides fragilis*; IL: interleukin; Treg: regulatory T cells; MS: multiple sclerosis; SFB: segmented filamentous bacteria; TLR2: Toll-like receptor 2; IgG: immunoglobulin G; Th17: T helper 17 cell; IFN-γ: interferon-gamma; MIP: macrophage inflammatory proteins; MCP-1: monocyte chemoattractant protein-1; IgA: immunoglobulin A; CSF: cerebrospinal fluid; *C. perfringens*: *Clostridium perfringens*; CD 40: cluster of differentiation 40; CD 154: cluster of differentiation 154; CNS: central nervous system; ETX: epsilon-toxin; pDC: proportion of days covered.

B. coprophilus in the intestinal microflora in patients with MS [61].

2.7. Effect of Flora on Cytokines in MS. An imbalance of intestinal bacteria, intestinal epithelial cells, and cells of the immune system in the intestinal mucosa can lead to overstimulation of the immune system. Suppression of excessive immune stimulation is controlled by Treg cells, a distinct population of CD4+ T cells produced in the thymus and peripheral organs of the immune system (e.g., GALT). Tregs have inhibitory effects on inflammatory cell populations and autoreactive effector cells. Treg function defects occur frequently in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and MS [65].

According to Berer and Krishnamoorthy [66], there is increasing evidence from animal studies of a relationship between the type of gut microflora and the progression of MS. Autoimmune reactions can be produced by molecular mimicry or by stimulating the production of lymphocytes. Metabolites produced by these bacteria can also affect the autoimmune system. Multiple sclerosis is a demyelinating disease, which coincides with inflammatory processes [67]. Whether bacterial pathogens act as initiators of MS is not entirely clear. The intestinal microflora can produce various metabolic by-products that affect the CNS, causing reduced autoimmunity. These by-products include short-chain fatty acids (acetic acid, butyric acid, and propionic acid) which bind to the anti-binding G protein coupled receptor (GPCR) and produce Treg, which suppresses proinflammatory cytokines and antigen presenting cells. Adenosine triphosphate stimulates the production of Th17 inflammatory cells, which can cause autoimmune reactions in the CNS [68]. This can adversely affect the commensal bacteria *Bacteroides fragilis* and cause T cell differentiation to Th1, IL-10, and Treg [69–71].

Farrokhi et al. [72] demonstrated unique lipodipeptide bacteria which originate from serine lipodipeptide, lipid 654, which is produced by some Bacteroidetes commensal species, and act as ligand-receptor, providing further evidence for an association between the bacteria and the MS. Kleinewietfeld et al. [73] demonstrated that lipid 654 is expressed at much lower levels in the serum of MS patients than in healthy controls. Lipid 654 may be biomarker of MS [72]. Furthermore, these bacteria produce a number of metabolic products known as the metabolome of by-products which could cause activation of the immune system.

2.8. Effect of the Flora on MS Symptoms. The earliest noticeable changes in the permeability of the blood-brain barrier are oligodendrocyte apoptosis and microglial activation [74]. At that time, demyelination is still visible [75–77]. Soluble toxins may be initiating factors in MS. Natural toxin of *Clostridium perfringens* or toxins B and D influence neurological symptoms [78–82]. The toxins are absorbed by the intestine, enter the bloodstream, and cause MS-like symptoms (e.g., blurred vision, lack of coordination, or spastic paralysis). Murrell et al. [83] first suggested that exotoxins could be a potential cause of MS, as humans are not natural hosts of *C. perfringens* types B or D [84–87]. Exotoxins bind to receptors present in the vascular system of the brain and in both myelinated and unmyelinated brain regions, such as the corpus callosum [88–91]. Osmolysis occurs upon binding to the receptor [92–96]. Dawson [97] first described the morphology of relapsing multiple sclerosis. Binding of retinal vein which forms a barrier to the CNS may explain the occurrence of periphlebitis retinae in MS patients [98, 99]. Toxins affect

the barrier veins, which may cause inflammation of the retina. Periventricular accumulation of monocytes is often observed [77]. During the test, the effect of intestinal microflora has been shown that the colonized mice had more symptoms become severe and inflammation [100]. This was demonstrated in another animal model of MS [101]. Dendritic cells (DCs) may not activate T cells to an autoantigen as effectively as DCs in colonized animals. Commensal bacteria can act as either the preferred symbiont or a disease-promoting pathobiont, depending on the target of the autoimmune disease, different effector mechanisms, and the composition of the intestinal flora [102].

2.9. Effect of Immunomodulatory Agents in MS on the Gut Flora. Further evidence of a link between bacteria in the gut and MS is the decreased concentration of *Faecalibacterium* in patients with MS compared with healthy subjects [103]. Butyrate production is associated with increased numbers of Treg cells, which suggests a potential mechanism through which changes in the intestinal microbiome may lead to predisposition for developing MS. Patients with MS treated with glatiramer acetate have less Bacteroidaceae, *Faecalibacterium*, *Ruminococcus*, Lactobacillaceae, *Clostridium*, and other members of the Clostridiales class compared with untreated MS patients. The study compared 3 groups: healthy subjects, patients with MS who are treated, and patients who are not treated. In response to vitamin D supplementation, only MS patients who did *not* undergo glatiramer acetate treatment showed an increase in the number of *Akkermansia*, *Faecalibacterium,* and *Coprococcus*. It was recently discovered that intestinal colonization by the *Clostridium perfringens* type B is associated with relapse in MS. Toxins produced by *C. perfringens* can lead to microvascular complications leading to neuronal and oligodendrocyte damage [104–107], which may serve as a trigger for future demyelinating events in susceptible individuals. Jhangi et al. [108] compared MS patients with healthy subjects and observed increased concentrations of Archaea (*Methanobrevibacter*) and decreased concentrations of *Butyricimonas* and Lachnospiraceae in MS patients.

According to Wekerle [8], MS also arises from a negative interaction between genetics and environmental factors. A large number of genes promote an autoimmune response against brain cells. New experimental and clinical studies indicate that autoimmune attacks are triggered by an interaction between immune cells of the brain and the gut microbiota. These findings may contribute to the development of new therapeutics that modulate gut microbiota.

Cannabinoid receptors in the intestinal tissue are activated by ligands of cannabinoids that may activate cannabinoid receptors in other local systems. Chiurchiù et al. [109] discovered that treatment of MS patients with anandamide affects the cannabinoid receptors and leads to a decrease in TNF-α and IL-6 production. Recent discoveries have shown that specific microorganisms of the intestinal microflora can improve the clinical symptoms of EAE; that is, strains of lactic acid bacteria can enhance the immunological activity of B cells and Treg by increased production of IL-10.

According to Miller et al. [110], MS is an autoimmune disease of the brain, because antagonists of TNF-l improve the condition of many patients with autoimmune disease (e.g., Crohn's disease, ankylosing spondylitis, and rheumatoid arthritis). TNFR2 deficiency causes spontaneous autoimmune-driven demyelination in the brains of transgenic female mice and increases the levels of IL-17, IFN-γ, and IgG2b. This is due to the composition of intestinal microflora, as autoimmunity persists after oral antibiotics. Another result of this study is the impact of the type of intestinal microflora (*Akkermansia muciniphila, Sutterella stercoricanis, Oscillospira, Bacteroides acidifaciens*, and *Anaeroplasma*) in male mice in the Alpine resort, where the microflora resulted in a protective effect.

However, the microflora of female mice mainly consisted of *Bacteroides* sp., *Bacteroides uniformis,* and *Parabacteroides,* which influenced the brain's autoimmune system. Commensal gut bacteria affect TNFR2 levels, which can cause autoimmune-driven demyelination in mice. Therefore, microflora can contribute to the autoimmune response of TNF in autoimmune diseases of the brain.

Messaoudi et al.[111] reported that EAE CD4+ cells directed against self-antigens pass into the CNS and cause demyelination. Many animals were treated with antibiotic therapy in order to reduce the intestinal microbiota. Antibiotics change the amount and composition of the intestinal flora and can reduce the clinical symptoms of autoimmune diseases of the brain. In the EAE model, broad-spectrum antibiotics reduced the susceptibility to EAE by altering the population of T cells in the GALT and peripheral lymphoid tissues. The growth of lymphocytes provided a protective role against EAE. CD5+ and CD19+ in the distant lymphoid organs decrease the levels of IFN-γ, MIP-1a, MIP-1b, MCP-1, IL-17, and IL-6 and increase IL-10 and IL-13 [112]. Small changes in Th1 and Th17 lead to an anti-inflammatory Th2 response. Th1 and Th17 contribute to MS progression, while FOXP3+ Treg cells play a protective role. These results indicate that the change in the composition of the intestinal flora due to antibiotic therapy may be beneficial in the treatment of MS [113]. Segmented filamentous bacteria are related to the introduction of Th17 and have been shown to influence autoimmune diseases [114].

2.10. The Effect of Diet on Gut Flora. Diet plays an important role in shaping the gut microbiome. In adults, a change in diet can produce a change in the intestinal microflora. One study found a plant-based diet, which leads to an increase in the population of Firmicutes (*Roseburia*, *Ruminococcus bromii*, and *Eubacterium rectale*) and the transition to a meat-based diet leads to an increase in the number of *Alistipes*, *Bilophila,* and *Bacteroides* [115]. Diet may influence the course of MS. Intestinal microbiota can modulate the effects of autoimmune diseases in humans [57, 116]. Th17 cells were shown to induce divided filamentous bacteria, which play an important role in the pathogenesis of autoimmune disease [117–119]. Some metabolites of commensal bacteria introduce FOXP3 positive Treg cells into the colon [120]. The gut has its own mechanism for the elimination of proinflammatory Th17 cells [12]. Studies have shown that consumption of *Candida* could be a new therapeutic strategy for the autoimmune system. This study

suggested that dietary yeast might be an important treatment for autoimmune diseases. Pathological examination showed that the amount of MNC (mononuclear cells) in the spinal cord of mice was lower than those in the control group, which was not treated.

Riccio and Rossano [121] reported that diet can influence the severity of MS in both relapsing-remitting MS and primary progressive MS. Diet can have an impact on symptoms of MS by controlling metabolic processes, inflammatory cells, and the composition of commensal intestinal microflora. Increased permeability of the intestinal barrier can lead to cross-reactivity between proteins, leading to the production of IgG and IgA. A diet high in salt, animal fat, red meat, sugar-sweetened beverages, carbohydrates, and fiber, in addition to a lack of physical activity, may increase symptoms, as these all influence metabolism and can lead to dysbiotic intestinal microflora, low-grade systemic inflammation, and increased permeability of the intestinal barrier. A low calorie diet of vegetables, fruits, legumes, fish, prebiotics, and probiotics can reduce symptoms by maintaining a healthy intestinal microflora symbiosis. These results indicate that microflora changes may cause activation of proinflammatory agents and may contribute to relapse in MS [122].

Burkitt [123] first described the potential protective effect of diet on bowel disease.

While working in Africa in 1960, he noticed a remarkable lack of noncommunicable diseases in the native Africans who consumed the traditional diet, which was rich in fiber. Antimicrobial peptides such as alpha-defensins, which are released by Paneth cells, may also have a significant effect on endogenous bacterial flora.

The reduction in the amount of microorganisms in the intestine is most likely to be an adverse effect of globalization, which leads to the consumption of generic, nutrient-rich, uncontaminated food. Both in the Western world and in developing countries, a diet high in fat, protein, and sugar and low in absorbable fibers is associated with a rapid increase in the incidence of noninfectious intestinal diseases [124].

The transition from a diet rich in fiber and low in fat to a high-fat diet alters the composition of the bacterial flora as early as after day 1 [41]. The intestinal flora of children from nonurban areas of Africa is rich in species, including *Prevotella* and *Xylanibacter*, which break abundant fiber. The resulting distribution of short-chain fatty acids in the flora has immunomodulating properties, which may explain the lower incidence of autoimmune diseases and asthma in these environments [125]. In contrast, animals given "Western" diets have high energy, due to the high sugar and fat content, lower levels of *Bacteroidetes*, and increased levels of Actinobacteria [41].

The relationship between the CNS, immune system, and gut microflora is associated with the development of MS. Possible interactions between the intestinal microflora and immune system can be examined by altering the endocannabinoid system to understand the interactions in the brain-gut axis [126].

3. Summary

The etiopathogenesis of MS is not fully known. Studies suggest that risk factors include viral infections, vitamin D deficiency, and smoking. There is also evidence to suggest that gut microflora could be important in the pathogenesis of MS. Recent studies have shown that replacing some of the bacterial population in the gut can lead to a proinflammatory state, indicating a potential mechanism causing MS in humans [53]. Furthermore, the intestinal microflora in MS patients has greater interindividual variability than that of healthy controls [62–64]. In contrast to other commensal bacteria, their antigenic products may protect against inflammation within the CNS. These results indicate that the intestinal microflora in patients with MS is characterized by moderate dysbiosis. Miyake et al. in their study also showed a decrease in the percentage of several *Bacteroides*, including *Bacteroides stercoris*, *Bacteroides coprocola*, and *Bacteroides coprophilus* in the intestinal microflora in patients with MS [61]. The results suggest a negative correlation between *Prevotella copri* and the pathogenesis of MS [61]. According to Ochoa-Repáraz et al., intestinal flora may affect the pathogenesis of MS, as changes in the gut microbiome lead to an irregular immune response, in both the colon and distal regions of the immune system, that is, the CNS. Specific bacterial antigens, such as PSA from *Bacteroides fragilis*, mediate the migration of the CNS, indicating a substantial interaction between the intestinal mucosa and the brain [69]. These observations suggest that the population of effector cells and regulatory cells involved in the pathogenesis of MS in humans may be associated with the lymphoid tissue of the gut [53]. Patients with MS had reduced concentrations of *Faecalibacterium* [103]. Furthermore, MS patients treated with glatiramer acetate showed an increase in Bacteroidaceae, *Faecalibacterium*, *Ruminococcus*, Lactobacillaceae, *Clostridium*, and other members of the class Clostridiales when compared with untreated MS patients.

Abbreviations

ATP: Adenosine triphosphate
CCL4: Chemokine ligand 4
CCL5: Chemokine ligand 5
CD4: Cluster of differentiation 4
CD8: Cluster of differentiation 8
CD39: Cluster of differentiation 39
CNS: Central nervous system
CRP: C-Reactive protein
DNA: Deoxyribonucleic acid
EAE: Experimental autoimmune encephalomyelitis
EBV: Epstein-Barr virus
ELISA: Enzyme-linked immunosorbent assay
FOXP3: Forkhead box P3
GALT: Gut-associated lymphoid tissue
G-CSF: Granulocyte colony-stimulating factor
GF: Germ-free
GPRS: Binding G protein coupled receptor
GWAS: Genome-wide association studies
HOMA-IR: Homeostasis Model Assessment of Insulin Resistance

HPA: Hypothalamic-pituitary-adrenal
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
IgG2b: PE anti-mouse antibody
IP-10: Interferon-gamma-induced protein 10
LPS: Lipopolysaccharide
MCP-1: Monocyte chemoattractant protein-1
MHC: Major histocompatibility complex
MIP-1: Macrophage inflammatory protein 1
MS: Multiple sclerosis
p: Probability value
PSA: Polysaccharide A
ROS: Reactive oxygen species
RNS: Reactive nitrogen species
SCFA: Short-chain fatty acid
SLE: Systemic lupus erythematosus
SNP: Single-nucleotide polymorphism
Th1: Th cells
TLR: Toll-like receptors
TNF: Tumor necrosis factor
TNFR2: Tumor necrosis factor receptor 2
Treg: Regulatory T lymphocytes
UVR: Ultraviolet radiation.

Competing Interests

The authors declare that they have no competing interests.

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