Interaction between *Clostridium* species and microbiota to progress immune regulation

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REVIEW ARTICLE

ABSTRACT

Gut microbiota can interact with the immune system through direct or indirect pathways. In the indirect pathway, gut microbiota produces metabolites such as short chain fatty acids (SCFAs), which may modulate the immune response. SCFAs reduce inflammation, repair intestinal barrier, and induce propagation of specific immune cells, e.g., T regulatory cells (Treg), which can suppress reactive cells such as macrophage and dendritic cells (DCs). As one of the most dominant members of microbiota, *Clostridium* produces SCFAs. As one of SCFA members, butyrate plays an important role in the modulation of immune cells. Through butyrate production, *Clostridium* helps to generate aryl hydrocarbon receptor (AhR). AhR interacts with many proteins inside the cytoplasm including Heat Shock Protein 90 (HSP 90), HSP 23, and chaperone. Activation of AhR leads to its translocation inside the nucleus and gene expression, which yields cell differentiation, energy metabolism, microbial defense, and immune cell propagation. Moreover, it may interact with other cells like B-cell and epithelial cell, which are responsible for modulation and maturation, respectively. AhR causes upregulation in the co-stimulatory marker in the DCs and interacts with nuclear factor KB (NF-κB) to modulate cell function. Butyrate induces Treg (iTreg) propagation and upregulates the Forkhead box p3 (FOXP3) as a special marker of Treg cell. It may also yield signaling through G-protein coupled receptors (GPRs) which, in turn, facilitates polymorphonuclear (PMN) chemotaxis.

The interaction between microbiota and non-immune cells, such as Paneth cells, leads to the secretion of antimicrobial substance, erection of barriers against bacterial pathogens, and regulation of microbiota composition via feedback effect. In addition, the components released from microbiota, such as peptidoglycan, reinforce the maturation of both the immune system and non-immune tissue development. Moreover, microbiota can directly activate the effector cells, e.g., macrophage, to secrete cytokines and propagate Treg cells.

KEYWORDS

Clostridium, immune regulation, microbiota, SCFAs, AhR

INTRODUCTION

Human gastrointestinal tract is a shelter organ for many bacterial species called microbiota. Composition of microbiota plays an important role in human health and any variation in this mixture causes multiple diseases. Although microbiota can be transmitted vertically, they can be transmitted horizontally via pathogenic potential [1]. In this regard, the microbial community may make some changes to the mentioned transmission, yielding an effect called dysbiosis. Dysbiosis is explained as disruption or change in the microbial community that yields the impairment of normal activities, including resistance against the invading pathogens. Sometimes, normal microbiota may undergo variations and mediate damage to the host, hence the emergence of pathobionts. Pathobionts can be either beneficial and protective

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or harmful, depending on the remaining composition of microbiota [2]. Virulence of these bacteria may occur only in a susceptible host. The pathogenic potential defines a host that becomes symptomatic after infection. The most abundant microbiota belongs to three groups of strict anaerobes, namely *Clostridium* cluster XIVa, *Clostridium* cluster IV, and *Bacteroides* [3]. *Clostridium* is a gram-positive, rod-shaped spore producer and many species in this group produce toxins. *Clostridium* cluster XIVa consists of *Clostridium*, *Ruminococcus*, *Coprococcus*, *Acetitomaculum*, *Anaerocolumna*, *Roseburia*, *Faecalicatena*, and *Hungatella* genera. Another cluster, i.e., cluster IV, consists of species belonging to *Clostridium*, *Ruminococcus*, *Eubacterium*, *Caproiciproducens, Faecalibacterium, Papillibacterium,* and *Subdoligranulum* genera [4–6]. This cluster produces many degrative enzymes that mediate digestion of oligosaccharides and polysaccharides, generating acetic acid, butyric acid, and propionic acid called short chain fatty acids (SCFAs). One of the most important metabolites that ensures a balanced gut is SCFA, which plays a significant role in the prevention of metabolic syndrome, colon cancer, and bowel disorder [7]. Microbiota inhibits the growth of pathogens by the occupation of intestinal niches, competitive metabolic interactions, production of SCFAs, and induction of host immune systems. Microbiota may regulate gut barrier through the inducement of IgA production and REGIII as an antimicrobial molecule, upregulation of IL-1β, and upregulation of mucus layer [8].

Clostridium species along with Lactobacillaceae, Bacteroidaceae, and Enterococcaceae inhabit the ascending colon. *Clostridium* members can be detected within a week after birth, changing from Cluster I in infants to Clusters IV and XIVa in adults [9, 10]. However, in addition to the beneficial role of *Clostridium* in health, some of Clostridia such as *C. perfringens*, *C. botulinum*, and *C. difficile* may cause infection. *C. perfringens* are divided into five types according to major α, β, ε and ι toxins. In addition to these toxins, another minor toxin and enterotoxin are produced by *C. perfringens*. The major problems with *C. perfringens* include gas gangrene, β-pore-forming toxin, and necrotizing enteritis [11].

This study focuses on the role of *Clostridium* spp. and their metabolites in immune modulation.

A. SCFA production and transportation

Many factors including dietary bacterial species composition, substrate availability, intestinal transit, and environment are involved in SCFA production. The majority of SCFAs are produced following the breakdown of carbohydrates in a proximal large intestine. Hydrogen metabolism and inorganic electron acceptors, such as sulfate and nitrate, facilitate the oxidization of SCFAs to form acetate. Other important factors involved in SCFAs production include growth rate and bioavailability of carbon [12]. The major pathway to glucose catabolism is the Embden-Meyerhof-Parnas (EMP) that initiates CO2 production. Acetate is produced via the fermentation of carbohydrate which is mostly formed from CO2 through the WOOD-Ljungdahl pathway [13]. SCFAs are mainly made of different polysaccharides, propionate, and acetate as products of arabinogalactan, butyrate from starch, and acetate from pectin [14].

Metabolism of carbohydrates by enteric bacteria produces SCFAs, which are composed of 1–6 carbons. The major SCFAs include acetate (C2), propionate (C3), and butyrate (C4) as well as isobutyrate (C4), valerate (C5), and hexanoate (C6), which are longer SCFAs produced with lower amounts in the colon [15]. SCFAs are absorbed in the colon by ionization or protonation mechanism and are affected by pH. Absorption across the cell membrane occurs through the following mechanism. Diffuse absorption may contribute to the removal of proton from SCFA. In the next step, the removed proton is exported by a monocarboxylate transporter or sodium/hydrogen exchange. The introduction of SCFA to the cytosol may occur by ion-exchange with bicarbonate. This interaction obviously stabilizes pH through the secretion of bicarbonate or proton removal [16].

B. Microbiota

Microbiota consists in a complex community of bacterial species in human intestines. A trillion of bacterial cells belonging to at least 21 different genera colonize the human gastrointestinal tract. Most of these strains include up to 9 different bacterial phyla, up to 90% of which include Bacteroidetes and Firmicutes [17, 18]. The preliminary roles of microbiota include resistance against pathogenic infection, food digestion, development of Gut-Associated Lymphoid Tissue (GALT), and regulation of the level of antimicrobial peptides [19]. The gastrointestinal tract must detect and confront bacterial pathogens and tolerate many bacteria, called microbiota, given that not all microbiota members are totally friendly and some may play the role of opportunistic pathogens [20]. Moreover, the presence of specific types of microbiota in the gastrointestinal tract may be conducive to the existence of closely related pathogens. Accordingly, the existence of a large number of *Escherichia coli* causes greater susceptibility to *Salmonella enterica*, while the existence of a large number of Lactobacilli causes greater susceptibility to *Lactobacillus reuteri* colonization due to some particular metabolite products and secretion of specific bacteriocins [21].

One of the important roles of microbiota is to maintain intestinal homeostasis by secretion of many important metabolites such as butyrate. The existence of butyrate initiates epithelial signaling through Peroxisome proliferator-activated receptorϒ (PPAR-ϒ), without which the expression of Nos2 gene as a nitric oxide synthesis is upregulated. On the other hand, PPAR-ϒ signaling mediates the consumption of oxygen by colonic epithelial cells, leading to inhibited availability for use by pathogenic bacteria [22]. GPR109a is another receptor of butyrate as well as of niacin in colonic cells. Signaling through GPR109a in the macrophages and DCs facilitates the differentiation of Treg cells. Macrophages and DCs are appropriate for differentiating naive T cells to Treg cells through anti-inflammatory Aldh1a, IL-10, and TGF-β expression [23]. Butyrate upregulates GPR109a and the tight junction proteins including Occludin, Claudin-3, and Zonula

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Occludens, thus facilitating recovery from diarrhea by reducing intestinal permeability and promoting the expression of p38, ERK1/2, and Akt phosphorylation [24]. The major role of tight junction proteins is in inhibiting the luminal passage of antigen and material across the paracellular space. Butyrate induces mRNA expression of claudins-3 through phosphorylation and Akt activation, thus upregulating protein expression [25]. It can also mediate the induction of c-Jun and Bcl-xS proteins, causing apoptosis by morphological changes in human gastric cancer [26]. Another receptor of butyrate is GPR43, which mediates butyrate signaling. Decrease in butyrate leads to the reduction of GPR43 expression, as a result of which the secretion of pro-inflammatory cytokines, such as IL-6 and TNF-α, is enhanced [27]. GPR43, in addition to butyrate signaling, is present in the PMNs and is associated with the modulation of neutrophil recruitment. Following the GPR43 deficiency, the number of neutrophils in the lamina propria increases [28]. Another important role of microbiota, especially Clostridia, is to provide resistance to the colonization of bacterial pathogens, such as *Salmonella typhymurium*, without stimulating the innate immune system or adaptive immunity [29].

B-1: Molecular identification of microbiota. To identify the microbiota, many molecular techniques can be applied, each of which is characterized by its own specific advantage and disadvantage, importance, and applicability (See Table 1). Since most of microbiota cannot be cultivated, the applicable methodologies are culture independent. Culture-independent techniques such as PCR, RT-PCR, NGS and sequencing of the 16S rRNA allow detecting microbiota genetics, pathogenic potential, and metabolite and protein products [30]. Molecular techniques such as metagenomics and genomics may identify new microbiota. Microbiota may exhibit few manifesting symptoms in culture and are subject to ambivalent need for growth or cultivation due to Viable but nonculturable (VBNC) state [31, 32].

B-2: Probiotic strains and interaction with microbiota. The existence of some species, their type and number, and the interaction between them may maintain one's health or induce a chronic or acute disease. Identification of an appropriate microbiota composition is associated with personal health. Manipulation of gut microbiota can be done through prebiotics, probiotics, postbiotics, and symbiotics. Prebiotics are identified as food components utilized to propagate beneficial bacteria. They are benign living bacteria that are eaten to colonize the colon [55]. Postbiotics are identified as metabolites or components such as enzymes, cell surface proteins, and peptidoglycan that are released by probiotics [56]. Colonization of probiotics is dependent on person-specific factors such as age, probiotic supplementation, diet, and microbiome. To the best of our knowledge, use of probiotics requires host data such as immune profiling, genetics, microbiome data, and factors involved in colonization resistance [57]. However, since *Clostridium* is one of the most important members of microbiota, using it as a probiotic may be successful. *Clostridium* intrinsically has a probiotic role because, first, it is already a commensal bacterium. Second, Clostridia, especially clusters IV and XIVa, may maintain intestinal health upon inducing an anti-inflammatory effect. Third, *Clostridium* shifts to spore formation and resists harassing conditions [5]. One of the important points about the selection of *Clostridium* as a probiotic is to consider toxin-producing species. Except for the toxin-producer Clostridia, species with protein fermentation activities and antibiotic-resistant genes should receive greater attention [58].

Microbiota existing on the mucus layer, called parietal microbiota, are reorganized through ingested probiotics [59]. However, probiotics such as *Lactobacillus rhamnosus* can be retrieved from stool without affecting or changing the microbiota composition [60]. Recent data have shown that although microbiota composition remains unchanged, the metabolic shift can be observed. Some bacteria such as *Lactobacillus acidophilus* are able to modify the metabolic profiles without changing the microbiota composition. With an increase in the local concentration of branched-chain fatty acid, *L. acidophilus* ensures the maintenance of gut physiology following an increase in the number of Bifidobacteria and Lactobacilli [61]. Bifidobacteria may produce substances that are consumed by microbiota. *Bifidobacterium bifidum* releases sialic acid from the host cell, which is utilized by microbiota *Bifidobacterium breve* [62, 63].

C. Immune modulation

C-1: Microbiota modulated immune system through IL-10 and Treg propagation. Gut microbiota contributes to immune regulation by inducing macrophage to produce IL-10. In the case of germ-free mice, macrophage is normally present, but the amount of IL-10 production is critically low. However, in the presence of microbiota the production of IL-10 increases. Increase in IL-10 production with the help of microbiota negatively regulates the immune response against lipopolysaccharide (LPS) [64]. Microbiota always interacts with epithelial cells including goblet cells, M cells, columnar epithelial cells, and Paneth cells. These intestinal epithelial cells play an important role in protection against pathogenic bacteria, opportunistic bacteria, and viruses. They also have other important roles such as absorbing nutrients, sampling antigens, producing digestive enzymes, and regulating pluripotent stem cells in the Peyer's patches and the mucosa-associated lymphoid tissue through the secretion of pro-inflammatory cytokines [65]. Intraepithelial cells express a variety of carbohydrates and highly secrete glycosylated mucins into the intestinal lumen to create a physical barrier. However, the microbiota evolves to use carbohydrates and can force the host cells to produce specific carbohydrates. One of the important carbohydrates found in the terminal residues of epithelial cells is Fucose [66]. *Bacteroides thetaiotamicron* is found in human colon and it actively induces fucose expression in the epithelial cells. Fucose is utilized as a nutrient by *B. thetaiotamicron* and regulates bacterial genes. On the other hand, *Bacteroides fragilis* utilizes fucose in the synthesized polysaccharide capsules, contributing to preferential colonization [67].

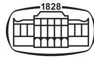

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Table 1. Molecular identification of microbiota and its pros and cons

Method	Activity	Pros and cons	References
Culture	Sample prepared and cultivated in pre-enriched or selective media	- Slow growth. - Difficulty detecting the specimen with few bacteria. - Phenotypes used for detection may not be expressed. - Most of microbiota are unculturable.	[33-35]
16S rRNA sequencing	Amplification of a highly conserved small subunit of the ribosome and sequencing of the products may cause a distinction among bacteria	- Costly - Biased in DNA extraction and primer - Results dependent on the read curation method - Dependence of results on the DNA sequencing method + Identification of bacterial lineages + Determination of unculturable bacteria	[36-38]
Denaturing gradient gel electrophoresis (DGGE)	Detection of variant in the microbial community	- Low reproducibility - Difficulty in choosing the best time and gel composition + Quick detection + Appropriate culture independent in the mixed sample	[39, 40]
Human intestinal tract chip (HITchip)	Phylogenetic microarray, over 4,800 oligonucleotide probes were designed based on the hypervariable region of small subunit ribosomal RNA	- Difficulty in finding the pattern + Cost and speed + Collection of many microbial profiles	[41, 42]
Matrix-assisted laser desorption/ ionization time of flight (MALDI-TOF)	Detection of metabolite/detection of a large number of proteins	- Time consuming - Low sensitivity - Difficulty detecting the small number of proteins - Requiring sample purification + Automated system + Analysis of the whole cell protein of tested bacteria + Favored tools for microbial communities + Low cost	[43, 44]
Machine learning classification of flow cytometry data (Cellcognize)	Analysis of cell diversity data from flow cytometry	- Need for more data and studies	[45]
Gas chromatography	Determination of metabolite	- Need to couple with other methods to provide a higher peak - Sample handling affecting the data + In combination with mass spectrometry to quantify SCFAs	[46, 47]
Real-time PCR	Amplification of target DNA for detection of organisms	- Costly - Special equipment - Different methods to analyze amplification curve + Quantification of a small amount of DNA, RNA	[48, 49]
PCR	Amplification of a special gene for detection of bacteria	- Activity depending on primer design - Detection of single gene per reaction - Presence of non-target DNA in specimen - Quality of the extracted DNA + Multiplication of the desired gene to a large extent + Simple and almost cheap	[50, 51]

(continued)

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Table 1. Continued

Method	Activity	Pros and cons	References
Fluorescence in situ hybridization (FISH)	Detection of target RNA or DNA by fluorescence	- Occasionally, RNA or DNA is not fluorescently labeled - Stray probe may be wrongly detected as RNA + Combination of methods such as NanoSIMS and micro-autoradiography to facilitate structure and function analysis	[52, 53]
Raman activated cell sorting (RACS)	Detection of unlabeled compound in the presence of deuterated water	- Limited function - Need for complementary technique + Linking phenotype to genotype directly	[54]

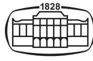
B. fragilis helps immune modulation by activating Toll like receptor-2 (TLR-2), which leads to the higher secretion of IL-10 and inhibition of inflammation [68]. Another type of TLR, namely TLR-9, recognizes unmethylated Cytidine-Phosphate-Guanosine (CPG) motif, which is abundant in prokaryotic DNAs. However, microbiota has costimulatory signals with oligodeoxynucleotides in the CpG motif to T-cells. This costimulatory signal induces CD28 on the T-cells, providing a modulated balance between Treg and effector T-cells [69]. Microbiota can balance the immune response through the regulation of Treg/Th17 proportion. Th17 induces immune response and summon effector cells to combat microbial pathogens. On the other hand, Treg plays a major role in suppressing and eliminating the immune response initiated by Th17. Composition of microbiota has an important role in immune modulation. Due to the changes of microbial composition and a reduction in beneficial bacteria such as Clostridia, dysbiosis may cause many diseases [70]. The greater the diversity of the strains of Clostridia, the higher the differentiation of Treg cells, and vice versa. This explains why a greater diversity of microbiota, especially Clostridia, results in better immune modulation [71].

C-2:* Clostridium, *TGF-β, and immunoglobulin production. *Clostridium*, among all species, induces TGF-β production and propagate a subset of T-cells as regulators by CD4$^+$ Foxp3$^+$ marker. Clostridia in their early years of life reinforce the resistance against systemic IgE response and colitis. Also, TGF-β as immunosuppressive cytokine can facilitate the development of Dendritic cells (DCs), B-cells, and natural killer (NK) cells [72]. The existence of $\alpha_v\beta_8$ integrin with DCs is essential to activating TGF-β to induce and maintain Treg cells in the colonic tissue [73]. TGF-β also has an important role in IgA production by B cells and the lack of TGFβR inhibits IgA production [74]. Initial activation of B cells, which is induced by TLRs or co-stimulatory receptor, can be enhanced by SCFAs in the gut-associated lymphoid tissue or it may reach the bloodstream and stimulate B-cell in the extra-intestinal tissue. SCFAs enhance the synthesis of ATP, fatty acids, and acetyl-CoA in B-cells, contributing to the differentiation of the plasma cells. As a result, secretion of IgG and IgA, attached to the microbiota, occurs that plays a determining role in microbial population adjustment [75].

IgA production occurs in both T-dependent and T-independent ways. However, microbiota including Clostridia, which colonize the epithelium surface, evoke the response of the T-cell-dependent pathway, which helps the differentiation of mature cells migrating to the lamina propria and secretion of high-affinity IgA against composition change in the microbiota. Foxp3$^+$ Treg ameliorates the diversity of microbiota, especially Clostridia, which contribute to the propagation of Treg cells. Through the feedback mechanism, Clostridia are further maintained and propagated [76].

C-3: Regulation of T-cell propagation. Most of the Treg cells in the lamina propria have a nuclear hormone receptor, namely RORϒt, which mediates transcription. This subset of Treg cells expresses more IL-10 and most of them express Foxp3+ marker, as well. Microbiota may induce T-RORϒt and Th17 and make a balance between them, provoking immune regulation [77]. Microbiota and availability of nutrients are two important factors in the differentiation of T-cells. Differentiation of Treg cells is dependent on oxidative phosphorylation; however, Th17 differentiation relies on the glycolytic-lipogenic metabolic pathway. Upregulation of glycolytic pathway can also be induced by the hypoxia-inducible factor 1α as a transcription factor, which finally induces Th17 and inhibits Foxp3+ expression [78]. Extracellular ATP and hypoxia both increase in the inflammatory site and inhibit AhR activation and Treg differentiation [79]. An important microbiota group is segmented filamentous bacteria (SFB), which are involved in immune regulation by attenuating the bacterial-inducer colitis. SFB also regulate the intestinal barrier function through the IL-23R/IL-22 pathway. Destruction of the intestinal barrier leads to the leakage of microbial products, which induce IL-23 production for intestinal barrier reformation. The production of IL-23, in turn, improves IL-22 production, leading to greater serum amyloid A making by epithelial cells [80]. Finally, serum amyloid mediates IL-17 expression in T-RORϒt, thus improving Th17 differentiation [81].

Clostridia reinforce IL-22 production by type-3 Innate lymphoid cells (ILC3), contributing to the recovery of intestinal barrier [82]. Microbiota helps macrophage to secrete



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IL-1β, releasing colony-stimulating factor 2 (Csf) from ILC3. Csf2 reinforces DCs and macrophage activity for IL-10 and retinoic acid production, resulting in propagating and maintaining Treg cells. Microbiota can be sensed by TLR which helps activate Myd88 as an important adaptor protein in the immune cells. Activation of Myd88 contributes to the transcription and release of IL-1β to induce production of Csf2 by ILC3. In other words, IL-1β can act as an inducer in Csf2 production [83].

C-4: Microbiota and innate immune system. Microbiota can also regulate innate immune response by pattern recognition receptors (PRRs) found on the surface of immune cells such as macrophage and DCs. PRRs include TLRs, RIG-I-like Receptors (RLRs), C-type lectin Receptors (CLRs), and nucleotide-binding oligomerization domain like Receptors (NLRs), which are recognized by their Pathogen-Associated Molecular Patterns (PAMPs) [84]. Regulation of the immune response is affected by microbial composition. Diet can make changes to the microbial composition. Western diet evokes an innate immune response by reprogramming myeloid progenitor through IL-1R and NLRP3 [85]. Through the modification of chromatin, methylate, and acetylate DNA as well as activation of STAT, MAPK, and NF-κB, microbiota helps to express the inflammatory cytokines [86, 87].

Microbiota indirectly affects NK cells through interaction with antigen presenting cells (APCs) such as macrophage and DCs. DCs express IL-15 in response to the type-1 interferon (IFN), hence their early activation. DCs transpresent IL-15 to the NK cells [88]. However, in the case of germ-free mice, the process of differentiation and maturation of NK cells is not affected by microbiota. Translocation of NK cells from the germ-free mice to normal ones was accompanied by normal activities, proving that the deficiency in NK cells activity had extrinsic origins and was dependent on the deficiency of APCs, specifically DCs. In other words, DCs in germ-free mice were not induced by microbiota to produce inflammatory cytokines [89]. Activation of DCs in the conventional mode by microbiota methylates H3K4 in the histones found around the inflammatory genes. However, in germ-free mice, it causes less histone modifications and low expression of inflammatory genes [90]. NOD-2 methylates H3K4 and promote the expression of inflammatory genes. NOD-2 is implicated in the autophagy formation in the DCs, macrophage, and epithelial cell that limit the dispersal of bacteria to the subintestinal tissue [91]. A-defensin is secreted by Paneth cells which stimulates the host's defense against bacterial pathogens. It also affects microbiota and changes their composition. NOD-2 signaling mediates secretion of α-defensin by Paneth cells. It has been observed that in the case of NOD-2 deficient mice, the level of α-defensin secretion is lower. Change to microbiota composition, especially SFB, by α-defensin alters the T-cells balance [92]. In addition to NOD-2, Paneth cells can secrete antimicrobial peptides such as phospholipase A2 and lysozyme, which regulate microbiota [93]. Sensing peptidoglycan of microbiota by NOD-2 leads to the secretion of IL-23 by DCs that would force Th17 to secrete IL-22 (See Fig. 1).

IL-22 provides protection and surveillance upon regenerating islet-derived protein IIIϒ (REGIII) and antimicrobial peptides [94]. One of the important roles of REGIII as antimicrobial lectin is producing a safe zone between intestinal epithelial cells and microbiota. This safe zone controls immune response against microbiota. However, the lack of REGIII helps microbiota colonize the epithelial surface, resulting in better activation and response of the adaptive immune system. When microbiota colonizes the adherent epithelial cells, signaling through MyD88 and more expression of REGIII occur [95]. As a consequence, the position of the microbiota is balanced, epithelial barrier is recovered, and the immune response is reduced.

C-5: NOD and Peptidoglycan. Through the recognition of peptidoglycan, NOD-1 induces the formation of intestinal lymphoid tissue. Formation of isolated lymphoid Follicles (ILFs) needs the sensing of bacteria and in the case of germ-free mice, the formation of ILFs is attenuated. ILFs are responsible for the generation of B-cells that secrete IgA. Thus, microbiota cause the formation of ILFs and in return, ILFs propagate IgA-producing B-cells that support microbial homeostasis [96]. This interaction between microbiota and B-cells creates negative feedback and intestinal homeostasis [97]. Nod-1 also induces bone marrow to produce inflammatory cells such as neutrophil and monocyte. In the case of germ-free mice, the number of neutrophils and their effectiveness are reduced against microbial pathogens such as *Staphylococcus aureus* [98]. NOD-1 also mediates the secretion of IL-17A by ϒδ-T cells, which modulate myeloid cell activity.

Peptidoglycan recognition protein (PGLYRP) as a receptor of peptidoglycan can combine with LPS and make it a multi-functional protein that recognizes both gram-positive and gram-negative bacteria. Moreover, it can combine with protein sensor histidine kinase inside the bacteria and activate a stress response to bacterial killing [99]. PGLYRP-2 contains the amidase domain and needs minimum muramyl tripeptide for its activity. In this regard, PGLYRP-2 is not as fluent as NOD-2 in recognizing muramyl dipeptide. Moreover, it interferes with NOD-2 by cleaving glycan, which is essential to NOD-2 recognition activity. This change in the tripeptide reduces recognition by NOD-2 and changes the balance towards NOD-1 [100]. Peptidoglycan recognition protein 4 (PGLYRP4) has antimicrobial activities and provides protection against both gram-positive and gram-negative bacteria. The lack of PGLYRPs is evident in severe inflammation, as a result of which more neutrophil recurrent to the infection site occurs. It has been observed that in the mice without PGLYRPs, the composition of microbiota is altered and lower SCFAs are detected. This may explain the lower amount of anti-inflammatory cytokines and greater pro-inflammatory cytokine production [101] (See Fig. 2).

NOD-1 specifically recognizes ϒ-D-glutamyl-meso-diaminopimelic acid, which is present in both gram-negative

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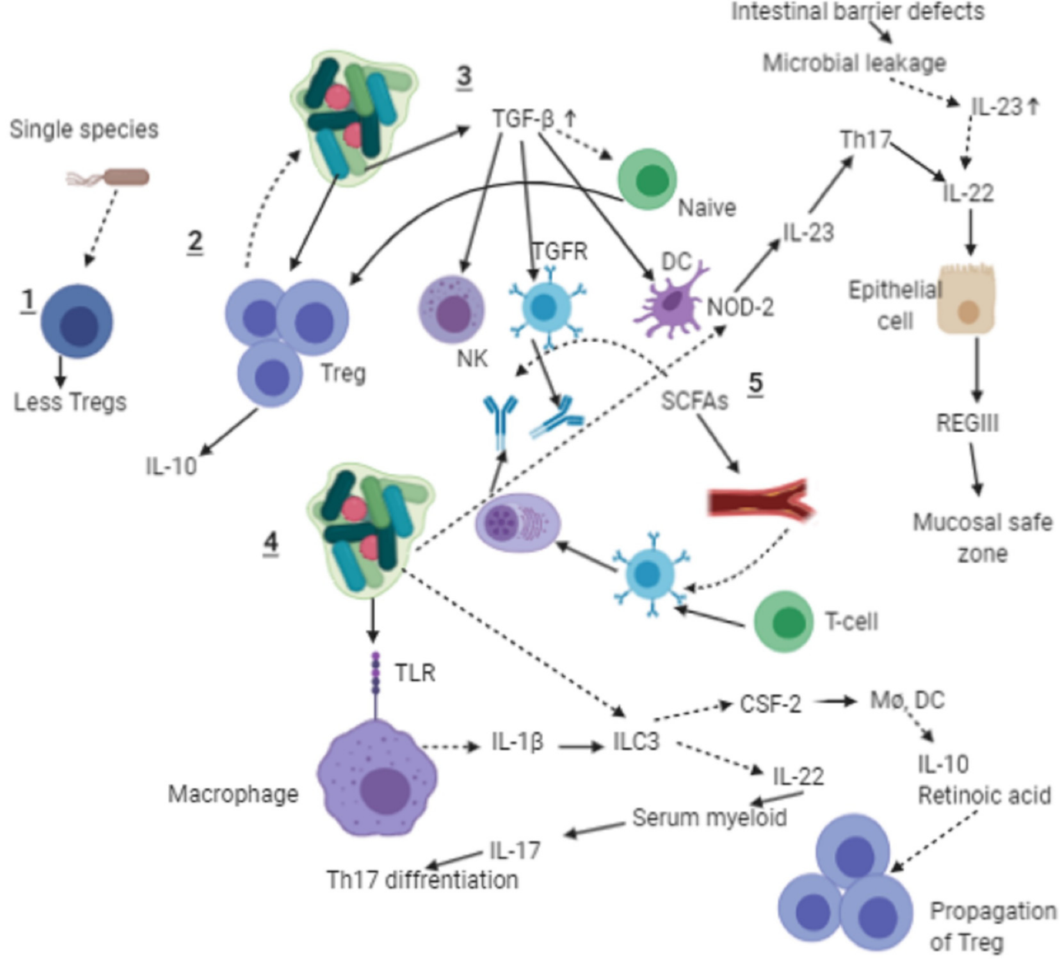

Fig. 1. Effect of microbiota on the T-cell differentiation.
Single species from microbiota causes less Treg differentiation. 2. Heterogeneous gut microbiota mediates more Treg differentiations. 3. Microbiota induces TGF-β production that leads to the conversion of Naïve T-cell into Treg. TGF-β affects NK, DC, and B-cells. 4. Sensing of the microbiota by macrophage through TLR causes IL-1β secretion and ILC3 formation. 5. Heterogeneous gut microbiota leads to NOD-2 signaling that mediates IL-23 secretion. IL-23 forces Th17 to secrete of the IL-22 which affects epithelial cells

and gram-positive bacteria. While NOD-1 is found in various cell types including epithelial cells, endothelial cells, hepatocyte, and stromal cells, NOD-2 is found only in the immune cells [102].

Peptidoglycan recognition can be done in many ways such as transport across cell membranes via the receptor of the host cell or via secretion system of the bacterial cell. Other ways include degradation of phagocytic bacteria, endocytosis, uptake of outer membrane vesicle, and internalization from the neighboring infected cell [94].

Peptidoglycan is disseminated to the distal tissues of brain and bone marrow. In the bone marrow, peptidoglycan directs myelopoiesis towards lymphopoiesis and erythropoiesis, which are dependent on IL-23 production through intestinal inflammation [103]. However, peptidoglycan from microbiota can evoke an immune response by being internalized in the epithelial cell or disseminated to the blood. In addition, it changes tissue functioning and development [104].

Circulation of peptidoglycan may vary in different people. In patients with autoimmune diseases such as systemic lupus erythematosus, the level of peptidoglycan circulation is significantly higher than that in normal people; hence, the immune system and a higher inflammatory response can be detected [105].

In *drosophila*, a peptidoglycan sensor can be found in the enterocyte cytosolic, which initiates NF-κB signaling or makes cleavage in peptidoglycan. Regulation of this enzyme through microbiota modulates the dissemination of peptidoglycan to the extra-intestinal tissue [106]. Peptidoglycan dissemination reaches the brain and is sensed through specific receptors, namely NLRs and peptidoglycan-recognition proteins, as shown and highly expressed in the developed brain. Peptide transporter 1 (pepT1) mediates translocation of peptidoglycan inside colonic cells and has a vital role in the development of the brain [107].

CLOSTRIDIUM AND SCFAS

In the first days of the life of breastfed infants, bacterial flora is heterogeneous and mostly colonized by the aerobic species. Then, oxygen consumption by aerobic bacteria

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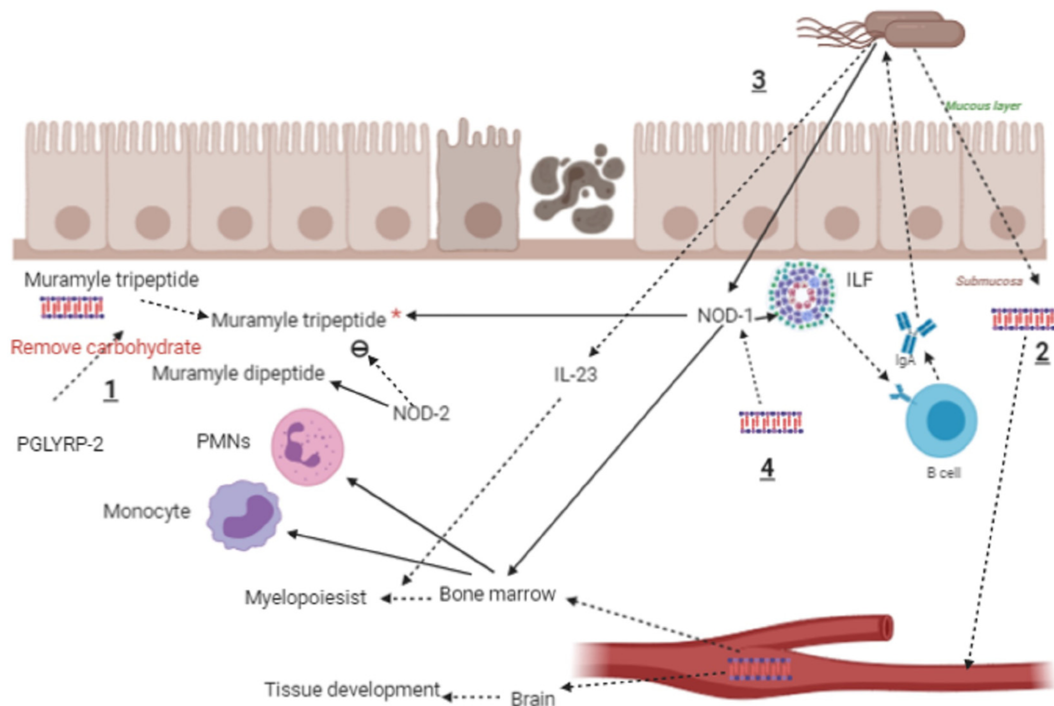
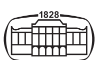


Fig. 2. Effect of recognition of cell wall on the Myelopoiesis.
PGLYRP-2 by amidase domain removed glycan from Muramyle tripeptide and converted it to the Muramyle tripeptide* which could not be recognized by NOD-2. However, this conversion is the reason NOD-1 take the recognition charge rather than NOD-2. 2. Dissemination of peptidoglycan from the microbiota to the bone marrow and brain occurs through bloodstream. 3. Microbiota can directly cause IL-23 secretion and myelopoiesist. 4. Muramyle tripeptide through NOD-1 recognition could mediate ILF formation, and at the next step B-cell activation and IgA secretion controlled the microbiota

provides optimal conditions for both obligate anaerobic and facultative aerobic bacterial growth [108]. Intestinal lumen is divided into two regions: the mucosal region, where *Clostridium* cluster is present, and the region occupied by *Bacteroidaceae* [109]. Butyrate as an important SCFA has many important functions and can be produced by Clostridia. It has immunomodulatory effects, is a good energy source for colonocyte cells, maintains intestinal barrier function, and decreases pH of the colon. Decrease in pH reduces ammonia adsorption and improves mineral absorption [110]. Butyrate also induces normal colon epithelial cells, has a protective role against colon cancer, inhibits the growth of bacterial pathogens, and stimulates mucin production [111]. Butyrate is made from pyruvate in pyruvate-ferredoxin oxidoreductase, the process of which produces carbon dioxide and reduces ferredoxin [112]. Acetyl-CoA as a median component is converted to butyryl-CoA via a four-step pathway. Production of butyrate leads to additional amount of ATP and regenerates NAD^+ from NADH [113]. Of note, the comparison of butyrate production in pathogens and commensal bacteria points to the differences between these two groups. Commensal bacteria such as Clostridia are abundant in human intestines and increase butyrate production [114]. In abnormal gut conditions, other genera such as *Anaerococcus*, *Streptococcus*, *Rothia*, and *Escherichia* are found in abundance due to the shortage of microbiota such as Clostridia [115]. Another important difference between these two groups stems from the fact that butyrate pathways do not exist in pathogenic bacteria such as *Streptococcus*, *Enterococcus*, and *Campylobacter*, while the pathogenic Clostridia possess all the four pathways for butyrate production [116]. Clostridia regulate the immune system by their metabolites and induce production of $\alpha\beta$ T-cell intraepithelial lymphocyte (IEL) and B cells, which are effective in providing a response to antigens [117]. Butyrate as an SCFA and an important metabolite of Clostridia initiates signaling through G protein-coupled receptors (GPRs), which are abundant in PMN and monocytes. GPRs activation is followed by the activation of ERK1/2 and intracellular Ca2+ that have important roles in PMN chemotaxis [118]. Butyrate also contributes to the differentiation of macrophage, improves antimicrobial production, makes shift in metabolism, and induces light chain 3 α(LC3)-associated production. In other words, exposure of macrophage to the butyrate during differentiation leads to more antimicrobial activities. In addition, butyrate inhibits mTOR as a master regulator of autophagy. Calprotectin is an antimicrobial protein that kills bacteria through sequestration of $zinc^{2+}$ and $manganese^{2+}$. This protein is also upregulated in the presence of butyrate and it enhances the antimicrobial activity of macrophage [119]. Butyrate stimulates the NaCl transport and inhibits Cl^- secretion. Blocking Cl^- inhibits the stimulation effect of cholera toxin, prostaglandin E2, and phosphocholine. The inhibitory effect is enhanced through the reduced production of cAMP. Butyrate helps absorb the Na-dependent fluid through SCFA/HCO3, Na-H, and



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SCFA/Cl exchange [120]. Therefore, upon reversing the impact of pathogenic bacteria, butyrate lowers inflammation and restores intestinal balance.

BUTYRATE AND ARYL HYDROCARBON RECEPTOR (AHR)

Aryl hydrocarbon Receptor (AhR) plays a significant role in regulating metabolic processes and an immune response in the intestinal tract. It is produced by butyrate and it has the ability to be localized in both nuclear and nuclear exports. Notably, it can also shuttle between nucleus and cytoplasm. In cytoplasm, AhR can interact with many proteins such as heat shock rotein 90 (HSP), HSP p23, tyrosine kinase Src, and chaperon Cdc37 [121]. Activation of AhR brings about conformational change, leading to its release from the neighbor proteins, hence its translocation to the nucleus. This translocation induces many genes like *Cyp1a1*, *Cyp1b1*, and AhRR, which is a detoxifying enzyme, as well as the genes that cause immune cell differentiation [122]. AhR is also vital to microbial defense, energy metabolism, chemical defense, reproduction, and immunity. It induces the macrophage to kill engulfed bacteria via the prolonged production of reactive oxygen species (ROS) and to survive by inhibiting the apoptosis process [123]. AhR also induces gene expression of $p40^{phox}$, which is a member of NADPH oxidases, for ROS production as an antimicrobial agent [124]. It helps produce the Apoptosis inhibitor of macrophage (AIM) and ensures macrophage survival in the microbial challenge [125] (See Fig. 3).

AHR AND DENDRITIC CELL (DC)

AhR contributes to the differentiation and functioning of DC upon controlling transcriptional response. AhR interacts with many transcriptional factors such as Nuclear Factor Kappa B (NF-κB) and activator protein-1 that contribute to functional modulation [126]. It can also modulate the inflammatory response, cytokine expression, and specific DC marker by TLR. The RelB member in NF-κB interacts with AhR signaling pathway to transcribe genes and produce cytokines [127]. Activation of AhR significantly promotes the expression of co-stimulatory markers such as CD86 and CD80 [128].

AHR AND MACROPHAGE

LPS may induce macrophage to produce tumor necrosis factor (TNF)-α and IL-6 in large amounts. However,

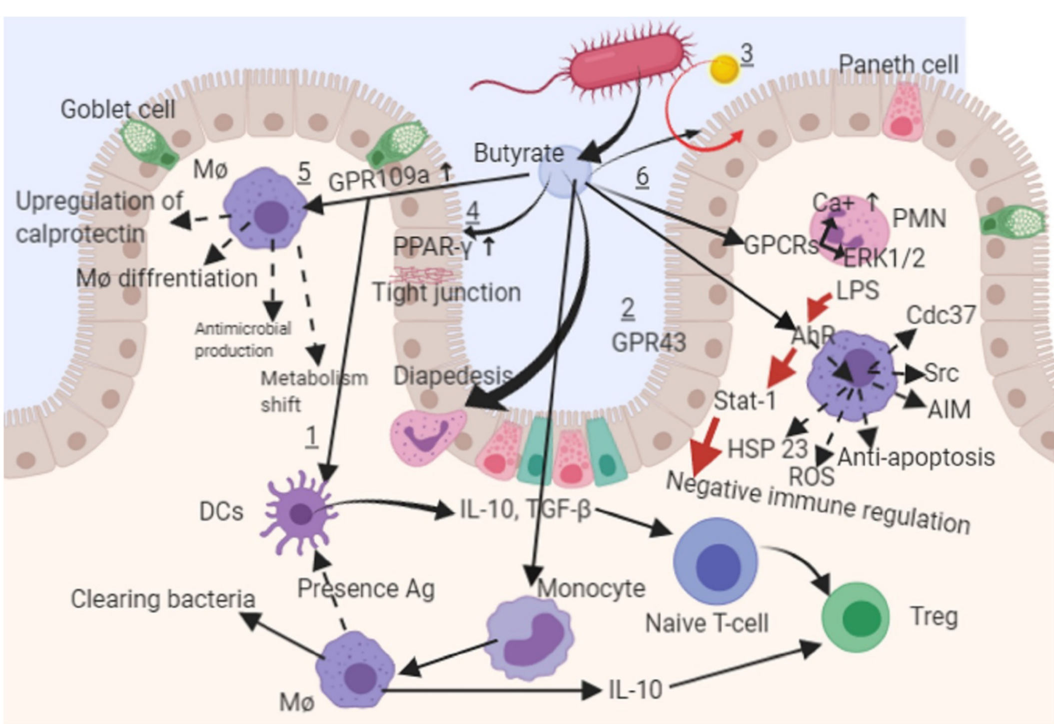

Fig. 3. Effect of SCFAs on the immune modulation.
Butyrate mediated the upregulation of GPR109a in the DCs and macrophage ($M_{\emptyset}$). At next step, DCs and macrophage through the secretion of IL-10 and TGF-β cause conversion of the naïve-T cell to the Treg cell. 2. Butyrate modulated recurrence of PMN. 3. Epithelial cell signaling by butyrate leads to the consumption of oxygen that is essential to the pathogenic bacteria. 4. Butyrate also mediates PPAR-ϒ upregulation that results in the upregulation of tight junction proteins and recovery from diarrhea. 5. Other effects of butyrate on the macrophage include antimicrobial production and metabolism shift and differentiation of macrophage caused by the upregulation of GPR109a. 6. Butyrate also leads to the ERK1/2 signaling through GPCRs

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endotoxic tolerance limits the immune response to higher LPS stimulation in order to inhibit the overabundant response. AhR modulates the macrophage response towards LPS through the negative regulation of inflammatory response. It can interact with signal transducer and activator of transcription-1 (Stat1) and regulate the LPS-induced inflammatory response by combining with NF-κB [129]. AhR-Stat1 complex interacts with NF-κB, through which its transcriptional activity is inhibited. However, this inhibition does not suppress DNA-binding activities. On the other hand, exposure of macrophage to SCFAs prevents histone deacetylase and downregulates LPS-inducing pro-inflammatory cytokines [130]. In intestines, SCFAs differentiate monocyte to a specific macrophage by AhR. This specific macrophage is found in the lamina propria and it is responsible for translocating antigens to DCs, clearing translocated bacteria, and producing modulatory cytokines such as IL-10, which is an important factor in the expansion of $CD4^+$Treg subset [131]. The macrophage in lamina propria maintains the epithelial barrier by clearing apoptotic epithelial cells and secretes bone morphogenic protein 2 (BMP2), which helps maintain enteric neurons [132]. Butyrate enhances LPS-induced secretion of prostaglandins E2 (PGE2) and PGD2 by modulating transcription through hyperacetylation of histone H3 and enhancing PGEs gene expression [133]. Secretion of PGEs leads to the formation of anti-inflammatory macrophage that inhibits Th17 and Th1 activities. On the other hand, it contributes to epithelial barrier recovery by secretion of IL-10 and annexin A1 [134]. Nitiric oxide (NO) as free radical facilitates cell proliferation, tumorigenesis, and cell migration. Butyrate may inhibit NO production in the macrophage as well as inflammatory response [135].

CLOSTRIDIUM AND AHR

Clostridium may utilize tryptophan through tryptophan decarboxylases. Tryptophan is used as a precursor for Indole-3-acetaldehyde, which can act as AhR ligands. Indole-3-acetaldehyde itself is used as a precursor for indole-3-ethanol through alcohol dehydrogenase. These derivates by *Clostridium* activate AhR and help facilitate gut barrier regulation [136]. *Clostridium sporogenes* extract indole-3-pyruvate from tryptophan which itself acts as a precursor for many AhR agonists such as indole-3-acetic acid, indole-3-acetaldehyde, and indole-3-aldehyde [137]. Other Clostridia such as *C. putrefaciens*, *C. sticklandii*, *C.subterminale*, and *C. lituseburense* produce indole-3-acetic acid from tryptophan. Indole-3-acetic acid activates AhR signaling pathway [138].

CLOSTRIDIA SIGNALING

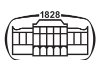
Like other gram-positive bacteria, *Clostridium* has a peptidoglycan, which is an important component of the cell wall. Peptidoglycan can enhance the innate immune system and release cytokines and chemokines [139]. It can be recognized by Toll-like receptor-2 (TLR), which contributes to gene expression and transcription. TLR as a transmembrane protein transduces a danger signal in response to the bacterial cell. The structure of TLR belongs to the Toll/IL-1 receptor (TIR) family. However, it has leucine rich repeats (LRRs) unlike the immunoglobulin-like domain in the IL-1 [140]. TLRs have an important role in detecting, recognizing, and initiating immune response against microbial pathogens [141]. TLR-2 is able to recognize the peptidoglycan, activate signal cascade, and secrete interleukins (ILs). The interaction between TLR-2 and peptidoglycan upregulates TLR-2 and secretes IL-6 [142]. *Clostridium* has an important role in enhancing the immune response of the host. *Clostridium* as a member of microbiota can induce special lymphocyte populations in the lamina propria such as CD4+ regulatory T cells. Clostridia appear after weaning and persist in adulthood for a long time. The existence of *Clostridium* clusters IV and XIVa is more common in the cecum where Treg cells are located, suggesting that *Clostridium* has an important role in this process [143]. Treg cells are categorized into two types, namely natural Treg (nTreg) and induced Treg cells (iTreg). nTreg has a specific marker, i.e., Helios, which is expressed in the thymus-derived Treg cells. It has been observed that a significant number of Treg cells are Helios-negative in the *Clostridium*-colonized mice, suggesting that most of the Treg cells are induced-Treg [144]. One of the important mechanisms that differentiates naive cells from Treg cells is SCFA. Among SCFAs, butyrate upregulates the transcription factors including Foxp3, which is a specific marker of Treg cells. This is thought to be due to histone H3 acetylation, which increases by exposure to butyrate [145].

Clostridium perfringens

The presence of *C. perfringens* in the gut lumen upregulates TLR-2 expression. TLR-2 signaling causes cascade signaling through Myd-88, TRIf, and TRAF6 [146]. Recognition of *C. perfringens* by innate immune response infiltrates polymorphonuclear (PMN) in the gut lumen. The interaction between bacteria and PMN induces intraepithelial lymphocyte in the lamina propria, thus upregulating gene transcription of IL-1β, IL-10, and IFN-ϒ [147].

Toxins. In addition to the cell wall components, *C. perfringens* have many toxins that improve the immune response. There are four main and many other accessory toxins that cause food poisoning and gas gangrene. Alpha toxin, as a major *C. perfringens* toxin, upregulates the expression of IL-8, IL-6, nucleotide-binding oligomerization domain-1 (NOD), and inducible nitric oxide synthase (iNOS) [148].

***Interaction between microbiota and* C. perfringens.** One of the important components produced by microbiota that enhances protection against microbial pathogens is bacteriocins. Bacteriocins have many classes of antibiotics. One of the major modified bacteriocins is called lantibiotic. Lantibiotic, as an antimicrobial peptide, is produced by gram-



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positive bacteria under post-translation modification and it has an unusual amino acid [149]. Nicin as a subclass of lantibiotics causes pore forming on cell membrane and inhibits cell wall biosynthesis [150]. Another type of bacteriocins, called trypsin-dependent lantibiotic, is produced by human fecal microbiota. Many microbiotas can produce trypsin-dependent lantibiotics. Ruminococcin A, including *Ruminococcus gnavus*, *Ruminococcus hansenii*, and *Clostridium nexile*, is a member of *Clostridium coccoides* phylogenetic group [151]. Among these bacterial strains, *R. gnavus* strain E1 and clostridial strains including *Clostridium orbiscindens* LEMH9 and *Clostridium hathewayi* LEMC7 have bactericidal effects against *C. perfringens*. RumA as a lantibiotic has bactericidal activities against *C. perfringens* and it is produced by *R. gnavus* strain E1 [152]. One important group of antimicrobial peptides produced by microbiota is called ribosomally synthesized and post-translationally modified peptides (RiPPs) [153]. RiPPs are produced by Firmicutes, Cyanobacteria, Proteobacteria, and Bacteroidetes. The post-translation process comprises dehydration of Ser and Thr residues. In case of cyclic process, the dehydrated residue includes Cys to form lanthipeptides [154]. *R. gnavus* E1 can also produce Ruminococcin C that consists of two antimicrobial fractions. Ruminococcin C has anti-*C. perfringens* activity and it is as effective as metronidazole in antimicrobial activity [155]. Prior to the activation of Ruminococcin C against *C. perfringens*, it should be treated by protease and the leader peptide should be removed to achieve its active form. S-adenosyl-L-methionine (SAM) enzyme helps post-translation modification by carbon-carbon bond, methylation, and thioether. This is possible in Ruminococcin A, shaping the active form [156]. Difficulty in dealing with *C. perfringens* in the chicken leads to the imbalance in the microbiota community. However, pre-treatment of chicken by *Bacillus licheniformis* provides resistance against the disruptive power of *C. perfringens* [157]. One important role of *C. perfringens* is changing the production of SCFAs. This change decreases the production of acetate, formate, and propionate. On the other hand, it improves the production of lactate. Enrichment with *L. acidophilus* increases the concentration of butyrate. The existence of *C. perfringens* also increases genus *Escherichia* and *Shigella* as a pathogenic bacterium [158]. Studies have shown that the presence of *C. perfringens* in the cecum of chicken hinders any increase in the number of Clostridia over time. It also inhibits Clostridial-related bacteria such as *Blautia*. *Blautia* as a non-pathogenic Clostridia can ferment carbohydrates and produce SCFAs. Therefore, supplementing sodium butyrate may modulate the composition of cecal microbiota, improve barrier function, and modulate the immune system, hence promoting pathogen control [159]. Neuromyelitis optica (NMO) as an autoimmune disease affects the central nervous system. NMO is associated with humoral immune response against aquaporin-4, the immunodominant protein in the water channel. This humoral response is T-cell-dependent and polarizes Th-17. Th-17 may cross-react with adenosine triphosphate-binding cassette transporter (ABC) of *C. perfringens*. in addition, in NMO patients, microbial composition is different from that in a normal human and the presence of *C. perfringens* is overexpressed. Overloaded *C. perfringens* produce pro-inflammatory cytokines, unbalancing the microbiota and hence, differentiating Th-17. Moreover, molecular mimicry of *C. perfringens* ABC to aquaporin-4 leads to the production of pro-inflammatory cytokines and T-cell proliferation [160].

CONCLUSION AND DISCUSSION

Gastrointestinal microbiota has many significant effects on the immune system. The interaction between microbiota and immune system provides normal conditions and any change in the microbiota composition leads to immunological disorders. Microbiota can be sensed by PRR, which induces an innate immune response. Sensing is done through recognized microbiota components such as peptidoglycan, MDP (Muramyl dipeptide), and lipoproteins. The innate immune response comprises the secretion of antimicrobial peptide, IgA, defensin, RegIIIϒ, and cytokines such as IL-10. Secretion of IL-10 propagates specific T regulatory cells by FOXP3+ marker, inhibiting inflammation and reducing immune response. TGF-β is another cytokine that is induced by microbiota, especially Clostridia, and it converts T cells to Treg cells. Another important role of microbiota, specifically Clostridia, is the production SCFAs, which provides regulatory signaling. SCFAs signaling occurs through GPRs and leads to intracellular signaling and its consequences. Clostridia represent a major group of microbiota and play an important role in immune modulation. Butyrate is an important metabolite produced by Clostridia that can induce AhR as a receptor. The induction has many outcomes including the production of heat shock proteins, ROS; conversion of monocytes to macrophage; and exertion of anti-apoptosis effects.

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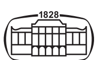

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