# VDR inhibits NLRP3 signal in lupus nephritis by competitively binding with importin 4 to suppress NF-κB nuclear translocation

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# Abstract

**Rationale**: Lupus nephritis (LN) is a major risk factor for morbidity and mortality in systemic lupus erythematosus patients, and lupus nephritis treatment is limited to immunosuppressive therapy with many problems. Vitamin D receptor (VDR) can regulate NLRP3 inflammasome which plays critical roles in LN pathogenesis.

**Objectives**: This study was designed to explore the therapeutic effect of VDR agonist on LN and its potential mechanisms, aiming to elucidatethe optimal therapy for LN.

**Findings**: In vivo, treatment of MRL/lpr mice since 8 weeks of age with VDR agonist paricalcitol for 8 weeks decreased disease pathogenesis of LN with markedly improved renal pathological changes, decreased urine protein and serum anti-ds-DNA antibody level in a time-depended manner. In MRL/lpr mice of 16 weeks of age with LN, the expression of NLRP3/caspase-1/IL-1β/IL-18 axis was upregulated detecteded by ELISA, RT-PCR, western blot and immunohistochemistry, while when treated with VDR agonist paricalcitol, expression of this axis was decreased significantly. Further, it is proved that VDR agonist paricalcitol modulated NLRP3/caspase-1/IL-1β/IL-18 axis via inhibiting NF-κB, in addition, co-immunoprecipitation results showed that VDR agonist suppressed NF-κB nuclear translocation by competitively binding with importin 4. In vitro, anti-dsDNA antibody induced apoptosis and upregulation of NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis in mRTECs, which could be reversed by VDR agonist paricalcitol.

**Conclusions**: Vitamin D receptor agonist may be a promising novel therapeutic strategy for patients with lupus nephritis, which paves the way for future preclinical/clinical studies.


# Introduction

Lupus nephritis (LN) is a major risk factor for morbidity and mortality in systemic lupus erythematosus (SLE) patients, renal-limited lupus nephritis has been reported as well, and 10% of patients with LN will develop to end stage renal disease (ESRD) [1]. Importantly, 10-year survival improves from 46–95% if disease remission can be achieved, lupus nephritis treatment is limited to immunosuppressive therapy, and existing problems include inadequate therapeutic response, medication related side effects, relapses of lupus nephritis, the optimal therapy for LN remains to be elucidated, so it is important to find potential effective disease remission strategies [2].

LN is immune complex-mediated glomerulonephritis with renal tubular dysfunction and tubulointerstitial inflammation [3, 4], recently, it is reported that tubulointerstitial inflammation may be the initiator of chronic renal injury and may predict response to therapy in LN [5]. Immunity/inflammation mechanism is a key component during the pathogenesis of LN [6], it is reported that over 110 immune- genes were differentially expressed between LN and healthy control kidney biopsies [7]. Immune cell infiltration participates in the pathogenesis of LN and associates with clinical features. Immune cells, including monocytes, B cells, and T cells, are recruited to kidney tissue and produce cytokines and chemokines

resulting tissue damage [8, 9]. Besides, plenty of inflammatory mediators have been implicated in the development and pathogenesis of lupus nephritis, such as IL-6, TNF-*α*, IFNs, and hyaluronan, which further drive the inflammatory processes [10]. A variety of immune mechanisms are involved in the onset and amplification of the inflammatory response in LN. Among them, inflammasome machinery plays an important role in promotion of renal damage during LN development, most inflammasomes involve NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), inflammasome NLRP3-generated cytokines aggravate nephritis in various murine models of lupus via NF-κB/NLRP3/caspase-1/IL-1β/IL-18 [11].

Vitamin D receptor (VDR) is an ancient nuclear receptor, vitamin D (VD) deficiency is implicated in various diseases, including SLE/LN [12]. VDR plays critical roles in transcriptional regulation, immunity, inflammation and proliferation, it relates with chronic tubulointerstitial changes, such as, cortical interstitial expansion, inflammation and fibrosis [13]. VDR signaling reduces inflammation by suppressing inflammatory signaling pathway, promoting anti-inflammatory cytokines secretion and regulating T-cell different [14, 15]. Recently, it is reported that VDR expression was downregulated in renal tissues of LN patients and was negatively correlated with disease activity and severity [16]. Combined with the pathogenesis mechanisms of LN, researchers proposed that VDR has the potential to be a novel therapeutic target for LN [17].

Untill now, the therapeutic effect of VDR agonist on LN has not been investigated, and the potential mechanisms are also unclear. Recently, it is reported that VDR inhibits NLRP3 activation which participated in LN development as described above in other diseases [18–20], at present the following issues need to clarify: 1) whether VDR agonist alleviates LN though inhibiting NLRP3 signal; 2) the molecular mechanism through which VDR inhibits NLRP3 signal.

Here, in this study, we try to test if an orally active VDR agonist would decrease disease pathogenesis in lupus-prone MRL/lpr mice. Additionally, we seek to delineate the cellular and molecular mechanism of action of VDR agonist in LN. We hope to provide the theoretical basis for potential clinical applications of vitamin D/VDR in LN.

# Materials And Methods

### Mice and paricalcitol treatment

The experimental animals were approved by the Institutional Animal Care and Use Committee of Xian jiaotong University. Female MRL/lpr mice (7 weeks of age) and age- and sex-matched C57BL/6 mice were purchased from Model Animal Research Center Of Nanjing University (Nanjing, China). C57BL/6 mice were used as control group, lupus-prone MRL/lpr mice were used as mouse model of LN [21]. The experimental animals were placed in a special pathogen-free environment (24 ± 1 °C, 50 ± 5% relative humidity and normal 12-h light/12-h dark cycle) in the Animal Experiment Center of Xian jiaotong University. All the mice were kept in controlled conditions for one week before the experiment. After 1 week acclimatization, all the MRL/lpr mice were continuously administrated intraperitoneally for 8 weeks

with VDR agonist paricalcitol (19-nor-1,25-dihydroxyvitamin D2, PAL, Abbott Laboratories, CA, USA, 300 ng/kg/mouse per dose, 5 times a week), the control groups were given equal dosage of saline. The amount of 24 h proteinuria were respectively obtained from mice using metabolic cages, and was assessed weekly, quantitative analysis of mouse urine protein was performed by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, MA, USA). All mice survived to the end of treatment, the mice were sacrificed at 0 week and 8 weeks after intervention (8 weeks old or 16 weeks old), respectively. Serum collected from mouse orbit was obtained by the centrifuge at 3000 rpm for 10 min at 4 °C, and stored at -20 °C for future ELISA use. Fresh Renal tissues were frozen at -80 °C for Western blot and PCR analysis. Renal tissues were fixed in 4% neutral-buffered formalin and embedded in paraffin for histopathological and immunohistochemistry analysis.

### Cells Culture

Mouse renal tubular epithelial cells (mRTECs) were obtained from Jennio-bio (Guangzhou, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C and 5% $CO_2$ in a humidified incubator after resuscitation. After starvation for 24 h, cells were stimulated with vehicle or PAL (0.2 ng/ml) for 24 h. In anti-dsDNA antibody stimulation experiment, mRTECs were treated with serum (the concentration of anti-dsDNA antibody was adjusted to 10 ng/ml) obtained from MRL/lpr mice with LN, for control group, mRTECs were treated with serum of same dilution obtained from normal C57BL/6 mice.

### Plasmid Construction

VDR overexpression plasmid was constructed. In brief, specific primers used to amplify the coding region of mouse VDR (NM_009504.04) were Mus-VDR-Forward: 5’-CCCAAGCTTATGGAGGCAATGGCAGCCAG-3’ and Mus-VDR-Reverse: 5’-CCGCTCGAGTCAGGAGATCTCATTGCCGA-3’. The above PCR products were TA subcloned into pcDNA3.1-Hygro(+) vector. The inserts were inserted into the XhoI and Hind III sites of the vector, plasmids containing the insert were isolated for sequencing.

### Cell Transfection

Cells were plated onto 6-well or 96-well plates at 50% confluence. Transient transfection of siRNA or plasmids of VDR was carried out using Lipofectamine 2000 reagent following the manufacturer’s procedure. Briefly, mRTEC cells were washed with serum-free medium and cultured in serum-free medium without antibiotics. The transfection complex (siRNA/plasmids and the transfection reagent mixture) were added to the medium in a drop-wise manner and mixed gently by rocking the media back and forth. After 4–6 h, the cell culture medium was changed back to DMEM containing serum and antibiotics and incubated at 37 °C for 48 h before proliferation assay, Western blot analysis, or PCR experiments.

The sequences of siRNA were as follows:

NFκB: sense: 5’-GCAGGUAUUUGACAUACUATT-3’;

anti-sense: 5’-UAGUAUGUCAAAUACCUGCTT-3’

NLRP3: sense: 5’- CCAACUGGUCAAGGAGCAUTT – 3’

anti-sense: 5’- AUGCUCCUUGACCAGUUGGTT – 3’

Negative control (NC): sense: 5’-UUCUCCGAACGUGUCACGUTT-3’;

anti-sense: 5’-ACGUGACACGUUCGGAGAATT-3’.

**RT-PCR**

Total RNA was extracted from Renal tissue or cultured cells using Trizol reagents (Sigma-Aldrich, USA). Reverse transcription was performed using Superscript III First-strand Synthesis System (Invitrogen, USA) and quantitative, real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems, UK). The relative abundance of target mRNA was normalized to that of the GAPDH, by a comparative cycle threshold method ($2^{-\Delta\Delta CT}$). The primer sequences were as listed below:

mus GAPDH: Forward: 5’-ATGGGTGTGAACCACGAGA-3’, and Reverse: 5’-CAGGGATGATGTTCTGGGCA-3’.

mus NLRP3: Forward: 5’-CTGGTCTGCTGGATTGTGTG-3’, and Reverse: 5’-AGAGCCCCTGTAGGTAGTCA-3’.

mus caspase1: Forward: 5’-GGAGGGAATATGTGGGACCA-3’, and Reverse: 5’-TTGTTTCTCTCCACGGCATG-3’.

mus VDR: Forward: 5’-CTTTGACCGGAATGTGCCTC-3’, and Reverse: 5’- GTGATGCGGCAATCTCCATT-3’.

mus NF-κB: Forward: 5’-CACCGGATTGAAGAGAAGCG-3’, and Reverse: 5’-AAGTTGATGGTGCTGAGGGA-3’.

mus importin-4: Forward: 5’-AAGCTACTGGGCCTCCTTTT-3’, Reverse: 5’-AGGAAGCTGAAGAGGTGACC-3’.

**Western blot**

Western Blots were performed to quantify the expression of GAPDH (rabbit, 1:1000, Goodhere Biological Technology, AB-P-R 001, Hangzhou, China), Caspase1 (rabbit, 1:1000, proteintech, 22915-1-AP, Wuhan, China), VDR (mouse, 1:500, Santa Cruz, SC-13133, Texas, USA), NF-κB (mouse, 1:500, Santa Cruz, SC-166588, Texas, USA), NLRP3 (rabbit, 1:300, Novus, NBP2-12446, CO, USA), Importin4 (rabbit, 1:10000, abcam, ab181046, MA, USA). Briefly, protein samples were loaded in equal amounts into individual wells of separated on a SDS-PAGE gel and subsequently blotted onto a nitrocellulose membrane (Millipore, MA, USA), and nonspecific sites on the membrane were blocked with 5% dried milk in Tris-Buffered Saline containing 0.05% Tween-20 (TBST buffer) for X-ray film. The membranes were incubated overnight at 4 °C with optimized dilutions of primary antibody as mentioned above. After the overnight incubation, membranes were washed with TBST buffer and re-incubated with a goat anti-rabbit or mouse IgG-HRP secondary antibody. The membranes were washed with TBST buffer and reacted with the electrochemiluminescence (ECL) substrate (Thermo, MA, USA) and exposed to X-ray film.

### Enzyme-linked Immunosorbent Assay (elisa)

The concentrations of IL-1β, IL-18 and anti-dsDNA antibody in serum of mice or cell supernatant were measured using mouse IL-1β (Elabscience Biotechnology, E-EL-M0038c, Wuhan, China), IL-18 (Elabscience Biotechnology, E-EL-M0730c, Wuhan, China) and dsDNA (CUSABIO, CSB-E11194m,Wuhan, China) ELISA kits following the manufacturer's protocol.

### Immunohistochemistry

Protein expression levels were detected in paraffin-embedded lung sections using the Importin4 (1:50 dilution), NF-κB (1:200 dilution), NLRP3 (1:150 dilution), VDR (1:2000 dilution), Caspase1 (1:200 dilution) antibodies, which were visualized using HRP-conjugated goat anti-rabbit/anti-mouse secondary antibody. Slides were counterstained with hematoxylin, dehydrated and mounted.

### Histopathologic Analysis

Renal tissues were collected and fixed in 10% neutral buffered formalin usually for 48–96 hours. Tissues were then routinely processed, embedded, sectioned (~4 μm), and stained with routine H&E stains for general examination. Periodic acid Schiff (PAS) stains were selected for observing renal glomerular basement membrane and the mesangial area.

### Flow Cytometry

Apoptosis of cells was determined by flow cytometric analysis using the AnnexinV-APC/7-AAD Assay kit (BD Biosciences, CA, USA) according to the manufacturer's instructions. Briefly treated mRTEC cells were harvested with trypsin, washed in cold PBS twice, and resuspended in binding buffer. A volume of 100 μL of the solution was transferred to a 5 mL culture tube and 5 μL AnnexinV-APC and 5 μL 7-AAD were added and incubated at room temperature for 15 minutes in the dark. Then, 400 μL of binding buffer was added to the culture tube. Samples were analyzed using CytoFLEX (Beckman Coulter, CA, USA) within 1hour after staining. Apoptotic cells were defined as AnnexinV-APC-positive, 7-AAD-negative cells.

### Co-Immunoprecipitation (Co-IP)

Cells were collected and lysed with lysis buffer on ice for 10–15 min and centrifuged at 10000 rpm at 4 °C for 10 min. The supernatant fractions were collected and incubated with appropriate antibody at 4 °C overnight and precipitated with protein A/G-agarose beads (Santa Cruz Biotechnology, sc-2003, Texas, USA) for another 3–6 h at 4 °C. The beads were washed with the lysis buffer 3 times by centrifugation at 3000 rpm for 5 min at 4 °C. The immunoprecipitated proteins were separated by SDS-PAGE, and western blotting was performed as previously described. The primary antibodies were as follows: anti-importin 4 (rabbit, abcam, Ab181046, MA, USA), anti-NF-κB (mouse, Santa Cruz, SC-166588, Texas, USA), anti-NLRP3 (mouse, Santa Cruz, SC-13133, Texas, USA). The second antibodies were HRP-goat anti-rabbit secondary antibody (BOSTER biological technology, BA1054, Wuhan, China) and HRP-goat anti-mouse secondary antibody (BOSTER biological technology, BA1051, Wuhan, China).

### Statistical analysis

Data were presented as means ± SEM. Comparison between the groups of data was evaluated using the Student's unpaired *t*-test. For multiple comparisons, one-way ANOVA was used with a Bonferroni post hoc test. A $p$ value < 0.05 was considered statistically significant.

# Results

### 1. Treatment of MRL/lpr mice with VDR agonist decreases disease pathogenesis of LN.

To test if VDR agonist paricalcitol would improve diesease in MRL/lpr mice with LN, MRL/lpr mice received 300 ng/kg/mouse of paricalcitol (Pari) or saline 5 times a week at 8 weeks of age for 8 weeks. Age- and sex-matched C57BL/6 mice were used as non-nephritic controls. Effects of Pari treatment on kidney disease were analyzed by assessing renal pathology and measuring proteinuria, serum anti-ds-DNA antibody. Compared with control group, renal pathology in the model group was deteriorated, as showed in Fig. 1A and 1B, we can see that glomerular mesangial cells and endothelial cells proliferated significantly, glomerular volume was increased compensatoryly, glomerular capillary wall damage and swollen renal tubular epithelial cells were observed, in addition, abundant inflammatory cells were infiltrated in renal tissues, while Pari treatment markedly improved the renal pathology in MRL/lpr mice with LN. Compared with controls, urine protein of MRL/lpr mice was increased gradually in a time-depended manner and reached the highest-level at 16 weeks of age, which could be reversed by Pari treatment (Fig. 1C). The same phenomenon was observed in the change of serum anti-ds-DNA antibody, as showed in Fig. 1D, at 8 weeks of age, there was no difference among the three groups, while at 16 weeks of age, the serum anti-ds-DNA antibody concentration was increased significantly compared with controls, and Pari treatment could lower the increased level of serum anti-ds-DNA antibody in MRL/lpr mice. Here we conclude that MRL/lpr mice presented with LN at 16 weeks of age, and Pari treatment could reduce disease severity of LN.

### 2. VDR agonist prevents LN by targeting NLRP3/caspase-1/IL-1β/IL-18 axis.

Next, we tested the role of NLRP3/caspase-1/IL-1β/IL-18 axis in MRL/lpr mice with LN and the effect of Pari treatment on this pathway axis. The expression of NLRP3/caspase-1/IL-1β/IL-18 axis in renal tissue among the three groups at 8 weeks of age showed no difference (Fig. 2A). Figure 2B showed that NLRP3/caspase-1/IL-1β/IL-18 axis was upregulated over time when the LN presented, compared with control mice, the expression of NLRP3/caspase-1/IL-1β/IL-18 axis was increased abviously in MRL/lpr mice at 16 weeks of age, and after treated with Pari, the expression of the axis was decreased significantly. Immunohistochemistry showed that the immunoreactivity of NLRP3 and caspase-1 significantly increased in the renal tissue from MRL/lpr mice at 16 weeks of age compared with those from control mice, and decreased after Pari treatment (Fig. 2C). NLRP3 and caspase-1-positive signals were predominantly found along the renal tubule epitheliums (Fig. 2C, e-f). These results suggest that NLRP3/caspase-1/IL-1β/IL-18 axis participated in the pathogenesis of LN, and it is a potential target for VDR agonist to prevent LN.

### 3. VDR agonist inhibits NLRP3/caspase-1/IL-1β/IL-18 axis through downregulating NF-κB expression in MRL/lpr mice with LN.

Then we investigated the upstream molecule through which VDR agonist Pari inhibited NLRP3/caspase-1/IL-1β/IL-18 axis in MRL/lpr mice with LN, and NF-κB that plays an important role in immunity and inflammation process was tested. Similar trend was observed on the expression of NF-κB as that of NLRP3/caspase-1/IL-1β/IL-18 axis. As showed in Fig. 2A and 2B, among the three groups at 8 weeks of age, the expression of NF-κB showed no difference. Compared with control mice, the expression of NF-κB was increased in MRL/lpr mice at 16 weeks of age significantly, and could be downregulated after treated with Pari. In addition, immunohistochemistry (Fig. 3C) showed that the immunoreactivity of NF-κB significantly increased in the renal tissue from MRL/lpr mice at 16 weeks of age compared with those from control mice, and decreased after Pari treatment. Together, these findings suggest that VDR agonist improved MRL/lpr mice with LN through NF-κB/NLRP3/caspase-1/IL-1β/IL-18 pathway.

### 4. VDR agonist suppresses NF-κB nuclear translocation by competitively binding with importin4.

As we know, tubulointerstitial inflammation may be the initiator of chronic renal injury, and VDR relates with chronic tubulointerstitial changes, our results also showed that NLRP3 were predominantly found along the renal tubule epitheliums, then we further explored the regulation mechanism of NF-κB inactivation by VDR agonist in mRTECs. From the Fig. 4A, we can see that both the total protein and nuclear protein of NF-κB were upregulated in mRTECs when treated with anti-dsDNA (10 ng/ml) that extracted from serum of MRL/lpr mice with LN, and Pari treatment could offset this effect, while the opposite trend was observed in the case of VDR expression. The obove findings suggest that expression of VDR and NF-κB had a "trade-off" relationship, including their nuclear translocation. Co-Immunoprecipitation results (Fig. 4B) showed that both VDR and NF-κB could combine with importin-4 to form a complex, and after overexpressing VDR, the binding of NF-κB and importin-4 was significantly reduced, indicating that VDR and NF-κB competitively binded with importin 4 to translocate into nucleus.

### 5. VDR agonist reverses anti-dsDNA-induced upregulation of NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis in mRTECs.

To investigate the mechanism of VDR activation for alleviating LN in vitro further, the effect of VDR agonist Pari on NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis of anti-dsDNA-treated mRTECs was observed. As showed in Fig. 5, compared with control group, after treated with anti-dsDNA (10 ng/ml) that extracted from serum of MRL/lpr mice with LN, NLRP3/caspase-1/IL-1β/IL-18 axis in mRTECs was upregulated. Pari treatment could reverse the increased expression of NLRP3/caspase-1/IL-1β/IL-18 axis induced by anti-dsDNA, and NF-κB/NLRP3 knockdown decreased its expression further.

### 6. VDR agonist inhibits mRTECs apoptosis via NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis.

It is reported that anti-dsDNA antibody could induce apoptosis of resident renal cells [22, 23]. In our study, the percentage of early apoptotic cells (APC+/7-AAD−, the lower right quadrant of density plot) of

mRTECs treated with anti-dsDNA antibody was clearly higher than that of control group (Fig. 6A), while after Pari treatment, the increased percentage of early apoptotic cells induced by anti-dsDNA antibody could be reversed. Knockdown of NF-κB/NLRP3 decreased the percentage of early apoptotic cells further, indicating that NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis, as a target of VDR, involved in mRTECs apoptosis. Moreover, in vivo experiments, the expression of Bax that functions as an apoptotic activator was increased in MRL/lpr mice with LN, which could be reversed by Pari treatment, and further decreased when knockdown NF-κB/NLRP3. For the expression of Bcl-2, which is an antiapoptotic protein and a member of the Bcl-2 family, opposite trend was observe compared with Bax (Fig. 6B). This indicates that VDR agonist inhibits anti-dsDNA antibody-induced mRTECs apoptosis via NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis.

# Discussion

To our knowledge, this is the first study to reveal the therapeutic effect of VDR agonist on LN and its potential mechanism. In the present study, we investigated the effects of paricalcitol on LN in MRL/lpr mice and demonstrated that 1) VDR agonist prevents LN in MRL/lpr mice and inhibits anti-dsDNA antibody-induced mRTECs apoptosis significantly by targeting NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis. 2) VDR agonist suppresses NF-κB nuclear translocation by competitively binding with importin 4, then inhibits NLRP3/caspase-1/IL-1β/IL-18 axis.

Nowdays many clues showed that VDR agonist-vitamin D analogues have the potential to be clinical treatment candidates for many kinds of dieases, such as, cancer and chronic renal diseases. Several studies have suggested that low circulating levels of vitamin D are associated with both an increased risk of developing cancer and poor outcome in patients with cancer, including renal cell carcinoma (RCC), especially colorectal, oral and breast cancer. VDR had anticancer effects, and supplementation with vitamin D analogues also could enhance response to standard therapies [24, 25], in addition, VDR activation was reported to ameliorate chemotherapy drug cisplatin-induced acute tubular necrosis [26]. Preclinical experiments suggest that vitamin D has the therapeutic potential for RCC through suppressing renal cancer cell proliferation and enhancing cancer cell death [27, 28]. For chronic kidney diseases, VDR agonists play a protective role in several models of renal disease. In dietary fat-induced renal disease mouse model, VDR agonists have been shown to prevent proteinuria, podocyte injury, mesangial expansion, accumulation of extracellular matrix proteins, infiltration with macrophages, and activation of markers of oxidative stress, inflammation, and fibrosis [29]. Recent clinical trials demonstrated that oral calcitriol can decrease proteinuria in IgA nephropathy patients, in its rats model, use of vitamin D can alleviate renal tissue damage by regulating immune response and NF-κB/TLR4 pathway [30]. A clinical observational study showed that supplementation with oral cholecalciferol for 6 months attenuate hypertension and proteinuria and delay the progression of polycystic kidney disease [31]. Together, VDR would be a novel potential therapeutic target for kidney diseases.

Vitamin D/VDR is involved in SLE pathogenesis, vitamin D3 could reduce the severity of SLE in MRL/l mice [32], it is reported that vitamin D deficiency could be a significant predictor of nephritis in SLE, and

has a direct relationship with increased disease activity and nephritis [33, 34]. Until now, the treatment effect of Vitamin D/VDR on LN has not be researched yet, in our study, we demonstrated that VDR agonist could prevent LN in MRL/lpr mice by improving renal pathological changes and decreasing proteinuria and serum anti-ds-DNA antibody. In vitro, we also found that VDR acitivation could ameliorate anti-dsDNA antibody-induced mRTECs apoptosis. All of these results indicate that VDR agonist could decrease disease pathogenesis of LN, which has the potential for clinical application.

Recent studies indicate that VDR is involved in the immune/inflammation regulation, several clues show that VDR can regulate NLRP3 inflammasome, VDR as a a nuclear receptor could translocate into nucleus to form a complex with NLRP3 and then block the formation of inflammasome [35]. While the mechanisms of VDR inhibiting NLRP3 inflammasome pathway remains unclear, especially in LN. According previous papers in other diseases, BRCC3-mediated deubiquitination of NLRP3 could be inhibited by VDR, and then NLRP3 activation is inhibited [18]. It has been proved that yes-associated protein 1 (YAP1) inhibit NLRP3 activation [36, 37], and VDR agonist could negatively regulate NLRP3 inflammation activation through activating YAP1 [20]. NLRP3 inflammasome has a crosstalk with Aryl hydrocarbon receptor (AhR) and NF-κB [38], vitamin D3 can increase the activation of AhR signaling, AhR blocks NF-κB binding sites in the NLRP3 promoter region, which result to suppress NLRP3 inflammasome activation [39]. In this study, we found a novel mechanism through which VDR inhibiting NLRP3 inflammasome. We revealed the crosstalk among VDR, importin 4 and NF-κB, nuclear translocation of VDR or NF-κB was decided by the histone nuclear import protein importin 4 that is known to shuttle between the cytoplasm and nucleus. Importin 4, identified as a interactive partner of VDR, is responsible for the ligand-independent nuclear translocation of VDR [40]. NF-κB-importins interaction mediates NF-κB translocation and NF-κB-dependent gene transcription [41, 42], while the interaction of NF-κB and importin 4 has not clarified yet. Our study showed that both VDR and NF-κB interact with importin 4 to complete nuclear transcription, and VDR could bond importin4 competitively to suppress NF-κB nuclear translocation and NF-κB-dependent NLRP3 transcription.

In conclusion, this work implicates the mechanism of VDR involved in LN pathogenesis and VDR agonist could be a potential novel anti-inflammation therapeutic strategy in clinical treatment of LN.

# Declarations


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### Author Contributions

Jing Huang, Ping Fan, Zhiming Hao, Lan He and Lei Wang designed research; Jing Huang, Bomiao Ju and Qi An analyzed data; Jing Huang, Bomiao Ju, Qi An, and Jing Zhang performed research; Jing Huang and Lei Wang wrote the paper.

### Declaration of interest

No conflicts of interest, financial or otherwise, are declared.

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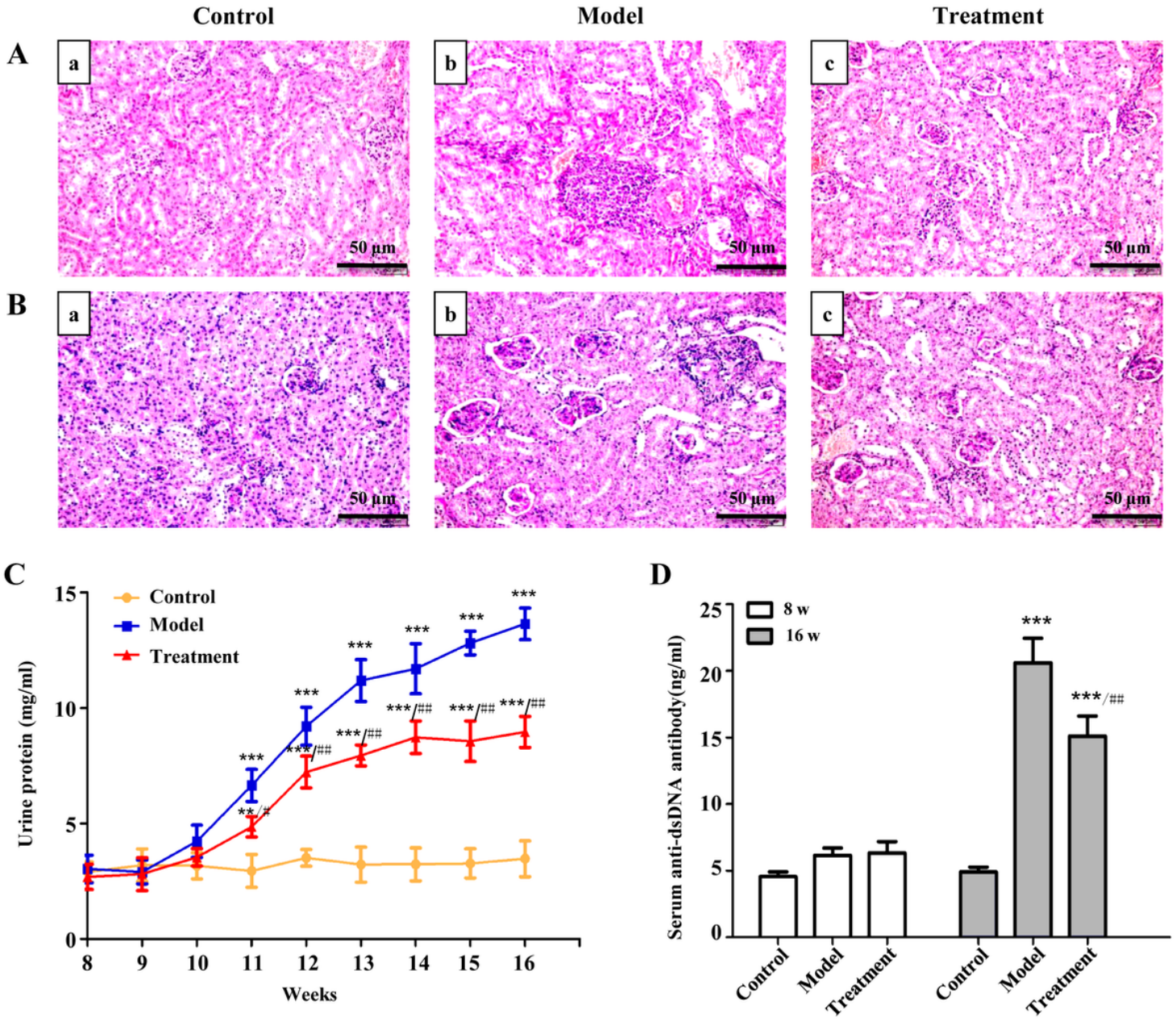
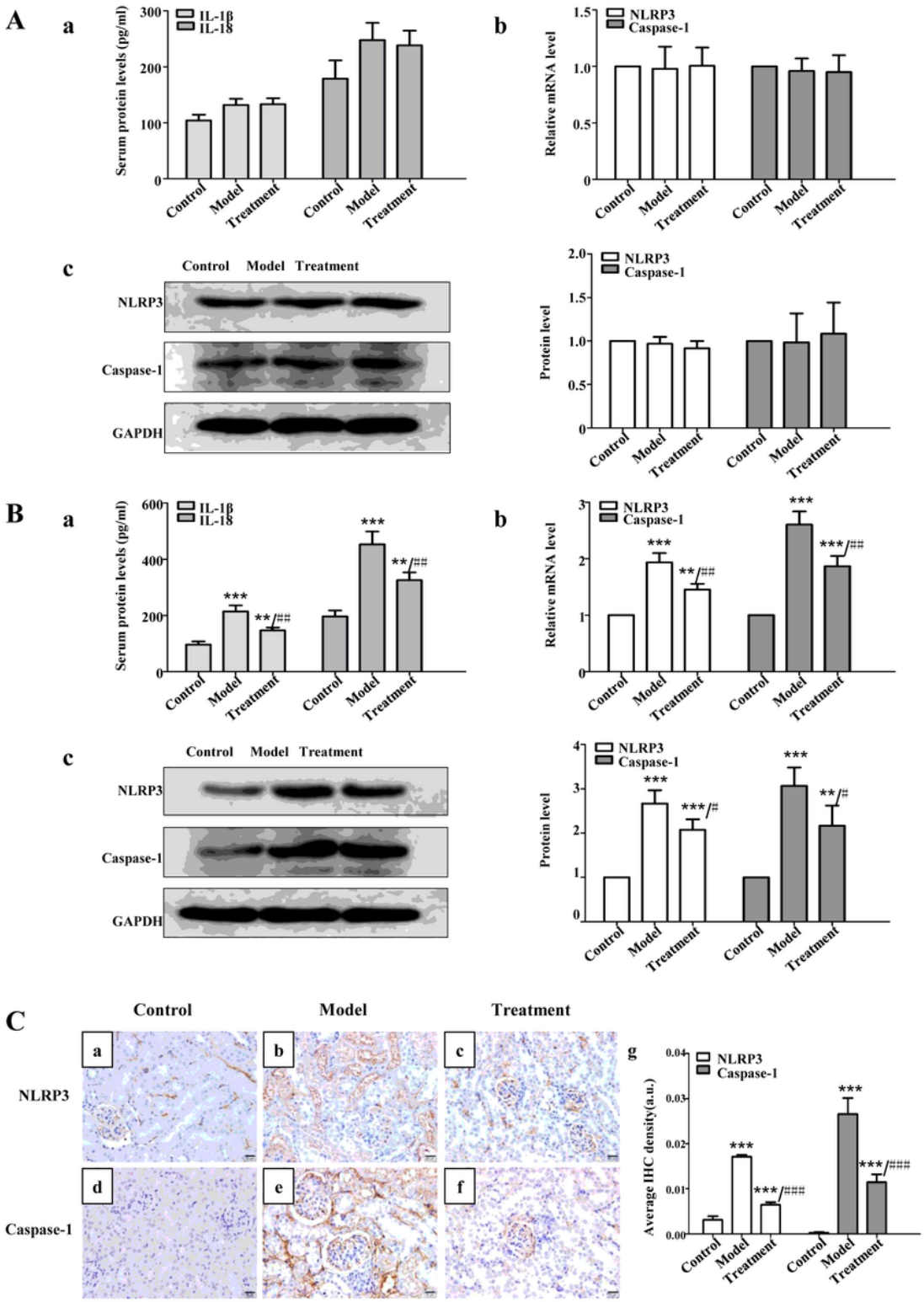
# Figures




## Figure 1

Renal disease measures are significantly improved by Pari treatment in MRL/lpr mice. (A, a-c) Renal sections stained with H&E from C57BL/6 mice (control), MRL/lpr saline-treated (model) and Pari-treated mice (treatment). Scale bars: 50 μm. (B, a-c) PAS staining of renal sections from control, model and treatment group. Scale bars: 50 μm. (B) The changes of urine protein level during the 8 weeks treatment in the three groups. *** $p<0.001$ (vs control); ** $p<0.01$ (vs control); ## $p<0.01$ (vs model); # $p<0.05$ (vs model). (C) Serum anti-dsDNA antibody level in 8 weeks old mice and 16 weeks old mice. *** $p<0.001$ (vs control); ## $p<0.01$ (vs model).




## Figure 2

Increased NLRP3/caspase-1/IL-1β/IL-18 axis in MRL/lpr mice with LN which could be blocked by VDR agonist. (A) The expression of NLRP3/caspase-1/IL-1β/IL-18 axis in MRL/lpr mice of 8 weeks old age. (a) Concentrations of IL-1β and IL-18 in the serum detected by ELISA. (b) mRNA level of NLRP3 and caspase-1 in renal tissue. (c) Protein level of NLRP3 and caspase-1 in renal tissue. (B) The expression of NLRP3/caspase-1/IL-1β/IL-18 axis in MRL/lpr mice of 16 weeks old age. (a) Concentrations of IL-1β and

IL-18 in the serum detected by ELISA. *** p<0.001; **/## p<0.01. (b) mRNA level of NLRP3 and caspase-1 in renal tissue. *** p<0.001; **/## p<0.01. (c) Protein level of NLRP3 and caspase-1 in renal tissue. *** p<0.001; ** p<0.01; # p<0.05. (C) Immunoreactivity for NLRP3 and caspase-1 (brown) in renal tissues of mice. (a-f) Immunohistochemistry staining of NLRP3 and caspase-1 in renal tissue sections from mice. Scale bars: 50 μm. (g) Bar charts for NLRP3 and caspase-1 staining of renal tissue sections from mice. ***/### p<0.001.

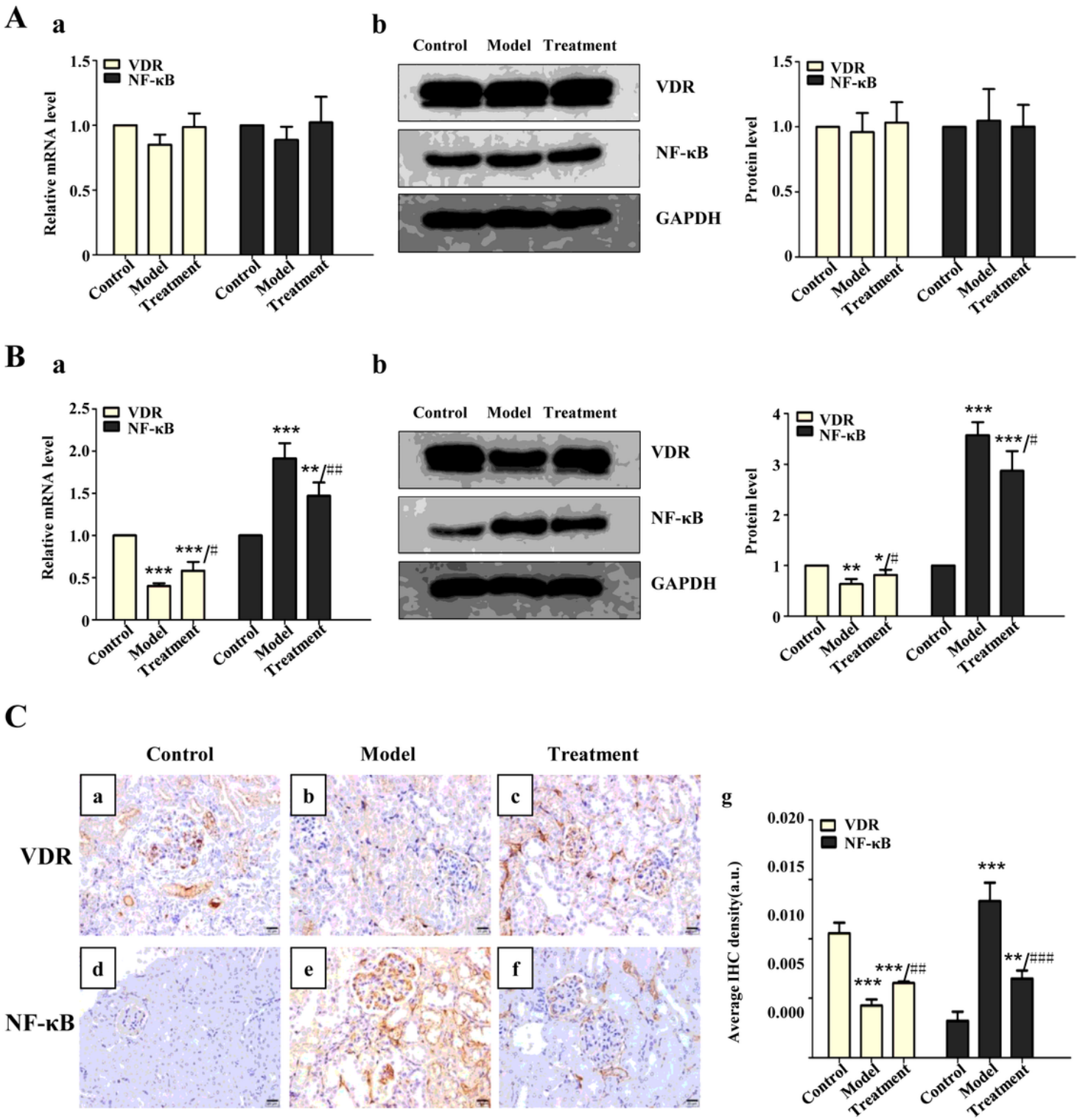
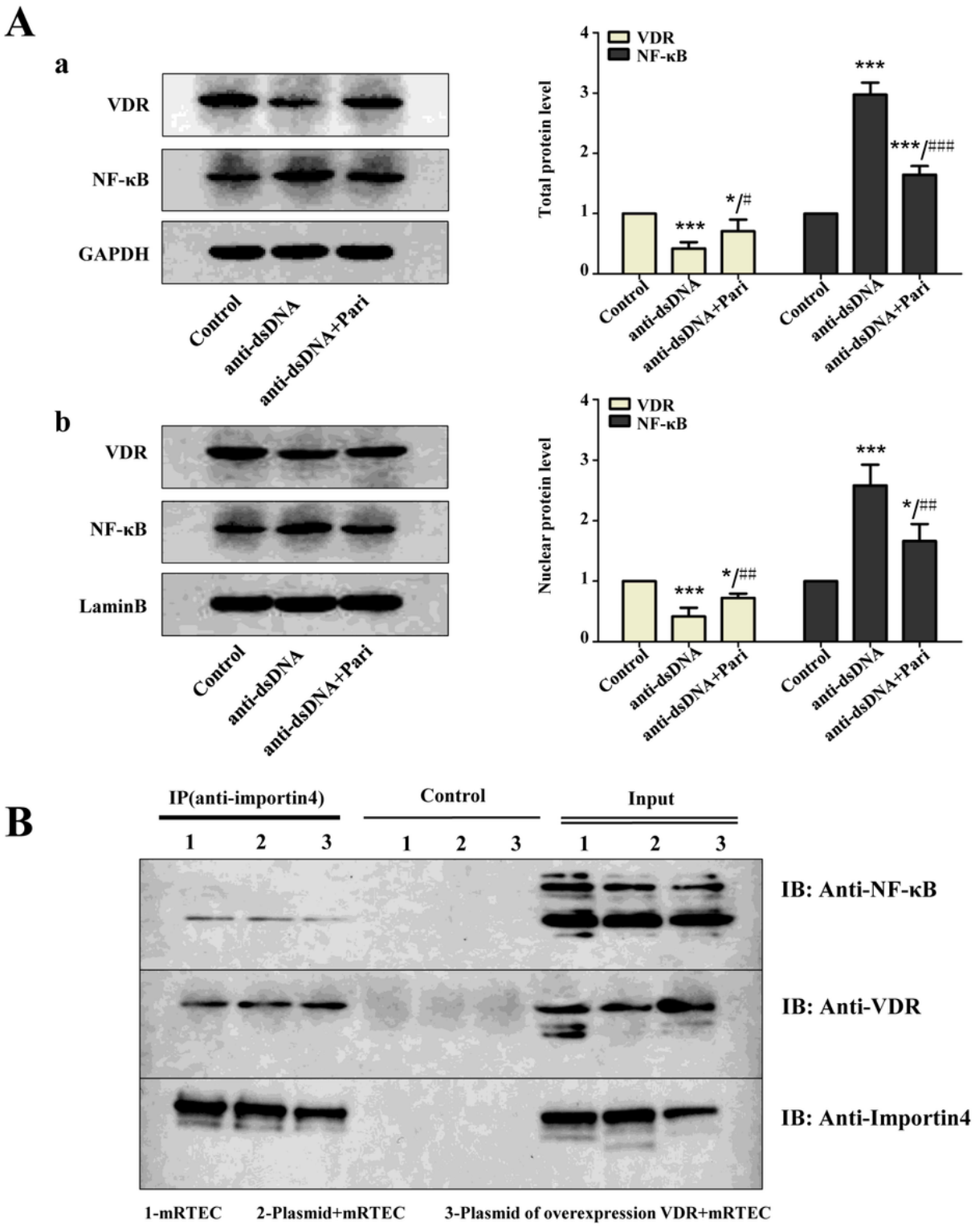



Figure 3

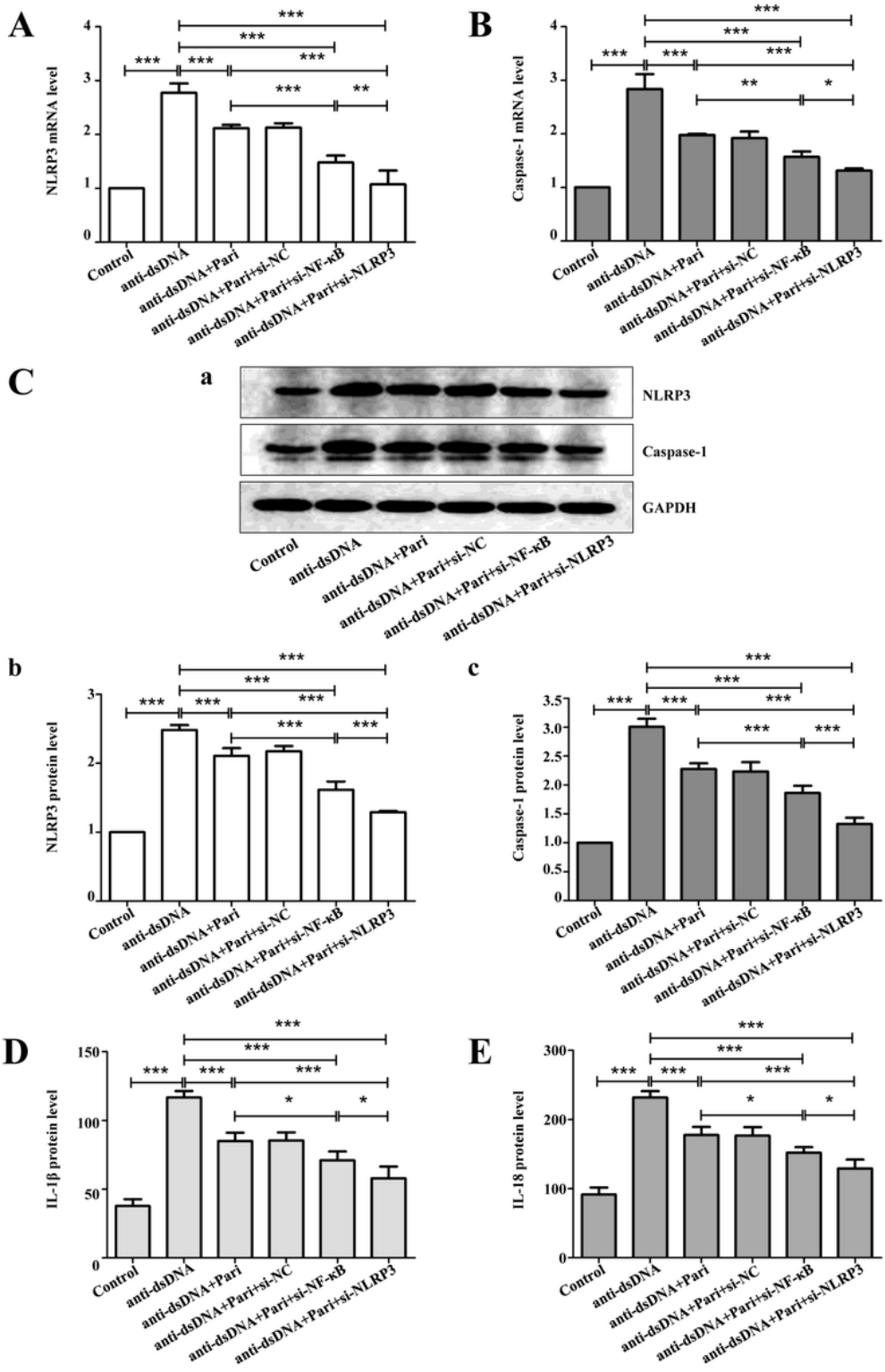
The expression pattern of VDR and NF-κB in control and model mice and the effect of VDR agonist on their expression. (A) The expression of VDR and NF-κB in MRL/lpr mice of 8 weeks old age. (a) mRNA level of VDR and NF-κB in renal tissue. (b) Protein level of VDR and NF-κB in renal tissue. (B) the expression of VDR and NF-κB in MRL/lpr mice of 16 weeks old age in mRNA level (a) and protein level (b). *** $p<0.001$; **/## $p<0.01$; */# $p<0.05$. (C) Immunoreactivity for VDR and NF-κB (brown) in renal tissues of mice. (a-f) Immunohistochemistry staining of VDR and NF-κB in renal tissue sections from mice. Scale bars: 50 μm. (g) Bar charts for VDR and NF-κB staining of renal tissue sections from mice. ***/### $p<0.001$; **/## $p<0.01$.




## Figure 4

VDR and NF-κB competitively bind importin 4 to translocate into nucleus. (A) The expression pattern of VDR and NF-κB in control and anti-dsDNA-treated mRTECs and the effect of VDR agonist (anti-dsDNA+Pari) on their expression. (a) Total protein of VDR and NF-κB in the three goups in mRTECs. *** p<0.001; ## p<0.01; */# p<0.05. (b) Nuclear protein of VDR and NF-κB in the three goups in mRTECs. *** p<0.001; ## p<0.01; * p<0.05. (B) Both VDR and NF-κB interact with importin 4. Western blot analysis of

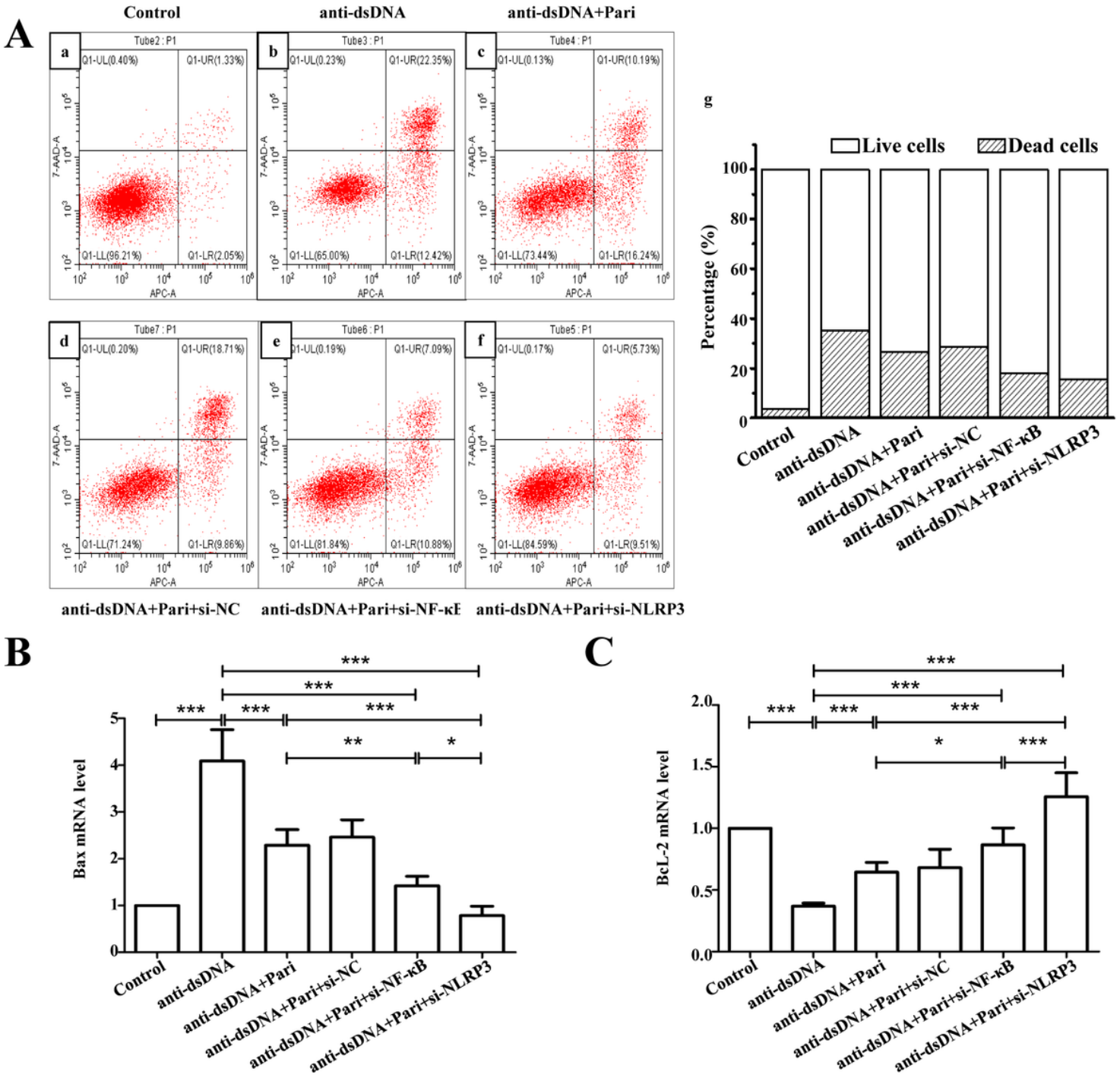
the co-immunoprecipitation experiment with three samples: mRTEC, mRTEC with control plasmid and mRTEC with VDR overexpression plasmid. Co-immunoprecipitation with anti-importin 4 was performed and VDR, NF-κB and importin 4 were visualized by western blotting.




## Figure 5

Anti-dsDNA-induced upregulation of NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis in mRTECs could be reversed by VDR agonist. (A, B) mRNA level of NLRP3 and caspase-1 in mRTECs with different treatments

respectively. *** p<0.001; ** p<0.01; * p<0.05. (C) Protein level of NLRP3 and caspase-1 in mRTECs with different treatments respectively. *** p<0.001. (D, E) Concentrations of IL-1β and IL-18 in the cell supernatant of mRTECs with different treatments detected by ELISA. *** p<0.001; * p<0.05.




## Figure 6

VDR agonist inhibits mRTECs apoptosis by targeting NF-κB/NLRP3/caspase-1/IL-1β/IL-18 axis. (A) mRTECs apoptosis after different treatments. (a-f) Density plot of cell flow cytometry of mRTECs with different treatments. For each panel, the lower left quadrant shows viable cells, the lower right quadrant represents early apoptotic cells, the upper right quadrant represents necrotic cells, and the upper left

quadrant represents dead cells. (g) Bar charts for percentage of live and dead cells respectively. (B) mRNA expression levels of Bax. *** $p<0.001$; ** $p<0.01$; * $p<0.05$. (C) mRNA expression levels of Bcl-2. *** $p<0.001$; * $p<0.05$.

## Supplementary Files

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