1,25-dihydroxyvitamin D3 ameliorates lupus nephritis through inhibiting NF- κ B and MAPKs signaling pathways in MRL/lpr mice

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Abstract

Lupus nephritis (LN) is a common and serious complication of systemic lupus erythematosus (SLE). However, the etiology and pathogenesis of LN remain unknown. 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂-VitD₃) is the active form of vitamin D, which has been known to have important functions in inflammation and immune diseases. In this study, its protective effects and underlying mechanism with time were investigated in MRL/lpr mice, a well-studied animal model for lupus. Briefly, MRL/lpr mice were randomly divided into VitD₃-treated group and control group. Both groups were treated for 3 weeks. Then at four time points after treatment, the skin lesions, histological changes, inflammatory factors and immunological examinations with time were analyzed between the groups. Furthermore, the NF-κB and MAPKs signaling pathways with time were also detected to explicate the underlying mechanism. Compared to the control group, mice in the VitD₃-treated group showed less skin lesions, less kidney injury, lower serum anti-ds DNA antibody, lower inflammatory cytokines TNF-α, IL-17 and higher serum complement C3; they also had less deposition of IgG, IgM and C3 within glomeruli. Moreover, the expressions of NF-κB and MAPKs signaling pathways were decreased, while those levels were increased with time. This study shows that 1,25-dihydroxyvitamin D₃ exerts a protective effect against lupus nephritis via inhibiting pro-inflammatory cytokines and regulating the NF-κB and MAPKs signaling pathways, which will be developed as a potential agent for the treatment of lupus nephritis. And the relationship between lupus activity and NF-κB and MAPKs signaling pathways over time was revealed.

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease of unknown etiology that is characterized by production of pathogenic autoantibodies and deposition of immune complexes (ICs) (1). Lupus nephritis (LN) is one of the most common and clinically challenging complications of SLE, with significant morbidity and mortality. Although some effective therapies for LN have been used in the clinical setting, chronic kidney damage still occurs in many patients (2). Even in patients having a relatively better clinical response to positive immunosuppression, kidney injury still accumulates rapidly (3). For this reason, the development of new effective therapeutic approaches of LN remains imperative. Active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂-VitD₃) is reported to regulate calcium and phosphorus metabolism (4). In recent years, 1,25-dihydroxyvitamin D₃ has become an important regulator of the immune system and it has immunomodulatory properties in different inflammatory diseases (5). It has been reported that the positive rate of A-ds DNA decreased significantly in SLE patients with vitamin D as a supplementary therapy (6). Thus, in this study, we investigated 1,25-dihydroxyvitamin D₃ protective effects and underlying mechanism of lupus nephritis.

Animal models have played an important role in probing the pathogenesis, progressive mechanisms, and clinical treatments of LN in vivo. The MRL/lpr mouse is universally acknowledged as spontaneous model of LN (7), which is characterized by numerous lymphadenopathy, massive autoantibodies and circulation

immune complexes (ICs) (8). Most MRL/lpr mice develop lupus nephritis between 12 and 24 weeks of age (7). The typical animal model provides a guarantee for the experiment.

The pathogenesis of LN is not yet clear. And the presence of autoantibodies that recognize various self-molecules and complement overactivation have been demonstrated to be related to the pathogenesis of LN (2, 3). Anti-double-stranded(ds) DNA antibody plays a significant role in kidney damages due to indirect or direct bind with glomerular antigens (9). Autoreactivity to complement components may have considerable pathological consequences, for example, complement C3. The degree of reduction in serum levels of C3 and deposition of C3 in glomerulus are related to pathogenesis of LN and associated with lupus activity (10). Besides, IgG and IgM antibodies are key components of adaptive humoral immunity but can lead to kidney damage if they bind self-antigen, as occurs in the autoimmune disease LN (11).

Pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α) is produced by monocytes and macrophages, exerting a variety of physiologic and pathogenic effects. And it is a key switch in the initiation and perpetuation of inflammatory responses (12). TNF- α contributes to the pathogenesis of autoimmune disease LN (13). In addition, interleukine-17 (IL-17) is the mainly signature of Th17 cells and it is also produced by double negative (DN) T-cells, macrophages, and neutrophils (14). IL-17 is crucial for the development of various autoimmune diseases by promoting Th17 cell-mediated tissue inflammation, including LN (15).

It has been confirmed that the intracellular signaling pathways, especially the NF- κ B and MAPAs signaling pathways, are involved in the process of LN due to their ability to regulate immune responses (16, 17). The NF- κ B (Nuclear Factor kappa B) transcription factor is an essential regulator of the regulation of numerous biological processes including development of the immune system, inflammation, and innate and adaptive immune responses (18). Many studies have shown that the activation of NF- κ B pathway is related to inflammatory reaction, and autoimmune activation is closely associated with NF- κ B pathway as well (19). That is, the NF- κ B pathway may play a significance role in LN. Furthermore, the extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway is involved in inflammatory and autoimmune diseases. This signaling pathway activation can regulate many cellular functions, including cell proliferation, oxidative stress and inflammation, leading to an aggravation of LN (17). Given the above, the two signaling pathways play crucial roles in the processes of LN, which may be the important targets to LN. However, existing literatures do not elaborate the relationship between lupus activity and NF- κ B and MAPKs signaling pathways with time. Thus, in the present study, we should investigate the regulative effect of 1,25-dihydroxyvitamin D₃ on the NF- κ B and MAPKs pathways to expound how it reduced the inflammatory and autoimmune responses with time, elucidating the underlying mechanism of 1,25-dihydroxyvitamin D₃ on LN.

Materials And Methods

Animals and treatments

Forty-eight female MRL/lpr mice (weighing 25.5 ± 3.0 g at 8 weeks old) were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and the quality certificate number: SCXK hu 2017-0005. All experiments were performed according to the institutional ethical guidelines on animal care and approved by the Institute Animal Care and Use Committee at the Weifang Medical University, Shandong, China. The mice were maintained under specific pathogen-free (SPF) conditions with free access to standard diet and tap water, and kept in a controlled temperature (20-24 °C) and humidity (50-55%) environment with a 12h light-dark cycle. All the mice were acclimatized to the environment for 2 weeks prior to the experiments.

At the age of 11 weeks, forty-eight MRL/lpr mice were randomly divided into 8 groups with 6 mice per group: the VitD3-treated groups and control groups. Mice in the VitD3-treated groups received 4μg/kg 1,25-dihydroxyvitamin D3 (Sigma, USA) in 1 dimethyl sulfoxide (DMSO, Sigma, USA) intraperitoneal injection twice a week for 3 weeks (mice were executed at 0,2,4,6 weeks after treatment); mice in the control groups received 1 DMSO intraperitoneal injections for 3 weeks (mice were executed at 0,2,4,6 weeks after injections). Then, the animals were weighed and anesthetized under 1% pentobarbital (35 ml/kg i.p.), and the blood and kidney samples were collected respectively at planned intervals (Figure 1).

Histological and immunohistochemistry analysis

The kidney samples were fixed in 4% paraformaldehyde solution and embedded in paraffin, sectioned into 5-μm slices. Hematoxylin and eosin (H&E) and Masson's trichrome stains were used for histological analysis. After deparaffinization, antigen retrieval, blocking by 3% H₂O₂ and 10% goat serum, antibodies against C3 (Proteintech, China) were used for immunohistochemistry staining. After the secondary antibody was incubated with biotin streptavidin HRP conjugate, the antibody staining color was displayed with DAB substrate solution. Besides, immunofluorescence detections of IgG and IgM in the kidney were implemented using fluorescent-dye conjugated antibodies against mouse IgG (Abcam, MA) and mouse IgM mu chain (Abcam, MA). All the images were obtained through a microscope equipped with a color camera (Nikon Eclipse Ni, Japan). The stains were scored by a renal pathologist blinded to the mice grouping. The scores of mesangial proliferation, interstitial congestion and fibrosis, C3 deposition were 0 to 4 (0, absent; 1, mild; 2, mild-moderate; 3, moderate; 4, severe). The grades of crescents and tubular lesions (atrophy, casts, dilatation, inflammatory infiltrates) were each scored from 0 to 4 (0, absent; 1, in < 25% of the section; 2, in 25–50% of the section; 3, in 50–75% of the section, and 4, in > 75% of the section). The maximum score for each mouse was 20 (mesangial hyperplasia, C3 deposits, crescent, tubulopathy, interstitial congestion and fibrosis were 4 points, respectively). Five random glomeruli in each section were measured for fluorescence intensity of IgG/IgM expressions by ImageJ.

Enzyme linked immunosorbent assay (ELISA) analysis

Serum samples were acquired from whole blood by centrifugation (4°C, 4000r, 10 min) and stored at -80°C to avoid freeze-thaw cycles for further examinations. Following the manufacturer's protocols, the

levels of serum, A-ds DNA, C3, TNF-α and IL-17 were detected by ELISA (Wuhan Boster Bio-engineering, China).

Western blotting analysis

Total protein was extracted from kidney samples in RIPA lysing buffer (Thermo Fischer Scientific, USA). And after BCA protein assay kit (Pierce, USA), the protein samples were mixed with loading buffer and boiled for 5 minutes. Then the protein samples were separated on electrophoresis gels and transferred onto PVDF membranes (Millipore, USA). The membranes were incubated with the following antibodies overnight at 4°C under agitation: NF-κBp65(1:1000, Cell Signaling Technology, USA), p-NF-κBp65(1:1000, Cell Signaling Technology, USA), IKBα(1:1000, Abcam, UK),p-IKBα(1:1000, Abcam, UK), IKKα/β (1:1000, Abcam, UK), p-IKKα/β(1:1000, Cell Signaling Technology, USA), ERK1/2 (1:1000, Cell Signaling Technology, USA), p-ERK1/2 (1:1000, Cell Signaling Technology, USA), JNK(1:1000, Abcam, UK), p-JNK(1:1000, Cell Signaling Technology, USA), p38(1:1000, Cell Signaling Technology, USA), p-p38(1:1000, Cell Signaling Technology, USA) and GAPDH (1:10,000, Abcam, UK), followed by incubation with the horseradish peroxidase-conjugated secondary antibody for 2h at room temperature under agitation. After ECL Detection Kit, the protein blots were collected and measured with ImageJ software.

Real-time quantitative PCR analysis

Total RNA was isolated from kidney samples by Trizol reagent (Takara Biotechnology, China). The cDNA was generated by using reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara Biotechnology, China) following the manufacturer’s protocols. RT-PCR was carried out for NF-κBp65, p38, ERK1, JNK, and GAPDH using LightCycler480 II (Roche). The levels of genes expression were analyzed by the equation $2^{-(\Delta\Delta Ct)}$. PCR primer sequences were listed in Table 1.

Table1. Primer sequences used in the study. (Primer Premier 5.0)

Genes	Forward primers (5'–3')	Reverse primers (5'–3')
NF-κBp65	CAGCATCCCTCAGCACCAT	CCATGGCTGAGGAAGGGA
p38	GACCTAAAGCCCAGCAACCT	CAGCCACGGACCAAATAT
ERK1	GCCTTCCAATCTGCTTATCAA	GATTTGGTGTAGCCCTTGGA
JNK	CAGTTTTACAGTGTGCAGGTGG	CTTTGCGTGCGTTTGTT
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Statistical analysis

Results were expressed as the mean ± standard deviation. SPSS statistical software (Version 20, USA) was used for statistical analysis. Data were analyzed by t-test and one-way analysis of variance (ANOVA) and p < 0.05 was considered to be statistically significant.

Results

General conditions

Eight-week-old MRL/lpr mice began to develop skin damage at different degrees. The skin lesions of mice and enlarged lymph nodes in control groups were obvious at the age of 20 weeks. In contrast, the mice treated by 4µg/kg 1,25-dihydroxyvitamin D3 twice weekly presented less skin damage and no enlarged lymph nodes (Figure 2). There was one mouse died in control group unexpectedly and no unscheduled death in VitD3-treated groups before 20 weeks age.

1,25-dihydroxyvitamin D3 ameliorated glomerular, tubular, and interstitial injuries in MRL/lpr mice

Lupus nephritis was observed by HE staining and Masson's trichrome staining. Light-microscopy analyses of kidney tissues revealed pathological alterations. In Figure 3, the stains of mice kidney showed that MRL/lpr mice in the control groups had significant nephritis than that in the VitD3-treated groups. The main pathological features include proliferation of endothelial and mesangial cells, basement membrane thickening, infiltration of inflammatory cells, ICs deposition, and even crescent formation. In HE stains, the control mice showed significant glomerulonephritis, increased number of matrix and cells in glomerulus, degeneration of renal tubular epithelial cells and necrosis of renal tubules, infiltration of numerous inflammatory cells, expansion of a small number of renal tubules, filling with eosinophils and forming tubular type, renal interstitial congestion and fibrosis. It was showed in Masson's trichrome stain that massive philoazaleine protein was deposited in strips under the capillary endothelium, resulting in basement membrane thickening. In addition, to explore the effect of 1,25-dihydroxyvitamin D3 on deposition of complement C3, immunohistochemistry was performed using renal tissue sections. As shown in Figure3, the deposition of complement C3 in all VitD3-treated groups were significantly reduced compared with the mice in control groups. Besides, the kidney damages of the older mice were more serious than that of the younger mice. These evidences illustrated that 1,25-dihydroxyvitamin D3 inhibits the inflammatory reactions in kidney, thus ameliorating glomerular, tubular, and interstitial injuries in MRL/lpr mice.

1,25-dihydroxyvitamin D3 decreased deposition of IgG/IgM in MRL/lpr mice

The deposition of antibodies plays a crucial role in the pathogenesis of lupus nephritis. We analysed the depositions of IgG and IgM in the glomeruli of mice by immunofluorescence. Then we found significantly decreased depositions of IgG and IgM in the glomeruli of VitD3-treated mice by immunofluorescence. Furthermore, IgG/IgM depositions on glomerulus increased with time in all groups. These results indicated that lupus activity increased with time and 1,25-dihydroxyvitamin D3 could alleviate lupus activity, which in turn reduced the symptoms of lupus. (Figure 4,5)

1,25-dihydroxyvitamin D3 reduced lupus activity and inhibited the pro-inflammatory cytokines

In general, the abnormal activation of autoimmunity was significantly lower in MRL/lpr mice treated with 1,25-dihydroxyvitamin D3. From 14 weeks to 20 weeks, the serum A-ds DNA level in MRL/lpr mice treated

with 1,25-dihydroxyvitamin D3 at all was significantly lower than that in control groups (Figure 6), there was no significant difference between the VitD3-treated groups but it increased with the age of weeks in the control groups. And as shown in figure 5 the level of serum C3 complement was increased in MRL/lpr mice with 1,25-dihydroxyvitamin D3 compared to the mice in control groups, whereas decreased with time.

In SLE patients with active nephritis, pro-inflammatory cytokines such as TNF- α and IL-17, are elevated in the serum, which are involved in the progression of renal damage. To determine the effect of 1,25-dihydroxyvitamin D3 on such pro-inflammatory cytokines, we detected TNF- α and IL-17 present in the serum. As shown in fig6, the levels of TNF- α and IL-17 are significantly reduced in the VitD3-treated groups compared to the control groups, whereas increased with time. The result demonstrated that lupus activity increased with time and 1,25-dihydroxyvitamin D3 has the capacity in inhibiting the excessive release of inflammatory cytokines, reducing lupus activity, and protecting renal function.

1,25-dihydroxyvitamin D3 inhibited the NF- κ B signaling pathway

NF- κ B signaling pathway has been shown to be involved in the regulation of inflammatory and immune responses, thereby playing an important role in LN. To determine the effect of 1,25-dihydroxyvitamin D3 on NF- κ B signaling pathway, the expressions of the NF- κ B pathway-related genes and proteins with time were measured. Real-time PCR assay showed that the mRNA expression levels of NF- κ BP65 in the control groups were higher than those of the VitD3-treated groups. Similarly, as shown in the Fig6, the Western blotting analysis showed that the protein expression levels of NF- κ Bp65, IKK α / β , I κ B- α and the phosphorylation of NF- κ Bp65, IKK α / β , and I κ B- α in the control groups were higher than those of the VitD3-treated groups (Figure 7). In order to detect the relationship between lupus activity and the expression level of NF- κ B signaling pathway, the MRL/lpr mice were executed for four time points. The results showed that the expression of NF- κ B signaling pathway was significantly increased along with time. Together, these data confirmed that 1,25-dihydroxyvitamin D3 could inhibit the activation of the NF- κ B pathway at the same time point in LN of MRL/lpr mice.

1,25-dihydroxyvitamin D3 blocked the MAPKs signaling pathway

To further explain the inhibitory mechanisms of 1,25-dihydroxyvitamin D3 on LN, we also analyzed the MAPKs signaling pathway in kidney tissue. Real-time PCR assay showed that, compared with the control groups, VitD3-treated groups significantly decreased the mRNA expression levels of ERK, JNK and P38, whereas increased with time (Figure 8). Additionally, compared to the control groups, the phosphorylation of ERK, JNK and P38 in the VitD3-treated groups were significantly decreased. However, there was no significant difference in the level of the ERK, JNK and P38 between the groups at the same time points. Furthermore, the expression of MAPKs signaling pathway was significantly increased along with time at the four time points (Figure 8). These results suggested that 1,25-dihydroxyvitamin D3 suppressed the activation of the MAPKs pathway which increased with time in LN.

Discussion

In the present study, we explored the effect of 1,25-dihydroxyvitamin D3 on lupus nephropathy with the widely used MRL/lpr lupus mice. Our study showed that 1,25-dihydroxyvitamin D3 was able to protect in MRL/lpr mice from lupus nephropathy as presenting lower levels of inflammatory and immunological makers, improved renal function and pathological changes. Furthermore, this study would explore the regulative effect of 1,25-dihydroxyvitamin D3 on the NF- κ B and MAPKs signaling pathways to expound its underlying mechanism of LN. And the relationship between lupus activity and NF- κ B and MAPKs signaling pathways with time was revealed.

Skin lesions are one of the typical symptoms of SLE and 72–85% of patients develop skin lesions over the course of SLE (20). Skin lesions are characterized by epidermal atrophy, hyperkeratosis, liquefactive degeneration at the layer of epidermal basal-cell, infiltration of inflammatory cells and free melanin pigment (21). It was observed that the MRL/lpr mice begin to have different degrees of skin lesions at the age of 8 weeks, especially in the skin of head, back, mouth and nose. However, 1,25-dihydroxyvitamin D3 treatment significantly attenuated the degree of skin lesions. The pathogenesis of skin lesions in SLE, which need to be further investigated, may be involved of skin deposited lupus IgG, immune cells, cytokines, intracellular molecules, and ultraviolet light (22).

The effect of 1,25-dihydroxyvitamin D3 on LN was confirmed by renal pathology and immunofluorescence of the MRL/lpr mice. The H&E and Masson's staining showed that the main pathological features include proliferation of endothelial and mesangial cells, basement membrane thickening, infiltration of inflammatory cells, ICs deposition, and even crescent formation. However, 1,25-dihydroxyvitamin D3 treatment significantly attenuated the degree of kidney injury.

There was study shown that anti-ds DNA autoantibodies were associated with lupus nephritis due to the facts that glomerular collagen is bound by DNA and nephritogenic antibodies are specific for DNA (23). Besides, anti-ds DNA antibodies can be eluted from the nephritic kidneys (24). The complement system plays an essential role in the occurrence, development and prognosis of LN as well. Complement factors are bound in kidney with deposited or formed immune complexes (25). The deposition of C3, which is component of the classical complement pathway, is characteristic of LN. Owing to activation by C1q and other unknown mechanisms, complement factor C3 is deposited in kidney (26). Moreover, another important factor that exaggerates complement activation is the activation of alternative pathway (27). These evidences suggested that not only classical pathway but also alternative pathway is involved in the pathogenesis of LN (28). The abnormally activated complement results in the deposition of complement in glomerulus, inducing the proliferation of glomerular mesangial cells, thereafter finally leads to nephropathy (29). Circulating C3 is usually depressed due to its consumption and is associated with disease (26). Furthermore, deposition of immune reactants plays a pivotal role in the occurrence, development, and prognosis of LN as well (30). The cross-reaction between immunoglobulin (IgG/IgM) and renal antigens plays key regulatory roles in many biological processes, such as activation of complement cascades, regulation of gene expression and cell proliferation (31). And it induces

phenotypic changes of glomerular resident cells, leading to kidney injury in LN (32). In our study, serum A-ds DNA and C3 activities showed markedly increased or decreased with time after onset, which may be associated with lupus activity; while 1,25-dihydroxyvitamin D3 treatment notably improved the activity of these serum markers, suggesting that 1,25-dihydroxyvitamin D3 ameliorated lupus nephritis and inhibited lupus activity. Additional evidence showed that 1,25-dihydroxyvitamin D3 treatment inhibited complement cascade and removed C3/IgG/IgM depositions on glomerulus to protect glomerular mesangial cells.

TNF- α and its receptors are involved in the pathogenesis of autoimmune diseases and TNF- α has both effects of checking autoimmunity and fostering inflammation (33). TNF- α contributes to the pathogenesis of LN as it promotes the activation and differentiation of macrophages, and its levels are increased in active LN and correlate with disease activity (34). IL-17 has a potential to induce the production of additional inflammatory cytokines and chemokines and to promote recruitment of inflammatory cells such as monocytes and neutrophils to the inflamed organ, being involved in the activation of many proinflammatory pathways (35). It has shown significant hyperactivation and participates in kidney damage in LN by inducing inflammation that trigger LN development (14). High serum level of IL-17 has been demonstrated in active LN and correlate with lupus activity (36). In the MRL/lpr mice, mice lacking IL-17 showed greatly improved survival and were largely protected from development of glomerulonephritis (37). In our study, the level of TNF- α and IL-17 was gradually upregulated with time in serum of MRL/lpr mice, demonstrating that TNF- α and IL-17 may be responsible for lupus activity. However, TNF- α and IL-17 were significantly reduced in VitD3-treated groups compared to the control groups. These finding demonstrated that 1,25-dihydroxyvitamin D3 has a protective effect against LN by suppressing pro-inflammatory cytokines release, to some extent.

To further investigate the underlying mechanism of 1,25-dihydroxyvitamin D3 against inflammation, the activation of the NF- κ B pathway was examined. The NF- κ B pathway is known to trigger the inflammatory responses, which controls the expression of several genes such as pro-inflammatory cytokines including TNF- α and is essential to self-reactive (16). The NF- κ B family of inducible transcription factors includes RelA (p65), RelB, c-Rel, p50 and p52. NF- κ B proteins usually exist as components of inactive cytoplasmic complexes bound by members of the inhibitor of κ B (I κ B) family that includes I κ B α and several structurally related proteins (38). In resting cells, I κ B α prevents the translocation of p65 into the nucleus. I κ B kinase (IKK) complex are activated by various stimuli, afterwards phosphorylate and degrade the I κ B α protein, releasing the p65 heterodimer to regulate transcription of various genes (39). In our study, the expression of NF- κ B signaling pathway was markedly upregulated with time in the kidneys of MRL/lpr mice, demonstrating that activation of NF- κ B signaling pathway is an important mediator of kidney injury in MRL/lpr mice and may be responsible for lupus activity. However, 1,25-dihydroxyvitamin D3 treatment evidently inhibited the expressions of NF- κ Bp65, I κ B α and IKK α / β , and markedly reduced the phosphorylations of NF- κ Bp65, I κ B α and IKK α / β , suggesting that 1,25-dihydroxyvitamin D3 attenuates inflammation responses mainly through inhibiting the NF- κ B signaling pathway. At the same time, we found the expression of pro-inflammatory cytokine TNF- α in serum were significantly reduced in VitD3-treated groups compared to the control groups, whereas increased with time. To a certain extent, our data

has further proved that the NF- κ B signaling pathway and downstream pro-inflammatory cytokine TNF- α , which may be associated with lupus activity, were inhibited by 1,25-dihydroxyvitamin D3.

Mitogen-activated protein kinases (MAPKs) are a family of protein-serine/threonine kinases, which mediate the basic biological process of responding to external stress signals, known with the extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK(1–3)) and p38(α , β , γ and δ) families (17). They are considered to be important regulators of inflammatory and innate immune responses. In response to inflammatory signals, MAPKs are activated by phosphorylation of ERK1/2, JNK and p38 (40). Furthermore, NF- κ B may be downstream of MAPKs and there are studies have shown that P38MAPKs signaling pathway is involved in the activation of NF- κ B (41). There may be a cross-talk between the signaling pathways. Under normal conditions, NF- κ B is located in the cytoplasm. Once stimulated by exogenous inducers or inflammatory cytokines, NF- κ B is separated from the cytoplasmic complex and transferred to the nucleus to regulate the expression of inflammatory mediators (42). Meanwhile, in our study, we found that MAPKs signaling pathway was also markedly upregulated with time in the kidneys of MRL/lpr mice, suggesting that activation of MAPKs signaling pathway is another important mediator of LN in MRL/lpr mice and may be associated with lupus activity as well. Therefore, decreasing the levels of the MAPKs and NF- κ B signaling pathways may be beneficial to LN. In this study, 1,25-dihydroxyvitamin D3 can significantly induce the expression of the phosphorylation of ERK1/2, JNK1/2 and p38, while there was no significant difference in the level of the ERK1/2, JNK1/2 and P38 between the groups at the same time point. Thus, our data showed that 1,25-dihydroxyvitamin D3 significantly inhibited NF- κ B and MAPKs signaling pathways to relieve lupus activity. All in all, NF- κ B and MAPKs signaling pathways should be potential targets for LN treatment.

Conclusion

In summary, this study demonstrated that NF- κ B and MAPKs signaling pathways markedly upregulated with time in LN in the classical MRL/lpr mice, suggesting that activation of NF- κ B and MAPKs signaling pathway are important mediators of LN and may be associated with lupus activity. Subsequent studies proved that 1,25-dihydroxyvitamin D3 can effectively ameliorate LN, which may be responsible for the inhibition of NF- κ B and MAPKs signaling pathway expressions and the suppression of pro-inflammatory cytokines release. At the same time, 1,25-dihydroxyvitamin D3 has little side effects or toxicity, suggesting that it may be developed as a potential natural medicine for the treatment of LN.

Declarations

Data Availability

All the data generated or analyzed during this study are included in this published article.

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This article is mainly written by XWL. YZ and NX wrote part of the manuscript and proofread the manuscript. EL helped us collect literature information and draw pictures. XLL reviewed the manuscript and proposed final revisions. All authors contributed to the article and approved the submitted version.

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Ethics declarations

Animal Ethics Approval

All animal experiments were approved by Institutional Animals Care and Use Committee at Weifang Medical University.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Figure 1

The experiment schedule. At the age of 11 weeks, Mice received 1,25-dihydroxyvitamin D3 or DMSO intraperitoneal injection twice a week for 3 weeks. The blood and kidney samples were collected respectively at 14, 16, 18, 20 weeks.

Figure 2

Treatment of MRL/lpr mice with 1,25-dihydroxyvitamin D3 inhibited the appearance of skin lesions and enlarged lymph nodes. (A) Before (11-week-old) and after (20-week-old) treatment of the same mouse with 1,25-dihydroxyvitamin D3. (B) 20-week-old MRL/lpr mice in VitD3-treated groups C. 20-week-old MRL/lpr mice in control groups show that treatment of MRL/lpr mice with 1,25-dihydroxyvitamin D3 inhibited the appearance of skin lesions and enlarged lymph nodes. (The blue arrow points to lymph nodes of mice in the control group. The red arrow points to lymph nodes of mice in the VitD3-treated group)

Figure 3

1,25-dihydroxyvitamin D₃ ameliorated glomerular, tubular, and interstitial injuries. (A) Histopathological features of the studied mice renal tissues determined by HE, Masson and immunohistochemistry (400×). (B) Scoring results. MRL/lpr mice in the VitD₃-treated groups revealed less renal damage. Scale bar = 50 μm. Values are expressed as means ± SD. * VS. the control group at the same time. # VS.

the control group at 14 weeks. Δ VS. the treated group at 14 weeks. $*\# \Delta p < 0.05$, $**\#\# \Delta \Delta p < 0.01$, $***\#\#\Delta \Delta \Delta p < 0.001$.

Figure 4

1,25-dihydroxyvitamin D3 decreased deposition of IgG in MRL/lpr mice. (A) The intensity of immunofluorescence. (B, C) Immunofluorescence findings for renal tissues from MRL/lpr mice (400×).

MRL/lpr mice in the VitD3-treated groups revealed less immunological deposition. Scale bar = 50 μ m. Values are expressed as means $\pm$ SD. * VS. the control group at the same time. # VS. the control group at T1. Δ VS. the treated group at T1. *# Δ p < 0.05, **## $\Delta\Delta$ p < 0.01, ***### $\Delta\Delta\Delta$ p < 0.001.

Figure 5

1,25-dihydroxyvitamin D3 decreased deposition of IgM in MRL/lpr mice. (A) The intensity of immunofluorescence. (B, C) Immunofluorescence findings for renal tissues from MRL/lpr mice (400×). MRL/lpr mice in the VitD3-treated groups revealed less immunological deposition. Scale bar = 50 μm. Values are expressed as means ± SD. * VS. the control group at the same time. # VS. the control group at T1. ΔVS. the treated group at T1. *#Δ p < 0.05, **##ΔΔp < 0.01, ***###ΔΔΔp < 0.001.

Figure 6

1,25-dihydroxyvitamin D3 reduced lupus activity and inhibited the pro-inflammatory cytokines. The concentration of A-ds DNA, C3, TNF-α and IL-17 were determined by ELISA. Values are expressed as means ± SD (n = 6/group). * VS. the control group at the same time. # VS. the control group at 14 weeks. ΔVS. the treated group at 14 weeks. *#Δ p < 0.05, **##ΔΔp < 0.01, ***###ΔΔΔp < 0.001.

Figure 7

1,25-dihydroxyvitamin D₃ inhibited the NF-κB signaling pathway. (A) The mRNA expression level of NF-κBp65 were determined by RT-PCR. (B) Protein levels were determined by Western blot, bands 1–8 represent the control groups at 14, 16, 18, 20 weeks and the VitD₃-treated groups at 14, 16, 18, 20 weeks respectively. Data are presented as means ± SD (n = 6/group). * VS. the control group at the same time. # VS. the control group at 14 weeks. Δ VS. the treated group at 14 weeks. *# Δ p < 0.05, **## Δ Δ p < 0.01, ***### Δ Δ Δ p < 0.001, ****#### Δ Δ Δ Δ p < 0.0001.

Figure 8

1,25-dihydroxyvitamin D₃ inhibited the MAPKs signaling pathway. (A) The mRNA expression levels of MAPKs were determined by RT-PCR. (B) The protein levels were determined by Western blot, bands 1–8 represent the control groups at 14, 16, 18, 20 weeks and the VitD₃-treated groups at 14, 16, 18, 20 weeks respectively. Data are presented as means $\pm$ SD ($n = 6/\text{group}$). * VS. the control group at the same time. # VS. the control group at 14 weeks. Δ VS. the treated group at 14 weeks. * $\# \Delta$ $p < 0.05$, ** $\# \Delta \Delta$ $p < 0.01$, *** $\# \Delta \Delta \Delta$ $p < 0.001$, **** $\# \Delta \Delta \Delta \Delta$ $p < 0.0001$.