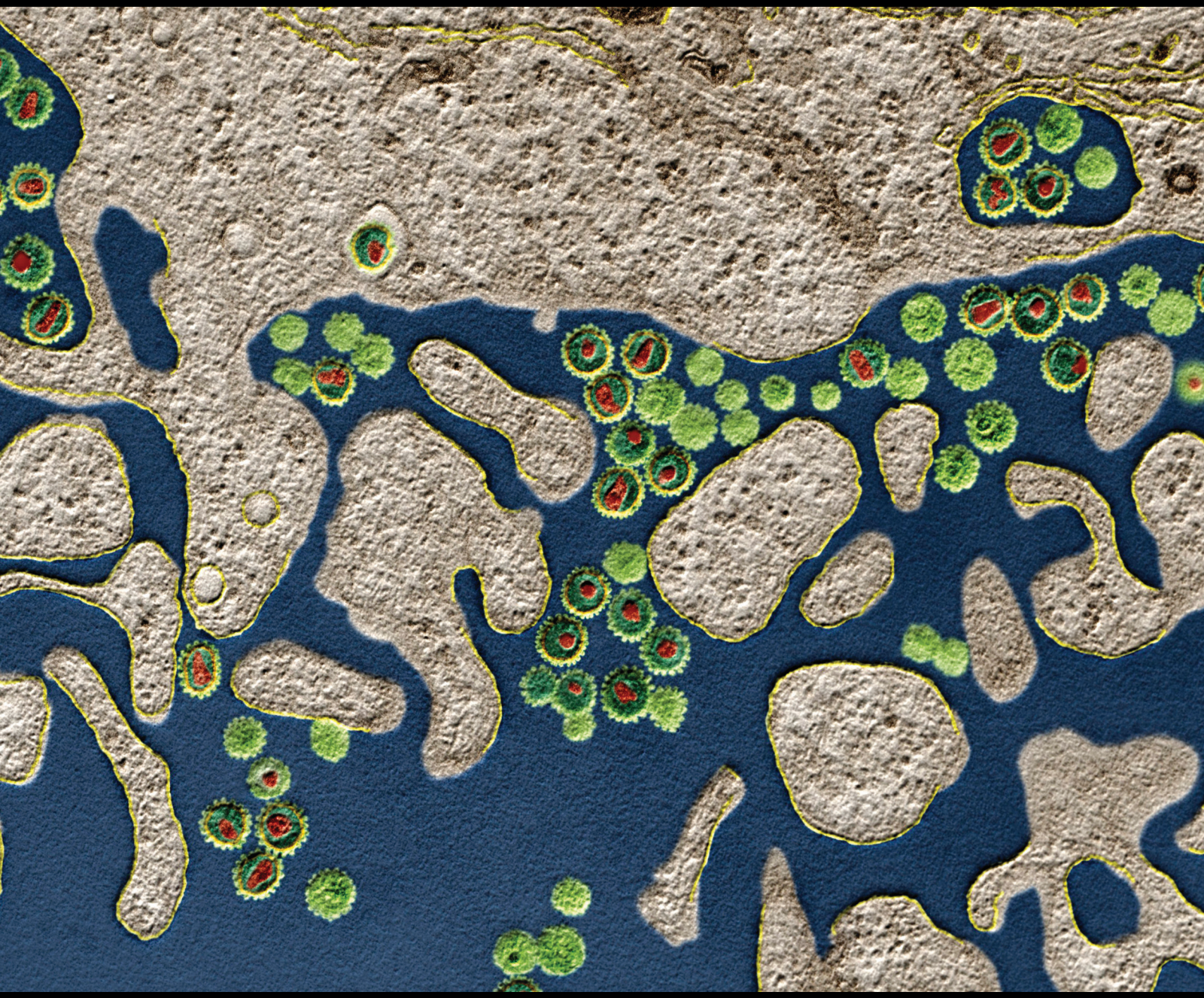
Journal of Immunology Research

The Interactions between Innate Immunity and Microbiota in Gastrointestinal Diseases

Guest Editors: Rossella Cianci, Anis Larbi, Danilo Pagliari, and Ciriaco A. Piccirillo



The Interactions between Innate Immunity and Microbiota in Gastrointestinal Diseases

Journal of Immunology Research

The Interactions between Innate Immunity and Microbiota in Gastrointestinal Diseases

Guest Editors: Rossella Cianci, Anis Larbi, Danilo Pagliari, and Ciriaco A. Piccirillo

Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 898297, 3 pages
http://dx.doi.org/10.1155/2015/898297

Editorial

The Interactions between Innate Immunity and Microbiota in Gastrointestinal Diseases

Danilo Pagliari,[1] Ciriaco A. Piccirillo,[2] Anis Larbi,[3] and Rossella Cianci[1]

[1]*Department of Medical Sciences, Catholic University of Rome, Largo A. Gemelli 8, 00168 Rome, Italy*
[2]*Department of Microbiology & Immunology and Medicine, Montreal General Hospital, McGill University and Research Institute of McGill University Health Centre (MUHC), Montreal, QC, Canada*
[3]*Singapore Immunology Network (SIgN), Singapore*

Correspondence should be addressed to Rossella Cianci; rossellacianci@gmail.com

Received 5 January 2015; Accepted 5 January 2015

The gut microbiota refers to the collection of microbial populations that reside in the gastrointestinal tract. It is characterized by an interplay between different cell types and their defense systems, food particles, molecules derived from digestion, and the vast array of residing microbial species with their secretory products. These microorganisms present in the gut lumen, which can be classified as probiotics, commensals, or pathogens, form the microbiota, which exerts several physiological functions, that is, the absorption and digestion processes, tolerance to non-self-food antigens, and defense from pathogens. The human microbiota can weigh up to 2 kg in total and contains tens of trillions of microorganisms, a number 10 to 20 times greater than the total number of cells in the human body, and includes at least 1000 different bacterial species [1]. This rich gut microbial community has coevolved in a symbiotic relationship with the human intestinal mucosa in such a way that the indigenous microbiota is essential for gut homeostasis. This ecosystem acts as a functional unit; thus, microbiota is considered a "superorganism" and is an integral part of the gastrointestinal tract [2].

The human intestine has the dual function of nutrient absorption and protection against intestinal pathogens. These two tasks have conflicting demands in the body. Optimal absorption requires a large surface area and selective permeability to nutrients through a thin epithelial layer. However, a large surface area increases exposure to luminal bacteria, and the thinness of epithelium increases the possibility of bacterial invasion. The intestinal immune system acts in order to avoid bacterial invasion of the epithelial layer. At the same time, it must not initiate inappropriate immune responses towards the commensal bacteria that reside in the intestinal lumen, as these responses may result in inflammatory diseases, such as Inflammatory Bowel Diseases (IBD), Celiac Disease (CD), and Diverticular Disease. Accordingly, it is important for the intestinal immune system to maintain an appropriate balance of pro- and anti-inflammatory mechanisms to ensure tolerance to commensal and dietary antigens and intestinal homeostasis [3].

The relationship between gut flora and humans is not merely commensal but rather a symbiotic one. This relationship is constantly challenged by many factors, such as the rapid turnover of the gastrointestinal epithelium and mucus, the exposure to the peristaltic activity, the presence of molecules resulting from digestion, the pancreatic and biliary secretions, the host defense, the presence of drugs or medications, the changes in pH and redox potential, and the exposure to transient organisms from the oral cavity and esophagus. Alternatively, numerous beneficial functions are ascribed to the microbiota in the human gut such as fermenting unused energy substrates, educating the immune system, preventing growth of harmful, pathogenic bacteria, regulating gut development, producing vitamins for the host, such as biotin and vitamin K, and producing hormones to direct the host to store fats [4]. Furthermore, the gut microbial community is akin to a safeguard of our health because the microbiota competes for space and nutrients with potential pathogens and induces the secretion of antimicrobial peptides through interaction with intestinal

epithelial cells [1]. The gut microbiota can also stimulate the differentiation and proliferation of epithelial cells, which regulate intestinal homeostasis [5].

According to the beneficial functions of gut microbiota mentioned above, it is clear that several bacteria that form microbiota confer a health benefit on the host. These bacteria have been mentioned as "probiotics." Thus, in recent years, experimental and clinical researchers have identified probiotics as a potential therapeutic target for several gastrointestinal diseases, as reported in the review by G. Giorgetti et al. Quali-quantitative changes in bacterial strains suggest that gut microbiota may be a therapeutic target based on probiotics and on probiotic-driven metabolic products, called "postbiotics." New therapeutic approaches based on probiotics are now available, and further treatments based on postbiotics will come in the future.

A principal function of the microbiota is to protect the intestine against colonization by exogenous pathogens and potentially harmful indigenous microorganisms. The specific mechanisms by which commensal bacteria achieve this function remain poorly understood, but there is evidence to indicate that both direct and indirect mechanisms might be involved. These mechanisms include the direct competition for limited nutrients, the promotion of mucosal barrier function, the modulation of innate immunity to pathogens and infections, the promotion of adaptive immunity, and even the protective role of the commensal microbiota against systemic infection [1]. However, in certain conditions, some species of bacteria are thought to cause disease by producing infection or increasing the risk of cancer in the host. In fact, particular bacterial populations that are typically found in very low abundance can acquire pathogenic properties. These conditions include inherent immune defects as well as changes in diet and/or acute inflammation and can result in the disruption of the normal balanced state of the gut microbiota, a condition referred to as dysbiosis [6]. Dysbiosis involves the abnormal accumulation or increased virulence of certain commensal populations of bacteria, thereby transforming former symbionts into "pathobionts." Pathobionts are typically colitogenic in that they can trigger intestinal inflammation [1]. Hence, the breakdown of the normal microbial community contributes to increase of the risk of pathogen infection, the overgrowth of harmful pathobionts, and inflammatory disease. Thus, understanding the interaction of the microbiota with pathogens and the host might provide new insights into the pathogenesis of disease, as well as novel avenues for preventing and treating intestinal and systemic disorders [1].

A prototype to understand the breakdown of the normal gut microbial community leading to dysbiosis is *Clostridium difficile* infection (CDI). In this special issue, S. Bibbò et al. describe the epidemiological and clinical characteristics of CDI and discuss how the intestinal microbiota modifications and the modulation of innate immune response can promote and exacerbate CDI. In this infection, the alteration of intestinal homeostasis promotes the development of an ecological niche that allows the growth of *Clostridium difficile*. Moreover, intestinal dysbiosis can promote a proinflammatory environment, whereas *Clostridium difficile* itself modulates the innate immunity through both toxin-dependent and toxin-independent mechanisms.

CDI causes intestinal inflammation leading to a subsequent reduction of intestinal microbiota diversity. The proportion between *Clostridium difficile* and the other gut bacteria has been related to the severity of inflammation. The work of C. Vincent et al. demonstrates how fecal excretion of abundant quantities of human DNA is associated with intestinal inflammation and with incipient CDI; thus, fecal excretion of host DNA should be investigated as a potential clinical marker of intestinal inflammation and CDI risk.

The specific composition of the microbiota is influenced by immune cells as well as external environmental factors, especially the use of antibiotics and diet [7]. For the preservation of gastrointestinal homeostasis, the intestine maintains control over the microflora, discriminating between pathogenetic and nonpathogenetic organisms by means of local innate immunity [1].

Initiation of innate immune responses in the intestine is triggered by pathogen-recognition receptors (PRRs) which serve as sensors of pathogen-associated molecular patterns (PAMPs) from the intestinal lumen. The most studied PRRs are the Toll-like receptors (TLRs) [8]. In this special issue, two reviews illustrate the major role of TLRs in the interaction between microbiota and immune system. In particular, M. Valentini et al. focus the attention on the possibility that microbiota acts through TLRs expressed by adaptive T cells providing regulatory signals. S. Frosali et al. discuss the role of TLRs polymorphism in human gastrointestinal pathology and the role of TLRs and their interactions with human microbiota in the pathogenesis of Inflammatory Bowel Diseases.

TLRs may be involved in the pathogenesis and clinical course of the diverticular disease. In the paper by R. Cianci et al., it has been demonstrated that variations before and after the antibiotic therapy with Rifaximin exist. This suggests that TLRs are modified by the presence of pathogenic flora in UDD. The role of pathogenic flora is supported by the finding that Rifaximin acts in the gut mucosa homeostasis by limiting the activation of TLRs. Furthermore, Rifaximin may also have a luminal anti-inflammatory function modulating the adaptive immune response in an inhibitory sense. Rifaximin keeps under control TLRs expression in peripheral blood suggesting that, in addition to its activity in gut mucosa, it may also have a systemic action on immune system.

The intestinal barrier regulates intestinal homeostasis throughout innate and adaptive immune responses. Unfortunately, in some cases, the innate immune system's attempt to protect the host fails and chronic inflammation and intestinal autoimmunity occur, such as in the case of IBD and Celiac Disease (CD). In this special issue, in the paper of D. Pagliari et al. it is clarified how CD may be considered as a model to explain the pathogenesis of several other autoimmune diseases. In fact, during this disease, the steady state condition homeostasis due to the complete balance between pro- and anti-inflammatory factors is altered. The breakdown of the interaction among microbiota, innate immunity, and genetic and dietary factors leads to the disruption of homeostasis leading to inflammation and tissue damage. Thus, focusing

the attention on this interaction and its breakdown may allow a better understanding of the CD pathogenesis and finally get novel translational avenues for preventing and treating this widespread disease.

The contributions of the gut microbiota to the development of the immune system have been extensively characterized. There is a coordinated cross talk between the gut microbiota and the immune system allowing the host to tolerate the large amount of antigens present in the gut. Hence, recent data have demonstrated that microbiota plays a pivotal role in orchestrating the immune response in gut mucosa and in regulating the balance between pro- and anti-inflammation factors. Gut flora has a continuous and dynamic effect on the host's gut and systemic immune systems. Bacteria are key in promoting the early development of the gut's mucosal immune system. The immune system recognizes and fights harmful bacteria, but, throughout the mechanism of tolerance induction within the first days of life, permits the colonization and growth of helpful species of bacteria. On the other hand, the immune-gastrointestinal flora interface plays a pivotal role in maintaining gut homeostasis with resident microbial communities, thus ensuring that the symbiotic nature of the host-microbial relationship is maintained [2].

According to what has been described so far, the interaction between human gut microbiota and intestinal immune system can be considered as the pivotal axis that contributes to the maintenance of intestinal homeostasis and allows proper defense to infections. As the intestinal immune system is influenced by many factors, including dietary components and commensal bacteria, strategies that restore a healthy gut microbial community by affecting the microbial composition are being developed as new therapeutic approaches to treat several gastrointestinal diseases.

We hope that this special issue will contribute to a better understanding of the strong interactions between innate immunity and microbiota in developing gastrointestinal diseases. These new acquisitions will be fundamental to finding new therapeutical strategies.

Danilo Pagliari
Ciriaco A. Piccirillo
Anis Larbi
Rossella Cianci

References

[1] N. Kamada, S.-U. Seo, G. Y. Chen, and G. Núñez, "Role of the gut microbiota in immunity and inflammatory disease," *Nature Reviews Immunology*, vol. 13, no. 5, pp. 321–335, 2013.

[2] L. V. Hooper, D. R. Littman, and A. J. Macpherson, "Interactions between the microbiota and the immune system," *Science*, vol. 336, no. 6086, pp. 1268–1273, 2012.

[3] F. Pandolfi, R. Cianci, D. Pagliari, R. Landolfi, and G. Cammarota, "Cellular mediators of inflammation: tregs and T_H17 cells in gastrointestinal diseases," *Mediators of Inflammation*, vol. 2009, Article ID 132028, 11 pages, 2009.

[4] F. Guarner and J.-R. Malagelada, "Gut flora in health and disease," *The Lancet*, vol. 361, no. 9356, pp. 512–519, 2003.

[5] J. M. Wells, O. Rossia, M. Meijerink, and P. van Baarlen, "Epithelial crosstalk at the microbiota-mucosal interface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, supplement 1, pp. 4607–4614, 2011.

[6] K. Honda and D. R. Littman, "The microbiome in infectious disease and inflammation," *Annual Review of Immunology*, vol. 30, pp. 759–795, 2012.

[7] D. Pagliari, R. Cianci, S. Frosali et al., "The role of IL-15 in gastrointestinal diseases: a bridge between innate and adaptive immune response," *Cytokine & Growth Factor Reviews*, vol. 24, no. 5, pp. 455–466, 2013.

[8] L. Shang, M. Fukata, N. Thirunarayanan et al., "Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria," *Gastroenterology*, vol. 135, no. 2, pp. 529–538, 2008.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 246203, 7 pages
http://dx.doi.org/10.1155/2015/246203

Research Article

Excretion of Host DNA in Feces Is Associated with Risk of *Clostridium difficile* Infection

Caroline Vincent,[1,2] Sudeep Mehrotra,[2,3] Vivian G. Loo,[4] Ken Dewar,[2,3,4] and Amee R. Manges[5]

[1] *Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada H3A 2B4*
[2] *McGill University and Génome Québec Innovation Centre, Montreal, QC, Canada H3A 0G1*
[3] *Department of Human Genetics, McGill University, Montreal, QC, Canada H3A 1B1*
[4] *The Research Institute of the McGill University Health Centre, Montreal, QC, Canada H3H 2R9*
[5] *School of Population and Public Health, The University of British Columbia, Vancouver, BC, Canada V6T 1Z3*

Correspondence should be addressed to Ken Dewar; ken.dewar@mcgill.ca and Amee R. Manges; amee.manges@ubc.ca

Received 12 August 2014; Revised 11 September 2014; Accepted 15 September 2014

Academic Editor: Rossella Cianci

Clostridium difficile infection (CDI) is intricately linked to the health of the gastrointestinal tract and its indigenous microbiota. In this study, we assessed whether fecal excretion of host DNA is associated with CDI development. Assuming that shedding of epithelial cell increases in the inflamed intestine, we used human DNA excretion as a marker of intestinal insult. Whole-genome shotgun sequencing was employed to quantify host DNA excretion and evaluate bacterial content in fecal samples collected from patients with incipient CDI, hospitalized controls, and healthy subjects. Human DNA excretion was significantly increased in patients admitted to the hospital for a gastrointestinal ailment, as well as prior to an episode of CDI. In multivariable analyses, human read abundance was independently associated with CDI development. Host DNA proportions were negatively correlated with intestinal microbiota diversity. *Enterococcus* and *Escherichia* were enriched in patients excreting high quantities of human DNA, while *Ruminococcus* and *Odoribacter* were depleted. These findings suggest that intestinal inflammation can occur prior to CDI development and may influence patient susceptibility to CDI. The quantification of human DNA in feces could serve as a simple and noninvasive approach to assess bowel inflammation and identify patients at risk of CDI.

1. Introduction

In healthy individuals, the intestinal microbiota is characterized by a highly complex and dynamic microbial community which contains as many as 1,000 bacterial species [1]. This microbial community constitutes an important metabolic organ that provides numerous beneficial functions to the host, including the digestion of complex carbohydrates, production of vitamins, maturation of the immune system, regulation of gastrointestinal transit, and stimulation of epithelial cell turnover [2]. The indigenous microbiota also has the ability to outcompete opportunistic microorganisms and enteric pathogens like *Clostridium difficile* through a process known as colonization resistance [3].

Human fecal matter is composed of a mixture of water, undigested food, microorganisms, and epithelial cells released from the walls of the gastrointestinal tract [4]. The desquamation of intestinal epithelial cells can be quantified by measuring the abundance of human DNA excreted in feces. Although the intestinal epithelium undergoes rapid turnover and is completely renewed every 4-5 days [5], typically very low amounts of human DNA can be detected in fecal matter [6]. However, when intestinal homeostasis is perturbed due to the presence of infectious agents or inflammation, greater amounts of damaged and dead epithelial cells are exfoliated from the intestinal wall, resulting in higher quantities of human cells shed in feces [7]. In sequenced-based studies of the fecal microbiome, the excretion of

host DNA has not been well-characterized. Studies using directed PCR and sequencing of phylogenetically informative regions of the bacterial 16S ribosomal RNA gene (rDNA) do not interrogate human DNA, and studies based on whole-genome shotgun (WGS) sequencing often apply bioinformatic filters to remove low quality reads, host DNA, and other contaminants prior to analyses.

C. difficile is the major etiological agent of infectious diarrhea and pseudomembranous colitis in hospitalized patients. The main risk factor for *C. difficile* infection (CDI) is antibiotic exposure and the overall risk increases with prolonged and combined use of antibiotics [8]. Broad-spectrum antibiotics have profound detrimental effects on the structure and diversity of the intestinal microbiota [9, 10]. These alterations can result in loss of colonization resistance, thereby providing an opportunity for *C. difficile* proliferation. *C. difficile* can also elicit intestinal inflammation during colonization as a way to further disrupt the indigenous microbiota and overcome colonization resistance [11].

Despite advances in infection control practices and the development of new treatment options, there has been a steady increase in the incidence and severity of CDI in the last two decades and outbreaks continue to occur in hospitals and healthcare institutions worldwide [12, 13]. As there is presently no vaccine for CDI, the development of new strategies for early identification of high-risk patients and earlier diagnosis of patients undergoing CDI would aid in infection prevention and patient management. In order to achieve this task, there is a need for an improved understanding of the intestinal ecosystem, including factors that maintain intestinal homeostasis and colonization resistance in the face of constantly changing environmental pressures.

The objective of this study was to investigate the relationship between intestinal epithelial cell shedding, microbiota composition, and subsequent development of nosocomial CDI. We used WGS sequencing to compare the proportions of human DNA and evaluate bacterial content in fecal samples obtained from (i) patients prior to the onset of CDI (cases), (ii) hospitalized controls, and (iii) nonhospitalized healthy subjects. Our results provide evidence that the excretion of high quantities of host DNA in feces is a general outcome of intestinal inflammation and is associated with CDI risk in hospitalized patients.

2. Materials and Methods

2.1. Subject Recruitment and Sample Collection. Between September 2006 and May 2007, a total of 599 hospitalized patients were enrolled in a prospective cohort study at the Royal Victoria Hospital in Montreal. A detailed description of the study population and definitions are available in Loo et al. [14]. A single rectal swab was obtained from each patient within 7 days of admission to the hospital. A questionnaire was administered to all study subjects to collect information on demographics, known risk factors for CDI, and use of various medications in the 8 weeks prior to hospital admission and during hospitalization. During the study period, 31 patients experienced one or more CDI episodes. After excluding patients with a history of previous CDI, fecal samples collected prior to CDI diagnosis were available for 18 patients (cases). Thirty-six controls were selected from patients who did not develop CDI, either during hospitalization or up to 60 days after discharge. Case patients were matched to controls in a 1 : 2 ratio based on sex, age (±5 years), and date of hospitalization (±2 months). All participants provided informed written consent. The human subjects' protocols for the cohort and case-control studies were approved by the Royal Victoria Hospital Internal Review Board as well as the McGill University Institutional Review Board (BMB 05-014). As nonhospitalized healthy controls, we included intestinal microbiome sequence data from 88 adults generated as part of the Human Microbiome Project (HMP) [6]. To ensure our sequencing data was comparable to that of the HMP, we also included two fecal samples obtained from nonhospitalized healthy adults living in Montreal.

2.2. WGS Sequencing and Data Analysis. Fecal DNA was extracted using the DNA IQ System (Promega) and subjected to whole-genome amplification using the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) according to the manufacturers' protocols. Due to limited amounts of fecal material available on the rectal swabs, whole-genome amplification was necessary in order to ensure sufficient DNA quantities for subsequent steps. Multiplexed DNA libraries were prepared according to a previously described protocol [15]. WGS sequencing was performed at the McGill University and Génome Québec Innovation Centre and the Illumina HiSeq 2000 instrument was used to generate 150-nucleotide sequence reads. Eleven to 15 samples were pooled in each sequencing lane and we obtained a median of 18.0 million reads per sample (range, 6.4–91.4 million). In each fecal sample, the proportion of reads derived from the human genome was determined with BMTagger [16]. In order to assess microbial diversity, we retrieved all reads containing the V1–V3 reverse primer sequence (which targets a segment of the 16S rRNA gene) [17] and their $3'$ sequences in order to obtain 55-mers originating with the primer sequence. We compiled the occurrence and frequency of these 16S rDNA motifs to measure the diversity of each sample using the inverse Simpson index. MetaPhlAn [18] was used to infer genus-level taxonomic abundances and assess the presence of *C. difficile* in fecal samples. All samples contained at least 1,000 reads with a hit to MetaPhlAn's marker database.

2.3. Statistical Analyses. Kernel density estimation and all other statistical analyses were performed with the R software [19]. LEfSe [20] was used to perform linear discriminant analysis and identify bacterial genera that discriminate between three categories of human DNA abundance.

3. Results and Discussion

We compared the proportions of human DNA in fecal samples collected from 18 CDI cases, 36 hospitalized controls, and 90 nonhospitalized healthy controls. Among case patients, the median interval of time between stool collection and CDI diagnosis was 10.5 days. The proportion of human reads detected in fecal samples differed significantly between the

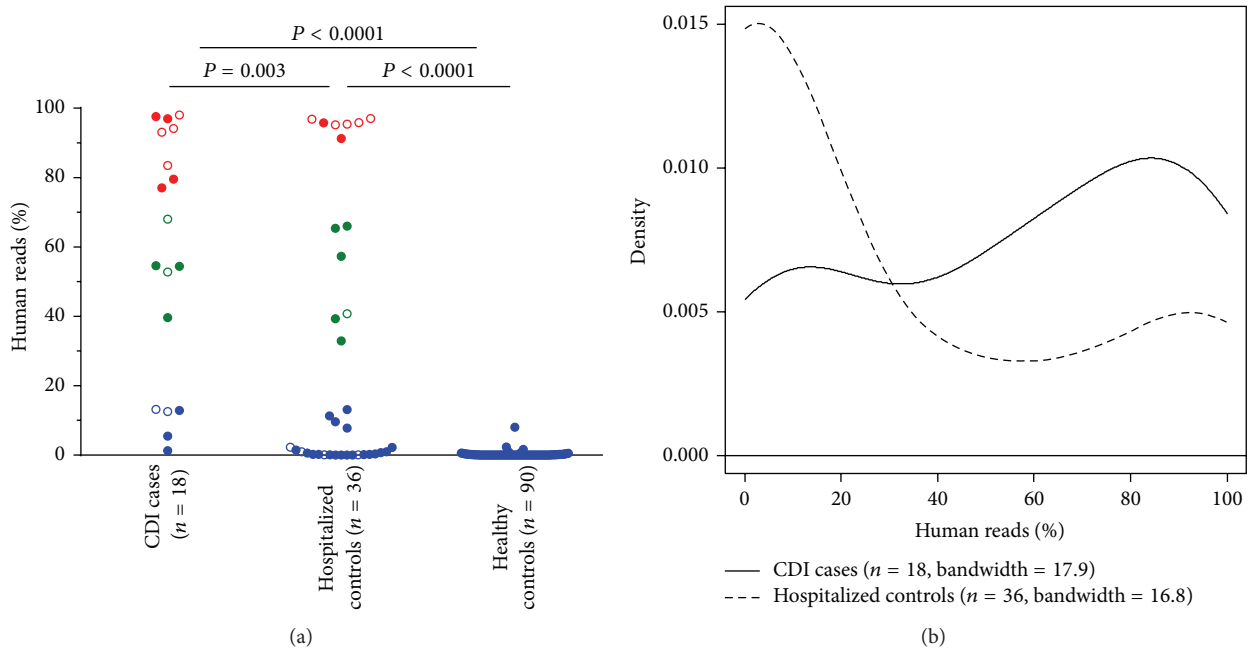


Figure 1: Distribution of human read proportions in fecal samples from CDI cases, hospitalized controls, and healthy subjects. (a) Scatter plot showing the proportions of human reads in each subject group. Patients admitted to the hospital for gastrointestinal problems (8 cases and 10 controls) are displayed as open circles. Three subgroups representing low (<30%, shown in blue), intermediate (30–70%, shown in green), and high (>70%, shown in red) amounts of human DNA were apparent from the sample distributions. P values were determined by Mann-Whitney U test. (b) Kernel density plot showing the distribution of human read proportions in CDI case and hospitalized control groups.

three subject groups (Figure 1(a)). In hospitalized patients (cases and controls), the proportion of human reads was highly variable, with values ranging from 0 to 98%. In contrast, the vast majority (95.5%) of stool samples from healthy subjects, including two samples sequenced in this study, contained less than 1% of human reads. Overall, samples from incipient CDI cases contained significantly higher proportions of human reads than samples from hospitalized and healthy controls ($P \leq 0.003$ by Mann-Whitney U test; Figure 1(a)). We acknowledge that variations in sample collection procedures (i.e., whole stool for healthy subjects versus rectal swabs for hospitalized patients) may have contributed to the observed differences in human read proportions between subject groups. To better evaluate and compare the complex underlying distributions of the incipient CDI case and hospitalized control groups, we generated a kernel density plot (Figure 1(b)). While fecal samples with low amounts (<30%) of human DNA were predominant in hospitalized controls (64% of samples), samples from incipient CDI cases displayed a shift towards intermediate (30–70%) and high (>70%) levels of host DNA content (72% of samples had >30% of human reads).

A review of patient records indicated that 18 (8 cases and 10 controls) out of 54 patients were admitted to the hospital for a gastrointestinal surgery or infection (other than CDI) (Figure 1(a), shown as open circles). The fecal samples obtained from patients admitted for a gastrointestinal ailment had significantly higher proportions of human reads compared to patients admitted for other reasons ($P = 0.01$ by Mann-Whitney U test). Increased excretion of human DNA has also been observed in patients with active ulcerative colitis and colorectal cancer, as well as in patients undergoing pelvic radiotherapy [21–25]. Taken together, these observations indicate that increased fecal excretion of host DNA is a general marker of intestinal insult.

In order to distinguish between the contributions of non-CDI related gastrointestinal ailment (based on hospital admission information) and incipient CDI and how they affect the level of human DNA excretion, we performed a multivariable analysis and found that human read abundance remains significantly associated with subsequent CDI development when admission for gastrointestinal problems is controlled for in the logistic regression model ($P \leq 0.02$). This indicates that while excretion of large amounts of human DNA is a general indicator of gastrointestinal insult and inflammation, it is also independently associated with CDI development. We were particularly intrigued by the increase in the proportion of CDI patients with intermediate levels (30–70%) of human reads in their fecal sample and consider two scenarios that could explain their importance in CDI. In the first case, the observation of intermediate levels of human DNA may reflect a low-grade, chronic inflammatory state. If so, then the presence of intermediate to high levels of human DNA provides another measure of intestinal health that aids in assessing patient risk to CDI. The presence of inflammation in the gut can have deleterious effects on the intestinal microbiota and may create a permissive environment for *C. difficile* colonization, thereby increasing patient susceptibility

to CDI. Alternatively, intermediate levels of host DNA excretion may reflect an early inflammation stage elicited by *C. difficile* colonization and thus represent an early sign of CDI. Although we cannot distinguish whether increased shedding of intestinal epithelial cells is a risk marker for CDI or whether it is inherent to CDI development, the observation that only 2 out of 18 cases had DNA sequences corresponding to *C. difficile* in their stool and the absence of a relationship between human read abundance and time to CDI diagnosis ($r = 0.30$, $P = 0.22$ by Spearman correlation) supports the idea that increased amounts of human DNA in feces is a risk marker for CDI. Larger studies using longitudinal sample collection are needed to clarify this question.

Since intestinal epithelium integrity, intestinal inflammation, and resident microbiota are intricately related, we assessed the diversity and composition of the fecal microbiota with respect to human DNA content. As there was little to no human DNA in samples from healthy subjects, we restricted our microbiome analyses to the hospitalized cases and controls. In order to estimate bacterial diversity from WGS datasets, we employed an rDNA data mining approach. 16S rDNA motifs (defined as 55-mer DNA segments starting with the V1–V3 primer sequence) were recovered after partitioning human DNA sequences. Three samples (one from a CDI case and two from hospitalized controls) with very high human DNA content (≥95.8% of reads) contained less than 15 rDNA motifs and were excluded from the diversity analyses. Of the 51 remaining samples, four contained more than 95.8% of human reads but still had enough rDNA motifs to allow a reliable estimation of bacterial diversity. In hospitalized controls, we observed a trend of decreasing microbial diversity with increasing proportions of human DNA ($r = -0.50$, $P = 0.003$ by Spearman's rank correlation test; Figure 2). This trend was not observed in CDI cases ($r = -0.14$, $P = 0.6$). Patients with incipient CDI exhibited overall low levels of microbial diversity, a feature that is already associated with CDI susceptibility [26]. Nonetheless, intestinal inflammation could be the common link between increased epithelial cell shedding and reduced microbial diversity.

Bacterial genomes typically range in size from 0.5 to more than 9 Mb and contain between 1 and 15 copies of the 16S rRNA gene [27, 28], so the naive expectation for pure bacterial samples would be to observe approximately one 16S rDNA read per Mb of sequence data. Within our population of 54 hospitalized patients and two healthy subjects, the frequency of 16S rDNA motifs ranged from 0.1 to 0.7 occurrences per Mb of total sequence data after accounting for host DNA. In a comparable analysis of a variety of individually sequenced bacterial species (*Listeria monocytogenes*, *Vibrio fluvialis*, *Salmonella enterica*, and *Campylobacter jejuni*) with genome sizes ranging from 1.8 to 6.7 Mb and 16S rDNA copy numbers ranging from 1 to 8, rDNA motifs were observed at a frequency of 1 to 3 occurrences per Mb. Therefore, 16S rDNA motif recovery rates differed by an order of magnitude between purified bacterial cultures and fecal microbiome samples. Whereas fecal DNA samples have been considered to be predominantly of bacterial origin, our results imply that other sources of DNA that are not of bacterial or human origin (e.g., food, fungi, protozoa, bacteriophages, and other viruses) account for a substantial fraction of the fecal microbiome and likely play a role in the interplay between the microbial community and the host. Nielsen et al. have also recognized that the microbiome is composed of a wide variety of microbes and approaches assessing the full genetic diversity are needed [29]. Even with the paucity of robust tools to identify these other sources of DNA, the use of methods that survey the whole community rather than focusing only on bacteria, such as WGS sequencing, offers a starting point for taking a census of the entire microbiome.

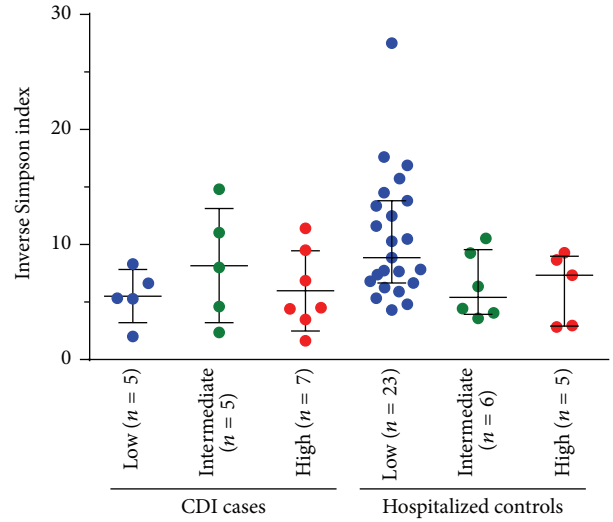


Figure 2: Intestinal microbiota diversity in hospitalized cases and controls as a function of host DNA abundance. Each patient group was divided into three subgroups with low (<30%, shown in blue), intermediate (30–70%, shown in green), and high (>70%, shown in red) proportions of human reads. One sample from the CDI group and two samples from hospitalized controls were excluded because they contained less than 15 reads with a 16S rDNA motif. Horizontal lines represent the median and interquartile range.

We also examined whether specific bacterial genera are associated with fecal excretion of human DNA in hospitalized patients. In the incipient CDI group, *Prevotella* was more abundant in patients with low levels of host DNA. This genus is a common member of the fecal microbiota in adults [30]. In the hospitalized control group, *Enterococcus* and *Escherichia* were enriched in patients with high proportions of host DNA, while *Ruminococcus* and *Odoribacter* were depleted (Figure 3(b)). Increased levels of *Enterococcus* and *Escherichia* have been previously associated with reduced colonization resistance, intestinal inflammation, and Crohn's disease [31–34]. Reduced abundance of *Ruminococcus* and *Odoribacter* has also been observed in patients with inflammatory bowel disease [31, 34]. These genera are important producers of short-chain fatty acids, which are metabolized by epithelial cells in the colon and play a major role in the regulation of colonocyte differentiation and proliferation [35, 36]. Therefore, a decrease in their abundance may have detrimental effects on the integrity of the intestinal epithelium and on the regulation of inflammation.

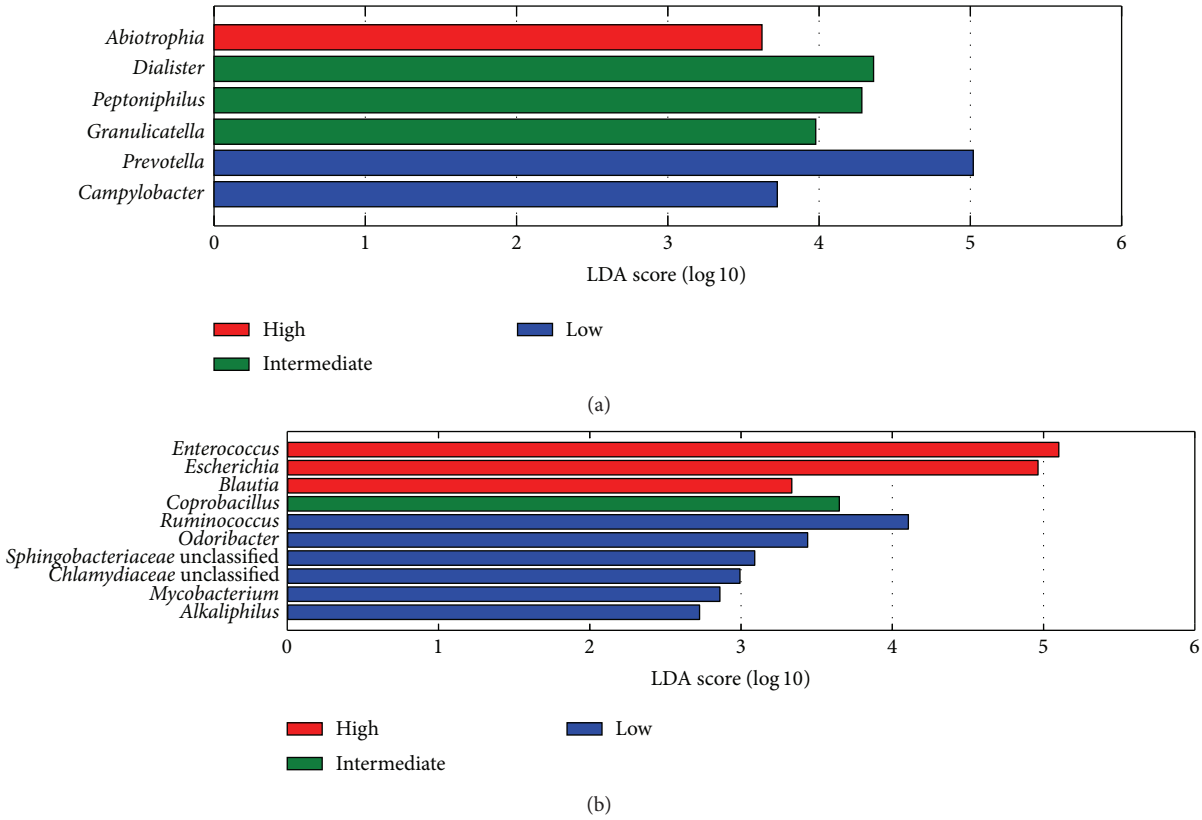


Figure 3: Bacterial genera that discriminate between low (<30%, shown in blue), intermediate (30–70%, shown in green), and high (>70%, shown in red) proportions of human DNA in (a) CDI cases and (b) hospitalized controls. The histograms show bacterial genera with an absolute log linear discriminant analysis (LDA) score of at least 2.0.

Although we did not directly measure the presence of intestinal inflammation in our population, a growing body of evidence (including this study) suggests that host DNA excretion is a general outcome of intestinal insult [21–24]. While earlier studies have directly targeted exfoliated colonocytes or human DNA via quantitative PCR, we believe WGS sequencing provides a comprehensive and unbiased way to assess fecal DNA content. Moreover, as the measurements are obtained from fecal samples, it provides a noninvasive and straightforward approach for monitoring intestinal health status. Whereas our study generated millions of sequences to support the characterization of intestinal microbiota, the monitoring of host DNA content could be achieved with much smaller datasets, thereby reducing costs and decreasing the time to results.

Our results also have implications for how different genomic approaches for studying the intestinal microbiome can affect informed consent and human subject protocols. Fecal samples used in WGS studies, especially for individuals with gastrointestinal diseases, provide information on the host genome as well as the microbiome. In instances of elevated host cell excretion, where host DNA constitutes more than 50% of the sequencing data, consent protocols should indicate that the data produced may be comparable to directly sequencing the individual's genome.

4. Conclusions

We have shown that fecal excretion of abundant quantities of human DNA is significantly associated with incipient CDI and appears to be an outcome of intestinal inflammation. High levels of human DNA were associated with a reduction of intestinal microbiota diversity, an increase in opportunistic microorganisms, and a depletion of short-chain fatty acid-producing bacteria. Fecal excretion of host DNA should be investigated as a potential clinical marker of intestinal inflammation and CDI risk.

Conflict of Interests

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

Acknowledgments

The authors would like to thank Dr. Nandini Dendukuri and Ian Schiller for their assistance with data management, Dr. Marcel Behr for providing useful suggestions and advices, and Dr. Simon Gravel for his valuable help with kernel density estimations. They also thank Bart Weimer and the organizers, instructors, and students of the 2013 Cold Spring Harbor

Laboratory course on Advanced Sequencing Technologies and Applications for the sequencing of the four bacterial genomes. This work was supported by a Pfizer Aspire Award [WS1954106 to Amee R. Manges] and a Doctoral Research Award from the Canadian Institutes of Health Research [GSD-113375 to Caroline Vincent].

References

[1] I. Sekirov, S. L. Russell, L. Caetano M Antunes, and B. B. Finlay, "Gut microbiota in health and disease," *Physiological Reviews*, vol. 90, no. 3, pp. 859–904, 2010.

[2] L. V. McFarland, "Normal flora: diversity and functions," *Microbial Ecology in Health and Disease*, vol. 12, no. 4, pp. 193–207, 2000.

[3] B. Stecher and W. D. Hardt, "Mechanisms controlling pathogen colonization of the gut," *Current Opinion in Microbiology*, vol. 14, no. 1, pp. 82–91, 2011.

[4] D. Mesko, "Stool," in *Differential Diagnosis by Laboratory Medicine: A Quick Reference for Physicians*, D. Mesko, Ed., pp. 522–530, Springer, Berlin, Germany, 2002.

[5] L. G. van der Flier and H. Clevers, "Stem cells, self-renewal, and differentiation in the intestinal epithelium," *Annual Review of Physiology*, vol. 71, pp. 241–260, 2009.

[6] Human Microbiome Project Consortium, "A framework for human microbiome research," *Nature*, vol. 486, no. 7402, pp. 215–221, 2012.

[7] M. Kim, H. Ashida, M. Ogawa, Y. Yoshikawa, H. Mimuro, and C. Sasakawa, "Bacterial interactions with the host epithelium," *Cell Host and Microbe*, vol. 8, no. 1, pp. 20–35, 2010.

[8] G. E. Bignardi, "Risk factors for Clostridium difficile infection," *Journal of Hospital Infection*, vol. 40, no. 1, pp. 1–15, 1998.

[9] L. Dethlefsen, S. Huse, M. L. Sogin, and D. A. Relman, "The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16s rRNA sequencing," *PLoS Biology*, vol. 6, no. 11, article e280, 2008.

[10] L. Dethlefsen and D. A. Relman, "Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, supplement 1, pp. 4554–4561, 2011.

[11] B. Stecher and W. D. Hardt, "The role of microbiota in infectious disease," *Trends in Microbiology*, vol. 16, no. 3, pp. 107–114, 2008.

[12] E. J. Kuijper, B. Coignard, P. Tüll et al., "Emergence of *Clostridium difficile*-associated disease in North America and Europe," *Clinical Microbiology and Infection*, vol. 12, supplement 6, pp. 2–18, 2006.

[13] C. P. Kelly and J. T. LaMont, "Clostridium difficile—more difficult than ever," *The New England Journal of Medicine*, vol. 359, no. 18, pp. 1932–1940, 2008.

[14] V. G. Loo, A.-M. Bourgault, L. Poirier et al., "Host and pathogen factors for *Clostridium difficile* infection and colonization," *The New England Journal of Medicine*, vol. 365, no. 18, pp. 1693–1703, 2011.

[15] M. Meyer and M. Kircher, "Illumina sequencing library preparation for highly multiplexed target capture and sequencing," *Cold Spring Harbor Protocols*, vol. 2010, no. 6, 2010.

[16] R. Agarwala and K. Rotmistrovsky, "BMTagger".

[17] Provisional 16S 454 protocol, http://www.hmpdacc.org/tools_protocols/tools_protocols.php.

[18] N. Segata, L. Waldron, A. Ballarini, V. Narasimhan, O. Jousson, and C. Huttenhower, "Metagenomic microbial community profiling using unique clade-specific marker genes," *Nature Methods*, vol. 9, no. 8, pp. 811–814, 2012.

[19] The R Project for Statistical Computing, http://www.r-project.org/.

[20] N. Segata, J. Izard, L. Waldron et al., "Metagenomic biomarker discovery and explanation," *Genome Biology*, vol. 12, no. 6, article R60, 2011.

[21] F. Casellas, M. Antolín, E. Varela et al., "Fecal excretion of human deoxyribonucleic acid as an index of inflammatory activity in ulcerative colitis," *Clinical Gastroenterology and Hepatology*, vol. 2, no. 8, pp. 683–689, 2004.

[22] C. H. W. Klaassen, M. A. F. Jeunink, C. F. M. Prinsen et al., "Quantification of human DNA in feces as a diagnostic test for the presence of colorectal cancer," *Clinical Chemistry*, vol. 49, no. 7, pp. 1185–1187, 2003.

[23] E. Varela, M. Antolín, F. Guarner, R. Verges, J. Giralt, and J.-R. Malagelada, "Faecal DNA and calprotectin as biomarkers of acute intestinal toxicity in patients undergoing pelvic radiotherapy," *Alimentary Pharmacology and Therapeutics*, vol. 30, no. 2, pp. 175–185, 2009.

[24] A. Loktionov, I. K. O'Neill, K. R. Silvester, J. H. Cummings, S. J. Middleton, and R. Miller, "Quantitation of DNA from exfoliated colonocytes isolated from human stool surface as a novel noninvasive screening test for colorectal cancer," *Clinical Cancer Research*, vol. 4, no. 2, pp. 337–342, 1998.

[25] F. Casellas, N. Borruel, M. Antolín et al., "Fecal excretion of deoxyribonucleic acid in long-term follow-up of patients with inactive ulcerative colitis," *Inflammatory Bowel Diseases*, vol. 13, no. 4, pp. 386–390, 2007.

[26] C. Vincent, D. A. Stephens, V. G. Loo et al., "Reductions in intestinal Clostridiales precede the development of nosocomial *Clostridium difficile* infection," *Microbiome*, vol. 1, no. 18, pp. 1–11, 2013.

[27] J. A. Klappenbach, P. R. Saxman, J. R. Cole, and T. M. Schmidt, "Rrndb: the ribosomal RNA operon copy number database," *Nucleic Acids Research*, vol. 29, no. 1, pp. 181–184, 2001.

[28] T. R. D. Gregory, "Comparative genomics in prokaryotes," in *The Evolution of the Genome*, T. R. Gregory, Ed., pp. 585–675, Elsevier Academic Press, 2004.

[29] H. B. Nielsen, M. Almeida, A. S. Juncker et al., "Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes," *Nature Biotechnology*, vol. 32, no. 8, pp. 822–828, 2014.

[30] M. Arumugam, J. Raes, E. Pelletier et al., "Enterotypes of the human gut microbiome," *Nature*, vol. 473, no. 7346, pp. 174–180, 2011.

[31] X. C. Morgan, T. L. Tickle, H. Sokol et al., "Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment," *Genome Biology*, vol. 13, no. 9, article R79, 2012.

[32] M. Baumgart, B. Dogan, M. Rishniw et al., "Culture independent analysis of ileal mucosa reveals a selective increase in invasive *Escherichia coli* of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum," *ISME Journal*, vol. 1, no. 5, pp. 403–418, 2007.

[33] E. J. Vollaard and H. A. L. Clasener, "Colonization resistance," *Antimicrobial Agents and Chemotherapy*, vol. 38, no. 3, pp. 409–414, 1994.

[34] S. Kang, S. E. Denman, M. Morrison et al., "Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom

phylogenetic microarray," *Inflammatory Bowel Diseases*, vol. 16, no. 12, pp. 2034–2042, 2010.

[35] J. M. W. Wong, R. de Souza, C. W. C. Kendall, A. Emam, and D. J. A. Jenkins, "Colonic health: fermentation and short chain fatty acids," *Journal of Clinical Gastroenterology*, vol. 40, no. 3, pp. 235–243, 2006.

[36] M. Göker, S. Gronow, A. Zeytun et al., "Complete genome sequence of odoribacter splanchnicus type strain (1651/6 T)," *Standards in Genomic Sciences*, vol. 4, no. 2, pp. 200–209, 2011.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 489821, 12 pages
http://dx.doi.org/10.1155/2015/489821

Review Article

How the Intricate Interaction among Toll-Like Receptors, Microbiota, and Intestinal Immunity Can Influence Gastrointestinal Pathology

Simona Frosali, Danilo Pagliari, Giovanni Gambassi, Raffaele Landolfi, Franco Pandolfi, and Rossella Cianci

Department of Medical Science, Institute of Internal Medicine, "A. Gemelli" University Hospital, Catholic University of the Sacred Heart, Largo A. Gemelli, 8-00168 Rome, Italy

Correspondence should be addressed to Franco Pandolfi; pandolfi@rm.unicatt.it

Received 30 April 2014; Revised 1 October 2014; Accepted 27 October 2014

Academic Editor: Ciriaco A. Piccirillo

The gut is able to maintain tolerance to microbial and food antigens. The intestine minimizes the number of harmful bacteria by shaping the microbiota through a symbiotic relationship. In healthy human intestine, a constant homeostasis is maintained by the perfect regulation of microbial load and the immune response generated against it. Failure of this balance may result in various pathological conditions. Innate immune sensors, such as Toll-like receptors (TLRs), may be considered an interface among intestinal epithelial barrier, microbiota, and immune system. TLRs pathway, activated by pathogens, is involved in the pathogenesis of several infectious and inflammatory diseases. The alteration of the homeostasis between physiologic and pathogenic bacteria of intestinal flora causes a condition called dysbiosis. The breakdown of homeostasis by dysbiosis may increase susceptibility to inflammatory bowel diseases. It is evident that environment, genetics, and host immunity form a highly interactive regulatory triad that controls TLR function. Imbalanced relationships within this triad may promote aberrant TLR signaling, critically contributing to acute and chronic intestinal inflammatory processes, such as in IBD, colitis, and colorectal cancer. The study of interactions between different components of the immune systems and intestinal microbiota will open new horizons in the knowledge of gut inflammation.

1. Introduction

The human gastrointestinal tract is colonized by different microbial populations including bacteria, fungi, and viruses [1]. Bacteria represent the largest population of intestinal microbiota, comprising 500–1000 different species [1, 2]. Surprisingly, the intestine is able to maintain tolerance to this antigenic burden, showing a symbiotic host relationship, and it may provide protective inflammatory responses against invading enteric pathogens. In fact, the intestinal tract has developed several strategies allowing a symbiotic relationship with microbiota and restricting the invasion of microorganisms through the gut epithelial barrier.

The intestine minimizes the number of harmful bacteria by shaping the microbiota through a symbiotic relationship. The commensal microbiota competes with pathogenic invaders and limits the colonization of the intestinal tract by pathogens [3, 4]. Furthermore, thick mucus layers composed of mucin glycoproteins secreted from Goblet cells create a physical barrier, which separates bacterial flora and the intestinal epithelial cells [5, 6]. Moreover, several antibacterial factors are secreted by mucosal cells and can directly regulate the microbiota's growth. For instance, Paneth cells at the base of the crypts are specialized cells that produce and secrete multiple antibacterial molecules, including α-defensins C-type lectins, lysozyme, and phospholipase A2 [7]. Secreted IgAs bind intestinal microorganisms, preventing their invasion through epithelial cell layers and controlling commensals. The IgA pools are antigen-specific [8] and are produced by plasma cells that migrate from Peyer's patches or other mucosal-associated lymphoid tissues, in response to epithelial signals.

In healthy human intestine, a constant homeostasis is maintained by the perfect regulation of microbial load and

Table 1: Human TLRs: an overview on their pathophysiology.

	Ligand(s)	Cell types	Genetic defect	Association
TLR 1	(i) Triacyl lipopeptides	(i) Monocytes/macrophages (ii) Dendritic cells (iii) B lymphocytes	TLR1-R80T	Ulcerative colitis, pancolitis
TLR 2	(i) Glycolipids (ii) Lipopeptides (iii) Lipoproteins (iv) Lipoteichoic acid (v) HSP-70 (vi) Zymosan (vii) Others	(i) Monocytes/macrophages (ii) Neutrophils (iii) Myeloid dendritic cells (iv) Mast cells	TLR2-R753Q	Ulcerative colitis, pancolitis
TLR 6	(i) Diacyl lipopeptides	(i) Monocytes/macrophages (ii) Mast cells (iii) B lymphocytes	TLR6-S249P	Decreased incidence of proctitis in IBD
TLR 4	(i) Lipopolysaccharide (ii) Heat shock proteins (iii) Fibrinogen (iv) Heparan sulfate (v) Hyaluronic acid (vi) Nickel (vii) Various opioid drugs	(i) Monocytes/macrophages (ii) Neutrophils (iii) Myeloid dendritic cells (iv) Mast cells (v) B lymphocytes (vi) Intestinal epithelium	TLR4-D299G	Increased susceptibility to IBD
TLR 5	(i) Flagellin	(i) Monocyte/macrophages (ii) Dendritic cells (iii) Intestinal epithelium	TLR5-STOP	Decreased susceptibility to IBD
TLR 9	(i) Unmethylated CpG (ii) Oligodeoxynucleotide DNA	(i) Monocytes/macrophages (ii) Plasmacytoid dendritic cells (iii) B lymphocytes	TLR9-SNPs: −1237T/C −2848A/G	Susceptibility to Crohn's disease

the immune response generated against it. Failure of this harmonized balance may result in various pathological conditions in the intestines. The alteration of the homeostasis between physiologic and pathogenic bacteria of intestinal flora causes a condition called dysbiosis. Thus, gut dysbiosis is a pathological condition characterized by an alteration of the normal bacterial flora that normally secretes vitamins, collaborates in digestion, regulates the permeability of the intestinal barrier, protects from infections, and prevents proliferation of pathogens. Consequently, the breakdown of homeostasis by dysbiosis or dysregulation of immune responses may increase susceptibility to Inflammatory Bowel Diseases (IBD) [9–11].

It is clear that innate immune sensors, such as Toll-like receptors (TLRs), play an important role in shaping intestinal microbiota. TLRs may be considered an interface among intestinal epithelial barrier, microbiota, and immune system. Moreover, TLRs pathway, activated by pathogens, is involved in the pathogenesis of several infectious and inflammatory diseases, such as IBD. In this review, we summarize the effects of TLRs on mucosal homeostasis and discuss the interaction between TLRs and human microbiota in the pathogenesis of gut diseases.

2. Toll-Like Receptors (TLRs)

TLRs are germline-encoded type I transmembrane receptors, expressed on numerous cell types including macrophages, dendritic cells (DCs), T lymphocytes, and intestinal epithelial cells. They act as pathogen recognition receptors (PRRs), identifying microbe-associated molecular pattern (MAMP), that are specific for microbes and essential for their survival [12]. TLR's name is derived from their similarity to the protein coded by the Toll gene identified in *Drosophila* in 1985. TLRs together with the Interleukin-1 receptors form a receptor superfamily, known as the "interleukin-1 receptor/Toll-like receptor superfamily" (Table 1).

A total of 10 TLRs are expressed in humans. Each TLR responds to distinct MAMPs, leading to the activation of specific signaling pathways. TLRs are characterized by the presence of an extracellular leucine-rich repeat domain (LRR) and an intracellular Toll/IL-1 receptor (TIR) domain [13]. LRRs are found on a diverse number of proteins and are involved in ligand recognition and signal transduction [14]. The TIR domain of the TLR is required for intracellular signaling and activation. This domain comprises about 200 amino acids, with varying degrees of sequence similarity among family members.

Three subgroups of TIR domains exist. Proteins of subgroup 1 are receptors for interleukins that are produced by macrophages, monocytes, and DCs; all these receptors have extracellular Immunoglobulin (Ig) domains. Proteins of subgroup 2 are considered a classical type of TLRs and bind directly or indirectly microbial molecules. Proteins of subgroup 3 are adaptor proteins, exclusively cytosolic, that mediate signals from proteins of subgroups 1 and 2 [14].

Activation of TLRs by their ligands induces several intracellular signaling cascades resulting in the production

of cytokines and chemokines and in the transcription of other genes important for the control of infection. Two major signaling pathways have been detailed. The first pathway, which is the principal one, is activated by most TLRs and leads to activation of the transcription factor NF-κB and the mitogen-activated protein (MAP) kinases p38 and JNK. These signaling cascades increase the expression of many proinflammatory genes. The second pathway is activated only by TLR3 and TLR4 and leads to activation of both NF-κB and interferon regulatory factor 3 (IRF3) that is a transcription factor and induces an additional set of genes including antiviral genes, such as interferon-beta [14].

A central role in the TLR signaling is played by the adaptor molecules MyD88, MAL (also known as TIRAP), TRIF (also known as TICAM1), and TRAM (also known as TICAM2 or TIRP). MyD88 is used by all TLRs, except TLR3. MyD88 recruits IRAKs (IL-1R-associated kinase family), leading to the activation of MAP3 kinases. Two of MAP3 kinases have been identified, MEKK3 and TAK1; these activate NF-κB, MAP kinases p38, and JNK. Activation of TAK1 by IRAKs requires TRAF6, as well as the ubiquitination of both TRAF6 and TAK1. BTK and PI3K also participate in TLR signaling.

Studies on MyD88-deficient mice have shown that signaling via TLRs plays an important role in intestinal homeostasis. This signaling is responsible for microbial recognition, induction of antimicrobial products, and modulation of the adaptive immune response [15, 16]. In fact, MyD88 knockout mice were susceptible to a greater number of bacterial infections caused by lack response of TLRs to MAMPs [17]. Moreover, recognition of commensal microbiota in a MyD88-dependent manner has been shown to be required for epithelial cell homeostasis [18], response to injury [19], and induction of antimicrobial peptides [20, 21].

3. TLR2 and Its Coreceptors (TLR1 and TLR6)

TLR2 is functionally expressed by distinct cell types in the intestinal mucosa and is constitutively expressed in the murine gastrointestinal epithelium, although this expression varies along the gut [22]. TLR2 recognizes a large spectrum of microbes, thanks to its ability to respond to molecular patterns such as lipoproteins [23, 24], lipoteichoic acid [25], and zymosan [26]. Ligand-induced activation of TLR2 leads to recruitment of TIRAP and MyD88, which results in activation of NF-κB, and production of cytokines and chemokines [27, 28].

In healthy gut, the role of TLR2 in epithelial cells is to maintain tolerance to ubiquitous commensal lipoproteins. As a low TLR2 expression is important for tolerance, there are several mechanisms which contribute to maintain a low expression: (1) increased expression of negative regulators, such as Tollip and A20 [29]; (2) activation of cell signaling pathways [30] inducing production of anti-inflammatory IL-10. IL-10 inhibits macrophage and DC effector functions, limits immune responses [31], and promotes the local differentiation and activation of T-regulatory cells (Tregs).

In inflammatory disease, increase of TLR2 expression induces NF-κB activation leading to exaggerated immune responses with production of inflammatory cytokines and such happens in Crohn's disease in which NOD2 is mutated [32].

Moreover, it is reported that TLR2 signaling conferred protection only against acute intestinal injury or inflammation [33], probably through maintenance of tight junction integrity [31]. In chronic inflammation, TLR2 showed moderate effects on regulation of sustained inflammatory processes [34, 35].

Furthermore, TLR2 signaling is critical for the acquisition of tissue-specific functional properties by gut-associated DCs, including their capacity to produce retinoic acid, to imprint gut-homing lymphocytes [36], and to activate Tregs. There is growing evidence about involvement of TLR2 in modulating T-cell functions both directly and indirectly. TLR2 stimulation can also promote T helper 17 cells (Th17) responses [37] and can reduce the suppressive function of Tregs by promoting a shift toward IL-17 production [38]. Notably, TLR2-induced mechanism of regulation of T-cell function could enhance microbial clearance and/or increase the risk of autoimmune reactions. However, commensal bacteria use a similar mechanism to enhance colonization of the gut and thereby establish host-microbial tolerance. For example, *B. fragilis* through TLR2, induces the production of the anti-inflammatory IL-10 in T-cells restraining Th17 responses [39]. Thus, TLR2, inducing pro- and anti-inflammatory effects, have a controversial action. The ability of TLR2 signaling to produce pro- and/or anti- inflammatory responses is influenced by the intestinal immunological niche, in which immune response, inflammation, and local homeostasis are modulated [40–42].

The complex response of TLR2 is further complicated by its ability to interact with multiple coreceptors [43], including TLR1 [44], TLR6 [44], Dectin-1 [45], CD36 [46], and CD14 [47].

For example, TLR6 associated with TLR2 uniquely induces IL-10 production by DCs and type-1 regulatory T-cells (Tr1). In contrast, TLR1 associated with TLR2 promotes differentiation of IL-12p40 production by DCs and inflammatory IFN-gamma T-cells (Th1) [48]. Furthermore, bacteria can also modulate the immune response based on the activation of TLR2 [49]. For example, bacterial triacylated lipoproteins activate TLR2/1, whereas bacterial diacylated lipoproteins activate TLR2/6, resulting in triggering different immune responses.

Thus, it is evident that the tissue microenvironment, bacteria composition, and metabolism all contribute to modulate the immune response.

4. TLR4

TLR4 is the best characterized pathogen-recognition receptor. Both immune cells and enterocytes express TLR4 [50]. Although there is a common signaling pathway and subsequent release of NF-κB and IFN-beta [51], the downstream effects of TLR4 are varied. TLR4 is involved in both defense against pathogens and maintaining tolerance to commensal bacteria. Continuous recognition of selective commensals by TLR4 under steady-state conditions is essential in mucosal protection against exogenous injury [18].

Intestinal mucosa expresses low concentrations of TLR4 protein at baseline [52]. However, TLR4 expression is significantly increased in intestinal epithelial cells (IECs) and *lamina propria* mononuclear cells in association with acute inflammation, such as in IBD [22, 53, 54]. The presence of inflammatory cytokines such as IFN-gamma and TNF-alpha strongly upregulates TLR4 expression in IECs [55, 56]. During disruption of the epithelium, activation of TLR4 elicits inflammatory cytokine and chemokine expression with recruitment of innate and adaptive immune cells to limit bacterial invasion [57]. The absence of TLR4 signaling during injury results in a pattern of severe mucosal damage with impaired epithelial proliferation, attenuated inflammatory response, and marked bacterial translocation [58]. TLR4 signaling is important for induction of repair of the injured gut, so that increase in TLR4 expression may serve a protective role. However, in necrotizing enterocolitis, TLR4 activation has a strong role in the induction of mucosal injury in the newborn small intestine via increased enterocyte apoptosis and an inhibition in mucosal repair, through decreased enterocyte proliferation and migration [59, 60]. Moreover, Ungaro et al. have recently shown in a study using chimeric mice that TLR4 signaling in colonic epithelial cells worsened intestinal inflammation [61].

TLR4 signaling has been shown to affect the intestinal flora. Regulation of the microbiota by TLR4 appears to be attributable to alterations in gastrointestinal motility that drives clearance of pathogens and maintenance of commensal populations [62], differentiation of goblet cells [63], and expression of antimicrobial peptides. In mice, in response to alterations in the microbial flora of the gut, TLR4 may directly regulate transcription of defensin genes [64].

TLR4 can be affected by modification in diet. A high-fat diet induces dysregulation of the gut microbiota and activation of the TLR4 signaling pathway with consequent increased intestinal permeability [65].

Thus, the effects of TLR4 on intestinal mucosa are complicated. The appropriate or inappropriate TLR4 signaling is linked to a variety of factors, including the involved cells, cytokines and chemokines, and microenvironment.

5. TLR5

TLR5 is expressed on epithelial cells, endothelial cells, macrophages, DCs, and T-cells. TLR5 recognizes flagellin, the main protein of bacterial flagella, and is crucial for the detection of invasive flagellated bacteria at the mucosal surface [66]. TLR5 plays an important role in maintaining intestinal homeostasis by regulating host defense against enterobacterial infections. However, regulation of TLR5 expression and its function in the intestine have not been fully elucidated.

The work of Feng et al. has compared the expression of TLR5 in various human tissues. In particular, in intestinal mucosa, DCs express high levels of TLR5 with respect to splenic tissue. These differences are due to the different microenvironment of each tissue. In mucosa, host-derived factors such as retinoic acid and stromal cell products alter TLR5 expression [67]. Moreover, activation of TLR5 signaling induces differentiation of naive B-cells into plasma cells producing IgA and promotes development of antigen-specific Th1 and Th17 cells [67]. More recently, it has been reported that activation of TLR5 signaling induces mucosal production of IL-17 and IL-22; these interleukins promote early defenses against pathogen invasion of host tissues.

Furthermore, TLR5 signaling restricts Tregs generation but promotes effector T-cells. Therefore, high expression levels of TLR5 on *lamina propria* DCs give these cells a crucial role in the induction of effector T-cell responses against invading flagellated pathogens. On the other hand, DCs not expressing TLR5 may be responsible for maintenance of intestinal homeostasis, through induction of Tregs.

The recognition of flagellin by TLR5 is the principal mechanism through which the intestinal epithelia activate proinflammatory pathways in response to infections, such as *Salmonella enterica* [68]. However, studies on TLR5 knockout (TLR5KO) mice have shown that TLR5KO are resistant to *Salmonella* infection. This resistance has been attributed to changes in the basal phenotype of TLR5KO mice [68]. The small intestine and colon of these mice exhibit elevated levels of host defense genes that mediate innate and adaptive immunity in the gut. This includes changes in the basal phenotype of antimicrobial peptides and an increase in serum and fecal IgA and IgG and transport proteins in the gut [69]. TLR5KO mice have a homeostatic shift in microbiota composition with an increase in enterobacterial species, including *E. coli*, that was observed in proximity to the gut epithelium [70].

Thus, the absence of TLR5 signaling leads to increased resistance to infections and dysbiosis and leads to alterations in gene expression which then impact host metabolism. TLR5KO mice exhibit the hallmark features of a metabolic syndrome that includes hyperlipidemia, hypertension, insulin resistance, and increased adiposity. TLR5KO mice have insulin resistance even when on a calorie-restricted diet. It has been demonstrated that the transfer of TLR5KO microbiota to wild-type germ-free mice conferred many aspects of the TLR5KO phenotype, suggesting that the altered microbiota contributes to the development of the metabolic syndrome [71]. However, whether the altered microbiota is the cause or the effect in TLR5KO mice remains yet to be determined.

6. TLR9

TLR9 is expressed in antigen-presenting cells (APCs) including macrophages, DCs, and B lymphocytes. TLR9 is localized in the endosomal compartment and recognizes intracellular bacteria by binding unmethylated cytosine phosphate guanine (CpG) dinucleotides [72]. These nucleotides are expressed at high levels in prokaryotic DNA of commensal microbiota.

Activation of intracellular TLR9 drives the production of numerous proinflammatory cytokines, including TNF, IL-6, and IL-12, leading to a strong induction of the Th1-immune response [73]. Several studies have shown that TLR9 is effective in reducing apoptosis in gastrointestinal inflammatory disease. Studies examining the localization of TLR9 in intestinal epithelial cells have suggested that activation can

occur via basolateral and apical surface domains of TLR9 [74]. These studies suggest that the signaling of TLR9 on the apical or basolateral surfaces determines whether the response is tolerogenic or inflammatory, respectively. Apical activation of TLR9 does not induce NF-κB. In contrast, basolateral activation of TLR9 activates NF-κB and ultimately induces IL-8 production [74].

The apical surface interacting with the intestinal lumen and coming into contact with commensal bacteria and probiotic DNA suppresses inflammation and it is protective in models of colitis. In fact, in models of experimental colitis, the administration of CpG significantly reduced the proinflammatory cytokine expression of IFN-gamma and IL-6, increased anti-inflammatory IL-10, and reduced disease severity [75]. In contrast to commensal bacteria, pathogenic bacteria that have breached the epithelium would stimulate basolateral TLR9 to produce inflammatory mediators and initiate the immune response. It was reported that TLR9 activation could limit TLR4 signaling in the gut, leading to reduced proinflammatory cytokines and apoptosis, thus ameliorating intestinal disease [76]. In the absence of TLR9, there is an increase in Tregs within the small intestine, leading to an inability to protect from infection [77].

7. TLRs Polymorphism in Human Gastrointestinal Pathology

Some data suggest that the human ability to respond to TLR ligands may be impaired by genetic variation within TLR genes, resulting in an altered susceptibility to infectious or inflammatory disease.

Genetic variations in TLRs may alter interaction between host and commensal bacteria. A defect in TLRs protein structure may influence ligand recognition, mucosal immune tolerance, and commensal composition, leading to innate/adaptive immune hypo- or hyperreactivity.

Several studies have evaluated the functional impact of TLR polymorphisms in IBD susceptibility. Although TLR polymorphism may not predict overall disease risk, they may influence phenotype severity in subgroups of patients with IBD [78].

The TLR variants are relatively rare. A number of variants in the *TLR1*, *TLR2,* and *TLR6* genes have been associated to distinct disease phenotypes of IBD. The polymorphisms TLR1-R80T and TLR2-R753Q in ulcerative colitis patients are associated with increased risk to develop pancolitis [79]. The SNPs TLR6-S249P was associated with a slightly decreased incidence of proctitis in IBD [79].

Allelic variants of the TLR4 gene may induce functional dysregulation of the lipopolysaccharide (LPS) receptor, exhibiting hyper- or hyposensitivity to LPS stimuli [80].

In active IBD, the allelic variants D299G and T399I exhibit proinflammatory effects in response to physiological concentrations of LPS [81, 82]. Increased susceptibility to IBD has been associated with the coexistence of TLR4 and/or NOD2 and BPI mutated alleles [11, 83]. An association between the TLR4-D299G polymorphism and sepsis has been also investigated. Two studies demonstrated that TLR4-D299G polymorphism increases the risk of gram-negative infections [84, 85] and another study linked this polymorphism to an increased incidence of systemic inflammatory response [86].

Several studies investigating associations between genetic variants of TLR genes and IBD have shown controversial results about TLR5 in human Crohn's disease.

Several mutations may induce an overrecognition of flagellin by TLR5 leading to intestinal inflammation. This could also explain the high prevalence of anti-flagellin antibodies in Crohn's disease patients compared to healthy controls. Recently, in one study, a partial functional dominant negative of TLR5 was associated with protection against Crohn's disease [87]; however the complete loss of TLR5 ($TLR5^{-/-}$ mice) displays a high risk to develop colitis [88].

The gene encoding for TLR9 is mapped on chromosome 3p21.3 in the vicinity of a shared susceptibility locus for Crohn's disease and ulcerative colitis. Torok showed that genetic variation in TLR9 is associated with IBD [89]. The interactions between TLR9 polymorphisms and allelic variants in *NOD2* and *IL23R* differentially modulate susceptibility to Crohn's disease [90].

In addition, it should be noted that other genetic mutations and polymorphisms associated with genes and proteins involved in pathogenesis of gastrointestinal diseases such as NOD2, IL-10, MDR1-alpha, and STAT3 exist. They could interact with TLR polymorphisms, increasing the complexity of IBD.

8. The Role of TLRs and Its Interactions with Human Microbiota in the Pathogenesis of Inflammatory Bowel Diseases

IBD, comprising Crohn's disease and ulcerative colitis, are chronic and multifactorial diseases affecting the gastrointestinal tract. IBD are characterized by idiopathic intestinal inflammation, resulting from predisposing genetic (genes encoding proteins relevant to both innate and adaptive immunity: NOD2, STAT3, IL-23 receptor, etc.) and environmental factors (specific TLRs, ligands, and antigens derived from commensal bacteria) acting on the immunoregulatory system. IBD may be result of an imbalance of proinflammatory- and regulatory-T-cells responses [91]. The pathogenetic mechanism is still unknown. However, in genetically predisposed individuals there is an abnormal and inappropriate immune response against luminal agents (bacteria, viruses, and food), with the production of cytokines and other mediators of inflammation. Both humoral and cell-mediated immunity are involved in the pathogenesis of IBD. Then, cell-mediated immunity induces the activation of T-cells, macrophages, neutrophils, and other leukocytes.

Available evidence suggests that both dysregulated innate and adaptive immune pathways contribute to the aberrant intestinal inflammatory response in patients with IBD [92]. Most studies conducted in the last thirty years have focused on the role of abnormal adaptive immune responses in the pathogenesis of IBD. In particular, while Crohn's disease has

long been considered to be driven by a Th1 response, ulcerative colitis has been rather associated with a nonconventional Th2 response [93]. Finally, it is important to consider that the innate immune response represents our first line of defense against pathogens [92].

It can be assumed that IBD are associated with an imbalance in the composition and function of intestinal bacterial flora. This involves, as a result of intestinal barrier dysfunction, a translocation of bacteria flora in the *lamina propria* and the activation of a strong inflammatory response following the activation of TLRs and of NF-κB pathway, responsible for the transcription of various proinflammatory cytokines and chemokines [40, 92, 94]. This process is amplified by a decrease of the innate immune response that, in turn, determines a greater translocation of bacterial flora thorough the intestinal membrane. Overall, the progression of these diseases is due to a defect in immune regulation and immune tolerance in response to the initial inflammatory insult [94].

It has been noted that IBD probably have genetic components; they are not inherited in a Mendelian fashion and are thus probably due to a complex set of factors rather than solely to a gene. However, neither bacterial colonization nor genetics is sufficient to cause the disease, bacteria probably play a role in these disorders. Some suspect that IBD is due to a reduction in immune tolerance and subsequent overreaction of the host's immune system to harmful or nonharmful bacteria. Bacteria in the digestive tract may have pathogenic properties in addition to their health-inducing ones: they can produce toxins and carcinogens and have been implicated in such conditions as multisystem organ failure, sepsis, colon cancer, and IBD [2]. A major factor in health is the balance of bacterial numbers; if the numbers grow too high or low, it will result in harm to the host. The host has enzymes to regulate this balance. Some genera of bacteria, such as *Bacteroides* and *Clostridium*, have been associated with an increase in tumor growth rate, while other *genera*, such as *Lactobacillus* and *Bifidobacterium,* are known to prevent tumor formation [2].

On the other hand, some evidence demonstrated that bacteria help train the immune system; in addition, some forms of bacteria can prevent inflammation. Thus, the constant exposure of the intestinal mucosal surface to commensal derived TLR ligands induces a basal state of activation of downstream signaling pathways that ensures mucosal homeostasis through limited inflammatory responses and accelerated restitution and healing in the healthy intestine. Commensal composition and tolerance represent essential mechanisms of maintaining hyporesponsiveness of the intestinal immune system. The composition of the commensal microbiota depends on host immunity, genetics, and environment [78]. In return, the composition of the commensal microbiota actively shapes mucosal and systemic immune homeostasis of the host at multidimensional levels. The presence of commensals modulates TLR expression in the intestinal mucosa. The complexity of the commensal composition is critical in augmenting protective mucosal immunity [95]. Changes in the commensal composition may differentially modulate mucosal TLRs responsiveness, thus subverting immune responses to a predominantly proinflammatory phenotype. Both quantitative and qualitative changes in the microbial composition have been reported in IBD [96]. These bacterial changes in IBD patients contain abnormal compositions of the intestinal microbiota, characterized by reduced bacterial diversity, temporal instability, and depletion of distinct commensal species (members of the phyla Firmicutes and Bacteroidetes). The latter includes a lower proportion of *Faecalibacterium prausnitzii*, an anti-inflammatory commensal that counterbalances dysbiosis [78, 97]. Several causal scenarios are plausible in IBD pathogenesis but remain to be directly proven: genetic defects and/or aberrant immune-mediated modulation of specific TLRs may diminish antimicrobial activities and disturb bacterial clearance, leading to a colitogenic commensal composition. Changes in the commensal composition may subvert the mucosal innate immune system, leading to TLR-mediated hyper- or hyporeactive immune responses. Dysbiosis may inhibit effective TLR recognition and bactericidal activation [78].

Some bacteria have a pathogenic effect on gut homeostasis and infections may contribute to IBD pathogenesis. In fact, episodes of *Salmonella/Campylobacter* gastroenteritis have been associated with increased risk of developing IBD. Loss-of-function mutations in the TLR4 gene can predispose to these Gram-negative bacteria and increase susceptibility to enteric infection, which may represent an essential disease trigger in IBD pathogenesis. Pathogenic infections may change the commensal composition and disrupt commensal tolerance [98]. *Campylobacter jejuni* may directly promote the internalization and translocation of commensal bacteria [99].

On the other hand, several negative control mechanisms that ensure tolerance to abundant resident microbiota and regulated activation via TLRs in the intestinal mucosa have recently been described: decreased surface receptor expression which limits frontline recognition, high expression levels of the downstream signaling suppressor Tollip, which inhibits IRAK activation, ligand-induced activation of PPARc (peroxisome proliferator-activated receptor c), which uncouples NF-κB-dependent target genes in a negative feedback loop, negative regulation of proinflammatory IL-1R/TLR4 signaling through SIGIRR (single immunoglobulin IL-1R-related molecule; also known as TIR8), which abolishes exaggerated immune responses to commensal bacteria in colitis, ubiquitination of key TLR signaling components via ubiquitin-editing enzymes, such as A20, or E3 ubiquitin protein ligases, such as TRIAD3A, and selective induction of transcriptional repressors, such as Bcl-3, which limits proinflammatory responses via NF-κB [78, 100]. Thus, inflammation in IBD may result from persistent commensal intolerance because of altered pattern recognition and TLR signaling. Accordingly, there is genetic evidence showing that the impaired recognition and killing of commensal bacteria also contribute to IBD development as has been suggested by the fact that many of the identified IBD-susceptibility genes regulate host-microbial interactions [94]. NOD2, which is an intracellular sensor of bacterial peptidoglycan, was identified as a susceptibility gene for Crohn's disease, and Crohn's

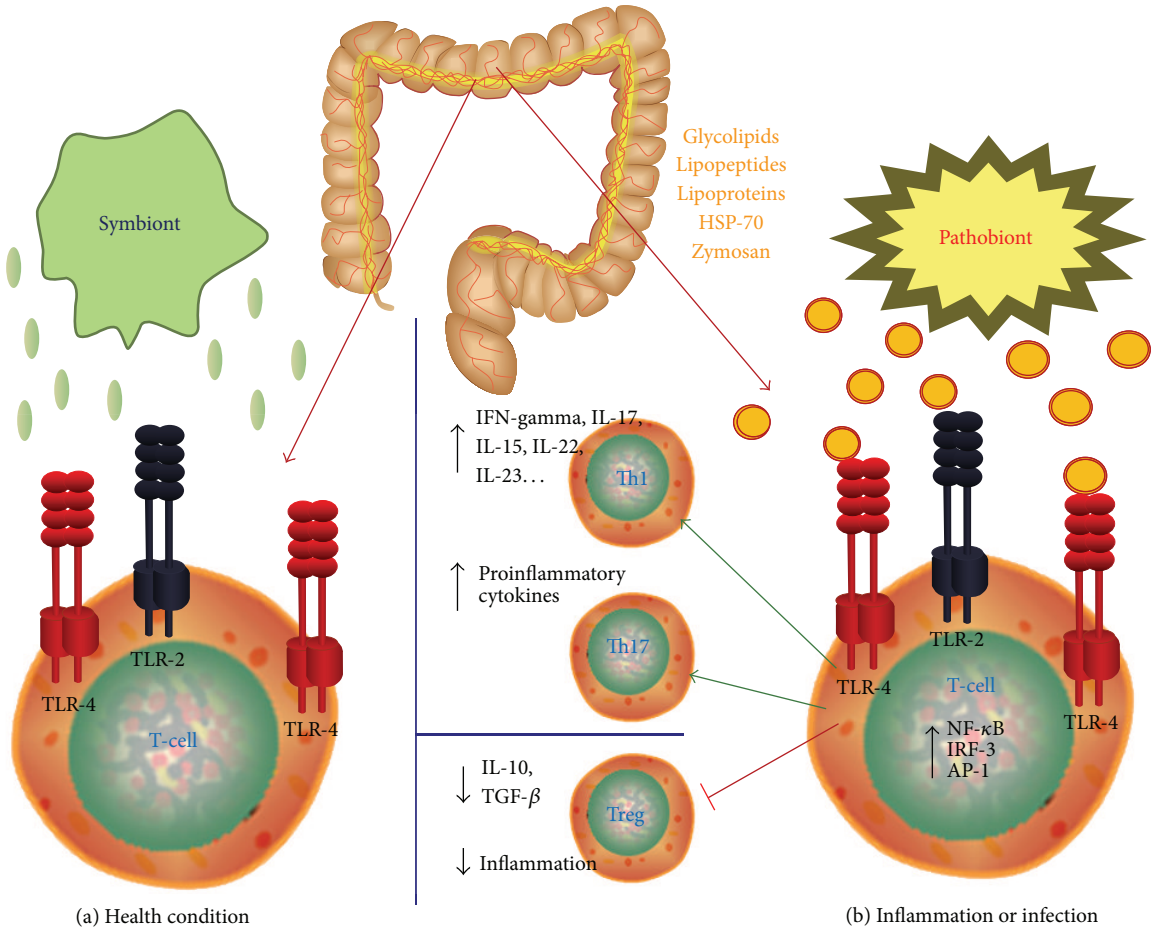


Figure 1: TLRs in human gastrointestinal pathology: health condition and inflammation. (a) In healthy human intestine, a constant homeostasis is maintained by the perfect regulation of microbial load and the immune response generated against it. (b) Failure of this balance may result in various pathological conditions. TLRs may be considered an interface among intestinal epithelial barrier, microbiota, and immune system. TLRs pathway, activated by pathogens, is involved in the pathogenesis of several infectious and inflammatory diseases. TLR signaling critically contributes to acute and chronic intestinal inflammatory processes.

disease-associated NOD2 mutations are associated with a loss of function of the protein [92, 94]. Three uncommon SNPs in NOD2 have been associated with susceptibility to ileal CD with an odds ratio equal to 2.4 in heterozygote individuals and 17.1 in homozygotes or compound heterozygotes, representing the strongest association with IBD to date [92]. Thus, defects in host mechanisms that recognize and clear bacteria are associated with the development of human IBD. How genetic defects lead to chronic colitis in patients with IBD remains unknown, but it is possible that impaired NOD2 or autophagy function might result in the accumulation of intestinal commensal bacteria that have the capacity to locally invade the intestinal mucosa and to trigger an abnormal inflammatory response [94].

Amongst the NOD family, in fact, NOD2 is crucially involved in IBD pathogenesis. It is expressed in the epithelium and senses muramyl dipeptide (MDP), which is a constituent of Gram-positive and Gram-negative bacteria [101]. Specific mutations of the NOD2 gene (Arg702Trp, Gly908Arg, and leu1007fsinsC) are linked to an increased susceptibility to ileal Crohn's disease [102]. The risk of developing ileal Crohn's disease is increased twofold to fourfold and 20-fold to 40-fold, respectively, for heterozygous and homozygous carriers of these NOD2 mutations [103]. In patients with NOD2 mutations, the activation of NF-κB in response to MDP is defective, enabling bacteria to trigger inflammation [104]. TLRs detect microbiota and damage-associated molecular patterns and are involved in the maintenance of the commensal flora and mucosal homoeostasis. In the healthy intestine, TLRs are expressed in small amounts not only by epithelial cells, but also by monocytes, macrophages, and DCs [105].

IBD are also linked to good hygiene in youth, lack of breastfeeding, and consumption of large amounts of sucrose and animal fat [2]. In fact, in accordance with the fact that hygiene and the rate of infections in youth are connected to lifestyle and environment, the incidence and prevalence of IBD are high in industrialized countries with a high standard of living and low in less economically developed countries, having increased in developed countries throughout the

twentieth century. IBD incidence is inversely linked to poor sanitation during the first years of life and consumption of fruits, vegetables, and unprocessed foods. Also, the use of antibiotics, which kill native gut flora and harmful infectious pathogens alike, especially during childhood, is associated with IBD.

Differential alteration of TLRs expression in IBD was first described at the beginning of the 21st century [78]. For example, TLR3 is downregulated in active Crohn's disease but not in ulcerative colitis and TLR5 is upregulated in both forms of IBD [78]. Essentially, these receptors provide a danger signal, which, amongst other effects, stimulates the formation of alpha- and beta-defensins [104, 106].

In conclusion, the impact of TLR signaling on commensal-host interactions appears to be context-dependent. Environment, genetics, and host immunity modulate TLRs in the intestinal mucosa (Figure 1). Conversely, mucosal TLR signaling influences outcome of environmental signals, genetic functions, and immune responses in the intestine. There is an important dichotomy in TLRs regulation and function between healthy and inflamed intestinal mucosa, reflecting a fine line between host protection and destruction. In the healthy host, basal TLR signaling is significantly involved in protective host defense and tissue repair responses, crucially maintaining mucosal and commensal homeostasis. In the IBD-susceptible host, aberrant TLR signaling may contribute to destructive host responses and chronic inflammation, disturbing mucosal and commensal homeostasis and leading to many different clinical phenotypes. Hyperactivation of the adaptive immune system, secondary to TLRs deficiency, may drive tissue damage and progressive inflammation in IBD [78].

9. Conclusion

The small intestine has an enormous surface area that is continuously exposed to dietary and microbial antigens. These antigens need to be tolerated by the immune system to maintain homeostasis. This important role is played by immune sensors such as TLRs. Unfortunately, in some cases the innate immune system fails to protect the host, and chronic inflammation and other disorders occur.

It is evident that environment, genetics, and host immunity form a multidimensional and highly interactive regulatory triad that controls TLR function in the intestinal mucosa. Imbalanced relationships within this triad may promote aberrant TLR signaling, critically contributing to acute and chronic intestinal inflammatory processes, such as in IBD, colitis, and colorectal cancer.

Changes in intestinal microbiota through genetics and environment may contribute to defective host immune response.

The gut microbiota has been studied for a long time. Recent studies have shown ever-expanding roles for these microscopic organisms in health and disease. Despite the complexity of microbial population present in gut, a delicate balance between host and bacteria populations exists. The disruption of this balance leads to dysbiosis and, consequently, to decreased resistance to pathogen colonization, to the favored growth of pathobionts, and to pathological immune responses by the host.

In many diseases, including IBD, dysbiosis is an important immunologic pathogenenic process. Dysbiosis and immune dysregulation might have a greater influence in young children than adolescents or adults. However, it is not clear whether dysbiosis contributes to the development of IBD or is instead a consequence of the disease. Indeed, antibiotics are not effective in the treatment of IBD, except in specific circumstances. For this reason, a better knowledge of the mechanisms underlying the intestinal innate immune response is crucial for developing of new therapies and vaccines to protect against pathogens and chronic inflammation.

Moreover, the understanding of host-microbial immune mutualism is fundamental because it is intimately connected with human health. Thus, the study of interactions between different components of the innate and adaptive immune systems, especially in relationship with the intestinal microbiota, will open new horizons in the knowledge of gut inflammation mechanisms.

Conflict of Interests

The authors declared that there is no conflict of interests.

Acknowledgment

The authors would like to gratefully and sincerely thank Dr. Estelle E. Newton (CytoCure LLC, Beverly, MA, USA) for her patience in English revision.

References

[1] L. V. Hooper and J. I. Gordon, "Commensal host-bacterial relationships in the gut," *Science*, vol. 292, no. 5519, pp. 1115–1118, 2001.

[2] F. Guarner and J. R. Malagelada, "Gut flora in health and disease," *The Lancet*, vol. 361, no. 9356, pp. 512–519, 2003.

[3] L. V. Hooper and A. J. MacPherson, "Immune adaptations that maintain homeostasis with the intestinal microbiota," *Nature Reviews Immunology*, vol. 10, no. 3, pp. 159–169, 2010.

[4] A. J. MacPherson and T. Uhr, "Compartmentalization of the mucosal immune responses to commensal intestinal bacteria," *Annals of the New York Academy of Sciences*, vol. 1029, pp. 36–43, 2004.

[5] M. E. V. Johansson, J. M. Holmén Larsson, and G. C. Hansson, "The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 1, pp. 4659–4665, 2011.

[6] M. E. V. Johansson and G. C. Hansson, "Keeping bacteria at a distance," *Science*, vol. 334, no. 6053, pp. 182–183, 2011.

[7] R. L. Gallo and L. V. Hooper, "Epithelial antimicrobial defence of the skin and intestine," *Nature Reviews Immunology*, vol. 12, no. 7, pp. 503–516, 2012.

[8] J. Benckert, N. Schmolka, C. Kreschel et al., "The majority of intestinal IgA^+ and IgG^+ plasmablasts in the human gut are antigen-specific," *The Journal of Clinical Investigation*, vol. 121, no. 5, pp. 1946–1955, 2011.

[9] R. J. Xavier and D. K. Podolsky, "Unravelling the pathogenesis of inflammatory bowel disease," *Nature*, vol. 448, no. 7152, pp. 427–434, 2007.

[10] R. B. Sartor, "Microbial influences in inflammatory bowel diseases," *Gastroenterology*, vol. 134, no. 2, pp. 577–594, 2008.

[11] C. Abraham and J. H. Cho, "Inflammatory bowel disease," *The New England Journal of Medicine*, vol. 361, no. 21, pp. 2066–2078, 2009.

[12] C. A. Janeway Jr., "The immune system evolved to discriminate infectious nonself from noninfectious self," *Immunology Today*, vol. 13, no. 1, pp. 11–16, 1992.

[13] F. L. Rock, G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan, "A family of human receptors structurally related to *Drosophila* Toll," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 2, pp. 588–593, 1998.

[14] B. Kobe and J. Deisenhofer, "A structural basis of the interactions between leucine-rich repeats and protein ligands," *Nature*, vol. 374, no. 6518, pp. 183–186, 1995.

[15] M. Schnare, G. M. Barton, A. C. Holt, K. Takeda, S. Akira, and R. Medzhitov, "Toll-like receptors control activation of adaptive immune responses," *Nature Immunology*, vol. 2, no. 10, pp. 947–950, 2001.

[16] R. Medzhitov, P. Preston-Hurlburt, E. Kopp et al., "MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways," *Molecular Cell*, vol. 2, no. 2, pp. 253–258, 1998.

[17] K. Takeuchi, S. Kawauchi, H. Araki, S. Ueki, and O. Furukawa, "Stimulation by nizatidine, a histamine H_2-receptor antagonist, of duodenal HCO_3^- secretion in rats: relation to anticholinesterase activity," *World Journal of Gastroenterology*, vol. 6, no. 5, pp. 651–658, 2000.

[18] S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, 2004.

[19] N. P. Malvin, H. Seno, and T. S. Stappenbeck, "Colonic epithelial response to injury requires Myd88 signaling in myeloid cells," *Mucosal Immunology*, vol. 5, no. 2, pp. 194–206, 2012.

[20] S. Vaishnava, C. L. Behrendt, A. S. Ismail, L. Eckmann, and L. V. Hooper, "Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 52, pp. 20858–20863, 2008.

[21] A. Menendez, B. P. Willing, M. Montero et al., "Bacterial stimulation of the TLR-MyD88 pathway modulates the homeostatic expression of ileal paneth cell α-defensins," *Journal of Innate Immunity*, vol. 5, no. 1, pp. 39–49, 2013.

[22] C. F. Ortega-Cava, S. Ishihara, M. A. K. Rumi et al., "Strategic compartmentalization of Toll-like receptor 4 in the mouse gut," *Journal of Immunology*, vol. 170, no. 8, pp. 3977–3985, 2003.

[23] H. D. Brightbill, D. H. Libraty, S. R. Krutzik et al., "Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors," *Science*, vol. 285, no. 5428, pp. 732–736, 1999.

[24] A. O. Aliprantis, R. B. Yang, M. R. Mark et al., "Cell activation and apoptosis by bacterial lipoproteins through Toll- like receptor-2," *Science*, vol. 285, no. 5428, pp. 736–739, 1999.

[25] R. Schwandner, R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning, "Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2," *Journal of Biological Chemistry*, vol. 274, no. 25, pp. 17406–17409, 1999.

[26] D. M. Underhill, A. Ozinsky, A. M. Hajjar et al., "The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens," *Nature*, vol. 401, no. 6755, pp. 811–815, 1999.

[27] T. Horng, G. M. Barton, R. A. Flavell, and R. Medzhitov, "The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors," *Nature*, vol. 420, no. 6913, pp. 329–333, 2002.

[28] M. Yamamoto, S. Sato, H. Hemmi et al., "Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4," *Nature*, vol. 420, no. 6913, pp. 324–329, 2002.

[29] D. L. Boone, E. E. Turer, E. G. Lee et al., "The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses," *Nature Immunology*, vol. 5, no. 10, pp. 1052–1060, 2004.

[30] E. Cario, G. Gerken, and D. K. Podolsky, "Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function," *Gastroenterology*, vol. 132, no. 4, pp. 1359–1374, 2007.

[31] E. Cario, "Barrier-protective function of intestinal epithelial Toll-like receptor 2," *Mucosal Immunology*, vol. 1, no. 1, pp. S62–S66, 2008.

[32] T. Watanabe, A. Kitani, P. J. Murray, and W. Strober, "NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses," *Nature Immunology*, vol. 5, no. 8, pp. 800–808, 2004.

[33] M. M. Heimesaat, A. Fischer, B. Siegmund et al., "Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via toll-like receptors 2 and 4," *PLoS ONE*, vol. 2, no. 7, article e662, 2007.

[34] W. Strober, A. Kitani, I. Fuss, N. Asano, and T. Watanabe, "The molecular basis of NOD2 susceptibility mutations in Crohn's disease," *Mucosal Immunology*, vol. 1, supplement 1, pp. S5–S9, 2008.

[35] O. Boulard, M. J. Asquith, F. Powrie, and K. J. Maloy, "TLR2-independent induction and regulation of chronic intestinal inflammation," *European Journal of Immunology*, vol. 40, no. 2, pp. 516–524, 2010.

[36] S. Wang, E. J. Villablanca, J. de Calisto et al., "MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting properties," *The Journal of Immunology*, vol. 187, no. 1, pp. 141–150, 2011.

[37] J. M. Reynolds, B. P. Pappu, J. Peng et al., "Toll-like receptor 2 signaling in $CD4^+$ T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease," *Immunity*, vol. 32, no. 5, pp. 692–702, 2010.

[38] M. H. Nyirenda, L. Sanvito, P. J. Darlington et al., "TLR2 stimulation drives human naive and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function," *The Journal of Immunology*, vol. 187, no. 5, pp. 2278–2290, 2011.

[39] J. L. Round, S. M. Lee, J. Li et al., "The toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota," *Science*, vol. 332, no. 6032, pp. 974–977, 2011.

[40] D. Pagliari, R. Cianci, S. Frosali et al., "The role of IL-15 in gastrointestinal diseases: a bridge between innate and adaptive immune response," *Cytokine & Growth Factor Reviews*, vol. 24, no. 5, pp. 455–466, 2013.

[41] R. Cianci, G. Cammarota, G. Frisullo et al., "Tissue-infiltrating lymphocytes analysis reveals large modifications of the duodenal "immunological niche" in coeliac disease after gluten-free diet," *Clinical and Translational Gastroenterology*, vol. 3, article e28, 2012.

[42] R. Cianci, D. Pagliari, R. Landolfi et al., "New insights on the role of T cells in the pathogenesis of celiac disease," *Journal of Biological Regulators and Homeostatic Agents*, vol. 26, no. 2, pp. 171–179, 2012.

[43] A. Ozinsky, D. M. Underhill, J. D. Fontenot et al., "The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13766–13771, 2000.

[44] R. William DePaolo, K. Kamdar, S. Khakpour, Y. Sugiura, W. Wang, and B. Jabri, "A specific role for TLR1 in protective TH17 immunity during mucosal infection," *Journal of Experimental Medicine*, vol. 209, no. 8, pp. 1437–1444, 2012.

[45] B. N. Gantner, R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill, "Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2," *Journal of Experimental Medicine*, vol. 197, no. 9, pp. 1107–1117, 2003.

[46] K. Hoebe, P. Georgel, S. Rutschmann et al., "CD36 is a sensor of diacylglycerides," *Nature*, vol. 433, no. 7025, pp. 523–527, 2005.

[47] M. Hirschfeld, G. J. Kirschning, R. Schwandner et al., "Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by toll-like receptor 2," *Journal of Immunology*, vol. 163, no. 5, pp. 2382–2386, 1999.

[48] R. W. DePaolo, F. Tang, I. Kim et al., "Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis," *Cell Host & Microbe*, vol. 4, no. 4, pp. 350–361, 2008.

[49] K. O. Omueti, J. M. Beyer, C. M. Johnson, E. A. Lyle, and R. I. Tapping, "Domain exchange between human Toll-like receptors 1 and 6 reveals a region required for lipopeptide discrimination," *The Journal of Biological Chemistry*, vol. 280, no. 44, pp. 36616–36625, 2005.

[50] M. D. Neal, C. Leaphart, R. Levy et al., "Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier," *Journal of Immunology*, vol. 176, no. 5, pp. 3070–3079, 2006.

[51] M. Fukata, Y. Hernandez, D. Conduah et al., "Innate immune signaling by toll-like receptor-4 (TLR4) shapes the inflammatory microenvironment in colitis-associated tumors," *Inflammatory Bowel Diseases*, vol. 15, no. 7, pp. 997–1006, 2009.

[52] E. Cario and D. K. Podolsky, "Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease," *Infection and Immunity*, vol. 68, no. 12, pp. 7010–7017, 2000.

[53] M. Hausmann, S. Kiessling, S. Mestermann et al., "Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation," *Gastroenterology*, vol. 122, no. 7, pp. 1987–2000, 2002.

[54] J. C. I. Singh, S. M. Cruickshank, D. J. Newton et al., "Toll-like receptor-mediated responses of primary intestinal epithelial cells during the development of colitis," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 288, no. 3, pp. G514–G524, 2005.

[55] M. T. Abreu, E. T. Arnold, L. S. Thomas et al., "TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells," *Journal of Biological Chemistry*, vol. 277, no. 23, pp. 20431–20437, 2002.

[56] M. Suzuki, T. Hisamatsu, and D. K. Podolsky, "Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex," *Infection and Immunity*, vol. 71, no. 6, pp. 3503–3511, 2003.

[57] T. Furuta, T. Kikuchi, S. Akira, N. Watanabe, and Y. Yoshikawa, "Roles of the small intestine for induction of toll-like receptor 4-mediated innate resistance in naturally acquired murine toxoplasmosis," *International Immunology*, vol. 18, no. 12, pp. 1655–1662, 2006.

[58] M. Fukata, K. S. Michelsen, R. Eri et al., "Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 288, no. 5, pp. G1055–G1065, 2005.

[59] C. L. Leaphart, J. Cavallo, S. C. Gribar et al., "A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair," *Journal of Immunology*, vol. 179, no. 7, pp. 4808–4820, 2007.

[60] C. P. Sodhi, X.-H. Shi, W. M. Richardson et al., "Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis," *Gastroenterology*, vol. 138, no. 1, pp. 185–196, 2010.

[61] R. Ungaro, M. Fukata, D. Hsu et al., "A novel Toll-like receptor 4 antagonist antibody ameliorates inflammation but impairs mucosal healing in murine colitis," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 296, no. 6, pp. G1167–G1179, 2009.

[62] M. Anitha, M. Vijay-Kumar, S. V. Sitaraman, A. T. Gewirtz, and S. Srinivasan, "Gut microbial products regulate murine gastrointestinal motility via toll-like receptor 4 signaling," *Gastroenterology*, vol. 143, no. 4, pp. 1006.e4–1016.e4, 2012.

[63] C. P. Sodhi, M. D. Neal, R. Siggers et al., "Intestinal epithelial toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice," *Gastroenterology*, vol. 143, no. 3, pp. 708.e5–718.e5, 2012.

[64] P. Vora, A. Youdim, L. S. Thomas et al., "β-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells," *The Journal of Immunology*, vol. 173, no. 9, pp. 5398–5405, 2004.

[65] K.-A. Kim, W. Gu, I.-A. Lee, E.-H. Joh, and D.-H. Kim, "High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway," *PLoS ONE*, vol. 7, no. 10, Article ID e47713, 2012.

[66] F. Hayashi, K. D. Smith, A. Ozinsky et al., "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5," *Nature*, vol. 410, no. 6832, pp. 1099–1103, 2001.

[67] S. Uematsu, K. Fujimoto, M. H. Jang et al., "Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5," *Nature Immunology*, vol. 9, no. 7, pp. 769–776, 2008.

[68] S. Uematsu, M. H. Jang, N. Chevrier et al., "Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c$^+$ lamina propria cells," *Nature Immunology*, vol. 7, no. 8, pp. 868–874, 2006.

[69] M. Vijay-Kumar, J. D. Aitken, A. Kumar et al., "Toll-like receptor 5-deficient mice have dysregulated intestinal gene expression and nonspecific resistance to Salmonella-induced typhoid-like disease," *Infection and Immunity*, vol. 76, no. 3, pp. 1276–1281, 2008.

[70] F. A. Carvalho, O. Koren, J. K. Goodrich et al., "Transient inability to manage proteobacteria promotes chronic gut inflammation in TLR5-deficient mice," *Cell Host & Microbe*, vol. 12, no. 2, pp. 139–152, 2012.

[71] M. Vijay-Kumar, J. D. Aitken, F. A. Carvalho et al., "Metabolie syndrome and altered gut microbiota in mice lacking toll-like receptor 5," *Science*, vol. 328, no. 5975, pp. 228–231, 2010.

[72] K. J. Ishii and S. Akira, "Innate immune recognition of, and regulation by, DNA," *Trends in Immunology*, vol. 27, no. 11, pp. 525–532, 2006.

[73] A. M. Krieg, "Immune effects and mechanisms of action of CpG motifs," *Vaccine*, vol. 19, no. 6, pp. 618–622, 2000.

[74] J. Lee, J.-H. Mo, K. Katakura et al., "Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells," *Nature Cell Biology*, vol. 8, no. 12, pp. 1327–1336, 2006.

[75] F. Obermeier, N. Dunger, U. G. Strauch et al., "Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis," *Clinical & Experimental Immunology*, vol. 134, no. 2, pp. 217–224, 2003.

[76] S. C. Gribar, C. P. Sodhi, W. M. Richardson et al., "Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis," *The Journal of Immunology*, vol. 182, no. 1, pp. 636–646, 2009.

[77] J. A. Hall, N. Bouladoux, C. M. Sun et al., "Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses," *Immunity*, vol. 29, no. 4, pp. 637–649, 2008.

[78] E. Cario, "Toll-like receptors in inflammatory bowel diseases: a decade later," *Inflammatory Bowel Diseases*, vol. 16, no. 9, pp. 1583–1597, 2010.

[79] M. Pierik, S. Joossens, K. van Steen et al., "Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 12, no. 1, pp. 1–8, 2006.

[80] E. Lorenz, M. Jones, C. Wohlford-Lenane et al., "Genes other than TLR4 are involved in the response to inhaled LPS," *American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 281, no. 5, pp. L1106–L1114, 2001.

[81] S. Kiechl, E. Lorenz, M. Reindl et al., "Toll-like receptor 4 polymorphisms and atherogenesis," *The New England Journal of Medicine*, vol. 347, no. 3, pp. 185–192, 2002.

[82] D. Franchimont, S. Vermeire, H. El Housni et al., "Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis," *Gut*, vol. 53, no. 7, pp. 987–992, 2004.

[83] I. Petermann, C. Huebner, B. L. Browning et al., "Interactions among genes influencing bacterial recognition increase IBD risk in a population-based New Zealand cohort," *Human Immunology*, vol. 70, no. 6, pp. 440–446, 2009.

[84] D. M. Agnese, J. E. Calvano, S. J. Hahm et al., "Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections," *Journal of Infectious Diseases*, vol. 186, no. 10, pp. 1522–1525, 2002.

[85] E. Lorenz, J. P. Mira, K. L. Frees, and D. A. Schwartz, "Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock," *Archives of Internal Medicine*, vol. 162, no. 9, pp. 1028–1032, 2002.

[86] N. J. A. Child, I. A. Yang, M. C. K. Pulletz et al., "Polymorphisms in Toll-like receptor 4 and the systemic inflammatory response syndrome," *Biochemical Society Transactions*, vol. 31, no. 3, pp. 652–653, 2003.

[87] A. T. Gewirtz, M. Vijay-Kumar, S. R. Brant, R. H. Duerr, D. L. Nicolae, and J. H. Cho, "Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 290, no. 6, pp. G1157–G1163, 2006.

[88] M. Vijay-Kumar, C. J. Sanders, R. T. Taylor et al., "Deletion of TLR5 results in spontaneous colitis in mice," *The Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3909–3921, 2007.

[89] H.-P. Török, J. Glas, L. Tonenchi, G. Bruennler, M. Folwaczny, and C. Folwaczny, "Crohn's disease is associated with a toll-like receptor-9 polymorphism," *Gastroenterology*, vol. 127, no. 1, pp. 365–366, 2004.

[90] H. P. Török, J. Glas, I. Endres et al., "Epistasis between toll-like receptor-9 polymorphisms and variants in NOD2 and IL23R modulates susceptibility to crohn's disease," *The American Journal of Gastroenterology*, vol. 104, no. 7, pp. 1723–1733, 2009.

[91] F. Pandolfi, R. Cianci, D. Pagliari, R. Landolfi, and G. Cammarota, "Cellular mediators of inflammation: tregs and T_H17 cells in gastrointestinal diseases," *Mediators of Inflammation*, vol. 2009, Article ID 132028, 2009.

[92] A. Geremia, P. Biancheri, P. Allan, G. R. Corazza, and A. di Sabatino, "Innate and adaptive immunity in inflammatory bowel disease," *Autoimmunity Reviews*, vol. 13, no. 1, pp. 3–10, 2014.

[93] I. J. Fuss, M. Neurath, M. Boirivant et al., "Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease: Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerative colitis LP cells manifest increased secretion of IL-5," *The Journal of Immunology*, vol. 157, no. 3, pp. 1261–1270, 1996.

[94] N. Kamada, S.-U. Seo, G. Y. Chen, and G. Núñez, "Role of the gut microbiota in immunity and inflammatory disease," *Nature Reviews Immunology*, vol. 13, no. 5, pp. 321–335, 2013.

[95] B. Stecher, S. Chaffron, R. Käppeli et al., "Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria," *PLoS Pathogens*, vol. 6, no. 1, Article ID e1000711, 2010.

[96] J. O. Shim, "Gut microbiota in inflammatory bowel disease," *Pediatric Gastroenterology, Hepatology & Nutrition*, vol. 16, no. 1, pp. 17–21, 2013.

[97] D. N. Frank, A. L. St. Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace, "Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 34, pp. 13780–13785, 2007.

[98] K. O. Gradel, H. L. Nielsen, H. C. Schønheyder, T. Ejlertsen, and B. Kristensen, ""Increased short- and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis," *Gastroenterology*, vol. 137, no. 2, pp. 495–501, 2009.

[99] L. D. Kalischuk, G. D. Inglis, and A. G. Buret, "Campylobacter jejuni induces transcellular translocation of commensal bacteria via lipid rafts," *Gut Pathogens*, vol. 1, no. 1, p. 2, 2009.

[100] E. Cario and D. K. Podolsky, "Intestinal epithelial TOLLerance versus inTOLLerance of commensals," *Molecular Immunology*, vol. 42, no. 8, pp. 887–893, 2005.

[101] L. Peyrin-Biroulet and M. Chamaillard, "Invited review: NOD2 and defensins: Translating innate to adaptive immunity in Crohn's disease," *Journal of Endotoxin Research*, vol. 13, no. 3, pp. 135–139, 2007.

[102] J.-P. Hugot, M. Chamaillard, H. Zouali et al., "Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease," *Nature*, vol. 411, no. 6837, pp. 599–603, 2001.

[103] A. P. Cuthbert, S. A. Fisher, M. M. Mirza et al., "The contribution of NOD2 gene mutations to the risk and site of disease in

inflammatory bowel disease," *Gastroenterology*, vol. 122, no. 4, pp. 867–874, 2002.

[104] M. Gersemann, J. Wehkamp, and E. F. Stange, "Innate immune dysfunction in inflammatory bowel disease," *Journal of Internal Medicine*, vol. 271, no. 5, pp. 421–428, 2012.

[105] J. K. Yamamoto-Furusho and D. K. Podolsky, "Innate immunity in inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 13, no. 42, pp. 5577–5580, 2007.

[106] J. Wehkamp, J. Harder, M. Weichenthal et al., "NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α-defensin expression," *Gut*, vol. 53, no. 11, pp. 1658–1664, 2004.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 501361, 7 pages
http://dx.doi.org/10.1155/2015/501361

Review Article

Interactions between Innate Immunity, Microbiota, and Probiotics

GianMarco Giorgetti,[1] Giovanni Brandimarte,[2] Federica Fabiocchi,[3] Salvatore Ricci,[4] Paolo Flamini,[4] Giancarlo Sandri,[3] Maria Cristina Trotta,[5] Walter Elisei,[6] Antonio Penna,[7] Piera Giuseppina Lecca,[2] Marcello Picchio,[8] and Antonio Tursi[9]

[1]*Digestive Endoscopy & Nutrition Unit, "S. Eugenio" Hospital, Piazzale dell'Umanesimo 10, 00144 Rome, Italy*
[2]*Division of Internal Medicine and Gastroenterology, "Cristo Re" Hospital, Via delle Calasanziane 25, 00167 Rome, Italy*
[3]*Clinical Nutrition Unit, "S. Eugenio" Hospital, Piazzale dell'Umanesimo 10, 00144 Rome, Italy*
[4]*Clinical Nutrition Unit, "Umberto I" Hospital, Viale del Policliniuco 185, 00186 Rome, Italy*
[5]*Division of Internal Medicine, "S. Eugenio" Hospital, Piazzale dell'Umanesimo 10, 00144 Rome, Italy*
[6]*Division of Gastroenterology, ASL Roma H, Via Borgo Garibaldi 12, Albano Laziale, 00041 Rome, Italy*
[7]*Division of Gastroenterology, "S. Paolo" Hospital, Contrada Capo Scardicchio 82, 00123 Bari, Italy*
[8]*Division of General Surgery, "P. Colombo" Hospital, ASL Roma H, Via Orti Ginnetti 7, Velletri, 00049 Rome, Italy*
[9]*Gastroenterology Service, ASL BAT, Via Torino 49, 76123 Andria, Italy*

Correspondence should be addressed to Antonio Tursi; antotursi@tiscali.it

Received 17 July 2014; Accepted 14 November 2014

Academic Editor: Anis Larbi

The term "microbiota" means genetic inheritance associated with microbiota, which is about 100 times larger than the guest. The tolerance of the resident bacterial flora is an important key element of immune cell function. A key role in the interaction between the host and the microbiota is played by Paneth cell, which is able to synthesize and secrete proteins and antimicrobial peptides, such as α/β defensins, cathelicidin, 14 β-glycosidases, C-type lectins, and ribonuclease, in response to various stimuli. Recent studies found probiotics able to preserve intestinal homeostasis by downmodulating the immune response and inducing the development of T regulatory cells. Specific probiotic strain, as well as probiotic-driven metabolic products called "postbiotics," has been recently recognized and it is able to influence innate immunity. New therapeutic approaches based on probiotics are now available, and further treatments based on postbiotics will come in the future.

1. Introduction

The term "microbiota" means genetic inheritance associated with microbiota, which is about 100 times larger than the guest. The functions of the microbiota are many and until now it is considered a virtual organ of the human body [1].

Main functions of microbiota are amino acids and vitamins synthesis and energy extraction from nonabsorbable polysaccharides. It also contributes to the intestinal wall integrity, acting against pathogens and supporting immune system growth [1].

Assessing the human microbiota is one of main topics in biology and medicine regarding the relationships emerging between types of microbiota and development of important diseases. From an evolutionary point of view, we know that microbiota accompanied the evolution of the species for 400 million years, and the human race would probably never have existed without this close symbiosis. In fact the human microbiota gives the human race genes that human have progressively lost in their evolutionary cycle, creating a real superorganism made up of human and microbiotic characteristics. This superorganism has 10^{14} cells arising from microbiota and 10^{13} cells arising from the human race, a biological mass composed of 98% human material, but with 3^{105} genes arising from microbiota and 3^{10} genes from human genes [1]. Our microbiota begins immediately after birth,

remaining relatively constant until adult age and reducing in old age. The "core" of intestinal microbiota remains constant and unmodified almost like our fingerprints. It is mainly represented by bacteria but is also composed of virus, fungus, and protozoa [1].

In the last 10 years the interest in intestinal microbiota has grown rapidly following molecular biology techniques that have overcome the limits of the old culture techniques. Microbiota lives in close contact with our intestine. Both intestine and microbiota form an ecological system that has many cellular and molecular components that work to maintain an adequate fast and efficient immune response which respects the morphological and functional integrity of the bowel.

2. Intestinal Microbiota

The identification of the resident intestinal bacterial species is mainly based on the analysis of 16S ribosomal RNA subunit obtained from the amplification by polymerase chain reaction of nucleic acids extracted from the lumen of the intestinal mucosa and faeces [1]. Using molecular biology we are able to overcome the relatively long period of growth of microorganisms and to isolate approximately 60–80% of the commensal bacterial species. The distribution of the microbiota along the digestive tract is not uniform: it is very low in the stomach (0–10%) due to the bactericidal action of the chlorhydric acid (real barrier in the entrance of many bacterial species in digestive tract), increases gradually in the small intestine ($10^7 \times 10^8$), and reaches very high concentrations in the colon ($10^{11} \times 10^{12}$) where the microbiota is represented mainly by Gram-negative anaerobic bacteria. Until now, between 1000 and 1150 bacterial species have been identified; among them every adult hosts about 160. Concentrations and bacterial biodiversity (composition) in different intestinal tracts depend on several factors. Some of these are intrinsic to the host, such as acid secretion, intestinal motility, and immune response, while others are exogenous such as diet, taking antibiotics, PPI, laxatives, and opioids. The microbiota changes at different stages of life. At birth, the gut is sterile, and it is colonized by the vaginal and intestinal microbiota of the mother or by the skin for those born by caesarean delivery. Intestinal microbiota changes with breastfeeding and weaning and in adulthood it remains stable but changes again in the elderly [2].

It also plays an important role in metabolic activities in humans, considering that the microbiota biomass is comparable to that of the liver. Among the main functions are the synthesis of essential amino acids and vitamins (K, B_2, B_1, folic acid, biotin, and pantothenic acid) and extraction of energy from components in the diet as some are not digestible polysaccharides of plant origin. Moreover, it contributes 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. Although 50 bacterial *phyla* have been identified, just three are living in our colon: Firmicutes, Bacteroidetes, and Actinobacteria. Actions taken by microbiota in normal conditions are reported in Figure 1.

3. Mucosal Layer

Microbiota is separated from the epithelial cells by a network of glycans (glycocalyx and mucus layer) mainly produced by mucipare cells.

The intestinal epithelial barrier is composed of several layers of defense as follows (see Figure 2):

(a) the mucus that opposes static hindrance to the bacteria;

(b) the epithelial layer which is composed of absorptive enterocytes, goblet cells, Paneth cells, tuft and cup cells, M cells, and enterochromaffin cells;

(c) immune cells such as intraepithelial $\gamma\delta$ and $\alpha\beta$ lymphocytes, retinoic acid receptor-related orphan receptor (ROR)$\gamma\tau$+, lymphoid-tissue inductor (LTI), and NKP46T innate immune cells; others have direct access to the lumen-like antigen, presenting cells or neutrophils after infection.

Paneth cells are secretory cells specialized in the production of antimicrobial peptides (AMP). These include defensins, lisozymes, C-type lectins, and cathelicidins. ROR$\gamma\tau$+ cells release IL-22, which is required for both epithelial cell repair and antibacterial activity.

The mucus layer is fundamental to guarantee the integrity of the intestinal wall. It is rapidly improved through an average daily production of 5 liters. Its main constituent is the mucin, a glycoprotein mainly represented by subtype 2 (MUC2) [2]. The mucus of the stomach and the colon is particularly thick and is made from two layers: an inner (50/200 μm) compact and with small pores that prevent the physical penetration of bacteria [2] and one external (70–150 μm) composed primarily of glycans. Both these layers contribute to the defensive effect. For example, mice with MUC2 deficiency spontaneously develop an inflammatory process located in the intestinal mucosa [3].

Only some bacteria are able to be located in the niche of the layer of mucus. This is because the microbiota develops some systems of adaptation such as the production of enzymes that degrade the mucus. This is an important factor for the survival of the bacteria in this habitat, since this activity allows the production of nutrients for the bacteria themselves. Microbiota also contributes to the production of mucus and the thickness of the mucus layer. This occurs also through the stimulation of the synthesis of mucin by some bacterial components such as lipopolysaccharide (LPS) and short chain fatty acids (SCFA) [4].

4. Interaction between Epithelium and Microbiota and Occurrence of the Intestinal Inflammation

The intestinal mucosa is a major constituent of the human immune system. In fact, small intestine contains about 1×10^{10} plasma cells per meter, approximately 80% of all plasma cells contained in the whole body. In addition, the daily production of IgA in the intestinal lumen is higher than the total daily production of IgG [1].

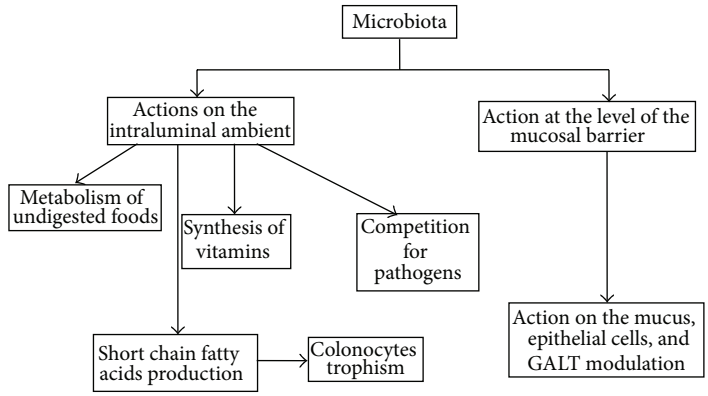


Figure 1: Action taken by microbiota in normal conditions.

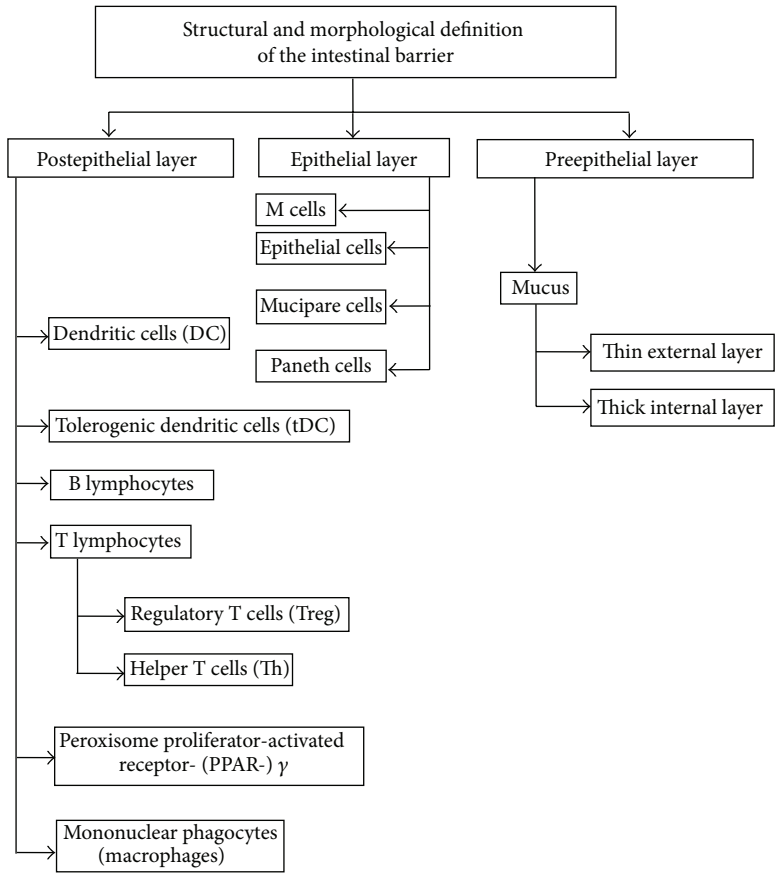


Figure 2: Structural and morphological definition of the intestinal barrier.

Considering that the intestinal lamina propria is infiltrated by lymphocytes, plasma cells, and macrophages, we can affirm that occurrence of inflammatory cells may represent a physiological phenomenon in the human intestine of human. This infiltration, called "physiological inflammation" of the normal intestinal mucosa, occurs and develops after the massive stimulation of the mucosal immune system by luminal antigens. In fact intestinal mucosa is constantly exposed to an enormous antigen load present in the lumen, by which the resident bacterial flora contributes predominant. This means that a range of structures and cells are responsible for the control and the maintenance of an adequate immune response in the normal intestinal mucosa and that a harmful immune response against exposure to luminal antigens is not happening. Due to the massive stimulation by luminal antigens, the immune cells that physiologically infiltrate the intestinal mucosa, which are mainly memory T cells, are both increased and activated and develop a local immune response [1]. The tolerance of the resident bacterial flora is therefore an important key element of immune cell function. In fact the

excessive degradation of the mucin layer by bacteria facilitates the access of luminal antigens and then the activation of the immune response. For example, it has been shown that alterations in the mucus layer contribute to the pathogenesis of inflammatory bowel disease (IBD) [5]. If we compare the healthy people with IBD patients, we can observe a larger amount of bacteria in contact with the epithelial cells in IBD patients than in healthy controls. The reason why this occurs has been clarified. Although leukocyte reaction tries to contain the bacteria living in the bowel lumen, thanks to its antimicrobial activity exerting in the external layer, some bacteria reach the mucosa owing to impairment of mucosal layer causing inflammation [6]. This has been confirmed by Ganesh et al. These authors have demonstrated that, in an animal model of mice with microbiota consisting essentially of eight bacterial species, the presence of a commensal bacterium (*Akkermansia muciniphila*) is able to degrade the mucin and exacerbate the inflammation and the severity of symptoms due to *Salmonella enterica* Typhimurium infection [7].

5. Interaction between Innate Immunity, Paneth Cells, and Toll-Like Receptors

A key role in the interaction between the host and the microbiota is played by Paneth cell. These are specialized typical epithelial cells of the small intestine but may be detected in lower concentrations in the stomach and colon. The Paneth cells are able to synthesize and secrete proteins and antimicrobial peptides, such as α/β defensins, cathelicidin, 14 β-glycosidases, C-type lectins, and ribonuclease, in response to various stimuli, including components of the bacterial surface and toll-like receptors (TLR) agonists [8, 9]. Defensin 5 (HD-5) is also produced by the Paneth cells, playing a role both in protection against pathogens and in determining the composition of the microbiota [10]. In particular, this second activity works by controlling the number of microorganisms, in this way contributing to host defense. In fact the transgenic mice for the HD-5 indeed show a greater resistance to oral *Salmonella* Typhimurium [11].

The epithelial cells have an important role in the control of complex interactions between the host and the microbiota, because those cells express receptors that are able to recognize selectively specific microbial patterns. The most characterized receptors are the TLR that are activated by the nuclear factor-κB (NF-κB) system and by production of cytokines, chemokines, and effectors of innate immunity [12, 13]. TLRs are able to recognize the characteristic structures of bacteria and viruses. For example, TLR-4 is essential in recognizing lipopolysaccharides (LPS) [12, 13], TLR-5 is essential in recognizing bacterial flagellin [12, 13], and TLR-9 is important in recognizing CpG islands of DNA [12, 13].

It has been demonstrated that all TLRs are expressed in the human colon and small intestine, but their functions are not well known yet. The TLRs are able to trigger an immune response against bacteria but also play an important role in protecting intestine from generical damage. For example, experimental studies in hosts knockout for TLR9, TLR4, and TLR2 have shown increased susceptibility to the development of a colitis induced by dextran sodium sulfate (DSS) [12, 13], as well as the protective role against DSS colitis induced by agonists of TLR2 and TLR4 supplement [12, 13].

It would seem that some strains of the microbiota also have a modulatory effect on the immune system of the GALT (gut associated lymphoid tissue), increasing the functionality of innate immunity, activating the dendritic cells, and stimulating NK cells by direct cytochemical action by pathogens penetrating into mucosa [12]. The intestinal microbiota can also regulate the activity of regulatory T cells (T-REG) that produce immunomodulatory interleukin and have an anti-inflammatory action and can activate T cells. Finally, helper T cells release IL-17 and IL-22, which have protective effects against enteropathogens genes [14]. There is great evidence that some patients suffering from irritable bowel syndrome (IBS) have an activation of the mucosal immune system [14]. In fact, some recent data show a significant increase of gene expression of TLR-4 on the colonic mucosa of the IBS patients compared to healthy subjects, although it is less than that observed in the colonic mucosa of patients with ulcerative colitis [15]. Recently, Martínez et al. [16] showed alterations in the integrity of the jejunal mucosal barrier in patients with IBS with diarrhea (IBS-D) and in particular at the apex junctional complex with an increased expression of Caudina 2, with a reduced phosphorylation of occludin and increased expression of myosin kinase. These changes are correlated with the activation of mast cells and abdominal pain reported by patients. However, the microbiota present in the intestinal lumen and in stools is different than the microbiota on the superficial layer of mucus covering the epithelium [17]. The most represented bacterial strains detected in the superficial layer of mucus are *Lactobacilli*, which are widely used as probiotics, and the *Clostridia* [17]. Some recent studies have shown that supernatants from cultures of some of the strains living in the intestinal mucus have an anti-inflammatory activity [17, 18]. These soluble substances produced by microorganisms are called postbiotics [18]. The first postbiotic described, obtained from the culture of *Faecalibacterium prausnitzii*, shows an anti-inflammatory effect in experimental model of colitis [19]. Similar effects were observed in supernatants obtained from cultures of *Lactobacillus paracasei*, which opposes the inflammation of the human intestinal mucosa induced by *Salmonella* Typhimurium [20]. Finally, also the supernatant obtained from a culture of *Lactobacillus rhamnosus* opposes the muscular alterations of the human colon induced by LPS of a pathogen strain of *Escherichia coli* [20]. This effect is mediated by activation of TLR-2 on the myocytes membrane and plays a protective effect in cardiac fiber cells [21].

In conclusion, the mesenchymal cells (fibroblasts and myocytes) that are located, respectively, in the lamina propria and *muscularis mucosae* of the intestinal epithelium recognize bacterial antigens circulating using the Toll-like receptors expressed in their cell membranes. Probably the activation of some of them by the microbiota and/or its secretory products can activate an anti-inflammatory response in the same intestinal mesenchymal cells, which are able to oppose a preinflammatory insult resulting from an alteration of intestinal permeability.

6. Interaction between Innate Immunity, Microbiota, and Probiotics

Probiotics are defined as bacteria having beneficial effects on the host. As most of them are driven from the gut microbiota, understanding how probiotics interact with the host can clarify how the microbiota interacts with the host. The mechanisms of action of probiotics have recently been explored [22]. As the microbiota, probiotics can be classified as inflammatory or anti-inflammatory according to their capacity to stimulate immune and nonimmune cells [23]. Probiotics may help preserve intestinal homeostasis by modulating the immune response and inducing the development of T-REG cells [23–25]. Similar to the microbiota, probiotics can be classified as inflammatory or anti-inflammatory depending on their capacity to stimulate immune and nonimmune cells [23].

One mechanism of probiotic action has been proposed based on the hypothesis that Crohn's disease (CD) susceptibility is linked to a defective initial innate immune response [26]. It has been demonstrated that the probiotics mixture *VSL#3* can induce NF-κB nuclear translocation in epithelial cells followed by release of TNF-α and that this correlates with reduced epithelial permeability and susceptibility to CD-like ileitis in the SAMP1/YitFc mice that spontaneously develop the disease [27].

Although unexpected, this observation is very interesting. It has been recently shown that TNF-α can stimulate epithelial cell proliferation, and this occurs only when, in combination with IFN-γ, TNF-α induces epithelial cell apoptosis [28]. Hence, it is possible that, by upregulating TNF-α, probiotics may participate in epithelial barrier regeneration. The interaction of inflammatory bacteria with epithelial cells may be beneficial to host by bacterial ability to simulate innate immunity that protects against chronic inflammation. However, the same bacteria cannot improve overt disease in mice [27], and it may be deleterious as shown in other systems by using inflammatory probiotics [23]. Schlee et al. found that the probiotic *E. coli* strain *E. coli* Nissle 1917 induces beta defensin 2 upregulation in Caco-2 cells only when flagellin activity is restored [29]. A similar result was obtained in healthy volunteers using a cocktail of two probiotic *E. coli* strains [30]. A 78% upregulation was evident after three weeks of treatment, while defensin levels in fecal samples were still significantly elevated 9 weeks after the end of the treatment, indicating a more long-lasting effect.

Lactobacillus plantarum v299 is able to induce an increase in *Muc3* expression in the jejunum and ileum of rats. However, this effect was only evident when live, but not heat-killed, bacteria were administered [31]. This suggests that metabolic activity of the bacteria is necessary for this action. The same strain upregulates *Muc3* expression and secretion on *HT29* cells, while at the same time limits adherence of *E. coli* E2348/49 strain [32].

Probiotic-driven metabolic products (called postbiotics) have been shown to enhance barrier function in a number of cases [33]. Culture supernatant of *S. boulardii*, but not *S. cerevisiae*, was able to improve significantly the epithelial cells ability in obtaining wound healing and migration *in vitro* and *in vivo* by $\alpha 2\beta 1$ integrin collagen receptors activation [34].

Probiotic derived polyphosphate is able to protect mice against DSS-induced colitis, acting on the integrin-p38 MAPK pathway and suppressing oxidant-induced intestinal permeability by preventing F-actin and E-cadherin degradation [35]. In another study, the authors identified the p40 molecule, produced and secreted by *Lactobacillus GG*, as the main mediator for ameliorating DSS and oxazolone-induced inflammation, through its binding to epidermal growth factor receptor (EGFR). EGFR activation by p40 was sufficient to reduce cytokine-induced intestinal epithelial cells (IEC) apoptosis *in vitro* and *in vivo*. Further, the authors succeeded in administering the p40 molecule specifically in the colon, where once again they observed a subsequent activation of EGFR. Providing p40 was therefore able to both prevent and cure DSS-induced colitis [36]. This effect has never been observed before for live probiotic strains, as most might exert strain-specific preventive actions but are of little help once inflammation is manifested [27–37].

Mileti et al. have observed that *L. paracasei* strain B21060 has preventive effect in the DSS colitis model, whereas the culture supernatant exerts a prominent anti-inflammatory effect on the explants from the same patients [23]. Although the active component has not yet been identified, the protective action of the supernatant might in part be linked to epithelial barrier strengthening. In fact Mileti et al. have shown that when preconditioned with supernatant, healthy colonic mucosa explants are significantly more resistant to *Salmonella* infection [23].

These studies show clearly that shielding the intestinal barrier and preventing IEC apoptosis can be an important regulatory mechanism, which can influence the course of etiopathogenetic events both at the onset and during disease.

7. Conclusions

Understanding the relationship between intestinal microbiota and intestinal epithelium has increased our knowledge on pathophysiological conditions in the gastrointestinal and extraintestinal diseases.

Intestinal microbiota shows therefore a significant adaptation to different environmental stimuli. Microbiota is mandatory for several activities, ranging from the growth of the immune system to the synthesis of some amino acids and vitamins. It is hypothesized that the vast majority of those activities is mediated by diet and that changing in microbiota composition may permit the adaptation to host's metabolic and immunologic activities according to environmental changes. When this partnership is impaired, we have a significant risk to develop a disease. It seems to be particularly true not only for the occurrence of IBS, which affects large percentages of western population, but also for the occurrence of severe, disabling diseases, such as IBD.

Quali-quantitative changing in bacterial strains suggests that intestinal microbiota may be a therapeutic target based on "good" bacteria, called "probiotics," and on probiotic-driven metabolic products, called "postbiotics." New therapeutic approaches based on probiotics are now available,

and further treatments based on postbiotics will come in the future.

Conflict of Interests

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

References

[1] S. R. Gill, M. Pop, R. T. DeBoy et al., "Metagenomic analysis of the human distal gut microbiome," *Science*, vol. 312, no. 5778, pp. 1355–1359, 2006.

[2] S. Cucchiara, V. Iebba, M. P. Conte, and S. Schippa, "The microbiota in inflammatory bowel disease in different age groups," *Digestive Diseases*, vol. 27, no. 3, pp. 252–258, 2009.

[3] J. P. Ouwerkerk, W. M. de Vos, and C. Belzer, "Glycobiome: bacteria and mucus at the epithelial interface," *Best Practice and Research: Clinical Gastroenterology*, vol. 27, no. 1, pp. 25–38, 2013.

[4] M. E. V. Johansson, M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G. C. Hansson, "The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 39, pp. 15064–15069, 2008.

[5] A. Baghaei, H. Esmaily, A. H. Abdolghaffari, M. Baeeri, F. Gharibdoost, and M. Abdollahi, "Efficacy of Setarud (IMOD), a novel drug with potent anti-toxic stress potential in rat inflammatory bowel disease and comparison with dexamethasone and infliximab," *Indian Journal of Biochemistry and Biophysics*, vol. 47, no. 4, pp. 219–226, 2010.

[6] A. Swidsinski, V. Loening-Baucke, and A. Herber, "Mucosal flora in Crohn's disease and ulcerative colitis—an overview," *Journal of Physiology and Pharmacology*, vol. 60, no. 6, pp. 61–71, 2009.

[7] B. P. Ganesh, R. Klopfleisch, G. Loh, and M. Blaut, "Commensal *Akkermansia muciniphila* exacerbates gut inflammation in *Salmonella* Typhimurium-infected gnotobiotic mice," *PLoS ONE*, vol. 8, no. 9, Article ID e74963, 2013.

[8] C. Rumio, D. Besusso, M. Palazzo et al., "Degranulation of Paneth cells via Toll-like receptor 9," *The American Journal of Pathology*, vol. 165, no. 2, pp. 373–381, 2004.

[9] C. Rumio, M. Sommariva, L. Sfondrini et al., "Induction of Paneth cell degranulation by orally administered Toll-like receptor ligands," *Journal of Cellular Physiology*, vol. 227, no. 3, pp. 1107–1113, 2012.

[10] N. H. Salzman, K. Hung, D. Haribhai et al., "Enteric defensins are essential regulators of intestinal microbial ecology," *Nature Immunology*, vol. 11, no. 1, pp. 76–83, 2010.

[11] N. H. Salzman, D. Ghosh, K. M. Huttner, Y. Paterson, and C. L. Bevins, "Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin," *Nature*, vol. 422, no. 6931, pp. 522–526, 2003.

[12] J. Lee, J.-H. Mo, K. Katakura et al., "Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells," *Nature Cell Biology*, vol. 8, no. 12, pp. 1327–1336, 2006.

[13] S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, 2004.

[14] I. I. Ivanov, K. Atarashi, N. Manel et al., "Induction of intestinal Th17 cells by segmented filamentous bacteria," *Cell*, vol. 139, no. 3, pp. 485–498, 2009.

[15] C. Cremon, L. Zecchi, M. R. Barbato et al., "843 Mucosal immune biomarkers in patients with irritable bowel syndrome," *Gastroenterology*, vol. 144, no. 5, supplement 1, p. S-150, 2013.

[16] C. Martínez, B. Lobo, M. Pigrau et al., "Diarrhoea-predominant irritable bowel syndrome: an organic disorder with structural abnormalities in the jejunal epithelial barrier," *Gut*, vol. 62, no. 8, pp. 1160–1168, 2013.

[17] I. Sekirov, S. L. Russell, L. Caetano M Antunes, and B. B. Finlay, "Gut microbiota in health and disease," *Physiological Reviews*, vol. 90, no. 3, pp. 859–904, 2010.

[18] K. Tsilingiri, T. Barbosa, G. Penna et al., "Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model," *Gut*, vol. 61, no. 7, pp. 1007–1015, 2012.

[19] H. Sokol, B. Pigneur, L. Watterlot et al., "*Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 43, pp. 16731–16736, 2008.

[20] F. Ammoscato, A. Scirocco, A. Altomare et al., "*Lactobacillus rhamnosus* protects human colonic muscle from pathogen lipopolysaccharide-induced damage," *Neurogastroenterology and Motility*, vol. 25, no. 12, pp. e984–e777, 2013.

[21] T. Ha, Y. Hu, L. Liu et al., "TLR2 ligands induce cardioprotection against ischaemia/reperfusion injury through a PI3K/Akt-dependent mechanism," *Cardiovascular Research*, vol. 87, no. 4, pp. 694–703, 2010.

[22] M. G. Gareau, P. M. Sherman, and W. A. Walker, "Probiotics and the gut microbiota in intestinal health and disease," *Nature Reviews: Gastroenterology & hepatology*, vol. 7, no. 9, pp. 503–514, 2010.

[23] E. Mileti, G. Matteoli, I. D. Iliev, and M. Rescigno, "Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for in vivo efficacy," *PLoS ONE*, vol. 4, no. 9, Article ID e7056, 2009.

[24] L.-L. Chen, X.-H. Wang, Y. Cui et al., "Therapeutic effects of four strains of probiotics on experimental colitis in mice," *World Journal of Gastroenterology*, vol. 15, no. 3, pp. 321–327, 2009.

[25] C. Di Giacinto, M. Marinaro, M. Sanchez, W. Strober, and M. Boirivant, "Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-β-bearing regulatory cells," *The Journal of Immunology*, vol. 174, no. 6, pp. 3237–3246, 2005.

[26] G. M. Cobrin and M. T. Abreu, "Defects in mucosal immunity leading to Crohn's disease," *Immunological Reviews*, vol. 206, pp. 277–295, 2005.

[27] C. Pagnini, R. Saeed, G. Bamias, K. O. Arseneau, T. T. Pizarro, and F. Cominelli, "Probiotics promote gut health through stimulation of epithelial innate immunity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 1, pp. 454–459, 2010.

[28] P. Nava, S. Koch, M. G. Laukoetter et al., "Interferon-gamma regulates intestinal epithelial homeostasis through converging beta-catenin signaling pathways," *Immunity*, vol. 32, no. 3, pp. 392–402, 2010.

[29] M. Schlee, J. Wehkamp, A. Altenhoefer, T. A. Oelschlaeger, E. F. Stange, and K. Fellermann, "Induction of human β-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin," *Infection and Immunity*, vol. 75, no. 5, pp. 2399–2407, 2007.

[30] M. Möndel, B. O. Schroeder, K. Zimmermann et al., "Probiotic *E. coli* treatment mediates antimicrobial human β-defensin synthesis and fecal excretion in humans," *Mucosal Immunology*, vol. 2, no. 2, pp. 166–172, 2009.

[31] N. S. Dykstra, L. Hyde, D. Adawi et al., "Pulse probiotic administration induces repeated small intestinal Muc3 expression in rats," *Pediatric Research*, vol. 69, no. 3, pp. 206–211, 2011.

[32] D. R. Mack, S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth, "Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 276, no. 4, pp. G941–G950, 1999.

[33] K. Tsilingiri and M. Rescigno, "Postbiotics: what else?" *Beneficial Microbes*, vol. 4, no. 1, pp. 101–107, 2013.

[34] A. Canonici, C. Siret, E. Pellegrino et al., "Saccharomyces boulardii improves intestinal cell restitution through activation of the $\alpha 2\beta 1$ integrin collagen receptor," *PLoS ONE*, vol. 6, no. 3, Article ID e18427, 2011.

[35] S. Segawa, M. Fujiya, H. Konishi et al., "Probiotic-derived polyphosphate enhances the epithelial barrier function and maintains intestinal homeostasis through integrin-p38 MAPK pathway," *PLoS ONE*, vol. 6, no. 8, Article ID e23278, 2011.

[36] F. Yan, H. Cao, T. L. Cover et al., "Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an EGFR-dependent mechanism," *The Journal of Clinical Investigation*, vol. 121, no. 6, pp. 2242–2253, 2011.

[37] D. Corridoni, L. Pastorelli, B. Mattioli et al., "Probiotic bacteria regulate intestinal epithelial permeability in experimental ileitis by a TNF-dependent mechanism," *PLoS ONE*, vol. 7, no. 7, Article ID e42067, 2012.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 123653, 10 pages
http://dx.doi.org/10.1155/2015/123653

Review Article

The Interaction among Microbiota, Immunity, and Genetic and Dietary Factors Is the *Condicio Sine Qua Non* Celiac Disease Can Develop

D. Pagliari,[1] R. Urgesi,[2] S. Frosali,[1] M. E. Riccioni,[3] E. E. Newton,[4] R. Landolfi,[1] F. Pandolfi,[1] and R. Cianci[1]

[1] *Institute of Internal Medicine, Catholic University, 00168 Rome, Italy*
[2] *Gastroenterology and Digestive Endoscopy Unit, Belcolle Hospital, 01100 Viterbo, Italy*
[3] *Digestive Endoscopy Unit, Catholic University, 00168 Rome, Italy*
[4] *CytoCure LLC, Beverly, MA 01915, USA*

Correspondence should be addressed to F. Pandolfi; pandolfi@rm.unicatt.it

Received 29 April 2014; Accepted 14 October 2014

Academic Editor: Ciriaco A. Piccirillo

Celiac disease (CD) is an immune-mediated enteropathy, triggered by dietary wheat gluten and similar proteins of barley and rye in genetically susceptible individuals. This is a complex disorder involving both environmental and immune-genetic factors. The major genetic risk factor for CD is determined by HLA-DQ genes. Dysfunction of the innate and adaptive immune systems can conceivably cause impairment of mucosal barrier function and development of localized or systemic inflammatory and autoimmune processes. Exposure to gluten is the main environmental trigger responsible for the signs and symptoms of the disease, but exposure to gluten does not fully explain the manifestation of CD. Thus, both genetic determination and environmental exposure to gluten are necessary for the full manifestation of CD; neither of them is sufficient alone. Epidemiological and clinical data suggest that other environmental factors, including infections, alterations in the intestinal microbiota composition, and early feeding practices, might also play a role in disease development. Thus, this interaction is the *condicio sine qua non* celiac disease can develop. The breakdown of the interaction among microbiota, innate immunity, and genetic and dietary factors leads to disruption of homeostasis and inflammation; and tissue damage occurs. Focusing attention on this interaction and its breakdown may allow a better understanding of the CD pathogenesis and lead to novel translational avenues for preventing and treating this widespread disease.

1. Introduction

Celiac disease (CD) is an immune-based enteropathy triggered by dietary wheat gluten and similar proteins in barley and rye in genetically susceptible individuals. Recently, the ESPGHN (European Society of Paediatric Gastroenterology, Hepatology and Nutrition) proposed that CD may be defined as an immune-mediated systemic disorder elicited by gluten and related prolamins in genetically susceptible individuals and characterized by a variable combination of gluten-dependent manifestations, CD-specific antibodies, and HLA-DQ2 or HLA-DQ8 haplotypes, causing duodenal chronic inflammation [1]. CD is a T-cell mediated disease, in which gliadin-derived peptides activate lamina propria infiltrating T lymphocytes [2]. These latter represent the pivotal cells that orchestrate tissue damage. This leads to the release of proinflammatory cytokines, such as IFN-γ and IL-15 [3] which are responsible for the activation of the cytotoxic activity of intraepithelial lymphocytes (IELs) that leads to a profound tissue remodeling [4]. This is a complex disorder, with environmental and immune-genetic factors contributing to its etiology. The main genetic influence on CD is the HLA locus [5], specifically MHC class II genes that encode HLA-DQ2 (HLA-DQ2.5 and HLA-DQ2.2) and HLA-DQ8 heterodimers. The prevalence of disease is usually reported to be about 1% in the general US population [6] but there

are emerging data suggesting an increase in some countries. Although there is a strong genetic predisposition, resulting from the presence of the HLA gene DQ2/DQ8 in the development of CD, and gluten is the main environmental factor responsible for the signs and symptoms of this disorder, neither genetic nor environmental factors show 100% correlation. Thus other immune, genetic, and environmental factors must be involved in CD onset [7].

2. Pathogenesis and Pathogenic Model of Celiac Disease

CD is considered an autoimmune disorder with both genetic and environmental components. Evidence for a genetic component is best exemplified by the strong dependence on the presence of the HLA-DQ2 (encoded by the alleles DQA1*05 and DQB1*02) and HLA-DQ8 (DQA1*03 and DQB1-*0302) haplotypes. More than 95% of those with CD express HLADQ2 while the rest express HLA-DQ8. However, about 30–40% of the general population expresses HLA-DQ2, so while these HLA genes are necessary, they are not sufficient for developing disease and clearly non-HLA genes are also involved. At least 39 non-HLA genes have been identified through genome-wide association studies as strongly associated with CD [8].

Most of these genes are involved in control of the innate and adaptive immune response.

However, the role of these genes in the development of CD is not completely clear. The primary environmental trigger is gluten, the protein fractions of wheat, barley, and rye. The incomplete digestion of this protein by humans, due to high concentrations of glutamine and proline, results in the formation of residual partially digested peptides. These peptides are responsible for innate and adaptive immune responses underlying the disease in genetically predisposed subjects. Other trigger factors may play a role in precipitating disease. Currently, some studies are evaluating the role of the modifications of gut microbiota as a factor contributing to the onset of disease [9].

3. Immunopathogenesis

Although the precise immune mechanisms that are involved in the progressive destruction of the small intestinal mucosa remain to be elucidated, the hallmark of CD is an immune-mediated enteropathy that involves both of the innate and adaptive immune system. In relation to innate immune system response, we should consider that some gluten peptides can induce tissue damage by directly activating components of innate immunity [10]. The peptide (α-gliadin 31-43) p31-43/49 has been shown to activate the production of IL-15 and the NK receptor-mediated cytotoxicity by IELs [11, 12], independent of TCR specificity, and to induce apoptosis of enterocytes, upregulate MHC class I molecules, activate MAP kinase pathway, and upregulate the expression of CD83, a maturation marker of dendritic cells [13]. The presence of a receptor for p31-43/49 in intestinal epithelial cells has not been found yet and, thus, the molecular mechanism underlying the biological effects observed as a consequence of the interaction of this peptide with the gut mucosa remains still unclear [14]. The DQ2 and DQ8 molecules confer susceptibility to CD by presenting disease-related peptides to T-cells in the small intestine or by shaping the T-cell repertoire during T-cell development in the thymus. Initially, paracellular passage of gliadin peptides is due to an increase of gut permeability which, in turn, is due to an upregulation of zonulin, an intestinal peptide involved in epithelial tight junction control [15]. Also, IL-15 contributes to promoting the CD4+ T-cell adaptive immune response [3, 16]. IL-15 up-regulates both CD94/NKG2C and NKG2D NK receptors boosting their ability to lyse enterocytes [14]. The adaptive immune response in CD is characterized predominantly by production of the proinflammatory cytokine interferon-γ (IFN-γ) from gluten-specific CD4+ T-helper cells triggered by gluten-derived peptides recognized by HLA-DQ2 or HLA-DQ8 heterodimers of antigen-presenting cells (APCs) [17]. Tissue transglutaminase-2 (TTG2) converts noncharged glutamine into negatively charged glutamic acid through a process called deamidation. In fact, the peptide-binding motifs of DQ2 and DQ8 predict a preference for negative charges at anchor positions of the bound peptides. DQ2 has a preference for negatively charged residues at the P4, P6, and P7 anchor positions [18], whereas DQ8 has a preference for negatively charged residues at anchor positions P1, P4, and P9 [19]. Generally, gluten proteins contain few negatively charged residues but in active CD the level of expression of enzyme TG2 is increased and the ratio of deamidation to transamidation is markedly increased. In active CD TG2 is expressed at the epithelial brush border, as well as being expressed extracellularly in the subepithelial region. The pH in the proximal small intestine is ~6.6 which should allow marked TG2-mediated deamidation of peptides in the brush border [20]. This process increases the negative charge on the peptide molecule and enhances binding of the peptide within the peptide binding groove of the HLA-DQ2 molecule on the surface of the APCs. This is a prerequisite for a gluten-specific T-cell response as well as a B-cell response that results in production of the anti-TTG antibodies that represents an epiphenomenon of the CD pathogenesis and may be used in the diagnostic pathway [15, 21]. Furthermore, deamidated gluten peptides are presented to CD4+ T-cells that release proinflammatory cytokines activating cytotoxic CD8 IELs (CD8+ TCR$\alpha\beta$+ and TCR$\gamma\delta$+ T-cells) contributing to a profound tissue remodeling and damage [13, 22]. While IgA antibodies against either gluten or the autoantigen TG2 serve as a highly useful means of testing for CD, their precise role in the immunopathogenesis of the condition remains yet unknown. Whether they are byproducts of the intestinal adaptive immune response or they play a direct role in CD pathogenesis remains unclear. There is evidence that IL-21 plays a role in the pathogenesis of CD as high levels of this cytokine can be demonstrated in biopsies from those with active disease that are not on treatment [23]. However, the mechanism whereby IL-21 is produced and the precise role it plays in the disease process remains unexplained. More recently, it has been proposed that, in the intestine of CD subjects, normal gluten peptides may be complexed to intraluminal secretory

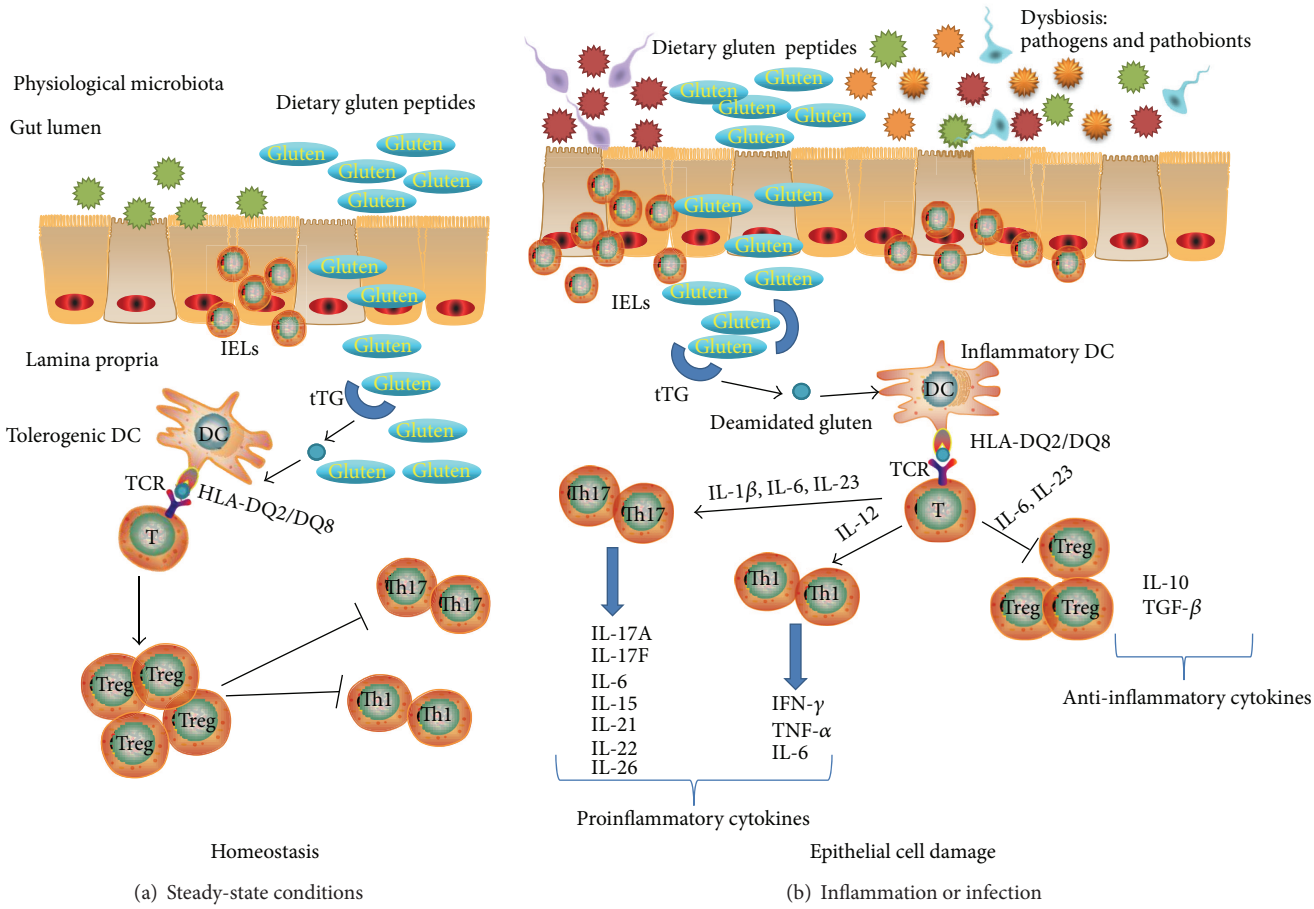


FIGURE 1: The complex interconnection among immune system, microbiota, and environmental factors (including dietary food antigens and/or infection) in the pathogenesis of celiac disease. (a) Steady-state condition. Dietary antigens and physiologic microbiota are in symbiotic relationship with host mucosal cells; thereby a harmonized balance between pro- and anti-inflammatory factors is achieved (homeostasis). (b) Inflammation or infection. The breakdown of the normal microbial community contributes to dysbiosis. In CD patients, gluten derived peptides are recognized by antigen presenting cells, with T-cells response. Deaminated gluten peptides are presented to T-cells with subsequent release of proinflammatory cytokines. In this way, Tregs are suppressed. This fact leads to a break of balance with consequent epithelial cell damage.

IgA, bound to an IgA receptor and transported, and protected from lysosomal degradation by a specific transcytosis pathway involving the transferrin receptor CD71. Compared to nonceliac subjects or CD subjects on a gluten-free diet, CD71 expression is increased in CD sufferers and colocalizes with IgA at the apical enterocyte membrane [24].

In summary, the disease develops as a result of an abnormal CD4+ T-cell-initiated immune response to gluten. Generally, gluten gains access to Peyer's patches physiologically via M cells or supraphysiologically during periods of increased epithelial permeability [25]; thus, it is processed by Peyer's patches dendritic cells [26] and presented to CD4+ T-cell [27]. In nonceliac subjects the presentation of gluten peptides on HLA-DQ2/DQ8 induces a Th2 response [28]; in celiac subjects in the presence of TTG2 activity and deamidation of gluten peptides in the APCs the response is biased towards Th1 response [29]. Presentation of gluten to T-cells could be carried out not only by dendritic cells but also by macrophages, B-cells, and even enterocytes that express HLA class II [30]. Enterocytes can present antigens to lipoprotein lipase (LPL) via evaginations through the basement membrane and can express costimulatory molecules under inflammatory conditions [31]. The primed CD4+ T-cells would then recirculate to the lamina propria [32, 33], and subsequent contact with gluten would induce their activation and proliferation, with production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-15, IL-17A, IL-17F, IL-21, IL-22, IL-23, and IL-26 (Figure 1) [34, 35]. This would result in synthesis and release of metalloproteases MMP-1, MMP-3 [36], and Keratinocyte Growth Factor (KGF) by stromal cells [37], which would induce cryptal hyperplasia [38]. The next stage, villous atrophy, would be due to enterocyte death induced by IELs [39, 40]. In fact CD8+ IELs from CD patients have been shown to respond to gluten peptides presented by HLA-A2 [41]. Additionally, there is an overexpression of membrane bound IL-15 on enterocytes in active CD which induces the expression of the NK receptors CD94 [13] and NKG2D by CD3+ IELs. The ligand for NKG2D and

MIC-A is overexpressed on enterocytes in active CD, and this supports the involvement of the MIC-A/NKG2D pathway in the epithelial atrophy of CD [12].

4. Association between Microbiota and Dietary Factors in Development of Innate and Adaptive Immunity

Gut microbiota is the collection of microbial populations that reside in the gastrointestinal tract. It is characterized by an interplay between different cells and their defense systems, food particles, molecules derived from digestion, and the vast array of residing microbial species with their secretory products. These microorganisms present in the gut lumen form the microbiota that performs several physiological functions, that is, the absorption and digestion processes, tolerance to non-self-food antigens, and defense from pathogens.

The composition of main bacterial populations does not stabilize until after the first few years of life. In this period, the microbiota gradually colonizes the mucosal and skin surfaces of the neonate and exerts the greatest effect on the development of the immune system [42]. Components of the intestinal microbiota play a crucial role in the postnatal development of the immune system. During the early postnatal period, the intestinal microbiota stimulates the development of both local and systemic immunity, while later on these components evoke inhibitory regulatory mechanisms intended to keep both mucosal and systemic immunity in check [43]. In this postnatal period, components of the normal microbiota induce a transient physiological inflammatory response in the gut associated with enlargement of the mucosal-associated lymphatic tissue and increases in its cellularity [43, 44]. Many studies have shown that microbial colonization of animals living in germ-free conditions results in an increase in immunoglobulin levels, the production of specific antibodies, and substantial changes in mucosal-associated lymphocyte tissues and cell populations [45, 46]. Interestingly, the microbial colonization of germ-free mice also speeds up the biochemical maturation of enterocytes, resulting in a shift in the specific activities of brush-border enzymes nearly to the extent found in conventional mice. Moreover, a similar introduction of microorganisms alters the synthesis of sugar chains in membrane-associated glycoproteins, which could influence the gut barrier function [47–49]. In some cases, impaired function of the intestinal barrier leads to an increase in antibodies directed against antigens present in the intestinal lumen. It was recently shown that the appearance of these antibodies or/and autoantibodies in individuals lacking clinical symptoms may have important predictive value for the development of inflammatory and autoimmune diseases [50, 51]. In the case of autoimmune diseases, considerable effort has been made to understand mechanisms leading to the loss of self-tolerance.

A principal function of the microbiota is to protect the intestine against colonization by exogenous pathogens and potentially harmful indigenous microorganisms. However, in certain conditions, some species of bacteria are thought to be capable of causing disease by producing inflammation, infection, or increasing cancer risk for the host [52]. Infectious agents are considered possible environmental factors triggering autoimmune diseases. Particular bacterial populations that are typically found in very low abundance can acquire pathogenic properties. These conditions include inherent immune defects as well as changes in diet and/or acute inflammation and can result in the disruption of the normal balanced state of the gut microbiota, which is referred to as dysbiosis [53]. Dysbiosis involves the abnormal accumulation or increased virulence of certain commensal populations of bacteria, thereby transforming former symbionts into "pathobionts." Pathobionts are typically colitogenic and can trigger intestinal inflammation [52]. The breakdown of the normal microbial community contributes to increase in the risk of pathogen infection and the overgrowth of harmful pathobionts and inflammatory disease.

The intestinal dysbiosis of CD patients is characterized by increases in numbers or proportions of *Bacteroides* spp. and reductions in those of *Bifidobacterium* spp. and *B. longum*, which were not completely normalized after patient adherence to a gluten-free diet (GFD) [54–56]. *E. coli* and *Staphylococcus* numbers were also higher in feces and biopsies of untreated CD patients than in those of controls, but the differences were normalized after gluten withdrawal [55]. The analyses of the prevalence of bacterial species associated with duodenal biopsies also revealed a reduction in *Bacteroides* species diversity in patients, untreated and treated with the GFD, in comparison with controls [57].

In addition to dysbiosis, infections may also disrupt the intestinal homeostasis leading to chronic inflammation and tissue damage, which could eventually contribute to reduced gluten tolerance [58]. In fact, infections have often been considered to initiate the process in genetically predisposed individuals. Infections can influence the host's immune tolerance by different mechanisms. These include polyclonal lymphocyte activation, increased immunogenicity of organ autoantigens secondary to infection-mediated inflammation, or antigen mimicry molecular mechanisms [59]. One major hypothesis explaining how infectious components can cause autoimmune reactions is based on the concept of cross-reactivity, also known as "molecular mimicry," that is the similarity between the epitopes of autoantigens and epitopes of harmless environmental antigens [60]. The adjuvant activity of microbial components may participate in the stimulation of APCs, such as dendritic cells, that leads to the abnormal processing and presentation of self-antigens. Homeostasis of the intestinal mucosa may be disturbed by pathogenic microorganisms and toxins attacking the intestine or by inadequately functioning components of the immune system, as observed in immunodeficiency or in cases of dysregulated mechanisms of the mucosal immune system. The intestinal mucosa can be affected as a consequence of either insufficient activity or exaggerated activation of the immune system [61]. Various complex diseases may occur as a consequence of disturbances of mucosal barrier function or of changes in mechanisms regulating mucosal immunity to food or component of the microbiota [62, 63]. The complexity and interindividual variation of the gut microbiota composition

in humans represent a confounding factor in the efforts to determine the possible significance of individual commensal microbial organisms in disease pathogenesis.

Recent data have described a possible link between CD onset in susceptible patients and diverse infectious agents, which may have occurred as early as during the perinatal period. Plot et al. [64] demonstrated a possible protective role that infections with EBV, CMV, and Rubella may have on susceptible individuals; it seems that encountering certain infections may establish a particular immunological background that disfavours the evolution of autoimmune conditions. Moreover, several intestinal viral triggers including adenovirus, hepatitis C virus (HCV), and rotavirus and bacterial infections capable of initiating or augmenting gut mucosal responses to gluten were suggested to play a role in the pathogenic mechanism of CD [65]. An environmental factor, such as an infectious agent, is thought to precipitate the disease, via various pathogenic mechanisms, such as molecular mimicry, resulting in modulation of the host's immune tolerance. Several other gastrointestinal pathogens have been associated with the development of CD, with varying outcomes; most are isolated case reports. Other pathogens such as *Campylobacter jejuni*, *Giardia lamblia*, *Rotavirus* infection, and *Enterovirus* infection have also been associated with development of CD [65].

Abnormal components found among the microbial inhabitants adhering to the diseased jejunal mucosa have been described and recently analyzed using new microbiological methods [66]. Bacteria were identified by 16S rDNA sequencing in DNA extracted from biopsies. Profound changes in the fecal and duodenal microbiota composition of patients with active disease who are on a gluten-free diet have also been demonstrated [56]. *Bacteroides* and *Clostridium leptum* groups were more abundant in faeces and biopsies of CD patients than in controls regardless of the stage of the disease. *Escherichia coli* and *Staphylococcus* counts were also higher in faces and biopsies of nontreated CD patients than in those of controls. Interestingly, some commensal bacteria, such as *Escherichia coli*, promoted the activation of innate immune cells by gliadin, whereas others such as *Bifidobacteria* exerted inhibitory effects [67]. Dietary factors seem particularly relevant at early stages when the immature neonate's gut is acquiring and shaping its own microbiota and undergoing major physiological and immunological developments up to the point when the immune system acquires full competence and tolerance to nonharmful antigens [68]. The period in which the human host is most acutely influenced by the microbiota is the postnatal period, during which the germ-free neonate moves from the sterile environment of its mother's uterus into a world full of microorganisms and during which the neonate's mucosal and skin surfaces become gradually colonized. Particularly relevant seems to be the era in which we introduce the gluten in the diet. As early as the seventies, it had become clear that the introduction of gluten in the diet after the fourth month of life would reduce the incidence of CD onset [69]. Infants who carry either the HLA-DR3 or DR4 alleles or who have a first-degree relative affected by CD [70] have a fivefold increased risk of developing CD autoimmunity with the presentation of positive tissue transglutaminase (tTG) autoantibody if they are exposed to gluten in the first three months of life. Infants introduced to gluten at 7 months or later also had an increased risk of CD compared with those exposed between 4 and 6 months.

Some explanations have been reported by Norris et al. [70] for the increased risk of CD when first gluten exposure occurs in younger and older children instead of at the age of 4–6 months. On the one hand, in younger children, early introduction of solid foods (i.e., before the intestinal immune system reaches a certain level of maturation) may lead to intolerance [71]. The increased risk of CD in children introduced to gluten at 7 months or older might be due to the larger amounts of gluten intake at the first exposure [70]. The ESPGHAN Committee on nutrition has outlined possible practical suggestions on the introduction of complementary feeding to avoid both early (<4 months) and late (≥7 months) introduction of gluten and to gradually introduce small amounts of gluten whilst the infant is still breast-fed [72] in order to reduce the predisposition to CD later in life [73]. However, the time of first exposure to potentially allergenic foods in infants differs significantly between countries and occurs much earlier than recommended in some countries, as reported in [74]. A recent meta-analysis of observational retrospective studies was used to analyze the protective role of breast-feeding against CD onset and concluded that increased duration of breastfeeding is associated with a reduced risk of CD [75]. A study of 627 cases with confirmed CD revealed that the risk of CD was reduced in a group of children aged <2 years if they were still being breast-fed when dietary gluten was introduced and the risk increased when the gluten was introduced in the diet in large amounts [76].

It is also important to examine further whether favorable infant dietary patterns postpone CD onset or in fact reduce the overall lifetime risk of the disease [76]. Other recent studies have pointed to the role of breast-feeding in delaying CD in infancy. D'Amico et al. [77] showed that children with CD who had been exclusively breast-fed had a delayed onset and less severe disease symptoms than those who had not been exclusively breast-fed [77]. In spite of all the evidence reported, it remains unclear whether those children breast-fed during the introduction of gluten are more likely to develop an extraintestinal (atypical) CD [78]. A series of 162 celiac children registered by the University of Chicago revealed that children breast-fed at the time of gluten introduction were just as likely to develop both intestinal and extraintestinal symptoms, whereas children who were not breast-fed when weaned with gluten had a much higher chance of developing intestinal symptoms [79]. Human milk provides many bioactive factors, including antimicrobial and anti-inflammatory agents, enzymes, hormones, and growth factors, many of which are involved in gut maturation and development of the infant's innate and acquired immunity [80]. Breast-feeding might affect tolerance induction in infants because of the possible transfer of small amounts of gluten and gluten-specific IgA antibodies through breast milk and the presence of factors in breast milk that affect immune system maturation and responses [81]. Also, it seems that the lymphocyte subset profile of breast-fed infants is associated

with a better response to gliadin after gluten introduction. Other reasons that could explain a protective effect of breastfeeding against CD development could be related to the role human milk plays in defining microbiota composition [82] and in the incidence of infections.

There is evidence that microbiota may enhance innate immunity to pathogens. The interaction between microbiota and innate immunity develops in the gut mucosa. In particular, innate immunity cells, located in the lamina propria, promote immunological unresponsiveness to commensal bacteria, which is important for maintaining gut homeostasis. Specifically, gut-resident phagocytes are hyporesponsive to microbial ligands and commensal bacteria, and they do not produce biologically significant levels of proinflammatory molecules upon stimulation. However, the microbiota is essential for upregulating the production of pro-IL-1β, the precursor to IL-1β, in resident innate immunity cells. It has been shown that IL-1β may have a protective role in intestinal immunity. This role is, at least partly, mediated by its ability to induce the expression of endothelial adhesion molecules that contribute to neutrophil recruitment and pathogen clearance in the intestine [52].

Initiation of the innate immune response in the intestine is triggered by pathogen-recognition receptors (PRRs). These PRRs serve as sensors of pathogen-associated molecular patterns (PAMPs) from the intestinal lumen. The most studied PRRs are the Toll-like receptors (TLRs). TLRs are transmembrane proteins that are typically expressed by intestinal epithelial cells either on the cell surface or in endosomes. TLR signaling in the intestine is involved in epithelial cell proliferation, immunoglobulin A (IgA) production [83], and antimicrobial peptide expression, functions that are crucial for maintaining a healthy epithelial barrier [84]. PRRs are also expressed by other immune cells in the lamina propria and they can activate an inflammatory response involving both innate and adaptive immune system [85]. The intestinal barrier regulates intestinal homeostasis throughout innate and adaptive immune responses. Unfortunately, in some cases the innate immune system's attempts to protect the host fail and chronic inflammation and intestinal autoimmunity occur, such as in the case of celiac disease and IBD [86].

Recent experimental data demonstrated the existence of a strong and direct interaction between TLRs and intestinal microbiota. For example, Cheng et al. demonstrated that pediatric CD patients have lower duodenal expression of TLR2 and higher expression of TLR9 as compared to healthy controls confirming that microbiota may have a role in CD [87].

Higher TLR9 does not directly correlate to microbiota. In fact, Cheng and colleagues concluded that the overall composition, diversity, and the estimated microbe associated molecular pattern (MAMP) content of microbiota were comparable between CD and healthy subjects, but a subpopulation profile comprising eight genus-like bacterial groups was found to differ significantly between healthy subjects and CD. In healthy subjects, increased TLR2 expression was positively correlated with the expression of tight junction protein ZO-1. In CD, the expression of IL-10, IFN-gamma, and CXCR6 was higher as compared to healthy subjects.

Other recent data [87] comparing the composition of microbiota between CD patients and healthy controls demonstrated that TLRs activation by intestinal microbiota may determine different effects on the human gut of CD patients and healthy controls. These data suggest that microbiota and altered expression of mucosal receptors have a role in CD. In CD subjects, the increased expression of IL-10 and IFN-gamma may have partly resulted from the increased TLR9 expression and signaling [88].

Furthermore, recently Eiro et al. [89] studied the expression of TLRs and cytokines and revealed that they occur in duodenal mucosa and are elevated in both children and adult celiac patients. In particular, TLR4 expression was increased twofold in CD patients compared to controls. CD patients with high levels of TLR4 also showed high levels of proinflammatory cytokines (IL-1, IL-6, IL-8, and IL-17) as well as transcription factors (IRAK4, MyD88, and NF-κB) [89]. These data support the hypothesis that a unique pattern of TLR expression is associated with CD independently of age at diagnosis. The evidence that pediatric and adult patients have a similar inflammatory profile will encourage treatment of both with the same immunological therapy in the future.

5. Dietary and Microbiota Changes and Immunomodulatory Approaches in Celiac Disease: Translational Applications

The only approved treatment for CD is the lifelong complete exclusion of the gluten from the diet. But, dietary compliance is difficult to achieve in several patients for several reasons. Indeed, we need novel strategies, targeting various factors at different levels in CD pathogenesis.

For example, the reduction of gluten antigenicity and/or elimination of toxic peptides from gluten could be walkable; in this manner, the food gluten would not have the negative effect on activation of host immune system and the consequential gut inflammation. These modifications of gluten antigenicity may be achieved in several manners, from the selection of natural cereal cultivars to the genetic modification of gluten peptide sequences or more likely producing genetically modified organisms, in which toxic sequences are deleted or silenced [90].

The other translational strategy of CD therapy derives from the knowledge of immunomodulatory mechanisms of disease. In fact, in CD, food gluten peptides activate lamina propria T-cells to produce inflammatory cytokines leading to tissue destruction. Thus, a way to block this pathogenetic event in CD could be a specific modulation of gluten reactive pathogenetic target T-cells. This modulation in gluten related to T-cell reactivity may be borrowed from the actual evidences on other immune hypersensitivity related diseases, such as allergy and autoimmune diseases. In fact, in these diseases, such as in CD, there is a pivotal role of CD4+ T-helper cells in driving and orchestrating gut inflammation and tissue destruction. The goal of pathogenetic translational therapy may be to develop vaccines consisting of synthetic peptides representing T-cells epitopes that may have the function to restore the balance between inflammatory and regulatory

responses to the causative antigens [91, 92]. In CD, this could be achieved using a therapeutic vaccination constructed with the panel of most immunodominant gluten epitopes. The efficacy of vaccine therapy has been just demonstrated in allergy in which it is able to reduce allergen sensitivity and improve regulatory T-cell function.

Furthermore, there are recent findings in the alterations of the microbiota in various medical conditions, such as CD, inflammatory bowel disease, allergy, and colorectal cancer. Even though changes of the microbiota could be linked to the etiopathogenesis of these diseases, further studies are needed to understand how to modulate microbiota to ameliorate the morbidity of CD.

6. Conclusion

Chronic inflammatory diseases have multifactorial etiologies that involve environmental components and many immune and genetic factors. CD represents a particularly informative model for chronic inflammatory diseases. An environmental factor that precipitates disease is known (gluten); the HLA molecules that confer predisposition to the disease have been identified (HLA-DQ2/HLA-DQ8); and access to the small intestine is simple. Thus, understanding the interaction of the microbiota with pathogens and host might provide new insights into the pathogenesis of disease, as well as novel avenues for preventing and treating intestinal and systemic disorders [52].

The high increase in incidence of autoimmune disorders cannot be explained only by genetic drift and is thought to be the result of changes in the environmental factors and in their complex interaction with innate and adaptive immunity. Although there is strong support for the role of microorganisms as triggers of innate immune activation, there is no evidence of a role for molecular mimicry in CD. In other words, we do not have any evidence so far that CD pathogenesis is the result of a T-cell response against a microbial peptide cross-reacting with gluten [93].

The gut microbiota has been studied for more than a century; however, we have only recently begun to understand the ever-expanding roles for these microscopic organisms in health and disease. Despite the complexity of the gut microbiota, there is a delicate balance in bacterial populations such that any disruption in this balance leads to dysbiosis and, consequently, to decreased resistance to pathogen colonization, to the favoured growth of pathobionts, and to pathological both innate and adaptive immune responses [52]. So, in genetically predisposed individuals, gluten in association with microbial antigens can stimulate and modulate innate and adaptive immune response, sustaining a chronic mucosal inflammation, underlining this chronic disease.

In summary, CD may be considered as a model to explain the pathogenesis of several other autoimmune diseases. In particular, how in the steady state condition homeostasis is maintained due to the complete balance between pro- and anti-inflammatory factors whereas, during disease, this balance is altered. The breakdown of the interaction among microbiota, innate immunity, and genetic and dietary factors leads to the disruption of homeostasis leading to inflammation and tissue damage. Thus, focusing the attention on this interaction and its breakdown may allow a better understanding of the CD pathogenesis and finally get novel translational avenues for preventing and treating this widespread disease.

Conflict of Interests

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

Authors' Contribution

D. Pagliari and R. Urgesi contributed equally to the work.

References

[1] S. Husby, S. Koletzko, I. R. Korponay-Szabó et al., "European society for pediatric gastroenterology, hepatology, and nutrition guidelines for the diagnosis of coeliac disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 54, no. 1, pp. 136–160, 2012.

[2] R. Cianci, G. Cammarota, G. Frisullo et al., "Tissue-infiltrating lymphocytes analysis reveals large modifications of the duodenal "immunological niche" in coeliac disease after gluten-free diet," *Clinical and Translational Gastroenterology*, vol. 3, article e28, 2012.

[3] D. Pagliari, S. Frosali, R. Landolfi et al., "The role of IL-15 in human cancer: friend or foe?" *International Trends in Immunity*, vol. 1, no. 2, pp. 35–42, 2013.

[4] B. Meresse, J. Ripoche, M. Heyman, and N. Cerf-Bensussan, "Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis," *Mucosal Immunology*, vol. 2, no. 1, pp. 8–23, 2009.

[5] D. A. van Heel, L. Franke, K. A. Hunt et al., "A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21," *Nature Genetics*, vol. 39, no. 7, pp. 827–829, 2007.

[6] C. Gianfrani, S. Auricchio, and R. Troncone, "Adaptive and innate immune responses in celiac disease," *Immunology Letters*, vol. 99, no. 2, pp. 141–145, 2005.

[7] P. C. Dubois and D. A. Van Heel, "Translational mini-review series on the immunogenetics of gut disease: immunogenetics of coeliac disease," *Clinical and Experimental Immunology*, vol. 153, no. 2, pp. 162–173, 2008.

[8] V. Kumar, C. Wijmenga, and S. Withoff, "From genome-wide association studies to disease mechanisms: celiac disease as a model for autoimmune diseases," *Seminars in Immunopathology*, vol. 34, no. 4, pp. 567–580, 2012.

[9] M. Fernandez-Feo, G. Wei, G. Blumenkranz et al., "The cultivable human oral gluten-degrading microbiome and its potential implications in coeliac disease and gluten sensitivity," *Clinical Microbiology and Infection*, vol. 19, no. 9, pp. E386–E394, 2013.

[10] L. Maiuri, C. Ciacci, I. Ricciardelli et al., "Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease," *The Lancet*, vol. 362, no. 9377, pp. 30–37, 2003.

[11] D. Pagliari, R. Cianci, S. Frosali et al., "The role of IL-15 in gastrointestinal diseases: a bridge between innate and adaptive immune response," *Cytokine and Growth Factor Reviews*, vol. 24, no. 5, pp. 455–466, 2013.

[12] B. Meresse, Z. Chen, C. Ciszewski et al., "Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease," *Immunity*, vol. 21, no. 3, pp. 357–366, 2004.

[13] S. Hüe, J.-J. Mention, R. C. Monteiro et al., "A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease," *Immunity*, vol. 21, no. 3, pp. 367–377, 2004.

[14] J. M.-L. Tjon, J. van Bergen, and F. Koning, "Celiac disease: how complicated can it get?" *Immunogenetics*, vol. 62, no. 10, pp. 641–651, 2010.

[15] K. M. Lammers, R. Lu, J. Brownley et al., "Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3," *Gastroenterology*, vol. 135, no. 1, pp. 194.e3–204.e3, 2008.

[16] F. Tang, Z. Chen, C. Ciszewski et al., "Cytosolic PLA 2 is required for CTL- Mediated immunopathology of celiac disease via NKG2D and IL-15," *Journal of Experimental Medicine*, vol. 206, no. 3, pp. 707–719, 2009.

[17] E. M. Nilsen, K. E. A. Lundin, P. Krajci, H. Scott, L. M. Sollid, and P. Brandtzaeg, "Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon γ," *Gut*, vol. 37, no. 6, pp. 766–776, 1995.

[18] F. Vartdal, B. H. Johansen, T. Friede et al., "The peptide binding motif of the disease associated HLA-DQ (α 1(*) 0501, β 1(*) 0201) molecule," *European Journal of Immunology*, vol. 26, no. 11, pp. 2764–2772, 1996.

[19] K. H. Lee, K. W. Wucherpfennig, and D. C. Wiley, "Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type I diabetes," *Nature Immunology*, vol. 2, no. 6, pp. 501–507, 2001.

[20] D. F. Evans, G. Pye, R. Bramley, A. G. Clark, T. J. Dyson, and J. D. Hardcastle, "Measurement of gastrointestinal pH profiles in normal ambulant human subjects," *Gut*, vol. 29, no. 8, pp. 1035–1041, 1988.

[21] C. Briani, D. Samaroo, and A. Alaedini, "Celiac disease: from gluten to autoimmunity," *Autoimmunity Reviews*, vol. 7, no. 8, pp. 644–650, 2008.

[22] G. Cammarota, F. Pandolfi, R. Cianci, D. Pagliari, R. Landolfi, and S. Kunkel, "Cellular mediators of inflammation: tregs and T H 17 cells in gastrointestinal diseases," *Mediators of Inflammation*, vol. 2009, Article ID 132028, 11 pages, 2009.

[23] D. Fina, M. Sarra, R. Caruso et al., "Interleukin 21 contributes to the mucosal T helper cell type 1 response in coeliac disease," *Gut*, vol. 57, no. 7, pp. 887–892, 2008.

[24] T. Matysiak-Budnik, I. C. Moura, M. Arcos-Fajardo et al., "Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease," *Journal of Experimental Medicine*, vol. 205, no. 1, pp. 143–154, 2008.

[25] K. Sugi, M. W. Musch, M. Field, and E. B. Chang, "Inhibition of NA+,K+-ATPase by interferon γ down-regulates intestinal epithelial transport and barrier function," *Gastroenterology*, vol. 120, no. 6, pp. 1393–1403, 2001.

[26] B. L. Kelsall and F. Leon, "Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease," *Immunological Reviews*, vol. 206, pp. 132–148, 2005.

[27] S. Nagata, C. McKenzie, S. L. F. Pender et al., "Human Peyer's patch T cells are sensitized to dietary antigen and display a Th cell type 1 cytokine profile," *Journal of Immunology*, vol. 165, no. 9, pp. 5315–5321, 2000.

[28] R. P. Anderson, P. Degano, A. J. Godkin, D. P. Jewell, and A. V. S. Hill, "In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope," *Nature Medicine*, vol. 6, no. 3, pp. 337–342, 2000.

[29] R. Cianci, D. Pagliari, R. Landolfi et al., "New insights on the role of T cells in the pathogenesis of celiac disease," *Journal of Biological Regulators and Homeostatic Agents*, vol. 26, no. 2, pp. 171–179, 2012.

[30] A. Iwasaki and B. L. Kelsall, "Unique functions of CD11b+, CD8α+, and double-negative Peyer's patch dendritic cells," *Journal of Immunology*, vol. 166, no. 8, pp. 4884–4890, 2001.

[31] A. Nakazawa, M. Watanabe, T. Kanai et al., "Functional expression of costimulatory molecule CD86 on epithelial cells in the inflamed colonic mucosa," *Gastroenterology*, vol. 117, no. 3, pp. 536–545, 1999.

[32] T. Arstila, T. P. Arstila, S. Calbo et al., "Identical T cell clones are located within the mouse gut epithelium and lamina propria and circulate in the thoracic duct lymph," *Journal of Experimental Medicine*, vol. 191, no. 5, pp. 823–834, 2000.

[33] T. Shibahara, J. N. Wilcox, T. Couse, and J. L. Madara, "Characterization of epithelial chemoattractants for human intestinal intraepithelial lymphocytes," *Gastroenterology*, vol. 120, no. 1, pp. 60–70, 2001.

[34] E. G. De La Concha, M. Fernández-Arquero, P. Vigil et al., "Celiac disease and TNF promoter polymorphisms," *Human Immunology*, vol. 61, no. 5, pp. 513–517, 2000.

[35] B. Jabri and L. M. Sollid, "Tissue-mediated control of immunopathology in coeliac disease," *Nature Reviews Immunology*, vol. 9, no. 12, pp. 858–870, 2009.

[36] J. Satsangi, R. W. G. Chapman, N. Haldar et al., "A functional polymorphism of the stromelysin gene (MMP-3) influences susceptibility to primary sclerosing cholangitis," *Gastroenterology*, vol. 121, no. 1, pp. 124–130, 2001.

[37] V. M. Salvati, R. Troncone, M. Bajaj-Elliott et al., "Keratinocyte growth factor and coeliac disease," *Gut*, vol. 49, no. 2, pp. 176–181, 2001.

[38] L. Maiuri, C. Ciacci, S. Auricchio, V. Brown, S. Quaratino, and M. Londei, "Interleukin 15 mediates epithelial changes in celiac disease," *Gastroenterology*, vol. 119, no. 4, pp. 996–1006, 2000.

[39] S. F. Moss, L. Attia, J. V. Scholes, J. R. F. Walters, and P. R. Holt, "Increased small intestinal apoptosis in coeliac disease," *Gut*, vol. 39, no. 6, pp. 811–817, 1996.

[40] A. Di Sabatino, R. Ciccocioppo, S. D'Alò et al., "Intraepithelial and lamina propria lymphocytes show distinct patterns of apoptosis whereas both populations are active in Fas based cytotoxicity in coeliac disease," *Gut*, vol. 49, no. 3, pp. 380–386, 2001.

[41] C. Gianfrani, R. Troncone, P. Mugione et al., "Celiac disease association with $CD8^+$ T cell responses: identification of a novel gliadin-derived HLA-A2-restricted epitope," *Journal of Immunology*, vol. 170, no. 5, pp. 2719–2726, 2003.

[42] I. Adlerberth and A. E. Wold, "Establishment of the gut microbiota in Western infants," *Acta Paediatrica*, vol. 98, no. 2, pp. 229–238, 2009.

[43] H. Tlaskalová, V. Kamarýtová, L. Mandel et al., "The immune response of germ-free piglets after peroral monocontamination with living *Escherichia coli* strain 086. I. The fate of antigen, dynamics and site of antibody formation, nature of antibodies and formation of heterohaemagglutinins," *Folia Biologica*, vol. 16, no. 3, pp. 177–187, 1970.

[44] T. Hrncir, R. Stepankova, H. Kozakova, T. Hudcovic, and H. Tlaskalova-Hogenova, "Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: Studies in germ-free mice," *BMC Immunology*, vol. 9, article 65, 2008.

[45] H. Tlaskalova Hogenova, J. Sterzl, and R. Stepankova, "Development of immunological capacity under germfree and conventional conditions," *Annals of the New York Academy of Sciences*, vol. 409, pp. 96–113, 1983.

[46] R. Stepankova, J. Šinkora, T. Hudcovic, H. Kozáková, and H. Tlaskalová-Hogenová, "Differences in development of lymphocyte subpopulations from gut-associated lymphatic tissue (GALT) of germfree and conventional rats: effect of aging," *Folia Microbiologica*, vol. 43, no. 5, pp. 531–534, 1998.

[47] Y. Umesaki, K. Tohyama, and M. Mutai, "Biosynthesis of microvillus membrane-associated glycoproteins of small intestinal epithelial cells in germ-free and conventionalized mice," *Journal of Biochemistry*, vol. 92, no. 2, pp. 373–379, 1982.

[48] L. Bry, P. G. Falk, T. Midtvedt, and J. I. Gordon, "A model of host-microbial interactions in an open mammalian ecosystem," *Science*, vol. 273, no. 5280, pp. 1380–1383, 1996.

[49] J.-F. Bach, "The effect of infections on susceptibility to autoimmune and allergic diseases," *The New England Journal of Medicine*, vol. 347, no. 12, pp. 911–920, 2002.

[50] E. Israeli, I. Grotto, B. Gilburd et al., "Anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic antibodies as predictors of inflammatory bowel disease," *Gut*, vol. 54, no. 9, pp. 1232–1236, 2005.

[51] Y. Shoenfeld, B. Gilburd, M. Abu-Shakra et al., "The mosaic of autoimmunity: genetic factors involved in autoimmune diseases—2008," *Israel Medical Association Journal*, vol. 10, no. 1, pp. 3–7, 2008.

[52] N. Kamada, S.-U. Seo, G. Y. Chen, and G. Núñez, "Role of the gut microbiota in immunity and inflammatory disease," *Nature Reviews Immunology*, vol. 13, no. 5, pp. 321–335, 2013.

[53] K. Honda and D. R. Littman, "The microbiome in infectious disease and inflammation," *Annual Review of Immunology*, vol. 30, pp. 759–795, 2012.

[54] I. Nadal, E. Donant, C. Ribes-Koninckx, M. Calabuig, and Y. Sanz, "Imbalance in the composition of the duodenal microbiota of children with coeliac disease," *Journal of Medical Microbiology*, vol. 56, no. 12, pp. 1669–1674, 2007.

[55] M. C. Collado, E. Donat, C. Ribes-Koninckx, M. Calabuig, and Y. Sanz, "Imbalances in faecal and duodenal Bifidobacterium species composition in active and non-active coeliac disease," *BMC Microbiology*, vol. 8, article 232, 2008.

[56] M. C. Collado, E. Donat, C. Ribes-Koninckx, M. Calabuig, and Y. Sanz, "Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease," *Journal of Clinical Pathology*, vol. 62, no. 3, pp. 264–269, 2009.

[57] E. Sánchez, G. De Palma, A. Capilla et al., "Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by *Bacteroides* species," *Applied and Environmental Microbiology*, vol. 77, no. 15, pp. 5316–5323, 2011.

[58] T. Pozo-Rubio, M. Olivares, E. Nova et al., "Immune development and intestinal microbiota in celiac disease," *Clinical and Developmental Immunology*, vol. 2012, Article ID 654143, 12 pages, 2012.

[59] J. F. Bach, "Infections and autoimmune diseases," *Journal of Autoimmunity*, vol. 25, pp. 74–80, 2005.

[60] M. Blank, O. Barzilai, and Y. Shoenfeld, "Molecular mimicry and auto-immunity," *Clinical Reviews in Allergy and Immunology*, vol. 32, no. 1, pp. 111–118, 2007.

[61] H. Tlaskalova-Hogenova, R. Stepankova, L. Tuckova, and et al, "Autoimmunity, immunodeficiency and mucosal infections: chronic intestinal inflammation as a sensitive indicator of immunoregulatory defects in response to normal luminal microflora," *Folia Microbiologica*, vol. 43, no. 5, pp. 545–550, 1998.

[62] M. C. Abt and D. Artis, "The intestinal microbiota in health and disease: the influence of microbial products on immune cell homeostasis," *Current Opinion in Gastroenterology*, vol. 25, no. 6, pp. 496–502, 2009.

[63] I. Sekirov, S. L. Russell, L. Caetano M Antunes, and B. B. Finlay, "Gut microbiota in health and disease," *Physiological Reviews*, vol. 90, no. 3, pp. 859–904, 2010.

[64] L. Plot, H. Amital, O. Barzilai, M. Ram, B. Nicola, and Y. Shoenfeld, "Infections may have a protective role in the etiopathogenesis of celiac disease," *Annals of the New York Academy of Sciences*, vol. 1173, pp. 670–674, 2009.

[65] L. Plot and H. Amital, "Infectious associations of Celiac disease," *Autoimmunity Reviews*, vol. 8, no. 4, pp. 316–319, 2009.

[66] G. Ou, M. Hedberg, P. Hörstedt et al., "Proximal small intestinal microbiota and identification of rod-shaped bacteria associated with childhood celiac disease," *The American Journal of Gastroenterology*, vol. 104, no. 12, pp. 3058–3067, 2009.

[67] G. de Palma, J. Cinova, R. Stepankova, L. Tuckova, and Y. Sanz, "Pivotal advance: bifidobacteria and Gram-negative bacteria differentially influence immune responses in the proinflammatory milieu of celiac disease," *Journal of Leukocyte Biology*, vol. 87, no. 5, pp. 765–778, 2010.

[68] E. A. M. Westerbeek, A. van den Berg, H. N. Lafeber, J. Knol, W. P. F. Fetter, and R. M. van Elburg, "The intestinal bacterial colonisation in preterm infants: a review of the literature," *Clinical Nutrition*, vol. 25, no. 3, pp. 361–368, 2006.

[69] D. N. Challacombe, I. K. Mecrow, K. Elliott, F. J. Clarke, and E. E. Wheeler, "Changing infant feeding practices and declining incidence of coeliac disease in West Somerset," *Archives of Disease in Childhood*, vol. 77, no. 3, pp. 206–209, 1997.

[70] J. M. Norris, K. Barriga, E. J. Hoffenberg et al., "Risk of celiac disease autoimmunity and timing of gluten introduction in the diet of infants at increased risk of disease," *Journal of the American Medical Association*, vol. 293, no. 19, pp. 2343–2351, 2005.

[71] A. G. Cummins and F. M. Thompson, "Effect of breast milk and weaning on epithelial growth of the small intestine in humans," *Gut*, vol. 51, no. 5, pp. 748–754, 2002.

[72] C. Agostoni and R. Shamir, "Can a change in policy of complementary infant feeding reduce the risk for Type 1 diabetes and celiac disease?" *Pediatric Endocrinology Reviews*, vol. 6, no. 1, pp. 2–4, 2008.

[73] S. L. Prescott and M. L. K. Tang, "The australasian society of clinical immunology and allergy position statement: summary of allergy prevention in children," *Medical Journal of Australia*, vol. 182, no. 9, pp. 464–467, 2005.

[74] S. A. Schiess, V. Grote, S. Scaglioni et al., "Introduction of potentially allergenic foods in the infant's diet during the first year of life in five European countries," *Annals of Nutrition and Metabolism*, vol. 58, no. 2, pp. 109–117, 2011.

[75] A. K. Akobeng, A. V. Ramanan, I. Buchan, and R. F. Heller, "Effect of breast feeding on risk of coeliac disease: a systematic

review and meta-analysis of observational studies," *Archives of Disease in Childhood*, vol. 91, no. 1, pp. 39–43, 2006.

[76] A. Ivarsson, O. Hernell, H. Stenlund, and L. Å. Persson, "Breast-feeding protects against celiac disease," *The American Journal of Clinical Nutrition*, vol. 75, no. 5, pp. 914–921, 2002.

[77] M. A. D'Amico, J. Holmes, S. N. Stavropoulos et al., "Presentation of pediatric celiac disease in the United States: prominent effect of breastfeeding," *Clinical Pediatrics*, vol. 44, no. 3, pp. 249–258, 2005.

[78] M. Silano, C. Agostoni, and S. Guandalini, "Effect of the timing of gluten introduction on the development of celiac disease," *World Journal of Gastroenterology*, vol. 16, no. 16, pp. 1939–1942, 2010.

[79] S. Strobel, "Immunity induced after a feed of antigen during early life: oral tolerance v. sensitisation," *Proceedings of the Nutrition Society*, vol. 60, no. 4, pp. 437–442, 2001.

[80] S. Rautava and W. A. Walker, "Academy of breastfeeding medicine founder's lecture 2008: Breastfeeding—an extrauterine link between mother and child," *Breastfeeding Medicine*, vol. 4, no. 1, pp. 3–10, 2009.

[81] V. Verhasselt, "Neonatal tolerance under breastfeeding influence," *Current Opinion in Immunology*, vol. 22, no. 5, pp. 623–630, 2010.

[82] R. Troncone, A. Ivarsson, H. Szajewska, and M. L. Mearin, "Review article: future research on coeliac disease—a position report from the European multistakeholder platform on coeliac disease (CDEUSSA)," *Alimentary Pharmacology and Therapeutics*, vol. 27, no. 11, pp. 1030–1043, 2008.

[83] L. Shang, M. Fukata, N. Thirunarayanan et al., "Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria," *Gastroenterology*, vol. 135, no. 2, pp. 529–538, 2008.

[84] L. V. Hooper, D. R. Littman, and A. J. Macpherson, "Interactions between the microbiota and the immune system," *Science*, vol. 336, no. 6086, pp. 1268–1273, 2012.

[85] G. Harris, R. KuoLee, and W. Chen, "Role of toll-like receptors in health and diseases of gastrointestinal tract," *World Journal of Gastroenterology*, vol. 12, no. 14, pp. 2149–2160, 2006.

[86] R. Santaolalla and M. T. Abreu, "Innate immunity in the small intestine," *Current Opinion in Gastroenterology*, vol. 28, no. 2, pp. 124–129, 2012.

[87] J. Cheng, M. Kalliomäki, H. G. H. J. Heilig et al., "Duodenal microbiota composition and mucosal homeostasis in pediatric celiac disease," *BMC Gastroenterology*, vol. 13, no. 1, article 113, 2013.

[88] S. de Kivit, E. van Hoffen, N. Korthagen, J. Garssen, and L. E. M. Willemsen, "Apical TLR ligation of intestinal epithelial cells drives a Th1-polarized regulatory or inflammatory type effector response in vitro," *Immunobiology*, vol. 216, no. 4, pp. 518–527, 2011.

[89] N. Eiro, S. Gonzalez-Reyes, L. Gonzalez et al., "Duodenal expression of toll-like receptors and interleukins are increased in both children and adult celiac patients," *Digestive Diseases and Sciences*, vol. 57, no. 9, pp. 2278–2285, 2012.

[90] A. Camarca, A. del Mastro, and C. Gianfrani, "Repertoire of gluten peptides active in celiac disease patients: perspectives for translational therapeutic applications," *Endocrine, Metabolic and Immune Disorders—Drug Targets*, vol. 12, no. 2, pp. 207–219, 2012.

[91] J. D. Campbell, K. F. Buckland, S. J. McMillan et al., "Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression," *Journal of Experimental Medicine*, vol. 206, no. 7, pp. 1535–1547, 2009.

[92] D. C. Wraith, "Therapeutic peptide vaccines for treatment of autoimmune diseases," *Immunology Letters*, vol. 122, no. 2, pp. 134–136, 2009.

[93] L. M. Sollid and B. Jabri, "Triggers and drivers of autoimmunity: lessons from coeliac disease," *Nature Reviews Immunology*, vol. 13, no. 4, pp. 294–302, 2013.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 586939, 8 pages
http://dx.doi.org/10.1155/2014/586939

Review Article

Immunomodulation by Gut Microbiota: Role of Toll-Like Receptor Expressed by T Cells

Mariagrazia Valentini,[1] Alessia Piermattei,[1] Gabriele Di Sante,[1] Giuseppe Migliara,[1] Giovanni Delogu,[2] and Francesco Ria[1]

[1] *Institute of General Pathology, Università Cattolica del Sacro Cuore, Largo F. Vito 1, 00168 Rome, Italy*
[2] *Institute of Microbiology, Università Cattolica del Sacro Cuore, Largo F. Vito 1, 00168 Rome, Italy*

Correspondence should be addressed to Gabriele Di Sante; gabriele.disante@hotmail.it

Received 30 April 2014; Revised 1 July 2014; Accepted 2 July 2014; Published 24 July 2014

Academic Editor: Rossella Cianci

A close relationship exists between gut microbiota and immune responses. An imbalance of this relationship can determine local and systemic immune diseases. In fact the immune system plays an essential role in maintaining the homeostasis with the microbiota that normally resides in the gut, while, at the same time, the gut microbiota influences the immune system, modulating number and function of effector and regulatory T cells. To achieve this aim, mutual regulation between immune system and microbiota is achieved through several mechanisms, including the engagement of toll-like receptors (TLRs), pathogen-specific receptors expressed on numerous cell types. TLRs are able to recognize ligands from commensal or pathogen microbiota to maintain the tolerance or trigger the immune response. In this review, we summarize the latest evidences about the role of TLRs expressed in adaptive T cells, to understand how the immune system promotes intestinal homeostasis, fights invasion by pathogens, and is modulated by the intestinal microbiota.

1. TLRs and Microbiota

The gut is the largest defense barrier of our body. More than 60% of immune cells are in the gut mucosa, ready to identify and counteract the presence of potential aggressors and inhibit uncontrolled inflammatory reactions [1, 2].

A further protection is represented by the presence of various populations of microorganisms (each encompassing several strains) that condition both the mucosal immune response and the ability of the host to resist aggressive pathogens' attacks [3, 4].

The human gut microbiota is composed of microorganisms that include bacterial communities, yeasts, and bacteriophages (viruses that control the bacterial community, and in particular, the ability of bacteria to regulate our metabolism) all residing in the intestinal tract. This community encompasses trillions of bacteria with an estimated biomass of 1 kg [5].

Molecular and metagenomic approaches have allowed identifying the main bacterial communities present in the digestive tract, their role in health, and their relationships with specific diseases [6–8].

The gut immune system monitors the communities that flow in the lumen and, in healthy conditions, reacts against potentially pathogenic organisms by inducing inflammation, while maintaining tolerance towards most members of commensal microbiota [9–11].

Therefore, the defense of the organism requires a careful surveillance able to distinguish microbes with pathogenic potential (pathobionts) from nonpathogenic microorganisms (mainly symbionts) [12].

The ability of these cells to discriminate pathogens from commensals is mediated by pattern recognition receptors (PRRs) that include the families of toll-like receptors (TLRs), nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), C-type lectin receptors (CLRs), cytosolic DNA receptors (CDRs), and RIG-I-like receptors (RLRs). In particular, TLRs are mostly (but not exclusively) present on the membrane of immune and epithelial cells [13] and NODs are present in the cytoplasm of enteric cells [14]. TLRs and

NODs are capable of recognizing conserved molecular motives, generally divided in microbe-associated molecular patterns (MAMPS, expressed by resident microbiota) and pathogens-associated molecular patterns (PAMPS, produced by microbial invaders). Their engagement induces several intracellular signaling cascades resulting in the production of cytokines, chemokines, and transcription factors that are essential for the maintenance of the gut homeostasis and/or infection control [15].

Therefore, TLRs play an important role in suppressing the activation of the inflammatory cascade to maintain the balance of intestinal homeostasis and in promoting inflammatory responses to pathogens [16–18].

Eleven different transmembrane proteins belong to the TLR family. Although they are constantly exposed to a significant charge of commensal bacteria, they are able to restrain inflammation in steady-state conditions, keeping a tone of hyporesponsiveness against the intestinal flora [19].

Recent studies suggest that the mechanisms that limit immune activation belong to potential synergistic actions from both host and bacterial effector molecules. Such molecules are able to antagonize and modulate the signal transmission mediated by TLRs, acting along the signal transduction from the TLRs or at the level of production of effector molecules [20].

TLR2 is involved in the recognition of Gram-negative and Gram-positive bacteria and yeast. At the same time, different evidence proves that TLR2 is able to switch its ability to produce pro- and anti-inflammatory responses by dimerization with several coreceptors such as TLR2 itself, TLR1, TLR6, and TLR10 [21]. Recent studies suggest that TLR2/TLR6 dimerization activates the TLR2-MyD88-IRAK-TRAF-NIK-IKK-NF-κB signal transduction pathway that induces transcription of proinflammatory molecules, while TLR2/TLR1 dimerization promotes the anti-inflammatory pathway that leads to the expression of IL-10 and the trans-differentiation of Th17 and iTreg cells [22].

In order to maintain the immune homeostasis, the host uses several mechanisms that limit and inhibit the inflammatory responses mediated by TLR2. One of these is the modulation of TLR2 signaling through the expression of negative regulators such as the toll-interacting protein (TOLLIP). TOLLIP inhibits IRAK binding TLR2 or TLR4, thereby breaking down this proinflammatory pathway [23]. In addition, commensal bacteria provide other supplementary mechanisms through which they prevent gut colonization by pathogens, as exemplified by the action of *Bacteroides fragilis* through its unique surface polysaccharide (PSA) [24].

TLR4 is expressed at low levels on the surface of epithelial gut cells, where it plays a role in the intestinal mucosal defense against Gram-negative bacteria. TLR4, after activation by lipopolysaccharide (LPS) or endotoxin from Gram-negative bacteria, dimerizes with CD14 and MD-2 and induces the consequent signaling cascade that ultimately leads to the activation of a proinflammatory response. TLR4 signaling is regulated by the expression of the transmembrane protein ST2. ST2 sequesters MyD88 and TIRAP (adaptor proteins associated with TLR), thus antagonizing TLR4 functions and contributing to the persistence of the hyporesponsiveness to commensal microbial community [25, 26].

TLR5 is the innate immune receptor for bacterial flagellin. As the other TLRs, TLR5 is involved both in the recruitment of the adaptor MyD88, upregulating a signaling cascade of proinflammatory transcription factors, and in the maintenance of the gut microbiota homeostasis. Indeed, many commensal species that colonize the gut express flagellin. Activation of TLR5 signaling displays a proinflammatory effect by regulating the production of IL-17 and IL-22 that in turn promote antimicrobial defense essential for clearance of pathogens and protective effects. On the other side, the interaction between Tlr5 and flagellin also leads to the expression of antiapoptotic genes that are correlated with the protective effect of the receptor against normal commensal such as *E. coli* [27].

A large body of evidence shows that TLR9 engagement has contrasting effects on activation of nuclear factor-κB, depending on its expression on apical or basolateral surface of intestinal epithelial cells (IECs) and thereby playing an important role in the gut epithelial homeostasis. TLR9 recognizes intracellular bacteria, by binding the unmethylated CpG motifs of bacterial DNA. While the interaction with engagement of basolateral Tlr9 has been reported to enhance the activation of NF-κB, binding of CpG with apical Tlr9 seems to promote the ubiquitination of IκB that prevents the activation of NF-κB [28, 29].

2. TLRs and Adaptive T Cells: Activation and Functions

Naïve $CD4^+$ T cells migrate from the thymus to periphery, under environmental signals that induce their maturation and functions. Depending on microbial and host signals, T cells differentiate into pro- and anti-inflammatory subsets, such as Th1, Th2, Th17, and iTreg.

The presence of Th17 and iTreg cells in the healthy gut has been largely demonstrated. Th17 cells are a specific lineage of $CD4^+$ Th cells that produce inflammatory cytokines such as IL-17a, IL-17f, IL-21, and IL-22 [30, 31]. They promote the host defense against fungal and bacterial infections, such as *Candida albicans*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Streptococcus pneumonia,* and *Citrobacter rodentium* [32, 33]. Differentiation of $CD4^+$ T cells into Th17 in the gut depends on the stimulation by intestinal microbiota and their products, such as serum amyloid A (SAA), from segmented filamentous bacteria and extracellular ATP [34].

iTreg cells, also defined as inducible suppressor cells, are a subset of $CD4^+$ Th cells that express CD4, CD25, and Foxp3 (Forkhead Box 3, the nuclear transcription factor specifically involved in Treg differentiation) [35].

iTreg are capable of suppressing the activation of the immune system, regulating the homeostasis and tolerance to self-antigens. Several recent studies have demonstrated the presence of Treg cells that secrete IL-10, an anti-inflammatory cytokine [36]. These Treg subsets are not found in thymic environment but are present in peripheral tissues, as the gut [37].

Albeit activation of TLRs is the hallmark of the innate immune response, it has been demonstrated that TLRs are also important for adaptive immune cell function as regulation of B lymphocytes development [38] and antibody production [39]. It has also been demonstrated that certain TLRs are also expressed on T lymphocytes [40] and that TLRs ligands can modulate directly functions of T cell such as signaling in Treg cells [41] or development and effector functions of the various subsets of T helper cell [42].

2.1. TLR2 and T Cells. TLR2 is able to trigger proliferation and cytokine production (in particular IL-2 and IFN-γ) of effector T cells activated via TCR [43], thus regulating the host's immune system against pathogens. Mokuno et al. also reported that stimulation of TLR2 on $\gamma\delta$ T cells increases significantly their proliferative response [44]. In $CD8^+$ cells, TLR2 induces T-bet activity, IFN-γ [45], TNF-α, and other cytotoxic mediators [46, 47]. The same effects have been observed in natural killer T cells (NKT), where the stimulation of TLR2 enhances the expression of Fas-L [48]. Recently, the literature has illustrated the important role of TLR2 in T helper subsets for proliferation and survival [49], cell migration [50, 51], protection against tuberculosis and filarial infections [52, 53], and reduction of IL-4 production [54].

Tlr2 enhances also IL17 productions in $CD4^+$ T cells, promoting experimental autoimmune encephalomyelitis (EAE) pathogenesis and severity [55]. We observed that a polymorphism of Tlr2 modulates severity, remission, and lesion distribution during EAE, although it does not influence disease incidence (manuscript in preparation). An interesting point is that Tlr2 stimulation promotes the differentiation of iTregs into a Th17 [56], which may enhance microbial clearance but may also increase the risk of autoimmune reactions. This role of Tlr2 may be relevant in the pathogenesis of MS (and of its experimental model, the EAE), since iTregs protect from autoimmune aggressions, whereas Th17 cells expand in the periphery and accumulate in the CNS, where they support demyelination [22, 57].

It has been shown in human T cells [56] and we are confirming it in experimental models (manuscript in preparation) that engagement of TLR2 expressed on T cells modulates Fox-P3 mRNA, in a strain-dependent and activation status-dependent manner. These observations imply that products derived from microbiota or pathobiota can modulate directly T cell polarization, in addition to their mobility. Thus, we suggest that environmental infectious agents (mainly viruses and bacteria) can influence autoimmune diseases in terms of lesions distribution and severity of disease along a pathway that, through engagement of TLRs, involves CD44, its ligands, and T cell functions.

2.2. TLR3 and T Cells. TLR3 recognizes viral components and double-stranded RNA (dsRNA) generated as an intermediate during viral replication. One of the main consequences of its induced-signaling in innate immune cells is the secretion of massive amounts of type I IFNs which play an antiviral role. TLR3 localization in immune cells, including resting T lymphocytes, is mainly intracellular and is capable of recognizing phagocytosed foreign nucleic acids from extracellular space; however, it has been detected at the cell surface of T cells following activation [58], similarly to what happens to TLR2 after stimulation with anti-CD3 antibodies.

The mRNA specific for TLR3 has been found in human $CD8^+$ T cells [59], in both effector memory and effector cells, but not in naïve or central memory cells. Its expression did not affect the cytolytic activity but could costimulate $CD8^+$ T cells, increasing IFN-γ secretion; for example, it has been described also for TLR2 in $CD4^+$ T cells [60].

2.3. TLR4 and T Cells. Tlr4 promotes EAE and arthritis by increasing the secretion of IFN-γ and IL-17 [49, 61], but it has been shown to decrease IFN-γ and IL-17 in experimental colitis [62].

Similar to Tlr2, Tlr4 enhances the severity of autoimmune disorders (EAE) in mice, where it promotes IFN-γ and IL-17 production by $\gamma\delta$ T cells [63] and IL-2 secretion and proliferation of NKT [64]. However, while the role of Tlr4 in triggering autoimmune diseases is well established, its influence in cytokine production is still debated.

Cell trafficking plays a fundamental role in autoimmune diseases. It has been demonstrated that Tlr4 is directly involved in cell migration by its ability to bind fibronectin [65].

2.4. TLR9 and T Cells. TLR9 engagement is important for T cell survival by decreasing apoptosis, promoting entrance in cell cycle, and arresting the rate of dsDNA break repair, as showed in a study about radiotherapy [66]. It has been demonstrated that oligodeoxynucleotides containing CpG motifs (CpG-ODN) cause a costimulation of T cells similar to that obtained by stimulation of CD28, independent of APC. This intrinsic effect of CpG-ODN via Tlr9 on T cells may explain, at least in part, the powerful adjuvanticity of bacterial DNA and of CpG-ODN on antigen-specific T cell responses in vivo and the efficacy of DNA-based vaccines possessing immunostimulatory sequences [67].

3. Microbiota, TLR, and T Cell Modulation

Several studies have shown that individual species of the microbiota modulate the ratio among the different types of immune cells, such as Th17 cells and $Foxp3^+$ regulatory T cells, suggesting that the composition of the microbiota may have an important influence on the immune response. Numerous reports have shown that alterations in gut microbiota can induce the activation of effector T cells over iTregs and, consequently, trigger the development of autoimmune/inflammatory diseases [68]. These studies identified specific gut commensals that are able to induce either Th17 or Treg responses that are, respectively, associated with development or protection from disease [69].

It has been shown that mice lacking components of the TLR signaling machinery, as Tlr2, Tlr4, or MyD88, are

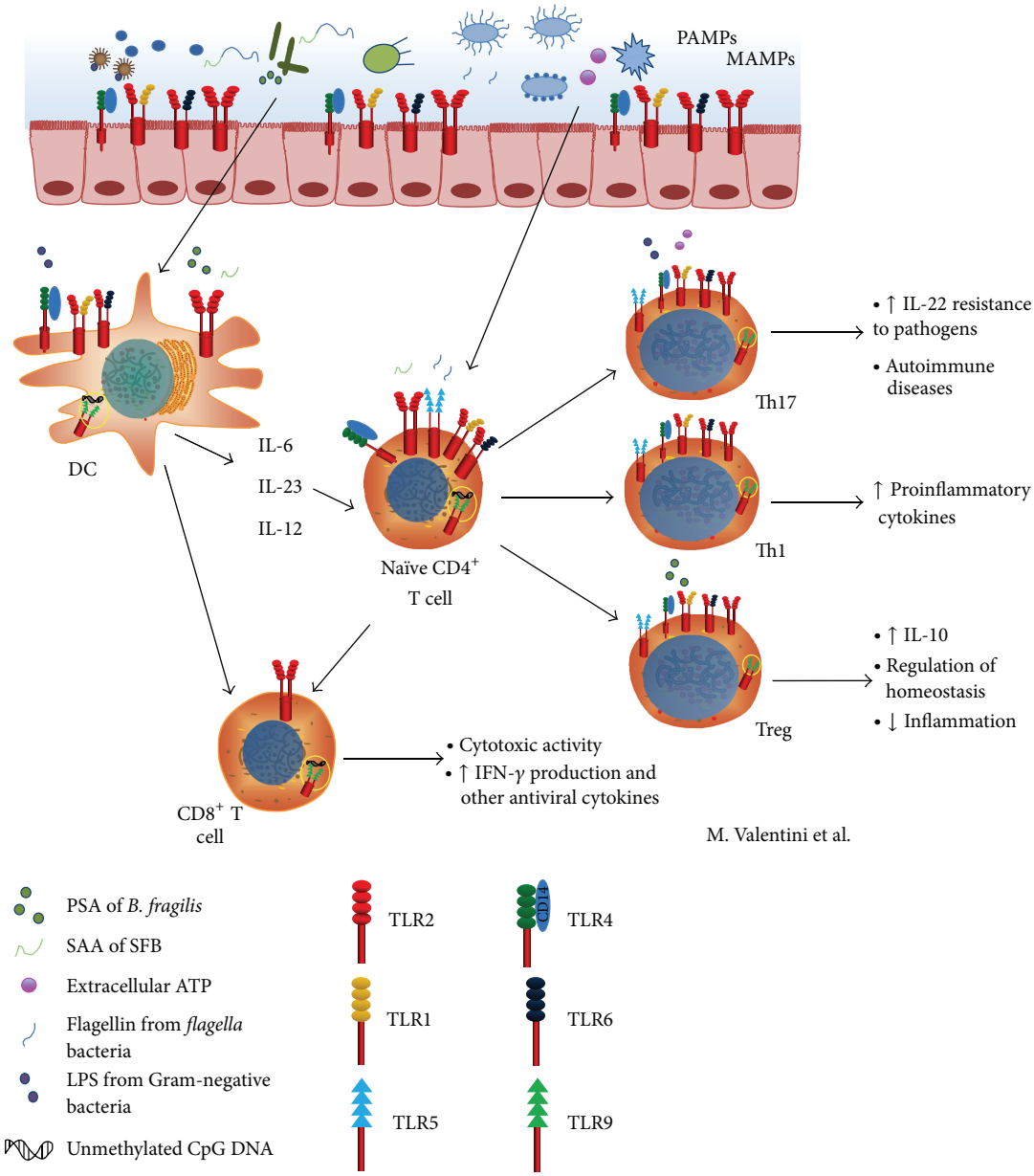


Figure 1: The mammalian gut microbiota is involved in the intestinal homeostasis and shapes the adaptive immune system. The interaction between TLRs and different ligands (such as polysaccharide A of *B. fragilis*, serum amyloid A protein of segmented filamentous bacteria, extracellular ATP from intestinal microbiota, flagellin, LPS, and unmethylated CpG of bacterial DNA) induces $CD8^+$ T activation and naïve $CD4^+$ T polarization towards Th17, Th1, and Treg subsets. The Th17 cells act against pathogens and promote autoimmune disease. The Th1 cells upregulate the production of proinflammatory cytokines, whereas Treg cells produce IL-10 and are involved in the maintenance of homeostasis and in a downregulation of inflammation. Moreover, $CD8^+$ T cells induce the IFN-γ and other cytotoxic mediators production.

highly susceptible to dextran sodium sulfate- (DSS-) induced intestinal inflammation [70, 71].

Microbiota, TLRs, and Tregs. In steady state, the gut is a rich source of TLR ligands from commensal bacteria, some of which have been recently associated with diseases in mouse models of colitis and in human inflammatory bowel diseases. One key antigen that drives gut pathology is flagellin, the major structural subunit of bacterial *flagella* [72]. Flagellin appears to play a central role in the balance and function of T-effector and iTreg cells [73]. In fact, flagellin acts as a TLR5 ligand on $CD4^+$ T cells. Low concentrations of flagellin enhance the expression of Foxp3 and the consequent suppressive effect of Treg, whereas high concentrations stimulate T-effector Tlr cell function.

Several studies on mouse models show that TLR2 is involved in regulatory immune responses in the gut. Minimal disruption of the epithelial barrier, resulting from the administration of ethanol or of AT1002 (*V. cholera zona occludens* toxin hexapeptide), leads to IL-10 secretion in

addition to the induction of persistent $CD4^+$ LAP^+ (latent TGF-β-associated with latency-associated peptide) cells [74]. The mechanism of induction of these cells is not yet clear, but it has been demonstrated to depend on the presence of an intact intestinal flora which acts, at least in part, via Tlr2 stimulation of *lamina propria* $CD11c^+$ DCs. Thus, it is likely that the activation of these cells promotes the maintenance of homeostasis against possible intestinal bacterial invasion, before $Foxp3^+$ iTreg cells reactions [75].

Bacteroides fragilis, a common member of the microbiota, prevents trinitrobenzene sulfonic acid- (TNBS-) induced colitis in mice by producing PSA (capsular polysaccharide A). PSA enhances Treg function via Tlr2 signaling directly in iTregs, promoting tolerance [76]. Administration of PSA prevents or reduces the severity of disease in model of TNBS-induced colitis, and $Tlr2^{-/-}$ animals treated orally with PSA are not protected from colitis [37, 77]. *B. fragilis* can also release PSA in outer membrane vesicles (OMVs) sensed by DC via Tlr2, inducing growth arrest and the production of DNA-damage-inducible protein (Gadd45a) in DC, and an increase in IL-10 production from $Foxp3^+$ iTreg cells [78]. IL-10, in turn, is required for the induction of homeostasis of effector T cell, since blocking the IL-10 receptor during colonization results in immune deviation [79].

Binding of TLR9 to DNA derived from the microbiota plays a critical role in iTreg/T-effector cells balance and in host defense against *Encephalitozoon cuniculi*, a microsporidian parasite that induces diarrheal, respiratory, and neurological diseases in immunocompromised humans [80].

The simultaneous engagement of multiple TLRs by products from microbial communities or invasive pathogens may vary signal strength [81] and effects. An example of this complex mechanism is that components of host's microbiota, once sensed through Tlr2, 4, and 9, activate a protective T cell responses to *Toxoplasma gondii* oral infection [82].

Microbiota, TLRs, and Th17. Despite the large body of works, the role of TLRs in the modulation of the adaptive Th17 cells in the gut is not unequivocal. It has been shown that TLR9-deficient mice have decreased numbers of *lamina propria* Th17 cells [80] and that the differentiation of intestinal Th17 cells is enhanced in vitro by the addition of flagellin, a Tlr5 ligand [83]. These results suggest a potential role for TLR5-dependent signaling also in Th17 differentiation. In contrast, MyD88 and TIR domain-containing adaptor inducing IFN-β (Trif) double deficient mice have normal numbers of LP Th17 cells in the small and large intestines [34, 84]. Thus, further studies are needed to clarify the role of TLRs in the induction of intestinal Th17 cells, in which other molecules signaling through MyD88 or Trif may play a role opposite to that of Tlr9 [85].

In addition to TLR ligands, intestinal bacteria have been shown to provide large amounts of extracellular ATP [12] that is a critical factor produced by intestinal commensal bacteria for the induction of the Th17 phenotype. It has been reported that the addition of the supernatant from intestinal commensal bacteria promotes the polarization of naïve Th cells into Th17 that is severely inhibited by the presence of the ATP degrading enzyme [34].

The presence of segmented filamentous bacteria (SFB) in the murine gut, for example, is associated with induction of Th17-mediated autoimmune/inflammatory diseases such as colitis, arthritis, and EAE [86, 87]. The mechanisms through which SFB-derived molecules induce IgA production and Th17 differentiation are still unknown. It is also unclear if SFB directly activate T and B cells or rather influence other intestinal cells, such as epithelial cells or DC. SFB protect from invasion by the pathogenic microorganism *Citrobacter rodentium* by inducing IL-22 production by Th17 cells that inhibits the growth of this microorganism [88]. Similarly, SFB protect from development of type 1-diabetes (T1D) the nonobese diabetes (NOD) mice [89], a spontaneous model of T1D, in an IL-17-dependent manner.

4. Conclusions

The role of microbiota in the activation and in the modulation of T cells functions is still under scrutiny. The specific mechanisms by which commensals trigger or hamper immune responses and immune-mediated diseases are still unknown. As summarized in Figure 1, we focused our attention on the evidence indicating the possibility that microbiota acts through TLRs expressed by adaptive T cells to provide regulatory signals.

Conflict of Interests

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

Acknowledgments

This work was partially supported by linea D1 (Catholic University of Rome) (Francesco Ria) and Grant 2010/R/34 from the Italian Foundation for Multiple Sclerosis (FISM) (Francesco Ria).

References

[1] L. V. Hooper and A. J. MacPherson, "Immune adaptations that maintain homeostasis with the intestinal microbiota," *Nature Reviews Immunology*, vol. 10, no. 3, pp. 159–169, 2010.

[2] I. Sekirov and B. B. Finlay, "The role of the intestinal microbiota in enteric infection," *The Journal of Physiology*, vol. 587, no. 17, pp. 4159–4167, 2009.

[3] M. J. Molloy, N. Bouladoux, and Y. Belkaid, "Intestinal microbiota: Shaping local and systemic immune responses," *Seminars in Immunology*, vol. 24, no. 1, pp. 58–66, 2012.

[4] N. H. Salzman, M. A. Underwood, and C. L. Bevins, "Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa," *Seminars in Immunology*, vol. 19, no. 2, pp. 70–83, 2007.

[5] The Human Microbiome Project Consortium, "Structure, function and diversity of the healthy human microbiome," *Nature*, vol. 486, pp. 207–214, 2012.

[6] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Microbiology: diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[7] G. A. Preidis and J. Versalovic, "argeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era," *Gastroenterology*, vol. 136, no. 6, pp. 2015–2031, 2009.

[8] J. Qin, R. Li, J. Raes et al., "A human gut microbial gene catalogue established by metagenomic sequencing," *Nature*, vol. 464, no. 7285, pp. 59–65, 2010.

[9] P. A. Bron, P. van Baarlen, and M. Kleerebezem, "Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa," *Nature Reviews Microbiology*, vol. 10, no. 1, pp. 66–78, 2012.

[10] C. L. Maynard, C. O. Elson, R. D. Hatton, and C. T. Weaver, "Reciprocal interactions of the intestinal microbiota and immune system," *Nature*, vol. 489, no. 7415, pp. 231–241, 2012.

[11] E. Slack, S. Hapfelmeier, B. Stecher et al., "Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism," *Science*, vol. 325, no. 5940, pp. 617–620, 2009.

[12] T. Tanoue, Y. Umesaki, and K. Honda, "Immune responses to gut microbiota-commensals and pathogens," *Gut Microbes*, vol. 1, pp. 224–233, 2010.

[13] T. Kawai and S. Akira, "TLR signaling," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 816–825, 2006.

[14] E. C. Lavelle, C. Murphy, L. A. J. O'Neill, and E. M. Creagh, "The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis," *Mucosal Immunology*, vol. 3, no. 1, pp. 17–28, 2010.

[15] S. Akira, S. Uematsu, and O. Takeuchi, "Pathogen recognition and innate immunity," *Cell*, vol. 124, no. 4, pp. 783–801, 2006.

[16] M. T. Bailey, "The contributing role of the intestinal microbiota in stressor-induced increases in susceptibility to enteric infection and systemic immunomodulation," *Hormones and Behavior*, vol. 62, no. 3, pp. 286–294, 2012.

[17] T. P. Monie, G. L. Hold, and I. Mukhopadhya, "Innate immune sensors and gastrointestinal bacterial infections," *Clinical and Developmental Immunology*, vol. 2011, Article ID 579650, 11 pages, 2011.

[18] J. L. Kubinak and J. L. Round, "Toll-like receptors promote mutually beneficial commensal-host interactions," *PLoS Pathogens*, vol. 8, no. 7, Article ID e1002785, 2012.

[19] T. Ichinohe, I. K. Pang, Y. Kumamoto et al., "Microbiota regulates immune defense against respiratory tract influenza a virus infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 13, pp. 5354–5359, 2011.

[20] F. Y. Liew, D. Xu, E. K. Brint, and L. A. J. O'Neill, "Negative regulation of toll-like receptor-mediated immune responses," *Nature Reviews Immunology*, vol. 5, no. 6, pp. 446–458, 2005.

[21] T. Horng, G. M. Barton, R. A. Flavell, and R. Medzhitov, "The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors," *Nature*, vol. 420, no. 6913, pp. 329–333, 2002.

[22] G. Melmed, L. S. Thomas, N. Lee et al., "Human intestinal epithelial cells are broadly unresponsive to toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut," *Journal of Immunology*, vol. 170, no. 3, pp. 1406–1415, 2003.

[23] L. L. N. Hubbard and B. B. Moore, "IRAK-M regulation and function in host defense and immune homeostasis," *Infectious Disease Reports*, vol. 2, no. 1, pp. 22–29, 2010.

[24] Y. Shen, M. L. G. Torchia, G. W. Lawson, C. L. Karp, J. D. Ashwell, and S. K. Mazmanian, "Outer membrane vesicles of a human commensal mediate immune regulation and disease protection," *Cell Host and Microbe*, vol. 12, no. 4, pp. 509–520, 2012.

[25] M. Fukata, Y. Hernandez, D. Conduah et al., "Innate immune signaling by toll-like receptor-4 (TLR4) shapes the inflammatory microenvironment in colitis-associated tumors," *Inflammatory Bowel Diseases*, vol. 15, no. 7, pp. 997–1006, 2009.

[26] L. Shang, M. Fukata, N. Thirunarayanan et al., "Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria," *Gastroenterology*, vol. 135, no. 2, pp. 529.el–538.el, 2008.

[27] F. Hayashi, K. D. Smith, A. Ozinsky et al., "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5," *Nature*, vol. 410, no. 6832, pp. 1099–1103, 2001.

[28] K. J. Ishii and S. Akira, "Innate immune recognition of, and regulation by, DNA," *Trends in Immunology*, vol. 27, no. 11, pp. 525–532, 2006.

[29] J. Lee, J. Mo, K. Katakura et al., "Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells," *Nature Cell Biology*, vol. 8, no. 12, pp. 1327–1336, 2006.

[30] D. R. Littman and A. Y. Rudensky, "Th17 and regulatory T cells in mediating and restraining inflammation," *Cell*, vol. 140, no. 6, pp. 845–858, 2010.

[31] T. Korn, E. Bettelli, M. Oukka, and V. K. Kuchroo, "IL-17 and Th17 cells," *Annual Review of Immunology*, vol. 27, pp. 485–517, 2009.

[32] L. Durelli, L. Conti, M. Clerico et al., "T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-β," *Annals of Neurology*, vol. 65, no. 5, pp. 499–509, 2009.

[33] P. R. Mangan, L. E. Harrington, D. B. O'Quinn et al., "Transforming growth factor-β induces development of the T H17 lineage," *Nature*, vol. 441, no. 7090, pp. 231–234, 2006.

[34] K. Atarashi, J. Nishimura, T. Shima et al., "ATP drives lamina propria TH17 cell differentiation," *Nature*, vol. 455, no. 7214, pp. 808–812, 2008.

[35] J. D. Fontenot and A. Y. Rudensky, "A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3," *Nature Immunology*, vol. 6, no. 4, pp. 331–337, 2005.

[36] M. Feuerer, Y. Shen, D. R. Littman, C. Benoist, and D. Mathis, "How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets," *Immunity*, vol. 31, no. 4, pp. 654–664, 2009.

[37] J. L. Round and S. K. Mazmanian, "Inducible Foxp3$^+$ regulatory T-cell development by a commensal bacterium of the intestinal microbiota," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 27, pp. 12204–12209, 2010.

[38] C. Pasare and R. Medzhitov, "Control of B-cell responses by Toll-like receptors," *Nature*, vol. 438, no. 7066, pp. 364–368, 2005.

[39] D. J. Rawlings, M. A. Schwartz, S. W. Jackson, and A. Meyer-Bahlburg, "Integration of B cell responses through Toll-like receptors and antigen receptors," *Nature Reviews Immunology*, vol. 12, no. 4, pp. 282–294, 2012.

[40] T. Matsuguchi, K. Takagi, T. Musikacharoen, and Y. Yoshikai, "Gene expressions of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes," *Blood*, vol. 95, no. 4, pp. 1378–1385, 2000.

[41] I. Caramalho, T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot, "Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide," *Journal of Experimental Medicine*, vol. 197, no. 4, pp. 403–411, 2003.

[42] A. Zanin-Zhorov and I. R. Cohen, "Signaling via TLR2 and TLR4 directly down-regulates T cell effector functions: the regulatory face of danger signals," *Frontiers in Immunology*, vol. 4, article 211, 2013.

[43] H. Liu, M. Komai-Koma, D. Xu, and F. Y. Liew, "Toll-like receptor 2 signaling modulates the functions of $CD4^+CD25^+$ regulatory T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 7048–7053, 2006.

[44] Y. Mokuno, T. Matsuguchi, M. Takano et al., "Expression of toll-like receptor 2 on γδ T cells bearing invariant Vγ6/Vδ1 induced by Escherichia coli infection in mice," *Journal of Immunology*, vol. 165, no. 2, pp. 931–940, 2000.

[45] A. Cottalorda, B. C. Mercier, F. M. Mbitikon-Kobo et al., "TLR2 engagement on memory CD8+ T cells improves their cytokine-mediated proliferation and IFN-γ secretion in the absence of Ag," *European Journal of Immunology*, vol. 39, no. 10, pp. 2673–2681, 2009.

[46] B. C. Mercier, A. Cottalorda, C. A. Coupet, J. Marvel, and N. Bonnefoy-Bérard, "TLR2 engagement on CD8 T cells enables generation of functional memory cells in response to a suboptimal TCR signal," *Journal of Immunology*, vol. 182, no. 4, pp. 1860–1867, 2009.

[47] N. Asprodites, L. Zheng, D. Geng, C. Velasco-Gonzalez, L. Sanchez-Perez, and E. Davila, "Engagement of Toll-like receptor-2 on cytotoxic T-lymphocytes occurs in vivo and augments antitumor activity," *The FASEB Journal*, vol. 22, no. 10, pp. 3628–3637, 2008.

[48] H. Shimizu, T. Matsuguchi, Y. Fukuda et al., "Toll-like receptor 2 contributes to liver injury by Salmonella infection through Fas ligand expression on NKT cells in mice," *Gastroenterology*, vol. 123, no. 4, pp. 1265–1277, 2002.

[49] T. Imanishi, H. Hara, S. Suzuki, N. Suzuki, S. Akira, and T. Saito, "Cutting edge: TLR2 directly triggers Th1 effector functions," *Journal of Immunology*, vol. 178, no. 11, pp. 6715–6719, 2007.

[50] C. Nicolò, G. Di Sante, A. Procoli et al., "*M tuberculosis* in the adjuvant modulates time of appearance of CNS-specific effector T cells in the spleen through a polymorphic site of TLR2," *PLoS ONE*, vol. 8, no. 2, Article ID e55819, 2013.

[51] J. M. Reynolds, G. J. Martinez, Y. Chung, and C. Dong, "Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 32, pp. 13064–13069, 2012.

[52] X. Chen, M. Zhang, X. Zhu et al., "Engagement of toll-like receptor 2 on $CD4^+$ T cells facilitates local immune responses in patients with tuberculous pleurisy," *The Journal of Infectious Diseases*, vol. 200, no. 3, pp. 399–408, 2009.

[53] M. M. Babu, M. L. Priya, A. T. Selvan et al., "A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins," *Journal of Bacteriology*, vol. 188, no. 8, pp. 2761–2773, 2006.

[54] T. Watanabe, T. Inoue, H. Ochi et al., "Lipid A directly inhibits IL-4 production by murine Th2 cells but does not inhibit IFN-gamma production by Th1 cells," *European Journal of Immunology*, vol. 29, no. 2, pp. 413–418, 1999.

[55] J. M. Reynolds, B. P. Pappu, J. Peng et al., "Toll-like receptor 2 signaling in $CD4^+$ T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease," *Immunity*, vol. 32, no. 5, pp. 692–702, 2010.

[56] M. H. Nyirenda, L. Sanvito, P. J. Darlington et al., "TLR2 stimulation drives human naive and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function," *Journal of Immunology*, vol. 187, no. 5, pp. 2278–2290, 2011.

[57] A. Rostami and B. Ciric, "Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination," *Journal of the Neurological Sciences*, vol. 333, no. 1, pp. 76–87, 2013.

[58] D. Wesch, S. Beetz, H. Oberg, M. Marget, K. Krengel, and D. Kabelitz, "Direct costimulatory effect of TLR3 ligand poly(I:C) on human γδ lymphocytes," *Journal of Immunology*, vol. 176, no. 3, pp. 1348–1354, 2006.

[59] V. Hornung, S. Rothenfusser, S. Britsch et al., "Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides," *Journal of Immunology*, vol. 168, no. 9, pp. 4531–4537, 2002.

[60] J. Tabiasco, E. Devêvre, N. Rufer et al., "Human effector CD8+ T lymphocytes express TLR3 as a functional coreceptor," *Journal of Immunology*, vol. 177, no. 12, pp. 8708–8713, 2006.

[61] J. A. Sullivan, E. H. Kim, E. H. Plisch, S. L. Peng, and M. Suresh, "FOXO3 regulates CD8 T cell memory by T cell-intrinsic mechanisms," *PLoS Pathogens*, vol. 8, no. 2, Article ID e1002533, 2012.

[62] J. M. González-Navajas, S. Fine, J. Law et al., "TLR4 signaling in effector $CD4^+$ T cells regulates TCR activation and experimental colitis in mice," *The Journal of Clinical Investigation*, vol. 120, no. 2, pp. 570–581, 2010.

[63] Y. Cui, L. Kang, L. Cui, and W. He, "Human γδ T cell recognition of lipid A is predominately presented by CD1b or CD1c on dendritic cells," *Biology Direct*, vol. 4, article 47, 2009.

[64] R. R. Kulkarni, A. I. Villanueva, I. Elawadli et al., "Costimulatory activation of murine invariant natural killer T cells by toll-like receptor agonists," *Cellular Immunology*, vol. 277, no. 1-2, pp. 33–43, 2012.

[65] A. Zanin-Zhorov, G. Tal-Lapidot, L. Cahalon et al., "Cutting edge: T cells respond to lipopolysaccharide innately via TLR4 signaling," *Journal of Immunology*, vol. 179, no. 1, pp. 41–44, 2007.

[66] L. Zheng, N. Asprodites, A. H. Keene, P. Rodriguez, K. D. Brown, and E. Davila, "TLR9 engagement on CD4 T lymphocytes represses (gamma)-radiation-induced apoptosis through activation of checkpoint kinase response elements," *Blood*, vol. 111, no. 5, pp. 2704–2713, 2008.

[67] A. Landrigan, M. T. Wong, and P. J. Utz, "CpG and non-CpG oligodeoxynucleotides directly costimulate mouse and human CD4+ T cells through a TLR9- and MyD88-independent mechanism," *Journal of Immunology*, vol. 187, no. 6, pp. 3033–3043, 2011.

[68] M. M. Kosiewicz, A. L. Zirnheld, and P. Alard, "Gut microbiota, immunity, and disease: a complex relationship," *Frontiers in Microbiology*, vol. 2, article 180, 2011.

[69] M. M. Kosiewicz, G. W. Dryden, A. Chhabra, and P. Alard, "Relationship between gut microbiota and development of T cell associated disease," *FEBS Letters*, 2014.

[70] S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, 2004.

[71] A. Araki, T. Kanai, T. Ishikura et al., "MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis," *Journal of Gastroenterology*, vol. 40, no. 1, pp. 16–23, 2005.

[72] T. S. Steiner, "How flagellin and toll-like receptor 5 contribute to enteric infection," *Infection and Immunity*, vol. 75, no. 2, pp. 545–552, 2007.

[73] M. E. Himmel, G. Hardenberg, C. A. Piccirillo, T. S. Steiner, and M. K. Levings, "The role of T-regulatory cells and Toll-like receptors in the pathogenesis of human inflammatory bowel disease," *Immunology*, vol. 125, no. 2, pp. 145–153, 2008.

[74] M. Boirivant, A. Amendola, A. Butera et al., "A transient breach in the epithelial barrier leads to regulatory T-cell generation and resistance to experimental colitis," *Gastroenterology*, vol. 135, no. 5, pp. 1612.e5–1623.e5, 2008.

[75] W. Strober, "Inside the microbial and immune labyrinth: gut microbes: friends or fiends?" *Nature Medicine*, vol. 16, no. 11, pp. 1195–1197, 2010.

[76] J. L. Round, S. M. Lee, J. Li et al., "The toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota," *Science*, vol. 332, no. 6032, pp. 974–977, 2011.

[77] K. Atarashi, T. Tanoue, T. Shima et al., "Induction of colonic regulatory T cells by indigenous *Clostridium* species," *Science*, vol. 331, no. 6015, pp. 337–341, 2011.

[78] C. L. Maynard, L. E. Harrington, K. M. Janowski et al., "Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10," *Nature Immunology*, vol. 8, no. 9, pp. 931–941, 2007.

[79] M. B. Geuking, J. Cahenzli, M. A. E. Lawson et al., "Intestinal bacterial colonization induces mutualistic regulatory T cell responses," *Immunity*, vol. 34, no. 5, pp. 794–806, 2011.

[80] J. A. Hall, N. Bouladoux, C. M. Sun et al., "Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses," *Immunity*, vol. 29, no. 4, pp. 637–649, 2008.

[81] W. S. Garrett, J. I. Gordon, and L. H. Glimcher, "Homeostasis and Inflammation in the Intestine," *Cell*, vol. 140, no. 6, pp. 859–870, 2010.

[82] A. Benson, R. Pifer, C. L. Behrendt, L. V. Hooper, and F. Yarovinsky, "Gut commensal bacteria direct a protective immune response against *Toxoplasma gondii*," *Cell Host and Microbe*, vol. 6, no. 2, pp. 187–196, 2009.

[83] S. Uematsu, K. Fujimoto, M. H. Jang et al., "Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5," *Nature Immunology*, vol. 9, no. 7, pp. 769–776, 2008.

[84] I. I. Ivanov, R. D. L. Frutos, N. Manel et al., "Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine," *Cell Host and Microbe*, vol. 4, no. 4, pp. 337–349, 2008.

[85] B. A. Duerkop, S. Vaishnava, and L. V. Hooper, "Immune responses to the microbiota at the intestinal mucosal surface," *Immunity*, vol. 31, no. 3, pp. 368–376, 2009.

[86] Y. K. Lee, J. S. Menezes, Y. Umesaki, and S. K. Mazmanian, "Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, supplement 1, pp. 4615–4622, 2011.

[87] W. Yi, N. P. Seth, T. Martillotti, K. W. Wucherpfennig, D. B. Sant'Angelo, and L. K. Denzin, "Targeted regulation of self-peptide presentation prevents type I diabetes in mice without disrupting general immunocompetence," *Journal of Clinical Investigation*, vol. 120, no. 4, pp. 1324–1336, 2010.

[88] I. I. Ivanov, K. Atarashi, N. Manel et al., "Induction of intestinal Th17 cells by segmented filamentous bacteria," *Cell*, vol. 139, no. 3, pp. 485–498, 2009.

[89] M. A. Kriegel, E. Sefik, J. A. Hill, H.-J. Wu, C. Benoist, and D. Mathis, "Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 28, pp. 11548–11553, 2011.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 696812, 11 pages
http://dx.doi.org/10.1155/2014/696812

Clinical Study

Uncomplicated Diverticular Disease: Innate and Adaptive Immunity in Human Gut Mucosa before and after Rifaximin

Rossella Cianci,[1] Simona Frosali,[1] Danilo Pagliari,[1] Paola Cesaro,[2] Lucio Petruzziello,[2] Fabio Casciano,[1] Raffaele Landolfi,[1] Guido Costamagna,[2] and Franco Pandolfi[1]

[1] *Department of Medical Science, Catholic University of the Sacred Heart, 'A. Gemelli' University Hospital, Largo A. Gemelli, 8-00168 Rome, Italy*
[2] *Endoscopy Unit, Catholic University of the Sacred Heart, 'A. Gemelli' University Hospital, Largo A. Gemelli, 8-00168 Rome, Italy*

Correspondence should be addressed to Guido Costamagna; gcostamagna@rm.unicatt.it

Received 16 April 2014; Revised 12 June 2014; Accepted 18 June 2014; Published 16 July 2014

Academic Editor: Anis Larbi

Background/Aim. Uncomplicated diverticular disease (UDD) is a frequent condition in adults. The pathogenesis of symptoms remains unknown. Bacteria are able to interact with Toll-like receptors (TLRs) and to induce inflammation through both innate immunity and T-cell recruitment. We investigated the pattern of TLRs 2 and 4 and the intestinal homing in patients with UDD before and after a course of Rifaximin. *Methods*. Forty consecutive patients with UDD and 20 healthy asymptomatic subjects were enrolled. Among UDD patients, 20 were assigned to a 2-month course of treatment with Rifaximin 1.2 g/day for 15 days/month and 20 received placebo. Blood sample and colonic biopsies were obtained from patients and controls. The samples were collected and analyzed at baseline and at the end of treatment. Flow cytometry was performed using monoclonal antibodies (CD3, CD4, CD8, CD103, TCR-gamma/delta, CD14, TLR2, and TLR4). *Results*. In UDD, TLR2 and TLR4 expression on immune cell subpopulations from blood and mucosa of the affected colon are altered as compared with controls. Rifaximin treatment induced significant modifications of altered conditions. *Conclusions*. Our data show the role of TLRs in the development of inflammation in UDD. TLRs distribution is altered in UDD and these alterations are reversed after antibiotic treatment. This trial is registered with ClinicalTrials.gov: NCT02068482.

1. Introduction

Colon diverticulosis is a frequent condition in adults in Western countries and several patients experience clinical symptoms even when diverticulosis is not complicated by diverticulitis. This condition is being referred to as uncomplicated diverticular diseases (UDD) [1]. UDD symptoms pathogenesis remains unknown, but alterations in the diet and gut microbiota may be involved and could be responsible for "chronic low mucosal inflammation" [2]. Intestinal motility exerts a major control on gut microflora through the sweeping of luminal contents [3]. An alteration of the intestinal motility, especially decreasing anaerobic bacteria, can influence intestinal inflammation and be beneficial in both clinical and experimental models of colitis [4]. Rifaximin is beneficial to mice colitis [5]. Animal data indicate that a reduced load of bacteria may be useful in the prevention of colitis in susceptible individuals [5]. Although there is no supporting evidence from placebo-controlled trials, recommendations for management of acute episodes of UDD include medical treatment with broad-spectrum antibiotics [6, 7]. We have recently shown that both central and mucosal immunity are altered in UDD with increased recruitment of CD103 lymphocytes; treatment with Rifaximin ameliorates clinical symptoms (when present) and reduces CD103 levels, suggesting decreased mobilization of mucosal homing [8].

Rifaximin is an antibiotic that acts locally in the gastrointestinal tract with a broad spectrum of antibacterial activity. Fecal concentrations of Rifaximin are known to largely exceed the minimum inhibitory concentration values of pathogenic enteric bacteria. Rifaximin, instead of other systemic absorbed antibiotics, acts in the gastrointestinal

tract modifying the gut microbiota (at the concentrations used, its action is mainly directed to pathobionts and much less to physiological gut flora). Furthermore, at the same time, Rifaximin, being a nonabsorbed antibiotic, has only little systemic effects [9].

To better understand the mechanisms involved, we reasoned that Rifaximin treatment may reverse immune system alteration by reducing bacteria related activation. Bacteria activate the immune system through specific receptors referred to as Toll-like receptors (TLRs) [10]. Bacterial lipopeptides (BLP) and lipopolysaccharides (LPS) are recognized by TLR2 and TLR4, respectively.

TLRs are involved in the generation of innate and adaptive immunity [11, 12]. Recent studies have shown that T cells also express certain types of TLRs [13, 14]. These TLRs can function as costimulatory receptors that complement T cell receptor- (TCR-) induced signals to enhance effector T cell activation [15]. Thus, TLRs also participate in adaptive immune response. No data have been reported on TLRs in humans affected by UDD. We therefore investigated TLR2, TLR4, and intestinal homing in UDD before and after a course of Rifaximin.

2. Material and Methods

The local Ethics Committee approved the study protocol and a written informed consent was obtained according to the principles of the Declaration of Helsinki (1983).

2.1. Patients' Recruitment. Over a period of 6 months, 40 consecutive patients with UDD (abdominal pain in the lower abdominal quadrant and change in bowel habit) and 20 healthy asymptomatic subjects undergoing screening colonoscopy for colorectal cancer were enrolled. The 40 patients with UDD were randomly assigned into two groups:

(i) 20 patients with UDD were assigned to a 2-month treatment with Rifaximin 1.2 g/day for 15 days/month.

(ii) 20 patients with UDD on the same time received *placebo*.

Colonoscopy with multiple biopsies and blood samples were taken from patients and controls and repeated in patients at the end of the 2-month Rifaximin treatment or *placebo* course. Control healthy group was matched for gender, age, and body mass index (BMI) to the other two groups of patients.

Inclusion criteria in UDD patients group were as follows: endoscopic evidence of extensive diverticula limited to the sigmoid and descending colon; continuous gastrointestinal symptoms for at least 3 months; and age ≥18 years.

Exclusion criteria were as follows: inflammatory bowel diseases (IBD); mucosal inflammation at endoscopy and histology; present or past episodes of acute diverticulitis; colonic surgery; intestinal and extraintestinal cancer; use of antibiotics, anti-inflammatory drugs, probiotics, PPI, steroids, or fibers in the previous 12 weeks; hematological diseases; and pregnancy.

Acute diverticulitis was excluded on the basis of clinical signs and laboratory data.

Each patient underwent an abdominal ultrasonography to exclude abdominal masses or abscesses. Patients' identification was performed by the presence of left colonic diverticula, whilst controls had a normal colon.

2.2. Colonic Mucosa. Bioptic tissue samples were incubated with 100 UI/mL of human recombinant interleukin-2 (IL-2) to allow lymphocyte growth as previously described [8]. When tissue infiltrating lymphocytes (TILs) reached a sufficient number to perform the immunophenotype, surface markers were studied by immunofluorescence.

2.3. Peripheral Blood. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque according to standard procedures.

Whole blood cells (WBC), for granulocytes analysis, were isolated from EDTA-treated blood through red cell lysis. Briefly, 1 mL of venous blood was incubated with 1 mL of PBS and 10 mL of isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, and pH 7.4) for 10 min. Next, after two washes in PBS, the cells were stained as described below.

2.4. Flow Cytometry. PBMC, WBC, and TILs were stained for 30 min at 4°C with the following anti-human monoclonal antibodies (mAb): PE-Cy7 or PE conjugated-CD3 (clone SK7), PE-Cy7 or PE conjugated-CD4 (clone SK3), PE-Cy7 or PE conjugated-CD8 (clone SK1), FITC conjugated-CD103 (clone Ber-ACT8), PE conjugated-TCRgd (clone 11F2), PE-Cy7 conjugated-CD14 (clone M5E2), Alexa 482 conjugated-TLR2 (clone 11G7) (all from BD, San Diego, CA), and APC conjugated-TLR4 (clone HTA125) (eBiosciences, San Diego, CA).

2.5. Gating Strategy. Each analysis was performed using at least 100.000 cells that were gated in the region of the lymphocyte-monocyte population or in granulocytes population, as determined by light-scatter properties (forward scatter versus side-scatter).

Flow analysis was performed by a standardized 3- or 4-colour analysis protocol that was gated on cell stained with one fluorochrome, followed by 2-colour analysis of cells stained with the prescribed remaining fluorochromes.

Quadrants of dot plot were set using appropriate isotype controls for each antibody. Appropriate fluorochrome-conjugated isotype-matched mAbs (Beckman Coulter) were used as control for background staining in each flow acquisition. In these assays, careful colour compensation was performed before cell analysis. Expression of TLRs in lymphocytes was performed analysing at least 500 events. Mean fluorescence intensity (MFI) was calculated only for positive events after subtraction of specific isotype control MFI. All samples were analyzed with a FACS Calibur and CellQuest software (BD, Franklin Lakes, NJ).

Table 1: Demographic and clinical characteristics of UDD patients and controls.

	UDD patients ($n = 40$)	Controls ($n = 21$)
Gender (male/female)	21/19	5/16
Age, median (range), years	67.5 (39–84)	53 (20–78)
Smoking habit (yes)	8	7
Alcohol intake (>60 g/day)	18	7
Weight, mean (range) (Kg)	70.38 (52–100)	66.14 (52–84)
Height, mean (range) (m)	1.66 (1.41–1.80)	1.67 (1.50–1.80)
Body mass index (range)	22.6 (17–24)	22 (17–22)

2.6. Statistical Analysis. The statistical analysis was carried out using the GraphPad 6.0 statistical package. The Mann-Whitney U test was applied to the continuous variables, the changes observed in unpaired groups, and the Wilcoxon test in paired groups.

The Kruskal-Wallis test H was applied to evaluate whether significant differences exist between different samples and also for comparisons between independent samples. When appropriate, the correction for multiple comparisons was applied to the statistical significance.

3. Results

Demographic and clinical features of UDD patients and controls are summarized in Table 1. No statistical differences in smoking habits, alcohol daily intake, and body mass index (BMI) were observed between the two groups.

Good compliance to Rifaximin (>90% of the appropriate dose) was observed in all patients. A statistically significant decrease in symptoms was observed in patients two months after treatment. No adverse events to the drug or complications were observed.

3.1. Expression of TLRs. In peripheral blood, at baseline, both the percentages of TRL2 and TRL4 lymphocytes were significantly higher ($P = 0.0004$ and $P < 0.0096$, resp.) in patients than in controls (Figures 1(a) and 1(b)).

After Rifaximin treatment (AR), the percentages of TRL2 and TRL4 lymphocytes in patients remained similar to basal values obtained before Rifaximin treatment (BR). However, TLR2 and TLR4 increased significantly ($P = 0.0003$ and $P = 0.0104$, resp.) in patients who received placebo treatment (after placebo or AP), as compared with patients who received Rifaximin treatment (Figures 1(a) and 1(b)). These data suggest that, in the lack of antimicrobial treatment, patient with UDD experiences an increase of TLRs expression.

In the sigmoid mucosa, at baseline, the percentages of TRL2 lymphocytes were lower ($P = 0.0091$) in patients than in controls, while TLR4 showed no significant differences (Figures 1(c) and 1(d)). In patients after Rifaximin, in a pattern similar to that observed in the peripheral blood, patients expressed significantly reduced percentages of both TLR2 and TLR4 ($P = 0.0424$ and $P = 0.0022$, resp.) in comparison to values observed in placebo treated patients (Figures 1(c) and 1(d)). Our data suggest that Rifaximin treatment contributes to maintain stable levels of TLRs and this is possibly related to the control of intestinal microflora.

Since the transverse mucosa of patients with UDD is supposed to be normal, lymphocytes in the transverse mucosa were studied as controls. In fact, no significant differences were observed before and after Rifaximin treatment (Figure 1(e)).

For a better understood role of TLR2 and TLR4 we also analyzed the subpopulations CD4 and CD8 lymphocytes as reported in Table 2.

TLR2 and TLR4 were analyzed in PBMC and sigma mucosa of same patient. To clarify the way in which the modulation of TLR2 and TLR4 in PBMC and sigma mucosa occurred, we compared the modulation of TLR2 and TLR4 expression between PBMC and gut in the different patient groups (BR and AR and BP and AP) (Figure 2). We observed the same trend of TLRs modulation in both the mucosa and the PBMC.

We observed coexpression of TRL2 and TLR4 on lymphocyte in both PBMC and sigma mucosa of the same patient. Moreover, we reported that the modulation of TLR2 and TLR4 correlated in PBMC (95% confidence interval 0.21 to 0.447; $r = 0.2366$; $P < 0.05$) and in transverse mucosa (95% confidence interval 0.052 to 0.687; $r = 0.4195$; $P < 0.05$), but not in sigma mucosa (95% confidence interval −0.334 to 0.256; $r = -0.043$; $P < 0.05$).

The phenotype of TLR lymphocytes was also evaluated for the CD4 and CD8 lymphocytes subpopulations to better understand the effect of Rifaximin in UDD patients; the results are summarized in Table 2. We show that both TLR2-CD8 and TLR2-CD4 cells were increased in patients PBMC as compared to controls. In the sigma mucosa, after Rifaximin, TLR4-CD4 cells were significantly reduced.

In peripheral blood, at baseline, no changes were found in TRL2 monocytes (CD14+) percentages. The percentages of TRL4 monocytes were lower ($P = 0.0019$) in patients with respect to controls (Figures 3(a) and 3(b)). After Rifaximin treatment the percentages of TRL2/CD14+ increased significantly ($P = 0.0039$) with respect to before Rifaximin (Figure 3(a)); no changes were found in TRL4 monocytes percentages (Figure 3(b)).

Analysis of MFI showed that, in monocytes, the TLR4 MFI baseline was significantly lower than control ($P =$

Table 2: TLRs phenotype in CD4 and CD8 lymphocytes subpopulation.

	PBMC				Sigma				Transverse			
	CD4 TLR2	CD8 TLR2	CD4 TLR4	CD8 TLR4	CD4 TLR2	CD8 TLR2	CD4 TLR4	CD8 TLR4	CD4 TLR2	CD8 TLR2	CD4 TLR4	CD8 TLR4
CTR	0.069 ± 0.016	0.094 ± 0.018	0.354 ± 0.206	0.090 ± 0.020	0.222 ± 0.048	0.250 ± 0.078	0.400 ± 0.11	0.356 ± 0.096	0.224 ± 0.03	0.255 ± 0.06	0.421 ± 0.10	0.45 ± 0.11
PZ	0.193 ± 0.041**	0.244 ± 0.037*	0.169 ± 0.035*	0.26 ± 0.059	0.191 ± 0.038	0.239 ± 0.069	0.371 ± 0.104	0.284 ± 0.078	0.171 ± 0.046	0.246 ± 0.10	0.308 ± 0.19	0.489 ± 0.28
BR	0.082 ± 0.015	0.276 ± 0.070	0.133 ± 0.042	0.233 ± 0.086	0.182 ± 0.055	0.258 ± 0.096	0.392 ± 0.17	0.285 ± 0.11	0.171 ± 0.05	0.246 ± 0.10	0.308 ± 0.19	0.489 ± 0.28
AR	0.115 ± 0.044	0.161 ± 0.045	0.197 ± 0.084	0.221 ± 0.074	0.092 ± 0.029	0.143 ± 0.057	0.076 ± 0.037*	0.194 ± 0.07	0.13 ± 0.06	0.232 ± 0.16	0.354 ± 0.21	0.488 ± 0.29
BP	0.251 ± 0.060	0.264 ± 0.058	0.205 ± 0.057	0.293 ± 0.082	0.371 ± 0.18	0.219 ± 0.101	0.592 ± 0.27	0.284 ± 0.12	ND	ND	ND	ND
AP	0.352 ± 0.084**	0.416 ± 0.081**	0.269 ± 0.066	0.293 ± 0.086	0.536 ± 0.21	0.419 ± 0.133*	1.482 ± 0.70*	1.442 ± 0.60	ND	ND	ND	ND

The values are reported as Mean ± SEM $P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ patients versus control (health or baseline); $P < 0.01$, $P < 0.05$ between treatments. Healthy control (CTR), UDD patients (PZ), before Rifaximin (BF), after Rifaximin (AR), before placebo (BP), and after placebo (AP).

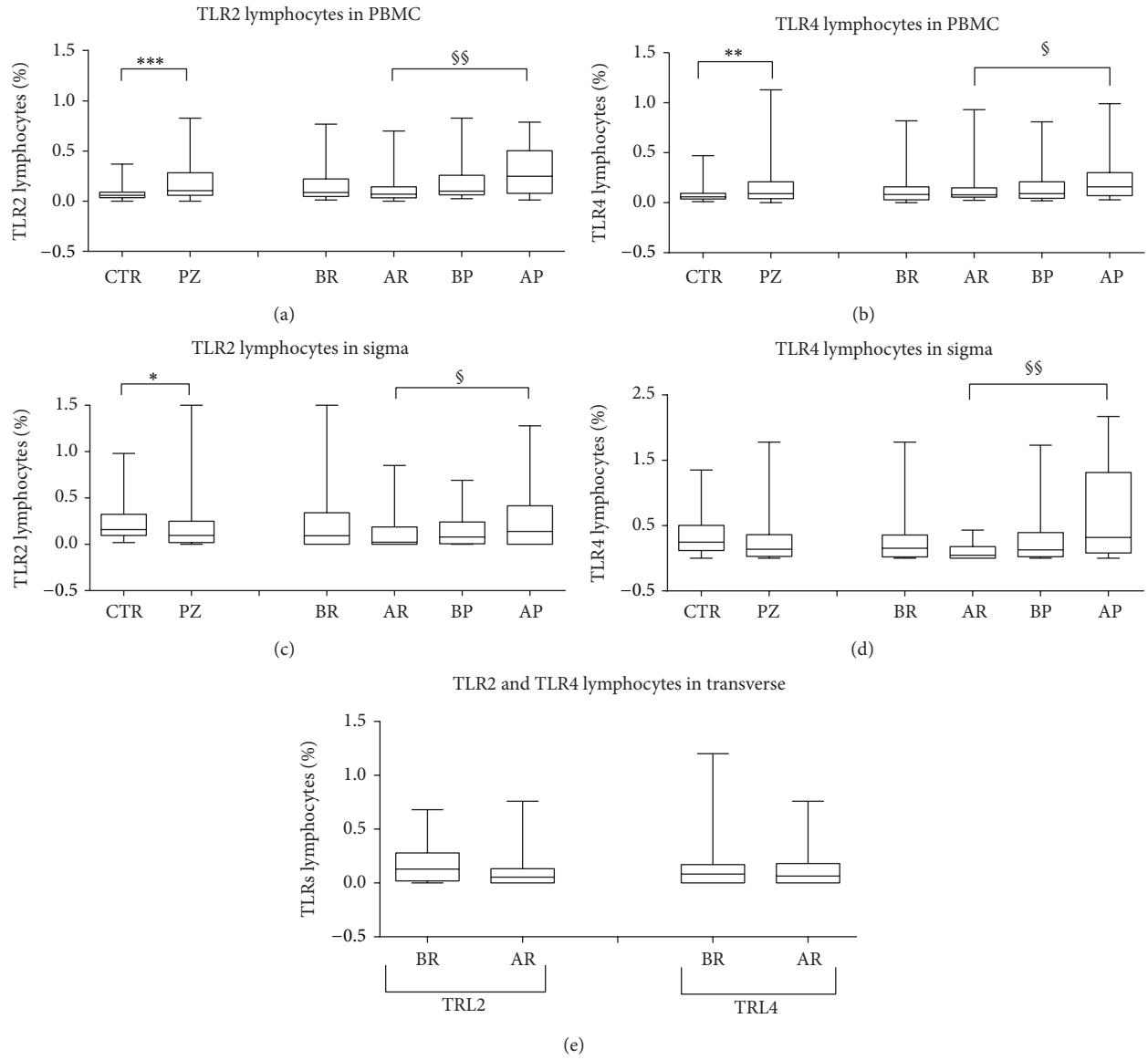


Figure 1: Expression of TLRs in lymphocytes. (a, b) TLR2 and TLR4 in peripheral blood lymphocytes, at baseline and after Rifaximin and placebo treatments. (c, d) TLR2 and TLR4 in sigma mucosa lymphocytes at baseline and after Rifaximin and placebo treatments. (e) TLR2 and TLR4 in transverse mucosa lymphocytes at baseline and after Rifaximin treatment. The values reported in box-and-whiskers plots show the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In particular, the whiskers go down to the smallest value (minimum) and up to the largest one (maximum), the top and bottom of the box are the 25th and 75th percentiles, and the line in the middle of the box is the median corresponding to 50th percentile ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ patients versus control (health or baseline)); $^{§§}P < 0.01$, $^{§}P < 0.05$ between treatments. Healthy control (CTR), UDD patients (PZ), before Rifaximin (BR), after Rifaximin (AR), before placebo (BP), and after placebo (AP).

0.0014), while after Rifaximin treatment no change was found (Figure 3(d)). On the other hand, TLR2 MFI was increased after placebo ($P = 0.0238$), while remained unchanged in the after Rifaximin patients. In granulocyte populations, at baseline TRL2 MFI decreased ($P = 0.0029$), while TLR2 MFI increased after placebo ($P = 0.0398$) (Figure 3(e)). All these findings suggest that Rifaximin treatment contributes to maintain stable levels of TLRs and possibly related to the control of intestinal microflora.

We did not find any correlation between TLRs expression in lymphocytes and monocytes; instead, we found correlations for both TLR2 and TLR4 in monocytes and granulocytes; in fact, the direct associations were found between MFI monocytes and MFI granulocytes in UDD patients at T0 (TLR2 correlation coefficient: 0.530, $P = 0.013$; TLR4 correlation coefficient: 0.478, $P = 0.033$).

3.2. CD103 Lymphocytes. CD103 is a gut homing receptor present on the surface of lymphocytes and marks gut-homing

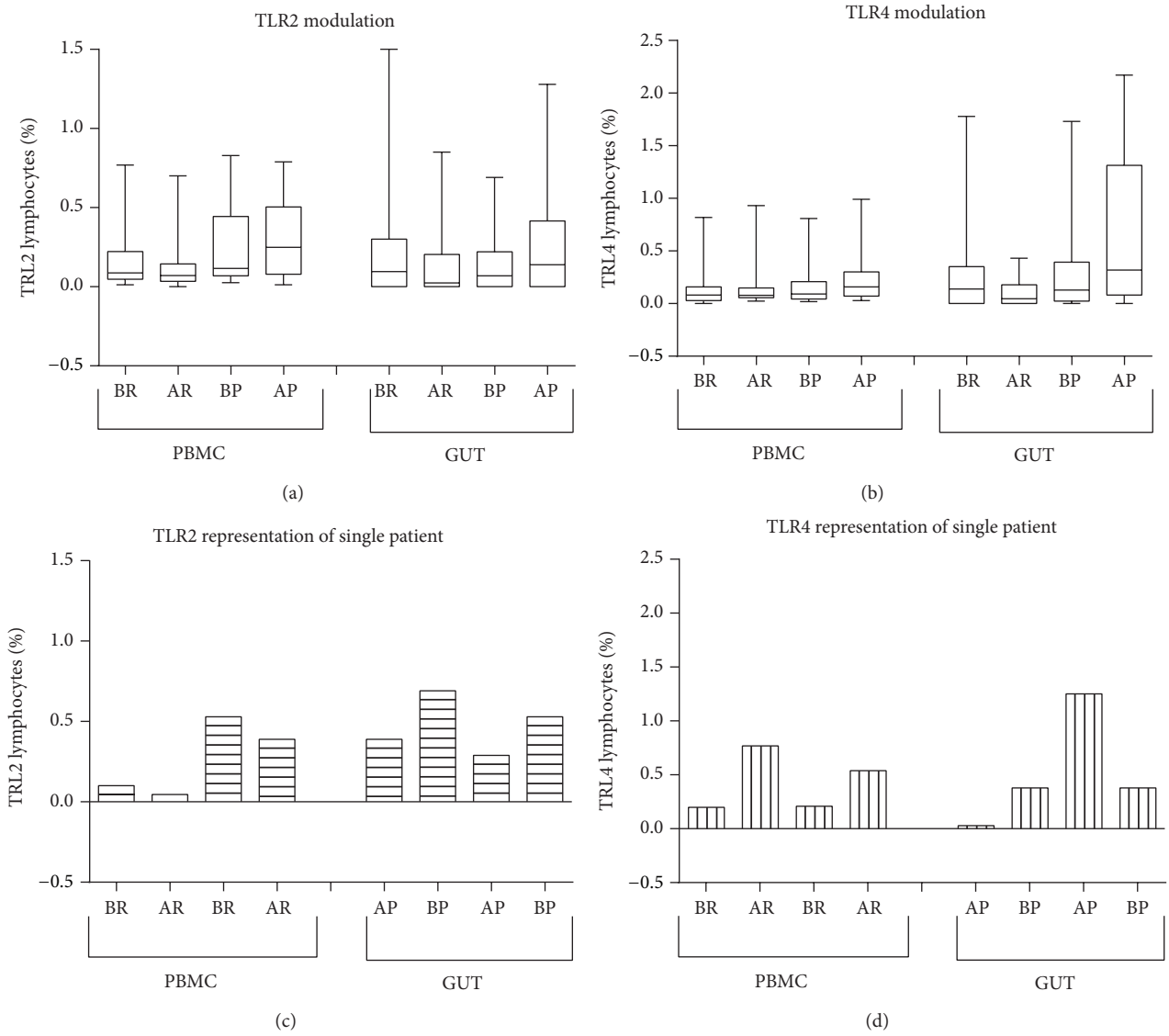


Figure 2: Modulation of TLR2 and TLR4 lymphocytes in PBMC and gut. (a, b) The comparison of modulation of TLR2 and TLR4 expression in the different patient groups between PBMC and gut (before and after placebo or Rifaximin). (c-d) Modulation of TLR2 and TLR4 expression observed in a single representative patient. The values reported in box-and-whiskers plots show the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In particular, the whiskers go down to the smallest value (minimum) and up to the largest one (maximum), the top and bottom of the box are the 25th and 75th percentiles, and the line in the middle of the box is the median, corresponding to 50th percentile, evaluated before Rifaximin (BR), after Rifaximin (AR), before placebo (BP), and after placebo (AP).

recruitment of lymphocytes. We analyzed CD103 expression in peripheral blood and in sigmoid and transverse mucosa TILs.

Considering both mucosal CD4 and CD8/CD103 cells, no significant variations were observed between patients and controls in colonic tissue (data not shown). In peripheral blood, in UDD patients, we found an increased recruitment of CD103 lymphocytes (as reported in our previous work (8)). When we evaluated CD4/CD103 and CD8/CD103 in sigma mucosa before and after Rifaximin treatment, we did not find any change. However, in patients treated with placebo the percentage of CD8/CD103 lymphocytes was lower after placebo ($P = 0.0072$) than before placebo (Figure 4(b)). This suggests that Rifaximin may keep a constant homing flux to the sigma of the CD8 cells.

The gamma/delta T cells are abundant in the gut mucosa. We therefore evaluated these cells in combination with the homing marker CD103 in TILs derived from sigma of UDD patients. vspace2.5pt

After treatment with Rifaximin the percentages of the sigma CD103 TCR-gamma/delta lymphocytes decreased significantly ($P = 0.0182$) with respect to the before Rifaximin percentages, while no differences were found in patients treated with placebo (Figure 4(c)).

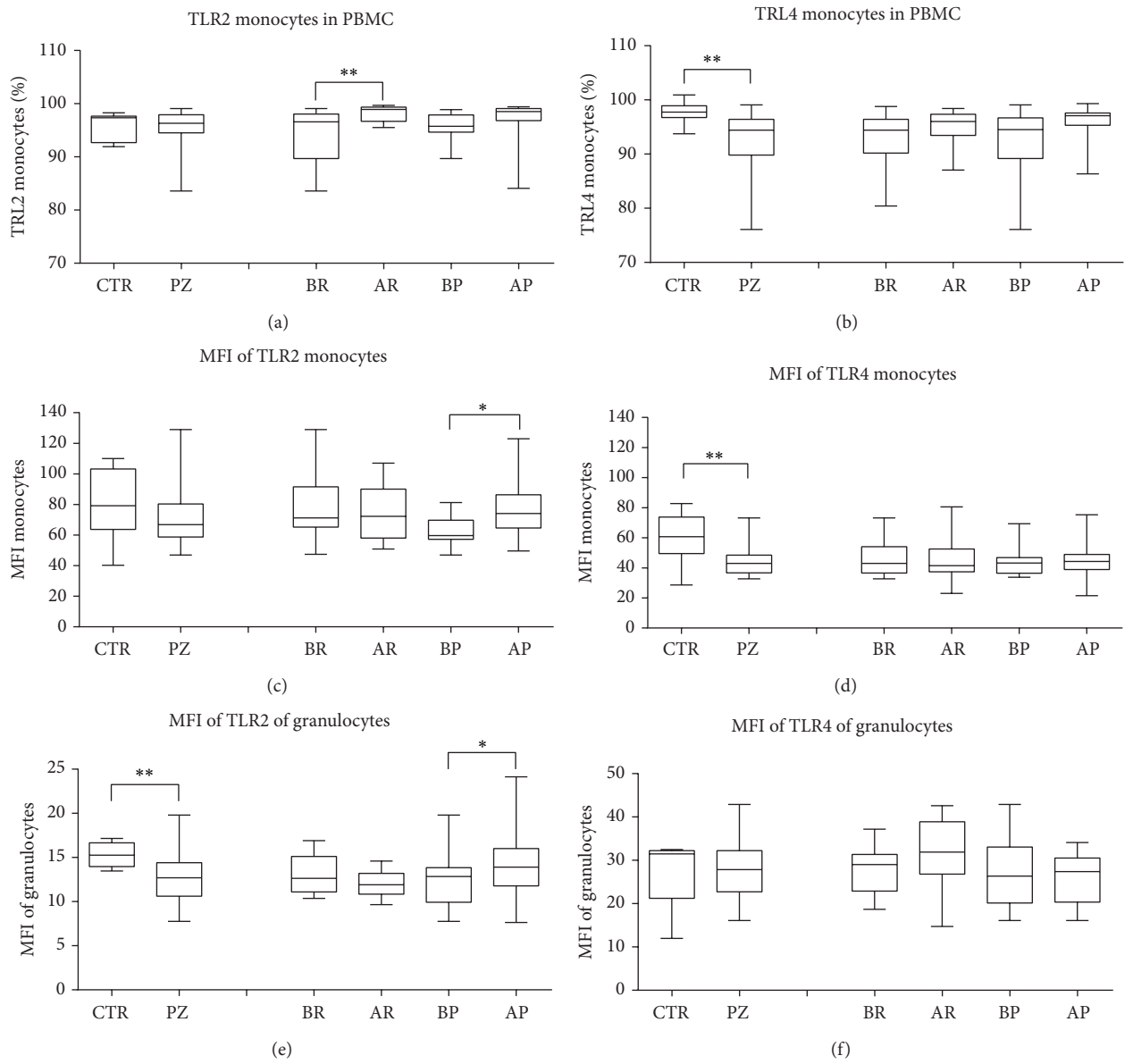


Figure 3: Expression of TLRs in monocytes and granulocytes. (a, b) Percentage of TLR2 and TLR4 monocytes in peripheral blood, at baseline and after Rifaximin and placebo treatments. (c, d) Median of fluorescence (MFI) of TLR2 and TLR4 monocytes in peripheral blood at baseline and after Rifaximin and placebo treatments. (e, f) MFI of TLR2 and TLR4 granulocytes in peripheral blood at baseline and after Rifaximin and placebo treatments. The values reported in box-and-whiskers plots show the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In particular, the whiskers go down to the smallest value (minimum) and up to the largest one (maximum), the top and bottom of the box are the 25th and 75th percentiles, and the line in the middle of the box is the median corresponding to 50th percentile ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ patients versus controls (health or baseline)). Healthy control (CTR), UDD patients (PZ), before Rifaximin (BR), after Rifaximin (AR), before placebo (BP), and after placebo (AP).

No correlations were found between TLRs and CD103 expression in TCR-gamma/delta lymphocytes.

4. Discussion

We have investigated bacterial ligands TLR2 and TLR4 and other markers in peripheral blood and mucosa and on lymphocytes, monocytes, and granulocytes of patients with UDD before and after antibacterial treatment. Several abnormalities of TLRs can be reversed by Rifaximin treatment.

We showed that UDD induces significant modifications of TLR2 and TLR4 expression on several immune system cell subpopulations isolated from both peripheral blood and affected mucosa as compared with controls. Since TLR2 and TLR4 are receptors for ligands present on bacterial walls, we reasoned that antibiotic treatment could reverse

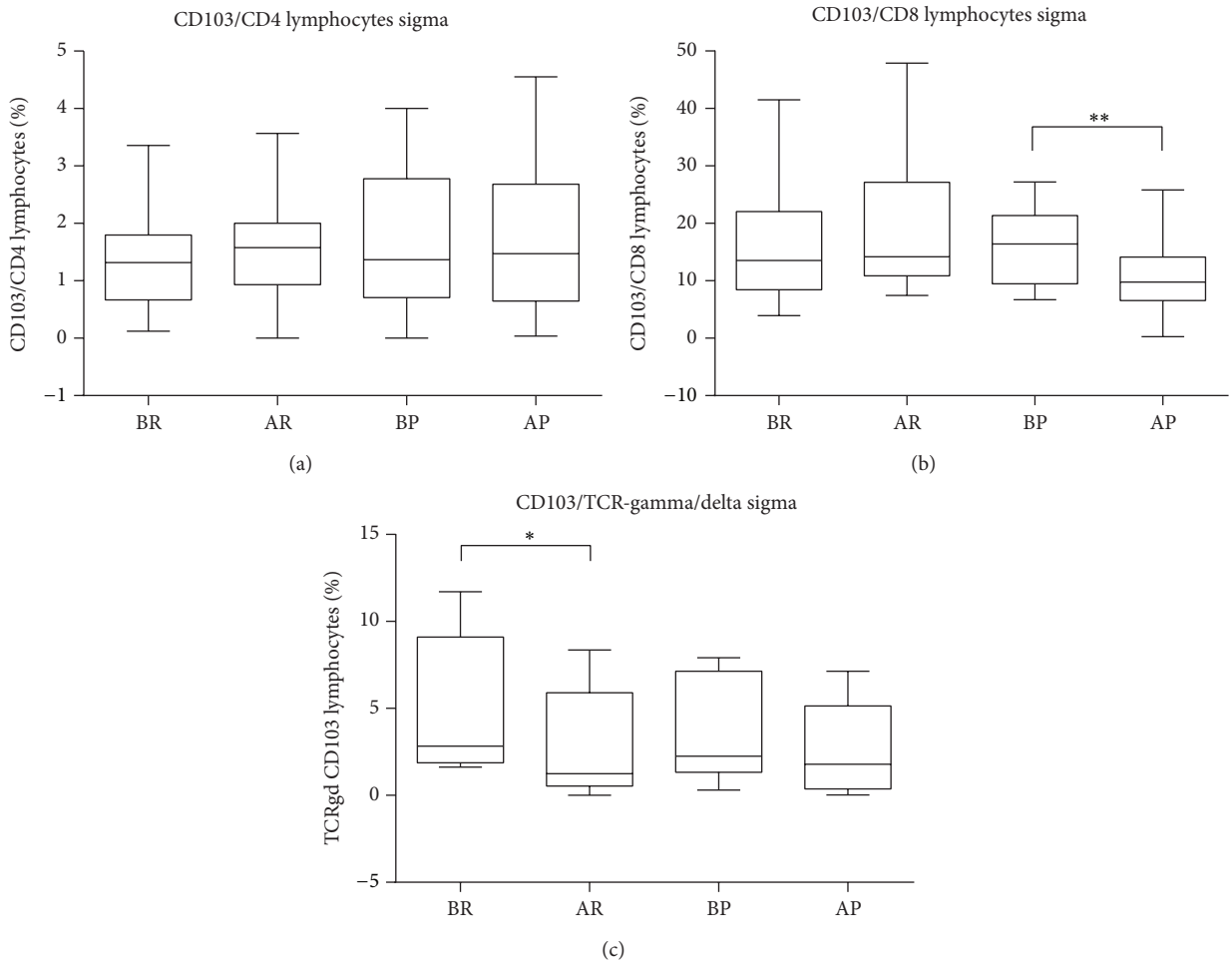


Figure 4: Expression of CD103 in lymphocytes of sigma mucosa. (a) Percentage of CD103 in CD4 lymphocytes subpopulation after Rifaximin and placebo treatments. (b) Percentage of CD103 in CD8 lymphocytes subpopulation after Rifaximin and placebo treatments. (c) Percentage of TCR-gamma/delta CD103 lymphocytes subpopulation after Rifaximin and placebo treatments. The values reported in box-and-whiskers plots show the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. In particular, the whiskers go down to the smallest value (minimum) and up to the largest one (maximum), the top and bottom of the box are the 25th and 75th percentiles, and the line in the middle of the box is the median corresponding to 50th percentile ($^{*}P < 0.05$ before versus after treatments in patients); before Rifaximin (BR), after Rifaximin (AR), before placebo (BP), and after placebo (AP).

colonic inflammation associated with UDD. Rifaximin treatment induced significant modifications of several altered conditions: either restoring the values observed in controls, or limiting the deviations from normal range observed after 2-month placebo treatment. Diverticula, even when not acutely inflamed, represent an anatomical abnormality where excessive growth of bacteria occurs. This may lead to clinical symptoms. We have previously shown that both clinical symptoms and immunological abnormalities can be ameliorated by a short course of Rifaximin [8].

TLR2 and TLR4 are not constitutively expressed on human T cell surface, but their expression requires activation of T cells by TCR complex [16]. We have previously reported increased CD25+ cells in UDD peripheral blood [8]. Accordingly, we showed that TLR2 and TLR4 lymphocytes in the peripheral blood are increased in patients versus controls suggesting more activated circulating T cells in peripheral blood.

TLRs are involved in the activation of NF-kB [17]. Moreover, NF-κB is a critical factor in antibacterial defense [18] and activates the secretion of proinflammatory cytokines resulting in a Th1 response [19]. Thus, Rifaximin keeping under control the activation of TLRs may also limit the triggering of Th1 adaptive immune response. These findings suggest that Rifaximin also has a systemic action on immune system.

In the sigmoid mucosa, percentages of TLR2 and TLR4 lymphocytes are decreased in patients and remain stable after Rifaximin, while we observed an increase of these populations after placebo. It may be suggested that in UDD immunotolerance to commensal bacteria and short-term TLRs activation impairment might be related to increased

inflammation that leads to the development of symptoms in UDD. No differences were observed in modulation of TLR2 and TLR4 lymphocytes between PBMC and sigma mucosa. However, data showing that TLR2 and TLR4 increase in time in after placebo patients suggest that the TLRs defect is not constitutive, but it is limited to the stage of early activation. This mechanism can be the result of a lack of lymphocytes activation since TLR2 is expressed in activated cells [16]. Alternatively, lymphocytes in UDD patients recognize the antigens through adaptive immune response, which requires time to be activated, rather than through innate immunity. Thus, our results of reduced TLR2 expression in sigmoid mucosa could be explained by the delayed activation. In fact, in after placebo patients TLR2 expression is increased. It is interesting to note that we observed a reduced TLR2 expression only in TILs and not in peripheral blood. Our data are similar to evidences in the pleural fluid of patients with tuberculosis infection [19] and filariasis [20], suggesting that downregulation of TLRs at the site of infection and not in the periphery may be connected with a reduction in the secretion of proinflammatory cytokines [19]. Alternatively, an involvement of the T regulatory cells (Tregs) could be proposed [21]. The agonists of TLRs may enhance Tregs proliferation, rendering Tregs transiently nonsuppressive [22].

Finally, we have shown as Rifaximin controls the activation of TLRs in TILs. This may limit the triggering of Th1 adaptive immune response. Limiting the activation of Th1 immune response, involved in the pathogenesis of several inflammatory diseases [23, 24], may be a mechanism by which Rifaximin acts as an anti-inflammatory agent in addition to its antibiotic effect [25].

We have also shown that both TLR2-CD8 and TLR2-CD4 cells were increased in patients PBMC as compared to controls. We hypothesize that, as in UDD bacterial infections are more frequent, lymphocytes expressing TLR2 and TLR4 are already mobilized and ready to mount the immune-response. After Rifaximin, TLR-CD4 cells are significantly reduced in sigmoid mucosa, thus confirming the indirect role of the antibiotic on mucosal immunity. In fact, the reduced number of TLR4-CD4 lymphocytes may be related to the fact that, after antibiotic treatment, there is a reduced need of TLR4-CD4 cells which are important in immune responses against bacteria. CD4 cells are instrumental in starting immune response to produce specific antibodies, cell to cell cross talking through production of cytokines, delivering activation signals and induction of activation markers. The TLR2-CD4 cells are also reduced, but this reduction is not significant. This suggests a similar trend for both TRL2-CD4 and TLR4-CD4 cells. However our data show that Rifaximin prevalently acts on TLR4-CD4 cells. Only further studies may clarify the different responses of CD4 cells to the antibiotic.

Monocytes and granulocytes are essential cells in the innate immune response and reflect the level of inflammation [26]; we therefore analyzed TLRs expression in peripheral blood monocytes and granulocytes.

In monocytes, TLR4 was decreased in peripheral blood both as percentages of cells and MFI, in patients versus controls. These conditions were not modified by Rifaximin. Then, we hypothesized that a defect in TLR4 expression predisposes to diverticular disease by impairing antibacterial defense. This could be related to genetic polymorphism of TLR4 as described by other authors [27, 28]. This reinforces the possibility that, in UDD, the increased bacterial load may contribute to the development of symptoms related to UDD.

Instead, both percentages and MFI of TLR2 monocytes in patients were similar to controls. After Rifaximin TLR2 monocytes were increased in percentage, while TLR2 expression was stable. These results suggest that Rifaximin limits the increase of TLR2 expression, probably in relation to its bactericide action on pathogenic flora. This was reinforced by the increase of MFI expression after placebo, both in monocytes and in granulocytes, which could be related to a stimulation of proinflammatory cytokine and TLR-ligands due to increased bacterial load. Our finding may reflect increased activation of monocytes, as reported [26].

Finally, CD103 cells are lymphocytes homing to the gut tissue [23]. Sigma's CD103 cells are normal in UDD (data not shown). After placebo, we observed a significant decrease of these cells, while after Rifaximin their percentage remained unchanged, suggesting that Rifaximin can maintain intestinal homing within the normal range. Moreover, the decrease of CD103 cells can be due to cell death, which may be related to insufficient recruitment.

Furthermore, we analyzed the involvement of gamma-delta T cells both in peripheral blood and in tissue. Gamma-delta T cells exert a regulatory function. These cells are a minor population in the peripheral blood but constitute a major population among intestinal intraepithelial lymphocytes [29]. Thus, we focused on gamma-delta T cells expressing the intestinal homing receptor CD103 [23].

After Rifaximin, mucosal CD103 positive gamma-delta T-cells were reduced and our data suggest that homing gamma-delta cells directly correlate with gut inflammation in UDD and their reduction supports the anti-inflammatory activity of Rifaximin either through the bacterial load reduction or because Rifaximin has been showed to have a direct anti-inflammatory activity, as already reported above. The gamma-delta T cells may be considered as a marker of inflammation, similar to Tregs, as reported in other papers of our group [21, 23, 30]. While our data clearly show the reduction of inflammatory-related features after Rifaximin, only further studies will demonstrate if these effects are due to reduced bacterial growth or due to intrinsic anti-inflammatory activity recently observed with Rifaximin [25]. It is interesting to note that, in the mice, gamma-delta activation is associated with Th17 cells acting through ligation of TLR2 and TLR4 [31].

In summary, we have described a vast immunological pattern of several innate and adaptive cells markers including TLRs, which may be involved in the pathogenesis and clinical course of the diverticular disease, and we have showed its variations before and after the antibiotic therapy with Rifaximin. We suggest that TLRs are modified by the presence of pathogenic flora in UDD. The role of pathogenic flora is supported by the finding that Rifaximin acts in the gut mucosa homeostasis by limiting

the activation of TLRs. Furthermore, Rifaximin may also have a luminal anti-inflammatory function modulating the adaptive immune response in an inhibitory sense. Rifaximin keeps under control TLRs expression in peripheral blood suggesting that, in addition to its activity in gut mucosa, it may also have a systemic action on immune system.

We have shown that, in UDD, TLRs and several immune cell populations are altered in patients with respect to controls, suggesting that in UDD inflammation is present even in the absence of severe clinical symptoms.

Abbreviations

AP: After placebo
AR: After Rifaximin
BP: Before placebo
BR: Before Rifaximin
MFI: Mean fluorescence intensity
TILs: Tissue infiltrating lymphocytes
TLRs: Toll-like receptors
UDD: Uncomplicated diverticular diseases
WBC: Whole blood cells.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contribution

Rossella Cianci, Danilo Pagliari, Simona Frosali, and Franco Pandolfi ideated the experiment, analyzed the samples, analyzed the data, and wrote and corrected the paper. Rossella Cianci and Franco Pandolfi coordinated the project. Paola Cesaro and Lucio Petruzziello recruited patients, performed colonoscopies, and collected histological samples. Fabio Cascciano analyzed part of samples. Raffaele Landolfi and Guido Costamagna participated in discussion of results and in writing and correcting the paper.

Acknowledgments

The authors wish to thank Dr. Bernardo Toraldo (Rome) for his help in the design and execution of the study. This work was supported in part by "linea D1" from Catholic University.

References

[1] W. Kruis, E. Meier, M. Schumacher, O. Mickisch, R. Greinwald, and R. Mueller, "Randomised clinical trial: Mesalazine (Salofalk granules) for uncomplicated diverticular disease of the colon—a placebo-controlled study," *Alimentary Pharmacology and Therapeutics*, vol. 37, no. 7, pp. 680–690, 2013.

[2] E. Isolauri, Y. Sutas, P. Kankaanpää, H. Arvilommi, and S. Salminen, "Probiotics: effects on immunity," *The American Journal of Clinical Nutrition*, vol. 73, supplement 2, pp. 444S–450S, 2001.

[3] G. Barbara, V. Stanghellini, G. Brandi et al., "Interactions between commensal bacteria and gut sensorimotor function in health and disease," *The American Journal of Gastroenterology*, vol. 100, no. 11, pp. 2560–2568, 2005.

[4] C. Fiocchi, "Inflammatory bowel disease: etiology and pathogenesis," *Gastroenterology*, vol. 115, no. 1, pp. 182–205, 1998.

[5] S. Fiorucci, E. Distrutti, A. Mencarelli, M. Barbanti, E. Palazzini, and A. Morelli, "Inhibition of intestinal bacterial translocation with rifaximin modulates lamina propria monocytic cells reactivity and protects against inflammation in a rodent model of colitis," *Digestion*, vol. 66, no. 4, pp. 246–256, 2002.

[6] A. A. Sheth, W. Longo, and M. H. Floch, "Diverticular disease and diverticulitis," *The American Journal of Gastroenterology*, vol. 103, no. 6, pp. 1550–1556, 2008.

[7] L. B. Ferzoco, V. Raptopoulos, and W. Silen, "Acute diverticulitis," *The New England Journal of Medicine*, vol. 338, no. 21, pp. 1521–1526, 1998.

[8] R. Cianci, F. Iacopini, L. Petruzziello, G. Cammarota, F. Pandolfi, and G. Costamagna, "Involvement of central immunity in uncomplicated diverticular disease," *Scandinavian Journal of Gastroenterology*, vol. 44, no. 1, pp. 108–115, 2009.

[9] C. Blandizzi, G. C. Viscomi, A. Marzo, and C. Scarpignato, "Is generic rifaximin still a poorly absorbed antibiotic? A comparison of branded and generic formulations in healthy volunteers," *Pharmacological Research*, vol. 85, pp. 39–44, 2014.

[10] S. Akira, "Toll-like receptor signaling," *The Journal of Biological Chemistry*, vol. 278, no. 40, pp. 38105–38108, 2003.

[11] D. Werling, O. C. Jann, V. Offord, E. J. Glass, and T. J. Coffey, "Variation matters: TLR structure and species-specific pathogen recognition," *Trends in Immunology*, vol. 30, no. 3, pp. 124–130, 2009.

[12] C. Pasare and R. Medzhitov, "Toll-like receptors: linking innate and adaptive immunity," *Advances in Experimental Medicine and Biology*, vol. 560, pp. 11–18, 2005.

[13] G. Liu and Y. Zhao, "Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells," *Immunology*, vol. 122, no. 2, pp. 149–156, 2007.

[14] I. Caramalho, T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot, "Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide," *Journal of Experimental Medicine*, vol. 197, no. 4, pp. 403–411, 2003.

[15] A. Cottalorda, B. C. Mercier, F. M. Mbitikon-Kobo et al., "TLR2 engagement on memory CD8+ T cells improves their cytokine-mediated proliferation and IFN-γ secretion in the absence of Ag," *European Journal of Immunology*, vol. 39, no. 10, pp. 2673–2681, 2009.

[16] M. Komai-Koma, L. Jones, G. S. Ogg, D. Xu, and F. Y. Liew, "TLR2 is expressed activated T cells as a costimulatory receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 9, pp. 3029–3034, 2004.

[17] I. Siddique and I. Khan, "Mechanism of regulation of Na-H exchanger in inflammatory bowel disease: role of TLR-4 signaling mechanism," *Digestive Diseases and Sciences*, vol. 56, no. 6, pp. 1656–1662, 2011.

[18] L. Zhao, M. Kwon, S. Huang et al., "Differential modulation of nods signaling pathways by fatty acids in human colonic epithelial HCT116 cells," *Journal of Biological Chemistry*, vol. 282, no. 16, pp. 11618–11628, 2007.

[19] C. Prabha, P. Rajashree, and D. D. Sulochana, "TLR2 and TLR4 expression on the immune cells of tuberculous pleural fluid," *Immunology Letters*, vol. 117, no. 1, pp. 26–34, 2008.

[20] S. Babu, C. P. Blauvelt, V. Kumaraswami, and T. B. Nutman, "Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection," *Journal of Immunology*, vol. 176, no. 7, pp. 3885–3889, 2006.

[21] G. Cammarota, F. Pandolfi, R. Cianci, D. Pagliari, R. Landolfi, and S. Kunkel, "Cellular mediators of inflammation: tregs and T H 17 cells in gastrointestinal diseases," *Mediators of Inflammation*, vol. 2009, Article ID 132028, 2009.

[22] P. T. Liu, S. Stenger, H. Li et al., "Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response," *Science*, vol. 311, no. 5768, pp. 1770–1773, 2006.

[23] R. Cianci, G. Cammarota, and G. Frisullo, "Tissue-infiltrating lymphocytes analysis reveals large modifications of the duodenal immunological niche in coeliac disease after gluten-free diet," *Clinical and Translational Gastroenterology*, vol. 3, p. e28, 2012.

[24] T. Olsen, R. Rismo, G. Cui, R. Goll, I. Christiansen, and J. Florholmen, "TH1 and TH17 interactions in untreated inflamed mucosa of inflammatory bowel disease, and their potential to mediate the inflammation," *Cytokine*, vol. 56, no. 3, pp. 633–640, 2011.

[25] A. Mencarelli, M. Migliorati, M. Barbanti et al., "Pregnane-X-receptor mediates the anti-inflammatory activities of rifaximin on detoxification pathways in intestinal epithelial cells," *Biochemical Pharmacology*, vol. 80, no. 11, pp. 1700–1707, 2010.

[26] R. Salomão, P. S. Martins, M. K. C. Brunialti et al., "TLR signaling pathway in patients with sepsis," *Shock*, vol. 30, supplement 1, pp. 73–76, 2008.

[27] A. Abu-Maziad, K. Schaa, E. F. Bell et al., "Role of polymorphic variants as genetic modulators of infection in neonatal sepsis," *Pediatric Research*, vol. 68, no. 4, pp. 323–329, 2010.

[28] D. M. Agnese, J. E. Calvano, S. J. Hahm et al., "Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections," *Journal of Infectious Diseases*, vol. 186, no. 10, pp. 1522–1525, 2002.

[29] A. S. Ismail, K. M. Severson, S. Vaishnava et al., "Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 21, pp. 8743–8748, 2011.

[30] R. Cianci, D. Pagliari, R. Landolfi et al., "New insights on the role of T cells in the pathogenesis of celiac disease," *Journal of Biological Regulators and Homeostatic Agents*, vol. 26, no. 2, pp. 171–179, 2012.

[31] A. Zuo, D. Liang, H. Shao, W. K. Born, H. J. Kaplan, and D. Sun, "In vivo priming of IL-17+ uveitogenic T cells is enhanced by Toll ligand receptor (TLR)2 and TLR4 agonists via $\gamma\delta$ T cell activation," *Molecular Immunology*, vol. 50, no. 3, pp. 125–133, 2012.

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 462740, 8 pages
http://dx.doi.org/10.1155/2014/462740

Review Article

Role of Microbiota and Innate Immunity in Recurrent *Clostridium difficile* Infection

Stefano Bibbò, Loris Riccardo Lopetuso, Gianluca Ianiro, Teresa Di Rienzo, Antonio Gasbarrini, and Giovanni Cammarota

A. Gemelli Hospital, Division of Internal Medicine and Gastroenterology, Department of Internal Medicine, School of Medicine and Surgery, Catholic University, 8, 00168 Rome, Italy

Correspondence should be addressed to Giovanni Cammarota; gcammarota@rm.unicatt.it

Received 12 February 2014; Accepted 20 May 2014; Published 5 June 2014

Academic Editor: Rossella Cianci

Recurrent *Clostridium difficile* infection represents a burdensome clinical issue whose epidemiology is increasing worldwide. The pathogenesis is not yet completely known. Recent observations suggest that the alteration of the intestinal microbiota and impaired innate immunity may play a leading role in the development of recurrent infection. Various factors can cause dysbiosis. The causes most involved in the process are antibiotics, NSAIDs, acid suppressing therapies, and age. Gut microbiota impairment can favor *Clostridium difficile* infection through several mechanisms, such as the alteration of fermentative metabolism (especially SCFAs), the alteration of bile acid metabolism, and the imbalance of antimicrobial substances production. These factors alter the intestinal homeostasis promoting the development of an ecological niche for *Clostridium difficile* and of the modulation of immune response. Moreover, the intestinal dysbiosis can promote a proinflammatory environment, whereas *Clostridium difficile* itself modulates the innate immunity through both toxin-dependent and toxin-independent mechanisms. In this narrative review, we discuss how the intestinal microbiota modifications and the modulation of innate immune response can lead to and exacerbate *Clostridium difficile* infection.

1. Introduction

Bacteria residing in the intestine consist of a real and essential organ known as commensal flora or microbiota. A morpho-functional entity, composed of intestinal microbiota, intestinal epithelium, and mucosal immune system, is responsible for the integrity and homeostasis of gastrointestinal tract. Gut microbial species composition differs greatly among individuals. Each person represents a unique collection of bacterial species, which is highly stable over the time. Variability of gut microbiota is based on the host organism's age, on genetic factors, and on environmental factors [1, 2].

Recent molecular techniques have identified 4 major microbial phyla which represent over 90% of the gut microbiota: *Firmicutes*, *Bacteroides*, *Proteobacteria*, and *Actinobacteria*. The most commensal bacteria present in human fecal flora are represented by two main groups of *Firmicutes*, subdivided in *Clostridium coccoides* (*Clostridium* cluster XIVa) and *Clostridium leptum* (*Clostridium* cluster IV) that are butyrate producers, and by the group of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) [3, 4].

Gut microbiota has metabolic and trophic functions. It has a direct role in the fermentation of dietary residuals and sugar, in the production of substances with antibiotic activity, in the metabolism of proteins, and in the synthesis of vitamins. In addition, it may have a role in the control of proliferation and differentiation of epithelial cells contributing to the formation of a protective barrier against pathogenic organisms [5, 6]. In particular, the fermentation mechanisms of carbohydrates have an important role in the production of short chain fatty acids (SCFA) that are the main source of energy for the enterocytes and are involved in the proliferation and in the differentiation of these cells.

Carbohydrates that arrive in the colon are, in the great part, fibers, and their degradation leads to the production of gas and SCFA such as acetate, propionate, and butyrate. Human body does not possess the majority of hydrolytic enzymes that are involved in these reactions, which are,

however, present in the bacterial species forming the gut microbiota [7, 8].

In this review, we will discuss how the intestinal microbiota modifications (intestinal dysbiosis) and the modulation of innate immune response can lead to and exacerbate *Clostridium difficile* infection (CDI).

2. Clinical Aspects of *Clostridium difficile* Infection

Clostridium (*C.*) *difficile* (*Clostridium* cluster XI) is a Gram-positive anaerobic spore-forming bacillus that lives in the environment (soil, water, and animal feces) and in the human gut where it can be a normal commensal [9]. Indeed, some people are carriers of the bacterium but do not develop the symptoms of the infection. We can refer to CDI only in the presence of symptoms [10, 11]. The disease is caused by toxin A and B expression that is responsible for gastrointestinal illness with a wide spectrum of severity, ranging from mild diarrhea to pseudomembranous colitis, that may progress to toxic megacolon, sepsis, and death [12].

There are several risk factors for *C. difficile*-associated diarrhea (CDAD). In particular, factors like the older age, the presence of comorbidities, an increased exposure to the spores of *C. difficile* during prolonged hospitalizations, and overall protracted and combined antimicrobial therapies can alter gut microbiota and promote CDI [13].

Diagnosis of CDI is based on a combination of clinical presentation signs confirmed by microbiological evidence of *C. difficile* toxin in the stools and, in certain cases, by a lower endoscopic exam that demonstrates pseudomembranous colitis [14].

Current treatment options for CDI are based on the use of oral antibiotics, fecal microbiota transplantation (FMT), or surgery for severe clinical pictures [15]. The antibiotics commonly used to treat CDI are metronidazole, vancomycin, and fidaxomicin. Patients with fulminant CDI who failed to respond to antimicrobial therapies and progress to systemic toxicity with peritonitis and toxic colonic dilatation require surgical intervention such as total colectomy [16]. In recent years, the restoration of healthy gut microbiota by FMT constitutes a suggestive effective therapeutic option for the management of recurrent CDI [17].

3. Interaction between Commensal Microbiota and *Clostridium difficile*

A great clinical problem related to CDI is the presence of relapses that are more difficult to treat. In fact, sometimes *C. difficile* may relapse despite a good adherence to the therapy. The meaning of this evidence is not well understood. There are many studies which indicate a role of the microbiota and its alteration in the development of the infection and in the resistance to antibiotic therapy [18, 19]. Intestinal dysbiosis may be due to several mechanisms such as the use of medication, diet, and physical and psychological stress [20] (Tables 1 and 2).

Table 1: This table shows the list of the main factors involved in the development of dysbiosis that promotes recurrent *Clostridium difficile* infection.

Dysbiosis promoting factors
(i) Antimicrobic agents
(ii) NSAIDs
(iii) Acid suppressing agents
(iv) Age
(v) Diet

Table 2: This table shows the list of pathogenetic factors generated by dysbiosis.

Pathogenetic factors resulting from dysbiosis
(i) SCFAs and other fermentative metabolites
(ii) Bacterial antimicrobic molecule
(iii) Bile acids metabolism
(iv) Competition for nutritional sources

Drugs most frequently implicated in the alteration of the intestinal microbiota are antimicrobic agents. It is proved that the administration of various types of antibiotics, in particular clindamycin, second and third generation cephalosporins, fluoroquinolones, and macrolides, can alter the ratio of different microbial communities. As described in several studies, there is a decrease in carbohydrate-fermenting and butyrate-producing bacteria members of *Bacteroides* and *Firmicutes* phyla [21–25].

A reduction of butyrate producers (such as *Roseburia* and *Ruminococcus*) is observed also in NSAIDs users, particularly in elderly subjects. These subjects, for their natural modification of the gut microbiota related to the age, have already an increased variability of microbial species and a relative decrease of *Firmicutes* and *Bacteroides* regardless of NSAIDs use [26].

Also acid-suppressing agents (H2-receptor antagonists and proton-pump inhibitors) can cause a change in the bacterial flora of the gastrointestinal tract. In particular, there is an increase of gastric and duodenal contamination with a possible minor degradation of *Clostridium* spores by gastric juices [27, 28]. The significance of this observation in the development of CDI is, however, still controversial. In fact, not all researchers recognize a primary role of acid suppression in establishing conditions that favor the *Clostridium* growth [29]. Furthermore, nutrition can have a direct role in modifying the intestinal microbiota and in creating a favorable environment for the growth of *C. difficile*. In particular, a prolonged elemental diet, poor in fibers, which are a substrate for some beneficial bacteria, can support the development of an alteration in the ratio of normal commensal bacteria [24, 30].

Overall, these environmental factors and the consequent intestinal dysbiosis disrupt and alter the protective effect exerted by the gut microbiota against recurrent CDI. The loss of this protective barrier allows for the formation of

an ecological niche where *C. difficile* can develop and better resist to antimicrobial therapies.

This niche concept is even more important if we consider that *C. difficile* multiplication and development, facilitated by dysbiosis, are necessary for CDAD [31, 32]. Consequently, intestinal dysbiosis is very important in the pathogenesis of the disease, especially when specific changes in the composition of the gut microbiota occur. CDI patients have a greater diversity of bacterial species and a reduced concentration of some commensal species, in particular the most represented phyla such as *Bacteroides* and *Firmicutes*. *Bacteroides*, which appear to be extremely reduced in these patients, are mainly responsible for the digestion of carbohydrates in the intestinal lumen, resulting in the production of substrates essential for the homeostasis of colonocytes. The reduced concentration of these commensal bacteria has been therefore associated with a higher frequency of relapse of CDI [23, 33, 34].

Also, the components of *Firmicutes* phylum are less represented in CDAD patients with respect to healthy subject. At family level, *Lachnospiraceae* and *Ruminococcaceae*, that are important butyrate producers, are significantly unrepresented in CDI, whereas *Deltaproteobacteria*, that are sulfate-reducing bacteria, are depleted. In contrast, several genera are enriched in association with CDI, such as *Veillonella*, *Enterococcus,* and *Lactobacillus*.

This evident dysbiosis generates an altered production of substrates fermented by the anaerobic gut microbiota, including butyrate, other SCFAs, acetate, and lactate that are critical to the homeostasis of the intestinal epithelial cells [35]. Butyric acid has an important anti-inflammatory molecule and is the preferred source of energy of colonocytes. Other SCFAs are known to decrease intestinal permeability and to increase the production of antimicrobial substances and mucin [36, 37]. Furthermore, a direct role of SCFAs in the inhibition of the growth of *C. difficile* was also assumed. This hypothesis has been confirmed by *in vitro* experiments, but results of *in vivo* studies do not seem to fully confirm this hypothesis [38, 39].

Higher concentration of some species of *Firmicutes* such as *Ruminococcus gnavus*, *Ruminococcus hansenii,* and *Clostridium nexile* was associated with a greater risk of recurrence and development of CDI. These bacterial species are producers of a trypsin-dependent antimicrobial substance (ruminococcin A) that has a low activity against *C. difficile* but can contribute to the disruption of the normal intestinal flora [40]. Another bacterial species that is capable of producing a substance with antimicrobial activity is the *Bacillus thuringiensis*. This bacteria strain produces the Thuricin CD that *in vitro* models proved to inhibit the growth of *C. difficile*. The efficacy of this molecule is effective as well as metronidazole [41, 42].

A further mechanism that gut microbiota uses against the *C. difficile* is the metabolization of bile that is proven to have a role in both the spores germination and the growth of the vegetative form [19]. Commensal flora plays two important roles in bile transformation. A first mechanism is represented by the action of bile salt hydrolase enzymes produced by bacteria. These enzymes transform bile acids by cleaving their glycine and taurine; the metabolites obtained can stimulate the germination of spores. A second mechanism is mediated by the enzyme 7-dehydroxylase that is also produced by the bacterial flora; this enzyme converts primary bile acids, cholate, and chenodeoxycholate into secondary biliar acids: deoxycholic and lithocholic acids, respectively. It is not yet well known which bacterial species operate on the transformation of bile acids [43, 44].

Deoxycholate is a potent germinant but is highly toxic to vegetative cells; cholate stimulates spore germination and vegetative *C. difficile*, whereas chenodeoxycholate has a strong inhibitory effect on spore germination. An alteration in the ratio of the different bile acids, caused by a change in the gut microbiota composition, may promote or inhibit the growth of *C. difficile* [45–47].

In a recent paper, it was demonstrated that the conjugated bile salt taurocholate is able to inhibit *C. difficile* toxins A and B activities in an *in vitro* assay. These results suggest that the mechanism of taurocholate-mediated inhibition modulates toxin activity. Indeed, taurocholate does not appear to affect *C. difficile* growth and toxin production [48].

An additional mechanism that commensal flora uses against the *C. difficile* colonization is represented by the competition for energy sources, in particular carbon source, between toxigenic *Clostridium* and nontoxigenic *Clostridium*. In animal models, it has been shown that nontoxigenic *Clostridium*, prevailing in this competition, crowds out *C. difficile* by ecological niche preventing its growth. Unfortunately, little is still known about this interesting aspect [19, 49, 50].

4. *Clostridium difficile* and Innate Immune Response

Several studies on commensal *Clostridia* showed that high levels of metabolite products, and their colonization in close proximity to the intestinal mucosa, are able to exert a strong influence on the host immune system [4]. Indeed, it has been shown that *Clostridia* can promote the development of $\alpha\beta$ T-cell receptor intraepithelial lymphocytes (IEL) and immunoglobulin A (IgA-) producing cells in the large intestine [51]. IEL, IgA-producing cells within the lamina propria, and intestinal epithelial cells are key players in determining the nature of the immunological response to antigens or pathogens ingested. Umesaki et al. assessed that germ-free mice inoculated with 46 strains of *Clostridia* singly isolated from conventional mice showed an increase in the ratio of $CD4^-$ $CD8^+$ cells to that of $CD4^+$ $CD8^-$ in $\alpha\beta$IEL within the large intestine. Conversely, the number and phenotype of IEL were similar to those in conventionally housed mice. The number of IgA-producing cells in the colons of mice treated with *Clostridia* was slightly increased compared to that in germ-free mice [51]. Thus, *Clostridia* appear to be involved in the promotion of immunological development [51] in the large intestine, but not in the small intestine. Moreover, commensal *Clostridia* are able to normalize cecal size when they are associated with germ-free mice [52]. How the immune system fundamentally senses *Clostridia* remains unclear. In this context, it has been suggested that the presence or gradient of SCFAs and secondary bile acids

produced by *Clostridia* may be sensed by epithelial cells and, in turn, may be associated with the initiation of immunological signaling [51], due to the cross-talk between epithelial and immune cells. For example, IL-7 secreted by epithelial cells can activate IL-7 receptor-bearing IEL on their progenitors [53, 54]. Furthermore, IL-6 [55] and transforming growth factor β [56] produced by the epithelia during infection can stimulate the development of Peyer's patches and IgA production [57].

Notably, elevated levels of *Clostridium* clusters *XIVa* and *IV* in mice lead to resistance to allergy and intestinal inflammation in experimental models [58]. Conversely, the microbiota of individuals with chronic inflammation shows lower bacterial diversity and it has been determined that *Clostridium* clusters *IV*, particularly *F. prausnitzii*, and *XIVa* are significantly less abundant in IBD patients compared to healthy subjects [59–61]. It is still unknown whether the decrease in *Clostridia* is a cause or a consequence of chronic inflammation in IBD patients and in autoimmunity, but we can speculate that they are necessary for immune homeostasis, contributing to the suppression of autoimmunity and deleterious inflammation in humans.

4.1. Effects of C. difficile Toxins Associated with Acute Colitis. In animal models the challenge of ileal loops with *C. difficile* toxin A produces an intense inflammatory response characterized by fluid accumulation, edema, increased mucosal permeability, mast cell degranulation, epithelial cell death, and neutrophil recruitment.

Toxins are able to trigger fluid secretion, to induce the production of reactive oxygen intermediates, IL-8 from colonic epithelial cells [62], and to downregulate mucin exocytosis from mucin-producing colon cells [63].

Moreover, toxins lead to the production of multiple proinflammatory cytokines and chemokines including IL-12, IL-18, interferon g (IFN-g), IL-1b, TNF-a, macrophage inflammatory protein 1 a (MIP-1a), MIP-2, IL-8, and leptin [64]. These factors can exacerbate the inflammation and may be responsible for host damage and many of the histopathological features of *C. difficile*-associated diseases.

Intestinal mast cells also play an important role in the toxin-mediated inflammatory responses. Both toxins A and B lead to activation, degranulation, and the release of inflammatory mediators from mast cells [65]. The inhibition of mast cell degranulation and the blockade of mast cell-derived histamine were associated with a decrease in inflammatory responses to toxin A [66]. Mast cell-deficient mice show severe inflammation and neutrophilic infiltration compared with wild-type mice in response to *C. difficile* toxin A [67]. These studies suggest that, like neutrophils, mast cells propagate the inflammatory response in *C. difficile*-associated diseases. To be noted, a part of the toxin A mediated neutrophil recruitment in rat ileal loops is dependent on mast cell activation [67].

The role of other immune cells, including macrophages, monocytes, and dendritic cells, has generally been extrapolated from *in vitro* and *ex vivo* studies using human and mouse cell lines, human monocytes, and monocyte-derived dendritic cells. Emerging evidence showed also that *C. difficile* toxins can stimulate the release of proinflammatory cytokines and chemokines from macrophages, monocytes, and dendritic cells with a mitogen-activated protein kinase (MAPK-) and p38-dependent pathway [68]. Furthermore, toxin A leads to NF-κB-mediated IL-8 production from human monocytes [69].

4.2. Effects on the Innate/Adaptative Immune System Predisposing to Recurrence of CDI. C. difficile is able to modulate intestinal innate immune responses and several groups studied this process. *Clostridium difficile* is able to modulate host innate immunity via toxin-independent and dependent mechanisms [70, 71]. The innate immune mechanisms against the toxins produced by *C. difficile* include the endogenous microbial flora, the mucus barrier, intestinal epithelial cells, and the mucosal immune system. Furthermore, *C. difficile* infection triggers the release of multiple proinflammatory mediators (cytokines, chemokines, and neuroimmune peptides) and the recruitment and activation of several innate immune cells (Figure 1).

Interestingly, *C. difficile* toxins activate both surface and intracellular innate immune sensors, including the inflammasome and the TLR4, TLR5, and NOD1 signaling pathways [72]. TLR4- and MyD88-dependent signaling pathways produce an enhanced inflammatory response [73]. The deficiency of these pathways increases the bacterial burden and the worsening of the disease [73].

C. difficile shows a proteinaceous cell surface layer, which is composed of an array of proteins arranged in a crystalline lattice. The surface layer proteins have the ability to activate proinflammatory signaling through TLR4 expressed on the surface of host cells. Engagement of TLR4 initiates downstream signaling of NF-κB and interferon regulatory factor 3, resulting in subsequent production of inflammatory cytokines and immune cell activation. Surface layer proteins induce dendritic cell maturation and activation *in vitro*, as demonstrated by increased expression of major histocompatibility complex class II, CD40, CD80, CD86, and production of IL-12p70, tumor necrosis factor-a, IL-23, and IL-6 [73]. Moreover, surface layer proteins were found to activate NF-κB, but not interferon regulatory factor 3. This indicates that the signaling is myeloid differentiation primary 12 response gene 88 (MyD88)-dependent. In fact, TLR4-deficient and MyD88-deficient mice were more susceptible to infection and exhibited greater pathology than wild-type mice [73]. Increased mucosal damage and inflammation in MyD88-deficient mice were attributed to a lack of neutrophil recruitment to the site of infection [74]. Neutrophils were shown to be critical in preventing bacterial dissemination through damaged mucosa [74]. In the case of TLR5 signaling, exogenous stimulation of TLR5 signaling was protective against *C. difficile* infection [75].

The intracellular innate immune sensors NOD1 and the IL-1b/inflammasome are also activated after *C. difficile* infection [72]. *C. difficile*-induced NOD1 activation triggered chemokine production and NOD1-deficient mice have lower chemokine production, less neutrophil recruitment, and

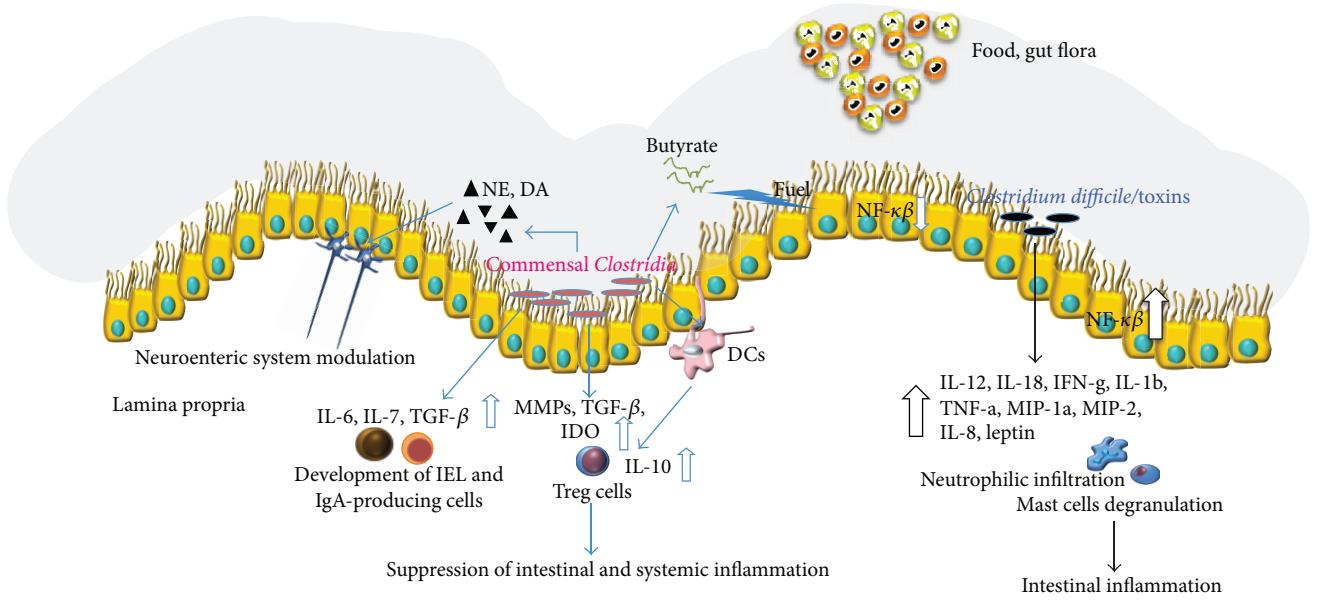


Figure 1: Commensal *Clostridia* have a peculiar role in modulating gut homeostasis. Establishing a close relationship with gut cells (interfold region), *Clostridia* spp. exert a strong influence on the host immune system. On the other hand, *C. difficile* and its toxins lead to the production of multiple proinflammatory cytokines and chemokines including IL-12, IL-18, interferon g (IFN-g), IL-1b, TNF-a, macrophage inflammatory protein 1 a (MIP-1a), MIP-2, IL-8, and leptin [66]. These factors can exacerbate the inflammation and may be responsible for host damage and many of the histopathological features of *C. difficile*-associated diseases.

more severe disease [72]. In fact NOD1-deficient mice have a higher *C. difficile* burden [72]. *C. difficile* toxins stimulate IL-1b release by activating inflammasomes in both mouse macrophages and human colon biopsy specimens [76].

Activation of the innate immune sensors and the release of cytokine and chemokine mediators are followed by an intense local neutrophilic infiltration [77]. This neutrophilic infiltration is one of the major pathological findings after *C. difficile* infection. Local recruitment and systemic proliferation of neutrophils are seen in *C. difficile*-associated diseases [77]. Indeed, induction of neutropenia in rats was associated with less severe disease [78].

5. Conclusions

In recent years, several studies analyzed the role of gut microbiota in human physiology and in maintaining gut immune homeostasis. One of the most interesting aspects involves CDI and CDAD.

Intestinal dysbiosis and impaired innate immune response are crucial players in triggering *C. difficile* colonization and related symptoms. In these conditions this Gram-positive anaerobic spore-forming bacillus finds an ecological niche where it can grow and better resist antimicrobial therapies.

In this scenario, gut microbiota modulation and the consequent control of the innate immune response represent a valuable and interesting tool to treat CDI-related diseases.

Conflict of Interests

Stefano Bibbò, Loris Riccardo Lopetuso, Gianluca Ianiro, Teresa Di Rienzo, and Giovanni Cammarota have no conflict of interests to declare. Antonio Gasbarrini is in the speaker's bureau of Alfa Wassermann, Bayer, Janssen, Gilead, MSD, BMS, Angelini, and Sanofi.

References

[1] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Microbiology: diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[2] J. Marchesi and F. Shanahan, "The normal intestinal microbiota," *Current Opinion in Infectious Diseases*, vol. 20, no. 5, pp. 508–513, 2007.

[3] P. D. Cani, "Metabolism in 2013: the gut microbiota manages host metabolism," *Nature Reviews Endocrinology*, vol. 10, no. 2, pp. 74–76, 2014.

[4] L. R. Lopetuso, F. Scaldaferri, V. Petito, and A. Gasbarrini, "Commensal Clostridia: leading players in the maintenance of gut homeostasis," *Gut Pathogens*, vol. 5, no. 1, article 23, 2013.

[5] F. Guarner, "Enteric flora in health and disease," *Digestion*, vol. 73, supplement 1, pp. 5–12, 2006.

[6] I. Sekirov, S. L. Russell, L. Caetano M Antunes, and B. B. Finlay, "Gut microbiota in health and disease," *Physiological Reviews*, vol. 90, no. 3, pp. 859–904, 2010.

[7] A. Bernalier-Donadille, "Fermentative metabolism by the human gut microbiota," *Gastroenterologie Clinique et Biologique*, vol. 34, supplement 1, pp. S16–S22, 2010.

[8] A. Cuervo, N. Salazar, P. Ruas-Madiedo, M. Gueimonde, and S. González, "Fibers from regular diet are directly associated with fecal short-chain fatty acid concentrations in the elderly," *Nutrition Research*, vol. 33, no. 10, pp. 811–816, 2013.

[9] H. Brüggemann and G. Gottschalk, "Comparative genomics of clostridia: link between the ecological niche and cell surface properties," *Annals of the New York Academy of Sciences*, vol. 1125, pp. 73–81, 2008.

[10] C. Eckert, G. Jones, and F. Barbut, "Diagnosis of Clostridium difficile infection: the molecular approach," *Future Microbiology*, vol. 8, pp. 1587–1598, 2013.

[11] K. Sánchez-Hurtado and I. R. Poxton, "Enhancement of the cytotoxic activity of *Clostridium difficile* toxin A by surface-associated antigens," *Journal of Medical Microbiology*, vol. 57, part 6, pp. 739–744, 2008.

[12] G. Dobson, C. Hickey, and J. Trinder, "*Clostridium difficile* colitis causing toxic megacolon, severe sepsis and multiple organ dysfunction syndrome [3]," *Intensive Care Medicine*, vol. 29, no. 6, p. 1030, 2003.

[13] V. G. Loo, A.-M. Bourgault, L. Poirier et al., "Host and pathogen factors for *Clostridium difficile* infection and colonization," *New England Journal of Medicine*, vol. 365, no. 18, pp. 1693–1703, 2011.

[14] S. H. Cohen, D. N. Gerding, S. Johnson et al., "Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA)," *Infection Control and Hospital Epidemiology*, vol. 31, no. 5, pp. 431–455, 2010.

[15] S. B. Debast, M. P. Bauer, and E. J. Kuijper, "European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection," *Clinical Microbiology and Infection*, vol. 20, supplement 2, pp. 1–26, 2014.

[16] C. M. Surawicz, L. J. Brandt, D. G. Binion et al., "Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections," *The American Journal of Gastroenterology*, vol. 108, no. 4, pp. 478–499, 2013.

[17] E. van Nood, M. G. W. Dijkgraaf, and J. J. Keller, "Duodenal infusion of feces for recurrent *Clostridium difficile*," *The New England Journal of Medicine*, vol. 368, no. 22, pp. 407–415, 2013.

[18] J. Bien, V. Palagani, and P. Bozko, "The intestinal microbiota dysbiosis and *Clostridium difficile* infection: is there a relationship with inflammatory bowel disease?" *Therapeutic Advances in Gastroenterology*, vol. 6, no. 1, pp. 53–68, 2013.

[19] R. A. Britton and V. B. Young, "Interaction between the intestinal microbiota and host in *Clostridium difficile* colonization resistance," *Trends in Microbiology*, vol. 20, no. 7, pp. 313–319, 2012.

[20] J. A. Hawrelak and S. P. Myers, "The causes of intestinal dysbiosis: a review," *Alternative Medicine Review*, vol. 9, no. 2, pp. 180–197, 2004.

[21] D. A. Antonopoulos, S. M. Huse, H. G. Morrison, T. M. Schmidt, M. L. Sogin, and V. B. Young, "Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation," *Infection and Immunity*, vol. 77, no. 6, pp. 2367–2375, 2009.

[22] C. Jernberg, S. Löfmark, C. Edlund, and J. K. Jansson, "Long-term ecological impacts of antibiotic administration on the human intestinal microbiota," *ISME Journal*, vol. 1, no. 1, pp. 56–66, 2007.

[23] A. R. Manges, A. Labbe, V. G. Loo et al., "Comparative metagenomic study of alterations to the intestinal microbiota and risk of nosocomial clostridum difficile-associated disease," *Journal of Infectious Diseases*, vol. 202, no. 12, pp. 1877–1884, 2010.

[24] S. J. D. O'Keefe, "Tube feeding, the microbiota, and *Clostridium difficile* infection," *World Journal of Gastroenterology*, vol. 16, no. 2, pp. 139–142, 2010.

[25] Å. Sullivan, "Effect of antimicrobial agents on the ecological balance of human microflora," *Lancet Infectious Diseases*, vol. 1, no. 2, pp. 101–114, 2001.

[26] H. Mäkivuokko, K. Tiihonen, S. Tynkkynen, L. Paulin, and N. Rautonen, "The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition," *The British Journal of Nutrition*, vol. 103, no. 2, pp. 227–234, 2010.

[27] M. A. Aldeyab, S. Harbarth, N. Vernaz et al., "Quasiexperimental study of the effects of antibiotic use, gastric acid-suppressive agents, and infection control practices on the incidence of *Clostridium difficile*-associated diarrhea in hospitalized patients," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 5, pp. 2082–2088, 2009.

[28] S. Dial, J. A. C. Delaney, A. N. Barkun, and S. Suissa, "Use of gastric acid-suppressive agents and the risk of community-acquired *Clostridium difficile*-associated disease," *Journal of the American Medical Association*, vol. 294, no. 23, pp. 2989–2995, 2005.

[29] M. M. Nerandzic, M. J. Pultz, and C. J. Donskey, "Examination of potential mechanisms to explain the association between proton pump inhibitors and *Clostridium difficile* infection," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 10, pp. 4133–4137, 2009.

[30] S. J. D. O'Keefe, "Nutrition and colonic health: the critical role of the microbiota," *Current Opinion in Gastroenterology*, vol. 24, no. 1, pp. 51–58, 2008.

[31] Y. C. Ju, D. A. Antonopoulos, A. Kalra et al., "Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea," *Journal of Infectious Diseases*, vol. 197, no. 3, pp. 435–438, 2008.

[32] K. H. Wilson, "The microecology of *Clostridium difficile*," *Clinical Infectious Diseases*, vol. 16, supplement 4, pp. S214–S218, 1993.

[33] E. Goldberg, I. Amir, M. Zafran et al., "The correlation between Clostridium-difficile infection and human gut concentrations of Bacteroidetes phylum and clostridial species," *The European Journal of Clinical Microbiology & Infectious Diseases*, vol. 33, no. 3, pp. 377–383, 2014.

[34] M. J. Hopkins and G. T. Macfarlane, "Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection," *Journal of Medical Microbiology*, vol. 51, no. 5, pp. 448–454, 2002.

[35] V. C. Antharam, E. C. Li, A. Ishmael et al., "Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhea," *Journal of Clinical Microbiology*, vol. 51, no. 9, pp. 2884–2892, 2013.

[36] S. I. Cook and J. H. Sellin, "Review article: short chain fatty acids in health and disease," *Alimentary Pharmacology and Therapeutics*, vol. 12, no. 6, pp. 499–507, 1998.

[37] J. M. Wong, R. de Souza, C. W. Kendall, A. Emam, and D. J. Jenkins, "Colonic health: fermentation and short chain fatty acids," *Journal of Clinical Gastroenterology*, vol. 40, no. 3, pp. 235–243, 2006.

[38] R. D. Rolfe, "Role of volatile fatty acids in colonization resistance to *Clostridium difficile*," *Infection and Immunity*, vol. 45, no. 1, pp. 185–191, 1984.

[39] W. J. Su, M. J. Waechter, P. Bourlioux, M. Dolegeal, J. Fourniat, and G. Mahuzier, "Role of volatile fatty acids in colonization resistance to *Clostridium difficile* in gnotobiotic mice," *Infection and Immunity*, vol. 55, no. 7, pp. 1686–1691, 1987.

[40] F. Marcille, A. Gomez, P. Joubert et al., "Distribution of genes encoding the trypsin-dependent lantibiotic ruminococcin A among bacteria isolated from human fecal microbiota," *Applied and Environmental Microbiology*, vol. 68, no. 7, pp. 3424–3431, 2002.

[41] M. C. Rea, A. Dobson, O. O'Sullivan et al., "Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, supplement 1, pp. 4639–4644, 2011.

[42] M. C. Rea, C. S. Sit, E. Clayton et al., "Thuricin CD, a post-translationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 20, pp. 9352–9357, 2010.

[43] J. M. Ridlon, D.-J. Kang, and P. B. Hylemon, "Bile salt biotransformations by human intestinal bacteria," *Journal of Lipid Research*, vol. 47, no. 2, pp. 241–259, 2006.

[44] K. H. Wilson, "Efficiency of various bile salt preparations for stimulation of *Clostridium difficile* spore germination," *Journal of Clinical Microbiology*, vol. 18, no. 4, pp. 1017–1019, 1983.

[45] J. L. Giel, J. A. Sorg, A. L. Sonenshein, and J. Zhu, "Metabolism of bile salts in mice influences spore germination in *Clostridium difficile*," *PLoS ONE*, vol. 5, no. 1, Article ID e8740, 2010.

[46] J. A. Sorg and A. L. Sonenshein, "Bile salts and glycine as cogerminants for *Clostridium difficile* spores," *Journal of Bacteriology*, vol. 190, no. 7, pp. 2505–2512, 2008.

[47] J. A. Sorg and A. L. Sonenshein, "Chenodeoxycholate is an inhibitor of *Clostridium difficile* spore germination," *Journal of Bacteriology*, vol. 191, no. 3, pp. 1115–1117, 2009.

[48] C. Darkoh, E. L. Brown, H. B. Kaplan, and H. L. DuPont, "Bile salt inhibition of host cell damage by *Clostridium difficile* toxins," *PLoS ONE*, vol. 8, no. 11, Article ID e79631, 2013.

[49] M. M. Merrigan, S. P. Sambol, S. Johnson, and D. N. Gerding, "Prevention of fatal *Clostridium difficile*-associated disease during continuous administration of clindamycin in hamsters," *Journal of Infectious Diseases*, vol. 188, no. 12, pp. 1922–1927, 2003.

[50] S. P. Sambol, M. M. Merrigan, J. K. Tang, S. Johnson, and D. N. Gerding, "Colonization for the prevention of *Clostridium difficile* disease in hamsters," *Journal of Infectious Diseases*, vol. 186, no. 12, pp. 1781–1789, 2002.

[51] Y. Umesaki, H. Setoyama, S. Matsumoto, A. Imaoka, and K. Itoh, "Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system," *Infection and Immunity*, vol. 67, no. 7, pp. 3504–3511, 1999.

[52] K. Itoh and T. Mutsuoka, "Characterization of clostridia isolated from faeces of limited flora mice and their effect on caecal size when associated with germ-free mice," *Laboratory Animals*, vol. 19, no. 2, pp. 111–118, 1985.

[53] K. Fujihashi, J. R. McGhee, M. Yamamoto, J. J. Peschon, and H. Kiyono, "An interleukin-7 internet for intestinal intraepithelial T cell development: knockout of ligand or receptor reveal differences in the immunodeficient state," *European Journal of Immunology*, vol. 27, no. 9, pp. 2133–2138, 1997.

[54] M. Watanabe, Y. Ueno, T. Yajima et al., "Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes," *Journal of Clinical Investigation*, vol. 95, no. 6, pp. 2945–2953, 1995.

[55] D. W. McGee, K. W. Beagley, W. K. Aicher, and J. R. McGhee, "Transforming growth factor-β enhances interleukin-6 secretion by intestinal epithelial cells," *Immunology*, vol. 77, no. 1, pp. 7–12, 1992.

[56] J. A. Barnard, G. J. Warwick, and L. I. Gold, "Localization of transforming growth factor β isoforms in the normal murine small intestine and colon," *Gastroenterology*, vol. 105, no. 1, pp. 67–73, 1993.

[57] K. W. Beagley, J. H. Eldridge, F. Lee et al., "Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells," *Journal of Experimental Medicine*, vol. 169, no. 6, pp. 2133–2148, 1989.

[58] K. Atarashi, T. Tanoue, T. Shima et al., "Induction of colonic regulatory T cells by indigenous *Clostridium* species," *Science*, vol. 331, no. 6015, pp. 337–341, 2011.

[59] D. N. Frank, A. L. St. Amand, R. A. Feldman, E. C. Boedeker, N. Harpaz, and N. R. Pace, "Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 34, pp. 13780–13785, 2007.

[60] H. Sokol, B. Pigneur, L. Watterlot et al., "Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 43, pp. 16731–16736, 2008.

[61] B. Willing, J. Halfvarson, J. Dicksved et al., "Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease," *Inflammatory Bowel Diseases*, vol. 15, no. 5, pp. 653–660, 2009.

[62] Y. R. Mahida, S. Makh, S. Hyde, T. Gray, and S. P. Borriello, "Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: induction of interleukin 8 production and apoptosis after cell detachment," *Gut*, vol. 38, no. 3, pp. 337–347, 1996.

[63] J.-E. Branka, G. Vallette, A. Jarry, C. Bou-Hanna, P. Lemarre, and C. L. Laboisse, "Early functional effects of *Clostridium difficile* toxin A on human colonocytes," *Gastroenterology*, vol. 112, no. 6, pp. 1887–1894, 1997.

[64] Y. Ishida, T. Maegawa, T. Kondo et al., "Essential involvement of IFN-γ in *Clostridium difficile* toxin A-induced enteritis," *Journal of Immunology*, vol. 172, no. 5, pp. 3018–3025, 2004.

[65] G. K. A. Meyer, A. Neetz, G. Brandes et al., "*Clostridium difficile* toxins A and B directly stimulate human mast cells," *Infection and Immunity*, vol. 75, no. 8, pp. 3868–3876, 2007.

[66] I. Kurose, C. Pothoulakis, J. T. LaMont et al., "*Clostridium difficile* toxin A-induced microvascular dysfunction. Role of histamine," *Journal of Clinical Investigation*, vol. 94, no. 5, pp. 1919–1926, 1994.

[67] B. K. Wershil, I. Castagliuolo, and C. Pothoulakis, "Direct evidence of mast cell involvement in *Clostridium difficile* toxin A-induced enteritis in mice," *Gastroenterology*, vol. 114, no. 5, pp. 956–964, 1998.

[68] M. Warny, A. C. Keates, S. Keates et al., "p38 MAP kinase activation by *Clostridium difficile* toxin A mediates monocyte necrosis, IL-8 production, and enteritis," *Journal of Clinical Investigation*, vol. 105, no. 8, pp. 1147–1156, 2000.

[69] K. K. Jefferson, M. F. Smith Jr., and D. A. Bobak, "Roles of intracellular calcium and NF-κB in the *Clostridium difficile* toxin A-induced up-regulation and secretion of IL-8 from human monocytes," *Journal of Immunology*, vol. 163, no. 10, pp. 5183–5191, 1999.

[70] N. V. Jafari, S. A. Kuehne, C. E. Bryant et al., "*Clostridium difficile* modulates host innate immunity via toxin-independent and dependent mechanism(s)," *PLoS ONE*, vol. 8, no. 7, Article ID e69846, 2013.

[71] R. Madan and W. A. Petri Jr., "Immune responses to *Clostridium difficile* infection," *Trends in Molecular Medicine*, vol. 18, no. 11, pp. 658–666, 2012.

[72] M. Hasegawa, T. Yamazaki, N. Kamada et al., "Nucleotide-binding oligomerization domain 1 mediates recognition of *Clostridium difficile* and induces neutrophil recruitment and protection against the pathogen," *Journal of Immunology*, vol. 186, no. 8, pp. 4872–4880, 2011.

[73] A. Ryan, M. Lynch, S. M. Smith et al., "A role for TLR4 in *Clostridium difficile* infection and the recognition of surface layer proteins," *PLOS Pathogens*, vol. 7, no. 6, Article ID e1002076, 2011.

[74] I. Jarchum, M. Liu, C. Shi, M. Equinda, and E. G. Pamer, "Critical role for myd88-mediated neutrophil recruitment during *Clostridium difficile* colitis," *Infection and Immunity*, vol. 80, no. 9, pp. 2989–2996, 2012.

[75] I. Jarchum, M. Liu, L. Lipuma, and E. G. Pamer, "Toll-like receptor 5 stimulation protects mice from acute *Clostridium difficile* colitis," *Infection and Immunity*, vol. 79, no. 4, pp. 1498–1503, 2011.

[76] J. Ng, S. A. Hirota, O. Gross et al., "*Clostridium difficile* toxin-induced inflammation and intestinal injury are mediated by the inflammasome," *Gastroenterology*, vol. 139, no. 2, pp. 542–552, 2010.

[77] C. P. Kelly, S. Becker, J. K. Linevsky et al., "Neutrophil recruitment in *Clostridium difficile* toxin A enteritis in the rabbit," *Journal of Clinical Investigation*, vol. 93, no. 3, pp. 1257–1265, 1994.

[78] B. Qiu, C. Pothoulakis, I. Castagliuolo, S. Nikulasson, and J. T. LaMont, "Participation of reactive oxygen metabolites in *Clostridium difficile* toxin A-induced enteritis in rats," *The American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 276, no. 2, part 1, pp. G485–G490, 1999.