Bovine Immunity and Vitamin D_3

Subjects: Immunology

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is an environmentally hardy pathogen of ruminants that is transmitted via the fecal-oral route. Transition from subclinical to clinical infection is a dynamic process led by MAP, which survives and replicates in host macrophages. Hallmark clinical symptoms include granulomatous enteritis, watery diarrhea, and significant loss of body condition. Clinical stage disease is accompanied by dysfunctional immune responses and a reduction in circulating vitamin D_3. The immunomodulatory role of vitamin D_3 in infectious disease has been well established in humans, particularly in *Mycobacterium tuberculosis* infection. However, significant species differences exist between the immune system of humans and bovines, including effects induced by vitamin D_3.

vitamin D | Mycobacterium avium subsp. paratuberculosis | cattle | macrophage | PBMC | endosomal trafficking

1. Johne’s Disease Overview

Johne’s disease, also called paratuberculosis, is a chronic, progressive enteritis affecting ruminants and has economic importance particularly within the dairy industry. The etiological agent, *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is an acid-fast bacillus that is classified as an obligate intracellular pathogen [1]. Estimates of MAP’s impact on economic losses have varied through the years, with the most recent values reaching $198 million dollars per year in the United States alone [2][3].

Persistence of MAP within the environment is achieved as a result of its thick, waxy cell wall composed of long mycolic acids. These fatty acids provide a uniquely impermeable barrier that protects the bacterium from desiccation and damage by chemical or biological agents [4][5]. Pasture-based grazing systems face unique challenges in the control of paratuberculosis, as MAP can be isolated from grass and top layers of soil following manure application [6]. Environmental conditions have been shown to impact MAP longevity with dry, shaded areas of land testing positive up to 55 weeks [7] and water also potentially being a prominent environmental reservoir, testing positive for up to 36 weeks [8]. Other barriers to eradication and control include the potential for a wildlife reservoir, such as deer, that can continue the disease cycle and infect domesticated livestock [9][10].

2. Transmission

MAP infection rates in young calves increase with exposure to larger numbers of MAP, and infection is most commonly established via the fecal–oral route for upwards of a year following birth [11][12]. Transmission of MAP through colostrum and milk by infected dams has been shown to occur and detection rates, along with bacterial burden, appear to coincide with the level of fecal shedding by the dam [13]. Further, rates of *in utero* transmission also appear to relate to disease severity and MAP burden in the feces [14][15]. These routes of infection represent important steps in biosafety protocol during efforts of herd infection control, and largely revolve around maintaining clean, dry housing environments.

Fecal shedding patterns are intermittent in animals naturally infected with MAP categorized in the subclinical stage of disease. Dynamics of fecal shedding have been shown to be largely different between experimentally and naturally infected cattle, with experimentally infected animals shedding after one year and alternating between positive and negative status several times while also fluctuating between high and low levels [16]. Most of these animals reached high levels of shedding, which could be impacted by MAP infection dose and administration route. This entry also showed those animals that were naturally infected at a young age had a propensity to begin shedding approximately 3 years following infection, had little to no switching between shedding status, and those that did shed intermittently had a reduced chance of becoming a high shedder compared to those who shed MAP consistently. Other reports have also shown similar disease outcomes related to shedding level and frequency [17]. Additionally, naturally infected cattle that began shedding high levels frequently continued to do so until being removed from the herd [16].

Cattle that progress to clinical stage paratuberculosis show symptoms including watery diarrhea, weight loss, and in severe cases submandibular edema. Gross pathology shows marked inflammation of the intestinal wall, which impairs nutrient absorption from the diet [18]. This can lead to hypoproteinemia, reducing oncotic pressure of capillary vessels allowing fluid to leak from the blood vessels and accumulate beneath the jaw in grazing animals. The clinical stage is often accompanied by shedding of high levels of MAP and a significant reduction in milk production for lactating animals [19].

3. Immune Responses to MAP

Macrophage phenotype and resulting effector functions are influenced by the cell's cytokine milieu, pathogen signaling, and immune cell interactions. Phenotypic differences can be broadly represented by two groups according to cell surface receptors and cytokines produced [20]. A pro-inflammatory T helper 1 (Th1) cytokine profile, namely including IFN-γ, promotes a host defense (M1) macrophage phenotype with bactericidal activity that contributes to pathogen elimination. These cells have been found to produce high levels of nitric oxide and express high levels of CD80 and CD86 [21][22]. Binding of CD40 on the macrophage with CD40L (CD154) on activated T cells is essential for T cell proliferation and induction of pro-inflammatory responses including IL-12 expression and nitric oxide production [23][24][25]. In bovine monocyte-derived macrophages (MDMs) infected with MAP, defects in CD40-CD40L signaling resulted in abrogated gene expression for IL-12p40 and inducible nitric oxide synthase [26]. In contrast, uninfected MDMs were able to upregulate these responses in addition to increased IL-6, TNF-α, IL-8, and IL-10. The subclinical stage of disease is often associated with greater capacity to produce pro-inflammatory

cytokines such as IFN-γ and TNF-α [27][28]. In the first year of infection with MAP, it has been shown CD4+CD45RO+ memory T cells are the main antigen responders, and significantly upregulate activation markers CD25 and CD26 following *in vitro* stimulation [29]. This entry also observed that while CD8+CD45RO+ comprised approximately 30% of CD8+ T cells in the first year, they did not begin to have significant responses to *in vitro* stimulation until 18 months post infection, at which time they increased their expression of activation markers CD25 and CD26 [29].

Importantly, prior activation of the macrophage can have a profound effect on intracellular viability of the invading bacterium, which has been shown in IFN-γ pretreatment of macrophage *in vitro* [30][31]. Stimulation with a pro-inflammatory mediator after infection, however, does not confer the same protective effects. Furthermore, physiological responses such as polarization and signaling of T cells following antigen presentation rely on a variety of factors including antigen dose, its affinity for the T cell receptor (TCR), duration of binding, and production of anti-inflammatory IL-4 or pro-inflammatory IFN-γ [32][33].

The resolution and repair (M2) macrophage facilitates an environment in which a T helper 2 (Th2) cytokine profile predominates, defined by an anti-inflammatory response with increased IL-10 along with high levels of CD163 [34][35][36]. In the case of bovine paratuberculosis, the greater proportion of M2 macrophages present in the intestinal tissue of cows in clinical stage of disease ultimately hinders MAP clearance and disease resolution within the host [37][38]. This stage is characterized by a reduction in IFN-γ production, a cytokine which is protective for the host and functions by activating macrophages [28]. While the specific mechanisms are unresolved in this transition of immune function from subclinical to clinical cows, it has been shown that CD4+CD25- naïve T cells are largely unresponsive to MAP antigen and do not develop a regulatory T cell cytokine profile [39]. MAP-induced Th1 cytokine gene expression in these CD4+CD25- T cells was observed to be reduced in subclinical cows as well, but to a lesser extent. Furthermore, IL-10 facilitates reduction in IFN-γ and IL-12 and, has also been shown to reduce major histocompatibility complex II (MHCII) on monocytes, collectively resulting in reduced cell activation and antigen presentation [36][40][41]. Signaling through TLR2 has been implicated in facilitating pathogenic mycobacteria's ability to upregulate IL-10 [36][42]. T cell subsets largely contributing to IL-10 production are CD4+CD25+ regulatory cells, and in the absence of CD25+ cells IFN-γ production is significantly enhanced [43]. Fresh, unstimulated PBMCs from cows with clinical paratuberculosis have been shown to have significantly lower CD25 expression compared to subclinical and control cows but following MPS activation no differences among infection status groups were observed [44]. When T cell subsets were parsed out, MPS stimulated PBMCs from subclinical and clinical cows had significantly higher CD4+CD25+ T cells, and this observation has been replicated [45].

An additional T cell subset found in significant proportions in the bovine is the γδ T cell. This subset can constitute up to 60% of all T cells, and are speculated to bridge responses between innate and adaptive immunity. They can express cytokines such as IL-2, IL-10, IL-12, IL-15, and IFN-γ [46] and have been found to have regulatory function through spontaneous IL-10 secretion which has negative effects on proliferation for CD4+ and CD8+ T cells [47]. In the presence of *Mycobacterium bovis* (*M. bovis*) infected dendritic cells, γδ T cells upregulate production of pro-

inflammatory IFN-γ and IL-12 [48]. Additionally, γδ T cells have been reported to be significantly lower in cattle with clinical paratuberculosis [44][49].

Furthermore, investigation of γδ T cell distribution in granulomatous tissue in the bovine has shown significantly higher amount of these cells localized to late stage lesions in naïve calves experimentally infected with MAP when compared to vaccinated calves, which also had higher lesion scores [50]. This experiment begs the question of whether γδ T cells are lower in clinical cows due to them exiting the periphery to assist in controlling bacteria at the site of infection. Granulomatous lesions in paratuberculosis are largely unorganized and resemble that of Type II lepromatous granulomas, with disordered structure and macrophages containing high amounts of bacteria [51][52]. Granuloma formation at the site of infection is dependent upon pro-inflammatory TNF-α expression, and blocking TNF-α expression can result in downregulation of IFN-γ, IL-12, IL-10, IL-17, and nitric oxide production [53]. In *M. bovis* infected cattle, antigen-specific responses by T cells show CD4+ T cells are significant producers of IL-22, while γδ T cells are the main source of IL-17A but can also concurrently produce IL-22 [54]. IL-17A has been associated with early responses in mycobacterial infections [55] and can be produced by CD4+ and CD8+ T cells outside of antigen presenting cell stimulation [56].

4. Vitamin D

4.1. History

The story of vitamin D began in the early 1900′s during a time when vitamin deficiencies were more common, and their underlying root cause elusive to physicians. Accessory dietary requirements that were shown to prevent a variety of clinical manifestations led scientists to subscribe to the concept of “vital amines” [57][58][59]. As a result, conventional wisdom of simply balancing dietary proportions of protein, carbohydrates, fats, and salts began to evolve.

Soon after the discovery of Vitamins A, B, and C, McCollum et al. discovered that feeding cod liver, oxidized or not, healed rickets [60]. He also made the observation that developing clinical symptoms of rickets in preparation for disease resolution experiments takes significantly longer in the summer than the winter, one of the first hints that sunlight is important in the mechanism. Work done in the 1930′s confirmed this observation, showing ultraviolet rays convert 7-dehydrocholesterol to vitamin D_3 in the skin of hogs [61].

4.2. Metabolism and Signaling

Two isoforms of vitamin D_3 are known currently. Vitamin D_2 is converted from ergocalciferol found in plant material and is known to be a less potent regulator of serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentration in humans [62][63]. Animal derived vitamin D_3 originates from 7-dehydrocholesterol and can be absorbed through the diet or converted in the skin through exposure to ultraviolet rays from sunlight. Further steps in activation of 7-dehydrocholsterol occur through multiple hydroxylation reactions. $25(OH)D_3$, also called previtamin D_3, is formed in the liver through action of several cytochrome P450 hydroxylases, of which the most common is thought to be

CYP27A1 [64][65]. Specifically in cattle, CYP27A1 and CYP2J2 have been associated with regulating incidence of milk fever, which results from dysregulation of calcium homeostasis [66][67]. Next, 25(OH)D_3 is shuttled through the periphery for local conversion by 1α-hydroxylase (CYP27B1) [68]. Activation of 25(OH)D_3 by CYP27B1 occurs at a variety of cellular sites, including the kidney [69][70], maternal uterine tissue [71], bone cells [72][73], macrophages [74], and skin cells [75][76]. A visual summary of vitamin D_3 metabolism is presented in **Figure 1**.

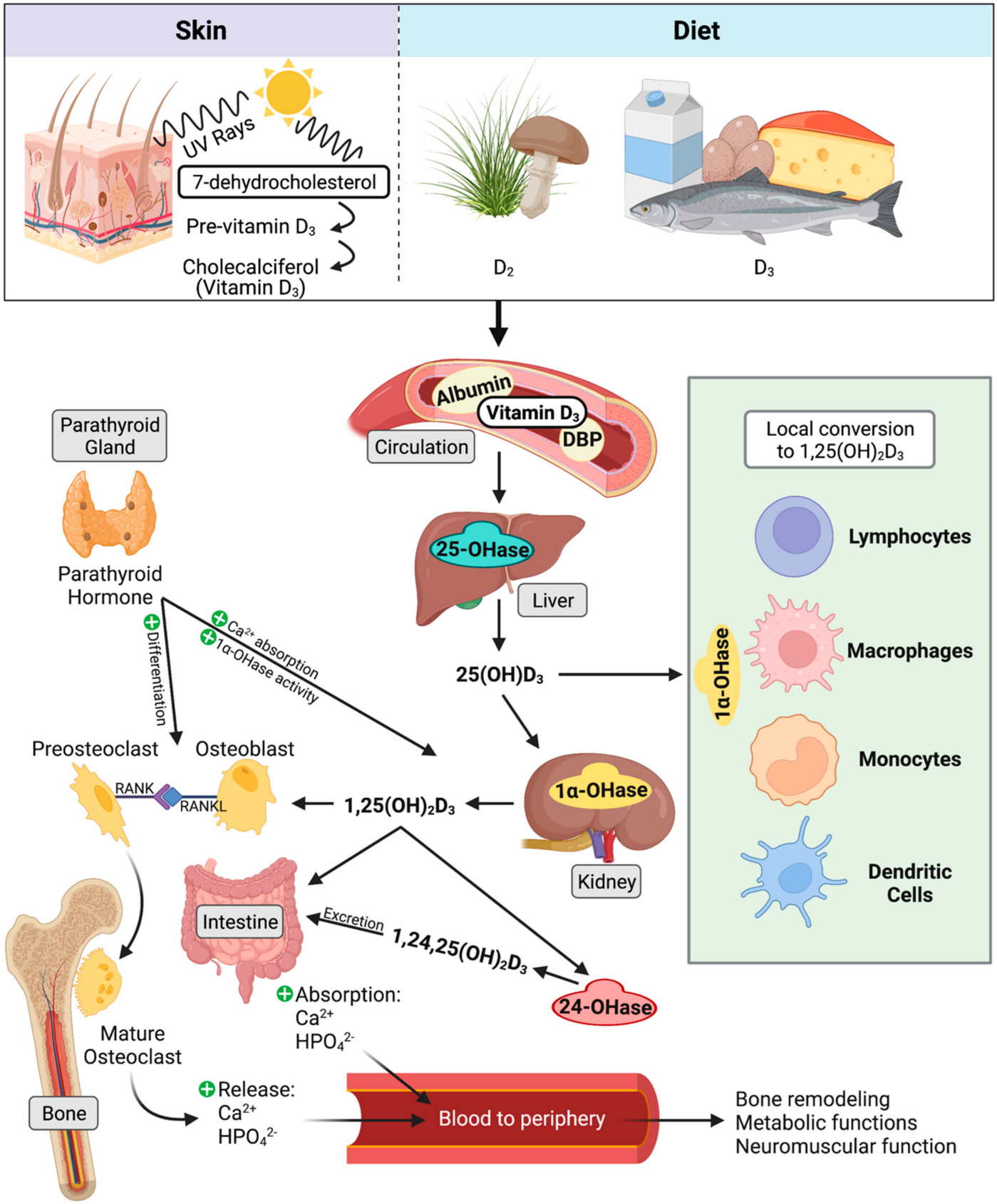

Figure 1. Vitamin D is synthesized in the skin or obtained from the diet. The most common and more potent form, vitamin D_3, is focused on in this entry. Vitamin D_3 is converted to $25(OH)D_3$ in the liver. Classical effects on calcium homeostasis begin in the kidney, where $25(OH)D_3$ is converted to bioactive $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ increases

osteoclast differentiation and activation, and upregulates calcium and phosphate absorption in the gut. Concurrently with $1,25(OH)_2D_3$, parathyroid hormone stimulates osteoclasts. It additionally upregulates 1α–OHase (CYP27B1) activity and calcium reabsorption in the kidney. $25(OH)D_3$ and $1,25(OH)_2D_3$ are both inactivated by 24–OHase (CYP24A1) and are excreted in the bile. Non-classical signaling of vitamin D_3 involves local activation of $25(OH)D_3$ to $1,25(OH)_2D_3$ by 1α–OHase (CYP27B1) in cells of the immune system. Figure created using BioRender.com (accessed on 28 July 2022).

The half-life of $25(OH)D_3$ is estimated to be around 15 days, allowing for this form to be a reliable indicator of vitamin D_3 status in the host [77][78]. Comparably, the half-life of $1,25(OH)_2D_3$ is a fraction of that, estimated at 4–6 h [78]. As a result, concentrations of $25(OH)D_3$ can be found over 1000 times greater compared to $1,25(OH)_2D_3$ [79]. Additionally, $25(OH)D_3$ is highly stable in serum under proper storage conditions and is largely unaffected by exposure to up to 4 freeze–thaw cycles [80].

Circulation of $25(OH)D_3$ and $1,25(OH)_2D_3$ in the blood is facilitated largely by vitamin D binding protein (DBP). DBP belongs to the serum albumin protein family. Additionally, a small proportion of $1,25(OH)_2D_3$ and $25(OH)D_3$ can be transported by serum albumin [81]. Properties of binding affinity to DBP and albumin vary among vitamin D_3 metabolites, with $25(OH)D_3$ showing the highest affinity but proportions of bound $25(OH)D_3$ and $1,25(OH)_2D_3$ are similar [81]. However, vitamin D_2 and its metabolites have a lower affinity for DBP, with an accompanying increased rate of plasma clearance [82]. $25(OH)D_2$ is cleared from the circulation 11 times faster than $25(OH)D_3$, and even greater differences are seen for bioactive $1,25(OH)_2D_2$ which has been shown to be cleared up to 33 times faster than its D_3 counterpart [83]. Collectively, these observations are likely attributed to vitamin D_2's inferior ability to influence serum $25(OH)D_3$ levels [62]. Execution of biological activity is inhibited by the presence of DBP [84][85][86], so following uptake of the bound molecules by endocytosis, acidification of the compartment facilitates disruption of the DBP-vitamin D bond freeing it for chaperone protein-mediated transport to the mitochondria for activation by CYP27B1 [87]. While considered a minority population, unbound vitamin D_3 can freely diffuse across cellular membranes [88].

Physiologic activity of $1,25(OH)_2D_3$ is facilitated through its binding of the nuclear hormone vitamin D receptor (VDR) (**Figure 2**). Collectively, VDR has been shown to have over 1000 target genes and is found in most tissues [88]. Genomic signaling pathways result in VDR dimerizing with retinoid X receptor (RXR) [89]. This complex directly binds the promoter of genes that possess vitamin D response elements, directly modulating their transcription. In a mouse VDR knockout model, animals observed abnormally increased $1,25(OH)_2D_3$ serum levels, overexpression of CYP27B1, and nearly undetectable expression of vitamin D_3-inactivating hydroxylase CYP24A1. Addition of $1,25(OH)_2D_3$ did not downregulate CYP27B1 or increase CYP24A1 expression in the absence of VDR, indicating that when bound to its ligand VDR helps modulate essential hydroxylase expression [68]. Furthermore, non-genomic signaling of $1,25(OH)_2D_3$ is thought to be facilitated through the binding of modified membrane VDRs, one of which is called protein disulphide isomerase family A member 3 (PDIA3) [90]. Uptake of the vitamin D_3 bound PDIA3 complex has been shown to be a result of caveolae-mediated endocytosis [91]. Vitamin D_3 signaling through these membrane VDRs are thought to modulate rapid responses to $1,25(OH)_2D_3$ and signaling through PDIA3 can

initiate cellular responses through the pro-inflammatory nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways [92][93].

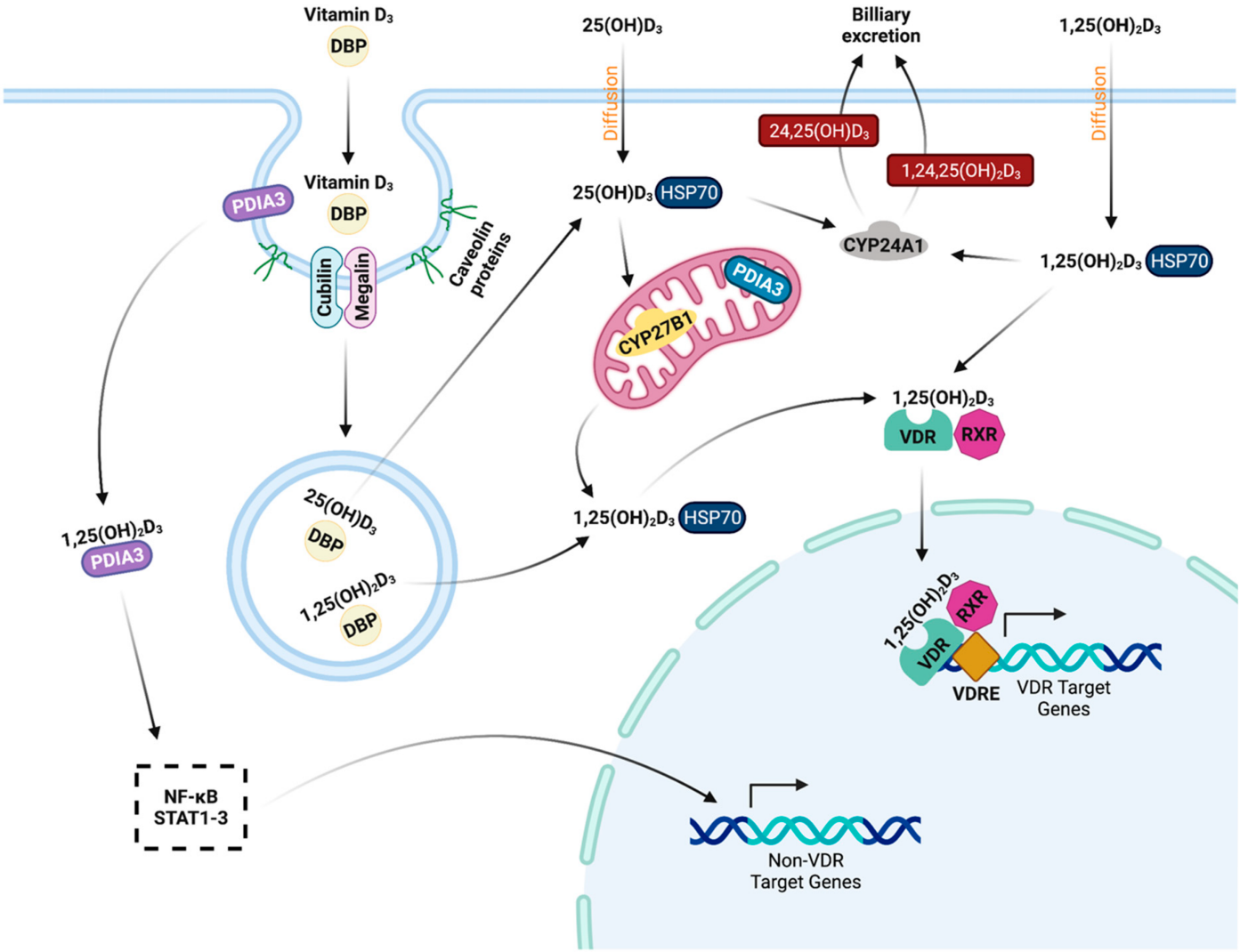

Figure 2. 25(OH)D_3 and 1,25(OH)$_2D_3$ travel in the circulation mainly bound to vitamin D binding protein (DBP). They are taken up by caveolae mediated endocytosis, where DBP then disassociates. Free 25(OH)D_3 and 1,25(OH)$_2D_3$ are lipophilic and can diffuse across the cell membrane. 25(OH)D_3 is activated by 1α-OHase (CYP27B1) in the mitochondria. Activated 1,25(OH)$_2D_3$ binds the vitamin D receptor (VDR) and forms a complex with retinoid X receptor (RXR). Together, they bind vitamin D response elements (VDREs) in vitamin D target gene promoters to facilitate gene expression. Additionally, alternate receptors at the cell membrane have been shown to bind 1,25(OH)$_2D_3$. One such receptor, protein disulphide isomerase family A member 3 (PDIA3) can bind 1,25(OH)$_2D_3$ and interact with NF-κB and STAT1–3 pathways, showing 1,25(OH)$_2D_3$ can also indirectly influence gene expression outside of VDR target genes. PDIA3 is also expressed in the mitochondria, but its full role is currently unknown. Figure created using BioRender.com (accessed on 28 July 2022).

CYP24A1, the 24-hydroxylase, functions as a regulator of 1,25(OH)$_2D_3$ by hydroxylating the number 24 carbon, which inactivates the molecule and prevents further physiological activity. Additionally, 25(OH)D_3 is also a substrate

for this enzyme and can similarly be inactivated [94]. Ultimately, the newly inactivated metabolites become more polar and water soluble, allowing for excretion in the bile [95][96]. In target cells, CYP24A1 expression and activity is highly induced by increasing amounts of $1,25(OH)_2D_3$ [97][98][99]. A mouse CYP24A1 knockout model showed dysregulation of $1,25(OH)_2D_3$ metabolism resulting in excessively high concentrations in the serum [100].

4.3. Classical Function

$25(OH)D_3$ is considered a prohormone, as it shares similar steroid chemical structure as adrenal and sex hormones [101]. When calcium levels are insufficient, bioactive $1,25(OH)_2D_3$ facilitates upregulation of calcium transport mechanisms to increase calcium and phosphate absorption by the intestine and renal tubule cells [67]. $1,25(OH)_2D_3$ facilitates bone growth and remodeling by stimulating differentiation and maturation of osteoblasts and osteoclasts, while remineralization is downregulated by high phosphate and osteopontin levels [102]. Reduced serum calcium concentrations stimulate production of parathyroid hormone from the parathyroid gland. $1,25(OH)_2D_3$ together with parathyroid hormone also facilitates demineralization of bone to release stored calcium by increasing activity of osteoclasts [103]. When sufficient serum calcium levels have been achieved, calcium inhibits production of parathyroid hormone, which then leads to inhibition of $1,25(OH)_2D_3$ synthesis by CYP27B1 in the kidney [104]. While these classical functions of vitamin D_3 are generally well understood in several species, much still stands to be elucidated about vitamin D_3's immunomodulatory role, particularly in cattle.

4.4. Hydroxylase Expression in Immune Cells

Evidence of $25(OH)D_3$ conversion to $1,25(OH)_2D_3$ was first shown in macrophages from humans suffering from sarcoidosis [105] and is shown not to be regulated by parathyroid hormone and calcium [106]. Stimulation of $1,25(OH)_2D_3$ production in monocytes and macrophages is a product of cellular activation through pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β [107][108][109]. Activation of peripheral bovine monocytes by lipopolysaccharide (LPS) induces *CYP27B1* expression, but the concurrent addition of $1,25(OH)_2D_3$ to cell cultures interrupts expression, bringing it back closer to baseline [110]. Gene expression for both *CYP27B1* and *CYP24A1* have been shown to be reduced by $1,25(OH)_2D_3$ in bovine PBMCs and MDMs activated by MAP sonicate or live MAP [45][111]. The reduction in vitamin D inactivating hydroxylase in this model may highlight a mechanism that enables monocytes and macrophages to maintain $1,25(OH)_2D_3$ levels during infection. Cattle with clinical stage paratuberculosis experience abrogated expression of CYP27B1 in the ileum, which may be a feature of inadequate access to its substrate $25(OH)D_3$ as a result of significantly reduced circulating levels in these animals. Interestingly, increased levels of IFN-γ have been associated with upregulation of *CYP27B1* activity [112][113]. Additionally, a *Streptococcus uberis* (*S. uberis*) bovine mastitis model observed localized expression increased for *CYP27B1* in milk CD14+ cells during active mastitis, while CD14- cells saw increased *CYP24A1* [114]. Bovine peripheral blood monocytes treated with $1,25(OH)_2D_3$ show increased *CYP24A1* transcripts, which are then reduced following activation with LPS [115]. Similar observations have been made in *M. bovis*-BCG vaccinated calves, where secondary purified protein derivative (PPD) antigen exposure abrogated the $1,25(OH)_2D_3$ induced upregulation of *CYP24A1* [116]. Intramammary treatment of healthy cows with $1,25(OH)_2D_3$ upregulates expression of *CYP24A1* in total milk somatic cells as early as 4 h post

treatment [117], as well as increases *CYP24A1* expression in milk macrophages and neutrophils [115]. Furthermore, the *S. uberis* infected mammary gland overall expressed increased *VDR* as well [114], however VDR expression does not undergo changes in the bovine ileum from MAP infected cows [113][118]. These studies show vitamin D signaling pathways and metabolism may be differentially mediated depending on the offending pathogen.

4.5. Host Vitamin D Status and Cathelicidins

Minimum thresholds of $25(OH)D_3$ concentrations reflecting deficiency, insufficiency, and therapeutic immune function have not been concretely established for neither humans nor cattle. An estimation for minimum levels required for immune function is speculated to be 30 ng/mL, whereas baseline concentrations for proper calcium signaling mechanisms is 20 ng/mL [119][120][121][122]. As previously mentioned in the case of bovine paratuberculosis, circulating levels of $25(OH)D_3$ have been shown to be significantly reduced in animals with late stage clinical disease [113]. Work in cattle has shown concentrations of serum $25(OH)D_3$ in adult dairy cows from across the United States at various stages of lactation and supplemented with 30,000–50,000 IU of vitamin D_3 per day vastly fall between 40–100 ng/mL, with an average of 68 ng/mL [123]. Additionally, seasonality has been shown to impact $25(OH)D_3$ status, with serum concentrations being higher in the summer [124]. This entry also reported a high incidence of $25(OH)D_3$ deficiency in calves following birth, observing concentrations mainly between 3–17 ng/mL and over 80% having lower than 20 ng/mL. These data highlight the importance of vitamin D_3 supplementation beginning from birth, especially during a time of greatest susceptibility to MAP infection.

Efficiency of vitamin D-induced antimicrobial activity is determined by the vitamin D status of the host, as availability of $25(OH)D_3$ substrate is ultimately a limiting factor. Evidence for this observation is reported in human monocytes and macrophages activated by *M. tb* or LPS signaling via TLR2/1 heterodimer or TLR4 binding, where serum with low levels of $25(OH)D_3$ had reduced ability to induce cathelicidin expression [125][126]. Cathelicidins are small cationic peptides that can upregulate expression of chemokines by macrophages, along with increasing phagocytosis of bacteria [127][128]. One functional cathelicidin gene has been identified in humans and mice, while 7 out of 11 cathelicidin genes in cattle have been shown to produce active protein [129][130]. Three of the bovine genes have purported vitamin D response elements, but studies thus far have shown their expression is not induced by $1,25(OH)_2D_3$ [110]. Other immunologic species differences exist. Cattle are estimated to have over 100 defensin genes while human and mice are estimated to have 39 and 52, respectively [131][132].

4.6. Macrophage Phagocytosis and Phenotype

Enhanced capacity for phagocytosis has been observed to be induced in monocytes and macrophages following treatment with $1,25(OH)_2D_3$ [111][133][134]. In another study, phagocytosis was upregulated in macrophages from healthy individuals that had a low phagocytic index, but those with a current pulmonary tuberculosis infection did not see a benefit [135]. This study used cultured PBMCs in the presence of $1,25(OH)_2D_3$ for 48 h followed by infection with *M. tb* for 3 h, so in considering the short half-life of $1,25(OH)_2D_3$ it is possible any $1,25(OH)_2D_3$-induced effects were not captured for this cell culture method. Small et al. showed increased phagocytosis of *Staphylococcus aureus* and *Candida albicans* with a concurrent upregulation of complement receptor

immunoglobulin (CRIg) in human macrophages treated with $1,25(OH)_2D_3$ [136]. This study utilized complement opsonized microbes; however, CRIg can function as an innate macrophage pattern recognition receptor (PRR) and directly bind some Gram-positive bacteria through recognition of lipoteichoic acids [137]. This posits the question of whether CRIg can also directly recognize any cell wall constituents in mycobacterial species; however, more definitive studies need to be performed to validate the PRR functions of CRIg outside of recognizing opsonized particles.

A study using human monocytes differentiated to macrophages by pro-inflammatory IL-15 showed antimicrobial effects against intracellular *Mycobacterium leprae* (*M. leprae*) were induced by the presence of $25(OH)D_3$ during differentiation [138]. Compared to IL-10 differentiated macrophages, IL-15 differentiated macrophages expressed lower amounts of CD163 and higher amounts of CD209, possibly indicating a more M2-like phenotype. This study did not observe any changes in phagocytosis related to $25(OH)D_3$ treatment, further indicating a M1-like phenotype, as M2 macrophages have been shown to have better phagocytic capabilities [139]. Macrophage phenotype markers CD163 (M1) and CD80 (M2) have recently been shown to have vitamin D_3-induced changes in expression in bovine MDMs. When infected *in vitro* with MAP, MDMs from cows with subclinical and clinical stage paratuberculosis exhibit reduced CD80 expression following treatment with $1,25(OH)_2D_3$ [140]. In control cow MDMs infected *in vitro* with MAP, both $1,25(OH)_2D_3$ and $25(OH)D_3$ reduced CD80. CD163 was largely upregulated by $1,25(OH)_2D_3$ treatment regardless of activation with MAP. An exception was observed in subclinical cows, which showed $1,25(OH)_2D_3$ and $25(OH)D_3$ reduced CD163 expression [140]. To fully understand changes elicited by vitamin D_3, further work including a more expansive macrophage phenotype panel is needed.

4.7. Cytokines, Nitric Oxide, and β-Defensins

Modulation of immune responses by exogenous vitamin D_3 during infectious disease has shown positive outcomes following treatment. PBMCs from *M. bovis* infected cattle treated *in vitro* with $1,25(OH)_2D_3$ show antigen-specific recall responses through increased nitric oxide production, although pro-inflammatory IFN-γ production was inhibited [141]. A similar reduction in IFN-γ expression has been observed in stimulated PBMCs treated with $1,25(OH)_2D_3$ from *M. bovis* vaccinated cattle [142]. Nelson et al. also showed a concurrent reduction in gene expression after vitamin D_3 treatment for IFN-γ, IL-17A, and IL-17F, although only significant for IL-17F during $1,25(OH)_2D_3$ treatment [116]. Contrasting previous reports, $1,25(OH)_2D_3$ treatment and activation with MAP has shown to induce a significant increase in *IFNG* transcripts and a concurrent reduction in IFN-γ secretion in PBMC-MDM co-cultures from cattle with naturally acquired paratuberculosis [111]. Similar observations have been reported in PBMCs, with these disparate effects being highlighted in cows in the clinical stage of Johne's disease [45]. This may highlight a key defense mechanism employed by MAP; however, in another perspective, by reducing IFN-γ production the host may be protected from unnecessary tissue damage while it employs other antimicrobial mechanisms.

Activation of monocytes from healthy cattle by LPS upregulates pro-inflammatory IL-1β (*IL1B*), but the effect is independent of $1,25(OH)_2D_3$ exposure [110]. In MAP activated PBMC-MDM co-cultures, $1,25(OH)_2D_3$ significantly upregulates production of *IL1B* transcripts and IL-1β, with an accompanying reduction in *IL10* and IL-10 [111].

However, when PBMCs were cultured alone and activated with MAP sonicate, $1,25(OH)_2D_3$ increased *IL10* transcripts but reduced IL-10 secretion in cows with paratuberculosis [45]. Similarly activated PBMCs from cows with clinical paratuberculosis observed reduced *IL10* and IL-10 following $25(OH)D_3$ treatment [45]. These data could indicate that vitamin D_3-induced effects are more efficiently deployed when PBMCs, especially T cells, have the opportunity for crosstalk with macrophages.

In addition to the previously discussed cathelicidin induction, human tuberculosis patients also observe increased nitric oxide production in alveolar macrophages following treatment with vitamin D_3, but this mechanism is not thought to be regulated by TLR signaling [143]. Human promyelocytic cell line HL-60 has also reported upregulation of nitric oxide production, *NOS2* expression, and a resulting inhibition of intracellular *M. tb* growth following treatment with $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ also reduces levels of intracellular *M. tb* in human monocytes and macrophages [144][145]. To further highlight the critical role of vitamin D_3 in infectious disease, *NOS2* knockout mice infected with *M. bovis* experience increased capacity to kill the bacteria if they have sufficient circulating $25(OH)D_3$ [146]. Work in cattle has shown that dietary supplementation with $25(OH)D_3$ results in a greater proportion of peripheral blood neutrophils with antimicrobial activity, as measured by oxidative burst [147].

$1,25(OH)_2D_3$ has recently been shown to induce nitric oxide production along with upregulating *NOS2* expression in macrophages from healthy cattle that have underwent *in vitro* infection with *M. bovis* [133]. In contrast, MAP activated PBMCs and PBMC-MDM co-cultures from dairy cattle with naturally acquired paratuberculosis experience reduced *NOS2* expression following $1,25(OH)_2D_3$ treatment [45][111]. Curiously, $1,25(OH)_2D_3$ significantly upregulated nitrite production, measured as an indicator of iNOS activity, in the activated PBMC-MDM co-cultures from cows with subclinical and clinical paratuberculosis [111]. At this 24 h timepoint, live MAP may target $1,25(OH)_2D_3$-induced signaling events to disrupt availability of transcripts for protein translation.

In bovine PBMCs activated with MAP sonicate, *CCL5* expression has been shown to be reduced by both $1,25(OH)_2D_3$ or $25(OH)D_3$ [45]. Other work in peripheral bovine monocytes from healthy dairy cattle has shown upregulated gene expression at 24 h for inducible nitric oxide synthase (iNOS/*NOS2*) and RANTES/*CCL5* in bovine monocytes coordinated by $1,25(OH)_2D_3$, an effect that is greatly enhanced by concurrent LPS activation [110]. This entry also showed *CCL5*, *NOS2*, and nitrite production increase in a dose-dependent manner for $1,25(OH)_2D_3$ [110]. In a model utilizing PBMCs from calves vaccinated with *M. bovis*-BCG, secondary exposure to *M. bovis* PPD and *in vitro* treatment with $1,25(OH)_2D_3$ or $25(OH)D_3$ resulted in RANTES/*CCL5* gene expression upregulation [116]. A recent report has similarly shown induction of *NOS2* by $1,25(OH)_2D_3$ following LPS activation and increased nitrite production [148].

Peripheral blood monocytes from healthy cows treated *in vitro* with $1,25(OH)_2D_3$ increase expression of various β-defensins, including *DEFB3*, *DEFB6*, *DEFB7*, *DEFB10*, along with *NOS2*, with the effect enhanced further in LPS stimulated cells [115]. Opposite observations were seen in *DEFB5*, whose expression was reduced, and the effect enhanced by LPS. This experiment used an 18 h timepoint, and a follow up experiment using a 4 h timepoint shows peripheral blood monocytes upregulated all bovine β-defensin genes investigated following treatment with $1,25(OH)_2D_3$, indicating these cellular responses are more robust early in antigen exposure. Recently, studies have

shown PBMCs from cattle at different stages of paratuberculosis infection do not have significantly different levels of *DEFB4* and *DEFB7* expression, and there were no notable vitamin D_3 induced effects [45]. Infection status effects on expression of these β-defensin genes were also not present in co-cultures of PBMCs and MDMs; however, in this model $1,25(OH)_2D_3$ was shown to significantly reduce *DEFB7* in control and subclinical cows. In milk neutrophils, LPS activation alone at 18 h increased transcripts of *DEFB3*, *DEFB4*, *DEFB7*, and *DEFB10*. The authors further show that $1,25(OH)_2D_3$ treatment of the mammary gland facilitates upregulation of *DEFB7* at 8 h in milk macrophages [115].

Follow up studies by Merriman et al. show direct treatment of mammary gland with $1,25(OH)_2D_3$ in healthy cows upregulated expression of *NOS2* and *DEFB7* in total milk somatic cells at 4 h following treatment [117]. When cows with subclinical mastitis had their mammary gland treated with $1,25(OH)_2D_3$, upregulation was enhanced further and increased expression was observed for *DEFB4* and *DEFB7* along with *NOS2*, with a significant treatment effect being seen by 24 h [117]. $1,25(OH)_2D_3$ induces localized expression of *NOS2* in milk CD14+ cells and increased *CCL5* in CD14- cells during experimentally induced mastitis caused by *S. uberis* [114]. In a similar study, treatment of mammary gland with $25(OH)D_3$ in cattle with LPS induced mastitis showed total milk somatic cells having upregulated expression of *NOS2*, *RANTES*/*CCL5*, *DEFB3*, *DEFB4*, *DEFB7*, *DEFB10*, *IL1B*, and *IL8*, with most effects being observed between 4–8 h following treatment [149]. Further isolating the source of these responses, the authors showed milk macrophages expressing significantly greater amounts of *NOS2* transcripts when compared to untreated controls, but the significant upregulation of other responses were sourced from neutrophils [149]. Another *S. uberis* induced model of mastitis has shown a resulting decrease in bacterial load in the mammary gland, along with clinical symptoms, after directly treating the gland with $25(OH)D_3$ following each milking [150]. Collectively, these studies may indicate that the type of pathogen and its preferred tissue may heavily influence antimicrobial responses facilitated by vitamin D_3.

4.8. Macrophage Endosomal Trafficking

The desire to understand mechanisms of intracellular MAP survival have driven recent studies to investigate the relationship between vitamin D_3 and endosomal trafficking markers at different stages of Johne's disease. $1,25(OH)_2D_3$ and $25(OH)D_3$ have no significant effects on early endosomal marker Rab5 expression in bovine MDMs; however, control cow MDMs infected with live MAP and treated with either form of vitamin D_3 experienced significantly reduced Rab5 expression [140]. A notable observation was made in MDMs from cows with subclinical and clinical paratuberculosis, which had significantly reduced Rab5 expression upon *in vitro* MAP infection when compared to MDMs from control cows. Late endosomal marker Rab7 expression was consistently reduced by $1,25(OH)_2D_3$ treatment in MDMs from control and subclinical cows, regardless of *in vitro* MAP infection [140]. The lack of downregulatory effect in clinical cows posits the question if $1,25(OH)_2D_3$ treatment has therapeutic potential for these animals, especially considering previous reports of animals in this severe stage of disease having reduced circulating $25(OH)D_3$. Expression of endosomal markers in ileal macrophages from cows in different stages of paratuberculosis have also been recently investigated. Rab5 expression in clinical cow macrophages was shown to be significantly reduced compared to other groups [118]. Total macrophage Rab7 expression was not different among groups, but when colocalized with intracellular MAP subclinical cows showed no detectable

association [118]. This may indicate MAP employs virulence mechanisms during early infection to inhibit recruitment of Rab7 to the intracellular compartment it is contained in, which would provide opportunity for MAP's replication and facilitation of the chronic subclinical phase. Further studies are needed to fully elucidate MAP's ability to interrupt the phagosomal maturation pathway and the role vitamin D_3 plays.

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