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1-Alpha-Calcidol Modulates Major Human Monocyte Antigens and Toll-like Receptors TLR 2 and TLR4 *in vitro**

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Abstract: Since vitamin D derivatives are known to interfere with the cellular immune response, we analysed the possibile effect of 1-alpha-calcidol (AC) on major monocyte antigens CD14 (an endotoxin receptor), HLA-DR, and toll-like recptors 2 and 4 (TLR2, TLR4). Peripheral blood monocytes were isolated from healthy donors and cultured by standard protocol followed by incubation with various concentrations of AC in unstimulated and LPS-activated cells. After 24, 48 and 72 hours cells were harvested and analysed for the expression of antigens by flowcytometry. Compared to the controls AC increased the expression of CD14 in a dose and time dependent manner (after 72 hours culture time $p<0.01$). AC was capable of further stimulating CD14 expression in LPS activated monocytes ($p<0.05$). Both LPS and AC downmodulated HLA-DR dramatically after 24 ($p<0.05$), 48 ($p<0.01$) and 72 hours ($p<0.0001$). The expression of TLR2 but not of TLR4 was inhibited by 10^{-7}M AC. The data reveal that AC significantly modulates the expression of CD14, HLA-DR as well as of TLR2, all involved as targets and effector molecules in antigen recognition and processing, relevant to overcome infections and organ lesions.

Key words: monocytes, antigen modulation, nephropharmacology, immuno-pharmacology, 1-alpha-calcidol, CD14, HLA-DR, TLR2, TLR4, cell cultures

Abbreviations: CD14 = pleiotropic monocyte receptor for LPS, Lipoteichonic acid, modified Lipids; IL = interleukin; kDa = kilo-Dalton; LPS = Lipopolysaccharide, TLR = Toll-like receptor; HLA-DR = major human histocompatibility class II antigen (complex); TNF = tumor necrosis factor.

Introduction

Studies from our laboratory showed that patients suffering from impaired renal function revealed an increased *proinflammatory cellular* activity of antigen presenting cells [11, 13, 14, 18]. One of the monocyte constituents involved is CD14, a myeloid differentiation antigen of 53 kDa bound to the cell membrane by a glycophosphatidyl-inositol anchor [15]. Beside the cell surface form CD14 exists in a soluble form, sCD14, which was shown to circulate in serum of patients with inflammatory and systemic diseases at an increased rate [11, 16, 17]. Soluble and membrane bound CD14 are closely associated with the *toll-like receptor* 2 (TLR2), where CD14 acts as a "co-receptor" molecule together with MD-2, a molecule which is part of the extracellular domain of toll-like receptor 4 (TLR4) [6, 8, 10]. TLR constitute "pattern recognition molecules" primarily for bacterial and viral products, and are integral components of the innate immune system [1, 25]. Dysregulation of the genetic and immune responses where CD14 and TLR are involved may facilitate increased susceptibility to infections, sepsis and other inflammatory lesions including increased risk for cardiovascular events [2, 7, 19, 26]. Under the presence of microbial products, e.g. lipopolysaccharide (LPS) TLR and CD14 cooperate and coordinate the immune response by activating monocytes to produce and secrete proinflammatory *cytokines* such as IL-1ß, IL-6, and TNF-alpha. *Activated monocytes* are main effector and target cells to promote and accelerate *atheromatosis* and tissue interstitial *fibrosis* [15, 17].

However, not only microbial but also *non-pathogen* derived ligands of TLR were recently described [22]. Thus, we were interested to evaluate if whether or not TLR and other major monocyte antigens are affected by known immunomodulatory substances: *Vitamin D* derivatives interact with cellular immune responses as well as with cell adhesion molecules revealing anti-inflammatory activities [3, 2, 24]. All these studies were performed applying calcitriol, a 1-25-dihydroxy-vitamin D3 [21 – 24]. In the present paper we analysed the possible effect of *1-alpha-calcidol*, a 1-hydroxy-vitamin D3, on monocyte antigens CD14, HLA-DR, TLR2, and TLR4 under *in vitro* conditions.

Material and Methods

Whole blood was collected from normal healthy donors in EDTA tubes (Sarsted Monovetes; Numbrecht, Germany) and mixed in a ratio 10: 1 with a so-

* This work is dedicated to Prof. Dr. Wilhelm Schoeppe, Frankfurt Main, on behalf of his 75th birthday.

lution of 6 % Dextran T500 (Pharmacia, Germany) suspended in phosphate-buffered saline (PBS) and incubated at 25 °C for 40 min. Leucocyte rich plasma was then carefully layered on top of the Ficoll gradient medium. As an alternative NycoPrep 1.068, (Nycomed, Norway) was used. After centrifugation for 15 minutes at 600 x g the monocyte ring was collected and the pooled monocyte fractions were washed twice with ice-cold PBS. The purity of CD14 positive monocytes was assessed by flowcytometry analysis. Cell numbers were counted in a Neubauer micro- chamber. Up to 66-68 % of the isolated cells were CD14 positive monocytes.

Cells were cultured at a density of 2 x 10^5 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Life Technologies, Germany). 200 mM glutamine was added to the medium, no antibiotics were added. Cell cultures were performed in 24-well plates (Falcon, Heidelberg, Germany) with 0.5 ml cell suspension per well [12, 13].

In order to analyse the possible effect of 1-alpha-calcidol (1-alpha-hydroxycholecalciferol, Leo Pharmaceutical Products Ltd, Ballerup, Denmark), cells were plated in 1-alpha-calcidol containing medium (10^{-4} – 10^{-12} M 1-alpha-calcidol), and incubated for 24, 48, 72 and 96 hours. In a second series of studies cells were preincubated with 100 ng/ml LPS (E.coli serotype 128:B12, Sigma Chemicals), washed threetimes with RPMI 1640, then alpha-calcidol was added, and subsequently cultured for 3 days (stimulated cells). Two different concentrations of 1-alpha-calcidol (Mol. weight 400.64 Da) were chosen (10^{-9} and 10^{-7} M). Cells were preincubated with medium free of 1-alpha-calcidol. Culture plates were prepared for cytoflowmetry in 24 hour intervals.

For analysis of the expression of monocyte antigens CD14, CD16, HLA DR, TLR2 and TLR4 adherent monocytes were detached from the culture plates, samples were analysed on a FACScan flow-cytometer (Becton Dickinson, Heidelberg, Germany). 100 microliters of cell suspensions were incubated with 20 microliters of FITC-or PE-labeled isotype control (mouse IgG2a, Biocarta, Hamburg). Monocytes were first gated by their forward and side scatter profile, then mean fluorescence channel intensity of the gates was measured by fluorochrome-labelled monoclonal antibodies against CD14, HLA-DR, TLR, including isotype control staining (RFC = relative fluorescence channel). Antibodies against CD14, and HLA-DR were from Becton Dickinson, Heidelberg, antibodies against TLR2 (clonotype TL2, IgG2a) and TLR4 (clonotype HTA-125, IgG2a) were from Biocarta, Hamburg.

Results were calculated on the basis of triplicates of cell culture analyses. In order to avoid donor specific changes monocytes were prepared from different donors. In addition, reproducibility of cell culture data was assessed by preanalytic inter-and intraassay series. Statistical analyses was performed applying nonparametric tests (Wilcoxon test for two paired samples and Mann-Whitney-Wilcoxon test). Data were recorded as median and values of $p < 0.05$ were considered significant. All analyses were accomplished using the SPSS software.

Results

1-Alpha-Calcidol and Expression of CD14 by Unstimulated and LPS Activated Monocytes

The expression of CD14 from (untreated) unstimulated monocytes slowly rose within culture time, indicating spontaneous upregulation of the LPS-receptor molecule. After incubation of cells with 1-alpha-calcidol the expression of CD14 further increased within the culture period compared to the controls (Fig.1.); reaching a level of $p<0.01$ after 48 and 72 hours culture time.

Presence of LPS alone also induced an increase of monocyte membrane bound CD14, which was significantly higher compared to that found with calcidol alone ($p< 0.01$). However, *1-alpha-calcidol* was capable of *further upregulating* the endotoxin receptor molecule in LPS pre-activated monocytes ($p< 0.05$). Thus, an additional or synergistic increase of CD14 expression induced by LPS and vitamin D emerged (Fig. 1, Fig. 2).

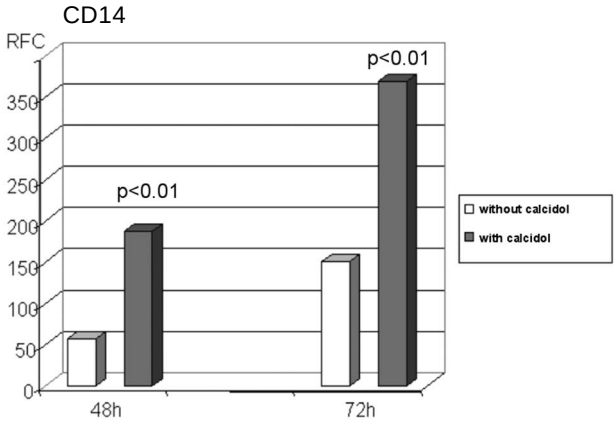

Fig.1. Influence of 1-alpha-calcidol (10^{-7}M) on monocyte expression of CD14 (endotoxin receptor) compared to the controls. Mann-Whitney- Wilcoxon-test.

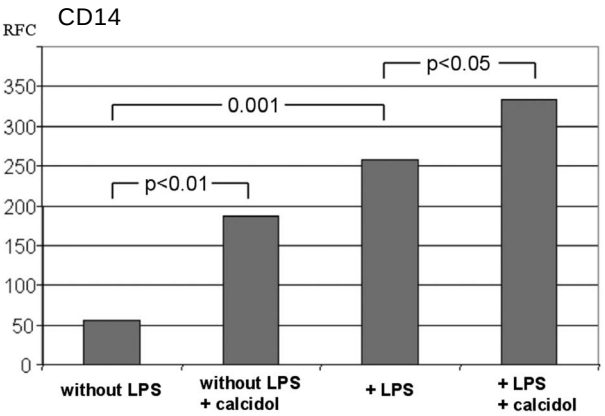

Fig 2. Modulation of CD14 expression by LPS and 1-alpha-calcidol. Synergistic increase of CD14 by LPS and Vitamin D. For details see text. Mann-Whitney-Wilcoxon-test.

Effect of 1-Alpha-Calcidol on HLA-DR Expression

In unstimulated cells HLA-DR expression rose continuously within culture time. This effect remained un-

changed in LPS stimulated cells. HLA-DR expression was inhibited dramatically by higher but physiological doses (10^{-7}M) of 1-alpha-calcidol (Fig. 3). A statistically significant difference was observed even after 24 hours ($p = 0.04$) and rose within culture time of 48 ($p<0.01$) and 72 hours ($p<0.0001$). In similar manner, 10 - 100 ng LPS downmodulated monocytic HLA-DR nearly completely, which was further influenced by addition of 1-alpha-calcidol (data not shown).

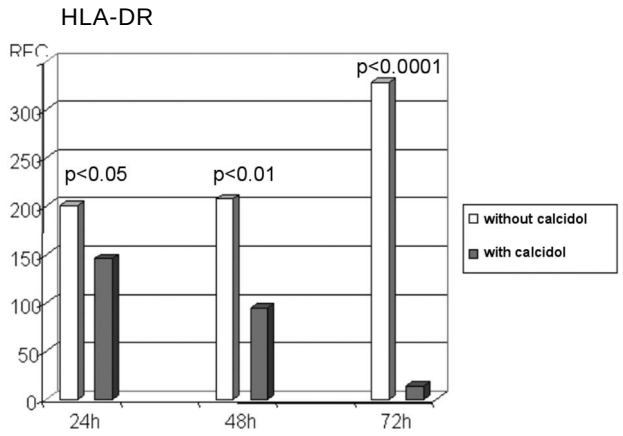

Fig 3. Downregulation of HLA-DR expression of cultured monocytes (24- 72 hours) by 1-alpha-calcidol. Mann-Whitney-Wilcoxon-test.

Effect of 1-Alpha- Calcidol on TL2 and TLR 4 Expression

The monocyte expression of TLR2, but not of TLR4, was inhibited by 10^{-7} M 1-alpha-calcidol (Fig. 4); after 72 hours culture time p reached < 0.01 compared to the controls. This effect was not observed after preincubation with LPS, however: Thus, endotoxin abolished the interaction of 1-alpha-calcidol on TLR2 expression. Low (10^{-9}M) as well as higher doses (10^{-7}M) of 1-alpha-calcidol upregulated insignificantly the expression of TLR4 in unstimulated cells (data not shown).

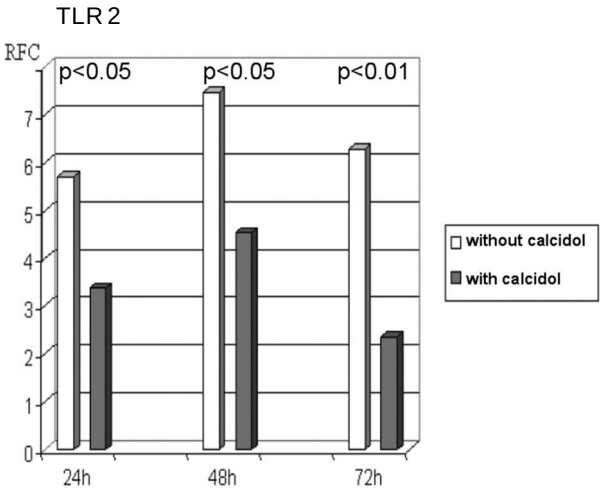

Fig 4. Modulation of toll-like receptor 2 (TLR2) expression of cultured monocytes by 1-alpha-calcidol. Isotype control versus calcidol (10^{-7} M) treated cells. Mann-Whitney-Wilcoxon-test.

Discussion

As confirmed by the results vitamin D derivatives disclose immunomodulatory potency. We placed emphasize on the possible interaction of 1-alpha-calcidol on monocyte CD14, HLA-DR, TLR2 and TLR4. Calcidol increased the expression of monocyte CD14 in unstimulated as well as in cells activated by endotoxin. LPS and calcidol interacted synergistically. By contrast, in the presence of glucocorticoids monocytes nearly completely downregulate membrane and soluble CD14 in vivo and in vitro, as previously shown [12, 13, 16].

In addition, 10^{-7} M calcidol reduced TLR2 expression in unstimulated monocytes. Since CD14 is a coreceptor of TLR2 (and for TLR4) we speculate a negative feedback of TLR2 under these circumstances [6, 9, 10]. The results further support the view that not only microbial components interact with TLR [22]. Thus, apart from Gram-negative bacteria, heat shock protein-70, and components of the extracellular matrix also 1-hydroxylvitamin D3 can modulate TLR, this is TLR 2, as shown here for the first time.

Calcidol dramatically downregulated HLA-DR expression, similarly as observed in monocytes cultures after incubation with LPS or in patients suffering from severe bacterial infections [5]. Downmodulation of HLA-DR by microbial ligands is equivalent with a status of relative immunosuppression and unresponsiveness of antigen-presenting cells. Thus, 1-alpha-calcidol mimics the biological effect of endotoxin or immunosuppressive-like agents. In this connection it is worthy to mention that a daily dose of 0.5 microgramm of 1-alfacalcidol was associated with a reduced risk for cardiovascular death in a cohort of 162 haemodialysis patients compared to 80 nonusers [20]. Monocyte TLR are under close regulatory control of proinflammatory ligands such as bacterial and viral products, heat shock proteins and other cell constituents [2, 22, 25]. Furthermore, reduced HLA-DR expression by vitamin-D might *antagonize microinflammatory* and potential *growth promoting* agents. Thus, our findings shed light on the interpretation of a previous study where calcitriol (1-25-Vit D3) given in a dose of 2 microgramm intraveneously improved anaemia and decreased the need for erythropoietin in haemodialysis patients [4]. As shown in fig 1 and 2 calcidol upregulated *CD14* expression in unstimulated and LPS-activated cultured monocytes, apparently by the same or a similar receptor as shown for 1-25-dihydroxy-vitamin D3 [23]. Peripheral blood monocytes from patients with impaired renal function without vitamin D supplementation disclose a significantly reduced expression of CD14 [21]. Cell-membrane expression of CD14 and binding capacity for LPS were directly correlated as described recently [13]. We conclude that monocytes in patients with renal failure lack cellular reactivity towards endotoxin and that vitamin D derivates e.g. calcitriol and 1-alpha-calcidol may restore this defect to a certain extent.

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