

Vitamin D₃ Mediated Molecular Mechanisms for the Function of the Alveolar-Capillary Barrier in Vitro

Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

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Erstgutachterin: Prof. Dr. Sabine Fuchs

Zweitgutachter: Prof. Dr. Thomas Roeder

Tag der mündlichen Prüfung: 12.07.2024

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Abstract

Background: The bio-active Vitamin D₃, 1- α -25-dihydroxy Vitamin D₃ (1 α ,25(OH)₂D₃), is well known for its functions in the preservation of bone homeostasis. A sufficient level of Vitamin D₃ in the human blood serum is also required to sustain the function and immunity of human organs, including the lung.

The alveolar-capillary barrier plays a critical role in the respiration function of the lung. However, the effect of Vitamin D₃ on the maintenance of the function properties of the alveolar-capillary barrier and its effect on the protection of the alveolar-capillary barrier from lung function disorders caused by pathogen infections still need to be verified. Furthermore, the specific physiological biomolecular mechanisms underlying its effects still need to be elucidated. Therefore, this study was motivated to clarify these subjects.

Methods: Instead of investigating in vivo lung tissues, this study used an in vitro co-culture model of alveolar epithelial cells and capillary microvascular endothelial cells in Transwell® to simulate the alveolar-capillary barrier in vivo.

This study also performed the mono-culture models of individual cell types including alveolar epithelial cells and capillary microvascular endothelial cells respectively. The mono-culture of alveolar epithelial cells in Transwell® was also applied to mimic the alveolar epithelial barrier in vivo. The lung bronchial adenocarcinoma epithelial cell lines including NCI-H441 cells, Calu-3 cells, and A549 cells were used as the alveolar epithelial cells in this study. The isolated outgrowth endothelial cells (OECs), were implemented as the capillary microvascular endothelial cells in this study.

Results: This study first investigated the impact of Vitamin D₃ on the properties of the alveolar-capillary barrier, which was evaluated through Trans-epithelial, endothelial Resistance (TEER) measurement. The results demonstrated that Vitamin D₃ (10 nM) significantly increased the TEER values of the mono-culture model of alveolar epithelial cell lines including NCI-H441 cells and Calu-3 cells in Transwell® and the co-culture model of NCI-H441 cells and OECs in Transwell®. Moreover, Vitamin D₃ (10 nM) significantly increased and maintained the TEER values in the co-culture model of NCI-H441 cells and OECs during the challenge with the bacterial endotoxin (LPS) treatment. Vitamin D₃ (10 nM) also maintained the properties of OECs- modeled capillary microvascular endothelial barrier

and sustained its properties in confront to LPS treatment, which was demonstrated with the Electric Cell-substrate Impedance Sensing (ECIS) measurement.

Real-time PCR analysis demonstrated that Vitamin D₃ (10 nM) attenuated the LPS-induced increased gene expression of the pro-inflammatory cytokine IL6 in NCI-H441 cells and attenuated LPS-induced significant gene expression of IL-1 β , IL6, and IL-8 in OECs. Vitamin D₃ (10 nM) significantly increased the expression of the anti-microbial peptide, Cathelicidin LL-37, in NCI-H441 cells. In addition, Vitamin D₃ (10 nM) increased the protein expression of intercellular junction proteins including tight junction proteins ZO-1, Occludin, and adherens junction protein E-Cadherin in NCI-H441 cells, and increased their integrity on the cell membrane barrier of NCI-H441 cells, which were demonstrated through Western-Blot and Immunofluorescence staining analysis.

Moreover, Vitamin D₃ (10 nM) significantly elevated the expression of Doublecortin-like kinase 1 (DCLK1) in NCI-H441 cells at both gene and protein levels. The previous mass spectrometry proteomic analysis in collab with Professor Andreas Tholey at the Univerity of Kiel (CAU, Kiel) demonstrated that Vitamin D₃ (10 nM) significantly increased the protein abundance of DCLK1 in NCI-H441 cells. However, the impact and concrete mechanisms of DCLK1 in maintaining the function properties of the alveolar epithelial barrier are still barely reported. This study implemented the DCLK1 specified inhibitor Leucine-rich repeat kinase 2 inhibitor 1 (LRRK2-IN-1) for the investigation of the role of DCLK1 in the properties maintenance of the alveolar epithelial barrier. The results demonstrated that the moderate inhibition of the expression of DCLK1 with the DCLK1 specified inhibitor, LRRK2-IN-1 (500 nM), even enhanced the positive effect of Vitamin D₃ (10 nM) in improving the properties of the NCI-H441 cells-assembled alveolar epithelial barrier. In particular, LRRK2-IN-1 (500 nM) increased the expression of E-Cadherin in NCI-H441 cells at both gene and protein levels.

Conclusion: This study verified the positive effects of Vitamin D₃ in strengthening the function properties of the alveolar-capillary barrier. Vitamin D₃ exerts its effect in maintaining and improving the function properties of the alveolar-capillary barrier for the defense against LPS infection through its positive effects in increasing the protein expression and integrity maintenance of junction proteins in alveolar epithelial cells, and its significant effect in increasing the expression of LL-37 in alveolar epithelial cells. Moreover, Vitamin

D₃ maintains the function properties of the alveolar-capillary barrier against LPS infection through its effect in attenuating the LPS-induced significantly increased expression of the pro-inflammatory cytokines in alveolar epithelial cells and capillary microvascular endothelial cells. These findings suggest the potential molecular mechanism of Vitamin D₃ in the properties maintenance and protection of the alveolar-capillary barrier for the prevention of LPS-induced disruption of the alveolar-capillary barrier. The adequate inhibition of the Vitamin D₃-induced expression of DCLK1 in alveolar epithelial cells further enhances the effects of Vitamin D₃ in improving the function properties of the alveolar epithelial barrier.

Keywords: Vitamin D₃, LPS, Alveolar-Capillary Barrier, Barrier Properties, DCLK1 Inhibition

Zusammenfassung

Hintergrund: Das bioaktive Vitamin D₃, 1- α -25-Dihydroxy-Vitamin D₃ (1 α ,25(OH)₂D₃), ist für seine Funktionen bei der Erhaltung der Knochenhomöostase bekannt. Ein ausreichender Vitamin-D₃ Spiegel im menschlichen Blutserum ist auch erforderlich, um die Funktion und Immunität menschlicher Organe, einschließlich der Lunge, zu erhalten.

Die Alveolar-Kapillaren-Schranke (Alveolar-Kapillaren Barriere) spielt eine entscheidende Rolle für die Atmungsfunktion der Lunge. Die Wirkung von Vitamin D₃ auf die Aufrechterhaltung der Funktionseigenschaften der Alveolar-kapillare Barriere und seine Wirkung auf den Schutz der Alveolar-Kapillaren Barriere vor Lungenfunktionsstörungen, die durch Pathogeninfektionen verursacht werden, müssen jedoch noch überprüft werden. Darüber hinaus müssen die spezifischen physiologischen biomolekularen Mechanismen, die seinen Wirkungen zugrunde liegen, noch aufgeklärt werden. Daher wurde diese Studie durchgeführt, um diese Fragen zu klären.

Methoden: Anstatt Lungengewebe in vivo zu untersuchen, wurde in dieser Studie ein in vitro Co-Kulturmodell von Alveolarepithelzellen und kapillaren mikrovaskulären Endothelzellen in Transwell® verwendet, um die Alveolar-Kapillaren Barriere in vivo zu simulieren.

In dieser Studie wurden auch Monokulturmodelle einzelner Zelltypen, einschließlich Alveolarepithelzellen und kapillare mikrovaskuläre Endothelzellen, durchgeführt. Die Monokultur von Alveolarepithelzellen in Transwell® wurde auch zur Nachahmung der Alveolarepithelbarriere in vivo verwendet. Die Epithelzelllinien des bronchialen Adenokarzinoms der Lunge, darunter NCI-H441-Zellen, Calu-3-Zellen und A549-Zellen, wurden in dieser Studie als Alveolarepithelzellen verwendet. Die isolierten Outgrowth-Endothelzellen (OECs) wurden in dieser Studie als kapillare mikrovaskuläre Endothelzellen eingesetzt.

Ergebnisse: In dieser Studie wurden zunächst die Auswirkungen von Vitamin D₃ auf die Eigenschaften der Alveolar-Kapillaren Barriere untersucht, die durch die Messung des trans-epithelialen, endothelialen Widerstands (TEER) bewertet wurden. Die Ergebnisse zeigten, dass VitaminD₃ (10 nM) die TEER-Werte des Monokulturmodells von Alveolarepithelzelllinien einschließlich NCI-H441-Zellen und Calu-3-Zellen in Transwell® und des

Co-Kulturmodells von NCI-H441-Zellen und OECs in Transwell® signifikant erhöhte. Darüber hinaus erhöhte Vitamin D₃ (10 nM) die TEER-Werte im Co-Kulturmodell von NCI-H441-Zellen und OECs während der Behandlung mit bakteriellem Endotoxin (LPS) signifikant und hielt sie aufrecht. Vitamin D₃ bewahrte auch die Eigenschaften der von OECs modellierten kapillaren mikrovaskulären Endothelbarriere und hielt deren Eigenschaften gegenüber der LPS-Behandlung aufrecht, was mit der ECIS-Messung (Electric Cell-substrate Impedance Sensing) nachgewiesen wurde.

Realtime-PCR-Analysen zeigten, dass Vitamin D₃ (10 nM) die LPS-induzierte erhöhte Genexpression des pro-inflammatorischen Zytokins IL6 in NCI-H441-Zellen abschwächte und die LPS-induzierte signifikante Genexpression von IL-1 β , IL6 und IL-8 in OECs abschwächte. Vitamin D₃ (10 nM) erhöhte signifikant die Expression des antimikrobiellen Cathelicidin-Peptids LL-37 in NCI-H441-Zellen. Darüber hinaus erhöhte Vitamin D₃ die Proteinexpression der interzellulären Verbindungsproteine, einschließlich der Tight Junction-Proteine ZO-1, Occludin und des Adherens Junction-Proteins E-Cadherin, in NCI-H441-Zellen und erhöhte ihre Integrität auf der Zellmembranbarriere von NCI-H441-Zellen, was durch Western-Blot und Immunfluoreszenz-Färbearanalysen nachgewiesen wurde.

Darüber hinaus, erhöhte Vitamin D₃ (10 nM) signifikant die Expression der Doublecortin-like kinase 1 (DCLK1) in NCI-H441-Zellen sowohl auf Gen- als auch auf Proteinebene. Die vorangegangene massenspektrometrische Proteomanalyse in Zusammenarbeit mit Professor Andreas Tholey, an der Universität Kiel (CAU, Kiel) zeigte, dass Vitamin D₃ (10 nM) die Proteinhäufigkeit von DCLK1 in NCI-H441-Zellen signifikant erhöhte. Die genaue Rolle von DCLK1 bei der Aufrechterhaltung der Funktionseigenschaften der Alveolarepithelbarriere ist jedoch noch kaum erforscht. In dieser Studie wurde der DCLK1-spezifische Inhibitor, Leucine-rich repeat kinase 2 inhibitor 1 (LRRK2-IN-1), für die Untersuchung der Rolle von DCLK1 bei der Aufrechterhaltung der Eigenschaften der Alveolarepithelbarriere eingesetzt. Die Ergebnisse zeigten, dass die moderate Hemmung der Expression von DCLK1 mit dem DCLK1-spezifischen Inhibitor LRRK2-IN-1 (500 nM) sogar die positive Wirkung von Vitamin D₃ (10 nM) bei der Verbesserung der Eigenschaften der aus NCI-H441-Zellen bestehenden Alveolarepithelbarriere verstärkte. Insbesondere erhöhte LRRK2-IN-1 (500 nM) die Expression von E-Cadherin in NCI-H441-Zellen sowohl auf Gen- als auch auf Proteinebene.

Schlussfolgerung: Diese Studie belegt die positiven Auswirkungen von Vitamin D₃ auf die Stärkung der Funktionseigenschaften der Alveolar-Kapillaren Barriere. Vitamin D₃ übt seine Wirkung bei der Aufrechterhaltung und Verbesserung der Funktionseigenschaften der Alveolar-Kapillaren Barriere für die Abwehr von LPS-Infektionen durch seine positiven Effekte bei der Erhöhung der Proteinexpression und der Aufrechterhaltung der Integrität von Verbindungsproteinen in alveolaren Epithelzellen und seine signifikante Wirkung bei der Erhöhung der Expression von LL-37 in alveolaren Epithelzellen aus. Darüber hinaus erhält Vitamin D₃ die Funktionseigenschaften der alveolar-Kapillaren Barriere gegen LPS Infektion aufrecht, indem es die LPS-induzierte, signifikant erhöhte Expression der pro-inflammatorischen Zytokine in alveolären Epithelzellen und kapillaren mikrovaskulären Endothelzellen abschwächt. Diese Ergebnisse deuten auf einen potenziellen molekularen Mechanismus von Vitamin D₃ bei der Aufrechterhaltung und dem Schutz der Alveolar-Kapillaren Barriere zur Verhinderung einer LPS-induzierten Störung der Alveolar-Kapillaren Barriere hin. Die adäquate Hemmung der Vitamin D₃-induzierten Expression von DCLK1 in Alveolarepithelzellen verstärkt die Wirkung von Vitamin D₃ bei der Verbesserung der Funktionseigenschaften der Alveolarepithelbarriere.

Stichwörter: Vitamin D₃, LPS, Alveolar-Kapillaren Barriere, Barriereeigenschaften, DCLK1 Hemmung

Acronyms

| | |
|--------------|---|
| AJs | Adherent Junctions |
| AT I Cs | Alveolar Epithelial Type 1 Cells |
| AT-II Cs | Alveolar Epithelial Type 2 Cells |
| ALI | Acute Lung Injury |
| AMP | Antimicrobial Peptides |
| ARDS | Acute Respiratory Distress Syndrom |
| BSA | Bovine Serum Albumin |
| cDNA | Complementary DNA |
| CD31 | Cluster of Differentiation 31 |
| CD44 | Cluster of Differentiation 44 |
| CD74 | Cluster of Differentiation74 |
| COPD | Chronic Obstructive Pulmonary Diseases |
| CTSS | Cathepsin S |
| CF | Cystic Fibrosis |
| DCLK1 | Doublecortin-like Kinase 1 |
| DMSO | Dimethyl Sulfoxide |
| E-Cadherin | Epithelial Cadherin |
| ECIS | Electric Cell-substrate Impedance Sensing |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal Growth Factor |
| EGM | Endothelial Cell Growth Medium |
| EMT | Epithelial-Mesenchymal Transition |
| Epis | Eppendorf Tubes |
| FBS | fetal Bovine Serum |
| ICAM-1 | Intercellular Adhesion Molecule-1 |
| IL-1 β | Interleukin-1 β |
| IL-6 | Interleukin-6 |

| | |
|----------------|---|
| IL-8 | Interleukin-8 |
| IFNs | Interferons |
| JAMs | Junction Adhesion Molecules |
| LDS | Lithium dodecyl Sulfate Loading Buffer |
| LL37 | Leucine Leucine-37 |
| LPS | Lipopolysaccharide |
| LPB | LPS Binding Proteins |
| LRRK2-IN-1 | Leucine-rich Repeat Kinase 2 Inhibitor 1 |
| MAP | Microcomputers-associated Protein |
| MIF | Macrophage Migration Inhibitory Factor |
| MCP-1 | Monocyte Chemoattractant Protein 1 |
| mL | Milliliter |
| μ L | Microliter |
| mG | Milligram |
| mM | Millimolar |
| MS | Mass Spectrometry |
| μ M | Micromolar/Liter |
| MD2 | Myeloid Differentiation Protein 2 |
| NF- κ B | Nuclear Factor Kappa B |
| NKs | Natural Killing Cells |
| nM | Nanomolar/Liter |
| nG | Nanogram |
| OEC | Outgrowth Endothelial Cells |
| PCR | Polymerase Chain Reaction |
| PAMP | Pathogen-associated Molecular Patterns |
| PBS | Phosphate-Buffered Saline |
| PECAM-1 | Platelet Endothelial Cell Adhesion Molecule 1 |
| PFA | Paraformaldehyde |
| PKCA | Protein Kinase C A (α) |
| PKCB | Protein Kinase C B (β) |
| PKCH | Protein Kinase C H (η) |

| | |
|-----------------|---|
| PVDF membrane | Polyvinylidene Fluoride |
| RPMI | Roswell Park Memorial Institute cell culture medium |
| RT-PCR | Real-time Polymerase chain reaction |
| RXR | Retinoid X Receptor |
| SP-A | Surfactant Protein A |
| SP-B | Surfactant Protein B |
| SP-C | Surfactant Protein C |
| SP-D | Surfactant Protein D |
| SCCs | Solitary chemosensory cells |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| TBS/T Buffer | Tris Buffered Saline with Tween Buffer |
| TEER | Trans Epithelial/Trans Endothelial Electrical Resistance |
| TGFs | Transforming Growth Factors |
| TJs | Tight Junctions |
| TLRs | Toll-like receptors |
| TNF- α | Tumor Necrosis Factor- α |
| VD ₃ | 1- α -25-dihydroxy Vitamin D ₃ |
| VDR | Vitamin D Receptor |
| VDRE | Vitamin D Responsive Elements |
| ZO-1 | Zona Occludens-1 |

1 Introduction

1.1 Background

The pulmonary alveolar-capillary barrier, which is formed by pulmonary alveolar epithelial cells and pulmonary capillary microvascular endothelial cells, is vital for the gas exchange in the alveoli during respiratory processes.

Previous studies have demonstrated that 1- α -25-dihydroxy Vitamin D₃ (Vitamin D₃) is also essential for the integrity maintenance of the alveolar epithelial barrier [1]. People with Vitamin D₃ deficiency in their blood serum may have a higher risk of suffering from acute lung injuries (ALI), acute respiratory distress syndromes (ARDS), and chronic obstructive pulmonary diseases (COPD) caused by pathogen infections, such as bacterial endotoxin lipopolysaccharide (LPS) [6, 7]. The disruption of the alveolar-capillary barrier (alveolar epithelial-pulmonary capillary microvascular endothelial barrier) induced by bacterial and viral infections causes high numbers of clinical death cases worldwide.

However, the effect of Vitamin D₃ on maintaining the function properties of the alveolar-capillary barrier still needs to be verified and the concrete physiological biomolecular mechanisms involved in the effects of Vitamin D₃ are still not fully elucidated. Moreover, many factors including the complicated micro-environment context, multiple cellular interactions in lung alveoli, and technical restrictions hamper the investigation of the alveolar-capillary barrier *in vivo*.

Under this condition, this study established a co-culture model of alveolar epithelial cells and capillary microvascular endothelial cells in Transwell® *in vitro* to simulate the lung alveolar epithelial-pulmonary capillary microvascular endothelial barrier (alveolar-capillary barrier) *in vivo*. This study also set up mono-culture models of individual cell types of alveolar epithelial cells and capillary microvascular endothelial cells. These established *in vitro* cell culture models simplify the study of the impact of the agents, in particular, Vitamin D₃ and the bacterial endotoxin, lipopolysaccharide (LPS) on the function properties of the alveolar-capillary barrier.

These in vitro cell culture models also simplify the investigation of the expression of the specific associated target molecules in the individual cell types. This strategy enables this study to indicate the physiological biomolecular mechanisms, that contribute to the impact of Vitamin D₃ and LPS etc. on the function properties of the alveolar-capillary barrier.

1.2 Structures of Lung and Alveolar-Capillary Barrier

The lung is a vital organ for the performance of respiration. During the breath, the oxygen (O₂) in the air is delivered into the lung, which is closely associated with its peripheral blood vessel. The inhaled oxygen is absorbed by blood and delivered to all parts of the human body through the bloodstream. Meanwhile, the bloodstream circularly delivers carbon dioxide (CO₂) to the lung. Then the CO₂ is diffused into pulmonary airspace and breathed out through the trachea.

The Lung is constituted of the left lung and right lung. The trachea branches are divided into the two main bronchi, which are connected to each lung's airways [12]. The pulmonary airway has a character with its fractional structure. The trachea is initially divided into the bronchi, which are further subdivided several times into smaller bronchioles subdivisions [13]. The pulmonary acini are the airway terminal unit which is constituted of respiratory bronchioles, alveolar ducts, and air sacs or alveoli [13].

The alveoli are also the termination sites of the inhaled air. The pulmonary acinus is defined as the respiratory unit) which is the gas exchange area during respiration. It has been found that there are about 30,000 acini in the human lung [13]. The diameter length of each acinus is 3.6 micrometers, and each acinus is constituted of approximately 10,000 alveoli [12]. Alveoli are the terminal branchings of the respiratory airways and are the most important respiratory unit for the gas exchange of human lung [14].

The alveolar-capillary barrier is an extremely thin interface barrier (2 μm) of the alveolar air space and the pulmonary capillaries which has a large surface area (in the human lung with an area of approximately 140 m²) [14]. The diffusion character of the alveolar-capillary barrier enables rapid gas exchange during respiration. During the gas exchange, the oxygen

diffuses into the blood in the pulmonary capillaries through the pressure gradient of the alveolar epithelium, and carbon dioxide reversely diffuses into the alveoli from the peripheral pulmonary capillaries [15].

The alveolar-capillary barrier is one of the most critical tissue barriers in human organs. The interface of the alveolar-capillary barrier is structured with the pulmonary alveolar epithelial barrier (alveolar epithelium) and pulmonary capillary microvascular endothelial barrier (pulmonary capillary endothelium) (Fig. 1.1). The alveolar epithelial barrier interface is composed of two alveolar epithelial cell types including the differentiated squamous alveolar epithelial type I cells (AT-I cells), and the cuboidal alveolar epithelial type II cells (AT-II cells) [16]. Approximately 93% area of the alveolar epithelium surface is covered by AT-I cells, and AT-I cells occupy an appropriate 33% of the alveolar epithelial cell numbers [16]. AT-II cells cover only 7% area of the alveolar epithelium surface, and AT-II cells occupy approximately 67% of the alveolar epithelial cell numbers. The ratio of AT-II cells in the structure of the alveolar epithelium surface reflects the biochemical importance of AT-II cells in the alveoli [16].

The major functions of AT-II cells include the synthesis of surfactants, the mediation of transepithelial transport of water and ions, metabolism of xenobiotics, and the regeneration of damaged alveolar epithelium interface after the pathogen-caused lung injury [17-19]. AT-II cells also act as the progenitors of AT-I cells [17]. The main functions of AT-I cells are related to the transport of lung liquid and ions and also the maintenance of the integrity of the alveolar epithelium interface [20, 21].

The main cell type in the pulmonary capillary endothelium is pulmonary capillary microvascular endothelial cells. Alveoli are immersed in the complex microenvironment. Besides the pulmonary alveolar epithelial cells and pulmonary capillary microvascular endothelial cells, the alveoli also contain alveolar macrophages in the alveolar interstitium and neutrophils in the alveolar blood capillaries [8]. Moreover, pulmonary fibroblasts are also present in the peripheric matrix of alveoli [8]. The structure of lung alveoli and the alveolar-capillary barrier are schematically displayed in Figure 1.1.

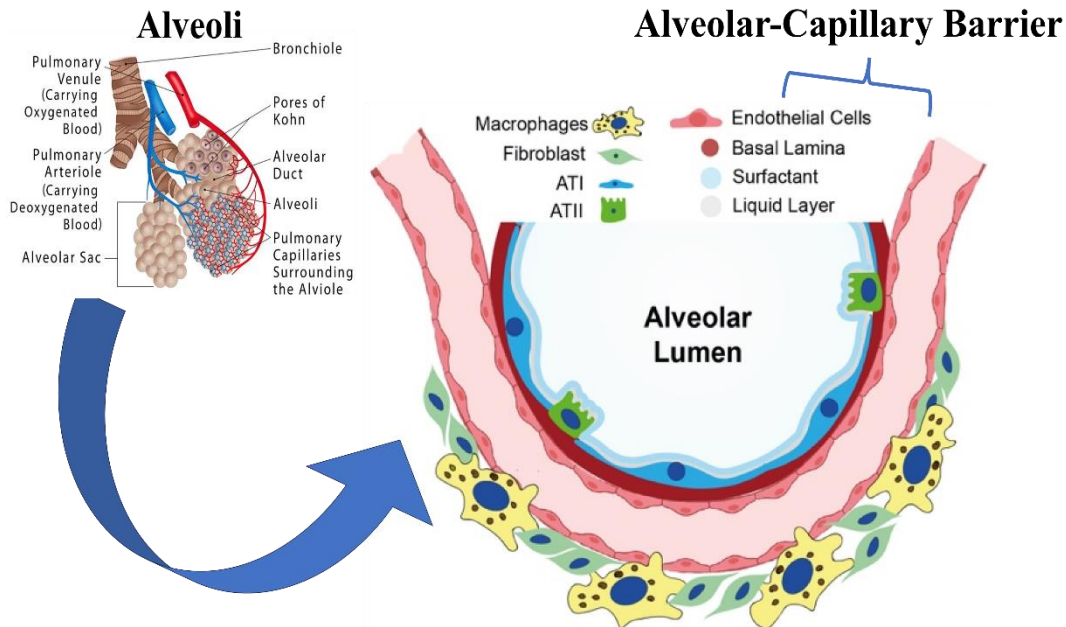


Figure 1.1: Schematic demonstration of the structure of alveoli and alveolar-capillary barrier in the lung tissue (The figure is cited and modified from www.therespiratorysystem.com/alveoli and [8])

1.3 Junction Proteins of Pulmonary Epithelial and Endothelial Cells

1.3.1 Junction proteins structure of alveolar epithelial cells

Both the alveolar epithelial cells and pulmonary capillary microvascular endothelial cells are intercellularly connected by multifunctional transmembrane junction protein complexes which include tight junction proteins (TJs), and adherens junction proteins (AJs) [22, 23]. The junction protein complexes exert distinctive functions for the contacts of neighboring cells [22, 23]. The intercellular junction proteins mechanically connect the adjacent alveolar epithelial cells which preserves the alveolar epithelial barrier intact. The intercellular junction proteins mainly consist of tight junction proteins (TJs) adherens junction proteins (AJs), and (Hemi) desmosomes [9].

Tight junction proteins are mainly located at the apical side of the cells [9]. Desmosomes are located on the basolateral side of the cells which adhesively bind the adjacent epithelial cells through their filament structure [9].

Tight junctions consist of a group of transmembrane protein complexes including Zona Occludens-1 (ZO-1), Occludin, Claudins, and junction adhesion molecules (JAMs) [24]. ZO-1 is partially located in the cytoplasm and combined with the cytoskeleton to maintain the stability of cell structure [24]. Occludin and Claudins perform their function in the regulation of the permeability of the cellular barrier [24]. It has been reported that tight junction proteins are also the main regulators of the permeability of the epithelial barrier [24].

The major component of adherens junction proteins of epithelial cells is E-Cadherin (epithelial cadherin) which is also a transmembrane protein [24]. The extracellular domain of E-Cadherin links to the homotypical neighboring cells [24]. The intracellular domain of E-Cadherin is associated with a network of microtubules including, p120-Catenin, β -Catenin, and α -Catenin proteins that bind the intracellular domain of E-Cadherin with cytoskeleton that mechanically support multiple intracellular signaling pathways [25, 26].

E-Cadherin plays a critical role in the formation of all of other intercellular junction proteins. The disrupted expression and loss of E-Cadherin in epithelial cells can also lead to the delocalization of tight junction proteins [25, 26]. It also has been reported that the disordered expression of E-Cadherin, β -Catenin, ZO-1, and Occludin also contributes to the impaired barrier function of alveolar epithelial cells, that can be observed among asthma patients from their airway epithelium [27, 28].

Moreover, in studies with murine experiments, it has been found that the degradation of tight junction proteins ZO-1, Occludin, and Claudins-5, -8, -18, and -23 occurs in the disease including eosinophilic, neutrophilic, and mixed granulocyte asthma [29]. The construction of junction proteins of alveolar epithelial cells is schematically displayed in Figure 1.2.

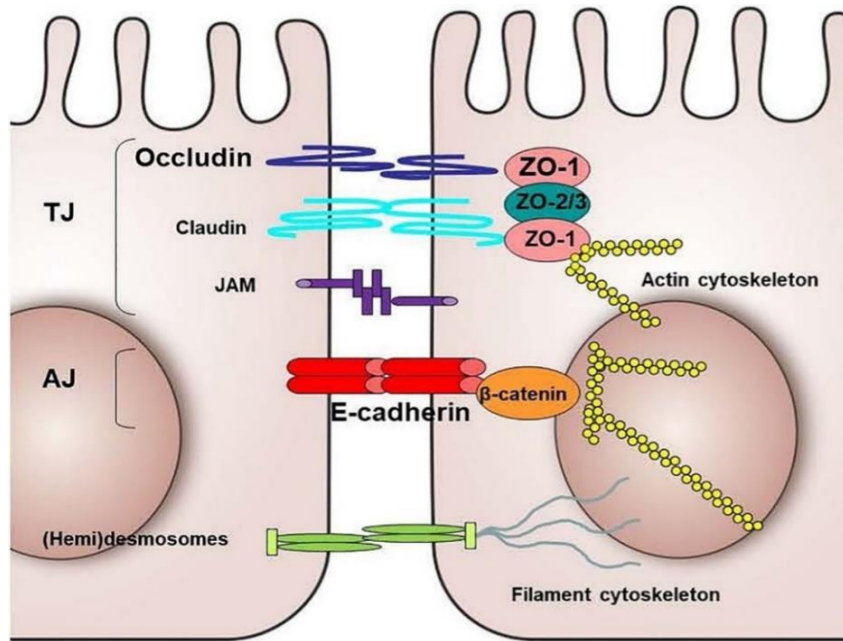


Figure 1.2: Schematic demonstration of the structure and constitution of junction proteins in alveolar epithelial cells (The figure is cited from [9]).

1.3.2 Junction proteins structure of vascular endothelial cells

Similar to the alveolar epithelial cells, the junction proteins in vascular endothelial cells are also composed of tight junction proteins (TJs), which consist of ZO-1, Occludin, Claudins, and adherens junction proteins (AJs) [10].

The adherens junction proteins of vascular endothelial cells are majorly composed of vascular endothelial cadherin (VE-Cadherin) (which is also known as Cadherin5 or CD144), and a variety of other adhesion molecules including Platelet endothelial cell adhesion molecule 1 (PECAM-1), which is also known as CD31, and nectins [10]. Nectins are linked to the actin cytoskeleton through different molecular acceptors [10]. VE-cadherin is an essential transmembrane protein of vascular endothelial cells that plays a critical role in the formation of other intercellular junction proteins, and the preservation of vascular endothelial barrier [30]. VE-Cadherin and CD31 are solely expressed in vascular endothelial cells [30]. VE-Cadherin and PECAM-1 (CD31) are essential for the facilitation and maintenance of the connection of vascular endothelial cells with neighboring homotypical cells.

Introduction

The VE-Cadherin and CD31-mediated cell-to-cell connection of vascular endothelial cells is mechanically supportive for the structure maintenance of the vascular endothelial barrier and the stability conservation of junction proteins in vascular endothelial cells [31].

VE-Cadherin is intracellularly linked to actin filaments via a catenin complex network of α - and β -Catenins, γ -Catenin, p120-Catenin, vinculin, and α -actinin [10, 32]. The intracellular linkage of VE-Cadherin with actin filaments is not only critical for the function stability conservation of junction proteins but also essential for the regulation of the dynamic functions of the junction proteins of vascular endothelial cells [33].

The junction proteins are functional for the vascular endothelial cells to mechanically form a semi-permeable vascular endothelial barrier of blood vessels for the separation of the bloodstream from other underlying tissues, which is essential for the regulation of the exchange of tissue fluids, solutes, and tissue cells between blood vessels and other tissues [10]. It has been reported that bacterial infection can also result in the loss and translocation of VE-Cadherin and tight junction proteins, especially ZO-1, which leads to the disruption and damage of the vascular endothelial barrier [34]. The construction of vascular endothelial junction proteins is schematically displayed in Figure 1.3.

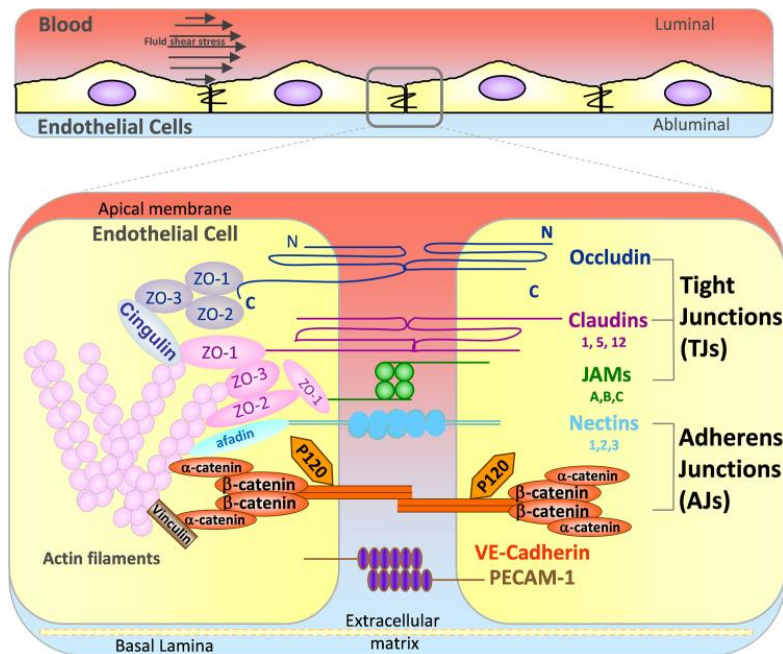


Figure 1.3: Schematic demonstration of the structure and constitution of junctional proteins in vascular endothelial cells (The figure is cited from [10]).

1.4 Surfactants in Alveolar Epithelial Cells

It is widely known that Surfactants are mainly synthesized by alveolar epithelial type II cells (AT-II cells) and Clara cells [15, 35]. The components of surfactants are consisted of approximately 80% phospholipids, 10% proteins, and 10% neutral lipids [36].

Surfactants are classified into two groups of specific surfactant-associated proteins. One group of surfactants is hydrophilic which is the association of surfactant protein A (SP-A) (34 - 36 kDa) and surfactant protein D (SP-D) (43 kDa). SP-A and SP-D are immunity-related surfactant proteins and play important roles in the defense against pathogens [36, 37]. Another group of surfactants is intensely hydrophobic and is composed of surfactant protein B (SP-B) (17 kDa) and surfactant protein C (SP-C) (4 kDa).

SP-B and SP-C interact with surfactant phospholipids to optimize airway epithelium surface tension and functions [36, 37]. SP-A and SP-D are water-soluble surfactant proteins and are classified in the collectin subgroup of the superfamily of C-type lectins [38].

Surfactants are essential for the maintenance of lung functions and lung formation. It has been demonstrated that surfactant deficiency and alteration (namely the surfactant inactivation), caused by surfactant nitration and oxidation can result in lung disruption and lead to a wide spectrum of lung diseases including acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and asthma, etc. [39,40,41].

SP-A and SP-D are also involved in -the defense-first line in the immune system of alveolar air space [40]. SP-A and SP-D interact with alveolar macrophages or phagocytic cells during the recognition and clearance of a wide range of microbial pathogens, such as the bacterial endotoxin lipopolysaccharide (LPS), and viruses. These pathogens are responsible for severe pulmonary inflammation and lung damage [42, 43]. SP-A and SP-D are also remarkable lung disease markers. Lung diseases and lung dysfunction can be indicated through the low concentration levels of SP-A, and SP-D in the human serum [40].

SP-B and SP-C are also essential for lung homeostasis maintenance. SP-B and SP-C are functional to reduce lung surface stress tension from lung respiration stretching, which is beneficial to preventing the alveoli from collapsing at end-respiration [44]. Therefore, it

indicates that both SP-B and SP-C are functional in the stability maintenance of alveoli. SP-B and SP-C are also required to support the functions of surfactants [45]. The adsorption of the surfactant phospholipid film at the alveolar air space-capillary interface is also critically mediated through the hydrophobic SP-B and SP-C [46].

SP-B and SP-C are essential to maintain lung function and alveoli formation. SP-B deficiency will result in the development of lethal respiratory failure, while SP-C deficiency will lead to the pathogenesis of lung fibrosis [47]. It can occur that SP-B deficiency will probably also lead to SP-C deficiency [48]. In a word, the supplement with surfactant proteins is a potentially promising therapy for the healing of lung diseases [36, 49].

AT-II cells are the only epithelial cell type of the lung that synthesize and release all of the four surfactants, namely, SP-A, SP-B, SP-C, and SP-D. Because SP-A, SP-B, and SP-D can be also synthesized by Clara cells in the airway [11], whereas, SP-C can be uniquely synthesized by AT-II cells [50]. Therefore, SP-C is also identified as the biomarker of AT-II cells. The production system of surfactant proteins is schematically displayed in Figure 1.4.

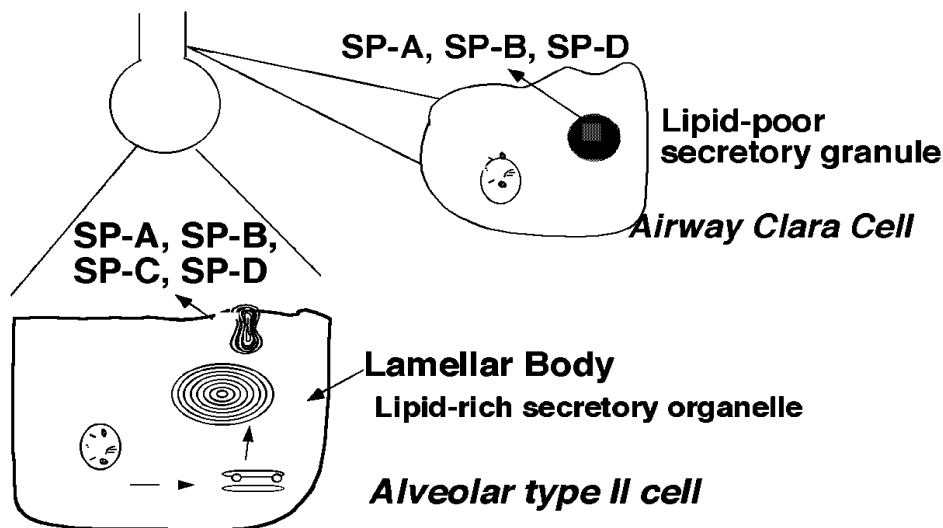


Figure 1.4: Schematic demonstration of the involved cells in the production system of surfactant proteins (The figure is cited from [11]).

1.5 Introduction to Protein Kinase C Isozymes (PKCs)

Protein Kinase Cs (PKCs) belong to a family of serine-threonine kinases and are also involved in the regulation of the cellular barrier function of epithelial cells [51].

There are at least 11 different PKC isozymes (PKCs) are identified. The PKC isozymes are classified into three different types based on their structure, biochemical properties, domain structure, and their responsiveness activators [52]. The types of PKC isozymes include classic PKC isozymes (α , $\beta 1$, $\beta 2$, and γ) which are both Ca^{2+} and diacylglycerol (DAG)-dependent, novel PKC isozymes (δ , ϵ , θ , η , and μ), which are Ca^{2+} independent but DAG-dependent, and atypical PKC isozymes (ι λ and ζ) which are sensitive to other lipid second messengers such as phosphatidylserine but are neither Ca^{2+} nor DAG-dependent [53].

Among the isozymes, the classic PKC isozymes, including PKC- α and PKC- $\beta 1$, and the novel PKC isozyme, PKC- η , are also known as PKCA, PKCB, and PKCH respectively. The cellular signaling of PKC isozymes participates in the mediation of multiple cellular physiological processes, including, cell cycle, cell division, cell migration, cell growth, cell apoptosis, and also cell proliferation and differentiation [54]. Some PKC isozymes are ubiquitously expressed while others display higher degrees of tissue specificity [55].

The overlap in expression of PKC isozymes is cell-type specific, allowing for a multiplicity of context-dependent functions of PKC isozymes [55]. This contextual dependency on the functions of PKC isozymes often makes it difficult to determine the precise roles of PKC isozymes in normal and aberrant cellular processes [55].

Previous studies have demonstrated, that PKC isozymes also play the role of disease-trigger of tumors, and lung cancer [54, 56]. PKC isozymes are also involved in the regulation of inflammation, and the translocation of the tight junction proteins of alveolar epithelial cells which result in hyperpermeability of the alveolar epithelial barrier and disruption of the pulmonary vascular endothelial barrier, which further leads to the lung disruption and the acute respiratory distress syndrome (ARDS) [55]. Therefore, the development of the PKC isozymes targeted inhibitors can be a potential therapeutic strategy, for lung disease treatment.

1.6 Caveolin-1 in Lung Epithelial Cells

The omega (Ω)-shaped Caveolae (size: 50 nM to 100 nM) are cellular plasma membrane invaginations [57]. The components of Caveolae are Caveolin-1, Caveolin-2, and Caveolin-3 in the protein family of Caveolins [57].

It has been found that Caveolae are involved in multiple cellular processes, including membrane trafficking and cell signaling, endocytosis, and intracellular cholesterol transport [58]. Indeed, Caveolae and Caveolins probably play a broad role in human physiology and pharmacology [58]. Caveolae are found in many cell types, including structural cells of the lung [59]. The presence of Caveolae depends on the expression of specific Caveolins in the family of Caveolin proteins in the cells [59]. The Caveolins are cholesterol-binding inserts of the inner leaflet of the plasma membrane [59]. Caveolin-1 (21-26 kDa) is the most important caveolin protein in the caveolin family for the formation of Caveolae.

Caveolae and Caveolins are integral membrane components and are located at the basolateral membrane surface of ciliated epithelial cells [60]. The expression and location of Caveolin-1 were observed in tracheal and bronchial epithelial cells, tracheal and bronchial smooth muscle cells, vascular endothelial and smooth muscle cells, airway fibroblasts, and alveolar epithelial type I cells (AT-I cells), but were absent from SP-C positive alveolar epithelial type II cells (AT-II cells) which are the progenitor of AT-I cells, and airway epithelial cells in the small bronchi [60].

Caveolin-1 is in linkage with different intracellular membrane structures, which include endoplasmic reticulum, Golgi, and plasma membranes [61]. Caveolin-1 is mainly involved in the regulation of the extracellular transport of macromolecules and the transport of macromolecules to the intracellular plasma [61]. It has been found that the knockout of Caveolin-1 (Caveolin-1 deficiency) in AT-I cells leads to the thickened alveolar septa and the aggregated higher volume of the extracellular matrix, which results in the hypertension of alveolar epithelium accompanied by the hyperpermeability of alveolar epithelium and the lung diseases of edema and asthma [62, 63].

Caveolin-1 is also involved in the functional regulation of the alveolar epithelial barrier via its mediated expression of adherens junction proteins including, E-Cadherin, and β -Catenin [64]. The reduced expression of Caveolin-1 will subsequently lead to abnormal and defective expression of E-Cadherin and β -Catenin [64]. Moreover, the loss of Caveolin-1 on the cell membrane is also attributed to Caveolin-1 internalization in the cell plasma, which will result in the redistribution of E-Cadherin [64]. Both of these two issues will contribute to the aberrant function of the alveolar epithelial barrier, which will make a higher risk of asthma-induced lung function disorder [64].

In contrast to the beneficial role of Caveolin-1 in the preservation of the function of the alveolar epithelial barrier, the endocytosis function of Caveolae and Caveolin-1 can also lead to the endocytosis of several pathogenic microorganisms, which can selectively via Caveolae enter into cells [65]. Caveolin-1 is also relevant in the pathogenesis of acute lung injury (ALI) and its severer form, acute respiratory distress syndrome (ARDS) [61,65]. The previous studies verified that mice with Caveolin-1 deficiency have a reduced inflammatory response which is induced by the LPS via NF κ B-mediated pathways [66]. Another investigation demonstrated that the excessive expression of Caveolin-1 exacerbates the LPS-induced inflammation in mouse alveolar epithelial type I cells and more intensively induces the production of pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor (TNF) [67]. Therefore, the regulation for the moderate expression of Caveolin-1 in epithelial cells is a potential therapeutical target for the treatment of lung diseases.

1.7 Epidermal Growth Factor (EGF) in Alveolar Epithelial Cells

Epidermal Growth Factor (EGF) is a protein, which has a molecular weight of 6 kDa and is composed of 53 amino acid residues [69]. EGF combined with its cognate receptor (EGFR) regulates the involved cell signaling for the moderation of cell proliferation, differentiation, mitogenesis growth, and also the survival of all epithelial cell types including alveolar epithelial cells [68, 69]. EGF is relevant in the regulation of lung maturation and development, and also the secretion of SP-A on the alveolar epithelium surface [70, 71].

Previous studies have found that low levels of EGF in the human lung plasma will lead to the development of chronic lung disease (CLD) [72]. A study also found that low concentration levels of EGF were detected in the pulmonary plasma of people who suffered from chronic obstructive pulmonary disease (COPD) [73]. It also has revealed that EGF together with its receptor EGFR in cell signaling also participates in lung repair and remodeling after the lung damage induced by inflammation [74]. Therefore, the appropriate modulation of EGF expression can be also a therapeutical strategy for lung disease treatment.

1.8 Overview of Lung Infection Caused by Bacterial Endotoxin Lipopolysaccharide (LPS)

The lipopolysaccharide (LPS)-induced lung infection is a common cause of lung diseases. LPS also known as bacterial endotoxin is an outer membrane component of Gram-negative bacteria. The components of LPS include a hydrophilic polysaccharide (O-antigen) an oligosaccharide core, and a highly toxic lipid A [75].

1.8.1 Mechanism, and consequence of LPS-induced lung inflammation

The LPS infection progresses through the cellular pathways of LPS signaling. LPS is first recognized by specific pattern-recognition receptors (PRRs). These receptors are composed of an extracellular domain and an intracellular domain [76].

The extracellular domain of these receptors recognizes the associated molecular patterns of specific pathogens, and the intracellular domain of these receptors is involved in the transduction of the intracellular signaling pathways of pathogens [76]. It is found that Toll-like receptors (TLRs) play the most important roles among the PRRs [5]. In cell culture studies, it has been demonstrated that Toll-like receptor 4 (TLR4) is responsible for the mediation of endotoxin (LPS)-induced immune responses [77].

Besides TLR4, LPS-binding protein (LBP) is another molecule that is also involved in the initial recognition of LPS and has been discovered by prior research in AT-I cells [78]. LPS-binding protein (LBP) recognizes LPS and binds the lipid part of LPS, from bacteria, which

solubilizes LPS, and the solubilized LPS is then transferred to glycoprotein CD14 [78, 79]. Because of this process, it implies that LBP together with CD14 facilitates the binding of LPS to TLR4 [80]. Therefore, it indicates that the binding of CD14 to LPS is crucial for the further activation of TLR4 for the transduction of the LPS signaling pathway [80].

TLR4 together with myeloid differentiation protein 2 (MD2) mediates cellular responses to LPS [81]. Therefore MD2 is considered to play an important role in the recognition of LPS by TLR4 [5]. The structure of the intracellular domain of TLR4 is homologous to the family of interleukin-1 receptors (IL-1R) which is also recognized as Toll/IL-1R homology (TIR) domains [82]. TIR-domain-containing adaptor protein (TRIF), and TRIF-related adaptor molecules (TRAM) are the involved adaptors in the LPS signaling pathway [83].

The activated TLR4-MD2 complex can trigger two signal transduction pathways, namely (1) MyD88-dependent [84], and (2) MyD88-independent pathway [85]. Mal (malonaldehyde) is required in the MyD88-dependent pathway for efficient signaling transduction and leads to rapid activation of the NF- κ B signaling pathway [84]. TRIF and TRAM mediate the MyD88-independent pathway, which leads to the phosphorylation and dimerization of interferon regulatory factor 3 (IRF3) [85]. This process induces the production of interferon β (IFN- β) [85]. The MyD88-independent pathway can also activate the NF- κ B signaling pathway but at a later time [85].

The activation NF- κ B signaling pathway leads to the production of various pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), and interleukin cytokines (IL), which include IL-1 β , IL-6, IL-8, and chemokines, which include monocyte chemoattractant protein 1 (MCP-1), and Macrophage inflammatory protein 3 α (MIP-3 α) [84, 85].

The interleukin pro-inflammatory cytokines are beneficial for the immune system to defend against invading microbial pathogens. However, the excessive production of the interleukin pro-inflammatory cytokines can result in the septic shock of tissue cells [86, 87]. The LPS signaling pathways are referred to alveolar epithelial cells, including AT-I cells and AT-II cells, vascular endothelial cells, and alveolar macrophages [5]. The factors including LPS-induced pulmonary over-inflammation response and LPS-triggered increased production of

intracellular nitric (NO) oxide will lead to the apoptosis of pulmonary epithelial and endothelial cells [88-90]. These processes destructure the alveolar-capillary barrier, namely integrity impairment of the alveolar-capillary barrier, which increases the barrier permeability and leads to the diffusion of edema blood fluid into alveoli and hypertension of the alveolar-capillary barrier surface, and increases the proliferation of lung fibrosis [88-90]. Moreover, the excessive inflammation response also causes the dysfunction and degradation of surfactants. As a result, these factors will contribute the serious lung diseases including acute respiratory distress syndromes (ARDS) and chronic obstructive pulmonary diseases (COPD), which result in high clinical mortality of infected patients worldwide [88-90]. The mechanism of the LPS signaling pathway is schematically displayed in Figure 1.5.

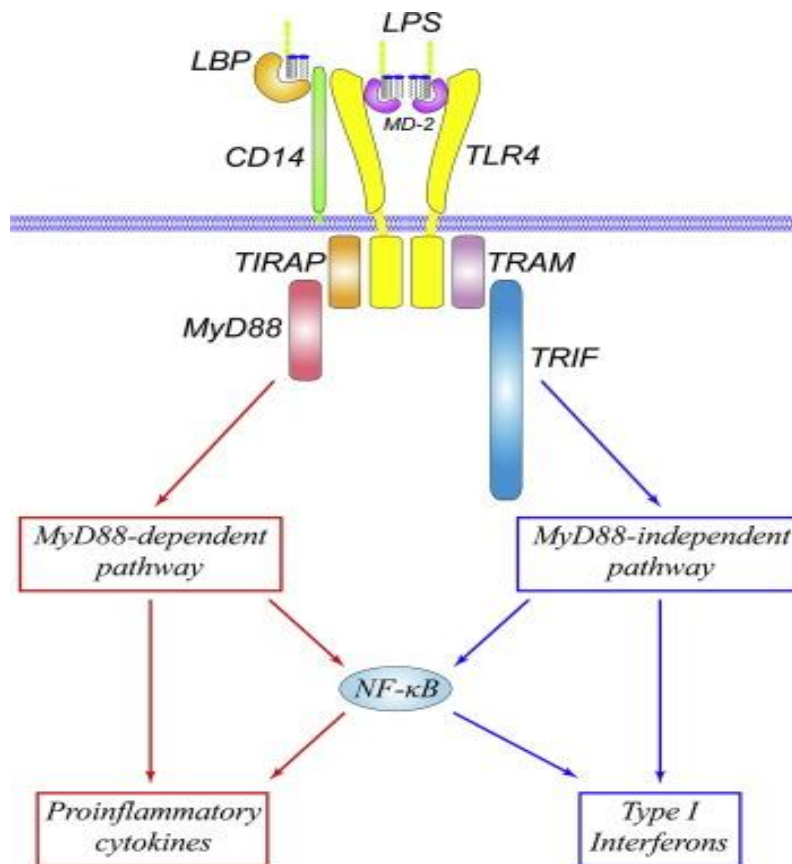


Figure 1.5: Schematic demonstration of the mechanism of LPS cellular signaling pathway in the alveolar cells (The figure is cited from [82])

1.8.2 Involved molecules in lung infection diseases

The immune response in the lung which is caused by pathogens, including LPS infection, is also mediated by cellular adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) in endothelial cells, and Cathepsin S. These proteins are associated with LPS infection, as well. These related molecules in lung infection diseases will be introduced in the following text.

The cell membrane adhesion protein molecules, inclusive intercellular adhesion molecule-1 (ICAM-1-CD54, molecular weight: 90-110 kDa), and vascular adhesion molecule-1 (VCAM-1-CD106, molecular weight: 100-110 kDa) belong to the immunoglobulin superfamily of cell adhesion molecules (CAMs) [110]. These molecules are expressed in the endothelial cells and leukocytes [110]. These two types of adhesion proteins are also known as endothelial cell adhesion molecules (ECAMs) when they are expressed in endothelial cells [110].

ICAM-1 and VCAM-1 are functionally involved in the recruitment of immune cells, such as leukocytes and neutrophils, which migrate to the LPS and pro-inflammatory cytokines-induced inflammatory sites [111]. ICAM-1 and VCAM-1 mediate the migration and adhesion of leukocytes and neutrophils to the endothelial cells and the alveolar-capillary interface [110-112]. ICAM-1 and VCAM-1 have distinct functions in the recruitment of immune cells. VCAM-1 is responsible for the recruitment and adhesion of monocytes, lymphocyte eosinophils, and basophils to the endothelial cells [113]. While, ICAM-1 is responsible for the adhesion of polymorphonuclear cells (PMNs), monocytes, and lymphocytes to the endothelium [114]. The migration of leukocytes and neutrophils into tissue is part of the host-immune resistant response to protect organs against tissue damage. However, it is also harmful and participates in the pathogenesis caused by excessive inflammation response [111].

It is found that ICAM-1 plays a critical role in LPS-induced lung inflammation, which leads to acute lung injury, in which ICAM-1 mediates the recruitment of neutrophils into the alveolar-capillary interface [115]. The degrees of recruitment and migration of immune cells

to the tissue inflammatory sites are dependent on the content levels of ICAM-1 and VCAM-1 on the cell membrane of endothelial cells [115, 116]. Therefore, a strategy for the moderate regulation of the expression of ICAM-1 and VCAM-1 is a feasible method of medicine development for the treatment of lung inflammatory diseases [116].

Cathepsin S (CTSS) (molecular weight 23.9 kDa) is one member of the cysteine proteinase family. The cysteine lysosomal proteinases consist of Cathepsin S, B, L, H, S, O, and K [91]. Among the Cathepsin kinase family, only Cathepsin S (CTSS) retains its activity after prolonged incubation at neutral pH, whereas all the other Cathepsin molecules are irreversibly inactivated [91]. Therefore, this fact makes Cathepsin S is the unique and appropriate kinase in the Cathepsin family for the study of issues of the destruction and remodeling of extracellular matrix (ECM), and tissue destruction in nonacidic extracellular environments [92].

Cathepsin S can be majorly detected from the endosomal compartments of the lysosome of antigen-presenting cells, such as B cells, macrophages, and dendritic cells [93]. Cathepsin S is also expressed in epithelial cells, smooth muscle cells (SMCs), endothelial cells, macrophages, and neutrophils [93]. The production of Cathepsin S is regulated by several factors, such as pro-inflammatory cytokines including IL-1 β , TNF- α , IL-4, and IL-13 which have been reported to have an effect in inducing the expression of Cathepsin S in the cells [94].

Cathepsin S is closely involved in various intracellular and extracellular processes, such as proteolysis [95], and the immune responses mediated by major histocompatibility complex (MHC) class II [96]. The extracellular Cathepsin S also participates in matrix remodeling through its degradation effect on collagen and elastin which are the structural components of the extracellular matrix [97]. Cathepsin S is also closely involved in several biological processes such as tissue remodeling through its activities in the lysis processes of elastin and collagen [98].

The structure of the extracellular matrix is organized and maintained by a balance of the synthesis of the extracellular matrix and the degradation of the components of the extracellular matrix which is regulated by Cathepsin S-mediated proteolysis [97, 98]. The Cathepsin S-mediated organization and degradation of extracellular matrix (ECM) can extracellularly and intracellularly take place. The cells can also internalize the extracellular degraded ECM residues through endocytosis [99]. Moreover, it has been found that Cathepsin S is also involved in the breakup of non-matrix substrates including cytokines [100], chemokines [101], and antimicrobial proteins (AMPs) [102].

In addition, it has been reported that the alveolar secreted Cathepsins B, L, and S also participate in the degradation of defensins (β -defensin family), antibacterial catalytic proteins, lactoferrins, and SP-A, which lead to the decreased anti-microorganism activity of alveolar epithelial cells during pathogenetic microorganisms infection, prolonged inflammation, and aggravation of lung fibrosis [103-105].

Due to the elastolysis and proteolytic effects of Cathepsin S, Cathepsin S can degrade various components of the membrane basement of pulmonary cells [106], which causes the over-diffusion of extracellular fluid into the lung cells. As a result, it will lead to lung hypertension [107]. Cathepsin S is also involved in the regulation of autophagy autoimmunity and inflammation in a variety of human organs, including lung [108, 109]. The dysregulation and overexpression of Cathepsin S in alveolar epithelial and endothelial cells contribute to the outcome of the development of various lung diseases, including bronchopneumonia, asthma, and acute lung injury (ALI), chronic obstructive pulmonary diseases (COPD), and also lung cancer [109]. Therefore, it can implicate that the expression level of Cathepsin S is also a potential diagnostic indicator of lung diseases.

1.9 Introduction of Vitamin D₃

1.9.1 The metabolism of Vitamin D₃

Vitamin D is an organic compound consisting of fat-soluble secosteroids. It is well-known that the function of Vitamin D is mainly involved in the preservation of bone function

homeostasis, calcium levels conservation, and calcium phosphorylation, via Vitamin D regulated calcium absorption, which is the classical or traditional function of Vitamin D [117]. Besides the classical functions, more comprehensive research found that Vitamin D is also involved in the regulation of more bio-physiological processes, including cellular proliferation, differentiation, apoptosis, and innate and adaptive immunity, which are classified as the novel functions of Vitamin D [118].

Vitamin D is classified into two major isoforms including Vitamin D₂ (ergocalciferol) and Vitamin D₃ (cholecalciferol) [118]. Vitamin D₂ is synthesized through the irradiation of ergosterol in yeast and fungi, which is triggered by ultraviolet B (UVB) [119]. The irradiated ergosterol (Vitamin D₂) can be obtained from some vegetal foods including UVB-radiated mushrooms and plants, and medical supplements [120]. While, Vitamin D₃ can be gained from the UVB-triggered photochemical reaction in the skin and the dietary source from the intake of animal-based foods (such as cod liver oil, egg yolks, and oily fish) [120].

The major source of Vitamin D₃ is the photochemical and thermal conversion of 7-dehydrocholesterol in the skin, which are induced by UVB irradiation under sunlight exposure [120]. The absorption of UVB radiation with the wavelength from 290 nm to 315 nm (wavelength peak: 297 nm) to the skin under sunlight exposure results in the opening of ring B of 7-dehydrocholesterol, which contributes to the formation of a thermodynamically unstable pre-Vitamin D₃ (9, 10-sitosterol) [120]. Afterward, this pre-Vitamin D₃ is thermally isomerized into a more stable Vitamin D₃ (cholecalciferol) [118].

The formation of Vitamin D₃ either occurs in the skin or is performed through absorption from the diet in the small intestine [118]. The obtained Vitamin D₃ is then delivered to the liver. Then, Vitamin D₃ is converted to 25-hydroxy Vitamin D₃ (25(OH) D₃) through Vitamin D 25-hydroxylase (CYP2R1) catalyzed hydroxylation [121, 122]. 25-hydroxy Vitamin D₃ (25(OH) D₃) or calcidiol is also defined as a biomarker to indicate the status of Vitamin D₃ [121, 122]. Afterward, 25(OH) D₃ is delivered to the kidneys. In the kidneys the 25(OH) D₃ is further converted to its biologically active form, 1- α -25-dihydroxy Vitamin D₃ (1,25(OH)₂ D₃) through 1-hydroxylase (CYP27B1) catalyzed hydroxylation [121,122]. The 1- α -25-

dihydroxy Vitamin D₃ (1 α ,25(OH)₂ D₃), is a secosteroid and can be also named as bioactive Vitamin D₃ (Vitamin D₃), and known as calcitriol [118].

It is found that during the metabolic process of Vitamin D₃, the synthesized bioactive Vitamin D₃ is initially bound to Vitamin D binding protein (VDBP), and is delivered to target cells together with its metabolites [118]. It has been found that, during the metabolic cycle of Vitamin D₃, 25(OH) D₃ and 1,25(OH)₂ D₃ can be also metabolically deactivated through 24-hydroxylase (CYP24A1) catalyzed hydroxylation [123]. The 1 α , 25(OH)₂ D₃ (Vitamin D₃) level in human blood serum is closely regulated by the feedback of the circulatory procedures of several factors including the metabolisms of calcium, phosphorous, 25(OH) D₃, parathyroid hormone (PTH), and fibroblast growth factor 3 [124, 125].

According to the assessment from previous studies, it has been found that the concentration levels of Vitamin D₃ in the blood serum below 20 ng/mL (50 nmol/L) are defined as a serious deficiency of Vitamin D₃. The blood serum with Vitamin D₃ concentration levels between 50 ng/mL and 70 ng/mL (125 nmol/L-175 nmol/mL) in the blood serum, is generally accepted as the adequate level of Vitamin D₃. However, the blood serum with a Vitamin D₃ concentration level over 80 ng/mL (200nmol/mL) is evaluated as Vitamin D₃ excess [126, 127]. The metabolism procedure of Vitamin D is schematically displayed in Figure 1.6.

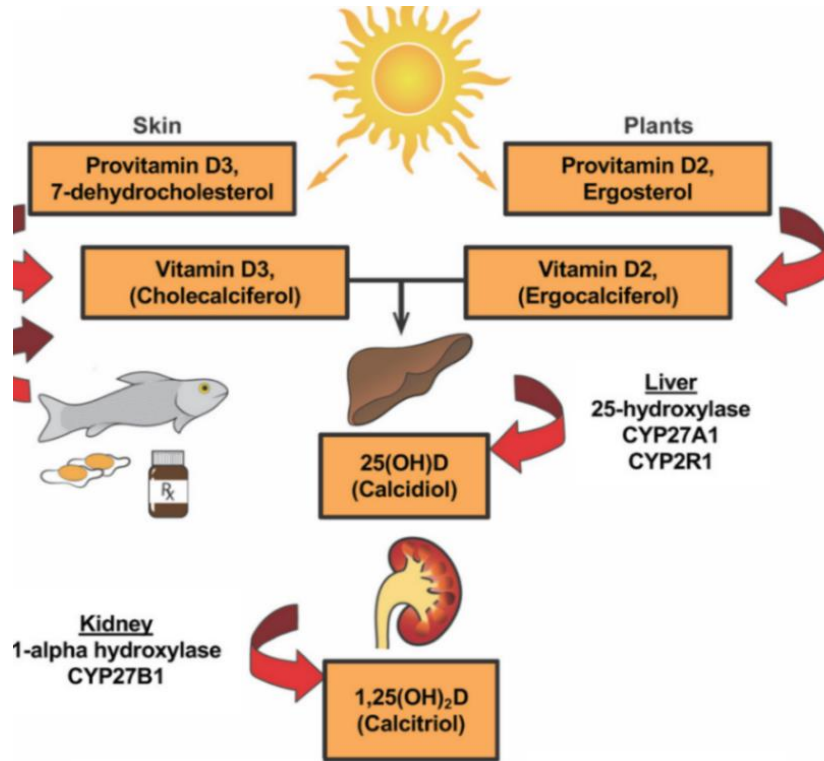


Figure 1.6: Schematic demonstration of the metabolic procedure of Vitamin D (The figure is cited from [4]).

1.9.2 The molecular signaling pathway of Vitamin D₃

During the Vitamin D₃ metabolic cycle, the bioactive form of Vitamin D₃ (1 α ,25(OH)₂ D₃) in the blood serum initially binds to the Vitamin D binding protein (VDBP). Then, VDBP transports the bioactive form of Vitamin D₃ (1 α ,25(OH)₂ D₃) to the nuclei of the specific target cells [128]. It has been found that Vitamin D₃ performs its biological functions through regulation by the Vitamin D receptor (VDR) mediated regulation of the Vitamin D₃ signaling pathway [3]. Vitamin D receptor (VDR) is the cognate receptor of Vitamin D₃ which also belongs to the family of the cellular nuclear receptor [3].

In the Vitamin D₃ signaling pathway, Vitamin D₃ first binds to VDR, then the VDR is heterodimerized with RXR (Retinoid X Receptor) [3]. Afterward, the VDR is subsequently activated by other co-activators including the DRIP complex, which facilitates the formation of the Vitamin D transcriptional regulatory unit [3]. Then the Vitamin D transcriptional

regulatory unit binds to the Vitamin D responsive element (VDRE) in the promoter region of the genes of target molecules [3]. This binding results in the regulation of gene expression of the target molecules in specific cells [3]. During the regulation of the gene expression process, the activated VDR directly binds to the Vitamin D-responsive elements (VDRE) in the promoters of the genes of target molecules [3].

The expression of the target molecules that are regulated by the activated Vitamin D₃ (or Vitamin D)/VDR signaling pathways is relevant for Vitamin D₃ to exert its physiological functions. The classical functions and roles of Vitamin D₃ are involved in the regulation of the homeostasis of calcium and phosphate levels for the preservation of skeletal function and integrity [2]. Vitamin D₃ is also reported to perform its novel functions in the promotion of cell differentiation, immunosuppression, and the inhibition of cell proliferation and cell growth [2]. These functions of Vitamin D₃ are supposed to be regulated by the Vitamin D/VDR signaling pathway. The molecular signaling pathway of Vitamin D₃ is schematically displayed in Figure 1.7.

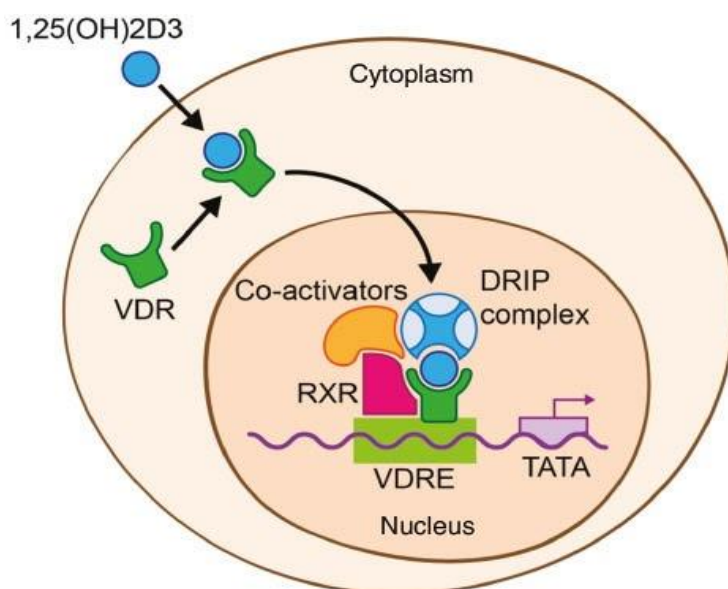


Figure 1.7: Schematic demonstration of the Vitamin D/Vitamin D Receptor (VDR) signaling pathway of Vitamin D₃ (The figure is cited from [3]).

Previous studies revealed that the transcriptional regulation of the gene expression of the target molecules through the Vitamin D/VDR signaling pathway is mediated in a tissue and cell-type-specific manner [129]. Therefore, the specific functions of the Vitamin D/VDR

signaling pathway should be investigated and elucidated in-depth in specific tissues and cell types. Due to the anti-cell Proliferation cell growth function of Vitamin D₃, Vitamin D₃ is extensively applied as a nutritional therapy for anti-cancer treatment [130].

1.9.3 Vitamin D₃ and lung diseases

Vitamin D₃ is essential to maintain the functions of a variety of human organs including, the kidney, colon, brain, skin, and lung [131]. Therefore, it indicates the importance of an adequate level of Vitamin D₃ in the human blood serum for the sustainment of human health. It is reported that Vitamin D₃ deficiency, which results from inadequate nutritional intake and insufficient sunlight exposure, is prevalent among adults worldwide [130,132]. The Vitamin D/VDR signaling pathway is also relevant in the regulation of disease pathogenesis [133]. People with Vitamin D₃ deficiency or loss of the normal form of Vitamin D receptor (VDR) are more susceptible to suffering from lung diseases including, viral and bacterial respiratory infection, asthma, cystic fibrosis, chronic obstructive pulmonary disease (COPD), tuberculosis, and lung cancer [131,132].

Moreover, the Vitamin D/VDR signaling pathway is also essential for the maintenance of the integrity of the lung alveolar-capillary barrier. People with vitamin D₃ deficiency have a higher risk of suffering from higher barrier permeability and lung dysfunction caused by LPS or other pathogens, which also lead to acute respiratory distress syndromes (ARDS) [134].

In the aspect of the novel functions of Vitamin D₃, Vitamin D₃ regulates the immunity of target tissues and cells. In the lung, Vitamin D₃ regulates the autoimmune response of bronchial epithelial cells and lung immune cells during pathogens infection [136]. During the regulation, Vitamin D₃ plays a vital role in the adaptive immune response for its prevention of excessive immune response, which stimulates serious lung inflammation and leads to aberrant lung functions [135].

It has been found that the Vitamin D/VDR signaling pathway interferes with the key transcription factors for the expression and secretion of pro-inflammatory cytokines in the nuclear factor kappa B (NF-κB) pathway [136]. Hereby, Vitamin D₃ down-regulates the

secretion of inflammatory cytokines, such as IL-8, and IL-6, and chemokines, such as leucocyte attracting C-X-C motif chemokine 10 (CXCL10) from stimulated epithelial and endothelial cells by modulating the NF κ B signaling pathway axis [136].

While Vitamin D₃ suppresses the excessive pulmonary immune response, Vitamin D₃ also facilitates innate defense mechanisms against invading respiratory pathogens [132]. It has been found that Vitamin D₃ stimulates the gene expression of Cathelicidin, which encodes an antimicrobial peptide, LL-37, in humans [137]. LL37 mediates the killing of invading pathogens in the human lung [138]. It has been found that the LL-37-expressing gene also has been identified in pulmonary epithelial cells [139]. Through the overview of the association of Vitamin D₃ with lung diseases, it can be conjectured that the supplementation of Vitamin D₃ at a sufficient level in human blood serum is helpful in alleviating the risk of the pathogenesis of various lung diseases.

1.10 The Antimicrobial Peptide Cathelicidin LL-37 in Lung Epithelial Cells

In the defense mechanisms, antimicrobial peptides (AMPs), or cationic host defense peptides (CHDPs) are essential for tissue protection from tissue infection caused by invading pathogens [140]. It has been found that infectious pathogens can be inactivated by AMPs. AMPs inactivate the invading pathogens, through the AMPs-mediated spores formation of the cell membrane of infectious agents [140]. It is possible that during inactivation of the microbial pathogens, the negatively charged phospholipids of the microbial membrane interact with positively charged CHDPs or AMPs, which enables CHDPs or AMPs to kill a wide spectrum of infectious microbes, which comprises Gram-positive, and Gram-negative bacteria, viruses, fungi, or protozoa in a very short time [141, 142].

In mammals, defensins and Cathelicidins are the two major groups of AMPs [140]. Defensins are subdivided into three subgroups including α , β , and θ [140]. The family of Defensins can be found in humans, monkeys, pigs, and several rodent species [140]. While, another antimicrobial peptides group, Cathelicidins, can be identified in rabbits, horses, pigs,

rats, monkeys, cattle, and humans [140]. The different antimicrobial peptides are characterized by their various structures and molecular weights.

The genes that encode Cathelicidins are approximately 2 kb and contain four-exon/three-intron organization, and Cathelicidins are synthesized from their initial inactive precursors [140]. The structure of the precursors of Cathelicidins is constructed with a highly conserved N-terminal segment, a cathelin domain, and a C-terminal domain with a varied sequence and length [143]. The N-terminal domain consists of residues of 29-30 amino acids which promotes the secretion of the biofunctional active Cathelicidins peptides [143]. The cathelin domain of Cathelicidins consists of residues of 98-114 amino acids. However, the functions of the cathelin domain still have not been exhaustively enunciated [143]. The C-terminal domain which consists of residues of 12-100 amino acids is essential for the mature Cathelicidins to demonstrate their broad antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses, and fungi [143]. The cells including neutrophils, epithelial cells, keratinocytes, macrophages, mast cells, natural killing (NK) cells, dendritic cells, and lymphocytes are reported to express and produce Cathelicidins [143, 144].

It has been found that in the Cathelicidin family, only 37 leucine-leucine-(LL37) is expressed in humans [145]. LL37 is known as a human cationic antimicrobial peptide (CAP), with a peptide length or molecular weight of 18 kDa [145]. Therefore, LL-37 is also named as CAP18. LL-37 has been demonstrated to be expressed in neutrophils, bone marrow, and tissue epithelial cells of organs including salivary glands, oral cavity, urethra, and gastrointestinal and pulmonary bronchia tracts [146, 147]. LL-37 is a member of Cathelicidin-related antimicrobial peptides (CRAMPs). The expression of LL-37 can be affected by the development of microorganisms stimulated-tissue inflammation [148]. Human Cathelicidin plays a role in the promotion of wound healing and also acts as direct and indirect antimicrobial factors for the modulation of adaptive immunity [149].

LL-37 generally has an anti-inflammatory effect, in which LL-37 inhibits the production of pro-inflammatory cytokine from TLR2 and TLR4-stimulated monocytic cells [140]. Moreover, LL-37 has been found to inhibit the LPS-induced gene expression of the pro-

inflammatory cytokines in endothelial cells through its interference with the toll-like receptor 4 (TLR-4) ligand and nuclear factor (NF)- κ B for the defense against LPS-induced excessive inflammation [150].

However, it has been demonstrated that people with a low concentration level of LL-37 in the serum and sputum are more susceptible to developing lung diseases including cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), asthma, etc. [151]. The impaired wound healing is also directly related to decreased LL-37 levels [152]. On another hand, it has been reported that the bioactive Vitamin D₃ up-regulated expression and secretion of LL-37 is beneficial in defense against the pathogenesis of tuberculosis, as well as a wide spectrum of other infectious lung diseases [151, 153], and also lung cancer [154]. Moreover, the increased expression of LL-37 can facilitate wound healing through its effects on the anti-biofilm formation on the invading pathogens-infected sites[151].

Due to the multi-functions of the antimicrobial peptide, LL-37, in the defense against disease development, LL-37 is a promising molecule for the development of novel therapeutic methods for the healing and treatment of various lung diseases.

1.11 Introduction of Doublecortin-Like Kinase 1 (DCLK1)

Doublecortin-like kinase 1 (DCLK1, 82 kDa) is a serine/threonine protein, which belongs to the family of doublecortin protein kinases. DCLK1 which is also known as DCAMKL1 is a Ca²⁺/calmodulin signaling pathway-dependent protein kinase and is encoded by the DCLK1 gene [155]. DCLK1 has a homogenous property with microtubule-associated protein (MAP). DCLK1 also has been identified as a biomarker of tuft cells or brush cells in the small intestine [156].

DCLK1 is also found to act as a biomarker of various types of tumors and cancer stem cells of the intestine and pancreas [156]. Some studies have demonstrated that DCLK1 is overexpressed in a variety of cancer cells, including colon, pancreas, liver, renal, esophageal, and lung cancer cells [157, 158]. On the other side, DCLK1 was reported to be initially identified in the human brain [159]. DCLK1 was also demonstrated to be relevantly involved

in neurogenesis and neuronal migration [160, 161]. Therefore, Based on the expression of DCLK1 has been identified in radial glial cells and neuronal precursor cells, it implies the important role of DCLK1 in brain development [162].

DCLK1 is a kind of microtubule-associated protein (MAP), which catalyzes the polymerization of tubulin dimmers and the formation of aster-like microtubule structures [160]. However, it has been reported that DCLK1 is involved in tumorigenesis and cancerogenesis of malignant cancers in a wide range of human tissues [163].

DCLK1 is tightly associated with epithelial-mesenchymal transition (EMT) and supports the EMT process, which is important to accelerate the metastatic processes of many tumors, and the self-renewal of cancer cells for the development and progression of cancer cells [157, 164]. Because DCLK1 is a biomarker of cancer cells in the initiation of the proliferation and metastasis of cancer and tumor cells, the inhibition of DCLK1 is a novel therapeutic target for cancer treatment [165].

Nevertheless, DCLK1 also has its beneficial function. DCLK1 known as a biomarker tuft or brush cells, also has been identified in solitary chemosensory cells (SCCs) [166]. A study has found that the cell numbers of SCCs with a high expression level of DCLK1 are increased at the impaired sites of alveoli for lung repair, after the tissue damage, which is caused by influenza infection [166]. It also has been reported that the deletion or ablation of DCLK1 gene in the tissue cells will lead to the failure of tissue repairment and even more server tissue injury, after pathogen infection [167].

However, on the other hand, the abnormal DCLK1-mediated EMT process contributes to a variety of pathologic occurrences, including tissue fibrosis cancer initiation and progression [168], and also the survival of cancer cells, which make cancer cells escape from apoptosis and immune surveillance [169], even though, the DCLK1-supported EMT process is required for normal tissue development and tissue repair after tissue injury.

Moreover, the DCLK1-supported network is involved in the loss of the polarity epithelial cells on the cell apical and basolateral sides and declined expression of epithelial cell-to-cell junction proteins, especially E-Cadherin, which is associated with cytoskeleton structure

[170], and the degradation of extracellular matrix [171, 172], which facilitates the migration and invasive process for cancer cells' metastasis [171, 171]. Because DCLK1 is a biomarker of cancer stem cells, therefore, the development of inhibitors that are targeted to the production of DCLK1 is a promising therapeutic method for cancer treatment.

It has been found that the usage of leucine-rich repeat kinase 2 inhibitor 1 (LRRK2-IN-1), which is a small molecule and has a molecular weight of 0.57 kDa, is a useful and effective pharmacologic strategy in the treatment of Parkinson's disease, which is genetically predisposed and sporadic [173]. LRRK2-IN-1 is also an effective inhibitor for the inhibition of DCLK1 production, which demonstrated a significant specific and relatively selective affinity for DCLK1 [174]. Through the inhibition or lockdown of DCLK1 expression, LRRK2-IN-1 is reported to display a potential anti-cancer activity, which is involved in the inhibition of proliferation, migration, and metastasis of cancer cells, and also the induction of the accelerated cell cycle and apoptosis of cancer cells [165]. Moreover, LRRK2-IN-1 controls the epithelial-mesenchymal transition (EMT) process and the initiation of the progression of cancer cells [165]. Therefore, the development of LRRK2-IN-1 can be used as a potential novel therapeutic method for the treatment of cancers.

2 Goal Setting and Experiment Design

2.1 Goal Setting for the Study

It is known that people with Vitamin D₃ deficiency in their blood serum are more susceptible to suffering from pathogens especially, LPS-induced lung diseases. This fact indicates the important role of Vitamin D₃ in the maintenance of lung health. However, the effect of Vitamin D₃ in the maintenance of the function of the alveolar-capillary barrier remains to be proved, and the biomolecular physiological mechanisms, that are involved in the effects of Vitamin D₃ on the function maintenance of the alveolar-capillary barrier, are still not fully clarified.

Therefore, this study aimed to deeply explore the involved biomolecular mechanisms underlying the effects of Vitamin D₃ in different aspects, including the impact of Vitamin D₃ on the conservation of properties and integrity of the alveolar-capillary barrier, and the effect of Vitamin D₃ on the function maintenance of the alveolar-capillary barrier in exposure to LPS. In addition, this study also aimed to deeply define associated molecules with LPS-induced inflammation in the alveoli.

Furthermore, a previous proteomics study which was performed in collab with Professor Andreas Tholey at the University of Kiel (CAU, Kiel), demonstrated that Vitamin D₃ significantly increased the protein abundance of DCLK1 in the cell line of alveolar epithelial cells, NCI-H441 cells via Tandem Mass Spectrometry (MS/MS) analysis. However, the impact and concrete mechanisms of DCLK1 on the maintenance of the alveolar epithelial barrier are still elusive. Therefore, the clarification of this subject was also aimed to investigate in this study.

2.2 Experimental Design

This study applied the in vitro co-culture model of alveolar epithelial cells and capillary microvascular endothelial cells in Transwell®, which was used to simulate the alveolar-capillary barrier in vivo. This study also implicated the mono-culture model of the individual

Goal Setting and Experiment Design

cell types of alveolar epithelial cells and capillary microvascular endothelial cells to investigate the role of involved molecules in the regulation of the barrier properties in specified cell types. The bronchial epithelial cell lines, including NCI-H441 cells, Calu-3 cells, and A549 cells were implemented as the alveolar epithelial cells in this study. The isolated outgrowth endothelial cells (OECs) were implemented as the capillary microvascular endothelial cells in this study. The barrier properties were evaluated with the measurement of transepithelial transendothelial electrical resistance (TEER) using the EVOM instrument in the co-culture model of alveolar epithelial cells and capillary microvascular endothelial cells, and the mono-culture model of alveolar epithelial cells in Transwells®.

The barrier properties of capillary microvascular endothelial cells were assessed by the measurement with the Electric Cell-substrate Impedance Sensing (ECIS) instrument, which specifically indicates the electrical barrier impedance of vascular endothelial cells.

The procedure of the cell culture cycle was carried out for 7 days. After the cell culture, the expressions of the target molecules were evaluated at the gene and protein levels. Semi-quantitative Real-time PCR was applied to analyze the RNA expression level of the involved target molecules. Western-Blot and Immunofluorescence Staining were implemented to test the protein expression levels. For the study of the molecular mechanisms of Vitamin D₃ in the maintenance of the alveolar-capillary barrier, this study investigated the effects of Vitamin D₃ on the expression of the related molecules, including, intercellular junction proteins, Protein Kinase C isozymes (PKCs), Caveolin-1, epidermal growth factor (EGF), LL-37, etc. in the alveolar epithelial cells. This study also performed the expression analysis of IL1- β , IL-6, IL-8, SP-A, Cathepsin S, and ICAM-1 in NCI-H441 cells and OECs after treatment with Vitamin D₃ and LPS. For the study of the role of DCLK1 on the preservation of the barrier properties of alveolar epithelial cells, this study treated the Vitamin D₃ pre-treated alveolar epithelial cells (NCI-H441 cells) with DCLK1 specified inhibitor, Leucine-rich repeat kinase 2 inhibitor 1 (LRRK2-In-1) in the experiments

The cell culture models, cell culture procedure, and the TEER and ECIS measurements for evaluation of the function properties of the alveolar-capillary barrier, alveolar epithelial

Goal Setting and Experiment Design

barrier, and capillary microvascular endothelial barrier are graphically displayed in Figure 2.1 and Figure 2.2 respectively.

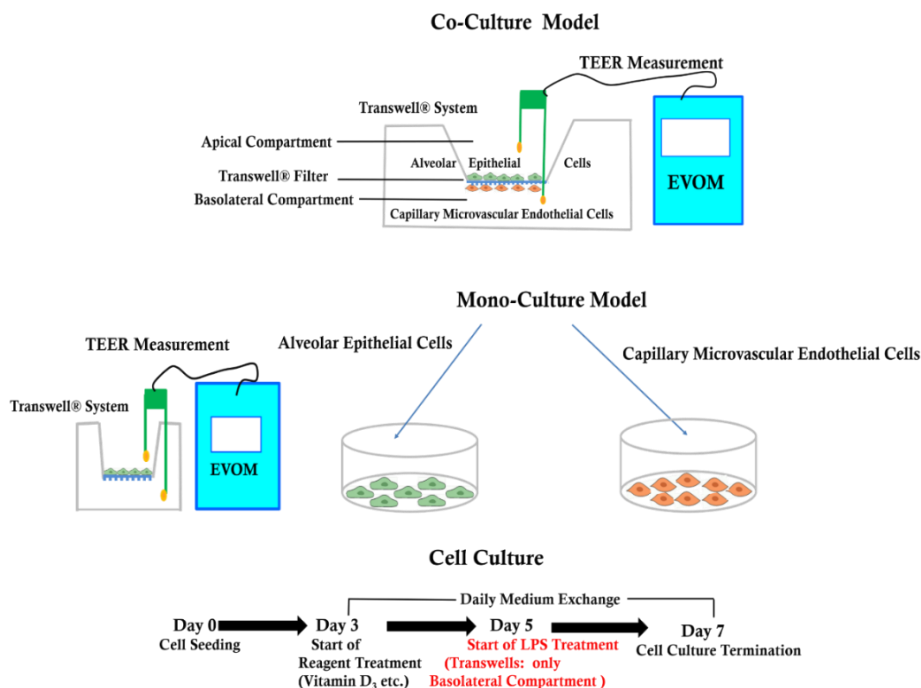


Figure 2.1: Graphic demonstration of the TEER Measurement co-culture model of alveolar epithelial cells and capillary microvascular endothelial cells, mono-culture model of individual cell types, and the cell culture process in the study

ECIS Measurement

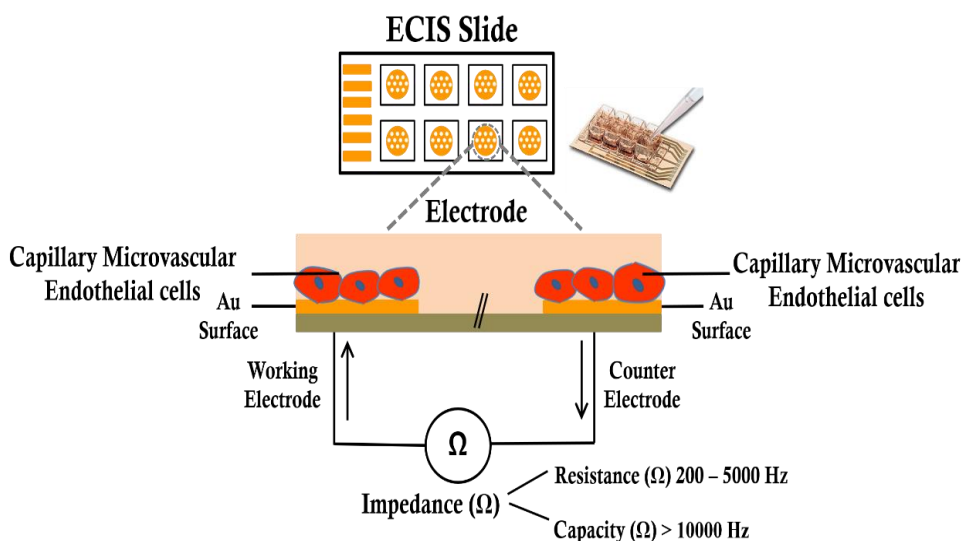


Figure 2.2: Graphic demonstration of the ECIS Measurement for the barrier impedance evaluation of the capillary microvascular endothelial cells in the study.

3 Materials and Methods

3.1 Cells for the Study

The pulmonary epithelial cell lines of NCI-H441 cells, A 549 cells, and Calu-3 cells, which are purchased from the ATCC (American Type Culture Collection, United States) were the applied alveolar epithelial cells in this study. The outgrowth endothelial cells (OECs) were applied as the capillary microvascular endothelial cells in this study. The cell lines of NCI-H441 cells Calu-3 cells and A549 cells are derived from human lung adenocarcinoma bronchial epithelial cells which show similar biological characteristics to alveolar epithelial type II cells (AT-II cells). Therefore, these cell lines are commonly used as suitable cell lines to simulate the alveolar epithelial cells in the research in alveolar epithelial cells. The OECs were isolated from the peripheral blood mononuclear cell fraction of different donors from the Universität Klinikum Schleswig Holstein (UKSH), Kiel. The isolation of OECs was performed according to the previously established protocols [175-177] The acquisition of the blood cell fractions for the isolation of OECs was implemented with the approval of the donors and the ethical authorization of UKSH.

3.2 Medium Preparation

For the cell culture, the cell culture media were prepared beforehand with different supplements for the cultivation of different cell types in the study. NCI-H441 cells and A549 cells were cultured with ibco RPMI-1640 (Roswell Park Memorial Institute 1640) medium (tissue culture medium) (Gibco®/Life Technologies, Paisley, UK) which was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, KS, USA), antibiotics (1% Penicillin and 1% Streptomycin) (pen/strep) (Biochrom GmbH, Berlin, Germany) and 1% L-Glutamines (Gibco®/Life Science, Germany). Calu-3 cells were cultured with DMEM/Ham's F-12 liquid medium (DMEM medium) with stable glutamines (Gibco, by Life Technologies, UK) which was supplemented with 10% FBS, and antibiotics [1% Penicillin (100 U/mL), and 1% streptomycin (100 µg/mL)]. OECs were cultured with the endothelial

cell growth medium-2 (EGM-2) (Lonza, Walkersville, MD, USA) which was supplemented with 5% FBS and antibiotics [1% Penicillin (100 U/mL) and 1% Streptomycin (100 µg/mL)] and also with the kit of full supplements (2 mL hFGF, 0.5 mL VEGF, 0.5 mL IFG-1, 0.5 mL ascorbic acid, 0.5 mL hFGF, 0.5 mL Heparin, 0.2 mL Hydrocortisone)

3.3 Cell Culture

3.3.1 Cultivation of NCI-H441 cells, A549 cells, and Calu-3 cells

The NCI-H441 cells, A549 cells, and Calu-3 cells used in this study were within the range passage numbers from 55 to 62 for NCI_H441 cells, from 15 to 20 for A549 cells, and from 34 to 43 for Calu-3 cells respectively. The cells were initially stored in the vials in the liquid nitrogen vapor phase (-150°C). After cell thawing in the water bath at 37°C, the cells suspensions (1 mL) of NCI-H441 cells and A549 cells were transferred into T75 cell culture flasks (flask area: 75 cm²) (Sarstedt, Nümbrecht, Germany) which were beforehand filled with 13 mL RPMI-1640 medium. The cell suspension of Calu-3 cells was transferred into the T75 flask which was beforehand filled with 13 mL DMEM DMEM /Ham's F-12 medium. The medium was refreshed at intervals every two days. The incubation of cell culture was carried out at 37°C in a 95% humidified atmosphere with 5 % CO₂. When the cells were approximately over 90% confluent, the cells were rinsed once with 5 mL phosphate-buffered saline (PBS) and trypsinized with 2.5 mL trypsin/EDTA to detach the cells from the flask surface. After 5 minutes of incubation at 37°C, 10 mL stop solution (10 % FBS in 1x PBS) was added to deactivate the function of trypsin/EDTA. The cells were collected into a 50 mL centrifugation tube. After 5 minutes of centrifugation at 400 g, the supernatant was aspirated and the cell pellet was resuspended with 5 mL RPMI-1640 medium. The cell number was quantified with the instrument of cell Counter CASY (CASYII TOLS, Bremen, Germany). After that, the total cell numbers in each T75 flask were calculated.

3.3.2 Cultivation of outgrowth endothelial cells (OECs)

The OECs used in the study were within the passage numbers from 5 to 14 from different donors. The cells were initially stored in the vials in the liquid nitrogen vapor phase (-150°C). After cell thawing in the water bath at 37°C, the cell suspensions (1mL) were transferred in a 15 mL Falcon tube, which was filled with 5mL EGM-2 medium to dilute the cell suspension. The Falcon tube was then centrifugated at 400g for 5 minutes. After that, the supernatant of the cell suspension was discarded to remove the Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), which was used for cell freezing but is deleterious and harmful to the cells. Then, the cell pellets were resuspended with 1 mL EGM-2 medium. After that, the cell suspension was transferred into a T75 cell culture flask (flask area: 75 cm²), which was coated with 1:100 diluted fibronectin (10µg/mL) (Millipore, Temecula, CA, USA) beforehand and filled with 10 mL EGM-2 medium. The medium was refreshed at intervals of every two days. The incubation of cell culture was carried out at 37°C in a 95% humidified atmosphere with 5 % CO₂. When the cells were approximately 100 % confluent, the cells were rinsed twice with 5 mL 1x PBS and trypsinized with 4 mL trypsin/EDTA to detach the cells from the flask surface. After 10 minutes of incubation at 37°C, 10 mL stop solution (10 % FBS in 1x PBS) was added to deactivate the function of trypsin/EDTA. The cells were collected into a 50 mL centrifugate tube. After 5 minutes of centrifugation at 400 g, the supernatant was aspirated and the cell pellet was resuspended with 4mL EGM-2 medium. The cell number was quantified with the instrument of the cell counter (CASYII TOLS, Bremen, Germany). After that, the total cell numbers in each T75 flask were calculated.

3.3.3 Reagents in the study for the cell culture

This study used the following reagents for the experimental treatments in the cell culture models. 1- α -25-dihydroxy Vitamin D₃ (Vitamin D₃) (Sigma-Aldrich, MO, USA) reconstituted with 95% ethanol to 10 µM as a stock solution in storage at -20°C. lipopolysaccharide (LPS) (Sigma-Aldrich, MO, USA) stored at -20°C. Cathepsin S inhibitor (Apoptosis and Epigenetics Company, Houston, USA) reconstituted with DMSO in storage

at -20°C. LL-37 (Tocris Bioscience, Bristol, UK) in storage at -20°C. leucine-rich repeat kinase 2 inhibitor 1 (LRRK2-IN-1) (Calbiochem, Darmstadt, Germany) reconstituted with dimethyl sulfoxide (DMSO) to 5 mM as a stock solution in storage at -20 °C. The reagents were purchased for the application of treatments to the cells in the cell culture experiments.

3.3.4 Mono-culture of NCI-H441, Calu-3, and A549 cells

The 24-well cell culture plates (TPP, Trasadingen, Switzerland) (well area: 2 cm²) were used for the mono-culture model of NCI-H441 cells, Calu-3 cells, and A549 cells. The NCI-H441 cells and A549 cells were suspended with RPMI-1640 medium. The Calu-3 cells were suspended with DMEM medium. The cells were seeded with a cell density of 7.5 x 10⁴/cm² into 24-well cell culture plates (well area 2 cm²/well) with 1 mL medium/well. The alveolar epithelial cell lines, including NCI-H441 cells and Calu-3 cells, were also seeded with a cell density of 7.5 x 10⁴/cm² with 200 µL medium/well on the apical sides of Transwell® inserts' filters (Corning, Lowell, Massachusetts, USA) on the 12-well Transwell® cell culture plates (filters' area 0.33 cm²/well) for the TEER measurement experiments. The NCI-H441 cells which were directly cultured in the T75 flasks were used for the preparation of the cell lysates for the Western Blot analysis. The cells were divided into different treatment groups. The required number of wells in the cell culture plates and the number of T75 flasks were dependent on the number of treatment groups. On the third day after the cell seeding, the medium was exchanged with the fresh RPMI-1640 medium supplemented with 1µM dexamethasone (Sigma, St. Louis, MO, USA). (for Calu-3 cells the medium was refreshed only with DMEM medium without dexamethasone). The treatments for the cells also started on day 3. The reagents for the treatments, for example, Vitamin D₃ (10 nM), were supplemented in the medium during the medium exchange. For the LPS treatment, LPS was subsequently supplemented in the medium during the medium exchange from day 5. The LPS treatment was sustained for 48 hours. The group of cells without any treatment was set as the control group. The cell culture procedure was terminated on day 7. Then, the cell lysates from each group were collected for the expression analyses of different target molecules, including Real-time PCR and Western Blot analyses. During the cell culture, the

medium was refreshed daily from day 3 to day 7 with the supplement of the reagent compounds for each treatment. The cells were cultured in the incubator at 37 °C in 95% humidity with 5 % CO₂. The mono-culture of the alveolar epithelial cell lines was carried out with the repetitions of at least three independent experiments ($n \geq 3$) with the usage of different cell passage numbers of the alveolar epithelial cell lines: NCI_H441 cells (passages from 55 to 62), A549 cells (passages from 15 to 20), and Calu-3 cells (passages from 34 to 43).

3.3.5 Mono-culture of OECs

The 24-well cell culture plates (TPP, Trasadingen, Switzerland) (well area: 2 cm²) were used for the mono-culture model of OECs, which were pre-coated with 1:100 diluted fibronectin (10 µg/mL) beforehand. The OECs were homogeneously suspended in the EGM-2 medium and were seeded with a cell density of $5.0 \times 10^4/\text{cm}^2$ into each well with 1 mL medium /well (1.0×10^5 cells/well). The cells were divided into different treatment groups. The group of cells without any treatment was set as the control group for the experiments. The seeded cells were treated with the reagents, e.g., Vitamin D₃, on day 3 after the cell seeding. The treatment with Vitamin D₃ (10 nM) was supplemented in the EGM-2 medium during the medium exchange for the Vitamin D₃ and Vitamin D₃ + LPS treatment groups. For the LPS treatment, LPS was subsequently supplemented in the medium on day 5 for the LPS and Vitamin D₃ + LPS treatment groups. The LPS treatment was also sustained for 48 hours during the cell culture. The cell culture procedure was terminated on day 7. Then, the cell lysates from each group were collected for expression analyses of different target molecules. including, Real-time PCR analysis. During the cell culture, the medium was refreshed daily from day 3 to day 7 with the supplement of the reagent compounds for each treatment. The cells were cultured in the incubator at 37°C in a 95% humidified atmosphere with 5 % CO₂. The mono-culture of OECs was carried out with the repetitions of at least three independent experiments ($n \geq 3$) with the usage of different donors and cell passage numbers (passages from 5 to 14) of OECs.

3.3.6 Co-culture of NCI-H441 cells and OECs

The Transwell® inserts (Corning, Lowell, Massachusetts, USA) were used for the co-culture model of NCI-H441 cells and OECs. The filter of the Transwell® inserts is constructed with a semi-permeable polycarbonate membrane with many micropores (membrane thickness: 10 μm , filter area: 0.33 cm^2 , pore size: 0.4 μm). The polycarbonate filter is available for the attachment of cells on both apical and basolateral sides, which makes it feasible to study cell-to-cell communication and the integrity and properties of the cell barrier. Before the cell seeding, the basolateral side of the filters was coated with 1:100 diluted fibronectin (10 $\mu\text{g/mL}$). After the coating, the OECs were seeded on the basolateral side with EGM-2 medium (80 $\mu\text{L}/\text{filter}$) with a cell density of 5×10^4 cells/ cm^2 (1.7×10^4 cells per filter). 2 hours of incubation at 37°C was required for the stable attachment of OECs on the basolateral surface of the filter. After that, 800 μL EGM-2 was gently filled into the basolateral compartment of each Transwell®. The NCI-H441 cells were then seeded with 200 μL RPMI-1640 medium on the apical compartment with a cell density of 1.5×10^5 cells/ cm^2 (5×10^4 cells per filter). The co-culture model of NCI-H441 cells and OECs was hereby established after cell seeding. The cells were also divided into different treatment groups. The group of cells without any treatment was set as the control group. On day 3 after cell seeding, the media in both apical (RPMI-1640) and basolateral (EGM-2) compartments were exchanged with fresh medium. For the apical compartments 200 μL RPMI-1640 medium which was supplemented with 1 μM dexamethasone, was refreshed for NCI-H441 cells. For the basolateral compartments, 800 μL EGM-2 medium was refreshed for OECs. Moreover, the treatments for the cells were also initiated on day 3. The reagents for the treatments were supplemented in the medium during the medium exchange for the treatments. It is important to note that the treatment with LPS was started on day 5. For the LPS treatment, LPS was supplemented in the EGM-2 medium which was applied only for the basolateral compartments. The LPS treatment on OECs lasted for 48 hours. The co-culture model of NCI-H441 cells and OECs was terminated on day 7. Then, the cell lysates of NCI-H441 cells on the apical compartment were collected from each treatment group for the expression

analyses of different target molecules, including Real-time PCR. During the cell culture, the medium was changed from day 3 to day 7 with the supplement of the reagent compounds for each treatment. The cells were incubated at 37°C in a 95% humidified atmosphere with 5 % CO₂. The co-culture was carried out with the repetitions of at least three independent experiments ($n \geq 3$) with the usage of different cell passage numbers of the NCI-H441 cells (passages from 55 to 62) and different cell passage numbers of the isolated OECs (passages from 5 to 14) from different donors.

3.4 Assessment of the Cellular Barrier Properties through TEER Measurement

The properties of the simulated alveolar-capillary barrier were evaluated through the measurement of transepithelial/transendothelial electrical resistance (TEER) which was implemented in the co-culture model of NCI-H441 cells and OECs in Transwell® (Corning, Lowell, Massachusetts, USA) (Transwell® filter size: 0.33 cm² pore size: 0.4 µm). Moreover, the TEER measurements were also implemented in the mono-culture model of NCI-H441 cells, and Calu-3 cells, which were established on the Transwell® filters (Corning, Lowell, Massachusetts, USA) respectively (Transwell® filter size: 0.33 cm² pore size: 0.4 µm). The measured TEER values were interpreted as Ohm × cm² ($\Omega \times \text{cm}^2$) and measured by using an EVOM volt-ohm meter (World Precision Instruments, Berlin, Germany) which is equipped with a pair of STX-2 chopstick electrodes. The electrodes were inserted into the apical and basolateral compartments without contact with the filter surface, and the values of (Ω) were read from the volt-ohm meter. The TEER values were documented from day 3 to day 7. The final TEER values were shown as the mean resistance × filter area ($\Omega \times \text{cm}^2$) measured from at least three independent cell culture experiments ($n \geq 3$) of the cell culture models. The electrical resistance of the blank Transwell® filter (104 Ω) as the background TEER value of the Transwell® was subtracted during the analysis. Additionally, the measured TEER values were multiplied by the area of the Transwell® filter (filter area: 0.33 cm²) to calculate the exact TEER values of the barrier ($\Omega \times \text{cm}^2$).

3.5 Assessment of the Barrier Properties of Capillary Microvascular Endothelial Cells with ECIS Measurement

The barrier properties of capillary microvascular endothelial cells were evaluated using the Electric Cell-substrate Impedance Sensing (ECIS) instrument. The ECIS of array slide electrodes can provide sophisticated impedance measuring circuits, which enable the ECIS instrument to simultaneously measure the impedance and resistance value of the cell layer of the confluent vascular endothelial cells. The 400 μL isolated OECs with the cell density of 4×10^4 cells/ cm^2 were seeded on each well of the 8 well 10 gold film electrode array slides (8W10E) (well area: 0.8 cm^2) (Applied Biophysics Inc., NY, USA), which were pre-coated with fibronectin (10 $\mu\text{g/mL}$) beforehand. The gold pads at the edge of the array slides were connected to the array station of the ECIS instrument, which was placed in the incubator at 37°C in a 95% humidified atmosphere with 5 % CO_2 . The Real-time impedance values (Ω) of the cell barrier were monitored. The cell culture procedure was implemented according to the mono-culture procedure of OECs. The blank value measured from the well that was only filled with medium without cell layer was subtracted from the measured values of the wells with cell layers at different time points.

3.6 Gene Expression Assessment

For the gene expression analysis, the NCI-H441 cells, from both mono-culture and co-culture models and the OECs from the mono-culture models, were collected and lysed with RNA Lysis Buffer T (Peqlab, Darmstadt, Germany) on day 7. The procedures of RNA isolation, RNA to cDNA reverse transcription, and Real-time PCR analysis were performed according to the manufacturer's protocols of the applied kits (Peqlab, Darmstadt, Germany)

3.6.1 RNA isolation

The RNA isolation was performed using the peqGOLD Total RNA Kit (Peqlab, Darmstadt, Germany). The cell lysates were prepared with RNA Lysis Buffer T for RNA isolation. The cell lysates of each sample with a volume of approximately 500 μL were transferred in the

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DNA Removing Columns, which were placed on the 2.0 mL Collection Tubes. The column and tube assemblies were centrifugated at 12.000 g for 1 minute at room temperature. After that, the DNA Removing Columns were discarded, and 70 % ethanol was added, which was equal to the volume of each sample. The lysates and ethanol were thoroughly mixed well by vortex, and the solutions were transferred in Perfect Bind RNA Columns, which were placed on the new 2.0 mL Collection Tubes. After the centrifugation at 10.000 g for 1 minute, the flow-through was discarded and 500 μ L RNA Wash Buffer I was added to each column. The column and tube assemblies were centrifugated at 10.000 g for 15 seconds, and the Perfect Bind RNA Columns were placed on the new 2.0 mL Collection Tubes. Then, 75 μ L DNase I Digestion Mixture per sample (73.5 μ L DNase I Digestion Buffer + 1.5 μ L 20x RNase-free DNase I) was directly pipetted onto the surface of the resins in each Perfect Bind RNA Column. The samples were incubated at room temperature for 15 minutes, and the columns were subsequently added with 400 μ L RNA Wash Buffer I and incubated for 5 minutes at room temperature. Then, the assemblies were centrifugated at 10.000 x g for 15 seconds. Then, the flow-through was discarded and 600 μ L RNA Wash Buffer II per sample was added to the column and centrifugated at 10.000 x g for 15 seconds. This washing process with RNA Wash Buffer II was repeated once more. After the washing, the columns were placed on the new 2.0 mL Collection Tubes and centrifugated at 10.000 x g for 2 minutes to remove ethanol and completely dry the column matrix. For the elution of the isolated RNA, the Perfect Bind RNA Columns were placed onto new 1.5 mL Eppendorf Tubes (Eppendorf, Hamburg, Germany) and pipetted with 30 μ L sterile RNase-free H₂O (20 μ L for OECs) and then centrifugated at 10.000 x g for 1 minute. The centrifugation of the assembly can be repeated one more time for the insurance to elute all the RNA-containing filtrate from the columns. After the final centrifugation, the Perfect Bind RNA Columns were discarded, and the RNA from each sample was extracted with the flow-through and collected in the Eppendorf Tubes.

3.6.2 Reverse transcription of RNA to cDNA

1.5 μL of the isolated RNA from each sample was pipetted to the detector of Nanodrop 2000 (Pecolab, Erlangen, Germany) to determine the RNA concentration. 1 μg RNA per sample was used for the reverse transcription with the RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, USA), in which 10 μL 2x TagMan RT Buffer and 1 μL 20x RT Enzyme Mix were mixed well for each sample. After the addition of an adequate amount of Nuclease-Free H_2O up to the total volume of 20 μL , the samples were mixed well and centrifugated. The samples were incubated in the Thermomixer Eppendorf, Hamburg, Germany) at 37°C for 1 hour. After that, the reaction was terminated through incubation in the Thermomixer at 95°C for 5 minutes. The solutions of each sample were finally filled with Nuclease-Free H_2O up to the volume of 100 μL with the cDNA concentration of 10 ng/ μL .

3.6.3 Semi-quantitative real-time polymerase chain reaction (Real-time PCR)

The expression level of the targeted genes was quantified through Semi-quantitative Real-Time polymerase chain reaction (Real-time PCR), which enables this study to evaluate the semi-quantitative values of the expressed mRNA (Message RNA) levels of the genes of the investigated molecules in this study. The Real-time PCR analyses were performed using the iCycler iQ Real-time Detection System (Bio-Rad, Hercules, CA, USA). For the Real-time PCR analyses, 16.8 μL master mix per sample [constituted with 12 μL QuantiTect™ SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany), 2 μL QuantiTect™ SYBR® Green Primer Assay (Qiagen, Hilden, Germany), and 2.8 μL Nuclease-Free H_2O], together with 3.2 μL cDNA (10 ng/ μL) per sample were supplemented in a total volume of 20 μL per each sample were implemented for the Real-time PCR reaction. The Real-time PCR reaction was executed with the usage of Rotor-Gene Real-time PCR Cycler (Qiagen Hilden Germany) using the standard thermal-cycling program of SYBR® Green Master Mix according to the following steps: uracil-DNA glycosylase (UDG) activation 2 minutes at 50 °C, DNA polymerase activation 2 minutes at 95°C, and 40 cycles of DNA denaturation 15 sec at 95 °C and the following DNA annealing and extension steps for 1 minute at 60°C. The reaction was

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finally ended and held at 20°C. A housekeeper gene RPL13A was used as a reference gene to normalize the gene expression level in the analysis. The gene expression was ultimately determined through the $\Delta\Delta C_t$ method, in which the C_t values of each gene were subtracted from the C_t values of the housekeeper gene RPL13A during the assessment. Primers applied in the analysis are displayed in Table 1.

Table 1: Primer list of the target genes for Real-time PCR analysis

| Gene Name | Primer | Catalog Number |
|----------------------------------|--------------------|------------------------|
| 60S Ribosomal Protein13a RPL13A) | Hs_RPL13A_1_SG | QIAGEN/QT089915 |
| IL-1 β (IL1Beta) | Hs_IL1B_1_SG | QIAGEN/QT00021385 |
| IL-6 (IL-6) | Hs_CXCL6_1_SG | QIAGEN/QT 330701 |
| IL-8 (CXCL8) | Hs_IL8_1_SG | QIAGEN/QT00000322 |
| SP-A (SFTPA2) | Hs_SFTPA2_1_SG | QIAGEN/QT00072926 |
| ZO-1 (TJP1) | Hs_TJP1_1_SG | QIAGEN/QT00077308 |
| Occludin (OCLN) | Hs_OCLNva.1_SG | QIAGEN/QT01327739 |
| Claudin-5 (CLDN5) | Hs_CLDN5_1_SG | QIAGEN/QT00232197 |
| E-Cadherin (CADH1) | Hs_CADH1_1_SG | QIAGEN/QT00080143 |
| Epidermal Growth Factor (EGF) | Hs_EGF 01099996_m1 | Thermo Fisher/ 4351372 |
| ICAM-1 (ICAM-1) | Hs_ICAM_1_SG | QIAGEN/QT00074900 |
| LL-37 (CAMP) | Hs_CAMP_1_SG | QIAGEN/QT00010458 |
| Cathepsin S (CTSS) | Hs_CTSS_1_SG | QIAGEN/QT00040404 |
| DCLK1 | Hs_DCLK_1_1_SG | QIAGEN/QT00071323 |
| Vitamin D Receptor(VDR) | Hs_VDR_1_SG | QIAGEN/QT01010177 |
| PKCA PKC (Alpha) | _PRKCA_1_SG | QIAGEN / QT330001 |
| PKCB (PKC (Beta) | Hs_PRKCB_1_SG | QIAGEN / QT249900 |
| PKCH (PKC (Eta) | Hs_PRKCH_1_SG | QIAGEN/QT 0381029 |
| Caveolin 1 | Hs_CAV1_1_SG | QIAGEN/QT00012607 |

3.7 Immunofluorescence Cell Staining and Imaging

3.7.1 Preparation of the cells for the staining

The protein expression levels of the target molecules in the cells were photographically analyzed through Immunofluorescence cell staining. For the immunofluorescence staining, the cells were prepared via the mono-culture procedure. The cells were cultured on the apical side of Transwell® filters or were cultured on the Thermanox coverslips, which were covered on the 24-well cell culture plates or cultured on the 8-well Ibidi slides (well area: 1 cm², Ibidi, Martinsried, Germany). The coverslips and the Ibidi slides were pre-coated with 1:100 diluted fibronectin (10 µg/mL). The cells were treated solely with Vitamin D₃ (10 nM) or treated with conditioned medium (CM) which were derived from the mono-culture of OECs with the treatments of Vitamin D₃ (10 nM) and LPS (100ng/mL). The usage of a conditioned medium enabled this study to investigate the impact of Vitamin D₃ (10 nM) and LPS (100ng/mL) on the morphological properties and integrity of the tight junctional protein ZO-1 in the alveolar epithelial cells in the context of the co-culture model.

3.7.2 Immunofluorescence staining procedure and fluorescence microscope detection

The cell culture processes were finished on day 7. Afterward, the cells were fixed with 500 µL/well 4% paraformaldehyde (PFA) (for Transwell® 200 µL/well) with incubation for 10 - 15 minutes at room temperature. After that, the PFA was aspirated, and the cells were 3 times washed with 1x PBS (500 µL /well, for Transwell® 200 µL/well) with 5 minutes of incubation at room temperature for each time. After that, 0.5 % Triton®X-100 was added to the cells (500 µL /well, for Transwell® 200 µL/well) for the permeabilization of the cells with 10 minutes of incubation at room temperature. After the incubation, the 0.5 % Triton®X-100 was aspirated, and the cells were repeatedly washed with 1x PBS, which was performed as the washing procedure described in the former text. After the wash, primary antibodies with adequate dilution in 1 % (bovine serum albumin) BSA buffer were added to the cells (300 µL/well, for Transwell® 150 µL/well), and the cells were incubated at room temperature

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for 1.5 hours. Then the washing procedure with 1x PBS was repeated. After that, 300 µl fluorescent conjugated secondary antibody with 1: 1000 dilution in 1 % BSA (2 µg/ml) was added to the cells (300 µL/well, for Transwell® 150 µL/well). Then, the cells were placed in the dark condition to avoid light exposure after the staining with secondary antibody and incubated at room temperature for 1 hour. After the incubation, the washing procedure with 1x PBS was repeated. After the washing, the cell nuclei were counterstained with Hoechst (300 µL/well, for Transwell® 150 µL/well), which was 1:500 diluted in 1x PBS with a concentration of 2 µg/mL. The cells were incubated in the dark condition at room temperature for 10 minutes. Then, the washing procedure was repeated. After the final washing, the coverslips were carefully removed from the 24-well plate and carefully put on the sample slides. For the microscopy analysis of the cells cultured in Transwell®, the transwell filters were carefully chipped off from the Transwell® and carefully put on the sample slides after the staining. Then, the cells were mounted with several drops of Fluoromount. Aqueous Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA), and the slides were finally covered by glass coverslips. After the drying, confocal laser scanning microscopy (CLSM) detection was performed, in which the samples were observed using a Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss Oberkochen Germany) to detect the fluorescent signals of specific molecules or cellular structures in the cells. For the immunofluorescence detection of the cells cultured in the 8-well Ibidi slides, the Ibidi slides with the cells were directly put on the Eppendorf tube-fluorescent Evos FL Auto 2 microscope (Life Technologies, Grand Island, USA) without mounting for fluorescence detection. The implemented antibodies and the dilution of the antibodies in the staining experiments of the target proteins in the cells are displayed in Table 2.

Table 2: Antibody list of the target proteins for Immunofluorescence Staining

| Primary Antibodies | | | |
|-----------------------------|---------|----------|------------------------|
| Antibodies | Species | Dilution | Manufacturers |
| Anti- ZO-1 | Rabbit | 1:100 | Invitrogen (USA) |
| Anti-Occludin | Rabbit | 1:100 | Invitrogen (USA) |
| Anti-E-Cadherin | Goat | 1:100 | R&D Systems (USA) |
| Anti- VE-Cadherin | Goat | 1:50 | R&DSsystems (USA) |
| Anti-SP-A | Mouse | 1:100 | Abcam (UK) |
| Anti-SP-C | Rabbit | 1:20 | Abcam (UK) |
| Anti-CD74 | Mouse | 1:10 | Abcam (UK) |
| Anti-CD31 | Mouse | 1:50 | ako,Glostrup (Denmark) |
| Anti-LL-37 | Mouse | 1:50 | HycultBiotech (USA) |
| Secondary Antibodies | | | |
| Alexa Fluor 488 anti-mouse | Rabbit | 1:100 | Invitrogen (USA) |
| Alexa Fluor 555 anti-rabbit | Donkey | 1:1000 | Invitrogen (USA) |
| Alexa Fluor 555 anti-goat | Donkey | 1:1000 | Invitrogen (USA) |
| Alexa Fluor 488 anti-rabbit | Donkey | 1:1000 | Invitrogen (USA) |

3.8 Western-Blot Analysis

3.8.1 Cell lysates preparation for the Western-Blot

The protein expression levels of the specific molecules were also evaluated through Western-Blot analysis. For the analysis, the proteins from the cell lysates were prepared. For the cell lysate preparation, the NCI-H441 cells were cultured in the T75 flasks. The cell culture procedure was executed according to the mono-culture procedure of NCI-H441 cells. On day 7 the cell culture was terminated. Then, the NCI-H441 cells were collected for the cell lysates preparation. This procedure is performed as the following described below. Firstly, the medium was removed from the T75 flasks. Afterward, the cells were 2 times washed with 5 mL PBS. After the wash, the PBS buffer was removed, and 2.5 mL 1× trypsin/EDTA, was added to the flasks. The cells were incubated in the incubator for 5 minutes. Then 10 mL stop solution (10 % FBS in PBS) was added to each flask. Afterward, the cells were collected into 15mL Falcon tubes and the cell suspensions were centrifugated at 400 g for 5 to 10 minutes.

The supernatants were discarded and the cell pellets were then resuspended with PBS (2mL/treatment group). The cell suspensions from each treatment group were transferred into 1.5 mL Eppendorf tubes (2x1.5 mL Eppendorf tubes for each treatment group with 1 mL cell suspension in each Eppendorf tube). The Eppendorf tubes were centrifugated at 400 g for 10 minutes. After the centrifugation, the PBS supernatant was discarded. The cell pellets were then lysed with 250 μ L RIPA Buffer + Protease and Phosphatase Inhibitors in each Eppendorf tube, and cell lysates were merged together from the two Eppendorf tubes of each treatment group (500 μ L/treatment group). Then, the cell lysates in the Eppendorf tubes were incubated on the ice for 30 min. During the incubation, the cell lysates were 5 times sonicated for 10 seconds at the lowest power. After the sonication, the Eppendorf tubes were centrifuged for 20 minutes, at 14000 g at 4°C. Finally, the supernatants with the cell lysates from each treatment were transferred into new Eppendorf tubes (500 μ L/ Eppendorf tube). The cell lysate samples were frozen in the freezer at -80 °C to store the samples for the measurement of the protein concentrations.

3.8.2 Protein concentration measurement

The protein concentrations of the isolated cell lysates were tested with the application of the BCA Protein Assay Reagent Kit (Pierce Biological Technology, Rockford, USA), using the microplate procedure. For the protein concentration measurement, the standard bovine serum albumin (BSA) diluents were pre-prepared. The preparation protocol of the set of standard diluents is displayed in Table 3.

Table 3: Protein Standard dilution procedure for protein concentration determination

| Standard | RIPA Buffer (μL) | BSA (μL) | Final Concentration (μg/mL) |
|----------|------------------|--------------------|-----------------------------|
| A | 0 μL | 100μL Stock | 2000 |
| B | 30 μL | 90 μL Stock from A | 1500 |
| C | 50 μL | 50 μL from A | 1000 |
| D | 50 μL | 50 μL from B | 750 |
| E | 50 μL | 50 μL from C | 500 |
| F | 50 μL | 50 μL from E | 250 |
| G | 50 μL | 50 μL from F | 125 |
| H | 40 μL | 10 μL from G | 25 |
| I | 50 μL | 0 μL | 0 |

After the preparation of the standard diluent series, the BCA Working Reagent (WR) was prepared for the test. The required volume was calculated with the formula: (9 standards + X samples) × 3 (triplicates) = (27 + 3 x) × 200μL (200μL working reagent/well). The working reagent buffer was prepared with the mixture of reagent A and Reagent B in the ratio of Reagent A: Reagent B (50:1). After the preparation of WR, the WR was stored in a closed Falcon Tube at room temperature. The samples were 1:10 diluted with RIPA buffer (4 μL original sample + 36 μL RIPA Buffer and in total 40 μL/sample). After that, the samples were incubated on ice. Then, the standard diluents and the samples (10 μL/well) were added to a 96-well plate. Afterward, the Working Reagent which was placed in a reagent reservoir was added to the 96-well plate with a multi-channel pipette (200 μL/well). The 96-well plate was then shaken for 10 seconds at the lowest shaking speed. Afterwards, the plate was incubated for 30 minutes, at 37 °C. After the incubation, the plate was held for 10 minutes. The optical density of the samples in the plate was immediately measured with an automatic microplate reader (Apollo LB 911 TECAN, Berthold Technologies, Austria) at the wavelength 560 nm. The measured data were finally analyzed, through MS Excel for the calculation of the protein concentrations of each sample.

3.8.3 Protein expression level evaluation with Western-Blot

An equal amount of the protein extracts of each sample (from 60 μg to 150 μg) were supplemented with non-reducing lithium dodecyl sulfate loading buffer (LDS Sample Buffer,

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Thermo Fisher Scientific, Rockford, IL, USA) in the ratio of 3:1. Then, the protein in each sample was denatured with the incubation in the Thermomixer at 70 °C for 2 minutes. After the denaturation procedure, the protein samples together with 5µL marker (Precision plus protein western C, Bio-Rad, Hercules, USA) were orderly added into each channel of SDS-PAGE Gels (Invitrogen, Carlsbad, CA, USA) which was prewashed with SDS buffer (Gel Electrophoresis running buffer) and was stabilized with the holder of Electrophoresis chamber (Invitrogen, Eugene, USA), and was also filled up with SDS buffer. Then, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the protein samples was performed for 1 or 1.5 hours at 200 voltage to separate the proteins. (the gel running time and the voltage size were dependent on the protein size). After the electrophoresis, the SDS gels were removed from the electrophoresis chamber and equilibrated with transfer buffer (20% Methanol in 1:10 diluted SDS buffer) for 5-10 minutes. Meanwhile, the Gel-PVDF membrane (Immobilon, Germany) was prepared with the equilibration with methanol (Carl Roth, Karlsruhe, Germany). Then, the Western-Blot sandwiches assembly was prepared for the Western-Blot running, which was constituted of 1 layer of Sponge, - 2 layers of Whatman paper, SDS-Gel - PVDF membrane 2 layers of Whatman 1 paper, and 1 layer of sponge. The gel was faced to the cathode side of the Western-Blot cassette holder (Mini Blot Module, Invitrogen, Eugene, USA), while, the membrane was faced to the anode side of the Western-Blot cassette holder. After the preparation of the Western-Blot sandwich cassette, the cassette was filled with transfer buffer and placed into the electrophoresis chamber, and the chamber was also filled with distilled water. Then run the Western Blot at a constant voltage of 20 V for 1 hour. Then, the proteins were transferred from the SDS Gels onto the PVDF membranes. After the running procedure, the PVDF membrane was removed from the Western-Blot cassette, and the membrane was blocked with 20mL in 1xTBS/T buffer (Thermo Fisher Scientific, USA) with 3 % solved skimmed milk (BIO-RAD, Hercules, USA) with incubation for 1 hour on the roller at room temperature. After the block, the supernatant was discarded, and the membranes were transferred into 50 mL falcon tubes. The PVDF membranes were then covered over with 10 mL TBS/T buffer with 3 % solved skimmed milk, which was

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supplemented with appropriately diluted primary antibodies of the specific proteins in this study. Then, the membrane was incubated on the roller overnight at 4 °C. On the next day, the membranes were 3 times washed with TBS/T buffer. After the washing, the membranes were covered over with 3% skimmed milk solved in TBS/T buffer, which was supplemented with the appropriately diluted secondary antibodies, which were conjugated with transmembrane protein (Protein TM), and Strep-Tactin HRP against the primary antibodies. Afterward, the membrane on the roller was incubated for 1 hour at room temperature. Afterward, the membranes were 3 times washed with 20mL TBS/T Buffer. After the washing, the whole membranes were exposed to Pierce ECL Western Blotting Substrate mix or ECL mix plus (Thermo, Rockford, USA) with incubation from 1 to 5 minutes. The protein signals were detected using High-Performance Chemi-luminescence Films (GE Healthcare, Little Chalfont, UK) in the dark atmosphere. The quantification of the density of the bands indicates the protein signals were analyzed via the software Image Studio Lite (LI-COR, Lincoln, USA). A housekeeper protein β -actin was used for the normalization of the protein expression levels for the analysis. The applied primary and secondary antibodies and the dilution of the antibodies in the Western-Blot experiment are listed in Table 4.

Table 4: Antibody list of the target Proteins for Western-Blot analysis.

| Primary Antibodies | | | |
|-----------------------|---------|----------------|---------------------------|
| Antibodies | Species | Dilution | Manufacturers |
| Anti-DCKLK1 | Rabbit | 1:5000 | Abcam (UK) |
| Anti-ZO-1 | Rabbit | 1:200 | Invitrogen (USA) |
| Anti-Occludin | Rabbit | 1:200 | Invitrogen (USA) |
| Anti-E-Cadherin | Goat | 1:400 | R&D system (USA) |
| Anti-actin | Rabbit | 1:1000 | Cell Signaling Tech (USA) |
| Secondary Antibodies | | | |
| SC-516102 anti- mouse | Goat | 1:5000-1:10000 | Santa Cruz (USA) |
| SC-2004 anti-rabbit | Goat | 1:5000-1:10000 | Santa Cruz (USA) |
| HAF109 anti-goat | Donkey | 1:1000 | R&D System (USA) |

3.9 Statistical Analysis

The data from the experiments were calculated and analyzed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, USA). The data in all of the graphs were demonstrated as mean \pm standard deviation of the mean (SD). The statistical analyses of the experimental data were evaluated with the usage of T-test and One- or Two-way ANOVA. In the statistical analysis of the experimental data, Turkey or Bonferroni post-tests were applied for the calculation and evaluation of the significance of the differences between each data group. The statistical confidence level was set to be significant when the p-values were less than 0.05 (* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3.10 Summary of the Invested Subjects in the Study

3.10.1 Mono-culture of the alveolar epithelial cell lines with Vitamin D₃ treatment

In the mono-culture model of NCI-H441 cells, Calu-3 cells, and A549 cells, the cell mono-culture procedures were implemented according to the previously described in section 3.3.6. Besides, dexamethasone (1 μ M), NCI-H441 cells, and A549 cells were only treated with Vitamin D₃ (10 nM). Calu-3 cells were only treated with Vitamin D₃ (10 nM) without dexamethasone. During the cell culture, the TEER measurement was performed as pre-described in section 3.4. After the cell culture, the gene expression levels of LL-37, DCLK1, a group of Protein Kinase C isozymes (PKC A, PKC B, PKC H), SP-A, intracellular junction proteins (including ZO-1 Occludin, Claudin-5, E-Cadherin), and Vitamin D receptor (VDR), Caveolin-1 were evaluated in NCI-H441 cells, Calu-3 cells, and A549 cells with Real-time PCR analysis. This procedure was conducted according to the described in section 3.6. The protein expression level of DCLK1 was tested in NCI-H441 cells with Western-Blot (as described in section 3.8). Moreover, the protein expression levels of ZO-1 Occludin, SP-A, LL-37, and DCLK1 in NCI-H441 cells were visually and morphologically analyzed through immunofluorescence staining and immunofluorescence microscopy (as described in section 3.7).

3.10.2 Study of the involved molecules in the LPS infection-induced inflammation

This study performed the co-culture model of NCI-H441 cells and OECs in Transwell® and the mono-culture models of the individual cell types. The cells were treated with Vitamin D₃ (10 nM) and LPS (100 ng/mL, 10 µg/mL). The cell culture process of the co-culture of NCI-H441 cells and OECs, the mono-culture of NCI-H441 cells, and the mono-culture of OECs were performed as pre-described in section 3.3.8, section 3.3.6, and section 3.3.7, respectively. The effects of Vitamin D₃ and LPS on the properties of the alveolar-capillary barrier in the co-culture model and the barrier properties of OECs-modeled capillary endothelial barrier were evaluated with TEER, and ECIS measurements respectively, which were carried out as pre-described in sections 3.4 and 3.5. After the cell culture, the gene expression levels of IL-1β, IL-6, IL-8, Surfactant protein A (SP-A), Vitamin D receptor (VDR), VE-Cadherin, Cathepsin S, and intercellular adhesion molecule (ICAM-1) were analyzed in NCI-H441 cells and OECs, with Real-time analysis as described in section 3.6. For a deeper study, the treatments with Cathepsin S specified inhibitor and LPS (100 ng/mL, 10 µg/mL) were also executed in the mono-culture model of NCI-H441 cells in Transwell® and the mono-culture model of OECs, and the co-culture model of NCI-H441 cells and OECs in Transwell®. Furthermore, the TEER measurement as described in section 3.4 was also carried out in the mono-culture model of NCI-H441 cells in Transwell® and the co-culture of NCI-H441 cells and OECs in Transwell®.

3.10.3 Study of LL-37 on NCI-H441 cells

The study of LL-37 on NCI-H441 cells was carried out in the mono-culture model of NCI-H441 cells in Transwell®, and also in the co-culture model of NCI-H441 cells and OECs in Transwell®. In the mono-culture of NCI-H441 cells, the cells were treated with LL-37 (0.1 ng/mL, 1 ng/mL, and 10 ng/mL). TEER measurement was also performed during the cell culture. The mono-culture process of NCI-H441 cells in Transwell® and TEER measurements were conducted as pre-described in section 3.3.6 and section 3.4, respectively. After the cell culture, the gene expression levels of the junctional protein group (ZO-1,

Occludin, Claudin-5, and E-Cadherin) and a group of Protein Kinase C isozymes (PKC A, PKC B, PKC H) were evaluated with Real-time PCR. The LL-37 study was also performed in the co-culture model of NCI-H441 cells and OECs in Transwell®. The cells were treated with LL-37 and LPS (100 ng/mL, 10 µg/mL). The barrier properties in the cell culture models were documented with TEER measurement during the cell culture. The co-culture of NCI-H441 cells and OECs in Transwell® and TEER measurements were carried out as pre-described in section 3.3.8 and section 3.4 respectively. After the cell culture, the gene expression levels of the interleukin cytokine group (IL-1β, IL-6, IL-8), Surfactant protein A (SP-A), and tight junction group (ZO-1, Occludin, Claudin-5) were analyzed through Real-time PCR performed as pre-described in section 3.6.

3.10.4 Further study of DCLK1 on NCI-H441 cells

The study of DCLK1 was investigated in the mono-culture model of NCI-H441 cells, which was performed with 24 cell culture plates, Transwells, and T75 flasks. The cells were treated with Vitamin D₃ (10 nM) and DCLK1 inhibitor (500 nM). TEER measurement was implemented during the mono-culture of NCI-H441 cells in Transwell®. The mono-culture of NCI-H441 cells and TEER measurement were conducted as pre-described in section 3.3.6 and section 3.4 respectively. The cells were treated with Vitamin D₃ (10 nM) on Day 3. The treatment with DCLK1 inhibitor LRRK2-IN-1 (500 nM) was initiated from day 5. After the cell culture, the gene expression levels of DCLK1, junction protein group (ZO-1, Occludin, Claudin-5, E-cadherin), EGF, and Caveolin-1 were analyzed with Real-time PCR which was performed as pre-described in section 3.6. Moreover, the protein expression levels of DCLK1, ZO-1 Occludin, and E-Cadherin were assessed through Western-Blot (the procedure is described in section 3.8). For a deeper study, NCI-H441 cells were treated with Vitamin D₃ (10 nM), and DCLK1 inhibitor (500 nM, 5µM). TEER measurement was also executed during the cell culture on the Transwell®. After the cell culture, the gene expression levels of DCLK1, junction protein group (ZO-1, Occludin, Claudin-5, E-Cadherin) were analyzed with Real-time PCR. which was performed as pre-described in section 3.6.

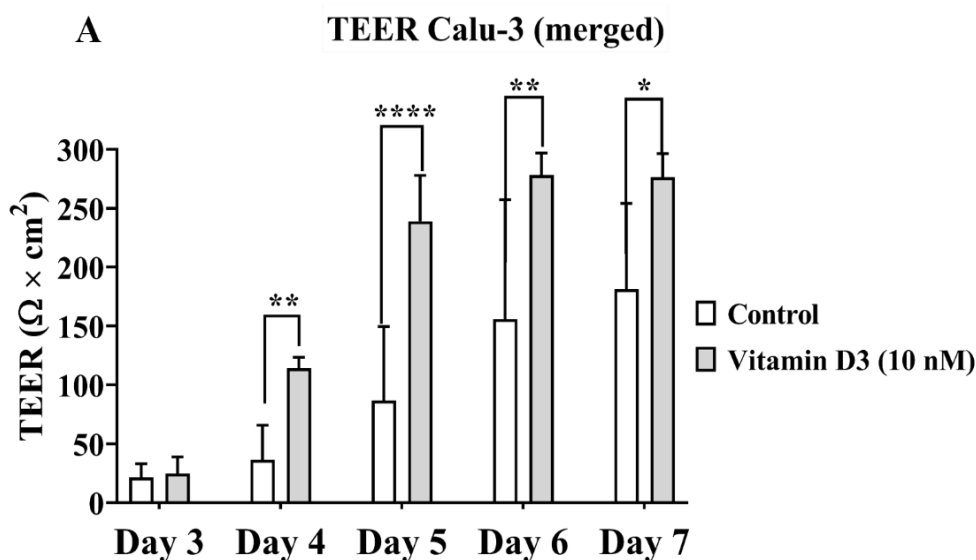
4 Experimental Results

4.1 Effects of Vitamin D₃ on the Function of the Alveolar Epithelial Barrier and the Expression of Related Molecules in the Alveolar Epithelial Cell Lines

This study first explored the effects of Vitamin D₃ on the maintenance of the barrier properties of the alveolar epithelial barrier and also the expression of the genes of the involved molecules in the cell lines of alveolar epithelial cells. The results are demonstrated as follows.

4.1.1 Impact of Vitamin D₃ on the barrier properties of alveolar epithelial cells

This study performed the mono-culture of NCI-H441 cells and the mono-culture of Calu-3 cells in the Tranwells. The Calu-3 cells and NCI-H441 cells in the mono-culture model were treated with Vitamin D₃ (10 nM). The barrier properties of the alveolar epithelial barrier in the mono-culture models of the alveolar epithelial cell lines in Transwell® were evaluated with the implementation of TEER measurement.



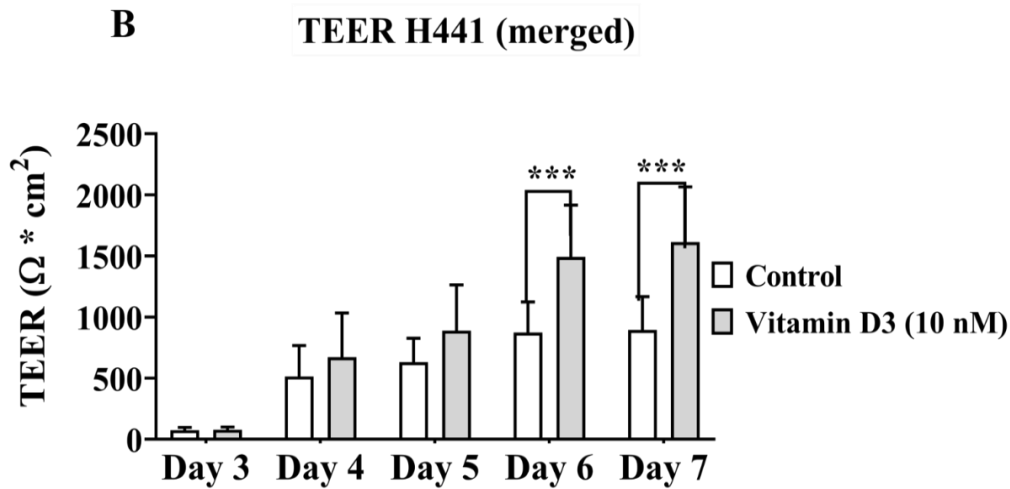


Figure 4.1: TEER measurement of (A) the mono-culture model of Calu-3 cells in Transwell® with VitaminD₃ (VD₃) (10 nM) treatment, and (B) the mono-culture model of NCI-H441 cells in Transwell® with Vitamin D₃ (VD₃) (10 nM) and treatments. The data were statistically analyzed with Tow-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

From the results of TEER measurement of the mono-culture model of Calu-3 cells and mono-culture model of NCI-H441 cells in Transwell®, Vitamin D₃ (10 nM) showed a significant effect in increasing the TEER values of the mono-culture models of Calu-3 cells and NCI-H441 cells in Transwell® in comparison with the control group. (Fig. 4.1 A, B). These results indicate the significant effect of Vitamin D₃ on the improvement of the properties of the alveolar epithelial barrier.

4.1.2 Effects of Vitamin D₃ on the expressions of intercellular junction proteins in alveolar epithelial cell lines

The former experiments verified that Vitamin D₃ has an effect on improving the barrier properties of alveolar epithelial cells (Fig. 4.1). The gene expressions of intercellular junction proteins, including the tight junction proteins ZO-1, Occludin, Claudin-5, and the adherens junction protein, E-Cadherin, were further tested in the cell lines of alveolar epithelial cells. These junction proteins are also essential for maintaining the barrier integrity of alveolar epithelial cells.

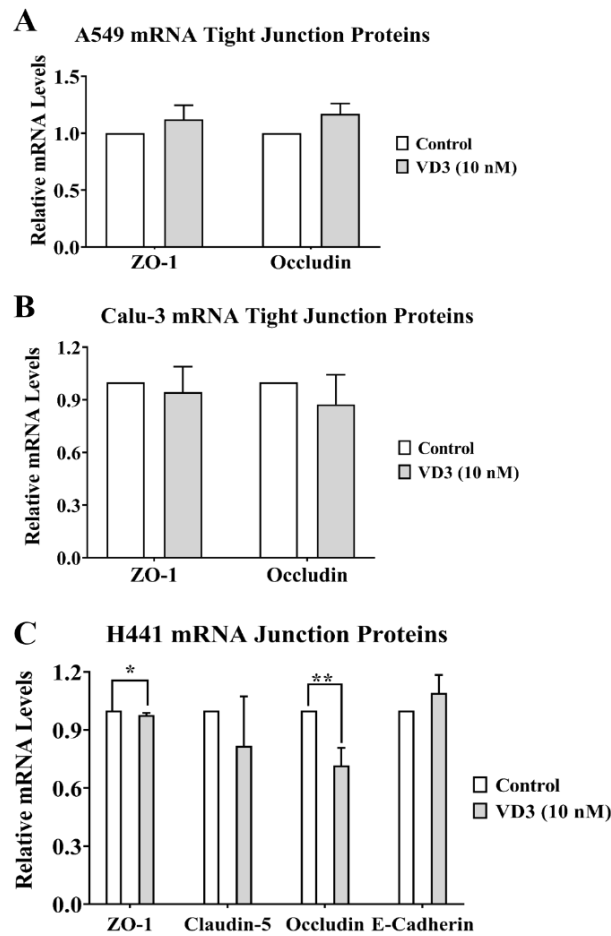


Figure 4.2: Real-time PCR analysis of the gene expression levels of junction proteins in alveolar epithelial cell lines, including the tight junction proteins, ZO-1, Occludin in Calu-3 cells (A), A549 cells (B), and the junction proteins, including, ZO-1, Occludin, Claudin-5, and E-Cadherin in NCI-H441 cells (C). The NCI-H441 cells and A549 cells were treated with Vitamin D₃ (VD₃) (10 nM) and Dexamethasone (Dexa) (1 μ M). Calu-3 cells were only treated with Vitamin D₃ (VD₃) (10 nM). The data were statistically analyzed with Two-way ANOVA (C) (n \geq 3). Significance: *P \leq 0.05, **P \leq 0.01.

From the results of the gene expression analysis of the intercellular junction molecules in alveolar epithelial cell lines, Vitamin D₃ (10 nM) generally declined the gene expression levels of the intercellular junction proteins in the alveolar epithelial cell lines (Fig. 4.2). The gene expression of tight junction proteins (ZO-1 and Occludin) was not prominently affected by Vitamin D₃ (10 nM) in Calu-3 cells (Fig. 4.2 B). Even though the gene expression levels of ZO-1 and Occludin showed an increasing trend in A549 cells with Vitamin D₃ (10 nM) treatment (Fig. 4.2 A). The gene expression levels of the intercellular junction proteins also

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showed a decreasing trend in NCI-H441 cells with Vitamin D₃ (10 nM) treatment (Fig. 4.2 C). Especially, Vitamin D₃ (10 nM) significantly repressed gene expression of the tight junction proteins, ZO-1 and Occludin, in NCI-H441 cells (Fig. 4.2 C). Whereas, the gene expression of adherens junction protein, E-Cadherin, was increased in NCI-H441 cells with Vitamin D₃ (10 nM) treatment (Fig. 4.2 C).

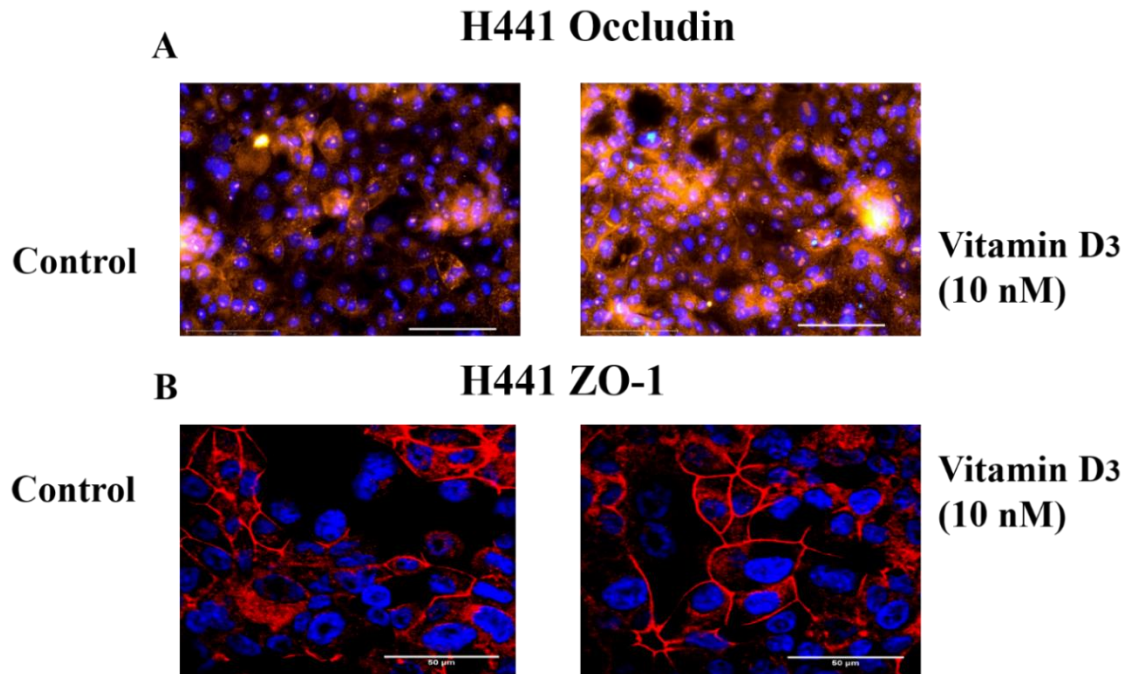


Figure 4.3: Immunofluorescence staining and microscopy for the detection of tight junction proteins, Occludin, and ZO-1, in NCI-H441 cells. (A) The fluorescent microscope EVOs were used for the detection of Occludin (40x magnified scale bar: 100 μm). (B) ZO-1 was detected with a confocal fluorescence microscope (60x magnified scale bar: 50 μm). Red: A. Occludin and B. ZO-1, Blue: nuclei.

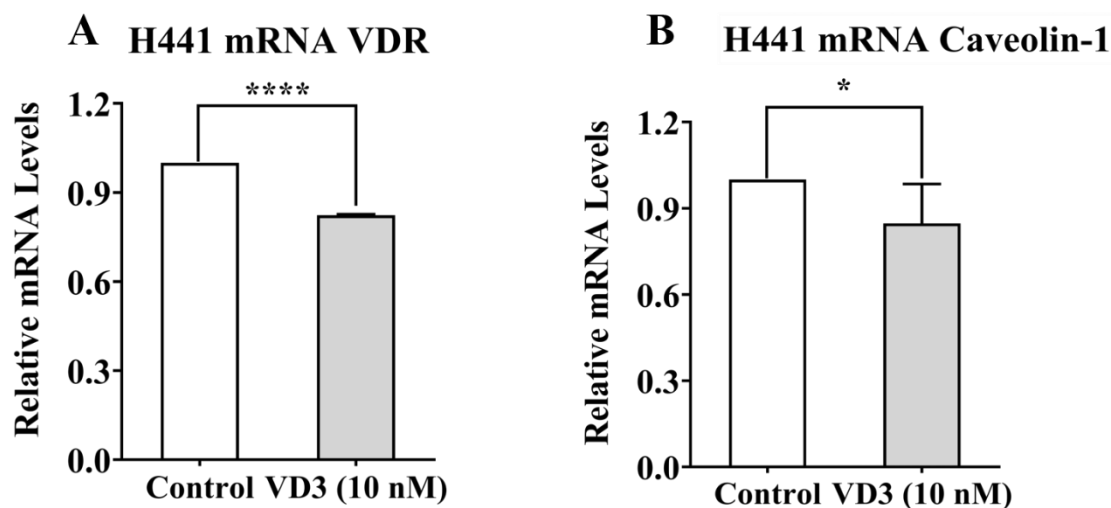
The protein expression levels of the tight junction proteins, ZO-1, and Occludin were further tested in NCI-H441 cells via immunofluorescence staining, which enabled the visual determination of the expression of the tight junction proteins, ZO-1, and Occludin, in NCI-H441 cells. According to the fluorescence detection of Occludin, and ZO-1 in NCI-H441 cells, the cells with Vitamin D₃ (10 nM) treatment showed more intensive fluorescent signals of Occludin and ZO-1 than the untreated cells (Fig. 4.3 A, B). The cells with Vitamin D₃ (10 nM) treatment demonstrated a more clear cell membrane structure, which was observed from

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the location of ZO-1, and Occludin in NCI-H441 cells (Fig. 4.3 A, B). These results indicate that Vitamin D₃ has a positive effect in improving protein expression of Occludin and ZO-1 in alveolar epithelial cells. These results also imply that Vitamin D₃ also has a significant effect in maintaining and strengthening the integrity of the tight junction proteins on the alveolar epithelial barrier.

4.1.3 Effects of Vitamin D₃ on the gene expression of additional involved molecules in alveolar epithelial cells

Besides, the effects of Vitamin D₃ on the expression of intercellular junction proteins in alveolar epithelial cells, this study also investigated the gene expression of the Protein Kinase C isozymes including, Protein Kinase C α , β , and η (PKC A, B, and H) and the gene expression of Caveolin-1, and Vitamin D receptor (VDR) in NCI-H441 cells, which were treated with Vitamin D₃ (VD₃) (10 nM).



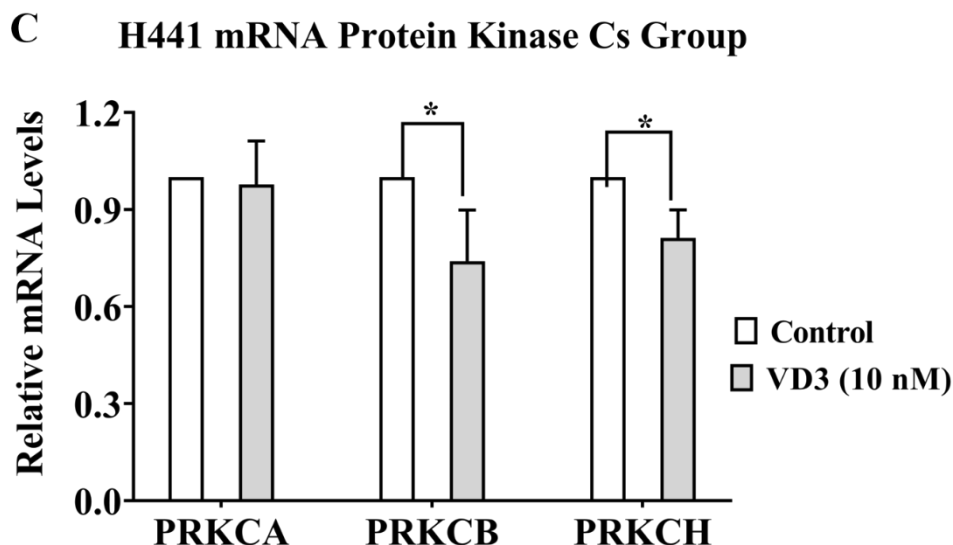


Figure 4.4: Real-time PCR analysis of the gene expression of (A) Vitamin D receptor (VDR) (B) Caveolin-1, and (C) the group of Protein Kinase C isozymes (PCK, A, B, H), in NCI-H441 cells with Vitamin D₃ (VD₃) (10 nM) treatment. The data were statistically analyzed with Two-way ANOVA (C), and T-test (A, B) (n ≥ 3). Significance: *P ≤ 0.05, ****P ≤ 0.0001.

The result generally displayed the effects of Vitamin D₃ (10 nM) in declining in the gene expression levels of Caveolin-1, the group of Protein Kinase C isozymes (PKC A, PKCB, PKCH), and Vitamin D receptor (VDR) in NCI-H441 cells (Fig. 4.4). Especially, the gene expressions of PKCB, and PKCH were significantly decreased in NCI-H441cells with Vitamin D₃ (10 nM) treatment (Fig. 4.4 C).

4.1.4 Test of the impact of Vitamin D₃ on the expression of antimicrobial peptide, Cathelicidin (LL-37) in alveolar epithelial cells

It has been demonstrated Vitamin D₃ facilitates the expression of Cathelicidin, LL-37, in tissue epithelial cells. This study was also performed to investigate the expression of LL-37 in alveolar epithelial cells, including, NCI-H441 cells, Calu-3 cells, and A549 cells, which were treated with Vitamin D₃ (10 nM)

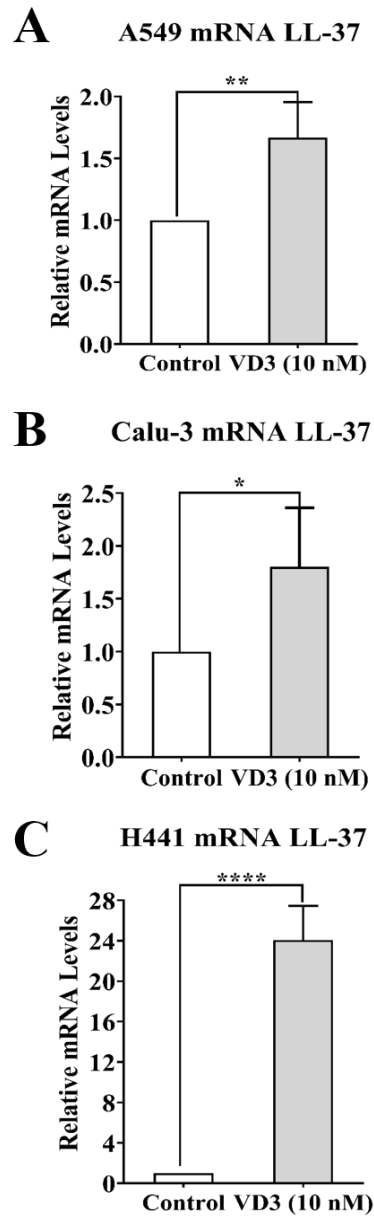


Figure 4.5: Real-time PCR analysis of the gene expression of LL-37 in the alveolar epithelial cell lines, including, (A) Calu-3 cells, (B) A549 cells, and (C) NCI-H441 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM). The data were statistically analyzed with T-test ((n ≥ 3). Significance: *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001.

The results of gene expression analysis of LL-37 showed that Vitamin D₃ (10 nM) significantly increased the gene expression of LL-37 in all of the alveolar epithelial cell lines including NCI-H441 cells, Calu-3 cells, and A549 cells (Fig. 4.5). This result indicated the

significant effect of Vitamin D₃ in increasing the gene expression of LL-37 in alveolar epithelial cells.

H441 LL-37

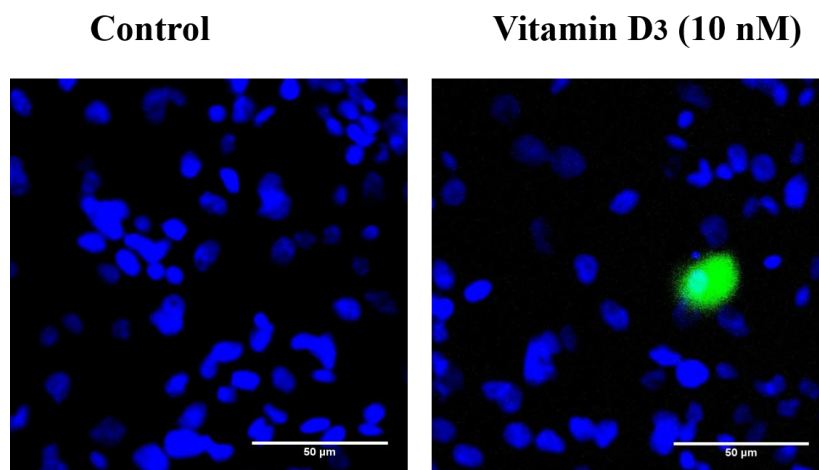


Figure 4.6: Immunofluorescence staining and Confocal microscopy for the detection of LL-37 in NCI-H441 cells with 60x magnification (scale bar: 50 µm). **Green:** LL-37. **Blue:** nuclei.

Based on the gene expression analysis. This study also investigated the effect of Vitamin D₃ (10 nM) on the protein expression level of LL-37 in NCI-H441 cells, with immunofluorescence staining. The result of the immunofluorescence staining of LL-37 in NCI-H441 cells displayed that the cells with Vitamin D₃ (10 nM) treatment showed a significant intensiver fluorescence signal of LL-37. Whereas there was almost no fluorescent signal of LL-37 detected in untreated cells (Fig. 4.6). This result suggested that Vitamin D₃ also has a significant effect in triggering the expression of Cathelicidin, LL-37, in alveolar epithelial cells at the protein level.

4.1.5 Effect of Vitamin D₃ on the expression of SP-A in alveolar epithelial cells

It is known that alveolar epithelial type II cells (AT-II Cs) are responsible for the production of surfactants. This study further investigated the effect of Vitamin D₃ on the expression of Surfactant protein A (SP-A) in the alveolar epithelial cell lines. SP-A is known to play roles in immune defense and lung tension elevation for the maintenance of lung functions This

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study tested the gene expression of SP-A in NCI-H441 cells and Calu-3 cells, which are also AT-II-like cell lines.

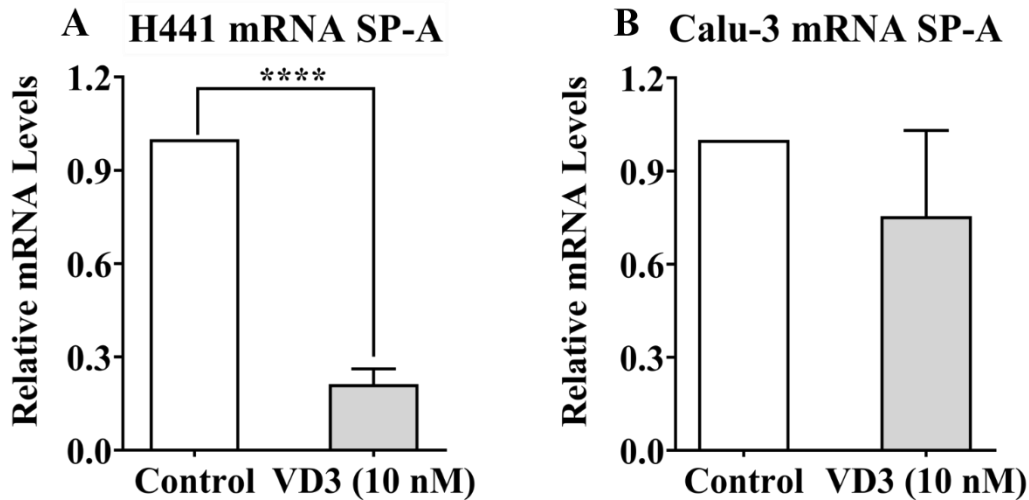


Figure 4.7: Real-time PCR analysis of the gene expression of Surfactant protein A (SP-A) in the alveolar epithelial cell lines, **(A)** Gene expression analysis of SP-A in NCI-H441 cells. **(B)** Gene expression analysis of SP-A in Calu-3 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM). The data were statistically analyzed with T-test ($n \geq 3$). Significance: **** $P \leq 0.0001$.

The gene expression analysis of SP-A showed that the expression of SP-A was significantly suppressed in NCI-H41 cells with Vitamin D₃ treatment (10 nM) (Fig. 4.7 A, B). Moreover, the result also demonstrated that the expression of SP-A showed a repressed trend in Calu-3 cells which were treated with Vitamin D₃ (10 nM) (Fig. 4.7 B). Besides gene expression tests of SP-A, the protein expression level of SP-A was visually tested with its immunofluorescent staining in NCI-H441 cells.

H441 SP-A, E-Cadherin

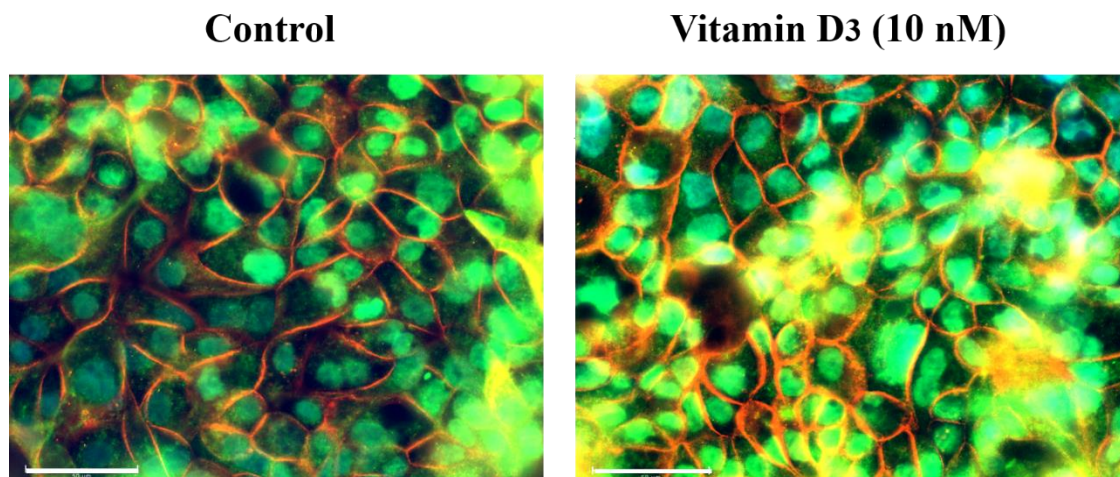


Figure 4.8: Immunofluorescence staining and microscopy for the detection of SP-A in NCI-H441 cells, via the EVOs fluorescent microscope 60 x magnified with the scale bar: 50 μm. **Red:** E-Cadherin, **Green:** SP-A, **Blue:** nuclei.

The result of immunofluorescence detection of SP-A in NCI-H441 cells displayed that the cells with Vitamin D₃ (10 nM) treatment showed a more noticeable fluorescent signal of SP-A (Fig. 4.8). This result suggests that Vitamin D₃ has a significant effect in increasing the expression of SP-A in alveolar epithelial cells at the protein level. Moreover, the NCI-H441 cells treated with Vitamin D₃ (10 nM) showed a more intensive and strengthened fluorescence signal of E-Cadherin on the cell membrane barrier of NCI-H441 cells (Fig.4.8). This result also indicates that Vitamin D₃ has an effect in enhancing the protein expression and the integrity of E-Cadherin in alveolar epithelial cells.

4.1.6 Effects of Vitamin D₃ on the expression of Doublecortin-Like Kinase 1 (DCLK1) in epithelial cells

This study also explored the effect of Vitamin D₃ on the molecular expression of doublecortin-like kinase 1 (DCLK1) in alveolar epithelial cell lines

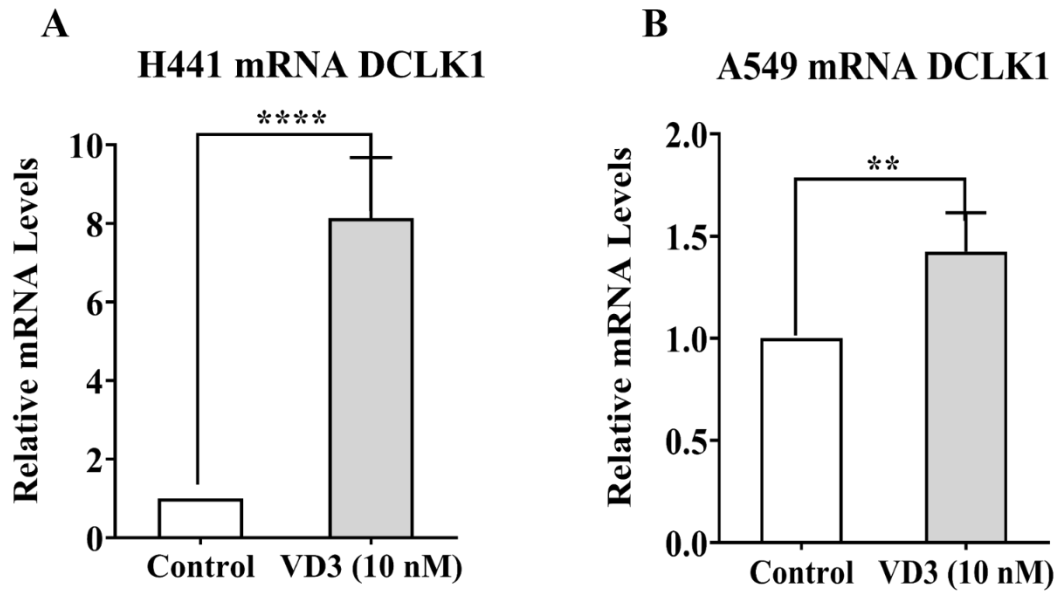


Figure 4.9: Real-time PCR analysis of the gene expression of DCLK1 in the alveolar epithelial cell lines, including (A) NCI-H441 cells and (B) A549 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM). The data were statistically analyzed with T-test ($n \geq 3$). Significance: ** $P \leq 0.01$, **** $P \leq 0.0001$.

From the Real-time PCR analysis, the gene expression of DCLK1 was significantly increased in NCI-H441 cells (Fig. 4.9 A) and A549 cells, which were treated with Vitamin D₃ (10 nM). (Fig. 4.9 B).

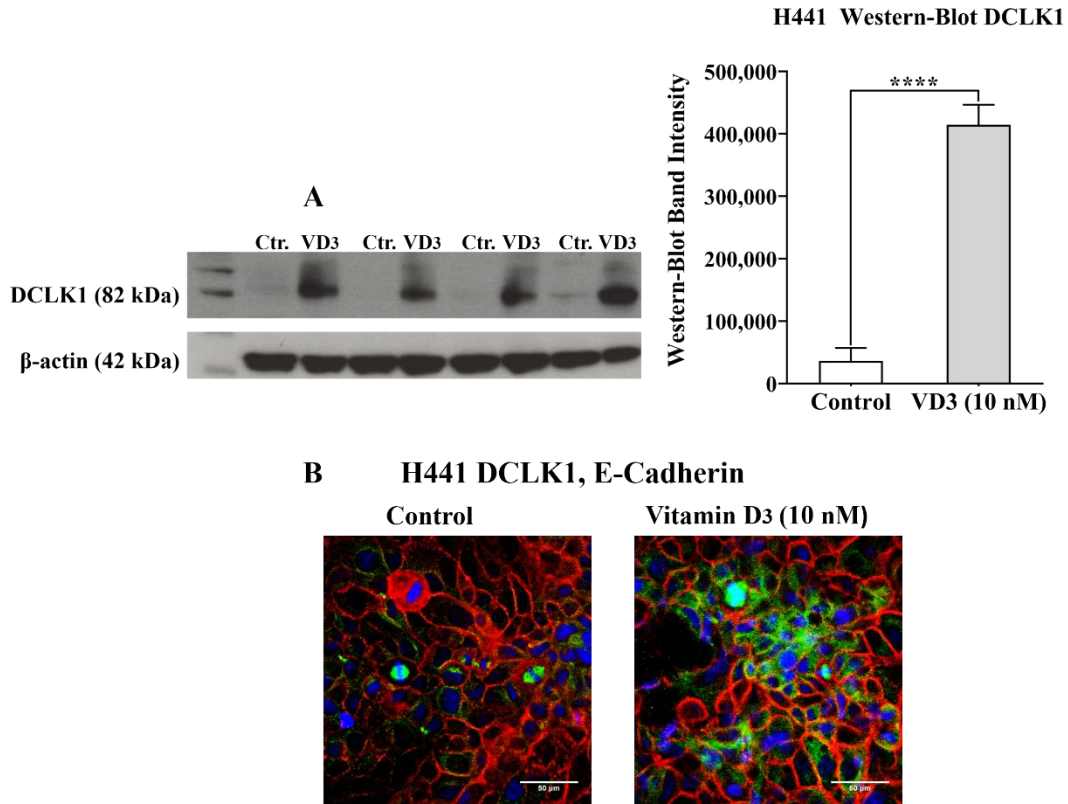


Figure 4.10: Protein expression analysis of DCLK1 in AT-II cell lines, NCI-H441 cells. **(A)** Western-Blot test of DCLK1 in NCI-H441 cells (n = 4). **(B)** Immunofluorescence microscopy of the detection of DCLK1 in NCI-H441 cells, which was detected with confocal fluorescence microscopy (40x magnification, scale bar: 50 μm), **Red:** E-Cadherin, **Green:** DCLK1, **Blue:** nuclei. The cells were treated with Vitamin D₃ (VD₃) (10 nM). The data were statistically analyzed with T-test. Significance: ****P ≤ 0.0001.

Based on the gene expression analysis, the effect of Vitamin D₃ on the expression of DCLK1 was further tested at the protein level through the analysis of Western-Blot, and immunofluorescent staining in NCI-H441 cells. From the Western-Blot analysis, Vitamin D₃ (10 nM) distinctly increased the protein expression of DCLK1 in NCI-H441 cells (Fig. 4.10 A). Moreover, Vitamin D₃ (10 nM) remarkably strengthened the intensity of the fluorescent signal of DCLK1 in NCI-H441 cells (Fig. 4.10 B). These results verified that Vitamin D₃ also has a significant effect in increasing the expression of DCLK1 in alveolar epithelial cells at the protein level. Moreover, Vitamin D₃ (10 nM) distinctively increased the integrity and strengthened the fluorescent signal of E-Cadherin around the cell membrane barrier of NCI-H441 cells (Fig. 4.10 B).

4.2 Study in the Co-Culture Model of the Alveolar-Capillary Barrier with LPS Treatment

4.2.1 Effect of Vitamin D₃ on the maintenance of the alveolar-capillary barrier after LPS treatment

From the former experiment of TEER measurement, Vitamin D₃ (10 nM) significantly improved the properties of the alveolar epithelial barrier in the mono-culture model of the alveolar epithelial cell lines including NCI-H441 cells, and Calu-3 cells. This study was further carried out to investigate the effect of Vitamin D₃ on the preservation of function of the alveolar-capillary barrier in the challenge with LPS treatment in the co-culture model of NCI-H441 cells and OECs in Transwell®. This study also investigated the involved molecular mechanism in LPS-induced lung inflammation. For the determination of the barrier properties of the capillary microvascular endothelial cells, (OECs), this study implemented the measurement of the electrical cell-substrate Independence Sensing (ECIS) to monitor the barrier electric impedance of OECs.

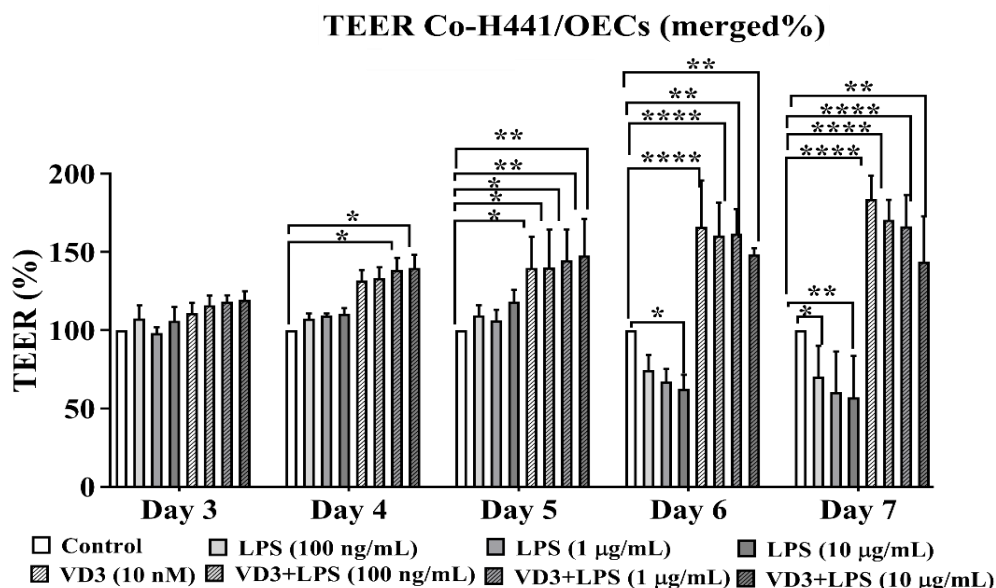


Figure 4.11: TEER measurements of the alveolar-capillary barrier of the properties of the co-culture model of NCI-H441 cells and OECs in Transwell®. The cells were treated with Vitamin D₃ (VD₃) (10 nM) from Day 3 and LPS (100 ng/mL, 1 µg/mL, 10 µg/mL) from Day 5 (only in basolateral compartments). The data were demonstrated as the relative TEER values in the percentage ratio of control (%). The data were statistically analyzed with Two-way ANOVA (n ≥ 3). Significance: *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001.

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From the results of TEER measurement, Vitamin D₃ (10 nM) remarkably increased the TEER values of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® (Fig. 4.11). Whereas, the TEER values were significantly decreased with LPS (100 ng/mL, 1 µg/mL, 10 µg/mL) treatments (Fig. 4.11). Nevertheless, Vitamin D₃ (10 nM) still demonstrated a significant effect on the improvement and maintenance of the TEER values of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell®, even in the challenge with LPS (100 ng/mL, 1 µg/mL, 10 µg/mL) treatment (Fig. 4.11).

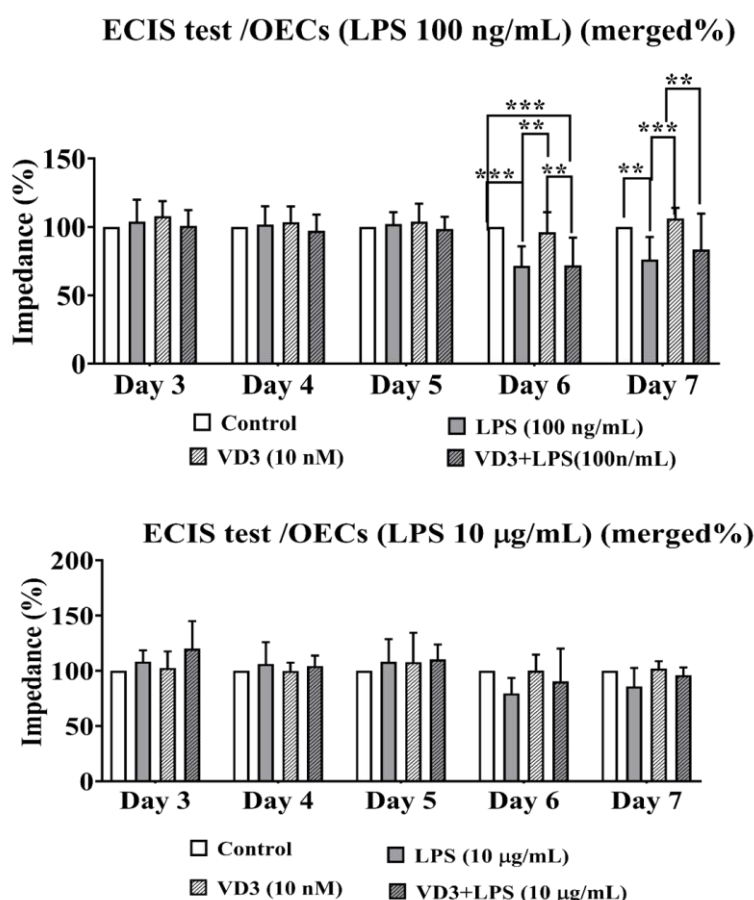


Figure 4.12: ECIS determining the barrier electrical impedance (Ω) of the capillary microvascular endothelial cells, OECs, with Vitamin D₃ (VD₃) (10 nM) and LPS (100 ng/mL and 10 µg/mL) treatments. The data were demonstrated as the relative barrier ECIS values in the percentage ratio of control (%). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$). Significance: ** $P \leq 0.01$, *** $P \leq 0.001$.

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This study also performed the ECIS measurement to specifically assess the electric impedance values of OECs cultured on the ECIS slides. The ECIS values also reveal the properties of the capillary microvascular endothelial barrier in the mono-culture of OECs on the ECIS slides. From the result of the ECIS measurement, the ECIS values of the capillary microvascular endothelial barrier in the mono-culture model of OECs on ECIS slides demonstrated an increasing trend with Vitamin D₃ (10 nM) treatment (Fig.4.12). LPS (100 ng/mL, 10 µg/mL) significantly decreased the ECIS values of the capillary microvascular endothelial barrier in the mono-culture model of OECs on ECIS slides (Fig. 4.12) Whereas, Vitamin D₃ (10 nM) still maintained the ECIS values of the mono-culture model of OECs on ECIS slides in the challenge with LPS (100 ng/mL, 10 µg/mL) treatment (Fig. 4.12).

H441 ZO-1

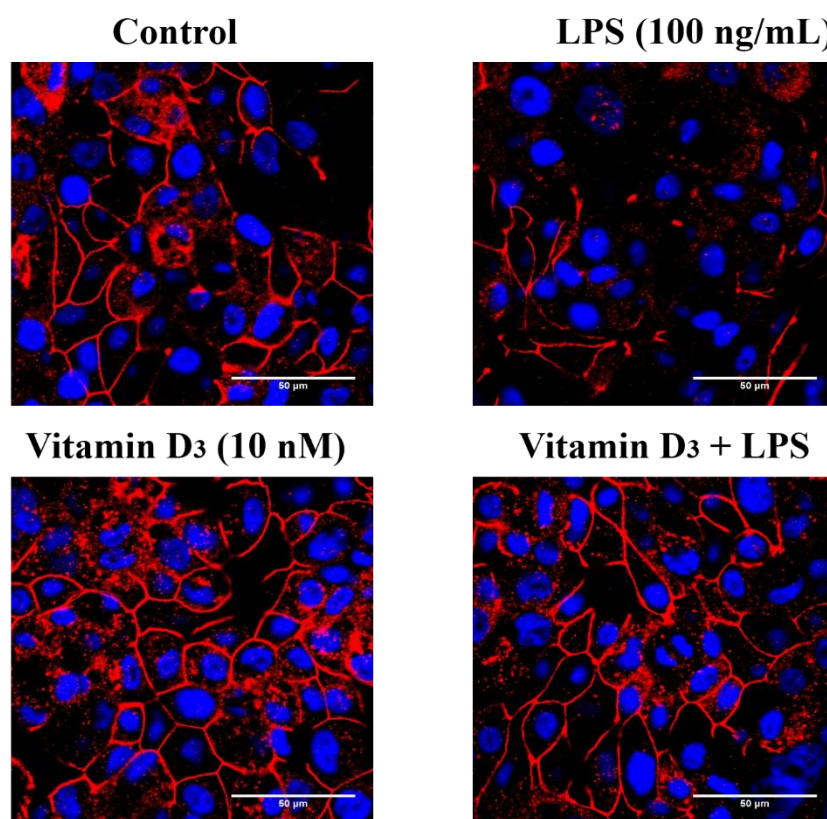


Figure 4.13: Immunofluorescence staining and Confocal fluoresces microscopy of ZO-1 in NCI-H441 cells treated with the conditioned medium from the mono-culture of OECs with Vitamin D₃ (10 nM), LPS (100 ng/mL) and Vitamin D₃ (10 nM) plus LPS (100 ng/mL) treatments (63x magnification scale bar: 50 µm). **Red:** ZO-1, **Blue:** nuclei.

In the fluorescence microscopy test, LPS (100 ng/mL) largely devastated the integrity of the cell-to-cell membrane barrier of NCI-H441 cells and caused a significant loss of the tight junction protein ZO-1 in NCI-H441 cells (Fig. 4.12). On the other hand, the cells with Vitamin D₃ (10 nM) treatment demonstrated a more intensive cell membrane integrity and enhanced the fluorescent signal of ZO-1 in the cells (Fig. 4.13). Moreover, Vitamin D₃ (10 nM) still maintained the integrity of ZO-1 and cell membrane barrier of NCI-H441 cells in the challenge with LPS (100ng/mL) treatment (Fig. 4.13).

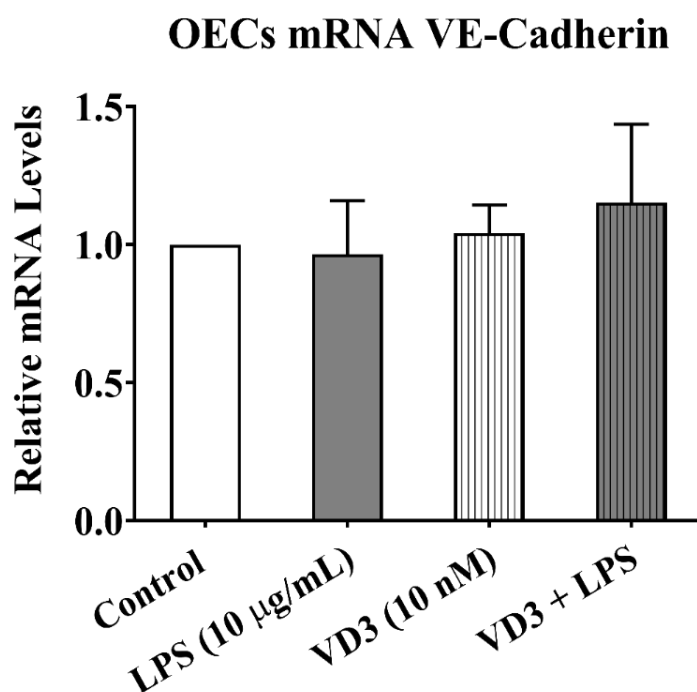


Figure 4.14: Real-time PCR analysis for the gene expressions of VE-Cadherin in OECs. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and LPS (10 µg/mL). The data were statistically analyzed with One-way ANOVA ($n \geq 3$).

From the gene expression analysis of vascular endothelial cadherin (VE-Cadherin) in OECs, the gene expressions of VE-Cadherin in OECs were not distinctively affected by Vitamin D₃ (10 nM) and LPS (10 µg/mL) (Fig. 4.14). Even though the gene expression of VE-Cadherin in OECs was decreased with LPS (10 µg/mL) treatment, while Vitamin D₃ (10 nM) showed the effect in increasing the gene expression of VE-Cadherin in OECs and maintained its gene expression levels in OECs in the exposure to LPS (10 µg/mL) treatment (Fig. 4.14).

4.2.2 Associated molecules in LPS-induced inflammation of the alveolar-capillary barrier.

The prior study proved the destructive effect of LPS on the alveolar epithelial barrier and vascular endothelial barrier (Fig. 4.11, 4.12, 4.13). The gene expressions of the involved molecules in the LPS-caused inflammation were further investigated in this study.

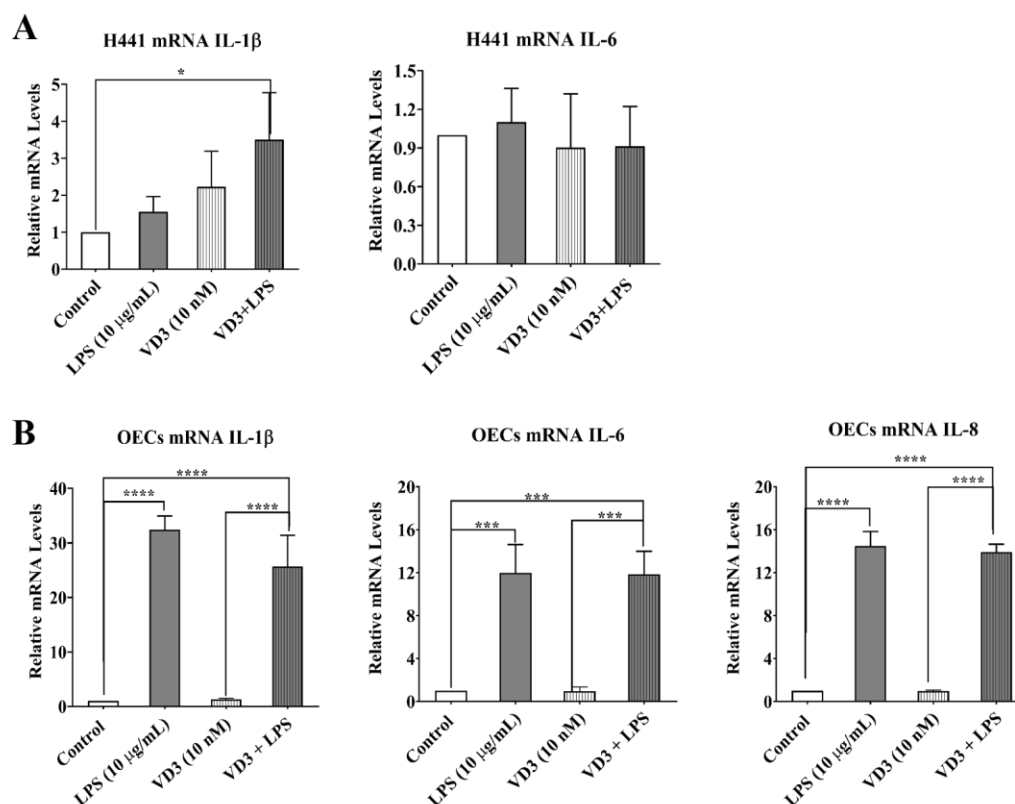


Figure 4.15: Real-time PCR analysis for the gene expressions of cell interleukin pro-inflammatory cytokines in (A) NCI-H441 cells and (B) OECs. The cells were treated with Vitamin D₃ (10 nM) and LPS (10 μ g/mL). The data shown were statistically analyzed with One-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

From the gene expression analyses, the gene expressions of the interleukin pro-inflammatory cytokines (IL-1 β , IL-6, and IL-8) were generously increased in NCI-H441 cells and OECs with LPS (10 μ g/mL) treatment (Fig. 4.15 A, B). In particular, LPS (10 μ g/mL) significantly increased the gene expression of the IL-1 β , IL-6, and IL-8 in OECs (Fig. 4.15 B). The expression of IL-6 in NCI-H441 cells showed a decreasing trend with the treatment with

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Vitamin D₃ (10 nM). Vitamin D₃ (10 nM) also alleviated the increased gene expression of IL-6 in NCI-H441 cells which was induced by LPS (10 µg/mL) treatment (Fig. 4.15 A). However, the expression of IL-1β in NCI-H441 cells even showed a higher level with Vitamin D₃ (10 nM) treatment (Fig. 4.15). Vitamin D₃ (10 nM) showed the effect in alleviating the significantly increased gene expressions of interleukins in OECs, which were induced by LPS (10µg/mL) treatment (Fig. 4.15 B).

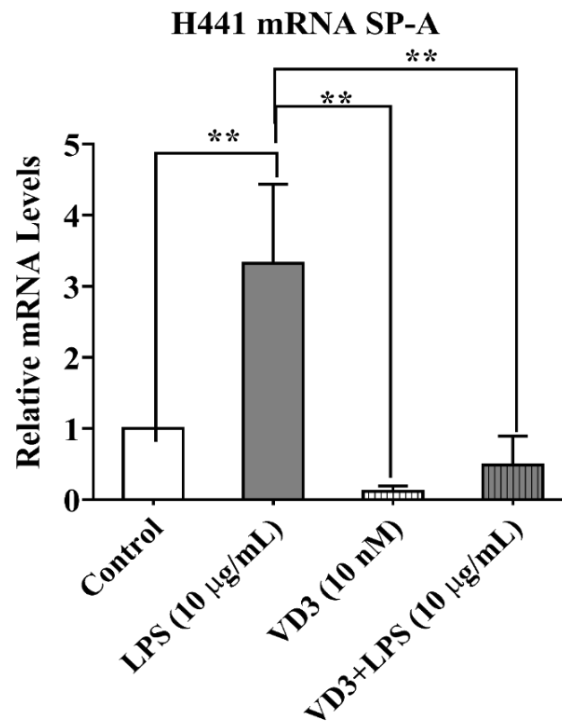


Figure 4.16: Real-time PCR analysis for the gene expressions of Surfactant Protein A (SP-A) in NCI-H441 cells. The cells were treated with Vitamin D₃ (10 nM) and LPS (10 µg/mL). The data shown were statistically analyzed with One-way ANOVA (n ≥ 3). Significance: **P ≤ 0.01.

According to the result of gene expression analysis, Vitamin D₃ (10 nM) significantly decreased the gene expression of SP-A in NCI-H441 cells (Fig. 4.16). Whereas, the gene expression of SP-A was significantly increased in NCI-H441 cells with LPS (10 µg/mL) treatment (Fig. 4.16).

4.2.3 Gene expression analysis of more associated molecules in the LPS-induced inflammation

Besides the interleukins, this study investigated the gene expression of more molecules in NCI-H441 cells and OECs. The cells were also treated with Vitamin D₃ (10 nM) and LPS (10 µg/mL). The molecules in the analysis included the gene expressions of, Vitamin D receptor (VDR), in OECs, and the gene expression of Cathepsin S in both NCI-H441 cells and OECs.

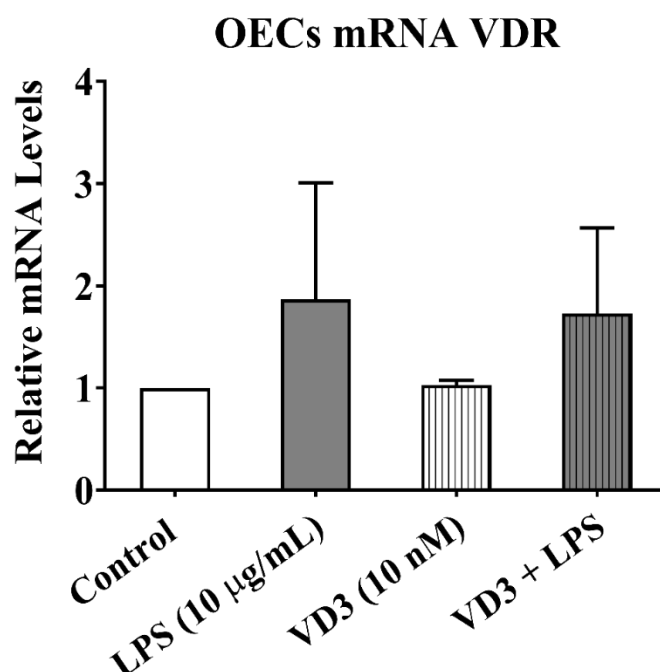


Figure 4.17: Real-time PCR analysis for the gene expressions of Vitamin D Receptor (VDR) in OECs. The cells were treated with vitamin D₃ (VD₃) (10 nM) and LPS 10 µg/mL). The data were statistically analyzed with One-way ANOVA ($n \geq 3$).

From the gene expression analysis of Vitamin D receptor (VDR) in OECs, the gene expression of VDR in OECs was not distinctively affected by Vitamin D₃ (10 nM). Whereas, the gene expression of Vitamin D Receptor was increased in OECs with LPS (10 µg/mL) treatment (Fig. 4.17).

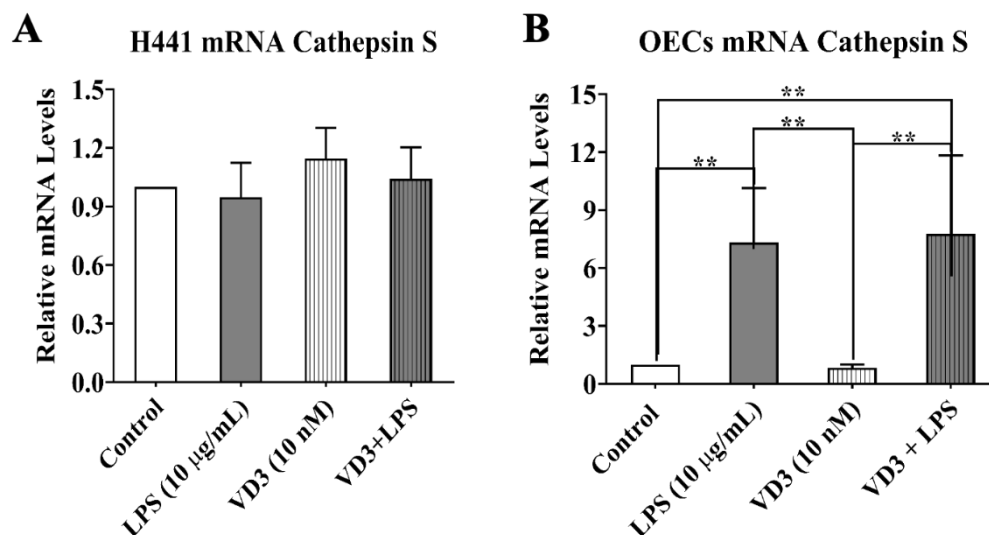
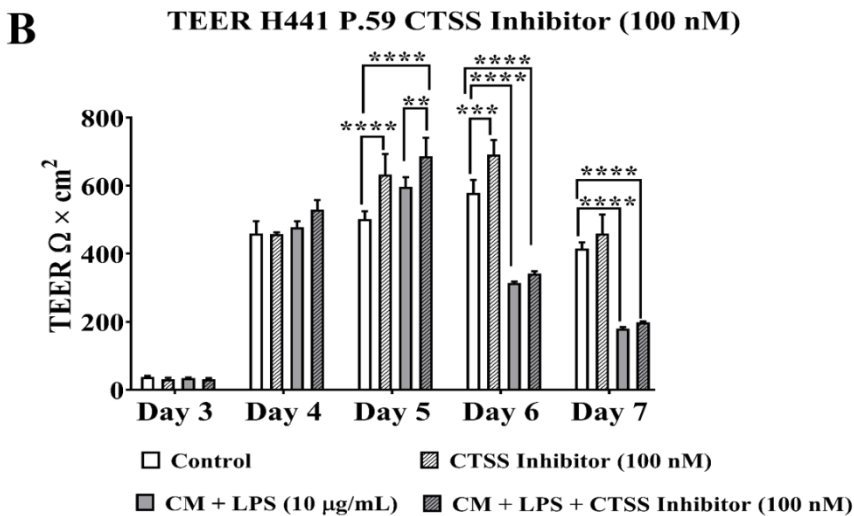
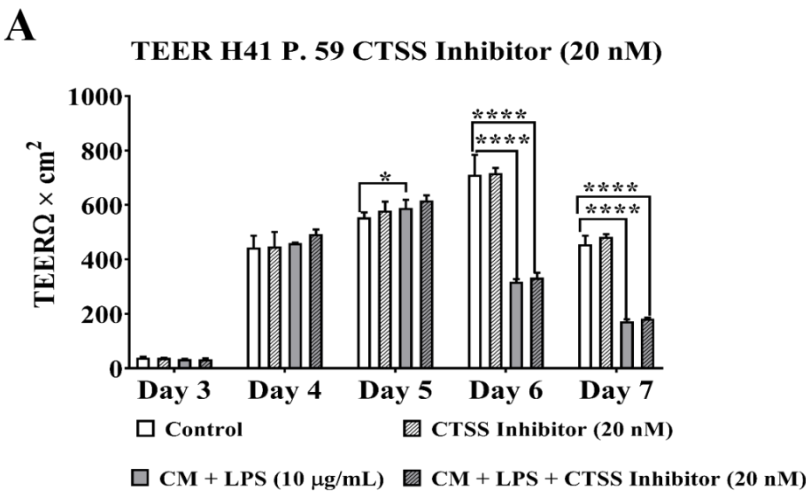


Figure 4.18: Real-time PCR analysis for the gene expressions of Cathepsin S in (A) NCI-H441 cells and (B) OECs. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and LPS (10 µg/mL). The data were statistically analyzed with One-way ANOVA (n ≥ 3). Significance: **P ≤ 0.01.

From the gene expression analysis of Cathepsin S, the gene expression of Cathepsin S was not distinctively influenced by Vitamin D₃ (10 nM) and LPS (10 µg/mL) in NCI-H441 cells (Fig. 4.18 A). Whereas, the gene expression of Cathepsin S was significantly stimulated by LPS (10 µg/mL) treatment in OECs (Fig. 4.18 B). Therefore, it can imply that the vascular endothelial cells are more responsive to LPS to stimulate the expression of Cathepsin S.

4.2.4 Study on the role of Cathepsin S in the LPS-induced dysfunction of the alveolar-capillary barrier

A former experiment in this study found that LPS significantly stimulated the gene expression of Cathepsin S (CTSS) in OECs (Fig. 4.18). This study was further carried out with the inhibition of Cathepsin S expression by using the Cathepsin S specified inhibitor to gain a deeper understanding of the role of Cathepsin S in the LPS-induced lung inflammation, which leads to the damage of the alveolar-capillary barrier. In this study, TEER measurement and Real-time PCR analysis were carried out in mono-culture and co-culture models of the cells for the analysis. The cells were treated with the Cathepsin S (CTSS) specified inhibitor and LPS (100 ng/mL, 10 µg/mL) during the cell culture process.



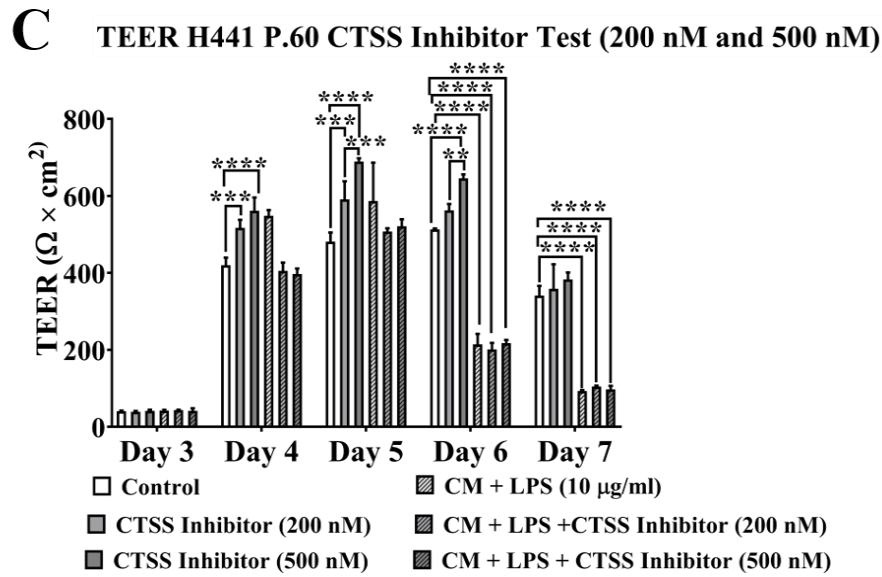


Figure 4.19: TEER measurement of the mono-culture model of NCI-H441 cells in Transwell® for the inhibition test of Cathepsin (CTSS) with different concentrations of Cathepsin S specified inhibitor. (A, B) TEER measurement of the mono-culture model of NCI-H441 cells with cell passage number 59 (P.59) in Transwell® with the treatment with Cathepsin S (CTSS) inhibitor (20 nM, and 100 nM) from Day 3 and Conditioned medium (CM) from the mono-culture model of OECs with LPS (10 µg/mL) treatment from Day 5 (only in basolateral compartments) (three replications). (C) TEER measurement of NCI-H441 cells with cell passage number 60 (P.60) in Transwell® with the treatment with Cathepsin S (CTSS) inhibitor (200 nM, and 500 nM) from day 3 and Conditioned medium (CM) from the mono-culture model of OECs with LPS (10 µg/mL) treatment from Day 5 (only in Basolateral compartments) (three replications). The data were statistically analyzed with Two-way ANOVA. Significance: * $p \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

This study firstly tested the effect of different concentrations of Cathepsin S (CTSS) inhibitor on the barrier properties of the alveolar epithelial cell line, NCI-H441 cells under the condition of LPS (10 µg/mL) treatment. This test was also aimed at selecting an appropriate concentration of CTSS inhibitor for further study. From the results of TEER measurement, LPS (10 µg/mL) remarkably decreased the TEER values of the NCI-H441 cells-modeled alveolar epithelial barrier (Fig. 4.18 A, B, C). The effects of the CTSS inhibitor on the barrier properties showed a dose-dependent manner. (Fig. 4.19 A, B, C) The higher the concentration of CTSS inhibitor, the higher the barrier TEER values of the alveolar epithelial barrier in the mono-culture model of NCI-H441 in Transwell® (Fig. 4.19 A, B, C). The CTSS inhibitor

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with concentrations of 100 nM and 500 nM showed the most significant effect in increasing the TEER values of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells (Fig. 4.19 B, C). Although the concentration of CTSS inhibitor (100 nM) is lower than 500 nM, the concentration of CTSS inhibitor (100 nM) seemed to display a more effective effect in increasing the TEER values which reflect the barrier properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.19 B, C). Therefore, the CTSS inhibitor with a concentration of 100 nM was selected for further investigations in the study of the role of Cathepsin S in the LPS-induced disruption of the alveolar-capillary barrier.

TEER co-H441/OECs CTSS Inhibitor LPS Test (merged)

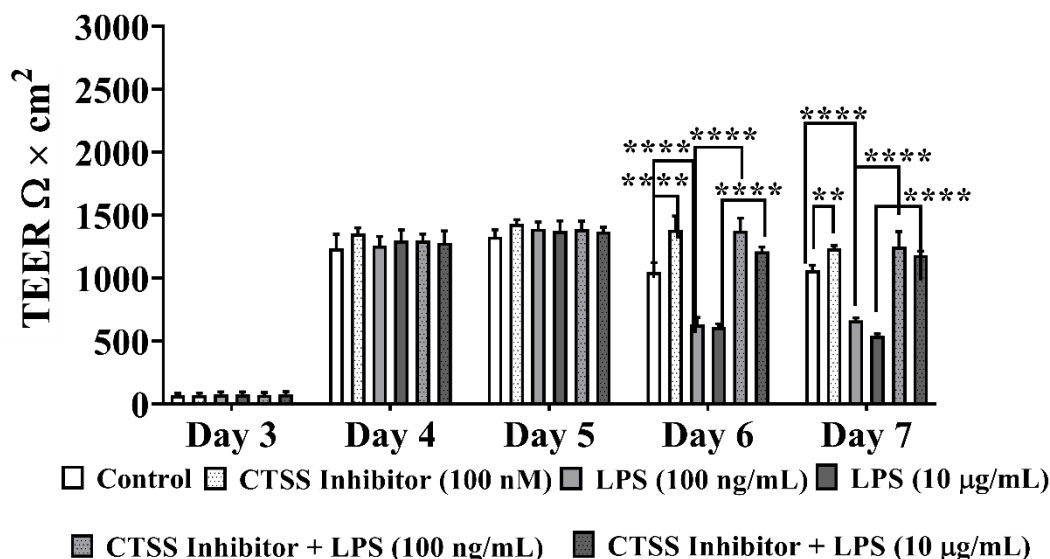


Figure 4.20: TEER measurement of the co-culture model of NCI-H441 cells and OECs in Transwell® for the test of the inhibitor of Cathepsin S (CTSS) from Day 3 with different LPS concentrations (100 ng/mL, 10 µg/mL) from Day 5 (only in basolateral compartments). The cells were treated with CTSS inhibitor (100 nM), and LPS (100 ng/mL, 10 µg/mL). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$). Significance: ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

This study further investigated the impact of Cathepsin S (CTSS) inhibitor (100 nM) on the properties of the alveolar-capillary barrier in the exposure to LPS (100 nM, 10 µg/mL) in the co-culture model of NCI-H441 cells and OECs in Transwell®. From the result of the TEER

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measurement, LPS (100 ng/mL, 10 μ g/mL) significantly decreased the TEER values of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® (Fig. 4.20). On the other side, the CTSS inhibitor (100 nM) significantly increased the TEER values of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® (Fig. 4.20) and demonstrated a significant effect in maintaining the TEER values of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® even under the treatment of LPS (100 ng/mL, 10 μ g/mL) (Fig. 4.20). This result suggests that the LPS-stimulated secretion of Cathepsin S is one of the major factors, which contribute to the LPS-induced destruction of the alveolar-capillary barrier. This study further investigated the effects of CTSS inhibitor (100 nM) on the gene expressions of the molecules in the mono-culture model of OECs. The molecules are also relevant in LPS-induced inflammation.

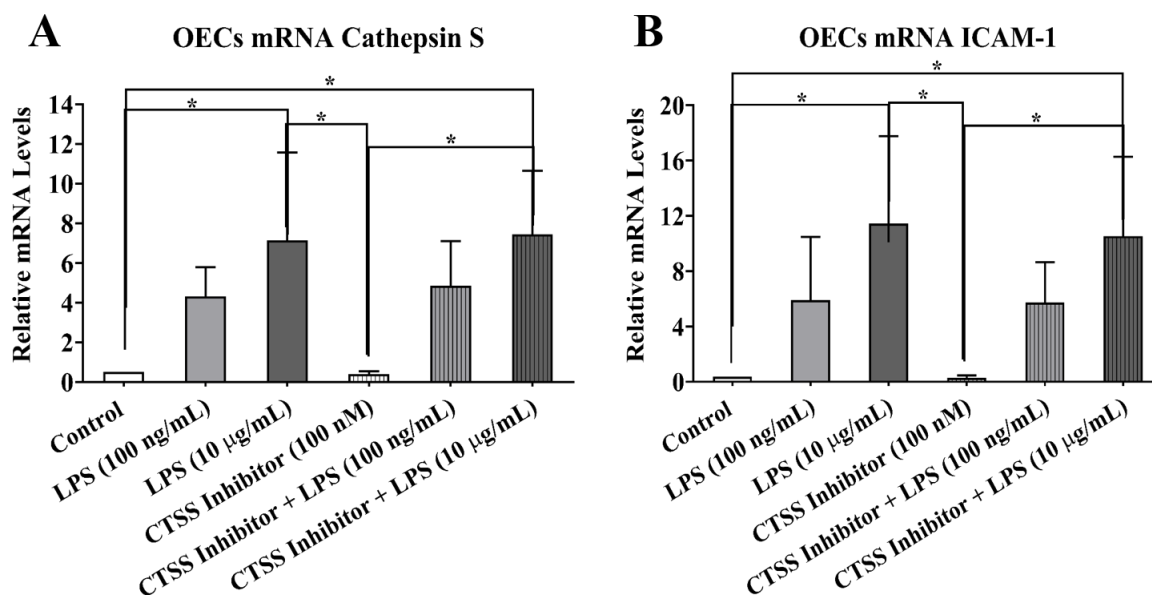


Figure 4.21: Real-time PCR analysis for the gene expressions of (A) Cathepsin S and (B) ICAM-1 in OECs. The cells were treated with Cathepsin S (CTSS) inhibitor (100 nM) and LPS (100 ng/mL, 10 μ g/mL). The data were statistically analyzed with One-way ANOVA ($n \geq 3$). Significance: $*P \leq 0.05$.

From the gene expression analysis, the gene expressions of Cathepsin S and ICAM-1 (intercellular adhesion molecule-1) in OECs were increased with the LPS treatment (100

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ng/mL, 10 μ g/mL), and the increasing trend showed an LPS concentration-dependent manner. A higher concentration of LPS treatment showed a higher gene expression level of Cathepsin S, and ICAM-1 (Fig. 4.21 A, B). The gene expressions of Cathepsin S and ICAM-1 were significantly increased in OECs with LPS (10 μ M) treatment (Fig. 4.21 A, B). Although the Cathepsin S inhibitor (100 nM) did not show any distinctive effect on the gene expression level of Cathepsin S, and ICAM-1 in OECs (Fig. 4.20 A, B), Cathepsin S inhibitor (100 ng/mL) mildly reduced the LPS (10 μ M)- induced gene expression of ICAM-1 in OECs (Fig. 4.21)

OECs mRNA Pro-inflammatory Cytokines

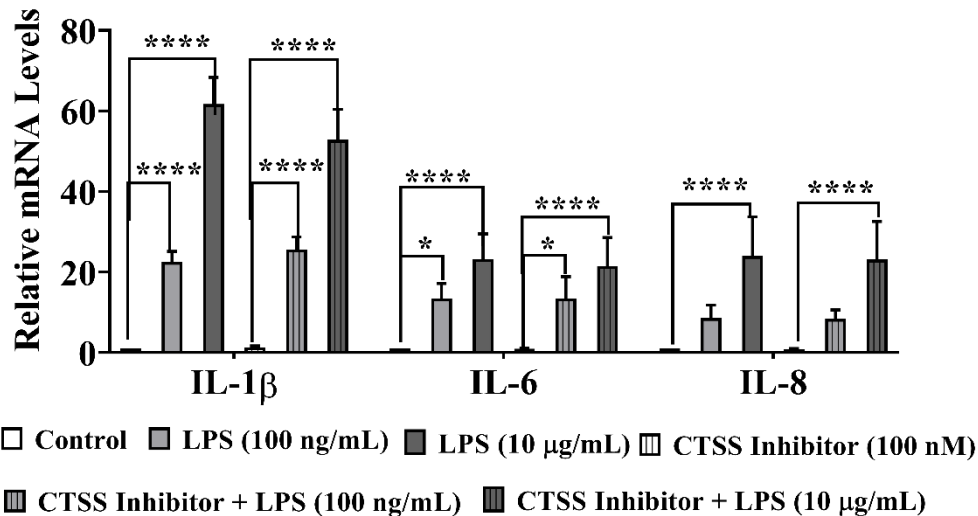


Figure 4.22: Real-time PCR analysis for the gene expressions of the interleukin pro-inflammatory cytokines, namely the interleukin group (IL-1 β , IL-6, IL-8) in OECs. The cells were treated with Cathepsin S (CTSS) inhibitor (100 nM) and LPS (100 ng/mL, 10 μ g/mL). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, **** $P \leq 0.0001$.

The gene expression analysis demonstrated that LPS (100 ng/mL, 10 μ g/mL) significantly triggered the gene expressions of the interleukin pro-inflammatory cytokines (IL-1 β , IL-6, and IL-8) in OECs (Fig. 4.22). The increasing trend of the gene expression levels of interleukins in OECs showed an LPS concentration-dependent manner (Fig. 4.22). Nevertheless, the Cathepsin S inhibitor (100 nM) treatment alleviated the LPS (100 ng/mL,

10 $\mu\text{g/mL}$)- induced gene expressions of interleukin pro-inflammatory cytokines in OECs (Fig. 4.22).

4.3 Study of the Role of Cathelicidin (LL-37) in the Maintenance of the Alveolar-Capillary Barrier

4.3.1 Impacts of LL-37 on the properties of the alveolar-capillary barrier

The former experimental results revealed that Vitamin D₃ (10 nM) significantly induced the expression of LL-37 in the cell lines of alveolar epithelial cells (Fig. 4.5, Fig. 4.6). This study further investigated the role of LL-37 in the maintenance of the properties of the alveolar-capillary barrier.

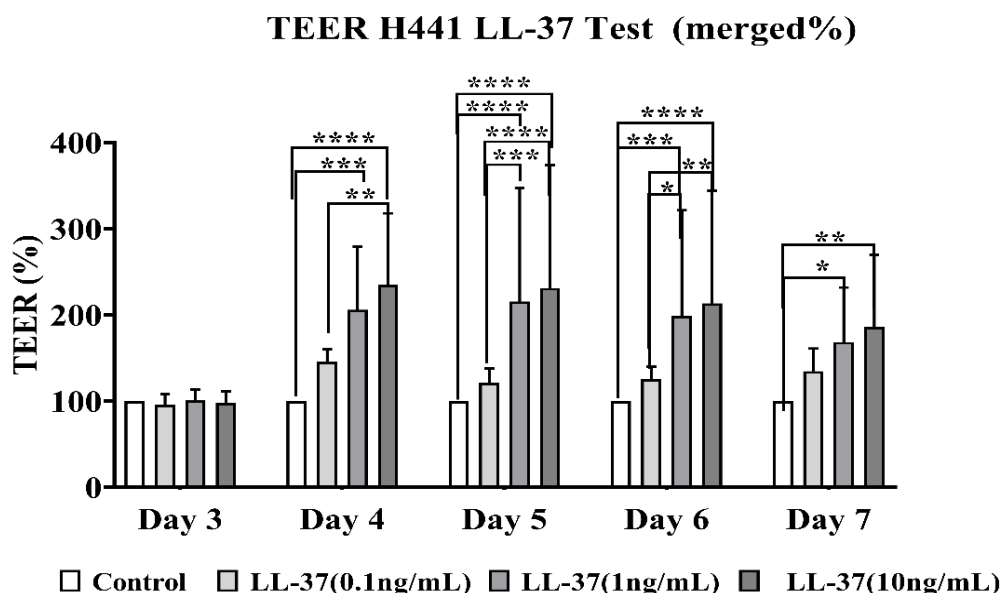


Figure 4.23: TEER measurement of the mono-culture model of NCI-H441 cells in Transwell® for the test of effects LL-37 on the barrier properties of alveolar epithelial cells. The cells were treated with LL-37 with a concentration series (0.1ng/mL, 1 ng/mL, and 10 ng/mL) from Day 3. The TEER values were demonstrated as the relative TEER values in the percentage ratio of control (%). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

From the analysis of TEER measurement, LL-37 (0.1 ng/mL, 1 ng/mL, 10 ng/mL) prominently increased the TEER values of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in Transwell®. The increased TEER value showed a LL-37

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concentration-dependent manner (Fig. 4.23). The higher the concentration of LL-37, the higher the TEER values of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.22). The LL-37 treatment with a concentration of 10 ng/mL showed the most significant effect in improving the TEER values (Fig. 4.23). Therefore, a higher concentration of LL37 (10 ng/mL) was chosen for further investigations. This result revealed that LL-37 also has a significant effect in improving the properties of the alveolar epithelial barrier

TEER Co-H441/OECs LL-37 LPS Test (merged%)

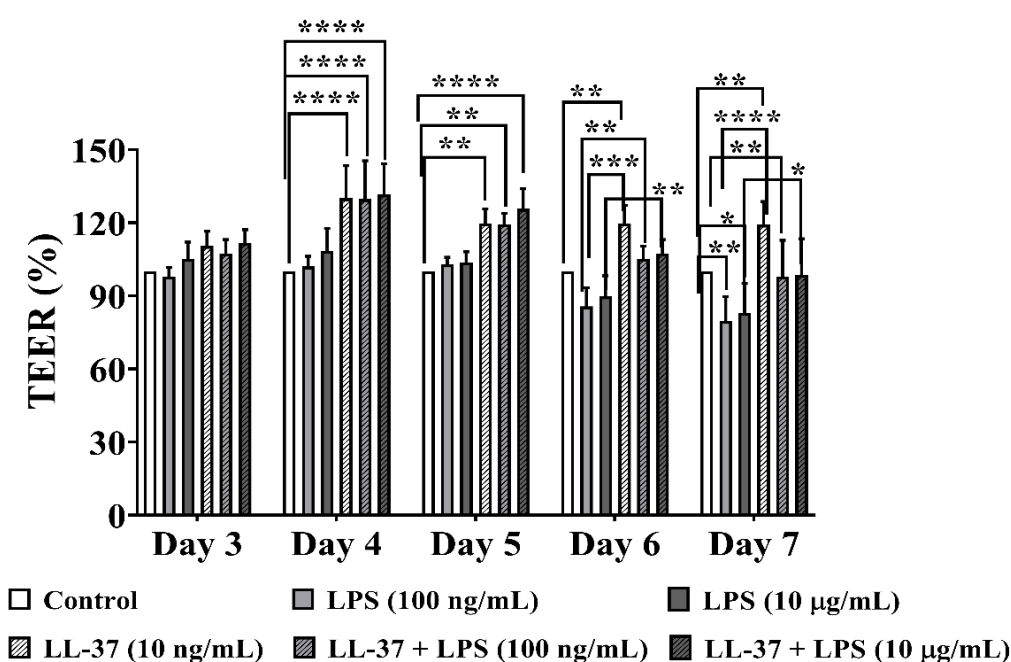


Figure 4.24: TEER measurement of the co-culture model of NCI-H441 cells and OECs in Transwell® for test of the effects of LL-37 on the barrier properties of alveolar-capillary barrier. The cells were treated with LL-37 (10 ng/mL) from Day 3, and LPS (100 ng/mL, and 10µg/mL) from Day 5(only in basolateral compartments). The TEER values were demonstrated as the relative TEER values in the ratio of control (%). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

From the result of TEER measurement, LL-37 (10 ng/mL) significantly increased the barrier properties of the co-culture model of NCI-H441 cells and OECs in Transwell®, which were revealed from the TEER values (Fig. 4.23). The TEER values of the co-culture model were significantly decreased in exposure to LPS (100 ng/mL, 10 µg/mL) (Fig. 4.24). Nevertheless,

LL-37 (10 ng/mL) still significantly maintained the TEER values of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® in the challenge with LPS (100 ng/mL, 10 µg/mL) treatment. This result suggested that LL-37 has a positive effect on maintaining and improving the properties of the alveolar-capillary barrier for defense against bacterial infection.

4.3.2 Effects of LL-37 on the gene expressions of the related molecules in the barrier function and infection

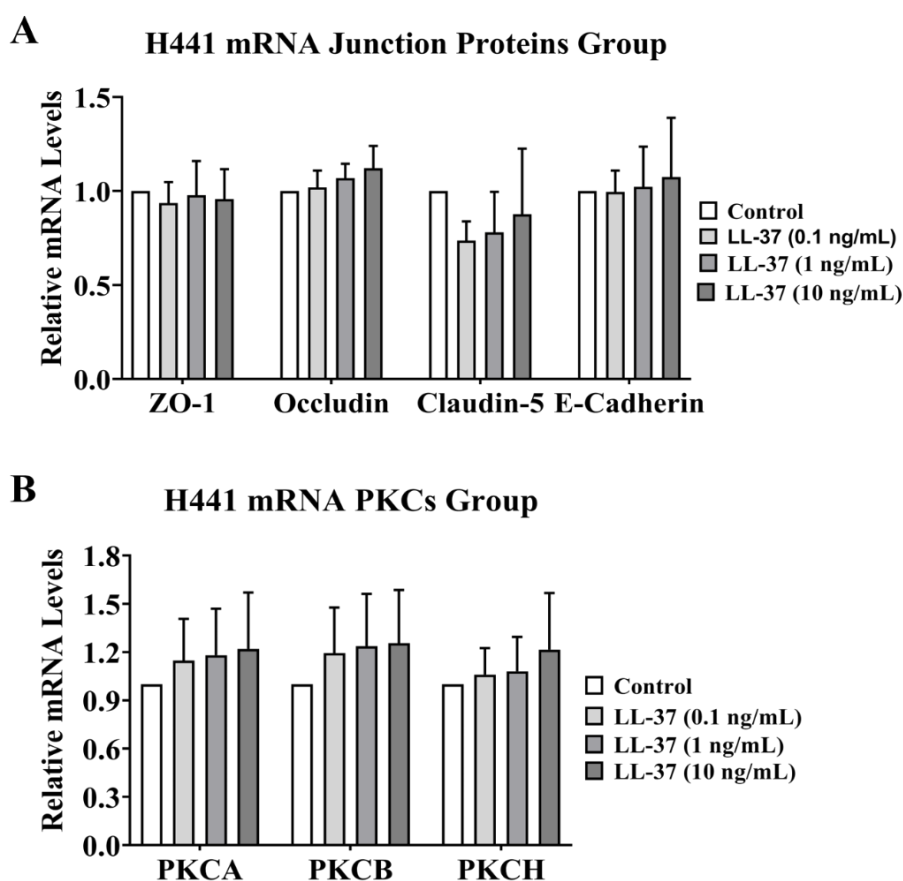


Figure 4.25: Real-time PCR analysis for the gene expressions of (A) Functional Proteins (ZO-1, Occludin, Claudin-5, and E-Cadherin) and (B) group of Protein Kinase C isozymes (PKCA, PKCB, PKCH) in the mono-culture model model of NCI-H441 cells in Transwell®. The cells were treated with a concentration series of LL-37 (0.1 ng/mL, 1 ng/mL, 10 ng/mL). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$).

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Based on the TEER measurement in the mono-culture model of NCI-H441 cells, and the co-culture model of NCI-H441 cells and OECs in Transwell®, this study further performed the gene expression analysis of the junction proteins, and the molecules involved in the LPS-caused inflammation in NCI-H441 cells. The results of the gene expression analysis revealed that the gene expressions of the junction proteins (ZO-1, Occludin, Claudin-5, and E-Cadherin) and Protein Kinases C (PKCA, PKCB, PKCH) in NCI-H441 cells were not significantly affected by LL-37 (0.1 ng/mL, 1ng/mL, 10ng/mL) (Fig. 4.25). Nevertheless, the gene expression of junction proteins and Protein Kinase Cs in NCI-H441 cells showed an increasing trend along with the increased concentration of LL-37 (0.1 ng/mL, 1 ng/mL, 10 ng/mL) treatment (Fig. 4.25 A, B).

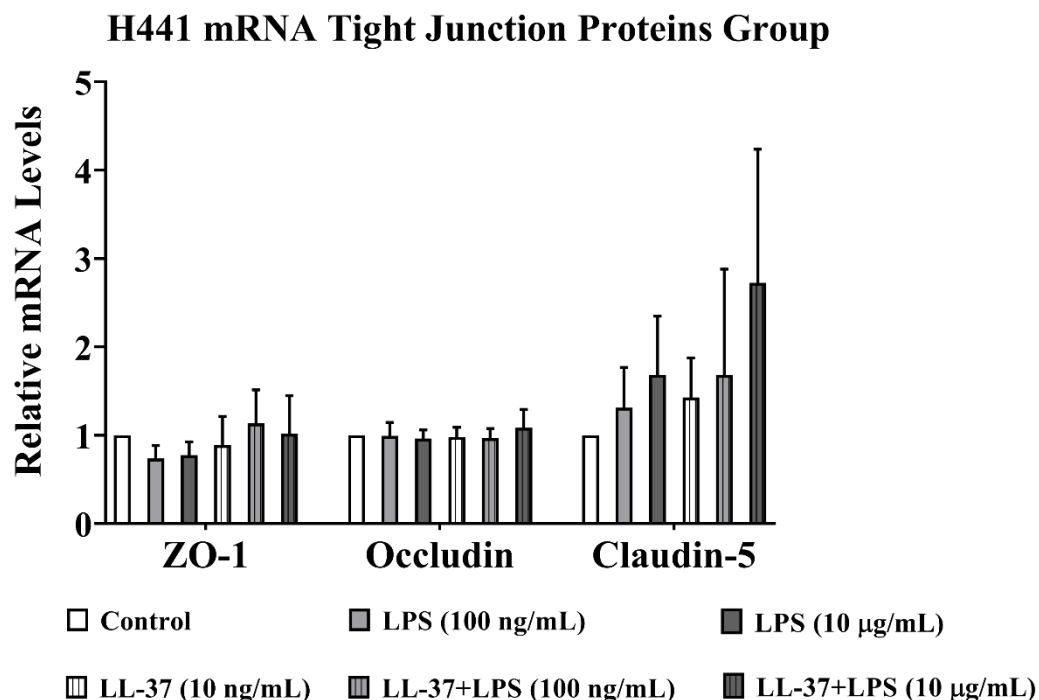


Figure 4.26: Real-time PCR analysis for the gene expressions of tight junction proteins (ZO-1, Occludin, Claudin-5,) in NCI-H441 cells, in the co-culture model of NCI-H441 and OECs in Transwell®. The cells were treated with LL-37 (10 ng/mL), and LPS (100 ng/mL, 10µg/mL). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$).

From the gene expression analysis, LL-37 (10 ng/mL) and LPS (100 ng/mL, 10 µg/mL) generally did not significantly affect the gene expression of the tight junction proteins (ZO-1, Occludin, and Claudin-5) in NCI-H441 cells, in the co-culture model. The gene

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expressions of ZO-1 and Occludin displayed a decreasing trend in NCI-H441 cells with LPS (100 ng/mL, 10 μ g/mL) treatment (Fig. 4.26). Whereas LL-37 (10 ng/mL) showed an effect in increasing and still maintaining the gene expression of the tight junction proteins, especially Claudin-5, in NCI-H441 cells in the exposure to LPS (100 ng/mL, 10 μ g/mL) treatment (Fig. 4.26).

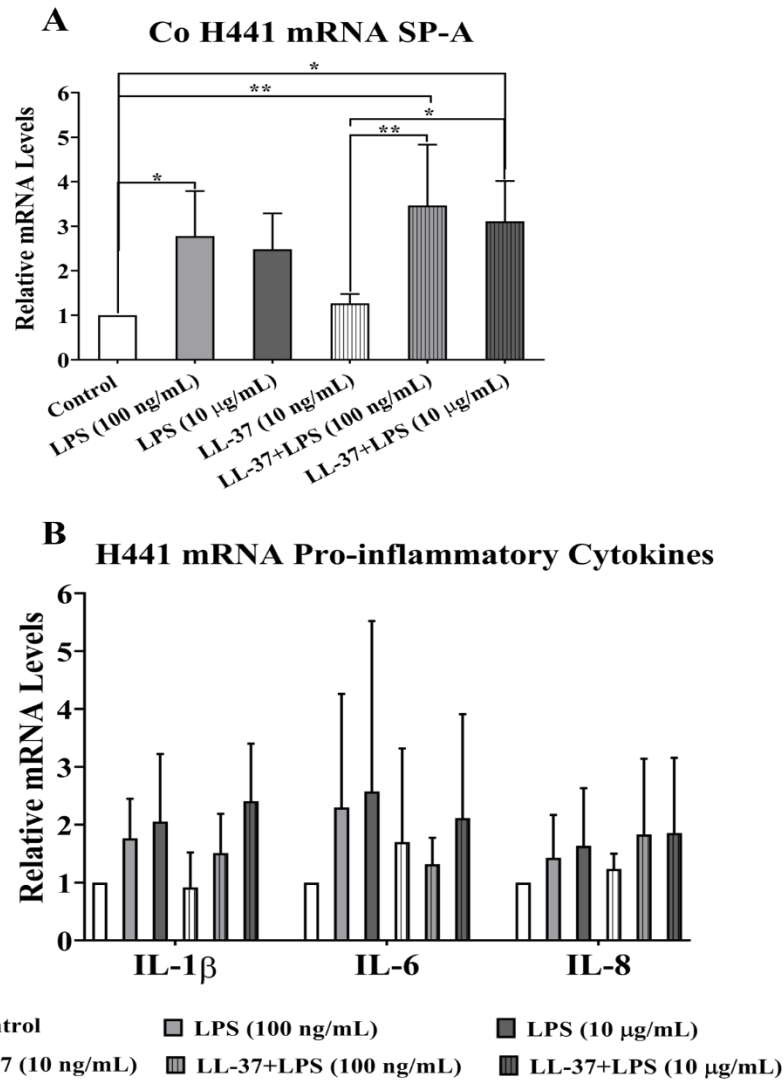


Figure 4.27: Real-time PCR analysis for the gene expressions of (A) Surfactant Protein A (SP-A) and (B) pro-inflammatory cytokines (IL-1 β , IL-6, IL-8) in NCI-H441 cells in the co-culture model of NCI-H441 cells and OECs in Transwell®. The cells were treated with LL-37 (10 ng/mL) and LPS (100 ng/mL, 10 μ g/mL). The data were statistically analyzed with One-way ANOVA (A) and Two-way ANOVA (B) ($n \geq 3$). Significance: * $P \leq 0.05$, ** $P \leq 0.001$.

From the gene expression analysis, LPS (100 ng/mL, 10 µg/mL) increased the gene expression of the interleukin pro-inflammatory cytokines (IL-1 β , IL-6, IL-8) and SP-A in NCI-H441 cells in the co-culture model with OECs in Transwell® (Fig. 4.27 A, B). The gene expressions of the pro-inflammatory cytokines and SP-A in NCI-H441 cells were generally not distinctively affected by LL-37 (10 ng/mL). Nevertheless, the gene expression of IL-1 β , IL-6, and IL-8 in NCI-H441 cells showed an increasing trend with the LL-37 (10 ng/mL) treatment (Fig. 4.26 B). The gene expression of the pro-inflammatory cytokines, and SP-A in NCI-H441 cells, even demonstrated a higher level with LL-37 (10 ng/mL) plus LPS (100 ng/mL, 10 µg/mL) treatment (Fig. 4.27 A, B).

4.4. Investigation of the Role of DCLK1 in the Barrier Maintenance of Alveolar epithelial barrier

The previous experiment found that Vitamin D₃ (10 nM) significantly increased the expression of Doublecortin Like Kinase 1 (DCLK1) in the alveolar epithelial cell line, NCI-H441 cells at both gene and protein levels (Fig. 4.9, Fig. 4.10). The role of DCLK1 in the maintenance of the alveolar epithelial barrier was further investigated with the usage of the DCLK1 specified inhibitor, Leucine-rich repeats kinase 2 inhibitor (LRRK2-In-1). The NCI-H441 cells were pretreated with Vitamin D₃ (10 nM) on day 3 and then treated with the inhibitor LRRK2-IN-1 on Day 5 during the cell culture procedure.

4.4.1 Impact of the inhibition of DCLK1 expression on the barrier properties of alveolar epithelial cells

For the investigation of the impact of the inhibition of DCLK1 expression on the function of the alveolar epithelial barrier, this study performed the TEER measurement experiments for the analysis. The NCI-H441 cells were cultured in Transwell® and treated with Vitamin D₃ (10 nM) from Day 3, and treated with DCLK1 inhibitor LRRK2 (500 nM) from Day 5, after the cell seeding in Transwell®.

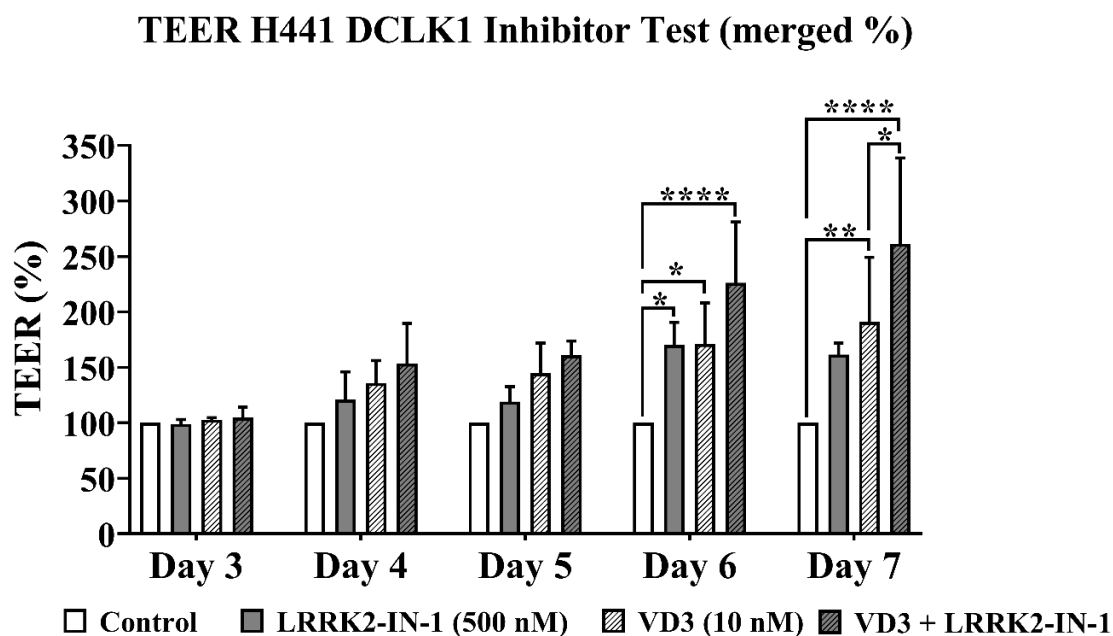


Figure 4.28: TEER measurement of the mono-culture model of NCI-H441 cells in Transwell® for the test of the impact of the inhibition of DCLK1 in NCI-H441 cells on the barrier properties of the alveolar epithelial barrier. The cells were treated with Vitamin D₃ (VD₃) (10 nM) from Day 3, and DCLK1 inhibitor, LRRK2-IN-1 (500 nM) from Day 5. The TEER values were demonstrated as the relative TEER values in the percentage ratio of control (%). The data were statistically analyzed with Two-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

From the TEER measurement, Vitamin D₃ (10 nM), and the DCLK1 inhibitor LRRK2-IN-1(500 nM) significantly increased the TEER values of the alveolar epithelial barrier in the mono-culture of NCI-H441 cells in Transwell® (Fig. 4.28), the TEER values of Vitamin D₃ (10 nM)-pretreated NCI-H441 cells in the mono-culture model were elevated to an even higher level after the treatment with LRRK2-IN-1 (500 nM) (Fig. 4.28). This result suggested that the DLK1 inhibitor, LRRK2 -IN-1 (500 nM) is effective in enhancing the positive effects of Vitamin D₃ on the improvement of the properties of the alveolar epithelial barrier.

4.4.2 Effects of the DCLK1 inhibitor on the expression of the relevant molecules in the maintenance of the alveolar epithelial barrier

Based on the TEER measurement, this study further investigated the expression of the relevant molecules in NCI-H441 cells, which were treated with Vitamin D₃ (10 nM) and

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LRRK2-IN-1 (500 nM). The molecules in the expression analysis in NCI-H441 cells included the junction proteins (ZO-1, Occludin, Claudin-5, and E-Cadherin), Caveolin-1, and the Epidermal growth factor (EGF). The investigation of the expression of these molecules in NCI-H441 cells is helpful to more clearly understand the role of DCLK1 in the preservation of the function properties of the alveolar epithelial barrier.

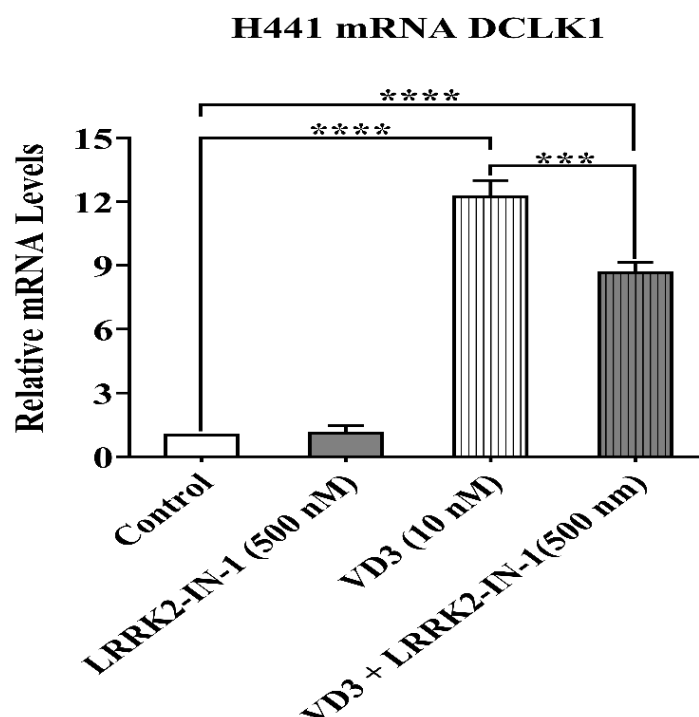


Figure 4.29: Real-time PCR analysis for the gene expressions of DCLK1 in the mono-culture of NCI-H441 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and DCLK1 inhibitor LRRK2-IN-1 (500 nM). The data were statistically analyzed with One-way ANOVA (n ≥ 3). Significance: ***P ≤ 0.001, ****P ≤ 0.0001

This study first investigated the effects of Vitamin D₃ and the DCLK1 inhibitor, LRRK2-IN-1, on the expression of DCLK1 at gene and protein levels. According to the gene expression analysis, the gene expression of DCLK1 significantly increased in NCI-H441 cells with Vitamin D₃ (10 nM) treatment (Fig. 4.29). Whereas, the DCLK1 inhibitor, LRRK2-IN-1 (500 nM) significantly decreased the gene expression of Vitamin D₃ (10 nM)-induced gene expression of DCLK1 in NCI-H441 cells (Fig. 4.29).

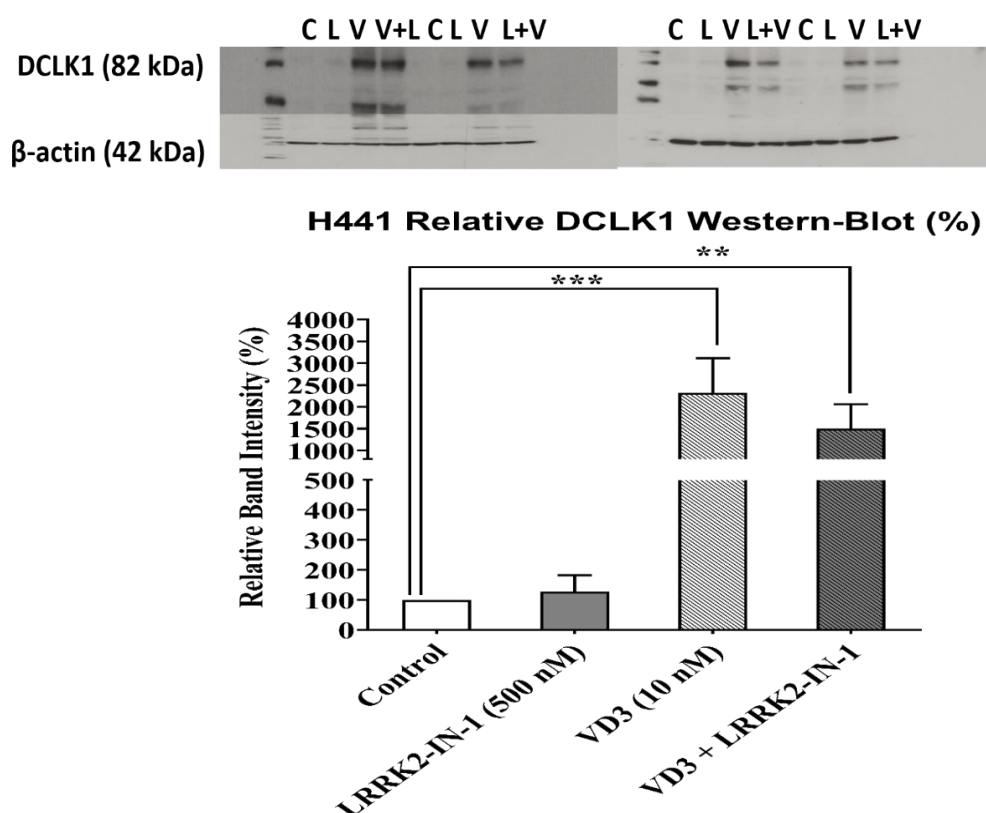


Figure 4.30: Western-blot test for protein expression analysis of DCLK1 in the mono-culture of NCI-H441 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and DCLK1 inhibitor LRRK2-IN-1 (500 nM). The data were demonstrated as the relative values of the quantification of the protein band intensity of DCLK1 in the percentage ratio to the control (%). The data were statistically analyzed with One-way ANOVA (n = 4). Significance: **P ≤ 0.01, ***P ≤ 0.001. Abbreviation: C: control, L: LRRK2-IN-1(500 nM), V: Vitamin D₃(10 nM), V+L: Vitamin D₃ (10 nM) +LRRK2-IN-1 (500 nM).

The Western-Blot protein expression analysis demonstrated that Vitamin D₃ (10 nM) significantly increased the protein expression of DCLK1 in the alveolar epithelial cell line, NCI-H441 cells (Fig. 4.30). Whereas the Vitamin D₃ (10 nM)-induced protein expression level of DCLK1 was distinctively repressed by the DCLK1 inhibitor, LRRK2-IN-1(500 nM), in NCI-H41 cells (Fig. 4.30). These results suggested that Vitamin D₃ has a significant effect in increasing the expression of DCLK1 in alveolar epithelial cells at both protein and gene levels. The results verified that the DCLK1 inhibitor, LRRK2 -IN-1, is effective in decreasing the expression of DCLK1 in alveolar epithelial cells at both gene and protein levels.

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This study also tested the effects of Vitamin D₃ and LRRK2-IN-1 on the expression of junction proteins including ZO-1, Occludin, and E-Cadherin in NCI-H441 cells at both gene and protein levels.

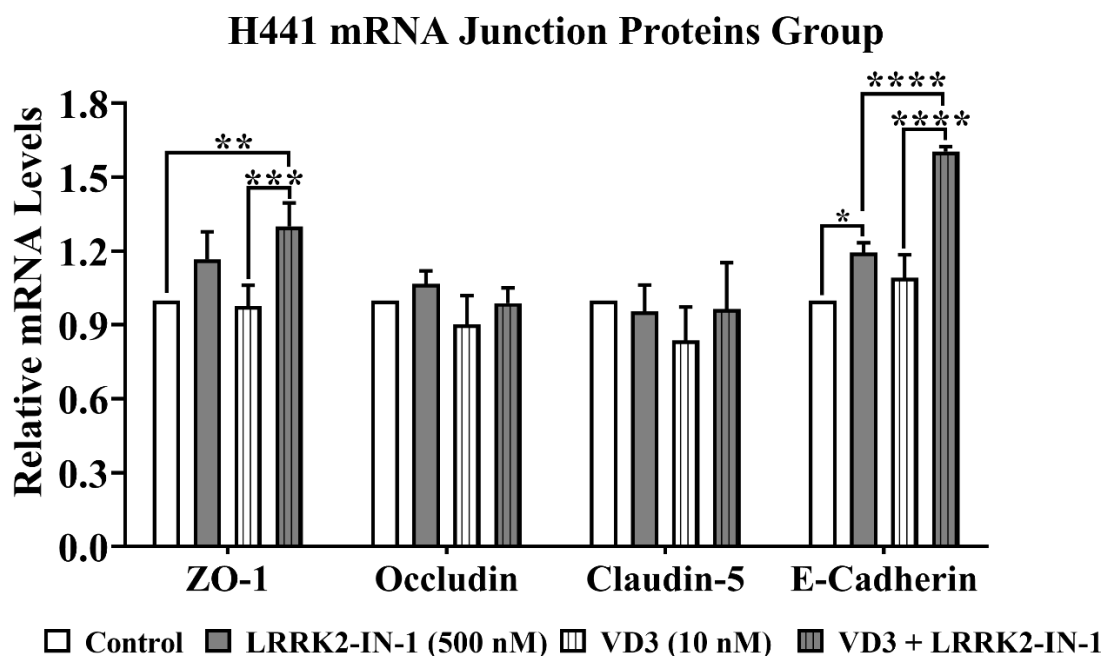


Figure 4.31: Real-time PCR analysis for the gene expressions of the junction protein molecules (ZO-1, Occludin, Claudin-5, and E-Cadherin in the mono-culture model of NCI-H441 cells in Transwell®. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and DCLK1 inhibitor LRRK2-IN-1 (500 nM). The data were statistically analyzed with Two-way ANOVA (n ≥ 3). Significance: *p ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

This study further tested the effects of Vitamin D₃ (10 nM) and LRRK2-IN-1 (500 nM) on the expression of the junction proteins in NCI-H441 cells. From the gene expression analysis, Vitamin D₃ (10 nM) did not distinctively affect the gene expression of the junction proteins in NCI-H441 cells (Fig. 4.31). Whereas, the DCLK1 inhibitor, LRRK2-IN-1 (500 nM), increased gene expression of the junction proteins in NCI-H441 cells. In particular, the gene expressions of ZO-1 and E-Cadherin were significantly increased in NCI-H441 cells, which were treated with LRRK2-IN-1 (500 nM) (Fig. 4.31).

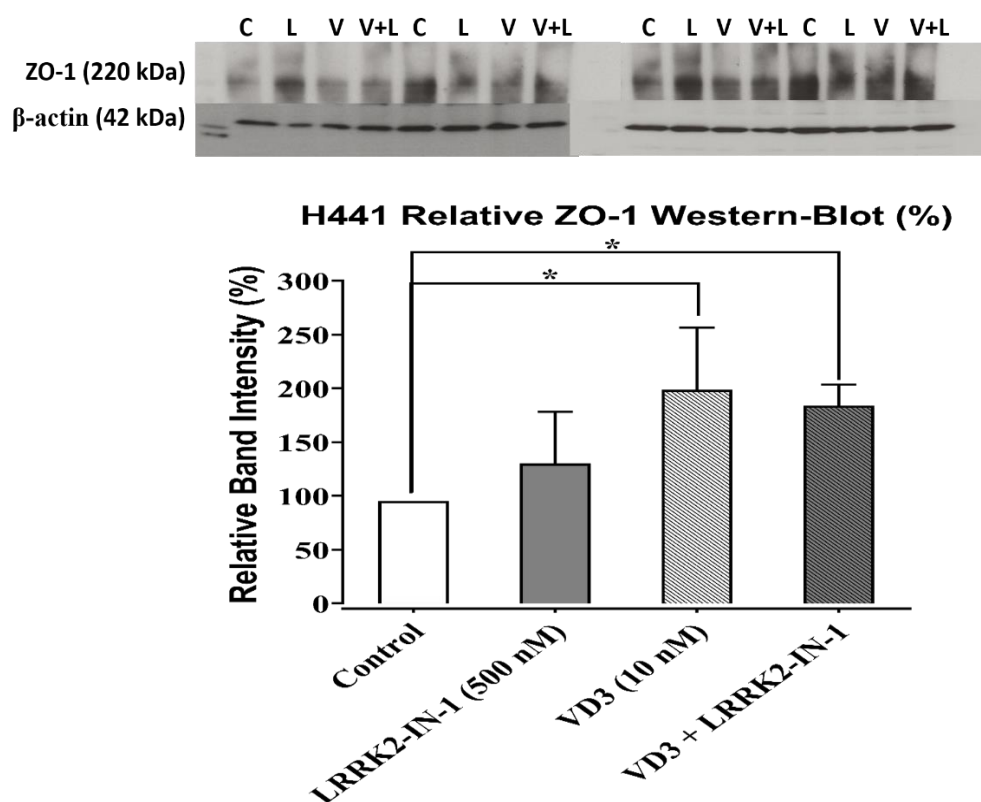


Figure 4.32: Western-Blot test for protein expression analysis of ZO-1 in the mono-culture of NCI-H441 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and DCLK1 inhibitor, LRRK2-IN-1 (500 nM). The data were demonstrated as the relative values of the quantification of the protein band intensity of DCLK1 in the percentage ratio to the control (%). The data were statistically analyzed with One-way ANOVA (n = 4). Significance: *P ≤ 0.05. Abbreviations: C: control, L: LRRK2-IN-1(500 nM), V: Vitamin D₃ (10 nM), V+L: Vitamin D₃ (10nM)+LRRK2-IN-1 (500 nM).

From the protein expression analysis of DCLK1 in NCI-H441 cells, the protein expression level of ZO-1 was not prominently influenced by LRRK2-IN-1 (500 nM) (Fig. 4.32). Even though the protein expression of ZO-1 showed an increasing trend in NCI-H441 cells with LRRK2-IN-1 (500 nM) treatment (Fig. 4.32). Meanwhile, Vitamin D₃ (10 nM) significantly increased the protein expression of ZO-1 in NCI-H441 cells (Fig. 4.32).

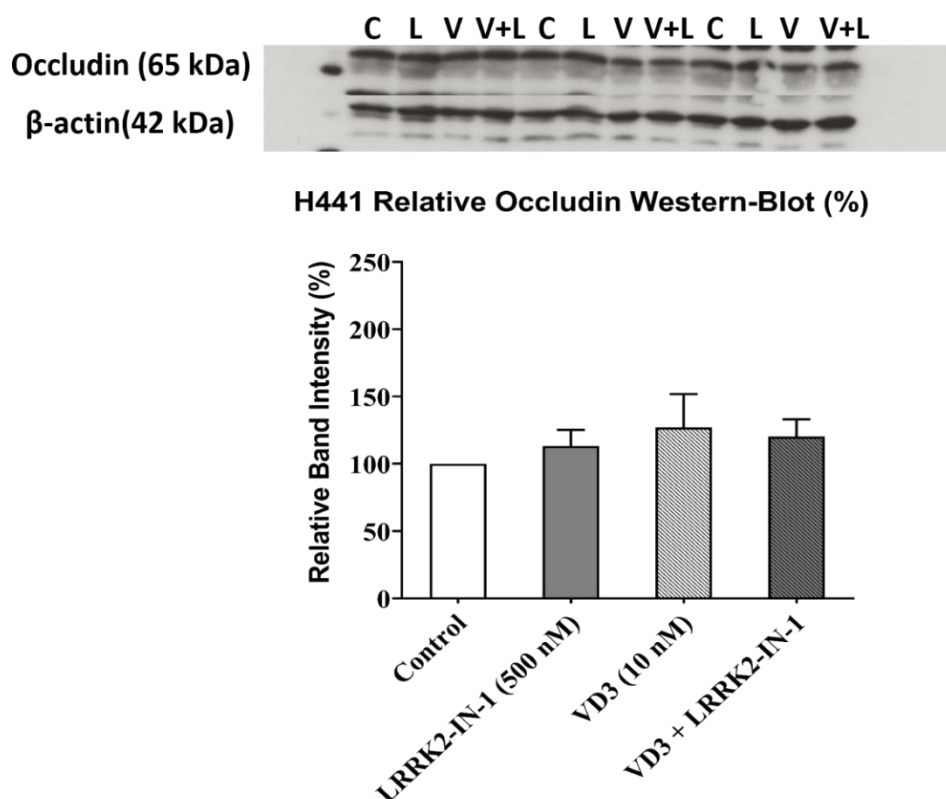


Figure 4.33: Western-Blot test for protein expression analysis of Occludin in the mono-culture of NCI-H441 cells. The cells were treated with Vitamin D₃ (VD₃) (10 nM), and DCLK1 inhibitor, LRRK2-IN-1 (500 nM). The data were demonstrated as the relative values of the quantification of the protein band intensity of DCLK1 in the percentage ratio to the control (%). The data were statistically analyzed with One-way ANOVA (n = 3). Significance: * P ≤ 0.05. Abbreviations: C: control, L: LRRK2-IN-1 (500 nM), V: Vitamin D₃ (10 nM), V+L: Vitamin D₃ (10 nM +LRRK2-IN-1 (500 nM)

From the protein expression analysis, the protein expression level of Occludin generally displayed an increasing trend in NCI-H441 cells, which were treated with both Vitamin D₃ (10 nM) and LRRK2-IN-1 (500 nM) (Fig. 4.33).

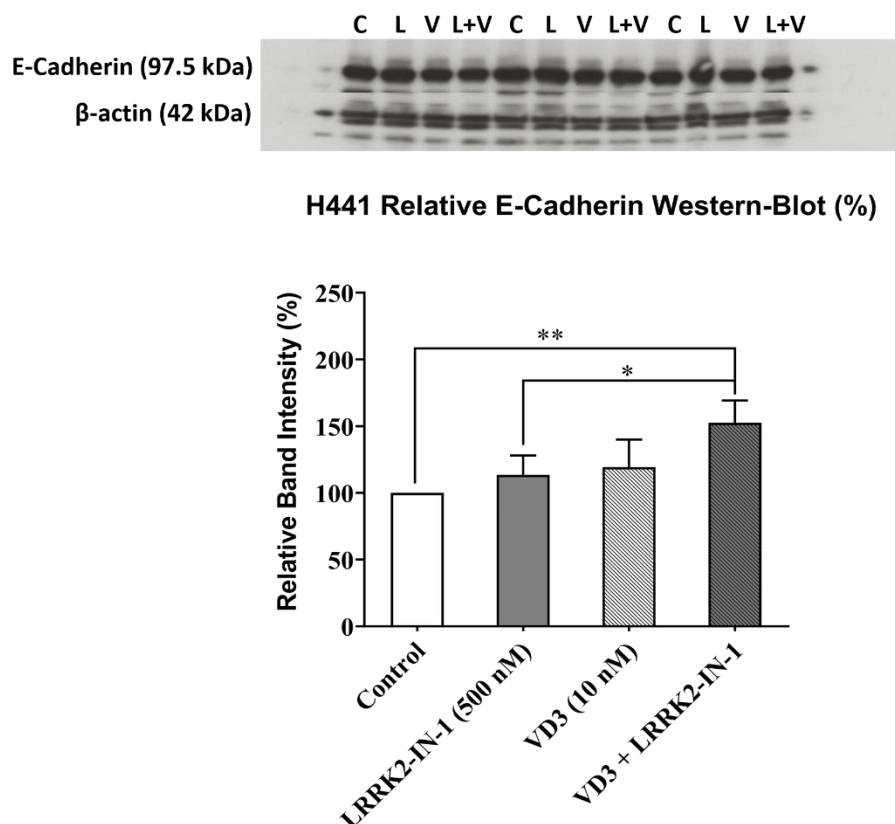


Figure 4.34: Western-Blot test for protein expression analysis of E-Cadherin in the mono-culture of NCI-H441 cells the cells were treated with Vitamin D₃ (VD₃) (10 nM), and DCLK1 inhibitor, LRRK2-IN-1 (500 nM) The data were demonstrated as the relative values of the quantification of the protein band intensity of DCLK1 in the percentage ratio to the control (%). The data were statistically analyzed with One-way ANOVA (n = 3). Significance: *P ≤ 0.05. **P ≤ 0.01 Abbreviations: C: control, L: LRRK2-IN-1(500 nM), V: Vitamin D₃ (10 nM), V+L: Vitamin D₃ (10 nM) +LRRK2-IN-1 (500 nM).

According to the protein expression analysis of E-Cadherin in NCI-H441 cells, Vitamin D₃ (10 nM), and LRRK2-IN-1 (500 nM) showed the effects in increasing the expression of E-Cadherin in NCI-H441 cells (Fig.4.34). In particular, the protein expression of E-Cadherin was significantly increased in NCI-H441 cells with Vitamin D₃ (10 nM) plus DCLK1 (500 nM) treatments (Fig. 4.34). This result suggested that the DCLK1 inhibitor, LRRK2-IN-1 is effective in enhancing the effect of Vitamin D₃ on the increase of the protein expression of E-Cadherin in alveolar epithelial cells.

Experimental Results

Besides the investigation of the expressions of the junctional protein in NCIH441 cells, this study also investigated the effects of the DCLK1 inhibitor, LRRK1-IN-1, on the expression of other molecules, which are related to the functionality of the alveolar epithelial barrier. These molecules included the epidermal growth factor (EGF), and Caveolin-1.

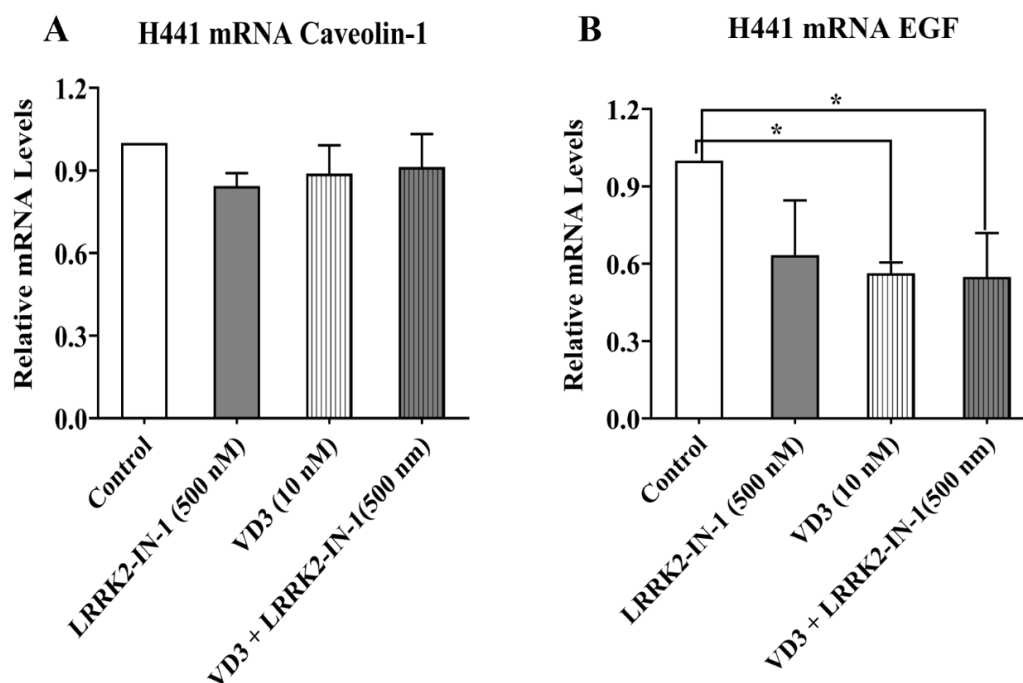


Figure 4.35: Real-time PCR analysis for the gene expressions of Cavolin-1(A) and epidermal growth factor (EGF) (B) in the mono-culture model of NCI-H441 cells in Transwell®. The cells were treated with Vitamin D₃ (VD₃) (10 nM) and DCLK1 inhibitor LRRK2-IN-1 (500 nM). The data were statistically analyzed with One-way ANOVA ($n \geq 3$). Significance: * $p \leq 0.05$.

According to the gene expression analysis, the gene expressions of Caveolin-1 and epidermal growth factor (EGF) showed a decreasing trend in NCI-H441 cells with Vitamin D₃ (10 nM) and LRRK2-IN-1 (500 nM) treatments (Fig. 4.35). Vitamin D₃ (10 nM) significantly decreased the gene expression of epidermal growth factor (EGF) in NCI-H441 cells (Fig. 4.35) This result indicated that Vitamin D₃ and LRRK2-IN-1 have a repressive effect on the gene expression of Caveolin-1 and EGF in alveolar epithelial cells.

4.4.3 Further study of the inhibition of DCLK1 in NCI-H441 cells

This study further investigated the impact of the inhibition of DCLK1 on the barrier properties of alveolar epithelial cells via the usage of a higher concentration of DCLK1 inhibitor, LRRK2-IN-1 (5 μ M). This study implemented TEER measurement in the mono-culture model of NCI-H441 cells in Transwell® and the analysis of the gene expression of the junctional proteins (ZO-1, Occludin, Claudin-5, and E-Cadherin) in NCI-H441 cells with the treatment using a gradient concentration of LRRK2-IN-1 (500 nM, 5 μ M) in the cell culture process.

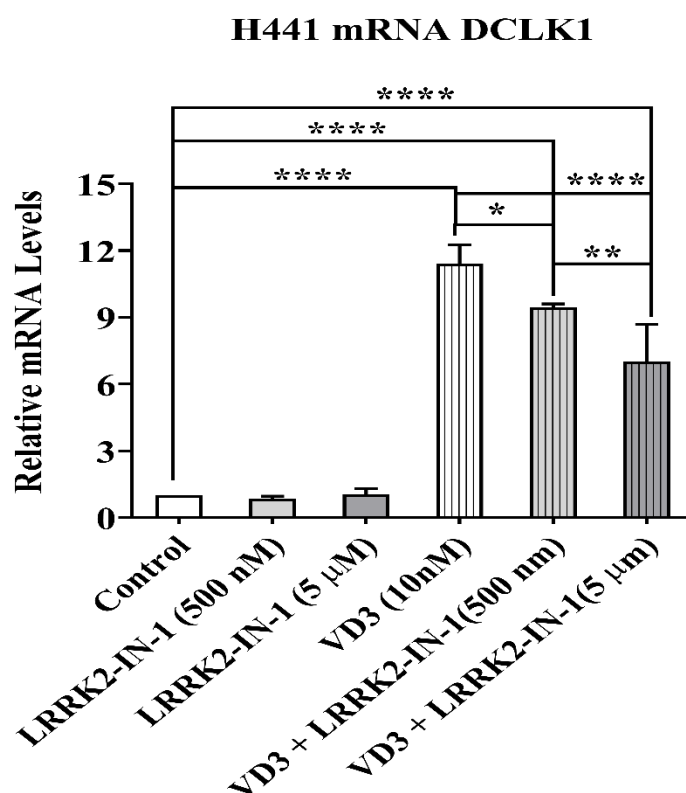


Figure 4.36: Real-time PCR analysis for the gene expressions of DCLK1 in the mono-culture of NCI-H441 cells in Transwell®. The cells were treated with Vitamin D₃ (VD₃) (10 nM), and DCLK1 inhibitor, LRRK2-IN-1 (500 nM, 5 μ M). The data were statistically analyzed with One-way ANOVA (n \geq 3). Significance: *p \leq 0.05, **P \leq 0.01, ****P \leq 0.0001

From the result of gene expression analysis, Vitamin D₃ (10 nM) still significantly increased the gene expression of DCLK1 in NCI-H441 cells (Fig. 4.36). LRRK2-IN-1 (500 nM) showed a significant effect in decreasing the gene expression of DCLK1 in NCI-H441 cells

Experimental Results

(Fig. 4.36). The gene expression of DCLK1 in NCI-H441 cells was more significantly decreased with the higher concentration of DCLK1 inhibitor, LRRK2-IN-1 (5 μ M) treatment (Fig. 4.36).

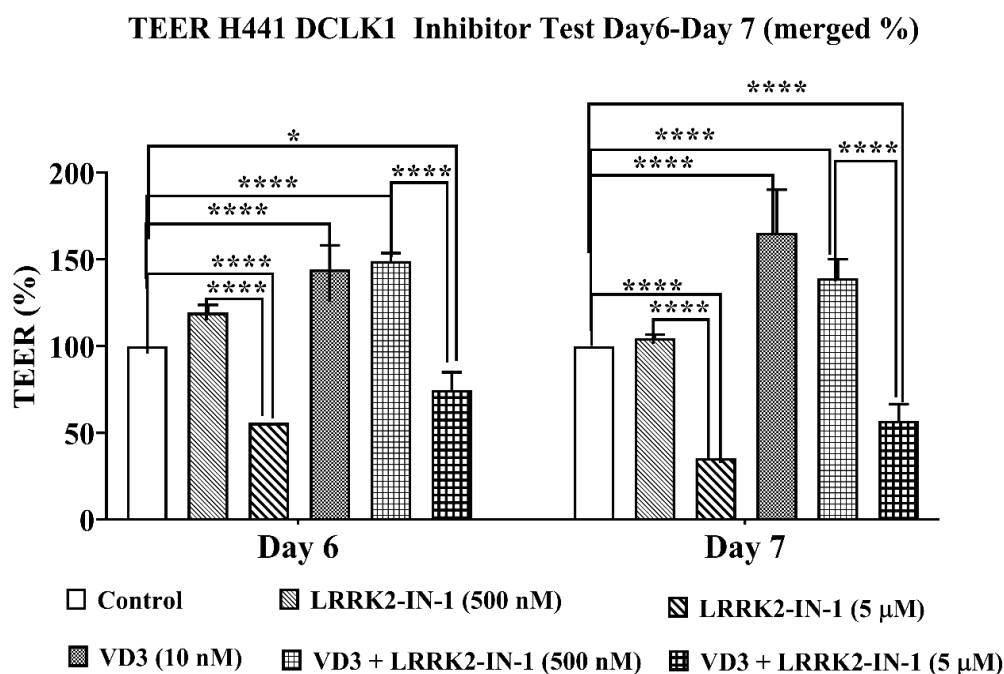


Figure 4.37: TEER measurement on Day 6 and Day 7 of the mono-culture of model NCI-H441 cells in Transwell® for the test of the effects of the inhibition of DCLK1 in NCI-H441 cells on the barrier properties of the alveolar epithelial barrier. The cells were treated with Vitamin D₃ (VD₃) (10 nM) from Day 3, DCLK1 inhibitor, and LRRK2-IN-1 (500 nM, 5 μ g/mL), from Day 5. The TEER values were demonstrated as the relative TEER values in the percentage ratio to the control (%). The data were statistically analyzed with Tow-way ANOVA ($n \geq 3$). Significance: * $P \leq 0.05$, **** $P \leq 0.0001$

From the results of the TEER measurement, Vitamin D₃ (10 nM) significantly increased the TEER values of the mono-culture model NCI-H441 cells in Transwell® (Fig. 4.37). The effects of the DCLK1 inhibitor, LRRK2-IN-1, on the barrier properties of the mono-culture model NCI-H441 cells in Transwell® showed a concentration-dependent manner. LRRK2-IN-1 (500 nM) showed an effect in increasing the TEER values (Fig. 4.37). However, on the contrary, the higher concentration of DCLK1 inhibitor, LRRK2-IN-1 (5 μ M), even significantly decreased the TEER values (Fig. 4.37). This result indicated that moderate

inhibition of DCLK1 expression in alveolar epithelial cells is beneficial to increase the function properties of the alveolar epithelial barrier.

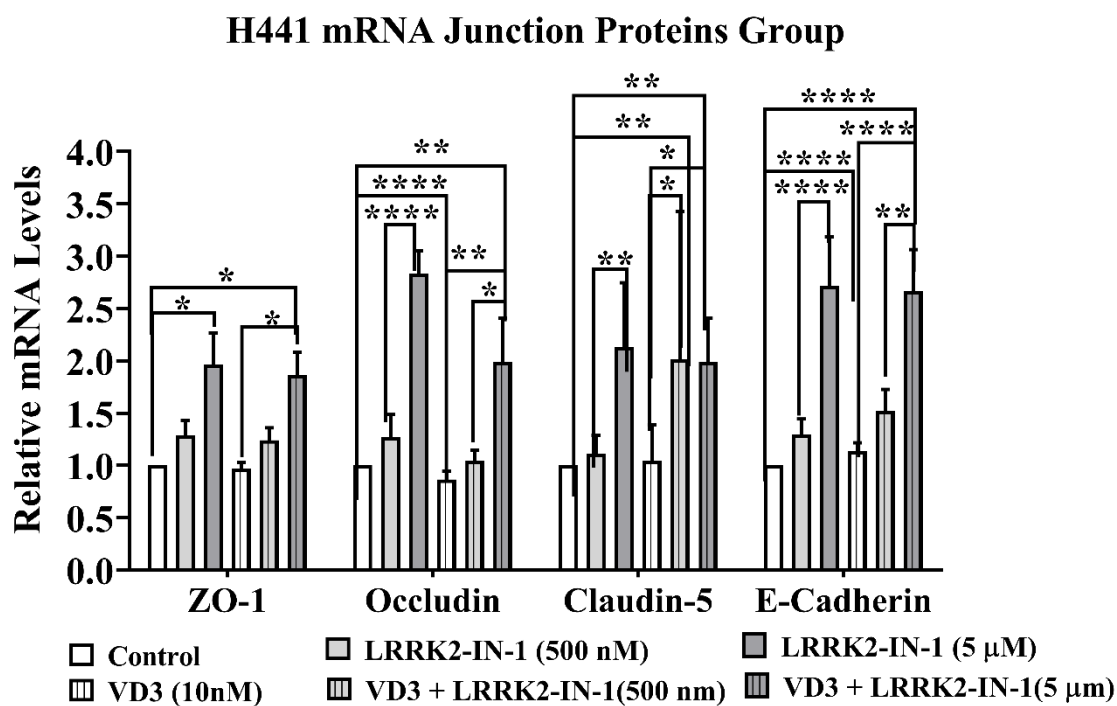


Figure 4.38: Real-time PCR analysis for the gene expressions of the junction protein molecules (ZO-1, Occludin, Claudin-5, and E-Cadherin) in the mono-culture model of NCI-H441 cells in Transwell®. The cells were treated with Vitamin D (10 nM) (VD₃), and DCLK1 inhibitor, LRRK2-IN-1 (500 nM, 5 μM). The data were statistically analyzed with Two-way ANOVA (n ≥ 3). Significance: *p ≤ 0.05, ** P ≤ 0.01, ****P ≤ 0.0001

According to the result of the gene expression analysis of the junctional proteins in NCI-H441 cells, Vitamin D₃ (10 nM) showed a mild effect on the gene expression of the junction proteins in NCI-H441 cells (Fig. 4.38). The impacts of LRRK2-IN-1 (500 nM, 5 μM) on the improvement of the gene expressions of the junction proteins displayed a concentration-dependent manner (Fig. 4.38). The gene expressions of the junction proteins were more significantly increased in NCI-H441 cells with LRRK2-IN-1 (5 μM) treatment, than in the treatment with LRRK2-IN-1 (500 nM) (Fig. 4.38).

5 Discussion

5.1 Effect and the Involved Mechanisms of Vitamin D₃ on the Maintenance of the Functions of the Alveolar-Capillary Barrier

5.1.1 Vitamin D₃ showed positive effects in increasing the protein expression of the junction proteins in alveolar epithelial cells

This study proved that Vitamin D₃ is effective in improving the properties of the alveolar-capillary barrier, especially the properties of the alveolar epithelial barrier were significantly increased with Vitamin D₃ treatment (Fig. 4.1, Fig. 4.11) and Vitamin D₃ (10 nM) also increased the barrier impedance of vascular endothelial cells, OECs (Fig. 4.12).

Concerning the junction proteins, Vitamin D₃ (10 nM) decreased the gene expression of ZO-1 in the alveolar epithelial cell line, NCI-H441 cells (Fig. 4.2 C). Vitamin D₃ (10 nM) also decreased the gene expression of Occludin in NCI-H441 cells (Fig. 4.2 C). Nevertheless, the Western-Blot analysis showed that vitamin D₃ (10 nM) increased the expression of ZO-1, and Occludin in NCI-H441 cells at the protein level (Fig. 4.32, Fig. 4.33). Vitamin D₃ (10 nM) also increased the expression of E-Cadherin in NCI-H441 cells at both gene and protein levels (Fig. 4.2 C, Fig. 4.34).

According to the immunofluorescence staining test, VitaminD₃ (10 nM) increased the intensity and integrity of the Occludin on the cell membrane barrier of NCI-H441 cells (Fig. 4.3). Vitamin D₃ (10 nM) also enhanced the integrity and intensity of ZO-1 and E-Cadherin on the cell membrane barrier of NCI-H441 cells (Fig. 4.3, Fig. 4.10).

It has been demonstrated that Vitamin D₃ performs its functions and modifies the gene expression of intercellular junction proteins including ZO-1, Occludin, and E-Cadherin, which increases their protein expression in tissue epithelial cells through Vitamin D/Vitamin D receptor (VD/VDR) signaling pathway [178,180].

However, this study demonstrated the different expression trends between the gene and protein expression of ZO-1 and Occludin in NCI-H441 cells with Vitamin D₃ (10 nM) treatment (Fig. 4.2 C, Fig. 4.3, Fig. 4.32, Fig. 4.33). It can be assumed that the effects of Vitamin D₃ on the gene expression of ZO-1 and Occludin are cell-type dependent. It is also

possible that protein abundance of ZO-1 and Occludin can also reversely influence their mRNA expressions in NCI-H441 cells through post-translation gene editing.

This study demonstrated the positive effect of Vitamin D₃ on the increase and maintenance of the integrity of intercellular junction proteins including tight junction proteins ZO-1, Occludin, and adherens junction protein E-Cadherin on the cell membrane barrier of alveolar epithelial cells. This study also demonstrated the positive effect of Vitamin D₃ on the increase of the protein expression of ZO-1, Occludin, and E-Cadherin in alveolar epithelial cells. These factors can be the essential molecular mechanisms of Vitamin D₃ in the improvement of the function properties of the alveolar epithelial barrier.

5.1.2 Other involved molecules: VDR, PKCA, PKCB PKCH, EGF, and Caveolin-1 in alveolar epithelial cells

According to the result of the gene expression analysis of the involved molecules, Vitamin D₃ decreased the gene expression of Vitamin D receptor (VDR), Protein Kinase C isozymes (PKCA, PKCB, and PKCH), epidermal growth factor (EGF), and Caveolin-1 in the alveolar epithelial cell line, NCI-H441 cells (Fig. 4.4, Fig. 4.35). The related mechanisms are more deeply explicated in the flowing text.

It has been demonstrated that the Vitamin D receptor (VDR), is a member of the steroid thyroid hormone superfamily of high nuclear affinitive receptors [178]. VDR exists and is expressed in almost all human cells and is required for the exerting of the functions of Vitamin D₃ [179]. VDR is also essential for the expression and production of biologically active Vitamin D₃ [178].

On the other side, it has been found that the supplementation of Vitamin D₃ (100 IU/kg) benefits the expression of VDR in monocytes, and in that way, it is beneficial to decrease the risk of inflammation and reducing the fat level of monozygotic twins [179]. It is also found that the Vitamin D/VDR signaling pathway is involved in the regulation of the expression and organization of epithelial junction proteins, including E-Cadherin, ZO-1, and Occludin in tissue epithelial cells [180] The regulation of Vitamin D/VDR signaling on the expression

of junction proteins contributes to the conservation of the integrity and functions of tissue epithelial barriers [180]. A study found that Vitamin D₃ deficiency decreased both mRNA and protein expression of VDR in bronchial smooth muscle cells (BSMCs) in mouse lungs [181].

However, it is inconsistent from this study, that Vitamin D₃ (10 nM) even decreased the gene expression of VDR in the cell line of alveolar epithelial cells, NCI-H441 cells (Fig. 4.4). This could be deduced that the effect of Vitamin D₃ on the expression of VDR is cell type-dependent. It is also possible that VDR belongs to some molecules, whose gene and protein expressions are not concordant and even versus to each other, because of the post-gene transcription and post-protein translation-mediated gene expression editing of these molecules during the gene expression process [182, 183]. Moreover, it has been found that the protein abundance level of some specific molecules can also reversely influence the expression of their mRNA expression levels [183].

Therefore, it indicates that the protein expression levels of some molecules don't demonstrate a consistent trend with their mRNA expression levels [184]. Therefore, it can be implicated that although Vitamin D₃ decreases the gene expression of VDR in NCI-H441 cells, Vitamin D₃ has the effect of increasing the protein expression of VDR in NCI-H441 cells. This conjecture was also verified by previous studies from other groups demonstrating that the protein expression levels of VDR were significantly increased in human alveolar epithelial cells with Vitamin D₃ treatment [180].

The results of this study demonstrated that Vitamin D₃ (10 nM) significantly decreased the gene expression of Protein Kinase C isozymes including PKCA (PKC α), PKCB (PKC β), and PKCH (PKC η) in alveolar epithelial cell line NCI-H441 cells (Fig. 4.4).

Previous studies demonstrated that Protein Kinase Cs are related to the mediation of a wide range of diseases including carcinomatosis, tumors, tissue disorders, and pulmonary respiratory diseases [185]. Pulmonary respiratory diseases are mainly caused by the disruption of the alveolar-capillary barrier, which is also induced by the Protein Kinase C

isozymes-mediated degradation and disorganization of intercellular junction proteins [185]. Previous studies displayed that the isozymes of Protein Kinase C (PKCs) mediate the functions and integrity of tight junction proteins in epithelial cells, through their progressive effects on the phosphorylation of the C-terminal domain of Occludin, and translocation of ZO-1 [185]. The activation of PKCs was shown to increase the barrier tension of many cell types, including the alveolar epithelial type II cells (AT-II Cs), which also increases the permeability of epithelial barriers, even the permeability of the whole lung [186].

A scientific group found that the activated PKCs together with an increased Calcium level also inhibit the expression of Vitamin D receptor in 3T3 cells [187]. It has been demonstrated that PKCs interact with Toll-like Receptor 2/4 (TLR 2/4) in the NF- κ B signaling pathway [188]. A study found that the interaction of the activated PKCs with the TLR signaling pathway triggers the increased expression and over-secretion of the pro-inflammatory cytokines such as IL-6 and IL-8 from lung epithelial cells in response to the fungal infection that enhanced the induction of lung inflammation [189].

A study also found that PKCs-mediated excessive immune response also leads to acute respiratory distress syndrome (ARDS) [190]. This research found that Vitamin D₃ showed a repressive effect on the gene expressions of the classic PKCs, PKCA, PKCB, and novel PKCs, PKCH (Fig. 4.4). This result implied that the suppressive effect of Vitamin D₃ on the expression of PKCs is also a potential molecular mechanism of Vitamin D₃ for the conservation of lung functions and lung health for the defense against lung cancers and microbial pathogens-induced lung infection diseases.

This study demonstrated that Vitamin D₃ (10 nM) decreased the gene expression of the Epidermal Growth Factor (EGF) in the cell line of the alveolar epithelial cell line, NCI-H441 cells (Fig. 4.35).

EGF was found to be involved in lung repair from a lung injury [69]. EGF, as a mitogen is known to stimulate the transcription of cyclin D1 [191]. It also has been demonstrated that EGF exerts its function through the binding with epithelial growth factor receptor (EGFR)

for the mediation of the metagenic process in the cell cycle [192]. However, the overexpression or the aberrant expression of EGF promotes the pathogenesis of cancer in various tissues, including lung carcinoma [187].

This study showed that Vitamin D₃ (10 nM) decreased the gene expression of EGF in NCI-H441 cells (Fig. 4.35). Former studies revealed that the inhibition of EGF in lung epithelial cells through vaccination with EGF antibodies significantly improved the survival of patients with non-small cell lung cancer [193]. Vitamin D₃ was also demonstrated to affect the inhibition of EGFR expression in the intestinal epithelial cell line [194]. This effect is an essential antimitogenic mechanism of Vitamin D₃ for the risk reduction of human colon carcinoma [194]. Therefore, the inhibition of the expression of EGF is a therapeutic target for cancer treatment.

The suppressive effect of Vitamin D₃ on the gene expression of EGF in NCI-H441 cells implied that Vitamin D₃ regulates the moderate expression of EGF in alveolar epithelial cells. This effect also can be a mechanism of Vitamin D₃ for the inhibition of cell proliferation, which can also indicate the anti-cancer viability of Vitamin D₃ to prevent the progression of metastasis proliferation of lung cancer cells.

This study showed that Vitamin D₃ (10 nM) decreased the gene expression of Caveolin-1 in NCI-H441 cells (Fig. 4.4).

Caveolin-1 (22 kDa) is a cell membrane and cytoplasm-associated scaffold protein and is a critical component for the structure of Caveolae [195]. Caveolin-1 can be universally expressed in various cell types and organ tissues. In the lung, Caveolin-1 can be prominently found in epithelial cells, endothelial cells adipocytes, macrophages, and fibroblasts [195, 196]. Former studies found that Caveolin-1 mediates the barrier functions of lung epithelial cells. The repress or knockdown of Caveolin-1 expression results in the loss and translocation of adherens junction proteins, including E-Cadherin and β -Catenin in the lung epithelial cell lines, which disrupts the function of the alveolar epithelial barrier and further induces the risk of Asthma [197] and increases the activity of tumor and cancer cells invasion [198].

Caveolin-1 regulates cellular endocytosis and cell signaling, which enables Caveolin-1 to be involved in many cellular metabolic processes, including cell proliferation, differentiation, cell senescence, and apoptosis. A study found that Caveolin-1 promotes the senescence of fibroblasts and tumor cells, which is beneficial for tumor suppression [199, 200].

It has been found that Caveolin-1 also plays a role in the regulation of immune response. Caveolin-1 is demonstrated to reduce the secretion of the inflammasome in lung macrophages and neutrophils and prevent pathogen-induced pulmonary inflammation and fibrosis [200].

However, besides the beneficial role of Caveolin-1 for lung health preservation, it is controversial that Caveolin-1 is also demonstrated to be one of the critical regulators of lung injury [61]. Caveolin-1 is known as a cell membrane-associated protein that also induces vesicle barrier formation on the cell membrane and regulates the transcytosis of macromolecules [201]. LPS was reported to significantly induce the expression and phosphorylation of Caveolin-1, which significantly increases the transcytosis of macromolecules and even increases the pathogen stimuli uptake into host cells [202].

This process was also reported to induce the hyperpermeability of human pulmonary microvascular endothelial cells and pulmonary epithelial cells [61, 204]. As a result, it can lead to the diseases of lung edema, acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) [61]. It also has been found that the deletion of Caveolin-1 in lung epithelial cells can increase the survival of lung epithelial cells from Caveolin-1-mediated cell apoptosis in response to pathogen-induced lung infection [61, 203].

Therefore, the appropriate regulation of the expression of Caveolin-1 is a therapeutic target for lung disease treatment. This study found that Vitamin D₃ (10 nM) showed a depressive effect on the gene expression of Caveolin -1 in lung epithelial cells, which suggests that Vitamin D₃ can effectively control the expression of Caveolin-1 to a moderate level in alveolar epithelial cells for the conservation of the barrier functions of alveolar epithelial cells for the defense against pathogen infection.

5.1.3 Effects of Vitamin D₃ on the expression of immune modulation involved molecules, SP-A, and LL-37, in alveolar epithelial cells

According to the results of this study, Vitamin D₃ (10 nM) significantly decreased the gene expression of surfactant protein A (SP-A) in both NCI-H441 cells and Calu-3 cells (Fig. 4.7). Whereas, Vitamin D₃ (10 nM) increased the expression of SP-A in NCI-H441 cells at the protein level which is verified through the immunofluorescence staining of SP-A in NCI-H441 cells (Fig. 4.8).

Vitamin D₃ (10 nM) demonstrated significant effect in increasing the gene expression of the cathelicidin (LL-37) in all of the cell lines of alveolar epithelial cells, including NCI-H441 cells and Calu-3 cells (Fig. 4.5). Moreover, according to the immunofluorescence staining of LL-37, Vitamin D₃ (10 nM) also significantly increased the expression of LL-37 in NCI-H441 cells at the protein level (Fig. 4.6).

This study revealed a suppressive effect of Vitamin D₃ (10 nM) on the gene expression of SP-A in NCI-H441 cells. Former studies displayed that, in the presence of Vitamin D₃, the gene transcription of surfactant proteins is interfered by the activation of retinoic acid X receptor (RXR), and Vitamin D receptor (VDR) in the Vitamin D/VDR signaling pathway [204]. It has been found that the flanking region of the promotor of the human surfactant protein gene sequence, especially the DNA of SP-A contains the binding site of VDR and the Vitamin D Responsive element (VDRE) [205]. The interaction of VDR RXR with the promoter regions of surfactant proteins is an essential factor that leads to the altered expressions of the surfactant proteins in alveolar epithelial type II cells (AT-II cells) [205, 207]. This process can be one of the main mechanisms of the reduction effect of Vitamin D₃ on the gene expression of SP-A in AT-II cells.

Nevertheless, from the immunofluorescence staining of SP-A, Vitamin D₃ (10 nM) increased the protein expression level of SP-A in NCI-H441 cells (Fig. 4.8). A prior study from another group displayed that Vitamin D₃ increased the protein expression level of SP-B in AT-II cells

[206]. Prior research from other groups found that Vitamin D₃ improved the synthesis secretion of surfactant phospholipids in rat lung tissues [208].

A study also displayed that a low level of Vitamin D₃ in blood serum resulted in a decrease in the synthesis and secretion of SP-A and SP-D from human fetal lung tissue, which leads to abnormal lung development of human fetal [208]. These findings support the result of this study that Vitamin D₃ showed a positive effect in increasing the protein expression of SP-A in NCI-H44 cells (Fig. 4.8). It is known that SP-A is one of the essential defense molecules in the human immune system [209]. Therefore, the effect of Vitamin D₃ in increasing the biosynthesis of SP-A can be a critical mechanism of Vitamin D₃ to facilitate human lung development and the improvement of lung immunity for the defense against pathogen infection.

From the results of this study, Vitamin D₃ (10 nM) significantly increased the gene expression of the anti-microbial peptide, Cathelicidin LL-37, in all of the alveolar epithelial cell lines, NCI-H441 cells, Calu-3 cells, and A549 cells (Fig. 4.5). Vitamin D₃ (10 nM) also increased the protein expression of LL-37 in NCI-H441 cells according to the result of immunofluorescence staining of LL-37 in NCI-H441 cells (Fig. 4.6).

It has been found that the expression of LL-37 is regulated by the biologically active form of Vitamin D₃ [1 α ,25,(OH)₂D₃], which requires the conversion of 25(OH) D₃ into 1 α ,25(OH)₂ D₃, through the hydroxylase (CYP27B1)-induced hydroxylation in the Vitamin D₃ metabolic pathway [210]. It also has been found that the active form of Vitamin D₃ further affects the transcription of LL-37 in the lung tissue through its interaction with the Vitamin D Responsive Element (VDRE) on the promotor of the LL-37 gene in the pulmonary alveolar epithelial cells and immune cells [138]. This mechanism contributes to the significantly increased expression and synthesis of LL-37 from the alveolar epithelial and immune cells in the alveolar airway [210, 138].

Several studies found that Vitamin D₃ mediates the up-regulation of LL-37 expression in airway epithelial cells and macrophages for the modulation of the innate and adaptive

immune response [138, 211]. This process can be one of the critical mechanisms of Vitamin D₃ for the improvement of lung immunity for the defense against pathogens-induced lung diseases. The positive effects of Vitamin D₃ on the expression of the Cathelicidin, LL-37 in the alveolar epithelial cells were also verified in this study.

5.1.4 Vitamin D₃ increases the expression of Doublecortin-Like Kinase 1 (DCLK1) in alveolar epithelial cells

A previous proteomics study which was performed in collab with Professor Andreas Throley (CAU, Kiel) found that Vitamin D₃ significantly increased the protein abundance of a serine/threonine protein, doublecortin-like kinase 1 (DCLK1) in the protein components of NCI-H441 cells through proteomics analysis using Tandem Mass Spectrometry (MS/MS).

This study verified that Vitamin D₃ (10 nM) also significantly induced the expression of DCLK1 at both gene and protein levels in the alveolar epithelial cell line, NCI-H441 cells (Fig. 4.9, Fig. 4.10). This result suggests that Vitamin D₃ has distinctive effects in increasing the expression of DCLK1 in alveolar epithelial cells.

At present, the effect of Vitamin D₃ on the expression of DCLK1 in alveolar epithelial cells is still barely reported. In the aspect of the impact of Vitamin D₃-induced DCLK1 expression in alveolar epithelial cells on the function of the alveolar epithelial barrier is even less elucidated. It is known that Vitamin D₃ inhibits the proliferation metastasis, and development of cancer cells, through its inhibition of the induction of epithelial-mesenchymal transition (EMT) [212, 213]. Whereas, DCLK1 is a biomarker of cancer stem cells and is a critical regulator for epithelial-mesenchymal transition (EMT), which contributes to the progression growth and metastasis of cancer/tumor [214].

However, according to the result of this study, it is inconsistent that Vitamin D₃ (10 nM) even significantly increased the expression and production of DCLK1 in NCI-H441 cells. Previous studies found that tuft or brush cells can be recognized through their expression of DCLK1[215]. Therefore, DCLK1 can also be a biomarker for the identification of tuft or brush cells [215].

Some studies revealed the beneficial effects of DCLK1, for self-renewal and tissue repair and regeneration for the injured tissue cells in response to pathogen-induced tissue inflammation, including intestine and lung [216, 217]. Vitamin D₃ is also demonstrated to have effects on the repair, regeneration, and healing of pathogen-damaged organ tissues. It has demonstrated that Vitamin D₃ up-regulates the proliferation of organ cells, especially the tuft or brush cells in response to inflammation, which helps to attenuate tissue injury and inspire the regeneration of damaged tissues [218, 219].

Regarding lung tissue, it has been found that Vitamin D₃ shows a positive effect on the proliferation of AT-II cells in response to LPS infection, which eliminates LPS-induced lung tissue damage and promotes lung tissue repair [220]. Therefore, according to the results of this study, it can implicate the significant effect of Vitamin D₃ on the increase of DCLK1 expression in alveolar epithelial cells is also a potential molecular mechanism of Vitamin D₃ in the facilitation of the repair and regeneration of alveolar epithelial cells for the preservation of the function of the alveolar epithelial barrier.

5.2 Protective Effect of Vitamin D₃ on the Alveolar-Capillary Barrier and the Relevant Mechanisms of LPS-induced Disruption of the Alveolar-Capillary Barrier

5.2.1 Vitamin D₃ maintains the barrier properties of the alveolar-capillary barrier after LPS treatment

From the results of this study, Vitamin D₃ (10 nM) significantly strengthened the barrier properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells and Calu-3 cells in Transwell® according to the TEER measurement test (Fig. 4.1). According to the ECIS test, Vitamin D₃ (10 nM) also improved the barrier properties of the OECs-modeled capillary microvascular endothelial barrier (Fig. 4.12). Moreover, Vitamin D₃ (10 nM) significantly improved the function properties of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® (fig. 4.11).

However, LPS (100 ng/mL, 10 μ g/mL) decreased both the barrier properties of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs and the barrier properties of OECs-modeled capillary microvascular endothelial barrier (Fig. 4.11, Fig. 4.12). Nevertheless, Vitamin D₃ (10 nM) still conserved the barrier properties of the alveolar-capillary barrier in the co-culture model at a higher level in the exposure to LPS (100 ng/mL, 1 μ g/mL, 10 μ g/mL) (Fig.4.11). Vitamin D₃ also maintained the barrier properties of the OECs-modeled capillary microvascular endothelial barrier in the challenge with LPS (100 ng/mL, 10 μ g/mL) treatment (Fig. 4.12).

From the immunofluorescence staining of the tight junction protein ZO-1 on NCI-H441 cells (Fig. 4.13), Vitamin D₃ (10 nM) strengthened the intensity of the fluorescence signal of ZO-1 on the cell membrane barrier of NCI-H441 cells. The treatment with the conditioned medium with LPS (100 ng/mL) damaged the NCI-H441 cells-modeled alveolar epithelial barrier and induced the loss of ZO-1 from the cell membrane barrier of NCI-H441 cells (Fig. 4.13). Nevertheless, Vitamin D₃ (10 nM) still significantly maintained the integrity and morphological features of the NCI-H441 cells-modeled alveolar epithelial barrier and maintained the integrity of ZO-1 on the cell membrane barrier of NCI-H441 cells (Fig. 4.13). Moreover, LPS (100 ng/mL) decreased the gene expression of the tight junction proteins, ZO-1, and Occludin in NCI-H441 cells in the co-culture model with OECs in Transwell® (Fig. 4.26). In addition, this study displayed that LPS (10 μ g/mL) decreased the gene expression of VE-Cadherin in OECs (Fig.4.14). Whereas, Vitamin D₃ (10 nM) still maintained the gene expression of VE-Cadherin in OECs in the exposure to LPS (10 μ g/mL) treatment (Fig. 4.14).

The decreased TEER and ECIS values and the impaired alveolar epithelial cell membrane structure with LPS treatment revealed that LPS causes damage to the pulmonary alveolar-capillary barrier, which increases the permeability and disrupts the functions of the alveolar-capillary barrier. These results also verified the significant effects of Vitamin D₃ on the improvement of the properties of the alveolar-capillary barrier and verified the positive

effects of Vitamin D₃ on the maintenance of the integrity and function properties of the alveolar-capillary barrier in challenge with LPS infection.

Therefore, the decreased barrier properties of the alveolar-capillary barrier of the cell culture model in this study with the LPS test can be attributed to the decreased expression of tight junction proteins in alveolar epithelial cells the loss of ZO-1 from the cell membrane barrier of alveolar epithelial cells and the decreased expression of VE-Cadherin in capillary microvascular endothelial cells in exposure to LPS.

Moreover, some studies also demonstrated that LPS induces the shedding and loss of the adherens junction protein, such as E-Cadherin from the lung epithelial cells, which also leads to the disruption and destabilization of tight junction proteins and further results in the disruption of lung epithelial barrier [221]. Some studies found that LPS induces the loss, disorganization, and reduced expression of the adherens junction proteins, such as VE-Cadherin from the cell membrane of lung endothelial cells [222, 223]. Therefore, these mechanisms can be the major factors, that contribute to the LPS-induced disruption of the alveolar-capillary barrier.

5.2.2 LPS disrupts the alveolar-capillary barrier by inducing lung inflammation

According to the results from this study, LPS (10 µg/mL) increased the expression of interleukin pro-inflammatory cytokines (IL-1 β, IL-6, and IL-8) in both NCI-H441 cells and OECs (Fig. 4.15 A, B). In particular, LPS (10 µg/mL) more significantly increased the gene expression of IL-1 β, IL-6, and IL-8 in OECs (Fig. 4.15 B). Nevertheless, Vitamin D₃ (10 nM) attenuated the LPS (10 µg/mL)-induced increased gene expression of IL-6 in NCI-H441 cells and attenuated the significantly increased gene expression of IL-1β, IL-6, and IL-8 in OECs, which were induced by LPS (10 µg/mL) treatment (Fig. 4.15 A, B).

Previous experiments from our laboratory group demonstrated that IL-6 significantly decreased the barrier properties of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell®, whereas, Vitamin D₃ still significantly maintained

the properties of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in exposure to IL-6 treatment [224]. These results indicate that LPS-induced significant expression of IL-6 is one of the factors that contribute to the LPS-induced destruction of the alveolar-capillary barrier, and Vitamin D₃ also maintains the function of the alveolar-capillary barrier through its anti-inflammation effect on the LPS-induced inflammation [224].

However, this study showed that Vitamin D₃ (10 nM) increased the gene expression of IL-1 β in NCI-H441 cells (Fig. 4.15 A). Interestingly LPS (10 μ g/mL) even significantly increased the gene expression of Vitamin D receptor (VDR) in OECs (Fig. 4.17).

Moreover, the gene expressions of Cathepsin S (CTSS) and intercellular adherent molecule-1 (ICAM-1) in OECs were significantly increased with LPS (10 μ g/mL) treatment (Fig. 4.18, Fig. 4.21 B). However, the gene expression of Cathepsin S in NCI-H441 cells was almost unaffected with LPS (10 μ g/mL) treatment (Fig. 4.18 A). These results suggest that vascular endothelial cells are more responsive than epithelial cells to demonstrate the inflammatory reaction in exposure to LPS infection.

It is reported that the increased expression and secretion of the proinflammatory cytokines (IL-1 β , IL-6, and IL-8) in tissue epithelial cells and endothelial cells is caused by the activation of the NF- κ B signaling pathway in the tissue cells in response to LPS [225]. It also has been demonstrated that LPS also increases the expression of ICAM-1 through Toll-like Receptor 4 (TLR4) transduction pathways [226]. Moreover, cytokines such as tumor necrosis factor- α (TNF- α), and IL-1 β are also declared to increase the expression of ICAM-1 in endothelial cells [227]. The up-regulation of interleukin pro-inflammatory cytokines and ICAM-1 in lung vascular endothelial cells in exposure to LPS contributes to LPS infection-induced lung inflammation and lung diseases [228].

The result showed that LPS significantly increased the expression of Cathepsin S in OECs (Fig. 4.18, B). It is known that Cathepsin S (CTSS) is expressed in several cell types including macrophages, neutrophils, smooth muscle cells, tissue epithelial cells, and endothelial cells [229, 230]. Previous studies revealed that several factors regulate the expression and

secretion of Cathepsin S. The pro-inflammatory cytokines, including L-1 β , tumor necrosis factor α (TNF- α), IL-6, IL-4, and IL-13 are known to induce the expression of Cathepsin S [93, 231].

It also has been demonstrated that Cathepsin S is also a relevant mediator of lung inflammation which induces lung tissue damage and lung disease [232]. Therefore, it suggests that the LPS-increased expression of Cathepsin S in pulmonary vascular endothelial cells also contributes to LPS-induced lung function disorder.

From this study, LPS even increased the gene expression of Vitamin D Receptor (VDR) in OECs (Fig. 4.17). Although Vitamin D₃ is known for its anti-inflammatory effect under pathogenic infection, it is still controversial in the studies with experimental models to demonstrate the exact function of Vitamin D₃ in lung disease development. Previous experimental studies verified that Vitamin D₃ treatment can be beneficial have no effect or even be harmful in the modulation of inflammation and lung disease development processes [233-235]. A study found that the knockout of VDR in immune cells splenocytes, and hematopoietic cells decreased the LPS infection-induced lung inflammation and lung diseases [236]. Therefore, according to this study, the increased expression of VDR in vascular endothelial cells can be also a potential molecular mechanism, which contributes to LPS-induced lung inflammation as well.

Vitamin D₃ (10 nM) decreased the LPS (10 μ g/mL)-induced gene expression of IL-6 in NCI-H441 cells and alleviated the LPS (10 μ g/mL) significantly stimulated gene expression of IL-1 β , IL-6, and IL-8 in OECs, (Fig. 4.15 A, B).

These results indicated the anti-inflammation effect of Vitamin D₃ in the alveolar-capillary barrier for the defense against LPS infection. The anti-inflammation effect of Vitamin D₃ has been demonstrated to be attributed to the Vitamin D₃-mediated interference and inhibition of LPS-triggered activation of the NF- κ B signaling pathway [135].

Interestingly, this study also demonstrated that Vitamin D₃ (10 nM) even showed an effect in increasing the gene expression of IL-1 β in NCI-H441 cells (Fig. 4.15 A). Although IL-1 β is known as a pro-inflammatory cytokine, IL-1 β is beneficial in improving the host resistance

capacity and survival against bacterial infection [237, 238]. A previous study also found that Vitamin D₃ elevates the expression of secretion of IL-1 β in pulmonary epithelial cells to regulate the innate immune system [239]. This mechanism is essential to stimulate the anti-microbial capacity of pulmonary epithelial cells for the defense against bacterial infection [238]. Therefore, the increased expression of IL-1 β in pulmonary alveolar epithelial cells with Vitamin D₃ supplementation also contributes to the effect of Vitamin D₃ on the improvement of lung immunity against pathogen infections.

5.2.3 LPS significantly induces the expression of SP-A in alveolar epithelial cells

From this study, LPS significantly induced the expression of Surfactant Protein-A (SP-A) in NCI-H441 cells (Fig. 4.16).

SP-A is known to be involved in the regulation of the immune system for the defense against pathogen infections. Previous studies demonstrated that LPS interacts with and alters the expression of surfactant proteins in alveolar epithelial type II cells (AT-II cells) [240, 241]. This study demonstrated the significant effect of LPS in increasing the gene expression of SP-A in alveolar epithelial cells (Fig. 4.16).

This result is also supported by previous studies. It also demonstrated that the bacterial endotoxin LPS induced the gene expression of SP-A, in AT-II cells, whereas the expression of SP-D in AT-II cells was not substantially affected by LPS [240, 242, 243]. However, LPS decreased the protein expression level of SP-A in AT-II cell line A549 cells [240, 242, 243].

LPS is reported to induce the expression of toll-like receptor 2 (TLR2) in AT-II cells [244]. Then, TLR2 mediates the activation of the gene transcription factor cJun and its translocation from the cytoplasm to nuclei through cJun N-terminal kinase-phosphorylation. This process further enhanced the gene expression of SP-A in AT-II cells in exposure to LPS [243]. However, on the contrary, it also has been found that LPS also decreases the expression of SP-B and SP-C in AT-II cells. As a result, it further leads to the dysfunction of SP-A

[244,245,249,]. This mechanism is also one of the factors that contribute to LPS-induced acute lung injury (ALI) [245, 246].

It has been found that SP-A which is also known as a lung collectin, suppresses LPS-induced lung inflammation, and reduces the bioactivity of LPS through its bindings and interactions with myeloid differentiation protein 2 (MD2), CD14, TLR2, and TLR4 in the LPS signaling pathway in response to the LPS infection [247-249]. Therefore, the significant increase of SP-A in AT-II cells in exposure to LPS can be an immunologic defense mechanism of AT-II cells in response to LPS infection.

5.2.4 Inhibition of Cathepsin S (CTSS) expression attenuates LPS-induced disruption of the alveolar-capillary barrier

Due to the significant increase in the gene expression of Cathepsin S (CTSS) in the capillary microvascular endothelial cells, OECs with LPS treatment (Fig. 4.18 B), Cathepsin S was further investigated as a target molecule to study the LPS-induced lung inflammation. This study applied the Cathepsin S (CTSS) specified inhibitor in the investigation.

Firstly, the effect of CTSS inhibition on the barrier properties of alveolar epithelial cells was tested with different concentrations of CTSS inhibitor (20 nM, 100 nM, 200 nM, 500 nM). According to the results from this study, CTSS inhibitor (20 nM, 100 nM, 200 nM, 500 nM) displayed the effect in increasing the barrier properties of NCI-H441 cells-modeled alveolar epithelial barrier (Fig. 4.19). The effect of the CTSS inhibitor on the improvement of the properties of NCI-H441 cells-modeled alveolar epithelial barrier showed a concentration-dependent manner. The higher the concentration of the CTSS inhibitor, the stronger the barrier properties of NCI-H441 cells (Fig. 4.19). In particular, the barrier properties of NCI-H441 cells significantly increased with CTSS inhibitor (100 nM, 200 nM, 500 nM) treatment (Fig. 4.19 B, C).

However, the properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in Transwell® were significantly decreased after the treatment with the conditioned medium contained with LPS (10 µg/mL) (Fig.4.19). CTSS inhibitor (20 nM, 100

nM, 200 nM, 500 nM) showed a slight effect to alleviate the LPS-caused decrease of the properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.19). Nevertheless, the CTSS inhibitor (100 nM) demonstrated a more effective effect in maintaining the properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in exposure to LPS in comparison with the CTSS inhibitor in other concentrations (Fig. 4.19).

CTSS inhibitor (100 nM) also showed a remarkable effect in improving the barrier properties of the alveolar-capillary barrier in the co-culture model of NCI-H441 cells and OECs in Transwell® (Fig. 4.20). Furthermore, CTSS inhibitor (100 nM) also demonstrated a significant effect on the maintenance of the properties of the co-culture model of the alveolar-capillary barrier even in the challenge with LPS (100 ng/mL, 10 µg/mL) treatment (Fig. 4.20). Previous studies, studies have demonstrated that Cathepsin S regulates the induction of the expression of pro-inflammatory factors [107].

Cathepsin S is also related to the pathogenesis of lung disease, especially chronic obstructive pulmonary disease (COPD) through its induction of cell apoptosis and cell fibrosis.[104]. Cathepsin S shows its elastolytic and collagenolytic activity in the neutral pH intracellular or extracellular microenvironment [250, 251]. It has been demonstrated that Cathepsin S has a significant effect in degrading the major structural components, such as elastin and collagen of the extracellular matrix (ECM) [96].

Cathepsin S is also found to reveal the effect in shedding and releasing the end domains of intercellular adherens junction proteins, such as E-Cadherin, VE-Cadherin, and membrane receptors [252]. Therefore, the degradation effect of Cathepsin S on the protein components of the structure of pulmonary cells contributes to lung tissue disorder. Moreover, previous studies found that in the Cathepsin S signaling pathway, Cathepsin S induces the transgenic overexpressing of β -epithelial Na^+ (sodium) channel ($\beta\text{ENaC-Tg}$) via its mediation of the expression and activity of a protease-activated receptor 2 (PAR2) in the tissue cells, such as lung tissue cells [251, 253]. These molecular mechanisms result in Cathepsin S-caused cystic

fibrosis of pulmonary cells and lung inflammation which further lead to lung damage and pathogenesis of severe lung diseases [254].

5.2.5 Inhibition of Cathepsin S expression decreased the expression of other inflammation-associated molecules in endothelial cells

According to the results of this study, Cathepsin S (CTSS) inhibitor (100 nM) showed the effect in diminishing the LPS (100 ng/mL, 10 µg/mL)-induced significant expression of interleukin pro-inflammatory cytokines, (IL-1 β , IL-6, and IL-8), and intercellular adhesion molecules-1 (ICAM-1) in the capillary microvascular endothelial cells OECs (Fig. 4.21 B, Fig. 4.22).

Previous studies from other groups found that Cathepsin S interferes and increases the expression of pro-inflammatory cytokines, including IL-6, IL-8, IL-1 β , Tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) in the patients who suffered from eye tissue inflammation and injury partially through its mediation to protease-activated receptor 2 (PAR 2) [255, 256]. It also has been demonstrated that the inhibition of Cathepsin S expression suppresses PAR 2 expression and attenuates the pro-inflammatory cytokines-induced inflammation and tissue disruption [255].

Moreover, this study also demonstrated that the inhibition of Cathepsin S expression attenuated the LPS-significantly-induced gene expression of ICAM-1 in capillary microvascular endothelial cells OECs (Fig. 4.21 B). Therefore, these results can imply that the inhibition of Cathepsin S expression can be a potentially effective therapeutic strategy to treat pathogens eg. bacterial endotoxin (LPS)-induced lung diseases, through its significant effects on the improvement of the function properties of the alveolar-capillary barrier, and its effects on the diminution of the pathogen-induced excessive inflammation.

5.3 Role and Effects of Cathelicidin, LL-37, on the Function of the Alveolar-Capillary Barrier in the Defense against LPS Infection

5.3.1 LL-37 increased the properties of the alveolar-capillary barrier

This study demonstrated that Vitamin D₃ (10 nM) significantly increased the expression of the antimicrobial peptide, Cathelicidin LL-37, in all of the alveolar epithelial cell lines in the experiments (NCI-H441 cells, Calu-3 cells, and A549 cells) at both gene and protein levels (Fig. 4.5, Fig. 4.6).

LL-37 is known for its tissue wound healing and immune defense effects in the conflict against the challenge of invading pathogens. The effects and molecular mechanisms of LL-37 on the integrity and function maintenance of the alveolar epithelial barrier were further investigated in this study.

According to the study results, LL-37 (0.1 ng/mL, 1 ng/mL, 10 ng/mL) treatment significantly increased the properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in a concentration-dependent manner, which was demonstrated through the elevated TEER values from the mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.23). LL-37 (10 ng/mL) showed the most significant effect in improving the properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.23).

Furthermore, LL-37 (10 ng/mL) not only significantly improved the properties of the alveolar-capillary barrier in the co-culture model of NC-IH441 cells and OECs in Transwell® but also effectively maintained the properties of alveolar-capillary barrier in the co-culture model at a higher level even in exposure to LPS (100 ng/mL, 10 µg/mL) treatment (Fig. 4.24). From the gene expression analysis of the intercellular junction proteins in NCI-H441 cells, LL-37 (10 ng/mL) also increased the gene expression of intercellular junction proteins (ZO-1, Occludin, Claudin-5, and E-Cadherin) in NCI-H441 cells (Fig. 4.25 A). Whereas, LPS (100 ng/mL, 10 µg/mL) decreased the gene expression of the tight junction proteins, ZO-1 and Occludin (Fig. 4.26). Nevertheless, the gene expressions of the tight junction proteins

were even increased at a higher level in NCI-H441 cells with LL-37 (10 ng/mL) plus LPS (100 ng/mL, 10 µg/mL) treatment (Fig. 4.26).

It has been demonstrated that, besides the effect of LL-37 on the regulation of immune defense, LL-37 also has a function in the maintenance of the function of the pulmonary epithelial barrier.

Previous studies found that LL-37 selectively increased the expression of tight junction-related proteins including Occludin and a group of junction proteins in the Claudin protein family, which is beneficial for LL-37 to up-regulate the barrier properties of human epidermal keratinocytes [257]. LL-37 has also been demonstrated to have the effect of improving the stiffness of pulmonary epithelial cells through its up-regulation of F-actin in the cortical region of keratinocytes of pulmonary epithelial cells, meanwhile decreasing the barrier permeability of pulmonary epithelial cells for the prevention against bacterial pathogen invasion [258].

Moreover, previous studies, revealed that LL-37 maintains and up-regulates the functions of the airway epithelial barrier, and conserves the expression and constitution of the components of tight junction proteins (ZO-1, Occludin, and a group of Claudins) and adherens junction protein (E-Cadherin) in the airway epithelial barrier [97]. Furthermore, a study from other groups found that LL-37 also attenuated the significantly reduced properties of the airway epithelial barrier and maintained the expression of intercellular junction proteins in the pulmonary epithelial barrier in exposure to cigarette smoke extract (CSE) [259]. This mechanism indicates the beneficial role of LL-37 in the prevention of the pathogenesis of severe lung diseases, including, chronic obstructive pulmonary disease (COPD) and asthma during the defense against pathogens [260].

These findings are supportive of the results of this study about the significant positive effect of LL-37 on the improvement of the function of the alveolar-capillary barrier through its effect in increasing and maintaining the expression of intercellular tight junction and adherens junction proteins, for the defense against LPS infection

5.3.2 Role of LL-37 in immune modulation of the pulmonary alveolar epithelial cells

Regarding the issue of the role of LL-37 in immune regulation, this study demonstrated that LPS (100 ng/mL, 10 µg/ mL) and LL-37(10 ng/mL) increased the gene expression of the interleukin pro-inflammatory cytokines (IL-1 β, IL-6, and IL-8) and Surfactant protein A (SP-A) in the alveolar epithelial cell line, NCI-H441 cells (Fig. 4.27), and the gene expressions of IL-1β, IL-6, IL-8, and SP-A showed even higher levels in NCI-H441 cells with LPS (100 ng/mL, 10 µg/mL) plus LL-37 (10 ng/mL) treatment (Fig. 4.27). Interestingly, this study showed that LL-37 increased the gene expression of the group of Protein Kinase C isozymes (PKCA, PKCB, and PKCH) in NCI-H441 cells (Fig. 4.25 B).

It has been shown that LL-37 induces the increase of Ca²⁺ levels in the intracellular plasmid and up-regulates the expression and activation of PKC isozymes through the stimulation of LL-37 binding proteins [261]. This molecular process increases the expression and secretion of pro-inflammatory cytokines (IL-1 β, IL-6, and IL-8), and chemokines in monocyte tissue epithelial cells, and nerve cells [262, 263]. The expressions of pro-inflammatory cytokines (IL-1 β, IL-6, and IL-8) were also reported to be induced in the cells in exposure to LPS [263, 264]. Therefore, these processes imply that LL-37 can even enhance the inflammatory response of tissue cells during the invasion of pathogens. Therefore, these findings are also supportive of explaining the results of this study.

This study also found that LL-37 (10 ng/mL) even enhanced the gene expression of SP-A in NCI-H441 cells by exposure to LPS (100 ng/mL, 10 µg/mL) (Fig. 4.27). A prior study found that LPS can promote the secretion of interleukins including, IL-6 and IL-8 in NCI-H441 cells and OECs [224]. It also has been found that IL-6 and IL-8 also promote the expression and secretion of surfactant proteins including, SP-A, SP-B, and SP-C of the fetal lung, this process promotes the lung maturation and healing after IL-6 and IL-8-caused lung damage [265]. Therefore, it suggests that the increased expressions of IL-6, and IL-8 which are induced by both LL-37 and LPS led to even higher expression of SP-A in NCI-H441 cells with LL-37 plus LPS treatment, in comparison with LPS treatment alone (Fig. 4.27).

Therefore, this study suggests that the increased expressions of the interleukins pro-inflammatory cytokines are beneficial for LL-37 to improve the immune response of tissue cells for the defense against pathogen infection through the recruitment of immune cells. Moreover, it also implies that the increased expression of SP-A in alveolar epithelial cells is also beneficial for LL-37 to improve its antimicrobial and tissue healing capacities for the defense against pathogen invasion, and facilitation of lung tissue healing after pathogen-induced lung injury through the cooperation with SP-A.

5.4 Role and Mechanisms of Doublecortin-Like Kinase 1 (DCLK1) in the Function Maintenance of the Alveolar Epithelial Barrier

5.4.1 The appropriate usage of DCLK1 inhibitor significantly increased the function of the alveolar epithelial barrier

This study found that Vitamin D₃ (10 nM) significantly increased the gene expression of doublecortin-like kinase 1 (DCLK1) in alveolar epithelial cell lines (NCI-H441 cells and 549 cells (Fig. 4.10). Vitamin D₃ (10 nM) also significantly increased the protein expression of DCLK1 in NCI-H441 cells (Fig. 4.10). Therefore, the role of DCLK1 in the function and function maintenance of the alveolar epithelial barrier was further investigated through the usage with DCLK1 specified inhibitor LRRK2-IN-1(Leucine-rich repeat kinase 2 inhibitor 1) in this study.

According to the results, the DCLK1 inhibitor, LRRK2-IN-1 (500 nM,), showed a significant effect in decreasing the expression of DCLK1 in NCI-H441 cells at both gene and protein levels(Fig. 4.29, Fig. 4.30). Furthermore, the higher concentration of DCLK1 inhibitor, LRRK2-IN-1 (5 μ M), showed a more significant effect in suppressing the gene expression of DCLK1 in NCI-H441 cells in comparison with the lower concentration of DCLK1 inhibitor (500 nM) (Fig. 4.36). This result suggested that the higher the concentration level

of the DCLK1 inhibitor, LRRK2-IN-1, can result in the lower expression level of DCLK1 in alveolar epithelial cells.

From the results of TEER measurement in the mono-culture model of NCI-H441 cells, Vitamin D₃ (10 nM) and DCLK1 inhibitor LRRK2-IN-1 (500 nM) significantly increased the properties (TEER values) of the NCI-H441 cells-modeled alveolar epithelial barrier (Fig. 4.28). In particular, the DCLK1 inhibitor, LRRK2-IN-1 (500 nM) even demonstrated a significant effect in strengthening the positive effect of Vitamin D₃ (10 nM) in increasing the TEER values of the alveolar epithelial barrier in the in vitro mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.28). However, the effect of the DCLK1 inhibitor, LRRK2-IN-1, on the properties of the alveolar epithelial barrier demonstrated a concentration-dependent manner. It is interesting that the higher concentration of DCLK1 inhibitor, LRRK2-IN-1 (5 µM), on the contrary even significantly decreased the properties of the alveolar epithelial barrier (TEER values) in the mono-culture model of NCI-H441 cells in Transwell® (Fig. 4.37).

It has been found that leucine-rich repeat kinase 2 (LRRK2) is related to the pathogenesis of Parkinson's disease, which results from the abnormal mutation of LRRK2 [96]. Therefore the usage of the LRRK2 inhibitor can be a pharmaceutical option for Parkinson's disease treatment [266].

However, previous studies demonstrated that severe LRRK2 deficiency and loss of LRRK2 from tissue cells can lead to the abnormality of the phenotype and morphology of various tissues, including the kidney, lung, and liver [267]. It was found that the rat lung tissue with the knock-out of LRRK2 expression demonstrated the increased accumulation of lamellar bodies in the larger area of alveolar epithelial type II cells in comparison with wild type, which was attributed to the higher degradation of the components of alveolar epithelial cells in the LRRK2 knock-out rat [267]. This process can result in a higher risk of lung disorder and lung tissue function deficit [267, 268].

Therefore, it is also crucial for the pharmaceutical development of LRRK2 inhibitors to control the inhibition of LRRK2 at a moderate and safe level [268]. Therefore, these findings

are helpful in explaining that the properties of the alveolar epithelial barrier in the mono-culture model of NCI-H441 cells were even significantly decreased with the usage of a higher concentration of DCLK1 inhibitor LRRK2-IN-1 (5 μ M).

5.4.2 DCLK1 inhibitor, LRRK2-IN-1, up-regulated the expression of intercellular junction proteins in alveolar epithelial cells

DCLK1 is known as a biomarker of cancer cells. It has been found that the abnormally high expression of DCLK1 can lead to a reduced protein level of intercellular adherens junction proteins, such as E-Cadherin, that can further promote the migration and metastasis of cancer cells [169]. Therefore, it implies that the inhibition of DCLK1 expression can improve the barrier properties of alveolar epithelial cells. Therefore, these findings are helpful in explaining the results of this study that demonstrated that the properties of the alveolar epithelial barrier (TEER values) in the in vitro mono-culture model of NCI-H441 cells were also significantly increased with DCLK1 inhibitor, LRRK2-IN-1 (500 nM), treatment, and the TEER values of the mono-culture model of NCI-H441 cells with Vitamin D₃ (10 nM) plus LRRK2-IN-1 (500 nM) treatment were even higher than the TEER values of the mono-culture model of NCI-H441 cells in with Vitamin D₃ (10 nM) treatment alone (Fig. 4.28).

From the results of gene expression analysis, Vitamin D₃ (10 nM) and LRRK2-IN-1 (500 nM) increased the gene expression of E-Cadherin in NCI-H441 cells (Fig. 4.31). Whereas, the gene expression of ZO-1 and Occludin showed a decreasing trend in NCI-H441 cells with Vitamin D₃ (10 nM) treatment (Fig. 4.31). Nevertheless, the positive effect of the inhibition of DCLK1 expression on the expression of junction proteins was also verified through the results of this study, that the DCLK1 inhibitor, LRRK2-IN-1 (500 nM) generally increased the gene expression of ZO-1, Occludin, Claudin-5 in NCI-H441 cells (Fig. 4.31). In particular, the DCLK1 inhibitor, LRRK2-IN-1 (500 nM), strengthened the effect of Vitamin D₃ (10 nM) in increasing the gene expression level of E-Cadherin in NCI-H441 cells (Fig. 4.31).

The effects of DCLK1 inhibitor LRRK2-IN-1 on the gene expression of the intercellular junction proteins (ZO-1, Occludin, Claudin-5, E-Cadherin) in NCI-H441 cells also

demonstrated a concentration-dependent manner. The higher concentration of DCLK1 inhibitor, LRRK2-IN-1 (5 μ M), demonstrated the effect in increasing the gene expression of the intercellular junction proteins (ZO-1, Occludin, Claudin-5, E-Cadherin) in NCI-H441 cells to an even significantly higher level than the treatment with the lower concentration of DCLK1 inhibitor LRRK2-IN-1 (500 nM) (Fig. 4.38).

According to the protein expression analysis, both Vitamin D₃ (10 nM) and LRRK2-IN-1 (500 nM) showed the effects in increasing the expression of ZO-1, Occludin, and E-Cadherin in NCI-H441 cells at the protein levels (Fig. 4.32, Fig. 4.33, Fig. 4.34). In particular, the Vitamin D₃ (10 nM)- increased protein expression level of E-Cadherin was further increased with the extra treatment with DCLK1 inhibitor LRRK2-IN-1 (500 nM) (Fig. 4.34).

Previous studies found that DCLK1, as a marker of tumor stem cells, and tuft cells (or brush cells), induces the activation of the epithelial-mesenchymal transcription (EMT) process [156]. The activation of the EMT pathway results in the up-regulation of several gene transcriptional repressor factors [156], that bind to the promoters of target genes [269], including the genes that encode the junction proteins, such as E-Cadherin, and ZO-1 [268, 270, 271]. As a result, it will lead to the loss of the junction proteins, especially E-Cadherin, breakdown of the intercellular junction of epithelial cells, and loss of cell polarity [268, 270]. Therefore, these findings are supportive to explain the results of this study that the inhibition of DCLK1 expression in epithelial cells even strengthened the effect of Vitamin D₃ in increasing the expression of E-Cadherin at even higher levels than Vitamin D₃ treatment alone.

However, a study also found that the tissue epithelial cells such as the intestine epithelial tuft cells with a lack of DCLK1 failed to progress self-renewing and reconstitution after radiation-induced intestine injury or damage. Moreover, the epithelial cells with the knock-out of DCLK1 expression demonstrated a significantly decreased protein expression of the tight junction proteins, including ZO-1, ZO-2, claudins, and the adherens junction protein, E-cadherin in intestinal epithelial cells [272]. Therefore, it can indicate that the expression of DCLK1 is also required to maintain the homeostasis of the constitution of the intercellular junction proteins in tissue brush epithelial cells, such as AT-II cells.

In this study, the gene expression of ZO-1 and Occludin in NCI-H441 cells was to some degree decreased with Vitamin D₃ (10 nM) treatment. Nevertheless, it is still in consensus with previous studies that this study also verified the positive effect of Vitamin D₃ on the improvement of the expression of ZO-1, Occludin, and E-Cadherin, in alveolar epithelial cells at the protein levels (Fig. 4.30, Fig. 4.31, Fig. 4.32). It has been found that Vitamin D₃ induces the protein expression levels of intercellular tight junction proteins ZO-1, Occludin, and adherent junction protein E-Cadherin, through the activation of Vitamin D receptor (VDR), and the interaction of Vitamin D₃ and VDR in the Vitamin D/VDR signaling pathway [272, 273]. whereas, the knock-out of VDR expression disrupts the expression and production of the junction proteins. As a result, it makes Vitamin D₃ fail to maintain the barrier integrity and decreases the barrier properties of epithelial cells [274].

According to the former text of the discussion, the high expression of DCLK1 in tuft epithelial cells causes the loss of E-Cadherin and other junction proteins [272]. Therefore, it implies that the Vitamin D₃-induced expression of DCLK1 attenuates the effect of Vitamin D₃ in the improvement of expression of junction proteins, in particular E-Cadherin in alveolar epithelial cells. Therefore, the inhibition of DCLK1 expression with the usage of the DCLK1 inhibitor, LRRK2-IN-1, is beneficial to increase the expression of junction proteins and strengthen the effect of Vitamin D₃ on the improvement of the properties of the alveolar epithelial barrier to higher levels.

However, the implementation with the high concentration of DCLK1 inhibitor LRRK2-IN-1, and excessive suppression of DCLK1 expression in epithelial cells is adverse for the integrity and function of the tissue epithelial barrier, including the alveolar epithelial barrier. Even though it showed a more significant effect on increasing the gene expression of the junction proteins (Fig. 4.38). It is assumed that. the even more significant effect of the higher degree of the inhibition of DCLK1 expression in increasing the gene expression of intercellular junction proteins in alveolar epithelial cells is for the rescue of the significant loss of the intercellular junction proteins in the alveolar epithelial barrier.

Therefore, in a word, the moderate inhibition of DCLK1 expression and appropriate usage of DCLK1 specified inhibitor, LRRK2-IN-1, are beneficial for the improvement of the expression of intercellular junction proteins in alveolar epithelial cells, which further strengthens the significant effect of Vitamin D₃ on the improvement of the function of the alveolar epithelial barrier.

6 Conclusion and Outlook

Conclusion:

1. This study verified the positive effects of Vitamin D₃ improving the properties of the alveolar epithelial barrier, capillary microvascular endothelial barrier, and the function of the alveolar-capillary barrier. Vitamin D₃ is also capable of maintaining the homeostasis of the alveolar-capillary barrier to prevent bacterial endotoxin LPS-induced disruption of the alveolar alveolar-capillary barrier. These results revealed that Vitamin D₃ is required for the maintenance of lung functions and lung health against pathogen infection.

2. Vitamin D₃ exerts its function through the Vitamin D/ Vitamin D Receptor (VDR) signaling pathway, which up-regulates the protein expression and integrity of the intercellular junction proteins including, ZO-1, Occludin, and E-Cadherin in alveolar epithelial cells, and improves the function of the alveolar epithelial barrier. Vitamin D₃ also maintains the function properties of the alveolar epithelial barrier for the defense against LPS invasion through its effect on the maintenance of tight junction proteins, such as ZO-1 in alveolar epithelial cells. Vitamin D₃ also conserves the properties of the capillary microvascular endothelial barrier against LPS challenge through its effect on the maintenance of the expression of adherens junction protein, VE-Cadherin in vascular endothelial cells.

3. The effect of Vitamin D₃ on the reduction of the gene expression of PKCA, PKCB, PKCH, Caveolin-1, and EGF to a moderate level in alveolar epithelial cells can be the potential molecular mechanisms of Vitamin D₃ for the exertion of its effects on the function maintenance of the alveolar epithelial barrier, and for the prevention of the inflammation-induced lung injury, and prevention of the pathogenesis of lung cancer. Therefore, it can indicate that the effects of Vitamin D₃ on the regulation of moderate expressions of PKCA, PKCB, PKCH, Caveolin-1, and EGF in alveolar epithelial cells are also the potentially involved molecular mechanisms of Vitamin D₃ in lung health maintenance.

4. LPS significantly increased the gene expression of IL-1 β , IL-6, IL-8, Cathepsin S (CTSS), and intercellular adhesion molecule 1 (ICAM-1) in OECs. LPS also mildly increased the gene expression of IL-1 β and IL-6 in NCI-H441 cells. These results infer that vascular endothelial cells are more reactive to LPS than alveolar epithelial cells for the stimulation of inflammation response, which results in the disruption of the alveolar-capillary barrier.

5. Vitamin D₃ demonstrated its anti-inflammation effects in the alveolar-capillary barrier through its attenuation effect on the LPS-induced increased expression of IL-6 in alveolar epithelial cells and the LPS (10 μ g/mL)-induced significant increased expression of IL-1 β , IL-6, and IL-8 in capillary microvascular endothelial cells.

6. Inhibition of Cathepsin S (CTSS) expression in alveolar epithelial cells and capillary microvascular endothelial cells is a potential therapeutic strategy to reduce LPS-induced inflammation response and improve the function of the alveolar-capillary barrier, which is beneficial to protect the alveolar-capillary barrier to prevent LPS-induced dysfunction of the alveolar-capillary barrier.

7. Vitamin D₃ significantly decreases the gene expression of surfactant protein A (SP-A) in alveolar epithelial cells. Nevertheless, Vitamin D₃ increases the synthesis of SP-A in alveolar epithelial cells. The improved synthesis of SP-A in alveolar epithelial cells indicates the positive effect of Vitamin D₃ on the improvement of lung immunity and the function maintenance of the alveolar epithelial barrier. The LPS-induced significant expression of SP-A in alveolar epithelial cells suggests the defense mechanism of alveolar epithelial cells for reducing the malignant toxicity of LPS to prevent serious lung diseases from LPS infection.

8. This study demonstrated that LL-37 significantly improved the properties of the alveolar epithelial barrier and further significantly improved the properties of the alveolar-capillary barrier. Moreover, LL-37 has also demonstrated a remarkable effect on the maintenance of the properties of the alveolar-capillary barrier in exposure to LPS challenge. Therefore, it implies that the Vitamin D₃-induced significant expression of LL-37 in alveolar epithelial

cells is also one of the molecular mechanisms of Vitamin D₃ in the improvement and maintenance of the function of the alveolar epithelial barrier.

9. Vitamin D₃ has a significant effect on the increase of the expression of Doublecortin-like kinase 1 (DCLK1) in alveolar epithelial cells. The increased expression of DCLK1 in alveolar epithelial cells is a potential molecular mechanism of Vitamin D₃ for the healing and repair of the damaged lung tissue caused by pathogen infection. The usage of DCLK1 inhibitor LRRK2-IN-1 (Leucine-rich repeat kinase 2 inhibitor 1) LRRK2-IN-1, with appropriate dosage, can strengthen the positive effects of Vitamin D₃ on the improvement of the function of the alveolar epithelial barrier, due to its effect in increasing the expression of the intercellular junction proteins, including ZO-1, Occludin, Claudin-5, and E-Cadherin in alveolar epithelial cells. The inhibition of Vitamin D₃-induced expression of DCLK1, with DCLK1 inhibitor, LRRK2-IN-1 has an effect in enhancing the expression of E-Cadherin in alveolar epithelial cells. The control for the dosage of DCLK1 inhibitor, LRRK2-IN-1, is important to demonstrate the beneficial effect of the inhibition of DCLK1 expression on the improvement of the function of the alveolar epithelial barrier.

In conclusion, this study verified that Vitamin D₃ has a significant effect on the improvement and maintenance of the function of the alveolar-capillary barrier, especially the alveolar epithelial barrier, for the defense against LPS infection. Vitamin D₃ reveals its positive effects on the increase of the function of the alveolar-capillary barrier through its mediated molecular mechanisms, including i) its upregulation of the protein expression and integrity of intercellular junction proteins including ZO-1, Occludin, and E-Cadherin; in alveolar epithelial cells; ii) its significant effect on the increase of the expression of the antimicrobial peptide Cathelicidin, LL-37 in alveolar epithelial cells; iii) its moderate suppressive effects on the gene expression of Protein Kinase C isozymes (PKCA, PKCB, PKCH), EGF, and Caveolin-1 in alveolar epithelial cells. Vitamin D₃ also has the effects of anti-pathogen-induced excessive inflammation response of the alveolar-capillary barrier via its effect in attenuating the pathogen-induced significant expression of the pro-inflammatory cytokines

including IL-1 β , IL-6, and IL-8, to avoid pathogen-induced damage of the alveolar-capillary barrier. The moderate inhibition of Vitamin D₃-induced significant expression of DCLK1 in alveolar epithelial cells with DCLK1 specified inhibitor, LRRK2-IN-1, has a positive effect in enhancing the expression of E-Cadherin in alveolar epithelial cells, which further strengthens the positive effects of Vitamin D₃ on the improvement of the function of the alveolar epithelial barrier.

Outlook:

This study used the in vitro co-culture model of alveolar epithelial cells and capillary microvascular endothelial cells in Transwell®. Further studies can implement the 3-D co-cell culture model with a curved surface for the cell culture of alveolar epithelial cells, and construct the air-liquid interface (ALI) cell culture conditions to make the in vitro cell culture more closely resemble the alveolar-capillary barrier in vivo. Because macrophages are also the essential immune cells in the alveolar air space, it is also advisable to include the immune cell macrophages in the in vitro cell culture model. This study only applied Vitamin D₃ with the concentration (10 nM) in the investigation. Further studies should investigate the effects of Vitamin D₃ on the function maintenance of the alveolar-capillary barrier with different concentrations of Vitamin D₃, which can be helpful for the selection of the optimal dosage of Vitamin D₃ in the therapeutic application for lung disease treatment. The results from this study indicate that the supplement with Vitamin D₃ is a promising strategy to deal with lung infection diseases from other pathogens including viruses, such as the COVID-19 epidemic. This promising strategy still needs to be investigated and verified in future studies.

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Appendix

Academic Conference Presentation

03/12/2015 → Junyu Xiong. Poster Presentation The protective effect of vitamin D₃ on the alveolar epithelial barrier and its anti-inflammatory mechanism. performed at Studierendentagung zu den Life Sciences in Kiel 2015. Life Science Nord. Kiel, Germany

28/10/2016 → Junyu Xiong. Selected presentation: Molecular effects of vitamin D₃ on the alveolar-capillary barrier during infection. Deutscher Kongress für Orthopädie und Unfallchirurgie (DKOU), Berlin, Germany

26/03/2018 → Junyu Xiong. Poster presentation: Model systems to assess site or cell-specific reactions in the alveolar region of the lung during inflammation and infection. International Symposium Precision Medicine in Chronic Inflammation of the Cluster of Excellence Inflammation at Interfaces, Hamburg, Germany

23/10/2018 → Junyu Xiong. Poster presentation: 1, 25-dihydroxy Vitamin D₃ induces Doublecortin-Like Kinase 1 Expression in Alveolar Epithelial Cells Enhancing the Barrier Resistance from Injury. Deutscher Kongress für Orthopädie und Unfallchirurgie (DKOU), Berlin, Germany

29/05/2019 → Junyu Xiong. Poster presentation: Cell-Specific Effects and Mechanisms Mediated by 1, 25-hydroxy Vitamin D₃ in the Protection of Alveolar-Capillary Barrier. 21st Transporter and Barrier Meeting. Bad Herrenalb, Germany

Academic Publications

Xiong Junyu; Kaleja Patrick; Ückert Larissa; Nezaratizadeh Niloufar; Krantz Stefanie; Friedrich Krause Martin; Fitschen-Oestern Stefanie; Seekamp Andreas; Cassidy Liam; Tholey Andreas; Fuchs Sabine: Alveolar–Capillary Barrier Protection In Vitro: Lung Cell Type-Specific Effects and Molecular Mechanisms Induced by 1 α , 25-Dihydroxyvitamin D3. In: International Journal of Molecular Science 24,7298 (2023)

Wang Fanlu, Xiao Yuejun, Neupane Sandesh, Helle Ptak Signe, Römer Ramona, Xiong Junyu, Ohmes Julia, Seekamp Andreas, Fretté Xavier, Alban Susanne, and Fuchs Sabine: Influence of Fucoidan Extracts from Different Fucus Species on Adult Stem Cells and Molecular Mediators in In Vitro Models for Bone Formation and Vascularization. In: Marine Drugs 19, 194 (2021)

Acknowledgement

I have benefited from a lot of direct and indirect help for my writing of this Ph.D. thesis. Here, I would like to express my gratitude for all of the help I have obtained. First of all, I would like to express my sincere thankfulness to my supervisor, Professor Sabine Fuchs, for her great kindness in accepting me and providing me the opportunity to finish my master's thesis and perform my Ph.D. study. I am also thankful to my supervisor for her provision of this interesting meaningful subject in my study. My supervisor gave me patient and helpful academic guidance in the explanation of my project and the experiment instruction of my study, which helped me to more profoundly understand and know this project of my Ph.D. study. My supervisor gave me the greatest support and help for my Ph.D. study. I also would like to give my sincere gratitude to Professor Thomas Roeder for his kindness and interest in serving as my second supervisor in my Ph.D. study.

I am also thankful to the technicians of our laboratory, Anna-Rose Nissen and Dijana Pavleska, who gave me a lot of help, support, and guidance for the performance of my experiments. I also very much appreciate my colleagues, Fanlu Wang, Julia Ohmes, and Yuejun Xiao in our laboratory, for their great cooperation, support, and help in my research work, which also make our laboratory filled with a pleasant and comfortable working atmosphere. I would like to express my deep thankfulness to the University of Kiel [Christian Albrecht Universität zu Kiel (CAU, Kiel)] and the University Hospital Schleswig-Holstein Campus Kiel [Universitätsklinikum Schleswig Holstein (UKSH) Campus Kiel] for their provision of the precious opportunity and platform for me to perform and finish my Ph.D. study.

Lastly, I would like to express my deepest gratitude to my parents. My parents have provided me with an adequate guarantee for my education from schools to universities. My parents also have given me a warm and comfortable familial atmosphere from my childhood. My parents also support me in studying biology, which is my most interested subject. The infinite and no ceasing loving help and support from my parents accompanied me all the time, which greatly encouraged me to move forward to arrive at my present achievement. In a word, I am deeply grateful in my heart to all the persons who have given me help and support to surmount the difficulties and hardships and helped me accomplish the writing of my Ph.D. thesis and my Ph.D. study.

Declaration and Erklärung

Declaration

I Junyu Xiong hereby confirm that my submitted dissertation was designed and exclusively accomplished by myself with the guidance of my supervisors of my Ph.D. study. This dissertation is accomplished only with the sources of the aids listed in the literature references of this dissertation. All of the results in my doctoral dissertation were collected from the experiments of my doctoral study on this topic, which were implemented by my work. My doctoral dissertation is only submitted to the mathematic-natural science faculty of the University of Kiel. My dissertation has never been submitted to other institutions for the graduation of my Ph.D. study. The thesis has not been published or submitted for publication. This thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation. No academic degree has been withdrawn from me.

Kiel, 2024

Junyu Xiong

Erklärung

Ich Junyu Xiong bestätigt hiermit, dass meine eingereichte Dissertation von mir selbst entworfen und ausschließlich unter Anleitung meiner Doktorbetreuerin angefertigt wurde. Diese Dissertation wurde nur mit den in den Literaturhinweisen dieser Dissertation aufgeführten Quellen der Hilfsmittel durchgeführt. Alle Ergebnisse in meiner Dissertation stammen aus den Experimenten meiner Promotionsstudie zu diesem Thema, die von mir durchgeführt wurden. Meine Dissertation wird nur an der mathematisch-naturwissenschaftlichen Fakultät der Universität Kiel eingereicht. Meine Dissertation wurde nie bei anderen Institutionen zur Graduierung meines Promotionsstudiums eingereicht. Die Dissertation ist nicht veröffentlicht oder zur Veröffentlichung eingereicht worden. Die Dissertation ist nach den Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt worden. Mir ist kein akademischer Grad entzogen worden.

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