Effect of 1,25-dihydroxy vitamin D_3 on toll-like receptor gene expression in neutrophils of pulmonary tuberculosis

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We studied the effect of 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] on toll-like receptor (TLR) gene expression in neutrophils cultured with *Mycobacterium tuberculosis* (*Mtb*) with or without $1,25(OH)_2D_3$ in pulmonary tuberculosis patients and healthy controls. $1,25(OH)_2D_3$ significantly increased the expression of TLR2, TLR9 and TIRAP genes compared to control cells in both the study groups ($P < 0.05$). In healthy controls, a significantly upregulated expression of TIRAP was observed in *Mtb* infected cells cultured with $1,25(OH)_2D_3$ compared to *Mtb* infected cells alone ($P < 0.05$). In conclusion, $1,25(OH)_2D_3$ upregulated the expression of TLR and TIRAP genes, which could lead to the activation of TLR signalling pathways and augment the innate immunity to tuberculosis.

Keywords: Gene expression, neutrophils, tuberculosis, toll-like receptors.

TUBERCULOSIS (TB) is a granulomatous disease caused by *Mycobacterium tuberculosis* (*Mtb*). Neutrophils are the predominantly infected cells at the site of infection and play a pivotal role in innate immunity by means of phagocytosis, production of reactive oxygen species and apoptosis[1]. Apart from macrophages and dendritic cells, neutrophils also express toll-like receptors (TLRs) which act as pattern recognition receptors (PRRs) and are important components of innate immunity. Neutrophils express all TLRs, except TLR3 (ref. 2). TLR2 has been shown to recognize antigenic components such as 19 kDa lipoprotein[3] and lipomannan[4] of *Mtb*. TLR4 recognizes the heat-labile and cell-associated components of *Mtb*[5]. TLR8 recognizes guanosine (G) and uridine (U)-rich ssRNA oligonucleotides and TLR9 recognizes CpG DNA[6,7]. TLR9 has also been shown to cooperate with TLR2 to elicit immune response against *Mtb*[8].

The stimulation of TLRs results in recruitment of cytoplasmic adaptor proteins such as myeloid differentiation primary response protein 88 (MyD88) or TIR domain-containing adaptor protein (TIRAP, also known as MAL-MyD88-adaptor-like), leading to the activation of signalling cascades. Activation of signalling cascades culminates in the production of cytokines[9] and induction of autophagy[10]. TLR activation also induces the upregulation of VDR target genes resulting in the expression of antimicrobial peptide genes such as cathelicidin and defensin that culminates with the killing of *Mtb*[11].

The biologically active form of vitamin D_3, $1,25(OH)_2D_3$, can modulate both innate and adaptive immune responses to tuberculosis. Many studies have investigated the effect of $1,25(OH)_2D_3$ on TLRs in macrophages infected with *Mtb*. There is lack of studies on the effect of $1,25(OH)_2D_3$ on the immune functions of neutrophils. In the present study, we investigate the effect of $1,25(OH)_2D_3$ on the expression of TLR2, 4, 8 and 9 and TIRAP genes in neutrophils of pulmonary tuberculosis (PTB) patients and healthy control (HC) subjects.

The study subjects consisted of 20 PTB patients (mean age ± standard deviation (SD), 36 ± 11.19) and 23 HCs (mean age ± SD, 27.26 ± 6.04). Patients included were clinically and radiologically diagnosed for PTB and confirmed by sputum smear positivity for *Mtb*. All the PTB patients were negative for HIV and recruited before the commencement of anti-tuberculosis treatment. The patients were recruited from the clinics of National Institute for Research in Tuberculosis (NIRT; formerly Tuberculosis Research Centre), Chennai and the Institute of Thoracic Medicine, Chennai. HCs consisted of students and staff of city colleges and university belonging to the same geographical area. HCs were asymptomatic for TB based on physical examination and information about their health status. An informed consent was obtained from all the study subjects before blood collection, and the study was approved by the Institutional Ethical Committee of NIRT, Chennai. The study was conducted during December 2009 to April 2011.

Neutrophils were isolated from 20 ml of heparinized venous blood by Ficoll–Hypaque gradient centrifugation followed by sedimentation in 3% Dextran (Sigma, USA). The neutrophil-rich supernatant was collected and the residual red blood cells were removed by hypotonic lysis, washed in Hanks balanced salt solution (HBSS) and suspended in 1 ml RPMI 1640 tissue culture medium (Sigma). This cell preparation contained more than 95% neutrophils as judged by flow cytometry with anti-CD16 FITC antibody and viability was > 95% as assessed by the trypan blue exclusion method. Also, 2×10^6 neutrophils/ml were cultured in a 24-well culture plate (Costar, USA) with 10% autologous serum and infected with live *Mtb* H37Rv at 1 : 10 multiplicity of infection (MOI) with or without 1,25 dihydroxyvitamin D_3 (Sigma Chemicals Co) at 10^{-7} M concentration for 18 h at 37°C and 5% CO_2 (ref. 12) in an incubator (Heraeus, Kendro Laboratories, Germany). Since $1,25(OH)_2D_3$ was dissolved in 95% ethanol (ETOH), the ethanol-treated cells were used as control and the final concentration of ethanol did not exceed 0.05% in the cultures.

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Total RNA was extracted from the neutrophils and converted to cDNA as described earlier[13]. The relative quantification of the target genes was done by TaqMan assay method. Two microlitre cDNA was used as a template per reaction in a total volume of 20 µl. Validated TaqMan assay primers and probes and TaqMan Universal polymerase chain reaction (PCR) master mix (Applied Biosystems, USA) were used for the expression analysis of target genes – TLR2 (Hs00152932_m1), TLR4 (Hs00152939_m1), TLR8 (Hs00607866_mH), TLR9 (Hs00370913_s1), TIRAP (Hs00364644_m1) and housekeeping gene, β-actin. The RT-PCR conditions were 50°C for 2 min, 90°C for 10 min (polymerase activation), followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All analyses were performed in triplicate in an ABI Prism® 7500 Sequence detection system (Applied Biosystems, USA). Target genes were normalized to β-actin content and relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

The relative quantification of target genes was expressed as fold change. The data were presented as mean ± standard error (SE). Student's *t*-test (two-tailed) was used for data analysis. *P* value less than 0.05 was considered statistically significant. All computations were done using GraphPad Prism software (version 4).

$1,25(OH)_2D_3$ significantly upregulated the expression of TLR2 mRNA compared to ethanol-treated neutrophils in both HCs ($P = 0.0123$) and PTB patients ($P = 0.0087$). However, $1,25(OH)_2D_3$ did not have any effect on *Mtb*-infected cells (Figure 1). The expression of TLR4 and TLR8 gene was not different between $1,25(OH)_2D_3$ treated and untreated neutrophils, irrespective of the stimulation with or without *Mtb*. $1,25(OH)_2D_3$ significantly enhanced TLR9 gene expression in patients ($P = 0.0110$). In HCs, though five-fold change in the expression of TLR9 gene was observed in the $1,25(OH)_2D_3$-treated neutrophils compared to ethanol treated neutrophils, it was not statistically significant ($P = 0.0567$). Neutrophils from HCs when stimulated with *Mtb* showed approximately about ten-fold change in the expression of TLR9 compared to unstimulated cells ($P = 0.013$), while neutrophils from PTB patients showed about five-fold change ($P = 0.0069$). However, $1,25(OH)_2D_3$ did not have significant effect on the expression of TLR9 gene in *Mtb* stimulated neutrophils from both study groups (Figure 2). $1,25(OH)_2D_3$ upregulated TIRAP gene expression in both HCs ($P = 0.022$) and PTB patients ($P = 0.012$) compared to ethanol-treated cells. In HCs, $1,25(OH)_2D_3$ strongly induced TIRAP gene expression in *Mtb*-infected neutrophils compared to neutrophils infected with *Mtb* alone ($P = 0.038$). However, $1,25(OH)_2D_3$ did not affect the expression of TIRAP gene in *Mtb*-infected neutrophils from PTB patients (Figure 3).

Neutrophils are mobile phagocytes and involved in innate immunity and initiation of adaptive immunity against mycobacterial infection. The present study investigated the influence of $1,25(OH)_2D_3$ on the expression of TLR genes that are crucial for innate immune responses against *Mtb* in neutrophils. TLRs are the major players in the activation of host-defence mechanisms. TLR2 complexes with TLR1 (TLR2/1) and TLR6 (TLR2/6) to recognize various mycobacterial components such as the 19 kDa lipoprotein[3,4]. It is reported that *Mtb*-induced neutrophil apoptosis is accelerated via TLR2 stimulation and activation of p38 MAPK pathway. This apoptotic programme helps reduce the tissue injury caused by neutrophil-mediated inflammatory responses[14].

The results of the present study reveal that $1,25(OH)_2D_3$ significantly upregulates TLR2 gene expression in both

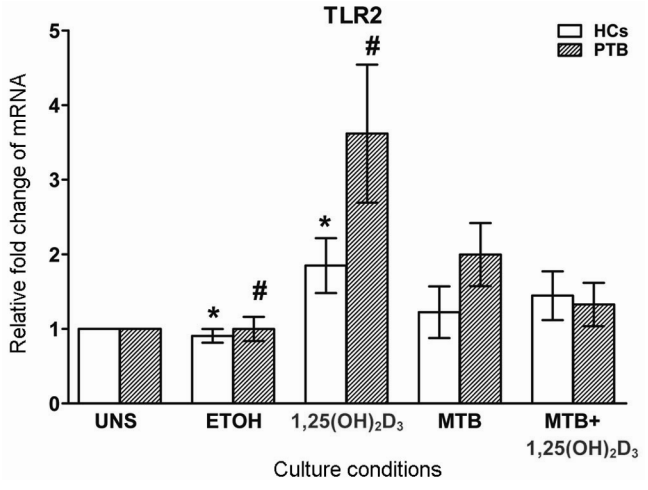

Figure 1. Effect of 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] at 10^{-7} M concentration on the relative expression of TLR-2 gene in neutrophil cultures of PTB patients ($n = 20$) and normal HCs ($n = 23$). TLR-2 gene expression was quantified (relative quantification) by real-time PCR and normalized to housekeeping gene, β-actin and fold induction over unstimulated cultures was calculated by ΔΔCT method. Results are expressed as mean ± SE. In EtOH versus $1,25(OH)_2D_3$: $^{*}P = 0.0123$, $^{\#}P = 0.0087$.

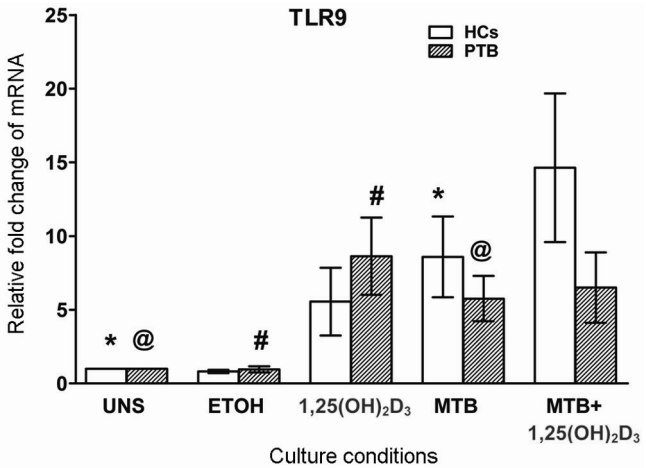

Figure 2. Effect of $1,25(OH)_2D_3$ on TLR-9 gene expression in neutrophil cultures of HCs ($n = 23$) and PTB patients ($n = 20$). In ETOH versus $1,25(OH)_2D_3$: $\#P = 0.0110$. In UNS versus *Mtb*: $^{*}P = 0.013$, $^{@}P = 0.0069$.

the study groups compared to ethanol-treated control cells. *Mtb* ligation with TLR2 triggers the vitamin D conversion from 25-hydroxy vitamin D_3 to $1,25(OH)_2D_3$ through the activation of VDR and subsequent induction of CYP27B1 (enzyme associated with $1,25(OH)_2D_3$ biosynthesis pathway) gene expression leading to induction of antimicrobial peptide cathelicidin in its active LL-37 form[11], which is associated with innate immunity. It has been suggested that increased expression of CYP27B1 on TLR2/1 stimulation depends on CAAT/enhancer binding protein (C/EBP)-β and subsequent activation of mitogen-activated protein kinase (MAPK)[15]. We did not observe any significant difference in TLR2 gene expression in *Mtb* with $1,25(OH)_2D_3$ stimulated cells compared to cells stimulated with *Mtb* alone. It has been shown that $1,25(OH)_2D_3$ downregulated TLR2 and TLR4 gene expression upon *Mtb* stimulation that resulted in the decreased production of proinflammatory cytokines[16]. The absence of increased TLR2 expression in *Mtb* with $1,25(OH)_2D_3$ stimulated cells in the present study suggests that during *Mtb* infection, $1,25(OH)_2D_3$ may repress the TLR2 gene expression leading to modulation of inflammatory cytokine response.

TLR2 and TLR4 are key sensors for *Mtb* and its various antigens[3–5]. However, in the present study, we did not observe any significant effect of $1,25(OH)_2D_3$ on expression of TLR4 and TLR8 genes in neutrophils. TLR9 gene expression was significantly increased on *Mtb* infection alone in both study groups. The study shows that DNA from mycobacteria contains stimulatory CpG motifs that activate TLR9 and upregulate Th1 cytokine response[8]. Stimulation of TLR9 induces autophagy by microtubule-associated protein 1A/1B light chain 3 (LC3) localization and phagosome maturation[17]. In the present study, we observed increased expression of TLR9 gene in $1,25(OH)_2D_3$ treated cells. The study shows that $1,25(OH)_2D_3$ is known to upregulate the expression of TLR9 gene through IL-10-dependent mechanism and adequate concentration of $1,25(OH)_2D_3$ is required to sustain the expression of both IL-10 and TLR9 genes[18]. Moreover, the ligation of TLR9 by CpG is shown to inhibit $1,25(OH)_2D_3$ mediated IL-10 synthesis, which may downregulate TLR9 gene expression[18]. This could be one of the reasons for the absence of increased TLR9 gene expression in *Mtb* with $1,25(OH)_2D_3$ stimulated cells. The results of the present study suggest that $1,25(OH)_2D_3$ upregulated TLR9 gene expression may enhance the antimicrobial activity of macrophages by inducing interferon-γ production[8].

The adaptor protein for Mal (MyD88 adaptor-like) encoded by the gene TIRAP is essential for MyD88-dependent signalling downstream of TLR2 and TLR4 (ref. 9). After stimulation of TLR2 or TLR4, Mal triggers a signalling cascade, which is involved in the activation of the transcription factor NF-kB and the subsequent activation of proinflammatory genes[9]. In the present study, $1,25(OH)_2D_3$ significantly enhanced the TIRAP gene expression in both the study groups compared to ethanol-treated control cells. Moreover, $1,25(OH)_2D_3$ significantly upregulated the expression of TIRAP gene in neutrophils stimulated with *Mtb* in HCs. The results suggest that $1,25(OH)_2D_3$ may induce TIRAP gene expression leading to activation of downstream signalling pathways associated with TLR stimulation. The lower expression in PTB patients compared to HCs in neutrophils infected with *Mtb* suggests that in PTB patients, genes might have become unresponsive due to repeated stimulation with *Mtb*. This may probably be due to host genetic factors or some other components of *Mtb* which might have downregulated the activation mechanism. The genetic variation in TIRAP gene attenuates TIRAP function and results in reduced activation of MAPK and NF-kB signalling cascades and impaired production of cytokines[19]. In an earlier study, we observed significantly increased frequency of T allele of TIRAP 975C/T220 polymorphism in PTB patients compared to HCs[20], which may be associated with defective signalling and reduced immune response. Hence, the genetic variations in the TIRAP gene could affect the expression of TIRAP observed in the present study.

In conclusion, $1,25(OH)_2D_3$ increased the expression of TLRs and TIRAP gene expression in neutrophils. This increased expression may enhance the TLRs signalling pathways, which could trigger the production of antimicrobial peptides and cytokines at the site of infection that may enhance the immunity against tuberculosis.

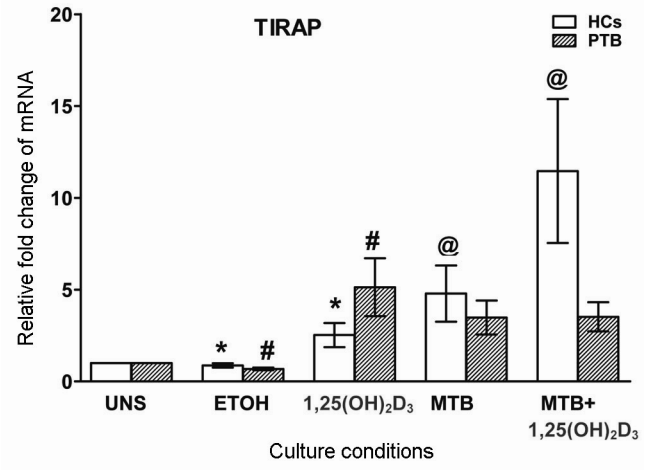

Figure 3. $1,25(OH)_2D_3$ and TIRAP gene expression in neutrophil cultures of HCs ($n = 23$) and PTB patients ($n = 20$). In ETOH versus $1,25(OH)_2D_3$: $^{*}P = 0.0220$, $^{\#}P = 0.0120$. In HCs, *Mtb* versus *Mtb* + $1,25(OH)_2D_3$: $^{@}P = 0.0387$.

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The essential oil of 'bhang' (*Cannabis sativa* L.) for non-narcotic applications

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***Cannabis sativa* L. (family Cannabaceae) is a medicinal and aromatic plant growing all over the world. The present study aims to investigate the essential oil composition and antimicrobial activity of *C. sativa* from the foothills of northern India. The hydrodistilled essential oil of *C. sativa* was studied by capillary gas chromatography/flame ionization detector (GC-FID) and GC-mass spectrometry (GC-MS) and evaluated against nine pathogenic bacterial strains using disc diffusion assay. A total of 57 constituents representing 90.5–93.1% of the total oil compositions were identified. Major constituents of the essential oil were (*E*)-caryophyllene (19.6–26.1%), limonene (4.1–15.8%), caryophyllene oxide (2.0–10.7%), (*E*)-β-farnesene (4.8–8.5%), α-humulene (5.4–7.8%), α-pinene (0.7–7.7%), myrcene (0.8–6.0%), terpinolene (0.2–6.0%) and β-selinene (1.8–5.4%). The oil showed moderate to good activity against most of the tested Gram-positive bacteria (*Staphylococcus aureus* (MTCC2940), *Staphylococcus aureus* (MTCC96) and *Streptococcus mutans*). The oil also showed moderate activity against a Gram-negative bacterium, *Salmonella typhimurium*. The chemical composition of the examined *C. sativa* essential oil was quite different from earlier reported compositions. The oil possessed moderate to good activity against most of the tested bacterial strains.**

Keywords: Antibacterial activity, *Cannabis sativa*, essential oil composition, non-narcotic applications.

CANNABIS sativa L., commonly known as 'bhang' or marijuana, an annual herb of the family Cannabaceae, is a hemp plant that grows freely throughout the world. In India, the plant is distributed throughout the Himalayan foothills and the adjoining plains, from Kashmir in the west to Assam in the east. It has become acclimatized in the plains of India and grows even in the warm climate of southern India[1]. It has been used to treat an array of ailments in the Indian traditional systems of medicine[1]. Bhang is being used as an anaesthetic and antiphlegmatic[2] since before to the 10th century BC. The *C. sativa* plant is known today as a potent psychoactive substance, but for many years it was harvested primarily for

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