

# Vitamin D, Cell Death Pathways, and Tuberculosis

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## Abstract

**Background:** *Mycobacterium tuberculosis* induces cellular necrosis that could promote spread of infection. The aim of this study is to analyze the effects of Vitamin D3 supplementation to improve the effectiveness of 2<sup>nd</sup>-line anti-tuberculosis (TB) drug therapy, especially in relation with cell death pathways. **Methods:** Mus musculus C3HeB/FeJ was randomly divided into four groups containing eight animals each. The 1<sup>st</sup> group (G1), consisting of mice that were intratracheally infected with multidrug-resistant strain of *M. tuberculosis* and sacrificed on 2-week postinfection to confirm successful infection. (G2) was a group of TB mice without therapy. Then, (G3) was a group of mice with the 2<sup>nd</sup>-line anti-TB therapy. The last group (G4) was a group of mice receiving not only the 2<sup>nd</sup>-line anti-TB therapy but also daily oral Vitamin D3 supplementation. Immunohistochemistry was used to measure expression of nuclear Vitamin D receptor, apoptosis marker cleaved caspase-3, cathelin-related antimicrobial peptide (CRAMP) and LC3B autophagy markers, necrosis marker RIPK3, and collagenase matrix metalloproteinase-1 (MMP1). The number of bacteria in the lung was calculated by colony forming units. The partial least square structural equation modeling with SmartPLS 3.2.6 software was used to analyze structural models among the variables. **Results:** Supplementation of Vitamin D3 on the 2<sup>nd</sup>-line anti-TB therapy increases Vitamin D3 receptor, CRAMP, LC3B, caspase-3 ( $P = 0.026$ ,  $P = 0.000$ ,  $P = 0.001$ ), presses MMP1, and the number of bacteria ( $P = 0.010$  and  $P = 0.000$ , respectively). The structural equation modeling analysis shows that increasing autophagy pathways reduces necrosis by lowering MMP1, whereas apoptosis reduces necrosis by decreasing the number of bacteria (each with indirect effects  $-0.543$  and  $-0.544$ ). **Conclusion:** A comprehensive analysis with the partial least square structural equation modeling shows decreasing necrosis requires increasing autophagy and apoptosis.

**Keywords:** Apoptosis, autophagy, matrix metalloproteinase-1, multidrug-resistant-tuberculosis, necrosis, Vitamin D3

## INTRODUCTION

One of the challenges of tuberculosis (TB) eradication program is the increasing multidrug-resistant-TB, whereas the effectiveness of 2<sup>nd</sup>-line anti-TB is very low.<sup>[1,2]</sup> There has been a lot of literature describing the role of Vitamin D in relation with the immunity of TB patients.<sup>[3-5]</sup> An active form of Vitamin D binds to the Vitamin D3 receptor on the membrane and or cell nucleus to begin its activity.<sup>[6]</sup> This study examines the role of Vitamin D3 supplementation in the TB cell death pathways and analyzes whether supplementation of Vitamin D can improve the effectiveness of 2<sup>nd</sup>-line anti-TB therapy as shown by decreasing matrix metalloproteinase-1 (MMP1) and the number of bacteria.

The viability of intracellular bacteria is influenced by cell death pathways. Apoptosis and autophagy are mycobactericidal, while necrosis precisely causes the bacteria to spread and infects the next cells.<sup>[7-9]</sup> *Mycobacterium tuberculosis*

induces apoptosis which involves caspase-3.<sup>[10]</sup> Autophagy is preceded by the formation of autophagy membranes with microtubule-associated protein 1A/1B-light chain 3 (LC3) precursors, expressed more cathelicidin antimicrobial peptides which are referred as cathelin-related antimicrobial peptide (CRAMP) in mice.<sup>[11,12]</sup> Cell necrosis occurs when cytosolic receptor interacting protein kinase 3 (RIPK3) undergoes translocation to mitochondria.<sup>[13]</sup>

The partial least square structural equation modeling has enabled researchers to simultaneously estimate such complex interrelationships of several variables and

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developed theory-based models. The use of structural equation modeling in the analysis of intracellular signals is not new.<sup>[14-17]</sup>

## METHODS

### Animals and experimental procedures

Mus musculus C3HeB/FeJ ( $n = 8$ ) aged 5–8 weeks were infected with 100  $\mu$ l intratracheal ( $10^5$  CFU/ml) and divided randomly into four groups. The 1<sup>st</sup> group (G1) was the group to examine the success of intratracheal infection. The mice in group 1 were euthanized 2-week postinfection and observed whether they have pulmonary TB. (G2) was the group of TB mice without therapy. (G3) was the group of mice with the 2<sup>nd</sup>-line anti-TB therapy recommended by the Indonesian National Tuberculosis Control Program. (G4) was the group of mice which was not only treated with the 2<sup>nd</sup>-line anti-TB therapy but also received daily oral Vitamin D3 supplementation for 6 months.

Kanamycin (Sigma K1876) was injected im 150 mg/kg body weight once a day in 5 days/week. Pyrazinamide (Sigma P7136) 150 mg/kg body weight, levofloxacin (Sigma 28266) 200 mg/kg body weight, ethionamide (Sigma E6005-5G) 50 mg/kg body weight, cycloserine (Sigma 30020-1G) 300 mg/kg body weight, and Vitamin D3 (Dvion Drops, Merck) 1.25 IU/g body weight were given per intragastric tube once a day in 7 days/week. At the end of treatment, the mice were euthanized. The left lung tissue was processed for immunohistochemistry and the number of bacteria was obtained from the right lung tissue culture.

### Immunohistochemistry

The used primary antibody included: anti-Vitamin D receptor antibody (ab3508, abcam), anti-cathelecidin antibody (ab64892 abcam), anti-LC3B antibody (ab63817, abcam), anti-MMP1 antibody (ab137332, abcam), anti-caspase3 (P17) (PA1961-1, BosterBio), and anti-RIPK3 (PA2242, BosterBio). The five different fields per section were analyzed by two independent investigators using the light microscope Olympus BX51 magnification 400x.

### Statistical analysis

The statistical analysis was performed by using IBM SPSS 20.0 for Windows. The normality test was done using Shapiro–Wilk. The different test among different groups was analyzed using the one-way ANOVA test or the Kruskal–Wallis test, followed by a *post hoc* test either with the Tukey’s test or the Mann–Whitney test. The two-sided  $P < 0.05$  were considered to indicate a statistically significant result.

Assessing the relationship among variables and predicting the theory-based structural model were done by applying the structural equation modeling test using the smartpls-3.2.6 GmbH

## RESULTS

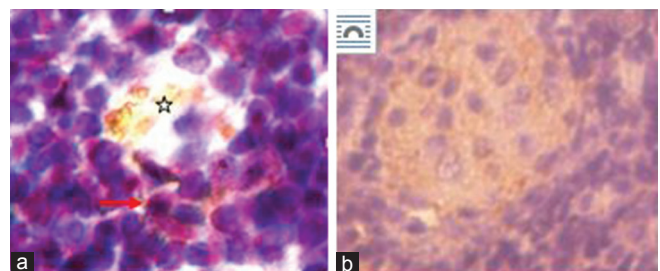
### Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis increases the VDR expression

Vitamin D is metabolized to 1,25-dihydroxyvitamin D. The hormonal form is the ligand for the Vitamin D receptor. Nuclear receptor-regulated transcription is cell specific. It is necessary to understand aspects of Vitamin D mechanisms of action to facilitate tissue-specific clinical application. Figure 1 visualizes Vitamin D3 receptor expression in lung tissue. Figure 2 shows supplementation of Vitamin D in the 2<sup>nd</sup>-line anti-TB which increases the Vitamin D3 receptor expression. *M. tuberculosis* can infect macrophages and alveolar epithelial type II pneumocytes.<sup>[18]</sup> Supplementation of Vitamin D3 promoted upregulation of Vitamin D-regulatory protein in infected cells.

### Cotreatment of Vitamin D3 and 2<sup>nd</sup>-line anti-tuberculosis drugs could enhance cathelin-related antimicrobial peptide and LC3B expression

Vitamin D may reduce the risk of infection through multiple mechanisms. With a Vitamin D response element within its promoter sequence, CRAMP further is a target gene of Vitamin D. Figure 3 shows immunohistochemical analysis of CRAMP in lung tissue. Immunohistochemistry is a technique for investigating protein expression and localization within tissues. Supplementation of Vitamin D increases the antimicrobial protein cathelicidin which is accordance with previous research [Figure 4].<sup>[19-21]</sup>

LC3 is an autophagy membrane precursor which is expressed into three variants/posttranslational isoforms of LC3A, LC3B, and LC3C. With lipidation, the posttranslation of LC3B transforms into an 18 kDa LC3I cytosolic form and moves into a 16 kDa LC3II membrane form. Provision of Vitamin D3 increases the conversion of LC3B-I to LC3B-II.<sup>[22]</sup> Figure 5 shows LC3B expression in lung tissue during *M. tuberculosis* infection. Figure 6 shows changes in LC3B expression following D3 administration. The expression of LC3B is higher among Vitamin D3-supplemented group.

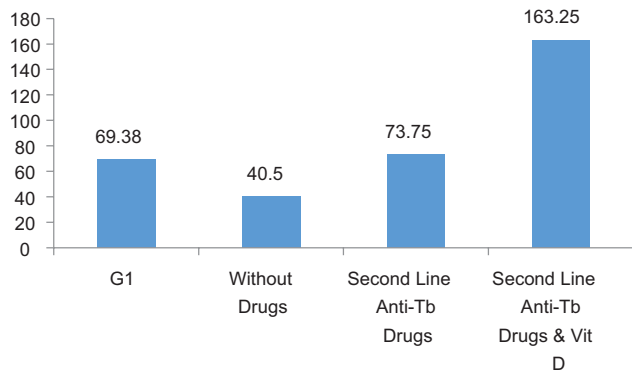


**Figure 1:** Immunohistochemistry of Vitamin D receptor in the lung. (a) Mice were treated with the only 2<sup>nd</sup>-line anti-tuberculosis drugs, immunoreactive VDR is noticed in macrophage (the star), activated lymphocyte (the red arrow) (b) mice were treated with the drugs in combination with the Vitamin D3. The number of Vitamin D receptor immunoreactive cells was increased by supplementation of Vitamin D (immunohistochemistry,  $\times 1000$ )

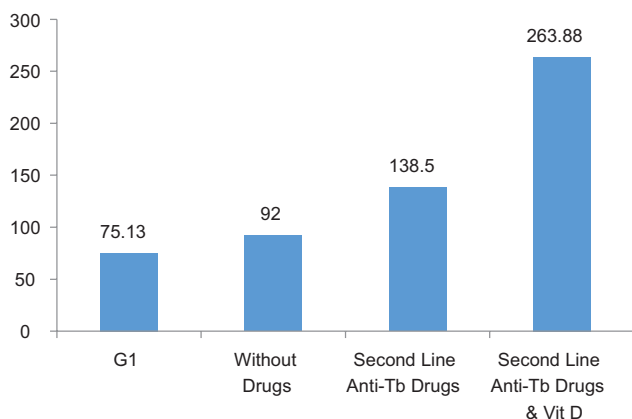
### Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis increases apoptosis

An apoptotic index is the number of positive caspase-3 cells in the cytosol per view field. Figure 7 shows that granulomas are dynamic lesions, both apoptosis and nonapoptosis macrophages are observed. Exogenous tumor necrosis factor (TNF) have been reported to elevate *M. tuberculosis*-mediated macrophage

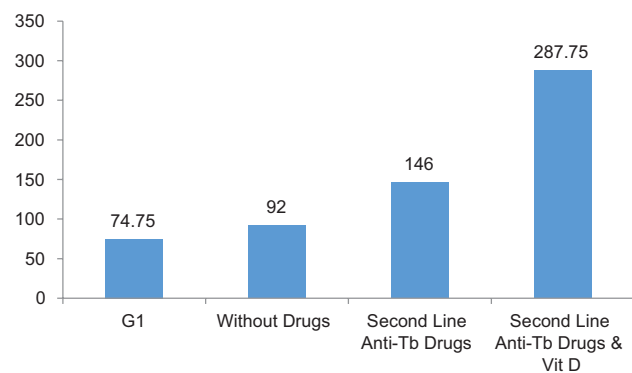
apoptosis.<sup>[23]</sup> Figure 8 shows Vitamin D3 induces caspase-3. This study demonstrates that exogenous Vitamin D upregulates caspase-mediated apoptosis. Apoptosis is a mechanism of infected cells to eliminate bacteria. It is the programmed cell death without causing an inflammatory reaction. Vitamin D has a beneficial role in the treatment of TB.



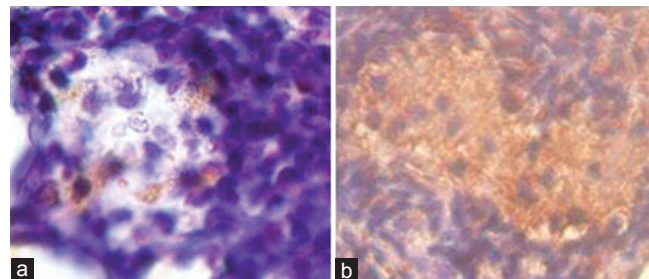
**Figure 2:** Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis increases the VDR expression



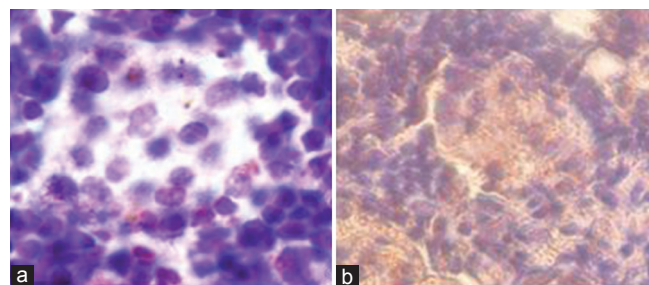
**Figure 4:** Supplementation of Vitamin D in the 2<sup>nd</sup>-line anti-tuberculosis increases the cathelicidin antimicrobial protein



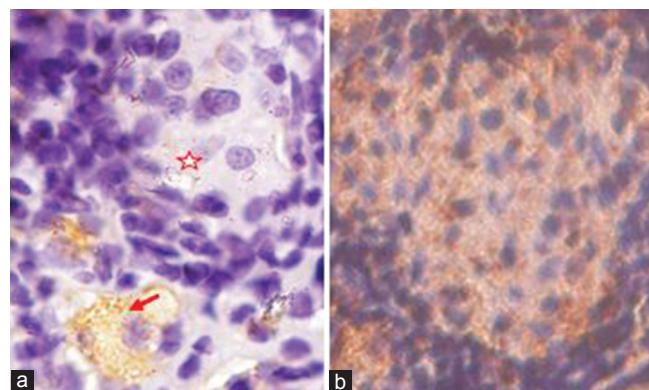
**Figure 6:** Supplementation of Vitamin D in the 2<sup>nd</sup>-line anti-tuberculosis increases the LC3B expression



**Figure 3:** Immunostaining for cathelin-related antimicrobial peptide in the lung section (a) mice were treated with the only 2<sup>nd</sup>-line anti-tuberculosis drugs and (b) mice were treated with the drugs in combination with the Vitamin D3. The number of cathelin-related antimicrobial peptide immunoreactive cells was increased by supplementation of Vitamin D (immunohistochemistry, ×1000)



**Figure 5:** Immunoreactivity of LC3B occurs in the lung. (a) Mice were treated with the only 2<sup>nd</sup>-line anti-tuberculosis drugs and (b) mice were treated with the drugs in combination with the Vitamin D3. The number of LC3B immunoreactive cells was increased by supplementation of Vitamin D (immunohistochemistry, ×1000)



**Figure 7:** (a) Caspase-3 expression in the lung tissue of mice treated with 2<sup>nd</sup>-line anti-tuberculosis drugs. The red arrow indicates an apoptotic macrophage and the star shows a nonapoptotic macrophage. (b) Mice were treated with the drugs in combination with the Vitamin D3. The number of Caspase-3 immunoreactive cells was increased by supplementation of Vitamin D (immunohistochemistry, ×1000)

**Supplementation of Vitamin D3 enhances the effectiveness of 2<sup>nd</sup>-line anti-tuberculosis therapy by lowering the number of bacteria, expression of pulmonary collagenase enzyme matrix metalloproteinase-1, and cell necrosis**

Figure 9 shows the mean bacterial count of the D3 supplemented group and the control group. Here, we show that Vitamin D3 have a positive impact on reducing bacterial load. The researcher noted that Vitamin D may help both innate and adaptive immunity.

Figure 10 shows immunohistochemical detection of MMP1 in lung tissue. The pulmonary tissue damage involves many factors. The study concludes that supplementation of Vitamin D decreases the expression of MMP1 [Figure 11], a collagenase enzyme that degrades types I, II, and III collagen. These data confirmed that Vitamin D regulate MMP1 expression in tissue where Vitamin D3 receptors are expressed.

The increased effectiveness of 2<sup>nd</sup>-line anti-TB was also measured by the decreasing the number of cell necrosis which causes bacteria to spread [Figure 12]. Necrosis is an inflammatory form of cell death. RIPK3 facilitates inflammation through damage-associated molecular patterns as well as nuclear factor-kappa B and result in the transcription of inflammatory cytokines. Figure 13 shows RIPK3 expression in response to D3 supplementation. Vitamin D as immunomodulator and-anti-inflammatory agent mitigate cell stress. A comprehensive analysis with structural equation

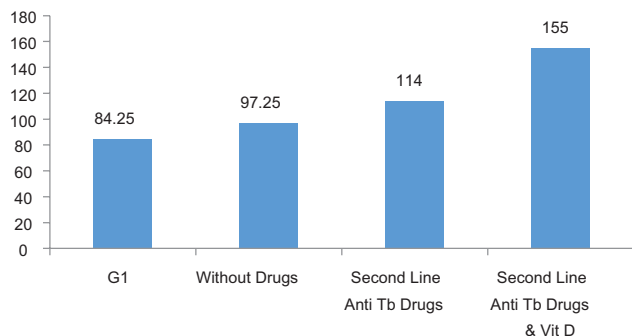
modeling concludes that supplementation of Vitamin D reduces cellular necrosis [Figures 14]. Autophagy reduce necrosis (RIPK3) by decreasing the pulmonary collagenase MMP1 with an indirect effect of -0.543. Autophagy has a protective effect on MMP-mediated cell injury.<sup>[24]</sup>

Apoptosis lowers RIPK3 by decreasing the number of bacteria with indirect effects of-0.544. Necrosis occurs when there is a high-intracellular bacillary load.<sup>[25]</sup> Apoptosis has control over the number of bacteria that is directly related to necrosis reduction.

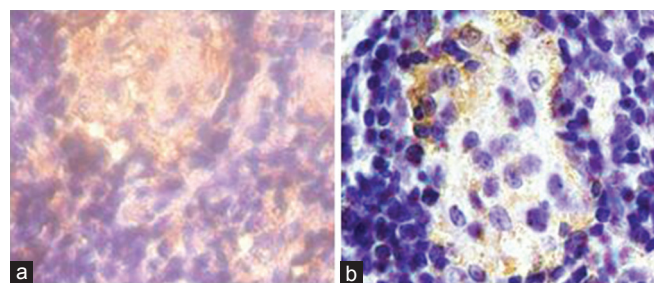
**DISCUSSION**

There has been a lot of literature showing the association of TB with Vitamin D deficiency.<sup>[26-29]</sup> It is proposed that the lower level result from chronic infection. Calcitriol, which is also referred as the Vitamin D-active form, binds to the Vitamin D3 receptor in the transcription process. The vitamin D receptor is important for adequate immune function. Intervention using a daily and constant dosage of vitamin D3 for 6 months might be appropriate to improve the effectiveness of 2<sup>nd</sup>-line anti-TB drug therapy. Our study shows that Vitamin D supplementation upregulates VDR expression.

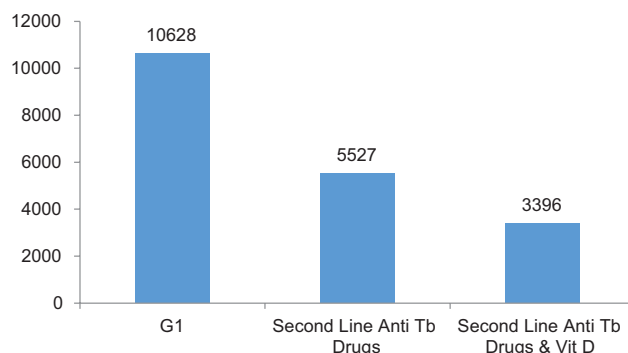
*M. tuberculosis* being trapped in immature phagosome. Vitamin D has mechanisms of controlling this evasion by inducing autophagy. The cathelicidin gene promoter region



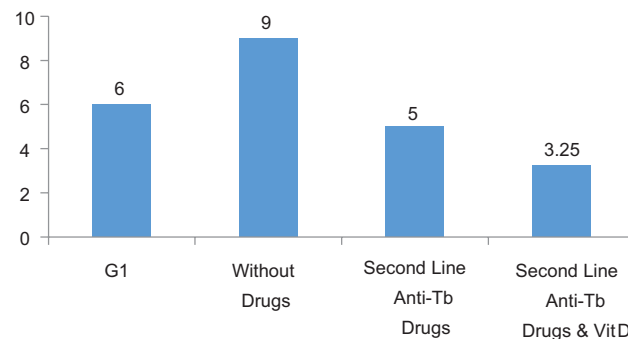
**Figure 8:** Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis improves caspase 3



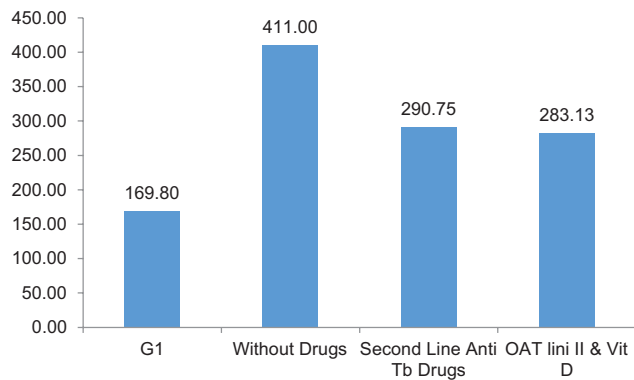
**Figure 10:** Matrix metalloproteinase-1 secretion in the lung. (a) Mice were treated with the only 2<sup>nd</sup>-line anti-tuberculosis drugs and (b) mice were treated with the drugs in combination with the Vitamin D3. The number of matrix metalloproteinase-1 immunoreactive cells was reduced by supplementation of Vitamin D (immunohistochemistry, ×1000)



**Figure 9:** Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis decreases the amount of *Mycobacterium tuberculosis* in the mice's lungs



**Figure 11:** Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis decreases the expression of pulmonary collagenase enzyme matrix metalloproteinase-1



**Figure 12:** Supplementation of Vitamin D3 in the 2<sup>nd</sup>-line anti-tuberculosis decreases cell necrosis

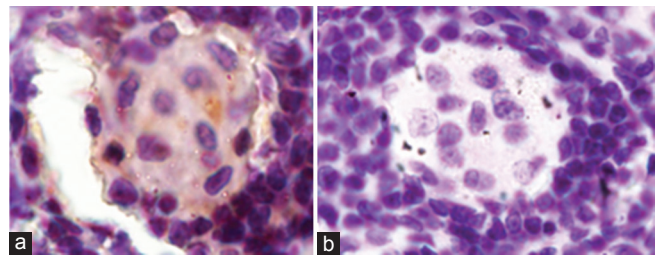
has 3 Vitamin D response element.<sup>[30-32]</sup> This study shows that oral supplementation with Vitamin D effectively increases cathelicidin expression in the lung. In conclusion, activation of Vitamin D receptor results in the expression of cathelicidin at both mRNA and protein level. Cathelicidin also requires Vitamin D (the Ca<sup>2+</sup> mobilizing agent) to reach the target antigen. This protein goes into autophagosome with the help of Ca<sup>2+</sup>/calmodulin-dependent kinase (CaMKK)- $\beta$ .<sup>[33,34]</sup>

Autophagy involves many factors. During autophagy, damages organelles or intracellular bacteria, is encapsulated in double-membrane autophagosome. The delivery of ubiquitin and antimicrobial peptide to the vesicles important in innate immunity. Colocalization LC3 with phagosome leads to the delivery of immature phagosome to lysosomes.<sup>[35]</sup> In this study, we report that Vitamin D upregulates LC3B expression. Previous study identified a conserved LC3-interacting region in mitogen-activated protein kinase 15 (MAPK15).<sup>[36]</sup> These data suggest that Vitamin D could induced LC3 activation through MAPK pathway. Although LC3 has several homologs, cytosol LC3B is most commonly used for autophagy marker. This is because LC3-II is present on autophagosome membranes, with the former being degraded.<sup>[37]</sup>

We demonstrate that Vitamin D enhances caspase-3 expression in murine model of TB. TNF-alpha promotes caspase activation.<sup>[38]</sup> It is possible that the interaction of TNF-alpha and calcitriol on mitochondria induces caspase-dependent apoptosis. The results of this study complete the data of other researchers which show that Vitamin D heightens apoptosis by increasing the activity of nitric oxide synthase. The increase of nitric oxide results potential changes in mitochondrial membrane and a release of cytochrome C.<sup>[39,40]</sup>

### Supplementation of Vitamin D3 enhances the effectiveness of 2<sup>nd</sup>-line anti-tuberculosis therapy by lowering the number of bacteria, expression of pulmonary collagenase enzyme matrix metalloproteinase-1, and cell necrosis

Supplementation of Vitamin D3 enhances the effectiveness of 2<sup>nd</sup>-line anti-TB therapy by lowering the number of bacteria. Since 1951, the antimycobacterial Vitamin D has



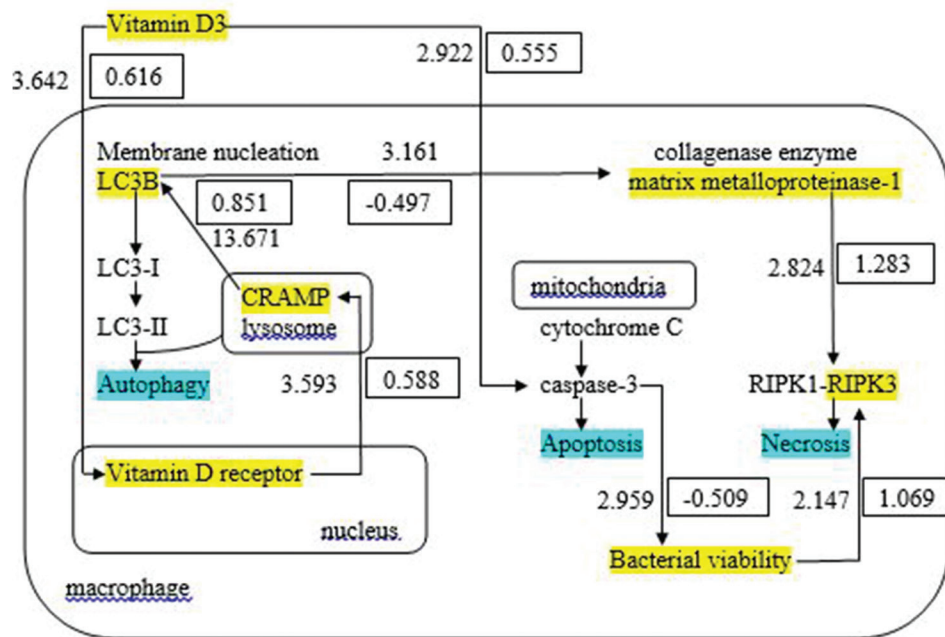
**Figure 13:** RIPK3 expressions in the lung granuloma. (a) Mice were treated with the only 2<sup>nd</sup>-line anti-tuberculosis drugs and (b) mice were treated with the drugs in combination with the Vitamin D3. The number of RIPK3 immunoreactive cells was reduced by supplementation of Vitamin D (immunohistochemistry,  $\times 1000$ )

been claimed<sup>[41]</sup> to inhibit *M. tuberculosis* growth directly<sup>[42]</sup> through upregulation of NO and NADPH oxidase, to induce maturation and activation of macrophages,<sup>[43]</sup> to increase fusion of phagolysosomes<sup>[44]</sup> and cathelicidin.<sup>[45]</sup> Together with the Vitamin A, it reduces the transcription of tryptophan-aspartate-containing coat protein.<sup>[46]</sup> The vitamin minimizes mycobacterium nutrition by inhibiting peroxisome proliferator-activated receptor  $\gamma$  which is responsible for differentiating macrophages into foam cells.<sup>[47]</sup>

Oxidative stress and tuberculosis are closely related. Oxidative stress is also implicated in activation of MMP1. Vitamin D is a secosteroid, an immunosuppressive steroid, the anti-inflammatory effect arising from immune suppression. The protective effect was associated with the induction of endogenous antioxidant and decrease of lipid peroxidation.<sup>[48]</sup> During treatment of pulmonary TB, we demonstrated that Vitamin D3 significantly inhibited MMP1 expression. Indeed, administration of Vitamin D also lowers MMP7, MMP9, increases TIMP,<sup>[49]</sup> and lowers granzyme A which hydrolyzes type IV collagen.<sup>[50]</sup>

Large level of reactive oxygen species and oxidative stress will induce cell death through necrotic pathway. The exacerbation of necrotic cell death and collagen destruction are a critical role causing caseous necrosis.<sup>[51]</sup> Structural equation modeling is used to analyze the relationship between Vitamin D supplementation and necrotic cell death. There is an association between Vitamin D supplementation, autophagy, MMP1 expression, and necrotic cell death. Vitamin D-induced autophagy helps control massive tissue damage by pulmonary collagenase. Adjunctive Vitamin D therapeutic approaches aimed at decreasing necrosis and improving diseases outcomes.

The structural relationship between apoptosis and necrosis is also confirmed. Apoptosis lowers RIPK3 by decreasing the number of bacteria. Apoptosis itself is not intrinsically bactericidal but requires phagocytic uptake of the apoptotic body. Necrosis is not dependent on bacterial virulence.<sup>[52]</sup> Necrosis was associated with the bacterial load. Necrosis occurs when there is a high-intracellular bacillary load.



**Figure 14:** Path analysis of the Vitamin D3 supplementation on the 2<sup>nd</sup>-line anti-tuberculosis. Numbers in the boxes are  $\beta$ : path coefficient. The amount of contribution of the independent variables affects the dependent variables. The boxless numbers are Tstat: signification. The Tstat value >1.96 indicates a meaningful relationship

## CONCLUSION

The cell death pathways are a fundamental process involved in the interaction between *M. tuberculosis* and infected cells. Decreasing necrosis requires increasing apoptosis and autophagy.

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## Conflicts of interest

There are no conflicts of interest.

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