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The Vitamin D–antimicrobial Peptide Pathway and Its Role in Protection against Infection



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Abstract and Introduction

Abstract

Vitamin D deficiency has been correlated with increased rates of infection. Since the early 19th century, both environmental (i.e., sunlight) and dietary sources (cod liver) of vitamin D have been identified as treatments for TB. The recent discovery that vitamin D induces antimicrobial peptide gene expression explains, in part, the 'antibiotic' effect of vitamin D and has greatly renewed interest in the ability of vitamin D to improve immune function. Subsequent work indicates that this regulation is biologically important for the response of the innate immune system to wounds and infection and that deficiency may lead to suboptimal responses toward bacterial and viral infections. The regulation of the cathelicidin antimicrobial peptide gene is a human/primate-specific adaptation and is not conserved in other mammals. The capacity of the vitamin D receptor to act as a high-affinity receptor for vitamin D and a low-affinity receptor for secondary bile acids and potentially other novel nutritional compounds suggests that the evolutionary selection to place the cathelicidin gene under control of the vitamin D receptor allows for its regulation under both endocrine and xenobiotic response systems. Future studies in both humans and humanized mouse models will elucidate the importance of this regulation and lead to the development of potential therapeutic applications.

Introduction

Vitamin D insufficiency/deficiency is a worldwide, public health problem in both developed and developing countries. Vitamin D promotes and maintains healthy bones and teeth, but with the near eradication of rickets in the early part of the 20th century by fortification of foods, chronic insufficiency has gone largely unrecognized. However, with the current reemergence of nutritional rickets among infants, recent evidence that low levels of circulating vitamin D are associated with increased risk and mortality from cancer, and evidence of the beneficial effects of vitamin D on multiple sclerosis, rheumatoid arthritis, diabetes, cardiovascular disease and microbial infections, there has been renewed interest in this vitamin. In 2007, *Time* magazine cited the "benefits of vitamin D" in its list of "Top 10 Medical Breakthroughs". Although extensive research has been done on vitamin D, the molecular and cellular mechanisms responsible for its many benefits have not been fully elucidated. With the discovery that vitamin D regulates the expression of an important antimicrobial peptide gene, exciting research findings are revealing and characterizing new pathways regulated by vitamin D and its receptor that may be essential for optimal immune function.

Vitamin D, Vitamin D Receptor & Immunity

Humans can obtain vitamin D in two different forms: vitamin D₃, or cholecalciferol, and vitamin D₂, or ergocalciferol. The former is synthesized in the skin by exposure to UVB radiation; the latter is produced in various plant materials, yeast and fungi when they are exposed to UVB radiation (Figure 1).^[1] Humans can obtain both forms in the diet by consumption of either animal or plant products that possess them. Both forms of vitamin D are hydroxylated by the cytochrome P450 enzyme CYP27A1 in the liver to 25-hydroxyvitamin D (25[OH]D) in a substrate-dependent reaction (Figure 2).^[1] The 25(OH)D circulates in the blood bound to the vitamin D-binding protein and is a reliable indicator of vitamin D status.^[2] To become fully activated, the 25(OH)D is converted into 1,25-dihydroxyvitamin D (1,25[OH]₂D) by the mitochondrial 1 α -hydroxylase enzyme (CYP27B1) (Figure 2). The majority of the body's 1,25(OH)₂D is synthesized

in the primary renal tubules of the kidney, but synthesis also occurs in numerous extrarenal sites in cells that express CYP27B1.^[1]

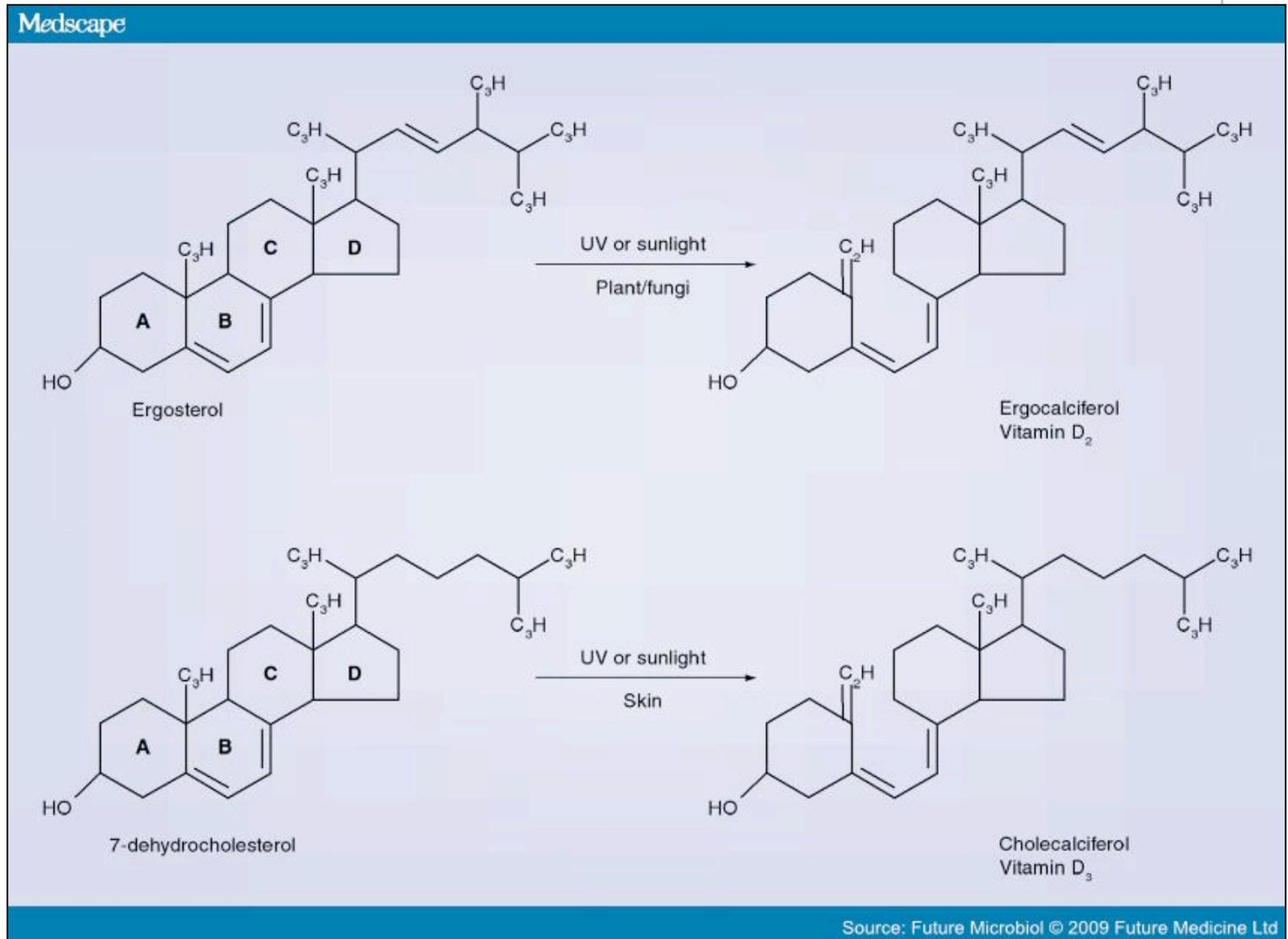
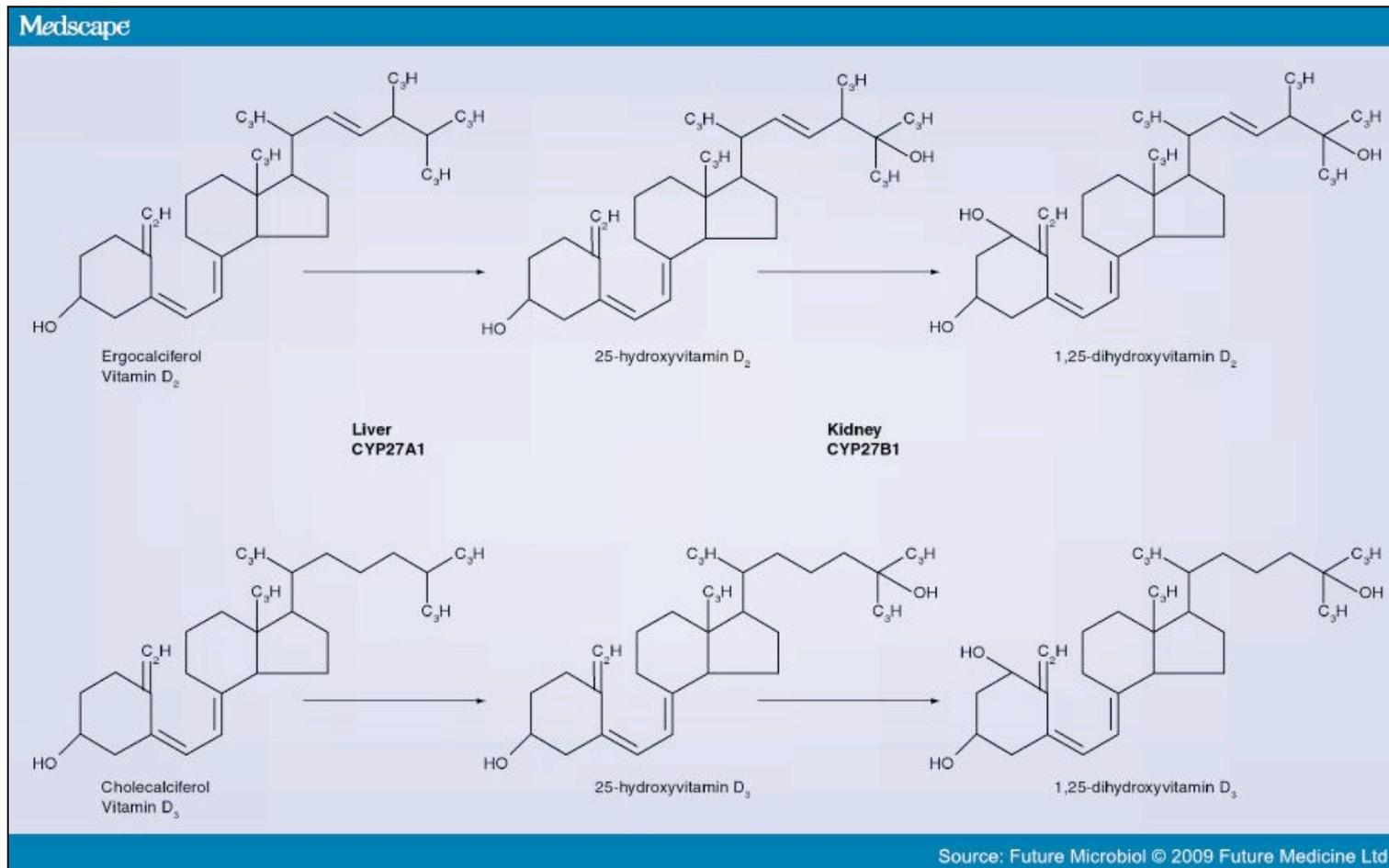


Figure 1. Photoproduction of vitamin D. In plants or fungi, sunlight or artificial UVB rays cleave ergosterol in the B-ring to produce ergocalciferol or vitamin D₂. In a very similar reaction in skin, 7-dehydrocholesterol is cleaved to produce cholecalciferol or vitamin D₃. Both forms can be used as sources of vitamin D.



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Figure 2. Conversion of vitamin D₂ or vitamin D₃ into active vitamin D. Vitamin D₂ or vitamin D₃ are hydroxylated in the liver by the enzyme CYP27A1 into 25-hydroxyvitamin D. This form of vitamin D circulates in the blood and is used to determine the vitamin D status of individuals (deficient: ≤ 20 ng/ml; insufficient: 21–29 ng/ml; sufficient: ≥ 30 ng/ml). When required by the body, the 25-hydroxyvitamin D form is converted by the CYP27B1 enzyme into the bioactive form, 1,25-dihydroxyvitamin D, which binds to the vitamin D receptor and activates gene expression. This primarily occurs in the kidney, but also occurs locally in cells that express CYP27B1. Immune-activated macrophages produce significant amounts of CYP27B1 and 1,25-dihydroxyvitamin D.

The genomic actions of 1,25(OH)₂D are modulated through the vitamin D receptor (VDR), a transcription factor belonging to the steroid/hormone receptor family.^[3] Target genes contain vitamin D response elements (VDREs) in their promoters, to which heterodimers of VDR and retinoid X receptors (RXRs) can bind and transactivate expression of the target genes.^[4] Most dividing cell types, normal and malignant, can express VDR and respond to 1,25(OH)₂D, and the VDR is expressed in at least 30 different target tissues.^[5,6]

Renal production of 1,25(OH)₂D occurs in response to decreased levels of circulating Ca²⁺, which stimulates the production of parathyroid hormone (PTH). PTH induces the production of CYP27B1 by primary renal tubules. As circulating levels of 1,25(OH)₂D rise, it suppresses its own production via a negative feedback loop in which the VDR binds to the CYP27B1 promoter to repress its expression. 1,25(OH)₂D increases the uptake of Ca²⁺ by the intestine, which leads to a decrease in PTH levels. In addition, 1,25(OH)₂D induces FGF-23 in osteocytes that represses PTH production. Furthermore, vitamin D induces the production of CYP24, a mitochondrial cytochrome P450 enzyme that catabolizes both 1,25(OH)₂D and 25(OH)D, thus limiting its own production.^[7]

Extrarenal production of vitamin D occurs in bone, epithelial cells of the skin, lung and colon, parathyroid glands and immune cells, especially activated macrophages. Control of its production in macrophages differs from renal tissues. Immune activation of macrophages with Toll-like receptor (TLR) ligands or IFN- γ leads to induction of CYP27B1 and production of 1,25(OH) $_2$ D and is dependent on the availability of 25(OH)D. CYP27B1 activity is not regulated by PTH or FGF-23 in macrophages and these cells synthesize an alternative splice variant of CYP24 that leads to the production of a dominant negative-acting protein that is catalytically inactive and prevents the catabolism of 1,25(OH) $_2$ D.^[1,8] Thus, unlike renal tubules, macrophages do not limit their production of 1,25(OH) $_2$ D and their continued activation may lead to accumulation of 1,25(OH) $_2$ D and contribute to human disease.^[8]

The importance of vitamin D and the active metabolite 1,25(OH) $_2$ D in immune function became apparent with the discovery of VDR expression in activated inflammatory cells.^[9,10] Also, the VDR is expressed in most cells of the immune system.^[11,12] Indeed, 1,25(OH) $_2$ D $_3$ directly modulates T-cell proliferation and cytokine production, decreases Th1 development, inhibits Th17 development and enhances the frequency of Th2 and regulatory T-cell production.^[13–17] A key mechanism in modulation of the adaptive immune system by vitamin receptor agonists involves their effects on myeloid dendritic cells (DCs).^[18] Numerous studies have demonstrated that 1,25(OH) $_2$ D impacts DC phenotype and function by downregulating expression of costimulatory molecules (CD40, CD80 and CD86) and cytokine IL-12 and upregulating IL-10 levels (recently reviewed in^[18]). The overall effect is to create tolerogenic myeloid DCs, which leads to decreased Th1 cell development, promotion of CD4+ suppressor T-cell activity and enhanced recruitment of regulatory T cells via increased expression of chemokine CCL22.^[19–21] Protolerogenic, plasmacytoid DCs are negligibly affected by VDR agonists; thus, their tolerogenic potential is less likely to be modified.^[18] The ability of 1,25(OH) $_2$ D to promote tolerance in DCs and T cells has prompted investigators to explore possible therapeutic treatments for a number of human autoimmune diseases.^[11,21–23] Vitamin D inhibits proliferation and production of immunoglobulin and slows differentiation of B-cell precursors into plasma cells.^[24] In addition to regulating their response to VDR agonists, macrophages, DCs and T cells can regulate the production and degradation of 1,25(OH) $_2$ D, which suggests an important biological role for 1,25(OH) $_2$ D in regulating innate and adaptive immunity.^[25–30] Suppression of the adaptive immune system and the inflammatory action of the nuclear factor (NF)- κ B pathway^[31–33] by vitamin D are probably beneficial for conditions that involve autoimmunity,^[34] however, it could prove detrimental for some infections.^[35,36]

Contrary to its suppressive effects on adaptive immunity, vitamin D has been known to be important for protecting against infection. Prior to the development of antibiotics, cod liver oil, sunlight (both sources of vitamin D) and pharmacologic doses of vitamin D were used to treat TB.^[37] This fell out of favor following the development of very effective antibiotic therapy. Recently, however, interest in using pharmacologic doses of vitamin D has been rekindled by discoveries made by our group and others that 1 α ,25-dihydroxyvitamin D can profoundly boost the innate immune system to combat pathogenic infections *in vitro*. The 'antibiotic' effect of vitamin D appears to be mediated, in part, by the induction of the human antimicrobial peptide genes.^[25,38–41]

Vitamin D is critical for the regulation of both the *CAMP* and *DEFB4* genes in both normal and transformed epithelial and hematopoietic cells.^[38–40,42–44] This regulation is biologically important for the response of the innate immune system to wounds and infection.^[25,43] Also, it may provide a mechanism to boost the innate immune system and counter the suppression of the adaptive immune system as described earlier.

Cathelicidins, Defensins & Innate Immunity

Cathelicidins are synthesized as a prepropeptide consisting of an N-terminal signal peptide, a conserved prosequence (cathelin domain) and a highly variable C-terminal cationic AMP.^[45] In neutrophils, proteolytic cleavage generates the mature AMP.^[46] While some mammals express numerous cathelicidins, humans possess a single cathelicidin gene^[47,48] called *CAMP*, also known as *hCAP18/LL-37/FALL39* (humans) and *CRAMP/ICNLP/MCLP* (mice). Throughout this paper the gene and mRNA is referred to as *CAMP*, the proprotein as cathelicidin or hCAP18 (human)

or CRAMP (mouse) and the processed peptide as LL-37 (human).

The hCAP18/CRAMP protein is synthesized and secreted in significant amounts by those tissues constantly exposed to environmental microbes,^[49] as well as other tissues^[50–53] and fluids,^[50,52,54–56] and is expressed by a wide array of immune cells.^[57–59] Other lines of evidence similarly indicate that hCAP18/CRAMP has important functions in host defense.^[60–62] Mice deficient in CRAMP are more susceptible to skin infection than wild-type mice,^[63] and CRAMP secretion is induced by bacteria and protects the murine urinary tract against invasive bacterial infection.^[64] Decreased expression of hCAP18 is reported in human diseases whose common denominator is enhanced susceptibility to infection.^[65–67] Collectively, these data strongly implicate a key role for *CAMP* in maintaining adequate host defenses.

The defensins are antimicrobial peptides with a characteristic β -sheet-rich fold resulting from a framework of six disulfide-linked cysteines.^[68] They are expressed by leukocytes and various epithelial cells that come in contact with environmental microbes. Expression is both constitutive and inducible depending on the tissue.^[68] Both cathelicidins and defensins disrupt the integrity of the bacterial cell membrane, resulting in the death of the microbe.^[68]

Vitamin D, TB & Other Infectious Diseases

Since the 1840s, both environmental (i.e., sunlight) and dietary sources (cod liver oil, eggs and liver) of vitamin D have been identified as treatments for TB.^[37] By the 1930s, vitamin D had been isolated from cod-liver oil and it was possible to begin using pharmacologic doses of vitamin D for treatment.^[37] Effective chemotherapeutic treatments decreased the interest in using cod-liver oil, sunlight and vitamin D for TB treatment, but studies over the last two decades have demonstrated that vitamin D deficiency is a risk factor for active TB and impaired antimycobacterial activity.^[25,69–71]

The mechanism of immune modulation that boosted antimycobacterial activity had remained an enigma. In the mid 1980s, vitamin D was found to boost the antimicrobial activity of human monocytes/macrophages against *Mycobacterium tuberculosis*.^[72] Most recently, we and others discovered that vitamin D strongly upregulated antimicrobial peptide gene expression.^[38,39] Subsequently, it was demonstrated *in vitro* that TLR2/1 signaling by a synthetic 19-kD *M. tuberculosis*-derived lipopeptide enhances the antimicrobial capacity of monocytes via a vitamin D and VDR-dependent pathway.^[25] This involved induction of the *CAMP* gene and its protein.^[25,37,73] In the model proposed, TLR2/1 activation of monocyte/macrophages induces the expression of CYP27B1 (25-hydroxyvitamin D-1 α -hydroxylase) which, in turn, leads to production of bioactive 1,25(OH)₂D from circulating inactive 25(OH)D.^[25] Also, the expression of VDR is increased and in the presence of locally high levels of 1,25(OH)₂D it activates the *CAMP* gene (Figure 3).^[25]

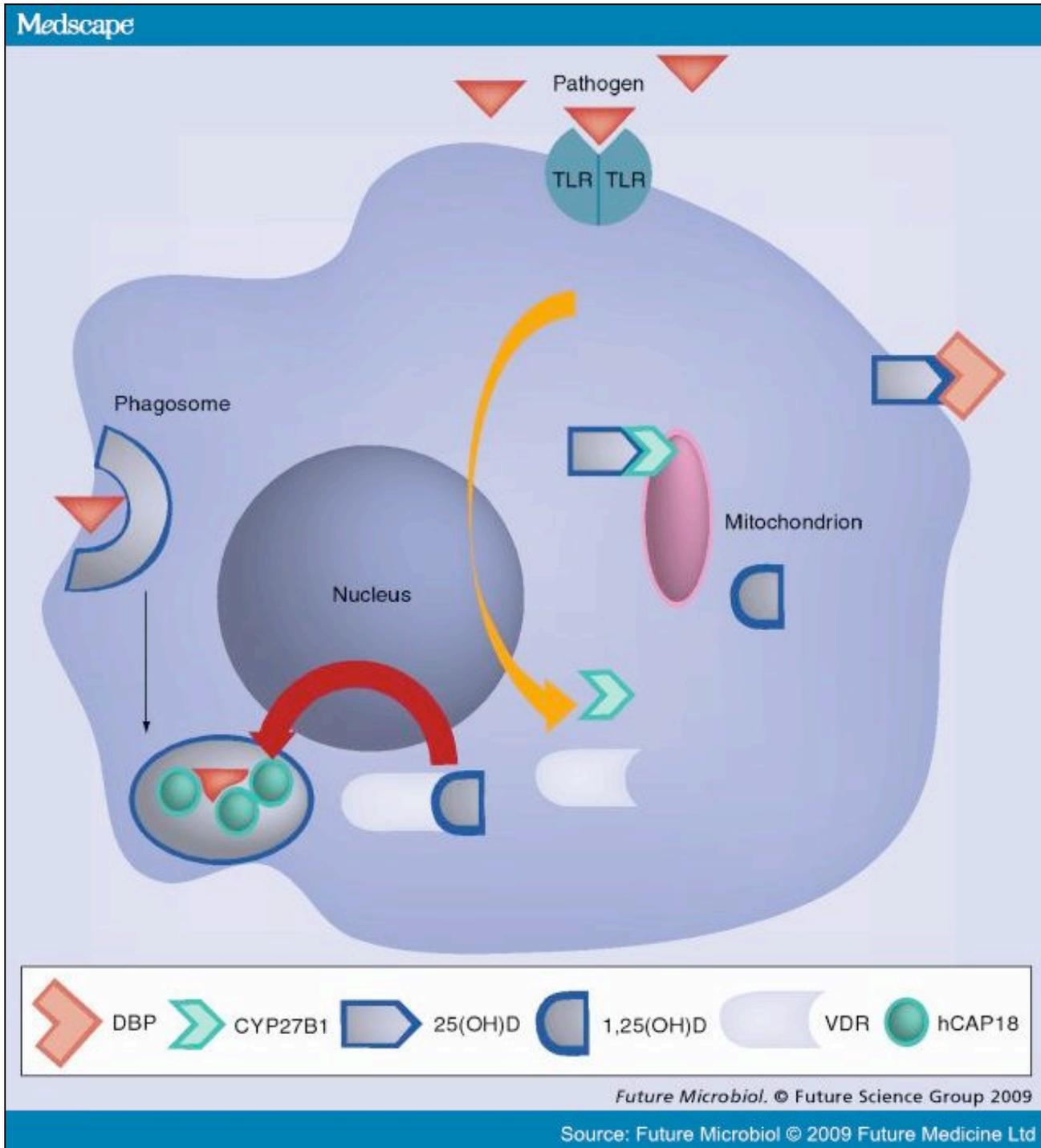


Figure 3. Vitamin D-dependent Toll-like receptor-activation of cathelicidin gene expression. The current model proposes that when a pathogen is detected by its respective TLR, VDR and YP27B1 gene expression are induced. This leads to 1- α -hydroxylation of 25(OH)D, which is taken up from the blood (in a complex with the D-binding protein) and subsequent binding of 1,25(OH)₂D to the VDR. The cathelicidin gene is activated and the protein (hCAP18/LL-37) is synthesized for use against the pathogen that has been engulfed in the phagosome of the macrophage.

TLR: Toll-like receptor; VDR: Vitamin D receptor.

While there is growing evidence that vitamin D boosts antimycobacterial immunity *in vitro*, it is essential to elucidate the

mechanism for immune modulation *in vivo*. In a review of three randomized controlled trials and ten case series in which vitamin D had been given to patients with pulmonary TB, it was concluded that the studies to date were flawed methodologically.^[74] Two small, randomized studies have suggested beneficial effects of vitamin D on treatment of TB.^[75,76] Also, administration of a single oral dose of vitamin D (2.5 mg) to individuals significantly enhanced the ability of their whole blood to restrict bacillus Calmette–Guérin–lux luminescence *in vitro* without affecting antigen-stimulated IFN- γ responses.^[77] In a larger recent double-blind, randomized, placebo-controlled trial it was found that vitamin D did not improve clinical outcome among patients with TB and no overall effect on mortality in patients with TB was observed.^[78] The authors went on to conclude that it is possible that the dose used was insufficient. Indeed, both the placebo and treated groups had similar levels of vitamin D at the start of the trial and at 2 and 8 months after the start of treatment.^[78] Further controlled, randomized, clinical trials need to be appropriately powered to detect modest effects and investigators should consider dose escalation, dosing frequency schedule and targeting populations that are severely deficient in vitamin D.^[79]

Vitamin D deficiency has been correlated with increased rates of other types of infection. Epidemiological studies demonstrate a link between vitamin D deficiency and increased rates of respiratory infections.^[80–83] It has been hypothesized that epidemic influenza may be the result of vitamin D deficiency.^[84] Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy.^[85] Low levels of vitamin D are described in HIV-infected individuals and this may have an impact on the progression of the disease.^[86] Consistent with the epidemiological data for respiratory infection, activation of the vitamin D pathway in response to respiratory syncytial virus infection occurs in lung cells via TLR3 signaling, resulting in the induction of the *CAMP* gene.^[87] Deficiency in vitamin D could impair this response by cells in the lung.

Regulation of *CAMP* Gene Expression by Vitamin D is a Primate-specific Adaptation

We demonstrated that regulation of the *CAMP* gene by VDR and its ligand 1,25(OH)₂vitamin D₃ is not evolutionarily conserved in mice, rats or dogs because the promoters of their *CAMP* genes lack a VDRE.^[38] The VDRE was found to be present in an AluSx short interspersed element present in the *CAMP* promoter of both chimps and humans, suggesting that this immune response is a primate-specific adaptation.^[38] Recently, we provided evidence for the evolutionary conservation of this regulation in humans, apes, Old World monkeys and New World monkeys, but not prosimians.^[88] This demonstrates that exaptation or co-option of vitamin D-mediated gene regulation by an AluSx short interspersed element provided a novel, biologically important innate immune response that is conserved in humans and nonhuman primates, but not other mammalian species. It is a convincing example of an evolutionarily-fixed, Alu-mediated divergence in steroid hormone nuclear receptor gene regulation between humans/primates and other mammals. This evolutionary conservation provides strong evidence that the TLR2/1–vitamin D–cathelicidin pathway evolved as a biologically important immune response mechanism for protecting human and nonhuman primates against infection.^[88]

What is the selective advantage of placing the primate *CAMP* gene under the regulation of the vitamin D pathway? The human *CAMP* gene is not induced consistently by pro-inflammatory stimuli.^[38,89–93] Additionally, infection of human macrophages with *M. tuberculosis* and other cell types with pathogens leads to the repression of the *CAMP* gene, whereas the murine *CAMP* gene is induced.^[37,94,95] Acquisition of the VDRE by ancestral primates that likely possessed high levels of vitamin D, similar to today's nonhuman primates,^[96] would have provided a pathway for induction of the *CAMP* gene in cells such as macrophages or epithelial barrier cells that are capable of activating the vitamin D pathway in response to infection or wounding.^[25,43] The activation of the vitamin D pathway provides a way for human macrophages to prevent the suppression of the *CAMP* gene when activated with TLR2 or TLR4 ligands.^[97] Thus, induction of the *CAMP* gene by 1,25(OH)₂D₃ provides a possible mechanism for primates to counteract pathogen-mediated suppression and modulate the immune response.

The mouse model is a powerful tool for understanding biological processes. The lack of vitamin D regulation of the cathelicidin gene makes it a poor model to study this mechanism. Our group is developing a 'humanized' mouse that

carries a portion of the human *CAMP* gene locus that allows for proper levels of *CAMP* expression in the appropriate tissues. In addition, the mouse will lack the murine *CAMP* gene.^[63] This mouse model will provide opportunities to study the importance of the vitamin D–cathelicidin pathway in infectious disease models while leveraging the power of crossing into other murine transgenics or knockouts.

Convergence of Additional Signal Pathways with Vitamin D Modulate Antimicrobial Peptide Gene Expression

Activation of TLRs promotes the release of immunomodulatory cytokines that play a role in the innate immune response. Once such cytokine, IL-15, was shown to be required for TLR2/1 induction of cathelicidin and also found to be sufficient to activate the vitamin D pathway in macrophages.^[98] The *in vivo* importance of IL-15 remains to be elucidated, but it may provide a mechanism to activate the vitamin D pathway in neighboring monocytes and macrophages, thus amplifying the innate immune response.^[98]

In conditions such as psoriasis, lesions contain significant levels of inflammatory cytokines. IL-17A is present at high levels in psoriatic lesions owing to an enrichment of Th17 cells, and LL-37 and HBD2 are present at high levels in psoriatic lesions and may contribute to the development of the disease.^[61,99] Recently, it was described that the LL-37 peptide converts self-DNA and self-RNA from dying host cells into a trigger of TLR9, -7 and -8 in human DCs, thus providing new insights into the mechanism that drives the autoimmune responses in psoriasis.^[99,100] In human keratinocytes it was found that IL-17A enhances expression of the *CAMP* gene via activation of Act1 and MEK–ERK in a process that is dependent on the presence of 1,25(OH)₂D.^[101] HBD2 and HBD3 have been shown to be regulated by IL-22 and -17A via JAK-STAT and NF-κB pathways.^[102,103] These findings elucidate a mechanism for cytokine-mediated inflammation in psoriatic skin through increased antimicrobial peptide expression. Interestingly, vitamin D analogs are used to successfully treat psoriasis and do not exacerbate the disease. Elucidation of the effects of vitamin D analogs on the cytokine milieu of the lesion or on keratinocyte differentiation may provide clues that explain this apparent paradox. Peric *et al.* recently reported that topical application of vitamin D and vitamin D analogs reduced expression of IL-17A, IL-17F and IL-8 in psoriatic lesions as well as the antimicrobial peptides HBD2 and HBD3.^[104] The vitamin D analogs blocked HBD2 induction by IL-17 by inhibition of NF-κB signaling.^[104] By contrast, levels of *CAMP* mRNA were increased in the lesions. In human primary keratinocytes *in vitro*, the unprocessed 18-kD form of the protein, hCAP18, was induced by vitamin D and its analogs, but the processed peptide LL-37, which plays a critical role in the development of psoriasis, was not detected by western blot analysis.^[104] The authors postulated that the induction of hCAP18 may not be accompanied by an increase in the active LL-37 peptide owing to a lack of processing. It remains to be determined if hCAP18 processing into LL-37 is altered in psoriatic skin lesions treated with active vitamin D and its analogs, but the hypothesis is testable and may explain why vitamin D does not exacerbate psoriasis.

While some cytokines may boost *CAMP* gene expression, others may inhibit it. In atopic dermatitis patients, the expression level of *CAMP* is found to be relatively low when compared with psoriasis patients.^[61] The reduced expression may be due to Th2-type cytokines, such as IL-4, -10 and -13, which are abundant in atopic dermatitis skin, inducing Bcl-3, a transcriptional regulator that represses *CAMP* gene expression.^[105]

The involvement of additional signaling pathways in the induction of antimicrobial peptide gene expression is especially important for induction of the *DEFB4* gene. The focus on cathelicidin gene expression has been due, in large part, to its robust and easily detected expression by 1,25(OH)₂D treatment alone. This was not the case for the *DEFB4* gene [GOMBART AF, UNPUBLISHED DATA]. A recent report demonstrated *in vitro* that induction of the *DEFB4* gene in macrophages requires TLR activation and the convergence of the IL-1β and vitamin D pathways.^[97]

The VDR & Beyond Vitamin D: Other Ligands

The VDR is a member of the steroid/hormone nuclear receptor superfamily that includes 48 ligand-activated

transcription factors.^[3] These factors bind to conserved elements in the promoters of genes as hetero- or homodimers through a zinc-finger motif and the adjacent ligand-binding domain activates or represses transcription upon binding to a small diffusible ligand. This allows nuclear receptors to function as intracellular detectors for endocrine hormones, dietary lipids and xenobiotic metabolites or compounds.

The nuclear receptor superfamily has been split into four groups based on class of ligand bound and whether DNA binding involves a hetero- or homodimer complex.^[106,107] The first class is the endocrine receptors that function as homodimers and bind steroid hormones produced by endocrine tissues, and include androgen, estrogen, progesterone, mineralocorticoid and glucocorticoid. The second group forms heterodimers with RXRs and ligands include dietary lipids, xenobiotics and various cholesterol metabolites. The third group heterodimerize with RXRs and include the thyroid hormone, retinoic-acid (vitamin A) and vitamin D receptors. The fourth group are those receptors that have unknown ligands and are referred to as orphan nuclear receptors.

Interestingly, the VDR was shown to possess characteristics of both the second and third groups with the discovery of its ability to function as an intestinal secondary bile acid receptor.^[108] Members of the second group include receptors for primary bile acids such as the farnesoid X receptor (FXR) and the xenobiotic receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR).^[106] Primary bile acids are major metabolites of cholesterol that are produced in the liver, excreted in the bile and subsequently reabsorbed in the intestine. Bile acids that are not reabsorbed are converted to secondary bile acids by the intestinal microflora.^[109] Secondary bile acids are toxic and must undergo detoxification by the body. FXR and PXR are receptors for secondary bile acids and promote their metabolism in the liver.^[108,110,111] VDR functions as a sensor for secondary bile acid lithocholic acid (LCA) and appears to be important for the detoxification of LCA in both the liver and intestine via the induction of CYP3A, a cytochrome P450 and known target gene of vitamin D, which mediates the catabolism of LCA.^[107–109,112,113] The importance of these nuclear receptors in maintaining a functional detoxification system in the intestinal tract has been nicely reviewed.^[107]

It is becoming clear that there is overlap both in the ligands that activate PXR, FXR, CAR and VDR and the genes that are regulated by them. Because vitamin D is an important immune regulator one may hypothesize that these other receptors could regulate some aspects of immunity, particularly gut immunity. Obstruction of bile flow leads to bacterial overgrowth in the small intestine, mucosal injury, barrier disruption and systemic infection. Oral administration of bile acids ameliorates the problems associated with bile-acid deficiency.^[114] Recently, in studies using mice, it was demonstrated that FXR plays a critical role in preventing bacterial overgrowth and maintaining the epithelial barrier in the intestine by upregulating several genes involved in mucosal defense of the gut.^[115] In addition, it was shown that bile salts may contribute to biliary tract sterility in humans by regulating the expression of the cathelicidin gene through the nuclear receptors VDR and FXR (Figure 4).^[116]

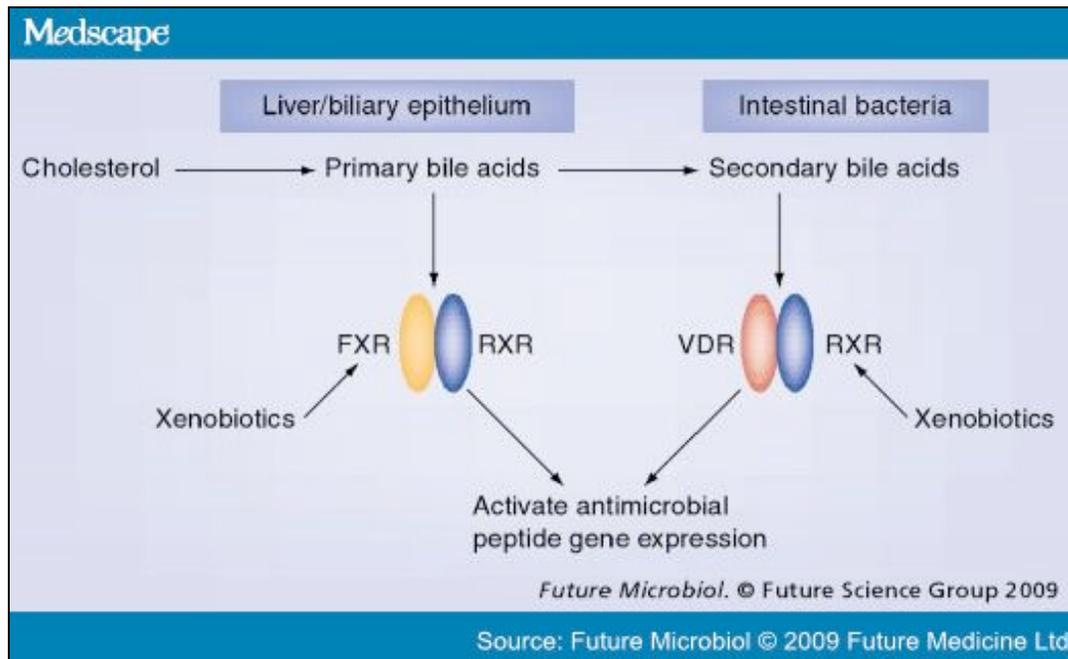


Figure 4. A role for enteric receptors in regulating the innate immune system by inducing antimicrobial gene expression. Intestinal detoxification receptors and the VDR share ligands and target genes. It has been shown that primary bile acids are capable of inducing cathelicidin gene expression in biliary epithelial cells through the FXR. Secondary bile acids that are byproducts of metabolism of primary bile acids by intestinal bacteria induce the cathelicidin gene via the VDR. The extensive crosstalk by these receptors possibly creates a system where bile acids, xenobiotic compounds and microbial byproducts can maintain critical gut immunity by inducing antimicrobial gene expression.

FXR: Farnesoid X receptor; VDR: Vitamin D receptor; RXR: Retinoid X receptor.

The accumulating evidence suggests that in the intestinal tract, the byproducts of gut microbes could be important for communicating with the epithelial cells, thus modulating the expression of the cathelicidin gene. This may be important for establishing a mucosal barrier to prevent contact of microbes and pathogens with the intestinal epithelium. This is nicely demonstrated by the ability of short-chain fatty acids such as sodium butyrate that are produced by fermentation of fiber by microbes in the colon to induce expression of the cathelicidin.^[92] The vitamin D pathway has been implicated in this induction.^[117] The production of secondary bile acids by microbes may modulate cathelicidin expression in the colon via the VDR. One could speculate that the selective force for placing the cathelicidin gene under the regulation of the VDR was so its expression could be regulated by both vitamin D and xenobiotic factors (Figure 4).

A recent study indicates that the VDR may act as a receptor for additional nutritional ligands, including curcumin and polyunsaturated fats such as α -linolenic acid, docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid.^[118] The *in vivo* relevance of these findings remains to be elucidated, but it is intriguing to consider that numerous nutritional compounds may modulate the expression of VDR target genes such as antimicrobial peptides.

Therapeutic Applications

Increasing endogenous cathelicidin (hCAP18/LL-37) expression may offer advantages over simple administration of synthetic antimicrobial peptides; transient expression of hCAP18 protein by adenovirus was tenfold more effective than synthetic peptide in preventing bacterial growth on second-degree burns in a mouse model.^[119] Also, induction of *CAMP* with butyrate treatment in *Shigella*-infected rabbits improved their outcome.^[120,121] Thus, hCAP18 may aid in clearance of bacterial infection and protect against sepsis. Administration of synthetic hCAP18/LL-37 may be beneficial,

but because butyrate-^[120] or adenoviral vector-induced increases in endogenous hCAP18 produced markedly improved outcomes, we propose that interventions that increase endogenous hCAP18 levels may be particularly useful in the treatment of infections.

Our own research has shown that high levels of hCAP18 in the blood of hemodialysis patients at the start of their treatment was indicative of a significant decrease in 1-year mortality.^[122] There was modest correlation with 1,25(OH)₂D levels, but not with 25(OH)D levels.^[122] In a recent study with sepsis patients, an association was found between severe illness and lower 25(OH)D, DBP and cathelicidin levels as compared with healthy controls.^[123] A positive correlation between 25(OH)D and cathelicidin levels was observed in all subjects.^[123] The question of whether increased supplementation of vitamin D-deficient individuals with 25(OH)D or therapeutic treatment of patients with active analogs of 1,25(OH)₂D will boost plasma levels of cathelicidin remains to be seen.

A study in atopic dermatitis patients demonstrated that supplementation with 4000 international units (IU)/day of oral vitamin D for 21 days increased cathelicidin expression in the lesional skin with a mild increase in the normal skin.^[124] We found that supplementation with 50,000 IU two times per week for 5 weeks had no effect on circulating levels of cathelicidin; however, it should be noted that the individuals in this study were relatively vitamin D replete and no patients were deficient. Thus, it is possible that circulating cathelicidin levels may be more dependent on vitamin D status at very low levels of serum 25(OH)D.^[97] In this same study, we did demonstrate that the ability of human macrophages to induce cathelicidin expression in response to TLR activation was directly proportional to serum vitamin D status. Cathelicidin induction was enhanced in vitamin D-insufficient patients given supplementary vitamin D.^[97] The studies to date would argue that it is important for individuals to have sufficient serum levels of 25(OH)D to allow cells in the body to synthesize cathelicidin when needed.

Conclusion

Deficiency in vitamin D is associated with numerous health conditions ranging from bone health to cancer, but with the discovery of antimicrobial peptide gene regulation by the vitamin D pathway a renewed interest in its impact on the immune system has ensued. It is particularly attractive to realize that adequate serum levels (>30 ng/ml) throughout life may alleviate many of the chronic ills that befall us as we age.

During the past 30 years or so, research has demonstrated the importance of vitamin D for optimal immune function, but many pieces of the puzzle are yet to be discovered and put in place. Both cathelicidin and defensin have activities beyond killing of microbes and can act as signaling molecules to activate the immune system.^[125,126] Numerous studies have established that α -defensins and cathelicidin act as chemoattractants for a variety of leukocytes, including DCs, T cells, monocytes and neutrophils.^[126] In addition, these antimicrobial peptides induce the expression of numerous cytokines and chemokines.^[126] This may provide another mechanism for vitamin D to exert additional effects on the adaptive immune system.

The overlap in the ligands and target genes of the VDR, FXR, PXR and CAR may reflect an important evolutionary redundancy that is critical for the detoxification and immunity of the gut. It also offers an abundance of new compounds that can be modified to produce potent ligands for the VDR. The therapeutic use of active vitamin D has been hampered by the toxic side effects of hypercalcemia. The development of synthetic analogs seeks to reduce the side effects while boosting the benefits. LCA can perform the *in vivo* functions of vitamin D by elevating serum calcium in rats^[127] and there is an interest in developing LCA derivatives that act as selective VDR ligands without inducing hypercalcemia.^[128]

The therapeutic use of vitamin D to boost immunity is an exciting possibility. While it is still not clear that we can increase the levels of cathelicidin in the blood with vitamin D or its synthetic analogs, we believe that higher levels of cathelicidin in the blood will be beneficial. We are particularly interested in testing this with the 'humanized' mouse model that we have developed.

Future Perspective

The future for the sunshine vitamin looks bright. Creative approaches involving animal models, human studies and genomic approaches to identify new targets of the VDR promise to shed even more light on the mechanisms by which vitamin D mediates its beneficial effects. Because vitamin D-mediated regulation of the antimicrobial peptide genes is a human/primate-specific process, it will be important to realize that animal models may not always reflect the human situation. This will require the generation of creative animal models and verification in human systems.

Many epithelial tissues such as the oral mucosa, intestinal tract, skin, urinary tract and reproductive organs are constantly exposed to the environment and the importance of the vitamin D–cathelicidin pathway in providing protection against pathogens in these tissues is already a major focus. Additional studies should reveal just how important vitamin D is in barrier defense of the body.

Considering that most people have insufficient levels of vitamin D and that nearly 1 billion people worldwide are deficient,^[129] properly designed supplementation studies in humans will be important for determining the benefits from raising serum levels of vitamin D on immune system function. It will be particularly interesting to determine if sufficient vitamin D levels will aid in treating patients with TB and HIV infection. These are classically deficient populations and vitamin D supplementation could be a potentially cheap adjuvant therapy for these conditions and is particularly attractive for impoverished countries where these diseases are rampant.

Sidebar

Executive Summary

Vitamin D, vitamin D receptor & immunity

- Renal production of active vitamin D is important for bone health by maintaining proper serum calcium and phosphorus levels.
- Extrarenal production of active vitamin D in immune cells is important for optimal immune response.
- Vitamin D modulates T-cell responses and has anti-inflammatory properties, but boosts innate immune responses by induction of the human gene for cathelicidin, *CAMP*.

Cathelicidins, defensins & innate immunity

- Cathelicidins and defensins are small peptides with amphipathic structures that allow them to disrupt the integrity of the pathogen cell membrane, resulting in its death.
- These proteins are expressed by most immune cells or those epithelial cells that are in contact with the environment.
- Deficiency in these peptides results in increased susceptibility to infection.
- Regulation of gene expression is not fully understood.

Vitamin D, TB & other infectious diseases

- Vitamin D deficiency is a risk factor for TB and, historically, sources of vitamin D were identified as treatments for TB.
- Vitamin D boosts the ability of macrophages to kill *Mycobacterium tuberculosis* via the vitamin D–cathelicidin pathway.
- Supplementation of humans with vitamin D has shown mixed results and future randomized, placebo-controlled trials need to be appropriately powered, consider dose escalation and frequency, and target severely deficient populations.
- Vitamin D deficiency has been correlated with increased respiratory tract infections, influenza and bacterial

vaginosis. Cause and effect is unknown.

Regulation of *CAMP* gene expression by vitamin D is a primate-specific adaptation

- A human/primate-specific transposable element provided a vitamin D receptor binding site in the promoter of the cathelicidin gene, thus conferring regulation of the cathelicidin gene by vitamin D to humans, apes, Old World and New World monkeys.
- This regulation is lacking in other mammals.
- This provides a possible mechanism for primates to counteract pathogen-mediated suppression and modulate the immune response.
- Our group is generating a humanized mouse model for understanding the biological importance of this regulation.

Convergence of additional signal pathways with vitamin D modulate antimicrobial peptide gene expression

- Activation of the cathelicidin gene by vitamin D is potentially modulated by cytokine signaling pathways.
- Vitamin D alone is not sufficient for induction of the human defensin $\beta 2$, but requires TLR activation and IL-1 β .
- The importance of additional signals needs to be examined.

The vitamin D receptor & beyond vitamin D: other ligands

- The vitamin D receptor (VDR) functions as a receptor for both vitamin D and secondary bile acids.
- The vitamin D receptor activates important detoxification genes that are targets of xenobiotic receptors.
- Xenobiotic receptors potentially activate VDR target genes and this cross-talk may play an important role in gut immunity.
- Evolutionary selection to place the cathelicidin gene under the regulation of the VDR potentially allows both endocrine and xenobiotic factors to regulate its expression.

Therapeutic applications

- The cathelicidin protein is present in the blood at significant levels and correlates with reduced mortality from infectious causes in hemodialysis patients.
- Severe sepsis patients have low vitamin D and cathelicidin levels.
- Supplementation with vitamin D increases cathelicidin expression in the lesions of atopic dermatitis patients and increases activated macrophage production of cathelicidin.
- The therapeutic potential of vitamin D and synthetic analogs against infection remain unexplored.

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Papers of special note have been highlighted as:

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