1,25-dihydroxyvitamin D₃ regulates LPS-induced cytokine production and reduces mortality in rats

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Abstract

AIM: To study the immunoregulatory effect of 1,25-dihydroxyvitamin-D₃ on dominant Th1 response in rats.

METHODS: Sixty adult Lewis rats were randomized into three groups. Rats in group 1 (n=25) were treated with 1,25-(OH)₂D₃ first and then challenged with LPS; rats in group 2 (n=25) were treated with vehicle first and then challenged with LPS. Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin, 24 h after the last administration of 1,25-(OH)₂D₃ and vehicle. Rats in group 3 (n=10) were treated with 1,25-(OH)₂D₃ only. Serum IL-12, IFN-γ, IL-2 and IL-4 levels were measured and target gene of 1,25-(OH)₂D₃ on Th cells was studied after 6 h. Gene abundance was verified by real-time quantitative PCR.

RESULTS: No death occurred in rats pretreated with 1,25-(OH)₂D₃ after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h. Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-(OH)₂D₃, as compared with those in rats pretreated with the vehicle. The serum content of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups.

CONCLUSION: 1,25-(OH)₂D₃ attenuates injury induced by the lethal dose of LPS, regulates Th1 and Th2 cells at the transcription level, and dominantly responds to cytokine production in rats.

INTRODUCTION

1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] is an active form of vitamin D, which not only regulates the dynamic balance of calcium and phosphorus metabolism but also participates in differentiation and regulation of the immune system[11,21]. In vitro study[22] showed that both antigen-presenting cells (APCs) and activated lymphocytes express vitamin D receptor (VDR), and that 1,25-(OH)₂D₃ acts on APCs (mainly dendritic cells) and helper T cells (Th) through VDR mediation[23], inhibits proliferation and differentiation of Th1 and cytokine production, and induces differentiation of Th2. The status of Th1/Th2 differentiation determines the type of immune response and the final outcome of body response[24]. Cytokine environment is the key factor for initiating Th1/Th2 differentiation[25].

Th1 immune response is not only associated with a variety of acute inflammatory responses but also plays a leading role in the development and progression of many autoimmune diseases and transplantation rejection[18-22]. Few in vitro studies reporting the influence of 1,25-(OH)₂D₃ on Th1 immune response are available, and the experimental results about cytokine regulation are conflicting or completely different[26-29]. The target gene in Th cells...
remains almost unknown\textsuperscript{[10,17]}. \textit{E. coli} endotoxin is a potent bacterial mitogen, able to promote maturity of immature dendritic cells (DC), directly activates T cells and induces Th1 immune response\textsuperscript{[18]}. We established a Th1 dominant response animal model and pretreated it with 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3}. The results of our study showed that 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3} was able to regulate the production of IL-12, IFN-\gamma and IL-4 in dendritic, Th1 and Th2 cells. The effector target point of regulation was at the gene transcription level. It is the regulation of 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3} on T cell polarization that attenuates injury induced by the lethal dose of LPS in rats and significantly reduces the mortality of rats.

\textbf{MATERIALS AND METHODS}

\textbf{Animals}

Inbred line Lewis rats (at the age of 3.5-4.5 mo, weighing 242 ± 14 g) were provided by Experimental Animal Center of the Chinese Academy of Medical Sciences (Beijing, China) and fed with normal chow containing 1.6% calcium, 0.9% phosphorus and 0.3% vitamin D (Nanjing Animal Technology Co., Ltd, Nanjing, China) with free access to water. The experiment protocol followed the institutional regulations of the Ministry of Health of the People's Republic of China concerning animal experimentation.

\textbf{Experiment protocol}

Sixty rats were randomized into three groups. Rats in group 1 (n = 25) as the study group, were administrated 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3} by gavage (GmbHcd&Go, Swiss) at 1 \textmu g/animal for 14 d\textsuperscript{[19]}; rats in group 2 (n = 25) as the positive control group were administrated the same dose of the vehicle for 14 d by gavage. Animals in groups 1 and 2 were injected intraperitoneally with \textit{E. coli} 0111, B4 (Sigma, USA). Rats in group 3 (n = 10) as the negative control group were administrated 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3} only by gavage at the dose of 1 \textmu g/animal for 14 d, and injected (i.p) with the same volume of normal saline (Sigma Chemical CO., St Louis, MO, USA).

Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin (10 mg/kg), 24 h after the last administration of 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3} and vehicle. Six hours after the injection, they were anesthetized with 50 mg/kg (i.p) pentobarbital (Sigma-Aldrich, USA) and used for drawing 5 mL blood from the abdominal major artery. The blood was centrifuged at 4°C for 15 min, and the serum was stored at -80°C for test. The spleen was removed aseptically, washed with PBS and stored in liquid nitrogen.

\textbf{Enzyme-linked immunosorbent assay (ELISA)}

Serum IL-12, IL-2, IFN-\gamma and IL-4 levels were measured with commercially available ELISA kits (Biosource CO., Camarillo, CA, USA) according to the manufacturer's instructions, and the quality control serum values were calculated.

\textbf{Ca\textsuperscript{2+}/NF-AT signaling pathway gene array}

Three spleen tissue samples were chosen randomly from rats in groups 1 and 2 for RNA extraction. UV absorption precipitation method and denaturing gel electrophoresis were used to test the quantity, quality and completion of RNA. The probe was synthesized by RT-PCR. Five \textmu g RNA was used to prepare annealing solution and mixed with RT solution to undergo reverse transcription reaction under the action of reverse transcriptase (M1701, Promega, USA).

Chip hybridization was conducted by using Ca\textsuperscript{2+}/NF-AT signaling pathway gene array chip (Super Array Bioscience CO., Cat.NO.HS-022 USA) and chemiluminescent assay kit (Super Array Bioscience CO., NO.D-01) according to the manufacturer's instructions. The chip was scanned with the ArtixScan 120tf scanner (Micro TEK CO., USA) and the original data were analyzed using the attached software GEArray analyzer. Each chip had 10 positive controls (2 for GAPDH, 4 for Ppia, 2 for RP113 and 2 for Actinb), three negative controls (PUC18DNA) and 3 blank controls. The original data were deduced by the background minimum value and then corrected by the content of home-keeping gene. The corrected data were analyzed for abundance of gene transcription between the two groups. The ratio \(\geq 2\) was considered up-regulation of the gene and \(\leq 0.5\) down-regulation\textsuperscript{[20]}.

\textbf{Verification of IL-2 gene expression by RT-PCR}

RNA extraction was done as previously described. The sample was RNA reverse transcripted to synthesized cDNA. The target gene and home-keeping gene of the sample were reacted by RT-PCR. A standard curve was plotted by measurement of the standard sample gradient to calculate the content of gene in the sample, which was corrected by the content of home-keeping gene to obtain the content of the related gene. All reagents used in the experiment were provided by Promega CO., USA. The sequences of \(\beta\)-actin (211 bp) and IL-2 (190 bp) are 5'-CCTGTAGCCACACAGTGCC-3' and 5'-ATACTCC TGCTTGCTGATCC-3', and 5'-CAGTGGCTTGTC CTCCCT-3' and 5'-TTCAATCTGTTGGCCTGCTT-3', respectively.

\textbf{Statistical analysis}

Data were represented as mean ± SD. SSPS 10.0 was used to perform \(t\)-test and \(F\)-test. \(P < 0.05\) was considered statistically significant.

\textbf{RESULTS}

\textbf{Mortality of rats after LPS injection and protective effect of 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3}}

No death occurred in rats pretreated with 1,25-(OH\textsubscript{2})\textsubscript{D}\textsubscript{3} after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h.
**DISCUSSION**

The purpose of the present experiment was to clarify the immune regulatory effect of 1,25-(OH)₂D₃ on Th1 dominant response in vivo. The results showed that 1,25-(OH)₂D₃ inhibited IFN-γ production of IL-12 and Th1 cytokines, suggesting that this inhibitory effect occurs at the transcription level. What implies in the results of the present experiment is the therapeutic effect of 1,25-(OH)₂D₃ on diseases mainly characterized by Th1 immune response (including autoimmune diseases) and transplantation rejection[6]. At the same time, as 1,25-(OH)₂D₃ affects the secretory profile of Th1 and Th2 cytokines[21,22], it inhibited the acute inflammatory reaction in the rats of group 1, indicating that 1,25-(OH)₂D₃ attenuates LPS lethal dose-induced injury in rats. The fact that all rats survived in group 1 suggests that 1,25-(OH)₂D₃ may also play a role in inhibiting the development and progression of acute inflammatory reaction.

IL-12 is a cytokine secreted by APCs and plays a central role in the growth of Th1 cells[23]. IL-12 has a potent biological function of inducing T cells to secrete IFN-γ. IFN-γ is a pleiotropic cytokine, promoting inflammatory reaction and inducing expression of main tissue surface compatible complex of multiple cells[23]. Most recent studies found that this cytokine promotes vascular disease of the transplanted organ at the late stage of transplantation[24]. The present experiment confirmed that 1,25-(OH)₂D₃ could inhibited IL-12 production in rats, suggesting that it is able to inhibit strong Th1 immune response via its action on APCs, thus reducing IFN-γ production. At the same time, 1,25-(OH)₂D₃ may also directly inhibit the differentiation and proliferation of Th1 cells, as the cytokines mainly secreted by Th1 cells are reduced, especially transcription of NF-κB is inhibited. NF-κB is a key mediator of gene expression in immune and inflammatory responses. We also found that 1,25-(OH)₂D₃ inhibited proliferation of splenic lymphocytes in rats challenged with LPS. We, therefore, think that the results of the above experiment suggest that differentiation and proliferation of 1,25-(OH)₂D₃ on Th1 may also have an inhibitory effect on proliferation of splenic lymphocytes and is able to selectively inhibit Th1 immune response.

IL-4 is a main factor influencing the development of T cells into Th2 cells[13,22]. Once IL-4 level is able to resist activation of IL-12 on Th cells and IFN-γ on IL-4, it promotes differentiation of juvenile T cells to Th2 cells[26]. It is controversial over the regulatory effect of 1,25-(OH)₂D₃ on IL-4. It was reported that the effect of 1,25-(OH)₂D₃ is mediated through IL-4[25] and that it is the up-regulation of IL-4 and TGF-β by 1,25-(OH)₂D₃ that inhibits the inflammatory reaction rather than by the reduction of Th1 cytokines IFN-γ and TNF-α[29]. It was also reported that 1,25-(OH)₂D₃ has no influence on the production of IL-4, or down-regulates IL-4[16,17]. We detected serum IL-4 levels in four batches of rats pretreated with 1,25-(OH)₂D₃ and

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**Table 1** 1,25-(OH)₂D₃-regulated LPS-induced cytokine production in rats (pg/mL, mean ± SD)

|         | 1,25-(OH)₂D₃ + LPS | Vehicle + LPS | 1,25-(OH)₂D₃ (n = 15) |
|---------|-------------------|--------------|-----------------------|
| IL-12   | 5986 ± 528        | 4610 ± 289   | 69.99 ± 3.99          |
| IFN-γ   | 4840 ± 802        | 5264 ± 524   | 4.24 ± 0.12           |
| IL-4    | 5.57 ± 1.79       | 3.76 ± 1.60  | 6.45 ± 3.02           |

*P < 0.05 vs vehicle + LPS; *P < 0.01 vs 1,25-(OH)₂D₃ + LPS and vehicle + LPS.

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**1,25-(OH)₂D₃ inhibited LPS-stimulated production of IL-12 and IFN-γ in rats**

Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-(OH)₂D₃ as compared with those in rats pretreated with the vehicle. The serum level of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups. As the serum IL-2 was below the limit of measurement in most rats 3 and 6 h after LPS attack, measurement was not done.

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**1,25-(OH)₂D₃ promoted IL-4 production in LPS-challenged rats**

Six hours after endotoxin stimulation, serum IL-4 level elevated significantly in rats pretreated with 1,25-(OH)₂D₃ as compared with that in rats pre-treated with the vehicle. As the serum IL-4 was below the limit of measurement in most rats that are not attacked by LPS, measurement was not done.

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**Quality control of RNA extraction**

Electrophoresis showed that RNA extracted from the rat spleen displayed two clear bands (18S and 28S), and the absorbance at 260 nm and 280 nm was between 1.8 and 2.0, indicating that no RNA degradation occurred and the extract outcome was good.

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**1,25-(OH)₂D₃ regulated expression of Th1 and Th2 cytokines and related transcription factors**

The gene chip used in the present experiment contains 95 target genes and other positive and negative controls.

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**Verification of down-regulation of IL-2 gene expression by RT-PCR**

The results of the experiment showed that gene expression level in rats pretreated with 1,25-(OH)₂D₃ was significantly lower than that in rats pre-treated with the vehicle (0.476 ± 0.023 vs 0.678 ± 0.038, P < 0.01).
those pretreated with the vehicle. Although we used inbred line Lewis rats with little individual variance in establishing the model, we still found a significant individual difference in serum IL-4 level of the same experiment group, where the IL-4 level was lower than the test baseline in some rats. Only when we expanded the sample capacity, were the statistically significant results obtained. The results of gene chip test also showed that there was a great difference in IL-4 expression level between the rats 6 h after LPS stimulation. Only in one of the three rats in the study group, was IL-4 mRNA expression up-regulated by stimulation. Only in one of the three rats in the study group, was IL-4 mRNA expression up-regulated by stimulation.

as there was still a tendency to up-regulate the gene expression of IL-4, IL-5 and IL-10 mainly secreted by Th2 cells, we performed another experiment, which confirmed again that 1,25-(OH)2D3 was able to up-regulate serum IL-10 level in rats challenged with LPS suggesting that 1,25-(OH)2D3 is able to promote the production of Th2 type cytokines and at the same time inhibit the extent and progression of Th1 type immune response, forming the so-called “immune deviation” phenomenon, which is believed to help establish peripheral tolerance and is of significance in inhibiting transplantation rejection.

IL-12 is an allodiploid consisting of two subunits (P35 and P40) encoded by two genes independently.

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Table 2 Genes down-regulated by 1,25-(OH)2D3 in rat spleens

| GenBank | Description | Gene name | Gene expression | Abundance (Exp/vehicle) |
|---------|-------------|-----------|-----------------|-------------------------|
| NM007596 | Calcium/calmodulin-dependent protein kinase II, beta | CamK II | 0.00E + 00 | 0.00E + 00 |
| NM007973 | Calcium/calmodulin-dependent protein kinase IV | CamK IV | 0.00E + 00 | 0.00E + 00 |
| NM009483 | Cytotoxic T-lymphocyte-associated protein 4 | CD152 | 9.20E - 02 | 0.00E + 00 |
| NM011162 | CD, antigen, zeta polypeptide | CD3Z antigen | 5.00E - 02 | 0.00E + 00 |
| NM007726 | Cannabinoid receptor 1 | cbl | 3.00E + 00 | 0.00E + 00 |
| NM009969 | Colony stimulating factor | GM-CSF | 1.88E - 01 | 1.38E - 01 |
| NM012413 | Epithelial calcium channel 2 | ECa2 | 0.00E + 00 | N/A |
| NM010118 | Early growth response 2 | Krox-20 | 2.99E - 02 | N/A |
| NM010184 | Fc receptor, IgE, high affinity 1, alpha polypeptide | FCεR-5 | 1.36E - 01 | 0.00E + 00 |
| NM016663 | FK506 binding protein 1b | FKBP1b/FKBP | 0.00E + 00 | 0.00E + 00 |
| NM019827 | Glycogen synthase kinase 3 | Gsk-3 | 2.31E - 01 | 1.05E + 00 |
| NM008284 | Sarcoma virus oncogene 1 | H-ras | 0.00E + 00 | 1.03E - 02 |
| NM008337 | Interferon gamma | IFN-γ | 3.44E - 01 | 1.02E + 00 |
| NM008366 | Interleukin 2 | IL-2 | 3.98E - 01 | 2.26E - 01 |
| NM008367 | Interleukin 2 receptor, alpha chain | CD25 | 1.99E - 01 | 2.11E - 01 |
| NM010591 | Jun oncogene | c-Jun | 0.00E + 00 | 0.00E + 00 |
| NM019486 | Kinase interacting protein 2 | Kip 2 | 1.84E - 01 | 8.26E - 03 |
| NM017746 | Mitogen activated protein kinase 8 | Cot | 1.50E - 02 | 0.00E + 00 |
| NM011951 | Mitogen activated protein Mus musculus Harvey rat | P38MAPK | 3.65E - 03 | 1.88E - 02 |
| NM016700 | Mitogen activated protein | JNK1 | 4.21E - 01 | 3.59E - 01 |
| NM021806 | Myogenic factor 5 | Myf 5 | 1.83E - 02 | 6.37E - 01 |
| NM019792 | Nuclear factor of activated T-cell, cytoplasmic 1 | NF-ATc | 3.59E - 01 | 1.38E - 01 |
| NM010256 | Protein phosphatase 3, catalytic subunit, gamma isoform | Calcineurin A gamma | 6.12E - 02 | 3.54E - 01 |
| NM013693 | Colony stimulating factor | GM-CSF | 1.88E - 01 | 0.00E + 00 |
| NM013188 | Fc receptor, IgE, Low affinity III | CD16 | 6.62E - 01 | 1.24E - 01 |
| NM24684 | Fas-like antigen 2 | fas-2 | 5.59E - 01 | 0.00E + 00 |
| NM011840 | Protein phosphatase 3, regulatory subunit B, alpha isoform | Calcineurin B | 2.23E + 00 | 4.73E - 01 |
| NM010177 | Tumor necrosis factor receptor (ligand) superfamily, member 6 | Fas | 1.02E + 00 | 1.88E - 01 |
| NM019408 | Nuclear factor of kappa light polypeptide gene enhancer in B-cell | NF-κB | 4.58E - 01 | 4.25E - 01 |

N/A: Gene expression level, 1,25-(OH)2D3 = 0 and vehicle ≠ 2.

Table 3 Genes up-regulated by 1,25-(OH)2D3 in rat spleens

| GenBank | Description | Gene name | Gene expression | Abundance (Exp/vehicle) |
|---------|-------------|-----------|-----------------|-------------------------|
| NM003548 | Interleukin 10 | IL-10 | 2.30E+00 | 1.69E+00 |
| NM010899 | Nuclear factor of activated T-cell, cytoplasmic 2 | NFAT1 (NFATP) | N/A | N/A |
| NM009192 | Src-like adaptor | SLA | 2.57E + 00 | 8.97E - 01 |
| NM013672 | Trans-acting transcription factor 1 | Sp1 | 2.03E + 00 | 4.87E - 01 |
| NM009505 | Vascular endothelial growth factor A | VEGF/ VEGI | 2.15E + 00 | N/A |
| NM012054 | FB osteosarcoma oncogene | c-fos | N/A | N/A |
| NM010510 | Interferon beta, fibrobast | IFNβ-1 | N/A | N/A |
| NM010583 | Mus musculus IL-2-inducible T-cell kinase | Tsk | 5.77E - 01 | 5.29E + 00 |
| NM012183 | Interleukin 4 | IL-4 | N/A | N/A |
| NM010558 | Interleukin 5 | IL-5 | N/A | N/A |

N/A: Gene expression level, 1,25-(OH)2D3 ≥ 2 and vehicle = 0.
It is known that the P_{40} gene initiator region contains a NF-κB combining site \[^{[13]}\]. The finding in the present experiment that 1,25-(OH)_{2}D_{3} downregulated the important transcription factor NF-κB, suggests that 1,25-(OH)_{2}D_{3} reduces the expression of IL-12P40 subunit by inhibiting NF-κB, thus down-regulating assembly and secretion of IL-12 protein \[^{[13]}\]. After activation of T cells, VDR is downregulated and also plays a major role in a variety of human autoimmune diseases and graft transplantation rejection.

**Peer review**

The manuscript \[^{1}\] presents experimental data from rats. The authors claim by precluding rats with 1,25-(OH)_{2}D_{3}, that the LPS response is shifted towards a Th2-associated cytokine response with reduced Th1-associated cytokine response, so ensuring increased survival. The topic is of high interest.

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