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Vitamin D attenuates lung injury via stimulating epithelial repair, reducing epithelial cell apoptosis and inhibits TGF-β induced epithelial to mesenchymal transition

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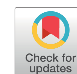

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ABSTRACT

Vitamin D regulates cell proliferation, inhibits cytokines release at sites of inflammation and reduces inflammatory responses. In this study, the aim was to investigate whether exogenous vitamin D attenuates LPS-induced lung injury via modulating epithelial cell proliferation, migration, apoptosis and epithelial mesenchymal transition (EMT). Murine and in vitro primary type II alveolar epithelial cell work were included in this study. In vivo, mice were mildly vitamin D deficient, 0.1, 1.5, 10 mg/kg 1,25$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 was administrated by means of an intra-gastric injection for 14 days pre-intra-tracheal (IT) LPS, which remarkedly promoted alveolar epithelial type II cells proliferation, inhibited ATII cells apoptosis and inhibited EMT, with the outcome of attenuated LPS-induced lung injury. In vitro, vitamin D stimulated epithelial cell scratch wound repair, reduced primary ATII cells apoptosis as well. Vitamin D promoted primary human ATII cells proliferation through the PI3K/AKT signaling pathway and activation of vitamin D receptor (VDR). Moreover, vitamin D inhibited EMT in response to TGF-β, which was vitamin D receptor dependent. In conclusion, vitamin D attenuates lung injury via stimulating ATII cells proliferation and migration, reducing epithelial cell apoptosis and inhibits TGF-β induced EMT. Together, these results suggest that vitamin D has therapeutic potential for the resolution of ARDS.

1. Introduction

Acute Respiratory Distress syndrome (ARDS) characterized by alveolar epithelial disruption, resulting in influx of protein-rich edema fluid [1] and refractory hypoxia causing acute respiratory failure. Many studies suggest that excessive inflammation resulting in profound alveolar epithelial cell injury is central to the pathogenesis of ARDS [2]. This results in extensive alveolar epithelial cell apoptosis and increases in alveolar capillary permeability thought to be mediated by soluble Fas ligand (sFasL) [3]. The degree of alveolar epithelial injury is an important determinant of the severity of ARDS [4]. Therefore, the timely repair of alveolar epithelium is also thought to be pivotal to the resolution phase of ARDS [5]. Disordered repair mechanisms such as epithelial to mesenchymal transition (EMT), may be involved producing the fibroproliferative response seen in some patients with ARDS [6,7].

Delicate alveolar sacs of the lung are composed of two main cells types, squamous alveolar type I (AT I) and cuboidal alveolar type II (AT II) cells [8]. AT I cells covering most of the alveolar surface area are more vulnerable in response to injury [9]. While AT II cells, acting as alveolar progenitors, proliferate, spread on the basement membrane, then transdifferentiate into AT I cells [10]. When ATII cells lose reparative mechanism, the alveolar epithelium damage will be repaired inpart by fibroblasts resulting in lung fibrosis at last [11]. Furthermore,

Abbreviations: ARDS, Acute respiratory distress syndrome; sFasL, soluble Fas ligand; AT II, Alveolar Type II; VDR, Vitamin D Receptor; EMT, Epithelial-Mesenchymal Transition; PCNA, Proliferating Cell Nuclear Antigen; α-SMA, (α-Smooth muscle actin

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Table1
Details of primers used in Real-time PCR.

Gene name	Accession number	Forward primer	Reverse primer
Homo sapiens Vimentin	NM_003380	CTTCAGAGAGAGGAAGCCGA	ATTCCACTTTGCGTTCAAGG
Homo sapiens snail-homolog-2 (SLUG)	NM_003068	CAGACCCTGGTTGCTTCAA	TGACCTGTCTGCAAATGCTC
Homo sapiens collagen, type I, alpha 1	NM_000088	CTTTGCATTCATCTCTCAAACTTAGTTTT	CCCCGCATGGGTCTTCA
Homo sapiens actin, alpha 2, smooth muscle, aorta (ACTA2)	NM_001613	CCGACCGAATGCAGAAGGA	ACAGAGTATTTGCGCTCCGAA
Homo sapiens cadherin 1, type 1, E-cadherin (epithelial) (CDH1)	NM_004360	GGTCTCTCTCACCACCTCCA	CCTCGGACACTTCCACTCTC
Homo sapiens cadherin 2, type 1, N-cadherin (epithelial) (CDH2)	NM_007664	CGTGAAGGTTTGCCAGTGT	CAGCACAAGGATAAGCAGGA

accumulative in-vitro and animal studies provide evidence that AT II cells contribute to rapid and extensive alveolar repair after injury [8,11,12]. Thus, promoting ATII cells proliferation and inhibiting ATII cells apoptosis may be a promising way to alleviate lung injury.

Traditionally, vitamin D is known to play a role in calcium and phosphorus homeostasis, bone mineralization. However, more and more evidence indicates that vitamin D is involved in regulating various cells of the innate and adaptive immune system [13]. Vitamin D has a broad role in regulating inflammatory responses in models of inflammations [14–16]. Vitamin D receptor knockout mice which were treated with LPS, displayed a more distinct inflammatory response than wildtype mice [17]. In addition, we previously demonstrated that vitamin D deficiency (VDD) contributed directly to the ARDS and that in a perioperative setting that vitamin D dosing reduced biomarkers of pulmonary vascular permeability index [18,19]. Taken together, vitamin D may play a potentially beneficial role in acute lung injury (ALI). Hence, we investigated the specific direct effect of 1,25$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 upon LPS-induced lung injury.

Concerning the protective effect of vitamin D on ALI, JUN XU et al have reported that a vitamin D receptor agonist, calcitriol alleviated LPS-induced acute lung injury [20]. Based on JUN XU's study, we further investigated the effect of 1,25$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 on LPS-induced acute lung injury, examined if mice were vitamin D deficient. Moreover, we treated the mice with a wide dose range of vitamin D. Importantly, we investigated if vitamin D could affect ATII cells proliferation, apoptosis and EMT abilities in vivo as well as in vitro. Our finding revealed that exogenous vitamin D could exert protective effects on human lung epithelial cell in a murine LPS-induced lung injury.

2. Material and methods

2.1. Reagents

1,25$(OH)_2$-vitamin D_3 (the biologically active form) or 25(OH)-vitamin D_3 (the main circulating precursor) were purchased from Sigma-Aldrich (Darmstadt, Germany). Recombinant human TGF-β was purchased from R&D (R&D Systems, Abingdon, UK). VDR ligands ZK159222 and ZK191784 were from Bayer Schering Pharma AG (Berlin, Germany). Antibody against caspase-3, AKT, phospho-AKT (Ser473) and α-SMA (48938) were obtained from Cell Signal Technology (Cell signal Technology, Boston, USA). Antibody against E-cadherin (ab1416), N-cadherin (ab18203) were obtained from Abcam (Abcam, Cambridge, UK). Antibody against β-actin (sc-47778) was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). 25(OH) Vitamin D_3 ELISA kit (ab213966) was purchased from Abcam (Abcam, Cambridge, UK). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich (Shanghai, China).

2.2. Animal and preparation

Specific pathogen-free male C57/BL6 mice, with 24 g of mean body weight, obtained from Slac laboratory animal (shanghai, China), were used in all experiments. The study was approved by animal studies ethics committee of WenZhou Medical University.

The LPS challenge model was performed as described previously [21]. Briefly, mice (male; randomized into 6 groups of 6 mice per group) were anaesthetized and instilled by intratracheal (IT) route as a model of direct lung injury with one dose (0.24 ± 0.01 mg) of LPS (Escherichia coli 055: B5, Sigma-Aldrich) for 24 h. No treatment control mice were treated with physiological saline. In certain groups, mice received supplementation of 0.1, 1.5, 10 mg/kg 1,25$(OH)_2$ -vitamin D_3 or 25(OH)-vitamin D_3 by means of an intra-gastric injection for 14 days pre-intratracheal (IT) LPS, while the control and LPS group mice were administered with physiological saline.

2.3. Hematoxylin and eosin (H&E) staining and lung injury score

Briefly, the middle lobe of the right lung was fixed with 10% paraformaldehyde for 24 h, embedded in paraffin wax, sectioned (5 µm thickness), stained with hematoxylin for 5 min, incubated in Scott's tap water for 5 min and stained with alcoholic eosin solution for 1 min. They were washed by tap water for 2 min after every step. Then tissues were immersed in 1% hydrochloric acid–ethanol each for 20 s. Tissues were then mounted by inversion onto glass slides dotted with Gel/Mount for conventional morphological evaluation under light microscope (Nikon eclipse 90i, Tokyo, Japan).

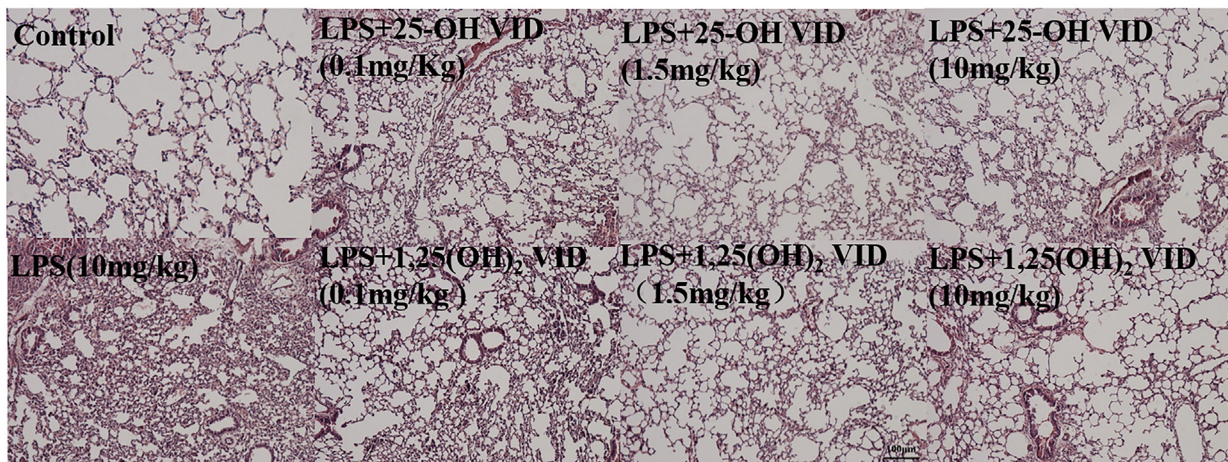
A semiquantitative scoring system was adopted to evaluate the lung injury including alveolar congestion, alveolar hemorrhage, infiltration or aggregation of neutrophils in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation and inflammatory cell infiltration. Lung injury scores were quantified by two investigators who was blinded to the treatment groups using Murray's standard: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%).

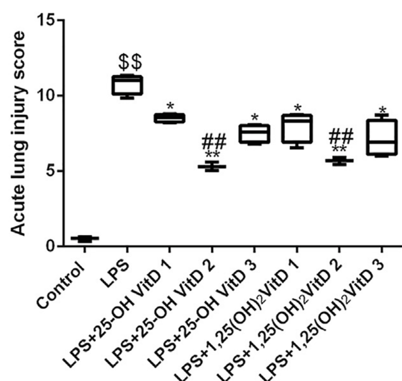
2.4. Wet/Dry weight ratio

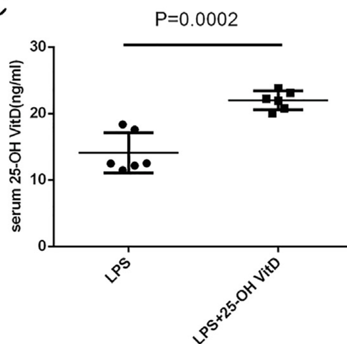
To quantify the magnitude of pulmonary edema, we evaluated the wet weight to dry weight (wet/dry) ratio of the lung as described previously [22]. Briefly, portions of the harvested wet upper lobes of the right lung were weighed, then placed in an oven for 72 h at 60 °C until weight was no longer changed. The dry lungs were weighted and the wet-to-dry (W/D) ratio was calculated.

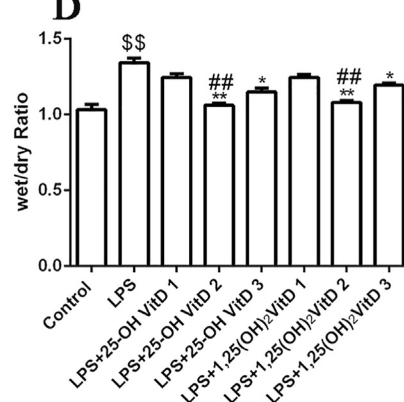
2.5. Pulmonary barrier permeability

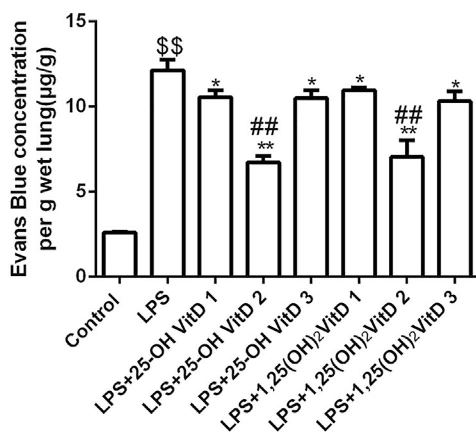
Pulmonary barrier permeability was assessed by using Evans blue dye. Evans blue dye (20 mg/kg) was injected via the caudal vein into mice 30 min prior to anesthesia. After sacrifice, the lung tissues were collected and washed with normal saline immediately. The lung tissues were weighed after drying with filter paper. The Evans blue dye was extracted following homogenization in 1 ml deionized formamide and pulverization using an ultrasonic liquid processor. A further 3 ml deionized formamide and 1 ml deionized formamide were added, and the mixture was incubated at 37 °C for 48 h. The supernatant was separated by centrifugation at 1,000 rpm for 5 min. The quantity of dye extracted was determined spectrophotometrically at 620 nm and calculated from a standard curve established with known amounts of











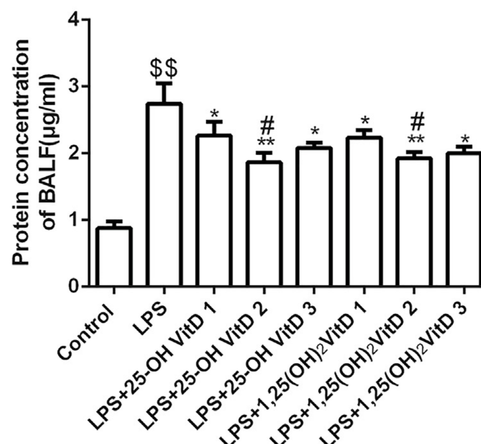


Fig. 1. Vitamin D protects pulmonary histopathological changes from LPS induced lung injury. C57BL6 mice were instilled by intra-tracheal (IT) route with N.S or LPS 10 mg/kg for 24 h. In vitamin D groups, mice received supplementation of 0.1, 1.5, 10 mg/kg 1,25(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ by means of an intra-gastric injection for 14 days pre-intra-tracheal LPS, while the control and LPS groups mice were administered physiological saline. A: lung sections were stained with Hematoxylin–Eosin (original magnification × 100). B: The injury scores of lung section were calculated as Murray injury score. The horizontal bar represents the median, and the boxes represent IQRs. Vertical lines show minimum–maximum range, n = 6C: The serum 25(OH)-vitamin D$_3$ levels in mice with or without 25(OH)-vitamin D$_3$ treatment. Each dot represents an individual mouse (n = 6 per group). Horizontal bars represent means. D, E, F: 1,25(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ alleviated pulmonary permeability in LPS induced acute lung injury. The protein concentration in BALF (D), W/D ratios(E) and the Evans blue leakage(F) were measured. Data are mean ± SD, n = 6. $$ $p < 0.01$ relative to control group, *$p < 0.05$, **$p < 0.01$ compared to LPS group, #$p < 0.05$, ##$p < 0.01$ compared to 0.1, 10 mg/kg 1,25(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ group. 1,2,3 in the B, D, E, F: different concentration of 0.1, 1.5, 10 mg/kg 1,25(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ respectively; BALF: Bronchoalveolar lavage fluid; W/D ratios: Wet/Dry Weight Ratios. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

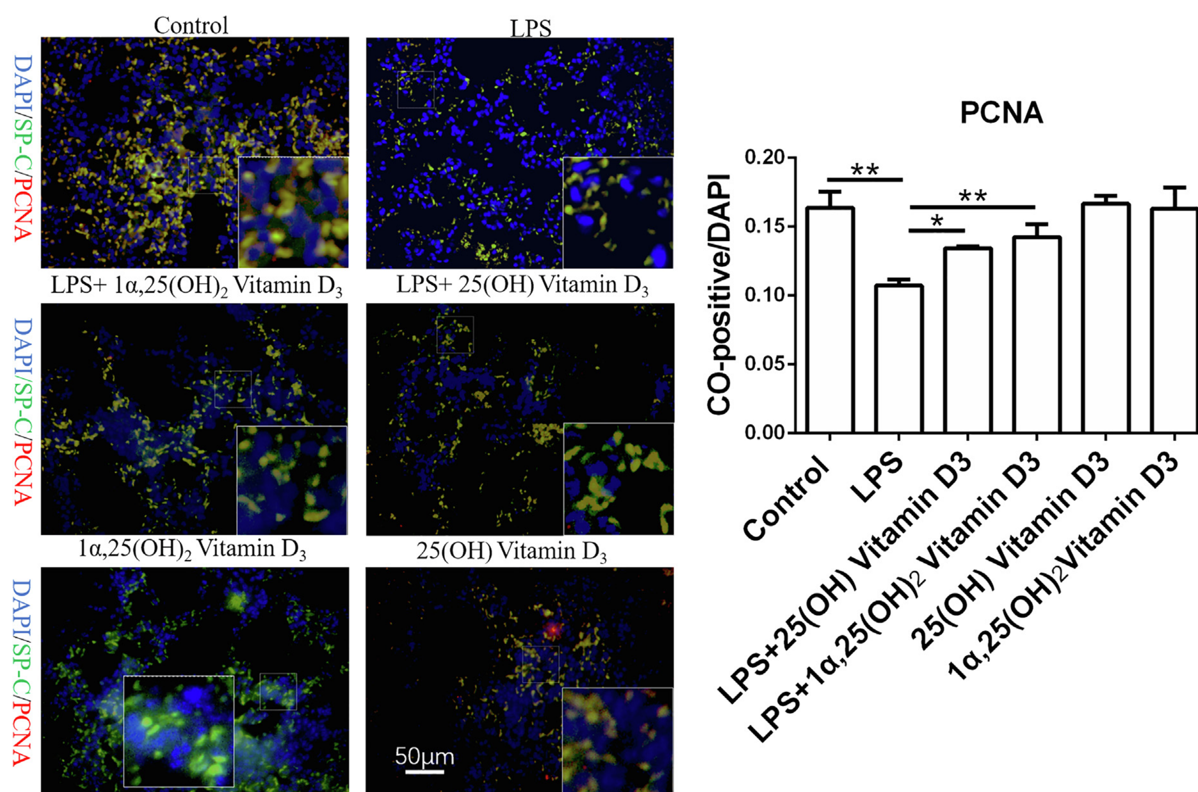


Fig. 2. Vitamin D promotes ATII cells proliferation induced by LPS in mice. C57BL6 mice were instilled by intratracheal (IT) route with N.S or LPS 10 mg/kg for 24 h. In vitamin D groups, mice received supplementation of 1.5 mg/kg 1,25(OH)2-vitamin D3 or 25(OH)-vitamin D3 by means of an intra-gastric injection for 14 days pre-intratracheal LPS, while the control and LPS groups mice were administered physiological saline. A, B: Immunofluorescence staining of formalin fixation lung section was shot by fluorescence microscope and calculated by positive goals compared to nuclear (DAPI, blue signal). We made a co-dying of (SP-C, green signal) and (PCNA, red signal) (×400). The merged images showed a decrease of SP-C and PCNA fluorescence signals after exposure to LPS. Vitamin D treatment promoted SP-C/PCNA double positive cells (proliferating ATII cells) in LPS induced lung injury. $^{*}p < 0.05$, $^{**}p < 0.01$, Data are mean ± SD. n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Evans blue dye. Results are expressed as mg of dye per weight of wet tissue.

2.6. Immunofluorescence microscopy

Paraffin-embedded murine lung tissue sections (5 μm thickness) were obtained. Double labelling fluorescence techniques were used to evaluate ATII cells proliferation, apoptosis and EMT in the lung tissue sections. Briefly, paraffin-embedded sections were baked overnight at 50 °C, deparaffinized with xylene and rehydrated with ethanol and water. Then the sections were ready for surfactant protein-C (rabbit polyclonal anti-SP-C, Sigma, USA, SAB4502837) and proliferating cell nuclear antigen (mouse monoclonal anti-PCNA, Abcam, Cambridge, UK, ab29) immunofluorescence double staining; surfactant protein-C and α-SMA (mouse monoclonal anti-α-SMA, Cell signal Technology, Boston, USA, #48938) immunofluorescence double staining or surfactant protein-C and TUNEL kit (In Situ Cell Death Detection Kit, POD Roche) immunofluorescence double staining. Nuclei were counterstained with 4′, 6-diamino-2-phenylindole (DAPI, Sigma-Aldrich). The intensity of the staining signal was measured and documented using Image-Pro Plus version6. 0 image analysis software. We counted double positive cells in a blinded manner on eight randomly generated visual fields at a high magnification field (×400).

2.7. The protein concentration in bronchoalveolar lavage fluid

The protein concentration in BALF was determined using a BCA protein assay kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, US).

2.8. Vitamin D status

Blood samples were collected from mice eyeball and centrifuged at 3000 rpm (soft) for 30 min at 4 ˚C. The serum in the upper layer was obtained. The levels of 25(OH) Vitamin D_3 in serum were analysis using ELISA kits (ab213966, Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.9. Cell culture

Lung tissue was obtained as part of the Midlands Lung Tissue Collaborative. All procedures in this study were carried out in accordance with approval from the local research ethics committees at the University of Birmingham (Birmingham, UK). All patients gave written informed consent for the use of their tissue and clinical data for research purposes. ATII cells were isolated from peripheral normal lung tissue distal from the tumor in patients undergoing lung cancer resection. We used cells from 14 donors for ATII cell extraction who had normal lung function (8 M:6F, mean age 62.7 years). The cells were isolated in accordance with approval from the local research ethics committees at the University of Birmingham (Birmingham, UK). Primary human alveolar type II (AT II) cells were extracted according to methods described previously [23]. Average yields of primary human alveolar type II cells were 30.2 million cells per resection with an average purity of 92% ATII-like cells. Cells were tested for primary human alveolar type II (AT II) cell phenotype by alkaline phosphatase staining, lysotracker lamellar body staining and by PCR expression of surfactant protein C—a type II cell marker with negative expression of aquaporin V (a type I cell marker) (data not shown) [23]. All

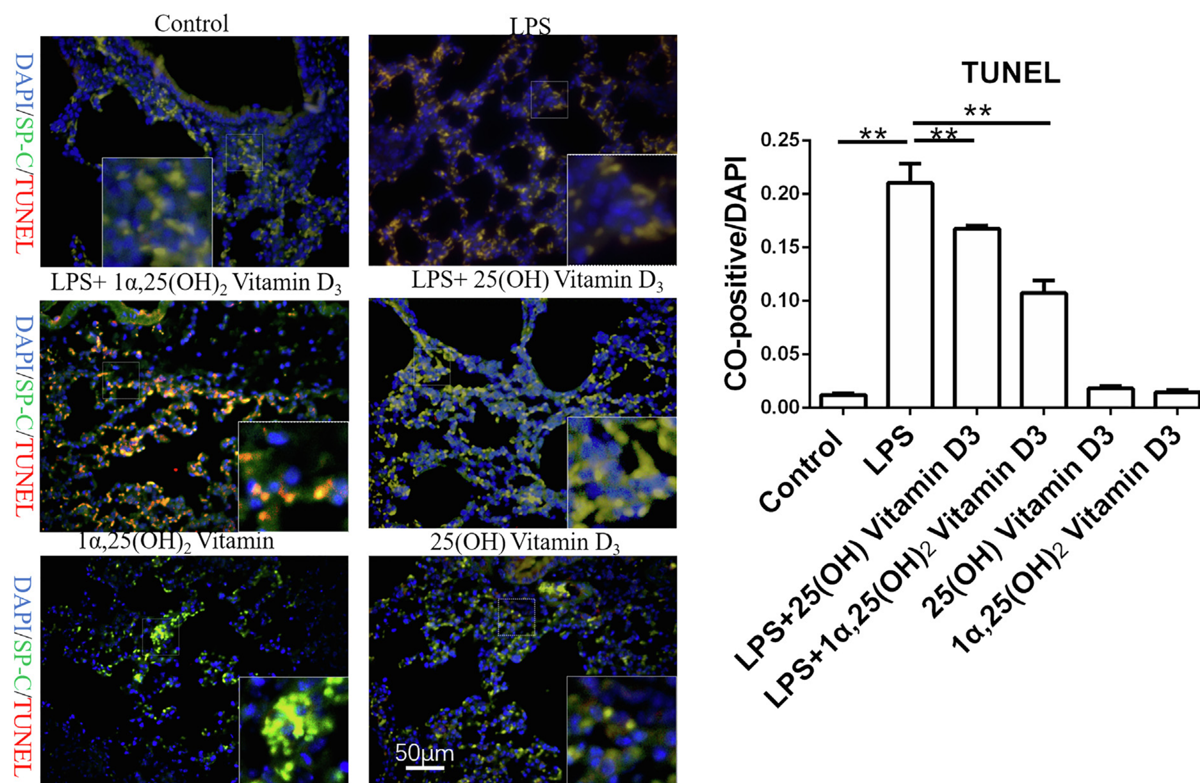


Fig. 3. Vitamin D reduces ATII cells apoptosis induced by LPS in mice. Mice were treated as in Fig. 2. A, B: Immunofluorescence staining of formalin fixation lung section was shot by fluorescence microscope and calculated by positive goals compared to nuclear (DAPI, blue signal). We made a co-dying of (SP-C, green signal) and (TUNEL, red signal) (×400). The merged images revealed that apoptotic epithelial type II cells remarkably increased by LPS. However, 1,25(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ significantly decreased apoptotic epithelial type II cells stimulated by LPS in mice. **$p < 0.01$. Data are mean ± SD. n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiments were repeated in triplicate.

2.10. *Scratch wound repair and proliferation assays were performed as described previously [24]*

2.10.1. *Flow cytometry*

Apoptosis of primary human alveolar type II cells was assessed as described previously using flow cytometry [24]. AT II cells were left in serum-free media for 24 h before exposure to 100 ng/ml Fas-ligand (R& D Sytems, Abingdon, UK). Apoptosis was determined by flow cytometry using the Annexin V and SyTOX antibody according to the manufacturer's recommendations (Molecular Probes, Eugene, OR) after 24 h exposure.

2.11. *Quantitative PCR*

Real time PCR was performed using total RNA from primary human alveolar type II (AT II) cells (RNeasy Mini Kit; Qiagen, Hilden, Germany), the cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany), RNA (1ug) was DNase treated at room temperature and reverse transcribed using superscript RTase and random primers, according to the manufacturer's protocol. mRNA expression was analyzed using Taqman primer/probe (Applied Biosystems) and multiplexed with 18S to account for total loading. Relative mRNA amounts were calculated using CT method [25] ΔCT = Ct target-Ct GAPDH, ΔΔCT = Ct treatment– Ct calibrator, where calibrator was the no-treatment group. Ct was then converted to fold change using the formula $2^{-\Delta\Delta CT}$. Quantitative PCR was performed using commercially obtained primers. Details of PCR primers are showed in Table 1.

2.12. *2.13 Western blot analysis*

Western blot analysis from cells lysates were performed as described previously [26]. After equal amounts of protein were electrophoresed on 10/12% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difloride membranes (Millipore, Billerica MA01821). Western blot analysis was performed using the Image Quant LAS 4000 mini (GE).

2.13. *Blinding method*

The present study adopted randomized, blinded methods. The randomization list of animals was computer-generated by the statistician using SAS/STAT software.

2.14. *Statistical analysis*

Data were expressed as mean ± SD unless otherwise indicated. Differences between three or more groups were analyzed using one-way analysis of variance followed by the Tukey's test for post hoc tests for variables with normal distribution or the Kruskal-Wallis test followed by the Dunn's test for those without a normal distribution. Differences between two groups were analyzed using the two-tailed unpaired Student's *t*-test for variables with a normal distribution or the Mann-Whitney test for variables without a normal distribution. A p value equal or < 0.05 was considered significant. The statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software).

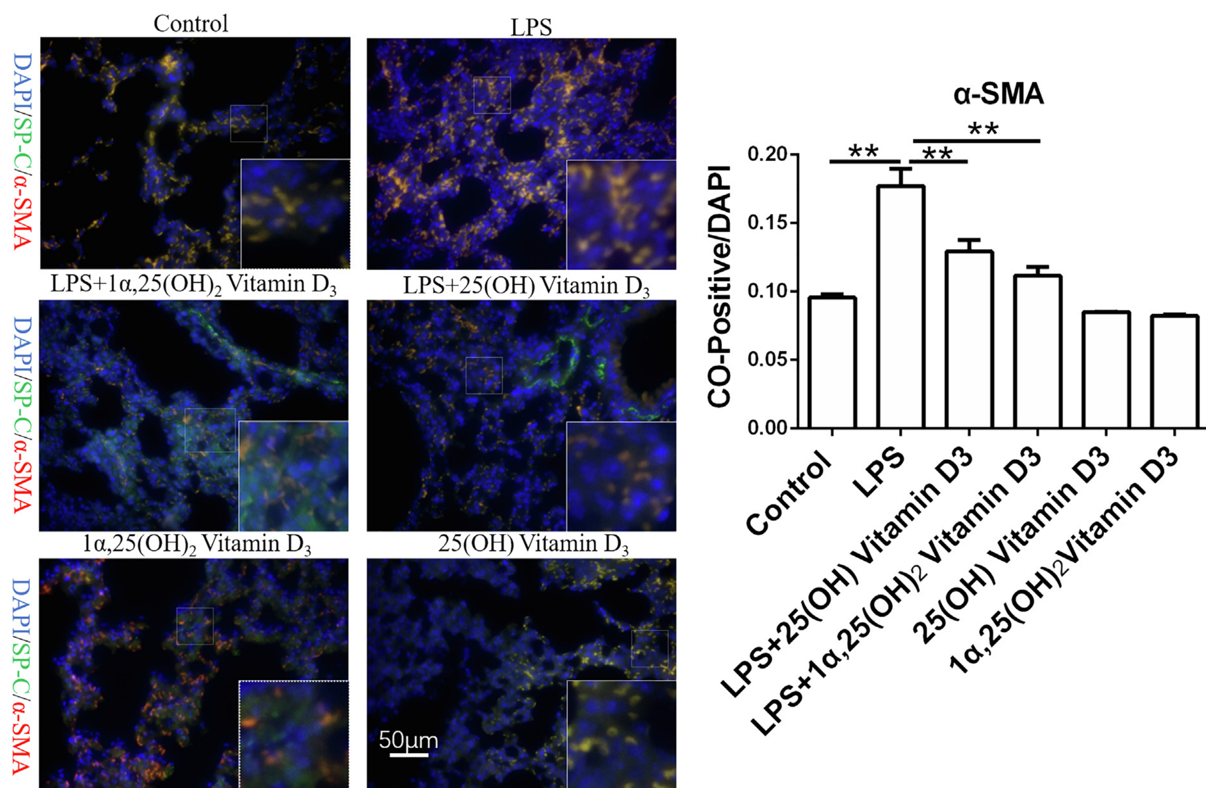


Fig. 4. Vitamin D reduces Epithelial–Mesenchymal Transition (EMT) induced by LPS in mice. Mice were treated as in Fig. 2. A, B: Immunofluorescence staining of formalin fixation lung section was shot by fluorescence microscope and calculated by positive goals compared to nuclear (DAPI, blue signal). We made a co-dying of (SP-C, green signal) and (α-SMA, red signal) (×400). The merged images showed that SP-C/α-SMA double positive signal cells remarkably increased by LPS treatment compared with the control group. However, 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 significantly inhibited SP-C/α-SMA double positive signal cells. ** $p < 0.01$. Data are mean ± SD. n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

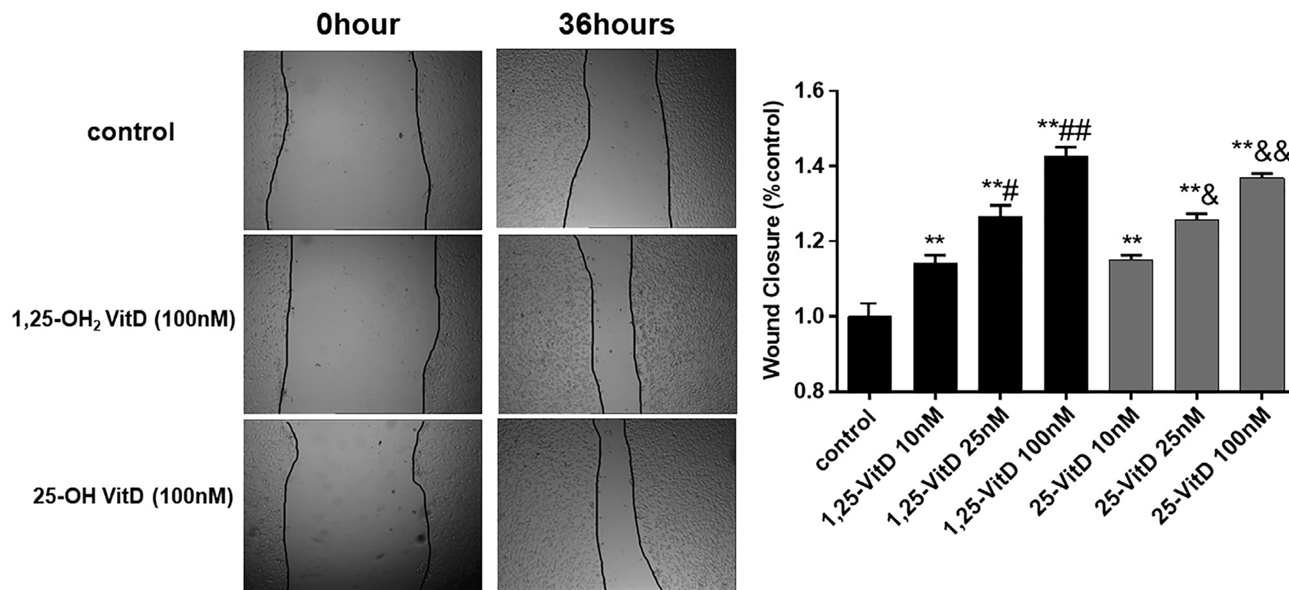


Fig. 5. Vitamin D stimulates primary human ATII cells scratch wound repair in vitro. $1,25(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 at different concentrations was added to monolayers of primary human ATII cells physically wounded with a 1-mL pipette tip. To allow for variability between cell batches, data are expressed as the mean (SE) percentage of the baseline wound size for each separate set of experiments for each culture condition. N = 6 for each culture condition, repeated using cells from 3 donors. *$p < 0.05$, **$p < 0.01$ relative to control group, #$p < 0.05$, ##$p < 0.01$ compared to 1,25vitD 10 nM group, &$p < 0.05$, &&$p < 0.01$ compared to 25vitD 10 nM group.

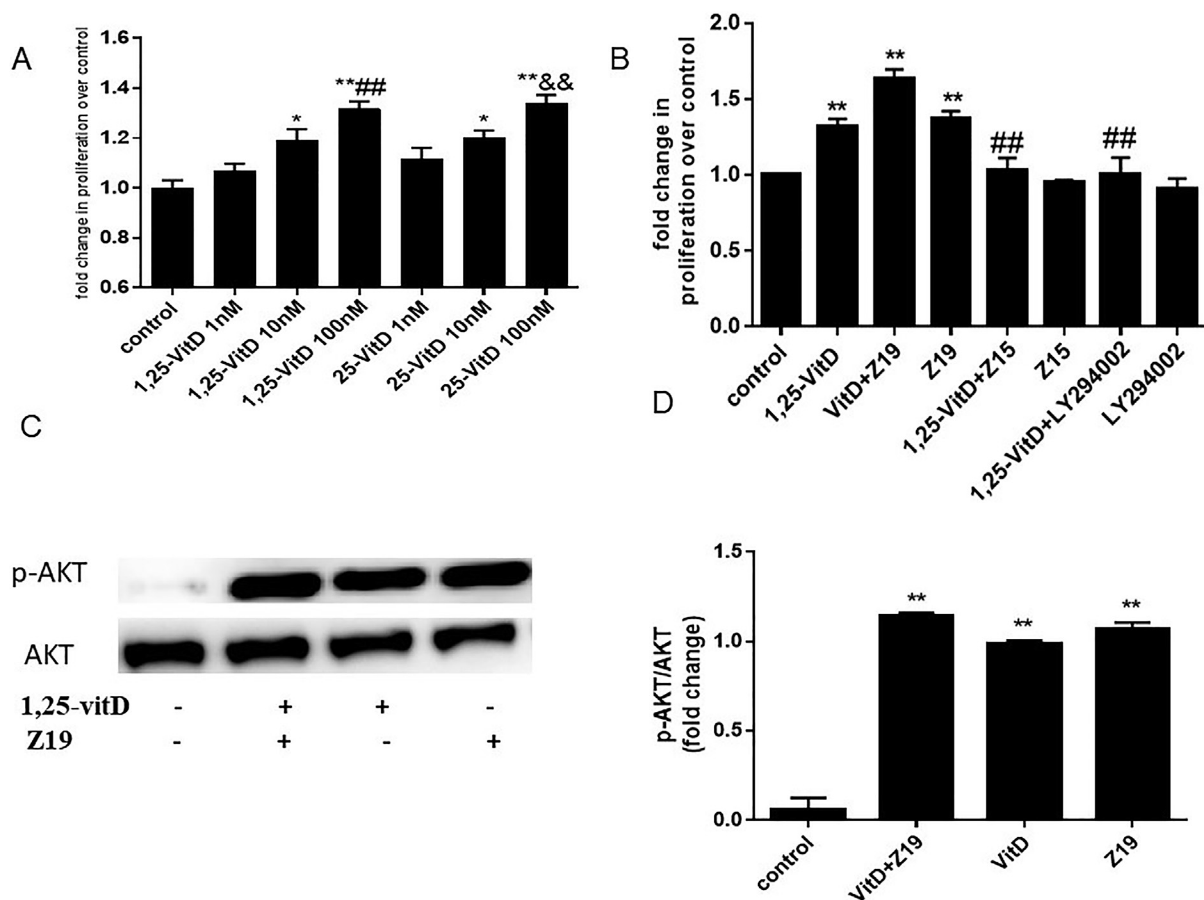


Fig. 6. Vitamin D promotes ATII cells proliferation through activation of vitamin D receptor, the PI3K/AKT signaling pathway. A: 1,25(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ at different concentrations stimulated the proliferation of primary human alveolar type II cells (ATII cells). Values of $>$ 1 fold of control reflect increased proliferation. N = 6 for each culture condition, repeated using cells from 3 donors. *p $<$ 0.05, **p $<$ 0.01 relative to control group, ##p $<$ 0.01 compared to 1,25vitD1nM group, &&p $<$ 0.01 compared to 25vitD 1 nM group. B: LY294002(a phosphatidylinositol 3′-kinase/Akt inhibitor, 10 μM), ZK159222 (VDR antagonist, 1 nM) and ZK191784 (VDR agonist, 1 nM) was respectively pre-incubated with primary human alveolar type II cells for 1 h before 1,25VitD treatment of AT II cells. N = 6 for each culture condition, repeated using cells from 3 donors. **p $<$ 0.01 relative to control group, ##p $<$ 0.01 compared to 1,25vitD group. C, D: ATII cells were incubated with 1,25(OH)$_2$-vitamin D$_3$ and(or) ZK191784 for 24 h. Western-blot analyses were repeated using cells from 3 donors. **p $<$ 0.01 relative to control group.

3. Results

3.1. Vitamin D status in mice

The serum vitamin D status were evaluated in mice with or without vitamin D treatment. As shown in Fig. 1C, in our study, the level of serum 25(OH)-vitamin D$_3$ in the no treatment mice was 14.1 ± 3.03 ng/L, and 21.98 ± 1.42 ng/L in mice treated with 25(OH)-vitamin D$_3$ (p = 0.0002).

3.2. Vitamin D alleviates inflammation and pulmonary permeability in the intratracheal LPS murine model of ALI/ARDS

Intratracheal instillation of LPS in mice resulted in inflammatory change (Fig. 1A, B), such as proteinaceous debris filling the airspaces, hemorrhage, the alveolar wall thickening, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score. These changes in mice were further accompanied by an increase of W/D ratios (Fig. 1D), the protein concentration in BALF (Fig. 1E) and the Evans blue leakage (Fig. 1F) compared with control mice treated with NS. Pre-treatment with different doses (0.1, 1.5, 10 mg/kg) of vitamin D significantly diminished histologic signs of LPS induced inflammation consistent with a decrease in lung injury score (Fig. 1A, B). Moreover, pre-treatment with different doses (0.1, 1.5, 10 mg/kg) of vitamin D dramatically decreased the protein concentration in BALF (Fig. 1E) and Evans blue leakage (Fig. 1F) stimulated by LPS. While higher concentrations of vitamin D (1.5 and 10 mg/kg) reduced the W/D ratio (Fig. 1D) compared with the LPS only group. The protective effect of moderate does (1.5 mg/kg) vitamin D on ALI was greater compared with other groups. These results suggested that vitamin D alleviates lung permeability damage induced by LPS.

3.3. Vitamin D promotes ATII cells proliferation induced by LPS in mice

In vivo, to determine whether vitamin D has an effect on proliferation of ATII cells in LPS-induced lung injury, lung specimens SP-C (a type II cell marker) and PCNA (Proliferating Cell Nuclear Antigen) double staining immunofluorescence were observed (Fig. 2). SP-C/PCNA double positive cells remarkably decreased by LPS treatment compared with the control group (Fig. 2B). However, 1,25-(OH)$_2$-vitamin D$_3$ or 25(OH)-vitamin D$_3$ significantly promoted SP-C/PCNA double positive cells (proliferating ATII cells) in LPS induced lung injury (Fig. 2B).

3.4. Vitamin D reduces ATII cells apoptosis induced by LPS in mice

Double staining using TUNEL in conjunction with

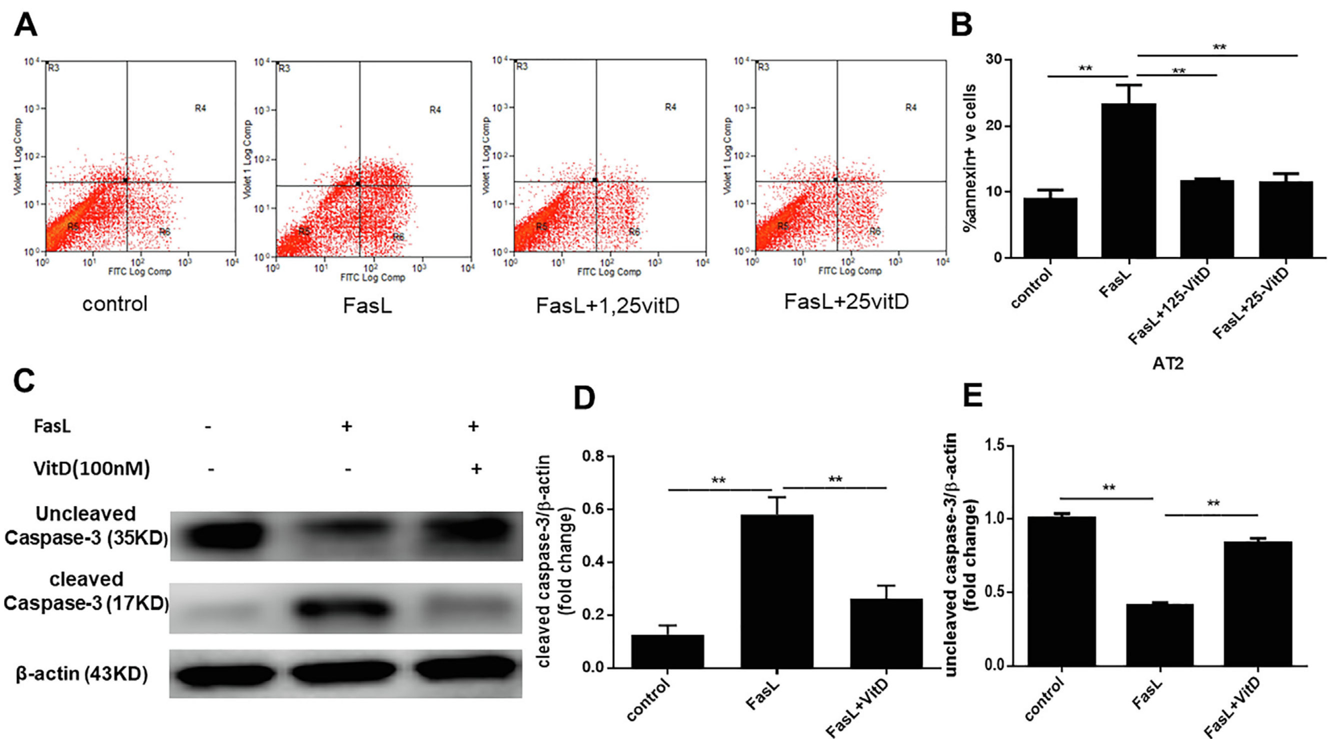


Fig. 7. Effect of vitamin D upon apoptosis and caspase-3 expression in primary human ATII cells. A, B: Primary human Alveolar Type II cells were incubated with 100 ng/mL sFasL and(or) vitamin D (100 nmol/ml) for 24 h. Apoptosis of the ATII cells were evaluated by flow cytometry analysis of annexin-positive cells. Flow cytometry analyses were using cells from 3 donors. $^{*}p < 0.05$, $^{**}p < 0.01$, Data are mean ± SEM. C, D, E: Western blots showing caspase-3 protein in primary human ATII cells treated with 100 ng/mL sFasL or/and 1,25VitD at 100 nM for 24 h. Caspase-3 protein was quantified and analyzed in the indicated groups. Western-blot analyses were repeated using cells from 3 donors. $^{**}p < 0.01$, Data are mean ± SEM.

immunofluorescence for the type II cell marker SP-C revealed that 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 significantly decreased apoptotic epithelial type II cells (double positive cells) in LPS induced lung injury in vivo (Fig. 3A, B). In addition, there is no significance between 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 groups and control group.

3.5. Vitamin D reduces Epithelial–Mesenchymal transition (EMT) induced by LPS in mice

To determine whether vitamin D or/and LPS affect EMT in mice, lung specimens SP-C (a type II cell marker) and α-SMA (α-Smooth muscle actin) double staining immunofluorescence were observed (Fig. 4). We showed that SP-C/α-SMA double positive cells were remarkably increased by LPS treatment (Fig. 4A, B). However, pre-treatment with 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 significantly decreased SP-C/α-SMA double positive cells in the intratracheal LPS murine model of ALI/ARDS (Fig. 4A, B).

3.6. Vitamin D stimulates primary human ATII cells scratch wound repair in vitro

Following on from previous wound repair assays [18] we assessed scratch wound repair over a wide dose range of vitamin D. As shown in Figs. 5, 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 at physiologically relevant concentrations stimulated scratch wound repair in ATII cells after 36 h compared with control group. Scratch wound repair can happen because of the migration and the proliferation of cells. So next we confirmed if 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 can affect primary human ATII cells proliferation.

3.7. Vitamin D promotes ATII cells proliferation through the PI3K/AKT signalling pathway and activation of VitD receptor

Cell proliferation studies in vitro demonstrated that 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 stimulated the proliferation of primary human ATII cells in a dose dependent manner (Fig. 6A). To confirm the effective involvement of vitamin D receptor (VDR) and PI3K/Akt pathway in the vitamin D proliferative response, we performed experiments in primary human ATII cells using the VDR antagonist ZK159222, VDR agonist ZK191784 and the PI3K/AKT inhibitor LY294002. Pre-treatment with LY294002 inhibited the effects of 1,25-$(OH)_2$-vitamin D_3 on ATII cells proliferation suggesting the pro-proliferation effects of 1,25-$(OH)_2$-vitamin D_3 are PI3-kinase dependent (Fig. 6B). ZK159222 pre-treatment inhibited the effects of 1,25-$(OH)_2$-vitamin D_3 on the proliferation of ATII cells while ZK191784 pre-treatment promoted the effects of 1,25-$(OH)_2$-vitamin D_3 on the proliferation of ATII cells suggesting the promoting proliferation effects of 1,25-$(OH)_2$-vitamin D_3 are VDR dependent (Fig. 6B).

Then, we investigated if vitamin D activated AKT Phosphorylation in primary human ATII cells. We found that vitamin D and (or) ZK191784 (VDR agonist) activated AKT phosphorylation (Fig. 6C, D).

3.8. Effect of vitamin D upon apoptosis and caspase-3 expression in primary human ATII cells

Compared with PBS treatment, treatment with Soluble Fas-ligand (sFasL, pro-apoptotic ligands) in primary human ATII cells increased the number of apoptotic cells from 9.05 ± 1.30% in PBS treatment to 23.28 ± 2.94% (P = 0.01). Rescue therapy with vitamin D reduced the number of apoptotic cells (Fig. 7A, B). In addition, vitamin D reduced cell death in response to sFasL and/or TNF-α (data not shown). Caspase-3 activation is the pivotal step in the pathways of apoptosis. Next, we determined if vitamin D and(or) Fas-ligand induced caspase-3

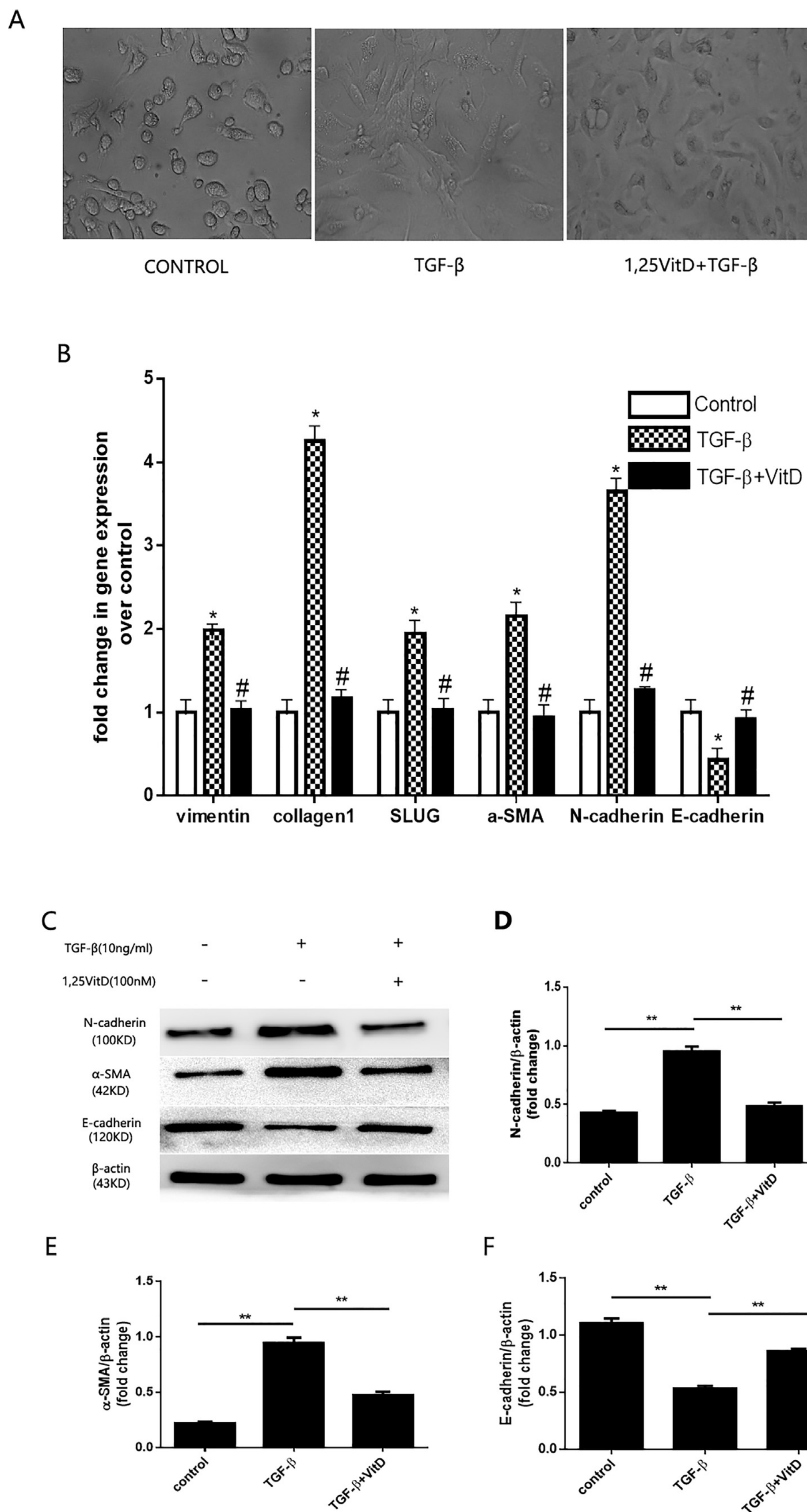


Fig. 8. Vitamin D inhibits TGF-β induced EMT in Primary human alveolar type II cells. Primary human ATII cells were pre-treated with vitamin D for 2 h. The cells were then cultured with TGF-β (10 ng/ml) for 48 h. The Real-time PCR and Western-blot analyses were repeated using cells from 3 donors. A: Phase-contrast images of ATII cells. After incubation, the cells were photographed using the Real time image system under a Leica microscope (Original magnification × 200). B: The mRNA expression of E-cadherin, N-cadherin, vimentin, type I collagen, SLUG, and α-SMA were assessed by Real-time Quantitative PCR Detecting System. *$p < 0.05$ relative to control group respectively, #$p < 0.05$ compared to TGF-β group respectively. C: The protein expression of E-cadherin, N-cadherin and α-SMA by Western Blot and analyzed by densitometry compared to β-actin. **$p < 0.01$.

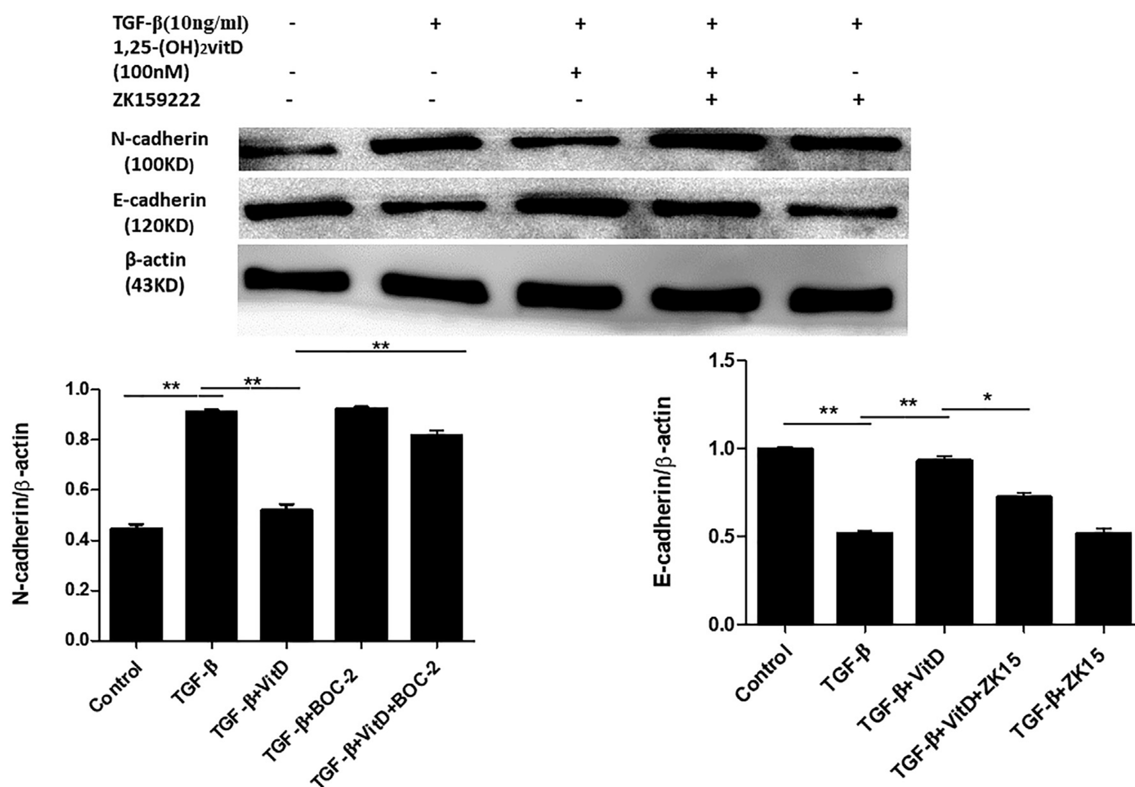


Fig. 9. Vitamin D inhibits TGF-β induced EMT in primary human alveolar type II cells via vitamin D receptor (VDR). ZK159222 (VDR antagonist, 1 nM) was preincubated with primary human alveolar type II cells for 1 h prior to 1,25VitD and TGF-β treatment. After ZK159222 treatment, primary human ATII cells were pretreated with vitamin D (100 nM/ml) for 2 h and then cultured with TGF-β (10 ng/ml) for 48 h. The protein expression of E-cadherin and N-cadherin by Western Blot and analyzed by densitometry compared to β-actin. Western-blot analyses were repeated using cells from 3 donors. $^{*}p < 0.05$, $^{**}p < 0.01$.

activation. The cleaved caspase-3 was detected by the western blotting analysis after the treatment of ATII cells with 100 ng/mL sFasL and(or) vitamin D for 24 h. Fas-ligand promoted caspase-3 activation, 1,25-$(OH)_2$-vitamin D_3 inhibited Fas-ligand induced caspase-3 activation in ATII cells (Fig. 7C, D, E).

3.9. Vitamin D inhibits TGF-β induced EMT in primary human alveolar type II cells through the activation of VitD receptor

Transforming growth factor- β (TGF-β), as a central inducer of the EMT, was used to stimulate EMT in our study. TGF-β treatment induced profound mesenchymal morphology changes (fibroblast-like) in ATII cells, and 1,25-$(OH)_2$-vitamin D_3 restored the epithelial morphology of the cells to a certain extent (Fig. 8A). As shown in Fig. 8B, 1,25-$(OH)_2$-vitamin D_3 promoted the mRNA expression of epithelial cell markers E-cadherin while inhibited the mRNA expression of mesenchymal cell markers including N-cadherin, vimentin, type I collagen, SLUG, and α-SMA. The effects of 1,25-$(OH)_2$-vitamin D_3 on the TGF-β-induced E-cadherin, α-SMA, N-cadherin protein expression of ATII cells were confirmed by western blot (Fig. 8C). The effects of vitamin D on EMT was abrogated by the preincubation of ATII cells with ZK159222 (the VitD receptor antagonist) suggesting that this effect is vitamin D receptor dependent (Fig. 9).

4. Discussion

In the present study, mice were mildly vitamin D deficient, vitamin D remarkedly promoted alveolar epithelial type II cells proliferation, inhibited ATII cells apoptosis and inhibited EMT, with the outcome of attenuated LPS-induced lung injury. In vitro, vitamin D also reduced primary ATII cells apoptosis, promoted alveolar type II (ATII) cell scratch wound repair and proliferation. Vitamin D also inhibited EMT in response to TGF-β through the activation of VitD receptor. Moreover, ZK159222 and LY294002 blocked the effects of vitamin D on the proliferation of ATII cells in vitro, indicating vitamin D promotes ATII cells proliferation through activation of vitamin D receptor, the PI3K/AKT signaling pathway.

Vitamin D_3, the natural form of vitamin D, is mainly formed in the skin from 7-dehydrocholesterol, which depends on the ultraviolet irradiation. In the liver, vitamin D is hydroxylated by the cytochrome *P*-450 enzymes including CYP2R1, CYP27A1, and CYP2D25 to 25(OH) vitamin D_3 [27]. 25(OH)vitamin D_3 (both free and bound to its binding protein) is the major circulating vitamin D_3 metabolite, which is widely used as one of the most reliable biomarkers of vitamin D status [27]. 25(OH)vitamin D_3 is hydroxylated by mitochondrial CYP27B1 to $1,25(OH)_2D_3$, active form of vitamin D which mediates the biological actions via the vitamin D receptor (VDR) [28]. Many studies have reported that CYP27B1 is expressed in extrarenal sites including other tissues (such as placenta, parathyroid gland) and some kinds of cells (such as epithelial cells and immune cells) [29,30]. Our study was based on these data, we found the role of both two forms of vitamin D (25(OH)-vitamin D_3 and $1,25(OH)_2$-vitamin D_3) in animal model and ATII cells were identical suggesting that ATII cells could convert 25(OH)vitamin D_3 to active form.

Several studies have reported that vitamin D deficiency was associated with ARDS [31,32]. Other studies have also suggested that the vitamin D/VDR signaling pathway had the protective function against ARDS induced by LPS [33,34]. In our animal model of LPS-induced lung injury, we have demonstrated that increased epithelial damage was evident in response to LPS challenge when vitamin D deficiency is present, and vitamin D diminished histologic signs of LPS induced alveolar inflammation, reduced the pulmonary epithelial barrier permeability suggesting that vitamin D has protective effects on the alveolar epithelium. Importantly, our data revealed that the beneficial effects of

moderate vitamin D were greater in those with vitamin D deficiency.

The degree of alveolar epithelial damage is an important predictor of outcome in ARDS. Injury to the alveolar epithelial barrier can activate certain apoptotic markers of caspases and results in the excessive apoptosis of alveolar epithelial cells during the development of ARDS [35,36].The restoration of the alveolar epithelial barrier is a critical factor for recovery in ARDS [37,38]. Previous studies indicated that vitamin D played a crucial role in maintaining the structure and function of epithelial barriers in many tissues [39–41]. Zhang H et al revealed that Vitamin D treatment greatly reduced the airway inflammatory response and cellular apoptosis in the lung tissue of a mouse model of asthma [42]. In our murine model, pre-treatment with 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 stimulated alveolar epithelial cells proliferation and restored epithelial barriers. In addition, we also showed that the apoptotic type II cells (SP-C/TUNEL double positive cells) induced by LPS were decreased by 1,25-$(OH)_2$-vitamin D_3 as well as 25(OH)-vitamin D_3.

In our murine model, we have provided the evidence of vitamin D in promoting proliferation and reducing apoptosis of ATII cells, but to predict the effect of vitamin D on permeability of the alveolar capillary barrier in human using an animal model, the species difference in lung metabolism should be taken into consideration. In our study, primary human alveolar type II cells were used to assess the effect of vitamin D upon proliferation, migration and apoptosis in vitro. Our study clearly demonstrated that 1,25-$(OH)_2$-vitamin D_3 or 25(OH)-vitamin D_3 promoted alveolar epithelial scratch wound closure and proliferation of primary alveolar epithelial cells in a dose dependent manner. It is well-known that the PI3K-AKT signaling pathway is a key regulator of proliferation. Our study suggested that 1,25-$(OH)_2$-vitamin D_3 promotes primary human ATII cells proliferation through activation of vitamin D receptor, the PI3K/AKT signaling pathway, which is consistent with previous study [43]. Furthermore, our study showed that vitamin D inhibited apoptosis in sFasL-treated primary human ATII cells, which seems to be related to the activation of caspase-3.

In ARDS, the alveolar epithelial cells undergo not only apoptosis but also epithelial–mesenchymal transition (EMT). EMT is considered as a key phenomenon occurring in the lung injury which could ultimately lead to the prognosis of ARDS and lung fibrosis [44]. In our animal model of LPS-induced lung injury, we showed that ATII cells gained mesenchymal biomarkers (α-SMA), which was decreased by treatment with vitamin D. we also confirmed the above results in vitro. In TGF-β-induced primary human ATII cells, 1,25-$(OH)_2$-vitamin D_3 promoted the expression of epithelial cell markers while inhibited the expression of mesenchymal cell markers. Our data is in keeping with recently published data in bronchial epithelia cells or Ovarian Cancer SKOV-3 cells treated with TGF-β [45,46]. Fischer et al also reported that a vitamin D agonist, calcitriol inhibited EMT induced by TGF-β in bronchial epithelia cells (BEAS-2B). Similarly, we also revealed 1,25-$(OH)_2$-vitamin D_3 inhibited EMT induced by TGF-β in primary ATII cells. Furthermore, we demonstrated that the effects of vitamin D on EMT was abrogated by the preincubation of ATII cells with ZK159222 (the vitamin D receptor antagonist) suggesting that this effect is vitamin D receptor dependent.

In conclusion, our findings indicate that exogenous vitamin D attenuates lung injury via stimulating ATII cells proliferation and migration, reducing ATII cells apoptosis and inhibits TGF-β induced EMT. Our findings reveal vitamin D has therapeutic potential for the resolution of ARDS which is associated with significant alveolar epithelial barrier damage.

CRediT authorship contribution statement

ShengXing Zheng: Data curation, Writing - original draft, Supervision. **JingXiang Yang:** Data curation, Writing - original draft, Software, Validation. **Xin Hu:** Data curation, Writing - original draft. **Ming Li:** Data curation, Writing - original draft. **Qian Wang:** Visualization, Investigation. **Rachel C.A. Dancer:** Writing - review & editing. **Dhruv Parekh:** Writing - review & editing. **Fang Gao-Smith:** Conceptualization. **David R. Thickett:** Conceptualization, Methodology, Software. **ShengWei Jin:** Conceptualization, Methodology, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

Lung tissue was obtained as part of the Midlands Lung Tissue Collaborative. All procedures in this study were carried out in accordance with approval from the local research ethics committees at the University of Birmingham (Birmingham, UK). All patients gave written informed consent for the use of their tissue and clinical data for research purposes.

All procedures were conducted in accordance with the guide for the Care and Use of Laboratory. Animals adopted by the national institutes of health and approved by Animal studies ethics committee of Wenzhou Medical University.

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