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Vitamin D and tuberculosis: where next?

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Abbreviations

TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; UVB, ultraviolet B; 25(OH)D, 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; VDR, vitamin D receptor; VDRE, vitamin D response element: VDBP, vitamin D binding protein; hCAP-18, human cathelicidin; CAMP, cathelicidin antimicrobial peptide; siRNA, small interfering RNA; TLR, toll-like receptor; iNOS, inducible nitric oxide synthase; MOI, multiplicity of infection; APC, antigen-presenting cell; DC, dendritic cell; Treg, regulatory T cell; NFAT, Nuclear Factor of

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Activated T cells; AP-1, Activator Protein 1, NF-κB, Nuclear Factor kappa B; PGE2, prostaglandin E2; peripheral blood mononuclear cells (PBMCs); TB-IRIS, tuberculosis-immune reconstitution inflammatory syndrome; MMP, matrix metalloproteinase; MDR-TB, multidrug-resistant TB; IU, international units; IOM, Institute of Medicine; ARI, acute respiratory infections; RSV, respiratory syncytial virus; NNT, numbers needed to treat; QFT, Quantiferon-TB Gold In-Tube; PET-CT, Positron Emissions Tomography-Computed Tomography.

Abstract

Tuberculosis (TB) has troubled mankind for millennia, but current treatment strategies are long and complicated and the disease remains a major global health problem. The risk of *Mycobacterium tuberculosis* (Mtb) infection or progression of active TB disease is elevated in individuals with vitamin D deficiency. High-dose vitamin D was used to treat TB in the pre-antibiotic era, and *in vitro* experimental data show that vitamin D supports innate immune responses that restrict growth of Mtb. Several randomised controlled trials have tested whether adjunctive vitamin D supplementation enhances the clinical and microbiological response to standard antimicrobial chemotherapy for pulmonary TB. The effects have been modest at best, and attention is turning to the question of whether vitamin D supplementation might have a role in preventing acquisition or reactivation of latent Mtb infection. In this article, we describe the effects of vitamin D on host immune responses to Mtb *in vitro* and *in vivo*, and review the results of clinical trials in the field. We also reflect on the findings of clinical trials of vitamin D supplementation for the prevention of acute respiratory tract infections, and discuss how these findings might influence the design of future trials to evaluate the role of vitamin D in the prevention and treatment of TB.

Keywords

antimicrobial peptides, clinical trials, immunotherapy, inflammation, tuberculosis, vitamins

Introduction

Mycobacterium tuberculosis (Mtb) is a highly successful intracellular bacterium that is an elusive challenge to the human immune system. Where Mtb manages to subvert host immunity, it is either maintained in a latent state or the infection progresses to cause active tuberculosis (TB) disease, which is most commonly manifested in the lung. A delicate immunological balance between the pathogen and the host will ultimately decide the outcome of infection and disease. Before the discovery of antibiotics with anti-mycobacterial activity, TB patients were isolated in sanatoria to receive open-air treatment with the rationale that bed-rest, fresh air, sunlight and good nutrition would strengthen the immune system and result in clinical improvement [1, 2]. Although no randomized clinical trials were ever performed to evaluate potential benefits of open-air treatment, clinical evidence of TB improvement was reported by many clinicians [1, 2]. An investigation from 1916 demonstrated that almost half of the TB patients who were admitted to a sanatoria died within 5 years, while the health condition in 56% of the patients was actually improved [1]. Likewise, an unblinded two-arm study including 1077 patients with a clinical diagnosis of pulmonary TB at the Royal Brompton Hospital in the UK in 1848, demonstrated that in half of the cohort who received a daily intake of cod liver oil, the disease was more often arrested and patient deterioration or death was reduced [3].

Nowadays, TB is treated using drugs with direct anti-mycobacterial activity ie. inhibition of cell wall synthesis, protein synthesis or RNA transcription etc. By contrast, host-directed therapies can influence clinical outcomes by regulating the immune response to Mtb. Vitamin D has received a lot of attention as a potential host-directed therapy by virtue of its immunomodulatory properties, including induction of innate antimicrobial effector responses as well as anti-inflammatory pathways that can dampen pathological inflammation in TB. While vitamin D has a well-established role to promote bone health and calcium homeostasis, a growing body of epidemiological evidence suggests that it also exerts 'non-classical effects' including modulation of immune responses [4]. Population studies indicate that low circulating levels of 25-hydroxyvitamin D (25(OH)D) in peripheral blood, indicating vitamin D deficiency or insufficiency, are common, and that low 25(OH)D associates independently with susceptibility to numerous clinical conditions [5-7]. Such associations have been reported for a wide range of infections [8], active TB included [9-13]. Importantly, intake of vitamin D occurs via exposure of the skin to UVB radiation in sunshine (which simulates cutaneous vitamin D synthesis) or oral ingestion of foods or supplements that contain vitamin D. In the tropics, sun exposure is usually enough to sustain adequate levels of 25(OH)D in the circulation year-round, but at higher latitudes UVB is of insufficient intensity to support cutaneous production of vitamin D during winter [14]. It is tempting to speculate that in the pre-antibiotic era, clinical recovery observed in many TB patients who received heliotherapy or cod liver oil supplements (rich in vitamin D), could partly be due to the positive effects of vitamin D on the host response to Mtb. This raises the question whether vitamin D supplementation might have a role in the treatment and/or prevention of TB in modern medicine.

Vitamin D-mediated effects on anti-mycobacterial immunity *in vitro*

As illustrated in Figure 1, vitamin D metabolism involves both endocrine and paracrine systems (Figure 1). *In vivo,* vitamin D produced in the skin or ingested in the diet undergoes hepatic conversion to its major circulating metabolite 25(OH)D, whose concentration in serum or plasma is the accepted measure of vitamin D status. 25(OH)D then undergoes a further hydroxylation step, catalyzed by the 25(OH)D converting enzyme 1α-hydroxylase (CYB27B1) to form the active metabolite and steroid hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) [15]. The 25(OH)D proform is primarily synthesized in the liver, although 25-hydroxylation has also been reported to occur in leucocytes [15]. Bioactive $1,25(OH)_2D$ is mainly produced in the kidney, but also in a number of other tissues including immune cells such as macrophages, lymphocytes and dendritic cells (DC) [15]. Vitamin D signaling is triggered by binding of active $1,25(OH)_2D$ to the intracellular vitamin D receptor (VDR), generating a transcription factor that can bind to specific sites in the DNA called vitamin D response elements (VDREs). The $1,25(OH)_2D$/VDR complex can modulate the expression of different target genes including the antimicrobial peptide, hCAP-18, which is the sole human cathelicidin [16-18]. Antimicrobial peptides are small cationic molecules with the ability to interact with and kill different pathogens primarily in skin and at mucosal surfaces [19]. LL-37 is the cleavage product produced from hCAP-18, which is an antimicrobial peptide that is largely produced by neutrophils and epithelial cells; it is also expressed in macrophages that are the main host cell in which Mtb resides. In human cells grown *in vitro,* 25(OH)D can support Mtb-induced expression of LL-37 in macrophages [9] and epithelial cells [20] and this increased expression of LL-37 restricts Mtb growth in alveolar macrophages in the lung. Interestingly, the VDREs present in the promoters of many human genes, i.e. cathelicidin (CAMP) are missing from rodent genes and therefore data on vitamin D signaling in mouse models may have limited relevance for humans [21].

In the mid-1980s, it was first discovered that 25(OH)D and 1,25$(OH)_2$D could reduce intracellular growth of Mtb in human monocytes [22] and macrophages [23] *in vitro*. Incubation of monocyte-derived macrophages with recombinant IFN-γ augmented the activity of 1α-hydroxylase in the cells, which promoted the conversion of the 25(OH)D proform to the active 1,25$(OH)_2$D metabolite [22]. Subsequently, it was reported that toll-like receptor (TLR) 2/1 activation on human Mtb-infected macrophages triggered an antimicrobial pathway that was dependent on both the endogenous production and action of 1,25$(OH)_2$D via the VDR [9]. Apparently, TLR 2/1 ligation up-regulated expression of the VDR as well as the 1α-hydroxylase genes, leading to induction of LL-37 and restricted growth of Mtb [9, 24]. In line with these *in vitro* findings, patients with chronic TB and severe vitamin D deficiency expressed low levels of LL-37 *in situ* in granulomatous tissue obtained from Mtb-infected lungs [25].

Inhibition of hCAP-18 expression in a vitamin D-treated human monocytic cell line using small interfering RNA (siRNA) demonstrated that growth of the avirulent Mtb strain H37Ra, was completely restored compared with a non-specific siRNA control, suggesting that LL-37 is essential for antimicrobial activity against Mtb in mononuclear phagocytes [24]. However, other studies have shown that vitamin D-mediated effects in human cells may involve other antimicrobial effector functions such as induction of nitric oxide synthase (iNOS) [26] or oxidative burst [26, 27], while vitamin D can also counteract Mtb-induced inhibition of phagolysosomal fusion [28, 29] in macrophages. In addition, 1,25$(OH)_2$D can induce IL-1β expression in Mtb-infected cells and promote an IL-1β-driven production of human β-defensin-2 by adjacent epithelial cells [30]. Another important defence mechanism promoted by vitamin D is autophagy [31]. Autophagy is a physiological process known to enhance

phagolysosomal maturation and degradation of intracellular pathogens including Mtb, and thus can overcome the phagosome-lysosome fusion arrest induced by Mtb [32]. In human monocytes, mycobacterial lipoproteins trigger autophagy by activation of TLR2/1, CD14 and VDR signaling [33]. In line with these findings, Rekha and colleagues recently demonstrated that $1,25(OH)_2D$, alone or in combination with the histone deacetylase inhibitor phenylbutyrate, counteracted Mtb-induced suppression of LL-37 as well as autophagy-related markers and restricted Mtb growth in human macrophages *in vitro* [34]. Notably, silencing of LL-37 expression blocked autophagy, suggesting that induction of autophagy was dependent on LL-37 [34]. Likewise, it has been shown that oral administration of vitamin D, or vitamin D in combination with phenylbutyrate, to healthy individuals reduced Mtb growth in monocyte-derived macrophages *ex vivo* [35]. Several *in vitro* findings support synergistic effects of combination treatment with vitamin D and phenylbutyrate including antimicrobial and anti-inflammatory responses to both enhance bacterial clearance and resolve immunopathology [20, 34, 36].

Overall, *in vitro* and *ex vivo* findings suggest that vitamin D-mediated innate immunity can contribute to control of Mtb infection via the induction of LL-37 and also other antimicrobial mechanisms, such as induction of reactive nitrogen and oxygen intermediates as well as autophagy in human macrophages. These pro-inflammatory actions have potential to cause immunopathology as well as protection: however, there is also evidence that vitamin D can simultaneously exert anti-inflammatory effects that have potential to regulate inflammation.

Vitamin D-mediated effects on anti-inflammatory responses

Apart from the described effects on antimicrobial immunity in TB, vitamin D has also been shown to have significant effects on adaptive immunity, primarily promoting anti-

inflammatory responses that could contribute to disease amelioration and decrease the risk of mortality (Figure 2). While $1,25(OH)_2D$ stimulates maturation and activation of human monocytes and macrophages *in vitro*, studies on other antigen-presenting cells such as DCs show opposite effects. VDR agonists including $1,25(OH)_2D$ [37], and its synthetic analogs [38, 39] inhibited the differentiation, maturation, activation as well as survival of monocyte-derived DCs. This resulted in decreased IL-12 and enhanced IL-10-production in mature DCs and reduced proliferation of alloreactive $CD4^+$ T cells [40]. Instead, it has been observed that $1,25(OH)_2D$-treated DCs can promote the development of $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells with suppressive properties that can be recruited to sites of inflammation [41, 42]. Tolerogenic DCs could also be induced by virulent Mtb strains and may induce functional inactivation of effector T cells [43]. Further studies investigating the role of $1,25(OH)_2D$ on modulation of DC function and Mtb-specific immune responses *in vivo* are warranted to understand the balance between good (anti-inflammatory) and bad (immunosuppressive) DC polarization.

In vitro studies have shown that vitamin D can inhibit transcription factors involved in cytokine gene regulation including Nuclear Factor of Activated T cells (NFAT), Activator Protein 1 (AP-1) and Nuclear Factor kappa B (NF-κB) [44-46], which result in less production of Th1 cytokines such as IFN-γ, IL-12, and TNF-α by T cells and macrophages [18, 47, 48]. Furthermore, IFN-γ up-regulated 1α-hydroxylase and thus enhanced production of $1,25(OH)_2D$, while IL-4 induced catabolism of 25(OH)D to the inactive metabolite $24,25D(OH)_2D$ via the 24-hydroxylase (CYP24A1) [49]. These results suggest that adaptive cell-mediated immune responses induced by vitamin D, may regulate innate antimicrobial mechanisms used to control Mtb infection. As such, $1,25(OH)_2D$ can suppress key effector functions of IFN-γ-activated macrophages, while no effect is seen on resting macrophages

[50]. Moreover, 1,25$(OH)_2$D may down-regulate pattern recognition receptors such as TLR2, TLR4, dectin-1 and mannose receptor on Mtb-infected macrophages [51], and instead promote induction of immunosuppressive mediators, such as IL-10 and prostaglandin E2 (PGE2) from Mtb-infected peripheral blood mononuclear cells (PBMCs) [52].

Studies on both human and mouse cells support that 1,25$(OH)_2$D contribute to the generation of *in vitro* induced IL-10 producing Treg cells [53]. PBMCs from TB patients and controls treated with Mtb-antigens and 1,25$(OH)_2$D *in vitro*, revealed an increased frequency of $CD4^+Foxp3^+$ Treg cells that were inversely correlated to the levels of inflammatory chemokines MCP-1, MIP-1β and CXCL10 [54]. High serum levels of IFN-γ-inducible CXCL10 is associated with enhanced T cell infiltration and progression of active TB [55] and CXCL10 is also a risk factor for development of TB-IRIS (TB-immune reconstitution inflammatory syndrome) [56]. Immunomodulatory effects of vitamin D supplementation *in vivo* involved enhanced resolution of lymphopenia, monocytosis, as well as a reduced pro-inflammatory cytokine/chemokine cascade [57]. Accordingly, vitamin D contributed to an elevated lymphocyte:monocyte ratio [58, 59] that has been shown to correlate with immune protection in TB. Vitamin D treatment also reduced a number of inflammatory mediators including CXCL9, CXCL10, MMP-9, LL-37, IL-6, IL-12, TNF and IL-10 [57]. A decrease of LL-37 and IL-10 contrasts *in vitro* data on vitamin D-induced effects, but may result as a consequence of enhanced TB control and reduced systemic inflammation. High serum levels of LL-37 in patients with active TB correlated with sputum smear positivity [60], which may reflect the acute phase response as a whole, rather than the induction of antimicrobial LL-37 at the site of Mtb infection in the lung.

In vitro studies using Mtb-infected leukocytes or PBMCs from TB patients and controls stimulated with Mtb-antigens, suggested that $1,25(OH)_2D$ can down-regulate Mtb-induced expression of several matrix metalloproteinases (MMPs) known to be associated with tissue remodeling and TB granuloma formation [52, 61]. In a recent study using an organotypic lung tissue model, it was also demonstrated that global inhibition of MMPs expressed by macrophages and epithelial cells could prevent early granuloma formation and Mtb growth in the tissue [62]. Another interesting *in vitro* study recently demonstrated that parent vitamin D as well as the 25(OH)D proform and active $1,25(OH)_2D$, contributes to maintaining or enhancing endothelial stability and barrier function in the presence of pro-inflammatory mediators via a mechanism that is not the result of conversion to $1,25(OH)_2D$, but independent from canonical VDR signaling [63]. Disruption of endothelial stability and an enhanced vascular leakage caused by inflammation, may enhance disease progression in several inflammatory disorders that are typically associated with low vitamin D status. It has also been suggested that vitamin D-induced effects are dependent on the multiplicity of Mtb infection (MOI), as $1,25(OH)_2D$ restricted bacterial growth by macrophages at low MOIs, while it mainly reduced host cytotoxicity at high MOIs [64].

These results support a role of active vitamin D in reducing host inflammation and tissue damage that could help in resolution of cavities in the Mtb-infected lung. But while it is important to reduce overt inflammation in TB, it is as important to enhance specific TB immunity and avoid local immunosuppression. Additional knowledge that could support our understanding of vitamin D-mediated effects include studies of Mtb-exposed DC and how these regulate T cell activation. More detailed analyses of the functional activity in central memory and effector memory T cells as well as the prevalence of Treg cells in vitamin D treated TB patients could bring further clarity into the lymphocyte dynamics in

response to vitamin D. While most in vitro studies involve the laboratory Mtb strain, H37Rv, testing clinical isolates and primarily the impact of multidrug-resistant TB (MDR-TB) on vitamin D responsiveness, and how this is affected by standard antibiotics also need to be resolved. However, in order to select the best regimen of vitamin D supplementation for clinical intervention studies, there are a number of issues relating to vitamin D metabolism and dosing that should be considered.

Vitamin D metabolites, blood concentrations and dosing

In recent years it has become clear that circulating concentrations of parent vitamin D (cholecalciferol or ergocalciferol) and the 25(OH)D proform may be important for efficient production of bioactive $1,25(OH)_2D$ [65]. The half-life of parent vitamin D is short i.e. 12-24h [66], and also the $1,25(OH)_2D$ form appears as a transient metabolite with a short half-life of 4-6h [67]. The 25(OH)D metabolite has a half-life of about 3 weeks, and is therefore the most abundant circulating form of vitamin D that is also used to assess vitamin D status in serum or plasma [68]. However, 25(OH)D is much more strongly bound to the plasma protein vitamin D binding protein (VDBP) compared to the parent form [69], and therefore cellular internalization in different tissues may become considerably restricted [70]. Another concern often brought up when interpreting observational studies in the vitamin D field relates to the lack of data regarding circulating concentrations of free 25(OH)D, as the majority of 25(OH)D is normally bound to VDBP in the circulation. The concentration of VDBP is one of the key determinants of the amount of free 25(OH)D, which – according to the 'free hormone hypothesis' - is the portion of total 25(OH)D that can be biologically active [71]. However, some studies imply that 25(OH)D complexed to VDBP can enter cells via receptor-mediated endocytosis to activate the VDR pathway [72]. It is known that black Americans have low levels of 25(OH)D in serum [73] and simultaneously, the incidence rate

for TB in non-Hispanic black Americans is eight times higher than for non-Hispanic whites [74]. The results from a highly cited study suggested that also levels of VDBP were lower in black Americans, which would result in similar levels of free 25(OH)D in black compared to white Americans [75]. However, a more recent report found a technical flaw in the ELISA-method used to measure serum levels of VDBP and instead show that the levels of free 25(OH)D were lower in black Americans compared to Caucasians [76]. With regard to TB, an *in vitro* study has reported that VDBP attenuates the ability of 25(OH)D to support antimicrobial responses, suggesting that concentrations of free 25(OH)D may have physiological relevance for mononuclear phagocyte function [77]. However, *in vivo* studies investigating the clinical significance of circulating concentrations of free and bioavailable vitamin D metabolites in patients with TB are still lacking.

Of importance is also the dosing of vitamin D in both health and disease, which currently is an area of debate. While the US Institute of Medicine (IOM) suggest that vitamin D levels >50 nmol/l is sufficient [78], the Endocrine Society instructs that >75 nmol/l is sufficient, 50-75 nmol/l is still insufficient and levels <50 nmol/l are defined as deficiency [79, 80]. To reach these blood levels of vitamin D, IOM recommends that an average daily intake of 400–800 IU is adequate for most individuals [78, 81], while the Endocrine Society suggests that a higher daily intake of 1000–10.000 IU may be required to maintain a sufficient vitamin D status [82]. The discrepancy in these opinions may be partly due to the fact that different 25(OH)D concentrations may be required to prevent or treat different conditions. Originally, a daily dose of 400 IU was given to sustain bone health and prevent rickets in children [83]. If baseline vitamin D status is low, supplementation with low doses may be efficient to reduce susceptibility to certain upper respiratory tract infections [84]. However, sometimes significantly higher vitamin D doses may be required to attain 25(OH)D levels associated

with optimal protection against respiratory pathogens [85]. A daily intake of 1120–1680 IU was required to maintain 25(OH)D levels above 75 nmol/l (30 ng/ml) in blood, while vitamin D deficient individuals needed 5.000 IU to reach these blood levels [86]. In the mid-20th century, case series reported that vitamin D2 given at doses of 100-150.000 IU/day promoted clinical recovery or cure in patients with lupus vulgaris (cutaneous TB) [87]. Such high pharmacological doses are more often administered less frequently as bolus doses, i.e. weekly, monthly or even yearly. Although it is convenient to give patients large bolus doses of vitamin D, these are not effective in achieving sustained elevation in circulating 25(OH)D concentrations if widely spaced [88]. Instead, as recently demonstrated in a meta-analysis of the effects of vitamin D in acute respiratory infections, a physiological dosing regimen of daily or weekly vitamin D is more likely to afford protection [8]. This way vitamin D could be readily used for cellular uptake in tissues and promote $1,25(OH)_2D$ conversion and VDR signaling that are required to reach clinical effects in different disease conditions.

Whereas vitamin D dosing and metabolism *in vivo* are important factors to consider when designing potential vitamin D supplementation regimens for the prevention or treatment of TB, many case-control studies performed in diverse geographical regions have also tried to elucidate whether vitamin D deficiency is associated with progression of active TB disease [9-13]. Since UVB radiation in sunshine is required for cutaneous synthesis, circulating 25(OH)D concentrations exhibits considerable seasonal variation outside of the tropics, through winter and spring. Vitamin D deficiency has also been reported to be common in certain groups such as breast-feeding mothers and their infants, young adults as well as elderly and people with dark skin [89-92]. A significant increase in TB incidence was observed in South African children during late winter and early spring, reported from 1983-1993 [93]. Consistent with these findings, seasonal variations in TB notifications was seen in

Cape Town, South Africa, that were inversely correlated to corresponding fluctuations in vitamin D status [11]. Vitamin D deficiency was highly prevalent among black Africans in this setting and correlated with susceptibility to active TB in both HIV-negative and HIV-positive individuals [11]. Increased melanisation of the skin affords physiological protection against adverse effects of UVB (skin cancer and degradation of folate), but it may increase the risk of vitamin D deficiency where UVB is limited [92, 94]. A high melanin content in the skin has been reported to reduce the capacity of UVB-exposed skin to produce 25(OH)D [95]. To synthesize the same amount of vitamin D, dark-skinned individuals require three to four times longer sun exposure than light-skinned persons because melanin efficiently absorbs UVB radiation [96]. Accordingly, two studies reported skin pigmentation as a significant predictor of vitamin D deficiency in Eastern and Northern Africa, respectively [97, 98]. Africans have lower neutrophil counts and lower circulating concentrations of antimicrobial peptides compared to south Asian and Caucasian people [99]. In addition, serum from African-American individuals was less effective than serum from Caucasians in supporting vitamin D-dependent induction of LL-37 mRNA in monocytes *in vitro* [9], a finding that may be attributable to ethnic differences in serum 25(OH)D content and/or VDBP genotype. As a consequence, African individuals may be more susceptible to TB [60, 100], perhaps due to a suboptimal induction of LL-37 and other important antimicrobial effector molecules at the site of Mtb infection in the lung [25].

Studies on the association between low levels of 25(OH)D and the risk of infection can always be criticized for reverse causality. While several studies have found a clear relationship between vitamin D deficiency and active TB, other studies have not observed such an association [101]. Thus, it is difficult to generalize these findings and conclude if there is a direct link between vitamin D status and susceptibility to active TB, and if so,

whether vitamin D deficiency in TB is a cause or consequence of clinical disease. A meta-analysis of observational studies between 1980-2006, found a 70% probability that a healthy individual would have higher 25(OH)D serum levels than a patient with untreated TB [102]. It has also been proposed that the susceptibility to TB is dependent on the degree of vitamin D deficiency, as serum vitamin D levels ≤ 25 nmol/l was significantly associated with an increased risk of TB, while 26-50 nmol/l provided an intermediately high TB risk and a range of 51-75 nmol/l did not enhance TB susceptibility [103]. To date, numerous studies have also been designed to address how genetic variations in proteins involved in the vitamin D signaling pathway may affect the susceptibility to TB, i.e. polymorphisms in the VDR or VDBP [12, 104]. Meta-analyses suggest that the VDR *FokI* polymorphism is associated with an increased risk of pulmonary TB in East Asians [105], while the *t* allele of the VDR *TaqI* polymorphism is significantly associated with an increased TB risk in South and West Asians [106]. Moreover, the Gc2/2 genotype of the VDBP was strongly associated with development of active TB in vitamin D deficient Gujarati Asians [104], while no such association was found in a South Asian cohort who presented an independent correlation between vitamin D deficiency and susceptibility to active TB [107].

Altogether, many studies support the notion that low 25(OH)D levels and polymorphisms in specific vitamin D signaling molecules can contribute to an enhanced susceptibility to active TB, at least in certain populations. Further studies exploring the association of bioavailable versus total 25(OH)D with susceptibility to Mtb infection or disease and how vitamin D metabolism changes with disease development, may provide valuable information about cellular vitamin D uptake and also learn us how to implement an effective dosing regimen. But altogether, can an improved vitamin D status in patients with pulmonary TB enhance bacterial clearance and clinical recovery?

Randomised controlled trials investigating the effects of vitamin D supplementation in active TB

Despite convincing *in vitro* data supporting an overall positive effect of vitamin D on innate immune functions and protection against TB, few larger clinical trials support such an effect using a microbiological outcome. A total of fifteen randomised placebo-controlled trials evaluating effects of adjunctive vitamin D on response to antimicrobial therapy for TB have been published to date (summarised in Table 1). Twelve of these clinical trials had clinical primary outcomes. Morcos and colleagues investigated the effects of 0.025 mg (1.000 IU) vitamin D daily in a 24 children in Egypt receiving antimicrobial therapy for TB, and showed no effect on body weight or resolution of symptoms [108]. Nursyam and colleagues subsequently conducted a trial of a daily dose of 0.25 mg (10.000 IU) vitamin D in 67 pulmonary TB patients in Indonesia [109]. In this study, adjunctive vitamin D enhanced sputum smear conversion at 6 weeks after initiation of antimicrobial therapy (34/34 vs. 25/33 smear-converted in intervention vs. control arm at 6 weeks, p=0.002), but no effect of the intervention was seen at 8 weeks [109]. The vitamin D status of participants was not assessed at either baseline or follow-up in this study, and details of safety monitoring, including monitoring of serum calcium concentrations, were not reported. Wejse and colleagues randomised 365 adult TB patients in Guinea-Bissau to receive three doses of 2.5 mg (100.000 IU) vitamin D_3 or placebo at initiation of antimicrobial therapy, and again at 5 and 8 months [110]. The intervention had no effect on the primary outcome measure, which was a composite clinical TB score [110]. Mean serum 25(OH)D concentrations at baseline showed generally sufficient levels of 78 nmol/L (31.2 ng/ml) versus 79 nmol/L (31.6 ng/ml) in the intervention compared to the control group [110]. Notably, the serum levels of 25(OH)D did not increase in the intervention group after vitamin D supplementation [110], which together with the widely-spaced dosing schedule may explain the lack of effect. A more recent study

conducted in 60 TB patients in Iran who were given a single oral dose (11.25 mg) of adjunctive vitamin D, reported a favorable effect on a similar TB score [111].

Martineau and colleagues investigated the effect of four 2-weekly doses of 2.5 mg (100.000 IU) vitamin D on time to sputum culture conversion in 146 patients with smear-positive pulmonary TB in the UK [58]. A 79 nmol/l (31.6 ng/ml) increase in serum 25(OH)D was seen among participants in the intervention arm of the study, which was associated with a trend towards faster sputum culture conversion (p=0.14) [58] and accelerated resolution of inflammatory responses in peripheral blood [57]. This finding was echoed by a subsequent study conducted in mice [112]. A sub-group analysis revealed that adjunctive vitamin D significantly hastened sputum culture conversion by more than 17 days in participants with the *tt* genotype of the *TaqI* VDR polymorphism (hazard ratio 8.09, 95% CI 1.36-48.01; p=0.02) [58], indicating that immunomodulatory effects of vitamin D supplementation are dependent on VDR genotype. Salahuddin and colleagues investigated the effects of two large intramuscular doses (600.000 IU) of vitamin D3 in 259 adults with pulmonary TB in Pakistan and found that the intervention enhanced weight gain and resolution of chest radiograph infiltrates, but did not influence sputum smear conversion [113].

Two subsequent trials employed a 2x2 factorial design to test the effects of vitamin D combined with other potential host-directed therapies. Ralph and colleagues investigated effects of a relatively low dose of vitamin D3 (2 x 1.25 mg) and the nitric oxide precursor L-arginine in 200 adults in Indonesia, and reported no effect of either intervention on sputum culture conversion [114]. Mily and colleagues investigated the effects of daily vitamin D_3 (5.000 IU) in conjunction with a twice-daily oral dose of 4-phenylbutyrate (500 mg) in 288 adults in Bangladesh and reported that administration of vitamin D enhanced sputum culture

conversion at eight weeks when all participants receiving vitamin D were compared with placebo [115]. Another study with vitamin D and phenylbutyrate conducted in Ethiopia using a similar dosing regimen to 348 pulmonary TB patients, showed a significant effect on clinical recovery assessed as a reduction in a composite TB score at week 8 compared to baseline (Clinicaltrials.gov ID: NCT01698476, invited revision). However, no effect on longitudinal sputum smear or culture conversion could be detected.

In the largest trial conducted to date, Datta and colleagues investigated effects of high-dose intramuscular vitamin D_3 and reported a trend towards reduced TB symptom score and higher rates of sputum smear conversion in participants randomised to the intervention arm of the trial: however, P values for inter-arm comparisons were not presented [116]. Three subsequent trials, one in the Republic of Georgia ([117], n=199), one in India ([118], n=247) and one in Mongolia ([59], n=390) investigated effects of high-dose oral vitamin D_3 on sputum culture conversion, and reported no significant effect on this outcome.

Three trials listed in Table 1, had biochemical primary outcomes, ie. calcium levels: two reported no hypercalcaemia in TB patients receiving either 0.125 mg (5.000 IU) vitamin D daily [119] or a single oral dose of 2.5 mg (100.000 IU) vitamin D [120], and one reported hypercalcaemia occurring in 19/30 TB patients receiving daily doses of 0.01 – 0.095 mg (400 – 3.800 IU) vitamin D daily [121]. However, this third study, by Narang and colleagues, also reported that a daily dose of 0.06 mg (2.400 IU) vitamin D elevated mean serum calcium in healthy controls – a finding which contrasts with other studies demonstrating that identical [122] or considerably higher [123] doses of vitamin D do not induce hypercalcaemia in healthy people. It is possible, therefore, that the actual doses of vitamin D administered in Narang's study were considerably higher than reported.

In summary, clinical trials of adjunctive vitamin D supplementation in patients with active TB have reported conflicting results. Meta-analysis of individual participant data from these studies has the potential to identify whether this heterogeneity arises from inter-trial differences in participant characteristics that may modify response to vitamin D supplementation, such as baseline vitamin D status or drug susceptibility. Such a meta-analysis is on-going: (http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42015020288), and its findings may identify sub-groups of TB patients who may derive benefit from adjunctive vitamin D. Overall, however, the conflict between largely positive *in vitro* data, and largely null *in vivo* data from treatment trials, presents something of a conundrum. What can we learn from these experiences in the continued design of new treatment and prevention trials?

Clinical evidence for positive effects of vitamin D in acute respiratory tract infections: Relevance for continued research on vitamin D to prevent TB

There are several potential explanations for the disconnect between positive results from *in vitro* studies and null results from treatment studies investigating the adjunct effects of vitamin D in TB. Some lessons can be learned from observing the role of vitamin D in the prevention of acute respiratory infections (ARIs) that has been intensively investigated during recent years. The rationale for a protective effect of vitamin D against viral and bacterial infections in the respiratory tract originates from several pieces of evidence. Similar to TB, results from observational studies support that vitamin D deficiency is a risk factor for acute respiratory infections [124]. There is a clear seasonal variation in the prevalence of ARI, peaking in the winter time when serum levels of 25(OH)D were low [125]. However, similar to TB, it is also possible that an enhanced susceptibility to frequent infections is an effect of

poor health in general, which is commonly associated with low vitamin D status due to lack of sun-exposure and/or an insufficient diet. In parallel to observational studies, numerous experimental models have supported a protective role for vitamin D against respiratory pathogens. For example, high local expression of the vitamin D converting enzyme, CYP27B1, has been found in bronchial epithelial cells, which promotes the generation of $1,25(OH)_2D$ that could alter immune function in the lung [126]. Consequently, vitamin D induced the expression of antimicrobial LL-37 in bronchial epithelial cells that exhibited activity against respiratory syncytial virus (RSV), rhinovirus as well as different airway bacteria [127-129]. In addition, vitamin D dampened inflammatory cytokine release from RSV-infected airway epithelial cells without increasing viral replication, which suggests that vitamin D-signalling ameliorate disease progression in virus-infected individuals [130].

The overall effect of vitamin D supplementation on the risk of ARIs was recently assessed in a meta-analysis using individual patient data obtained from 10,933 patients who participated in 25 different trials [8]. This meta-analysis also enabled detailed subgroup analysis of the various factors involved in the outcome of the included randomised trials. The results showed that vitamin D supplementation protected against ARI's overall, but that the effect was most pronounced in individuals with severe vitamin D deficiency (serum 25(OH)D < 25 nmol/l) at baseline, who received a daily or weekly dose, i.e. no bolus dosing [8]. Accordingly, the numbers needed to treat (NNT) in order to prevent one person from getting an ARI was considerably lower for individuals with vitamin D levels < 25 nmol/l who received daily or weekly vitamin D supplementation (NNT=4) compared to the all included study participants (NNT=33). This study identified two factors that independently modified the effect of vitamin D supplementation on the risk of ARIs: baseline vitamin D status and dosing frequency. A recent systematic review supports these evidence, as the results concluded a

beneficial effect of vitamin D supplementation in common ARIs and asthma, but found little effect against other non-skeletal outcomes, TB included [131]. Of note, in contrast to TB and several other conditions, the number of trials and amount of patient data available in vitamin D trials aiming to prevent ARIs are relatively high, which enables adequately powered subgroup analyses.

Can the findings on beneficial effects of vitamin D in ARIs teach us something about vitamin D-mediated effects in TB? Several relevant parallels can be made, highlighting differences as well as similarities. First, in the studies on ARIs, vitamin D has been given as a preventive intervention and not as a therapeutic drug. This is a major difference compared to most TB trials where vitamin D has been administered as adjunctive therapy together with standard anti-TB drugs to primarily sputum-positive patients with pulmonary TB. Although drug-susceptible TB has to be treated with multiple drugs every day for months, there is a rapid bactericidal effect already within the first few weeks of standard treatment characterized by rapid sputum conversion, radiological improvement and clinical recovery in most patients [132, 133]. This will probably mask relatively modest antimicrobial effects induced by vitamin D that were apparent in the pre-antibiotic era [134] This notion would be consistent with most clinical evidence showing a null effects in treatment studies (Table 1). The findings from ARI studies suggest that we might be more likely to detect an effect of vitamin D supplementation in TB prevention than for adjunctive treatment. Low 25(OH)D levels may enhance the risk of latent TB [135, 136] as well as progression of active TB [11, 137]. However, trials of vitamin D to prevent acquisition or reactivation of latent Mtb infection are considerably more challenging to conduct than those investigating ARIs, since event rates are much lower: thus, sample sizes need to be much larger, and follow-up much longer, in order to provide power to detect biologically plausible effect sizes.

Second, the ARI studies suggest that vitamin D supplementation is most beneficial if administered with short intervals, i.e. not as large bolus doses [8]. Interestingly, daily or weekly vitamin D could prevent ARIs particularly in vitamin D deficient patients (< 25 nmol/l), but also in individuals with higher (> 25 nmol/l) 25(OH)D levels at baseline [8]. In contrast, less frequent bolus dosing failed to prevent ARIs in the subjects, regardless of vitamin D status [8]. Administration of high-dose bolus vitamin D results in large fluctuations in circulating 25(OH)D concentrations [65], which may lead to a dysregulation of 1α-hydroxylase activity that ultimately reduce the conversion to active $1,25(OH)_2D$ available in the tissues [138]. This seemingly paradoxical phenomenon may be caused by a delay in the metabolic feed-back mechanism induced in responses to widely fluctuating 25(OH)D levels in blood, when the body is trying to adapt to interchangeably high and low concentrations of 25(OH)D [138]. Thus, although it is convenient to give patients large bolus doses of vitamin D to increase adherence, lower doses given more often may be required to maintain stable levels of 25(OH)D that could induce protective immunity and reach desired clinical effects. Altogether, these results suggest that a widely spaced bolus dosing regimen that has been frequently used to study the effects of vitamin D in randomised TB trials, should be avoided in trials of vitamin D for TB prevention.

Third, as described above, vitamin D supplementation appears to be more effective in preventing ARIs in individuals with profound vitamin D deficiency, while the positive effects may be less pronounced if baseline vitamin D status is high [8]. This is in line with sub-group analyses performed in two vitamin D supplementation trials indicating clinical improvement and enhanced sputum-smear conversion, particularly in TB patients with low baseline vitamin D levels [113] (Clinicaltrials.gov ID: NCT01698476, invited revision). This is logical, as people with the lowest levels of a micronutrient, will be the most likely to respond

to its replacement. Ideally, screening TB patients for vitamin D deficiency before inclusion in randomised vitamin D trials may enhance the likelihood to detect significant effects. However, there are both ethical and logistic problems associated with such an approach that have to be carefully considered.

Potential future approaches: Vitamin D supplementation to treat or prevent TB in certain high-risk groups

Current clinical evidence points to the fact that vitamin D-mediated effects in TB are slight or absent when administrated to a broad range of patients receiving antimicrobial therapy for pulmonary TB. But since vitamin D has documented antimicrobial as well as anti-inflammatory properties in experimental models, the rationale to use this cheap and safe intervention against TB in some form is still attractive. Most of the intervention trials performed with vitamin D in TB are too small to allow sub-group analyses to assess the importance of different variables as potential effect modifiers such as low baseline vitamin D status, HIV infection or other co-morbidities, MDR-TB or advanced cavitary TB disease, etc. These groups may be relevant to target in future treatment trials. Accordingly, a key area where adjunctive vitamin D could be useful, is in patients infected with MDR-TB. Since standard drugs are not expected to be as efficient, any true effect of vitamin D may be unmasked and should be possible to find in a reasonably large clinical trial. There are studies where MDR-TB patients were included, revealing a trend towards enhanced improvement of vitamin D in MDR-TB patients [59, 117]. But in most trials, MDR-TB has usually been an exclusion criterion or the numbers of MDR-TB patients in individual studies have not been large enough to make definite conclusions. Given the emerging threat of MDR-TB in certain regions in i.e. South-Africa and Eastern Europe, it should be possible to design a larger study that specifically address the effects of vitamin D in MDR-TB. Another vulnerable group is

TB patients with other co-morbidities including HIV-infection and diabetes. Here, daily supplementation of vitamin A (2000 IU), vitamin D (400 IU) or the combination was planned to be provided to 400 patients with active pulmonary TB and type 2 diabetes in Qingdao, China, assessing if adjunct anti-TB treatment could ameliorate glucose metabolism and improve immune and nutritional status [139] (Trial Registration: ChiCTR-TRC-12002546). The current progress of this study is unknown.

The effects of adjunctive therapy with vitamin D in TB may also be enhanced by combination treatment with other drugs or compounds such as arginine [114], phenylbutyrate [115] or statins [140], that may exert synergistic effects with vitamin D. In this context, the interaction of vitamin D with standard anti-TB drugs should be investigated in more depth, both *in vitro* and *in vivo*, as several studies suggest that anti-TB drugs can alter vitamin D-mediated immune pathways. Apart from their bactericidal or bacteriostatic actions, standard antibiotics can also modulate host immune responses such as phagocytosis, chemotaxis, antigen presentation, cytokine secretions and autophagy [141-143]. Furthermore, intensive-phase treatment with rifampicin and isoniazid have been shown to reduce baseline vitamin D levels in the majority of treated TB patients, which suggests that standard treatment alone may promote vitamin D deficiency or worsen an already low vitamin D status [144].

Whereas there is a need to reconsider the current approaches and try other innovative study designs, one approach that has yet to be formally tested in clinical trials is whether vitamin D can prevent Mtb infection and/or control latent TB and prevent the conversion to active, sputum-positive TB. One previous trial conducted in 120 Mongolian schoolchildren, concluded that 6 months vitamin D supplementation (800 IU/day) resulted in significantly improved vitamin D status while a trend towards reduced number of tuberculin skin conversion was also detected in the intervention group [145]. Most tuberculin conversions

occurred in children who remained vitamin D deficient [145], suggesting that vitamin D supplementation indeed may prevent latent TB infection. As a follow up to this pilot study, a much larger trial has been initiated, the Vitamin D supplementation in TB prevention trial, which aims to investigate the preventive role of vitamin D in 8000 school children in a similar high transmission setting in Mongolia (Clinicaltrials.gov ID: NCT02276755). Here, the acquisition of latent TB, assessed using the Mtb-specific test, Quantiferon-TB Gold In-Tube (QFT) test, as well as the development of active TB, will be monitored after three years of weekly vitamin D (14.000 IU). The notion is that children with the lowest vitamin D status at baseline will gain most from the intervention. Another large ongoing clinical trial is the VidiKids trial (Trial of Vitamin D Supplementation in Cape Town Primary Schoolchildren), conducted in Cape Town, South Africa, to determine whether a weekly oral dose (10.000 IU) of vitamin D_3 will reduce the risk of latent TB among 5.400 QFT-negative primary schoolchildren, who are followed for three years (Clinicaltrials.gov ID: NCT02880982). The large size of these ongoing trials will probably increase the likelihood to detect differences in QFT conversion between vitamin D and placebo groups. However, QFT is not a validated gold standard to monitor acquisition of Mtb infection, and will only detect an immune response towards Mtb bacteria but not the presence of bacteria in the lung. Moreover, the QFT test has technical limitations that may impede overall analysis, highlighting the need for new read-out systems used to monitor latent TB.

Two on-going randomised trials are investigating whether vitamin D supplementation might have a role in the prevention of incident or recurrent TB disease. The TOV4 trial (Trial of Vitamin D in HIV progression) have recruited 4.000 HIV-infected Tanzanian individuals who stated antiretroviral treatment and simultaneously were provided weekly (50.000 IU) and later daily (2.000 IU) vitamin D supplementation for 12 month until follow-up assessing all-

cause mortality and incidence of active TB (Clinicaltrials.gov ID: NCT01798680). The ResolveD-TB trial (Vitamin D to Resolve Inflammation After TB), is a small proof-of-concept study that aims to determine if vitamin D can prevent recurrent TB by enhanced resolution of pulmonary inflammation detected using 18F-FDG PET-CT scanning (Clinicaltrials.gov ID: NCT03011580). In this pilot study, 40 vitamin D-deficient patients with baseline 25(OH)D levels <50 nmol/l will be included if they have residual pulmonary lesions detected on PET-CT scanning after 6-months standard anti-TB treatment, to receive high-dose daily vitamin D (9.600 IU/day) or placebo for 8 weeks. The presence of PET-hot intra-thoracic lesions at the end of TB treatment has been posited as a biomarker for risk of post-treatment relapse [146]. If vitamin D supplementation can suppress PET-CT-detectable intra-thoracic inflammation in this patient group, it would provide a rationale to progress to a phase 3 randomised trial of vitamin D supplementation to prevent recurrrent TB.

Concluding remarks

Vitamin D is an immunomodulatory micronutrient that simultaneously supports anti-mycobacterial and anti-inflammatory activity *in vitro*. Attempts to use the immunomodulatory properties of vitamin D to treat pulmonary TB can be traced back to the 19th century. Despite vitamin D deficiency being common in individuals with active TB, clinical and bacteriological results from randomised controlled trials of adjunctive vitamin D supplementation in patients receiving antimicrobial therapy for pulmonary TB have not demonstrated consistent clinical benefits, despite showing immunomodulatory activity. This may represent 'masking' of favourable immunomodulatory activity by the actions of potent antimicrobial therapy in the context of drug-sensitive disease. Adjunctive vitamin D may yet have potential to benefit sub-groups of patients in whom antimicrobial therapy is less effective, such as those with multidrug-resistant disease, and clinical trials are needed in this

area. However, given the lack of consistent favourable results from trials of adjunctive vitamin D in TB treatment, increasing attention is focusing on the potential for vitamin D supplementation to prevent acquisition or reactivation of latent Mtb infection. Trials of vitamin D for the prevention of acute respiratory infections suggest that protective efficacy of this intervention is greatest when vitamin D is given in daily or weekly doses to individuals with low 25(OH)D levels at baseline. These findings have the potential to inform the design of future trials of vitamin D supplementation for the prevention and treatment of TB.

Conflict of interest statement

No conflict of interest to declare.

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Table 1 *Randomised placebo-controlled trials investigating effects of adjunctive vitamin D in patients with tuberculosis*

First author, year, reference	Sample size, setting	Vitamin D dose administered	Effect on serum 25(OH)D concentration	Primary outcome
Morcos 1998 [108]	24 children, Egypt	0.025 mg (1.000 IU) daily	Not reported	Body weight / symptoms: no change
Nursyam 2006 [109]	67 adults, Indonesia	0.25 mg (10.000 IU) daily	Not reported	Smear conversion: increased rate at 6 wks
Martineau 2009 [120]	25 adults, UK	1 x 2.5 mg (100.000 IU) D2 @ 0 months	22 nmol/L (8.8 ng/ml) increase in active arm	Serum 25(OH)D: small increase at 8 wks
Wejse 2009 [110]	365 adults, Guinea Bissau	3 x 2.5 mg (100.000 IU) D3 @ 0/5/8 months	25 nmol/L (10 ng/ml) increase both arms	TB score: no effect
Martineau 2011 [58]	146 adults, UK	4 x 2.5 mg (100.000 IU) D3 @ 0/2/4/6 weeks	79 nmol/L (31.6 ng/ml) increase in active arm	Culture conversion: no effect in study population as a whole, but effect seen in subgroup with *tt* genotype of the *TaqI* VDR polymorphism
Salahuddin 2013 [113]	259 adults, Pakistan	2 x 15 mg D3 i.m. @ 0/4 weeks	170 nmol/L ↑ in active arm	Body weight ↑, CXR infiltration ↓ with vitamin D. Smear conversion: no effect
Ralph 2013 [114]	200 adults, Indonesia	2 x 1.25 mg D3 p.o. @ 0/4 weeks (factorial with L-arginine)	Not reported	Culture conversion: no effect
Mily 2015 [115]	288 adults, Bangladesh	125 mg D3 p.o. daily for 2 months (factorial with phenylbutyrate)	80 nmol/L ↑ in active arm	Culture conversion: 123/127 (97%) vs. 110/122 (90%) culture converted at 8 wk in D3 vs. placebo arms respectively (P=0.04, Fisher's exact test)
Datta 2015 [116]	622 adults, India	2 x 15 mg D3 i.m. @ 0/3 months	Not ↑ in active arm, but ↓ in control arm	Trend towards lower 'TB score' and higher rates of smear conversion in active arm: P not presented.
Tukvadze 2015 [117]	199 adults, Georgia	3 x 1.25 mg D3 per wk for 8 wk, then 1.25 mg D3 alternate wks for 8 wk	200 nmol/L ↑ in active arm	Culture conversion: no effect. Trends towards efficacy in MDR sub-group.
Daley 2015 [118]	247 adults, India	4 x 2.5 mg (100.000 IU) D3 @ 0/2/4/6 weeks	14 nmol/L ↑ in active arm	Culture conversion: no effect.
Ganmaa 2017 [59]	390 adults, Mongolia	4 x 3.5 mg (100.000 IU) D3 @ 0/2/4/6 weeks	139 nmol/L ↑ in active arm	Culture conversion: no effect.
Farazi 2017 [111]	60 adults, Iran	1 x 11.25 mg @ 0 months	Not reported	TB score: reduced in intervention arm at follow-up
Gwinup 1981 [119]	23 adults, USA	0.125 mg (5.000 IU) daily	Not reported	Serum calcium: no change
Narang 1984 [121]	30 adults, India	0.01-0.095 mg (400 - 3.800 IU) daily	Not reported	Serum calcium: hypercalcaemia in 63%

Figure captions

Fig. 1 *Schematic illustration of vitamin D metabolism and signaling using the endocrine as well as the paracrine systems. Vitamin D is produced in the skin upon UVB exposure or ingested in the diet. Conversion to the 25(OH)D proform, which is the major circulating metabolite, occurs primarily in the liver. 25(OH)D in serum is present either in its free form or bound to the VDBP. 1-alpha hydroxylation of 25(OH)D occurs in the kidney but also in different immune cells, for production of the active metabolite 1,25(OH)$_2$D. 1,25(OH)$_2$D binds to the intracellular VDR to form a transcription factor complex that can regulate the expression of different target genes involved in the immune response to TB.*

Fig 2. *Effects of vitamin D on innate and adaptive immune responses. Active 1,25(OH)$_2$D up-regulates expression of antimicrobial peptides such as LL-37, hBD-1 as well as oxidative stress, autophagy and IL-1β in Mtb-infected macrophages. It also down-regulates differentiation and activation of DCs, which may result in expansion of Treg cells that can inhibit effector Th1 responses. In addition, 1,25(OH)$_2$D has direct inhibitory effects on the production of Th1 cytokines by CD4+ T helper cells.*