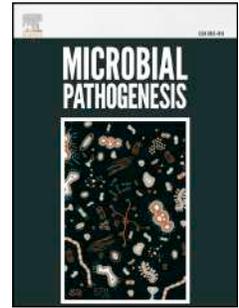


Accepted Manuscript

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PII: S0882-4010(19)30225-6

DOI: <https://doi.org/10.1016/j.micpath.2019.03.041>

Reference: YMPAT 3472

To appear in: *Microbial Pathogenesis*

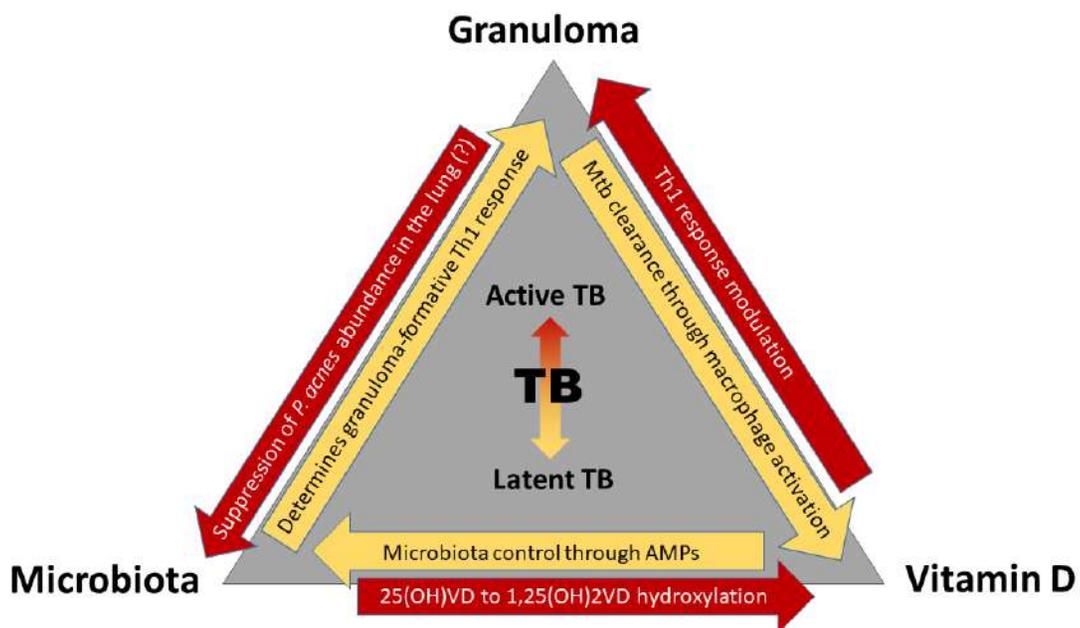
Received Date: 4 February 2019

Revised Date: 28 March 2019

Accepted Date: 29 March 2019

Please cite this article as: Balcells MaríElvira, Yokobori Noemí, Hong B-y, Corbett J, Cervantes J, The lung microbiome, vitamin D, and the tuberculous granuloma: A balance triangle, *Microbial Pathogenesis* (2019), doi: <https://doi.org/10.1016/j.micpath.2019.03.041>.

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ACCEPTED MANUSCRIPT

The lung microbiome, vitamin D, and the tuberculous granuloma: A balance triangle

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Abstract

Mycobacterium tuberculosis (Mtb) has the extraordinary ability to persist for decades within within granulomas in the human host. These histopathological structures involved in both protection and pathogenesis, are subject to various influences from the host systemically and through micro-niche environments. Despite the fact that vitamin D (VD) has a key role in macrophage activation and mycobacterial clearance in the early stages of Mtb infection, the overall role of VD in granuloma maintenance or functionality has been scarcely studied. VD deficiency has long time known to influence on gut microbiota composition, and recent studies have shown that it can also impact on respiratory microbiome. The human microbiota plays an important role in pathogen colonization resistance, and it has been proposed to play a potential role in TB pathogenesis.

In this article, we have reviewed current knowledge on the interaction between VD, the lung microbiome and TB, and propose mechanisms by which the tuberculous granuloma's outcome could be modulated by these two factors. The determinants of the final fate of lung granulomas are still unclear, and deciphering the underlying drivers of Mtb infection outcome within those structures is of critical importance.

Keywords: Granuloma; tuberculosis; Vitamin D; lung microbiota

1. Introduction

Tuberculosis (TB) remains a serious health threat worldwide, with more than 10 million new cases each year and almost 23% of the world's population infected (1). Although antibiotic treatment exists, the regimens involve multiple drugs administered for several months, with a global treatment success rate of only 82% (1). With BCG (*Bacillus Calmette-Guerin*), an attenuated strain of *Mycobacterium bovis* being a vaccine of questionable efficacy, better diagnostic, preventive, and therapeutic strategies are necessary to gain control of this disease, particularly in the light of the alarming emergence of multi and extended drug-resistant TB (2). Most of the research in TB pathogenesis has focused on host-pathogen interactions, and in the understanding of the innate and adaptive immune responses against this pathogen that has the extraordinary ability to persist for decades within human host. Persistence, which is the hallmark of TB infection, occurs within granulomas, hallmark histopathological structures developing in TB, and involved in both protection and pathogenesis. The determinants of the final fate of lung granulomas are still unclear, and deciphering the underlying drivers of *Mycobacterium tuberculosis* (Mtb) infection outcome within those structures is of critical importance. Vitamin D (VD) has a key role in macrophage activation in the early stages of Mtb infection and appears to be important in mycobacterial clearance (3). Despite macrophage and foam cells being key to the granulomas, and VD having been clearly involving in the maintenance of integrity of other cellular structures such as gut epithelium, the overall role of VD in granuloma maintenance or functionality has been scarcely studied for Mtb. VD deficiency has long time known to influence on gut microbiota composition, and recent studies have shown that it can also impact on respiratory microbiome (4). The human microbiota plays an important role in pathogen colonization resistance, and it has been proposed to play a potential role in TB pathogenesis (5-7). In this article, we reviewed current knowledge on the interaction between VD, the lung microbiome and TB, and propose mechanisms by which the tuberculous granuloma's outcome could be modulated by these two factors.

2. Granuloma formation in tuberculosis

A granuloma is an organized and structured collection of immune cells that forms in response to chronic antigenic stimulation. The granuloma is the classic histopathologic feature of TB, and functions both as a niche in which the bacilli can grow and persist and, an immunologic microenvironment in which cells with anti-mycobacterial functions interact to control and prevent dissemination of the infection (8). After being inhaled, Mtb encounters macrophages in the airways and lung alveoli of the human host. Infected macrophages may facilitate the spread of disease by extracellular dissemination after macrophage cell death, and via migration to distal sites. Release of inflammatory mediators and chemoattractants from infected macrophages recruit uninfected macrophages, monocytes, neutrophils, and primed T cells and B cells to lungs to ultimately form a granuloma (9). Although granulomas are composed of a variety of cell types, macrophages constitute the primary cellular component of their structure

and are the initiating cell type for granuloma formation (9) (Figure 1). There are a variety of cell phenotypes in granulomas with various functions, including antimycobacterial effector mechanisms, pro- and anti-inflammatory cytokines production, and secretion of chemokines and proteins associated with tissue remodeling (9).

Granulomas are observed in active, latent, and reactivation TB. In active TB, the host often has numerous granulomas that are incapable of controlling infection, with mycobacteria being present either extracellular or within macrophages or dendritic cells (9). In latent infection, there are commonly a few granulomas in lungs, with fewer inflammatory cells than in granulomas of active disease. Latent TB granulomas are usually calcified, as this represents a successful immune response (9).

The granuloma is a dynamic entity, maintained by a balance between a necessary protective response vs. a detrimental inflammatory response (10). Granulomatous inflammation is necessary to kill the mycobacteria or to prevent the spread of infection (11). Although some evidence supports a protective role of neutrophils in control of infection (well summarized in (8), disruption of the architecture of the granuloma observed in active disease, shows an increased neutrophil dominance (10), favoring host tissue damage and bacterial persistence (11). This granuloma disruption allows mycobacteria to, then, spread throughout the lung or disseminate to other organs, initiating new granuloma formation. The factors defining the type of granuloma that eliminates or controls over the infection are multiple, and may differ not only among individuals but even among granulomas in a single individual (9, 10). In chronic bacterial infections, hijacking of essential host's immunoregulatory mechanisms in order to minimize immune pathology, and activation of immune effectors occur (12). A high TNF responses from the host promotes a robust granuloma formation in the early events (13). However, excess of inflammatory mediators, like TNF, can cause granuloma and macrophage necrosis and release of mycobacteria into the extracellular space, where they can grow (8). Once containment is lost, a neutrophil-based inflammatory response leads to tissue destruction, mycobacterial growth, and transmission (12, 14). This, at least in part, explains the strong immune response in patients with active disease.

The local lung environment appears to shift from a host-protective nature towards one favorable to microbial persistence at the granuloma level (15). These changes promote a shift from M1 macrophage to an M2 polarization, and possibly affect B-cell function as well (15). Granuloma polarization dynamics are predictive of granuloma outcome, where a higher TNF presence in the first 40 to 80 days drives a higher granuloma polarization and containment, followed by a divergence between two granuloma outcome groups (containment vs. disseminated granulomas), at about 2 to 3 months post-infection (16). Rather than the spatial organization of M1 and M2 macrophages in the granuloma, the temporal dynamics of granuloma polarization ratios is what appears to correlate with the outcome. A spike in granuloma polarization ratio at 2 to 3 months post-infection occurs in granulomas that are able to contain infection, while those with disseminating infection remain flat. This early spike in M1 polarization limits excessive TNF expression and potentially later pathological inflammation (16).

3. Lung microbiota changes that could affect tuberculosis granuloma dynamics

The human microbiota has several functions including control over pathogens and education of the immune system. The lung is no exception and the lung microbiome has started to become the target of numerous studies, especially in TB (5). We have previously analyzed available data on the lung microbiota in TB and observed that several species that compose the lung microbiota are differentially abundant in patients with pulmonary TB vs. healthy controls (6). Lung microbiota changes induced by Mtb are not uniformly consistent amongst the different lung segments (17), and also differs depending on the stages of the infection, as shown by a recent study in macaques showing an initial increase in lung diversity at 1 month post-Mtb infection, which normalizes by the 5th month, demonstrating an Mtb-driven microbiota ecological reorganization in the lung (18).

The gut–lung axis involves the passage of bacteria, bacterial endotoxins, hormones, and cytokines into the bloodstream leading from the gut to the lung and vice-versa (19, 20). Changes in the composition of microbial communities in the respiratory tract (21) and the gut (22) have been linked to alterations in the immune responses and to disease development in the lungs. Similarly, Mtb lung infection has shown significant gut microbiota changes in mice (23). In an experimental model of intestinal inflammation, it has been shown that intestinal commensal bacteria act as activators of the intestinal innate immune system to instigate Th1 responses, which induces the development of granulomas (24). Depletion of intestinal microbiota leads to less inflammation and decreased intestinal granuloma development, in the case of intestinal infection with the parasite *Schistosoma mansoni* (25). Other human granulomatous diseases, such as Crohn disease, have shown microbiota clustering associated with mucosal granulomata (26). Studies in mice have shown that presence of intestinal commensal bacterium *Helicobacter hepaticus* early in life increases their susceptibility to challenge Mtb infection (27, 28). Epidemiological studies on humans have shown that *H. pylori* infection, on the other hand, may confer protection against TB disease (19, 29). *H. hepaticus* does, in fact, induce Th17 cells, which upregulate the Th1 program in the intestine (30, 31). IL-17 is an important cytokine in the induction of optimal Th1 response and protective immunity against mycobacterial infection (32, 33), and is necessary for granuloma formation (34).

It is possible that lung microbiota changes could also play a role in the inflammatory outcome that determines granuloma formation in the case of Mtb infection. Studies in other conditions have shown how commensals in the lung can affect granulomatous-disease progression. Sarcoidosis, an idiopathic disease presenting as non-caseating granulomas primarily in the lung, has been attributed in some animal models to may result from local *Propionibacterium acnes* infection (35). We have found *P. acnes* to be naturally present in the bacterial species signature in the lungs of healthy individuals (6). A study assessing the expression of Th1/Th2 responses in bronchoalveolar lavage fluid (BALF) of TB patients vs. lung cancer patients found that presence of some bacterial species such as *Haemophilus* induces a Th1-related gene expression (T-bet) in TB patients, while *Neisseria* influences Th1/Th2 responses in lung cancer patients (36). Despite the fact that these findings focused on only a few specific bacteria and examined subjects with lung cancers as controls, which can bear their own dysbiosis due to cancer (37), it is interesting that a link between presence of certain bacteria in the lung and granuloma-promoting Th1 responses does exist.

Finally, it has been shown in murine models that host microbiota dysbiosis after antibiotic treatment increases the risk of early lung colonization by *Mtb*, despite not altering immune cell populations nor the early inflammatory response to *Mtb* infection in the lungs (38). However, *Mtb* infection in the lungs of antibiotics-treated animals after 1 week was associated with a decrease in a subpopulation of microbiota-dependent T cells called lung mucosal associated invariant T cells (MAIT) (38). Finally, the long-term and dramatic effects of anti-TB drugs on the lung microbiome diversity and immunity (20, 39), could contribute to post-treatment susceptibility to reinfection, even to other mycobacteria species (40, 41).

All these recent reports seem to point out that the presence of commensals in the lung do play a role in the immune response elicited against pathogens like *Mtb*. More studies in the future, shall provide details on specific cell-bacteria interactions, and how they affect the outcome of TB disease.

4. The role of vitamin D in the immune response to tuberculosis and on tuberculous granuloma

Vitamins and micronutrients have a well-known role in immunity, with severe deficiencies translating into skin and mucosal damage and immune cells dysfunction. VD in particular, has effects of multifactorial nature at the cellular, molecular and metabolic level, in different cells of the innate and adaptive immune system (42). These pleiotropic effects of VD also include the modulation of the immune response, and it impacts both innate and adaptive immunity. The metabolically active form $1,25(\text{OH})_2$ VD has been shown to enhance the mycobactericidal activity of macrophages (43). Sera of individuals with low levels of $25(\text{OH})$ VD is inefficient for macrophage activation of VD pathway, and the anti-mycobacterial peptide cathelicidin (43, 44). In addition, VD has been proved to be necessary for the activation of human macrophages by $\text{IFN-}\gamma$, a cytokine of critical importance in the protective response *in vivo* (45) but which was known to be insufficient to activate mycobactericidal mechanisms *in vitro* (46). Their combination leads to the maturation of autophagosomes and the killing of intracellular bacilli (47).

In contrast, VD has a negative effect on Th1 response, which represents the protective adaptive immune response in TB. Numerous studies described that VD inhibits the production of $\text{IFN-}\gamma$ by Th1 lymphocytes, but the real impact of these observations in clinical TB disease outcome is not clear. It has recently been reported that VD does not block differentiation of CD4^+ cells to Th1 cells, and that IL-12 induce these Th1 cells to produce equal amounts of $\text{IFN-}\gamma$ in the presence of VD (48). Cathelicidin synthesis by dendritic cells relies on activation by VD but is independent of Th1 signaling, and this induction can overcome the negative effect of TLR2 ligands of *Mtb in vitro*. The lack of a cathelicidin analogue in mice has led to contradictory results. While some authors reported a reduced immunopathology with unaltered bacillary loads (49), others describe a negative effect on pathogen control (50). Interestingly, both studies describe a reduced contribution of lymphocytes in the structure of granuloma after administration of $1,25(\text{OH})_2$ VD. Histopathological analysis of human lung resection from patients with treatment refractory TB showed that, compared to distal healthy lung parenchyma, tuberculous lung lesions are enriched in FoxP3 positive Tregs and IgG secreting B

cells, and express lower levels of cathelicidin (51). Considering that all the studied patients had VD deficiency and the comparisons were made within different regions of the same lung samples, it is difficult to link the lower expression of LL37 with their VD status, but further studies in this line would be important to properly link these observations.

Another interesting evidence of a positive role of VD in the orchestration of granuloma structure in humans comes from leprosy, another mycobacterial disease. Tuberculoid leprosy and lepromatous leprosy are two well defined manifestations of the disease with distinctive immunological hallmarks (52). It has been shown that the enhanced ability to control bacillary burden in the former type of lesions correlated with their enhanced ability to synthesize 1,25(OH)₂ VD and to activate the VD-dependent antimicrobial pathway. In contrast, lepromatous lesions, enriched in IL-10-producing macrophages, are unable to metabolize 25(OH) VD to its active form (53). In addition, certain polymorphisms in the VD receptor (VDR) gene have been associated with tuberculoid leprosy (54), and protection against pulmonary TB (55).

These observations indicate that there is still a gap to fill between the widely accepted correlation of low serum VD levels with active disease and the apparent failure of its utilization as an adjuvant therapy. Taken together, although VD has been clearly shown to improve the innate control of *Mtb*, its impact in human adaptive immune response and granuloma formation is less clear.

As we pointed out earlier, the presence of commensals in the lungs of mice is important for an adequate number of MAIT cells (38). MAIT cells are believed to contribute to protection from pulmonary infections, and reduced MAIT cell frequency in peripheral blood is associated with more severe lung disease in cystic fibrosis (CF). A study suggests that VD might enhance migration of activated MAIT cells to the lungs, decreasing their numbers in peripheral blood, as adult patients with CF receiving VD had decreased expression of activation markers on CD4⁺ and CD8⁺ T cells in their lungs, and a decreased frequency of MAIT cells in blood (56).

5. The role of vitamin D in lung dysbiosis

VD is important into the protection of mucosal cells barrier and preventing inflammation, a process that has been extensively studied in the gastrointestinal mucosa (57-59). VD deficiency is not only associated with harmful changes to intestinal epithelial barrier but also with modifications in the composition of the intestinal microbiome in murine models (57, 59, 60). In humans, VD deficiency, as well as specific polymorphisms in the VDR gene, also associate with changes in gut microbiota composition or inflammatory markers as studied in different cohorts (61, 62). Conversely, maternal levels of 25(OH) VD and maternal VD supplementation, have been related with the abundance of several key bacterial taxa within the infant gut microbiota (63).

Several possible mechanisms have been proposed to explain the role of VD in the maintenance of microbiota homeostasis, involving both the innate and acquired immune response. A pertinent mechanism may relate to VD capacity to induce the expression of several antimicrobial peptides, such as cathelicidins and β -defensins by monocytes, macrophages and epithelial cells (64) (Figure 2). Accordingly, VD deficiency has been shown to lead to reduced

colonic expression of angiogenin-4 (Ang4), an antimicrobial peptide which plays an essential role in regulating gut microbial status by maintaining submucosal autophagy, lysosomal activity, and elimination of pathogens (65, 66). Mice lacking the expression of the 1α -hydroxylase enzyme (responsible for converting circulating 25(OH) VD to active $1,25(\text{OH})_2$ VD) display an increased gut inflammation and fewer tolerogenic CD103⁺ dendritic cells in the lamina propria (57). These CD103⁺ dendritic cells are responsible for shaping the regulatory T cell repertoire of the gut, with ongoing presentation of bacterial antigens required for the development of tolerance towards gut microbes (67). Thus, sustained VD deficiency may translate into reduced tolerance towards gut microbes, and increased mucosal inflammation, leading to the predominance of some microbial taxa upon others. This notion is supported by reports on the effect of high doses of VD in the modulation of intestinal microbiome (68), with a potential positive influence on inflammatory diseases and bacterial infections (69).

The study of the effects of VD in airway inflammation and respiratory microbiota is more challenging than in the gut, given the technical difficulties for obtaining sampling from the lungs in healthy humans, and much of the current knowledge has been provided from upper respiratory tract sampling or from animal models. Increased inflammatory cells in the BALF and increased bacterial load are found in mice with allergic airway disease and VD deficiency compared to control, and this abnormal status can be reversed by dietary VD supplementation (70). Mice fed with a VD-containing diet throughout life, do not show significantly affected bacterial diversity in the lungs nor increased BALF 25(OH) VD levels (71). However, a sex-specific effect in the lung microbial community is observed, with increased levels of *Acinetobacter* in female lungs compared to male lungs. Also, VD sufficiency was shown to limit the number of respiratory pathobionts like *Pseudomonas* rather than increasing the number of protective commensals in the lungs (71). Remarkably, dietary VD increases levels of antimicrobial murine β -defensin-2 (mBD2) in BALF of mice. The inverse relationship between lung *Pseudomonas* and serum 25(OH) VD could be related to increased levels of mBD2 in BALF of VD-sufficient mice.

In humans, VD is under scrutiny as a potential regulator of the development of respiratory diseases characterized by chronic lung inflammation, including asthma and chronic obstructive pulmonary disease. Epidemiological investigations have reported the associations of lower blood 25(OH) VD levels at birth or early infancy with an increased risk and severity of acute respiratory infections, including bronchiolitis (72). A meta-analysis of individual participant data from 25 trials demonstrated that VD supplementation is protective against acute respiratory infections in subjects with low baseline 25(OH) VD concentrations (73).

The effect of VD deficiency on the respiratory microbiome has seldom been investigated in humans. A multicenter prospective cohort study of more than a thousand infants with severe bronchiolitis, reported a significant association between circulating 25(OH) VD levels with nasopharyngeal microbiota composition, with a *Haemophilus*-dominant microbiota profile being associated with a significantly higher risk of intensive care use compared to a *Moraxella*-dominant profile (4).

Providing VD supplementation in humans has been associated with variable outcomes regarding gut microbiome composition, going from regional differences in the response of the gastrointestinal microbiome of healthy volunteers (68), reduced intestinal inflammation in

patients with active ulcerative colitis (74) and, changes in *Bacteroides* abundance in pre-diabetic individuals (75). Only one clinical trial so far has evaluated the impact of VD supplementation in human respiratory microbiome of patients with cystic fibrosis. This study showed that initial serum 25(OH) VD concentration levels correlated with sputum microbiota alterations with differential clustering based on VD status (76). Several taxa, especially of the genus *Bacteroides*, were enriched in the sputum samples of subjects with VD insufficiency compared with samples from subjects with VD sufficiency at baseline. There was also differential clustering of microbiota in subjects receiving VD supplementation. Although differences in microbiota composition were found, these observations may not be necessarily extrapolated to healthy subjects, given that pathogenic bacteria are more common in the lungs and associated respiratory tissue of patients with respiratory diseases.

6. Conclusions

Granuloma formation in tuberculosis involves multiple cells, and cell interactions. These cell responses are affected by the presence of commensals in the lung. It is possible that lung microbiota changes could affect tuberculous granuloma dynamics. VD plays an important role in the immune response to TB and in the formation of tuberculous granuloma. VD also plays a role in modulating both, the intestinal and the lung microbiome. Despite the limited amount of data, it seems very plausible that these three variables, i.e. granuloma, microbiome, and VD, interact with each other in the TB scenario, determining the outcome of infection and progression of disease. All this opens the doors to potential therapeutic strategies aiming to redirect disease outcome through modulation of the components of this balance triangle.

Funding

No funding sources to acknowledge.

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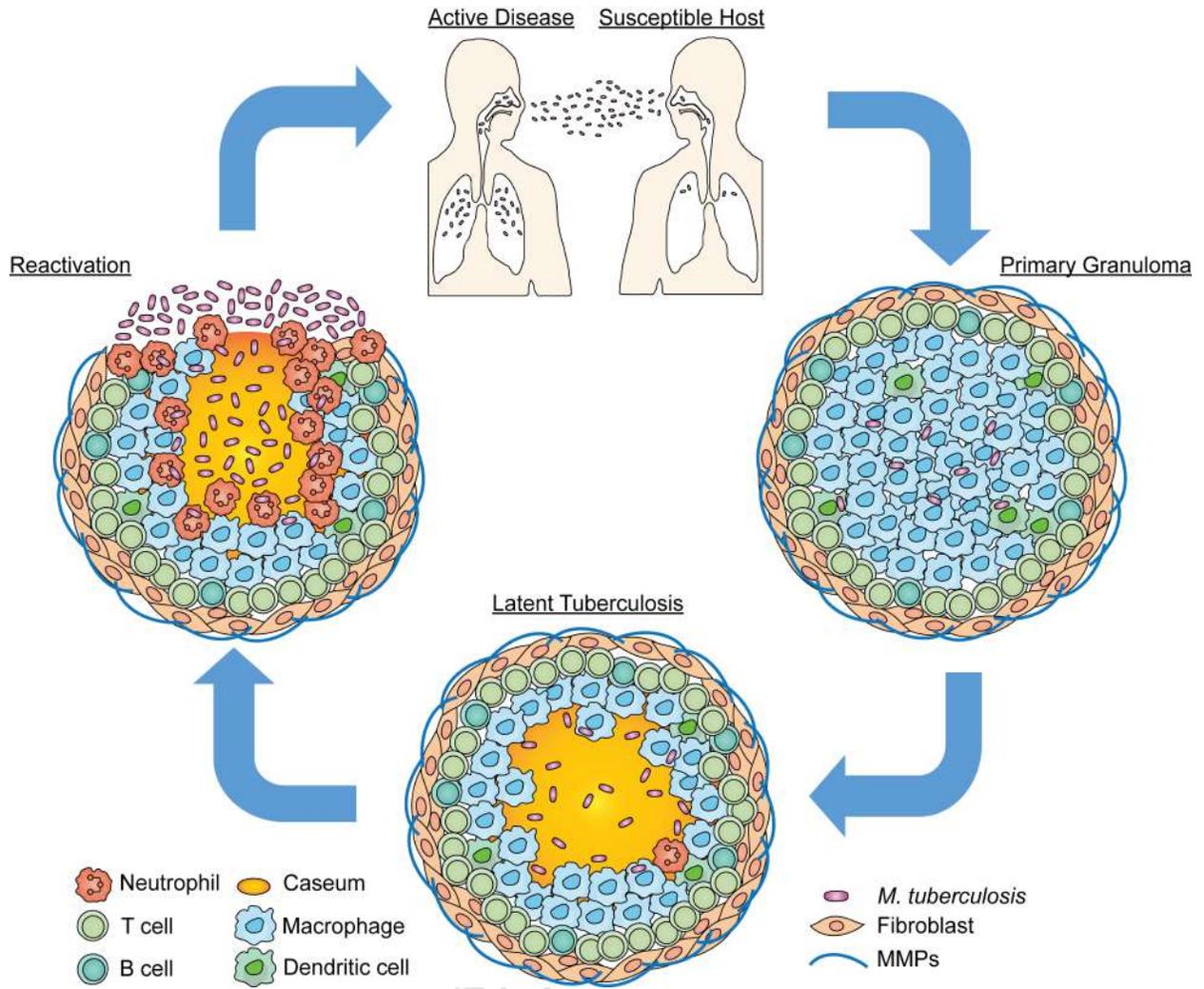
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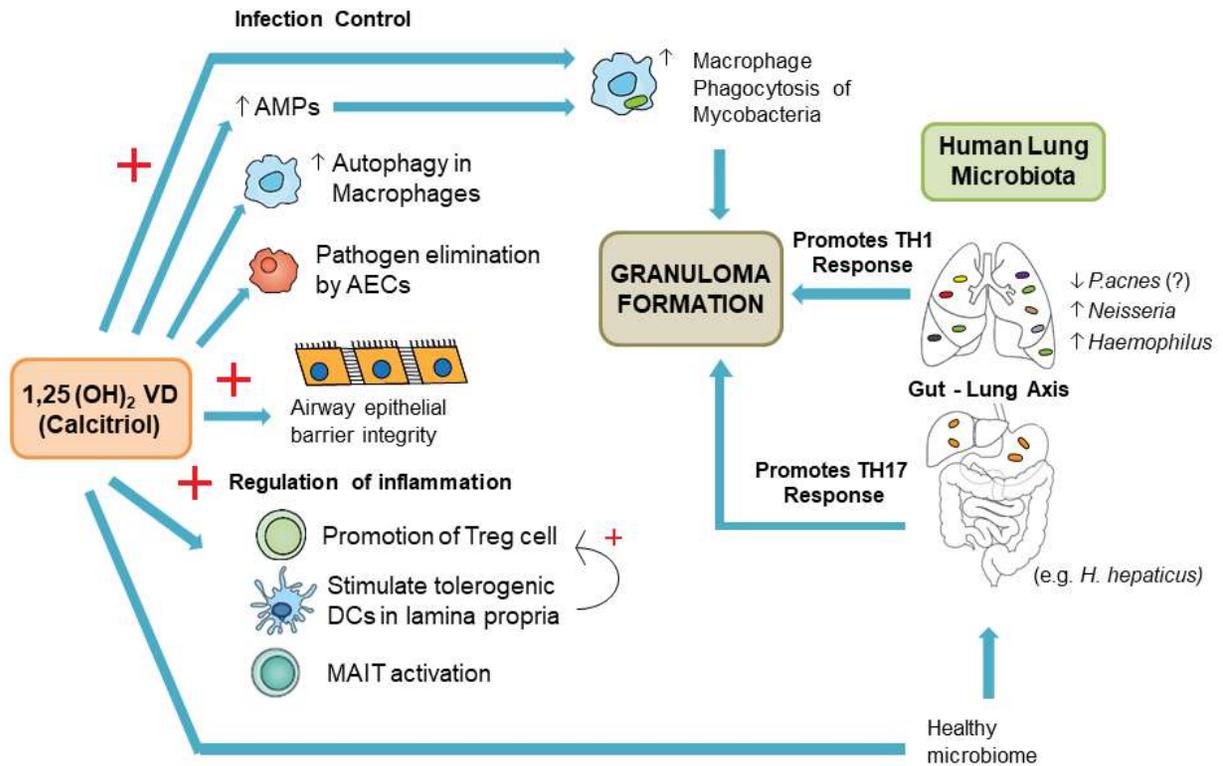
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Figure Legends

Figure 1. The granuloma in TB pathogenesis and transmission. Granulomas are composed of a variety of cell types, macrophages constitute the primary cellular component of their structure. Granulomas are observed in active, latent, and reactivation TB. The granuloma is a dynamic entity, maintained by a balance between a necessary protective response vs. a detrimental inflammatory response. Granulomatous inflammation is necessary to kill the mycobacteria or to prevent the spread of infection. In active TB, the host often has numerous granulomas that are incapable of controlling infection, with mycobacteria being present either extracellular or within macrophages or dendritic cells. In latent infection, there are commonly a few granulomas in lungs, with fewer inflammatory cells than in granulomas of active disease. Disruption of the architecture of the granuloma observed in active disease, shows an increased neutrophil dominance favoring host tissue damage and bacterial persistence. This granuloma disruption allows mycobacteria to, then, spread throughout the lung (accounting for human to human transmission) or disseminate to other organs, initiating new granuloma formation.

Figure 2. Vitamin D and the Lung microbiota play a key role in Mtb infection and granuloma formation. VD is involved in various mechanisms of mycobacterial infection control and granuloma formation. The human lung microbiome, and through comensal and immunological signals via the gut-lung axis is also involved in the formation of granulomas for Mtb infection containment,





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Highlights

- Granuloma formation in tuberculosis involves multiple cells, and cell interactions, which are affected by the presence of commensals in the lung.
- It is possible that lung microbiota changes could affect tuberculous granuloma dynamics.
- VD plays an important role in the immune response to TB and in the formation of tuberculous granuloma. VD also plays a role in modulating both, the intestinal and the lung microbiome.
- These three variables, i.e. granuloma, microbiome, and VD, interact with each other in the TB scenario, determining the outcome of infection and progression of disease.