Inflammation and Regeneration Vol.35 No.3 May 2015

Special Issue: Interaction between gut microbiota and host immune cells

Mini Review

Gut microbial short-chain fatty acids in host defense and immune regulation

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Our guts are inhabited by an enormous number of commensal bacteria, commonly referred to as the gut microbiota. Complex host-gut microbiota interactions are involved in the formation of the unique "gut ecosystem". This ecosystem is thought to play a variety of roles in host physiology and pathology, including the modification and shaping of the host immune system. We have proposed an integrated omics approach for a comprehensive understanding of the gut ecosystem, where different levels of cyclopedic analyses, such as (meta)transcriptome and metabolome, are combined with the metagenome. Using this approach, we have discovered the mechanism by which the gut microbiota-derived short-chain fatty acid acetate protects mice from enterohemorrhagic *Escherichia coli* O157-infectious death. We have also shown that butyrate produced by the gut microbiota promotes differentiation of peripherally derived regulatory T cells in colonic lamina propria through epigenetic modification. In this minireview, I will discuss recent advances in our understanding of the role of gut microbial short-chain fatty acids in host defense and immune regulation, focusing mainly on results from our laboratory.

Rec.2/9/2015, ACC.2/23/2015, pp114-121

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Key words gut microbiota, host defense, immune responses, integrated omics, regulatory T cells, short-chain fatty acids

Introduction

Enormous numbers of commensal bacteria, collectively referred to as the gut microbiota, reside inside the animal digestive tract. In human colon, the gut microbiota consists of ～ 500-1,000 bacterial species, and the total number of bacterial cells is estimated to easily exceed 100 trillion[1], which outnumbers the 40-60 trillion somatic cells in the human body[2]. When viewed in another way, the gut microbiota has about 600 thousand genes[3], far more than the 20 plus thousand genes encoded by the human

genome.

As such, the gut microbiota is in some ways like an organ, possessing its own metabolic system, often described as comparable to that of the liver, and it forms a unique and complex gut ecosystem by interacting with the host. The gut ecosystem is thought to play a variety of roles in host physiology and pathology, including the modification and shaping of the host immune system.

Until recently, there has been no suitable analytical means to understand this complex gut ecosystem. Explosive innovations in gene sequencing technology, with the development of next generation sequencers in this century, however, have enabled the establishment of the new field of "metagenome" analysis, where entire genomic DNAs of organisms in a given environment can be collectively shotgun sequenced. Metagenome analysis of the gut microbiota has been revealing its composition in humans and animals, as well as the individual genes possessed by this vast collection of microorganisms[4]. Nevertheless, metagenome analysis can provide only a catalogue of the kinds of microbes (microbiota composition) and their genes, but functional data have been very difficult to come by.

To overcome the problem, we have proposed an integrated omics approach for comprehensive understanding of the gut ecosystem, where different levels of cyclopedic analyses such as (meta)transcriptome and metabolome are combined with the metagenome (Fig. 1), and we have provided evidence that this approach is suitable for understanding of functional aspects of the gut ecosystem[5-7]. In this minireview, I will discuss recent advances in our understanding of the role of gut microbial short-chain fatty acids (SCFAs) in host defense and immune regulation, focusing mainly on results from our laboratory.

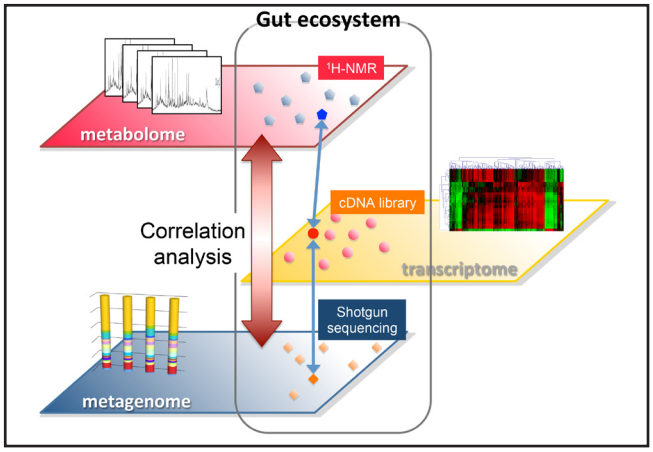

Fig. 1 Overview of the Integrated omics approach

Adapted from the webpage of the Laboratory for Intestinal Ecosystem, RIKEN Center for Integrative Medical Sciences (http://leib.rcai.riken.jp/riken/index.html).

Acetate can protect from death due to enterohemorrhagic Escherichia coli infection

Enterohemorrhagic *E. coli* O157:H7 is one of the most common causes of food poisoning in humans[8]. O157 possess virulence factors such as Shiga toxin and a type III secretion system and, upon oral ingestion by eating contaminated foods, the bacteria make attaching and effacing lesions on the colonic epithelium thereby causing epithelial cell death and detachment. This results in debilitating watery and/or bloody diarrhea; occasionally, Shiga toxin enters the blood circulation, leading to life-threatening systemic complications including hemolytic uremic syndrome.

Mice with a normal gut microbiota are usually unaffected by oral administration of O157, since the bacteria cannot cause attaching and effacing lesions of the intestinal epithelium in mice and are outcompeted for niche space by the commensal microbiota. However, in germ-free (GF) mice without competing microbiota, O157 proliferate well and the mice eventually die from the circulating Shiga toxin[9].

Prior association of GF mice with certain strains of *Bifidobacterium* protects them from subsequent O157-infectious death[10]. There are no differences between the mice colonized with "protective" and "non-protective" *Bifidobacterium* strains in terms of the number of O157 bacteria, the concentration of fecal Shiga toxin, the amount of mucin, or the pH of their feces. However, the serum concentration of Shiga toxin was significantly elevated in mice harboring the non-protective strain, reaching a lethal level, while it remained very low in mice with the protective strain[5].

Histological examination revealed that in the non-protective strain-associated mice apoptosis of the epithelium was modestly increased only in the most distal part of colon, accompanied by a weak inflammation, as early as one day after O157 oral infection. By contrast, no difference was observed between infected and non-infected protective strain-associated mice in the apoptotic cell number, i.e. there were only a small number of apoptotic cells on the mucosal surface resulting from physiological epithelial turnover[5].

We hypothesized that a difference in metabolite production between the protective and non-protective strains could result in the differential response of colonic epithelium to O157 infection. Indeed, metabolome analysis of the feces revealed that the amount of sugars was significantly lower, whereas that of acetate was significantly higher in the protective strain-associated mice[5]. *Bifidobacterium* is known to metabolize sugars to produce SCFAs such as lactate and acetate; however, among the SCFAs detected by the metabolome analysis, there was no significant difference in the concentration of SCFAs other than acetate in the feces of protective and non-protective strains.

To test whether acetate is responsible for protection of mice from O157-infectious death, at first acetate was fed in the drinking water of non-protective strain-associated mice. However, even levels as high as 200 mM of acetate (equivalent to about a 1/4 dilution of household vinegar) in the drinking water failed to raise the fecal concentration of acetate, likely because acetate is mostly absorbed by the small intestine. A higher concentration of acetate could not be used because the mice refused to drink the supplemented water and started dying from dehydration. Instead, therefore, acetylated high-amylose maize starch[12] (HAMSA) was fed to the mice, which raised the concentration of acetate in the colon as well as feces by sustained release of the covalently-bonded acetyl groups. The HAMSA-containing diet protected mice associated with the non-protective strain from O157-infectious death, strongly suggesting that acetate is, at least partly, involved in the protection mechanism[5].

Further functional and transcriptomic analyses *in vivo* and in an intestinal epithelial cell line have revealed that acetate can suppress apoptosis of epithelial cells upon exposure to O157, and that this is at least partly due to modulation of the gene expression profile of colonic epithelium, including upregulation of molecules in MAPK and NF-κB pathways (ref 5, and our unpublished observation).

Comparative whole genome analysis was performed to further address the difference between protective and non-protective bifidobacterial strains[5]. Two hypothetical gene loci, both of which encode putative ATP-binding cassette-type transporters for pentose/fructose, were only present in the genomes of the three protective strains analyzed, whereas both were completely or partially missing from the genomes of the two non-protective strains. These genes were confirmed to encode functional fructose transporters, in that the protective strains, but not non-protective strains,

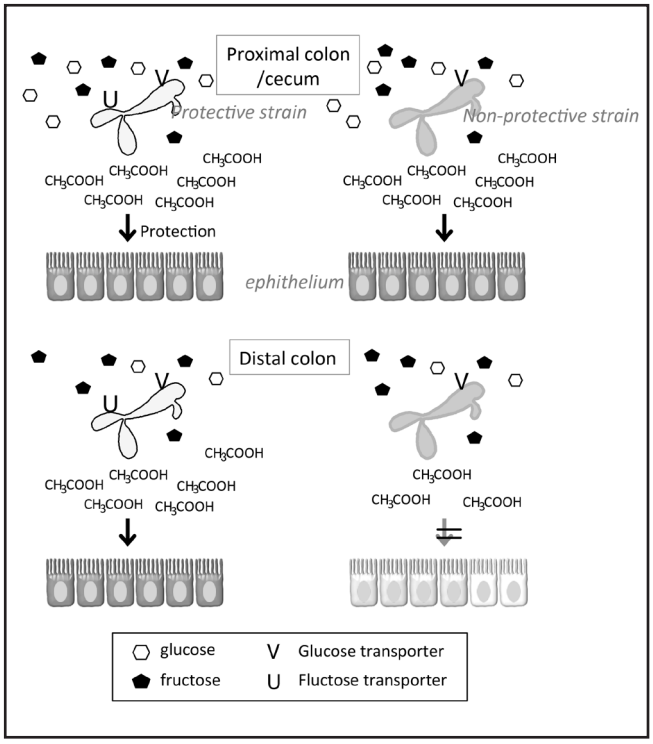

Fig. 2 Schematic view of the differences between *Bifidobacterium* strains that are protective or non-protective from *E. coli* O157-infectous death in mice

Refer to the text for a detailed explanation. Adapted from the webpage of the Laboratory for Intestinal Ecosystem, RIKEN Center for Integrative Medical Sciences (http://leib.rcai.riken.jp/riken/index.html).

efficiently utilized fructose to produce acetate *in vitro* when these *Bifidobacterium* strains were cultured with fructose as the sole carbon source[5]. Of note, both protective and non-protective strains produce equivalent amounts of acetate *in vitro* when glucose instead of fructose is used as the carbon source. This is consistent with the fact that glucose is abundant in the cecum but barely detectable in the feces of mice associated with both protective and non-protective strains, whereas fructose is readily detected even in the feces of these mice. The differential distribution of these carbohydrates is consistent with our finding that the slight gut inflammation in non-protective strain-associated mice upon O157 infection is only observed in the distal colon but not in the cecum. This is likely due to the production of sufficient acetate from glucose to prevent epithelial cell apoptosis in the cecum by both protective and non-protective strains but, by contrast, only the protective strains, which possess fructose transporters, can produce enough protective acetate from fructose in the distal colon (Fig. 2).

Collectively, certain strains of *Bifidobacterium* that possess fructose transporters can protect mice from O157-infectious death by metabolizing fructose to produce acetate. This study also provides evidence that the integrated omics approach can be a powerful tool to understand the very complex gut ecosystem.

Microbial butyrate epigenetically enhances colonic regulatory T-cell induction

Regulatory T cells (Tregs) are a subset of $CD4^+$ T lymphocytes expressing the transcription factor Foxp3, which is required for their differentiation. Tregs play a unique role in the maintenance of immune tolerance and homeostasis by modulating many aspects of the immune response via their immune suppressive function[13]. Two distinct pathways are known for Treg differentiation[14]. In the thymus, T-cell receptor (TCR) specificity of the developing thymocytes to self-antigen appears to be a primary determinant for Treg lineage commitment. In addition, *de novo* generation of Tregs from $Foxp3^-$ naïve T cells in peripheral tissues also occurs. These two Treg subsets are now called thymus-derived Treg (tTreg) and peripherally derived Treg (pTreg), respectively[15].

The pTregs are especially abundant in the intestine and exogenous antigens are thought to be important for their differentiation, since the number of colonic pTregs is reduced in GF compared to conventional mice[16]. It has been shown by Kenya Honda's group that a mixture of bacteria belonging to class *Clostridia* both from murine[16] and human[17] feces can augment colonic pTregs in GF mice.

The cecum of GF mice is distended because of the retention of the water-retentive, viscous indigestible dietary fibers. Association of *Clostridia* normalizes the cecum size, indicating that these microbes can digest such dietary fibers. This raises the possibility that *Clostridia* metabolites of otherwise indigestible dietary fibers could be involved in colonic Treg induction. Comparative metabolome analysis of the cecal content from *Clostridia*-associated mice fed a high fiber or low fiber diet detected SCFAs (acetate, propionate, and butyrate) and certain amino acids (l-leucine, l-isoleucine, and γ-aminobutyric acid) were especially enriched in the high fiber diet group[7]. Among these metabolites, only butyrate strongly increased the number of $Foxp3^+$ Tregs[7] when added to *in vitro* Treg induction cultures[18]. Furthermore, colonic pTregs were increased in mice fed butyrylated high-amylose maize starch[12] (HAMSB),

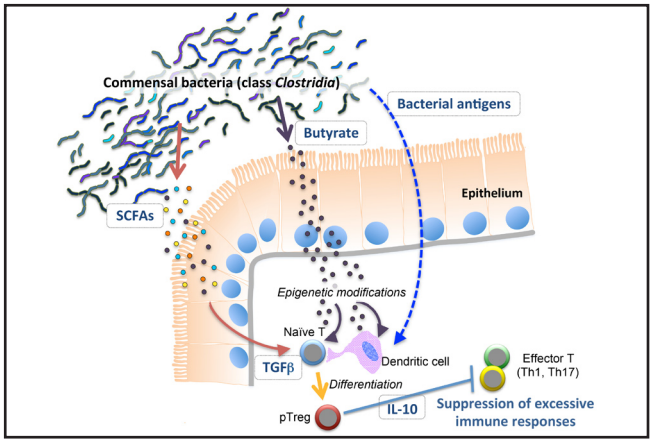

Fig. 3 Gut microbe-derived butyrate for induction of colonic pTregs to maintain gut immune homeostasis

Refer to the text for a detailed explanation. Adapted from the webpage of the Laboratory for Intestinal Ecosystem, RIKEN Center for Integrative Medical Sciences (http://leib.rcai.riken.jp/riken/index.html).

suggesting that butyrate can enhance the differentiation of colonic pTregs (Fig. 3).

How then does butyrate enhance pTreg differentiation? Butyrate is a well-known potent inhibitor of histone deacetylases (HDACs)[19, 20], which negatively regulate transcription through a specific epigenetic modification, deacetylation of lysine residues in the C-terminal histone tail[20]. Histones are the main protein component of chromatin, around which the DNA winds. Histone acetylation is regulated by the enzymatic balance of histone acetyltransferases and HDACs. DNA can wind tightly onto deacetylated histones and this reduces the accessibility of this region of the chromosome to transcriptional regulators; by contrast, histone acetylation loosens the winding of the DNA, which promotes accessibility to these factors. Genome wide analysis of histone acetylation in Treg induction cultures in the presence or absence of butyrate was performed by chromatin immunoprecipitation-sequencing using an acetylated histone H3 monoclonal antibody. These studies revealed that the promoter regions of a subset of transcription factor genes, notably including *Foxp3*, were significantly more acetylated and that, concomitantly, the transcription of these genes was specifically upregulated in the presence of butyrate[7]. That the histone acetylation was only observed in a subset of chromatin regions is consistent with the notion that HDAC inhibition generally affects the expression level of as few as ～ 2% of total genes[20]. The limited effect of butyrate on histone acetylation observed

here could be at least partly explained by the fact that butyrate can inhibit only a subset of HDACs (unpublished observation). Not only the promoter region, but histone acetylation was also upregulated for the consensus noncoding sequences 1-3 in the *Foxp3* gene locus, important for transcriptional activation and maintenance of expression of this gene[7]. Thus, butyrate can enhance Treg induction by epigenetic regulation of Foxp3 transcription (Fig. 3). HDACs catalyze the removal of acetyl functional groups from the lysine residues of both histone and non-histone proteins[21]. Foxp3 itself is known to be acetylated and, as a result, stabilized to exert enhanced function[22]. Indeed, a higher Foxp3 protein level per cell was observed in butyrate-treated compared to non-treated $CD4^+$ T cells[23]. In addition to its direct effect on $CD4^+$ T cells, indirect effects of butyrate have also been reported; e.g., butyrate-pretreatment confers dendritic cells with an enhanced Treg differentiation ability, probably also via HDAC inhibition[23] (Fig. 3).

G-protein-coupled receptors (GPRs) such as GPR41[24], GPR43[25,26] and GPR109A[27] have also been reported to sense SCFAs and to exert immunomodulatory functions. However, studies using mice deficient for these GPRs exclude their possible involvement in butyrate-mediated colonic pTreg differentiation[23, 28].

Colonic pTregs are induced when GF mice mono-associated with *Bacteroides thetaeotaomicron*, a dominant species of the gut microbiota that do not themselves produce butyrate, are fed with HAMSB. However, there is no colonic pTreg induction when GF mice are fed with HAMSB, even though this diet increases the fecal butyrate concentration. Therefore, some sort of bacterial component(s) is required for colonic pTreg induction. However, microbial pattern recognizing Toll-like receptor (TLR) signaling is dispensable for colonic pTreg induction, since HAMSB can still induce colonic pTregs in MyD88/TICAM1 (Trif)-double deficient mice in which TLRs cannot transduce activation signals[7]. Taken together, it seems likely that bacterial antigens should be presented to antigen-specific naïve T cells for pTreg induction in the colonic lamina propria.

Butyrate-induced colonic pTregs are functional in that they produce the anti-inflammatory cytokine IL-10[7]. Furthermore, butyrate-induced colonic pTregs ameliorate colitis in mice. Colitis can be induced when naïve $CD4^+$ T cells are adoptively transferred into immunodeficient mice because of the lack of Tregs[29, 30]. HAMSB, but not the control HAMS, increases colonic pTregs and ameliorates colonic inflammation in these colitis model mice[7]. Treg depletion negates the HAMSB-mediated amelioration of colitis, indicating that butyrate-induced colonic pTregs can suppress excessive immune responses by $CD4^+$ effector T cells, most likely reactive with gut commensal microbiota, to maintain immune homeostasis in the gut (Fig. 3). The effect of butyrate in the containment of colitis in mice is consistent with observations in human inflammatory bowel disease (IBD). For example, the concentration of butyrate[31] and butyrate-producing bacteria[32, 33] are decreased in IBD. In addition, butyrate enema has been reported to have anti-inflammatory effects in patients with the IBD ulcerative colitis[34, 35].

Suppressive regulation of colonic macrophages by microbial butyrate

Macrophages are the most abundant immune cells in colonic lamina propria[36]. Macrophages in the colon are different from those in the other tissues in that they are more anti-inflammatory, with a lower sensitivity to TLR ligand stimulation[37]. This hyposensitivity to TLR signaling has been reported to be due to epigenetic regulation through HDAC inhibition by microbial butyrate[38]. Of interest, in this case enhanced lysine acetylation of histone H3 in the promoter region of genes encoding pro-inflammatory cytokines IL-6 and IL-12 recruits Mi-2β, a component of the Mi-2/NuRD repressor complex, which results in the decreased expression of these cytokines[38].

Immunoregulatory functions of SCFAs via GPR43

Gut microbiota-derived acetate has also been reported to exert a systemic anti-inflammatory effect[39]. Oral administration of dextran sulphate sodium (DSS) in mice induces a colitis resembling human ulcerative colitis[40], and this colitis is exacerbated in GF mice[39]. Acetate in the drinking water prevented this exacerbation and, concomitantly, levels of the pro-inflammatory cytokine TNFα and the neutrophil inflammation mediator myeloperoxidase decreased in the colonic tissue. GPR43-deficient mice suffered from exacerbated DSS colitis similar to GF mice, but in this case oral acetate did not ameliorate the disease, indicating that the effect of acetate is mediated by GPR43. Of note, inflammations outside of the intestine, such as in experimental arthritis and asthma, becomes more severe in GF, as well as GPR43-deficient, mice, and these inflammations in GF mice can also be ameliorated with acetate in drinking water. GPR43 has previously

been reported to act as a chemoattractant receptor for neutrophils[41]. Maslowski et al. observed that acetate was both a chemoattractant as well a potent inducer of neutrophils apoptosis[39]. Acetate is rapidly absorbed from the intestinal lumen into the bloodstream[42] and therefore could act systemically on neutrophils to retain them in the vascular system and to induce apoptosis, which would suppress peripheral inflammatory responses.

It has also been reported that supplementation of drinking water with acetate and propionate, but not butyrate, recruits already existing systemic tTregs to the intestine through GPR43 expressed on tTregs by upregulating the expression of GPR15[43], an orphan receptor that plays a role in gut homing[44]. This observation is consistent with the notion that orally administered SCFAs are mostly absorbed in the small intestine before arriving in the colon, thus exerting a systemic effect rather than acting on the colonic tissue[42], and that butyrate is a weaker agonist than acetate and propionate for GPR43[45].

Conclusion

As highlighted in this brief review, SCFAs exert a range of functions in immune and inflammatory responses by acting on diverse cell populations using several different mechanisms. Our knowledge on the functions of SCFAs is still quite limited, but comprehensive understanding of the roles of SCFAs in our physiology should allow us to utilize a combination of microbes and SCFAs for preventive and therapeutic use in the future.

Acknowledgement and Source of funding

I would like to thank Dr. Peter D. Burrows for critical reading and English editing of the manuscript. The original work described in the present review article was done with many collaborators, and supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology-Japan, and the Japan Science and Technology Agency.

Conflict of interests

None

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