J Gastroenterol (2015) 50:928–939
DOI 10.1007/s00535-015-1084-x

The Japanese Society of Gastroenterology

REVIEW

# A breakthrough in probiotics: *Clostridium butyricum* regulates gut homeostasis and anti-inflammatory response in inflammatory bowel disease

**Takanori Kanai[1] · Yohei Mikami[1] · Atsushi Hayashi[1,2]**

Received: 10 April 2015 / Accepted: 18 April 2015 / Published online: 5 May 2015


**Abstract** Intestinal immune homeostasis is regulated by gut microbiota, including beneficial and pathogenic microorganisms. Imbalance in gut bacterial constituents provokes host proinflammatory responses causing diseases such as inflammatory bowel disease (IBD). The development of next-generation sequencing technology allows the identification of microbiota alterations in IBD. Several studies have shown reduced diversity in the gut microbiota of patients with IBD. Advances in gnotobiotic technology have made possible analysis of the role of specific bacterial strains in immune cells in the intestine. Using these techniques, we have shown that *Clostridium butyricum* as a probiotic induces interleukin-10-producing macrophages in inflamed mucosa via the Toll-like receptor 2/myeloid differentiation primary response gene 88 pathway to prevent acute experimental colitis. In this review, we focus on the new approaches for the role of specific bacterial strains in immunological responses, as well as the potential of bacterial therapy for IBD treatments.

**Keywords** *Clostridium butyricum* · Probiotics · Macrophages · Interleukin-10

✉ Takanori Kanai
takagast@z2.keio.jp

[1] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan

[2] Research Laboratory, Miyarisan Pharmaceutical Co. Ltd, Tokyo 114-0016, Japan

## Introduction

Crohn's disease and ulcerative colitis, the two major types of inflammatory bowel disease (IBD), are relapsing and remitting conditions characterized by chronic intestinal inflammation caused by aberrant immune responses against the patient's own organs. Genome-wide association studies of IBD have implicated genes connected to the host interaction with microbiota in its pathogenesis, such as *NOD2*, *CARD9*, and *ATG16L1* [1, 2]. These genetic studies support the idea that perturbations of the host commensal microbiota and host immune system contribute to the pathogenesis of IBD.

In humans, the gastrointestinal tract is home to $10^{14}$ commensal bacteria from many thousands of individual strains [3]. The number of bacterial cells present in the gut ranges from $10^{1}$–$10^{3}$ per gram of contents in the stomach and duodenum, to $10^{4}$–$10^{7}$ bacteria per gram in the small intestine, and $10^{11}$–$10^{12}$ per gram in the colon [4]. In addition, the gut microbial composition differs among gastrointestinal tract sites. Compared with the colon, the small intestine is enriched with sequences of the genus *Bacillus* of the phylum *Firmicutes* [5]. In contrast, the phylum *Bacteroidetes* and the family *Lachnospiraceae* of the phylum *Firmicutes* are more prevalent in colonic samples [5]. Methods based on 16S ribosomal RNA gene sequences show that the human gut microbiota is dominated by only two bacterial divisions: the phyla *Bacteroidetes* and *Firmicutes*, which constitute more than 90 % of the known phylogenetic categories [6]. The gut microbiota plays a key role in human health and disease.

Many studies have revealed that an imbalance in the bacterial composition (dysbiosis) provokes proinflammatory immune responses and induces inflammatory diseases in the host [7, 8]. Recent progress in gnotobiotic

technology and metagenomics has made possible analysis of the role of specific bacterial strains in immunological responses. One recent study showed that the 17 strains of clostridia from clusters IV, XIVa, and XVIII derived from the healthy human microbiota induced accumulation of interleukin (IL)-10-producing induced regulatory $T_{reg}$ (-$T_{reg}$) cells, which suppressed colitis in a dextran sodium sulfate (DSS)-induced colitis model [9]. Emerging evidence shows that specific commensal bacteria can affect the differentiation of mucosal $T_{reg}$ cells under normal conditions [9–12], but it has remained largely unclear which specific bacteria influence mucosa-specific antigen-presenting cell phenotypes such as macrophages and dendritic cells. Unlike these commensal *Clostridium* strains, the probiotic strain *Clostridium butyricum* MIYAIRI 588 (CBM588) primarily induced IL-10 production from colonic $F4/80^{+}CD11b^{+}$ intestinal macrophages rather than T cells, and prevented experimental colitis by an IL-10-dependent mechanism [13].

Macrophages are essential for maintaining intestinal homeostasis, and are involved in the pathogenesis of IBD. In this review, we discuss the role of $T_{reg}$ cells and macrophages in gut homeostasis and inflammation, and describe how the gut microbiota, especially clostridia, influences the immune system and colitis.

## Gut microbiota and IBD

IBD is a chronic and relapsing inflammatory disorder caused by a dysregulated immune response to host intestinal microbiota. The target organ of ulcerative colitis is limited to the large intestine, whereas Crohn's disease may involve any part of the gastrointestinal tract, from the mouth to the anus [14]. Accumulation of epidemiological and clinical evidence and recent genetic studies have shown the relationship between IBD and several environmental factors, such as modern lifestyle, diet, antibiotic use, genetic background, and immunological factors [2, 14, 15]. Some of these factors may affect the composition of the gut microbiota [2, 15].

The gut microbiota consists of a complex community of microorganisms that live in the gastrointestinal tract. Several techniques have been used to analyze the composition of the gut microbiota, such as culture methods, fluorescence in situ hybridization, and terminal restriction fragment length polymorphism. These studies of the human IBD microbiota have been limited to alterations of the fecal microbiota, thus preventing the possibility that different subpopulations exist within IBD classifications. However, recent advances in next-generation sequencing technology have allowed in-depth characterization of various alterations in the composition of the microbiota in disease states. Most studied have indicated reduced diversity of the gut microbiota in IBD patients, which is due to a reduction in the diversity of *Firmicutes*. For example, Frank et al. [5] demonstrated that the abundances of bacterial members of the phyla *Firmicutes* and *Bacteroidetes* are reduced and those of *Actinobacteria* and *Proteobacteria* are increased in IBD. In particular, family *Lachnospiraceae*, belonging to *Clostridium* clusters IV and XIVa, is significantly less abundant in IBD patients than in healthy individuals [5]. Sokol et al. [16, 17] showed a reduction in representation of the phylum *Firmicutes*, and particularly *Clostridium* cluster IV members, *Faecalibacterium prausnitzii*, in the feces of Crohn's disease patients. However, it remains controversial whether the IBD-associated dysbiosis in the gut microbiota is a cause or a consequence of inflammation, but it is likely to be important in maintaining the diversity of the clostridia to prevent IBD.

No specific pathogenic bacteria associated with IBD have been identified to date. *Mycobacterium avium* ssp. *paratuberculosis* has long been suspected as a causative pathogen of Crohn's disease [18]. However, a 2-year clinical trial of an antitubercular drug administered to Crohn's disease patients revealed no efficacy [19]. Other bacterial genera such as *Listeria* and *Mycoplasma* have been investigated in relation to Crohn's disease [20, 21]. Adherent-invasive *Escherichia coli* has been isolated from ileal biopsy samples from patients with Crohn's disease. Pathogenic adherent-invasive *Escherichia coli* can colonize the ileal mucosa by adhering to intestinal epithelial cells, and is also a true invasive pathogen that is able to invade intestinal epithelial cells [22]. Adherent-invasive *Escherichia coli* also replicates in macrophages and stimulates production of tumor necrosis factor (TNF)-α by macrophages [22]. In ulcerative colitis patients, *Fusobacterium varium* attaches to inflamed mucosa and invades ulcerated mucosa [23]. Thus, some specific bacteria might be associated with IBD; however, a role for these bacteria in the pathogenesis of IBD has not been confirmed.

## Gut microbiota and experimental models of IBD

Many different animal models of colitis have been established to investigate the different factors in IBD, by inducing acute or chronic intestinal inflammation. The DSS-induced colitis model exhibits many phenotypic characteristics of human ulcerative colitis. DSS-induced colitis in BALB/c mice was first described by Okayasu et al. [24]. Mice given DSS in their drinking water for several days developed acute inflammation limited to the colon, with weight loss, bloody diarrhea, and histopathological changes. Importantly, acquired immunity is dispensable for this model. Severe combined immunodeficiency mice [25, 26] and mice

deficient in recombination activating gene (RAG)-1 [27, 28], lacking T and B cells, develop colitis after DSS treatment, suggesting innate immune cells are sufficient in the acute phase of DSS-induced colitis, such as macrophages, dendritic cells, neutrophils, and innate lymphoid cells. Although DSS-induced colitis occurs without adaptive immune cells, wild-type mice are more susceptible than RAG-deficient mice to DSS-induced colitis [28]. How DSS initiates colonic inflammation is not fully understood, but some studies have shown that DSS has a direct effect on the inner mucus layer, allowing bacteria to invade the epithelial cells and thus trigger the characteristic inflammatory reaction [29, 30]. In the acute phase, colonic inflammation is characterized by increased levels of T helper ($T_h$) 1–$T_h$17-mediated inflammatory cytokines such as TNF-α, IL-6, and IL-17 [31]. In contrast, a $T_h$1–$T_h$2-mediated inflammatory response is found in the chronic phase [31, 32]. IL-17A is an important proinflammatory cytokine that is secreted by $T_h$17 cells, and macrophages also produces this cytokine [33]. In a previous study, DSS-induced colitis was ameliorated by administration of IL-17A-neutralizing antibody [34], suggesting that deficiency of IL-17A could relieve the inflammation. It has been shown that the susceptibility of DSS-induced colitis is increased in mice lacking the genes that affect Toll-like receptor (TLR) signaling, such as *Tlr2*, *Tlr4*, and *Myd88*, which encodes myeloid differentiation primary response gene 88 (MyD88), an adaptor molecule of TLRs [35, 36]. These studies have indicated important protective roles of the TLR/MyD88 pathway during the development of colitis.

Patients with long-standing ulcerative colitis have an increased risk of colorectal cancer [37]. Chronic inflammation induced by DSS in combination with azoxymethane (AOM), a classic chemical carcinogen, results in malignancy. This model has also been used to analyze colitis-associated colorectal cancer [38]. IL-1β and IL-18 have key roles in inducing colorectal tumor formation [39–41]. One carcinogenic mechanism of this AOM/DSS model may be that IL-1β induced by neutrophils leads to macrophage production of IL-6, as a tumor promoter [42]. In addition, AOM/DSS *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice are more susceptible to colitis and polyp formation than wild-type mice are [41]. Another study showed that *Myd88*$^{-/-}$ mice subjected to multiple injections of AOM had reduced colon tumor growth, suggesting that an MyD88-dependent signaling pathway plays a crucial role in carcinogen-induced colonic tumorigenesis [35].

Colitis induced by oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) is one of the models suitable for the analysis of the $T_h$2-dependent immune response in progressive intestinal inflammation resembling ulcerative colitis [43, 44]. Intrarectal administration of a single dose of oxazolone leads to acute inflammation through the infiltration of lymphocytes and neutrophils in the distal colon and particular mucosal layers [43]. In addition, repeated intrarectal administration of oxazolone induces chronic inflammation, and IL-13 produced by natural killer (NK) T cells in the lamina propria (LP) is a key pathogenic cytokine in the induction of colitis. Intrarectal administration of oxazolone to CD1-deficient mice and Jα281-deficient mice lacking NK T cells does not result in colitis, and neutralization of IL-13 prevents induction of colitis [44], suggesting that these data have important implications for understanding the treatment of ulcerative colitis.

Another IBD model is 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, which mimics patients with Crohn's disease. TNBS-induced colitis is mainly characterized by an increased $T_h$1-mediated response, such as IL-12p40 and interferon (IFN)-γ, and decreased IL-4 levels, and by infiltration of the LP by T cells and macrophages, with diarrhea, weight loss, and rectal prolapse [31, 45]. Administration of IL-12p40-neutralizing antibody to TNBS-treated mice led to marked improvement in the clinical and histopathological aspects of the disease [45]. LP $CD4^+$ T cells isolated from anti-IL-12-treated mice failed to induce IFN-γ production [45]. IL-12 plays a critical role in promoting $T_h$1 responses and cell-mediated immunity. The IL-12 family of cytokines, which includes IL-12, IL-23, IL-27, and IL-35, are key mediators of induction of inflammation. IL-12 consists of a heterodimer of p35 and p40, and IL-12 and IL-23 share a p40 subunit [46]. Several studies have shown that IL-23 is essential for T-cell-mediated colitis and promotes inflammation via IL-17 and IL-6 [47]. However, IL-23p19-deficient *lacZ* knock-in mice are more susceptible to the development of experimental T-cell-mediated TNBS colitis than wild-type mice are, suggesting that production of IL-12 is regulated by other IL-12 family members, such as IL-23, during $T_h$1-mediated TNBS-induced colitis [48].

Besides these chemically induced models of colitis, there are several spontaneous colitis models. IL-10 and its receptor are reported as susceptibility genes for IBD [1]. IL-10 acts as an immunoregulator to suppress excessive host immune responses against commensal bacteria, and altered function of IL-10 can lead to development of autoimmune diseases. Mice deficient in IL-10 develop spontaneous colitis [49]. However, IL-10 null mice under germ-free conditions do not develop colitis [50], indicating the significance of microbes in this model. Loss of T-bet and RAG genes also induces spontaneous colitis [7]. Wild-type mice co-housed with T-bet and *Rag1* double knockout mice (TRUC mice) also develop colitis. This suggests that the alteration of microbiota in TRUC mice can cause transmissible colitis.

The transfer colitis model was first reported in 1993. $CD4^+CD45RB^{high}$ T cells isolated from spleens of donor mice transferred to immunodeficient recipient mice

(*Rag2*$^{-/-}$ or severe combined immunodeficiency mice) caused aggressive inflammation in the colon and wasting disease [51, 52]. In contrast, reconstitution of *Rag2*$^{-/-}$ mice with CD4$^{+}$CD45RB$^{high}$ and CD4$^{+}$CD45RB$^{low}$ T cells did not result in development of wasting disease or colitis [53]. Furthermore, *Rag2*$^{-/-}$ mice with adoptive transfer of CD4$^{+}$CD45RB$^{low}$ T cells from diseased IL-10 knockout mice developed colitis [54], suggesting that IL-10 from CD45RB$^{low}$ T cells is required for the prevention of colitogenic cells [55]. $T_h$ cell subsets are supposed to contribute to the pathogenesis of this model, such as $T_h1$, $T_h17$, and $T_{reg}$ cells. $T_h1$ cells have long been thought to be necessary for pathogenesis of colitis. Naïve T cells deficient in T-bet [56] or signal transducer and activator of transcription 4 [57], key $T_h1$-associated molecules, fail to induce colitis after transfer into immunodeficient mice. However, T-cell-induced IFN-γ is dispensable for this model [57]. $T_h17$ is important in the pathogenesis of colitis in mice, but its role is still controversial. O'Connor et al. [58] demonstrated that *Rag1*$^{-/-}$ mice into which *Il17a*$^{-/-}$ CD4$^{+}$CD45RB$^{high}$ T cells were transferred developed severer colitis and wasting diseases, with significantly increased colon IFN-γ production, and concluded that IL-17A blocks differentiation of $T_h1$ cells via IL-17 receptor α on CD4$^{+}$ T cells. Adoptive transfer of naïve T cells from *Il17a*$^{-/-}$, *Il17f*$^{-/-}$, or *Il22*$^{-/-}$ mice into *Rag1*$^{-/-}$ mice caused severe colitis that was indistinguishable from that caused by wild-type cells [59]. In contrast, naïve T cells deficient in the transcription factor retinoic acid receptor related orphan receptor (ROR) γ [59] or signal transducer and activator of transcription 3 [60], which regulate $T_h17$ differentiation, fail to increase mucosal IL-17 cytokine levels and do not induce colitis when they are transferred into *Rag1*$^{-/-}$ mice. The role of the $T_h17$/$T_h1$ subsets in this experimental colitis is controversial. To assess this matter, we co-transferred colitogenic CD4$^{+}$ T cells isolated from CD4$^{+}$CD45RB$^{high}$ T cells from *Rag2*$^{-/-}$ mice and *Il10*$^{-/-}$ mice into *Rag2*$^{-/-}$ mice [61]. Mice that underwent co-transfer developed colitis to an extent similar to that in mice into which one type of cell was transferred [61]. The proportions of IFN-γ- and IL-17A-expressing CD4$^{+}$ T cells in mice that underwent co-transfer were significantly decreased compared with those in mice with single transfer, suggesting colitogenic $T_h1$ and $T_h17$ cells are in competition in this form of colitis [61]. We investigated the plasticity between $T_h1$ and $T_h17$ cells in vivo. When CD4$^{+}$CD45RB$^{high}$ T cells derived from RORγt reporter mice were transferred into *Rag2*$^{-/-}$ mice, the latter mice developed $T_h1$-dependent colitis [62]. RORγt$^{+}$ cells are induced to express T-bet and differentiate to IL-17A$^{-}$ IFN-γ$^{+}$ alternative $T_h1$ cells via IL-17A$^{+}$ IFN-γ$^{+}$ $T_h17$/$T_h1$ cells and IL-17A$^{-}$IFN-γ$^{+}$RORγt$^{+}$ $T_h1$-like cells [62]. In addition, we recently indicated that both alternative $T_h1$ cells and $T_h1$-like cells have the potential to be colitogenic in an adaptive transfer model [63]. These data suggest a critical role for the plasticity of these T cell types in the development of experimental colitis.

## Regulation of immune system by the intestinal microbiota

Recent advances in metagenomics, proteomics and gnotobiotic technology have made possible analysis of the role of specific bacterial strains in immunological responses [64, 65]. With use of these approaches, a recent study showed that commensal bacteria promote immune responses and specific T cell subsets, such as $T_h17$ cells and $T_{reg}$ cells (Table 1).

Segmented filamentous bacteria (SFB) are Gram-positive anaerobic bacteria and are members of the phylum *Firmicutes* and are similar to clostridia [66]. SFB associate with the intestinal epithelium and enhance IgA production by B cells, serum amyloid A dependent $T_h17$ cell differentiation in the LP of the terminal ileum, and production of proinflammatory cytokines, such as IL-17 and IL-22 [67, 68]. Mice colonized with SFB show amelioration of infection with *Citrobacter rodentium*, but SFB can also induce colitis in genetically susceptible animals, suggesting immune activation by SFB can control infection but they can cause inflammation [69]. Specific-pathogen-free C57BL/6 mice from Taconic Biosciences (formerly Taconic Farms) have increased levels of IL-17-producing T cells in the LP of the small intestine compared with mice from the Jackson Laboratory [67]. Study of germ-free mice colonized with cecal contents from Taconic Biosciences B6 or Jackson Laboratory B6 mice revealed that mice colonized with feces of Taconic Biosciences B6 mice led to recovery of normal proportions of LP $T_h17$ cells, but that this did not occur in mice colonized with feces from Jackson laboratory B6 mice [67]. SFB are important for induction of $T_h17$ cells and promotion of $T_h17$-mediated autoimmune disease in animal models; however, it still unclear how the bacteria modulate $T_h17$ cell homeostasis. A recent study reported that presentation of SFB antigens on MHC II in the small intestinal LP CD11c$^{+}$ dendritic cells led to the induction of SFB-specific intestinal $T_h17$ cells [70, 71]. Goto et al. [71] demonstrated SFB-mediated $T_h17$ cell induction in the gut involves SFB-derived antigens and generation of SFB-specific $T_h17$ cells. SFB-induced intestinal $T_h17$ cells recognize SFB antigen presentation by MHC II in the periphery. In contrast, SFB-independent $T_h17$ cells are generated even in the absence of MHC II. Thus, expression of MHC II on CD11c$^{+}$ cells is necessary and sufficient for SFB-induced $T_h17$ cell differentiation. Yang et al. [70] also demonstrated that the

**Table 1** Gut microbes promote immune response and specific T cell subsets

| Microbes | Cell type | Mechanisms and functions | References |
|---|---|---|---|
| SFB | $T_h17$ cells (IL-17, IL-22) | SAA-dependent $T_h17$ cell differentiation in the small intestine<br>SFB-induced $T_h17$ cells recognize SFB antigens produced by dendritic cells | [67, 68] |
| *Clostridium* clusters XIVa and IV | $Foxp3^+$ $T_{reg}$ cells (IL-10) | *Clostridium* induces TGF-β in intestinal epithelial cells to promote IL-10-producing $T_{reg}$ cells | [9, 10] |
| Chloroform-resistant bacteria | $T_{reg}$ cells (IL-10) | Commensal-microbe-derived butyrate induces colonic $T_{reg}$ cells in an FFAR2 (GPR43)-dependent manner | [93] |
| *Faecalibacterium prausnitzii* (*Clostridium* cluster IV) | $T_{reg}$ cells? | Metabolite (short-chain fatty acid? or active molecule?) increases secretion of IL-10 | [17] |
| *Bacteroides fragilis* | $Foxp3^+$ $T_{reg}$ cells (IL-10) | PSA interacts with TLR2-expressing T cells to induce colonic $T_{reg}$ cells | [11, 12, 90] |

*FFAR2* free fatty acid receptor 2, *Foxp3* forkhead box p3, *GPR43* G-protein-coupled receptor 43, *PSA* polysaccharide A, *SAA* serum amyloid A, *SFB* segmented filamentous bacteria, *TGF* transforming growth factor, *$T_h17$ cells* T helper 17 cells, *TLR* Toll-like receptor, *$T_{reg}$ cells* regulatory T cells

T-cell antigen receptor repertoire of intestinal $T_h17$ cells is restricted to SFB antigens, which implies that microbial antigens could decide the differentiation of antigen-specific T cells.

IL-22 is another important $T_h17$ cytokine also produced by innate lymphoid cells [72]. A human NK cell subset provides an innate source of IL-22 for mucosal immunity [73–77]. IL-22 is a member of the IL-10 cytokine family. IL-22 binds to a heterodimeric receptor composed of two subunits, IL-10R2 and IL-22R1. IL-10R2 is ubiquitously expressed, but IL-22R1 is exclusively expressed on non-hematopoietic cells [78]. IL-22 induces various genes of antimicrobial peptides, mucin production, and cellular differentiation, and maintains mucosal barrier function [79–81]. IL-22 prevents host infection with the Gram-negative enteric bacterium *Citobacter rodentium* [82–84], or peripheral dissemination of commensal bacteria [85]. Although IL-22 promotes inflammation in some models, such as *Toxoplasma gondii*, IL-22 is one of the key cytokines to maintain the balance between gut and commensal bacteria.

Intestinal IgA is involved in limiting the invasion of pathogenic bacteria into host tissue and regulating the composition and function of the commensal microbiota. In animals deficient in activation-induced cytosine deaminase, the absence of hypermutated IgA shows that these mice display lymphoid hyperplasia of the gut and an altered microbiota, and therefore IgA is a key factor for regulation of bacterial composition and represents the immune system involved in maintenance of homeostasis [86]. Impaired IgA selection results in uncontrolled T cell proliferation that severely affects the balance of microbial composition and disease in the intestine [86]. IgA responses are involved in maintaining host–bacterium mutualism by limiting innate immune responses to a specific gut microbiota [87]. A recent study by Kawamoto et al. [88] demonstrated an important role of $T_{reg}$ cells in promoting the diversity of the microbiota through regulation of IgA selection and in facilitating bacterial species responsible for gut homeostasis. To assess which $CD4^+$ T cell subsets contribute to the diversity and composition of the gut microbiota, naïve $CD4^+$ T cells or forkhead box p3 $(Foxp3)^+$ T cells were transferred into mice lacking T cells ($Cd3e^{-/-}$). The transfer of naïve $CD4^+$ T cells alone decreased microbiota complexity even in the absence of overt inflammation. By contrast, co-transfer of naïve $CD4^+$ T cells and $Foxp3^+$ T cells, or transfer of $Foxp3^+$ T cells alone increased bacterial diversity and modified the composition of the microbiota to the degree of the wild-type mice [88]. In addition, $Foxp3^+$ T cells can promote the diversification of *Firmicutes*, including *Clostridium* clusters IV and XIVa, which are inducers of $T_{reg}$ cells [9, 10, 88]. These results strongly suggest that clostridia can not only induce Foxp3 expression, but $Foxp3^+$ T cells can induce the expansion of these bacteria, which are required for the establishment of microbial communities in the gut under normal and inflammatory conditions. Kawamoto et al. [88] also demonstrated a mutual relationship between the adaptive immune response and the gut microbiota. Colonization of germ-free mice with the feces from $Cd3e^{-/-}$ mice into which with naïve $CD4^+$ T cells or $Foxp3^+$ T cells were transferred showed that $Foxp3^+$ T cells induce T follicular helper cells and generate more IgA-producing cells and $T_{reg}$ cells as compared with naïve $CD4^+$ T cells [88]. These findings may have important consequences in disease related to dysbiosis of IBD.

*Bacteroides fragilis* is a Gram-negative bacterium and belongs to the phylum *Bacteroidetes*. Polysaccharide A (PSA) from *Bacteroides fragilis* was originally identified to mediate establishment of a proper $T_h1/T_h2$ balance for the host [89]. Further study showed that colonization of germ-free mice by *Bacteroides fragilis* or treatment with purified

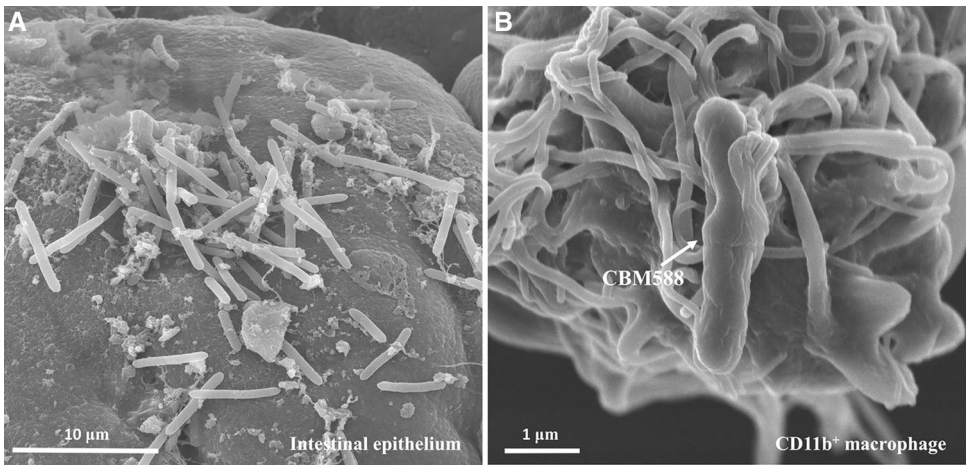


**Fig. 1** Morphology of *Clostridium butyricum* MIYAIRI 588 (CBM588). CBM588 belongs to cluster I, and is a Gram-positive rod-shaped obligate anaerobe. **a** Germ-free mice were colonized with CBM588 ($1 \times 10^8$ cells) for 3 weeks. The scanning electron microscopy image shows the proximal colon of CBM588-monoassociated mice. **b** $CD11b^+$ macrophages from dextran sodium sulfate treated $Rag2^{-/-}$ mice were co-cultured with heat-killed CBM588. The scanning electron microscopy image shows phagocytosis of CBM588 by colitic $CD11b^+$ macrophages.

PSA can protect against the induction of experimental colitis [90]. Conversely, colonization with a mutant strain that lacks expression of PSA from *Bacteroides fragilis* does not prevent colitis. Oral treatment of mice with purified PSA protects against TNBS-induced colitis, increases IL-10 production, decreases the levels of the proinflammatory cytokines TNF-α and IL-17, and inhibits epithelial cell hyperplasia and neutrophil infiltration into the gut [90]. To provide a mechanistic basis for PSA-mediated protection, it was shown that colonization of germ-free animals with *Bacteroides fragilis* increased the number of IL-10-producing $Foxp3^+$ $T_{reg}$ cells in the gut via TLR2 [11]. Thus, functional $Foxp3^+$ $T_{reg}$ cells are also produced by PSA during intestinal inflammation through TLR2 signals acting directly on $Foxp3^+$ $T_{reg}$ cells to promote IL-10 production [11, 12]. Induction of $T_{reg}$ cells is not limited to *Bacteroides fragilis*, and they are also induced in intestinal clostridia. Atarashi et al. [10] reported that a complex mixture of 46 strains of *Clostridium* induced transforming growth factor (TGF)-β in intestinal epithelial cells that promoted the subsequent accumulation of IL-10-producing induced $T_{reg}$ cells, which suppressed colitis in a DSS-induced colitis model. Consistent with these data, germ-free mice were colonized with a mixture of bacterial species, referred to as altered Schaedler flora species, which includes *Clostridium* species that induce mucosal $T_{reg}$ cells specifically in the LP of the colon [64]. To identify $T_{reg}$-cell-inducing bacteria from human stool samples, germ-free mice were colonized with the 17 strains of *Clostridium* belonging to clusters IV, XIVa, and XVIII, derived from a healthy human microbiota [9]. These clostridia induced IL-10-producing $T_{reg}$ cells that attenuated colitis in mice [9]. *Faecalibacterium prausnitzii* belongs to *Clostridium* cluster IV, and its abundance is significantly decreased in Crohn's disease patients as compared with healthy individuals [91]. In addition, oral administration of either live *Faecalibacterium prausnitzii* or its supernatant markedly reduced the severity of TNBS-induced colitis through the induction of IL-10 [17]. Thus, certain anti-inflammatory commensal microbes might harbor a specific structure to promote IL-10 production by T cells, regulating gut homeostasis and intestinal inflammation. Recent studies have reported that short-chain fatty acids bind to G-protein-coupled receptor 43 and prevent experimental colitis [92, 93]. Butyrate is produced by commensal microbiota during starch fermentation and promotes extrathymic $T_{reg}$ cell generation [94]. Histone deacetylase inhibitory activity of butyrate decreases proinflammatory cytokine expression within dendritic cells to promote $T_{reg}$ cell induction [94].

All of these findings have demonstrated how the commensal microbiota contributes to modulate colonic $T_{reg}$ and $T_h17$ cell differentiation and homeostasis, and how the immune system in turn affects the composition of the microbiota.

## *Clostridium butyricum* induces IL-10-producing macrophages

CBM588 is a Gram-positive rod-shaped obligate anaerobe and spore-forming bacterium [95]. Unlike pathogenic clostridia, such as *Clostridium difficile*, *Clostridium perfringens*, and *Clostridium botulinum*, CBM588 is non-pathogenic and has been used for treatment of human gastrointestinal disease in clinical settings [96, 97]. Several reports have revealed that CBM588 has a potentially

beneficial anti-inflammatory effect in animals [98], improving nonalcoholic fatty liver disease [99, 100] and preventing *Clostridium difficile* infection [101, 102]. Although CBM588 has a protective role in some diseases, the precise mechanism by which it exerts its protective anti-inflammatory effect remains unknown. We have reported the anti-inflammatory effect of CBM588 and its mechanism in more detail [13]. Using gnotobiotic techniques, we analyzed whether colonization by a single strain of *Clostridium butyricum* has an immunomodulatory role in intestinal inflammation (Fig. 1a). Monocolonization of CBM588 exhibited a protective effect in an acute DSS-induced colitis model, accompanied by an increase in IL-10 production in the LP mononuclear cells from inflamed intestine. These data suggest that IL-10 production in the intestine contributes to the regulation of colitis. The association between IL-10 and IBD has been demonstrated in several human studies and animal models. IL-10 has been identified as a immunosuppressive cytokine secreted by $T_h2$ cells that inhibits $T_h1$ cell function [103]. Many IL-10-expressing cell subsets have been found, such as $T_h1$ cells [103–105], $Foxp3^+$ $T_{reg}$ cells [106–108], $Foxp3^-$ $T_{reg}$ cells [109], B cells [110, 111], macrophages [112–114], dendritic cells [115–117], neutrophils [118], and eosinophils [119]. IL-10-deficient mice are well established as a genetic model for IBD. They developed colitis when they were kept under conventional conditions, but failed to develop colitis when they were raised under germ-free conditions [50, 120]. Furthermore, IL-10-deficient mice developed inflammation restricted to the colon, suggesting that the resident microbiota plays a role in the development of spontaneous colitis [50]. Consistent with this observation, antibiotic therapy can prevent the development of colitis in IL-10 knockout mice [121, 122]. In addition, germ-free IL-10 knockout mice developed colitis after they were colonized with *Enterococcus faecalis* [123]. A genome-wide association study in humans has shown that a single-nucleotide polymorphism in the *Il10* gene is closely associated with IBD [124]. As IL-10 is a key immunoregulatory cytokine, we questioned whether neutralization of IL-10 in DSS-treated, specific-pathogen-free mice with CBM588 could suppress development of colitis. CBM588 treatment cured DSS-induced colitis under specific-pathogen-free conditions; however, the protective effects of CBM588 treatment were completely abrogated by co-administration of anti-IL-10 monoclonal antibody.

$T_{reg}$ cells play important roles in the establishment and maintenance of immune homeostasis and suppress inflammatory immune responses and autoimmunity [125–127]. A recent study demonstrated that cluster IV and XIVa clostridia induce IL-10-producing $T_{reg}$ cells in the intestine and prevent experimental colitis [9, 10]. We have examined whether the anti-inflammatory effect of CBM588 was mediated through $T_{reg}$ cells. In inflamed colonic LP mononuclear cells, IL-10 expression was significantly enhanced in intestinal $CD11b^+$ macrophages by CBM588 administration, but not in $CD4^+$ $T_{reg}$ cells. To address whether the anti-inflammatory effect of CBM588 was conserved in $T_{reg}$ null mice, $CD4^+CD25^+$ $T_{reg}$-cell-depleted mice were given DSS with or without CBM588 treatment. CBM588 administration suppressed colitis even in $T_{reg}$-cell-depleted mice, suggesting that CBM588 suppresses intestinal inflammation by a $T_{reg}$-cell-independent anti-inflammatory mechanism. However, CBM588 administration did not fully prevent the development of colitis in the absence of $T_{reg}$ cells, indicating that $T_{reg}$ cells also have a partial role in the CBM588-mediated anti-inflammatory effect. Furthermore, CBM588 treatment significantly prevented DSS-induced colitis in RAG-2-deficient mice that lack functional T and B cells.

TLRs, which recognize specific molecular patterns of microbial components, play an important role in immune and nonimmune functions in the intestine [128, 129]. Macrophages induce IL-10 following stimulation of specific pattern recognition receptors [130–132], and TLR2 signaling is essential for the induction of IL-10 production by macrophages [133]. CBM588 failed to increase the production of IL-10 by intestinal macrophages in the steady state, whereas it directly stimulated intestinal macrophages through the TLR2/MyD88 signaling pathway and induced IL-10 production by macrophages in inflamed conditions (Fig. 1b). Platt et al. [134] identified two subsets of colonic macrophages in mice, whose proportions differed in healthy and inflamed intestine. Under steady-state conditions, most $F4/80^+$ macrophages lacked TLR2 expression in response to stimulation. However, during experimental colitis, monocyte-derived chemokine CC receptor (CCR) 2 dependent macrophages expressed TLR2 [134, 135]. Thus, this difference in responsiveness to microbial ligands between resident and inflammatory macrophages might explain the failed induction of macrophage-mediated IL-10 production by CBM588 under physiological conditions. We have indicated that IL-10 is the important factor in the anti-inflammatory effect of CBM588, and that CBM588 induces IL-10 production by intestinal macrophages rather than T cells. To confirm the direct effect of macrophage-produced IL-10 in preventing colitis following CBM588 treatment, CBM588 was administered to DSS-treated macrophage-specific IL-10 knockout mice. CBM588-fed macrophage-specific IL-10-deficient mice failed to improve DSS-induced colitis in terms of weight loss and histological score, suggesting IL-10-producing macrophages induced by CBM588 are essential for the prevention of colitis. These findings clarify the role of a probiotic strain of *Clostridium butyricum*, which regulates

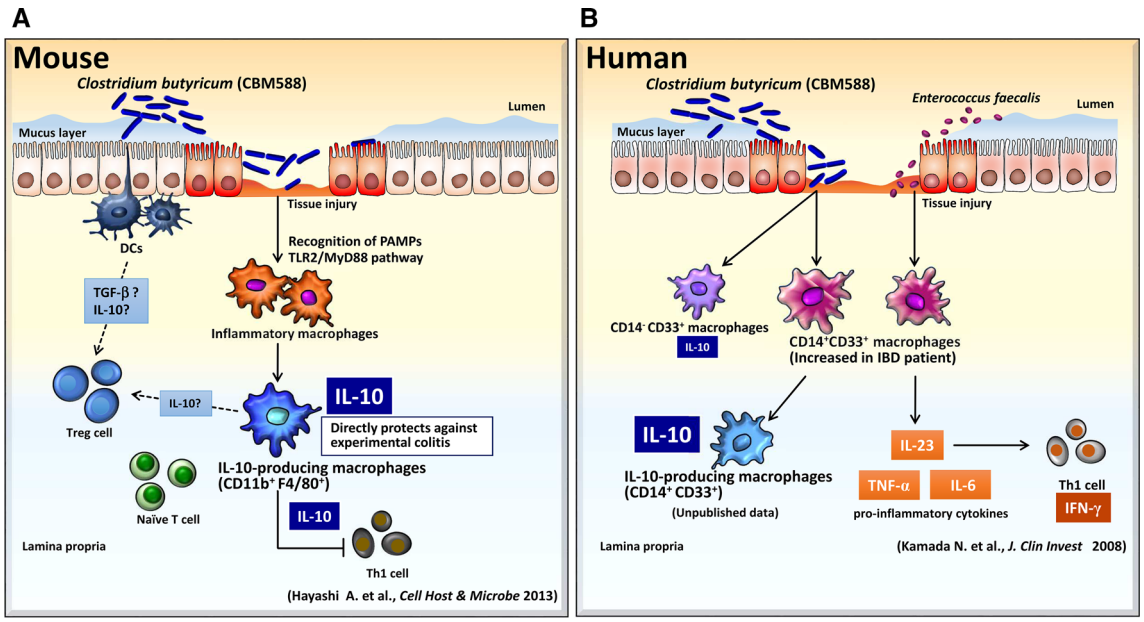


**Fig. 2** **a** Dextran sodoim sulfate treatment of mice allowed bacteria to invade the colon mucus layer and penetrate the epithelial barrier into the lamina propria. In the inflamed condition, *Clostridium butyricum* MIYAIRI 588 directly stimulated intestinal macrophages through the Toll-like receptor 2 (*TLR2*)/myeloid differentiation primary response gene 88 (*MyD88*) signaling pathway and induced interleukin (*IL*)-10 production by macrophages in mice. **b** $CD14^{+-}$ $CD33^{+}$ macrophages isolated from human lamina propria stimulated with *Enterococcus faecalis* induced proinflammatory cytokines, such as IL-12/IL-23p40, IL-23, tumor necrosis factor α (*TNF-α*), and IL-6. However, *Clostridium butyricum* MIYAIRI 588 stimulation induced higher levels of IL-10 in $CD14^{+}CD33^{+}$ lamina propria macrophages from human inflammatory bowel disease (*IBD*) tissue. *DCs* dendritic cells, *IFN* interferon, *PAMPs* pathogen-associated molecular patterns, *TGF* transforming growth factor, *Th1 cell* T helper 1 cell, *Treg cell* regulatory T cell

IL-10 production by intestinal macrophages in inflamed mucosa, and prevents the development of colitis. Control of inflammatory macrophages in IBD is crucial for the therapeutic strategy. Consistent with the data showing that intestinal macrophages in mice do not express TLRs in normal conditions in response to bacterial stimulation, human intestinal macrophages lack or express low levels of innate response receptors, including TLRs and lipopolysaccharide co-receptor CD14 [136–138]. Resident intestinal macrophages also do not produce proinflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, which mediate inflammatory reactions [136]. Our group previously reported that the levels of CD14-expressing macrophages were increased in the intestinal mucosa of patients with IBD [139]. The levels of $CD14^{+}CD33^{+}$ macrophages expressing chemokine receptors such as CCR1, CCR2, CCR4, CCR9, chemokine CXC receptor (CXCR) 4 and CX3CR1 were increased in IBD patients, especially in those with Crohn's disease [139]. To analyze the response to commensal bacterial stimulation, $CD14^{+}CD33^{+}$ and $CD14^{-}CD33^{+}$ cells isolated from LP were stimulated with *Enterococcus faecalis*, and inflammatory cytokine production were assessed. $CD14^{+}CD33^{+}$ cells but not $CD14^{-}CD33^{+}$ cells from Crohn's disease patients induced IL-12/IL-23p40, IL-23, TNF-α, and IL-6 production [139]. TNF-α and IL-23 are major proinflammatory cytokines secreted by macrophages; therefore, these inflammatory macrophages contribute to the pathogenesis of the disease. In addition, LP macrophages stimulated with bacteria induced IFN-γ production from $CD4^{+}$ T cell via IL-23 [139, 140]. CBM588 regulates IL-10 production by intestinal macrophages in inflamed mucosa in animal experiments; therefore, we tried to establish the involvement of the anti-inflammatory effect of CBM588 in human tissue. $CD14^{+}$ monocytes from peripheral blood mononuclear cells or $CD14^{+}$ LP macrophages were stimulated with or without heat-killed CBM588 or *Enterococcus faecalis*, and production of IL-10 in culture supernatants was measured. CBM588 stimulation induced IL-10 production in $CD14^{+}$ monocytes of human peripheral blood mononuclear cells as compared with *Enterococcus faecalis* stimulation, but decreased TNF-α production (unpublished data). Moreover, CBM588 stimulation also induced higher levels of IL-10 in $CD14^{+}CD33^{+}$ LP macrophages from human IBD tissue as compared with *Enterococcus faecalis* (unpublished data). Although IL-10 therapy for IBD has not been successful, owing to ineffective delivery to the inflammatory site in the LP, bacterial therapy that induces high amounts of IL-10 directly in the intestinal mucosa

may be a new therapeutic approach for IBD. Therefore, CBM588 may have potential as an immunomodulatory probiotic for both maintenance and induction of remission in IBD, by targeting intestinal macrophages (Fig. 2).

## Conclusion

The gut immune system interacts with the gut microbiota and maintains intestinal homeostasis. Accumulating evidence supports the idea that imbalance in this complicated network between the host and the microbiota is involved in the pathogenesis of gut inflammatory diseases, including IBD. Recently, various studies have explored adjustment of dysbiosis, using probiotics or fecal microbiota transplantation [2, 141–143]. However, it is unclear how these treatments act on the host immune system and commensal bacteria. CBM588 has been long used in clinical settings in Japan. Our recent findings using animal models suggest that CBM588 may induce IL-10-producing immunoregulatory macrophages and have potential as a safer therapeutic option for gut inflammatory diseases. Further clinical evidence of targeting the gut microbiota or combination with conventional therapy will be gathered from clinical and basic research into the microbiota and disease.

**Acknowledgments** We thank Shinta Mizuno, Hiroki Kiyohara, Mari Arai, Mina Kitazume, Kozue Takeshita, Keiichiro Saigusa, Makoto Naganuma, Katsuyoshi Matsuoka, Toshiro Sato, and Tadakazu Hisamatsu (Keio University) for technical assistance and for valuable discussion. This study was supported in part by Grants-in-Aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Japanese Ministry of Health, Labour and Welfare, and the Keio University Medical Fund.

**Conflict of interest** Atsushi Hayashi is an employee of Miyarisan Pharmaceutical. The other authors declare that they have no conflict of interest.

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