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Maturation of Paneth Cells Induces the Refractory State of Newborn Mice to *Shigella* Infection

Maria-Isabel Fernandez,²,³,*† Béatrice Regnault,²‡ Céline Mulet,*† Myriam Tanguy,*† Philippe Jay,§ Philippe J. Sansonetti,⁴,*† and Thierry Pédon*†

The intestinal tract of adult mice is naturally resistant to infection by *Shigella*, the causative agent of bacillary dysentery in humans. Conversely, newborn mice are highly susceptible to intragastric *Shigella* infection and develop inflammatory lesions of the jejunal mucosa, very similar to those observed in the colon of dysenteric patients. However, the susceptibility period is short and one week after birth, animals have acquired a status of resistance characteristic of adult animals. To identify the developmental changes controlling the switch from disease susceptibility to resistance, we performed global gene expression analysis on noninfected and injected intestinal tissues taken from 4-day- and 7-day-old animals. Transcriptomic analysis of 4-day-old mice infected with the invasive *Shigella* strain showed a profile reflecting a strong inflammatory response with no evidence for retrocontrol, suggesting that the invasive process had occurred, whereas inflammation had been controlled after infection with the noninvasive strain. Differences in gene expression profiles between noninfected 4-day- and 7-day-old mice corresponded mainly to genes encoding anti-microbial peptides and proteases, suggesting that these molecules could be candidates for host antimicrobial resistance in the course of shigellosis. Indeed, expression of genes specific of Paneth cells was higher in 7-day- than in 4-day-old mice, and histological analysis indicated that Paneth cells were present only at day 7. Finally, using Sox9<sup>flo</sup>/<sup>Cre</sup>-vill-cre mice, we showed that depletion of Paneth cells restored the susceptibility to *Shigella* of 7-day-old mice, clearly indicating that Paneth cells development is crucial for the clearance of intestinal infection.


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*Shigella* is characterized by an acute intestinal inflammation in response to expression of the *Shigella* invasive phenotype. Understanding the mechanisms of rupture, invasion and inflammatory destruction of the epithelial barrier is essential to analyze the disease process. For this, a relevant animal model is required and mice would by far be the most suitable model. However, the adult mouse intestine is refractory to *Shigella* infection. This could be due to the lack of a surface receptor specifically mediating *Shigella* adhesion/invasion on murine epithelial cells, as described for murine E-cadherin which is unable to bind internalin of *Listeria monocyogenes* unlike its human homologue (1). However, this seems not to be the case because murine intestinal epithelial cell lines and primary murine enterocytes are efficiently invaded by *Shigella* (2), and bacteria are seen invading the epithelium in a model of streptomycin-treated mouse, even though invasion does no lead to a destructive polymorphonuclear leukocytes (PMN)<sup>3</sup> infiltrate (3). If the defect in induction of the inflammatory response is not the consequence of a deficiency in epithelial invasion, it could indeed be due either to poor bacterial sensing or to inefficient production of the combination of chemokines and cytokines required to elicit a strong inflammatory infiltrate, particularly PMN recruitment. Relevant differences may be considered such as hNod1 that requires a tripeptide for efficient intracellular sensing of peptidoglycan, whereas mNod1 recognizes a tetrapeptide (4), or absence in the mouse of the gene encoding IL-8, a chemokine that plays a key role in *Shigella*-induced inflammation in human and rabbit. As a matter of fact, local administration of IL-8 during *Shigella* infection elicits a strong PMN infiltrate in the murine colonic mucosa similar to what is observed in human disease (5). As previously shown, the only instance in which mice appear susceptible to infection with invasive *Shigella* is when inoculation is conducted intragastrically in the immediate postnatal period (i.e., newborn mice <1 wk old). These animals develop acute, lethal enteritis (mostly jejunal). This model does not anatomically reproduce the rectocolitis observed in humans, but it still mimics the human disease because epithelial invasion, inflammation, rupture, detachment, and epithelial apoptosis are observed (6). This model may also unravel new factors governing mouse resistance to intestinal infection since those previously considered (i.e., IL-8 and nod) are unlikely to be involved in this age-dependent susceptibility process. In contrast, this model is all the more interesting, due to the susceptibility window that closes before the end of the first week of life, indicating that the susceptibility phenotype of the mouse small intestine to invasive *Shigella*

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реди названіх у цьому праці: PMN, polymorphonuclear leukocyte; DibAC<sub>4</sub>-bis(1,3-dibutylbarbituric acid)-trimethine oxanol; CD, Crohn’s disease.

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depends on developmental properties. Thus, we decided to analyze this model to better understand basic mechanisms that may dictate susceptibility of intestinal mucosa to *Shigella*. In this model, it appears that the maturation of epithelial anti-microbial defense systems is at the heart of the developmentally regulated switch between a susceptible to a refractory state to *Shigella* infection.

**Materials and Methods**

**Bacterial strains**

Two *S. flexneri* strains were used in this study: *M90T* (INV\(^+\)), an invasive isolate belonging to serotype 5a; and *BSI76* (INV\(^-\)), its isogenic, non-invasive derivative that has been cured of the virulence plasmid pWR100 (7). For intragastric (i.g.) inoculation of mice, an overnight culture at 37°C on agar plates was diluted in sterile saline.

**In vivo infection experiments**

Newborn BALB/c mice (Janvier) 4- or 7-day-old and 7-day-old *S. flexneri* wild-type mice were used in this study. Mice were separated from their mothers 2 h before the experiment and inoculated i.g. with 50 μl of bacterial suspensions containing 5.10⁸ INV\(^+\) or INV\(^-\) bacteria. Control animals received an equal volume of saline. Newborn mice were maintained at a temperature of 30°C during the experimental course. After 2 or 4 h of infection, mice were sacrificed and a 1 cm-long jejunal section of their intestine was removed for RNA extraction and preparation of tissues for histopathological analysis. For the dithizone experiments, newborn mice were separated from their mothers and injected i.p. with 100 μl of physiological water, LiCO₃ (25 mM), or dithizone (6 mg/ml in LiCO₃). Five hours later, mice were inoculated i.g. with 50 μl of the bacterial suspension containing 5.10⁷ bacteria. Three or five hours later, intestinal tissues were removed and processed for histological staining.

**Microarray analysis of gene expression**

Three to four biological replicates were used in this study. After infection, a section of intestine was removed, and homogenized in 1 ml of Trizol (Invitrogen) with Ultra-Turrax. Then, total RNA was extracted according to the manufacturer’s instructions, and cleaned with RNeasy Kit (Qiagen). RNA quantity and quality were analyzed by the Agilent Bioanalyzer 2100. Synthesis, hybridization, and labeling of cRNA were done as described (8). A total of 10 μg of cRNA were fragmented and hybridized to the Affymetrix MOE430a probe array cartridge. After staining using PE-streptavidin, arrays were scanned at 488 nm using an Argon-ion laser. Preprocessed data were robust multi-chip analysis (RMA) (9) applied to process individual probe values (perfect match) and to generate summary values for each probe set (transcript). Comparison between the 4- and 7-day-old noninfected samples was computed with an unpaired Welch t test with a p value threshold of 0.05 and filtered to a signal log₂ ratio threshold of 1. Hierarchical clustering of proinflammatory genes during infection of newborn mice infection with *Shigella*.

**Histological processing and staining of tissue samples**

Intestinal tissues were fixed overnight at 4°C in 4% paraformaldehyde in PBS and processed for paraffin embedding. Thin section of 5 μM were cut, dried, and stored at 4°C. Sections were rehydrated, permeabilized in 0.1% Triton X-100, washed in PBS. Endogenous peroxidase activity was quenched with 3% H₂O₂ in water for 10 min, washed in PBS and sections were saturated by 1% BSA. For detection of bacteria, samples were then incubated with a rabbit polyclonal serum against *S. flexneri* 5a LPS for 1 h at room temperature. After washing, sections were incubated with a peroxidase-labeled goat anti-rabbit Ig for 1 h at room temperature. After washing, a drop of the substrate chromogen DakoCytomation, AEC\(^+\) (Dako) was placed on the section for 1 min. and hematoxylin was used as counterstain. Slides were observed on a Nikon, Eclipse E800 microscope. For the detection of neutrophils, a rat IgG (MCA771G; Serotec) directed against mouse polymorphonuclear neutrophils (PMN) was revealed using a goat anti-rat Ab. For lysozyme detection, a goat IgG (W-20; Santa Cruz Biotechnology) directed against Lysozyme C was revealed using a biotinylated rabbit anti-goat Ab, followed by peroxidase-streptavidin incubation. Other sections were processed using periodic acid-Schiff staining and hematoxylin to visualize Goblets and Paneth cells. Detection of apoptosis on histological sections was performed using the TACS TdT kit (R&D Systems).

**Measurement of tissue antimicrobial activity**

After separation from their mothers, 4- or 7-day-old mice were inoculated i.g. with 3·10⁸ bacteria. Three hours post infection, a 5 cm-long intestinal section was removed and perfused with 0.5 ml of PBS. Flushed bacteria were incubated with an anti-LPS 5a rabbit polyclonal serum followed by incubation with Cy5-labeled goat anti-rabbit Ig. Bacteria were then incubated with bis-(1,3-dibutylbarbituric acid)-trimethoxine oxanil (DiBAC\(_4\); Molecular Probes) at 10 μg/ml for 10 min at room temperature before acquisition (11). Analysis of DiBAC\(_4\)-fluorescent bacteria was performed on Shigella-LPS 5a positive gated bacteria with a FACScanCalibur apparatus using a CellQuestPro software.

**Results**

Intestinal gene expression and modulation during newborn mice infection with *Shigella*

When infected with an invasive (INV\(^+\)) strain, 4-day-old mice develop an acute jejunitis followed by death of animals, whereas 4-day-old mice infected with a noninvasive (INV\(^-\)) strain, and...
7-day-old mice infected either with INV\(^+\) or with INV\(^-\) are resistant (6). We first attempted to correlate the brisk appearance of the resistance status to a certain pattern of gene expression in intestinal tissues. For this purpose, samples were taken from 4-day- and 7-day-old mice infected for 2 and 4 h with INV\(^+\) or INV\(^-\) strains. RNA corresponding to the respective samples was extracted and hybridized on a microarray displaying 22,000 murine transcripts representing 15,000 genes. Redundant probe sets targeting a single gene were removed. Fig. 1 shows the number of genes whose expression was up- or down-modulated during infections. Although neither macroscopic nor microscopic alteration of the tissues was observed during infection with INV\(^-\), the intestinal epithelium showed a response pattern to this strain. It was dominated by an increase in expression of a set of proinflammatory genes, likely due to expression of pattern recognition receptors on intestinal epithelial cells recognizing PAMPs expressed by inoculated microorganisms. A higher number of modulated genes (i.e., 246 up- or down-regulated genes) appeared in 4-day-old mice infected during 4 h with the invasive strain of \textit{Shigella}, compared with only 24 genes modulated in similar experimental conditions in 7-day-old mice. Among the modulated genes, a large cluster of genes encoding cytokines and chemokines, mainly proinflammatory genes, was regulated during bacterial infection of 4-day-old animals, as observed in a previous study using macroarrays dedicated to these two families of molecules (6). Hierarchical clustering of proinflammatory genes expression represented in Fig. 2 clearly shows that after 2 h of infection these genes were up-regulated in 4-day-old animals, then their expression was rapidly controlled and returned to basal level 4 h after infection with INV\(^-\). Conversely, these genes continued to be highly expressed after 4 h of infection with INV\(^+\). This was particularly the case for the main proinflammatory genes CXCL1 and CXCL2, and for CCL20. The level of expression of the genes encoding, IL-1\(\beta\) and IL-6 decreased between 2 and 4 h of infection in the 4-day-old mice infected with INV\(^+\) or INV\(^-\). Conversely, there was absolutely no variation in this cluster of genes in 7-day-old animals infected either with INV\(^+\) or with INV\(^-\), reflecting the appearance of a gut refractory state to infection/inflammation by \textit{Shigella}.

**Expression of Paneth cells related genes is higher in 7-day- than in 4-day-old mice**

The striking difference in the number of modulated genes during infection of 4- and 7-day-old mice led us to compare gene expression levels in intestinal tissues of noninfected 4- and 7-day-old mice to determine whether a particular transcriptional signature could be correlated with the switch from a susceptible to a refractory state in \textit{Shigella}-infected newborn mice. Expression of 29 genes appeared higher in 4-day-old mice than in 7-day-old mice, whereas 25 genes were more expressed in the later than in the former condition. Interestingly, comparison of gene expression patterns between 4- and 7-day-old control mice showed that the expression of genes primarily expressed in Paneth cells (i.e., \(\alpha\)-5 defensin, trypsin 2, elastase, pancreatic lipase-related protein) was significantly higher in 7-day-old than in 4-day-old mice (Fig. 3A and data not shown). Paneth cells are localized at the bottom of the intestinal crypts of 7-day-old mice. After treatment with Dithizone or its vehicle (LiCO3), mice were infected with the invasive or noninvasive strains of \textit{Shigella}. Five hours postinfection sections of intestine were removed and processed to immunostaining as described in Materials and Methods section. A, Bacteria staining using anti-LPS polyclonal Ab. B, Polymorphonuclear staining.
crypts of Lieberkühn in the small intestine and produce α-defensins (cryptdins), lysozyme, lectin RegIII, and phospholipase A2 (12, 13), as well as a large number of serine proteases and serine protease inhibitors (14). They are thought to play a role in innate immunity by protecting the host against infection by secreting this set of anti-microbial peptides and associated molecules, an important consequence being the decrease in concentration of bacteria in the intestinal stem cells immediate environment (13). The present gene expression study suggested that maturation of Paneth cells between 4- and 7-day-old mice could explain the occurrence of resistance of mice to Shigella infection. Immuno-histological staining of sections from the intestine of 4-day- and 7-day-old animals using an anti-lysozyme Ab, which stains basic Paneth cells granules, confirmed that these cells were absent in the former (Fig. 3B) and present in the later (Fig. 3D) as confirmed also by staining with periodic acid-Schiff’s dye (Fig. 3, C and E).

Depletion of Paneth cells restores susceptibility to Shigella infection of 7-day-old mice

To functionally confirm the above correlation, we studied whether 7-day-old mice would become Shigella-susceptible if their Paneth cells were made nonfunctional. Paneth cells are functionally zinc-dependent. Indeed, zinc deficiency is common in developing countries and zinc supplementation reduces morbidity due to diarrheal disease in toddlers (15). Kelly et al. (16) postulated that zinc deficiency, in conditions of malnutrition, impairs the function of Paneth cells whereas the other intestinal epithelial lineages remain unaltered. To study the functionality of Paneth cells, and confirming that absence of these cells was indeed permissiveness was a consequence of the lack of these cells.

As our initial results suggested that these cells may play a key role in the control of Shigella infection in newborn mice, we injected 7-day-old mice i.p. with 100 μl of 6 mg/ml dithizone, with its vehicle (LiCo3, 25 mM) alone, or with saline as controls. Five hours later, we infected mice i.g. with Shigella. Three or five hours postinfection, jejunal segments were removed and fixed in paraformaldehyde for histological processing. When infection was performed with INV', bacteria appeared massively present in the lumen and in the intestinal tissue (Fig. 4A, upper panel), as observed in 4-day-old animals infected with INV' (6). This was not observed when mice were treated with saline or with LiCo3, the dithizone vehicle. Moreover, bacteria were barely observed in dithizone-treated mice infected with INV' (Fig. 4A, lower panel). In addition, to monitor inflammation characterized by an increasing infiltrate of PMN, immunostaining was performed using an Ab directed against mouse PMNs. No PMN was seen in control mice, in contrast to the massive PMN infiltrate observed in dithizone-treated, INV' infected, 7-day-old mice (Fig. 4B, upper panel). When 7-day-old mice were treated with dithizone and infected with INV', the PMN infiltrate was not observed (Fig. 4B, lower panel).

To confirm that the phenotype of Shigella susceptibility observed in 7-day-old dithizone-treated mice was actually due to absence of antimicrobial molecules produced by Paneth cells, and not by adjacent effects on other components of the gut innate immune system, we performed infections of 7-day-old Sox9<sup>flox/flox</sup> -vil-cre mice with an anti-lysozyme Ab revealed the absence of this molecule, suggesting that the crypts were devoid of Paneth cells (Fig. 5A). After infection of 7-day-old Sox9<sup>flox/flox</sup> -vil-cre mice with INV', bacteria were seen in the epithelium (Fig. 5B) and a massive PMN infiltrate was observed (Fig. 5C) similar to what was seen in 7-day-old dithizone-treated mice, indicating the relevance of this model to study the functionality of Paneth cells, and confirming that acquired permissiveness was a consequence of the lack of these cells.

Shigella induces epithelial apoptosis in dithizone-treated mice

It has been previously described that treatment of rats with the zinc chelator diphenylthiocarbazone (dithizone) selectively destroys Paneth cells (17, 18). Zinc was also described as a potent modulator of immune response. For example, zinc deficiency in conditions of malnutrition, impairs the function of Paneth cells between 4- and 7-day-old mice could explain the occurrence of resistance of mice to Shigella infection. Immuno-histological staining of sections from the intestine of 4-day- and 7-day-old animals using an anti-lysozyme Ab, which stains basic Paneth cells granules, confirmed that these cells were absent in the former (Fig. 3B) and present in the later (Fig. 3D) as confirmed also by staining with periodic acid-Schiff’s dye (Fig. 3, C and E).

Table I. Modulated genes involved in apoptosis following infection of 4-day-old mice with INV' for 4 h

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Gene Description</th>
<th>SLR</th>
<th>FC</th>
<th>Adjusted p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1420772_a_at</td>
<td>glucocorticoid-induced leucine zipper</td>
<td>-1.66</td>
<td>-3.16</td>
<td>0.001916</td>
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<tr>
<td>1419004_s_at</td>
<td>B cell leukemia/lymphoma 2 related protein A1a</td>
<td>1.16</td>
<td>2.23</td>
<td>0.004949</td>
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<tr>
<td>1421392_a_at</td>
<td>caspase 8</td>
<td>1.35</td>
<td>2.55</td>
<td>0.001251</td>
</tr>
<tr>
<td>1424552_a_at</td>
<td>caspase 8</td>
<td>1.35</td>
<td>2.55</td>
<td>0.001251</td>
</tr>
<tr>
<td>1419004_s_at</td>
<td>B cell leukemia/lymphoma 2 related protein A1a</td>
<td>1.16</td>
<td>2.23</td>
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<tr>
<td>1421392_a_at</td>
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<td>1424552_a_at</td>
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<td>2.55</td>
<td>0.001251</td>
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<tr>
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<td>3.38</td>
<td>10.41</td>
<td>0.000061</td>
</tr>
</tbody>
</table>

<sup>a</sup>Signal log ratio and fold change indicate the difference in gene expression observed in a log2 or in a linear scale, respectively. SLR, Signal log ratio; FC, fold change.
The killing of Shigella is more important in the 7-day-old animals, where Paneth cells are functional and secrete antimicrobial products, than in the 4-day-old animals, where Paneth cells are not present or still immature.

Taken together, our findings support the concept that Paneth cells secrete key antibacterial effectors that maintain pathogens under a threshold of density that prevents their triggering of the infectious process. This concept can easily be extended to control of commensal density, particularly in the immediate vicinity of the epithelium surface. This is likely to be sufficient to account for the lack of inflammation and epithelial apoptosis observed in 7-day-old mice. Whether Paneth cells also produce anti-inflammatory and anti-apoptotic molecules needs further evaluation.

**Discussion**

We have investigated the events leading to resistance of mice to Shigella, the causative agent of human bacillary dysentery. The switch from susceptibility in 4-day-old mice to resistance in 7-day-old mice, that persists in adult animals led us to ask two major questions: 1) what is the gene expression profile of 4- and 7-day-old mice infected with noninvasive (INV-) or invasive (INV+) Shigella strains and 2) could gene expression analysis suggest clues to explain the switch between the susceptible and resistant states. The difference of susceptibility during infection following the age of infected mice was already described and interestingly found to depend of the pathogen. Indeed, whereas neonates were more sensitive than adults to Listeria monocytogenes infection (24), they were resistant to i.g. infection with Yersinia enterolitica (25). To gain insight into these questions during infection of mice with Shigella, DNA chip hybridizations coupled to immunohisto logical and flow cytometry studies were performed. Our results confirmed that the intestinal epithelium of 7-day-old mice was less affected by Shigella: only 24 genes modulated after 4 h of infection with INV+, instead of 246 in similar experimental conditions in 4-day-old mice. In addition, results have shown that the noninvasive strain was recognized by the intestinal epithelium and that the expression of proinflammatory genes, which were modulated at early time of infection, was rapidly controlled. Conversely, expression of these genes was high throughout the time course of infection with the invasive strain. The noninvasive strain was probably recognized by the innate immune system via TLRs sensing through the release of PAMPs in the jejunum. Massive inflammation was not observed, suggesting that the inflammatory process was rapidly controlled, perhaps to the induction of a tolerance state, as shown by Lotz et al. (26). With the invasive strain, tissue destruction and massive inflammation of the jejunum did not make this control possible.

Concerning the second point, transcriptomic analysis and histological staining, indicated that major differences existed between 4- and 7-day-old mice regarding expression of genes that are essentially encoded by Paneth cells. Moreover, destruction of the Paneth cells of 7-day-old mice with dithizone, a zinc chelator, led these animals to become susceptible to Shigella infection. These results corroborate previous studies showing that full Paneth cells differentiation occurred few days after birth (27, 28). Moreover infection of mice invalidated for the transcription factor SOX9, essential for Paneth cell differentiation, confirmed our results obtained with dithizone-treated mice.

Among genes modulated upon infection of 4-day-old mice with invasive Shigella we identified a set of genes encoding proteins involved in induction of the apoptosis process, such as Gadd45γ, caspase 8, and BH3-interacting domain death agonist. Apoptosis of intestinal epithelial cells following bacterial infection was observed previously in vitro on a human cell line infected with S.
enterica serovar Dublin (29) and also in vivo, in a mouse model of shigellosis after treatment of mice with streptomycin to deplete the intestinal microflora before infection (3). Using a neonatal mouse model of Cryptosporidium parvum infection, Sasahara et al. (30) observed an induction of apoptosis in the small intestine. In the present study, the depletion of Paneth cells with dithizone was found to restore the capacity of Shigella to induce apoptosis in 7-day-old mice, showing again the crucial role of Paneth cells secreted products as a shield upon infection.

Wilson et al. (31) observed that a lack of α-defensin maturation in the intestine, following targeted disruption of the matrilysin gene, increases susceptibility to bacteria. In contrast, transgenic mice overexpressing the human α-defensin 5 appeared resistant to virulent S. typhimurium i.g. infection (11). The present study clearly shows that the maturation of Paneth cells, thus the expression and secretion of antimicrobial molecules, was followed by the death of bacteria reaching the jejenum, as suggested by the difference of membrane polarization potential of the bacteria recovered from the intestinal lumen of 7-day- and 4-day-old infected mice. This observation is consistent with data showing that an increase of the number of E. coli recovered from jejunal and ileal lavages is significantly higher in dithizone-treated neonatal rats, than in controls (18). It should be noted that in dithizone-treated mice infected with the noninvasive strain, only few bacteria were observed in the lumen, in contrast to what happened with the invasive strain, suggesting that inflammation induced by the invasive phenotype allowed growth/multiplication of the bacteria.

In humans, a decrease of α-defensin expression in intestinal biopsies is associated with a higher risk of infectious diarrhea (32). In line with these observations, our results highlight the critical role played by Paneth cells and their associated effectors in the control of intestinal infection, in agreement with the fact that shigellosis appears only in the human colon and not in the small intestine where Paneth cells are abundant. Secretion of antimicrobial peptides by Paneth cells is therefore likely to control the density of bacteria direct expression of an intestinal bactericidal lectin.

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Disclosures

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