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Original Article

Synergistic action of vitamin D and retinoic acid restricts invasion of macrophages by pathogenic mycobacteria

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Background and Purpose: Phagosomal maturation arrest is known to play a central role in the survival of pathogenic mycobacteria within macrophages. The maturation arrest of mycobacterial phagosome results from the retention of tryptophan-aspartate-containing coat protein (TACO) on this organelle, enabling successful replication of the pathogen. We have shown earlier that vitamin D_3 and retinoic acid (RA) down-regulate TACO gene transcription in a dose-dependent manner.

Methods: In this study, we analyzed the promoter region of TACO gene using bioinformatics tools and observed that the vitamin D receptor (VDR)/retinoid-X-receptor (RXR) response sequence was highly functional. We also evaluated the effect of treatment with vitamin D_3/RA on *Mycobacterium tuberculosis* entry and survival in cultured human macrophages.

Results: TACO gene down-regulation observed with vitamin D_3/RA treatment occurred through modulation of this gene via the VDR/RXR response sequence present in the promoter region of TACO gene. Treatment of macrophages with vitamin D_3/RA allows maturation of mycobacterial phagosome, leading to degradation of the pathogen.

Conclusions: Our results elucidate the mechanism of TACO gene down-regulation observed with vitamin D_3/RA. Furthermore, the results revealed that vitamin D_3/RA treatment inhibits mycobacterial entry as well as survival within macrophages, possibly through rescue of phagosome maturation arrest. The developing knowledge in this area suggests that vitamin D_3/RA may be of importance in the treatment of intracellular infection, particularly tuberculosis.

Key words: Down-regulation; DNA-binding proteins; Macrophages; *Mycobacterium tuberculosis*; Tretinoin; Vitamin D

Introduction

The survival of pathogenic mycobacteria is tightly linked to the successful establishment of an intracellular niche within the host. Macrophages have a unique paradoxical role in tuberculosis infection, serving as a first line of defense against such infection and also creating the primary site for mycobacterial replication and dissemination [1].

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Mycobacterial survival within macrophage is achieved primarily by convoluting the mycobacterial phagosome with the endogenous macrophage protein tryptophan-aspartate-containing coat protein (TACO), also known as coronin-1, that specifically restricts phagosomes containing pathogenic mycobacteria from entering the late endosomal/lysosomal pathway [2,3]. In fact, this strategy of survival within TACO/coronin-1-coated phagosomes is initiated during the entry itself, and is made possible by the presence of multiple receptors on the phagocyte surface [4-7].

TACO/coronin-1 is an actin-binding protein known to associate with cholesterol within the plasma membrane [8-11]. The preference of *Mycobacterium tuberculosis* for

cholesterol-rich domains within the plasma membrane ensures that mycobacteria are subsequently sequestered within TACO/coronin-1-coated phagosomes, and therefore evade lysosomal fusion.

Historically, administration of vitamins (A or D) has been considered beneficial for the treatment of tuberculosis [12]. It has been shown experimentally that the immunologically active compound retinoic acid (RA) increases the resistance of cultured human macrophages to experimental infection with virulent *M. tuberculosis* [13]. Several studies have shown the role of vitamin D in tuberculosis. Studies have revealed a low vitamin D status in tuberculosis patients [14,15]; correlation between vitamin D receptor (VDR) gene polymorphism and human susceptibility to mycobacteria [16,17]; and nitric oxide (NO)-mediated mycobacterial killing by 1,25-dihydroxyvitamin D_3 [18,19].

We have shown earlier that vitamin D_3 and RA when combined down-regulate TACO gene transcription in a dose-dependent fashion [20]. Since vitamin D_3 and RA act through ligand-dependent transcription factor (TF) heterodimeric complex VDR/retinoid-X-receptor (RXR), we analyzed the promoter region of TACO gene using bioinformatics tools. In the present study, we investigated the mechanism of this transcriptional repression using reporter assay, and also the effect of this TACO gene suppression on *M. tuberculosis* entry and survival within cultured human macrophages.

Methods

Cells and materials

THP-1 (human acute monocytic leukemia) and Jurkat cell lines were obtained from National Centre for Cell Science (Pune, India). Human monocytes were isolated from blood of healthy volunteers. 1,25-dihydroxyvitamin D_3, RA, culture media and fetal bovine serum were obtained from Sigma (St. Louis, MO, USA). The cDNA synthesis kit was purchased from MBI Fermentas Inc. (Burlington, ON, Canada) and the polymerase chain reaction core kit was obtained from Qiagen (Hilden, Germany). Tri-n-octylphosphine oxide (TOPO)-reporter kit, Lipofectamine 2000 and beta (β)-galactosidase assay kit were purchased from Invitrogen (Carlsbad, CA, USA).

In silico deduction of VDR/RXR response sequence

We extracted a 2-kb promoter sequence upstream of TACO gene from GenBank DB [21]. The analysis of TF binding sites in this sequence was performed using the program MatInspector Professional (Genomatix Suite, Version 7.2.2 release of April, 2004; Genomatix Software GmbH, München, Germany) that searches TRANSFAC (transcription factor) database [22,23]. An individual score for each sequence fragment is generated, and matches for that score above a user-defined threshold are reported. Since default similarity values (core similarity: 0.75) of MatInspector may produce high numbers of false-positives, we used vertebrate matrix with strict threshold values (core similarity: 1.0; matrix similarity optimized) for efficient analysis of promoter sequences.

β-Galactosidase reporter plasmid assay

Transfection and reporter assay was done as previously described [24]. In order to ascertain whether or not vitamin D_3/RA modulation of TACO gene is mediated through VDR/RXR TF, we used a Jurkat cell line (a pure CD4+ T-cell line with an intrinsic enhanced expression of c-myc gene), and designed a reporter cassette that contained a c-myc response sequence together with a VDR/RXR response sequence. As a result, the effect observed with vitamin D_3/RA treatment was specific and selective.

The VDR/RXR response element 'CGG GCA CCG AGG CCT GG' present in the TACO gene promoter and c-myc response sequence 'GTCACGT GCCTT' was incorporated in the TOPO vector using a TOPO cloning reaction as per the manufacturer's instructions. TOPO vector (without insert) was used as a control. Cells were seeded at the desired density in Dulbecco's modified Eagle medium (DMEM) and the reporter cassette transfected using Lipofectamine 2000. The cells were then treated with 1 μM each of vitamin D_3 and RA. The culture was followed for 72 h and the supernatant assayed for β-galactosidase reporter activity. The experiment was repeated three times and the results are represented as relative β-galactosidase activity.

Monolayer culture and treatment

THP-1/mononuclear cells were cultured and differentiated into macrophages as described earlier [25,26]. The purity of macrophages was checked by giemsa staining and found to be >95%. Macrophages in culture wells were either: (1) pretreated, i.e., vitamin D_3/RA treatment before *M. tuberculosis* H37Rv infection; or (2) post-treated, i.e., vitamin D_3/RA treatment after *M. tuberculosis* H37Rv infection. In both conditions,

vitamin D_3 and RA were used at fixed concentrations of 1 μM each.

M. tuberculosis phagocytosis assay

Invasion of macrophage cultures with *M. tuberculosis* was done as described earlier [25,27,28]. *M. tuberculosis* virulent strain H37Rv was added to macrophage monolayers in each well for 2 h at 37°C, to obtain a multiplicity of infection of 10:1. Assays were terminated by washing once with phosphate-buffered saline (PBS) and replacing the overlying medium supplemented with 100 μg/mL of gentamicin (that kills extracellular bacteria to remove any nonspecific adhesion or internalization) [29]. After 2 h incubation with gentamicin at 37°C, the cell monolayers were washed once with PBS and processed for immunolabeling and flow cytometry as described below.

Immunoflourescent labeling

At each measurement time point, the control and vitamin D_3/RA-treated cells were removed from tissue culture wells and washed three times with PBS. Cells were then fixed and permeabilized for 20 min with 4% paraformaldehyde/0.1% Triton-X 100 in PBS. Primary antibody against lipoarabinomannan (LAM), a major component of the outer surface of mycobacteria, was then added at appropriate dilution (1:1500) in PBS for 1 h at 37°C. Monocyte-derived-macrophage (MDM) monolayers were then washed three times in PBS and overlaid with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin G, diluted 1:50 in PBS, for 1 h at 37°C [27,28].

Bacterial enumeration by flow cytometry and colony counts

The cells were enumerated for mycobacterial association by fluorescence-activated cell sorter (FACS; FACSCalibur™ [Becton, Dickinson and Company, Franklin Lakes, NJ, USA]), by measurement of mean fluorescence intensity associated with each sample. The results are shown as FACS histograms with percentage LAM fluorescence (indicating number of bacteria present). Each experiment was performed at least three times. This experiment was duplicated in THP-1 macrophages, and the number of viable *M. tuberculosis* was enumerated by colony counts (colony-forming units [CFU]) as described previously [30]. The experiment was repeated three times and the results presented as percent survival ± standard deviation.

Direct effect of vitamin D_3/RA on *M. tuberculosis*

M. tuberculosis H37Rv was grown in 7H9 medium supplemented with albumin dextrose catalase enrichment. When the culture was in the log phase of growth, an appropriately tested concentration of each stimulus was added to different culture flasks. The flasks were incubated at 37°C with shaking, and 100 μL was plated at 24 h and 48 h. The experiment was repeated three times and the results presented as mean CFU ± standard deviation.

Results

TACO gene promoter contains VDR/RXR response sequence

We have shown earlier that vitamin D_3 and RA in combination down-regulate TACO gene expression in a concentration-dependent manner [20]. Vitamin D_3 and RA are known to act through the TF VDR/RXR heterodimeric complex. Using the MatInspector program to scan promoter sequences for putative TF binding sites, we observed a VDR/RXR response element within 2 kb of the translation start site (Fig. 1).

Functional status of VDR/RXR binding site

β-Galactosidase reporter plasmid carrying VDR/RXR binding sequence from TACO gene promoter showed a decrease in β-galactosidase activity of more than 15-fold compared to control cells that were not stimulated by vitamin D_3/RA (Fig. 1).

Vitamin D_3/RA inhibits mycobacterial entry and survival within macrophages

Down-regulation of TACO gene by vitamin D_3/RA would be expected to rescue phagosome maturation arrest, leading to degradation of the pathogen within lysosomes. To investigate this, we studied the entry/survival of pathogenic *M. tuberculosis* within THP-1/MDM cultures for different time periods (Fig. 2 and Fig. 3). To investigate the entry within TACO down-regulated macrophages, we pretreated these cultures with vitamin D_3/RA and then infected them with *M. tuberculosis*. FACS analysis of these cultures to detect FITC-labeled LAM (indicating number of bacteria present) revealed that mycobacterial entry was restricted by 33% (Fig. 2). At 12 h after infection, 59% of the mycobacteria were found within macrophages (Fig. 2). To investigate bacterial survival when TACO gene is down-regulated, we post-treated *M. tuberculosis*-infected MDM with vitamin D_3/RA.

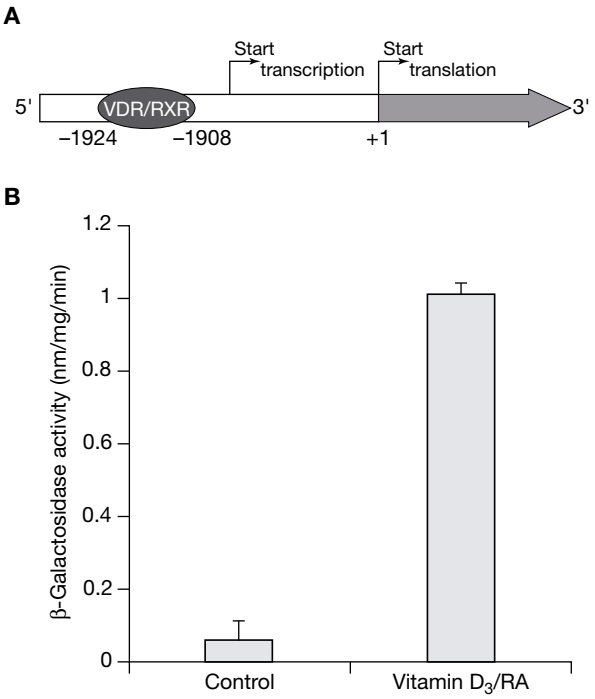

Fig. 1. In silico analysis and reporter assay for vitamin D receptor (VDR)/retinoid-X-receptor (RXR) response sequence present within tryptophan-aspartate-containing coat protein (TACO) gene promoter. (A) Predicted VDR/RXR binding site in the promoter region of TACO gene on the basis of in silico analysis using MatInspector version 7.2.2 with the TRANSFAC (transcription factor) database. (B) TACO promoter-dependent beta (β)-galactosidase reporter activity in Jurkat T-cells exposed to 1 μM each of vitamin D_3 and retinoic acid (RA). Each bar represents mean ± standard deviation of the experiments done in triplicate.

Flow cytometric analysis of such cultures revealed 88% bacterial survival at 12 h (Fig. 2). By 48 h, only 26% of the bacteria were found within macrophages (Fig. 2). However, CFU count of the duplicate set of this experiment in THP-1 macrophages revealed no growth in any of the pretreated or post-treated cultures (Fig. 3).

Viability of free-growing bacilli

To confirm that the observed effect on mycobacterial growth is not due to a vitamin D_3/RA effect on mycobacterial viability per se, we incubated free-growing H37Rv bacilli with an appropriate dose of the stimulus and followed the culture for 48 h. Although a marginal decrease in growth was observed, it was insignificant. The results (Fig. 4) confirmed that the effects on mycobacterial entry and survival results from down-regulation of TACO expression and subsequent maturation of these organelles into phagolysosomes.

Discussion

Intracellular pathogens such as *M. tuberculosis* lie within phagosomes that are plasma-membrane-derived organelles resistant to fusion with late endocytic compartments [31-35]. Instead of following their usual fate of fusion with late endosomes and lysosomes (hydrolytic machinery of the host cell), live *M. tuberculosis* containing phagosomes fail to mature into phagolysosomes [36,37]. During maturation, phagosomes gradually lose and acquire new proteins, leading to the biogenesis of phagolysosomes [38-40]. Mycobacterial phagosomal progression along the endocytic pathway is hindered at the early phagosome, a condition known as phagosome maturation arrest. Different mechanisms have been shown to contribute to this maturation arrest, e.g., failure to shuttle between Rab5 and Rab7 [34,41]; exclusion of vacuolar adenosine triphosphate complex responsible for acidification [42,43]; interference in calcium and phosphatidylinositol-3-kinase (PI3K) signaling pathways [44]; inhibition of actin assembly [45]; secretion of eukaryotic-like protein kinase G [46]; and retention of TACO/coronin-1 on the mycobacterial phagosome [2,47]. TACO is an actin-binding protein involved in cytoskeletal modulation [48], cytokinesis and intracellular membrane transport [49,50]. TACO is associated with cholesterol on the cytosolic face of the plasma membrane and is acquired by the phagosome during the early stages of its formation [10,51].

Increased recruitment and retention of TACO have been observed in *Helicobacter pylori* infection [26]. Therefore, it is possible that TACO may be of importance in the intracellular infection process in general, and modulation of the interaction between the pathogen and its host in particular. However, one study observed recruitment of TACO during the early stages of phagocytosis, but no retention/persistence in phagosomes carrying low numbers of bacteria [25]. It may be possible that TACO is not retained on phagosomes containing relatively lower numbers of bacteria.

We have shown earlier that vitamin D_3 and RA act synergistically to down-regulate TACO gene expression at the transcriptional level [20]. This may be important, in view of the increased retention of TACO in intracellular infection [26]. Vitamin D_3 and RA have long been known to have antimycobacterial properties [13,52]. Different studies have shown that: (1) VDR gene polymorphism is associated with tuberculosis

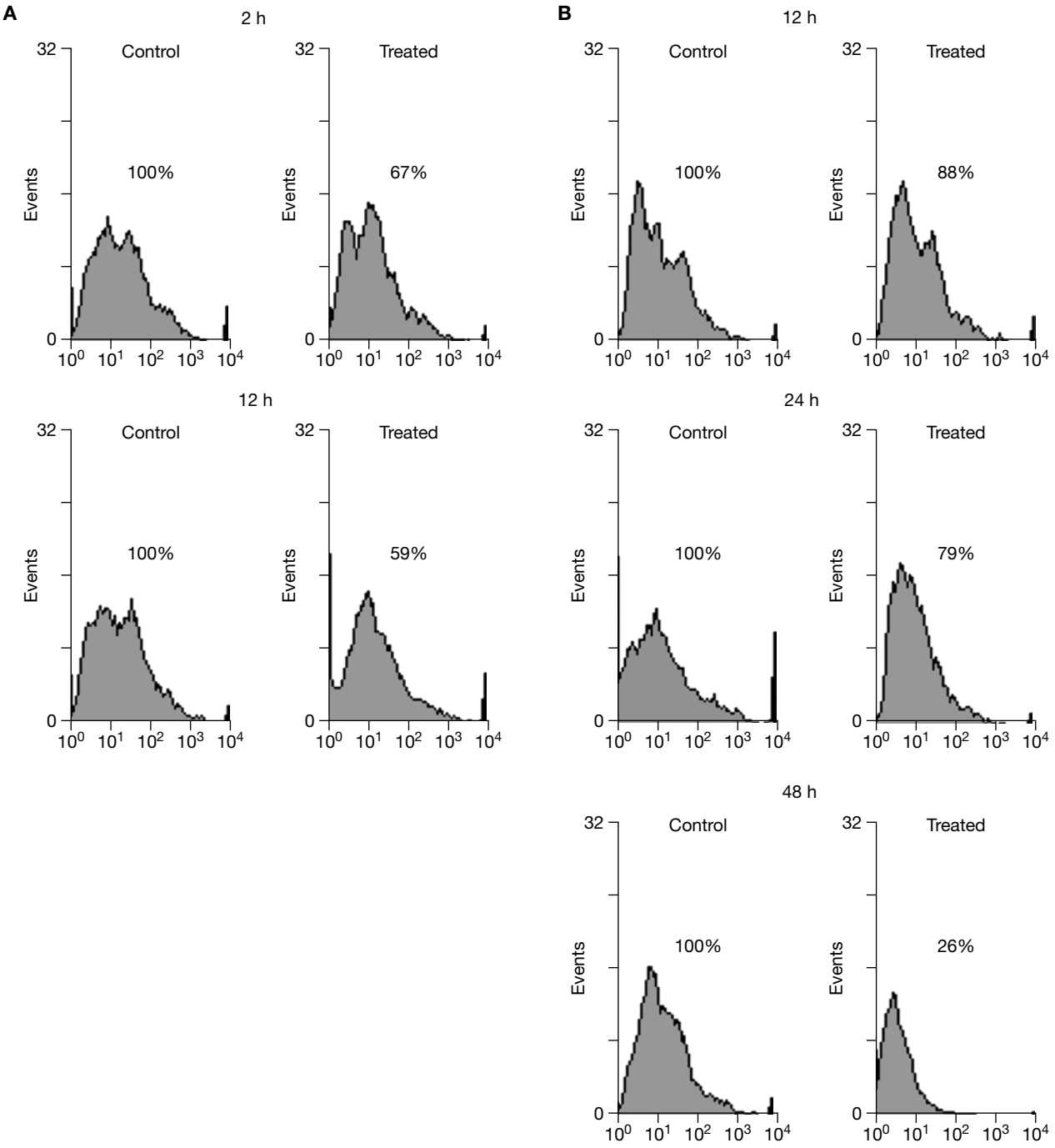

Fig. 2. Percent lipoarabinomannan (LAM) fluorescence of *Mycobacterium tuberculosis* within monocyte-derived macrophages at indicated time periods. (A) Mycobacteria in macrophages exposed to vitamin D_3/retinoic acid for 24 h prior to infection. (B) Mycobacteria in macrophages exposed to vitamin D_3/RA for 24 h after infection. Percentage labels indicate relative fluorescence of control and treated macrophages.

[53]; (2) vitamin D_3 deficiency is associated with increased risk for tuberculosis [19]; (3) vitamin D_3 has immunomodulatory functions in tuberculosis [53,54]; (4) vitamin D_3 restricts intracellular growth of *M. tuberculosis* [55]; and (5) RA restricts *M. tuberculosis* infection in cultured human macrophages [13].

The biological effects of vitamin D_3 are mediated by VDR, a member of the superfamily of nuclear hormone receptors [56]. Ligand binding to VDR induces conformational change, which promotes heterodimerization with RXR [54,56]. This heterodimeric complex then binds to VDR/RXR response element in target genes,

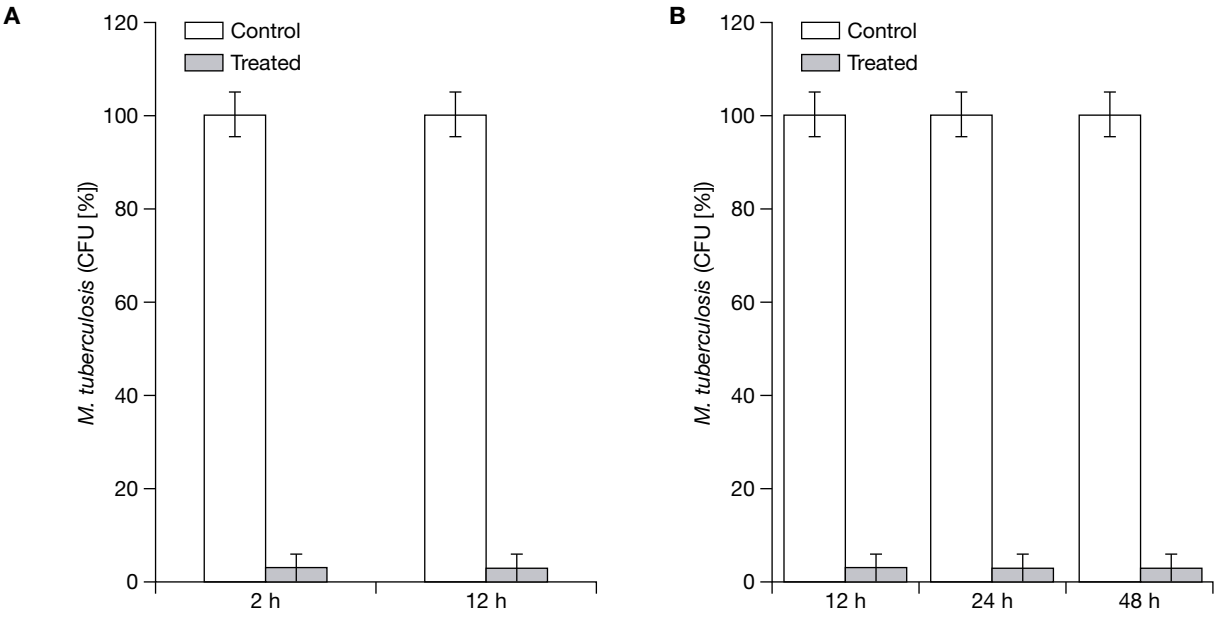

Fig. 3. Percent colony forming units (CFU) of *Mycobacterium tuberculosis* in THP-1 (human acute monocytic leukemia) macrophages at indicated time periods. (A) Mycobacteria in THP-1 macrophages exposed to vitamin D_3/retinoic acid (RA) for 24 h prior to infection. (B) THP-1 macrophages exposed to vitamin D_3/RA for 24 h after *M. tuberculosis* infection.

thus modulating their expression. In order to evaluate the modulation of TACO gene by vitamin D_3/RA, we analyzed the promoter region of TACO gene using a bioinformatics approach and found a VDR/RXR binding sequence (Fig. 1). In order to confirm that TACO gene down-regulation results from activation of VDR/RXR TF and its subsequent binding to the observed response element, we performed reporter assay using β-galactosidase reporter plasmid (carrying c-myc sequence coupled with VDR/RXR response sequence) in homogenous Jurkat T-cells and observed a significant decrease (>15 fold) in β-galactosidase activity.

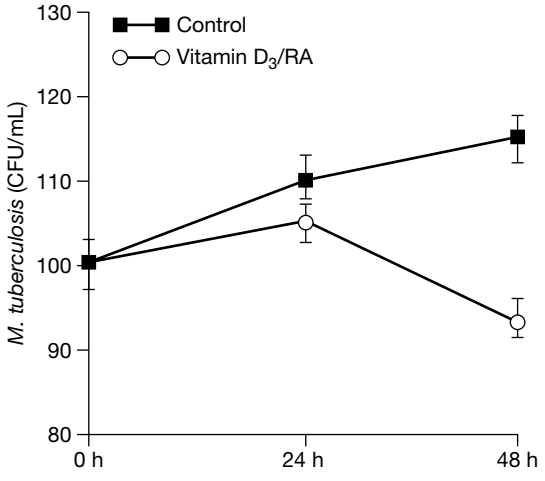

Fig. 4. Effect of vitamin D_3/retinoic acid (RA) each at a concentration of 1 μM on free-growing *Mycobacterium tuberculosis* H37Rv bacilli over a 48 h period.

The observed TACO gene down-regulation should lead to rescue of phagosome maturation arrest, leading to fusion of mycobacterial phagosome to lysosome, resulting in degradation of the pathogen. To test this hypothesis, we performed *M. tuberculosis* entry and survival experiments in THP-1/MDM, pretreated or post-treated with vitamin D_3/RA (Fig. 2 and Fig. 3). These experiments revealed that vitamin D_3/RA pretreatment decreased the mycobacterial entry by 33% as analyzed by flow cytometry (FACS) detection of FITC-labeled LAM. In the case of vitamin D_3/RA post-treatment, mycobacterial survival was restricted to only 26% (Fig. 2). However, the same experiment in THP-1 macrophages when analyzed by CFU revealed no mycobacterial growth at any time point (Fig. 3).

The apparent ambiguity in flow cytometry results and CFU can be resolved in that FACS data is a reflection of LAM-fluorescence associated with macrophages. LAM is a cell wall antigen of mycobacteria and could remain associated with macrophages even if mycobacteria are killed or are being processed within lysosomes. Vitamin D_3 has been shown to initiate a signaling pathway in human mononuclear phagocytes, involving activation of PI3K [57]. Similarly, another study [58] reported that vitamin D_3 treatment of *M. tuberculosis*-infected THP-1 cells activated PI3K. PI3K has also

been shown to be essential for the formation of the phagosomal cup (type-IA PI3K) and for fusion with lysosomes (type-III PI3K, hVPS34) in normal cells [59,60]. Studies using PI3K inhibitors have reported a role for this enzyme in regulating phagosome fusogenicity in response to vitamin D_3 [52]; vitamin D_3 treatment resulted in rescue of phagolysosome fusion arrest, the authors reporting a differential functional role of PI3K specifically in vitamin D_3-induced phagosome fusion with lysosomes.

Treatment with vitamin D_3 in experimental tuberculosis has been reported to cause a concentration-dependent decrease in interferon-gamma production and enhanced mycobacterial killing by NO production [61]. However, experiments in mice have disputed the exclusive role of NO-dependent effects of vitamin D_3 against tuberculosis [19]. Furthermore, one study reported that neither THP-1 human macrophages nor human MDM macrophages produced nitrite in response to vitamin D_3 [58]. In contrast, vitamin D_3 treatment of infected RAW mouse macrophages resulted in significant secretion of nitrite [58]. These findings demonstrate that the antimycobacterial action of vitamin D_3 is NO-independent in human macrophages, whereas the converse is true in murine cells.

The results reported in this study were observed in MDM and THP-1 human macrophages. Further, it is pertinent to note that the observed effects of vitamin D_3/RA on TACO gene expression were not evident when either of these was used alone [20]. This implies that vitamin D_3/RA act synergistically to activate VDR/RXR TF, that down-regulates TACO gene expression through the observed response sequence. This leads to maturation of the mycobacterial phagosome and subsequent killing of the pathogen. Furthermore, the results revealed that vitamin D_3/RA treatment inhibits mycobacterial entry as well as survival within macrophages, possibly through rescue of phagosome maturation arrest. The developing knowledge in this area suggests that vitamin D_3/RA may be of importance in the treatment of intracellular infection, particularly tuberculosis.

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