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Vitamin D Receptor Negatively Regulates Bacterial-Stimulated NF-κB Activity in Intestine

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Vitamin D receptor (VDR) plays an essential role in gastrointestinal inflammation. Most investigations have focused on the immune response; however, how bacteria regulate VDR and how VDR modulates the nuclear factor (NF)-κB pathway in intestinal epithelial cells remain unexplored. This study investigated the effects of VDR ablation on NF-κB activation in intestinal epithelia and the role of enteric bacteria on VDR expression. We found that $VDR^{-/-}$ mice exhibited a pro-inflammatory bias. After *Salmonella* infection, $VDR^{-/-}$ mice had increased bacterial burden and mortality. Serum interleukin-6 in noninfected $VDR^{+/+}$ mice was undetectable, but was easily detectable in $VDR^{-/-}$ mice. NF-κB p65 formed a complex with VDR in noninfected wild-type mouse intestine. In contrast, deletion of VDR abolished VDR/P65 binding. P65 nuclear translocation occurred in colonic epithelial cells of untreated $VDR^{-/-}$ mice. VDR deletion also elevated NF-κB activity in intestinal epithelia. VDR was localized to the surface epithelia of germ-free mice, but to crypt epithelial cells in conventionalized mice. VDR expression, distribution, transcriptional activity, and target genes were regulated by *Salmonella* stimulation, independent of 1,25-dihydroxyvitamin D3. Our study demonstrates that commensal and pathogenic bacteria directly regulate colonic epithelial VDR expression and location *in vivo*. VDR negatively regulates bacterial-induced intestinal NF-κB activation and attenuates response to infection. Therefore, VDR is an important contributor to intestinal homeostasis and host protection from bacterial invasion and infection. *(Am J Pathol 2010, 177:686–697; DOI: 10.2353/ajpath.2010.090998)*

Vitamin D receptor (VDR) is a nuclear receptor[1] that mediates most known functions of 1,25-dihydroxyvitamin D ($1,25[OH]_2D_3$), the hormonal form of vitamin D. VDR and $1,25(OH)_2D_3$ are involved in calcium homeostasis,[2] electrolyte and blood pressure regulation,[3,4] immune response,[5] and anti-inflammation activity.[6,7] Different ligand shapes of 1, $25(OH)_2D_3$ act through VDR in different cellular locations.[8,9] The target genes of the VDR signal pathway include the enzyme Cyp24 and antimicrobial peptides β-defensin[10,11] and cathelicidin.[12]

Nuclear factor-κB (NF-κB) is a family of transcription factors that play an essential role in innate and adaptive immune responses. NF-κB is active in the nucleus, and its activity is inhibited by the inhibitor of κBα (IκBα).[13] VDR physically interacts with NF-κB subunit p65 in human osteoblasts[14] and mouse embryonic fibroblast cells,[15] but the functional relevance of this VDR/p65 interaction in regulating intestinal inflammation remains unclear.

Human intestinal epithelial cells are constitutively exposed to commensal microbiota and pathogenic bacteria. Enteric commensal bacteria play a crucial role in the pathogenesis of many diseases such as inflammatory bowel diseases.[16–18] and colon cancer.[19,20] Several studies have implicated vitamin D in inflammatory bowel disease (IBD). Low vitamin D levels have been reported in patients with IBD.[21,22] In animal models, $1,25(OH)_2D_3$ suppressed the development of experimental intestinal inflammation.[23,24] Both local and endocrine synthesis of $1,25(OH)_2D_3$ affect murine colitis[25] and VDR status affects the development of murine colitis.[26] VDR mediates T cell homing to the gut.[23] VDR expression is significantly decreased in IBD patients.[22,27] However, the majority of studies of vitamin D, VDR, and inflammation are focused on immunoregulation, with little emphasis on assessing the effects of VDR in intestinal epithelial cells. It is un-

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known how intestinal VDR signaling responds to commensal and pathogenic bacterial stimulation.

In the present study, we hypothesize that *Salmonella typhimurium* infection can induce colonic VDR expression, alter location of VDR *in vivo*, and stimulate VDR expression, transcription, and signaling in colonic epithelial cell lines. We found that mice lacking VDR are in a pre-inflammatory or pro-inflammatory state. Hence, our current study investigated the effect of VDR ablation on NF-κB activation using a VDR knockout mouse model infected with pathogenic *Salmonella* or human commensal *Escherichia coli*. F18. We also examined intestinal VDR expression in normal, germ-free (GF), and conventionalized wild-type mice. Our data demonstrate that intestinal VDR is directly involved in suppression of bacteria-induced NF-κB activation and that commensal bacterial colonization affects intestinal epithelial VDR expression and distribution.

Materials and Methods

Bacterial Strains and Growth Condition

S. typhimurium wild-type ATCC14028, commensal *E. coli* F18, and nonpathogenic *Salmonella* mutant strain PhoP^{c28} were used in this study. Non-agitated micro-aerophilic bacterial cultures were as follows: nonagitated micro-aerophilic bacterial cultures were prepared by inoculation of 10 ml of Luria-Bertani broth with 0.01 ml of a stationary phase culture, followed by overnight incubation (~18 hours) at 37°C, as previously described.[29] Bacterial overnight cultures were concentrated 33-fold in Hanks' balanced salt solution (HBSS) supplemented with 10 mmol/L HEPES, pH 7.4.

Streptomycin Pretreated Mouse Model

Animal experiments were performed by using specific-pathogen-free female C57BL/6 mice (Taconic, Hudson, NY) that were 6 to 7 weeks old[30] and VDR knock out C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) as previously described.[31] The protocol was approved by the University of Rochester University Committee on Animal Resources. Water and food were withdrawn 4 hours before oral gavage with 7.5 mg/mouse of streptomycin (100 μl of sterile solution or 100 μl of sterile water as control). Afterward, animals were supplied with water and food. Twenty hours after streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 1×10^7 colony forming units (CFU) of *S. typhimurium* (100-μl suspension in HBSS) or treated with sterile HBSS (control) by oral gavage as previously described.[32] For the mouse survival experiment, 1×10^8 CFU of *S. typhimurium* was used.

GF and Conventionalized Mouse Colon

GF mice (129/SvEv) were obtained from the Center for Gastrointestinal Biology and Disease Gnotobiotic Core Facility and the National Gnotobiotic Rodent Resource Center, University of North Carolina, Chapel Hill. Mice were analyzed at the University of North Carolina National Gnotobiotic Rodent Resource Center. Sterility of germ-free mice was documented on a monthly basis by fecal Gram stains and aerobic and anaerobic cultures of the feces and bedding. For selected mice, sterility of cecal contents was documented Gram stain and cultures at the time of necropsy. Conventionalized mice were originally GF mice that were infected with a commensal enteric bacteria by orally swabbing fecal slurries from specific pathogen-free (SPF) mice, placing fecal slurries on their food and then housing them in SPF conditions.

Cell Culture

Caco2BBE and HCT116 cells were grown in Dulbecco's Modified Eagle Medium (high glucose, 4.5g/L) containing 5% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin. Monolayers of Caco2BBE cells were grown on permeable supports (0.33 or 4.67 cm^2, 0.4 μm pore. Costar, Cambridge, MA) and used 6 to 14 days after being plated.

Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated from embryonic day 13.5 embryos generated from VDR$^{+/-}$ × VDR$^{+/-}$ mouse breeding as previously described.[15] Cells from each embryo were genotyped by PCR using genomic DNA isolated from the cells. VDR$^{+/-}$ and VDR$^{-/-}$ MEFs were used in experiments after more than 15 passages when they had been immortalized.

Bacterial Colonization in Cultured Cells in Vitro

Human colonic epithelial cells were colonized with equal numbers of the indicated bacteria for 30 minutes, washed with HBSS, and incubated in Dulbecco's Modified Eagle Medium containing gentamicin (500 μg/ml) for the times indicated in our previous studies.[29,33] The first 30-minute incubation allowed bacteria to contact the surface of the epithelial cells and inject the effectors in the host cells. After extensive HBSS washing, the extracellular bacteria were washed away. Incubation with gentamicin inhibited the growth of bacteria. In this way, we focused on the effects of the bacterial effectors injected to the host cells.

Immunoblotting

Mouse colonic epithelial cells were collected by scraping from mouse colon including proximal and distal regions as previously described.[30] Mouse epithelial cells were lysed in lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA pH 8.0, 0.2 mmol/L sodium ortho-vanadate, and protease inhibitor cocktail). MEFs were rinsed twice in ice-cold HBSS, lysed in protein loading buffer (50 mmol/L Tris, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Immunoblot was performed with primary antibodies: anti-VDR, anti-p65,

anti-IκBα (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-phospho-IκBα, anti-phospho-p65 (ser536) (Cell Signal, Beverly, MA) or anti-β-actin (Sigma-Aldrich, Milwaukee, WI) antibodies and visualized by enhanced chemiluminescence.[15,34]

Co-Immunoprecipitation Assay

Cells were rinsed twice in ice-cold HBSS and lysed in cold immunoprecipitation buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA pH 8.0, 0.2 mmol/L sodium ortho-vanadate, protease inhibitor cocktail (Boehringer Mannheim). Samples were immunoprecipitated with the anti-VDR antibody (Santa Cruz) as previously described.[15] Blots were probed with anti-VDR antibody (Transduction Laboratories), stripped, and reprobed with anti-p50 and anti-p65 antibodies (Santa Cruz Biotechnology, CA) and visualized by enhanced chemiluminescence.

Nuclear Protein Fraction

Mouse colon epithelial mucosa was washed with HBSS three times and lysed. The nuclear proteins were extracted using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein fraction was used for the co-immunoprecipitation assay.

S. typhimurium *Invasion of MEFs Monolayers*

Infection of MEFs was performed by the method described previously.[32] Bacterial solution (~20 bacteria/epithelial cell) was added and bacterial invasion was assessed after 1 hour. Cell-associated bacteria represent bacteria adhered to and/or internalized into the monolayers were released by incubation with 100 μl of 1% Triton X-100 (Sigma). For cell-associated bacteria, 0.9 ml of Luria broth was added, mixed, and quantified by plating on MacConkey agar medium for CFU.

Salmonella *Burden in Intestine*

Cecum was dissected from mouse, and put into 14 ml tube with 5 ml sterile PBS, cut into pieces with scissors, then homogenized adequately using homogenizer (Polytron PT2100, Kinematica, Switzerland). Homogenate was diluted at 1000 to 10,000 with Luria-Bertani broth. One hundred μl of diluted homogenate was plated out on MacConkey agar plates and incubated at 37°C overnight. CFU were quantified.

Histology of Mouse Colon

Mouse colons were harvested, fixed in 10% formalin (pH 7.4), processed, and paraffin embedded. "Swiss rolls" were prepared as described.[35] Sections (5 μm) were stained with H&E. For immunostaining, antigens were retrieved by 10-minute boiling in 10 mmol/L citrate (pH 6.0). The slides were stained with anti-VDR antibody (Santa Cruz) as previously described.[30] *Salmonella* distribution in intestine was determined by immunostaining using anti-*Salmonella* lipopolysaccharide antibody (Santa Cruz). Blinded histological inflammatory scores were performed by a validated scoring system[36] by a trained pathologist.

Salmonella-*Induced Mouse Interleukin-6 Secretion*

Mouse blood samples were collected by cardiac puncture and placed in tubes containing EDTA (10 mg/ml).[37,38] Mouse interleukin (IL)-6 was measured using the TiterZyme Enzyme Immunometric Assay kits (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions.

Vitamin D-Responsive Element Transcriptional Activity

Cells were grown in triplicate and transfected with Cignal Vitamin D Reporter (luc) Kit (SABiosciences, Frederick, MD) using Surefect reagent (SABiosciences, Frederick, MD). The plasmid for the VDR reporter is a mixture of inducible Vitamin D-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1). The negative control is a mixture of noninducible firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1). After transfection for 24 hours, cells were colonized with *Salmonella* for 30 minutes, washed, incubated in Dulbecco's Modified Eagle Medium with gentamicin (500 μg/ml) for 12 hour or 16 hours. Luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Quantitative Real-Time PCR Analysis

Total RNA was extracted from epithelial cell monolayers using TRIzol reagent (Invitrogen, Carlsbad, CA). The reverse transcription cDNA reaction products were subjected to quantitative real-time PCR using the MyiQ single-color real-time PCR detection system (Bio-Rad) and iQ SYBR green supermix (Bio-Rad) according to the manufacturer's directions.[38] All expression levels were normalized to the β-actin levels of the same sample. Percent expression was calculated as the ratio of the normalized value of each sample to that of the corresponding untreated control cells. All real-time PCR reactions were performed in triplicate. All PCR primers (Table 1) were designed using Lasergene (DNAStar, Madison, WI).

Statistical Analysis

Data are expressed as mean ± SD. Differences between two samples were analyzed by Student's *t*-test. *P* values of 0.05 or less were considered significant.

Table 1. Real-Time PCR Primers

Gene Name	Primer
hVDR F	5′-GGACTGCCGCATCACCAA-3′
hVDR R	5′-TCATCTCCCGCTTCCTCT-3′
hCathe F	5′-TGCCCAGGTCCTCAGCTAC-3′
hCathe R	5′-GTGACTGCTGTGTCGTCCT-3′
hCyp24 F	5′-GCCTGGCAGAGCTTGAATT-3′
hCyp24 R	5′-ACAGTCCGGGTCTTGGGT-3′
hDEFB4 F	5′-ATTCCTGATGCCTCTTCC-3′
hDEFB4 R	5′-GTGCCAATTTGTTTATACCT-3′
h β-actin F	5′-AGAGCAAGAGAGGCATCCTC-3′
h β-actin R	5′-CTCAAACATGATCTGGGTCA-3′
m β-actin F	5′-TGTTACCAACTGGGACGACA-3′
m β-actin R	5′-CTGGGTCATCTTTTCACGGT-3′

F: forward; R: reverse.

Results

The Pre-Inflammatory State of the VDR$^{-/-}$ Mouse

Because NF-κB is a key regulator involved in the synthesis of inflammatory cytokines, we measured the production of IL-6, a well-known NF-κB target gene, as a readout of the biological effect of VDR expression on NF-κB pathway *in vivo*. The synthesis of IL-6 was compared in the serum of the VDR$^{+/+}$ and VDR$^{-/-}$ mice under unstimulated (noninfected) and stimulated (pathogenic *Salmonella* infection) conditions. Whereas serum IL-6 in specific pathogen-free VDR$^{+/+}$ mice was undetectable (<0.01pg/ml), it was easily detectable in VDR$^{-/-}$ mice (IL-6 200 pg/ml, $P < 0.0001$ vs. wild-type mice) (Figure 1, VDR$^{-/-}$ noninfected control). When the mice were infected with *S. typhimurium*, serum levels of IL-6 in VDR$^{-/-}$ mice (IL-6 1200 pg/ml) was increased from baseline six-fold compared with VDR$^{+/+}$ mice (200 pg/ml, $P < 0.0001$) (Figure 1A).

VDR Null Mutant Mice Have Worse Outcomes Following Salmonella-Induced Infection

We used the *Salmonella* infection model to investigate the role of VDR in intestinal homeostatic responses to infection. We compared colitis development in VDR$^{+/+}$ and VDR $^{-/-}$ mice infected with *Salmonella*. Figure 1, B–E show experiments in which VDR$^{+/+}$ and VDR$^{-/-}$ mice were infected with *S. typhimurium*. Cecal inflammation is the key feature in the *Salmonella*-colitis mouse model.[39] We found that ceca of the VDR$^{-/-}$ mice infected with *S. typhimurium* looked smaller and more red compared with the wild-type mice infected with *S. typhimurium*. The length of ceca in VDR$^{-/-}$ group infected with *Salmonella* was significantly decreased comparing to the wild-type mice (Figure 1B). H&E staining data showed more severe

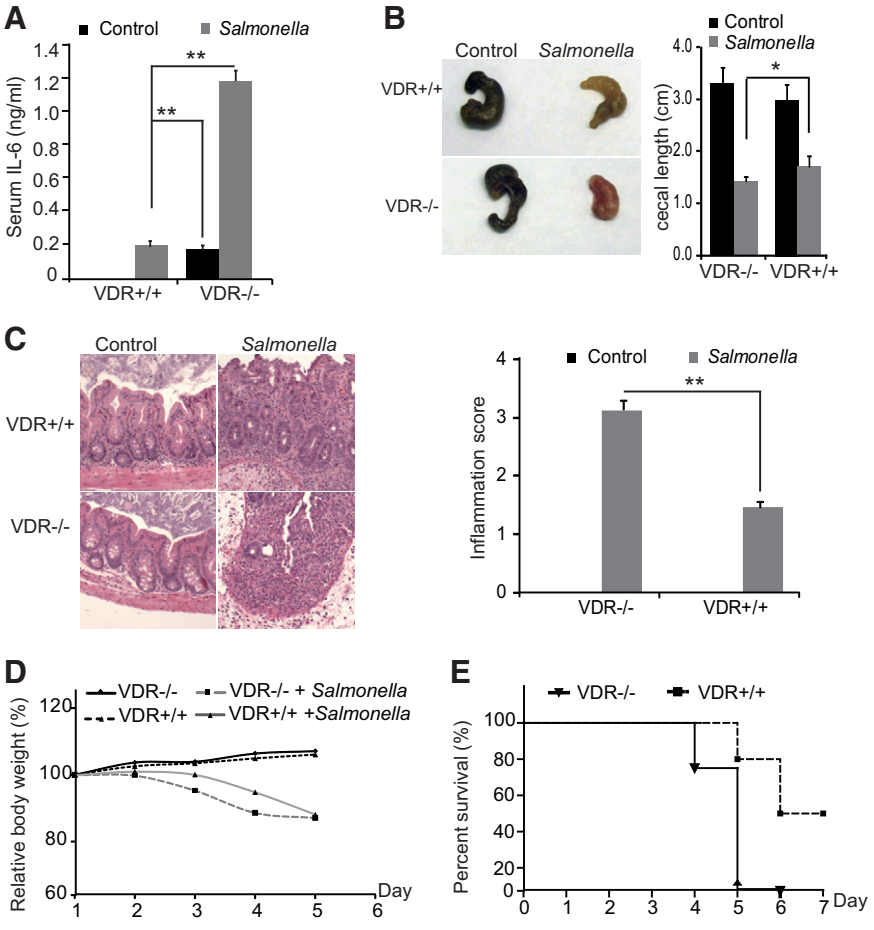

Figure 1. VDR-null mutant mice have worse outcomes with *Salmonella*-induced infection. **A:** Serum IL-6 increased in VDR deficient mice. Normal mice (VDR$^{+/+}$) and VDR-deficient mice were infected with wild-type *S. typhimurium* for six hours. Mouse serum was collected for IL-6 enzyme-linked immunosorbent assay. Data are presented as the mean ± SD from a single experiment assayed in triplicate. *$P < 0.05$. **B:** Cecum was dissected out from the indicated mouse and photographed. Cecum shortening was found in the mouse intestine with *Salmonella* infection. **C:** H&E staining and scores of the mouse intestine with or without *Salmonella* infection. **D:** Relative body weight change in mice with or without *Salmonella* infection. **E:** Survival percentage of the VDR$^{+/+}$ and VDR$^{-/-}$ mice administrated *S. typhimurium*. wild-type group $n = 10$, VDR$^{-/-}$ group $n = 12$. *$P < 0.05$, **$P < 0.01$.

intestinal inflammation in VDR$^{-/-}$ mice infected with *S. typhimurium* compared with the wild-type mice (Figure 1C). Next, the level of intestinal inflammation was quantified histologically in a blinded fashion by a trained pathologist. The inflammation score in the *Salmonella*-infected VDR$^{-/-}$ mice was significantly higher than the *Salmonella*-infected VDR$^{+/+}$ mice (Figure 1D). Moreover, the body weight of the *Salmonella*-infected VDR$^{-/-}$ mice dropped much earlier than the VDR$^{+/+}$ mice (Figure 1D). Ninety percent of VDR$^{-/-}$ mice infected with *Salmonella* died within 5 days and 100% by 6 days (Figure 1E). In contrast, VDR$^{+/+}$ mice were relatively resistant at *Salmonella*; with higher survival rates (60%) seen within the 7-day observation period (Figure 1E). Hence, these data indicate that VDR contributes to host protection against detrimental outcomes to pathogenic invasive enteric bacterial infection.

VDR Expression Protects against Salmonella *Colonization and Mucosal Invasion*

We also examined whether VDR expression contribute to protect cells from *Salmonella* invasion. Immunofluorescence assay showed more *Salmonella* staining (green) in the VDR$^{-/-}$ mouse intestine. We further counted the numbers of *Salmonella* invading the intestine. VDR$^{-/-}$ mice had increased *Salmonella* invasion of the intestine, whereas the VDR$^{+/+}$ mouse had significantly fewer *Salmonella* (Figure 2A). Culture of the cecum showed significantly higher concentrations of *Salmonella* in the VDR$^{-/-}$ mice than in VDR$^{+/+}$ mice (Figure 2B). Furthermore, we used MEFs to investigate the effect of VDR ablation on *Salmonella* invasion. We used VDR$^{+/-}$ and VDR$^{-/-}$ MEFs, as we wanted to compare the difference between VDR-null and one allele of the *Vdr* gene. *Salmonella* invasion was increased in the VDR$^{-/-}$ MEFs (Figure 2C). These *in vitro* data are consistent with our *in vivo* findings (Figure 2, A and B).

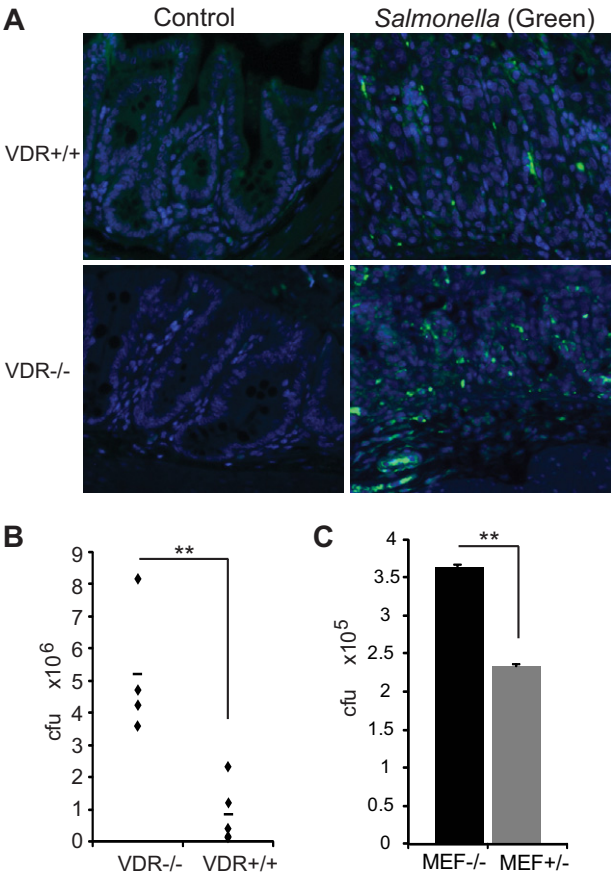

Figure 2. VDR expression reduces *Salmonella* invasion in cells. **A:** *Salmonella* location (green) in the VDR$^{+/+}$ and VDR$^{-/-}$ mouse cecum. Normal mice and VDR deficient mice were infected with wild-type *Salmonella typhimurium* for five days. **B:** *Salmonella* concentrations in the VDR$^{+/+}$ and VDR mouse ceca. **C:** *Salmonella* invasion in infected VDR$^{+/-}$ and VDR$^{-/-}$ MEFs. MEFs were stimulated with wild-type *Salmonella* for 30 minutes, washed, and incubated in fresh Dulbecco's Modified Eagle Medium for 30 minutes. The mean ± SD is from three replicate experiments. $^{**}P < 0.01$.

Absence of VDR Permits Translocation of NF-κB p65 to the Nucleus

We then assessed the level of NF-κB p65 protein, the most abundant NF-κB subunit, in epithelial cells. It is known that VDR physically binds to p65.[14,15] Total p65 was reduced in colonic epithelial cells of noninfected VDR$^{-/-}$ mice (Figure 3, A and B). To examine the possibility that cross-regulation of the VDR and NF-κB signaling pathways may occur through physical interaction, we determined whether VDR can form a complex with NF-κB *in vivo*. Using a co-immunoprecipitation assay, we found that the NF-κB p65 subunit formed a complex with VDR in unstimulated wild-type mouse colonic epithelial cells. VDR deletion totally abolished the VDR/p65 binding in freshly isolated VDR$^{-/-}$ mouse colonic epithelial cells, whereas there is no interaction of VDR with nonspecific IgG (Figure 3C). Additionally, we found no apparent interaction of VDR with p50, a NF-κB subunit (data not shown) in wild-type mice indicating that the physical interaction between VDR and NF-κB p65 is specific.

The IP results were also confirmed by cell fraction of VDR/p65 levels in the cytosolic and nuclear compartments of cells in mouse colon. As shown in Figure 3D, VDR/p65 binding was found in cytosolic compartments in normal mouse colon. The VDR/p65 physical interaction was undetectable in the nuclear fraction of mouse colon epithelial cells. Thus our data demonstrate a clear physical interaction between VDR and NF-κB in basal colonic epithelia.

Absence of VDR Permits Translocation of NF-κB p65 to the Nucleus

Because of the lack of VDR, the physical interaction between VDR and p65 was absent in VDR$^{-/-}$ cells. VDR knockout also reduced the levels of the NF-κB inhibitor, IκBα, in colonic epithelial cells.[40] Consequently, these alterations combined may led to free p65 and increase p65 translocation to the nuclei. Immunostaining data showed a marked increase in nuclear p65 in VDR$^{-/-}$

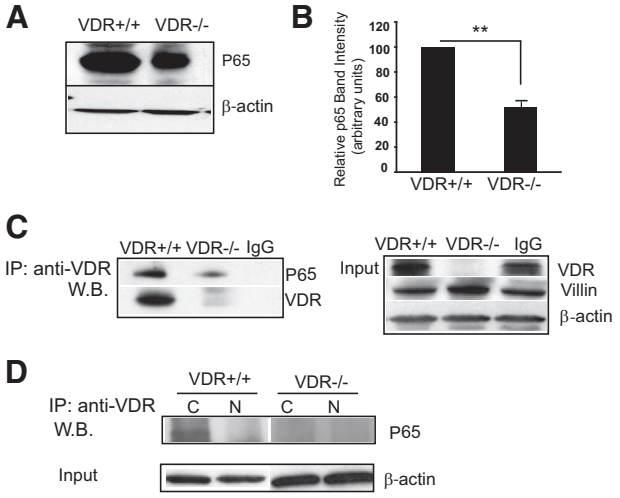

Figure 3. The expression of NFκB p65 in intestinal epithelial cells in VDR knockout mice. **A:** NF-κB p65 expression in normal mice and VDR-deficient mice. Colonic epithelial cells were collected and lysates were immunoblotted with antibody against NF-κB p65. **B:** Relative NF-κB p65 band intensity in normal and $VDR^{-/-}$ mice. Data are presented as the mean ± SD from three repeated experiments. **C:** VDR physically interacts with NF-κB p65 in intestinal epithelial cells *in vivo*. VDR deletion abolishes this interaction. Mouse intestinal epithelial cells were collected. Cell lysates were immunoprecipitated with anti-VDR antibody, then the precipitated complex was probed with anti-p65 antibody by Western blot (W.B. left). Note that the background level of p65 was detectable in $VDR^{-/-}$ cells. An aliquot of the cell lysates was probed with antibodies against VDR, villin, and β-actin to confirm equal input (right). **D:** VDR/p65 binding in mouse colon using cell fraction. Mouse colon epithelial mucosa was lysed and the nuclear proteins were extracted. Cell fraction was used for co-immunoprecipitation assay using anti-VDR antibody. N: nuclei; C: cytoplasm. $^{**}P < 0.01$.

mouse colon without any stimulation (Figure 4A). In contract, p65 was located in the cytosol and absent in the nuclei in the $VDR^{+/+}$ mouse colon. Moreover, we examined the cell fraction of p65 level in the cytosolic and nuclear compartments of cells in the mouse colon. There was detectable nuclear p65 in the $VDR^{-/-}$ colon, whereas no p65 was found in the nuclei of the $VDR^{+/+}$ mice (Figure 4B). Equal loading of the epithelial cells was by Villin, the epithelial cell marker. The loading of nuclear proteins was confirmed by the level of Sp1, a nuclear protein absent in the cytosol. In the MEFs, the expression level of p65 in the nuclear extracts was higher in $VDR^{-/-}$ cells than in $VDR^{+/-}$ cells (Figure 4C). These data are also consistent with our previous observation in $VDR^{-/-}$ MEFs.[15] Taken together, our *in vivo* and *in vitro* data show that absence of VDR permits translocation of NF-κB p65 to the nucleus.

VDR Expression Negatively Regulates Transcriptional NF-κB Activity

We further hypothesize that the NF-κB activity is high in cells lacking VDR expression. Transcriptional activity of NF-κB is controlled by phosphorylation of p65 at serine 536.[41,42] Constitutive phospho-p65 levels were also significantly increased in $VDR^{-/-}$ MEFs without stimulation (Figure 4D). We found that $VDR^{-/-}$ mouse colonic epithelial cells had significantly elevated phospho-p65, over 2 times of the p-p65 concentrations in $VDR^{+/+}$ epithelial cells (Figure 4E). Overall, absence of VDR led to elevated levels of phospho-p65 in the fibroblasts and colonic epithelial cells without any bacterial stimulation.

VDR Distribution in the Normal Mouse Colon

There has been very little information on the VDR distribution in colonic epithelial cells, which are consistently exposed to the enteric bacteria. We investigated the distribution of VDR from the proximal colon to the distal colon in the normal mouse intestine by making a colonic "Swiss roll" (Figure 5A). We demonstrated less VDR staining (green) in the distal colon with heavier staining in the proximal colon. In contrast, 4,6-diamidino-2-phenylindole staining (blue) is even throughout the colon. Under high magnification, we identified VDR distributed at the top of the epithelial cell crypts in the proximal colon. Most of the enhanced VDR staining is in the cytosol; some of the staining is in the nuclei (indicated with white arrows in Figure 5B).

Western blot analysis of VDR levels in colon also confirmed the immunostaining observation (Figure 5C). Data from 4 individual mouse colon samples showed that the VDR expression is significantly higher in the proximal colon than that in the distal colon (Figure 5D).

Salmonella *Infection Increased the VDR Protein Expression in Mouse Colon* in Vivo

Furthermore, we examined the expression of VDR in pathogenic bacteria-induced inflammation using normal mice. Interestingly, we found that the total amount of VDR protein increased in the colonic epithelium of mice 6 hours after *Salmonella* infection (Figure 6A). To confirm that the increased VDR expression is induced specifically by pathogenic wild-type *Salmonella* infection, we gavaged the mice with wild-type *Salmonella* or commensal *E. coli* F18. Western blot (Figure 6B) showed that only wild-type *Salmonella* was able to increase the expression of VDR, whereas commensal *E. coli* F18 colonization did not change the VDR expression in the mouse colonic epithelial cells. These results indicate that colonic epithelial responses to specific pathogenic bacteria such as wild-type *Salmonella* are determined in part through the VDR signaling pathway.

VDR Relocation in the Mouse Colon after Bacterial Infection Colonization

To further examine the distribution of VDR in the mouse colon with bacterial infection, we used the *Salmonella* infection model in normal mice. Histological examination demonstrates that VDR was located on the surface of colonic epithelial cells in normal mice without any treatment (Figure 6C control mouse). Pathogenic *Salmonella* colonization in mice increased VDR staining at the surface of the crypts, but also induced VDR expression in the middle and at the bottom of the crypts in mice infected with wild-type *Salmonella* (Figure 6C *Salmonella*).

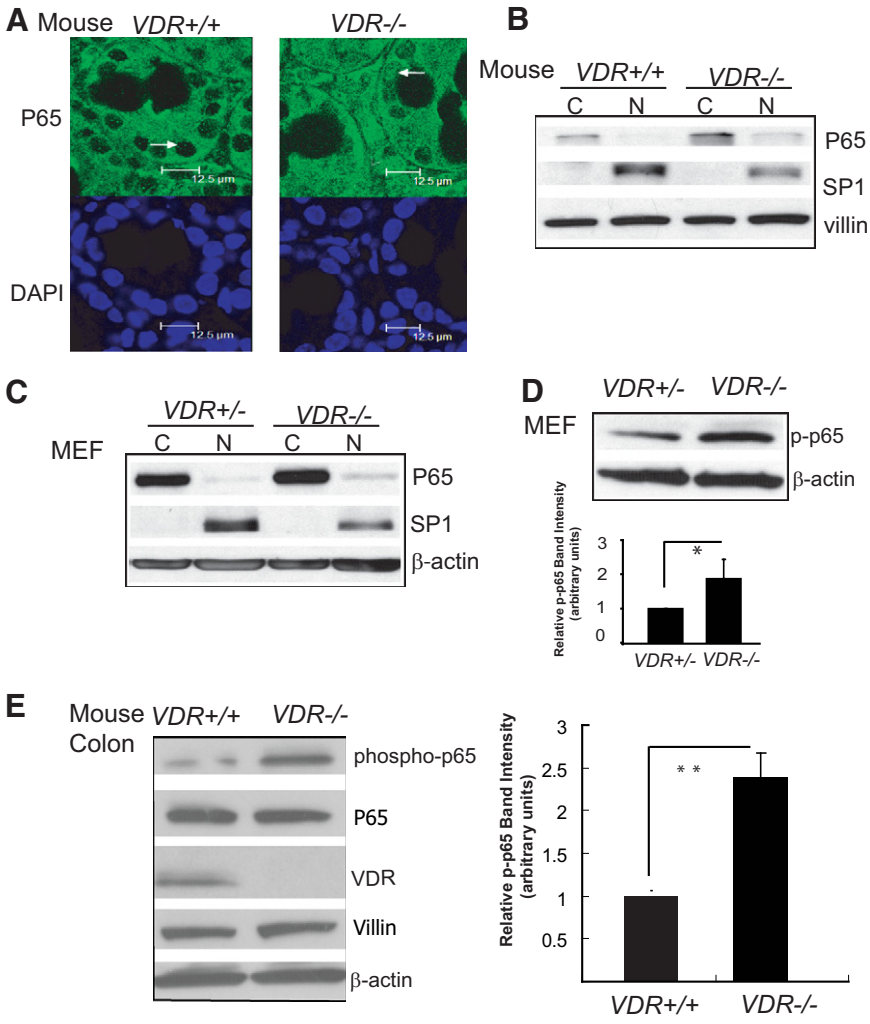

Figure 4. The location and activity of NFκB p65 in intestinal epithelial cells in VDR knockout mice. **A:** NF-κB p65 location in colon in normal mice ($VDR^{+/+}$) and $VDR^{-/-}$ mice without any treatment. **Arrows** indicate the p65 nuclear location (green). **B:** Western blot analysis of p65 levels in cytosolic (C) and nuclear (N) extracts isolated from $VDR^{+/+}$ and $VDR^{-/-}$ mouse colons. **C:** The p65 level in cytosolic and nuclear extracts isolated from $VDR^{+/-}$ and $VDR^{-/-}$ MEFs. The nuclear protein Sp1, which is absent in the cytosolic fraction, serves as a nuclear protein loading control. **D:** Higher phospho-p65 level in the $VDR^{-/-}$ MEFs without any treatment. $^{*}P < 0.05$. **E:** Increased phospho-p65 level in the $VDR^{-/-}$ mouse colonic epithelial cells without any treatment. Relative phospho-p65 band intensity in normal and $VDR^{-/-}$ mice is shown. Villin is used as a marker for epithelial cells and an internal control. β-actin is also used as an internal control. The mean ± SD is from three repeats. $^{**}P < 0.01$.

Our data indicate that pathogenic *Salmonella* infection changed the location of VDR in colonic epithelial cells.

We also compared GF and conventionalized mice (GF mice colonized with SPF fecal bacteria) to explore the effect of bacteria on modulating VDR distribution. Our data showed that VDR was localized at the top of the crypts of the GF mouse intestine by immunostaining (Figure 6D). Bacterial colonization of ex-GF conventionalized mice for 7 days induced VDR relocation to the middle and even at the bottom of the crypts. In addition, monoassociation of GF mice with *E. coli* F18 increased the epithelial VDR protein expression just like the *Salmonella* infection in the SPF mice (Figure 6E).

Salmonella *Directly Increased VDR Protein Expression and VDR Transcriptional Activity Independent of Vitamin D3*

The classical VDR pathway is initiated on 1,25-dihydroxyvitamin D3 binding to VDR. Recent studies demonstrate that VDR was activated through increased protein expression independent of classical transcriptional regulation.[43–45] Therefore, it is possible that bacteria alone modulate VDR expression and subsequent activity in the absence of 1,25-dihydroxyvitamin D3. We cultured human intestinal epithelial cells in medium with or without 1,25-dihydroxyvitamin D3, then exposed them to *Salmonella typhimurium*. We found that bacterial colonization of Caco2 BBE cells for only 1 hour increased VDR expression in the absence of 1,25-dihydroxyvitamin D3 (Figure 7A). In cells stimulated by 1,25-dihydroxyvitamin D3 for 24 hours, the level of VDR increased, but there was no additional increase with *Salmonella* stimulation. We further investigated VDR transcription activity under bacterial stimulation in two intestinal epithelial cell lines, Caco2 BBE and HCT116. In cells transfected with the VDR reporter plasmid, *Salmonella* colonization for 12 hours and 16 hours induced the VDR transcriptional activity significantly compared with cells without bacteria. The relative fold increase was compared with cells transfected with the negative control plasmid (Negative) (Figure 7B). Additionally, the expression of VDR target genes Cyp24, β-defensin, and cathelicidin increased significantly after *Salmonella* infection in the human epithelial cells without

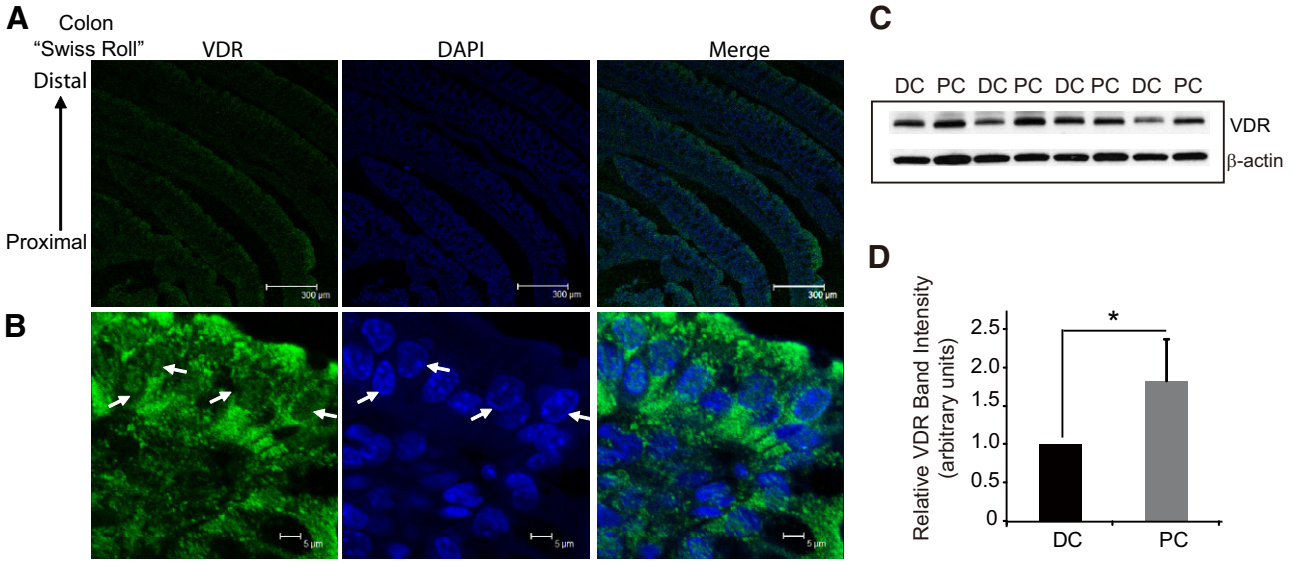

Figure 5. The distribution of VDR in normal mouse colon. **A:** VDR distribution in the "Swiss roll" made with the SPF mouse colon. **Arrow** indicates the direction of the proximal and distal colon. **B:** Higher magnification of VDR distribution in the normal mouse proximal colon. **White arrow** indicates the nuclear staining of VDR at the **top** of colonic crypts. **C:** VDR protein expression in the proximal and distal colon determined by Western blot. DC: distal colon; PC: proximal colon. **D:** Relative VDR band intensity in normal mice. Data are presented as the mean ± SD; n = four mice per group. $^{*}P < 0.05$.

1,25-dihydroxyvitamin D3 in media (Figure 7C). Taken together, these data indicate that *S. typhimurium* increased VDR protein expression, VDR transcriptional activity and VDR- mediated gene transcription independent of 1,25-dihydroxyvitamin D3.

Discussion

In the current study, we demonstrate that VDR contributes to host protection against detrimental outcomes of pathogenic enteric bacterial infection (Figure 8). In the

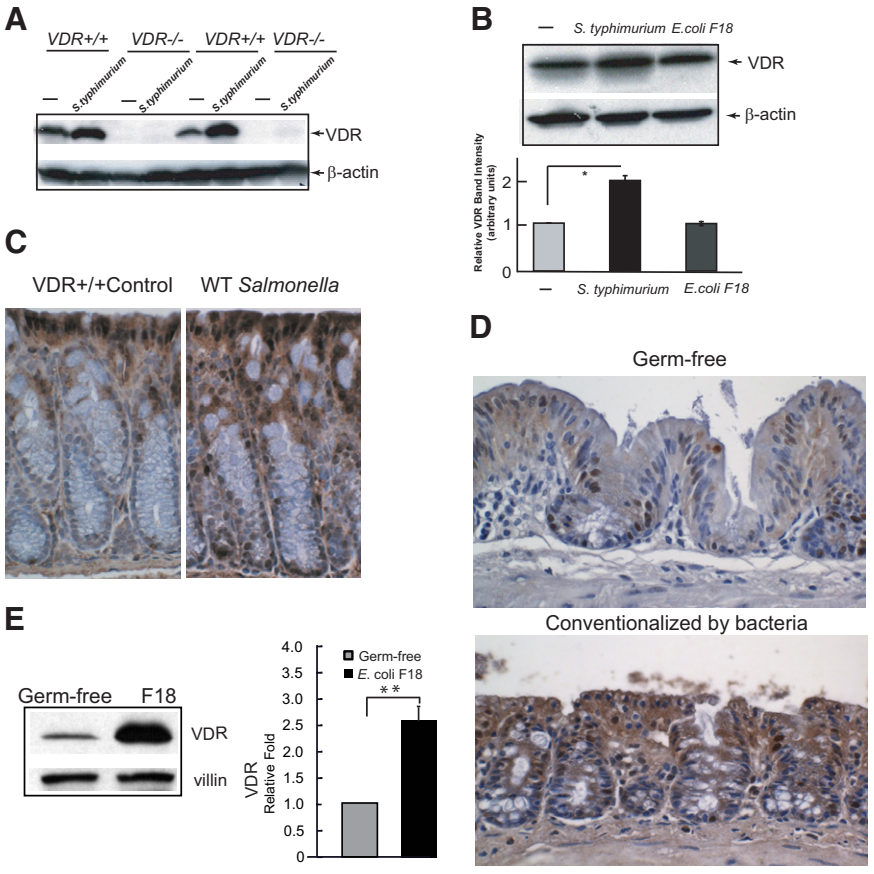

Figure 6. The expression and location of VDR protein in mouse intestinal epithelial cells infected with or without bacteria. **A:** Total VDR protein in mouse colonic epithelial cells increased after infection with pathogenic *Salmonella typhimurium* for six hours. **B:** Normal mice were gavaged without (−) or with either wild-type *Salmonella typhimurium* or *E. coli* F18 for six hours. Colonic epithelial cell lysates were immunoblotted with antibodies against VDR or β-actin. Data are from a single experiment and are representative of three separate mouse cells. $^{*}P < 0.05$. **C:** Relocation of VDR after *Salmonella* for six hours. **D:** VDR distribution in germ-free and conventionalized mouse intestine seven days after colonization with SPF slurries. **E:** Total VDR protein in mouse colonic epithelial cells increased after GF mice mono-associated with *E.*coli F18 for six days. $^{**}P < 0.01$.

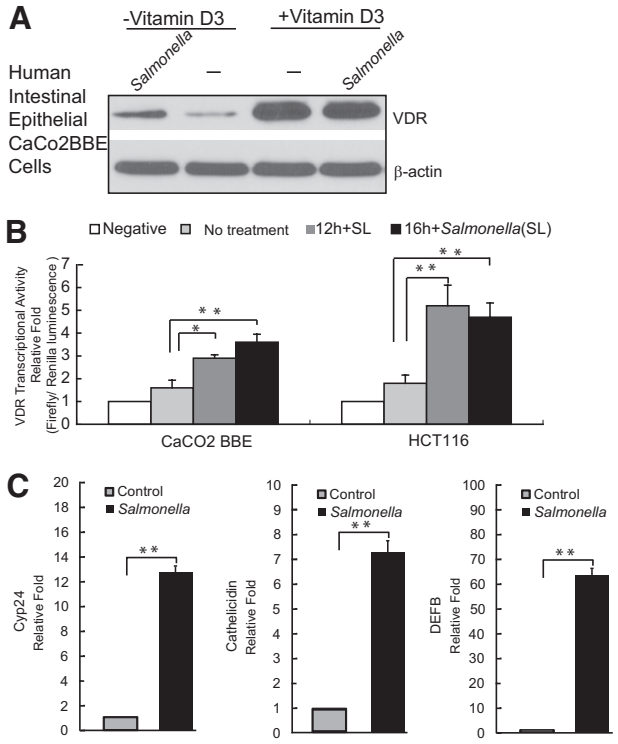

Figure 7. *Salmonella* directly increases VDR expression and transcriptional activity in human intestinal epithelial cells. **A:** *Salmonella* directly increases VDR protein expression in human intestinal epithelial CaCo2 BBE cells in the absence of 1,25-dihydroxyvitamin D3 (−). 1,25-Dihydroxyvitamin D3 (20 nmol/L) stimulated VDR protein was measured by Western blot. **B:** *Salmonella* colonization increased the VDR transcription activity in human epithelial cells. The cells were transfected with Cignal Vitamin D Reporter (luc) Kit. After transfection for 24 hours, cells were colonized with *Salmonella* for indicated time and lysed, and luciferase activity was determined. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the activity was expressed as relative luminescence units. **C:** *Salmonella* colonization increased the mRNA level of the VDR target genes in human epithelial cells. $^{*}P < 0.05$; $^{**}P < 0.001$. Data are from a single experiment and are representative of two to three separate experiments.

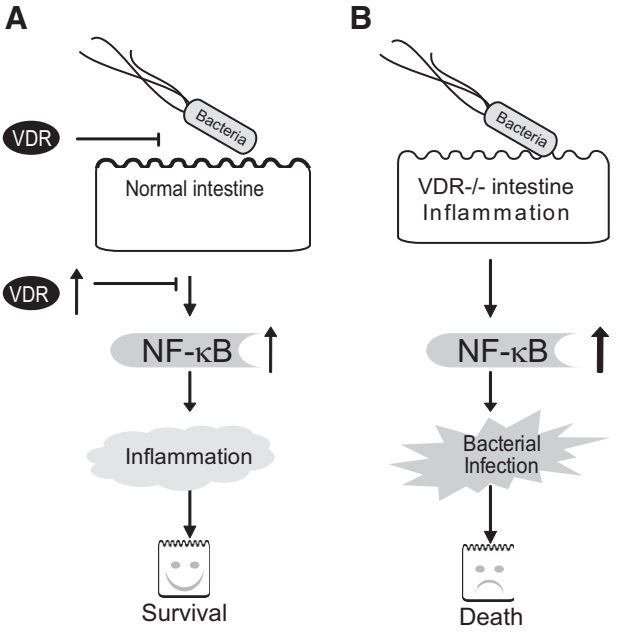

Figure 8. Working model of VDR in regulation of bacterial invasion, NF-κB activity, host protection, and intestinal homeostasis.

wild-type mouse with VDR expression in the intestine, VDR is able to reduce *Salmonella* invasion comparing to the $VDR^{-/-}$ cells. Therefore, there is less bacterial burden in the wild-type intestine. Moreover, VDR expression increases after *Salmonella* infection in intestine. VDR plays a role as a negative regulator of the NF-κB activity and modulates inflammatory responses of the host, thus allowing mice to survive the enteric bacterial infection (Figure 8A). In contract, $VDR^{-/-}$ cells have more *Salmonella* invasion in the intestine. Mice lacking VDR have a pro-inflammatory state due to intrinsically high NF-κB activity. VDR deletion leads to more aggressive gross and histological colonic injury, increases serum IL-6 levels as a marker of systemic inflammation, and enhances mortality after *Salmonella* infection (Figure 8B). Hence, VDR is an important contributor to host protection and intestinal homeostasis.

We postulate that increased NF-κB activity in $VDR^{-/-}$ intestinal cells is a consequence of the absence of VDR, which leads to free p65 and elevated phospho-p65, in part due to the reduction in IκBα.[40] Consistent with these findings, the induction of serum IL-6 by pathogenic bacterial infection was much more robust in $VDR^{-/-}$ than in $VDR^{+/+}$ mice. Interestingly, basal IL-6 serum levels in noninfected $VDR^{-/-}$ mice were comparable to those of *Salmonella* infected $VDR^{+/+}$ mice. This is consistent with our demonstration that NF-κB activity is increased in MEFs lacking the vitamin D receptor, leading to a pre-inflammatory or pro-inflammatory state.[15] Take together; our *in vitro* and *in vivo* data suggest that VDR negatively regulates NF-κB activity and suppresses inflammatory responses to a common invasive enteric bacterial pathogen.

We demonstrate intracellular complexes of VDR and p65 in freshly isolated colonic epithelial cells. Likewise, VDR physically interacts with NF-κB p65 in osteoblasts[14] and fibroblasts[15]. In tubular cells, VDR/p65 formed a complex, thus inhibiting the ability of p65 to transactivate gene transcription.[46] In the present study, we show that VDR is able to negatively modulate NF-κB activity through physical binding with NF-κB p65, decreasing translocation of p65 in the nucleus, and reducing p65 phosphorylation. Intestinal VDR concentrations are increased by enteric *Salmonella* infection and are directly involved in the regulation of the NF-κB pathway in intestinal epithelial inflammation *in vivo*. Consistent with a host protective role of intestinal VDR during intestinal infection, VDR null mutant mice have worse outcomes following *Salmonella*-induced infection.

Our study also demonstrates that enteric bacterial infection directly increases VDR protein expression and distribution in the colon and that commensal enteric bacteria also influence VDR distribution in epithelial cells. In the normal mouse colon, VDR locates to differentiated colonic epithelial cells at the top of crypts. Pathogenic *Salmonella* infection increases VDR expression and changes VDR distribution in mouse colonic epithelial cells *in vivo* to cells lower in the crypt. VDR was localized at the top of the crypts of the GF mouse colon. Commen-

sal bacterial colonization of GF mice (conventionalization) induced VDR relocation in the colon and similar to cecum (data not shown) that seen with pathogenic *Salmonella* infection in conventional mice. The small intestine contains relatively few bacteria compared with the colon; therefore, bacterial colonization in GF mice did not change the VDR distribution in small intestine (data not shown). VDR exerts multiple effects, differentiation,[9] growth inhibitory[31,47] and anti-inflammatory actions.[7,14] The relocation of VDR in intestine may relate to transient activation of NFκB and other signaling pathways by colonization of the GF colon by commensal bacteria[48] or infection in specific-pathogen-free mice[49] or possibly by replacement of the infected epithelial cells and host defense against the *Salmonella*-induced damage.

Using normal mice, we also identified a gradient for distribution of VDR from the proximal colon to the distal colon. In the normal proximal colon, bacterial fermentation is very intense with high production of short-chain fatty acids, an acid environment (pH 5 to 6), and rapid bacterial growth.[50] By contrast, the distal colon has less substrate availability, neutral pH and slow bacterial growth.[50] Interestingly, we found that in the proximal colon where the bacterial growth and metabolism is high, VDR expression is enhanced, whereas in the distal colon with less commensal bacterial growth, less VDR expression was detected. Our data on the distribution of VDR expression in the gut are correlated with bacterial density. VDR plays a critical role in protecting the colon from injury and maintaining intestinal integrity.[26,51] Previous studies indicated that bacterial products, such as butyrate, regulate VDR expression.[52–56] Therefore, bacterial growth and metabolic products may contribute to the gradient distribution of VDR in the colonic epithelium in normal mice and be responsible for redistribution of VDR to the base and middle of colonic epithelial crypts after colonization of GF mice with complex enteric commensal microbiota.

Toll-like receptors are key contributors to bacterial-induced inflammation and commensal bacteria- driven epithelial homeostasis.[57–59] Recent studies show that activation of toll-like receptor-2 on human macrophages up-regulated the expression of VDR and induced the human antimicrobial peptide CAMP and killing of intracellular *Mycobacterium tuberculosis*.[6] It is known that the CAMP gene is strongly up-regulated in myeloid cells by $1,25(OH)_2D_3$.[12] This process was dependent on both the endogenous production and action of $1,25(OH)_2D_3$ through the VDR. It will be interesting to investigate the influence of intestinal toll-like receptor signaling on VDR expression in a future study.

Our data indicate that VDR expression, location, transcriptional activity, and target gene expression are regulated by intestinal bacteria, which may be independent of 1,25-dihydroxyvitamin D3. Classically, VDR is considered to be a ligand-activated transcription factor. VDR binds to the vitamin D-response element in the target gene promoter to regulate gene transcription.[60] Several studies demonstrate that VDR signaling can be activated independent of 1,25-dihydroxyvitamin D3.[34,45,61] Our study suggested that bacteria directly activate VDR signaling by increasing protein level of VDR. We have not addressed this issue *in vivo*, but did show 1,25-dihydroxyvitamin D3 independent induction of VDR by *Salmonella* in colonic cell lines *in vitro*.

In summary, we demonstrate that an invasive enteric pathogen, *S. typhimurium*, can induce colonic VDR expression and location *in vivo*, and stimulate VDR expression, transcription, and signaling in colonic epithelial cell lines and MEFs. Our current study also highlights the importance of VDR in negatively regulating bacterial-stimulated NF-κB activity in intestine. The presence of VDR in various tissues along with its ability to exert differentiation,[9] growth inhibitory[31,47] and anti-inflammatory actions,[7,14] set the stage for therapeutic exploitation of VDR ligands for the treatment of various intestinal inflammatory conditions and cancers.[62] Our results and other investigators' studies demonstrate that VDR has multiple critical functions in regulating response to enteric invasive pathogen, intestinal homeostasis,[26,51] tight junction structure,[26] bacterial infection, and commensal bacterial colonization. Given the crucial role of enteric microbiota in the pathogenesis of IBD[16–18,57] and the therapeutic and pharmacological potential of probiotics in the treatment of inflammatory bowl disease,[63,64] understanding how pathogenic bacteria and probiotics regulate the VDR pathway may facilitate the development of targeted interventions to control intestinal inflammation.

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