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Vitamin D enhances IL-1β secretion and restricts growth of *Mycobacterium tuberculosis* in macrophages from TB patients

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ABSTRACT

The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (MTB), the bacterium responsible for tuberculosis (TB), has rekindled the interest in the role of nutritional supplementation of micronutrients, such as vitamin D, as adjuvant treatment. Here, the growth of virulent MTB in macrophages obtained from the peripheral blood of patients with and without TB was studied. The H37Rv strain genetically modified to express *Vibrio harveyi* luciferase was used to determine the growth of MTB by luminometry in the human monocyte-derived macrophages (hMDMs) from study subjects. Determination of cytokine levels in culture supernatants was performed using a flow cytometry-based bead array technique. No differences in intracellular growth of MTB were observed between the different study groups. However, stimulation with 100 nM 1,25-dihydroxyvitamin D significantly enhanced the capacity of hMDMs isolated from TB patients to control the infection. This effect was not observed in hMDMs from the other groups. The interleukin (IL)-1β and IL-10 release by hMDMs was clearly increased upon stimulation with 1,25-dihydroxyvitamin D. Furthermore, the 1,25-dihydroxyvitamin D stimulation also led to elevated levels of TNF-α (tumor necrosis factor-alpha) and IL-12p40. It was concluded that vitamin D triggers an inflammatory response in human macrophages with enhanced secretion of cytokines, as well as enhancing the capacity of hMDMs from patients with active TB to restrict mycobacterial growth.

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Introduction

Tuberculosis (TB), once considered a disease of the past, owing to the discovery of effective antibiotics, is today the number one cause of death from bacterial infections worldwide. In the pre-antibiotic era, the infection was primarily treated in sanatoria, where a nutritious diet and outdoor rest were important components of the therapy. The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (MTB), the bacterium responsible for the disease, has rekindled the interest in nutritional aspects of TB treatment in recent years. Several groups, including this one, have performed studies to investigate the role of vitamins (vitamin A and D), minerals (zinc) and amino acids like L-arginine as adjuvants to the standard chemotherapy of TB [1–4]. In 2006, Liu et al. showed that vitamin D enhances the ability of human monocytes to control intracellular growth of MTB [5], and that it was a process depending on an increased expression of the vitamin D receptor (VDR) and enhanced production of the anti-microbial peptide cathelicidin, upon engagement of Toll-like receptors (TLRs) 2 and 1. Cathelicidin was later shown to be crucial for the control of mycobacterial growth in THP-1 cells [6]. These mechanistic studies on the relationship between vitamin D and the ability of immune cells to control MTB encouraged further clinical and experimental studies on the role of vitamin D-deficiency as a risk factor for TB. Clinical studies have so far been inconclusive and have shown both an effect of vitamin D in subgroup analysis of patients with specific VDR polymorphisms [1], and a lack of any clinical improvement or effects on mortality from vitamin D supplementation [4]. Most experimental studies have been performed with monocytes or macrophage-like cell lines, and so far none has studied the ability of vitamin D to enhance the antimycobacterial capacity of monocyte-derived macrophages isolated from patients with active TB. Here, the growth of virulent MTB was studied in hMDMs from patients carefully categorized with having active, previous or latent TB and how this was affected by stimulation with 1,25-dihydroxyvitamin D3 (the active form of vitamin D, hereafter referred to as 1,25D3).

Study population and methods

Study population

Patients who were investigated because of persistent cough and/or exposure to TB were included in the study after informed and written consent. The study protocol was approved by the Ethical Committee of Linköping University, Sweden. Patients were classified by a senior pulmonary physician and a senior thoracic radiologist as non-TB, previous TB, current TB or latent TB (LTB) according to published guidelines [7]. The classification was performed without knowledge of the experimental results. Non-TB were patients with negative PPD/IGRA-test, normal chest X-ray and/or high resolution computer tomography (HRCT), and bronchoscopy (performed because of persistent cough) with negative mycobacterial cultures (C1–C3, C5–C9, C12–C14; $n = 11$). Previous TB were patients with a medical history of previous TB and/or radiological findings on chest X-ray and/or HRCT consistent with previous TB, supported by a positive TB-skin test (TST) and/or IGRA-test, but negative cytology and negative mycobacterial cultures/PCR by bronchoscopy (PTB1–PTB7; $n = 7$). Current TB were patients treated for TB because of a positive mycobacterial culture and/or clinical and radiological symptoms suggestive of active TB (TB1–TB8, TB10–12, $n = 11$). TB-exposed subjects exhibiting positive TST and/or positive IGRA, but no clinical and radiological signs of TB were categorized as latent TB (LTB1–LTB4, LTB6–LTB7 $n = 6$). At least two of three criteria were required for a LTB diagnosis. A positive TST was defined as $\geqslant 10$ mm, since most of the subjects included displayed a scar after previous vaccination. None of the patients investigated was treated with TB drugs when blood samples were collected. All patients were HIV-negative. The patient characteristics are presented in Table 1.

Preparation of macrophages

Human monocyte-derived macrophages (hMDMs) were prepared from monocytes isolated from heparinized blood as previously described [8] using routine methods, with the modification that differentiation was performed during 5–7 days in DMEM with 10% human serum (pooled from five healthy donors); hMDM were seeded in 96-well plates (Sarstedt) one day prior to infection in DMEM free of antibiotics (100,000 cells/well).

Bacteria

The virulent MTB strain H37Rv (American Type Culture Collection) harboring a pSMT1-plasmid encoding *Vibrio harveyi* luciferase [9,10] were grown in Middlebrook 7H9 broth supplemented with TWEEN 80 and oleic acid-albumin-dextrose-catalase (OADC) (BD) for 2 to 3 weeks at 37 °C with 100 μg/ml hygromycin for selection before being re-inoculated in fresh broth and incubated for 7 days to reach early log phase. Prior to infection, bacteria were prepared as previously described [11]. Briefly, bacterial suspension was washed and re-suspended in DMEM and a single-bacillus suspension was obtained via a sterile syringe equipped with a 27-gauge needle. The concentration was determined by using optical density at 600 nm (OD_{600}) as a function of colony-forming units (CFU)/ml.

Infection procedure

A pulse-chase approach was used to infect hMDMs, where bacteria were added to the hMDMs in a serum-free medium at a multiplicity of infection (MOI) of 10 and incubated at 37 °C for 1 h. The infection was followed by a change of medium to serum-containing antibiotic-free DMEM with 100 nM 1,25D3 (Sigma–Aldrich). Control wells were given medium without added 1,25D3.

Quantiferon assay

In the QuantiFERON®-TB Gold in-Tube assay (Cellestis, Australia), blood samples were collected in tubes provided by the manufacturer. Samples were shaken and incubated at

Table 1 – Characteristics of the population studied. Data are presented as means ± SD. Significant difference from "non-TB" subjects is indicated.

	Non-TB subjects	Previous TB infection	Active TB infection	Latent TB infection
Subjects	11	7	11	6
Mean age, year	51 ± 16	52 ± 13	42 ± 19	51 ± 12
Females/males	7/4	4/3	6/5	3/3
Ethnicity	Caucasians 10 Asians 1	Caucasians 4 Africans 3	Caucasians 7 Africans 3 Asians 1	Caucasians 4 Africans 2
Current smokers (%)	3 (27%)	0 (0%)	4 (36%)	2 (33%)
Present BCG scar (%)	10 (91%)	4 (57%) Unknown in two cases	4 (36%) Unknown in two cases	4 (66%)
TB skin test, mm	3 ± 5 ($n = 8$)	14 ± 5** ($n = 6$)	18 ± 3*** ($n = 8$)	17 ± 6*** ($n = 6$)
BMI (kg/m^2)	24 ± 4 ($n = 11$)	28 ± 3 ($n = 7$)	21 ± 3 ($n = 11$)	24 ± 5 ($n = 5$)
Serum-albumin (g/l)	42 ± 4 ($n = 11$)	40 ± 4 ($n = 7$)	38 ± 5 ($n = 11$)	41 ± 4 ($n = 5$)
Radiologic findings	Normal 10 Small[a] benign tumor 1	Possibly inactive 6 Possibly active 1	Possibly inactive 2 Possibly active 6 Active 3	Normal 6
Serum 25-OH Vitamin D (nmol/l)	34.5 ± 25.9	27.2 ± 21.8	27.4 ± 26.9	45.8 ± 52.7
Serum quantiferon positive (%)	1 (11%)[b]	6 (86%)	11 (100%)	5 (83%)
Subjects w/cough	8	5	8	0
Haemoptysis	0	2	4	0
Night sweats	1	3	5	1
Heart rate > 100	0	0	2	0
Body temp > 38 °C	0	0	4	0
Anemia, Hb < 120		3	0	0

** $p < 0.01$ (Students t-test, unpaired analysis).
*** $p < 0.001$ (Students t-test, unpaired analysis).
[a] Later diagnosed as a benign tumor.
[b] IGRA level in the grey zone (0.93 IU/ml), close to the cut off value for a positive test (0.35 IU/ml).

37 °C overnight and subsequently analyzed by ELISA for IFN-γ (IU/ml) according to the instructions from the manufacturer.

Measurement of 25-hydroxyvitamin D in serum

The major circulating metabolite of vitamin D, 25-hydroxyvitamin D, was measured by ELISA (Immmundiagnostik, Germany) according to the manufactureŕs instructions.

Measurement of bacterial growth

The number of viable bacteria was acquired through the measurement of flash-luminescence with a GloMax® Multi-Detection System (Promega) as previously described [11]. It was previously shown that this method, based on flash-luminescence emitted from the luciferase-expressing bacteria when given the luciferase substrate *n*-decanal, correlates well to CFUs and displays less variation than classic CFU assays [11]. Measurements were performed one hour after the pulse-chase and after two days of incubation. Results are expressed as arbitrary luminescence units (ALU) or as fold-change of MTB growth (Day 2/Day 0).

Measurement of cytokine release

To quantify the concentrations of cytokines produced by MTB-infected hMDMs, BD™ Cytometric Bead Array Flex Set (BD) was used according to the manufacturer's instructions. Briefly, culture supernatants from infected hMDMs were collected at 24 h post-infection, aliquoted and kept at −70 °C until further use. For analysis, samples were centrifuged at 10,000*g* for 10 min prior to dilution and mixing with cytokine-specific beads. The supernatant-bead suspensions were incubated for 1 h at RT, followed by 2 h of incubation in the presence of cytokine-specific PE-conjugated detection antibodies. To sterilize samples, 4% paraformaldehyde (PFA) was added for 1 h before the final wash step. The quantification was performed by flow cytometry (Gallios Flow Cytometer, Beckman Coulter) where each bead population was separated by a unique ratio of APC and APC-Cy7, and the fluorescence intensity of PE corresponded to the concentration of the specific cytokine, derived from a standard curve of known cytokine concentrations.

Statistics

To evaluate the effects of 1,25D3 stimulation within each group and comparison of the total non-stimulated with stimulated samples, a paired Student's t-test was used. The means between different study groups were compared using ANOVA with Bonferroni's post hoc test. Differences between groups are shown as *($p < 0.05$) or **($p < 0.01$) or ***($p < 0.001$).

Results

Clinical observations of patients

Patients were classified by a senior pulmonologist into non-TB, previous TB, current TB and latent TB, as described in Table 1. The mean age of the patients ranged from 42 to

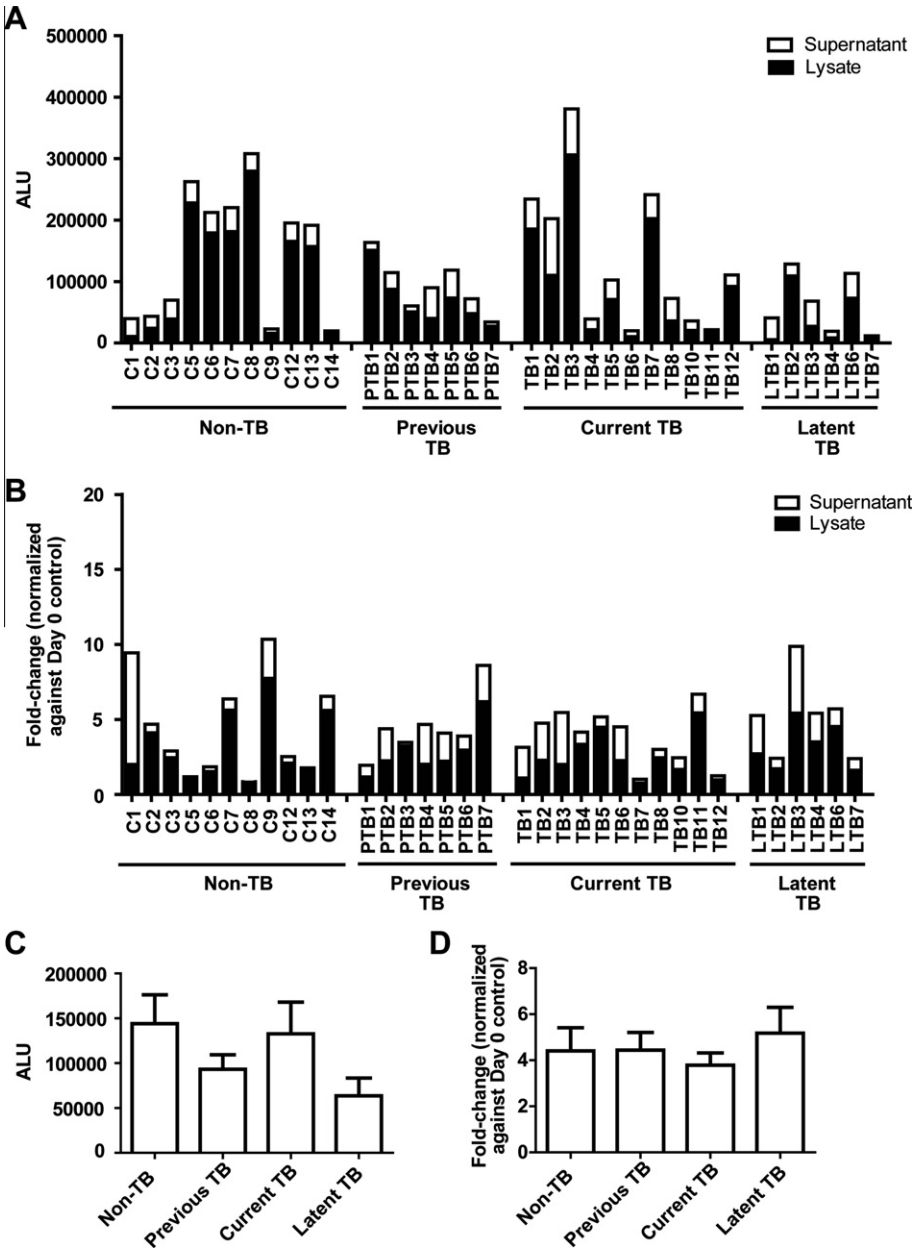

Fig. 1 – Phagocytosis and growth of H37Rv in hMDMs. Cells from the four studied groups of patients were infected with H37Rv at MOI 10 as described, before luminescence was determined 1 h after pulse-chase (D0 value) as a measure of phagocytosis. After an additional 48 h of incubation, luminescence was measured again and growth was evaluated (D2 value). (A) Luminescence (expressed as arbitrary luminescence units, ALU) emitted from lysate- and supernatant-associated bacteria after 1 h after pulse-chase. (B) Relative growth of H37Rv (D2/D0) in the cells of individual patients, with indicated proportions of the relative growth in the supernatant (open bars) and lysate (black bars). (C) The mean ALU (intracellular + extracellular) value of each of the groups 1 h after pulse-chase (phagocytosis). (D) The mean relative growth (intracellular + extracellular) at day 2 post-infection from each group is shown. Non-TB (n = 11), previous TB (n = 7), current TB (n = 11) and latent TB (n = 6). Bar graphs show the mean and SEM. The means were compared using ANOVA with Bonferroni's post hoc test.

52 years, and there was an even distribution of sex and ethnicity (Caucasians constituting the majority). There were no significant differences in mean age or body mass index (BMI) between the groups. The mean levels of serum 25-OH vitamin D ranged from 27.4 nmol/l in the group of patients with previous TB to 48.1 nmol/l in patients with latent TB with no significant differences detected (Table 1).

Uptake and growth of MTB

For *ex vivo* analysis of the capacity of the patient and control hMDMs to control MTB, cells were infected with H37Rv. It was previously shown that the bacterial load of hMDMs is a critical determinant of the ability of macrophages to control the infection [12,13]. Therefore, the phagocytic capacity of the

hMDMs was investigated first. The cells were challenged with luciferase-expressing H37Rv in a pulse-chase approach, and the number of cell-associated and free bacteria was determined using luminometry. As shown in Fig. 1A and C, the phagocytic capacity of the cells (expressed as arbitrary luminescence units [ALU] at Day 0 [D0]) varied between individuals, but showed no significant differences between study groups, with the non-TB and current TB group being very similar. Next, the relative intracellular growth of MTB in hMDMs (Fig. 1B and D) was determined after two days of infection, but no differences could be observed between hMDMs obtained from non-TB patients compared to cells from patients with previous, latent or active TB.

Effect of 1,25D3 on bacterial growth

To assess the effects of 1,25D3 on the capacity of the macrophages to control bacterial growth, the cells were exposed to 100 nM 1,25D3 immediately after the pulse-chase infection, and the bacterial load was analyzed at day 2. Fig. 2 shows that stimulation of the cells with 1,25D3 significantly reduced bacterial growth in hMDMs from patients with current TB ($p = 0.044$), whereas there was no significant reduction in the other groups (Fig. 2) or when cells from all groups were evaluated together as non-stimulated or 1,25D3-stimulated (not shown). Initial experiments included stimulation of hMDMs with 100 nM of the inactive metabolite 25-hydroxyvitamin D, but this did not result in any significant effect on bacterial growth in cells obtained from patients with current TB ($p = 0.36$, data not shown), and was therefore excluded for further experiments.

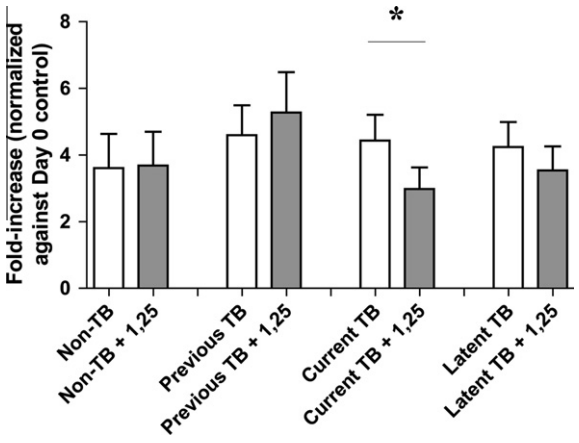

Fig. 2 – Effects of 1,25D3 on bacterial growth. hMDMs were infected with H37Rv for two days at MOI 10. Measurements were performed at 1 h after pulse-chase (D0 value) and at day 2 post-infection (D2 value). The figure shows the relative growth (intracellular + extracellular) of H37Rv (D2/D0) in the absence or presence of 100 nM 1,25D3 in the four study groups. All patients where stimulation experiments were performed are shown. Non-TB ($n = 10$), previous TB ($n = 6$), current TB ($n = 6$) and latent TB ($n = 5$). Bar graphs show the mean and SEM. Differences in growth with or without 1,25D3 stimulation within each group was tested by a paired Student's *t*-test.

Cytokine analysis

To evaluate how macrophages from patients with and without TB responded to MTB in the presence or absence of 1,25D3, both pro-inflammatory (interleukin [IL]-1β, tumor necrosis factor-α [TNF-α] and IL-12p40) and anti-inflammatory cytokines (IL-10) in cell supernatants one day after infection were analyzed. Stimulation with 1,25D3 significantly increased the levels of IL-1β in hMDMs from non-TB subjects (Fig. 3A, $p = 0.039$) and of IL-10 in subjects with current TB (Fig. 3C, $p = 0.028$). TNF-α and IL-12p40 were not significantly altered in the different study groups. There was a tendency towards an increase in IL-1β-production upon 1,25D3 stimulation in hMDMs from patients in all the TB-associated groups, and when the cytokine data from all study groups were combined, there was a significant increase of the 1,25D3-stimulated levels of IL-1β and IL-10 ($p = 0.0017$ and $p = 0.0008$, respectively, Fig. 3E). The combined analysis further revealed that the concentration of IL-12p40 ($p = 0.046$) and TNF-α ($p = 0.082$) in the supernatant slightly increased upon 1,25D3 stimulation (Fig. 3E).

Discussion

In the present study, the capacity of hMDMs from patients with or without TB to control the growth of MTB and whether or not such growth inhibition could be enhanced in the presence of 1,25D3 was investigated. Although the first observations of vitamin D stimulation of monocyte and macrophage anti-mycobacterial activity is more than 25 years old [14,15], this is the first study where this activity of hMDMs from patients with current TB was investigated.

Due to its relatively short half-life, one dose of 100 nM 1,25D3 was used throughout the study; 1,25D3 in this range has previously been shown to be required for effective stimulation of primary monocytes [16]. It was observed that hMDMs from patients with active TB, when stimulated with 1,25D3, were more effective in controlling MTB infection. This effect was not seen in hMDMs from the other groups, despite the fact that all groups showed a tendency to respond to 1,25D3 by enhancing IL-1β production, with a significant increase in the non-TB group. Enhanced production of IL-1β by 1,25D3 has been demonstrated before, with a necessary lipopolysaccharide priming step [17]. The pro-inflammatory properties of IL-1β are mediated through the IL-1 receptor 1 (IL1R1) and is important in the protection against MTB [18,19]. IL1R1 shares signaling partners, including Myd88, with TLRs, and *in vivo* studies have suggested that mice lacking Myd88 are highly susceptible to MTB not because of the inability to signal through TLRs, as earlier thought, but rather because of the inability to signal through IL-1R1 [20]. It is possible that the *in vivo* preconditioning of monocytes in hosts with replicating MTB, as is the case in the patients with active TB, primes the monocytes for a more efficient antimicrobial response. In fact, studies have shown that TLR1/2 engagement, in addition to the role of up-regulation of VDR expression, is also able to sensitize cells to IL-1β stimulation. This sensitization together with 1,25D3 stimulation leads to the expression of the antimicrobial peptide, beta-Defensin 4 [16]. It is tempting to

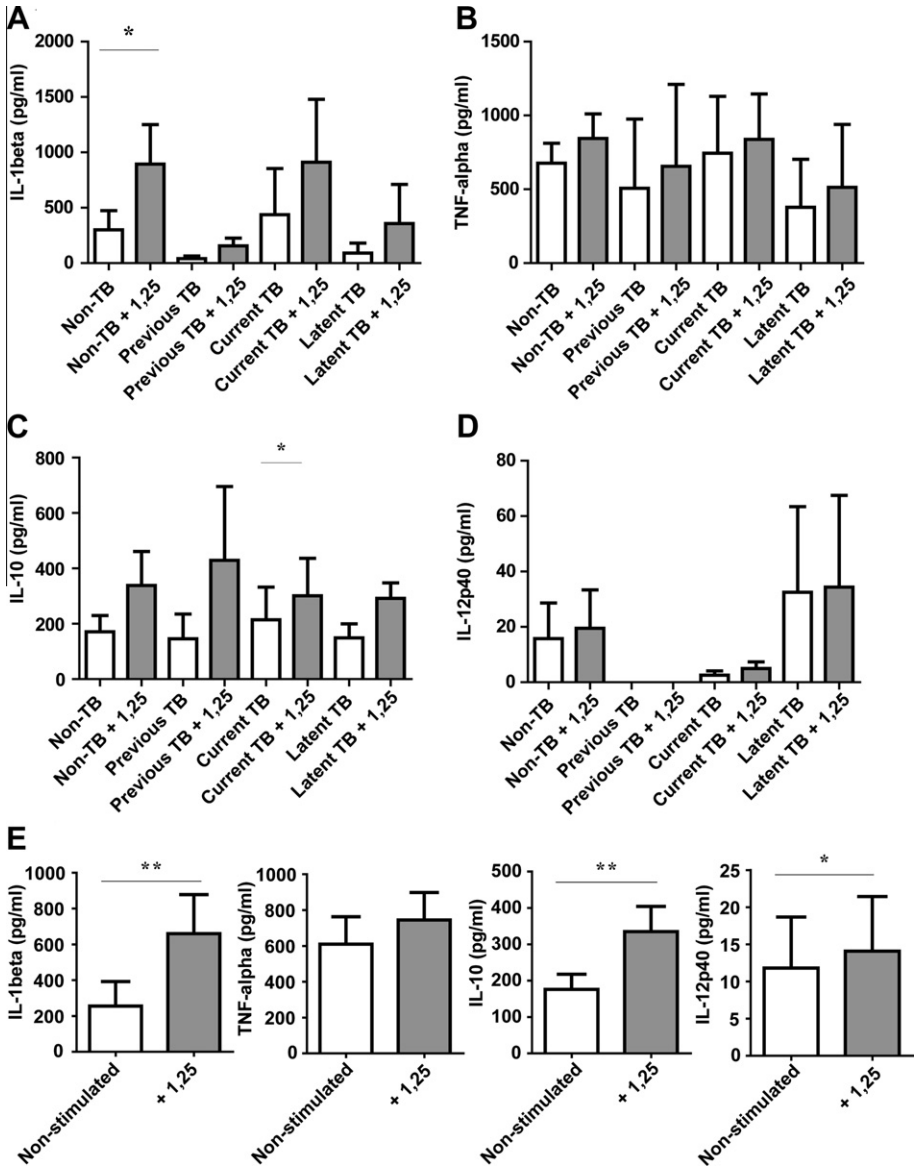

Fig. 3 – Cytokine release upon infection by H37Rv with and without stimulation by 1,25 D3. hMDMs were infected with H37Rv at MOI 10, before supernatants were harvested 24 h post-infection and concentrations of TNF-α, IL-β, IL-10 and IL-12p40 were determined by BD CBA Flex Set. The figure shows the mean concentrations of (A) IL-β, (B) TNF-α, (C) IL-10 and (D) IL-12p40 from each group, with and without 1,25D3. (E) The panel shows the total mean concentration of the respective cytokine between all non-stimulated and stimulated samples. Non-TB (*n* = 5), previous TB (*n* = 3), current TB (*n* = 6) and latent TB (*n* = 3). Bar graphs show the mean and SEM. The means between different groups were compared using ANOVA with Bonferroni's post hoc test. To evaluate the effects of 1,25D3 stimulation within each group and comparison of the total non-stimulated with stimulated samples, a paired Student's *t*-test was used.

speculate that the potential priming event in TB patients could render the macrophages more sensitive to inflammatory signals, such as 1,25D3-induced IL-1β, leading to increased expression of anti-microbial peptides and the decreased bacterial growth in the active TB group. It would be of interest to measure the expression of anti-microbial peptides in response to 1,25D3 stimulation in future studies and to investigate if this is truly mediated through IL-1β.

Furthermore, there was a clear induction of IL-10 in hMDMs from the non-TB and current TB group. IL-10, together with the IL-10 receptor, has been shown to be induced by 1,25D3 [21,22]. This 1,25D3-induced up-regulation of IL-10 has been proposed to represent a protective mechanism in TB, where IL-10 inhibits the increased expression of matrix metalloproteinases by MTB, thereby limiting the tissue damage and subsequent pulmonary cavitation [23]. However, the effects of IL-10 *in vitro* might be more detrimental to the cells since it can inhibit the phagosomal maturation process of macrophages infected with MTB [24], a process counteracted by pro-inflammatory signals or effector molecules. Blocking IL-10 signaling in the *in vitro* system would help to clarify whether or not IL-10 is indeed counteracting the effects of

IL-1β and if IL-10 is able to affect the expression of antimicrobial peptides in hMDMs.

Although this study displays a small sample size and the number of cells obtained from the patients is limited, the included study subjects are well characterized clinically, and these study groups were categorized in a blinded fashion, with no knowledge of experimental data. No differences between the different study groups were seen in regard to sex, ethnicity, age, BMI, or other general confounder. Of interest, no significant differences were seen in the serum levels of 25-hydroxyvitamin D in the limited study population and as reported elsewhere [25]: levels varied considerably between subjects with no obvious pattern between study groups. So far, there is no proven causality between low serum vitamin D levels and susceptibility to TB, as both healthy individuals and TB patients may have low serum levels of vitamin D. However, this data suggests that unrelated to the levels of vitamin D in serum, there might be clear differences in how cells from both individual patients and different patient groups respond to the supplementation of vitamin D. It would be of interest to study if hMDMs would become less responsive to 1,25D3 stimulation after patients have undergone treatment, thereby lowering the inflammatory state of the host.

Today, there is no "gold standard" for diagnosis of latent TB; TST and chest X-ray remain the primary screening tools [7]. In the present study, all latent TB subjects had a history of a previous TB contact and a normal chest X-ray; 5 of 6 displayed a TST $\geqslant$ 10 mm, and the remaining case had a positive IGRA. One subject in the non-TB group was IGRA-positive. However, lacking all other indications of TB, it was decided to define this subject as a non-TB subject.

This study has several limitations, including the small study sample, and the impact of 1,25D3 stimulation on bacterial growth is, although clear, relatively small. Considering the slow growth of MTB, this relatively small effect might be influenced by the early time point at which bacterial growth was measured. It was chosen to measure bacterial growth at day 2 post-infection, since it was previously shown that beyond this time point, less than 10% of hMDMs are viable when infected at an MOI of 10 [13]. However, if possible, it would be of interest to extend the incubation time to evaluate if differences between 1,25D3 stimulated and non-stimulated cells become more pronounced with respect to both bacterial growth and cytokine responses. Another factor that might hide possible differences in the ability to control growth is the MOI. It was previously shown that the MOI greatly influences the outcome of MTB infection in hMDMs [12], and it would be of interest to lower the bacteria-to-cell ratio in order to investigate the effect of 1,25D3 stimulation under such conditions. For future studies, it would also be interesting to investigate whether or not 1,25D3 stimulation affects cell death of hMDMs and how this might potentially relate to bacterial growth.

In a parallel study, the anti-mycobacterial activity of alveolar macrophages obtained from broncho lavage fluid from some of the patients in the present study was investigated. As opposed to the finding that hMDMs from patients with current TB were better suited to controlling MTB infection in this study, alveolar macrophages from patients with a history of TB were in fact less capable of limiting bacterial growth at MOI 10 two days post-infection compared with the control group (unpublished observation). An important difference between hMDMs (as in the present study) and isolated alveolar macrophages is that the latter are differentiated *in vivo*, whereas hMDMs are differentiated *in vitro* with serum pooled from five healthy donors. The fact that the same serum was used for differentiation of cells from all study groups may contribute to masking possible differences.

Taken together, the current study suggests that circulating monocytes from TB patients might be primed and sensitized to the effects of 1,25D3 stimulation, such as increased IL-1β secretion, while the parallel study suggests that when these monocytes leave circulation and migrate into the lungs of (current or previous) TB patients, the microenvironment in the lungs of these particular patients renders macrophages less able to control growth of MTB, overriding the beneficial effects of the possible priming event.

Conclusion

It was concluded that vitamin D triggers an inflammatory response in human macrophages with enhanced secretion of IL-1β, and that in patients with ongoing TB, hMDMs can restrict MTB growth more effectively upon vitamin D stimulation. Further studies are needed to clarify the importance of the presence of infection in the host and its immune status for later antimicrobial response of *in vitro*-differentiated macrophages.

Conflict of interest

We have no conflict of interest to declare.

Acknowledgments

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