Original Paper

Roles of 1,25(OH)₂D₃ and Vitamin D Receptor in the Pathogenesis of Rheumatoid Arthritis and Systemic Lupus Erythematosus by Regulating the Activation of CD4⁺ T Cells and the PKCδ/ERK Signaling Pathway

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Key Words

Systemic lupus erythematosus • Rheumatoid arthritis • 1,25(OH)₂D₃ • Vitamin D receptor • Protein kinase C δ • Extracellular signal-regulated kinase • CD4⁺ T cells

Abstract

Background/Aims: The study aims to elucidate the roles of 1,25(OH)₂D₃ and vitamin D receptor (VDR) in the pathogenesis of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) by regulating the activation of CD4⁺ T cells and the PKCδ/ERK signaling pathway. **Methods:** From January 2013 to December 2015, a total of 130 SLE patients, 137 RA patients and 130 healthy controls were selected in this study. Serum levels of 1,25(OH)₂D₃ and VDR mRNA expression were detected by ELISA and real-time fluorescence quantitative PCR (RT-qPCR). Density gradient centrifugation was performed to separate peripheral blood mononuclear cells (PBMCs). CD4⁺ T cells were separated using magnetic activated cell sorting (MACS). CD4⁺ T cells in logarithmic growth phase were collected and assigned into 9 groups: the normal control group, the normal negative control (NC) group, the VDR siRNA group, the RA control group, the RA NC group, the VDR over-expressed RA group, the SLE control group, the SLE NC group, and the VDR over-expressed SLE group. The mRNA and protein expressions of VDR, PKCδ, ERK1/2, CD11a, CD70 and CD40L were detected by RT-qPCR and Western blotting. Bisulfite genomic sequencing was conducted to monitor the methylation status of CD11a, CD70 and CD40L. **Results:** Compared with healthy controls, serum 1,25(OH)₂D₃ level and VDR mRNA expression in peripheral blood were decreased in SLE patients and RA patients. With the increase of concentrations of 1,25(OH)₂D₃ treatment, the VDR mRNA expression and DNA methylation levels of CD11a, CD70 and CD40L were declined, while the expressions of PKCδ, ERK1/2, CD11a, CD70 and CD40L were elevated in SLE, RA and normal CD4⁺ T cells. Compared

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with the SLE contro, RA control, SLE NC and RA NC groups, the expressions of PKC δ , ERK1/2, CD11a, CD70 and CD40L decreased but DNA methylation levels of CD11a, CD70 and CD40L increased in the VDR over-expressed SLE group and VDR over-expressed RA group. However, compared with the normal control and normal NC groups, the expressions of PKC δ , ERK1/2, CD11a, CD70 and CD40L increased, but DNA methylation levels of CD11a, CD70 and CD40L decreased in the VDR siRNA group. Compared with the normal control group, the expressions of PKC δ , ERK1/2, CD11a, CD70 and CD40L increased, but DNA methylation levels of CD11a, CD70 and CD40L decreased in the SLE control and RA control groups. **Conclusion:** Our study provide evidence that 1,25(OH)₂D₃ and VDR could inhibit the activation of CD4⁺ T cells and suppress the immune response of SLE and RA through inhibiting PKC δ /ERK pathway and promoting DNA methylation of CD11a, CD70 and CD40L.

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Introduction

Systemic lupus erythematosus (SLE) is a chronically complex systemic autoimmune disease which affects women 10 times more frequently than men, especially women of child-bearing age [1]. Generally speaking, many organs and tissues in the human body are likely to be involved in SLE, skin, joints, hematopoietic system, kidneys, central nervous system, and heart in particular thereby resulting in a large variety of symptoms [2]. A wide range of factors have been found to be associated with the etiology of this multisystem disease, including genetic predisposition, the influence of female sex hormones as well as such environmental factors as infections and ultraviolet (UV) exposition [3]. Recently, researches demonstrated that abnormalities of B cells (such as B10 cells), T lymphocytes and macrophages have been considered as leading bases of SLE [4, 5]. Current treatments for SLE relies on anti-malarials, steroids, cytotoxic drugs and immunosuppressive agents, which, in spite of their curative effects, lead to a good many deleterious side effects such as therapy-resistant disease symptoms and infection [6]. The hallmark of SLE is characterized by the release of autoantibodies which react with self-nuclear and cytoplasmic antigens, resulting in immunologic attacks to body organs [7]. Rheumatoid arthritis (RA) is also a common autoimmune disease which can result in systemic complications, early death, progressive disability and socioeconomic costs [8]. A previous study elucidates that deregulation of the mechanisms, which properly control the production of cytokines secreted by CD4⁺T cells, may indicate a common pathogenic mechanism which underlies the development of RA and SLE [9]. Thus, it is gaining increasing momentum to address the disease from genetic perspective.

It has been reported that abnormalities in T lymphocytes are frequently found in SLE patients, represented by exaggerated CD4⁺T cell activities, the exploration of whose mechanisms has led to the finding of several new defects in signal transduction and thus generated the study on their molecular basis [10]. CD4⁺T cells play a crucial part in the pathology of SLE by actively suppressing the activation and expansion of self-reactive T cells [11]. The steroid hormone 1,25(OH)₂D₃ regulates gene transcription through a nuclear receptor called vitamin D receptor (VDR) and initiation of rapid cellular responses through a putative plasma membrane-associated receptor (VDR_{mem}) [12]. It has been revealed that the suppression of extracellular signal-regulated kinase (ERK) phosphorylation is sensitive to oestrogen, a female sex hormone that binds to nuclear receptors and alters the rate of gene transcription, in patients with inactive or mild symptoms [13]. Moreover, the disease was found to be related to the expression of protein kinase C (PKC) δ in SLE monocytes [14]. It has been reported that blocking the PKC δ /ERK signaling pathway can consequently reduce migration and invasion of human breast carcinoma cells [15]. However, no studies have been carried out to explore the function of molecular in SLE. Therefore, the study aims to explore the roles of 1,25(OH)₂D₃ and VDR in the pathogenesis of RA and SLE by regulating the activation of CD4⁺ T cells and the PKC δ /ERK signaling pathway.

Materials and Methods

Study subjects

From January 2013 to December 2015, 130 SLE patients were selected from the Second Xiangya Hospital, Central South University. These SLE patients included 118 females and 12 males with a mean age of 34.32 ± 12.40 years (range from 15 to 70 years). All SLE patients were conformed to the diagnostic criteria for SLE of American Rheumatism Association (ARA) and revised in 1997 [16]. Furthermore, a total of 137 RA patients were included as a disease control group (RA group), including 118 females and 19 males with a mean age of 35.98 ± 12.24 years (range from 16 to 70 years), excluded of other systemic diseases). All RA patients were conformed to the diagnostic criteria for RA of American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) [17]. Meanwhile, 130 healthy controls underwent physical examination were selected, including 121 females and 9 males with a mean age of 33.35 ± 11.03 years (range from 16 to 72 years). This study was approved by the Ethics Committee of the Second Xiangya Hospital, Central South University, and informed consent was obtained from all study subjects.

ELISA assay

Venous blood (4 ml) was collected from all subjects, with 2 ml peripheral blood for PCR detection of VDR mRNA expression, 2ml placed in refrigerator for 30~60 min at 4°C. The blood was centrifuged for 15 min at a speed of 3000 rpm after coagulation. Supernatant was collected cautiously for detection of serum 1,25(OH)₂D₃ level. Serum 1,25(OH)₂D₃ levels in healthy controls, RA patients and SLE patients were detected strictly in accordance with ELISA kit (purchased from Westang Biotechnology Co., Ltd., Shanghai, China). Firstly, 10 standard holes were set in enzyme-labeled coated plate where samples were treated with gradient dilution to draw standard curve. Samples to be detected were added into holes on enzyme-labeled coated plate and got mixed with gentle shake before being incubated for 30 min at 37°C. Liquid in holes was removed, and cleaning solution was added to rinse it for 30 s, which was repeated for 5 times before drying the holes; enzyme-labeled reagent of 50 µl was added to incubate for 30 min at 37°C; liquid in holes was removed, and cleaning solution was added to rinse it for 30 s, which was repeated for 5 times before drying the holes; chromogenic agent A and B of 50 µl each were added successively and was incubated in dark place for 15 min at 37°C after mixture, ending with 50 µl elimination agent. Blank holes were used for zero setting and optical density (OD) value (450 nm) in each hole was detected using microplate reader (Bio-Rad Company, USA) within 15 min. After that, standard curve was drawn with concentration of standard samples as abscissa and OD value as ordinate, followed by counting the corresponding concentration of samples with their OD value plugged in the standard curve equation.

CD4⁺ T cells isolation and culture

Peripheral blood of 5 ml was collected from healthy controls, RA patients and SLE patients and mixed evenly with Phosphate Buffered Saline (PBS). Appropriate amount of lymphocyte separation solution (Sigma Company) was added. It was centrifuged horizontally at 18°C for 30 min at a speed of 2000 rpm, dividing it into 3 layers. White mist layer in the interface between upper layer and middle layer was extracted with caution into centrifuge tube. PBS of 5 times larger size was used to rinse mononuclear cells which were later centrifuged for 10 min at 1600 rpm. With supernatant removed, the rinse procedure was carried out again. PBS of 1 ml was used to resuspend cells, one drop of which was placed on cell counting chamber for counting. Mononuclear cells suspension weighing 400 g was centrifuged for 10 min at 4°C, after which, with supernatant abandoned, 1×10^7 cells were selected to be added into 80 µl magnetic activated cell sorting (MACS) buffer and 20 µl MACS CD4 superbead (MiltenyiBiotec Company) for incubation for 15 min at 4°C. Next, 1~2ml MACS buffer was added to rinse cells weighing 300 g, followed by 10 min centrifugation, with supernatant abandoned, 500 µl MACS buffer was added to resuspend cells which were later placed in LS separator column and were classified in MiniMACS separator. LS separator column was rinsed with 500 µl buffer for 3 times and 1ml MACS buffer was used to separate CD4⁺T cells from LS column when it was removed from magnetic field. CD4⁺T cells selected with MACS were added into RPMI-1640 culture medium containing 10% fetal bovine serum (FBS) (Hyclone Company) and 1% mycillin (Hyclone Company), and placed in 5% CO₂ incubator at 37°C for incubation. As to CD4⁺T cell detection, CD4-FITC-labeled antibody (eBioscience Company) was added into CD4⁺T cells extracted with MACS, which was incubated in the dark at room temperature for 30 min after mixture. PBS of 2 ml was employed to resuspend cells which

were centrifuged for 5 min at 1000 rpm. With supernatant abandoned, appropriate amount of flow buffer solution was used for resuspension and selected CD4⁺T cells purity was detected using flow cytometry (Becton Dickinson Company).

CD4⁺T cells treated with different dose of 1,25(OH)₂D₃

Absolute ethanol was added into 1,25(OH)₂D₃ (Sigma Company) solution with an initial concentration of 1 × 10⁻³ mmol/L, which was diluted into 4 concentration gradient: 1 × 10⁻⁶ mmol/L, 1 × 10⁻⁷ mmol/L, 1 × 10⁻⁸ mmol/L and 1 × 10⁻⁹ mmol/L. CD4⁺T cells in SLE group and control group were mixed fully with RPMI-1640 culture medium (Hyclone Company) containing 10% FBS (Hyclone Company) and 1% mycillin (Hyclone Company), after which, 1 ml, some 10⁶ cells, was transplanted into each hole on 24-hole culture plate. In RPMI-1640 culture medium, 1,25(OH)₂D₃ of 0 mmol/L, 1 × 10⁻⁶ mmol/L, 1 × 10⁻⁷ mmol/L, 1 × 10⁻⁸ mmol/L and 1 × 10⁻⁹ mmol/L was added respectively, and it was placed at 37°C in 5% CO₂ incubator (SANYO Company, Japan) to incubate for 72 h.

Construction of human VDR cDNA overexpression vector

Total RNA in healthy controls was extracted using Trizol and human VDR cDNA was obtained using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Forward primer (CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA) and reverse primer (AAC CAG CGG GAA GAG GTC AAG GG) synthesized by Sangon Biotech Co., Ltd., Shanghai were employed for PCR amplification, after which, obtained PCR products received agarose gel electrophoresis and target segments were cut from gel. QIAquick PCR purification recovery kit (QIAGEN Company) was used for purification recovery, after which, purified and recovered VDR cDNA was instilled into pcDNA3.1 vector that had been cut by *Hind III* and *Xho I* enzymes. Products were connected to be transformed into Escherichia coli DH5α competent cells, which were cultured on culture plates containing ampicillin for one night at 37°. The next day, colony in single colony was selected for PCR and sequencing, the result of which was compared to National Center of Biotechnology Information (NCBI) VDR sequences, so as to substantiate VDR gene sequences' presence in high expressed plasmids.

Construction of VDR-siRNA interference vector

According to sequences of VDR cDNA in GenBank, the Basic Local Alignment Search Tool (BLAST) software was used to design VDRsiRNA sequences. In conformity with requirements of enzyme restriction site of PLKO.1-sp6-GFP, expression plasmids of siRNA, sequences encoding siRNA cDNA were synthesized, whose structure was Age I + positive-sense strand (5'-TGC TGA ACA TGA TCA CCT CAA TGG CAG TTT GGC CAC TGA CTG ACT GCC ATT GGT GAT CAT GTT-3') + loop + antisense strand (5'-CCT GAA CAT GAT CAC CAA TGG CAG TCA GTC AGT GGC CAA AAC TGC CAT TGA GGT GAT CAT GTTC-3') + termination signal + EcoR I, with strands synthesized by GenePharma Company, Shanghai. Expression plasmids of VDR siRNA that had been successfully constructed were transferred to Escherichia coli DH5α competent cells before being cultured in culture plates containing ampicillin for one night at 37°C. The next day, monoclonal colony was selected for scale-up culture. Eventually, regular-PCR was used for detection with U6: 5'-ATT TGC GTG TCA TCC TTGCG-3' and Sp6: 5'-AAT TTA GGT GAC ACT ATAG-3' employed as detection primers, after which, plasmids shown correct in detection were sent to BGI TECH SOLUTIONS CO., LTD. (Shenzhen, China) for sequencing.

Cell transfection and grouping

CD4⁺T cells in logarithmic growth phase were collected and assigned into 9 groups: the normal control group (normal CD4⁺T cells without any transfection), the normal negative control (NC) group (normal CD4⁺T cells transfected with empty vector plasmid), the VDR siRNA group (normal CD4⁺T cells transfected with VDR siRNA expression plasmid), the RA control group (RA CD4⁺T cells without any transfection), the RA NC group (RA CD4⁺T cells transfected with empty plasmid), the VDR over-expressed RA group (RA CD4⁺T cells transfected with VDR overexpression vector), the SLE control group (SLE CD4⁺T cells without any transfection), the SLE NC group (SLE CD4⁺T cells transfected with empty vector plasmid), and the VDR over-expressed SLE group (SLE CD4⁺T cells transfected with VDR expression vector). With 0.25% trypsin digestion and centrifugation for 5 min at 1000 r/min, they were resuspended in culture medium, and 5 × 10⁶ cells were added into 2 mm electric cup, followed by addition of 10 μg VDR overexpression vector and VDR-siRNA respectively. With voltage set at 500 V and electroporation performed at 1 ms, it was next placed

Table 1. Primers sequences for reverse transcription-polymerase chain reaction (RT-PCR). VDR, Vitamin D receptor; PKC δ , protein kinase C δ ; ERK1/2, extracellular signal-regulated kinase 1/2; F, forward; R, reverse

Gene	Sequence (5'-3')
VDR	F: CAACCAAGACTACAAGTACCGCGTCAGTGA R: AACCAGCGGGAAGAGGTCAAGGG
PKC δ	F: GTCATCCAGATTGTGCTAATGCG R: TCTTGTGGATGGCAGCGTTCA
ERK1/2	F: GCAAGCACTACCTGGATCAGCTC R: TCGGGCCTTCATGTTGATGATA
CD11a	F: TGAGAGCAGGCTATTTGGGTTAC R: CGGCCCATGTGCTGGTAT
CD70	F: TGCTTTGGTCCCATTGGTCG R: TCCTGCTGAGGTCCTGTGTGATTG
CD40L	F: AAAGGAAAGTTTGAAGTGAAT R: TGAGTAAGGTGGTTATTTTGATAGT
β -actin	F: GCACCACACCTTCTACAATGAGC R: GGATAGCACAGCCTGGATAGCAAC

statically for 10 min at 4°C, after which, RPMI-1640 culture medium containing 10% serum was added to culture for another 24 h. Electrotransfer was purchased from Bio-Rad Company. During the process of culture, cell morphology was observed under inverted phase contrast microscope (OLYMPUS Company).

Real-time fluorescence quantitative PCR (RT-qPCR)

With CD4⁺T cells collected from each group, total RNA was extracted using Trizol. RNA of 500 ng was selected for reverse transcription in accordance with instructions on Reverse Transcription System A3500 reverse transcription kit purchased from Promega Company. According to gene sequences released publicly by Genbank, Primer 5.0 was employed to design primers included in Table 1 respectively which were synthesized by Sangon Biotech Co., Ltd., Shanghai. Reaction condition for RT-qPCR (SYBR GREEN method): 1) at 95°C for 15 min once; 2) at 95°C for 30 s, at 55°C for 45 s, at 72°C for 1 min, 40 times; reaction system: Premix Ex Taq or SYBR Green Mix 12.5 μ l, Forward Primer 1 μ l, Reverse Primer 1 μ l, DNA template 1-4 μ l and ddH₂O up to 25 μ l. With glyceraldehyde phosphate dehydrogenase (GAPDH) as internal control gene and the standard for control group set at 1, Ct values of target genes (inflection point of amplification power curve) were obtained and Relative Quantification (RQ) values of target genes were calculated for statistical analysis in accordance with $RQ = 2^{-\Delta\Delta Ct}$. RT-qPCR equipment, whose model number was iQ5, was purchased from BioRad Company.

Western blotting

CD4⁺T cell protein was collected from each group and its concentration was examined based on bicinchoninic acid (BCA) kit (Beyotime Biotechnology Research Institute). 5 $\times$ loading buffer (Beyotime Biotechnology Research Institute) was added into protein and boiled for 10 min at 95°C. Samples of 30 μ g were placed into each hole, and electrophoresis (Bio-RAD Company) with 10% polyacrylamide gel (Boster Company, Wuhan) was used for separation. Transmembrane was performed with Polyvinylidene Fluoride (PVDF) (Amresco Company), and it was kept sealed off at room temperature for 1 h with 5% bovine serum albumin (BSA) (Sino-American Biotechnology Co., Ltd., Beijing). Tris-Buffered Saline and Tween 20 (TBST) (Biomix Laibo Technology Co., Ltd., Beijing), diluted primary antibody of β -actin (ab8226, 1: 1000), VDR (ab8756, 1: 100), pPKC δ (ab76181, 1: 2500) and pERK1/2 (ab54230, 1 μ g/ml), which were purchased from Abcam Company and kept for one night at 4°C. Membrane was rinsed with TBST for three times, 5 min each time. Secondary antibody (Abcam Company) was added for reaction at 37°C for 2 h. Membrane was rinsed and chromogenic reagent of substrate A was mixed with B in 1: 1 ratio (Promega Company) for developing at room temperature for 1 min. Then, it, wrapped up with preservative film, was transferred to dark room, and received developing and photographic fixing with X-ray after exposure. Gel-Pro analyzer 4.0 was used to analyze bands and target protein expression level was presented in grey value ratio of target protein to β -actin.

Flow cytometry

With mononuclear cells isolated from peripheral blood, mononuclear cells of 10⁶/L were selected from SLE group and control group respectively. Cells of 100 μ l were added into flow cytometry tube where antibodies labeled by 20 μ l of CD4-PE+CD11a-FITC, CD4-PE+CD70-FITC, CD4-PE+CD40L-FITC respectively

Table 2. Primers

sequences for nested-polymerase chain reaction. F, forward; R, reverse

Gene	Sequence (5'-3')
CD11a 1 round	F: GAAGAATTCGTAGGTTGGTTTGAGTGTAGTGGTGTAAAA R: CTCTCTAGAACTAAAACCAAAATACATACCACCATACTAA
CD11a 2 round	F: TTAAAGTAGTTGGGATTATAGGTAT R: Cy3-ATTAATAACTAATTATACTCCATTA
CD70 1 round	F: GGTGAATTCTTTAAGGTTAGGAGTTTAAAGTTTAGTT R: CAATCTAGAACTACACATTTATTAATAAATTAATTA
CD70 2 round	F: Cy3-GTTGAATTCGGTTAATATGGTGAAATTTATTTTAT R: CACTCTAGATACAACAAACATCCAAAAATTAATAA
CD40L 1 round	F: GAAGAATTCAGTTGATGGGATATTAGTTATAAAATTAATTT R: AAATCTAGACCCAATCATCTAAATAATAAAAAAACAA
CD40L 2 round	F: AAAGGAAGTTTGAAGTGAAT R: TGAGTAAGGTGGTTATTTTGATAGT

(eBioscience Company) were added. In control tube of the same type, 20 µl CD4-PE, CD11a-FITC, CD70-FITC and CD40L-FITC were added respectively, each repeated 5 times and incubated in the dark at room temperature for 3 min after even mixture. PBS of 2 ml was used to resuspend cells which were centrifuged at 1000 rpm for 5 min. Supernatant abandoned, appropriate amount of flow buffer solution was used for resuspension and CD11a, CD70 and CD40L protein expressions were detected using flow cytometry (Becton Dickinson Company).

Bisulfite genomic sequencing

Blood/tissue/genomic DNA extraction kit (TIANamp Genomic DNA Kit) was used to extract CD4⁺T cells in SLE patients and control group respectively, the DNA of which received Sodium Bisulfite with EpiTectBisulfite kit (Qiagen Company) in strict accordance with instructions on kit. Then, nested PCR was carried out, employing Go Taq hot star polymerase System of Promega Company, and nested PCR primers could be seen in Table 2. PCR products underwent agarose gel electrophoresis, and agar gel with target band was cut under ultraviolet lamp, followed by purification recovery of DNA using QIAquick Gel Extraction Kit of Qiagen Company. After that, pGEM-T Easy Vector Systems (Promega Company) were employed to connect purification-recovered nested PCR products to T Vector, which were transformed into Escherichia coli DH5α competent cells. Positive colonies were filtered based on blue-white screening, where plasmids were extracted using QIAprepMiniprep Kit (Qiagen Company) and sent to BGI TECH SOLUTIONS CO., LTD. (Shenzhen, China) for sequencing.

Statistical analysis

Statistical software of SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis of data, with measurement data presented in mean ± standard deviation ($\bar{x} \pm s$). Independent sample *t* test was employed for the comparison between groups while One-Way analysis of variance (ANOVA) with least significant difference (LSD)-*t* was used for comparison among groups, *P* < 0.05 being statistically significant.

Results

Baseline characteristics of healthy controls, RA patients and SLE patients

The baseline characteristics of the healthy controls, RA patients and SLE patients were shown in Table 3. No significant difference was revealed in age, height and weight among healthy controls, RA patients and SLE patients (all *P* > 0.05). The body mass index (BMI) was also not significantly different among healthy controls, RA patients and SLE patients (*P* > 0.05).

Comparisons of serum 1,25(OH)₂D₃ level and VDR mRNA expression among healthy controls, RA patients and SLE patients

Serum 1,25(OH)₂D₃ levels and VDR mRNA expressions of RA patients and SLE patients were significantly lower than those of healthy controls (all *P* < 0.05). However, there was no

Table 3. Baseline characteristics of healthy controls, RA patients and SLE patients. RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; BMI: body mass index

Characteristic	Healthy control (n = 130)	RA patients (n = 137)	SLE patients (n = 130)	P
Age (years)	33.35 ± 11.03	35.98 ± 12.24	34.32 ± 12.40	0.191
Gender (Male / Female)	9 / 121	19 / 118	12 / 118	0.157
Height (m)	1.62 ± 0.07	1.64 ± 0.08	1.62 ± 0.08	0.085
Weight (kg)	55.81 ± 9.43	55.37 ± 9.79	53.68 ± 9.74	0.171
BMI (kg/m ²)	21.30 ± 4.13	20.72 ± 4.11	20.59 ± 4.29	0.345

Fig. 1. Comparisons of serum 1,25(OH)₂D₃ levels and VDR mRNA expression of healthy controls, RA patients and SLE patients. Note: (A) Serum 1,25(OH)₂D₃ levels in healthy controls, RA patients and SLE patients detected by ELISA; (B) VDR mRNA expressions in healthy controls, RA patients and SLE patients detected by real-time fluorescence quantitative PCR (RT-qPCR); *, $P < 0.05$ compared with control group.

Fig. 2. Scatter plots of CD4⁺ T cells separated using magnetic activated cell sorting (MACS) and detected by flow cytometry. Note: (A) Normal CD4⁺ T cells; (B) RA CD4⁺ T cells; (C) SLE CD4⁺ T cells; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

difference in serum 1,25(OH)₂D₃ levels and VDR mRNA expressions between RA patients and SLE patients (both $P > 0.05$) (Fig. 1).

Identification of normal CD4⁺T cells, RA CD4⁺T cells and SLE CD4⁺T cells

CD4⁺T cells sorted out from MACS were labeled with anti-CD4-FITC flow monoclonal fluorescent antibody and CD4⁺T cell purity was detected by flow cytometry. Normal CD4⁺T cell purity was 82.88%, RA CD4⁺T cell purity was 85.03% and SLE CD4⁺T cell purity was 83.19% by FCS express V3 software analysis (Fig. 2).

Fig. 3. The mRNA and protein expressions of VDR, PKC δ and ERK1/2 in normal CD4⁺ T cells, RA CD4⁺ T cells and SLE CD4⁺ T cells after treated with different doses of $1,25(\text{OH})_2\text{D}_3$ detected by RT-qPCR and Western blotting. Note: *, $P < 0.05$ compared with normal CD4⁺ T cells at the same doses of $1,25(\text{OH})_2\text{D}_3$; #, $P < 0.05$ compared with SLE CD4⁺ T cells treated with 0 mol/l $1,25(\text{OH})_2\text{D}_3$.

Fig. 4. The mRNA expressions of CD11a, CD70, and CD40L in normal CD4⁺ T cells, RA CD4⁺ T cells and SLE CD4⁺ T cells after treated with different doses of $1,25(\text{OH})_2\text{D}_3$ detected by RT-qPCR. Note: *, $P < 0.05$ compared with normal CD4⁺ T cells at the same doses of $1,25(\text{OH})_2\text{D}_3$; #, $P < 0.05$ compared with SLE CD4⁺ T cells treated with 0 mol/l $1,25(\text{OH})_2\text{D}_3$.

Expressions of VDR, PKC δ and ERK1/2 in CD4⁺ T cells treated with different doses of $1,25(\text{OH})_2\text{D}_3$

All CD4⁺ T cells were treated with 0, 10^{-6} , 10^{-7} , 10^{-8} , and 10^{-9} mol/L $1,25(\text{OH})_2\text{D}_3$, respectively. The results showed that VDR expressions in normal/RA/SLE CD4⁺ T cells increased with the increase doses of $1,25(\text{OH})_2\text{D}_3$, and reached a stable stage at 10^{-6} mol/L. VDR expressions in SLE CD4⁺ T cells and RA CD4⁺ T cells were lower than those in normal CD4⁺ T cells (all $P < 0.05$). However, PKC δ and ERK1/2 expressions decreased with the increase doses of $1,25(\text{OH})_2\text{D}_3$, and also reached a lowest level at 10^{-6} mol/L. PKC δ and ERK1/2 expressions in SLE CD4⁺ T cells and RA CD4⁺ T cells were higher than those in normal CD4⁺ T cells (all $P < 0.05$). However, there were no significant differences between SLE CD4⁺ T cells and RA CD4⁺ T cells in the expressions of VDR, PKC δ and ERK1/2 after treated with same doses of $1,25(\text{OH})_2\text{D}_3$ (all $P > 0.05$) (Fig. 3).

The mRNA expressions of CD11a, CD70 and CD40L in CD4⁺ T cells treated with different doses of $1,25(\text{OH})_2\text{D}_3$

In comparison with CD4⁺ T cells treated with 0 mol/L $1,25(\text{OH})_2\text{D}_3$, the mRNA expressions of CD11a, CD70 and CD40L decreased in normal/RA/SLE CD4⁺ T cells after

Fig. 5. DNA methylation levels in transcriptional regulation region of CD11a, CD70, and CD40L in CD4⁺ T cells in normal CD4⁺ T cells, RA CD4⁺ T cells and SLE CD4⁺ T cells after treated with different doses of $1,25(\text{OH})_2\text{D}_3$ detected by Bisulfite genomic sequencing. Note: *, $P < 0.05$ compared with normal CD4⁺ T cells at the same doses of $1,25(\text{OH})_2\text{D}_3$; #, $P < 0.05$ compared with SLE CD4⁺ T cells treated with 0 mol/l $1,25(\text{OH})_2\text{D}_3$.

Fig. 6. The mRNA and protein expressions of PKC δ and ERK1/2 in CD4⁺ T cells after transfected with VDR overexpression vector and VDR siRNA vector. Note: *, $P < 0.05$ compared with the VDR siRNA group; #, $P < 0.05$ compared with the VDR overexpressed RA and overexpressed SLEA group.

treated with other doses of $1,25(\text{OH})_2\text{D}_3$, in which those expressions were lowest after treated with 10^{-6} mol/L $1,25(\text{OH})_2\text{D}_3$. The mRNA expressions of CD11a, CD70, CD40L expressions in SLE CD4⁺ T cells and RA CD4⁺ T cells were higher than those in normal CD4⁺ T cells (all $P < 0.05$). However, there were no significant differences between SLE CD4⁺ T cells and RA CD4⁺ T cells in the mRNA expressions of CD11a, CD70, CD40L after treated with same doses of $1,25(\text{OH})_2\text{D}_3$ (all $P > 0.05$) (Fig. 4).

DNA methylation levels of CD11a, CD70 and CD40L in CD4⁺ T cells treated with different doses of $1,25(\text{OH})_2\text{D}_3$

The Sodium Bisulfite sequencing results showed that DNA methylation levels in transcriptional regulation region of CD11a, CD70, and CD40L in normal/RA/SLE CD4⁺ T cells increased with the increase doses of $1,25(\text{OH})_2\text{D}_3$. Compared with CD4⁺ T cells treated with 0

Fig. 7. The mRNA expressions of CD11a, CD70, and CD40L in CD4⁺ T cells after transfected with VDR overexpression vector and VDR siRNA vector. Note: *, $P < 0.05$ compared with the VDR siRNA group; #, $P < 0.05$ compared with the VDR overexpressed RA and overexpressed SLEA group.

Fig. 8. DNA methylation levels in transcriptional regulation region of CD11a, CD70, and CD40L genes in CD4⁺ T cells after transfected with VDR overexpression vector and VDR siRNA vector. Note: *, $P < 0.05$ compared with the VDR siRNA group; #, $P < 0.05$ compared with the VDR overexpressed RA and overexpressed SLEA group.

mol/L 1,25(OH)₂D₃, other doses of 1,25(OH)₂D₃ could significantly increase DNA methylation levels of CD11a, CD70, and CD40L. Among them, DNA methylation level was the highest at 10⁻⁶ mol/L 1,25(OH)₂D₃. But in contrast with normal CD4⁺ T cells, DNA methylation levels of CD11a, CD70, and CD40L in SLE CD4⁺ T cells and RA CD4⁺ T cells were down-regulated (all $P < 0.05$). However, no significant difference was found in DNA methylation levels of CD11a, CD70, and CD40L between SLE CD4⁺ T cells and RA CD4⁺ T cells (all $P > 0.05$) (Fig. 5).

Expressions of PKC δ and ERK1/2 in CD4⁺ T cells after transfected with VDR overexpression vector and VDR siRNA vector

The VDR overexpression vectors were transfected into SLE CD4⁺ T cells and RA CD4⁺ T cells. Compared with the SLE control, RA control, SLE NC and RA NC groups, the PKC δ and ERK1/2 expressions decreased in the VDR over-expressed SLE and VDR over-expressed RA groups (all $P < 0.05$). VDR siRNA vectors were transfected into normal CD4⁺ T cells. The PKC δ and ERK1/2 expressions in the VDR siRNA group were higher than those in the normal control group and normal NC group (all $P < 0.05$). Furthermore, the PKC δ and ERK1/2 expressions in SLE CD4⁺ T cells and RA CD4⁺ T cells were higher than those in normal CD4⁺ T cells (all $P < 0.05$). However, no significant difference was found in the expressions of PKC δ and ERK1/2 in SLE CD4⁺ T cells and RA CD4⁺ T cells (all $P > 0.05$) (Fig. 6).

The mRNA expressions of CD11a, CD70, and CD40L in CD4⁺ T cells after transfected with VDR overexpression vector and VDR siRNA vector

The results of RT-qPCR and flow cytometry illustrated that the expressions of CD11a, CD70 and CD40L reduced in the VDR over-expressed SLE and VDR over-expressed RA groups in comparison to the SLE control, RA group, SLE NC group and RA NC groups (all

$P < 0.05$). The expressions of CD11a, CD70, and CD40L in the VDR siRNA group were higher than those in the normal control and normal NC groups (all $P < 0.05$). In addition, the expressions of CD11a, CD70, and CD40L in SLE CD4⁺T cells and RA CD4⁺T cells were higher than those in normal CD4⁺T cells (all $P < 0.05$). However, no significant difference was found in the expressions of CD11a, CD70 and CD40L between SLE CD4⁺T cells and RA CD4⁺T cells (all $P > 0.05$) (Fig. 7).

DNA methylation levels of CD11a, CD70, and CD40L genes in CD4⁺T cells after transfected with VDR overexpression vector and VDR siRNA vector

The results of Sodium Bisulfite sequencing indicated that DNA methylation levels in transcription regulation region of methylation sensitive genes CD11a, CD70, and CD40L genes increased in the VDR over-expressed SLE and VDR over-expressed RA groups in comparison to the SLE control, RA control, SLE NC and RA NC groups (all $P < 0.05$). DNA methylation levels of CD11a, CD70, and CD40L genes in the VDR siRNA group were lower than those in the normal control and normal NC groups (all $P < 0.05$). Also, DNA methylation levels of CD11a, CD70, and CD40L genes in SLE CD4⁺T cells and RA CD4⁺T cells were lower than those in normal CD4⁺T cells (all $P < 0.05$). However, no significant difference was found in DNA methylation levels of CD11a, CD70, and CD40L genes between SLE CD4⁺T cells and RA CD4⁺T cells (all $P > 0.05$) (Fig. 8).

Discussion

SLE was a chronic and severe systemic autoimmune disease with heterogeneous clinical manifestations, which affected almost all organs of the body [18, 19]. Although SLE risk of the genetic contribution was high, it was actually a genetically complex disease [20]. Therefore, an increased understanding of the genome structure could cause more discoveries that would be helpful to unravel the mechanisms involved [21].

In our study, we found that 1,25(OH)₂D₃ and VDR could play an important role in the activate regulation of CD4⁺T cells and the SLE pathogenesis by mediating the PKCδ/ERK pathway, because serum 1,25(OH)₂D₃ levels and VDR expressions were significantly reduced in SLE patients. Also, inhibiting PKCδ/ERK pathway and increasing DNA methylation levels were conducive to inhibit the CD4⁺T cells excessive activation so as to ease SLE autoimmune reaction. As the active form of vitamin, 1,25(OH)₂D₃ had anti-proliferative and pro-differentiation effects in many malignant cells, which could reduce the development and growth of tumors in preclinical models [22, 23]. It was also found that 1,25(OH)₂D₃ could regulate the expression of many apoptosis factors or mediators and directly induce apoptosis through the activation of Caspase, which had been proved that it could reduce the immunostimulatory effects of the SLE [24, 25]. Carvalho et al. had illustrated vitamin D, acting through VDR expressed in all immune cells, played an important role in the immune system homeostasis, and VDR polymorphisms and SLE had positive association [26]. In addition, some immunosuppressive effects were mediated by the interaction of hormone and nuclear receptor, and it had found that VDR could regulate gene expressions of sensitive tissues in vitamin D [27], which was consistent with our results. PKCδ was a serine kinase, being implicated in the control of cell proliferation and tissue remodeling [28]. Study had concluded that PKCδ deficiency could lead to a genetic defect of apoptosis leading to SLE, which accorded to our study [29]. ERK pathway was a kinase signaling cascade ubiquitously expressed in eukaryotic cells, which was initially thought to be primarily regulated by cell growth and proliferation, but was also believed to mediate inflammatory responses [30]. Being sufficient to cause SLE, CD4⁺T cells from SLE patients showed ERK signaling pathway decreased and could result in changes of gene expression, which proved that environmentally-mediated T cell PKCδ inactivation played a causative role in SLE. In addition, patients with active SLE could lead to the decrease of T cell methylation, which was consistent with our study result [31]. In addition, ERK1/2 was critical for T cell activation, therefore the

methylation defect in T cells from SLE patients was traced to inhibit ERK pathway signaling [30], which proved our results from the side.

Our study also found that the increase doses of 1,25(OH)₂D₃ could increase DNA methylation levels of CD11a, CD70 and CD40L in SLE CD4⁺T cells and decrease the expressions of PKCδ, ERK1/2, CD11a, CD70 and CD40L. When SLE CD4⁺T cells were transfected with VDR overexpression vector, PKCδ, ERK1/2, CD11a, CD70 and CD40L expressions decreased, but CD11a, CD70 and CD40L methylation levels increased; when normal CD4⁺T cells were transfected with VDR siRNA vector, PKCδ, ERK1/2, CD11a, CD70 and CD40L expressions increased, but CD11a, CD70 and CD40L methylation levels decreased. Ivanova et al. pointed out that sera from SLE patients had differential immuno-reactivity to RNA, mitochondrial DNA (mtDNA) and total DNA, and mtDNA might play a vital role in the pathogenesis of SLE [32]. As the active form of vitamin D, 1,25(OH)₂D₃ was proved to regulate calcium and phosphate metabolism, cell differentiation, which had immunosuppressive effects [33]. Also, VDR was expressed in the mammary gland and vitamin D had been shown to display anti-cancer properties [34]. A previous study had concluded that with the increasing of serum 1,25(OH)₂D₃ level in human primary cell myoblasts, VDR expression could significantly increase [35]. It had been reported that CD40 stimulation was essential in the control of intracellular killing of pathogens and 1,25(OH)₂D₃ had been stimulated with the CD40L ligand. Therefore, 1,25(OH)₂D₃ could inhibit the expression of CD40 [36]. Also, VDR overexpression vector could reduce the activities of the collagen promoter in transfected cells [37]. Previous study had illustrated that evaluation of fibroblast VDR has revealed three general classes of molecular defects, and one was decreased or absent 1,25(OH)₂D₃ binding [38].

In conclusion, our study provide evidence that 1,25(OH)₂D₃ and VDR could inhibit the activation of CD4⁺ T cells and suppress the immune response of SLE and RA through inhibiting PKCδ/ERK pathway and promoting DNA methylation of CD11a, CD70 and CD40L. However, the way of signal pathways to affect SLE and the measurements of CD11a, CD70 and CD40L were still unclear, more studies needed to be conducted.

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Disclosure Statement

The authors declare no conflicts of interest.

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Erratum

In the original article by He et al. entitled “Roles of $1,25(\text{OH})_2\text{D}_3$ and Vitamin D Receptor in the Pathogenesis of Rheumatoid Arthritis and Systemic Lupus Erythematosus by Regulating the Activation of CD4^+ T Cells and the $\text{PKC}\delta/\text{ERK}$ Signaling Pathway” [Cell Physiol Biochem 2016;40:743-756 (DOI: 10.1159/000453135)] is necessary for authors to add the funding:

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