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REVIEW

Immunoregulation by the gut microbiota

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Abstract The human intestinal mucosa is constantly exposed to commensal microbiota. Since the gut microbiota is beneficial to the host, hosts have evolved intestine-specific immune systems to co-exist with the microbiota. On the other hand, the intestinal microbiota actively regulates the host's immune system, and recent studies have revealed that specific commensal bacterial species induce the accumulation of specific immune cell populations. For instance, segmented filamentous bacteria and *Clostridium* species belonging to clusters XIVa and IV induce the accumulation of Th17 cells in the small intestine and Foxp3$^+$ regulatory T cells in the large intestine, respectively. The immune cells induced by the gut microbiota likely contribute to intestinal homeostasis and influence systemic immunity in the host.

Keywords Gut microbiota · *Clostiridium* · Segmented filamentous bacteria · Regulatory T cells · Th17 · Innate lymphoid cells

Abbreviations

APRIL	A proliferation-inducing ligand
AID	Activation-induced cytidine deaminase
AMM	Antimicrobial molecule
ASC	Apoptosis-associated speck-like protein
BAFF	B cell activating factor
CSR	Class switch recombination
DC	Dendritic cell
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
Fut2	Fucosyltransferase 2
GF	Germ-free
GALT	Gut-associated lymphoid tissue
IKK	IκB kinase
IL-10R	IL-10 receptor
Ig	Immunoglobulin
iTreg	Inducible Treg
IBD	Inflammatory bowel diseases
ILC	Innate lymphoid cell
IECs	Intestinal epithelial cells
ILFs	Isolated lymphoid follicles
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
nTreg	Natural Treg
NOD	Non-obese diabetic
NLR	Nucleotide-binding oligomerization domain-like receptor
PRR	Pattern recognition receptor
PP	Payer's patche
pIgR	Polymeric Ig receptor
PSA	Polysaccharide A
RAG	Recombinase-activating gene
RegIIIγ	Regenerating islet-derived IIIγ
Treg	Regulatory T
RORγt	Retinoic acid receptor-related orphan receptor gamma t
rDNA	Ribosomal DNA
SIgA	Secretory IgA
SFB	Segmented filamentous bacteria
STAT3	Signal transducer and activator of transcription 3
SHM	Somatic hypermutation
SPF	Specific pathogen-free
TCR	T cell receptor

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TD	T cell-dependent
TI	T cell-independent
Th	T helper
TSLP	Thymic stromal lymphopoietin
TLR	Toll-like receptor
TGF-β	Transforming growth factor-β
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
T1D	Type 1 diabetes
VRE	Vancomycin-resistant *Enterococcus*

Introduction

The intestinal mucosa is constantly exposed to the microbiota, which is composed of more than 100 trillion commensal bacteria comprising more than 1,000 species. These bacteria are more than coincidental companions. We provide the microbiota with a secure habitat and a constant source of nutrition. In turn, the gut microbiota helps our digestion and metabolism and protects us from intestinal intrusion by foreign pathogenic microorganisms via competition for space and nutrition. Therefore, the host–microbiota relationship is mutually beneficial. To utilize the benefits, the gut mucosa has evolved a multi-component system to co-exist with the microbiota. Intestinal epithelial cells (IECs), which directly interface with the microbiota, form tight junctions and constitutively produce mucus gels and antimicrobial agents to form a physical barrier [1, 2]. IECs also constitutively produce immune regulatory cytokines. Consequently, IECs have critical roles in sequestering the microbiota in the lumen and in achieving immunological tolerance toward the microbiota. Beneath the epithelium, the intestinal mucosa is equipped with multiple lineages of cells, including innate and adoptive immune cells, that also cooperate to establish a robust symbiotic system with the microbiota [3–6].

An accumulating body of evidence suggests that the commensal microbiota actively regulates the host immune system. Using germ-free (GF) and/or gnotobiotic techniques and metagenomic approaches by massive parallel sequencing, it is becoming clear that the composition of the gut microbiota affects the structure of gut-associated lymphoid tissues (GALTs) and the activation/differentiation status of immune cells and IECs [7, 8]. Furthermore, specific commensal bacterial species induce the accumulation of specific immune cell populations [9–14]. Therefore, the alteration of the bacterial composition may result in a disruption of immune homeostasis between the host and the microbiota.

In this article, we summarize the host immune system by discussing several important cell subsets and then discuss the interactions of the microbiota with the host mucosal immune system and the consequences of these interactions (although many are still unknown). In particular, we focus on how the host immune system is adapted to co-exist with the commensal microbiota and how the commensal microbiota regulates the host immune system. A variety of cell populations and mechanisms appear to be involved in the establishment of these complicated but sophisticated relationships.

Features of the host immune system to allow co-existence with the gut microbiota

Multiple different lineages of cells in the intestines, including IECs and innate and adoptive immune cells, have their own roles to allow co-existence with commensal bacteria while protecting the host from invasion by pathogenic microbes. Recent advances have elucidated the roles of each cell population and, further, have identified several new cell populations.

Intestinal epithelial cells (IECs)

IECs are located at the interface between the host and the commensal microbiota. Accordingly, they have a critical role in sensing physical and molecular stimulation by the microbiota. IECs express multiple pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and C-type lectin receptors, to sense pathogenic bacteria and commensal bacteria, both of which contain microorganism-associated molecular patterns in their structures. TLRs are expressed at low levels in normal IECs, but are upregulated in inflammatory bowel diseases (IBD) patients. Inflammatory cytokines induced by TLRs, such as interferon (IFN)-γ and tumor necrosis factor-α, can act as precipitating factors for IBD by modifying tight junction function in IECs and increasing flux across the leak pathway [15]. Therefore, a dysregulated expression and function of TLRs in IECs may be involved in IBD pathogenesis.

On the other hand, PRR signaling in IECs has been shown to contribute to the enhancement of tight junctions, epithelial cell proliferation, and antimicrobial peptide production, leading to the enhancement and maintenance of the mucosal barrier system [1]. For example, when mice kept in specific pathogen-free (SPF) conditions are treated with broad-spectrum antibiotics, they become highly susceptible to dextran sulfate sodium (DSS)-induced intestinal inflammation [16]. This is, at least in part, due to reduced constitutive TLR signaling in response to the microbiota, as oral administration of lipopolysaccharide (LPS, a TLR4

ligand) or lipoteichoic acid (a TLR2 ligand) restores resistance of antibiotic-treated mice to DSS-associated injury of the colonic epithelium [16]. Consistently, mice lacking either TLR2, TLR4, TLR9, or MyD88 are highly susceptible to DSS colitis [16]. TLR2 activation redistributes the tight junction protein zonula occludens-1 (ZO-1) in IEC cells, resulting in the enhancement of tight junctions [17, 18].

The production of antimicrobial molecules (AMMs), such as α-defensins, β-defensins, cathelicidins, C-type lectins, and lipocalin-2, is one of the pivotal roles of IECs in protecting the host from pathogenic microbiota. The expression of the C-type lectin regenerating islet-derived IIIγ (RegIIIγ) is induced in the mouse small intestine, particularly in Paneth cells, by commensal bacteria, such as *Bacteroides thetaiotaomicron*, whereas it is downregulated by *Bifidobacterium longum* [19]. This commensal-mediated induction of RegIIIγ depends on MyD88-dependent TLR activation. Indeed, IEC-specific MyD88-deficient mice have impaired production of RegIIIγ, and the transgenic expression of MyD88 specifically in Paneth cells restores the expression of RegIIIγ [20]. The antibacterial function of RegIIIγ is dependent upon binding bacterial targets through interactions with peptidoglycan, which is expressed on the surface of Gram-positive bacteria but is buried in the periplasmic space of Gram-negative bacteria [21]. Therefore, RegIIIγ has a critical role in the host defense against pathogenic Gram-positive bacteria, including vancomycin-resistant *Enterococcus* (VRE) [21, 22]. Furthermore, RegIIIγ is critical for generating the 'microbiota-free zone', which is filled with mucus gels containing AMMs, including RegIIIγ, that physically separate IECs from the luminal microbiota in small intestine during steady-state conditions [23]. Therefore, TLR and RegIIIγ have critical roles in maintaining intestinal homeostasis by preventing unnecessary direct contact between IECs and commensal bacteria.

It is interesting to note that IECs also produce other families of soluble-type lectins, including galectins. The production of some galectins, such as galectin-9, is upregulated by TLRs. Galectins bind luminal β-galactoside containing glycans and modulate the mucosal and systemic immune response. Interestingly, dietary supplementation with prebiotic galacto- and fructo-oligosaccharides and *Bifidobacterium breve* enhances galectin-9 expression by IECs, which associates with the prevention of allergic symptoms [24].

The production of AMMs in IECs is also indirectly regulated by TLRs via cytokines, such as IL-22 [25]. Systemic injection of flagellin stimulates TLR5 on hematopoietic cells, which leads to an increase in the concentration of IL-22 in the intestine. IL-22 then stimulates IECs to produce RegIIIγ and lipocalin-2, resulting in

resistance to infection by pathogens such as VRE [25]. IECs have also been shown to produce IL-17C, which is a member of the IL-17 family of cytokines. IL-17C signals through the IL-17RE and IL-17RA complex, which is expressed on IECs, in an autocrine manner, leading to the induction of AMMs, such as RegIIIβ, RegIIIγ, lipocalin-2, S100A8, and S100A9, during *Citrobacter rodentium* infection and DSS-induced colitis [26, 27]. IL-17C functions in synergy with IL-22 to induce these AMMs [26]. In the DSS-induced colitis model, IL-17C mRNA is induced soon after DSS administration [27], suggesting that IL-17C production is critical for the rapid production of AMMs to cope with a massive bacterial burden.

IECs have another important role as messengers that relay the signal from bacteria to the immune cells that reside underneath the IECs. For instance, IEC-conditioned intestinal dendritic cells (DCs) drive Th2 response in both human and mouse [28], suggesting that a Th2-skewed response might be required for intestinal homeostasis. IECs constitutively express thymic stromal lymphopoietin (TSLP), which prevents DCs from producing IL-12 and differentiating naïve T cells into Th1 cells [28]. Consistently, mice with the IEC-specific ablation of IκB kinase (IKK)-β exhibit decreased expression of TSLP by IECs and consequently increased production of IL-12/IL-23p40 and TNFα by intestinal DCs, which is accompanied by severe colitis characterized by increased numbers of T helper (Th) 1 and Th17 cells [29]. In addition to TSLP, IECs express IL-25 and promote the accumulation of an innate lymphocyte population in the GALT that promotes Th2 cytokine responses [30]. IECs also constitutively produce transforming growth factor-β (TGF-β) and retinoic acid, which were reported to convert human CD103⁻ DCs into CD103⁺ DCs to induce de novo generation of Treg cells with gut-homing properties [31].

TLR stimulation in IECs contributes to the production of IgA, which is secreted into the lumen to directly neutralize bacteria. IECs express a proliferation-inducing ligand (APRIL) upon stimulation with TLR ligands, such as lipopolysaccharide and flagellin, or with whole bacteria [32]. APRIL is functionally related to CD40L and can promote activation-induced cytidine deaminase (AID) expression in B cells. Thus, APRIL expression by IECs induces IgA class switching in B cells in a CD40L-independent manner. In addition, IECs promote IgA class switching by producing TSLP in response to TLR ligands or whole bacteria. TSLP activates DCs to produce APRIL and IL-10, which together promote switching to IgA [33].

NLR proteins, including NLRP1, NLRP3, NLRC4, and NLRP6, form cytoplasmic multiprotein complexes called inflammasomes. Upon sensing bacterial components, one of the NLRPs forms the inflammasome by associating with apoptosis-associated speck-like protein (ASC) and

pro-caspase-1 in cytoplasm. Inflammasome formation triggers the activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 to generate the effector inflammatory cytokines IL-1β and IL-18. Recently, NLRP3 and NLRP6 in IECs have emerged as critical regulators of intestinal homeostasis. Several groups have shown that NLRP3$^{-/-}$ mice are susceptible to DSS-induced colitis [34–36]. Mice deficient in ASC or caspase-1 are similarly susceptible to experimental colitis [34–37]. NLRP6 is thought to form inflammasomes on the basis of the similarity of its molecular structure to NLRP3, although its ligands have yet to be identified. A recent report showed that deficiency in NLRP6 in mouse IECs results in a colitogenic-skewed commensal microbiota that is characterized by expanded *Prevotellaceae* and TM7 [38]. Indeed, the abundance of *Prevotellaceae* and TM7 have been implicated in human IBD [39, 40]. Mice deficient in ASC, caspase-1, or IL-18 also exhibit a significant increase in *Prevotellaceae* in their intestinal commensal microbiota, indicating the impact of NLRP6 and other inflammasome components on the commensal microbiota. More importantly, wild-type mice co-housed with NLRP6$^{-/-}$ mice acquire susceptibility to DSS-induced colitis characterized by the alteration of the microbiota toward a profile that is similar to the co-housed knockout mice, suggesting that the colitogenic microbiota of the NLRP6$^{-/-}$ mice is horizontally transmissible [38]. NLRP3 or NLRP6 deficiency in IECs lead to reduced IL-18 production by the IECs, and IL-18- or IL-18 receptor-deficient mice are susceptible to DSS-induced colitis [41, 42]. IEC-specific IL-18-deficient mice also display high susceptibility to DSS-induced colitis accompanied by colitogenic-skewed microbiota. However, the commensal microbiota profile in these mice is distinct from that of NLRP6-, ASC- or caspase-1-deficient mice [38], suggesting an additional NLRP6-mediated and IL-18-independent pathway of microflora regulation. Although the link between NLRP6 deficiency in IECs and the shift toward a colitogenic community is currently unknown, the studies above showed that host's microbiota-sensing NLRPs have a critical role in maintaining the balance of commensal components.

Dendritic cells (DCs)

DCs are specialized antigen-presenting cells that have a critical role in activating T cells by presenting bacterial or food-derived antigens in both steady-state and infectious/inflammatory conditions. Although different groups differently classified intestinal lamina propria DCs according to the use of different surface markers, at least two populations (CD103$^+$ DCs and CX3CR1$^+$CD103$^-$ DCs) are consistent among the different classification methods.

These two populations are differentiated from different precursors [43, 44], localize differently in the intestinal lamina propria, and have been reported to have different functions [45, 46]. The CX3CR1$^+$CD103$^-$ DCs are derived from circulating Ly6C^{hi} monocytes [44], and extend their dendrites to penetrate the IEC layer and to sample antigens from pathogenic and commensal bacteria [45, 47]. The extensions of the CX3CR1$^+$ DCs markedly increase upon infection with pathogens, such as *Salmonella*, and MyD88-dependent TLR signaling by non-hematopoietic (epithelial) elements has an important role in the DC extension response [48]. Several studies show that intestinal lamina propria CX3CR1$^+$ DCs induce Th17 cells [49, 50]. CD70$^+$CD11c$^+$CX3CR1$^+$ DCs expresses ATP receptors, and can differentiate naïve T cells into Th17 cells in the presence of ATP [50]. On the other hand, intestinal CX3CR1^{high} CD11b$^+$ CD11c$^+$ cells have recently been shown to inhibit CD4$^+$ T cell proliferation in a cell contact-dependent manner and prevent T cell-dependent colitis [51]. Therefore, CX3CR1$^+$ cells include several functionally distinct subsets.

The CD103$^+$ DCs arise from pre-DCs [44], localize in the core of the villi and migrate to the draining mesenteric LNs [46], where they induce the gut-homing receptor CCR9 and $\alpha 4\beta 7$-integrin on the responding T cells [52]. As discussed later, the CD103$^+$ DCs may contribute to the accumulation of Treg cells induced by the gut microbiota in the steady-state colon. Indeed, CD103$^+$ DCs express retinal dehydrogenase to synthesize retinoic acid, which in cooperation with TGF-β induces the development of Treg cells [53, 54]. In addition to CD103$^+$ DCs, mucosal macrophages have been shown to drive the generation of Foxp3$^+$ Treg cells within the lamina propria [55]. On the other hand, the CD103$^+$ DCs from colitic mice exhibit impaired induction of Treg cells and instead favor the induction of IFN-γ-producing CD4$^+$ T cells [56]. Furthermore, a recent study showed that mice with the DC-specific ablation of Notch2 exhibit a loss of CD11b$^+$CD103$^+$ DCs in the intestinal lamina propria and fewer Th17 cells than wild-type mice, indicating that CD103$^+$ DCs are involved in the development of Th17 cells in the intestine [57]. These reports suggest that CD103$^+$ DCs functionally adapt to different environmental conditions.

Interestingly, GF mice exhibit reduced CX3CR1$^+$ CD103$^-$ DCs in the colon lamina propria compared with SPF mice, although the number of CD103$^+$ DCs remains unchanged [58]. Moreover, the number of extended dendrites on CX3CR1$^+$CD103$^-$ DCs is greatly decreased in GF mice or antibiotic-treated mice [58]. Therefore, the presence of the gut microbiota affects the populations and functions of DCs, thereby affecting the adaptive immune system in the intestine.

IgA-producing cells

More than 80 % of the body's effector B cells are known to reside in the intestinal mucosa. B cells develop in the bone marrow and subsequently migrate to the peripheral lymphoid organs, including GALT such as Payer's patches (PPs) and isolated lymphoid follicles (ILFs). In these organs, the B cells mature through two processes, somatic hypermutation (SHM) and class switch recombination (CSR). The B cells acquire further diversity, and, subsequently, high-affinity immunoglobulin (Ig) mutants are selected. IgA is the dominant Ig isotype in the intestinal mucosa. Oral administration of cholera toxin induces specific IgA with high affinity; thus, this experimental system has been frequently used to study IgA responses in vivo [59]. The induction of cholera toxin-specific IgA is T cell-dependent (TD) because T cell- or MHC class II-deficient mice have a defect in the specific IgA response [60, 61]. In contrast, mice with a deficiency of T cells or MHC class II and CD40-deficient mice still have relatively abundant intestinal IgA [60], suggesting the existence of T cell-independent (TI) IgA. Upon TLR stimulation, myeloid and epithelial cells produce B cell activating factor (BAFF, also known as BLyS) and/or APRIL, which promote CSR to IgA by binding the receptors, BAFF-R and transmembrane activator, and calcium modulator and cyclophilin ligand interactor (TACI), which are expressed on B cells [32, 33, 62]. Given that IgA production via the TD pathway takes several days following antigen encounter, a faster, TI IgA response may be required to compensate for the temporal gap in the intestinal mucosa, which is constantly exposed to the microbiota. Intestinal IgA-producing plasma cells are 10- to 100-fold lower in GF mice than in SPF mice, indicating that the gut microbiota promotes the generation of IgA-producing cells [7].

IgA is secreted as a dimer or oligomer, and this dimer/oligomer binds the polymeric Ig receptor (pIgR) expressed on the basolateral surface of IECs, resulting in basolateral-to-apical transcytosis of secretory IgA (SIgA) to directly neutralize microbial toxins or pathogens [63]. PIgR-deficient mice, which lack the ability to transport IgA into the intestinal lumen, display low-grade enteropathy [64, 65], implying that IgA contributes to mucosal protection from commensal bacteria. Furthermore, the lack of activation-induced cytidine deaminase (AID), which results in a defect in CSR and thereby a lack of IgA-producing plasma cells in the intestine, leads to the excessive proliferation of anaerobic bacteria in the small intestine, particularly the segmented filamentous bacteria (SFB), accompanied by the hyperplasia of ILFs [66, 67]. The addition of IgA prevents the aberrant SFB expansion and ILF hyperplasia in the small intestine in AID-deficient mice. Importantly, mice expressing AID^{G23S}, which have normal levels of IgA but have a defect in SHM, have large amounts of intestinal bacteria, particularly anaerobic bacteria, that is accompanied by germinal center B cell hyperplasia in gut lymphoid tissues [68]. Therefore, TD high-affinity IgA production in GALTs is indeed required for gut homeostasis.

IL-17-producing cells, including Th17 cells and innate lymphoid cells

Th17 cells are characterized by the production of IL-17A (also referred to as IL-17), IL-17F, and IL-22. Both the small and large intestines of SPF mice harbor far more Th17 cells under steady-state conditions compared with other lymphoid or non-lymphoid organs [50]. GF mice have markedly fewer Th17 cells than SPF mice, suggesting a specific and important role of Th17 cells in intestinal mucosa immunity in the context of co-existence with commensal bacteria. In addition to Th17 cells, $\gamma\delta$ T cells and several different innate lymphoid cell (ILC) populations are producers of high levels of IL-17 in the intestines. These cells share many characteristics with Th17 cells: the cytokines they produce, triggers for activation, and transcription factors required for their development.

Upon encountering antigens, naïve CD4 T cells are activated through the T cell receptor (TCR) and undergo differentiation into different types of effector Th cells in the presence of specific cytokines that are produced by innate cells. Th17 cells are differentiated in the presence of TGF-β and IL-6. TGF-β and IL-6 activate the signal transducer and activator of transcription 3 (STAT3), which induces the retinoic acid receptor-related orphan receptor gamma t (RORγt). RORγt is a master regulator that is essential for Th17 differentiation [69]. Other transcription factors, such as aryl hydrocarbon receptor (Ahr) and basic leucine zipper transcription factor ATF-like (BATF), also contribute to IL-17 differentiation [70, 71]. During differentiation, Th17 cells proliferate in response to IL-21, which is produced by the Th17 cells themselves [72]. IL-21 also induces the IL-23 and IL-1 receptors, which allow Th17 cells to be responsive to IL-23 and IL-1β [73]. Therefore, in the presence of highly proinflammatory cytokines (such as IL-23 and IL-1β), Th17 cells are activated to become fully inflammatory cells.

$\gamma\delta$ T cells are constitutively activated and have the potential to produce IL-17 [74]. Mice deficient in fully functional Treg cells develop spontaneous colitis accompanied by an expansion of $\gamma\delta$ T cells, including an IL-17-expressing population [75, 76]. The disease is abrogated in $\gamma\delta$ T cell-deficient and antibiotic-treated mice, suggesting that the activation of $\gamma\delta$ T cells by commensal bacteria is responsible for the colitis [76]. Therefore, Treg cells restrain the excessive activation of $\gamma\delta$ T cells so as not to lead to damage of the host tissue. On the other hand,

TCR$\delta^{-/-}$ mice exhibit more severe colitis induced by DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS), suggesting that $\gamma\delta$ T cells also have an important role in immuno-regulation [77, 78]. Therefore, $\gamma\delta$ T cells are likely constitutively activated by commensal bacteria to prevent the invasion of intestinal bacteria and to maintain the front-line mucosal barrier system.

Several ILCs have been identified as another important source of IL-17 in the intestines. When an agonistic anti-body against CD40 is injected into recombinase-activating gene (RAG)-deficient mice, which lack lymphocytes, including Th17 cells and $\gamma\delta$T cells, the mice develop colitis accompanied by a local elevation in IL-23 and IL-17 [79]. IL-23p40 and RAG double-deficient mice do not develop colitis upon the same treatment, suggesting that ILCs other than Th17 cells and $\gamma\delta$T cells produce IL-17 upon IL-23 stimulation in the intestine. Following this report, several new IL-17-producing ILC populations have been identified in the lungs, skin, and intestines, i.e., the organs that are continuously exposed to the environment [80]. New pop-ulations of IL-17-producing ILCs, including CD4$^+$ lymphoid tissue inducer (LTi) cells and Thy1$^+$S-CA1$^+$CD3$^-$CD4$^-$c-KIT$^-$ cells, have been characterized. These IL-17-producing ILCs share common characteristics with differentiated Th17 cells. All the IL-17-producing ILCs require RORγt for their differentiation [69, 81–83]. Aryl hydrocarbon receptor is also expressed in $\gamma\delta$ T cells, CD4$^+$ LTi cells, and Thy1$^+$SCA1$^+$CD3$^-$CD4$^-$c-KIT$^-$ cells [70, 81, 84]. Many IL-17-producing ILCs constitu-tively express the IL-23 receptor and the IL-1β receptor, both of which allow them to produce IL-17 or IL-22 rap-idly in the presence of IL-23 or IL-1β [81, 85, 86].

Thy1$^+$SCA1$^+$CD3$^-$CD4$^-$c-KIT$^-$ cells accumulate and express IL-17 in the colon lamina propria in RAG-deficient mice infected with *Helicobacter hepaticus*, an innate colitis model [83]. The cells markedly expand and express IL-17, IL-22, and IFN-γ during inflammation [83]. As a similar cell population to mouse Thy1$^+$SCA1$^+$CD3$^-$CD4$^-$ c-KIT$^-$ cells, CD3$^-$CD19$^-$CD14$^-$CD16$^-$CD56$^-$CD127$^+$ cells have been reported to be increased in the inflamed ileum and colon of patients with Crohn's disease [87]. CD3$^-$NKp46$^+$ cells, which are another RORγt-expressing ILC population, are present in high numbers in the intestine and express IL-22, but not IL-17. Currently, how these newly discovered IL-17- and IL-22-producing ILCs con-tribute to intestinal inflammation or homeostasis remains largely unknown.

Th17 cells, IL-17-producing ILCs, and their associated factors (such as IL-23, IL-17, IL-17F, IL-22, or RORγt) have been implicated in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, uveitis, and IBD. Indeed, inflamed colon tissues in IBD patients highly express IL-23 [88], and a genome-wide study has revealed that IL23R gene is a susceptibility gene for IBD [89]. However, many studies have also shown that IL-17-pro-ducing cells have a critical role in protection rather than inflammation in the context of infection [90]. Furthermore, the transfer of IL-17A-deficient CD45RB^{hi} CD4$^+$ T cells has been shown to elicit a more aggressive colitis in lymphopenic mice than the transfer of wild-type cells [91], suggesting that IL-17 mediates a protective effect in intestinal inflammation. Although IL-17 has been the most intensively studied of the Th17 cytokines, the functions of other cytokines, including IL-17F and IL-22, are becoming apparent. IL-22 receptor expression is exclusively restric-ted to non-hematopoietic cells, particularly IECs, such that IL-22 promotes the host defense and tissue protection activities of IECs, such as the secretion of AMMs. IL-22 has protective roles in T cell transfer-induced colitis and DSS-induced colitis; transfer of IL-22-deficient naïve T cells induces more severe colitis than wild-type T cell transfer, and IL-22-deficient mice are more susceptible to DSS-induced colitis than wild-type mice [92]. During infection with the enteric pathogen *C. rodentium*, IL-22 protects the host by promoting the production of RegIIIβ and RegIIIγ by IECs [93]. IL-23p40-deficient mice are severely impaired in IL-22 production, leading to high susceptibility to *C. rodentium* infection [93]. These find-ings suggest that IL-22 has a more protective than pro-inflammatory role in the intestinal mucosa. In contrast, IL-17F-deficient mice exhibit milder DSS-induced colitis than wild-type mice, whereas IL-17-deficient mice have more severe colitis than wild-type mice [94], suggesting that IL-17F and IL-17 are pro-inflammatory and anti-inflammatory, respectively, during DSS-induced colitis. The individual cytokines associated with Th17 cells and IL-17-producing ILCs are probably produced at different levels and with different kinetics, which are dependent on the conditions of the mucosa, i.e., whether it is in a steady, inflammatory or bacteria-intruded state.

Regulatory T (Treg) cells

Multiple regulatory lymphoid and myeloid cell subsets are present in the intestinal mucosa. Among them, Foxp3$^+$ Treg cells are essential for maintaining peripheral tolerance and for preventing autoimmune and inflammatory diseases. Foxp3 is a key transcription factor that is required for the development, maintenance, and function of Treg cells [95, 96]. Human IPEX (immune dysregulation, polyendo-crinopathy, enteropathy, X-linked) patients and scurfy mice, which harbor mutations in the Foxp3 gene on the X-chromosome, exhibit multi-organ inflammation via massive infiltration of immune cells [97–99]. The two subsets of Foxp3$^+$ Treg cells differ in terms of the location of their differentiation: natural Foxp3$^+$ Treg (nTreg) cells

are differentiated in the thymus, and induced Foxp3$^+$ Treg (iTreg) cells are differentiated in the periphery. Although a small fraction of Foxp3$^+$ Treg cells are thought to be iTreg cells according to TCR sequence analysis [100–103], their role in physiological conditions is still unclear. The in vivo conversion of naïve cells has been observed in only a limited number of models [104–107]. Most of the models utilize foreign antigen-specific TCR-transgenic mice, such as OVA-specific TCR-transgenic mice crossed to RAG$^{-/-}$ mice. These mice have TCR$\alpha\beta^+$ T cells that express only a single clonotype TCR but have no Treg cells because of the lack of an endogenous antigen. When the antigen is administered by feeding, injection, or transplantation, antigen-specific iTreg cells emerge from naïve CD4$^+$ T cells. For example, when OVA-specific TCR transgenic RAG$^{-/-}$ mice are fed with OVA, Foxp3$^+$ Treg cells appear in the mesenteric lymph node or the small intestine lamina propria [104, 107]. iTreg cells are found in lymphopenic mice, such as RAG$^{-/-}$ or TCR$\alpha^{-/-}$ mice, after naïve T cells are transferred because the lymphopenic animals have an empty niche for Treg cells and, therefore, allow the conversion of naïve T cells into Treg cells to fill the niche [108].

Among several mechanisms for the regulation of other immune cells by Treg cells [109], IL-10 production by Treg cells has a critical role in maintaining intestinal immune tolerance. This role is demonstrated in IL-10-deficient or IL-10 receptor (IL-10R)-deficient mice, which exhibit spontaneous colitis, but not systemic autoimmune diseases [110]. The blockade of IL-10 signals skews intestinal CD4$^+$ T cells toward the Th1 and Th17 lineages in commensal bacteria-colonized mice [111], suggesting that IL-10 restrains the basal activation of these Th cell subsets in the steady state. On the basis of the report that the genetic ablation of IL-10 specifically in Foxp3$^+$ Treg cells elicits colitis, IL-10 produced by Treg cells has a critical role in intestinal homeostasis [112]. However, the incidence of colitis in these mice is slightly less and the onset is delayed compared with IL-10$^{-/-}$ mice, suggesting that IL-10 produced by other cells also contributes to the maintenance of intestinal homeostasis. Indeed, CD11b$^+$F4/80$^+$CD11c$^-$ macrophages in the small intestinal lamina propria are capable of producing IL-10 and involved in maintenance of mucosal homeostasis [49]. Furthermore, patients lacking a functional IL-10R develop colitis with an earlier onset and a higher penetrance compared with IPEX patients [113]. Therefore, IL-10 secretion by both Treg and non-Treg cells likely has a role in the control of intestinal immune homeostasis. Mice with a Treg-specific ablation of IL-10R or STAT3, which is a downstream signaling molecule of the IL-10R, develop spontaneous colitis with dysregulated Th17 cell responses [114, 115]. These results indicate that IL-10 signaling in Treg cells is critical for suppressing Th17 cells. IL-10R is also expressed by intestinal Th17 cells and required for the suppression of excess Th17 responses [116]. CTLA-4, which is highly expressed on intestinal Treg cells [9], is another molecule that is involved in intestinal homeostasis. On the basis of studies showing that CTLA-4 blockade abolishes Treg cell-mediated inhibition of colitis in the CD45RB^{hi}CD4$^+$ T cell transfer colitis model [117], and that the clinical application of an anti-CTLA-4 antibody (tremelimumab) for cancer treatment induces colitis as a side effect [118], CTLA-4 appears to be an indispensable molecule for maintaining intestinal homeostasis.

Intestinal Treg cells have suppressive activity against not only effector CD4$^+$ T cells but also other types of cells. As mentioned above, Treg cells regulate TCR$\gamma\delta$ T cells at steady state to maintain enteric immune tolerance [76]. Colon Foxp3$^+$ Treg cells facilitate IgA responses to the microbiota-derived antigen flagellin, which is one of the TLR5 ligands [119]. This IgA induction could be another mechanism for the maintenance of intestinal homeostasis through Treg cells. Thus, intestinal Treg cells appear to contribute greatly to intestinal homeostasis via various mechanisms and via targeting different cell subsets.

Immune regulation by the gut microbiota

Studies using GF and gnotobiotic animals have revealed the enormous impact of commensal bacteria on a host's systemic immune system. GF mice have smaller PPs and ILFs with fewer germinal centers, decreased IgA$^+$ cells and lamina propria T cells, and reduced production of AMMs by IECs compared with SPF mice [21]. All these observations suggest that active immune responses occur in the intestinal mucosa in the presence of the commensal microbiota. Although the purpose of the 'classical' immune response is to eliminate pathogens to protect the host, the active immune response in the steady-state intestine is somehow oriented toward co-existence with the commensal microbiota. In other words, host immune cells likely function to contain the commensal bacteria to local sites and regulate local inflammation possibly elicited by the commensal bacteria.

Because more than half the intestinal microbiota is not culturable, the composition of the intestinal microbiota was until recently poorly defined. Culture-independent bacterial sequencing has now been extended to include metagenomics, which refers to studies of the structures and functions of microbial communities as well as their interactions with the habitats they occupy [120]. This analysis includes the sequencing of microbial 16S ribosomal DNA (rDNA) and the genome to determine the lineage and genes of the microbiota composing the community, and also

includes characterizing the community's expressed RNA and protein products and metabolic networks [120]. Based on this concept, the international human microbiome project has been initiated. This project has revealed that most bacteria belong to two phyla, Firmicutes and Bacteroidetes, which dominate among all the human adult commensal bacteria analyzed, and that enormous diversity exists in the types and proportions of species within the phyla [120]. The results also revealed considerable interpersonal variation [121–123].

Accumulating evidence suggests that deviations in the balance of the composition of commensal bacteria affect local and systemic diseases. Although the precise mechanisms of IBD remain unclear, the gut microbiota are thought to contribute to pathogenesis [5]. Several studies attempting to identify the causative microbiota composition for IBD have reported that the phylum Firmicutes appeared with less frequency in patients than healthy controls [122, 124–126]. Given that the samples were from patients in whom disease was established, whether the reduction in Firmicutes is a cause or a consequence of IBD is unclear.

IL-10$^{-/-}$ mice are often used as an IBD model, which have no colitis when they are raised under GF conditions [127]. This finding clearly demonstrates that commensal bacteria contribute to intestinal inflammation. In another IBD model, T-bet$^{-/-}$RAG2$^{-/-}$ (TRUC) mice develop spontaneous colitis, which is horizontally and vertically transmissible to wild-type mice when they are co-housed with the TRUC mice [128], indicating that altered commensal bacteria (dysbiosis) alone have the potential to cause intestinal inflammation [128]. More recent studies have shown that mice deficient in NLRP6 have altered commensal bacteria and exhibit high sensitivity to DSS-induced colitis. The colitogenic microbiota is also transmissible to wild-type mice [38]. A 16S rDNA-based analysis revealed that TRUC mice have an increase in *Klebsiella pneumoniae* and *Proteus mirabilis* whereas NLRP6-deficient mice have an increase in *Prevotellaceae* and TM7 in their fecal microbiota [38, 129]. Collectively, these findings imply that the composition of the microbiota can dictate the status of local host immune system.

Induction of Th17 cells by segmented filamentous bacteria

Segmented filamentous bacteria (SFB) are yet-to-be cultured, Gram-positive, and spore-forming bacteria that are commonly considered to be nonpathogenic. SFB are host-specific members of the gut microbiota in numerous species, including mammals, birds, fishes, and insects (although whether SFB are present in the human intestine is currently unclear). Very recently, three independent groups reported the complete genome sequence of SFB indigenous to mice, and revealed that the bacteria have a highly reduced genome and are highly dependent on and utilize the host for nutrition and metabolism [130–132]. SFB colonize mainly the small intestine and exhibit a characteristic long filamentous morphology comprising multiple segments with well-defined septa. Each bacterium is likely to originate from a single, holdfast-bearing cell that tightly adheres to, and even embeds itself in, the microvilli on the IEC surface. The attachment of SFB induces morphological changes in the IECs, such as the accumulation of actin around the attachment site [133, 134]. SFB activate IECs to induce MHC class II molecules and fucosyltransferase 2 (Fut2) [135]. Fut2 fucosylates host glycoproteins on the IECs, such as asialo GM1 glycolipids. SFB are also potent stimuli of the IgA response and of the recruitment of TCRαβ$^+$ IELs in the small intestine [136].

C57BL/6 mice from Taconic Farms have a substantial population of Th17 in the small intestine lamina propria whereas the mice from the Jackson Laboratory have only a few [12]. By comparing the components of the intestinal microbiota between the mice from the two vendors, SFB were found to be responsible for the induction of Th17 cells in the small intestine [137]. This finding was confirmed by the accumulation of Th17 cells in the small intestines of SFB-monocolonized mice [11, 137]. Furthermore, transgenic mice expressing the human antimicrobial peptide α-defensin, which exhibit the loss of SFB of the intestinal microbiota, have fewer Th17 cells in the lamina propria of the small intestine [138]. The mechanisms by which intestinal Th17 cells are induced by SFB are not at present clear. TGF-β and IL-6, which are critical for the early stages of differentiation of Th17 cells, are likely to be required for the accumulation of Th17 in the small intestine in SPF mice [12]. However, how TGF-β and IL-6 are induced by SFB colonization remains unclear. Given that SFB directly attach to IECs and induce the cytoplasmic accumulation of polymerized actin in IECs [139], SFB may have specific mechanisms for inducing TGF-β and IL-6 through direct interaction with IECs.

Induction of Treg cells by the commensal microbiota

Foxp3$^+$ Treg cells distribute to essentially all organs, and the frequencies of Foxp3$^+$ cells are approximately 10 % within the CD4$^+$ cell subset. In contrast, the frequency of Foxp3$^+$ Treg cells in the gut lamina propria is notably higher (>30 % within CD4$^+$ T cell) than in other organs [9]. Therefore, the intestinal microbiota likely has a critical role in the accumulation of intestinal Treg cells. Indeed, we have shown that, in the colon lamina propria, Treg cell numbers decreased in GF mice and antibiotic-treated mice [9]. However, the analyses of the number of colon Treg

cells in various studies have provided discordant results. Some studies, including those from our laboratory, show a decrease in GF mice [9, 140, 141], and other studies show normal numbers of Treg cells in GF mice [111, 142–144] relative to SPF mice. These different results might be produced by different methods of isolating lymphocytes from gut lamina propria. The discrepancies could also be attributed to differences in the components of the intestinal microbiota in mice among various SPF animal facilities. Furthermore, there might be differences in the stringency of cleanliness between GF facilities. Importantly, many reports have consistently shown that the percentage and number of Helios-negative Tregs was markedly reduced in GF mice compared with SPF mice [9, 111, 143]. In SPF mice, Helios-negative and Helios-positive cells are present at approximately a 1:1 ratio within colon Treg cells [9, 111]. Helios is a putative marker for thymically derived nTreg cells [145]. Thus, Helios$^-$ Treg cells in the colon lamina propria may be iTreg cells. Although more detailed studies are required to fully characterize Helios-negative Treg cells in the intestine (discussed later), the presence of the microbiota likely affects this population of Treg cells.

The human symbiont *Bacteroides fragilis* is commonly isolated from human fecal samples [146, 147]. Colonization of GF mice with *B. fragilis* suppresses colitis by the opportunistic pathogen *H. hepatics*. This suppression is dependent on polysaccharide A (PSA), which is a component of *B. fragilis* [13]. Administration of purified PSA is sufficient to increase IL-10 production by Treg cells and to suppress IL-17 production during intestinal inflammation [142, 148]. The consumption of another human commensal species, *Bifidobacterium infantis*, also increases Foxp3$^+$ Treg cells during pathogenic *Salmonella typhimurium* infection, leading to a reduction in disease severity and the systemic translocation of the bacteria with an attenuation of local NF-κB activation [14].

Among the microbiota that is indigenous to the murine colon, spore-forming components, particularly those of the genus *Clostridium* belonging to clusters XIVa and IV, are potent inducers of colon Treg cells [9]. Some species of *Clostridium*, such as *C. perfringens* (a member of *Clostridium* cluster I), *C. difficile* (a member of cluster XI), and *C. tetani* (a member of cluster I), produce toxins and are pathogenic, but most of the *Clostridium* clusters XVIa and IV maintain a commensal relationship with the host. The colonization of GF mice with a defined mixture of 46 strains of *Clostridium* clusters XIVa and IV, which were originally isolated from the chloroform-treated fecal material (sporulated fraction) of conventionally reared mice [149], is sufficient to induce Treg cells [9]. Importantly, the 46 strains of *Clostridium* spp. do not include pathogenic clostridia, such as *C. difficile*. Perhaps consistent with the importance of commensal *Clostridium*

clusters XIVa and IV, colonization with altered Schaedler flora (ASF), which includes *C. clostridioforme* (*Clostridium* cluster XIVa), results in Treg cell accumulation in the colon LP [111]. Similar to mice reared in SPF conditions, Treg cells in the colon of mice colonized with *Clostridium* clusters XIVa and IV contain a large number of Helios$^-$ Foxp3$^+$ cells [9]. Therefore, *Clostridium* clusters XIVa and IV may induce iTreg cell conversion in the colon. In addition, a large subset of the Treg cells induced by colonization by *Clostridium* clusters XIVa and IV expresses high levels of IL-10 and CTLA-4 [9]. Therefore, *Clostridium* clusters XIVa and IV may influence the qualitative properties of colon Treg cells in addition to the quantity [9].

The mechanisms by which certain bacteria induce the intestinal accumulation of Treg cells are still unknown. Given that Helios is a candidate marker for nTreg cells [145], conversion from local naïve CD4 T cells may be responsible for the accumulation of Helios$^-$Foxp3$^+$ Treg cells in the colon (Fig. 1a). In addition, the intestinal microenvironment is rich in TGF-β and bacteria-derived antigens and, therefore, might promote iTreg cell induction (Fig. 1a). Alternatively, Helios$^-$Foxp3$^+$ Treg cells could be a further activated or differentiated subset of thymus-derived nTreg cells that pre-exist in the colon (Fig. 1b). The steady-state colon microenvironment might induce nTreg cells to differentiate into IL-10-producing Treg cells, resulting in the downregulation of Helios. Another possibility is that the microbiota activates the host IECs or other cells to produce chemokines to recruit Helios$^-$ Treg cells into the colon lamina propria (Fig. 1c). In support of the first scenario (the induction of iTreg cells in the colon), one recent study provided evidence that many clones of Treg cells that localize specifically in the colon react to the intestinal bacterial content in vitro [143]. Therefore, specific commensal bacteria likely induce naïve CD4$^+$ T cells to differentiate into antigen-specific colon Treg cells that, presumably, enforce immune system tolerance toward those bacteria.

Impacts of commensal bacteria on systemic immune responses and diseases

Several models of autoimmunity have been tested in GF conditions to assess the influence of commensal bacteria on the diseases. Non-obese diabetic (NOD) mice and BioBreeding rats, which are models of type 1 diabetes (T1D), display accelerated and severe diabetes under GF conditions [150, 151]. In contrast, myelin oligodendrocyte glycoprotein (MOG)-specific TCR-transgenic mice, which spontaneously develop experimental autoimmune encephalomyelitis (EAE) in a model of multiple sclerosis, exhibit decreased severity of the disease under GF
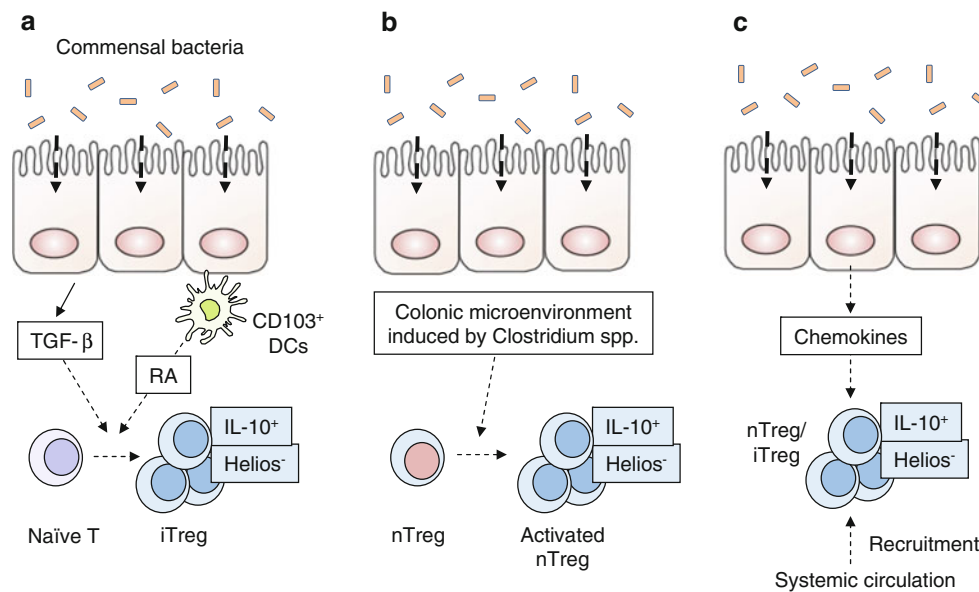
Fig. 1 Possible mechanisms for the induction of Helios⁻ IL-10⁺ Treg cells by commensal bacteria in the colon lamina propria. **a** Upon colonization with commensal bacteria, the colon epithelial cells produce TGF-β, which then contributes to the conversion of naive Treg cells to iTreg cells. Retinoic acid (RA) production by CD103⁺ DCs in the lamina propria may also contribute to the conversion. **b** Alternatively, the colon microenvironment that is generated in response to the commensal microbiota may contribute to the activation, differentiation, and proliferation of nTreg cells to become Helios⁻ IL-10⁺ Treg cells. **c** Another possibility is that upon interaction with the microbiota, IECs or other intestinal cells produce chemokines that recruit Helios⁻ IL-10⁺ Treg cells from other organs into the colon lamina propria

conditions [10]. The commensal bacteria-dependent activation of MOG-specific CD4⁺ T cells leads to an increase in MOG-specific antibody production by the draining lymph node B cells as a mechanism of altering the severity of disease by commensal bacteria [10]. For rheumatoid arthritis, different models yield different outcomes of the disease related to colonization by commensal bacteria. GF conditions ameliorate arthritis in IL-1 receptor antagonist-deficient mice and KBxN mice, both of which develop spontaneous arthritis under SPF conditions [152, 153]. In addition, GF mice have slightly less severe disease in an adjuvant-based arthritis model than SPF mice [154]. Collectively, these results imply that commensal bacteria affect autoimmune disease and that the outcome differs between the diseases.

Because SFB induce Th17 cells in the small intestine, several studies have focused on the influence of the bacteria on infections or autoimmune diseases. SFB-colonized mice are resistant to infection with *C. rodentium* [137]. In KBxN mice, the disease is attenuated under GF conditions, and this attenuation is accompanied by a loss in autoreactive IgG production by B cells that results from a decrease in Th17 cells [153]. Monocolonization with SFB reverts arthritis through an increase in splenic Th17 cells and IgG production [153]. Similar to the KBxN arthritis model, the severity of EAE is greatly attenuated under GF conditions [10, 155]. However, in SFB-monocolonized mice, the level

of infiltration of Th17 cells in the spinal cord and the disease severity are equivalent to these measures in SPF mice [155]. These two autoimmune disease models (KBxN arthritis and EAE) have been shown to require Th17 cells for disease development, because mice with an IL-17 deficiency or subjected to IL-17-blocking treatment display attenuated disease symptoms [153, 156]. Therefore, SFB likely induce Th17 cells in the intestine, and these cells then migrate to, and accumulate in, the target organs of the autoimmune disease. NOD mice spontaneously develop a disease similar to T1D, which is a Th1-mediated disease. There is a significant difference in incidence of disease development between SFB-colonized and uncolonized NOD mice [157]. SFB-colonized NOD mice develop diabetes at a greatly reduced frequency. Interestingly, SFB colonization does not have an effect on the development of insulitis [157], suggesting that SFB colonization cannot overcome the genetic influence on the susceptibility to insulitis. However, the bacteria can modify the progress of the disease, presumably by affecting the progress of insulitis and/or the metabolism of insulin and glucose [157]. As a mechanism, the report suggests that SFB colonization directs the Th1/Th17 balance toward a Th17-skewed response and consequently suppresses the Th1 response [157]. However, how intestinal Th17 cell accumulation elicits a systemic Th17 response in the EAE, arthritis and T1D models remains unclear.

Oral inoculation of SPF mice with 46 strains of *Clostridium* clusters XIVa and IV decreased the severity of DSS-induced colitis, and the Th2 response, including OVA-specific IgE production, upon immunization [9]. In humans, a reduction in the number of *Faecalibacterium prausnitzii*, which is a member of *Clostridium* cluster IV, was shown to be associated with a high risk of postoperative recurrence of Crohn's disease [158]. The supernatants from *F. prausnitzii* cultures increase the production of IL-10 by peripheral blood mononuclear cells in vitro. In addition, the proportion of *Clostridium* clusters XIVa and IV in the fecal community was shown to be smaller in IBD patients than in healthy controls [122]. These reports raise the possibility that the *Clostridium*-dependent constitutive induction of Treg cells and their expression of IL-10 and CTLA-4 may contribute to the suppression of local and systemic autoimmunity and deleterious inflammation in humans. They further suggest that the prophylactic administration of human-gut-resident *Clostridium* clusters XIVa and IV could reduce susceptibility to chronic disease. However, it still remains unclear whether the defects in Treg cells actually contribute to human IBD. Interestingly, serological analysis of colitic C3H/HeJBir mice has revealed that a flagellin protein called CBir1, which contains sequences related to the flagellins of *Clostridium* cluster XIVa, is the dominant antigen responsible for the colitis. The adoptive transfer of a CBir1 flagellin-specific CD4$^+$ T cell line into immunodeficient mice induced colitis in the recipients [159]. Furthermore, Crohn's disease patients have an increased number of CD4$^+$ T cells that are reactive to CBir1 flagellin [160]. Therefore, depending on the circumstances, clostridia-derived antigens could be causative factors for inflammatory diseases.

Concluding remarks

Considerable progress has been made in improving our understanding of intestine-residing immune cell populations and the influences of the composition of the gut microbiota on the immune system. Specific species of the gut microbiota affect the numbers and activities of intestinal immune cells. However, we are still far from achieving a comprehensive understanding of the mechanisms underlying how IECs and immune cells, including the newly discovered cells, collaborate to protect the host from pathogenic bacteria and at the same time develop tolerance to commensal bacteria. It also remains unclear whether and how the intestinal immune cells affect the systemic immune responses. In addition, it is also unclear how the host immune system maintains the diversity and balance of the microbiota. More intensive studies will be required to answer these questions.

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