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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.

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

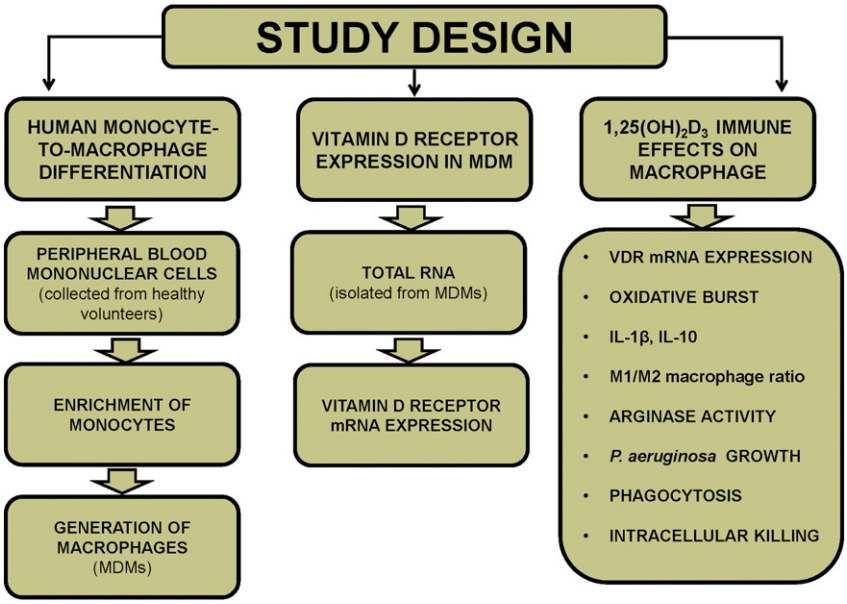

Fig. 1. Study flow-chart. MDM: monocyte-derived macrophages, VDR: vitamin D receptor, IL: interleukin, M1: classically activated macrophages, M2: alternatively activated macrophages.

technologies, New Zealand, USA), according to the manufacturer's recommendations. The reverse transcription was carried out using RT Master Mix (5× first-stand Buffer, dTT, 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′-ACCAAGGACAACCGACGCCA-3′, anti-sense 5′-GGCAATGATGCGCTGCTGCT-3′ and 18S RNA sense 5′-AAAC GGCTACCACATCCAAG-3′, anti-sense 5′-CCTCCAATGGATCCTCGTTA-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 H_2O_2 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 H_2O_2 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 $MnCl_2$ 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 (t_0) and 1 h (t_1) on a mixture of macrophages and *P. aeruginosa* cells at a MOI of 30 or bacterial cells alone as controls. Briefly, 5×10^5 of MDMs were cultured in 24-well culture plates, infected by bacteria and incubated with or without 10^{-7} M of $1,25(OH)_2D_3$ for 1 h at 37 °C in 5% CO_2. 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:

$$\%\text{Phagocytosis} = \text{Mt0} - 100 \times \frac{\left(\frac{\text{NEC}}{\text{NC1}/\text{NC0}}\right)}{\text{Mt0}}$$ [28,29], in which M_{t0} is the number of bacteria in the mixture assay sample at t_0, NEC is the number of extracellular bacteria in the mixture assay sample at t_1, NC_0 and NC_1 correspond to control sample at t_0 and t_1, respectively.

2.11. Bacterial killing assay

To kill extracellular bacteria, *P. aeruginosa*-infected phagocytic cells were washed 3 times with 1× 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 (t_0). Others were incubated for 1 h (t_1) [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 gentamycin (<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: $\%\text{bacterial killing} = 100 \times \left(\frac{\text{Nt0}-\text{Nt1}}{\text{Nt0}}\right)$ [27–29], in which N_{t0} and N_{t1} correspond to the number of viable intracellular bacteria at t_0 and t_1, 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 $1,25(OH)_2$ D_3 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 $1,25(OH)_2D_3$-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 $1,25(OH)_2D_3$ on macrophage oxidative burst

The NO production and H_2O_2 were evaluated as macrophage oxidative burst biomarkers. The NO production was measured in culture supernatants by the sensitive Griess reaction. The H_2O_2 levels were measured spectrophotometrically in the macrophage cell lysates.

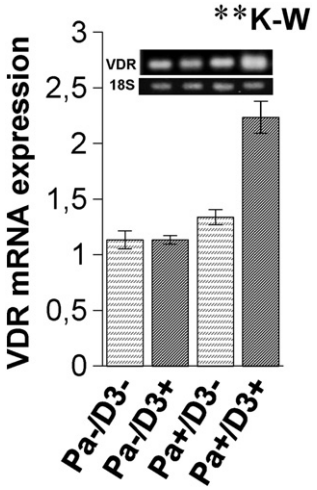

Fig. 2. Relative expression of vitamin D receptor mRNA in macrophages cultures. A real-time PCR was performed to check whether *P. aeruginosa* would have no adverse effect on the expression of VDR in macrophage. To this end, total RNA was isolated from macrophages, infected or not by *P. aeruginosa*, and treated or not with 1,25$(OH)_2D_3$, using Trisol reagent. The reverse transcription was carried out using RT Master Mix. Reactions were expressed as mean ± SEM ΔCt values, after normalization using 18S RNA primers. PCR: polymerase chain reaction, SEM: standard error of mean, VDR: vitamin D receptor, K-W: Kruskal–Wallis test, Pa −/D3 −: macrophages not cultured with *P. aeruginosa* and not treated with 1,25$(OH)_2D_3$, Pa −/D3 +: macrophages not cultured with *P. aeruginosa* but treated with 1,25$(OH)_2D_3$, Pa +/D3 −: macrophages cultured in the presence of *P. aeruginosa* but not treated with 1,25$(OH)_2D_3$, Pa +/D3 +: macrophages cultured in the presence of *P. aeruginosa* and treated with 1,25$(OH)_2D_3$. $^{**}p < 0.01$ by Kruskal–Wallis test. Statistical differences highlighted among different groups are defined as follows: Pa −/D3 − *vs.* Pa +/D3 + or Pa −/D3 + *vs.* Pa +/D3 + or Pa +/D3 − *vs.* Pa +/D3 +, $p < 0.01$ by Mann–Whitney U test.

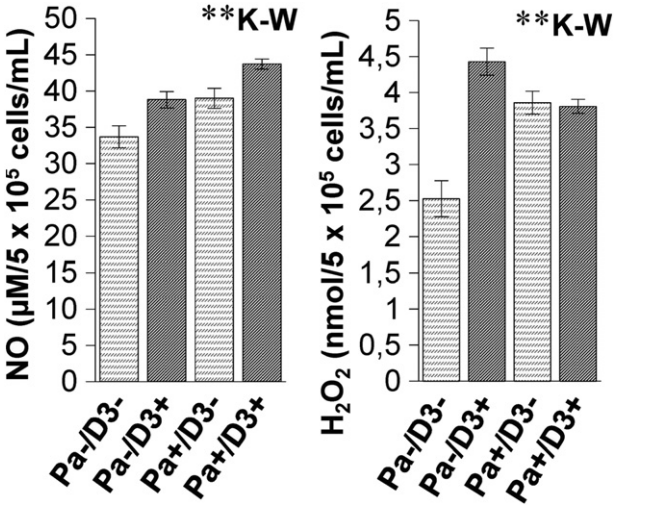

Fig. 3. Effect of 1 alpha,25$(OH)_2D_3$ on macrophage respiratory burst activation against *Pseudomonas aeruginosa*. The assay was quarried out by means of spectrophotometric determination of NO production and H_2O_2 levels. H_2O_2: hydrogen peroxide, NO: nitric oxide [NOx, nitrite (NO_2-) and nitrate (NO_3-)], K-W: Kruskal–Wallis test, Pa −/D3 −: macrophages not cultured with *P. aeruginosa* and not treated with 1,25$(OH)_2D_3$, Pa −/D3 +: macrophages not cultured with *P. aeruginosa* but treated with 1,25$(OH)_2D_3$, Pa +/D3 −: macrophages cultured in the presence of *P. aeruginosa* but not treated with 1,25$(OH)_2D_3$, Pa +/D3 +: macrophages cultured in the presence of *P. aeruginosa* and treated with 1,25$(OH)_2D_3$. $^{**}p < 0.01$ by Kruskal − Wallis test. Statistical differences highlighted among different groups are given as follows by Mann–Whitney U test: (i) NO; Pa −/D3 − *vs.* Pa +/D3 +, $p < 0.05$, Pa −/D3 + *vs.* Pa +/D3 +, $p < 0.01$, Pa +/D3 − *vs.* Pa +/D3 +, $p < 0.05$, (ii) H_2O_2; Pa −/D3 − *vs.* Pa −/D3 + or Pa −/D3 − *vs.* Pa +/D3 − or Pa −/D3 − *vs.* Pa +/D3 +, $p < 0.05$.

As shown in Fig. 3, 1,25$(OH)_2D_3$ 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 H_2O_2 levels were significantly increased in uninfected and 1,25$(OH)_2D_3$-treated macrophages compared to the uninfected and untreated cells ($p = 0.011$ by Mann–Whitney U test). Whereas, the H_2O_2 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 H_2O_2.

3.3. Effect of 1,25$(OH)_2D_3$ 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$(OH)_2D_3$-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$(OH)_2D_3$-treated macrophages when compared with uninfected and not 1,25$(OH)_2D_3$-treated cells ($p = 0.000$ by Mann–Whitney U test). Additionally, *P. aeruginosa* in the absence of 1,25$(OH)_2D_3$ decrease the M1/M2 ratio. Inversely, treatment of *P. aeruginosa*-infected macrophages with 1,25$(OH)_2D_3$ 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.

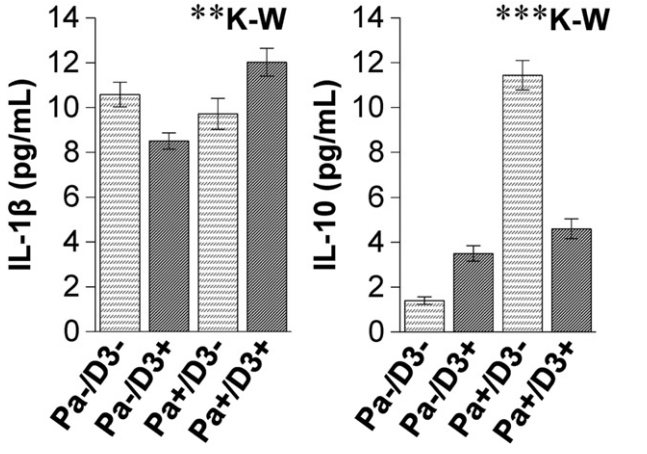

Fig. 4. Effect of 1 alpha,25$(OH)_2D_3$ on macrophage IL-1β and IL-10 in the presence of *Pseudomonas aeruginosa*. The two macrophage cytokines levels were measured using sandwich enzyme-linked immunosorbent assay (ELISA). K-W: Kruskal–Wallis test, Pa −/D3 −: macrophages not cultured with *P. aeruginosa* and not treated with 1,25$(OH)_2D_3$, Pa −/D3 +: macrophages not cultured with *P. aeruginosa* but treated with 1,25$(OH)_2D_3$, Pa +/D3 −: macrophages cultured in the presence of *P. aeruginosa* but not treated with 1,25$(OH)_2D_3$, Pa +/D3 +: macrophages cultured in the presence of *P. aeruginosa* and treated with 1,25$(OH)_2D_3$. $^{**}p < 0.01$, $^{***}p < 0.001$ by Kruskal–Wallis test. Statistical differences highlighted among different groups are given as follows by Mann–Whitney U test: (i) IL-1β; Pa −/D3 − *vs.* Pa −/D3 +, $p < 0.05$, Pa −/D3 + *vs.* Pa +/D3 +, $p < 0.01$, Pa +/D3 − *vs.* Pa +/D3 +, $p < 0.05$, (ii) IL-10; Pa −/D3 − *vs.* Pa −/D3 +, $p < 0.01$, Pa −/D3 − *vs.* Pa +/D3 −, $p < 0.001$, Pa −/D3 − *vs.* Pa +/D3 +, $p < 0.01$, Pa −/D3 + *vs.* Pa +/D3 −, $p < 0.001$, Pa −/D3 + *vs.* Pa +/D3 +, $p < 0.01$.

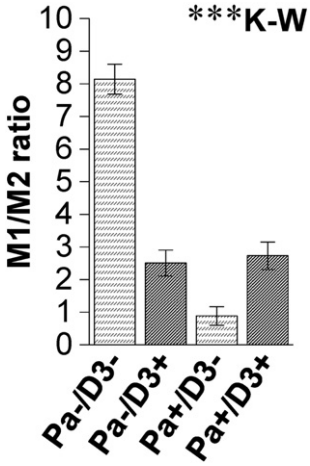

Fig. 5. Effect of 1 alpha,25$(OH)_2D_3$ on M1/M2 macrophage ratio in the presence of *Pseudomonas aeruginosa*. The M1/M2 ratio was estimated from the interleukin 1β (IL-1β)/IL-10 ratio. M1: classically activated macrophages, M2: alternatively activated macrophages, K-W: Kruskal–Wallis test, Pa −/D3 −: macrophages not cultured with *P. aeruginosa* and not treated with 1,25$(OH)_2D_3$, Pa −/D3 +: macrophages not cultured with *P. aeruginosa* but treated with 1,25$(OH)_2D_3$, Pa +/D3 −: macrophages cultured in the presence of *P. aeruginosa* but not treated with 1,25$(OH)_2D_3$, Pa +/D3 +: macrophages cultured in the presence of *P. aeruginosa* and treated with 1,25$(OH)_2D_3$. ***$p < 0.001$ by Kruskal–Wallis test. Statistical differences highlighted among different groups are defined as follows by Mann–Whitney U test: Pa −/D3 − *vs.* Pa-D3 +, $p < 0.001$, Pa −/D3 − *vs.* Pa + D3 −, $p < 0.001$, Pa −/D3 − *vs.* Pa + D3 +, $p < 0.001$, Pa −/D3 + *vs.* Pa + D3 −, $p < 0.05$, Pa +/D3 − *vs.* Pa + D3 +, $p < 0.05$.

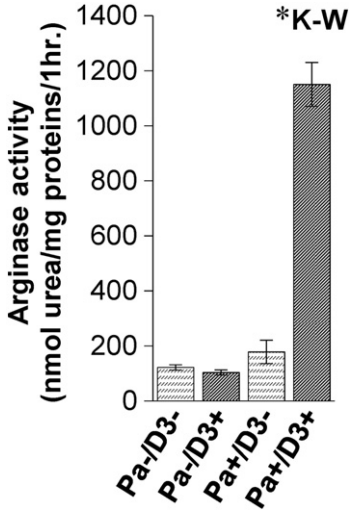

Fig. 6. Effect of 1 alpha, 25$(OH)_2D_3$ on macrophage arginase activity in the presence of *Pseudomonas aeruginosa*. Arginase activity was measured in macrophage cell lysates using a spectrophotometric technique based on the determination of the production of urea. K-W: Kruskal–Wallis test, Pa −/D3 −: macrophages not cultured with *P. aeruginosa* and not treated with 1,25$(OH)_2D_3$, Pa −/D3 +: macrophages not cultured with *P. aeruginosa* but treated with 1,25$(OH)_2D_3$, Pa +/D3 −: macrophages cultured in the presence of *P. aeruginosa* but not treated with 1,25$(OH)_2D_3$, Pa +/D3 +: macrophages cultured in the presence of *P. aeruginosa* and treated with 1,25$(OH)_2D_3$. *$p < 0.05$ by Kruskal–Wallis test. Statistical differences highlighted among different groups are defined as follows: Pa −/D3 − *vs.* Pa +/D3 + or Pa −/D3 + *vs.* Pa +/D3 + or Pa +/D3 − *vs.* Pa +/D3 +, $p < 0.001$ by Mann–Whitney U test.

3.4. Effect of 1,25$(OH)_2D_3$ on arginase activity

There were no significant difference in arginase activity levels between uninfected and 1,25$(OH)_2D_3$-treated macrophages compared to the uninfected but not treated cells; while arginase activity was significantly increased in infected and 1,25$(OH)_2D_3$-treated macrophages compared to the infected and not treated cells ($p = 0.000$ by Mann–Whitney U test. For all comparisons, $p = 0.016$ by Kruskal–Wallis test) (Fig. 6). Additionally, we observed that the arginase activity seems the be up-regulated in untreated *P. aeruginosa*-infected macrophages compared to that in uninfected macrophages, treated or not with 1,25$(OH)_2D_3$, but the difference did not reach statistical significance.

3.5. Effect of 1,25$(OH)_2D_3$ on macrophage intracellular growth of P. aeruginosa

We see in Fig. 7 that treatment with 1,25$(OH)_2D_3$ 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$(OH)_2D_3$ 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 (t_0) and 1 h (t_1).

As shown in Fig. 8, both phagocytosis and *P. aeruginosa* killing were significantly increased in 1,25$(OH)_2D_3$-treated macrophages than in not 1,25$(OH)_2D_3$-treated 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

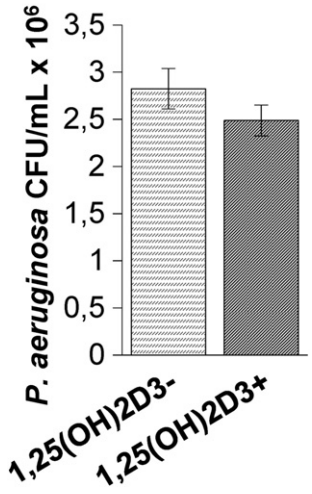

Fig. 7. Effect of 1 alpha, 25$(OH)_2D_3$ on intracellular *P. aeruginosa* growth within macrophage. The effect of 1,25$(OH)_2D_3$ on MDMs to control *P. aeruginosa* growth was examined to complete assays on phagocytosis and bacterial killing. No asterisk mark indicates that the difference did not quite achieve significance ($p > 0.05$ by Mann–Whitney U test).

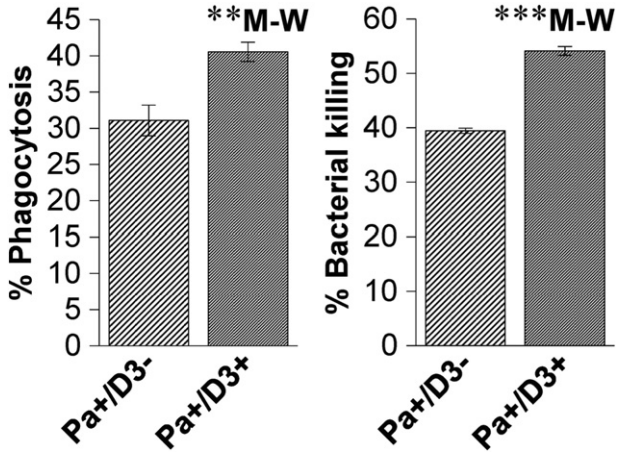

Fig. 8. Effect of 1 alpha, 25$(OH)_2D_3$ on macrophage phagocytosis and killing of *Pseudomonas aeruginosa*. The bactericidal activity of macrophages was assayed using the antibiotic protection method. After infection, intracellular live bacteria were analyzed by the colony forming unit (CFU) method at two time points after infection, *i.e.* at 0 (t0) and 1 h (t1). M-W: Mann–Whitney U test. $^{**}p < 0.01$, $^{***}p < 0.001$ by Mann–Whitney U test.

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$(OH)_2D_3$ [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.

M1 macrophages exhibit powerful microbicidal activity as well as proinflammatory cytokines, including IL-1β, which release through activation of the caspase-1/nucleotide-binding domain and leucine-rich repeat receptor containing pyrin domain 3 (NLRP3) inflammasome [35]. In contrast, M2 macrophages appear to be involved in the resolution of inflammation by upregulation of anti-inflammatory cytokine IL-10, and tissue repair [36]. They are poorly microbicidal [20] and may promote the production of arginase [37]. In this context, we tested the effect of active vitamin D on M1/M2 ratio in the presence of *P. aeruginosa*.

4.1. VDR mRNA expression

Numerous *in vitro* experiments have largely studied the anti-infective 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$(OH)_2D_3$-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$(OH)_2D_3$ can upregulate VDR expression [39]. In the same context, it has recently been demonstrated [40] that 1,25$(OH)_2D_3$ induces intracellular redistribution and stabilization of the VDR in human CD4+ T cells by protecting it from proteasomal degradation cells.

4.2. Effect of 1,25$(OH)_2D_3$ 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 H_2O_2, 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 H_2O_2 responses to 1,25$(OH)_2D_3$ in *P. aeruginosa*-infected macrophages. We found that vitamin D increased significantly NO production, but it had no significant effect on H_2O_2 production in the presence of *P. aeruginosa*. Therefore, previous reports suggest that NO can decrease H_2O_2 production by formation of catalase and helps macrophages to survive in environment rich with oxidants [43].

4.3. Effect of 1,25$(OH)_2D_3$ on macrophage IL-1β and IL-10 production and M1/M2 ratio

Activated macrophages and monocytes secret IL-1β, a pro-inflammatory cytokine that exerts multiple biological activities in controlling host immune responses. The precursor molecule of IL-1β (proIL-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 controling 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$(OH)_2D_3$ 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$(OH)_2D_3$ 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$(OH)_2D_3$ on arginase activity

NO can be synthetised 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$(OH)_2D_3$-treated macrophages compared with *P. aeruginosa*-infected and not 1,25$(OH)_2D_3$-treated macrophages, we observed a significant increase in arginase activity. Conversely, in the absence of *P. aeruginosa*, 1,25$(OH)_2D_3$ 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)$_2$D$_3$ 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)$_2$D$_3$ may somehow have an effect on macrophages to control *P. aeruginosa* growth. Accordingly, it has recently been demonstrated that 1,25(OH)$_2$D$_3$ 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)$_2$D$_3$ should be used to identify the optimum useful dose required for the maximum level of *P. aeuginosa* 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)$_2$D$_3$ 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)$_2$D$_3$ 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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References

[1] E.G. Lavoie, T. Wangdi, B.I. Kazmierczak, Innate immune responses to *Pseudomonas aeruginosa* infection, Microbes Infect. 13 (2011) 1133–1145, http://dx.doi.org/10.1016/j.micinf.2011.07.011.
[2] E. Amiel, R.R. Lovewell, G.A. O'Toole, D.A. Hogan, B. Berwin, *Pseudomonas aeruginosa* evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression, Infect. Immun. 78 (2010) 2937–2945, http://dx.doi.org/10.1128/IAI.00144-10.
[3] J.W. Chung, Z.H. Piao, S.R. Yoon, M.S. Kim, M. Jeong, S.H. Lee, et al., Pseudomonas aeruginosa eliminates natural killer cells via phagocytosis-induced apoptosis, PLoS Pathog. 5 (8) (2009), e1000561http://dx.doi.org/10.1371/journal.ppat.1000561.
[4] L.D. Thomas, A.W. Cripps, J.M. Kyd, Immune response mechanisms against *Pseudomonas aeruginosa* associated with mucosal immunization with protein antigens in a rat model of acute lung infection, Vaccine 27 (2009) 3324–3330, http://dx.doi.org/10.1016/j.vaccine.2009.01.085.
[5] G.B. Pier, *Pseudomonas aeruginosa* lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity, Inter. J. Med. Microbiol. 297 (2007) 277–295.
[6] T.L. Van Belle, C. Gysemans, C. Mathieu, Vitamin D in autoimmune, infectious and allergic diseases: a vital player? Best Pract. Res. Clin. Endocrinol. Metab. 25 (2011) 617–632, http://dx.doi.org/10.1016/j.beem.2011.04.009.
[7] C. Aranow, Vitamin D and the immune system, J. Investig. Med. 59 (2011) 881–886, http://dx.doi.org/10.231/JIM.0b013e31821b8755.
[8] F. Baeke, C. Gysemans, H. Korf, C. Mathieu, Vitamin D insufficiency: implications for the immune system, Pediatr. Nephrol. 25 (2010) 1597–1606, http://dx.doi.org/10.1007/s00467-010-1452-y.
[9] M. Djukic, M.L. Onken, S. Schütze, S. Redlich, A. Götz, U.K. Hanisch, et al., Vitamin D deficiency reduces the immune response, phagocytosis rate, and intracellular killing rate of microglial cells, Infect. Immun. 82 (6) (2014) 2585–2594, http://dx.doi.org/10.1128/IAI.01814-14.
[10] S.V. Ramagopalan, A. Heger, A.J. Berlanga, L.M.R. MaugeriNJ, A. Burrell, et al., A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution, Genome Res. 20 (2010) 1352–1360, http://dx.doi.org/10.1101/gr.107920.110.
[11] G. Chandra, P. Selvaraj, M.S. Jawahar, V.V. Banurekha, P.R. Narayanan, Effect of vitamin D3 on phagocytic potential of macrophages with live *Mycobacterium tuberculosis* and lymphoproliferative response in pulmonary tuberculosis, J. Clin. Immunol. 24 (2004) 249–257.
[12] P. Selvaraj, P. Anand, M. Harishankar, K. Alagarasu, Plasma 1,25 dihydroxy vitamin D3 level and expression of vitamin D receptor and cathelicidin in pulmonary tuberculosis, J. Clin. Immunol. 29 (2009) 470–478, http://dx.doi.org/10.1007/s10875-009-9277-9.
[13] L. Larcombe, P. Orr, E. Turner-Brannen, C.R. Slivinski, P.W. Nickerson, N. Mookherjee, Effect of vitamin D supplementation on *Mycobacterium tuberculosis*-induced innate immune responses in a Canadian Dené First Nations cohort, PLoS ONE 7 (2012), e40692http://dx.doi.org/10.1371/journal.pone.0040692.
[14] P. McNally, C. Coughlan, G. Bergsson, M. Doyle, C. Taggart, L. Adorini, et al., Vitamin D receptor agonists inhibit pro-inflammatory cytokine production from the respiratory epithelium in cystic fibrosis, J. Cyst. Fibros. 10 (2011) 428–434.
[15] S.K. Jain, D. Micinski, Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes, Biochem. Biophys. Res. Commun. 437 (2013) 7–11, http://dx.doi.org/10.1016/j.bbrc.2013.06.004.
[16] T.T. Wang, F.P. Nestel, V. Bourdeau, Y. Nagai, Q. Wang, J. Liao, et al., Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression, J. Immunol. 173 (5) (2004) 2909–2912.
[17] N. Wang, H. Liang, K. Zen, Molecular mechanisms that influence the macrophage m1-m2 polarization balance, Front. Immunol. 5 (2014) 614, http://dx.doi.org/10.3389/fimmu.2014.00614. eCollection 2014.
[18] Z. Huang, Q. Luo, Y. Guo, J. Chen, G. Xiong, Y. Peng, et al., *Mycobacterium tuberculosis*-induced polarization of human macrophage orchestrates the formation and development of tuberculous granulomas *in vitro*, PLoS One 10 (6) (2015), e0129744http://dx.doi.org/10.1371/journal.pone.0129744.
[19] J. Khan, P.K. Sharma, A. Mukhopadhaya, Vibrio cholera porin OmpU mediates M1-polarization of macrophages/monocytes via TLR1/TLR2 activation, Immunobiology (2015) pii S0171-2985(15)30006-1. doi: 10.1016/j.imbio.2015.06.009.
[20] M. Benoit, B. Desnues, J.L. Mege, Macrophage polarization in bacterial infections, J. Immunol. 181 (2008) 3733–3739.
[21] S.E. Jang, M.J. Han, S.Y. Kim, D.H. Kim, *Lactobacillus plantarum* CLP-0611 ameliorates colitis in mice by polarizing M1 to M2-like macrophages, Int. Immunopharmacol. 21 (2014) 186–192, http://dx.doi.org/10.1016/j.intimp.2014.04.021.
[22] A. Richardson, S. Fedoroff, Quantification of cells in culture, in: A. Richardson, S. Fedoroff (Eds.), Protocols for Neural Cell Culture, Springer Science & Business Media, New York 1997, pp. 219–233.

[23] I. Guevara, J. Iwanejko, A. Dembińska-Kieć, J. Pankiewicz, A. Wanat, P. Anna, et al., Determination of nitrite/nitrate in human biological material by the simple Griess reaction, Clin. Chim. Acta 274 (1998) 177–188.
[24] A. Rouzaut, M.L. Subirà, C. Miguel, E. Domingo-de-Miguel, A. Gonzàlez, E. Santiago, et al., Co-expression of inductible nitric oxide synthase and arginases in different humain monocyte subsets. Apoptosis regulated by endogenous NO, Biochim. Biophys. Acta 1451 (1999) 319–333.
[25] D. Porto, N. Cifani, S. Guarnieri, E.G. Di Domenico, M.A. Mariggiò, F. Spadro, et al., Dysfunctional CFTR alters the bactericidal activity of human macrophages against *Pseudomonas aeruginosa*, PLoS One 6 (5) (2012), e19970http://dx.doi.org/10.1371/journal.pone.0019970.
[26] Z.H. Piao, M.S. Kim, M. Jeong, S. Yun, S.H. Lee, H.N. Sun, et al., VDUP1 exacerbates bacteremic shock in mice infected with *Pseudomonas aeruginosa*, Cell. Immunol. 280 (2012) 1–9, http://dx.doi.org/10.1016/j.
[27] Nibbering PH, Broug-Holub E, Bezemer AC, Jansen R, van de Winkel JG, Geertsma MF. Phagocytosis and intracellular killing of serum-opsonized *Staphylococcus aureus* by mouse fibroblasts expressing human Fcgamma receptor type IIa (CD32). Front Biosci1996; 1:a25-33.
[28] M. Aribi, W. Meziane, S. Habi, Y. Boulatika, H. Marchandin, J.L. Aymeric, Macrophage bactericidal activities against *Staphylococcus aureus* are enhanced *in vivo* by selenium supplementation in a dose-dependent manner, PLoS One 10 (9) (2015), e0135515http://dx.doi.org/10.1371/journal.pone.0135515.
[29] J. Mehrzad, L. Duchateau, C. Burvenich, Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation, Vet. Microbiol. 134 (1–2) (2009) 106–112, http://dx.doi.org/10.1016/j.vetmic.2008.09.001.
[30] M.L. Manni, L.P. Tomai, C.A. Norris, L.M. Thomas, E.E. Kelley, R.D. Salter, J.D. Crapo, et al., Extracellular superoxide dismutase in macrophages augments bacterial killing by promoting phagocytosis, Am. J. Pathol. 178 (6) (2011) 2752–2759, http://dx.doi.org/10.1016/j.
[31] C.D. Nelson, T.A. Reinhardt, D.C. Beitz, J.D. Lippolis, *In vivo* activation of the intracrine vitamin D pathway in innate immune cells and mammary tissue during a bacterial infection, PLoS One 5 (2010), e15469http://dx.doi.org/10.1371/journal.pone.0015469.
[32] R.D. Junkins, A. Shen, K. Rosen, C. McCormick, T.J. Lin, Autophagy enhances bacterial clearance during *P. aeruginosa* lung infection, PLoS One 8 (2013), e72263http://dx.doi.org/10.1371/journal.pone.0072263.
[33] M. Hewison, Vitamin D and innate and adaptive immunity, Vitam. Horm. 86 (2011) 23–62, http://dx.doi.org/10.1016/B978-0-12-386960-9.00002-2.
[34] D.A. Youssef, C.W. Miller, A.M. El-Abbassi, D.C. Cutchins, C. Cutchins, W.B. Grant, et al., Antimicrobial implications of vitamin D, Dermato. Endocrinol. 4 (2011) 220–229, http://dx.doi.org/10.4161/derm.3.4.15027.
[35] P. Pelegrin, A. Surprenant, Dynamics of macrophage polarization reveal new mechanism to inhibit IL-1beta release through pyrophosphates, EMBO J. 28 (2009) 2114–2127, http://dx.doi.org/10.1038/emboj.2009.163.
[36] A. Covarrubias, V. Byles, T. Horng, ROS sets the stage for macrophage differentiation, Cell Res. 23 (2013) 984–985, http://dx.doi.org/10.1038/cr.2013.88.
[37] A.P. Ralph, G. Waramori, G.J. Pontororing, E. Kenangalem, A. Wiguna, E. Tjitra, et al., L-arginine and vitamin D adjunctive therapies in pulmonary tuberculosis: a randomised, double-blind, placebo-controlled trial, PLoS One 8 (2013), e70032http://dx.doi.org/10.1371/journal.pone.0070032.
[38] A. Brosbøl-Ravnborg, B. Bundgaard, P. Höllsberg, Synergy between vitamin D(3) and Toll-like receptor agonists regulates human dendritic cell response during maturation, Clin. Dev. Immunol. 2013 (2013) 807971, http://dx.doi.org/10.1155/2013/807971.
[39] D. Tiosano, G. Wildbaum, V. Gepstein, O. Verbitsky, Y. Weisman, et al., The role of vitamin D receptor in innate and adaptive immunity: a study in hereditary vitamin D-resistant rickets patients, J. Clin. Endocrinol. Metab. 98 (2013) 1685–1693, http://dx.doi.org/10.1210/jc.2012-3858.
[40] M. Kongsbak, M.R. von Essen, L. Boding, T.B. Levring, P. Schjerling, Vitamin D up-regulates the vitamin D receptor by protecting it from proteasomal degradation in human CD4+ T cells, PLoS One 9 (2014), e96695http://dx.doi.org/10.1371/journal.pone.0096695.
[41] L. Aussel, W. Zhao, M. Hébrard, A.A. Guilhon, J.P. Viala, S. Henri, et al., Salmonella detoxifying enzymes are sufficient to cope with the host oxidative burst, Mol. Microbiol. 80 (2011) 628–640, http://dx.doi.org/10.1111/j.1365-2958.2011.07611.x.
[42] R.A. Miller, B.E. Britigan, Role of oxidants in microbial pathophysiology, Clin. Microbiol. Rev. 1 (1997) 1–18.
[43] Y. Yoshioka, T. Kitao, T. Kishino, A. Yamamuro, S. Maeda, Nitric oxide protects macrophages from hydrogen peroxide-induced apoptosis by inducing the formation of catalase, J. Immunol. 176 (2006) 4675–4681.
[44] C.A. Dinarello, Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme, Ann. N. Y. Acad. Sci. 856 (1998) 1–11.
[45] M.J. Nicklin, J.L. Barton, M. Nguyen, M.G. FitzGerald, G.W. Duff, K. Kornman, A sequence-based map of the nine genes of the human interleukin-1 cluster, Genomics 79 (2002) 718–725.
[46] K.W. Moore, M.R. de Waal, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.
[47] N. Cole, M. Krockenberger, F. Stapleton, S. Khan, E. Hume, A.J. Husband, M. Willcox, Experimental *Pseudomonas aeruginosa* keratitis in interleukin-10 gene knockout mice, Infect. Immun. 71 (2003) 1328–1336.
[48] X.L. Rudner, K.A. Kernacki, P.B. Ronald, L.D. Hazlett, Prolonged elevation of IL-1 in *Pseudomonas aeruginosa* ocular infection regulates macrophage-inflammatory protein-2 production, polymorphonuclear neutrophil persistence, and corneal perforation, J. Immunol. 164 (2000) 6576–6582.
[49] M. Verway, M. Bouttier, T.T. Wang, M. Carrier, M. Calderon, B.S. An, et al., Vitamin D induces interleukin-1β expression: paracrine macrophage epithelial signaling controls *M. tuberculosis* infection, PLoS Pathog. 9 (2013), e1003407http://dx.doi.org/10.1371/journal.ppat.1003407.
[50] D. Eklund, L.H. Persson, M. Larsson, A. Welin, J. Idh, J. Paues, et al., Vitamin D enhances IL-1β secretion and restricts growth of *Mycobacterium tuberculosis* in macrophages from TB patients, Int. J. Mycobacteriol. 2 (2013) 18–25.
[51] M.L. Xue, H. Zhu, A. Thakur, M. Willcox, 1α,25-Dihydroxyvitamin D3 inhibits pro-inflammatory cytokine and chemokine expression in human corneal epithelial cells colonized with *Pseudomonas aeruginosa*, Immunol. Cell Biol. 80 (2002) 340–345.
[52] R.S. Koti, J. Tsui, E. Lobos, W. Yang, A.M. Seifalian, B.R. Davidson, Nitric oxide synthase distribution and expression with ischemic preconditioning of the rat liver, FASEB J. 19 (2005) 1155–1157.
[53] L.E. Sousse, C.C. Jonkam, D.L. Traber, H.K. Hawkins, T.L.D. RehbergSW, et al., *Pseudomonas aeruginosa*is associated with increased lung cytokines and asymmetric dimethylarginine compared to methicillin-resistant *Staphylococcus aureus*, Shock 36 (2011) 466–470, http://dx.doi.org/10.1097/SHK.0b013e3182336b45.
[54] A. Mehl, P. Ghorbani, D. Douda, H. Huang, N. Palaniyar, F. Ratjen, et al., Effect of arginase inhibition on pulmonary L-arginine metabolism in murine *Pseudomonas pneumonia*, PLoS ONE 9 (2014), e90232http://dx.doi.org/10.1371/journal.pone.0090232.
[55] Y.Z. KulkarniNN, C. Huehnken, B. Agerberth, G.H. Gudmundsson, Phenylbutyrate induces cathelicidin expression *via* the vitamin D receptor: linkage to inflammatory and growth factor cytokines pathways, Mol. Immunol. 63 (2015) 530–539.
[56] T.H. Kim, B. Lee, E. Kwon, S.J. Choi, Y.H. Lee, G.G. Song, et al., Regulation of TREM-1 expression by 1,25-dihydroxyvitamin D3 in human monocytes/macrophages, Immunol. Lett. 154 (2013) 80–85, http://dx.doi.org/10.1016/j.imlet.2013.08.012.
[57] L. McMahon, K. Schwartz, O. Yilmaz, E. Brown, L.K. Ryan, G. Diamond, Vitamin D-mediated induction of innate immunity in gingival epithelial cells, Infect. Immun. 79 (2011) 2250–2256, http://dx.doi.org/10.1128/IAI.00099-11.