ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH
INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation
Vol 9, Issue 5, 2016
Online - 2455-3891
Print - 0974-2441
Research Article

# EFFECTS OF *IN VITRO* 1,25 DIHYDROXYVITAMIN D ON MATURATION OF DENDRITIC CELLS IN GRAVES' DISEASE PATIENTS

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*Received: 06 June 2016, Revised and Accepted: 08 June 2016*

**ABSTRACT**

**Objective:** The autoimmune reaction in Graves' disease (GD) is induced by self-antigen, which is presented by dendritic cells (DCs). DCs in GD have more active immune responses than those in healthy subjects. The ability of DC as antigen-presenting cell is determined by its maturity level. In GD, vitamin D level is inversely proportional to antibody titer and proportionally associated with remission status. Studies on healthy subjects and autoimmune patients (systemic lupus erythematosus (SLE), multiple sclerosis (MS), and Crohn's disease) have demonstrated immunoregulatory effects of vitamin D, mainly through inhibition of DC maturation, which may decrease the DC's immunogenic profile. This study aims to identify the effect of 1,25-D3 *in vitro* on DC maturation in patients with GD.

**Methods:** This is an experimental study, which was conducted in 12 GD patients with thyrotoxicosis. Monocyte-derived DC of GD patients was cultured, with or without 1,25-D3 *in vitro* at monocytic phase. The DC maturation was then stimulated by lipopolysaccharide (LPS) and evaluated based on the expression of DC markers (human leukocyte antigen-D-related [HLA-DR], CD80, CD40, CD83, CD14, and CD206) and the ratio of cytokine interleukin-12 (IL-12)/IL-10 levels in the supernatants.

**Results:** Following the LPS stimulation, DC with 1,25-D3 showed lower expressions of HLA-DR, CD80, CD40, and CD83, and higher expressions of CD14 and CD206 compared to DC without 1,25-D3. DC with 1,25-D3 had lower ratio of IL-12/IL-10 levels than those without 1,25-D3.

**Conclusion:** *In vitro* 1,25-D3 supplementation inhibits DC maturation in patients with GD.

**Keywords:** Vitamin D, Graves' disease, Dendritic cells.

 DOI: http://dx.doi.org/10.22159/ajpcr.2016.v9i5.13287

## INTRODUCTION

Graves' disease (GD), the most common cause of hyperthyroidism, is an autoimmune disease which is characterized by the presence of autoantibody (thyroid receptor antibody [TRAb]). In GD, inflammatory cytokines also increase, in which the interleukin-12 (IL-12) is the major one related to maturation and activation of dendritic cells (DCs) [1,2]. Mature DCs are potent antigen-presenting cells (APCs) and will induce an adaptive immune response to produce autoantibody.

DCs, especially intrathyroidal, are more immunogenic in GD than in healthy subjects [3]. The number and activity of DC are influenced by thyroid hormone (triiodothyronine [T3] and thyroxine[T4]) [4]; therefore, thyroid hormone will increase DC activity and *vice versa*, which results in continuous adaptive immune response.

In autoimmune disease, the level of DC maturation has an essential role since it determines its immunogenic properties. Mature DCs act as strong APCs, which are able to expose self-antigen to naïve T-cells and stimulate differentiation of T-cells. Immature DC (iDC) has the main capacity to capture and process antigen, but they are weak APC. Mature DC produces a high level of pro-inflammatory cytokine IL-12 and low level of anti-inflammatory IL-10 [5]. DC maturation is affected by genetic and environmental factors. One of the environmental factors which suppress DC maturation is vitamin D [6].

In association with GD, both experimental and epidemiological studies have shown an association between vitamin D deficiency, vitamin D receptor, and vitamin D binding protein polymorphisms with the development of GD [7,8]. Clinically, vitamin D level is related with remission status [9] and inversely related to the TRAb titer in patients with GD [10]. In GD patients, vitamin D analog supplementation (1a-OH) together with antithyroid drugs (ATDs) reduce thyroid hormone level faster than those who receive ATD alone [11]. The reduction of thyroid hormone level is due to Vitamin D-induced alteration of thyroid hormone synthesis, while the immunomodulatory effect of vitamin D on the reduction of thyroid hormone level has not been studied yet.

In term of autoimmune process, inhibition of DC maturation is the main target of vitamin D supplementation. Studies in healthy subjects and those with autoimmune diseases (systemic lupus erythematosus [SLE], multiple sclerosis [MS], and Crohn's disease) demonstrate that vitamin D inhibits DC maturation in monocyte-derived DC (MDDC) cultures [12-15]. This study wants to know the effect of *in vitro* 1,25-D3 supplementation on DC maturation in patients with GD.

## METHODS

### Design

We enrolled 12 GD patients with thyrotoxicosis at the endocrinology clinic of Dr. Cipto Mangunkusumo Hospital in Jakarta, Indonesia. The diagnosis of GD was established based on clinical manifestations (tachycardia, fine tremor, excessive sweat, diffuse struma, and ophthalmopathy) and laboratory data (low thyroid-stimulating hormone [TSH] level, increased fT4 level, and positive TRAb). Patients who took vitamin D supplementation, pregnant, or having comorbidities (diabetes mellitus, chronic kidney disease, and other autoimmune diseases) were excluded from the study. This study has been approved by The Ethics of Committee of the Faculty of Medicine, Universitas Indonesia no.489/H2.F1/ETIK/2014. To be included in the study, subjects had to sign a written informed consent form.

Right after 30 ml venous blood drawing, MDDC cultures were performed with two interventions: Without 1,25-D3 and with 100 nM 1,25-D3 at monocytic phases. MDDC cultures, which consisted of peripheral blood mononuclear cell (PBMC) isolation, monocytes isolation, and lipopolysaccharide (LPS) stimulation, were performed according to study protocol conducted by Budiyati [16]. Some blood specimens were centrifuged, and the serum was stored at −80°C for hematologic analysis. Supernatants were stored at −20°C before IL-12 and IL-10 evaluations.

**Measurement**

TSH, fT4, and TRAb levels were measured using commercial kits as follows: TRAb (DRG), TSH, and fT4 (ROCHE).

**MDDC culture: Mo-DC differentiation**

The PBMC isolation was performed by the method of density differences using Ficoll-Paque premium (GE health care), and the specimens were suspended in the Roswell Park Memorial Institute (RPMI) medium at cell density of $1\times10^6$/mL. In the 12-well culture plate, isolation of monocytes was performed using the adherence method. After the monocytes adhered to the bottom of plate, we added 1 mL of DC culture media, which was a mixture of RPMI 1640 medium, 10% fetal bovine serum (FBS, Gibco), 800 U/mL of granulocyte-macrophage colony-stimulating factor (BD Bioscience Pharmingen), and 1000 U/mL of IL-4 (BD Bioscience Pharmingen). The culture plate was then incubated at 37°C with $CO_2$ 5% for 5 days.

**1,25-D3 stimulation**

The powder of 1,25-D3 (Sigma-Aldrich) was dissolved in 95% ethanol, and a stock solution was made at the concentration of 1 M and was stored at −80°C. Prior to use, the stock solution of 1,25-D3 was diluted until the concentration of 100 nM was reached.

**MDDC culture: DC maturation and harvesting**

After being incubated for 5 days, 500 ng LPS was added (Sigma-Aldrich) and incubation was continued for 2 days. Cell harvesting was performed twice, i.e., before adding LPS (D5) and after being incubated for 2 days with LPS (D7). Cell harvesting was done by light spray at the bottom of the wells, and the DC solution was then processed to separate supernatant from cells. The cells can be processed for monoclonal antibody (mAb) staining.

**Flow cytometry analysis**

The fluorochrome-conjugated antibodies used in flow cytometry technique were obtained from BD Biosciences Pharmingen with the following details: Human leukocyte antigen-D-related (HLA-DR) fluorescein isothiocyanate (FITC) (G46-6, mouse IgG2aκ); CD40 phycoerythrin (PE) (5C3, mouse IgG1κ); CD80 PE-Cyanine5 (PECy5) (L307,4, mouse IgG1κ); CD83 PECy5 (HB15e, mouse IgG1κ); CD206 PE (19,2, mouse IgG1κ), and CD14 FITC (M5E2, mouse IgG2aκ). The flow cytometry analysis was performed using FACSCalibur (BD Biosciences).

The expressions of HLA-DR, CD80, CD40 and CD206 were measured in the form of mean fluorescence intensities; meanwhile the expressions of CD14 and CD83 were presented in numbers of positive cells.

**Enzyme-linked immunosorbent assay (ELISA) analysis**

Cytokine levels (IL-12 and IL-10) in supernatant were measured using human IL-12 p70 Quantikine HS ELISA Kit - R&D Systems kit and human IL-10 Quantikine ELISA Kit - R&D Systems according to instruction in the kit manual.

**Statistical analysis**

Data were presented in mean value±standard deviation when they were normally distributed and in median value (minimum and maximum value) when they were not normally distributed. The comparison of mean or median value for DC marker expression and ratio of IL- 12/ IL-10 cytokines of DC between those with and without 1,25-D3 treatment was conducted using paired t-test when the data had normal distribution and Wilcoxon test when the data did not have a normal distribution.

## RESULTS

Subject characteristics can be seen in Table 1.

**Effects of *in vitro* 1,25-D3 on the expression of DC markers and the ratio of cytokines IL-12/IL-10 in MDDC cultures of GD patients**

Effects of *in vitro* 1,25-D3 supplementation on DC maturation were observed in the cultures on the 5th and 7th day. On the 7th day, the expressions of HLA-DR, CD80, CD40, and CD83 in the cultures with 1,25-D3 were lower than those cultures without 1,25-D3 ($p=0.016$; $p<0.01$; $p=0.056$; $p=0.019$); the expression of CD14 in the cultures with 1,25-D3 was higher than those cultures without 1,25-D3 ($p<0.01$), while the expression of CD206 in the cultures with 1,25-D3 was maintained ($p=0.3375$) (Fig. 1).

On the 5th day, the expression of HLA-DR in the cultures with 1,25-D3 was lower than the cultures without 1,25-D3 ($p<0.01$), while the expression of CD14 in the cultures with 1,25-D3 was higher than the cultures without 1,25-D3 ($p<0.01$) (Fig. 2).

On the 5th day of culture, 1,25-D3 supplementation did not change the expression of CD206 ($p=0.3375$). The ratio of IL-12/IL-10 in the cultures with 1,25-D3 was significantly lower than those cultures without 1,25-D3 (Fig. 3).

## DISCUSSION

As a natural immunomodulator, vitamin D increases innate immune response and regulates excessive adaptive immune response such as found in autoimmune disease [17]. DCs are immune cells that initiate and maintain the autoimmune response [5]. One of the important effects of vitamin D in autoimmune is inhibition of DC maturation. The present study provides additional information regarding the immunoregulator effect of vitamin D, particularly on inhibition of DC maturation in GD patients. This is the first study reporting the effect of *in vitro* vitamin D on inhibition of DC maturation in GD.

**Effects of *in vitro* 1,25-D3 on the expression of DC markers in MDDC cultures of GD patients**

This study shows that *in vitro* 1,25-D3 supplementation inhibits DC maturation in MDDC cultures of GD patients, which is characterized by lower expression of major histocompatibility complex class II

**Table 1: Subject characteristics (n=12)**

| Characteristics | Mean value±SD | Median (minimum-maximum) |
|---|---|---|
| Age (years) | 35.83±10.74 | |
| Duration of illness (months) | | 20 (2-204) |
| TSH (μIU/L) | 0.01 | |
| fT4 (ng/dL) | | 6.61 (2.69-7.77) |
| TRAb (U/L) | 28.17±25.33 | |
| Vit 25-D3 (ng/mL) | 26.99±6.89 | - |

Data are presented in mean value±SD when they were normally distributed and in median value (minimum and maximum value) when they did not have normal distribution. SD: Standard deviation, TSH: Thyroid stimulating hormone, TRAb: Thyroid receptor antibody

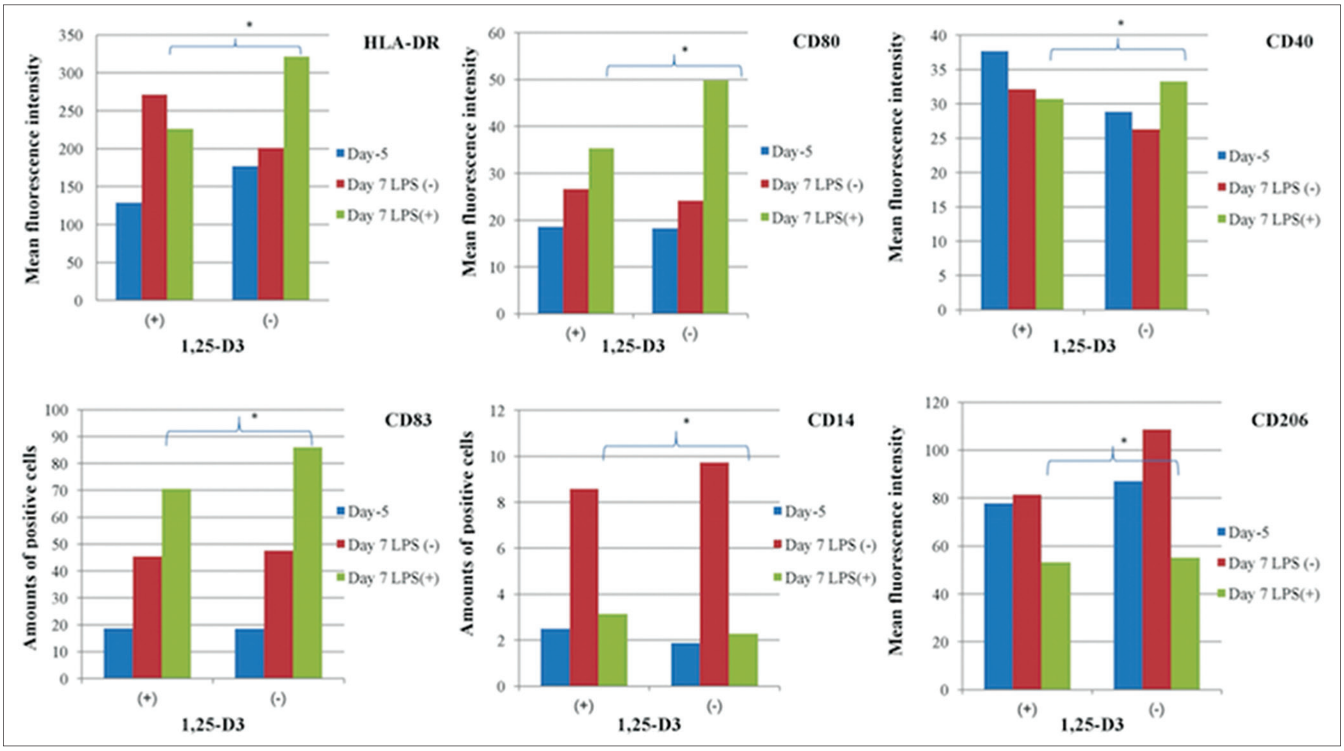


**Fig. 1: The expressions of DC molecules in cultures day 7. *The expressions of DC molecules on day 7 (LPS+) in cultures without and with 1,25-D3, n=12**

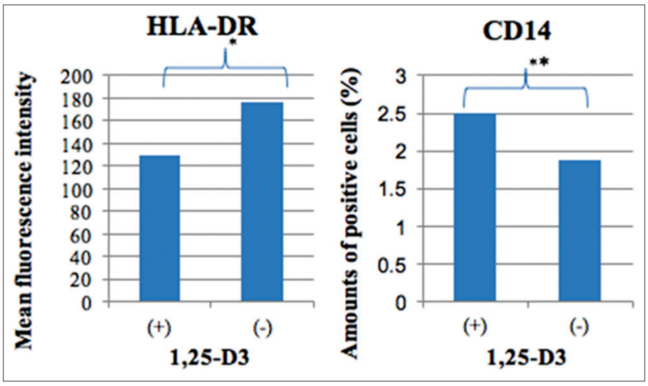


**Fig. 2: The expressions of human leukocyte antigen-D-related (HLA-DR) and CD14 in cultures day 5. *The expression of HLA-DR in cultures day 5 with and without 1,25-D3 *in vitro* (p<0.05, paired *t*-test), n=12. **The expression of CD14 in cultures day 5 with and without 1,25-D3 *in vitro* (p<0.05, Wilcoxon test), n=12**

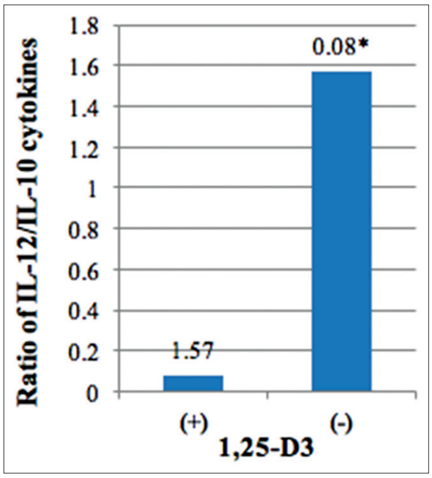


**Fig. 3: The ratio of interleukin-12 (IL-12)/IL-10 in cultures with and without 1,25-D3. *p<0.01, Wilcoxon test, n=12**

(HLA-DR) molecules, costimulatory molecules (CD80 and CD40), and marker of mature DC (CD83) ($p<0.05$). Any disruption on DC maturation may result in weak immunogenicity of DC, which may lead to lower DC ability as APC and activator of naïve T-cells. The *in vitro* 1,25-D3 supplementation also increases monocyte marker (CD14) and maintain MR expression (CD206), which are major markers for monocytes and iDC; therefore, it demonstrates the capacity of 1,25-D3 to maintain DC immaturity status. Those results are consistent with other experimental studies among healthy subjects, SLE, and Crohn's disease [12,14].

Other findings in our study are the change of DC molecules expression on the 5th day. At iDC phase, the expressions of HLA-DR and CD14 have already changed but other markers have not. *In vitro* 1,25-D3 in monocytic phase reduced the expression of HLA-DR ($p<0.01$) and increased the expression of CD14 ($p<0.01$) of iDC on the 5th day, while the expressions of CD80, CD40, CD83, and CD206 showed no difference ($p>0.05$).

HLA-DR molecule is very important since polymorphism or genetic mutation of HLA-DR has a role in the pathogenesis of some autoimmune diseases including GD [18,19]. The complex HLA-DR gene is the major genetic factor in AITD in addition to the gene of Treg cells (CTLA4) and specific genes of thyroid [19]. It explains why the expression of HLA-DR in MDDC culture has been affected since the iDC phase by *in vitro* 1,25-D3 supplementation, while the expression of CD80, CD40, CD83, and CD206 was not affected. The role of CD14 in GD still cannot be explained as clearly as the role of HLA-DR. The change of CD14 expression is earlier and probably due to the essential role of the molecule in the GD progression. It needs further studies considering that there is still no study reporting the correlation between the genetic defect of CD14 molecule and GD.

**Effects of *in vitro* 1,25-D3 on the ratio of cytokines IL-12/IL-10 levels in MDDC cultures of GD patients**

The balance between cytokines IL-12 and IL-10 is required to maintain tolerance [20]. This study used IL-12/IL-10 ratio to provide a better description of both cytokines interaction [20]. It shows that *in vitro* vitamin D supplementation in MDDC cultures of GD patients decreases the ratio of IL-12/IL-10 cytokines. This is the first study which demonstrates the effect of *in vitro* 1,25-D3 supplementation on IL-12/IL-10 ratio in patients with autoimmune disease. Lower IL-12/IL-10 ratio suggests lower DC immunogenicity.

If each cytokine is evaluated separately in this study, *in vitro* 1,25-D3 supplementation decreases IL-12 cytokine level significantly, while IL-10 cytokine level is relatively maintained. It indicates that *in vitro* 1,25-D3 supplementation can suppress IL-12 production and maintain the role of IL-10 on immune response initiated by DC. Low inflammatory cytokines level will reduce T-cells activation leading to prevention of persistent immune response in autoimmune cases [5,20].

The effect of *in vitro* vitamin D supplementation on IL-12 or IL-10 cytokines has been previously reported for SLE, MS, and Crohn's disease. Studies on Crohn's disease did not demonstrate the effect of *in vitro* vitamin D supplementation on the decrease of IL-12 level because they used less sensitive ELISA kit. Studies on SLE and MS only evaluated either IL-12 or IL-10, therefore, may not provide balanced description of inflammatory and anti-inflammatory cytokines produced by DC [12,13].

## CONCLUSION

*In vitro* 1,25-D3 supplementation inhibits DC maturation in patients with GD.

## ACKNOWLEDGMENTS

Author wants to acknowledge DIKTI (The Directorate General of Higher Education) Research Grants 2014 program for grants given to support this research. The content is solely the responsibility of authors and does not necessarily represent general view of the University of Indonesia.

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