

Vitamin D/VDR signaling attenuates lipopolysaccharide-induced acute lung injury by maintaining the integrity of the pulmonary epithelial barrier

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Abstract. Vitamin D and its receptor have a protective effect on epithelial barriers in various tissues. Low levels of vitamin D are associated with numerous pulmonary diseases, including acute lung injury (ALI) and acute respiratory distress syndrome. The present study investigated whether the vitamin D/vitamin D receptor (VDR) pathway may ameliorate lipopolysaccharide (LPS)-induced ALI through maintaining the integrity of the alveolar epithelial barrier. This was investigated by exposing wild-type (WT) and VDR knockout C57BL/6J mice to LPS, then comparing the healthy and LPS-treated mice lungs and bronchoalveolar lavage fluid (BALF). More specifically, lung histology, mRNA levels of proinflammatory cytokines and chemokines, and protein expression levels of tight junction proteins were determined. In addition, a vitamin D analog (paricalcitol) was administered to WT mice in order to investigate the effect of vitamin D on the alveolar epithelial barrier following exposure to LPS. VDR knockout mice exhibited severe lung injuries ($P<0.001$), increased alveolar permeability [demonstrated by a higher wet-dry ratio of lung weight ($P<0.05$), greater expression levels of BALF protein ($P<0.001$) and fluorescein isothiocyanate-conjugated 4 kDa dextran ($P<0.001$) leakage into the alveolar space], elevated proinflammatory cytokine and chemokine mRNA levels, as demonstrated by reverse transcription-quantitative polymerase chain reaction ($P<0.05$), and decreased protein and mRNA expression levels of occludin ($P<0.01$) and zonula occludens-1 (ZO-1; $P<0.01$) compared with WT mice.

Paricalcitol treatment partially inhibited these pathological changes in WT mice by maintaining the mRNA and protein expression levels of occludin ($P<0.01$) and ZO-1 ($P<0.05$). A lack of VDRs in the pulmonary epithelial barrier appeared to compromise its defense, leading to more severe LPS-induced lung injury. Furthermore, vitamin D treatment alleviated LPS-induced lung injury and preserved alveolar barrier function. Therefore vitamin D treatment may present as a potential therapeutic strategy in ALI and acute respiratory distress syndrome.

Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), contribute to morbidity and mortality in critically ill patients, with rates of morbidity and mortality of 75/100,000 and 40-60%, respectively (1,2). In particular, gram-negative sepsis often leads to ALI/ARDS (3). ALI/ARDS are characterized by an extensive inflammatory process leading to diffuse alveolar damage, an influx of neutrophils, activation of proinflammatory cytokines and chemokines, macrophages and protein-rich exudate in the alveolar space due to the disruption of the alveolar epithelial barrier (1,2).

The active form of vitamin D, termed calcitriol or 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], binds to the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily (4). In addition to regulating calcium, vitamin D acts as a regulator of multiple biological processes, including anti-inflammation, immunomodulation and barrier function maintenance (5). Furthermore, it has been suggested that the production of 1,25-(OH)₂D₃ is evidence of a local paracrine/autocrine action in various tissues (6), including pulmonary epithelial cells (7). There is growing evidence in support of associations between vitamin D deficiency, and impaired pulmonary function (8), an increased incidence of ALI/ARDS (9-12) and inflammatory diseases, including asthma (13), tuberculosis (14) and chronic obstructive pulmonary disease (COPD) (8,15). Previous studies have suggested that vitamin D deficiency is also common in critically ill patients (9,10), and is often associated with increased morbidity and mortality, including that caused by

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ALI/ARDS (9-12,16,17). However, the underlying mechanism of vitamin D/VDR signaling and sepsis-induced ALI/ARDS has yet to be investigated.

Vitamin D/VDR signaling is important for the integrity of tissue barriers and anti-inflammatory functions (5,6). It has been demonstrated to regulate the components of tight junctions and maintain the integrity of epithelial barriers in multiple organs, including the skin (18), eyes (19) and large intestine (20). In lungs, the permeability of the alveolar epithelial barrier is largely regulated by the intercellular junctions that seal the paracellular space (21,22). Disruption of the epithelial barrier may increase alveolar permeability and result in paracellular movements of fluid from the interstitium to the pulmonary airspace, as well as infiltration of inflammatory cells. Subsequent pulmonary edema impairs blood-gas exchange, leading to ARDS. Preserving or restoring these barriers may provide a therapeutic strategy for preventing or treating ALI/ARDS (23,24). However, to the best of our knowledge, there has been no previous investigation regarding vitamin D/VDR signaling regulation of the alveolar epithelial barrier in the lungs.

The aim of the present study was to investigate the effect of VDR knockout on lipopolysaccharide (LPS)-induced ALI and to assess the effect of vitamin D treatment on LPS-induced ALI in a mouse model. The results suggested that mice lacking VDR had a compromised alveolar epithelial barrier and aggravated ALI. Furthermore, it was identified that vitamin D treatment may sustain the integrity of the barrier and thus attenuate ALI.

Materials and methods

Ethics statement. All experimental procedures were reviewed and approved by the Institutional Ethics Committee of China Medical University (Shenyang, China).

Animals. VDR heterozygous (VDR^{+/-}) mice with a C57BL/6J background (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). VDR heterozygous males and females were bred to generate wild-type (WT; VDR^{+/+}) and VDR knockout (KO; VDR^{-/-}) mice for the experiment. All mice were kept in pathogen-free cages with a 12-h light/dark cycle, and fed a rescue diet high in calcium, phosphate and lactose (Envigo; Madison, WI, USA) to maintain a normal plasma calcium level in VDR KO mice (25).

Genotyping. Total DNA was harvested from the mouse tails and primers were provided by The Jackson Laboratory. Polymerase chain reaction (PCR) was performed as follows: Pre-denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 40 sec, 72°C for 10 min, and held at 4°C until electrophoresis. Agarose gel electrophoresis was performed with 2% agarose (in TAE buffer; Beyotime Institute of Biotechnology) at 100 V until the bands were clearly separated.

Treatment groups. Two mice (age, 8-12 weeks) per gender were selected for each treatment group. LPS [Lipopolysaccharides from *Escherichia coli* 0111:B4, dissolved in phosphate-buffered saline (PBS)] from Sigma-Aldrich (St. Louis, MO, USA)

was injected once intratracheally (10 mg/kg) in WT and KO mice. The control group was comprised of KO and WT mice injected with the same volume of sterile PBS. Bronchoalveolar lavage fluid (BALF) was collected. Briefly, following anesthesia by intraperitoneal injection with a cocktail of xylazine (Rompun 2%; Bayer AG, Leverkusen, Germany) and ketamine (Ketavest; 100 mg/ml; Pfizer, Inc., New York, NY, USA), the trachea was exposed and the lungs were lavaged three times with 0.2 ml sterile saline per wash. The BALF was stored at 4°C and samples of lung tissue were harvested for RNA, protein and histological studies 12 h after treatment, and refrigerated at -80°C.

WT mice were randomly divided into four treatment groups. Two vitamin D treatment groups (one PBS group and one LPS group) were treated with the vitamin D analog paricalcitol (Sigma-Aldrich) dissolved in propylene glycol:ethanol, 90:10; 0.5 µg/kg body weight) through intraperitoneal injection 30 min prior to LPS or PBS treatment. The two vehicle groups received the dissolvent only prior to LPS or PBS treatment.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the lung tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNAs were synthesized using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) and PCR was performed with the 20-µl volume reaction mixture using a SYBR-Green PCR reagent kit (Clontech Laboratories, Inc., Mountainview, CA, USA) on a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). The cycling conditions were as follows: 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of: 95°C for 15 sec, 55°C for 15 sec, 72°C for 1 min and 72°C for 1 min. Relative transcripts of mRNA were calculated using the quantification cycle (Cq) 2^{-ΔΔCq} formula (26). β-2 microglobulin served as an internal control. Sequences of the PCR primers are provided in Table I.

Western blot analysis. Following anesthesia, the chests of the mice were opened and the lung was harvested and homogenized in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). The supernatant was used to measure protein concentration according to the BCA method (Bio-rad Laboratories, Inc., Hercules, CA, USA). SDS (3X) was subsequently added and the mixture was heated to 95°C for 5 mins. The proteins (50 µg per lane) were electrophoresed on 10% SDS-PAGE (80 V for 30 min in condensed gel and 120 V for 90 min in dissociated gel), separated by 10% SDS-PAGE gels and electroblotted onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) at 90 V for 90 min. The density of the bands were quantitated using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA) and normalized to that of β-actin. The following primary antibodies were used: Polyclonal, anti-rabbit VDR (cat. no. C20; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), monoclonal, anti-mouse β-actin (cat. no. A5316; 1:10,000 dilution; Sigma-Aldrich), monoclonal, anti-mouse zonula occludens-1 (ZO-1; cat. no. 339100; 1:2,000 dilution; Cell Signaling Technology, Inc., Danvers MA, USA) and monoclonal,

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Primer name	Forward (5'→3')	Reverse (5'→3')
Mouse TNF- α	ATGAGCACAGAAAGCATGA	AGTAGACAGAAGAGCGTGGT
Mouse IL-6	CCTCTGGTCTTCTGGAGTACC	ACTCCTTCTGTGACTCCAGC
Mouse IL-1 β	AATGAAAGACGGCACACCCA	TGCTTGTGAGGTGCTGATGT
Mouse IFN- γ	TTCTTCAGCAACAGCAAGGC	TCAGCAGCGACTCCTTTTCC
Mouse MCP-1	GCTCAGCCAGATGCAGTTAA	TCTTGAGCTTGGTGACAAAACT
Mouse MIP-2	TGAAGTGCCTGTCAATGC	GCTTCAGGGTCAAGGCAAAC
Mouse occludin	CTACGGAGGTGGCTATGGAG	AGCGCTGACTATGATCACGA
Mouse ZO-1	ACGATCTCCTGACCAACGTT	GCTTTGGGTGGATGATCGTC
Mouse B2M	CGGCCTGTATGCTATCCAGA	GGGTGAATTCAGTGTGAGCC

TNF- α , tumor necrosis factor; IL-6, interleukin; IL-1 β , interleukin β ; IFN- γ , interferon; MCP-1, monocyte chemoattractant protein; MIP-2, macrophage inflammatory protein; ZO-1, zonula occludens-1; B2M, β 2 microglobulin.

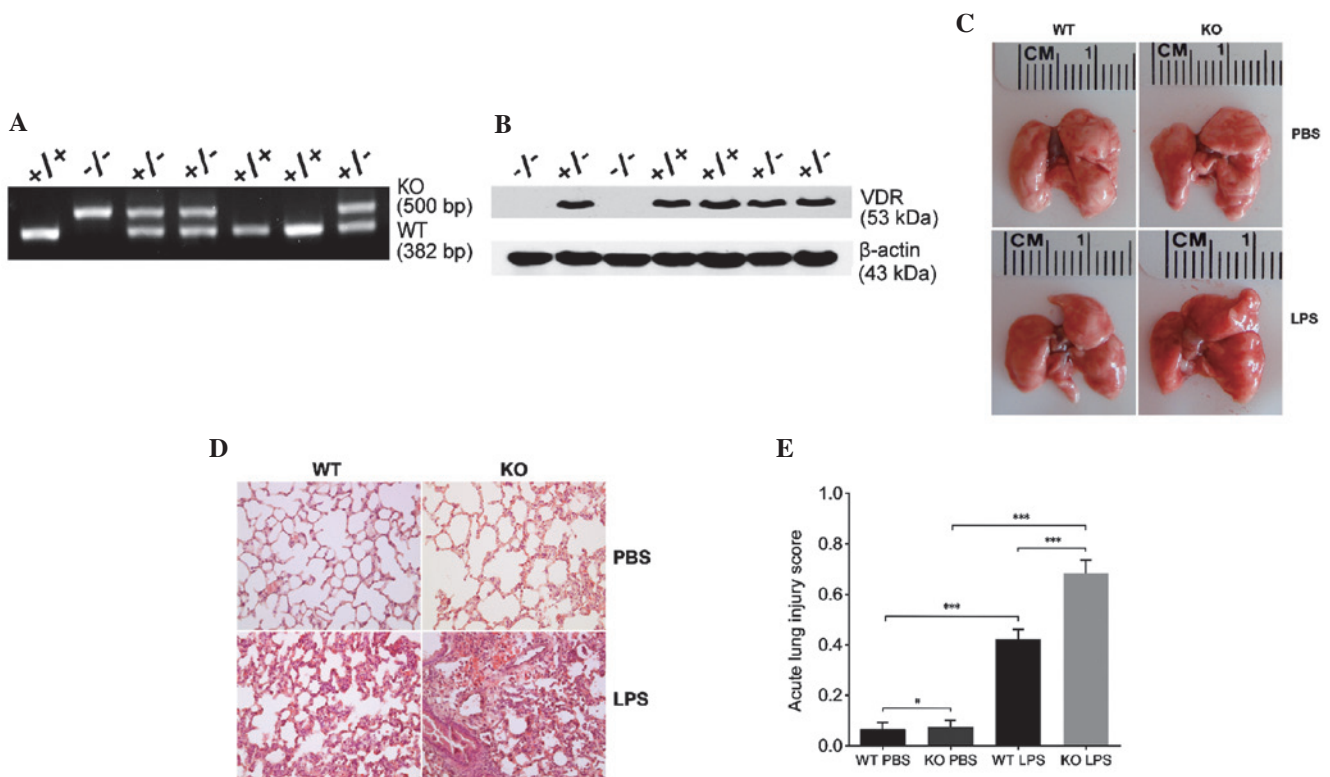


Figure 1. VDR KO mice exhibit more severe acute lung injury (ALI) than WT mice after LPS treatment. (A) Mouse genotyping of VDR [WT(+), KO(-)]. (B) Western blotting of lung lysates from WT (VDR^{+/+}), heterozygous (VDR^{+/-}) and KO (VDR^{-/-}) mice with anti-VDR antibody. Following exposure to LPS or PBS, the (C) morphology and (D) histology of lung tissue sections from WT and VDR KO mice were investigated. Hematoxylin and eosin stain; magnification, $\times 200$. (E) ALI scores according to microscopic examination for the two mouse models. Data are presented as mean $\pm$ standard deviation (n=5). *P<0.05, ***P<0.001. KO, knockout; WT, wild-type; VDR, vitamin D receptor; PBS, phosphate buffered saline; LPS, lipopolysaccharides.

anti-mouse occludin (cat. no. 331500; 1:2,000 dilution; Cell Signaling Technology, Inc.). The PVDF membranes were incubated at room temperature for 1 h in 5% Tris-buffered saline and Tween-20 (TBST) with non-fat milk to block non-specific binding, then incubated overnight at 4°C with the primary antibodies. After washing three times in 0.1% TBST, the membranes were incubated at room temperature for 60 min with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and anti-mouse IgG-HRP; cat. nos. sc-2004 and sc-2005, respectively; 1:2,000 dilution;

Santa Cruz Biotechnology, Inc.). The membrane was then washed three times in 0.1% TBST.

Histology and immunofluorescence. The mice lungs were harvested and the right lung was placed in 4% formalin overnight, dehydrated with graded alcohol, placed in xylene for 1 h and then embedded in paraffin at 60°C. Sections of the lung tissues (4 μ m) were stained with hematoxylin and eosin (H&E; Beyotime Institute of Biotechnology) at room temperature. The lung morphology resulting from the

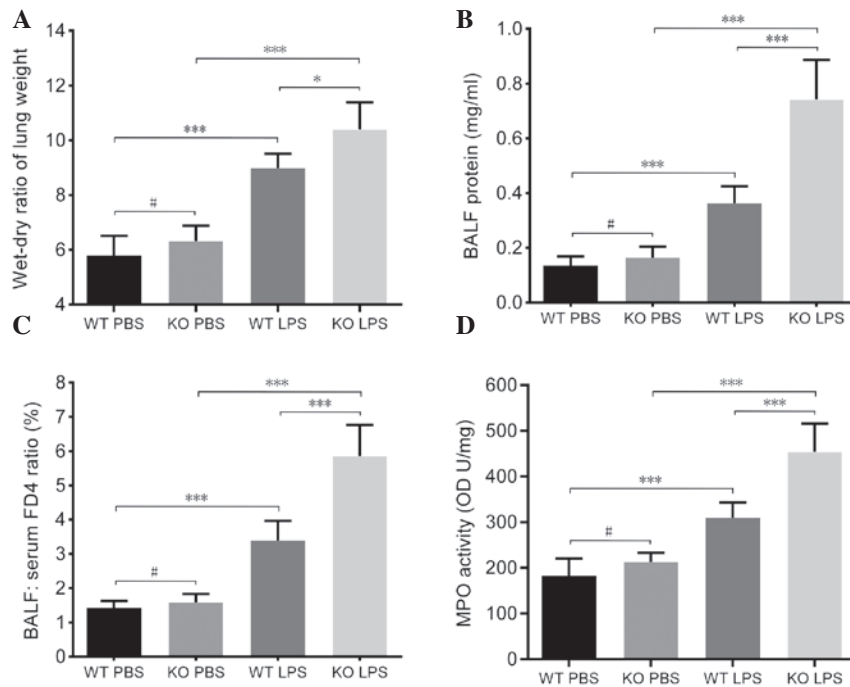


Figure 2. VDR KO mice exhibit greater pulmonary edema than VDR WT mice. WT and KO mice were treated with PBS or LPS intratracheally. Lung samples were harvested and analyzed 12 h later. (A) Wet-dry ratio of lung weight, (B) BALF protein concentration, (C) BALF to serum FD4 ratio and (D) MPO activity of lung lysates of different mice. Data are presented as mean $\pm$ standard deviation. # $P>0.05$, * $P<0.05$, *** $P<0.001$. (n=5-6 per group). WT, wild-type; PBS, phosphate-buffered saline; KO, knockout; LPS, lipopolysaccharides; FD4, fluorescein isothiocyanate-conjugated 4 kDa dextran; MPO, myeloperoxidase.

different treatments was scored according to H&E stained slides using a novel acute lung injury scoring system (27). A total of five random fields were selected and the evaluation was completed by two pathologists blinded to the study design. To localize the expression of tight junction proteins, sections were incubated with the anti-ZO-1 (1:200 dilution) or anti-occludin (1:200 dilution) antibodies, and were subsequently conjugated with Alexa Fluor 555 or 488 secondary antibodies (Invitrogen; Thermo Fisher Scientific, Inc.). Antigens were visualized using a Leica DFC425 fluorescence microscope [Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland].

Assessment of lung injury. The wet-dry ratio of lung weight was measured, as previously described (21). Briefly, the left lung was excised and the wet weight was measured using a Genimi-20 Portable Milligram Scale (American Weigh Scales, Inc., Norcross, GA, USA), the lung was then dried in an oven at 85°C for 72 h and weighed dry. The wet-dry ratio was calculated as follows: Wet lung weight/dry lung weight. The BALF protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Myeloperoxidase (MPO) activity was analyzed using a Myeloperoxidase Activity Assay kit, according to the manufacturer's protocol (CytoStore, Inc., Alberta, Canada) and an M200 microplate system (Tecan Group Ltd., Männedorf, Switzerland) was used to measure the optical density. In addition, the BALF to serum fluorescence ratio of fluorescein isothiocyanate-conjugated 4 kDa dextran (FD4; Sigma-Aldrich) was calculated to evaluate the pulmonary permeability, as previously described (21).

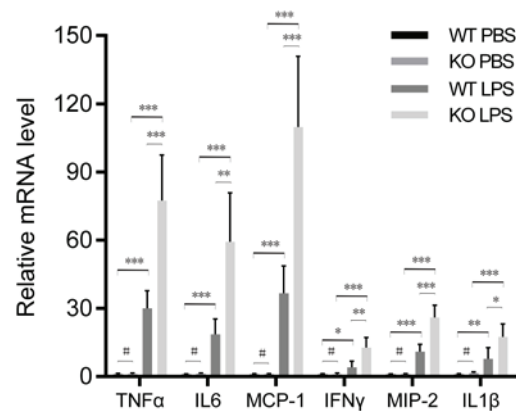


Figure 3. Quantified mRNA expression of proinflammatory cytokines and chemokines is higher in KO compared with WT mice following LPS or PBS treatment. Data are presented as mean value $\pm$ standard deviation. # $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (n=6). WT, wild-type; PBS, phosphate-buffered saline; KO, knockout; LPS, lipopolysaccharides; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; IFN γ , interferon γ ; MIP-2, macrophage inflammatory protein-2; IL-1 β , interleukin-1 β .

Statistical analysis. All continuous data are presented as the mean $\pm$ standard deviation. Statistical comparison of continuous variables between groups was performed using Student's t-test or one-way analysis of variance with GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA) and Statistical Product and Service Solutions (version 17.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ indicated a statistically significant difference.

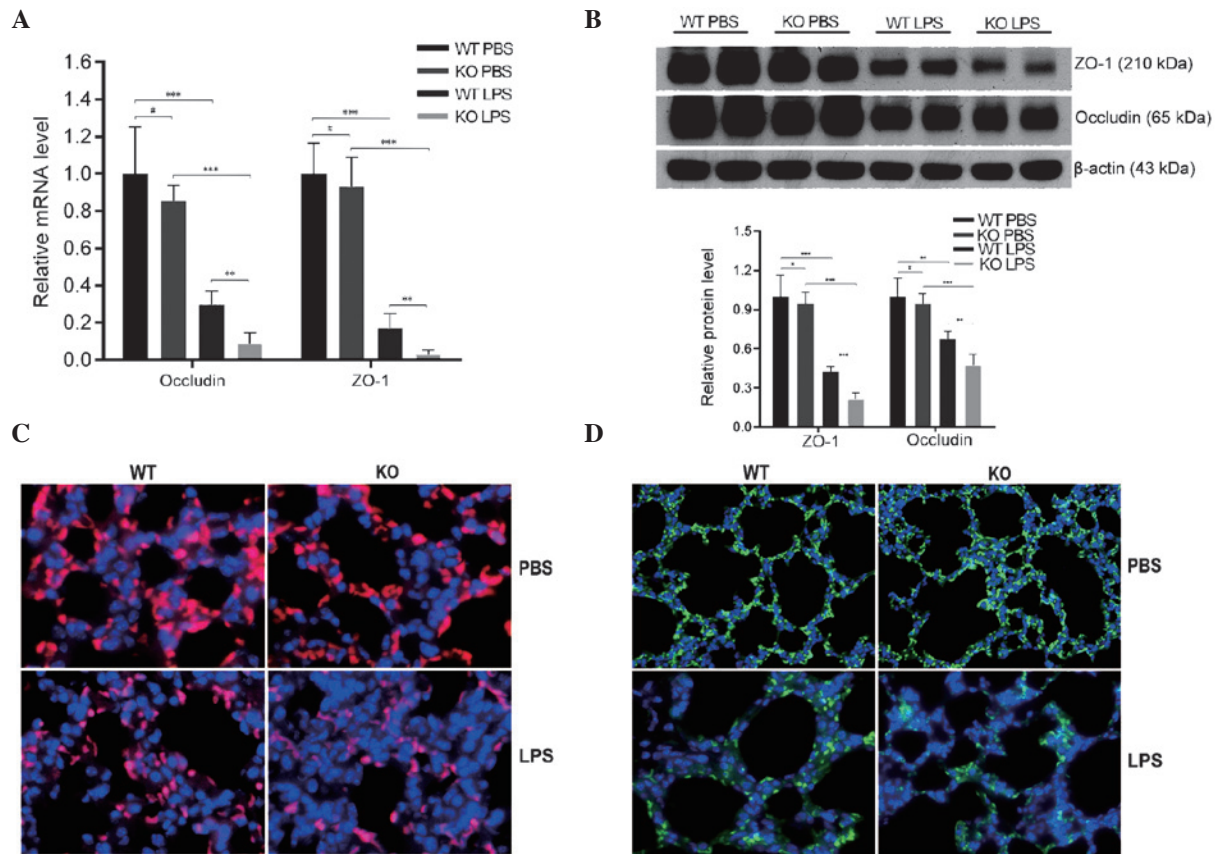


Figure 4. KO mice exhibit reduced occludin and ZO-1 expression following LPS treatment. (A) Quantification of occludin and ZO-1 mRNA expression in WT and KO mice following LPS or PBS treatment. (B) Western blots and quantification of ZO-1 and occludin protein levels in lung lysates. (C) Occludin (red, occludin and Alexa Fluor 488; blue, DAPI) and (D) ZO-1 (blue, DAPI) and (D) ZO-1 and Alexa Fluor 488) immunofluorescence staining. Data are presented as the mean $\pm$ standard deviation $^{\#}P>0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (n=5-6 per group). WT, wild-type; PBS, phosphate-buffered saline; KO, knockout; LPS, lipopolysaccharides; ZO-1, zonula occludens-1.

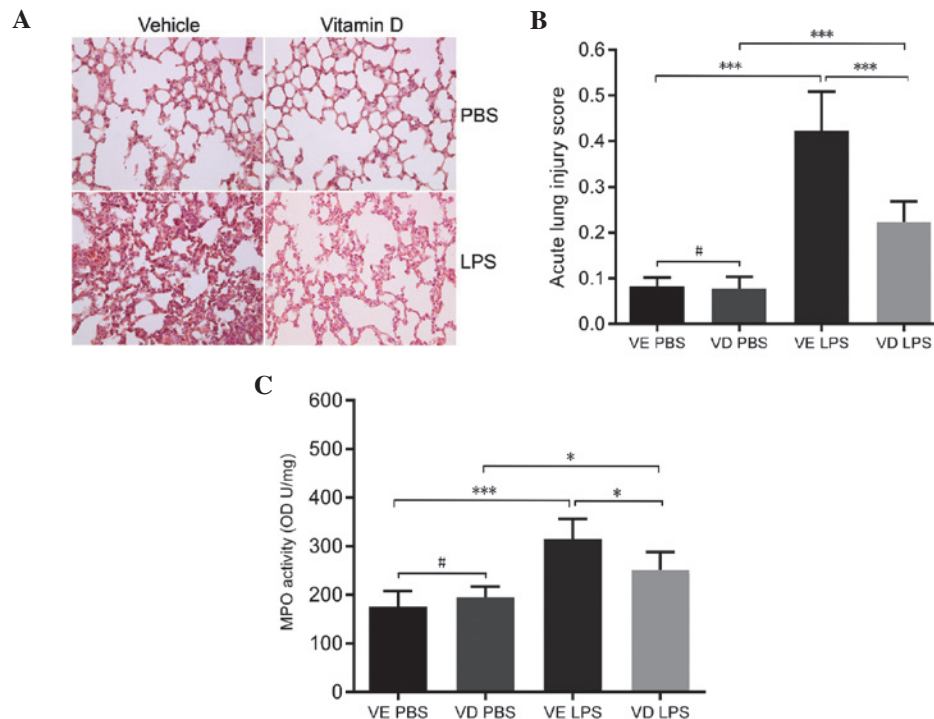


Figure 5. VD attenuates LPS-induced acute lung injury (ALI). (A) Representative hematoxylin and eosin staining of lung sections (magnification, x200). (B) ALI score and (C) MPO activity in wild-type and knockout mice treated with VD or VE. Data are represented as mean $\pm$ standard deviation. $^{\#}P>0.05$, $^{*}P<0.05$, $^{***}P<0.001$ (n=5-6 per group). PBS, phosphate-buffered saline; LPS, lipopolysaccharides; VE, vehicle; VD, vitamin D (paricalcitol); MPO, myeloperoxidase; OD, optical density.

Results

VDR KO mice exhibit more severe LPS-induced ALI than VDR WT mice. VDR WT and KO mice were bred and genotyped according to the protocol of The Jackson Laboratories. Genotype and VDR expression were confirmed using the lung lysates (Fig. 1A and B). The lungs of VDR KO mice appeared more injured and congested following LPS treatment compared with VDR WT mice (Fig. 1C). VDR KO mice also exhibited more severe interstitial edema, alveolar wall thickening and inflammatory cells infiltration compared with WT mice (Fig. 1D). Furthermore, ALI scores of VDR KO mice were significantly higher than VDR WT mice following LPS treatment ($P<0.001$; Fig. 1E).

In mice lacking VDR, pulmonary permeability was increased and MPO activity was higher. More specifically, a significantly higher wet-dry lung ratio was observed ($P<0.05$; Fig. 2A) in VDR KO mice subsequent to LPS exposure compared with WT mice, indicating that more fluid was retained in the VDR KO lung. VDR KO mice were also identified to have higher expression levels of BALF protein (Fig. 2B; $P<0.001$), and greater FD4 leakage from the lung interstitium or capillaries into the alveolar space (Fig. 2C; $P<0.001$) compared with VDR WT mice. Furthermore, exposure to LPS induced significantly higher MPO activity in the lungs of VDR KO mice compared with VDR WT mice ($P<0.001$; Fig. 2D).

VDR KO leads to severe lung inflammation. To further investigate the anti-inflammatory effect of VDR in lungs, the levels of proinflammatory cytokines and chemokines were determined. RT-qPCR indicated that the mRNA expression levels of proinflammatory cytokines and chemokines were significantly higher in VDR KO mice compared with VDR WT mice following LPS treatment (Fig. 3).

VDR KO decreases the expression of pulmonary epithelial tight junction proteins, occludin and ZO-1. Following exposure to LPS, mRNA (Fig. 4A) and protein (Fig. 4B) expression levels of occludin and ZO-1 were significantly lower in VDR KO mice compared with VDR WT mice ($P<0.01$). Furthermore, LPS treatment resulted in lower levels of expression of occludin (Fig. 4C) and ZO-1 (Fig. 4D) in the alveolar epithelial cells of all mice treated with LPS, with VDR KO mice demonstrating a more severely disrupted expression pattern than VDR WT mice. Therefore, the lack of VDR may compromise the function of the pulmonary barrier by decreasing the expression of occludin and ZO-1.

Vitamin D analog treatment alleviates LPS-induced ALI by preserving occludin and ZO-1 expression. Histological examination of LPS-exposed vehicle mice revealed a considerable infiltration of inflammatory cells and thickening of the alveolar wall. However, in mice treated with vitamin D analog prior to LPS exposure, pulmonary inflammation and the thickening of the alveolar wall was less apparent (Fig. 5A). This was accompanied by a significantly decreased ALI score in the vitamin D pretreated mice compared with the vehicle treated mice ($P<0.001$; Fig. 5B). MPO activity was also significantly lower in VD mice ($P<0.05$; Fig. 5C). The induction of proinflammatory cytokine and chemokine

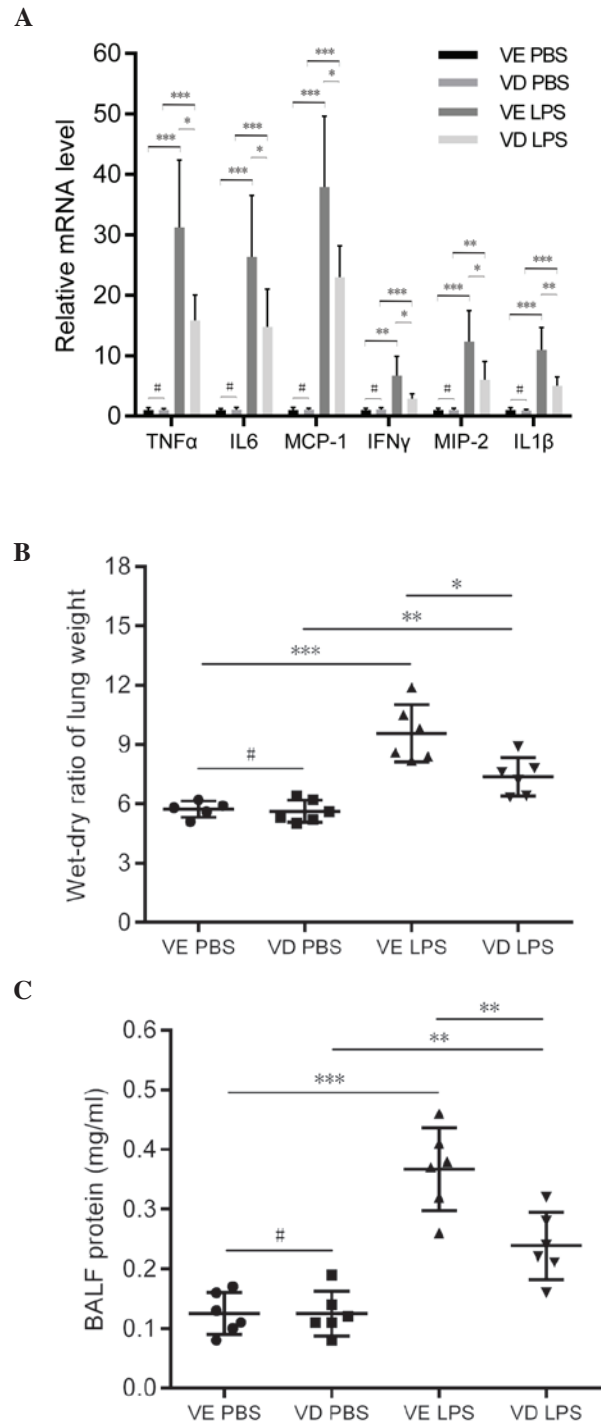


Figure 6. Vitamin D treatment inhibits lung inflammation and alleviated pulmonary edema. (A) Reverse transcription-polymerase chain reaction quantitation of proinflammatory cytokines and chemokines. (B) Wet-dry ratio of lung weight. (C) BALF protein concentration. Data are presented as mean value $\pm$ standard deviation. # $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ($n=4-6$ per group). VE, vehicle; VD, vitamin D (paricalcitol); PBS, phosphate-buffered saline; LPS, lipopolysaccharides; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; IFN γ , interferon γ ; MIP-2, macrophage inflammatory protein-2; IL-1 β , interleukin-1 β ; BALF, bronchoalveolar lavage fluid.

expression was significantly suppressed by vitamin D treatment (Fig. 6A and B). Furthermore, animals pretreated with vitamin D experienced significantly less severe pulmonary edema ($P<0.05$; Fig. 6C) and lower levels of BALF protein

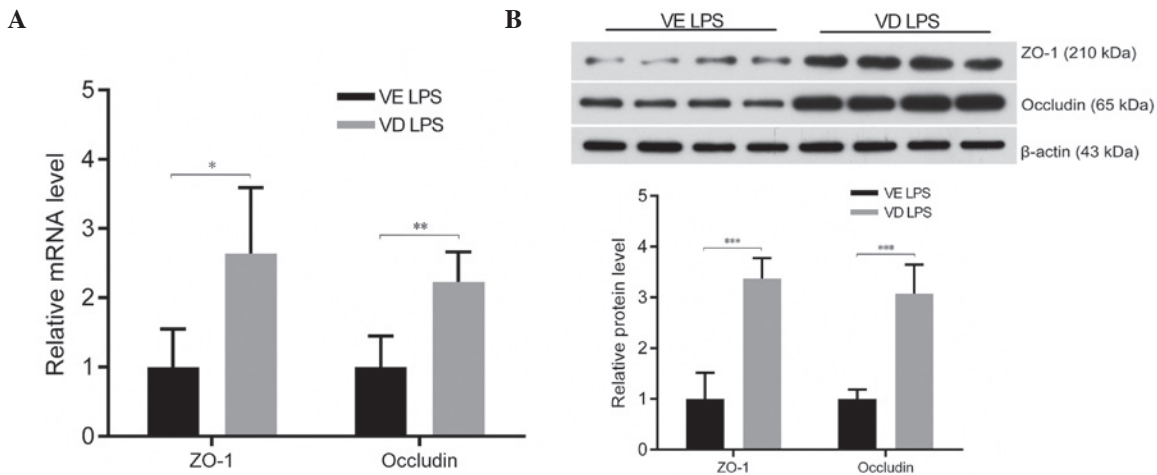


Figure 7. Vitamin D treatment preserves occludin and ZO-1 expression. (A) Reverse transcription-polymerase chain reaction quantitation of occludin and ZO-1 mRNA levels. (B) Western blot and quantification of ZO-1 and occludin protein levels in lung lysates. Data are presented as the mean $\pm$ standard deviation. $^{\#}P>0.05$, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ ($n=5-6$ per group). VE, vehicle; VD, vitamin D (paricalcitol); LPS, lipopolysaccharides; ZO-1, zonula occludens-1.

debris entering the alveolar space ($P<0.01$; Fig. 6D) compared with VE mice. Vitamin D pretreated mice exhibited significantly higher mRNA (Fig. 7A) and protein (Fig. 7B) expression levels of occludin and ZO-1 compared with vehicle treated mice. This may be due to VDR decreasing the permeability of the pulmonary epithelial barrier, thus limiting the pathological changes that occur during ALI.

Discussion

Vitamin D deficiency impairs lung function and has been associated with a number of lung diseases, including COPD, asthma, tuberculosis, ALI and its severe form, ARDS (8-17); however the underlying mechanisms of these associations remain unclear. The present study demonstrated that VDR null mice exhibit more severe LPS-induced ALI, primarily due to deterioration of the alveolar epithelial tight junctions through a decrease in occludin and ZO-1 expression. By contrast, vitamin D treatment alleviated lung injury through maintenance of the pulmonary barrier. To the best of our knowledge, this was the first study to investigate the protective function of the vitamin D/VDR signaling pathway on the pulmonary epithelial barrier.

ARDS remains a major cause of morbidity and mortality in critically ill patients (1-3). It is characterized by the disruption of the endothelial and epithelial barriers of alveoli, leading to increased barrier permeability (1,2). The alveolar epithelial barrier consists of a monolayer of epithelial cells with intercellular junctions that seal the paracellular space and regulate barrier permeability (21,22). Preserving or restoring the barrier function of alveolar epithelial cells may be a novel treatment for sepsis-induced ARDS. Tight junction complexes are composed of integral membrane proteins, cytoplasmic plaque proteins and cytoskeletal proteins (28). Among these, occludin and ZO-1 are key components that regulate paracellular permeability. They are indispensable in alveolar epithelial barrier function and fluid clearance (21). Reduced or dysmorphic expression of occludin and ZO-1 may compromise the alveolar barrier function and result in increased alveolar permeability, thus impairing blood-gas exchange. In a

previous study, hyperoxia was identified to disrupt the pulmonary epithelial barrier in newborn rats by decreasing occludin and ZO-1 levels (21). Therefore, disruption of the alveolar epithelial barrier is a critical factor in the pathogenesis of lung injuries and subsequent pathological changes; however, the molecular mechanisms that influence components of the tight junction remain poorly understood.

The active form of vitamin D is $1,25-(OH)_2D_3$, and is important in maintaining the structure and function of epithelial barriers in multiple tissues (18-20). The administration of $1,25-(OH)_2D_3$ as part of a therapeutic regimen may revert proteinuria and inhibit glomerular podocytes injury, partially through improving the barrier function in the kidneys (29). VDR null mice are more susceptible to colonic injury induced by dextran sulfate sodium compared with WT mice, as the lack of VDR compromises the intestinal epithelial barrier structure (20). Thus, the vitamin D/VDR signaling pathway may be a target in the treatment of various inflammatory diseases through preserving or restoring epithelial barrier function. In the present study, VDR KO mice exhibited more severe ALI induced by LPS compared with WT mice. Furthermore, occludin and ZO-1 levels in VDR KO mice were lower compared with WT mice. This led to greater infiltration of inflammatory cells, release of proinflammatory cytokines and chemokines, and fluid retention. However, vitamin D treatment may partly reverse this pathological process. The present study indicates that vitamin D/VDR signaling may inhibit endotoxin-induced ALI through maintaining the integrity of pulmonary epithelial barrier.

Neutrophil recruitment is important in the progression of ALI and ARDS (30). The infiltration of neutrophils may be evaluated through MPO activity. In the present study, VDR KO mice exposed to LPS exhibited higher MPO activity in the lung lysates compared with WT mice. When vitamin D pretreatment was applied, lower MPO activity was observed. In agreement with these results, a previous study used a hamster model of ALI to identify that $1,25-(OH)_2D_3$ inhibits neutrophil recruitment by 40%, due to its inhibitory effect on interleukin-8 (IL-8) (30). The alveolar barrier limits neutrophil infiltration, thus the inhibitory effect of neutrophil recruitment

by vitamin D/VDR signaling may be partly due to the maintenance of the integrity of the alveolar barrier.

Furthermore, lung epithelial cells had been identified to express high baseline levels of 1α -hydroxylase, which activates vitamin D, and low levels of inactivating 24-hydroxylase (7). Active vitamin D generated by lung epithelial cells is important for innate immune functions (7). During sepsis, pro-inflammatory cytokines and chemokines are produced by activated alveolar macrophages in the air space. Tumor necrosis factor- α (TNF- α), IL-6 and IL-1 β proteins are also important as they mediate, amplify and promote the process of lung inflammation (1). These cascades of immune responses result in various stages of alveolar epithelial injury (1). Furthermore, $1,25\text{-(OH)}_2\text{D}_3$ may ameliorate seawater-aspiration induced ALI through the inhibition of nuclear factor- κ B and the RhoA/Rho kinase signaling pathways (31). In addition, it may also alleviate lung damage secondary to ischemia reperfusion injury (32). The present study identified that LPS treatment increased the expression levels of the following cytokines and chemokines in WT mice: TNF- α , IL-6, IL-1 β , interferon- γ , monocyte chemoattractant protein-1 and macrophage inflammatory protein-2. This effect was even more apparent in VDR KO mice, thus confirming that the VDR KO lung was markedly more inflamed than the WT lung. By contrast, vitamin D treatment substantially inhibited the build-up of chemokines and attenuated LPS-induced lung injury. Furthermore, the present study determined that active vitamin D was capable of triggering an anti-inflammatory defense and preserving the structural barrier in the lungs.

In conclusion, the current study highlights that lack of VDR may compromise the pulmonary epithelial barrier defense, leading to a more severe LPS-induced lung injury. Furthermore, vitamin D treatment may preserve the alveolar barrier function and therefore alleviate LPS-induced lung injury. These observations provide further insight into the pathogenesis of ALI/ARDS and emphasize that vitamin D may be a novel treatment for ALI/ARDS.

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