Paneth cell alertness to pathogens maintained by vitamin D receptors

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Short title: Vitamin D receptor in Paneth cells protects from pathogens

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Author Contributions

RL, YZ, and JZ performed the cellular and animal studies, the detailed analyses of the results, prepared the figures and the draft text; YX contributed to the statistical analysis of data and the draft text; AK and RB provided materials and critical intelligence contributions; and JS obtained funds, designed the study, and directed the project. All authors contributed to the writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Abstract

**Background** Vitamin D exerts a regulatory role over mucosal immunity via the vitamin D receptor (VDR). While 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 and Results** We identified the decreased intestinal VDR significantly correlated with reduction of an IBD risk gene ATG16L1 and Paneth cell Lysozymes in patients with Crohn’s Disease. We generated a Paneth cell specific VDR knockout (VDR$^{\Delta PC}$) mice to investigate the molecular mechanisms. 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$^{loxp}$ mice made the VDR$^{\Delta PC}$ less vulnerable to the DSS-colitis, suggesting the transmission of protective bacterial from the VDR$^{loxp}$ mice. Thus, a lack of VDR in Paneth cells leads to impaired anti-bacterial 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. **Conclusion** We provide new insights into the tissue-specific functions of VDRs in maintaining the 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.

**Keywords**

Autophagy, Bacteria, Crohn’s Disease (CD), Defensin, dysbiosis, inflammatory bowel diseases, inflammation, infection, *Lactobacillus*, *Salmonella*, Microbiome, Nonsteroidal anti-inflammatory drug (NSAID), nuclear receptor, Paneth cells, Vitamin D, Vitamin D receptor.

**Abbreviations used in this paper:**
AMP: antimicrobial peptides
ANOVA: Analysis of variance
ATG16L1: autophagy related 16 like1
CD: Crohn’s disease
CFU: colony-forming unit
DSS: dextran sulfate sodium
GFP: Green fluorescent protein
H&E: hematoxylin and eosin
IBD: inflammatory bowel diseases
IEC: intestinal epithelial cells
IF: Immunofluorescence
IHC: immunohistochemical
GEO: Gene Expression Omnibus
LCN2: Lipocalin-2
LPS: Lipopolysaccharides
Loxp (or Lox): locus of x-over, P1
Lyz: lysozyme
NSAID: nonsteroidal anti-inflammatory drugs
PC: Paneth cells
PCoA: Principal coordinate analysis
PERMANOVA: permutational multivariate ANOVA
qRT-PCR: quantitative RT-PCR
VDR: Vitamin D receptor
Introduction

Vitamin D deficiency is associated with various diseases, including infections and IBD \(^{1-6}\). This is in part, because vitamin D exerts a regulatory role in mucosal immunity and host defenses, via VDRs \(^{6}\). VDRs belong to the nuclear receptor superfamily. They 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 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 (AMP) cathelicidin \(^{15}\) and \(\beta\) defensin \(^{16}\). Several of the 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 that was induced by VDR deletion, would lead to abnormal anti-bacterial 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 (VDRΔPC) 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, the VDRΔPC mice had significantly higher levels of inflammation post Salmonella infection. The lack of VDR in the Paneth cells led to impaired anti-bacterial abilities and inflammatory responses. VDRΔPC mice also showed high susceptibility to small intestinal injury induced by indomethacin, a NSAID. Furthermore, we examined the dysbiosis in the VDRΔPC mice. Co-housing of VDRΔPC and VDRlox mice made the VDRΔPC less vulnerable to the DSS-colitis, suggesting the transmission of protective bacterial from the VDRlox mice. Thus, our study fills an existing gap in the knowledge by characterizing the precise role of the tissue specific VDRs in regulating the Paneth cells in host defenses, from the enteric pathogens.

**Materials and Methods (Details in the Supplement Documents)**

**Human intestinal biopsies**

Slides containing paraffin-embedded small intestinal biopsy samples of patients with CD and healthy controls 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 previous publication 12.

**Gene expression datasets**
For expression analyses, we used microarray data reported in the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/) (GEO accession number GSE102134) reported on ileum of patients with Crohn’s Disease and normal ileum from control individuals.

Experimental Animals

Paneth cell VDR specific knockouts (VDR$^{ΔPC}$) were obtained by crossing VDR$^{loxP/loxP}$ mice with DEFA6-cre mice. The mice were provided with water ad libitum and maintained in a room with a 12 h 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 adhere to the ARRIVE guidelines and was approved by the University of Illinois at Chicago Committee on Animal Resources and ethical guidelines were followed with the treatment.

Bacterial strains and growth conditions

The *Salmonella* strain used in this study was *S. Typhimurium* 14028. Bacterial cultures were prepared by inoculating 10 ml of LB broth with 0.01 ml of a stationary-phase culture, followed by overnight incubation at 37 °C.

*Salmonella*-infected mouse model

Animal experiments were performed using VDR$^{loxP/loxP}$ and VDR$^{ΔPC}$ mice (male and female, 2–3-month-old). Water and food were withdrawn 4 h before oral gavage, with 7.5 mg/mouse of streptomycin. Afterwards, the animals were supplied with water and food ad libitum. Twenty hours after the streptomycin treatment, the water and food were withdrawn again for 4 h, before the mice were infected with $1*10^6$ colony-forming units of *Salmonella* (100-ml suspension in HBSS by gavage.

Co-housing of VDR$^{lox}$ and VDR$^{ΔPC}$ mice and Induction of DSS-colitis
Two-to-three-month-old female VDR\textsuperscript{lox} and VDR\textsuperscript{ΔPC} mice were co-housed in new cages according to previously published methods\textsuperscript{8}. One cage contained three VDR\textsuperscript{lox} and two VDR\textsuperscript{ΔPC}, another one contained two mice each. The mice were fed with the same food and water. After 4 weeks of co-housing, 5% DSS dissolved in filter-purified water was administered to the mice. Animals were weighed daily. At day 7 after 5% DSS (MW = 40–50 kDa; USB Corp. Cleveland, OH) administration, mice were sacrificed\textsuperscript{8}.

**Induction of small-intestinal lesions**

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

**Statistical analyses** (details 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. The microbiome data were analyzed by using R packages of ampvis2, microbiome, phyloseq, and vegan, as did in our book on statistical analysis of microbiome data\textsuperscript{24}.

**RESULTS**

Reduced VDR positively correlated with the reduction of ATG16L1 and Paneth cell lysozyme in human CD.
We found that the mRNA expression levels of VDR and ATG16L1 genes were both significantly reduced in human CD, using the GEO database GSE102134\(^1\) (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 IHC staining using small intestine tissues from healthy controls and CD patients. As shown in Figure 1C, there is lower ATG16L1 expression in the CD patients than that in the normal small intestine. We found the significantly lower VDR expression in the small intestine of CD patients, compared to 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 the 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 have established a VDR\(^{\Delta PC}\) model by crossing VDR\(^{\text{loxP/loxP}}\) (VDR\(^{\text{lox}}\)) mice with DEFA6-cre mice (Figure 2A). We found more granules in Paneth cells in the small intestine tissues of the VDR\(^{\text{lox}}\) mice than in the VDR\(^{\Delta PC}\) mice, via H&E staining (Figure 2B). To verify that the VDR gene was deleted in Paneth cells, the Paneth cells from the ileum tissue were laser captured and microdissected for qPCR.
The VDR expression in the VDRΔPC Paneth cells was significantly reduced compared to that in the VDRlox Paneth cells (Figure 2C). Furthermore, there was no detectable VDR expression in the intestinal Paneth cells of the VDRΔPC mice by IF staining (Figure 2D). These data confirmed that the Paneth cell VDR knock out 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. We found lower levels of the ATG16L1 expression in the small intestine tissues of the VDRΔPC mice compared with those of the VDRlox mice, by IHC staining (Figure 2E). Paneth cells are specialized intestinal epithelial cells that secrete AMPs, sense commensal bacteria, and maintain homeostasis at the intestinal-microbial interface. We evaluated the status of the Paneth cells by lysozyme detection. Lower expression levels of the lysozymes in the VDRΔPC mice were found by IF staining, when compared with the VDRlox mice (Figure 2F). We found the percentage of the normal Paneth cells (D0) was much lower in the VDRΔPC mice (Figure 2G). Based on the lysozymes patterns, abnormal Paneth cells were grouped as D1-D3. Abnormal Paneth cells were significantly higher in the VDRΔPC mice. These results were further confirmed by testing the lysozyme (Lyz) expression by western blotting and real time PCR (Figure 2H&I). In the VDRΔPC mice, Lyz was significantly reduced at the protein and mRNA levels. The structure of the Paneth cells observed by a transmission electron microscopy (TEM) also revealed more abnormal cells (e.g. less granules) in the VDRΔPC mice, when compared to the VDRlox 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 beta-catenin in intestinal stem and progenitor cells through phosphorylation at Ser552 (P-beta-catenin552) \(^{27}\). We found that P-beta-catenin552 was decreased in the small intestine of the VDR\(^{\Delta PC}\) mice, when compared to the VDR\(^{lox}\) mice (Figure S1). We also examined the stem cell markers (Igr5 and bmi1). However, there was no significant change between VDR\(^{\Delta PC}\) and VDR\(^{lox}\) mice (data not shown).

**Salmonella infections were worse in the 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). The 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 post-infection (Figure 3C). Higher inflammation scores were also found in the cecum tissues of the VDR\(^{\Delta PC}\) mice 4-days post-infection, by H&E staining (Figure 3 E & F). The lengths of the small intestine were measured, but no significant differences were found between the two mouse models. Both the liver and spleen were involved in the *Salmonella* infections, and so we measured their post-infection 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 unit (CFU) were determined by plating on agar plates to determine the *Salmonella* colonies. The fecal and spleen samples, 4 days post-infection, showed more *Salmonella* in the VDR\(^{\Delta PC}\) mice, compared with the VDR\(^{lox}\) mice (Figure 3 G & H).

Lipocalin-2 (LCN2) is a pleiotropic mediator of various inflammatory processes \(^{31}\). We found an increased expression level of lipocalin2, and its expression was higher in the VDR\(^{\Delta PC}\) group 8-hour post infection, compared to VDR\(^{lox}\) mice (Figure 4A). Lipopolysaccharides (LPS) are
characteristic components of the cell walls of Gram-negative bacteria. The LPS increased in the VDRΔPC mice compared to the VDRlox mice 8-hour post infection (Figure 4B). The α-defensin6 is expressed in the Paneth cells of the ileum. Its expression in the ileum tissues of the VDRΔPC mice was significantly lower than that in the VDRlox mice, even before Salmonella infection (Figure 4C). The percentage of the normal Paneth cells was further reduced in the VDRΔPC mice postinfection (Figure 4D). With the decrease in the number of the normal Paneth cells, the expression of the defensin4 was significantly downregulated in the VDRΔPC mice after the Salmonella treatments (Figure 4E). Taken together, the biologically significance of the VDRΔPC mice in a Salmonella-colitis model was measured by several readouts, including bodyweight, intestinal inflammation, bacterial burden, bacterial translocation, LCN2, LPS, and cellular changes of Paneth cells.

**VDRΔPC mice showed reduced Paneth cells during small intestinal injuries**

To further test the effects of VDRΔPC mice in response to small intestinal injury, we treated mice with indomethacin, a NSAID. It is a relevant Crohn's 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 the VDRΔPC 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 AMPs32. To test their antimicrobial activity in a well-controlled condition, the 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, the 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 the Paneth cells led to significantly decreased expressions of the ATG16L1 and LYZ (Figure 5B). These data also validated the successful deletion of VDR in Paneth cells in the VDR\textsuperscript{\textasciitilde\textalpha} mice. Then, we tested the antibacterial functions of the purified Paneth cells. Isolated Paneth cells were incubated with GFP-\textit{Salmonella} for 5 hours. The Paneth cells in the VDR\textsuperscript{\textalpha} group reduced the bacterial clearance ability and had more growth of the \textit{Salmonella}, compared to the VDR\textsuperscript{\textasciitilde} Paneth cells (Figure 5C). The lack of \textit{VDR} genes caused the VDR\textsuperscript{\textasciitilde} Paneth cells to lose their anti-bacterial 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 Lyz gene was lower in the VDR\textsuperscript{\textasciitilde} supernatant than in the VDR\textsuperscript{\textasciitilde} supernatant (Figure 5D). We then added \textit{Salmonella} into these supernatant samples. After 5 hours of incubation, we collected the medium to determine the number of CFU. At 0 hour, the supernatant samples were clear, but after 5 hours we found that only two samples remained clear, and these two samples were from the supernatant of the Paneth cells (Figure 5E). Paneth cells can secrete AMPs and proteins to inhibit the growth of \textit{Salmonella}. The VDR\textsuperscript{\textasciitilde} group had a stronger antibacterial ability than the VDR\textsuperscript{\textasciitilde} (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\textsuperscript{\textasciitilde} non-paneth cells) and purple line (VDR\textsuperscript{\textasciitilde} non-paneth cells) show the similar trend, no significant difference of cfu (Figure 5E). Taken together, these experiments have confirmed the decreased antibacterial ability of the VDR\textsuperscript{\textasciitilde} Paneth cells without VDR.

\textbf{Lack of VDR in the intestinal Paneth cells leads to increased inflammatory responses in the setting of \textit{Salmonella} infection}
Inflammatory cytokines play a complex role in intestinal inflammation. We thus investigated the profile of the cytokines in the serum of the mice. The cytokines we tested included interleukin (IL)-1b, IL-6, IL-17A, IL-18, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. For IL-6, significant differences were also found between the VDRΔPC and VDRlox mice after 8 hours and 4 days post-infection. Four days post-infection, significant differences in the IL-1β, IL-17A, IL-18, IFN-γ and TNF-α were found in the salmonella treated mice, especially the VDRΔPC mice (Figure 6A). The increased expressions of the IFNγ, IL6, and TNFα in the ileum tissue of VDRΔPC mice after the Salmonella treatments were also detected by real time PCR (Figure 6B), indicating the enhanced local inflammation in the small intestine of VDRΔPC mice. The TNF-alpha is a multifunctional proinflammatory cytokine. Paneth cells were treated with Salmonella for 1 hour and the supernatant was collected to detect the TNFα by ELISA. The expression of the TNFα was higher in the VDRΔPC supernatant than that in the VDRlox supernatant (Figure 6C).

In the VDRΔPC Paneth cells, the expression of the VDR decreased with the lower expression levels of the ATG16L1 (Figure 6D&6E). The defensin4 expression in the VDRΔPC Paneth cells was also lower than that of the VDRlox cells post Salmonella infection (Figure 6F). We detected the autophagy marker of the Paneth cells with the CYTO-ID® Autophagy detection kit. After 1-hour of the Salmonella treatment, we found stronger LC3 expression in the VDRlox cells, compared to the VDRΔPC cells (Figure 6G), suggesting reduced autophagic responses due to 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. We examined the bacterial abundance in feces of VDRΔPC and VDRlox mice, using the shotgun metagenomic sequencing. We found that bacterial homeostasis in the VDRΔPC mice was
changed, compared with the VDR\textsuperscript{lox} mice without any treatment (Figure 7A&B). Taxa abundance and diversity of bacteria of VDR\textsuperscript{ΔPC} and VDR\textsuperscript{lox} mice were shown in Figure 7A. The relative bacterial abundance in species level (s$_{__;}$) was shown with the top 10 species. 

*Ralstonia solanacearum* and *Faecalibaculum rodentium* were enriched, and *Lactobacillus* was depleted in the VDR\textsuperscript{ΔPC} mice. PCoA showed that overall the VDR\textsuperscript{lox} and VDR\textsuperscript{ΔPC} mice were separated each other with only partially overlapped, and two principal coordinate axes could explain 72.1\% of variances (Figure 7B). Furthermore, we performed the permutational multivariate ANOVA of Bray-Curtis dissimilarity, which confirmed the VDR\textsuperscript{lox} and VDR\textsuperscript{ΔPC} mice have different dissimilarities (p=0.027). We also confirmed that the dissimilarity of bacteria in VDR\textsuperscript{ΔPC} mice was significantly decreased, compared with VDR\textsuperscript{lox} mice (Figure 7C).

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

Because the microbiota may play a role in the vulnerability of the VDR\textsuperscript{ΔPC} mice to the 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 the co-housing decreased the disease activity index of VDR\textsuperscript{ΔPC} mice to a level similar to that seen in the VDR\textsuperscript{lox} mice (Figure 7E&7F). Inflammation scores in the colon tissues of the VDR\textsuperscript{ΔPC} mice were reduced after co-housing with VDR\textsuperscript{Lox} mice (Figure S2 A & B). *Lactobacillus* in the VDR\textsuperscript{Lox} mice increased after co-housing treatment (Figure 7G), and *Lactobacillus* strains have been shown to decrease the
inflammatory response in the intestine. Interestingly, the numbers of Paneth cells per crypt were restored in the VDR\textsuperscript{ΔPC} mice after co-housing with the VDR\textsuperscript{lox} mice (Figure S2C). The percentage of normal Paneth cells (D0) in the VDR\textsuperscript{ΔPC} mice was significantly increased after co-housing with the VDR\textsuperscript{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 have identified the decreased intestinal VDR significantly correlated with reduction of ATG16L1 and Paneth cell Lysozymes in patients with Crohn’s Disease. We have demonstrated that a lack of VDR in Paneth cells leads to impaired anti-bacterial activities and increased inflammatory responses in the setting of an enteropathogenic exposure. The lysozymes in Paneth cells was significantly decreased in the VDR\textsuperscript{ΔPC} mice compared to the VDR\textsuperscript{lox} mice. The inhibition of the bacterial growth by the VDR\textsuperscript{ΔPC}/CD24+ cells was significantly weakened, and the VDR\textsuperscript{ΔPC} mice had high inflammation levels post *Salmonella* infection. We found the dysbiosis in the VDR\textsuperscript{ΔPC} mice. Interestingly, co-housing of VDR\textsuperscript{ΔPC} and VDR\textsuperscript{lox} mice made the VDR\textsuperscript{ΔPC} less vulnerable to the DSS-colitis, suggesting the transmission of protective bacteria community from the VDR\textsuperscript{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. 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 \(^{15}\), \(\beta\) 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 of VDR leads to decreased number of Paneth cells, abnormal Paneth cells in differentiation, defects in AMPs secretion, and reduced pathogen clearance. VDR\(^{\Delta \text{PC}}\) mice also showed susceptibility to small intestinal injury induced by a NSAID. 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-beta-catenin 552 was decreased in the small intestine of the VDR\(^{\Delta \text{PC}}\) mice. Previous studies have shown that Wingless activate beta-catenin in intestinal stem cells through P-beta-catenin 552 \(^{27}\). Salmonella infection is also known to involve the beta-catenin pathway \(^{36}\). In the future study, we will investigate the effect and mechanism of VDR deletion in Paneth cells 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 controls \(^{38}\). 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 against chronic inflammation and intestinal injury.

Here, we have established a unique experimental model with the Paneth-cell-VDR deletion. In vitro, we have established a system to study the purified Paneth cells in an anti-bacterial 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 the small intestinal samples. Salmonella typically targets intestinal epithelial cells, but not the Paneth cells, presumably due to their significant levels of AMP secretion and autophagy. As autophagy is linked to defensin secretion, the diminished autophagy observed may contribute to diminished AMP secretion and increased Salmonella that resulted. Thus both diminished AMP production and secretion due to diminished autophagy probably explain 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 biological 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 the Paneth cells and responses to microbes may serve as a novel paradigm for understanding the mechanisms of host-bacterial interactions in IBD and infection.

Figure Legends

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 were expressed as mean ± SEM; Normal, n=11; CD, n=51; Welch’s t test, * P < 0.05, ** P < 0.01.

(B) Significantly coordinated expression of VDR and ATG16L1 in CD patients. We performed a regression of VDR against ATG16L1 and conducted a scatter plot with a regression line. Values for healthy controls were in blue color and values for CD patients were in red color. GEO database GSE102134 Normal, n=11; CD, n=51; the coefficient is 0.0247 with P = 0.0498 in linear regression model.

(C) Lower ATG16L1 expression in the small intestine tissues of the CD patients than that of the normal, as determined by IHC staining. Images were representative of experiments in triplicate; Data were expressed as mean ± SD, Normal, n=6; CD, n=8; Welch’s t test; ***P < 0.001.

(D) Significantly lower VDR expression in the small intestine of CD patients, compared with normal small intestine. Images were representative of experiments in triplicate; Data were expressed as mean ± SD, Normal, n=6; CD, n=8; Welch’s t test; ***P < 0.001.

(E) Numbers of Paneth cells per crypt and percentage of normal Paneth cells (D0) were lower in the CD patients compared with normal small intestine. The percentage of the Paneth cells that displayed normal (D0) and abnormal (D1 to D3) patterns of lysozyme expression. Images were representative of experiments in triplicate; Data were expressed as mean ± SD, Normal, n=6; CD, n=8; Welch’s t test or two way ANOVA test, respectively; **P < 0.01, ***P < 0.001.

Figure 2. The expression of VDR was decreased in the VDRΔPC mice

(A) By crossing the VDRlox mice with DEFA6-cre mice, we generated Paneth cell VDR specific knockouts (VDRΔPC).

(B) HE staining shows more granules in the small intestine tissues of the VDRlox mice than in the VDRΔpc mice.

(C) RNAs of the Paneth cells from the laser captured micro-dissected ileum were collected for qPCR. The VDR expression in the VDRΔPC mice was significantly reduced compared to the
VDRlox mice. Each single experiment was performed in triplicate. The data are expressed as mean ± SEM. n = 6, Student’s t-test, *P < 0.05.

(D) There is lower VDR expression in the small intestines of the VDRΔPC mice than that of the VDRlox mice. Immunostaining images are from a single experiment and representative of 6 mice/group. Data are expressed as means ± SEM. n = 6, Student t test. ***P < 0.001.

(E) Lower ATG16L1 expression in the small intestine tissues of the VDRΔPC mice than the VDRlox mice. Images are from a single experiment and are representative of 6 mice/group. Data are expressed as means ± SEM. n = 6, Student t test. ***P < 0.001.

(F) Lower expression of the lysozymes in the VDRΔPC mice by IF staining. Yellow arrows: normal lysozyme expression in the Paneth cells. Images are from a single experiment and are representative of 6 mice/group.

(G) The percentage of normal Paneth cells was lower in the VDRΔPC mice. Paneth cells displayed normal (D0) and abnormal (D1-D3) patterns of lysozyme (n = 10/ group). The data are expressed as mean ± SEM. Student’s t-test, *P < 0.05.

(H) Lower expression of lysozymes in the VDRΔPC mice by western blotting. Each single experiment was performed in triplicate. The data are expressed as the mean ± SEM. Student’s t-test, *P < 0.05.

(I) RNA of the ileum tissues was collected for qPCR. The lysozyme expression in the VDRΔPC mice was significantly reduced compared to VDRlox mice. Each single experiment was performed in triplicate. The data are expressed as the mean ± SEM. n = 6, Student’s t-test, *P < 0.05.

(J) More abnormal Paneth cells in the VDRΔPC mice compared to the VDRlox mice by TEM. The Paneth cells were identified by the presence of the cytoplasmic granules. Ultrastructural changes with the w were used for quantification. The data are expressed as the mean ± SEM. n = 6, Student’s t-test, **P < 0.01.
Figure 3. *Salmonella* infections were worse in the VDR\textsuperscript{ΔPC} mice

(A) A *Salmonella* infection model.

(B) Changes in the relative body weights after *Salmonella* infections. Data are expressed as mean ± SEM. n = 10, two-way ANOVA test, *P < 0.05.

(C) The length of cecum and colon for the VDR\textsuperscript{ΔPC} group were shorter, compared with the VDR\textsuperscript{lox} mice, 4-days post-infection. Data are expressed as mean ± SEM. n = 10, two-way ANOVA test, *P < 0.05.

(D) Large spleens were found in the VDR\textsuperscript{ΔPC} mice 4-days post-infection. Data are expressed as mean ± SEM. n = 10, two-way ANOVA test, *P < 0.05.

(E) & (F) Higher inflammation scores in the cecum of the VDR\textsuperscript{ΔPC} mice 4-days post-infection by HE staining. Data are expressed as mean ± SEM. n = 10, one-way ANOVA test, **P < 0.01.

(G) Quantification of the *Salmonella* in the feces 4 days post-infection. Data are expressed as mean ± SEM. n = 10, one-way ANOVA test, *P < 0.05.

(H) Quantification of the *Salmonella* in the spleen and liver 4 days post-infection. Data are expressed as mean ± SEM. n = 10, one-way ANOVA test, *P < 0.05. Each single experiment was assayed in triplicate.

Figure 4. Lack of VDR in Paneth cells leads to increased inflammatory responses

(A) The expression of the lipocalin2 was higher in the feces of the VDR\textsuperscript{ΔPC} mice. Each ELISA was assayed in triplicate. The data are expressed as mean ± SEM. n = 10, two-way ANOVA test, *P < 0.05.

(B) More serum LPS from the of the VDR\textsuperscript{ΔPC} mice after 8 h infection. Each single experiment was assayed in triplicate. The data are expressed as the mean ± SEM. n = 10, two-way ANOVA test, *P < 0.05.
(C) The expression of α-defensin6 in the ileum tissue in the VDR\textsuperscript{ΔPC} mice was lower than that in the VDR\textsuperscript{lox} mice by ELISA. Each single experiment was assayed in triplicate. The data are expressed as mean ± SEM. n = 10, two-way ANOVA test, *P < 0.05, **P < 0.01.

(D) The percentage of normal Paneth cells was reduced post Salmonella infection, especially in the VDR\textsuperscript{ΔPC} mice. Data are expressed as mean ± SEM. n = 20, two-way ANOVA test, *P < 0.05, **P < 0.01, ***P < 0.001.

(E) Lower expression of the defensin4 in the VDR\textsuperscript{ΔPC} mice post Salmonella infection. n = 6-9, one-way ANOVA test, **P < 0.01, ***P < 0.001.

(F) The percentage of normal Paneth cells was reduced after the indomethacin treatment, especially in the VDR\textsuperscript{ΔPC} mice. Data were expressed as mean ± SD. n = 6-9, one-way ANOVA test, *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5. Lack of VDR in the Paneth cells leads to impaired anti-bacterial abilities

(A) Small intestine was collected and digested into single cells. After staining with the anti-CD24 Ab, the cells were detected by flow cytometry. The Paneth cells and non-Paneth cells were isolated. Each single experiment was performed in triplicate.

(B) Lack of VDR in the Paneth cells lead to decreased expression of the ATG16L1 and LYZ. Western blots were done using the isolated Paneth cells from VDR\textsuperscript{lox} and VDR\textsuperscript{ΔPC} mice. Each single experiment was performed in triplicate. The data are expressed as mean ± SEM. n = 6, Student’s t-test, **P < 0.01, ***P < 0.001.

(C) Isolated Paneth cells incubated with GFP-Salmonella for 5 hours. The Paneth cells in the VDR\textsuperscript{lox} group could inhibit the Salmonella growth. Each single experiment was performed in triplicate. Data are expressed as mean ± SEM. n = 20, Student’s t-test, **P < 0.01.

(D) The expression of the Lyz was lower in the VDR\textsuperscript{ΔPC} supernatant than the VDR\textsuperscript{lox} supernatant. Paneth cells and non-Paneth cells were incubated overnight and the supernatant was collected. Each experiment was performed in triplicate.
(E) The supernatant from VDR\textsuperscript{lox} Paneth cells had stronger antibacterial abilities than that of the VDR\textsuperscript{ΔPC} group. \textit{Salmonella} was added into the supernatant. After 1, 3, and 5 hours, the medium was collected for counting the number of \textit{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, two-way ANOVA test, ***P < 0.001.

\textbf{Figure 6. Lack of VDR in Paneth cells leads to impaired inflammatory responses}

(A) Serum inflammatory cytokines were significantly increased, especially in the VDR\textsuperscript{ΔPC} mice with the infection. Each single experiment was assayed in triplicate. Data are expressed as mean ± SEM. n = 6, one-way ANOVA test, *P < 0.05, **P < 0.01.

(B) The expression of the IFN\textgamma, IL1\beta, and TNF\alpha were increased in the VDR\textsuperscript{ΔPC} ileum post infection. Each single experiment was assayed in triplicate. The data are expressed as mean ± SEM. n = 6, one-way ANOVA test, *P < 0.05, **P < 0.01.

(C) The expression of TNF\alpha was increased in VDR\textsuperscript{ΔPC} supernatant post \textit{Salmonella} infection, as determined by ELISA. Each single experiment was assayed in triplicate. The data are expressed as mean ± SEM. n = 10, one-way ANOVA test, ***P < 0.001.

(D) The expression of the VDR was decreased in the VDR\textsuperscript{ΔPC} Paneth cells. Data are expressed as mean ± SEM. n = 10, Student’s t-test, ***P < 0.001.

(E) The expression of the ATG16L1 was decreased in the VDR\textsuperscript{ΔPC} Paneth cells. Data are expressed as mean ± SEM. n = 10, Student’s t-test, ***P < 0.001.

(F) Defensin4 expression in the VDR\textsuperscript{ΔPC} Paneth cells was low. Each single experiment was assayed in triplicate. The data are expressed as mean ± SEM. n = 10, one-way ANOVA test, ***P < 0.001.
(G) After the *Salmonella* treatment for 1-hour, stronger autophagy signals were found in the VDR\(^{\text{lox}}\) cells compared to VDR\(^{\Delta PC}\) cells. Each single experiment was assayed in triplicate. The data are expressed as mean ± SEM. n = 10, one-way ANOVA test, ***P < 0.001.

**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\(^{\text{lox}}\) mice. The relative bacterial abundance of top 10 species(s) was shown. All the unidentified and other identified species were grouped into “others”. Species were colored using the key as list in the right side. Each bar represents individual mouse. n=10 per group.

(B) PCoA visualized the sample differences between VDR\(^{\Delta PC}\) and VDR\(^{\text{lox}}\) mice. The samples were collected from VDR\(^{\Delta PC}\) (red) and VDR\(^{\text{lox}}\) 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\(^{\text{lox}}\) and VDR\(^{\Delta PC}\). n=10 per group. P = 0.025.

(D) Relative body weight changes in mice with DSS. VDR\(^{\Delta PC}\) mice have worse outcomes with DSS-induced colitis. Data are expressed as mean ± SEM. n = 6, Two-way ANOVA test, **P<0.01, * P<0.05.

(E) Cecum shorten was found in VDR\(^{\Delta PC}\) mice with colitis, but not for co-housing mice. After 4 weeks of co-housing, the mice were administered with 5% DSS for 7 days to induce colitis. Data are expressed as the mean ± SEM. n = 6, Student’s t-test compared between VDR\(^{\text{lox}}\) and VDR\(^{\Delta PC}\) mice, * P < 0.05.

(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\(^{\text{lox}}\) and VDR\(^{\Delta PC}\) mice, * P < 0.05.
(G) *Lactobacillus* in VDR<sup>ΔPC</sup> mice was enhanced after cohousing with VDR<sup>Lox</sup> mice. Data are expressed as means ± SEM. Each experiment was assayed in triplicate. n = 6, one-way ANOVA test, ***P < 0.001.

(H) The percentage of normal Paneth cells in the VDR<sup>ΔPC</sup> mice was increased after co-housing with VDR<sup>Lox</sup> mice. IF images were representative of experiments in triplicate; Data were expressed as mean ± SD, n = 5-6; one way ANOVA test; *P < 0.05, ****P < 0.001.

**Acknowledgement**

We thank Figen Seiler at the UIC Electron Microscopy Core for assistance with transmission electron microscopy. We thank the UIC DNAS facility for assistance with shotgun metagenomic sequencing. We thank Lorraine Holland and Svetlana Saveljeva for helping with human small intestinal samples.

**References**

**Table S1. Primers for real-time PCR**

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**Table S2. Bacterial 16S rDNA Real-time PCR primers.**

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## STAR METHODS KEY RESOURCES TABLE

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| **Chemicals, Peptides, and Recombinant Proteins** | | |
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| TRIzol | Thermo Fisher Scientific | Cat # 15596026 |
| Pierce™ BCA protein assay kit | Thermo Fisher Scientific | Cat # 23225 |
| iScript cDNA synthesis kit | BioRad | Cat # 1708840 |
| SYBR Green PCR kit | BioRad | Cat # 1708880 |
| Phosphate-Buffered Saline | Corning | Cat#21-040-CV |
| Triton X-100 | Fisher BioReagents | BP151-100 |
| 16% Formaldehyde | Fisher BioReagents | Cat#28908 |
| sucrose | Fisher BioReagents | Cat#15503022 |
| LB Broth, Miller | Fisher BioReagents | Cat#BP1426 |
| LB Agar, Miller | Fisher BioReagents | Cat#BP1425 |
| TNF alpha Mouse ELISA Kit | Thermo Fisher Scientific | Cat # BMS607-3 |
| LAL Chromogenic Endpoint Assay | Hycult Biotech | Cat# HIT302 |
| Lcn-2 ELISA kit | R&D Systems | Cat# DY1857 |
| ProcartaPlexTM Multiplex kit | Thermo Fisher Scientific | Cat# 26088-901 |
| RNAqueous-Micro Kit | Thermo Fisher Scientific | Cat#AM1931 |
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### Oligonucleotides

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### Software and Algorithms

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Figure S1: Proliferation marker PCNA and β-catenin (p-Ser552) are downregulated in the VDR\textsuperscript{ΔPC} mice, compared with the VDR\textsuperscript{Lox} mice.

(A) The expression of PCNA in the ileum tissue in the VDR\textsuperscript{ΔPC} mice was lower than that in the VDR\textsuperscript{lox} mice, as determined by IHC staining. Images are representative of experiments in triplicate; Data are expressed as mean ± SD, n=6; Welch’s t test; \(*P < 0.05\).

(B) The expression of p-β-catenin (Ser552) in the ileum tissue in the VDR\textsuperscript{ΔPC} mice was lower than that in the VDR\textsuperscript{lox} mice, as determined by IHC staining. Images were representative of experiments in triplicate; Data were expressed as mean ± SD, n=6; Welch’s t test; \(*P < 0.05\).

Figure S2. Lack of VDR in intestinal Paneth cells affected gut microbiota and sensitivity to DSS damage.

(A) H&E of colon tissues from VDR\textsuperscript{ΔPC} mice and VDR\textsuperscript{LoxP} mice with or without co-housing in DSS-colitis. Images were representative of experiments in triplicate.

(B) Inflammation scores in the colon tissues of the VDR\textsuperscript{ΔPC} mice were reduced after co-housing with VDR\textsuperscript{LoxP} mice. Data were expressed as mean ± SD, n = 5-6; one way ANOVA test; \(**P < 0.01\).

(C) The numbers of Paneth cells per crypt in the VDR\textsuperscript{ΔPC} mice were restored after co-housing with VDR\textsuperscript{LoxP} mice. Data were expressed as mean ± SD, n = 5-6; one way ANOVA test; \(*P < 0.05, **P < 0.001\).
S. Fig. 2

A

VDR Loxp DSS
VDR ΔPC DSS
VDR ΔPC co-housing DSS
VDR ΔPC co-housing DSS

B

Inflammation Score

VDR ΔPC DSS
VDR ΔPC co-housing DSS
VDR ΔPC co-housing DSS

C

Paneth cells / Crypt

VDR ΔPC DSS
VDR ΔPC co-housing DSS
VDR ΔPC co-housing DSS
METHOD DETAILS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Jun Sun (Junsun7@uic.edu).

Human intestinal biopsies

Slides containing paraffin-embedded small intestinal biopsy samples of patients with CD and healthy controls were obtained from Dr. Arthur Kaser, University of Cambridge, Cambridge, U.K. After obtaining written informed consent, human intestinal biopsies from the terminal ileum of CD patients and healthy controls were collected upon colonoscopy and stored in optimal cutting temperature compound. All protocols were approved by Cambridgeshire 4 Research Ethics Committee (reference 03/5/012). Patient characteristics are outlined in previous publication 1.

Gene expression datasets

For expression analyses, we took advantage of microarray data reported in the Gene Expression Omnibus 2 repository (https://www.ncbi.nlm.nih.gov/geo/). In particularly, gene expression data in biopsy of human intestinal tissues were obtained from the dataset (GEO accession number GSE102134) reported on ileum of patients with Crohn’s Disease and normal ileum from control individuals 3. Gene expression data for VDR and ATG16L1 from CD patients (n=51) and normal (n=11) were extracted and further analyzed in our study.

Experimental Animals
VDR\textsuperscript{loxP/loxP} mice were originally developed by Dr. Geert Carmeliet \textsuperscript{4}. DEFA6-cre mice were from Dr. Richard Blumberg \textsuperscript{5}. Paneth cell VDR specific knockouts (VDR\textsuperscript{APC}) were obtained by crossing VDR\textsuperscript{loxP/loxP} mice with DEFA6-cre mice. The mice were provided with water ad libitum and maintained in a room with a 12 h 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 adhere to the ARRIVE guidelines and was approved by the University of Illinois at Chicago Committee on Animal Resources and ethical guidelines were followed with the treatment.

**Bacterial strains and growth conditions**

The *Salmonella* strain used in this study was *S.* Typhimurium 14028 \textsuperscript{6}. Bacterial cultures were prepared by inoculating 10 ml of Luria–Bertani broth with 0.01 ml of a stationary-phase culture, followed by overnight incubation (> 18 h) at 37 °C, as previously described \textsuperscript{7,8}.

**Salmonella-infected mouse model**

Animal experiments were performed using VDR\textsuperscript{loxP/loxP} and VDR\textsuperscript{APC} mice (male and female, 2–3-month-old). Water and food were withdrawn 4 h before oral gavage, with 7.5 mg/ mouse of streptomycin. Afterwards, the animals were supplied with water and food ad libitum. Twenty hours after the streptomycin treatment, the water and food were withdrawn again for 4 h, before the mice were infected with \(1 \times 10^6\) colony-forming units of *Salmonella* (100-ml suspension in HBSS by gavage, as previously described \textsuperscript{7,8}.

**Co-housing of VDR\textsuperscript{lox} and VDR\textsuperscript{APC} mice**
Two-to-three-month-old female VDR\textsuperscript{lox} and VDR\textsuperscript{ΔPC} mice were co-housed in new cages according to previously published methods\textsuperscript{9}. One cage contained three VDR\textsuperscript{lox} and two VDR\textsuperscript{ΔPC}, another one contained two mice each. The mice were fed with the same food and water. After 4 weeks of co-housing, 5% DSS dissolved in filter-purified water was administered to the mice. Animals were weighed daily. At day 7 after DSS administration, mice were sacrificed under anesthesia.

**Induction of DSS-colitis**

Mice were administered 5% DSS (MW = 40–50 kDa; USB Corp. Cleveland, OH) dissolved in filter-purified and sterilized water ad libitum for the experimental period. Animals were weighed daily. At day 7 after DSS administration, mice were sacrificed under anesthesia. Severity of colitis was quantified by a disease activity index, determined by weight loss, fecal blood and diarrhea.

**Induction of small-intestinal lesions**

To induce small-intestinal injury, 10 mg/kg indomethacin (Sigma Chemical, St. Louis, MO, USA) was subcutaneously given to non-fasted animals. The animals were killed 24 h after anesthetization. The jejunum and ileum were then removed, opened along the anti-mesenteric attachment, and examined for lesions under a dissecting microscope with square grids. The area (mm\textsuperscript{2}) of visible lesions was macroscopically measured, summed per small intestine and expressed as an ulcers core\textsuperscript{10}.

**EXPERIMENTAL DETAILS**

**Salmonella-infected mouse model**

Animal experiments were performed using VDR\textsuperscript{loxP/loxP} and VDR\textsuperscript{ΔPC} mice (male and female, 2–3-month-old). Water and food were withdrawn 4 h before oral gavage, with 7.5
mg/ mouse of streptomycin (100 ml of sterile solution). Afterwards, the animals were supplied with water and food ad libitum. Twenty hours after the streptomycin treatment, the water and food were withdrawn again for 4 h, before the mice were infected with 1*10^6 colony-forming units of Salmonella (100-ml suspension in HBSS (Hank’s Balanced Salt Solution) or treated with sterile HBSS (control) by oral gavage, as previously described 7, 8. After the Salmonella gavage, the tissue samples were collected after 8 hours and 4 days. The mice were sacrificed under anesthesia. The severity of the colitis was quantified by a disease activity index, determined by weight loss, fecal blood, and diarrhea. The intestines were harvested, fixed in 10 % formalin (pH 7.4), processed, and embedded in paraffin. Sections (5 µm) were stained with H&E. Blinded histological inflammatory scores were performed by a validated scoring system by a trained pathologist 9.

**Immunoblotting**

Mouse intestine tissues were lysed in lysis buffer (1 % Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM sodium orthovanadate, and protease inhibitor cocktail), and the protein concentrations were measured. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted with anti-ATG16L1 (Abcam, Cambridge, MA, USA), anti-Lysozyme (Cell Signal, Beverly, MA), and anti-VDR antibodies (Santa Cruz Biotechnology Inc., CA) as previously described 11, 12.

**Real-time quantitative PCR analysis**

Total mRNA was extracted from the scraped mouse colonic epithelial cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA). cDNA was then subjected to real-time PCR (SYBR
Green PCR kit, BioRad) with primers (Table S1). The percent expression was calculated as the ratio of the normalized value of each sample relative to that of the corresponding untreated control samples. All real-time PCR reactions were performed in triplicate.

**Immunohistochemistry**

Intestinal tissues were fixed in 10 % buffered formalin and processed the next day with standard techniques as previously described \(^7, 12-14\). The slides were stained with anti-VDR (Santa Cruz Biotechnology Inc., CA) and anti-ATG16L1 (Abcam, Cambridge, MA, USA). Immunohistochemistry of VDR and ATG16L1 staining was initially assessed as a product of staining intensity (0, no staining; 1, minimal; 2, slight; 3, moderate; 4, marked intensity). We randomly took 3 scores derived from each samples. The final analytical sample consisted of 18 normal and 24 CD samples.

**Immunofluorescence**

Ileum tissues were freshly isolated and embedded in paraffin wax after fixation with 10 % neutral buffered formalin. Immunofluorescence was performed on paraffin-embedded sections (4 µm), after preparations of the slides, as described previously \(^15\), followed by incubation for 1 hour in a blocking solution (2 % bovine serum albumin, 1 % goat serum in HBSS) to reduce the non-specific background of staining. The tissue samples were incubated overnight with primary antibodies at 4 °C. The following antibodies were used: anti-ATG16L1 (Abcam, Cambridge, MA, USA), anti-Lysozyme (Cell Signal, Beverly, MA), anti-Defensin 4, and anti-VDR antibodies (Santa Cruz Biotechnology Inc., CA). Slides were washed 3 times for 5 minutes each at room temperature in the wash buffer. The samples were then incubated with secondary antibodies (goat anti-rabbit Alexa Fluor 488, Molecular Probes, CA; 1:200) for 1 hour at room temperature. Tissues were mounted with SlowFade Antifade Kit (Life technologies, s2828, Grand Island, NY, USA),
followed by a coverslip, and the edges were sealed to prevent drying. Specimens were examined with a Zeiss laser scanning microscope (LSM 710, Carl Zeiss Inc., Oberkochen, Germany).

**Laser capture microdissection of Paneth cells**

Laser capture microdissection (LCM) of individual Paneth cells was performed with the PixCell I LCM System (Arcturus Engineering, Mountain View, CA), as previously described \(^{16}\). The LCM was performed on a Zeiss Axiovert 200M microscope equipped with PALM RoboSoftware (Carl Zeiss, Thornwood, NY), and the total area of the tissue collected per slide was tracked and recorded. The RNA was isolated from the dissected tissue by following the protocol provided by the RNA queous-Micro kit (Ambion, Austin, TX) via column purification \(^{17}\).

**Transmission electron microscopy**

For transmission electron microscopy (TEM), small intestines were fixed in 4 % paraformaldehyde / 3 % glutaraldehyde, in 10 mM sodium phosphate buffer (pH 7.4) for 48 h. All samples were post-fixed with 1 % osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) on ice for 1 h. Samples were then treated with 0.5 % aqueous uranyl acetate, dehydrated in graded alcohol, treated with propylene oxide, and embedded in Embed 812 (Electron Microscopy Sciences). The resin was polymerized in a 60 °C oven for 2–3 d. The samples were sliced into 1 × 2 × 2 mm pieces and examined with a Philips CM 100 electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 80 KV for images.

**Lipopolysaccharides (LPS) detection**
LPS in serum samples was measured with Limulus amebocyte lysate (LAL) chromogenic endpoint assays (HIT302, Hycult Biotech, Plymouth Meeting, PA, USA), according to the manufacturer's indications. The samples were diluted 1:4 with endotoxin-free water and then heated at 75 °C for 5 min, in a warm plate to denature the protein before the reaction. A standard curve was generated and used to calculate the concentrations, which were expressed as EU/ml, in the serum samples.

**Quantification of serum 1,25-dihydroxyvitamin D3 by ELISA**

Mouse blood samples were collected by cardiac puncture and placed in tube containing EDTA (10mg/ml). The serum was collected after centrifugation. The level of serum 1,25-dihydroxyvitamin was detected by a mouse 1, 25-dihydroxyvitamin D3 (DVD/DHVD3) ELISA Kit (Biomatik, Delaware, USA).

**Quantification of fecal lipocalin 2 (Lcn-2) by ELISA**

Freshly collected fecal samples were reconstituted in PBS containing 0.1 % Tween 20 (100 mg/ml) and vortexed for 20 min to get a homogenous fecal suspension. These samples were then centrifuged for 10 min at 12 000 rpm and 4 °C. The clear supernatants were collected. The fecal Lcn-2 level was estimated using Duoset murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, MN).

**Quantification of fecal defensin-6 by ELISA**

Ileum tissue samples were collected from VDR<sup>Cox</sup> and VDR<sup>∆PC</sup> mice. 100mg tissue was rinsed with 1X PBS, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernatant was collected and assayed immediately. The defensin-6 level was estimated using Mouse defensin-6 (DEFA6) ELISA Kit (MyBioSource, San Diego, CA).
**Autophagy activity**

Autophagy activity were quantified using the commercial Cyto ID® autophagy detection kit (ENZO Life Sciences, ENZ-51,031-K200), in accordance with the manufacturer’s protocol. The kit contained a 488 nm excitable green fluorescent detection reagent that became brightly fluorescent when incorporated into the vesicles produced during autophagy. Then the specimens were examined with a Zeiss laser scanning microscope (LSM 710 (Carl Zeiss Inc., Oberkochen, Germany).

**Isolation of Paneth cell**

The small intestines were harvested. The intestines were flushed with cold PBS to remove the large debris and then cut lengthwise to open the intestinal segment. They were then placed in ice-cold PBS and rocked for 5 min in a cold room. The PBS was carefully removed and Buffer #1 added (2mM EDTA in PBS). They were then rocked for 30 min in a cold room, Buffer #1 was then carefully removed and Buffer #2 added (54.9 mM D-sorbitol and 43.4 mM sucrose in PBS) in 15 ml conical tubes. They were shaken for 2~3 min vigorously by hand to dissociate the crypts from the intestine as much as possible. They were then put in a 100 µm cell strainer on top of a 50 ml conical tube on ice and Buffer #2 was pouted on top of the contents. The tubes were then centrifuged at 150 g for 10 min at 4 ℃ and the supernatant removed as much as possible without disturbing the pellet. The crypts were resuspended with TrypLE Express supplement with DNAse I (200 U/ml), and incubated at 37 ℃, with gentle shaking every 5 min. The solution was filtered through a 70 µm filter and centrifuged at 200 g for 10 min at 4 ℃. The pellet was resuspended with a flow washing buffer (2 mM EDTA and 1 % FBS in PBS), incubated with CD24-PE Ab in the dark at 4 ℃ for 15 min. It was then centrifuged
at 150 g for 5 min, then the cells were washed 2 times with the washing buffer. The pellet was then resuspended with the washing buffer for flow sorting.

**Real-Time PCR Measurement of Bacterial DNA**

Mice feces sample DNA was extracted using the stool DNA Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's instructions. 16S ribosomal DNA PCR reactions used the MyiQ single-color real-time PCR detection system (Bio-Rad Laboratories) and iTaq Universal SYBR green supermix (1725121; Bio-Rad Laboratories) according to the manufacturer's directions. Primers specific to 16S ribosomal RNA were used as an endogenous control to normalize loading between samples. The relative amount of 16S ribosomal DNA in each sample was estimated using the ΔΔCT. Primer sequences were designed using Primer-BLAST or were obtained from the Primer Bank primer pairs listed in Table S2.

**Shotgun metagenomic sequencing**

We used whole-genome shotgun sequencing to sequence fecal samples. Genomic DNA was fragmented into relatively small pieces prior to sequencing. Sequencing was performed using a recent Illumina HiSeq system\(^\text{18}\). Basic processing of the raw data for all samples were performed including the quality checking, filtering the reads, removing noisy sequences, metagenomic assembly, gene calling and binning\(^\text{19}\). We filtered the resulting assemblies to exclude contigs shorter than 1,000 nucleotides or *base pairs (bp)* and classified all remaining contigs with Centrifuge\(^\text{20}\), an efficient metagenomic classifier capable of indexing the entirety of nucleotide (nt), searching for the comprehensive NCBI Genbank non-redundant nucleotide database to obtain a taxonomic classification.
of each contig(genes) (as described in https://merenlab.org/2016/06/18/importing-taxonomy). Identical sequences with ≥99% identity of each other have been removed to make it nonredundant, but even after this reduction, in a total of 10 samples, this data set contains over 70 million reads, with an average of 7,025,354 reads per sample. Of which, total 24,739,969 reads are taxonomic alignments, with an average of 2,473,997 reads per sample.

Statistical Analysis

The results are presented as the mean value ± SD or SEM (the standard error of the mean). All the tests were two-tailed and the differences were considered statistically significant when P-value < 0.05. The normality distribution test of each variable was performed using Shapiro-Wilk’s test. The differences between the two samples were analyzed by a Student’s t test or Welch-t-test based on whether the two samples have unequal variances and/or unequal sample sizes.

The differences among three or more groups were analyzed using parametric one-way ANOVA or non-parametric Kruskal–Wallis test, depending on whether the variable is normally distributed or not. For two factors of samples, a two-way ANOVA test was used. For the human data in CD and control patients, we performed a regression of VDR against ATG16L1 and conducted a scatter plot with a regression line. For microbiome data, we first performed PCoA to visualize the Bray-Curtis dissimilarities between groups; then we performed the permutational multivariate ANOVA (PERMANOVA) to detect the statistical differences of Bray-Curtis dissimilarities between groups, followed by a variance homogeneity assumption testing to ensure the reliability of the PERMANOVA results. Next, a nonparametric procedure analysis of similarity
(ANOSIM) based on a permutation test was used for analyzing between- and within-group similarities. To correct multiple comparisons, a Tukey method was used to adjust for p-values for experimental data. The statistical analyses of experimental data were performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The regression and scatter plot of VDR against ATG16L1 were performed using SAS version 9.4 (Cary, NC: SAS Institute Inc.). The microbiome data were analyzed by using R packages of ampvis2, microbiome, phyloseq, and vegan, which were implemented with the latest version of R, as did in our book on statistical analysis of microbiome data 19.

References


