2020 Doctoral Thesis

Food factor and nutrients induce α-defensin secretion from Paneth cells and influence intestinal environment

（食品成分・栄養素は Paneth 細胞のαディフェンシン分泌を
誘導して腸内環境制御に関わる）

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Table of contents

Chapter 1. General Introduction

1. Background and Aim

The intestine plays a role in digestion and absorption of ingested foods, and at the same time, functions as immune organ that eliminates pathogens ingested with foods and responds to various antigens from foods or intestinal microbiota. One layers of the intestinal epithelial cells, which form the structure of crypts and villi, are constantly exposed to the intestinal microbiota stimuli, so that the intestinal epithelial cells separate "inside", the intestinal tissue, from "outside", the intestinal lumen. Furthermore, the intestine plays a role in signal transduction with recognition of ingested food factors and nutrients. Intestinal epithelial cells are composed of absorptive epithelial cells, goblet cells, enteroendocrine cells, and Paneth cells that all differentiated from crypt base columnar stem cells.

Paneth cells secrete antibacterial peptide, α-defensin, in response to bacterial stimuli and contribute to innate enteric immunity by eliciting potent microbicidal activities [1]. α-Defensin, a typical antibacterial peptide in mammals, is cationic and 3 to 4 kD having 3 disulfide bonds, and Cryptdin (Crp) 1 to 6 in mice and HD5 and HD6 in humans are known. It has been reported that Paneth cell α-defensin not only kills pathogens but also participates in symbiosis with microbiome to regulate the intestinal microbiota composition by its selective bactericidal activities, potent killing against pathogens while no or less killing against commensal bacteria [2]. In addition, α-defensin affects the composition of the intestinal microbiota in the small intestine [3], and secreted α-defensin exists in the lumen of the large intestine with bactericidal activities, contributing to the large intestinal microbiota composition [4,5]. Furthermore, Paneth cell depletion or abnormalities cause imbalance of the intestinal microbiota composition, dysbiosis, by decreased or degraded α-defensin secretion, and is involved in the onset and progression of certain diseases such as obesity, Crohn's disease, and graft-versus-host disease (GVHD) [6].

The human intestine has been known to harbor about one hundred-trillion bacteria to create the intestinal microbiota and affect gut immunity together with their metabolites [7]. Keeping normal intestinal microbiota leads to maintaining intestinal homeostasis, whereas dysbiosis leads to the onset or progress of various diseases such as inflammatory bowel disease, obesity, diabetes, and allergies. Aging, lifestyle, drugs, stress and diet etc., have been known as factors that affect the intestinal microbiota [8]. Among them, diet is an important factor that serves as a nutrient source for the intestinal microbiota and affects their composition. In addition, the intestinal bacterial metabolites are also known to affect host immune system. It is also known that food factors, nutrients, and nutritional status such as intake of high-fat diet and malnutrition affect the balance

of intestinal microbiota [9,10]. Thus, it is suggested that the intestinal environment maintains its homeostasis by the interaction and balance of diet, intestinal microbiota, and host immunity.

In addition, Paneth cells are known to reside adjacent to intestinal stem cells (ISCs) in the base of crypts in the small intestine to create stem cell niche and control regeneration and differentiation of the intestinal epithelial cells [11]. It has been reported that feeding with calorie-restricted diet promotes proliferation of ISCs and regeneration of the intestinal epithelia. [12] It has also been reported that the number of Paneth cells increases by calorie restriction, and Paneth cells monitor the caloric intake status and control the proliferation of ISCs by reducing mechanical target of rapamycin (mTORC1) activities in response to energy restriction to increase self-renewal of ISCs. α-Defensin secreted from Paneth cells protects ISCs and regulates the intestinal microbiota to maintain homeostasis of the intestinal environment, suggesting that Paneth cell α-defensin greatly contributes to health and disease prevention [13]. However, the relationship between Paneth cells and food factors or nutrients, which are intestinal environmental factors, has not been known yet. This study aims to clarify that Paneth cells recognize not only intestinal bacteria but also food factor and nutrients and secrete α-defensin to control intestinal environment via the innate enteric immunity, providing a novel function of foods.

Chapter 2. Food factor and nutrients induce α-defensin secretion from Paneth cells

2.1 Introduction

As foods that regulate the intestinal environment, probiotics such as *Lactobacillus* and *Bifidobacterium* and prebiotics including indigestible food factors such as oligosaccharides and dietary fibers have been known. Probiotics is reported to balance microorganisms on the surface of the intestinal mucosa and improve immune function [14]. Prebiotics are substances that promote growth of the intestinal microbiota in the small intestine and the large intestine. [15] It has been reported that both probiotics and prebiotics improve the balance of composition and function of the intestinal microbiota by increasing useful bacteria and decreasing harmful bacteria to the host [16]. Furthermore, it has been known that *Lactobacillus* influences directly to the host immunity, improving intestinal barrier function by increasing expression of tight junction (TJ) proteins and mucin production [17].

Recently, it has become clear that nutrients such as vitamins and minerals directly affect intestinal immunity. Retinoic acid, a metabolite of vitamin A, induces regulatory T cells (Treg) and suppresses Th17 cell differentiation [18]. It has also been reported that niacin (vitamin B3) induces Treg differentiation via G-protein-coupled receptor (GPCR) GPR109A and suppresses proinflammatory activities of macrophages and dendritic cells in the colon [19]. Furthermore, it has been reported that zinc acts as a ligand for zinc-sensing receptor GPR39 [20], and is involved in the expression of TJ proteins and regulates the intestinal barrier function [21]. On the other hand, it has also been reported that metabolites of the intestinal microbiota affect host immunity. Tryptophan, an amino acid, is metabolized by the intestinal bacteria to indole-3-aldehyde (IAld), which activates intestinal aryl-hydrocarvon receptor (AhR) and promotes interleukin 22 (IL-22) secretion [22]. Furthermore, short-chain fatty acids (SCFAs) produced by intestinal bacteria using diet-derived indigestible polysaccharides as substrates have been reported that not only be used as energy for proliferation of intestinal epithelial cells, mucosal secretion, and absorption of water and minerals[23], but also affect intestinal immunity. Among SCFAs, butyric acid contributes to suppression of inflammation by epigenetically inducing Treg via Forkhead bx P3 (Foxp3) and suppressing the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [24]. In addition to the previously reported actions of prebiotics and probiotics, it has been known that food factors and metabolites of the intestinal microbiota derived from food factors directly influence on intestinal immunity.

Paneth cells secrete α-defensin in response to cholinergic stimuli, Gram-positive and Gram-negative bacteria, and bacterial antigens such as lipopolysaccharide (LPS) [1]. Regarding the relationship between Paneth cells and food factors or nutrients, it has been reported that excessive

intake of high-carbohydrate and high-fat diets reduces the number of Paneth cells in rat duodenum and jejunum [25]. On the other hand, α-defensin secretion into the lumen of mouse gastrointestinal tract is higher in the ileum, where the number of intestinal microbiota increases dramatically compared to the duodenum and jejunum, and Paneth cells in the duodenum and jejunum, where digestion and absorption occur, also secrete α-defensin [13]. It has also been reported that mTORC1 activities are decreased in response to energy restriction in Paneth cells and result in activation of ISC self-renewal [12], suggesting that Paneth cells respond to food and its digestive and absorptive processes. Therefore, I hypothesize that Paneth cells secrete α-defensin by recognizing not only bacteria but also food factors and nutrients in the innate enteric immunity to maintain intestinal environment, focusing on a novel Paneth cell function regarding the relationship between food factor recognition and intestinal immunity.

This study investigated α-defensin secretion from Paneth cells in response to SCFAs, which are both food factors and food-derived metabolites of the intestinal microbiota, and amino acids as nutrients. Because SCFAs serve not only as energy sources for intestinal epithelial cells but also ligands for GPCRs in signal transduction, and amino acids serve not only as nutrients from proteins but also signaling molecules, it is hypothesized that SCFAs and amino acids could trigger secretion of α-defensin from Paneth cells.

2.2 Materials and methods

2.2.1 Animals

Crlj: CD1 (ICR) male mice were purchased from Charles River Japan (Kanagawa, Japan). All animal experiments were approved by the Committee of Animal Experimentation at Hokkaido University.

2.2.2 Reagents

SCFAs, Propionic acid (PA) and butyric acid (BA), and amino acids, L-glycine (Gly), L-alanine (Ala), L-serine (Ser), L-threonine (Thr), L-cysteine (Cys), L-methionine (Met), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-phenylalanine (Phe), L-tryptophan (Trp), L-aspartic acid (Asp), L-glutamic acid (Glu), L-asparagine (Asn), L-glutamine (Glu), L-histidine (His), L-asparagine monohydrate (Arg), L-tyrosine (Tyr), and L-ascorbic acid were purchased from FUJIFILM Wako (Osaka, Japan). A SCFA, Acetic acid (AA), and an amino acid, L-lysine (Lys), and sodium butyrate were purchased from Nacalai Tesque (Kyoto, Japan), and an amino acid, proline (Pro) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade.

2.2.3 Crypt isolation

2.2.3.1. Crypt isolation for sandwich enzyme-linked immunosorbent assay (ELISA) and bactericidal assay

Crypts were isolated from the small intestine, as previously described [1]. The small intestinal lumen of adult mice was rinsed with ice-cold water, yielding the ileum segments. The segments were everted and shaken in cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 30 mM EDTA to detach the crypts. Villi and crypts detached for 7-min intervals were deposited by centrifugation at 700 g for 5 min at 4 °C and resuspended in phosphate-buffered saline (PBS). For experiments using >10,000 crypts, the numbers were estimated by using hemocytometer.

2.2.3.2. Crypt isolation for quantitative polymerase chain reaction (qPCR) and enteroid culture

For crypt isolation, mouse small intestine was flushed with cold $Ca2^{+}$- and Mg^{2+}-free PBS and cut open lengthwise in ~10 cm long pieces. The villi were scraped off using a scalpel blade and washed with cold PBS. The tissue fragments were incubated in 30 mM EDTA with HBSS for 10 min at 25 °C. The solution was removed, and the tissue was shaken vigorously for ~300 times in fresh HBSS. Intact tissue was discarded, then dissociated crypts were pelleted by centrifugation at 440 g for 4 min at 4 °C.

2.2.4 Stimulation and collection of Paneth cell secretions

The crypt fractions obtained in Section 2.2.3.1 were incubated at 37 °C for 30 min to stimulate secretion of α-defensin from Paneth cells by adjusting the final concentration to 100 μM SCFAs or 1 μM amino acids and PBS control. Supernatants were collected by centrifugation at 700 g for 5 min at 4 °C. Supernatants were adjusted to 30% acetic acid, and proteins were extracted using a 1000 Da dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) overnight at 4 °C. The solution after the dialysis was lyophilized and stored at −80 °C until use.

2.2.5 Sandwich ELISA

The materials obtained in Section 2.2.4 were dissolved in 200 μL of PBS, and Crp1, which is a major isoform of mouse α-defensin, was measured by sandwich ELISA as previously described [26]. Briefly, microtiter plate wells were coated overnight at 4 °C with 100 μL of the capture antibody (77-R5) at a concentration of 1 μg/mL in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6). The plate was then washed with PBS-T and blocked at 25 °C for 1 h with 200 μL of 25% Block Ace (DS Pharma Biomedical, Osaka, Japan). Next, 100 μL of Crp1 or samples were added to the wells and incubated at 25 °C for 2 h. After washing in PBS-T, 100 μL biotinylated detection antibody (77-R20, 0.5 μg/mL) was added at 25 °C for 1 h. Subsequently, the wells were incubated with 100 μL of streptavidin-horseradish peroxidase conjugate (GE Healthcare, Little Chalfont, UK) in a 1:5000 dilution at 25 °C for 1 h. Following the final washing, 100 μL of TMB chromogen substrate buffer was added and incubated at 25 °C for 30 min. The reaction was stopped by adding 100 μL of 0.6 N H_2SO_4, and absorbance values were determined at 450 nm using a microplate reader (Multiscan FC, Thermo Fisher Scientific, Waltham, MA, USA).

2.2.6 Bactericidal assay

The bactericidal assay was performed as previously described [1]. Briefly, secretions collected from crypts exposed to PBS, 100 μM butyric acid, and 1 μM leucine obtained in Section 2.2.4 were analyzed for bactericidal activities against 1×10^3 colony-forming units of *Salmonella typhimurium* PhoP- (*S. typhimurium*) by co-incubation at 37 °C for 1 h. Surviving colonies were quantified by evaluating their growth on semi-solid media at 37 °C overnight. Bacterial survival rates were determined from surviving colonies relative to the PBS control.

2.2.7 Quantification of Paneth cell granule secretion in enteroids

Enteroid culture was performed as previously described [27]. The pellets, as obtained in Section 2.2.3.2, were resuspended in HBSS supplemented with 10 μM Y-27632 (Sigma-Aldrich), the crypts were counted, and a fraction of >80% crypt purity was used. The fraction was centrifugated at 400 g for 4 min at 4 °C, and 150 crypts were resuspended in 30 μL of Matrigel (Corning, Inc., Corning, NY,

USA), followed by plating in 48-well plates (Thermo Fisher Scientific). After Matrigel polymerization, 250 μL of enteroid culture medium (Advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, 1× GlutaMAX, 1× N2, 1× B27 (all from Thermo Fisher Scientific), and 1 μM N-acetylcysteine containing growth factors 50 ng/mL EGF (PeproTech, Rocky Hill, NJ, USA), 50 ng/mL HA-R-Spondin1-Fc (produced using Cultrex® HA-R-Spondin1-Fc 293T Cells, Trevigen, Gaithersburg, MD, USA; #3710-001-01), and 100 ng/mL Noggin (PeproTech) were overlaid. The culture medium was changed every other day.

At day 5–7 of culture, enteroids were harvested by dissolving Matrigel with cold Advanced DMEM/F12 and transferred onto a Cell Imaging Dish (Eppendorf, Hamburg, Germany) at 150 enteroids/dish. The dish was kept on ice for 5 min to allow the enteroids to settle on the bottom of the dish. After Matrigel polymerization, enteroid culture media pre-warmed to 37 ℃ were added to the dish 30 min before microinjection.

Microinjection was performed while scanning the enteroid at 15 frames/s by confocal microscopy in a microscope cage incubator (37 ℃, 5% CO2, Tokken, Kashiwa, Japan). The needle (Femtotips, Eppendorf) was inserted into the enteroid by using Coarse and Fine Three-axis Oil Hydraulic Micromanipulator and One-axis Oil Hydraulic Micromanipulator (MN-4, MMO-202ND, MMO-220A, NARISHIGE), and reagents were introduced into the enteroid lumen using a Pneumatic Microinjector (IM-11-2, NARISHIGE) at a final concentration of 50 mM sodium butyrate, 1 μM L-leucine, and 1 μM L-threonine.

Differential interference contrast time-lapse imaging was performed using confocal microscope (A1, Nikon) equipped with a 0.95 NA objective lens (CFI Apo LWD 20X WI λS, Nikon) and a resonant scanner at 15 frames/s from the start of injection to 30 min after the introduction of reagents. To quantify Paneth cell granule secretion, the number of granules secreted in 30 min after the introduction of reagents was counted by observing the images displayed by the frame-by-frame playback with an image analysis software, NIS-Elements AR (Nikon). For sodium butyrate, L-leucine, and L-threonine, microinjection was performed on five enteroids, and the number of secreted granules in one experiment represents the total number of secreted granules in five microinjections. The experiment was repeated three times.

2.2.8 Statistical analysis

All statistical computations were performed using GraphPad Prism 6.07 software (GraphPad, Inc., San Diego, CA, USA). Data comparing two groups were analyzed by the two-tailed unpaired Student's t-test. Data comparing several treatments were analyzed by one-way analysis of variance, followed by

Tukey’s post hoc test for multiple comparisons and the Kruskal–Wallis test. Differences between groups were considered significant if p-values were <0.05.

2.3 Results

2.3.1 Paneth cells secrete α-defensin in response to butyric acid among SCFAs

First, whether SCFAs induce α-defensin secretion from Paneth cells was examined. This study tasted acetic acid, propionic acid, and butyric acid among SCFAs, which have been known as the intestinal environmental factors produced by the intestinal microbiota and ingested from food, by measuring the amount of Crp1 secretion using sandwich ELISA. Among SCFAs, butyric acid significantly induced Crp1 secretion in Paneth cells compared to in the PBS control (Figure 1C). In contrast, no significant secretion was observed with either acetic acid or propionic acid compared to PBS control (Figure 1A, 1B). 100 μM ascorbic acid, a nutrient tested for comparison with SCFAs, did not induce Crp1 secretion (Figure 1D). Butyric acid further showed a tendency to induce α-defensin secretion in a concentration-dependent manner when the dose of butyric acid was changed from 1 to 100 μM (Figure 2).

2.3.2 Paneth cells secrete α-defensin in response to leucine among twenty amino acids

Next, Crp1 secretion-inducing activities from Paneth cells were examined for all twenty amino acids. This study examined amino acids because they are all important nutrients from food including essential nine amino acids especially and the intestinal environmental factors. Of the twenty amino acids, leucine significantly induced Crp1 secretion compared to PBS control (Figure 3). In contrast, the remaining nineteen amino acids examined did not induce Crp1 secretion. When the secretion induction activities of leucine were tested in different doses from 100 nM to 10 μM, no significant difference was observed at any of the doses despite the significant secretion induced in Paneth cells by leucine (Figure 4).

2.3.3 Paneth cell secretions induced by butyric acid and leucine killed pathogenic bacteria

To test whether the secretions induced by butyric acid and leucine elicit bactericidal activities against pathogenic bacteria, bactericidal assays against *S. typhimurium* were conducted. The secretions collected from Paneth cells induced by both butyric acid and leucine elicited significant strong bactericidal activities against *S. typhimurium*, indicating that secreted α-defensins in response to butyric acid and leucine are functional (Figure 5A, 5B).

2.3.4 Butyric acid and leucine induce α-defensin secretion in enteroids

Finally, to confirm the results obtained with isolated small intestinal crypts, this study further tested the stimulatory effects of butyric acid and leucine on the secretion of Paneth cell granules containing α-defensins using enteroids, which are three-dimensional cultures of small intestinal epithelial cells. Secretions were quantitatively analyzed by administering butyric acid or leucine, as well as threonine, which did not induce α-defensin secretion in the isolated small intestinal crypts compared to PBS as a control, into the lumen of enteroids using the microinjection method. A significant Paneth cell granule secretion induced by butyric acid compared to the PBS control was observed (Figure 6A). Also, leucine significantly induced Paneth cell granule secretion in enteroids compared to both PBS and threonine treatment (Figure 6B). Comparison of granule secretion before and after butyric acid (Figure 7A), leucine (Figure 7B), threonine (Figure 7C) and PBS (Figure 7D) injection were shown decrease area by butyric acid and leucine. These results reproduced and confirmed the results obtained with the isolated crypts. Furthermore, granules were not secreted by Paneth cell stimulation from the basolateral side (Figure 8).

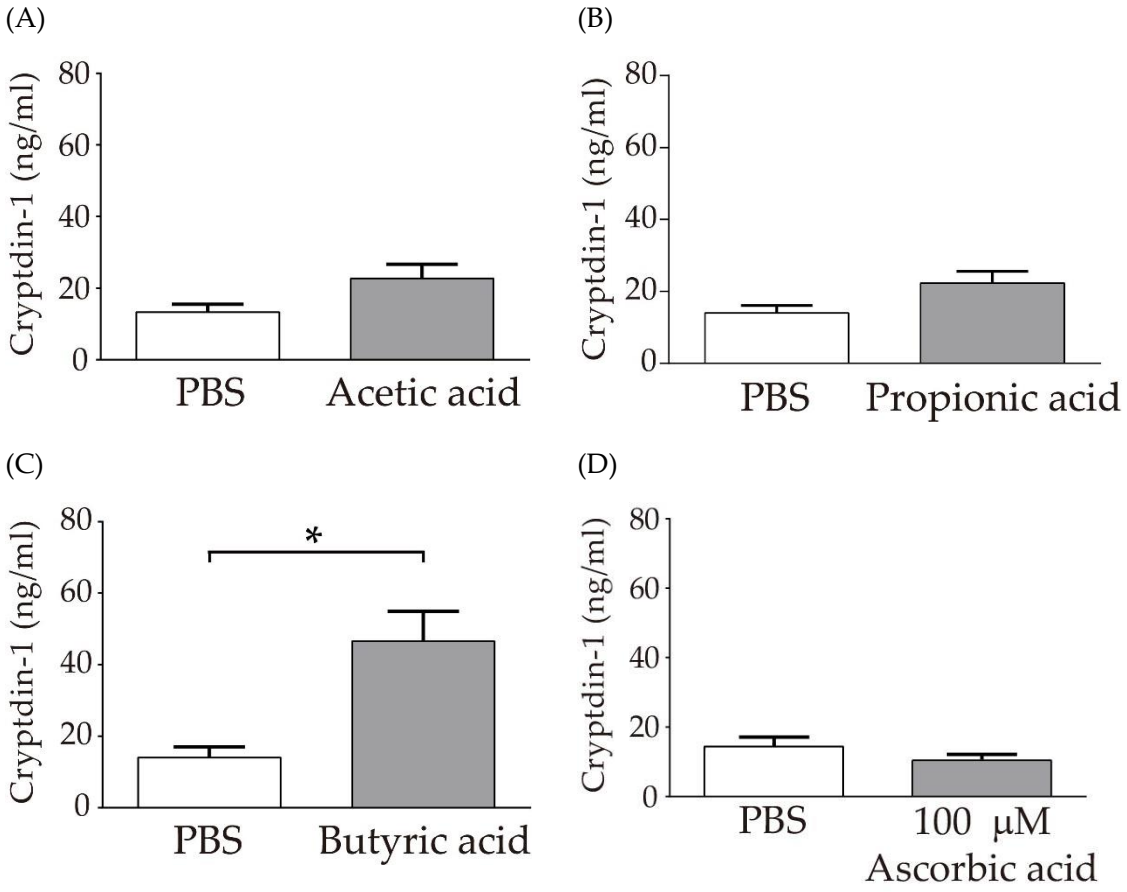


Figure 1. Induction of Crp1 secretion from Paneth cells by SCFAs using isolated crypts of the mouse small intestine: Induction of Crp1 secretion from Paneth cells in response to 100 μM (A) Acetic acid, (B) Propionic acid, and (C) Butyric acid. Data were expressed as the means ± SEM (n = 5). *$p < 0.05$ by Student's *t*-test. Analysis of Crp1 secretion induction from Paneth cells in response to 100 μM Ascorbic acid (D). Data were expressed as means ± SEM (triplicate).

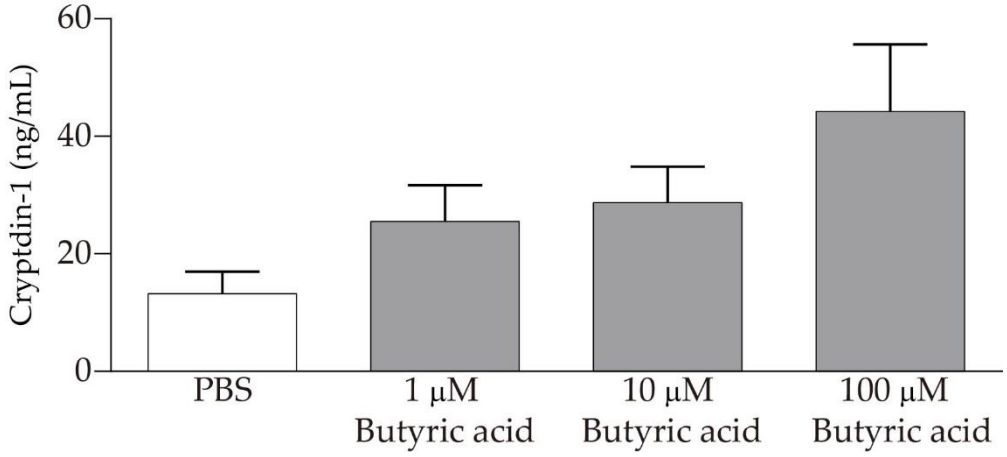


Figure 2. Analysis of Crp1 secretion from Paneth cells in response to 1–100μM Butyric acid. Data were expressed as the means ± SEM (n = 4).

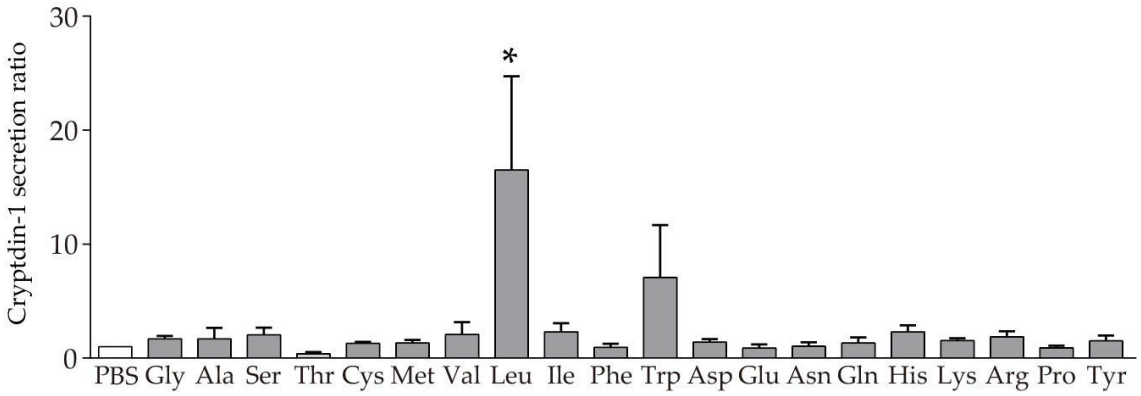


Figure 3. Induction of Crp1 secretion from Paneth cells by amino acids using isolated crypts of the mouse small intestine. The results were shown as a relative ratio of Crp1 concentration compared to PBS. Data are expressed as the means ± SEM (n = 3–6).
* $p < 0.05$ by Tukey's multiple comparisons test.

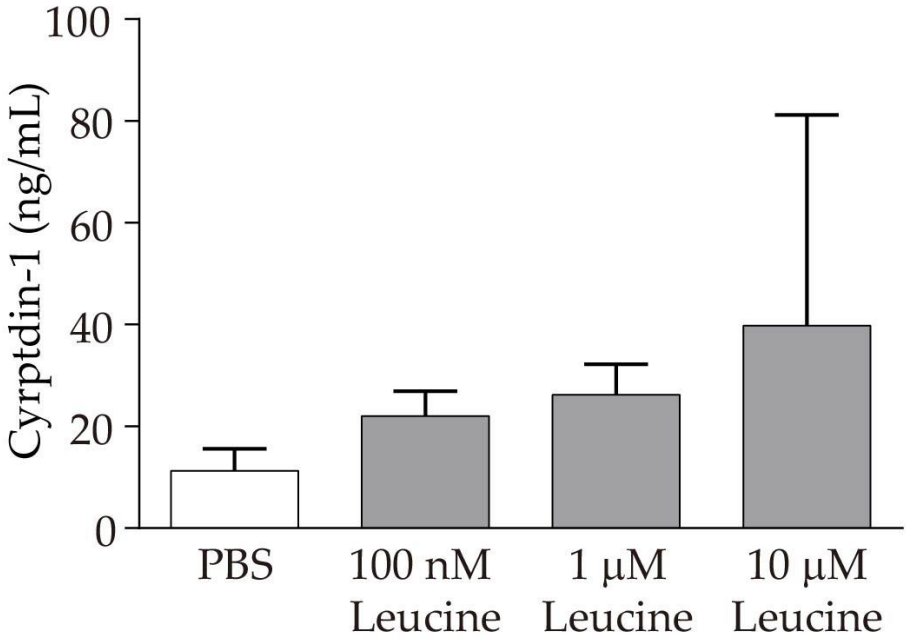
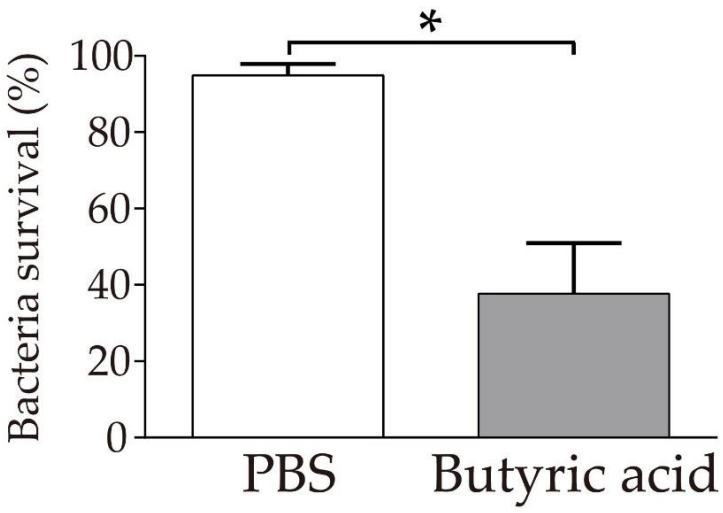


Figure 4. Examination of Crp1 secretion from Paneth cells in response to 100 nM–10 μM Leucine. Data were expressed as the means ± SEM (n = 5).

(A)



（B）

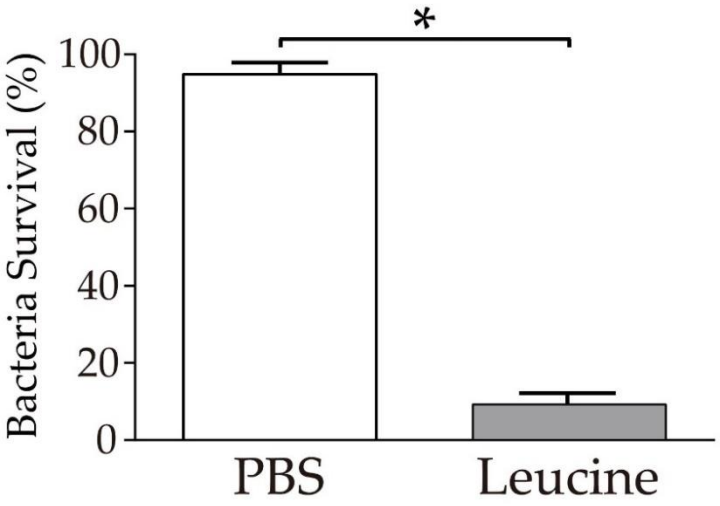


Figure 5. Bactericidal activities of Paneth cell secretions stimulated by Butyric acid and PBS control (A), Leucine and PBS (B) against *S. typhimurium*. Data were expressed as the means ± SEM (n = 3). * $p < 0.05$ by Student's t-test.

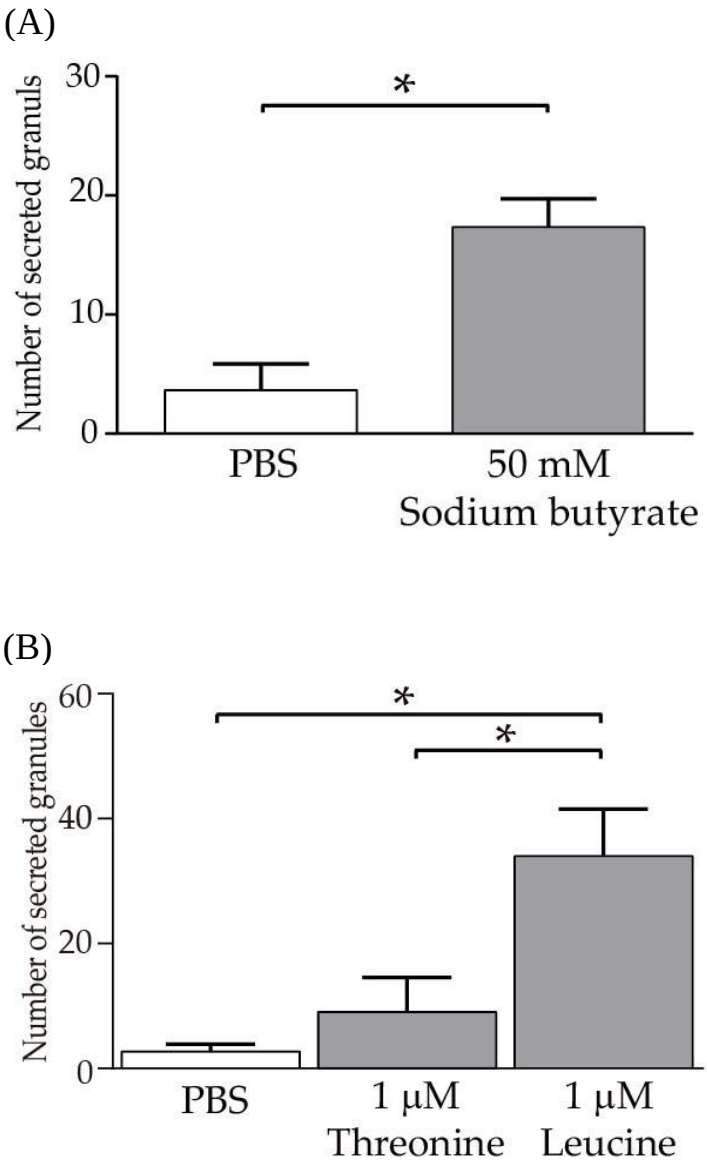


Figure 6. Quantification of secreted Paneth cell granules in response to butyric acid or leucine: (A) Enteroids at days 5 to 7 were injected with sodium butyrate (right bar, 17.3 ± 4.2) or PBS (left bar, 3.7 ± 3.8). Secreted granules were counted for 30 min after injection of reagents. Data are expressed as the total number of secreted granules from five Paneth cells ± SEM (n = 3). * $p < 0.05$ by Student's t-test. (B) Secreted granules after the introduction of L-threonine (middle bar, 9.0 ± 9.6) and L-leucine (right bar, 34.0 ± 13.1) were compared to PBS (left bar, 2.7 ± 2.1). Data are expressed as the total number of secreted granules from five Paneth cells ± SEM (n = 3). * $p < 0.05$ by Tukey's multiple comparisons test.

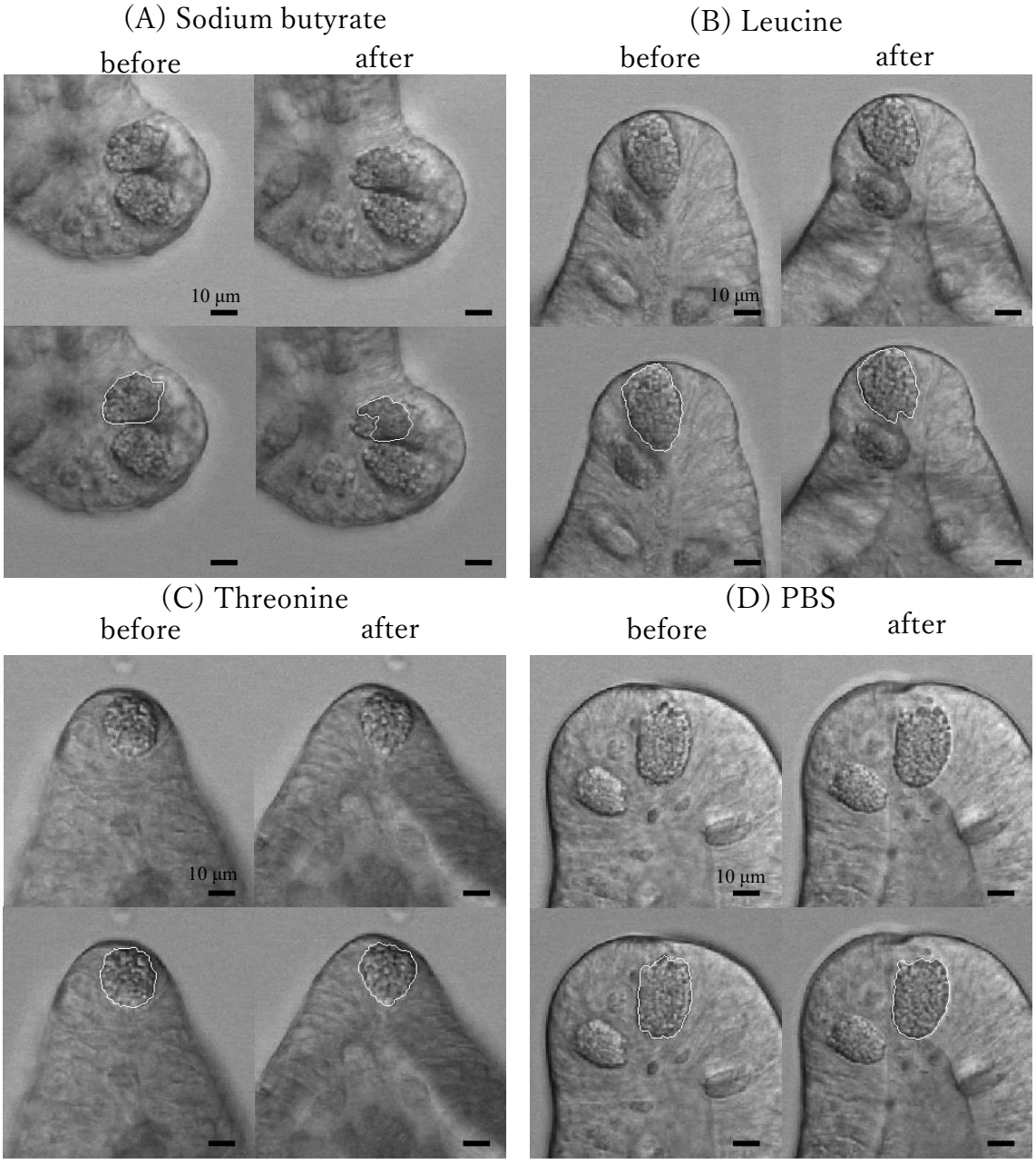


Figure 7. Comparison of granule secretion before and after injection of butyric acid or amino acids into enteroid lumen: (A) Sodium butyrate; (B) Leucine; (C) Threonine; (D) PBS control. Granule area before and after injecting substances were surrounded by white lines. The granule area was decreased after introduction of butyric acid or leucine into the enteroid lumen. In contrast, no change was observed with introduction of PBS or threonine into the enteroid lumen.

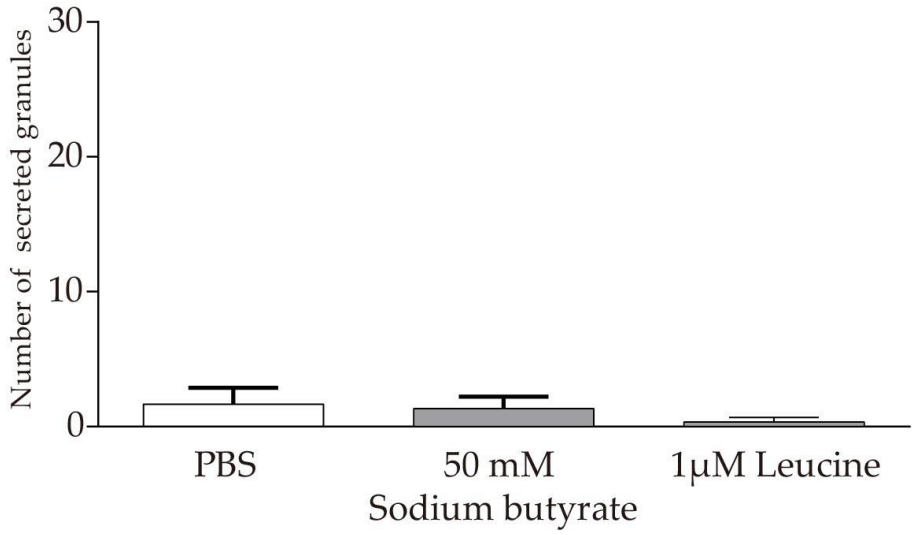


Figure 8. Quantification of granule secretion by Paneth cells stimulated from basolateral side. Data were expressed as means ± SEM (triplicate).

2.4 Discussion

In this chapter, whether food factors and nutrients i.e., SCFAs and amino acids induce α-defensin secretion from Paneth cells was investigated, and revealed that butyric acid and leucine induce α-defensin secretion from Paneth cells. SCFAs are carboxylic acids having 6 or less carbon atoms, and acetic acid, propionic acid, and butyric acid are known. These are supplied from foods by dairy products and various fermented foods, as well as major metabolites produced by the intestinal microbiota using indigestible polysaccharides derived from foods as a substrate [23]. Among the SCFAs, butyric acid has been reported to preferentially serve as an energy source for colonic epithelial cells and promote the proliferation of small intestinal and colonic epithelial cells [28]. In addition, SCFAs have been known to activate certain GPCRs, which are receptors on the cell membrane, and propionic acid and butyric acid are ligands for GPR41, and acetic acid and propionic acid are ligands for GPR43 [29,30]. Butyric acid also activates GPR109A in dendritic cells, and promotes the production of retinoic acid and IL-10 and induces IL-10-producing Treg [30], further activates *Foxp3* transcription by inhibiting histone deacetylase (HDAC) activity to induce differentiation into Treg [24]. Furthermore, it has been reported that the intestinal microbiota of Crohn's disease (CD) patients are decreased in butyric acid-producing bacteria such as *Clostridium* compared to healthy subjects [31], and it has been becoming clear that butyric acid contributes to anti-inflammatory effects in the intestine with its various physiological activities. In addition, in the amyotrophic lateral sclerosis (ALS) model mice, the number of Paneth cells per crypt and the mRNA expression levels of *HD5* and *lysozyme* were significantly reduced compared to wild type mice, whereas when the ALS model mice was administered 2% butyric acid by drinking water, this reduction is recovered to the same levels as wild type [32]. Butyric acid has also been reported to increase promoter activities of *HD5* [33], suggesting association between Paneth cells and butyric acid. Thus, the induction of Paneth cell α-defensin secretion by butyric acid revealed in this study added previously unknown function of butyric acid in the innate enteric immunity, in addition to its conventional activities in immunity and metabolic regulation in the intestine.

Amino acids are constituents of proteins and among twenty types of amino acids, nine of them are the essential amino acids that cannot be produced in human body and must be obtained from foods. Leucine, which induced α-defensin secretion from Paneth cells in this study, is an essential amino acid presenting the largest amount in human tissues, and the required amount is 39 mg / kg / day, which is also the largest among essential amino acids [34]. While SCFAs are the energy source for the large intestine, it has been known that amino acids such as glutamine and glutamic acid are the energy source for the small intestinal epithelial cells [35]. In addition, regarding amino acids, it has been reported that glutamine protects intestinal epithelial cells [36] and histidine

suppresses enteritis by inhibiting Tumor Necrosis Factor (TNF) -α production from macrophages [37].

In this study, it was revealed that α-defensin secreted from Paneth cells induced by butyric acid and leucine kills the pathogenic bacteria, *Salmonella*, and further confirmed granule secretion responses of Paneth cells by injecting butyric acid and leucine into the lumen of enteroids, indicating that Paneth cells recognize butyric acid and leucine from the lumen side, i.e., apical side. It is considered that Paneth cells recognize butyric acid and leucine as feeding signals and secrete α-defensin in surveillance of the entire intestine to contribute to maintaining intestinal homeostasis. It has been reported that mice under total parenteral nutrition have decreased granules in Paneth cells and reduced responsiveness to LPS compared to normally fed mice [38]. In addition, parenteral nutrition reduced *Crp4*, regenerating islet-derived protein 3γ (*Reg IIIγ*), and *lysozyme* mRNA, also decreased Firmicutes and increased Bacteroidetes compared to normally fed mice [39]. The intestinal microbiota composition of mice under total parenteral nutrition was similar to that in mice deficient in the α-defensin activating enzyme matrix metalloproteinase-7 (MMP7), suggesting that the state in which the intestine is not stimulated by feeding in parenteral nutrition is similar to the state of activated α-defensin deficient in the intestine [6].

In this study, it was confirmed that the nutritional status in the intestine affects Paneth cells and α-defensin secretion, showing butyric acid and leucine directly induce secretion of α-defensin secretion from Paneth cells. Butyric acid and leucine are the energy source of intestinal epithelial cells and influence the development of intestinal epithelial cells. Since they are rarely deficient in a normal diet, it is speculated that butyric acid and leucine reflect general nutritional status in the intestine and may act as the stimulator of α-defensin secretion to control intestinal environment. However, the precise reason why only butyric acid and leucin induce α-defensin secretion from Paneth cells remain unknown and further studies are needed.

Taken together, in this study, it was clarified for the first time that Paneth cells recognize not only intestinal bacteria but also food factors and nutrients as intestinal environmental factors, showing that butyric acid and leucine secrete α-defensin from Paneth cells. Furthermore, Paneth cell secretions induced by butyric acid and leucine showed bactericidal activities against pathogen, indicating previously unknown insights into the novel function of food factors and nutrients in maintaining homeostasis of the intestinal environment.

Chapter 3. Paneth cells express receptors and transporters that recognize food factor and nutrients

3.1 Introduction

In Chapter 2, it was shown that only butyric acid among SCFAs and leucine among amino acids induce α-defensin secretion using isolated crypts from small intestine. Further, using enteroids, it was clarified that butyric acid and leucine are recognized from the luminal side and induce α-defensin secretion. Paneth cells recognize not only bacteria in the intestine but also food factors and nutrients and secrete α-defensin, suggesting that food factors and nutrients contribute to maintaining the intestinal homeostasis by activating the innate immunity in the small intestine. Therefore, next, the mechanism how Paneth cells recognize butyric acid and leucine to induce α-defensin secretion was investigated by analyzing expression of receptors and transporters that recognize butyric acid and leucine on Paneth cells. It has been reported that SCFAs ingested as food or produced by the intestinal microbiota as metabolites are not only used as energy sources for the host but also regulate energy metabolism and inflammation as signal transduction molecules via GPCR [40]. Since GPR41 (FFAR3), GPR43 (FFAR2), and GPR109A have been identified as GPCRs binding SCFAs including butyric acid as ligands [28,29,41], the expression of these GPCRs on Paneth cells was analyzed to understand the mechanism of α-defensin secretion from Paneth cells. It has been known that GPR41 and GPR43 are expressed in the intestinal secretory cells, and GPR41 is highly expressed in sympathetic ganglia to maintain energy homeostasis [42], also GPR43 is highly expressed in white adipocytes to suppress fat accumulation [43]. Furthermore, GPR109A is expressed in dendritic cells and macrophages in addition to colonic epithelial cells and adipocytes, and its involvement in intestinal inflammation has been reported [19]. Thus, it has been becoming clear that SCFAs are involved in the energy metabolism and immune function of host via GPCRs.

On the other hand, because amino acids are taken up into cells via amino acid transporters existing on the cell membrane, amino acid transporters known to recognize leucine were analyzed the expression on Paneth cells. As amino acid transporters, more than 60 families and more than 400 molecules of solute carrier family (SLC) have been reported (SLC TABLES; http://slc.bioparadigms.org/). Amino acids have different properties depending on types of the side chain, and there are many amino acid transporters to transport amino acids with high diversity. Families of SLC1, SLC3, SLC6, SLC7, SLC16, SLC17, SLC25, SLC36, SLC38, and SLC43 have been reported as human amino acid transporters [44]. Leucine, a neutral amino acid, is known to be taken up in the small intestine via SLC7A6, SLC7A7, SLC7A8, SLC7A9, SLC6A19, SLC6A14, and SLC43A2, etc. [45] It has been known that amino acid transporters are abundant

in the absorptive epithelial cells of the small intestine, which absorbs nutrients, and the kidney, which reabsorbs amino acids from urine. Furthermore, it has also been reported that amino acid transporters contribute to sensing amino acids and eliciting various functions with regulation of mTORC1 signal, in addition to absorbing amino acids into cells [46]. The ingested nutrients are used as materials and energy sources for cells and tissues. Furthermore, it has been known that nutrients also act as intracellular signal transduction molecules to control various cell functions. The receptors and transporters play a role of sensors that recognize nutrients as cell signals in addition to transport ingested nutrients into cells.

On the other hand, it has been reported that Paneth cells contribute to proliferation and differentiation of intestinal epithelial stem cells by creating stem cell niche. Because the proliferation and differentiation of intestinal epithelial stem cells are affected by calorie-restriction and high-fat diet [12, 47], food factors and nutrients may be involved in Paneth cell function. However, the expression and function of receptors and transporters involved in the recognition of food factors and nutrients on Paneth cells remain completely unknown. Therefore, this study analyzes the expression of receptors and transporters recognizing butyric acid and leucine on Paneth cells to understand mechanisms for α-defensin secretion. By clarifying that Paneth cells recognize not only intestinal bacteria but also food factors and nutrients and metabolites of the intestinal microbiota as intestinal environmental factors via receptors and transporters and secrete α-defensin, this study may contribute to understanding novel function of Paneth cells in maintaining the intestinal homeostasis.

3.2. Materials and Methods

3.2.1 qPCR analysis of receptor and transporter gene expression

The small intestinal crypts which obtained in section 2.2.3.2 were resuspended in HBSS 300 U/mL collagenase (Sigma-Aldrich), 10 μM Y-27632 (Sigma-Aldrich), and 1 mM N-acetylcysteine (Sigma-Aldrich) at 37 °C, and shaken at 180 rpm for 5 min on a horizontal shaker (TAITEC, Kyoto, Japan). Next, 50 μg/μL DNase I (Roche, Basel, Switzerland) was added and the sample was mixed by pipetting. Cells were pelleted at 500 g for 5 min at 4 °C and resuspended in washing buffer (DMEM/F12, 10 μM Y-27632, 1 mM N-acetylcysteine). Then, cells were passed through a 40-μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and washed with washing buffer. For Paneth cell enrichment, Paneth cell granules were stained with 10 μM Zinpyr-1 (Santa Cruz Biotechnology, Dallas, TX, USA) in washing buffer at 37 °C for 10 min and washed with washing buffer. After Zinpyr-1 staining, the cells were passed through a 35-μm-pore-size filter Cell Strainer cap (BD Falcon) prior to cell sorting. Zinpyr-1+ cells were sorted by flow cytometry (JSAN; Bay Bioscience, Kobe, Japan). Single cells were gated by forward scatter and side scatter. Sorted cells were collected in washing buffer.

For Paneth cell isolation, Paneth cells were identified as Zinpyr-1+ granular cells in PBS on a poly (HEMA)-coated glass slide under a confocal microscope (A1; Nikon, Tokyo, Japan). Each Paneth cell was aspirated individually using a 50-μm glass micropipette (1-GT50S-5; NEPAGENE, Ichikawa, Japan) with micromanipulators (MN-4, MMO-202ND; NARISHIGE, Tokyo, Japan) and an electronic pipette (PicoPipet; NEPAGENE), and placed into 4 μL of lysis buffer from SingleShotTM Cell Lysis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in the flat optical caps of Vari-Strip™ low profile 8 strip tubes (NIPPON Genetics, Tokyo, Japan). Each cell lysate containing 50 Paneth cells was placed in a 0.2-mL PCR tube (NIPPON Genetics).

After cell isolation, the cell lysates were incubated at 25 °C for 10 min and boiled at 75 °C for 5 min. cDNA was synthesized with the iScriptTM Advanced cDNA synthesis kit for RT-qPCR (Bio-Rad Laboratories) using 10 μL of a reaction mixture containing 4 μL of cell lysate, 2 μL of 5× iScript Advanced reaction mix, and 0.5 μL of iScript Advanced reverse transcriptase. The complete reaction was cycled at 42 °C for 30 min and at 85 °C for 5 min. Thermal cycling was conducted using a Veriti Thermal cycler (Thermo Fisher Scientific). PCR primers used are listed in Supplementary Table S1. The relative mRNA levels were calculated according to the 2ΔCt method, using β-actin as a reference.

3.2.2. Western blot

Pooled crypts were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) in the presence of a protease inhibitor cocktail (Nacalai Tesque) using a Nippi Biomasher (Nippi, Tokyo, Japan) for 1 h at 4 °C. Homogenized crypts were centrifuged at 20,000 g for 30 min to

obtain supernatants. Protein concentrations in the supernatants were measured using a BCA protein assay kit (Thermo Fisher Scientific). Samples, including 10 mg of protein and 25 or 50 ng of mouse kidney lysate (positive control), were separated on an SDS-PAGE, following which proteins were transferred to nitrocellulose membranes. The membrane was blocked with StabilGuard (SurModics, Eden Prairie, MN, USA) for 1 h at 25 °C and then incubated at 4 °C overnight with 1 μg/mL anti-FFAR3/GPR41 (ab236654; Abcam, Cambridge, UK), anti-FFAR2/GPR43 (ABC299; Merck Millipore, Darmstadt, Germany), and anti-LAT2/Slc7a8 antibody (ab75610; Abcam) antibodies. After the membranes were washed, they were incubated for 1 h at 25 ℃ with goat anti-rat IgG-HRP (Imgenex, San Diego, CA, USA). After another wash, the proteins were detected using a chemiluminescent substrate (Chemi-Lumi One, Nacalai Tesque).

3.2.3. Immunofluorescence staining

The ileum tissue from CD1 (ICR) mice were fixed in 10% neutralized buffered formalin, embedded in paraffin, and placed on poly-L-lysine-pretreated slides. For immunofluorescent staining, after deparaffinization and rehydration, the antigens were retrieved in an autoclave at 105 ℃ for 20 min with Tris–EDTA (ethylenediaminetetraacetic acid) buffer (pH 9.0). After the antigen retrieval, nonspecific binding was blocked with 5% goat serum. Primary antibody reaction was performed with 1 μg/mL rat monoclonal anti-cryprdin-1 (clone: 77-R63, produced by our laboratory), 25 μg/mL anti-FFAR3/GPR41 (Abcam), 10 μg/mL anti-FFAR2/GPR43 (Merck Millipore), and 50 μg/mL anti-LAT2/Slc7a8 antibody (Abcam) diluted by PBS at 4 ℃ overnight. After rinsing in PBS, tissue sections were incubated with Alexa Fluor 488 goat anti-Rabbit IgG and Alexa Fluor 594 goat anti-Rat IgG (dilution 1:400, Thermo Fisher Scientific) diluted by PBS. Tissue sections were also counterstained with 5 μg/mL staining 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for 5 min at 25 ℃ to visualize nuclei and were mounted with Aqua Poly/Mount (Polysciences, Warrington, PA, USA). Pictures were taken using a confocal microscope (A1, Nikon).

3.2.4 Inhibition assay in enteroids

Enteroid culture was performed as described in 2.2.7 section. Binding inhibition assay (butyrate binding to Gpr41) was performed by adding culture media containing 1 mM beta-hydroxybutyrate (BHB) (Sigma-Aldrich), an antagonist to Gpr41 [42], to the dish 30 min before microinjection. For L-leucine and L-threonine injection, culture media containing Advanced DMEM/F12, which lacks individual essential amino acids (leucine or threonine, respectively) (Research Institute for the Functional Peptides, Yamagata, Japan), were added respectively, and the enteroids were microinjected with these compounds (nutrients or antagonist) after 12 h of culture. For the L-leucine transporter Slc7a8 inhibition assay, culture media (lacking L-leucine) containing 6 mM 2-amino-2-norbornane-

carboxylic acid (BCH) (R & D system, Minneapolis, MN, USA), an antagonist of Slc7a8 [48], were added to the dish for 12 h before microinjection.

Microinjection was performed while scanning the enteroid at 15 frames/s by confocal microscopy in a microscope cage incubator (37 °C, 5% CO2, Tokken, Kashiwa, Japan). The needle (Femtotips, Eppendorf) was inserted into the enteroid by using Coarse and Fine Three-axis Oil Hydraulic Micromanipulator and One-axis Oil Hydraulic Micromanipulator (MN-4, MMO-202ND, MMO-220A, NARISHIGE), and reagents were introduced into the enteroid lumen using a Pneumatic Microinjector (IM-11-2, NARISHIGE) at a final concentration of 50 mM sodium butyrate, 1 µM L-leucine, and 1 µM L-threonine. For the Gpr41 inhibition assay, sodium butyrate and BHB were co-injected at a final concentration of 50 and 1 mM, respectively. For the Slc7a8 inhibition assay, L-leucine and BCH were co-injected at a final concentration of 1 and 6 mM, respectively.

3.3. Results

3.3.1 Paneth cells express genes and proteins for butyric acid receptors and amino acid transporters

Secretion from Paneth cells was induced by limited components, i.e., butyric acid and leucine from among three SCFAs and twenty amino acids, respectively. Thus, further analyses of whether Paneth cells express receptors and transporters that recognize butyric acid and leucine were conducted using purified Paneth cells. The expression of GPCR for SCFAs, SLC family transporter for amino acids, and Ca^{2+}-sensing receptor (CaSR) in highly purified Paneth cells was quantified by real-time PCR (Figure 9, Table 1). Paneth cells expressed SCFA receptors, *Gpr41*, *Gpr43*, and *Gpr109a* genes. Paneth cells also expressed *Slc7a8* (L-type amino acid transporter-2: LAT-2), which use neutral amino acids including leucine other than proline as substrates. These results indicated that Paneth cells express *Gpr41*, *Gpr43*, and *Gpr109a*, which recognize SCFAs, and *Slc7a8*, which recognize leucine, and further suggest that Paneth cells secrete α-defensin by recognizing butyric acid and leucine via these receptors and transporters.

Next, western blot analyses were performed for Gpr41 (FFAR3), Gpr43 (FFAR2), and Slc7a8 whose mRNA expression was detected by qPCR. Bands with the molecular weight of 39 kDa corresponding to Gpr41, 47 kDa to Gpr43, and 58 kDa to Slc7a8 were observed (Figure 10). Furthermore, immunohistochemical analysis confirmed that Paneth cells express Gpr41, Gpr43, and Slc7a8 in the ileum (Figure 11).

3.3.2 Inhibitors of Gpr41 and Slc7a8 attenuate granule secretion from Paneth cells

Finally, inhibition assays were performed using inhibitors for GPR41 and Slc7a8. Inhibition assays revealed that BHB, which is an antagonist for Gpr41, significantly suppresses Paneth cell granule secretion induced by butyric acid (Figure 12A) and BCH, an antagonist for Slc7a8, also inhibited leucine-induced Paneth cell granule secretion in enteroids (Figure 12B).

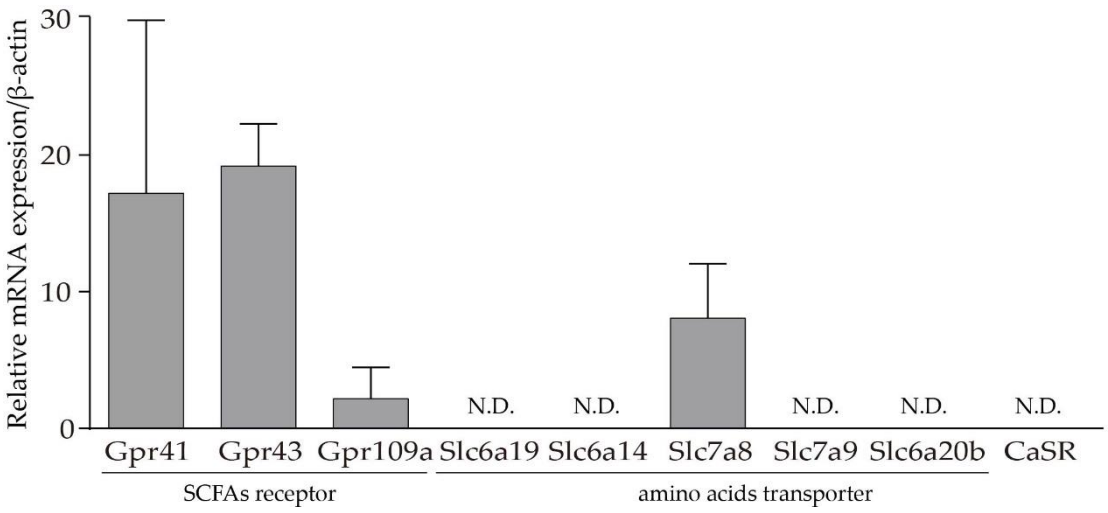


Figure 9. Expression of receptors and transporters recognizing SCFAs or amino acids in Paneth cells. Relative expression of mRNA is shown by the 2ΔCt method as the means ± SEM (n = 3).

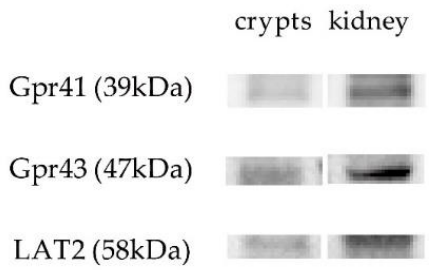


Figure 10. Expression of receptors and transporters recognizing SCFAs or amino acids in Paneth cells. Western blot analysis of Gpr41, Gpr43, and Slc7a8 protein expression. Proteins were extracted from crypts of the mouse ileum and kidney.

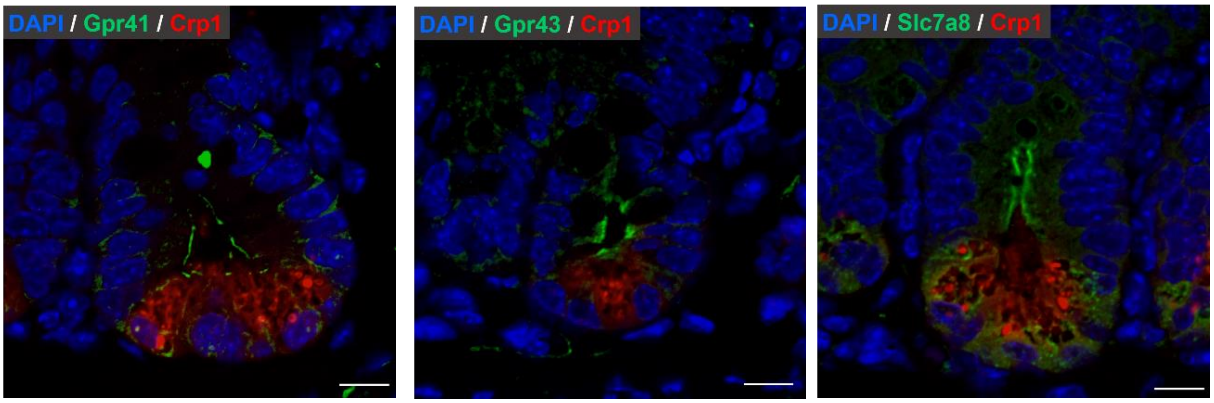


Figure 11. Expression of receptors and transporters recognizing SCFAs or amino acids in Paneth cells. Immunofluorescence staining of Gpr41, Gpr43, and Slc7a8 with Crp1 and 4’,6-diamidino-2-phenylindole (DAPI) in the mouse ileum. Scale bar: 10 μm.

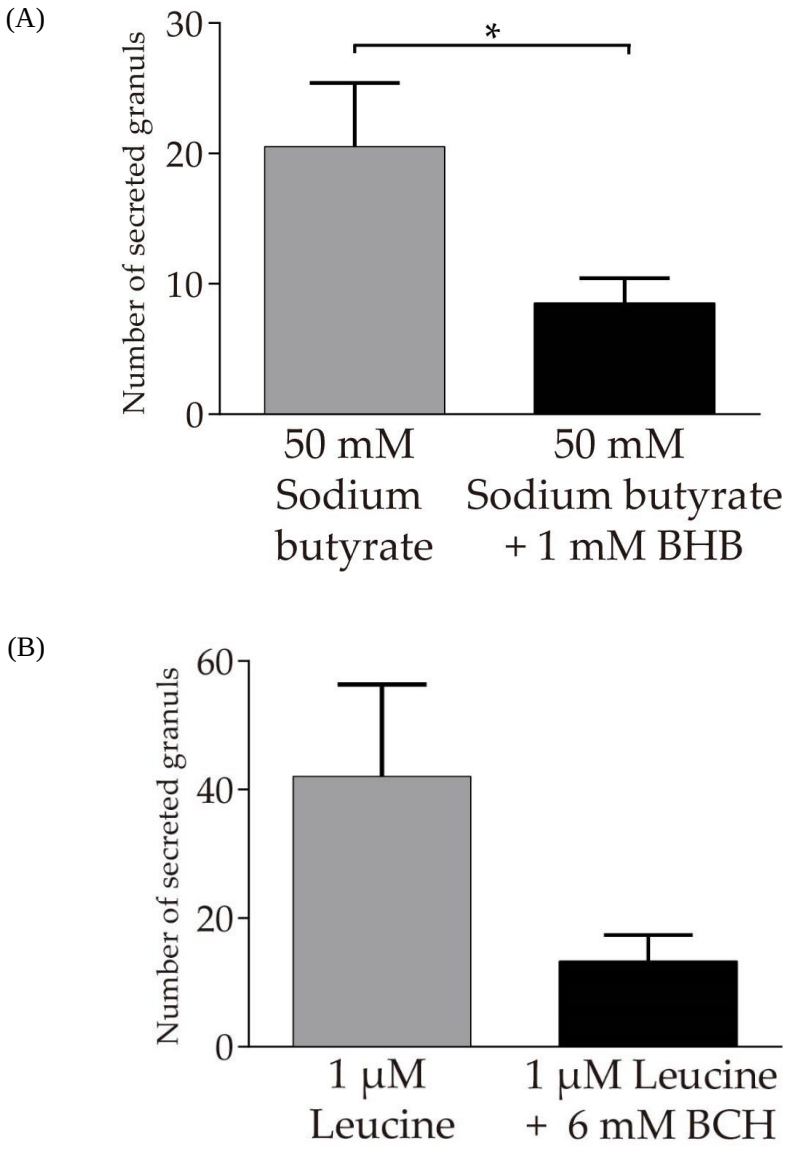


Figure 12. Quantification of secreted Paneth cell granules in inhibition assays: (A) For the Gpr41 inhibition assay, sodium butyrate (50 mM) and beta-hydroxybutyrate (BHB) (1 mM) were co-injected. BHB significantly inhibited butyrate granules secretion. (B) For the Slc7a8 inhibition assay, L-leucine (1 µM) and 2-amino-2-norbornane-carboxylic acid (BCH) (6 mM) were co-injected. BCH showed a tendency to inhibit granule secretion induced by leucine. Secreted granules were counted for 30 min after injection of reagents. Data are expressed as the total number of secreted granules from five Paneth cells ± SEM (n = 4). * $p < 0.05$ by Student's t-test.

Table.1 List of qPCR primer sets

Target gene	UPL	Primer sequence
Ffar3	#12	(F) GTGCACTCACAAGGACTCTCC (R) AAATTCGGGGTTTATGAGAGG
Ffar2	#27	(F) GAGAGAAGGAGGGAGTCG (R) TGGGATGTTCCACATGTCAC
Hcar2	#13	(F) CTGCTCAGGCAGGATCATCT (R) CCCTCTTGATCTTGGCATGT
Slc6a19	#25	(F) CTTCCCCTACCTATGCCAGA (R) AAGGATGAGGAATGGGATCA
Slc6a14	#22	(F)GGAGCAAAAAGATGGATATTCTG (R) ACCAGTGACCAGACTAGAATTGC
Slc7a8	#41	(F) GCTACCCCTCATTCCTCTCA (R) GAGAGAGAGAGACAATAATTCAAGGAG
Slc7a9	#64	(F) TTCACAGTGATGACCCCAAC (R) AACACGGTCTCCGAAGGTC
Slc20b	#78	(F) TCCTCACCCCTCTGACTGAC (R) GGTGAACACCATGCCAATG
Casr	#38	(F) TGCAGACATCAAGAAGGTTGA (R) CCGCACTCATCGAAGGTC
α-defensin	#68	(F) CCAAAACACAGATGAAGAGACTAAAA (R) GCATCCAGATCTCTCAACGATT
β-actin	#64	(F) CTAAGGCCAACCGTGAAAAG (R) ACCAGAGGCATACAGGGACA

3.4 Discussion

In this chapter, to clarify the mechanism that butyric acid and leucine induce α-defensin secretion from Paneth cells, GPCR and amino acid transporter expression on Paneth cells are analyzed by qPCR and Western blot, also the localization of the GPCR and amino acid transporter was determined by fluorescent immunostaining of ileal tissues. It was revealed that Gpr41, which recognize butyric acid, and Slc7a8, which recognize amino acids including leucine, are involved in α-defensin secretion from Paneth cells.

Since SCFAs are GPCR ligands and have been reported to regulate intestinal immune responses via GPCRs, GPCR expression on Paneth cells as butyric acid receptor was investigated. Receptor expression analyses of purified Paneth cells revealed for the first time that butyric acid-recognizing Gpr41, Gpr43, and Gpr109a are expressed on Paneth cells. It has been reported that GPCRs can be activated by the concentrations of SFCAs in the blood (dozens to several hundreds of μM *in vivo*) [49], so that the butyric acid concentration, 100 μM, used *ex vivo* in this study is reasonable to activate the GPCRs in Paneth cells and induce α-defensin secretion. Furthermore, this study showed that BHB, an Gpr41 antagonist, significantly inhibits butyric acid-induced granule secretion from Paneth cells using enteroids. GPR41 expressed in the sympathetic ganglion has been known to recognize SCFAs produced by the intestinal microbiota as a signal related with increasing energy and activate sympathetic nerves to increase energy consumption. In addition, BHB synthesized in the liver during energy deficient status reduces energy consumption by inhibiting GPR41-mediated sympathetic nerve activation. Thus, GPR41 recognizes *in vivo* energy balance and regulates energy consumption [42]. Small intestinal epithelial cells have been known to express a wide variety of receptors and transporters and efficiently obtain energy by identifying food factors and nutrients in the lumen, and it is possible that Paneth cells are also involved in intestinal energy metabolism via Gpr41.

On the other hand, regarding leucine recognition, among five types of amino acid transporters examined, the expression of Slc7a8 was detected on Paneth cells. Furthermore, BCH, an antagonist of Slc7a8, inhibited leucine-induced Paneth cell granule secretion in this study. Because Slc7a8 is a transporter that widely recognizes neutral amino acids, it remains possibilities that leucine may compete with other amino acids or be recognized by other receptors or transporters. Although it remains to be determined why only leucine induces α-defensin secretion from Paneth cells among twenty amino acids, one characteristic of leucine is to activate mTORC1. Several amino acids have been known to function as signal transduction molecules controlling metabolism, and leucine, arginine, glutamine, and tryptophan have been reported to contribute to mTORC1 activation [50-52]. Also, it has been reported that among amino acids, only leucine is

involved in both mTORC1 activity-mediated protein synthesis and autophagy [50,53]. This study also suggested that amino acid transporters such as Slc7a8 may be involved in regulation of Paneth cell function.

Paneth cells, which locate base of the crypt, expressing GPCRs and amino acid transporters are not likely to use their receptors and transporters for simply efficient nutrient absorption like absorptive epithelial cells. It is possible that Paneth cells express these receptors and transporters because of involvement in differentiation and proliferation of intestinal epithelial cells in the stem cell niche. Since it has been reported that a zinc receptor ZIP7 is expressed in Paneth cells and contributes to proliferation of intestinal epithelial cells and maintenance of stem cells [21], similar effects may be able to think for Gpr41 and Slc7a8. In the future, by elucidating the entire mechanisms in Paneth cells from recognition of food factors and nutrients to α-defensin secretion, further contribution of Paneth cells to maintaining the intestinal homeostasis will be clarified.

Chapter 4. Summary

Results obtained in this study were summarized in Figure13. Butyric acid as both a food factor and a metabolite of the intestinal microbiota and leucine as a food factor and nutrient induce α-defensin secretion from Paneth cells. Although BHB and BCH are not quite specific antagonists to Gpr41 and Slc7a8, respectively, our results indicated that Paneth cells recognize butyric acid and leucine via Gpr41 and Scl7a8, respectively and induce secretion by conducting inhibition assay. This study illustrates a new role of nutrients in maintenance of the intestinal homeostasis. Abnormalities in α-defensin secretion are known to cause health problems and relate to onset of certain diseases via dysbiosis, disruption of the intestinal microbiota [14]. In addition, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) mutations have been reported to lead to a decrease in α-defensin production in Paneth cells in patients with CD [54] and it has also been reported that immunoreactive α-defensin decreases in obese people [55]. Furthermore, in GVHD model mice, severe dysbiosis due to disappearance of Paneth cells could be rescued by administration of Wnt agonist R-Spondin1 via leading to increase of Paneth cell numbers and recovery of α-defensin secretion, resulting in significant improvement of the disease [56-58].

It has been known that food factors and nutrients contribute to maintaining the intestinal environment by supporting growth of the symbiotic microbiota such as probiotics. Recently, it has become clear that some food factors and nutrients directly affect intestinal immunity. For the first time, this study revealed that butyric acid and leucine induce innate immunity in the small intestine by inducing Paneth cell α-defensin secretion. This study provides previously unknown insights into functions of food factors and nutrients and further intestinal bacterial metabolites in maintenance of intestinal homeostasis via Paneth cell secretion (Figure 13). In the future, to understand a big picture of the relationship between food factors and Paneth cells, further studies testin g sugars, medium- and long-chain fatty acids, vitamins, minerals, etc., on induction of α-defensin secretion from Paneth cells are needed. It will be a next goal of my research to show that not only the effect of single food factor or single nutrient but also food as itself ingested by the host contributes to maintenance of the intestinal homeostasis and further host health by interacting with Paneth cells and the intestinal microbiota.

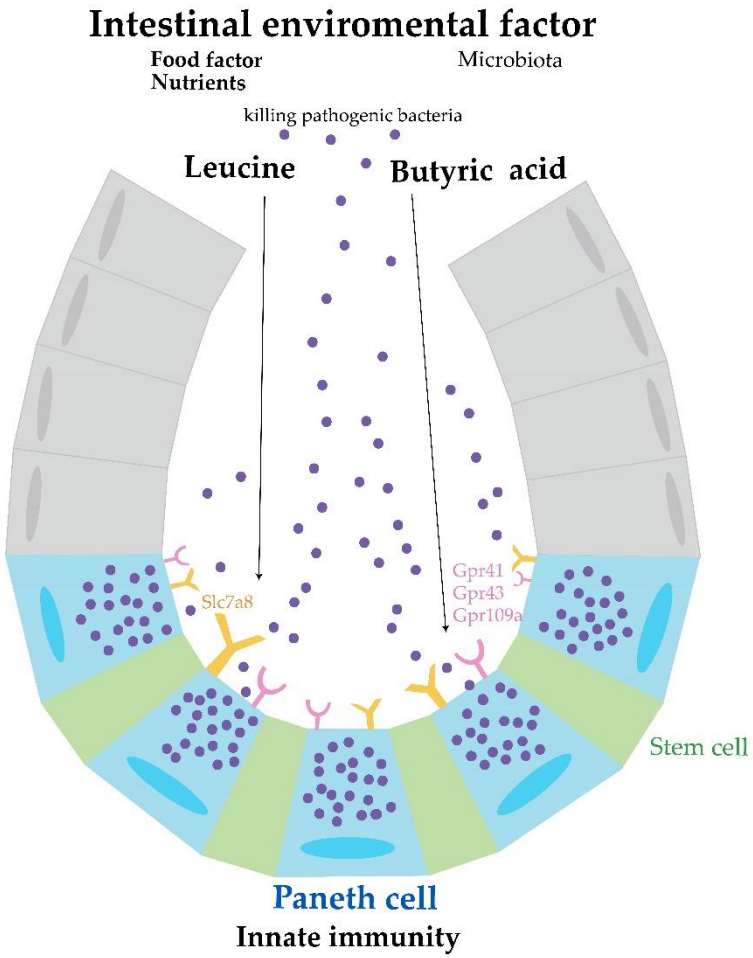


Figure 13. Paneth cell α-defensin secretion induced by food factor and nutrients in a possible regulation of intestinal homeostasis. This study indicates that the nutrients butyric acid and leucine induce α-defensin secretion from Paneth cells through recognition by receptors and transporters on Paneth cells and further suggests that these nutrients play previously unknown roles in regulating intestinal homeostasis.

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