The effects of vitamin D on cellular responses, molecular immunity and mycobacterial killing in cattle

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I. STATEMENT OF ORIGINAL AUTHORSHIP

I hereby certify that the submitted work is my own work, was completed while registered as candidate for the degree on the Title page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

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II. ABSTRACT

The positive effect of vitamin D (vit D) on health and resistance against infectious diseases, particularly tuberculosis (TB) is well recognized. However, research has predominantly focused on murine and human species and functional data in bovines is limited. The aims of this project were: **chapter 2&3)** To investigate the effect of $1,25(OH)_2D_3$ on the microbicidal and immunoregulatory activities in peripheral blood leukocytes (PBL) and on neutrophils. **Chapter 4)** To develop a model to drive divergent 25(OH)D circulating levels in dairy calves, and **Chapter 5)** To investigate the influence of differential 25(OH)D circulating levels on the microbicidal and immunoregulatory activities of PBL following and ex-vivo BCG challenge.

Results from **chapter 2** showed that $1,25(OH)_2D_3$ supplementation significantly increased BCG killing on PBL to 65.7% compared to 49.1% in the untreated blood. Serial cell subset depletion showed that depletion of granulocytes had the greatest impact on BCG growth and lead to a significant enhancement of bacterial colonies. Significantly enhanced bacterial killing was observed in $CD14_{neg}$ PBLs treated with $1,25(OH)_2D_3$ and a similar trend was observed in $Gran_{neg}$ subsets ($P = 0.06$). In contrast, depletion of $CD4^+$ or $CD8^+$ T cells individually or in combination ($CD3^+$) had no impact on mycobacterial control. Data also showed that $1,25(OH)_2D_3$ stimulation increased reactive oxygen species (ROS) production and decreased overall BCG growth in PBLs. $1,25(OH)_2D_3$ increased the expression of a cluster of genes including *DEFB7, TAP, ELANE, CCL2, CXCL10, IFNB, IL33* and *PKR* implicated in activation of the innate immune response to mycobacteria. Results from **chapter 3** confirmed the microbicidal effects of $1,25(OH)_2D_3$ on neutrophils showing an enhanced killing against BCG and *M. bovis*.

Then, in **chapter 4** calves were supplemented with vit D_3 from birth to 7 months age. Two control groups (Ctl-In, Ctl-Out) received a diet containing 6,000 IU/Kg of vit D_3 in milk replacer and 2,000 IU/Kg in rations, and two groups (VitD-In, VitD-Out) were fed with 10,000 IU/Kg of vit D_3 in milk replacer and 4,000 IU/Kg in rations. After weaning, Ctl-Out and VitD-Out groups were moved outdoors, whereas Ctl-In and VitD-In groups were kept indoors. Results showed a high incidence of vitamin D deficiency (VDD) at birth with mean 25(OH)D of 7.64 ±3.206. Significant elevated 25(OH)D concentrations were observed after weaning, with the maximal 25(OH)D level achieved in VitD-Out reaching 60.86±7.318. Greatest divergence in haematology profile was observed between Ctl-In and VitD-In groups, with Ctl-In calves showing an elevated count of neutrophils, eosinophils, and basophils associated with reduced 25(OH)D concentrations. Neither IL-8 nor ROS production in serum were significantly different between calves. In **chapter 5** the assessment of the microbicidal activity and immunoregulatory effect to an *ex-vivo* BCG challenge was performed on Ctl-In and VitD-In

groups. Results showed higher bacterial killing in the VitD-In group (48.0%) in contrast to Ctl-In group (29.5%). Besides, increased production of ROS and NO was detected in VitD-In calves. Whereas serum concentrations of IL-1β and IL-8 were significantly lower. Likewise, a significant downregulation of a cluster of genes including *IL1B, IL1R1, TNFA, CXCL1, CXCL2, CXCL5, CXCL8* was detected in VitD-in calves relative to Ctl-In animals.

Collectively, results showed that $1,25(OH)_2D_3$ improved mycobacterial killing in bovine PBLs via the synergistic activity of monocytes and granulocytes and enhanced activation of innate immunity. Evidence indicates that vit D_3 supplementation boosts antimicrobial and innate immune responses but modulates excessive inflammation which may be detrimental for the outcome to infection. Finally, results identified neutrophils as one of the principal targets of vit D modulatory effects.

III. DEDICATION

A mi papá, Lucio Flores

Por tus enseñanzas que me ayudan a superarme día a día, tu incansable apoyo y dedicación hicieron de este sueño una realidad. Tu memoria vive en cada una de estas páginas.

Nos vemos en los espejos

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V. LIST OF FIGURES

VI. LIST OF TABLES

VII. COLLABORATIONS

Dr. Aude Remot, INRAE Centre Val de Loire, France.

- Fluidigm gene expression analysis (Chapter 2 & 5)
- Analysis of neutrophil modulation by $1,25(OH)_2D_3$ (Chapter 3)

Dr. Seán Lacey, Munster Technological University, Ireland.

- Statistical analysis (Chapter 4)

VIII. LIST OF ABBREVATIONS

$1,25(OH)_2D_3$	1, 25-dihydroxyvitamin D_3
25(OH)D	25 hydroxyvitamin D
AM	Alveolar macrophage
BCG	*Mycobacterium bovis* bacillus Calmette-Guérin
BTB	Bovine tuberculosis
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
CYP27B1	Cytochrome P450 family 27 subfamily B member 1
CYP2R1	Cytochrome P450 family 2 subfamily R member 1
FGF23	Fibroblast growth factor 23
HDP	Host defence peptides
M. bovis	*Mycobacterium bovis*
MAP	*Mycobacterium avium* ssp. paratuberculosis
M. tuberculosis	*Mycobacterium tuberculosis*
NO	Nitric oxide
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cell
PTH	Parathyroid hormone
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RXR	Retinoid X receptor
TB	Tuberculosis
TLR	Toll-like receptors
VDBP	Vitamin D binding protein
VDD	Vitamin D deficiency
VDR	Vitamin D receptor
Vit D	Vitamin D
Vit D_2	Ergocalciferol
Vit D_3	Cholecalciferol
WB	Whole blood

1. CHAPTER 1. General Introduction

1.1. Bovine tuberculosis (BTB)

Zoonotic and livestock diseases are a major threat to human and animal health and represent an unsustainable cost on livestock production systems. Their control is essential for safeguarding and securing global food supplies and for improving the livelihood of rural communities in developing countries [1]. A zoonotic disease is an infection or disease that can be transmitted from vertebrate animals to humans or from humans to animals. It is estimated that more than 60% of human pathogens are zoonotic in origin. Zoonotic diseases are caused by a wide range of pathogens, that includes bacteria, viruses, fungi, protozoa, parasites and mycoplasma [2].

Among the bovine zoonoses of significant public health significance, tuberculosis is considered the most important. Bovine tuberculosis (BTB) is caused by *Mycobacterium bovis* (or rarely *M. caprae*) [3]. BTB is accountable for economic loses to agriculture globally, due to trade restrictions, non-movement of animals and costs associated with the test and culling control strategies, with some authors estimating annual losses close to $3 billion [4]. However, estimating the true economic cost of BTB is challenging due to differences in epidemiological situations, livestock systems, natural reservoirs, and control strategies between countries [5]. For example, in 2020 €97 million was spent on the BTB eradication program in Ireland, and if no reductions in the disease incidence is achieved by 2030 the total cost is expected to reach €1 billion [6].

Although cattle are considered the main host for *M. bovis*, other livestock and wildlife species can be affected making it difficult to eradicate, this being the case of badgers in the United Kingdom and Ireland, brushtail possums in New Zealand, white-tailed deer in the United States of America, wild board in Spain, and the African buffalo and March antelope in Africa [7]. Eradication programs have reduced the infection in cattle in most countries and cases of *M. bovis* infections in humans are now rare. However, in developing countries BTB is still a risk factor for communities living in close contact with livestock [8]. The global prevalence of zoonotic TB was estimated to be around 1.4%, however, the true prevalence is likely to be an underestimate due to diagnostic limitations [9]. Furthermore, large differences in prevalence between studies have been reported. For example, an epidemiological study from all the TB cases in USA during 2006-2013 showed that the annual percentage of TB cases attributed to *M. bovis* was within 1.3 to 1.6% [10]. Another study in Mexico revealed that *M. bovis* was responsible of approximately 30% of all TB cases from 2000 to 2015 [11]. From these studies, children, and females from low-income backgrounds were among the most affected. This data indicates that people, particularly from countries with limited resources, are still at risk of *M. bovis* infection.

M. bovis is naturally resistant to pyrazinamide, one of the four medications used in the standard tuberculosis treatment for humans. Commonly laboratory procedures do not differentiate between *M. tuberculosis* and *M. bovis,* and usually healthcare providers initiate treatment without drug susceptibility testing. Therefore, identification of the bacilli strain causing disease is necessary to provide an adequate treatment to vulnerable populations [12].

TB disease results from an interdependence of human, agricultural and wildlife factors. Thus, collaborative, multisectoral and transdisciplinary tactics are necessary to tackle the health and economic impact of this disease in a One Health approach.

1.1.1. The prevalence of BTB

Geographically, BTB is distributed worldwide (Figure 1-1). According to the Worldwide Animal Health Information Database from the OIE, 62.2% of the countries reported the presence of BTB in livestock; 35.4% reported the disease in both cattle and wildlife, and 2.4% reported its presence only in wildlife [13].

A recent meta-analysis estimated the global prevalence of BTB in different livestock species and showed an overall prevalence around 10% (except for farmed wild boar). This study included the analysis of 182 articles published between 1993 and 2020 and encompassed eight host groups: cattle, goat, pig, buffalo, camelids, sheep, farmed cervids and farmed wild boar [14]. Using a random-effects model, the pooled prevalence of worldwide BTB in cattle was estimated as 13.12% (95% CI: 11.24% - 15.26%). Moreover, the prevalence was higher in female (8.7%) than male cattle (4.8%), which reflects differences in the production systems. Dairy farms are associated with more intensive systems and longer animal lifetime, factors identified as a risk for increased BTB incidence [14, 15].

In the European Union (EU) in 2019 eleven members reported an heterogenous prevalence of BTB from 0.48% in Portugal to 11.29% in the UK [16]. After Brexit, Ireland is the member with the highest level of BTB within the EU, and it has been experiencing a sustained increase in BTB levels from 2016, with herd incidence going from 3.27% to 4.37% in 2020 [6].

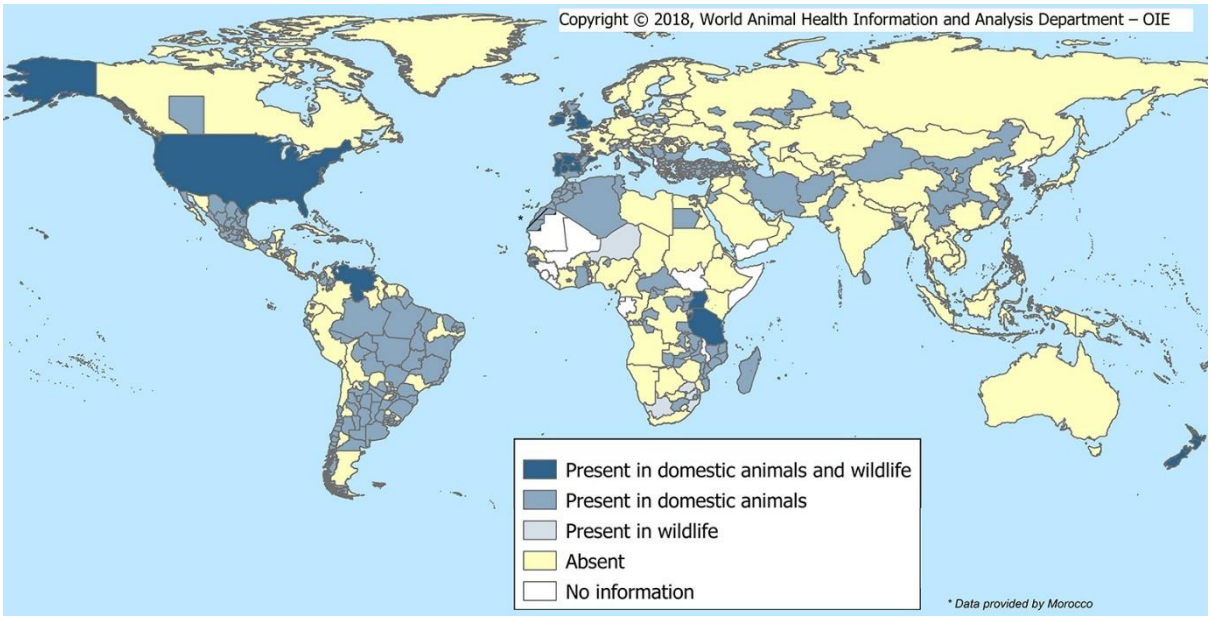


Figure 1-1 Global distribution of BTB in 2017 and first semester of 2018.
Taken from Worldwide Animal Health Information Database of OIE [13].

1.1.2. Control and eradication strategies for BTB

Control and eradication programmes have been carried out in many countries for decades with estimations showing a gradual decline in the global BTB burden. However, the disease is still a significant problem in developing countries, but also in the United States of America (USA), New Zealand, UK, and Ireland [17]. Generally, the eradication programs are based on test and slaughter schemes. Therefore, the detection of infected animals is crucial for the effectiveness of control programs. The infectious status of animals is assessed by their cellular immune response to mycobacterial antigens. The intradermal tuberculin skin tests, in single or comparative versions, is used as the primary screening test due to its low cost and well-documented use [18]. Whereas the interferon gamma (IFN-γ) assay is used as complementary test or in specific situations [18]. These control measures are supplemented with abattoir surveillance and comprehensive epidemiological investigations of outbreaks [19].

Additional control strategies are under investigation, including vaccination of cattle against BTB, together with the use of a DIVA test, to differentiate vaccinated from infected animals. Where wildlife reservoirs of *M. bovis* exist, measures such as culling, or hunting are applied to reduce the population density [20]. Furthermore, the use of genetic selection to improve resistance to BTB is emerging as a potential tool to control the disease [21].

In Ireland, the Department of Agriculture has committed to eradicating BTB by 2030, and with the support of the Bovine TB Stakeholder Forum has established different policies to achieve this goal. The key component of the BTB programme is to reduce cattle-to-cattle transmission [6]. However, it has been pointed out that additional actions, such as reducing the risk of persistent TB due to presence of infected but undetected animals in the herds, would be needed to achieve the intended goal [22]. Nutrition has a profound impact on animal health, and micronutrients (vitamins and minerals) play a determining role in regulating and shaping the immune response [23]. Understanding the interaction between nutrients and the immune response, represent an opportunity to develop low-cost strategies to decrease the incidence of infectious diseases like BTB.

1.1.3. The pathogenesis of BTB

BTB is as a chronic granulomatous caseous-necrotising disease that primarily affects the lungs and their draining lymph nodes, but it also affects a variety of other organs depending on the bacilli's point of entry [24]. The pathology of BTB is very complex, including several interactions between the bovine host and *M. bovis*. The same cells required for protection are implicated with BTB pathology; hence, the quality and quantity of the inflammatory response determines disease control, progression and pathology [25].

The pathophysiology of BTB is well understood as a result of research in natural and experimental *M. bovis* infection in cattle, and from extrapolation from human and mouse models [24]. The most frequent route of infection is inhalation of *M. bovis* infected droplets, thus lesions are predominantly distributed in the lower respiratory tract, including the lungs and related lymph nodes. The ingestion of *M. bovis* from contaminated pastures, feed, or water usually causes lesions of the mesenteric lymph nodes [24]. Lesions can persist, with or without progression, and stay localised, at this stage the disease can remain subclinical for months or years, until the lesion spreads to adjacent tissues and organs causing functional impairment of the organ. Currently, advanced forms of BTB are rare and the majority of infected animals are detected in the early stages of infection [24, 26].

M. bovis reaches the respiratory mucosa after inhalation, which is the first line of defence against pathogens. A layer of alveolar epithelial cells (AECs) and a supporting lamina propria constitute the respiratory mucosa. AECs present antigens to mucosal associated invariant T-cells (MAITs) in the lamina propria. MAIT cells rapidly respond to infection, and release IFN-γ to launch the inflammatory response. Eventually, *M. bovis* reaches the alveolae, where it encounters the alveolar macrophages (AM) and dendritic cells (DC) [27]. Although there is no direct proof that AMs are the first phagocyte to contact *M. bovis in vivo*, their localization

supports this theory. In response to infection AM upregulate the expression of IL-23 and TNF-α [28]. These signals stimulate the recruitment of other cells such as monocytes, neutrophils, and NK cells, increasing their numbers by 20-30 fold [29]. Data from mouse models showed that at 14 days post-infection, there was an equal proportion of AM, myeloid DC and neutrophils infected, but by day 19 neutrophils became the predominant population of infected cells [30]. This suggest that cell populations vary at specific phases of infection and *M. bovis* may face diverse micro-environments within these cells. However, the impact of these altered environments for disease progression or control remains unclear [31].

The recruited cells serve as the foundation for the granuloma, the pathological hallmark of tuberculosis infection. The granuloma is defined as an aggregate of different types of cells, including macrophages and neutrophils surrounded by a cuff of lymphocytes and delineated by fibrin and other extracellular matrix components [32]. Inside the granuloma, macrophages can fuse to form multinucleated giant cells, or accumulate lipids to become foam cells or specialize to epithelioid histiocytes [33]. The role of each specific macrophage subset and the signals that promote their formation and differentiation, are, nevertheless, poorly understood. Foamy macrophages are often observed surrounding the necrotic lipid-rich core and it has been shown that the cholesterol present in the lipid droplets is essential for bacterial growth [34]. After chronic antigen stimulation and persistent IFN-γ stimulation, multinucleated giant cells are formed by the union of multiple macrophages. These cells express higher levels of anti-inflammatory cytokines (TGF-β, IL-10) and have reduced phagocytic abilities, yet their absence indicates an inadequate immune response [20].

The formation of the granuloma is dependent on a robust cellular immune response against the bacteria. However, the adaptive immune response in TB infection is delayed, with the first T cells arriving after several weeks of infection [35]. In fact, a proportion of animals may control the infection without the participation of the T cell response [36]. In fact, despite having an optimal T cell response, MyD88 and TLR-2 knockout mice succumb to *M. tuberculosis* infection faster than wild-type mice. The latter suggest that components of the innate immune response play a critical role in the effective immune response against mycobacteria [37]. Therefore, research into the mechanism that contribute to an effective innate immune response against *M. bovis* has been a focus in recent years (Figure 1-2).

1.1.4. Host innate immune mechanisms to *M. bovis* challenge

The innate immune system is the first line of defence against pathogens, and it includes a variety of mechanisms from physical barriers to cellular components. The most relevant cellular components in the innate defence against *M. bovis* infection are macrophages and neutrophils. These professional phagocytes share common features and have dual and opposing function in *M. bovis* control, contributing to host resistance, while also providing a potential niche for infection [38]. Both cells are capable of implementing diverse microbicidal mechanisms to combat bacterial infections, including the engulfment of the bacilli and its enclosure in a hostile environment where toxic agents and enzymes can destroy the pathogen, restrict essential nutrients for bacterial growth, produce antimicrobial peptides and cytokines, as well induce autophagy and efferocytosis that eliminate the bacilli [39].

Mycobacteria are recognized by phagocytes via their pattern recognition receptors (PRR), including C-type lectins, mannose receptor (MR), complement receptors (CR), scavenger receptors, DC-SIGN, Toll-like receptors (TLR) [i.e.,TLR2,4,8 and 9], and nucleotide-binding oligomerization domain-like receptors (NLRs) [i.e.,NOD1, NOD2]. Following PPR activation, signal transduction cascades induce phagocytosis as well as an array of proinflammatory responses such as antimicrobial effector synthesis, and cytokine and chemokine production [40].

Phagocytosis is a critical process for bacilli elimination that results in the formation of phagosomes, which following multiple maturation steps, merge with lysosomes to form phagolysosomes. These intracellular organelles have a pH close to 5 and contain around 60 hydrolases capable of destroying and killing the bacilli [41]. NADPH oxidase complexes (NOXs) are also found in phagolysosomes, where they produce reactive oxygen species (ROS) including superoxide anion (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). Myeloperoxidase is used by neutrophils and monocytes to further combine H_2O_2 with Cl^- to form hypochlorite, which aids in the elimination of bacteria [41, 42]. The oxidative stress response also includes the production of reactive nitrogen species (RNS) including nitric oxygen (NO). ROS and RNS can combine to produce different oxidative molecules such as peroxynitrite (ONOO−) and nitrogen dioxide (NO_2) [42]. In conjunction, these reactive species cause oxidative damage in molecules like DNA, lipids, and proteins which facilitates clearance of pathogens. Moreover, ROS and RNS are modulators of the immune response, they can regulate type 1 interferon signalling, autophagy and the NLRP3 inflammasome [43]. The effect of ROS on the immune system are mainly ascribed to H_2O_2 which can pass through membranes and have effects both intracellularly and extracellularly. The major targets of H_2O_2 are protein tyrosine kinases and protein tyrosine phosphatases. ROS also affects other key

immune regulators such as caspases, HIF-1, ERKs, JNKs, which are regulated by redox levels and can have both activating or inhibitory effects [44].

Autophagy is another mechanism employed by professional phagocytes to eliminate intracellular pathogens like *M. bovis*. This is a recycling system of senescent or damaged organelles that promote the fusion of lysosomes with autophagosomes resulting in the degradation of the intracellular cargo. TLR2, TLR4 and TLR7 activation promotes autophagy and activates several downstream signalling molecules. NADPH-oxidase activation and the production of ROS are critical for efficient autophagic targeting of mycobacteria. In addition, autophagy is regulated by IFN-γ, which promotes activation of the macrophage to overcome phagosome maturation block by an *IRGM*-dependent mechanism [40].

Although macrophages and neutrophils have similar functions, they have unique roles in mycobacteria defence. Neutrophils have higher microbicidal activity than macrophages; for example, neutrophils have higher oxidative burst capacity than macrophages and are armed with different type of granules full of lytic enzymes and antimicrobial peptides. Phagosome maturation in neutrophils occurs simultaneously with phagocytosis and it is a faster process than in macrophages. Furthermore, the fusion of the granules to the phagosome happens during the closure of the phagocytic cup which can cause the release of granules into the extracellular space [45]. Therefore, degranulation is an important microbicidal mechanism by neutrophils for mycobacterial killing. Granules are released sequentially, leaving the granules with the most pro-inflammatory proteins (elastase, myeloperoxidase, and cathepsin and defensins) at the end to prevent tissue damage. Neutrophil granules also enhance the microbicidal activity of macrophages, which are taken directly by the macrophage or after ingesting dying neutrophils. In this sense, the roles of lactoferrin, myeloperoxidase and HNP-1 has been suggested as important mediators of this type of cooperation between neutrophils and macrophages [39, 45]. Additionally, neutrophils can release extracellular traps (NETs) which are composed of DNA-histone complexes and anti-microbial proteins. These structures help to limit bacterial spread and aids in the killing of bacteria by exposing them to high levels of microbicidal effectors. NETs can be induced by cytokines (IL-8, TNF-α), ROS, elastase and myeloperoxidase translocation to the nucleus, and histone modification [46]. NETs induce a pro-inflammatory response in alveolar macrophages inducing the release of IL-6, TNF-α, and IL-1β, which promotes the recruitment of more cells to the site of infection.

Macrophages and neutrophils work together in the coordination of the innate immunity by the common secretion of proinflammatory cytokines (IL-1α, IL-1β, IL-18, IL-23, IL12-p40), chemokines (CXCL8, CXCL1/2/3, CCL2/3/4), and their receptors (CXCR2, CXCR1, CCR1,2,4), creating a feedback loop that amplifies and sustains their activation [39, 47].

Synthesis of IFN-γ and TNF-α plays an important role in phagocyte activation. The proinflammatory environment provided by the cytokines IL-12, IL-18, and IL-23 released by phagocytes and DC, activates lymphocytes producing IFN-γ, which then increases production of TNF-α by macrophages. IFN-γ is produced mainly by $CD4^+$ T helper cells (Th1), $CD8^+$ cytotoxic lymphocytes, natural killer (NK) cells and γδ T cells. Both cytokines are essential for mycobacterial resistance, but excessive production can cause tissue damage. TNF-α is essential for granuloma formation as well as for trans-endothelial migration of cells to the site of infection. In synergy with IFN-γ, TNF-α promotes phagocyte activation to improve their microbicidal mechanisms and regulate cytokines and chemokine production. Moreover, TNF-α regulates apoptosis of infected macrophages. IFN-γ also promotes phagosome maturation, ROS generation and autophagy [48, 49].

Another crucial effector of the immune defence against mycobacteria are DC which main function is antigen presentation. In contrast to other phagocytes, activation of DC by antigen stimuli results in their maturation and migration to draining lymph nodes where they interact with T cells and initiates the adaptive immune responses. Mycobacteria uptake results in DC maturation and upregulation of MHC-I, MHC-II, and the co-stimulatory molecules CD40, CD54, CD58, and CD80. Data showed that DC activity varied depending on the mycobacterial strain; for example, infection of DC with hypervirulent *M. tuberculosis* strains resulted in suboptimal antigen presentation, low production of IFN-γ ,TNF-α, and poor T cell activation. In contrast, infection with less virulent strains promoted higher apoptosis in DC which served as antigenic source for additional DC or macrophages allowing an efficient priming of $CD4^+$ T cells [50]. The above results suggest that multidirectional interactions between macrophages, DC, neutrophils, and lymphocytes are orchestrated in the infected tissues, highlighting the complexity of TB infection.

Recently, there has been a renewed interest of the role of epithelial cells and innate-like lymphocytes such as NK cells and γδ T cells in the host defence against mycobacteria. *M. tuberculosis* can infect AECs and initiates pro-inflammatory responses. However, it can also infect bronchial epithelial cells from the upper respiratory track and induce chemokine and antimicrobial peptide secretion. One study showed that M cells (epithelial cells specialized to deliver mucosal particles to APC) were associated with *M. tuberculosis* dissemination to lymph nodes. The authors suggested that TB prophylactic strategies should involve improving the innate activity of upper airway epithelial cells [51, 52].On the other hand, it has been demonstrated that NK cells recognize *M. tuberculosis*-infected cells and inhibit bacterial replication in a granzyme dependent way. The cytotoxic mechanisms used by NK cells are similar to those used by CD8+ T cells, and includes the release of granzymes, the usage of

perforin, and the activation of death receptors. Perforin form holes in the membranes of target cells, causing apoptosis and allowing granzyme to enter. Granzyme is capable of cleaving caspases and triggering inflammasome activation and apoptosis. NK cells also influence T cell activity through IFN-γ signalling. Furthermore, a larger frequency of circulating NKT cells (which express TCR receptor) is linked to better *M. tuberculosis* clearance and recovery from TB infection. Therefore, they have become an intriguing targets for preventing and treating mycobacterial infection [25, 53].

1.1.1. Host adaptive immune mechanisms to *M. bovis* challenge

Following activation of the innate immune response and failure to control infection, the host T cell response is critical for an effective control of *M. bovis* infection. Multiple T cell populations are required to mediate protection against TB, including Th1, Th2, and Th17 $CD4^+$ effector T cells, $CD4^+$ T regulatory cells (Treg), and $CD8^+$ cytotoxic T cells. CD4+ T cells are essential to control mycobacteria growth, as demonstrated by the increased TB susceptibility in patients with HIV-1 infection. However, in TB the onset of T cells responses is delayed, and the first CD4+ T cells arrive in the lung 2-3 weeks after infection. This delay could be to a number of factors, including slow bacterial growth, reduced apoptosis of infected cells, and delayed activation and migration of DCs, all of which allow mycobacteria to establish a persistent infection in the lung [54].

T cells contribute to TB resistance by generating IFN-γ which actives microbicidal functions in phagocytes. According to research, polyfunctional T cells with the capacity to produce IFN-γ, TNF-α, and IL-2 are superior effectors when compared to mono of bifunctional T cells. This is because trifunctional cells have been associated to latent TB infection rather than active TB infection and are seen at higher frequencies following BCG vaccination, and effective antibiotic treatment [55]. Cytokine production in polyfunctional T cells occurs sequentially, with evidence demonstrating that the numbers of cells secreting IL-2 and IFN-γ rose over time, whereas those secreting TNFα decreased [56].

IL-17 is another major inflammatory cytokine that can be produced by antigen-specific T cells. IL-23 is required for IL-17 responses, which are produced by $CD4^+$ T cells and γδ T cells. In humans, Th17 responses are detected in vaccinated individuals but are absent in patients with active TB. However, the role of Th17 cells in protection against mycobacteria remains unclear although is associated with granuloma development, Th1 cell recruitment and induction of CXC chemokines. Uncontrolled Th17 responses are associated with pathology due to an excessive neutrophil recruitment. Although during the early stages of infection IL-17 is required for neutrophil recruitment to the site of infection and mycobacterial control [55, 57].

T regs can impair antimycobacterial T-cell responses and contribute to disease during the early stages of infection but they can also restrict overt inflammation in chronic stages of infection. Studies in mouse showed that TB disease progression and pathology was differential in strains with divergent TB susceptibility. TB infection in resistant mouse resulted in less pathology and was associated with increased T reg frequencies. Whereas susceptible mouse succumbed rapidly to infection and had fewer T regs in the lung. T reg suppression strategies include contact dependent and independent mechanism, with some of key anti-inflammatory cytokines released by T regs being IL-10, TGF-β, and IL-35. IL-10 inhibits macrophage function, reduce DC migration and block T cell proliferation and activation.

B cells also play an important role in determining the immune response to mycobacteria. B cells can present antigens, as well as secrete immunoglobulins and cytokines that influence the function of diverse cells including T cells, macrophages, neutrophils, and DC. The ability of B cells to present antigens increases vaccine effectiveness, and the co-stimulatory molecules (CD80 and CD86) expressed by B cells are essential for $CD4^+$ T cell activation and proliferation [58]. Mycobacteria-specific antibodies can opsonize extracellular bacilli and enable their uptake and recruitment by APC, as well as produce immune complexes that fix complement which can boost opsonization and phagocytosis of mycobacteria through CR [59]. Furthermore, the inflammatory environment in which B cells interact with other cells, influences their development into different subsets such as B effector-1 (Be1) cells [expressing IFN-γ, IL-12, TNF-α, IL-10 and IL-6], and B effector-2 (Be2) cells [secreting IL-2, lymphotoxin, IL-4, and IL-13, IL-10, and IL-6]. Thus, each Be1 and Be2 subsets can influence the development of T cells into Th1 or Th2 effectors, respectively [60]. Lastly, B cells are a prominent component of granulomas, and evidence suggests that they aggregate within lesions to form germinal centers which are critical for the development of effective and long-lived humoral immunity [58, 59].

Because TB is a disease that develops as a result of a suboptimal immune response, it is critical to investigate intercellular interactions and how the microenvironment influences such interactions in order to gain a complete understanding of the responses that can promote bacteria clearance or proliferation.

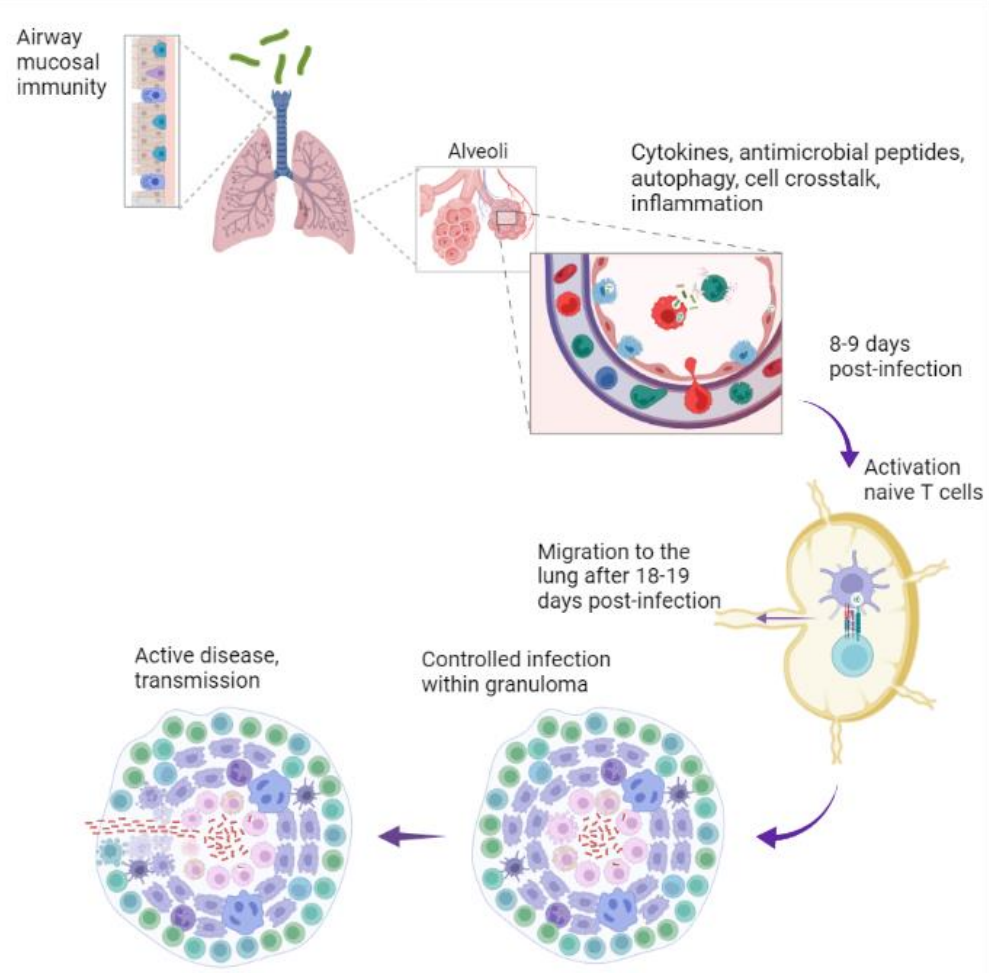


Figure 1-2. The pathology of *M. bovis* infection.

After inhalation, *M. bovis* is recognized by epithelial cells from the trachea, bronchi, and bronchioles, which together with MAIT cells, and innate lymphocytes play a role in the airwall mucosal immunity against mycobacteria. Once in the alveoli, *M bovis* is fought by type I and II alveolar epithelial cells (AT-I and AT-II), as well as pulmonary resident alveolar macrophages, which produces IL-23, IL-6, TNF-α and other pro-inflammatory cytokines to stimulate neutrophil, monocyte, NK cell and lymphocyte recruitment. Here, the immune defence involves a variety of cooperative mechanisms among cells that conjunctly enhances the microbicidal capacities of phagocytes and orchestrates the initiation of the inflammatory response. After 8-9 days post-infection, APC migrate to draining lymph nodes to active naïve T cells, this effector T cells migrate to the lung 2-3 weeks post-infection. Eventually, the aggregation of these immune cells leads to the formation of granulomas which serve to limit the spread of the bacilli. Containment fails when the immune status changes as a consequence of an impaired function of innate and T cells, resulting in granuloma rupture and bacterial dissemination (Created with BioRender.com).

1.2. Vitamin D

Vitamin D (vit D) is the collective term used to describe a group of closely related fat-soluble steroids. Vit D is photosynthesized in all forms of life and it is an essential steroid for life in higher animals [61]. The main biological function of vit D is to maintain serum calcium and phosphorous concentrations within the normal range by enhancing the efficiency of the small intestine to absorb these minerals from the diet. The two most prominent forms of vitamin D are ergocalciferol (Vit D_2) and cholecalciferol (Vit D_3). Ergocalciferol is derived from the plant steroid, ergosterol, whereas cholecalciferol is produced in the skin following exposure to UVB rays from the sun [62]. Cattle can obtain vitamin D through dermal synthesis and dietary sources (hay, silage, milk replacers, etc.). The content of Vit D_2 in alfalfa and silage is highly variable, and green grass is a poor source of Vit D_2; thus, for grazing ruminants, almost all vit D comes from dermal synthesis. Although, Vit D_3 is provided in feed concentrates, differences in husbandry practices causes variation in the vit D status of cattle between farms and throughout the year [63].

1.2.1. Vitamin D metabolism

Vit D itself does not have biologic activity; regardless of the source it must first be hydroxylated twice to become the active hormone 1, 25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], also named calcitriol. After ingestion or synthesis, vit D is bound to the vit D binding protein (VDBP) for systemic transport, although a small proportion can be circulating free or unbound [64]. The first hydroxylation occurs in the liver by action of cytochrome P450 enzymes, mainly *CYP2R1* and *CYP2J2* [65]. This step produces 25(OH)D or calcidiol, the major circulating form of vitamin D; this form is relatively stable and has a half-life of approximately 2 to 3 weeks. Consequently, the concentration of 25(OH)D in serum is used as a marker of vitamin D status [66]. The second hydroxylation occurs in the kidney, where the conversion of 25(OH)D to 1,25$(OH)_2D_3$ is catalysed by the *CYP27B1* enzyme (25-hydroxyvitamin-D 1α-hydroxylase), a mitochondrial enzyme closely regulated by the parathyroid hormone (PTH). The *CYP27B1* enzyme, is induced by the PTH which results in an increase of 1,25$(OH)_2D_3$ to promote the intestinal absorption of calcium and osteoclastic activity [67]. The activity of the enzyme *CYP27B1* is tightly controlled by the PTH, fibroblast growth factor 23 (*FGF23*) and by the enzyme *CYP24A1* [62]. The *CYP24A1* enzyme adds a hydroxyl group at the 24 position of both metabolites, 25$(OH)_2D$ and 1,25$(OH)_2D_3$ to inactivate them. The *CYP24A1* transcription is induced by 1,25$(OH)_2D_3$, forming a negative feedback look that controls vitamin D signalling [62] (Figure 1-3).

After synthesis in the kidney, $1,25(OH)_2D_3$ is released into the circulation to regulate gene expression in their target cell. $1,25(OH)_2D_3$ exerts its actions by binding to the vitamin D receptor (VDR) which heterodimerizes with the retinoid X receptor (RXR). This complex acts as a transcription factor to regulate genes with vitamin D response elements (VDRE) in their promoter [68]. The best-known targets for VDR regulation are genes associated with calcium and phosphorus homeostasis; however, the wide distribution of the VDRE has shown that $1,25(OH)_2D_3$ regulates the expression of genes not only involved in mineral homeostasis and bone metabolism. These non-classical responses of $1,25(OH)_2D_3$ involve antimicrobial and immunoregulatory effects [69]. Remarkably, many cells that express VDR also express vitamin D activating enzymes such as *CYP27B1*, hence allowing the local activation of vitamin D [70].

1.2.1.1. $1,25(OH)_2D_3$ synthesis in the immune system

Extra-renal synthesis of $1,25(OH)_2D_3$ was first described in patients with sarcoidosis, where macrophages from affected tissues were shown to be the source of *CYP27B1* [71]. Contemporary work has reported extra-renal *CYP27B1* expression in epithelial cells (epidermis, prostate, colon, breast, endometrium), macrophages, monocytes and dendritic cells and lymphocytes [72]. The discovery of the local synthesis of $1,25(OH)_2D_3$ along with the fact that the *VDR* is expressed in almost all cells of the immune system suggest that locally produced $1,25(OH)_2D_3$ contributes in the regulation of cells from the innate and adaptive immune system [73].

In macrophages the production of $1,25(OH)_2D_3$ is linked to pathogen recognition by activation of the TLR signalling pathway. Activation of the heterodimer TLR1 and TLR2 (TLR2/1) results in the transcriptional induction of *VDR* and *CYP27B1* [74]. $1,25(OH)_2D_3$ bound to the *VDR* act as a transcriptional factor to induce the expression of the antimicrobial peptides such as *CAMP* and *DEFB4*, as well as the expression of *NOD2*, *IL1B* and *IL8* [68]. Translocation of the VDR to the nucleus also promotes autophagy and the expression of *HAMP* to restrict circulating levels of iron [75]. Thus, in conjunction, $1,25(OH)_2D_3$ enhances the antimicrobial activity of macrophages [75, 76] (Figure 1-3).

The regulation of *CYP27B1* in macrophages differs from renal expression. Whereas the production of $1,25(OH)_2D_3$ in the kidney is regulated by PTH, FGF23 and itself, in macrophages *CYP27B1* is not responsive to these mechanisms [77]. Expression of *CYP27B1* is regulated by an immune circuit between Th1 and Th2 cytokines. Activation of TLR 2/1 pathway in the macrophage results in the upregulation of IL-15 and IL-1β. IL-15 is a strong inducer of *CYP27B1* and *VDR* and together with IL-1β promotes Th1 cell proliferation and

IFN-γ production. The expression of IFN-γ by Th1 cells enhances *CYP27B1* expression. As a result, the amplification loop enhances *CYP27B1* and *VDR* expression [78] (Figure 1-4).

While $1,25(OH)_2D_3$ promotes the innate immune response, it has the opposite effect on the Th1 cell function, providing a negative feedback regulation. The rise in $1,25(OH)_2D_3$ production by the activated macrophage, allows its release to the local inflammatory environment to act in a paracrine way to other bystander cells. $1,25(OH)_2D_3$ inhibits the proliferation of IFN-γ derived Th1 and Th17 cells. Moreover, inhibition of Th1 cell function causes an enhancement of Th2 cell response, where IL-4 and IFN-β inhibits *CYP27B1* activation and block the extra-renal synthesis of $1,25(OH)_2D_3$ [79] (Figure 1-4).

The immune metabolism of vit D is linked to the renal metabolism through the 25(OH)D metabolite. Circulating 25(OH)D synthetized in the liver by *CYP2R1* is the main vit D substrate for immune cells. Therefore, the vit D status of the animal influence the activation of the vitamin D pathway in the immune system [74] (Figure 1-3).

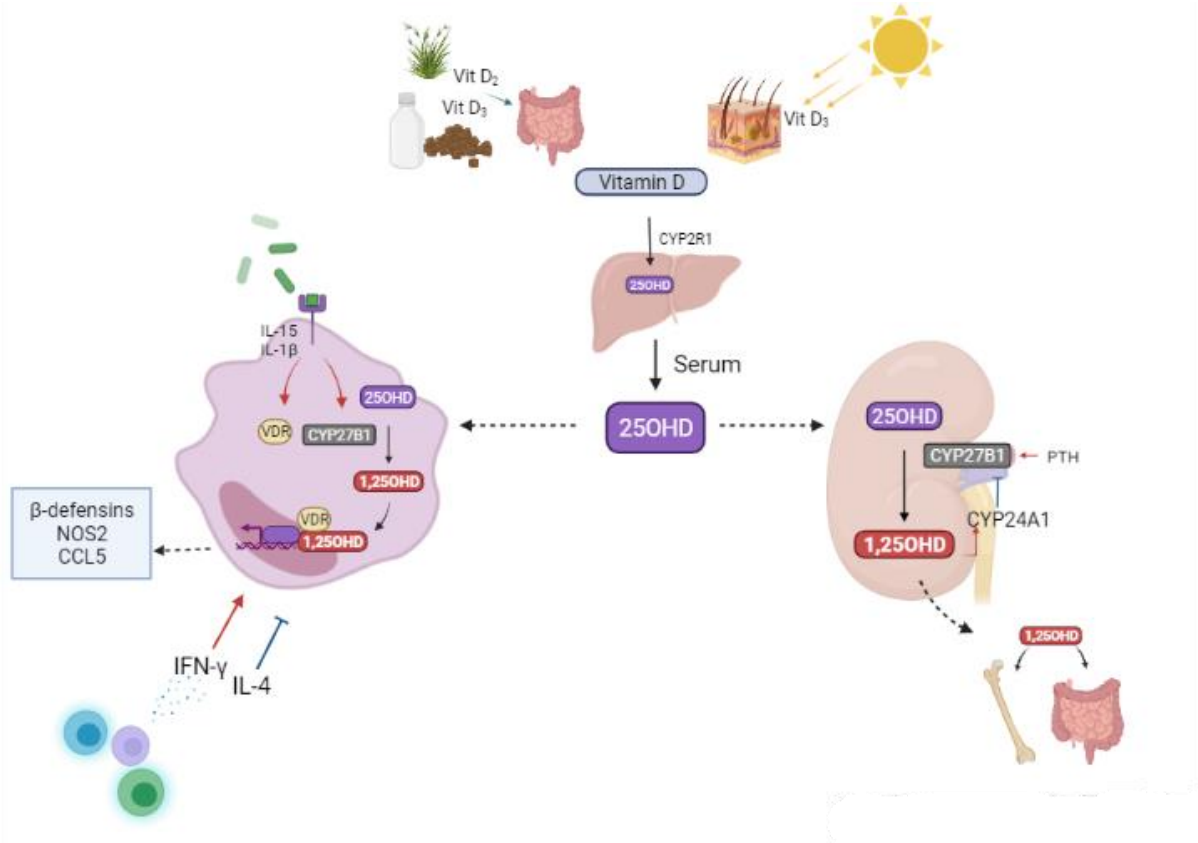


Figure 1-3. Renal and extra-renal metabolism of vitamin D in cattle.

After synthesis in the skin by action of the UVB rays or by ingestion through the diet, vit D (D_2 and D_3) is hydroxylated in the liver by the *CYP2R1* to form 25(OH)D. Subsequently, 25(OH)D is transported to the kidney and other organs or cells. **Renal metabolism:** In the kidney, *CYP27B1* is induced by action of the parathyroid hormone (PTH) to converts $25(OH)_2D$ to $1,25(OH)_2D_3$. The latter is released in the circulation to exert its function in the musculoskeletal system and intestine. *CYP27B1* renal expression is regulated by $1,25(OH)_2D_3$ through induction of *CYP24A1*. At the same time, high concentrations of $1,25(OH)_2D_3$ supress synthesis of PTH and induces *FGF23* that supress the activity of *CYP27B1* in the kidney. **Extra-renal metabolism:** activation of TLR by pathogens like *M bovis* leads to MyD88 and NK-κB transcription, and upregulation of IL-15 and IL-1β. This promotes the co-expression of *VDR* and *CYP27B1*. The latter catalyse the conversion of serum 25(OH)D to $1,25(OH)_2D_3$. Finally, the complex VDR-$1,25(OH)_2D$ acts as transcription factor to regulate the transcription of genes like *NOS2*, *CCL5*, and several β-defensins. Regulation of *CYP27B1* in macrophages is carried out by an immune circuit between IFN-γ and IL-4. (*Red arrows indicate positive regulation; blue arrow shows inhibition*). Created with BioRender.com

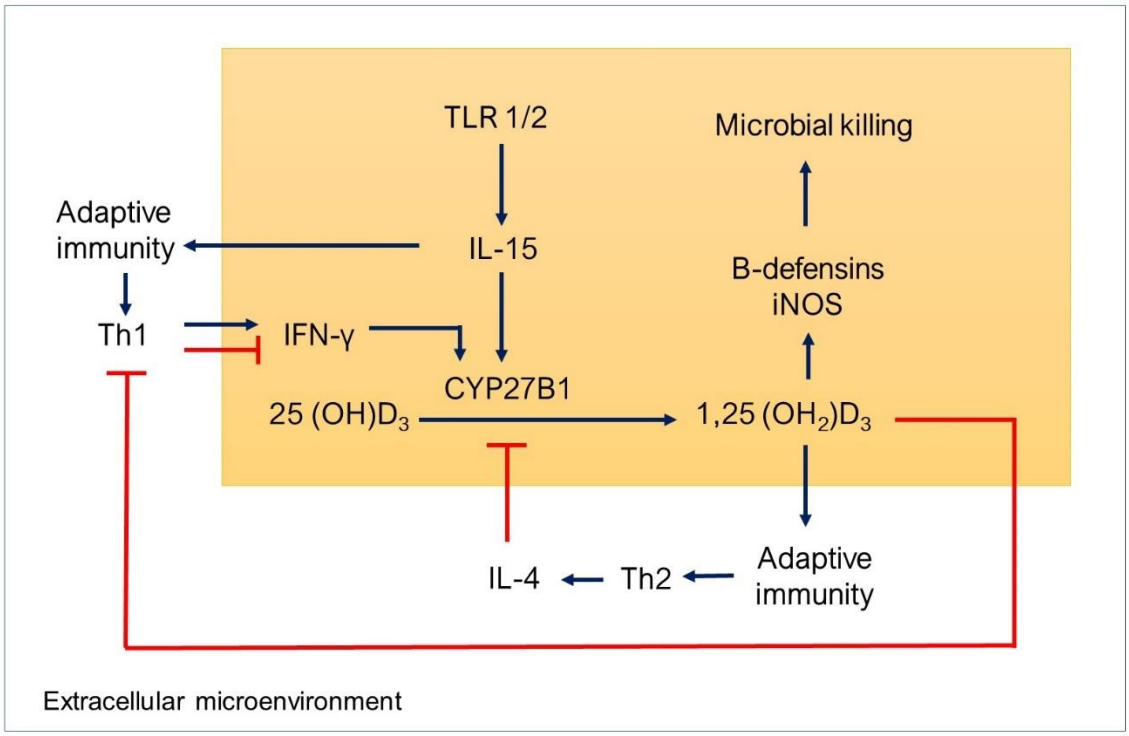


Figure 1-4. Regulation of the vitamin D pathway by the immune system.

Activation of TLR leads to upregulation of IL-15 and Th1 cell proliferation to promote IFN-γ production. This enhances *CYP27B1* expression and conversion of 25(OH)D to $1,25(OH)_2D_3$. The intracrine effects of $1,25(OH)_2D_3$ results in an enhancement of the microbicidal activities of innate cells (yellow area). When intracellular levels of $1,25(OH)_2D_3$ increases, its release from the cells (white area) acts on Th1 and Th2 cells to interrupt the activation of CYP27B1 (*Red arrows indicate positive regulation; blue arrow shows inhibition*) Adapted from [78].

1.2.1.2. Vitamin D signalling in the bovine immune system

Although the general mechanism of $1,25(OH)_2D_3$ on the innate immune response are conserved across species, gene targets of vit D in non-primate animals are different in comparison with humans. The main target of $1,25(OH)_2D_3$ in the human macrophage is the antimicrobial protein cathelicidin (*CAMP*) and β-defensin 4A (*DEFB4A*) [76]. However, a similar motif of the VDRE from primates has not been described in other mammals. Thus, *CAMP* regulation by $1,25(OH)_2D_3$ has only been observed in human macrophages, whereas bovine cathelicidins (*CATH 4, 5* and *6*) are not responsive to vitamin D [80]. Instead, $1,25(OH)_2D_3$ promotes the expression of bovine β-defensins [81]. Merriman *et al.* [81] showed that addition of $1,25(OH)_2D_3$ increased the expression of *BNBD3*, *BNBD4*, *BNBD6*, *BNBD7* and *BNBD10* in monocytes, but not in neutrophils or mammary epithelial cells. Besides, addition of LPS had an additive effect on β-defensin expression in all cells, suggesting that activation of TLR4 was required to promote β-defensin production. However, unlike *DEFB4A* regulation by $1,25(OH)_2D_3$ in humans, β-defensin expression in cattle seems to be a secondary response to $1,25(OH)_2D_3$ and not a direct target of the VDR [81].

In bovine monocytes, $1,25(OH)_2D_3$ induces *NOS2* and *CCL5* expression, and similarly to β-defensin expression, activation of TLR augments their production [80, 82]. Moreover, expression of *NOS2* and *CCL5* was dose-dependent on $1,25(OH)_2D_3$ and $25(OH)_2D$ concentrations. Upregulation of *NOS2* was associated with higher NO production, which has been associated with mycobacterial killing in bovine macrophages [80, 83].

Effects on the adaptive immune response by $1,25(OH)_2D_3$ are similar between human and cattle. $1,25(OH)_2D_3$ modulates the proliferation and differentiation of bovine lymphocytes, it promotes cell proliferation of T $CD8^+$ cells, whereas it inhibits $CD4^+$ T cell proliferation [84]. Inhibitory effects on T cell proliferation seems to be cell-specific since the effect was only observed on $CD4^+$ T cells but not on γδ T cells [82]. Likewise, $1,25(OH)_2D_3$ inhibits IFN-γ secretion by mitogen and antigen activated $CD4^+$ T cells [82, 85]. *IL17F* expression was also downregulated by 25(OH)D on $CD3^+$ lymphocytes co-cultured with PBMC from BCG vaccinated cattle [86]. Therefore, $1,25(OH)_2D_3$ induce an anti-inflammatory effect by inhibition of $CD4^+$ T proliferation and downregulation of IFN-γ and IL-17 responses on antigen-stimulated PBMC [87].

1.2.2. Immunomodulatory effects of vitamin D

The discovery that most cells of the immune system express the VDR and the vit D metabolizing enzymes paved the way for research into the vit D immunoregulatory properties. It is now recognized that locally synthetized $1,25(OH)_2D_3$ can act in an intracrine or paracrine fashion, impacting both the function of the cells that synthesizes it and the activity of surrounding cells [73, 75].

The majority of previous research has focused on the role of vit D in monocytes and macrophages. As previously stated, activation of the vit D pathway in innate cells is linked to pathogen recognition. Stimulation of the TLR 2/1 by mycobacterial antigens led to the induction of *VDR* and *CYP27B1* [74]. VDR activation causes the generation of downstream gene products as well as pro-differentiative and immunomodulatory effects [76]. According to studies, $1,25(OH)_2D_3$ increases the differentiation of monocytes into mature phagocytic macrophage [88]. Monocytes have higher levels of VDR on their surface than macrophages, and as they differentiate into macrophages the levels of VDR decreases. The change in VDR expression suggest that the high content of VDR in monocytes provides a faster pathway for cell maturation into macrophages [89]. On the contrary, expression of *CYP27B1* increase as monocytes differentiate into macrophage, improving their capacity for $1,25(OH)_2D_3$ synthesis (Figure 1-5) [77, 89].

As outlined earlier, in monocytes and macrophages $1,25(OH)_2D_3$ promotes the expression of host defence peptides (HDP) such as cathelicidin and β-defensins. These HDP exhibit a broad-spectrum of antimicrobial activities including direct microbial killing. When the HDP comes into contact with the bacterial membrane, it self-assembles to form amphiphilic structures causing increased membrane permeability and release of ions and metabolites. This causes the transmembrane potential to depolarize, culminating in membrane rupture and cell lysis [90]. HDP have also immunoregulatory functions, such as cell recruitment and regulation of inflammation. Cathelicidin, for example, is chemoattractant to monocytes, macrophages, DC, and neutrophils, favouring the recruitment of antigen presenting cells. HDP also promotes neutrophil degranulation and NET formation helping in the resolution of infection [90, 91]. Moreover, $1,25(OH)_2D_3$ induction of cathelicidin promotes the generation of autophagosomes enhancing bacterial killing [76, 92]. Vit D also regulates ROS and NO production of phagocytes via activation of the NAPDH oxidase system and *NOS2* expression [83, 93, 94].

$1,25(OH)_2D_3$ also modulates the expression of diverse cytokines and chemokines crucial for the immune defence against infection. Of these, $1,25(OH)_2D_3$ was found to influence IL-1β

expression. Verway M. *et al*., [95] showed that 1,25$(OH)_2D_3$ amplified IL-1β signalling from *M. tuberculosis* infected macrophages cultured in conjunction with epithelial cells. The above, resulted in an enhanced expression of DEFB4 from epithelial cells and reduced mycobacteria load in macrophages. Results from this study showed that the actions of 1,25$(OH)_2D_3$ expands beyond macrophages by regulating paracrine signalling and function of bystander cells [95]. Studies had also shown that 1,25$(OH)_2D_3$ induce an anti-inflammatory profile after bacterial challenge [96]. Stimulation of PMBC and monocytes with 1,25$(OH)_2D_3$ and 25(OH)D led to a reduction of TNF-α, IFN-γ, IL-1β, IL-6, and IL-8. Whereas an opposing effect was observed for IL-10 production [96]. In conjunction, these studies suggest that vit D enhances the microbicidal activity of innate cells while controlling the development of an excessive inflammatory response. The physiological significance of this immunoregulatory property was shown on mice. Pathology of pulmonary tuberculosis was attenuated in mice fed with vit D_3. Dietary vit D_3 reduced the proinflammatory gene expression in lung while still permitting *M. tuberculosis* control, reducing the negative impact of the inflammatory response to infection [97].

The dual effect of vit D (anti or pro-inflammatory) might depend on the cell phenotype. For example, treatment with 1,25$(OH)_2D_3$ on monocyte-derived macrophages (MDM) from patients with cystic fibrosis decreased IL-8 production after bacterial challenge, whereas a reverse effect was observed in cells from healthy patients. Cells from cystic fibrosis patients showed a hyperinflammatory phenotype, producing higher concentrations of IL-8 before and after bacterial stimulation, in opposition to healthy patients. Consequently, it was suggested that effects of vit D varies according to the inflammatory state of the cells [98]. Modulation of cytokine production by 1,25$(OH)_2D_3$ is associated to regulation of the NF-κB transcription factor. The complex 1,25$(OH)_2D_3$-VDR binds to a subunit of the IKK kinase and blocks IκB phosphorylation, resulting in a decreased NF-κB transcriptional activity [99].

On the other hand, it has been suggested that neutrophils do not express a functional *CYP27B1* [75]. However, evidence indicates that IFN-γ promotes activation of *CYP27B1* on human neutrophils in a dose dependent manner. And in synergy with TLR activation, expression of *CYP27B1* and *VDR* is upregulated [100]. Likewise, neutrophils express the *VDR* at levels comparable to monocytes, and stimulation with 1,25$(OH)_2D_3$ facilitates the production of IL-8 which boost the recruitment of most cells to the site of infection [101, 102]. Similar to macrophages, 1,25$(OH)_2D_3$ promotes *CAMP* expression from neutrophils and due to its abundance, they appear to be the major source of cathelicidin at infection sites. Treatment with vit D also induced the expression of *DEFB2* and *NGAL*, the later has been implicated in iron siderophores sequestering which limits bacterial growth [103]. Moreover, 1,25$(OH)_2D_3$

regulates excessive inflammation in neutrophils by promoting the expression of *SOCS* proteins in a IL-4 dependent manner, which results in the downregulation of *TRAF6* and NF-κB [100]. Overall, these evidence points to vit D playing a role in suppressing neutrophil-driven inflammatory responses, while still encouraging pathogen clearance.

$1,25(OH)_2D_3$ has complex effects on DC, it increases the expression of molecules involved in antigen presentation such as CD14, CD32 and mannose receptor, while inhibiting costimulatory molecules CD80 and CD40. Moreover, $1,25(OH)_2D_3$ supressed DC maturation, as revealed by the null rise in the expression of MHC-I, MHC-II, CD80, CD86, CD40, and CD83 and by the impaired stimulatory capacity for T lymphocytes after exposure to LPS [104]. Effects of DC maturation by $1,25(OH)_2D_3$ are associated to an inhibition of NF-κB p65 subunit phosphorylation and nuclear translocation, an effect that was only observed in myeloid DC as opposed to plasmacytoid DC [105]. A reciprocal organization of *VDR* and *CYP27B1* its also observed in DC. Mature DC had lower VDR expression than immature DC, while *CYP27B1* expression increases as DC differentiates towards a mature phenotype. This allows an initial presentation of antigen to T cells whilst preventing continued maturation of DCs and over-stimulation of T cells [77, 106].

Contrary to its role in promoting innate immune responses, $1,25(OH)_2D_3$ exerts a downregulatory effect on lymphocyte function. $1,25(OH)_2D_3$ inhibits IL-2, IFN-γ and IL-17 transcription, altering the T cell phenotype and function. Effects are commonly described as an inhibition of Th1 response and promotion of Th2 cell differentiation, with a reduction in IFN-γ and upregulation of IL-4, IL-5, and IL-13 [107]. Also, it was shown that addition of IL-12 reversed the inhibitory effect of $1,25(OH)_2D_3$, and increased IFN-γ production to a level comparable to non-vit D treated cells [108].

Effects of $1,25(OH)_2D_3$ on Th2 cells are less consistent, with some studies revealing an increase on IL-4 levels and other showing no effect. For example, one study showed an upregulation of IL-4 by $1,25(OH)_2D_3$ only when T cells were previously polarized with IL-4. Whereas other study found a decrease in IL-4 production in the presence of $1,25(OH)_2D_3$ and IL-4. Differences between mouse and human cells, as well as culture methods might explain the contradictory results observed. However, it seems that the response on Th2 cells is modest in comparison to the effects on Th1 cells [107, 108]. Effects of $1,25(OH)_2D_3$ on T cell proliferation are also conflictive, with some studies revealing anti-proliferative effects and others showing increased proliferation [107, 109]. Therefore, some authors have suggested that the antiproliferative effects of $1,25(OH)_2D_3$ are due to the reduction on IL-2 production, and others have linked it to the vit D effects on antigen-presenting cells that indirectly suppress T cell proliferation [75, 77, 107].

$1,25(OH)_2D_3$ stimulates the development of Treg cells and the production of IL-10 in $CD4^+$ and $CD8^+$ T cells, while inhibiting the production of pro-inflammatory Th1, Th17 cytokines [110]. The generation of a Tregs by $1,25(OH)_2D_3$ is related to direct activation of genes such as *FOXP3* and *CTLA4* as well as concurrent downregulation of *IFNG* and *IL17A.* Moreover, a synergistic effect with TGF-β has also been observed in Treg development. Furthermore, it seems that Tregs are not responsive to $1,25(OH)_2D_3$ stimulation due to the low level of VDR expression [75, 77]. However, as part of the Th17-Treg axis, a reduction in Th17 cells occurs in parallel to Treg development. Additionally, $1,25(OH)_2D_3$ acts on Th17 cells to reduce the production of IL-17, IL-22, TNF-α, IFN-γ, and the chemokine receptor CCR6, inhibiting Th17 cell migration to the site of inflammation [75].

Similar to T cells, *VDR* expression switches from low to high level with B cell activation. This also holds true for *CYP27B1* expression, which has also been found expressed in T-cells. Therefore, lymphocytes are able to respond to and synthetize $1,25(OH)_2D_3$ to modulate their microenvironment [111]. $1,25(OH)_2D_3$ affects B cell proliferation and Ig production by interfering with the differentiation of plasma cells and class switched memory cells. In addition, formation of memory B cells was inhibited by $1,25(OH)_2D_3$ [112]. However, the inhibitory effects of vit D on immunoglobulin production has not been observed *in vivo* [113]. It was hypothesized that differences between long-lived (memory) plasma cells and short-lived plasma cells might explain the differences observed between *in vitro* and *in vivo* supplementations. Stimulation of B cells *in vitro* reflects effects of vit D on short-lived, but not long-lived plasma cells, whereas *in vivo* circulating IgG levels are mainly produced by long-lived plasma cells. Because changes in IgG levels have not been observed *in vivo*, this suggests that vit D does not affect the generation of memory plasma cells [114].

In summary, vit D promotes a tolerogenic response. While it enhances the innate immune response to pathogens, vit D simultaneously limits the development of an overzealous immune response by maintaining a balance between the inflammatory Th1/Th17 cells and immunosuppressive Th2/T reg cell (Figure 1-5) [111].

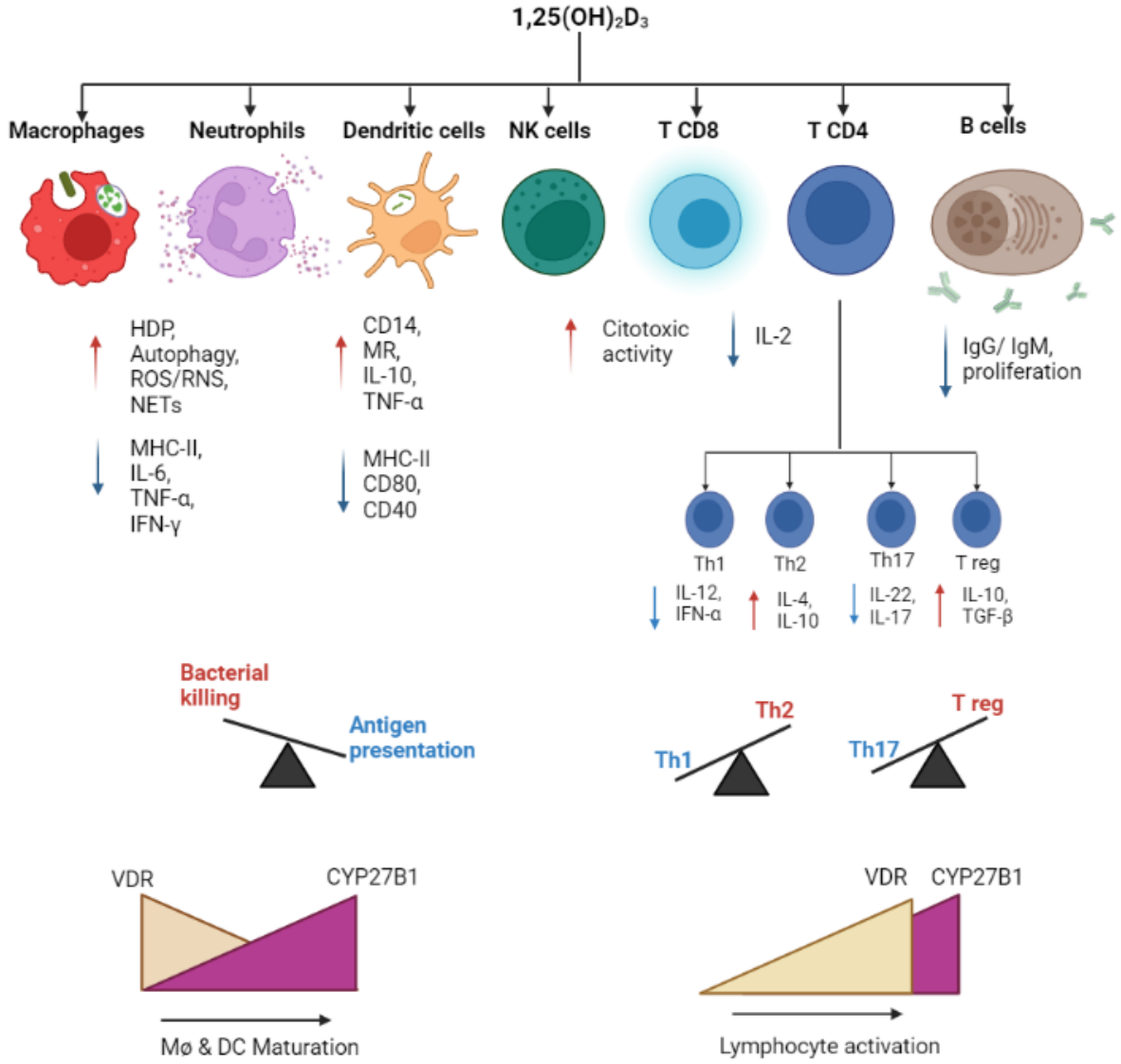


Figure 1-5. Regulatory effects of vitamin D in the immune system.

$1,25(OH)_2D_3$ has diverse effects on cells of the innate and adaptive immune system. It increases HDP synthesis, ROS and RNS production, autophagy, NET formation and cytolytic activity in macrophages, neutrophils, NK cells, and $CD8^+$ T cells, enhancing their microbicidal functions. $1,25(OH)_2D_3$ downregulates antigen presentation and maturation in DC, favouring Treg development. It also promotes a Th2 environment by increasing IL-4, IL-10 and TGF-β production. $1,25(OH)_2D_3$ inhibits synthesis of IL-17 and the migration of Th17 cells. In B cells, $1,25(OH)_2D_3$ reduces immunoglobulin production. As a result, $1,25(OH)_2D_3$ boost innate immune responses and induces a tolerogenic response. *VDR* and *CYP27B1* expression profile differ with macrophage and DC maturation, although expression of both rises with lymphocyte activation (Created with BioRender.com).

1.2.3. Vitamin D status

Vit D status is measured in the serum as 25(OH)D, the major circulating form of vit D with a half-life of 2 to 3 weeks. Definition of the normal or sufficient range of 25(OH)D level has been a difficult task in humans and bovines. The serum concentrations achieved in settings with abundant exposure to sunlight are between 30 to 100 ng/ml, and are considered an indication of the sufficient range of 25(OH)D for optimal health in humans [66]. However, several different 25(OH)D thresholds have been used to define the ranges for vit D sufficiency, deficiency, or insufficiency (Figure 1-6). Variations in the interpretation of the data attributing vit D status to an specific health outcome in a particular population have contributed to the differences [115]. For example, the Endocrine Society define sufficient 25(OH)D levels above 30 ng/ml, levels between 20-29 ng/ml indicates insufficiency, and levels below 20 ng/ml are deficient [116]. Whereas for the US Institute of Medicine (IOM) sufficient 25(OH)D levels are at least 20 ng/ml and levels above 50 ng/ml are a reason of concern, risk of inadequacy is between 12-20 ng/ml, and levels below 12 ng/ml represent a risk for deficiency [117]. For the European Food Safety Authority (EFSA) 20 ng/ml are adequate to all population groups [118]. The lack of consensus on the definition of the optimal 25OHD concentration have generated different recommendations about vit D intakes which might have serious implications in the clinical practice [115].

Biological effects of vit D deficiency (VDD) were also used to define a sufficient level of 25(OH)D [119]. Elevation of PTH is observed in cases of osteoporosis and rickets, therefore it is used as a marker for vit D deficiency. By analysing the relationship between PTH and 25(OH)D levels in healthy adults, it was shown that PTH levels started to plateau when 25(OH)D levels were between 30-40 ng/ml [120]. Furthermore, studies on calcium absorption showed that intestinal calcium transport was more efficient in women with mean $25(OH_2D$ levels >32ng/ml [119]. Therefore, for the Endocrine Society the IOM recommendation might be inadequate for individuals with underlying conditions or receiving medications that put them at risk of VDD. Thus, the Endocrine Society suggested that the optimal serum concentration of 25(OH)D to be considered at least 30 ng/ml [116].

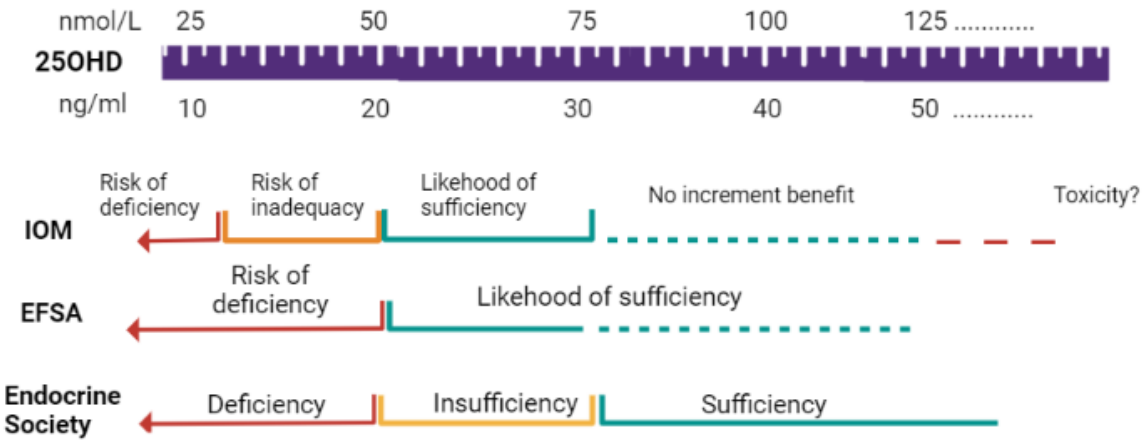


Figure 1-6. Serum 25(OH)D concentration thresholds to define the vitamin D status of an individual.

Thresholds defined by the Institute of Medicine (IOM), the European Food Safety Authority (EFSA), and the Endocrine Society. Edited and modified from [115] (Created with BioRender.com)

In cattle a review report done in 1994 indicated that in healthy cows 25(OH)D plasma levels ranged between 20-50 ng/ml, VDD was defined as levels below 5 ng/ml, whereas the serum threshold for vitamin D toxicity was proposed to be 200 ng/ml [67, 121]. However, vit D toxicity in bovines is very rare, and 25(OH)D levels up to 270 ng/ml have been observed after vit D supplementation, with no signs of hypercalcemia or other adverse effects [122, 123]. Definitions of the vit D status in bovines are lacking, but based on the similarities in the vit D metabolism between humans and cattle, the cut-offs recommended by Endocrine Society have been adopted for bovines, with 25(OH)D concentrations < 30 ng/ml considered deficient [87, 124].

Diverse environmental and host factors affect the vit D status in cattle. A summary of them is described in Table 1-1. The evidence indicates that calves have different vit D requirements than adult cattle [63, 125] Besides, the stage of lactation might influence the vit D requirements in dairy cows [126]. Furthermore, the optimal 25(OH)D concentration for immunity has not been determined yet, and vit D requirement might vary according to the health status. For example, it was observed that calves facing an acute viral response showed a rapid decrease in vitamin D status, which suggested a higher requirement of vitamin D in response to infection [127]. The above indicates that further research is needed to fully elucidate how these factors affect the overall health and wellbeing of cattle.

Table 1-1. Environmental and host factors that influence the vit D status in cattle.

Factor	25(OH)D serum concentration	Reference
Sex	Higher in females than in males	[128]
Age	Lower in calves than in adults. Higher in 2nd lactation cows than in 3rd lactation cows	[63, 125] [126]
Breed	Higher in *Bos taurus* than in *Bos indicus*	[129]
Hair color & coverage	No differences observed between cows with black or white hair color. Skin synthesis occurs in all the body	[130, 131]
Season	Higher in summer than in winter	[125, 131-133]
Latitude	Lower at higher latitudes (>50°N)	[63, 125, 131]
Time of UVB exposition	Rise of 1 ng/ml per day after exposition to UVB light (280-415 nm) for 120 min	[134]
Husbandry practices & diet	Dietary vit D is fed a higher concentration in American than European livestock systems. Thus, higher 25(OH)D levels are observed in intensive systems than in pasture-based systems.	[63, 125] [135]

1.2.4. Vitamin D deficiency and disease

VDD is considered a major public health concern worldwide, affecting people of all ages but being more prevalent among children and elderly [115]. Prevalence rates of 25(OH)D levels < 20 ng/ml have been reported as 24% in the USA, 37% in Canada, and 40% in Europe [136]. VDD results in abnormalities in calcium, phosphorus, and bone metabolism. But it also has been associated to an increased risk of a variety of health outcomes, including all-cause mortality, cancer, type 1 diabetes mellitus, neurological and cardiovascular disorders, as well as infectious diseases including sepsis and respiratory infections like influenza, covid-19 and tuberculosis [115, 136, 137].

In cattle, a similar association between VDD and disease has been suggested and supplementation studies have shown the physiological relevance of vit D in animal health. Feeding prepartum transition cows with vit D_3 or 25(OH)D reduced the incidence of retained placenta, metritis and reduced the proportion of cows affected with multiple diseases in early lactation [138]. Likewise, Poindexter M. *et al* [139]. demonstrated that feeding 25(OH)D improved the vit D status on dairy cows and altered the mammary immune response to a *Streptococcus uberis* challenge. In this study, cows fed with 25(OH)D had a delayed start of symptoms and lower severity of mastitis. Moreover, the leukocyte marker CD11b and CD62L was increased on macrophages and neutrophils from milk, suggesting that vit D promoted leukocyte recruitment to the mammary gland during infection [139]. Intramammary administration of 25(OH)D with experimental mastitis, reduced bacterial loads, somatic cell counts and symptomatology [140].

Studies on animals naturally infected with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) showed that 25(OH)D serum concentrations were lower in animals seropositive to MAP in comparison to their seronegative heard mates [141]. Furthermore, reduced 25(OH)D levels were associated with increased severity to MAP infection [142]. A similar observation has recently been made in animals naturally infected with *M. bovis*. Lower levels of 25(OH)D were observed in cattle reactor to the tuberculin skin test, in comparison with tuberculin negative animals [143].

These studies suggest that low serum concentration of 25(OH)D are associated with bacterial diseases in cattle as it is in humans, however the incidence of VDD and its impact on health and disease in bovines is unknown.

1.3. Vitamin D and BTB

The role of vit D in the antibacterial activity against TB in humans has been studied widely [76, 144, 145]. In fact, in the 1900's exposure to sunlight (heliotherapy) was the only therapy known to treat TB [146]. In 1903 the Nobel Prize in Medicine was awarded to Dr. Neils Finsen for his therapy against cutaneous tuberculosis using a lamp to refract short wave UV light onto the skin of infected patients. Then, in the 1940's reports on dietary procedures with vit D_3 to treat cutaneous tuberculosis were published providing evidence of its safety and efficacy [147]. Likewise, in 1946 Dr. Walter Raab reported the successful use (in 4 of 6 patients) of intrapleural injections of high doses of vit D_3 to treat pulmonary tuberculosis. He also demonstrated a bacteriostatic effect of vit D_3 on cultures of *M. tuberculosis* and *Proteus vulgaris* [148]. However, the use of vi D for TB treatment was discarded due to the discovery of streptomycin in 1952 [147]. In 1986 and 1987, it was reported by two different groups that $1,25(OH_2)D_3$ had bactericidal effects on human monocytes against *M. tuberculosis* [149, 150]. Authors also reported that monocytes were able to synthesize 25(OH)D to $1,25(OH_2)D_3$ with or without the addition of IFN-γ [149]. However, it was not until the early 2000's that the molecular mechanism of the antimycobacterial actions of vit D were described [74, 151].

Vit D induces pro- and anti-inflammatory effects against mycobacteria that restrain the bacilli grow and limit an excessive inflammatory response. The proinflammatory activities are associated with the production of cathelicidin and β-defensins which can directly damage the bacterial wall. Another way in which vit D modulates the microbicidal activity of macrophages is by upregulation of the ROS and NO production, as well as by the induction of autophagy in infected cells [80, 82, 83, 86, 94, 143, 152, 153]. On the other hand, the anti-inflammatory actions of vit D are linked with the reduction of pro-inflammatory cytokines like IFN-γ and IL-1β, as well as with the decrease of chemokine production, and with expansion of T reg lymphocytes which in turn limit the activity of Th1 cells [97, 154-156].

Given this evidence, diverse studies have analysed the link between 25(OH) concentrations and the risk of TB. One of the most comprehensive meta-analyses that assessed the multiple aspects of TB disease showed that VDD was associated with an increased risk to develop TB (OR =2.57; 95% CI =[1.74, 3.80]). Moreover, VDD was positively associated with an increased risk to develop active TB in close-contact individuals of active TB. In this work, a total of 23 studies, with 3,491 TB cases and 3,259 controls were included [157]. In addition, analysis of polymorphisms in the *VDR* have shown that the *FokI-ffI* polymorphism contributes to the risk of TB, especially in the HIV- negative patients and among Asian patients [158]. Although these studies suggest a direct link between vit D and TB susceptibility, randomised controlled trials have failed to show a benefit of supplementation

[159]. Multiple factors can be attributed to the contradictory results, including a lack of consensus in the definition of supplementation regimens (dose and duration), selection of individuals with adequate vit D status at baseline which can have limited the benefit of vit D supplementation, and trial designs with low statistical power to detect meaningful differences [160]. Moreover, it has been suggested that cofounding factors such as body weight, age, sex, ethnicity, and socio-economic context can influence the responsiveness to vit D [161].

In cattle, the vit D pathway is activated in the same manner as it is in humans, and research suggest that after *M. bovis* exposure there is a rapid mobilization of $1,25(OH)_2D_3$ in blood, which might be necessary for granuloma formation [162]. Studies on bovine macrophages have suggested that $1,25(OH)_2D_3$ stimulates NO and β-defensin production which restrain *M. bovis* replication [80, 83]. Similar studies on MAP infected cattle have shown the benefits of $1,25(OH)_2D_3$ on limiting MAP survival [163]. Therefore, these finding suggest that vit D has a high potential for increasing cattle's intrinsic resistance against mycobacteria. Because of the similarities between cattle and humans, the bovine model provides an opportunity to dive into the complexities of the vit D effects in response to mycobacterial infections, which might have positive consequences for public health and the agriculture sector.

1.4. Thesis hypothesis and aims

This thesis proposes that vit D influences cellular and molecular process in bovine leukocytes which affects their microbicidal efficacy against *Mycobacterium bovis* BCG (BCG). The main objectives were as follows:

1. To investigate the effect of $1,25(OH)_2D_3$ on the microbicidal and immunoregulatory activities in peripheral blood leukocytes after BCG challenge.
2. To investigate the effect of $1,25(OH)_2D_3$ on the microbicidal and immunoregulatory activities in neutrophils after BCG challenge.
3. To develop a model to drive divergent 25(OH)D circulating levels in dairy calves.
4. To investigate the influence of differential 25(OH)D circulating levels on the microbicidal and immunoregulatory activities of peripheral leukocytes following an *ex-vivo* BCG challenge.

2. CHAPTER 2. Microbicidal activity and immunoregulatory effect of $1,25(OH)_2D_3$ on peripheral blood leukocytes (PBL)

2.1. Introduction

Mycobacteria are obligate intracellular pathogens, that survive and grow inside macrophages, hence analysis of the mechanics of the macrophage response has been the predominant focus of tuberculosis research [164]. The macrophage model of infection usually involves the use of cell lines or primary cells. The most common cell lines used are the RAW264.7 and THP-1 macrophages, from murine and human origin respectively, and the preferred primary cells used are either monocyte-derived macrophages (MDMs) or alveolar macrophages [164]. *In vitro* infections with mycobacteria have revealed multiple aspects of the host-pathogen interaction which are crucial for mycobacterial destruction, including phagosome maturation, production of antimicrobial peptides and cytokines, induction of cell death pathways and autophagy [165]. Crucial to the antimicrobial activity of macrophages is their ability to synthesize toxic anti-microbial molecules such as nitric oxide (NO) and reactive oxygen intermediates, and recent studies have shown that NO synthesis is a critical effector mechanism against mycobacteria. Furthermore, many of these studies have shown that the vitamin D signalling pathway is essential for macrophage mycobactericidal activity [149-151, 153, 166].

Activation of the vitamin D pathway in macrophages is initiated by pathogen recognition via TLR causing the transcriptional induction of *VDR* and *CYP27B1* leading to the induction of the HDP cathelicidin (*CAMP*) resulting in antimicrobial activity [74, 103, 151]. Seminal work by Liu *et al*. showed that $1,25(OH)_2D_3$ treatment of THP-1 cells reduced the intracellular viability of *M. tuberculosis* H37Ra by 30-50% via *CAMP* production. The use of a VDR agonist blocked *CAMP* induction and reduced antimicrobial activity by 70%, illustrating direct dependence between *CAMP* production and *VDR* expression. Additionally, the authors observed the co-localization of *CAMP* with bacteria-containing vacuoles only in cells treated with $1,25(OH)_2D_3$, describing for the first time a mechanism by which vit D influences the antimicrobial activity of macrophages [151].

Subsequently, authors of the same study showed that the vit D mediated antimycobacterial activity was enhanced with the activation of the IL-1 pathway [167]. IL-1β is essential for host defence against mycobacteria and its proinflammatory actions are required for cell recruitment and activation [168]. This research showed that TLR2/1 activation promoted upregulation of IL-1β and its receptor, with the synergic effect of VDR and TLR signalling pathways upregulating the antimicrobial peptide *DEFB4*. A similar requirement for IL-1 activation was not observed for *CAMP* mRNA expression. However, expression of both antimicrobial peptides required activation of the VDR. Furthermore, *CAMP* and *DEFB4* peptides showed

direct antimicrobial activity against *M. tuberculosis* H37Ra and inhibition of its mRNA expression with small interfering RNA reduced by almost 100% the antimicrobial activity in human monocytes [167].

Cathelicidin is a short cationic peptide produced by phagocytes and epithelial cells. It is stored in granules as an inactive proform, which after cell activation is processed by proteolytic cleavage into its bioactive form. The antimicrobial activity of cathelicidin its associated with its pore-forming ability in bacterial membranes and the induction of autophagy [92]. Autophagy is a host defence mechanism which promotes phagosome maturation through the generation of autophagosomes and their subsequent fusion with lysosomes [144]. Yuk *et al.* [92] showed that $1,25(OH)_2D_3$ induced autophagy in human monocytes infected with *M. tuberculosis* in a cathelicidin-dependent manner through the modulation of autophagy-related genes *BECN1* and *ATG5*. The authors demonstrated that treatment with $1,25(OH)_2D_3$ was essential for this cathelicidin recruitment to autophagosomes. In addition, the use of autophagy inhibitors blocked the $1,25(OH)_2D_3$ mediated antimicrobial activity in a similar way illustrated by the inhibition of *CAMP* mRNA transcription [92].

Overall, these studies have shown that the antimicrobial activity by $1,25(OH)_2D_3$ in human macrophages depends on cathelicidin expression and induction of autophagy. In the bovine linage, the cathelicidin locus is expanded [169]. However, unlike humans and mice, bovine cathelicidins (*CATH4, CATH5,* and *CATH6*) are not responsive to vit D treatment; instead, $1,25(OH)_2D_3$ induces the expression of an alternative class of HDP, the β-defensins and also *NOS2* [80, 81]. $1,25(OH)_2D_3$ treatment of LPS-stimulated bovine monocytes was shown to increase the expression of β-defensins *BNBD3, BNBD4, BNBD6, BNBD7*, and *BNBD10* [81]. In cattle, an expansion of β-defensin gene cluster was also reported, with a family repertoire of up to 57 β-defensins genes. Like cathelicidins, β-defensins have antimicrobial and immunoregulatory functions, which make them interesting potential targets to improve bovine health [170].

Phagocytes produce NO via upregulation of the *NOS2* gene. In cattle, NO production by macrophages has been associated with intracellular killing of *M. bovis* [171]. Nelson, *et al* [80] previously showed that $1,25(OH)_2D_3$ treatment induced *NOS2* production in bovine monocytes, an effect that was increased after TLR stimulation. *NOS2* mRNA expression and subsequent NO production increased when higher $1,25(OH)_2D_3$ concentrations were used. Moreover, upregulation of *NOS2* was also observed when using the vit D metabolite 25(OH)D in a dose dependent manner [80]. Likewise, NO modulation by $1,25(OH)_2D_3$ was associated with a reduction in *M. bovis* replication in bovine macrophages [83].

Although the macrophage model has been fundamental for understanding the molecular mechanisms induced by vit D, neither antimycobacterial killing, nor the effects of vitamin D are limited to monocytes and macrophages. Modulation by vit D has been observed in other myeloid and epithelial cells including neutrophils, mammary epithelial cells, dermal fibroblasts, $CD4^+$ T cells and γδ T cells [86, 172, 173]. For example, bovine neutrophils, express the *VDR* and *CYP27B1* genes upon LPS treatment, which suggest they are a major contributor to $1,25(OH)_2D_3$ local synthesis [172]. However, the antimicrobial effects of vit D on neutrophils are not well understood. This represents a significant limitation given the abundance of this cell type in mammalian blood and their reported activity against mycobacteria. Macrophages and neutrophils share a complex relationship, and together orchestrate an enhanced immune response against mycobacteria. Considering that the interaction between them influence each other's function, the analysis of the vit D effects in both cells is warranted [38, 40].

2.2. Specific aims

The main aim on this chapter was to examine the microbicidal activity and immunoregulatory role of $1,25(OH)_2D_3$ on a heterogenous cell population of peripheral blood leukocytes (PBL) against *Mycobacterium bovis* BCG (BCG). The analysis of the microbicidal activity by PBL was studied using two models: A) Whole blood (WB); and B) Peripheral blood leukocytes cultured after red blood cell lysis, thereafter, referred to as the PBL model.

The specific objectives were to:

1) To investigate the effect of $1,25(OH)_2D_3$ on the mycobacterial activity by PBL.
   a) To compare $1,25(OH)_2D_3$ mycobacterial effects on WB and PBL.
   b) To investigate the effect of depletion of specific cell types on the mycobacterial activity by PBL and to determine their response to $1,25(OH)_2D_3$.
   c) To investigate the effect of $1,25(OH)_2D_3$ on the mycobacterial activity by PBL from cattle reactor to the tuberculin skin test (BTB+).
2) To investigate the effect of $1,25(OH)_2D_3$ on the phagocytic activity and oxidative stress response by PBL.
3) To investigate the effect of $1,25(OH)_2D_3$ on gene expression after BCG challenge on PBL.

2.3. Materials and Methods

2.3.1. Animal ethics committee approval

All experimental procedures were approved by the Teagasc Ethics Committee (TAEC190-2018) and were conducted under experimental license (AE19132/P085) from the Health Products Regulatory Authority in accordance with the cruelty to Animals Act (Ireland 1876) and European Community Directive 2010/63/EU.

2.3.2. Blood sampling, haematology, and serum 25(OH)D analysis

Ten 7-month-old Holstein-Friesian male calves were used for all the assays conducted in this chapter. Animals were housed indoors for the duration of the experiments. The number of calves used for each experiment ranged from 4 to 7 and is specified in the figure legends.

Blood samples were collected via the jugular vein into vacutainer tubes (Becton Dickinson). A sample of 6 ml blood was collected in an EDTA tube used for haematology analysis on the ADVIA 2120 haematology system. Another 10 ml serum separator tube was utilized for serum collection. Tubes were centrifuged at 2500×g for 15 min for serum separation within 1 h of sample collection and biobanking. Serum samples were transferred into microtubes and stored frozen at −20 °C. The serum samples were analysed for concentrations of total 25(OH)D using an ELISA (Human 25-OH Vitamin D ELISA, Eagle Biosciences, Nashua, NH) and carried out as per the manufacturer's instructions using bovine standards, prepared as previously described [174].

2.3.3. 1,25(OH)$_2$D$_3$ preparation and treatment

A 10 µg stock of 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) [Sigma-Aldrich] was prepared by dissolving in 1 ml of pure ethanol. The stock was stored in 50 µl aliquots of 10 µg/ml at -20°C. For cell stimulation, the 1,25(OH)$_2$D$_3$ stock solution was further diluted in RPMI medium to achieve the desired concentration. A treatment of ethanol alone, prepared in the same way as 1,25(OH)$_2$D$_3$ was used as a control.

2.3.4. M. bovis BCG growth and maintenance

One frozen aliquot (1 ml) of *Mycobacterium bovis* BCG Denmark (BCG) or BCG-GFP was diluted in 9 ml Middlebrook 7H9 broth (Becton Dickinson) supplemented with 0.2% glycerol, 0.5% BSA, 0.2% glucose, 0.085% NaCl (Sigma Aldrich), in a 50 ml falcon tube. The tube was incubated at 37°C for 12 days. Then, the culture was scaled up for a larger volume by

transferring 4 ml of the started culture to 46 ml of 7H9 media, supplemented as before. The bacterial culture was grown with constant, gentle rotation (60 rpm) in roller bottles (Corning) at 37°C until they reached an optical density of 0.5-0.8 at 600nm (approximately 2 weeks). Then, 1 ml aliquots from logarithmically growing cultures were frozen in fresh 7H9 media and stored at -20°C. Three representative vials were thawed and enumerated for viable colony forming units (CFU) on Middlebrook 7H11 plates (Becton Dickinson) containing 0.2% glycerol, 0.5% BSA, 0.2% glucose and, 0.085% NaCl.

Mycobacteria handling was performed in a Class II microbiological safety cabinet. All material used was sprayed with 5% Biocleanse before sterilization by autoclave and final disposal.

2.3.5. Assessment of $1,25(OH)_2D_3$ microbicidal effects on PBL

PBL microbicidal effects were assessed on whole blood (WB) and on PBL after serum removal and red blood cell lysis. The WB protocol was adapted from Pepponi I. *et al*, [175]. Briefly, 300 µL of whole blood was placed in a 2 mL tube. Then, cells were stimulated or not with 4 ng/ml of $1,25(OH)_2D_3$ and incubated for 2 h at 37ºC on a rotatory platform. After this time, 300 µL of RPMI medium containing 1 x 10^6 CFU of BCG was added. The tubes were placed again in the rotatory platform and incubated at 37ºC for 24 h. Then, the tubes were centrifugated at 500 *x* g for 5 min and the supernatant was removed. The red blood cells were lysed with 1.5 mL of High Yield Lysis Solution (Thermo Fisher Scientific) and incubated at room temperature for 10 min. Tubes were centrifuged at 500 *x* g for 5 min and the cell pellets were washed twice with 2 ml of PBS. Finally, cells were lysed with 200 µL of H_2O with 0.05% tween 80. The cell pellet was mixed to facilitate cell lysis and incubated at room temperature for 15 min. This lysate was used to determine the bacteria load by CFU counting.

For PBL evaluation, red blood cells were lysed with High Yield Lysis Solution (Thermo Fisher Scientific) and incubated at room temperature for 10 min. Cell pellets were washed twice with PBS and cells were counted using a haematocytometer. Then, 1x10^6 cells/ml were placed on a 24 well plate and incubated with (or without) 4 ng/ml of $1,25(OH)_2D_3$ for 2 h. Next 1x10^6 CFU/ml of BCG were added and incubated for 24 or 48 h. Plates were centrifuged at 500 *x* g for 5 min and the cell pellets were lysed with 200 µL of H_2O with 0.05% tween 80. The cell pellet was mixed up and down to facilitate cell lysis and incubated at room temperature for 15 min. This lysate was used to determine the bacteria load by CFU counting.

The cell lysate was diluted in ten-fold serial dilutions with 7H9 media. Then, 50µl of each serial dilution was plated on a 7H11 agar petri dish. Plates were put inside a plastic bag (to

prevent them drying out) and stored at 37°C for 2-3 weeks, until visible colonies were counted. All samples were run in duplicate. The CFU/ml was determined with the formula:

CFU/ml = (number of colonies x dilution factor)/ volume of culture plate

2.3.5.1. Determination of the multiplicity of infection

The absolute numbers of white blood cells (WBC), lymphocytes, neutrophils, monocytes, eosinophils, and basophils determined by the haematology analysis were used to estimate the multiplicity of infection (MOI) [bacteria:cell ratio]. First, the total number of cells in each model was estimated by multiplying the number of each cell type ($x10^3$/µl) for the volume of blood or PBL used. Thus, for the WB model, cells number were multiplied by 300 µl and for PBL for $1x10^6$ cells. Then, the MOI was determined by dividing the number of bacteria used ($1x10^6$ CFU) by the total number of cells in each model.

2.3.6. Cell depletion of $CD14^+$, neutrophils, $CD3^+$, $CD4^+$ and $CD8^+$ T cells.

Specific depletion of $CD14^+$, $CD3^+$, $CD4^+$ and $CD8^+$ T cells was performed by magnetic cell sorting. Briefly, 9 ml of blood was taken in acid citric dextrose vacutainers, the red blood cells were lysed with 30 ml of High Yield Lysis Solution (Thermo Fisher Scientific), after 10 min of incubation at room temperature, tubes were centrifugated at 300 *x* g for 10 min. Then, the cell pellet was washed with 5 ml of MACS buffer (D-PBS 1x with 0.5% BSA and 2 mM EDTA). Cells were centrifuged as before and resuspended in 1 ml of MACS buffer. Next, 2 µl (1:500 dilution) of the corresponding antibody (Table 2-1) was added, cells were mixed and incubated for 15 min at 4°C. Cells were washed as before and resuspended in 80 µl of MACS buffer per 10^7cells.Then, 20 µl of anti-mouse microbeads (Miltenyi Biotec) was added per 10^7cells. Cells were incubated for 15 min at 4°C. To assess the % of depletion, 1 µl (1/200 dilution) of goat anti-IgG-FITC-labelled secondary antibody was added in the last 5 min of incubation. Cells were washed as before and resuspended in 1 ml of MACS buffer. Next, cells were passed through LS columns (Miltenyi Biotec) supported in magnets (MidiMACS Separation Unit, Miltenyi Biotec). The flow through containing the cell depleted subset was collected. The flow through was washed twice with PBS. An aliquot of 100 µl of each cell depleted subset was taken, then cells were resuspended in RPMI medium supplemented with 2mM of L-Glutamine, 10 mM HEPES and 1% BSA (Sigma Aldrich).

Depletion of granulocytes was done by PBMC purification by density gradient. For this purpose, 15 ml of Histopaque-1077 (Sigma Aldrich) was added to a Leucosep™ tubes (Greiner Bio-One) and centrifuged at 1000 *x* g for 30 sec. Then, 8 ml of blood was mixed with

8 ml of PBS and transferred to the Leucosep tube, that was centrifuged at 1,200 *x* g for 20 min. Next, the PBMC layer was removed and transferred to a new tube. The PBMC (granulocyte depleted subset) was washed twice with PBS and resuspended in RPMI medium supplemented with 2mM of L-Glutamine, 10 mM HEPES and 1% BSA (Sigma Aldrich).

A peripheral blood leukocyte (PBL) subset was prepared by red blood cell lysis of 9 ml of blood with 30 ml of High Yield Lysis Solution (Thermo Fisher Scientific). The percentage of cell depletion was assessed by flow cytometry, using as a control the PBL that contained all the cell subsets that were depleted. For this purpose, 6 aliquots of 100 µl of PBL was taken and transferred to a 96 well plate. 1 PBL aliquot was used as unstained control, whereas the rest was incubated with the corresponding primary antibody (1:500) described in Table 2-1 for 15 min at 4°C. Then, the plate was centrifuge at 300 *x* g for 5 min, and FITC-labelled secondary antibody (1:200) was added. Cells were incubated as before and washed twice with PBS-BSA 1%. The percentage of cell depletion for each cell subset was done by comparing the % of cells present in the PBL before and after depletion. The cell subsets with depletion of $CD14^+$ cells, granulocytes, $CD3^+$, $CD4^+$, $CD8^+$ T cells were labelled as: $CD14_{neg}$, Gra_{neg}, $CD3_{neg}$, $CD4_{neg}$ and $CD8_{neg}$, respectively.

2.3.7. Analysis of $1,25(OH)_2D_3$ microbicidal effects in cell depleted PBL

For this assay, the cell subsets PBL, $CD14_{neg}$, Gra_{neg}, $CD3_{neg}$, $CD4_{neg}$ and $CD8_{neg}$, were plated in duplicated in two 96 well plate at 1 x 10^5 cells per well. One plate was used for phagocytosis analysis (2 h of incubation) and one for survival (24 h of incubation). The cells were treated with (or without) 4 ng/ml of $1,25(OH)_2D_3$ for 2 h at 37ºC with 5% CO_2. After this time, cells were challenged with BCG at MOI 1 (1 x 10^5 CFU/ml). The plates were placed again in the incubator at 37ºC with 5% CO_2 for 2 h to allow bacterial uptake. Next, the supernatant of the wells was removed, and cells were washed trice with PBS. Fresh RPMI media (supplemented as describe before) was added to the survival plate and returned to the incubator for 24 h. The cells in the phagocytosis plate were lysed with 200 µL of H_2O with 0.05% tween 80. The cell pellet was mixed to facilitate cell lysis and incubated at room temperature for 15 min. The lysate was plated on 7H11 petri dishes to quantify the mycobacterial CFUs. After 24 h, the survival plate was treated similarly. The supernatant was removed and was plated on 7H11 petri dishes to quantify the number of extracellular bacteria, and the cell lysate was used to determine the number of intracellular bacteria. The 7H11 petri dishes were place inside a plastic bag and stored at 37°C until visible colonies were counted (2-3 weeks). The CFU/ml was determined as before. Results are presented as CFU/ml and as the percentages. The percentage of phagocytosis was calculated as the fold change

between the initial inoculum and bacterial load at 2 h; and the percentage of survival represents the fold change between the bacterial load at 24 h and 2 h.

2.3.8. Analysis of cell viability

To analyse the cell viability after BCG infection, 100 µl of sample was taken after 24 h of infection. The red blood cells were lysed and incubated with a live/dead violet fixable stain (Invitrogen) at 1:1000 dilution, in PBS for 30 min incubation at room temperature. Then, samples were washed with PBS and fixed with 4% formaldehyde (Invitrogen) for 20 min. Next, samples were analysed by flow cytometry using an unstained, uninfected sample as a control.

To determine the degree of cell death, a lactate dehydrogenase (LDH) assay (Pierce™) was carried out according to manufacturer's instructions. The degree of cytotoxicity was determined by comparing the OD_{490} of LDH in the supernatant of infected cells against the OD_{490} of LDH in the supernatant of no-infected cells. Thus, the results are represented as fold change.

2.3.9. BCG-GFP phagocytosis assay

The phagocytosis assay was done using a *M. bovis* BCG strain that constitutively expresses GFP (BCG-GFP, a gift from Maximiliano Gutierrez from The Francis Crick Institute, UK). Briefly, 100 µL of blood per animal was placed in a 96 well plate. Then, 4 ng/ml of $1,25(OH)_2D_3$ was added and the blood was incubated for 2 h at 37ºC. After this time, blood was challenged with $1x10^7$ CFU/ml BCG for 30 min at 37ºC. Then, RBCs were lysed and resuspended in PBS-BSA 1% for antibody labelling.

2.3.10. Phagotest assay

The Phagotest™ assay (Glycotope Biotechnology GmbH, Germany) was performed according to the manufacturer's instructions except that the temperature was kept at 38.5 °C and 50 µL of blood was used, these changes were proved to produce repeatable results for bovine blood [176]. Briefly, 50 µL of heparinised blood was incubated at 38.5 °C for 10 min with 20 µL of bacterial suspension. Then, the samples were put on ice and 100 µL of quenching solution was added. The erythrocytes were lysed, and cells were analysed by flow cytometry in the Attune Focusing Flow Cytometer. A total of 30,000 events in the gate set for leukocytes was collected. The results are reported as the percentage of cells that performed phagocytosis (% of phagocytosis) and the number of ingested bacteria by their mean fluorescence intensity.

2.3.11. Measurement of intracellular ROS

The intracellular ROS production was measured in PBL with the CellROX green kit (Thermo Fisher Scientific). Cells were placed in a 96 well plate at 1 x 10^5 cells per well, and they were treated with (or without) 4 ng/ ml of $1,25(OH)_2D_3$ for 2 h at 37°C with 5% CO_2. Then BCG was added at 1 x 10^7 CFU/ml and cells were incubated for 1 h. Next, 10μM of the CellROX reagent was added and the cells were incubated for 1 additional hour. Then, the plate was centrifugated at 300 *x* g for 5 min and labelling of $CD14^+$ cells and granulocytes was performed.

2.3.12. Cell labelling and flow cytometry analysis

Cells were labelled with the primary and secondary antibodies described in Table 2-1 (CD14 at 1:100 and G1 at 1:500). Incubation with each antibody were done for 20 min at 4°C in PBS with 1% BSA. The live/dead violet fixable stain (Invitrogen) was used at 1:1000 dilution, for 30 min incubation at 4°C. Next, cells were washed twice with PBS and fixed with PFA 4% for 30 min. Finally, cells were washed and resuspended in PBS and flow cytometry acquisition was performed using the Attune Focusing Flow Cytometer. Data were collected from 30,000 events. Results from unstained sample and single stained AbC anti-mouse beads (Thermo Fisher Scientific) were used to calculate the compensation matrix. Cell doubles were excluded using side scatter area versus side scatter height parameters. Voltages were used as follows: Forward Scatter (FSC) = 2800, Side Scatter (SSC) = 3950, BL1, BL2, BL3, VL1 = 850.

Table 2-1. Antibodies used for cell depletion and flow cytometry assays

	Target	Clone	Supplier	Task	Dye
Primary antibodies	CD4	CACT138A IgG1	Washington State University	MACS	--
	CD8	CACT80C IgG1	Washington State University	MACS	--
	CD3	MM1A	Washington State University	MACS	--
	CD14	MM61A IgG1	Washington State University	MACS & Flow cytometry	--
	Granulocyte (G1)	CH138A IgM	Washington State University	Flow cytometry	--
Secondary antibodies	IgM	AB-934045	Invitrogen	Flow cytometry	PE
	IgG1	AB-2536635	Invitrogen	Flow cytometry	PE-Cy 5.5
	IgG	AB-2794319	Southern Biotech	Flow cytometry	FITC
	Viability dye	--	Invitrogen	Flow cytometry	Violet

2.3.13. High performance real-time PCR analysis using the Fluidigm system

RNA was isolated from cells using a combination method of Trizol and the NucleoSpin® Mini kit (Macherey-Nagel). Chloroform was added to cells containing Trizol in a 2 mL microfuge and shaken vigorously. The solution was then centrifuged at 12,000 x g for 15 min at 4°C. The aqueous layer containing RNA was transferred to a clean microfuge tube. An equal amount of 70% ethanol was then added and mixed vigorously. This solution was then transferred to a kit column, and from this point the manufacturer's instructions were followed. RNA quantity was measured using the NanoDrop system. However, RNA yield was below the 5 ng/µl requirement for analysis with the bioanalyzer nano kit. Thus, RNA integrity was not evaluated. However, an RNA pool made with 8 samples with RNA integrity >8 was used as positive control for following analysis. These samples were whole blood stimulated or not with LPS obtained as part of the PhD project from another student. cDNA was synthesised using the iScript™ cDNA synthesis kit (Bio-Rad) with 60 ng of total RNA. Then, gene expression was assessed with the Fluidigm Biomark HD system. This is a high-performance real-time PCR system that uses microfluidics technology to process samples at nanolitre-scale volumes. The equipment uses non-fluidic chips for qPCR, a 96.96 chip format (96 samples x 96 targets/genes) allows for 9,216 PCR reactions in a single qPCR run. For this assay, a panel of 96 primers (Appendix 1) was designed to cover a large set of cytokines, chemokines,

enzymes, antimicrobial peptides, surface receptors, transcription factors and vitamin D genes. The genes were selected based on their relevance to mycobacteria infection and for having been identified as vit D targets in studies on human and cattle cells.

Briefly, a pool of 96 primers was prepared by combining 1 µl of each 100 µM stock of forward and reverse primers, then a DNA suspension buffer (Fluidigm) is added to make the final volume of 200 µl with a final concentration of 500 nM. Then, a pre-mix is prepared by adding 1 µL of PreAmp Master Mix (Fluidigm), 0.5 µl of the pool of primers and 2.25 µl of water. Next, 1.25 µl of the cDNA (at 1:5 dilution) for each sample was added to the pre-mix and a pre-amplification is performed in a thermal cycler. The cycling conditions for the pre-amplification consisted of 1 cycle at 95°C for 2 min, followed by 14 cycles at 95°C for 15 sec, with an extension step at 60°C for 4 min. The preamplification allows for multiplex amplification of the targets of interest and it is used to increase the number of copies to a detectable level. Therefore, the cDNA is amplified without a bias for most of the genes. Then, an Exonuclease-1 (Fluidigm) treatment is performed to remove the unincorporated primers. Next, the pre-amplified samples were loaded in the right-hand side of the 96.96 IFC plate, whereas, in the left-hand side one pair of primer per well (96 assays) was added. Then, the IFC run was carried out in the Biomark HD Fluidigm. Data was analysed with Fluidigm Real-Time PCR software to determine the cycle threshold (Ct) values. Gene expression was normalized to the mean expression of two reference genes (PPIA and ACTB) to obtain the ΔCt value. For each animal, values from infected, treated or not with 1,25(OH)D were normalized to the uninfected-unstimulated sample to obtain the relative gene expression or fold change using the method $2^{-\Delta\Delta Ct}$ [177]. Genes that were not detected or low expressed were removed before statistical analysis. Thus, statistical analysis was done with 61 genes (Table 2-2).

For statistical analysis the fold change values were Log_2 transformed to assess differences between treatments by multiple Wilcoxon paired T test with p-adjusted to 0.1 with the Benjamini-Hochberg method. For principal component analysis (PCA) and heatmap visualization with hierarchical clustering the Log_2-fold change was standardized by mean centring and scaling to unit variance. The hierarchical clustering analysis was done by computing the spearman correlation distance between the observations followed by the Ward's linkage method for definition of clusters. Analysis for gene expression were done in R studio (Version 4.0.3) with the packages *rstatix, factoextra, cluster, tidyverse* and *ComplexHeatmap.*

Table 2-2. List of genes selected for analysis of 1,25(OH)$_2$D$_3$ effects on PBL.

Gene	Function	Gene	Function
DEFB10	Antimicrobial peptide	*IL1A*	IL1 Pathway
DEFB3	Antimicrobial peptide	*IL1B*	IL1 Pathway
DEFB4	Antimicrobial peptide	*IL1R1*	IL1 Pathway
DEFB6	Antimicrobial peptide	*IL1RN*	IL1 Pathway
DEFB7	Antimicrobial peptide	*IL33*	IL1 Pathway
LAP	Antimicrobial peptide	*NLRP3*	IL1 Pathway
S100A12	Antimicrobial peptide	*COX2*	Inflammation
S100A8	Antimicrobial peptide	*ELANE*	Inflammation
S100A9	Antimicrobial peptide	*MMP9*	Inflammation
TAP	Antimicrobial peptide	*PKR*	Inflammation
CD14	Cell adhesion	*C5AR1*	Innate immunity
ITGAM	Cell adhesion	*IFITM3*	Interferon
CCL2	Chemokine & Receptors	*IFNA*	Interferon
CX3CR1	Chemokine & Receptors	*IFNAR1*	Interferon
CXCL1	Chemokine & Receptors	*IFNB*	Interferon
CXCL10	Chemokine & Receptors	*MX1*	Interferon
CXCL2	Chemokine & Receptors	*OAS1Z*	Interferon
CXCL5	Chemokine & Receptors	*NCF1*	Oxidative Stress
CXCL8	Chemokine & Receptors	*TLR1*	Pathogen recognition
CXCR1	Chemokine & Receptors	*TLR2*	Pathogen recognition
IL10	Cytokine Anti-Inflammatory	*TLR3*	Pathogen recognition
IL13	Cytokine Anti-Inflammatory	*TLR4*	Pathogen recognition
IL15	Cytokine Anti-Inflammatory	*TLR5*	Pathogen recognition
IL5	Cytokine Anti-Inflammatory	*TLR6*	Pathogen recognition
TGFB1	Cytokine Anti-Inflammatory	*HIF1A*	Transcription factor
IL12B	Cytokine Proinflammatory	*STAT1*	Transcription factor
IL6	Cytokine Proinflammatory	*CYP24A1*	VD metabolism
IL6R	Cytokine Proinflammatory	*CYP27A1*	VD metabolism
TNFA	Cytokine Proinflammatory	*RXRA*	VD metabolism
CASP1	IL1 Pathway	*VDR*	VD metabolism
CASP13	IL1 Pathway		

2.3.14. Statistical Analysis

GraphPad Prism software version 8 was used for data presentation and statistical analysis, unless otherwise is stated. Variables were tested for normality and if necessary, a log transformation was used to normalize the data. Data is presented as mean ± SEM, unless otherwise is stated. Statistical test used and the number of samples for each experiment are indicated in the figure legends. Evidence of statistical significance was considered at $P \leq 0.05$ and tendency was considered at P values ≤ 0.10.

2.4. Results

2.4.1. Microbicidal activity by 1,25$(OH)_2D_3$ on peripheral blood leukocytes

Analysis of the microbicidal activity by 1,25$(OH)_2D_3$ was done on WB and PBL. To reduce variability of circulating 25(OH)D concentration between animals, calves were kept indoors during the duration of the experiments. Animals were vit D deficient at the moment of infection with mean serum 25(OH)D levels of 18.89 ± 2.027 ng/ml.

For the initial infection experiment, changes in BCG viability were assessed in 4 animals at 24- and 48-h post infection. There were no significant differences between timepoints in any model, and although no conclusions can be drawn from this data due to the small sample size, this pilot experiment showed a biological trend towards a reduction in the number of viable bacilli in cells stimulated with 1,25$(OH)_2D_3$ after 24 h of infection (Figure 2-1).

The viability of the cells after infection was determined by flow cytometry (FC) or Lactate dehydrogenase (LDH) activity. In the case of the WB model, cell viability was analysed by FC using a fixable live/dead stain. For the PBL model, LDH activity was measure in the supernatant of infected and non-infected cells. Both methods, FC and LDH activity are used to measure the proportion of live cells within a population, but the inherent LDH activity in serum caused a background signal in WB, that did not allow discrimination between infected and non-infected cells. Results showed that cell death was minimal in WB and PBL after BCG infection in both, control and 1,25$(OH)_2D_3$ treated cells (Figure 2-2). Cell death was not significantly different between timepoints.

Having determined the time of infection (24 h) the BCG challenge was repeated with higher number of animals (n=7/6 per treatment). The results showed that stimulation with 1,25$(OH)_2D_3$ led to a significant reduction in the number of viable bacilli in both models (Figure 2-3). For WB, a drop of 5 $x10^5$ CFU/ml from control to 3.4 $x10^5$ CFU/ml from 1,25$(OH)_2D_3$ stimulated blood was observed (Figure 2-3 A). Results are also presented as % of bacteria killing, a significant difference of 16.57% of bacteria killing was observed when cells were stimulated with 1,25$(OH)_2D_3$ (49.14 % for control vs 65.71% for 1,25$(OH)_2D_3$ treated blood) [Figure 2-3 C]. Results were similar in PBL, with a significant reduction in bacteria growth observed in 1,25$(OH)_2D_3$ treated cells, from 7 $x10^5$ to 4.3 $x10^5$ CFU/ml between control and 1,25$(OH)_2D_3$ cells (Figure 2-3 B). Likewise, a higher % of bacteria killing was observed in vit D treated PBL. The difference between control and 1,25$(OH)_2D_3$ stimulated PBL was 26.67% (29.8% for control and 56.5% for 1,25$(OH)_2D_3$ P) [Figure 2-3 D].

Then, to test if higher concentrations of 1,25$(OH)_2D_3$ conferred more protection, three concentrations of vit D were tested using the WB model. Infections were done as before, but blood was stimulated with 4, 40 and 80 ng/ml of 1,25$(OH)_2D_3$. Results showed a reduction in the number of viable bacilli with the two lowest vit D concentrations tested. Blood treated with 4 and 40 ng/ml of 1,25$(OH)_2D_3$ had significant ($P < 0.05$) and close to significant ($P = 0.0532$) lower number of viable bacteria than control (4 ng/ml = 3.1×10^4, 40 ng/ml = 3.5×10^4, control = 5.3×10^4 CFU/ml). The decrease in the number of bacilli with 80 ng/ml was not significant (Figure 2-4 A). When expressed as % of bacteria killing, results suggest a significantly greater proportion of BCG killing when using 4 or 40 ng/ml of 1,25$(OH)_2D_3$ (Figure 2-4 B).

The MGIA is optimized to use a small inoculum (~10^4 CFU), which is 100 times lower than the inoculum used in this assay (~10^6CFU) [175]. The differences in the inoculum imply a different multiplicity of infection (MOI) that could impact the results. Therefore, MOI (bacteria:cell ratio) for each model was estimated. The cell profile when using 300µl of blood or $1x10^6$ PBL/ml is shown in Figure 2-5. Based on the cell profile values in the WB model, the CFU translates to an approximate MOI of 1.31± 0.28 for neutrophils and 10.55 ±3.4 for monocytes. In the case of the PBL model, the MOI for neutrophils and monocytes was 3.92 ±1.049 and 34.33 ± 8.265, respectively (Figure 2-5). The MOI for each cell type in both models is also shown in the Table 2-3. Therefore, despite both models were challenged with the same number of bacilli, a low MOI was achieved with WB, whereas a higher MOI was reached in PBL.

Collectively, these results show that 1,25$(OH)_2D_3$ significantly decreases the number of viable bacilli after 24 h post-infection and increases the microbicidal activity of PBL at low and high MOI of BCG infection. Furthermore, differences in the proportion of dead bacteria were not observed when using higher concentrations of 1,25$(OH)_2D_3$.

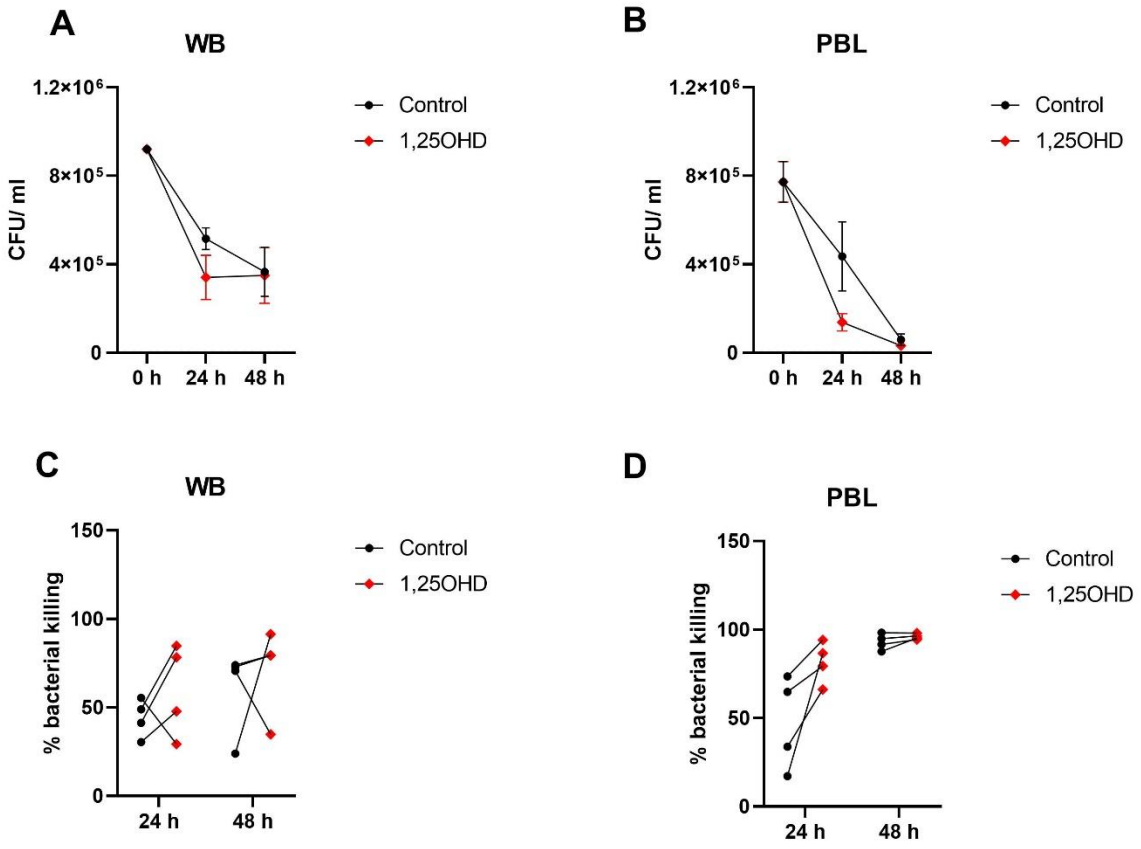


Figure 2-1. Infection of WB and PBL with *M. bovis* BCG for 24 and 48 h.

(A, C) 300 µl of blood was stimulated or not with 4 ng/ml 1,25$(OH)_2D_3$ and infected with 1x10^6 CFU/ml of BCG. (B, D) 1x10^6 PBL/ ml were stimulated or not with 4 ng/ml 1,25$(OH)_2D_3$ and infected with BCG. After 24 and 48 h post-infection, cells were washed and lysed to determine the number of viable bacilli following serial dilution on 7H11 agar plates. Percentage of bacterial killing was calculated by the ratio of bacteria used for challenge between those that were killed after 24 h. Wilcoxon paired test was used to assess differences between control and 1,25$(OH)_2D_3$ stimulated samples. The results shown are the mean± SEM of n= 4.

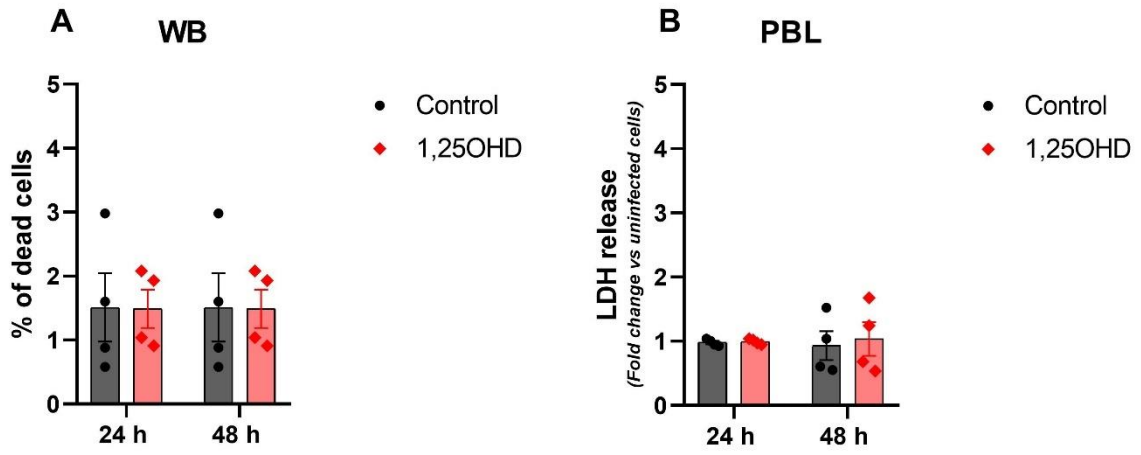


Figure 2-2. Cell viability in WB and PBL after *M. bovis* BCG infection for 24 and 48 h. Cells were stimulated or not with 4 ng/ml of $1,25(OH)_2D_3$ and challenge with *M. bovis* BCG over 48 h. A) RBC's cells lysed, then cells were stained with a live/dead violet fixable stain and fluorescence was measure by flow cytometry. B) Levels of LDH were measure as a proxy for cell death. Results are represented as fold change between uninfected-unstimulated cells. Wilcoxon paired test was used to assess differences between control and $1,25(OH)_2D_3$ stimulated samples. The results shown are the mean± SEM of n=4.

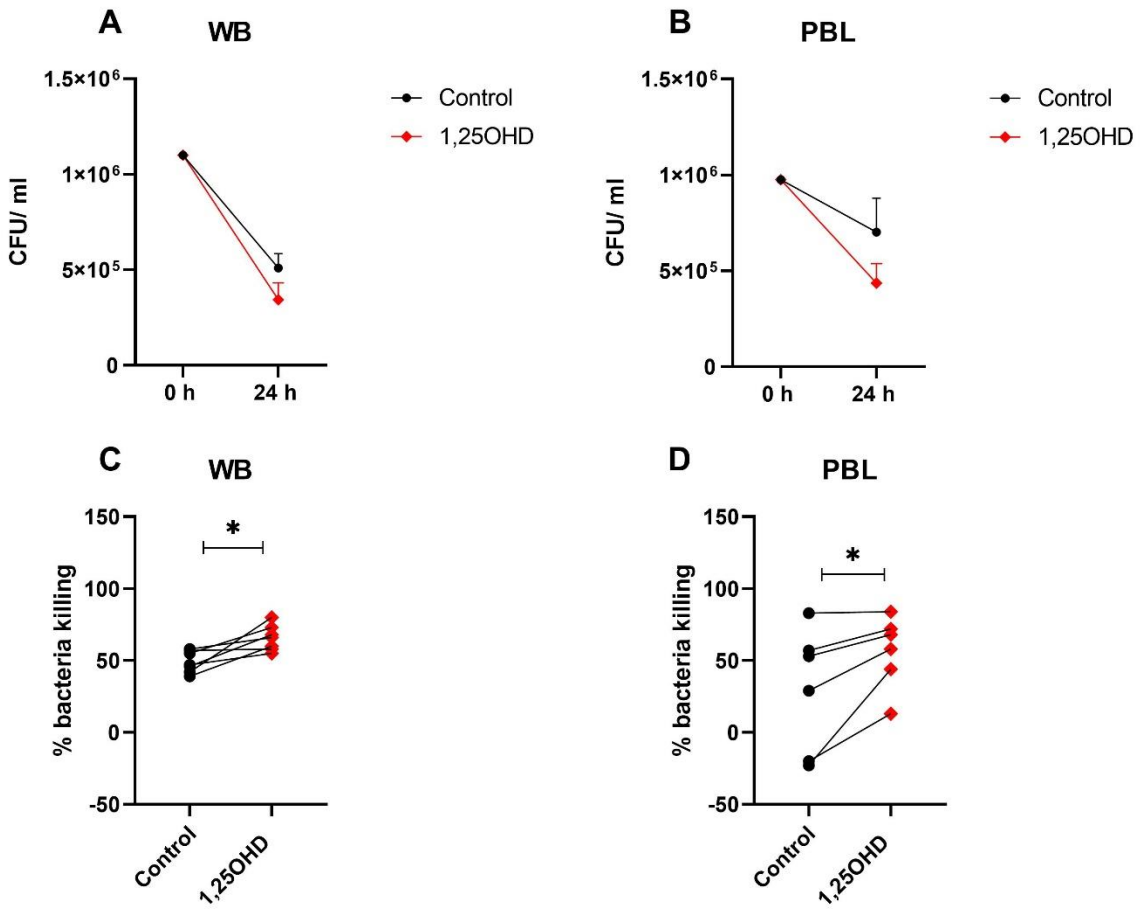


Figure 2-3. Microbicidal activity of WB and PBL stimulated with 1,25(OH)D.

(A, C) 300 µl of blood was stimulated or not with 4 ng/ml $1,25(OH)_2D_3$ and infected with $1x10^6$ CFU/ml of BCG. (B, D) $1x10^6$ PBL/ ml were stimulated or not with 4 ng/ml $1,25(OH)_2D_3$ and infected with $1x10^6$ CFU/ml BCG. After 24 h post-infection, cells were washed and lysed to determine the number of viable bacilli following serial dilution on 7H11 agar plates. Percentage of bacterial killing was calculated by the ratio of bacteria used for challenge between those that were killed after 24 h. Wilcoxon paired test was used to assess differences between control and $1,25(OH)_2D_3$ stimulated samples. The results shown are the mean± SEM of PBL, n= 6 and WB n= 7animals. *P < 0.05 was considered statistically significant.

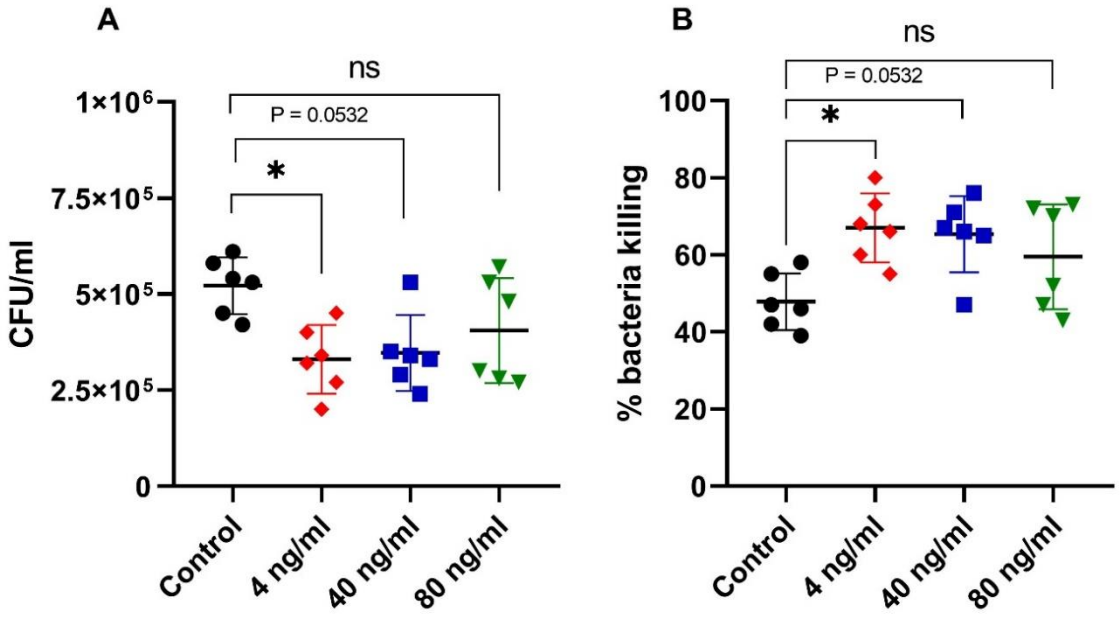


Figure 2-4. Microbicidal activity of WB stimulated with 4, 40 and 80 ng/ml of 1,25(OH)$_2$D$_3$. 300 µl of blood was stimulated or not with 4, 40 and 80 ng/ml of 1,25(OH)$_2$D$_3$ and infected with 1x10^6 CFU/ml. After 24 h post-infection, cells were washed and lysed to determine the number of viable bacilli following serial dilution on 7H11 agar plates. (A) CFU/ml after 24 h post-infection and (B) Percentage of bacterial killing calculated by the ratio of bacteria used for challenge between those that were killed after 24 h. Kruskal-Wallis test with Dunn's multiple comparison test was used to assess differences between control and the different concentrations of 1,25(OH)$_2$D$_3$. The results shown are the mean± SEM of n= 6 animals. *P < 0.05 was considered statistically significant.

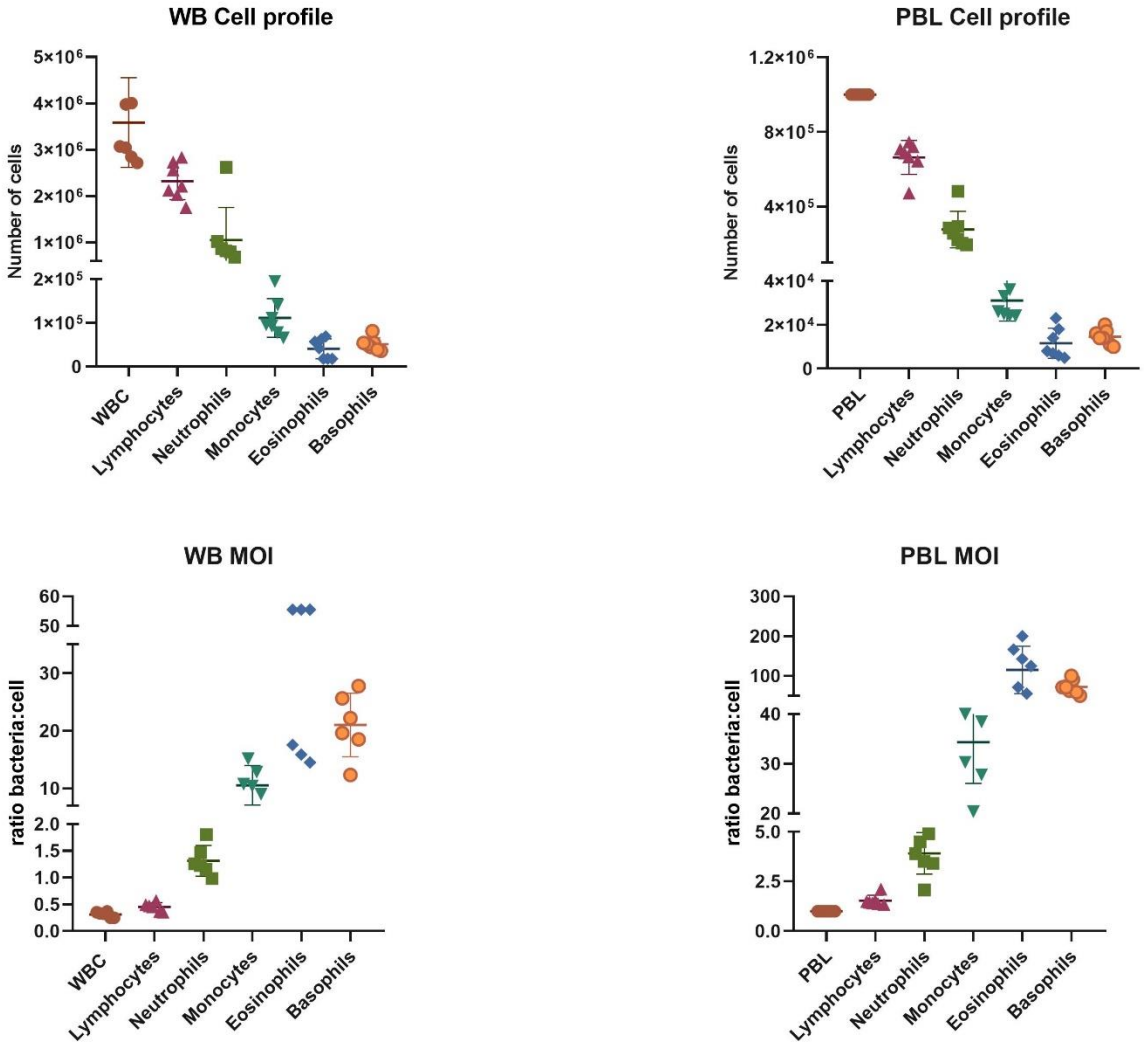


Figure 2-5.Haematological profile and MOI used in the WB and PBL models of BCG challenge.

Cell profile was determined using the ADVIA 2120 haematology analysis system. The MOI for white blood cells (WBC), lymphocytes, neutrophils, monocytes, eosinophils, and basophils was estimated by the ratio of $1x10^6$ CFU/ml of BCG and the total numbers of each cell type in 300 µl of blood and 1 ml of PBL.

Table 2-3. Estimation of the MOI used in the WB and PBL models of BCG challenge according to the total number of cells in each model.

	WBC		Neutrophils		Lymphocytes		Monocytes		Eosinophils		Basophils	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
WB	0.3127	0.05044	1.315	0.2856	0.4513	0.08214	10.55	3.411	35.76	21.7	21.02	5.513
PBL	ND	ND	3.92	1.049	1.538	0.2655	34.33	8.265	115	59.61	72.16	17.75

2.4.2. Analysis of the contribution of monocytes, granulocytes, lymphocytes CD3+, cells T CD4+ and T CD8+ T on the microbicidal activity by $1,25(OH)_2D_3$

2.4.2.1. Impact of cell depletion on BCG growth control

Having seen that $1,25(OH)_2D_3$ improved the microbicidal activity of PBL, the next step was to study the contribution of each cell type in the control of BCG growth. For this purpose, a cell depletion assay was performed. Depletion of granulocytes ($Gran_{neg}$) was performed by gradient centrifugation, depletion of monocytes ($CD14_{neg}$), T lymphocytes ($CD3_{neg}$), T $CD4^+$ cells ($CD4_{neg}$) and T $CD8^+$ cells ($CD8_{neg}$) were carried by magnetic cell separation. Infections were done as previously, analysis of the infection included CFU counts of intracellular and extracellular bacteria after 2 and 24 h of infection, and cell death measured by LDH release. The earliest time point was selected to analyse the proportion of bacilli that were engulfed (% phagocytosis), and the latest time point was used to analyse the proportion of bacteria killed (% bacterial killing).

The percentage of cell depletion for each cell subset was determined by flow cytometry by comparing the proportion of each cell in the PBL sample. The median cell depletion and range for each cell subset is shown in Table 2-4. The percentage of cell depletion was between 97 to 98 % per cell subset.

While there was no significant difference on bacteria counts at 2 h post infection (Figure 2-6 A & B), results showed than granulocyte depletion ($Gran_{neg}$) has the greatest impact on BCG growth in control and $1,25(OH)_2D_3$ stimulated cells at 24 h post infection (Figure 2-6 C & D). No significant difference was observed after cell depletion of the other cell subsets. This data suggest that neutrophils have a principal role in the mycobacterial activity in PBL.

Table 2-4. Median cell depletion efficacy per cell subset measured by flow cytometry		
Cell subset	**Depletion (%)**	**Range**
$CD14_{(neg)}$	98.8	97.3 - 98.9
$Gran_{(neg)}$	98.3	96.8 - 98.8
$CD3_{(neg)}$	97.15	86.7 - 98.3
$CD4_{(neg)}$	98.4	97.9 - 98.9
$CD8_{(neg)}$	97.85	96.3 - 98.9

*Values are the percentage of each cell subset depleted by the procedure compared to the PBL sample from the same animal.

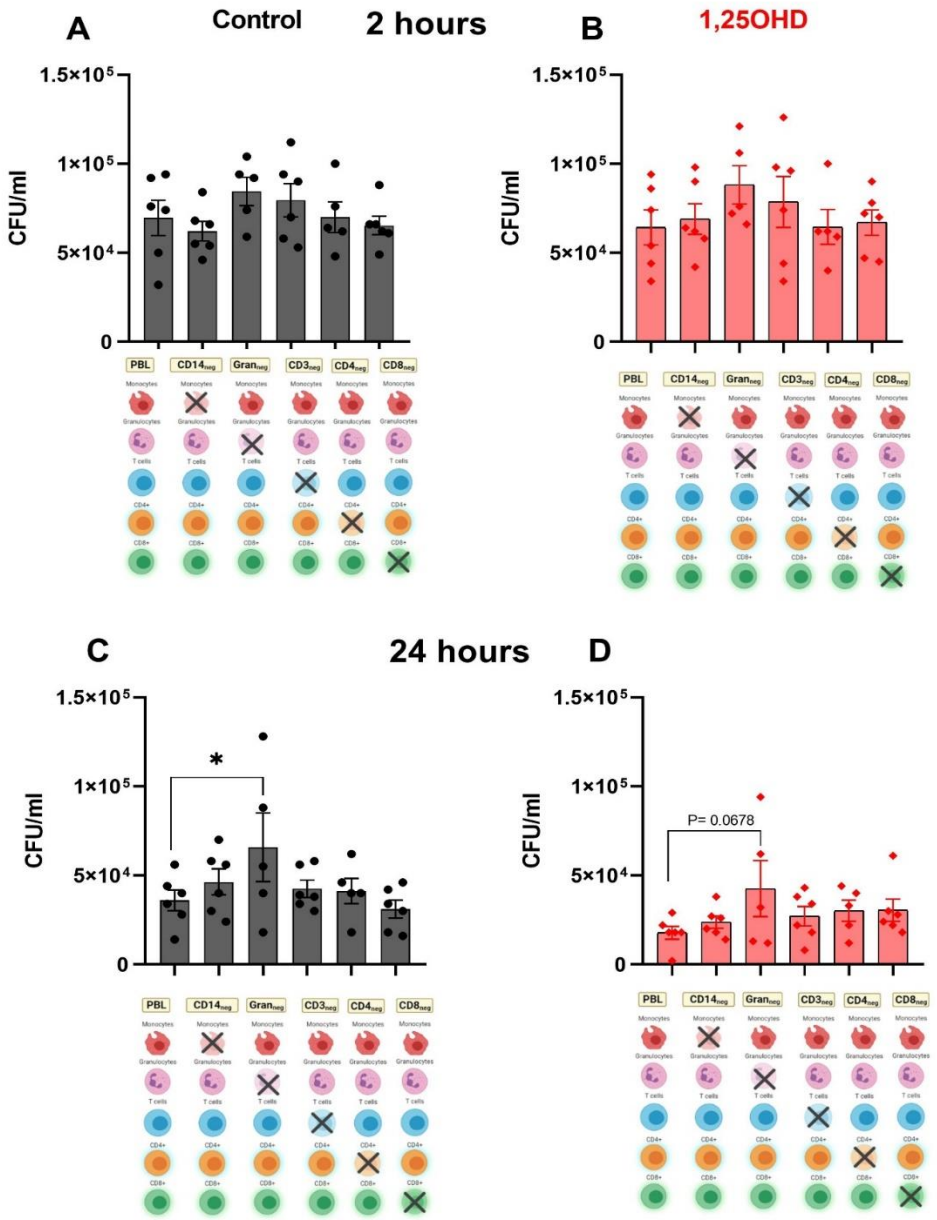


Figure 2-6. Impact of cell depletion on control of BCG growth.

$1x10^6$ cells/ml of PBL and the cell depleted subsets of monocytes ($CD14_{neg}$), granulocytes ($Gran_{neg}$), lymphocytes ($CD3_{neg}$), T CD4+ ($CD4_{neg}$) and T CD8+ ($CD8_{neg}$) were stimulated with (or without) 4 ng/ml of $1,25(OH)_2D_3$ and infected with $1x10^6$ CFU/ml of BCG for 2 and 24 h. After each timepoint cells were lysed and CFU counts were determined following serial dilution on 7H11 agar plates. CFU/ml at 2 h post infection of (A) control and (B) $1,25(OH)_2D_3$ samples. CFU/ml at 24 h post infection of (C) control and (D) $1,25(OH)_2D_3$ samples. Results are shown as the mean ±SEM of n=5/6. Friedman test with Dunn's correction for multiple comparations was performed to analyse the differences between PBL and each cell subset. *P < 0.05 was considered statistically significant.

2.4.2.2. Microbicidal activity by 1,25(OH)$_2$D$_3$ of cell depleted subsets at 2 and 24 h post-infection.

The analysis of the microbicidal activity was done at 2 and 24 h post-infection to analyse the percentage of phagocytosis and the proportion of bacterial killing. Thus, results are presented as CFU/ml and percentage of phagocytosis (Figure 2-7 A & B), and as CFU/ml and percentage of bacteria killing (Figure 2-7 C & D). There was no significant difference between control and 1,25(OH)$_2$D$_3$ stimulated cell subsets after 2 h post infection (Figure 2-7 A & B). The average percentage of phagocytosis was 47- 48 % for control and 1,25(OH)$_2$D$_3$ treatment. The percentage of phagocytosis for each cell subset is shown in Table 2-5.

Similar to previous results, 1,25(OH)$_2$D$_3$ significantly reduced the number of viable bacilli after 24 h of infection in PBL. When represented as percentage of bacterial killing, 1,25(OH)$_2$D$_3$ significantly increased bacterial killing to 69% in comparison to 40% killing in control PBL (Figure 2-7 C & D). In general, the biological trend shows that 1,25(OH)$_2$D$_3$ treatment increases the microbicidal activity in all the cells subsets. There was significantly greater bacterial killing in CD14$_{neg}$ treated with 1,25(OH)$_2$D$_3$ and close to significant in Gran$_{neg}$ subsets (P = 0.0625) (Figure 2-7 C & D). Results for each cell subset are presented in Table 2-5.

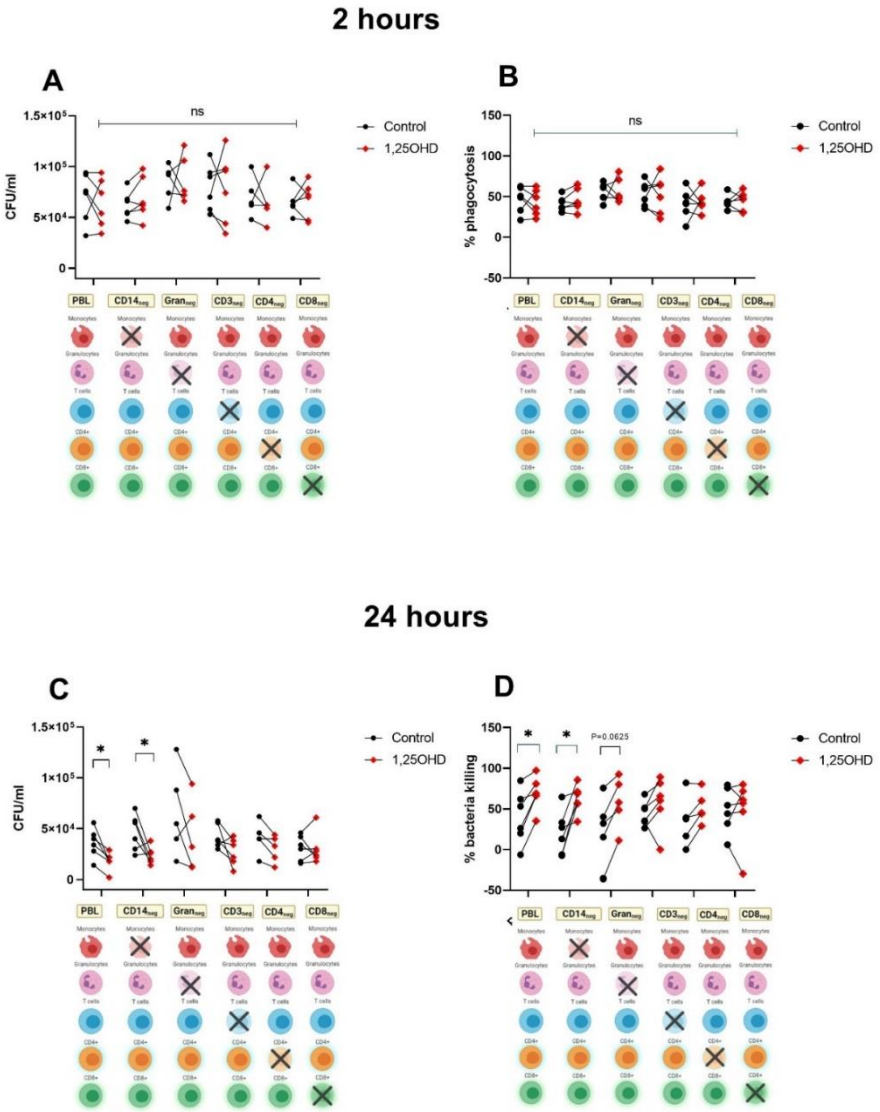


Figure 2-7. Effect of 1,25(OH)$_2$D$_3$ on BCG growth after cell depletion of monocytes, granulocytes, and lymphocytes.

1x10^6 cells/ml of PBL and the cell depleted subsets of monocytes (CD14$_{neg}$), granulocytes (Gran$_{neg}$), lymphocytes (CD3$_{neg}$), T CD4 (CD4$_{neg}$) and T CD8 (CD8$_{neg}$) were stimulated with 4 ng/ml of 1,25(OH)$_2$D$_3$ and infected with 1x10^6 CFU/ml of BCG for 2 and 24 h. Then, cells were lysed and CFU counts were determined following serial dilution on 7H11 agar plates. (A, B) CFU/ml and % of phagocytosis at 2 h post infection. (C, D) CFU/ml and % bacteria killing at 24 h post infection. Phagocytosis was calculated by the ratio of bacteria used for challenge between those counted at 2 h. Bacteria killing was calculated by the ratio of bacteria at 2 h between those that were killed after 24 h. Results are shown as the mean ±SEM of n=5/6. Wilcoxon test was performed between control and 1,25(OH)$_2$D$_3$ per cell subset at each timepoint. *P < 0.05 was considered statistically significant.

Table 2-5. Mean percentage of BCG phagocytosis and bacterial killing after cell depletion							
			Control		$1,25(OH)_2D_3$		
Phagocytosis	**Cell subset**	**n**	**Mean**	**SD**	**Mean**	**SD**	***P value**
	PBL	6	46.44	16.21	42.89	16.01	0.8125
	$CD14+_{(neg)}$	6	41.44	8.968	46	14	0.3125
	$Gran_{(neg)}$	5	55.22	11.08	58.8	24.21	0.6875
	$CD3+_{(neg)}$	6	53	15.29	52.44	23.35	0.8438
	$CD4+_{(neg)}$	5	41.11	17.9	43.89	13.16	0.9999
	$CD8+_{(neg)}$	6	43.56	8.492	44.67	11.81	0.8125
	Average		**47**	**12.99**	**48**	**17.09**	
Bacteria killing	PBL	6	40.03	32.85	69.25	20.44	0.0313
	$CD14+_{(neg)}$	6	20.81	27.22	62.28	17.33	0.0313
	$Gran_{(neg)}$	5	25.46	40.88	58.16	31.43	0.0625
	$CD3+_{(neg)}$	6	44.33	15.11	57.85	31.71	0.3125
	$CD4+_{(neg)}$	5	13.44	59.85	46.36	21.88	0.125
	$CD8+_{(neg)}$	6	48.75	27.64	47.82	39.74	0.8438
	Average		**32**	**33.93**	**57**	**27.09**	

*Wilcoxon test between control and $1,25(OH)_2D_3$ treatments in each cell depleted subset

2.4.2.3. Analysis of extracellular and intracellular bacterial counts at 2 and 24 h of post-infection.

Previous results showed that 1,25$(OH)_2D_3$ treatment decreased the number of viable bacilli in PBL. The CFU counts for those assays were analysed in cell pellets which correspond to intracellular bacteria. However, the release of bacteria to the supernatant can overestimate the number of intracellular bacteria being killed. Thus, extracellular CFU counts were analysed in supernatants after infection alongside intracellular CFU counts in cell pellets. Results showed that in both treatments, control and 1,25$(OH)_2D_3$, there was a reduction in the intracellular and extracellular CFU counts at 24 h post infection in each cell depleted subset (Figure 2-8). This suggests that the decline in intracellular bacteria was not associated to an increase in extracellular bacteria for its release by cell death, suggesting that the bacilli were being killed by the cells. Furthermore, no significant difference in cell viability was observed between treatments in each cell depleted subset. The LDH release expressed as the fold change between infected and non-infected cells, was similar between treatments (Figure 2-9).

Collectively, these results suggest that 1,25$(OH)_2D_3$ modulates the activity of monocytes, neutrophils, and lymphocytes.

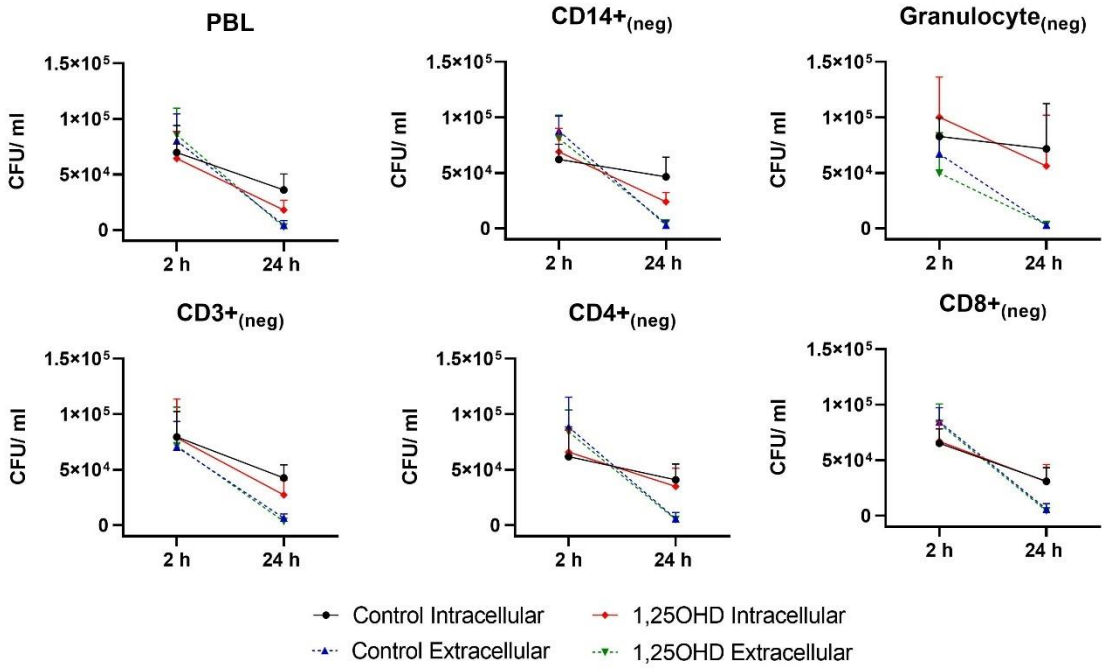


Figure 2-8. Extracellular and intracellular CFU counts after 2 and 24 h of BCG infection. 1x10^6 cells/ ml of PBL and the cell depleted subsets of monocytes ($CD14_{neg}$), granulocytes ($Gran_{neg}$), lymphocytes ($CD3_{neg}$), T CD4 ($CD4_{neg}$) and T CD8 ($CD8_{neg}$) were stimulated with 4 ng/ml of $1,25(OH)_2D_3$ and infected with 1x10^6 CFU/ml of BCG for 2 and 24 h. The supernatants (dotted lines) were removed to determine the extracellular bacteria. Cells were lysed (solid lines) to analyse the intracellular bacteria. The results are shown as the mean ±SEM of n=6.

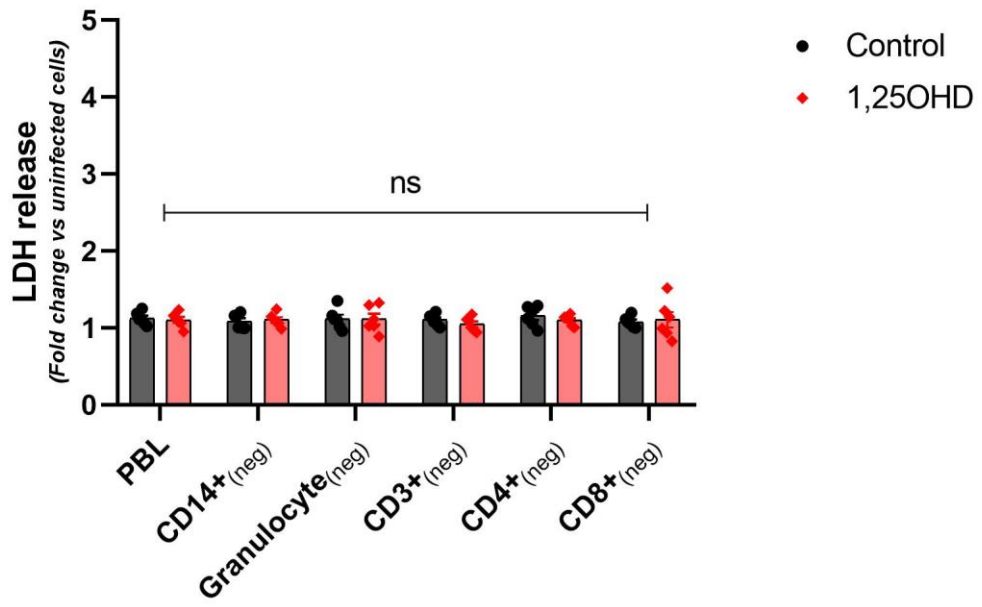


Figure 2-9. Cell viability in PBL and cell depleted subsets after 24 h of BCG infection. Cell viability was determined by LDH release. Results are represented as fold change between uninfected-unstimulated cells. Wilcoxon paired test was used to assess differences between control and 1,25$(OH)_2D_3$ stimulated samples per each cell subset. The results shown are the mean± SEM of n=6.

2.4.3. Analysis of the microbicidal activity by 1,25$(OH)_2D_3$ on PBL for control of BCG infection in BTB- and BTB+ cattle

To characterize the effect of vit D on the microbicidal activity on animals infected naturally with *M. bovis*, samples from cattle reactor (n= 8) to the tuberculin skin test (BTB+) were selected. Age-sex matched control animals (n=7) were chosen from a farm with no history of bovine tuberculosis infection which were negative to the tuberculin skin test (BTB-). The study includes analysis of the cell profile, circulating levels of 25(OH)D and microbicidal activity measured as percentage of phagocytosis and bacteria killing between BTB- and BTB+ cattle.

Analysis of the haematologic profile showed no difference in the cell profile between BTB+ and BTB- cattle (Figure 2-10). Also, there were no significant differences on the 25(OH)D levels in circulation, although, BTB+ animals were vit D deficient, with serum 25(OH)D levels below the 30 ng/ml threshold for optimal levels. The mean 25(OH)D concentration for BTB+ animals was 22.18±5.736 ng/ml, whereas BTB- cattle had 33.13 ±19.21 ng/ml 25(OH)D levels (Figure 2-11).

Comparison of the phagocytic and bacterial killing activity between BTB- and BTB+ animals showed that BTB- cattle ingested higher proportion of bacilli than BTB+ (Figure 2-12 A & B). However, despite the differences in BCG phagocytosis observed, there were no differences in the bacterial killing activity after 24 h of infection between BTB- and BTB+ cattle (Figure 2-12 C & D).

Overall, treatment with 1,25$(OH)_2D_3$ resulted in significant increase of BCG phagocytosis (Figure 2-13 A & B) and bacterial killing (Figure 2-13 C & D) in both groups of animals. Interestingly, the higher rise in bacterial killing was observed in BTB+ animals with 25(OH)D serum levels below 20ng/ml (shown as blue stars) (Figure 2-13 D).

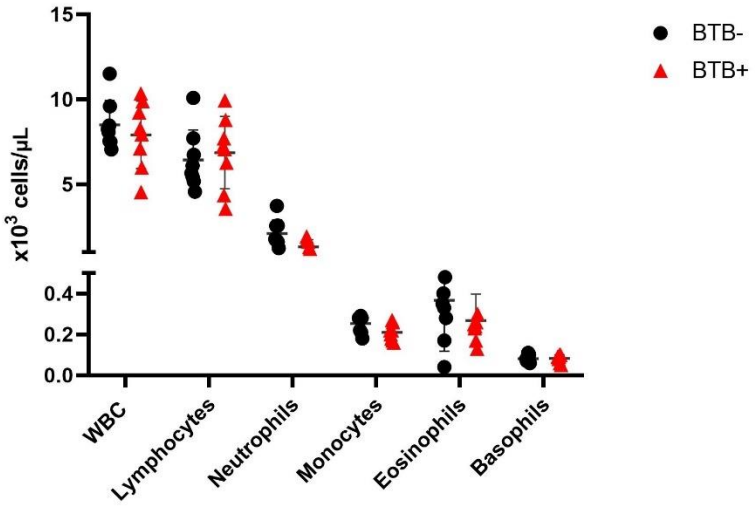


Figure 2-10. Haematology profile in BTB- and BTB+ cattle.

Blood samples were taken via neck venepuncture and processed by the ADVIA 2120 system. Results are represented as absolute counts x10^3 cells/µl of n= 7 BTB- and 8 BTB+. Welch's t-test was used to analyse differences between BTB- and BTB+ animals in each cell type.

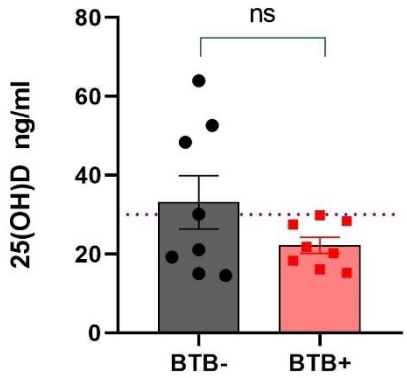


Figure 2-11. Serum 25(OH)D concentrations in BTB- and BTB+ cattle.

Serum samples were analyzed by ELISA. The purple dotted line indicate the optimal 25(OH)D level of 30 ng/ml. Concentrations are shown as mean ± SEM of n= 7 BTB- and 8 BTB+. Mann-Whitney test was used to determine differences between BTB- and BTB+ animals. ns= not significant.

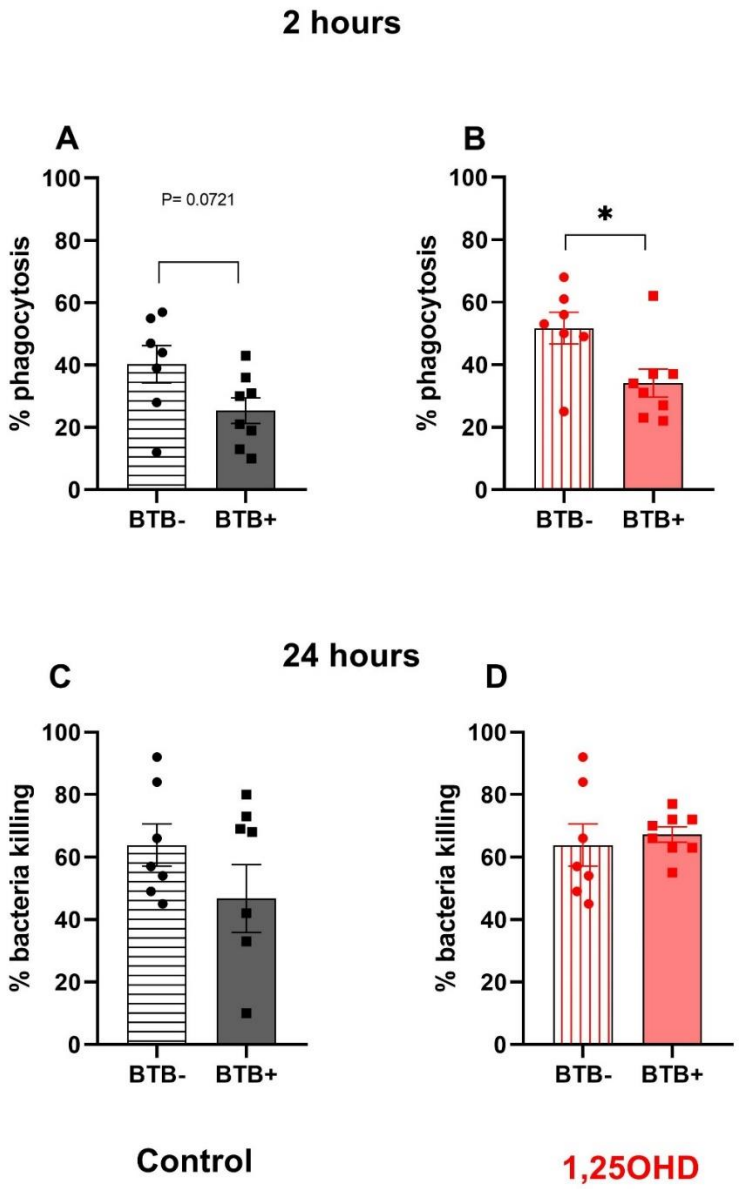


Figure 2-12. Comparison of phagocytic and bacterial killing activity in BTB- and BTB+ animals.

PBL was stimulated or not with 4 ng/ml of $1,25(OH)_2D_3$ and infected with $1x10^6$ CFU/ml of BCG for 2 and 24 h. Then, cells were washed and lysed to determine the number of viable bacilli following serial dilution on 7H11 agar plates. Percentage of phagocytosis in (A) control and (B) $1,25(OH)_2D_3$ treated PBL. Percentage of bacterial killing in (C) control and (D) $1,25(OH)_2D_3$ treated cells. Phagocytosis was calculated by the ratio of bacteria used for challenge between those counted at 2 h. Bacterial killing was calculated from the ratio of bacteria at 2 h between those that were killed after 24 h. Wilcoxon test was used to assess differences between control and $1,25(OH)_2D_3$ treatments in each BTB group. The results shown are the mean± SEM of n= 7 BTB- and 8 BTB+. *P < 0.05 was considered statistically significant.

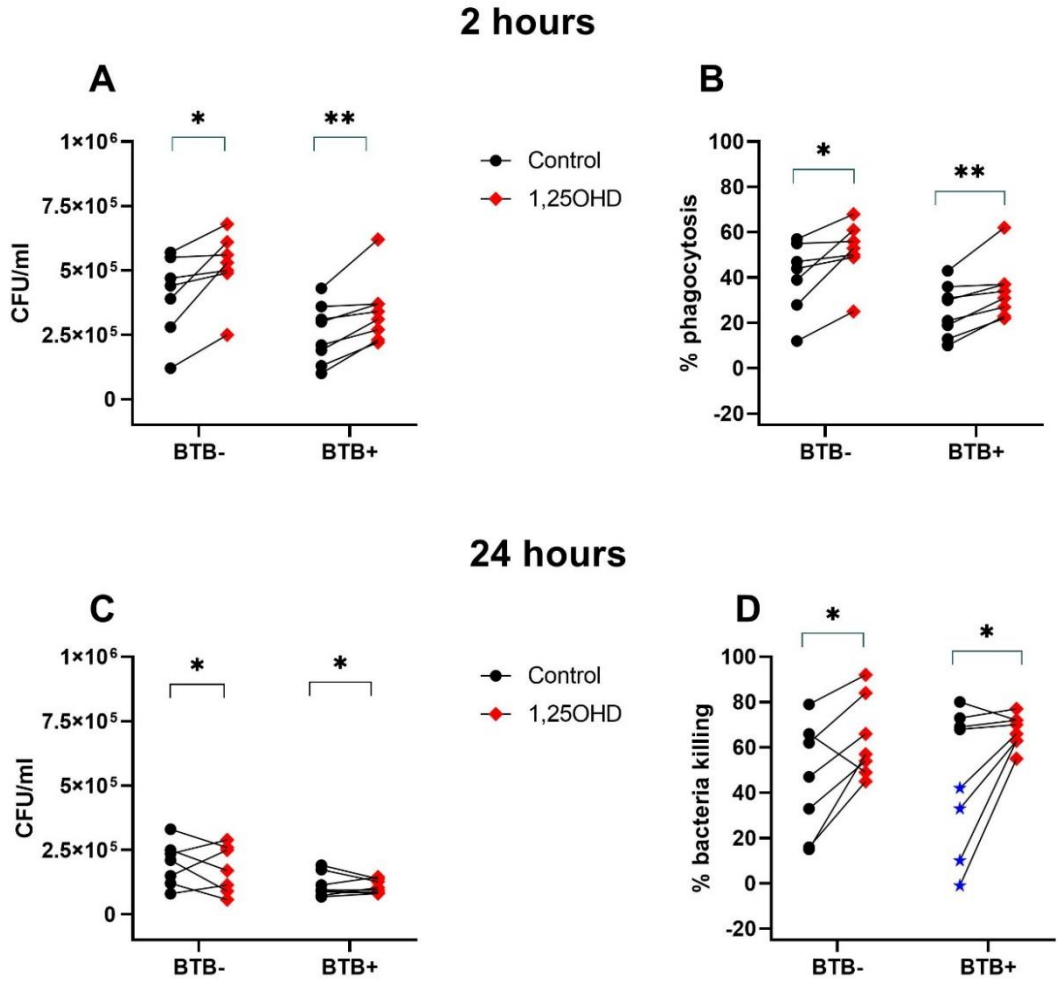


Figure 2-13. Microbicidal activity by 1,25(OH)$_2$D$_3$ on PBL from BTB- and BTB+ animals. PBL was stimulated or not with 4 ng/ml of 1,25(OH)$_2$D$_3$ and infected with 1x10^6 CFU/ml of BCG for 2 and 24 h. Then, cells were washed and lysed to determine the number of viable bacilli following serial dilution on 7H11 agar plates. (A, B) CFU/ml and % of phagocytosis at 2 h post infection. (C, D) CFU/ml and % bacteria killing at 24 h post infection. Phagocytosis was calculated by the ratio of bacteria used for challenge between those counted at 2 h. Bacteria killing was calculated by the ratio of bacteria at 2 h between those that were killed after 24 h. Wilcoxon test was used to assess differences between control and 1,25(OH)$_2$D$_3$ treatments in each BTB group. The results shown are the mean± SEM of n= 7 BTB- and 8 BTB+. *P < 0.05, ** P < 0.001 was considered statistically significant.

2.4.4. Analysis of the phagocytic activity by $1,25(OH)_2D_3$ on PBL.

Analysis of phagocytosis of BCG-GFP was performed by flow cytometry using the gating strategy shown in Figure 2-14. Results are presented as percentage of total cells which were fluorescent for GFP (% BCG-GFP$^+$ cells) and the proportion of monocytes (CD14$^+$ BCG-GFP$^+$) and granulocytes (G1$^+$ BCG-GFP$^+$) GFP positives (Figure 2-15). No significant differences in the percentage of BCG-GFP+ cells were observed in total leukocytes, nor monocytes or granulocytes (Figure 2-15). However, a great proportion of BCG-GFP+ cells were observed in the double negative gate (Figure 2-14). This suggests that most cells were adhered extracellularly to other cells rather than being ingested. However, this assay does not allow us to explore the intracellular location of the bacteria.

Analysis of the proportion of monocytes (CD14$^+$) and granulocytes (G1$^+$) before and after BCG infection, showed a significant decrease in the proportion of each phagocyte. The reduction in the identification of CD14$^+$ and G1$^+$ cells was higher in $1,25(OH)_2D_3$ treated cells (Figure 2-16).

The Phagotest™ assay was used to confirm the previous result. The Phagotest™ assay, is one of the most robust methods used to test phagocytosis in human blood. This method employs an opsonized *E.coli*-FITC labelled bacteria, and have been optimized for use in bovine blood [176]. Thus, the $1,25(OH)_2D_3$ stimulation was performed as before for 2, 4 and 6 h. There was no difference in the percentage of total leukocytes that ingested bacteria at any time point evaluated (Figure 2-17). In addition, treatment with 4, 40 or 80 ng/ml of $1,25(OH)_2D_3$ did not modify *E. coli* phagocytosis (Figure 2-18).

Taken together these results suggest that $1,25(OH)_2D_3$ does not significantly affect the phagocytic activity of leukocytes.

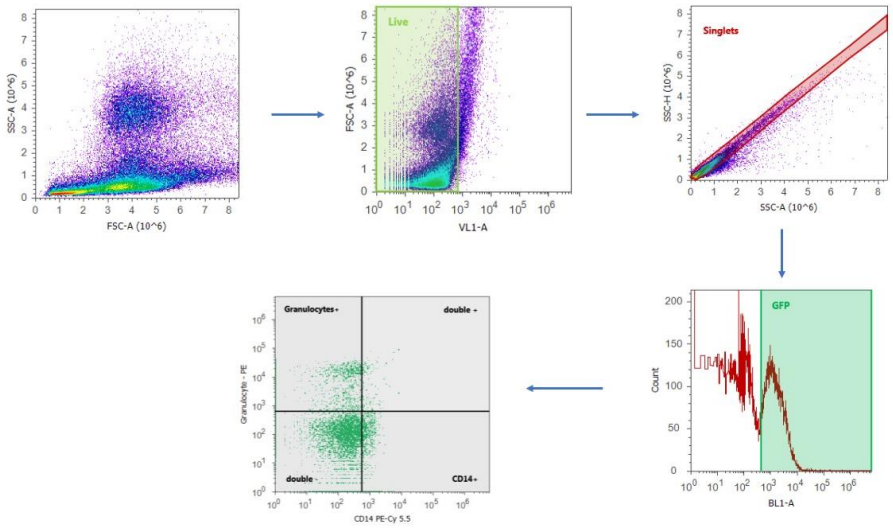


Figure 2-14. Gating strategy used to assess BCG-GFP phagocytosis by 1,25(OH)$_2$D$_3$.
The first gate was created for live cells only, then cells were gated for single events. BCG-GFP+ cells were selected based on their fluorescence in the BL1-A channel. Monocytes were identified based on the surface expression of CD14$^+$ marker, whereas granulocytes were selected based on the surface expression of G1$^+$ marker. 30,000 events were analysed by flow cytometry.

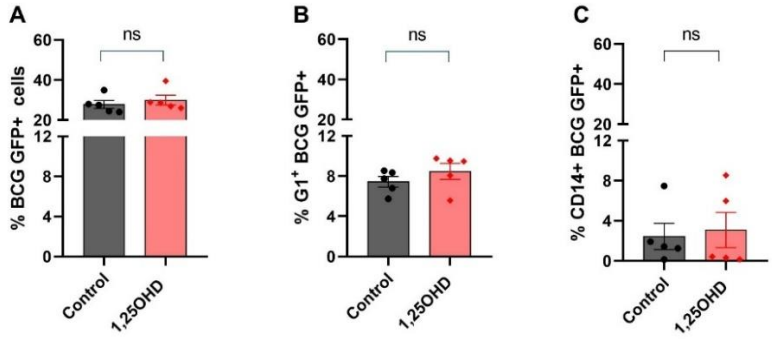


Figure 2-15. Effect of 1,25(OH)$_2$D$_3$ on BCG-GFP phagocytosis.
(A) Percentage of BCG-GFP+ cells in total leukocytes (B) granulocytes and (C) monocytes. 300 µl of blood was incubated or not with 4 ng/ml of 1,25(OH)$_2$D$_3$. Then, 300 µl RPMI containing 1x10^7 CFU/ml of BCG-GFP was added. After 1 h, RBC were lysed, and leukocytes were washed and labelled with markers for monocytes (CD14+) and granulocytes (G1+) in addition to live/dead stain. Then, cells were fixed and analysed by flow cytometry. Results shown are the mean ±SEM of n =5. Wilcoxon paired test was used to assess differences between control and 1,25(OH)$_2$D$_3$ treatments. *ns* = not significant.

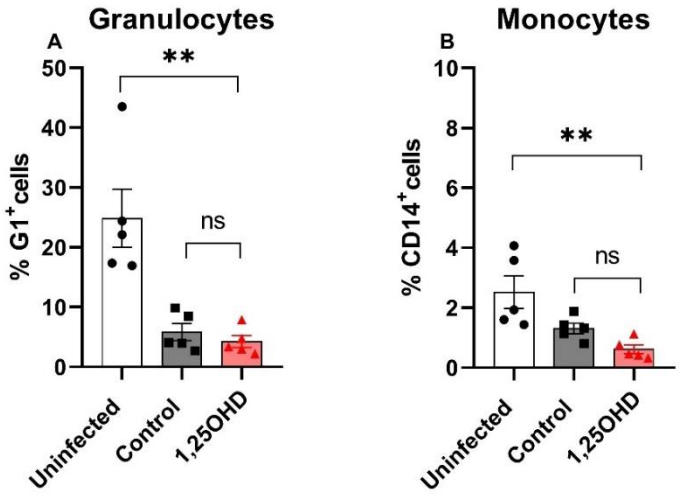


Figure 2-16. Proportion of monocytes and neutrophils identified before and after BCG-GFP phagocytosis.

(A) Percentage of granulocytes and (B) monocytes in non-infected blood, and after BCG-GFP phagocytosis in control and 1,25$(OH)_2D_3$ treated blood. 300 µl of blood was incubated or not with 4 ng/ml of 1,25$(OH)_2D_3$ Then, 300 µl RPMI containing 1x10^7 CFU/ml of BCG-GFP was added. After 1 h, RBC were lysed, and leukocytes were washed and labelled with markers for monocytes (CD14+) and granulocytes (G1+) in addition to live/dead stain. Then, cells were fixed and analysed by flow cytometry. Results shown are the mean ±SEM of n =5. Friedman test was used to assess differences between noninfected and BCG infected control, and 1,25$(OH)_2D_3$ treatments. *ns* = not significant, ** P<0.001 was considered statistically significant.

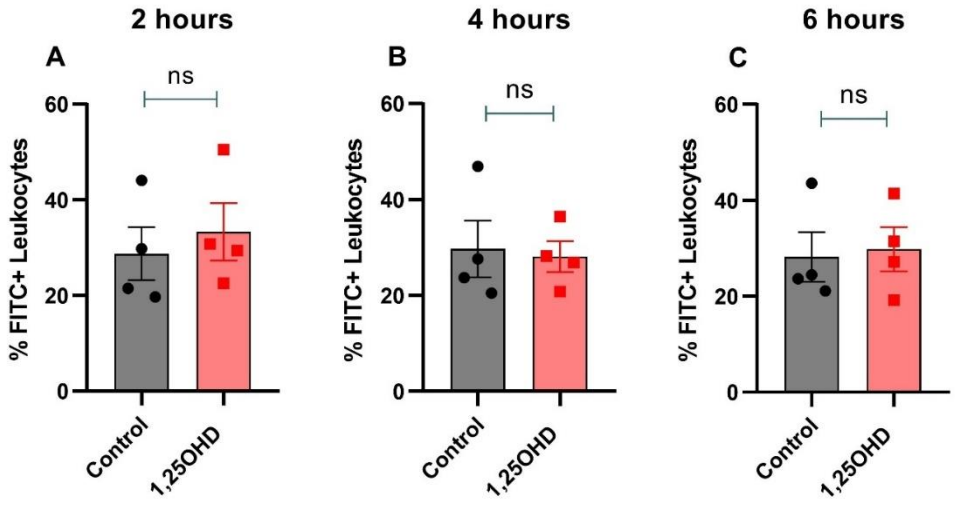


Figure 2-17. Effect of $1,25(OH)_2D_3$ on *E. coli*-FITC phagocytosis.

1 ml of blood was stimulated with 4 ng/ml of $1,25(OH)_2D_3$ for 2, 4 and 6 h. Then 50 µl was used to perform the Phagotest™ assay and analysed by flow cytometry. Results shown are the mean ±SEM of n= 4. Difference between treatments were assessed by Wilcoxon paired test. *ns* = not significant.

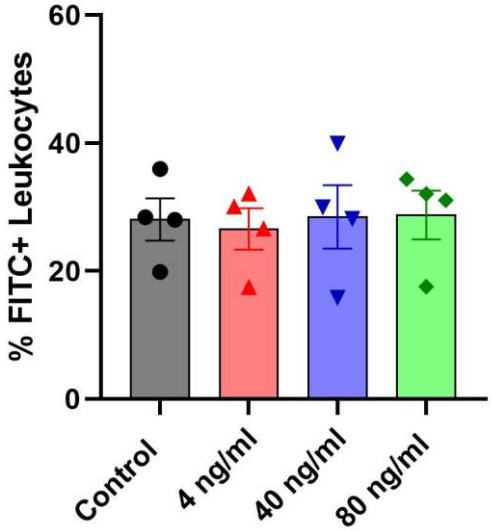


Figure 2-18. Effect of 4, 40 and 80 ng/ml of $1,25(OH)_2D_3$ on *E. coli*-FITC phagocytosis.

1 ml of blood was stimulated with 4, 40 or 80 ng/ml of $1,25(OH)_2D_3$ for 2 h. Then 50 µl was used to perform the Phagotest™ assay and analysed by flow cytometry. Results shown are the mean ± SEM of n= 4. Difference between treatments were assessed by Wilcoxon paired test.

2.4.5. Analysis of the ROS production by 1,25(OH)$_2$D$_3$ on PBL

Generation of ROS has been implicated in a variety of physiologic responses from bacterial killing to cell proliferation and apoptosis. Furthermore, the production of ROS after *M. tuberculosis* phagocytosis has been associated with the immune response to mycobacteria [42]. Thus, to assess the effect of 1,25(OH)$_2$D$_3$ on ROS production in blood, samples were analysed by flow cytometry with the CellROX green reagent. The gating strategy was similar to the BCG phagocytosis analysis, and it is shown in Figure 2-19. A significant higher proportion of ROS+ leukocytes was observed in 1,25(OH)$_2$D$_3$ treated blood, in comparison with control. However, no differences in ROS production were observed for granulocytes nor monocytes (Figure 2-20). Like in the previous BCG phagocytosis assay, a great proportion of ROS+ cells were observed in the double negative gate. This could suggest that 1,25(OH)$_2$D$_3$ promotes ROS production in leukocytes beyond granulocytes or monocytes. However, this could be also the result with a problem of cell markers detection after BCG infection. This issue will be discussed further in the discussion session.

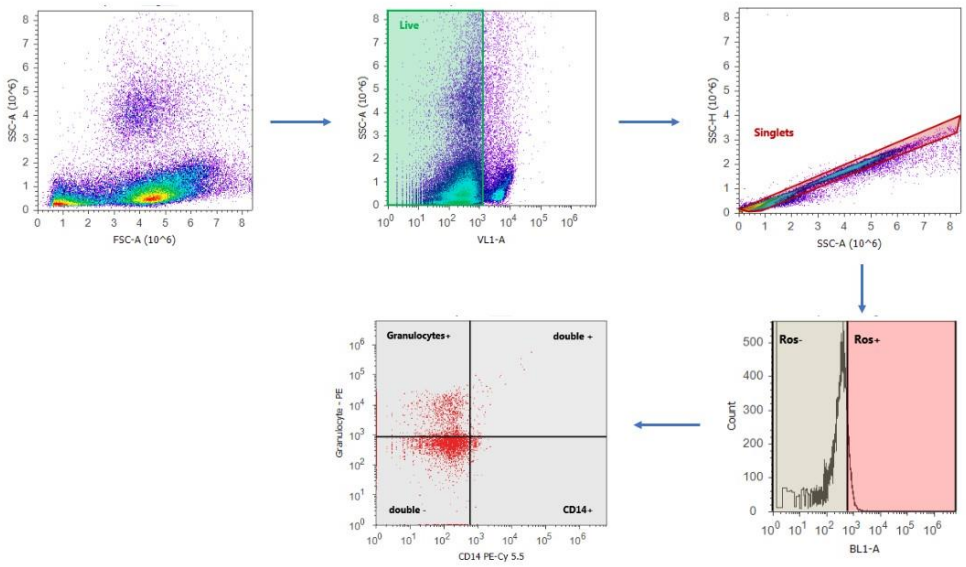


Figure 2-19. Gating strategy to assess ROS production by 1,25(OH)$_2$D$_3$.
The first gate was created for live cells only, then cells were gated for single events. ROS+ cells were selected based on their fluorescence in the green (BL1-A) channel. Monocytes were identified based on the surface expression of CD14+ marker, whereas granulocytes were selected based on the surface expression of G1+ marker. 30,000 events were analysed by flow cytometry.

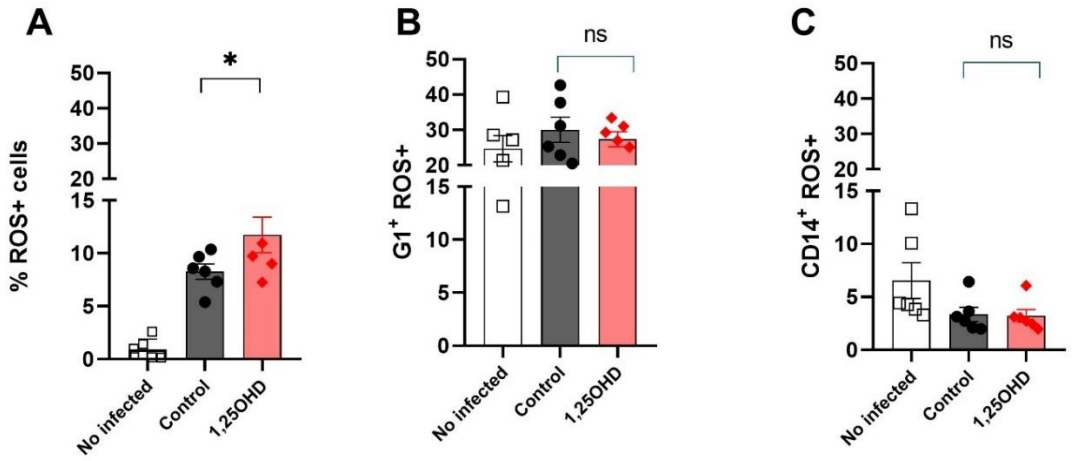


Figure 2-20. Effect of $1,25(OH)_2D_3$ on ROS production in whole blood.

(A) Percentage of ROS+ cells in total leukocytes, (B) percentage of granulocytes ROS+ and (C) monocytes ROS+ detected in the ROS+ total cells. Blood was incubated or not with 4 ng/ml of $1,25(OH)_2D_3$ and infected with 1 x 10^7 CFU/ml of BCG for 1 h. Then, CellROX green reagent was added. After 1 h RBC were lysed, and leukocytes were washed and labelled with markers for monocytes (CD14+) and granulocytes (G1+) in addition to live/dead stain. Then, cells were fixed and analysed by flow cytometry. Data shown is the mean ±SEM of n =6. Wilcoxon paired test was used to assess differences between control and $1,25(OH)_2D_3$ treatments. *ns* = not significant, *P<0.05 was considered statistically significant.

2.4.6. Analysis of the gene expression by 1,25$(OH)_2D_3$ on PBL after BCG challenge.

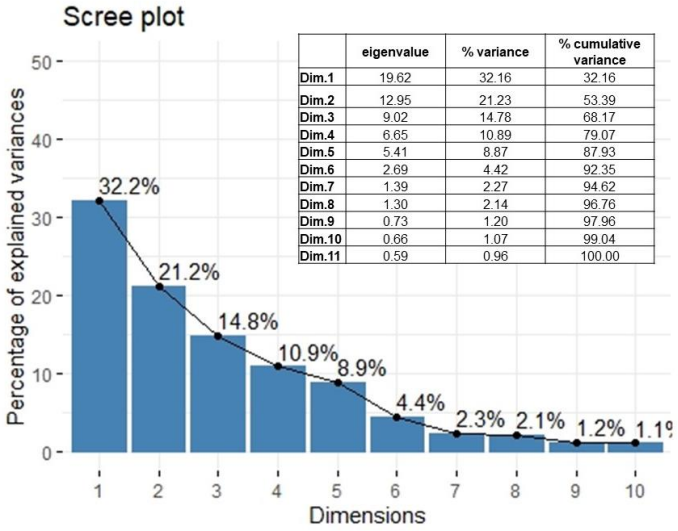
Analysis of the immunoregulatory role by 1,25$(OH)_2D_3$ was done on replicate samples from previous BCG infections on PBL. Gene expression of 96 genes was assessed by RT- qPCR using the Fluidigm Biomark HD system. Low expressed or non-detected genes were removed from the dataset; therefore, analysis was done with sixty-one genes.

A PCA was performed to analyse the overall structure of the dataset. The plot of the eigenvalues showed that analysis of four dimensions was necessary to explain 79% of the variance (Figure 2-21). Figure 2-22 shows the plot of individuals based on their similarity where each group is enclosed in a convex ellipse, the line encircles each sample within their corresponding ellipse. Each sample is represented by their number, where C1 corresponds to control sample 1 and V1 corresponds to their 1,25$(OH)_2D_3$ stimulated sample. Results showed that the overall gene expression between control and 1,25$(OH)_2D_3$ treatment to BCG challenge overlap along dimension 1 to 4 suggesting few divergences between treatments. Furthermore, differences between groups correspond to a subset of samples, such as V1, V3 or V4 for dimension 1&2. Therefore, a high variability between animals in response to 1,25$(OH)_2D_3$ was observed.

The biplots show the relationship between individuals and variables (genes) within dimension 1 to 4 (Figure 2-23 A & B). For the representation, only the top 20 genes that contributed the most to each dimension are shown. Thus, *TGFB1* and *PKR* influence the most to dimension 1, whereas *IL1R1* and *IL6* contribute to dimension 2. *IL1A* and *IL12B* influences dimension 3, and *IL10* and *COX2* contributes to dimension 4. The genes that are grouped together are positively correlated (e.g., *DEFB10, S100A8*, etc), whereas negatively correlated genes are represented on opposed quadrants (e.g., IL1R1 vs IL6). The biplot shows that differences between groups are along dimension 2 and 4 represented mostly by antimicrobial peptides genes such as *DEFB10, DEFB8, DEFB7, DEFB3, LAP, S100A8.* Other genes, including *IL10, COX2, IL1A, CXCL10* contributed also to the observed differences.

To identify clusters of genes differentially expressed between treatments a hierarchical cluster analysis with heatmap visualization was performed. The heatmap at Figure 2-24 showed that the gene expression in response to BCG challenge is highly variable. The dendrograms along the columns show that animals are grouped in 3 clusters with high, mid, or low response along all the clusters of genes. Six clusters of genes were detected, with cluster 4 showing a pattern of differential expression between control and 1,25$(OH)_2D_3$ PBL. Cluster 4 formed by genes such as *CCL2, CXCL1, ELANE, IFNB, IFNA, IFNAR1, IL33, PKR,*

TAP and *DEFB7* showed a higher expression in $1,25(OH)_2D_3$ treated PBL in comparison to control samples (Figure 2-26). Finally, a bar plot representation of antimicrobial peptides genes is shown in Figure 2-25. Although there were no differences between control and $1,25(OH)_2D_3$ treatment, there is a biological effect of higher antimicrobial peptide production by $1,25(OH)_2D_3$. No statistical differences were observed in any gene analysed, mean and *P* adjusted values genes for all genes are shown in Appendix 2.



	eigenvalue	% variance	% cumulative variance
Dim.1	19.62	32.16	32.16
Dim.2	12.95	21.23	53.39
Dim.3	9.02	14.78	68.17
Dim.4	6.65	10.89	79.07
Dim.5	5.41	8.87	87.93
Dim.6	2.69	4.42	92.35
Dim.7	1.39	2.27	94.62
Dim.8	1.30	2.14	96.76
Dim.9	0.73	1.20	97.96
Dim.10	0.66	1.07	99.04
Dim.11	0.59	0.96	100.00

Figure 2-21. Percentage of explained variance from PCA of gene expression effects by $1,25(OH)_2D_3$ on PBL.

Scree plot from the eigenvalues ordered from the largest to the smallest, showing the percentage of explained variance per dimension.

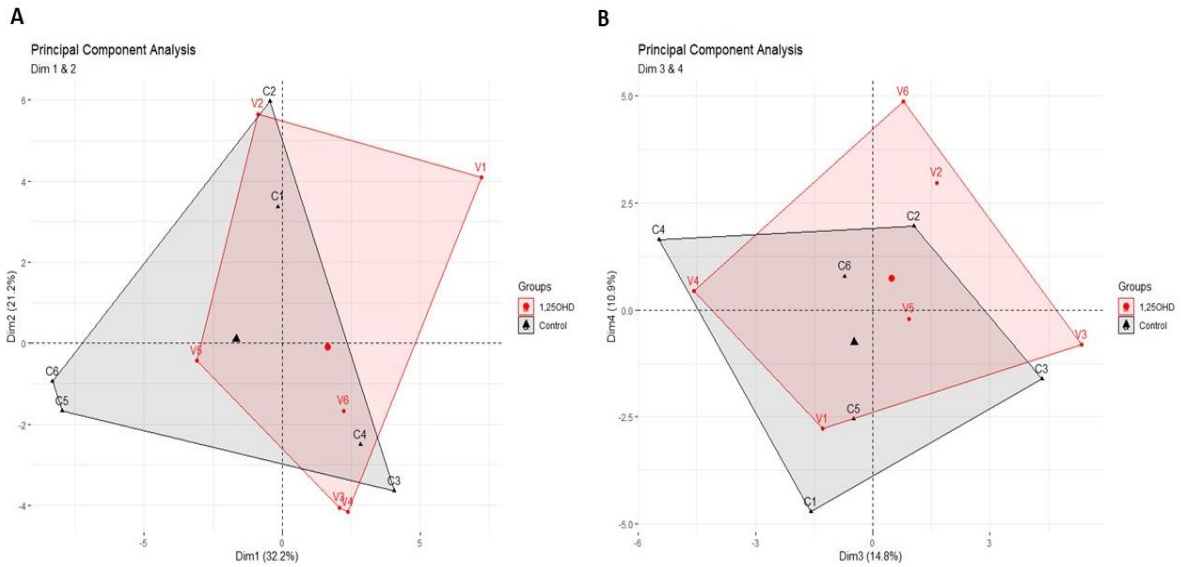


Figure 2-22. Plot of individuals from PCA of gene expression effects by 1,25(OH)$_2$D$_3$ on PBL.

(A) PCA plot for dimensions 1 and 2 and (B) dimensions 3 and 4. Individuals are represented as C1 to C6 for control and their paired sample V1 to V6 for 1,25(OH)$_2$D$_3$ treatment. Animals are grouped by a convex hull to show the clustering of the groups.

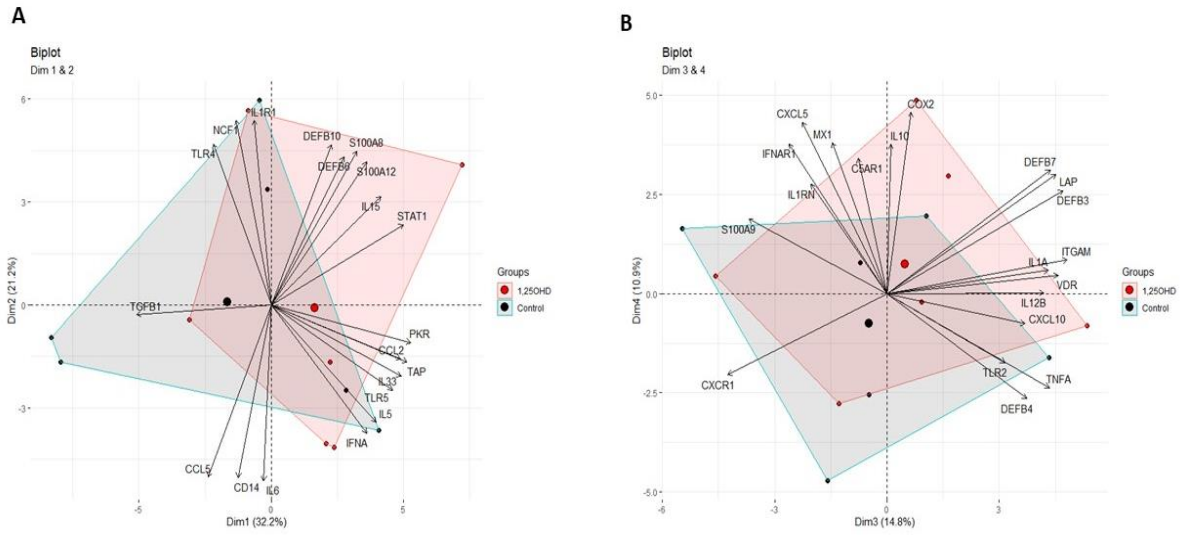


Figure 2-23. Biplot of individuals from PCA of gene expression effects by 1,25(OH)$_2$D$_3$ on PBL.

(A) Biplots for dimension 1 and 2, and (B) dimension 3 and 4, showing the representation of individuals and variables within the principal component. Animals are grouped by a convex hull to show the clustering of the groups.

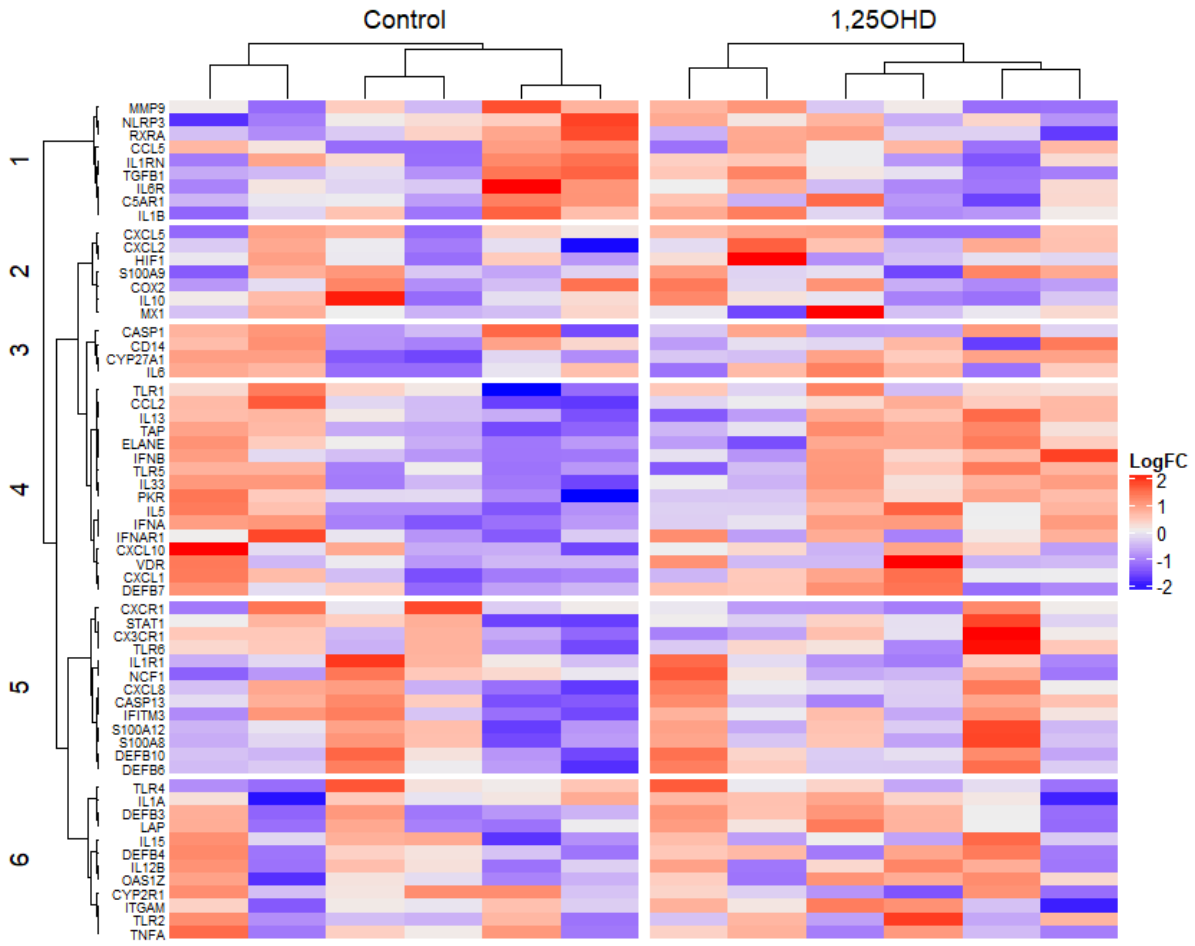


Figure 2-24. Heatmap of the gene expression effects by $1,25(OH)_2D_3$ on PBL.

Results represent the standardized-Log_2 relative fold change. Hierarchical clustering with spearman correlation distance and Ward's linkage method was used to identify clusters of genes and samples with similar patterns. Division of genes in 6 clusters was done by splitting the dendrogram in clusters with the same height.

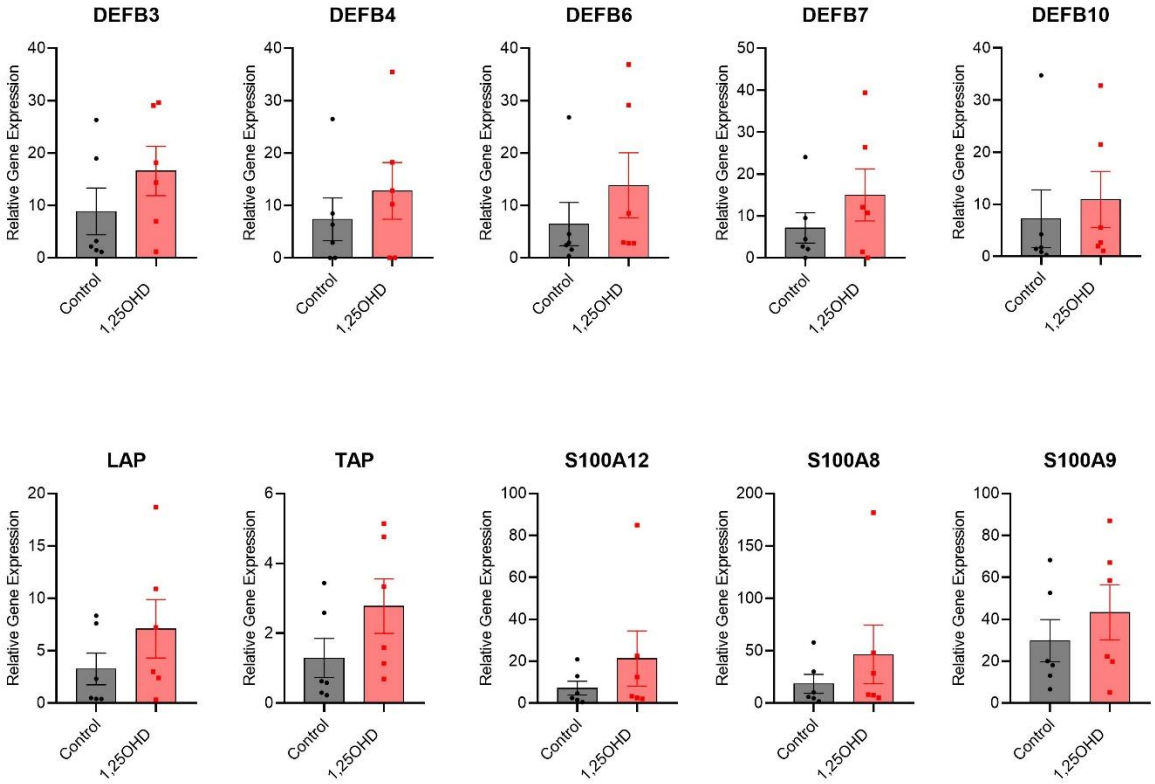


Figure 2-25. Relative gene expression of antimicrobial peptides in control and $1,25(OH)_2D_3$ treated PBL.

Results are expressed as relative gene expression compared to a non-infected sample. Data shown is the mean ± SEM of n= 6. Difference between groups were assessed by multiple Wilcoxon paired T test with p-adjusted to 0.1 with the Benjamini-Hochberg method.

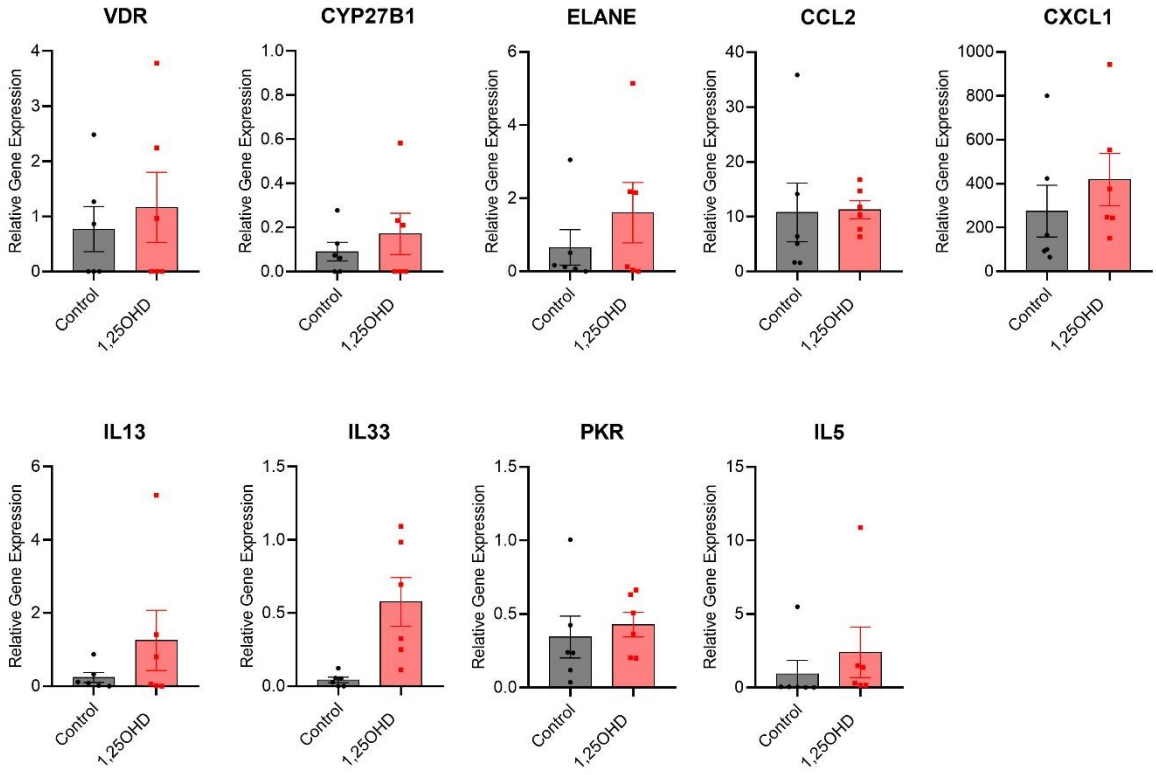


Figure 2-26. Relative gene expression of genes in cluster 4 in control and 1,25(OH)$_2$D$_3$ treated PBL.

Results are expressed as relative gene expression compared to a non-infected sample. Data shown is the mean ± SEM of n= 6. Difference between groups were assessed by multiple Wilcoxon paired T test with p-adjusted to 0.1 with the Benjamini-Hochberg method.

2.5. Discussion

The host immune response to mycobacteria is a complex process that requires the coordinate action of multiple cell types. The interactions between the host and bacilli will determine whether the bacilli are destroyed, or if they survive and replicate. In this sense, the immunomodulatory effects by vit D have gained remarkable attention in the last two decades [75, 87, 178]. Vit D enhances the antimicrobial response by macrophages, whereas it elicits an anti-inflammatory and tolerogenic response. This dual role of vit D helps the immune system to limit bacterial growth but also to dampen an excessive inflammatory response [77]. In cattle, *in vitro* and *in vivo* studies have shown the activation of the vit D pathway in response to mycobacterial antigens [82, 83, 86, 162]. $1,25(OH)_2D_3$ promotes the upregulation of *NOS2* and β-defensins through TLR signalling [80]. Although *NOS2* modulation by $1,25(OH)_2D_3$ has been associated with a reduction of *M. bovis* survival in bovine macrophages, evaluation of vit D microbicidal effects on heterogenous cell populations has not been previously examined in either humans or cattle. Therefore, it was aimed to examine the microbicidal and immunoregulatory role of $1,25(OH)_2D_3$ on bovine PBL using *M. bovis* BCG as a model.

Our results showed that $1,25(OH)_2D_3$ improved microbicidal killing activity by PBL against BCG after 24 h of infection. The analysis was done in parallel in both WB and PBL (after RBC lysis and serum removal) to consider the conversion of serum 25(OH)D to $1,25(OH)_2D_3$ by blood leukocytes. Blood is the natural environment of PBL which consist mainly of monocytes, neutrophils, lymphocytes, eosinophils, and basophils, and it is routinely used in the mycobacterial growth inhibition assay (MGIA) for TB vaccine research [179]. The main advantage of using blood is that it allows the assessment of cell function in a natural environment, it requires minimal sample processing and cell manipulation is reduced. Furthermore, the use of WB or PBL, instead of PBMC allows the assessment of vit D effects on neutrophils, a phagocyte that is essential during the early stage of infection [180]. Results showed that BCG killing was increased by 65 % in WB and 57% in PBL in $1,25(OH)_2D_3$ treated cells, confirming the previously demonstrated antimycobacterial effects of vit D in human macrophages [94, 151, 153, 181]. Although a previous study in bovine macrophages showed a reduction of *M. bovis* survival by $1,25(OH)_2D_3$, these assays were done with a single bovine donor, decreasing the power of the study. Our results demonstrate an important animal variability effect, with some animals showing no additive effect of $1,25(OH)_2D_3$ treatment and others seen more benefited with the addition of vit D. These results suggest a differential inter-individual vit D responsiveness, a concept described in humans vit D supplementation trials [182]. In this study individuals were distinguished as high, mid, and low responders to diverse molecular parameters such as changes in gene expression and chromatin accessibility, and

although resistance to infections was not assessed in this study, it is likely that individuals with high vit D response index will benefit more from vit D treatments [182].

It was also investigated whether increasing $1,25(OH)_2D_3$ concentrations improved the microbicidal activity in PBL. Previous research showed an upregulation of *NOS2* gene expression in a dose dependent manner, where 4 ng/ml of $1,25(OH)_2D_3$ was the highest concentration tested [80]. Our results show no additive effect in the microbicidal activity with concentrations higher than 40 ng/ml. This is similar to previous studies in human macrophages, where the reduction in *M. tuberculosis* growth plateaued at 40 ng/ml (10^{-7}M) [149]. However, our results represent the effect of $1,25(OH)_2D_3$ added at once during the infection process. It is therefore likely that greater bactericidal activity could be achieved if $1,25(OH)_2D_3$ was replenished at different time points after infection. Indeed, Crowle A.J. *et al*. [181] showed a decrease in mycobacterial growth when cells were treated 24 h before and after infection with 4 µg/ml of $1,25(OH)_2D_3$. Nonetheless, the $1,25(OH)_2D_3$ concentrations used in this study were higher than the physiological ranges (5-20 pg/ml) [124], and the local concentration of $1,25(OH)_2D_3$ at the site of infection is unknown. In addition, the circulating 25(OH)D levels determine the amount of $1,25(OH)_2D_3$ that can be generated locally, consequently dose determinations with 25(OH)D or *in vivo* studies with animals with divergent vit D status are more physiologically relevant to assess the optimal range of vit D for resistance to infections [124].

To determine the contribution of each phagocyte to the antimycobacterial response of $1,25(OH)_2D_3$, we compared the ability of cell depleted subsets to restrict BCG growth relative to complete PBL. Depletions for monocytes, neutrophils, $CD3^+$, $CD4^+$ and $CD8^+$ T cells were carried out. As shown in human studies, depletion of neutrophils from PBL affected the control of BCG leading to an enhancement of mycobacteria growth. This effect is consistent with results reported in human blood, where neutrophil depletion had the greatest impact on bacilli growth than the depletion of other cell types such as dendritic cells, monocytes and NK cells [183]. Moreover, the concentration of the antimicrobial peptides HNP-1, LL-37 and lipocain-2 was lower in neutrophil depleted blood, suggesting that these neutrophil peptides were necessary for bacterial destruction [180]. Addition of $1,25(OH)_2D_3$ to PBL and each cell depleted subset showed a similar biological effect, where vit D treatment improved BCG killing in all cell subsets. A statistical difference was only observed when monocytes were depleted ($CD14_{neg}$) suggesting an important effect of modulation by vit D on neutrophils. Likewise, in the granulocyte ($Gran_{neg}$) depleted subsets the effect of $1,25(OH)_2D_3$ approached our significance threshold (P= 0.0625). Having observed a similar effect in the $CD14_{neg}$ and $Gran_{neg}$ depleted subsets suggest that $1,25(OH)_2D_3$ modulates the bactericidal activity in

monocytes and neutrophils. Indeed, relevant other data indicates that $1,25(OH)_2D_3$ enhances the transcription of β-defensins in bovine neutrophils [81]. Defensins are stored in granules and are released upon neutrophil activation by exocytosis, becoming an antimicrobial supply for macrophages. Transference of neutrophil granules to macrophages enhances the antimicrobial capacity of macrophages [39]. Thus, a possible hypothesis of vit D microbicidal mechanism in PBL is that enhanced β-defensin production in neutrophils may promote vesicle trafficking from neutrophils to macrophages, resulting in a boost of the bactericidal activity. In fact, gene ontology analysis from transcriptome datasets on THP-1 cells stimulated with $1,25(OH)_2D_3$ for 24 h identified neutrophil activation and neutrophil degranulation as one of the main biological pathways activated by $1,25(OH)_2D_3$ treatment, suggesting that cross-talk between macrophages and neutrophils is a mechanism promoted by vit D [184].

Additionally, we assessed blood samples from cattle which positively react to the intradermal skin test (BTB+) as well as to age-sex matched animals negative to the tuberculin skin test (BTB-). Although there were no statistical differences in the 25(OH)D serum levels between BTB+ and BTB- animals , all BTB+ cattle were classified as vit D deficient. Research has shown that individuals with chronic infections like TB had low levels of circulating 25(OH)D [157]. But whether vit D deficiency is a cause or consequence of the infection is still controversial. In cattle, a similar effect has been observed in animals seropositive to *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Cows with positive serum MAP antibody ELISA had significantly lower serum 25(OH)D levels than seronegative animals [141]. Recently an analogous association was described in BTB+ cattle. Although, animals were not vit D deficient, BTB+ animals had lower serum 25(OH)D concentration than BTB- cattle from the same herd [143]. In contrast to the above study, the mean 25(OH)D concentration from our BTB- and BTB+ animals ranged between 22.18 and 33.13 ng/ml, which are borderline with the recommended level of 30 ng/ml. Whereas Lopez-Constantino, *et al.* [143] reported 25(OH)D levels between 46.86 and 95.96 ng/ml in BTB+ and BTB- cattle. This has led to speculation that the lower 25(OH)D levels in chronically infected animals is the result of higher use of 25(OH)D to maintain an adequate $1,25(OH)_2D_3$ concentration at the site of infection [141]. Differences in husbandry practices, diet and geographic location between studies can explain the dissimilarities observed, but it suggests that infected cattle might have a higher requirement of vit D and therefore benefit from additional supplementation in their diet.

Our results also showed higher phagocytosis and mycobacterial killing in BTB- animals in comparison to BTB+ cattle. This suggests that *M. bovis* infection dampens the immune response to mycobacteria or that BTB+ cattle had an inherent lower ability to restrain *M. bovis* growth and thereby become infected [185]. Although we did not observe differences in the

25(OHD) concentrations between groups, the vit D status was lower in BTB+ animals which opens the question on whether the described divergences in 25(OH)D levels between BTB+ and BTB- animals are associated with the differential microbicidal activity [143]. Furthermore, addition of $1,25(OH)_2D_3$ improved the phagocytosis and BCG killing in both group of animals, but a higher effect was observed in BTB+ animals with the lowest 25(OH)D concentrations (< 20 ng/ml). Although this requires confirmation with higher number of animals, our data suggests that vit D supplementation in BTB+ cattle could improve the containment of *M. bovis* and potentially reduce bacterial shedding and dissemination. This is consistent with previous studies on MAP infected cattle, where addition of $1,25(OH)_2D_3$ or 25(OH)D reduced MAP growth in macrophages from cows with clinical MAP by increasing IL-1β production as well as IFN-γ, and nitric oxide [163]. Collectively, results suggest that vit D has a putative role in controlling mycobacterial dissemination in infected cattle.

Bacterial killing is a multifactorial process that requires the coordinated effort of several cellular activities such as phagocytosis. Phagocytosis is the first step for bacterial elimination but also an essential stage for mycobacteria dissemination [165]. Therefore, the analysis of the effects of $1,25(OH)_2D_3$ on phagocytosis is another crucial aspect to assess in leukocyte function. Our results showed that neither BCG nor *E. coli* phagocytosis was modulated by $1,25(OH)_2D_3$. Furthermore, increasing $1,25(OH)_2D_3$ concentrations did not affect the phagocytic activity of the cells. Previous studies on phagocytosis modulation by $1,25(OH)_2D_3$ have reported contradictory results, showing either a decrease or an increase in phagocytosis by mononuclear phagocytes [186, 187]. Chandra G *et al.* [186], showed a rise in *M. tuberculosis* ingestion by $1,25(OH)_2D_3$ treated macrophages, but only on cells from patients with low phagocytic index (<10%). Whereas no effect was observed in macrophages with a phagocytic proportion higher than 20% [186]. In our study, the proportion of phagocytosis was above 20% for both BCG, and *E. coli*, thus the effect of $1,25(OH)_2D_3$ in animals with lower phagocytic activity is yet to be determined.

In the same way, there were no differences in the proportion of BCG-GFP$^+$ associated to monocytes (CD14$^+$) and granulocytes (G1$^+$) after $1,25(OH)_2D_3$ treatment. However, we observed that around 80 percent of BCG-GFP$^+$ events were in the double negative gate, which are not neutrophils or monocytes. This could suggest that the majority BCG-GFP$^+$ events were unspecific bonded to the lymphocyte population or that the method did not allow an accurate identification of both phagocytes after infection. Previously, it was shown that the identification of monocytes by flow cytometry was diminished due to CD14$^+$ downregulation after BCG infection. Moreover, changes in the size and granularity after infection preclude identification of neutrophils by the side scatter measurement [188]. Our results showed a similar pattern,

where the proportion of neutrophils and monocytes decreased after BCG infection in comparison to non-infected cells. $1,25(OH)_2D_3$ caused a higher decrease of $CD14^+$. Transcriptomic analysis on THP-1 cells has shown that *CD14* is upregulated by $1,25(OH)_2D_3$ and BCG [184, 189]. And in fact, our gene expression analysis showed an upregulation of *CD14* after BCG infection, therefore, it is probable that the decline in CD14+ is due to its internalization upon TLR stimulation, and a similar effect may have happened with the $G1^+$ marker [190]. Thus, a higher internalization of surface markers could be the result of increased phagocytosis in both cell types. Given the previous results, it is likely that $1,25(OH)_2D_3$ modulates phagocytosis in neutrophils and monocytes, as it enhances their microbicidal activities.

Recognition of bacteria by phagocytes leads to ROS and RNS production. These are very versatile molecules with diverse roles from direct antimicrobial activity to regulation of immune signalling and inflammasome activation [191]. Enzymes from the NADPH oxidase family, like *NOX2* are the main source for ROS generation, while inducible nitric oxide synthase (*NOS2*) is the enzyme responsible for RNS production. Although the relevance of ROS in mycobacteria killing is controversial, research in human monocytes have shown that *NOX2* interaction with TLR2 is essential for cathelicidin production and the subsequent antimicrobial activities [94]. In our study the increase of ROS by $1,25(OH)_2D_3$ was modest but significant, a possible explanation is that we missed the peak of maximum ROS production. Previous research showed that ROS production in response to $1,25(OH)_2D_3$ occurred within the first hour of *M. tuberculosis* challenge whereas at latter time points very low or undetectable levels were detected [93]. Our assay had a total duration of 2 h, thus it is possible that shorter time points are necessary to analyse the effects of ROS production by $1,25(OH)_2D_3$. Also, we did not observe differences between neutrophils and monocytes. This suggests that $1,25(OH)_2D_3$ promotes the production of ROS in other cells such as NK cells. NK cells are key effectors of the innate immune response capable of killing not only infected macrophages, but also capable of killing mycobacteria directly [192, 193]. This result reinforces the assertion that $1,25(OH)_2D_3$ influence the function of multiple cells and the collective result of this interactions can determine the killing or survival of *M. bovis*.

On the other hand, NO is known to be one of the major antimycobacterial molecules produced by the host. In cattle, modulation of NO by $1,25(OH)_2D_3$ has been associated to *M. bovis* killing [80, 83, 171]. However, in our study, NO determinations were below the range of detection after all experimental infections, and *NOS2* gene expression was not detected either. This might have been because of differences in the model of infection, the use of PBL in contrast to macrophage/monocyte models; or the time post-infection analyzed. For example,

by treating PBMC with $1,25(OH)_2D_3$ Waters *et al*. showed a slight increase in NO production after 24 h but a substantial difference after 48 and 72 h [82]. Likewise, data indicates that concentration of NO is higher in assays using monocyte or macrophages (μM range) than when using PBMC (nM range) [80, 82, 83]. This suggests that interactions between cells in PBMC or PBL influences NO dynamics and require the use of more sensitive methods of detection. Thus, we cannot rule out the effect of NO in the microbicidal activity by $1,25(OH)_2D_3$.

Finally, the gene expression pattern was very similar between control and $1,25(OH)_2D_3$ treatment. A possible explanation is that the strong response to BCG challenge surpassed any influence of the vit D signal, or that the differences in gene expression occurred at earlier timepoints. In fact, a previous study showed that the major transcriptomic changes in response to bovine tuberculin (PPDB) occurred after 3 h of stimulation [194]. This, along with the fact that vit D induces ROS at early time points, suggests that analysing transcriptomic alterations by $1,25(OH)_2D_3$ during mycobacterial infection necessitates consideration of earlier time points [93]. Nevertheless, results from the PCA points out that the gene expression of antimicrobial peptides explains some of the divergence observed between treatments, with a trend for higher β-defensin expression induced by $1,25(OH)_2D_3$. This is consistent with reports in cattle showing that $1,25(OH)_2D_3$ modulates the expression of β-defensins on LPS-stimulated monocytes. Remarkably, in this study the maximum β-defensin gene expression was observed after 12 h of stimulation, and it started to decreased after 24 h, which might explain the narrow differences observed in our study [81]. This suggests that vit D stimulates β-defensin production as an antimycobacterial mechanism. Several authors have proposed the use of β-defensins as therapeutic or prophylactic tools against bacterial diseases [170, 195]. And their effectiveness has been tested by diverse ways; for example, authors showed that a transgenic cattle expressing human β-defensin 3 had reduced susceptibility to *M. bovis* infection [196]. Therefore, vit D might be an option to boost the β-defensin production and promote disease resistance in cattle.

Our results also showed a cluster of genes with divergent pattern of expression, from which genes grouped in cluster 4 were upregulated by $1,25(OH)_2D_3$. Cluster 4 includes chemokines (*CCL2, CXCL1, CXCL10),* cytokines (*IL13, IL33* and *IL5*), antimicrobial peptides (*DEFB7, TAP*), the neutrophil elastase (*ELANE*) and the protein kinase R (*PKR*), genes with key functions during the innate immune response to infection. *PKR* is a host sensor of diverse stress signals including viral and non-viral pathogens, ROS, mechanical stress, calcium and cytokines [197]. Importantly, PKR is required for autophagic destruction of *M. tuberculosis,* and its overexpression promoted mycobacterial killing in THP-1 cells [198]. Therefore, it is tempting to speculate that $1,25(OH)_2D_3$ -mediated *PKR* activation stimulated autophagy as a

microbicidal mechanism in PBL. Furthermore, the pattern of expression of cluster 4 suggest that vit D promoted leukocyte recruitment and activation of innate cells. *IL33* was one of the genes with higher expression induced by $1,25(OH)_2D_3$. IL-33 is a member of the IL-1 family with anti- and pro- inflammatory functions. As pro-inflammatory cytokine, IL-33 plays a crucial role in innate immunity, and it is involved in host defence against bacteria by promoting production of IL-6 and IL-1 [199]. Overall, our results showed that $1,25(OH)_2D_3$ enhances HDP production, promotes leukocyte recruitment and favours activation of innate cells.

Finally, a limitation from our transcriptomic data is the low RNA yield obtained that could have influenced the results. The average yield was 60 ng of total RNA, whereas a systematic analysis showed that the RNA yield with 10^6 PBMC is around 0.3 to 2 µg [200]. According to the cell viability results, cells were viable after 24 h of infection, thus, it is highly probable that a mistake in the cell centrifugation occurred causing the loss of cells and low RNA yield. However, the Fluidigm system has higher sensitivity than conventional real time PCR, requiring cDNA quantities at picogram levels from less than 100 cells per assay [201]. Thus, our results are still biologically relevant, although confirmation with samples with better RNA quality is recommended.

To summarize, our findings indicates that $1,25(OH)_2D_3$ increases antimycobacterial activity in bovine PBL via the synergistic activity of monocytes and granulocytes, and by enhancing ROS production. Data also showed that $1,25(OH)_2D_3$ stimulation increased innate immune activation by boosting the expression of HDP, *PKR, IL33, IL13, CCL2, CXCL1 and CXCL10*, all of which are key genes for the host defence against bacteria. Additionally, it was shown that $1,25(OH)_2D_3$ supplementation on infected cattle may offer an opportunity to control mycobacterial growth and prevent disease progression.

3. CHAPTER 3. Microbicidal activity and immunoregulatory effect of $1,25(OH)_2D_3$ on neutrophils.

3.1. Introduction

Neutrophils are among the earliest cells to migrate to the infection site and deploy early defence mechanism against mycobacteria [180]. They circulate in blood as quiescent cells ready to work when and where they are required; as a result, neutrophil activation is tightly controlled. Upon a sterile or infectious stimulus, neutrophils migrate through the capillary walls and enter the connective tissue, where they become activated. Neutrophils can be activated when they encounter microbial products and also by stimulation with chemokines and cytokines mainly, IL-8, IL-17, IL-1β, TNF-α, G-CSF and GM-CSF [39, 202]. Neutrophil activation leads to expression of powerful antimicrobial activities like the release of granule components, secretion of chemokines and cytokines, phagocytosis, ROS production and NET formation [203]. At the same time, neutrophils shape the response to infection by modifying the function of other immune cells like macrophages via release of granule proteins but as well via synthesis of cytokines and chemokines, and direct cell contact [204].

Neutrophil interactions with mycobacteria are mediated by opsonization and direct recognition of the microbe occurs through pattern recognition receptors such as TLR2 and TLR4, but as well by complement receptors (CR) and C-type lectins receptors (CLRs) [205]. The mycobacterial killing ability of neutrophils can be affected by diverse host and bacterial factors which, when combined could decide the outcome of a mycobacterial challenge. Thus, whereas effective killing controls the early bacterial growth, a killing failure will lead to bacterial dissemination to other sites and ultimately disease progression. Indeed, data from a whole blood model of infection showed that neutrophils contributed significantly to resistance to *M. tuberculosis* infection, activity that was linked to the production of neutrophil antimicrobial peptides [180]. Whereas an inverse relationship was observed in patients with active tuberculosis, neutrophils provided a more permissive site for bacterial replication and were the most common cell type infected with mycobacteria [206]. Due to similar findings, some authors consider neutrophils as a “Trojan horse” favouring the growth of the bacilli and allowing their spread to distal sites [202].

Mycobacterial killing by neutrophils is a controversial topic with some studies reporting killing and others not. Lowe *et al*. [202] reported that comparisons between studies are difficult due differences in the mycobacterial strain used, as well as in the multiplicity of infection, and time point after infection when killing was assessed. However, although the experimental variability between studies can potentially explain the divergent results reported, research has shown that mycobacterial killing by neutrophils occurs at a differential rate than killing of other Gram-positive or Gram-negative bacteria [207]. By assessing the phagocytosis and killing rate

of *M. smegmatis* in human neutrophils, authors showed that the half-life of *M. smegmatis* within neutrophils was 30 min, which was 5 times longer than reported for *S. aureus* ($t_{1/2}$= 6 min) and 15 times longer than *E. coli* ($t_{1/2}$= 2 min) [207]. This finding confirms that although neutrophils can kill mycobacteria, the killing rate is slow.

Moreover, other studies on the mycobacterial killing ability of neutrophils have reported a large donor-specific variability. For instance, neutrophils stimulated with TNF-α showed a range of *M. tuberculosis* killing from 50 to 95% and when neutrophils were not activated with TNF-α, two of six donors did not kill bacteria at all [208]. In other study, when neutrophils were stimulated with the *M. tuberculosis* 19-kDa lipoprotein responses were consistently observed in cells from a subset of donors, whereas neutrophils from other donors did not respond despite showing responsiveness to LPS [209]. The latter results can contribute somewhat to the variation observed between different studies but also opens the question as to why neutrophils are not unequivocally bactericidal.

Oxidative and non-oxidative pathways have been proposed to explain how neutrophils kill or limit the growth of mycobacteria. Oxidative burst is activated after bacilli ingestion, which leads to the activation of NADPH oxidase and production of ROS. Although few studies support the role of ROS as direct killing mechanism, ROS contributes to neutrophil migration and magnifies neutrophil responses, such as NET formation and release of pro-inflammatory cytokines [210]. Non-oxidative bactericidal mechanisms are mainly assigned to antimicrobial peptide production. Cattle have multiple cathelicidins and β-defensins stored in large or tertiary granules [211]. Both, cathelicidin and β-defensins have demonstrated bactericidal activity against mycobacteria. Recombinant bovine neutrophil β-defensin 4 (BNBD4) was shown to exhibit direct bactericidal activity against *M. smegmatis* and *M. bovis*. Moreover, addition of BNBD4 to THP-1 macrophages reduced intracellular survival of both mycobacteria strains in comparison to non-treated cells [212]. Human cathelicidin has been shown to restrict the growth of *M. tuberculosis* in neutrophils [180]. Relevantly, research has shown that $1,25(OH)_2D_3$ promotes cathelicidin expression in human neutrophils [74].

Neutrophils express the VDR at levels comparable to monocytes, and IFN-γ stimulates the activation of *CYP27B1,* the enzyme responsible for converting the pro-form 25(OH)D to the active form $1,25(OH)_2D_3$ [100, 213]. As a result, neutrophils can respond to the intracrine and paracrine actions of $1,25(OH)_2D_3$. Moreover, due to its abundance, neutrophils appear to be a significant source of $1,25(OH)_2D_3$ in infections sites. Evidence indicates that $1,25(OH)_2D_3$ stimulates the oxidative burst activity and β-defensin production in neutrophils [81]. Furthermore, Subramanian *et al.* [100] showed that $1,25(OH)_2D_3$ treatment enhanced pneumococcal killing by human neutrophils. Bactericidal killing was associated with

cathelicidin production but also to α-defensin (HNP1-3) expression. And, when neutrophils were supplemented with 25(OH)D, a similar rise in bactericidal killing was observed in IFN-γ activated cells. Interestingly, authors showed that $1,25(OH)_2D_3$ downregulated the production of proinflammatory cytokines IL-6, IL-8, and IL-12, and upregulated the anti-inflammatory cytokine IL-4 in neutrophils infected with pneumococci [100]. Recently, it was shown that $1,25(OH)_2D_3$ induces the formation of NETs. However, the NETs induced by $1,25(OH)_2D_3$ did not show the same morphology and shape as the NETs induced by the positive control PMA. Although vit D upregulated the transcription of *ELANE*, and *PAD-4* genes whose products are associated with the antibacterial innate responses mediated by NETs [214]. Therefore, these results suggest that $1,25(OH)_2D_3$ can boost the antimicrobial activity of neutrophils while limiting an excessive proinflammatory response.

Significant number of studies suggest the presence of distinct neutrophil subsets during homeostatic and pathologic conditions. These subsets display divergent phenotype and function that can affect their role during the activation of the immune response [215]. Recently, a new subset of regulatory neutrophils was described in cattle [216]. A detailed characterization by flow cytometry allowed the identification of two subsets of neutrophils, a main population that did not express MHC-II on their surface (MHC-II^{neg}) and a small population of neutrophils expressing MHC-II (MHC-II^{pos}). The transcriptional profile of both neutrophil subsets, in a steady-state was opposing, with MHC-II^{pos} neutrophils showing lower gene expression in comparison to the MHC-II^{neg} subset. Furthermore, although both subsets of neutrophils displayed similar bacterial phagocytosis and killing activity, only the MHC-II^{pos} population suppressed T cell proliferation under contact-dependent mechanisms. Therefore, authors defined the MHC-II^{neg} subset as classical neutrophils and, the MHC-II^{pos} subset as regulatory neutrophils [216].

For all the above, neutrophils are now considered a global player in the immune response, not only providing protection against infections, but also guiding and supporting the innate and adaptive immune response via interaction with other cell types. Thus, a better understanding of the complexity of the immune responses to mycobacteria, requires investigation into the factors that affect neutrophil function.

3.1.1. Specific aims

Results from chapter 2 showed that neutrophils are an important mediator in BCG growth control and that $1,25(OH)_2D_3$ can modulate its bactericidal activity. Furthermore, the discovery of distinct neutrophil subsets in cattle, as well as their regulatory features raises the question of how vit D modulates these innate cell subsets. Therefore, this chapter was carried out in

collaboration with the research group from INRAE who characterized the regulatory and classical neutrophil subsets described above.

The main aim for chapter 3 was to assess the immunoregulatory role and bactericidal activity of 1,25$(OH)_2D_3$ on neutrophils in response to *M. bovis* challenge. For this study, the response to two mycobacteria strains was assessed, BCG and *M. bovis* AF2122/97.

The specific objectives were:

1. To investigate the microbicidal activity on classical neutrophils stimulated with 1,25$(OH)_2D_3$ in response to BCG and *M. bovis* challenge.
2. To investigate the oxidative stress response on classical neutrophils stimulated with 1,25$(OH)_2D_3$ in response to BCG and *M. bovis* challenge.
3. To investigate the immunoregulatory effects of 1,25$(OH)_2D_3$ on classical and regulatory neutrophils in response to BCG and *M. bovis* challenge.

3.2. Materials and Methods

All the experimental procedures for chapter 3 were performed at INRAE, Centre Val de Loire, France. In collaboration with Dr. Aude Remot and the IBIR team (Bacterial Infections and Ruminant Immunity). Experimental assays with *M. bovis* AF2122/97 were performed at the BSL3 laboratory of Dr. Remot.

3.2.1. Animal ethics committee approval

Animal protocol for blood sampling was approved by the “Val de Loire” Ethics Committee for Animal Experimentation and were registered to the French National Committee for Animal Experimentation under N°2016040410531486. Experimental protocol complied with French law (Décret: 2001–464 29/05/01) and European directive 2010/63/UE for the care and use of laboratory animals and to be carried out under authorization for experimentation on laboratory animals number E 37-175-2 (UE-PAO, INRAE Centre Val de Loire). Therefore, an exemption for full ethical review by Animal Research Ethics Committee from UCD was granted.

3.2.2. Blood sampling

Experiments were carried out in six 6-month-old female Holstein-Friesian calves, housed indoors. Blood samples were collected via the jugular vein into vacutainer tubes (Becton Dickinson). 10 ml serum separator tube was utilized for serum collection. For neutrophil cell sorting four 9-ml EDTA tubes were collected, and 1 ml sample of blood was used for haematology analysis using the ADVIA 2120 haematology system. Serum samples were stored frozen at -20°C for 25(OH)D analysis.

3.2.3. Neutrophil sorting

The preparation of neutrophils was done accordingly to the method developed by the IBIR team [216, 217]. Briefly, blood samples were centrifuged at 1000 *x* g for 10 min at 20°C, then the plasma layer, buffy coat and one third of the blood were removed. The remaining blood was treated with 10 ml of red blood cell lysing buffer (Sigma Aldrich) for 5 min incubation at room temperature. Cells were washed twice in D-PBS with 2mM EDTA. Next neutrophils were labelled with primary anti-G1 (clone CH138A, Monoclonal Antibody Center, Washington State University), and anti-MHC-II Ab (clone CAT82A, Monoclonal Antibody Center, Washington State University) at 1:500 dilution, followed by secondary goat-anti mouse Alexa Fluor 647 and Alexa Fluor 488 conjugated Ab (Invitrogen) at 1:200 dilution. For purification of neutrophil subsets, cell concentrations were adjusted to 10^7 cells/ml and sorted with a MoFlo AstriosEQ Flow cytometer (Beckman Coulter, Inc., Brea,CA, USA) placed under a class II biological

safety cabinet. For the flow cytometry sorting strategy, granulocytes were defined by their size and granularity in the R1 gate, and dead cells were excluded by the Fixable Viability Dye eFluor780 (Thermo Fisher Scientific) staining in gate R2. In R1, not R2 gate, neutrophils highly expressing the G1 marker ($G1^{high}$) were selected (R3 gate), eosinophils were identified as $G1^{low\ or\ int}$ (R4). Then, within the $G1^{high}$ neutrophils in R3 gate, the MHC-II^{neg} and MHC-II^{pos} subsets were sorted. The MHC-II^{neg} neutrophils represented the main population (93%), whereas the MHC-II^{pos} were a minor population (1-1.5%).

3.2.4. Infection of neutrophils with *M. bovis* AF2122/97 and BCG

Sorted MHC-II^{neg} neutrophils were resuspended in RPMI medium supplemented with 2mM of L-Glutamine, 10 mM HEPES and 1% BSA (Sigma Aldrich) and were (or not) incubated for 2 h with 4 ng/ml of $1,25(OH)_2D_3$. After this time, neutrophils were plated in duplicated in two 96 well plate at 1 x 10^5 cells per well. Cells in one plate were challenged with BCG Pasteur at MOI 5, whereas the cells in the second plate were infected with *M. bovis* AF2122 at MOI 1. Cells were incubated at 37 ºC with 5% CO_2 overnight. Next day, the plates were centrifuged at 300 *x* g for 5 min at 20 ºC. Supernatant was removed and stored at -20 ºC for further analysis. The cells were lysed with 200 µL of H_2O with 0.05% tween 80 per well, the cell pellet was mixed to facilitate cell lysis and incubated at room temperature for 15 min. The lysate was plated on 7H11 petri dishes to quantify the mycobacterial CFUs.

3.2.5. Measurement of oxidative stress response in neutrophils

Sorted MHC-II^{neg} neutrophils were treated (or not) with $1,25(OH)_2D_3$ and infected (or not) with BCG and *M. bovis* AF2122 as described in the previous section; however, cells were plated in a 96 black non-treated-sterile microwell plate. After overnight incubation, plates were centrifuged at 300 *x* g for 5 min at 20 ºC and supernatant was removed. Then, 50 µl of the following fluorochromes diluted in PBS (all from Sigma Aldrich) were added: DHE (1/300), H_2DCFDA (1/1000), and DAF_2DA (1/750). Concentration of fluorochromes were: DHE (25mg in 1mL of DMSO), H_2DCFDA (50mg in 500uL of DMSO), and DAF_2DA (1mg in 500uL of DMSO). The plate was placed immediately in a plate reader with the following wavelength settings (excitation/emission): DHE: 485/610; H_2DCFDA 485/530; and DAF_2DA 485/530. Results are reported as ratio between infected vs non-infected cells.

3.2.6. Quantification of cytokine and chemokine expression

Sorted MHC-II^{neg} and MHC-II^{pos} neutrophils were treated (or not) with $1,25(OH)_2D_3$ and infected (or not) with BCG and *M. bovis* AF2122 as described in the previous section. After

overnight incubation, supernatant was removed and stored at -20°C until analysis. Multiplex analysis of 15 cytokines and chemokines (CCL2, CCL3, CCL4, IFN-γ, IL-10, IL-17a, IL-1ra, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IP-10 and TNF-α) was done with MILLIPLEX® Bovine cytokine/chemokine panel 1 (SPRCUS706, Merck) at the Unité Virologie et Immunité Moléculaire, INRAE Domaine de Vilvert. Data were acquired using a MagPix instrument (Luminex) and analysed with Bio-Plex Manager software (Bio-Rad).

3.2.7. RNA extraction

RNA was extracted from 1, 2 and 3 x 10^6 neutrophils using the RNeasy Mini Kit (Qiagen), Total RNA purification kit (Norgen Biotek) according to manufacturer instructions. RNA extraction was also performed with the standard phenol-chloroform method. Briefly, 0.8 ml of trizol was added, cell pellets were mixed to allow cell lysis and were incubated at RT for 5 min. Then, 0.2 ml of chloroform was added to cells containing trizol and shaken vigorously. The solution was then centrifuged at 12,000 x g for 15 min at 4°C. The aqueous layer containing RNA was transferred to a clean microfuge tube. Next, 0.5 ml of isopropanol was added, and the RNA pellet was incubated 90 min at -20°C. Tubes, were centrifuged at 12,000 x g for 5 min at 4°C. The RNA pellet was washed trice with ethanol 75%. Finally, the RNA was air dried for 10 min and then dissolved in 30µl of RNase-free water. RNA quantity was measured using the NanoDrop system and RNA integrity was assessed using the RNA 6000 Nano Kit (Agilent Technologies) on the Bioanalyser according to manufacturer instructions.

3.2.8. Statistical analysis

GraphPad Prism software version 8 was used for data presentation and statistical analysis. Data is presented as mean ± SEM, unless otherwise is stated. Statistical test and number of samples for each experiment is indicated in the figure legends. Evidence of statistical significance was considered at $P \leq 0.05$ and tendency was considered at $P \leq 0.10$.

3.3. Results

3.3.1. Microbicidal activity against mycobacteria induced by $1,25(OH)_2D_3$.

The recently described classical and regulatory subsets of neutrophils were purified by fluorescence activated cell sorting (FACS) using the G1 and MHC-II antibodies [216, 217]. The main population of neutrophils, defined as classical neutrophils was identified as $G1^{hi}$ + $MHC\text{-}II^{neg}$. The regulatory subset represents a minor proportion of $G1^{hi}$ neutrophils and are classified as $G1^{hi}$ + $MHC\text{-}II^{pos}$ neutrophils. Figure 3-1 shows the gating strategy used for their purification.

After purification, the analysis of the microbicidal activity by $1,25(OH)_2D_3$ was performed on classical ($MHC\text{-}II^{neg}$) neutrophils. There were insufficient number of regulatory ($MHC\text{-}II^{pos}$) neutrophils for analysis of the microbicidal activity. Analysis of the main effect of infection, showed that non-treated neutrophils were better at controlling BCG growth than *M. bovis.* When $1,25(OH)_2D_3$ was added, the microbicidal activity against both strains of mycobacteria improved similarly (Table 3-1). Results shown a significant drop in the number of viable bacteria and a corresponding increase in the percentage of bacterial killing to BCG and *M. bovis* by $1,25(OH)_2D_3$. The mean percentage of BCG killing was 33.67% in control cells in comparison to 57.87% in $1,25(OH)_2D_3$ treated neutrophils. Whereas the proportion of *M. bovis* killing went from 0.58% to 40.20% (Figure 3-2).

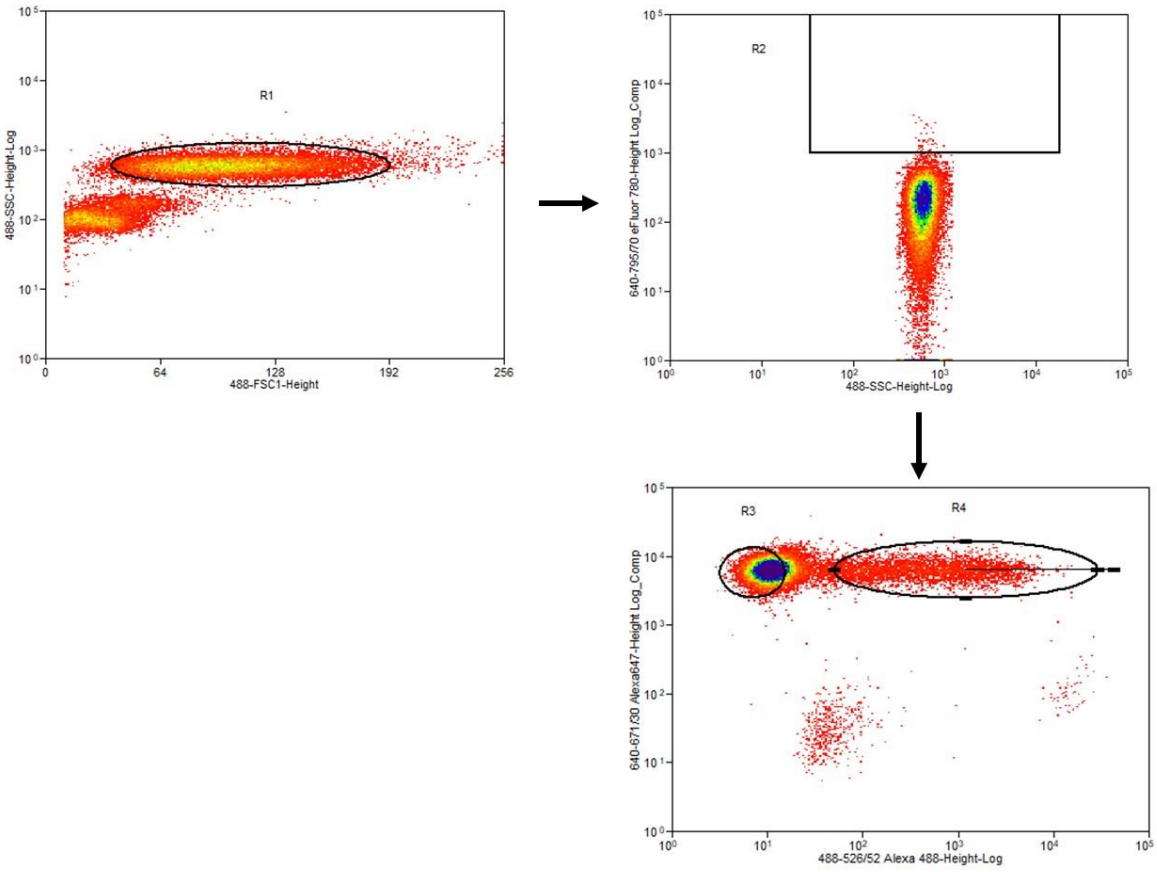


Figure 3-1. Gating strategy for purification of MCH-II^{neg} and MCH-II^{pos} neutrophil subsets.

Neutrophils were defined by their size and granularity in the R1 gate, and dead cells were excluded by the Fixable Viability Dye eFluor780 staining in gate R2. In R1, not R2 gate, neutrophils highly expressing the G1 (G1^{high}) marker labelled with Alexa Fluor 647 were selected. Then, within the G1^{high} population the MHC-II^{neg} (R3) and MHC-II^{pos} (R4) subsets were sorted.

Table 3-1. Mean percentage of BCG and *M. bovis* killing by neutrophils.				
		BCG	***M. bovis***	**P value***
Control	Mean	33.67	-0.5804	0.0435
	SD	50.24	33.19	
1,25(OH)$_2$D$_3$	Mean	57.83	40.2	0.3027
	SD	34	30.39	

*Main single effect was analysed by 2-way ANOVA with Bonferroni's correction.

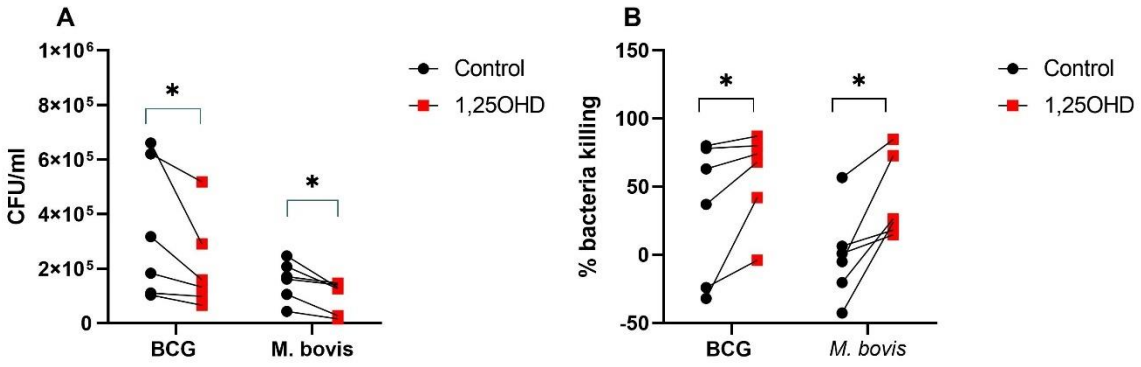


Figure 3-2. Microbicidal activity induced by 1,25(OH)$_2$D$_3$ on neutrophils.

MHC-II^{neg} neutrophils were stimulated or not with 4 ng/ml of 1,25(OH)$_2$D$_3$ and infected with BCG (MOI 5) or *M. bovis* (MOI 1) for 24 h. Then, cells were washed and lysed to determine the number of viable bacilli following serial dilution on 7H11 agar plates. (A) CFU/ml and (B) percentage of bacteria killing. Bacterial killing was calculated by the ratio of bacteria used for challenge between those that were killed after 24 h. Wilcoxon test was used to assess differences between control and 1,25(OH)$_2$D$_3$ treatments by each mycobacterial strain (n=6 animals) *P < 0.05 was considered statistically significant.

3.3.2. Effect of $1,25(OH)_2D_3$ on the oxidative stress response in neutrophils

In order to examine the effect of $1,25(OH)_2D_3$ on the oxidative stress response in neutrophils, the analysis of the superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) production was performed after 24 h of infection on classical neutrophils (MHC-II^{neg}). Analysis of the main effects showed that H_2O_2 is the major oxygen specie produced after BCG and *M. bovis* infection. The second oxygen specie produced after *M. bovis* infection was O_2^- and a similar trend (P= 0.0811) was observed in BCG infected cells. No differences were observed for NO production (Table 3-2). Likewise, there were no significant differences in the oxidative stress response by $1,25(OH)_2D_3$ to any mycobacteria strain (Figure 3-3).

Table 3-2. Main effect of BCG and *M. bovis* infection on O_2^-, H_2O_2, and NO production on neutrophils.

		Control		$1,25(OH)_2D_3$		
		Mean	SD	Mean	SD	P value*
BCG	O_2^-	1.316	0.2262	1.085	0.1873	0.0811
	H_2O_2	1.754	0.5639	1.334	0.2633	0.0001
	NO	1.209	0.2401	1.167	0.1062	0.1055
M. bovis	O_2^-	1.417	0.2828	1.239	0.1137	0.0268
	H_2O_2	1.775	0.6884	1.684	0.3586	0.0001
	NO	1.222	0.03422	1.224	0.112	0.1457

Values are represented as fold change between infected and non-infected samples.
*Main single effects analysed by 2-way ANOVA with Dunnett's correction to non-infected sample.

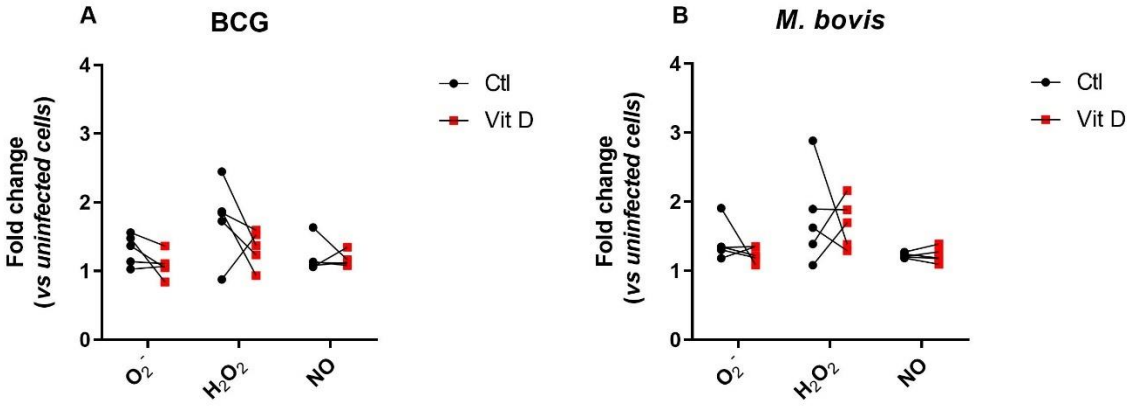


Figure 3-3. Production of O_2^-, H_2O_2, and NO on neutrophils infected with BCG and *M. bovis*.

(A) Production of each oxygen specie by BCG and (B) *M. bovis* after 24 h of infection. Results are shown as fold change between infected and non-infected sample. Comparison between control and $1,25(OH)_2D_3$ treatments were done by Wilcoxon test in each oxygen species of n=5.

3.3.3. Effect of $1,25(OH)_2D_3$ on cytokines and chemokines production by neutrophils in response to mycobacterial infection

The supernatants from previous experiments were analysed by a Multiplex ELISA. From the 15 cytokines and chemokines analysed only CCL3, CCL4, IL-1ra and TNF-α were detected. However, the analysis was done only on 4 animals and in some cases the proteins were only detected in 2-3 animals, which precluded any statistical analysis. However, it is interesting to note that IL1-ra concentration was higher in *M. bovis* infected cells in comparison to BCG infected neutrophils in both neutrophil subsets (Figure 3-4).

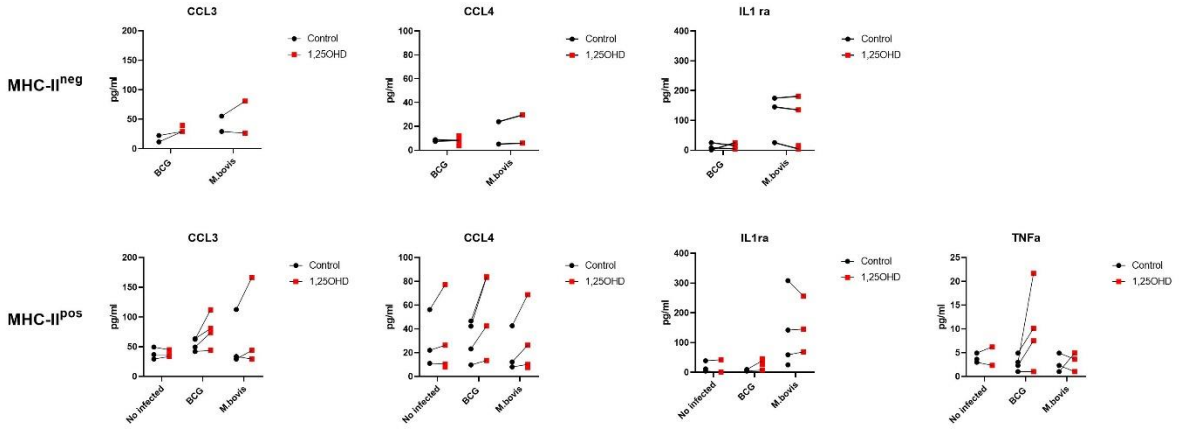


Figure 3-4. Cytokine and chemokine levels on neutrophils infected with BCG and *M. bovis*.

The protein levels were measure in supernatants from MHC-II^{neg} and MHC-II^{pos} neutrophils by Multiplex ELISA after 24 h of infection. n=2/4.

3.3.4. Comparison of methods for RNA extraction from neutrophils

RNA extraction from three cell concentrations were assessed using three methods. The methods evaluated were the RNeasy Mini Kit from Qiagen, Total RNA purification kit from Norgen Biotek, and the standard phenol-chloroform method. RNA extraction was done with 1, 2 and 3 x 10^6 neutrophils. Figure 3-5 shows that the Qiagen kit was the best in terms of RNA quality and yield, giving on average 0.15 µg (5 ng/µl) of total RNA per 10^6 cells.

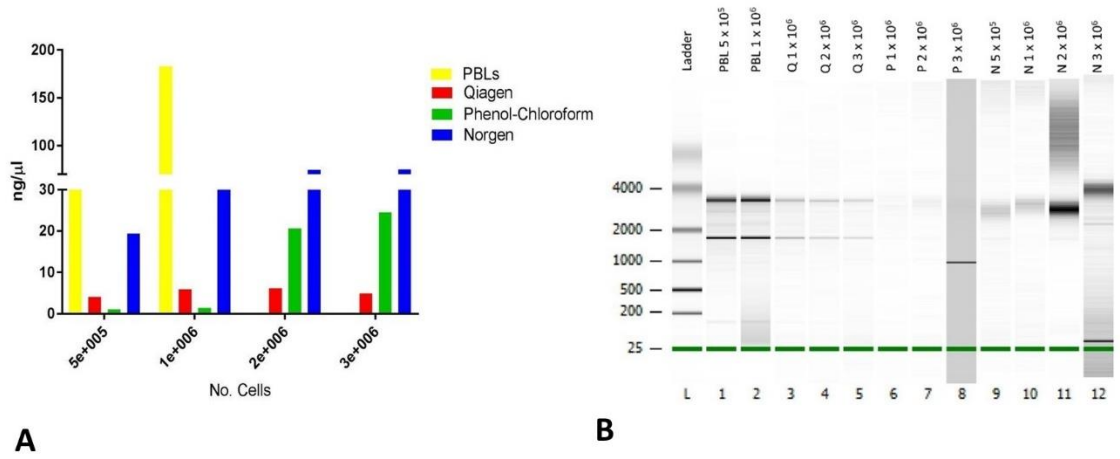


Figure 3-5. Comparison of methods for RNA extraction from neutrophils.

A) RNA concentration was determined with the Nanodrop system, B) RNA integrity was analysed using the Agilent 2100 Bioanalyzer system. 5x10^5 and 1x10^6PBL was used as positive control using the RNeasy mini kit.

3.4. Discussion

Neutrophils are key immune cells during the host response to mycobacterial infection. They are the major cell type recruited during the early stages of granuloma formation and are present in granulomas of all stages [218, 219]. Neutrophil activation can lead to an effective killing of *M. bovis* by oxidative killing, degranulation, and NET formation. However, during an excessive inflammatory response this bactericidal response can lead to tissue damage and bacterial proliferation [219]. In fact, a study on naturally infected cattle found a positive correlation between neutrophil numbers and bacterial load, and it was suggested that neutrophil recruitment was detrimental for control of *M. bovis* growth [220]. Vit D can regulate the excessive inflammation while boosting the innate immune response to mycobacteria. Effects of vit D have been largely studied on macrophages and monocytes and information about its effects on other myeloid cells like neutrophils is limited. Thus, the aim of this chapter was to assess the bactericidal activity and immunoregulatory role of $1,25(OH)_2D_3$ on neutrophils in response to *M. bovis* challenge.

Our results show that neutrophils were better at killing BCG than *M. bovis*. Previous research showed that bovine neutrophils were unable to kill *M. bovis* but were capable of reducing the bacterial growth of *M. smegmatis* by 75% within the first 9 h of infection [221]. A similar effect was observed in studies on human neutrophils, where killing was more effective against attenuated *M. tuberculosis* strains [202]. Treatment with $1,25(OH)_2D_3$ improved the bactericidal killing against BCG and *M. bovis* by 24% and 40%, respectively. To our knowledge this is the first study to assess the microbicidal effects of $1,25(OH)_2D_3$ against mycobacteria in neutrophils, in both human and cattle. Data also showed that H_2O_2 was the predominant ROS specie produced in response to mycobacterial infection, which is more stable and can diffuse membranes contributing to cell signalling [191]. Results from this work opens the way to further investigation into the potential mechanisms and contribution of neutrophil vit D-mediated effects. Because of their abundance in blood and global contribution to the innate and adaptive immune system, further studies are necessary to evaluate the role of vit D on neutrophil function and disease susceptibility.

Whereas the microbicidal activity by $1,25(OH)_2D_3$ has only been investigated in human neutrophils [100], previous data have shown that vit D stimulates the oxidative burst activity and β-defensin production in bovine neutrophils. A single intervention with 300 µg of $1,25(OH)_2D_3$ in dairy cows increased the percentage of neutrophils in blood with higher oxidative burst activity and phagocytosis [222]. Likewise, cows fed with a prepartum diet containing 3 mg of 25(OH)D showed a reduced incidence of retained placenta and metritis, which resulted in a reduction of mortality. The authors associated these effects with increased

oxidative burst activity of neutrophils from cows receiving a 25(OH)D supplemented diet [138]. Similarly, intramammary administration of 25(OH)D modulated the percentage of neutrophils and somatic cells in milk and increased the expression of *NOS2*, and β-defensin genes in monocytes and neutrophils. Animals treated with 25(OH)D showed a reduced severity of the inflammatory response in the mammary gland [140, 172]. Therefore, these studies suggest that vit D modulates neutrophil function and limits the inflammatory response.

Emerging research have shed light into the vit D-mediated effects on neutrophils, in addition to enhancing HDP production, $1,25(OH)_2D_3$ upregulates the expression of TLR2 and NOD2 [100]. A rise in IL-8 expression was also observed in LPS-stimulated neutrophils, although no effect on the phagocytosis of *E. coli* was observed [101]. Furthermore, one study found that $1,25(OH)_2D_3$ decreased IL-8, MIP-1β and VEGF production in adult neutrophils, but not in neonatal cells. Authors hypothesised that the effect was associated to the lower expression of *VDR* and *CYP27B1* in neonatal neutrophils in comparison to adults [223]. Results from this study suggest that responsiveness to vit D in neonatal cells is diminished which might contribute to the increased disease susceptibility in young animals. Therefore, the study of vit D immunomodulation in neonatal and young animals requires further attention.

The immunoregulatory effects of $1,25(OH)_2D_3$ were then assessed on supernatants using the Multiplex system. However, a low signal was observed, and a limited number of chemokines and cytokines were detected. This can be explained by the low number of cells used per treatment since the expression of diverse cytokines by neutrophils has been previously validated [224, 225]. CCL3 and CCL4 are chemokines produced by neutrophils upon mycobacteria stimulation, and are required for chemotaxis of neutrophils, monocytes and NK cells [226]. Whereas neutrophils produce large quantities of the interleukin-1 receptor antagonist (IL-1ra) [227]. Concentration of the IL-1ra was higher after *M. bovis* challenge in comparison to BCG. IL-1ra has been described as an important mediator of *M. tuberculosis* susceptibility driven by type-1 IFNs [228]. Thus, our results point out a possible mechanism for the reduced killing of *M. bovis* observed. Although no differences were observed by $1,25(OH)_2D_3$ treatment, research has shown that $1,25(OH)_2D_3$ and 25(OH)D modulate the IL-1 pathway in human macrophages and epithelial cells without affecting the NLRP3 inflammasome, suggesting that vit D heighten the innate immune responses, without causing an excessive inflammation [95].

One of the main goals for this chapter was to analyse the transcriptomic differences between MHC-II^{neg} and MHC-II^{pos} neutrophils after $1,25(OH)_2D_3$ treatment. Thus, the few MHC-II^{pos} neutrophils obtained were used for RNA extractions after BCG and *M. bovis* infections. Unfortunately, the RNA yield was too low (below the nanodrop range) and it is

suspected that RNA quality was not good either because of the null signal found by real time PCR and with the Fluidigm system. In previous research, the French team was able to obtain RNA from both neutrophils subsets, and to perform real time PCR analysis using the Fluidigm system [216]. However, the RNA extraction for that study was performed on neutrophils obtained immediately after purification, which could have prevented RNA degradation.

In comparison with macrophages, neutrophils have 10 to 20 times less RNA per cell and the high concentration of RNases make RNA extraction from neutrophils difficult [224]. Transcriptomic studies on human neutrophils have shown that the analysis of 10^6 to 10^7 neutrophils is necessary to obtain at least 0.1 µg of total RNA [229]. In our study RNA extraction was done with $2x10^5$ cells which explains the low RNA yield. By comparing three RNA extraction methods we determined that a minimum of 10^6 cells is required to obtain on average 0.15 µg (5 ng/µl) of total RNA using the Qiagen kit. However, compared to humans, bovine neutrophils are not the most prevalent leukocyte in blood as they represent less than 50% of total leukocytes in circulation [211]. Thus, a greater proportion of blood is required to achieve the proportion of cells recommended for all the treatments evaluated. Furthermore, the low proportion of MHC-II^{pos} neutrophils in circulation prevented obtaining those cell numbers. Therefore, a standardization of the RNA extraction method is required to improve the yield and quality of RNA obtained from less than 10^6 neutrophils.

The results for this chapter were severely impacted by the Covid-19 pandemic. Laboratory closures and restrictions to international travel delayed the start of experimental work and precluded the optimization of the experiments for better assessment of the effects of vit D on neutrophil function. However, preliminary data from this study suggest that investigation into the vit D modulation of neutrophil function represents an opportunity to enhance the host immune response to mycobacteria.

4. CHAPTER 4. *In vivo* supplementation of vitamin D_3 (cholecalciferol) in Spring-born dairy calves

4.1. Introduction

The main source of vitamin D comes via skin synthesis after sunlight exposition. Photochemical conversion of dermal 7-dehydrocholesterol by ultraviolet radiation generates pre-vit D_3 in the skin which is then converted to vit D_3 by a thermal reaction [130]. However, in current livestock production systems, a large proportion of cattle are housed with consequently little or no exposure of sunlight. Cattle that are not confined indoors are subject to seasonal variation in sunlight exposure [131]. Thus, cattle usually rely on supplementation of their diets to fulfil their vit D requirements. Dietary sources of vit D can provide vit D_2 (ergocalciferol) or vit D_3 (cholecalciferol). Vit D_2 is obtained from the roughage used for cattle feeding (e.g., hay and silage) and Vit D_3 is supplied as a synthetic supplement in the feed. Although both molecules, are metabolized in the same way, Vit D_2 is less physiologically effective and less efficient at ensuring sufficient blood concentrations of vit D in cattle [230]. Vit D is biologically inert and must be activated by two sequential hydroxylations. The first hydroxylation occurs in the liver to form 25-hydroxyvitamin D [25(OH)D], which is the main circulating form used to determine vit D status; and this is converted to the active metabolite 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$] in the kidney but also in many peripheral tissues and immune cells [111].

The vit D requirements for cattle are listed in the National Research Council (NRC) publications, however these recommendations are based on data which are more than 50 years old, originally set to prevent vit D deficiency symptoms like rickets or milk fever rather than to improve overall health and wellbeing [231]. The definition of vit D requirements in cattle is a difficult task because the 25(OH)D status is affected by multiple non-exclusive factors including season, UVB exposure, husbandry practices, productive stage, age, sex, and breed. For example, oral Vit D_3 supplementation of *Bos taurus* and *Bos indicus* steers during the last 8 days of a 123-days feed program, showed a differential rise on plasma 25(OH)D levels, with higher 25(OH)D levels observed in *Bos taurus* steers in comparison to *Bos indicus* animals [232]. Likewise, Blakely *et al.* showed sex differences in the 25(OH)D levels from Holstein calves. Supplementation with vit A, D and E (ADE) for 28 days resulted in higher serum 25(OH)D concentration in heifers than in supplemented bulls, indicating a potential interaction with steroid hormone levels or body mass [128, 233]. Differences in the vit D status implies that animals require diets with vit D content accordingly to their age, productive stage, sex and breed.

The optimal concentration of circulating 25(OH)D is still a matter of debate, particularly in non-human animals. In healthy cows a range from 20 to 100 ng/ml of serum 25(OH)D levels has been observed, and although this levels might be enough to support bone and mineral

homeostasis, the optimal circulating 25(OH)D concentration for immune has not been determined [87]. Epidemiological studies in humans suggest that 25(OH)D concentrations above 30 ng/ml is recommended to prevent respiratory infections [116]. Given the similarities in the vit D metabolism between humans and cattle, the same threshold has been tentatively adopted for cattle [87]. Information of the vitamin D status of calves is limited. Data is available for US systems [174, 234] but different genetics and systems of feeding management means these values are not directly transferrable to calves under less intensive production systems and in different geographical locations. For example, in Ireland, milk replacer usually contains 6,000 IU/Kg of Vit D_3, and in previous studies, our research group observed a high prevalence of low 25(OH)D serum concentration in calves during the first 5 months of life; moreover, the seasonal vit D profile was negatively correlated with the expression of the pro-inflammatory cytokine interleukin-8 (IL-8) suggesting immune relevant consequences [125].

Adequate vit D is now viewed as vital for optimal health with research showing important associations between 25(OH)D blood concentration and immune function. Studies have shown important immunomodulatory and antimicrobial effects of vit D on innate cells including macrophages and neutrophils [75, 235]. Furthermore, in humans low 25(OH)D circulating levels have been associated with higher risk for upper respiratory infections including influenza and tuberculosis [157]. And in cattle, a similar relationship has been hypothesised [143, 236]. It is therefore probable that sub-optimal vit D status could have negative consequences for optimal calf immune system development and consequently disease susceptibility.

4.1.1. Specific aims

Previous results from chapters 2 and 3 showed that $1,25(OH)_2D_3$, the active form of vit D enhances the microbicidal activities of phagocytes. Bactericidal effects were not modified by increased $1,25(OH)_2D_3$ concentrations but required the cooperative activity of monocytes and neutrophils. Synthesis of $1,25(OH)_2D_3$ by the immune cells is influenced by the availability of 25(OH)D on circulation. Thus, to investigate how variation in the circulating concentration of 25(OH)D affected the immune response in dairy calves, an *in vivo* vit D_3 supplementation study was carried out.

Vit D supplementation was conducted under the current European supplementation guidelines with Vit D_3, the only authorised source of supplemental vit D for cattle. The EU guidelines set the maximum permitted levels at 10,000 IU/Kg for milk replacer and 4,000 IU/Kg for feed [135]. The main aim of this chapter was to develop a model to drive divergent vit D status and to investigate how variations in the circulating concentrations of 25(OH)D affected the immune response of dairy calves.

The specific objectives were:

1. To develop a model to drive divergent vit D status under the current European supplementation guidelines. Comparison between animals kept indoors or outdoors was performed to model the influence of sunlight exposure.
2. To investigate how the variation in the circulating concentration of 25(OH)D affected cellular immunity over time.
3. To investigate how variation in the circulating concentration of 25(OH)D affected the production of IL-8 and ROS.

4.2. Materials and Methods

4.2.1. Animal ethics committee approval

All experimental procedures were approved by the Teagasc Ethics Committee (TAEC237-2019) and were conducted under the experimental license (AE19132/P105) from the Health Products Regulatory Authority in accordance with the cruelty to Animals Act (Ireland 1876) and the European Community Directive 2010/63/EU.

4.2.2. Animals and housing

The study was conducted at Teagasc, Grange in Ireland (53°N) between February and October 2020. Forty-eight Holstein-Friesian bull calves born between February and March were enrolled in the experiment. Calves were removed from the dam and fed 6 L of colostrum within 4 h of birth and were transported to the research farm within 24-48 h. Transfer of adequate passive immunity was assessed in serum by optical refractometry, calves had a mean Brix value of 9 % [237]. All calves were group housed and fed in buckets with milk replacer (MR). Calves were fed with 3 L of MR from 0 to 14 days, 6 L from 15 to 60 days of age, and then 3 L from 60 to 70 days. Ad libitum access to starter pellets and water was provided via bucket. Weaning occurred at 70 days of age on average, then a commercial pellet was offered once a day. Outdoors (Ctl-Out, VitD-Out) groups were moved to outside areas after weaning and were rotationally grazed from May to October. Indoors (Ctl-In, VitD-In) groups were kept in confinement during all the duration of the trial and were offered hay and silage *ad libitum*.

4.2.3. Experimental design and treatments

The experiment was a randomized complete block design with a two-by-two factorial arrangement of treatments, with calves randomly assigned to one of 4 treatments. Treatments were arranged as a factorial with two sunlight access (indoors=In, or outdoors=Out) and two vitamin D_3 diets (Ctl and VitD). Therefore, the four treatments were: Ctl-In: Indoors and 6,000 IU/Kg in MR + 2,000 IU/Kg of vit D_3 in ration; VitD-In: Indoors and 10,000 IU/Kg in MR + 4,000 IU/Kg of vit D_3 in ration; Ctl-Out: Outdoors and 6,000 IU/Kg in MR + 2,000 IU/Kg of vit D_3 in ration; VitD-Out: Outdoors and 10,000 IU/Kg in MR + 4,000 IU/Kg of vit D_3 in ration. A single time injection of 50,000 IU of vit D_3 was administered subcutaneously to all calves at birth, except Ctl-In, which received a vehicle injection with ethanol.

A commercial milk replacer with 6,000 IU/Kg and a commercial pellet with 2,000 IU/Kg were used for the Ctl diets. For the vit D_3 diets the MR and pellet were supplemented with vit D_3 to achieve 10,000 IU/Kg and 4,000 IU/Kg, respectively. The vit D_3 was prepared from dry powder concentrate (Rovimix D3 500, DSM Nutritional Products) containing 500,000 IU per gram of vit D_3 by adding 0.5 g of the concentrate to distilled water. The supplements were prepared fresh weekly and stored at 4°C. Supplements were added once daily to the MR, and top dressed on the pellets after weaning. The ration was provided once a day at a rate of 1 to 4 kg per day from 70 to 220 days of average age. The feed was offered to calves at approximately 0800 h to ensure consumption of the concentrate.

4.2.4. Haematology, serum 25-hydroxyvitamin D and IL-8 ELISA

Blood samples were collected via the jugular vein into vacutainer tubes (Becton Dickinson). A sample of 6 ml blood collected in an EDTA tube was used for haematology analysis using the ADVIA 2120 haematology system. Another 10 ml serum separator tube was utilized for serum collection. Tubes were centrifuged at 2,500 x *g* for 15 min for serum separation within 1 h of sample collection. Serum samples were transferred into microtubes and stored frozen at -20°C. Serum samples were taken at the beginning of the trial (T1), at 15 days (T2) and every 30 days relative to the start of the experiment (T3 to T9). Haematology analysis was carried out at the same time points except for T2.

The serum samples were analysed for concentrations of total 25(OH)D using an ELISA (Human 25-OH Vitamin D ELISA, Eagle Biosciences, Nashua, NH) and was carried out as per the manufacturer's instructions using bovine standards, prepared as previously described [174]. The bovine IL-8 ELISA used to measure IL-8 concentration was carried out as previously described by Cronin et al [238].

4.2.5. Determination of reactive oxygen metabolites

The reactive oxygen metabolites (ROM) were quantified with the standardized d-ROM test (Diacron International, Grosseto, Italy). This test determines hydroperoxides (breakdown products of lipids and other organic substrates generated by the oxidative attack of ROS), through their reaction with the chromogen N,N-diethylparaphenylenediamine. The results are expressed in arbitrary 'Carratelli Units' (CarrU), where 1 CarrU is equivalent to the oxidising power of 0.08 mg H_2O_2/dL.

4.2.6. Global solar radiation

Information of the monthly solar radiation from Teagasc, Grange's meteorological station (located on the research farm) was obtained from The Irish Meteorological Service, available at www.met.ie/climate/available-data/monthly-data.

4.2.7. Statistical analysis

Of the 48 calves enrolled in the trial, 1 calf from group Ctl-In died at 5 months of age due to a pneumonia. Additionally, calves with missing values were removed from the analysis. Therefore, 3 animals were removed for the 25(OH)D analysis (n=44) and 1 for the haematology data (n=46).

All statistical analysis were performed in RStudio (version 4.0.3). After assessing that the ANOVA assumptions were met a within-between subject design was used for analysing the effects of treatment, sunlight, and time and its interactions on the 25(OH)D serum levels. Individual calves were included as a random effect and weigh difference as a covariate. Differences between treatments were tested by pairwise comparison with Bonferroni correction. This analysis was done using *tidyverse, rstatix,* and *psych,* packages.

A multiple factor analysis (MFA) was performed on the cell counts for neutrophils (Neu), eosinophils (Eos), basophils (Bas), monocytes (Mon), and lymphocytes (Lym) collected from the haematology analysis. The haematology profile was done over 8 time points through the curse of the trial. Therefore, the cell counts ($x10^3$µl/ml) for each cell type from all the time points was gather in one group. Thus, the group "Neu" was formed with the neutrophil cell counts from the 8 sampling points, and the same procedure was done with each cell. Then, MFA was done with 5 groups of cells (Neu, Mon, Bas, Eos and Lym), whereas treatment was used as categorical supplementary variable. The MFA was done with 46 individuals and 41 variables using the *FactoMineR* and *Factoextra* packages [239].

The collective differences between groups in each cell type was further analysed by multivariate analysis of variance (MANOVA) using Pillai's test. Data was assessed for normality, multicollinearity, and homogeneity of variance. A statistically significant difference on the combined dependent variable was followed by Welch's ANOVA test, pairwise comparisons were done by Games-Howel test. Eosinophil data was analysed by Kruskal-Wallis test followed by Dunn's Test. All statistical tests were interpreted using a 5% level of significance. This analysis was done using *tidyverse, rstatix,* and *psych* packages.

4.3. Results

4.3.1. Analysis of the circulating 25(OH)D levels after Vit D_3 supplementation

In this study we analysed the effects of vit D_3 supplementation on the neonatal vit D deficiency and its impact on the bovine immune response in early life. The average circulating concentrations of 25(OH)D across all calves at birth (T1) was 7.64 ±3.21 ng/ml, indicating vit D deficiency. Despite a sub-dermal injection of vit D_3 (50,000 IU) at birth and inclusion of supplementary vit D_3 in milk replacer (6,000 IU/Kg in Ctl groups and 10,000 IU/Kg in VitD groups), a change in serum 25(OH)D concentration at 15 days post-vit D_3 injection (T2) was not detected (Figure 4-1).

Insufficiency continued in all treatment groups until calves were on average 3 months of age (T4) (Figure 4-1). Average values for T2 were 19.86 ± 7.84 ng/ml, T3 were 24.61 ± 8.22 ng/ml and for T4 were 27.55± 7.74ng/ml. During this period, calves were on an exclusively milk diet and no statistically significant differences due to vit D_3 supplementation was evident pre-weaning (Table 4-1).

After weaning (T5, Figure 4-1), one control group (Ctl-Out) of calves (maintained on the industry standard concentrate containing 2,000 IU/Kg of vit D_3) and one treatment (VitD-Out) group (received vit D_3 supplementation to 4,000 IU/Kg of vit D_3) were moved outdoors to grass. While groups Ctl-In and VitD-In were kept indoors. Circulating concentrations of 25(OH)D increased over time in VitD-In, Ctl-Out and VitD-Out groups. Whereas concentrations of 25(OH)D in the Ctl-In group remained the lowest after weaning (T5-T8). The slight increase across time points in Ctl-In is likely due to incidental sun exposure indoors (Figure 4-1). The increase in circulating 25(OH)D in Ctl-Out and VitD-Out groups coincided with the highest solar radiation recorded for the year during May and started to decline after peak sun exposure at T8 (Sept) [Figure 4-1].

The main effect of treatment and sunlight was statistically significant in all groups at T6, T7 and T9 (Table 4-1). A significant interaction between treatment and sunlight was observed at T6, although no significant interaction was detected at T7 or T9 (Table 4-1). At T6 no statistical difference in the 25(OH)D concentration was observed between Ctl-Out and VitD-Out groups. In contrast, a significant divergence in 25(OH)D levels was observed between Ctl-In and Vit-In groups (Figure 4-2). The highest 25(OH)D concentration was observed at T7 in VitD-Out group, with an average 25(OH)D concentration of 60.86 ± 7.32 ng/ml in comparison with 51.36 ± 7.92 ng/ml in Ctl-Out group (Table 4-1). As the increase was lower

in VitD-In than in the outdoor supplemented group (VitD-Out), results show evidence for an additive effect between dietary supplementation and sun exposure (Figure 4-2).

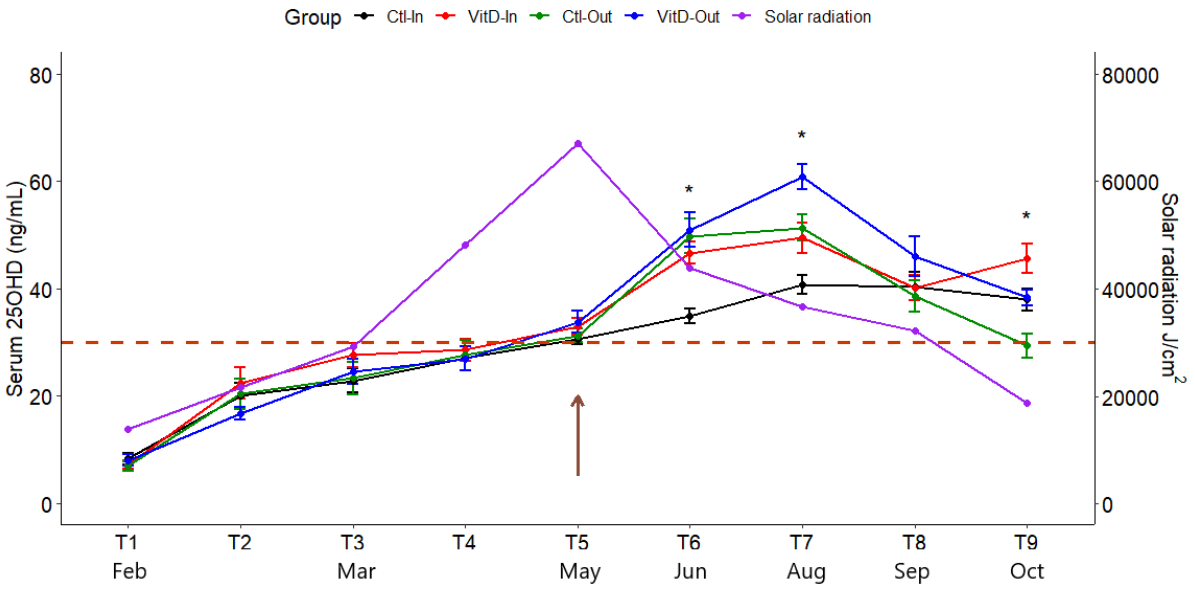


Figure 4-1. Vitamin D3 supplementation and sunlight exposure increases 25(OH)D circulating levels in calves.

Ctl-In (n = 11) and VitD-In (n = 12) groups were kept indoors and fed with 6000 IU/kg in MR + 2000 IU/kg of Vit D3 in ration; or 10,000 IU/kg in MR + 4000 IU/kg of Vit D_3 in ration, respectively. Whereas Ctl-Out (n = 11) and VitD-Out (n = 10) groups were move outdoors after weaning and fed with 6000 IU/kg in MR + 2000 IU/kg of Vit D_3 in ration; or 10,000 IU/kg in MR + 4000 IU/kg of Vit D_3 in ration, respectively. All calves received a single time injection of 50,000 IU of Vit D3 at T1, except Ctl-In, which received a vehicle injection with ethanol. Serum samples were taken at the beginning of the trial (T1), after 15 days (T2) and at 30, 70, 90, 130, 160, 200 and 230 days after T1 (T3-T9). Data represent mean and standard error of serum 25(OH)D profile in calves for each time point within each group. The main effect of sunlight and treatment was significant ($P < 0.05$) at T6, T7 and T9. Red dotted line shows the recommended 25(OH)D serum level of 30 ng/ml. Brown arrow shows the time where Ctl-Out and VitD-Out groups were moved outside. Right Y-axis represent the solar radiation during the time points where 25(OH)D concentrations were analysed.

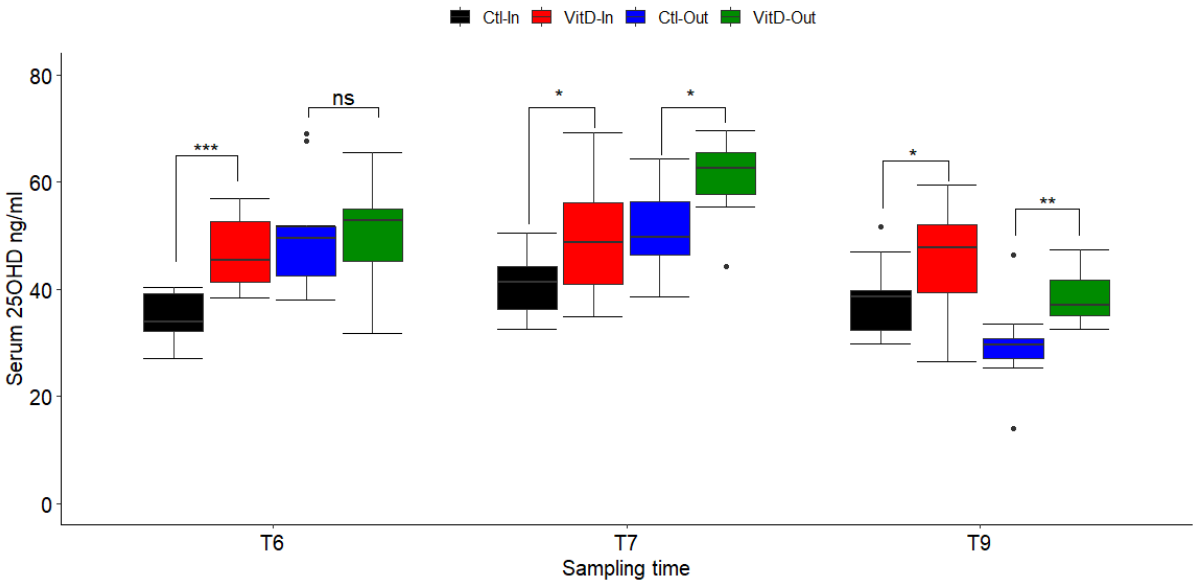


Figure 4-2. Maximum 25(OH)D levels are achieved by dietary VitD3 supplementation and sunlight exposure.

Boxplots of serum 25(OH)D levels in calves within each group at time T6, T7 and T9. Differences between groups were calculated by a 2x2 factorial design as described in the material and methods section. Dots represent outlier values. * P < 0.05, ** P < 0.01, *** P < 0.001, ns= not significant. Ctl-In (n=11), VitD-In (n=12), Ctl-Out (n=11) and VitD-Out (n=10).

Table 4-1. Serum 25(OH)D concentrations (ng/ml) within each group for all time points assessed.

	Ctl-In (n=11)		VitD-In (n=12)		Ctl-Out (n=11)		VitD-Out (n=10)				*p*-value[3]		
Time	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Overall mean***	**Overall SD***	**Treatment[1]**	**Sunlight[1]**	**Treatment x Sunlight[2]**
T1	8.34	3.619	7.24	2.769	6.94	3.054	8.04	3.382	7.64	3.206	0.908	0.6541	0.217
T2	20.00	8.027	22.34	10.222	20.42	9.406	16.68	3.699	19.86	7.839	0.533	0.578	0.094
T3	22.90	7.654	27.62	7.72	23.37	10.059	24.55	7.441	24.61	8.219	0.32	0.758	0.382
T4	27.03	7.584	28.58	6.846	27.62	9.279	26.96	7.253	27.55	7.741	0.948	0.896	0.404
T5	30.70	3.421	32.88	5.465	31.21	4.194	33.83	6.589	32.16	4.917	0.195	0.436	0.82
T6	34.96	4.412	46.71	6.769	49.81	10.554	50.99	9.921	NA	NA	0.021	0.000411	0.033
T7	40.79	5.765	49.46	9.945	51.36	7.921	60.86	7.318	NA	NA	0.00075	0.0000891	0.879
T8	40.42	8.798	40.13	8.217	38.66	9.592	46.04	11.654	NA	NA	0.198	0.597	0.159
T9	37.98	6.788	45.71	9.517	29.38	7.577	38.33	4.821	NA	NA	0.001	0.002	0.86

* When no statistical difference was observed between treatments, an overall mean and SD was obtained for all groups, NA = not applicable; n = number of animals

[1]Main effects of Treatment and Sunlight

[2]Interaction between Treatment and Sunlight

[3]Interaction among treatment, sunlight and time was significant ($p < 0.05$)

4.3.2. Analysis of the effects of divergent circulating 25(OH)D levels on cellular immunity

To analyze the potential relationship between circulating levels of 25(OH)D and hematological profile, a multiple factorial analysis (MFA) was performed using values for predominant cell profiles of neutrophils, eosinophils, basophils, lymphocytes, and monocytes (Neu, Eos, Bas, Lym, and Mon) on all calves across all time points (Figure 4-3). MFA is a generalization of the PCA analysis, and it allows to reduce the dimensionality of several sets of variables that had been measured on the same set of individuals at different occasions [239]. Thus, the cell counts for Neu, Eos, Bas, Lym, and Mon for each time point assessed were gathered in one group. Then, the contribution of each variable within each group was normalized and a generalized PCA was computed. The result is the representation of the observations as points such as the distances in the map best reflect the similarities or differences between the observations [240]. Therefore, in the individual factor map (Figure 3) groups are projected on the side of variables for which they have a high value, and opposite those variables for which they have low value. The correlation circle Figure 4-4 shows the contribution of each variable to the principal component, thus; Lym, Bas and Mon are well represented in dimension 1 (Dim1), while Neo and Eos are represented in dimension 2 (Dim2).

Overall, the cell profile of Ctl-Out and VitD-Out groups was similar; the barycentre (overall mean) from these groups are situated close together compared to indoors groups. Whereas Ctl-In and VitD-In groups show a distance relative to the outdoor groups, indicating a change in overall circulating cell composition across time; therefore, its within inertia values for Dim2 were higher (Table 4-2). The most divergent group were Ctl-In, which separate away from the other groups along dimension 2, which explains 11.22% of the phenotypic variation (Figure 4-3). Finally, the MFA shows that the cells making the most significant contribution to the divergence between Ctl-In and VitD-In are Neu, Eos, and Bas indicated by the greater distance between the partial points for each group (Figure 4-3).

Multivariate statistical analysis of covariance within each cell type (Figure 4-6) shows that the principal significant differences in Neu, Eos, and Bas was at T6 (p <0.05). This time point (T6) was one of the times where we observed a high divergence in the blood 25(OH)D concentration between Ctl-In and VitD-In groups (Figure 4-2); the analysis of the cell profile at T6 shows that calves from Ctl-In group had higher number of Neu, Eos, and Bas in comparison with animals from VitD-In (p <0.05 (Figure 4-5). Notably, these cell counts were increased relative to reference values for cattle which is indicative of neutrophilia. These results suggest that low 25(OH)D is associated with increased numbers of circulating leukocytes.

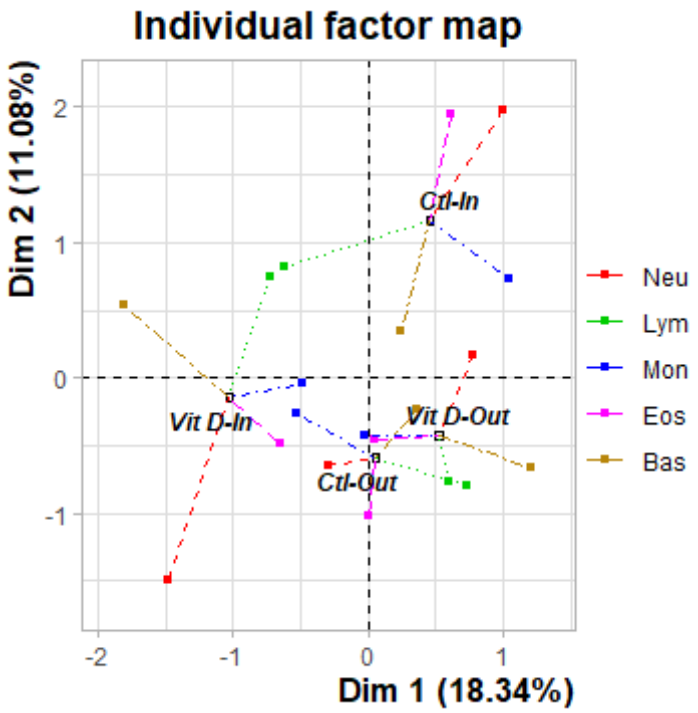


Figure 4-3. Changes in overall cell composition across time in all groups.

The haematology profile for all time points was analysed by MFA as described in material and methods section. The individual factor map shows each group represented at the barycentre of their individuals. Individuals are projected on the side of the group of variables for which they have a high value, and opposite those variables for which they have low value. Lym, Bas and Mon are represented in Dim1, whereas Neu, Eos are represented in Dim2. Ctl-In (n=11), VitD-In (n=12), Ctl-Out (n=12) and VitD-Out (n=11).

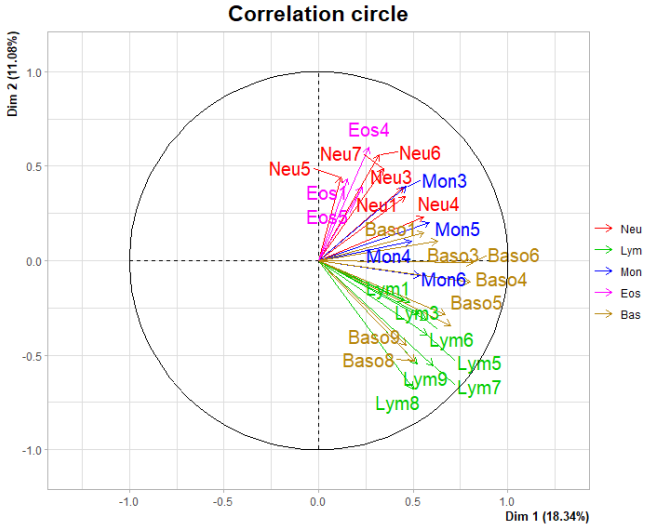


Figure 4-4. Correlation circle from MFA analysis of the cell profile observed between groups.

The haematology profile for all time points was analysed by MFA as described in material and methods section. The correlation circle shows the relationship between variables and how they are represented based in the quality of its representation (cos2=0.2). Lym, Bas and Mon are well represented in Dim1, whereas Neu, Eos are represented in Dim2. Ctl-In (n=11), VitD-In (n=12), Ctl-Out (n=12) and VitD-Out (n=11).

Table 4-2. Multiple Factor Analysis results for within individual inertia of the groups[1]

	Dim.1	Dim.2
Vit D-Out	2.295542309	1.380713741
Ctl-Out	2.417169815	1.014944399
Vit D-In	2.832678364	7.508729728
Ctl-In	3.813751515	5.328866525

[1]Within individual inertia measures the heterogeneity of the groups along each dimension. The groups with similar values are closely to each other for that dimension

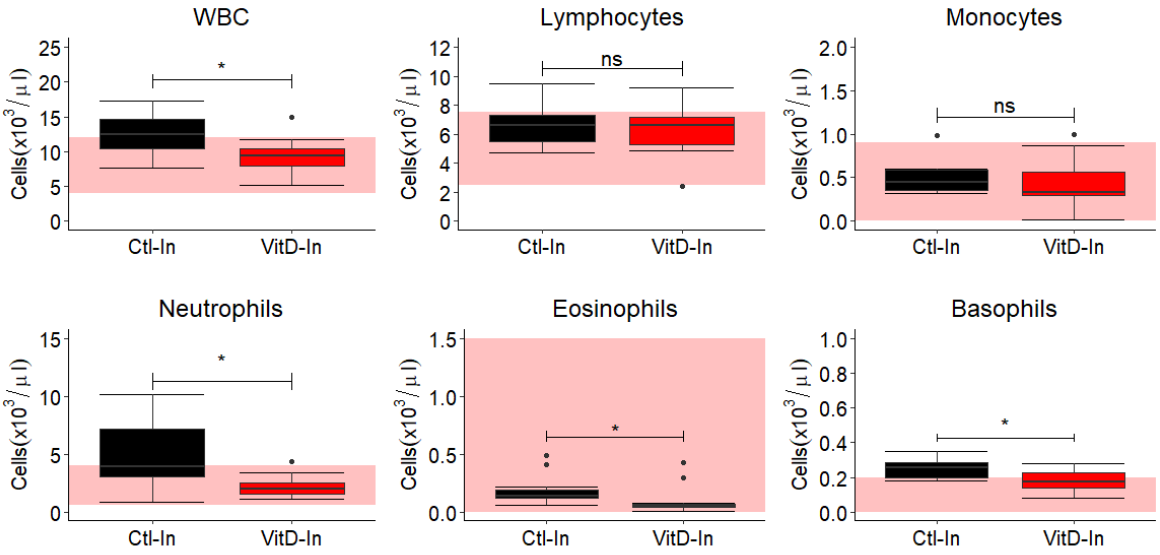


Figure 4-5. Collective differences in the cell profile between indoor groups at T6.

Data shows the boxplot of WBC (white blood cells), neutrophils, eosinophils, basophils, lymphocytes, and monocytes of Ctl-In (n=11) and VitD-In (n=12) groups at T6. Collective differences between groups were analysed by MANOVA as described in material and methods section. Pink area shows references values according to Merck Veterinary Manual. Dots represent outlier values. * $P < 0.05$, ns= not significant.

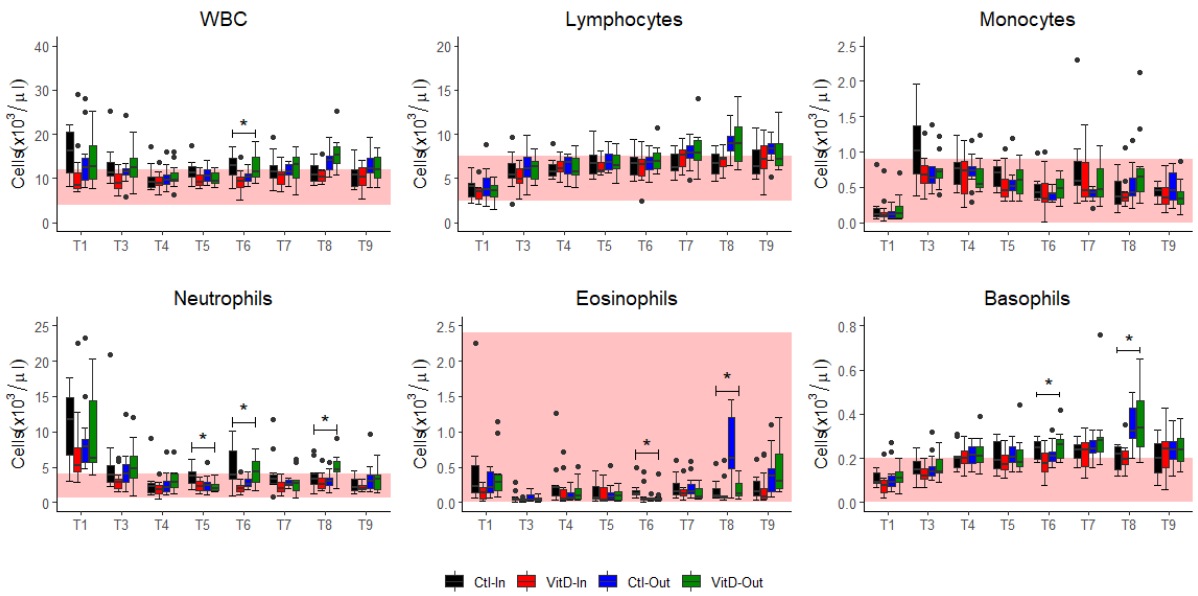


Figure 4-6. Collective differences in the cell profile within each group at all time points. Data shows the boxplot of WBC, neutrophils, eosinophils, basophils, lymphocytes, and monocytes within each group at all time points. Collective differences between groups were analysed by MANOVA as described in material and methods section. Pink area shows references values Merck Veterinary Manual. Dots represent outlier values. * $P < 0.05$. Ctl-In (n=11), VitD-In (n=12), Ctl-Out (n=11) and VitD-Out (n=10).

4.3.3. Analysis of the effects of divergent circulating 25(OH)D levels on IL-8 expression and ROS production.

To investigate potential mechanisms by which elevated circulating 25(OH)D may suppress numbers of circulating immune leukocytes, IL-8 expression and ROS production in serum were evaluated in the most 25(OH)D divergent groups (Ctl-In and VitD-In). The serum concentrations were evaluated at time point 4 (T4) where the vit D status was similar between calves, and at time point 6 (T6) the most differing time point (Figure 4-1). We did not observe a statistically significant difference in IL-8 expression levels in calves from Ctl-In and VitD-In groups at any of the time points evaluated (Figure 4-7 A). Similarly, we did not observe a statistical difference in the ROS production between groups at any time point, although there is a trend of higher ROS levels in VitD-In animals (Figure 4-7 B).

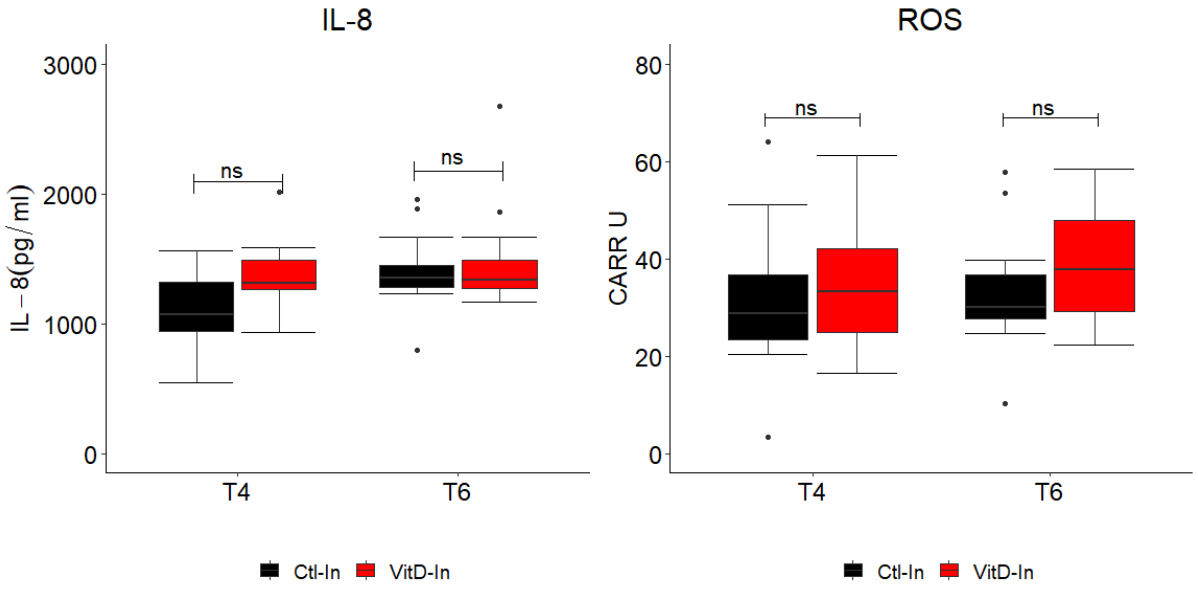


Figure 4-7. Serum concentration of IL-8 and ROS in calves from indoor groups.
Data shows the boxplot of serum concentration of IL-8 and ROS at T4 and T6 in calves from Ctl-In (n=11) and VitD-In (n=12) groups. A) IL-8 protein levels were measure by ELISA. B) ROS were measured in serum with the d-ROM test. Data was analysed by ANOVA test. Dots represent outlier values. ns= not significant.

4.4. Discussion

Neonatal mortality and morbidity remains a significant issue for the global dairy industry [241]. Nutritional strategies to support optimal immune system development may hold significant promise to reduce these losses, particularly in artificially reared dairy calves. Our work has recently identified that Spring-born dairy calves have a low level of circulating vit D in serum [125]. However, the consequences of such concentrations for the development of the immune system have not been clearly defined. The current definition of vit D requirement in cattle diets is based on concentrations required to maintain the bone and mineral homeostasis [231]; however, the optimal 25(OH)D concentration for immunity has not been conclusively determined. Given the similarities in vit D metabolism between humans and cattle, vit D requirements for humans are adopted in cattle; thus, vitamin D deficiency is defined as circulating 25(OH)D concentrations below 20 ng/ml and insufficiency as levels between 20-29 ng/ml. Therefore, a 25(OH)D blood level above 30 ng/ml is deemed to be the target to improve the health and resistance to infections [87, 116]. Furthermore, as levels of vit D supplementation is tightly regulated within the EU, it is critical to understand if circulating levels of vit D in calves under current dairy production systems are sufficient.

In this study, calves at birth were vit D deficient, with 25(OH)D levels below 10 ng/ml. These blood levels did not reach the optimal threshold until animals were on average 3 months of age, thereby exposing them to a potentially prolonged period of disease susceptibility. The 25(OH)D levels reported here are significantly lower than previous reports in US dairy studies, where calves had 25(OH)D levels within the range of 15 to 40 ng/ml at birth [63, 242]. Our results show a failure of supplemental injection of 50,000 IU of vit D_3 to impact circulating levels of 25(OH)D, despite sub-dermal injections having previously been shown to effectively improve vit D concentrations in calves at birth [63, 242]. This is likely explained by the divergent concentrations used as previous work used an initial injection of 150,000 IU of vit D_3 followed by 5,000 IU daily, which resulted in an increase of 25(OH)D levels from 30 ng/ml at birth to near 100 ng/ml after 14 days in calves raised indoors [242]. In addition, in our study, supplemented milk replacer, at maximal levels did not significantly influence circulating 25(OH)D levels. Nevertheless, the neonatal vit D concentrations in our calves were significantly lower than in this previous study, suggesting that a concentration of more than 150,000 IU of vit D_3 is likely required for Irish calves to reach a target of 30 ng/ml 25(OH)D serum levels at birth.

At weaning the average 25(OH)D concentration was close to the optimal level of the desired 30 ng/ml threshold in all calf groups. However, it could be considered low according to the concentrations observed on US dairy calves raised indoors and fed with a similar vit D_3

rate (6,600 IU/Kg of DM) with a mean serum 25(OH)D concentration near 60 ng/ml at 6 weeks of age [63]. Thus, calves under more intensive dairy systems have considerably elevated vit D concentrations than pasture-based calves born in Spring. One factor that could contribute to the divergent circulating 25(OH)D concentrations in calves between studies is the concentration of vit D in the dam. Weiss *et al*., showed a positive correlation with the serum 25(OH)D concentrations in calves at birth with concentrations of 25(OH)D in the serum of their dams [122]. This therefore raises important questions regarding the vitamin D status of pasture-based dairy cows, and indeed suggests a potential strategy whereby dietary supplementation of the dam could be a possible solution to vitamin D deficiency in Spring born dairy calves. However, this avenue requires detailed further study.

Dietary supplementation in the post-weaning period did successfully increase 25(OH)D concentrations in this study. Peak 25(OH)D concentrations were achieved using a combination approach of dietary supplementation and sun exposure which occurred in the month following peak solar exposure. The maximum 25(OH)D blood levels were observed at time point 7, with average 25(OH)D concentration of 60.86 ± 7.32 ng/ml in vit D supplemented calves exposed to sunlight, whereas vit D supplemented calves kept indoors had 49.46 ± 9.95 ng/ml. This is similar to levels observed in 5-6 month old beef calves grazing on the summer in central US with a mean serum 25(OH)D concentration between 50 to 60 ng/ml with minimal vit D_3 supplementation (~80 to 190 IU/kg of DM) [243]. Nevertheless, 25(OH)D blood levels up to 100 ng/ml had been reported in cows grazing at 30° N with an estimated intake of 2, 800 IU of vit D_3 per day [174]. Although there are no data regarding the optimal range of 25(OH)D levels, evaluation of the macrophage responses *in vitro* had shown a linear benefit to increasing 25(OH)D concentrations to 100 ng/ml [80].

Multiple factors are known to regulate vit D_3 skin synthesis, including latitude, altitude, and time of exposition to sunlight [134]. As in humans, seasonal variation in the vit D status on grazing calves (with minimal or null vit D_3 supplementation) has been reported, with low 25(OH)D concentration in winter and early spring [243, 244]. Thus, at northern latitudes (>40°N) cutaneous production of vit D_3 is not sustained throughout the year. The above is supported by our results which suggest that vit D_3 skin synthesis in pasture-based calves raised in Ireland (53°N) is limited from autumn to early spring. Furthermore, our results show that calves without access to sunlight require a vit D_3 supplementation of at least 4,000 IU/Kg to maintain 25(OH)D concentration similar to sunlight exposition during summer.

The vit D status of an animal is the result of the skin vit D_3 synthesis and diet supplementation, but cutaneous vit D_3 synthesis is regulated by endogenous mechanisms, therefore an excessive sunlight exposure does not result in vit D intoxication. Thus, the initial

blood 25(OH)D level restrict the vit D_3 skin synthesis by UV-light exposure [245]. Yet, 25(OH)D blood levels of 70 to 100 ng/ml had been reported in cows grazing in late summer at 30° N with an estimated intake of 2, 800 IU of Vit D_3 per day [174]. Therefore, we do not think we observed a plateau in the serum 25(OH)D levels, but a limitation in the oral supplementation. Higher 25(OH)D blood levels could be achieved with higher vit D_3 supplementation. Although, the serum 25(OH)D levels found in our study are within the sufficient range [124], whether higher values are beneficial for an optimal immune responses remains unknown.

In this study, a clear effect of divergent vitamin D profile on circulating immune cell populations was demonstrated. Specifically, we detected that the animals with low 25(OH)D concentration had an increase in the overall circulating cell composition across time in comparison to calves with higher 25(OH)D levels. Our results show that the main changes in leukocytes were significant in neutrophils, eosinophils, and basophils. Previous short-term vit D_3 supplementation studies for 30 days did not report changes in the leukocyte populations [139, 222, 246] and no long-term VitD_3 supplementation studies had previously assessed the effects on the immune response in cattle.

Haematological profiles in dairy calves are limited [247, 248], and research had shown that the use of adult cows' reference interval values is inaccurate to neonatal calves in the first 5-8 weeks of life [249]. However, in this study all the average values obtained after this period were within the values reported for healthy beef and dairy calves with differences only observed in calves with low 25(OH)D levels. [247, 250]. These immune cell changes could have important consequences for disease susceptibility. Neutrophilia is associated with stress and inflammatory process such as infectious diseases [251]. However, all the calves were clinically healthy when the increase in the immune cells was evident. IL-8 is a potent chemotactic agent for neutrophil recruitment and inflammation, and previously, we observed an inverse relationship between circulating 25(OH)D levels and IL-8 expression in calves with two different IL-8 haplotypes [125]. In this study, IL-8 expression was not associated with the changes in 25(OH)D levels. Therefore, our results suggest that although IL-8 genotype shape vit D responses, other molecular factors are associated with the vit D status in cattle. In fact, a recent genome wide association study in African calves had found that the serum 25(OH)D concentration is under polygenic control [252]. Therefore, environmental and host factors can contribute to changes in the vit D status in cattle. Another potential mechanism underlying the neutrophilia reported here is an increase release of cells from the bone marrow. The exit of mature neutrophils from the bone marrow is tightly regulated by a cytokine network, which includes IL-17, [253]. IL-17 production is modulated by vitamin D [107], and vit D restriction

results in the overexpression of IL-17 [254], which identifies potentially productive avenues for future research.

Reactive oxygen and reactive nitrogen species (ROS/RNS) are key players in cellular signalling and regulation of oxidative stress. Neutrophils are known to produce a large amount of ROS and RNS, and although it is a highly regulated process, under certain circumstances, such as chronic inflammatory diseases, neutrophils can be triggered to release ROS/RNS causing damage to host tissues [255]. Vit D is one of the key controllers of systemic inflammation and oxidative stress, and its deficiency may contribute to the dysregulation of ROS signalling pathways [256]. We measured the reactive oxidative metabolites (ROMs) in serum as a reference for ROS production [257]. However, we did not observe differences in ROS production between animals with divergent 25(OH)D concentrations. The lack of difference in our results could be due to the lower ROS production in calves in comparison with adult cattle, with previously published reports of ROS concentration 64% lower in calves as compared to adults [258]. Furthermore, while the serum analysis of ROMs has been validated in dairy cattle for the analysis of free radicals of oxygen [257], ROS and RNS represent a broad range of molecules with distinctive properties and are challenging to detect [259]. Therefore, the assessment of the oxidative stress index (based on the ratio between ROS and serum antioxidant capacity) has been suggested as a more accurate approach to determine the oxidative status [260]. Thus, future studies should assess the production of antioxidant factors to better define the oxidative status in calves with divergent 25(OH)D levels. Previous work showed that vit D modulates nitric oxygen production via NOS2 gene expression in cattle [80, 83]. Our results show a trend for higher ROS levels in calves with high 25(OH)D levels. Merriman *et al.,* [172] showed that 25(OH)D induced elevated NOS2 gene expression, and its expression was upregulated upon LPS stimulation in macrophages and neutrophils from milk. Whether high ROS/RNS levels under noninflammatory conditions provides resistance to infections remains to be determined.

Studies on the vit D profile on young calves are sparse and to our knowledge this is the first study of a long term vit D_3 supplementation in calves from birth to 7 months of age. Our results identify vit D deficiency in Spring-born dairy calves which significant perturbs the cellular immune response. Deficiency of vit D could have important implications for calf health, not only on immune system development and the microbiome [261] but also in terms of bone development [262]. Sub-optimal immune system development in early life will inevitably contribute to a failure to thrive and potentially a lifetime of disease susceptibility as well as an overdependence on antibiotic usage [241]. We have also demonstrated that vit D_3 supplementation within the current EU guidelines is not sufficient to improve the vit D status

of calves during the pre-weaning period. The antimicrobial and immunoregulatory role of vit D may offer a low-cost and effective supplement to boost natural disease resistance in cattle [235] and this offers and intriguing area for further investigation.

5. CHAPTER 5. *Ex-vivo* characterisation of the microbicidal and immunoregulatory effects of *in-vivo* supplementation with vitamin D_3.

5.1. Introduction

Vitamin D deficiency (VDD) is a major public health issue that affects people from all age groups living in countries from diverse latitudes [137]. VDD plays a direct role in the development of rickets, osteoporosis and osteomalacia, but it also has been linked to a variety of infectious diseases, including sepsis, influenza, tuberculosis, human immunodeficiency virus (HIV), hepatitis C virus (HCV) and recently COVID-19 [263].

The role of vit D in tuberculosis has been widely investigated with data showing VDD as an independent risk factor for TB infection [157]. Vit D supplementation as an adjunct to anti-tuberculosis treatment has been analyzed in multiple studies; however, few of these have investigated the effects of vit D supplementation as a prophylaxis [264]. A double-blind randomized controlled trial conducted in 192 healthy adults receiving a single oral dose of 2.5 mg vit D_2 or placebo showed that individuals in the supplemented group had higher ability to restrict BCG growth in an *ex-vivo* whole blood assay [265]. In contrast, a randomized controlled trial of vitamin D supplementation in schoolchildren living in Mongolia did not result in a lower risk of TB disease. In this study 8851 children with negative results to *M. tuberculosis* infection (assessed by an interferon-γ assay, QFT) were randomly assigned to a group receiving or not a weekly oral dose of 14,000 IU (0.35 mg) of vit D_3 for 3 years. At the end of the trial the mean 25(OH)D serum concentration in the vit D group was 31.0 ng/ml vs 10.7 ng/ml in the placebo group. However, TB disease was found at similar percentages in both groups and the proportion of children with positive QFT results was similar between the vit D and placebo group [159]. A systematic review of 25 randomized controlled trials however did reveal a highly significant protective effect of vit D supplementation for prevention of acute respiratory infections. And the analysis of potential factors that affected the efficacy of vit D supplementation showed that daily administrations of 400-1000 IU were more effective than weekly doses [266]. Given the contradictory results published, whether higher 25(OH)D concentrations are required to promote resilience to disease, remains an open question.

In cattle, the consequences of VDD on disease susceptibility are unknown. And although it has been suggested that low 25(OH)D serum concentrations are associated with BTB whether the reduction in circulating vit D levels are the cause or consequence of the disease remains unclear [143].

5.1.1. Specific aims

In chapter 4 we showed that low circulating concentrations of 25(OH)D were associated with changes in circulating immune cell populations, with animals from group Ctl-In showing higher counts of neutrophils, eosinophils, and basophils. However, effects on disease susceptibility are yet to be elucidated. Thus, the main aim from this chapter was to assess the effect of divergent 25(OH)D circulating levels on the microbicidal activity and immune response to an *ex-vivo M. bovis* BCG challenge.

The specific objectives were:

1. To investigate the microbicidal activity of PBL after a whole blood BCG challenge
2. To investigate the differences in ROS and NO production after a whole blood BCG challenge
3. To investigate the differences in IL-1β and IL-8 production after a whole blood BCG challenge
4. To investigate the differences of gene expression after a whole blood BCG challenge

5.2. Materials and Methods

5.2.1. BCG Whole Blood Infection

The BCG whole blood infection was performed as described in chapter 2, except for the stimulation with 1,25(OH)D. Briefly, 300 µL of whole blood was placed in a 2 mL tube and were challenged with 300 µL of RPMI medium containing 1 x 10^6 CFU of BCG Denmark. The tubes were placed in the rotatory platform and incubated at 37ºC for 24 h. Then, the tubes were centrifugated at 500 *x* g for 5 min the supernatant was removed and stored at -20 °C. The cell pellets were lysed with 200 µL of H_2O with 0.05% tween 80 and used to determine the bacteria load by CFU counting. Similar aliquot was prepared for RNA extraction, but the cell pellet was lysed with 0.8 ml trizol and stored at -80°C until analysis. A non-infected (null) sample was prepared in the same way and used as negative control.

5.2.2. Bacterial killing in serum

Analysis of the bacterial killing in serum was done with two types of bacteria, *E. coli* and BCG. The assay was conducted on a 96 well plate in a total volume of 100 µl, 10^8 CFU of bacteria was incubated with 90% serum and incubated at 37°C for 1 h. Then, number of viable bacteria was determined by serial dilution on agar plates. Analysis was done by duplicate using PBS as a control.

5.2.3. Determination of reactive oxygen metabolites and nitric oxide concentrations.

The analysis of ROS production was carried out using the d-ROM assay as previously described in chapter 4. This assay measures the reactive oxidative metabolites in serum as a reference for ROS production and results are expressed as arbitrary ‘Carratelli Units’ (CarrU).

Concentration of nitric oxide (NO) was analysed using the Griess reagent system (Promega) following the manufacturer’s recommendations. This system measures nitrite (NO_2) as an indicator of NO production. Absorbance at 550 nm was measured using microplate reader (Clariostar Plus, BMG Labtech). The micromolar concentration (µM) of NO2 was calculated with a nitrites’ standard curve using fresh RPMI medium enriched with 10% FBS.

5.2.4. Determination of IL-1β and IL-8 by ELISA

The bovine IL-8 ELISA used to measure IL-8 concentration was carried out as previously described by Cronin et al [238]. Whereas the IL-1 beta bovine uncoated ELISA kit (ThermoFisher) was used as per manufacturer's instructions.

5.2.5. RNA extraction, cDNA synthesis and Fluidigm analysis

All these procedures were done similarly as described in chapter 2 section 2.3.13. with the same panel of genes listed in Appendix 1. However, RNA integrity was assessed using the RNA 6000 Nano Kit (Agilent Technologies) on the Bioanalyser according to manufacturer instructions. All samples had a RIN value ≥ 7.5, and cDNA synthesis was performed with 15 ng/µl of RNA. Lowly expressed or non-detected genes were removed before statistical analysis, the remaining 50 genes are shown in Table 5-1.

5.2.6. Statistical analysis

GraphPad Prism software version 8 was used for data presentation and statistical analysis, except for analysis of Fluidigm data. Data is presented as mean ± SEM, unless otherwise stated. Statistical test and number of samples for each experiment is indicated in the figure legends. Evidence of statistical significance was considered at $P \leq 0.05$ and tendency was considered at P values ≤ 0.10.

For analysis of fluidigm data, multiple T tests, PCA and hierarchical clustering were done in R studio (Version 4.0.3) with the packages rstatix, factoextra, cluster, tidyverse and ComplexHeatmap. Differences between groups were assessed on Log_2 fold change by unpaired T test with p-adjusted to 0.1 with the Benjamini-Hochberg correction for multiple testing. Standardized Log_2 fold change was used for principal component analysis (PCA) and heatmap visualization. Hierarchical clustering analysis was done by computing the spearman correlation distance between the observations followed by the Ward's linkage method for definition of clusters.

Table 5-1. List of genes selected for analysis of 25(OH)D effects *ex-vivo*.

Gene	Function	*Gene*	Function
DEFB10	Antimicrobial peptide	*TNFA*	Cytokine Proinflammatory
DEFB3	Antimicrobial peptide	*CASP13*	IL1 Pathway
DEFB4	Antimicrobial peptide	*IL1A*	IL1 Pathway
DEFB6	Antimicrobial peptide	*IL1B*	IL1 Pathway
DEFB7	Antimicrobial peptide	*IL1R1*	IL1 Pathway
LAP	Antimicrobial peptide	*IL1RN*	IL1 Pathway
S100A12	Antimicrobial peptide	*NLRP3*	IL1 Pathway
S100A8	Antimicrobial peptide	*COX2*	Inflammation
S100A9	Antimicrobial peptide	*MMP9*	Inflammation
CD14	Cell adhesion	*PKR*	Inflammation
ITGAM	Cell adhesion	*C5AR1*	Innate immunity
CCL2	Chemokine & Receptors	*IFITM3*	Interferon
CX3CR1	Chemokine & Receptors	*IFNAR1*	Interferon
CXCL1	Chemokine & Receptors	*IFNB*	Interferon
CXCL10	Chemokine & Receptors	*IFNG*	Interferon
CXCL2	Chemokine & Receptors	*MX1*	Interferon
CXCL5	Chemokine & Receptors	*OAS1Z*	Interferon
CXCL8	Chemokine & Receptors	*NCF1*	Oxidative Stress
CXCR1	Chemokine & Receptors	*TLR1*	Pathogen recognition
IL10	Cytokine Anti-Inflammatory	*TLR2*	Pathogen recognition
IL15	Cytokine Anti-Inflammatory	*TLR4*	Pathogen recognition
TGFB1	Cytokine Anti-Inflammatory	*TLR6*	Pathogen recognition
IL12B	Cytokine Proinflammatory	*HIF1A*	Transcription factor
IL6	Cytokine Proinflammatory	*STAT1*	Transcription factor
IL6R	Cytokine Proinflammatory	*RXRA*	VD metabolism

5.3. Results

5.3.1. Microbicidal activity on vitamin D supplemented calves

For these experiments, samples from animals of indoors groups, Ctl-In and VitD-In were used. Calves from these groups were kept indoors and had the greatest 25(OH)D divergence during the trial (Figure 4-1 and Table 4-1). Samples were analyzed at three time points: T3, T5 and T9, which correspond to the age of 1-3 and 7 months old.

As shown in chapter 4, no differences in the circulating 25(OH)D levels were observed at T1 nor T5. However, at T9 animals from VitD-In group have significantly higher 25(OH)D concentrations, with a mean of 46 ng/ml in comparison to 38 ng/ml in Ctl-In group (Figure 5-1 A). Differences in microbicidal activity followed the same pattern, with no significant differences in the number of viable bacteria nor the proportion of bacteria killing at T1 and T5, but at T9 a significant drop in the CFU counts and the corresponding rise in the bacterial killing was detected in the VitD-In group. The percentage of bacteria killed in the VitD-In calves was on average 48.0% in contrast to 29.5% in the Ctl-In group (Figure 5-1). Changes in the bacterial killing activity was not observed throughout the time in any group, although a trend (P= 0.07) of increased bacterial killing from T5 to T9 in the VitD-In group was observed (Table 5-2).

A bactericidal assay with serum was carried out to assess the microbicidal activity by serum components. For this assay, bacteria (*E. coli* and BCG) were incubated with 90% serum for 24 h, then bacteria viability was evaluated by agar plating. Results shown that there were no differences in bacterial growth between serum from Ctl-In and VitD-In calves (Figure 5-2). Therefore, this result suggests that the bactericidal activity against BCG was due to cellular components in the blood.

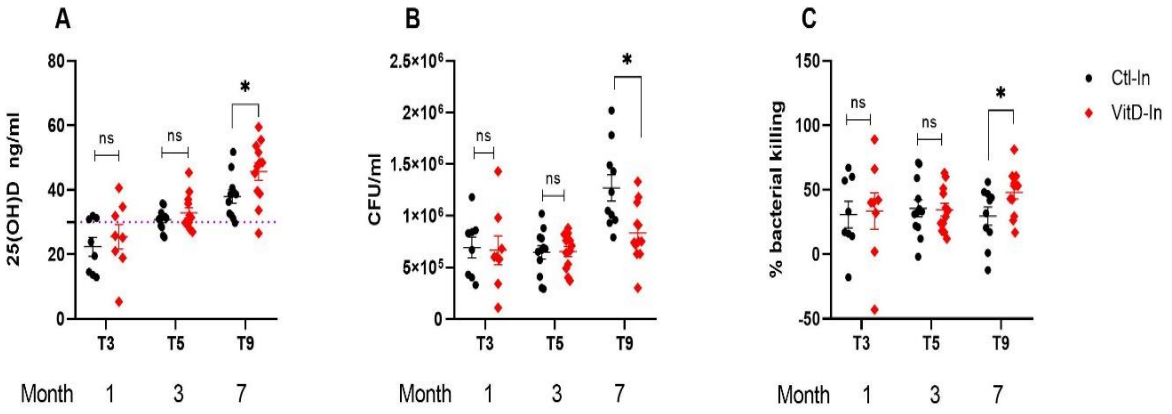


Figure 5-1. Microbicidal activity of PBL in Ctl-In and VitD-In calves at T1, T5 and T9. Blood from Ctl-In and VitD-In groups was infected with $1x10^6$ CFU/ml of BCG, after 24 h cells were washed and lysed to determine the number of intracellular bacteria. Analysis was done at T1, T5 and T9 that corresponds to 1-, 3- and 7-months age. (A) Serum 25(OH)D levels. (B) number of viable bacteria and (C) percentage of bacteria killing at 24 h post-infection. Data is shown as mean ± SEM of n= 8-12. Mixed effect model with Bonferroni correction to assess differences between Ctl-In and VitD-In groups *P < 0.05 was considered statistically significant.

Table 5-2. Microbicidal activity of PBL on Ctl-In and VitD-In calves									
	Ctl-In			VitD-In			*P value		
Time	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**[1]Group**	**[2]Time Ctl-In**	**[3]Time VitD-In**
T3 (1 month)	8	30.75	29.1	8	33.5	39.89	0.877	0.605	0.945
T5 (3 months)	12	35.58	22.26	12	34.58	17.13	0.903	0.923	0.315
T9 (7 months)	10	29.5	22.35	12	47.97	17.59	0.049	0.612	0.074

[1]Comparison between groups at each time point with Bonferroni correction

[2]Effect of time in Ctl-In group. T3 vs T5, T3 vs T9, T5 vs T9

[3]Effect of time in VitD-In group. T3 vs T5, T3 vs T9, T5 vs T9

*There was no interaction between time and group P > 0.05

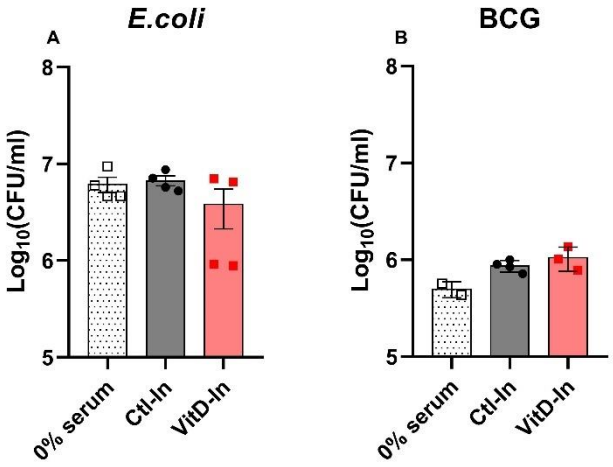


Figure 5-2. Bacterial killing activity in serum from Ctl-In and VitD-In calves.

E. coli and BCG were incubated with 90% serum for 24 h, then bacteria counts were assessed by agar plating. Data is shown as mean ± SEM of n=4. Kruskal-Wallis test with Dunn's correction was carried out to analyse differences between treatments.

5.3.2. Analysis of ROS, NO, IL-1β and IL-8 production in serum from Ctl-In and VitD-In calves after BCG challenge

Results showed than ROS and NO production was significantly higher in non-infected (null) samples from VitD-In calves. Mean ROS production in null samples from VitD-In animals was 123.6 CARR units, in contrast to 54.2 CARR units in Ctl-In calves. Whereas null NO levels were on average 1 μM in Ctl-In and 5.6 μM in VitD-in group. Differences between groups after BCG infection were only significant for ROS production, but not for NO. However, there was no effect of BCG challenge in ROS or NO production since there were no differences between the null and BCG treatment within any group (Figure 5-3).

Levels of IL-1β and IL-8 in null samples was not significantly different between Ctl-In and VitD-In groups; however, after BCG challenge concentration of both cytokines increased although a significantly lower concentration in VitD-In calves. After BCG challenge the mean concentration of IL-1β in Ctl-In was 2.0 ng/ml in comparison to 0.41 ng/ml in VitD-In group. For IL-8 a similar pattern was observed, with mean concentrations of 25.02 ng/ml and 14.85 ng/ml in Ctl-In and VitD-in calves, respectively (Figure 5-4).

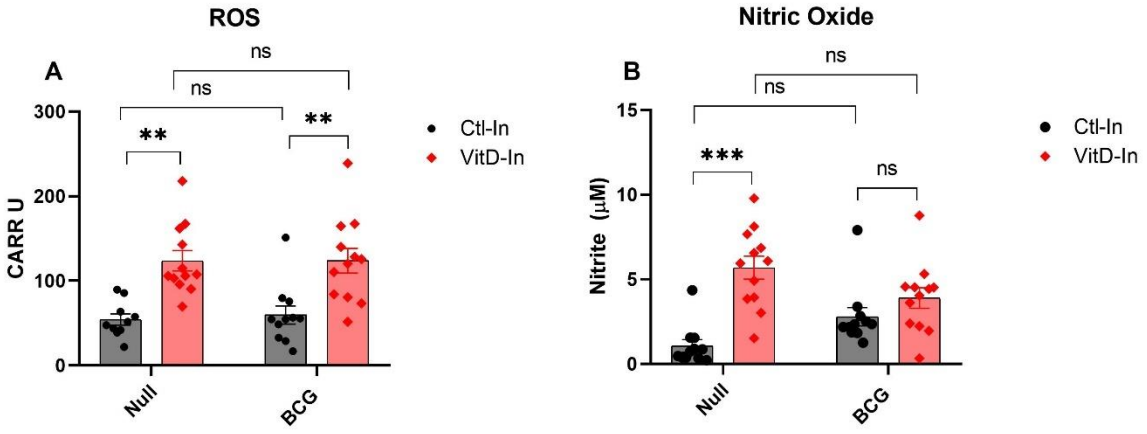


Figure 5-3. Oxidative stress response on Ctl-In and Vit-D calves after BCG challenge. (A) ROS and (B) NO production was measured in supernatant taken after BCG infection using the ROM test and Griess reagent system, respectively. A non-infected (null) sample was used as a control. Results are shown as mean ± SEM of n= 11/12. Mixed effects analysis with Tukey correction was used to assess differences between treatments. ns= not significant, **P< 0.001, ***P < 0.0001, was considered statistically significant.

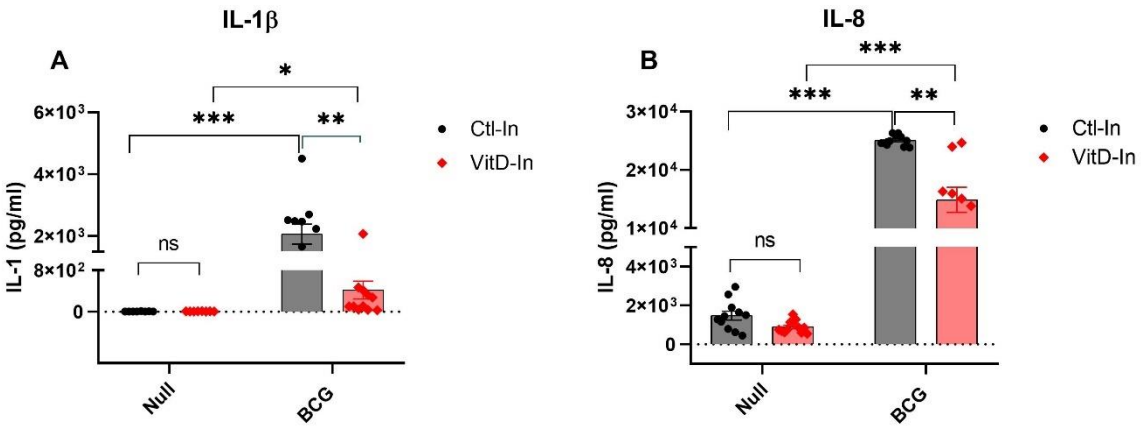


Figure 5-4. IL-1 and IL-8 production on Ctl-In and Vit-D calves after BCG challenge.

(A) IL-1 and (B) IL-8 levels were measured in supernatants taken after BCG infection by ELISA. A non-infected (null) sample was used as a control. Results are shown as mean ± SEM of n= 11/12. Mixed effects analysis with Tukey correction was used to assess differences between treatments. ns= not significant, *P < 0.05, **P< 0.001, ***P < 0.0001 was considered statistically significant.

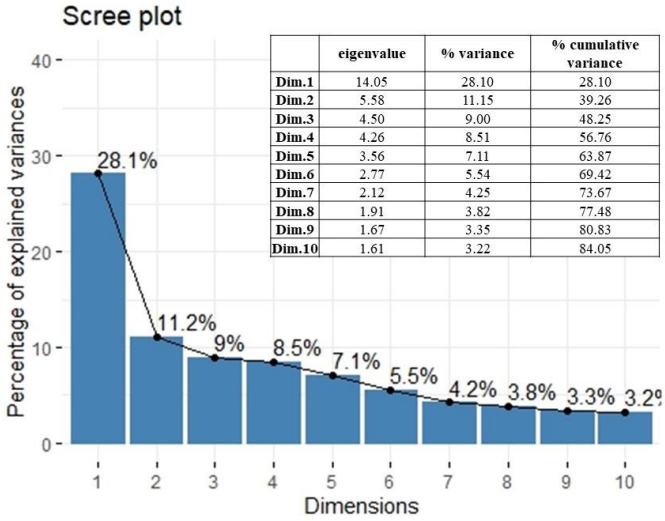
5.3.3. Analysis of the gene expression on Ctl-In and Vit-D calves after BCG challenge

Gene expression was analysed with the Fluidigm Biomark HD system using the same panel of 96 genes as described in chapter 2. After removal of lowly expressed or non-detected genes, analysis with 50 genes was carried out.

The plot from eigenvalues from the PCA analysis showed that nine dimensions are necessary to explain 80% of the variance (Figure 5-5). However, for clarity of visualization and interpretation, results from the first 4 dimensions that explain 57% of the variance is shown. The PCA plot of the individuals showed an overlap in the response between animals from Ctl-In and VitD-In group along dimension 1. A group of animals from Ctl-In treatment separates alongside dimension 2 that explains 11% of the variance (Figure 5-6 A). Whereas VitD-in group is distinguished from Ctl-In along dimension 3 (Figure 5-6 B). The biplot shows the correlation between animals and genes. Thus, separation along dimension 2 from Ctl-In group is explained by their differential response to *CXCL2, CXCL1, CXCL8, IL1B, COX2* and *TNFA* (Figure 5-7 A). Whereas response to genes such as *DEFB7, DEFB4, DEFB6* and *NCF1* explain the divergence between Ctl-In and VitD-In groups along dimension 3 (Figure 5-7 B).

The heatmap at Figure 5-8 reveals a similar pattern, with some animals from Ctl-In and VitD- group having a parallel gene expression profile. For example, animals C2, C4, C7 and C9 from group Ctl-In are clustered in a group with similar profile than animals V6, V4 and V11 from VitD-In group. Six clusters of genes were distinguished from which cluster 1 containing genes such as *IL1B, IL1RN, CXCL1, CXCL2, IL12B* and *TNFA* were significantly downregulated in VitD-In calves in comparison to Ctl-In calves. Scatter plots of fold change differences in the expression of cytokines and chemokines that were differentially regulated are shown in Figure 5-9.

Similarly, fold change differences from genes associated with the IL-1 pathway are shown in Figure 5-10. *IL1B* and *IL1R1* was significantly downregulated in VitD-In calves in comparison to Ctl-In group. Whereas no differential response was observed for *IL1A* and *IL1RN*. On the other hand, data showed a biological trend for higher expression of *IFNB, IL10, NLRP3* and *CASP13* in VitD-In calves; but animal variability did not allow to reach statistical significance after P adjustment. Mean and p adjusted values for all genes are shown in Appendix 3.



	eigenvalue	% variance	% cumulative variance
Dim.1	14.05	28.10	28.10
Dim.2	5.58	11.15	39.26
Dim.3	4.50	9.00	48.25
Dim.4	4.26	8.51	56.76
Dim.5	3.56	7.11	63.87
Dim.6	2.77	5.54	69.42
Dim.7	2.12	4.25	73.67
Dim.8	1.91	3.82	77.48
Dim.9	1.67	3.35	80.83
Dim.10	1.61	3.22	84.05

Figure 5-5. Percentage of explained variance from PCA of gene expression effects on Ctl-In and VitD-In groups after BCG challenge.

Scree plot from the eigenvalues ordered from the largest to the smallest, showing the percentage of explained variance per dimension.

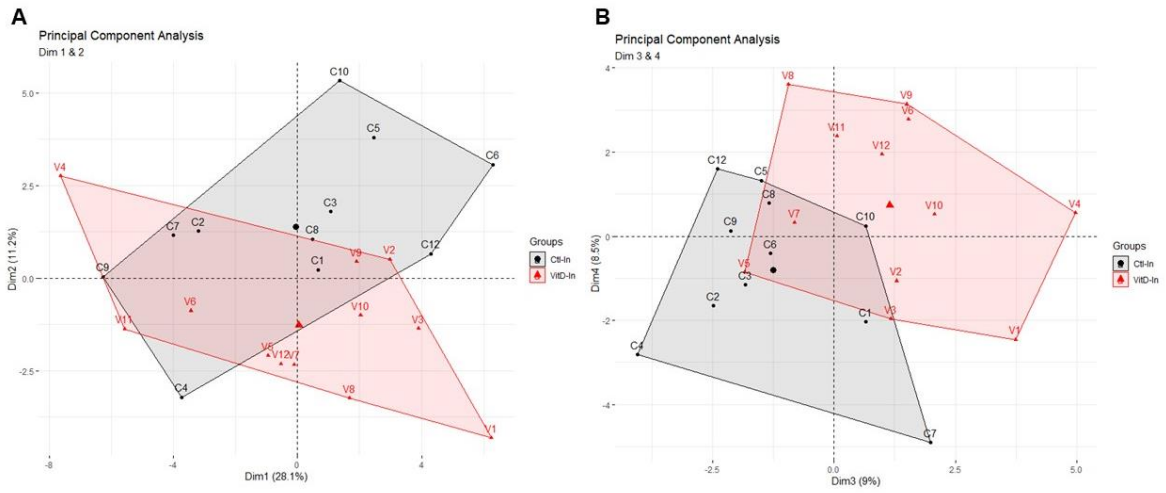


Figure 5-6. Plot of individuals from PCA of gene expression effects on Ctl-In and VitD-In groups after BCG challenge.

(A) PCA plot for dimensions 1 and 2 and (B) dimensions 3 and 4. Individuals are represented as C1 to C12 for Ctl-In and as V1 to V12 for VitD-In group. Animals are grouped by a convex hull to show the clustering of the groups.

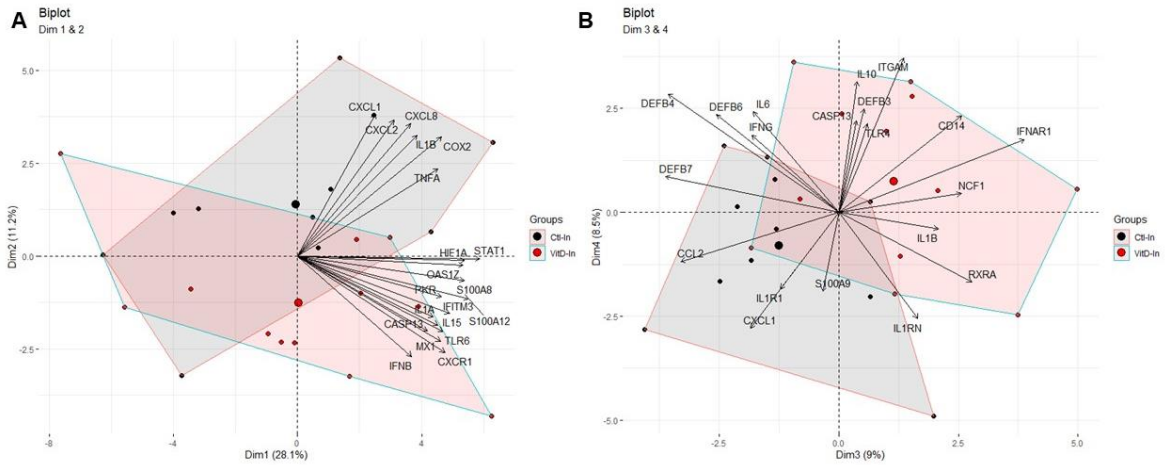


Figure 5-7. Biplot of individuals from PCA of gene expression effects on Ctl-In and VitD-In groups after BCG challenge.

(A) Biplots for dimension 1 and 2, and (B) dimension 3 and 4, showing the representation of individuals and variables within the principal component. Animals are grouped by a convex hull to show the clustering of the groups.

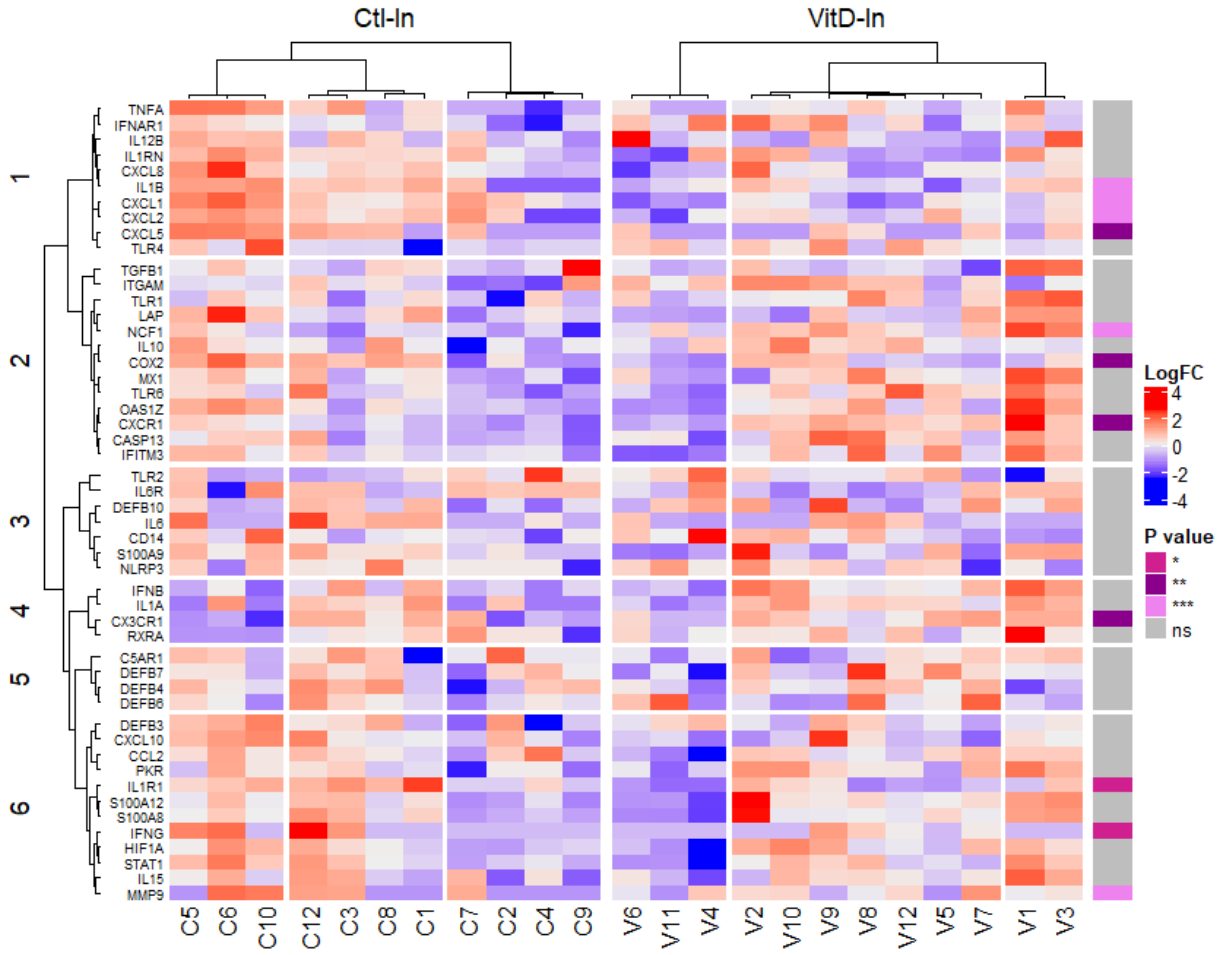


Figure 5-8. Heatmap from the gene expression analysis on Ctl-In and VitD-In groups after BCG challenge.

Results represent the standardized-Log_2 relative fold change. Hierarchical clustering with spearman correlation distance and Ward's linkage method was used to identify clusters of genes and samples with similar patterns. Division of clusters was achieved by splitting the dendrogram in clusters with the same height. P value shows the difference between groups assessed by multiple paired T-test, significance is after p-adjustment by Benjamini-Hochberg method. *P <0.1, **P <0.05. ***P <0.01, ns= not significant.

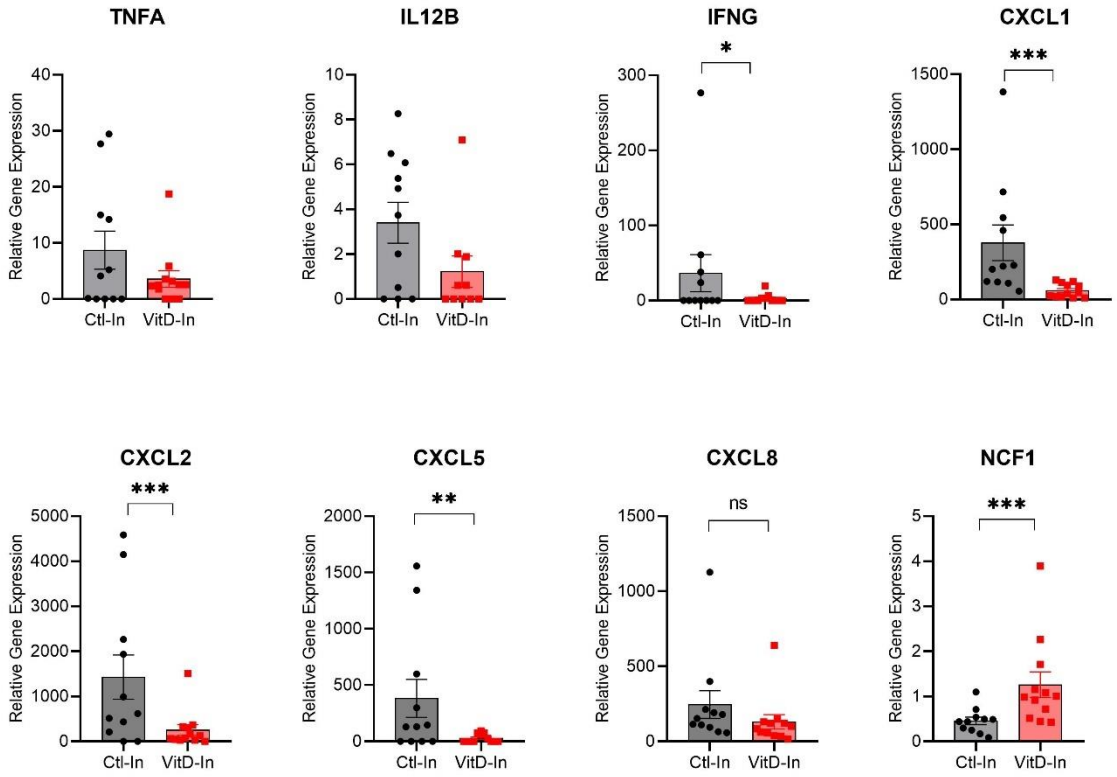


Figure 5-9. Gene expression of cytokines and chemokines on Ctl-In and VitD-In groups after BCG challenge.

Results are expressed as relative gene expression compared to a non-infected sample. Data is represented as mean ± SEM of Ctl-In (n=11), VitD-In (n=12). Difference between groups were assessed by multiple paired T-test, significance is after p-adjustment by Benjamini-Hochberg method. ns= not significant, *P < 0.1, **P < 0.05. ***P < 0.01 was considered statistically significant.

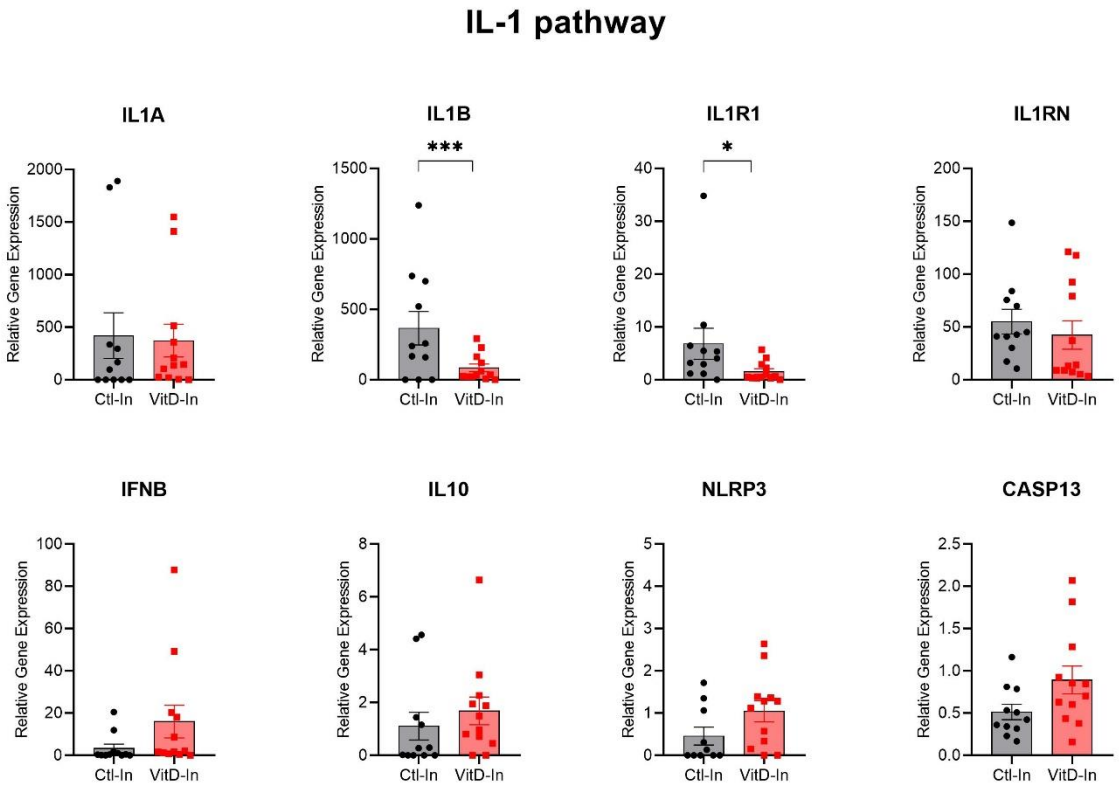


Figure 5-10. Gene expression of genes associated to the IL-1 pathway on Ctl-In and VitD-In groups after BCG challenge.

Results are expressed as relative gene expression compared to a non-infected sample. Data is represented as mean ± SEM of Ctl-In (n=11), VitD-In (n=12). Difference between groups were assessed by multiple paired T-test, significance is after p-adjustment by Benjamini-Hochberg method. ns= not significant, *P < 0.1, **P < 0.05. ***P < 0.01 was considered statistically significant.

5.4. Discussion

Despite the growing appreciation of the beneficial role of vitamin D for health and resistance against infectious diseases, research has predominantly focused on murine and human species. This represents a significant gap in the bovine immunology research, and particularly in relation to BTB. In this chapter we used an *ex-vivo* BCG challenge model to assess the antimycobacterial activity and immunoregulatory effects in calves fed with standard or high vit D_3 diets for 7 months. Our results demonstrate that dietary vit D_3 modulates the immune response to BCG by boosting the microbicidal activity and controlling excessive inflammation which may be detrimental for the outcome to infection.

Few studies have previously analysed the effects of vit D supplementation on disease susceptibility in cattle. Sacco E., *et al.* [236] did not observe an effect on the pathogenesis of respiratory syncytial virus (RSV) infection in calves with differential 25(OH)D circulating levels. In this study, new-born calves were fed with a milk replacer containing low or high vit D (1,700 or 17, 900 IU of vit D/ Kg, respectively), after 10-weeks calves were challenged with RSV. Animals in the high vit D group had on average 177.3 ng/ml of serum 25(OH)D, in contrast to 32.5 ng/ml 25(OH)D levels in the low vit D group. Despite the divergent vit D status, there were no differences in severity of lung lesions between groups. Nonetheless, authors showed that expression of *IL8, IL12B* and *IFNG* were upregulated in the vit D group relative to the low vit D group. However, it remained unclear whether the increased production of pro-inflammatory cytokines would be beneficial in the long term [236]. Another study found no differences in the severity of MAP infection in calves fed with colostrum supplemented or not with vit D (or vit ADE) for 14 days [242]. However, in this study calves were challenged with MAP after one day of birth, and differences in 25(OH)D circulating levels were observed after 7 days of birth. Thus, it is still uncertain if differential 25(OH)D circulating concentrations are protective against MAP infection [242].

In comparison with our model, the above studies have provided vit D during short time periods, from days to maximum 10 weeks. However, data from clinical trials in humans have shown that standard dosage interventions for 6 to 12 months are more beneficial to prevent acute respiratory infections [266, 267]. Our vit D supplementation study is in line with this evidence and shows that animals receiving a diet with high vit D_3 concentrations (VitD-In) for 7 months had better ability to restrict BCG than animals fed with low vit D_3 concentrations (Ctl-In). We did not observe differences in the microbicidal killing through the time but differences in the serum 25(OH)D levels were not evident until 7 months. This opens the question on whether if increasing the vit D status of calves at younger ages will improve their resilience to infections during the pre-weaning period [268]. For instance, Martineau A, *et al*. shown that a

single high dose (2.5 mg) of Vit D_2 enhanced the ability of PBL from TB contact patients to restrict BCG growth. This dose of supplementation corrected VDD in all individuals and reduced BCG-lux luminescence after 24 h of infection [265]. Thus, results from this study suggest that raising 25OHD serum levels is an effective intervention to enhance immunity to mycobacteria, effect that might be extended against other bacteria.

Analysis of potential microbicidal mechanisms regulated by vitamin D included the assessment of NO and ROS production. Our results showed that calves in the VitD-In group have higher oxidative stress response than Ctl-In calves. In addition, our results showed that *NCF1* (which encodes for a component of the NADPH oxidase) was significantly upregulated in Vit-D group. In previous chapter, we did not observe a modulation of ROS production in serum, which represent a steady-state condition. Thus, collectively these results suggest that after a stressful condition like hypoxia or bacterial challenge, animals with higher serum 25(OH)D concentrations had a differential regulation of the redox signalling pathway which could be advantageous for bacterial control. Besides their direct bactericidal killing effect on mycobacteria, ROS and RNS work as secondary messengers on diverse signalling pathways including NF-κB, AP-1, MAPK, PI3K, influencing the expression of genes involved in inflammation, cell proliferation, differentiation and apoptosis [269]. Moreover, ROS plays an important role in regulating autophagy and cathelicidin production, thereby potentially influencing the vit D mediated antimicrobial effects [94]. Although autophagy was not evaluated in this work, research has shown that this defence mechanism inhibits *M. bovis* survival in bovine neutrophils [221]. Thus, modulation of ROS production seems an important avenue to understand the antimicrobial mechanism by vit D in cattle.

Our results also showed that dietary vit D enhanced NO production in VitD-In calves in comparison to Ctl-In animals; however, in contrast to other studies, we did not observe expression of *NOS2* in our study. This might be explained by the different model of infection used, PBL instead of macrophages. Previous research has shown that vit D enhances *NOS2* expression in macrophages and monocytes. Data from these studies showed several fold change differences in gene expression between control and $1,25(OH)_2D_3$ stimulated cells (60 to 300 times more) [80, 83]. Whereas gene expression data from PBMC showed no differences after $1,25(OH)_2D_3$ treatment, and an increase of 1 to 1.5 relative fold changes [86]. Therefore, it is possible that the use of PBL (or PBMC) allows the crosstalk between monocytes, neutrophils and T cells which influence the dynamics of *NOS2* gene expression. Nonetheless, our data supports the evidence by other research groups which suggest that that vit D enhances *M. bovis* killing in bovine cells via NO-dependent mechanism [80, 82, 83, 86, 143, 152].

The pattern of gene expression in PBL after BCG challenge showed that animals from both groups can be clustered into three groups from low, mid, and high response. However, the correlation analysis did not show a relationship with 25OHD serum concentrations. This effect is similar to the response observed after short and long term oral vit D supplementations in humans [182, 270, 271]. Authors showed that the characterization of the response to up to 36 parameters including changes in gene expression, chromatin accessibility and serum proteins and metabolites, allowed the segregation of individuals into high, mid, and low responders to vit D. However, this classification was based on the response inducible by vit D and did not reflect the 25OHD serum level. Thereby, an individual with high vit D index is a participant that showed response to most of the parameters evaluated [182, 270, 271]. Our results suggest that calves could be classified by a similar method, but the analysis of more parameters would be necessary to confirm our findings. If it were true, identification of animals with high vit D responsiveness would be of interest since they might be less affected by low 25OHD serum levels.

Analysis of the gene expression showed an anti-inflammatory profile in VitD-In calves in comparison to Ctl-In animals after BCG infection. Remarkably, a significant lower production of IL-1β and IL-8 was observed at protein and mRNA level in the VitD-In group. IL-1β is recognized for its role in mediating control of *M. tuberculosis* infection although an overactive response can contribute to pathology and disease severity [95, 167]. IL-1β production is regulated by multiple mechanisms including IFNs and the inflammasome NLRP3. Studies *in vitro* have shown that $1,25(OH)_2D_3$ and 25(OH)D boost IL-1β production in a bimodal fashion, by increasing IL1B transcription and by promoting the maturation of the protein through the NLRP3 inflammasome [95, 272]. On the other hand, studies in mice showed that IFN-β inhibit IL-1β production in macrophages and dendritic cells by an upregulation of the IL-1R antagonist (IL-1Ra) and by promoting IL-10 expression [273]. Likewise, data from patients with multiple sclerosis have shown that vit D synergises with IFN-β to reduce the inflammatory response by $CD4^+$ T cells lymphocytes [274]. Overall, these data suggest that there is a complex interplay between vit D, type I IFN signalling, inflammasome and cytokine expression that conjunctively promotes a controlled inflammatory immune response. Our results suggest a similar biological mechanism, where VitD-In calves had a reduction in *IL1B* response but not a complete inhibition. IL-1β production was significantly upregulated after BCG challenge in both Ctl-In and VitD-In in comparison to non-infected treatments. However, dietary vit D promoted a controlled IL-1β response in VitD-In calves. *NLRP3* was upregulated in VitD-In but not in Ctl-In which suggest that inflammasome activation induced an efficient IL-1β maturation adequate to control BCG growth. Besides, *IL1B* expression was regulated by *IFNB* and *IL10* in VitD-In calves, whereas an uncontrolled IL-1β response was observed in Ctl-In animals. However,

analysis of the IL-1 modulation by vit D for disease outcome and severity requires further investigation.

The anti-inflammatory profile observed in VitD-In calves was not only limited to the IL-1 pathway, but to a cluster of genes including *TNFA, IL12B, CXCL8, CXCL1, CXCL2* and *CXCL5*. Suppression of CXC chemokines by $1,25(OH)_2D_3$ was reported in PBMCs from patients with active TB [156]. The reduction in chemokine and IL-8 production in VitD-In calves suggest a differential regulation of leukocyte recruitment upon infection that can favour the anti-inflammatory environment. Moreover, dietary vit D promoted a decrease in *IFNG, TNFA* and *IL12B*, essential components of the innate immune response against mycobacteria that can also play a role in the pathology of BTB [48]. Although analysis of the protein levels was not assessed, previous studies have validated the decrease of IFN-γ, TNF-α, and IL-12 by $1,25(OH)_2D_3$ and 25(OH)D in supernatants [86, 275]. A systematic review on the impact of vit D on inflammation indicate that $1,25(OH)_2D_3$ and 25(OH)D decrease the inflammatory status of human-derived immune cells through suppression of the NFκB transcriptional activity but as well by non-genomic pathways such as modulation of intracellular calcium levels, and activation of secondary messengers like phospholipase C and G protein-coupled receptors [276].

Finally, given the low statistical significance of BCG killing between VitD-In and Ctl-In groups (P = 0.049), another plausible explanation is that the result occurred by chance (type 1 error). However, based on the results with $1,25(OH)_2D_3$ from previous chapters and data from other studies on bovine and human cells [74, 94, 95, 103, 143, 151, 277], the results suggest that the increased BCG killing in VitD-In calves reflects a meaningful enhancement of global antimycobacterial host response. And even though the results in bacterial killing were modest, the effects on the inflammatory immune response were substantially different between groups. These differences could shape the outcome to infection by dampening the potentially harmful inflammatory T cell responses.

Collectively our results have shown that vit D mediates protection against BCG by enhancing microbicidal activity of innate cells through a ROS/RNS mechanism. Likewise, dietary vit D exerts anti-inflammatory effects by inducing a controlled production of inflammatory agents including *IL1B, IL8, IFNB, TNFA* and CXC chemokines.

6. CHAPTER 6. General Discussion

Infectious diseases constitute a severe economic constraint in the dairy industry due to the direct cost of animal losses, resources needed for control, limitations in trade, and repercussions on human health and welfare [278]. Additionally, during the neonatal period infectious diseases can have long-term consequences on animal performance [279]. The immune system of calves is immature, and although there are higher number of phagocytes in circulation, these cells have decreased activity, with data showing that the immune response in calves is slower and weaker than in adults [280]. Furthermore, the metabolic challenge that dairy cows face during and after parturition can increase their susceptibility to infectious diseases. Although antibiotics and vaccinations are essential for safeguarding animal health, the rise in antibiotic resistance demands additional research into new immune-boosting strategies. Feeding the immune system provides an avenue to minimize the impact of infectious diseases in cattle. Several micronutrients have shown to influence the immune response, from which vit D has gained remarkable attention due to its association with infectious and non-infectious diseases in humans.

Vit D is required to maintain bone and calcium homeostasis, and multiple studies have identified its participation in several cellular processes including cellular proliferation, differentiation, wound healing, host defence, and inflammation [68, 75, 281]. Several studies have shown that the active metabolite of vit D, $1,25(OH)_2D_3$, enhances the microbicidal activity of macrophages against *M. tuberculosis*, and epidemiological studies have linked VDD with a higher risk for developing TB. However, clinical trials have failed to show a benefit of supplementing vit D to prevent or treat TB in humans. The discrepancy between *in vitro* and *in vivo* interventions suggests that the interaction between vit D and the immune system is very complex and that better designed *in vivo* interventions are needed to further understand this relationship [160, 282].

Studies in cattle are limited, but data indicates a similar association. Research has shown that $1,25(OH)_2D_3$ improves killing of *M. bovis* and *M. avium* ssp. *paratuberculosis* in bovine macrophages. Besides, low levels of 25(OH)D have been associated with BTB and MAP infections. However, the consequences of VDD are unknown. Thus, it was aim from this project to investigate the role of vit D on the microbicidal, cellular and immunomodulatory activity against BCG. For this purpose, we analysed the effects of vit D using an *in vitro* and *in vivo* approach. First, we looked into how $1,25(OH)_2D_3$ modulated the response of PBL (chapter 2) and neutrophils (chapter 3). Then, we established an *in vivo* model to drive divergent circulating levels of 25(OH)D in calves and investigated how these variations affected the immune response in steady-state conditions (chapter 4). Lastly, we assessed the

effects of these divergent 25(OH)D levels on the microbicidal and immunoregulatory role to an *ex-vivo* BCG challenge (chapter 5).

Overall, the results showed that *in vitro* and *in vivo* vit D interventions improved the mycobacterial killing activities of circulating leukocytes. *In vitro* data showed that PBLs and neutrophils stimulated with $1,25(OH)_2D_3$ had higher killing of BCG and *M. bovis* than non-stimulated cells. Also, we showed that neutrophils are a major contributor to BCG growth control and that the modulation of $1,25(OH)_2D_3$ on both, monocytes, and neutrophils contributed to limit BCG survival. Moreover, we showed that $1,25(OH)_2D_3$ increased ROS production and the gene expression of antimicrobial peptides (*DEFB7, TAP*), chemokines (*CCL2, CXCL10*), cytokines (*IFNB, IL33*) and enzymes (*ELANE, PKR*), with key functions in the innate immune response to mycobacteria. Furthermore, $1,25(OH)_2D_3$ improved BCG growth control on PLBs from infected cattle (BTB+) suggesting that vit D could be a tool to limit the infection and reduce BTB dissemination.

To analyse the effect of $1,25(OH)_2D_3$ we used 4 ng/ml (10nM) which is the most common concentration used in studies *in vitro*. However, this is a supra-physiological concentration that is not reached in circulation. Whether it is reached in the granuloma or at infection sites is currently unknown. Besides, addition of $1,25(OH)_2D_3$ allows to analyse the last step on the activation of the vit D pathway [$VitD_3 \rightarrow 25(OH)D \rightarrow 1,25(OH)_2D_3$] which is still biologically relevant but means that the responses might happened at early time points after infection. This could explain the small differences observed between treatments with respect to ROS production and gene expression. Studies on $1,25(OH)_2D_3$-stimulated monocytes/ macrophages have shown significant differences in *NOS2* expression after 24 h of BCG or LPS challenge [80, 81, 83]. However, this might not hold true for stimulations on complex cells populations like PBMC and PBL [86, 194]. Therefore, analysis of the dynamics of $1,25(OH)_2D_3$ effects at earlier time points are necessary to fully elucidate the molecular mechanisms associated with mycobacterial killing.

A limitation of our *in vitro* stimulations is the lack of inhibitory experiments to confirm the effects of $1,25(OH)_2D_3$. For example, the analysis of ROS mediated effects could be assessed in $1,25(OH)_2D_3$ stimulated cells using an inhibitor of the NADPH oxidase, such as DPI (Diphenyleneiodonium chloride). This type of experiment could help to determine if ROS is required for vit D modulation of bovine cells, as it is in human macrophages [94]. Inhibitory experiments can also be performed by blocking the VDR receptor. Currently, there are no commercially available antibodies against the bovine VDR, but the high similarity (~90%) between the bovine and human VDR at protein level suggest that the human antibody could be used instead. Standardization of these experiments is time consuming, and our ultimate

goal was to assess the *in vivo* effects of vit D thus the decision was made to continue with the *in vivo* model that could provide more biologically relevant data.

The *in vivo* trial was carried out with vit D_3 (cholecalciferol) the most common metabolite used in clinical trials in humans and the only authorised source of vit D for cattle in the EU [135]. Supplementation was done from birth to 7 months with the standard vit D_3 concentration used on Irish farms and the maximum dose permitted within the European guidelines. Also, comparison between animals kept indoors or outdoors was performed to model the influence of sunlight exposure. Effect of vit D_3 supplementation was analysed as changes in the 25(OH)D serum levels. And the effect on the *ex-vivo* BCG challenge was assumed to be the result of the conversion of 25(OH)D to 1,25$(OH)_2D_3$ by immune cells. Thus, this model allowed a better physiological analysis of the vit D pathway [VitD_3→25(OH)D→1,25$(OH)_2D_3$]. Although the analysis of each metabolite was not performed, research has shown that changes in the circulating levels of vit D_3 and 1,25$(OH)_2D_3$ are minimal with the dose regimen provided [139]. Nonetheless, profiling these and other vit D metabolites can reveal individual characteristics on the vit D status that could be applied in a targeted approach. For example, the measurement of 24,25$(OH)_2D_3$ (catabolic product of *CYP24A1*) along with 25(OH)D_3 has been shown to provide a better assessment of the vit D status. These metabolites are used to determine the vitamin D metabolite ratio (VMR) which is used as a marker to identify type 2 diabetes complications [283]. Also, biological effects by other vit D metabolites have been described; for example, the 20S(OH)D_3 metabolite synthetized by the enzyme *CYP11A1* on placenta, adrenal glands, and epidermal keratinocytes showed proliferative and anti-inflammatory properties on epithelial cells [284]. Thus, although the profiling of the vit D metabolome in animals is challenging due to the lack of standardized methods for veterinary species, a comprehensive investigation of the vit D metabolites will aid in the development of more efficient vit D supplementations [285].

The *in vivo* vit D_3 supplementation trial showed that VDD perturbed the proportion of circulating leukocytes. Animals with low 25(OH)D levels (Ctl-In) had higher number of neutrophils, eosinophils, and basophils in circulation. Besides, Ctl-In calves showed decreased mycobacterial activity and an increased production of IL-1β and IL-8 than animals with higher 25(OHD) levels (VitD-In). This could suggest that vit D controls the number of neutrophils in circulation and its functionality, which contribute towards an uncontrolled inflammatory response in a vit D deficient state. In fact, studies on mice have shown that VDD compromise the pulmonary resistance and immunity against fungus infections by increasing the migration of neutrophils into airways and by an enhanced and sustained pro-inflammatory response resulting in an aggravated pathology [286]. Similarly, dietary vit D_3 reduced the

pulmonary pathology during *M. tuberculosis* infection by altering the accumulation of leukocytes in granulomatous regions [97]. Collectively, our results of the *in vitro* and *in vivo* studies suggests that neutrophils are one of the primary targets of vit D, and as previously stated, neutrophils are key regulators of the immune response who play an essential role in maintaining host homeostasis. Thus, research into the environmental factors that influence this cell's response is essential to understand their role as a friend or foe [46].

The microbicidal effect by vit D observed in the *in vitro* and in the *ex-vivo* BCG challenge was modest. This could suggest that in some animals, the main effect of vit D is not to improve *M. bovis* killing, but to enhance the activation of the innate immunity and to protect the host from the negative impact of the inflammatory response. For example, data from *in vitro* and *in vivo* vit D interventions indicate that the regulation of the oxidative stress response is differentially regulated by vit D, thus an enhanced cell activation could be achieved by influencing the activation of the NF-κB pathway through ROS and RNS production [269]. Our results also identified a high inter-animal variability in response to vit D. This can be explained by differences in the *VDR* that affected the responsiveness to vit D. In humans, the *FokI* polymorphism of the *VDR* is associated with an increased risk to TB [158]. However, in cattle little is known about the *VDR* and a complete characterization of the gene has not been performed [287, 288]. Furthermore, it has been shown that the combined effect of genetics and epigenetics can explain the inter-individual differences to vit D [182] with data showing that methylation of the VDR influences the immune function by dampening the TLR signalling [289]. Thus, it will be interesting to identify functional sequence variants of the VDR as well as changes on the VDR epigenome to understand their relationship with disease susceptibility in bovines.

With respect to the gene expression results, similarities and differences between the responses to *in vitro* and *in vivo* vit D supplementations were analysed with a heatmap. This was performed by comparing the results of the 50 genes that were equally detected in both datasets from chapter 2 and 5. This analysis shows that vit D *in vivo* had lower expression than vit D *in vitro* (Figure 6-1). However, this comparison might not be correct, for three reasons: 1) different dose and vit D metabolite used, 2) duration of exposure, and 3) intervention time. As previously stated, the vit D metabolite used in both interventions was different, and while $1,25(OH)_2D_3$ is responsible for the microbicidal and immunoregulatory effects, the concentration of it is not necessarily the same in both models. Besides, little is known about the dynamics of $1,25(OH)_2D_3$ synthesis. It is possible that conversion of $25(OH)D \rightarrow 1,25(OH)_2D_3$ by the cells is not a single time process and occur continuously during the infection process, resulting in different concentration and exposition time. Likewise, results

from the *in vivo* trial represent a long-term vit D_3 exposition time performed on animals from birth to 7 months. Whereas the *in vitro* study represents a single time exposition to $1,25(OH)_2D_3$ in calves of 7 month-age. Thus, results are not comparable. However, these show some of the possible explanations of the discrepancy between *in vitro* and *in vivo* vit D studies in humans [282].

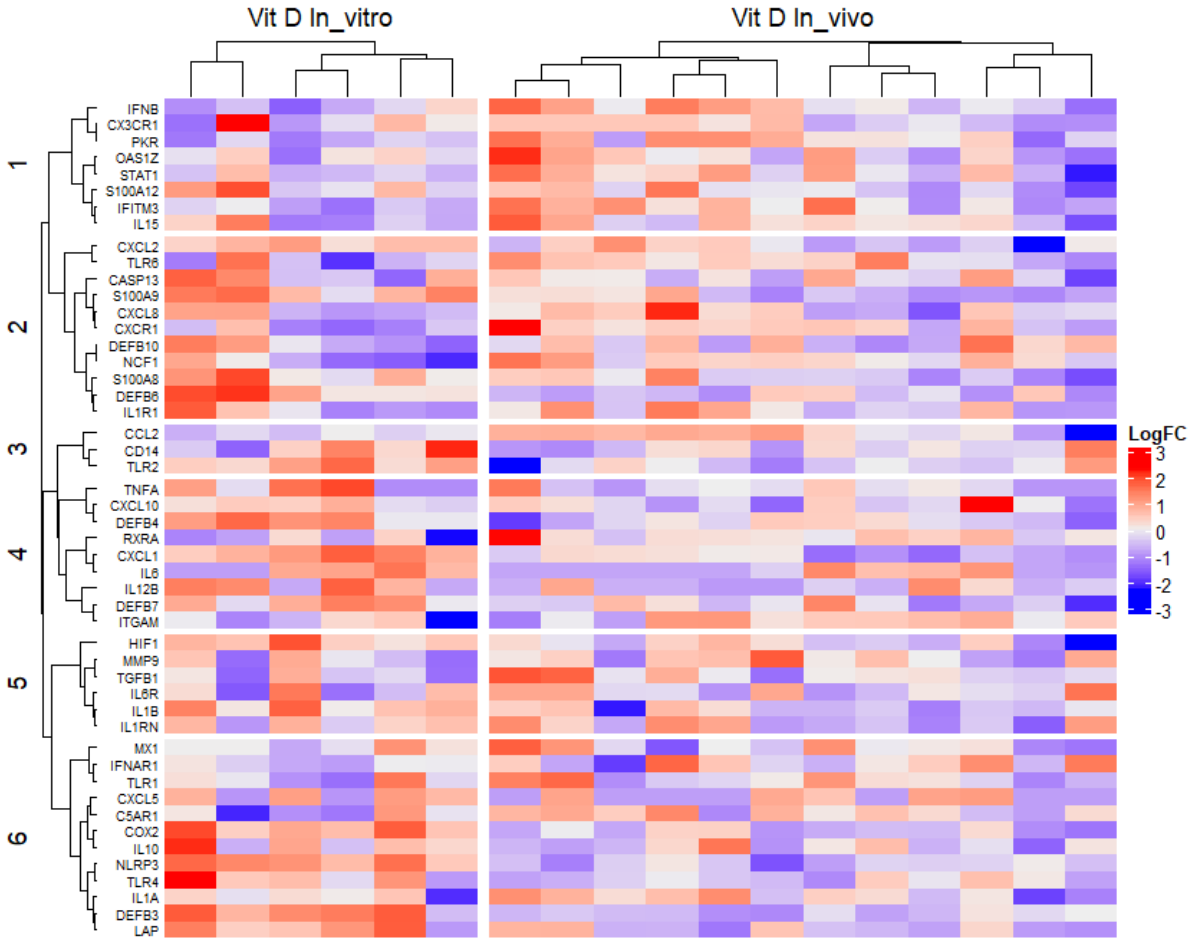


Figure 6-1. Comparison of the gene expression of vit D *in vitro* and *in vivo*.
Comparison was done between the *in vitro* effects of $1,25(OH)_2D_3$ from chapter 2, and the *in vivo* effects of vit D_3 supplementation from chapter 5. Results represent the standardized-Log_2 relative fold change. Hierarchical clustering with spearman correlation distance and Ward's linkage method was used to identify clusters of genes and samples with similar patterns. Division of clusters was achieved by splitting the dendrogram in clusters with the same height.

Despite the limitations of the *in vitro* studies, they are very informative to elucidate mechanistic pathways. Future work based on the results presented here should focus on the effects of both 1,25$(OH)_2D_3$ and 25(OH)D on cells with microbicidal killing capacity, including neutrophils, NK cells, and γδ T cells. These mechanistic studies can include their interactions with macrophages. Studies like this can help to fully contextualise the impact of vit D on BTB. Additionally, it is clear that VDD can influence the susceptibility to disease, thus comparing the activity and efficiency of innate cells from animals with sufficient and deficient 25(OH)D circulating levels will aid in determining the impact of VDD. However, based on results from this work and evidence from supplementation trials in humans, long-term vit D interventions are better at showing prophylactic effects [266]. Thus, it will be interesting to assess the effects of long-term vit D deficiency and sufficiency on the function of innate cells. Besides, studies on cattle have shown that dietary 25(OH)D is more efficient alternative than vit D_3 at improving the vit D status, and its therapeutic benefit has been shown in the treatment of mastitis on dairy cows [122, 139, 172, 290]. Our results showed a high incidence of VDD in calves at birth; thus, dietary, or parenteral 25(OH)D represent an alternative to boost the vit D status of calves in an efficient and rapid way, which could increase the resilience to disease during the neonatal period.

The basic premise of this thesis was that vit D influenced cellular and molecular processes in bovine leukocytes hence influencing their microbicidal efficiency against *Mycobacterium bovis* BCG. The work presented here shown that vit D modulates ROS production, as well as the expression of genes that activate innate cells. Though, more research is required to demonstrate that these mechanisms are responsible of the reported microbicidal effects. Additionally, results showed that vit D_3 supplementation in cattle is an avenue to improve the microbicidal activity against mycobacteria and promoting a controlled inflammatory response which can increase the resilience to BTB. Thus, the described actions of vit D on the innate immune system provide exciting opportunities to improve disease resistance of cattle through better nutrition. However, many challenges remain, and future work should focus on the following:

1. Determination of the most appropriate cut-offs of the vit D concentrations in cattle based on empirical evidence from the different livestock production systems accounting for the effects of age, sex ,and breed. This will aid in the identification of the optimal 25(OH) thresholds for immunity.
2. Determination of the vit D status of pasture-based calves. Our data showed a high incidence of VDD in calves at birth, which could have severe implication in disease

susceptibility. This will aid to implement strategies to correct and prevent VDD during the neonatal period which can have widespread potential relevance for other diseases.

3. Determination of the vit D status in BTB+ and BTB- cattle. There is an association between low 25(OH)D and BTB+, and our findings suggest that 1,25$(OH)_2D_3$ improves BCG killing in BTB+ cattle. Therefore, vit D supplementation in BTB+ animals represent an opportunity to aid in the control of BTB in low-income countries, where eliminating infected animals is economically difficult. Furthermore, retrospective studies in cattle can help to clarify if VDD is a cause or consequence of BTB which could have important implications for both human and animal health.
4. Determination of the genomic and epigenomic effects of vit D in cattle. Polymorphisms in VDR and hydrolases enzymes involved in vit D metabolism, can result in differential responses to vit D supplementation, which may explain the inter-individual differences observed. Thus, research into vitamin D-regulated epigenome and transcriptome modifications will help in the discovery of the mechanisms associated with VDD and disease.

The link between vit D and the immune system is undisputable, but as demonstrated throughout this thesis, research in cattle is scarce, with the majority of the papers published in the last 7 years [285]. This represents a significant gap in the veterinary knowledge but also an opportunity to conduct research in a collaborative and interdisciplinary approach to improve the sustainability of livestock systems and protect both human and animal health.

7. APPENDIX A

7.1. Graduate modules

Current Concepts in Infection Biology A (VET40090) 5 ECT
Current Concepts in Infection Biology B (VET40100) 5 ECT
Flow Cytometry: Principles and Practice (CNWY40130) 2.5 ECT
Data Programming with R [Online] (STAT40730) 5 ECT
Data Analysis: Biological Science (CNWY41130) 2.5 ECT
Advances in Infection Biology (VET40080) 5 ECT
PhD Skills (FDSC40670) 5 ECT

7.2. Presentations

Poster presentations:
AVTRW, Irish Branch Annual Meeting 2019.
Keystone eSymposia: Tuberculosis: Science Aimed at Ending the Epidemic, 2020
7th European Veterinary Immunology Workshop, 2021
Seventh International Conference on *Mycobacterium bovis* 2022.

Oral presentations:
9°Simposio Becarios Conacyt en Europa, 2020
AVTRW, Irish Branch Annual Meeting 2021
Conway Festival of Research & Innovation, 2021
An All Island One Health Vitamin D workshop, 2021
UCD One Health Conference, 2022.

7.3. Publications

Flores-Villalva S., O'Brien M.B., Reid C., Lacey S., Gordon S.V., Nelson C., Meade K. G. Low serum vitamin D concentrations in Spring-born dairy calves are associated with elevated peripheral leukocytes. *Scientific Reports, 2021*. 11(1): p. 18969.

Flores-Villalva S., Remot A., Gordon S.V., Meade K. G. Vitamin D Induced Microbicidal Activity Against *Mycobacterium bovis* BCG is Dependent on Synergistic Activity of Bovine Peripheral Blood Cell Populations. *In preparation*

Flores-Villalva S., Remot A., Reid C., Gordon S.V., Meade K. G. Effects of Divergent Vitamin D Concentrations *in vivo* on Microbicidal and Immunoregulatory Responses to *Mycobacterium bovis* BCG in Dairy Calves. *In preparation*

7.4. Associated publications

Vigors S., **Flores-Villalva S.,** Meade. K.G. The impact of vitamin D_3 supplementation on the faecal and oral microbiome of Holstein-Freisian calves housed indoors or at pasture. *Submitted to Animal Microbiome*

Reid C., Flores-Villalva S., O'Farrelly C., Meade K.G. Long-term in vivo vitamin D supplementation in calves modulates IL-1, inflammasome and chemokine signaling in unstimulated and PAMP stimulated whole blood. *In preparation*

8. APPENDIX B

Appendix 1. List of primers used for gene expression analysis with the Fluidigm system.

Gene	Reference Sequences	Primer Forward	Primer Reverse
ACTB	NM_173979.3	ACGGGCAGGTCATCACCATC	AGCACCGTGTTGGCGTAGAG
C5AR1	NM_001007810	ATACCGTCCTTTGTGTTCCG	ATTGTAAGCGTGACCAGCG
CASP1	XM_024975697	CTCCCACCTGGCAGGAATAC	AGGAGCTGGAAAAGGAGGGA
CASP13	NM_176638.5	TCCGGACATTCAACAACCGT	ACCCACAATTCCCCACGATT
CASP8	NM_001045970.2	AATATTGGGGAGCAGCTGGG	AGGCATCCTTGATGGGTTCC
CAT	NM_001035386.2	AGATGGACACAGCCACATGA	ACTGCCTCTCCATTTGCATT
CATH4	NM_174827	GACCCACCTCCCAAGGATAATG	TTGAAGTCACACTGCTCCGC
CATH5	NM_174510	GGAGAATGGGCTGCTGAAAG	CACAGCACAGGTGATGTCG
CATH6	NM_I 74832	TCAGTTCAATGAGCGGTCCT	TTGAAGTCACACTGCTCTGC
CCL2	NM_174006	GCTCGCTCAGCCAGATGCAA	GGACACTTGCTGCTGGTGACTC
CCL20	NM_174263	TTCGACTGCTGTCTCCGATA	GCACAACTTGTTTCACCCACT
CCL5	NM_175827	CTGCCTTCGCTGTCCTCCTGATG	TTCTCTGGGTTGGCGCACACCTG
CCR1	NM_001077839.1	ATGTCTTTGTCGGCGAGAGG	TCTGTGGACAGGAAGGGGAA
CD14	NM_174008	TCCACAGTCCAGCCGACAAC	AACGGCGCTAGACCAGTCAG
CD209	NM_001145756.1	CACCCTCGACCACTACACAG	TGAAGAAGCCCAGTGAGACG
COX2	AF004944	CATGGGTGTGAAAGGGAGGAA	ATTTGTGCCCTGGGGATCAG
CX3CR1	NM_001102558	TGGCCTTGGGAACTGTCTTC	TGGCTGTTGATGAGGGCAAA
CXCL1	NM_175700.2	CCAAACCGAAGTCATAGCC	TCAGTTGGCACTAGCCTTGTTTAGC
CXCL10	NM_001046551	TTCAGGCAGTCTGAGCCTAC	ACGTGGGCAGGATTGACTTG
CXCL2	NM_174299.3	GTGTCTCAACCCCGCCGCTC	TCCAGATGGCCTTAGGAGGTGG
CXCL3	NM_001046513.2	AGCGATGCTGCTCCTGCTCCT	CCATGGGAGCTTCAGGGTTGAG
CXCL5	NM_174300.2	TGTTTAACCACCACACCGGG	AGGTGGCTATCACTTCCACC
CXCL8	NM_173925.2	TGAAGCTGCAGTTCTGTCAAG	TTCTGCACCCACTTTTCCTTGG
CXCR1	NM_174360	ACATGGTTGGTGACTCAGTCTT	CGTGCCGCTGTAATTTCCAA
CXCR2	NM_001101285	ACAGGTGACAAGCCCAGAATC	CGACCAATCCGGCTGTATAA
CXCR3	NM_001011673	CCACAGGACTTCAGCCTCAA	CGACTGCCACGATGCCATTA
CYP24A1	NM_001191417.1	GAAGACTGGCAGAGGGTCAG	CAGCCAAGACCTCGTTGATT
CYP27A1	NM_001083413.2	GGGCAGCTACGCCTCTTATT	ATCTGAGGCCCTACTCGGTT
CYP27B1	NM_001192284.1	TGGGACCAGATGTTTGCATTCGC	TTCTCAGACTGGTTCCTCATGGCT
CYP2R1	XP_002707855.2	CCTGGTCCTTCATCAGTTTGTGA	CGGTTGGAGAAGATTTGGCCT
DEFB10	NM_001115084.1	CTCCTCCTGCTCCTCTTGGT	TGCCAATCTGTCTCATGCGT
DEFB3	NM_001282581.1	CTCCTCGCACTCCTCTTCCT	GCATCTTCGCCTTCTTCTACCACGA
DEFB4	NM_174775.1	TCCTCGCAGTCCTCTTCCT	GGCACAAGAACGGAATACAGA
DEFB6	XR_240313.1	CTTCTCTAGCATCAGCCGCA	CGCATCTTCGCCTTCTTTTACCTCC

DEFB7	NM_001102362.2	TCTTCCTGGTCCTGTCTGCT	GGTGCCAATCTGTCTCCTGT
ELANE	NM_001105653.1	CGATTCCTTCATCCGTGGGG	GCGCCGGATGATAGAGTTGA
GAPDH	NM_001034034.2	GGCATCGTGGAGGGACTTATG	GCCAGTGAGCTTCCCGTTGAG
GM-CSF	NM_174027	CAGCTTCTCCGCACCTACTC	TGCAGGCCGTTCTTGTACAG
HIF1A	NM_174339	ACCCTGCACTCAACCAAGAA	TGGGACTGTTAGGCTCAGGT
IFITM3	NM_001078141	CCTGAACATCTGCTCCCTGG	CTCGGAGACTGCTTGAACGA
IFNA	NM_001017411.1	TGGCTGTGAGGAAATACTTCCAGAG	CAGTCCTTTCTCCTGAAACTCTCCTG
IFNAR1	NM_174552.2	TCCTTTGCCACGTGTCAAGT	AGTAGCGTGAGGGAGACAGA
IFNB	XM_005209900	GCTACAGCTTGCTTCGATTC	TGTGCTGGAGCATCTCATAC
IFNG	NM_174086	ACCAGGTCATTCAAAGGAGCAT	TCTGCAGATCATCCACCGGA
IL10	NM_174088.1	GTGATGCCACAGGCTGAGAA	TGCTCTTGTTTTCGCAGGGCAG
IL12A	NM_174355	ACAGAAGGCCAGACAAACTC	AGCCAGACAATGCCCATTAG
IL12B	NM_174356	CACCAGCAGCTTCTTCATCA	CTTGTGGCATGTGACTTTGG
IL13	NM_174089.1	CATGGCGCTCTTATTGACCG	AATGAGCTCCTTGAGGGCTG
IL15	NM_174090	AACAGCGATGCAGTGCTTTC	TCCTCCAGTTCCTCACATTC
IL17A	NM_001008412	GCCCACCTACTGAGGACAAG	GCTGGATGGTGACAGAGTTC
IL17C	617538	TGACGTCCACCAGCGCTCCATC	CTGGACCAGCGGCACTGAGTTG
IL17F	NM_001192082	CACTCTGGAGGACCACATTG	GAGTTCAGGGTCCTGTCTTC
IL1A	NM_174092	CTGAAGAAGAGACGGTTGAG	ATGCATTCCTGGTGGATGAC
IL1B	NM_174093.1	CTCTCACAGGAAATGAACCGAG	GCTGCAGGGTGGGCGTATCACC
IL1R1	NM_001206735	TCCTTCTCTGGAGGCTGATAA	GGCGTCGTGCTGTCATTTTT
IL1RN	NM_174357.3	CTTCTCCTCTTCCTGTTCCGTT	TTGACATCCCAGATCCTGAAGGC
IL23A	NM_001205688	GATGGCTGTGATCCACAAGG	TGGGAATAGGGCTTGGAGTC
IL26	NM_001205424	CAGAGCAACGATTCCAGAAG	TCTGCCTGAGGCTATGAAAG
IL33	NM_001075297.1	GATGGTGGCAGTCATCGGAA	GTAGCTCCACAGAGTGCTCC
IL4	NM_173921.2	GCCACACGTGCTTGAACAAA	CTTGTGCTCGTCTTGGCTTC
IL5	NM_173922.1	CAAACTGCACAAGGGGATGC	ATCTTTCTCCTCCACACTTCCT
IL6	EU276071	TGCTGGTCTTCTGGAGTATC	GTGGCTGGAGTGGTTATTAG
IL6R	NM_001110785.3	TGCTGGTCTTCTGGAGTATC	GTGGCTGGAGTGGTTATTAG
IRF3	NM_001029845.3	GGAAGGATAAGCCCGACCTG	GAGTCCTTGCTGTGGTCCTC
IRF7	NM_001105040.1	AAGTCTACTGGGAGGTGGGG	CCGAAGTCAAAGATGGGCGT
ITGAM	NM_001039957.1	TTGAGGCGACGATGGAGTTC	ACTTTCACCTGCCCAGCAAT
LAP	NM_203435	TGCTCCTTGCGCTCCTCTTC	CTCCGAGACAGGTGCCAATC
MIF	NM_001033608	GCAAGCCGGCACAGTACATC	CCGCGTTCATGTCGCAGAAG
MMP2	NM_174745	CCAAGGGTACAGCCTGTTCC	GGCCGGTGCCAGTATCAATG
MMP9	NM_174744	CGTTCCGACGACATGCTCTG	CATTGCCGTCCTGGGTGTAG
MX1	NM_173940.2	GGCCACATCCCCTTGATCAT	CGTACTGGTCCTTGTCCTGG
NCF1	NM_174119.4	GCAGGACCTGTCTGAGAAGG	GATGTCCCCCGCCTCAATAG
NLRP3	NM_001102219.1	CTCAGTGGCAATACCCTGGG	AGCACTGTCCCAACCACAAT
NOD1	NM_001256563.1	TGGTCACTCACATCCGAAAC	AGGCCTGAGATCCACATAAG
NOD2	NM_001002889	CCCAGGGGCTCAGAACTAACA	CCTTCATCCTGGACGTGGTTC
NOS2	NM_001076799	CTTGAGCGAGTGGTGGATGG	ATCTGAGGGCTGGCATAGGG
OAS1Z	NM_001029846.2	CCAATGGTTCTTCTGCCCCT	GGCAGGAGGTGGTCTTTGAT
PKR	NM_178109.3	TTTTCGCCTCCTCCTCATGC	AACGAATACAGGCTCGCAGA

PPIA	NM_178320.2	TCCGGGATTTATGTGCCAGGG	GCTTGCCATCCAACCACTCAG
RXRA	NM_001304343.1	GTCCTCTTCAACCCCGACTC	CTGCTCGGGGTACTTGTGTT
S100A12	NM_117 1651	GCTGAAGCAGCTGATCACAA	TCTTTATCGGCATCCAGGTC
S100A7	NM_174596	CAGCTTGAGCAGGCCATTAC	CGTGGCTGTGGTTGTGATAG
S100A8	NM_001113725	CTCCCTGATTGACGTCTACC	TCCAGGCCCACCTTTATCAC
S100A9	NM_001046328	TGACACCCTGATCCAGAAAG	GCCACCAGCATAATGAACTC
SAA3	NM_181016	CCTCAAGGAAGCTGGTCAAG	TACCTGGTCCCTGGTCATAC
STAT1	NM_001077900.1	CAAAGGAAGCCCCAGAGCCTAT	GCCACTCTTCTGTGTTCACTTAC
TAP	NM_174776.1	GTAGGAAATCCTGTAAGCTGTG	GTGTCTTGGCCTTCTTTTAC
TGFB1	XM_024977949	CCTGAGCCAGAGGCGGACTAC	GCTCGGACGTGTTGAAGAAC
TLR1	NM_001046504.1	ACCCTACTCTGAACCTCAAG	GACTGCACACTGGATTTCTG
TLR2	NM_174197.2	ACTGGGTGGAGAACCTCATGGTCC	ATCTTCCGCAGCTTACAGAAGC
TLR3	NM_001008664.1	TTTGCCTGGCTTCCACATCT	GGCGTCTCAAGTTGGAAAGC
TLR4	NM_174198.6	GCATGGAGCTGAATCTCTAC	CAGGCTAAACTCTGGATAGG
TLR5	NM_001040501.1	TTCCTGCAACCTCACCCAAG	CTGAGATTGGGCAGGTTTCG
TLR6	NM_001001159	CTCCGGGAGATAGTCACTTC	GGCCCTGGATTCTATTATGG
TNFA	NM_173966.3	TCTTCTCAAGCCTCAAGTAACAAGC	CCATGAGGGCATTGGCATAC
VDR	NM_001167932.1	GAGGGGAACCGTCCTTTGAG	GAGAAGCTGGTTGGCTCCAT

Appendix 2. Mean relative fold change differences in PBL treated or not with $1,25(OH)_2D_3$.

	Control		1,25OHD			
Genes	**Mean**	**SD**	**Mean**	**SD**	**P value**	**P adjusted**
C5AR1	0.77	0.52	0.71	0.64	0.844	0.899
CASP1	0.45	0.68	0.24	0.30	1.000	1.000
CASP13	1.24	1.17	1.42	1.19	0.313	0.562
CCL2	10.79	13.10	11.27	4.00	0.313	0.562
CCL5	13.28	12.97	10.31	9.60	0.855	0.899
CD14	7.01	6.26	6.59	9.65	0.438	0.652
COX2	244.40	260.70	228.90	248.80	0.844	0.899
CX3CR1	0.32	0.57	3.16	6.58	1.000	1.000
CXCL1	274.50	288.80	418.70	291.60	0.219	0.534
CXCL10	6.67	11.66	2.86	2.18	0.844	0.899
CXCL2	267.30	159.30	479.10	255.10	0.438	0.652
CXCL5	25.45	26.83	35.92	31.69	0.584	0.766
CXCL8	51.59	50.38	89.66	74.14	0.156	0.476
CXCR1	2.31	2.83	1.05	1.47	0.059	0.476
CYP27A1	0.09	0.10	0.17	0.23	0.281	0.562
CYP2R1	0.08	0.10	0.10	0.12	0.106	0.476
DEFB10	7.22	13.56	10.92	13.14	0.156	0.476
DEFB3	8.89	10.94	16.56	11.51	0.094	0.476
DEFB4	7.37	9.96	12.80	13.25	0.361	0.629
DEFB6	6.44	10.08	13.85	15.20	0.031	0.476
DEFB7	7.14	8.88	15.01	15.24	0.281	0.562
ELANE	0.65	1.19	1.61	2.02	0.787	0.899
HIF1	5.75	1.82	6.97	4.40	0.844	0.899
IFITM3	0.36	0.31	0.45	0.19	0.313	0.562
IFNA	0.16	0.35	0.23	0.37	0.059	0.476
IFNAR1	0.50	0.23	0.54	0.17	0.563	0.766
IFNB	0.09	0.07	1.03	1.35	0.156	0.476
IL10	5.88	7.02	3.28	3.42	0.106	0.476
IL12B	19.66	27.55	36.22	39.49	0.100	0.476
IL13	0.24	0.33	1.25	2.02	0.787	0.899
IL15	0.20	0.27	0.50	0.92	0.844	0.899
IL1A	79.49	72.65	116.60	88.93	0.059	0.476
IL1B	2.64	3.94	1.71	2.63	0.563	0.766
IL1R1	0.04	0.05	0.58	0.41	0.031	0.476
IL1RN	0.94	2.23	2.39	4.20	0.156	0.476
IL33	15.90	15.55	27.14	32.06	0.219	0.534
IL5	2.12	3.56	0.54	0.51	0.031	0.476
IL6	620.10	811.80	539.30	622.70	0.584	0.766
IL6R	71.84	58.62	36.90	21.46	0.313	0.562
ITGAM	0.39	0.15	0.57	0.36	0.438	0.652

LAP	3.26	3.73	7.09	6.84	0.156	0.476
MMP9	18.66	27.84	9.69	11.48	0.281	0.562
MX1	0.49	0.16	0.70	0.72	1.000	1.000
NCF1	0.42	0.42	0.53	0.64	0.844	0.899
NLRP3	17.16	20.93	13.30	7.72	0.688	0.856
OAS1Z	0.37	0.31	0.76	0.42	0.156	0.476
PKR	0.34	0.35	0.43	0.21	0.438	0.652
RXRA	0.64	0.99	0.33	0.41	0.106	0.476
S100A12	7.15	8.07	21.28	32.18	0.156	0.476
S100A8	18.39	21.86	46.44	68.29	0.438	0.652
S100A9	29.77	24.67	43.29	32.10	0.313	0.562
STAT1	1.57	1.03	2.29	2.20	0.438	0.652
TAP	1.29	1.37	2.77	1.91	0.219	0.534
TGFB1	0.70	0.58	0.55	0.39	0.688	0.856
TLR1	0.13	0.13	0.34	0.32	0.563	0.766
TLR2	5.24	2.47	6.74	3.75	0.156	0.476
TLR4	4.44	4.88	4.24	5.04	0.590	0.766
TLR5	0.04	0.08	1.24	1.85	0.281	0.562
TLR6	0.36	0.32	1.27	2.26	0.219	0.534
TNFA	15.71	20.39	9.48	10.96	0.100	0.476
VDR	0.77	1.00	1.17	1.56	0.181	0.526

Appendix 3. Mean relative gene expression from Ctl-In and VitD-In after BCG challenge.

	Ctl-In		VitD-In			
Genes	**Mean**	**SD**	**Mean**	**SD**	**P value**	**P adjusted**
LAP	3.39	6.77	1.48	1.74	0.373	0.984
DEFB10	5.13	4.25	11.18	12.49	0.562	0.989
DEFB3	2.11	1.84	1.29	0.90	0.987	0.999
DEFB4	2.66	3.16	1.11	1.19	0.475	0.989
DEFB6	1.22	0.84	1.82	1.82	0.638	0.989
DEFB7	1.36	1.14	5.19	12.86	0.941	0.999
S100A12	2.80	2.32	8.30	16.07	0.504	0.989
S100A8	5.90	6.23	12.42	24.75	0.895	0.999
S100A9	5.21	2.63	6.42	9.38	0.367	0.984
CCL2	79.13	107.20	39.27	29.91	0.229	0.235
CXCL1	377.10	393.40	58.72	46.14	0.000	0.002
CXCL10	15.64	21.14	25.65	84.27	0.125	0.235
CXCL2	1427.00	1630.00	253.30	415.00	0.001	0.005
CXCL5	382.10	559.00	28.00	37.62	0.007	0.040
CXCL8	245.90	307.30	129.70	166.00	0.085	0.234
CX3CR1	0.06	0.09	0.27	0.37	0.008	0.041
CXCR1	1.24	0.82	5.95	11.67	0.010	0.041
IL1A	420.00	723.10	373.00	540.00	0.203	0.364
IL1B	365.40	392.90	84.17	96.21	0.001	0.003
CASP13	0.51	0.30	0.89	0.57	0.077	0.275
NLRP3	0.73	1.09	1.04	0.87	0.802	0.802
IL1RN	55.07	38.60	42.34	46.52	0.113	0.301
IL1R1	6.82	9.73	1.55	1.82	0.004	0.017
IL10	1.11	1.75	1.69	1.82	0.233	0.796
IL12B	3.40	3.03	7.72	16.29	0.930	0.981
IL15	0.48	0.64	0.83	1.60	0.536	0.954
IL6	118.80	265.90	14.15	24.59	0.104	0.536
IL6R	0.30	0.57	0.35	0.48	0.862	0.981
TGFB1	1.24	1.55	1.05	1.03	0.639	0.954
TNFA	8.71	11.27	3.59	5.07	0.381	0.909
IFNB	3.36	6.67	16.02	26.77	0.135	0.517
IFNG	36.33	82.34	2.68	5.65	0.014	0.092
PKR	0.84	0.98	2.20	2.03	0.150	0.517
IFITM3	1.04	0.52	1.59	1.48	0.777	0.950
MX1	0.35	0.33	1.11	1.64	0.221	0.527
OAS1Z	1.25	1.45	2.17	4.06	0.822	0.950
IFNAR1	0.47	0.28	1.03	1.01	0.090	0.434
NCF1	0.46	0.28	1.26	0.98	0.002	0.004
MMP9	108.00	174.30	24.49	41.52	0.001	0.003
COX2	60.49	78.30	16.98	17.91	0.037	0.037

C5AR1	1.35	1.49	1.07	0.71	0.512	0.943
CD14	1.29	1.89	2.01	3.07	0.539	0.943
ITGAM	0.62	0.41	1.02	0.51	0.052	0.416
TLR1	0.20	0.12	0.35	0.35	0.096	0.598
TLR2	2.87	3.66	2.72	2.19	0.884	0.987
TLR4	1.12	1.53	1.43	0.91	0.226	0.785
TLR6	1.04	1.25	1.70	1.83	0.191	0.774
HIF1A	3.04	1.59	3.23	1.86	0.899	0.987
STAT1	10.85	13.14	7.46	8.66	0.358	0.891
RXRA	0.62	0.85	1.83	3.58	0.135	0.686

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