Inflammatory Bowel disease

Lactic acid bacteria isolated from Korean kimchi modulate the VDR-autophagy signaling pathways

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Abstract:

Background: Probiotics Lactic acid bacteria 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. Our previous study has demonstrated that human *vdr* gene is a key host factor to shape gut microbiome. Further, our study has showed that 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 (*Lactobacillus paracasei* DKL128, DK119, DKLLa5, DKL109 and DKL121) were isolated from Korean Kimchi. Conditional medium was used to treat a human cell line HCT116 and measured the expression of VDR and autophagy. Mouse embryonic fibroblast (MEF) cells with or without VDR were also used to test the change of the VDR-autophagy signaling. To test the role of LAB in anti-inflammation, $VDR^{+/+}$ organoids were treated with 121-CM prior to infection with *Salmonella* Enteritidis. *In vivo*, the role of LAB in regulating VDR autophagy signaling was tested, using 121-CM orally administrating to VDR^{Loxp} and $VDR^{\Delta IEC}$ mice. **Results**: LAB-CM treated groups showed higher mRNA expression of VDR and its target genes cathelicidin, compared to the control group. LAB treatment also enhanced Beclin-1, ATG16L1 expression and changed the ratio of LC3B I and II, indicating the activation of autophagic responses. Furthermore, 121CM treatment prior to *Salmonella* infection dramatically increased VDR and ATG16L1 and inhibited the inflammation. Administration of 121CM 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 P40 and P75 in the LAB CM contributed to the anti-inflammatory function by increasing VDR. **Conclusions:** probiotic LAB exert the anti-inflammation activity and induces autophagy. These effects depend on

the VDR expression. Our data highlight the beneficial effects of these five LAB strains isolated from food in anti-infection and anti-inflammation, suggesting that they may have therapeutic and possibly preventive efficacy in IBD.

Key Words: Autophagy, Lactic acid bacteria Inflammation, intestine, 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]. Probiotics and their fermented food products are beneficial for health [2]. Researchers have reported success with in the treatment of some human diseases. However, the clinic outcomes were not consistent and the mechanisms of probiotics are still not well-understood [3-6]. 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 is related to the stabilization of the intestinal microbiota, which is a very difficult and subjective parameter to evaluate, and specially, to measure [7].

Vitamin D receptor (VDR) is a nuclear receptor that mediates most functions of vitamin D[8]. Vitamin D deficiency has been implicated in patients with IBD.[9-13] It also plays a protective role in infection and inflammation.[14] *Vdr* is identified as an IBD risky gene.[15-19]. 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. Hence, restoration of the function of VDR to control inflammation and infection is desirable.

Five LAB strains (*Lactobacillus paracasei* DKL128, DK119, DKLLa5, DKL109 and DKL121) were isolated from Korean Kimchi [20]. Our previous study has reported that evidence that heat-killed DK128 had protective effects against influenza virus [21]. However, it is not clear whether these LAB strains have protective roles in intestinal inflammation and infection.

In the current study, we hypothesized that conditional medium [19] from LAB could enhance the VDR expression, thus enhancing the protective functions again inflammation and infection. We used a human cell line (HCT116), mouse embryonic fibroblast (MEF) cells with or without VDR, ad also organoinds in vitro to test the changes of the VDR-autophagy signaling. To test the role of LAB in anti-infection, $VDR^{+/+}$ organoids were treated with 121-CM prior to infection with *Salmonella* Enteritidis. *In vivo*, the role of LAB in regulating VDR autophagy signaling was tested, using 121-CM orally administrating to VDR^{Loxp} and $VDR^{\Delta IEC}$ mice. Our study highlighted the beneficial effects of these five LAB strains from a beneficial food *in vitro* and *in vivo*, suggesting that they may have therapeutic and possibly preventive efficacy in colitis. Mechanistically, we had evidence to support the anti-inflammation activity of these LAB strains is through P40 and P75, depending on the VDR expression.

Materials and Methods

Animals $VDR^{+/-}$ and $VDR^{-/-}$ mice on a C57BL6 background were obtained by breeding heterozygous $VDR^{+/-}$ mice.[22] VDR^{LoxP} mice were originally reported by Dr. Geert Carmeliet.[22] $VDR^{\Delta IEC}$ mice were obtained by crossing the VDR^{LoxP} mice with villin-cre mice (Jackson Laboratory, 004586), as we previously reported[23]. Experiments were performed on 2–3 months old mice including male and female. Mice were provided with water ad libitum and maintained in a 12 h 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) 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* DKL128, DK119, DKLLa5, DKL109 and DKL121) were isolated from Korean Kimchi [20] and cultured in Lactobacillus MRS broth (Difco, MD) at 37℃ overnight. As the bacterial density reached to 10^9 U/ml. All the conditional medium [21] were collected and filtered using 0.22μm filters. The supernatants were stored at -80℃for use.

Culture of mouse small intestinal organoids

Organoids from small intestine of $VDR^{+/-}$and $VDR^{-/-}$ mice were prepared and maintained as we previously described.[24-26] 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 EGF. Each condition was examined in triplicate with multiple (>10) organoids in each sample. At day 7 after passage, organoids were colonized by *Salmonella* for 30 min, washed, and incubated for 1 hour in Mini gut medium with Gentamicin (500 μg/ml).

Cell culture Human epithelial HCT116 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (Penicillin, 100 I.U./ml/Streptomycin, 100μg/ml), and L-glutamine (4.5g/L), as previously described.[27, 28] Mouse embryonic fibroblasts (MEFs) were cultured in high glucose DMEM with 5% FBS (vol/vol) and streptomycin-penicillin [23, 29].

***Salmonella* Enteritidis infection**

Salmonella Enteritidis wild-type (*S.*E-*WT*) was used in the process of infection, as we descripted in a previous study [30]. Nonagitated microaerophilic bacteria were recovered in LB broth (1:100, vol/vol). After overnight incubation (about 16~18h) at 37℃according to the previous study. The bacterial culture suspensions were centrifuged at 5,000 g for 10min. Hank's balanced salt solution (HBSS) with 10 mM HEPES (pH 7.4) was used to resuspend the bacterial cultures. Then the resuspension of *S.*E-*WT* (0.75×10^7 CFU) was added in the cell culture plate of HCT116, MEFs or organoid. Half an hour later, the bacteria were removed and washed for three times by HBSS. After that, cells were incubated with routine medium containing gentamicin (500 μg/ml) for 1h to control the extracellular bacteria.

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.[27, 31] 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 protein concentration was measured using the BioRad 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. Mouse colonic epithelial cells were collected by scraping the tissue from the colon of the mouse, including the proximal and distal regions. [27, 31] The cells were sonicated in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 8.0, 1% Triton X-100) with 0.2 mM sodium ortho-vanadate, and protease inhibitor cocktail. The protein concentration was measured using the BioRad Reagent (BioRad, Hercules, CA, USA). 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), anti-Beclin-1, anti-LC3B, anti-ATG16L1, anti-TNF-α (Cell Signal, Beverly, MA), anti-P40 and anti-P75 (kindly provided by Prof. F. Yan) (Yan et al., 2007) , anti-p-IκBα, anti-IκBα (Cell Signal, Beverly, MA, USA), anti-Villin, and anti-VDR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-β-actin (Sigma-Aldrich, Milwaukee, WI, USA) antibodies and were visualized by ECL. Membranes that were probed with more than one antibody were stripped before re-probing.

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

Real Time quantitative PCR

Total cellular RNA was obtained by TRIzol reagent (Thermo Fisher, USA) according to the manufacturer's protocol. VDR and its target genes CYP24, Cathelicidin, autophagic markers ATG16L1 and LC3B were 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 2-ΔΔCt method.

Autophagy activity culture[32]

Autophagy activity were 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., Oberkochen, Germany).

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 was carried out in as described before (Bäuerl et al 2019). Briefly, specific primers were designed to amplify the target regions of L. casei BL23 chromosomal DNA and to 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 following standard laboratory manuals (Sambrook and Russell, 2001). 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 1mM IPTG was added to induce expression. Then bacteria were collected by centrifugation, lysed by sonication, 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, Uppsala, Sweden). 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 (50mM tris pH 8.0, 50mM NaCl, 30mM sucrose y 0.01% tween 80) and dried in a Virtis Genesis freeze drier (SP Scientific).

Statistical Analysis

All of the data are expressed as the mean ± SD. All statistical tests were 2-sided. The p values <0.05 were considered statistically significant. Differences between samples were analyzed using Student's t-test for 2 groups, one-way ANOVA for more than 2 groups with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA USA), respectively. Multiple comparisons of mean body weight were performed using two-way ANOVA. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

121-CM 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 (0.5h, 1h, 2h, 3h), which presented a time-dependent trend. 121-CM upregulated the level of cathelicidin and the level of cyp24 was decreased at 0.5h, but increased at 2h (**Fig. 1A**). Meanwhile, the autophagy response was measured, which presented that the mRNA levels of LC3 and ATG16L1 were elevated at 0.5h and 3h (**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 **Fig. 1C**, 121-CM could reverse the effect of downregulating VDR and its target genes expression caused by actinomycin D treatment. Above results confirmed that 121-CM promoted VDR at the RNA level and its target gene expression.

121-CM elevates protein levels of VDR and autophagy regulators in HCT116

To determine whether 121-CM affect 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 **Fig. 2A** and **Fig. 2B**, the protein levels of VDR and Beclin1 were increased in 121-CM treated HCT116 cells at 0.5, 1h, 2h and 3h. 121-CM treatment could downregulate the protein expression of LC3B at 0.5h and 1h, while it increased the LC3B protein level at 2h and 3h. At 3h, 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-CM 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 $VDR^{\Delta IEC}$ mice. Compared to vehicle control (non-treated group), 121-CM upregulated the level of VDR and LC3B after administrating the 121-CM for 24h in $VDR^{\Delta IEC}$ mice. In $VDR^{loxp/loxp}$ mice, VDR expression was increased at 24h. Meanwhile, it also upregulated the levels of LC3B at 12h, while decreased at 24h **(Fig. 3**). The data further indicated that 121-CM significantly promoted VDR and autophagy *in vivo*.

Four other LAB strains have the similar effects on VDR expression

To detect the cellular function of four other LAB strains on VDR expression in HCT116 cells, we analyzed the mRNA expression of VDR in HCT116 at different time (0.5h, 1h, 2h and 3h) after incubation with the conditional medium from four other LAB strains (109-CM, 119-CM, 128-CM and La5-CM). As shown in **Fig. 4A**, VDR expression was significantly increased in LAB-CM treated cells. Autophagy activity were also detected by CYTO-ID® Autophagy detection kit. 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 Beclin1 and LC3B depending VDR, we chose $VDR^{+/+}$ MEF and $VDR^{-/-}$ MEF cells to coculture with LAB-CM. VDR expression was upregulated in 119-CM, 121-CM, and La5-CM treated $VDR^{+/+}$ MEFs, which was similar with the effect of positive control LGG-CM treated group. It also showed that autophagy was modulated by LAB-CM. The expression of Beclin1 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 was happened in 119-CM, 121-CM 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 on the promotion of VDR and activation of autophagy.

121-CM increased expressions of VDR in VDR$^{+/+}$ organoids and suppresses *Salmonella* (*S.E.*) induced inflammation

We have reported a *Salmonella* Typhimurium infected organoid culture system suitable for studying host–bacterial interactions.[33] Here, we further used the *Salmonella* Enteritidis strain in the infected organoids (**Fig. 6A**). 121-CM post incubation apparently induced the expression of VDR and ATG16L1 in VDR$^{+/+}$ organoids after treating with 121-CM for 3h. This result was accordance in the above data, which further confirmed the function of 121-CM on promoting VDR-ATG16L1 expression.

We further hypothesized that 121-CM can protect the host from *Salmonella* infection. A *Salmonella*-colitis model was used in organoid isolated from the ileum of VDR$^{+/+}$ mouse. Prior to S.E.-WT infection, 121-CM was used to stimulate the organoids for 3h. We found that the levels of VDR and ATG16L1 in S.E.-WT infection plus 121-CM treated organoids were higher than that in S.E.-WT infected group. S.E.-WT infection could obviously promote the expression of TNF-α. Interestingly, 121-CM downregulated the infection-induced TNF-α (Fig. 6B and 6C, remove TLR4 data and add stat data for Fig. 6A). 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 protein P40 and P75 are known to be key functional proteins in inhibiting inflammation [34, 35]. According to their PAGE migration, these two proteins have apparent sizes of 40 kDa and 75 kDa and they were first isolated from LGG culture supernatants. P40 has more potent effects on intestinal epithelial cells, as compared to P75. P40 protein

activates 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 also have purified proteins to treat the organoids and determine the change of VDR. Protein P40 and P75 proteins were detected in the LAB strains (**Fig. 7A**). APRIL is a known cytokine secreted by intestinal epithelial cells to trigger IgA production. P40 upregulated the mRNA level of April (**Fig. 7B**). We also treated the organoids with P40 or P75 (100ng/ml) for 24h. The expressions of VDR was 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 were 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. LAB-CM -treated groups showed higher VDR and its target genes cathelicidin, compared to the control group. LAB also enhanced autophagy responses in vitro an in vivo. Furthermore, 121CM treatment prior to *Salmonella* infection dramatically increased VDR and ATG16L1 and inhibited the inflammation. Furthermore, we identified that P40 and P75 in the LAB CM contributed to the anti-inflammatory function by increasing VDR. 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.

A previous study has shown that Probiotic LGG treatment protected host from *Salmonella* Typhimurium infection. Here, we found that probiotics also protect host from *Salmonella* Enteritidis-induced inflammation and infection. These protective effects depend on VDR.

VDR regulates host response to invasive pathogens, commensal bacteria, and probiotics in innate and adaptive immunity.[36-44] Absence of VDR in intestine leads to activation of NF-κB and higher risk of chronic inflammation.[45, 46] VDR deletion led to more severe inflammation and bacterial invasion. Here, we find the LAB CM treatment increased VDR and autophagy responses. Mechanistically, we identified the soluble protein P40 and P75 from LAB strains contribute to enhance VDR and inhibit inflammation. Thus, this study highlights an important mechanism for probiotics through VDR/autophagy regulation.

Probiotic bacteria have specific and particular features that can distinguish the mode of action of different species and even strains from the same species. They may use various strategies to interact with the host cells. In IBD patients, the responses to probiotics treatment and clinical outcomes are inconsistent [3-6]**.** 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 five 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 [47]. Specific probiotic strains exert specific effects in IBD therapy, however, the anti-inflammatory role of probiotics remains unclear [48]. Elucidating how probiotics specifically regulate signaling pathways, including VDR, will advance our understanding of bacterial-host interaction in inflammation.

Our and other's studies support the critical role of intestinal VDR in maintaining intestinal homeostasis with its dysregulation possibly contributing to human IBD[23, 49]. 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.

The beneficial role of the Lactic acid bacteria isolated from Korean kimchi provide positive evidence of ingestion of probiotics from food. It is important to point out, ingestion of probiotics has many forms, such as in foodstuff (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, incorporated in fermented or, just 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 in order to optimize probiotic effects.

In conclusion, we have tested five LAB strains from Korean Kimchi in anti- infection and inflammatory responses**.** Using conditional $VDR^{\Delta IEC}$ mice, organoids, and cultured human intestinal epithelial cells, we perform 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.[50] Our findings reveal a novel role of LAB from nutriment factors in the food in regulating VDR and autophagy responses in inflammation and infectious diseases. This knowledge can be exploited to define novel strategies to prevent and treat various human diseases, including IBD and infectious diseases.

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Author contributions

RL, MS, YZ, and YJ: acquisition, analysis and interpretation of data, and drafting of the manuscript; YX: statistical analysis and drafting of the manuscript: JS: study concept and design; obtained funding; study supervision; analysis and interpretation of data; and writing the manuscript; CK and SK: critical materials and intelligent contribution to probiotic strains. All authors read the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1: Real-time PCR Primers

Primers name	Sequence
mβ-actinF	5′-TGTTACCAACTGGGACGACA-3′
mβ-actinR	5′-CTGGGTCATCTTTTCACGGT-3′
mVDRF	5′-GAATGTGCCTCGGATCTGTGG-3′
mVDRR	5′-ATGCGGCAATCTCCATTGAAG-3′
mCathelicidinF	5′-GGCTGTGGCGGTCACTATC-3′
mCathelicidinR	5′-GTCTAGGGACTGCTGGTTGAA-3′
mCyp24F	5′-GCTGATGACCGACGGTGAG-3′
m Cyp24R	5′-GTGCGGTACAGAGCTTCCAG-3′
mLC3F	5′-GACCGCTGTAAGGAGGTGC-3′
m LC3R	5′-CTTGACCAACTCGCTCATGTTA-3′
mATG16L1F	5′-CAGAGCAGCTACTAAGCGACT-3′
mATG16L1R	5′-AAAAGGGGAGATTCGGACAGA-3′
mAprilF	5′-CTTTCGGTTGCTCTTTGGTTG-3′
mAprilR	5′-CGACAGCACAAGTCACAGC-3′

Figure Legends

Fig 1. mRNA expression of VDR and autophagy marker in 121-CM treated HCT116 compared with non-treated cells.

(A) mRNA levels of VDR and its target genes in 121-CM treated HCT116 for 0.5h,1h,2h,3h. Data are expressed as mean ± SEM. n = 3, one-way ANOVA test, *P < 0.05, **P < 0.01; (B) mRNA expression of LC3 and ATG16L1 in HCT116 after 121-CM treatment for 0.5h,1h,2h,3h. Data are expressed as mean ± SEM. n = 3, one-way ANOVA test, *P < 0.05, **P < 0.01; (C) Transcriptional level of VDR and its target genes in actinomycin (10μg/ml) treated HCT116 prior to co-culturing with 121-CM for 3h. Data are expressed as mean ± SEM. n = 3, one-way ANOVA test, *P < 0.05, **P < 0.01, compared with no treatment.

Fig 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 0.5h,1h,2h,3h; (B) The graph represents the quantification of VDR, LC3BII/I, and Beclin-1 protein levels. Data are expressed as mean ± SEM. n = 3, one-way ANOVA test, *P < 0.05, **P < 0.01, compared with no treatment.; (C) Expression of ATG16L1 and LC3 were increased after 121-CM treated (3h) in the HCT116 cells.

Fig 3. Expression of VDR and autophagy-related LC3B in ileum of VDR^{loxp} and $VDR^{\Delta IEC}$ mice oral administrated with 121-CM for 12h and 24h.

(A) Changes of VDR and LC3B expression after 121 administration for 12h and 24h. (B) The graph represents the quantification of VDR, and LC3BII/I ratio. Data are expressed as mean ± SEM. n = 6, one-way ANOVA test, *P < 0.05, **P < 0.01, compared with normal (No treatment).

Fig 4. mRNA expression of VDR in HCT116 treated with four other LAB stains for different time.

(A) VDR expression in HCT116 treated with conditional medium of four other LAB stains DKL109, DKL119, DKL128, DKLLa-5. Data are expressed as mean ± SEM. n = 6, one-way ANOVA test, *P < 0.05, **P < 0.01, compared with normal (No treatment). (B) Autophagy activity were increased after LAB strains treated. Autophagy activity were detected by CYTO-ID® Autophagy detection kit.

Fig 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 $VDR^{+/-}$ and $VDR^{-/-}$ MEFs. (B) The graph represents the quantification of VDR, Beclin-1 and LC3BII/I ratio. Data are expressed as mean ± SEM. n=3, one-way ANOVA test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with no treatment; student's t-test, *P < 0.05, **P < 0.01.

Fig 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 3h. (B) Expression of VDR, ATG16L1, and inflammation molecules in *S.E.*-infected organoids prior to 121-CM treatment. VDR and ATG16L1 expression in S.E.-infected (0.5h) organoids prior to 121-CM treatment for 3h. Data are expressed as mean ± SEM. n=3, one-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.E., Data are expressed as mean ± SEM. n=3, one-way ANOVA test, *p<0.05, **p<0.01, compared with control (No treatment).

Fig 7. 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 ± SEM. n=3, one-way ANOVA test, *P < 0.05, **P < 0.01. (C) The expression of VDR in organoids were upregulated after P40 or P75 treatment (100ng/ml for 24h). Data are expressed as mean ± SEM. n=3, one-way ANOVA test, *P < 0.05. (D) Organoid from ileum tissue were treated with P40 or P75(100ng/ml) for 24h. Then added with TNF-α 5ng/ml for 30min. 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 ± SEM. n=3, one-way ANOVA test, *P < 0.05, **P < 0.01. (E) a working model of probiotic upregulation of VDR through P40 and P75 to stimulate autophagic responses and inhibit inflammation.

Figure 1

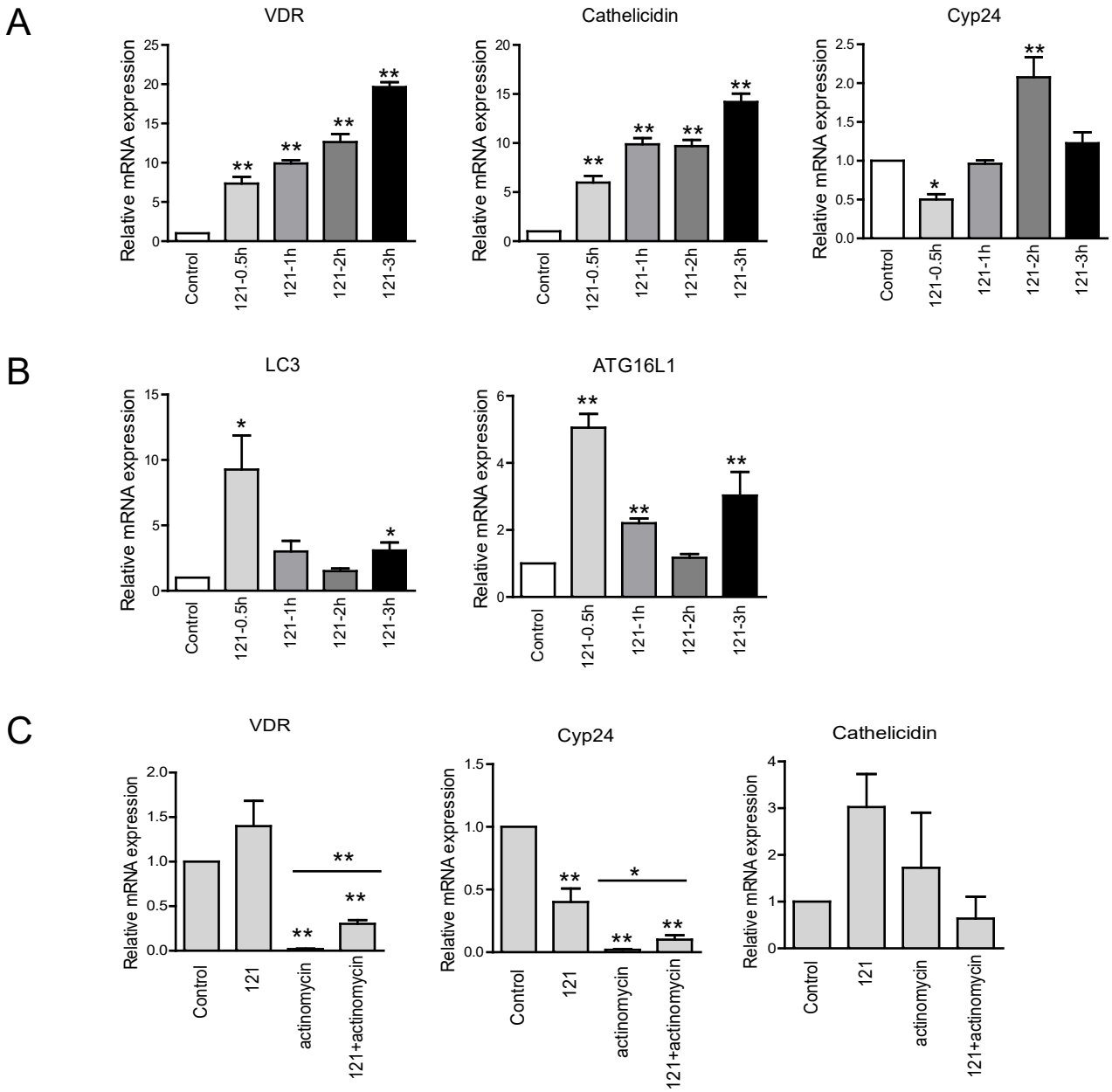

A
VDR
Cathelicidin
Cyp24
Relative mRNA expression
Control
121-0.5h
121-1h
121-2h
121-3h
B
LC3
ATG16L1
C
VDR
Cyp24
Cathelicidin
Control
121
actinomycin
121+actinomycin

Figure 2

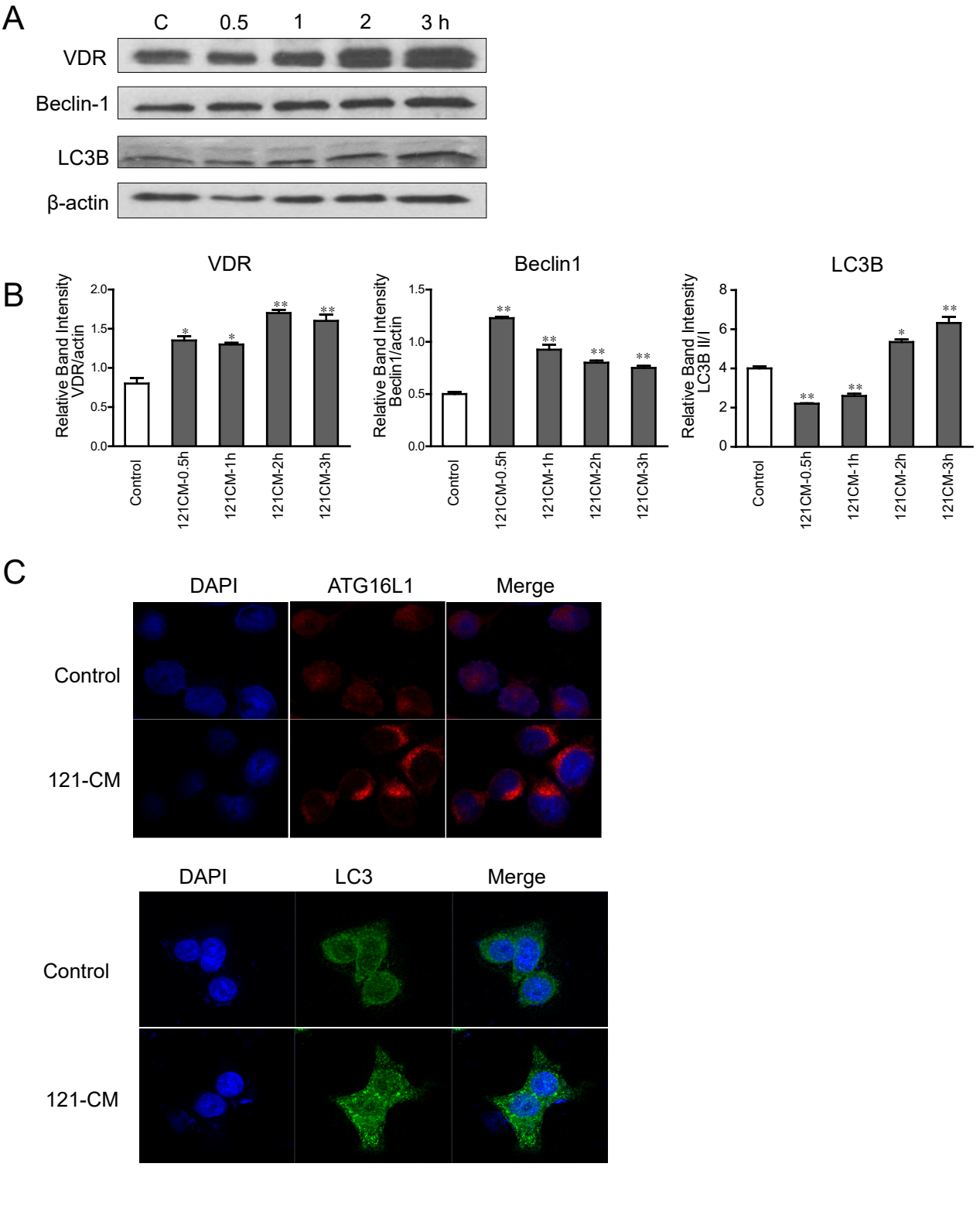

A
C
0.5
1
2
3 h
VDR
Beclin-1
LC3B
β-actin
B
VDR
Beclin1
LC3B
Relative Band Intensity VDR/actin
Relative Band Intensity Beclin1/actin
Relative Band Intensity LC3B II/I
Control
121CM-0.5h
121CM-1h
121CM-2h
121CM-3h
C
DAPI
ATG16L1
Merge
Control
121-CM
DAPI
LC3
Merge
Control
121-CM

Figure 3

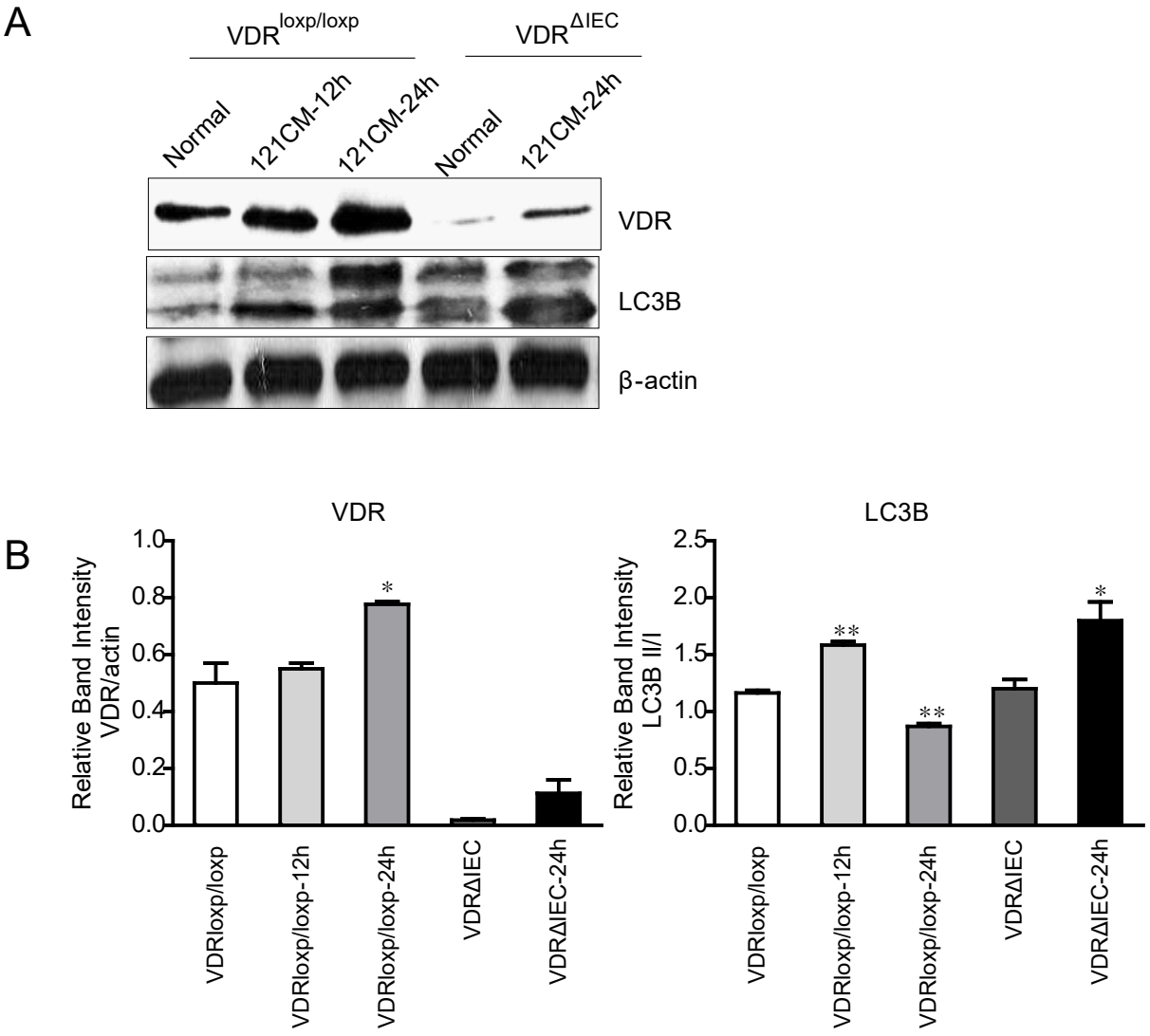

A
VDR loxp/loxp
VDR ΔIEC
Normal
121CM-12h
121CM-24h
Normal
121CM-24h
VDR
LC3B
β-actin
B
VDR
Relative Band Intensity VDR/actin
1.0
0.8
0.6
0.4
0.2
0.0
*
VDRloxp/loxp
VDRloxp/loxp-12h
VDRloxp/loxp-24h
VDRΔIEC
VDRΔIEC-24h
LC3B
Relative Band Intensity LC3B II/I
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
VDRloxp/loxp
VDRloxp/loxp-12h
VDRloxp/loxp-24h
VDRΔIEC
VDRΔIEC-24h

Figure 4

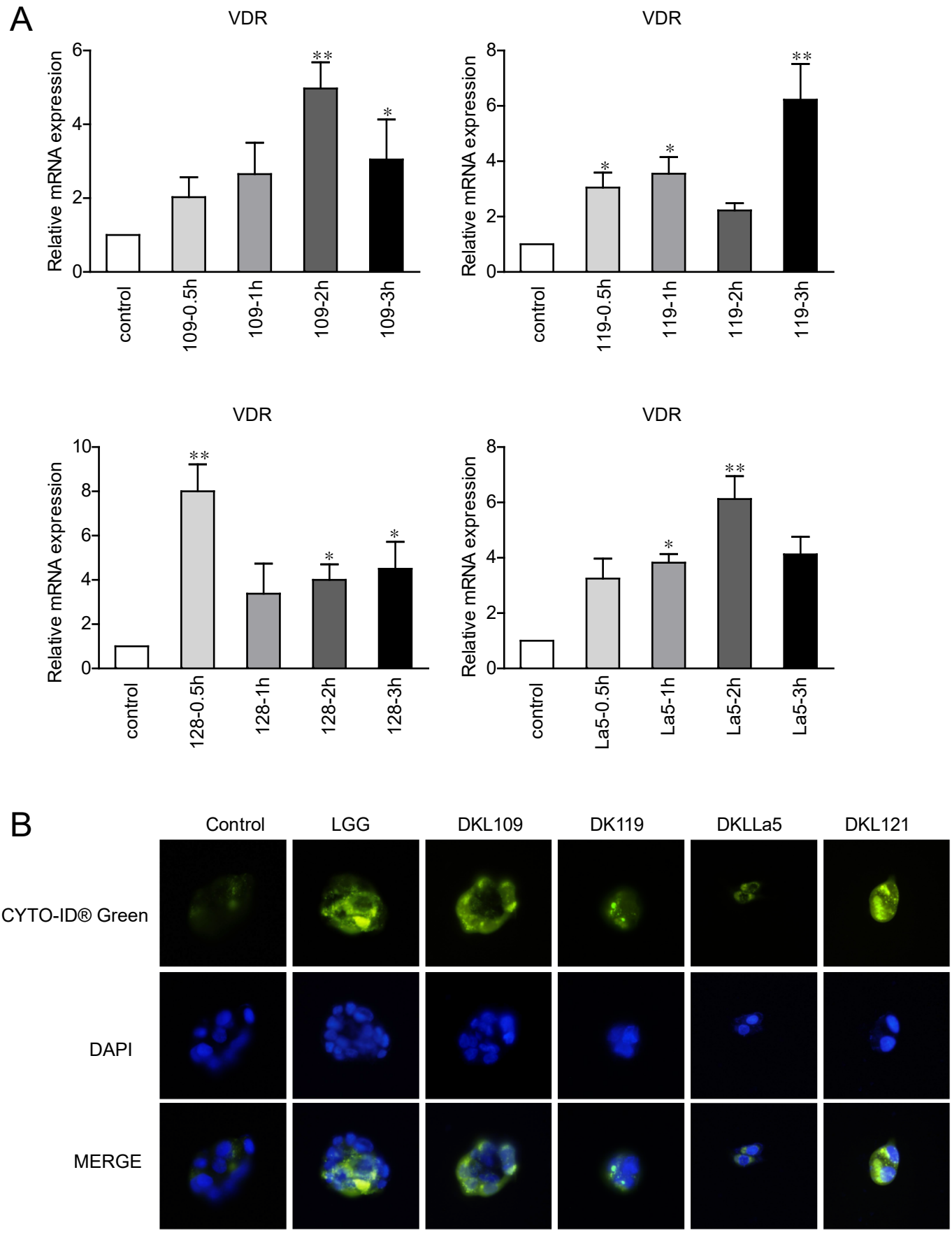

A
VDR
Relative mRNA expression
control
109-0.5h
109-1h
109-2h
109-3h
VDR
Relative mRNA expression
control
119-0.5h
119-1h
119-2h
119-3h
VDR
Relative mRNA expression
control
128-0.5h
128-1h
128-2h
128-3h
VDR
Relative mRNA expression
control
La5-0.5h
La5-1h
La5-2h
La5-3h
B
Control
LGG
DKL109
DK119
DKLLa5
DKL121
CYTO-ID® Green
DAPI
MERGE

Figure 5

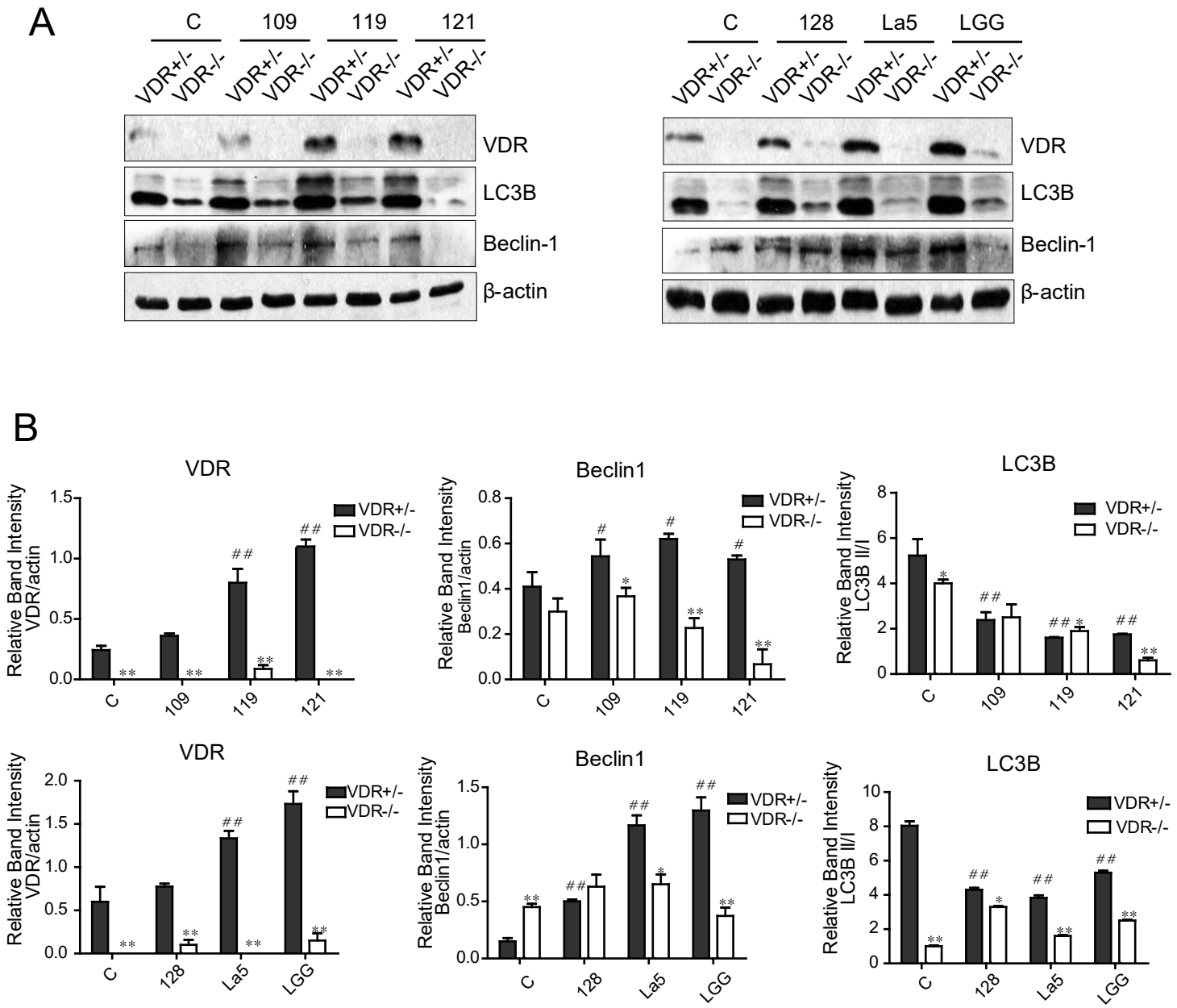
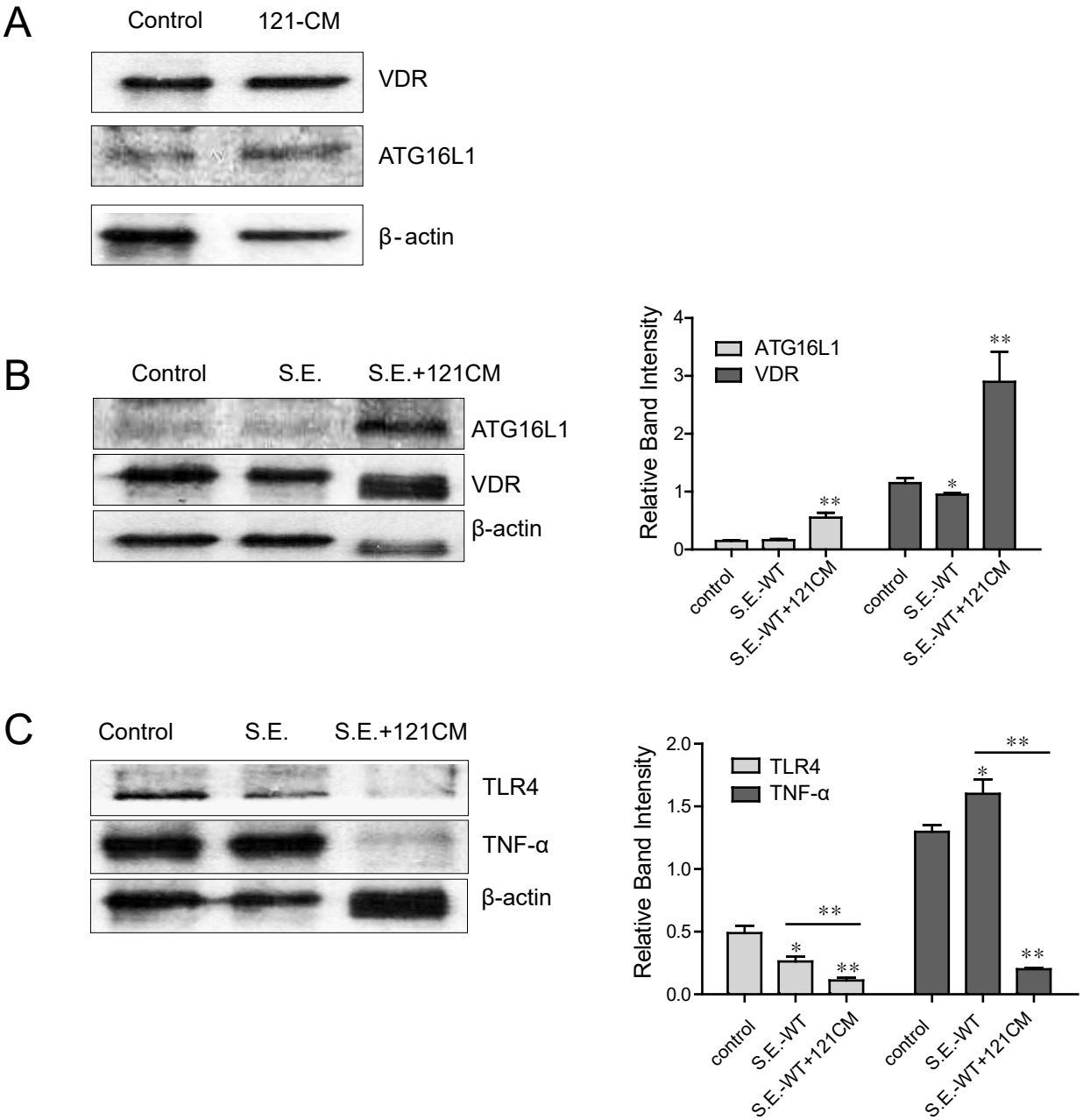

A
C
109
119
121
VDR+/-
VDR-/-
VDR
LC3B
Beclin-1
β-actin
C
128
La5
LGG
B
VDR
Beclin1
LC3B
Relative Band Intensity
VDR/actin
Beclin1/actin
LC3B II/I
VDR+/-
VDR-/-
C
109
119
121
128
La5
LGG

Figure 6


A
Control
121-CM
VDR
ATG16L1
β-actin
B
Control
S.E.
S.E.+121CM
ATG16L1
VDR
β-actin
Relative Band Intensity
ATG16L1
VDR
control
S.E.-WT
S.E.-WT+121CM
C
Control
S.E.
S.E.+121CM
TLR4
TNF-α
β-actin
Relative Band Intensity
TLR4
TNF-α
control
S.E.-WT
S.E.-WT+121CM

Image

Figure 7