Increased expression of herpesvirus entry mediator in 1,25-dihydroxyvitamin D3-treated mouse bone marrow-derived dendritic cells promotes the generation of $CD4^+CD25^+Foxp3^+$ regulatory T cells

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Abstract. Dendritic cells (DCs) can initiate immune responses or induce immune tolerance. 1,25-dihydroxyvitamin D3 [$1,25(OH)_2D3$] is a secosteroid hormone that can induce tolerogenic dendritic cells favoring the induction of regulatory T cells (Treg). The present study revealed a tolerogenic effect of $1,25(OH)_2D3$ on the phenotype and function of ovalbumin (OVA)-activated mouse bone marrow-derived DCs. Three inhibitory molecules associated with tolerogenic DCs, programmed death-ligand-1 (PD-L1), PD-L2 and herpesvirus entry mediator (HVEM) and the expression of co-stimulatory molecules (CD80, CD86 and CD40) on $1,25(OH)_2D3$-treated DCs were examined. The levels of interleukin (IL)-2, IL-6 and IL-10 secreted by $1,25(OH)_2D3$-treated DCs were analyzed. The capability of $1,25(OH)_2D3$-treated DCs to induce $CD4^+CD25^+Foxp3^+$ Treg and the stimulation of allogeneic $CD4^+$ T-cell proliferation in mixed lymphocyte reaction (MLR) was studied. $1,25(OH)_2D3$-treated DCs induced up to 21.0-fold upregulation of the HVEM expression and 4.1-fold enhancement of the HVEM expression upon activation with OVA [OVA-D3/immature (im)DCs]. PD-L1 was not affected by $1,25(OH)_2D3$-treated DCs and downregulated PD-L2 expression on $1,25(OH)_2D3$-treated DCs and OVA-D3/imDC. The expression of co-stimulatory molecules (CD80, CD86 and CD40) was downregulated in $1,25(OH)_2D3$-treated DCs and OVA-D3/imDC. Furthermore, $1,25(OH)_2D3$-treated DCs secreted much higher levels of IL-10, however lower levels of IL-2 and IL-6 compared with the activated control DCs. Together with this pattern of cytokines, $1,25(OH)_2D3$-treated DCs exhibited low allogeneic $CD4^+$ T-cell stimulatory activity and a higher number of $CD4^+CD25^+Foxp3^+$ cells in the MLR cultures but not in the activated control DCs. These findings indicate that $1,25(OH)_2D3$ possesses the immunosuppressive properties by upregulating the expression of the inhibitory molecules, HVEM, which may be therapeutically useful in controlling chronic immune and/or inflammatory diseases.

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Introduction

The activated form of vitamin D, 1,25-dihydroxyvitamin D3 [$1,25(OH)_2D3$] has, in addition to its central function in calcium and bone metabolism (1), important effects on the growth and differentiation of a number of cell types, and immunoregulatory properties. The biological effects of $1,25(OH)_2D3$ are mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors functioning as a ligand-activated transcription factor that binds to specific DNA sequence elements in vitamin D responsive genes and affects their rate of RNA polymerase II-mediated transcription (2). A number of studies have demonstrated an immunoregulatory effect for this steroid hormone (3-7). Antigen presenting cells (APCs), in particular DCs, express the VDR and are key targets of VDR agonists. Numerous studies have demonstrated that $1,25(OH)_2D3$ inhibits the differentiation and maturation of DCs (8-12). These studies have shown that $1,25(OH)_2D3$-treated DCs downregulated expression of the co-stimulatory molecules CD40, CD80, CD86 and MHC class II, decreased IL-12 and enhanced IL-10 production, resulting in decreased T-cell activation *in vitro*.

Besides the co-stimulatory molecules, inhibitory molecules are crucial to the tolerogenic capacity acquired by DCs. Members of the B7 family, programmed death ligand-1 (PD-L1) and PD-L2 have been found to be important in DC-mediated immune tolerance (13). In addition, PD-L1 signaling regulates the generation of $CD4^+Foxp3^+$ regulatory T cells (Treg) (14).

Chen *et al* (15) found that PD-L1 and PD-L2 contribute to the poor stimulatory capacity of immature DCs (imDCs) via engagement of the inhibitory PD-1. However, PD-1 ligand blockade could not endow the same stimulatory capacity of imDCs as mature DCs (mDCs), which implied that other negative molecules could be involved in this process.

Herpesvirus entry mediator (HVEM), also known as tumor necrosis factor receptor superfamily, member 14 (TNFRSF14), is a member of the TNFR, is expressed by several types of cells, including T cells, B cells and DCs (16-18), can regulate the differentiation of T cells and DCs. HVEM can also regulate DC-mediated T-cell immune responses. Certain studies have demonstrated that HVEM$^{-/-}$ mice were more susceptible to autoimmune diseases and APCs from HVEM$^{-/-}$ mice were more active in stimulating T cells compared with those from wild type (WT) mice (19). HVEM overexpression on APCs inhibited ovalbumin (OVA) peptide-dependent T-cell proliferation (20). HVEM overexpression on DCs produced a regulatory cytokine, IL-10, which had further effects on the induction of IL-10 producing CD4$^+$ T cells (21). These findings supported the role of HVEM as an inhibitor molecule involved in DC-mediated immunological tolerance. However, the expression of HVEM on tolerogenic dendritic cells (tDCs) induced by VDR agonists remain incompletely characterized. Therefore, the expression of inhibitory molecules HVEM, PD-L1 and PD-L2 on 1,25(OH)$_2$D3-treated mouse bone marrow-derived DCs was analyzed.

The data of the present study verified that the upregulated expression of the inhibitory molecule HVEM in 1,25(OH)$_2$D3-treated DCs induced CD4$^+$CD25$^+$Foxp3$^+$ Treg and arrested allogeneic CD4$^+$ T-cell proliferation. All these results further support the critical role of the inhibitory molecule HVEM in DC-mediated immune tolerance.

Materials and methods

Experimental animals. Female C57BL/6J and Balb/c mice were used at ages 6-8 weeks (Chongqing Experimental Animal Co., Chongqing, China). All the mice were bred under specific pathogen-free conditions. All the experiments were approved by an Ethics Committee (The Ethics Committee, Xinqiao Hospital, Third Military Medical University, Jiangyin, Jiangsu, China).

Preparation of DCs from mouse bone marrow. Murine bone marrow-derived DCs were prepared as described previously with minor modifications. Briefly, bone marrow mononuclear cells were prepared from C57BL/6 mouse tibia and femur suspensions by depletion of red cells and cultured at a density of 2x10^6 per well in six-well plates in RPMI-1640 medium supplemented with 10% fetal calf serum and 10 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor and 1 ng/ml recombinant murine IL-4 (22). Non-adherent cells were gently washed out on the third day of culture; the remaining loosely adherent clusters were cultured for a further 4 days as non-treated (NT) DCs. To produce the different subsets of DCs, imDCs were harvested on day 5 or 6 of culture. 1,25(OH)$_2$D3-treated DCs (D3/imDCs) were generated by adding 10^{-8} 1,25(OH)$_2$D3 on day 3 of NT-DCs culture and harvested on day 8. OVA-D3/imDCs were generated by adding 100 μg/ml OVA on day 7 of the D3/imDCs culture for 24 h and harvested on day 8. Mature DCs (OVA/imDCs) was generated by adding 100 μg/ml OVA on day 7 of the NT-DCs culture for 24 h and harvested on day 8. DCs prepared in this manner consisted of ~80% CD11c$^+$ cells, however, higher purities were required thus the cells were sorted on CD11c$^+$ by MACS beads (Miltenyi Biotec, Germany) to yield ~90% purity.

Flow cytometric analysis. DCs were triple-stained with APC-anti-CD11c and either PE-CD80, -MHC class II, -HVEM (CD270), -PDL1 (CD274) or FITC-CD86, -CD40, -PDL2 (CD273) for phenotypic analysis. The incidences of positive cells were determined by flow cytometry using a FACS Calibur (BD Biosciences, San Jose, CA, USA). Appropriately conjugated isotype-matched control antibodies were used as negative controls.

Mixed lymphocyte reaction.
DCs. Varying numbers of mitomycin C (25 mg/ml)-treated DCs were seeded in triplicate in a flat-bottom 96-well plate (Corning, Tewksbury, MA, USA) for use as stimulator cells. T cells were prepared from spleens and isolated by CD4$^+$ T cell magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the purity of the CD4$^+$ T cells was >95%, according to the manufacturer's instructions. In total, 1x10^5/well of T cells from BALB/c mice were added to the DC cultures, with the final MLR occurring in 200 μl of RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Life Technologies). The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 for 4 days, and pulsed with 1 μCi [^{3}H] thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the last 18 h of the culture. The cells were harvested onto glass fiber filters, and the radioactivity incorporated was quantitated using a Beckman liquid scintillation counter (Beckman Coulter, Miami, FL, USA). The results were expressed as the mean cpm of triplicate cultures ± standard error of the mean.

In vitro differentiation assessment of CD4$^+$CD25$^+$Foxp3$^+$ Treg. Naïve CD4$^+$CD25-T cells were purified from Balb/c spleen cells by magnetic-activated cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec). In total, 1x10^6 allogeneic CD4$^+$ CD25$^-$ T cells were cocultured with mitomycin C (25 mg/ml)-treated 1x10^5 OVA/imDCs and OVA-D3/imDCs for 96 h. These CD4$^+$-T cells were then surface stained with FITC-anti-CD4 and PE-anti-CD25 mAbs and resuspended in Fix/Perm buffer (eBioscience, San Diego, CA, USA). Intracellular staining with PEcy5-anti-Foxp3 mAb (eBioscience) and estimation of the incidence of CD4$^+$CD25$^+$Foxp3$^+$ Treg was then determined by flow cytometry.

Enzyme-linked immunosorbent assay. Different subsets of DCs or DC coculture with naïve CD4$^+$CD25$^-$-T cells supernatants were stored at -80˚C. The levels of IL-2, IL-6 and IL-10 were measured using ELISA kits (Cusabio Biotech Co., Ltd., Hubei, China), according to the manufacturer's instructions. The sensitivity limits for IL-2, IL-6 and IL-10 were 3.9 pg/ml, 0.39 and 0.8 pg/ml, respectively.

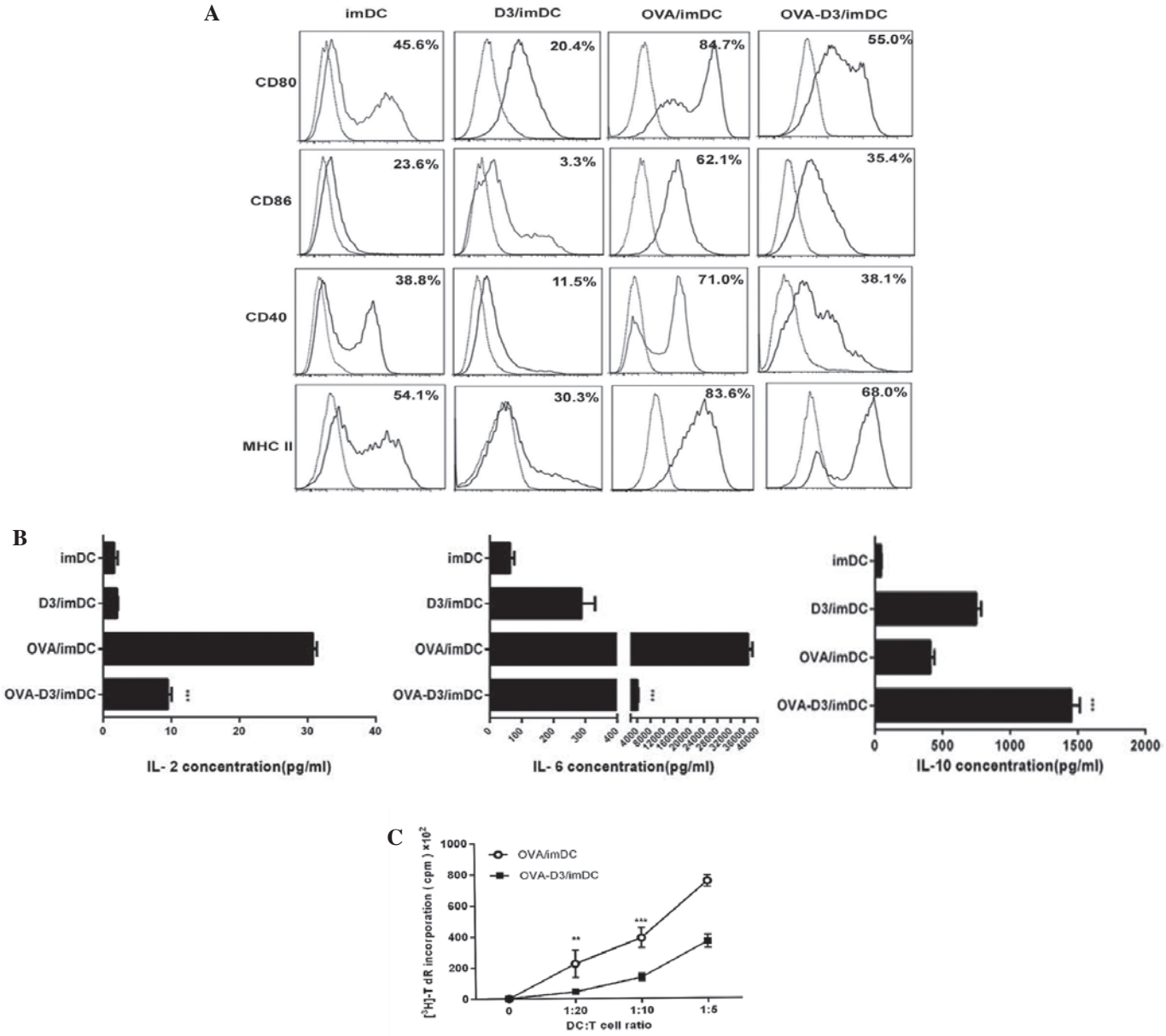

Figure 1. 1,25(OH)$_2$D3-treated bone marrow-derived dendritic cells exhibit tolerogenic features. (A) Expression of co-stimulatory molecules (CD80, CD86 and CD40) and MHC class II on DCs was measured by flow cytometry. Dashed lines represent isotype control mAb-staining. Solid lines represent surface molecule staining. Results are shown from a representative experiment out of 3-5 independent experiments performed. (B) IL-2, IL-6 and IL-10 levels. Results are shown from a representative experiment out of 3 independent experiments performed. (C) The allostimulatory capacities of D3/imDCs and OVA/imDCs were measured by MLR. **P<0.01 and ***P<0.001 indicate significant differences in comparison with controls. Results are shown from a representative experiment out of 3 independent experiments performed. MHC, major histocompatibility complex; DCs, dendritic cells; IL, interleukin; imDCs, immature DCs; OVA, ovalbumin; MLR, mixed lymphocyte reaction.

Statistical analysis. The data are presented as the mean ± standard deviation. Statistical comparisons were performed using analysis of variance and the χ^2 test. P<0.05 was used to indicate a statistically significant difference. Data analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

1,25(OH)$_2$D3-treated bone marrow-derived dendritic cells exhibit features of tolerogenic DCs. The phenotype and T-cell stimulatory capacity of 1,25(OH)$_2$D3-treated BMDCs activated by OVA (OVA-D3/imDCs) were analyzed and compared with those of mature DCs (OVA/imDCs). 1,25(OH)$_2$D3-treated DCs (D3/imDCs) demonstrated immature phenotype, which downregulated the expression of the co-stimulatory molecules (CD80,CD86 and CD40) and MHC class II compared with those of immature DCs (all P<0.001). Upon activation by OVA, D3/imDCs demonstrated a semi-mature phenotype, with higher expression of co-stimulatory molecules CD86 and MHC class II compared with those of imDCs (all P<0.05), but lower than OVA/imDCs (all P<0.001) (Fig. 1A). This indicates that 1,25(OH)$_2$D3 inhibits DC maturation. Next, the cytokine production pattern of the OVA-D3/imDCs was examined. D3/imDCs secreted low levels of IL-2, IL-6 and intermediate levels of IL-10. Upon activation by OVA, D3/imDCs produced more

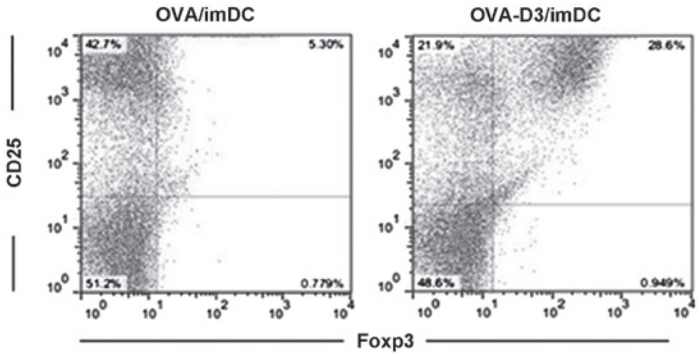

Figure 2. 1,25(OH)$_2$D3-treated bone marrow-derived dendritic cells induce CD4$^+$CD25$^+$Foxp3$^+$ Treg. OVA/imDCs or OVA-D3/imDCs were co-cultured with CD4$^+$CD25$^-$ T cells for 96 h. The expression percentage of CD4$^+$CD25$^+$Foxp3$^+$ (Treg) and CD4$^+$CD25$^+$Foxp3$^-$ T (Teff) cells were detected by flow cytometry. Histograms gated on CD4$^+$CD25$^+$Foxp3$^+$ cells. The results are shown from a representative experiment out of 3 independent experiments performed. OVA, ovalbumin; imDCs, immature dendritic cells.

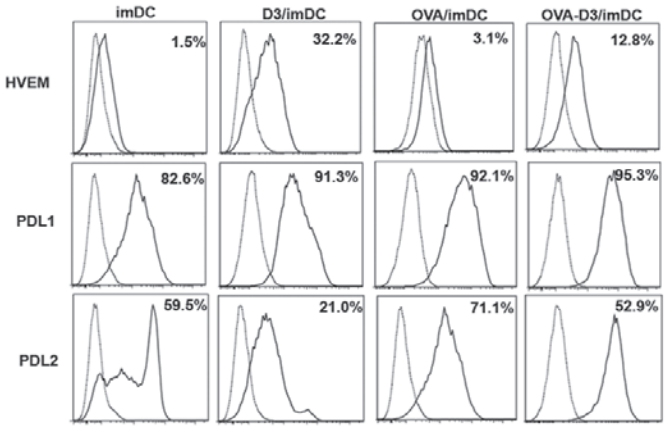

Figure 3. Expression of inhibitory molecules HVEM, PDL1 and PDL2 on 1, 25(OH)$_2$D3-treated bone marrow-derived dendritic cells. The expression of HVEM, PD-L1 and PD-L2 on imDCs, D3/imDCs, OVA/imDCs and OVA-D3/imDCs were measured by flow cytometry. Dashed lines represent isotype control mAb staining. Solid lines represent surface molecule staining. The results are shown from a representative experiment out of 3 independent experiments performed. HVEM, herpesvirus entry mediator; PDL, programmed death ligand; imDC, immature dendritic cells; mAB, monoclonal antibody.

IL-10 and less IL-2 and IL-6 compared with those produced by mature DCs (all P<0.001) (Fig. 1B). These results indicate that 1,25(OH)$_2$D3 inhibits the secretion of pro-inflammatory cytokines but promotes the secretion of anti-inflammatory cytokines. OVA-D3/imDCs exhibited a semi-mature phenotype and promoted secretion of anti-inflammatory cytokines. We speculated that OVA-D3/imDCs would be less capable of allogeneic CD4$^+$ T-cell stimulation. Indeed, OVA-D3/imDCs demonstrated poor capacity to stimulate CD4$^+$ T-cell proliferation compared with OVA/imDCs at different DC:T cell ratio in a primary allogeneic MLR (P<0.01 or 0.001; Fig. 1C).

All these results support that 1,25(OH)$_2$D3-treated dendritic cells possess tolerogenic phenotypes.

1,25(OH)$_2$D3-treated bone marrow-derived dendritic cells promote expansion of CD4$^+$CD25$^+$Foxp3$^+$ Treg. 1,25(OH)$_2$D3-treated BMDCs exhibit features of tolerogenic DCs. Next, whether the tolerogenic DCs may be correlated with specific interactions with CD4$^+$CD25$^+$Foxp3$^+$ Treg was investigated. OVA-D3/imDCs or OVA/imDCs were cultured with CD4$^+$CD25$^-$-T cells for 96 h, and then the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg was analyzed by flow cytometry. As expected, a significantly high incidence of CD4$^+$CD25$^+$Foxp3$^+$ Treg and lower incidence of CD4$^+$CD25$^+$Foxp3$^-$ effector-T cells (Teff) were detected from OVA-D3/imDC cultures compared with those of OVA/imDCs (Fig. 2). This indicates that 1,25(OH)$_2$D3-treated dendritic cells promote expansion of CD4$^+$CD25$^+$Foxp3$^+$ Treg and inhibit CD4$^+$CD25$^+$Foxp3$^+$ Teff proliferation.

1,25(OH)$_2$D3 induces the expression of the inhibitory molecules HVEM and PD-L1 on bone marrow-derived dendritic cells. Inhibitory molecules HVEM, PD-L1, PD-L2 are known to negatively regulate T-cell responses (19,23,24). Therefore, the expression of HVEM, PD-L1 and PDL2 on 1,25(OH)$_2$D3-treated dendritic cells (D3/imDCs) were investigated by flow cytometric analysis. D3/imDCs induced up to 21.0-fold upregulation of HVEM expression and 1.1-fold upregulation of PD-L1 expression. OVA-D3/imDCs led to a 4.1-fold enhancement of HVEM expression and a slight 1.0-fold upregulation of PD-L1 expression (all P<0.001). Conversely, D3/imDCs led to 2.8-fold downregulation of PD-L2 expression and OVA-D3/imDCs led to 1.5-fold downregulation of PD-L2 expression, respectively (Fig. 3). These data implied that HVEM, but not PD-L2, is involved in tDCs induced by VDR agonists and promotes production of Treg.

Discussion

In the present study, $1,25(OH)_2D3$-treated bone marrow-derived dendritic cells were verified to be tolerogenic DCs, which induced Treg generation and inhibited allogeneic $CD4^+$ T-cell proliferation. In addition, $1,25(OH)_2D3$ was observed to markedly upregulate the expression of the inhibitory molecule HVEM, which may be associated with tolerogenic DCs.

DCs not only initiate T-cell responses but are also involved in the silencing of T-cell immune responses. Mature DCs induce differentiation of effector T cells (Th1, Th2) (25). Immature and semi-mature DCs appear to induce the differentiation of Treg and thus promote tolerance (26). The data of the present study confirmed the findings of previous studies (8-9) revealing that $1,25(OH)_2D3$-treated DCs significantly downregulated the co-stimulatory molecules CD86, CD80, CD40 and MHC II, which show an immature phenotype. Upon activation by OVA, $1,25(OH)_2D3$-treated DCs show a semi-mature phenotype, with higher expression of co-stimulatory molecule CD86, and MHC class II and decreased IL-2, IL-6 and enhanced IL-10 production. Furthermore, $1,25(OH)_2D3$-treated DCs demonstrate a poor capacity to stimulate $CD4^+$ T-cell proliferation in a primary MLR as previous studies have proved (10,27,28). Indeed, $1,25(OH)_2D3$-treated DCs converted naïve $CD4^+$ T cells into $Foxp3^+$ Treg and suppressed allogeneic $CD4^+$ T-cell proliferation. All these results exhibited the tolerogenic properties of $1,25(OH)_2D3$-treated dendritic cells.

Besides the co-stimulatory molecules, it is observed that inhibitory molecules, including PD-L1 and PD-L2 (members of the B7 family) are involved in DC-mediated immune tolerance (15,29). Unger *et al* (30) found PD-L1 was significantly upregulated on $1,25(OH)_2D3$-treated DCs and regulated the generation of Treg, as the blockade of PD-L1 eradicated the suppressive capacity of Treg. This indicates that the inhibitory molecule is critical for tDCs in the generation of Treg induced by $1,25(OH)_2D3$. However, the results of the present study identified that PD-L1 was hardly affected by $1,25(OH)_2D3$ and PD-L2 was downregulated on $1,25(OH)_2D3$-treated DCs, which implied that other negative molecules could be involved in this process.

HVEM, also known as TNFRSF14, is a member of the TNF family and their specific receptors (TNFR), which as an inhibitory molecule is also involved in DC-mediated immune tolerance. Studies have found that $HVEM^{-/-}$ mice were more susceptible to autoimmune diseases and APCs in $HVEM^{-/-}$ mice were more active in stimulating T cells compared with WT mice (15). HVEM overexpression on APCs inhibits OVA peptide-dependent T-cell proliferation (20). HVEM overexpression on DC induced IL-10 producing $CD4^+$-T cells, and immune adoption of these DCs *in vivo* can protect against experimental autoimmune myocarditis (EAM) (21). However, very little is known regarding the role of HVEM in the induction of Treg by $1,25(OH)_2D3$-treated BMDCs.

The present study's observations that HVEM was significantly upregulated in $1,25(OH)_2D3$-treated DCs indicates that HVEM may be involved in the induction of Treg by $1,25(OH)_2D3$. However, it remains unclear what the exact underlying mechanism is. Kuipers *et al* (31) found that by triggering PD-L1 on DCs using soluble PD-1 immunoglobulin resulted in a decreased expression of the positive co-stimulatory molecules CD80, CD86 and CD40 and increased IL-10 production. An explanation may be reverse signaling by HVEM into DCs, resulting in a suppressive DC-phenotype. All these findings are based on the hypothesis that vitamin D regulates immune responses by controlling the expression of CYP27B1 and VDR (32-36).

In conclusion, increased HVEM on $1,25(OH)_2D3$-treated mouse-BMDCs induced $CD4^+CD25^+Foxp3^+$ Treg and impaired allogeneic $CD4^+$ T-lymphocyte proliferation, which further supported the important role of inhibitory molecules in DC-mediated immune tolerance.

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