Calcitriol enhances pyrazinamide treatment of murine tuberculosis

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Abstract

Background: Tuberculosis is a leading cause of morbidity and mortality in humans worldwide. There is an urgent need for new and effective drugs to treat tuberculosis and shorten the duration of tuberculosis therapy. 1, 25-dihydroxy vitamin D_3 (1,25 $(OH)_2D_3$) has been reported to have a synergistic effect with pyrazinamide (PZA) in killing tubercle bacilli *in vitro*. The addition of 1,25 $(OH)_2D_3$ to standard tuberculosis treatment should benefit patients if the adjunctive drug has a synergistic effect *in vivo*. Thus, in this study, calcitriol (bioactive 1,25 $(OH)_2D_3$) was administered to mice undergoing treatment for *Mycobacterium tuberculosis* (*M.tb*) infection with PZA, a first-line anti-tuberculosis drug, to determine whether vitamin D3 enhances the therapeutic effect.

Methods: C57BL/6 female mice were infected with the *M.tb* H37Rv strain through aerosol exposure. Calcitriol and PZA, either alone or in combination, were orally administered to the *M.tb* infected mice. The effect of calcitriol on PZA activity was determined by evaluating the bacterial burden and analyzing the histopathological lesions in the lungs and spleen. To investigate the expression of inflammatory cytokines and anti-microbial peptide genes, we determined the transcriptional levels of interferon-γ (*IFN-γ*), interleukin-4 (*IL-4*), mouse β-defensin-2 *(mBD2)*, and cathelicidin *LL-37* through real-time quantitative polymerase chain reaction. The protein levels of *IFN-γ* were detected by enzyme-linked immunosorbent assay. Differences between groups were analyzed with independent samples *t*-test or one-way analysis of variance.

Results: Calcitriol alone had little effect on tuberculosis infection, whereas PZA, compared with saline control treatment, decreased the bacterial burden (spleens: PZA *vs.* saline, 4.82 ± 0.22 *vs.* 5.22 ± 0.40 Log_{10} colony-forming units [CFU]/gram, $t = 2.13$, $P < 0.05$; lungs: PZA *vs.* saline, 5.55 ± 0.15 *vs.* 6.83 ± 0.46 Log_{10} CFU/gram, $t = 6.56$, $P < 0.01$) and pathological lesions in the lungs. Simultaneous administration of calcitriol with PZA, compared with PZA alone, decreased the bacterial load (spleen: calcitriol + PZA *vs.* PZA, 4.37 ± 0.13 *vs.* 4.82 ± 0.22 Log_{10} CFU/gram, $t = 4.36$, $P < 0.01$; lung: calcitriol + PZA *vs.* PZA, 5.03 ± 0.32 *vs.* 5.55 ± 0.15 Log_{10} CFU/gram, $t = 3.58$, $P < 0.01$) and attenuated the lung lesions (gross pathological score: calcitriol + PZA *vs.* PZA, 3.25 ± 0.50 *vs.* 2.50 ± 0.58, $t = 1.96$, $P < 0.05$; affected area of total lung area: calcitriol + PZA *vs.* PZA, 30.75% ± 6.50% *vs.* 21.55% ± 2.99%, $t = 2.66$, $P < 0.05$). Further studies demonstrated calcitriol significantly increased the expression of anti-inflammatory cytokine IL-4 but suppressed production of the pro-inflammatory cytokine *IFN-γ* (*IL-4*: calcitriol *vs.* saline, 5.69 ± 0.50 *vs.* 2.80 ± 0.56 fold of control, $t = 6.74$, $P < 0.01$; *IFN-γ*: calcitriol *vs.* saline, 1.36 ± 0.11 *vs.* 4.13 ± 0.83 fold of control, $t = 5.77$, $P < 0.01$). In addition, calcitriol alone or in combination with PZA significantly enhanced the transcriptional level of anti-microbial peptides (cathelicidin *LL-37*: calcitriol *vs.* saline, 10.59 ± 1.03 *vs.* 2.80 ± 0.90 fold of control, $t = 9.85$, $P < 0.01$; *mBD2*: calcitriol *vs.* saline, 7.92 ± 0.62 *vs.* 1.79 ± 0.45 fold of control, $t = 13.82$, $P < 0.01$), whereas PZA exerted a negative effect on anti-microbial peptide gene expression.

Conclusions: Calcitriol as adjunctive treatment can result in beneficial treatment outcomes in *M.tb* infection by suppressing the inflammatory response and up-regulating the expression of anti-microbial peptides. These results indicate the feasibility of using calcitriol adjunctively with standard chemotherapy for the treatment of *M.tb* infection.

Keywords: *Mycobacterium tuberculosis*; Calcitriol; Pyrazinamide; Vitamin D

Introduction

Tuberculosis (TB) is a major health threat, particularly in developing countries. Continuously high TB mortality rates and the emergence of multidrug-resistant *Mycobacterium tuberculosis* (*M.tb*) strains emphasize the need for improved treatment approaches. Moreover, mortality is partially associated with a failure to limit immunopathological inflammatory damage.[1-3] Given that numerous studies have demonstrated that immunomodulatory agents can promote the resolution of inflammation, immunomodulatory agents should be investigated as potential adjunctive immunotherapies to improve treatment outcomes.[4,5]

Previous studies have indicated that vitamin D has both direct and indirect anti-microbial effects through restricting

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intra-cellular *M.tb* growth, as well as immunomodulatory effects.[6,7] In addition, *in vitro* studies have shown that calcitriol, the active metabolite of vitamin D, has anti-microbial activity and inhibits pro-inflammatory cytokine responses.[8] Furthermore, calcitriol functions in host defense against mycobacterial infection through the induction of anti-microbial peptides (AMPs) and/or autophagy.[6] As the evidence on the regulatory role of vitamin D accumulated, interest in treatments with vitamin D supplementation grew. Multiple studies have recognized the effect of vitamin D supplementation as an adjunct to anti-TB treatment with different degrees of efficacy, on the basis of accelerating sputum smear conversion or improving the resolution of active TB.[7,9-13] However, the role of vitamin D supplementation in TB treatment is controversial: some studies have shown better treatment responses, whereas others have revealed conflicting results. Therefore, further investigations are needed to definitively identify the link between vitamin D supplementation with standard anti-TB drugs and the alleviation of disease severity. Because the immunomodulatory properties of vitamin D in mice inoculated with *M.tb* and pathological similarities between mice and humans have been identified,[14-16] a mouse model of TB was therefore considered to be an attractive tool for investigating the unclear effects of vitamin D supplementation on host resistance to *M.tb* and disease outcome.

Pyrazinamide (PZA) is a first-line anti-TB agent that kills non-replicating or slow-growing bacilli under acidic pH conditions. Ultraviolet light has been found to enhance the killing activity of PZA against *M.tb in vitro*.[17] Calcitriol (bioactive vitamin D) has a synergistic effect with PZA in killing tuberculosis bacilli in cultured human macrophages.[18] However, there is a lack of published data with respect to the effect of vitamin D combined with a first-line medication against TB in an experimental TB mouse model.[16] In the current study, we therefore sought to determine whether adjunctive therapy with vitamin D might have beneficial effects in improving the outcomes of *M.tb* infection.

Methods

Animals

C57BL/6 female mice (6- to 8-week old) were supplied by the Center for Animal Experiment of Wuhan University (Wuhan, China; SCXK [e] 2014-0004) and were maintained in specific-pathogen-free conditions until infection with *M.tb*, when they were transferred and maintained in animal biosafety level III (ABSL-III) laboratory facilities. The ABSL-III has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal study protocols were performed and approved by the Institutional Animal Care and Use Committee of Wuhan University School of Medicine.

M. tuberculosis strain

The *M.tb* H37Rv strain (ATCC 93009) was obtained from the Research Institute of Tuberculosis (Tokyo, Japan). To ensure virulence for *in vivo* studies, the H37Rv strain of *M.tb* was passaged twice in mice and stored as frozen aliquots. Prior to infection, stocks were thawed and used to prepare fresh sub-cultures in Middlebrook 7H9 broth (Difco, BD, San Jose, CA, USA) as described previously.[19]

Aerosol infection with M.tb

M.tb aerosol infection was performed as described previously.[20] Briefly, 6- to 8-week-old female C57BL/6 mice were exposed to *M.tb* H37Rv using the aerosol inhalation system (Glas-Col, Terre Haute, IN, USA) to deliver approximately 100 bacilli into the lungs of each mouse. Three *M.tb*-infected mice were euthanized 24 h post-infection and their lung homogenates were plated to confirm the viability and the actual numbers of bacilli inoculated in the lungs.

Treatments

Forty mice were randomly assigned to four treatment groups: (1) saline only (control group), (2) 0.02 μg/kg calcitriol (Roche, Reinach, Switzerland), (3) 150 mg/kg PZA (Sigma, MD, USA), and (4) 0.02 μg/kg calcitriol + 150 mg/kg PZA. Calcitriol and PZA were dissolved in 0.2 mL of sterile saline and administered via oral gavage. The treatment was initiated 4 weeks after aerosol infection and continued for 6 weeks (daily and 5 days per week). The dosage of calcitriol was selected for similarity to the recommended human amount for treatment of osteoporosis and renal osteodystrophy (up to 0.5 μg per day). The dose of PZA was chosen to match the area under the concentration-time curve value obtained with the recommended dosage in humans.[21,22]

Determination of bacterial burden

Two days after the conclusion of treatments, mice were sacrificed by carbon dioxide narcosis. Total right lung and spleen were weighed and homogenized in 2 mL of phosphate-buffered saline (PBS) using a tissue homogenizer (Pro 250, Pro Scientific, Oxford, CT, USA). The tissue suspension was serially diluted 10-fold, and a 0.1 mL of each dilution was plated on Middlebrook 7H11 (Difco) agar supplemented with 10% oleic albumin dextrose catalase (Difco) and incubated at 37°C with 5% CO_2. The numbers of colony-forming units (CFU) on the agar were determined 4 weeks later; the tissue bacterial burdens were calculated in the form of CFU per gram of organ.

Histopathological assessment

At specified time points following the aseptic removal of organs, the remaining left lung was aseptically removed and fixed in 10% neutral buffered formalin that was routinely processed and embedded in paraffin. Lung tissues were cut into 5-μm-thick sections and stained with hematoxylin and eosin (H&E). The sections were then examined for histopathological changes using a light microscope (AKIOSKOP40, Zeiss, Germany). The nature and severity of microscopic lesions were evaluated subjectively and scored by board-certified pathologists, who were blinded to the experimental groups, evaluating the lesions at least twice to verify the reproducibility of the

observation. The gross histopathological scoring and histometric analysis of lung lesions were performed as described previously.[23]

Preparation of single-cell suspensions from infected spleens

Briefly, the spleen was aseptically harvested and passed through a 70 μm cell strainer (BD, San Jose, CA, USA) to harvest single splenocytes. The single-cell suspension was incubated with Ammonium-Chloride-Potassium (ACK) lysing buffer (GIBCO, Carlsbad, CA, USA) to lyse the remaining red blood cells. The cells were washed twice with PBS and re-suspended in Roswell Park Memorial Institute 1640 medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum for *in vitro* stimulation and RNA isolation.

In vitro stimulation of single splenocytes

Single spleen cell suspensions were plated at a density of 0.5×10^6 cells per well in a 24-well plate for this study. For detecting the interferon-γ (IFN-γ) level of cell culture supernatants, cells were stimulated with either a tuberculin-purified protein derivative (TB-PPD) (5 μg/mL) or phorbol 12-myristate 13-acetate (5 μg/mL, Sigma, St. Louis, MO, USA) as a positive control for 72 h, and then the supernatants were frozen in −80°C for an enzyme-linked immunosorbent assay (ELISA) test. Untreated cells were included as a negative control (NC). For determining the AMP and pro-inflammatory cytokines of splenocytes at the transcriptional level, cells were treated with TB-PPD (5 μg/mL) for 20 h, then subjected to the RNA extraction and real-time polymerase chain reaction (PCR) assay.

RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction

Total cellular RNA was extracted from splenocytes using Tri-reagent (Molecular Research Center Inc, Cincinnati, OH, USA) according to the manufacturer's protocol. The concentration and purity of isolated RNA were determined spectrophotometrically with nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA was synthesized from isolated RNA using the reverse transcription system from Promega Corporation (Madison, WI, USA). The real-time quantitative polymerase chain reaction (RT-PCR) was performed with the brilliant SYBR Green PCR master mix (Bio-rad Laboratory, Hercules, CA, USA). The oligonucleotide primers were synthesized by Invitrogen (Shanghai, China). Each PCR run included a non-template control. All samples were assayed in triplicates. All specific oligonucleotide primers were designed by Premier 5.0 and the information on the forward and reverse primers are shown in Table 1.

Cytokine detection by ELISA

The protein expression of IFN-γ was evaluated by ELISA. The concentration of IFN-γ in the culture supernatants harvested in the *in vitro* splenocytes stimulation experiment were assayed using Ready-SET-Go ELISA kits (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Statistical analysis

Prism 6.0 software (GraphPad, San Diego, CA, USA) was used to perform all statistical analyses. Quantitative data are presented as the mean ± standard deviation. Differences between groups were analyzed using independent *t*-test. *P* values of <0.05 were considered to be statistically significant.

Results

Treatment with calcitriol plus PZA ameliorates pathological lesions in mice infected with M.tb

We sought to determine whether PZA treatment supplemented with calcitriol might attenuate the pathological damage resulting from *M.tb* infection. The PZA treatment alone and the combined treatment, compared with saline treatment, resulted in a significantly greater decrease in the number and size of lung lesions, on the basis of gross pathological examination, whereas calcitriol had little effect on pathological lesions [Figure 1A and 1B]. Compared with the PZA group, the calcitriol plus PZA group showed further decreased lung lesions. The histopathological analysis of lung sections demonstrated the alleviation of pathological damage through combination therapy. Consistently with these findings, histopathological examination of the lungs from the saline group displayed aggravated lung lesions characterized by coalescing lobar granulomatous pneumonia and consolidation. Little improvement in lung tissue damage was observed in the calcitriol treatment group. In contrast, the affected area of the lungs in the PZA treatment group was significantly smaller characteristic of minor pneumonia lesions and no granuloma formation. Moreover, the pathological damage in the combination treatment group was less than that in the PZA group, and the difference between the two groups was statistically significant (gross pathological score: calcitriol + PZA *vs*. PZA, 3.25 ± 0.50

Table 1: Real-time PCR primers and relevant parameters.

Genes	Forward primer	Reverse primer
IFN-γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCTC
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
mBD2	AAGTATTGGATACGAAGCAG	TGGCAGAAGGAGGACAAATG
LL-37	CTTCAACCAGCAGTCCCTAGACA	TCCAGGTCCAGGAGACGGTA

PCR: Polymerase chain reaction; *IFN-γ*: Interferon-γ; *IL-4*: Interleukin-4; *mBD2*: Mouse β-defensin-2; *LL-37*: Cathelicidin *LL-37*.

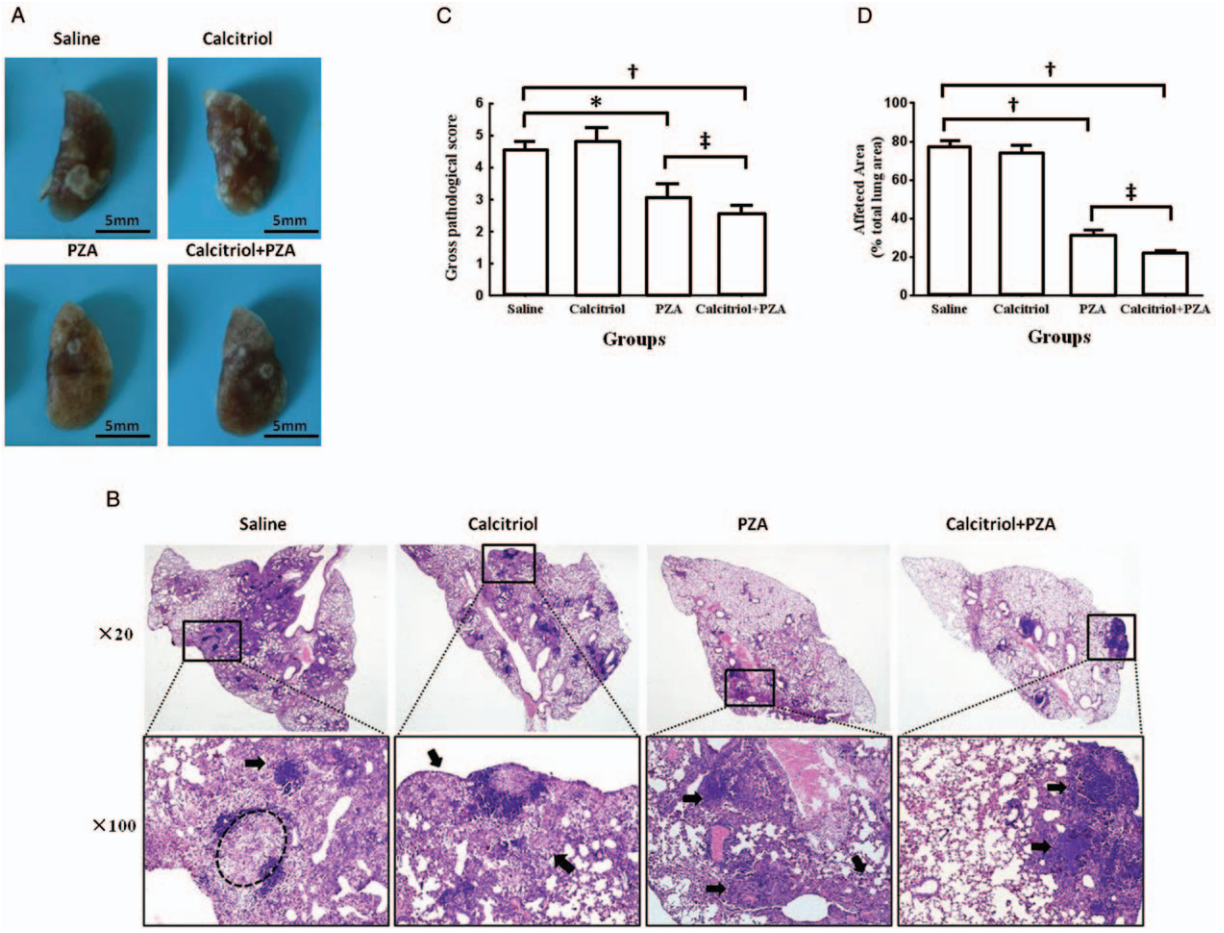

Figure 1: Combination treatment with calcitriol and PZA attenuated pathological lesions in lungs of *M.tb*-infected mice. C57BL/6 mice were infected with aerosolized *M.tb* H37Rv strain. At 4 weeks post-infection, mice were treated with calcitriol, PZA or calcitriol + PZA for 6 weeks. The saline group received an equal volume of saline as a control. (A) Representative photographs of lung lobes of mice from various groups. The granulomas were seen on the surface of lung tissue from *M.tb*-infected mice. (B) Representative photographs of lung sections stained with H&E. The black arrows denote consolidation or inflammatory lesions containing epithelioid macrophages and lymphocytes hyperplasia. The black circle indicates a granuloma. (C) Gross pathology scores of lungs from *M.tb*-infected mice. (D) Affected area over total lung area of *M.tb*-infected mice from four groups. $^{*}P < 0.05$; $^{\dagger}P < 0.01$, as compared with the saline control; $^{\ddagger}P < 0.05$, compared with PZA-treated alone. H&E: Hematoxylin & eosin; *M.tb*: *Mycobacterium tuberculosis*; PZA: Pyrazinamide.

vs. 2.50 ± 0.58, $t = 1.96$, $P < 0.05$; affected area of total lung area: calcitriol + PZA *vs.* PZA, 30.75 ± 6.50% *vs.* 21.55 ± 2.99%, $t = 2.66$, $P < 0.05$) [Figure 1C and 1D]. Together, these results suggest that calcitriol in combination with PZA treatment in mice attenuates *M.tb* driven lesions.

Calcitriol supplementation enhances the effectiveness of PZA therapy by decreasing the bacterial burden in the spleen and lungs in M.tb-infected mice

Compared with saline treatment, PZA treatment alone and combined treatment resulted in significantly lower bacterial burden in the spleen (PZA *vs.* saline, 4.82 ± 0.22 *vs.* 5.22 ± 0.40 Log_{10} CFU/gram, $t = 2.13$, $P < 0.05$; calcitriol + PZA *vs.* saline, 4.37 ± 0.13 *vs.* 5.22 ± 0.40 Log_{10} CFU/gram, $t = 4.99$, $P < 0.01$) or lungs (PZA *vs.* saline, 5.55 ± 0.15 *vs.* 6.83 ± 0.46 Log_{10} CFU/gram, $t = 6.56$, $P < 0.01$; calcitriol + PZA *vs.* saline, 5.03 ± 0.32 *vs.* 6.83 ± 0.46 Log_{10} CFU/gram, $t = 7.89$, $P < 0.01$), although calcitriol treatment alone appeared to have no effect on the number of bacterial CFU counts [Figure 2]. Furthermore, the combined treatment group, compared with the PZA treatment group, showed a significantly lower bacterial burden in the *M.tb* infected organs (spleen: calcitriol + PZA *vs.* PZA, 4.37 ± 0.13 *vs.* 4.82 ± 0.22 Log_{10} CFU/gram, $t = 4.36$, $P < 0.01$; lung: calcitriol + PZA *vs.* PZA, 5.03 ± 0.32 *vs.* 5.55 ± 0.15 Log_{10} CFU/gram, $t = 3.58$, $P < 0.01$) [Figure 2]. These results demonstrated that calcitriol supplementation with PZA treatment of *M.tb*-infected mice had a synergistic therapeutic effect, as evidenced by decreased bacterial loads.

Combination treatment with calcitriol and PZA suppresses IFN-γ production while enhancing interleukin-4 gene expression

To further determine the immunomodulatory effects of vitamin D, we investigated its influence on the production of PPD-specific Th1 cytokine *IFN-γ* and Th2 cytokine interleukin-4 (*IL-4*) in mice infected with *M.tb*. As shown in Figure 3, PZA treatment increased *IFN-γ* expression (PZA *vs.* saline, 6.15 ± 0.67 *vs.* 4.13 ± 0.83 fold of control, $t = 3.30$, $P < 0.05$), whereas calcitriol treatment inhibited *IFN-γ* expression (calcitriol *vs.* saline, 1.36 ± 0.11

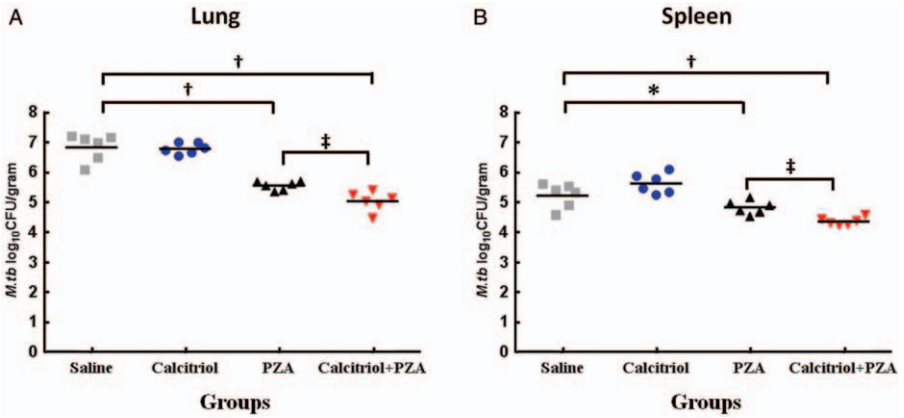

Figure 2: Combination treatment with calcitriol and PZA decreased bacterial burden in *M.tb*-infected organs of mice. The bacterial burden is measured in CFU per gram in spleen and lung tissues. Mice in four groups were challenged with *M.tb* H37Rv strain. (A) Bacterial burdens in the lungs, (B) Bacterial burdens in the spleens. $^{*}P < 0.05$, $^{\dagger}P < 0.01$ as compared with saline control; $^{\ddagger}P < 0.01$, compared with PZA-treated alone. CFU: Colony formation units; *M.tb*: *Mycobacterium tuberculosis*; PZA: Pyrazinamide.

vs. 4.13 ± 0.83 fold of control, $t = 5.77$, $P < 0.01$). Moreover, the combination treatment group showed significant down-regulation of *IFN-γ* expression compared with that in the PZA treatment group (calcitriol + PZA *vs.* PZA, 1.59 ± 0.12 *vs.* 6.15 ± 0.67 fold of control, $t = 11.69$, $P < 0.01$) [Figure 3A]. In addition, the suppressive effect of calcitriol was further confirmed by the ELISA results (calcitriol *vs.* saline, 246.67 ± 15.28 *vs.* 451.67 ± 27.54 pg/mL, $t = 11.28$, $P < 0.01$; calcitriol + PZA *vs.* saline, 286.67 ± 25.66 *vs.* 451.67 ± 27.54 pg/mL, $t = 7.59$, $P < 0.01$) [Figure 3C]. The Th2 cytokine *IL-4* increased both in the calcitriol alone group and in the combination treatment group, as compared with the saline group (calcitriol *vs.* saline, 5.69 ± 0.50 *vs.* 2.80 ± 0.56 fold of control, $t = 6.74$, $P < 0.01$; calcitriol + PZA *vs.* saline, 6.06 ± 0.51 *vs.* 2.80 ± 0.56 fold of control, $t = 7.54$, $P < 0.01$), whereas the PZA treatment group showed a slight but insignificant effect on the expression of *IL-4* [Figure 3B]. Further analysis indicated that *IL-4* was significantly elevated in the combination treatment group compared with the PZA treatment group (calcitriol + PZA *vs.* PZA, 6.06 ± 0.51 *vs.* 4.15 ± 0.70 fold of control, $t = 3.85$, $P < 0.05$) [Figure 3B].

Combination treatment with calcitriol plus PZA enhances AMP gene expression

Because *in vitro* studies have indicated that vitamin D is a potential inducer of AMPs, we sought to examine the effect of vitamin D on AMP expression *ex vivo*. Calcitriol alone or combination treatment both significantly increased the expression of AMP genes, such as cathelicidin *LL-37* (calcitriol *vs.* saline, 10.59 ± 1.03 *vs.* 2.80 ± 0.90 fold of control, $t = 9.85$, $P < 0.01$; calcitriol + PZA *vs.* saline, 11.92 ± 1.13 *vs.* 2.80 ± 0.90 fold of control, $t = 10.96$, $P < 0.01$) [Figure 4A] and mouse β-defensin-2 (*mBD2*) (calcitriol *vs.* saline, 7.92 ± 0.62 *vs.* 1.79 ± 0.45 fold of control, $t = 13.82$, $P < 0.01$; calcitriol + PZA *vs.* saline, 9.59 ± 1.09 *vs.* 1.79 ± 0.45 fold of control, $t = 11.45$, $P < 0.01$) [Figure 4B], as compared with either the saline control or the PZA treatment, whereas PZA treatment had no effect on AMP expression.

Discussion

Numerous *in vitro* studies have demonstrated that vitamin D has anti-microbial and immunomodulatory effects in the treatment of TB. Although these studies are promising, clinical trials on vitamin D supplementation therapy have revealed contrasting results. Hence, we investigated the effects of vitamin D combined with the first-line anti-TB drug PZA in a mouse model in this study. Our data demonstrated the enhancement of PZA activity in the TB treatment. PZA alone suppressed bacterial loads and ameliorated pathological damage in the *M.tb* infected lungs and spleens. With supplementation with calcitriol at a relevant dose, additional clearance of viable bacteria and alleviation of pathological lesions were observed. In summary, calcitriol protects the host against TB by increasing the activity of PZA and differentially modulating the production of cytokines in response to *M.tb* infection. Additionally, calcitriol alone had little effect on the bacterial load. Our observations support the previously reported findings that vitamin D is ineffective in alleviating *M.tb* burden *in vivo*.[15]

To understand the mechanism underlying the synergistic effects of calcitriol combined with PZA in treating *M.tb* infection, we examined two key inflammation-associated cytokines (*IFN-γ* and *IL-4*). Compared with the PZA group, the combination treatment group had a significantly lower expression of the *M.tb* specific *IFN-γ* and higher expression of the *M.tb* specific *IL-4*, accompanied by a lower bacillary burden and slightly mitigated pathological lesions in the *M.tb*-infected organs. The results are consistent with those from several early *in vivo* studies indicating that vitamin D inhibits the pro-inflammatory cytokine response and attenuates pulmonary immunopathology.[14,15,24] Considering the similar findings, it is likely that calcitriol enhances PZA activity by suppressing *M.tb*-induced inflammation to a level that is sufficient to limit the growth of *M.tb* but that does not lead to tissue damage. Our findings provide additional evidence supporting that a balance between pro-inflammatory and anti-inflammatory responses plays a key role in TB disease progression, and excessive aggressive pro-inflammatory

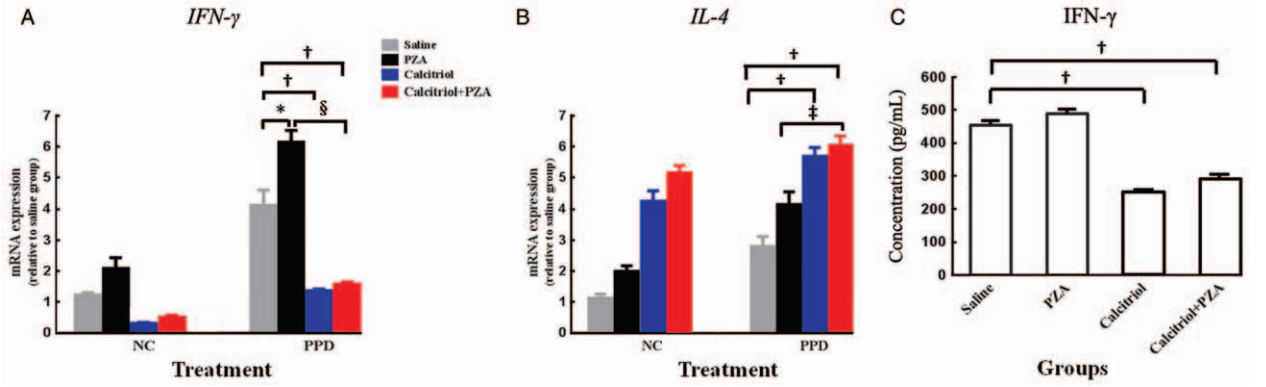

Figure 3: Combination treatment with calcitriol and PZA suppressed IFN-γ production while enhanced *IL-4* gene expression. Spleen single-cell suspensions harvested at the experiment endpoint were plated at a density of 0.5×10^6 cells per well in a 24-well plate. For the experiment detecting the IFN-γ level of cell culture supernatants, cells were stimulated with tuberculin-PPD (5 μg/mL) as the inductor for 72 h. The NC without PPD stimulation was also used for comparison. Total RNA was isolated from the splenocytes stimulated with/without PPD from *M.tb*-infected mice. The gene expression level of *IFN-γ* and *IL-4* was evaluated by RT-PCR. Data were expressed as the mean ± standard deviation of three independent experiments. (A) The gene expression level of *IFN-γ*; (B) The gene expression level of *IL-4*. (C) The level of IFN-γ was determined by ELISA. $^{*}P < 0.05$; $^{\dagger}P < 0.01$, as compared with the saline control; $^{\ddagger}P < 0.05$; $^{\S}P < 0.01$, compared with PZA-treated alone. ELISA: Enzyme-linked immunosorbent assay; *IFN-γ*: Interferon-γ; *IL-4*: interleukin-4; *M.tb*: *Mycobacterium tuberculosis*; NC: Negative control; PPD: Purified protein derivative; PZA: Pyrazinamide; RT-PCR: Real-time quantitative polymerase chain reaction.

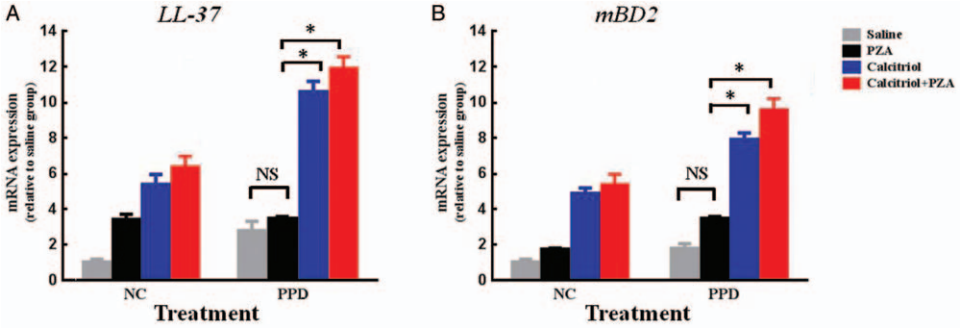

Figure 4: Combination treatment with calcitriol plus PZA enhances anti-microbial peptide gene expression. Spleen single-cell suspensions harvested at the experiment endpoint were plated at a density of 0.5×10^6 cells per well in a 24-well plate. For the experiment detecting the *LL-37*and *mBD2* level of cultured cell, cells were stimulated with TB-PPD (5 μg/mL) for 72 h. A NC without PPD stimulation was also used for comparison. Total RNA was isolated from the splenocytes stimulated with/without PPD from *M.tb*-infected mice. The gene expression level of *LL-37* and *mBD2* was evaluated by RT-PCR. Data were expressed as the mean ± standard deviation of three independent experiments. (A) The gene expression level of *LL-37*; (B) The gene expression level of *mBD2*. $^{*}P < 0.01$, compared with PZA-treated alone. *LL-37*: Cathelicidin *LL-37*; *mBD2*: Mouse β-defensin-2; *M.tb*: *Mycobacterium tuberculosis*; NC: Negative control; NS: No significance; PPD: Purified protein derivative; PZA: Pyrazinamide; TB-PPD: Tuberculin-purified protein derivative.

conditions or a shift toward an anti-inflammatory cytokine response may worsen the host's ability to contain the infection and the progression of severe disease.[25]

In many related immunological research studies, vitamin D has been found to increase the expression of AMPs, including cathelicidin and β-defensin-2, which can restrict the intra-cellular growth of *M.tb*.[16,26,27] Human cathelicidin *LL-37* has a key role in the immune defense against *M.tb* infection.[28-30] Similarly to *LL-37*, β-defensin-2 has been reported to possess anti-microbial activities, including against *M.tb*.[31,32] We found that the production of both the *M.tb* specific *LL-37* and *mBD2* was significantly enhanced in the calcitriol plus PZA combination group compared with the PZA group. Our findings are in line with those from previous studies showing that vitamin D is crucial for up-regulating *LL-37* induction.[28] Our results demonstrated that PZA has no effect on *LL-37* levels in the presence of calcitriol, in agreement with the findings from several reports.[33-35]

We are aware of the limitations of this pilot study. Calcitriol plus standard chemotherapy was not tested. Therefore, we do not know whether the effect of calcitriol might be even greater. The duration of treatment and the number of experimental animals in our study were limited. Further studies are required in mouse models with various clinical *M.tb* strain challenges, treatment combinations and duration to evaluate the interaction between vitamin D and the host immune response and to comprehensively define the underlying mechanisms.

In summary, our study provides compelling *in vivo* experimental evidence that vitamin D adjunctive therapy results in beneficial outcomes in TB treatment by regulating the balance between the pro-inflammatory and anti-inflammatory response and elevating the levels of AMPs. It may be possible to take advantage of this enhancement effect to further design and investigate calcitriol as an adjunct to standard chemotherapy for *M.tb* infection.

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Conflicts of interest

None.

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