ORIGINAL ARTICLE

Basic Study

Vitamin D differentially regulates *Salmonella*-induced intestine epithelial autophagy and interleukin-1β expression

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Abstract

AIM

To investigate the effects of active vitamin D3 on autophagy and interleukin (IL)-1β expression in *Salmonella*-infected intestinal epithelial cells (IECs).

METHODS

Caco-2 cells, NOD2 siRNA-, Atg16L1 siRNA- or vitamin D receptor (VDR) siRNA-transfected Caco-2 cells were pretreated with 1,25-dihydroxyvitamin D3 (1,25D3), and then infected by wild-type *S. typhimurium* strain SL1344. The conversion of LC3-I to LC3-II was detected by Western blot analysis and LC3+ autophagosome was analyzed by immunofluorescence. Caco-2 cells or VDR siRNA-transfected cells were pretreated with 1,25D3, and then infected by SL1344. Membrane protein and total RNA were analyzed by Western blot and RT-PCR for VDR and Atg16L1 protein and mRNA expression, respectively. Atg16L1 siRNA-transfected Caco-2 cells were pretreated by 1,25D3 and then infected with SL1344. Total RNA was analyzed by RT-PCR for IL-1β mRNA expression.

RESULTS

The active form of vitamin D, 1,25D3, showed enhanced VDR-mediated Atg16L1 mRNA expression, membranous Atg16L1 protein expression leading to enhanced autophagic LC3II protein expression and LC3 punctae in *Salmonella*-infected Caco-2 cells which was counteracted by Atg16L1 and VDR siRNA, but Atg16L1 mediated suppression of IL-1β expression. Thus, active vitamin D may enhance autophagy but suppress inflammatory IL-1β expression in *Salmonella*-infected IECs.

CONCLUSION
Active vitamin D might enhance autophagic clearance of *Salmonella* infection, while modulation of inflammatory responses prevents the host from detrimental effects of overwhelming inflammation.

Key words: Vitamin D; Atg16L1; Autophagy; Interleukin-1β; *Salmonella*; Intestinal epithelia

Core tip: In this study, the enhanced autophagy expression and down-regulation of inflammatory responses in *Salmonella*-infected intestinal epithelial cells by active vitamin D provide a rationale of its alternative therapy for invasive bacterial infection and other inflammatory disorders.

INTRODUCTION
The incidence of food-borne human infections caused by *Salmonella enteritidis* and by multi-drug-resistant strains of *S. typhimurium* has increased substantially[1], with similar trends being reported from Europe[2] and Taiwan[3]. Intestinal epithelial cells (IECs) serve as not only a barrier to bacteria colonizing the gut but also rather as an integral and essential component of the innate mucosal immune system of the host.

Although clear evidence of the beneficial effects of vitamin D on a variety of systemic diseases exists, there has been renewed interest in this vitamin, which has a broad range of activities on microbial infections. Recent research has begun to unravel important roles of vitamin D in the regulation of innate immunity[4]. Vitamin D may play a role in protecting against infection during pregnancy and bacterial vaginosis. Four studies included in a systemic review[5] demonstrated the therapeutic effect of vitamin D supplementation for colitis. Thus, supplementation with vitamin D3 could provide a novel strategy to reduce antibiotic use and indirectly prevent the emerging epidemic of bacterial resistance.

The role of autophagy has been expanded in recent years to include diverse immunological effector and regulatory functions. Increasing evidence indicates the potential of autophagy in controlling infections by directing intracellular or ingested pathogens to lysosomes, leading to their destruction[6]. Several studies have linked autophagy to host defense against several intracellular bacterial pathogens that use different strategies to establish infection, such as *Listeria monocytogenes*, *Shigella flexneri*[7] and *S. enterica* serovar *typhimurium*[8]. Therefore, dissecting the molecular mechanisms by which *Salmonella* utilizes autophagy has the potential to lead to the identification of novel drug candidates to prevent and treat *Salmonella* infection and related intracellular infections.

Autophagy plays an essential role in the clearance of *Salmonella* by alveolar macrophages. Previous studies have linked nucleotide-binding oligomerization domain-containing protein 2 (NOD2) function to autophagy[9,10]. NOD2 is critical for the autophagic response to invasive bacteria, which recruits autophagy-related protein 16-like 1 (ATG16L1) to the plasma membrane at bacterial entry sites. The observation that NOD2 is a vitamin D target gene[11,12] and vitamin D receptor (VDR) transcriptionally regulates *ATG16L1* as a VDR target gene[13,14] also links vitamin D signaling to autophagy. Atg16L1 is required for autophagy in IECs and protection of mice from *Salmonella* infection[15]. 1,25-dihydroxyvitamin D3 (1,25D3), the active form of vitamin D, up-regulates NOD2 mRNA expressions in *Salmonella*-infected IECs[16], and the interaction of NOD2 and Arg16L1 plays a critical role in *Salmonella*-induced autophagy in IECs[17]. These results strongly suggest that stimulation of NOD2 and Atg16L1 expression in human epithelial cells by 1,25D3 would recruit Atg16L1 to boost autophagy, which would then contribute to innate immune responses of vitamin D to bacterial infection.

Therefore, this study was designed to investigate the effects of active vitamin D3 on *Salmonella*-induced autophagy *via* enhanced NOD2 and Atg16L1 mRNA expression and membranous recruitment of proteins in IECs.

MATERIALS AND METHODS
Bacterial strains
The wild-type *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) strain used in this work was SL1344. *Salmonella* inoculum was prepared as described previously[16,18]. Bacteria were grown overnight in static cultures with minimal aeration in LB medium. The bacteria were collected by centrifugation at 14000 g for 5 min, washed with sterile PBS, and resuspended in tissue culture medium without antibiotics.

Cell culture and infection
Caco-2 cells were purchased from the American Type Culture Collection (Manassas, VA, United States) and were cultured as described previously[16,18,19]. Briefly, Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 atmosphere at 37 ℃. Passage 10-30 was used for all experiments. The cells were seeded in tissue culture

plates and grown to 60%-75% confluence. About 1 h before addition of bacteria, the cells were washed and placed in antibiotic-free medium.

Cell fractionation
Cytosolic, membranous and nuclear extracts from untreated and treated cultured cells were prepared by the method previously described[16,19,20]. Protein concentrations in cell fractions were determined using a Bio-Rad assay kit.

Western blotting
Equal amounts of total protein from colon tissue or cultured cells were separated by SDS-PAGE and then transferred to nitrocellulose membranes by semi-dry blotting as previously described[16,19,20]. After blocking the membranes with 5% non-fat dry milk, they were probed with antibodies to either ATG16L1 (Cell Signaling, Beverly, MA, United States), NOD2 (Cayman Chemical, Ann Arbor, MI, United States), LC3B (Cell Signaling) or total GAPDH (Santa Cruz Biotechnology, Dallas, TX, United States). After washes, the membranes were incubated with appropriate horseradish peroxidase-associated secondary antibodies before signals were visualized with the enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, United States).

RNA isolation and cDNA synthesis
Total RNA was prepared from control or infected cells with the Trizol reagent (Invitrogen Corporation, Carlsbad, CA, United States), following the manu-facturer's directions. The RNA was reverse transcribed using the GeneAmp kit (Roche Diagnostics, Nutley, NJ, United States) as described in detail earlier[16].

Real-time reverse transcription PCR
Real-time reverse transcription PCR analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics) as described pre-viously[16,19,20] to determine the NOD2 and Atg16L1 and interleukin-1β (IL-1β) mRNA expression levels using the comparative threshold cycle ($\Delta\Delta$Ct) method of relative quantitation.

Immunofluorescence
The immunofluorescence study of LC3 was performed as previously described[16,19,20]. Briefly, cultured cells after treatment and infection were washed, fixed and permeabilized and then incubated with rabbit anti-LC3B (Cell Signaling Technology). Secondary antibody was goat anti-rabbit IgG conjugated with Alexa Fluor 594 fluorochrome (Invitrogen Molecular Probes, Eugene, OR, United States). After extensive washing, the nucleic acid stain 4,6-diamidino-2-phenyindole dihydrochloride (DAPI) was added to visualize the nucleus. Then, the coverslips were examined under immunoflourescence conditions by using a Zeiss

Axio Observer Z1 (Carl Zeiss, Jena, Germany). The percentage of cells with endogenous LC3 punctate was determined by counting the number of positively stained cells from 100 randomly chosen cells from three separate experiments.

RNA interference in cultured cells
RNA interference (RNAi) experiments in cultured cells were performed as described previously[16,19,20]. Cells were transfected according to the manufacturer's protocol, which was modified in our Lab. After 48 to 72 h incubation in a 37 ℃ incubator, the cells were infected by bacteria, and then the cells were lysed and RNA or proteins extracted over ice for further experiments.

Cell viability and morphologic features
Representative cell populations from each condition were examined under light microscopy. No significantly morphologic change was observed under any con-dition. Cell viability in untreated or treated cells was found to be > 90% as analyzed by trypan blue exclusion (data not shown).

Statistical analysis
All above experiments were carried out at least twice with similar results. Statistical analysis was performed using the paired Student's *t*-test and ANOVAs (StatView; SAS Institute, Cary, NC, United States). *P* values < 0.05 were considered significant.

RESULTS

1,25 D3 enhanced the autophagy expression in Salmonella-infected Caco-2 cells
In order to explore if active vitamin D plays a role in *Salmonella*-induced autophagy in IECs, Caco-2 cells were pretreated with 1,25D3, and then infected by wild-type S. typhimurium strain SL1344. The conversion of LC3-I to LC3-II was detected by Western blot analysis and LC3^{+} autophagosome was analy-zed by immunofluorescence. As shown in Figure 1, *Salmonella*-induced autophagy in Caco-2 cells was accompanied by an increase in the conversion of LC3-I to LC3-II (Figure 1A and B) and increased LC3 punctae-containing cells in immunofluorescent analysis (Figure 2C and D), while 1,25D3 enhanced *Salmonella*-induced LC3-II protein and LC3^{+} autophagosome expressions in Caco-2 cells. This suggested that 1,25D3 up-regulated the autophagic process in *Salmonella*-infected Caco-2 cells.

Involvement of VDR in 1,25D3-enhanced Atg16L1 mRNA expressions in Salmonella-infected Caco-2 cells
To examine if 1,25D3 up-regulates *Salmonella*-induced Atg16L1 mRNA expression in IECs, Caco-2 cells were untreated or pretreated with 1,25D3 and then infected with wild-type *Salmonella* strain SL1344 for 1 h. Total
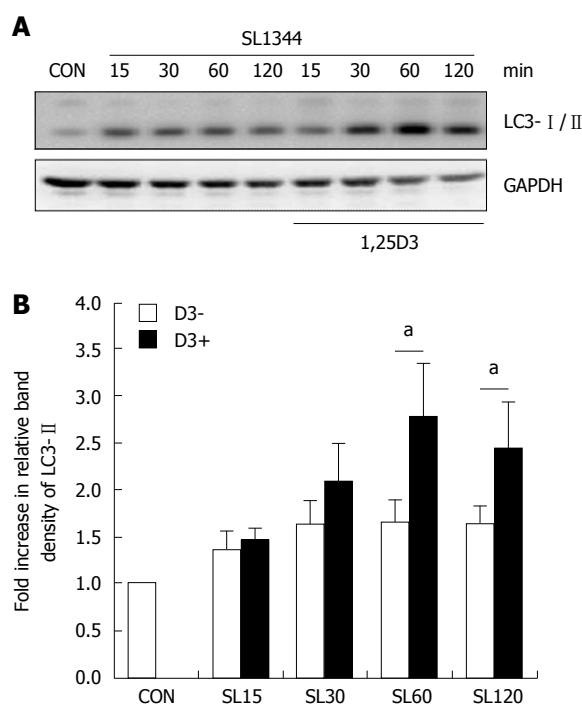
A

B

Figure 1 Effect of 1,25D3 on autophagy expression in *Salmonella*-infected Caco-2 cells. Caco-2 cells were untreated (CON) or treated with 1,25D3 (D3) and then infected by *S. typhimurium* wild-type strain SL1344 (SL). Immunoblots were performed on whole cell lysates with antibody to detect autophagy LC3-Ⅰ/Ⅱ protein expression. The results shown are representative of three separate experiments. GAPDH was detected for normalization of cytosolic protein. Representative immunoblots (A) and densitometric quantification of immunoreactive bands (B) are shown. The relative band intensities of LC3-Ⅱ in untreated (white) and treated (black) Caco-2 cells were quantified as fold-increases compared with the control cells.

RNA was analyzed by real-time quantitative PCR for Atg16L1 mRNA expression. As shown in Figure 3B, 1,25D3 enhanced Atg16L1 mRNA expression in *Salmonella*-infected Caco-2 cells compared to *Salmonella* infection alone.

To investigate if VDR was involved in the 1,25D3-mediated up-regulation of Atg16L1 mRNA in *Salmonella*-infected cells, VDR siRNA-transfected cells were untreated or treated with 1,25D3 for 6 h and infected by *S. typhimurium* wild-type strain SL1344. Knockdown of VDR was confirmed by Western blot (Figure 3A). Following knockdown of VDR, the 1,25D3-enhanced Atg16L1 mRNA expression in *Salmonella*-infected cells was diminished in VDR-silenced cells (Figure 3B), but not in control siRNA-silenced cells. Therefore, specific suppression by siRNA targeting VDR attenuated the enhanced effect of 1,25D3 on Atg16L1 mRNA expression in *Salmonella*-infected Caco-2 cells.

1,25D3 up-regulated membranous Atg16L1 protein expression in Salmonella-infected Caco-2 cells, depending on VDR

A previous study demonstrated that 1,25D3 enhanced NOD2 mRNA and membranous protein expression in IECs[16]. Likewise, we proceeded to investigate the effect of 1,25D3 on Atg16L1 protein expression in

IECs. Western blot of Atg16L1 proteins expression was analyzed in membranous extract of *Salmonella*-infected Caco-2 cells in the presence or absence of 1,25D3. As shown in Figure 4, 1,25D3 enhanced the membranous Atg16L1 protein expression in *Salmonella*-infected Caco-2 cells compared to *Salmonella* infection only. Following knockdown of VDR, the 1,25D3-enhanced Atg16L1 membranous protein expression in *Salmonella*-infected cells was diminished in VDR-silenced cells (Figure 4), but not in control siRNA-silenced cells. Therefore, specific suppression by siRNA targeting VDR attenuated the enhanced effect of 1,25D3 on Atg16L1 membranous protein expression in *Salmonella*-infected Caco-2 cells.

Involvement of NOD2 and Atg16L1 in 1,25D3-mediated enhancement of autophagy expression in Salmonella-infected Caco-2 cells

Based on the above observation that 1,25D3 enhanced NOD2 and Atg16L1 mRNA expressions in *Salmonella*-infected Caco-2 cells and the interaction of NOD2 and Arg16L1 in *Salmonella*-induced autophagy in IECs[17], we investigated the involvement of NOD2 or Atg16L1 in 1,25D3-enhanced autophagy in *Salmonella*-infected IECs. NOD2 siRNA- or Atg16L1 siRNA-transfected Caco-2 cells were untreated or pretreated with 1,25D3 and then infected with wild-type *Salmonella* strain SL1344 for 1 h. Knockdown of NOD2 or Atg16L1 with specific siRNA in Caco-2 cells was confirmed by Western blot in a previous report[21]. The conversion of LC3-I to LC3-II was detected by Western blot analysis (Figure 2A and B). The Caco-2 cells were fixed, permeabilized, immunostained with antibody to endogenous LC3 and visualized by fluorescence microscopy. Immunofluorescence study (Figure 2C) showed numerous LC3 punctae (red) in *Salmonella*-infected cells. Pre-treatment of the cells with 1,25D3 significantly increased the LC3II protein as above, while NOD2 siRNA- or Atg16L1-transfected Caco-2 cells significantly diminished 1,25D3-enhanced conversion of LC3 (Figure 2A and B) to a level below that detected in the SL1344 infection only group. The 1,25D3-enhanced percentage of cells showing accumulation of LC3 punctae was significantly diminished by NOD2 or Atg16L1 siRNA (Figure 2C and D). Our results suggest that NOD2 and Atg16L1 become involved in the effect of 1,25D3 on autophagy expression in *Salmonella*-infected Caco-2 cells.

1,25D3 up-regulated VDR mRNA and protein expressions in Salmonella-infected Caco-2 cells

In order to determine if 1,25D3 up-regulates VDR mRNA and protein expressions in *Salmonella*-infected Caco-2 cells, cultured cells were left untreated or stimulated with 1,25D3 for 6 h, followed by wild-type *S. typhimurium* strain SL1344 infection. Immunoblots were performed on whole cell lysates with antibody to detect VDR protein and real-time quantitative PCR to
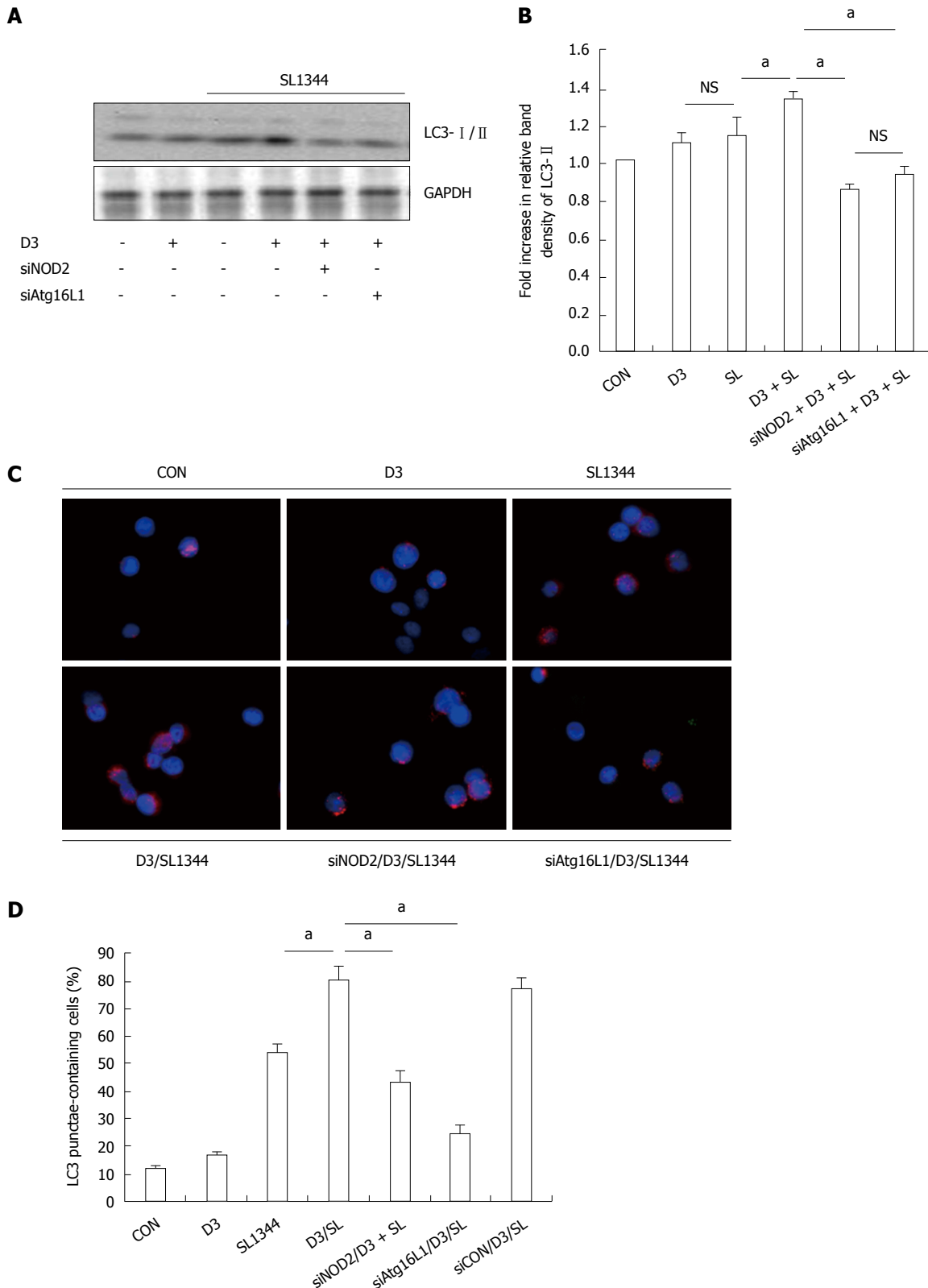
Figure 2 Involvement of NOD2 and Atg16L1 in 1,25D3-enhanced autophagy expression in *Salmonella*-infected Caco-2 cells. Caco-2 cells were transfected with control siRNA, NOD2 siRNA or Atg16L1 siRNA (siCON: non-target control siRNA; siNOD2: siRNA to NOD2; siAtg16L1: siRNA to Atg16L1) for 48 h. The transfected cells were left uninfected (CON) or infected by wild-type *S. typhimurium* strain SL1344 (SL) in the absence or presence of 1,25D3 (D3). Knockdown of NOD2[21] and Atg16L1[18] was confirmed previously by Western blot. The transfected cells were pretreated with 1,25D3 prior to wild-type *S. typhimurium* strain SL1344 infection for 1 h. Immunoblotting was performed on whole cell lysates with antibody to detect autophagy LC3-I/II protein expression. The results shown are representative of three separate experiments. GAPDH was detected for normalization of cytosolic protein. Representative immunoblots (A) and densitometric quantification of immunoreactive bands (B) are shown. The relative band intensities of LC3-II in Caco-2 cells are quantified as fold-increases compared with the control cells. The cells were fixed, permeabilized and stained with anti-LC3 (red), and LC3 puncta formation was detected by a confocal microscope; representative images of LC3 punctae are depicted. Scale bar = 10 μm (C); The percentage of cells showing accumulation of LC3 punctae is reported (D). Data are mean ± SEM of three independent experiments. $^aP < 0.05$.

A

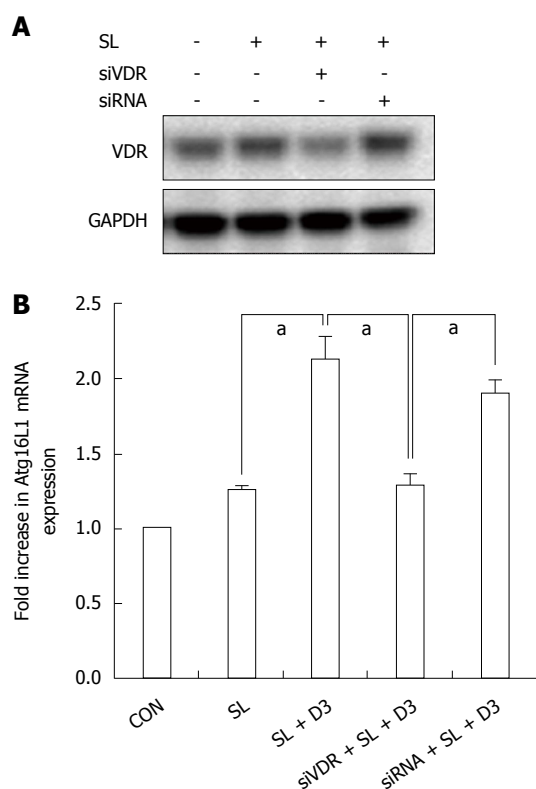| SL | - | + | + | + |
| siVDR | - | - | + | - |
| siRNA | - | - | - | + |

VDR

GAPDH

B

Figure 3 Effect of 1,25D3 on Atg16L1 mRNA expressions in *Salmonella*-infected Caco-2 cells and involvement of vitamin D receptor in the effect. Caco-2 cells were transfected with control siRNA and vitamin D receptor (VDR) siRNA (siRNA: non-target control siRNA; siVDR: siRNA to VDR) for 48 h. The transfected cells were left uninfected (CON) or infected by wild-type *S. typhimurium* strain SL1344 in the absence or presence of 1,25D3. Western blots probed with antibodies against VDR and GAPDH confirmed knockdown of VDR (A). Total RNA was prepared after infection and analyzed by real-time quantitative PCR to estimate amounts of Atg16L1 transcript. The amount of Atg16L1 mRNA produced and normalized to the corresponding amount of GAPDH transcript is shown as the fold-increase over uninfected control cells (B). Results are presented as mean ± SEM for at least three determinations from independent experiments. [a]$P < 0.05$.

detect mRNA expression. As shown in Figure 5, VDR mRNA and protein were up-regulated in *Salmonella*-infected cells and even higher in 1,25D3-treated cells.

Involvement of VDR in 1,25D3-enhanced autophagy expression in Salmonella-infected Caco-2 cells

To investigate the role of VDR in 1,25D3-enhanced autophagy expression in *Salmonella*-infected Caco-2 cells, VDR siRNA-transfected Caco-2 cells were untreated or pretreated by 1,25D3 and then infected with wild-type *Salmonella* strain SL1344 for 1 h. The conversion of LC3-I to LC3-II was detected by Western blot analysis and LC3^{+} autophagosome was analyzed by immunofluorescence. 1,25D3 enhanced *Salmonella*-induced LC3-II protein and LC3^{+} autophagosome expressions in Caco-2 cells, while VDR siRNA-transfected Caco-2 cells significantly diminished the enhancement of 1,25D3 on the conversion of LC3 (Figure 6A and B) or the percentage of LC3 punctae (Figure 6C and D). Our results suggest that VDR is involved in the effect of 1,25D3 on autophagy
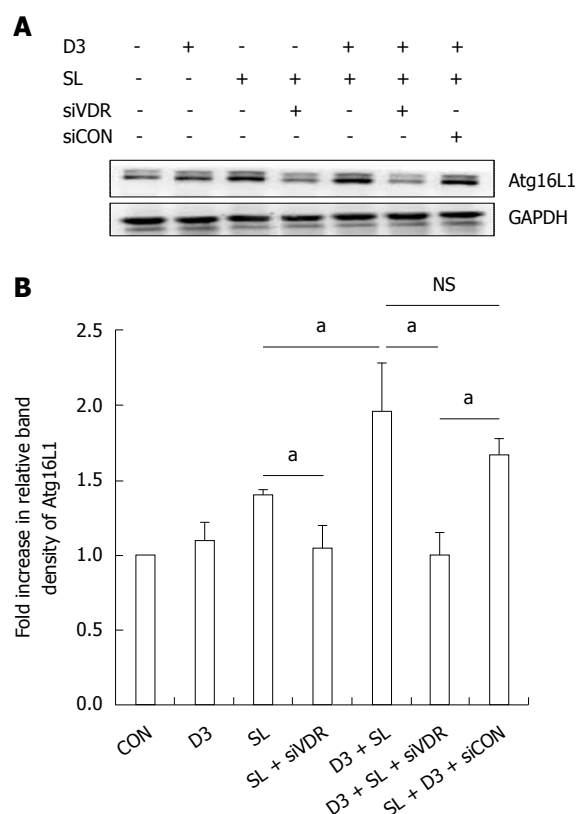
A

D3	-	+	-	-	+	+	+
SL	-	-	+	+	+	+	+
siVDR	-	-	-	+	-	+	-
siCON	-	-	-	-	-	-	+

Atg16L1

GAPDH

B

Figure 4 Effect of 1,25D3 on the membranous Atg16L1 protein expression in *Salmonella*-infected Caco-2 cells *via* vitamin D receptor. Caco-2 cells were transfected with control siRNA and vitamin D receptor (VDR) siRNA (siCON: non-target control siRNA; siVDR: siRNA to VDR) for 48 h. The transfected cells were left uninfected (CON) or infected by wild-type *S. typhimurium* strain SL1344 (SL) in the absence or presence of 1,25D3 (D3). Immunoblots were performed on membrane lysates with antibody to detect Atg16L1 protein expression, and E-cadherin for normalization of membrane proteins. Representative immunoblots (A) and densitometric quantification of immunoreactive bands (B) are shown. The relative band intensities of Atg16L1 in Caco-2 cells were quantified as fold-increases compared with the control cells. Each value represents the mean ± SEM of three independent experiments. [a]$P < 0.05$.

expression in *Salmonella*-infected Caco-2 cells.

Involvement of Atg16L1 in 1,25D3-mediated suppression of Salmonella-induced IL-1β mRNA expression in Caco-2 cells

In order to determine the effect of 1,25D3 on IL-1β mRNA expression in *Salmonella*-infected Caco-2 cells and the involvement of Atg16L1, Atg16L1 siRNA-transfected Caco-2 cells were untreated or pretreated by 1,25D3 and then infected with wild-type *Salmonella* strain SL1344 for 1 h. Knockdown of Atg16L1 with specific siRNA in Caco-2 cells was confirmed previously. Total RNA was analyzed by real-time quantitative PCR for IL-1β mRNA expression. As shown in Figure 7, *Salmonella*-induced IL-1β mRNA expression in Caco-2 cells was down-regulated by 1,25D3. Following knockdown of Atg16L1, the 1,25D3-mediated suppression of IL-1β mRNA expression in *Salmonella*-infected cells was diminished in Atg16L1-silenced cells, but not in control siRNA-silenced cells (data not shown). Therefore, specific suppression by
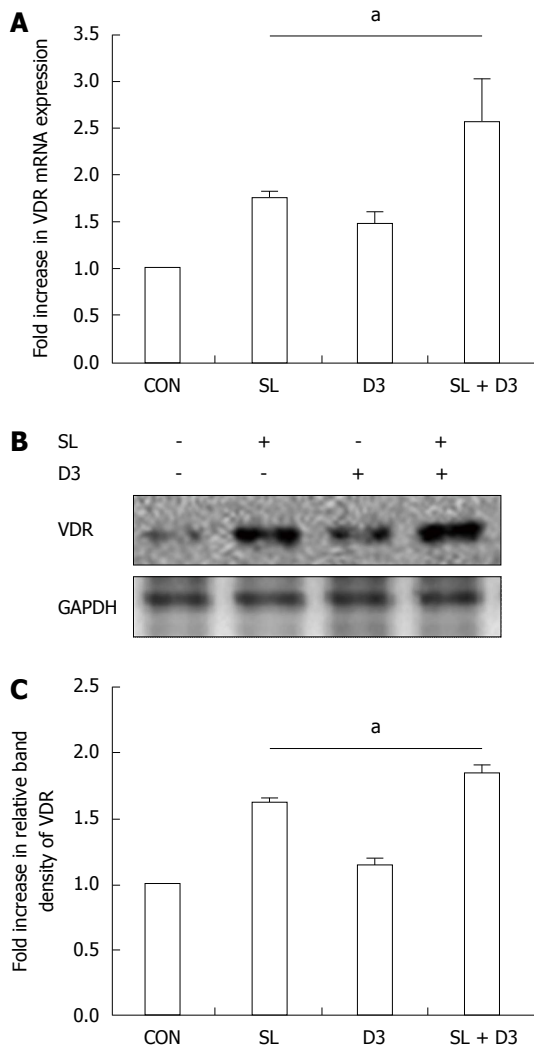
Figure 5 1,25D3 up-regulates the vitamin D receptor protein or mRNA expression in *Salmonella*-infected Caco-2 cells. Caco-2 cells were uninfected (CON) or infected by wild-type *S. typhimurium* strain SL1344 (SL) at indicated times, in the presence or absence of 1,25D3 (D3). Total RNA was prepared after infection and analyzed by real-time quantitative PCR to estimate amounts of vitamin D receptor (VDR) transcript (A). The amount of VDR mRNA produced, normalized to the corresponding amount of GAPDH transcript, is shown as the fold-increase over uninfected control cells. Results are represented as mean ± SEM for at least three determinations from independent experiments. Immunoblotting was performed on whole cell lysates with an antibody to detect VDR protein expression, or GAPDH for normalization of proteins. Representative immunoblots (B) and densitometric quantification of immunoreactive bands (C) are shown; The relative band intensities of VDR (C) in Caco-2 cells were quantified as fold-increases compared with the control cells. Each value represents the mean ± SEM of three independent experiments. [a]$P < 0.05$.

siRNA targeting Atg16L1 attenuated the suppressive effect of 1,25D3 on *Salmonella*-induced IL-1β mRNA expression in Caco-2 cells.

DISCUSSION

Vitamin D is an important mediator of intestinal epithelial defenses against infectious agents. Vitamin D deficiency predisposes to more severe intestinal injury in an infectious model of colitis[22]. However, the

mechanism is unknown. Based on previous reports that 1,25D3 signaling induces NOD2 expression in human IECs[16] and activated NOD2 recruits ATG16L1 that control autophagy[23] to kill *Salmonella*[17], we investigated the effect of vitamin D on autophagy expression in IECs after *Salmonella* infection and the involvement of NOD2 and Atg16L1. We observed that 1,25D3 increased the Atg16L1 mRNA (Figure 3) and membranous protein expression (Figure 4) in *Salmonella*-infected Caco-2 cells and the enhancement of 1,25D3 on autophagy expression in *Salmonella*-infected Caco-2 cells (Figure 1) is NOD2- and Atg16L1-dependent (Figure 2). It suggests vitamin D enhances autophagy in *Salmonella*-infected IECs, depending on enhanced NOD2 and Atg16L1 expression. Atg16L1 is required for autophagy in IECs and protection of mice from *Salmonella* infection[15]. Additionally, the association of autophagy-related Atg16L1 gene polymorphism with sepsis severity in patients with sepsis and ventilator-associated pneumonia[24] provides the rationale to investigate the role of Atg16L1 gene polymorphism in patients with septic complication in *Salmonella* colitis and potential therapy of active vitamin D on invasive microorganisms.

ATG16L1 is a VDR target gene and VDR regulates autophagic activity through ATG16L1[13]. Intestinal epithelial VDR deletion leads to defective autophagy in a colitis model[14]. Lack of intestinal epithelial VDR down-regulates expression of Atg16L1 and the resulting microbial dysbiosis may sensitize the colonic mucosa to chemical-induced colitis. Low levels of intestinal epithelial VDR correlated with reduced ATG16L1 and dysbiotic microbial ecology dominated by intestinal *Bacteroides* known to contribute to the pathogenesis of inflammatory bowel disease (IBD). An increased risk of IBD following enteric infections with *Salmonella* was reported[25]. The abnormalities in the handling of intracellular bacteria through autophagy might play a role in Crohn's disease pathogenesis[17,26-29]. This indicates the fundamental relationship between VDR, autophagy, and gut microbial assemblage that is essential for maintaining intestinal homeostasis, but also in contributing to the pathophysiology of IBD. 1,25D3 up-regulates the VDR through stabilization of the receptor in rat intestinal epithelial cells (IEC-6)[30] or the activation of gene expression in cells other than IECs[31]. Pretreatment with 1,25D3 can increase the VDR protein expression in Caco-2 cells[32]. Colonic epithelial VDR levels are markedly reduced in patients with inflammatory bowel diseases or in experimental colitis models, whereas vitamin D analog therapy that ameliorates colitis up-regulates epithelial VDR[33].

Thus, active vitamin D may enhance VDR expression to maintain intestinal homeostasis, induce autophagic defense against *Salmonella* infection and prevent IBD attack. We demonstrated that 1,25D3 enhances VDR mRNA and protein expression (Figure 5), which is involved in 1,25D3-enhanced Atg16L1 mRNA (Figure 3)
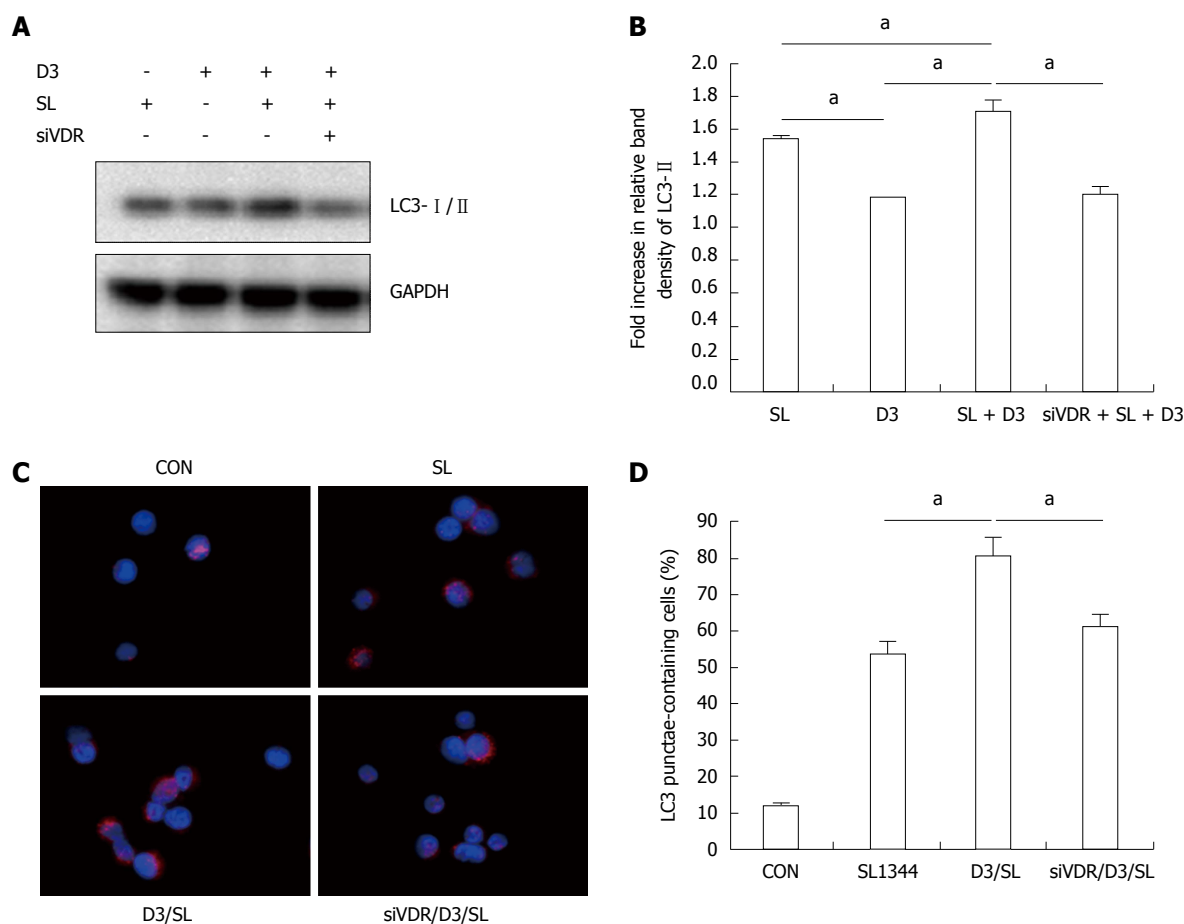
Figure 6 Involvement of vitamin D receptor in the enhanced effect of 1,25D3 on autophagy expression in *Salmonella*-infected Caco-2 cells. Caco-2 cells were transfected with control siRNA and vitamin D receptor (VDR) siRNA (siVDR: siRNA to VDR) for 48 h. The transfected cells were left uninfected (CON) or infected by wild-type *S. typhimurium* strain SL1344 (SL) in the absence or presence of 1,25D3 (D3). Immunoblots were performed on whole cell lysates with antibody to detect autophagy LC3- I / II protein expression. The results shown are representative of three separate experiments. GAPDH was detected for normalization of cytosolic protein. Representative immunoblots (A) and densitometric quantification of immunoreactive bands are shown. The relative band intensities of LC3- II in Caco-2 cells are quantified as fold-increases compared with the control cells (B). The cells were fixed, permeabilized and stained with anti-LC3 (red), and LC3 puncta formation was detected by a confocal microscope. Representative images of LC3 punctae are depicted (C). Scale bar = 10 μm. The percentage of cells showing accumulation of LC3 punctae is reported (D). Each value represents the mean ± SEM of three independent experiments. $^aP < 0.05$.

and protein expression (Figure 4) in *Salmonella*-infected Caco-2 cells, subsequently resulting in enhanced Atg16L1-mediated autophagy (Figure 2). Collectively, our study suggests that 1,25D3 may have applicability for infectious diseases and autoimmune diseases through its actions on ATG16L1 and VDR.

Mice lacking VDR are much more susceptible to dextran sodium sulfate (DSS)-induced mucosal injury, leading to extensive ulceration and early death[34]. Chemical-induced colitis in the VDR knockout mice was accompanied by high colonic expression of TNF-α, IL-1α and IL-1β[35]. VDR knockout mice were hyper-responsive to exogenously injected lipopolysaccharide, and cultures of the peritoneal exudates of moribund DSS-treated VDR knockout mice were positive for bacterial growth. This suggests that VDR may suppress overwhelming inflammation and maintain intestinal epithelial integrity to prevent bacterial invasion during colitis. Treatment of IL-10 knockout mice with 1,25D3 resulted in the suppression of IBD symptoms[36]. 1,25D3 in the diet or delivered rectally decreased

the severity and extent of inflammation in wild-type mice[35]. Furthermore, human studies documented some benefits of supplemental vitamin D in maintaining remission of Crohn's disease[37]. However, the mechanism is not clear. Vitamin D hormone not only can induce VDR expression[38] but also can suppress TNF-α production[39]. Here, we observed that the enhancement of 1,25D3 on Atg16L1 expression depends on VDR (Figures 3 and 4) while 1,25D3 suppressed Atg16L1-mediated IL-1β expression (Figure 7). Thus, gut epithelial VDR signaling enhanced by active vitamin D could be a useful therapeutic target in the management of IBD and appears to play an essential role in controlling mucosal inflammation.

Sorbara *et al*[40] showed that Atg16L1 suppresses inflammatory cytokines induced by NOD2 signaling in an autophagy-independent manner. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production[41]. Atg16L1 polymorphisms exhibit an excessive production of IL-1β in human peripheral mononuclear cells[42]. Thus, in theory,
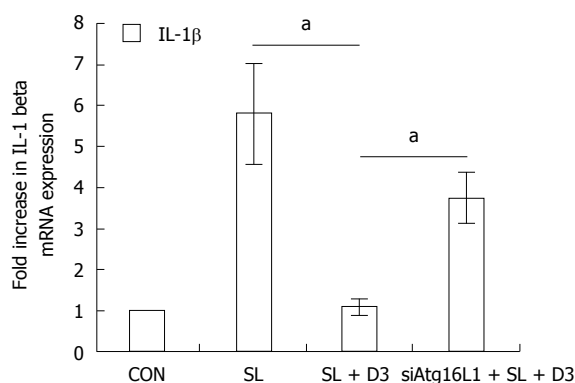
Figure 7 Involvement of Atg16L1 in suppressed *Salmonella*-induced IL-1β mRNA expression in Caco-2 cell by 1,25D3. Caco-2 cells were transfected with control siRNA or Atg16L1 siRNA (siAtg16L1: siRNA to Atg16L1) for 48 h. Transfected Caco-2 cells were pretreated with 1,25D3 (D3) prior to wild-type *S. typhimurium* strain SL1344 (SL) infection for 1 h. Total RNA was prepared after infection and analyzed by real-time quantitative PCR to estimate amounts of IL-1β transcript. The amount of IL-1β mRNA produced, normalized to the corresponding amount of GAPDH transcript, is shown as the fold-increase over uninfected control cells (CON). Results are represented as mean ± SEM for at least three determinations from independent experiments. [a]$P < 0.05$.

vitamin D therapy can shift the balance to favor inhibition of inflammation. Here, we showed that 1,25D3 suppresses *Salmonella*-induced IL-1β mRNA expression depending on Atg16L1 (Figure 7). IL-1β is one of the key mediators of intestinal inflammation in IBD, with a role in amplifying mucosal inflammation[43], consistent with the finding that IL-1β is up-regulated in IBD patients[44] and animal models[45]. The IL-1β-induced increase in intestinal epithelial tight junction permeability has been postulated to be an important pathogenic mechanism contributing to intestinal inflammation. This could be a mechanism by which vitamin D therapy ameliorates IBD.

Limitations

Almost all of the current data on how vitamin D can influence innate immune function has stemmed from studies of human cells. A limited number of animal models have been utilized with varying results, and future studies will need to improve this lack significantly. Finally, intestinal VDR expression was lower in patients with active Crohn's disease despite having adequate serum levels of vitamin D[46], suggesting that epithelial responses to vitamin D may be diminished during active IBD and this may not be altered by increasing serum vitamin D alone. Thus, more clinical trials are needed to determine how vitamin D affects infection *in vivo*, and whether or what dose of active vitamin D administered orally or rectally can act as a therapy to ameliorate *Salmonella* colitis or IBD.

Conclusively, the study provides a link between vitamin D3-induced autophagy and anti-inflammation, both of which target the intracellular pathogen for eradication and prevention of overwhelming inflammation. The ability of the active form of vitamin D, 1,25D3 to induce autophagy has provided a new approach to treat invasive microbial infections in humans. Vitamin D therapy can shift the balance to favor inhibition of inflammation and enhancement of IEC autophagy against *Salmonella* infection, leading to the identification of novel drug candidates to treat *Salmonella* infection and prevent IBD. Perhaps, in the future, we might be able to treat or prevent certain infectious diseases with safe and inexpensive substances that induce expression of endogenous autophagy.

COMMENTS

Background

Salmonella spp. remain a major public health problem for the entire world. Intestinal epithelial cells (IECs) play an essential role in mucosal innate immunity of the host and autophagy has been highlighted as an innate immune defense against intracellular pathogens. Accumulated studies have suggested a critical role for vitamin D in mucosal innate immunity. NOD2 and Atg16L1, both vitamin D target genes, are required for autophagy in IECs. Therefore, we investigated the effects of active vitamin D3 on autophagy expression in *Salmonella*-infected IECs.

Research frontiers

Previous study has demonstrated that 1,25-dihydroxyvitamin D3 (1,25D3) up-regulates NOD2 mRNA expressions in *Salmonella*-infected IECs and that the interaction of NOD2 and Arg16L1 plays a critical role in *Salmonella*-induced autophagy in IECs. However, the effect of vitamin D on Atg16L1-mediated autophagy and inflammation in *Salmonella*-infected IECs is not clear.

Innovations and breakthroughs

This is the first study to demonstrate the different effects of vitamin D on autophagy and IL-1β expression in *Salmonella*-infected IECs *via* Atg16L1 and VDR.

Applications

This study not only provides the mechanistic pathway for the effect of active vitamin D on *Salmonella* colitis but a rationale for its alternative therapy for invasive bacterial infection and other inflammatory disorders.

Terminology

Autophagy is a unique process of membrane trafficking in which the membrane compartment (autophagosomes) engulfs both organelles and cytosolic macromolecules and delivers them to the lysosome for degradation. It is a well-characterized process that protects host cells from nutrient deprivation and other cellular stresses.

Peer-review

The authors demonstrated that active vitamin D enhances autophagy but suppresses inflammatory IL-1β expression in *Salmonella*-infected Caco-2. These results are interesting and novel. Previous studies have established the relationship between vitamin D, vitamin D receptor (VDR), NOD2 and Atg16L1. However, the effect of vitamin D on autophagy and inflammation in *Salmonella*-infected IECs *via* Atg16L1 and VDR has not been elucidated.

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