Mycobacterium tuberculosis DNA Increases Vitamin D Receptor mRNA Expression and the Production of Nitric Oxide and Cathelicidin in Human Monocytes

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Abstract

Background: The innate immune response to tuberculosis infection may involve the increased production of nitric oxide and cathelicidin due to the up-regulated expression of the vitamin D receptor (VDR), though this proposed mechanism remains controversial. The aim of this study was to determine how the exposure of human monocytes to Mycobacterium tuberculosis (*M. tuberculosis*) DNA affects the production of nitric oxide and cathelicidin, as well as the expression of VDR.

Methods: This study was performed using monocytes obtained from healthy donors. After 24 h incubation, monocytes were stimulated with *M. tuberculosis* DNA for 18 h to determine the expression of VDR mRNA and the production of nitric oxide and cathelicidin versus non-stimulated cells (the control group).

Results: The expression of VDR mRNA was higher in the monocytes exposed to *M. tuberculosis* DNA compared to the control group ($P = 0.020$). Monocytes exposed to *M. tuberculosis* DNA also showed significantly increased production of nitric oxide and cathelicidin compared to the control group ($P = 0.0001$; $P = 0.028$).

Conclusion: The stimulation of human monocytes with *M. tuberculosis* DNA increases the expression of the VDR mRNA and the production of nitric oxide and cathelicidin.

Keywords: monocytes, Mycobacterium tuberculosis DNA, nitric oxide, cathelicidin, tuberculosis

Introduction

Host defence mechanisms against pathogenic microorganisms consist of innate and acquired immunity. The activation of innate immunity occurs several minutes after infection and is responsible for defence during the initial hours and days of the infection (1). One important factor in innate immunity is the role of toll-like receptors (TLRs). TLRs are mediators of innate immunity that are essential for microbial recognition by monocytes/macrophages and for bridging the innate and acquired immune responses (1). Previous studies have shown that stimulation of TLRs by microbial components triggers the induction of inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12 (2,3). In mice, inducible nitric oxide synthase (iNOS) is capable of catalysing production of a large amount of nitric oxide (NO) when cells are induced by mediators, such as interferon gamma (IFN)-γ, TNF-α, IL-1, IL-2, IL-6, lipopolysaccharide (LPS), and a number of microorganisms (4). However, under similar conditions, human macrophages produce either low levels of NO (5,6) or none at all (7,8).

In addition to increasing NO production, TLR stimulation by its ligands also increases cathelicidin production. Liu et al., demonstrated that TLR2/1 activation in human macrophages

up-regulates the expression of the vitamin D receptor and the vitamin D1–hydroxylase genes, leading to the induction of the antimicrobial peptide cathelicidin and the killing of intracellular Mycobacterium tuberculosis (*M. tuberculosis*) (9). In contrast to the study of Liu et al., our study focused on the response of NO and cathelicidin production, as well as VDR mRNA expression, to stimulation with the TLR9 ligand *M. tuberculosis* DNA.

Therefore, we studied the expression of vitamin D receptor mRNA and the production of NO and cathelicidin in human monocytes after stimulation with *M. tuberculosis* DNA, demonstrating increases in all of these parameters.

Materials and Methods

Human monocyte donors

Monocyte donors consisted of 12 healthy subjects that were recruited at the Faculty of Medicine, University of Brawijaya in Malang, Indonesia. Exclusion criteria were as follows: acquired immune deficiency syndrome (AIDS), diabetes mellitus, malignancy, corticosteroid treatment, pregnancy, breastfeeding, renal failure, liver dysfunction, positive acid fast bacilli (AFB) on sputum smear examination or an abnormal chest X-ray.

Study design

The study was conducted at the Biomedical Laboratory of the Faculty of Medicine, University of Brawijaya. Monocyte culture was performed with and without the addition of *M. tuberculosis* DNA (5 µg/mL). Cells were cultured for 18 h prior to determining the production of NO by the Griess reaction, the expression of vitamin D receptor mRNA by real-time polymerase chain reaction (rt-PCR) and the production of cathelicidin by immunocytochemistry.

Culture of Mycobacterium tuberculosis

Mycobacterium tuberculosis H37Rv was used for the study and stored on Lowenstein-Jensen medium at 4 °C. The bacteria were cultured in Middlebrook 7H9 broth medium at 37 °C. After four weeks, the culture was centrifuged at 5,000 rpm for 5 min and its concentration adjusted spectrophotometrically to a density of 2×10^9 bacteria/mL phosphate buffer saline (PBS).

Mycobacterium tuberculosis DNA isolation and preparation

Mycobacterium tuberculosis DNA was extracted using the Genomic DNA Mini Kit (Geneaid, Cat. No. GB 100/300) following the manufacturer's instructions. Briefly, a bacterial cell culture (up to 1×10^9 bacteria/mL) was transferred to a 1.5 mL micro-centrifuge tube and centrifuged for 1 min at 14 000× g. The supernatant was discarded, and the pellet was re-suspended in 200 µL of GT buffer with vigorous shaking and incubated at room temperature for 5 min. For the lysis step, 200 µL of GB buffer was added to the cell suspension and vigorously shaken for 5 sec then incubated at 60 °C for at least 10 min. The cell suspension was transferred to a GD column and centrifuged at 14 000× g for 2 min. After placing the GD column into a new collection tube, 400 µL of W buffer was added, and the column was centrifuged at 14 000× g for 30 sec. The dried GD column was transferred to a clean, 1.5 mL microcentrifuge tube, and 100 µL of pre-heated Elution buffer was added to the centre of the column matrix. The column was left standing at least 3 min to ensure that the Elution buffer was absorbed by the matrix. Finally, the column was centrifuged at 14 000× g for 30 sec to elute the purified DNA. The concentration and purity of the DNA was determined spectrophotometrically using a UV-Visible Spectrophotometer (UV-1601, Shimadzu).

Culture of monocytes

Blood was drawn from donors with a heparinised vacutainer. The blood was then diluted 1:1 with PBS, layered on Ficoll-Hypaque 1077 (MP Bio, Lymphocyte Separation Medium, Cat. No. 50494X) and centrifuged for 30 min at 400× g. Peripheral blood mononuclear cells (PBMCs) were isolated from the interface and washed with PBS. The pellet was suspended in 10% foetal bovine serum (FBS)-containing RPMI medium, adjusted to 5×10^5 cells/mL, placed in 24-well culture plates and incubated overnight at 37 °C and 5% CO_2. Next, non-adherent cells were removed by washing with PBS, and adherent cells were retained using 0.2% Trypsin EDTA. A total of 95% of the resulting cells were monocytes as determined by flow cytometry using a mode of Cellquest pro with CD 14PE (Biolegend, Cat. No. 325605). Monocytes were incubated in RPMI and 10% FBS and cultured for 18 h with or without *M. tuberculosis* DNA (5 µg/mL). The culture supernatant was used to determine NO production. Adherent cells from the culture plate were removed by administering 0.2% Trypsin EDTA solution and used for immunostaining (to monitor cathelicidin production) and DNA isolation (to measure the expression of vitamin D receptor mRNA using rt-PCR).

Expression of vitamin D receptor mRNA

RNA from up to 3×10^6 monocytes was extracted using the Total RNA Mini Kit (Geneaid, Cat. No. RB 100) following the manufacturer's instructions. The RNA was used to produce cDNA using the Transcriptor First Strand complementary deoxyribonucleic acid (cDNA) Synthesis Kit (Roche, Cat. No. 04896866001) following the manufacturer's instructions. Briefly, reverse transcription was performed using the template RNA, 2.5 µM oligo(dT), 2.5 µM specific primer, 20 units RNase inhibitor (20 unit/µL), 1 mM deoxynucleotides and 1× Transcriptor Reverse Transcriptase Reaction Buffer. The cDNA was used to determine the expression of the VDR gene using a Fast Start Universal Sybr Green rt-PCR kit (Roche, Cat. No. 04913850001). For rt-PCR, 2.3 µL (50 ng) of the template cDNA was added to 9 µL PCR-grade water, 1 µL forward primer (5 pmol/µL) (5"-CTTCAGGCGAAGCATGAAGC-3") (10), 1 µL reverse primer (5 pmol/µL) (5"-CCTTCATCATGCCGATGTCC-3") (10), and 4 µL Master Mix. The rt-PCR mixture was denatured at 95 °C for 10 min (1 cycle), amplified at 72 °C for 10 min and cooled at 40 °C for 30 min, followed by measurement using a rt-PCR machine. To detect contamination, a negative control was included in each PCR run. Standard curves of PCR product dilutions were generated in each PCR run. The data are presented as absolute copies of cDNA.

Production of nitric oxide

As a measure of the production of NO, the concentration of nitrite produced by the monocytes was determined at 540 nm using Griess reagent (R&D Systems Cat. No. KGE 001, SKGE 001, PKGE 00) in a spectrophotometer. Briefly, 50 µL of supernatant was removed from the monocyte culture wells, centrifuged at 400× g for 10 min to remove cells and incubated with equal volumes of Griess reagent I (Sulfanilamide in 2 N hydrochloric acid) and Griess reagent II (N-(1-Naphthyl) ethylenediamine in 2 N hydrochloric acid) at room temperature for 10 min. The concentration of nitrite was determined using sodium nitrite as a standard.

Production of cathelicidin

Immunocytochemistry was used to monitor the production of cathelicidin in human monocytes. Briefly, monocytes were incubated with primary antibody LL-37 (Santa Cruz, D-5 mouse monoclonal IgG, Cat. No. sc-166770) and stained with an immunostaining kit (DakoLSAB+System HRP, Cat. No. K0679, Cat. No. K0690) following the manufacturer's instructions. The slides were counterstained with Mayer's haematoxylin 10%. The resulting slides were blinded and observed by a competent specialist. The percentage of monocytes expressing cathelicidin was calculated as the average of 20 fields (×400).

Ethics

This research has been approved by the research ethics committee of the Faculty of Medicine, University of Brawijaya, Malang, Indonesia.

Statistical analysis

The results are presented as the mean (SD). The independent t test was used to assess the differences between two groups when a normal distribution of the data was present (Shapiro-Wilk test, > 0.05). Differences were considered statistically significant at $P < 0.05$ (2-sided). Statistical analysis was performed with IBM SPSS Statistics 20.

Results

Expression of vitamin D receptor mRNA in monocytes exposed to M. tuberculosis DNA

Figure 1 and table 1 show the expression of vitamin D receptor mRNA, which was determined using rt-PCR, in monocytes exposed to *M. tuberculosis* DNA versus the control group. The monocytes that were exposed to *M. tuberculosis* DNA have higher expression levels of vitamin D receptor mRNA (cDNA copies 34.7×10^6 (SD 29.9×10^6) compared to the control group (cDNA copies 5.6×10^6 (SD 3.4×10^6); $P = 0.020$).

Production of nitric oxide in monocytes exposed to M. tuberculosis DNA

Figure 2 and table 1 show the production of NO, which was determined using Griess reagent, in monocytes exposed to *M. tuberculosis* DNA versus the control group. Monocytes exposed to *M. tuberculosis* DNA showed significantly increased production of NO 13.75 µmol/L (SD 4.36) compared to the control group 5.72 µmol/L (SD 3.31); $P = 0.0001$.

Production of cathelicidin in monocytes exposed to M. tuberculosis DNA

Figure 3 and table 1 show the percentage of cathelicidin-positive cells by immunocytochemistry in monocytes exposed to *M. tuberculosis* DNA versus the control group. Monocytes exposed to *M. tuberculosis* DNA have a higher percentage of positive cells 70.12%

Table 1: Expression of vitamin D receptor mRNA, production of NO and cathelicidin in the stimulated monocyte cells and controls

	Stimulated monocyte cells	Controls	*P* value (2-tailed)
NO Productions (μmol/L)	13.75 (SD 4.36)	5.72 (SD 3.31)	0.0001*
Cathelicidin Productions (% positive cells)	70.12 (SD 29.54)	43.09 (SD 26.55)	0.028*
Expression of VDR mRNA (cDNA copies)	34.7×10^6 (SD 29.9×10^6)	5.6×10^6 (SD 3.4×10^6)	0.020*

* Independent *t* test, significantly different ($P < 0.05$).
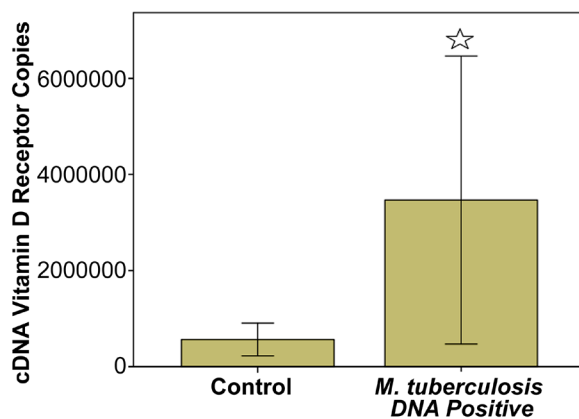Data are means (SD$_S$).

Figure 1: Expression of vitamin D receptor mRNA. Monocytes which were exposed to *M. tuberculosis* DNA for 18 h have higher expression compared to control group. Data shown are means (SD$_S$) and representative of 12 individual experiments. * *P* = 0.020.
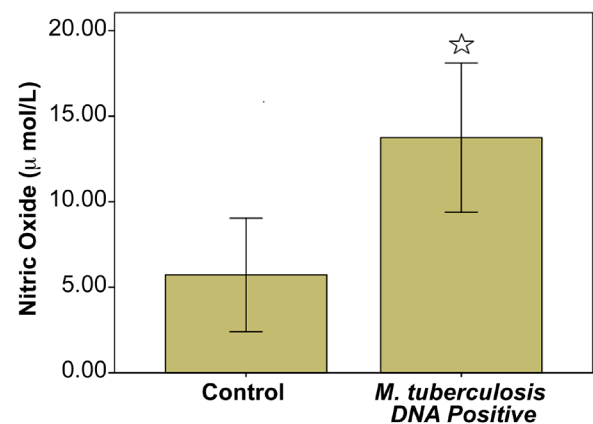

Figure 2: The production of nitric oxide after stimulation of monocyte with *M. tuberculosis* DNA for 18 h. The production of NO was significantly higher in monocytes exposed to *M. tuberculosis* DNA compared to control group. Data shown are means (SD$_S$) and representative of 12 individual experiments. * *P* = 0.0001.

(SD 29.54%) compared to the control group 43.09% (SD 26.55%); *P* = 0.028. Figure 4a, show that immunostaining was very strong in monocyte exposed to *M. tuberculosis* DNA, in contrast, immunostaining was weaker in the control group (Figure 4b).

Discussion

In our study, we have found that normal human monocytes are capable of producing a small amount of NO, but this production increases when human monocytes are stimulated by *M. tuberculosis* DNA. Thus, bacterial components are able to induce a pathway that can increase production of nitric oxide. Previously reported differences in the apparent ability of monocytes to produce NO could be explained by the different bacterial components used for stimulation. Previous research by Sook Lee et al., reported that human macrophages stimulated with the Purified Protein Derivate (PPD) antigen of *M. tuberculosis* produce low levels of NO, under 10 μmol/L (11). However, another report indicated that stimulation of human monocytes with *M. tuberculosis* H37Rv components, such as whole cell lysate (WCL), culture filtrate protein (CFP), and lipoarabinomannan (LAM), produces a large
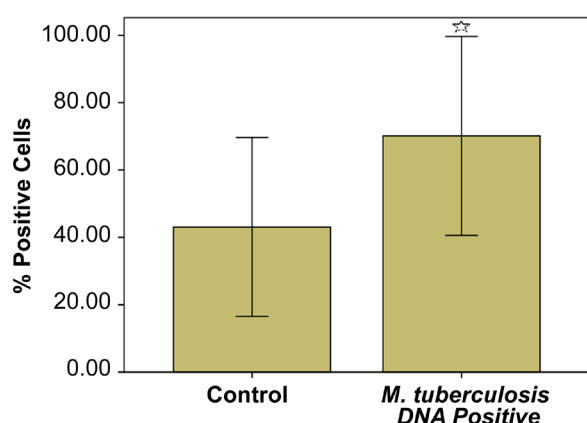
Figure 3: Percentage of cells positive for cathelicidin detected by immunochemistry. Monocytes which exposed with *M. tuberculosis* DNA for 18 h have higher cathelicidin production compared to control group. Data shown are means (SD$_S$) and representative of 12 individual experiments. * P = 0.028.
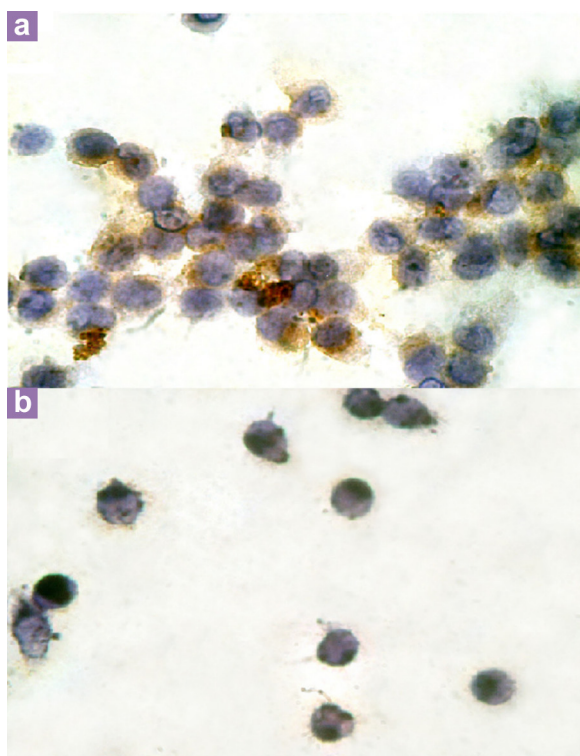

Figure 4: Monocytes exposed with *M. tuberculosis* DNA showed the higher percentage of positive cells for cathelicidin detected by immunocytochemistry (a); compared to control (b). The immunostaining for the monocytes exposed with *M. tuberculosis* DNA was stronger than control (400× magnifications).

amount of NO (47 μmol/L, 47 μmol/L, and 42 μmol/L, respectively) (12). The increased NO production is explainable because *M. tuberculosis* DNA is a known ligand of TLR9, and the observed effect could be associated with the stimulation of this receptor (3,13,14). Lee et al., revealed that TLRs, cytokines, and NOS act cooperatively in the innate immune response towards otitis media with effusion (15). Another previous study showed that elements of the innate immune response, such as pattern recognition receptors (PRRs), TLR-2, TLR-9, cytokines, and NOS, are increased in inflammatory diseases, as in the case of patients with endometriosis (16). It has been reported that ligand-stimulated TLRs initiate signalling pathways; the signal transduction is initiated from the Toll/interleukin-1 receptor (TIR) domain of TLRs after pathogen recognition and culminates with the activation of the transcription factor nuclear factor kappa B (NF-kB (1,17), which controls the expression of inflammatory cytokine genes such as TNF-α and IL-12 (18–20). Chan et al., reported that iNOS is capable of catalysing production of NO when cells are induced by mediators such as TNF-α and IL-12 (4). Based on these previous reports, it is possible that activation of TLR9 by *M. tuberculosis* DNA will induce the activation of NF-kB, promoting the transcription of inflammatory cytokines with the consequent induction of NO production.

In addition to increasing NO production, TLR activation also triggers direct antimicrobial activity in innate immunity (21,22). Our results showed that monocytes exposed to *M. tuberculosis* DNA have increased expression of VDR mRNA and production of cathelicidin compared to the control. This finding is similar to a previous study that showed that TLR2/1 activation of monocytes/macrophages up-regulated the expression of the VDR and the vitamin D hydroxylase genes, leading to the induction of cathelicidin production and the killing of intracellular *M. tuberculosis* (9). Liu et al., showed that treatment of THP-1 cells with 1,25-dihydroxyvitamin D$_3$ up-regulated cathelicidin mRNA expression, which was correlated with increased expression of the cathelicidin protein (23). With respect to cathelicidin production, our findings are also consistent with a previous study showing that production of cathelicidin in human monocyte-

derived macrophages exposed to *M. tuberculosis* DNA is increased (24).

Conclusion

This study shows that stimulation of human monocytes with *M. tuberculosis* DNA increases the expression of VDR mRNA and the production of NO and cathelicidin, which could be important for defence against *M. tuberculosis*. These results suggest that TLR9 could be an important element in the innate immune response to *M. tuberculosis* upon activation with mycobacterial components, such as DNA. Future studies using cells with and without TLR9 expression should be carried out to validate this hypothesis.

Conflict of Interest

None.

Authors' Contributions

Conception and design, analysis and interpretation of the data, critical revision of the article for the important intellectual content: SS, KH, LEF, SRP
Drafting of the article, statistical expertise: SS, LZ
Final approval of the article: SS, LEF
Provision of study materials or patient, administrative, technical or logistic support, collection and assembly of data: LZ

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