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Paneth Cell Alertness to Pathogens Maintained by Vitamin D Receptors

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Abstract

BACKGROUND AND AIMS: Vitamin D exerts a regulatory role over mucosal immunity via the vitamin D receptor (VDR). Although Paneth cells and their products are known to regulate the commensal and pathogenic microbiota, the role that VDRs in Paneth cells play in these responses is unknown.

METHODS: We identified the decreased intestinal VDR significantly correlated with reduction of an inflammatory bowel disease risk gene *ATG16L1* and Paneth cell lysozymes in patients with

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The authors disclose no conflicts.

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Supplementary Material
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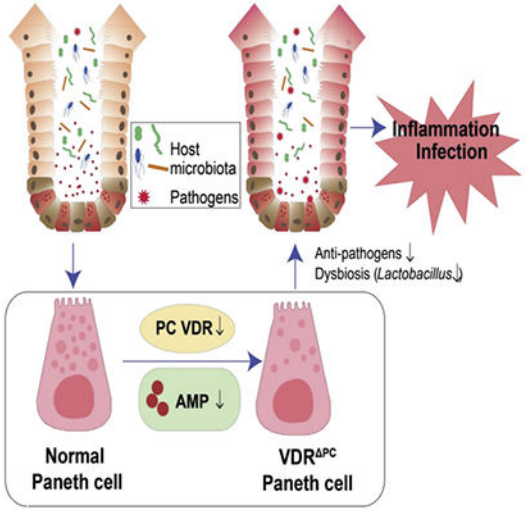
Crohn's disease. We generated Paneth cell–specific VDR knockout ($VDR^{\Delta PC}$) mice to investigate the molecular mechanisms.

RESULTS: Lysozymes in the Paneth cells were significantly decreased in the $VDR^{\Delta PC}$ mice. Isolated $VDR^{\Delta PC}$ Paneth cells exhibited weakened inhibition of pathogenic bacterial growth and displayed reduced autophagic responses. $VDR^{\Delta PC}$ mice had significantly higher inflammation after *Salmonella* infections. $VDR^{\Delta PC}$ mice also showed high susceptibility to small intestinal injury induced by indomethacin, a nonsteroidal anti-inflammatory drug. Co-housing of $VDR^{\Delta PC}$ and VDR^{lox} mice made the $VDR^{\Delta PC}$ less vulnerable to dextran sulfate sodium colitis, suggesting the transmission of protective bacterial from the VDR^{lox} mice. Thus, a lack of VDR in Paneth cells leads to impaired antibacterial activities and consequently increased inflammatory responses. Genetically and environmentally regulated VDRs in the Paneth cells may set the threshold for the development of chronic inflammation, as observed in inflammatory bowel diseases.

CONCLUSIONS: We provide new insights into the tissue-specific functions of VDRs in maintaining Paneth cell alertness to pathogens in intestinal disorders. Targeting the VDR affects multiple downstream events within Paneth cells that inhibit intestinal inflammation and establish host defense against enteropathogens.

Graphical Abstract



Gastroenterology

Keywords

Autophagy; Bacteria; Crohn's Disease (CD); Defensin

Vitamin D deficiency is associated with various diseases, including infections and inflammatory bowel disease (IBD).[1-6] This is in part because vitamin D exerts a regulatory role in mucosal immunity and host defenses via vitamin D receptors (VDRs).[6] VDRs belong to the nuclear receptor superfamily and regulate the transcription of many target genes, including *ATG16L1*, an IBD susceptibility gene involved in autophagy.[7-11] Impaired *ATG16L1*-dependent autophagy drives ileal inflammation in Crohn's disease (CD).[12] We have previously demonstrated that intestinal epithelial VDR deficiencies lead to impaired

function of the autophagy pathway because of the reduced expression of *ATG16L1* and an abnormal morphology of Paneth cells.[8]

Paneth cells are important in both initiating and preventing inflammatory disorders. Paneth cell–specific abnormalities in the unfolded protein response may serve as the origin for intestinal inflammation.[13] Paneth cells also through their secretion of antimicrobial factors regulate commensal microbe composition and protect the host from enteropathogens.[14] The mechanisms that underlie Paneth cell regulation in the latter context are still unknown. We considered the potential role played by VDRs in Paneth cells, given the importance of vitamin D in host responses involving the epithelium. Importantly, VDR target genes include antimicrobial peptides (AMPs) cathelicidin[15] and β defensin.[16] Several IBD polymorphisms are phenotypically manifested in Paneth cells, altering AMPs and autophagy. Paneth cell abnormalities in human subjects are associated with mucosal dysbiosis in CD.[17] Deletion of the intestinal epithelial VDRs contributes to abnormal Paneth cells and reduced autophagy responses.[8] We thus hypothesized that the Paneth cell dysfunction induced by VDR deletion would lead to abnormal antibacterial functions and a loss of intestinal mucosal and microbial homeostasis and aimed to elucidate these mechanisms.

We identified the decreased intestinal VDR significantly correlated with reduction of *ATG16L1* and Paneth cell lysozymes in patients with CD. To investigate the molecular mechanism of VDR regulation of Paneth cells, we generated Paneth cell VDR specific knockout ($VDR^{\Delta PC}$) mice. Furthermore, we established a new method to isolate the intestinal Paneth cells by flow cytometry. The antibacterial ability of the isolated Paneth cells and their expression of the VDR and lysozymes were evaluated. In the *Salmonella* colitis model, $VDR^{\Delta PC}$ mice had significantly higher levels of inflammation post-*Salmonella* infection. The lack of VDR in the Paneth cells led to impaired antibacterial abilities and inflammatory responses. $VDR^{\Delta PC}$ mice also showed high susceptibility to small intestinal injury induced by indomethacin, a nonsteroidal anti-inflammatory drug. Furthermore, we examined the dysbiosis in the $VDR^{\Delta PC}$ mice. Co-housing of $VDR^{\Delta PC}$ and VDR^{lox} mice made the $VDR^{\Delta PC}$ less vulnerable to dextran sulfate sodium (DSS) colitis, suggesting the transmission of protective bacterial from the VDR^{lox} mice. Thus, our study fills an existing gap in the knowledge by characterizing the precise role of tissue-specific VDRs in regulating Paneth cells in host defenses from enteric pathogens.

Methods

More details can be found in the supplementary documents.

Human Intestinal Biopsies

Slides containing paraffin-embedded small intestinal biopsy samples of patients with CD and healthy control subjects were obtained from Dr Kaser, University of Cambridge. All protocols were approved by Cambridgeshire 4 Research Ethics Committee (reference 03/5/012). Patient characteristics are outlined in a previous publication.[12]

Gene Expression Datasets

For expression analyses we used microarray data registered in the Gene Expression Omnibus[18] repository (https://www.ncbi.nlm.nih.gov/geo/; Gene Expression Omnibus accession number GSE102134) reported on the ileum of patients with CD and normal ileum from control individuals.[19]

Experimental Animals

$VDR^{\Delta PC}$ mice were obtained by crossing $VDR^{loxP/loxP}$ mice[20] with DEFA6-cre mice.[13] Mice were provided with water ad libitum and maintained in a room with a 12-hour dark–light cycle. Multiple breeding pairs were set up within a specific vivarium room where environment, cage changes, and dietary schedules are more uniform. All animal work complied with ARRIVE guidelines and was approved by the University of Illinois at Chicago Committee on Animal Resources; ethical guidelines were followed for treatment.

Bacterial Strains and Growth Conditions

The *Salmonella* strain used in this study was *S typhimurium* 14028.[21] Bacterial cultures were prepared by inoculating 10 mL Lysogeny broth broth with 0.01 mL of a stationary-phase culture, followed by overnight incubation at 37°C.[22]

Salmonella-*infected Mouse Model*

Animal experiments were performed using $VDR^{loxP/loxP}$ and $VDR^{\Delta PC}$ mice (male and female, 2–3 months old). Water and food were withdrawn 4 hours before oral gavage, with 7.5 mg per mouse of streptomycin. Afterward, the animals were supplied with water and food ad libitum. Twenty hours after the streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 1×10^6 colony-forming units of *Salmonella* (100-mL suspension in Hank's balanced salt solution by gavage).[22]

Co-housing of VDR^{lox} and $VDR^{\Delta PC}$ Mice and Induction of DSS Colitis

Two- to 3-month-old female VDR^{lox} and $VDR^{\Delta PC}$ mice were co-housed in new cages according to previously published methods.[8] One cage contained 3 VDR^{lox} and 2 $VDR^{\Delta PC}$ mice, another cage contained 2 mice each. The mice were fed with the same food and water. After 4 weeks of co-housing, 5% DSS (molecular eight, 40–50 kDa; USB Corp, Cleveland, OH) dissolved in filter-purified water was administered to the mice. Animals were weighed daily. At day 7 after 5% DSS administration mice were killed.[8]

Induction of Small-intestinal Lesions

To induce small-intestinal injury, 10 mg/kg indomethacin (Sigma Chemical, St Louis, MO) was subcutaneously given to nonfasted animals. The animals were killed 24 hours after anesthetization. The jejunum and ileum were then removed, opened along the antimesenteric attachment, and examined for lesions under a dissecting microscope with square grids. The area (mm^2) of visible lesions was macroscopically measured, summed per small intestine, and expressed as an ulcers core.[23]

Statistical Analyses

More details are also provided in the supplement document. The statistical analyses of experimental data were performed with GraphPad Prism 5. The regression and scatter plot of VDR against *ATG16L1* were performed using SAS version 9.4 (SAS Institute). Microbiome data were analyzed by using R packages ampvis2, microbiome, phyloseq, and vegan, as previously reported.[24]

Results

Reduced VDR Positively Correlated With the Reduction of ATG16L1 *and Paneth Cell Lysozyme in Human CD*

We found that the messenger RNA expression levels of *VDR* and *ATG16L1* genes were both significantly reduced in human CD, using the Gene Expression Omnibus database GSE102134[18] (Figure 1A). The regression line indicated the positive correlation of *VDR* and *ATG16L1* expression (Figure 1B). Thus, we identified a significantly coordinated downregulated gene expression of *VDR* and *ATG16L1* in patients with CD.

To investigate the changes of VDR and *ATG16L1* at the protein level, we did immunohistochemical staining using small intestine tissues from healthy control subjects and CD patients. As shown in Figure 1C, a lower *ATG16L1* expression was found in CD patients than that in the normal small intestine. We found significantly lower *VDR* expression in the small intestine of CD patients compared with the normal small intestine (Figure 1D). Reduction of VDR may lead to impaired Paneth cells and consequently increased inflammatory responses.

We then evaluated the status of the Paneth cells using a previously reported method for lysozyme detection.[8] We found that the percentage of normal Paneth cells were lower in CD patients compared with the normal small intestine (Figure 1E). Abnormal Paneth cells were grouped as D1 (disordered), D2 (depleted), and D3 (diffuse).[8,9] Paneth cells in the small intestine of CD patients also displayed a higher percentage of abnormal patterns of lysozyme expression (Figure 1E). Thus, we showed that *VDR* and *ATG16L1* were markedly downregulated in patients with CD. The reduction of *ATG16L1* and abnormal Paneth cells is correlated with the reduction of VDR in small intestinal samples from human colitis.

Establishing an Intestinal $VDR^{\Delta PC}$ Model

To investigate the molecular regulation of VDR in Paneth cells, we established a $VDR^{\Delta PC}$ model by crossing $VDR^{loxP/loxP}$ (VDR^{lox}) mice with DEFA6-cre mice (Figure 2A). We found more granules in Paneth cells in the small intestine tissues of the VDR^{lox} mice than in the $VDR^{\Delta PC}$ mice using hematoxylin and eosin staining (Figure 2B). To verily that the *VDR* gene was deleted in Paneth cells, the Paneth cells from the ileum tissue were laser captured and microdissected for quantitative polymerase chain reaction. The VDR expression in the $VDR^{\Delta PC}$ Paneth cells was significantly reduced compared with that in the VDR^{lox} Paneth cells (Figure 2C). Furthermore, there was no detectable VDR expression in the intestinal Paneth cells of the $VDR^{\Delta PC}$ mice by immunofluorescence staining (Figure 2D). These data confirmed that the Paneth cell VDR knockout was established. We also tested the serum

level of 1,25-vitamin D. However, we found that the lack of VDR in Paneth cells did not cause the changes of serum 1,25-vitamin D in vivo (data not shown).

Absence of VDR Expression Leads to Abnormal Paneth Cells

The IBD susceptibility gene *ATG16L1* is involved in autophagy and contributes to inflammation and dysbiosis.[7-9] We found lower levels of *ATG16L1* expression in the small intestine tissues of the $VDR^{\Delta PC}$ mice compared with those of the VDR^{lox} mice with immunohistochemical staining (Figure 2E). Paneth cells are specialized intestinal epithelial cells that secrete AMPs, sense commensal bacteria, and maintain homeostasis at the intestinal–microbial interface.[25] We evaluated the status of the Paneth cells by lysozyme detection.[8] Lower expression levels of the lysozymes in the $VDR^{\Delta PC}$ mice were found by immunofluorescence staining when compared with the VDR^{lox} mice (Figure 2F). We found the percentage of the normal Paneth cells (D0) was much lower in the $VDR^{\Delta PC}$ mice (Figure 2G). Based on the lysozymes patterns, abnormal Paneth cells were grouped as D1–D3.[8,9] Abnormal Paneth cells were significantly higher in the $VDR^{\Delta PC}$ mice. These results were further confirmed by testing the lysozyme expression by Western blotting and real-time polymerase chain reaction (Figure 2H and I). In the $VDR^{\Delta PC}$ mice, lysozyme was significantly reduced at the protein and messenger RNA levels. The structure of the Paneth cells observed by a transmission electron microscopy also revealed more abnormal cells (eg, less granules) in the $VDR^{\Delta PC}$ mice when compared with the VDR^{lox} mice (Figure 2J).

Paneth cells may support some aspects of the stem cell niche. We then examined the cell proliferation of small intestinal epithelial cells. Previous studies have identified serine/threonine protein kinase Akt[26] signaling cooperates with Wingless to activate β-catenin in intestinal stem and progenitor cells through phosphorylation at Ser552 (P-β-catenin552).[27] We found that P-β-catenin552 was decreased in the small intestine of $VDR^{\Delta PC}$ mice when compared with VDR^{lox} mice (Supplementary Figure 1). We also examined the stem cell markers (lgr5 and bmi1). However, there was no significant change between $VDR^{\Delta PC}$ and VDR^{lox} mice (data not shown).

Salmonella *Infections Were Worse in* $VDR^{\Delta PC}$ *Mice*

Salmonella infections, known to induce intestinal damage[28] and involve Paneth cells,[29] were conducted using VDR^{lox} and $VDR^{\Delta PC}$ mice (Figure 3A). Body weights were decreased after 4 days of the *Salmonella* treatment, significantly in the $VDR^{\Delta PC}$ mice compared with the VDR^{lox} mice (Figure 3B). Cecal shortening and inflammation are key features in the *Salmonella* colitis mouse model.[30] The length of the cecum and colon were shorter in the $VDR^{\Delta PC}$ mice than the VDR^{lox} mice 4 days after infection (Figure 3C). Higher inflammation scores were also found in the cecum tissues of the $VDR^{\Delta PC}$ mice 4 days after infection by hematoxylin and eosin staining (Figure 3E and F). The lengths of the small intestine were measured, but no significant differences were found between the 2 mouse models. Both the liver and spleen were involved in the *Salmonella* infections, and so we measured their postinfection weights. After 4 days the *Salmonella*-treated mice had heavier spleens than the control group, especially for the $VDR^{\Delta PC}$ mice (Figure 3D). The numbers of colony-forming units were determined by plating on agar plates to determine

the *Salmonella* colonies. The fecal and spleen samples, 4 days after infection, showed more *Salmonella* in the $VDR^{\Delta PC}$ mice compared with the VDR^{lox} mice (Figure 3G and H).

Lipocalin-2 is a pleiotropic mediator of various inflammatory processes.[31] We found an increased expression level of lipocalin-2, and its expression was higher in the $VDR^{\Delta PC}$ group 8 hours after infection compared with VDR^{lox} mice (Figure 4A). Lipopolysaccharides are characteristic components of the cell walls of gram-negative bacteria. The lipopolysaccharide increased in $VDR^{\Delta PC}$ mice compared with VDR^{lox} mice 8 hours after infection (Figure 4B).

α-Defensin6 is expressed in the Paneth cells of the ileum. Its expression in the ileum tissues of $VDR^{\Delta PC}$ mice was significantly lower than that in VDR^{lox} mice, even before *Salmonella* infection (Figure 4C). The percentage of normal Paneth cells was further reduced in $VDR^{\Delta PC}$ mice postinfection (Figure 4D). With the decreased number of normal Paneth cells, the expression of defensin4 was significantly downregulated in $VDR^{\Delta PC}$ mice after *Salmonella* treatments (Figure 4E). Taken together, the biological significance of $VDR^{\Delta PC}$ mice in a *Salmonella* colitis model was measured by several readouts, including body weight, intestinal inflammation, bacterial burden, bacterial translocation, lipocalin-2, lipopolysaccharide, and cellular changes of Paneth cells.

$VDR^{\Delta PC}$ Mice Showed Reduced Paneth Cells During Small-Intestinal Injuries

To further test the effects of $VDR^{\Delta PC}$ mice in response to small-intestinal injury, we treated mice with indomethacin, a nonsteroidal anti-inflammatory drug. It is a relevant CD environmental trigger that induces small-intestinal injury. We found that the percentage of normal Paneth cells was significantly reduced after the indomethacin treatment, especially in $VDR^{\Delta PC}$ mice (Figure 4F). This observation suggested that VDR deletion in Paneth cells made the mice susceptible to small-intestinal injury induced by indomethacin.

Lack of VDR in the Paneth cells leads to impaired anti-bacterial abilities

Paneth cells can control the intestinal growth of bacterial pathogens through the secretion of AMPs.[32] To test their antimicrobial activity in a well-controlled condition, Paneth cells were isolated from the small intestine. We collected small-intestine tissues and digested them into single cells. After staining with anti-CD24 Ab, $CD24^{+}$ Paneth cells and $CD24^{™}$ non-Paneth cells were purified by flow cytometry (Figure 5A). We were able to test the protein expression in the isolated Paneth cells by Western blots. We found that the lack of VDR in Paneth cells led to significantly decreased expressions of *ATG16L1* and LYZ (Figure 5B). These data also validated the successful deletion of VDR in Paneth cells in the $VDR^{\Delta PC}$ mice. Then, we tested the antibacterial functions of the purified Paneth cells. Isolated Paneth cells were incubated with green fluorescent protein *Salmonella* for 5 hours. The Paneth cells in the $VDR^{\Delta PC}$ group reduced the bacterial clearance ability and had more growth of *Salmonella* compared with the VDR^{lox} Paneth cells (Figure 5C). The lack of *VDR* genes caused the $VDR^{\Delta PC}$ Paneth cells to lose their antibacterial qualities.

The antibacterial components secreted by Paneth cells also exist in the supernatants. The Paneth and non-Paneth cells were therefore cultured overnight and the supernatant collected. We determined that the expression of the lysozyme gene was lower in the $VDR^{\Delta PC}$

supernatant than in the VDR^{lox} supernatant (Figure 5D). We then added *Salmonella* into these supernatant samples. After 5 hours of incubation, we collected the medium to determine the number of colony-forming units. At 0 hours the supernatant samples were clear, but after 5 hours we found that only 2 samples remained clear, and these 2 samples were from the supernatant of the Paneth cells (Figure 5E). Paneth cells can secrete AMPs and proteins to inhibit the growth of *Salmonella*. The VDR^{lox} group had a stronger antibacterial ability than the $VDR^{\Delta PC}$ group (Figure 5E). However, both tubes with the non-Paneth cell fraction were cloudy after incubation for 5 hours, indicating bacterial growth. The green line (VDR^{lox} non-Paneth cells) and purple line ($VDR^{\Delta PC}$ non-Paneth cells) show the similar trend, with no significant difference of colony-forming units (Figure 5E). Taken together, these experiments confirmed the decreased anti-bacterial ability of $VDR^{\Delta PC}$ Paneth cells without VDR.

Lack of VDR in Intestinal Paneth Cells Leads to Increased Inflammatory Responses in the Setting of Salmonella Infection

Inflammatory cytokines play a complex role in intestinal inflammation.[33] We thus investigated the profile of cytokines in the serum of the mice. The cytokines we tested included interleukin (IL)1b, IL6, IL17A, IL18, interferon gamma, and tumor necrosis factor (TNF)-α. For IL6, significant differences were also found between the $VDR^{\Delta PC}$ and VDR^{lox} mice 8 hours and 4 days after infection. Four days after infection, significant differences in the IL1β, IL17A, IL18, interferon gamma, and TNF-α were found in the *Salmonella* treated mice, especially $VDR^{\Delta PC}$ mice (Figure 6A). The increased expressions of the interferon gamma, IL6, and TNF-α in the ileum tissue of $VDR^{\Delta PC}$ mice after *Salmonella* treatments were also detected by real-time polymerase chain reaction (Figure 6B), indicating the enhanced local inflammation in the small intestine of $VDR^{\Delta PC}$ mice. TNF-α is a multifunctional proinflammatory cytokine. Paneth cells were treated with *Salmonella* for 1 hour, and the supernatant was collected to detect TNF-α by enzyme-linked immunosorbent assay. The expression of TNF-α was higher in the $VDR^{\Delta PC}$ supernatant than that in the VDR^{lox} supernatant (Figure 6C).

In the $VDR^{\Delta PC}$ Paneth cells, the expression of VDR decreased with the lower expression levels of *ATG16L1* (Figure 6D and E). Defensin4 expression in $VDR^{\Delta PC}$ Paneth cells was also lower than that of the VDR^{lox} cells after *Salmonella* infection (Figure 6F). We detected the autophagy marker of the Paneth cells with the CYTO-ID Autophagy detection kit (Enzo Life Sciences). After 1 hour of *Salmonella* treatment, we found stronger LC3 expression in the VDR^{lox} cells compared with the $VDR^{\Delta PC}$ cells (Figure 6G), suggesting reduced autophagic responses because of the lack of VDR in Paneth cells.

Lack of VDR in Intestinal Paneth Cells Affects Intestinal Microbiota and Sensitivity to Chemical Damage

Paneth cell abnormalities in human subjects are associated with mucosal dysbiosis.[17] We examined the bacterial abundance in feces of $VDR^{\Delta PC}$ and VDR^{lox} mice using shotgun metagenomic sequencing. We found that bacterial homeostasis in $VDR^{\Delta PC}$ mice was changed compared with VDR^{lox} mice without any treatment (Figure 7A and B). Taxa abundance and diversity of bacteria of $VDR^{\Delta PC}$ and VDR^{lox} mice are shown in Figure

7A. The relative bacterial abundance in species levels was shown with the top 10 species. *Ralstonia solanacearum* and *Faecalibaculum rodentium* were enriched and *Lactobacillus* depleted in the $VDR^{\Delta PC}$ mice. Principal coordinate analysis showed that overall VDR^{lox} and $VDR^{\Delta PC}$ mice were separated from each other with only partial overlap, and 2 principal coordinate axes could explain 72.1% of variances (Figure 7B). Furthermore, we performed the permutational multivariate analysis of variance of Bray-Curtis dissimilarity, which confirmed that VDR^{lox} and $VDR^{\Delta PC}$ mice have different dissimilarities ($P = .027$). We also confirmed that the dissimilarity of bacteria in $VDR^{\Delta PC}$ mice was significantly decreased compared with VDR^{lox} mice (Figure 7C).

To investigate the biologic effects of VDR in response to injury, we used a DSS colitis model. Our data showed that $VDR^{\Delta PC}$ mice were susceptible to DSS-mediated inflammation. The $VDR^{\Delta PC}$ mice had significant loss of body weight after DSS treatment for 7 days (Figure 7D). The cecum length was significantly reduced in the $VDR^{\Delta PC}$ mice with DSS compared with VDR^{lox} mice (Figure 7E). In $VDR^{\Delta PC}$ mice with DSS, fecal blood was more obvious and stools less formed. Accordingly, the Disease Activity Index was significantly increased compared with the VDR^{lox} group (Figure 7F).

Because microbiota may play a role in the vulnerability of $VDR^{\Delta PC}$ mice to DSS colitis, we examined the transmissibility of the phenotype by performing a co-housing experiment and then challenging the mice with DSS. We found that co-housing decreased the Disease Activity Index of $VDR^{\Delta PC}$ mice to a level similar to that seen in VDR^{lox} mice (Figure 7E and F). Inflammation scores in the colon tissues of $VDR^{\Delta PC}$ mice were reduced after co-housing with VDR^{Lox} mice (Supplementary Figure 2A and B). *Lactobacillus* in VDR^{Lox} mice increased after co-housing treatment (Figure 7G), and *Lactobacillus* strains have been shown to decrease the inflammatory response in the intestine.[34] Interestingly, the numbers of Paneth cells per crypt were restored in the $VDR^{\Delta PC}$ mice after co-housing with the VDR^{Lox} mice (Supplementary Figure 2C). The percentage of normal Paneth cells (D0) in the $VDR^{\Delta PC}$ mice was significantly increased after co-housing with the VDR^{Lox} mice (Figure 7H). These suggest that lack of VDR in Paneth cells affects gut microbiota and sensitivity to colitis.

Discussion

Genetically regulated VDR in the Paneth cells may set the threshold for maintaining the microbial homeostasis and alertness to pathogens. Here, we identified decreased intestinal VDR significantly correlated with reduction of *ATG16L1* and Paneth cell lysozymes in patients with CD. We demonstrated that a lack of VDR in Paneth cells leads to impaired antibacterial activities and increased inflammatory responses in the setting of an enteropathogenic exposure. The lysozymes in Paneth cells were significantly decreased in $VDR^{\Delta PC}$ mice compared with VDR^{lox} mice. The inhibition of bacterial growth by $VDR^{\Delta PC}/CD24^{+}$ cells was significantly weakened, and $VDR^{\Delta PC}$ mice had high inflammation levels after *Salmonella* infection. We found dysbiosis in $VDR^{\Delta PC}$ mice. Interestingly, co-housing of $VDR^{\Delta PC}$ and VDR^{lox} mice made the $VDR^{\Delta PC}$ less vulnerable to DSS colitis, suggesting the transmission of protective bacteria community from the VDR^{lox} mice. Thus, our data

suggest that VDR is critical in maintaining the alertness of the Paneth cells in response to pathogens.

Paneth cells play a key role in host innate immune responses and in shaping the gut microbiome.[13,14,35] Paneth cells are found throughout the small intestine at the base of the intestinal glands and in the colon during infection and inflammation in a process called intestinal metaplasia. In patients with ileal CD, there is lower expression of Paneth cell defensins, increased inflammation, and reduced cell defense. Several IBD polymorphisms are phenotypically manifested in Paneth cells, altering AMPs and autophagy. VDR target genes include AMP cathelicidin,[15] β defensin,[16] and *ATG16L1*.[8] Our data indicate a critical role of VDR in shaping these activities. In IBD patients, it is critical to maintain the level of VDR to suppress inflammation diseases.[1-6] Here, we have shown that lacking VDR leads to decreased numbers of Paneth cells, abnormal Paneth cells in differentiation, defects in AMP secretion, and reduced pathogen clearance. $VDR^{\Delta PC}$ mice also showed susceptibility to small-intestinal injury induced by a nonsteroidal anti-inflammatory drug. Our study further indicates the importance of VDR in supporting host defenses and protecting from intestinal injury through effects on Paneth cells.

VDR deletion in Paneth cells may reduce cell development and proliferation of small intestinal epithelial cells. P-β-catenin552 was decreased in the small intestine of $VDR^{\Delta PC}$ mice. Previous studies have shown that Wingless activate β-catenin in intestinal stem cells through P-β-catenin552.[27] *Salmonella* infection is also known to involve the β-catenin pathway.[36] In future studies we will investigate the effect and mechanism of VDR deletion in Paneth cell differentiation and intestinal stem cells.

We report a significant correlation of reduced VDR and *ATG16L1* in the CD patients. VDR directly regulates its target gene *ATG16L1* in colitis.[8] In the pathogenesis of CD, the *ATG16L1* variant plays a crucial role in pathogen clearance, resulting in imbalanced cytokines, and is linked to endoplasmic reticulum stress.[37] Thus, we could consider VDR deficiency as a multifunctional susceptibility factor in CD. Evidence has demonstrated that vitamin D deficiency is a critical factor in the pathology associated with infection, IBD, and other diseases.[1-6] Vitamin D administration leads to a shift of the intestinal bacterial composition in CD patients but not in healthy control subjects.[38] To move forward, we should have well-designed therapeutic studies to examine if enhanced vitamin D/VDR will restore functions of Paneth cells and protect again chronic inflammation and intestinal injury.

Here, we established a unique experimental model with the Paneth cell VDR deletion. In vitro, we established a system to study purified Paneth cells in an antibacterial condition. These tools allowed us to establish the mechanisms by which Paneth cell VDR regulates the host–microbial interactions in vitro and in vivo. We rarely detected the entrance of the *Salmonella* into the Paneth cells in vivo using small-intestinal samples. *Salmonella* typically targets intestinal epithelial cells but not Paneth cells, presumably because of their significant levels of AMP secretion and autophagy.[14,35] Because autophagy is linked to defensin secretion,[14] the diminished autophagy observed may contribute to diminished AMP secretion and increased *Salmonella* that resulted. Thus, both diminished AMP production

and secretion because of diminished autophagy probably explains our findings as a consequence of VDR deletion.

In summary, we characterize the precise role of VDR in regulating intestinal homeostasis and microbiome by changing the biologic function of the Paneth cells. We propose that targeting the VDR affects multiple downstream events within Paneth cells that establishes increased host defense against enteropathogens. Insights gained from the understanding of how the VDR pathway is integrally involved in regulating Paneth cells and responses to microbes may serve as a novel paradigm for understanding the mechanisms of host–bacterial interactions in IBD and infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

AMP	antimicrobial peptide
CD	Crohn’s disease
DSS	dextran sulfate sodium
IBD	inflammatory bowel disease
VDR	vitamin D receptor

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WHAT YOU NEED TO KNOW:

BACKGROUND AND CONTEXT

While Paneth cells are known to regulate the commensal and pathogenic microbiota, the role that vitamin D receptor (VDR) in Paneth cells play in bacterial infections and Crohn's disease (CD) is unknown.

NEW FINDINGS

Decreased intestinal VDR significantly correlates with reduction of levels of ATG16L1 and Paneth cell Lysozymes in patients with CD. The lack of VDR in the Paneth cells lead to impaired anti-bacterial abilities and inflammatory responses, dysbiosis, and high susceptibility to small intestinal injury induced by indomethacin.

LIMITATIONS

No human therapeutic studies to examine if enhanced vitamin D/VDR will restore Paneth cells and protect again chronic inflammation and intestinal injury.

IMPACT

We provide new evidence of the tissue-specific roles of the VDR in intestinal and microbial homeostasis. Targeting the VDR affects multiple downstream events within Paneth cells in host defense and anti-inflammation.

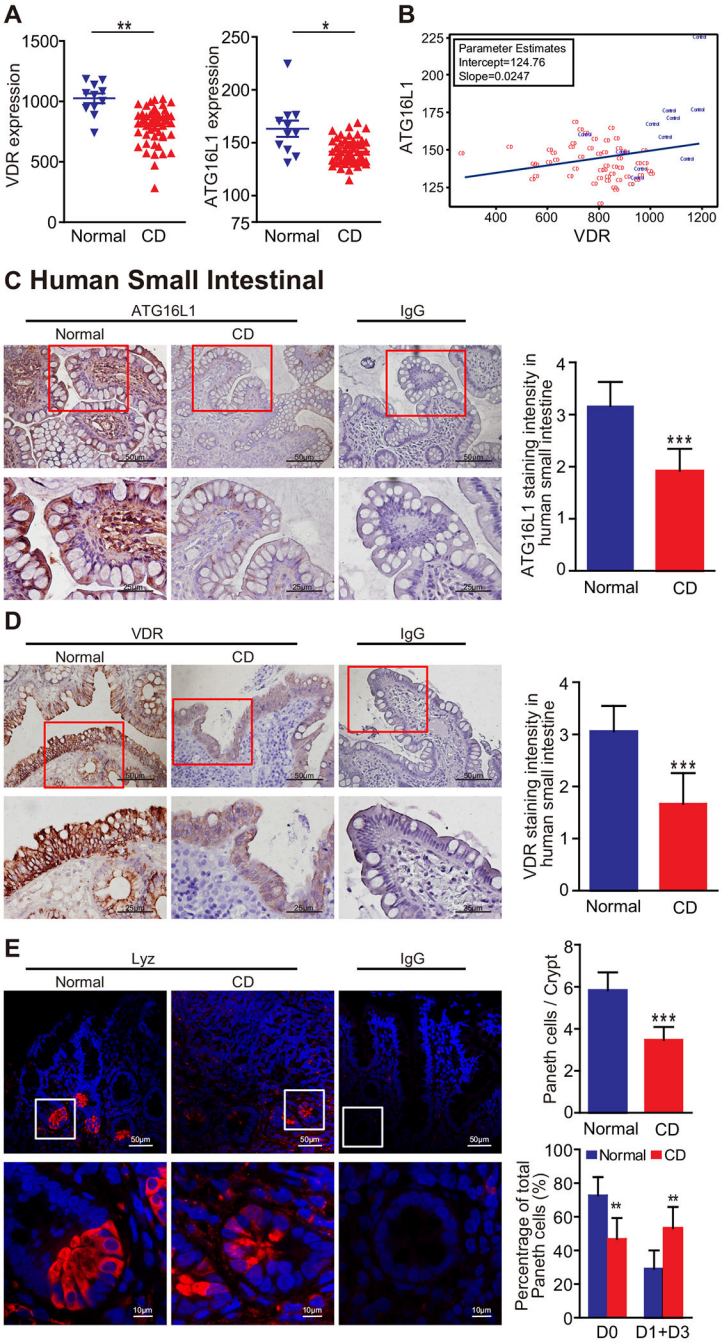

Figure 1.

The expression of VDR, *ATG16L1,* and lysozymes is downregulated in CD patients. (*A*) Reduced VDR and *ATG16L1* expression in patients with CD. Data are expressed as mean ± SEM; normal, n = 11; CD, n = 51; Welch's *t* test, **P* < .05, ***P* < .01. (*B*) Significantly coordinated expression of VDR and *ATG16L1* in CD patients. We performed a regression of VDR against *ATGT6L1* and conducted a scatter plot with a regression line. Values for healthy control subjects are in *blue* and values for CD patients in *red*. Gene Expression Omnibus database GSE102134 normal, n = 11; CD, n = 51; the coefficient is 0.0247

with $P = .0498$ in the linear regression model. (*C*) Lower *ATG16L1* expression in the small intestine tissues of the CD patients than that of normal tissues, as determined by immunohistochemical staining. Images are representative of experiments in triplicate. Data are expressed as mean ± SD, normal, n = 6; CD, n = 8; Welch's *t* test; ***$P < .001$. (*D*) Significantly lower VDR expression in the small intestine of CD patients, compared with normal small intestine. Images are representative of experiments in triplicate. Data are expressed as mean ± SD, normal, n = 6; CD, n = 8; Welch's *t* test; ***$P < .001$. (*E*) Numbers of Paneth cells per crypt and percentage of normal Paneth cells (D0) were lower in CD patients compared with normal small intestine. The percentage of Paneth cells that displayed normal (D0) and abnormal (D1–D3) patterns of lysozyme expression. Images are representative of experiments in triplicate. Data are expressed as mean ± SD, normal, n = 6; CD, n = 8; Welch's *t* test or 2-way analysis of variance test, respectively; **$P < .01$, ***$P < .001$.

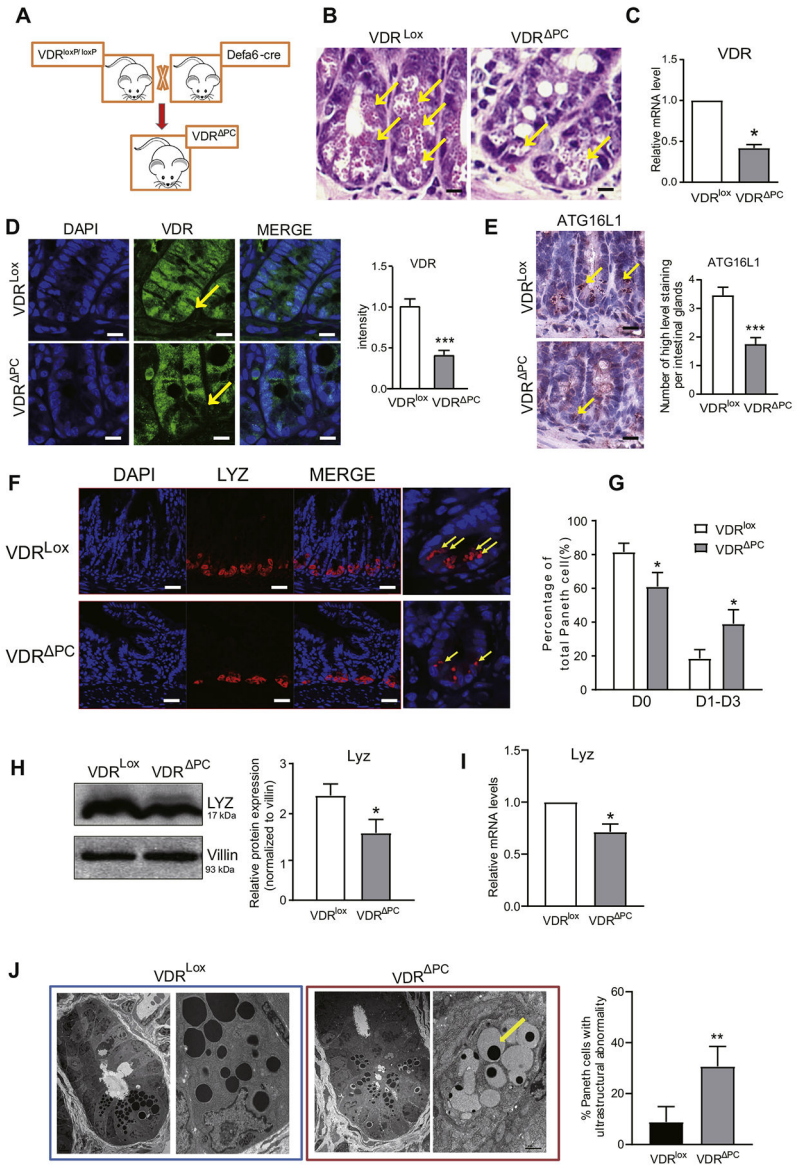

Figure 2.

The expression of VDR was decreased in VDR$^{\Delta PC}$ mice. (*A*) By crossing VDR^{lox} mice with DEFA6-cre mice, we generated Paneth cell VDR specific knockouts (VDR$^{\Delta PC}$). (*B*) Hematoxylin and eosin staining shows more granules in the small-intestine tissues of VDR^{lox} mice than in VDR$^{\Delta PC}$ mice. (*C*) RNAs of Paneth cells from the laser-captured micro-dissected ileum were collected for quantitative polymerase chain reaction. VDR expression in VDR$^{\Delta PC}$ mice was significantly reduced compared with VDR^{lox} mice. Each single experiment was performed in triplicate. Data are expressed as mean ± SEM, n = 6, Student's *t* test, **P* < .05. (*D*) There is lower VDR expression in the small intestines of VDR$^{\Delta PC}$ mice than that of VDR^{lox} mice. Immunostaining images are from a single experiment and representative of 6 mice per group. Data are expressed as means ± SEM, n = 6, Student *t* test. ****P* < .001. (*E*) Lower *ATG16L1* expression in the small-intestine tissues of the VDR$^{\Delta PC}$ mice than in the VDR^{lox} mice. Images are from a single experiment

and are representative of 6 mice per group. Data are expressed as means ± SEM, n = 6, Student *t* test. ***$P < .001$. (*F*) Lower expression of the lysozymes in the $VDR^{\Delta PC}$ mice by immunofluorescence staining. *Yellow arrows* represent normal lysozyme expression in the Paneth cells. Images are from a single experiment and are representative of 6 mice per group. (*G*) The percentage of normal Paneth cells was lower in $VDR^{\Delta PC}$ mice. Paneth cells displayed normal (D0) and abnormal (D1–D3) patterns of lysozyme (10 per group). Data are expressed as mean ± SEM. Student's *t* test, *$P < .05$. (*H*) Lower expression of lysozymes in $VDR^{\Delta PC}$ mice by Western blotting. Each single experiment was performed in triplicate. Data are expressed as the mean ± SEM. Student's *t* test, *$P < .05$. (*I*) RNA of the ileum tissues was collected for quantitative polymerase chain reaction. The lysozyme expression in the $VDR^{\Delta PC}$ mice was significantly reduced compared with VDR^{lox} mice. Each single experiment was performed in triplicate. Data are expressed as the mean ± SEM, n = 6, Student's *t* test, *$P < .05$. (*J*) More abnormal Paneth cells in $VDR^{\Delta PC}$ mice compared with VDR^{lox} mice by transmission electron microscopy. Paneth cells were identified by the presence of cytoplasmic granules. Data are expressed as the mean ± SEM, n = 6, Student's *t* test, **$P < .01$.

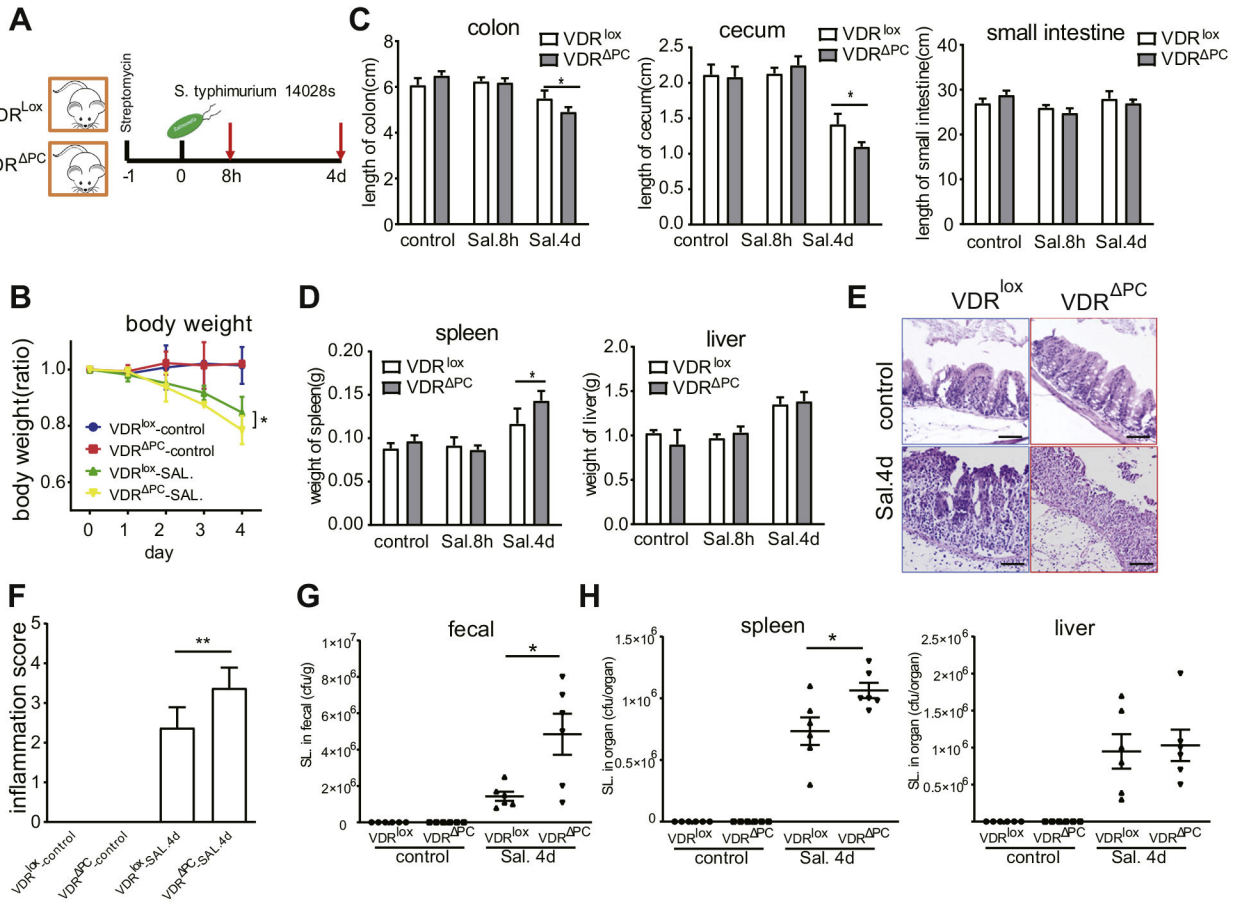

Figure 3.

Salmonella infections were worse in VDR$^{\Delta PC}$ mice. (*A*) A *Salmonella* infection model. (*B*) Changes in the relative body weights after *Salmonella* infections. Data are expressed as mean ± SEM, n = 10, 2-way analysis of variance (ANOVA) test, **P* < .05. (*C*) The length of cecum and colon for the VDR$^{\Delta PC}$ group were shorter, compared with the VDR^{lox} mice, 4 days after infection. Data are expressed as mean ± SEM, n = 10, 2-way ANOVA test, **P* < .05. (*D*) Large spleens were found in VDR$^{\Delta PC}$ mice 4 days postinfection. Data are expressed as mean ± SEM, n = 10, 2-way ANOVA test, **P* < .05. (*E* and *F*) Higher inflammation scores in the cecum of the VDR$^{\Delta PC}$ mice 4 days after infection by hematoxylin and eosin staining. Data are expressed as mean ± SEM, n = 10, 1-way ANOVA test, ***P* < .01. (*G*) Quantification of *Salmonella* in feces 4 days postinfection. Data are expressed as mean ± SEM, n = 10, 1-way ANOVA test, **P* < .05. (*H*) Quantification of the *Salmonella* in the spleen and liver 4 days postinfection. Data are expressed as mean ± SEM, n = 10, 1-way ANOVA test, **P* < .05. Each single experiment was assayed in triplicate.

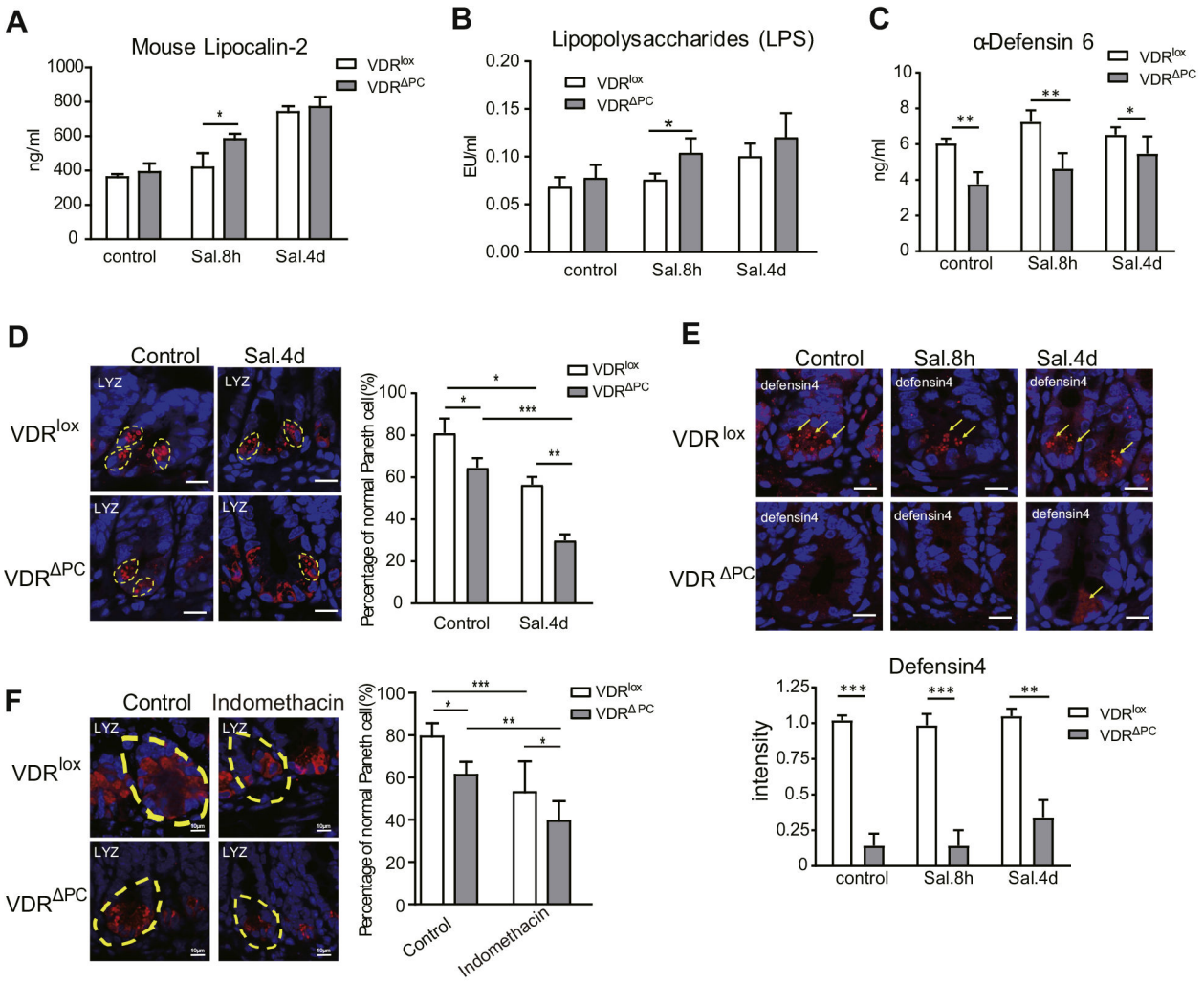

Figure 4.

Lack of VDR in Paneth cells leads to increased inflammatory responses. (*A*) Expression of lipocalin-2 was higher in feces of VDR$^{\Delta PC}$ mice. Each enzyme-linked immunosorbent assay (ELISA) was assayed in triplicate. Data are expressed as mean ± SEM, n = 10, 2-way analysis of variance (ANOVA) test, $*P < .05$. (*B*) More serum lipopolysaccharide from VDR$^{\Delta PC}$ mice 8 hours after infection. Each single experiment was assayed in triplicate. Data are expressed as the mean ± SEM, n = 10, 2-way ANOVA test, $*P < .05$. (*C*) Expression of α-defensin6 in the ileum tissue in VDR$^{\Delta PC}$ mice was lower than that in VDR^{lox} mice by ELISA. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM, n = 10, 2-way ANOVA test, $*P < .05$, $**P < .01$. (*D*) The percentage of normal Paneth cells was reduced after *Salmonella* infection, especially in VDR$^{\Delta PC}$ mice. Data are expressed as mean ± SEM, n = 20, 2-way ANOVA test, $*P < .05$, $**P < .01$, $***P < .001$. (*E*) Lower expression of the defensin4 in VDR$^{\Delta PC}$ mice after *Salmonella* infection, n = 6–9, 1-way ANOVA test, $**P < .01$, $***P < .001$. (*F*) The percentage of normal Paneth cells was reduced after indomethacin treatment, especially in VDR$^{\Delta PC}$ mice. Data are expressed as mean ± SD, n = 6–9, 1-way ANOVA test, $*P < .05$, $**P < .01$, $***P < .001$.

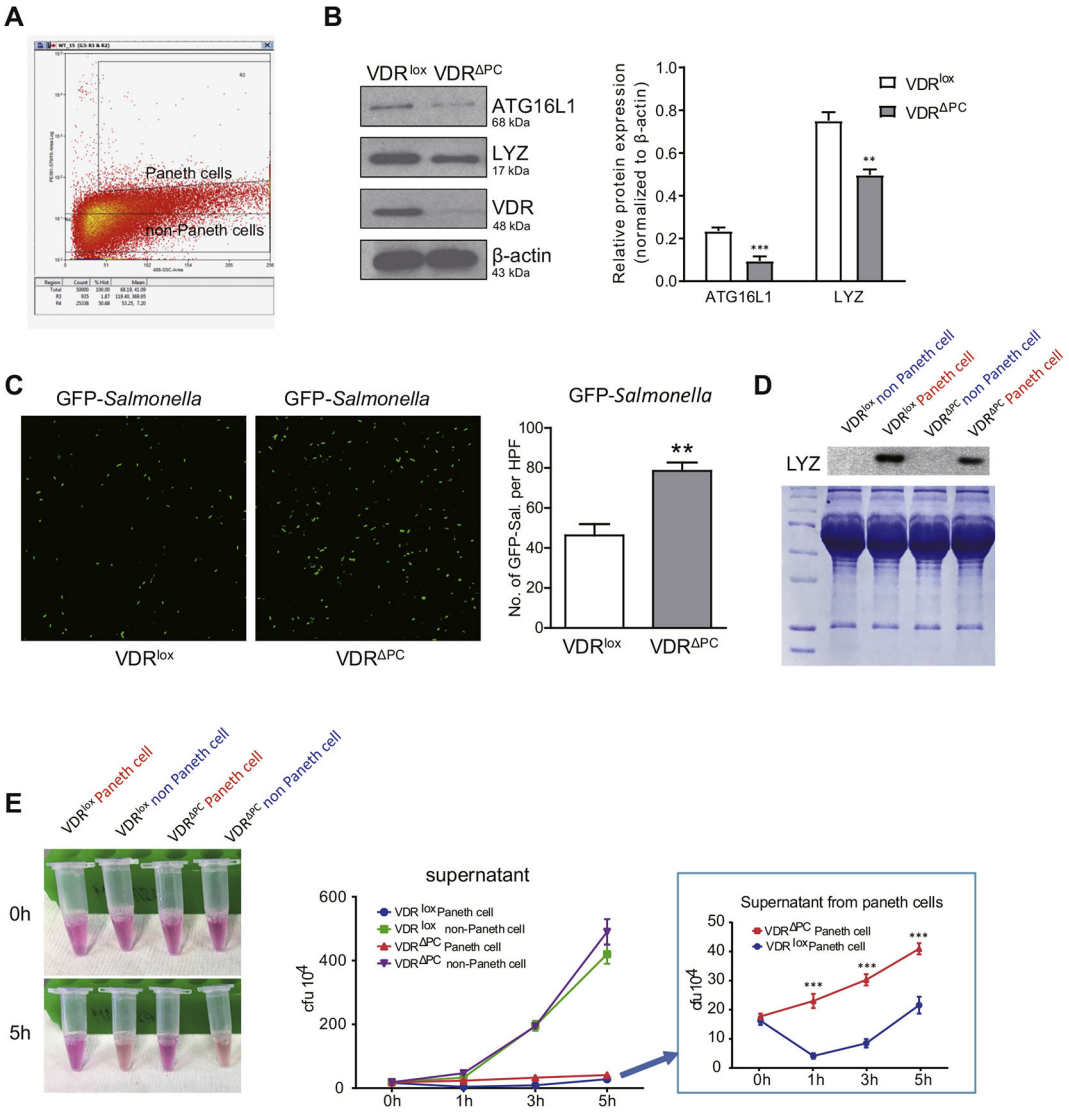

Figure 5.

Lack of VDR in Paneth cells leads to impaired antibacterial abilities. (*A*) Small intestine was collected and digested into single cells. After staining with the anti-CD24 Ab, cells were detected by flow cytometry. Paneth cells and non-Paneth cells were isolated. Each single experiment was performed in triplicate. (*B*) Lack of VDR in Paneth cells led to decreased expression of *ATG16L1* and LYZ. Western blots were done using isolated Paneth cells from VDR^{lox} and $VDR^{\Delta PC}$ mice. Each single experiment was performed in triplicate. Data are expressed as mean ± SEM, n = 6, Student's *t* test, $^{**}P < .01$, $^{***}P < .001$. (*C*) Isolated Paneth cells incubated with green fluorescent protein *Salmonella* for 5 hours. Paneth cells in the VDR^{lox} group could inhibit *Salmonella* growth. Each single experiment was performed in triplicate. Data are expressed as mean ± SEM, n = 20, Student's *t* test, $^{**}P < .01$. (*D*) Expression of the lysozyme was lower in the $VDR^{\Delta PC}$ supernatant than the VDR^{lox} supernatant. Paneth cells and non-Paneth cells were incubated overnight and the supernatant collected. Each experiment was performed in triplicate. (*E*) The supernatant from VDR^{lox} Paneth cells had stronger antibacterial abilities than that of the $VDR^{\Delta PC}$ group. *Salmonella*

was added into the supernatant. After 1, 3, and 5 hours, the medium was collected for counting the number of *Salmonella.* The supernatant from the Paneth cells stayed clear for 5 hours because of the antibacterial ability. Each single experiment was performed in triplicate. Data are expressed as mean ± SEM, n = 20, 2-way analysis of variance test, ***$P < .001$.

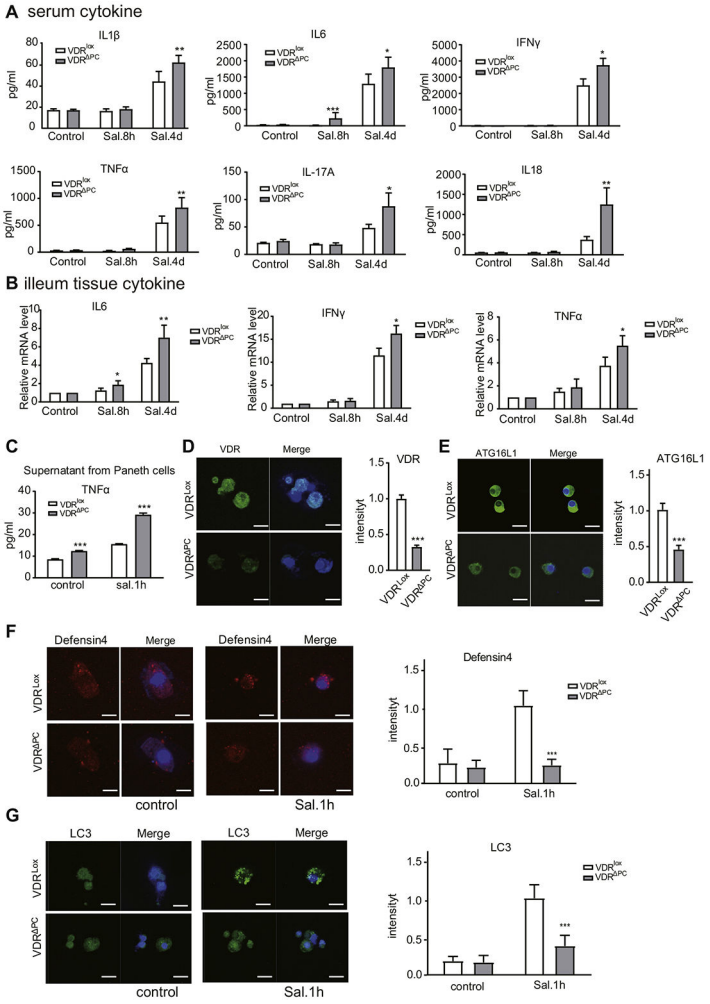

Figure 6.

Lack of VDR in Paneth cells leads to impaired inflammatory responses. (*A*) Serum inflammatory cytokines were significantly increased, especially in $VDR^{\Delta PC}$ mice with the infection. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM, n = 6, 1-way analysis of variance (ANOVA) test, $*P < .05$, $**P < .01$, $***P < .001$. (*B*) Expression of interferon gamma, IL1β, and TNF-β were increased in the $VDR^{\Delta PC}$ ileum postinfection. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM, n = 6, 1-way ANOVA test, $*P < .05$, $**P < .01$. (*C*) The expression of TNF-α was increased in $VDR^{\Delta PC}$ supernatant after *Salmonella* infection, as determined by enzyme-linked immunosorbent assay. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM, n = 10, 1-way ANOVA test, $***P < .001$. (*D*) Expression of the VDR was decreased in $VDR^{\Delta PC}$ Paneth cells. Data are expressed as mean ± SEM, n = 10, Student's *t* test, $***P < .001$. (*E*) Expression of *ATG16L1* was decreased in $VDR^{\Delta PC}$ Paneth cells. Data are expressed as mean ± SEM, n = 10, Student's *t* test, $***P < .001$. (*F*) Defensin4 expression in $VDR^{\Delta PC}$ Paneth cells was low. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM, n = 10, 1-way ANOVA test, $***P < .001$. (*G*) After *Salmonella* treatment for 1 hour, stronger autophagy signals were found in VDR^{lox}

cells compared with $VDR^{\Delta PC}$ cells. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM, n = 10, 1-way ANOVA test, ***$P < .001$.

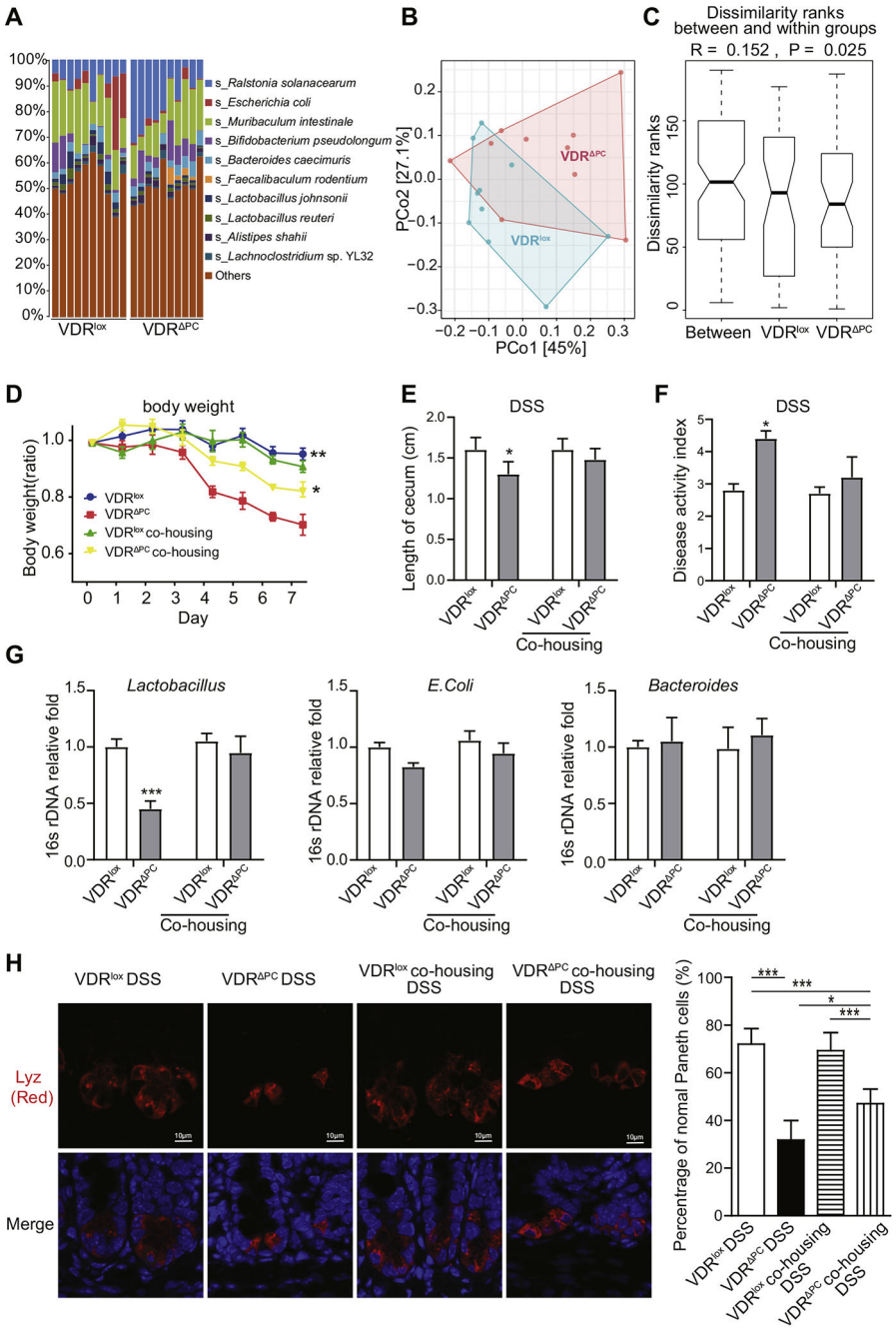

Figure 7.

Lack of VDR in Paneth cells affects gut microbiota and sensitivity to chemical damage. (*A*) Bacterial abundance in feces of VDR$^{\Delta PC}$ and VDR^{lox} mice. The relative bacterial abundance of top 10 species (s_) is shown. All unidentified and other identified species were grouped into “others”. Species were colored using the key as list seen on the right side. Each bar represents individual mouse, n = 10 per group. (*B*) Principal coordinate analysis visualized the sample differences between VDR$^{\Delta PC}$ and VDR^{lox} mice. Samples were collected from VDR$^{\Delta PC}$ (*red*) and VDR^{lox} mice (*light blue*). Two axes explain 72.1% (45% + 27.1%) of total sample variations, n = 10 per group. (*C*) Plots of between- and within-mean ranks of

Bray-Curtis dissimilarity. The analysis of similarity was performed to test the Bray-Curtis dissimilarity on between- and within-groups of VDR^{lox} and $VDR^{\Delta PC}$. n = 10 per group, $P = .025$. (*D*) Relative body weight changes in mice with DSS. $VDR^{\Delta PC}$ mice had worse outcomes with DSS-induced colitis. Data are expressed as mean ± SEM, n = 6, 2-way analysis of variance (ANOVA) test, $^{**}P < .01$, $^{*}P < .05$. (*E*) A shortened cecum was found in $VDR^{\Delta PC}$ mice with colitis but not for co-housing mice. After 4 weeks of co-housing, mice were administered 5% DSS for 7 days to induce colitis. Data are expressed as the mean ± SEM, n = 6, Student's *t* test compared between VDR^{Lox} and $VDR^{\Delta PC}$ mice, $^{*}P < .05$. (*F*) Disease Activity Index of colon from mice with DSS treatment. Data are expressed as the mean ± SEM, n = 6, Student's *t* test compared between VDR^{Lox} and $VDR^{\Delta PC}$ mice, $^{*}P < .05$. (*G*) *Lactobacillus* in $VDR^{\Delta PC}$ mice was enhanced after co-housing with VDR^{Lox} mice. Data are expressed as means ± SEM. Each experiment was assayed in triplicate, n = 6, 1-way ANOVA test, $^{***}P < .001$. (*H*) Percentage of normal Paneth cells in $VDR^{\Delta PC}$ mice was increased after co-housing with VDR^{Lox} mice. Immunofluorescence images are representative of experiments in triplicate. Data are expressed as mean ± SD, n = 5–6, 1 way ANOVA test, $^{*}P < .05$, $^{***}P < .001$.