LPLUNC1 Modulates Innate Immune Responses to *Vibrio cholerae*

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**Background.** Recent studies demonstrate that long palate, lung, and nasal epithelium clone 1 protein (LPLUNC1) is involved in immune responses to *Vibrio cholerae*, and that variations in the LPLUNC1 promoter influence susceptibility to severe cholera in humans. However, no functional role for LPLUNC1 has been identified.

**Methods.** We investigated the role of LPLUNC1 in immune responses to *V. cholerae*, assessing its effect on bacterial growth and killing and on innate inflammatory responses to bacterial outer membrane components, including purified lipopolysaccharide (LPS) and outer membrane vesicles. We performed immunostaining for LPLUNC1 in duodenal biopsies from cholera patients and uninfected controls.

**Results.** LPLUNC1 decreased proinflammatory innate immune responses to *V. cholerae* and *Escherichia coli* LPS. The effect of LPLUNC1 was dose-dependent and occurred in a TLR4-dependent manner. LPLUNC1 did not affect lipoprotein-mediated TLR2 activation. Immunostaining demonstrated expression of LPLUNC1 in Paneth cells in cholera patients and controls.

**Conclusions.** Our results demonstrate that LPLUNC1 is expressed in Paneth cells and likely plays a role in modulating host inflammatory responses to *V. cholerae* infection. Attenuation of innate immune responses to LPS by LPLUNC1 may have implications for the maintenance of immune homeostasis in the intestine.

Cholera is a severe diarrheal disease that results from infection with the bacterium *Vibrio cholerae*. The World Health Organization (WHO) estimates that there are 3–5 million cases of cholera annually, with more than 100 000 deaths [1]. Strains of *V. cholerae* can be differentiated serologically by the O side chain of lipopolysaccharide (LPS). *V. cholerae* serogroup O1 causes the majority of cases, and the El Tor biotype is responsible for the current global pandemic of cholera. *V. cholerae* O1 strains can be further differentiated into 2 major serotypes, Ogawa and Inaba. Two key virulence factors of *V. cholerae* are cholera toxin (CT), an ADP-ribosylating toxin that stimulates intestinal fluid secretion [2], and the toxin coregulated pilus (TCP), a type IV pilus essential for intestinal colonization [3]. CT is a protein exotoxin consisting of a single, active A subunit associated with 5 B (CtxB) subunits. The CtxB pentamer binds to ganglioside GM1 on eukaryotic cells; the A subunit is then translocated intracellularly, where it elevates cAMP and leads to secretory diarrhea that may be fatal in over 50% of untreated cases [4].

Cholera is considered a prototypical noninflammatory, toxigenic diarrhea. In acute cholera, there are no gross changes in the intestinal mucosa, and histopathology of duodenal biopsies from cholera patients does not demonstrate changes in the integrity of mucosal tissue [5]. However, acute *V. cholerae* O1 infection triggers mucosal

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in innate immune responses with infiltration of neutrophils, degranulation of mast cells and eosinophils, and expression of proinflammatory molecules, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in the intestinal mucosa [5–7]. Gene expression data from duodenal biopsies of cholera patients demonstrate up-regulation of many genes with established or possible roles in innate immunity. In particular, long palate, lung, nasal epithelium clone protein 1 (LPLUNC1) is the most highly up-regulated transcript during acute cholera, as compared with convalescence [8]. An association between LPLUNC1 and cholera is further supported by a recent candidate gene association study. In a family-based study in Bangladesh, a significant association between LPLUNC1 and cholera, confirmed by Western blotting in our laboratory with both anti-DDK and anti-LPLUNC1 antibody (Sigma).

**METHODS**

**Bacteria, Cells, and Reagents**

*V. cholerae* O1 El Tor serotype Inaba strain N16961 and classical serotype Ogawa strain O395 were grown in LB media plus streptomycin 100 µg/mL. *E. coli DH5α* Δpir strain (Sigma) was used for the bactericidal assay. Preparation and purification of *V. cholerae* Ogawa or Inaba LPS was conducted as described elsewhere [17]. *V. cholerae* outer membrane vesicles (OMV) were isolated and prepared as described elsewhere [18].

HEK 293 cells stably expressing yellow fluorescence protein (YFP)-tagged TLR4/MD2 (HEK-TLR4/MD2) were cultured in Dulbecco modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). SZ10 cells stably expressing an NF-κB luciferase reporter, as well as TLR2 and CD14, were also used for reporter studies and were maintained in DMEM with 10% FBS. The use of these cells has been described in previous studies [19–21].

Human recombinant LPLUNC1 protein was purchased from Origene. According to the manufacturer, the protein was prepared by transfection of HEK cells with a Myc, DDK (Flag)-tagged LPLUNC1 plasmid. Supernatants were purified using anti-DDK affinity column, and the expression of LPLUNC1 was confirmed by Western blotting in our laboratory with both anti-DDK and anti-LPLUNC1 antibody (Sigma).

**Bacterial Growth and Bactericidal Assays**

To test for growth-inhibiting effects of LPLUNC1, stationary phase cultures of *V. cholerae* N16961 and O395 were diluted 100-fold in LB broth in a 96-well plate and exposed to a 2-fold dilution series of LPLUNC1. For bactericidal assays, organisms were cultured overnight in Luria-Bertani (LB) broth at 37°C. For assays of cells from log-phase cultures, overnight cultures were diluted 1:100 in LB medium and grown to an optical density at 600 nm (OD600) of 0.5. Cells were washed twice, and a 20 µL volume of bacterial suspension was added to 96-well plates. Zero to 10 µg/mL of LPLUNC1 was incubated with cells for 1 hour at 37°C.

**Transfection and Reporter Gene Assays**

Transfection of HEK-TLR4/MD2 cells was performed using GeneJuice Transfection Reagent (Novagen). The cells were seeded in 96-well plates at 30 000 cells/well and transfected 24 hours later with a total of 0.3 µg DNA per well. The transfected DNA included 80 ng of NF-κB-driven firefly luciferase plasmid and 20 ng of HSV-TK promoter-driven *Renilla* luciferase plasmid (Promega), along with a human CD14 construct cloned into pCDNA3 at a concentration of 10 ng/well. Empty pCDNA3 vector was used to bring the total amount of transfected DNA to 0.3 µg per well. After 24 hours, recombinant LPLUNC1 or PBS was added to the cells 1 hour prior to stimulation with LPS purified from either *E. coli* or *V. cholerae* O1 serotype Inaba or Ogawa. Cells were lysed after 6 hours using 50 µL Passive Lysis Buffer (Promega) and firefly and *Renilla* B-dependent firefly luciferase activity to NF-κB-independent reporter, as well as TLR2 and CD14, were also used for reporter studies and were maintained in DMEM with 10% FBS. The use of these cells has been described in previous studies [19–21].

**ELISA**

Cell-free supernatants were collected and assayed via enzyme-linked immunosorbent assay (ELISA) for IL-8 (R&D Systems) or TNF-α (BD Biosciences) against a standard of recombinant IL-8 or TNF-α according to the manufacturer’s protocol.

**Limulus Amebocyte Lysate Test**

The Limulus Amebocyte Lysate (LAL) test (Associates of Cape Cod) was used to assay the ability of LPLUNC1 to bind and
neutralize LPS. Briefly, 0.5 EU/mL LPS from *E. coli* O113:H10 was incubated with a 2-fold dilution series of polymyxin B or LPLUNC1 protein in a 96-well plate format for 1 hour at 37°C, then assayed for conversion of the chromogenic substrate according to the manufacturer’s guidelines. Apparent LPS concentration was then calculated in EU/mL based on absorbance values of a standard curve using LPS only.

**Immunohistochemistry**

Human experimentation guidelines of the US Department of Health and Human Services were followed during the conduct of this research. Approval for collection of duodenal biopsy samples from cholera patients was obtained from the Research and Ethical Review Committees of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) and from the Partners Human Research Committee at Massachusetts General Hospital; all participants provided written informed consent. Serial sections (5 μm) of tissue were cut from paraffin-embedded biopsy samples. Slides were dried at 60°C for 30 minutes, and sections were deparaffinized in xylene and graded ethanol. The sections were then rinsed in tris-buffered saline/0.05% Tween 20 where necessary. Slides were incubated with rabbit polyclonal anti-LPLUNC1 antibody at a 1:200 dilution (Sigma) diluted in 1% BSA/TBS and stained the following day with EnVision+ anti-rabbit/horseradish peroxidase (HRP) polymer as a secondary antibody (Dako) at room temperature. Then 3,3-Diaminobenzidine (DAB) was used for color development and slides were counterstained with hematoxylin.

**Double Fluorescence Immunostaining of LPLUNC1 and β-Defensin 5**

Paraffin slides were baked for 30 minutes at 60°C and then deparaffinized in xylene and graded ethanol to water. After antigen retrieval, the slides were blocked with goat serum for 20 minutes at room temperature. The rabbit polyclonal anti-LPLUNC1 antibody was applied, followed by biotinylated goat anti-rabbit IgG antibody (Vector Laboratories). After rinsing in PBS, Alexa Fluor Streptavidin 488 (Molecular Probes) was applied for 30 minutes at room temperature. Slides were rinsed in PBS, and rabbit serum was applied for 20 minutes at room temperature. Slides were incubated with the goat polyclonal anti-β-defensin 5 antibody (Santa Cruz Biotechnology) diluted 1:500 in PBS/BSA, followed by biotinylated rabbit anti-goat IgG antibody (Vector labs), for 30 minutes at room temperature. After rinsing in PBS, slides were incubated for 30 minutes with Streptavidin Cy3 (Molecular Probes) at a 1:5000 dilution. Slides were counterstained with DAPI. Images were captured with a digital CCD camera (Hamamatsu).

**Statistical Analyses**

Statistical analyses were performed by the Mann–Whitney student’s *t* test (GraphPad Prism 4 software, Graphpad, Inc). Differences were considered significant if a 2-tailed *P* < .05.

**RESULTS**

**Recombinant LPLUNC1 Does Not Kill *V. cholerae* In Vitro**

Bactericidal assays were performed to test for the ability of LPLUNC1 to kill *E. coli* or strains of the El Tor or classical biotypes of *V. cholerae*. We used polymyxin B as a positive control for the killing of Gram-negative bacteria; in previous reports, polymyxin B was shown to induce killing of a classical strain of *V. cholerae*, but not an El Tor strain [22]. The recovery of *V. cholerae* and *E. coli* incubated in LPLUNC1 did not differ from the recovery observed for bacteria incubated in PBS buffer alone, suggesting that LPLUNC1 does not have bactericidal properties under these in vitro conditions (Figure 1Aa). We also performed a bacterial growth assay to test if LPLUNC1 inhibits bacterial growth. There was no significant difference in *V. cholerae* growth with or without LPLUNC1 (Figure 1B and C).

**V. cholerae** LPS Stimulates TLR4 Signaling in Transfected HEK293 Cells

In the absence of a direct inhibitory effect on bacterial survival, we hypothesized that LPLUNC1 might act as an immunomodulatory protein and affect the induction of TLR signaling pathways. HEK293 cells lack expression of endogenous TLR2, TLR4, TLR9, MD2, and CD14 and are commonly used to study TLR pathways [19]. HEK293 cells stably expressing human TLR2 and MD2 (HEK-TLR4/MD2 cells) were transiently transfected with CD14, then stimulated with increasing amounts of purified *E. coli* or *V. cholerae* LPS. Activation of NF-κB was examined using a luciferase reporter assay. Similar to findings recently described by Matson and colleagues [22], LPS purified from *V. cholerae* increased NF-κB response in a TLR4-dependent manner. Although of lesser magnitude than the *E. coli* LPS-driven response, a significant increase (2–2.5 fold) in NF-κB activation was observed following stimulation with purified LPS (100 ng/mL) from either Ogawa or Inaba serotypes of *V. cholerae* (Figure 2A). Furthermore, this response was enhanced by CD14 transfection (Figure 2B). These results demonstrate the ability of *V. cholerae* O1 LPS to stimulate the TLR4-mediated signaling pathway.

**LPLUNC1 Suppresses TLR4 Signaling in Response to *V. cholerae* and *E. coli* LPS**

To study the effect of LPLUNC1 on LPS-driven TLR4 signaling, we treated HEK TLR4/MD2 cells transfected with CD14 with LPLUNC1 protein prior to stimulation with LPS from *E. coli* or *V. cholerae*. Pretreatment with LPLUNC1 (100 ng/mL) for 1 hour significantly inhibited TLR4-driven NF-κB production in response to LPS (40% reduction in *E. coli*, 53% reduction in *V. cholerae* O1 serotype Ogawa, 42% reduction in *V. cholerae* O1 serotype Inaba) (Figure 3A). Pretreatment with LPLUNC1 (100 ng/mL) for 1 hour was also associated with a significant inhibition of LPS-driven IL-8 production (14% reduction in
E. coli, 20% reduction in *V. cholerae* O1 serotype Ogawa, 19% reduction in *V. cholerae* O1 serotype Inaba) (Figure 3B).

We then used THP-1 cells, a human monocyte derived lineage, to evaluate whether LPLUNC1 suppresses the proinflammatory pathway in antigen presenting cells that naturally express TLR4. We observed an LPLUNC1-mediated dose-dependent decrease of TNF-α secretion in response to *E. coli* LPS (24% reduction for LPLUNC1 100 ng/mL) (Figure 3C). Consistent with the results in
HEK293 cells, the results in THP-1 cells suggest that LPLUNC1 suppresses the inflammatory response mediated by TLR4 recognition of LPS.

**LPLUNC1 Neutralizes LPS Activation in a Dose-Dependent Manner**

We confirmed that recombinant human LPLUNC1 had 0.1 EU per 1 µg of endotoxin using the LAL assay. We then demonstrated that increasing concentrations of LPLUNC1 neutralized *E. coli* activation of LAL in a dose-dependent manner similar to polymyxin B (Figure 4A and B).

**V. cholerae OMV–Mediated Activation of the TLR2 Signaling Pathway Is Not Suppressed by LPLUNC1**

Components of *V. cholerae* are known to be recognized by different TLRs. TLR5 senses *V. cholerae* flagellin [23], whereas TLR4 recognizes *V. cholerae* LPS [22]. We sought to determine if components of *V. cholerae* cause activation of the TLR2 signaling pathways. OMV of *V. cholerae* have been shown to induce robust antibacterial and mucosal protective immunity in a mouse model [18]. We evaluated the capacity of OMV of *V. cholerae* O1 serotype Ogawa and Inaba to activate the TLR2 signaling pathway. HEK293 cells stably expressing TLR2 and CD14 (SZ10 cells) were stimulated with various concentrations of OMV (0.1–1000 ng/mL). OMVs from both *V. cholerae* O1 Ogawa and *V. cholerae* O1 Inaba caused up-regulation of NF-κB in a TLR2/CD14-dependent manner, implicating the involvement of the TLR2 pathway in *V. cholerae* sensing (Figure 5A).

We next examined the specificity of LPLUNC1-mediated suppression of proinflammatory signaling. Addition of LPLUNC1 (100 ng/mL) did not affect NF-κB production in response to synthetic TLR2 ligands in HEK293 cells stably expressing TLR2 and CD14 (Figure 5B). We also examined if LPLUNC1 modulates *V. cholerae* OMV-mediated TLR2 signaling. Addition of LPLUNC1 (100 ng/mL) did not affect *V. cholerae* OMV-driven NF-κB activation (Figure 5C), suggesting the inhibitory effect of LPLUNC1 is specific to LPS mediated responses by the TLR4 signaling pathway.

**LPLUNC1 Is Expressed in Paneth Cells of Intestinal Mucosa of Cholera Patients**

To localize the expression of LPLUNC1, we performed immunohistochemistry on duodenal biopsy specimens collected from 7 cholera patients at the acute (day 2 after onset of symptoms) and convalescent (day 30 after onset of symptoms) phases of disease. We observed LPLUNC1 protein in the mucosal crypts of duodenal tissue sections; representative images are shown in Figure 6. Although gene expression data suggest increased expression of *LPLUNC1* during the acute phase of cholera [8], we observed no quantitative difference in the acute and convalescent staining patterns of LPLUNC1 protein by immunohistochemistry, or between healthy North American or Bangladeshi controls (data not shown).

Cells that stained positive for LPLUNC1 were highly granular and were located at the base of intestinal crypts, suggesting that the localization of LPLUNC1 was within Paneth cells. To confirm this, we performed double immunofluorescence staining for LPLUNC1 and human β defensin-5, an antimicrobial peptide that is secreted specifically by Paneth cells. Both LPLUNC1 and β defensin-5 were observed to be associated with each other in intestinal crypts (Figure 7), demonstrating that LPLUNC1 is a secreted product of Paneth cells.
LPLUNC1 is the most highly up-regulated transcript in duodenal tissue during acute cholera [8], and a variant in the LPLUNC1 promoter region is associated with susceptibility to severe cholera in a Bangladeshi population [9]. However, no biological function of LPLUNC1 has been previously described. Here, we found that LPLUNC1 is expressed in Paneth cells during cholera and that it inhibits the TLR4 signaling pathway in response to *V. cholerae* O1 and *E. coli* LPS. The demonstration of LPLUNC1 protein in Paneth cells is the first observation of LPLUNC1 outside of the respiratory tract and oral cavity in humans; previous studies have identified LPLUNC1 in the stomach and GI tract of mice, but at very low levels [24, 25]. In addition, our results are the first to our knowledge to identify an immunomodulatory role for a member of the PLUNC family, and the first to identify a potential role for LPLUNC1 in response to an enteric bacterial infection. Our findings suggest that LPLUNC1 may act in vivo to modulate immune responses to *V. cholerae*.

The identification of an immunomodulatory role for a PLUNC family protein in response to a mucosal infection is consistent with the predicted function of this family of proteins. The PLUNC family is a rapidly evolving protein family that includes 9 adjacent genes on human chromosome 20q11.21. These are classified as long PLUNC proteins (LPLUNC1-4, 6) or short PLUNC proteins (SPLUNC1-3, and BASE) [26, 27]. PLUNC family members are expressed abundantly in the oro-nasopharynx and respiratory tract and share predicted structural similarity to the immunomodulatory LPS binding proteins, BPI and LBP. Specifically, the long PLUNC proteins share homology with both the N- and C-terminal domains of LBP/BPI, while the short PLUNC proteins share predicted structural homology only with the N-terminal domain [28]. For both LBP and BPI, the N-terminal portion is responsible for lipid A binding and the immunomodulatory effects [16, 29], while the C-terminal domain is required for bacterial opsonization and enhances phagocytosis [30, 31]. The structural similarity to BPI and LBP and the prominent expression of PLUNC proteins at mucosal surfaces have led to predictions of their role in mucosal immunity.

We initially hypothesized that LPLUNC1 might function as an antimicrobial protein. However, under standard in vitro growth conditions, LPLUNC1 demonstrated no bactericidal or
bacteriostatic effect against *V. cholerae* O1 or *E. coli*. Although LPLUNC1 might have an antimicrobial function in the intestinal milieu, the lack of a demonstrable bactericidal activity in vitro suggests the possibility of alternative in vivo roles for this protein. In support of this, we found that LPLUNC1, similar to BPI, has a robust immunomodulatory effect on the interaction between TLR4 and LPS. Together with the constitutive expression of LPLUNC1 in goblet cells in the upper aerodigestive tract [11], this suggests that LPLUNC1 may promote tolerance to oral antigens by limiting LPS-induced signaling via TLR4. Whether this effect depends on LPS structure or is specific for certain pathogens bears further investigation.

Although cholera has been considered paradigmatic of a non-inflammatory infection, there is significant up-regulation of proinflammatory molecules and an increase in inflammatory cells in the duodenal tissue of cholera patients [5, 8, 32]. In vitro studies suggest mechanisms by which *V. cholerae* may activate critical innate immune signaling pathways involved in microbial pattern and danger signal recognition, including TLR4 [22] and TLR5 signaling pathways [23, 33], and the NLRP3 inflammasome [34]. These data support a role for the innate immune system in mediating initial host responses to *V. cholerae*.

**Figure 4.** LPLUNC1 neutralizes LPS in a Limulus Amebocyte Lysate (LAL) assay. LPS from *E. coli* O113:H10 was incubated with polymyxin B (A) or LPLUNC1 protein (B) for 1 hour at 37°C and then assayed for apparent endotoxin activity with the LAL test. The results are shown as the average ± standard deviations (SD) of duplicate wells and are representative of at least 2 independent experiments.

**Figure 5.** *V. cholerae* OMV activate NF-κB pathway via TLR2, but LPLUNC1 has no effect on TLR2 signaling. HEK293 cells stably expressing TLR2, CD14, and an NF-κB-driven firefly luciferase reporter gene (SZ10 cells) were stimulated with OMV from either *V. cholerae* O1 serotype Ogawa or Inaba (0.1–1000 ng/mL) (A). SZ10 cells were incubated with LPLUNC1 (100 ng/mL) and stimulated with TLR2 ligands, Pam3CSK4 (100 ng/mL) or Pam2CSK4 (100 ng/mL) (B) or Ogawa or Inaba *V. cholerae* OMV (100 ng/mL) (C). Luciferase production was measured at 6 hours post-infection. The results are shown as the average ± SD of duplicate wells and are representative of 3 independent experiments. *P* value between PBS buffer control and LPLUNC1-treated group was not significant.
Although we have shown that LPLUNC1 is expressed during acute cholera and decreases in vitro signaling via TLR4 in response to *V. cholerae* LPS, the reason why variations in the LPLUNC1 promoter affect susceptibility to cholera remains a question [9]. Most likely, polymorphisms in the LPLUNC1 promoter region affect LPLUNC1 expression in response to acute *V. cholerae* infection. Given the high levels of expression of LPLUNC1 in the duodenum of cholera patients with severe disease [8], it is possible that attenuation of the immune response by LPLUNC1 may result in failure to clear infection rapidly and more severe disease. This hypothesis is consistent with the recent finding that *V. cholerae* O1 produces a sheathed flagellum that allows evasion of TLR5 mediated NF-κB signaling [35]. Thus, factors that affect innate immune recognition and signaling via the NF-κB activating pathways may be important in the pathogenesis of cholera.

Our study has limitations. Although we identified the presence of LPLUNC1 in Paneth cells, we were unable to identify quantitative differences in LPLUNC1 expression between the acute and convalescent phases of cholera and between cholera patients and healthy controls, despite the fact that transcriptional profiling studies indicate that LPLUNC1 is up-regulated during the acute phase of cholera [8]. Given the reproducible transcriptional data (array and quantitative reverse-transcription polymerase chain reaction), this likely reflects the limitations of immunohistochemistry for measuring protein abundance, especially in the setting of Paneth cell degranulation [36]. A mass-spectrophotometry-based analysis of duodenal specimens might identify quantitative differences in protein expression and assess physiologic concentrations of LPLUNC1.

In conclusion, we identified LPLUNC1 as a Paneth cell-expressed immunomodulatory protein that decreases TLR4-mediated innate immune responses to *V. cholerae* LPS. The maintenance of homeostasis between pro- and anti-inflammatory effects in the intestinal environment is crucial, and it is possible that LPLUNC1 serves to reduce intestinal

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**Figure 6.** LPLUNC1 expression in intestinal mucosa of cholera patients. Immunohistochemistry was performed on duodenal biopsy samples using rabbit polyclonal anti-LPLUNC1 antibody, followed by anti-rabbit HRP polymer. The slides were counterstained with hematoxylin. Biopsy samples were collected from 7 cholera patients on day 2 and day 30. A representative example of the results are shown for day 2 (B) and day 30 (D). Secondary antibody controls are shown for day 2 (A) and day 30 (C). Prominent LPLUNC1 staining is seen in granules of intestinal crypts (E). The original magnification of images was 40×.

**Figure 7.** Association of LPLUNC1 and human β-defensin 5 in Paneth cells. Duodenal biopsy samples of cholera patients were double-stained for LPLUNC1 and β-defensin 5. The localization of LPLUNC1 protein was detected by rabbit polyclonal anti-LPLUNC1 antibody, followed by anti-rabbit Alexa Fluor Streptavidin 488 (A, green). β-defensin 5 was detected by goat polyclonal anti- β-defensin 5 antibody, followed by anti-goat Streptavidin Cy3 antibody (B, red). DAPI shows nuclear staining (C, blue). D shows a merged image of LPLUNC1, β-defensin 5, and DAPI. The arrow indicates areas of LPLUNC1 and β-defensin 5 association.
inflammation to enteric pathogens. Further studies of the anti-inflammatory effect of LPLUNC1 using more complex in vivo models of mucosal host-pathogen interactions would be of interest.

Notes

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