THE INTERACTION BETWEEN INTESTINAL COMMENSAL MICROBIOTA AND HOST IMMUNE SYSTEM IN HEALTH AND DYSBIOSIS

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Abstract

Although mucosal surfaces of the human body are colonized with innumerable microorganisms, immunological tolerance towards commensal bacteria dominates in these environments. Bidirectional communication between intestinal commensal microbiota and host immune system is essential for continuing the mutualistic partnership and intestinal homeostasis. Therefore, absence of these microorganisms or perturbations in the commensal microbiota composition, called dysbiosis, could have profound deleterious effects for the host. Here, the effect of commensal microbiota on host immune response is discussed. The enteric flora modulates immune establishment as well as function of individual immune cell types, thereby preventing pathological conditions. Identification of microbiota-derived factors that modulate host immunity would be useful for developing new therapies for dysbiosis-associated diseases.

1. Introduction

Mucosal surfaces of the skin, and urogenital, respiratory, and gastrointestinal tracts are abundantly colonized by a variety of organisms, including bacteria, fungi, viruses, and archaea. The gastrointestinal tract alone contains 10^{14} microorganisms, predominantly bacteria, representing 100 times more genes than our genome [1-3]. These microorganisms range from beneficial to harmful for the host. Lifelasting colonization with microbiota at mucosal surfaces of newborn is initiated during birth upon contact with the microbial ecosystem of the mother. The host is highly adjusted to resident commensal bacteria and both parties take advantage of the established mutualistic partnership. Commensal bacteria profit from the broad availability of nutrients in the gut and, in turn, serve as supplier for multiple essential factors for the host, including vitamins [4]. In addition to improvement of the digestive capacity of ingested food [5], inhabitant microbiota limit the invading capacity of pathogens through limiting nutrient availability and niches competition [6]. However, the mutualistic partnership might be abolished in case of changes in the dynamic habitat. Due to their pivotal role in shaping the host immune system, both local and systemic, shifts in microbiota composition or a life devoid of commensal bacteria could have deleterious effects. Continue regulation of several immune cell subsets by the commensal microbiota is required in order to maintain a proper immune function and to avoid dysbiosis-driven pathological conditions. On the other hand, the host immune system itself is involved in controlling and shaping the microbiota composition, emphasizing that bidirectional communication between host and microbiota is essential. Alterations in the makeup of microbiota can be ascribed to several physiological conditions,

such as intake of antibiotics [7], smoking [8], and eating habits [9]. Dysbiosis, referred to disrupted microbiota equilibrium, results in an altered microbiota metabolism and overgrowth of pathogenic bacteria [10], which makes the host more susceptible to multiple pathological conditions, including rheumatoid arthritis [11], inflammatory bowel disease [12], cancer [13], and allergic disorders [14]. In this paper interactions between commensal microbiota and host immune system will be discussed. Since intestinal microbiota - host immune communication was extensively studied over the past decades, this paper will focus on the interactions that occur in the gastrointestinal tract.

2. Commensal bacteria influence immune response against pathogens

The mutualistic partnership between host immune system and commensal bacteria results in a homeostatic balance of all the immune cell subsets, enabling immunological tolerance towards commensal bacteria whereas outgrowth of injurious pathogens is prevented. Commensal bacteria are not only involved in the onset of the immunological response against pathogens, but also function as an adjuvant in order to expand the ongoing immune response. The use of antibiotic treated (ABX) experimental animals is a powerful tool to access the effect of commensal bacteria on the host immune response against harmful pathogens. Adaptive and innate immunity against lymphocytic choriomeningitis virus was suboptimal in ABX treated mice and resulted in diminished viral clearance [15]. Furthermore, ABX-mediated microbiota depletion in mice resulted in a reduced intestinal production of IL-17 and INF-γ upon infection with *Encephalitozoon cuniculi* [16]. Reversibility of the immune response was observed upon reconstitution with microbiota DNA and resulted in ameliorated *E. cuniculi* clearance [16]. ABX-mediated perturbation of microbiota prior to infection also caused an increased susceptibility to enteric infections, such as *Citrobacter rodentium* or *Salmonella* [17-19]. In accordance with these data, long-term usage of antibiotics facilitates overgrowth of vancomycin resistant *Enterococcus*, abbreviated as VRE [20]. Ubeda *et al.* showed in mice that administration of antibiotics facilitated VRE overgrowth upon a VRE challenge through perturbations of the intestinal commensal microbiota [21]. Introduction of anaerobic microbiota components into ABX treated mice diminished VRE overgrowth [22]. VRE clearance was achieved in ABX treated mice upon introduction of *Barnesiella* species [22]. This suggests that anaerobic *Barnesiella* bacteria are major participants in the suppression of VRE colonization. It was reported that antibiotic-mediated VRE overgrowth might eventually results in Bacteremia [21] A significant number of patients also develop VRE Bacteremia after undergoing an allogeneic hematopoietic stem cell transplantation (allo-HSCT) [23] VRE bloodstream infections after allo-HSCT can be ascribed to shifts in microbiota composition, as during allo-HSCT microbiota stability and diversity is altered [23]

Also germ-free (GF) animal models were used to access the effect of commensal bacteria on the host immune response against injurious pathogens. Mice in a germ-free state were highly

susceptible to a variety of pathogens, including *Coxackie B*, *Bacillus antheracis*, *Listeria monocytogenes*, and *Shigella flexneri* [24-25]. In addition, GF mice were unable to eradicate *C. rodentium* upon inoculation [26]. This might be explained by an increased availability of luminal nutrient sources and reduced niche competition upon commensal microbiota depletion [26]. On the other hand, pathogens itself might increase their invading capacity by modulating resident commensal bacteria. For example, subspecies I of *Salmonella enterica* (serovar Typhimurium) control intestinal commensal bacteria composition and growth through induction of the host immune response, thereby facilitating establishment of infection [27]. Also extra-intestinal commensal bacteria are involved in regulating the host immune response against injurious pathogens. An impaired immunological response upon cutaneous *Leishmania major* infection was observed in GF mice [28]. Correction of the GF environment though intestinal enrichment with cutaneous commensals restored *L. major*-specific immunity in GF mice, whereas reestablishment of the immune response was lacking upon colonization with intestinal-resident commensals [28]. All these studies suggest that commensal bacteria play a pivotal role in influencing the host immune response against pathogens. In the next few chapters the effect of intestinal microbiota on immune homeostasis will be discussed in more detail.

3. Commensal bacteria influence development of the host immune system

Studies with GF mice showed that intestinal commensal microorganisms directly influence host lymphoid structure development. Impaired development of gut-associated lymphoid tissue (GALT) and reduced number and structurally different mesenteric lymph nodes and Peyer's patches were observed in GF mice [29]. Postnatally, lymphoid tissue inducer cells (LTi) are involved in the establishment of secondary lymphoid structures [30]. At the newborn state, LTi-like cells cluster together in the lamina propria in cryptopatches, which eventually transform into mature isolated lymphoid follicles (ILFs) [31-32]. Intestinal commensal bacteria are required in order to form mature ILFs from cryptopatches [33]. Deprivation of the enteric flora in mice resulted in impaired formation of ILFs [34]. Boeshkra *et al*. showed that commensal bacteria might regulate ILF formation through the recognition of gram-negative-derived Nod1 ligands by Nod1 receptors present in epithelial cells [35]. These Nod1 receptors are intracellular pattern recognition receptors, which will be further discussed in chapter 5.

Besides their involvement in the establishment of mature lymphoid tissues, the intestinal microbiota also influences development of multiple immune cell subtypes. Expression of Toll-like receptors (TLR), which are involved in immune cell activation, is reduced on intestinal epithelial cells (IECs) of GF mice [36]. Furthermore, in GF mice the portion of intestinal intraepithelial lymphocytes

(IELs) and intestinal TCRγδ T cells dramatically expanded upon microbiota enrichment [37-38]. Also colonic Foxp3$^+$ T_{regs} were significantly diminished in microbiota-deprived mice [39]. In addition, the proportion of CD4$^+$ T cells in GF mice was diminished in the lamina propria as well at extra-intestinal sides such as the spleen, suggesting a role for the enteric flora in controlling systemic immunity [40-41]. The quantity of CD4$^+$ T cells was restored upon introduction with *Bacteroides fragilis*, indicating that commensal bacteria possess immunomodulatory capacity and influence immune development [41]. The influence of intestinal microbiota on mononuclear phagocytes, innate lymphoid cells, invariant NK T cells, TCRγδ T cells, intraepithelial lymphocytes, T_H17, and regulatory T cells will be further discussed in chapter 6.

4. Commensal bacteria influence immune mediators

Besides their modulating capacity on a variety of immune cell types, intestinal commensal bacteria also influence several immune mediators. In order to avoid bacterial overgrowth and interaction between invading pathogens and IECs, intestinal commensal bacteria regulate the production of immune mediators, including mucus, Immunoglobin A (IgA), short-chain fatty acids (SCFA), and antimicrobial peptides (AMPs).

4.1 Mucus layer

Gastrointestinal commensal bacteria might combat against injurious pathogens through maintaining mucus layer integrity. Mucin glycoproteins, produced by Goblet cells from IEC layer, assemble and form the mucus layer. This mucus layer serve as a protective shield in order to prevent direct interaction of invading pathogens with the epithelial layer, as the inner mucus layer is devoid of pathogens [42]. However, some pathogens developed mechanisms to be able to pass the protective mucus layer, such as *Campylobacter jejuni* and *Helicobacter pylori* [43-44]. The role of microbiota in mucus production was shown in GF animals. In animals devoid of microbiota the mucus layer was twice as small as the mucus layer of colonized animals and the mucus composition was attenuated [45-46]. The mechanism behind the modulation of the intestinal mucus layer by intestinal microbiota is currently unknown and remains to be elucidated.

4.2 IgA

In the germinal centers of Peyer's patches production of IgA-secreting cells takes place with help of T lymphocytes [47]. Upon encountering antigens, lymphocytes migrate into the mesenteric lymph node where differentiation into IgA-producing plasma cells takes place [47]. In the lamina propria IgA secretion can be elicited in a T lymphocyte-independent manner [48]. Production of IgA is

required in order to establish sufficient local as well as systemic immune homeostasis. Therefore, IgA serves as a host defense mechanism that prevents pathogen entrance. Absence of microbiota in GF mice resulted in reduced numbers of IgA secreting cells in the lamina propria and diminished secretion of IgA, suggesting that intestinal commensal bacteria influence secretion of IgA [47, 49]. It has been described that particular intestinal-resident bacterial species, including *Clostridia* and segmented filamentous bacteria (SFB), play a role in the development of IgA-secreting cells, probably via TLR signaling [47, 50]. However, the immunomodulatory effect on the secretion of IgA differs between commensal bacteria species. For example, *Bacteroides* species are more potent to induce IgA secretion in Peyer's patches than *Lactobacillus* species [49]. It was also reported that IgA is induced by commensal-carrying intestinal dendritic cells and prevent penetration of commensal bacteria into the mucosal layer [51]. This implies that, besides tolerating them, the host immune system is able to sense and regulate indigenous commensal bacteria. Therefore, it is likely that IgA elicited upon interaction with commensal bacteria contributes in maintaining the mutualistic partnership between host and commensal bacteria [52].

4.3 SCFAs

The breakdown of dietary fibers by commensal bacteria results in the release of SCFAs. The kind of fermentation product that is released in the host's intestines is determined by the composition of the intestinal microbiota. Elevated quantities of butyrate are formed by *Firmicutes*, whereas propionate and acetate are secreted in high concentrations by *Bacteriodetes* species [53]. In patients suffering from severe ulcerative colitis and Crohn's disease, which is associated with microbiota alterations, diminished levels of fecal SCFAs are found, in particular acetate [54]. In agreement with this observation, severity of dextran sulphate sodium-induced colitis in GF mice was significantly decreased upon acetate treatment [55]. G-protein-coupled receptors were identified to serve as receptors for SCFAs [56]. Maslowski *et al.* reported that interaction between G-protein-coupled receptor 43 located on innate immune cells, abbreviated as GPR43, and SCFAs influenced host immune response [55]. SCFAs proportions were strongly diminished in GF mice [55, 57]. These mice showed the same phenotype as *Grp43* deficient mice, which had an impaired regulation of the inflammatory response [55]. Augmented proportions of $CD4^+$ T cells, but not of T_H17 and T_H1 subtypes were reported in mice as an effect of SCFA release [58]. In addition, SCFAs modulated both function and extend of the T_{reg} population in the colon of mice in a GRP42-dependent manner, suggesting that SCFAs are involved in maintaining homeostasis [58]. The interaction between SCFAs and GPF43 might be a mechanism by which commensal bacteria affect host immune response and homeostasis thereby keeping up the mucosal barrier function. Therefore, the fact that intestinal dysbiosis results in impaired homeostasis, which can be partly ascribed to reduced SCFA levels. This knowledge might be useful as a step towards the development of new therapeutic options.

4.4 Antimicrobial peptides

In order to prevent adherence and entering of pathogenic microbes, AMPs are abundantly secreted in response to microbial communities by a variety of cell types, such as intestinal goblet cells, paneth cells, enterocytes, and cutaneous keratinocytes [59]. Skin commensal bacteria are able to stimulate the production of AMPs (RNase7 and HBD-3) by human keratinocytes through the activation of EGFR, TLR-2, and NF-κB [60]. Intestinal enrichment with *Bacteriodes thetaiotaomicron* in GF mice resulted in Ang4 upregulation in Paneth cells [61]. In addition to Ang4, intestinal commensal bacteria are also involved in induction of bactericidal C-type lectin (RegIIIγ) expression by IECs [62]. Both mouse RegIIIγ and their human ortologue HIP/PAP possess bactericidal effects to gram-positive bacteria via binding to their surface-located peptidoglycan [62]. It has been shown that GF mice with *Bifidobacterium longum* reconstruction show diminished RegIIIγ expression, whereas expression is increased upon *B. thetaiotaomicron* colonization, suggesting that the commensal community structure is involved in differential expression of RegIIIγ [63]. The same phenomenon was observed in studies with antibiotic-driven changes in microbiota composition [64]. The hypothesis that commensal bacteria modulate RegIIIγ production is strengthened by the fact that a combination of antibiotics (vancomycin, metronidazole, and neomycin sulphate) diminished the expression of RegIIIγ in IECs and Paneth cells of mice [65]. In these mice an increased susceptibility to vancomycin-resistant *Enterococcus* [65] intestinal colonization was observed. Experiments with mice also showed impaired clearance of intestinal *Listeria monocytogenes* and diminished RegIIIγ expression in the small intestine upon loss of MyD88 signaling, implying that induction of RegIIIγ by commensal bacteria is TLR-mediated [66]. Flagellin-induced TLR-5 stimulation as well as LPS-induced TLR-4 stimulation restored RegIIIγ expression in IECs of microbiota-depleted mice [65, 67]. It has been shown that RegIIIγ also controls the physical separation of commensal microbiota from the intestinal epithelium [68]. Lack of RegIIIγ function in mice was associated with an increased bacterial colonization of intestinal mucosal surfaces and an enhanced adaptive immune response [68]. This suggests that RegIIIγ is involved in host-bacterial segregation, thereby promoting the mutualistic partnership between commensal microbiota and host. Vaishnava *et al.* reported that gut commensal bacteria are also involved in secretion of RegIIIβ (an AMP that interacts with peptidoglycan), α-defensins (possess antimicrobial activity), RELMβ (a modulator of inflammation), and CRP-ductin (binds gram-positive and gram-negative bacteria) in Paneth cells upon TLR activation [69].

Expression of AMPs also affects the commensal microbiota makeup. Dysbiosis might be driven by constitutive expression of intestinal α-defensins [70]. Dysbiosis-mediated alterations in IL-17^+ $CD4^+$ T lymphocyte number exert skewing of the immune response [70]. Thus, AMPs are also involved in immunomodulation.

5. Commensal bacteria influences signaling through pattern recognition receptors

Despite the existence of an intestinal physical and biological barrier, some pathogens are able to pass this barrier and to interact with IECs. Pattern Recognition Receptors (PRRs) on IECs are important components of the host immune surveillance system and have the ability to sense microorganisms. These receptors of innate immunity sense components derived from microorganisms known as microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) [71]. In the gastrointestinal tract two classes of PRR can be distinguished: Toll-like receptors and Nod-like receptors, also abbreviated as TLRs and NLRs respectively [71]. NLRs are located in the cytosol of the cell, whereas TLRs are membrane-spanning receptors associated with the plasma membrane or membranes of intracellular organelles [71]. PRR signaling eventually results in T cell activation, upregulation of several molecules (e.g. adhesion and adaptor molecules), and cytokine secretion through NF-κB. Since the gastrointestinal tract is abundantly colonized with microorganisms, excessive activation of PRRs would be expected. However, several negative regulators of PRR signaling have been described that dampen the host immune response [72]. In addition, since PRR are not abundantly expressed at the apical side of the intestinal epithelial cell layer, interaction with non-invasive (commensal) bacteria is not likely [72]. Nevertheless, interaction between microbiota and host PRRs remain required in order to establish a proper intestinal immune homeostasis [73]. Thus, PRRs located on epithelial cells facilitate communication between microbiota and epithelial cells. With the use of GF or MyD88 knockout mice PPR-microbiota interactions were investigated, as MyD88 is a downstream signaling molecule involved in almost all TLR signaling pathways and NLR signaling is abrogated in MyD88 knockout mice [74].

In contrast to GF animals, mice lacking MyD88 have a normal formation of gut-associated lymphoid tissue [75]. This argues against a role for PRR signaling in the development of lymphoid tissue. However, the establishment of (immune) cell populations might be affected by microbiota-PRR interactions. For example, both GF and MyD88 $^{-/-}$ mice showed significantly diminished numbers of intestinal IELs, suggesting that IELs are modulated upon microbiota-PRR interaction [37, 76]. In addition, Polysaccharide A (PSA) is a factor derived from the intestinal commensal *B. fragilis* that interacts with TLR-2, which caused induction of T_{regs} [77]. The induced T_{reg} population has a suppressive effect on the T_H17 pool, as FoxP3 expression by T_{regs} has an antagonizing effect on RORγt action, which is required for differentiation of naive T cells into T_H17 lymphocytes [78]. Through TLR-2 signaling, PSA stimulates secretion of IL-10 by $CD4^+$ T cells, which inhibits expansion and activity of the T_H17 pool [77]. By doing so, PSA creates a more regulatory intestinal environment, as proinflammatory T_H17 lymphocytes are suppressed and T_{regs} are induced. Diminished IL-17 production through *B. fragilis*-mediated TLR activation enhanced symbiotic colonization of *B. fragilis* [77]. This is in contrast with pathogens, as TLR signaling triggered by pathogenic microorganisms result in

pathogen clearance [77]. These data revealed insight into a potential mechanism by which the host immune system can differentiate between beneficial commensals and harmful pathogens [77].

GF animals have an abrogated intestinal barrier function, which is observed as diminished proliferation of epithelial cell and a higher permeability, and it is likely that a lack of PRR signaling is responsible for this effect [74]. However, abrogation of the intestinal barrier was not TLR mediated, as MyD88 deficient mice did not show an aberrant intestinal barrier [74]. Although disruption in the intestinal barrier appeared not TLR mediated, intestinal commensal bacteria are able, through activation of macrophages, to induce proliferation of colonic epithelial progenitors [79]. This process is MyD88 depended and thus indeed PRR mediated [79].

Besides their modulating activity on (immune) cell types, recognition of commensal microflora through PRR also regulates the production of several other factors. As mentioned previously, the induction of antimicrobial peptides and IgA by commensal microbiota depends on TLR signaling. Furthermore, the overgrowth of (commensal) bacteria is prevented through TLR-mediated production of AMPs, suggesting that TLR deficiencies might be associated with alterations in microbiota composition. However, Ubeda *et al.* recently reported that modifications in microbiota composition due to abrogated TLR function are marginal and that enteric flora changes observed in TLR-deficient animals can be ascribed to housing [86]. In addition to AMPs, MyD88-mediated induction of colonic cytokine production (TNF, IL-6, and KC-1) was completely absent in ABX mice, suggesting the ability of microbes to stimulate PRR at steady state [73]. In addition, mice lacking MyD88 signaling were unable to respond to cytokines produced upon NLR signaling, suggesting that NLR signaling is abrogated in these mice [74].

NLRs, the other class of PRRs, are important components of the inflammasome. Inflammasomes are polyprotein complexes consisting of one of several Nod-like receptors proteins (NLRPs), which serve as sensors for MAMPs. Inflammasomes are able to elicit innate immune responses through the activation of the caspase-1 cascade, which leads to the maturation of IL-1β and IL-18 [80]. Intestinal mononuclear phagocytes induced maturation of IL-1β from pro-IL-1β through NLRC4 inflammasome activation by pathogenic *Pseudonomas aeruginosa* or *Salmonella* [81]. In contrast, mononuclear phagocytes hyporesponsiveness was observed upon encountering commensal microbiota-derived PRR ligands [81]. This suggests that lamina propria-located phagocytes distinguish between harmful pathogens and beneficial commensal bacteria through inflammasome-mediated maturation of IL-1β.

Inflammasomes itself are able to modify commensal bacteria composition [82]. Mice with NLRP6 inflammasome component deficiency developed an altered intestinal microbiota composition, which drives a colitogenic phenotype [82]. The candidate phylum, TM7, possibly representing Gram-positive bacteria [148], and members of the *Prevotellaceae* family were more abundantly present in microbiota of NLRP6$^{-/-}$ mice compared to microbiota of conventionally raised animals [82]. These bacterial phylotypes have been linked to multiple human diseases, including inflammatory bowel disease (IBD) and periodonditis [83, 84, 148]. These data suggest that inflammasome-driven dysbiosis

might be the underlying condition of several diseases. In agreement with this study, several deficiencies in NLRs have been shown to be associated with alterations in commensal bacteria composition [35, 85]. On the other hand, Ubeda *et al.* recently reported that modifications in microbiota composition due to abrogated TLR function are marginal and that enteric flora changes observed in TLR-deficient animals can be ascribed to housing [86].

6. Commensal bacteria influence multiple immune cell types

6.1 Mononuclear phagocytes

Phagocytes, such as $CD103^+/CX_3CR1^+$ dendritic cells and macrophages, are abundantly present in the intestinal lamina propria and Peyer's patches and are involved in sampling antigens. Mononuclear phagocytes are important participants in maintaining the 'immunological tolerance/immune response balance', as tolerance against commensals and immunity against injurious pathogens is induced. Studies with GF mice were used in order to investigate the role of commensal microbiota on intestinal phagocytes. The proportion of CX_3CR1^+ dendritic cells in the lamina propria was diminished in GF mice, indicating the involvement of commensal bacteria in proportion enlargement of the CX_3CR1^+ phagocyte population [87]. Besides their involvement in phagocyte population size, commensal bacteria have the ability to act on CX_3CR1^+ phagocyte transport to the mesenteric lymph nodes. The enteric flora prevents this CX_3CR1^+ phagocyte-mediated trafficking of both harmful pathogens and beneficial gut bacteria [88]. This process is MyD88 mediated and allows tolerance towards enteric microbiota [88]. These data suggest that the inhibitory effect on trafficking might be eliminated due to dysbiosis, facilitating immunologic priming and inflammation. Thus, loss of microbiota will result in enhanced clearance of invading pathogens, but also might contribute to immune disease pathogenesis.

As mentioned previously, phagocyte responsiveness to microbiota and injurious pathogens is differential, as mononuclear phagocytes secreted IL-1β upon infection with pathogens, whereas intestinal commensals induce an anergic response [81]. Konstantinov *et al.* reported that the enteric flora actively alters dendritic cells function [89]. *Lactobacillus acidophilus*, a member of the commensal microbiota community, interacts with DC-SIGN on dendritic cells through surface layer protein A resulting in increased IL-10 and diminished IL-12 secretion [89]. *L. acidophilus* affects both T cell and dendritic cell function through dendritic cell-mediated alterations in the cytokine profile, suggesting a role of commensal bacteria in stimulating tolerance [89]. Commensal microbiota-driven modulation of dendritic cell function into a more tolerogenic state can also occur indirectly through stimulation of PRRs on IECs [90]. Zeuthen *et al.* suggested that under influence of commensal microbiota TSLP and TGF-β secretion of IECs is increased, creating a tolerogenic setting in the intestine [91]. Soluble factors secreted by commensals might also serve as mediators in commensal-

dendritic cell communication. *Lactobacillus planetarium* secretes sTP, which prime dendritic cells, resulting in expanded secretion of dendritic cell derived-IL-10 [92].

Since the intestinal flora plays a pivotal role in phagocyte function, it is likely that changes in enteric flora composition have detrimental effects on phagocyte action. Intestinal dendritic cells of subjects suffering from Crohn's disease secrete significantly higher quantities of IL-6 and IL-12, generating a more pro-inflammatory environment [93]. This cytokine profile secreted by dendritic cells of Crohn's disease patients was associated with diminished proportions of *Faecalibacterium prausnitzii* (*Clostridium* group IV) and an increased intestinal bacteriodes/bifidobacteria ratio [93]. Thus, the cytokine secretion profile of dendritic cells was correlated with commensal microbiota composition. This suggests that individual components of the commensal microbiota might influence dendritic cell function.

6.2 Innate Lymphoid cells

Commensal bacteria are able to shape innate lymphoid cells (ILCs), an innate immune cell population, which includes natural killer (NK) cells and T-bet$^+$ ILCs, GATA3$^+$ ILCs, and RORγt$^+$ ILCs [94]. ILCs are located at the lamina propria in close contact with dendritic cells and their main function is to contribute to host defense through the expression of cytokines [94]. Although the establishment of most of the ILCs populations was not impaired in GF mice [95, 96], commensal bacteria-derived signals are able to affect ILC function.

Commensal bacteria might directly regulate ILCs via Toll-like receptor signaling, as the presence of functional TLR-2 was demonstrated on RORγt$^+$ ILCs [97], and both functional TLR-2 and TLR-9 are present on NK cells [98, 99]. In addition to TLRs, bacteria are able to bind to Natural Cytotoxicity Receptors (NCR) present on particular ILC population [100]. This suggests that commensal bacteria might also regulate ILCs via NCR signaling. Furthermore, Aryl hydrocarbon receptor (AhR) signaling in RORγt$^+$ ILCs is associated with ILC expansion, decreased susceptibility to *C. rodentium* infection, and the establishment of intestinal lymphoid follicles [101]. Thus, intestinal commensal bacteria might be able to induce AhR signaling as commensal bacteria generate AhR ligands during metabolizing tryptophan [102].

Besides direct regulation of ILCs via TLR, NCR, and AhR, ILCs can also integrate indirectly commensal-derived signals. Intestinal commensal bacteria control myeloid cell cytokine production and thereby modulate RORγt$^+$ ILC responses [103]. Intestinal lactic acid bacteria drive NK cell responses through dendritic cell stimulation [104]. In addition, the enteric flora provides signals that act on mononuclear phagocytes resulting in the expression of IFN-I, which enhance NK cell priming and induce an antiviral immune response [96]. Furthermore, commensal bacteria are critical for the induction of IL-7 production in IECs, a cytokine required for proper function of RORγt$^+$ and GATA3$^+$ ILCs [103, 105, 106]. Sawa *et al.* reported a negative regulation of RORγt$^+$ ILC-IL-22 production by the intestinal microbiota through epithelial induction of IL-25 [107]. IL-22 is a cytokine crucial for

maintaining intestinal barrier homeostasis and the induction of IEC-derived AMPs, including RegIIIγ [108-109]. Modifying or depleting intestinal commensal bacteria communities in mice resulted in the induction of RORγt$^+$ ILC-IL-22 production [107]. The role of commensal bacteria on the production of other intestinal epithelial cell-derived cytokines with ILC modulating properties, including IL-33, IL-18, and IL-1β, remains to be elucidated [103].

6.3 Invarient NKT cells

Invariant NKT cells, abbreviated as iNKT cells, belong, together with γδ T lymphocytes, to the family of innate-like lymphocytes and serve as a bridge between adaptive and innate immunity. iNKT cells are characterized by expression of the same surface markers as found on T and NK cells [146]. Invariant T cell receptors on iNKT cells interact with MHC I-presented (glyco)lipids [110]. This is in contrast with T cells, as these T cell receptors respond to presented peptide antigens [110]. Upon activation iNKT cells produce multiple proinflammatory cytokines, suggesting their role in stimulating inflammation [110]. In germ-free conditions both pulmonary and colonic- iNKT cell populations expanded, which was related to an augmented disease severity in allergic asthma and IBD animal models. [111]. This phenomenon was reversible through intestinal enrichment of commensal microbiota shortly after birth, but not through reconstruction at adult age [111]. These data indicates that commensal bacteria dampen the host inflammatory response and induce intestinal homeostasis at an age-dependent manner.

6.4 γδ T lymphocytes

TCRγδ$^+$ T lymphocytes are components of intraepithelial lymphocytes, but can also be found in the lamina propria and peritoneum. TCRγδ$^+$ T lymphocytes have the ability to sense a variety of ligands, including microbial-derived ligands, and are involved in host defense, as they produce cytokines and AMPs upon infection. For example, dendritic cells infected with *Mycobacterium tuberculosis* secreted IL-23, which contributes to the secretion of TCRγδ$^+$ T lymphocytes-derived IL-17 [112]. This suggests that commensal bacteria might influence the IL-17 output of TCRγδ$^+$ T lymphocytes. Maintenance of IL-1R-expressing TCRγδ$^+$ T lymphocytes was severely impaired in GF mice, but restored upon microbiota reconstruction [38]. Therefore, expansion of the TCRγδ$^+$ T lymphocyte subset by commensals allows rapid IL-17 induction, which is required for protection against invading pathogens [38]. In addition, mice lacking *pdk1*, a CD4$^+$ T lymphocyte activator gene, suffered from severe inflammatory colitis [113]. TCRγδ$^+$ T lymphocytes were identified as major players in establishing colitis, as TCRγδ$^+$ T cells were highly expanded in this phenotype and colitis severity was abrogated in absence of TCRγδ$^+$ T lymphocytes [113]. The colitis phenotype was also abolished upon ABX-mediated perturbation of the intestinal microbiota, suggesting that commensal bacteria are able to activate and extend the proportion of TCRγδ$^+$ T lymphocytes [113]. Introduction of T_{regs} in *pdk1*$^{-/-}$ mice diminished the number of TCRγδ$^+$ T lymphocytes [113]. Indeed, T_{regs} had an

inhibitory effect on TCRγδ$^+$ T lymphocytes though secretion of IL-10 [113], indicating that T_{regs} play a role in maintaining intestinal tolerance through their inhibitory effect on TCRγδ$^+$ T lymphocytes [113]. The role of T_{regs} in immune homeostasis will be detailed in paragraph 6.7.

6.5 IELs

IELs are early-activated T lymphocytes resident within the IEC layer possessing cytotoxic activity, which is important in host defense [114]. The enteric flora profoundly affects both function and development of IELs. As aforementioned, the portion of IELs was severely reduced in GF mice [37]. Especially the pool of TCRαβ$^+$ IELs was diminished in GF mice, whereas the proportion of TCRγδ$^+$ intestinal IELs was limited affected [115]. Both *Clostrisium spp.* and SFB were able to restore proportions of TCRαβ$^+$ intestinal IELs [35]. The exact mechanism behind this observation still requires clarification. Although TCRγδ$^+$ IELs numbers were limited influenced in GF mice, upon stimulation with enteric commensals TCRγδ$^+$ small intestinal IELs secreted antimicrobial substances (RegIIIβ and RegIIIγ) at a MyD88-dependent manner, thereby inhibiting entrance of invading pathogens [116]. By doing so, TCRγδ$^+$ intestinal IELs maintain homeostasis between host intestinal immune system and commensal microbiota. Not all components of the commensal flora are evenly potent in inducing AMP expression in TCRγδ$^+$ intestinal IELs. For example, RegIIIγ expression by TCRγδ$^+$ IELs was absent upon *B. thetaiotaomicron* enrichment [116]. In addition, stimulation with SFB also did not lead to TCRγδ$^+$ IELs-derived RegIIIγ expression [116]. On the other hand, *E. coli* is a potent inducer of RegIIIγ secretion by intestinal TCRγδ$^+$ IELs [116].

Besides commensal-mediated influences on IELs, IELs, in turn, are also able to influence commensal microbiota composition. IELs express AhR at high level [117]. Both intestinal and cutaneous proportions on IELs are maintained through AhR signaling, as impaired numbers of IELs were observed in absence of AhR ligands or AhR deficiency [117]. Absence of AhR signaling modulated both commensal microbiota composition and numbers, allowing maintenance of intestinal homeostasis [117].

6.6 Th17 cells

T helper 17 (T_H17) cells, a subgroup of CD4$^+$ lymphocytes, are present at mucosal surfaces, where they express cytokines, such as IL-17, IL-17F, and IL-22, that are important mediators in tissue repair and remodeling and secretion of AMPs [118]. The association between T_H17 cells and disease susceptibility has been suggested in several pathological conditions, such as inflammatory bowel disease [119] and rheumatoid arthritis [120]. Due to their pivotal role in IL-17 and IL-22 secretion, T_H17 cells are essential in the protection against invading microorganisms, including *C. rodentium, Staphylococcus aureus*, and *Candida albicans* [121, 122]. Secreted IL-23, IL-21, IL-6, and TGF-β drive differentiation of T_H17 cells both *in vitro* and *in vivo* through the induction of RORγt expression [119, 123]. Since TGF-β induces expression of both RORγt and FoxP3, this indicates that upon TGF-β

stimulation naive T cells can differentiate in either T_H17 or T_{reg} cells as FoxP3 inhibits differentiation into T_H17 cells [78]. Since TGF-β without the presence proinflammatory cytokines favors T_{reg} differentiation, whereas TGF-β with additional IL-6 or Il-21 favors T_H17 differentiation, additional proinflammatory cytokines are required in order to drive differentiation of T_H17 cells [78]. In addition to proinflammatory cytokines, AhR signaling is an important player in differentiation of T_H17 cells [124, 125]. As mentioned previously, commensal bacteria generate AhR ligands during metabolizing tryptophan, which might influence T_H17 cell differentiation. The role of TLR and other PRR in the establishment of T_H17 subset is currently unknown. Although conventionally mice and mice lacking MyD88 have similar proportions of T_H17 cells in the lamina propria of the small intestine [119], flagellin-mediated TLR-5 stimulation in mice enhanced T_H17 differentiation [126], and TLR-9 deficiency in mice resulted in reduced proportions of intestinal T_H17 [16].

The role of commensal bacteria in the development of T_H17 subset was shown in GF mice. Proportions of large intestinal-located T_H17 cells were increased in mice lacking commensal microbiota [127]. This observation can be explained by commensal bacteria-driven induction of IL-25 expression in IECs, which restricted the number of intestinal T_H17 cells through limiting the secretion of IL-23 from macrophages [127]. In contrast to augmented T_H17 population in the large intestine of GF mice, Ivanov *et al.* showed that small intestinal T_H17 differentiation from the naive T cell population was abrogated in GF mice and this phenomenon was not caused by deficiency of the immune system [119]. Providing commensal microbiota to GF mice restored the number of T_H17 in the lamina propria of the small intestine, suggesting that commensal bacteria are important stimulators of small intestine-located T_H17 differentiation [119]. Besides the number of T_H17 cells, levels of adenosine 5'-triphosphate (ATP) in the intestinal lumen of GF mice were also significantly diminished [128]. The diminished proportion of T_H17 cells in the small intestine of GF mice was reversible upon ATP intake, indicating that commensal-derived ATP, like AhR ligands, induces T_H17 differentiation from its naive precursor [128].

Experiments with antibiotic-mediated specific depletion of commensal microbiota in mice revealed that specific components of the enteric flora are involved in T_H17 differentiation [119]. Since the absence of the T_H17 pool in the small intestine population in GF animals resulted in an enlarged $FoxP3^+ T_{reg}$ subset in the lamina propria, it is likely that these specific components of the enteric flora are regulators of the T_H17/T_{reg} balance [119]. Several bacteria, including *B. thetaiotaomicron* and *Bacteriodes distasonis,* were unable to establish T_H17 cell responses [129], indicating that the potency of bacteria to affect T_H17 polarization differs. Ivanov *et al.* showed that exclusively the commensal SFB was able to enhance the proportion of small intestinal IL-17- and IL-22- secreting T_H17 cells at a TLR signaling-independent manner [130]. SFB was identified in the gastrointestinal tract of multiple vertebrates including man [131]. In agreement with the study of Ivanov *et al.*, loss of SFB was associated with reduced numbers of T_H17 cells in the small intestinal lamina propria in mice [70]. Since ATP levels in the intestinal lumen of GF mice were not augmented upon SFB mono-colonization, differentiation into T_H17 cells by SFB was ATP-independent [130]. After intestinal introduction of SFB

in GF mice, alterations in intestinal gene expression occurred. One of the effects was that the degree of expression of Serum Amyloid A was increased [130]. Recombinant Serum Amyloid A drived differentiation of the T_H17 subset *in vitro* [130], indicating that SFB establish the T_H17 population through upregulation of Serum Amyloid A. It is plausible that upregulated Serum Amyloid A drive differentiation of the T_H17 subset by acting on dendritic cells. Also antimicrobial peptide-related genes were upregulated upon introduction with SFB [130]. In addition, overexpression of human α-defensins in mice caused diminished intestinal colonization of SFB [70]. Therefore, it is plausible that SFB is able to modulate the host immune response. Indeed, a reduced susceptibility to *C. rodentium* was observed upon SFB colonization [130]. However, reduced *C. rodentium* susceptibility was not directly linked to SFB-induced T_H17 induction [130]. Nevertheless, induction of T_H17-derived cytokines and AMPs is a plausible mechanism by which SFB limits infection susceptibility [130]. Further research is required to investigate whether SFB provide protection against invading pathogens also via other mechanisms. Interestingly, besides their involvement in intestinal T_H17 induction, intestinal SFB also affect T_H17 numbers in the central nervous system [132]. GF animals showed resistance to the establishment of experimental autoimmune encephalomyelitis (EAE), which is a multiple sclerosis animal model [132]. This is probably due to absence of immune priming by commensal bacteria, as GF animals with SFB reconstitution showed EAE disease progression [132]. These observations points out that SFB also can induce inflammation/regulate the T_H17 pool in other tissues than the intestine [132]. In agreement with this observation, Wu *et al.* reported increase of autoimmune arthritis severity upon colonization with SFB in GF mice [133]. Thus, besides positive effects of SFB-induced T_H17 differentiation on defense against invading pathogens, SFB-driven T_H17 induction can also have detrimental consequences for the host. Although the exact mechanism by which T_H17 elicit a protective or deleterious effect has to be clarified, IL-1β and IL-23 are suitable candidates [47].

Fungi also may influence induction of the T_H17 pool. Fungal-derived β-glucans are able to act on Dectin-1, a non-TLR receptor mainly present on myeloid cells [134]. β-Glucan-Dectin-1 interaction leads to altered secretion of multiple cytokines, including upregulation of IL-23 and downregulation of IL-12, which might favor T_H17 responses [134]. In addition, contact of β-glucans with Dectin-1 present on dendritic cells drives T_H17 differentiation at a TLR-independent manner [135].

6.7 Regulatory T cells

Since $FoxP3^+$ regulatory T cells (T_{regs}) enforce inhibition of multiple immune cells (e.g. NK cells, B cells, $CD4^+$/$CD8^+$ T cells), they have a controlling effect on host immunity [136]. Two distinct T_{reg} populations can be distinguished: thymic-differentiated T_{regs} and peripheral-differentiated T_{regs}, also called induced T_{regs} [137]. In mice, T_{regs} are not evenly distributed among tissues, as they were predominantly found in mucosal tissues of the colon [39]. Daily intake of probiotics decreased disease severity in mice suffering from colitis [138]. Since these probiotics extended both IL-10 production and proportion of the $CD4^+$ T_{reg} population, it is likely that function and extent of the T_{reg} pool depends, at least in part, on the microbiota [138]. Indeed, the T_{reg} pool in the lamina propria of the

colon was significantly reduced in GF mice [39]. In contrast, T_{reg} numbers were increased or unaffected in the small intestinal lamina propria of GF mice [39, 119]. This suggests that commensal bacteria are involved in extending the T_{reg} pool in the lamina propria of the colon, but not in the lamina propria of the small intestine. In accordance with this, multiple studies reported that T_{reg} populations were unaffected in GF mice, except the T_{reg} pool in the colon [52, 129]. Thus, GF mice have a reduced colonic T_{reg} pool, but an induced T_H17 pool. As mentioned before, this can be explained by the fact that naive T cells can develop in either T_{reg} lymphocytes or T_H17 lymphocytes, as FoxP3 expression inhibits differentiation into T_H17 lymphocytes. It is plausible that commensal bacteria affect a single type of T_{reg} (thymic-differentiated T_{regs} or peripheral-differentiated T_{regs}). Expression of the transcription factor Helios on T_{regs} can be used to discriminate between thymic-differentiated T_{regs} and peripheral-differentiated T_{regs}, as Helios is only expressed by Thymus-derived T_{regs} [137]. Multiple studies showed that in GF mice especially Helios-negative FoxP3$^+$ T_{reg} cells were diminished [39, 129], suggesting that commensal bacteria mainly affect induced T_{regs} [137]. In contrast, it was recently reported that the establishment of tolerance towards commensal-derived products was induced by thymic T_{regs} rather than induced T_{regs} [139].

Like it was the case for IELs and T_H17 cell populations, some commensal species affect the FoxP3$^+$ T_{reg} population more than others. T_{reg} numbers were exclusively reduced in mice receiving vancomycin therapy, emphasizing a pivotal role for gram-positive enteric bacteria in extending colonic T_{reg} numbers, like resident intestinal commensal bacteria belonging to gram-positive *Clostridium spp.*, which are potent inducers of the IL-10-secreting T_{reg} population in the murine lamina propria of the colon [39]. Numbers of colonic FoxP3$^+$ T_{regs} were also augmented upon introducing altered Schaedler flora into GF mice, a mixture containing multiple species of the murine microbiota including *Clostridium* [129]. A cocktail of *Clostridium spp.* did not modulate T_{reg} numbers in the lamina propria of the small intestine [39]. In addition, SFB and *Lactobacillus* had negligible effects on the extent of the colonic T_{reg} pool [39]. In contrast, induction of the proportion of FoxP3$^+$ T_{regs} in the spleen and mesenteric lymph nodes was observed upon colonization with *Lactobacillus reuteri* [140]. Also the use of several probiotics, such as *Bifidobacterium infantis* [141] and *L. acidophilus* [142], might influence the T_{reg} pool. Subjects with diminished *F. prausnitzii* proportions have a higher probability to experience Crohn's disease recurrence postoperatively [143]. Since *F. prausnitzii* was highly capable to exert IL-10 responses upon culturing with peripheral blood mononuclear cells, it is plausible that *F. prausnitzii* is involved in modulating FoxP3$^+$ T_{regs}, resulting in the inhibition of excessive inflammation [143].

Also microbiota components can act directly on T_{reg} lymphocytes. One of these components is PSA located at the surface of *B. fragilis* [144]. Introduction of *B. fragilis* in GF mice augmented the portion of IL-10-secreting T_{regs} and this effect was completely abrogated in GF mice and upon colonization with *B. fragilis* lacking PSA [144]. PSA-mediated induction of IL-10 protected mice against the development of colitis [145]. In fact, mono-colonization of *B. fragilis* made GF mice less susceptible for *Helicobacter hepaticus*-induced colitis compared to animals devoid of *B. fragilis*

colonization [145]. However, introduction of *B. fragilis* lacking PSA resulted in severe *H. hepaticus*-induced colitis and augmented secretion of proinflammatory cytokines [145]. PSA-induced T_{regs} possessed a suppressive effect on both activation and expansion of other T cell subtypes [144]. In accordance with this, upon *B. fragilis* introduction in GF mice PSA-induced FoxP3$^+$ T_{regs} limit expansion of the T_H17 pool [77]. As mentioned previously, TLR-2 signaling was required in order to achieve the inhibitory effect of PSA on T_H17, as PSA stimulated secretion of IL-10 by CD4$^+$ T lymphocytes at a TLR-2-dependent manner [77]. TLR-2 signaling was also essential to augment the portion of T_{regs} [144]. In contrast to pathogenic bacteria that use TLR-2 signaling to establish inflammation and facilitate pathogenic clearance, PSA use TLR-2 signaling to allow long-lasting colonization of *B. fragilis* to mucosal surfaces [77].

Although commensal bacteria have a regulatory effect on intestinal T_{regs}, the mechanism behind this observation is currently unknown. Nishio *et al.* reviewed potential mechanisms that could be used by enteric bacteria [137]. As mentioned previously, it is likely that peripheral-differentiated T_{regs} rather than thymus-differentiated T_{regs} are affected by commensal microbiota. It is possible that commensal bacteria drive naive T cell into an induced-T_{reg} phenotype through promoting TGF-β secretion and other T_{reg} stimulatory cytokines in IECs. In favor of this hypothesis is the observation that a cocktail of *Clostridium* stains induced TGF-β secretion through SCFAs, which drives T_{reg} induction [146]. It is also possible, although not proven, that components of the intestinal microbiota affect retinoic acid dehydrogenase expression on dendritic cells [147]. This enzyme is involved in the secretion of retinoic acid, which stimulates T_{reg} induction and inhibits T_H17 lymphocyte stimulation at a TGF-β-dependent manner [147]. Alternatively, commensal-driven chemokine secretion by IECs might attract Helios-negative FoxP3$^+$ T_{reg} cells into the colonic lamina propria. Furthermore, the enteric microbiota could affect further activation of thymus-differentiated T_{regs}, thereby inducing a Helios-negative phenotype. Further research is required in order to identify mechanism(s) used by commensal bacteria to augment induced-T_{reg} populations in the colon.

7. Concluding remarks

Mucosal surfaces of the human body are the natural habitat of millions of microorganisms. Most of these microorganisms are beneficial for the host, but could also have deleterious effects. The microbiota and host have co-evolved over time, allowing the formation of a complex ecosystem. Since the gastrointestinal tract, but also other mucosal tissues, encounters countless microbes, it remains challenging to keep up intestinal homeostasis. An inadequate immune response might result in opportunistic infections, whereas dysbiosis might occur in response to an excessive immune response. Therefore, a carefully fine-tuned immune response is necessary. Fully functional (healthy) intestinal microbiota affects the host immune system in such way that intestinal homeostasis is

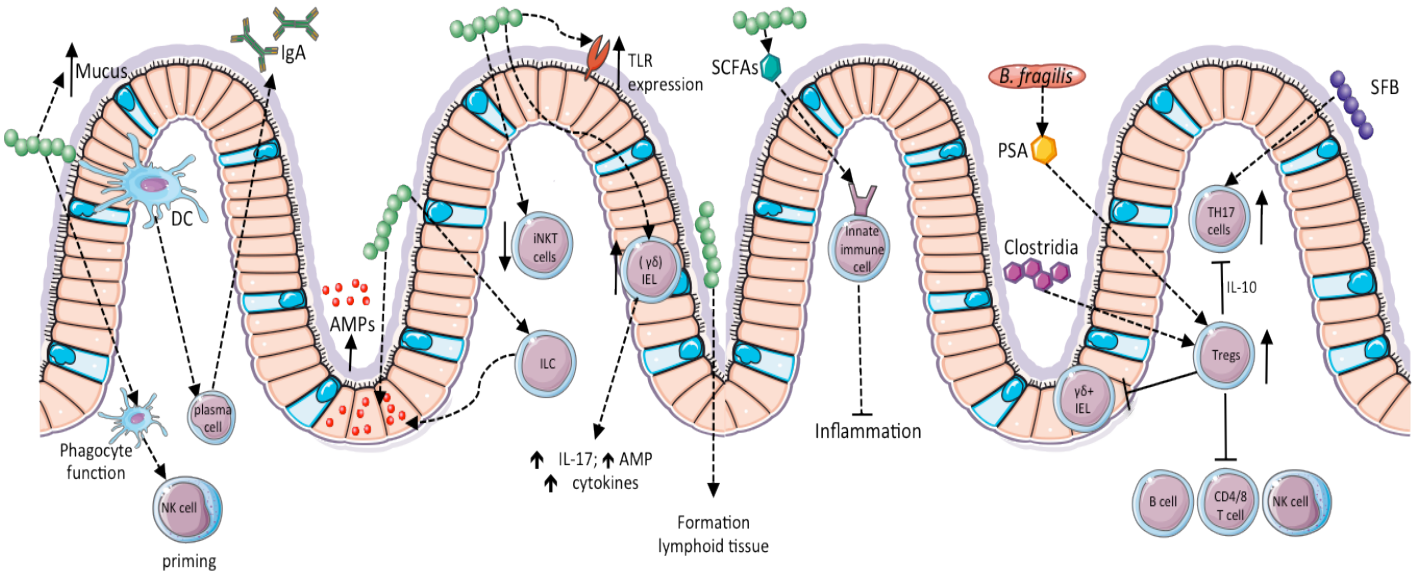

Figure 1: Intestinal commensal bacteria have an immunomodulatory function and contribute to immune homeostasis. Mechanisms by which commensal bacteria modulate host immunity and that are discussed in this paper are summarized in this figure. Gut commensal bacteria contribute to enhanced TLR expression on IECs and augmented secretion of several immune mediators: mucus, IgA, AMPs, and SCFAs. Furthermore, commensal bacteria influence phagocyte development and function, which might result in priming of NK cells. In addition, the enteric flora reduces expansion of iNKT cells and affect both function and development of IELs. PSA and Clostidia augment the proportion of IL-10-secreting T_{regs}, which, in turn, have an inhibitory effect on the activation and expansion of multiple immune cell types, including T_H17. On the other hand, SFB enhance the proportion of the intestinal T_H17 pool.

AMP: antimicrobial peptides; DC: dendritic cell; ILC: innate lymphoid cell; IEL: intraepithelial cell; IECs: intestinal epithelial cells; iNKT cells: invariant NK T cells; NK cell: natural killer cell; SCFAs: short-chain fatty acids

achieved. This means that healthy commensal bacteria enforce the equilibrium of proinflammatory T_H17 and regulatory T cell actions. However, this fine-tuned equilibrium might be disrupted due to either an augmented proinflammatory environment or a diminished anti-inflammatory environment, resulting in a higher susceptibility to pathological conditions. Several lifestyle changes, such as worldwide antibiotic consumption and changes in diet, have modulating effects on commensal microbiota composition. The increasing prevalence of multiple autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, might be a consequence of developed dysbiosis. However, although it is suggested in animal experiments, the actual link between dysbiosis and disease onset in human is still not proven and is currently under investigation.

Literature clearly showed that interaction between commensal microbiota and host is bidirectional: commensal microorganisms influence the host immune system, but the host immune system also shapes the both the number and composition commensal microbiota. In this paper several mechanisms were discussed by which commensal bacteria affect host immunity. These mechanisms are summarized in *Figure 1*. Commensal bacteria maintain IEC barrier function and prevent adherence of invading pathogens to the intestinal epithelial cell layer through establishing a proper mucus barrier and through induction of AMPs, SCFAs, and IgA. In addition, commensal

bacteria influences several immune cell subtypes (phagocytes, innate lymphoid cells, innate-like lymphocytes, and multiple other subsets of lymphocytes) at a PRR-dependent and PRR-independent manner to accelerate clearance of pathogens and to retain intestinal homeostasis. Probiotic therapy might restore altered microbiota and aberrant immune function in individuals with dysbiosis. In addition, it is likely that treatment with antibiotics targeting specific proinflammatory microorganisms also restores the disrupted equilibrium. However, as the contribution of individual constituents of the microbiota on host immune response is limitedly unraveled, development of proper therapeutic options for some diseases might be challenging. In addition, for most of the microbiota components it is currently unknown which commensal-derived factors are actually involved in modulating the immune system. Therefore, it is likely that identification of other important immune modulators would directly improve the availability of new therapeutic agents.

8. References

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