Effects of 1,25(OH)2D3 and vitamin D receptor on peripheral CD4+/CD8+ double-positive T lymphocytes in a mouse model of systemic lupus erythematosus

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Abstract

This study aims to explore effects of 1,25(OH)2D3 and vitamin D receptor (VDR) on peripheral CD4+/CD8+ double-positive (DP) T lymphocytes in systemic lupus erythematosus (SLE). MRL-Lpr/Lpr mice with SLE (n = 20) and normal MRL mice (n = 20) were assigned into the control group (normal mice, without feeding with 1,25(OH)2D3), the 1,25(OH)2D3 group (SLE mice, feeding with 1,25(OH)2D3), the VDR-knock-in + 1,25(OH)2D3 group (SLE mice, VDR-knock-in, feeding with 1,25(OH)2D3) and the VDR-knockout group (normal mice, VDR-knockout, without feeding with 1,25(OH)2D3) (n = 10 per group). Levels of T lymphocytes were measured by flow cytometry. The mRNA and proteins expressions of inflammatory factors were measured by qRT-PCR and ELISA. Extracellular signal-regulated kinase-1/2 (ERK1/2) expression was measured by Western blotting.

Compared with normal mice, SLE mice showed reduced levels of CD4+, CD4+/CD8+ ratio, and DP lymphocytes. The levels of SLE-related indicators all increased significantly, followed with severe skin ulcers and urinary system infection. With the increase in time, skin ulcers and urinary system infection were significantly improved, levels of CD4+, CD4+/CD8+ ratio, and DP lymphocytes increased, and levels of SLE-related indicators all decreased in the 1,25(OH)2D3 group. There were no significant changes in bioindicators in the control and the VDR-knock-in + 1,25(OH)2D3 groups. The symptoms of SLE gradually occurred in the VDR-knockout group. This study demonstrates that VDR and 1,25(OH)2D3 could elevate CD4+/CD8+ DP T lymphocytes and reduce expressions of inflammatory factors, thus inhibiting the development and progression of SLE.

Keywords: 1,25(OH)2D3 – vitamin D receptor – systemic lupus erythematosus – CD4+ – CD8+ – T lymphocytes – mouse model

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder of connective tissues affecting multiple systems and is associated with a variety of clinical manifestations [1]. It is estimated that the incidence of SLE in Western Europe is 2.2 to 5 per 100,000 people, and the prevalence rate is 20.5 to 91 per 100,000 people [2]. Symptoms of SLE include neurologic complications, hair loss, ulcers, intermittent fevers, rash and arthritis [3]. However, majority of SLE patients have no obvious etiological reasons, and the long-term fluctuations of the disease cannot be fully explained, which may involve genetic (risk alleles), environmental, drug (anti-allergic drugs) or viral factors (retrovirus) [4, 5]. At present, a standard therapy of SLE includes using immunosuppressive drugs to suppress the immune response and excessive inflammation, among which the vitamin D (VD) is an ideal regulator of immune responses [6].

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The most active vitamin D metabolite is 1,25(OH)₂D₃, a steroid hormone whose synthesis involves the catalytic 1α-hydroxylase that transforms 25(OH)D₃ to 1,25(OH)₂D₃, a key factor in maintaining autoimmune tolerance, and participates in a variety of cell functions including differentiation, proliferation and apoptosis [7]. The VD insufficiency is associated with the pathogenesis of many autoimmune diseases including SLE, and it has been shown that 1,25(OH)₂D₃ inhibited the maturation of dendritic cells (DCs) and the activation of Th1 cells in SLE patients [8]. Vitamin D receptor (VDR), a member of the nuclear receptor superfamily, is also a DNA ligation and transcription factor, which is considered as a chromatin protein member of the nuclear receptor superfamily, is also a DNA ligation and transcription factor, which is considered as a chromatin protein.

Materials and methods

Ethical statement

Animal experiments were designed with the consent of the animal ethics committee, and all the studies were conducted strictly in accordance with international standards [11].

Model establishment and grouping

Six- to eight-week-old MRL-LPr/LPr mice with SLE and normal MRL mice (SLC Inc., Sizuoka, Japan) were selected. There were 20 mice for each type, with half females and half males, weighing 20–24 g. All mice were housed in clean animal rooms at 22–25°C, with normal circadian rhythm. And all mice were given free access to food and drinking water. SLE VDR-knock-in mice and normal VDR-knockout mice models were established and identified by Seajet Scientific Inc. (Beijing, China). The grouping of mice is shown in Table 1, with 10 mice in each group.

Specimen collection and processing

After fed for 8, 16 and 24 weeks, the body weights of all mice were measured. Then, the mice were anaesthetized with intraperitoneal injection of 10% chloral hydrate in saline (Boster, Wuhan, China). The blood samples were drawn from retinal venous plexus. Heparin was added into the blood samples to prevent clotting. The samples were divided into two parts, one was used for the determination of T lymphocytes by flow cytometry, and the other was used to detect the expressions of inflammatory cytokines and biochemical indicators. Both kidneys in the mice were collected. Part of the kidney samples was used to detect the expressions of inflammatory cytokines by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. The other part of kidney samples was fixed in 10% neutral formalin, washed with PBS, dehydrated with gradient alcohol, processed with xylene and embedded in paraffin (Thermo Fisher Scientific Inc., Waltham, MA, USA). The embedded samples were cut into 5-μm-thick slices and were used for the observation of tissue morphology.

Isolation of peripheral blood T lymphocytes

Tissue diluent (Invitrogen Inc., Carlsbad, CA, USA) was mixed with blood samples with a 1:1 ratio. Equal volume of lymphocyte separation medium (Invitrogen Inc.) was added carefully. The mixture was centrifuged at 4°C in a Thermo centrifuge. After the centrifugation, there were four layers of visible cells in the centrifuge tube. The second layer that contained milky lymphocytes was collected and washed by adding 5 ml cell washing buffer (Invitrogen Inc.). The samples were then fully mixed and centrifuged for 10 min. at 2000 r.p.m. The supernatant was discarded, and the remaining precipitate was mononuclear cells. The cells were resuspended in 80 μl of immunomagnetic buffer solution and 20 μl of magnetic beads (Miltenyi biotec, Bergisch Gladbach, Germany) were added into the tube. The suspension was maintained at 4°C in the dark for 15 min. and centrifuged at 1000 r.p.m. for 10 min. The supernatant was discarded, and 500 μl of buffer solution were added to resuspend the cells. The cells were then transferred into a magnetic separation column (Miltenyi biotec) which was washed twice with the buffer solution. At the end, the magnetic field was removed and the T lymphocytes were collected with 1 ml of RPMI-1640 medium (Hyclone, Thermo Fisher Scientific Inc.).

Flow cytometry

The collected T lymphocytes were diluted to 1 × 10⁶/ml. A volume of 100 μl cell suspension was collected in the Eppendorf tube for the measurement. Fluorescein isothiocyanate (FITC)-labelled anti-IgG1 antibody (IgG1-FITC; Becton, Dickinson and Company, BD Bioscience, Sam lose, CA, USA) and phycoerythrin (PE)-labelled anti-IgG antibody (IgG-PE; Becton, Dickinson and Company) of 10 μl each were added into

| Group               | Treatment                                                                 |
|---------------------|----------------------------------------------------------------------------|
| VDR-knock-in + 1,25(OH)₂D₃ group | MRL-LPr/LPr mice, VDR-knock-in, feeding with 1,25(OH)₂D₃                  |
| Control group       | Normal mice, feeding without 1,25(OH)₂D₃                                  |
| VDR-knockout group  | Normal mice, VDR-knockout, feeding without 1,25(OH)₂D₃                   |

VDR, vitamin D receptor.
the negative control. In the testing samples, 10 μl of monoclonal antibody for CD4 (Becton, Dickinson and Company) or CD8 (Becton, Dickinson and Company) was added and incubated in the dark for 20 min. A volume of 100 μl haemolysin (Becton, Dickinson and Company) was subsequently added and incubated in the dark for 15 min. The mixture was centrifuged again, and the supernatant was discarded. The samples were then measured using flow cytometry (Becton, Dickinson and Company). The cells were sorted with a flow rate of 4000 cells per second. The number of analysed cells should not be less than 2000. The results were analysed with the CellQuest software. The changes in CD4+, CD4+CD8+, DP, CD8+ and double-negative (DN) cells were analysed with the CS2000 software.

Detection of serum biochemical indexes

Blood urea nitrogen (BUN) was detected by continuously monitoring of the urease level according to the manufacturer’s instruction of a reagent kit (Rongsheng biotech, Shanghai, China). In brief, 10 μl of samples was added into a test tube, and 10 μl of calibration solution was added into a control tube. The colorimetric values of the two tubes were immediately measured after mixing using a Thermo spectrophotometer. The absorbance of the mixture was measured at 30 sec. (A1) and 90 sec. (A2), and the serum level of BUN (mmol/l) was calculated as:

\[
\text{BUN} = \frac{A1}{A2} \times C_{\text{calibration solution}}
\]

The serum creatinine (Cr) was detected by a picric acid method, according to the manual of the reagent kit (Rongsheng biotech). In brief, 100 μl of sample solution was added into a test tube, and 100 μl of calibration solution was added into a control tube. The tubes were incubated in a 37°C water bath for 30 sec., and the initial absorbance (A1) and the absorbance at 90 sec. (A2) were measured at the wavelength of 505 nm. The level of Cr (μmol/l) was calculated as:

\[
\text{Cr} = \frac{A1}{A2} \times C_{\text{calibration solution}}
\]

Enzyme-linked immunosorbent assay (ELISA)

The ELISA measurements were conducted in strict accordance with the manual of the experimental kit (eBioscience, San Diego, CA, USA). In brief, the ELISA kit was equilibrated at room temperature for 20 min. before using it to prepare the experimental solutions. After the standard was dissolved, 100 μl of standard solution was added into the reaction plate to produce the standard curve. A volume of 100 μl sample solution was added into each well, and the plate was incubated at 37°C for 90 min. After washing the plate, 100 μl of promptely worked solution containing biotinylated antibodies was added into the wells and incubated at 37°C for 60 min. After the second washing, 100 μl of promptly made solution containing enzyme-bound reagents were added and incubated in the dark at 37°C for 30 min. The plate was then washed consecutively for three times, and 100 μl of substrate solution were added and incubated in the dark at 37°C for 15 min. Finally, stop solution was quickly added into the plate to terminate the reactions, and within 3 min., the optical density (OD) values were measured at a wavelength of 450 nm using a multifunction plate reader (Synergy 2; Bio Tek Instruments, Inc., Winooski, VT, USA). The standard curve was drawn based on the measured OD values, and the plasma levels of interleukin-4 (IL-4), IL-10, IL-17 and interferon-γ (INF-γ) were calculated from the standard curve.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRizol (Invitrogen Inc.) was used to extract mice renal RNA. The RNA concentration and purity were measured with NanoDrop2000 (Thermo Fisher Scientific Inc.). According to the gene sequences published in the Genbank database, PCR primers were designed using the Primer5.0 software and were synthesized (GenePharma Co., Ltd., Shanghai, China). The primer sequences are shown in Table 2. The PCR was performed using the PRISM 7500 PCR System (Applied Biosystems, Foster City, CA, USA). The PCR system included 12.5 μl of SYBR premix ex Taq II (Takara Holdings Inc., Kyoto, Japan), 1 μl each of forward and reverse primers, 2 μl of DNA template and 8.5 μl of sterile water. The β-actin was used as internal control. The PCR conditions were as follows: 94°C pre-denaturation for 3 min.; 40 cycles of 94°C denaturation for 30 sec., 54°C annealing for 30 sec. and 72°C extension for 60 sec. The reliability of the PCR results was evaluated using the dissolution curve. The cycle threshold (Ct) values were obtained to calculate the relative gene expressing using 2^{-ΔΔCt}, where ΔΔCt = Ct (target gene) – Ct (internal control), and ΔΔCt = ΔCt (sample group) – ΔCt (control group) [12].

Western blotting

Mouse renal proteins were extracted and the protein concentrations were measured by a BCA Kit (Boster). The extracted proteins were denatured by adding a buffer solution and boiled for 10 min. at 95°C.

Table 2 Primer sequences for the qRT-PCR

| Gene | Sequence |
|------|----------|
| IL-4 | Forward Primer 5’-GCTATTGATGGTCTCACACC-3’ |
| IL-10 | Reverse Primer 5’-CAGGAGCTCAAGGTACAGGA-3’ |
| IL-17 | Forward Primer 5’-GGTTGCAAGCCTTATCGGA-3’ |
| INF-γ | Reverse Primer 5’-ACCTGTCCTCCAGCTTGCCTG-3’ |
| β-Actin | Forward Primer 5’-CAAGACTGAAACCCGACTAAG-3’ |
| INF-γ | Reverse Primer 5’-TCTCCAAGGAGGCTGA-3’ |
| IL-4 | Forward Primer 5’-GAGGGATCCATGAAATATACAAGCTAT-3’ |
| IL-10 | Reverse Primer 5’-GACGAATTCTTACGTTGATGCTCTCC-3’ |
| IL-17 | Forward Primer 5’-CGAGGATCCATAGAAATACAAGCTAT-3’ |
| INF-γ | Reverse Primer 5’-GATCAGAATTCTTACGTTGATGCTCTCC-3’ |

qRT-PCR, quantitative real-time polymerase chain reaction; IL, interleukin; INF-γ, interferon-γ.
Measurement data were presented as mean ± standard deviations. SPSS18.0 statistical software was used for statistical analyses. Differences of measurement data of the normal distributions among multiple groups were analysed using one-way analysis of variance (ANOVA), and the differences between two groups were compared using LSD t-test. \( P < 0.05 \) indicated the difference was statistically significant.

**Results**

**Comparisons of general conditions of mice in each group**

The weight of mice in the 1,25(OH)\(_2\)D\(_3\) group was elevated with the increase in time. The general conditions of mice were significantly improved, and the severity of skin ulcers and urinary system infections were significantly reduced. In the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) group, the weight of mice was also raised with the increase in time, but the difference was not significantly different. In addition, the severity of loosening hair, skin ulcers and urinary system infection was not significantly improved. In the control group, the weight of mice was increased along with the time, and there was no skin ulcer or urinary system infection. In the VDR-knockout group, the weight of mice was decreased with the increase in time, and the fur became loose. In addition, skin ulcers and urinary tract infections occurred (Figure 1).

**Comparisons of T lymphocyte parameters of mice in each group**

Compared with normal mice, the levels of CD4\(^+\)CD8\(^-\) single-positive (CD4\(^+\)SP), CD4\(^+\)/CD8\(^+\) and CD4\(^+\)CD8\(^+\) DP T lymphocytes were decreased in SLE mice, while the levels of CD8\(^+\)CD4\(^-\) single-positive (CD8\(^+\)SP) and CD8\(^+\)/CD8\(^-\) DN T lymphocytes were increased in SLE mice (all \( P < 0.05 \)). In the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) group, the levels of CD4\(^+\)SP, CD4\(^+\)/CD8\(^-\) and DP T lymphocytes were elevated, while the levels of CD8\(^+\)SP and DN T lymphocytes were

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**Haematoxylin–eosin (HE) staining**

The pathological morphology of sample tissues was observed by HE staining. Paraffin sections of renal tissues were deparaffinized and hydrated using gradient alcohol. After staining with haematoxylin (Boster) for 10 min., the slides were washed with water. The slides were subsequently differentiated with a hydrogen chloride and ethanol solution for 30 sec., and stained in blue using ammonia (Boster) for 30 min. Finally, the slides were washed twice with water, counterstained using eosin for 3 min., dehydrated with alcohol and made transparent using xylene (Boster). The slides were mounted in neutral resin and placed under a microscope (Cx31, Olympus, Japan) to observe the pathology of renal tissues.

**Immunofluorescence assay**

Immunoglobulins IgG, IgA, IgM, C3 and C1q were labelled with FITC. During PAGE, each well was added with 40 µg of samples, and the electrophoresis voltages were 80 V in concentrated gels and then 120 V in separation gels. The transferring membrane was polyvinylidene fluoride (PVDF). Wet transfer was used with a transferring voltage of 100 mV. The operation time of transfer was 60 min. and was polyvinylidene fluoride (PVDF). Wet transfer was used with a transferring voltage of 100 mV. The operation time of transfer was 60 min. and was then mounted by adding a glycerol buffer (DAKO Company). In order to wash the samples, the samples were rinsed three times by a Tris-buffered saline and Tween-20 (TBST) solutions, with each rinse taking 5 min. Secondary antibodies were then added and incubated at room temperature for 1 hr. The samples were rinsed, and then washed with water. The slides were subsequently reacted with a chemical luminescence reagent, and the grey values of the target bands were analysed by the ImageJ software using β-actin as the internal control.
reduced along with the time (all \( P < 0.05 \)). At 24 weeks after the treatment, there were no significant differences between the 1,25(OH)\(_2\)D\(_3\) and the control groups. In the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) and the control groups, the T lymphocyte parameters had no significant changes along with the time (all \( P > 0.05 \)). However, in the VDR-knockout group, the levels of CD4\(^{+}\)SP, CD4\(^{+}\)/CD8\(^{-}\) and DP T lymphocytes were decreased, while the levels of CD4\(^{+}\)/CD8\(^{-}\) and DN T lymphocytes were increased along with time (all \( P < 0.05 \)) (Table 3).

### Comparisons of blood biochemical indexes of mice in each group

Compared with the normal mice, the levels of BUN and Cr were significantly increased in SLE mice (both \( P < 0.05 \)). The levels of BUN and Cr were significantly reduced along with the time in the 1,25(OH)\(_2\)D\(_3\) group. At 24 weeks after the treatment, there were no significant changes in levels of BUN and Cr between the 1,25(OH)\(_2\)D\(_3\) and the control groups. In the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) and the control groups, the levels of BUN and Cr had no significant changes along with the time (all \( P > 0.05 \)). However, in the VDR-knockout group, the levels of BUN and Cr were increased along with time (all \( P < 0.05 \)) (Table 4).

### Comparisons of expressions of IL-4, IL-10, IL-17 and INF-\(\gamma\) in each group

The expressions of inflammatory cytokines were measured by ELISA. Compared with normal mice, the expressions of IL-4, IL-10, IL-17 and INF-\(\gamma\) were significantly increased in SLE mice (all \( P < 0.05 \)). In the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) group, the expressions of IL-4, IL-10, IL-17 and INF-\(\gamma\) were significantly reduced along with the time (all \( P < 0.05 \)). At 24 weeks after the treatment, there were no significant changes in expressions of IL-4, IL-10, IL-17 and INF-\(\gamma\) between the 1,25(OH)\(_2\)D\(_3\) and the control groups. In the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) and the control groups, the expressions of IL-4, IL-10, IL-17 and INF-\(\gamma\) had no significant changes along with the time (all \( P > 0.05 \)). However, in the VDR-knockout group, the expressions of IL-4, IL-10, IL-17 and INF-\(\gamma\) were significantly reduced along with the time (all \( P < 0.05 \)).

### Table 3: Comparisons of different subsets of T lymphocytes in each group (%)

|                | CD\(^{+}\)/SP (%) | CD\(^{+}\)/CD8\(^{-}\) (%) | CD4\(^{+}\)/CD8\(^{-}\) (%) | DP (%) | DN (%) |
|----------------|-------------------|-----------------------------|-----------------------------|---------|--------|
| **1,25(OH)\(_2\)D\(_3\)** group |                   |                             |                             |         |        |
| 8 weeks        | 30.09 ± 3.92 \(^i\) | 18.06 ± 3.53 \(^i\)         | 2.72 ± 0.59 \(^i\)          | 0.64 ± 0.13 \(^i\) | 3.76 ± 0.69 \(^i\) |
| 16 weeks       | 36.58 ± 4.28 \(^\#\) | 14.03 ± 3.28 \(^\#\)         | 3.68 ± 0.74 \(^\#\)         | 1.21 ± 0.06 \(^\#\) | 2.68 ± 0.35 \(^\#\) |
| 24 weeks       | 49.85 ± 5.16 \(^\#\) | 10.81 ± 2.96 \(^\#\)         | 4.79 ± 1.64 \(^\#\)         | 2.54 ± 0.12 \(^\#\) | 1.38 ± 0.24 \(^\#\) |
| **VDR-knock-in + 1,25(OH)\(_2\)D\(_3\)** group |                   |                             |                             |         |        |
| 8 weeks        | 29.64 ± 6.30 \(^*\) | 17.23 ± 3.65 \(^*\)         | 2.37 ± 0.81 \(^*\)         | 0.60 ± 0.23 \(^*\) | 3.62 ± 1.00 \(^*\) |
| 16 weeks       | 29.83 ± 4.90 \(^\#\) | 18.15 ± 2.95 \(^\#\)         | 2.46 ± 0.29 \(^\#\)         | 0.68 ± 0.20 \(^\#\) | 3.73 ± 0.72 \(^\#\) |
| 24 weeks       | 30.50 ± 9.00 \(^\#\) | 18.36 ± 5.10 \(^\#\)         | 2.62 ± 1.11 \(^\#\)         | 0.71 ± 0.40 \(^\#\) | 3.79 ± 0.91 \(^\#\) |
| **Control group** |                   |                             |                             |         |        |
| 8 weeks        | 53.12 ± 8.2       | 10.35 ± 2.32                | 5.43 ± 1.69                 | 2.70 ± 0.51 | 1.03 ± 0.24 |
| 16 weeks       | 51.14 ± 9.7       | 10.29 ± 1.39                | 5.09 ± 1.31                 | 2.65 ± 0.41 | 1.09 ± 0.38 |
| 24 weeks       | 50.98 ± 10.2      | 10.96 ± 4.1                 | 5.19 ± 1.92                 | 2.59 ± 0.26 | 0.97 ± 0.24 |
| **VDR-knockout group** |                   |                             |                             |         |        |
| 8 weeks        | 48.56 ± 5.32      | 11.04 ± 2.68                | 5.02 ± 0.41                 | 2.69 ± 0.59 | 1.22 ± 0.34 |
| 16 weeks       | 39.95 ± 4.85 \(^\#\) | 19.65 ± 3.74 \(^\#\)       | 3.78 ± 0.39 \(^\#\)        | 1.16 ± 0.31 \(^\#\) | 2.93 ± 0.60 \(^\#\) |
| 24 weeks       | 31.92 ± 3.62 \(^\#\) | 23.65 ± 5.30 \(^\#\)       | 1.75 ± 0.24 \(^\#\)        | 0.65 ± 0.14 \(^\#\) | 3.49 ± 0.59 \(^\#\) |

*indicating \( P < 0.05 \) as compared to the values at 8 weeks; \(^i\)indicating \( P < 0.05 \) as compared to the values at 16 weeks; \(^\#\)indicating \( P < 0.05 \) as compared to the VDR-knock-in + 1,25(OH)\(_2\)D\(_3\) group; \(^*\)indicating \( P < 0.05 \) as compared to the control group; SP, single-positive; DP, double-positive; DN, double-negative.
Table 4 Comparisons of blood biochemical indexes in each group

|                          | BUN (µmol/l) | Cr (µmol/l) |
|--------------------------|--------------|-------------|
| 1,25(OH)2D3 group        |              |             |
| 8 weeks                  | 23.01 ± 3.65  | 88.81 ± 8.65 |
| 16 weeks                 | 16.53 ± 2.32  | 76.31 ± 7.14 |
| 24 weeks                 | 9.36 ± 1.29   | 49.12 ± 5.18 |
| VDR-knock-in + 1,25(OH)2D3 group |      |             |
| 8 weeks                  | 22.03 ± 2.36  | 86.71 ± 7.26 |
| 16 weeks                 | 21.98 ± 1.27  | 85.89 ± 5.62 |
| 24 weeks                 | 20.52 ± 2.36  | 83.32 ± 9.20 |
| Control group            |              |             |
| 8 weeks                  | 9.15 ± 1.02   | 50.15 ± 4.21 |
| 16 weeks                 | 8.95 ± 0.96   | 46.89 ± 3.09 |
| 24 weeks                 | 10.01 ± 2.63  | 49.31 ± 3.21 |
| VDR-knockout group       |              |             |
| 8 weeks                  | 9.29 ± 0.29   | 47.65 ± 4.31 |
| 16 weeks                 | 14.57 ± 1.21*  | 58.25 ± 5.26* |
| 24 weeks                 | 19.32 ± 1.32*  | 86.78 ± 6.13* |

*indicating $P < 0.05$ as compared to the values at 8 weeks; †indicating $P < 0.05$ as compared to the values at 16 weeks; ‡indicating $P < 0.05$ as compared to the VDR-knock-in + 1,25(OH)2D3 group; §indicating $P < 0.05$ as compared to the control group; BUN, blood urea nitrogen; Cr, creatinine.

knockout group, the expressions of IL-4, IL-10, IL-17 and INF-γ were increased along with time (all $P < 0.05$) (Figure 2).

Comparisons of ERK1/2 expression and phosphorylation in each group

Compared with normal mice, the p-ERK1/2 expression was increased, while the t-ERK1/2 expression was decreased in SLE mice, and the ratio of p/t-ERK1/2 was significantly increased (all $P < 0.05$). In the 1,25(OH)2D3 group, the p-ERK1/2 expression was reduced, the t-ERK1/2 expression was elevated, and the ratio of p/t-ERK1/2 was decreased along with the time (all $P < 0.05$). At 24 weeks after the treatment, there were no significant changes in p-ERK1/2 expression between the 1,25(OH)2D3 and the control groups. In the VDR-knock-in + 1,25(OH)2D3 and the control groups, the expressions of p-ERK1/2 and t-ERK1/2 as well as the ratio of p/t-ERK1/2 had no significant changes along with the time (all $P > 0.05$). However, in the VDR-knockout group, the p-ERK1/2 expression was gradually increased, the t-ERK1/2 expression was gradually decreased, and the ratio of p/t-ERK1/2 was elevated along with the time (all $P < 0.05$) (Figure 4).

Comparisons of histopathological observation of mice in each group

In normal mice, the morphology of renal tissues showed no histopathological changes. In SLE mice, severe renal injuries were observed, the glomerular volume and the number of cells were both increased, the glomerular lobules formed with thickening of the basement membrane, inflammatory cell infiltration was seen in glomerular and interstitial tissues, and mononuclear cell infiltration was seen in glomerular capillaries. In the 1,25(OH)2D3 group, renal injuries were improved, and inflammatory cell infiltration was reduced along with the time (both $P < 0.05$). At 24 weeks after the treatment, renal injuries of SLE mice were improved, and there were no significant changes in inflammatory cell infiltration between the normal and SLE mice. In the VDR-knock-in + 1,25(OH)2D3 and the control groups, renal injuries and inflammatory cell infiltration had no significant changes along with the time (all $P > 0.05$). However, in the VDR-knockout group, the degree of renal injuries and inflammatory cell infiltration were increased along with the time, and SLE symptoms gradually occurred in the mice of VDR-knockout group (all $P < 0.05$) (Figure 5).

Comparisons of histopathological changes in mice tissues in each group

Compared with the normal mice, the levels of immunoglobulin and complement deposition increased in SLE mice, and the immunofluorescence staining of the renal tissues showed positive signals for IgG, IgA, IgM, C3 and C1q at the same time, which is a characteristic immune pathological change for lupus nephritis. In addition, microscopically visible immune complex deposition in a variety of different sizes was seen in the glomerular...
endothelium, epithelium, mesangial and tubular basement membrane. In the 1,25(OH)2D3 group, the levels of immunoglobulin and complement deposition were decreased gradually along with the time (all \(P < 0.05\)). At 24 weeks after the treatment, there were no significant changes in the levels of immunoglobulin and complement deposition between the normal and SLE mice. In the VDR-knock-in + 1,25(OH)2D3 and the control groups, the level of complement deposition had no significant changes along with the time (all \(P > 0.05\)). However, in the VDR-knockout group, the level of complement deposition was gradually increased along with the time, and SLE symptoms gradually occurred in the mice of VDR-knockout group (all \(P < 0.05\)) (Figure 6).

**Discussion**

The purpose of this study is to elucidate the effects of 1,25(OH)2D3 and VDR on peripheral blood T lymphocytes in SLE. The results showed that 1,25(OH)2D3 and VDR were associated with the occurrence of SLE, suggesting that they may become new targets for the treatment of SLE.

SLE is a systemic autoimmune disease with clinical heterogeneity, and some specific genes can increase the susceptibility to SLE [13]. The VD deficiency may play an important role in the occurrence of some autocrine diseases [14]. SLE is usually accompanied by abnormal immune responses including inadequate regulation of B cells by T cells and excessive secretion of
autoimmune antibodies, and the VD of many SLE patients is deficient, with the level of 1,25(OH)2D3 negatively related to the prognosis of SLE [15].

In this study, feeding with 1,25(OH)2D3 significantly improved the health of SLE mice, while the health conditions of normal mice which are insufficient in VDR deteriorate gradually along with the time. 1,25(OH)2D3, the active form of vitamin D, mainly functions on calcium homeostasis and bone metabolism. It is located in 12q of the chromosome and includes more than 470 sites of single nucleotide polymorphism (SNP), with some SNPs upregulating the level of 1,25(OH)2D3 [16]. The risk of VD deficiency is higher in SLE patients than in the general population, and VD deficiency can lead to B-cell hyperactivation and production of autoimmune antibodies in genetically susceptible populations, and VDR is expressed in all types of cells of the immune system [17]. Mechanisms of transmembrane signal transduction can cause rapid biological effects of 1,25(OH)2D3, which are mainly activated by 1,25(OH)2D3 inducing the phosphorylation of some signal transduction molecules, such as protein kinase C (PKC) and ERK1/2; 1,25(OH)2D3 can modulate the PKC activity, thus activating the Raf in the upstream of the ERK1/2 pathway, which lead to the phosphorylation of ERK1/2 [18–20]. Boyan et al. [21] found that 1,25(OH)2D3 can rapidly induce the phosphorylation of PKC and ERK1/2 in growth zone cells, and the induction of 1,25(OH)2D3 on ERK1/2 phosphorylation is mediated by PKC. According to Deeb et al., [22] rapid actions of 1,25(OH)2D3 involve 1,25(OH)2D3 in the growth zone cells.

Fig. 3 Expressions of IL-4, IL-10, IL-17 and INF-γ mRNA in each group. (A), IL-4 mRNA expression at different time-points in each group; (B), IL-10 mRNA expression at different time-points in each group; (C), IL-17 mRNA expression at different time-points in each group; (D), INF-γ mRNA expression at different time-points in each group; * indicating P < 0.05 as compared to the values at 8 weeks; # indicating P < 0.05 as compared to the values at 16 weeks; $ indicating P < 0.05 as compared to the VDR-knock-in + 1,25(OH)2D3 group; $ indicating P < 0.05 as compared to the control group; IL, interleukin; INF-γ, interferon gamma.
The binding of vitamin D3 (1,25(OH)2D) with the cytosolic and membrane VDR activates PKC through phosphorylation, thus activating the ERK pathway through the Ras-Raf-MEK-ERK cascade. A previous study demonstrated that 1,25(OH)2D3 interferes with the ERK signalling pathway to inhibit tumour cell growth [23]. It has been shown that during the treatment of SLE patients, the increase in 1,25(OH)2D3 dosage inhibited the activity of SLE [24], similar to the results from this study. In this study, the mice with VDR deficiency showed deteriorating health conditions, indicating that VDR deficiency may increase the risk of SLE.

In this study, as compared to normal mice, the levels of CD4+SP, CD4+/CD8+ and DP in SLE mice were decreased, while the number of CD8+SP and DN cells was increased, indicating certain immune abnormalities. The ability of 1,25(OH)2D3 to suppress inflammation has been linked to its

(H)2D3 binding with the cytosolic and membrane VDR to activate PKC through phosphorylation, thus activating the ERK pathway through the Ras-Raf-MEK-ERK cascade. A previous study demonstrates that 1,25(OH)2D3 interferes with the ERK signalling pathway to inhibit tumour cell growth [23]. It has been shown that during the treatment of SLE patients, the increase in 1,25(OH)2D3 dosage inhibited the activity of SLE [24], similar to the results from this study. In addition, a close association has been found between VDR polymorphisms and SLE severity [10]. In this study, the mice with VDR deficiency showed deteriorating health conditions, indicating that the VDR deficiency may increase the risk of SLE.

In this study, it was found that as compared to normal mice, the levels of CD4+SP, CD4+/CD8+ and DP in SLE mice were decreased, while the number of CD8+SP and DN cells was increased, indicating certain immune abnormalities. The ability of 1,25(OH)2D3 to suppress inflammation has been linked to its
capacity to regulate DC and T-cell functions [25]. Autoreactive T lymphocyte activation can lead to abnormal numbers of CD4+ and CD8+ T cells, and these changes can further induce SLE; in SLE patients, the number of CD4+ T cells was decreased, the number of CD8+ T cells was increased, and the ratio of CD4+/CD8+ T cells was continued to decline [26].

In this study, it was also shown that as compared with normal mice, the protein and mRNA expressions of IL-4, IL-10, IL-17 and INF-γ in SLE mice were significantly increased. Mature CD4+ T cells can be divided into two distinct subgroups (Th1 and Th2) based on different expressions of cell factors [27]. In SLE patients, the serum levels of Th1 and Th2 cytokines increased, suggesting that they may participate in the inflammation and tissue damage of SLE [28]. INF-γ is produced by Th1 cells, while IL-4 and IL-10 are produced by Th2 cells, and IL-17 is mainly secreted by Th1 [29]. Secretion of IL-17 promotes inflammation, and its expression in T cells is associated with the activity of SLE. Due to the positive association between IL-17 and INF-γ, the increase in INF-γ in the serum of SLE patients reasonably upregulated the IL-17 expression and it has been shown that IL-17 plays an important role in various autocrine diseases, including SLE and rheumatoid arthritis [30, 31].

In summary, this study found that 1,25(OH)2D3 and VDR participated in the occurrence of SLE by regulating cytokine secretion in different subsets of T cells, and they may become new drug targets during the treatment of SLE. However, there are still limitations in this research, and the exact mechanisms of 1,25(OH)2D3 and VDR remain elusive, which require further studies.

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Competing interests
None.

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