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)2D3) 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)2D3 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)2D3-treated macrophages compared to controls (p < 0.01). Treatment with 1,25(OH)2D3 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)2D3 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)2D3-treated compared to the infected, but not 1,25(OH)2D3-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.

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 D3 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 (C27H44O2) or calcidiol is metabolized in the kidney to the active form 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) 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)2D3 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)2D3 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)2D3 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
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 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)2D3 on the levels of macrophage nitric oxide (NO) production, hydrogen peroxide (H2O2), 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 H2O2 assays. MDM lysates were used to measure the effect of 1,25(OH)2D3 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 × 107) were resuspended in phosphate-buffered saline (PBS) with 1 mM ethylenediaminetetraacetic 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 cell viability was evaluated by Trypan bleu counting as described [22].

2.5. Ex vivo generation of monocyte-derived macrophages and 1,25(OH)2D3 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 × 105 cells/mL in 24-well culture plate at 37 °C and 5% CO2 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% CO2 for 24 h in RPMI 1640 medium with 10% autologous serum in both presence and absence of 1,25(OH)2D3 (Sigma Chemical Co., St. Louis, USA), at a dose of 10−7 M [12]. 1,25(OH)2D3 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)2D3 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.
technologies, New Zealand, USA), according to the manufacturer's recommendations. The reverse transcription was carried out using RT Master Mix (5 x first-stand Buffer, dTTP, M-MLV, random primers, dNTPs, RNase OUT) prepared by reagents obtained from Invitrogen (Life technologies, USA). The polymerase chain reaction (PCR) was performed using SYBR Green PCR Master Mix (Bio-Rad, Singapore) in StepOnePlus Real-Time PCR (RT-PCR) systems (Applied Biosystems). Reactions were expressed as mean ± standard error of mean (SEM) ΔCt values, after normalization using 18S RNA primers. The primers (Invitrogen, Life technologies, USA) employed in this study are: VDR sense 5′-ACCAAGGACAAACGAGCCGA-3′, anti-sense 5′-GGCAATGATGCGCTGCTGCT-3′ and 18S RNA sense 5′-AAAC GGCCTACACATCGACG-3′, anti-sense 5′-CTCCATTGATCCTGGTTA -3′. Such assays were performed in the INSERM UMR 866 (University of Burgundy, France).

2.7. Oxidative burst assay

Oxidative/respiratory burst assay was quarried out by measurement of NO production (NOx, nitrite and nitrate) and H2O2 levels. NO production levels in cell culture supernatants were measured spectrophotometrically using the sensitive colorimetric Griess method at 540 nm as previously described [23]. The concentration of NO was determined from linear standard curve established by 0–150 μmol/L sodium nitrite. Levels of H2O2 in cell lysates were measured by spectrophotometer using a commercial kit (Sigma Chemical Co., St. Louis, USA), according to instructions of the manufacturers.

2.8. Cytokines assays and M1/M2 ratio

On the basis of the respective M1 and M2 macrophage signature cytokines, the M1/M2 ratio was estimated from the IL-1β/IL-10 ratio. IL-1β- and IL-10 levels were measured in the cell culture supernatants using the quantitative enzyme-linked immunosorbent assay (ELISA), with respective commercial kits, as per the manufacturer’s instructions (Sigma Aldrich Co., St. Louis, USA). Limits of detection were 0.3 pg/ml for IL-1β and 1 pg/ml for IL-10.

2.9. Arginase activity assay

Arginase activity was measured in MDM cell lysates. The assay was performed using a spectrophotometric technique based on the determination of the production of urea as previously described [24]. Briefly, cells were lysed in 500 μL of 0.1% Triton X 100 for 30 min. A mixture of Tris–HCl and MnCl2 was added and after activation by heating for 10 min, a volume of activated lysates was incubated with the same volume of arginine (0.5 M) (1 h at 37 °C). Four hundred μL of an acidic mixture were added to stop the reaction. Finally, after addition of 9% HCl and MnCl2 was added and after activation by heating for 10 min, a volume of activated lysates was incubated with the same volume of arginine (0.5 M) (1 h at 37 °C). Four hundred μL of an acidic mixture were added to stop the reaction. Finally, after addition of 9% isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 100 °C, the urea was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea/min.

2.10. Phagocytosis assay

Phagocytosis assay was studied as described in detail with minor modifications [25,26]. Assays were made at 0 (t0) and 1 h (t1) on a mixture of macrophages and P. aeruginosa cells at a MOI of 30 or bacterial cells alone as controls. Briefly, 5 x 105 of MDMs were cultured in 24-well culture plates, infected by bacteria and incubated with or without 10–7 M of 1,25(OH)2D3 for 1 h at 37 °C in 5% CO2. After incubation, supernatants were taken and serial dilutions were prepared in order to determine the number of viable extracellular bacteria by counting bacterial colony forming units (CFUs) on Cetrimide agar. The results were determined using the percentage decrease in the number of extracellular bacteria [27] as follows:

% Phagocytosis = M0 – 100 × (M0 – Mt1) / Mt0 [28,29], in which M0 is the number of bacteria in the mixture assay sample at t0, NEC is the number of extracellular bacteria in the mixture assay sample at t1, NC0 and NC1 correspond to control sample at t0 and t1, respectively.

2.11. Bacterial killing assay

To kill extracellular bacteria, P. aeruginosa-infected phagocytic cells were washed 3 times with 1 x PBS and cultured in media supplemented with 100 μg/mL gentamicin. After incubation for 1 h and washing, 1% Triton X-100 in PBS was added in some wells to lyse macrophage cells and determine the number of intracellular bacteria at time 0 (t0). Others were incubated for 1 h (t1) [30] in antibiotic-free medium. The lysis was stopped at the indicating times by addition of TSB. Intracellular and extracellular bacteria were counted after plating serial dilutions on Cetrimide agar and incubated overnight at 37 °C. The next day, CFUs count was related to the original bacterial suspension. Controls including wells of uninfected macrophages or inoculated with bacteria only were required for all assays. Two conditions were taken into consideration: (i) total absence of bacterial colonies in uninfected macrophage lysates, (ii) death of all bacteria incubated alone for one hour with gentamicin (<10 CFUs left). Trypan blue counting test was performed to check the viability of macrophages after infection [25,26]. The percentage of intracellular bacterial killing was calculated as follows: % bacterial killing = 100 × (NC0 – Nt1) / NC0 [27–29], in which NC0 and Nt1 correspond to the number of viable intracellular bacteria at t0 and t1, respectively.

2.12. Statistical analysis

All experiments were repeated at least four times and the data are presented as mean ± SEM. Differences in mean values between groups were performed by Kruskal–Wallis test for comparison of four groups or Mann–Whitney U test for comparison of two groups. Statistical analyses were performed with SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). P-values less than 0.05 were considered statistically significant.

3. Results

In the present study, we examined the ex vivo effect of vitamin D on the modulation of macrophage activation against P. aeruginosa. Human MDMs were stimulated with live P. aeruginosa for 3 h, followed by treatment with the active vitamin D [1,25-dihydroxyvitamin D3 (1,25D3)] for 24 h at a concentration of 10−7 M.

3.1. VDR mRNA expression

Knowing that the presence of VDR is necessary for 1,25(OH)2D3 to exert its effects on gene expression [31], we first performed a Real-Time quantitative PCR to confirm whether or not P. aeruginosa would have no adverse effect on the expression of VDR in macrophage. Thus, relative quantification of mRNA revealed that P. aeruginosa had no substantial effect on the expression of VDR mRNA. Additionally, relative VDR expression was significantly increased in infected and 1,25(OH)2D3-treated macrophages compared to the infected and non-treated cells (p = 0.008 by Mann–Whitney U test. For all comparisons, p < 0.01 by Kruskal–Wallis test) (Fig. 2).

3.2. Effect of 1,25(OH)2D3 on macrophage oxidative burst

The NO production and H2O2 were evaluated as macrophage oxidative burst biomarkers. The NO production was measured in culture supernatants by the sensitive Griess reaction. The H2O2 levels were measured spectrophotometrically in the macrophage cell lysates.

As shown in Fig. 3, 1,25(OH)2D3 significantly increased NO production concentration in treated P. aeruginosa-infected macrophages compared to the untreated and infected condition (p < 0.05 by Mann–Whitney U test). The treated and uninfected macrophage cells showed also increased NO production when compared to the untreated and uninfected macrophage cells, but the difference did not reach statistical significance level (p < 0.05 by Mann–Whitney U test). Additionally, the H2O2 levels were significantly increased in uninfected and 1,25(OH)2D3-treated macrophages compared to the uninfected and untreated cells (p = 0.011 by Mann–Whitney U test). Whereas, the H2O2 levels were not different between the two groups of P. aeruginosa-infected macrophages. The Kruskal–Wallis test gave p-value = 0.001 for NO production and p-value = 0.008 for H2O2.

3.3. Effect of 1,25(OH)2D3 on macrophage IL-1β and IL-10 production and M1/M2 ratio

We observe in Fig. 4 that in the absence of P. aeruginosa, the active vitamin D induced a significant increase in IL-10 levels and a significant decrease in those of IL-1β (respectively, p = 0.002 and p = 0.020 by Mann–Whitney U test). Similarly, the presence of P. aeruginosa without active vitamin D leads to a highly significant increase in IL-10 levels compared to uninfected macrophages, supplemented or not with active vitamin D (For the two comparisons, p = 0.000 by Mann–Whitney U test). Conversely, the active vitamin D induced a highly significant decrease of IL-10 levels and a significant increase of IL-1β levels in infected macrophages compared to infected but not 1,25(OH)2D3-treated macrophages (respectively, p = 0.006 and p = 0.048 by Mann–Whitney U test). For the two cytokines using Kruskal–Wallis test, p-value was 0.006 for IL-1β and p-value was 0.000 for IL-10.

As shown in Fig. 5, M1/M2 ratio was significantly decreased in uninfected and 1,25(OH)2D3-treated macrophages when compared with uninfected and not 1,25(OH)2D3-treated cells (p = 0.000 by Mann–Whitney U test). Additionally, P. aeruginosa in the absence of 1,25(OH)2D3 decrease the M1/M2 ratio. Inversely, treatment of P. aeruginosa-infected macrophages with 1,25(OH)2D3 induced an increase in the M1/M2 ratio when compared to P. aeruginosa-infected and not treated cells (p < 0.05 by Mann–Whitney U test). For all comparisons using Kruskal–Wallis test, p-value was 0.000.
3.6. Effect of 1,25(OH)2D3 on Pseudomonas aeruginosa phagocytosis and M1/M2 ratio. Statistical differences highlighted among different groups are determined by the Kruskal-Wallis test. For all comparisons, p = 0.000 by Mann-Whitney U test. For all comparisons, p = 0.000 by Mann-Whitney U test. For all comparisons, p = 0.000 by Mann-Whitney U test. For all comparisons, p = 0.000 by Mann-Whitney U test.

3.4. Effect of 1,25(OH)2D3 on arginase activity

There were no significant differences in arginase activity levels between uninfected 1,25(OH)2D3-treated macrophages compared to the infected but not treated cells; while arginase activity was significantly increased in infected 1,25(OH)2D3-treated macrophages compared to the infected but not treated cells (p = 0.000 by Mann–Whitney U test). All comparisons, p = 0.001 by Kruskal–Wallis test (Fig. 6). Additionally, we observed that the arginase activity seems to be up-regulated in untreated P. aeruginosainfected macrophages compared to that in uninfected macrophages, treated or not with 1,25(OH)2D3, but the difference did not reach statistical significance.

3.5. Effect of 1,25(OH)2D3 on macrophage intracellular growth of P. aeruginosa

We see in Fig. 7 that treatment with 1,25(OH)2D3 induced a decrease in the intracellular growth of P. aeruginosa within macrophages compared to the untreated macrophages. Nevertheless, the difference was not statistically significant (p = 0.259 by Mann–Whitney U test).

3.6. Effect of 1,25(OH)2D3 on Pseudomonas aeruginosa phagocytosis and killing

Macrophages are considered as the first line of defense against infections and the major phagocytic cells [26]. The number of extracellular bacteria was determined after 1 h of incubation with P. aeruginosa. After infection, intracellular live bacteria were counted at two time points after infection, i.e. at 0 (t0) and 1 h (t1).

As shown in Fig. 8, both phagocytosis and P. aeruginosa killing were significantly increased in 1,25(OH)2D3-treated macrophages compared to the untreated macrophages (respectively, p = 0.009 and p = 0.000 by Mann–Whitney U test).

4. Discussion

Formerly known as Bacillus pyocyaneus, P. aeruginosa is an opportunistic pathogen that could be dangerous and difficult to treat, especially in cystic fibrosis patients. Usually considered as an extracellular organism, but could also invade specific cells during infection course [32]. P. aeruginosa has also the ability to evade the host immune response and to persist during chronic infections. Therefore, the strengthening of the innate immune response against this bacterium may then be able to eradicate it in a more efficient manner.

In addition to its crucial role in physiology and in maintaining bone homeostasis, vitamin D can have various beneficial effects on both innate and adaptive immune responses regarding the fight against...
bacterial infections. Its antibacterial activity can be maintained only following the final converting step in the kidney of precursor 25-hydroxyvitamin D (25OHD) to active 1,25(OH)2D3 [33]. It also can boost innate immunity by modulating expression of potent antimicrobial peptides (AMPs) and cytokine response [34]. Thus, we for our part, highlighted for the first time the ex vivo effects of the active form of vitamin D on the stimulation of macrophage against infection with P. aeruginosa. In order to properly evaluate its effects, the VDR expression analysis was a prerequisite for subsequent experiments.

4.1. VDR mRNA expression

Numerous in vitro experiments have largely studied the anti-inflammatory roles of vitamin D on the modulation of host immune responses in different infectious diseases. These roles are exerted through VDR. In this study, expression of VDR mRNA was evaluated in macrophage under different culture conditions using real-time PCR. Our results revealed that P. aeruginosa-infected macrophages were with no adverse effect on VDR expression. Additionally, VDR expression was significantly increased in infected and 1,25(OH)2D3-treated macrophages. Therefore, infection with P. aeruginosa and vitamin D supplementation may provide a synergistic effect on the VDR expression. Hence, such effect has been recently observed with toll-like receptor agonists, like LPS, which may be present on P. aeruginosa [38]. Moreover, our results are consistent with previous data showing that 1,25(OH)2D3 can upregulate VDR expression [39]. In the same context, it has recently been demonstrated [40] that 1,25(OH)2D3 induces intracellular redistribution and stabilization of the VDR in human CD4+ T cells by protecting it from protozoal degradation cells.

4.2. Effect of 1,25(OH)2D3 on macrophage oxidative burst

The oxidative burst, an important mechanism of host cells against pathogens, is produced by the NADPH oxidase complex [41]. Through activation of NADPH oxidase, activated macrophages produce various reactive oxygen or nitrogen species, including NO and H2O2, all of which have strong cytotoxic activities against microorganisms [42].

To the best of our knowledge, this is the first study that shows NO and H2O2 responses to 1,25(OH)2D3 in P. aeruginosa-infected macrophages. We found that vitamin D increased significantly NO production, but it had no significant effect on H2O2 production in the presence of P. aeruginosa. Therefore, previous reports suggest that NO can decrease H2O2 production by formation of catalase and helps macrophages to survive in environment rich with oxidants [43].

4.3. Effect of 1,25(OH)2D3 on macrophage IL-1β and IL-10 production and M1/M2 ratio

Activated macrophages and monocytes secrete IL-1β, a pro-inflammatory cytokine that exerts multiple biological activities in controlling host immune responses. The precursor molecule of IL-1β (pro-IL-1β) has to be cleaved to generate the active form of IL-1β [44, 45]. IL-10, a most important anti-inflammatory cytokine, can be produced by B cells, macrophages, dendritic cells, and CD4+ T cells, including forkhead box P3 (Foxp3)+CD4+ T cells, antigen-induced type 1 regulatory T cells (Tr1), and T helper 1 (Th1) and Th2 cells. This cytokine has regulatory functions in controlling inflammation by inhibition of costimulatory molecules CD80 and CD86 expression on monocytes and macrophages and limiting pro-inflammatory cytokines/chemokines production [46]. Both IL-1β and IL-10 can play an important antimicrobial activities in corneal response to P. aeruginosa infection [47-48].

In our study, we observed that in the absence of P. aeruginosa, 1,25(OH)2D3 decreased levels of IL-1β and M1/M2 ratio but in contrast increased IL-10. Conversely, in the presence of P. aeruginosa, vitamin D increased levels of IL-1β and M1/M2 macrophage ratio but decreased IL-10. These results clearly demonstrate the immunomodulatory effect of vitamin D during P. aeruginosa infection. Our results corroborate recent data showing that vitamin D enhances IL-1β secretion by human MDMs during infection with Mycobacterium tuberculosis [49,50]. However, previous data reported that 1,25(OH)2D3 down-regulates the levels of pro-inflammatory cytokines including IL-1β in a dose-dependent manner in human corneal epithelial cells colonized with P. aeruginosa [51]. These contradictory results may in part reflect the differences in the dose of vitamin D3 and cell types used. Knowing that no study has been undertaken in the same context of our work, the comparison with other data is not yet possible. So discussion of our observations will require further investigations.

4.4. Effect of 1,25(OH)2D3 on arginase activity

NO can be synthesised directly by the enzymatic conversion of L-arginine by type II inducible NO synthase (iNOS). iNOS and arginase are coinduced in activated macrophages. They compete for their common substrate, L-arginine. The enzymatic action of arginase on L-arginine results in the generation of L-ornithine and urea. Hence increased levels of arginase activity might limit the amount of arginine for NO synthesis [52].

It has been reported that the predominance of M2 macrophage phenotype can promote the production of arginase [37]. In our study, despite the predominance of M1 phenotype in P. aeruginosa-infected and 1,25(OH)2D3-treated macrophages compared with P. aeruginosa-infected and not 1,25(OH)2D3-treated macrophages, we observed a significant increase in arginase activity. Conversely, in the absence of P. aeruginosa, 1,25(OH)2D3 has no significant effect on the activity of arginase. Therefore, vitamin D may predispose to an overproduction of arginase activity in infection caused by P. aeruginosa. Nevertheless, these data are still relatively uncommon and their importance should be checked as early as possible with dose-dependent effect studies. Finally,
our results support previous works suggesting that arginase expression and activity are increased in *P. aeruginosa* lung infection [53,54].

4.5. Effect of 1,25(OH)2D3 on macrophage intracellular growth of *P. aeruginosa*

In order to identify its antibacterial properties, the active vitamin D3 form was screened for its ability to inhibit bacterial growth. Our results show that 1,25(OH)2D3 may somehow have an effect on macrophages to control *P. aeruginosa* growth. Accordingly, it has recently been demonstrated that 1,25(OH)2D3 significantly reduced *Mycobacterium tuberculosis* growth in human MDMs from patients with current tuberculosis (*p < 0.05*) [50]. Additionally, other results show that co-treatment of the human bronchial epithelial cell line (VA10) with vitamin D3 and phenylbutyrate may inhibit growth of *P. aeruginosa* [55]. Our preliminary findings should pay close attention to this issue, but may need to be detailed by additional *ex vivo* and *in vivo* immunopharmacological studies. To this end, various concentrations of 1,25(OH)2D3 should be used to identify the optimum useful dose required for the maximum level of *P. aeruginosa* growth control in macrophage cells without any obvious adverse effect.

4.6. *Pseudomonas aeruginosa* phagocytosis and killing

In addition to the results on the control of bacterial growth, we highlighted the potent immunotherapeutic effects of active vitamin D3 on *P. aeruginosa* phagocytosis and intracellular killing by human MDMs. Although currently there has, to our knowledge, been no study conducted on *P. aeruginosa* in exactly the same way, and the most assays have been performed on *Mycobacterium tuberculosis*, our results agree with recent report [56] suggesting that 1,25(OH)2D3 enhances the ability of macrophages to phagocytose and kill of pathogens. Among the main molecular mechanisms involved in enhancing such activities should be the upregulation of triggering receptor expressed on myeloid cells-1 (TREM-1) expression, which is considered as a pivotal amplifier of the innate immune response in macrophages [57].

4.7. Conclusions and future prospects

In conclusion, our findings supported the hypothesis that vitamin D3 may modulate innate immune response and prevent host against *P. aeruginosa* infection. This study deserves further investigations, and among others, it would be interesting to: i) evaluate protein levels of VDR to confirm the quantitative RT-PCR-based analysis results, ii) try other approaches such as flow cytometry or immunofluorescence staining to identify molecular determinants of polarized M1-M2 macrophages as well as phagocytic and microbicidal capacity, and iii) study of molecular mechanisms of action of vitamin D and its main immunological effects on macrophage antimicrobial peptides production. Finally, future works would involve improving the treatment response through the use of animal models and various concentrations of 1,25(OH)2D3 to identify the optimum useful dose required for the effective eradication of *P. aeruginosa* by boosting an adequate number of macrophages with high phagocytic and bactericidal potential in the context of translational medicine and therapeutics.

Conflict of interest

The authors have no financial conflicts of interest.

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