ORIGINAL RESEARCH ARTICLE—BASIC SCIENCE

Lactic Acid Bacteria Isolated From Korean Kimchi Activate the Vitamin D Receptor–autophagy Signaling Pathways

Rong Lu, MD, PHD,*,a Mei Shang, MD, PHD,*,a Yong-Guo Zhang, PHD,* Yang Jiao, PHD,* Yinglin Xia, PHD,*
Shari Garrett, MS,* Danika Bakke, PHD,* Christine Bäuerl, PHD,† Gaspar Perez Martinez, PHD,†
Cheol-Hyun Kim, PHD,‡ Sang-Moo Kang, PHD,§ and Jun Sun, PHD*,¶

Background: Probiotic lactic acid bacteria (LAB) have been used in the anti-inflammation and anti-infection process of various diseases, including inflammatory bowel disease (IBD). Vitamin D receptor (*VDR*) plays an essential role in pathogenesis of IBD and infectious diseases. Previous studies have demonstrated that the human *VDR* gene is a key host factor to shape gut microbiome. Furthermore, intestinal epithelial *VDR* conditional knockout (VDR$^{\Delta IEC}$) leads to dysbiosis. Low expressions of *VDR* is associated with impaired autophagy, accompanied by a reduction of *ATG16L1* and LC3B. The purpose of this study is to investigate probiotic effects and mechanism in modulating the *VDR*-autophagy pathways.

Methods: Five LAB strains were isolated from Korean kimchi. Conditional medium (CM) from these strains was used to treat a human cell line HCT116 or intestinal organoids to measure the expression of *VDR* and autophagy. Mouse embryonic fibroblast (MEF) cells with or without *VDR* were used to investigate the dependence on the *VDR* signaling. To test the role of LAB in anti-inflammation, *VDR*$^{+/+}$ organoids were treated with 121-CM before infection with *Salmonella enterica* serovar Enteritidis. In vivo, the role of LAB in regulating *VDR*-autophagy signaling was examined using LAB 121-CM orally administered to VDR^{Loxp} and VDR$^{\Delta IEC}$ mice.

Results: The LAB-CM-treated groups showed higher mRNA expression of *VDR* and its target genes cathelicidin compared with the control group. LAB treatment also enhanced expressions of Beclin-1 and *ATG16L1* and changed the ratio of LC3B I and II, indicating the activation of autophagic responses. Furthermore, 121-CM treatment before *Salmonella enterica* serovar Enteritidis infection dramatically increased *VDR* and *ATG16L1* and inhibited the inflammation. Administration of 121-CM to VDR^{Loxp} and VDR$^{\Delta IEC}$ mice for 12 and 24 hours resulted in an increase of *VDR* and LC3B II:I ratio. Furthermore, we identified that probiotic proteins P40 and P75 in the LAB-CM contributed to the anti-inflammatory function by increasing *VDR*.

Conclusions: Probiotic LAB exert anti-inflammation activity and induces autophagy. These effects depend on the *VDR* expression. Our data highlight the beneficial effects of these 5 LAB strains isolated from food in anti-infection and anti-inflammation.

Key Words: autophagy, lactic acid bacteria, inflammation, organoids, probiotics, salmonella enteritidis, vitamin D, vitamin D receptor

INTRODUCTION

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[1] It is known that probiotics and their fermented food products are beneficial for health.[2] Researchers have reported success with in the probiotic treatment of inflammatory bowel disease (IBD).[3, 4] However, the clinical outcomes were not consistent, and the mechanisms of probiotics are still not well understood.[5–8] In IBD, for example, the intestinal microbiota composition showed a decreased *Clostridium* IXa and IV groups and *Bifidobacterium* and an increase in members of a detrimental microbiota like *Escherichia coli* and sulfate-reducing bacteria. The beneficial effects attributed to specific probiotic strains in this case are related to the stabilization of the intestinal microbiota, which is a very difficult and subjective parameter to evaluate—or measure.[9]

Vitamin D receptor (*VDR*) is a nuclear receptor that mediates most functions of vitamin D.[10] Vitamin D deficiency has been implicated in patients with IBD.[11–15] It also plays a protective role in infection and inflammation.[16] Vitamin D

receptor is identified as an IBD risky gene.[17–21] Because *VDR* exerts cell differentiation, growth, anti-inflammatory actions, and microbiome in the intestine, there is extensive therapeutic exploitation of *VDR* ligands for the treatment of inflammatory conditions. Dysfunction of vitamin/*VDR* signaling is reported in patients with chronic inflammation.[22, 23] Hence, restoration of the function of *VDR* to control inflammation and infection is desirable.

The purpose of this study is to investigate probiotic effects and mechanisms in modulating the *VDR*-autophagy pathways. Five lactic acid bacteria (LAB) strains (*Lactobacillus paracasei* DKL121, *L. casei* DK128,[24] *L. plantarum* DKL109 and DKL119,[24] and *L. acidophilus* DKLLa5) were isolated from Korean kimchi.[24] Our previous study reported that the heat-killed DK128 had protective effects against the influenza virus.[25] However, it is not clear whether these LAB strains have the protective roles in intestinal inflammation and bacterial infection. In the current study, we hypothesized that conditional medium from LAB could enhance the *VDR* expression, thus enhancing the protective functions against inflammation and infection. We generated conditional medium (CM) from different LAB strains for the in vitro and in vivo studies because we hoped to identify the universal beneficial factor(s) in the LAB strains. We used the CM in the current study to avoid potential variation in bacterial cultures. Our data indicate that LAB induces autophagy and exerts anti-inflammation and anti-infection activities. These effects depend on the *VDR* expression.

MATERIALS AND METHODS

Animals

The *VDR*^{+/-} and *VDR*^{−/−} mice on a C57BL6 background were obtained by breeding heterozygous *VDR*^{+/−} mice.[26] The VDR[LoxP] mice were originally reported by Dr. Geert Carmeliet.[26] The VDR[ΔIEC] mice were obtained by crossing the VDR[LoxP] mice with villin-cre mice (Jackson Laboratory, 004586), as we previously reported.[27] Experiments were performed on mice that were 2 to 3 months old, including male and female. Mice were provided with water ad libitum and maintained in a 12-hour dark/light cycle. The animal work was approved by the UIC Office of Animal Care. Euthanasia method was sodium pentobarbital (100 mg per kg body weight) intraperitoneal injection (I.P.) followed by cervical dislocation. All experiments were carried out in accordance with the approved guidelines.

Lactic Acid Bacteria Culture

Five LAB strains (*Lactobacillus paracasei* DKL121, *L. casei* DK128,[24] *L. plantarum* DKL109 and DKL119[24], and *L. acidophilus* DKLLa5) were isolated from homemade or commercial Korean kimchi from different regions in Korea, as reported in a previous study.[24] Bacteria were cultured in

Lactobacillus MRS broth (Difco, MD, USA) at 37°C overnight. As the bacterial density reached 10⁹ U/mL, all the conditional medium[25] were collected and filtered using 0.22µm filters. The supernatants were stored at −80°C for indicated studeis.

Cell Culture

Human epithelial HCT116 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (Penicillin, 100 IU/mL, Streptomycin, 100µg/mL), and L-glutamine (4.5 g/L), as previously described.[28, 29] Mouse embryonic fibroblasts (MEFs) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 5% FBS (vol/vol) and streptomycin-penicillin.[27, 30]

Salmonella Enteritidis Infection

Salmonella enterica serovar Enteritidis wild-type (SE-WT) was used in the process of infection, as we described in a previous study.[31] Nonagitated microaerophilic bacteria were recovered in Luria-Bertani (LB) broth (1:100, vol/vol) after overnight incubation (about 16~18 h) at 37°C according to the previous study. The bacterial culture suspensions were centrifuged at 5000 g for 10 minutes. Hank's balanced salt solution (HBSS) with 10 mM HEPES (pH 7.4) was used to resuspend the bacterial cultures. Then the resuspension of SE-WT (0.75 x 10⁷ CFU) was added in the cell culture plate of HCT116, MEFs, or organoid. Half an hour later, the bacteria were removed and washed 3 times by HBSS. After that, cells were incubated with routine medium-containing gentamicin (500 µg/mL) for 1 hour to control the extracellular bacteria.

Protection of 121 Conditional Medium on *Salmonella* Invasion in Cultured Organoids

Organoids from small intestines of *VDR*^{+/+} and *VDR*^{-/-} mice were prepared and maintained as previously described.[16, 32, 33] Mini gut medium (advanced DMEM/F12 supplemented with HEPES, L-glutamine, N2, and B27) was added to the culture, along with R-Spondin, Noggin, and epidermal growth factor (EGF). Organoid cells (6 days after passage) were incubated with or without 10% 121 conditional medium (121-CM) in mini gut media for 2 hours and washed with HBSS. After 121-CM pretreatment, organoids were infected with SE-WT for 30 minutes, washed with HBSS, and incubated in mini gut media containing gentamicin (500 mg/mL) for 1 hour. After extensive HBSS washing, the samples were collected for Western blots.

Mouse Colonic Epithelial Cells

Mouse colonic epithelial cells were collected by scraping the tissue from the colon of the mouse, including the proximal and distal regions.[28, 34] The cells were sonicated 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 ortho-vanadate,

and protease inhibitor cocktail). The total protein concentration in the samples was measured using the BioRad Protein Assay Dye Reagent (BioRad, Hercules, CA, USA).

Immunoblotting

Cultured cells were rinsed twice with ice-cold HBSS, lysed in protein-loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and then sonicated. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with primary antibodies. The membrane was incubated with the following primary antibodies: anti-VDR (Santa Cruz Biotechnology Inc., CA, USA), anti-Beclin-1, anti-LC3B, anti-*ATG16L1*, anti-tumor necrosis facto (TNF)-α (Cell Signal, Beverly, MA, USA), anti-P40, and anti-P75 (kindly provided by Prof. F. Yan),[35] anti-IκBα (Cell Signal, Beverly, MA, USA), and anti-β-actin (Sigma-Aldrich, Milwaukee, WI, USA) antibodies and visualized by ECL. Membranes that were probed with more than 1 antibody were stripped before reprobing.

Immunofluorescence

Cells were fixed with 100% ethanol for 30 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. Normal goat serum was performed to block the nonspecific antigen. The primary antibody against *ATG16L1* was added to incubate the cells overnight at 4°C. After incubating with Alexa Fluor 549 anti-Rabbit secondary antibody for 1 hour, coverslips were mounted by fluoromount-G (SouthernBiotech, AL, USA). Images were photographed using Zeiss laser scanning microscope LSM 710 (Carl Zeiss Inc., Oberkochen, Germany).

Real Time Quantitative Polymerase Chain Reaction

Total cellular RNA was obtained by TRIzol reagent (Thermo Fisher, MA, USA) according to the manufacturer's protocol. Vitamin D receptor and its target genes *CYP24*, cathelicidin, autophagic markers *ATG16L1*, and LC3B were measured by real-time polymerase chain reaction (PCR). APRIL is a protein of the TNF superfamily and measured by real-time PCR. The sequences of the primers were shown in Table 1. All the gene expression levels were normalized to GAPDH through the 2-ΔΔCt method.

Autophagy Activity Culture[36]

Autophagy activity was quantified using the commercial Cyto ID autophagy detection kit (ENZO Life Sciences, ENZ-51,031-K200) while following the manufacturer's protocol. This kit contains a 488 nm excitable green fluorescent detection reagent that becomes 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.).

TABLE 1. Real-time PCR Primers

Primers name	Sequence
β-actin F	5′-TGTTACCAACTGGGACGACA-3′
β-actin R	5′-CTGGGTCATCTTTTCACGGT-3′
VDR F	5′-GAATGTGCCTCGGATCTGTGG-3′
VDR R	5′-ATGCGGCAATCTCCATTGAAG-3′
Cathelicidin F	5′-GGCTGTGGCGGTCACTATC-3′
Cathelicidin R	5′-GTCTAGGGACTGCTGGTTGAA-3′
Cyp24 F	5′-GCTGATGACCGACGGTGAG-3′
Cyp24 R	5′-GTGCGGTACAGAGCTTCCAG-3′
LC3 F	5′-GACCGCTGTAAGGAGGTGC-3′
LC3 R	5′-CTTGACCAACTCGCTCATGTTA-3′
ATG16L1 F	5′-CAGAGCAGCTACTAAGCGACT-3′
ATG16L1 R	5′-AAAAGGGGAGATTCGGACAGA-3′
April F	5′-CTTTCGGTTGCTCTTTGGTTG-3′
April R	5′-CGACAGCACAAGTCACAGC-3′
IFNγ F	5-GCCACGGCACAGTCATTGA-3
IFNγ R	5-TGCTGATGGCCTGATTGTCTT-3
IL1β F	5-GAAATGCCACCTTTTGACAGTG-3
IL1β R	5-TGGATGCTCTCATCAGGACAG-3
IL8 F	5-TTTTGCCAAGGAGTGCTAAAGA-3
IL8 R	5-AACCCTCTGCACCCAGTTTTC-3

P40 and P75 Proteins

Cloning of the encoding genes in vector pQE80e over expression in *E. coli* BL21 and purification of the complete mature proteins P40 and P75 were carried out in as described before.[37] Briefly, specific primers were designed to amplify the target regions of *L. casei* BL23 chromosomal DNA and clone them in the BamHI and SmaI restriction sites of pQE80e that carried a RGS-His encoding region. They were then ligated and cloned in an intermediate *E. coli* DHB10 host, and after checking the sequences, they were subcloned in *E. coli* BL21(BE3)-[pLysS]. Nucleic acid manipulation and cloning procedures were carried out as previously described.[16] For protein purification, *E. coli* BL21 clones carrying the selected plasmids were grown in batches of 500 mL of LB at 37ºC 100 µg/mL ampicillin and 20 µg/mL chloramphenicol until OD600 reached 0.4, when 1 mM IPTG was added to induce expression. Then bacteria were collected by centrifugation, lysed by sonication, and centrifuged, and supernatants were loaded onto HisTrapTM FF Crude Column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in an ÅktaPrimeTM Plus chromatography system (GE Healthcare Bio-Sciences AB). His-tagged P40 and P75 were separated, eluted, and collected according to the instructions of the manufacturer. For the correct preservation of the proteins, they were subject to lyophilization. For this purpose, 0.5 mL aliquots of both proteins were prepared at 0.5 mg/mL in freeze drying buffer (50 mM

tris pH 8.0, 50 mM NaCl, 30 mM sucrose, and 0.01% tween 80) and dried in a Virtis Genesis freeze drier (SP Scientific, Stone Ridge, NY, USA).

To study the protective role of P40 and P75 proteins, the organoid from ileum tissue were pretreated with P40 or P75 (100 ng/mL) for 24 hours. Then the organoids were added with TNF-α 0.5ng/mL for 30 minutes. The expressions of *VDR* and IκBα were detected by Western blots.

Statistical Analysis

All data are expressed as the mean ± SD. All statistical tests were 2-sided. The *P* values <0.05 were considered statistically significant. Differences between 2 groups were analyzed using 2-sample Welch *t* test, 1-way ANOVA for more than 2 groups, and 2 time points, respectively. For multiple comparisons, either the Tukey method or the Dunnett test was used for adjusting the *P* values, depending on if the pairwise or treatment (follow-up time points) vs control (baseline) was compared. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

121 Conditional Medium Upregulates mRNA Levels of *VDR* and Autophagy Regulators in HCT116

To determine the effect of the conditional medium from *Lactobacillus paracasei* DKL121 (121-CM) on *VDR* expression in HCT116 cells, we firstly assessed whether 121-CM alters the transcriptional levels of *VDR* and its target gene cyp24, Cathelicidin in human HCT116 cells. As compared with the control group, 121-CM treatment increased *VDR* mRNA levels at different times (0.5 h, 1 h, 2 h, 3 h), which presented a time-dependent trend. The 121-CM upregulated the level of cathelicidin over all time points, and the level of cyp24 was decreased at 0.5 hour but increased at 2 hours (Fig. 1A). Meanwhile, the autophagy response was measured, which presented that the mRNA levels of LC3 and *ATG16L1* were elevated at 0.5 hour and 3 hours (Fig. 1B). These findings indicate that 121-CM might induce *VDR* upregulation in vitro. To further verify the action of 121-CM, the DNA inhibitor actinomycin was added to suppress mRNA levels of *VDR*. As shown in Figure 1C, 121-CM could reverse the effect of downregulating *VDR* and its target gene expression caused by actinomycin D treatment. These results confirmed that 121-CM promoted *VDR* at the RNA level and its target gene expression.

121 Conditional Medium Elevates Protein Levels of *VDR* and Autophagy Regulators in HCT116

To determine whether 121-CM affects the *VDR* and autophagic markers at the protein levels, we examined the expression of *VDR*, Beclin-1, and LC3B after 121-CM treatment by Western blots. As presented in Figure 2A and Figure 2B, the protein levels of *VDR* and Beclin-1 were increased in 121-CM-treated HCT116 cells at 0.5, 1 , 2, and 3 hours. The 121-CM treatment could downregulate the protein expression of LC3B at 0.5 and 1 hour, whereas it increased the LC3B protein level at 2 and 3 hours. At 3 hours, 121-CM increased *ATG16L1* and LC3 abundance in human HCT116 cells (Fig. 2C). These results demonstrated that autophagic response was activated during the process of 121-CM upregulating *VDR* expression.

121 Conditional Medium Administration Upregulates *VDR* and Autophagy in Ileum of VDR^{loxp/loxp} Mice

To determine the function of 121-CM-induced *VDR* expression in vivo, we measured the effect of 121-CM administration on *VDR* expression and the activation of autophagy in the ileum of VDR^loxp/loxp and VDR^ΔIEC mice. Compared with vehicle control (nontreated group), 121-CM upregulated the level of *VDR* and LC3B after administrating the 121-CM for 24 hours in VDR^ΔIEC mice. In VDR^loxp/loxp mice, *VDR* expression was increased at 24 hours. Meanwhile, it also upregulated the levels of LC3B at 12 hours, though decreased at 24 hours (Fig. 3). The data further indicated that 121-CM significantly promoted *VDR* and autophagy in vivo.

Four Other LAB Strains Have Similar Effects on *VDR* Expression

To detect the cellular function of 4 other LAB strains on *VDR* expression in HCT116 cells, we analyzed the mRNA expression of *VDR* in HCT116 at different times (0.5 hour, 1 hour, 2 hours and 3 hours) after incubation with the conditional medium from 4 other LAB strains (109-CM, 119-CM, 128-CM, and La5-CM). As shown in Figure 4A, *VDR* expression was significantly increased in LAB-CM-treated cells. Autophagy activity was also detected by CYTO-ID Autophagy detection kit for the autophagy marker LC3. We found that the autophagy activity was increased after treatment with the LAB strains (Fig. 4B).

LAB-CM-enhanced *VDR* and Beclin-1/LC3B Is Abolished in the *VDR*^{-/-} MEF Cells

To analyze whether LAB-CM enhanced Beclin-1 and LC3B depending *VDR*, we chose *VDR*^+/- MEF and *VDR*^-/- MEF cells to coculture with LAB-CM. The *VDR* expression was upregulated in 119-CM-treated, 121-CM-treated, and La5-CM-treated *VDR*^+/- MEFs, which was similar with the effect of positive control lactobacillus rhamnosus GG (LGG)-CM-treated group. It also showed that autophagy was modulated by LAB-CM. The expression of Beclin-1 was elevated after LAB-CM (109-CM, 119-CM, 121-CM, 128-CM, and La5-CM) stimulation in *VDR*^+/- MEFs. Elevation of LC3B II/I happened in 119-CM-treated, 121-CM-treated,
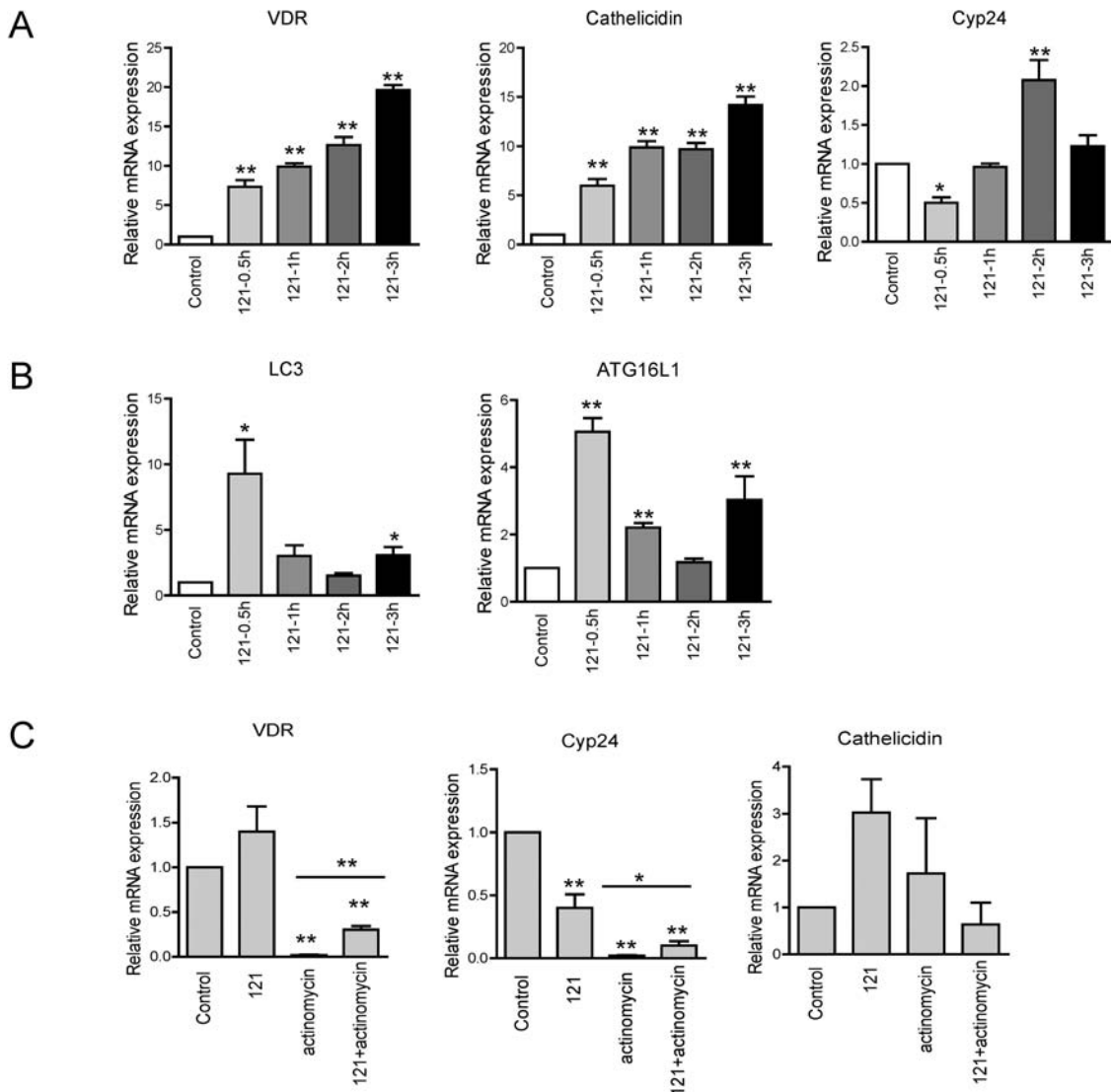
FIGURE 1. mRNA expression of *VDR* and autophagy marker in 121-CM-treated HCT116 compared with nontreated cells. A, mRNA levels of *VDR* and its target genes in 121-CM-treated HCT116 for 0.5 hour, 1 hour, 2 hours, and 3 hours. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control group. B, mRNA expression of LC3 and *ATG16L1* in HCT116 after 121-CM treatment for 0.5 hour, 1 hour, 2 hours, and 3 hours. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control group. C, Transcriptional level of *VDR* and its target genes in actinomycin (10 μg/mL)-treated HCT116 before coculturing with 121-CM for 3 hours. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with no treatment.

and LGG-CM-treated *VDR*^{+/-} MEFs (Fig. 5). Otherwise, *VDR* deletion in VDR^{-/-} MEF cells could abolish the effect of LAB-CM on *VDR* expression and autophagy response. These data illustrated that *VDR* was required in the action of LAB-CM for the promotion of *VDR* and activation of autophagy.

We further tested the effects of LAB on the *VDR* expression and activation of autophagy in macrophages (Supplemental Figs. 1 and 2). However, we did not observe the robust responses that we observed in the epithelial cells. Thus, we focused on the intestinal epithelial studies using organoids and intestinal tissues.

121 Conditional Medium Increased Expressions of *VDR* in *VDR*^{+/+} Organoids and Suppresses *Salmonella*-induced Inflammation

We have reported a *Salmonella* Typhimurium–infected organoid culture system suitable for studying host-bacterial interactions.[16] Here, we further used the *Salmonella enterica* serovar Enteritidis wild-type (SE-WT) strain in the infected organoids (Fig. 6A). The 121-CM postincubation apparently induced the expression of *VDR* and *ATG16L1* in *VDR*^{+/+} organoids after treating with 121-CM for 3 hours. This result
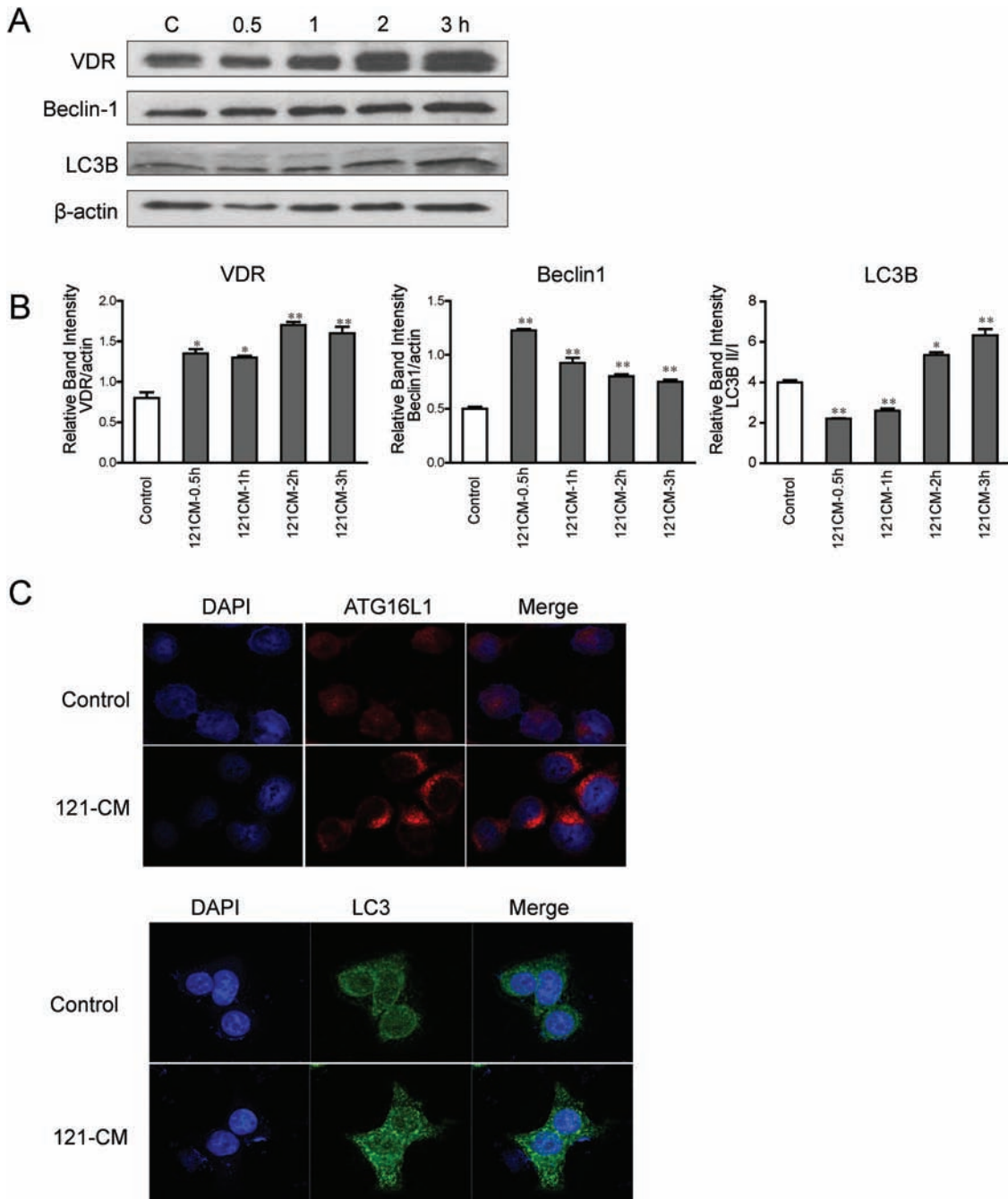
FIGURE 2. Protein expression of *VDR* and autophagy regulators in 121-CM-treated HCT116. A, Protein levels of *VDR*, LC3B and Beclin-1 in 121-CM-treated HCT116 for 0.5 hour, 1 hour, 2 hours, and 3 hours. B, The graph represents the quantification of *VDR*, LC3BII/I, and Beclin-1 protein levels. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with no treatment. C, Expression of *ATG16L1* and LC3 were increased after 121-CM treatment (3 hours) in the HCT116 cells.

was accordance with the previously described data, which further confirmed the function of 121-CM on promoting *VDR-ATG16L1* expression.

We then hypothesized that 121-CM protects the host from *Salmonella* infection. A *Salmonella*-infected model was used in an organoid isolated from the ileum of *VDR*^{+/+} mouse.

Before SE-WT infection, 121-CM was used to stimulate the organoids for 3 hours. We found that the levels of *VDR* and *ATG16L1* in SE-WT infection plus 121-CM-treated organoids were higher than that in the group infected with SE-WT. The SE-WT infection could obviously promote the expression of TNF-α. Interestingly, 121-CM downregulated the infection-induced TNF-α (Figs. 6B and 6C). The 121-CM
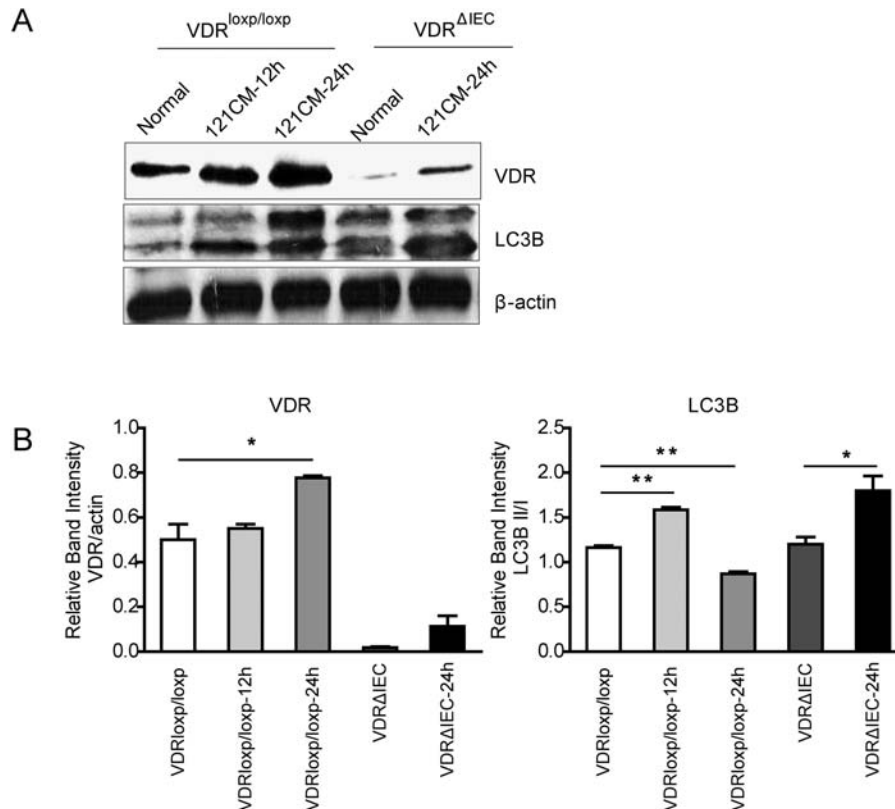
FIGURE 3. Expression of *VDR* and autophagy-related LC3B in ileum of VDR^loxp^ and VDR^ΔIEC^ mice orally administrated with 121-CM for 12 hours and 24 hours. A, Changes of *VDR* and LC3B expression after 121 administration for 12 hours and 24 hours. B, The graph represents the quantification of *VDR* and LC3B II:I ratio. Data are expressed as mean ± SD; n = 6, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with normal (No treatment).

treatment also reduced the expression levels of interleukin (IL)-1β and IFNγ (Fig. 6D). Thus, our data indicate that 121-CM not only increased *VDR* and *ATG16L1* but also inhibited the inflammatory response induced by *Salmonella* infection.

P40 and P75 of the LAB Strains Can Protect the Organoids from Inflammatory Response Induced by TNF-α

Probiotic LGG proteins P40 and P75 are known to be key functional proteins in inhibiting inflammation.[35, 38] According to their polyacrilamide gel electrophoresis (PAGE) migration, these 2 proteins have apparent sizes of 40 kDa and 75 kDa, and they were first isolated from LGG culture supernatants. The P40 protein has more potent effects on intestinal epithelial cells as compared with P75. The P40 protein activates epidermal growth factor receptor (EGFR), leading to Akt. It is not clear whether P40 or P75 directly regulate *VDR* expression. We have tested the expression levels of P40 and P75 in the conditional medium of probiotic strains. We have also purified proteins to treat the organoids and determine the change of *VDR*. Proteins P40 and

P75 were detected in the LAB strains (Fig. 7A). APRIL is a known cytokine secreted by intestinal epithelial cells to trigger IgA production. The P40 protein upregulated the mRNA level of APRIL (Fig. 7B). We also treated the organoids with P40 or P75 (100 ng/mL) for 24 hours. The expressions of *VDR* were increased by P40 or P75 treatment (Fig. 7C). Excitingly, P40 and P75 can protect the organoids from inflammatory response induced by TNF-α (Fig. 7D). The expression of IκBα was not reduced by TNF-α in organoids with P40 or P75 treatment.

DISCUSSION

In the current study, we demonstrate the protective role of LAB strains from Korean kimchi in inflammatory responses. We found a robust increase of *VDR* and autophagy signaling when cells treated with the conditional medium from these probiotic strains. The LAB-CM treatment induced higher *VDR* and its target genes cathelicidin compared with the control group. Lactic acid bacteria also enhanced autophagy responses in vitro and in vivo. The 121-CM treatment before *Salmonella* infection dramatically increased *VDR* and *ATG16L1* and inhibited the
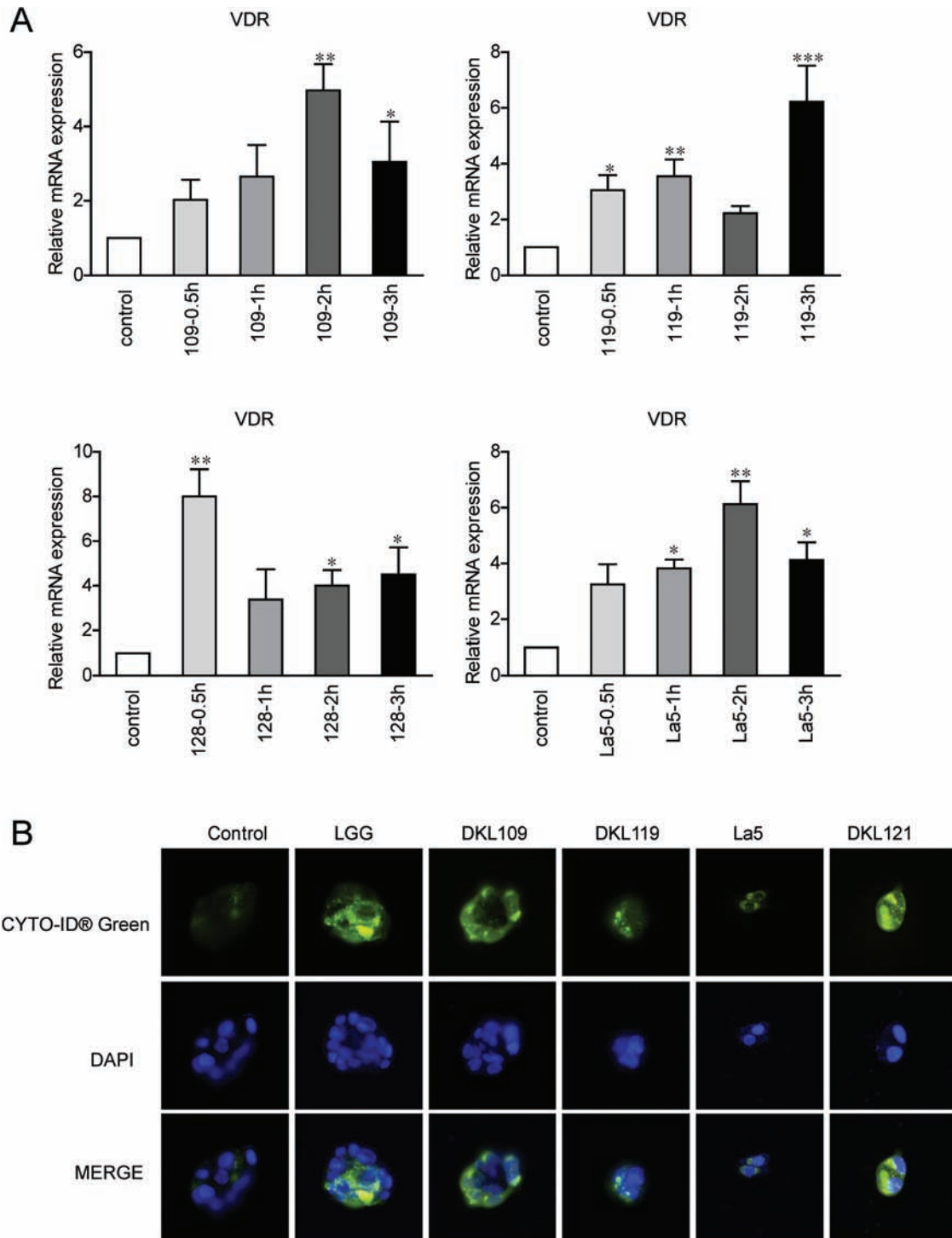
FIGURE 4. mRNA expression of *VDR* in HCT116 treated with 4 other LAB stains for different time. A, *VDR* expression in HCT116 treated with conditional medium of 4 other LAB stains: DKL109, DKL119, DKL128, and DKLLa-5. Data are expressed as mean ± SD; n = 6, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with normal (no treatment). B, Autophagy activity was increased after LAB strains treated. Autophagy activity was detected by CYTO-ID Autophagy detection kit.

inflammation. Furthermore, we identified that P40 and P75 in the LAB-CM contributed to the anti-inflammatory function by increasing *VDR* (Fig. 7E). Our study highlights the beneficial effects of probiotics from food in anti-infection and anti-inflammation, suggesting that they may have therapeutic and possibly preventive efficacy in IBD.
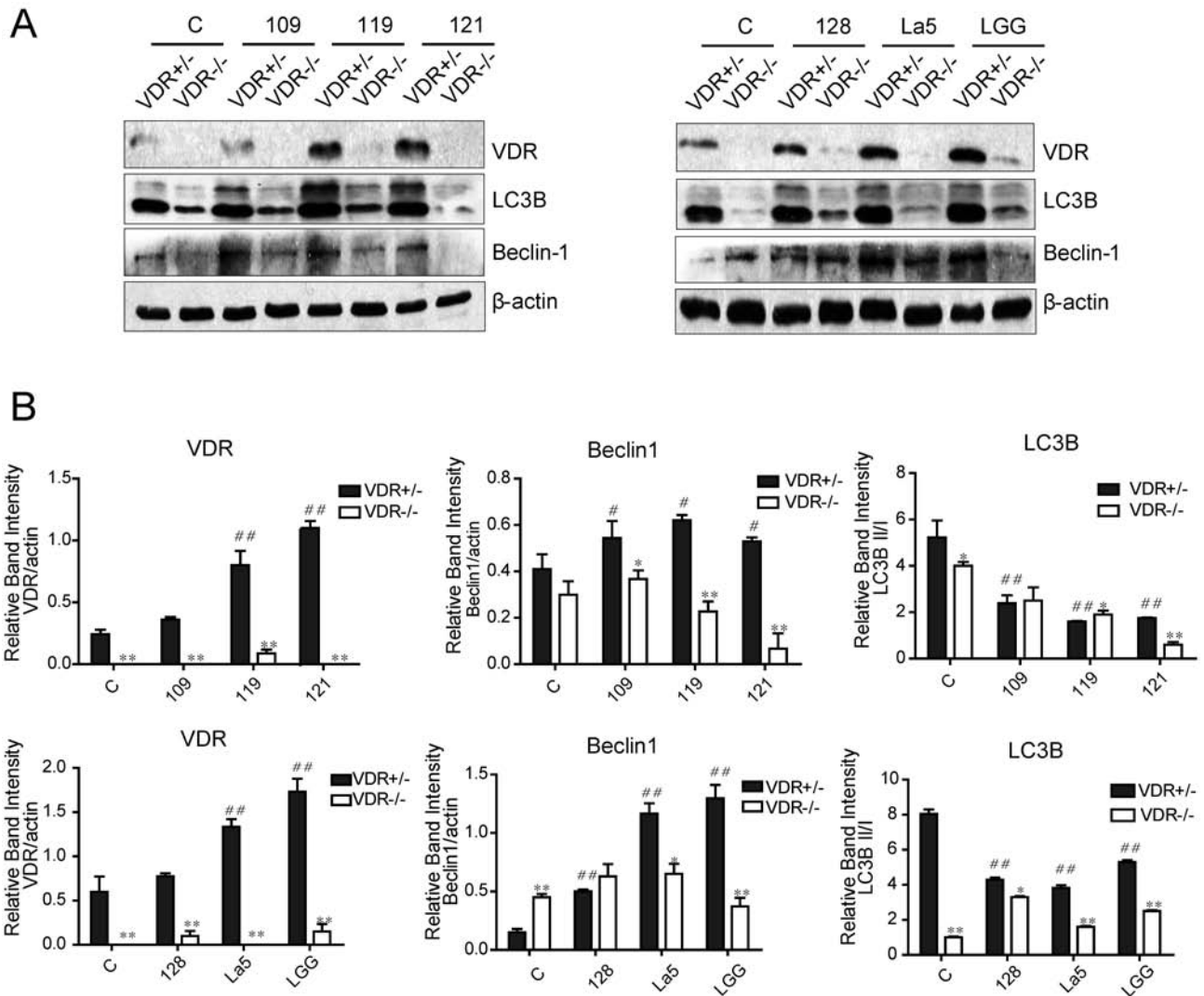
FIGURE 5. Effects of *VDR* deletion on 121-CM-induced increase of *VDR* and autophagy. A, *VDR*, LC3B, and Beclin-1 expression in 121-CM-treated *VDR*⁺ᐟ⁻ and *VDR*⁻ᐟ⁻ MEFs. B, The graph represents the quantification of *VDR*, Beclin-1, and LC3B II:I ratio. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, #$P < 0.05$, ##$P < 0.01$ compared with no treatment; Student t test, *$P < 0.05$, **$P < 0.01$.

Our study has shown that probiotic LGG treatment protected hosts from *Salmonella* Typhimurium infection.[39] Here, we found that probiotics also protect hosts from *Salmonella* Enteritidis–induced inflammation and infection. These protective effects depend on *VDR*. Vitamin D receptor regulates host response to invasive pathogens, commensal bacteria, and probiotics in innate and adaptive immunity.[40–48] Absence of *VDR* in the intestine leads to activation of NF-κB and higher risk of chronic inflammation.[49, 50] Vitamin D receptor deletion leads to more severe inflammation and bacterial invasion. Here, functionally, we find the LAB-CM treatment increased *VDR* and autophagy responses. Mechanistically, we identified that the soluble proteins P40 and P75 from LAB strains contribute to enhance *VDR* and inhibit inflammation. Thus, this study highlights an important mechanism for probiotics through the *VDR*/autophagy regulation.

Probiotics are not equal; they may use various strategies to interact with the host cells. They have specific features that can distinguish the mode of action of different species and even strains from the same species. In IBD patients, the responses to probiotic treatment and clinical outcomes are inconsistent.[5–8] What remains unknown is how probiotics specifically work on *VDR* signaling and effectively play the anti-inflammatory role. Our data indicate that the probiotic function of 5 LAB strains involves *VDR* signaling. A VSL3#, a mixture of 8 probiotics, resulted in upregulation of antagonists of NF-κB inflammatory pathways, including the *VDR* signature.[51] Specific probiotic strains exert specific effects in IBD therapy; however, the anti-inflammatory role of probiotics remains unclear.[52] Elucidating how probiotics specifically regulate signaling pathways, including *VDR*, will
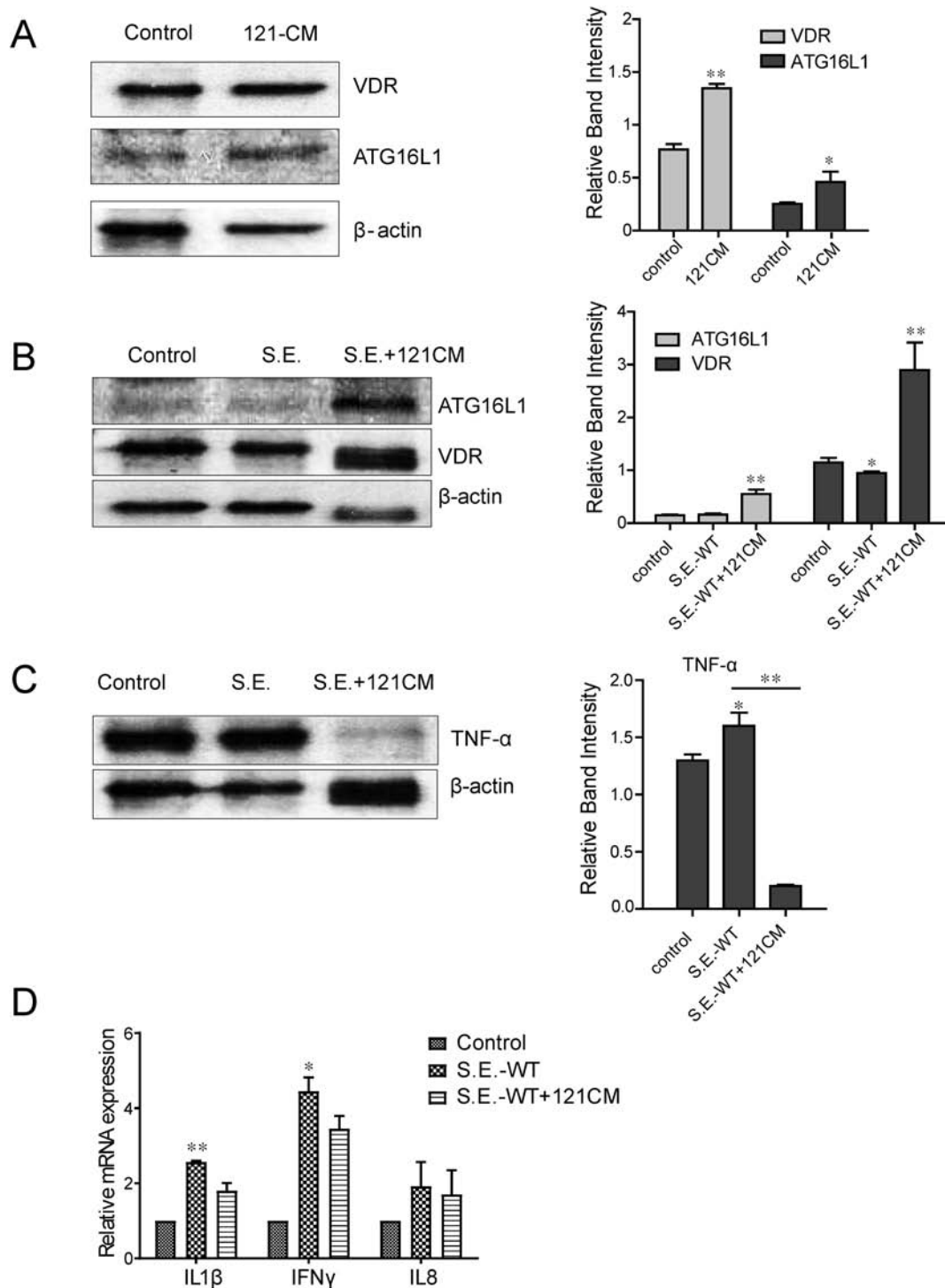
FIGURE 6. Protein level of *VDR* and *ATG16L1* expression in the 121-CM-treated organoids. A, *VDR* and *ATG16L1* expression is determined by Western blots after treatment with 121-CM for 3 hours. Data are expressed as mean ± SD; n = 3, Student *t* test, *$P < 0.05$, **$P < 0.01$ compared with control group. B, Expression of VDR, *ATG16L1*, and inflammation molecules in organoids infected with *Samonella enterica* serovar Enteritidis before 121-CM treatment. *VDR* and *ATG16L1* expression in (0.5 hour) organoids infected with *S*. Enteritidis before 121-CM treatment for 3 hours. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with control (no treatment). C, TNF-α protein levels in organoids after treatment with 121-CM plus *S*. Enteritidis Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with control (no treatment). D, mRNA levels of IL1β, IFN γ, and IL8 gene expression in the organoids after treatment with 121-CM plus *S*. Enteritidis Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with 121 CM treatment.
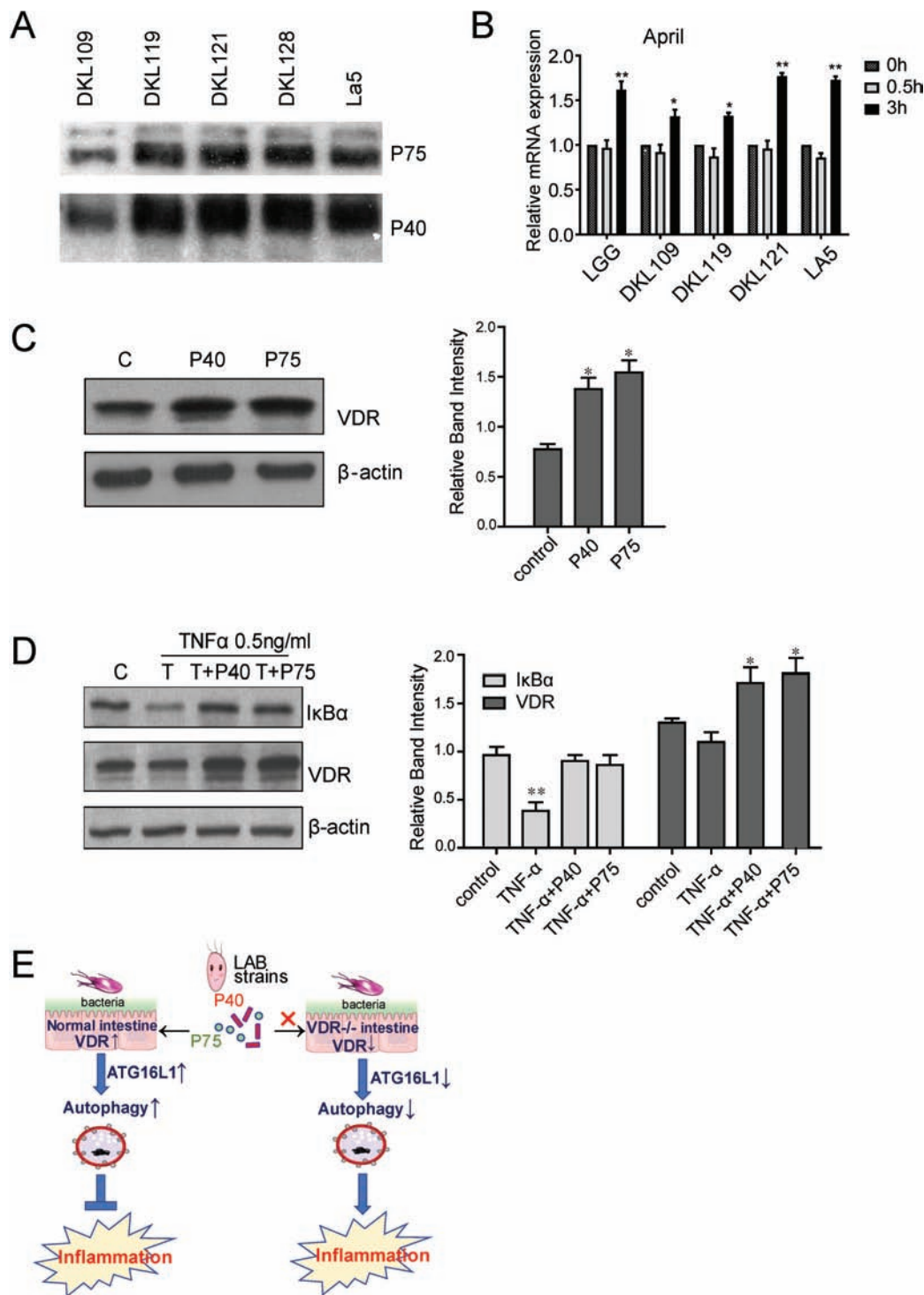
FIGURE 7. The P40 and P75 of the LAB strains increase *VDR* and protect the organoids from inflammatory response induced by TNF-α. A, P40 and P75 proteins can be detected in the LAB strains. B, LAB increased the expression of APRIL. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with 0h group. C, The expression of VDR in organoids were upregulated after P40 or P75 treatment (100ng/mL for 24 hours). Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$ compared with control group. D, Organoid from ileum tissue WAS treated with P40 or P75 (100 ng/mL) for 24 hours, Then added with TNF-α 0.5 ng/mL for 30 minutes. Proteins P40 and P75 upregulated the expression of *VDR*. The expression of IκBα was decreased after TNF-α treatment but not in the groups with P40 or P75 treatment. Data are expressed as mean ± SD; n = 3, 1-way ANOVA test, *$P < 0.05$, **$P < 0.01$ compared with control group. E, A working model of probiotic upregulation of *VDR* through P40 and P75 to stimulate autophagic responses and inhibit inflammation.

advance our understanding of bacterial-host interaction in inflammation.

Ours and others' studies support the critical role of intestinal *VDR* in maintaining intestinal homeostasis, with its dysregulation possibly contributing to human IBD.[27, 53] Based on our study here, changes in the expression of the *VDR* can be a sensitive measurable parameter to evaluate clinical outcomes of probiotics health effects.

It is important to point out ingestion of probiotics has many forms, such as in foodstuff (eg, cheese, yogurt, fermented milk, fruit juice, or chewing gum) or as a constituent of tablets and capsules. Probiotic activity depends very much on a daily and continued ingestion. It should be part of an individual's diet and, as such, either incorporated in fermented or supplemented food products. Moreover, some food ingredients might protect probiotic strains against the stressful digestive conditions, increase their activity, or act as gene promoters and should be tested to optimize probiotic effects. Our study found functional proteins P40 and P75 in both probiotic strains and culture media. The functional components of probiotics are present in both culture media of LAB and ingested kimchi. The beneficial role of the lactic acid bacteria isolated from Korean kimchi provides positive evidence of ingestion of probiotics from food. It was reported that probiotics isolated from kimchi can be used in yogurt manufacturing as a starter culture.[24] These probiotic strains could be mixed into dairy products to benefit diets.

In conclusion, we tested 5 LAB strains from Korean kimchi in anti-infection and inflammatory responses. Using conditional VDR^ΔIEC mice, organoids, and cultured human intestinal epithelial cells, we performed a series of molecular and biochemical experiments in vivo and in vitro to investigate probiotic regulation of *VDR* and autophagy signaling in infection and inflammation. We investigated the mechanisms on how *VDR* and autophagy responses are related in probiotic-treated cells. We highlight the complex role of *VDR* in bacterial infection and chronic inflammation.[54] Our findings reveal a novel role of LAB from nutriment factors in food in regulating *VDR* and autophagy responses in inflammation and infectious diseases. This knowledge can be expanded to define novel strategies to prevent and treat various human diseases, including IBD and infectious diseases.

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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