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Vitamin D3 enhances bactericidal activity of macrophage against *Pseudomonas aeruginosa*

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ABSTRACT

Background: The bioactive form of vitamin D3, *i.e.*1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) vitamin D has been shown to modulate monocytes/macrophages physiology and its response against bacterial infections. *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic bacterial pathogen that can most frequently be fatal in immunocompromised infected people.

Methods: We investigated the *ex vivo* effect of $1,25(OH)_2D_3$ on monocyte-derived macrophages function against *P. aeruginosa* infection.

Results: Relative vitamin D receptor (VDR) mRNA expression was significantly increased in infected and $1,25(OH)_2D_3$-treated macrophages compared to controls ($p < 0.01$). Treatment with $1,25(OH)_2D_3$ markedly resulted in up-regulation of nitric oxide (NO) and IL-1β production and down-regulation of IL-10 levels (respectively, $p = 0.029$, $p = 0.048$ and $p = 0.008$). Additionally, $1,25(OH)_2D_3$ significantly increased M1/M2 macrophage ratio ($p < 0.05$) and slightly reduced intracellular bacterial development. Furthermore, the arginase activity, *P. aeruginosa* phagocytosis and killing were significantly increased in cells that were both infected and $1,25(OH)_2D_3$-treated compared to the infected, but not $1,25(OH)_2D_3$-treated macrophages (respectively, $p < 0.001$, $p < 0.01$ and $p < 0.001$).

Conclusions: We show in this study that bioactive from of vitamin D [1,25-dihydroxyvitamin D3 (1,25D3)] can enhance M1 macrophage polarization and their bactericidal protective activity against *P. aeruginosa*. Future works would involve improving the treatment response through dose-dependent effect studies, both in *ex vivo* and *in vivo* models.

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1. Introduction

P. aeruginosa is a pathogenic and opportunistic Gram-negative bacterium responsible for a range of acute and chronic diseases [1,2]. *P. aeruginosa* is well known for its ability to rapidly adapt to varying environmental conditions, but also for its resistance to innate antimicrobial immune defense [3,4]. *P. aeruginosa* can exhibit a battery of virulence factors, including lipopolysaccharide (LPS) that mediate both bacterial pathogenesis and host responses [5].

Vitamin D or calcitriol is a steroid hormone, which is obtained *via* diet or synthesized from 7-dihydrocholesterol in the skin upon ultraviolet rays exposure. B cells, T cells and antigen-presenting cells (APCs) are able to synthesize bioactive vitamin D metabolites by two subsequent hydroxylation steps [6,7]: 1) Vitamin D_3 is converted by hepatic 25-hydroxylase into biologically inactive circulating metabolite 25 OH vitamin D3 (25(OH)D3). 2) The inactive form of vitamin D, vitamin D3 ($C_{27}H_{44}O_2$) or calcidiol is metabolized in the kidney to the active form 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) by 1-α-hydroxylase encoded by the gene CYP27B1 [8], which is also expressed by extrarenal tissues (including those of the immune system) [9]. $1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR) in the nucleus of target cells. The VDR forms a complex with the retinoid X receptor (RXR) and modulates multiple gene expression by binding to the vitamin D response element (VDRE) [10].

In addition to its classical function in homeostasis and bone metabolism, vitamin D can modulate the innate and adaptive immune responses [7]. It has been shown that $1,25(OH)_2D_3$ decreases the risk of *Mycobacterium tuberculosis* infections and boosts the innate immune response through various mechanisms including antimicrobial peptides production and cytokines response [11–13].

Furthermore, *in vitro* studies have demonstrated that $1,25(OH)_2D_3$ can reduce the production of pro-inflammatory cytokines/chemokines, such as IL-6 and CXCL8 after infection by *P. aeruginosa* [14]. Moreover, vitamin D supplementation decreases reactive oxygen species (ROS) levels in monocytes [15] and modulates the phagocytic ability of macrophages [11], as well as increases expression and secretion of antimicrobial peptides against *P. aeruginosa* [16].

Macrophages are heterogeneous cells of innate immunity that, depending to the microenvironment signals, can be divided into two

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major subpopulations, classically activated macrophages (M1) and alternatively activated macrophages (M2) [17]. The polarized macrophages toward the M1 and M2 phenotypes play an important role for host's defense during bacterial infection. M1 macrophages induce secretion of pro-inflammatory cytokines such as interleukin (IL) 1β, tumor necrosis factor alpha, (TNFα), IL-12, and IL-18 and increase bactericidal properties [18,19], while M2 macrophages produce anti-inflammatory mediators, like IL-10, which participate in the resolution of inflammation [20,21].

In the current study, we investigated the impact of the bioactive from of vitamin D3 on M1/M2 ratio and bactericidal activities of human monocyte-derived macrophages (MDMs) against *P. aeruginosa* infection.

2. Materials and methods

2.1. Ethical statement

This study was approved by the local ethics committee of Tlemcen University. Volunteer's healthy donors provided their written informed consent in accordance with the Helsinki Declaration.

2.2. Study design

Experiments were performed on MDMs, whole cell lysates, and total RNA. Firstly, macrophages were generated from monocytes enriched from peripheral blood mononuclear cells (PBMCs) by negative selection. VDR mRNA expression was examined on total RNA isolated from infected or not infected MDMs with *P. aeruginosa*. The effect of $1,25(OH)_2D_3$ on the levels of macrophage nitric oxide (NO) production, hydrogen peroxide (H_2O_2), IL-1β, IL-10, M1/M2 macrophage ratio, bacterial growth, phagocytosis and bacterial killing were carried out on a mixture of MDMs and *P. aeruginosa*. The oxidative burst assay was performed using the NO production and H_2O_2 assays. MDM lysates were used to measure the effect of $1,25(OH)_2D_3$ on the macrophage arginase activity. Each experiment was repeated at least four times (Fig. 1).

2.3. Bacterial strains

Assays were performed using the referent strain of *P. aeruginosa* ATCC 27853 provided from the American Type Culture Collection (Manassas, Va). Bacteria were grown over night at 37 °C in tryptic soy broth (TSB) (Fluka Analytical, Sigma Aldrich Co., St. Louis, USA). Number of bacteria was determined spectrophotometrically at 600 nm [3].

2.4. Cells preparation

Venous blood samples was collected from healthy volunteers into heparinated *vacutainer* tubes (BD, Belliver Industrial Estate, UK) and PBMCs were isolated using gradient density centrifugation on Histopaque-1077 (Sigma Aldrich Co., St. Louis, USA). Monocytes were enriched from PBMCs by negative selection using the EasySep Human Monocyte Enrichment Kit (STEMCELL Technologies, Vancouver, Canada), according to the manufacturer's instructions. Briefly, PBMCs (7×10^7) were resuspended in phosphate-buffered saline (PBS) with 1 mM ethylenediamineteraacetic acid (EDTA) and 2% fetal bovine serum (FBS) and were incubated with enrichment antibody cocktail (50 μL per mL) at 2–8 °C for 10 min. Magnetic Particles (50 μL per mL) were added for an additional 5 min. Finally, monocytes were collected from the negative fraction by washing (400 ×*g* for 10 min), and cells viability was evaluated by Trypan bleu counting as described [22].

2.5. Ex vivo generation of monocyte-derived macrophages and $1,25(OH)_2D_3$ treatment

To generate MDMs, monocytes were seeded in RPMI 1640 media (Sigma Chemical Co., St. Louis, USA) supplemented with 2 mM L-glutamine, 50 μg/mL gentamycin and 10% autologous serum at a concentration of 5×10^5 cells/mL in 24-well culture plate at 37 °C and 5% CO_2 for 48 h. After incubation, the MDMs were infected with *P. aeruginosa* at a MOI (multiplicity of infection) of 30, *i.e.* 30 bacteria for one macrophage. After incubation for 3 h at 37 °C, cells were washed three times with PBS to remove uningested bacteria. Thereafter, the MDMs were cultured at 37 °C and 5% CO_2 for 24 h in RPMI 1640 medium with 10% autologous serum in both presence and absence of $1,25(OH)_2D_3$ (Sigma Chemical Co., St. Louis, USA), at a dose of 10^{-7} M [12]. $1,25(OH)_2D_3$ was dissolved in 95% ethanol. The final concentration of ethanol did not exceed 0.5% (v/v) in the culture medium.

2.6. Vitamin D receptor mRNA expression

Total RNA was isolated from $1,25(OH)_2D_3$ treated and untreated *P. aeruginosa*-infected or not infected MDMs using Trisol reagent (life

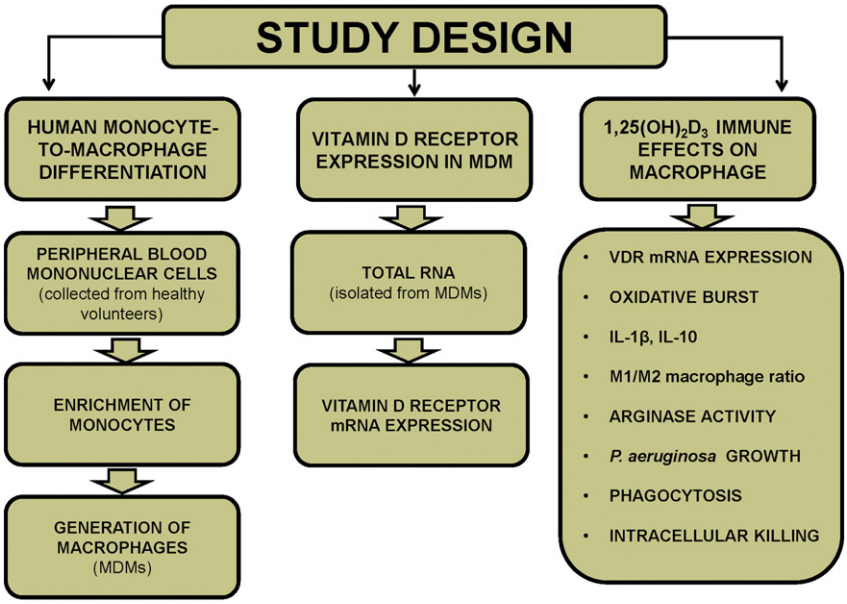

Fig. 1. Study flow-chart. MDM: monocyte-derived macrophages, VDR: vitamin D receptor, IL: interleukin, M1: classically activated macrophages, M2: alternatively activated macrophages.

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