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Cutting Edge: Progesterone Directly Upregulates Vitamin D Receptor Gene Expression for Efficient Regulation of T Cells by Calcitriol

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The two nuclear hormone receptor ligands progesterone and vitamin D (vit.D) play important roles in regulating T cells. The mechanism that connects these two hormones in regulating T cells has not been established. In this study, we report that progesterone is a novel inducer of vit.D receptor (VDR) in T cells and makes T cells highly sensitive to calcitriol. At the molecular level, the induction by progesterone is mediated by two progesterone receptor-binding elements in the intron region after the first noncoding exon of the human VDR gene. Increased expression of VDR by progesterone allows highly sensitive regulation of T cells by vit.D even when vit.D levels are suboptimal. This novel regulatory pathway allows enhanced induction of regulatory T cells but suppression of Th1 and Th17 cells by the two nuclear hormones. The results have significant ramifications in effective regulation of T cells to prevent adverse immune responses during pregnancy. The Journal of Immunology, 2015, 194: 000–000.

The biologically active form of vitamin D (vit.D; 1,25-dihydroxyvitamin D₃, also called calcitriol) is a nuclear hormone ligand mediating its effects mainly through the vit.D receptor (VDR)–retinoid X receptor nuclear hormone receptor system (1). VDR is widely expressed in the body. Within the immune system, T cells express VDR and are an important target of calcitriol for immune regulation (2). Calcitriol induces regulatory T cells (Tregs) but suppresses the generation of effector T cells (3–8). Vit.D metabolites inhibit the maturation of dendritic cells and make them tolerogenic cells (9, 10). Progesterone (P₄) is another nuclear hormone critical for preparation and maintenance of pregnancy (11). Whereas vit.D is obtained through dietary absorption and UV-induced synthesis in the skin and sequentially activated in the liver and kidneys to become calcitriol, P₄ is produced at high levels directly from ovariies and placenta and at low levels in the adrenal gland and the testes (12). In a manner similar to vit.D, P₄ increases Tregs but suppresses inflammatory effector T cells (13).

In this study, we report that P₄ induces VDR expression in CD4⁺ Th cells, an effect mediated by P₄-induced binding of P₄ receptor (PR) to canonical PR-binding elements (PREs) in the human VDR (hVDR) gene. VDR induction by P₄ allows T cells to be more efficiently regulated by calcitriol for enhanced promotion of Tregs but suppression of potentially inflammatory effector T cells.

Materials and Methods

Cell isolation and animal study

Cord blood (CB) CD4⁺CD25⁻ and adult peripheral blood CD4⁺CD25⁺CD45RO⁻CD69⁻ naive CD4⁺ T cells were isolated as described before (14). Total spleen CD4⁺ T cells were isolated from pregnant mice (15–18 d postcoitus), and naive CD4⁺ T cells were isolated from the spleen of nonpregnant mice as described before (15). Female C57BL/6 mice were injected s.c. with medroxyprogesterone (2 mg; Depo-Provera from Pfizer) and sacrificed 4 d later. All human subject and animal studies were approved by Institutional Review Committees at Purdue University.

In vitro differentiation of T cells and hVDR knockdown with small interfering RNA

Human naive CD4⁺ T cells were activated with anti-CD3/28 beads (5 μl/ million cells; Miltenyi Biotec) and IL-2 (25 U/ml) cultured in Th1/Th17/Treg cytokine conditions in phenol red–free RPMI 1640 supplemented with 10% charcoal/dextran-treated FBS (HyClone) or in vit.D-free X-VIVO 15 medium (Lonza) as described before (14). CB T cells, activated for 24 h with anti-CD3/28 beads and human IL-2, were transfected with control or hVDR small interfering RNA (siRNA; 20 pmol/4 million cells, Santa Cruz Biotechnology) using an Amaxa Nucleofector (Lonza). Cells were cultured with P₄ (2 μg/ml) and/or calcitriol (1–100 nM) for 3 d for VDR mRNA expression or 5–6 d for the expression of Foxp3, CD25, CD38, latency-associated peptide/TGF-β1, IL-17, and/or IFN-γ (13).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CB, cord blood; CBP, chromatin immunoprecipitation; hVDR, human VDR; P₄, progesterone; PB, peripheral blood; P₄, P₄ receptor; PRE, PR-binding element; sRNA, small interfering RNA; Treg, regulatory T cell; vit.D, vitamin D; VDR, vitamin D receptor.
In vitro T cell suppression assay
CB naive CD4+ T cells were stained with CFSE and 5 × 10^6 cells were added to 96-well plates in the presence of anti-CD3/CD28–coated beads as target cells along with in vitro–differentiated Tregs generated with IL-2 (25 U/ml) and calcitriol (100 nm) and/or P4 (2 μg/ml). CFSE dilution was determined by flow cytometry on day 4.

Microarray, RT-PCR, and Western blotting of VDR expression
The microarray analysis of CB naive CD4+ T cells was performed previously (13). Quantitative RT-PCR was performed with the primers for human or mouse VDR gene (Supplemental Table I). CB naive CD4+ T cells, activated with anti-CD3/CD28 and IL-2 with or without P4 (2 μg/ml) for 3–5 d, were examined for VDR protein expression with an mAb to hVDR (R&D Systems) and HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology).

Promoter analysis and chromatin immunoprecipitation assay
PREs on the hVDR gene were identified with TESS. A chromatin immunoprecipitation (ChIP) assay was performed as described before with the primers listed in Supplemental Table I (15). CB CD4+ T cells, activated with anti-CD3/CD28–coated beads in the presence of P4 (2 μg/ml) for 3–4 d, were processed and immunoprecipitated using 4 μg rabbit mAb to human PR (Abnova).

Mutagenesis and reporter assay
PRE no. 1 (5′-AGAAGCT-3′) and PRE no. 2 (5′-GGGACA-3′) in the regulatory region of VDR gene cloned in pGL3-VDR (+909 to +1267) (16) were mutated to 5′-AAGAGT-3′ and 5′-GAGGGA-3′, respectively, with a site-directed mutagenesis kit (Clontech Laboratories). Mutant pGL3-VDR vectors (20 μg) were electroporated into MCF-7 cells (310 V, 950 μF; Bio-Rad). The transfected cells were rested overnight, activated with PMA (50 ng/ml) in the presence or absence of P4 (2 μg/ml) for 6 h, and then assayed for luciferase activity with a Synergy HT reader (BioTek).

Statistical analysis
Significant differences between indicated groups were determined by a Student paired two-tailed t test.

Results and Discussion
P4 induces VDR expression in T cells
Analysis of microarray data on human CB naive CD4+ T cells activated with P4 (13) revealed that one of the major genes induced by P4 is the VDR gene (Fig. 1A). To verify this finding, CB naive CD4+ T cells were activated in the presence of P4, and VDR expression at mRNA and protein levels was examined. P4 substantially increased the expression of VDR mRNA in activated T cells in a dose-dependent manner (Fig. 1B). Optimal induction occurred at ~2 μg/ml P4, concentrations that were detected in placental tissues during pregnancy (17). Thus, this concentration range is physiologically relevant. The induction of VDR gene expression was suppressed by RU486, a PR antagonist (Fig. 1C). A similar induction was observed in adult peripheral blood CD4+ T cells. P4 also induced the expression of VDR protein in CB T cells (Fig. 1D). The VDR induction by P4 occurred in Treg-, Th1-, and Th17-polarizing cytokine conditions (Fig. 1E). The VDR induction by P4 was observed in mouse T cells as well (Fig. 1F). It was also increased in spleen CD4+ T cells and uterus in pregnant or progesterin-injected mice (Fig. 1F). These results indicate that P4 induces VDR gene expression in T cells in heterogeneous conditions or species.

P4 responsive elements on the hVDR gene mediate P4-induced VDR expression
To gain insights into the molecular mechanism of the VDR induction by P4, we examined the DNA sequence of the VDR gene for the presence of the canonical PREs. We found five PREs in the 5′ regulatory region of the VDR gene spanning into the first exon and intron regions (Fig. 2A). A luciferase reporter containing this regulatory region was highly responsive to P4 (Fig. 2B), suggesting that this region contains functional PREs. A ChIP assay revealed that only one site (site D) between exon 1A and 1B had a clear PR binding activity (Fig. 2C). When the two PREs in site D were mutated, the VDR gene promoter activity was largely abolished (Fig. 2D).

P4-induced VDR potentiates the effect of calcitriol on suppression of Th1 and Th17 cells
Vit.D metabolites suppress the generation of Th1 and Th17 cells (5, 8, 18). VDR directly binds the IFNG gene promoter to suppress Th1 cells (19) and induce C/EBP protein expression to suppress Th17 cells (8). We found that P4 decreases the effective concentration of calcitriol in suppressing the induction of Th1 and Th17 cells (Fig. 3A). Interestingly, the suppression by calcitriol in the presence of P4 was largely abolished when the VDR gene was knocked down with siRNA (Fig. 3B, 3C). Thus, P4-induced VDR functions to increase the sensitivity of T cells to calcitriol.
P4-induced VDR enhances the function of calcitriol in inducing Tregs

We next examined the effect of calcitriol on induction of Tregs expressing Foxp3, CD38, and/or latency-associated peptide/TGF-β1 (20, 21) in the presence or absence of P4. P4 increased T cell sensitivity to calcitriol in upregulating the Treg-associated molecules (Fig. 4A). Moreover, the Tregs generated with calcitriol and P4 were more suppressive than were the Tregs generated with P4 or calcitriol alone (Fig. 4B). The Treg-inducing activity of calcitriol in the presence of P4 was greatly abolished when the VDR gene was knocked down with siRNA (Fig. 4C).

Our results revealed the presence of a novel regulatory pathway linking P4 and vit.D in regulating T cells. The VDR induction in T cells is mediated by two PRE sites after the noncoding first exon of the VDR gene, which contains a number of other cis-acting elements (22). It is known that VDR expression in human T cells is upregulated upon TCR signaling (23), and we think that P4 and calcitriol are probably involved in the induction because culture media including animal sera generally contain these two nuclear hormones. Vit.D is important for normal function of the reproductive system, and its deficiency is common in pregnant females and is linked to pregnancy complications such as preeclampsia and miscarriage and to defective immune tolerance in the newborn (24–27). The VDR upregulation may allow T cells to sense low levels of vit.D and become effectively regulated to prevent inflammation when vit.D levels are decreased in the body. Therefore, the P4-induced VDR in T cells would be important to prevent adverse immune responses involved in pregnancy complications. The regulatory pathway may be active in other cell types as well because it has been observed that VDR expression was increased in endometrial tumor cells cultured with P4 (28) and in the uterus in pregnant females, as determined in this study. In summary, our findings reveal a novel role of P4 in expression with calcitriol and P4 were more suppressive than were the Tregs generated with P4 or calcitriol alone (Fig. 4B). The Treg-inducing activity of calcitriol in the presence of P4 was greatly abolished when the VDR gene was knocked down with siRNA (Fig. 4C).

FIGURE 2. PR binding to cis-acting elements in the VDR gene promoter drives gene expression. (A) Putative PREs and ChIP sites in the hVDR gene are shown. (B) A luciferase assay was performed with a reporter vector containing the VDR gene promoter. (C) A ChIP assay was performed to identify PR binding sites in the 5′ regulatory region of the hVDR gene. (D) A reporter assay with null mutations in the PRE sites in the VDR gene. A representative dataset of three to four separate experiments is shown. *p < 0.05 between indicated groups. n.d., not detectable.

FIGURE 3. Impact of P4 and induced VDR on T cell response to calcitriol in suppression of Th1 and Th17 cells. (A) CB CD4<sup>+</sup> naive T cells were activated in Th1 (IL-2, IL-12, and anti-IL-4) or Th17 (IL-6, IL-21, IL-23, IL-1β, TGF-β1, anti-IL-4, and anti–IFN-γ) polarization conditions in a vit.D-free medium containing P4 (2 μg/ml) and/or calcitriol for 5–6 d. (B) Expression of hVDR mRNA after siRNA knockdown. (C) Impact of VDR knockdown on T cell differentiation into effector T cells in response to calcitriol (1 nM) and/or P4 (2 μg/ml). Pooled data with SEM are shown (n = 4). *p < 0.05 versus controls.
of the VDR gene in T cells for highly sensitive regulation of T cell activity by vit.D metabolites.

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Disclosures

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References