1,25 (OH)_2D_3 treatment alters the granulomatous response in M. tuberculosis infected mice

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Induction of cathelicidin-mediated antimicrobial pathway against intracellular M. tuberculosis by 1,25-dihydroxyvitamin D_3 (1,25(OH)_2D_3), the active form of vitamin D, has been documented in vitro. However, in in vivo studies related to inflammatory disorders, 1,25(OH)_2D_3 has been demonstrated to induce an anti-inflammatory response. We therefore examined whether in the murine model of tuberculosis, the anti-inflammatory effects of 1,25(OH)_2D_3 would affect the outcome of M. tuberculosis infection. We show here that administration of 1,25(OH)_2D_3 to M. tuberculosis infected mice led to a change in lung granuloma architecture, characterized by a marked decrease in B cell lymphocytic aggregates. Consistent with the altered granulomas, 1,25(OH)_2D_3-treated mice also exhibited significantly higher bacterial burden in the lungs compared to the control group. These findings highlight the need to further investigate the effect of vitamin D on host immunity to M. tuberculosis in the context of the granulomatous response.

Vitamin D exerts regulatory control over a multitude of biological functions, including immune regulation. The synthesis of the physiological form of vitamin D, vitamin D_3 (cholecalciferol) is initiated with the photolysis of 7-dehydrocholestrol in the skin. Upon UVB radiation exposure, 7-dehydrocholesterol is converted to pre-vitamin D_3 which is subsequently isomerized to vitamin D_3. Conversion of vitamin D_3 to 25-hydroxyvitamin D_3 (25(OH)D_3) takes place in the liver, and subsequently to 1,25-dihydroxyvitamin D_3 (1,25(OH)_2D_3) in the kidney. Extra-renal conversion of 25(OH)D_3 to 1,25(OH)_2D_3 occurs in other tissue types including immune cells such as dendritic cells, macrophages and T cells. 1,25(OH)_2D_3, the bioactive form of vitamin D mediates its effect in various cell types by binding to its nuclear receptor known as vitamin D receptor (VDR) which functions as a heterodimer with the retinoid X receptor and together with co-regulatory complexes, affects the transcription of target genes.

Previous studies have noted that 1,25(OH)_2D_3 restricts intracellular Mycobacterium tuberculosis (Mt) replication via the induction of anti-microbial peptides. The convergence of Interleukin (IL-1β and VDR signaling pathways in antimicrobial response has also been reported. The vitamin D-mediated anti-microbial pathway can be activated by CD40L and IFNγ. 1,25(OH)_2D_3 inhibits intracellular growth of Mt by interfering with the accumulation of infection-induced lipid droplets. In contrast to its antimicrobial host protective functions, 1,25(OH)_2D_3 also down-modulates proinflammatory adaptive immune responses by inhibiting the differentiation of dendritic cells resulting in the suppression of the proinflammatory cytokine IL-12 and an increase in the anti-inflammatory cytokine IL-10. Similarly, a number of in vitro studies suggest that 1,25(OH)_2D_3 induces differentiation of T regulatory cells. In murine models of autoimmune diseases, 1,25(OH)_2D_3 has been reported to suppress the generation of Th1 and Th17 effector cells, the same T cell subsets that are involved in protection against tuberculosis. With the identification of many immunomodulatory properties of 1,25(OH)_2D_3, interest in vitamin D supplementation as a therapeutic approach to treat chronic inflammatory diseases is gaining momentum. Although a definite link between vitamin D supplementation and amelioration of disease severity is yet to be established, several studies show an improvement in clinical outcome. Treatment of patients with remitting multiple sclerosis (MS) with a 6 month supplementation of high dose dietary vitamin D resulted in beneficial immunomodulatory effects. In another randomized placebo controlled trial, high dose supplementation of 1,25(OH)_2D_3 resulted in reduced relapse rate and slower progression of the disease. These findings highlight the value of vitamin D in the treatment of chronic inflammatory diseases. However, the potential of vitamin D as a therapeutic agent for the treatment of tuberculosis is yet to be explored.

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vitamin D supplementation led to decreased inflammatory cytokine levels and moderate clinical improvement in patients with systemic lupus erythematosus (SLE)31. A randomized, double-blind, placebo-controlled trial in adults with chronic obstructive pulmonary disease (COPD) indicated that vitamin D3 supplementation reduced the risk of severe exacerbations32. Because 1,25(OH)2D3 can initiate potent anti-inflammatory response in the host and suppress Th1 and Th17 responses26,27,33, it becomes imperative to ascertain whether 1,25(OH)2D3 treatment will have any repercussions on the induction of host protective response against Mtb infection.

Cathelicidin and β defensin genes, that have been shown to play a key role in anti-mycobacterial mechanisms in humans34, are not regulated by VDR in rodents due to the lack of vitamin D response elements (VDRE)34. However, a large number of VDR responsive lymphoid and myeloid cell functions have been studied in murine models of infection and autoimmune disease. Administration of 1,25(OH)2D3 was shown to protect against experimental autoimmune encephalomyelitis (EAE) as well as against experimental inflammatory bowel disease (IBD)25. Vitamin D was also reported to suppress proinflammatory immune response in experimental cerebral malaria in mice35. 1,25(OH)2D3 treatment resulted in increased susceptibility to C. rodentium infection27 and its infusion in M. paratuberculosis-infected mice led to exacerbation of the disease resulting in increased bacterial burden36. Overall these studies suggest that the murine model can be used to investigate the impact of vitamin D on host resistance against Mtb, which has remained undetermined.

In this study, we therefore, investigated whether administration of 1,25(OH)2D3, the active form of vitamin D, would affect host immunity to Mtb in a murine model of tuberculosis. We report here that Mtb-infected mice treated with 1,25(OH)2D3 exhibited altered pulmonary granuloma formation and reduced ability to contain bacterial burden in the lung as compared to the control group of mice. These findings are significant since they provide a framework to further explore the potential role of vitamin D in disrupting the inflammatory networks involved in granuloma formation and control of Mtb growth.

Results
Administration of 1,25(OH)2D3 during Mtb infection alters cellular recruitment to the lungs. In a low dose aerosol exposure model, Mtb infection leads to an increased recruitment of various immune cell types to lungs that reaches a peak at four weeks post infection24,37. Therefore, in order to investigate the impact of vitamin D on lung cellularity during this acute phase of Mtb-infection, 1,25(OH)2D3-treated and control mice were sacrificed at four weeks post infection and flow cytometric analysis of lung single cell suspensions was carried out. The total number of cells recruited to the lungs of Mtb infected mice was observed to be the same in both the groups (Fig. 1a). Characterization of these cell populations was carried out via Trypan blue dye exclusion method (a). Percentage of CD4⁺, CD8⁺, B220⁺ and CD11b⁺ Gr1⁺ cells in the lungs were quantitated by flow cytometry (b). Five mice were used in each group and data are presented as mean ± SD.
of B220+ B cells, albeit not statistically significant and a significant increase in the percentage of CD11b+Gr1+ neutrophils in the lungs of 1,25(OH)2D3-treated mice compared to the control group (Fig. 1b).

**1,25(OH)2D3 administration alters the organization of pulmonary granulomas.** In the murine model of tuberculosis, the acute phase of infection is followed by establishment of a granuloma that consists of small foci of lymphocytic aggregates interspersed with macrophages and other cell types38–40. The granulomatous response leads to containment of inflammation and controls bacterial growth41. Since at four weeks post infection lung single cell suspension derived from the two groups of mice exhibited unique cellular pattern, we therefore compared the subsequent development of inflammatory lesions in the lungs of 1,25(OH)2D3-treated mice to that of control mice at six weeks following Mtb infection. A similar level of inflammation was observed in both groups of mice (Fig. 2a). However, lymphocytic aggregates in the inflammatory zones were significantly smaller in size in the 1,25(OH)2D3-treated lungs compared to the control group (Fig. 2b). Immunohistochemical evaluation of lung sections showed that these lymphocytic clusters are rich in CD20 positive B cells (3A) and are associated with Ly6G (3B) positive neutrophils. The granulomas of 1,25(OH)2D3-treated mice exhibited an overall decrease in CD20 (3D) cell staining but showed enhanced Ly6G+ cells (Fig. 3E).

**Mice treated with 1,25(OH)2D3 exhibit reduced ability to contain bacterial burden during the chronic phase of Mtb infection.** We next compared the bacterial burden in the two groups of mice at four and six week time intervals following Mtb infection. In the control group, as expected, there was an increase in bacterial burden at four weeks, and at six weeks as mice entered the chronic phase of infection, the bacterial burden decreased significantly (Fig. 4). In the 1,25(OH)2D3-treated mice, a similar increase in bacterial burden was also seen at four weeks, but, unlike in the control group, these mice did not exhibit a decrease in bacterial burden at six weeks and there was a significant difference in the bacterial burden in the two groups of mice at this stage (Fig. 4).

**1,25(OH)2D3-treated mice exhibit an overall increase in inflammatory gene expression.** We next evaluated the gene expression profile of key inflammation-related genes including Cyp24a, at four weeks post infection by real-time PCR. We observed that Cyp24a expression was induced in 1,25(OH)2D3 treated group of animals (Fig. 5a). Increased expression of Ifng, Nos2, Il17, and Tnf was observed in 1,25(OH)2D3-treated mice compared to control mice (Fig. 5b). The 1,25(OH)2D3-treated mice also exhibited significantly elevated gene expression for Il10 and Arg1 (Fig. 5c), indicating that the overall increase in both pro- and anti-inflammatory genes is likely a reflection of the increased bacterial burden in the lungs of these mice. Immunofluorescence staining confirmed the increased expression of arginase -1 in the lungs of 1,25(OH)2D3-treated mice (Fig. 5d).

**Discussion**

Host resistance to Mtb infection is critically dependent on the complex interplay between innate and adaptive immune responses to the pathogen42. Although proinflammatory responses are key to the host’s ability to contain the infection, anti-inflammatory immune response pathways are critical for the prevention of excessive
inflammation-induced damage to the host. Vitamin D deficiency and VDR polymorphism are associated with increased susceptibility or progression to tuberculosis disease. It has also been suggested that vitamin D as an adjunctive therapy during tuberculosis treatment may accelerate clinical recovery and inflammation resolution. Although a number of studies conducted in vitro strongly suggest that 1,25(OH)₂D₃ directly and indirectly activates host antimicrobial pathways, there is a lack of in vivo studies that focus on immunomodulatory role of vitamin D in the context of Mtb infection. The key innate and adaptive immune response pathways...

Figure 3. B cell rich lymphocytic cluster are diminished in the granulomas of 1,25(OH)₂D₃-treated mice. Immunohistochemistry was performed on lung tissue sections at six weeks post infection to detect CD20⁺ (A, D), Ly6G⁺ (B, E) and CD3⁺ (C, F) cells in the lymphocytic clusters. Tissue sections were stained individually with antibodies against indicated surface markers. Image (100X) was captured using Nikon Eclipse E 800 microscope. The boxed area in each section is presented at higher magnification (200X) in Fig. S2. Lungs from all the animals in each group were used for analysis and representative images from each group are shown here.

Figure 4. 1,25(OH)₂D₃-treated mice fail to control the bacterial burden. C57BL/6 mice were treated with 1,25(OH)₂D₃ or vehicle and infected via aerosol with ~100 CFU of Mtb Erdman strain. Mice were sacrificed at four and six weeks following infection and viable bacterial burden was determined by plating the lung homogenates on 7H11 agar plates. Each time point includes five mice per group. Experiment was performed twice and data from one experiment is presented as mean CFU ± SD.
generated in response to Mtb infection bear similarities in humans and mice. The availability of mice strains that exhibit disease pathology similar to humans is increasingly making this model an attractive tool in preclinical testing of drugs and vaccines. Therefore, in this study we sought to employ the murine model of tuberculosis to investigate the impact of 1,25(OH)2D3 on the outcome of Mtb infection. Our data indicating altered cellular recruitment to the lungs and change in granuloma architecture of 1,25(OH)2D3-treated mice, suggest an important role for vitamin D in regulating Mtb infection induced immune response. Particularly, the reduction in the size of B cell rich lymphocytic clusters in the 1,25(OH)2D3-treated lungs suggest that vitamin D may affect the immune mechanisms involved in B cell follicular response in the tuberculosis granuloma with consequent impact on the host's ability to control Mtb growth.

Several studies have indicated that B cell functions are modulated by 1,25(OH)2D3. It has been reported that although B cells do not express VDR constitutively, VDR expression is induced by B cell activating signals. Whether 1,25(OH)2D3 directly regulates B cells has been a matter of debate. It has been suggested that 1,25(OH)2D3 mediated inhibition of B cell function may be indirect, through the modulation of T cell or monocyte functions. B cell rich lymphocytic aggregates, bearing features of secondary lymphoid follicles, are characteristic of both human and murine tuberculosis granulomas. B cell follicles in tuberculosis granuloma have been suggested to provide a site for continuing cellular proliferation in response to Mtb antigens and have been implicated in the regulation of the granulomatous response. In a study by Chen et al., 1,25(OH)2D3 was shown to inhibit the proliferation of activated B cells and induce their apoptosis in vitro. However, we were not able to detect enhanced apoptosis in the lymphocytic aggregates in the 1,25(OH)2D3-treated mice compared to the control group. Mtb infection in B cell deficient mice resulted in an increased influx of neutrophils to the lungs, higher expression of IL-17 and increased bacterial burden. It is thus plausible that modulation of B cell responses by 1,25(OH)2D3, either directly or indirectly, may be a contributing factor to the observed increase in the number of neutrophils in the lungs of 1,25(OH)2D3-treated mice. Because of the altered granuloma environment, 1,25(OH)2D3-treated mice are subsequently unable to restrict bacterial burden in the lungs as efficiently as control animals as they enter the chronic phase of infection. Although we observed increased expression of several pro-inflammatory cytokines, expression of IL-10 and arginase-1 was also increased in 1,25(OH)2D3-treated mice. Macrophage-derived IL-10 induces arginase-1 in alternatively activated macrophages, and furthermore, arginase-1 controls Mtb growth and T cell mediated immunopathology. Therefore, future studies should evaluate how these cytokines modulate the inflammatory response in acute infection and also the immunopathology that is induced in chronic infection in 1,25(OH)2D3-treated mice.

In a recent study, Reeme et al. reported that high dietary vitamin D suppressed proinflammatory cytokine response, accompanied by mitigated pulmonary immunopathology in late stage Mtb infection in C3HeB/FeJ

Figure 5. Enhanced expression of cytokines in the lungs 5.1,25(OH)2D3-treated mice and in situ detection of arginase-1. Total RNA was isolated from the lungs of Mtb infected mice at four weeks post infection. Gene expression was assessed by real-time RT-PCR and fold induction in gene expression compared to uninfected lungs was determined (a, b, c). Immunofluorescence was used to detect arginase-1 expression in situ (d). Lung sections from mice infected with Mtb at four weeks post infection were stained for CD20 (red) and arginase-1 (green). Lungs from all the animals in each group were used for analysis. Representative sections from each group are shown. For RT PCR, five mice were used in each group and data are presented as mean ± SD.
mice. It is of interest that similar to this study\(^5\) we also observed increased proinflammatory cytokine response and neutrophil influx in 1,25(OH)\(_2\)D\(_3\)-treated mice. Although, in the Reeme study, the authors did not characterize B cells in the lymphocytic clusters, they observed a reduction in lymphocytic cluster size in the groups of mice that were fed high vitamin D diet. The reduction in B cell rich lymphocytic cluster size in response to 1,25(OH)\(_2\)D\(_3\) treatment suggests that vitamin D may modulate B cell mediated immune response to Mtb infection. Our future experiments will dissect the mechanisms involved in vitamin D mediated modulation of B cell function in Mtb infection. Another difference between our finding in the C57BL/6 mice and the C3HeB/FeJ data is that the latter study did not find increased bacterial numbers in the lung of mice fed high dietary vitamin D. The increased bacterial burden in response to 1,25(OH)\(_2\)D\(_3\) seen in our study may be the result of mouse geno-type specific differences. It is also possible that the strain of bacteria may influence the outcome of vitamin D or 1,25(OH)\(_2\)D\(_3\) treatment since our study used Mtb Erdman while the C3HeB/FeJ mice were infected with Mtb H37Rv. It is therefore critical that investigations are conducted on mice with varying genetic backgrounds such as the Diversity Outbred mice\(^6\) and with diverse clinical Mtb strains to delineate the effects of vitamin D on host immunity to Mtb. Another limitation of our study is that mice do not express human cathelicidin, which has been shown to be involved in antimicrobial activity against Mtb in vitro\(^7\). Further in vivo studies are needed in LL37 transgenic mice (expressing the human cathelicidin gene) to fully evaluate the interplay between vitamin D and the host immune response during Mtb infection and to dissect the underlying mechanisms.

**Methods**

**Mice.** C57BL/6 female mice (6–8 weeks old) were purchased from the National Cancer Institute (Frederick, MD, USA). Mtb-infected mice were housed in the animal BSL3 facility and guidelines from R-NJMS-Institutional Animal Care and Use Committee were followed in handling the animals. All experimental protocols in this study were approved by R-NJMS-Institutional Animal Care and Use Committee.

**Determination of pulmonary bacterial burden.** 1,25(OH)\(_2\)D\(_3\) was obtained from Cayman Chemical Company, (Ann Arbor, MI, USA). Mice were injected with 20 ng 1,25(OH)\(_2\)D\(_3\) or vehicle (90% 1,2-propanediol in ethanol) via subcutaneous route three times a week. The dose of 1,25(OH)\(_2\)D\(_3\) was chosen on the basis of previous in vivo studies with mice\(^8\). The treatment was initiated a day before the infection and continued until the completion of the study. Mice were infected with a low dose of Mtb Erdman strain (Trudeau Institute, Saranac, NY) in a whole body inhalation exposure system (Glus-Col, LLC, Terre Haute, IN). The number of bacteria deposited in the lungs were determined by plating the whole lung homogenates on 7H11 plates 24 hrs following aerosolization. At each time interval studied, infected animals were sacrificed by cervical dislocation and the right superior lobe of the lung was homogenized in PBS containing 0.05% Tween 80. Serial dilutions of the homogenates were plated onto 7H11 agar. The plates were incubated at 37 °C and colonies counted after 21 days. The rest of the lung was reserved for single cell preparation, RNA extraction and histological studies.

**Lung single cell preparation.** Lungs were perfused with 5 ml sterile PBS, cut into small pieces and incubated with 2 mg/ml collagenase D (Roche) for 30 min. The digestion was stopped by adding 5 mM EDTA. The digestion was continued by using heat induced epitope retrieval method as described previously\(^68\). The tissue sections were dipped in 70% ethanol until they were embedded in paraffin. Five micrometer sections were cut and stained using the standard H&E protocol. For immunohistochemistry, four to six micrometer sections were cut and mounted onto Superfrost/Plus microscope slides (Fisherbrand). Tissue sections were de-paraffinized with xylene and rehydrated with ethanol gradations and water. CD3, Ly6G, CD20 and arginase-1-epitopes were retrieved using heat induced epitope retrieval method as described previously\(^68\). The tissue sections were dipped in 10 mM citrate retrieval buffer (pH 6.0) and heated in a microwave pressure cooker (Nordic ware). Tissue sections were then blocked with Background Buster (Innovex Biosciences) for 30 minutes. Primary antibodies against CD20 (M-20), and arginase-1 (H-52) were obtained from Santa Cruz Biotechnology. CD3 (Rabbit polyclonal) was obtained from Abcam and Ly6G (1A8) was obtained from Biolegend. Sections were stained with these antibodies at 4 °C overnight. The sections were washed (PBS containing 0.5% tween 20) and reacted with biotinylated (1:100, Vector Laboratories) or fluorescent-labeled secondary antibody (Rabbit polyclonal) was obtained from Abcam and Ly6G (1A8) was obtained from Biolegend. Sections were then blocked with Background Buster (Innovex Biosciences) for 30 minutes. Primary antibodies against CD20 (M-20), and arginase-1 (H-52) were obtained from Santa Cruz Biotechnology. CD3 (Rabbit polyclonal) was obtained from Abcam and Ly6G (1A8) was obtained from Biolegend. Sections were stained with these antibodies at 4 °C overnight. The sections were washed (PBS containing 0.5% tween 20) and reacted with biotinylated (1:100, Vector Laboratories) or fluorescent-labeled secondary antibody (1:1000 Life Technologies) for 45 minutes at room temperature. For fluorescent detection of CD20 and arginase-1, Alexa 568 and Alexa 488 conjugated donkey – anti rabbit (Life Technologies) secondary antibodies were used. Relevant isotype controls were used for each primary antibody. The streptavidin horseradish peroxidase substrate (BioGenex) was used for immunodetection using DAB as a chromogen (BioGenex). The sections were counterstained with hematoxylin, and subsequently dehydrated in 95% and 100% ethanol followed by xylene. Finally, they were mounted on coverslips for microscopic visualization. Sections stained with fluorescent-labeled antibodies were directly mounted with ProLong antifade mounting media (Molecular Probes). Nikon Microphot-FXA, equipped with Objective Imaging Surveyor and a motorized microscope stage was used to capture tiled images. Granuloma area was measured using NIS Elements Advanced Research software. Fluorescent images were captured using NikonA1R laser scanning confocal microscope equipped with 20X planApo - numerical aperture 0.75.

**Flow cytometry.** The following anti-mouse mAbs used for the study: anti-CD4 (RM4–5), anti-CD8 (Ly-2), anti-B220 (RA3-6B2), anti-CD11b (M1/70) and anti-Ly-6G & Ly-6C (RB6-8C5) were purchased from BD Biosciences. All antibodies were directly conjugated to fluorochromes and isotype controls were included.
for each antibody type. Single cell suspensions from the lungs were made as described above and cell density was determined. Approximately one million cells were washed and re-suspended in FACS buffer containing appropriate concentrations of fluorochrome-conjugated antibodies. After thirty minutes incubation at 4°C, the cells were washed again in FACS buffer and fixed with 4% paraformaldehyde for 30 min. The cells were acquired on BD LSR II flow cytometer and data were analyzed using FlowJo (Tree Star).

**Real-Time RT PCR.** Lung tissues were homogenized in TRIZol® (Ambion™) and stored at −80 °C till further processing. RNA was extracted from TRIZol® and further purified using RNeasy kit (Qiagen) and reverse transcribed using High Capacity RNA to cDNA kit (Applied Biosystems™). cDNA was amplified using Taqman® reagents (Applied Biosystems™) on the ABI PRISM 7900 HT Sequence Detection System and fold induction in gene expression relative to uninfected tissue (RQ) was calculated by 2−ΔΔCt method69 by the Applied Biosystems software. Briefly, relative gene expression (fold induction) is calculated as 2−ΔΔCt, where ΔΔCt = Ct (gene of interest) − Ct (normalizer = β-actin) and the ΔΔCt = ΔCt (sample) − ΔCt (calibrator). The calibrator in our study is lung tissue obtained from uninfected mice. Due to the undetectable Cyp24a1 amplification in uninfected tissues, a Ct value of 40 was assigned to calculate relative fold induction. The following primer/probe sets from Applied Biosystems™ were used; Nos2 (Mm00440502_m1), Tnf (Mm00443260_g1), Ifng (Mm01168134_m1), Il17a (Mm00439618_m1), Il10 (Mm00439614_m1), Arg1 (Mm00475988_m1), Cyp24a1 (Mm00487244_m1), Cxcl1 (Mm04207460_m1), and β-actin (Mm00607939_s1).

**Statistical analysis.** For statistical analysis, GraphPad Prism Software (version 5) was used. The unpaired Student t test was used to determine statistical significance between the two groups. Values of *p* ≤ 0.05, **p** ≤ 0.01 and ***p*** ≤ 0.001 were considered significant.

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Acknowledgements
This study was supported by a grant from NJ Health Foundation Program and NIH grant AI-121621 awarded to P.S. & S.C. We thank Luke Fritzky (Confocal Imaging Facility, Rutgers - NJMS) for excellent assistance with confocal microscopy and Vaishali Veldurthy for helpful discussions.

Author Contributions
The study was designed and planned by P.S., S.C., K.B. and resources were secured by P.S., S.C. K.B., W.R. and N.S. carried out the experiments and contributed to data acquisition. Data analysis was carried out by K.B. and P.S. and S.C. K.B. wrote the original draft. P.S. and S.C. contributed to the review and editing of the manuscript. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Bhatt, K. et al. 1,25 (OH)2D3 treatment alters the granulomatous response in M. tuberculosis infected mice. Sci. Rep. 6, 34469; doi: 10.1038/srep34469 (2016).

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