Cell Biol Toxicol (2022) 38:427–450
https://doi.org/10.1007/s10565-021-09629-6

ORIGINAL ARTICLE

Vitamin D_3 decreases TNF-α-induced inflammation in lung epithelial cells through a reduction in mitochondrial fission and mitophagy

Yu-Chen Chen · Hsin-Ching Sung · Tzu-Yi Chuang · Tsai-Chun Lai · Tzu-Lin Lee · Chiang-Wen Lee · I.-Ta Lee · Yuh-Lien Chen

Received: 8 February 2021 / Accepted: 24 June 2021 / Published online: 13 July 2021

Abstract Previous work has shown an association between vitamin D_3 deficiency and an increased risk for acquiring various inflammatory diseases. Vitamin D_3 can reduce morbidity and mortality in these patients via different mechanisms. Lung inflammation is an important event in the initiation and development of respiratory disorders. However, the anti-inflammatory effects of vitamin D_3 and the underlying mechanisms remained to be determined. The purpose of this study was to examine the effects and mechanisms of action of vitamin D_3 (Vit. D) on the expression of intercellular adhesion molecule-1 (ICAM-1) in vitro and in vivo with or without tumor necrosis factor α (TNF-α) treatment. Pretreatment with Vit. D reduced the expression of ICAM-1 and leukocyte adhesion in TNF-α-treated A549 cells. TNF-α increased the accumulation of mitochondrial reactive oxygen species (mtROS), while Vit. D reduced this effect. Pretreatment with Vit. D attenuated TNF-α-induced mitochondrial fission, as shown

Y.-C. Chen · T.-C. Lai · T.-L. Lee · Y.-L. Chen (✉)
Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, No. 1, Sec 1, Ren-Ai Road, Taipei, Taiwan
e-mail: ylchenv@ntu.edu.tw

H.-C. Sung (✉)
Department of Anatomy, Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, No. 259, Wenhua 1st Rd., Guishan Dist., Taoyuan City, Taiwan
e-mail: hcs@mail.cgu.edu.tw

H.-C. Sung
Department of Dermatology, Aesthetic Medical Center, Chang Gung Memorial Hospital, Linkou, Taoyuan, Taiwan

T.-Y. Chuang (✉)
Division of Pulmonary Medicine, Department of Internal Medicine, Min-Sheng General Hospital, No. 168 Jin-Kuo Road, Taoyuan City, Taiwan
e-mail: revival_chuang@yahoo.com

T.-Y. Chuang
Department of Internal Medicine, College of Medicine and National Taiwan University Hospital, Taipei, Taiwan

C.-W. Lee
Department of Nursing, Division of Basic Medical Sciences, and Chronic Diseases and Health Promotion Research Center, Chang Gung University of Science and Technology, Chiayi, Taiwan

C.-W. Lee
Research Center for Industry of Human Ecology and Research Center for Chinese Herbal Medicine, Chang Gung University of Science and Technology, Taoyuan, Taiwan

C.-W. Lee
Department of Orthopaedic Surgery, Chang Gung Memorial Hospital, Chiayi, Taiwan

I.-T. Lee
School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan

by the increased expression of mitochondrial fission factor (Mff), phosphorylated dynamin-related protein 1 (p-DRP1), and mitophagy-related proteins (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, Bnip3) in A549 cells. Inhibition of DRP1 or Mff significantly decreased ICAM-1 expression. In addition, we found that Vit. D decreased TNF-α-induced ICAM-1 expression, mitochondrial fission, and mitophagy via the AKT and NF-κB pathways. Moreover, ICAM-1 expression, mitochondrial fission, and mitophagy were increased in the lung tissues of TNF-α-treated mice, while Vit. D supplementation reduced these effects. In this study, we elucidated the mechanisms by which Vit. D reduces the expression of adhesion molecules in models of airway inflammation. Vit. D might be served as a novel therapeutic agent for the targeting of epithelial activation in lung inflammation.

Keywords Adhesion molecules · ROS · Inflammation · Vitamin D_3 · Mitochondrial fission · Mitophagy

Abbreviations

Vit. D	Vitamin D_3
ROS	Reactive oxygen species
mtROS	Mitochondrial reactive oxygen species
ATP	Adenosine triphosphate
DRP1	Dynamin-related protein 1
Mff	Mitochondrial fission factor
Bnip3	BCL2/adenovirus E1B 19kDA protein-interacting protein 3
COPD	Chronic obstructive pulmonary disease
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
SDS	Sodium dodecyl sulfate

Introduction

Inflammatory lung diseases display high morbidity and mortality and pose a significant socioeconomic burden to society (Scherer and Chen 2016). In the lung, inflammation is caused by pathogens or exposure to irritants, air pollutants, and allergens (Moldoveanu et al. 2009). Inflammation is an important factor in the occurrence and progression of respiratory diseases, such as asthma and chronic obstructive pulmonary disease (Lee and Yang 2013). The complicated interactions between circulating polymorphonuclear leukocytes and constituent cells in lung tissues were involved in these inflammatory responses. During inflammation, intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin supergene family, which is upregulated in respiratory epithelial cells (Lee and Yang 2013; Roebuck and Finnegan 1999). Therefore, it is important to both mechanistically understand and attempt to inhibit the known inflammatory cascade that induces and amplifies lung inflammation.

Oxidative stress is a predictor and indicator of tissue damage and is involved in many biological and pathological diseases, such as inflammation and carcinogenesis (Cachon et al. 2014). Reactive oxygen species (ROS) are mainly produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family members in the plasma membrane and mitochondria (Forrester et al. 2018). An increasing body of evidence has shown that excessive ROS plays an important role in the pathogenesis of airway inflammation and tissue damage, so the neutralization of ROS or inhibition of the redox pathway may reduce inflammation (Babbar and Casero 2006; Kim et al. 2008, 2014Liu et al. 2018). However, the role of excessive mitochondrial ROS (mtROS) production in driving lung inflammation and ICAM-1 expression has not been fully elucidated.

Under physiological conditions, mitochondria are not only the "powerhouses" of cells, producing large amounts of adenosine triphosphate (ATP) necessary for cells to live, but are also a hub for sensing inflammatory signals and the starting point for inflammatory responses (Ng Kee Kwong et al. 2017). Mitochondria undergo balanced membrane remodeling through fusion and fission and form a dynamic interconnected intracellular network (Park et al. 2013). When stressed, healthy and damaged mitochondria are separated through a fission mechanism, which is regulated by dynamin-related protein 1 (DRP1) and mitochondrial fission factor (Mff) (Westermann 2010). Damaged mitochondria are mainly degraded by mitophagy, a selective form of autophagy (Harris et al. 2018). Mitophagy can preserve energy metabolism and reduce damage caused by external stimuli, thereby protecting the body. However, excessive mitophagy can lead to cell death (Ravikumar

et al. 2010). Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) is a receptor-mediated mitophagy-related factor located on the outer mitochondrial membrane that recruits LC3 interacting region (LIR) motifs and triggers mitophagy (Harris et al. 2018). However, the role of the mitophagy-associated proteins Bnip3 and LC3B in TNF-α-induced lung inflammation is still unclear.

Vitamin D_3 regulates adaptive and innate immune functions, proliferation and differentiation of many cell types, and airway remodeling (Hutchinson et al. 2018). Vitamin D_3 is primarily synthesized by 7-dehydrocholesterol in the skin upon exposure to ultraviolet B (UVB) radiation present in sunlight (Christakos et al. 2016; Dusso et al. 2005) but can also be obtained from the diet. Vitamin D_3 is not biologically active. The first metabolite of vitamin D_3, 25(OH)D_3, is the major circulating form of vitamin D_3 and the second metabolite of vitamin D_3, 1,25$(OH)_2D_3$, is the biologically active form of vitamin D_3. 25(OH)D_3 level in serum has become a reliable biomarker of vitamin D_3 status (Cashman et al. 2017). Vitamin D_3 deficiency is also closely related to the level of inflammatory biomarkers such as vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, and interleukin-6 (IL-6) (Dobnig et al. 2008). In addition, vitamin D_3 extensively modulates both innate and adaptive immune responses and could reduce systemic inflammation in patients (Dusso et al. 2005). Vitamin D_3 administration reduced ICAM-1 and VCAM-1 levels in hemodialysis patients in a placebo-controlled, double-blind clinical trial (Naeini et al. 2017). It has been reported that vitamin D_3 deficiency is associated with the occurrence of bronchiectasis, which is linked to disorders such as asthma and chronic obstructive pulmonary disease (COPD) (Ferri et al. 2019). Vitamin D_3 treatment could reduce morbidity and mortality of patients with chronic kidney disease and end-stage kidney disease by reducing inflammatory mechanisms (Assimon et al. 2012). However, the role of vitamin D_3 in inflammatory diseases is not fully understood. Herein, we studied the effects and mechanisms of action of vitamin D_3 on the expression of adhesion molecules. A better understanding of these processes may provide important insights for preventing airway inflammation.

Materials and methods

Cell culture

A549 cells (tumor transformed human type II epithelial alveolar cells) and THP-1 cells (human monocytic cells) were bought from the American Type Culture Collection (VA, USA). A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, BI, CT, USA) containing 10% fetal bovine serum (FBS, BI) and 1% penicillin/streptomycin (BI). THP-1 cells were cultured in RPMI-1640 medium (BI) containing 10% FBS and 1% penicillin/streptomycin/amphotericin B. Both types of cells were cultivated in a humidified, 5% CO_2 atmosphere at 37 °C.

Western blot

Cells were pretreated with 25-hydroxy vitamin D_3 (25(OH)D_3, 25VD_3, Vit. D; Cayman, NY, USA) or 1,25-dihydroxyvitamin D_3 (1–25$(OH)_2D_3$; 1-25VD_3; Cayman, NY, USA) for 24 h and then treated with 10 ng/mL TNF-α (PeproTech, NJ, USA). A uniform amount of protein samples (30 μg) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes (Millipore, Germany). The membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk for 1 h. Then, membranes were treated with primary antibodies against ICAM-1 (Santa Cruz, TX, USA; 1:2000 dilution); phospho-ERK, phospho-JNK, phospho-p38, phospho-AKT, phospho-p65, phospho-DRP1 (Ser616), DRP1, Mff, LC3B, Bnip3 (Cell Signaling, MA, USA; 1:2000 dilution); or HIF-1α (GeneTex, CA, USA; 1:2000 dilution) overnight at 4 °C. Anti-GAPDH or anti-β-actin antibodies (BIOTOOLS, Taiwan, 1:10,000 dilution) were used as loading controls. Furthermore, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, PA, USA; 1:2000 dilution) for 1 h at room temperature (RT). Protein levels were detected using Immobilon Western Chemiluminescent HRP substrate (Merck, Germany). Images were visualized on a UVP ChemStudio PLUS Touch imaging system (Analytik Jena, Germany). The intensity of each band

was quantified using ImageJ and normalized to that of GAPDH or β-actin.

Immunofluorescent staining

To detect the in situ expression of ICAM-1, confluent A549 cells (control cells or cells treated with Vit. D for 24 h) on sterile coverslips were treated with the absence or presence of 10 ng/mL TNF-α at 37 °C for 4 h. Then, the medium was removed and the cells were rinsed with PBS, fixed in 4% paraformaldehyde for 15 min at RT, and permeabilized with 0.01% Triton X-100 at RT for 1 min. The cells were further blocked with 1% bovine serum albumin (BSA) in PBS at RT for 1 h. Then, cells were treated with anti-ICAM-1 (diluted 1:50 in PBS containing 1% BSA, Jackson ImmunoResearch Laboratories) overnight at 4 °C. After washing off the unbound antibody, the cells were treated with Alexa Fluor 488-conjugated secondary antibody (diluted 1:200 in PBS containing 1% BSA) for 1 h. Nuclei were labeled with 1 μg/mL DAPI for 3 min, to observe and photograph these results with a fluorescence microscope (Leica, Germany). Further, to examine the expression of mitochondrial DRP1, Bnip3 or LC3B, control cells or 24 h Vit. D-treated cells on sterilized coverslips were incubated with 1 μM MitoTracker (Invitrogen, CA, USA) or with LysoTracker (Invitrogen) for 30 min and then treated with or without 10 ng/ml TNF-α at 37 °C for 1–2 h. The cells were then fixed and permeabilized by the same method as described above. Then, the cells were incubated with primary antibodies against DRP1, Bnip3, or LC3B (1:200 dilution in PBS containing 1% BSA) overnight at 4 °C. Then, the subsequent experimental steps are the same as above.

Leukocyte-epithelial cell adhesion assay

A549 cells cultured in 12-well plates were pretreated with or without Vit. D for 24 h and treated with 10 ng/mL TNF-α for 4 h. 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Invitrogen) labeled THP-1 cells were incubated with A549 cells for 1 h. Nonadherent THP-1 cells were removed by gentle washing with PBS. For each experiment, three randomly selected images were captured under a fluorescence microscope and the number of THP-1 cells adhered to A549 cells was counted in each image.

Detection of mitochondrial ROS

A549 cells were pretreated with Vit. D for 24 h and then stimulated with 10 ng/mL TNF-α for 4 h. MitoSOX Red (Invitrogen), a mitochondrial superoxide indicator, was used to detect mitochondrial ROS levels. A549 cells were incubated with 1.5 μM MitoSOX Red at 37 °C for 15 min. Fluorescence intensity was examined using a fluorescence microscope and a FACSCalibur flow cytometer (Becton, Dickinson and Company, BD, NJ, USA).

Mitochondrial membrane potential (ΔψM) determination

The change in mitochondrial transmembrane potential was analyzed using 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Invitrogen). Cells were pretreated with Vit. D for 24 h and then stimulated with 10 ng/mL TNF-α. JC-1 (1 μg/mL) was added to the culture media prior to TNF-α stimulation. At high ΔψM, JC-1 forms aggregates and emits red fluorescence; at low ΔψM, JC-1 exists as a monomer and emits green fluorescence. The fluorescence was measured by a fluorescence microscope and an LSRFortessa flow cytometer (BD).

Determination of adenosine triphosphate (ATP) levels

A Molecular Probes® ATP determination kit was used to examine the levels of cellular ATP (Invitrogen). The cells were collected in lysis buffer and centrifuged (14,500 × g) for 20 min at 4 °C. Subsequently, the supernatants were collected and twenty micrograms of each sample was added to a 96-well plate. Then, the reaction solution was added to the 96-well plate. ATP level was measured using a microplate luminometer (Berthold, Bad Wildbad, Germany) and calculated it based on the standard ATP curve.

Mitochondrial imaging

Confluent A549 cells (control cells or 24 h Vit. D-treated cells) on sterilized coverslips were incubated with 1 μM MitoTracker (Invitrogen) at 37 °C

for 30 min and then stimulated with or without 10 ng/ml TNF-α for 0–4 h at 37 °C. Mitochondria were observed and imaged using a Zeiss ApoTome (Zeiss, Germany). ImageJ software was used to measure mitochondrial length from 20 cells and at least 10 mitochondria per cell. Three replicates were performed for each biological sample.

Transmission electron microscopy

After pretreatment with Vit. D for 24 h, the cells were treated with 10 ng/mL TNF-α. After centrifugation, the cells were collected, washed with PBS, fixed with 2% paraformaldehyde and 2% glutaraldehyde in PBS at 4 °C for 24 h, and then post-fixed with 1% osmic acid at RT for 1 h. The cells were further dehydrated in graded ethanol, washed with propylene oxide, and embedded in epoxy resin. The ultrathin sections were cut by using a Reichert ultramicrotome, stained with aqueous uranyl acetate and lead citrate, and viewed using a HITACHI H-7100 at 100 kV.

Mitochondrial isolation assay

Whole A549 cell lysates were collected in trypsin–EDTA and then the mitochondrial and cytoplasmic parts were prepared using a mitochondria isolation kit (Thermo Scientific, CA, USA). The experimental procedure was carried out in accordance with the manufacturer's instructions. The mitochondrial and cytoplasmic parts were stored at − 20 °C for immunoblot analysis.

Acridine Orange (AO) staining

A549 cells were seeded on sterilized coverslips, preincubated with Vit. D for 24 h and then treated with TNF-α (10 ng/mL) for 2 h. AO reagent (1 μg/mL, Cayman) was added to the culture media prior to TNF-α stimulation. Cells were washed with PBS and then observed and photographed under a fluorescence microscope. Red fluorescence indicated acidic autophagic vesicles, while green fluorescence was observed in the cytoplasm and nucleus.

Coimmunoprecipitation assay

For coimmunoprecipitation, cells were collected in lysis buffer and centrifuged at 12,000 × g for 10 min. Then, the supernatant was collected, incubated with 1 μg of the appropriate antibody against protein of interest, and precipitated with GammaBind Plus-Sepharose beads (GE Healthcare, IL, USA) overnight at 4 °C. Wash the beads 3 times with PBS, and then centrifuge at 2500 × g for 5 min to collect the precipitated protein. The immunoprecipitated proteins were separated by SDS-PAGE and subjected to Western blot. P*rimary antibodies* used for immunoblot *were* as follows: *DRP1* and Bnip3. In addition, Mff and LC3B antibodies were used to check the purity of the precipitate.

Animal model

Male C57BL6/J wild-type mice were bought from National Taiwan University (Taipei, Taiwan). This study uses mice aged 8–12 weeks, weighing between 25 and 35 g. The mice were orally fed vitamin D_3 (10,000 IU/kg/day) for 14 days and then anesthetized by inhalation of 2% isoflurane. The neck of the mouse was shaved, and the surgical site was disinfected with 75% alcohol. Make a vertical 5 mm incision to expose the trachea. Use an insulin syringe to puncture the anterior wall of the trachea between the second and third tracheal cartilage rings at a 45° angle to avoid damage to the posterior wall. TNF-α (10 μg/kg) in sterile PBS was slowly infused into the trachea. Then suture the skin incision. After returning to normal behavior, the mouse was placed back into the cage. The next day, the mice were anesthetized via inhalation of isoflurane and sacrificed.

Part of lung tissue was fixed in 4% buffered paraformaldehyde and embedded in paraffin for immunohistochemical analysis and hematoxylin–eosin staining. The remaining part was quickly frozen in liquid nitrogen for protein separation to examine the levels of ICAM-1, DRP1, Mff, Bnip3, and LC3B expression by Western blot. In short, lung tissue was lysed in lysis buffer supplemented with phosphatase inhibitors and protease. The lysate was then centrifuged at 14,500 × g at 4 °C for 20 min. The supernatant was stored at − 80 °C for further study.

Immunohistochemistry

Five-micrometer-thick sections were cut from the paraffin blocks. The sections were placed in a 60 °C oven for 1 h for deparaffinization and then gradually rehydrated through graded alcohol: 100%, 95%, 85%, and 75% for 5 min each. After antigen retrieval using 10 mM sodium citrate, endogenous peroxidases were inactivated with 3% hydrogen peroxide for 10 min at RT. To check the ICAM-1 expression in lung tissues, the sections were incubated overnight with ICAM-1 antibody (1:200 dilution) at 4 °C. Subsequently, they were incubated with biotin-conjugated goat anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch Laboratories) at RT for 1 h. After washing with PBS, the sections were incubated with avidin–biotin peroxidase complex (VECTASTAIN® ABC-HRP Kit, Vector Laboratories, CA, USA) for 1 h at RT. The sections were then stained with 3′3-diaminobenzidine tetrahydrochloride (DAB; Vector, CA, USA) and H_2O_2, counterstained with hematoxylin, and examined by light microscopy.

In order to check whether ICAM-1 is related to type II alveolar epithelial cells, the sections were double stained with ICAM-1 and SP-D (a marker for type II alveolar epithelial cells, 1:100, Bioss, Beijing, China) antibodies. After washing with PBS, the sections were incubated with Alexa Fluor 488 (1:200 dilution) for ICAM-1 and Alexa Fluor 594 (1:100 dilution) for SP-D. Subsequently, the slides were counterstained with DAPI solution and detected by fluorescence microscopy.

Statistical analysis

Data are expressed as the fold change compared to the control value and expressed as the mean ± SEM. Statistical significance was determined through one-way analysis of variance (one-way ANOVA) using the Statistical Package for the Social Sciences (SPSS) with Fisher's least significant difference (LSD) test. Statistical analyses were performed using GraphPad Prism software (CA, USA). *$p < 0.05$ compared with the control group; †$p < 0.05$ compared with the TNF-α-stimulated cells.

Results

Vit. D reduces TNF-α-induced ICAM-1 expression in A549 cells

Vit. D is reported to have anti-inflammatory effects (Brito et al. 2020). Therefore, we investigated whether Vit. D suppressed the inflammatory response by affecting TNF-α-induced adhesion molecules. To analyze the effect of Vit. D on ICAM-1 expression under inflammatory conditions, A549 cells were preincubated with 0.1, 0.5, 1, 5, or 10 μM Vit. D for 24 h and then treated with 10 ng/mL TNF-α for 4 h. As shown in Fig. 1a, TNF-α significantly increased ICAM-1 expression, while 10 μM Vit. D reduced this effect. In addition, TNF-α induced ICAM-1 expression in a time-dependent manner, while 10 μM Vit. D reduced this expression (Fig. 1b). Based on this result, Vit. D was used at 10 μM in all following experiments evaluating the anti-inflammatory effect and molecular mechanism of Vit. D treatment. 1,25-dihydroxyvitamin D_3 (1–25$(OH)_2D_3$; 1-25VD_3) is an active, hormonal metabolite of vitamin D_3 (Dusso et al. 2005). To examine whether the protective effect of Vit. D (25VD_3) is similar to that of 1-25VD_3, A549 cells were pretreated with 0.1 or 10 μM 1-25VD_3 for 24 h and then treated with TNF-α for 4 h. As shown in Fig. 1c, the low dose of 1-25VD_3 had no significant effect on ICAM-1 reduction, while the high dose of 1-25VD_3 significantly decreased TNF-α-induced ICAM-1 expression. Similarly, ICAM-1 was strongly present in the cytoplasm of TNF-α-treated A549 cells (T) by fluorescence microscopy. In contrast, pretreatment with Vit. D reduced ICAM-1 expression in TNF-α-treated A549 cells (Vit. D/T) (Fig. 1d). Because cell adhesion molecules are essential for monocyte binding, the subsequent step was to check the effects of Vit. D about the adhesion of monocytes to epithelial cells. TNF-α-treated A549 cells (T) exhibited increased monocyte adhesion compared with confluent control cells (C). Pretreatment with Vit. D reduced the number of monocytes adhered to TNF-α-treated A549 cells (Fig. 1e). In addition, pretreatment with an ICAM-1 neutralizing antibody significantly reduced the monocyte binding to TNF-α-treated A549 cells (ICAM-1 Ab/T) (Fig. 1e). These results suggested that Vit. D reduces TNF-α-induced inflammatory responses in A549 cells.

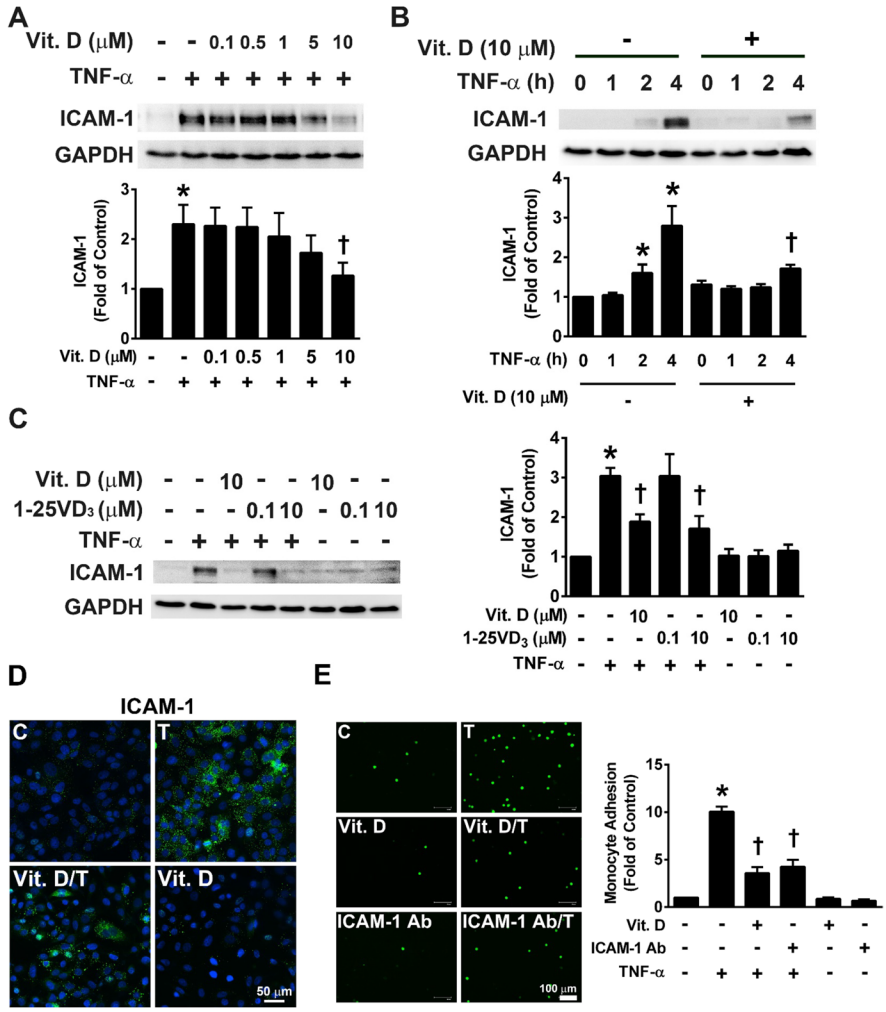


Fig. 1 Vit. D reduces TNF-α-induced ICAM-1 expression in A549 cells. **a** A549 cells were pretreated with Vit. D (0–10 μM) for 24 h and stimulated with TNF-α (10 ng/mL) for 4 h. Then, the expression of ICAM-1 was detected by Western blot. **b** A549 cells were pretreated with 10 μM Vit. D for 24 h and then treated with TNF-α for the indicated times. Then, ICAM-1 levels were measured by Western blot. **c** A549 cells were pretreated with Vit. D (10 μM) or 1-25VD_3 (0.1, 10 μM) for 24 h and then stimulated with TNF-α (10 ng/mL) for 4 h. Then, expression of ICAM-1 was detected by Western blot. **d** The distribution of ICAM-1 expression was detected by immunofluorescent staining. The nuclei were stained with DAPI. Bar = 50 μm. **e** Representative images and quantitative analysis of fluorescein-labeled THP-1 cells adhering to A549 cells. A549 cells were left untreated or were pretreated with 10 μM Vit. D or 2 μg/mL anti-ICAM-1 antibody for 24 h and then stimulated with TNF-α for 4 h. Then, a fluorescence microscope was used to photograph and count adherent cells. Bar = 100 μm. Data are reported as the mean ± SEM (n = 9). *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to TNF-α-treated cells

Vit. D reduces TNF-α-induced mitochondrial ROS (mtROS) production in A549 cells

We next wanted to examine whether Vit. D reduces TNF-α-induced inflammatory responses by decreasing mitochondrial oxidative stress. The production of ROS, which are predominantly derived from mitochondria, is a key event in the progression of many inflammatory disorders (Liu et al. 2018; Park et al. 2013). Hypoxia-inducible factor-1α (HIF-1α) has been recognized as a transcription factor that can act as a major regulator of oxygen homeostasis (Semenza 2014). Hypoxia-inducible factor-1α (HIF-1α) has been recognized as a transcription

factor that can act as a major regulator of oxygen homeostasis However, Haddad and Land reported that TNF-α plays a major role in mediating HIF-1α regulation under normoxia (Haddad and Land 2001). In this study, we examined the effect of Vit. D on TNF-α-induced HIF-1α expression. The data showed that TNF-α treatment significantly increased the expression of HIF-1α in a time-dependent manner, while pretreatment with Vit. D reduced TNF-α-induced HIF-1α expression (Fig. 2a). The levels of mtROS were evaluated using the MitoSOX Red assay. Cells treated with TNF-α for 4 h displayed significantly increased mtROS levels, as shown by both fluorescence microscopy (Fig. 2b) and flow cytometry (Fig. 2c). As shown in Fig. 2b, pretreatment with Vit. D significantly decreased TNF-α-induced mtROS production. MitoTEMPO, a mitochondrial-targeted antioxidant, was used to examine whether Vit. D affects ROS production in mitochondria. Pretreatment with MitoTEMPO significantly decreased TNF-α-induced mtROS production, as shown by fluorescence microscopy. The flow cytometry results were consistent with the fluorescence microscopy findings (Fig. 2c). TNF-α-induced HIF-1α expression was suppressed by mitoquinol (MitoQ), a mitochondrial-targeted antioxidant (Fig. 2d). We next evaluated whether the expression of ICAM-1 in TNF-α-treated A549 cells was caused by the production of mtROS. The data showed that pretreatment with MitoQ reduced the expression of ICAM-1 in TNF-α-treated A549 cells (Fig. 2e). Taken together, these data indicate that TNF-α-induced ICAM-1 expression is related to mtROS production in A549 cells.

Vit. D attenuates the TNF-α-induced decrease in mitochondrial membrane potential (ΔΨm) and increase in mitochondrial fission

Under normal conditions, mitochondria undergo dynamic and balanced membrane remodeling through cycles of fusion and fission. Once stimulated, healthy and damaged mitochondria are separated through a fission mechanism. Imbalances in mitochondrial fission and fusion play an important role in the progression of many diseases (Park et al. 2013; Westermann 2010). Due to the production of mtROS in TNF-α-stimulated A549 cells, we examined several mitochondrial functions, such as changes in membrane potential, ATP production and expression of mitochondrial dynamics-related proteins. The levels of ΔΨm and ATP are key parameters for cell and mitochondrial function. Cells treated with TNF-α for 1 and 2 h (T1h, T2h) displayed a marked change in fluorescence shift from red to green, as shown by the JC-1 analysis; this change indicates the collapse of ΔΨm. Cells pretreated with Vit. D displayed reduced green fluorescence emission, with a distribution type is similar to that of the control group (Fig. 3a). Moreover, JC-1 analysis of flow cytometry showed that TNF-α stimulation increased the ratios of green to red MFI (mean fluorescence intensity), while Vit. D pretreatment reversed this effect (Fig. 3b and Supplementary Fig. 1). ATP level was decreased after TNF-α treatment, while Vit. D treatment protected cells from this TNF-α-induced influence (Fig. 3c).

Mitochondria are highly dynamic cellular organelles with the ability to undergo fission and fusion. Mitochondrial dynamics play the important role in cellular and mitochondrial function (Scott and Youle 2010). As shown in Fig. 3d, DRP1 phosphorylation at residue Ser616 was significantly increased after 1 and 2 h TNF-α stimulation, while pretreatment with Vit. D reduced this activation. The expression of Mff, an adaptor of DRP1, was also decreased in TNF-α-treated A549 cells pretreated with Vit. D. Next, MitoTracker was used to monitor changes in mitochondrial morphology in A549 cells incubated with Vit. D for 24 h and then treated with TNF-α for 0–4 h. We found that mitochondrial length was significantly reduced in TNF-α-treated cells as compared to cells pretreated with Vit. D before TNF-α stimulation (Fig. 3e). TEM images also showed that mitochondrial length was reduced in TNF-α-treated cells, while pretreatment with Vit. D reversed these effects (Fig. 3f). We also found that there was increased expression of mitochondrial DRP1 and Mff in TNF-α-treated cells. These data showed that cells pretreated with Vit. D displayed reduced mitochondrial DRP1 and Mff levels after 1 h TNF-α stimulation (Fig. 3g). Consistently, immunostaining showed that TNF-α stimulation increased DRP1 expression, while the mitochondrial marker MitoTracker was used to demonstrate that the expression of DRP1 was localized to the mitochondria (Fig. 3h). These data indicate that TNF-α increases DRP1 expression in the mitochondria. The mitochondrial fission inhibitor Mdivi-1 was used to investigate whether the inflammatory responses are caused by TNF-α-induced

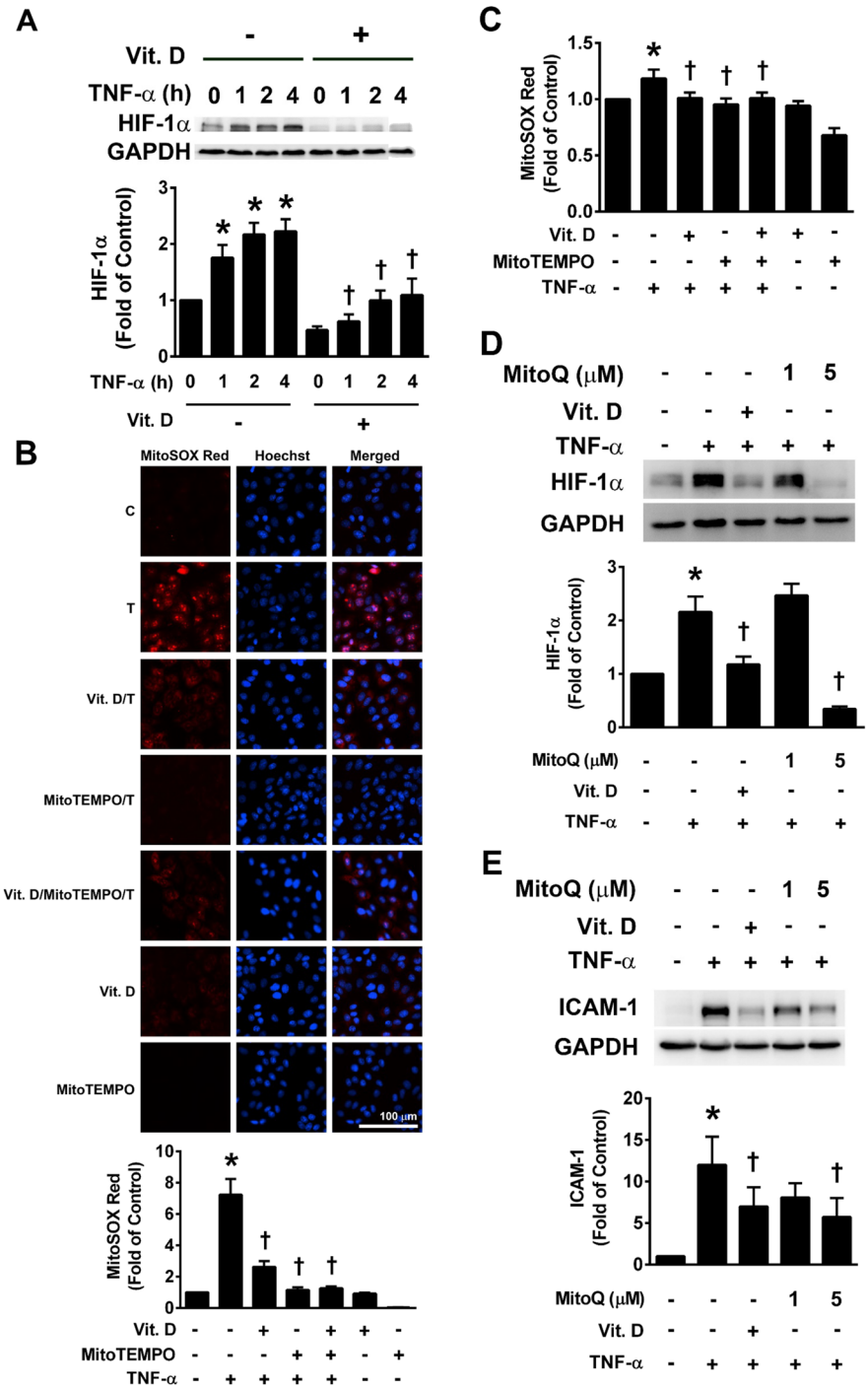


Fig. 2 The effect of Vit. D on TNF-α-induced mitochondrial ROS (mtROS) production in A549 cells. **a** A549 cells were pretreated with 10 μM Vit. D for 24 h and then treated with TNF-α for the indicated times. HIF-1α expression levels were measured by Western blot. **b**, **c** A549 cells were pretreated with 10 μM Vit. D or with 100 μM MitoTEMPO (a mitochondria-targeted antioxidant) for 24 h and then treated with TNF-α for 4 h. Mitochondrial ROS levels were detected using 1.5 μM MitoSox Red (a mitochondrial ROS indicator) by fluorescence microscopy (**b**) and flow cytometry (**c**). Bar = 100 μm. **d**, **e** A549 cells were incubated with 10 μM Vit. D or 1 or 5 μM mitoquinol (MitoQ, a mitochondria-targeted antioxidant) for 24 h and then stimulated with TNF-α for 4 h. Expression levels of HIF-1α and ICAM-1 were measured by Western blot. Data are reported as the mean ± SEM ($n = 9$). *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to TNF-α-treated cells

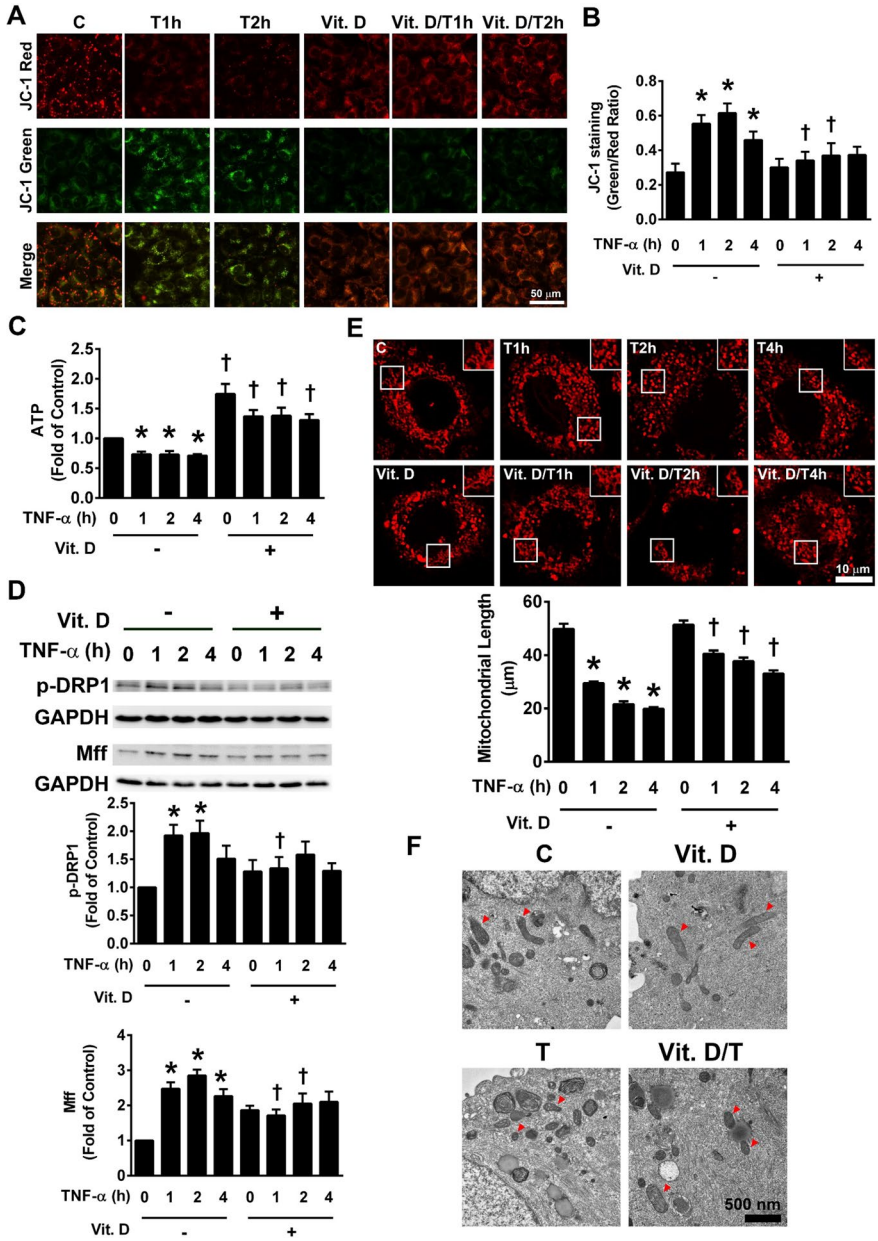
mitochondrial fission. Western blot analysis revealed that TNF-α-induced ICAM-1 expression was decreased following Mdivi-1 pretreatment (Fig. 3i). Pretreatment with Mdivi-1 also reduced the number of adherent monocytes (Fig. 3j). The efficiently reduced endogenous levels of DRP1 and Mff were indicated by Western blot (Fig. 3k). Knockdown of DRP1 or Mff significantly reduced TNF-α-induced ICAM-1 expression (Fig. 3l). These results suggested

that Vit. D reduces ICAM-1 expression by decreasing TNF-α-induced mitochondrial fission in A549 cells.

Vit. D reduces TNF-α-induced mitophagy

Mitochondrial dysfunction has been demonstrated to play a key role in several diseases, and the accumulation of dysfunctional mitochondria leads to oxidative stress and impairment of cellular functions (Bhatti et al. 2017). Mitochondrial dynamics and mitophagy have been identified as important mediators that control mitochondrial quality. Mitophagy appears to be an autophagic mechanism for the degradation of dysfunctional mitochondria (Harris et al. 2018). To investigate the involvement of mitophagy in A549 cells pretreated with Vit. D and then stimulated with TNF-α for the indicated times, we examined expression of the mitophagy-related protein Bnip3 by Western blot. As shown in Fig. 4a, TNF-α significantly increased both Bnip3 and LC3B expression in a time-dependent manner, while pretreatment with Vit. D reduced these TNF-α-induced effects. Interestingly, expression of the autophagy-related protein LC3B was significantly increased in the Vit. D pretreated cells. After 2 h of TNF-α stimulation, cells showed more acidic vesicular organelles by AO staining, which were detected as red-stained dots by fluorescence microscopy, compared with the 0 h group. Cells pretreated with Vit. D showed fewer red-stained dots (Fig. 4b). We then investigated whether Vit. D affected mitochondrial translocation of Bnip3 and LC3B in TNF-α-treated A549 cells. Vit. D pretreatment reduced mitochondrial translocation of Bnip3 when compared to that of TNF-α-treated cells, while mitochondrial fractions of LC3B expression showed no difference between TNF-α stimulation and Vit. D pretreatment with TNF-α stimulation (Fig. 4c). In addition, fluorescent staining revealed increased Bnip3 localization to mitochondria in TNF-α-treated cells, while Vit. D pretreatment reduced this effect (Fig. 4d). Coimmunoprecipitation was used to investigate interactions between Bnip3 and LC3B. As shown in Fig. 4e, Vit. D reduced TNF-α-induced Bnip3-LC3B interactions in A549 cells. These data suggested that Vit. D reduces TNF-α-induced Bnip3-mediated mitophagy. Mitophagy was identified in TNF-α-treated A549 cells with or without Vit. D pretreatment. Therefore, we next used bafilomycin A1 (Baf. A1) and chloroquine (CQ) to examine whether Vit. D reduced inflammation via inhibition of TNF-α-induced mitophagy (Fig. 4f). CQ inhibits autophagy because it raises the lysosomal pH, which results in the inhibition of autophagosome-lysosome fusion and lysosomal protein degradation whereas Baf. A1 inhibits the fusion between autophagosomes and lysosomes by inhibiting vacuolar H^+-ATPase, thereby preventing the maturation of autophagosomes (Mauthe et al. 2018; Mauvezin and Neufeld 2015). Baf. A1 pretreatment did not affect TNF-α-induced monocyte adhesion, however, CQ pretreatment significantly increased TNF-α-induced monocyte adhesion (Fig. 4g). Mdivi-1 was used to assess rates of mitophagy and revealed that increased mitophagy was correlated with excess TNF-α-induced mitochondrial fission. As shown in the results, pretreatment with Mdivi-1 reduced TNF-α-induced Bnip3 expression in A549 cells (Fig. 4h). These data suggested that

Fig. 3 Vit. D reduces excessive TNF-α-induced mitochondrial fission in A549 cells. **a** A549 cells were pretreated with 10 μM Vit. D for 24 h and then treated with TNF-α for the indicated times. Changes in mitochondrial membrane potential were detected using JC-1 staining and fluorescence microscopy. Bar = 50 μm. **b** Cells were incubated with 10 μM Vit. D for 24 h and then stimulated with TNF-α for the indicated times. Changes in mitochondrial membrane potential were examined using JC-1 staining and flow cytometry. **c** ATP production was measured using an ATP assay. **d** The levels of mitochondrial fission-related proteins, phosphorylated DRP1 (Ser 616) and Mff, were examined by Western blot. **e** Mitochondrial morphology was observed using a Zeiss ApoTome. The right panel shows a larger magnification of the indicated area in the left panel. Bar = 10 μm. The bar graph shows mitochondrial lengths in A549 cells as measured using ImageJ software. **f** A549 cells were pretreated with 10 μM Vit. D for 24 h and then stimulated with TNF-α for 2 h. Mitochondrial morphology (arrowhead) was examined using TEM. Scale bar = 500 nm. **g** A549 cells were pretreated with Vit. D and then stimulated with TNF-α for 1 h. The mitochondrial fraction was analyzed by Western blot. **h** Immunofluorescence was used to assess colocalization of DRP-1 (green) and MitoTracker (mitochondria, red) Bar = 10 μm. **i** The cells were pretreated with mitochondrial division inhibitor 1 (Mdivi-1) for 1 h and then stimulated with TNF-α for 4 h. The expression of ICAM-1 was detected by Western blot. **j** Photographs and quantitative analysis of fluorescein-labeled THP-1 cells adhered to A549 cells after Mdivi-1 pretreatment and TNF-α stimulation. Bar = 100 μm. **k** A549 cells were transfected with 50 nM DRP1 siRNA or 25 nM Mff siRNA for 48 h and then DRP1 and Mff expression was determined by Western blot. **l** A549 cells were transfected with 50 nM DRP1 siRNA or 25 nM Mff siRNA for 48 h and then stimulated with TNF-α for 4 h. The expression of ICAM-1 was determined by Western blot. Data are reported as the mean ± SEM ($n = 9$). *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to TNF-α-treated cells



mitophagy in A549 cells is caused by TNF-α-induced mitochondrial fission and that Vit. D could reduce these effects.

Vit. D reduces TNF-α-induced the expression of ICAM-1 in A549 cells through the AKT/p65 pathways

The inflammatory cytokines-induced ICAM-1 expression is mediated through multiple signaling pathways, including MAPKs, AKT, and p65 (Lee and Yang 2013). We first examined whether TNF-α-induced ICAM-1 expression was mediated by activation of MAPKs. ERK1/2, p38 and JNK phosphorylation was significantly increased in A549 cells after 5 and 15 min of TNF-α stimulation but declined beginning at 30 min. In order to determine the impact of Vit. D, cells were pretreated with Vit. D for 24 h, then treated with TNF-α for 5, 15, and 30 min. As shown in Fig. 5a, Vit. D treatment

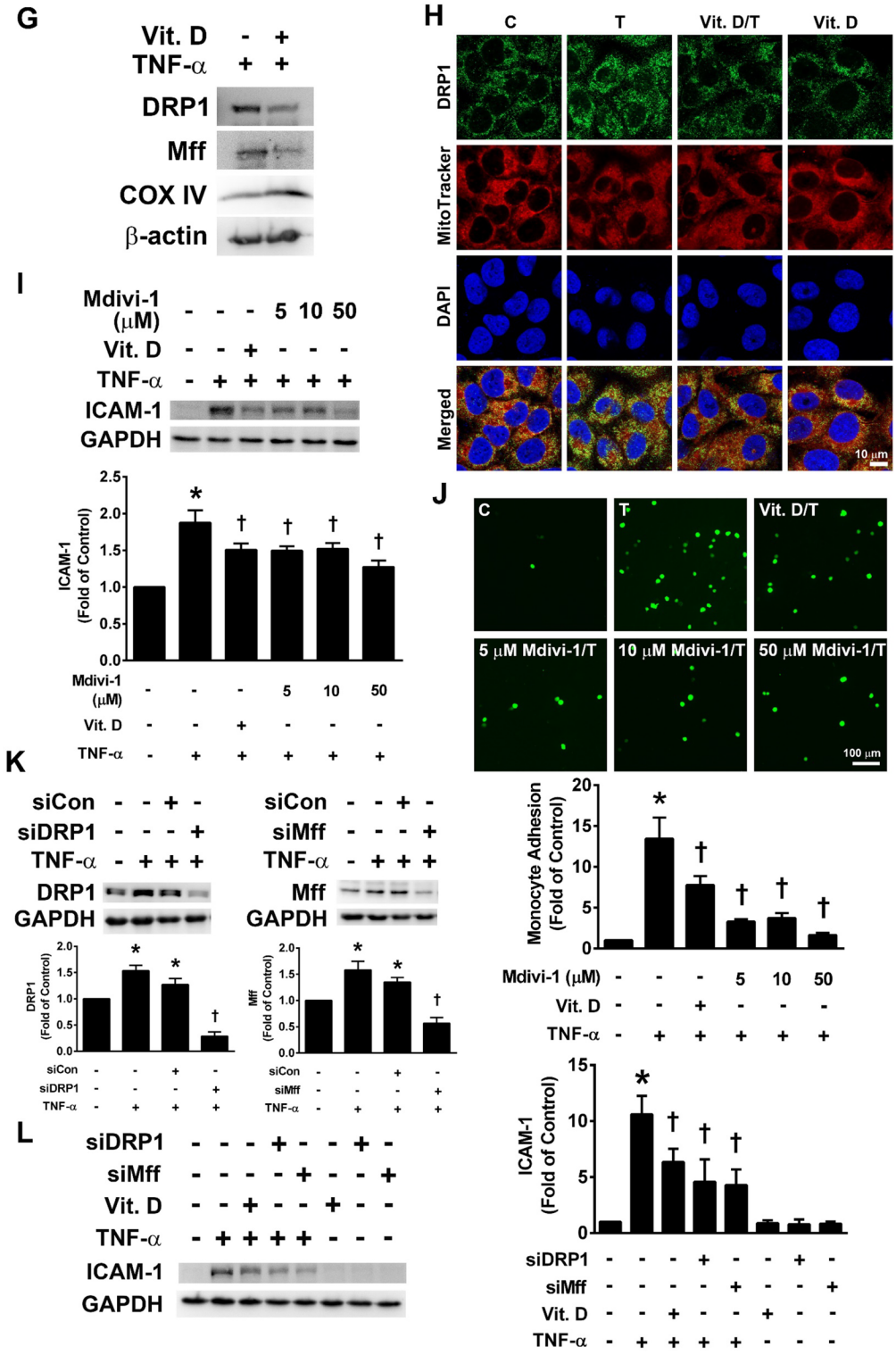


Fig. 3 (continued)

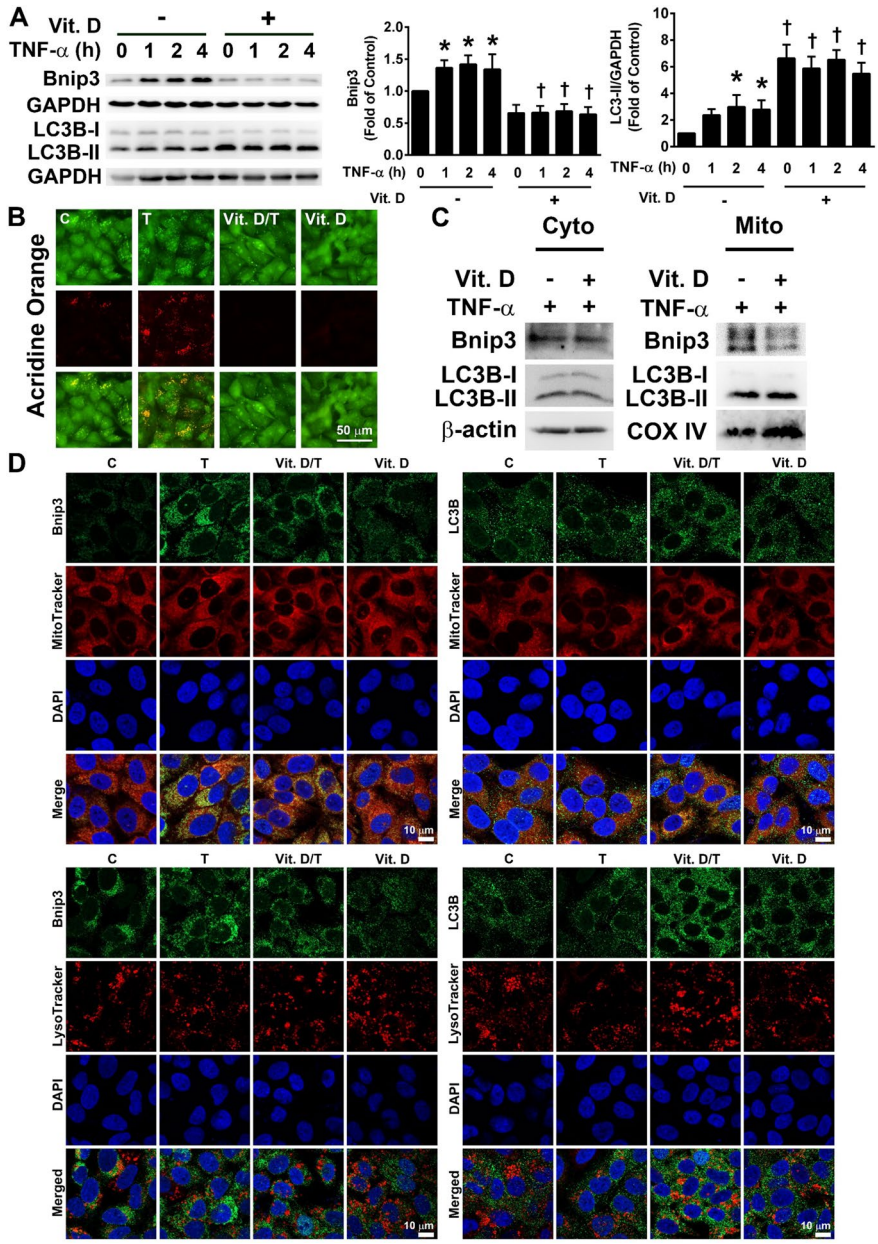


Fig. 4 Vit. D reduces TNF-α-induced mitophagy in A549 cells. **a** A549 cells were pretreated with Vit. D for 24 h and then treated with TNF-α for the indicated times. Bnip3 and LC3B expression levels in cell lysates were examined by Western blot. **b** A549 cells were pretreated with Vit. D for 24 h and then treated with TNF-α for 2 h. The formation of autophagosomes was examined via Acridine Orange (AO) staining and fluorescence microscopy. Bar = 50 μm. **c** A549 cells were pretreated with Vit. D for 24 h and then treated with TNF-α for 2 h. The cytoplasmic and mitochondrial fractions were examined for Bnip3 and LC3B expression by Western blot. **d** Immunofluorescence was used to assess colocalization of Bnip3 (green) or LC3B (green) and MitoTracker (mitochondria, red) or LysoTracker (lysosome, red). Bar = 10 μm. **e** Coimmunoprecipitation of Bnip3 and LC3B in A549 cells pretreated with Vit. D for 24 h and stimulated with TNF-α for 2 h. The left panel shows the Western blot analysis of whole cell lysates, and the right panel shows the analysis of immunoprecipitated samples. **f** A549 cells were pretreated with 100 nM bafilomycin A1 (Baf. A1) or 10 μM chloroquine (CQ) for 6 h, treated with or without Vit. D for 24 h, and then stimulated with TNF-α for 4 h. The expression of ICAM-1 was examined by Western blot. **g** Representative images and quantitative analysis of fluorescein-labeled THP-1 cells adhered to A549 cells. The adherent cells were photographed and counted under a fluorescence microscope. Bar = 100 μm. **h** A549 cells were pretreated with Mdivi-1 for 1 h and then stimulated with TNF-α for 2 h. Bnip3 and LC3B expression levels were analyzed by Western blot. Data are reported as the mean ± SEM ($n = 9$). *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to TNF-α-treated cells

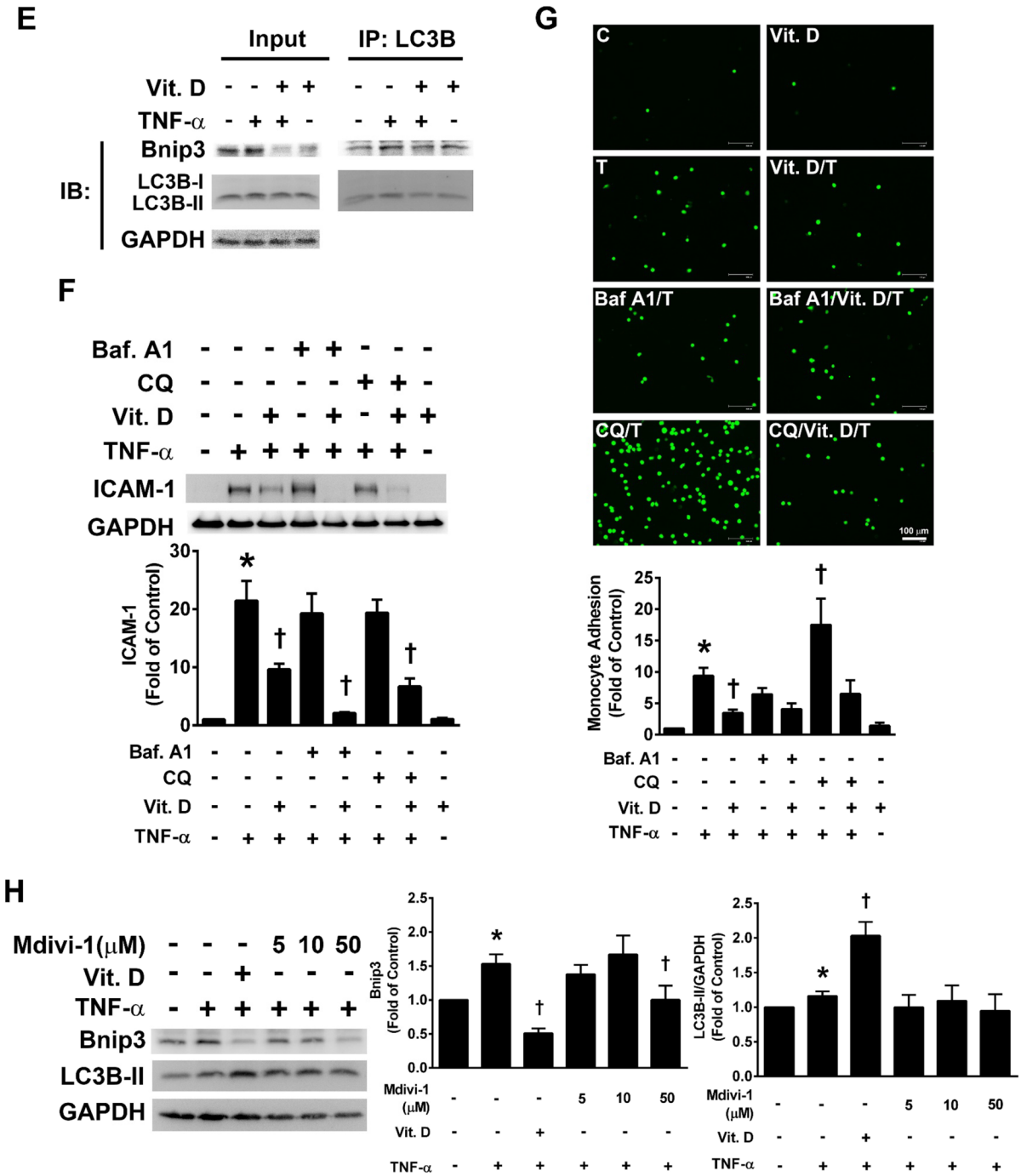


Fig. 4 (continued)

significantly decreased ERK, p38 and JNK phosphorylation levels. In addition, the levels of phosphorylated AKT and p65 expression were significantly induced after TNF-α stimulation, while Vit. D pretreatment remarkably reduced these effects (Fig. 5b). To further confirm the involvement of MAPKs, AKT, and p65 in TNF-α-induced mitochondrial fission and mitophagy, we used inhibitors

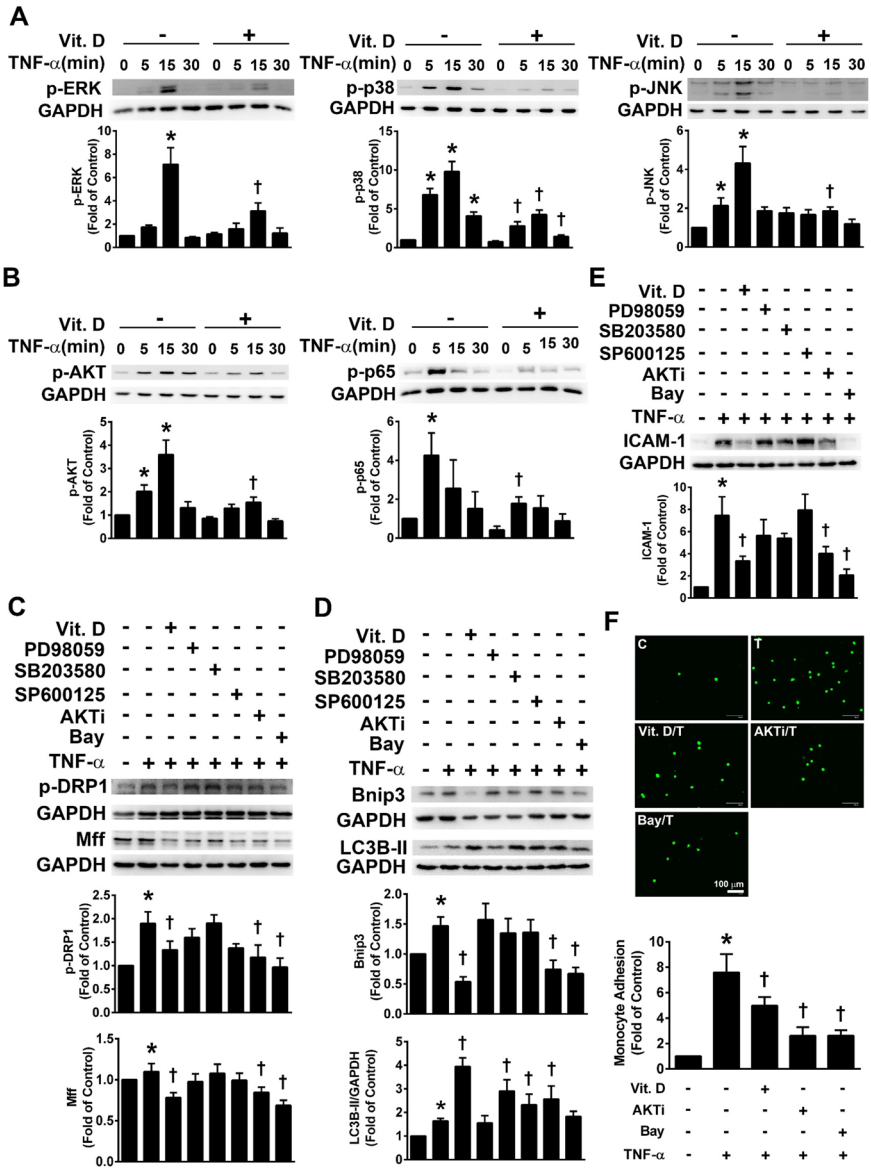


Fig. 5 Vit. D reduces TNF-α-induced mitochondrial fission, mitophagy, and ICAM-1 expression in A549 cells through the AKT/p65 pathways. **a**, **b** The effect of Vit. D treatment on TNF-α-induced phosphorylation of **a** ERK1/2, p38, JNK, **b** AKT and p65 in A549 cells. A549 cells were pretreated with 10 μM Vit. D for 24 h and then stimulated with TNF-α for 0–30 min. The levels of phosphorylated ERK1/2, p38, JNK, AKT and p65 were analyzed by Western blot. **c** A549 cells were pretreated with 50 μM PD98059 (an ERK1/2 inhibitor), 50 μM SB203580 (a p38 inhibitor), 50 μM SP600125 (a JNK inhibitor), 15 μM AKTi (an AKT inhibitor), or 15 μM Bay (a p65 inhibitor) for 1 h and then treated with TNF-α for 1 h. The expression of Ser616-phosphorylated DRP1 and Mff was analyzed by Western blot. **d** The effects of MAPKs, AKT, and p65 on the expression of Bnip3 and LC3B after 2 h of TNF-α stimulation were detected by Western blot. **e** A549 cells were pretreated with PD98059, SB203580, SP600125, AKTi, or Bay for 1 h and then treated with TNF-α for 4 h. Then, expression of ICAM-1 was examined by Western blot. **f** The effects of AKT/p65 inhibitors on TNF-α-induced adhesion of fluorescein-labeled THP-1 cells to A549 cells. The cells were left untreated or pretreated with AKTi or Bay for 1 h. Then, they were treated with TNF-α in the continued presence of the inhibitors for 4 h. Bar = 100 μm. **g**, **h** The effects of AKT/p65 inhibitors on mitochondrial length (**g**) and JC-1 staining (**h**). Bar = 10 or 50 μm as panel indicated. Data are reported as the mean ± SEM ($n = 9$). *$p < 0.05$ compared to untreated cells; †$p < 0.05$ compared to TNF-α-treated cells

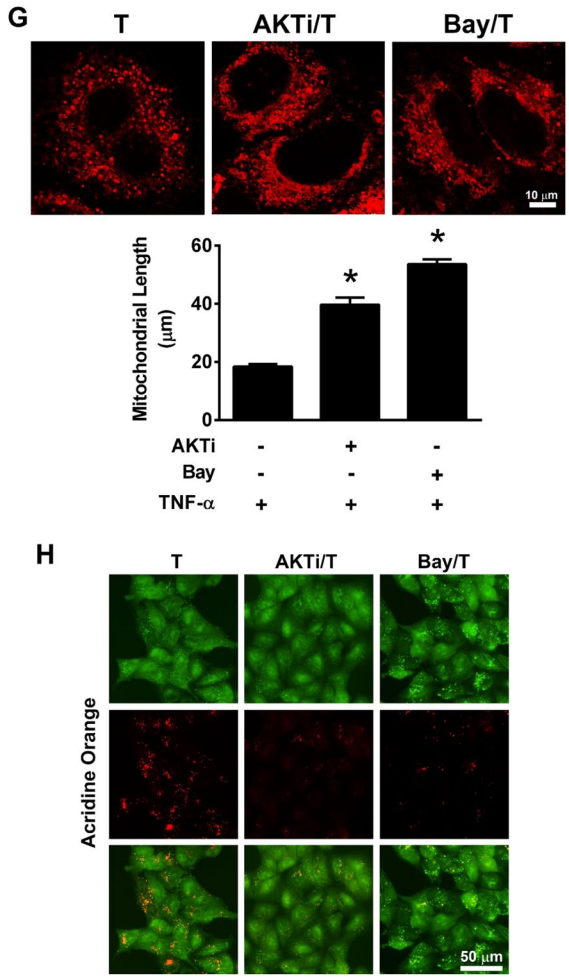


Fig. 5 (continued)

for specific signal transduction pathways. TNF-α-induced increases in p-DRP1, Mff, and Bnip3 expression was inhibited by pretreatment with AKTi (an AKT inhibitor) and Bay (an NF-κB inhibitor) but not with PD98059 (an ERK 1/2 inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor), as demonstrated by Western blot (Fig. 5c, d). Furthermore, we assessed whether the activation of MAPKs/AKT/p65 was related to the inhibition of TNF-α-induced ICAM-1 expression by Vit. D. TNF-α-induced ICAM-1 expression was inhibited by pretreatment with AKTi and Bay but not with PD98059, SB203580, or SP600125, as shown by Western blot analysis (Fig. 5e). Moreover, AKT and p65 specific inhibitors reduced TNF-α-increased monocyte binding (Fig. 5f), which further corroborated that sequential activation of the AKT/p65 pathway lead to the upregulation of ICAM-1 and subsequent monocyte binding. We also demonstrated that inhibitors specific for AKT and p65 enhanced TNF-α-induced reductions in mitochondrial length (Fig. 5g) and JC-1 staining (Fig. 5h). These data suggested that Vit. D decreased TNF-α-induced ICAM-1 expression, mitochondrial fission, and mitophagy via AKT and NF-κB pathways.

Vit. D reduces lung inflammation, mitochondrial fission, and mitophagy in TNF-α-treated mice

In order to examine the impact of Vit. D on the expression of ICAM-1 under inflammatory conditions in vivo, mice were orally fed Vit. D for 14 days and then stimulated for 24 h via intratracheal injection of TNF-α. At the light microscopic level, airspaces were inflated in healthy controls, and fewer blood cells were observed in the septal walls. 24 h of TNF-α stimulation caused thickening of distal airspaces and infiltration of many inflammatory cells, while preadministration of Vit. D reduced these effects (Fig. 6a). Western blot and immunohistochemical staining were performed to detect ICAM-1 expression levels in lung tissues. As shown in Fig. 6b, TNF-α significantly induced ICAM-1 expression in lung tissues, and pretreatment with Vit. D diminished these effects. Consistent with the Western blot results, stronger ICAM-1 staining was observed on epithelial cells in the TNF-α-treated group; in contrast, preadministration of Vit. D followed by 24 h of TNF-α stimulation resulted in weaker ICAM-1 staining and weak ICAM-1 expression in the control and Vit. D-treated groups (Fig. 6c). The expression of ICAM-1 was colocalized with type II epithelial cell maker SP-D (Fig. 6d). Expression of the mitochondrial fission-related proteins DRP1 and Mff and the mitophagy-related protein Bnip3 was significantly increased after TNF-α treatment, while Vit. D preadministration reduced mitochondrial fission and mitophagy (Fig. 6e). Furthermore, inhibitors specific for AKT and p65 reduced TNF-α-induced the expression of ICAM-1, Mff, and Bnip3, while did not affect the expression DRP1 and LC3B in lung tissues (Fig. 6f).

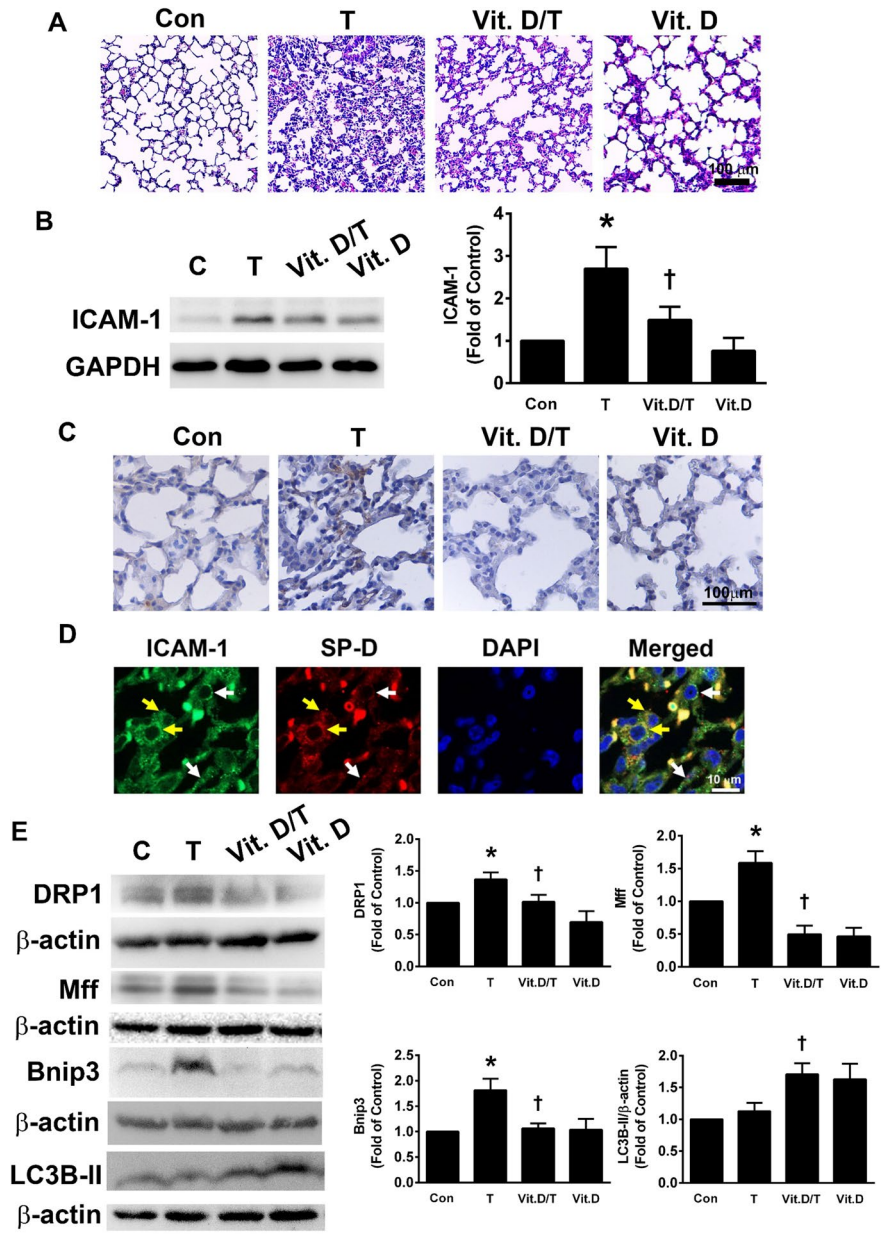


Fig. 6 Vit. D reduces lung inflammation, mitochondrial fission, and mitophagy in TNF-α-treated mice. C57BL/6 J mice were orally administered vitamin D_3 (10,000 IU/kg/day) for 14 days, and then TNF-α (10 μg/kg) was administered intratracheally. Mice were sacrificed 24 h after TNF-α treatment. **a** Representative light microscopic images of control (Con), TNF-α stimulated (T), Vit. D pre-administered and TNF-α stimulated (Vit. D/T), and Vit. D pre-administered (Vit. D) lung tissues. Bar = 100 μm. **b** The expression of ICAM-1 in lung tissues was examined by Western blot. **c** The localization of ICAM-1 expression in lung tissues was examined by immunohistochemistry. Bar = 100 μm. **d** ICAM-1 expression (white arrow) was localized to type II epithelial cells (yellow arrow) in lung tissues, as demonstrated by immunohistochemistry. Bar = 10 μm. **e** The expression levels of mitochondrial fission-related proteins (DRP1 and Mff), mitophagy-related protein (Bnip3), and autophagy-related protein (LC3B) in lung tissues were examined by Western blot. **f** The effects of AKT/p65 inhibitors on the expression of ICAM-1, Mff, Bnip3, DRP1, and LC3B were examined by Western blot. Data are reported as the mean ± SEM ($n = 5$). *$p < 0.05$ compared to the control group; †$p < 0.05$ compared to TNF-α-treated group

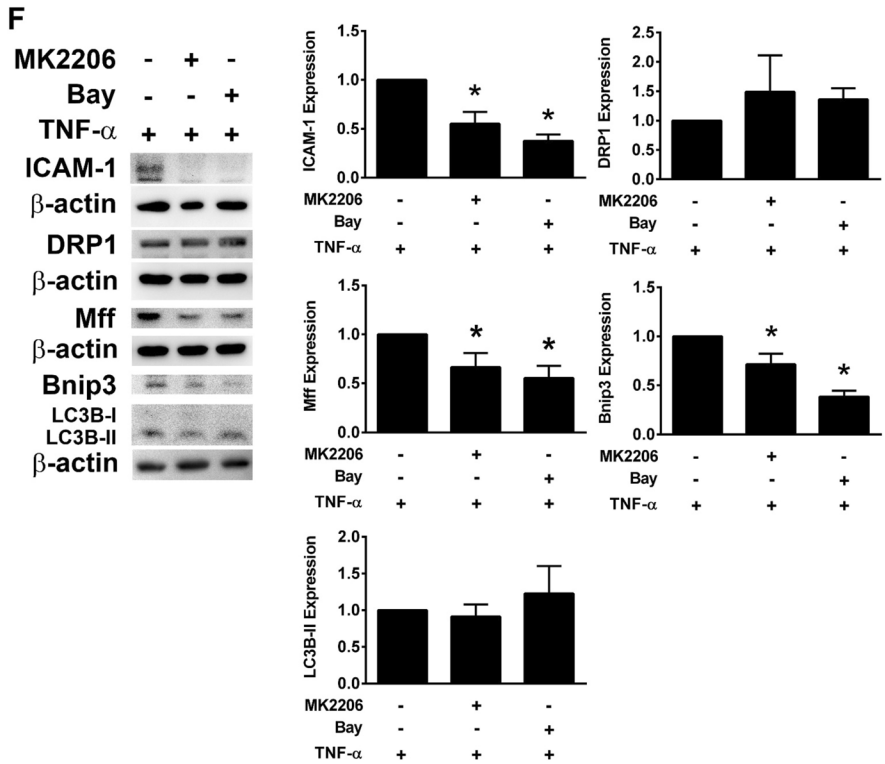


Fig. 6 (continued)

Discussion

Inflammation is considered a key factor in lung injury that leads to respiratory system failure. Therefore, novel research related to lung injury is urgently needed to generate novel insights into potential lung injury treatments. Our data showed that Vit. D treatment remarkably attenuated ICAM-1 expression and monocyte binding to TNF-α-treated A549 cells. This effect was mediated by inhibiting AKT/NF-κB phosphorylation. Moreover, pretreatment with Vit. D significantly reduced TNF-α-induced mitochondrial fission and mitophagy. Furthermore, Vit. D treatment attenuated ICAM-1 expression, mitochondrial fission and mitophagy in murine lung tissues following TNF-α stimulation. Therefore, the protection of pulmonary epithelial cells by Vit. D is due to the AKT/NF-κB-mediated reduction of ICAM-1 expression, mitochondrial fission and mitophagy.

Vit. D has been found to be an essential nutrient necessary for the optimal absorption of dietary calcium and phosphate. Recently, due to the adverse effects of vitamin deficiency on health, Vit. D has received more and more attention worldwide. Vit. D deficiency impairs lung function and aggravates hypertension and inflammatory diseases, including COPD, asthma, tuberculosis, acute lung injury and its more severe form, acute respiratory distress syndrome (Andersen et al. 2015; Shi et al. 2016). 1α,25-Dihydroxyvitamin D_3 treatment increased mitochondrial volume and mitochondrial oxygen consumption rate, which are likely to influence muscle strength in human skeletal muscle cells (Ryan et al. 2016). In addition, Vit. D extensively modulates both innate and adaptive immune responses and could reduce systemic inflammation in patients. Vit. D reduced inflammatory responses in both endothelial cells and db/db mice exposed to a diabetes-like environment (Einbinder et al. 2016). The addition of 1,25-dihydroxyvitamin D_3 reduced TNF-α-induced inflammation in human chondrocytes (Avcioglu et al. 2020). In this study, TNF-α treatment increased tumor necrosis factor receptor-2 (TNFR2) expression in A549 cells, while Vit. D pretreatment did not affect this

expression (Supplementary Fig. 2). Vit. D treatment reduces the morbidity and mortality of patients with chronic kidney failure and end-stage renal disease by reducing inflammatory mechanisms (Assimon et al. 2012). Our previous studies and other reports demonstrated that the inflammatory cytokines TNF-α and IL-1β increased ICAM-1 expression and monocyte adhesion in human lung epithelial cells and lung tissues (Oh and Kwon 2009; Sung et al. 2015, 2018; Peng et al. 2018). Furthermore, this study shows for the first time to show that Vit. D reduces the ICAM-1 expression in TNF-α-treated A549 cells and mouse lung tissue. In addition, Vit. D inhibits the adhesion of monocytes to A549 cells treated with TNF-α. ICAM-1 is an important regulator of respiratory epithelial cell inflammation (Lee and Yang 2013). Our experiments provide evidence that Vit. D can reduce ICAM-1 expression and monocyte adhesion; therefore Vit. D may be an effective therapeutic agent for lung inflammation.

Cell survival or death is closely tied to mitochondrial function (Reddy 2014). Healthy mitochondria produce ATP which is needed by cells, but damaged mitochondria produce pathological and excessive ROS (Tang et al. 2015). In this study, we demonstrated that TNF-α-treated A549 cells experienced mitochondrial dysfunction, as demonstrated by decreased ATP production and mitochondrial membrane potential, while Vit. D reversed these effects. In order to prohibit cell death and sustain mitochondrial function, harmful mitochondria may enter into degradation and fission. Oxidative stress affects mitochondrial fission (Kroemer et al. 2007) but may also cause excessive mitochondrial fission, leading to the changes of mitochondrial structure and function, and cell damage (Reddy 2014). Using MitoTracker and TEM, we observed shorter mitochondria, indicating upregulation of mitochondrial fission, in TNF-α-treated A549 cells, while Vit. D reduced this change. Mitochondrial fission is regulated by DRP1 and Mff (Westermann 2010). DRP1, a member of the conserved dynamin GTPase superfamily, is necessary for mitochondrial fission (Shen et al. 2018) and is mainly present in the cytosol, however activated DRP1 migrates from the cytosol to the outer mitochondrial membrane, where it interacts with adaptors after external stimulation (Zhou et al. 2019a). It has been suggested that the function of DRP1 is related to various cellular processes, including autophagy, apoptosis and necrosis (Kim et al. 2018). The activity of DRP1 is controlled by phosphorylation by various kinases at three different sites, including Ser 616, Ser 637 and Ser 693 (van der Bliek et al. 2013). Phosphorylation of Ser616 promotes mitochondrial fission, leading to inflammation and apoptosis (Deng et al. 2020a, 2020b). High levels of DRP1 have been detected in RAW264.7 cells and mouse models of lipopolysaccharide (LPS)-induced acute lung injury (Deng et al. 2020b). TNF-α induces cardiomyocyte death by increasing the level of p-DRP1 Ser616 and mitochondrial translocation (Shen et al. 2018). It is well known that inhibiting DRP1 can prevent mucosal damage (Mazumder et al. 2019). Our data showed that TNF-α treatment upregulated the levels of p-DRP1 and Mff expression, while Vit. D reduced these effects. Importantly, we demonstrated that TNF-α-induced increases in ICAM-1 expression and monocyte adhesion could be reversed by Vit. D or by Mdivi-1, a mitochondrial fission inhibitor. Our study showed that Vit. D can be used as a mitochondrial fission inhibitor to reduce ICAM-1 expression and monocyte adhesion in TNF-α-treated A549 cells. Furthermore, in this study, stimulation with TNF-α increased the levels of mitochondrial fission-related proteins and ICAM-1 in the lung tissues of mice, while Vit. D treatment significantly decreased these levels. The previous study showed that Vit. D deficiency is associated with increased COVID-19 risk via altered mitochondrial dynamics, oxidative stress, and inflammatory states (de Las Heras et al. 2020). Based on the findings and our results, we suggest that the therapeutic effects of vitamin D_3 are mediated through the downregulation of ROS production and the improvement of mitochondrial function.

Healthy mitochondria sustain the mitochondrial life cycle to generate energy and participate in cell signal transduction, while deleterious mitochondria are cleared mainly through the mitochondrial autophagy (mitophagy) pathway, which is a lysosomal catabolic process accompanied by the expression of LC3B and Bnip3 (Yang and Klionsky 2010; Novak 2012). The effects of autophagy on lung inflammation are controversial (Zhang et al. 2019a; Qu et al. 2019; Li et al. 2016, 2019). In our study, TNF-α treatment significantly increased mitophagy by increasing Bnip3 expression. Additionally,

we used AO staining to demonstrate that TNF-α increased autophagolysosome. Furthermore, we used fluorescence microscopy to show that MitoTracker-labeled mitochondria colocalized with Bnip3, indicating the presence of mitophagy in TNF-α-treated A549 cells. Finally, interactions between Bnip3 and LC3B were increased in TNF-α-treated A549 cells. These results indicate that mitophagy is significantly activated by TNF-α stimulation in pulmonary epithelial cells, while Vit. D treatment can attenuate this effect. ROS have been suggested to be involved in the activation of HIF-1α (Qutub and Popel 2008). It has been reported that the Bnip3 promoter contains a functional hypoxia response element (HRE) and can be activated under hypoxic environment (Chen et al. 2016; Zhang et al. 2019b; Fu et al. 2020). Thus, HIF-1α activates the downstream protein Bnip3 and induces mitophagy. In our study, the expression of HIF-1α and Bnip3 was significantly increased in TNF-α-treated A549 cells, while Vit. D reduced this effect. These results indicate that Vit. D attenuates TNF-α-induced HIF-1α expression and further reduces the occurrence of mitophagy. Interestingly, Vit. D treatment decreased the expression of the mitophagy-related protein Bnip3 but increased the expression of LC3B in A549 cells with or without TNF-α stimulation. Previous study indicated that Vit. D modulates autophagy via VDR by directly upregulating transcription (Hoyer-Hansen et al. 2010). This discrepancy may be caused by VDR-driven transcriptional activation of LC3B under Vit. D treatment (Tavera-Mendoza et al. 2017). Therefore, Vit. D attenuated the mitophagy through the downregulation of Bnip3, while Vit. D sustained the autophagy probably via transcriptional activation of LC3B. In this study, VDR expression was present in A549 cells and 10 μM of Vit. D significantly increased VDR expression (Supplementary Fig. 3). Vit. D significantly increased the expression of 25(OH)D 1α-hydroxylase (CYP27B1, Supplementary Fig. 4). The present study suggested that the function of Vit. D used in the present study may be mediated by the increased VDR expression and converted to $1,25(OH)_2D_3$ via the increased 25(OH)D 1α-hydroxylase expression. In addition, to comprehend the mechanism of Vit. D effect on TNF-α-induced mitophagy, we also examined another pathway about PINK1-Parkin. TNF-α significantly increased the expression of PINK1 and Vit. D reduced the effect. However, TNF-α did not affect the expression of Parkin and even with Vit. D pretreatment (Supplementary Fig. 5). The expression of PINK1 and Parkin was not consistent, and further study is needed. Furthermore, the levels of mitophagy-related proteins were also decreased in lung tissues of mice, showing the same trend as in vitro study. In conclusion, our data indicated that the reduction of mitophagy is related to the protective impact of Vit. D on TNF-α-induced lung inflammation.

MAPK, PI3K/AKT and NF-κB pathways play vital roles in regulating the expression of pro-inflammatory cytokines, leading to the occurrence and development of lung inflammation (Lee and Yang 2013; Sung et al. 2015, 2018). In our study, as reported in previous studies, MAPK, AKT and NF-κB were strongly phosphorylated in A549 cells treated with TNF-α (Lee and Yang 2013; Oh and Kwon 2009). It has been reported that Vit. D attenuates inflammatory responses by reducing NF-κB activity (Mousa et al. 2017). Vit. D attenuated the LPS-induced inflammatory response in endothelial cells by inhibiting the PI3K/AKT/NF-κB signaling pathway (Zhou et al. 2019b). However, the detailed protective mechanism of Vit. D has not yet been determined. Our results showed that Vit. D significantly reduced the TNF-α-induced phosphorylation of MAPKs, AKT, and NF-κB. In addition, the TNF-α-induced increase in ICAM-1 expression and monocyte adhesion was markedly suppressed in the presence of inhibitors of either AKT or NF-κB. In addition, there is increasing evidence that the activation of MAPKs, AKT, or NF-κB is involved in mitochondrial fission and mitophagy (Aravamudan et al. 2014; Feng et al. 2018; Mao and Klionsky 2011). In this study, we demonstrated that TNF-α induces mitochondrial fission and mitophagy in A549 cells through the activation of NF-κB, while Vit. D reduces this effect.

Taken together, our current study shows that Vit. D prevents TNF-α-induced inflammation by the regulation of mitochondrial function. Vit. D inhibits TNF-α-induced mitochondrial fission and mitophagy by the inhibition of the expression of DRP1, Mff and Bnip3. This effect is mediated through the AKT/NF-κB pathway. Due to these findings, Vit. D should be considered a new and novel therapeutic agent for the targeting epithelial activation in lung inflammation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10565-021-09629-6.

Author contribution Yuh-Lien Chen, Tzu-Yi Chuang, and Hsin-Ching Sung has full access to all data in the study and are responsible for the integrity of the data and the accuracy of data analysis. Yu-Chen Chen contributed to the research design, conducted experiments, assisted the analysis of samples, and participated in the interpretation of the results and the writing of the manuscript. Tsai-Chun Lai, Tzu-Lin Lee, Chiang-Wen Lee, and I-Ta Lee participated in the discussion of the results.

Funding This study was supported, in part, by grants from Chang Gung Memorial Hospital, Taiwan (CMRPD1H0311, CMRPD1J0181), Ministry of Health and Welfare, Executive Yuan, Taiwan (11008), and from the Ministry of Science and Technology of Taiwan (MOST 108–2320-B002-065-MY3).

Data availability All the data and materials are available. These are all openly available.

Declarations

Ethics approval and consent to participate All animal procedures described in this study were conducted in accordance with the guidelines for the care and use of laboratory animals approved by National Taiwan University (IACUC: CGU108-33).

Consent for publication No personal information is included in this study.

Conflict of interest The authors declare no competing interests.

References

Andersen LB, Przybyl L, Haase N, von Versen-Hoynck F, Qadri F, Jorgensen JS, Sorensen GL, Fruekilde P, Poglitsch M, Szijarto I, Gollasch M, Peters J, Muller DN, Christesen HT and Dechend R. Vitamin D depletion aggravates hypertension and target-organ damage. J Am Heart Assoc. 2015;4(2). https://doi.org/10.1161/JAHA.114.001417

Aravamudan B, Kiel A, Freeman M, Delmotte P, Thompson M, Vassallo R, Sieck GC, Pabelick CM, Prakash YS. Cigarette smoke-induced mitochondrial fragmentation and dysfunction in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol. 2014;306(9):L840–54. https://doi.org/10.1152/ajplung.00155.2013.

Assimon MM, Salenger PV, El-Fawal HA, Mason DL. Nutritional vitamin D supplementation in haemodialysis: a potential vascular benefit? Nephrology (carlton). 2012;17(3):237–42. https://doi.org/10.1111/j.1440-1797.2011.01555.x.

Avcioglu G, OzbekIptec B, Akcan G, Gorgun B, Fidan K, Carhan A, Yilmaz G, Kozaci LD. Effects of 1,25-dihydroxy vitamin D3 on TNF-alpha induced inflammation in human chondrocytes and SW1353 cells: a possible role for toll-like receptors. Mol Cell Biochem. 2020;464(1–2):131–42. https://doi.org/10.1007/s11010-019-03655-z.

Babbar N, Casero RA Jr. Tumor necrosis factor-α increases reactive oxygen species by inducing spermine oxidase in human lung epithelial cells: a potential mechanism for inflammation-induced carcinogenesis. Cancer Res. 2006;66(23):11125–30. https://doi.org/10.1158/0008-5472.CAN-06-3174.

Bhatti JS, Bhatti GK, Reddy PH. Mitochondrial dysfunction and oxidative stress in metabolic disorders-a step towards mitochondria based therapeutic strategies. Biochim Biophys Acta Mol Basis Dis. 2017;1863(5):1066–77. https://doi.org/10.1016/j.bbadis.2016.11.010.

Brito RBO, Rebello JF, Grabulosa CC, Pinto W, Morales A Jr, Elias RM, Moyses RMA, Dalboni MA. 25-vitamin D reduces inflammation in uremic environment. Sci Rep. 2020;10(1):128. https://doi.org/10.1038/s41598-019-56874-1.

Cachon BF, Firmin S, Verdin A, Ayi-Fanou L, Billet S, Cazier F, Martin PJ, Aissi F, Courcot D, Sanni A, Shirali P. Proinflammatory effects and oxidative stress within human bronchial epithelial cells exposed to atmospheric particulate matter (PM(2.5) and PM(>2.5)) collected from Cotonou. Benin Environ Pollut. 2014;185:340–51. https://doi.org/10.1016/j.envpol.2013.10.026.

Cashman KD, van den Heuvel EG, Schoemaker RJ, Preveraud DP, Macdonald HM, Arcot J. 25-Hydroxyvitamin D as a biomarker of vitamin D status and its modeling to inform strategies for prevention of vitamin D deficiency within the population. Adv Nutr. 2017;8(6):947–57. https://doi.org/10.3945/an.117.015578.

Chen J, Bai M, Ning C, Xie B, Zhang J, Liao H, Xiong J, Tao X, Yan D, Xi X, Chen X, Yu Y, Bast RC, Zhang Z, Feng Y, Zheng W. Gankyrin facilitates follicle-stimulating hormone-driven ovarian cancer cell proliferation through the PI3K/AKT/HIF-1alpha/cyclin D1 pathway. Oncogene. 2016;35(19):2506–17. https://doi.org/10.1038/onc.2015.316.

Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. Physiol Rev. 2016;96(1):365–408. https://doi.org/10.1152/physrev.00014.2015.

de Las Heras N, Martin Gimenez VM, Ferder L, Manucha W and Lahera V. Implications of oxidative stress and potential role of mitochondrial dysfunction in COVID-19: therapeutic effects of vitamin D. Antioxidants (Basel). 2020;9(9). https://doi.org/10.3390/antiox9090897

Deng S, Zhang L, Mo Y, Huang Y, Li W, Peng Q, Huang L and Ai Y. Mdivi-1 attenuates lipopolysaccharide-induced acute lung injury by inhibiting MAPKs, oxidative stress and apoptosis. Pulm Pharmacol Ther. 2020a;62:101918. https://doi.org/10.1016/j.pupt.2020.101918

Deng X, Liu J, Liu L, Sun X, Huang J, Dong J. Drp1-mediated mitochondrial fission contributes to baicalein-induced apoptosis and autophagy in lung cancer via

activation of AMPK signaling pathway. Int J Biol Sci. 2020b;16(8):1403–16. https://doi.org/10.7150/ijbs.41768.

Dobnig H, Pilz S, Scharnagl H, Renner W, Seelhorst U, Wellnitz B, Kinkeldei J, Boehm B, Weihrauch G, Maerz W. Independent association of low serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels with all-cause and cardiovascular mortality. Arch Intern Med. 2008;168(12):1340–9. https://doi.org/10.1001/archinte.168.12.1340.

Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. Am J Physiol Renal Physiol. 2005;289(1):F8–28. https://doi.org/10.1152/ajprenal.00336.2004.

Einbinder Y, Ohana M, Benchetrit S, Zehavi T, Nacasch N, Bernheim J, Zitman-Gal T. Glucagon-like peptide-1 and vitamin D: anti-inflammatory response in diabetic kidney disease in db/db mice and in cultured endothelial cells. Diabetes Metab Res Rev. 2016;32(8):805–15. https://doi.org/10.1002/dmrr.2801.

Feng M, Wang L, Chang S, Yuan P. Penehyclidine hydrochloride regulates mitochondrial dynamics and apoptosis through p38MAPK and JNK signal pathways and provides cardioprotection in rats with myocardial ischemia-reperfusion injury. Eur J Pharm Sci. 2018;121:243–50. https://doi.org/10.1016/j.ejps.2018.05.023.

Ferri S, Crimi C, Heffler E, Campisi R, Noto A, Crimi N. Vitamin D and disease severity in bronchiectasis. Respir Med. 2019;148:1–5. https://doi.org/10.1016/j.rmed.2019.01.009.

Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive oxygen species in metabolic and inflammatory signaling. Circ Res. 2018;122(6):877–902. https://doi.org/10.1161/CIRCRESAHA.117.311401.

Fu ZJ, Wang ZY, Xu L, Chen XH, Li XX, Liao WT, Ma HK, Jiang MD, Xu TT, Xu J, Shen Y, Song B, Gao PJ, Han WQ and Zhang W. HIF-1alpha-BNIP3-mediated mitophagy in tubular cells protects against renal ischemia/reperfusion injury. Redox Biol. 2020;36:101671. https://doi.org/10.1016/j.redox.2020.101671

Haddad JJ, Land SC. A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha. FEBS Lett. 2001;505(2):269–74. https://doi.org/10.1016/s0014-5793(01)02833-2.

Harris J, Deen N, Zamani S, Hasnat MA. Mitophagy and the release of inflammatory cytokines. Mitochondrion. 2018;41:2–8. https://doi.org/10.1016/j.mito.2017.10.009.

Hoyer-Hansen M, Nordbrandt SP, Jaattela M. Autophagy as a basis for the health-promoting effects of vitamin D. Trends Mol Med. 2010;16(7):295–302. https://doi.org/10.1016/j.molmed.2010.04.005.

Hutchinson K, Kerley CP, Faul J, Greally P, Coghlan D, Louw M, Elnazir B, Rochev Y. Vitamin D receptor variants and uncontrolled asthma. Eur Ann Allergy Clin Immunol. 2018;50(3):108–16. https://doi.org/10.23822/EurAnnACI.1764-1489.46.

Kim H, Hwang J, Woo C, Kim E, Kim T, Cho K, Seo J, Lee S, Kim J. TNF-α-induced up-regulation of intercellular adhesion molecule-1 is regulated by a Rac-ROS-dependent cascade in human airway epithelial cells. Exp Mol Med. 2008;40(2):167–75. https://doi.org/10.3858/emm.2008.40.2.167.

Kim SR, Kim DI, Kim SH, Lee H, Lee KS, Cho SH and Lee YC. NLRP3 Inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation. Cell Death Dis. 2014;5:e1498. https://doi.org/10.1038/cddis.2014.460

Kim YY, Yun SH, Yun J. Downregulation of Drp1, a fission regulator, is associated with human lung and colon cancers. Acta Biochim Biophys Sin (shanghai). 2018;50(2):209–15. https://doi.org/10.1093/abbs/gmx137.

Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev. 2007;87(1):99–163. https://doi.org/10.1152/physrev.00013.2006.

Lee IT, Yang CM. Inflammatory signalings involved in airway and pulmonary diseases. Mediators Inflamm. 2013;2013:791231. https://doi.org/10.1155/2013/791231.

Li G, Li Y, Zheng SF, Han YB, Bai QL, Zhao T. Autophagy in pulmonary macrophages mediates lung inflammatory injury via c-Src tyrosine kinase pathway activation during mechanical ventilation. Eur Rev Med Pharmacol Sci. 2019;23(4):1674–80.

Li Y, Yu G, Yuan S, Tan C, Xie J, Ding Y, Lian P, Fu L, Hou Q, Xu B, Wang H. 14,15-Epoxyeicosatrienoic acid suppresses cigarette smoke condensate-induced inflammation in lung epithelial cells by inhibiting autophagy. Am J Physiol Lung Cell Mol Physiol. 2016;311(5):L970–80. https://doi.org/10.1152/ajplung.00161.2016.

Liu CW, Lee TL, Chen YC, Liang CJ, Wang SH, Lue JH, Tsai JS, Lee SW, Chen SH, Yang YF, Chuang TY and Chen YL. $PM_{2.5}$-induced oxidative stress increases intercellular adhesion molecule-1 expression in lung epithelial cells through the IL-6/AKT/STAT3/NF-kB-dependent pathway. Part Fibre Toxicol. 2018;15(1):4. https://doi.org/10.1186/s12989-018-0240-x.

Mao K, Klionsky DJ. MAPKs regulate mitophagy in Saccharomyces cerevisiae. Autophagy. 2011;7(12):1564–5. https://doi.org/10.4161/auto.7.12.17971.

Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema KJ, Coppes RP, Engedal N, Mari M, Reggiori F. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. Autophagy. 2018;14(8):1435–55. https://doi.org/10.1080/15548627.2018.1474314.

Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. Autophagy. 2015;11(8):1437–8. https://doi.org/10.1080/15548627.2015.1066957.

Mazumder S, De R, Debsharma S, Bindu S, Maity P, Sarkar S, Saha SJ, Siddiqui AA, Banerjee C, Nag S, Saha D, Pramanik S, Mitra K, Bandyopadhyay U. Indomethacin impairs mitochondrial dynamics by activating the PKCzeta-p38-DRP1 pathway and inducing apoptosis in gastric cancer and normal mucosal cells. J Biol Chem. 2019;294(20):8238–58. https://doi.org/10.1074/jbc.RA118.004415.

Moldoveanu B, Otmishi P, Jani P, Walker J, Sarmiento X, Guardiola J, Saad M, Yu J. Inflammatory mechanisms in the lung. J Inflamm Res. 2009;2:1–11.

Mousa A, Naderpoor N, Johnson J, Sourris K, de Courten MPJ, Wilson K, Scragg R, Plebanski M, de Courten

B. Effect of vitamin D supplementation on inflammation and nuclear factor kappa-B activity in overweight/obese adults: a randomized placebo-controlled trial. Sci Rep. 2017;7(1):15154. https://doi.org/10.1038/s41598-017-15264-1.

Naeini AE, Moeinzadeh F, Vahdat S, Ahmadi A, Hedayati ZP, Shahzeidi S. The effect of vitamin D administration on intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 levels in hemodialysis patients: a placebo-controlled, double-blinded clinical trial. J Res Pharm Pract. 2017;6(1):16–20. https://doi.org/10.4103/2279-042X.200994.

Ng Kee Kwong F, Nicholson AG, Harrison CL, Hansbro PM, Adcock IM and Chung KF. Is mitochondrial dysfunction a driving mechanism linking COPD to nonsmall cell lung carcinoma? Eur Respir Rev. 2017;26(146). https://doi.org/10.1183/16000617.0040-2017

Novak I. Mitophagy: a complex mechanism of mitochondrial removal. Antioxidants & Redox Signaling. 2012;17(5):794–802. https://doi.org/10.1089/ars.2011.4407.

Oh JH, Kwon TK. Withaferin A inhibits tumor necrosis factor-alpha-induced expression of cell adhesion molecules by inactivation of Akt and NF-kappaB in human pulmonary epithelial cells. Int Immunopharmacol. 2009;9(5):614–9. https://doi.org/10.1016/j.intimp.2009.02.002.

Park J, Choi H, Min JS, Park SJ, Kim JH, Park HJ, Kim B, Chae JI, Yim M, Lee DS. Mitochondrial dynamics modulate the expression of pro-inflammatory mediators in microglial cells. J Neurochem. 2013;127(2):221–32. https://doi.org/10.1111/jnc.12361.

Peng HL, Huang WC, Cheng SC, Liou CJ. Fisetin inhibits the generation of inflammatory mediators in interleukin-1beta-induced human lung epithelial cells by suppressing the NF-kappaB and ERK1/2 pathways. Int Immunopharmacol. 2018;60:202–10. https://doi.org/10.1016/j.intimp.2018.05.004.

Qu L, Chen C, He W, Chen Y, Li Y, Wen Y, Zhou S, Jiang Y, Yang X, Zhang R, Shen L. Glycyrrhizic acid ameliorates LPS-induced acute lung injury by regulating autophagy through the PI3K/AKT/mTOR pathway. Am J Transl Res. 2019;11(4):2042–55.

Qutub AA, Popel AS. Reactive oxygen species regulate hypoxia-inducible factor 1alpha differentially in cancer and ischemia. Mol Cell Biol. 2008;28(16):5106–19. https://doi.org/10.1128/MCB.00060-08.

Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, Jimenez-Sanchez M, Korolchuk VI, Lichtenberg M, Luo S, Massey DC, Menzies FM, Moreau K, Narayanan U, Renna M, Siddiqi FH, Underwood BR, Winslow AR, Rubinsztein DC. Regulation of mammalian autophagy in physiology and pathophysiology. Physiol Rev. 2010;90(4):1383–435. https://doi.org/10.1152/physrev.00030.2009.

Reddy PH. Inhibitors of mitochondrial fission as a therapeutic strategy for diseases with oxidative stress and mitochondrial dysfunction. J Alzheimers Dis. 2014;40(2):245–56. https://doi.org/10.3233/JAD-132060.

Roebuck KA, Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J Leukoc Biol. 1999;66(6):876–88.

Ryan ZC, Craig TA, Folmes CD, Wang X, Lanza IR, Schaible NS, Salisbury JL, Nair KS, Terzic A, Sieck GC, Kumar R. 1alpha,25-Dihydroxyvitamin D3 regulates mitochondrial oxygen consumption and dynamics in human skeletal muscle cells. J Biol Chem. 2016;291(3):1514–28. https://doi.org/10.1074/jbc.M115.684399.

Scherer PM, Chen DL. Imaging pulmonary inflammation. J Nucl Med. 2016;57(11):1764–70. https://doi.org/10.2967/jnumed.115.157438.

Scott I, Youle RJ. Mitochondrial fission and fusion. Essays Biochem. 2010;47:85–98. https://doi.org/10.1042/bse0470085.

Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu Rev Pathol. 2014;9:47–71. https://doi.org/10.1146/annurev-pathol-012513-104720.

Shen YL, Shi YZ, Chen GG, Wang LL, Zheng MZ, Jin HF, Chen YY. TNF-α induces Drp1-mediated mitochondrial fragmentation during inflammatory cardiomyocyte injury. Int J Mol Med. 2018;41(4):2317–27. https://doi.org/10.3892/ijmm.2018.3385.

Shi YY, Liu TJ, Fu JH, Xu W, Wu LL, Hou AN, Xue XD. Vitamin D/VDR signaling attenuates lipopolysaccharide-induced acute lung injury by maintaining the integrity of the pulmonary epithelial barrier. Mol Med Rep. 2016;13(2):1186–94. https://doi.org/10.3892/mmr.2015.4685.

Sung HC, Liang CJ, Lee CW, Yen FL, Hsiao CY, Wang SH, Jiang-Shieh YF, Tsai JS, Chen YL. The protective effect of eupafolin against TNF-α-induced lung inflammation via the reduction of intercellular cell adhesion molecule-1 expression. J Ethnopharmacol. 2015;170:136–47. https://doi.org/10.1016/j.jep.2015.04.058.

Sung HC, Liu CW, Hsiao CY, Lin SR, Yu IS, Lin SW, Chiang MH, Liang CJ, Pu CM, Chen YC, Lin MS, Chen YL. The effects of wild bitter gourd fruit extracts on ICAM-1 expression in pulmonary epithelial cells of C57BL/6J mice and MicroRNA-221/222 knockout mice: involvement of the MiR-221/-222/PI3K/AKT/NF-κB pathway. Phytomedicine. 2018;42:90–9. https://doi.org/10.1016/j.phymed.2018.03.023.

Tang Y, Liu J, Long J. Phosphatase and tensin homolog-induced putative kinase 1 and Parkin in diabetic heart: role of mitophagy. J Diabetes Investig. 2015;6(3):250–5. https://doi.org/10.1111/jdi.12302.

Tavera-Mendoza LE, Westerling T, Libby E, Marusyk A, Cato L, Cassani R, Cameron LA, Ficarro SB, Marto JA, Klawitter J, Brown M. Vitamin D receptor regulates autophagy in the normal mammary gland and in luminal breast cancer cells. Proc Natl Acad Sci U S a. 2017;114(11):E2186–94. https://doi.org/10.1073/pnas.1615015114.

van der Bliek AM, Shen Q and Kawajiri S. Mechanisms of mitochondrial fission and fusion. Cold Spring Harb Perspect Biol. 2013;5(6). https://doi.org/10.1101/cshperspect.a011072

Westermann B. Mitochondrial fusion and fission in cell life and death. Nat Rev Mol Cell Biol. 2010;11(12):872–84. https://doi.org/10.1038/nrm3013.

Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol. 2010;22(2):124–31. https://doi.org/10.1016/j.ceb.2009.11.014.

Zhang L, Huang J, Dong R, Feng Y, Zhou M. Therapeutic potential of BLT1 antagonist for COPD: involvement of inducing autophagy and ameliorating inflammation. Drug Des Devel Ther. 2019a;13:3105–16. https://doi.org/10.2147/DDDT.S215433.

Zhang Y, Liu D, Hu H, Zhang P, Xie R, Cui W. HIF-1alpha/BNIP3 signaling pathway-induced-autophagy plays protective role during myocardial ischemia-reperfusion injury. Biomed Pharmacother. 2019b;120: 109464. https://doi.org/10.1016/j.biopha.2019.109464.

Zhou K, Wu J, Chen J, Zhou Y, Chen X, Wu Q, Xu Y, Tu W, Lou X, Yang G, Jiang S. Schaftoside ameliorates oxygen glucose deprivation-induced inflammation associated with the TLR4/Myd88/Drp1-related mitochondrial fission in BV2 microglia cells. J Pharmacol Sci. 2019a;139(1):15–22. https://doi.org/10.1016/j.jphs.2018.10.012.

Zhou W, Yuan G, Wang Q. Vitamin D attenuates lipopolysaccharide-induced inflammatory response in endothelial cells through inhibition of PI3K/Akt/NF-kappaB signaling pathway. Pharmazie. 2019b;74(7):412–7. https://doi.org/10.1691/ph.2019.9373.