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Research paper

$1,25(OH)_2D_3$ induces regulatory T cell differentiation by influencing the VDR/PLC-γ1/TGF-β1/pathway

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ABSTRACT

Vitamin D has been recommended as an immune modulator in recent years, in addition to regulating calcium-phosphorous-bone metabolism. Clinical studies on organ transplantation found that vitamin D sufficiency patients were less likely to develop acute cellular rejection within one year after transplantation compared to those with vitamin D deficiency. Thus, a high percentage of regulatory T cells might play a key role in preventing acute cellular rejection (ACR). In this report, we studied the specific effects of 1,25(OH)2D3 on human T cell diff ;erentiation, and determined the potential molecule mechanism behind. Results showed that 1,25(OH)2D3 induced the differentiation of T-regulatory cells (Treg cells), while inhibiting Th17 cell proliferation. In addition, 1,25(OH)2D3 promoted secretion of the anti-inflammatory cytokine, transforming Growth Factor beta1 (TGF-β1) but suppressed pro-inflammatory cytokines such as interleukin-17 (IL-17). Phospholipase C gamma 1 (PLC-γ1) is an indispensable signaling protein downstream of the classical TCR signaling pathway and was shown to play a crucial role in T cell activation, while Naive T cells expressed less PLC-γ1. Here we showed that Vitamin D could significantly upregulate PLC-γ1 expression, which then induced expression of TGF-β1. In summary, 1,25(OH)2D3 indirectly modulates the differentiation of Treg/Th17 cells by aff ;ecting the VDR/PLC-γ1/TGF-β1pathway. These results indicate that administration 1,25(OH)2D3 supplements may be a beneficial treatment for organ transplantation recipients.

1. Introduction

1,25-dihydroxy-vitamin D3 [$1,25(OH)_2D_3$], the active form of vitamin D, plays a key role in maintaining calcium and phosphorus homeostasis. However, increasing reports indicate that vitamin D also has important immunomodulatory properties (Chun et al., 2014; Wei and Christakos, 2015). In the adaptive immune system, vitamin D acts as an immune suppressor. Epidemiological studies have found that autoimmune diseases including Type 1 diabetes, multiple sclerosis, and systemic lupus erythematosus (SLE) are related to vitamin D deficiency (Gianfrancesco et al., 2017; Prietl et al., 2013). In addition, daily vitamin D supplementation could reduce the risk of type 1 diabetes and multiple sclerosis (Hypponen et al., 2001; Salzer et al., 2012). In the innate immune system, for instance, vitamin D could induce human monocyte-derived dendritic cells (DCs) to express a tolerogenic phenotype in vitro. $1,25(OH)_2D_3$-modulated tolerogenic DCs express low mature surface molecules including HLA-DR and costimulatory molecules (CD80, CD86), instead of secreting additional IL-10 (Ferreira et al., 2015). $1,25(OH)_2D_3$ acts on the vitamin D receptor (VDR), which belongs to the nuclear hormone receptor superfamily (Coussens et al., 2012). When interacting with $1,25(OH)_2D_3$, VDR heterodimerizes with retinoid X receptor (RXR) and binds target DNA sequences called vitamin D response elements (VDRE) to regulate the expression of interrelated downstream target genes.

PhospholipaseC-γ1 (PLC-γ1) is an important signaling protein participating in the classical TCR signaling pathway. PLC-γ1 plays a crucial role in T cells activation while Naive T cells express less PLC-γ1 (Bradl et al., 2014). The substrate of phosphorylated PLC-γ1 is phosphatidylinositol-4,5-bisphosphate (PIP2), hydrolyzed to inositol-1,4,5-trisphosph-e (IP_3) and diacylglycerol (DAG). IP_3 and DAG then act as second messengers regulating intracellular calcium mobilization. Thus, the increased Ca^+ concentration and Ca^+ signaling pathway induces several important transcription factors which trigger the expression of several genes involved in T-cell activation and cell-cycle entry (von Essen et al., 2010). Another important element related to $1,25(OH)_2D_3$ in our research is transforming growth factor beta 1 (TGF-β1) produced

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by many types of cells. TGF-β1 is a kind of immunosuppressive cytokine that plays a pleotropic role in regulating a variety of immune cells (Rubtsov and Rudensky, 2007). TGF-β1 suppresses the differentiation and function of effector T cells, while converting $CD4^+CD25^-$ T cells into $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells (Maywald et al., 2017) by inducing gene encoding forkhead box P3 (FOXP3) (Dardalhon et al., 2008; Marie et al., 2005). FOXP3 is the specific transcription factor found in Treg cells (Ren and Li, 2017).

Treg cells were first detected and identified as a master regulator of maintaining immune homeostasis in patients with autoimmune disease. Individuals who suffer from X-linked syndrome (IPEX) have an inadequate numbers of Treg cells (Bacchetta et al., 2016) and a mutated FOXP3, which could be alleviated or treated successfully by the adoptive transfer of Treg cells in mouse models (Tang et al., 2004). Furthermore, additional studies showed that defects in Treg cells function contributes to the development of Type 1 diabetes, rheumatoid arthritis, and IBD, which could be prevented or cured potentially by therapies directed at these defects. Treg cells mediate immune suppression through many mechanisms including secreting inhibitory cytokines (IL-10, TGF-β1), direct contact with effector T cells to disrupt metabolism, and targeting dendritic cells (Vignali et al., 2008). Th17 cells, which are characterized by the secretion of IL-17, IL-21/22, and expression of the specific transcription factor, the retinoid-related orphan receptor (RORγt), are instrumental in promoting auto-inflammatory diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and inflammatory bowel diseases (IBDs) (Noack and Miossec, 2014; Waite and Skokos, 2012). For example, elevated levels of IL-17 (Montes et al., 2009; Tzartos et al., 2008) have been found in cerebrospinal fluid (CSF) and serum from MS patients. Further evidence showed that increased levels of IL-17A in the peripheral blood were produced and detected as well as increased numbers of Th17 cells (Eastaff-Leung et al., 2010) within the intestinal mucosa of IBD patients.

Previous clinical and in vitro experiments demonstrated that high circulating levels of $1,25(OH)_2D_3$ could decrease the risk of acute cellar rejection(ACR) in patients with liver transplantation, and high percentage of FOXP3+ Treg cells might play a key role in preventing ACR. In the present study, differentiation of Treg cells was detected, as well as, Th17 and cytokines secretion (TGF-β1 and IL-17A), in the presence of $1,25(OH)_2D_3$ in vitro. It was found that $1,25(OH)_2D_3$ induced Treg cells, while suppressing the differentiation of Th17 cells. It was determined that PLC-γ1 played a key role in inducing the expression of TGF-β1. Briefly, $1,25(OH)_2D_3$ induced regulatory T cell differentiation by regulating the VDR/PLC-γ1/TGF-β1 pathway.

2. Materials and methods

2.1. Cell culture and activation

JE6.1 and Jγ1 (ATCC®CRL-2678™) cells were obtained from the Chinese Academy of Sciences and the American Type Culture Collection (Manassas, VA, USA). Human Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin. All cells were maintained at 37 °C in a 5% CO_2 incubator. 1,25(OH)2D3 (BMLDM200-0050) was obtained from Enzo Life Sciences, Inc., Ann Arbor, MI. Stock solutions of 1 mM $1,25(OH)_2D_3$ were prepared in anhydrous (≥95.0%) ethanol and stored at −20 °C. For stimulation, 1 × 106 cells were treated with anti-CD3 (clone CD3 UCHT1, BD Biosciences_555336) and anti-CD28 (clone CD28.2, BD Biosciences_555725) monoclonal antibodies, at a concentration of 5 μg/mL of each antibody, for 3 days, and the cells were re-stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) for another 3 days (5% CO_2 at 37 °C). CD3 clone UCHT1 is optimal for the immobilized format according to manufacturer's instructions, so the CD3 UCHT1 antibodies were coated onto a plate at 5 μg/mL at 4 °C overnight before stimulation.

2.2. Lentiviral vector construction and establishment of PLC-γ1 overexpression cells

Full-length cDNA encoding PLC-γ1 was ampifed by PCR with primer (F: 5′-AGGTCGACTCTAGAGGATCCCGCCACCATGGCGGGCGCCGCGTCCCCTTGCGCCAACG, R:5′-TCCTTGTAGTCCATACCGAGGCGGTTGTCTCCATTGACCCG AGTCC-3′). Subsequently, the purified PLC-γ1 PCR products were ligated to GV492 vector (GV492-PLC-γ1) (Genechem Co., Ltd, shanghai, China) according to the manufacturer's instructions. The GV492-PLC-γ1 expression construct or the empty GV492 vector was transfected into the 293FT cell line. Virus-containing supernatants were collected for subsequent transduction into Jurkat cell line. Cells were infected with the viruses at a multiplicity of infection (MOI) of 20. At 3 days after virus transduction, the transfected cells were cultured for 1 days with 5 μg/mL puromycin (OriGene) for the establishment of stable cells.

2.3. Western blots

Cells were lysed in an ice-cold lysis buffer (150 mM NaCl, 0.02% NaN3, 0.1% SDS, 50 mM TrisCl, pH 8.0, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1% Triton). Samples of lysate containing 25 μg of protein were separated on a 10% SDS-PAGE gel, and the protein bands were transferred onto PVDF membranes (Millipore; Billerica, MA, USA). The PVDF membranes were then blocked with TBST buffer containing 5% skim milk for 1 h. After washing with TBST buffer thrice for 15 min each, they were incubated with primary antibodies specific for PLC-γ1 (1:1000, Cell Signaling Technology_2822), TGF-β1 (1:1000, Cell Signaling Technology_3709), VDR (1:500, abcam_3508), P-PLC-γ1 (1:1000, Cell Signaling Technology_2821), TGF-β1 (1:1000, Cell Signaling Technology_3709), IL-17A (1:1000, R & D Systems_MAP3171), RORγt (1:1000, R & D Systems_562197), and Foxp3 (1:1000, absin_118887), GAPDH (1 : 2000, BBI Life Science_D110016). After washing with TBST buffer thrice for 15 min each, the gels were then counterstained with HRP-conjugated goat anti-rabbit secondary antibodies (Promab Biotechnologies, 1:1000; Richmond, CA, USA), The individual protein bands were detected with the enhanced chemiluminescence (ECL) reaction (Kibbutz Beit Haemek, Israel), and the staining intensity of each band was quantified using Quantity One software (BioRad Laboratories; Hercules, CA, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA) for cytokines

The concentrations TGF-β1 in culture supernatants were measured by ELISA kits (R & D Systems_DB100B), according to the manufacturer's instructions. The limits of detection were 6.25 pg/mL for TGF-β1.

2.5. Real-time polymerase chain reaction (PCR)

Total cellular RNAs were isolated using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from the purified RNA using a reverse-transcription system (Roche, Germany). Real-time PCR was performed on the Applied Biosystems™ ViiA™ 7 system (Thermo Fisher, USA) by using SYBR Green (Roche, Germany, Cat No: 4913914001) as a double-stranded DNA-specific binding dye. GAPDH was used to normalize the gene expression levels. The PCR primer sequences are listed in Table 1. The PCR reactions were operated for 40

Table 1
Primer sequences for real-time polymerase chain reaction.

Gene	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
VDR	GTGGACATCGGCATGATGAAG	GGTCGTAGGTCTTATGGTGGG
PLC-γ1	GGAAGACCTCACGGGACTTTG	GCGTTTTCAGGCGAAATTCCA
TGF-β1	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC
RORγt	CTTGCCGTAGGGATGTCTCG	GAAGTTCCGTCAGCCCGTT
Foxp3	GTGGCATCATCCGACAAGG	TGTGGAGGAACTCTGGGAAT
GAPH	GAGTCCACTGGCGTCTTC	GACTGTGGTCATGAGTCCTTC