REGULATION OF 25-HYDROXYVITAMIN D₃-1α-HYDROXYLASE AND 
PRODUCTION OF 1α,25-DIHYDROXYVITAMIN D₃ BY HUMAN DENDRITIC 
CELLS

Short title: 1α,25-Dihydroxyvitamin D₃ production by dendritic cells

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ABSTRACT
25-Hydroxyvitamin D₃-1α-hydroxylase (25(OH)D₃-1α-hydroxylase), the key enzyme of 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) production, is expressed in monocyte-derived macrophages (MAC). Here we show for the first time constitutive expression of 25(OH)D₃-1α-hydroxylase in monocyte-derived dendritic cells (DC) which was increased after stimulation with LPS. Accordingly, DC showed low constitutive production of 1,25(OH)₂D₃ but activation by LPS increased 1,25(OH)₂D₃ synthesis. In addition, 25(OH)D₃-1α-hydroxylase expression was found in blood-DC, but not in CD34⁺-derived DC. Next we analyzed the functional consequences of these results.
Addition of 1,25(OH)₂D₃ at concentrations comparable to those produced by DC inhibited the allo-stimulatory potential of DC during the early phase of DC differentiation. However, terminal differentiation decreased the responsiveness of DC to 1,25(OH)₂D₃.
In conclusion, DC are able to produce 1,25(OH)₂D₃ especially following stimulation with LPS. Terminal maturation renders DC unresponsive to the effects of 1,25(OH)₂D₃ but those cells are capable to suppress the differentiation of their own precursor cells in a paracrine way through the production of 1,25(OH)₂D₃.

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INTRODUCTION

The active form of vitamin D, 1,25(OH)2D3 positively modulates the differentiation of MAC 1, 2 whereas the functions lymphocytes and DC are suppressed 3, 4. At least in vitro, monocytes are precursor cells of both MAC and DC, and the specific microenvironment determines their fate, e.g., differentiation into either DC or MAC. Positive regulators of MAC differentiation like M-CSF or 1,25(OH)2D3, inhibit DC differentiation in vitro 5-9. Accordingly, VDR deficient mice which show an unresponsiveness to 1,25(OH)2D3 have increased numbers of DC in lymph nodes suggesting a physiologically relevant inhibition of DC differentiation by 1,25(OH)2D3 10.

The normal serum level of 1,25(OH)2D3 is relatively low (10^-10 M), whereas that of 25-hydroxyvitamin D3 (25(OH)D3), the major circulating vitamin D metabolite, is about 1000-fold higher. Synthesis of 1,25(OH)2D3 from its precursor 25(OH)D3 normally occurs in the kidney, however several reports describe extrarenal production of 1,25(OH)2D3 11, 12. This production is believed not to exert systemic effects but rather suggests a local paracrine function of 1,25(OH)2D3. We and others have shown that MAC convert 25(OH)D3 into 1,25(OH)2D3 13, 14 and have speculated that extrarenal 1,25(OH)2D3 production is involved in the autocrine/paracrine regulation of MAC differentiation. In light of recent publications on the inhibitory effect of 1,25(OH)2D3 on DC differentiation, the absence or presence of 1,25(OH)2D3 within the microenvironment could determine whether a monocyte becomes a MAC or DC. Therefore we were interested as to whether similar to MAC DC can also convert 25(OH)D3 into 1,25(OH)2D3 and found that DC are indeed a possible extrarenal source of 1,25(OH)2D3.
STUDY DESIGN

Cell separation and culture. DC and MAC were derived from human blood monocytes or CD34+ as described previously \(^{15,16}\). Terminal maturation was induced with 10 ng/ml tumor necrosis factor or 10 ng/ml LPS or a mixture containing 10 ng/ml TNF-\(\alpha\), 10 ng/ml IL-1\(\beta\), 1000 U/ml IL-6 and 1 \(\mu\)g/ml prostaglandin E\(_2\) \(^{17}\).

Northern blot analysis. A cDNA fragment of 25(OH)D\(_3\)-1\(\alpha\)-hydroxylase complementary to the nucleic acids +91 bp to +2073 bp (GenBank/EMBL Accession No. AB005989) was used for hybridization. As a loading control membranes were rehybridized with an 18S rRNA-oligonucleotide (5'-ACG GTA TCT GAT CGT CTT CGA ACC-3').

Immunohistochemistry. DC were fixed with glutaraldehyde and a standard APAAP staining was performed with a polyclonal sheep antibody against mouse and human 25(OH)D\(_3\)-1\(\alpha\)-hydroxylase (The Binding Site, Birmingham, UK), a secondary rabbit anti sheep antibody (Abcam, Cambridge, UK), APAAP reagent (Dianova, Hamburg, Germany) and Fast Red as a detection substrate (BioGenex, San Ramon, CA).

1\(\alpha\),25-Dihydroxyvitamin D\(_3\)-ELISA. Cells were seeded (10\(^6\) cells/well/ml) in RPMI medium in a 6-well plate. After incubation for 24 hours with 5x10\(^{-8}\) M 25(OH)D\(_3\) (Sigma, Deisenhofen, Germany) with or without 100 ng/ml LPS (Salmonella abortus equi, kindly provided by Dr. Chris Galanos, MPI, Freiburg, Germany) the supernatant and the cells were harvested. 1,25(OH)\(_2\)D\(_3\) was separated from other vitamin D metabolites by extraction columns (Immundiagnostik, Bensheim, Germany) and determined with a commercially available ELISA (Immundiagnostik). This ELISA is specific for 1,25(OH)\(_2\)D\(_3\) and does not recognize other vitamin D metabolites.

Mixed lymphocyte reaction (MLR). 10\(^5\) T cells were incubated with different amounts of immature and mature allogenic DC in RPMI containing 5% T cell-autologous
plasma. On day 6 of co-culture, 1µCi of $^3$H-methyl-thymidine/well was added and incorporated radioactivity was determined after 20 hours. All samples were performed in triplicates and values represent mean ± SEM.
RESULTS AND DISCUSSION

25(OH)D$_3$-1$\alpha$-hydroxylase, the mitochondrial cytochrome P450 enzyme that catalyzes the conversion of 25(OH)D$_3$, was detectable only in the late stages of DC differentiation and the level of expression was low independent of the culture condition (GM-CSF plus IL-4 vs. IFN$\alpha$)$^{16}$. Terminal differentiation of DC with LPS clearly upregulated the expression (Fig. 1A). In contrast, MAC cultured either in human AB-group serum or with FCS plus GM-CSF showed a strong expression of 25(OH)D$_3$-1$\alpha$-hydroxylase mRNA. CD34$^+$-derived DC$^{15}$ cultured with SCF, GM-CSF and TNF-$\alpha$ showed no 25(OH)D$_3$-1$\alpha$-hydroxylase mRNA expression (data not shown). At the protein level, immature DC on day 7 of culture were weakly positive for 25(OH)D$_3$-1$\alpha$-hydroxylase (data not shown) and terminal differentiation of DC markedly increased the expression (Fig. 1B). 25(OH)D$_3$-1$\alpha$-Hydroxylase expression was also found in freshly isolated blood-DC (Fig. 1E). The specificity of the 25(OH)D$_3$-1$\alpha$-hydroxylase antibody used has previously been demonstrated by Zehnder and colleagues$^{18}$ who detected extrarenal expression of 25(OH)D$_3$-1$\alpha$-hydroxylase in different tissues, e.g. lymph nodes. Parallel staining with CD68 indicated that 25(OH)D$_3$-1$\alpha$-hydroxylase is expressed in MAC and/or DC as both cell types express CD68$^{18,19}$.

Next we measured the 1,25(OH)$_2$D$_3$ levels in the supernatants of immature and mature DC. Low constitutive production was found in monocytes and immature DC up to day 4 of culture but stimulation with LPS always upregulated 1,25(OH)$_2$D$_3$ synthesis (Fig. 1C). Accordingly, terminal differentiation induced with LPS stimulated 1,25(OH)$_2$D$_3$ synthesis, but other inducers of terminal differentiation had no effect on 1,25(OH)$_2$D$_3$ production (Fig. 1D). Therefore the upregulation of 25(OH)D$_3$-1$\alpha$-hydroxylase by LPS represents an activation rather than a differentiation event.
Accordingly, positive regulation of 25(OH)D₃-1α-hydroxylase by LPS has already been shown by Reichel and colleagues in human MAC²⁰. Mature DC synthesize 1,25(OH)₂D₃ at a maximal concentration of 5×10⁻⁹M. To analyze the functional consequences of 1,25(OH)₂D₃ production by DC we added 5×10⁻⁹M 1,25(OH)₂D₃ or 5×10⁻⁸M of the precursor 25(OH)D₃ to DC on day 1 and day 6, respectively and performed mixed lymphocyte reaction. Even at this low concentration 1,25(OH)₂D₃ markedly inhibited antigen presentation capabilities when added in the early stage of DC differentiation on day 1 (Fig. 2A), but the precursor 25(OH)D₃ had no effect in DC cultured with 10% FCS. In contrast, DC generated with 2% autologous plasma were also suppressed by the precursor (Fig. 2B). This is in line with our finding, that the production of 1,25(OH)₂D₃ was extremely high under serum-free conditions or in the presence of autologous plasma, but low in the presence of FCS (data not shown) indicating an inhibitory effect of FCS on 25(OH)D₃-1α-hydroxylase activity. Terminal differentiation has been reported to render DC unresponsive to the actions of 1,25(OH)₂D₃ with regards to the expression of the maturation-associated antigen CD83⁶ and only these DC produce high amounts of 1,25(OH)₂D₃ comparable to MAC. Accordingly, we found a stable expression of maturation-associated antigens on terminally differentiated DC (data not shown) but exposure to both vitamin D₃ metabolites still slightly influenced their capacity for antigen presentation (Fig. 2A).

In summary, our results clearly show that myeloid DC are a possible extrarenal source of 1,25(OH)₂D₃. The high local 25(OH)D₃-1α-hydroxylase production of 1,25(OH)₂D₃ by DC (and MAC) after LPS stimulation may serve as a paracrine signal during bacterial infection and favour MAC differentiation but suppress DC differentiation and lymphocyte activation.
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LEGENDS

Figure 1: **Expression of 25(OH)D₃-1α-hydroxylase and production of 1,25(OH)₂D₃ by different types of immature and mature DC.** RNA of monocyte-derived DC (GM/IL-4, GM/IFNα) and MAC (AB, GM) cultured for 5 days was used for Northern blot analysis. Terminal differentiation of DC was induced by LPS (DC/LPS) (Fig. 1A). The expression of 25(OH)D₃-1α-hydroxylase protein in DC matured by stimulation with LPS (DC/LPS) was determined by immunohistochemistry. As negative control staining without the primary antibody is shown (Fig. 1B). 1,25(OH)₂D₃ synthesis was measured by ELISA. Freshly isolated monocytes or monocytes differentiated along the DC pathway for 3 or 6 days were harvested and cultured for another 24 hours under serum-free conditions with 25(OH)D₃ in the absence or presence of 100 ng/ml LPS. The values represent mean ± SEM of at least 3 experiments (Fig. 1C). The maturation of DC was induced on day 4 of culture for 72 hours with tumor necrosis factor (DC/TNF-α) or LPS (DC/LPS) or a mixture containing TNF-α, IL-1β, IL-6 and prostaglandin E₂ (DC/mix). The values represent mean ± SEM of 4 experiments (Fig. 1D). Blood-DC were isolated from mononuclear cells and the expression of 25(OH)D₃-1α-hydroxylase was investigated by immunohistochemistry (Fig. 1E).

Figure 2: **The effect of 1,25(OH)₂D₃ and 25(OH)D₃ on DC function.** 5x10⁻⁸M 25(OH)D₃ or 5x10⁻⁹M 1,25(OH)₂D₃ (kindly provided by Hoffmann-La Roche AG, Basel) were added on day 1 or day 6 of DC culture with or without induction of
terminal differentiation (Fig. 2A; n=3). In Figure 2B (n=2) we compared DC generated with either 10% FCS or 2% autologous plasma (aut. pl.). After 8 days antigen presentation was determined by MLR.
REFERENCES


Regulation of 25-hydroxyvitamin D₃-1α-hydroxylase and production of 1α,25-dihydroxyvitamin D₃ by human dendritic cells

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