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Paneth Cell Defects Induce Microbiota Dysbiosis in Mice and Promote Visceral Hypersensitivity

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BACKGROUND & AIMS: Separation of newborn rats from their mothers induces visceral hypersensitivity and impaired epithelial secretory cell lineages when they are adults. Little is known about the mechanisms by which maternal separation causes visceral hypersensitivity or its relationship with defects in epithelial secretory cell lineages. **METHODS:** We performed studies with C3H/HeN mice separated from their mothers as newborns and mice genetically engineered ($Sox9^{flox/flox}$-vil-cre on C57BL/6 background) to have deficiencies in Paneth cells. Paneth cell deficiency was assessed by lysozyme staining of ileum tissues and lysozyme activity in fecal samples. When mice were 50 days old, their abdominal response to colorectal distension was assessed by electromyography. Fecal samples were collected and microbiota were analyzed using Gut Low-Density Array quantitative polymerase chain reaction. **RESULTS:** Mice with maternal separation developed visceral hypersensitivity and defects in Paneth cells, as reported from rats, compared with mice without maternal separation. $Sox9^{flox/flox}$-vil-Cre mice also had increased visceral hypersensitivity compared with control littermate $Sox9^{flox/flox}$ mice. Fecal samples from mice with maternal separation and from $Sox9^{flox/flox}$-vil-cre mice had evidence for intestinal dysbiosis of the microbiota, characterized by expansion of *Escherichia coli*. Daily gavage of conventional C3H/HeN adult mice with 10^9 commensal *E coli* induced visceral hypersensitivity. Conversely, daily oral administration of lysozyme prevented expansion of *E coli* during maternal separation and visceral hypersensitivity. **CONCLUSIONS:** Mice with defects in Paneth cells (induced by maternal separation or genetically engineered) have intestinal expansion of *E coli* leading to visceral hypersensitivity. These findings provide evidence that Paneth cell function and intestinal dysbiosis are involved in visceral sensitivity.

Keywords: Stress; Antimicrobial Activity; Abdominal Pain; Lysozyme.

BASIC AND TRANSLATIONAL AT

The intestine is colonized by trillions of commensal microorganisms that constitute a complex microbial community.[1–3] These microorganisms live in symbiotic and mutualistic relationship with the host and, as such, the microbiota is essential for mediating physiology, metabolism, and host immune response. Intestinal homeostasis relies on a tightly regulated crosstalk between commensal bacteria, intestinal epithelial cells, and mucosal immune cells. Innate immunity provides the first line of defense against invading microorganisms and confers protection by triggering inflammatory and antimicrobial responses.

Paneth cells producing enteric antimicrobial peptides are important players in small intestine innate immunity. Paneth cells located at the bottom of crypts produce and secrete various antimicrobial proteins or peptides (AMP) like lysozyme, phospholipase A2 (PLA2), Reg3 lectins, and α-defensin named cryptdin in mice (for review see Bevins and Salzman[4]). Many studies performed in rodent models highlight Paneth cell contribution in establishment of an appropriate colonization with commensal microbiota[5] and host protection from enteric pathogens.[6,7] Studies show that both gut microbiota profile[8] and AMP repertoire[8,9] are dependent on mouse strain, suggesting the role of Paneth cell AMP in shaping intestinal microbiota. Even though some AMPs are dependent on microbiota colonization, Paneth cell–derived AMPs, such as cryptdin[10] lysozyme and PLA2,[11,12] are expressed independently of microbiota colonization.

A defect in Paneth cell numbers and/or antimicrobial activity (functionality) has been incriminated in susceptibility to enteric infections as well as in multifactorial organic gastrointestinal disorders like necrotizing enterocolitis (NEC)[13] and Crohn's disease (CD),[14,15] both characterized by intestinal microbiota dysbiosis. One hypothesis is that Paneth cell failure triggers microbiota dysbiosis in favor of opportunistic bacteria that might trigger intestinal disorders in predisposed individuals.

Furthermore, it is well accepted that genetic and environmental factors contribute to the development of organic gastrointestinal disorders like CD,[16,17] but also functional ones like irritable bowel syndrome (IBS). Despite high discrepancies concerning the severity of intestinal inflammation between CD and IBS, some common

Abbreviations used in this paper: **AMP, antimicrobial peptides; ANOVA, analysis of variance; AUC, area under the curve; CD, Crohn's disease; CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; EMG, electromyograph; GVHD, graft-versus-host disease; IBS, irritable bowel syndrome; IFNγ, interferon gamma; IL, interleukin; MS, maternal separation; NEC, necrotizing enterocolitis; PCR, polymerase chain reaction; PLS-DA, partial least-squares discriminant analysis; TNFα, tumor necrosis factor α; VIP, variable importance in projection.**

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EDITOR'S NOTES

BACKGROUND AND CONTEXT

Maternal separation in rats is known to impair Paneth cells and triggers visceral hypersensitivity. However, a potential link between a defect of Paneth cells and visceral hypersensitivity remained unknown.

NEW FINDINGS

Paneth cell defects lead to commensal *Escherichia coli* expansion in intestinal microbiota that are responsible for visceral hypersensitivity and that can be prevented with lysozyme gavage.

LIMITATIONS

Although this study suggests that MS-induced intestinal low-grade inflammation could be responsible for Paneth cell defect, it did not fully prove this hypothesis.

IMPACT

Paneth cells are an important contributor to visceral sensitivity. This study highlights antimicrobial compounds as novel therapeutic or preventive strategies to overcome the critical influence of fecal dysbiosis on visceral hypersensitivity.

pathophysiological features, such as visceral hypersensitivity[18] and microbiota dysbiosis[19] have been reported. Interestingly, among environmental factors affecting the course of these diseases, early life adverse events play a crucial role.[20,21] Indeed, maternal separation (MS) performed in rats decreases epithelial secretory cell lineages, including Paneth cells,[22] and induces visceral hypersensitivity.[23] However, a mechanistic link between Paneth cell defect and visceral hypersensitivity has never been investigated.

In the present study, by establishing a mouse model of MS and using various mouse models including $Sox9^{flox/flox}$-vil-Cre mice genetically engineered to be deficient in Paneth cells, we sought to assess the direct influence of Paneth cell antimicrobial functions on host fecal microbiota composition and subsequent consequences on intestinal pathophysiology.

Our results reveal for the first time a key role for Paneth cell–produced lysozyme on visceral sensitivity regulation through the prevention of *E coli* expansion in intestinal microbiota.

Materials and Methods

Mouse Models

All experimental protocols described in this study were approved by the local Animal Care Use Committee (Comité d'Ethique de Pharmacologie-Toxicologie de Toulouse - Midi-Pyrénées, France) registered as N°86 at the Ministry of Research and Higher Education (N° 0029/SMVT), and conducted in accordance with the European directive 2010/63/UE. All experiments were performed on Day (D) 50 mice. More details are in Supplementary Materials and Methods.

MS Protocol

To minimize cannibalism induced by perinatal stress, we used C3H/HeN mice known to be excellent breeders. Pups were separated from their dam and the rest of the litter 3 hours per day. MS was repeated for 10 working days, weekend excluded, between day (D)2 and D15. Control pups were left with their dam (Supplementary Figure 1*A*). More details are in Supplementary Materials and Methods.

$Sox9^{flox/flox}$-vil-cre Mice

Heterozygote Villin-Cre (vil-Cre) mice (kindly given by S. Robine) in which the Cre recombinase is expressed specifically in the intestinal epithelium were crossed with $Sox9^{flox/flox}$ mice (kindly given by T. Pedron), which have both *Sox9* alleles flanked by floxP sequences. This generated $Sox9^{flox/flox}$-vil-Cre mice, with an intestinal epithelium lacking Sox9 protein, indicating effective vil-Cre–mediated recombination of the $Sox9^{flox}$ allele. $Sox9^{flox/flox}$-vil-Cre mice developed as their control littermates ($Sox9^{flox/flox}$).

Oral Gavage of Commensal E coli Streptomycin Resistant

Live *Escherichia coli* were isolated from feces of naïve healthy C3H/HeN mice by culture on selective ChromID coli plates (Biomérieux, Marcy L'étoile, France). To facilitate monitoring of this commensal *E coli* isolate in feces of mice after gavage, a spontaneous streptomycin-resistant mutant of the commensal isolate was generated. More details are in Supplementary Materials and Methods.

Nulliparous female 35-day-old C3H/HeN mice (Janvier, Roubaix, France) were randomized in 2 groups: vehicle, which received 100 μL of bicarbonate buffer (0.2 M, pH 8.2) per os per day, and *E coli* gavage, which received 10^9 colony-forming units (CFUs) of streptomycin-resistant *E coli* in 100 μL of bicarbonate buffer per os per day. Animals were analyzed after 15 days of daily gavage (Supplementary Figure 1*B*).

Lysozyme Treatment

Mice submitted or not to the MS protocol received an oral gavage with lysozyme (ref L6876; SIGMA, Saint Quentin Fallavier, France) from D35 to D50. Animals were randomized in 2 groups: vehicle, which received 200 μL of bicarbonate buffer per os per day, and lysozyme gavage, which received 240 U of active lysozyme in 200 μL of bicarbonate buffer per os per day (Supplementary Figure 1*C*).

Visceral Sensitivity

Mice were equipped with 3 NiCr wire electrodes implanted into the abdominal external oblique muscle at D47 and kept individually after surgery. The electromyographic (EMG) activity was recorded and analyzed with a Powerlab Chart from AD instrument (Paris, France). EMG recordings began 3 days after surgery. Mice were placed in polypropylene tunnels. A balloon consisting of an arterial embolectomy catheter (Fogarty, 4F; Edwards Laboratories, Santa Ana, CA) was introduced into the rectum at 2.5 cm from the anus and fixed at the base of the tail. The balloon was progressively inflated during 15 seconds by step of 0.02 mL, from 0.02 to 0.1 mL, with 10 minutes wait between each step. The Fogarty embolectomy catheter balloon was calibrated using an electronic caliper gauge and the maximal pressure applied (corresponding to 0.1 mL) was calculated as 63.1 ± 1.7 mm Hg meaning that