

Original Article

Preventive effects of 1,25-(OH)₂VD₃ against ConA-induced mouse hepatitis through promoting vitamin D receptor gene expression

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Aim: To investigate the immunosuppressive effects of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂VD₃) on concanavalin A (ConA)-induced hepatitis and elucidate the action mechanism.

Methods: Female BALB/C mice were intravenously administered ConA (20 mg/kg) to induce acute immunological liver injury. Liver damage was evaluated in respect to serum alanine transaminase (ALT) level and liver histological changes. The proliferation of splenocytes was measured by using [³H]-thymidine incorporation. The cytokine level in the cultured splenocyte supernatant was determined by using enzyme-linked immunosorbent assays (ELISAs). The percentage of different splenic T cell subtypes was analyzed by using flow cytometry. The expression of splenic vitamin D receptor (VDR) mRNA and protein was detected by using real-time qRT-PCR and Western blot, respectively.

Results: 1,25-(OH)₂VD₃ (2.5 μg/kg, ip) significantly decreased the serum ALT levels and markedly attenuated the histological liver damage. The beneficial effect of 1,25-(OH)₂VD₃ was associated with: (i) inhibition of CD4⁺ T cell activation; (ii) reduction of interferon-γ (IFN-γ) and elevation of both IL-4 and IL-5 in supernatants of cultured splenocytes; and (iii) elimination of activated T cells by increasing VDR mRNA and protein expression in the spleen.

Conclusion: 1,25-(OH)₂VD₃ had a significant protective effect against ConA-induced hepatitis, and its mechanism of action was associated with down-regulation of T cell-mediated immunity and up-regulation of VDR gene expression.

Keywords: 1,25-dihydroxyvitamin D₃; concanavalin A; hepatitis; vitamin D receptor; interferon-γ; interleukin 4; interleukin 5

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Introduction

Autoimmune liver disease includes autoimmune hepatitis (AIH), a distinct form of acute and chronic inflammatory liver disease in which immune reactions against host antigens have been found to be the major pathological mechanism^[1]. AIH may lead to severe liver disease such as liver cirrhosis. The frequency of AIH among patients with chronic liver disease in North America is between 11% and 23%^[2]. With improvements in the clinical detection technology, the incidence of autoimmune liver disease has gradually increased in recent years. The major treatment option is immunosuppressive therapy, which is proven to be effective^[3].

In the pathogenesis of AIH, genetic factors predispose individuals to the development of the disease^[4]. Moreover, the environment contributes to development, and there is reason to believe that vitamin D may be an environmental factor that affects AIH. A previous study demonstrated that calcium and vitamin D supplementation is beneficial to patients with AIH^[5]. Vitamin D inadequacy is common in noncholestatic chronic liver disease such as AIH and correlates with disease severity^[6]. Compared with healthy people, patients with AIH display lower serum levels of vitamin D^[7].

1,25-(OH)₂VD₃ is one of the metabolites of vitamin D, and among all vitamin D metabolites, it has the strongest biological activity *in vivo* with the bio-active domain of non-protein binding free form^[8]. In addition to regulating calcium and phosphorus metabolism, 1,25-(OH)₂VD₃ was also recently found to have immunosuppressive effects. Its biological

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effects are primarily mediated by vitamin D receptor (VDR)^[9]. 1,25-(OH)₂VD₃ selectively inhibits Th1 cell proliferation and function^[10] by inhibiting interleukin (IL)-2 and interferon (IFN)- γ generation in Th1 cells^[11, 12] as well as IL-12 production in mononuclear cells^[13], thereby inhibiting the occurrence and progress of autoimmune diseases^[14] and organ transplant rejection^[15], which are caused by excessive activation of T cell-mediated immunity. The immunosuppressive effects of 1,25-(OH)₂VD₃ have been demonstrated in many animal models such as experimental autoimmune encephalomyelitis^[16], autoimmune diabetes^[17], rheumatoid arthritis^[18], systemic red spot lupus^[19], inflammatory bowel disease^[20] and allogeneic transplantation^[15]; however, its role in AIH has not previously been reported.

ConA-induced hepatitis is a commonly used mouse model of T cell immune-mediated liver injury that resembles viral and autoimmune hepatitis in humans^[21]. In the present study, we investigated whether 1,25-(OH)₂VD₃ could alleviate ConA-induced liver injury of mice *in vivo*, and we also investigated its immunosuppressive mechanisms of action.

Materials and methods

Mice

Female BALB/c mice (6–8 weeks old, 20–22 g) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences. The animals were housed in specific pathogen-free conditions. All mice were allowed to acclimatize in our facility for 1 week before any experiments were started. All experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai University of Traditional Chinese Medicine.

Induction of ConA-induced hepatitis and drug administration

ConA (Sigma, St Louis, USA) was dissolved in pathogen-free saline and filtered through 0.45 μ m filter (Millipore). Mouse hepatitis was induced by injection of the indicated doses of ConA (in 0.2 mL saline) into the tail vein. A stock solution of 1,25-(OH)₂VD₃ (Sigma, St Louis, USA) was prepared in ethanol and diluted in pathogen-free saline to 0.25 μ g/mL. 1,25-(OH)₂VD₃ was administered intraperitoneally (*ip* in 0.2 mL solvent) once daily on d -7, -6, -5, -4, -3, -2, -1, and 1 h before ConA injection on d 0.

Liver transaminase activity

Serum from individual mice was obtained 24 h after ConA injection. Alanine transaminase (ALT) activity was measured by the standard photometric method using a commercial kit (Jiancheng, Nanjing, China).

Histological examination

Livers were fixed in 10% formalin, embedded in paraffin, cut into 5 μ mol/L sections, deparaffinized in xylene and serially dehydrated in decreasing concentrations of ethanol. Sections were stained with hematoxylin-eosin and examined under light microscopy to evaluate tissue structure and pathological

grade. Necrosis was analyzed with a five-point score: 0, none; 1, single cell necrosis; 2, 1%–20% necrosis; 3, 20%–40% necrosis; and 4, >40% necrosis ($n=5$ mice/group).

Splenocyte preparation

Mice were killed, and their spleens were removed aseptically. A single spleen cell suspension was prepared, and cell debris and clumps were removed. Erythrocytes were lysed with Tris-buffered ammonium chloride. Mononuclear cells were washed and re-suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL).

Flow cytometric analysis

Splenocytes were harvested 24 h after ConA injection. To analyze the splenic T cell subtype percentage, splenocytes were stained with FITC conjugated anti-mouse CD4 and PE conjugated anti-mouse CD8 mAb (eBioscience) and analyzed on a fluorescence activated cell sort (FACS) Aria (Becton Dickinson, CA).

Proliferation assay

Splenocytes (5×10^5 cells/well) were cultured in triplicate with medium alone or stimulated with ConA (5 μ g/mL) for 24 h in 96-well plates in a 37 °C incubator with 5% CO₂. Cells were pulsed with 0.25 μ Ci of [³H]-thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux, PerkinElmer Life Sciences, Boston, MA).

Cytokine measurement

Splenocytes (5×10^5 cells/well) were cultured in triplicate in 96-well flat-bottom plates (Corning) in the presence of 5 μ g/mL ConA. After 24 h, culture supernatants were harvested and stored at -20 °C. The concentrations of IFN- γ , IL-4, and IL-5 were determined by enzyme-linked immunosorbent assays (ELISAs) (BD PharMingen, San Diego, CA, USA).

Real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR)

Splenocytes (5×10^6 cells/well) were stimulated with 5 μ g/mL ConA in 12-well plates for 24 h and lysed using Trizol reagent (GIBCO). Total RNA was isolated from each cell preparation and reverse transcribed into cDNA. Relative quantitation with real-time PCR was performed with SYBR Green PCR Reagents (Qiagen, Valencia, CA, USA) and a Continuous Fluorescence Detection System (MJ Research, USA) according to the manufacturer's instructions. The mRNA levels were normalized to those of GAPDH.

Gene-specific primers were as follows: VDR: (sense) 5'-AACGCTATGACCTGTGAAGGC-3', (anti-sense) 5'-CCTGTACTTACGTCTGCACGA-3'. GAPDH: (sense) 5'-AACTTTGGCATTGTGGAAGG-3', (anti-sense) 5'-GGATGCAGGGATGATGTTCT-3'.

The cDNA was amplified by 45 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 20 s and

then melting at 65 °C–95 °C.

Western blot

Splenocytes (5×10^6 cells/well) were stimulated with 5 $\mu\text{g}/\text{mL}$ ConA in 12-well plates for 24 h. Stimulated cells were lysed in lysis buffer (25 mmol/L Tris buffer, pH 7.4, containing 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L dithiothreitol, 50 $\mu\text{g}/\text{mL}$ each of leupeptin, aprotinin and phenylmethylsulfonylfluoride) by incubation on ice for 30 min. Lysates were then centrifuged at $13\,000 \times g$ at 4 °C for 10 min, and the supernatants were transferred to fresh tubes and stored at -80 °C until required. Proteins (50 μg per lane) were electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with 5% non-fat milk for 1 h to block non-specific binding and then rinsed and incubated with a panel of rabbit polyclonal antibodies against VDR (Santa Cruz Biotechnology, Inc) overnight at 4 °C. The membranes were then incubated with a 1:2000 dilution of HRP-conjugated anti-rabbit IgG (H+L) for 1 h. Immune complexes were detected with a chemiluminescence substrate (Pierce) and exposed to Kodak X-ray film (Kodak).

Statistical analysis

Data are expressed as mean \pm SD of indicated experiments. The Student's *t*-test was used to determine variances between groups where appropriate. A *P* value <0.05 was considered significant.

Results

1, 25-(OH)₂VD₃ effectively attenuated ConA-induced liver damage

To examine the effects of 1,25-(OH)₂VD₃ on ConA-induced mouse hepatitis, which engenders a T cell-dependent acute liver-specific injury that closely resembles human autoimmune hepatitis, 1,25-(OH)₂VD₃ (2.5 $\mu\text{g}/\text{kg}$) was intraperitoneally administered to mice. Serum ALT levels were measured 24 h after ConA injection in both the 1,25-(OH)₂VD₃ and vehicle-treated groups. As shown in Figure 1A, in the 1,25-(OH)₂VD₃-treated group, significantly lower serum ALT levels were detected.

Liver histological studies were also used to determine the effect of 1,25-(OH)₂VD₃ on ConA-induced liver injury (Figure 1B). Light microscopy examination showed massive necrosis in the livers from ConA-induced hepatitis mice 24 h after ConA injection, whereas mice pretreated with 1,25-(OH)₂VD₃ displayed only minor liver damage. The pathological grades also showed the significant preventative effects of 1,25-(OH)₂VD₃ on necrosis (Figure 1C).

1,25-(OH)₂VD₃ effectively reduced the ratio of CD4⁺/CD8⁺ T cells in the spleen

To test whether the inhibitory effect of 1,25-(OH)₂VD₃ was associated with the variation in percentages of T cell subtypes the spleens, FACS analysis was performed. Compared with vehicle control mice, treatment with 1,25-(OH)₂VD₃ signifi-

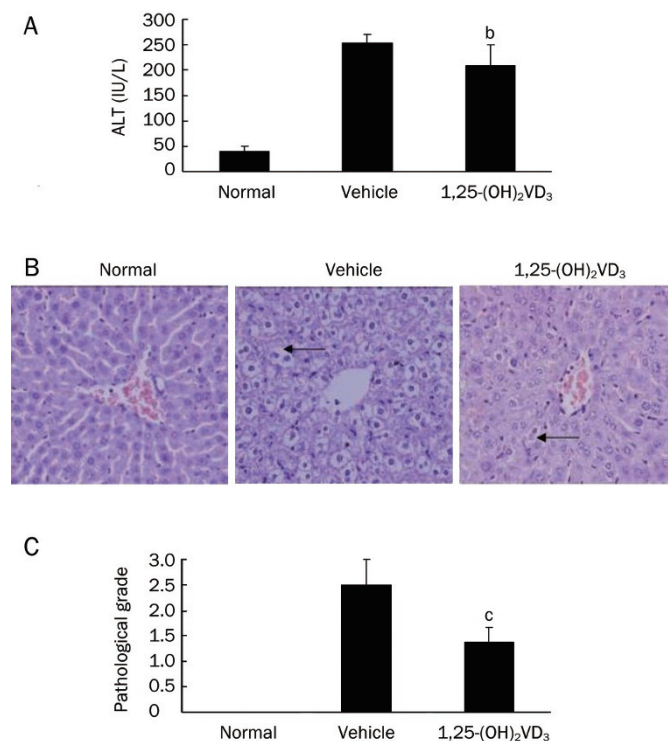


Figure 1. Effects of 1,25-(OH)₂VD₃ on ConA-induced hepatitis. (A) Effect of 1,25-(OH)₂VD₃ on ALT level; (B) Histological examination. H&E staining (×200). Necrotic liver injury was indicated by black arrows. (C) Pathological grade. Data were from a representative experiment. Three separate experiments were performed with similar results. *n*=5 mice/group. Mean \pm SD. ^b*P*<0.05, ^c*P*<0.01 vs vehicle-treated mice.

cantly reduced the percentage of CD4⁺ T cells and increased the percentage of CD8⁺ T cells. As a result, 1,25-(OH)₂VD₃ effectively reduced the ratio of CD4⁺/CD8⁺ T cells in the spleen (Table 1).

Table 1. Effect of 1,25-(OH)₂VD₃ on splenic T-cell subtype percentage. Data were from a representative experiment. Three independent experiments were performed with similar results.

	Normal	Vehicle	1,25-(OH) ₂ VD ₃
CD4 ⁺ T	20.1%	26.9%	22.2%
CD8 ⁺ T	10.2%	5.9%	8.2%
CD4 ⁺ /CD8 ⁺ ratio	1.97	4.56	2.71

1,25-(OH)₂VD₃ significantly impaired T lymphocyte proliferation

The proliferation response of lymphocytes after *in vivo* 1,25-(OH)₂VD₃ treatment was further investigated. When stimulated with ConA *ex vivo*, the proliferation of splenocytes from 1,25-(OH)₂VD₃-treated mice was dramatically inhibited compared with splenocytes from vehicle control mice (Figure 2).

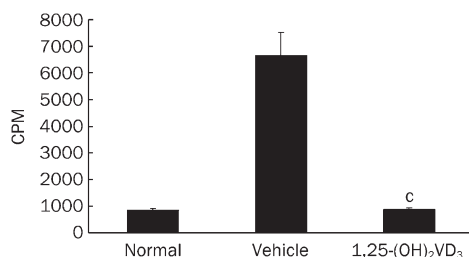


Figure 2. 1,25-(OH)₂VD₃ inhibited ConA-induced splenocytes proliferation. Data were from a representative experiment made in triplicate and expressed as mean±SD. Three independent experiments were performed with similar results. *n*=5 mice/group. ^c*P*<0.01 vs vehicle-treated mice.

1,25-(OH)₂VD₃ effectively reduced interferon-γ (IFN-γ) and increased IL-4 and IL-5 in culture supernatants of splenocytes

Immune responses are greatly influenced by the balance between Th1- and Th2-type cytokines. Th1 cytokines, including IFN-γ and IL-2, generally promote Th1 cell-mediated immune responses. By contrast, Th2 cytokines, which include IL-4 and IL-5, are generally anti-inflammatory and are involved in Th2 cell-mediated humoral immune responses^[22, 23]. To examine the immunosuppressive effect of 1,25-(OH)₂VD₃, the cytokine production of the splenocytes was assayed. Splenocytes separated from 1,25-(OH)₂VD₃-treated and vehicle-treated mice were activated with ConA. After 24 h in culture, the culture supernatant of the splenocytes was harvested, and the levels of IFN-γ, IL-4, and IL-5 were detected by ELISA. Our data show that IFN-γ was markedly reduced in 1,25-(OH)₂VD₃-treated mice compared with vehicle-treated mice, and the levels of IL-4 and IL-5 were significantly increased in 1,25-(OH)₂VD₃-treated mice compared with vehicle-treated mice (Figure 3).

1,25-(OH)₂VD₃ significantly increased the expression of vitamin D receptor mRNA and protein in spleens

To determine whether the immunosuppressive effect of 1,25-(OH)₂VD₃ was related to vitamin D receptor (VDR) expression, the effect of 1,25-(OH)₂VD₃ on VDR mRNA and VDR protein production in splenocytes was examined. Splenocytes separated from 1,25-(OH)₂VD₃-treated and vehicle-treated mice were activated with ConA (5 μg/mL). After 24 h in culture, total RNA and total protein were extracted,

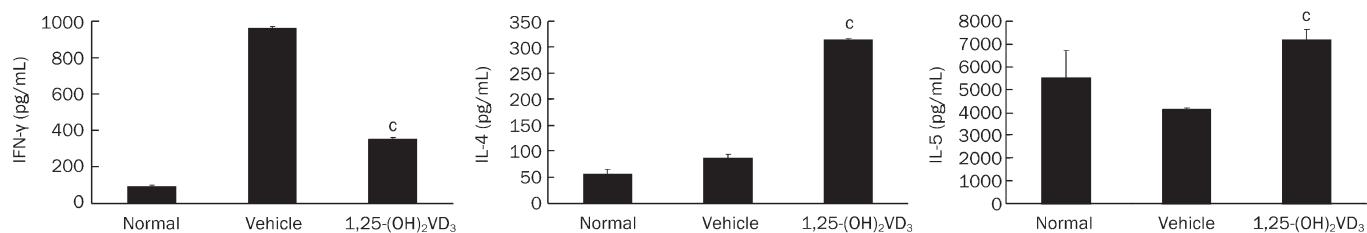


Figure 3. 1,25-(OH)₂VD₃ reduced IFN-γ and increased IL-4 and IL-5 levels in the supernatant of cultured splenocytes. Data of cytokines production were from a representative experiment made in duplicate and expressed as mean±SD. Three independent experiments were performed with similar results. *n*=5 mice/group. ^c*P*<0.01 vs vehicle-treated mice.

and the VDR mRNA and protein expression were detected by real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) and Western blot assays, respectively. Our results show that expression of VDR mRNA (Figure 4A) and VDR protein (Figure 4B) in splenocytes was markedly increased in 1,25-(OH)₂VD₃-treated mice compared with vehicle-treated mice.

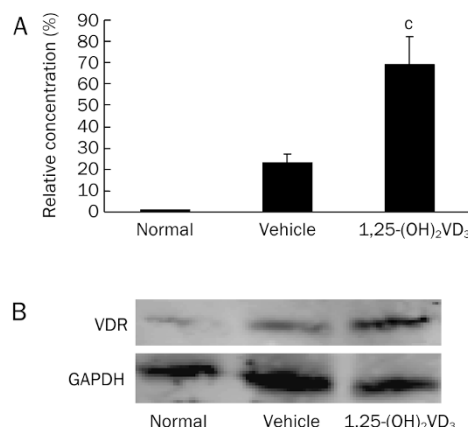


Figure 4. 1,25-(OH)₂VD₃ increased VDR mRNA and protein levels in spleen. (A) The VDR mRNA expression was detected by real-time qRT-PCR assay; (B) The VDR protein production detected by Western blot. Data were from a representative experiment. Three independent experiments were performed with similar results. *n*=5 mice/group. Mean±SD. ^c*P*<0.01 vs vehicle-treated mice.

Discussion

In the present study, we report that 1,25-(OH)₂VD₃ protected mice from immune-mediated liver injury in ConA-induced hepatitis (CIH), and the mechanism of action of 1,25-(OH)₂VD₃ was shown to be closely associated with its immunosuppressive effect on T cell-mediated immunity and its role in promoting VDR gene expression.

ConA-induced mouse hepatitis is due to T cell-mediated liver damage. Intravenous injection of ConA in mice can selectively cause liver injury; T helper (Th) cells and macrophages are the major effector cells in this pathological process^[24]. A large number of splenic T lymphocytes are activated by ConA and produce cytokines; activated T cells and

cytokines can damage liver cells directly or injure liver cells indirectly through activating macrophages. When mice are given 20 mg/kg ConA, the liver is almost entirely edematous and necrotic 24 h later. 1,25-(OH)₂VD₃ can significantly reduce the extent of liver necrosis, indicating its protective effect in immunological liver injury.

In the ConA-induced mouse hepatitis model, the activation of CD4⁺ T cell is closely related to liver injury. The research of Tiegs *et al* has shown that pretreatment with an antibody against T lymphocytes can fully protect mice against ConA-induced liver injury similar to a monoclonal anti-mouse CD4 antibody. However, monoclonal anti-mouse CD8 antibodies failed to protect against damage^[21]. Our study demonstrated that 1,25-(OH)₂VD₃ significantly reduced T cell proliferation *in vitro* and significantly reduced the generation of CD4⁺ T cells, thereby reducing the CD4⁺/CD8⁺ ratio. This indicates that 1,25-(OH)₂VD₃ can prevent mice from ConA-induced hepatitis by inhibiting CD4⁺ T cell activation.

CD4⁺ T cells are functionally classified into functional Th1 cells, Th2 cells and others (undifferentiated, nonfunctional T cells). Th1 cells induce T cell-mediated immune responses^[25]. Th1-type cytokines and pro-inflammatory cytokines play a major role in ConA-induced liver injury^[26], and IFN- γ and TNF- α are the most important cytokines that induce liver injury^[27, 28]. Th2 cells inhibit the generation and effector functions of Th1 cells via the release of cytokines such as IL-4 and IL-5, and consequently, both the reduction in Th1 and an increase in Th2 cell activity are associated with resolution of inflammation in autoimmune diseases^[29]. 1,25-(OH)₂VD₃ significantly reduced IFN- γ expression and increased IL-4 and IL-5 production, indicating that it can inhibit T cell-mediated immunity in ConA-induced mouse hepatitis by inhibiting Th1-cell function and enhancing Th2-cell function.

Vitamin D achieves its biological effects through the vitamin D receptor (VDR). Most cells in the immune system contain VDR, such as monocytes, tissue macrophages and activated T and B lymphocytes^[30]. Of all the vitamin D metabolites, 1,25-(OH)₂VD₃ has the strongest biological activity *in vivo*, and it can directly inhibit T cell proliferation and IL-2 and IFN- γ secretion^[31]. Our results show that 1,25-(OH)₂VD₃ significantly increased VDR mRNA and protein levels, and it also inhibited the production of IFN- γ and increased the generation of IL-4 and IL-5, increasing the proportion of CD8⁺ T cells and reducing the proportion of CD4⁺ T cells. These results indicate that 1,25-(OH)₂VD₃ can inhibit T cell-mediated immunity by increasing VDR gene expression.

In conclusion, 1,25-(OH)₂VD₃ has a strong inhibitory effect on T cell-mediated immunity. It may inhibit T cell immune responses partially through promoting VDR gene expression, thereby playing a role in the prevention and treatment of ConA-induced acute immunological liver injury. However, the inhibition mechanism of VDR gene expression against T cell activation needs to be further studied.

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Author contribution

Xu-dong HU and Ping LIU designed research; Xu-dong HU and Shi-li JIANG performed research; Cheng LIU, Ming-yu SUN and Gao-feng CHEN contributed new analytical tools and reagents; Xu-dong HU, Cheng-hai LIU and Yi-yang HU analyzed data; Xu-dong HU wrote the paper.

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