

Paneth cell α -defensins and enteric microbiota in health and disease

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Antimicrobial peptides are major effectors of innate immunity of multicellular organisms including humans and play a critical role in host defense, and their importance is widely recognized. The epithelium of the intestine is the largest surface area exposed to the outer environment, including pathogens, toxins and foods. The Paneth cell lineage of intestinal epithelial cells produces and secretes α -defensin antimicrobial peptides and functions in innate enteric immunity by removing pathogens and living symbiotically with commensal microbiota to contribute to intestinal homeostasis. Paneth cells secrete α -defensins, HD5 and HD6 in humans and cryptdins in mice, in response to bacterial, cholinergic and other stimuli. The α -defensins have selective activities against bacteria, eliciting potent microbicidal activities against pathogenic bacteria but minimal or no bactericidal activity against commensal bacteria. Therefore, α -defensins regulate the composition of the intestinal microbiota *in vivo* and play a role in homeostasis of the entire intestine. Recently, relationships between dysbiosis, or abnormal composition of the intestinal microbiota, and diseases such as inflammatory bowel disease and lifestyle diseases including obesity and atherosclerosis have been reported. Because α -defensins regulate the composition of the intestinal microbiota, Paneth cells and their α -defensins may have a key role as one mechanism linking the microbiota and disease.

Key words: Paneth cell, α -defensin, intestinal microbiota, innate immunity, dysbiosis, inflammatory bowel disease, lifestyle disease

INTRODUCTION

Antimicrobial peptides are gene-encoded effectors in innate immunity of multicellular organisms, from plants and insects to animals including humans, and generally they are cationic small peptides with broad microbicidal activities against bacteria, fungi, protozoa, and certain viruses [1–3]. More than 1,000 antimicrobial peptides have been found in nature. Among mammals, defensins and cathelicidins are the two major families [4–6].

The intestine absorbs nutrients necessary for life, and it also prevents invasion of pathogens, thus contributing to mucosal immunity. Intestinal epithelial cells occupy

the front line of the human interface with the external environment, which is under constant stimulation by foods and microbes, so they are important in a number of ways, including in nutrient absorption, regeneration and mucosal immunity [7, 8]. The epithelium of the intestine is the largest body surface exposed chronically to various pathogens, toxins, commensal microbiota and foods. More than 1×10^{14} bacteria live in the human intestinal lumen, and the normal intestinal microbiota is comprised of a huge number of symbiotic microorganisms [9, 10]. The epithelial cells that line the small intestine are organized into villi and crypts and consists of four major lineages of terminally differentiated cells: columnar cells, enteroendocrine cells, goblet cells, and Paneth cells. These cell lineages are generated by cells termed crypt base columnar (CBC) stem cells, which reside at the bottom of the crypt, adjacent to Paneth cells [11]. Intestinal epithelial cells, except Paneth cells, migrate toward the top of the villi as they differentiate, and they are renewed every three to four days [12]. In contrast, Paneth cells reside at the bottom of small intestinal crypts, establish physical contact with CBC stem cells,

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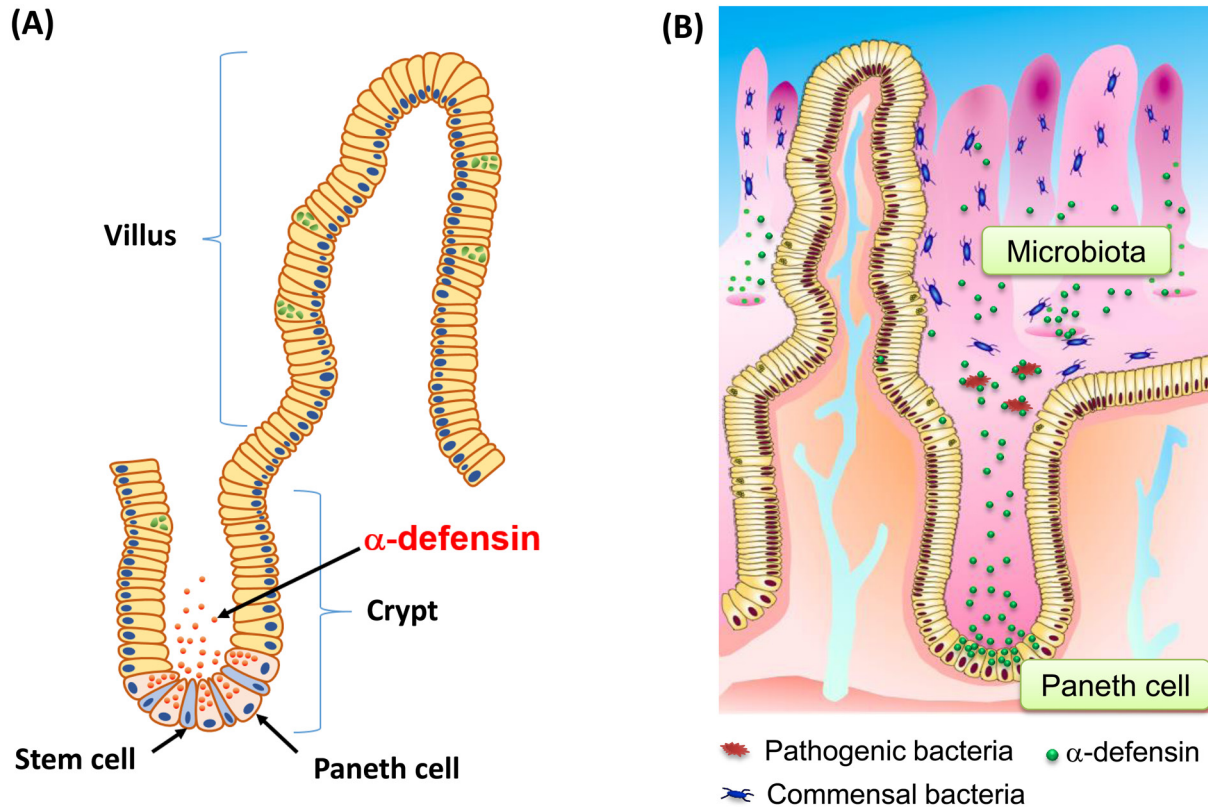


Fig. 1. Stem cells and Paneth cells, their α -defensins and the microbiota in the small intestine.

(A) Small intestinal epithelial cells form villus and crypt structures. Paneth cells and stem cells reside with physical contact at the bottom of crypt and create a stem cell niche. (B) Paneth cells secrete α -defensins in response to bacterial and various stimuli and contribute to innate enteric immunity as well as to regulation of the intestinal microbiota.

and live 24 days or more [13, 14] (Fig. 1A).

Recently, relationships between the intestinal microbiota and various diseases have become evident. One of the reasons for this is the rapid advances in metagenome analyses of the microbiota, which can detect and quantitate even non-culturable bacteria [15]. Similarly, the importance of antimicrobial peptides in regulating the microbiota as major effectors in innate immunity has also been widely recognized recently [16–20]. Paneth cells are the only intestinal epithelial cells expressing enteric α -defensins and secreting them onto the intestinal luminal surface in response to certain stimuli [16–18]. It is known that Paneth cell α -defensins are directly involved in not only removing pathogens but also in establishing symbiosis with the normal intestinal microbiota [19, 20]. In this review, we summarize recent advances in innate immunity related to Paneth cells and their α -defensins and consider possible links between the microbiota and disease.

PANETH CELLS, A LINEAGE OF INTESTINAL EPITHELIAL CELLS, SECRETE α -DEFENSINS

The intestine faces the outer environment with epithelial cells, which absorb nutrients, water and minerals while preventing invasion of pathogens or toxins by establishment of a physical barrier as well as innate immune responses. The intestinal cavity is an outside at an inside of the human body. It has long been known that the number of bacteria comprising the microbiota in the small intestine is much lower than that in the large intestine. To explain this, the contributions of the digestive juice, mucus and bowel peristalsis have been considered as physiologic factors in the small intestine, as has the contribution of the adaptive immune system including IgA and lymphocytes in Peyer's patches. In addition, the contribution of Paneth cells and secreted α -defensins in the innate enteric immunity has been recognized, as their roles have been clarified. In the innate immunity, recognition of microbes by Toll-

like receptors and other pattern-recognition receptors on host cells occurs first [21, 22]. Immediately after this step, microbicidal antimicrobial peptide synthesis and/or mobilization are triggered in affected epithelia or neutrophils, and their secretion by epithelia is activated. Antimicrobial peptides comprised of 18–45 amino acids in multicellular organisms are microbicidal cationic peptides and are capable of killing a variety of pathogens including bacteria, fungi, protozoa and viruses [3]. In mammals, defensins and cathelicidins are two major families of antimicrobial peptides. Among them, only α -defensins are constitutively expressed in intracellular granules of Paneth cells and secreted immediately in response to bacteria. Mammalian α -defensins are 3–4 kDa cationic peptides with 6 cys residues that form invariant 3 disulfide bonds [6, 23–25]. In Paneth cells, HD5 and HD6 in humans and cryptdins comprised of six major isoforms, cryptdin-1 to cryptdin-6, in mice are the major constituents of the intracellular granules [16, 17, 26, 27]. Mouse cryptdins are processed by matrix metalloproteinase 7 (MMP7) also called matrilysin, to the active form during granulogenesis [28]. In contrast, human Paneth cell α -defensins, HD5 and HD6, are packaged as pro- α -defensin and processed by one or more isoforms of trypsin during or after secretion [29]. Both in mouse and human Paneth cells, mature α -defensin segments are in the oxidized state so that internal digestion is prevented.

Paneth cell α -defensins are the major antimicrobial peptides produced and released by the small intestinal epithelium. As unique exceptions, it was reported that metaplastic Paneth cells found in the *Helicobacter pylori* infected stomach and in the colon in patients with ulcerative colitis produce α -defensins [30, 31]. Prevention of infection with pathogens by secretion of microbicidal α -defensins in immediate response to bacterial, cholinergic or other stimuli was revealed as an important role of Paneth cells in mucosal immunity [18, 32–39] (Fig. 1B). Paneth cells also secrete other antimicrobial peptides, such as Reg3 γ , and antimicrobial proteins including lysozyme and secretory phospholipase A₂ [40, 41]. Furthermore, Paneth cells are known to create a specific microenvironment called the stem cell niche together with CBC stem cells [42–47]. In the niche, Paneth cells produce growth factors and Wnt signaling molecules that lead to Wnt on and Notch off, which are delivered to CBC cells and induce differentiation of specific epithelial cell lineages. Therefore, Paneth cells function both in innate enteric immunity and in regeneration/differentiation of epithelial cells in the small intestine. They elicit even more multifunctional roles in

chemotaxis and metabolism [48–50]. However, it must be emphasized that Paneth cells contribute professionally to host defense by secreting α -defensins. The fact that Paneth cells, which are capable of rapidly responding to microbial invaders, reside back to back with stem cells in the intestine may be very important.

α -DEFENSINS ELIMINATE PATHOGENS BUT DO NOT KILL COMMENSAL BACTERIA TO MAINTAIN THE INTESTINAL ENVIRONMENT

Intestinal epithelial cells absorb nutrients and water and at same time create potent barriers against microbes including pathogens. When pathogens try to invade the host, innate immune mechanisms of intestinal epithelial cells are induced or activated immediately. Since potent microbicidal activities of cryptdins, mouse α -defensins, against pathogenic bacteria were reported, the importance of α -defensins in mucosal immunity has been widely recognized [51–53]. MMP7 processes and activates pro- α -defensins, pro-cryptdins, in mouse Paneth cells. MMP7-null mice lack activated cryptdins in Paneth cell granules, accumulating only inactive, non-microbicidal precursors. When mice were challenged orally with *Escherichia coli*, significantly greater numbers of surviving bacteria were recovered from the MMP-7-null mouse intestine as compared with the numbers recovered from wild type mice. Furthermore, when *Salmonella typhimurium* was orally administered, the MMP7-null mouse was more susceptible to systemic disease [28]. This was among the first evidence showing that antimicrobial peptides are involved in mammalian host defense *in vivo*. Paneth cells at the bottom of small intestinal crypts of the mouse secrete intracellular granules rich in cryptdins in immediate response to various stimuli including carbamylcholine, Gram-positive and Gram-negative bacteria, lipopolysaccharide, and muramyl dipeptide [18]. Secreted cryptdins kill pathogenic bacteria to contribute to innate immunity. The expression of a human Paneth cell α -defensin transgene, i.e., HD5, in mouse Paneth cells augmented mouse innate enteric immunity and conferred immunity against oral *Salmonella* infection by reducing bacterial numbers in the intestinal lumen and in feces, decreasing bacterial translocation and promoting high survival rates after lethal *Salmonella* challenge [54]. These results demonstrated that Paneth cell α -defensins contribute actively to enteric host defense *in vivo*. In addition, deficiency of another antimicrobial peptide in mouse Paneth cells, Reg3 γ , disrupts the physical barrier function of intestinal epithelial cells [55].

It has been known that the growth of germ-free mice is

poor and that these mice are immature or even deficient in mucosal immunity [56]. The commensal microbiota is able to elicit maturation of enteric mucosal immunity, including development of Peyer's patches, increasing IgA production; differentiation of regulatory T cells; and induction of certain antimicrobial peptides [10, 57–69]. Therefore, commensal bacteria are necessary for development and stability of the enteric immune response. It has been revealed that α -defensins not only kill pathogens but also contribute to intestinal homeostasis by maintaining the composition of the normal intestinal microbiota. For example, the ileal microbiota in MMP7-deficient mice, which lack activated cryptdins, contained a significantly higher percentage of *Firmicutes* and a significantly lower percentage of *Bacteroides* compared with wild-type mice [19]. Furthermore, in DEFA5^{+/+} mice, which express the human α -defensin HD5 transgene in Paneth cells, the microbiota composition in the small intestine was dramatically different from that of the wild-type strain, with significantly decreased *Firmicutes* and significantly increased *Bacteroidetes* [54]. Of the six most abundant mouse Paneth cell α -defensins, cryptdin4 is the most potent microbicide. Masuda et al. revealed that oxidized cryptdin4 with three disulfide bonds shows potent bactericidal activity against pathogenic bacteria but no or minimal bactericidal activity against commensal bacteria such as *Bifidobacterium bifidum*, *Lactobacillus casei* and *Bacteroides fragilis* [20]. In contrast, reduced cryptdins, which have no disulfide bonds, kill both pathogenic and commensal bacteria. These results suggest that Paneth cell α -defensins possess disulfide bond-dependent bactericidal activities and play a role in regulating the composition of the intestinal microbiota to maintain the intestinal environment (Fig. 2). It has been shown that Crp4 permeabilized the phospholipid bilayer and that the activity was dependent on the membrane composition [70]. It has also been reported that depolarization of the membrane potential in some noncommensal bacteria occurs via cryptdin4 [20]. However, the precise bactericidal mechanisms of native Crp4 have yet to be fully elucidated and may be bacteria dependent. Furthermore, a recent report clarified that activated cryptdins, which have been previously thought to play a role only in the small intestine, are present in the luminal content of the cecum and colon [71, 72]. Although the effects of α -defensins on the composition of the colonic microbiota remain unknown, it is possible that Paneth cell α -defensins may influence the microbiota of the distal gut. However, a lack of sensitive assays to quantify α -defensins released into the intestinal lumen has limited efforts to establish a relationship between

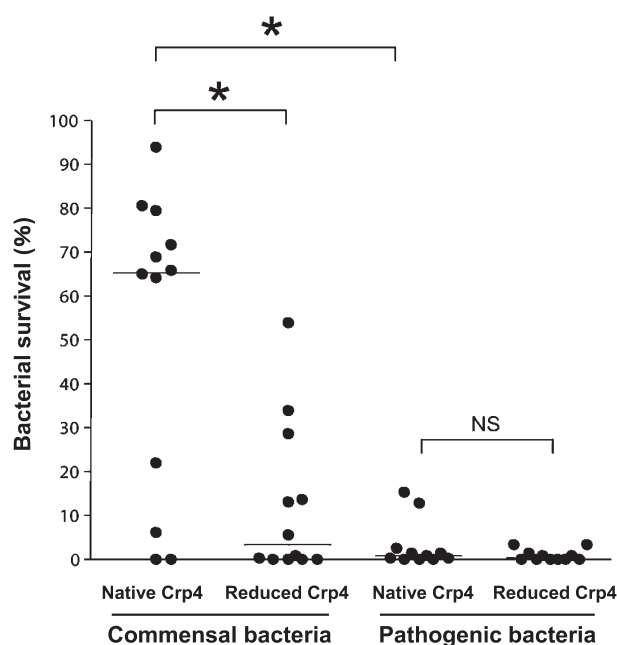


Fig. 2. Bactericidal activities of native (oxidized) cryptdin4 and reduced cryptdin4 against commensal bacteria and pathogenic bacteria.

The survival rates of twenty commensal bacteria and eleven pathogenic bacteria when each bacterium was exposed to either native cryptdin4 or reduced cryptdin4 are shown. Cryptdins regulate the intestinal microbiota by eliciting potent bactericidal activities against pathogenic bacteria and less potent bactericidal activities against most commensal bacteria. (Modified from the study of Masuda et al. [20].) * $p < 0.05$. NS: not significant.

levels of secreted α -defensin and disease. Nakamura et al. established a highly sensitive and specific sandwich ELISA for cryptdins recently [73]. The concentration of cryptdin4 was measured throughout the enteric lumen, and it was confirmed that the concentrations of secreted cryptdin4 increases along the proximal to distal axis, with the highest levels in the distal ileum. In addition, the concentration of cryptdin4 was determined in the luminal contents of the cecum and colon, where Paneth cells are absent. This is consistent with a previous finding indicating that processed cryptdins were recovered from the luminal contents of the colon and cecum, which was confirmed by both bactericidal assays and gel electrophoresis [73]. Therefore, secreted α -defensins may influence the intestinal microbiota not only in the small intestine but also in the colon. By testing fecal samples, the ELISA revealed an overall deficiency of this secreted α -defensin in the intestine of the IL10-null mouse, a mouse model of colitis, which is consistent with decreased α -defensin levels reported in CD patients [74].

Furthermore, the ability of this ELISA system to quantify total secreted cryptdin4 in feces may contribute to a better understanding of the relation of luminal α -defensin levels to diverse diseases, including inflammatory bowel disease (IBD), infectious diseases, diabetes and obesity.

PANETH CELL DISRUPTION INDUCES DYSBIOSIS AND IS LINKED TO CERTAIN DISEASES

Dysbiosis, disruption of the composition of the intestinal microbiota, has been associated not only with infectious diseases but also with the pathogenesis and pathophysiology of diverse diseases. Because of recent rapid advances in methods of genetic analysis of microbes such as terminal-restriction fragment length polymorphism (T-RFLP) analysis and metagenomic analysis targeted at bacterial 16S rRNA in addition to the gold-standard culture methods, the possible relationship between the microbiota and diseases has become a major topic in the biomedical sciences. For example, the relative proportion of *Bacteroidetes* and *Firmicutes* in obese people differs markedly from that of lean individuals, suggesting that obesity is associated with a particular microbial component [75, 76]. Since then, the number of reports regarding the composition of the microbiota determined by metagenomics analysis and diverse diseases has grown continuously. Such diseases include type II diabetes mellitus [77], metabolic syndrome [78, 79], atherosclerosis [80], nonalcoholic steatohepatitis [81, 82], autism [83], liver cirrhosis [84], liver cancer [85], IBD [86–88] and more [89, 90]. Moreover, fecal microbiota transplantation has therapeutic efficacies for recurrent *Clostridium difficile* infection, indicating that microbiota alteration also may be beneficial for other diseases such as IBD and metabolic syndrome [91]. However, the mechanisms underlying how dysbiosis is linked to these diseases have yet to be fully determined. We are currently working on a simplified model to understand possible mechanisms of the “intestinal environment,” which we hypothesized is associated with three factors: Paneth cell α -defensins, the intestinal microbiota and dietary factors (Fig. 3).

Mice lacking IL10 or IL2 induce colitis spontaneously; however, these mice have no or mild colitis if they are kept under germ-free conditions [92–94]. It is common for patients with IBD to frequently suffer local or systemic infection with bacterial overgrowth or dysbiosis [95]. The microbiota of patients with Crohn’s disease (CD) shows less diversity of *Firmicutes* than healthy subjects [86]. CD is a young-onset IBD that affects the entire digestive tract, especially the small intestine and causes

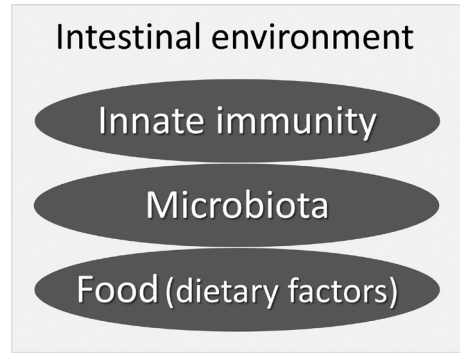


Fig. 3. Factors affecting the intestinal environment: a working hypothesis.

A simplified model: Three factors, innate immunity, microbiota and food, cross talk with each other and contribute to the intestinal environment in both the short and long term. Innate Immunity: Paneth cell α -defensins act as major effectors in innate immunity. Microbiota: Commensal bacteria form the normal intestinal microbiota. Food (dietary factors): Food and dietary factors act as orally administered extrinsic factors.

transmural enteritis, longitudinal ulcers and stenosis of the intestine. Many CD patients suffer relapse frequently after achieving remission through therapy, and the disease persists chronically, so the patients’ quality of life is poor and a big problem. Although our understanding of the pathogenesis of CD is still incomplete, it has been revealed that some of the many CD risk factors and susceptibility genes are related to recognition receptors of pathogen-associated molecular patterns, autophagy, molecular chaperones and ER stress response, many of which are expressed in Paneth cells. Furthermore, genetic defects of *NOD2* [96–100] or genes related to autophagy and ER stress affect Paneth cells selectively and contribute to IBD pathogenesis. In mice with genetic defects for the autophagy gene, *Atg16L1*, Paneth cells showed morphological abnormalities and were defective in secretion [101]. It was also reported that such Paneth cell disruption is dependent both on the mutated *Atg16L1* and on infection with a specific strain of norovirus as a so-called second hit [102]. This is taken as a clear example showing that a combination of both a genetic risk factor and an environmental factor, such as a specific infectious agent, is necessary for onset of IBD. Furthermore, deletion of *Xbp1* leads to an abnormal unfolded protein response and induces Paneth cell apoptosis and severe ileitis in the mouse [103]. Also, mice with deletion of the *Anterior Gradient 2* gene, a protein disulfide isomerase, have goblet cells depleted of Mucin 2, a disrupted Paneth cell morphology and severe terminal ileitis and colitis induced by ER stress [99, 104–106]. These results

indicate that genetic defects that lead to disruption of Paneth cell homeostasis could weaken innate enteric immunity by reducing α -defensin production and secretion. In addition, importantly, Paneth cell disruption could also eliminate or modify the stem cell niche, which is necessary for regeneration and differentiation of the entire intestinal epithelial monolayer via communication between Lgr5-positive CBC stem cells and Paneth cells to maintain small intestinal integrity [44].

Reduced mRNA expression of intestinal tissue HD5 compared with healthy subjects has been reported in some CD patients [107]. In addition, the HD5 precursor was reported to be reduced in certain CD patients [108]. Reduced pro-HD5 was sensitive to degradation by trypsin, a processing enzyme for HD5, and resulted in a diminished production of mature HD5. These results suggest that potent protease resistance of α -defensins due to their three disulfide bonds may contribute to the maintenance of a healthy intestinal environment and the pathogenesis of diseases such as IBD. Changes in Paneth cell α -defensin expression and/or secretion may contribute to CD, and changes in the intestinal microbiota may mediate the pathogenesis [109]. To test this, it is important to monitor sequential changes in the levels of α -defensins secreted into the intestinal lumen. Secreted cryptdin4 levels in feces were reduced significantly in IL10-null mice compared with wild type mice [72], suggesting that diminished Crp4 secretion led to dysbiosis. Failure of cryptdin production and/or cryptdin secretion in IL10-null mice may contribute to the reduction in secreted cryptdin. Whether or not decreased production or secretion of α -defensins in CD patients is an independent event in CD or secondary event due to severe inflammation with massive loss of small intestinal epithelia, including Paneth cells, is still controversial.

Paneth cells are selectively depleted during graft-versus-host disease (GVHD), resulting in a marked reduction in expression and secretion of α -defensins, which selectively kill pathogenic bacteria while preserving the commensal population [110]. In the mouse GVHD model, the diversity of the intestinal microbiota is lost, an overwhelming expansion of *Escherichia coli* occurs, and the GVHD mice die of *E. coli* septicemia [110]. These results revealed a direct link between Paneth cell loss in GVHD and a dramatic shift in the microbiota composition from commensals to specific pathogens. Thus, Paneth cell depletion with subsequent loss of α -defensins leads to vital dysbiosis [111, 112]. Furthermore, obese subjects reportedly show increased HD5 mRNA expression and decreased HD5 protein in comparison with healthy subjects [113].

Although it is not yet clear whether Paneth cells of obese people actually secrete less α -defensins relative to lean subjects, α -defensins and the intestinal microbiota composition may have a pathophysiological link with obesity. In addition, ischemia/reperfusion injury of the small intestine [106] and *Toxoplasma* infection [114] in mice have been shown to induce Paneth cell depletion, leading to dysbiosis and severe infection.

From recent advances regarding the roles of Paneth cells and their α -defensins, a consensus view has emerged that suggested that a reduction or excess of α -defensins or disruption of the α -defensin tertiary structure results in dysbiosis due to Paneth cell dysfunction. Paneth cells also have a critical role in establishing the stem cell niche contributing directly to epithelial regeneration and differentiation [115]. Thus, we suggest that varied Paneth cell abnormalities and their production and release of α -defensins are associated with various diseases including infectious diseases, IBD and obesity and that they may also contribute to morbidity in other diseases by failing to maintain eubiosis, thereby disrupting the intestinal environment. Further studies are necessary to understand Paneth cell biology and to prove links among Paneth cells, the intestinal microbiota and diverse diseases.

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