Low serum vitamin D levels in type 2 diabetes patients are associated with decreased mycobacterial activity

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Abstract
Background: Concurrent diabetes mellitus and tuberculosis represent a significant health problem worldwide. Patients with diabetes mellitus have a high risk of tuberculosis, which may be mediated by an abnormal innate immune response due to hyperglycaemia or low vitamin D levels.

Methods: In the present study, we evaluated inactive vitamin D serum levels and the monocyte response to infection with M. tuberculosis, including phagocytosis of M. tuberculosis, antimycobacterial activity, LL-37, human β defensin-2 and IL-10 gene expression and nitric oxide production, between type 2 diabetes mellitus patients (n = 51) and healthy volunteers (n = 38).

Results: Twenty-seven type 2 diabetes mellitus patients had inadequate inactive vitamin D levels (<50 nM). The percentages of M. tuberculosis phagocytosis between monocytes were similar across groups according to microscopy. Intracellular mycobacterial growth was similar in infected monocytes from both groups. However, M. tuberculosis growth was significantly higher in monocytes obtained from type 2 diabetes mellitus patients and lower vitamin D levels after 1-h (D0) and 72-h (D3) post-infection (p ≤ 0.05). LL-37, human β defensin-2 and IL-10 mRNA expression were similar between monocytes across groups; vitamin D serum levels and LL-37, human β defensin-2 and IL-10 expression were not correlated. Nitric oxide production was significantly higher in healthy volunteers than in type 2 diabetes mellitus patients with low vitamin D serum levels at D3 post-infection (p ≤ 0.05).

Conclusions: Our results show that monocytes from type 2 diabetes mellitus patients and low vitamin D serum levels show an impaired ability to control the intracellular growth of M. tuberculosis, which is not associated with significant decrease of LL-37 or human β defensin-2 expression. Vitamin D could be the link between diabetes and tuberculosis susceptibility.

Keywords: Type 2 diabetes mellitus, Vitamin D, Human monocytes, Tuberculosis, Mycobacterium tuberculosis

Background
Vitamin D insufficiency is a risk factor for osteomalacia in adults and rickets in children and is associated with a variety of conditions, such as various cancers, type 1 and 2 diabetes, hypertension, multiple sclerosis, metabolic syndrome and infectious diseases, including tuberculosis [1–4].

The human body can synthesize vitamin D from the precursor molecule 7-dehydrocholesterol, which is generated by the skin upon sufficient exposure to solar ultraviolet radiation. Vitamin D is also obtained from natural food, particularly from fish. Since the final effector molecule of vitamin D is produced in humans, vitamin D is also considered a pro-hormone [5]. Vitamin D status is determined by measuring circulating 25-hydroxyvitamin D (25OHD3), which is the predominant inactive form in plasma or serum and is the first hydroxylation product of vitamin D that is synthesized in the liver.
Currently, there is a debate regarding the definition of vitamin D insufficiency. Although the majority of Working Group members nominated 75 nM as the appropriate target concentration for older individuals in the International Osteoporosis Foundation position statement [6]. However, other groups have opted for a lower target of 50–75 nM. The North American Institute of Medicine has classified serum 25OHD3 levels of 50 to 125 nM as sufficient, levels between 30 and 49.99 nM as inadequate, levels below 30 nM as deficient and levels above 125 nM as potentially harmful [6].

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), influences pancreatic β-cells and insulin secretion [7]. Although a systemic review and meta-analysis that included clinical trials on the effect of vitamin D on glycaemic control in persons with diabetes mellitus (DM) have not shown an effect, previous reports have shown a clear association between low levels of vitamin D and DM.

In persons with established DM and in the general population, low levels of 25OHD3 are associated with higher fasting glucose, insulin resistance and metabolic syndrome [8].

Vitamin D plays a prominent role in innate immunity, and vitamin D receptors (VDR) are found on monocytes; these cells differentiate into macrophages under the influence of 1,25(OH)2D3 [9]. The vitamin D also is active in many other cells, such as keratinocytes, epithelial cells and natural killer cells [10, 11]. Vitamin D deficiencies are known to affect host immunity [12]. Vitamin D exerts regulatory activity to modulate memory and effector CD8+, CD4+, regulatory T cells and regulation of cytokines [3, 13]. In macrophages, this vitamin modulate phagocytosis and autophagy between others mechanism [14]. The antimicrobial response induced by Vitamin D involves recognition of bactericidal lipoproteins by Toll-like receptors (TLRs), induction of the 25-hydroxyvitamin D3–1α-hydroxylase (CYP27B1), which converts the vitamin D prohormone (25OHD3) into the active 1,25(OH)2D3 form and the activation of the VDR that directly modulate gene expression that favours antimicrobial peptides production [15].

Vitamin D restricts intracellular Mycobacterium tuberculosis replication via the induction of antimicrobial peptides such as cathelicidin (LL-37) and human β defensin-2 (HBD2) [15, 16]. Previous studies have reported that 1,25(OH)2D3 induces differentiation of the human monocytic THP-1 cell line and enhances the chemotactic and phagocytic activity of macrophages [17, 18]; 1,25(OH)2D3 also plays a role in acquired immunity directly by acting on T cells and indirectly by regulating dendritic cells; it also restricts Th1/Th17 cell differentiation and favours Th2 response [10].

In addition, previous research has shown that DM triples the risk of tuberculosis (TB) [19]. Several studies have shown an association between low levels of vitamin D and both TB and DM [7, 20, 21].

The high prevalence of DM in Mexico results in a considerable proportion of TB cases attributable to this disease [19]. The susceptibility to M. tuberculosis infection observed in persons with DM suggests an impaired immune response, and vitamin D could be the link between DM and TB susceptibility [22].

Therefore, in this work, we evaluate the correlation between serum levels of vitamin D in type 2 diabetes mellitus (T2D) patients and antimycobacterial activity.

**Methods**

**Participants**

Fifty-one T2D patients (17 man/34 woman) and 38 people healthy volunteers (12 man/26 woman) were enrolled in this study from 2008 to 2015. The T2D patients were invited to participate in the study at the Metabolic Syndrome Clinic of the National Institute of Respiratory Diseases in Mexico City. They were diagnosed according to the criteria of the American Diabetes Association (Diagnosis and Classification of DM) [23], and active TB was ruled out by clinical and chest X-ray findings. Additionally, healthy volunteers were invited to participate in this study. All participants provided written informed consent, and routine laboratory tests on blood, as well as chest X-rays and PPD tests, were conducted. The Ethics Committee of the National Institute of Respiratory Diseases approved this study.

**Determination of vitamin D**

Blood samples were drawn from all participants, and the serum samples were separated and stored at −80 °C until determination of the inactive form of vitamin D (25OHD3). Serum 25OHD3 levels were determined using a commercial serum vitamin D kit (Liaison, Diasonin MN, USA) according to the manufacturer’s instructions and reported as nM.

**Monocyte isolation**

Sixty millilitres of heparinized human peripheral venous blood was obtained from the participants, and peripheral blood mononuclear cells were obtained from whole blood by centrifugation using Lymphoprep® (Nycomed Pharma, Oslo, Norway) [24].

Monocytes were enriched by positive selection using MACS® magnetic beads coupled to anti-human CD14 antibodies (Miltenyi Biotech, Auburn, CA), according to the manufacturer’s recommendations. The purity of monocytes was assessed by conventional flow cytometry using anti-human CD14 antibodies. The cell preparations were routinely >95% pure, and viability was evaluated using the trypan blue exclusion assay, which revealed 99% viability.
Media and cell culture
Monocytes were cultured in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 50 μg/ml gentamicin sulfate, 2 mM L-glutamine and 10% heat-inactivated pooled human serum (Gemini Bioproducts, Sacramento, CA) at 37 °C under a 5% CO₂ atmosphere.

Preparation of M. tuberculosis H37Ra
The M. tuberculosis strain H37Ra (ATCC # 25177, Manassas, VA) was prepared in Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, MI) supplemented with 10% albumin-dextrose-catalase (ADC, Difco Laboratories) and 0.2% glycerol. The mycobacteria were incubated for 21 days at 37°C with 5% CO₂ on an orbital shaking incubator, and the M. tuberculosis suspension was harvested, centrifuged (1972 g, 15 min, 25 °C), resuspended in fresh 7H9 medium and aliquoted into one-millilitre samples that were stored at −70 °C until use. The mycobacteria stock concentration was determined by colony forming units (CFU) counts from ten-fold serial dilutions in 7H9 medium after 21 days of incubation on 7H10 agar. For infection assays, M. tuberculosis stock was thawed and centrifuged (5220 g, 8 min, 25 °C), and the supernatant was replaced by complete medium (supplemented medium with 10% non-heat-inactivated human pool serum). Then, mycobacteria were declumped by mixing and repeatedly forcing the sample out of a 27-G syringe needle, followed by a 1-min sonicatation (Untrastonic 28X, Ney Dental International, Yacaipa, CA) and centrifugation (82 g, 2 min, 25 °C). The supernatant containing disaggregated mycobacteria was recovered and used in infection assays.

Phagocytosis assay by microscopy
A total of 1 × 10⁶ monocytes/well were placed in a chamber slide (Nunc-Thermo Fisher Scientific, Rochester, NY) and were allowed to adhere for 1 h at 37 °C in 5% CO₂. The cells were subsequently incubated with either culture medium (RPMI 1640, 10% pool human serum) or infected with M. tuberculosis at multiplicity of infection (MOI) 10 (10 mycobacteria: 1 monocyte) and incubated for 1 h. Three washes with supplemented RPMI were performed to remove the extracellular mycobacteria, and the cells were fixed for 10 min with methanol. Then, the slide was stained with a TB stain kit-K (Becton-Dickinson, San Jose, CA) and analysed under a microscope (Carl-Zeiss, Gottingen, Germany) at 100X with immersion oil. We counted 300–400 monocytes, and the number of monocytes with intracellular mycobacteria was expressed as the phagocytosis percentage.

Mycobacterium tuberculosis infection and intracellular mycobacterial growth
A total of 2 × 10⁵ monocytes/well were infected with M. tuberculosis at MOI of 10 in a 96-well culture plate (Corning Costar Co., Corning, NY). Subsequently, the infected monocytes for 1 h (D0), 24 h (D1) and 72 h (D3) and mycobacterial intracellular growth was evaluated by serial dilution and CFU counts.

Quantifying CFU
The intracellular growth of M. tuberculosis was assessed by CFU. Briefly, the infected monocytes at D0, D1 and D3 were lysed as previously reported [25]. Ten-fold serial dilutions of the triplicates were performed, and the dilutions were seeded on 7H10 agar plates in triplicate. After 21 days at 37 °C under a 5% CO₂ atmosphere, the CFU were counted.

Gene expression by real-time quantitative PCR
After a 1-h (D0) or 72-h (D3) incubation, the uninfected (UN) and infected monocytes (M.tb) were lysed, and total RNA was extracted by column purification using a RNeasy Mini Kit (Qiagen Co., Strasse, Germany) following the manufacturer’s instructions. RNA was eluted in DEPC-water and used for cDNA synthesis with a Superscript First-Strand-cDNA Synthesis System (Invitrogen, Carlsbad, CA). Briefly, RNA with random hexamers and dNTPs was denatured (65 °C, 5 min), and a reverse transcription mix (25 mM MgCl₂, 2 mM DTT, RNAse Out inhibitor, PCR buffer 10X) was added. The synthesis was performed with Superscript II enzyme in a thermal cycler (iCycler, BioRad Co.). Quantitative real-time-PCR (qPCR) was performed using Taqman assays for LL-37 (CAMP, Hs00189038, Applied Biosystem, Foster City, CA), human IL-10 (Hs00961622_m1, Applied Biosystem) and ribosomal RNA 18S (rRNA 18S) as a housekeeping control was used for the normalization (Applied Biosystem). Briefly, a universal master mix (Applied Biosystem), TaqMan assay (LL-37-FAM or IL-10-FAM and rRNA18S -VIC), DEPC-water and cDNA were mixed and added to each well of a 96-well plate. The plates were sealed and centrifuged (300 g, 5 min). Amplification was performed in a StepOne Plus Sequence Detection System (Applied Biosystem) starting with 2 min at 50°C, 10 min at 95°C and 40 cycles of 30 s at 95°C and 60 s at 62°C. Data were analysed with 7500 Fast System SDS software version 1.4 (Applied Biosystem), and the results are reported as fold changes respective to UN monocytes. A relative quantification method (ΔΔCt) was used for gene expression analysis.

Gene expression by PCR
A 102 bp fragment of human β defensin-2 (HBD2) was amplified using the primers 5′-CAT CAG CCA TGA GGG TCT-3′ (forward) and 5′-AGG CAG GTA ACA GGA TCG-3′ (reverse) and a fragment of 496 bp from Hypoxanthine Phosphoribosyl Transferase (HPRT) as internal control using the primers 5′-TAT GGA CAG GAC TGA ACG TCT TGC-3′ (forward) and 5′-GAC...
ACAA AAC ATG ATT CAA ATC CCT CA-3 r’ (reverse). cDNA were mixed with PCR buffer, MgCl2 (3 mM), dNTPs (0.4 mM each one), primers (0.4 μM and the amplifications were done in a thermocycler by 35 cycles: 30 s of denaturation at 95 °C, 30 s of annealing primers at 55 °C (HBD2) or 60 °C (HPRT) and 1 min of extension at 72 °C. The amplification products were mixed with GelRed and analyzed on 2% agarose gel electrophoresis and the image captured in a ChemiDoc MP Imaging System (BioRad, Hercules, CA). The amplification band fluorescence was analyzed with Image Lab software and results were reported as arbitrary units.

Nitric oxide (NO) determination
After monocytes were infected with M. tuberculosis, the supernatants were recovered at 1 h (D0) and 72 h (D3) post-infection, and the NO concentration was determined as previously reported [26]. Briefly, the culture supernatant was added to a 96-well plate and mixed with Griess reagent (0.1% naphthyl-ethilenediamine dihydrochloride and 1% sulfanilamide in 1NHCl) and incubated for ten min. A standard curve with different NaNO2 concentrations was included, and the optical density was determined at 550 nm with a plate microreader (Labsystems Multiskan MCC/340; Labsystems, Helsinki, Finland). The NO concentration was reported in μM.

Statistical analysis
Comparisons between T2D patients and healthy volunteers were made using nonparametric Mann-Whitney Rank Sum test, Wilcoxon signed rank tests or Fisher exact test. For rank correlations, we used Spearman’s rank correlation coefficient on nonparametric data. For PPD, gender and the recruitment of people in the two groups during years and seasons, we used proportion tests. Statistical significance was defined as p ≤ 0.05. We used GraphPad Prism Software package, version 5.04 (2008). All data are expressed as the median.

Results
The clinical characteristics of the study groups are presented in Table 1. The T2D patients was significantly older and had higher levels of fasting glucose, glycated haemoglobin A (HbA1c), cholesterol, LDL and triglycerides than the group of healthy volunteers (p ≤ 0.05). Most T2D patients were taking metformin and glibenclamide, but none were prescribed angiotensin receptor blockers, insulin, or statins. Healthy volunteers did not exhibit any infections or inflammatory diseases at the time of blood donation for the experiments and did not take any medications during the study period. Complementary clinical characteristic and data base are presented in Additional file 1: Table S1 and S2.

Table 1 The clinical description of the study groups

|                   | T2D     | Healthy | *p ≤ 0.05 |
|-------------------|---------|---------|-----------|
| n                 | 51      | 38      |           |
| Gender (Men/Women)| 17/34   | 12/26   | ns        |
| Age (years)       | 49.4 ± 7.7 | 44.8 ± 8.0 | *         |
| Weight (kg)       | 69.4 ± 12.3 | 66.6 ± 10.3 | ns        |
| Height (m)        | 1.58 ± 0.08 | 1.59 ± 0.08 | ns        |
| BMI (Kg/m²)       | 27.4 ± 4.5 | 26.0 ± 2.7 |           |
| Fasting glucose (mg/dL) | 206.6 ± 82.9 | 93.5 ± 7.9 | *         |
| HbA1c (%)         | 9.4 ± 2.5 | 5.5 ± 0.2 | *         |
| Creatinine (mg/dL)| 0.73 ± 0.15 | 0.79 ± 0.15 | ns        |
| Cholesterol (mg/dL) | 208.9 ± 40.0 | 179.4 ± 23.4 | *         |
| HDL (mg/dL)       | 423.9 ± 9.1 | 436.3 ± 10.7 | ns        |
| LDL (mg/dL)       | 128.8 ± 34.7 | 111.9 ± 18.4 | *         |
| Triglycerides (mg/dL) | 271.3 ± 213.2 | 137.6 ± 46.5 | *         |
| Status PPD (+/-)  | 56.8% (29/22) | 57.8% (22/16) | ns        |

Vitamin D levels in T2D patients
Since the levels of vitamin D could have seasonal variations, we searched for seasonal differences among samples of T2D patients and healthy volunteers obtained at various times, and we did not find any differences (p>0.05). The serum levels of vitamin D in the T2D patients and healthy volunteers groups were significantly different. Our results show that 54% of T2D patients had an inadequate level of vitamin D (between 30 and 49.99 nM) (Fig. 1).

M. tuberculosis phagocytosis in monocytes from T2D patients
Vitamin D plays a role in innate immunity and has been described to affect mycobacterial phagocytosis [27, 28]. Therefore, we analysed the phagocytosis of M. tuberculosis by monocytes from T2D patients and healthy volunteers. Our results showed that the percentages of monocytes that phagocytosed M. tuberculosis were similar (p>0.05) in T2D patients (Fig. 2a) and healthy volunteers (Fig. 2b), and phagocytosis was not associated with levels of vitamin D (Fig. 2c).

Intracellular growth of M. tuberculosis was higher in monocytes from T2D patients and low vitamin D levels
Then, we explored the intracellular mycobacterial growth in monocytes obtained from T2D patients and healthy volunteers. No significant differences in mycobacterial growth were observed between our groups (p>0.05) (Fig. 3a). However, M. tuberculosis growth was significantly higher in monocytes obtained from T2D patients and inadequate...
levels of vitamin D (<50 nM) after 1 h (D0) and 72 h (D3) post-infection (p ≤ 0.05) (Fig. 3b), with a significant negative correlation of r = −0.39 and −0.44, respectively.

**LL-37, HBD2 and IL-10 expression and NO production are not associated with serum vitamin D levels**

Vitamin D directly and indirectly regulates the expression of the antimicrobial peptides cathelicidin (LL-37) and human β defensin-2 (HBD2), which exert bactericidal activity [15, 16]. Because low sera levels of vitamin D were associated with significantly higher intracellular mycobacterial growth, we decided to evaluate the expression of LL-37 and HBD2 in monocytes from T2D patients with lower sera vitamin D levels in comparison with monocytes from healthy volunteers with adequate serum vitamin D levels. We found that LL-37 (Fig. 4a) and HBD2 (Fig. 4b, Fig. 4c) expression was similar between monocytes from T2D patients with low serum levels of vitamin D and those healthy volunteers (p>0.05). We did not find a correlation between serum levels of vitamin D and LL-37 and HBD2 expression.

Then, we measured IL-10 gene expression in monocytes from T2D patients with low serum levels of vitamin D and healthy volunteers. We found no significant difference on IL-10 expression comparing T2D patients to healthy volunteers neither correlation of IL-10 expression with the levels of vitamin D (p>0.05) (Fig. 4d).

Additionally, vitamin D also participates in the regulation of NO production, which exhibits important antibacterial activity [29]. Our results revealed that NO concentrations produced by infected monocytes from T2D patients and healthy volunteers were similar at D3 (p>0.05) (Fig. 4e).

However, NO production was significantly increased in the supernatant of infected monocytes at D3 compared with D0 in T2D patients (Vitamin D <50 nM and ≥50 nM).
and healthy volunteers post-infection ($p \leq 0.05$). NO production was significantly higher in healthy volunteers than in T2D patients with low serum vitamin D levels (< 50 nM) ($p \leq 0.05$) (Fig. 4f).

**Discussion**

T2D patients have increased susceptibility to TB, and previous research has shown that low vitamin D levels are associated with both DM and TB. Because vitamin D is a molecule that exert immunoregulatory activities and it has been described that vitamin D play an important role in the immunity against pathogens as *M. tuberculosis*. We wondered whether vitamin D could be involved in antimycobacterial activity in T2D patients. Thus, we explored the levels of vitamin D and its correlation with phagocytosis of *M. tuberculosis* and antimycobacterial activity in monocytes.

Our results showed that 54% of the study participants with T2D had inadequate sera levels of vitamin D, and these results agree with previous reports. Previous studies have shown that T2D patients are more susceptible to TB [19, 30]. Then, we explored the association between low levels of vitamin D and the phagocytosis and intracellular growth of *M. tuberculosis* in monocytes from T2D patients. Our results showed no significant difference in the percentage of monocytes that phagocytosed *M. tuberculosis* from T2D patients compared with those from healthy volunteers, as determined by light microscopy. These results are inconsistent with a recent report describing a reduced percentage of monocytes from T2D patients associated with *M. tuberculosis*; this report also demonstrated that phagocytosis was strongest under higher autologous serum concentrations (20% versus 5%), and the authors concluded that the association involved complement factors [31]. We evaluated phagocytic activity in enriched monocytes using 10% of a pool of commercial non-autologous serum, and we did not study autologous complement components. However, the differences could be explained by variations in the experimental conditions. In the whole blood system, there may be other molecular and cellular mechanisms that participate in phagocytosis independent of the complement factors evaluated by others [31]. Our results also showed that phagocytosis of *M. tuberculosis* in T2D patients did not correlate with serum levels of vitamin D.

Next, we evaluated antimycobacterial activity in T2D patients and healthy volunteers and observed similar intracellular growth of *M. tuberculosis* between the groups. Since the percentage of infected monocytes was comparable between the groups, the magnitude of intracellular growth was similar. These results are also inconsistent with previous reports showing that monocyte bactericidal function was decreased in patients with poorly controlled non-insulin-dependent DM [32]. Although the T2D patients in this study were treated with metformin or glibenclamide, their disease was poorly controlled, as confirmed by the high HbA1c values. In addition, the mycobactericidal activity observed in T2D patients may be associated with metformin therapy. Previous studies have reported that metformin exhibits bactericidal activity and reduces the deleterious inflammation associated with immune pathology [33, 34].

In addition, we analysed the intracellular growth of *M. tuberculosis* in monocytes from T2D patients and associated it with the levels of vitamin D, we found that the mycobactericidal activity in T2D patients was significantly lower in monocytes from patients with inadequate
levels of vitamin D. These data indicate that low levels of vitamin D are associated with the decreased antimycobacterial activity observed in T2D patients. Since vitamin D induces the production of LL-37 and HBD2, antimicrobial peptides with antimycobacterial activity [16, 35, 36], we evaluated the expression of LL-37 and HBD2 and its association with vitamin D levels. However, the expression of LL-37 and HBD2 were not significantly decreased in T2D patients with low serum levels of vitamin D and was not correlated with serum vitamin D levels or antimycobactericidal activity. However, the induction of LL-37 was slightly lower in M. tuberculosis-infected monocytes from T2D patients with low serum vitamin D levels. Out of its participation in the regulation of antimicrobial peptides, vitamin D also regulates other human genes trough VDR-mediated response elements (Vitamin D Response Elements) and vitamin D inhibits the production of pro-inflammatory cytokines trough MKP-1 pathway [37]. With this rationale, we assessed IL-10 expression and analysed its correlation with vitamin D levels. We found not significant difference on IL-10 expression comparing T2D patients to healthy volunteers neither correlation of IL-10 expression with the levels of vitamin D. These results are consistent with our previous report where IL-10 levels were similar in T2D patients and healthy volunteers [38]. Other authors studied patients with rheumatoid arthritis and also found no correlation of vitamin D levels and the production of IL-10 [39]. Therefore, our results suggest that the decreased capacity to control the intracellular growth of M. tuberculosis by monocytes from T2D patients with low serum levels of vitamin D could be associated with another mechanism regulated directly or indirectly by vitamin D other than LL-37, HBD2 or IL-10.

NO is an innate metabolite that exhibits antibacterial activity, and previous reports have indicated that vitamin D affects the expression of mRNAs encoding inducible NO synthase (iNOS), iNOS protein expression and NO production [40, 41]. Therefore, we evaluated NO production and observed that NO production in response to M. tuberculosis infection in monocytes from T2D patients with serum low levels of vitamin D was significantly lower than that in monocytes from healthy volunteers at D3. Although lower NO production is in agreement with the decreased antibacterial activity observed in T2D patients with low serum levels of vitamin D, these results are inconsistent with a previous report.
showing that vitamin D-induced inhibition of NO release [41]. We speculate low serum levels of vitamin D in T2D patients could correlate with higher NO production. Nonetheless, we must also mention that determination of NO in human cells is not a sensitive assay, and these measures are not completely accurate. In addition, other bactericidal mechanisms directly or indirectly influenced by vitamin D remain functional in monocytes from T2D patients and are the responsible for the observed mycobactericidal activity. These mechanisms should be examined in future studies to define the role of vitamin D in the increased susceptibility of T2D patients to TB development.

Conclusions
The results of our study demonstrate that vitamin D could be the mediator between DM and TB susceptibility. Since monocytes from T2D patients and low serum levels of vitamin D show an impaired ability to control the intracellular growth of \textit{M. tuberculosis} (even though vitamin D levels are not associated with significant expression of LL-37 and HBD2), other mechanisms directly or indirectly regulated by vitamin D could be involved in the inability of monocytes from T2D patients to control \textit{M. tuberculosis} growth.

Additional file

Additional file 1: Complementary clinical characteristic and data base. 
Table S1. T2D patients. Table S2. Healthy volunteers. (XLSX 42 kb)

Abbreviations
1,25(OH)\textsubscript{2}D\textsubscript{3}: 1,25-dihydroxyvitamin D\textsubscript{3}; 25(OH)\textsubscript{2}D\textsubscript{3}: 25-hydroxyvitamin D; CFU: Colony forming units; DM: Diabetes mellitus; HbA1c: Glycated haemoglobin A; HBD2: Human B2 defensin-2; IL-10: Interleukin 10; INOS: Inducible NO synthase; MT: Mycobacterium tuberculosis; M:\textit{O}I: Multiplicity of infection; NO: Nitric oxide; T2D: Type 2 diabetes mellitus; TB: Tuberculosis

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Availability of data and materials
All data were added in Additional file 1.

Authors’ contributions
MTHB and YGH performed the experiments, collected the data, analysed and interpreted the data and wrote the manuscript. FHS performed the experiments and made valuable contributions to the study design, data analysis and interpretation and the written manuscript. GF performed the clinical analysis and wrote the discussion, and MT participated in the study design and wrote the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate
The protocol was approved by the Ethics Committee in Research of the Instituto Nacional de Enfermedades Respiratorias “Israelio Costo Villegas” with reference number: Protocol number B20–12. All participants provided written informed consent.

Consent for publication
Not applicable.

Competing interests
None of the authors have any competing interests.

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